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ABOUT COVER

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The primary aim of *World Journal of Diabetes* (*WJD*, *World J Diabetes*) is to provide scholars and readers from various fields of diabetes with a platform to publish high-quality basic and clinical research articles and communicate their research findings online.

WJD mainly publishes articles reporting research results and findings obtained in the field of diabetes and covering a wide range of topics including risk factors for diabetes, diabetes complications, experimental diabetes mellitus, type 1 diabetes mellitus, type 2 diabetes mellitus, gestational diabetes, diabetic angiopathies, diabetic cardiomyopathies, diabetic coma, diabetic ketoacidosis, diabetic nephropathies, diabetic neuropathies, Donohue syndrome, fetal macrosomia, and prediabetic state.

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REVIEW

Mesenchymal stem cells-based drug delivery systems for diabetic foot ulcer: A review

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Abstract

The complication of diabetes, which is known as diabetic foot ulcer (DFU), is a significant concern due to its association with high rates of disability and mortality. It not only severely affects patients' quality of life, but also imposes a substantial burden on the healthcare system. In spite of efforts made in clinical practice, treating DFU remains a challenging task. While mesenchymal stem cell (MSC) therapy has been extensively studied in treating DFU, the current efficacy of DFU healing using this method is still inadequate. However, in recent years, several MSCs-based drug delivery systems have emerged, which have shown to increase the efficacy of MSC therapy, especially in treating DFU. This review summarized the application of diverse MSCs-based drug delivery systems in treating DFU and suggested potential prospects for the future research.

Key Words: Diabetic foot ulcer; Mesenchymal stem cells; Drug delivery systems; Diabetes; Wound healing

Core Tip: Diabetic foot ulcer (DFU) is a significant concern due to its association with high rates of disability and mortality. Mesenchymal stem cell (MSC) therapy has been extensively studied in treating DFU, the current efficacy of DFU healing using this method is still inadequate. However, in recent years, several MSCs-based drug delivery systems have emerged, which have shown to increase the efficacy of MSC therapy, especially in treating DFU.

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INTRODUCTION

Diabetes, along with its associated metabolic complications, has emerged as one of the most rapidly growing global health emergencies in the 21st century[1-3]. Diabetic foot ulcer (DFU) is a multifaceted and troubling complication of diabetes that poses significant challenges to healthcare providers and patients. DFU is characterized by the development of chronic wounds on the feet, which can lead to severe morbidity and mortality rates if left untreated[4-6]. Approximately 19%-34% of diabetics experience DFU, a condition with a recurrence rate of 40% within one year. This poses a significant threat to patients' physical and mental well-being and places a substantial financial strain on both patients and their families[7]. The 10th edition of the International Diabetes Federation (IDF) Diabetes Atlas has revealed a sobering reality concerning the global burden of diabetes. With an estimated 536.6 million diabetic cases diagnosed worldwide in 2021, diabetes has become a significant public health crisis that demands immediate attention and action. Even more alarming is the prediction that this number will surge to a staggering 783 million by 2045 if current trends continue. Furthermore, it is anticipated that the healthcare costs associated with diabetes and its complications could globally reach a staggering 966 billion USD in 2021[8-10]. The reported statistics revealed that DFU, a condition that affects the skin tissue of the feet and can cause serious complications, has a prevalence of 6.3% among the global population. It is noteworthy that this condition is more frequently observed in men than in women, indicating a gender-based disparity in its incidence rate. Moreover, the data suggested that patients with type 2 diabetes (T2D), a chronic metabolic disorder, are at a higher risk of developing DFU, with an estimated prevalence rate of 6.4%, as opposed to those with type 1 diabetes, who have a lower rate of approximately 5.5%. The prevalence of DFU varies significantly across different regions of the world, as per available data. Specifically, North America has the highest recorded incidence rate of 13%, followed by Africa at 7.2%. Meanwhile, relatively lower rates of 5.1% and 5.5% were reported in Europe and Asia, respectively. The prevalence of DFU in Oceania is the lowest among the regions mentioned above, with a rate of 3.0%[11-13]. Previous studies have also demonstrated that DFU increases the risk of death by 2.5-fold in diabetics[14,15].

The synthesis of iodine (I)-polyvinyl alcohol (PVA)@polydopamine (PDA) microspheres was documented in Yang *et al*'s study[16]. The aim was to attain computed tomography images, drug loading and controlled release capabilities, as well as improved embolization of the liver portal vein. The *in vivo* embolization findings demonstrated the presence of focal necrosis in hepatocytes, along with necrotic cell fragments and infiltration of inflammatory cells in liver tissue. These observations provided evidence that the I-PVA@PDA microspheres exhibit a more potent embolization effect compared to PVA particles. Additionally, the I-PVA@PDA microspheres were utilized for the delivery and controlled release of 5-fluorouracil, a chemotherapeutic drug. The results showed an initial rapid release (29.74% released) within the first 24 h, followed by sustained release (34.48%) over a period of 72 h. In Ouyang *et al*'s research[17], a multifunctional bio-hemostatic hydrogel (CODM) was prepared based on hydrogen bonding and Schiff base bonding by using modified alginate, polyvinylpyrrolidone (PVP), and carboxymethyl chitosan. The amino group-modified montmorillonite was uniformly dispersed in the hydrogel through amido bond formation with the carboxyl groups of carboxymethyl chitosan and oxidized alginate. The catechol group (-CHO) and PVP were able to form hydrogen bonds with the tissue surface, resulting in firm tissue adhesion and wound hemostasis. The addition of montmorillonite-NH2 further improved the hemostatic ability, surpassing that of commercial hemostatic materials. Furthermore, the photothermal conversion capability (derived from polydopamine) was synergized with the phenolic hydroxyl group, quinone group, and protonated amino group, effectively eliminating bacteria both *in vitro* and *in vivo*. In a recent review[18], it was reported that a multifunctional CH hyaluronic acid three-dimensional (3D) hydrogel possesses a notable capacity for water absorption. This property holds potential for its application in managing inflammatory bowel diseases, with a concentration on various aspects, such as adhesion, synergistic therapy, pH sensitivity, particle size, and temperature sensitivity. A desirable polymer hydrogel for hemostasis is expected to possess the following characteristics[18]: (1) It should exhibit a rapid gelation rate to promptly stop bleeding and promote active wound healing; (2) in dynamic and humid environments, the hemostatic hydrogel should demonstrate adequate adhesion and exceptional mechanical properties to effectively seal the wound and prevent the displacement of the hemostatic hydrogel from the bleeding site; and (3) it should exhibit favorable biocompatibility. Furthermore, it is important for the hydrogel to exhibit controllable swelling behavior as overly swollen hydrogels may exert pressure on the surrounding tissue.

The physiological mechanism underlying wound healing encompasses a series of coordinated events, primarily involving inflammation, angiogenesis, and extracellular matrix (ECM) remodeling. However, DFU is associated with an abnormal microenvironment with prolonged inflammatory cell infiltration and slow angiogenesis and ECM remodeling,

leading to impaired wound repair *via* the vascular and neurotrophic pathways. This in turn impedes local tissue regeneration and greatly reduces wound healing[19-22]. In addition, due to decreased granulocyte functions and chemotaxis, DFU cases are prone to infections[23-25]. The challenge of healing wounds in DFU is a multifaceted issue that arises from a combination of different factors. These factors include peripheral arterial disorders, which can impair blood flow and delivery of essential nutrients to the wound site, peripheral neuropathy, which can affect nerve function and lead to reduced sensation and poor healing response, foot deformities that can create pressure points and limit mobility, and also bacterial infections that can further complicate the healing process[26-29]. Wound debridement, which is the standard treatment for DFU and involves surgical removal of thickened, necrotic, damaged or infected tissues, has been widely used in clinical practice; the wound is then covered with dressing and/or treated with antibiotics to prevent infection[30-32]. A wound dressing that meets the ideal standards should facilitate a moist wound environment, shield against secondary infections, eliminate wound exudates, regulate biofilm formation, and stimulate tissue regeneration[33, 34]. However, none of the existing dressings are able to meet all of these requirements, and due to the influence of various factors, traditional treatments can no longer achieve satisfactory outcomes[35,36]. DFU patients continue to face significant clinical hurdles when it comes to wound healing. Despite numerous chronic wound management techniques and treatments having been developed, including gene therapy, growth factor therapy, stem cell therapy, and biomaterial application, successfully repairing these wounds remains a formidable task[37,38]. Because of their diverse characteristics that involve producing numerous growth factors, cytokines, and chemokines, as well as regulating immune responses, supporting the development of new blood vessels, and restructuring tissue, mesenchymal stem cells (MSCs) have demonstrated significant therapeutic capabilities in improving wound healing for cases with DFU[39-41]. Most of the current cell-based therapies are administered *via* systemic or subcutaneous injection of cells[42,43]. However, MSCs cannot be delivered to the wound *via* the systemic route, as cells are mainly retained in the lung or liver[44,45]. Although intradermal injection of MSCs into the wound was reported to significantly improve healing[46-48], despite the promising potential of MSC therapy, the effectiveness of this approach is still hampered by challenges, such as inadequate cell localization and compromised cell viability at the injury site[49]. To eliminate these problems, researchers have utilized delivery systems to deliver stem cells to the site of injury, and these delivery systems have been suggested to significantly improve stem cell viability and wound implantation rates. Furthermore, the delivery system scaffold also provides a 3D structure for stem cell migration, proliferation, and differentiation[50]. Figure 1 illustrates the summary of the main content of this review.

In the context of MSCs-based treatment of DFU, we reviewed the advancements made in preclinical and clinical application of various delivery systems, as depicted in Figure 2.

POTENTIAL MECHANISM OF ACTION OF MSC THERAPY FOR DFU

Stem cells exhibited diverse developmental potentials, enabling them to be classified into three categories: Totipotent stem cells (comprising embryonic stem cells), pluripotent stem cells, and specialized stem cells (*e.g.*, hematopoietic stem cells and neural stem cells). Furthermore, stem cells can be distinguished from embryonic and somatic stem cells based on their developmental stage. Embryonic stem cells are derived from embryonic and fetal tissues, while somatic stem cells are extracted from the organs or tissues of postnatal individuals. The two types of stem cells each have advantages. However, the utilization of embryonic stem cells is subject to restrictions for several reasons, including the ethical controversy surrounding their use for medical purposes and the presence of legal constraints limiting their application. In addition, the sources of embryonic stem cells are limited. *In vitro* technologies for amplification and purification are still in an early stage of development. The utilization of allogeneic transplantation of embryonic stem cells in individuals of different genetic backgrounds is accompanied by the potential hazards of immune rejection and teratoma formation. In contrast, somatic stem cells, including hematopoietic stem cells, neural stem cells, liver stem cells, and MSCs, possess relatively lower immunogenicity and reduced risk of tumorigenesis, rendering them more appropriate for various clinical applications. The choice of stem cell type for a particular therapeutic intervention should be based on several important factors, including their safety profiles, efficacy, and compatibility with the recipient's immune system[51]. Clinical and animal experiments have indicated that there are two striking biological features of somatic stem cells. First, once transplanted, somatic stem cells undergo chemotaxis and are recruited to the site of damage in massive numbers. Second, once they reach the site of damage, somatic stem cells undergo induced differentiation into cells essential for damaged tissue repair in the local microenvironment. Site-specific differentiation is one of the mechanisms by which somatic stem cells promote damaged tissue repair, and researchers have revealed that MSCs promote reconstruction of the local microcirculation by releasing cytokines and growth factors through paracrine and endocrine effects; this is the main mechanism by which these cells accelerate wound healing[52,53]. These findings may expand the indications for clinical treatment using somatic stem cells and facilitate the development of somatic cell and tissue engineering approaches. MSCs are nonhematopoietic stem cells resulting from mesoderm differentiation. They are adherent cells *in vitro* and can be massively expanded and differentiated into mesenchymal cells, also known as mesenchymal progenitor cells. The latter can be further differentiated into various connective tissue cells, including adipose cells, osteocytes, chondrocytes, vascular endothelial cells, osteoblasts, myoblasts, and nerve cells[54]. MSCs are a versatile cell population that can be obtained from various sources, including bone marrow, umbilical cord and blood, peripheral blood, fat, liver, gingiva, oral mucosa, amniotic fluid, as well as interstitial and connective tissues of organs. Due to the abundance of MSCs in these sources and their ability to differentiate into multiple cell types, they are valuable tools in regenerative medicine and tissue engineering research. One of the major advantages of MSCs is that their isolation does not pose ethical issues, unlike some other stem cells. Furthermore, studies have demonstrated that treatment with MSCs is safe and can lead to

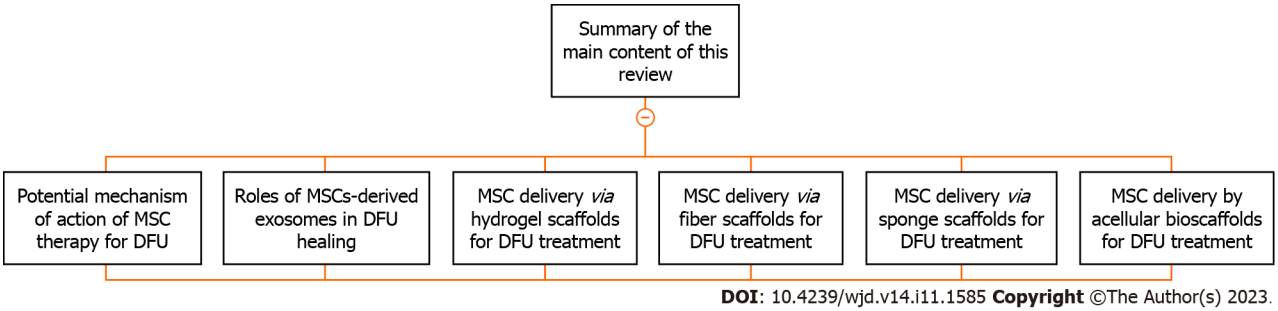

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Figure 1 The summary of the main content of this review. MSC: Mesenchymal stem cell; DFU: Diabetic foot ulcer.

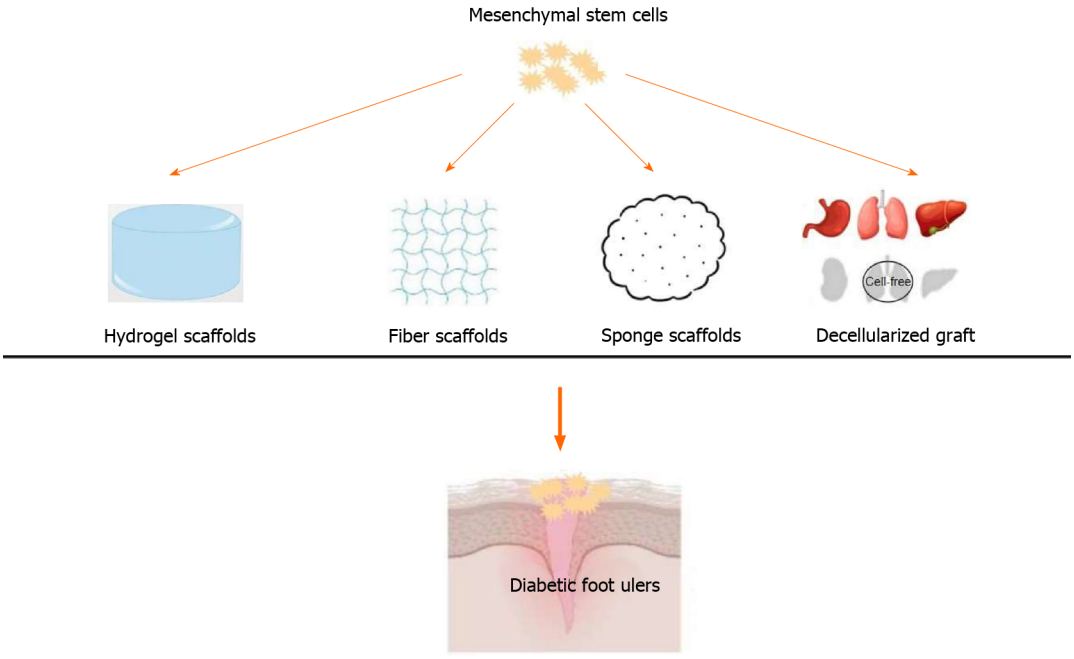

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Figure 2 Themesenchymal stem cells-based drug delivery system for the treatment of diabetic foot ulcer.

few side effects, providing another level of confidence in their use. The versatility and safety of MSCs make them an ideal candidate for utilization in tissue engineering investigations and clinical trials[55]. MSCs are easily isolated from different sources and have high proliferative potential and genetic stability. They migrate to damaged tissues, where they exhibit resistance to inflammation, influence the microenvironment, promote angiogenesis, and exert antifibrotic and antiapoptotic effects. Additionally, they release cytokines involved in damage repair and tissue regeneration, contributing to the healing process[56].

The intricate process of wound healing can be divided into four distinct overlapping phases, including homeostasis, inflammation, proliferation, and maturation. During the homeostasis stage, blood vessels in the affected area quickly constrict to minimize bleeding, while platelets form a plug to seal the injured site. During this stage, coagulation factors are activated, coordinating their efforts to form a fibrin clot that plays a vital role in stabilizing the wound. The inflammation phase is characterized by the influx of immune cells, such as neutrophils and macrophages, into the wound area. These cells are responsible for the removal of debris, pathogens, and damaged tissue. Additionally, they release growth factors and cytokines that initiate the subsequent phases of the healing process. In the proliferation stage, fibroblasts and endothelial cells start to proliferate and migrate into the wound bed, leading to the formation of new blood vessels and ECM. This stage also involves the deposition of collagen fibers, which provide structural support to the healing tissue. Finally, in the maturation phase, the newly formed tissue undergoes remodeling and maturation, resulting in a stronger and more organized scar. This process can take several months to complete, during which time the collagen fibers realign and cross-link to increase the tensile strength of the tissue[57]. MSCs exert therapeutic effects on DFU *via* several mechanisms. First, a key cause of failure of DFU healing is poor blood supply to the site of the ulcer and disrupted angiogenesis. MSCs, through their ability to secrete various growth factors, have been shown to enhance angiogenesis, referring to the process of forming new blood vessels from pre-existing ones. This effect is mediated *via* both autocrine and endocrine signaling pathways, leading to the upregulation of several growth factors that are crucial for promoting angiogenesis. Vascular endothelial growth factor (VEGF) is a critical growth factor that stands out among others because of its ability to stimulate the proliferation and differentiation of endothelial cells, leading to the formation of new blood

vessels. Basic fibroblast growth factor (bFGF), placental growth factor (PIGF), insulin-like growth factor 1 (IGF-1), and angiopoietin-1 (Ang-1) are also key players in promoting angiogenesis by inducing endothelial cell migration and proliferation. In addition to these growth factors, MSCs can also secrete stromal cell-derived factor-1 (SDF-1), erythropoietin, inducible nitric oxide synthase (iNOS), epidermal growth factor (EGF), and keratinocyte growth factor 2. These growth factors work synergistically to further enhance angiogenesis and promote wound healing. The ability of MSCs to increase the levels of these growth factors is particularly relevant in DFUs wherein impaired angiogenesis is a significant contributing factor to poor wound healing. By improving blood flow in the affected area, MSCs can significantly accelerate the repair of DFUs, thus offering a promising therapeutic option for this debilitating condition[58]. Second, MSCs are involved in immunoregulation *via* different pathways, and they can improve the microenvironment, reduce the inflammatory response and alleviate tissue injury[59]. (1) MSCs exert immunomodulatory effects by inhibiting T-cell activation[60]. T cells usually secrete a variety of proinflammatory factors after skin damage, delaying wound healing. MSCs secrete cytokines, including interferon-γ (IFN-γ), tumour necrosis factor(TNF)-α, interleukin (IL)-1α or IL-1β, and NOS, which inhibit T-cell activation. In addition, MSCs can block antigen-presenting cell maturation, thereby inhibiting the ability of T cells to respond and exert immunomodulatory effects; (2) MSCs inhibit proinflammatory T cells, and immunomodulatory effects are mainly mediated by Th17 cells and Treg cells[61]. One study showed that after the injection of bone-derived mesenchymal stem cells (BMSCs) into a mouse model of experimental allergic encephalomyelitis, Th17 cells were inhibited, accompanied by increases in the percentages of CD4+CD25+Foxp3+ Treg cells and IL-10-producing cells[62]. According to another report, MSCs modulate cytokine secretion by different T-cell subsets. Specifically, in experimental studies, it has been observed that the administration of MSCs results in a noticeable decrease in the secretion of certain proinflammatory cytokines, specifically IFN-γ and TNF-α. On the other hand, there is a concomitant increase in the secretion of anti-inflammatory cytokines, such as IL-4 and IL-10. Moreover, the percentage of Treg cells was reported to increase after MSC treatment[63]; (3) MSCs exert immunomodulatory effects by reducing the number of classically activated macrophages (M1-type, proinflammatory) and increasing the number of selectively activated macrophages (M2-type, anti-inflammatory)[64]. It has been shown that the coculture of MSCs and macrophages reduces the overall number of macrophages/monocytes, including M1 macrophages, but increases the percentage of M2 macrophages. In addition, MSCs induce M2 polarization of macrophages to exert immunomodulatory effects, enhancing wound repair; and (4) MSCs exert immunomodulatory effects by reducing reactive oxygen species (ROS) levels[65]. In damaged tissues, macrophages engulf bacteria, apoptotic inflammatory cells or cell fragments, thereby killing pathogens and eliminating other harmful factors. However, the prolonged presence of neutrophils after phagocytosis usually results in massive production of ROS, which ultimately causes a respiratory burst and tissue injury. MSCs prevent excessive or improper activation of oxidative metabolism in neutrophils, while preserving the phagocytic ability of neutrophils. MSCs also inhibit neutrophil apoptosis, reducing ROS generation and the intensity of the respiratory burst. In summary, MSCs have exhibited to exert immunomodulatory effects, leading to the alleviation of inflammatory responses and tissue injury, as well as the promotion of wound healing. Another important characteristic of MSCs is their ability to self-replicate and differentiate into different types of mature cells that possess distinct morphology, specific molecular markers, and specialized functions. This multidirectional differentiation potential allows for the generation of a diverse range of cell types, which has significant implications for regenerative medicine and other therapeutic applications involving tissue repair and regeneration[66]. MSCs can be divided into endothelial cells and keratinocytes that are involved in injury repair. Following transplantation of MSCs, there is a notable rise in the levels of angiogenic factors such as IGF-1, EGF, and IL-8. Moreover, the expression of keratinocyte-specific proteins and cytokeratin in wounds leads to the significant proliferation of various cell types, including epithelial cells and keratinocytes. These proteins expressed in wounds facilitate angiogenesis, epithelial cell regeneration, and wound healing. According to another study, in a rat model of DFU, MSCs were specifically localized to target ulcers, where keratin 19 secretion, formation of keratinocytes and ECM, and epithelial cell regeneration were promoted[67]. MSCs show promise for the treatment of DFU, and some encouraging results have been obtained from clinical trials. Further optimization is needed in terms of the following aspects of treatment with MSCs: The feasibility of treatment using autologous and allogeneic MSC transplantation in patients with DFU, factors related to transplantation efficiency, the standardization of MSC quality detection methods and assessment criteria, MSC delivery systems, and methods to determine the survival rate of transplanted MSCs and the effectiveness and long-term efficacy of MSC transplantation. MSC therapy has potential for promoting tissue regeneration and healing in DFU through the differentiation of MSCs into various cell types and the release of growth factors and cytokines. Furthermore, MSC therapy can enhance angiogenesis and blood vessel formation, increasing blood flow to the ulcerated area and promoting healing, while also preventing infections and reducing the need for antibiotics through its antimicrobial effects. Overall, these mechanisms suggest that MSC therapy may be a promising approach for treating DFU, and MSCs can provide neuroprotection by promoting nerve regeneration and reducing neuropathic pain associated with DFU. MSCs possess the capacity to produce and release neurotrophic factors, such as nerve growth factor and brain-derived neurotrophic factor, which are potent mediators of nerve growth and survival. The secretion of these factors by MSCs can contribute to the repair and regeneration of damaged nerve tissues by promoting neuronal cell proliferation and differentiation, enhancing axonal sprouting and myelination, and reducing neuronal apoptosis.

ROLES OF MSCS-DERIVED EXOSOMES IN DFUHEALING

Medical professionals have been perplexed by the limited efficacy of MSCs in promoting wound healing. Low survival and proliferation rates of MSCs due to micro-environmental factors, such as ischemia, hypoxia, and inflammation, further affect the efficacy of MSCs-based treatment. An increasing body of research suggests that the transplantation of MSCs

facilitates wound healing through two distinct mechanisms. One way is through direct differentiation, where MSCs differentiate into specific cell types such as fibroblasts, myofibroblasts, and endothelial cells, all of which contribute to tissue repair and angiogenesis. The other mechanism involves a paracrine effect, in which MSCs release various bioactive molecules, such as growth factors and cytokines, promoting the proliferation and migration of nearby cells involved in wound healing[68]. MSCs-derived exosomes act as mediators that deliver membrane receptors, proteins, mRNAs and microRNAs to receptor cells. Due to modulatory effects, gene expression and protein translation undergo changes in receptor cells, thereby influencing the biological activity of target cells. Exosome-based treatment provides a promising approach to overcome various limitations associated with stem cell-based treatment. These limitations include the challenge of large cell volume impeding capillary flow, the low dose and potency of stem cells, the potential presence of mutations or damaged DNA in stem cells, their potential to impair immunocompetence and the immune response, as well as their tendency to exhibit poor differentiation. Exosomes are paracrine products of stem cells and exert similar effects as stem cells. Exosomes are involved in a series of important processes during wound healing, including inflammatory regulation, angiogenesis, epithelial regeneration, and collagen deposition. Bone marrow is the most common site for harvesting MSCs. The utilization of BMSCs that exert their therapeutic effects through paracrine exosomes has been the subject of extensive investigation in the treatment of DFU. For instance, Wang *et al*[69] conducted a comprehensive analysis of the effects of exosomes originating from BMSCs on the tube-forming capabilities of endothelial progenitor cells (EPCs). Their results highlighted that BMSCs-derived exosomes exerted a significant modulatory influence on Nrf2, which ultimately led to reduced wound inflammation. Consequently, the exosomes played a vital role in promoting wound healing, re-epithelialization, collagen deposition, and angiogenesis in diabetic rats. Ding *et al*[70] carried out subcutaneous injection of exosomes into skin wounds on the backs of diabetic rats. Following the transplantation of exosomes, the wound healing rate was notably higher in the group after 7 and 14 d*vs* that in the control group. The findings suggest that exosomes derived from BMSCs triggered the PI3K/AKT signaling pathway *via* miRNA-126-mediated PTEN downregulation, leading to proangiogenic characteristics both *in vivo* and *in vitro*. Furthermore, adipose-derived stem cells (ADSCs) also facilitate the repair of diabetic ulcers. ADSC-derived exosome transplantation may be a new method for treating DFU. A group of researchers conducted a study in which they extracted exosomes from ADSC-conditioned medium and combined them with EPC cells. Their findings indicated that the exosomes derived from ADSCs had a regulatory impact on LINC00511. This, in turn, impeded Twist1 ubiquitination and degradation induced by PAQR3. Ultimately, this process encouraged the proliferation, migration, and angiogenesis of EPCs, thereby accelerating the healing of DFUs[71]. Li *et al*[72]revealed that ADSC-derived exosomes inhibited ROS and inflammatory cytokine production, thereby inhibiting EPC aging induced by high glucose and an oxidative microenvironment. Other effects included enhancing EPC viability, proliferation, and angiogenesis capacity and improving vascularization. Moreover, it was suggested that Nrf2-overexpressing ADSC-derived exosomes facilitated diabetic wound healing by enhancing collagen deposition, tissue fibrosis, and micro-angiogenesis. The mechanism may involve a promoting effect on vascularization and growth factor release and mitigation of the oxidative stress response. In addition, researchers have demonstrated the influences of human umbilical cord MSCs-derived exosomes on DFU healing. For instance, Hu *et al*'s research involved investigating the effects of exosomes derived from human umbilical cord MSCs on promoting angiogenesis and fibroblast functions, leading to improved skin wound healing. This was achieved through PTEN inhibition by miR-21-3p and SPRY1, which resulted in enhanced healing of skin wounds[73]. Liu *et al*[74] conducted a study to determine the efficacy of MSCs derived from human umbilical cords in improving wound healing and angiogenesis in a rat model of deep second-degree burn wounds. They observed a higher rate of wound closure and increased expression of CD31 *in vivo*. Furthermore, they found evidence suggesting that these MSCs facilitated the proliferation, migration, and tube formation abilities of human umbilical vein endothelial cells *via* exosome-mediated secretion of Ang-2. Despite these findings, there is a dearth of studies comparing the therapeutic potential of exosomes derived from MSCs of varied tissues in treating DFU, highlighting the need for further research in this area. There are still many problems to be resolved, such as the source and type of cells, isolation technique, dosage, transplantation method, and amplification method for MSCs-derived exosomes. Preclinical and clinical studies with large sample sizes are still needed in the future. Exosomes derived from MSCs have emerged as critical players in the process of wound healing acceleration and promotion for individuals afflicted with DFU. These minute vesicles are laden with a plethora of bioactive molecules, including growth factors, cytokines, and microRNAs, that intricately regulate multiple cellular processes crucial to the wound healing cascade. Their multifaceted mechanisms of action make them an attractive therapeutic avenue for DFUs, as they not only promote angiogenesis and cell proliferation, but also modulate inflammation and ECM remodeling. Overall, MSCs-derived exosomes offer a promising approach to treat DFU, as they provide a safe and effective alternative to the use of whole cells. They can be easily obtained from MSCs through noninvasive techniques, and their administration has minimal risks compared with the use of whole cells (Figure 3)[75].

MSC DELIVERY *VIA* HYDROGEL SCAFFOLDS FOR DFU TREATMENT

Hydrogels are complex structures made up of a mesh-like network of polymer chains that are chemically linked together. This unique arrangement allows hydrogels to absorb vast amounts of water, up to hundreds of times their own weight, while still maintaining their structural integrity. The implantation of hydrogels has been demonstrated to enhance surface cytocompatibility, antibacterial properties, and the preservation of cell viability at the targeted site, reflecting their capability in alleviating wound healing[76,77]. Naturally derived hydrogels are biocompatible and biodegradable, interact with innate immune cells, and they are structurally similar to natural human tissues[78-80]. However, there has been a growing interest in the development of hydrogels with tailored properties and performance. However, studies

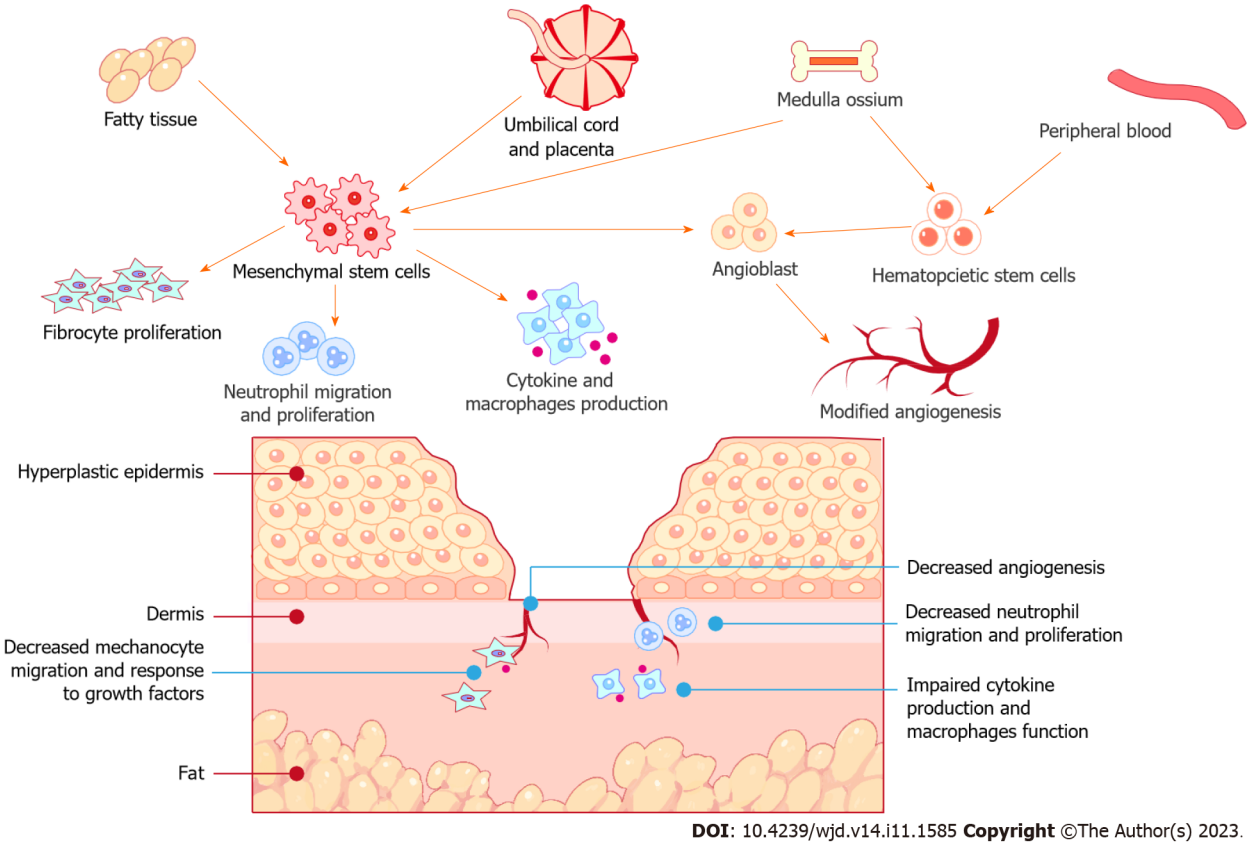

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Figure 3 Illustration of mesenchymal stem cells-derived exosomes in the healing of diabetic foot ulcer[75].

have demonstrated that natural hydrogels possess a limited set of mechanical characteristics and tend to have significant fluctuations in properties between batches[81,82]. This limitation has led to a shift towards the use of synthetic hydrogels, which offer numerous advantages over their natural counterparts. Synthetic hydrogels have significantly longer lifetimes, higher water absorption capacities, and greater gel strengths compared to natural hydrogels[83,84]. Furthermore, they are known to be stable even under severe temperature fluctuations, making them ideal for a wide range of applications[85, 86]. To capitalize on the unique properties of both types of hydrogels, composite hydrogels that combine natural and synthetic components have emerged as a promising alternative. These composite hydrogels offer the possibility of creating materials with controllable and customizable structures and functions, thereby expanding the range of potential applications[87,88]. Composite hydrogels are emerging as promising materials for tissue engineering due to their ability to be engineered with specific properties such as size, shape, surface activity, biodegradability, and biocompatibility. By carefully tailoring these characteristics, hydrogel scaffolds can provide a precise mechanical and biological environment to support cell growth and tissue regeneration[89]. New research has revealed that the use of type I rat tail collagen hydrogel to deliver murine BMSCs and adipose-derived MSCs (ADMSCs) could noticeably modulate immune and inflammatory responses at wound sites. This could be attained *via* upregulating the expression levels of growth factors, including VEGF, as well as by attracting macrophages, which play a crucial role in tissue repair. This approach has been particularly successful in promoting wound healing in diabetic mice, where impaired immune function and chronic inflammation mainly hinder the natural healing process[90-93]. It has been demonstrated that collagen hydrogels can successfully deliver MSCs to the wound site and improve healing. Murine ADMSCs delivered *via* a hyperbranched polyethylene glycol diacrylate-cross-linked gelatin hydrogel exhibit superior cell adhesion and are viable and metabolically active for 3 wk[94,95]. Db/db diabetic mice that are injected with an ADMSC-loaded hydrogel at the wound surface have a significantly improved cell retention rate in the wound, accelerated wound closure, enhanced angiogenesis, and attenuated inflammation[96]. In an effort to promote effective wound healing in the context of diabetes-induced impaired healing, innovative methods for delivering beneficial cells and substances have been investigated. Specifically, studies have revealed that the utilization of a biodegradable n-isopropylacrylamide thermo-sensitive hydrogel to deliver mouse BMSCs could remarkably improve wound healing in db/db mice. This delivery method has been demonstrated to enhance ECM deposition, angiogenesis, re-epithelialization, and granulation tissue formation within wounds. Additionally, it has exhibited the ability to regulate polarization of M1 and M2 macrophages at the wound site[97,98]. Additionally, the utilization of Pluronic F-127, a synthetic biocompatible hydrogel with unique thermo-sensitivity, offers an effective means of encapsulating and delivering numerous rat ADMSCs to the wound site. Such delivery has been found to stimulate angiogenesis and cell proliferation, ultimately leading to expedited wound healing in diabetic rats. These findings suggest the potential for novel therapeutic approaches utilizing these delivery methods to improve healing outcomes in individuals with diabetes-induced impairment[99,100]. Moreover, umbilical MSC implantation withPF-127 and sodium phosphate promotes wound healing and angiogenesis as well as improves dermal regeneration

and collagen deposition in diabetic rats[101]. Diabetic rats that received ADMSCs encapsulated in silk fibroin/chitosan hydrogel exhibited significantly increased re-epithelialization, granulosa tissue formation and capillary formation at the wound site 7 d after treatment[102]. Furthermore, the expression levels of epithermal growth factor (EGF), TGF-β, and VEGF were also upregulated in the wound tissues on day 14 post-treatment. Utilizing hydrogels composed of hyaluronic acid and N-carboxyethyl chitosan cross-linked by adipic acid dihydrazide as a delivery mechanism, rat BMSCs have been found to effectively inhibit chronic inflammation, promote granulosa tissue formation, collagen deposition, nucleated cell proliferation, and stimulate angiogenesis in diabetic rats. As a result, these hydrogels can significantly enhance wound healing outcomes in this population[103]. Efforts to improve wound healing outcomes in individuals with diabetes-induced impairment have spurred innovative research in the field of cell delivery mechanisms. Researchers have discovered that delivering rabbit BMSCs *via* a nitric oxide-releasing S-nitroso-N-acetyl-penicillamine-loaded chitosan/polyvinyl-alcohol hydrogel can significantly enhance wound healing rates, re-epithelialization, and collagen deposition in diabetic rabbits, as described in literature sources[104-106]. Jin *et al*[107] developed an injectable hydrogel with unique properties such as suitable electrical conductivity and sustained hypoxia that can upregulate HIF-1α and connexin-43 expression in loaded ADMSCs, ultimately facilitating wound closure in diabetic rats. This hydrogel has been found to enhance angiogenesis, promoting the reconstruction of blood vessels, hair follicles, and dermal collagen matrix, further contributing to improved wound healing outcomes[107]. Srifa *et al*[108] conducted a study wherein they administered VEGFA-overexpressing human BMSCs to wounds in db/db mice either through direct injection or embedding them within a HyStem HP hydrogel. The researchers discovered that both methods of cell delivery enhanced the rate of wound healing; however, between days 7-9 after treatment, the hydrogel group exhibited significantly better wound healing compared to the direct injection group[108]. In a phase II clinical trial of MSC delivery *via* a hydrogen scaffold (NTC02619877), the authors developed an allogeneic ADMSC hydrogel sheet that can maintain long-term stability under cryopreservation and has been approved for marketing by the Ministry of Food and Drug Safety of South Korea (Approval No. ALL-ASC-DFU-201). This trial showed that 82% of diabetic patients had complete wound closure at week 12 after receiving the allogeneic ADMSC hydrogel sheet compared with 53% of controls, and adverse reactions were not observed after treatment, demonstrating that ADMSC delivery *via* the hydrogel is effective and safe for diabetic wound healing[109]. An in-depth case study was undertaken to explore the implications of utilizing sodium alginate hydrogel-encapsulated placenta-derived MSCs as a topical treatment for foot ulcers in patients with T2DM. The findings were exceptionally promising, with complete wound healing observed three weeks post-treatment, along with marked improvements in foot pain and minimal toxicity. Furthermore, no recurrence was noted during the six-month follow-up period[110]. However, as it was a case study, further investigation is required.

At present, MSC delivery systems supported by hydrogels meet the need for local controlled release and create a 3D bionic environment. Hydrogel scaffolds not only increase bioavailability and antibacterial capacity by improving transport dynamics, but also provide a moist and stable wound repair environment for damaged ulcers, promoting synergy between skin cells and cytokines. Functional materials should be optimized in terms of the degree of crosslinking, porosity, swelling property, mechanical performance, cell adhesion, permeability, toxicity and cost efficiency. They should be synthesized with optimal healing strength to best mimic the ECM microenvironment to maintain the features and activity of each component. The development of hydrogel materials as MSC delivery systems or scaffolds holds significant potential for the future advancements in this field. In recent years, increasing research on the complex dynamics of biological systems has improved hydrogel material properties and hydrogel preparation methods and led to continual optimization of the interactions between organisms and scaffold materials. It can be concluded that the use of hydrogel scaffolds for the delivery of MSCs can significantly improve the therapeutic potential of MSCs-based treatments for DFU. Hydrogels offer a 3D framework that maintains the viability, proliferation, and specialization of cells, and simultaneously enables regulated discharge of growth factors that stimulate recovery of wounds and regeneration of tissues. Additionally, the mechanical properties of hydrogels can be modified to match the specific needs of DFU treatment, such as flexibility to accommodate weight-bearing or stiffness to support tissue regeneration. Overall, MSC-hydrogel therapies can effectively reduce inflammation, stimulate angiogenesis, and improve wound closure rates in human/animal models of DFU (Figure 4)[111]. However, there is an expectation for the development and application of a wider range of functional hydrogels that can perform distinct functions to facilitate DFU repair.

MSC DELIVERY VIA FIBER SCAFFOLDS FOR DFU TREATMENT

Fiber scaffolds are 3D structures primarily composed of micro- or nanoscale fibers prepared by electrospinning to simulate the structure of natural human tissues[112]. The utilization of fiber scaffolds has been observed in multiple domains of tissue engineering, such as bone, cartilage, skin, vascular, and neural tissue engineering[113-115]. The notable surface-to-volume ratio of fiber scaffolds provides an ideal setting for cell adhesion, although the limited pore size may pose a challenge to cell migration. Consequently, the properties of fiber scaffolds should be tailored based on the specific cell type being cultured[116,117]. The employment of fiber scaffolds for the purpose of wound healing has recently garnered significant attention, as they have demonstrated a remarkable potential in promoting cell-cell and cell-ECM interactions, while also directing the functions and behaviors (*e.g.*, cell morphology, proliferation, and differentiation) of diverse cells, including MSCs[118-121]. In the realm of diabetes wound healing, the utilization of fiber scaffolds for MSC transportation has been extensively employed. Chen *et al*[122] have devised a 3D scaffold that is comprised of vertically or radially aligned nanofibers that can be customized to fit the size, depth, and configuration of different T2D wounds. The scaffold itself possesses an impressive ability to regain its shape both in water and the atmosphere, even after undergoing compression. When infused with BMSCs, this 3D fiber scaffold has the potential to stimulate the

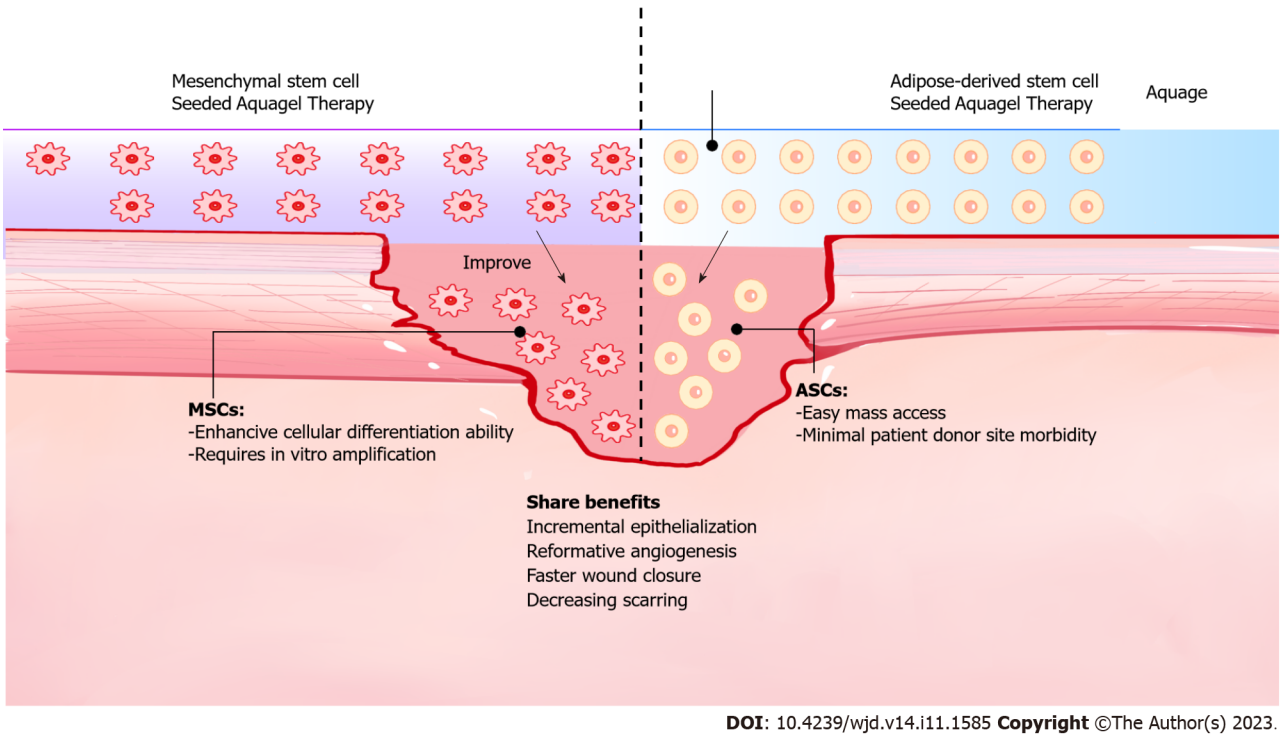

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Figure 4 Delivery of mesenchymal stem cells and adipose-derived stem cells by hydrogel scaffolds for diabetic foot ulcer[111]. MSC: Mesenchymal stem cells; ASC: Adipose-derived stem cells.

development of granulosa tissue, encourage angiogenesis, and facilitate collagen deposition within the T2D wound[122, 123]. Furthermore, Hou *et al*[124] developed a novel electrospun nanofibrous scaffold, which was composed of 80% polylactic acid, 10% silk, and 10% collagen. The scaffold was designed to deliver HO-1-overexpressing human BMSCs to wounds in diabetic mice. This hybrid scaffold has exhibited promising results in improving wound healing in diabetic mice. The authors found that this approach significantly improved angiogenesis and wound healing *via* the Akt signaling pathway[124]. He *et al*[125] delivered human BMSCs that overexpressed neurotrophic factors to wounds in diabetic mice *via* an electrospun biomaterial, and this method was found to significantly accelerate wound closure and increase angiogenesis[125]. In addition, delivery of human ADMSCs to wounds *via* silk fibroin scaffolds led to faster complete wound closure in db/db mice (d10) than in control mice (d15-17)[126-128]. It is easy to manipulate fiber scaffolds, but their preparation is very complicated. The dimensions and morphology of fiber scaffolds are affected by many factors, including solution viscosity, voltage, temperature, humidity, the distance between receiver and nozzle, and the loading flow rate of the solution. Ideal nanoscale fiber scaffolds can be fabricated only by systematically optimizing the above parameters. Fiber scaffolds offer a favorable setting for the growth and differentiation of MSCs, facilitate the regulated release of growth factors, and assist in the process of wound healing. Thus, MSC delivery *via* fiber scaffolds can improve wound closure rates, boost angiogenesis, reduce inflammation, and potentially offer better outcomes than other platforms for MSC delivery. Additional research is essential to enhance the design and production of fiber scaffolds for delivering MSCs, develop universally recognized procedures, and assess the enduring safety and efficacy of this technique.

MSC DELIVERY VIA SPONGE SCAFFOLDS FOR DFU TREATMENT

Scaffold sponges, which are widely used in tissue engineering and regenerative medicine, can be fabricated using a diverse range of techniques, involving porogen leaching, gas foaming, and freeze-drying. These methods enable the creation of scaffolds made from natural or synthetic polymers that possess a high degree of porosity and a uniform network of interconnected pores[129-131]. Despite the longstanding use of sponge scaffolds in the biomedical field, researchers have long been striving to generate an environment that can provide support for the ECM in autologous cells and tissues. A 3D system with the ability to modulate cell viability and customize the structural and architectural properties, such as porosity, pore size, and interconnected dimension offers a significant degree of freedom. These features synergistically contribute to the regulation of cell-material interactions and consequently promote tissue growth within the scaffold gap[132,133]. Sponge and hydrogel scaffolds mainly differ in their method of fabrication, which results in differences in the water content of the scaffold. In contrast to hydrogels, the creation of sponge scaffolds is a time-consuming process that necessitates surface and structural modifications based on the type of cell and host tissue being used, as stated in the original citation. However, sponge scaffolds offer several potential benefits for skin wound healing. First, their highly porous structure closely resembles that of the ECM, which aids in supporting cell migration to the site of injury[134-136]. Second, because of their water absorption and retention capabilities, sponge scaffolds can

absorb exudates from the wound site, providing a favorable environment for cell proliferation and migration[137-139]. The utilization of MSC scaffolds for diabetic wound healing frequently involves the use of sponge scaffolds made with collagen and chitosan. To create a collagen sponge scaffold, O'Loughlin *et al*[140] utilized freeze-drying techniques. Delivery of allogeneic BMSCs *via* topical application of the collagen sponge scaffold resulted in superior wound closure and angiogenesis on day 7 following implantation in diabetic rabbits when compared to the no treatment control group [140,141]. Tong *et al*[142] developed a collagen-chitosan sponge scaffold that is suitable for BMSC delivery by employing cross-linking and freeze-drying techniques, as mentioned in the original citation. This sponge scaffold has a 100 μm pore network and appropriate biodegradability and swelling ratio[142]. This type of scaffold creates an environment that is favorable for cell growth and stimulates hypoxia-pretreated rat BMSCs to produce higher levels of VEGF and platelet-derived growth factor, as well as upregulate expression of key transcription factors, including HIF-1α, while retaining cell viability. In STZ-induced diabetic rats, the BMSC-sponge scaffold group exhibited significantly improved wound closure, increased angiogenesis, and reduced inflammation (upregulated *IL-10* gene and protein expression on days 7 and 14 post-implantation) *vs* the control group. Furthermore, Ní Annaidh *et al*[143] fabricated a sponge scaffold made of collagen and chitosan that was infused with simvastatin. The scaffold had high porosity, with pore sizes ranging from 20-200 μm, and possessed sufficient mechanical strength while maintaining elasticity similar to human skin. Additionally, the release of simvastatin from the scaffold could be controlled[143-145]. It was previously shown that delivery of rat ADMSCs by a sponge scaffold made of glycol chitosan and polyurethane combined with acupuncture had a synergistic immunomodulatory effect on wounds in mice with STZ-induced diabetes. This combination therapy improved wound closure and promoted complete re-epithelialization within 8 d, in contrast with ADMSCs alone[146-150]. In addition, on day 8 after treatment, the wound displayed an increase in secretion of SDF-1 and TGFβ-1, while production of TNF-α and IL-1β was reduced. Additionally, sponge scaffolds have the potential to serve as a cell delivery system in conjunction with growth factors. Delivery of Balb/c mouse BMSCs by chitosan-alginate sponge scaffolds combined with EGF can enhance cell viability and transcription factor expression, maintain MSC pluripotency and self-renewal capability, and promote collagen deposition and angiogenesis by increasing granulosa tissue formation in the wounds of diabetic rats[151-154]. De Francesco *et al*[155] conducted a study to assess how effective autologous dermal micro-grafts, similar to MSCs, could be in treating DFUs by delivering them through collagen sponge scaffolds. The dermal micro-grafts were obtained through mechanical dissociation of small pieces of skin tissues and express MSC markers (*e.g.*, CD34, CD73, CD90, and CD105) *in vitro*. The results showed that the micro-grafts remained viable and proliferative in the collagen scaffold, indicating that MSC-loaded sponge scaffolds could remarkably improve ulcer wound closure and enhance patients' quality of life[155-157].

A pore size of a few hundred microns is usually considered most suitable to ensure that cells obtain the needed nutrients. In addition, the porosity is generally above 70%, which provides enough space for cell penetration and mass transfer. Initial cell attachment is also guaranteed by the adequacy of the materials. The size of interconnections is a crucial factor that affects the transport characteristics of the entire porous structure. There seems to be a consensus that the minimal interconnection size is approximately 50 μm to allow angiogenesis and cell migration. The regulation of cell differentiation and function can be influenced by physical parameters, involving material hardness, viscoelasticity, and pore curvature. However, it is important to acknowledge the significant role that endogenous factors play in promoting complete cell maturation. Sponge scaffolds have demonstrated their effectiveness as delivery vehicles for MSCs, providing a supportive environment for cell growth and promoting tissue regeneration at the site of the ulcer. The utilization of sponge scaffolds has the potential to regulate the immune response and alleviate inflammation at the site of injury, thereby facilitating the process of wound healing. MSCs delivered by sponge scaffolds can improve angiogenesis and blood vessel formation, increasing blood flow to the ulcerated area and promoting healing. The controlled release of MSCs from sponge scaffolds can provide sustained therapeutic effects over time, reducing the need for frequent treatments. Compared to other methods of MSC delivery, the use of sponge scaffolds may improve patient compliance and reduce the risk of infection. The potential of MSCs delivered *via* sponge scaffolds to promote DFU wound healing is notable. These natural polymer-based scaffolds provide a 3D environment that supports MSC survival, proliferation, and differentiation, facilitating interactions with surrounding tissues. Overall, compared with the delivery of MSCs *via* a silk fibroin sponge scaffold, utilizing chitosan-based sponge scaffolds for MSC delivery could potentially lead to faster healing rates and increased collagen deposition. Moreover, significant reduction in ulcer size and improvement in wound closure time could also be noted.

MSC DELIVERY BY ACELLULAR BIOSCAFFOLDS FOR DFU REPAIR

Acellular bioscaffolds refer to biological substances that are derived from human or animal organs or tissues, which undergo decellularization techniques for the removal of immunogenic cellular components[158-161]. Acellular bioscaffolds have exhibited favorable results in diverse tissues and organs and have garnered noticeable interest in the domain of tissue engineering. Mechanical (freezing or force), chemical (acid or Triton), and enzymatic (trypsin or pepsin) methods are decellularization techniques that can be employed. While combining multiple techniques is often more effective than using a single method, it is crucial to choose the appropriate decellularization approach based on the distinctive features of each tissue type[162]. Acellular bioscaffolds are composed mainly of ECM and other extracellular macromolecules (*e.g.*, collagen, elastin, fibronectin, laminin, and stromal cell proteins). They possess the unique characteristic of being nonimmunogenic 3D structures, distinguishing them from other synthetic scaffolds[160]. These characteristics are critical for the identification and development of implantable scaffolds for diabetic wounds. The utilization of acellular bioscaffolds has numerous benefits in treating diabetic wounds, involving the ability to replace damaged ECM

with a variety of proteins (*e.g.*, collagen, glycosaminoglycans, proteoglycans, and glycoproteins). Additionally, acellular bioscaffolds facilitate the infiltration of host cells and regulation of immune responses. They also promote angiogenesis and granulation tissue formation[163,164]. There are currently few commercially available acellular bioscaffolds for wound healing[165,166]. These scaffolds are manufactured differently and hence have differentmechanical properties and varying abilities to support skin regeneration[167]. Several studies have examined the delivery of MSCs by acellular bioscaffolds for the treatment of diabetic wounds. Shi *et al*[168] developed a decellularized dermal matrix scaffold, called book-shaped decellularized dermal matrix (BDDM), closely resembling native dermal tissues in terms of histology, microstructure, and composition. This noncytotoxic scaffold exhibited low immunogenicity and supported the attachment and proliferation of ADMSCs. The researchers also synthesized a recombinant growth factor, collagen-binding domain (CBD)-bFGF, by fusing a CBD-bFGF, and tethered it to the collagen fibers of the BDDM scaffold. This was resulted in the creation of a functional scaffold (CBD-bFF/BDDM), promoting endothelial cell inducibility more effectively. *In vitro* tests revealed that CBD-bFGF/BDDM scaffold can gradually release tethered bFGF and facilitate ADMSC interactions until endothelial differentiation is achieved. To evaluate the effectiveness of this scaffold, ADMSCs were cultured to create a cell sheet which was placed between PIGF, CBD-bFGF and BDDM before being transplanted into diabetic rats. Results from *in vivo* experiments showed that the implantation of ADMSC-loaded CBD-bFGF/BDDM scaffold promoted the formation of granulation tissue and angiogenesis. It also facilitated collagen deposition and remodeling. Zhang *et al*[169] developed a novel delivery system for exogenous cells using nanoparticles encapsulating IL-8 along with polylactic-co-glycolic acid (PLGA) loaded onto acellular matrix insulin-like growth factor 1. This efficient delivery medium, termed PLGA@IL-8/ADM, was found to promote significant proliferation and endothelial differentiation of the MSCs while increasing their survival rate. Moreover, PLGA@IL-8/ADM scaffold loaded with MSCs facilitated capillary construction, collagen deposition, and Ang-1 wound healing in skin wounds of mice with STZ-induced diabetes, thereby demonstrating its effectiveness as a therapeutic intervention for diabetic wounds. These findings highlight the promise of the PLGA@IL-18/ADM scaffold as a novel delivery system for exogenous cells that can aid in tissue regeneration. Chu *et al*[170] conducted a study, in which they loaded mouse BMSCs onto a decellularized dermal matrix scaffold obtained from normal mouse skin and applied it at full-thickness cutaneous wound sites in diabetic mice. The use of MSC-ADM for treating these wounds resulted in a noteworthy increase in the percentage of wound closure, a boost in type I collagen fiber synthesis, and an acceleration of both angiogenesis and re-epithelization [170-172]. Moreover, a cell delivery platform comprised of acellular dermal matrix and reduced graphene oxide has high stability and promising mechanical properties. Upon delivery of murine BMSCs to wounds in diabetic mice, this acellular bioscaffold provides a favorable milieu for BMSC adhesion and proliferation, promotes angiogenesis and collagen deposition, and accelerates wound healing[173,174]. A study using acellular dermal matrix to deliver human umbilical cord MSCs showed that MSC proliferation and differentiation were regulated by activation of Wnt signaling, which ultimately promoted wound healing in diabetic rats[175].

Matrix formed by the removal of cells from dermal tissues should be used to deliver MSCs to wounds, thereby resolving immune rejection of allografts. Research priorities related to the use of decellularized vascular bioscaffolds for MSC delivery and wound repair include cell growth promotion, vascularization, and appendage regeneration, which may represent the future of DFU treatment.

Acellular bioscaffolds have demonstrated their efficacy as a successful delivery platform for MSCs, facilitating a supportive environment for cell growth and driving tissue regeneration at the site of the ulcer. The use of acellular bioscaffolds can modulate the immune response and reduce inflammation at the wound site, aiding in the healing process. MSCs delivered by acellular bioscaffolds can improve angiogenesis and blood vessel formation, increasing blood flow to the ulcerated area and promoting healing. The controlled release of MSCs from acellular bioscaffolds can provide sustained therapeutic effects over time, reducing the need for frequent treatments. The use of acellular bioscaffolds may improve patient compliance and reduce the risk of infection compared with other methods of MSC delivery. Overall, MSCs can promote wound healing by secreting cytokines and growth factors, reducing inflammation, and stimulating angiogenesis. However, delivering MSCs directly to the site of DFU wounds can be challenging due to low survival rates and poor engraftment. Using an acellular bioscaffold as a delivery vehicle could potentially improve the viability and functionality of transplanted MSCs and enhance their therapeutic effects for DFUs. The bioscaffold provides a 3D microenvironment similar to the natural ECM, allowing for cell attachment and migration, promoting angiogenesis, and increasing nutrient and oxygen availability.

CONCLUSION

In conclusion, the increasing incidence of nontraumatic amputation and the poor therapeutic effect of currently available treatments make DFU one of the most important clinical challenges[176]. MSCs are widely utilized in the treatment of DFU, however, their efficacy needs to be improved. The application of different MSCs-based drug delivery systems for DFU and the relevant mechanisms were discussed (Table 1). Several preclinical investigations have exhibited impressive results, indicating that diverse MSCs-based drug delivery mechanisms can expedite wound healing and stimulate skin regeneration in DFU. However, there are still limited clinical data regarding the utilization of MSCs-based drug delivery systems for treating DFU. There is no consistent correlation between the results obtained in animal and human models. The safety, efficacy, and cost of different MSCs-based drug delivery systems should be deeply investigated in the future research. An interdisciplinary approach is required to develop cells-based drug delivery systems for the clinical treatment of DFU.

Table 1 Summary of four mesenchymal stem cells-based drug delivery systems for diabetic foot ulcer

Scaffold	Advantage	Disadvantage
Hydrogel	Excellent biocompatibility and biodegradability[33-36], low cytotoxicity[74-79], with similar structure as human tissues[77-82], longer lifetime, higher water absorption capacity[90-95], and greater gel strength[107-111]	Weak mechanical strength and high batch-to-batch variability[161-165]
Fiber	Well-mimicking the human tissues and excellent cell attachment[112-118]	Small pore size limiting cell migration[46-48]
Sponge	Appropriate microenvironment for cell attachment, migration, and nutrient transition[59-63]; Exceptional ability of loading, retaining and releasing of fluids [103-107]	Longer time for fabrication procedure[79-83]; Requirement to adjust according to cell type and host tissues[137-142]
Decellularization	Mimicking an optimal non-immune environment with native three-dimensional structures and various bioactive components[121-124]; Flexible mechanical properties[127-133]; Satisfactory mechanical strength[139-142]	Complete decellularization is complex and time consuming[152-156]

FOOTNOTES

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REVIEW

Mechanisms of action of natural products on type 2 diabetes

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Abstract

Over the past several decades, type 2 diabetes mellitus (T2DM) has been considered a global public health concern. Currently, various therapeutic modalities are available for T2DM management, including dietary modifications, moderate exercise, and use of hypoglycemic agents and lipid-lowering medications. Although the curative effect of most drugs on T2DM is significant, they also exert some adverse side effects. Biologically active substances found in natural medicines are important for T2DM treatment. Several recent studies have reported that active ingredients derived from traditional medicines or foods exert a therapeutic effect on T2DM. This review compiled important articles regarding the therapeutic effects of natural products and their active ingredients on islet β cell function, adipose tissue inflammation, and insulin resistance. Additionally, this review provided an in-depth understanding of the multiple regulatory effects on different targets and signaling pathways of natural medicines in the treatment of T2DM as well as a theoretical basis for clinical effective application.

Key Words: Type 2 diabetes; Natural product; β cell; Adipose tissue inflammation; Insulin resistance

Core Tip: This review compiled leading articles about the therapeutic effects of natural products and their active ingredients on islet β cell function, adipose tissue inflammation, and insulin resistance and provided an in-depth understanding of the multiple regulatory effects of different targets and signaling pathways of natural medicines in the treatment of type 2 diabetes mellitus.

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INTRODUCTION

According to the International Diabetes Federation, the number of patients with diabetes mellitus (DM) worldwide was 536 million in 2021, which is expected to reach 783 million by 2045[1]. Globally, China has the highest number of patients with DM, whose prevalence is increasing steadily. By 2045, the total number of patients with DM in China is expected to exceed 174 million[1]. Based on its etiology, mechanism, and clinical manifestations, DM can be classified into type 1 DM, type 2 DM (T2DM), specific types of DM due to other causes, and gestational DM[2]. In China, T2DM accounts for 90% of all DM cases[3]. T2DM is mainly caused by insulin resistance (IR) associated with obesity, deficiencies in insulin secretion (INS), and reduction in islet cell numbers due to apoptosis[3]. DM and its complications are serious health and economic problems that affect individuals worldwide and require urgent prevention and early intervention.

Through diet management, lifestyle changes, and oral use of biguanides and sulfonylureas, blood sugar levels can be effectively controlled to treat T2DM. Although these treatment modalities can relieve symptoms and improve patients' conditions to a certain extent, they cannot completely prevent the occurrence and progression of complications; moreover they exert toxic side effects[4]. Natural medicines have become a hotspot in the exploration of alternative treatments for DM owing to their minimal side effects. Natural products mainly refer to small or macromolecular active substances with pharmacological properties and are extracted from plants, animals, or microorganisms. They can be used to treat DM and its complications through multiple targets and pathways. The antidiabetic ingredients of natural products include monomeric compounds such as flavonoids, alkaloids, terpenes, polyphenols, saponins, and quinines[5].

The current literature on T2DM treatment with natural products is mostly based on their different active ingredients; however, reviews on their regulation mechanisms are lacking. This review aimed to summarize the mechanism of natural products and/or their monomers on T2DM treatment (Table 1). It also provided a theoretical basis for comprehensively understanding the mechanism and clinical application of natural medicines in the treatment of T2DM by summarizing the signal pathways involved in the regulation.

PROTECTION OF ISLET B CELLS

Inhibition of islet β cell function is a prerequisite for T2DM occurrence. β cell impairment and IR are crucial in the development and pathogenesis of T2DM[6]. During the course of the illness, islet β cell function failure is observed along with frequent episodes of exacerbation[7,8]. Natural products exhibit notable effectiveness in reducing the inflammation, promoting the regeneration, and inhibiting the apoptosis of islet β cells (Figure 1).

Reduction in the inflammation of islet β cells

The accumulation of intra-islet macrophages is observed in T2DM, which represents the primary source of proinflammatory cytokines within the islets[9]. Activated monocytes and macrophages release proinflammatory mediators, such as tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), and monocyte chemoattractant protein-1 (MCP-1)[10], which activate inflammatory signaling pathways, such as the inhibitor of kappa B kinase and c-Jun N-terminal kinase (JNK), and impair the insulin signaling pathway by regulating the levels of phosphoinositide 3-kinase (PI3K)and protein kinase B(Akt).

Flavonoids: Quercetin is one of the most important bioflavonoids found in vegetables, cereals, fruits, and other plants. It is widely detected in green tea, onions, and apples and exerts antioxidative, anti-inflammatory, and antifibrotic effects [11]. A previous study reported that the anti-inflammatory effect of quercetin is mediated by the upregulation of peroxisome proliferator-activated γ (PPAR-γ), which interferes with proinflammatory transcriptional factors, such as signal transducer and activator of transcription (STAT) and nuclear factor-kappa B (NF-κB), and reduces the expression of IL-1β, IL-6, and TNF-α[12]. Abdelkader *et al*[13] also demonstrated that the anti-inflammatory effect of quercetin decreased the expression of IκB-α by inhibiting the expression of IKK-α and IKK-β in islets β cells, thereby inhibiting NF-κB activation and decreasing TNF-α levels.

Naringin and hesperidin are abundant in citrus fruits and exert antioxidative, antidiabetic, lipid-lowering, anti-atherosclerotic, and anti-inflammatory effects[14,15] They can reduce the expression of TNF-α and IL-6, regulate the level of nitric oxide (NO), activate the JNK pathway, inhibit the PI3K/Akt pathway, inactivate the lipid peroxide reaction, and reduce the levels of free radicals in high-fat diet/streptozocin (HFD/STZ)-induced rats with diabetes[16].

Polyphenols: Curcumin is a bioactive molecule found in the rhizome of turmeric plants; it exhibits extensive pharmacological and biological activities, such as exerting anti-inflammatory and hypoglycemic effects, improving β cell function, preventing β cell death, and improving IR[17]. It has been reported that curcumin indirectly inhibits the NF-κB pathway to prevent inflammation by inhibiting IκB-α degradation as well as reduces the levels of IL-6, MCP-1, and TNF-α in the serum of rats with diabetes[18]. Another study reported that curcumin decreased the expression of JNK, cyclooxygenase-

Table 1 Mechanism of natural products in the treatment of type 2 diabetes mellitus

Classification, extracts/monomers	Model		Signaling pathway	Related genes/proteins	Improvement effect	Ref.
	In vivo	*In vitro*				
Flavonoids						
Quercetin	STZ-induced Wistar rats	-	IKK/NF-κB/TNF-α	Serum SOD and GSH ↑, TNF-α ↓	Lowered blood glucose, cholesterol, and triglyceride levels and restores the number of islet β cells	Abdelkader *et al*[13]
	Fructose-treated Wistar rats	INS-1 β cells	Akt/FoxO1	p-Akt, JAK2, and STAT3 ↑, Akt/FoxO1 and Socs3 ↓	Protected β cell mass and function	Li *et al*[35]
	STZ-induced Sprague–Dawley rats	-	-	Islet β cell number ↑, total cholesterol ↓	Caused regeneration of islets and increased insulin release	Vessal *et al*[39]
	Balb/c mouse	-	-	HO-1 and Bcl-2 ↑, NO, iNOS, and Bax ↓	Enhanced islet viability, reduced apoptosis	Kim *et al*[67]
	db/db mice	INS-1 cells	SIRT3-FoxO3a	SOD2, CAT, and Sirt3 ↑, cleaved-caspase-3 and Bax/Bcl-2 ratio ↓	Protected islet β cells against apoptosis	Wang *et al*[68]
	HFD-induced C57BL/6 mice	-	AMPKα1/SIRT1	GLUT4, AMPK, and SIRT1 ↑, TNF-α , IL-6, and MSP-1 ↓	Suppressed ATM infiltration and inflammation, increased insulin sensitivity, and decreased adipose tissue weight	Dong *et al*[103]
Hesperidin	STZ-induced Wistar rats	-	PI3K/Akt	FFA, p-IRS-1, Akt, IL-6, and TNF-α ↓	Enhanced the antioxidant defense system while inhibiting the production of proinflammatory cytokines	Mahmoud *et al*[16]
	STZ-induced Wistar rats	-	-	Antioxidative enzyme activities ↑, MDA, NO, and lipid peroxidation ↓	Decreased oxidative stress while preserving the integrity of β cells	Coskun *et al*[36]
	db/db mice	Palmitic acid-induced MIN-6 cells	ERK1/2	Bcl-2/Bax ratio↑, caspase-3, caspase-9, and caspase-12 ↓	Inhibited cell apoptosis, improved fat metabolism disorders, and reduced blood sugar levels	Zhuang *et al*[38]
Puerarin	HFD-induced C57BL/6J mice	High glucose-induced MIN-6 cells	-	GLP-1R ↑, PDX-1, caspase-3, and Foxo1 ↓	Improved glucose homeostasis and protected β cell survival	Yang *et al*[42]
	STZ-induced C57BL/6 mice	CoCl2-induced MIN-6 cells	PI3K/Akt/mTOR	Bcl-2/BAX ratio and SOD and GPX1 activity↑, caspase-3 ↓	Protected pancreatic β cell function and survival	Li *et al*[43]
3Cyanidin-3-glucoside	-	High glucose-induced MIN-6 cell	NF-κB/MAPK/caspase	β cell viability ↑, ROS, ERK, p-ERK, JNK, p-JNK, caspase-3, and Bax ↓	Decreased the generation of intracellular reactive oxygen species, DNA fragmentation, and apoptosis rate; prevented pancreatic β cell apoptosis	Lee *et al*[69]
Kaempferol	-	PA induced INS-1E cells	PDX-1/cAMP/PKA/CREB	β cell activity and Bcl-2 ↑, caspase-3	Promoted pancreatic β cell survival and	Zhang *et al*[73]

				and Bax ↓	function	
	-	High glucose-induced INS-1E β cells	cAMP/Akt/CREB	Bcl-2 ↑	Improved insulin secretory function and synthesis in β cells	Zhang and Liu[74]
Butein	-	3T3-L1 cells	NF-κB/AMPK	iNOS, NO, ERK, JNK, and p38MAPK ↓	Prevented adipose tissue inflammation and obesity-linked IR	Wang *et al*[96]
Naringin	-	3T3-L1 cells	NF-κB/ERK/TNF-α	TNF-α and IL-6 ↓	Repressed FFA secretion to alleviate IR induced by FFA	Yoshida *et al*[98]
	HFD-induced C57BL/6 mice	3T3-L1 cells	IκB-α/JNK/TNF-α	TNF-α, TLR2, and MCP-1 ↓	Decreased blood glucose levels	Yoshida *et al*[99]
Baicalin	HFD-induced C57BL/6 mice	-	-	β-cell activity ↑, HOMA-IR ↓	Improved IR by inhibiting macrophage-mediated inflammation	Na *et al*[115]
	HFD-induced C57BL/6 mice	-	-	MCP-1 ↓	Suppressed macrophage infiltration into the adipose tissue	Yoshida *et al*[100]
	HFD-induced C57BL/6J mice, C57BL/6 mice	-	IRS1/PI3K/Akt, AMPKα	MAPK, NF-κB, and p85 ↑, FFA, IRS1, and Akt ↓	Exerted an anti-inflammatory effect, inhibited IR	Pu *et al*[125]
Icariin	High-sugar HFD and STZ-induced SD rats	-	AMPK/GLUT-4	p-AMPK, and GLUT4 ↑, islets cell number ↓	Reduced hyperglycemia	Li *et al*[76]
Cyanidin-3-glucoside	-	H_2O_2-induced MIN-6 cells	-	Islet cell apoptosis, ERK, p38, and caspase-3 ↓	Prevented diabetes by inhibiting oxidative stress-induced β cell apoptosis	Lee *et al*[70]
Anthocyanins	STZ-induced SD rats	-	-	Caspase-3 ↓	Reduced IR and β cell apoptosis	Nizamutdinova *et al*[71]
Polyphenols						
Curcumin	STZ-induced SD rats	High-fructose-induced U937 monocytes	IKK/NF-κB/TNF-α	TNF-α, IL-6, and MCP-1 ↓	Reduced inflammation and oxidative stress levels	Jain *et al*[18]
	High fructose fed Wistar rats	-	IKK/NF-κB /COX-2	Proliferation of β cells and SOD ↑, TNF-α and COX-2 ↓	Reduced glucose intolerance and IR	Maithilikarpagaselvi *et al*[19]
	STZ-induced SD rats	PA and high fructose-induced INS-1 cells	-	Caspase-3 and Bax ↑	Inhibited apoptosis	Li *et al*[83]
	-	3T3-L1 and BV-2 cells	IKK/NF-κB/TNF-α	TNF-α, IL-1β, IL-6, and COX-2 ↓	Inhibited chronic inflammation	Gonzales *et al*[111]
Gallic acid and p-coumaric acid	STZ-induced Albino rats	-	IKK/NF-κB/iNOS	*PPARγ* mRNA and adiponectin ↑, TNF-α, IL-1, and IL-6 ↓	Decreased glucose and glycosylated hemoglobin levels, increased insulin level and body weight	Abdel-Moneim *et al*[23]
Resveratrol	HFD-induced SD rats	-	IKK/NF-κB/TNF-α	ICAM-1, MCP-1, IL-1, and TNF-α ↓	Improved IR and vascular permeability and attenuated inflammatory injury	Zheng *et al*[28]
	HFD + STZ-induced SD rats	PA-induced INS-1E cells	SIRT1/NF-κB/TNF-α	PPAR-γ, SIRT1, FOXO-3a, and TNF-α ↑	Decreased blood glucose and insulin levels	Cao *et al*[29]

	-	UA-induced MIN-6 cells	PI3K/Akt	miR-126 ↑, Bax, cleaved-caspase-3, and iNOS ↓	Enhanced cell viability, reduced cell apoptosis, and increased insulin secretion	Xin *et al*[77]
	Human islet cells	-	-	VEGF, insulin, and C-peptide secretion ↑, ROS and HIF-1α ↓	Diminished apoptosis and enhanced islet survival and function	Keshtkar *et al*[79]
Sargassum oligocystum	STZ-induced Wistar rats	-	-	-	Enhanced the number of insulin-positive β cells, facilitated the survival of islet β cells, and conserved islet mass	Akbarzadeh *et al*[46]
	HSHFD-induced SD rats	-	-	-	Decreased blood glucose levels, alleviated pancreas, liver, and kidney damage	Motshakeri *et al*[45]
Genistein	HF + STZ-induced C57BL/6 mice	-	-	-	Improved glycemic control, glucose tolerance, and insulin levels while enhancing islet β cell survival	Fu *et al*[47]
	HFD + STZ-induced Wistar rats	-	ERK1/2 /Akt	Bcl-2 and caspase-3 ↓	Regulated pancreatic β cell function, enhanced the morphology of pancreatic β cells, and mitigated cellular apoptosis	Yousefi *et al*[49]
Mangiferin	PPX C57BL/6J mice	-	-	Cyclins D1 and D2 and cyclin-dependent kinase 4 ↑, p27Kip1 and p16INK4a ↓	Stimulated β cell proliferation and suppressed β cell apoptosis	Wang *et al*[53]
Cranberries	-	3T3-L1 cells	-	*AP2*, *FAS*, *LPL*, *HSL*, and *PLIN1* mRNA ↓	Inhibited mass production of the adipose tissue	Kowalska *et al*[105]
	-	3T3-L1 cells	-	IL-6, PAI-1, McP-1, and leptin ↓	Exerted an anti-inflammatory effect	Kowalska and Olejnik [106]
Peanut skin extract	HFD-induced mice	-	-	TNF-α, IL-1β, IL-6, and PAI-1 ↓	Maintained the gut microbiota, inhibited inflammation, and reduced fasting blood glucose levels, body weight, and food intake	Xiang *et al*[109]
Luteolin	-	3T3-L1 cells	AMPK/SIRT1	p-p65 ↑, TNF-α, IL-6, and MCP-1 ↓	Inhibited inflammation and promoted glucose disposal	Xiao *et al*[112]
	HFD-induced C57BL/6N mice	-	-	IL-1β and PAI-1 ↓	Enhanced dyslipidemia, ameliorated hepatic steatosis, improved IR, and reduced inflammation	Kwon *et al*[131]
	HDF-induced C57BL/6J mice	-	-	PPARγ, SREBP1, SREBP2, ACC G6PD, Fas, ME, PAP, HMCGR, and ACAT ↓	Attenuated hepatic lipotoxicity and improved circulating fatty acid levels as well as hepatic insulin sensitivity	Kwon *et al*[130]
Mulberry anthocyanin extract	db/db mice	Palmitic acid and high-fructose-	PI3K/Akt	Proliferation of islet β cells, AKT, GSK-3β, and GYS-2 levels	Decreased fasting blood glucose, serum insulin, leptin, trigly-	Yan *et al*[123]

		induced HepG2 cells		↑, TC, TG, FOXO-1, and PGC-1α ↓	ceride, IR, and cholesterol levels and increased adiponectin levels	
Terpenoids						
Geniposide	HFD-induced C57BL/6J mice	MIN-6 cells	β-catenin/TCF7L2	TCF7L2 and GLP-1R ↑, GSK3 ↓	Promoted β cell survival by inducing proliferation and inhibiting apoptosis	Yao *et al*[56]
Paeoniflorin	-	INS-1 cells	MAPK/caspase	Bax, p38, JNK, caspase-3 activity ↓	Enhanced insulin secretion and inhibited β cell apoptosis	Liu *et al*[90]
	-	High-fructose-induced INS-1 cells	-	HO-1 and Bcl-2 ↑, caspase-3 and Bax ↓	Protected β cells and reduced apoptosis	Liu *et al*[88]
Alpha-mangostin	-	STZ-induced INS-1 cells	PI3K/Akt and ERK	Bax, p38, JNK, and caspase-3 activity ↓	Improved insulin secretion in pancreatic β cells and prevented apoptosis	Lee *et al*[87]
Ethanolic extracts of Pluchea indica	STZ-induced BALB/C mice	-	-	IFN-γ, TNF-α, IL-1β , caspase-3, caspase-8, and caspase-9 ↓	Maintained body weight, reduced hyperglycemia, restored islet function, and inhibited β cell apoptosis	Nopparat *et al*[89]
Dioscorea batatas extract	HDF-induced C57BL/6 mice	-	PI3K/Akt	p-Akt ↑, p-ERK, and p-S6K1 ↓	Reduced glucose and insulin levels and improved IR	Kim *et al*[132]
Alkaloids						
Rhizoma coptidis	HFD/STZ-induced Wistar rats	-	PI3K/p-Akt	PPAR-γ ↑, TNF-α, GLUT4, HOMA-IR, TC, TG, and p-Akt ↓	Enhanced insulin sensitivity of the adipose tissue, regulated adipogenesis, elevated glucose uptake in adipocytes, and preserved β cell function	Gandhi *et al*[127]
Berberine	db/db mice	PA-induced MIN6 cells	iPLA2β/OL/OPA1	TNF-α, IL-1, NO, PEG2, and CRP ↑	Prevented apoptosis of β cells and enhanced islet β cell function	Li *et al*[84]
Brucea javanica, luteolin, protocatechuic acid	NA/STZ-induced SD rats	-	-	TG, TC, IL-6, INF-γ, TNF-α, ROS, and MDA ↓	Improved hepatic glucose and carbohydrate metabolism, suppressed oxidative stress, and prevented inflammation	Li *et al*[83]
Coffee	STZ-induced C57BL/6J	-	-	Caspase-3 and Bax ↓	Reduced glucose levels and maintained pancreatic insulin contents	Kobayashi *et al*[85]
Caffeic acid, naringenin, and quercetin	-	INS-1 cells	PI3K/Akt	*HSP90* mRNA ↑, caspase-3 and Bax ↓	Enhanced glucose-induced insulin secretion and sensitivity and improved β cell survival and function	Kobayashi *et al*[86]
Quinones						
Thymoquinone	STZ-induced Wistar rats	-	-	Survivin CD31 and IL-10 ↑, caspase-3, IL-1β, and TBARSS ↓	Promoted β cell regeneration, mitigating inflammation and oxidative	El-Shemi *et al*[60]

	stress, suppressed apoptosis of β cells, and enhanced revascularization of islets

Akt: serine/threoninekinase; ATM: Adipose tissue macrophage; $CoCL_2$: Cobalt dichloride; FFA: Free fatty acids; GSH: Glutathione; HFD: High-fat diet; HSHFD: High sucrose-high fat diet; HOMA: Homeostasismodel assessment; IR: Insulin resistance; INS: Insulin; IL: Interleukin; MDA: Malondialdehyde; MCP: Membrane cofactor protein; NA: Nicotinamide; NF-κB: Nuclear factor-kappa B; NO: Nitric oxide; PA: Palmitic acid; PPAR: Peroxisome proliferator-activated receptors; PPX: Partial pancreatectomy; PI3K: Phosphatidylinositol-3-hydroxykinase; ROS: Reactive oxygen species; SOD: Superoxide dismutase; STAT: Signal transducer and activator of transcription; STZ: Streptozocin. TC: Total cholesterol; TG: Triglyceride; TNF: Tumor necrosis factor; UA: Uric acid; VEGF: Vascular endothelial growth factor.

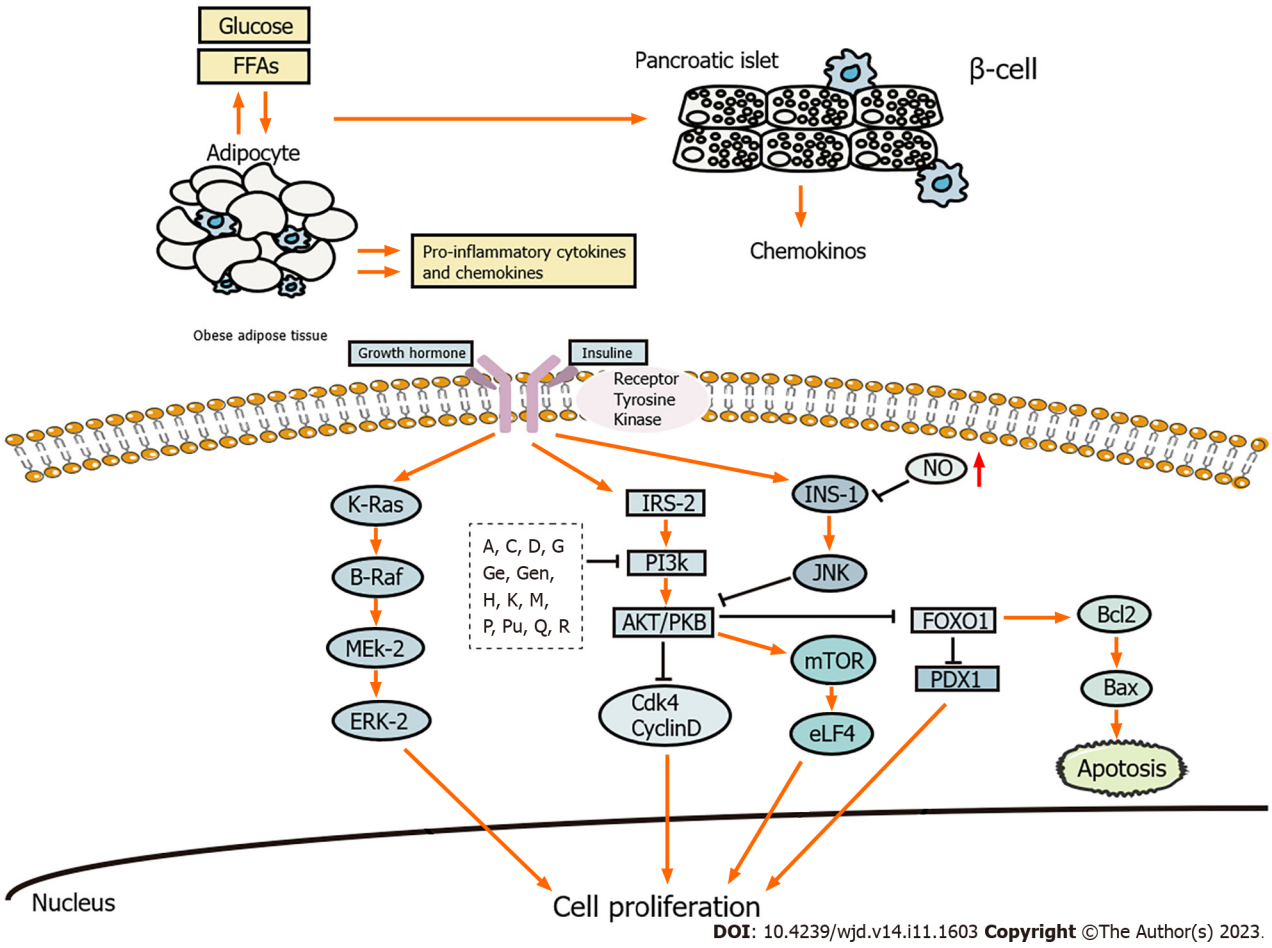

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Figure 1 Mechanism of natural products in promoting the regeneration and inhibiting the apoptosis of islet β cells. The letters inside the black squares refer to natural products. Inhibitory effects are shown by black pathways. A: Alpha-mangostin; C: Caffeic acid; D: Dioscorea batatas extract; G: Gallic acid; Ge: Genistein; Gen: Geniposide; H: Hesperidin; K: Kaempferol; M: Mulberry anthocyanin extract; P: Paeoniflorin; Pu: Puerarin; Q: Quercetin; R: Resveratrol; FFA: Free fatty acids; FOXO: Forkhead box class O; JNK: c-Jun N-terminal kinase; mTOR: Mammalian target of rapamycin; NF-κB: Nuclear factor-kappa B; NO: Nitric oxide; PI3K: Phosphoinositide 3-kinase; ROS/RNS: Reactive oxygen/nitrogen species.

2 (COX-2), protein kinase C, extracellular signal-regulated kinase (ERK), and p38, reduced the level of malondialdehyde (MDA), and prevented inflammation[19].

Gallic and p-coumaric acids are found in plants such as tea, mango, and cocoa; they exert anti-inflammatory, antioxidative, and antiobesity effects[20,21]. IL-1β reportedly induces NO production, increases NF-κB DNA binding, activates inducible NO synthase (iNOS) in islet β cells, and aggravates the inflammatory injury in islet β cells. Oral administration of gallic and p-coumaric acids also increases the expression of PPAR-γ[22], suppresses the expression of NF-κB, decreases the levels of proinflammatory cytokines (IL-1, IL-6, and TNF-α), iNOS expression, and nitrite production, and increases insulin sensitivity[23].

Luteolin is widely found in vegetables, fruits, and natural herbs, such as parsley, thyme and celery. It exerts various antitumor and anti-inflammatory effects by inducing cell apoptosis and inhibiting NF-κB activation, respectively[24]. Luteolin reportedly inhibits the NF-κB pathway and increases IL-10 levels in lipopolysaccharide-activated macrophage-

like cell lines, thus exerting its anti-inflammatory effect[25].

Resveratrol is detected in cereals, fruits, and plant derived-beverages. It exerts antidiabetic, anti-inflammatory, and antioxidative effects[26]. Resveratrol also inhibits the production of inflammatory factors by activating sirtuin 1 (SIRT1) and inhibiting p65/RelA acetylation, which results in decreased mRNA expression of *ICAM-1*, *MCP-1*, and *TNF-α*[27-29].

Genistein is an isoflavone found in legumes and herbs. It is a natural estrogen and tyrosine kinase inhibitor with potential hypolipidemic, antioxidative, and antiapoptotic effects[30]. Genistein reportedly inhibits p65 acetylation by activating SIRT1 to reduce the levels of IL-1β, IL-6, and TNF-α in ovariectomized rats with diabetes as well as the expression of NF-κB[31].

Alkaloids: Brucea javanica belongs to the bitter wood family, which is generally used for the treatment of DM[32]. A previous study showed that it effectively reduced the levels of TNF-α and IL-6 in rats, inhibited the NF-κB pathway, enhanced the expression of insulin receptor substrate-1 (IRS-1), and GLUT4 and played an anti-inflammatory role[33].

Promotion of β cell regeneration

β cells are key to maintaining balance in glucose metabolism. A decrease in the number of β cells leads to insufficient insulin production, which is one of the key factors in the pathogenesis of T2DM. β cell regeneration can be considered a new approach for treating T2DM[34].

Flavonoids: Quercetin promotes the differentiation and regeneration of β cells[13]. Previous studies have revealed that quercetin decreases the phosphorylation of Akt and FoxO1 in fructose-fed rat islets and increases the expression of nuclear FoxO1 in fructose-treated INS-1 cells[35]. Quercetin significantly decreases MDA and NO levels, increases antioxidative enzyme activities, and enhances insulin staining and β-cells preservation[36]. Oyedemi *et al*[37] reported that quercetin increased the number of pancreatic islets and β cells and can normalize the weight ratio of rat pancreas, suggesting that quercetin has the potential to regenerate pancreatic β cells. Furthermore, Zhuang *et al*[38] reported that quercetin improved the vacuolation of β cells and increased the number of pancreatic islets in db/db mice, consistent with the regeneration of pancreatic islets in STZ-induced rats with diabetes after 7 d of treatment with quercetin[39].

Puerarin, the dry root of pueraria, exerts neuroprotective, antioxidative, anti-inflammatory, and antiapoptotic effects [40]. It reportedly increases the mass and proliferation of mouse β cells, leading to the activation of glucagon-like peptide 1 receptor signaling[41]. Another study confirmed that puerarin protects pancreatic β cell function and promotes survival by mediating the PI3K/Akt pathway, thereby exhibiting resistance to the toxicity of cobalt chloride[42,43].

Polyphenols: *Sargassum* is a brown macroalgae found in shallow sea meadows. It exerts anti-inflammatory, antioxidative, and immune regulatory effects[44]. Pathological analysis of the islets revealed that the water extract of *Sargassum* can restore the damaged islet structure. Previous studies have revealed that the islet area and regeneration percentage increased and the regeneration function of pancreatic β cells improved after 30 d of supplementation with hydroalcoholic extract of *Sargassum*[45,46].

Genistein intake can improve hyperglycemia, increase insulin levels, and enhance glucose tolerance in mice with diabetes[47]. Akt and ERK1/2 are markers of cell proliferation and growth[48]. Genistein reportedly increases the expression of p-ERK1/2, p-Akt, and Bcl-2 and suppresses the expression of caspase-3, concomitant with improved morphology and mass of islet β cells[49].

Mangiferin is a polyphenolic compound isolated from *Anemarrhena*. C-glycoside, which is isolated from mango leaves, is a type of mangiferin exhibiting biological activities. It reduces blood glucose levels and contributes to the regeneration of pancreas and islet cells in rats with diabetes[50]. Neurogenin-3 (Ngn3) is a marker of new endocrine progenitor β cells [51]. A previous study reported that mangiferin increased the expression of Ngn3, FoxO-1, and PDX after partial pancreatectomy in mice and contributed to the proliferation of β cells. Mangiferin can also regulate the cell cycle through the activation of p16INK4a and promote islet regeneration in rats[52,53].

Terpenoids: Geniposide is widely found in herbs. It exhibits anti-inflammatory, antioxidative, and antidiabetic effects [54]. T cell transcription factor 7-like 2 (TCF7L2) is a key factor involved in the Wnt/β-catenin pathway, which is an important regulator of β cell survival and regeneration. Geniposide reportedly increases the expression of TCF7L2 by activating Wnt signaling[55]. Furthermore, it inhibits GSK3β activity as well as promotes the nuclear translocation of β-catenin and regeneration of β cells. Geniposide can also induce ductal cell differentiation by upregulating TCF7L2 expression and activating the JAK2/STAT3 pathway. Thus, it can promote β cell survival and regeneration by activating β -catenin/TCF7L2 signaling[56].

Astragalus belongs to the legume family and possesses many pharmacological properties, including antidiabetic, antioxidative, anti-inflammatory, and antiapoptotic effects[57]. A previous study reported that *Astragalus* strengthens the structure of pancreatic islet cells; the researchers also reported the appearance of new pancreatic islet cells and abundant capillaries around the islets, which promote β cell regeneration in HDF/STZ-induced Wistar rats with diabetes[58].

Quinones: Thymoquinone is the most abundant constituent in the volatile oil of *Nigella sativa* seeds. It exerts antioxidative, anti-inflammatory, and immunomodulatory effects[59]. In rats with diabetes, treatment with thymoquinone can efficiently ameliorate the histomorphological deteriorations of pancreatic islets, replenish the mass of β cells; and restore the function of β cells[60]. It has also been shown that thymoquinone inhibits COX-2 activity, relieves lipid peroxidation, and enhances antioxidative enzyme activity, thereby protecting pancreatic β-cells[61].

Inhibition of β cell apoptosis

β cell apoptosis is a common pathological feature of T2DM. Mass production of superoxide ions and endoplasmic

reticulum stress caused by high concentrations of free fatty acids lead to β cell apoptosis and dysfunction. Furthermore, the impaired balance between oxidation and antioxidation promotes β cell apoptosis and dysfunction[62,63]. Excessive production of reactive oxygen species (ROS) and reactive nitrogen species induces IR and chronic inflammation through abnormal changes in intracellular signaling pathways[64]. Inflammation also promotes β cell apoptosis and dysfunction [64,65].

Flavonoids: The decrease in mitochondrial membrane potential is an early indicator of apoptosis[66]. Previous studies have reported that quercetin reverses the decrease in mitochondrial membrane potential, inhibits the activation of caspase-3, caspase-9, and caspase-12, and increases the Bcl-2/Bax ratio, thereby suppressing apoptosis[38,67]. Quercetin also protects islet β cells from oxidation-induced apoptosis *via* SIRT3. After treating INS-1 cells and mice with diabetes were treated with quercetin, superoxide dismutase 2 and SIRT3 proteins levels increased, whereas the cleaved caspase-3 levels and Bax/Bcl-2 ratio decreased, along with reduced blood glucose levels and elevated insulin levels[68].

According to a previous study, cyanidin-3-glucoside decreased the apoptotic rate, intracellular ROS generation, and caspase-3 activity as well as reduced MAPK phosphorylation in MIN-6 cells treated with high levels of glucose[69]. The same results were observed in MIN-6 cells treated with H_2O_2[70]. A previous study revealed that anthocyanins protected the pancreatic tissue from STZ-induced apoptosis by regulating the levels of caspase-3, Bax, and Bcl-2 proteins in rats with diabetes[71].

Kaempferol is a flavanol compound found in various Chinese medicinal herbs[72]. It has been reported that kaempferol protects β cells and human islets from palmitate-induced apoptosis *via* the upregulation of the PDX-1/cAMP/PKA/CREB signaling cascade[73], increases the expression of Bcl-2 *via* CREB to activate the PI3K/Akt pathway, maintaining β-cell survival under high-glucose conditions, and reduces the expression of caspase-3[74].

Icariin is the main active ingredient of the natural medicine epimedium. It is considered a potential therapeutic agent for various diseases and is known to exert antioxidative, antineuroinflammatory, and antiapoptotic effects[75]. Icariin reportedly increases *GLUT4* mRNA expression and promotes AMP-activated protein kinase (AMPK) phosphorylation to reduce the loss of islets in the pancreatic tissue[76].

Puerarin promotes the proliferation and reduces the apoptosis of pancreatic β-cells. It also reverses the effect of impaired glucose tolerance[41,42]. Isoflavone glycosides (the main component of puerarin) inhibit apoptosis and protect β cells *via* Akt phosphorylation[43].

Polyphenols: Resveratrol reportedly alleviates uric acid-induced apoptosis, reduces the expression of Bax, cleaved-caspase-3, and iNOS, and activates the PI3K/Akt pathway by upregulating the expression of miR-126[77]. A previous study demonstrated that ROS overproduction affected cell apoptosis by destroying the mitochondrial membranes, releasing cytochrome C, and stabilizing HIF-1 and p53[78]. Previous research has also revealed that resveratrol inhibited the production of ROS and HIF-1α[79]. Another study showed that the PI3K/Akt pathway reduced ROS production and inhibited p53 expression and pancreatic islet cell apoptosis[80].

Curcumin possesses antiapoptotic activity and improves the function of pancreatic islets. On the one hand, it interferes with the interaction among Beclin1, Bcl-2, and Bim through the signal pathway mediated by JNK-1 and AMPK, thereby regulating the transition between apoptosis and autophagy[81,82]. On the other hand, it decreases palmitate-induced oxidative stress in pancreatic islet cells by regulating the NADPH pathway, increases insulin levels, reduces the expression of cleaved caspase-3 and Bax, and protects cells from apoptosis[83].

Alkaloids: In a previous study, overexpression of independent phospholipase A2β and treatment with berberine significantly attenuated palmitate-induced apoptosis. Furthermore, silencing independent phospholipase A2β partially abolished the antiapoptotic effect of berberine and inhibited cardiolipin/Opa1 signaling in MIN6 cells[84]. In another study, coffee ingestion protected β cells from STZ cytotoxicity, suppressed hyperglycemia, inhibited β cells apoptosis, and maintained the pancreatic insulin content by inhibiting the activity of poly ADP ribose polymerase[85]. Based on a previous research, caffeic acid, naringin and quercetin increased the expression of GLUT2, Ins1, β2, Pdx1, Akt1, Bcl2 and Hsp70/90, reduced the expression of caspase-3 and Bax, and inhibited apoptosis of INS-1E cells[86].

Terpenoids: Mangostin reduces ROS, p38, and JNK phosphorylation, restores the impaired secretory function of pancreatic β cells, and exerts its antiapoptotic effect on STZ-induced INS-1 cells[87]. Geniposide inhibits the apoptosis of INS-1 cells induced by high levels of glucose, thereby preventing caspase-3 cleavage. Further research demonstrated that AMPK siRNA attenuated the effects of geniposide on apoptosis-associated proteins and cell viability, suggesting that AMPK plays a key role in protecting β cells from high-glucose-induced apoptosis[88]. According to a previous study, pretreatment with licorice extract inhibited the expression of caspase-3, caspase-8, caspase-9, and other apoptotic factors as well as the expression of p-STAT1, thereby hindering STZ-induced β cell apoptosis[89].

Paeoniflorin is a glycoside extracted from the root of *Paeonia lactiflora* Pall. It inhibits the activation of the p38MAPK and JNK signaling pathway and reduces the phosphorylation of p38MAPK and ERK1/2 by increasing the expression of Bcl-2 and inhibiting the expression of Bax and caspase-3. It also increases the survival rate of STZ-induced INS-1 cells[90].

REDUCTION OF ADIPOSE TISSUE INFLAMMATION

Adipose tissue is an important endocrine organ that regulates insulin sensitivity and energy homeostasis throughout the body. It can secrete various hormones such as adiponectin, leptin, resistin, and visfatin as well as typical cytokines such as TNF-α and IL-6. It can also activate the MAPK and NF-κβ pathways[10,91]. Adipose tissue inflammation is a mechanistic pathogenesis of T2DM. Fat-infiltrated macrophages, basophils, and regulatory T cells cooperate with

adipocytes to mediate adipose tissue inflammation by secreting proinflammatory factors[92]. Activation of monocytes and release of MCP-1 cause the transformation of white fat cells into the proinflammatory phenotype[93]. MCP-1 recruits macrophages into adipose tissue, which in turn produce inflammatory cytokines. PPARα/γ agonists also reduce the expression of IL-6, CXC-L10, and MCP-1 in human adipocytes[94].

Flavonoids

Butein is isolated from the bark of the sumac tree. It exerts antioxidative, anti-inflammatory, antidiabetic, and neuroprotective effects[95]. It has been reported that pretreatment with butea results in the complete blockade of TNF-α-induced IκB-α degradation, prevents p65 phosphorylation at Ser311 and Ser536, and inhibits ERK, JNK, and p38MAPK phosphorylation in 3T3-L1 adipocytes[96]. These results are consistent with the previous findings, indicating that butein suppresses the expression of IL-6, TNF-α, and MCP-1, increases the expression of HO-1, and activates the p38MAPK/Nrf2/HO-1 pathway in the epididymal white adipose tissue of HFD-fed mice[97]. These findings suggest that butein plays an anti-inflammatory role in adipocytes *in vitro* and *in vivo*.

Naringin possesses strong antioxidative activity. Previous studies have demonstrated that naringin suppresses TNF-α–induced activation of NF-κB and ERK pathways in 3T3-L1 adipocytes[98]. Naringenin presumably exerts an anti-inflammatory effect by inhibiting IκB-α degradation and p-JNK expression, thereby inhibiting the expression of TLR2 in TNF-α induced adipocytes[99]. It was found to suppress macrophage infiltration into the adipose tissue by inhibiting MCP-1 production[100]. A recent study demonstrated that naringenin suppresses neutrophil infiltration into the adipose tissue by regulating MCP-3 expression and macrophage infiltration[101].

SIRT1 activators suppress inflammatory responses by promoting p65 deacetylation and inhibiting NF-κB activity in adipocytes[102]. Quercetin increases antioxidative activity as well as p-AMPK and SIRT1 expression in the adipose tissue of HFD-fed mice. Moreover, it reduces proinflammatory enzymatic activity and cytokine levels[103].

Polyphenols

Cranberry contains various types of bioactive components with high antioxidative and anti-inflammatory potentials. It also exerts beneficial effects on adipogenesis and lipid metabolism *in vitro*[104]. Cranberries reportedly reduce lipid accumulation during adipocyte differentiation by decreasing the levels of acid-binding protein, lipoprotein lipase, fatty acid synthase, and perilipin 1[105]. In addition, they reduce H_2O_2-induced inflammation in 3T3-L1 cells by decreasing the expression of IL-6, PAI-1, MCP-1, and leptin in adipose tissue[106].

Peanut skin extract is a rich source of polyphenols[107]. It is effective in the treatment of various diseases, such as DM, obesity, and inflammation[108-110]. A previous study reported that peanut skin extracts significantly alleviate adipose tissue inflammation by reducing the expression of TNF-α, IL-1β, IL-6, and PAI-1[109].

According to another study, the combined use of curcumin and resveratrol inhibited the activation of NF-κB, decreased the expression of IL-1β, TNF-α, IL-6, and COX2, and reduced the damage induced by chronic inflammation in adipocytes [111]. Based on a previous study, luteolin increases the expression of p-AMPK and SIRT1, suppresses the expression of p-p65, and decreases the mRNA expression of *TNF-α*, *IL-6*, and *MCP-1* in 3T3-L1 cells[112]. Studies have shown that SIRT1 inhibits NF-κB activation[113], and AMPK antagonizes inflammation through SIRT1[114].

NATURAL PRODUCTS CAN TREAT T2DM BY INHIBITING IR

IR usually refers to the reduction in insulin-induced glucose uptake and utilization efficiency in the muscle, body fat, and liver, leading to compensatory INS, which ultimately results in a series of clinical manifestations such as hyperglycemia, hyperinsulinemia, and dyslipidemia[115,116]. A previous study reported that lipid accumulation in the liver and adipose tissue accelerated IR in patients with T2DM[117]. Inflammatory factors such as TNF-α and IL-6 activate the NF-κB pathway and inhibit the expression of IRS-1 and GLUT4, thereby promoting IR[118,119]. IL-1β also inhibits the IRS-1 pathway and promotes IR[120]. In general, IR is related to the NF-κB, JNK, p38MAPK, and PI3K/Akt pathways. When the energy intake is high, the activation of the PI3K/Akt pathway can alleviate obesity and IR[121](Figure 2).

Flavonoids

Anthocyanins reportedly improve INS and IR[122]. A previous study showed that mulberry anthocyanin extract activates the PI3K/Akt pathway, increases the phosphorylation of its downstream target GSK3β, activates GYS2, and alleviates IR in HepG2 cells induced by high levels of glucose and palmitic acid. According to *in vivo* experiments, mulberry anthocyanin extract reduces the secretion of leptin and insulin and increases the levels of adiponectin in the serum, thereby improving IR[123].

According to a previous study, baicalein reduced the expression of TNF-α and F4/80, activated AMPK, p-AKT, and IRS-1, and induced dephosphorylation of ERK, NF-κB and JNK, thereby reducing IR[124]. A study by Pu *et al*[125] confirmed that the inhibitory effect of baicalein on IR was mediated by the inhibition of the MAPK pathway and activation of the IRS1/PI3K/Akt pathway.

Naringin possesses strong antioxidative activity. It reportedly increases the expression of GLUT4, adiponectin, and ChREBPβ in white adipocytes, promotes energy consumption and insulin sensitivity, and inhibits the proliferation of fat cells[126].

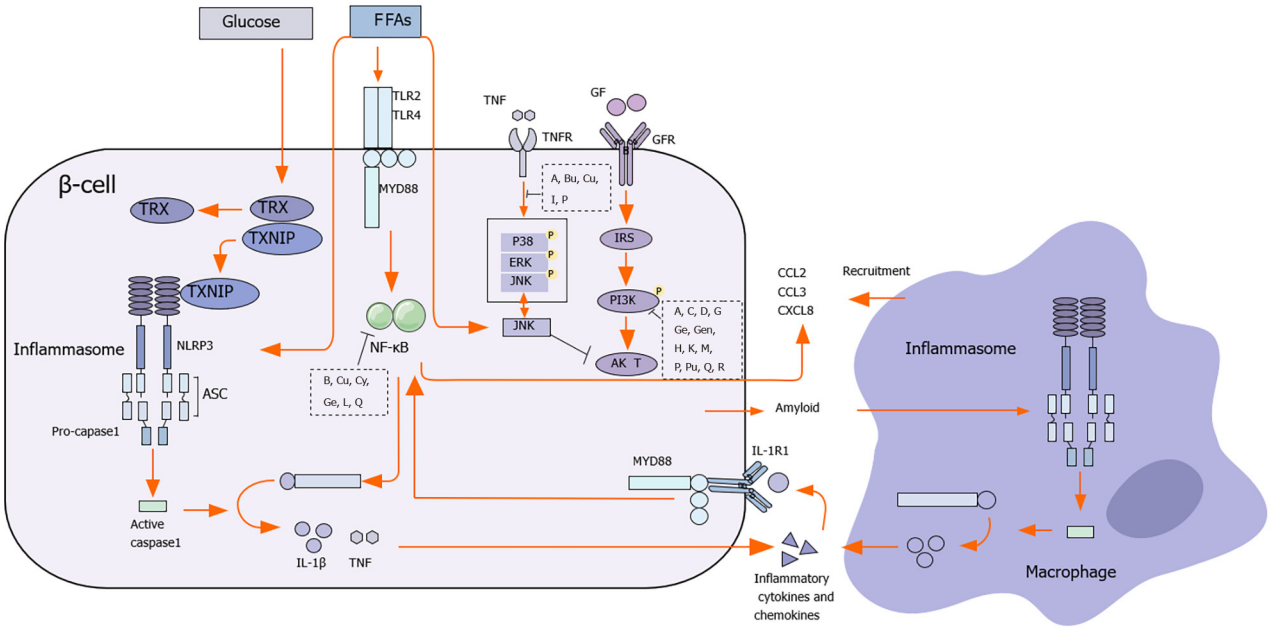

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Figure 2 The mechanism of natural products suppresses insulin resistance. The letters inside the black squares refer to natural products. A: Alpha-mangostin; B: Berberine; Bu: Butein; C: Caffeic acid; Cu: Curcumin; Cy: Cyanidin-3-glucoside; D: *Dioscorea batatas* extract; G: Gallic acid; Ge: Genistein; Gen: Geniposide; H: Hesperidin; I: Icarrin; K: Kaempferol; L: Leteolin; M: Mulberry anthocyanin extract; P: Paeoniflorin; Pu: Puerarin; Q: Quercetin; R: Resveratrol; CHOP: CCAAT-enhancer-binding protein homologous protein; ER stress: Endoplasmic reticulum stress; FFA: Free fatty acids; FOXO: Forkhead box class O; GFR: Growth factor receptor; GR: Growth factor; IKK: Inhibitor of nuclear factor-kappa B kinase; IL-1β: Interleukin 1β; IL-1: Interleukin 1; iNOS: Inducible nitric oxide synthase; IRAK: Interleukin 1 receptor-associated kinase; JNK: c-Jun N-terminal kinase; mTOR: Mammalian target of rapamycin; NF-κB: Nuclear factor-kappa B; NO: Nitric oxide; PI3K: Phosphoinositide 3-kinase; ROS/RNS: Reactive oxygen/nitrogen species; STAT: Signal transducer and activator of transcription; STAT1: Signal transducer and activator of transcription 1; STAT3: Signal transducer and activator of transcription 3; TNF-α: Tumor necrosis factor-alpha; TNFR: Tumor necrosis factor receptor.

Polyphenols

Gallic acid increases the expression of PPAR-γ in the adipose tissue, liver, and skeletal muscle, enhances tyrosine kinas activity, promotes IRS phosphorylation, and improves insulin-dependent glucose transport through GLUT4 in the PI3K/p-Akt dependent pathway in the adipose tissue, thereby improving IR in rats[127]. Adiponectin plays an important role in regulating insulin function as well as the occurrence and development of T2DM[128]. Gallic acid reduces the levels of serum total cholesterol and triglycerides by inhibiting adipogenesis and increasing adiponectin activity. The combined use of gallic acid and p-coumaric acid increases the levels and mRNA expression of PPAR-γ and reduces the levels of serum adiponectin in STZ-induced rats with diabetes[23].

Luteolin reportedly reduces blood lipid and glucose and improves hyperinsulinemia and IR through PPAR-γ[129]. It increases the absorption of circulating free fatty acids and reduces liver fat toxicity by increasing the protein expression of PPARγ in the adipose tissue[130]. In HFD-fed mice, luteolin reduces lipid formation, increases fatty acid oxidation, and significantly reduces the levels of IL-1, IL-6, and PAI-1, thereby improving obesity and metabolic disorders[131].

Terpenoids

HFD-induced IR in mouse visceral adipose tissue is characterized by increased p-ERK and decreased p-Akt expression. The therapeutic effect of the *Dioscorea batatas* extract decreased the protein expression of p-ERK and p-S6K1 and enhanced the translocation of GLUT4 to the plasma membrane of the visceral adipose tissue in mice. It has been speculated that the *Dioscorea batatas* extract attenuates IR by upregulating the expression of GLUT4 in the plasma membrane of the visceral adipose tissue in HFD-fed mice[132]. The discoloration mixture of *Astragalus membranaceus* and *Potentilla anserina* reportedly increases the mRNA expression of *PPARγ* and *PI3K* in the liver, reduces FPG levels, and improves IR in mice [133].

CLINICAL STUDY ON NATURAL PRODUCTS IN THE TREATMENT OF DM

To date, only a few clinical studies have been reported on natural medicines for treating DM. Most previous studies have focused on the addition of natural medicines to the diet to examine their effects on blood glucose levels, blood lipid levels, and body mass index in patients with T2DM. The addition of soluble fibers from psyllium to the normal diet of patients with T2DM significantly improved the levels of fasting blood sugar, hemoglobin A1c, C-peptide, Homeostasis Model Assessment-IR, and Homeostasis Model Assessment-B after 8 wk of administration[134]. A study by Noureddin *et al*[135] also showed that psyllium supplementation decreased the body weight, blood glucose levels, and cholesterol

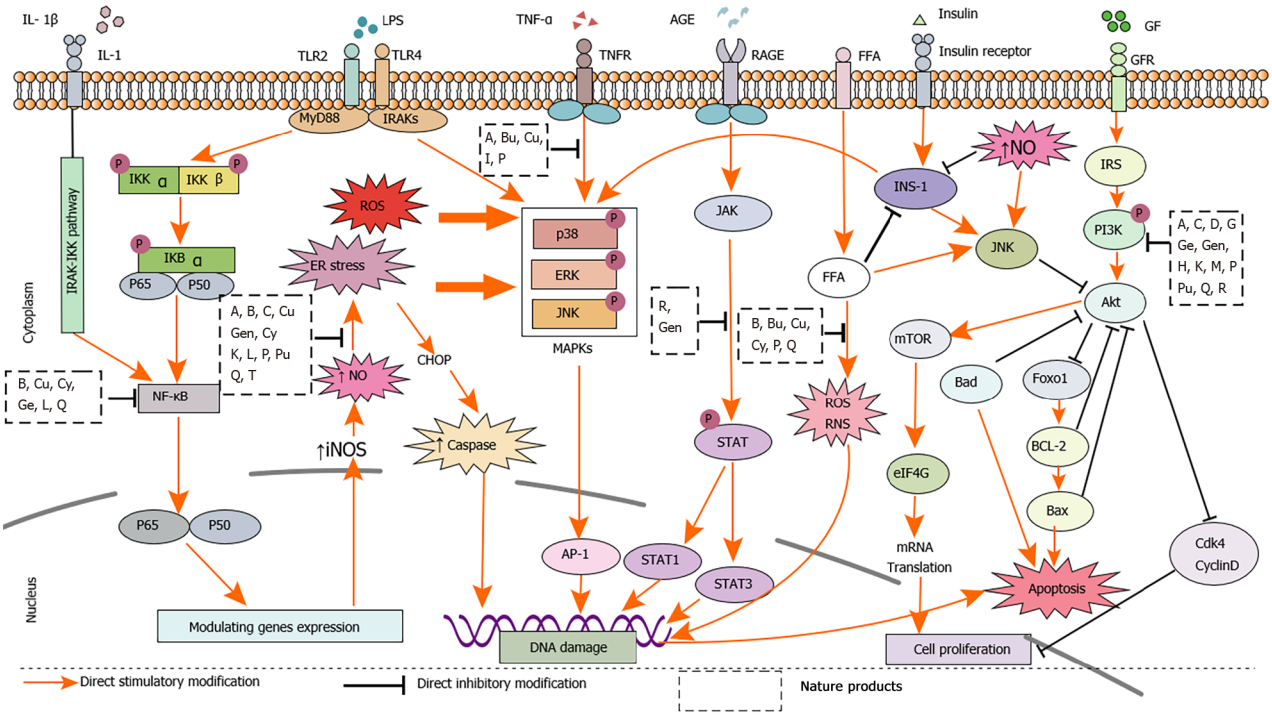

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Figure 3 Mechanisms of natural products for the treatment of type 2 diabetes mellitus. The letters inside the black squares refer to nature products. Inhibitory effects were shown by black pathways. A: Alpha-mangostin; B: Berberine; Bu: Butein; C: Caffeic acid; Cu: Curcumin; Cy: Cyanidin-3-glucoside; D: *Dioscorea batatas* extract; G: Gallic acid; Ge: Genistein; Gen: Geniposide; H: Hesperidin; I: Icarrin; K: Kaempferol; L: Leteolin; M: Mulberry anthocyanin extract; P: Paeoniflorin; Pu: Puerarin; Q: Quercetin; R: Resveratrol; Akt: serine/threoninekinase; AGE: Advanced glycation end products; AP-1: Activator protien-1; Bad: Bcl2 associated death promoter; Bax: BCL2-Associated X; Bcl-2: B-cell lymphoma-2; Cdk4: Cyclin dependent kinase 4; CHOP: CCAAT-enhancer-binding protein homologous protein; eIF4G: Eukaryotic translation initiation factor 4G; ER stress: Endoplasmic reticulum stress; FFA: Free fatty acids; FOXO: Forkhead box class O; GFR: Growth factor receptor; GR: Growth factor; IkBa: Inhibitory subunit of NF Kappa B Alpha; IKK: Inhibitor of nuclear factor-kappa B kinase; IL-1β: Interleukin 1β; IL-1: Interleukin 1; iNOS: Inducible nitric oxide synthase; IRAK: Interleukin 1 receptor-associated kinase; JNK: c-Jun N-terminal kinase; mTOR: Mammalian target of rapamycin; NF-κB: Nuclear factor-kappa B; NO: Nitric oxide; PI3K: Phosphoinositide 3-kinase; ROS/RNS: Reactive oxygen/nitrogen species; STAT: Signal transducer and activator of transcription; STAT1: Signal transducer and activator of transcription 1; STAT3: Signal transducer and activator of transcription 3; TNF-α: Tumor necrosis factor-alpha; TNFR: Tumor necrosis factor receptor.

levels and increased the high-density lipoprotein cholesterol levels in patients with T2DM. Similar results were reported in other clinical trials[136,137].

A previous study showed that dietary raspberries significantly reduced serum glucose levels at 2 h and 4 h after intake and decreased the serum levels of IL-6 and TNF-α[138]. These results indicated that propolis increased the serum activity of superoxide dismutase and GPx, decreased the levels of fasting blood sugar, 2-h postprandial glucose and insulin, and alleviated IR[139]. In a previous study, based on the results of the area under the curve, the consumption of bitter melon for 3 mo increased INS and decreased the body weight, body mass index, and glucose in patients with T2DM, possibly by increasing uncoupling protein expression or inhibiting PPAR-γ[140].

These results indicated that quercetin intake was inversely correlated with T2DM prevalence in the Chinese population. Moreover, quercetin intake reduced pancreatic β-cell inflammation, thus successfully treating T2DM[141].

CONCLUSION

Accumulating studies including clinical trials and animal experiments have confirmed the effectiveness of natural products. *In vivo* and *in vitro* studies have demonstrated that the active ingredients of monomeric compounds, such as flavonoids, polyphenols, alkaloids, terpenes, and quinones in natural medicines can inhibit the release of inflammatory mediators and reduce oxidative stress. Thus, reduction in IR and lipid accumulation can protect islet cells and treat T2DM. The mechanisms by which natural medicines treat T2DM include the following: (1) β cell inflammation was mainly inhibited by IKK/IκB/NF-κB, PI3K/Akt, and SIRT1/NF-κB pathways; (2) β cell regeneration was mainly promoted *via* ERK1/2/MDA, PI3K/Akt/mTOR, Wnt/β-catenin, and JAK2/STAT3/Ngn3 pathways; (3) β cell apoptosis was inhibited through MAPK/caspase-3, PI3K/Akt/caspase-3, and SIRT1/HIF-1/P53 pathways; (4) Adipose tissue inflammation was attenuated by PPAR-γ/SREBP, TGF-β/STAT3/Smad2/3, P38MAPK/Nrf2/HO-1, JNK/MCP-1, and AMPK/SIRT1 pathways; and (5) IR was alleviated mainly through IRS1/PI3K/Akt, TGF-β/Smad, LKB1/AMPK/PGC1α, and mTOR/S6K1 pathways (Figure 3).

Commonly used drugs for treating T2DM, such as α-glutaminase inhibitors, sulfonylureas, biguanides, and glitalactone, can be used alone or in combination to regulate blood glucose levels. However, the multiple side effects and high cost of these drugs have led to the urgent need to explore natural medicines to treat T2DM. In recent years, an increasing number of studies have explored various effective active ingredients of natural medicines for treating T2DM to discover a new alternative medicine. The plants and their main components reported in this review can alleviate the effects of T2DM on the body to a certain extent and provide a theoretical basis for the development of new drugs. Further studies in the following areas are still warranted: (1) The potential toxicity of natural medicines and the interactions between drug compatibilities remain unclear. Common adverse effects associated with the intake of natural medicines include gastrointestinal disturbances such as abdominal pain, diarrhea, constipation, nausea, and vomiting[142-145]. However, more severe toxicities may occur and affect patients' cardiovascular systems, auditory functions, or reproductive health[146,147]. Furthermore, the concomitant use of natural medicines and established antidiabetic drugs may increase the risk of hypoglycemia in patients with T2DM[148]. Thus, further studies are warranted on the specific mechanism of action and long-term toxic side effects of these natural products; and (2) Although some natural products have shown positive effects in cell and animal models, their activities have not yet been verified. Thus, further clinical studies are warranted to confirm the efficacy of natural medicines.

FOOTNOTES

Co-first authors: Tao Wang and Yang-Yang Wang.

Author contributions: Wang T and Wang YY reviewed and summarized the literature and wrote the paper; Shi MY revised the manuscript; Liu L designed and revised the manuscript; Liu L is the guarantor of this work. All authors were involved in the critical review of the results and have contributed to, read, and approved the final manuscript. Wang T and Wang YY contributed equally to this work as co-first authors. The reasons for designating Wang T and Wang YY as co-first authors are threefold. First, the research was performed as a collaborative effort, and the designation of co-corresponding authorship accurately reflects the distribution of responsibilities and burdens associated with the time and effort required to complete the study and the resultant paper. This also ensures effective communication and management of post-submission matters, ultimately enhancing the paper's quality and reliability. Second, the overall research team encompassed authors with a variety of expertise and skills from different fields, and the designation of co-corresponding authors best reflects this diversity. This also promotes the most comprehensive and in-depth examination of the research topic, ultimately enriching readers' understanding by offering various expert perspectives. Third, Wang T and Wang YY contributed efforts of equal substance throughout the research process. The choice of these researchers as co-first authors acknowledges and respects this equal contribution, while recognizing the spirit of teamwork and collaboration of this study. In summary, we believe that designating Wang T and Wang YY as co-first authors of is fitting for our manuscript as it accurately reflects our team's collaborative spirit, equal contributions, and diversity.

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MINIREVIEWS

Molecular mechanisms of noncoding RNA and epigenetic regulation in obesity with consequent diabetes mellitus development

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Abstract

Diabetes mellitus (DM) and obesity have become two of the most prevalent and challenging diseases worldwide, with increasing incidence and serious complications. Recent studies have shown that noncoding RNA (ncRNA) and epigenetic regulation play crucial roles in the pathogenesis of DM complicated by obesity. Identification of the involvement of ncRNA and epigenetic regulation in the pathogenesis of diabetes with obesity has opened new avenues of investigation. Targeting these mechanisms with small molecules or RNA-based therapies may provide a more precise and effective approach to diabetes treatment than traditional therapies. In this review, we discuss the molecular mechanisms of ncRNA and epigenetic regulation and their potential therapeutic targets, and the research prospects for DM complicated with obesity.

Key Words: Diabetes mellitus; Obesity; Noncoding RNA; Epigenetic regulation; Insulin resistance

Core Tip: Non-coding RNA (ncRNA) and epigenetic regulation play crucial roles in the pathogenesis of diabetes mellitus complicated by obesity. Identification of the involvement of ncRNA and epigenetic regulation in the pathogenesis of diabetes with obesity has opened new avenues. Targeting these mechanisms with small molecules or RNA-based therapies may provide a more precise and effective approach to diabetes treatment than traditional therapies.

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INTRODUCTION

The combination of diabetes mellitus (DM) and obesity has become a global health concern due to the high prevalence and serious consequences of these conditions. The pathogenesis of DM combined with obesity is complex and involves multiple mechanisms, including insulin resistance (IR), chronic inflammation, and adipokine dysregulation[1,2].

IR is a key factor in the development of both obesity and type 2 DM (T2DM)[3,4]. Adipose tissue, particularly visceral adipose tissue, produces a range of hormones, cytokines, and chemokines, collectively known as adipokines. In obesity, adipose tissue expands and produces increased amounts of proinflammatory adipokines, such as leptin, as well as decreased amounts of anti-inflammatory adipokines, such as adiponectin[5]. This leads to chronic inflammation, which exacerbates IR. Obesity and diabetes are associated with alterations in the gut microbiome, which can contribute to the pathogenesis of both conditions[6,7]. The gut microbiota of obese and diabetic individuals is distinct from that of healthy individuals, with reduced microbial diversity and altered microbial composition.

While the exact mechanisms underlying the development of DM complicated with obesity are still not fully understood, emerging evidence suggests that epigenetic modifications and noncoding RNA (ncRNA) play a critical role in its pathogenesis[8]. Epigenetic regulation refers to the modification of gene expression without changes to the underlying DNA sequence[9]. These modified activities can have a significant impact on gene expression and cellular function. Additionally, several genes are linked to an increased risk of developing these conditions, including genes involved in adipogenesis, lipid metabolism, and insulin signaling. Compounded obesity in DM is a multifactorial disorder that involves complicated interplay among genetic, environmental and lifestyle factors. It is vital to establish effective strategies for the prevention and treatment of these disorders by understanding these mechanisms.

ncRNAs, particularly microRNA (miRNA) and long noncoding RNA (lncRNA), have been shown to play critical roles in the development and progression of DM. Dysregulation of miRNA expression can lead to impaired glucose metabolism and IR[10]. For example, miRNA-29 regulates insulin signaling by targeting the insulin receptor substrate-1 (*IRS-1*) gene[11]. In obese mice, miRNA-29 expression is decreased, leading to increased *IRS-1* expression and improved insulin sensitivity[11]. Similarly, miRNA-223 has been shown to regulate glucose uptake by targeting GLUT4, a glucose transporter protein. lncRNA has also been implicated in the pathogenesis of DM[12]. In addition, lncRNA MEG3 controls insulin secretion by modulating gene expression involved in insulin synthesis and secretion[13]. lncRNA taurine-upregulated gene 1 regulates the proliferation and differentiation of pancreatic beta cells, which are responsible for insulin production[14].

DNA methylation can alter gene expression patterns. The promoter region of the insulin gene is hypermethylated in patients with T2DM, leading to decreased insulin production[15]. Similarly, the promoter region of the adiponectin gene is hypomethylated in obese individuals, leading to increased adiponectin expression and improved insulin sensitivity [16]. The augmentation of gene expression is linked to histone acetylation, whereas histone methylation may either stimulate or hinder gene expression, contingent on the location and extent of methylation[17].

Emerging evidence suggests that epigenetic modifications and ncRNA play a critical role in the development and progression of DM complicated with obesity (Figure 1). Dysregulation of miRNA and lncRNA expression, as well as altered DNA methylation and histone modifications, can lead to impaired glucose metabolism and IR[18]. Although much is still unknown about the mechanisms underlying these epigenetic changes, identification of these modifications as potential therapeutic targets offers new hope for the prevention and treatment of DM. Future research should elucidate the role of epigenetic regulation and ncRNA in diabetes pathogenesis and develop effective therapies targeting these pathways. The aim of this review is to explore the molecular mechanisms of ncRNAs and epigenetic regulation in the pathogenesis of DM complicated by obesity. We intend to discuss the potential therapeutic targets associated with these mechanisms and highlight the research prospects for DM complicated with obesity.

MOLECULAR MECHANISMS OF NCRNA IN THE PATHOGENESIS OF DM COMPLICATED WITH OBESITY

Role of lncRNAs

lncRNAs in obesity and DM: The utilization of cutting-edge bioinformatic techniques has facilitated the identification of lncRNAs associated with obesity and adipocyte differentiation[19]. Investigations of gain-of-function and loss-of-function have both strongly pointed to the pivotal participation of lncRNAs in adipogenesis. To date, various lncRNAs have been examined in a range of models and they are potent modulators of diverse genetic pathways linked to white adipose tissue (WAT) compartmentalization and activity[20].

The first adipogenesis-related lncRNA was a steroid receptor RNA activator (SRA), which acts as a coactivator of peroxisome proliferator-activated receptor (PPAR)γ[21]. Among the lncRNAs involved in adipogenesis, ASMER-1 and ASMER-2 are upregulated in subcutaneous adipose tissue (ScAT) and are linked to adipocyte-specific metabolism and IR

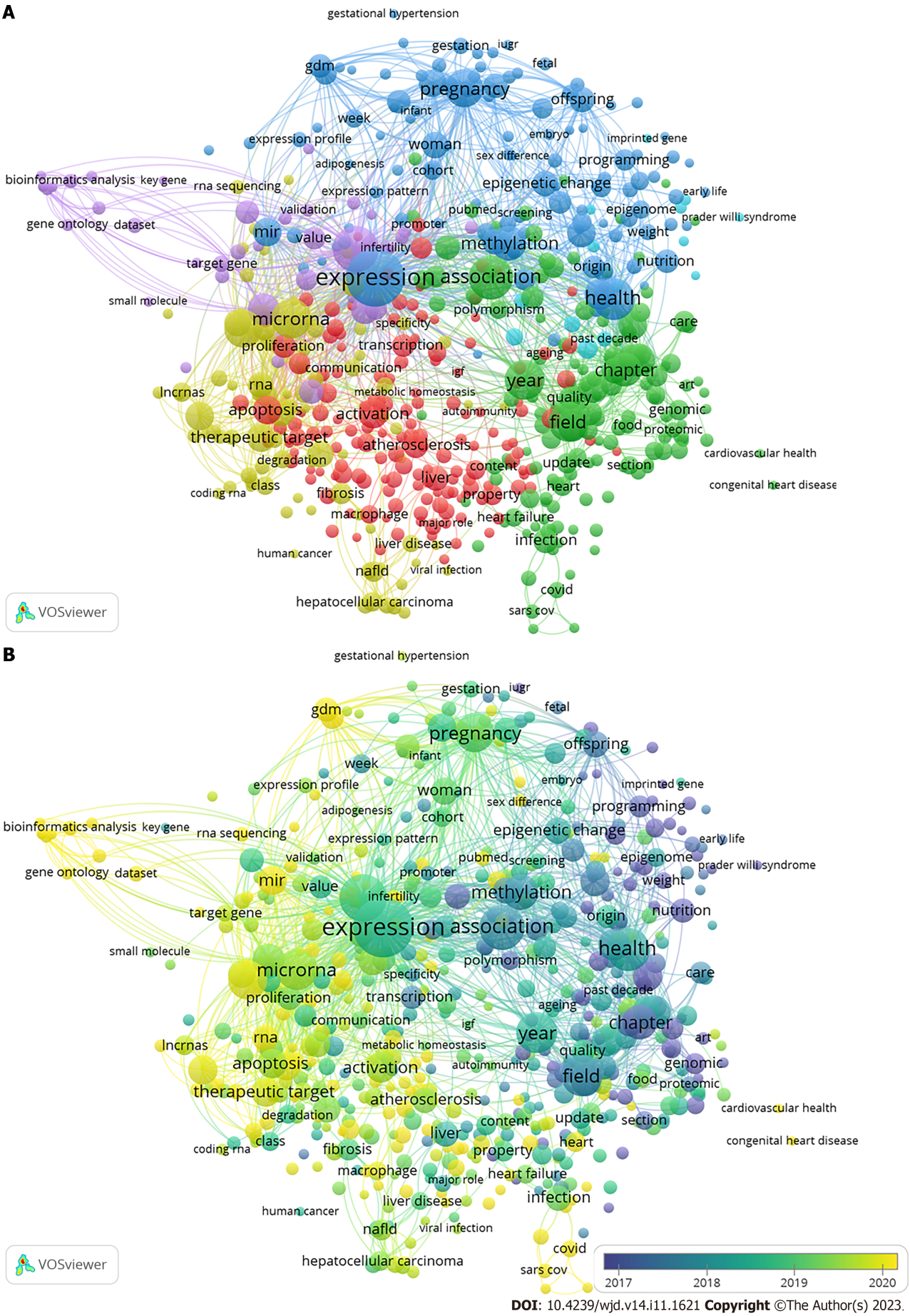

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Figure 1 Epigenetic modifications and noncoding RNA play a critical role in the development and progression of diabetes mellitus

complicated with obesity. A: Network visualization of the titles and abstracts related to the long noncoding RNAs and epigenetic regulation in the pathogenesis of diabetes mellitus complicated with obesity; B: Overlay visualization of the years of publication on long noncoding RNAs and epigenetic regulation in the pathogenesis of diabetes mellitus complicated with obesity. Different color dots are used to distinct the keywords that have appeared in the publications.

[20]. Several lncRNAs have roles in adipogenesis (the formation of fat cells), lipolysis (the breakdown of fat), and adiponectin secretion in human adipocytes (fat cells). ADNCR is an endogenous competitive RNA for miR-204, and overexpression of SIRT-1 inhibits adipocyte differentiation and impairs the PPARγ pathway *in vitro*. Finally, HOTAIR is implicated in preadipocyte differentiation[20,22-26].

Brown adipose tissue (BAT) is a specialized form of adipose tissue that is mainly responsible for thermogenesis and energy expenditure. It is characterized by the presence of uncoupling protein 1 (UCP1), leading to increased energy expenditure and weight loss[27,28]. Recent studies have identified several lncRNAs that are involved in BAT regulation, including brown fat lncRNA1 (Blnc1) and H19[25,29]. Research has indicated that Blnc1 plays a role in regulating thermogenic genes, resulting in an increase in the expression of UCP1 and mitochondrial genes[30]. Conversely, H19 has been found to have an inverse correlation with body mass index (BMI) and a positive correlation with browning markers. H19 is involved in modulating adipogenesis, oxidative metabolism, and mitochondrial respiration in BAT. Thus, the manipulation of lncRNA expression shows promise as a therapeutic approach for metabolic diseases. This could involve enhancing BAT activity or inducing browning in WAT[31]. Various studies have suggested the potential of different lncRNAs as biomarkers for diagnosing and managing obesity. For example, Sun *et al*[32] found reduced expression of three lncRNAs in obese but not in lean subjects. The expression of these lncRNAs was inversely correlated with waist-to-hip ratio, BMI and fasting plasma insulin levels. lncRNA-p19461 was upregulated following weight loss due to a 12-wk diet, suggesting that bariatric interventions could manage expressed lncRNA profiles. Alterations in the expression levels of lncRNAs were found following bariatric surgery in animals, particularly those engaged in digestive, absorptive and inflammatory pathways.

While the potential of lncRNAs as therapeutic targets for obesity management is promising, several challenges need to be addressed before their clinical application. One major challenge is the lack of understanding of the precise molecular mechanisms underlying the regulation of lncRNA expression in different tissues and under different physiological conditions[33]. The delivery of lncRNA-based therapeutics to specific tissues remains a major hurdle due to their large size and potential off-target effects[33,34]. Therefore, additional investigation is required to uncover the molecular pathways involved in the regulation of lncRNA and to develop delivery methods that can specifically target tissues while minimizing off-target effects.

lncRNAs in DM: In animal models and human islets, dysregulation of lncRNAs is engaged in various stages of insulin secretion and is implicated in the progression of IR[35,36] (Table 1). In addition, the genes that encode them are located near islet-specific chromatin domains that contain genes involved in β-cell function modulation[37]. The specific functions and action mechanisms of these lncRNAs are still not fully understood[36].

In T2DM, metabolic syndrome and low-level high-density lipoprotein, a decline in MALAT1 expression was found, along with overexpressed H19 in patients with worse glycemic control than those with glycated hemoglobin concentration < 7%[38]. Additionally, MALAT1 is related to angiogenesis in diabetic eyes and kidneys. A few dysregulated lncRNAs in diabetic subjects are positively correlated with transcriptional markers of IR, impaired glucose control, and aging. These lncRNAs were apparently relevant to DM, even after correction[39]. Newly diagnosed diabetic patients exhibited similar results, indicating that dysregulated lncRNAs control IR and inflammation, ultimately resulting in disrupted glucose homeostasis[40].

The role of lncRNAs in both microvascular and macrovascular complications of DM has been investigated. A widely studied lncRNA associated with diabetic complications is ANRIL, which is considered a potential biomarker[41,42]. Another is MALAT1 in association with elevated production of reactive oxygen species and proinflammatory cytokines, contributing to endothelial lesions in the microvasculature[35,41].

Dysregulation of specific genes has been identified in renal biopsies affected by diabetic nephropathy. Additionally, a study of diabetic patients with chronic complications found downregulation of CASC2 in the serum and renal tissue of DM patients with chronic kidney disease when compared to healthy controls[36,43]. Both MIAT and MALAT1 were found to be over-regulated in renal specimens from diabetic subjects and in animal models[36]. The effect of lncRNAs in diabetic patients with peripheral neuropathy has also been investigated. Specifically, NONRATT021972 was shown to be increased in T2DM subjects with exacerbated symptoms connected to neuralgia, together with an increase in tumor necrosis factor (TNF)-α levels. Furthermore, siRNA-NONRATT021972 alleviated neuropathic pain by decreasing TNF-α in rats, resulting in decreased blood glucose and inflammation, which paved the way for potential therapies of neuropathic pain[44]. MALAT1 is over-expressed in gastrointestinal spasms and in T2DM sufferers with signs related to gastric spasms, and its impact is likely associated with smooth muscle cells.

Function of miRNAs in DM with obesity: miRNAs prevent the translation of mRNA into protein, leading to mRNA degradation or translational repression. miRNAs have been shown to regulate various cellular processes. Dysregulated miRNA has been implicated in metabolic disorders, such as in obesity (Table 2).

The pathogenesis of metabolic diseases has been linked to the expression of various miRNAs. Kunej *et al*[45] found that 221 of the 1736 Loci associated with obesity coincided with miRNAs. It has been reported that miRNAs can modulate pathways that control adipogenesis[46,47], which is impaired in obesity. Consequently, miRNA dysregulation could be involved in metabolic processes that contribute to obesity[48,49].

Table 1 Transcription factors and long noncoding RNAs in insulin resistance

Factors related to the development of IR	Names of lncRNAs	Targeted nuclear proteins
Lipogenic activity	H19, MALAT1, MEG3, and MIAT↑	SREBP-1c, PPARγ, and FoxO1
Gluconeogenesis	MEG3 and H19↑	CRTC2/CREB, FoxO1, HNF4A and ATF4
Inflammation and oxidative stress	MALAT1 and H19↑	EZH2 and PRC2
Cellular dysfunction	MEG3, MALAT1 and MIAT↑	N/A

lncRNA: Long noncoding RNA; IR: Insulin resistance; SREBP-1c: Sterol reg-ulatory element binding protein-1c; PPARγ: Peroxisome proliferator-activated receptor γ; FoxO1: Forkhead box protein O1; CRTC2/CREB: CREB-regulated transcription coactivator 2; HNF4A: Recombinant hepatocyte nuclear factor 4 α; ATF4□Recombinant activating transcription factor 4; EZH2: Enhancer of zeste homolog 2; N/A: Not applicant.

Table 2 Circulating microRNA in obesity

Tissue or organs	Names of miRNAs	Targeted genes
Adipocytic tissue	miR-155, miR-27a, and miR-34a	*SOCS1, PPAR*
Liver	miR-99b and miR-155	*FGF21, PPAR*
Muscle	miR-27a, miR-155, and miR-130b	*PPAR, PGC1α*
Pancreas	miR-132 and miR-92a	*BTG2, PTBP1*
Cardiovascular system	miR-29a, miR-410-5p, and miR-194	*SMAD7*

miRNA: microRNA.

miRNA-375: The islet-specific miRNA-375 is expressed at high levels in pancreatic islets and regulates insulin secretion by modulating gene expression. The impact of miRNA-375 on glucose-stimulated insulin secretion (GSIS) and insulin gene transcription was investigated by Poy *et al*[50], who found that its overexpression suppressed GSIS and reduced insulin gene transcription, whereas its downregulation resulted in increased insulin secretion. This study confirmed the crucial role of miRNA-375 in the development of T2DM, as demonstrated by its higher expression in the pancreas of T2DM patients compared to healthy individuals. Dysregulation of miRNA-375 was observed 5 years prior to the start of T2DM and in prediabetes, indicating its potential use in the prediction and prevention of high-risk populations[51].

miRNA-130b: In prepubertal obesity, some miRNAs may become deregulated, as evidenced by a study which showed that the expression of miRNA-130b in plasma was upregulated and directly correlated with BMI and other indicators of obesity in children.

miRNA-200 family: The miRNA-200 family can contribute to protection against beta-cell apoptosis and dedifferentiation *in vitro*[52]. miRNA-200c is one of the most highly expressed miRNAs in beta cells, and partially protects against oxidative stress-induced beta-cell apoptosis, suggesting that the miRNA-200 family is essential in diabetes pathophysiology[53].

miRNA-7: Human islets are enriched in another miRNA named miRNA-7, which adversely modulates GSIS by restricting the expression of genes participating in the integration of insulin granules within the plasma membrane and the SNARE proteins[54]. The levels of hsa-miRNA-7-1-3p were reduced in pancreatic islets of individuals with T2DM compared to nondiabetic donors. The expression levels of hsa-miRNA-7-3-5p were increased in T2DM pancreatic islets [55].

miRNA-184: miRNA-184 is one of the miRNAs predominantly expressed in beta cells of pancreatic islets, regulating insulin secretion and beta-cell proliferation during IR[56]. Knockout of miRNA-184 in beta cells has been shown to increase their proliferation, resulting in improved insulin secretion following glucose stimulation. Blocking miRNA-184 in rat and human islets has been demonstrated to protect beta cells from apoptosis induced by prolonged exposure to proinflammatory cytokines and/or fatty acids.

Circular RNAs in obesity and DM

Role of circular RNAs: Adipose tissue is a complex and metabolically active organ, playing an essential role in energy storage and homeostasis. Adipocytes are the primary cell type in adipose tissue, and their differentiation and function are tightly regulated by multiple molecular mechanisms. In recent years, the role of circular RNAs (circRNAs) in adipose tissue has gained significant attention.

circRNA expression in carboxy-terminal region, prediabetic and T2DM patients showed 411 downregulated and 78 upregulated circRNAs[57]. Notably, 220 circRNAs demonstrated differential expression, including 107 upregulated and 113 downregulated circRNAs[58]. Of particular interest were the ci-INS and ci-Ins2 Lariats, derived from human INS and mouse Ins2, respectively in beta cells[59].

EPIGENETIC REGULATION AND ITS ROLE IN THE PATHOGENESIS OF DM COMPLICATED WITH OBESITY

Genetic variation is a crucial factor in the regulation of DNA methylation[60]. As methylated DNA predominantly arises on cytosine nucleotides after a guanine, it is evident additions or deletions of variants of cytosine-guanine dinucleotides(CG dinucleotides) affect the likelihood of methylated DNA at the loci[61]. Remarkably, roughly one-fourth of single nucleotide polymorphisms (SNPs) add or delete CpG site[62].

The presence of an SNP in NDUFB6 Led to the emergence of a CpG site that in turn affected DNA methylation and gene expression in human skeletal muscle, particularly age-related gene expression[63]. Although genetic variations can directly impact DNA methylation, it remains unclear whether they can affect methylation in more remote sites and, if so, what the underlying mechanism would be. The extent to which this phenomenon is widespread throughout the genome and its potential contribution to clinical phenotypes remain uncertain. Another study identified that nearly half of the genetic variations associated with diabetes introduce or remove a CpG site[64].

In 2014, a study extended previous research and provided a whole-genome description of genetic and epigenetically variations in human pancreatic islets[63]. Numerous *cis*- and *trans*-SNP–CpG pairs were determined, even though the machinery of the latter is still unclear[65]. Additionally, causal inference test established a catalytic interaction between SNPs, DNA methylation and genetic expression of annotated HLA regions highly correlated with type 1 DM[66]. More than 100000 DNA metylation quantitative trait loci (mQTLs) were identified by GWASs, which were linked to adipose-tissue gene expression, BMI, and insulin levels[67,68].

RESEARCH PROSPECTS

ncRNAs as early diagnostic markers

ncRNAs are involved in the development of both diabetes and obesity and may be potential early diagnostic markers for these conditions. miRNAs are small ncRNAs that play important roles in post-transcriptional gene regulation. These miRNAs are dysregulated in both DM and obesity and may serve as potential early diagnostic markers for these conditions[69]. For example, miRNA-126 has been shown to be downregulated in obese individuals and may serve as a potential early diagnostic marker in obesity[70]. Similarly, miRNA-375 has been shown to be upregulated in individuals with T2DM and may serve as a potential early diagnostic marker for this condition[71].

lncRNAs are longer ncRNAs that also play important roles in gene regulation, suggesting that lncRNAs are involved in the development of both diabetes and obesity and may serve as potential early diagnostic markers[19]. The lncRNAs HOTAIR and H19 have been shown to be upregulated in individuals with T2DM and may be early diagnostic markers [72,73].

circRNAs are a class of ncRNAs that form covalently closed circular RNA molecules, which have recently been observed in the dysregulation in both DM and obesity and may be early diagnostic markers[74]. For example, circRNA-000911 has been shown to be downregulated in individuals with T2DM, and may serve as a potential early diagnostic marker[75]. Similarly, serum and exosome circRNA-0000907 and circRNA-0057362 have been shown to be upregulated in patients with diabetic foot ulcer (DFU), indicating that they may have a potential role as early diagnostic markers for DFU [11].

ncRNAs have emerged as potential early diagnostic markers for both DM and obesity. Early diagnosis and management of DM and obesity are crucial to prevent complications and improve outcomes. Therefore, the identification of novel early diagnostic markers for these conditions is of utmost importance. ncRNAs may serve as valuable tools in this regard and may help improve patient outcomes. Therefore, further research is needed to validate the potential of ncRNAs in early diagnosis.

Possible treatment targets for DM with obesity

miRNAs are one of the best-studied classes of ncRNAs, and they have been implicated in the regulation of glucose homeostasis and insulin sensitivity. It was shown that miRNA-29a regulates insulin signaling by targeting IRS1 in adipocytes[76]. Additionally, miRNA-103 and miRNA-107 have been shown to promote IR by targeting the insulin receptor and GLUT4, respectively[77]. Another lncRNA, NEAT1, has been shown to regulate the expression of genes involved in the inhibition of high glucose-induced diabetic retinopathy[78]. Furthermore, S961-treated mouse sera reproduced beta-cell replication in pancreatic islets in an E2F1-dependent way, indicating that IR-induced adipocyte proliferation signaling activates E2F1 and is a potential target for promoting beta-cell compensation[79].

Epigenetic regulation has also emerged as an important contributor to the pathogenesis of DM with obesity. DNA methylation regulates motifs involved in glucose homeostasis and insulin signaling[80]. Histone modifications regulate the expression of key genes in the insulin signaling pathway[81].

In addition, lncRNAs are newly emerging and promising biomarkers, so we summarize the shared lncRNAs in both obesity and DM in order to provide further information (Table 3).

Identification of the pathogenesis of DM with obesity has opened new avenues. Targeting these mechanisms with small molecules or RNA-based therapies may provide a more precise and effective approach to DM treatment than traditional therapies. For example, miRNA-based therapies have already been tested in preclinical models of DM, with promising results.

CONCLUSION

The pathogenesis of DM complicated with obesity involves complex molecular mechanisms, including ncRNA and epigenetic regulation. Understanding the roles of ncRNA and epigenetic regulation in the pathogenesis of DM complicated with obesity provides new insights into the development of novel therapeutic targets and strategies. Future research should focus on exploring the potential of ncRNA and epigenetic regulation as biomarkers for diagnosis and prognosis, as well as precision medicine and personalized treatment strategies.

Table 3 The profiles of shared long noncoding RNAs in obesity and diabetes mellitus

lncRNA	Description	Expression in obesity	Expression in DM
SRA	Steroid receptor RNA activator	High	Low in patients with type II diabetic cardiovascular disease[74]
ASMER-1	Adipocyte-associated metabolic related lncRNA 1	High in ScAT	High expression related to IR[20]
ASMER-2	Adipocyte-associated metabolic related lncRNA 2	High in ScAT	High expression related to IR[20]
ADNCR	Adipocyte differentiation-associated lncRNA	Low	Low[23]
HOTAIR	HOX antisense intergenic RNA	High	High[71]
Blnc1	Brown fat lncRNA 1	High in high-fat-diet-fed mice[28]	High in the blood of patients with diabetic nephropathy[82]
H19	LncRNA H19	Low in obesity-associated inflammatory conditions[83]	Low[84]
MALAT1	Metastasis-associated lung adenocarcinoma transcript 1	High[85]	High expression in PBMCs from type 2 diabetes patients[39]

lncRNA: Long noncoding RNA; DM: Diabetes mellitus; ScAT: Subcutaneous adipose tissue; IR: Insulin resistance; PBMC: Peripheral blood mononuclear cell.

FOOTNOTES

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ORIGINAL ARTICLE

Observational Study

Reduced risk of dementia in patients with type 2 diabetes mellitus using Chinese herbal medicine: A nested case-control study

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Abstract

BACKGROUND

Dementia is a prevalent condition in type 2 diabetes mellitus (T2DM) patients. While Chinese herbal medicine (CHM) is often employed as complementary therapy for glycemic control, its effect in controlling likelihood of dementia has not yet been fully elucidated.

AIM

To compare the risk of dementia between T2DM patients with and without CHM treatment.

METHODS

We undertook a nested case-control study and obtained data on patients 20-70 years of age who received medical care for T2DM between 2001 and 2010 from the National Health Insurance Research database in Taiwan. Cases, defined as those with dementia that occurred at least one year after the diagnosis of T2DM, were randomly matched to controls without dementia from the study cohort at a 1:1 ratio. We applied conditional logistic regression to explore the associations between CHM treatment and dementia.

RESULTS

A total of 11699 dementia cases were matched to 11699 non-dementia controls. We found that adding CHM to conventional care was related to a lower risk of dementia [adjusted odds ratio (OR) = 0.51], and high-intensity CHM treatment was associated with an adjusted OR of 0.22.

CONCLUSION

This study shows that the cumulative CHM exposure was inversely associated with dementia risk in an exposure-response manner, implying that CHM treatment may be embraced as a disease management approach for diabetic patients to prevent dementia.

Key Words: Type 2 diabetes mellitus; Dementia; Chinese herbal medicine; Nested case-control study; Odds ratio

Core Tip: This population-based nested case-control study is the first to determine if integrating Chinese herbal medicine (CHM) into routine care of type 2 diabetes mellitus (T2DM) could aid in the prevention of subsequent dementia chance. In this report, we found that adding CHM to conventional care may reduce the subsequent risk of dementia for T2DM patients by 49%. Identification of an exposure-response manner, negative correlation between the days of CHM use and risk of dementia herein may further support the therapeutic benefit of CHM.

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INTRODUCTION

Chronic illnesses are a leading cause of present-day morbidity and mortality, thereby thwarting the healthcare system worldwide. Taking diabetes as an example, a report from the Centers for Disease Control showed that nearly 40 million Americans had diabetes in 2019, and nearly 95% of whom have type 2 diabetes mellitus (T2DM)[1]. In such a case, the disease may profoundly affect patients, their families, and society at large. By one estimate, the annual medical expenditures per T2DM patient in the United States was $16752 yearly, which was approximately 2.3 times higher than the average cost for a person without T2DM ($7151)[2]. The total national costs imposed by diabetes were expected to rise as well, from $322 billion in 2012 to $404 billion in 2017, an increase of 25% over the past several years[3].

Although recent medical innovations have greatly improved the prognosis of T2DM, its impact is still challenging for the survivors, especially regarding onset of dementia, which may cause serious long-term health problems. Unfortunately, dementia is a silent illness and thus affected persons may be unaware of their cognitive impairment. An earlier meta-analysis of 29 prospective observational studies reported a risk for all-cause dementia among T2DM patients as high as 73%[4]. Notably, T2DM patients experience twice the risk of dying after experiencing comorbid dementia[5,6]. Recent studies have presumed a link between T2DM and dementia that may include systemic insulin resistance and increased levels of circulating pro-inflammatory markers, both of which would lead to defects in insulin signaling pathway and changes in brain synaptic plasticity, thereby inciting chance of dementia[7]. Given the prominence of dementia in the patients with T2DM[5], it is critical to attenuate the likelihood of dementia while managing people with T2DM.

Today, Chinese herbal medicine (CHM) is often employed in treating a broad range of health conditions. Several active CHM ingredients were established to introduce favorable prognoses among patients with inflammatory conditions *via*

the regulation of specific circulating cytokines[8]. For example, Du-Huo-Ji-Sheng-Tang, one of CHM formulae used for bone diseases treatment clinically, has been proven to suppress the expression of cytokines by abating nuclear factor kappa beta (NF-κB) signaling[9], as well as inhibit several inflammatory mediators known to be involved in the pathogenesis of neurodegenerative illnesses[10,11]. In view of a growing body of evidence manifesting that abnormal inflammatory responses may be involved in the pathogenesis of T2DM and dementia[7,12], CHM may be a useful approach to prevent or delay onset of dementia among such groups.

After a thorough literature review, we found no studies that reported data on the association of CHM use with the probable subsequent dementia among T2DM patients. Accordingly, we carried out a nested case-control study based on a nationwide claims database to clarify this issue. Data from such a study would provide valuable information on the compatibility and clinical application of CHM, enabling healthcare providers to institute a more appropriate treatment for T2DM patients.

MATERIALS AND METHODS

Study design and data source

This nested case-control study was conducted using patient records from a national claim data held by the Bureau of National Health Insurance (NHI) in Taiwan[13]. As participation in this insurance is compulsory, more than 99% of the healthcare providers have contracted with NHI so far. The implementation of national insurance program would enable the residents to access the cost-effective and quality health care[13]. All analytical data were obtained from the Longitudinal Health Insurance Database between 2000 and 2013, which included the original claims data of 1 million insurants randomly extracted from all beneficiaries under the NHI program. This database holds the information on demographics, diagnoses, prescriptions, referrals, hospitalization for these patients covered by the NHI program. This work has been approved by the facility's institutional review board (No. B10004021-3). The institutional review board also waived the need for informed consent since the raw data used was on the basis of a retrospective claims data with encrypted attribution.

Underlying cohort establishment

Medical diagnoses and procedures in this claims database were recorded with the International Classification of Diseases, 9th Revision, Clinical Modification (ICD-9-CM). In accordance with the earlier rule[14,15], the present study recruited patients 20-70 years of age with T2DM who had at least three outpatient or a single inpatient claim with the ICD-9-CM code 250 between 2001 and 2010. Considering the temporal relationship for casual inference, we excluded patients with T2DM who had been diagnosed with dementia before or within 1 year after the onset of T2DM. Consequently, cases of this study had their first diagnosis of dementia during 2002-2013. Additionally, the primary outcome, namely incident dementia, was defined as having the first diagnosis of dementia (ICD-9-CM codes of 290, 294 and 331). To ensure the validity, only those diagnosed with dementia who had at least three outpatient service claims within one year, or at least 1 inpatient hospitalization claim were selected[16]. Date of when first dementia occurred was deemed the index date. We also employed one-year washout period to exclude the cases if they had any medical visits of dementia before T2DM (n = 30520), as well as those who had missing values or were followed up for less than one year after cohort entry (n = 2567); in sum, a total of 31219 new-onset T2DM patients were included.

Ascertainment of case and control groups

Among the recruited patients with T2DM, 17241 (55.2%) developed dementia before the end of 2013. Each case with dementia was randomly matched to one control who was not diagnosed with dementia, determined by age (within 2 years), sex, and comorbidities. Each member from the control group was assigned the same index date as the corresponding case with dementia, thus keeping a comparable probability of dementia onset during the follow-up period for each group. The flowchart of the study participants' selection is summarized in Figure 1.

Assessment of CHM exposure

Firstly, to identify individual exposure of CHM treatment, we reviewed the overall treatment records that occurred from cohort entry to index date. Based on former criteria[17,18], those receiving CHM treatment for more than 30 d due to T2DM were considered CHM users, whereas those who only seek healthcare from Western medical doctors were deemed non-CHM users. All participants were further classified into four groups, non-CHM use (< 31 d), low-intensity use of CHM (31-365 d), medium-intensity use of CHM (366-730 d), and high- intensity use of CHM (731 d or more) based on their cumulative days of CHM prescriptions. This approach would allow us to carefully determine the exposure-response effect of CHM in preventing dementia.

Information regarding covariates

Covariates in the statistical analysis included age, sex, monthly income, prior comorbidities, and urbanization of individual residential area. With regard to monthly income, we utilized the NHI premium payment as a proxy and transformed this indicator into a 3-level ordinal variable as follows: USD ≤ 623, 624-1394, and ≥ 1395[19]. Furthermore, the urbanization degree was classified into three types of settlements based on a previous study, which comprised cities, towns and semi-dense areas, and rural areas[20]. Baseline comorbidities were identified as occurring within one year preceding T2DM onset, and all of them were calculated by the established Charlson-Deyo comorbidity index (CCI)[21].

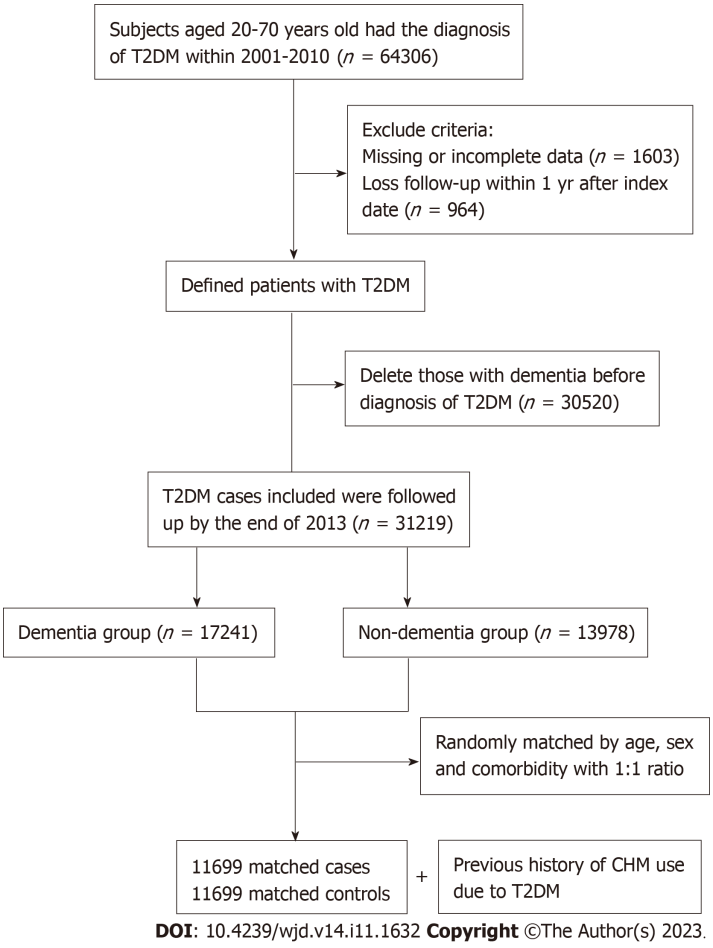

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Figure 1 Flowchart of patient screening. T2DM: Type 2 diabetes mellitus; CHM: Chinese herbal medicine.

The Deyo-adapted CCI incorporates 17 diseases, each which is rated on the scale of 1-6, with higher total scores indicating more severe burden due to comorbidities.

Analysis

In all comparisons, a P value < 0.05 was considered statistically significant. About the descriptive statistics, we reported continuous variables using mean ± SD, and categorical variables using frequency and percentage. The χ^2 test was used to determine differences in percentages of categorical variables, and student's t-test was adopted to evaluate differences in the means between two groups. Univariate and multivariate conditional logistic regressions were then applied to estimate odds ratio (OR) with 95% confidence intervals (CI) for association between CHM usage and sequent dementia. Subgroup analysis stratified by sex and age was performed as well. Sensitivity analysis, after excluding medical comorbidities, was conducted to determine the independent effect of CHM exposure on subsequent dementia risk. All of the analyses were carried out using SAS Version 9.3 for Windows (SAS Institute Inc., Cary, NC, United States).

RESULTS

A total of 11699 matched pairs of T2DM patients with and without dementia were identified from the cohort dataset. Male patients dominated the number of studied cases and matched controls. Mean age for the total sample was 51.6 years, with a mean follow-up period of 5.8 years. Also, nearly sixty percent of the participants were at the meddle income level and lived in cities. No significant baseline differences in terms of demographic data or comorbidities between two groups were detected. Details of the relevant variables at baseline were shown in Table 1.

During the study period, 22.6% of the cases and 35.6% of the controls had a history of CHM use. In the multivariate analyses of CHM use and dementia risk, we observed that those who had a history of CHM use exhibited a 49% reduced risk of dementia, in comparison to those who never used CHM (adjusted OR = 0.51; 95%CI: 0.48-0.53). Furthermore, those with high-intensity CHM treatment appear to have benefitted by attenuation of likelihood of dementia, which implied an exposure-res-ponse inverse association between duration of CHM use and dementia risk (Table 2). This exposure-response association still persisted following stratification by sex and age (Table 3). After excluding medical comorbidities as covariates, the sensitivity analysis revealed CHM usage was still tied to a lower risk of dementia, with an adjusted OR of 0.59 (95%CI: 0.51-0.69). Figure 2 displays the top-ten most commonly prescribed single-herb and multi-herb

Table 1 Demographic data and selected comorbidities of the two study groups

Variables	Number (%)	Cases	Controls	*P* value
		n = 11699 (%)	*n* = 11699 (%)	
Age (yr)				0.07
20-40	2307 (9.9)	1139 (9.7)	1168 (10.0)	
41-60	16457 (70.3)	8174 (69.9)	8283 (70.8)	
61-70	4634 (19.8)	2386 (20.4)	2248 (19.2)	
mean ± SD	51.6 ± 7.3	51.5 ± 7.2	51.6 ± 7.3	0.34
Duration of follow-up, yr (SD)	5.8 (3.4)	6.4 (3.2)	5.5 (3.4)	
Sex				0.99
Female	11243 (48.1)	5622 (48.1)	5621 (48.0)	
Male	12155 (51.9)	6077 (51.9)	6078 (52.0)	
Monthly income				0.15
Low	8561 (36.5)	4290 (36.7)	4271 (36.5)	
Median	13116 (56.1)	6587 (56.3)	6529 (55.8)	
High	1721 (7.4)	822 (7.0)	899 (7.7)	
Residential area				0.19
Urban	14005 (59.8)	6939 (59.3)	7066 (60.4)	
Suburban	3718 (15.9)	1869 (16.0)	1849 (15.8)	
Rural	5675 (24.3)	2891 (24.7)	2784 (23.8)	
CCI	4.3 (5.7)	4.3 (5.8)	4.3 (5.6)	0.68

SD: Standard deviation; CCI: Charlson-Deyo comorbidity index.

Table 2 The association between dementia onset and use of Chinese herbal medicine among type 2 diabetes mellitus patients

CHM exposure	Patients				Crude OR (95%CI)	Adjusted OR[1] (95%CI)
	Cases, *n* = 11699		Controls, *n* = 11699			
Non-CHM users	9053	77.4	7533	64.4	[1]	[1]
CHM users	2646	22.6	4166	35.6	0.53 (0.50-0.56)	0.51 (0.48-0.53)
Low intensity (31-365 d)	2350	20.1	3366	28.8	0.58 (0.55-0.62)	0.57 (0.53-0.60)
Medium intensity (366-730 d)	206	1.8	482	4.1	0.35 (0.30-0.42)	0.33 (0.28-0.39)
High intensity (731 d or more)	90	0.8	318	2.7	0.24 (0.19-0.30)	0.22 (0.17-0.28)

[1]Adjusted for potential confounders that contained age, residential area, monthly income and Charlson-Deyo comorbidity index.
CHM: Chinese herbal medicine; OR: odds ratio; CI: Confidence interval.

formulae for the treatment of T2DM, along with their associated OR. Uses of certain CHM products appeared to be correlated with a lower risk of dementia, which included Da-Huang, Hai-Piao-Xaio, Dan-Shen, Ge-Gen, Ye-Jaio-Teng, Bei-Mu, Chuan-Qi, Jia-Wei-Xiao-Yao-San (JWXYS), Ge-Gen-Tang (GGT), Shao-Yao-Gan-Cao-Tang (SYGCT), and Gan-Lu-Yin (GLY).

DISCUSSION

There is accumulating evidence manifesting that T2DM may elevate the risk of cognitive impairment, including dementia, due to the shared pathogenesis between the two diseases[7,12]. With no prophylactic options for dementia currently available for T2DM patients, CHM may provide a promising approach on the basis of suggestions drawn from

Table 3 Risk of dementia among type 2 diabetes mellitus patients with and without exposure to Chinese herbal medicine use stratified by sex and age

Variables	Patients, *n* (%)	Crude OR (95%CI)	Adjusted OR (95%CI)
Female			
Non-CHM users	4140 (73.6)	1	1
CHM users	1482 (26.4)	0.51 (0.47-0.55)	0.49 (0.45-0.53)[1]
Male			
Non-CHM users	4913 (80.8)	1	1
CHM users	1164 (19.2)	0.54 (0.50-0.59)	0.53 (0.48-0.57)[1]
Aged 20-40 yr			
Non-CHM users	1011 (77.6)	1	1
CHM users	291 (22.4)	0.46 (0.39-0.55)	0.45 (0.37-0.54)[2]
Aged 41-60 yr			
Non-CHM users	6487 (77.2)	1	1
CHM users	1921 (22.8)	0.53 (0.49-0.57)	0.51 (0.48-0.55)[2]
Aged 61-70 yr			
Non-CHM users	1555 (78.2)	1	1
CHM users	434 (21.8)	0.54 (0.47-0.61)	0.53 (0.46-0.60)[2]

[1]Adjusted for the age, residential area, monthly income and Charlson-Deyo comorbidity index.
[2]Adjusted for the sex, residential area, monthly income and Charlson-Deyo comorbidity index.
CHM: Chinese herbal medicine; OR: Odds ratio; CI: Confidence interval.

earlier research[8,9].

As of now, no study has been conducted to explore the long-term effect of CHM on the prevention of dementia among T2DM patients. The multi-variate regression in this study showed that receiving CHM in addition to conventional treatment was associated a 49% reduction in the risk of dementia (OR = 0.51), as compared to those without the use of CHM. Longer duration of CHM treatment was found to be associated with greater reduction in dementia risk, independent of gender and age. Specifically, receiving CHM treatment for more than two years was associated a 78% reduction in the risk of dementia (OR = 0.22). Despite a lack of comparable studies, the observed beneficial impact of CHM in preventing cognitive decline may contribute to a growing body of evidence indicating the clinical efficacy of CHM among individuals with chronic diseases[22,23]. We postulate that some herbal products may provide effective synaptic concentrations of monoamines for modulating hippocampal neurogenesis[24], hence supporting the positive impact of CHM shown in this research. For example, we found that usage of Hai-Piao-Xiao was associated with a reduced the risk of dementia, echoing an earlier study showing that this herb possessed a neuroprotective properties[25]. Another study described that extracts of Hai-Piao-Xiao slowed down lipid oxidation by chelating ferrous ions[26]. It is well known that oxidative stress is not only involved in the development of T2DM, but also in cognitive impairment[27]. Additionally, the elevated oxidative stress gradually triggered the release of neuroinflammatory cytokines and deposition of amyloid-â-peptide (Aâ), thus provoking development of dementia[4]. The prescriptions of Dan-Shen and Ge-Gen were associated with a decreased risk of dementia herein. Some animal models found that these two remedies could possibly elicit neurotherapeutic effects by inhibiting Aâ production and accumulation, thus decreasing the expression of proinflammatory cytokines[28,29].

We found those who received Ye-Jiao-Teng or Chuan-Qi experienced a nearly 50% lower risk of dementia compared to controls. An earlier murine model portrayed that the root of Ye-Jiao-Teng could downregulate nitric oxide and pro-inflammatory cytokines, such as tumor necrosis factor-á and interleukin (IL)-6, by abating NF-κB signaling[30]; both of these products had been implicated in triggering episodes of neurodegeneration. As for Chuan-Qi, recent pharmacological studies argued that the main component of this herbal formula has beneficial impact on the cardiovascular system through inhibition of pro-inflammatory mediators, such as IL-6 and matrix metalloproteinase-3[31,32]. These parameters are known to trigger cognitive dysfunction and decline[10].

Our findings also suggest that Bei-Mu and Da-Huang reduce the risk of dementia in T2DM patients. Emodin, an extract from Da-Huang, has been noted *in vitro* and *in vivo* to exert anti-inflammatory effect through restraining the NF-κB and p38MAPK pathways[33], which play key roles in regulating inflammatory cytokines and cellular reactions to stress[12]. Moreover, the effect of emodin on brain-derived neu-rotrophic factor (BDNF) could be responsible for its neuroprotective effects. Beneficial impact of emodin on neurological disorders may be related to the activation of intracellular signaling pathways, such as BDNF/tropomyosin receptor kinase B signaling[34].

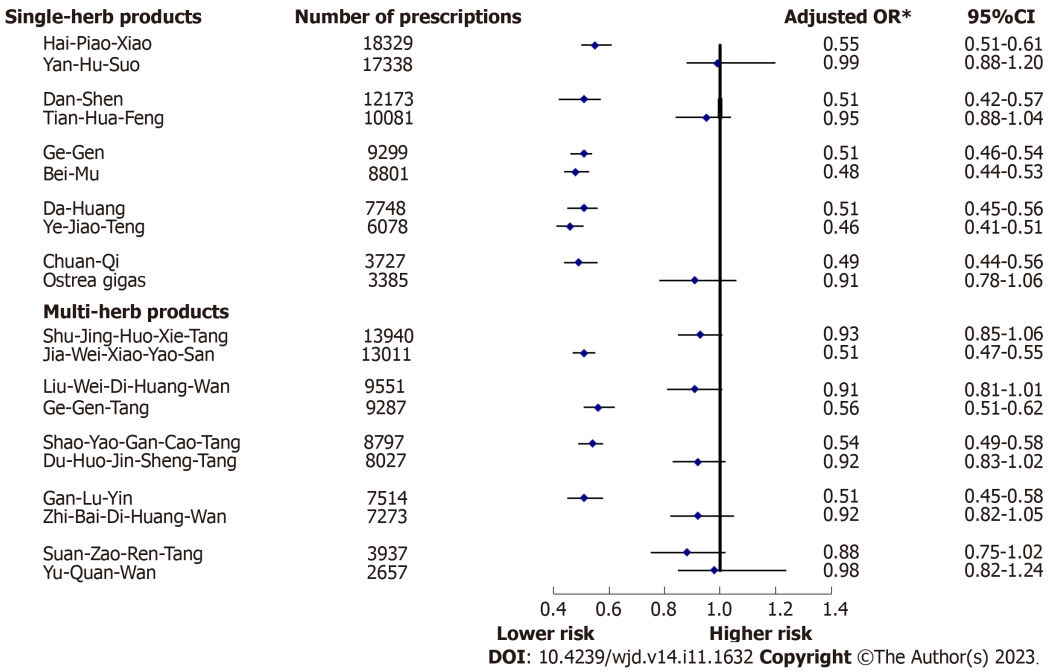

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Figure 2 Risk of dementia in relation to the ten commonly used single-herb and multi-herb products for subjects. OR: Odds ratio; CI: Confidence interval.

Of the commonly used multi-herb products to treat T2DM, we noticed that the prescriptions of SYGCT and JWXYS were correlated with reduction of dementia risk. One recent report posited that SYGCT might regulate the inflammatory state in a murine model by lessening the activation of Toll-like receptor 4/NF-κB signaling pathway[35]. The suppression of this pathway has been proven to inhibit Aâ-induced oxidative stress and inflammation[36], thus modifying risk of mitochondrial abnormalities[37]. JWXYS is often prescribed to relieve emotional and neuropsychological disorders in clinical practice, and its pharmacologic benefits in preventing dementia have been explored in animal studies. For example, Shen *et al*[38] noted that Angelica sinensis, a major compound in JWXYS, may enhance synaptic plasticity and upregulate the expression of hippocampal BDNF in depressed rats, induced by mild, chronic, and unpredictable stress. This herb can also inhibit abnormal tau phosphorylation, regulate the release of Aâ peptides, and decrease Aâ-induced neurotoxicity[39,40]. These underlying mechanisms may account for the positive impact of JWXYS found herein.

We also identified the benefit of GLY in reducing the subsequent predisposition to dementia among T2DM patients. As a classic CHM, GLY has been proven to exert the anti-inflammatory effect through the NF-κB dependent pathway[41]. Furthermore, *in vivo* research has shown that pretreatment of high glucose-stimulated cultured human umbilical vein endothelial cells with baicalin and baicalein, two flavonoids derived from GLY, remarkably inhibited glucose-induced inflammatory responses that mediate vascular permeability and production of reactive oxygen species (ROS)[42]. Activation of ROS may cause neuroinflammation that incites the Aâ generation[43]. We also noticed the positive effect of GGT in reducing dementia. Former evidence has shown that mice fed with GGT had profoundly lower levels of inflammatory-induced cytokines, as compared to untreated controls. Its anti-inflammatory effect may be correlated with down-regulation of NF-κB/p65 activation and the inhibition of NF-κB activity[44].

Despite being a pioneer study in exploring the effect of CHM on the risk of developing dementia in T2DM patients, this study should be considered in the context of several limitations. First, data used in this work were extracted from a claims-based database; accordingly, the information regarding biochemical data, family history, lifestyle behaviors, and body weight were not available. Thus, residual confounding by these factors might exist to partly bias the association herein. A larger cohort of T2DM patients created by prospective randomized trials, therefore, should be employed to further explore the potential mechanisms underlying the clinical benefits of CHM in preventing cognitive impairment. Second, participants in this study were assigned a diagnosis based on the ICD-9-CM coding only, which may carry a risk of inaccurate indication of true disease status. Thus, misclassification is possible. To deal with this issue, we recruited only those patients with either dementia or T2DM after they were recorded as having at least three outpatient claims or one inpatient record that indicated consistent diagnoses. It should also be acknowledged that the probability of exposure being misclassified was independent of disease status, and the probability of disease status being misclassified was independent of exposure status, which in turn may lead to an underestimate (dilution) of the true strength of an association between exposure and disease[45]. Third, information on T2DM severity was not available from the database, and failure to consider this factor might affect the veracity of the findings. As a result, we performed a sensitivity analysis in which we included the T2DM patients with no comorbidities only. This analysis indicated that those receiving CHM therapy still experienced a lower risk of dementia than the controls by 40%, which implied that the observed results were unlikely to be influenced by disease severity. Notwithstanding these limitations, this work is reflective of a population-based investigation that evaluates the association of CHM use with dementia risk among T2DM patients *via* a nationwide health claims program, thus leaving little room for non-response or loss to follow-up. Another strength of this work is the

long observation period. Facing with dementia being a major contributor to disability and the need for care, we had the advantages coming with the over 10-year observation period and the large sample size, which contributed to a better understanding of the link among diabetes, dementia and the benefits of CHM. Lastly, the nested case-control approach used is a rival alternative to a cohort analysis when studying time-varying exposure, such as patterns of drugs or treatments. Hence, the evidence from our study, using real-world health claims, may be comparable to findings derived from medical research using randomized controlled trials.

CONCLUSION

This population-based nested case-control study found that during conventional treatment for T2DM, the integration of CHM was associated with a reduction in the risk of deme-ntia by 49%. Moreover, the duration of CHM use had an inverse exposure-response association with the risk of dementia among T2DM patients. These findings suggested that a complementary therapy, specifically CHM, may contribute to the disease management of cognitive decline. Not only that, the results of our study further identified certain commonly prescribed herbal products that might be likely to lower dementia risk, hence paving the way for further pharmacological research to cure and control other health maladies. As far as clinical practice is concerned, we recommend that clinicians and people living with diabetes be aware of the increased risk of developing dementia and be vigilant in watching for early symptoms of this disease, so that appropriate diagnostic tests along with provable treatments could be administered when early symptoms of these conditions become observable.

ARTICLE HIGHLIGHTS

Research background

Over the last several years, patients afflicted with type 2 diabetes mellitus (T2DM) were found to have a nearly double likelihood of having dementia as compared to those without T2DM, which may take a critical toll on their health conditions.

Research motivation

Though the widespread use of Chinese herbal medicine (CHM) in the diabetic patients, to the knowledge of the authors, no population-level study has so far been done to assess if CHM use could be a potential disease management program in lowering risk of dementia among patients with T2DM.

Research objectives

To address this issue, a nested case-control study, aimed to compare dementia risk in T2DM patients with and without the use of CHM, was undertaken.

Research methods

Using a nationwide health insurance database, we identified incident patients diagnosed with T2DM between 2001 and 2010. Among them, each case, defined as who suffered from dementia occurring at least one year after T2DM onset, was randomly matched to one control without dementia. Relationship between CHM use and the risk of dementia was estimated by end of 2013 and evaluated using conditional logistic regression.

Research results

A total of 11699 dementia patients 20-70 years of age, were matched to 11699 non-dementia controls. Among them, use of CHM was correlated to a lower dementia risk (adjusted odds ratio = 0.51, 95% confidence interval: 0.48-0.53). Notably, those receiving high-intensity use of CHM had a 78% reduced the risk of dementia.

Research conclusions

Findings of this study indicated that add-on CHM formulae as part of T2DM care may be a potential treatment in preventing incident dementia.

Research perspectives

A large cohort of diabetic patients created by randomized trials are warranted to further explore the potential mechanisms underlying the clinical benefits of CHM on prevention of cognitive impairment.

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ensure the universal participation and equal opportunity of medical care. The interpretation and conclusions contained herein do not represent those of the Bureau of National Health Insurance, Department of Health, or National Health Research Institutes.

FOOTNOTES

Co-corresponding authors: How-Ran Guo and Tzung-Yi Tsai.

Author contributions: Liao HH, Livneh H, and Huang HL contributed equally to this work. Liao HH, Livneh H, and Huang HL were involved in the study design and drafted the manuscript; Livneh H, Lu MC and Tsai TY contributed to data analysis and revised the manuscript; Liao HH, Livneh H, and Hung JY contributed to the interpretation of data and provided comments on the final draft of the manuscript; Lu MC provided administrative support; Guo HR and Tsai TY were not only involved in design and conduct of the study, but also contributed to the software and validation of data; and all authors gave final approval of the version to be published, and agree to be accountable for all aspects of the work.

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ORIGINAL ARTICLE

Basic Study

Vascular endothelial growth factor B improves impaired glucose tolerance through insulin-mediated inhibition of glucagon secretion

Yu-Qi Li, Lu-Yang Zhang, Yu-Chi Zhao, Fang Xu, Zhi-Yong Hu, Qi-Hao Wu, Wen-Hao Li, Ya-Nuo Li

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Abstract

BACKGROUND

Impaired glucose tolerance (IGT) is a homeostatic state between euglycemia and hyperglycemia and is considered an early high-risk state of diabetes. When IGT occurs, insulin sensitivity decreases, causing a reduction in insulin secretion and an increase in glucagon secretion. Recently, vascular endothelial growth factor B (VEGFB) has been demonstrated to play a positive role in improving glucose metabolism and insulin sensitivity. Therefore, we constructed a mouse model of IGT through high-fat diet feeding and speculated that VEGFB can regulate hyperglycemia in IGT by influencing insulin-mediated glucagon secretion, thus contributing to the prevention and cure of prediabetes.

AIM

To explore the potential molecular mechanism and regulatory effects of VEGFB on insulin-mediated glucagon in mice with IGT.

METHODS

We conducted *in vivo* experiments through systematic VEGFB knockout and pancreatic-specific VEGFB overexpression. Insulin and glucagon secretions were

detected *via* enzyme-linked immunosorbent assay, and the protein expression of phosphoinositide 3-kinase (PI3K)/protein kinase B (AKT) was determined using western blot. Further, mRNA expression of forkhead box protein O1, phosphoenolpyruvate carboxykinase, and glucose-6 phosphatase was detected *via* quantitative polymerase chain reaction, and the correlation between the expression of proteins was analyzed *via* bioinformatics.

RESULTS

In mice with IGT and VEGFB knockout, glucagon secretion increased, and the protein expression of PI3K/AKT decreased dramatically. Further, in mice with VEGFB overexpression, glucagon levels declined, with the activation of the PI3K/AKT signaling pathway.

CONCLUSION

VEGFB/vascular endothelial growth factor receptor 1 can promote insulin-mediated glucagon secretion by activating the PI3K/AKT signaling pathway to regulate glucose metabolism disorders in mice with IGT.

Key Words: Vascular endothelial growth factor B; Insulin-mediated; Glucagon secretion; Prediabetes; Impaired glucose tolerance

Core Tip: Impaired glucose tolerance (IGT) is an abnormal metabolic stage between the normal state and diabetes, which belongs to prediabetes. Therefore, intervention in IGT can effectively reduce the incidence rate of diabetes. The pathological mechanism of IGT is related to glucose homeostasis imbalance and decreased insulin sensitivity. Currently, vascular endothelial growth factor B (VEGFB) has been reported to have the effect of restoring glucose tolerance and improving insulin sensitivity. Therefore, the use of VEGFB as a target for intervention has become the focus of current research. This research mainly illustrates the role of VEGFB in promoting insulin and glucagon secretion to alleviate IGT and its potential molecular mechanism.

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INTRODUCTION

Impaired glucose tolerance (IGT) is a homeostatic state between euglycemia and hyperglycemia, indicating a fasting blood glucose (FBG) level of < 7.0 mmol/L and/or postprandial blood glucose (PBG) level of 7.8-11.1 mmol/L after 2 h of oral glucose administration (75 g)[1]. IGT is considered a high-risk state in the early stage of type 2 diabetes (T2DM)[2]. Although only slight abnormal glucose metabolism is observed in this stage, the secretion function of β cells has been reported to be defective, leading to insulin resistance[3]. It is estimated that approximately 70% of IGT cases progress to T2DM[4]. When IGT occurs, insulin sensitivity decreases, causing reduced insulin secretion; decreased insulin levels can increase glucagon secretion[5]. Glucose metabolism disorders occur due to the abnormal production and release of insulin or glucagon[6].

Owing to the high reversibility of IGT, interventions to improve insulin sensitivity in patients with IGT can effectively prevent or delay the progression of IGT to T2DM, thereby reducing the incidence rate of diabetes and its complications [7]. Recently, vascular endothelial growth factor B (VEGFB) has been demonstrated to play a regulatory role in improving glucose metabolism and insulin sensitivity[8]. Animal experiments have confirmed that VEGFB can increase insulin supply and insulin sensitivity in high-fat diet (HFD)-fed mice[9]. Specific VEGFB overexpression in mice can affect insulin secretion and improve diabetes[10]. Notably, we previously reported a decline in insulin secretion in VEGFB knockout mice[11].

Various signal pathways participate in several biological activities such as glucose homeostasis, including the phosphoinositide 3-kinase (PI3K)/protein kinase B (AKT) signal pathway[12]. Activation of the PI3K/AKT pathway regulates insulin secretion in β cells[13]. All IGT types are accompanied with insulin resistance[14]. Alleviation of insulin resistance can promote insulin secretion and inhibit glucagon release, thereby improving IGT[15]. The combination of VEGFB and VEGF receptor 1 (VEGFR1) can activate downstream pathways, including the PI3K/AKT pathway related to the proliferation, differentiation, and metabolism of cells[16]. However, it remains unclear whether VEGFB regulates glucagon secretion and improves IGT *via* the PI3K/AKT pathway.

In this study, we established an HFD-induced mouse model of IGT and constructed VEGFB knockout and pancreatic-specific VEGFB overexpressed mice to determine the role of VEGFB in mice with IGT. This study aimed to explore the potential mechanism and regulatory effects of VEGFB on insulin-mediated glucagon secretion in mice with IGT. We observed an increase in glucagon secretion and blood glucose levels in mice with IGT and VEGFB knockout; the reduced

glucagon level in mice was due to the activated PI3K/AKT pathway. Therefore, we hypothesized that VEGFB can regulate insulin-medicated glucagon secretion in IGT.

MATERIALS AND METHODS

Experimental animals

Male C57BL/6 mice were purchased from Jinan PengYue Experimental Animal Breeding Co., Ltd. All mice were acclimatized to laboratory conditions (24 °C, 12 h/12 h light/dark, 50% humidity, ad libitum access to food and water) for 1 wk prior to experimentation. All animal experiments were under the approval of the animal ethics committee of Binzhou Medical University (IACUC protocol number: 2023-170). The VEGFB gene knockout mouse model was constructed by CRISPR/Cas 9[17]. Mouse genotypes were identified by agarose gel electrophoresis. The islet cell mass was extracted to detect the expression of VEGFB at the mRNA level in $VEGFB^{+/+}$ and $VEGFB^{-/-}$ mice (Figure 1A and B). Mice for the experiment were divided into 5 groups (n = 9, total = 45): Standard diet (SD), HFD-WT, HFD-KO, Con, and adeno-associated virus (AAV) group. $VEGFB^{+/+}$ mice and $VEGFB^{-/-}$ mice were selected randomly for the experiment. Nine $VEGFB^{+/+}$ mice fed with a standard diet were named the SD group. Twenty-seven $VEGFB^{+/+}$ mice and nine $VEGFB^{-/-}$ mice were fed with a HFD for 20 wk (Figure 1C). Nine $VEGFB^{+/+}$ and nine $VEGFB^{-/-}$ mice with HFD were defined as the HFD-WT and HFD-KO groups. Nine $VEGFB^{+/+}$ mice injected with targeting VEGFB186 AAV were named as AAV group, and nine $VEGFB^{+/+}$ mice injected with the non-targeting AAV were defined as the Con group. All mice were sacrificed and dissected in the 24th wk through cervical dislocation.

AAV injection of VEGFB in IGT mouse

The transcript and vector of AAV were constructed from OBIO Technology Co. LTD. (Shanghai, China). The VEGFB gene was overexpressed in the AAV group by injection of pAAV-CAG-$VEGFB^{186}$-P2A-EGFP-3xFLAG-WPRE vector, pAAV-CAG-EGFP-3xFLAG-WPRE was regarded as a control group. The frozen section of the pancreas with GFP green fluorescence was observed by fluorescence microscopy after 1 mo of injection to detect the efficiency of infection. The expression of VEGFB in islet cells was detected by Q-PCR (Figure 1D and E)[18].

IGT mouse model

FBG was tested after 12 h of starvation and PBG was tested 2 h after being fed. FBG and PBG were detected after 12 wk of HFD. Tail vein blood was detected from the 16th to the 24th wk. In the 16th wk, the FBG and PBG of IGT mice were higher than those of normal mice, but there are no differences between FBG, PBG, and oral glucose tolerance test (OGTT) of IGT mice in the rest four groups. Mice with 7.8 mmol/L < PBG < 11.1 mmol/L and 6.1 mmol/L < FBG < 7.0 mmol/L were regarded as the IGT animal model. The body weight and food intake of mice were measured once every two weeks from the 16th to the 24th wk (Figure 1F-H)[19].

Oral glucose tolerance test and intraperitoneal insulin tolerance test

In the oral glucose tolerance test (OGTT) experiment, mice were given glucose through gavage by a standard dosage of 2.0 mg/kg after fasting for over 12 h. While in the intraperitoneal insulin tolerance test (IPITT) experiment, mice were taken by intraperitoneal injection of insulin (0.5 U/kg) after over 6 h-starvation in advance. Both OGTT and IPITT curve was performed and the area under the curve (AUC) was calculated (Figure 1I and J)[20].

Isolation of islet cells

The pancreas was taken out, and the peripheral adipose tissue was isolated and placed in Hank's buffer after the mice's death. Injected 0.5 mg/mL collagenase P through the pancreatic duct and digested for 10 min after complete expansion of the pancreas. Hank's was pre-cooled at 4 °C to stop digestion, and cell mass was selected under the stereoscopic microscope (OLYMPUS-SZ61, Japan). Then the active stain of the islet cell mass was performed (Figure 1A)[21].

Hormonal analysis using Enzyme-linked Immunosorbent Assay

The blood was gained before the mice were sacrificed. And the serum was collected by centrifuge at 1000 g for 10 min to detect the glucagon, glycosylated hemoglobin (GHb), and serum insulin content using Enzyme-linked Immunosorbent Assay (ELISA) according to the instructions of the manufacturer. Measured the OD values at 450 nm with a microplate reader (BioTek, China)[22].

Hematoxylin and Eosin (H&E) staining

The pancreas tissue sample was washed with run water overnight in an embedding box to wipe off 4% paraformaldehyde which was fixed for 24 h. After gradient dehydration, the embedded box was put into soft wax for 1 h, and then into hard wax for 1 h. And then embedded tissue was sliced and baked at 60 °C for 1.5 h for gradient dewaxing and rehydration. Hematoxylin staining was performed for 5 min. Hydrochloric acid alcohol differentiation solution was used for 10 s and then followed by a water rinse. Stained with eosin for 1 min and then continue to gradient dehydration, sealed the cover glass for observation[23].

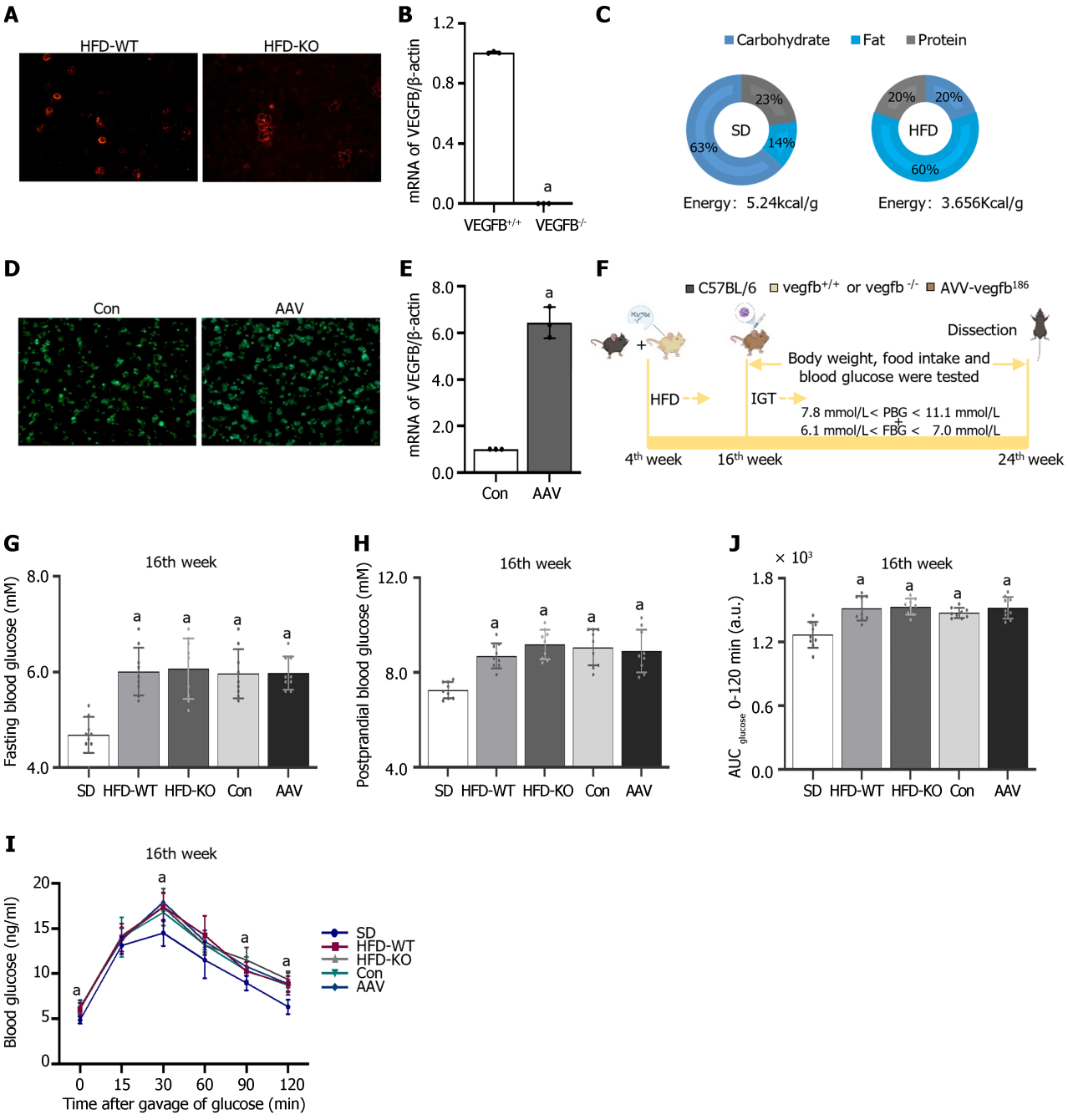

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Figure 1 The construction of impaired glucose tolerance mice with vascular endothelial growth factor B gene knockout and overexpressed. A: Active Stain of the islet cell mass in high-fat diet (HFD)-WT and HFD-KO mice; B: The mRNA expression of vascular endothelial growth factor B (VEGFB) in the islet of mice in the HFD-WT and HFD-KO group; C: The ingredients of standard diet (SD) and HFD; D: Green fluorescence staining of the islet with adeno-associated virus (AAV) infection in Con and AAV mice; E: The mRNA expression of VEGFB in the islet of mice in the Con and AAV group; F: The flow chart of animal experiments; G: Fasting blood glucose at the 16th wk; H: PBG at the 16th wk; I: Oral glucose tolerance test at the 16th wk; J: Area under the curve at the 16th wk. $^{a}P < 0.05$ *vs* vegfb$^{+/+}$; $^{a}P < 0.05$ *vs* Con; $^{a}P < 0.05$ *vs* SD. HFD: High-fat diet; AAV: Adeno-associated virus; SD: Standard diet; VEGF: Vascular endothelial growth factor.

Transmission electron microscopy

Pancreas tissue was fixed with 2.5% glutaraldehyde solution and washed with PBS, and repeated overnight. Washed and fixed with 1% osmotic acid fixed buffer, then gradient dehydrated with ethanol, soaked with acetone and embedding agent at room temperature overnight. Embedded at gradient temperature and polymerized the sample blocks for quick trimming and ultra-thin cutting. After staining with uranium diacetate and lead citrate, the section was photographed by the transmission electron microscope (JEM-1400, Japan)[24].

Immunofluorescence

The baked slide was dewaxed and rehydrated by using xylene, alcohol, and distilled water. And then immersed in the citric acid buffer in an antigen repair box to execute antigen repair at 100 °C. After washing with PBS, the sections were added with 3% hydrogen peroxide and incubated with serum. Added the primary antibody of insulin (1:200, Proteintech, 66198-1-I g) and glucagon (1:100, Abcam, ab92517) to the tissue. The next day, the tissue was completely covered by drops of fluorescence enzyme-labeled Goat anti-mouse (1:100, ZSGB-BIO, ZF-0314) in a wet box. Incubated with DAPI in a dark box and rinsed with PBS four times and observed under the fluorescence microscope (Zeiss, LSM880, Germany)[25].

Western blot

The pancreas tissue was weighed 20 mg and added RIPA (Solarbio, R0010, Beijing) lysate containing protease inhibitor cocktail (Solarbio, PO100, Beijing), centrifuged at 4°C at 12000 r/min after 30 min of ice lysed, the supernatant was taken and added the loading buffer (Solarbio, D1020-5, Beijing), heated it at 99°C for 10 min. The protein content was analyzed with a Bradford protein quantitative kit. The proteins were transferred onto the PVDF membrane. The PVDF membrane was sealed and incubated with the primary antibody overnight. Incubated secondary antibody and observed with ECL hypersensitivity exposure (Tanon5200, Tanon Science & Technology)[22].

RNA extraction and quantitative real-time PCR

The total RNA from the pancreas was extracted by isopropyl alcohol and RNA isolator (R401-01-AA, Vazyme Biotech Co., Ltd.). The genomic DNA removal reaction system was configured with 4 × gDNA wiper Mix (R223-01, Vazyme Biotech Co., Ltd.) and DEPC water according to the concentration of RNA samples. The reverse transcription reaction system was prepared using a 5 × HiScript II qRT SuperMix II. And then cDNA was executed with 2 × ChamQ Universal SYBR qPCR Master Mix (Q711-02, Vazyme Biotech Co., Ltd.) by PCR QuantStudio 3 (Thermo Fisher Scientific, Inc.). Primer sequences were: Forkhead box protein O1 (FOXO1), F: 5′-CCCAGGCCGGAGTTTAACC-3′, R: 5′-GTTGCT-CATAAAGTCGGTGCT-3′; Phosphoenolpyruvate carboxykinase (PEPCK), F: 5′- CTGCATAACGGTCTGGACTTC-3′, R: 5′-CAGCAACTGCCCGTACTCC-3′; glucose-6 phosphatase (G6Pase), F: 5′-CGACTCGCTATCTCCAAGTGA-3′, R: 5′-GTTGAACCAGTCTCCGACCA-3′[26].

Data analysis

By SPSS 20.0 statistics software to carry on the analysis, all data were calculated as mean ± SD. One-way analysis of variance (ANOVA) was used to compare the means of multiple samples and *t*-test was used to compare the means of two samples. Statistically, a significant difference was described as $P < 0.05$.

RESULTS

Effects of VEGFB on glucose metabolism, glucose tolerance, and insulin sensitivity in IGT mice

The body weight and food intake in HFD-KO were higher than in HFD-WT mice from the 22nd wk. Compared with mice in the Con group, the body weight and food intake of mice decreased after the injection of AAV (Figure 2A and B). FBG of HFD-KO mice was significantly higher than that of HFD-WT mice from the 22nd wk. After AAV injection, the FBG and PBG of mice were ameliorated dramatically from the 20th wk and the 22nd wk (Figure 2C and D). OGTT and IPITT showed that glucose tolerance and insulin sensitivity in the AAV group were increased, while in the HFD-KO group were decreased after 12 h fasting in the 24th wk (Figure 2E-H). Meanwhile, the contents of GHb, serum glucose, and serum glucagon increased in HFD-KO mice and decreased in AAV group significantly, while the content of serum insulin decreased in HFD-KO group and increased in the AAV group significantly (Figure 2I-L).

Effects of VEGFB on the morphology of pancreatic islets, number of secretory granules of islet α cells, and β cells

Scattered islet cell clusters with different sizes and shallow staining can be seen in the exocrine pancreas of mice. Compared with mice fed with SD, IGT mice have smaller islet cell clusters with irregular edges and smaller volumes (Figure 3A). It can be seen under the transmission electron microscope that the quantity of β cells is large and the volume is small, and the β cells contain different sizes of secretory granules. When IGT occurs, the number of secretory granules decreased in β cells. The α cells are relatively large in volume, and large secretory granules can be seen in the α cells (Figure 3B). After immunofluorescence labeling, red fluorescent-labeled insulin was mostly concentrated in the center of the islet, while green fluorescent-labeled glucagon was mostly located in the periphery of the islet (Figure 3C).

Compared with HFD-WT mice, the number, area, diameter, and mass of islets in HFD-KO mice decreased significantly. Compared with the Con mice, the number, area, diameter, and mass of pancreatic islets in AAV mice were significantly increased (Figure 4A-D). The number of mature and immature secretory granules decreases with the knockout of the VEGFB gene and increases after AAV injection (Figure 4E-G). Unlike β cells, after the VEGFB gene is knocked out, the secretory granules in α cells increased, while secretory granules declined after overexpression of VEGFB by AAV injection (Figure 4H).

The fluorescence intensity of insulin secreted by β cells and the mass of the β cells in HFD-KO mice reduced significantly, while in α cells the fluorescence intensity of glucagon secreted by α cells and the mass of the α cells significantly increased (Figure 4I-L).

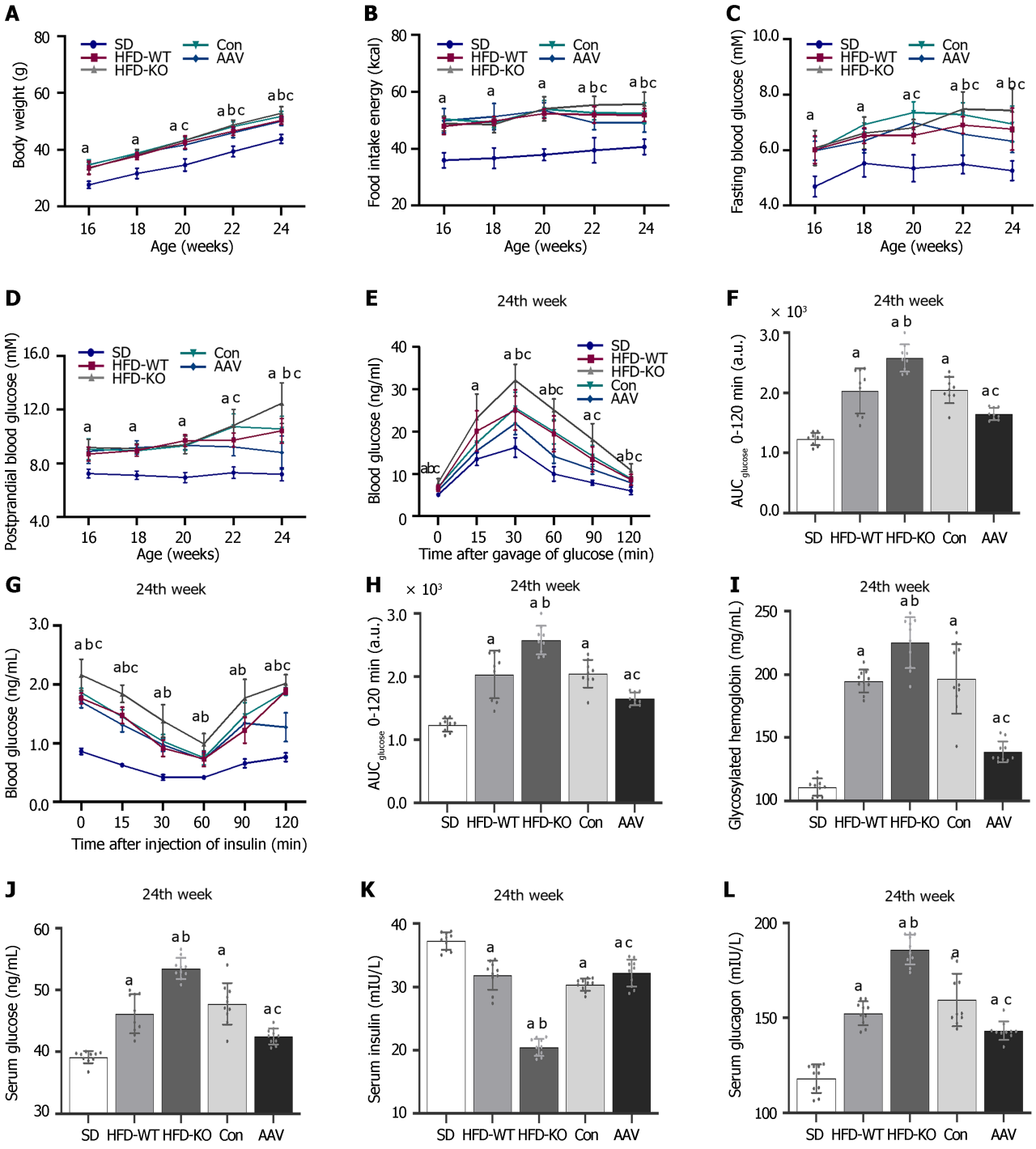

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Figure 2 Effects of vascular endothelial growth factor B on body weight and blood glucose level. A: Body weight from the 16th to the 24th wk; B: Food intake energy from the 16th to the 24th wk; C: Fasting blood glucose from the 16th to the 24th wk; D: PBG from the 16th to the 24th wk; E and F: Oral glucose tolerance test and area under the curve (AUC); G and H: Intraperitoneal insulin tolerance test and AUC; I: GHb; J: Serum glucose; K: Serum insulin; L: Serum glucagon. $^{a}P < 0.05$ *vs* standard diet; $^{b}P < 0.05$ *vs* high-fat diet-WT; $^{c}P < 0.05$ *vs* Con. HFD: High-fat diet; AAV: Adeno-associated virus; SD: Standard diet.

Protein-protein interaction network creation

To examine the most significant clusters of the diversely expressed genes (DEGs), the protein-protein interaction (PPI) network of DEGs was constituted by Search Tool for the Retrieval of Interacting Genes (STRING11.5; https://string-db.org/). There were 8 nodes and 21 edges shown in the PPI network, and which PPI enrichment *P* value is 1.4E-09 (Figure 5).

VEGFB/VEGFR1 regulates insulin secretion of β cells in IGT mice

In order to confirm that VEGFB plays a role by combining its receptor, VEGFR1, we detect the expression of VEGFB and VEGFR1 with the western blot method. We observed that the VEGFR1 expressed highly in the AAV group and had a low

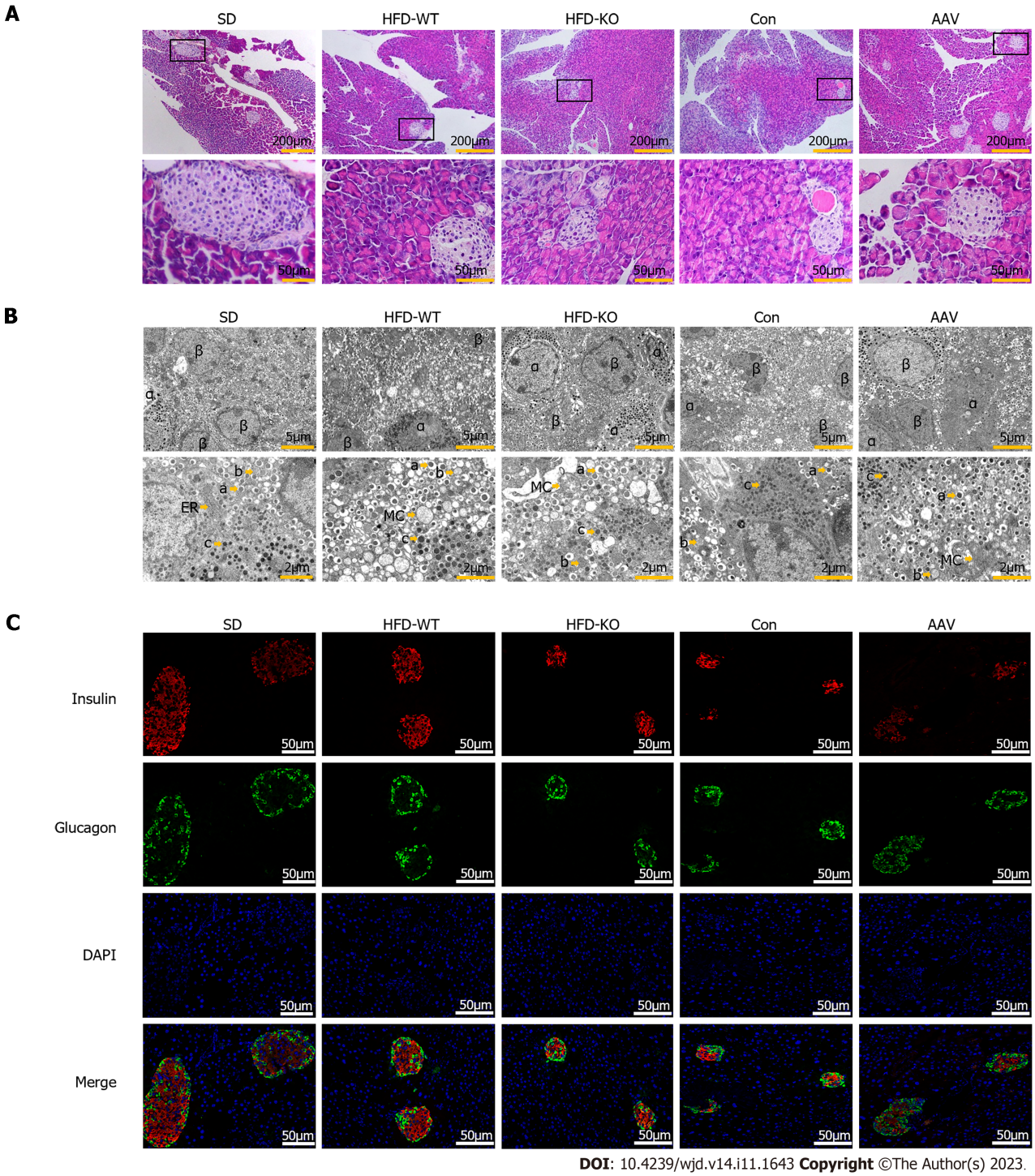

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Figure 3 Effects of vascular endothelial growth factor B on the cellular structure of islet. A: The HE staining of the pancreas under the light microscope (scale bar = 200 μm and 50 μm); B: The ultrastructure of the islet cell under the transmission electron microscope (scale bar = 5 μm and 2 μm); C: The immunofluorescence of islet cells under the fluorescence microscope (scale bar = 50 μm). HFD: High-fat diet; SD: Standard diet.

expression in HFD-KO mice as the VEGFB gene was overexpressed or knocked out (Figure 6A-C). We extracted the islet mass from the pancreas islet of IGT mice. We observed that the concentration of calcium and insulin declined in the isolated islet mass of HFD-KO mice, but went high in AAV mice with the glucose incubation when compared with Con mice (Figure 6D-G).

VEGFB regulates glucagon secretion of α cells via PI3K/AKT signal pathway in IGT mice

The protein expression of insulin resistance (IR) and insulin resistance substrate (IRS) was inhibited in IGT mice when compared with SD mice. IR and IRS expression were at the lowest levels in HFD-KO mice and increased dramatically after AAV injection. PI3K/AKT was considered to have a relationship with glucagon secretion, therefore we detected the relative protein expression levels through the signal pathway. PI3K and AKT expression levels remained no obvious

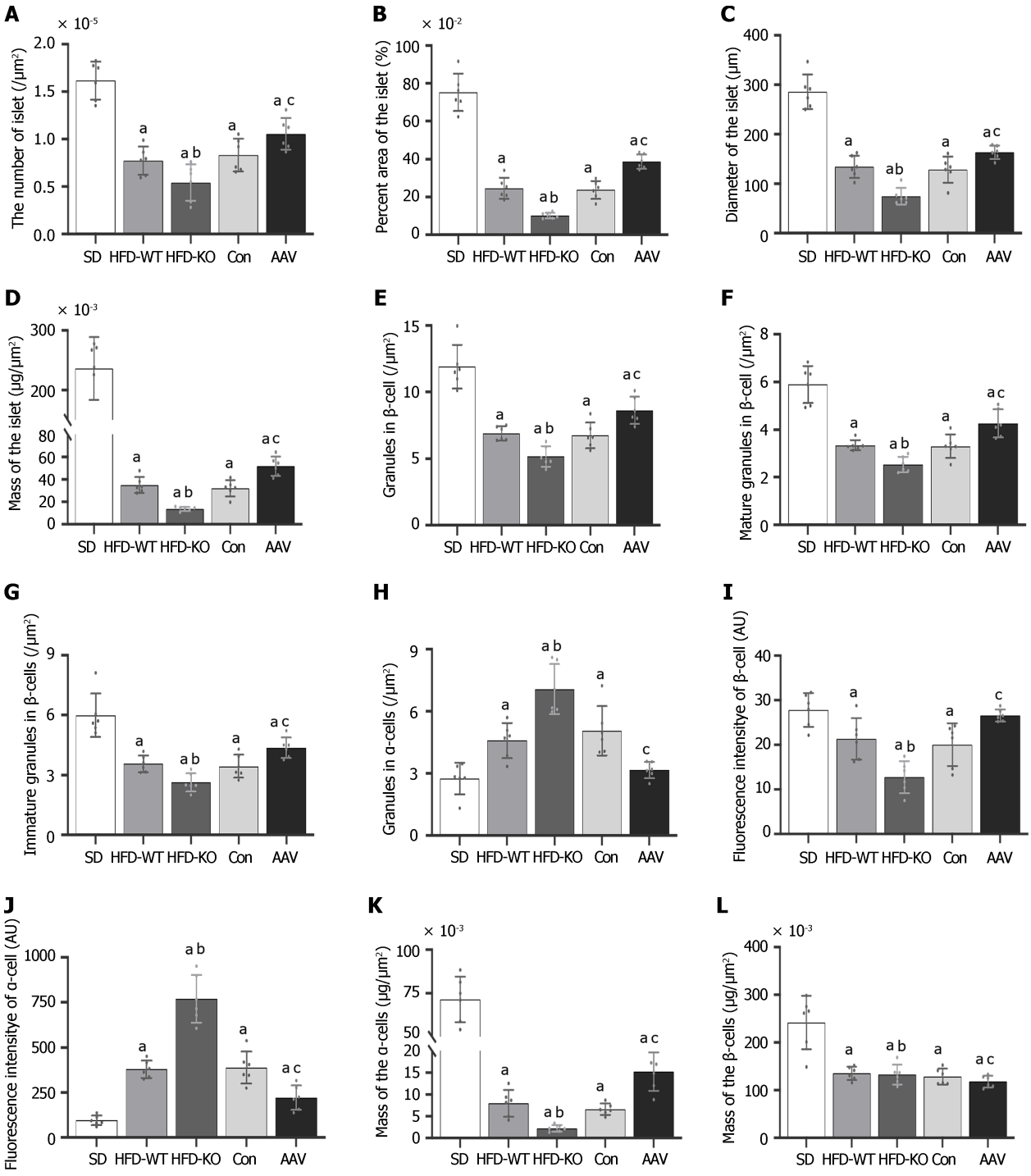

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Figure 4 The quantitative analysis of vascular endothelial growth factor B affects the cellular structure of islets. A: The number of islets; B: The relative area of the islet; C: Diameter of the islet; D: Mass of the islet; E: The number of granules in β cells; F: The number of mature granules in β cells; G: The number of immature granules in β cells; H: The number of granules in α cells; I: Mean fluorescence intensity of β cells; J: Mean fluorescence intensity of α cells; K: Mass of the β cells; L: Mass of the α cells. $^{a}P < 0.05$ *vs* standard diet; $^{b}P < 0.05$ *vs* high-fat diet-WT; $^{c}P < 0.05$ *vs* Con. HFD: High-fat diet; AAV: Adeno-associated virus; SD: Standard diet.

changes while the phosphorylation of PI3K and AKT changed obviously in HFD-KO and AAV mice (Figure 7A-E). Moreover, glucagon secretion-related genes like FOXO1, G6Pase, and PEPCK were examined by Q-PCR, which demonstrated high expressions level in HFD-KO mice and low expression in AAV mice compared with Con mice (Figure 7F).

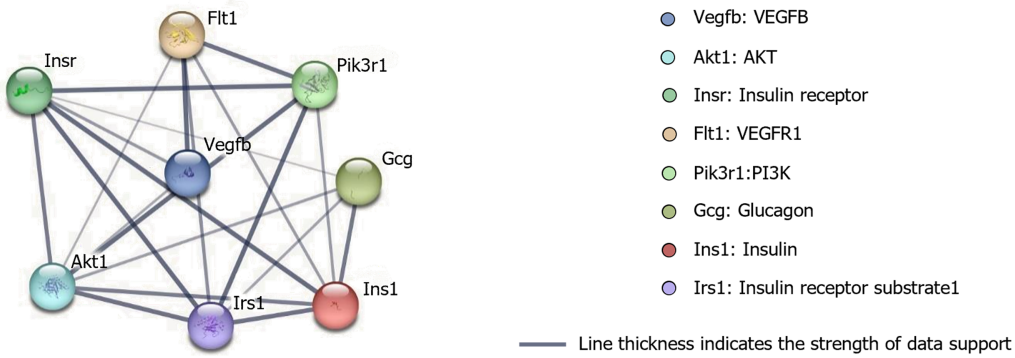

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Figure 5 Cluster analysis of the protein-protein interaction network. There were 8 nodes and 21 edges shown in the protein-protein interaction network. VEGF: Vascular endothelial growth factor.

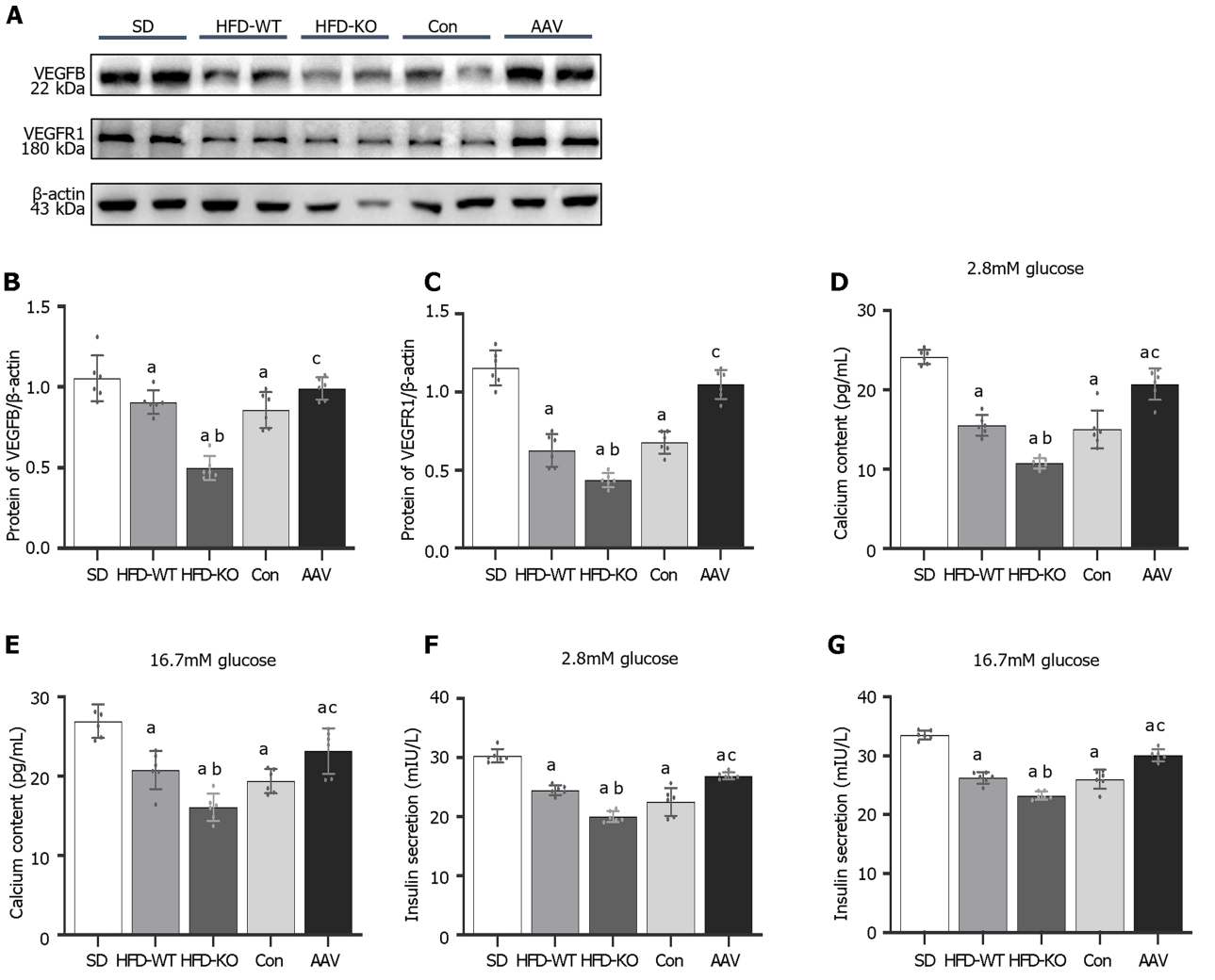

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Figure 6 Vascular endothelial growth factor B regulates the content of Ca^{2+} and insulin *via* combining with vascular endothelial growth factor receptor 1. A: The protein expression of vascular endothelial growth factor B (VEGFB); B: The protein expression of VEGFB/β-actin; C: The protein expression of VEGF receptor 1/β-actin; D and E: The content of Ca^{2+} with 2.8 mmol/L and 16.7 mmol/L glucose; F and G: The content of insulin with 2.8 mmol/L and 16.7 mmol/L glucose. $^{a}P < 0.05$ *vs* standard diet. $^{b}P < 0.05$ *vs* high-fat diet-WT; $^{c}P < 0.05$ *vs* Con. HFD: High-fat diet; AAV: Adeno-associated virus; SD: Standard diet.

DISCUSSION

Seven members of the VEGF family have been identified to date, including VEGFA, VEGFB, VEGFC, VEGFD, VEGFE, VEGFF, and placental growth factor. These subtypes play different roles in many biological activities. *VEGFA* and *VEGFC* deletion can cause pathological changes and biological function loss. VEGFC and VEGFD are mainly involved in the regulation of lymphangiogenesis.

VEGFB is primarily expressed in tissues such as the heart, skeletal muscle, vascular smooth muscle, and pancreas[27]. Unlike other members, the role of VEGFB in the vascular system remains unclear[28]. Recently, VEGFB has been reported to affect glucose metabolism and insulin sensitivity. Robciuc *et al*[9] revealed that after injection of AAV-VEGFB^{186}, glucose tolerance and peripheral insulin sensitivity improved significantly in HFD-fed mice. Moreover, Shang *et al*[10] demonstrated that specific overexpression of VEGFB in mice can enhance insulin sensitivity and secretion. The novel role of VEGFB in regulating glucose and insulin homeostasis provides an innovative approach for preventing the progression of IGT to T2DM.

We previously indicated that VEGFB has a similar regulatory effect on insulin secretion in MIN6 cells. After constructing recombinant VEGFB protein, the calcium content and insulin secretion in MIN6 cells increased with glucose stimulation[29]. Impaired fasting glucose (IFG) and IGT are currently considered to be the prediabetes state. According to the prediction of the International Diabetes Federation (IDF), > 470 million people will have prediabetes in 2030[30]. Active intervention in these two prediabetes states is of great significance to prevent the onset of diabetes and improve its long-term prognosis. Therefore, an increasing number of studies are focusing on the regulation mechanism of glucose tolerance in prediabetes.

According to the criteria of the American Diabetes Association, IFG refers to an FBG level of 5.6-6.9 mmol/L[31]. When IFG occurs, insulin resistance reduces the inhibitory effect of insulin on endogenous glucose production[32]. Although islet β cells undergo functional impairment, insulin secretion remains normal in the early stage[33]. After glucose stimulation, glucose abnormalities commonly occur during fasting conditions. The criteria for IGT include an FBG level of > 5.6 mmol/L and PBG level of 7.8-11.0 mmol/L[34]. An abnormal increase in PBG level is associated with peripheral insulin resistance[35]. After glucose stimulation, it is difficult to maintain PBG balance due to a significant decline in insulin secretion in the early stage, thus resulting in PBG abnormalities.

IGT is also associated with obesity[36]. Obesity leads to the excessive release of free fatty acids from adipocytes in the body, inducing insulin resistance, promoting lipid aggregation of islet β cells, and damaging the function of islet β cells. These changes further affect the secretion of glucose and insulin, leading to IGT[36]. Kim *et al*[37] revealed that patients with obesity and fatty insulin resistance are 4.3 times more likely to have IGT than normal patients. Bacha *et al*[38] reported that the visceral and subcutaneous fat proportion in adults with IGT was significantly higher than that in healthy adults[38]. We previously demonstrated that the mouse body weight as well as subcutaneous fat, inguinal fat, triglyceride, and cholesterol contents significantly increased after 10 wk of HFD feeding[17]. Moreover, significant increases in FBG and PBG levels were positively correlated with weight gain. At 20 wk of HFD feeding, mice with FBG levels of > 7 mmol/L and PBG levels of > 16.7 mmol/L developed T2DM. In the current study, we constructed the mouse model of IGT *via* HFD. After 12 wk of HFD feeding, FBG and PBG levels were measured in 16-wk-old mice. Mice with T2DM or IFG alone were excluded, and those with FBG levels of 5.6-6.9 mmol/L and PBG levels of 7.8-11.0 mmol/L were selected as mice with IGT. In mice with IGT and VEGFB knockout, FBG and PBG levels increased gradually with increasing body weight, whereas *VEGFB* overexpression significantly improved the body weight and blood glucose levels of mice with IGT.

IGT is associated with glucose tolerance and insulin sensitivity[39]. The OGTT and IPITT are used to examine glucose tolerance and insulin sensitivity in the clinical diagnosis of diabetes, respectively[40]. Currently, OGTT is considered the gold standard for evaluating IGT[41]. Using OGTT and IPITT, we further revealed that *VEGFB* knockout can increase the AUC values of OGTT and IPITT in mice with IGT, whereas *VEGFB* overexpression can significantly reduce the AUC values. Wu *et al*[42] revealed significant differences in plasma VEGFB expression between patients with IGT and those with normal glucose tolerance. Hagberg *et al*[43] confirmed that *VEGFB* regulates glucose tolerance in db/db mice, which is consistent with our results.

Abnormal elevation in glycosylated hemoglobin (GHb) levels is another measure of IGT[44]. Ye *et al*[45] reported a significantly strong association between VEGFB and GHb. We observed that the effect of VEGFB on GHb was consistent with that of FBG and PBG. After 8 wk of intervention with AAV overexpressing VEGFB in mice with IGT, the serum GHb levels decreased significantly. IGT is characterized by decreased insulin and glucagon secretion. Under hyperglycemic conditions, β cells release adequate insulin, but α cells release relatively reduced levels of glucagon, prompting α cells to absorb or utilize glucose from the blood[46]. During the abnormal production or release of insulin or glucagon, the body continues to remain hyperglycemic due to inconsistent physiological functions. Our research suggested that VEGFB improves blood glucose balance in mice with IGT. List *et al*[47] showed that after the occurrence of IFT, the average size of pancreatic islets decreased significantly in VEGFB$^{-/-}$ mice with IGT, along with a decrease in the number, size, area, and mass of pancreatic islets. After 4 wk of VEGFB overexpression, the size and number of pancreatic islets partially recovered as blood glucose levels improved.

Furthermore, fluorescence intensity analysis of β cells demonstrated that the effect of VEGFB was related to the number of islet cells. The IGT-induced decrease in insulin secretion is related to endocrine granules in β cells. β cells contain mature and immature secretory granules. Proinsulin and other soluble proteins are encapsulated in immature granules [48]. Immature granules are transformed into mature granules through a series of regulatory steps. Mature granules are stored in the cisterns or transported near the cell membrane[49]. When the level of blood glucose increases in the body, mature granules fuse with the cell membrane to release insulin. Similar to insulin, glucagon-related proteins accumulate in α cells in the form of granules to release glucagon in a paracrine manner[50]. Enhanced insulin secretion inhibits the

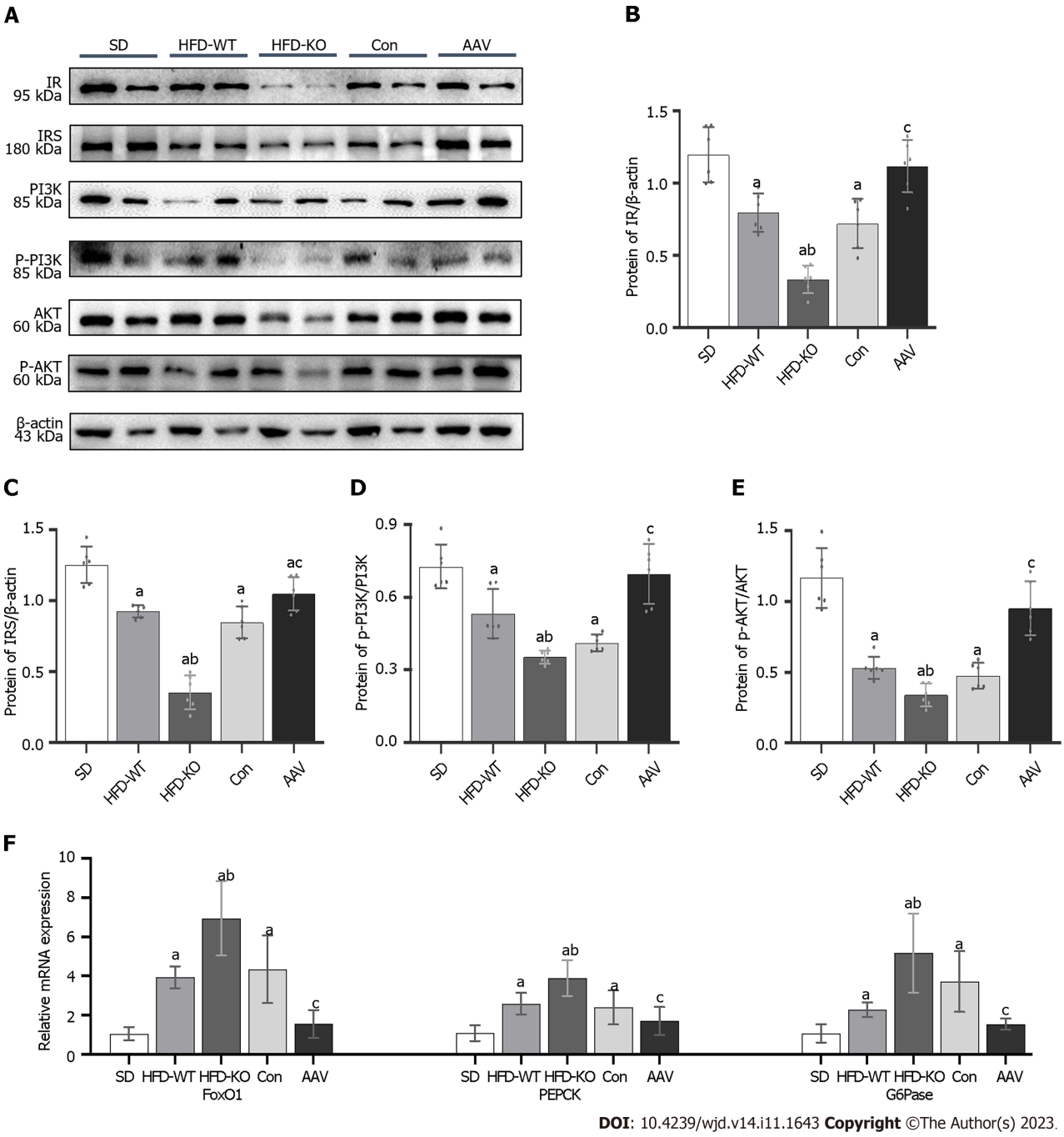

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Figure 7 Vascular endothelial growth factor B regulates glucagon secretion *via* PI3K/AKT signal pathway and relative genes. A: The protein expression of IR, insulin resistance substrate (IRS), PI3K, p-PI3K, AKT, and p-AKT; B: The protein expression of IR/β-actin; C: The protein expression of IRS/β-actin; D: The protein expression of p-PI3K/PI3K; E: The protein expression of p-AKT/AKT; F: The mRNA expression of forkhead box protein O1, PEPCK, and G6Pase. $^{a}P < 0.05$ *vs* standard diet; $^{b}P < 0.05$ *vs* high-fat diet-WT; $^{c}P < 0.05$ *vs* Con. HFD: High-fat diet; AAV: Adeno-associated virus; SD: Standard diet; IRS: Insulin resistance substrate; FOXO: Forkhead box protein O1.

secretion pathway of α cells, which reduces proglucagon synthesis in the endoplasmic reticulum, thereby reducing glucagon secretion. Studies have confirmed that insulin-mediated inhibition of glucagon secretion in patients with obesity is impaired as the severity of IGT is similar to that of insulin resistance and cellular dysfunction. We observed that the number of mature and immature granules in islet β cells decreased significantly. Furthermore, the number of granules in α cells increased significantly after VEGFB knockout, whereas the number of granules was significantly affected by VEGFB overexpression in mice with IGT. It is inferred that the effect of VEGFB on IGT hyperglycemia may be related to the activation of insulin-mediated inhibition of glucagon secretion.

Multiple signaling pathways regulate the secretion of insulin and glucagon[51]. Bioinformatics results regarding protein-protein interaction revealed that proteins related to insulin and glucagon secretion, such as insulin receptor (IR), IR substrate (IRS), PI3K, and AKT, are biologically related to VEGFB and VEGFR1. The combined signaling pathways of VEGFB and VEGFR1 can activate various biological reactions (*e.g.*, VEGFB promoting insulin secretion in MIN6 cells by binding to VEGFR1)[29]. Glucagon secretion in α cells critically depends on insulin secretion in β cells[52]. The PI3K/AKT

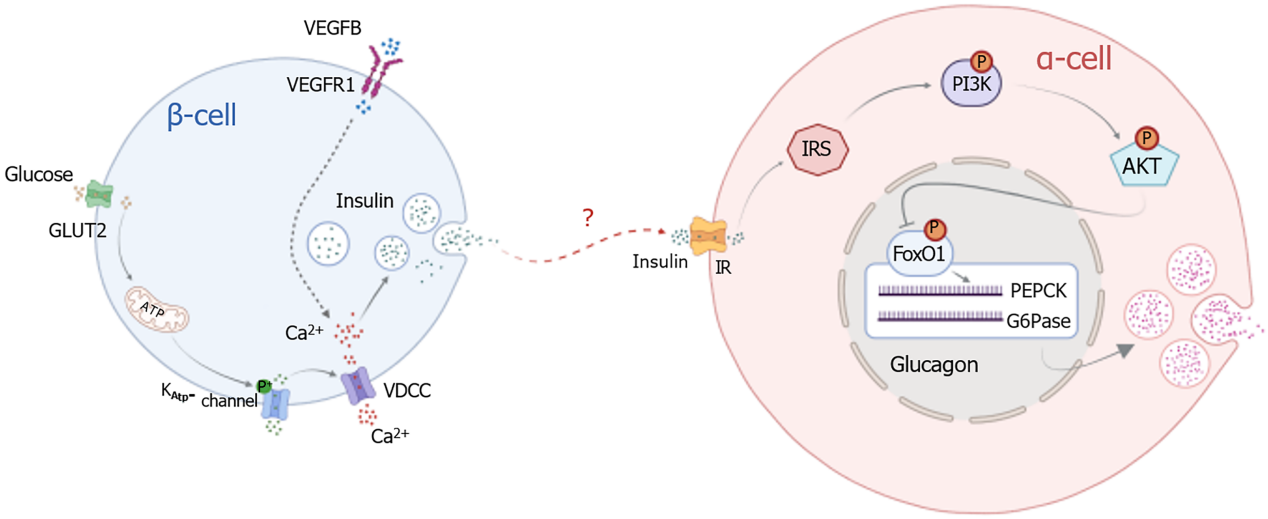

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Figure 8 Pattern diagram of vascular endothelial growth factor B regulates insulin-medicated glucagon secretion through PI3K/AKT signal pathway. IRS: Insulin resistance substrate; VEGF: Vascular endothelial growth factor; VDCC: Voltage dependent calcium channels.

pathway is a typical pathway in insulin signal and is regulated by multiple factors such as insulin and IR; moreover, it is involved in the regulation of glucagon secretion in α cells. When the glucose level increases, insulin is secreted in β cells.

After insulin binds to IR on the membranes of α cells, the conformation of IR changes, leading to the recruitment of IRS proteins[53]. P13K and AKT are downstream proteins of IRS[54]. When PI3K receives the upstream IRS protein signal, its subunit p85 binds to IRS to promote the phosphorylation of PI3K[55]. After PI3K phosphorylation, AKT aggregates in the cell membrane and changes its structure to induce activity, inhibiting the gene encoding glucagon through AKT phosphorylation, thus reducing glucagon secretion in α cells[26]. Mancuso *et al*[22] utilized *in vitro* α cell models and revealed that the activation of the PI3K/AKT pathway can inhibit glucagon secretion. In conclusion, VEGFB/VEGFR1 can promote β cells to secrete insulin and activate PI3K/AKT signaling pathways in α cells.

Hyperglycemia in T2DM caused by increased glucagon levels is often accompanied with abnormal gluconeogenesis [56]. PEPCK and G6Pase are two rate-limiting enzymes in gluconeogenesis[57]. Various hormones and signaling pathways affect the transcription and expression of these two key enzymes, thereby affecting glucagon secretion[58]. Among them, insulin affects the secretory function of glucagon in α cells *via* PEPCK and G6Pase regulation[59]. The PI3K/AKT signaling pathway and its downstream FOXO1 gene regulate the expression of PEPCK and G6Pase[60]. FOXO1 is an important member of the fork-helix transcription factor family, closely related to the proliferation, apoptosis, differentiation, and growth of cells[61]. FOXO1 regulates PEPCK and G6Pase by directly binding to its target DNA sequence and interacting with nuclear receptors[62]. Stavroula indicated that insulin activates the PI3K/AKT signaling pathway and inhibits FOXO1 activity, thereby promoting glucose metabolism[63].

CONCLUSION

Our research demonstrates that the binding of VEGFB to VEGFR1 promotes insulin release, and insulin binding to its receptor activates PI3K/AKT signaling to inhibit the expression of *FOXO1*, *PEPCK*, and *G6Pase*, thereby suppressing glucagon secretion. Our experiments confirmed that combining VEGFB and VEGFR1 can stimulate insulin-mediated glucagon secretion in α cells by activating the PI3K/AKT signaling pathway to regulate glucose metabolism disorders in mice with IGT (Figure 8). This provides not only new avenues for investigating the molecular mechanism of IGT but also a more theoretical and experimental basis for diagnosing and treating diabetes in the early stage and preventing its development into T2DM.

ARTICLE HIGHLIGHTS

Research background

The results showed that after vascular endothelial growth factor B (VEGFB) overexpression, serum glucose, glucose tolerance, and insulin sensitivity in impaired glucose tolerance (IGT) mice were improved, and the number of secretory granules of β cells was increased by activating the PI3K/AKT signal pathway.

Research motivation

VEGFB can promote insulin-mediated glucagon secretion by activating the PI3K/AKT signaling pathway to improve glucose metabolism in mice with IGT.

Research objectives

This study illustrated the role of VEGFB in regulating insulin-mediated glucagon secretion in mice with IGT by regulating the PI3K/AKT signal pathway, which indicated the regulatory function of VEGFB in improving the IGT condition of mice and preventing the onset of type 2 diabetes in the body. This study proved the molecular mechanism of VEGFB regulating IGT and provided theoretical basis for the treatment of prediabetes.

Research methods

The research was conducted by Crispr Cas9 and high-fat diet feeding to construct the animal model. Western blot and qRT-PCR were used to detect the expression of proteins and genes. Bioinformatics was used to analyze the correlation between relative proteins in the PI3K/AKT signal pathway.

Research results

To specify the underlying mechanism of VEGFB effects on insulin-mediated glucagon secretion in impaired glucose tolerance.

Research conclusions

Type 2 diabetes (T2D) can be prevented in pre-diabetic individuals with impaired glucose tolerance. Therefore, converting IGT into a normal condition is critical to prevent the onset of diabetes.

Research perspectives

Diabetes is a worldwide health problem, affecting about 415 million people globally. Among them, the number of patients with type 2 diabetes accounts for about 90% of the number of patients with diabetes with a population of 373 million. Pre-diabetes is the main risk factor for progression to type 2 diabetes. Impaired glucose tolerance (IGT) is a pre-diabetes state, and it is mainly manifested as fast blood glucose (FBG) level of<7.0 mmol/L and/or posttraumatic blood glucose (PBG) level of 7.8 – 11.1 mmol/L after 2 h of oral glucose administration. Long-term IGT will greatly increase the risk of type 2 diabetes. Therefore, precision intervention can improve the insulin sensitivity of patients with IGT and effectively prevent or delay the progression to type 2 diabetes. VEGFB, as a novel metabolic regulatory target, has received much attention for its role in regulating insulin sensitivity. Therefore, we successfully constructed a mouse model with IGT, and intervened in the upregulation and downregulation of the VEGFB gene at the gene level, so as to explore that VEGFB regulates insulin-mediated Glucagon secretion, and thus improves IGT symptoms in pre-diabetes.

FOOTNOTES

Author contributions: Li YQ and Li YN conceived and designed the study; Zhang LY, Zhao YC, and Xu F performed the experiments; Hu ZY and Wu QH analyzed the data; Li YQ wrote the manuscript; Li WH revised the manuscript; and all authors approved the final version of the article.

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ORIGINAL ARTICLE

Basic Study

Molecular targets and mechanisms of *Jiawei Jiaotai Pill* on diabetic cardiomyopathy based on network pharmacology

Yu-Juan Wang, Yan-Li Wang, Xiao-Fan Jiang, Juan-E Li

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Abstract

BACKGROUND

Jiawei Jiaotai Pill is commonly used in clinical practice to reduce apoptosis, increase insulin secretion, and improve blood glucose tolerance. However, its mechanism of action in the treatment of diabetic cardiomyopathy (DCM) remains unclear, hindering research efforts aimed at developing drugs specifically for the treatment of DCM.

AIM

To explore the pharmacodynamic basis and molecular mechanism of *Jiawei Jiaotai Pill* in DCM treatment.

METHODS

We explored various databases and software, including the Traditional Chinese Medicine Systems Pharmacology Database, Uniport, PubChem, GenCards, String, and Cytoscape, to identify the active components and targets of *Jiawei Jiaotai Pill*, and the disease targets in DCM. Protein-protein interaction network, gene ontology, and Kyoto Encyclopedia of Genes and Genomes analyses were used to determine the mechanism of action of *Jiawei Jiaotai Pill* in treating DCM. Molecular docking of key active components and core targets was verified using AutoDock software.

RESULTS

Total 42 active ingredients and 142 potential targets of *Jiawei Jiaotai Pill* were identified. There were 100 common targets between the DCM and *Jiawei Jiaotai*

Pills. Through this screening process, *TNF, IL6, TP53, EGFR, INS*, and other important targets were identified. These targets are mainly involved in the positive regulation of the mitogen-activated protein kinase (MAPK) MAPK cascade, response to xenobiotic stimuli, response to hypoxia, positive regulation of gene expression, positive regulation of cell proliferation, negative regulation of the apoptotic process, and other biological processes. It was mainly enriched in the AGE-RAGE signaling pathway in diabetic complications, DCM, PI3K-Akt, interleukin-17, and MAPK signaling pathways. Molecular docking results showed that *Jiawei Jiaotai Pill*'s active ingredients had good docking activity with DCM's core target.

CONCLUSION

The active components of *Jiawei Jiaotai Pill* may play a role in the treatment of DCM by reducing oxidative stress, cardiomyocyte apoptosis and fibrosis, and maintaining metabolic homeostasis.

Key Words: *Jiawei Jiaotai pill*; Diabetic cardiomyopathy; Mechanism of action; Enrichment analysis; Network pharmacology; Molecular docking

Core Tip: *Jiawei Jiaotai Pill* is composed of Rhizoma Coptidis, Cinnamon, Radix Astragali, and Puerariae Lobatae Radix. It is mainly used to treat disharmony between the heart and kidneys, insomnia, sore mouth, and the tongue. It is often used to improve apoptosis, increase insulin secretion, and improve blood sugar tolerance. However, there are no reports on the mechanism of *Jiawei Jiaotai Pills* in the treatment of diabetic cardiomyopathy. We used the network pharmacology method, starting from the drug target, focused on analyzing the biological processes and conducting enrichment analysis of the important targets, and used molecular docking technology to verify the results.

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INTRODUCTION

Diabetic cardiomyopathy (DCM) occurs in patients with diabetes. They can be distinguished from hypertensive heart disease, atherosclerotic heart disease of the coronary arteries, and other heart diseases. Its main clinical symptoms include congestive heart failure and angina. In severe cases, this can lead to reduced ventricular compliance, reduced cardiac function, and congestive heart failure[1]. The number of individuals with diabetes worldwide is predicted to reach 439 million by 2030[2]. The incidence of DCM is increasing annually and is becoming a leading cause of death in patients with diabetes[3]. Therefore, prevention and treatment of DCM is important.

According to Traditional Chinese Medicine (TCM), the fundamental pathogenesis of DCM is based on the concept of "deficiency." Specifically, it attributes the onset of the condition to deficiencies in qi (vital energy) and yin (nourishing essence). The core pathogenesis in this context is characterized by "heat," where phlegm and blood stasis play crucial roles. The influencing factor, referred to as "stasis," is associated with various manifestation such as cough, asthma, phlegm, difficulty sleeping at night, reduced appetite, nausea, constipation, and yellow greasy fur[4]. *Jiawei Jiaotai Pill* is a modified version of the traditional *Jiaotai pill*. It is composed of Rhizoma Coptidis (RC), Cinnamon (CM), Radix Astragali (RA), and Puerariae Lobatae Radix (PLR). It is primarily used to treat heart and kidney disorders, insomnia, and mouth and tongue sores. It is commonly used for treating insomnia, diabetes, depression, and palpitations[5,6]. Recently, many studies have found that *Jiawei Jiaotai Pill* can reduce improve cell apoptosis, increase insulin secretion, improve blood glucose tolerance, and has a good therapeutic effect on DCM[7,8]. As the research on the treatment of DCM with *Jiawei Jiaotai Pill* progresses, there is a need to further clarify the pharmacology and mechanism of action of the drug. There are many effective components of the *Jiawei Jiaotai Pill*. They may act *via* various targets and pathways. Network pharmacology is a new method of studying the mechanisms of drug action. It is extensively used in the study of various traditional Chinese medicinal compounds. The concept of the target pathway provides a new way to study the complex mechanisms of TCM[9]. Therefore, this study aimed to use the techniques and methods of network pharmacology to comprehensively and systematically analyze the main chemical components of *Jiawei Jiaotai Pill* and its mechanism of action in the therapy of DCM to provide a foundation for further study of its pharmacological mechanism.

MATERIALS AND METHODS

Jiawei Jiaotai Pill active ingredients and targets

Jiawei Jiaotai Pill was analyzed using the TCM Systems Pharmacology Database and Analysis Platform (TCMSP) for retrieving chemical components. The screening criteria included drug-like (DL) value ≥ 0.18 and oral bioavailability ≥ 30% for the pharmacokinetic parameters of the compounds[10]. We screened the main effective active ingredients in the *Jiawei Jiaotai Pill* and their corresponding target information. The target information was converted into a standard target name using the Uniport platform, and the active ingredient was used as a keyword to search Pub Chem to determine the structural information of *Jiawei Jiaotai Pill* components.

Identifying targets of Jiawei Jiaotai Pill for DCM treatment

The GeneCards database was searched using the search term "diabetic cardiomyopathy" to identify DCM targets. The targets corresponding to the active ingredients of *Jiawei Jiaotai Pill* were compared with the related targets of DCM to obtain the related targets of *Jiawei Jiaotai Pill* in the treatment of DCM.

Construction of protein-protein interaction network

The related targets of *Jiawei Jiaotai Pill* for treating DCM were uploaded to the STRING database. The organism type was set to "Homo sapiens," targets with interaction > 0.7 were selected and those without interaction were removed, and a protein-protein interaction (PPI) plot was generated. In this network diagram, nodes represent the intersection of target proteins, and the thickness and number of edges represent close interactions between targets[11]. The topological properties of the network were analyzed using the "Network Analyzer" function in the Cytoscape software 3.9.1, and the "Degree," "Closeness," and "Betweenness" were determined to screen the main target information. The degree represents the number of connections between a node and other nodes in the network graph. The higher the number of nodes, the closer the connection to other protein genes in the network graph. The degree is often used to evaluate the importance of a node. Closeness represents the degree of closeness between a node and other nodes in the network diagram. The closer a node is to the other nodes, the closer the connection between the two, and the greater the closeness value. Betweenness is the proportion of the shortest path through the node in all the shortest paths in the network. The shorter the paths through the node, the closer the connection with other nodes in the network and the higher the betweenness[12].

Construction of drug-active ingredient-target network

To further understand the relationship between the active components of *Jiawei Jiaotai Pill* and related targets, Cytoscape software 3.9.1 was used to establish the drug-active component-target network. The network analysis was performed by the analysis network plug-in, and the "Degree," "Closeness" and "Betweenness" values of each drug were obtained. The topological parameters were compared to screen the important active drugs[11].

Gene ontology and Kyoto Encyclopedia of Genes and Genomes pathway enrichment analyses

The Database for Annotation, Visualization, and Integrated Discovery online analysis tool was used to perform Gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analyses on the related targets for treating DCM in the active components of *Jiawei Jiaotai pill*. GO analysis included molecular function, cellular components, and biological processes. The above pathway analysis was screened using $P < 0.05$ as the standard[11].

Active ingredient-target molecular docking

The core active components of *Jiawei Jiaotai Pill* were molecularly docked with core functional targets. The mol2 format file of the active ingredient was obtained from the TCMSP database, and the key target structure file was obtained from the Protein Data Bank database. AutoDock Vina software was used for molecular docking and PyMOL software was used to visualize the results. In molecular docking, the drug acts as a ligand and the protein transcribed and translated by the core target acts as a receptor. When the binding energies of the ligand and receptor decrease, the binding ability improves. The lowest binding energy is generally considered to be < 5 kcal/mol, indicating better docking activity[12].

RESULTS

The main active components and targets of Jiawei Jiaotai pill

The active ingredients of *Jiawei Jiaotai Pill* were screened using a preliminary database and literature search, and 48 RC, 100 CM, 87 RA, and 18 PLR were obtained. A total of 42 chemical constituents with oral bioavailability ≥ 30% and DL ≥ 0.18 were screened out. Note that certain components in CM such as oleic and linoleic acids have a low DL value but have high content in the drug or significant pharmacological effects. In such cases the DL should be ≥ 0.10. After relevant literature searches, 34 candidate active ingredients were identified after deleting non-target compounds, including 11 RC, four CM, 17 RA, and four PLR, as presented in Table 1. After the Uniport platform conversion of the standard targets, 142 potential active ingredient targets of *Jiawei Jiaotai Pill* were obtained.

Table 1 Thirty-four active components with therapeutic effect on diabetic cardiomyopathy in *Jiawei Jiaotai pill*

ID	Compounds	Wolecular weight	OB (%)	DL	TCM
MOL001454	Berberine	336.39	36.86	0.78	RC
MOL002894	Berberrubine	322.36	35.74	0.73	RC
MOL002897	Epiberberine	336.39	43.09	0.78	RC
MOL002903	(R)-Canadine	339.42	55.37	0.77	RC
MOL002904	Berlambine	351.38	36.68	0.82	RC
MOL002907	Corchoroside A_qt	404.55	104.95	0.78	RC
MOL000622	Magnograndiolide	266.37	63.71	0.19	RC
MOL000785	Palmatine	352.44	64.6	0.65	RC
MOL000098	Quercetin	302.25	46.43	0.28	RC/RA
MOL001458	Coptisine	320.34	30.67	0.86	RC
MOL002668	Worenine	334.37	45.83	0.87	RC
MOL000131	EIC	280.50	41.9	0.14	CM
MOL002003	(-)-Caryophyllene oxide	220.39	32.67	0.13	CM
MOL000057	DIBP	278.38	49.63	0.13	CM
MOL000675	Oleic acid	282.52	33.13	0.14	CM
MOL000211	Mairin	456.78	55.38	0.78	RA
MOL000239	Jaranol	314.31	50.87	0.29	RA
MOL000296	hederagenin	414.79	36.91	0.75	RA
MOL000033	(3S,8S,9S,10R,13R,14S,17R)-10,13-dimethyl-17-[(2R,5S)-5-propan-2-yloctan-2-yl]-2,3,4,7,8,9,11,12,14,15,16,17-dodecahydro-1H-cyclopenta[a]phenanthren-3-ol	428.82	36.23	0.78	RA
MOL000354	isorhamnetin	316.28	49.6	0.31	RA
MOL000371	3,9-di-O-methylnissolin	314.36	53.74	0.48	RA
MOL000378	7-O-methylisomucronulatol	316.38	74.69	0.30	RA
MOL000379	9,10-dimethoxypterocarpan-3-O-β-D-glucoside	462.49	36.74	0.92	RA
MOL000380	(6aR,11aR)-9,10-dimethoxy-6a,11a-dihydro-6H-benzofurano[3,2-c]chromen-3-ol	300.33	64.26	0.42	RA
MOL000387	Bifendate	418.38	31.1	0.67	RA
MOL000392	formononetin	268.28	69.67	0.21	RA
MOL000417	Calycosin	284.28	47.75	0.24	RA
MOL000422	kaempferol	286.25	41.88	0.24	RA
MOL000433	FA	441.45	68.96	0.71	RA
MOL000439	isomucronulatol-7,2'-di-O-glucosiole	626.67	49.28	0.62	RA
MOL000442	1,7-Dihydroxy-3,9-dimethoxy pterocarpene	314.31	39.05	0.48	RA
MOL000392	formononetin	268.28	69.67	0.21	PLR
MOL000358	beta-sitosterol	414.79	36.91	0.75	PLR
MOL002959	3'-Methoxydaidzein	284.28	48.57	0.24	PLR
MOL003629	Daidzein-4,7-diglucoside	578.57	47.27	0.67	PLR

TCM: Traditional Chinese medicine; RC: Rhizoma Coptidis; CM: Cinnamon; RA: Radix Astragali; PLR: Puerariae Lobatae Radix.

Potential targets of Jiawei Jiaotai Pill in the treatment of DCM

Using GeneCards, 6031 DCM target genes were identified. Taking the intersection of the target genes of the active components in *Jiawei Jiaotai Pill*, 100 common targets were obtained, which are the potential targets of *Jiawei Jiaotai Pill* in the treatment of DCM- (Figure 1).

Construction and analysis of the PPI network

One hundred potential targets were imported into the STRING platform to construct the PPI network. The PPI network and associated data files were analyzed and imported into the Cytoscape software 3.9.1 for further analysis and display, as shown in Figure 2. There were only 87 target genes as nodes and 287 as edges in the network diagram. Combined with the topological parameter analysis, the average value of "Degree" of all targets is 6.92, and there were 33 targets that exceeded the average "Degree" value for the first time (Figure 3A). The average value of "Closeness" of all targets was 30.02, and 30 targets exceeded the average "Closeness" value in the second screening (Figure 3B). The average value of "Betweenness" of all targets was 146.39, and there were 18 targets that exceeded the average "Betweenness" value in the third screening (Figure 3C). The interaction of these core targets may be key to the effect of *Jiawei Jiaotai Pill* on DCM. The top five key target genes were tumor necrosis factor (TNF), interleukin-6 (IL-6), cellular tumor antigen p53 (TP53), epidermal growth factor receptor (EGFR), and insulin (INS).

Construction of drug-active ingredient-target network diagram

To better illustrate the relationship between drugs, related compounds, and targets, TCM and its components and corresponding targets were used to construct a TCM compound target network map using Cytoscape, including 11 components of RC, four components of CM, 17 components of RA, and four components of PLR, corresponding to 100 targets (Figure 4). The Cytoscape network analysis revealed 139 nodes and 502 edges. The average "Degree" value of all nodes was 7.22, the average "Closeness" value of all targets was 53.58, and the average "Betweenness" value of all targets was 226.75. The degree, closeness, and betweenness of quercetin, formononetin, kaempferol, 7-O-methylisomucronulatol, and isorhamnetin were higher than the average of all node topological parameters, indicating that these five compounds were the main chemical components of *Jiawei Jiaotai Pill* in the treatment of DCM. Quercetin is also a common component of several TCMs (Table 2).

GO biological function enrichment analysis

The GO enrichment analysis revealed 324 biological processes. It mainly involved positive regulation of the mitogen-activated protein kinase (MAPK) cascade, response to xenobiotic stimuli, response to hypoxia, positive regulation of gene expression, positive regulation of cell proliferation, negative regulation of apoptotic processes, and other biological processes. There were 59 cell components, and analysis of the cell components suggested that they were involved in the caveola, plasma membrane, membrane raft, cell surface, and other tissue structures. The results showed that these mainly included protease binding, RNA polymerase II transcription factor activity, ligand-activated sequence-specific DNA binding, protein domain-specific binding, and other molecular functions (Figure 5).

KEGG pathway analysis

A total of 131 pathways were identified through KEGG pathway enrichment analysis ($P < 0.05$), and a bar chart of the first 20 pathways is shown in Figure 6. These pathways were mainly enriched in the AGE-RAGE signaling pathway in diabetic complications, fluid shear stress, atherosclerosis signaling pathway, DCM signaling pathway, PI3K-Akt signaling pathway, cGMP-PKG signaling pathway, IL-17 signaling pathway, and TNF signaling pathway.

Molecular docking of active components and core targets

Molecular docking is an important tool in molecular simulations. The principle is to use spatial and energy matching between molecules to complete the recognition between two or more molecular structures. Taking the small chemical molecules and receptor proteins used in this study as examples, molecular docking can be used to predict their binding modes and estimate the strength of their binding ability, that is, the binding energy, also known as affinity. It is generally believed that the smaller the binding energy between the ligand and receptor, the more stable the molecular conformation of the ligand and receptor. From the PPI network diagram and TCM drug-core target network diagram, five core target proteins (TNF, IL-6, TP53, EGFR, and INS) and five core drugs (quercetin, formononetin, kaempferol, 7-O-methylisomucronulatol, and isorhamnetin) were selected for binding energy prediction. The molecular docking results are presented in Table 3. The results showed that TNF, IL-6, TP53, EGFR, and INS had good binding abilities to quercetin, formononetin, kaempferol, 7-O-methylisomucronulatol, and isorhamnetin. PyMOL software was used to visualize the partial docking results (Figure 7).

DISCUSSION

The *Jiaotai pill* is a TCM originally used by ancient practitioners to treat insomnia. Through ongoing research and development of secondary prescriptions in clinical practice, along with the application of the theory of treating different diseases with the same drugs and the use of yin and yang, the scope of prescription has expanded from insomnia and palpitations to include conditions such as depression and diabetes[5,6]. *Jiawei Jiaotai Pill* contains RA and PLR based on RC and CM. Astragalus polysaccharides found in RA partially improved myocardial glucose and lipid metabolism

Table 2 Information table of core active substances in *Jiawei Jiaotai Pill*

MOLID	Compound	Degree	Closeness	Betweenness
MOL000098	Quercetin	108	88.08	9136.98
MOL000392	Formononetin	48	69.17	2365.63
MOL000422	Kaempferol	31	71.83	1333.03
MOL000378	7-O-methylisomucronulatol	31	71.83	1309.63
MOL000354	Isorhamnetin	25	80	2758.62

Table 3 Key targets and component docking results

ID	Compounds	Minimum binding energy (kcal/mol)				
		TNF	IL-6	TP53	EGFR	INS
MOL000098	Quercetin	-7.61	-6.88	-7.43	-6.14	-7.72
MOL000392	Formononetin	-6.56	-6.75	-6.12	-7.11	-6.21
MOL000422	Kaempferol	-6.7	-6.78	-6.19	-6.21	-14.8
MOL000378	7-O-methylisomucronulatol	-6.95	-6.68	-6.42	-5.98	-5.75
MOL000354	Isorhamnetin	-4.62	-4.56	-4.48	-4.27	-4.46

TNF: Tumor necrosis factor; IL-6: Interleukin 6; EGFR: Epidermal growth factor receptor; TP53: Cellular tumor antigen p53; INS: Insulin.

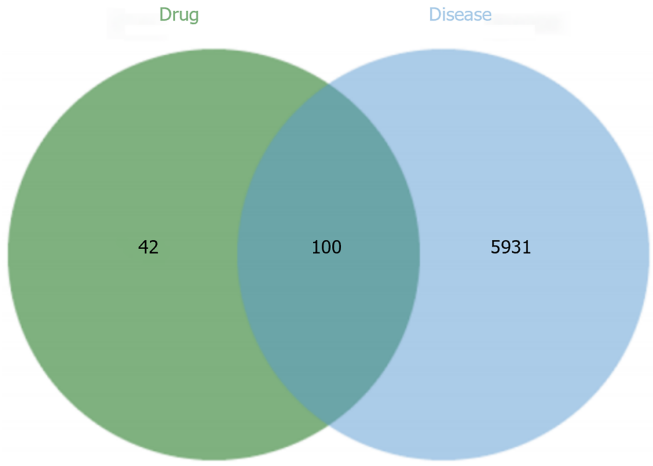

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Figure 1 Venn diagram of the common target of *Jiawei Jiaotai pill* and diabetic cardiomyopathy. Drug: *Jiawei Jiaotai pill*; Disease: Diabetic cardiomyopathy.

disorders in diabetic hamsters and have a protective effect on the myocardium[13]. Furthermore, PLR has good anti-inflammatory properties and maintains cardiovascular and cerebrovascular functions[14]. Although Jiawei Jiaotai pills are widely used in the treatment of diabetes and DCM in clinical practice, the pharmacological mechanism of *Jiawei Jiaotai Pill* remains unclear. Therefore, the application of the *Jiawei Jiaotai Pill* in treating DCM is clearer based on network pharmacology. The active ingredients and mechanism of action, and the systematic interpretation of the pathway at the molecular biology level provide a pharmacological basis for the clinical application of *Jiawei Jiaotai Pill* in treating DCM, thereby improving its curative effect.

After screening the active components of *Jiawei Jiaotai Pill*, the results showed that quercetin, formononetin, kaempferol, 7-O-methylisomucronulatol, and isorhamnetin had greater therapeutic effects, which may be the key active components in the treatment of DCM. In addition to oxidative stress, inflammation, myocardial cell death pathways, and neurohumoral mechanisms, the current understanding of the basic mechanisms of DCM in clinical research includes abnormalities in cardiac metabolism and physiological and pathophysiological signals such as abnormal changes in myocardial cells, myocardial insulin resistance, mitochondrial dysfunction, and abnormal oxidative stress[15]. Quercetin, a flavonoid, exerts antioxidant effects by inhibiting oxidative damage to low-density lipoproteins, chelating metal ions,

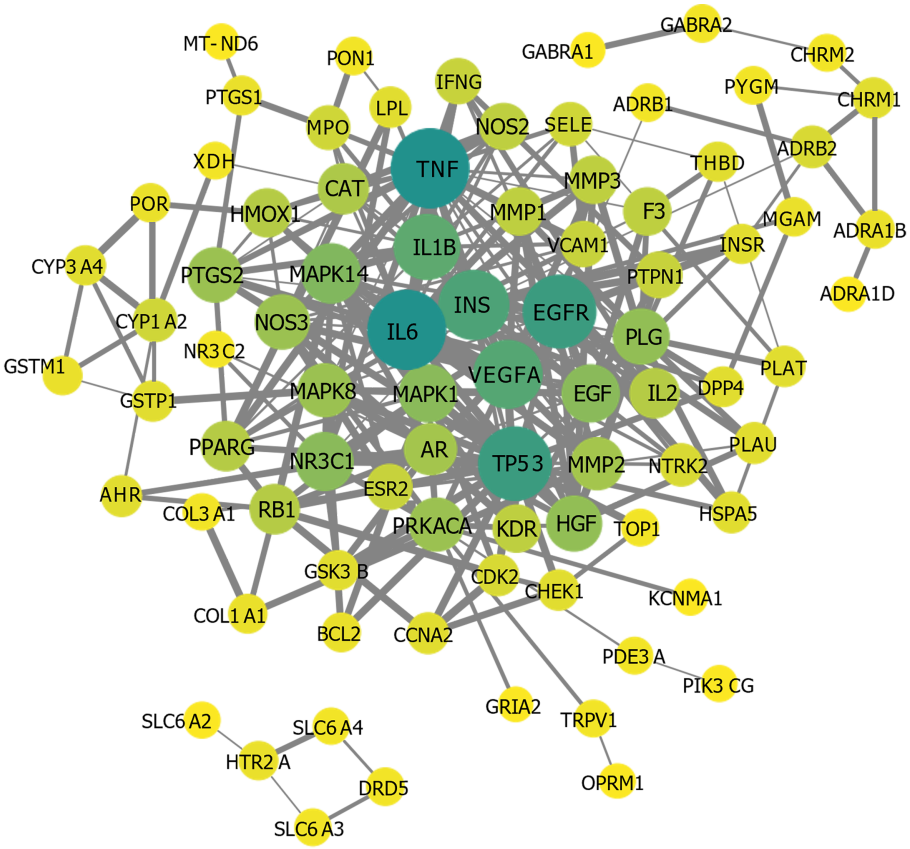

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Figure 2 Protein-protein interaction network diagram of *Jiawei Jiaotai pill* in the treatment of diabetic cardiomyopathy.

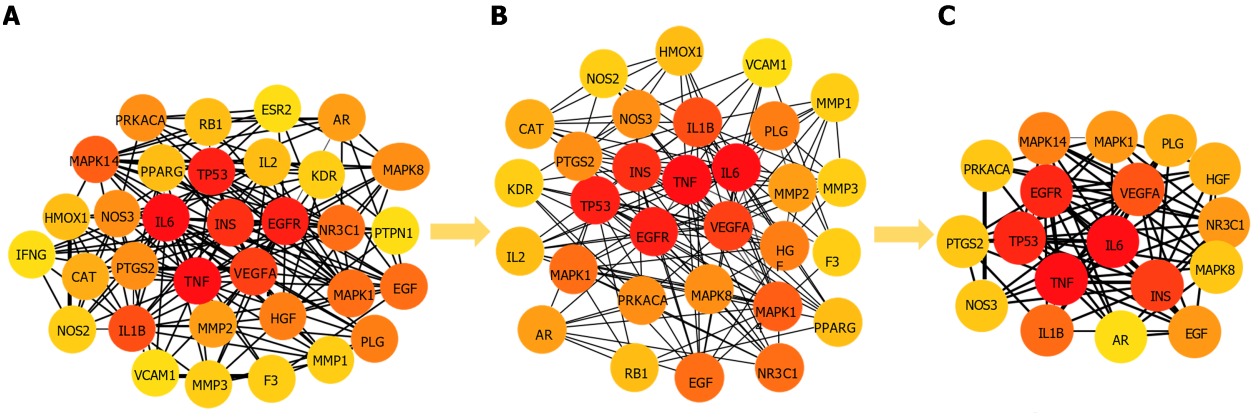

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Figure 3 Topology calculation process diagram of key targets. A: In the initial screening, 33 targets were found to exceed the average degree of freedom of all other targets; B: In the second screening, 30 targets were found that exceeded the average closeness of all targets; C: In the third screening, 18 targets were found that exceeded the average betweenness of all targets.

and directly scavenging reactive oxygen free radicals. It may also exert anti-inflammatory effects by regulating the production of inflammatory factors and inhibiting the nuclear factor-κB (NF-κB) and MAPK pathways. It also exerts hypoglycemic and lipid-lowering effects[16,17]. Formononetin is a polyphenolic compound that regulates lipid metabolism by activating the AMPK and PPARγ pathways[18,19]. Oza and Kulkarni[20] also found that 20 and 40 mg/kg formononetin could effectively improve blood lipid and glucose levels in diabetic rats by increasing the expression of human silent information regulator 1 in the pancreatic tissue. Kaempferol is a flavonol compound with anti-apoptotic, anti-inflammatory, and antioxidant properties[21]. Studies have shown that kaempferol significantly inhibits the expression of inflammatory cytokines and the production of reactive oxygen species induced by high glucose, resulting in reduced fibrosis and apoptosis in vitro. Concurrently, it mediates DCM protection by inhibiting NF-κB nuclear translocation and activating nuclear factor erythroid 2 p45-related factor-2[22]. Isorhamnetin is a natural small-molecule

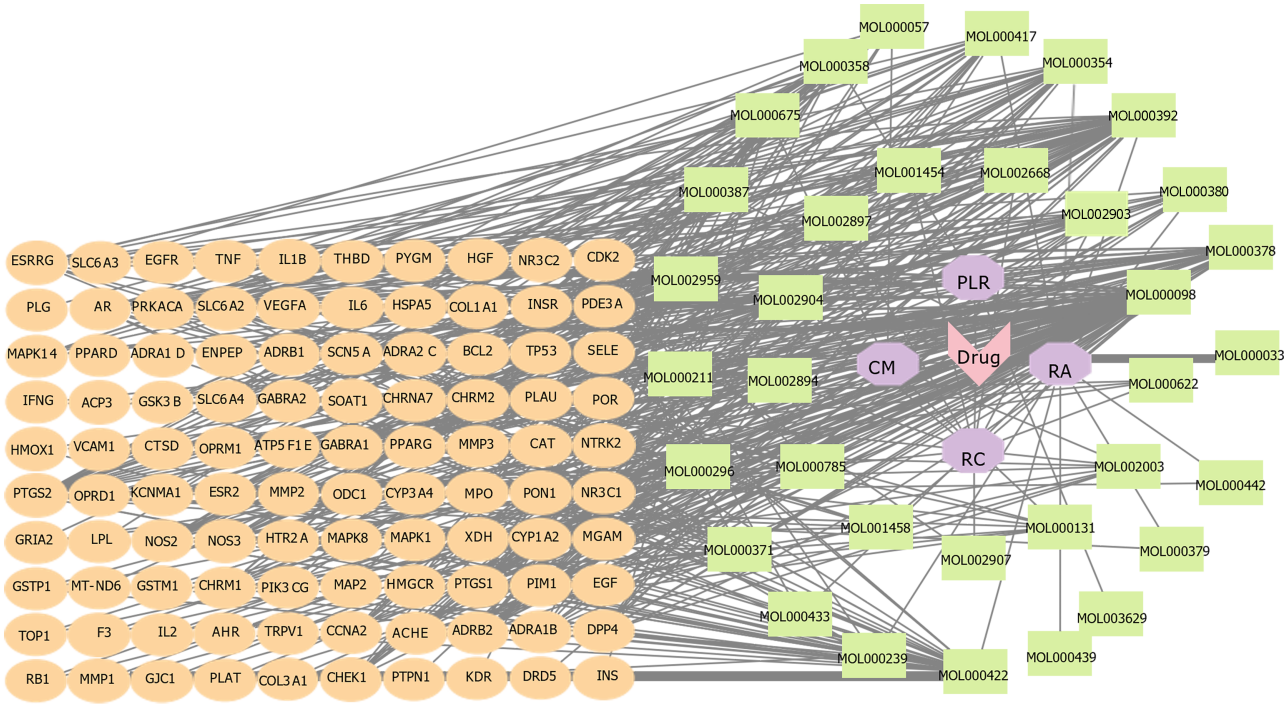

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Figure 4 "Chinese medicine-active ingredient-target" network diagram. Pink: Drug; Purple: Active ingredient; Green: Target protein; RC: Rhizoma Coptidis; CM: Cinnamon; RA: Radix Astragali; PLR: Puerariae Lobatae Radix; Drug: *Jiawei Jiaotai pill*.

flavonoid found in many plants. It has many biological functions, including anti-inflammatory, antiviral, anti-myocardial, hypoxia-ischemia, and lipid-lowering properties[23]. Isorhamnetin reduces myocardial injury by regulating the expression of autophagy and apoptosis pathway proteins in H9c2 cardiomyocytes. It also reduces the production of inflammatory mediators and decreases oxidative stress in diabetic rats by regulating NF-κB signaling activity[24,25].

The PPI and core target network diagrams revealed that the treatment of DCM with *Jiawei Jiaotai Pill* mainly involves genes such as *TNF, IL6, TP53, EGFR,* and *INS*. TNF-α is a major cytokine associated with obesity. Wu *et al*[26] reported that TNF-α can promote the regulation of glucose homeostasis by upregulating plasma TNF-α levels. TNF-α may play an active role in reducing INS resistance in diabetic mice through a TNF-α receptor 1-independent manner. IL-6 is a pro-inflammatory cytokine that is frequently involved in diabetes-related inflammatory responses and is currently considered an important biomarkers for the risk of developing diabetes[27]. IL-6 induces the expression of SOCS-3, a potential inhibitor of INS signal transduction, by controlling differentiation, migration, proliferation, and apoptosis, and impairs the phosphorylation of INS receptors and insulin receptor substrate-1, leading to insulin resistance[28]. TP53 mainly acts as a tumor suppressor, controlling numerous signaling pathways and preventing malignant transformation of cells[29]. Chen *et al*[30] suggested that pathological activation of the TP53 signaling pathway can induce myocardial fibrosis, apoptosis, heart failure, and premature death. The EGFR is a receptor tyrosine kinase that is widely expressed in various tissues, including the heart. Studies have shown that EGFR tyrosine kinase (EGFRtk) activity and endoplasmic reticulum (ER) stress increase in type 2 diabetic mice, leading to vascular dysfunction. Inhibition of EGFRtk and ER stress reduces apoptosis and inflammation and exerts cardioprotective effects. Therefore, targeting EGFRtk and ER stress may prevent myocardial infarction in patients with type 2 diabetes[31]. Metabolic disorders caused by INS resistance or a lack of INS signaling are closely related to the pathogenesis of DCM. An imbalance in INS expression can impair glucose oxidation, resulting in the diversion of glucose to other metabolic pathways with deleterious effects on myocardial cell function[32].

Target GO analysis results showed that *Jiawei Jiaotai Pill* is mainly involved in the positive regulation of the MAPK cascade, response to xenobiotic stimulus, response to hypoxia, positive regulation of gene expression, positive regulation of cell proliferation, negative regulation of apoptotic processes, and other biological processes through the caveola, plasma membrane, membrane raft, cell surface, and other organizational structures.

Among the 20 KEGG enrichment pathways of related targets, the main pathways enriched were the AGE-RAGE signaling pathway in diabetic complications, the DCM signaling pathway, the PI3K-Akt signaling pathway, the IL-17 signaling pathway, and the MAPK signaling pathway. Targeting the AGE-RAGE pathway is a potential therapeutic strategy to improve DCM[33]. Studies have shown that the accumulation of AGEs and activation of RAGE can induce continuous oxidative stress in vascular tissues, which may reduce the likelihood of diabetic macrovascular complications by inhibiting the AGE-RAGE pathway and subsequent oxidative stress[34]. The PI3K/AKT signaling pathway is essential for metabolic homeostasis. The PI3K family is involved in the regulation of various physiological processes, including cell growth, survival, differentiation, autophagy, chemotaxis, and metabolism[35]. AKT is downstream of PI3K in the INS signaling pathway and promotes a variety of cellular processes by targeting a large number of regulatory proteins that control glucose and lipid metabolism. Many studies have indicated that activation of the PI3K/Akt pathway may be the key mechanism for protection against DCM[36,37]. IL-17 is a pro-inflammatory cytokine synthesized by T helper cells,

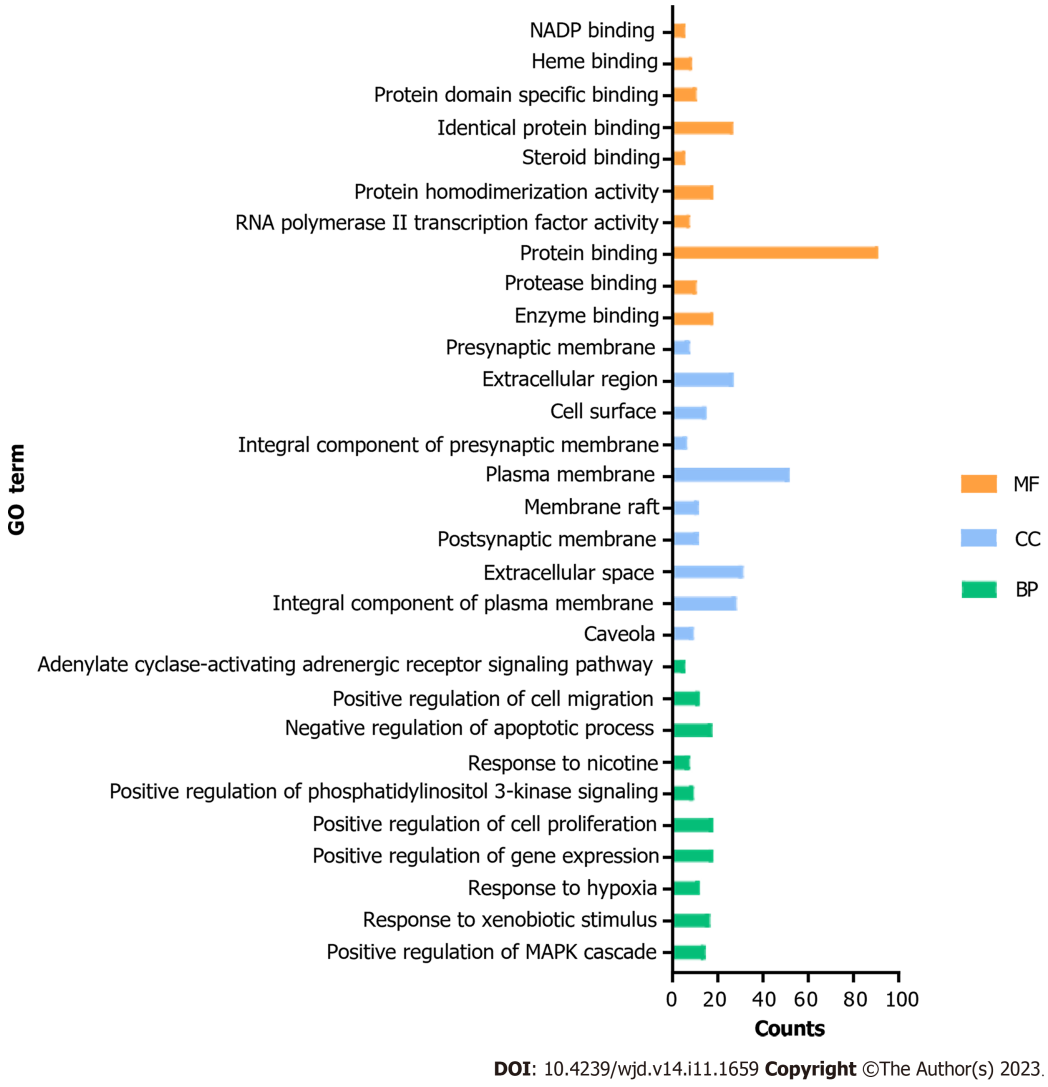

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Figure 5 GO pathway bar chart. MF: Molecular function; CC: Cellular component; BP: Biological process; NADP: Nicotinamide adenine dinucleotide phosphate; MAPK: Mitogen-activated protein kinases.

macrophages, dendritic cells, and natural killer cells. It promotes the expression of inducible nitric oxide synthase and induces cardiomyocyte apoptosis. Simultaneously, it activates matrix metalloproteinases, resulting in increased synthesis of the extracellular matrix in cardiomyocytes, leading to myocardial fibrosis and playing an important role in DCM. IL-17 Levels increase with the deterioration of cardiac function[38,39]. The MAPK pathway is activated by p38MAPK under high glucose conditions, and dysfunction occurs. Qian *et al*[40] found that inhibiting the expression of p38MAPK can rescue the MAPK pathway, thereby significantly ameliorating myocardial injury and dysfunction in diabetic mice. In summary, this study applied network pharmacology methods and molecular docking techniques to preliminarily explore the complex mechanism of multi-component, multi-target, and multi-pathways in the treatment of DCM through the active components in *Jiawei Jiaotai Pill*.

This study still has certain limitations. This study is only a preliminary theoretical determination of the molecular mechanism of the treatment of DCM with *Jiawei Jiaotai Pill* and is a predictive study. Future specific experiments are needed to further validate the results of this study.

CONCLUSION

The active components in *Jiawei Jiaotai Pill* include quercetin, formononetin, kaempferol, 7-O-methylisomucronulatol, and isorhamnetin, which act synergistically on target proteins such as TNF, IL-6, TP53, EGFR, and INS. It regulates the AGE-RAGE signaling pathway in diabetic complications, DCM pathway, PI3K-Akt signaling pathway, IL-17 signaling pathway, and MAPK signaling pathway to reduce the body's oxidative stress level, reduce myocardial cell apoptosis and fibrosis, and maintain metabolic homeostasis. The active components of *Jiawei Jiaotai Pill* mainly play a role in inhibiting inflammatory response, antioxidant response, anti-apoptosis, improving INS resistance, and stimulating INS secretion for the treatment of DCM.

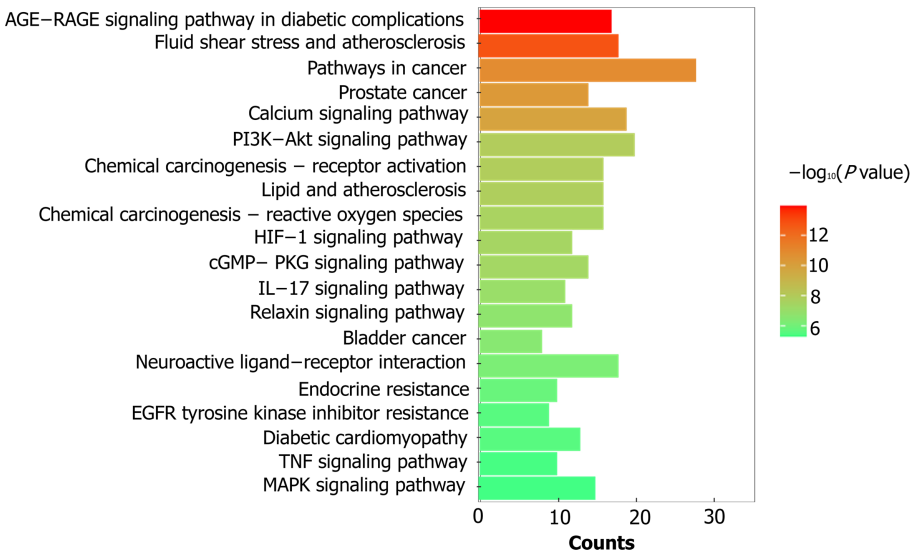

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Figure 6 Kyoto Encyclopedia of Genes and Genomes enrichment analysis bar chart. HIF: Hypoxia-inducible factor; IL: Interleukin; EGFR: Epidermal growth factor receptor; TNF: Tumor necrosis factor; MAPK: Mitogen-activated protein kinases.

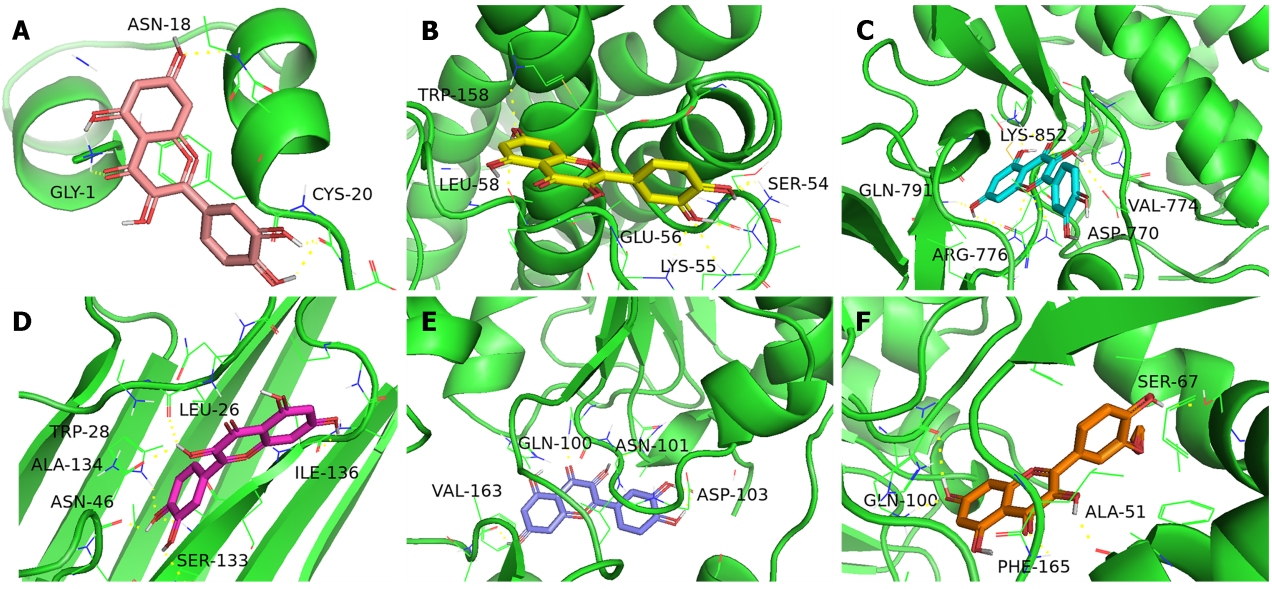

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Figure 7 Molecular docking 3D model of some key targets and active ingredients. A: Insulin-Quercetin; B: Interleukin-6-Quercetin; C: Epidermal growth factor receptor-Quercetin; D: Tumor necrosis factor-Quercetin; E: Tumor antigen p53 (TP53)-Quercetin; F: TP53-Isorhamnetin.

ARTICLE HIGHLIGHTS

Research background

Diabetic cardiomyopathy (DCM) is a type of cardiomyopathy independent of hypertension, coronary artery disease, and vascular complications. Traditional Chinese medicine (TCM) has unique advantages for treating this disease. In the current study, *Jiawei Jiaotai Pill* was widely used for the treatment of diabetes and its complications. *Jiawei Jiaotai Pill* has increased the use of Radix Astragali and Puerariae Lobatae Radix. It was also found that these two drugs had protective effects on the heart.

Research motivation

To improve the efficacy in DCM patients and further clarify the pharmacological basis of the *Jiawei Jiaotai Pill*, it is necessary to study the molecular mechanism of the *Jiawei Jiaotai Pill* in the treatment of DCM.

Research objectives
Based on the network pharmacology method and molecular docking technology, this study analyzed the effective active ingredients and important gene targets in *Jiawei Jiaotai Pill* and provided a reference for clinical treatment.

Research methods
The targets of the four TCMs in *Jiawei Jiaotai Pill* for DCM were identified using relevant databases. The core targets and compounds were identified using a protein-protein interaction network and a drug-active ingredient-target network. Gene ontology and Kyoto Encyclopedia of Genes and Genomes analyses were used to determine the related pathways of biological processes, and molecular docking was performed for verification.

Research results
The main components of *Jiawei Jiaotai Pill* used in the treatment of DCM are quercetin, formononetin, kaempferol, 7-O-methylisomucronulatol, and isorhamnetin. These components can act synergistically on disease-related target proteins such as tumor necrosis factor, interleukin-6 (IL-6), cellular tumor antigen p53, epidermal growth factor receptor, and insulin, and play therapeutic roles through the AGE-RAGE signaling pathway, PI3K/Akt, IL-17, and mitogen-activated protein kinase pathways. However, as predicted, the specific mechanism of *Jiawei Jiaotai Pill* requires further verification.

Research conclusions
The active ingredients of *Jiawei Jiaotai Pill* have a complex mechanism involving multiple components, targets, and pathways in the treatment of DCM, which may protect myocardial function by reducing the level of oxidative stress, reducing cardiomyocyte apoptosis and fibrosis, and maintaining metabolic homeostasis.

Research perspectives
Based on network pharmacology and molecular docking technology, the related mechanism of *Jiawei Jiaotai Pill* in the treatment of DCM was speculated, providing a reference for future experimental verification.

FOOTNOTES

Author contributions: Wang YJ and Wang YL contributed to the literature search; Jiang XF performed the data acquisition and statistical analysis; Li JE contributed to the manuscript preparation and editing; All authors have approved the final manual.

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ORIGINAL ARTICLE

Basic Study

Exploring the targets and molecular mechanism of glycyrrhetinic acid against diabetic nephropathy based on network pharmacology and molecular docking

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Abstract

BACKGROUND

Diabetic nephropathy (DN) stands as the most prevalent chronic microvascular complication of diabetes mellitus. Approximately 50% of DN patients progress to end-stage renal disease, posing a substantial health burden.

AIM

To employ network pharmacology and molecular docking methods to predict the mechanism by which glycyrrhetinic acid (GA) treats DN, subsequently validating these predictions through experimental means.

METHODS

The study initially identified GA targets using Pharm Mapper and the TCMSP database. Targets relevant to DN were obtained from the Genecards, OMIM, and TTD databases. The Venny database facilitated the acquisition of intersecting targets between GA and DN. The String database was used to construct a protein interaction network, while DAVID database was used to conducted Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis and Gene

Ontology (GO) analysis. Molecular docking experiments were performed using Autodock software with selected proteins. Experimental validation was conducted using renal proximal tubular cells (HK-2) as the study subjects. A hyperglycemic environment was simulated using glucose solution, and the effect of GA on cell viability was assessed through the cell counting kit-8 method. Flow cytometry was employed to detect cell cycle and apoptosis, and protein immunoblot (western blot) was used to measure the expression of proteins of the phosphatidylinositol 3-kinase (PI3K)/protein kinase B (AKT) signaling pathway and insulin resistance pathway, including insulin receptor (INSR), PI3K, p-PI3K, AKT, p-AKT, and glycogen synthase kinase-3 (GSK3).

RESULTS

A total of 186 intersecting targets between GA and DN were identified, which were associated with 144 KEGG-related enrichment pathways, 375 GO biological process entries, 45 GO cellular component entries, and 112 GO cellular function entries. Molecular docking demonstrated strong binding of GA to mitogen-activated protein kinase (MAPK)-1, SRC, PIK3R1, HSP90AA1, CASPASE9, HARS, KRAS, and MAPK14. *In vitro* experiments revealed that GA inhibited HK-2 cell viability, induced cell cycle arrest at the G2/M phase, and reduced apoptosis with increasing drug concentration. Western blot analysis showed that GA differentially up-regulated GSK3 protein expression, up-regulated AKT/p-AKT expression, down-regulated INSR, AKT, p-AKT, PI3K, and p-PI3K protein expression, and reduced p-PI3K/PI3K levels under high glucose conditions.

CONCLUSION

GA may protect renal intrinsic cells by modulating the PI3K/AKT signaling pathway, thereby inhibiting HK-2 cell viability, reducing HK-2 cell apoptosis, and inducing cell cycle arrest at the G0/G1 phase.

Key Words: Network pharmacology; Molecular docking; Diabetic nephropathy; Glycyrrhetinic acid; Mechanism of action

Core Tip: Diabetes nephropathy (DN) brings a huge burden to human health. Through network pharmacology, we found that glycyrrhetinic acid (GA) has a therapeutic effect on DN, and found 186 therapeutic targets. We speculated and verified that GA plays a role in treating DN by regulating phosphatidylinositol 3-kinase/protein kinase B signaling pathway, inhibiting the proliferation of HK-2 cells, blocking the cell cycle in the G2/M phase, and reducing apoptosis of HK-2 cells. This study provides a new development direction for the treatment of DN.

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INTRODUCTION

Diabetes mellitus (DM) is a prevalent condition characterized by either an absolute or relative deficiency in insulin secretion. In recent years, the incidence of DM has steadily increased due to the fast-paced nature of modern life, resulting in a rising number of DM cases since 1990[1]. The functional unit of the kidney, known as the nephron, comprises renal corpuscles and tubules. The kidneys play a crucial role in filtering blood and generating urine through processes involving glomerular filtration and tubular reabsorption. Elevated glucose levels can lead to microvascular damage in the kidneys, resulting in structural and functional impairments, ultimately giving rise to diabetic nephropathy (DN). DN stands as the most prevalent chronic microvascular complication of DM, often characterized by proteinuria, making 24-h urine albumin measurement a key diagnostic modality. Approximately 50% of DN patients progress to end-stage renal disease, posing a substantial health burden. Projections estimate that DN-related deaths will continue to rise, with an anticipated 224.2% increase, reaching 88803 deaths by 2030, as compared to 1990[2]. Primary treatment approaches focus on blood sugar regulation and the enhancement of renal function. Commonly used clinical medications to improve renal blood circulation include angiotensin converting enzyme inhibitors and sulfonylureas. However, these drugs may exhibit varying degrees of side effects on other organs, underscoring the need for natural compounds with minimal side effects. Glycyrrhetinic acid (GA) is a metabolite of glycyrrhizic acid primarily processed by the liver, existing in both α and β forms. Initially employed for liver fibrosis treatment, GA's medical utility has expanded to include various organ diseases, such as liver cancer and tumors. Studies have suggested that GA may safeguard glomeruli in diabetes by reducing transforming growth factor-β1 expression[3], mitigating podocyte injury[4], countering oxidative stress-induced glomerular mesangial cell hypertrophy and injury[5], and alleviating glomerular fibrosis. While the protective effect of GA on early to mid-stage renal fibrosis in kk-Ay diabetic mice has been demonstrated[6], no

specific drug targets or molecular mechanisms have been conclusively identified. Therefore, this study delves into the microscopic perspective of GA to explore its specific drug targets and molecular mechanisms in DN.

DN involves a multifaceted mechanism of pathogenesis, encompassing factors beyond insulin resistance (IR) and glucose metabolism disorders. It encompasses altered hemodynamics, inflammatory responses, oxidative stress, autophagy, and exosomal processes. Researchers have explored various molecular mechanisms to address DN. For instance, Hou *et al*[7] revealed that GA exerts preventive and therapeutic effects on DN by activating the kinase signaling pathway, specifically involving AMPK/SIRT1/PGC-1 (phosphorylated peroxisome proliferator-activated receptor-coactivator-1) in renal tissues. In a separate study, Ping-Ping Jia employed the Yi Qi, Nourishing Yin, and activating blood formula to mitigate inflammatory responses and reduce renal pathological injuries in DN rats. This was achieved by modulating the NLRP3/Caspase-1/GSDMD pathway, thus suppressing cellular pyroptosis[8]. Additionally, Tan *et al* [9] suggested that glycyrrhizidine may confer protection against DN in rats by inhibiting ferroptosis and modulating the vascular endothelial-derived growth factor/protein kinase B (AKT)/extracellular signal-regulated kinase pathway[9]. Interestingly, our exploration identified a research gap, as no prior studies had delved into the potential of GA as a therapeutic agent for DN treatment. Consequently, we elected to investigate this promising avenue.

Utilizing comprehensive databases such as PharmMapper, TCMSP, GeneCards, OMIM, and TTD, we meticulously searched for targets associated with both the drug GA and the disease DN. Subsequently, we identified common targets by taking their intersections. The pivotal target-pathway relationships were elucidated through a protein-protein interaction (PPI) network analysis. Furthermore, we conducted Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analyses to gain deeper insights. Molecular docking studies were performed employing GA constituents to provide a comprehensive understanding of potential interactions. Finally, we executed experimental validations to furnish a novel reference method for the treatment of DN (refer to Figure 1).

MATERIALS AND METHODS

GA structure and target acquisition

The study harnessed a combination of databases and tools to establish connections between diseases and drugs based on gene networks. This approach allowed us to predict the potential efficacy of drugs in treating specific diseases, subsequently subjecting these predictions to verification. The relevant information was procured from the PubChem database (https://pubchem.ncbi.nlm.nih.gov/). Herein, the keywords "alpha-glycyrrhetinic acid" and "beta-glycyrrhetinic acid" were employed to conduct searches, and the corresponding mol2 structure files for GA were duly downloaded. The acquisition of GA targets was executed by eliminating duplicates from datasets obtained from both the Pharm Mapper (http://www.lilab-ecust.cn/pharmmapper/) and TCMSP (https://old.tcmsp-e.com/tcmsp.php) databases.

Acquisition of DN targets

To identify targets associated with DN, searches were conducted in the GeneCards database (https://www.genecards.org/), OMIM database (https://www.genecards), and TTD database (https://www.genecards) using keywords such as "Diabetic Kidney Disease" and "Diabetic Nephropathy". The OMIM database (https://omim.org/) and TTD database (http://db.idrblab.net/ttd/) were instrumental in locating these DN targets, and any duplications within the obtained target lists were thoughtfully eliminated.

Acquisition of common targets of GA and DN

Employing the Venny 2.1.0 platform (https://bioinfogp.cnb.csic.es/tools/venny/), we performed an intersection analysis on the target sets for GA and DN, thus identifying the common targets shared between them.

Construction of PPI network

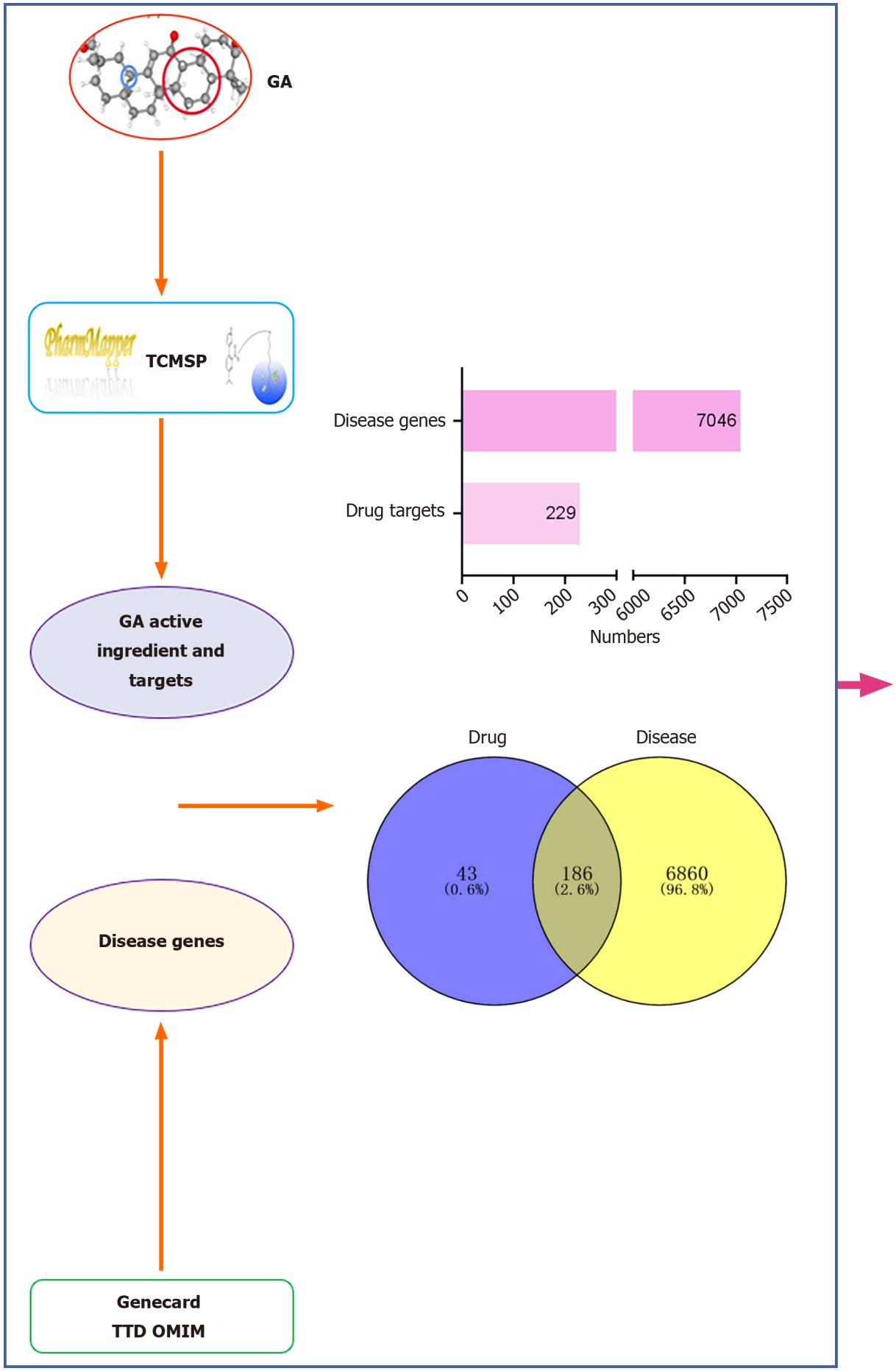
The common targets of GA and DN were imported into the STRING11.5 database (https://cn.string-db.org/) to assemble a PPI network. During this process, "Homo sapiens" was designated as the biological species, and a minimum interaction threshold of "highest confidence" (> 0.9) was applied. Isolated nodes were concealed, while the default settings were maintained to generate the protein interaction diagram and associated data. Subsequently, the TSV file containing PPI information was extracted and introduced into Cytoscape software. Here, the network analyzer plug-in facilitated the analysis of network properties, allowing for the identification of core targets within the PPI network based on node degree.

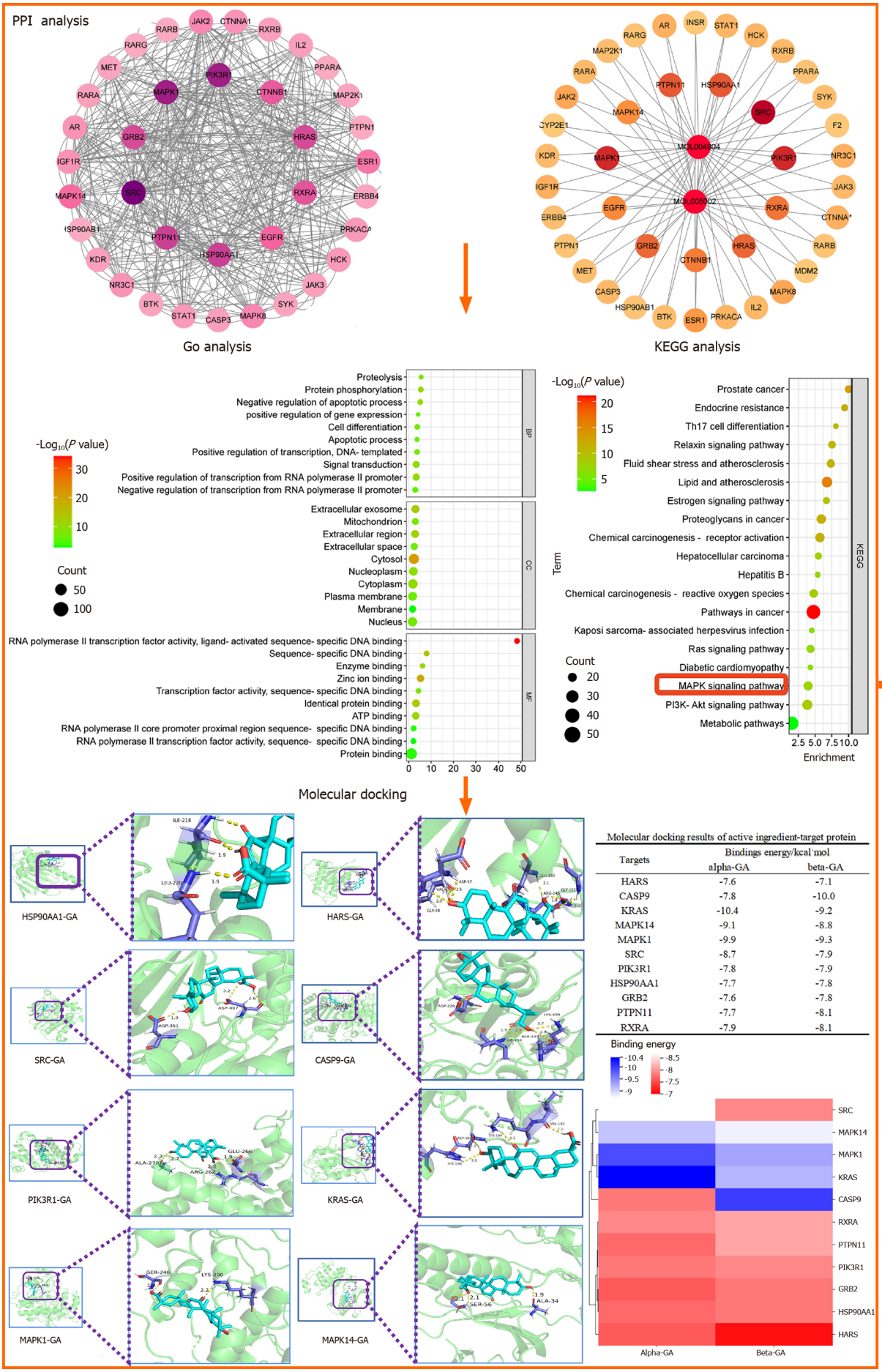
Composition and target network diagram construction

The assembled targets and active components of GA were used to construct a dedicated network within Cytoscape software. Subsequently, the network analyzer plug-in was harnessed to scrutinize the network's inherent characteristics, with a specific focus on the interaction between the active components of GA and the core targets, assessed based on node degrees.

GO and KEGG enrichment analysis

The shared targets of GA and DN underwent GO function and KEGG pathway enrichment analysis within the DAVID database (https://david.ncifcrf.gov/). The criteria for selection encompassed a P-value < 0.01 and false discovery rate <


GA
PharmMapper
TCMSP
GA active ingredient and targets
Disease genes
Genecard
TTD OMIM
Disease genes
7046
Drug targets
229
0
100
200
300
6000
6500
7000
7500
Numbers
Drug
Disease
43
(0. 6%)
186
(2. 6%)
6860
(96. 8%)


PPI analysis
Go analysis
KEGG analysis
Molecular docking
-Log10(P value)
Count
BP
CC
MF
KEGG
Term
Enrichment
Molecular docking results of active ingredient-target protein
Targets
Bindings energy/kcal·mol
alpha-GA
beta-GA
HARS -7.6 -7.1
CASP9 -7.8 -10.0
KRAS -10.4 -9.2
MAPK14 -9.1 -8.8
MAPK1 -9.9 -9.3
SRC -8.7 -7.9
PIK3R1 -7.8 -7.9
HSP90AA1 -7.7 -7.8
GRB2 -7.6 -7.8
PTPN11 -7.7 -8.1
RXRA -7.9 -8.1
Binding energy
HSP90AA1-GA
HARS-GA
SRC-GA
CASP9-GA
PIK3R1-GA
KRAS-GA
MAPK1-GA
MAPK14-GA
Alpha-GA
Beta-GA

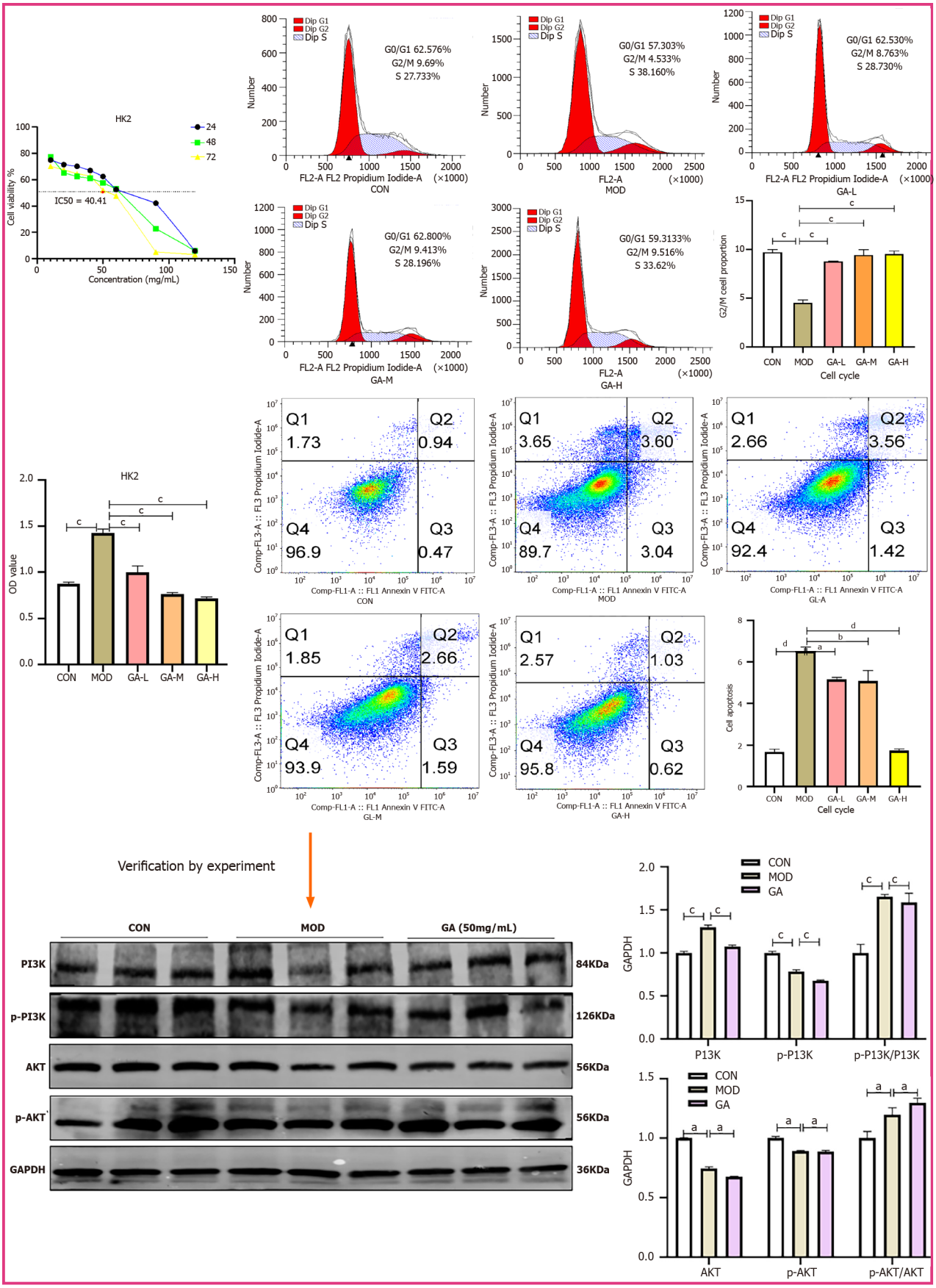


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Figure 1 Graphical abstract summarizing the network pharmacological predictions and experimental validations. GA: Glycyrrhetinic acid; PPI: Protein-protein interaction; GO: Gene Ontology; KEGG: Kyoto Encyclopedia of Genes and Genomes.

0.01. Subsequently, the identified items meeting these criteria were visually presented using the Micro Bioinformatics platform (http://www.bioinformatics.com.cn/).

Construction of component-target network diagram pertaining to signal pathway

The core pathway targets obtained post-KEGG enrichment were incorporated into the STRING11.5 database to establish a comprehensive PPI network. The resultant TSV file, representing PPIs, was retrieved and imported into Cytoscape software. Employing the network analyzer plug-in, an intricate component-target network diagram pertaining to the pathway was meticulously reconstructed. This reconstruction illuminated the interactions between the active components of GA and the target components, with the degree value of nodes serving as a crucial determinant.

Molecular docking

To facilitate molecular docking, the 3D structures of core targets were obtained from the PDB database (https://www.rcsb.org/). The selection criteria ensured that the protein 3D structures originated from humans, possessed a conformational resolution of less than 2.5 A, encompassed complete protein conformation sequences, and included small molecular ligands. Furthermore, the crystallization pH value closely approximated the normal physiological pH of the human body and was stored in PDB format. Subsequently, Pymol software was utilised to eliminate water molecules and small molecular ligands from the proteins. AutoDock software was then introduced to facilitate the necessary hydrotreatment, followed by molecular docking, employing the mol2 structure of GA's active ingredient. The resulting binding activity was subsequently evaluated.

Cell experiment verification

Cells: HK-2 cells (renal proximal tubular cells) were procured from Pronoxel (Catalog No. CL-0109).

Drugs and reagents: GA was obtained from Sigma Inc. (BCC0217; Table 1).

Cell culture: HK-2 cell cultivation involved the use of a complete medium comprising high-glucose DMEM, 10% foetal bovine serum, and a 1% penicillin-streptomycin mixture. This cultivation took place within an incubator set at 37 °C with 5% CO_2. The degree of growth confluence, typically reaching 70%-80%, was routinely observed under a microscope.

Determination of half inhibitory concentration of GA by cell counting kit-8 method: The determination of GA's half inhibitory concentration (IC_{50}) was carried out using the cell counting kit-8 (CCK-8) method. The WST-8 reagent in the CCK-8 kit can be reduced to produce orange formazan, and this transformation is linked to cell metabolic activity. Thus, CCK-8 can ascertain cell survival rates by measuring the concentration of formazan (http://keygentec.com.cn/uld/pdf/KGA317.pdf). In a previous study by Cao *et al*[10], the CCK-8 method revealed decreased cell viability in the ultrasound plus microbubble group. HK-2 cells in the logarithmic growth phase were subjected to trypsin digestion to prepare a cell suspension. These cells were counted using a 20 × microscope and subsequently divided into experimental groups. These groups included the control group (CON) (comprising complete cell culture medium), the model group (MOD) (consisting of 60 mmol/L high glucose culture medium), and the GA drug group. These cells were inoculated into 96-well plates at a density of 1 × 10^4 cells per well, with each group having five replicate wells. The drug was diluted to varying concentrations based on a drug concentration gradient. The cells were treated for 24 h, 48 h, and 72 h, respectively, with the addition of 100 μL of liquid to each well. Following the drug intervention, each well was incubated for 1 h with 10 μL of CCK-8 solution. The enzyme marker was activated, and the wavelength parameter was set to 450 nm to measure cell optical density (OD) for subsequent statistical analysis.

Detection of effect of GA on the cell cycle of HK-2 cells induced by high sugar by flow cytometry: The impact of GA on the cell cycle of HK-2 cells induced by high sugar was assessed through flow cytometry. HK-2 cells in the logarithmic growth phase were divided into five groups: The CON (cultured in complete cell culture medium), the MOD (grown in 60 mmol/L high-glucose culture medium), the low-dose group (cultivated in high-glucose culture medium + 30 mg/mL GA), the medium-dose group (grown in high-glucose culture medium + 40 mg/mL GA), and the high-dose group (cultivated in high-glucose culture medium + 50 mg/mL GA). In each group, three samples were prepared and inoculated into 6-well plates at a density of 1 × 10^5 cells per well. After 24 h of incubation in the culture, the cells were treated with serum-free medium for 12 h. Subsequently, each group received an additional 24 h of intervention. Cells were then digested and collected, followed by resuspension in pre-cooled 70% ethanol. After an overnight fixation, the cells were processed according to the cell cycle kit's instructions. Proportions of cells in each cycle were measured using flow cytometry, and statistical analysis was conducted accordingly.

Determination of effect of GA on apoptosis of HK-2 cells induced by high glucose by flow cytometry: A total of five groups of logarithmically growing HK-2 cells, identical to those in the cell cycle experiment group, were analysed. Each group comprised three samples, and 1 × 10^5 cells were inoculated per well in 6-well plates. Following 24 h of incubation in the culture, the corresponding culture medium was introduced to each group for a 24-h intervention period. After the cells were digested, collected, and processed using the apoptosis kit, flow cytometry was employed to detect the proportion of apoptosis for subsequent statistical analysis.

Detection of expression of insulin receptor, phosphatidylinositol 3-kinase, p-phosphatidylinositol 3-kinase, AKT, p-AKT, and glycogen synthase kinase-3 by Western blot: The expression of insulin receptor (INSR), phosphatidylinositol 3-kinase (PI3K), phosphorylated (p)-PI3K, AKT, p-AKT, and glycogen synthase kinase-3 (GSK-3) was assessed through western blot. HK-2 cells in the logarithmic growth phase were categorised into three groups: The CON, the MOD, and the

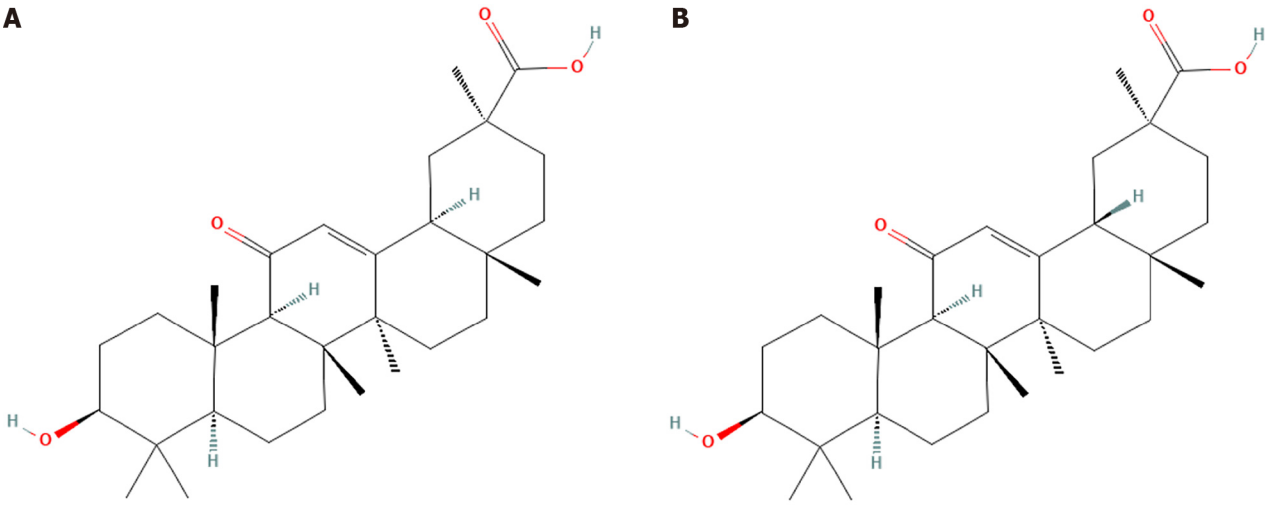

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Figure 2 The structure of drug active ingredient. A: Beta-glycyrrhetinic acid; B: Alpha-glycyrrhetinic acid.

high-dose group (50 mg/mL GA), each consisting of three samples. The cell intervention procedure remained consistent with the previous description. After cell digestion, 1.5 mL EP tubes were utilised to collect cells for each group, with 100 μL of RIPA lysis buffer added to each tube. The subsequent steps followed the instructions of the total protein extraction kit (http://keygentec.com.cn/uld/fct/103761.pdf). Upon successful protein extraction, protein content was determined. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was conducted based on the protein's quantitative results. Following TBST cleaning of the protein bands, they were subjected to rotation and sealing, and then incubated with primary antibodies diluted in TBST at 4 °C overnight. After 2 d, a 1-h incubation at room temperature was carried out, followed by a secondary antibody incubation for another hour. Subsequently, TBST cleaning was performed three times, and a luminescence solution was added for gel imaging. Image J software was used to measure the grey value of the obtained data, with statistical software employed for statistical analysis.

Statistical methods

Statistical analyses were conducted using GraphPad Prism 8.0 software, and intergroup comparisons were executed *via* one-way analysis of variance. A significance level of $P < 0.05$ indicated statistical significance.

RESULTS

GA structure and targets

The chemical composition of GA was ascertained through TCMSP. The structural data for the two GA subtypes were acquired from the PubChem database and downloaded in mol2 format (Table 2 and Figure 2). A total of 229 GA targets were identified *via* Pharm Mapper and TCMSP after eliminating duplicates.

Targets of DN

After eliminating duplicate entries from the GeneCards, OMIM, and TTD databases, we obtained 7046 targets associated with DN. These targets were visualized and compared with the previously identified GA targets using GraphPad Prism (Figure 3A).

Intersection of GA and DN targets

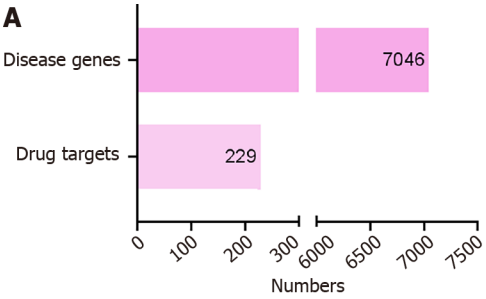
We employed the online tool Venny2.1.0 (https://bioinfogp.cnb.csic.es/tools/venny/) to create Venn diagrams that illustrated the intersection between the 229 potential GA targets and the 7046 DN targets. This analysis yielded 186 shared targets (Figure 3B).

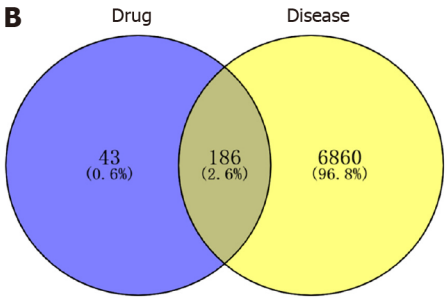
Construction of PPI network diagram

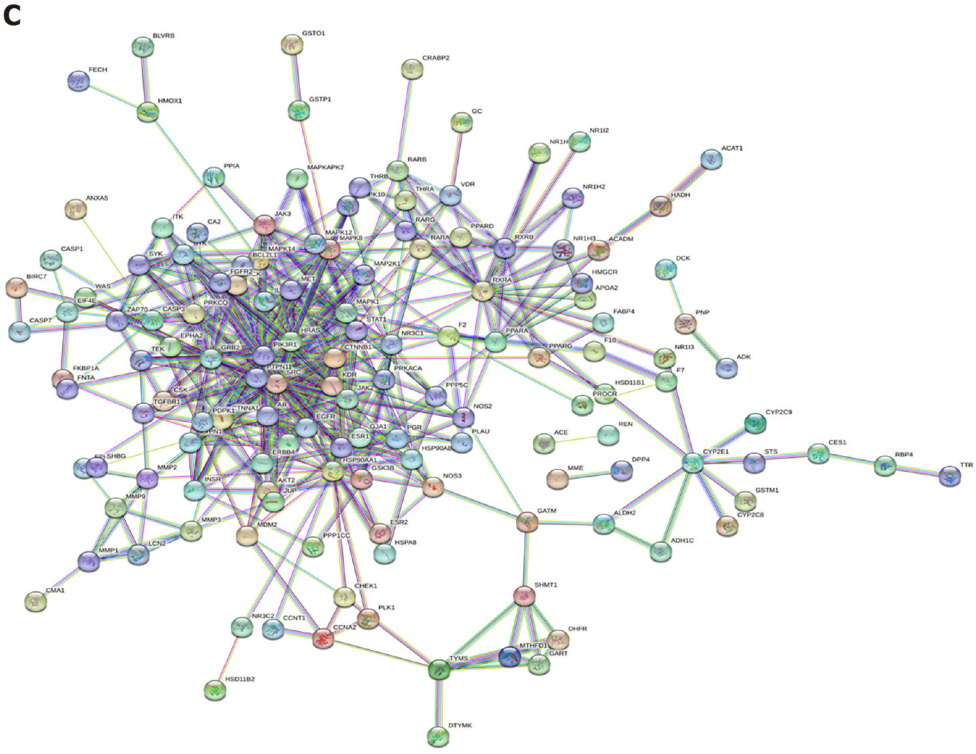
The intersection targets of GA and DN were utilized to construct a PPI network diagram by accessing the STRING database (Figure 3C). This network consisted of 186 nodes and 511 edges, with each node representing a specific protein.

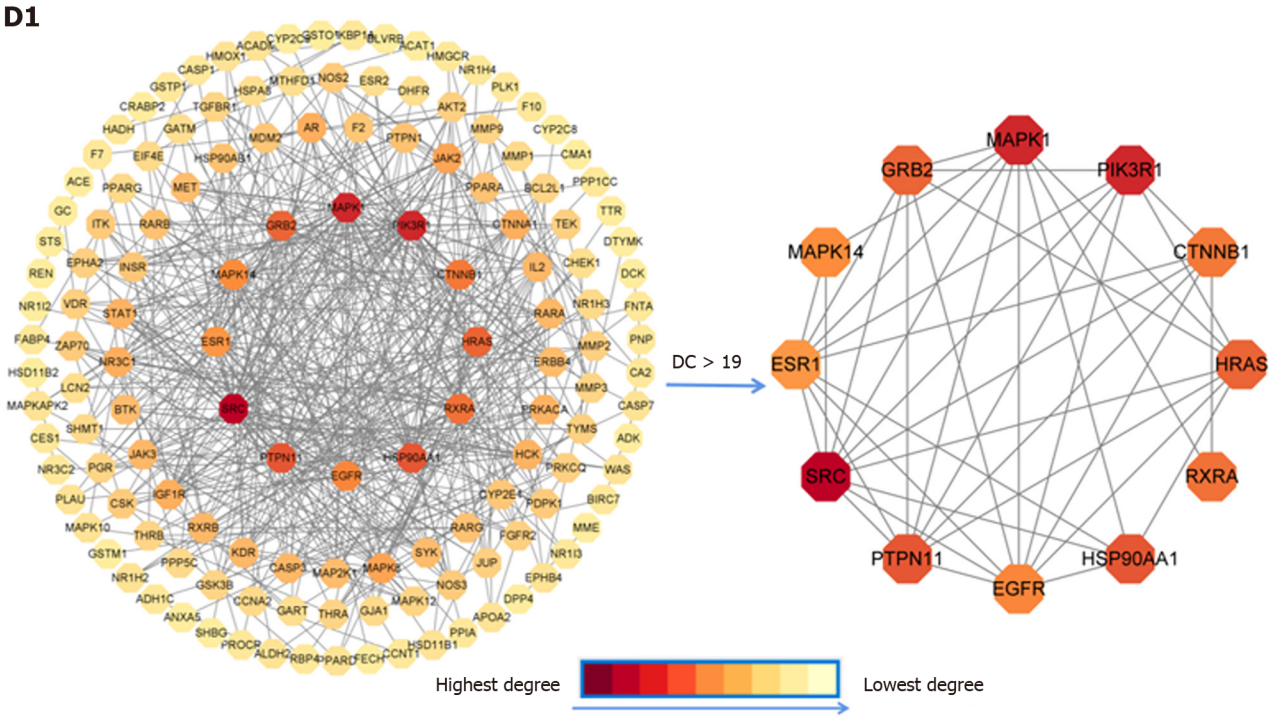
PPI network analysis

The PPI network diagram was analyzed using the network analyzer plug-in in Cytoscape, leading to the identification of 141 targets from the TSV file. Based on topological analysis results, we identified core targets for GA treatment of DN as nodes with a degree median greater than 19. These core targets included mitogen-activated protein kinase (MAPK)-1,


A
Disease genes
7046
Drug targets
229
0
100
200
300
6000
6500
7000
7500
Numbers


B
Drug
Disease
43
(0. 6%)
186
(2. 6%)
6860
(96. 8%)


C


D1
DC > 19
MAPK1
GRB2
PIK3R1
MAPK14
CTNNB1
ESR1
HRAS
SRC
RXRA
PTPN11
HSP90AA1
EGFR
Highest degree
Lowest degree

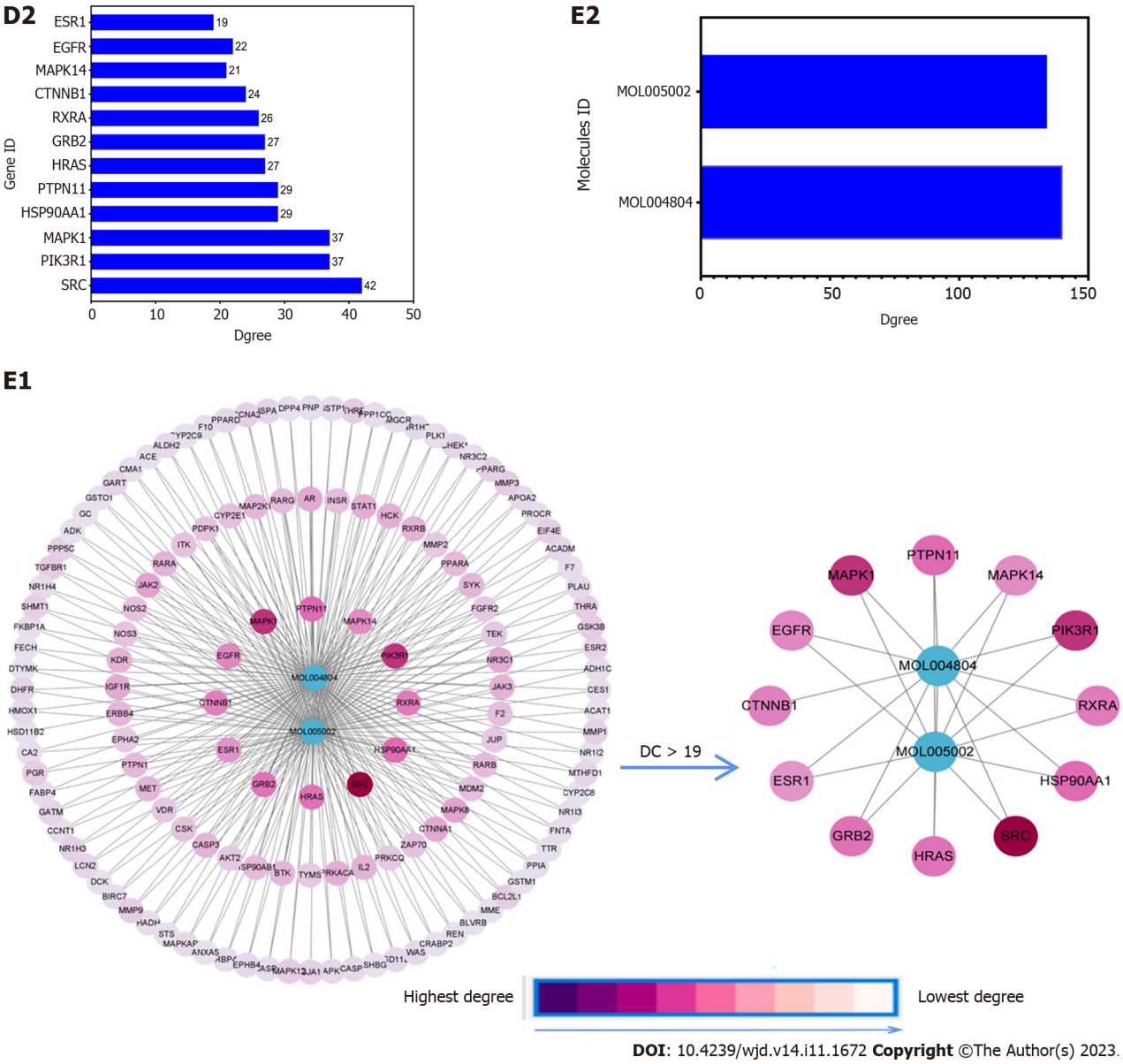


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Figure 3 Targets and protein-protein interaction network enrichment analysis. A: Drug targets and disease genes; B: Intersection of drug and disease targets; C: Protein-protein interaction (PPI) network diagram of intersection genes between glycyrrhetinic acid (GA) and the disease; D: Utilization of Cytoscape to construct a PPI network comprising 186 common targets and 2 core targets for potential disease treatment; E: Analysis results of selected core targets and GA's active components. Each node's color denotes its degree.

SRC, PIK3R1, HSP90AA1, PTPN11, HRAS, GRB2, RXRA, MAPK14, and epidermal growth factor receptor (EGFR), among others. The results were visualized using software tools such as GraphPad Prism and Cytoscape (Figure 3D).

Component-target network analysis

We established a network diagram connecting the selected PPI targets with the active components of GA. Through network analyzer plug-in analysis in Cytoscape software, we revealed interactions between the two active GA components and 141 common targets, along with degree values for each target. Notably, targets such as MAPK-1, SRC, PIK3R1, HSP90AA1, HRAS, GRB2, and EGFR, among others, were identified, and the results are visually presented (Figure 3E). The shading of nodes in the figure corresponds to the degree median, with darker colors indicating higher median values.

GO and KEGG enrichment analysis

The GO enrichment analysis revealed 375 entries pertaining to biological processes, with significant involvement in processes such as signal transduction, transcriptional regulation from the RNA polymerase II promoter, phosphorylation of proteins, and the regulation of apoptosis. Additionally, 45 cellular component items were identified, encompassing cellular locations such as cytosol, cytoplasm, nucleus, plasma membrane, and extracellular exosomes. Molecular function entries numbered 112, predominantly featuring functions related to protein binding and sequence-specific DNA binding. The first ten enriched results from each group are graphically represented (Figure 4A). The KEGG pathway enrichment

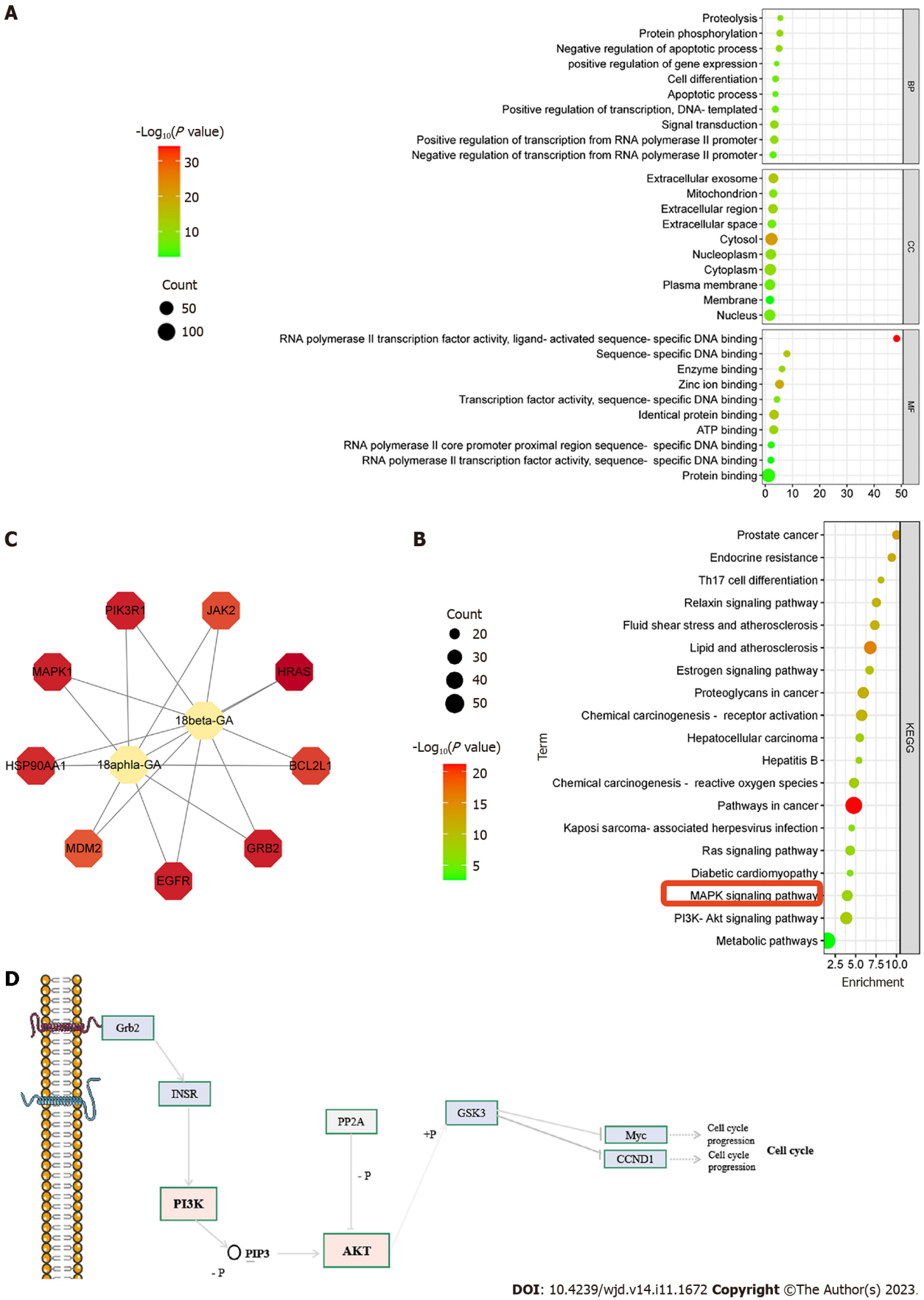

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Figure 4 Gene Ontology and Kyoto Encyclopedia of Genes and Genomes analysis. A: Results of Gene Ontology enrichment analysis; B: Enrichment outcomes for Kyoto Encyclopedia of Genes and Genomes pathways; C: Expression results of enriched core genes within the phosphatidylinositol 3-kinase (PI3K)/protein kinase B (AKT) signaling pathway; D: Correlation between the PI3K/AKT signaling pathway and the cell cycle.

analysis unveiled 90 enriched pathways, with notable involvement in signaling pathways such as PI3K/AKT, proteoglycans in cancer, and MAPK. Notably, the pathway associated with diabetic cardiomyopathy emerged as potentially significant in GA's treatment of DN. The initial 20 enrichment results are depicted graphically (Figure 4B). For the construction of the component-target network map within the signaling pathway, network analyzer from STRING11.5 and Cytoscape software were utilised, incorporating GA's active components. This analysis yielded core targets including MAPK-1, SRC, PIK3R1, HSP90AA1, HRAS, GRB2, and EGFR, among others, with the results visually presented (Figure 4C). The signaling pathway itself is also visualised (Figure 4D).

Molecular docking

The identified targets, namely, GASRC, MAPK1, PIK3R1, HSP90AA1, PTPN11, HRAS, GRB2, RXRA, MAPK14, and EGFR, underwent rigorous molecular docking analysis using Pymol software. The docking results are thoughtfully visualised and presented (Figure 5A). The assessment of binding activity was meticulously carried out, considering docking scores as the primary indicator. Notably, scores falling below -4.25 kcal/mol signified a certain level of binding activity, while scores less than -5.0 kcal/mol indicated strong binding activity. Visual representation of the binding activity between GA's active components and target proteins is facilitated (Figure 5B), employing the ChiPlot online tool for comprehensive visualization output (Figure 5C). It is noteworthy that the binding energy observed between GA's active components and the top 10 core target proteins consistently exceeded -5.0 kcal/mol, with KRAS-GA displaying the most favourable binding energy, registering at -10.4 kcal/mol.

Half inhibitory concentration of GA

Optical density values were recorded at three distinct time points: 24 h, 48 h, and 72 h. These values were then utilised in the calculation formula for inhibition rates. Subsequently, the IC_{50} values for HK-2 cells at each time point were calculated through fitting procedures employing Graphd Prism 8.0 software (Figure 6A). Notably, the experimental data indicated that after 72 h of drug intervention, the cell growth curves generated with the software closely approximated logarithmic curves. Therefore, the 72-h mark was chosen as the optimal duration for drug intervention. The calculated IC_{50} value at this time point was determined to be 40.41 mg/mL, subsequently rounded to 40 mg/mL. In the context of CCK-8-based viability assessment of HK-2 cells across various groups, noteworthy trends emerged: The MOD displayed significantly enhanced viability compared to the CON ($P < 0.05$). Furthermore, cell viability exhibited a substantial increase ($P < 0.01$) when compared to the CON. Conversely, HK-2 cell viability exhibited a noteworthy decrease upon GA intervention ($P < 0.01$) relative to the MOD. This reduction in viability displayed a dose-dependent relationship, signifying that higher doses of GA yielded more pronounced decreases in HK-2 cell viability (Figure 6B).

Effect of GA on cell cycle of HK-2 cells induced by high glucose

Following a 24-h exposure to GA, flow cytometry analysis was conducted to ascertain the impact of GA on the cell cycle of HK-2 cells. In comparison to the CON, the MOD exhibited a reduced proportion of cells in the G0/G1 phase ($P < 0.01$), with a concurrent increase in the proportion of cells in the G2/M and S phases ($P < 0.01$). Remarkably, GA intervention led to a higher proportion of cells in the G0/G1 phase and G2/M phase ($P < 0.01$) when compared to the MOD. These results underscore GA's ability to arrest HK-2 cells in the G2/M phase (Figure 6C; CON 9.693 ± 0.506, MOD 4.533 ± 0.487, GA-L 8.763 ± 0.085, GA-M 9.413 ± 0.964, and GA-H 9.517 ± 0.557).

Effect of GA on apoptosis of HK-2 cells induced by high glucose

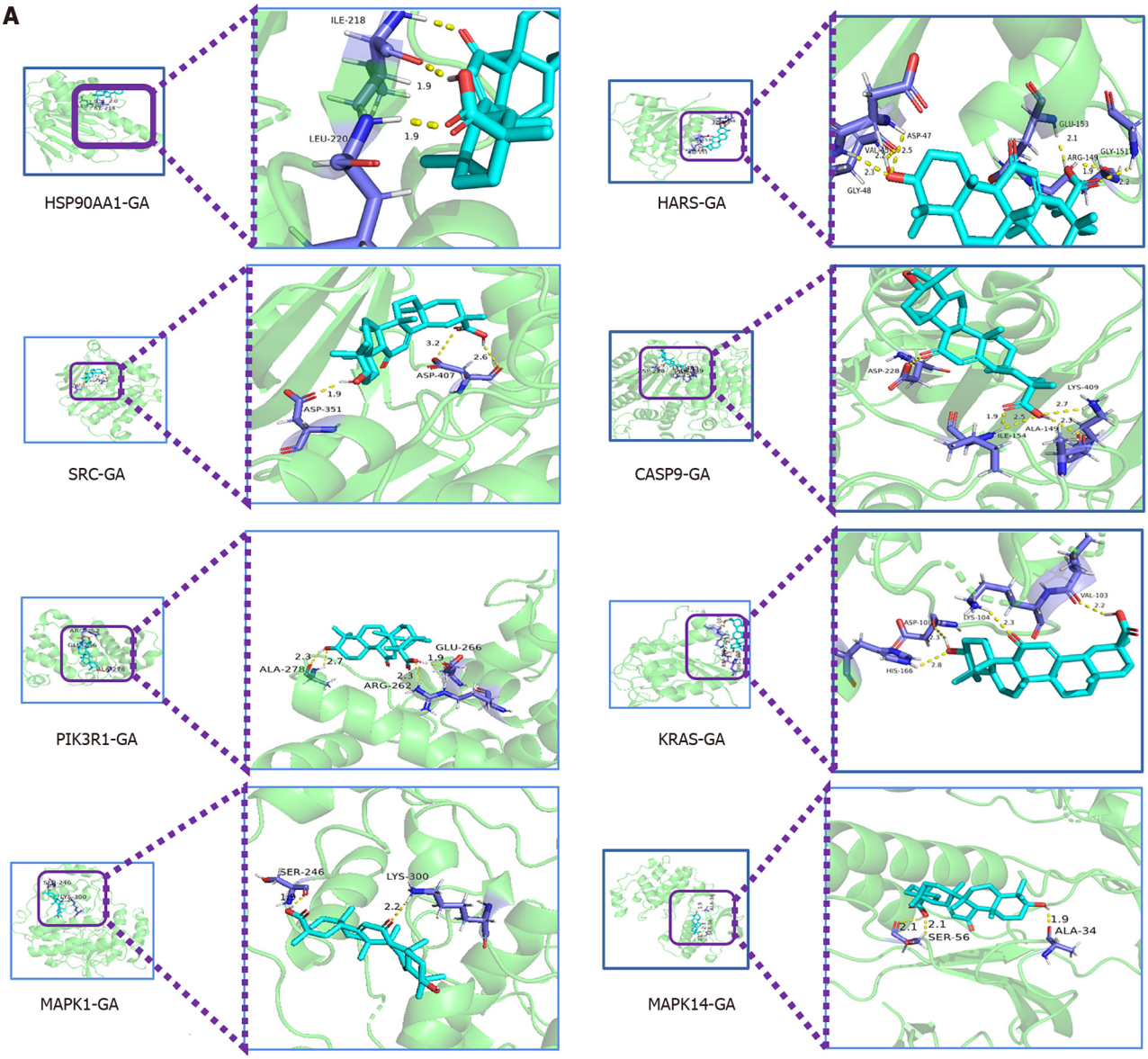
Following a 24-h exposure to GA, apoptosis of HK-2 cells was assessed using AV-PI double staining coupled with flow cytometry. Relative to the CON, the MOD exhibited a substantial increase in the percentage of apoptotic HK-2 cells ($P < 0.01$). In contrast, the GA dose groups displayed a notable reduction in the percentage of apoptosis when compared to the MOD ($P < 0.01$). This observed decrease in HK-2 cell apoptosis was particularly pronounced with increasing concentrations of GA (Figure 6D; CON 1.6733 ± 0.228, MOD 6.537 ± 0.327, GA-L 5.163 ± 0.172, GA-M 5.08 ± 0.878, and GA-H 1.747 ± 0.119).

Expression of INSR, PI3K, p-PI3K, AKT, p-AKT, and GSK-3 detected by western blot

Western blot analysis was employed to assess the protein expression levels of INSR, PI3K, p-PI3K, AKT, p-AKT, and GSK-3 in HK-2 cells after exposure to GA. The results revealed an up-regulation in the protein expression of INSR and PI3K, along with increased p-PI3K/PI3K and AKT/p-AKT ratios in the MOD when compared to the CON. Conversely, the expression levels of AKT, p-AKT, p-PI3K, and GSK3 proteins were down-regulated in the MOD. Subsequent to GA intervention, we observed an up-regulation in the expression of GSK3 protein, as well as elevated AKT/p-AKT and p-PI3K/PI3K ratios ($P < 0.01$ or $P < 0.05$). Conversely, the protein expression of INSR, AKT, p-AKT, PI3K, and p-PI3K was down-regulated, with a decrease in p-PI3K/PI3K expression ($P < 0.01$ or $P < 0.05$) (Figure 7).

DISCUSSION

Within the realm of Chinese medicinal and edible products, licorice, a staple of traditional Chinese medicine, holds a prominent position for its efficacy in treating digestive disorders. As is widely acknowledged, the advanced stages of DN, particularly the uremia stage, are often accompanied by distressing gastrointestinal symptoms such as vomiting and diarrhea. These symptoms arise in part due to the accumulation of peptides and guanidine compounds within the body.



B

Molecular docking results of active ingredient-target protein

Targets	Bindings energy/kcal·mol	
	alpha-GA	beta-GA
HARS	-7.6	-7.1
CASP9	-7.8	-10.0
KRAS	-10.4	-9.2
MAPK14	-9.1	-8.8
MAPK1	-9.9	-9.3
SRC	-8.7	-7.9
PIK3R1	-7.8	-7.9
HSP90AA1	-7.7	-7.8
GRB2	-7.6	-7.8
PTPN11	-7.7	-8.1
RXRA	-7.9	-8.1

C

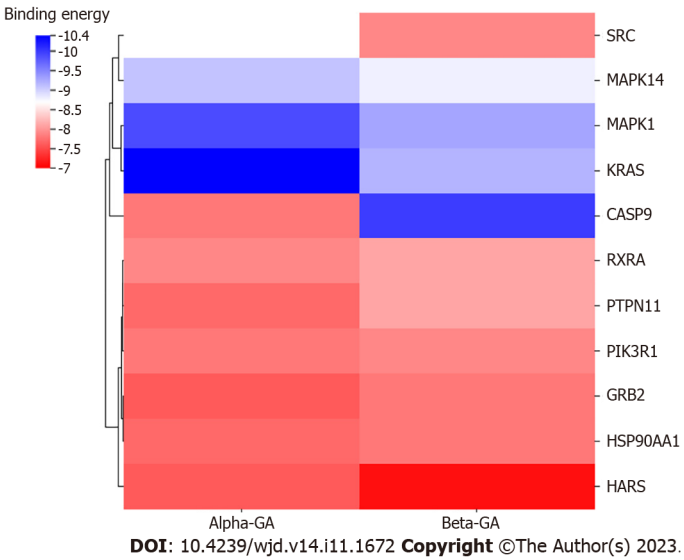


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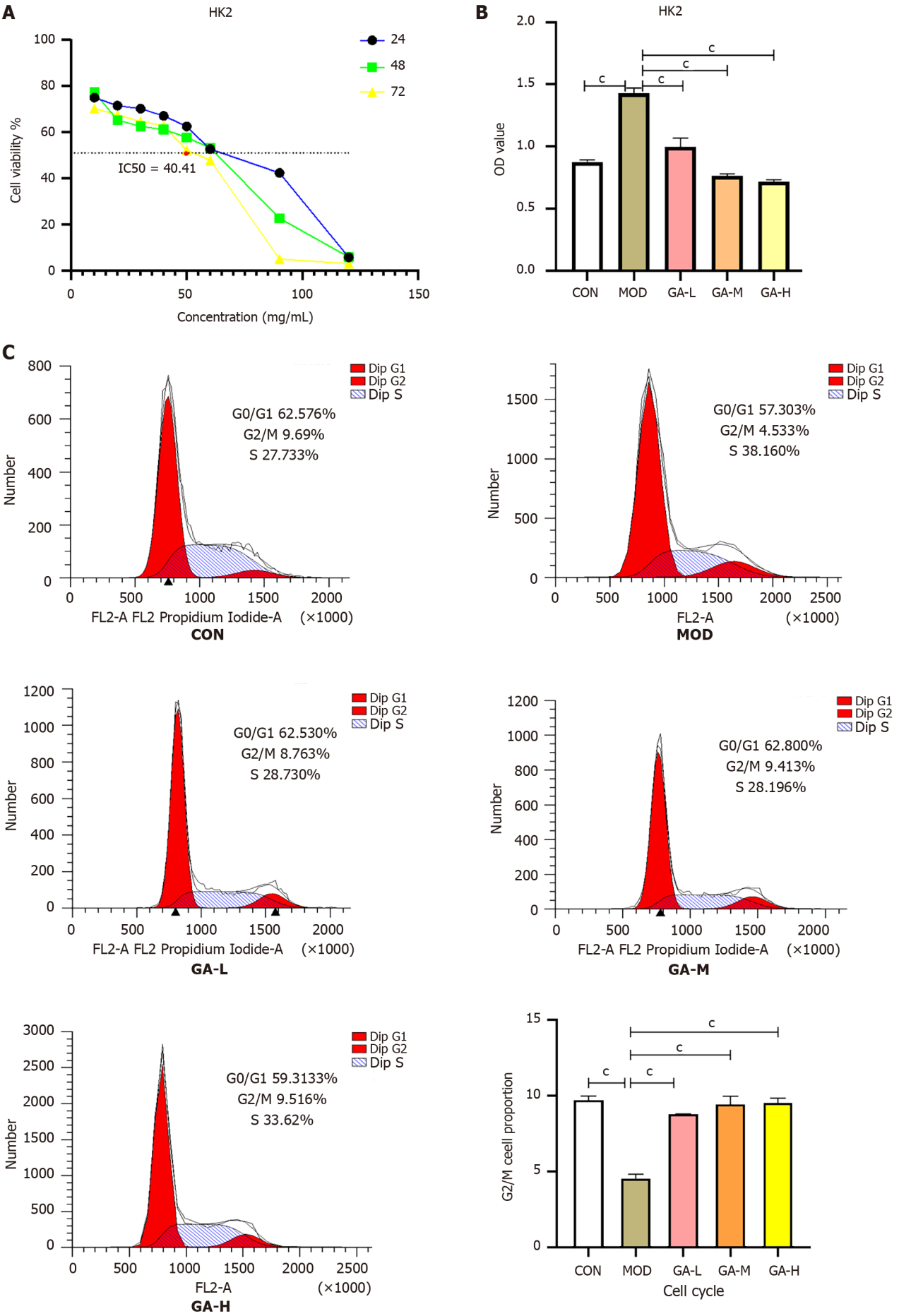
Figure 5 Molecular docking. A: Demonstration of docking outcomes between glycyrrhetinic acid (GA) components and core target molecules; B: Presentation of docking results and binding energies between GA components and core target molecules; C: Heat map display of molecular docking binding energy results. GA: Glycyrrhetinic acid.


A
HK2
Cell viability %
Concentration (mg/mL)
IC50 = 40.41
24
48
72
B
HK2
OD value
CON
MOD
GA-L
GA-M
GA-H
C
Dip G1
Dip G2
Dip S
G0/G1 62.576%
G2/M 9.69%
S 27.733%
FL2-A FL2 Propidium Iodide-A (×1000)
CON
G0/G1 57.303%
G2/M 4.533%
S 38.160%
FL2-A (×1000)
MOD
G0/G1 62.530%
G2/M 8.763%
S 28.730%
GA-L
G0/G1 62.800%
G2/M 9.413%
S 28.196%
GA-M
G0/G1 59.3133%
G2/M 9.516%
S 33.62%
GA-H
G2/M ceell proportion
Cell cycle

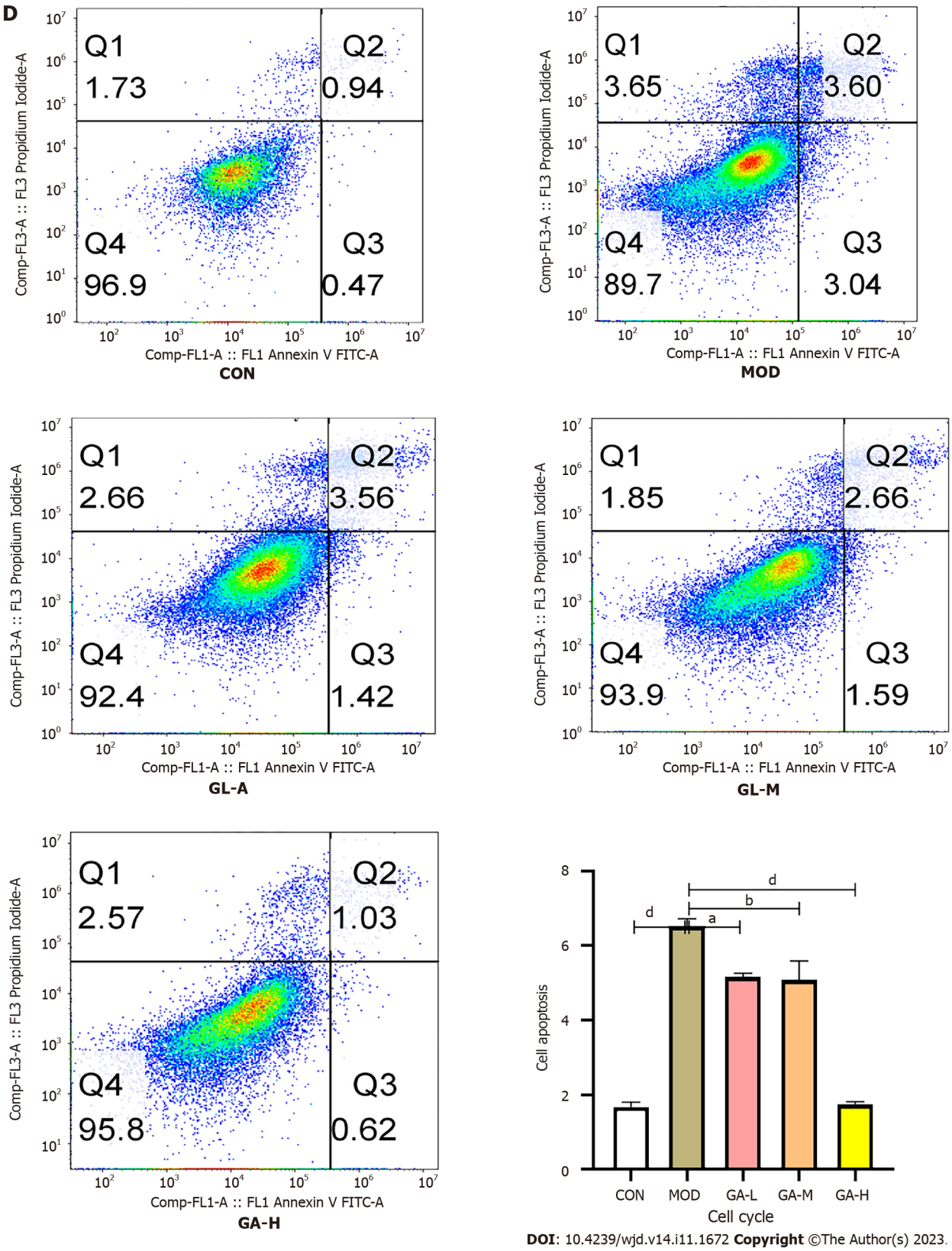


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Figure 6 Determination of IC_{50}, cell cycle, and apoptosis. A: Impact of glycyrrhetinic acid (GA) on the IC_{50} of HK-2 cells; B: Influence of GA on the activity of HK-2 cells; C: Effect of GA on the cell cycle of HK-2 cells; D: Effect of GA on apoptosis of HK-2 cells. The sequence from left to right represents the control group, the model group, and the GA group. $^{a}P < 0.05$, $^{b}P < 0.01$, $^{c}P < 0.001$, $^{d}P < 0.0001$. GA: Glycyrrhetinic acid; CON: Control; MOD: Model.

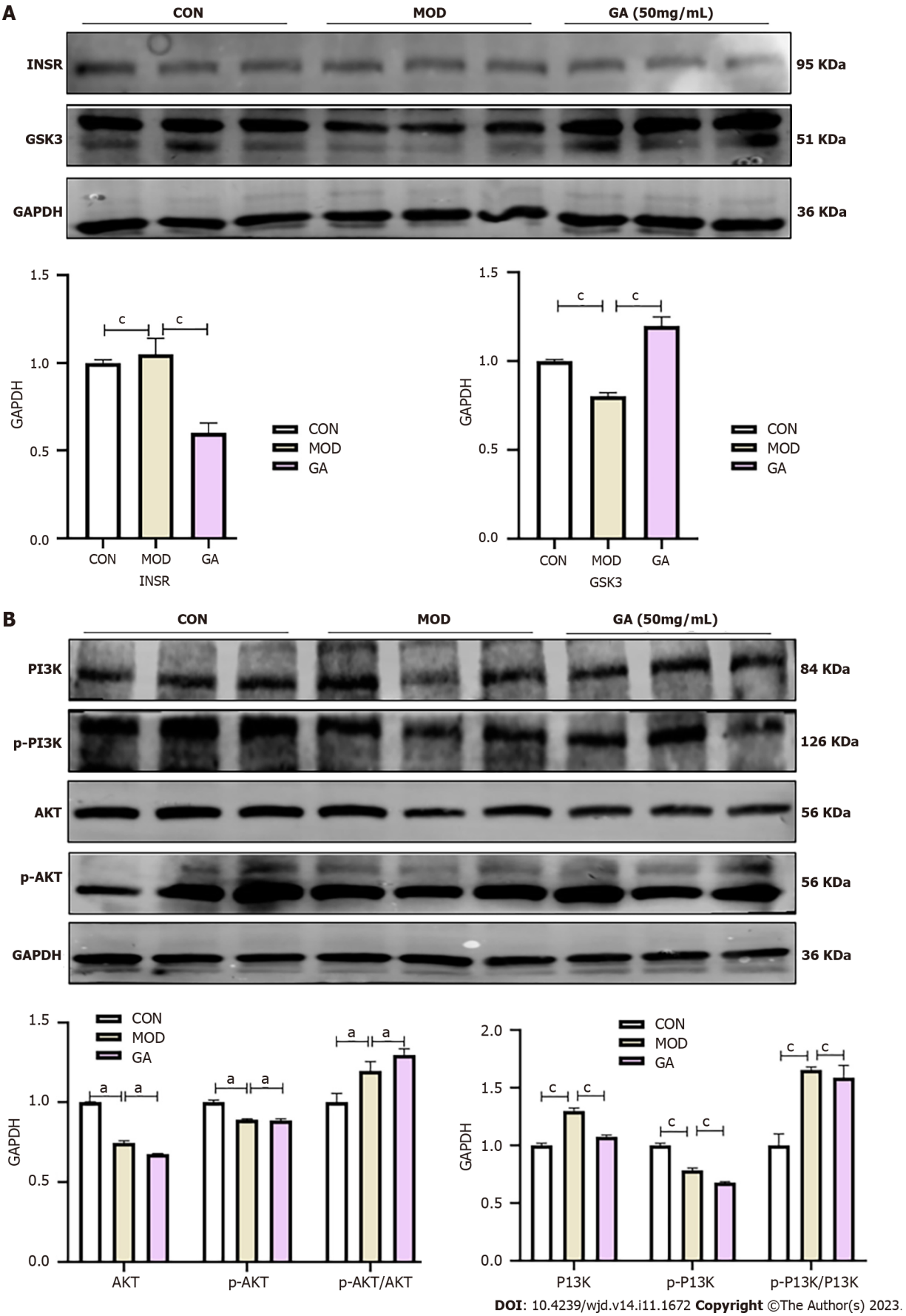

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Figure 7 Impact of glycyrrhetinic acid on signal proteins in the phosphatidylinositol 3-kinase/protein kinase B signaling pathway. A: Expression levels of insulin receptor and glycogen synthase kinase-3 proteins; B: Protein levels of phosphatidylinositol 3-kinase (PI3K)/p-PI3K and protein kinase B (AKT)/p-AKT. The sequence from left to right represents the control group, the model group, and the glycyrrhetinic acid group. $^{a}P < 0.05$, $^{b}P < 0.01$, $^{c}P < 0.001$. GA:

Glycyrrhetinic acid; CON: Control; MOD: Model; GSK3: Glycogen synthase kinase-3; INSR: Insulin receptor; AKT: Protein kinase B; PI3K: Phosphatidylinositol 3-kinase.

In light of this, our focus turned to the natural herb licorice. The primary bioactive constituent within licorice is glycyrrhizic acid, which undergoes hepatic metabolism to yield GA. Notably, GA exhibits anti-inflammatory, antiviral, anticancer, anti-cardiovascular disease, and antioxidant properties[6,11-14]. Given its multifaceted therapeutic potential, we selected GA as a candidate to predict its potential targets in the context of DN and subsequently validated these predictions through *in vitro* cellular assays.

This study delves into the mechanistic insights into GA's effects on DN, employing a network pharmacology approach. We identified a total of 186 GA targets associated with DN therapeutic effects. Utilizing a combination of GO analysis, KEGG analysis, and network interaction software, we revealed that GA may exert its influence on DN through the PI3K/AKT signaling pathway. Our predictions were substantiated *via in vitro* cellular experiments, where GA demonstrated a significant ability to inhibit the proliferation of HK-2 cells. Furthermore, it induced cell cycle arrest in the G2/M phase and resulted in a dose-dependent reduction in apoptosis in HK-2 cells. Western blot analysis unveiled that GA intervention led to the upregulation of p-AKT and GSK3 protein expression, with a concurrent increase in p-AKT/AKT ratio. Conversely, it downregulated the expression of INSR, AKT, PI3K, and p-PI3K proteins, along with decreased p-PI3K/PI3K ratio. These findings suggest that GA may hold promise in the treatment of DN by modulating the PI3K/AKT signaling pathway.

The PI3K/AKT signaling pathway assumes a pivotal role in insulin signaling[14]. PI3K initiates the activation of AKT, subsequently engaging the phosphorylated and activated AKT in a cascade of cellular processes including apoptosis, protein synthesis, metabolism, and inflammation. It orchestrates intracellular signal transduction and modulates insulin receptor substrate, thereby exerting a regulatory influence on insulin signal transduction. This can lead to IR, a condition implicated in the development of DN. Furthermore, within the diabetic milieu, the PI3K/AKT pathway is activated in renal tubule cells, governing cellular growth, epithelial-interstitial transitions, and lipid metabolism[15]. As a downstream messenger of PI3K/AKT, insulin receptor activation by receptor tyrosine kinase triggers substrate phosphorylation, activating PI3K. Subsequently, the activated AKT mediates cell proliferation, differentiation, and apoptosis by modulating a constellation of signaling molecules, including GSK3 and mechanistic target of rapamycin[16-18]. It mitigates the deleterious impact of elevated glucose levels on rat mesangial cells, ameliorates oxidative stress responses, reduces renal parenchymal cell damage, and enhances kidney function. Notably, compound Zhengjiao lipid regulating capsules have been demonstrated to effectively lower fasting glucose levels, urinary protein excretion, and creatinine in DN mice. This is achieved through the inhibition of the PI3K/AKT and STAT3 signaling pathways[19]. The therapeutic efficacy of Liuwei Dihuang Decoction in DN is linked to its ability to modulate glucose absorption, glycogen degradation, and synthesis *via* the PI3K/AKT signaling pathway[20]. Other investigations have underscored the potential of Tripterygium wilfordii-Gualsanthis Decoction in treating DN. This herbal remedy achieves its protective effects on renal intrinsic cells by inhibiting AKT activity, improving IR, curbing inflammation, and mitigating oxidative stress[21]. Furthermore, GA demonstrates the capacity to stimulate lipolysis through the activation of the PI3K/AKT/HSL pathways, reduce fatty acid synthesis, and regulate lipid metabolism by down-regulating the sregbp-1c/FAS/SCD1 pathway[22]. Consequently, these effects impinge upon glucose uptake, insulin secretion, diabetic vascular dysfunction, as well as the development of retinopathy and nephropathy[23]. In our experimental setup, we induced IR in HK-2 cells through high glucose exposure, followed by intervention with varying GA concentrations. Our findings revealed that GA effectively arrested HK-2 cells in the G2/M phase, underscoring its regulatory role in cell cycle progression from the late stage of DNA synthesis to mitosis completion in HK-2 cells. Moreover, GA resulted in a dose-dependent reduction in apoptosis among HK-2 cells. These effects were accompanied by a potential upregulation of GSK3 protein expression. Simultaneously, downregulation of INSR, AKT, PI3K, and p-PI3K proteins hinted at GA's capacity to activate or restore the impaired PI3K/AKT signaling pathway in a high-glucose environment. This cascade of events led to decreased fatty acid synthesis, reduced glucose absorption, lowered insulin secretion, and other pertinent biological processes, ultimately conferring a protective effect on human renal cortex proximal tubular epithelial cells exposed to high glucose conditions.

CONCLUSION

In summary, GA appears to modulate the PI3K/AKT signaling pathway, resulting in the inhibition of HK-2 cell proliferation and a reduction in HK-2 cell apoptosis. It also induces a G2/M phase arrest while upregulating GSK3 and p-AKT expression, and downregulating AKT, PI3K, and p-PI3K proteins (Figure 8). These actions potentially involve the reactivation or restoration of the impaired PI3K/AKT pathway in the presence of hyperglycemia, thereby affording protection to renal parenchymal cells. Subsequently, we plan to conduct genetic testing using high-throughput validation techniques such as whole transcriptome analysis, methylation studies, and acetylated proteome analysis. Gene silencing or overexpression experiments targeting the core genes will be employed to verify whether the predicted targets serve as viable drug targets. Additionally, we will perform pathway validation by employing transcriptional and translational inhibitors targeting upstream and downstream signaling proteins. Furthermore, experimental techniques including the assessment of transcription factors and DNA binding through dual luciferase assays and chromatin immunoprecipitation will be utilized to delve deeper into the therapeutic mechanism of GA in the context of DN.

Table 1 Drugs and reagents

Reagent	Manufacturer	Article number
DMEM high sugar medium	Gibco Corporation, United States	11960044
Fetal bovine serum	Corning Corporation, United States	35-081-CV
Phosphate buffer	Beijing Soleibao Company	SH30256.01
0.25% trypsin protein	Gibco Corporation, United States	T1320
Green streptomycin mixture	Beijing Soleibao Company	P1400-100
CCK-8 kit	Jiangsu Kaiji Biology	KGA317
Cell cycle kit	Jiangsu Kaiji Biology	KGA512
Apoptosis kit	Jiangsu Kaiji Biology	KGA101
BCA protein content detection kit	Jiangsu Kaiji Biology	KGP902
Total protein extraction kit	Jiangsu Kaiji Biology	KGP250
5× loading buffer	Jiangsu Kaiji Biology	KGP101
Chemiluminescence kit	Jiangsu Kaiji Biology	KGS133
10× WB washing solution	Jiangsu Kaiji Biology	KGP109
PVDF membrane	Millipore Corporation	IPVH00010
10× electrotransfer buffer	Jiangsu Kaiji Biology	KGP102
0.25% trypsin protein (EDTA not included)	Gibco Corporation	T1320
PI3K rabbit antibody	Abcam	ab302958
p-PI3K rabbit antibody	Abcam	ab278545
AKT rabbit antibody	Abcam	ab283852
Rabbit antibody to p-AKT	Abcam	ab38449
INSR Rabbit antibody	Wuhan Sanying	20433-1-AP
Rabbit antibody to GSK3	Wuhan Sanying	13419-1-AP
Rabbit IgG	Santa Cruzgong Company, United States	sc2004
GAPDH	Abcam	Ab8245

Table 2 Drug active ingredient

ID	Phytochemical name	OB (%)	DL
MOL005002	Beta-glycyrrhetinic acid	17.41	0.74
MOL004804	Alpha-glycyrrhetinic acid	22.05	0.74

OB: Oral bioavailability; DL: Drug-likeness.

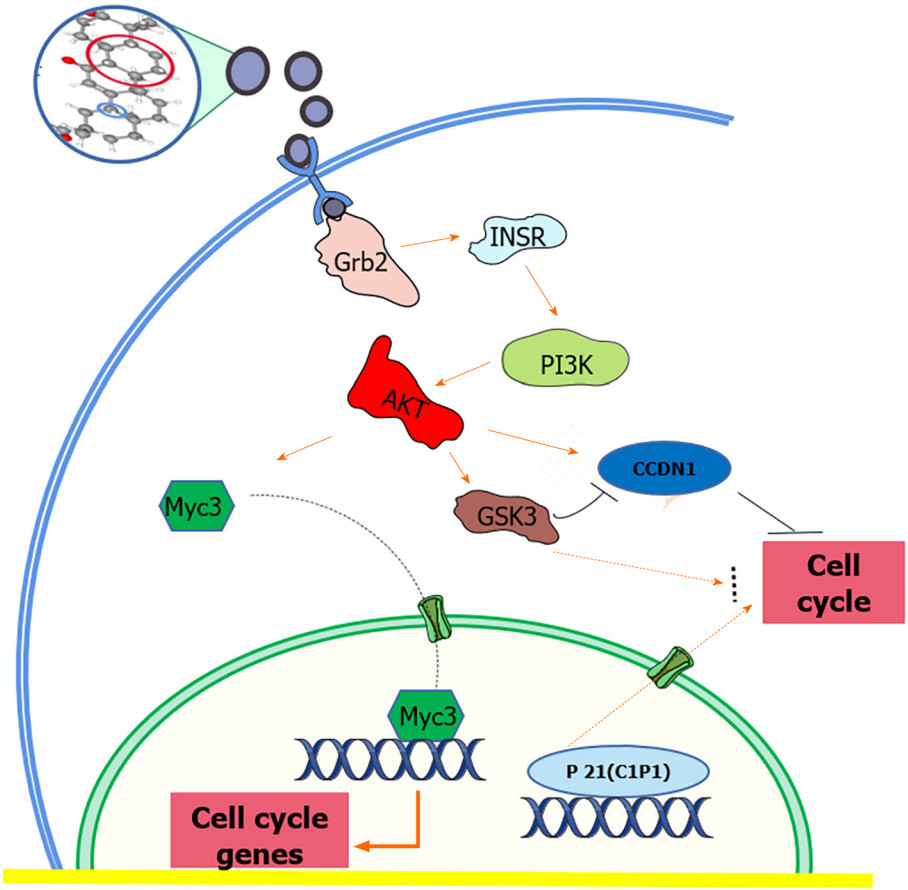

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Figure 8 Diagram of the effect of glycyrrhetinic acid on the PI3K/AKT signaling pathway. GSK3: Glycogen synthase kinase-3; INSR: Insulin receptor.

ARTICLE HIGHLIGHTS

Research background

The prognosis of diabetes nephropathy is poor. Its pathological changes are chronic progressive damage. The clinical symptoms appear late. Once there is persistent proteinuria, its renal function will inevitably decline and develop into end-stage renal failure, causing a huge health burden.

Research motivation

We executed experimental validations to furnish a novel reference method for the treatment of DN.

Research objectives

This study employed network pharmacology and molecular docking methods to predict the mechanism by which glycyrrhetinic acid (GA) treats DN, subsequently validating these predictions through experimental means.

Research methods

Utilizing comprehensive databases such as PharmMapper, TCMSP, GeneCards, OMIM, and TTD, we meticulously searched for targets associated with both the drug GA and the disease DN. Subsequently, we identified common targets by taking their intersections. The pivotal target-pathway relationships were elucidated through a protein-protein interaction network analysis. Furthermore, we conducted Gene Ontology and Kyoto Encyclopedia of Genes and Genomes enrichment analyses to gain deeper insights. Molecular docking studies were performed employing GA constituents to provide a comprehensive understanding of potential interactions.

Research results

In summary, GA appears to modulate the phosphatidylinositol 3-kinase (PI3K)/protein kinase B (AKT) signaling pathway, resulting in the inhibition of HK-2 cell proliferation and a reduction in HK-2 cell apoptosis. It also induces a G2/M phase cell cycle arrest while upregulating glycogen synthase kinase-3 and p-AKT expression, and downregulating AKT, PI3K, and p-PI3K proteins. These actions potentially involve the reactivation or restoration of the impaired PI3K/AKT pathway in the presence of hyperglycemia, thereby affording protection to renal parenchymal cells.

Research conclusions

This study found 186 therapeutic targets for DN with GA, and GA may act on DN through the PI3K/AKT signaling pathway. The results of *in vitro* cell experiments indicate that GA inhibits the proliferation of HK2 cells, blocks the cell cycle in the G2/M phase, and reduces the apoptosis of HK2 cells. GA can activate or restore the activity of the PI3K/AKT pathway damaged under high glucose conditions, thereby protecting inherent kidney cells.

Research perspectives

We plan to conduct genetic testing using high-throughput validation techniques. Gene silencing or overexpression experiments targeting the core genes will be employed to verify whether the predicted targets serve as viable drug targets. Additionally, we will perform pathway validation by employing transcriptional and translational inhibitors targeting upstream and downstream signaling proteins. Furthermore, experimental techniques including the assessment of transcription factors and DNA binding through dual luciferase assays and chromatin immunoprecipitation will be utilized to delve deeper into the therapeutic mechanism of GA in the context of DN.

FOOTNOTES

Author contributions: Meng FD, Yuan L, Xu DJ, Che MY, Hou SZ, and Lu DD designed the study; Meng FD, Yuan L, Lu DD, and Yi Nan conducted the study; Che MY and Hou SZ contributed new reagents and analytical tools; Meng FD, Yuan L, and Xu DJ analyzed the data and wrote the manuscript; and all authors have read and approved the final manuscript.

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SYSTEMATIC REVIEWS

Cellular and molecular overview of gestational diabetes mellitus: Is it predictable and preventable?

Pei-Qi Lim, Yen-Ju Lai, Pei-Ying Ling, Kuo-Hu Chen

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Abstract

BACKGROUND

In contrast to overt diabetes mellitus (DM), gestational DM (GDM) is defined as impaired glucose tolerance induced by pregnancy, which may arise from exaggerated physiologic changes in glucose metabolism. GDM prevalence is reported to be as high as 20% among pregnancies depending on the screening method, gestational age, and the population studied. Maternal and fetal effects of uncontrolled GDM include stillbirth, macrosomia, neonatal diabetes, birth trauma, and subsequent postpartum hemorrhage. Therefore, it is essential to find the potential target population and associated predictive and preventive measures for future intensive peripartum care.

AIM

To review studies that explored the cellular and molecular mechanisms of GDM as well as predictive measures and prevention strategies.

METHODS

The search was performed in the Medline and PubMed databases using the terms "gestational diabetes mellitus," "overt diabetes mellitus," and "insulin resistance." In the literature, only full-text articles were considered for inclusion (237 articles). Furthermore, articles published before 1997 and duplicate articles were excluded. After a final review by two experts, all studies (1997-2023) included in

the review met the search terms and search strategy (identification from the database, screening of the studies, selection of potential articles, and final inclusion).

RESULTS

Finally, a total of 79 articles were collected for review. Reported risk factors for GDM included maternal obesity or overweight, pre-existing DM, and polycystic ovary syndrome. The pathophysiology of GDM involves genetic variants responsible for insulin secretion and glycemic control, pancreatic β cell depletion or dysfunction, aggravated insulin resistance due to failure in the plasma membrane translocation of glucose transporter 4, and the effects of chronic, low-grade inflammation. Currently, many antepartum measurements including adipokines (leptin), body mass ratio (waist circumference and waist-to-hip ratio], and biomarkers (microRNA in extracellular vesicles) have been studied and confirmed to be useful markers for predicting GDM. For preventing GDM, physical activity and dietary approaches are effective interventions to control body weight, improve glycemic control, and reduce insulin resistance.

CONCLUSION

This review explored the possible factors that influence GDM and the underlying molecular and cellular mechanisms of GDM and provided predictive measures and prevention strategies based on results of clinical studies.

Key Words: Gestational diabetes mellitus; Overt diabetes mellitus; Insulin resistance; Diabetes mellitus

Core Tip: Maternal and fetal effects of uncontrolled gestational diabetes mellitus (GDM) include stillbirth, macrosomia, neonatal diabetes, and birth trauma. Risk factors are maternal obesity or overweight, pre-existing diabetes mellitus, and polycystic ovary syndrome. The complex pathophysiology involves genetic variants, pancreatic β cell depletion or dysfunction, aggravated insulin resistance due to glucose transporter 4 translocation failure, and chronic, low-grade inflammation. Antepartum measurements including adipokines (leptin), body mass ratio (waist circumference and waist-to-hip ratio), and biomarkers (microRNA in extracellular vesicles) are useful markers for predicting GDM. For preventing GDM, physical activity and diet (such as the Mediterranean diet) control are effective interventions.

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INTRODUCTION

Gestational diabetes mellitus (GDM) poses a severe and neglected threat to maternal and neonatal health. According to data from the International Diabetes Federation, approximately 223 million women globally are living with diabetes[1]. By 2045, this number is projected to increase to 343 million, and it is estimated that 1 in 6 births will be affected by GDM [1]. The majority of hyperglycemia cases during pregnancy occur in low-income and middle-income countries where access to maternal care is limited[1]. The rising incidence of obesity has contributed to an increase in the occurrence of GDM and related complications during pregnancy and the perinatal period[2]. Various risk factors for GDM include excessive weight gain during early adulthood and before 24 wk gestation, advanced maternal age, a history of prior GDM, a family history of overt diabetes, prepregnancy body mass index (BMI) ≥ 30 kg/m^2, prepregnancy polycystic ovary syndrome, and prior delivery of a newborn more than 4000 g[3-6]. Several races/ethnicities (Native and Hispanic American, Native Hawaiian, Native Alaskan, East or South Asian, and Pacific Islander) are also at a greater risk for the development of GDM[7-9].

Recommendations for universal screening of GDM advise conducting the screening between 24 wk and 28 wk of gestation, based on randomized controlled trials (RCTs) demonstrating improved maternal and perinatal outcomes with GDM treatment[10]. However, there is currently no consensus on the optimal diagnostic method for gestational diabetes. Two screening approaches (one-step *vs* two-step) are considered acceptable. Proposed by the International Association of the Diabetes and Pregnancy Study Groups (IADPSG) and used by the American Diabetes Association (ADA), the one-step approach employs a 2-h oral glucose tolerance test (OGTT) in all pregnant women. Despite the advantage of concurrent screening and diagnosis within a single visit, pregnant women have to fast before the examination and wait for a subsequent 2-h examination[10]. Recommended by the American College of Obstetricians and Gynecologists (ACOG), the two-step approach involves a non-fasting 1-h glucose challenge test and seems more convenient for the participants. If the patient passes the first step, further screening is usually unnecessary. However, those who fail the initial screening undergo the 3-h 100 g OGTT to confirm the diagnosis of GDM[10]. Despite the availability of these two

screening options, there is currently a lack of consensus regarding the preferred approach. The ACOG has not fully adopted the one-step process due to insufficient evidence regarding its impact on pregnancy outcomes[2]. Hillier *et al*[10] conducted a RCT and concluded that even the one-step approach can detect more cases of GDM in comparison with the two-step approach. There was no significant difference between the two groups regarding the risks of maternal and perinatal complications.

Maternal hyperglycemia stimulates fetal hyperglycemia, which in turn triggers the release of fetal insulin. As insulin possesses both metabolic and anabolic effects, fetal hyperinsulinemia can induce excessive fetal growth and subsequent adverse perinatal outcomes including neonatal shoulder dystocia, birth injury, prematurity, and death and an increased likelihood of cesarean section[11]. It was observed that 93% of women who exceeded the gestational weight gain guidelines set by the Institute of Medicine experienced excessive early gestational weight gain[11]. Excessive early gestational weight gain in low-risk nulliparous women was associated with the development of GDM and increased fetal growth[11]. The results of the Hyperglycemia and Adverse Pregnancy Outcomes (HAPO) study revealed close relations between maternal hyperglycemia and adverse perinatal outcomes, including preterm delivery, shoulder dystocia, or other birth injury, conversion to cesarean delivery, neonatal hypoglycemia or hyperbilirubinemia, and the need for intensive neonatal care. These associations were attributed to the clinical consequences of maternal hyperglycemia, namely fetal overgrowth and hyperinsulinemia[12]. The aforementioned findings further supported the role of glucose-lowering therapy as a priority for women who were diagnosed with GDM[12].

This review explored the underlying molecular and cellular mechanisms of GDM as well as the potential influencing factors. Additionally, we discussed predictive and preventive measures based on both basic and clinical studies that have investigated the etiology, pathophysiology, and management of GDM. Understanding the cellular and molecular mechanisms involved in GDM will enhance our knowledge of the current principles of drug action and may provide insights into identifying new treatment targets.

MATERIALS AND METHODS

Search terms and strategies in the literature

The objective of this review was to identify relevant studies in the literature that investigated the underlying cellular and molecular mechanisms of GDM as well as potential influencing factors, predictive measures, and preventive strategies. A systematic search was conducted in the Medline and PubMed databases using keywords such as "gestational diabetes mellitus," "overt diabetes mellitus," and "insulin resistance." The flowchart in Figure 1 illustrates the search terms, strategy, and the process of study screening and selection. Initially, only full-text articles were considered for further analysis, followed by the exclusion of articles published prior to 1997 and any duplicates. Out of the 237 articles initially identified, 111 articles published between 1997 and 2023 met the inclusion criteria.

Following the article selection process, two experts in the field conducted independent reviews of the selected articles, evaluating various aspects such as demographics, research designs, and outcomes to identify relevant basic and clinical studies for inclusion. Articles with inadequate research designs, questionable sampling methods, or incongruent outcomes were excluded at this stage. Any discrepancies between the experts were resolved through mutual communication to establish a consensus. All eligible studies included in this review adhered to the predefined search terms and strategy, which encompassed database identification, study screening, article selection, and final inclusion. In the end, a total of 79 articles were included in the review out of the initial 237 identified articles. Among these selected articles (n = 79), approximately 60% (n = 47) were published between 2018 and 2023, ensuring the novelty of the review.

EPIDEMIOLOGY

GDM is a worldwide health problem that threatens both maternal and fetal health, and its prevalence is increasing. Both global and regional prevalence of GDM are affected by different race/ethnicities, age, body composition, and geological regions[13,14]. Prevalence is also affected by variations between different screening strategies and diagnostic criteria[15]. Noted international organizations, including the ADA, Federation International of Gynecology and Obstetrics, and the World Health Organization, have all adopted the diagnostic criteria for GDM adopted by the IADPSG after the landmark HAPO study. According to the statistics and data collected by the International Diabetes Federation using the 2021 IADPSG diagnostic criteria, the global prevalence of GDM was 14%[15,16]. The regional prevalence of GDM was 20.7% in North America and Caribbean, 15.0% in Europe, 15.8% in South America and Central America, 13.0% in Africa, 14.0% in the Western Pacific, 25.9% in South-East Asia, and 14.1% in the Middle East and North Africa. A high prevalence of GDM was noted especially in Asia and North America[16].

ETIOLOGY AND RISK FACTORS

The specific and detailed cause of GDM is not fully known, although theories have been proposed for its etiology and pathophysiology. During pregnancy, the placenta supplies not only nutrition to the fetus but also a variety of hormones to maintain the pregnancy, such as human placental lactogen and estrogen. These hormones can cause insulin resistance, starting usually from 20-24 wk of pregnancy and becoming more prominent in the later stages of pregnancy. Thus, the β

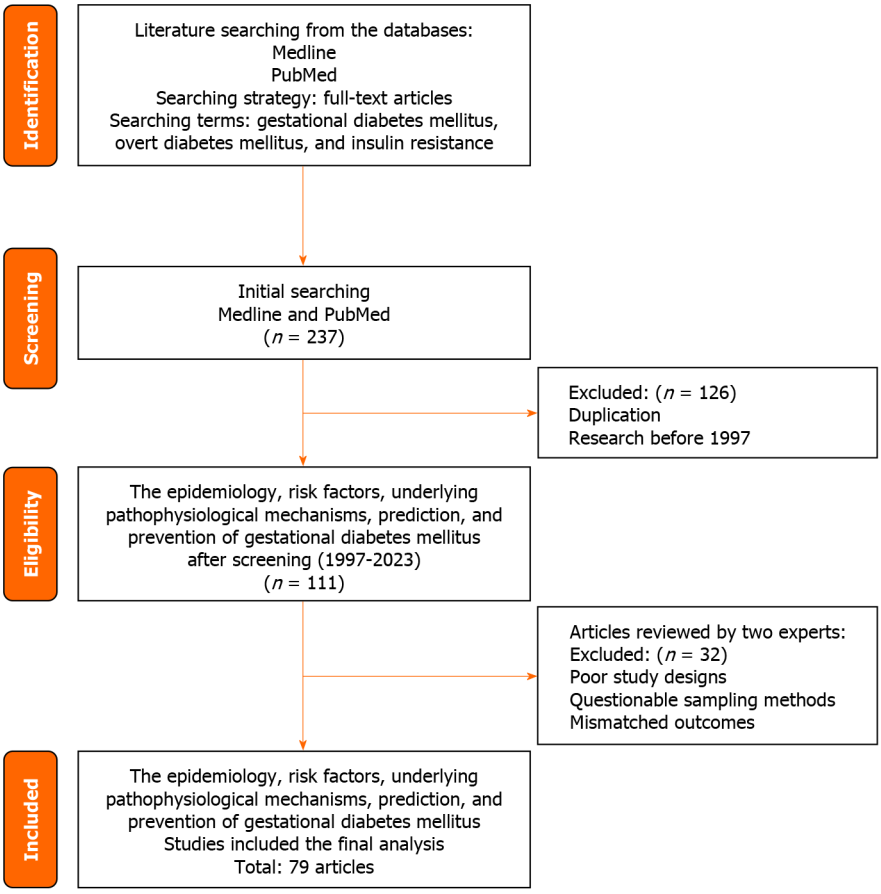

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Figure 1 PRISMA flow diagram of literature search, search strategy, screening, and selection of studies.

cells of the pancreas need to produce more insulin to overcome insulin resistance. Once this additional production of insulin is not enough to overcome the resistance, GDM occurs[17].

Many well-established risk factors are associated with GDM. Each of these risk factors is either directly or indirectly associated with impaired β cell function and insulin resistance. Several risk factors increase concurrently with the rising prevalence of GDM, including pregestational overweight and obesity, excessive gestational weight gain, and advanced maternal age[18-21]. Other risk factors include a previous history of GDM, a family history of DM, and endocrine diseases such as hypothyroidism and polycystic ovary syndrome (PCOS)[18,19]. Most of the identified factors can be categorized into several groups.

Risk factors related to the mother

Obesity is one of the key factors causing overt DM and GDM. BMI is commonly used to measure the severity of obesity. Therefore, pregestational BMI is one of the most important risk factors associated with GDM. According to a recent umbrella review and other studies, a convincing correlation was shown between BMI and GDM[18,19]. A meta-analysis in Asia has also shown that pregestational BMI ≥ 25 kg/m^2 increased the risk of GDM by more than three-fold compared to the risk in those with normal BMI before pregnancy[19]. Moreover, pregestational BMI and gestational weight gain, both of which result in elevated gestational BMI, are major risk factors for GDM. High rates of bodyweight gain, particularly during early pregnancy, has been shown to increase the risk of GDM. This is also more prominent in those who are already overweight/obese before pregnancy[22,23].

Advanced maternal age is another important risk factor for GDM. The risk is more prominent for women with a maternal age of 35 years or older, by more than three-fold. According to a systematic review and meta-analysis of over 120 million participants, for each 1-year increase in maternal age from 18 years, the risk of GDM increases by 7.90% for the overall population[24]. According to previous reports, the odds ratios (ORs) and 95% confidence intervals (CIs) for the risk of GDM acquired from this large-scale population of women aged 30-34 years, 35-39 years, and ≥ 40 years were 2.73 (95%CI: 2.28-3.27), 3.54 (95%CI: 2.88-4.34) and 4.86 (95%CI: 3.78-6.24), respectively[24,25]. These age-related results were attributed to decreased insulin sensitivity and pancreatic β cell function, which eventually led to glucose and lipid metabolism abnormalities during pregnancy.

Risk factors associated with personal and family history of GDM or DM

Experiencing a previous pregnancy complicated by GDM is a well-established risk factor for developing GDM in subsequent pregnancies, as highlighted in various studies, systematic reviews, and meta-analyses[19,26]. A meta-analysis

focusing on Asian populations found that pregnant women with a history of GDM were at least eight times more likely to develop GDM in their subsequent pregnancy compared to those without such a history[19].

In addition to a personal history of GDM, having a family history of DM is also significant as a risk factor. Females with a family history of DM, particularly in first-degree relatives, have a genetic predisposition and increased susceptibility to GDM. Multiple studies, including a systematic review and meta-analysis, have reported that pregnant women with a family history of DM are at least twice as likely to develop GDM compared to those without a family history[18,26-28].

PCOS is a complex condition characterized by irregular menstruation/oligo-ovulation, or anovulation, and excess androgens and/or small cysts on one or both ovaries[29]. Due to the hormone imbalance of hyperandrogenism, the majority of women with PCOS also manifest insulin resistance. This gives females with PCOS a higher risk of developing GDM. A recent systematic review and meta-analysis displayed the positive relationship between PCOS and GDM with an OR of 2.33 (95%CI: 1.72-3.17), consistent with the underlying pathophysiology[19]. This finding was further supported by an umbrella review in 2019[18]. Our previous study of a large population (> 1000000 nationals) also reported that prepregnancy PCOS was significantly associated with subsequent GDM (adjusted OR: 2.15; 95%CI: 1.96-2.37)[30].

Hypothyroidism disorders and GDM are among the most common endocrinopathies during pregnancy. A meta-analysis of 5995 cases revealed a significant association between hypothyroidism and GDM[31]. A recent umbrella review also provided convincing evidence to support this point of view[18]. Based on these results, routine screening of thyroid function appears to be necessary in pregnant women.

Other risk factors

Several risk factors that are less frequently documented yet identified by a number of studies and reviews include previous congenital anomalies of fetuses, previous macrosomia, a history of stillbirth, previous abortion, multiparity ≥ 2, and previous preterm delivery with ORs ranging from 1.37 to 4.25[19]. Other reported influence factors include ethnicity, lifestyles, and socioeconomic status[32,33].

PATHOPHYSIOLOGY: CELLULAR AND MOLECULAR MECHANISMS OF GDM

GDM is characterized by an imbalance in glucose control during pregnancy, involving complex interactions among insulin sensitivity, glucose production, and the body's cells. Throughout pregnancy, insulin requirements naturally increase due to factors such as heightened maternal caloric intake, maternal weight gain, and the influence of placental hormones like placental growth hormone and human placental lactogen[2]. Additionally, there is an approximate 50% decrease in maternal insulin sensitivity, which is compensated by a 250% increase in maternal insulin production to maintain normal blood sugar levels during pregnancy[34]. Figure 2 provides a concise overview of insulin signaling and subsequent translocation of glucose transporter 4 (GLUT4), illustrating the cellular and molecular mechanisms implicated in GDM.

Genetic components of GDM

GDM is influenced by a combination of genetic variants, nutritional factors, and environmental influences. The pathophysiological changes observed in GDM are similar to those found in type 2 DM (T2DM), characterized by impaired insulin secretion and increased insulin resistance. This indicates a strong association between the two forms of diabetes. Consequently, research on GDM has focused on investigating genetic variants associated with T2DM susceptibility. Several case-control studies and meta-analyses using single nucleotide polymorphisms have revealed common pathogenic pathways shared by GDM and T2DM. A genome-wide association study conducted on a South Korean cohort showed a significant association between GDM and specific single nucleotide polymorphisms in genes already known to be associated with T2DM susceptibility, highlighting the genetic overlap between the two forms of diabetes. Many of the genetic loci associated with GDM primarily impact β cell functions. Various genome-wide association studies have identified variants in the *HKDC1* and *BACE2* genes, which influence glycemic traits during pregnancy and exhibit specific associations with GDM in different ethnic groups[34].

β cell dysfunction

The mechanisms underlying β cell dysfunction and inadequate insulin production in GDM are intricate and diverse. Defects can occur at various stages of the process, including pro-insulin synthesis, post-translational modifications, granule storage, glucose sensing, and granule exocytosis. However, the majority of susceptibility genes associated with GDM primarily relate to β cell functions, such as potassium voltage-gated channel KQT-like 1 and glucokinase. Minor deficiencies in β cell machinery may only become evident during metabolic stress, such as pregnancy. Animal studies have demonstrated that the quantity of β cells plays a crucial role in glucose homeostasis. A reduction in β cell mass, associated with the epigenetic downregulation of pancreatic homeobox transcription factor, has been linked to GDM. Additionally, adequate β cell proliferation relies on prolactin, as evidenced in mouse models with prolactin receptor knockouts (Prlr-/-). Glucotoxicity is also believed to contribute to β cell apoptosis over time. Pancreatic samples from patients with T2DM showed a reduction in β cell mass ranging from 40% to 60%, while a loss of less than 24% has been reported after 5 years of the disease. Animal studies and limited post-mortem human studies suggest that reduced β cell hyperplasia may also play a role in the pathophysiology of GDM. Overall, a reduced β cell mass, decreased number of β cells, β cell dysfunction, or a combination of these factors contribute to GDM, depending on the individual[17].

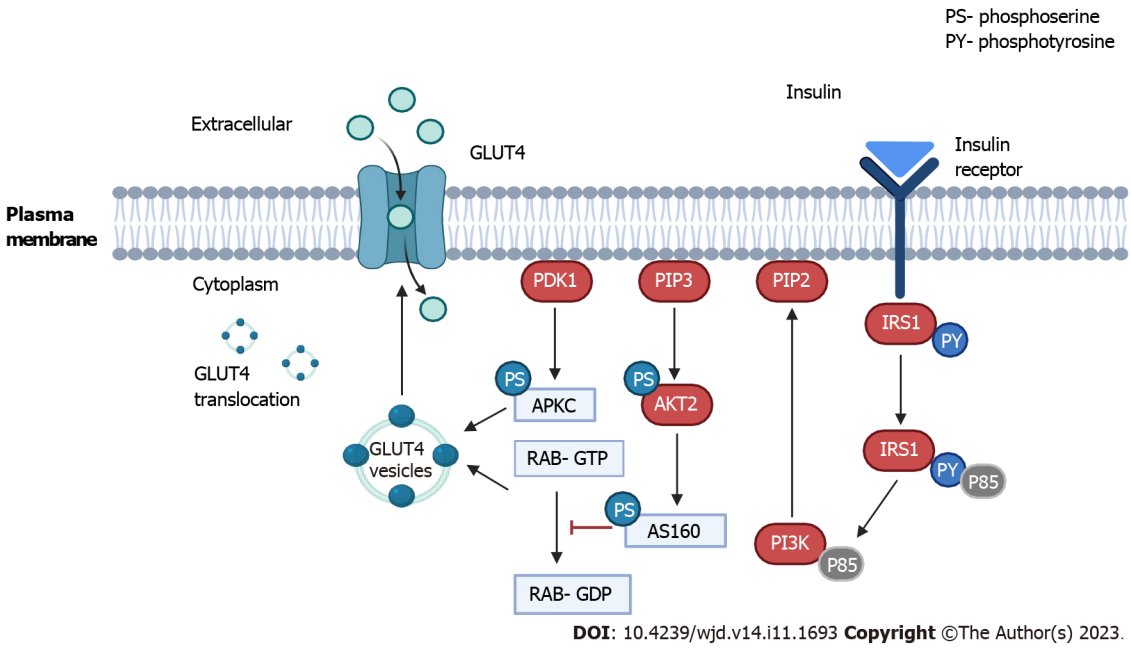

Figure 2 A brief summary of insulin signaling and subsequent glucose transporter 4 translocation that implicate the cellular and molecular mechanisms of gestational diabetes mellitus. AKT: Protein kinase B; APKC: Atypical protein kinases C; GDP: Guanosine diphosphate; GLUT4: Glucose transporter 4; GTP: Guanosine triphosphate; IRS: Insulin receptor substrate; PI3K: Phosphatidylinositol 3 kinase; PDK1: Phosphoinositide-dependent protein kinase 1.

Chronic insulin resistance

In clinical practice, insulin resistance refers to a state in which a given concentration of insulin is associated with a subnormal glucose response. At the molecular level, GLUT4 serves as the primary transporter responsible for bringing glucose into the cell to use as an energy source. Insulin resistance is usually a failure of insulin signaling, which is associated with inadequate plasma membrane translocation of GLUT4 (Figure 2)[17]. GLUT4 has the unique characteristic of a mostly intracellular disposition in the unstimulated state in storage vesicles called GLUT4 storage vesicles that are acutely redistributed in the plasma membrane in response to insulin and other stimuli, like exercise. The major insulin signaling pathway involved in GLUT4 storage vesicles translocation is the phosphatidylinositol 3 kinase/phosphoinositide-dependent protein kinase 1/protein kinase B pathway through phosphorylation of the AS160 substrate. AS160 is a guanosine triphosphatase-activating protein that blocks the exchange of guanosine triphosphate for guanosine diphosphate when small G proteins called RAB that are involved in membrane trafficking are activated by phosphorylation. Atypical protein kinases C isoforms also appear to be involved downstream of phosphoinositide-dependent protein kinase 1 but not through protein kinase B[35].

Dysfunction of leptin signaling pathways

Another notable etiology that is responsible for GDM is dysfunction of the leptin signaling pathways (Figure 3). Leptin signaling is mediated by the JAK2/signal transducer and activator of transcription 3 (STAT3) pathway to exert its anorexigenic effect. Binding to the leptin receptor, leptin activates JAK2, STAT3, and MAPK *via* phosphorylation of different sites on the leptin receptor and subsequent binding to downstream molecules[36,37]. Thus, JAK2 and STAT3 are phosphorylated. The activated STAT3 translocates to the nucleus and activates the transcription of the target genes, which mediates the anorexigenic effect of leptin. The negative regulators of JAK2, including suppressor of cytokine signaling 3 and protein tyrosine phosphatase 1B, which act as feedback inhibitors of leptin signaling, have been reported to promote obesity and diabetes. On the other hand, leptin also regulates MAPK and phosphatidylinositol 3 kinase signaling through phosphorylation of insulin receptor substrate. In addition, leptin inhibits appetite-stimulators neuropeptide Y and agouti-related peptide, thus activating the anorexigenic polypeptide pro-opiomelanocortin[36,37].

Inflammation underlying GDM: Nuclear factor-kappa B signaling pathway

During pregnancy, a regulated inflammatory profile is necessary for successful implantation and fetal development. However, conditions like GDM, obesity, and metabolic diseases are associated with chronic low-grade inflammation, leading to altered immune profiles and the promotion of a proinflammatory environment in various tissues, including adipose tissue, liver, kidney, heart, and pancreas. This chronic inflammation, characterized by increased levels of proinflammatory cytokines (IL-1β, IL-6, TNFα, and leptin) and decreased levels of anti-inflammatory molecules (IL-4, IL-10, and adiponectin), plays a significant role in the pathophysiology of GDM. Studies have shown that inflammation in pregnant women with obesity or GDM can affect fetal development. Experimental animal models and clinical studies provide evidence that maternal and placental inflammation associated with GDM and obesity can influence neurodevelopment and alter inflammatory responses in offspring[34].

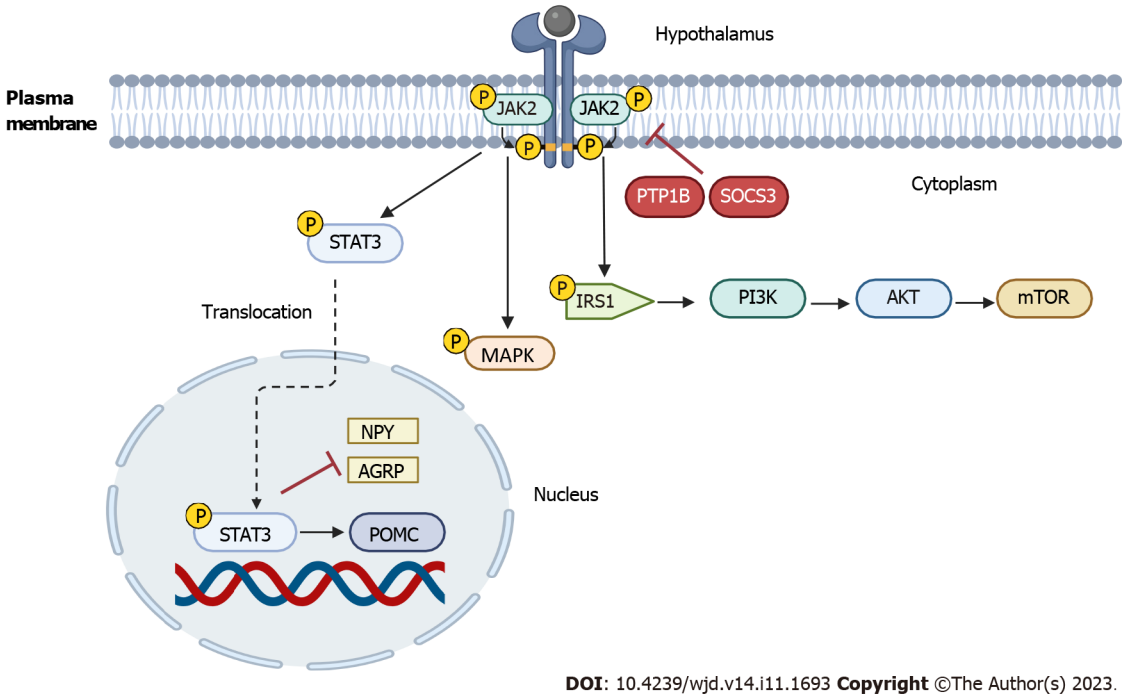

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Figure 3 Brief summary of leptin signaling pathways, in which leptin can bind to the leptin receptor and activate JAK2, signal transducer and activator of transcription 3, and MAPK *via* phosphorylation of different sites on the leptin receptor and subsequent reaction to downstream molecules to exert its anorexigenic effect. AgRP: Agouti-related peptide; AKT: Protein kinase B; IRS: Insulin receptor substrate; NPY: Neuropeptide Y; PI3K: Phosphatidylinositol 3 kinase; POMC: Polypeptide pro-opiomelanocortin; PTP1B: Protein tyrosine phosphatase 1B; SOCS3: Suppressor of cytokine signaling 3; STAT3: Signal transducer and activator of transcription 3.

The nuclear factor kappa B (NF-κB) signaling pathway is a central detailed mechanism in the inflammatory process (Figure 4). The proteins in the NF-κB family combine with each other to form homodimers or heterodimers to exert stimulative or repressive functions after transcription. As suppressors, the inhibitory regulators of NF-κB bind to the NF-κB dimers to form a complex that remains sequestered and inactive in the cytoplasm of non-stimulated cells[38]. Under the status of insulin resistance, proinflammatory cytokines, including IL-1β, IL-6, and TNFα, are increased to initiate the NF-κB signaling pathway. After receiving stimulation from the aforementioned cytokines and toll-like receptors, the inhibitory regulators of NF-κB s are rapidly phosphorylated, ubiquitinated, and then degraded, exposing a nuclear localization sequence on the NF-κB proteins. Thus, the NF-κB dimers translocate to the nucleus to regulate gene transcription and induce inflammatory cascades[38].

PREDICTING GDM

Recently, many antepartum measurements, including adipokines (leptin), body mass ratio [waist circumference (WC) and waist-to-hip ratio], and biomarkers (microRNA in extracellular vesicles) have been studied and confirmed to be useful as markers for predicting development of GDM during pregnancy. The HAPO study has provided compelling evidence of the associations between maternal BMI, hyperglycemia, and pregnancy complications. Both factors are correlated with higher rates of excessive fetal growth, primary cesarean birth, clinical neonatal hypoglycemia, fetal adiposity, neonatal hyperinsulinemia, and hypertensive disorders of pregnancy. The relationship between hyperglycemia and adverse outcomes generally follows a linear pattern, while BMI exhibits a quadratic pattern with diminishing increments in the highest BMI categories. Moreover, considering BMI and GDM together, the HAPO study has shown that they are associated with pregnancy complications.

GDM often serves as a precursor to the later development of T2DM and indicates a higher risk of (premature) cardiovascular disease in females. McIntyre *et al*[39] concluded that predictive models incorporating multiple clinical characteristics and early pregnancy glucose measurements exhibited the best performance. Research has also indicated that interventions initiated early in pregnancy can reduce the incidence of GDM in overweight and obese pregnant women[40].

Leptin

Adipokines, such as leptin, play a crucial role in regulating various processes in the human body, including glucose and lipid metabolism, insulin sensitivity, appetite, immune response, and inflammation, and may serve as potential targets for predictive and therapeutic strategies in different medical conditions[17]. Leptin, primarily secreted by adipocytes in response to adequate fuel stores, acts on neurons within the hypothalamic arcuate nucleus to decrease appetite and

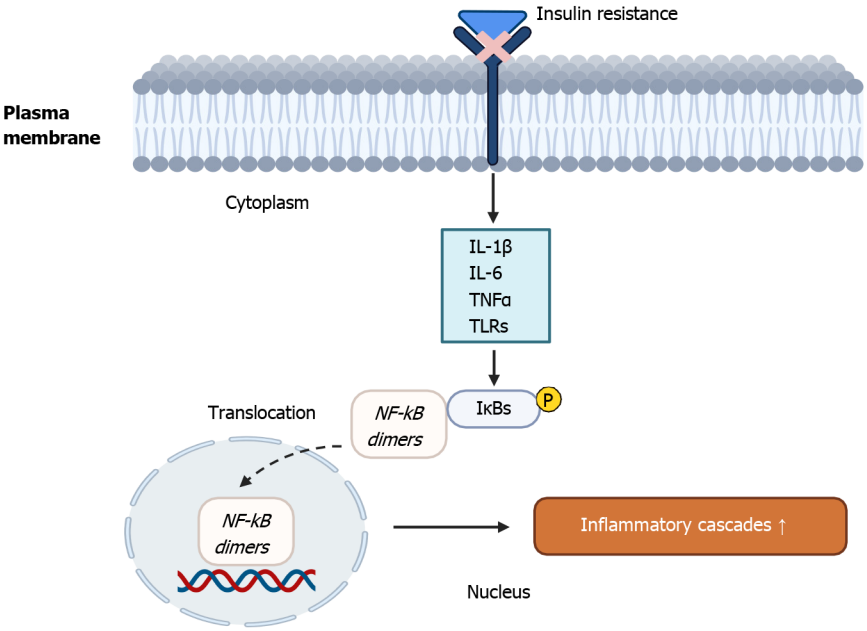

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Figure 4 Brief summary of the nuclear factor-kappa B signaling pathway. As suppressors, the inhibitory regulators of nuclear factor-kappa B (NF-κB) can bind to the NF-κB dimers to form a complex, which remains sequestered and inactive in the cytoplasm of non-stimulated cells. Under the status of insulin resistance, proinflammatory cytokines (IL-1β, IL-6, and TNFα) and toll-like receptors are increased to initiate the phosphorylation and degradation of inhibitory regulators of NF-κB to expose a nuclear localization sequence on the NF-κB proteins. Thus, the NF-κB dimers translocate to the nucleus to regulate gene transcription and induce inflammatory cascades. IκB: Inhibitory regulators of nuclear factor-kappa B; TLR: Toll-like receptor.

increase energy expenditure. During normal pregnancy, a certain degree of leptin resistance develops to ensure sufficient fat stores. However, in GDM, leptin resistance is further exacerbated, leading to hyperleptinemia. The placenta becomes the primary source of leptin during pregnancy and increases its production, facilitating the passage of amino acids to the fetus. Elevated leptin levels have been associated with obesity, GDM, and the risk of fetal macrosomia. Therefore, hyperleptinemia can serve as a marker for predicting the occurrence of GDM. Additionally, leptin has been implicated in placental neoformation, functioning as a growth factor, angiogenic factor, and immunomodulator[40].

GDM represents a stage of subclinical inflammation and serves as a risk factor for the subsequent development of T2DM and cardiovascular diseases. Leptin has been associated with vascular and metabolic changes in GDM, although findings regarding its involvement in maternal, perinatal, and future complications have been inconsistent and varied. Several studies have demonstrated significantly higher leptin levels in participants with GDM compared to controls. Moreover, leptin has been incorporated into first-trimester risk prediction models for GDM, and elevated levels have been observed in females who later develop GDM, confirming its predictive value. Leptin has also been identified as a promising diagnostic biomarker for GDM, showing high sensitivity and specificity in adequate sample sizes. During pregnancy, circulating leptin concentrations increase, reaching a peak around 28 wk of gestation and returning to prepregnancy levels postpartum. Pregnant women with GDM exhibit even higher leptin levels, further highlighting its potential as a predictive marker for GDM[40].

Obesity

Maternal obesity contributes to increased risks during pregnancy, including the development of GDM. Women with a BMI of 30 kg/m^2 or higher are considered at risk and should receive additional care. Heslehurst *et al*[41], in a systematic review, presented comprehensive evidence on the relationship between adiposity measures in early pregnancy and maternal health outcomes. WC was the most commonly studied adiposity measure during early pregnancy. Both meta-analyses and narrative syntheses consistently demonstrated that WC is a robust predictor of adverse maternal health outcomes. It consistently exhibits a significant association with GDM, hypertensive disorders, delivery-related outcomes, metabolic syndrome, and composite adverse pregnancy outcomes. Waist-to-hip ratios also show potential associations with these conditions, as they are significantly linked to GDM, hypertensive disorders, and delivery-related outcomes. Furthermore, several other measures such as fat mass, neck circumference, skinfold thickness, visceral fat measures, arm circumference, and waist-to-height ratio exhibit significant associations with various adverse outcomes[41].

Meta-analyses have indicated significantly increased odds of GDM in higher WC categories (OR: 1.40, 95%CI: 1.04-1.88) and per unit increase in WC (OR: 1.31, 95%CI: 1.03-1.67), suggesting that women with GDM have larger WC measurements compared to controls (mean difference: 6.18 cm, 95%CI: 3.92-8.44 cm). Although data on other adiposity measures are limited, fat mass, neck circumference, skinfold thickness, and visceral fat exhibit significant associations with adverse outcomes. This study emphasized the importance of exploring the utility of adiposity measures in predicting the risk of GDM during pregnancy beyond BMI alone, highlighting the need for personalized care based on specific requirements of pregnant women[41].

Other predictive factors

GDM is linked to abnormal placentation and early pregnancy markers commonly used for predicting aneuploidy, such as pregnancy-associated plasma protein A and free β human chorionic gonadotropin. These markers have been integrated into predictive models. Proteomic screening during early pregnancy has identified potential protein markers, including a cluster related to insulin secretion, binding, resistance, and signaling, which may have implications for the development of GDM later on.

Additionally, extracellular vesicles (ECVs) have been investigated as potential markers for GDM. ECVs primarily contain microRNAs and are associated with glucose metabolism. These circulating particles originate mainly from the placenta and adipose tissue during pregnancy and carry various protein and RNA molecules that can be transported to specific sites. James-Allan *et al*[42] demonstrated that specific small ECVs are associated with GDM and that infusing ECVs from females with GDM induced insulin resistance and reduced insulin secretion in rodents, aligning with the pathophysiology of GDM. Yoffe *et al*[43] conducted a case-control study and suggested that microRNA-223 and microRNA-23a in first-trimester blood samples strongly predict the development of GDM (area under the receiver operating characteristic curve: 0.91)[39]. Similarly, another recent cohort study confirmed similar findings for microRNA-233[36,39]. These cohort studies have examined various clinical, demographic, and molecular biomarkers, including early pregnancy glycemic measurements, to uncover complex networks of predictive factors. For molecular biomarkers to be clinically useful, they must outperform clinical risk factors and simple glucose measurements in predicting GDM and pregnancy outcomes[39].

Cohort studies have examined single or multiple clinical and demographic measures, including early pregnancy glycemic measurements, and have expanded their focus to include complex networks of molecular biomarkers. To be applicable in routine clinical practice, molecular biomarkers need to demonstrate superior performance compared to clinical risk factors and simple glucose measurements in predicting GDM and pregnancy outcomes[39].

PREVENTING GDM

Numerous reviews and studies have revealed that early intervention and prevention of GDM reduces the risk of perinatal and long-term complications. Therefore, it is important to provide early intervention to achieve optimal outcomes. Several meta-analyses have emphasized the effectiveness of lifestyle interventions, including both dietary interventions and physical exercise for prevention of GDM.

One meta-analysis involving 29 RCTs with around 11500 participants demonstrated that either diet or physical activity may result in an overall 18% reduction in the risk of GDM, especially in early pregnancy before the 15th gestational week [relative risk (RR): 0.80, 95%CI: 0.66-0.97][44]. A meta-analysis involving 47 RCTs with more than 15000 individuals showed that diet and exercise during pregnancy were preventive factors for GDM (RR: 0.77, 95%CI: 0.69-0.87)[45]. A review indicated that moderate-intensity exercise for 50-60 min twice a week reduced the incidence of GDM by about 24%[46]. Another systematic review and meta-analysis soliciting 45 studies also reported that dietary management and physical activity during pregnancy reduced the risk of GDM by 44% (RR: 0.56, 95%CI: 0.36-0.87) and 38% (RR: 0.62, 95%CI: 0.50-0.78), respectively[46].

In contrast, another systematic review and network meta-analysis pointed out that neither diet, physical activity, nor a combination of both offered significant benefit in preventing GDM in overweight/obese women, despite that these measures were all remarkably beneficial for gestational weight gain restriction. Although most studies concluded that combining diet with physical activity may still tend to prevent GDM development, more studies are required to compensate for the inconsistency between studies. Table 1 summarizes the preventive strategies for GDM.

Physical activity

Physical activity is an effective intervention for controlling body weight, improving glycemic control, and reducing insulin resistance. At one time, physical activity alone was considered not effective enough in reducing the risk of developing GDM. However, the American Dietetic Association and the American Nutrition Society published a study in 2009 that overweight and obese females who practiced moderate exercise during pregnancy not only gained significantly less fat but also reduced the risk of GDM[47]. Two meta-analyses in 2015 and 2016, which analyzed 2873 pregnant women from 13 RCTs and 11487 pregnant women from 29 RCTs, respectively, revealed that physical exercise during pregnancy decreased the risk of GDM by 31% (RR: 0.69; P = 0.009)[48] and 20% (RR: 0.80, 95%CI: 0.66-0.97)[42], respectively, especially during the early stage of pregnancy (before the 15th week)[42].

Moreover, a 2018 systematic review and meta-analysis that examined a total of 106 RCTs including 273182 participants with moderate-to-high-quality evidence revealed that exercise-only interventions were effective in reducing the risk of GDM by a remarkable 38% (OR: 0.62, 95%CI: 0.52-0.75)[49]. A recent umbrella review in 2022 focusing on the relationship between exercise during pregnancy and GDM prevention further confirmed the effectiveness of early initiated (first trimester), low-to-moderate intensity exercise in reducing the incidence of GDM[50].

In addition to the advantage of preventing GDM, physiological studies have reported that exercise in pregnant women improved cardiovascular functions, resulting in improved fitness, blood pressure, and peripheral edema. An ACOG report also showed that exercise improved the symptoms of constipation, bloating, fatigue, and insomnia in pregnant women. For the fetus, the benefits of moderate maternal exercise included an increase in amniotic fluid, increases in placenta viability and volume, improvement in neurological system development, and reduction in body fat percentage. However, excessive exercise may elevate the incidence of antepartum hemorrhage and the risk of preterm birth. Considering these disadvantages of excess exercise, physical exercise should be avoided in pregnant women with

Table 1 A summary of preventive strategies for gestational diabetes mellitus

Ref.	Study design	Patients	Intervention	Result
Song *et al* [44], 2016	Meta-analysis of 29 RCTs	11487 pregnant women	Diet or physical activity during pregnancy	(1) Intervention groups resulted in 18% (95%CI: 5%-30%) reduction in the risk of GDM (P = 0.0091); and (2) Intervention was effective especially before the 15th gestational week (RR: 0.80, 95%CI: 0.66-0.97)
Guo *et al* [45], 2019	Meta-analysis of 47 RCTs	15745 participants	Diet and exercise during pregnancy	(1) Combination intervention were preventive of GDM (RR 0.77, 95%CI: 0.69-0.87); and (2) Exercise of moderate intensity for 50-60 min twice a week could lead to an approximately 24% reduction in GDM
Bennett *et al* [46], 2018	Systematic review and meta-analysis of 45 studies	15293 participants	Diet and exercise during pregnancy	(1) Diet intervention reduced GDM risk by 44% (RR: 0.56, 95%CI: 0.36-0.87); (2) PA intervention reduced GDM risk by 38% (RR: 0.62, 95%CI: 0.50-0.78); (3) PA interventions in southern European group reduced GDM risk by 37% (RR: 0.63, 95%CI: 0.50-0.80); and (4) Diet and PA interventions in Asian group reduced GDM risk by 62% (RR: 0.38, 95%CI: 0.24-0.59) and 32% (RR: 0.68, 95%CI: 0.54-0.86), respectively
Sanabria-Martínez *et al*[48], 2015	Meta-analysis of 13 RCTs	2873 pregnant women	Physical exercise during pregnancy	(1) Physical exercise decreased the risk of GDM by 31% (RR = 0.69; P = 0.009); and (2) Decreases were also observed in maternal weight (WMD = -1.14 kg; 95%CI: -1.50 to -0.78; P < 0.001)
Davenport *et al*[49], 2018	A systematic review and meta-analysis including 106 studies	273182 participants	Exercise, alone or in combination (dietary + exercise)	(1) Exercise-only interventions, but not exercise + cointerventions, reduced odds of GDM (n = 6934; OR: 0.62, 95%CI: 0.52 to 0.75); and (2) Also reduced odds of gestational hypertension (n = 5316; OR: 0.61, 95%CI: 0.43 to 0.85) and pre-eclampsia (n = 3322; OR: 0.59, 95%CI: 0.37 to 0.9) compared with no exercise
Martínez-Vizcaíno *et al*[50], 2022	An umbrella review: 23 systematic reviews and meta-analyses and 63 RCTs were included		Single exercise interventions	(1) Single exercise interventions reduced GDM incidence in most systematic reviews and meta-analyses around 39%; (2) Single exercise interventions also reduced gestational hypertension incidence in most systematic reviews and meta-analyses around 47%; and (3) Particularly when supervised, with low-to-moderate intensity level, and initiated early during the first trimester of pregnancy
Griffith *et al* [55], 2020	An overview of Cochrane Reviews (11 Cochrane Reviews with 71 trials)	23154 women	Diet, exercise, and combination; dietary supplements such as vitamin D and probiotics, pharmaceuticals such as metformin	(1) A combination of exercise and diet of possible benefit in reducing the risk of GDM; (2) Unknown benefit or harm of a low glycemic index diet *vs* a moderate-high glycemic index diet on the risk of GDM; (3) Supplementation with vitamin D and metformin were of possible benefit in reducing the risk of GDM; and (4) There was insufficient high-quality evidence to establish the effect on the risk of GDM of diet or exercise alone, probiotics, vitamin D with calcium or other vitamins and minerals
Melero *et al* [61], 2020	RCT	284 participants	MedDiet *vs* control	MedDiet intervention reduced GDM rate with adjusted RR 0.77 (95%CI: 0.61-0.97, P = 0.008)
Assaf-Balut *et al*[62], 2017	RCT	874 participants	MedDiet *vs* control	MedDiet intervention reduced GDM rate with adjusted RR 0.75 (95%CI: 0.57-0.98; P = 0.039)
Al Wattar *et al*[63], 2019	Multicenter RCT	1252 participants	MedDiet *vs* control	MedDiet intervention reduced GDM rate with adjusted RR 0.65 (95%CI: 0.47-0.91, P = 0.01)
Tobias *et al* [64], 2012	Retrospective cohort	15254 participants	MedDiet, DASH	(1) MedDiet was associated with a 24% lower risk (RR: 0.76; 95%CI: 0.60-0.95; P = 0.004); and (2) DASH with a 34% lower risk (RR: 0.66; 95%CI: 0.53-0.82; P = 0.0005)
Tobias *et al* [64], 2012	Retrospective cohort	4413 participants	MedDiet, DASH	(1) MedDiet was associated with a 40% lower risk (RR: 0.60; 95%CI: 0.44-0.82; P = 0.002); and (2) DASH with a 46% lower risk (RR: 0.54; 95%CI: 0.39-0.73; P < 0.001)
Luoto *et al* [67], 2016	RCT	256 participants	Probiotic with/without dietary counseling *vs* control	One probiotic intervention reduced the frequency of GDM; 13% (diet/probiotics), 36% (diet/placebo), and 34% (control); P = 0.003
Callaway *et al*[68], 2019	RCT	411 overweight and obese pregnant women	Probiotic *vs* placebo	(1) GDM occurred in 12.3% in the placebo arm and 18.4% in the probiotics arm (P = 0.10); and (2) Probiotics did not prevent GDM in overweight and obese pregnant women
Davidson *et al*[69], 2021	7 RCTs	1647 participants	Probiotic with either placebo or diet	(1) It was uncertain if probiotics had any effect on the risk of GDM compared to placebo (RR: 0.80, 95%CI: 0.54-1.20) due to low-certainty evidence; and (2) High-certainty evidence suggested an increased risk of pre-eclampsia with probiotic administration (RR: 1.85, 95%CI: 1.04- 3.29) and increased the risk of hypertensive disorders of pregnancy (RR: 1.39, 95%CI: 0.96-2.01)
Mijatovic-	A systematic review	30871	A variety of dietary	(1) MedDiet, DASH were associated with 15%-38% reduced RR of

Vukas *et al* [70], 2018	and meta-analysis of 40 trials	pregnant women	regimens, prepregnancy and early pregnancy PA	GDM; and (2) Compared to no PA, any prepregnancy and early pregnancy PA was associated with 30% and 21%, respectively, reduced odds of GDM
De-Regil *et al*[71], 2016	Cochrane database systematic. review with 15 RCTs	2833 females	Vitamin D alone or in combination with other micronutrients during pregnancy	(1) Similar risk of GDM among those taking vitamin D supplements or no intervention/placebo (RR: 0.43, 95%CI: 0.05-3.45) very low quality evidence; and (2) Vitamin D supplements may lower risk of pre-eclampsia risk (RR: 0.52, 95%CI: 0.25-1.05) low quality evidence
Pérez-López *et al*[72], 2015	A systematic review and meta-analysis of 13 RCTs	2299 females	Vitamin D supplementation during pregnancy	Incidence of pre-eclampsia, GDM, weight, preterm birth, and cesarean section were not influenced by vitamin D supplementation

CI: Confidence interval; DASH: Dietary Approaches to Stop Hypertension; GDM: Gestational diabetes mellitus; MedDiet: Mediterranean diet; OR: Odds ratio; PA: Physical activity; RCT: Randomized controlled trial; RR: Risk ratio; WMD: Weighted mean differences.

restrictive lung disease, pre-eclampsia, persistent bleeding in the second or third trimester, incompetent cervix, placenta previa, hemodynamically significant heart disease, and higher-order multiple gestations (≥ triplets)[51].

Dietary approaches

Several types of diets have been devised and studied to reduce the risk of GDM. These diets included a low glycemic index (GI) diet, energy restriction diet, low carbohydrate content with high fat substitute, the Mediterranean diet (MedDiet), and Dietary Approaches to Stop Hypertension (DASH)[52-54]. The low GI diet ranks foods on a scale from 0 to 100 based on their effects on blood sugar. A food with a low GI was less likely to impact the blood sugar levels significantly. Although the diet was introduced to reduce insulin resistance and to decrease the risk of GDM, a meta-analysis from 2016 and an overview of Cochrane Reviews from 2020 displayed conflicting results of benefit from the low GI diet on reducing the risk of GDM[55,56]. Therefore, the effect of a low GI diet on reducing the incidence of GDM still remains inconclusive.

The restricted-energy diet and low carbohydrate diet have been suggested to minimize weight gain during pregnancy, while some approaches tried to replace dietary carbohydrates with fat. Excessive fat or a ketogenic diet may have an impact on maternal insulin resistance and will result in excessive fat accumulation in the fetus[57]. To date, no sufficient data are available to support the safety of the ketogenic diet in the GDM population[58]. In addition, these diets are not practical as there is still a minimum requirement of daily carbohydrates of at least 175 g to ensure appropriate fetal growth and development. The Institute of Medicine has recommended that 46%-65% of calories come from carbohydrates [59,60].

The MedDiet is considered one of the healthiest forms of nutrition and is confirmed safe for GDM[58]. It is composed of a high vegetable and fruit content along with legumes, olive oil, cereals, fish, and limited animal products. Olive oil is the main source of additional fat, and the consumption of processed meat is low. Several RCTs that studied 284, 874, and 1252 participants, respectively, have reported the positive impact of the MedDiet on lowering the incidence of GDM to around 35%[61-63].

DASH was originally developed for reducing hypertension. It consists of a high intake of vegetables, fruits, legumes, and nuts, with low consumption of sodium and sweets, moderate low-fat dairy products, and limited processed meat and animal protein. It was also shown to be effective in reducing the risk of GDM because the majority of its components overlapped with the MedDiet. In some studies, it was even considered superior to the MedDiet[64-66].

Two double-blind controlled studies have shown that probiotics may reduce the risk of GDM[67,68]. That conclusion was based on the hypothesis that probiotics affect intestinal microflora, thus they may influence host inflammatory pathways and result in better control of glucose and lipid metabolism. However, a recent Cochrane Review concluded that due to low-certainty evidence and limited trials no definitive benefit of probiotics was identified for GDM control[69,70].

Previous evidence indicated that insufficient supplementation of vitamin D in early pregnancy may correlate with an increased risk of GDM. However, the mechanism of reducing GDM risk by supplementing vitamin D was not fully understood. Similar to probiotics, it was suggested that vitamin D supplements in early pregnancy may also reduce the risk of GDM. Based on the available studies discussed in several meta-analyses, no compelling evidence was found to support this practice[70-72].

The functional advantages of low GI foods include lowering postprandial glucose, preventing excessive increases in postprandial insulin, and inducing satiety, all of which may contribute to weight loss. To date, however, low GI foods have shown no marked influence on obstetric, maternal, or fetal outcomes, including maternal weight gain, neonatal birth weight or the proportion of large-for-gestational-age, and macrosomia[73]. Current evidence has suggested that the low GI nutritional approach is reasonably safe in GDM. However, further research is needed to develop tools to facilitate patient adherence to treatment goals, individualize interventions and improve outcomes[74].

The low-carbohydrate diet results in a lower postprandial glucose, lower daytime mean glucose concentrations, lower area under the curve for 2-h postprandial glucose, and lower 24-h total glucose area under the curve when compared with the 60% carbohydrate diet. However, study results have revealed that lower carbohydrate intake will often lead to an increased intake of fat, which outside of pregnancy has been associated with an increase in serum fatty acids, insulin resistance, increased fetal fat accretion, and infant adiposity[59]. These disadvantages may further limit the use of the low-carbohydrate diet as a means of dietary intervention.

The MedDiet may reduce the risk of GDM by alleviating systemic oxidative stress[75]. Further, the MedDiet may downregulate circulating inflammatory biomarkers and favor glucose homeostasis, improving insulin sensitivity and glycemic postprandial response. Moreover, the MedDiet has a high fiber content, which increases satiety and controls weight gain[76]. Similar to the low GI MedDiet, the DASH diet in pregnant women with GDM is associated with a decreased number of macrosomic infants. The DASH diet also led to lower mean weight and head circumference and ponderal index newborns but did not affect the infants' body lengths and Apgar scores[77]. However, the disadvantages of dietary control, as mentioned above, lie in the degree of adherence to dietary interventions by pregnant women.

DISCUSSION

GDM is a complex condition of pregnancy with significant implications for both the mother and the fetus. Currently, no scientific consensus has been reached on how best to diagnose GDM. Expert professional organizations acknowledge two acceptable options: the IADPSG one-step screening approach (currently preferred by the ADA); and the two-step Carpenter-Coustan screening approach (recommended by the ACOG). Both organizations have noted the need for additional evidence related to outcomes. Each approach has advantages and disadvantages. The 1-step approach involves a 2-h OGTT for all participants; while screening and diagnosis can be completed in a single visit, all women must fast before screening and allow time for a 2-h visit. The 2-step approach includes an initial non-fasting 1-h glucose challenge test, which is logistically simpler for gravidas, and can easily be done as part of a scheduled prenatal visit; most women do not require further screening. However, approximately 20% of pregnant women fail this initial screening and must return for a 3-h fasting diagnostic OGTT. In addition, these two methods have different diagnostic cutoffs. In comparison to the 2-step approach, the 1-step approach identifies women with milder hyperglycemia as having GDM and expands the target population that will be further treated under the diagnosis of GDM. Although a clear linear relationship is shown between maternal hyperglycemia and maternal and perinatal outcomes, the specific effects on these outcomes of identifying and treating milder cases of GDM are not known[10].

The pathophysiology of GDM is complex and probably involves genetic variants that are responsible for insulin secretion and glycemic control, pancreatic β cell depletion or dysfunction, aggravated insulin resistance due to failure in the plasma membrane translocation of GLUT4, and the actions of chronic, low-grade inflammation. In practice, each factor or a combination of the aforementioned factors may contribute to the development of GDM.

Currently, many antepartum measurements, including adipokines (leptin), body mass ratio (WC and waist-to-hip ratio), and biomarkers (microRNA in ECVs) have been studied and confirmed as appropriate markers for predicting the occurrence of GDM during pregnancy. Leptin resistance is further augmented in GDM. Thus, the resultant hyperleptinemia can be used as a marker in predicting the occurrence of GDM.

For preventing GDM, physical activity is an effective intervention to control body weight, improve glycemic control, and reduce insulin resistance. On the other hand, several RCTs have reported the positive impact of dietary control (such as the MedDiet) on lowering the incidence of GDM to around 35%[61-63].

To update this overview of GDM, the current treatment for GDM is very frequently suboptimal. The most common oral pharmacological interventions that have been assessed include metformin, probiotics, and vitamin D administration. However, no single intervention appears to be universally superior to placebo/no intervention for preventing GDM[78]. Currently, insulin injection is the preferred medication for treating hyperglycemia in GDM. Metformin and glyburide are not regarded as first-line agents since both cross the placenta to the fetus. Even though sufficient data are available confirming the safety and effectiveness of metformin in pregnant women with GDM, data are very limited concerning the long-term effects of metformin on the offspring. Furthermore, glyburide should be used with caution, as it increases the risk of neonatal hypoglycemia. Some studies have also shown that glyburide increases the risk of macrosomia. Overall, oral agents may be a therapeutic option in women with GDM after the known risks have been reviewed along with the need for more long-term safety data in the offspring[78].

The INTERCOVID study investigated the role of overt or GDM and high BMI as risk factors for coronavirus disease 2019 (COVID-19) infection during pregnancy[79]. The study, conducted between March 2020 and February 2021 across 43 institutions in 18 countries, included 2184 pregnant women aged ≥ 18 years. Each pregnant woman diagnosed with COVID-19 was matched with two females without COVID-19 who received antenatal care or delivered at the same institution. The findings of the study indicated that COVID-19 was associated with pre-existing DM (RR: 1.94, 95%CI: 1.55-2.42), overweight or obesity (RR: 1.20, 95%CI: 1.06-1.37), and GDM (RR: 1.21, 95%CI: 0.99-1.46). Specifically, the association between COVID-19 and GDM was observed among women requiring insulin, regardless of their weight (RR: 1.79, 95%CI: 1.06-3.01) or being overweight or obese (RR: 1.77, 95%CI: 1.28-2.45). A somewhat stronger association with COVID-19 diagnosis was observed among women with pre-existing DM, regardless of their weight (RR: 1.93, 95%CI: 1.18-3.17) or being overweight or obese (RR: 2.32, 95%CI: 1.82-2.97). The study concluded that DM and overweight or obesity were risk factors for COVID-19 diagnosis during pregnancy, with insulin-dependent GDM being particularly associated with the disease. Therefore, it is crucial to prioritize the vaccination of women with these comorbidities[79].

Subsequently, a large prospective, observational study (INTERCOVID-2022) involving 41 hospitals across 18 countries, enrolled 4618 pregnant women from November 27, 2021 to June 30, 2022[80]. During pregnancy, each woman with a COVID-19 diagnosis was matched with two females without COVID-19. Overall, females with a COVID-19 diagnosis had an increased risk for morbidity and mortality index (RR: 1.16, 95%CI: 1.03-1.31), severe perinatal morbidity and mortality index (RR: 1.21, 95%CI: 1.00-1.46), and severe neonatal morbidity index (RR: 1.23, 95%CI: 0.88-1.71) compared to those without a COVID-19 diagnosis[80]. Moreover, severe COVID-19 symptoms in the total sample increased the risk of severe maternal complications (RR: 2.51, 95%CI: 1.84-3.43), perinatal complications (RR: 1.84, 95%CI: 1.02-3.34), and referral,

intensive care unit admission, or death (RR: 11.83, 95%CI: 6.67-20.97). Notably, vaccine effectiveness (all vaccines combined) for severe complications of COVID-19 in all females who completed the regimen was 48% (95%CI: 22-65) and 76% (47-89) after a booster dose[80]. The study conclusion stated that COVID-19 in pregnancy was associated with increased risk of severe maternal morbidity and mortality, especially among symptomatic and unvaccinated women. Females with complete or boosted vaccine doses had a reduced risk for severe symptoms, complications, and death. Accordingly, COVID-19 vaccination coverage among pregnant women remains a priority[80].

The present review of the literature has identified several cellular and molecular mechanisms associated with GDM as well as influence factors and risk factors that can affect its development. Additionally, we have identified a number of potential predictive and preventive measures that can be employed to help reduce the incidence of GDM. These measures include lifestyle modifications, such as dietary changes and increased physical activity, and screening for risk factors and early diagnosis. Advances in our understanding of the pathophysiology of GDM have enabled the development of more effective preventive strategies, which can have a significant impact on the health of both mother and child. It is our hope that the findings of this review will contribute to the ongoing effort to improve the management of GDM and ultimately lead to better health outcomes for women and their offspring.

CONCLUSION

GDM is a complex condition of pregnancy with significant implications for both the mother and the fetus. Currently, no scientific consensus has been reached on how best to diagnose GDM, either by one-step or two-step OGTT. The pathophysiology of GDM undoubtedly involves genetic variants, pancreatic β cell depletion or dysfunction, and aggravated insulin resistance due to failure in the plasma membrane translocation of GLUT4. GDM pathophysiology is also associated with the negative regulation of leptin signaling pathways and the actions of chronic low-grade inflammation, which involve the NF-κB signaling pathway. Currently, leptin and body mass ratio are used as markers for predicting the occurrence of GDM during pregnancy. For preventing GDM, physical activity and dietary control are substantial interventions. Nevertheless, many detailed cellular and molecular mechanisms underlying GDM, as well as prediction and prevention, remain unexplored and warrant further investigation.

ARTICLE HIGHLIGHTS

Research background

Defined as impaired glucose tolerance induced by pregnancy, gestational diabetes mellitus (GDM) may arise from exaggerated physiologic changes in glucose metabolism. Maternal and fetal effects of uncontrolled GDM include stillbirth, macrosomia, neonatal diabetes, birth trauma, and subsequent postpartum hemorrhage.

Research motivation

It is essential to find the potential target population and associated predictive and preventive measures for future intensive peripartum care.

Research objectives

To review studies that explored the cellular and molecular mechanisms of GDM as well as predictive measures and prevention strategies.

Research methods

The search was performed in the Medline and PubMed databases using the terms "gestational diabetes mellitus," "overt diabetes mellitus," and "insulin resistance." In the literature, only full-text articles were considered for inclusion (237 articles). Furthermore, articles published before 1997 and duplicate articles were excluded. After a final review by two experts, all studies (1997-2023) included in the review met the search terms and search strategy (identification from the database, screening of the studies, selection of potential articles, and final inclusion).

Research results

A total of 79 articles were collected for review. Reported risk factors for GDM included maternal obesity or overweight, pre-existing DM, and polycystic ovary syndrome. The pathophysiology of GDM involves genetic variants responsible for insulin secretion and glycemic control, pancreatic β cell depletion or dysfunction, aggravated insulin resistance due to failure in the plasma membrane translocation of glucose transporter 4, and the effects of chronic, low-grade inflammation. Currently, many antepartum measurements including adipokines (leptin), body mass ratio (waist circumference and waist-to-hip ratio], and biomarkers (microRNA in extracellular vesicles) have been studied and confirmed to be useful markers for predicting GDM. For preventing GDM, physical activity and dietary approaches are effective interventions to control body weight, improve glycemic control, and reduce insulin resistance.

Research conclusions

This review explored the possible factors that influence GDM and the underlying molecular and cellular mechanisms of

GDM and provided predictive measures and prevention strategies based on results of clinical studies.

Research perspectives

Many detailed cellular and molecular mechanisms underlying GDM, as well as prediction and prevention, remain unexplored and warrant further investigation.

FOOTNOTES

Author contributions: Lim PQ and Chen KH designed the research study; Lim PQ, Lai YJ, Ling PY, and Chen KH performed the research; Lim PQ, Lai YJ, and Chen KH analyzed the data; Lim PQ, Lai YJ, and Chen KH wrote the manuscript; All authors read and approved the final manuscript.

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CASE REPORT

Rapid correction of hyperglycemia: A necessity but at what price? A brief report of a patient living with type 1 diabetes

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Abstract

BACKGROUND

The correction and control of chronic hyperglycemia are the management goals of patients living with diabetes. Chronic hyperglycemia is the main factor inducing diabetes-related complications. However, in certain situations, the rapid and intense correction of chronic hyperglycemia can paradoxically favor the onset of microvascular complications.

CASE SUMMARY

In this case report, we describe the case of a 25-year-old woman living with type 1 diabetes since the age of 9 years. Her diabetes was chronic and unstable but without complications. During an unplanned pregnancy, her diabetes was intensely managed with the rapid correction of her hyperglycemia. However, over the following 2 years, she developed numerous degenerative microvascular complications: Charcot neuroarthropathy with multiple joint involvement, severe proliferative diabetic retinopathy, gastroparesis, bladder voiding disorders, and end-stage renal failure requiring hemodialysis.

CONCLUSION

In the literature to date, the occurrence of multiple microvascular complications following the rapid correction of chronic hyperglycemia has been rarely described in the same individual.

Key Words: Unstable diabetes; Chronic hyperglycemia; Microvascular complication; Type 1 diabetes; Case report

Core Tip: Our case describes a sad and rare development of multiple microvascular complications: Charcot neuroarthropathy with multiple joint involvement, severe proliferative diabetic retinopathy, gastroparesis, bladder voiding disorders, and end-stage renal failure. These devastating complications, which were probably due to the rapid correction of long-term hyperglycemia, severely impacted the quality of life of a young patient with type 1 diabetes.

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INTRODUCTION

The vascular complications of diabetes are generally linked to chronic hyperglycemia[1]. This is the case for patients living with both type 1 and type 2 diabetes[2,3]. Nevertheless, the intense treatment of hyperglycemia can favor the onset of macrovascular complications such as stroke and myocardial infarction[4]. Large randomized clinical studies have assessed the impact of the so-called intense correction of hyperglycemia on macrovascular complications in the subjects living with diabetes[5,6], although few studies have evaluated the consequences of this rapid correction in terms of microvascular complications. Several descriptive studies have highlighted the potential deterioration of diabetic neuropathy and retinopathy in the case of the rapid correction of chronic hyperglycemia in people living with type 1 diabetes[7,8]. However, these studies were unable to provide a clear explanation of the relation between the deterioration of these microvascular complications and the rapid correction of chronic hyperglycemia[7,8]. The authors described a few cases of patients who developed several complications simultaneously.

The understanding of this mechanism remains at the theoretical stage, particularly following the degradation of diabetic neuropathy after the rapid onset of chronic hyperglycemia[7]. Theorical research has described the presence of neuropathy induced by the treatment of diabetes, with the presence of arteriovenous shunts and hypervascularity in the extremities and a still-unexplained disruption of inflammation factors such as certain interleukins[9]. The contribution of the rapid correction of hyperglycemia to the worsening of retinopathy may be more easily understood due to the specific action of insulin on the retinal endothelium with the appearance of so-called florid retinopathy[10,11]. When describing the degradation of complications after the rapid correction of chronic hyperglycemia, the literature usually focuses on a single complication[12,13].

Our case describes a sad and rare development of multiple microvascular complications: Charcot neuroarthropathy with multiple joint involvement, severe proliferative diabetic retinopathy, gastroparesis, bladder voiding disorders, and end-stage renal failure. These devastating complications, which were probably due to the rapid correction of long-term hyperglycemia, severely impacted the quality of life of a young patient with type 1 diabetes.

CASE PRESENTATION

Chief complaints

Our 25-year-old patient had been diagnosed with type 1 diabetes at the age 9 years. She had no other medical history. Her sister, who was 3 years older, also had type 1 diabetes. The management of her diabetes was exacerbated by chronic hyperglycemia, which had been present since adolescence with this major imbalance continuing after the age of 20 years.

History of present illness

Her medical records provide the mean values of glycated hemoglobin (HbA1c) dosed using the high-performance liquid chromatography method[14]: 11.0% at 16 years, 12.5% at 17 years, 11.6% at 18 years, 10.9% at 20 years, 11.3% at 22 years, 12.8% at 23 years, 13.3% at 24 years, and 12.0% at 25 years. It should be noted that the young woman did not consult for diabetes but was hospitalized in 2010 due to acute metabolic ketoacidosis. Despite her high HbA1c levels, she systematically refused educational and therapeutic assistance as well as an interstitial glucose monitoring system.

History of past illness

After a lengthy period without consulting a diabetologist, our 25-year-old patient sought an evaluation and treatment in November 2017 at the Centre Hospitalier Sud Francilien in France. At the time, she was 2 mo into an unplanned pregnancy. Before receiving therapeutic care, a complete assessment was performed, detecting an HbA1c level of 12%. She measured 167 cm and weighed 62 kg. The clinical examination revealed peripheral neuropathy with paresthesia in the lower limbs. Monofilament test was abnormal, results showed microalbuminuria (289 mg/L) with a normal glomerular filtration rate (80 mL/min/1.73 m^2), which suggested incipient nephropathy. Therapeutic care was rapidly

implemented with a subcutaneous insulin infusion pump, leading to the rapid reduction of HbA1c to 7.4% *vs* 12.0% ($P < 0.01$), equivalent to a 60% reduction over 8 mo of pregnancy The patient's weight increased to 72.6 kg. Fundus examination performed at the start, middle, and end of the pregnancy did not reveal the presence of diabetic retinopathy. This pregnancy presented a new opportunity to manage this patient's disease due to her strong motivation and desire to achieve an HbA1c level below 7%.

In addition to insulin, the only other medication was Ramipril 10 mg, which was prescribed in the post-partum period due to the presence of early nephropathy.

Between the first consultation in November 2017 and March 2022, the patient presented multiple microvascular complications related to diabetes. These complications were not detected during the initial pregnancy assessments but instead occurred following the rapid correction of chronic hyperglycemia.

The complications began during pregnancy with a rare presentation of Charcot neuroarthropathy with multiple joint involvement in November 2017. This led to a left knee prosthesis with a transtibial amputation of the left lower limb in March 2018[9,10].

In January 2019, following a spontaneous decline in her visual acuity, the patient presented for ophthalmological consultation, which revealed severe diabetic pre-proliferative retinopathy with a bilateral macular edema. The diagnosis was confirmed by retinal angiography and optical coherence tomography. Her HbA1c level was 6.8%. The patient underwent more than 20 pan-photocoagulation sessions and received intravitreal injections of anti-vascular endothelial growth factor therapy.

When this retinopathy was discovered in June 2019 about 1.5 years after achieving an HbA1c level below 7%, the patient also complained of abdominal discomfort with the presence of the following symptoms: nausea, vomiting, meteorism and abdominal pain, full feeling after a few mouthfuls (early satiety), heartburn or gastroesophageal reflux, and excessive weight loss. C reactive protein was 5 mg/L, and hepatic function was normal. The abdominopelvic ultrasound did not reveal any anomalies. Given the persistence of symptoms, an endoscopy of the upper gastrointestinal tract was performed, confirming the presence of non-erosive gastritis. Persistent symptoms were managed with a gastric emptying scintigraphy. During the scintigraphy, planar acquisitions were taken immediately after a meal comprised of 120 mg egg white labelled with 33 MBq of Technetium 99m-human albumin nanocolloids, two slices of sandwich bread, 30 g jam, and/or 250 mL fruit juice, and then after 15 min, 30 min, 45 min, 1 h, 2 h, and 4 h. The findings revealed an extremely slow progression of the radiolabeled meal in the stomach as well as substantial proximal and distal gastric retention due to decreased fundic tone. The findings pointed to diabetic gastroparesis (Figure 1). The patient was treated in the gastroenterology department, and prokinetic therapy was prescribed to improve gastric emptying and antiemetic agents.

Simultaneous with the onset of the digestive disorders, the patient described urinary symptoms with a decreased need to urinate along with less micturition, reduced flow rate, delayed urine flow with only a few drops, and the feeling of incomplete emptying, which sometimes led to overflow incontinence. The urodynamic assessment performed in response to the urinary symptoms confirmed the involvement of the bladder and highlighted potential complications. This assessment also revealed significant anomalies associated with diabetic cystopathy.

From June 2018, which was 1 year since the patient's HbA1c level had been stabilized below 7%, the creatinine threshold showed a rapid upward trend with a marked increase in the microalbuminuria rate and a continuous decrease in the glomerular filtration rate (Figure 2).

Unfortunately, the management of the patient by nephrologists was unable to slow down the rapid development of end-stage renal failure over a period of 3 years from late 2017 to June 2020 when it was diagnosed, requiring treatment with thrice-weekly hemodialysis sessions. All diagnostic and therapeutic options were implemented following the initiation of care in nephrology. Other causes of this renal failure were eliminated *via* a kidney biopsy, with the histological results showing advanced nephropathy of metabolic origin. The early management of the ionic disorders by correcting the phosphate levels with calcium supplements and vitamin D aimed to reduce the excessive phosphate load and limit the protein intake.

Supplementary Figure 1 shows the timeline of each diabetes-related complication along with the time of diagnosis.

The patient currently lives with a left leg prosthesis and a left prosthetic knee. She uses a urinary catheter for her urinary incontinence and has tunnel vision due to her reduced visual field. She is on the national waiting list for a double kidney-pancreas transplant, and in the meantime, she undergoes peritoneal dialysis three times per week. Since December 2022, she has benefited from a hybrid closed-loop insulin delivery system (Medtronic 780 G).

Personal and family history

Our 25-year-old patient had been diagnosed with type 1 diabetes at the age 9 years. Her sister, who was 3 years older, also had type 1 diabetes.

Physical examination

She measured 167 cm and weighed 62 kg. The clinical examination revealed peripheral neuropathy with paresthesia in the lower limbs. Monofilament test was abnormal.

Laboratory examinations

Glycated hemoglobin (HbA1c) dosed using the high-performance liquid chromatography method[14]: 11.0% at 16 years, 12.5% at 17 years, 11.6% at 18 years, 10.9% at 20 years, 11.3% at 22 years, 12.8% at 23 years, 13.3% at 24 years, and 12.0% at 25 years.

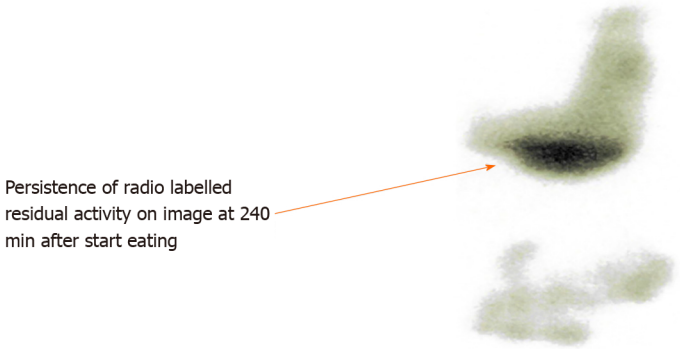

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Figure 1 Image of gastric scintigraphy showing a severe delay in gastric emptying.

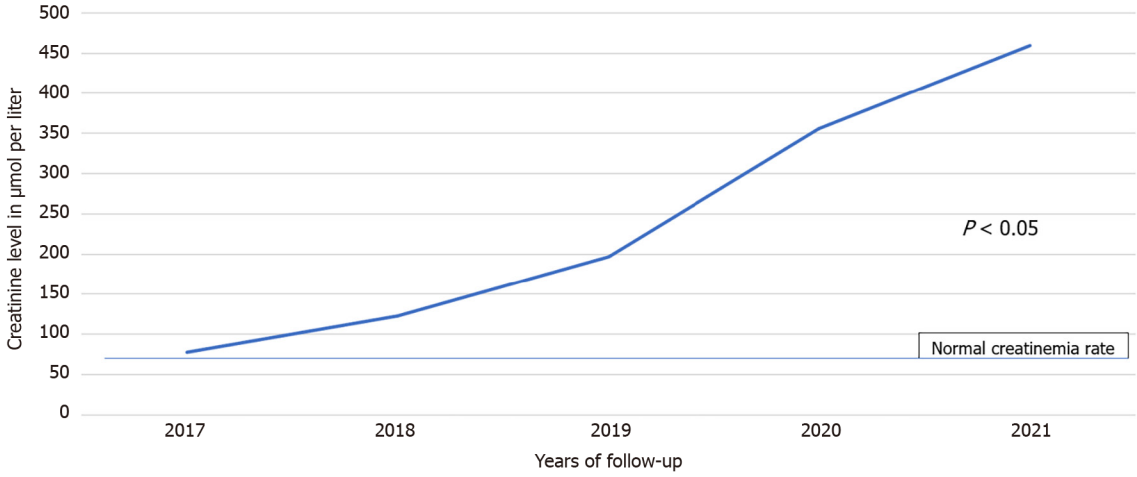

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Figure 2 Flow draft. From June 2018, which was 1 year since the patient's HbA1c level had been stabilized below 7%, the creatinine threshold showed a rapid upward trend with a marked increase in the microalbuminuria rate and a continuous decrease in the glomerular filtration rate.

Imaging examinations

Gastric scintigraphy, planar acquisitions were taken immediately after a meal comprised of 120 mg egg white labelled with 33 MBq of Technetium 99 m-human albumin nanocolloids, two slices of sandwich bread, 30 g jam, and/or 250 mL fruit juice, and then after 15 min, 30 min, 45 min, 1 h, 2 h, and 4 h. The findings revealed an extremely slow progression of the radiolabeled meal in the stomach as well as substantial proximal and distal gastric retention due to decreased fundic tone.

FINAL DIAGNOSIS

Type 1 diabetes with severe microvascular complications.

TREATMENT

Hybrid closed-loop insulin delivery system (Medtronic 780 G).

OUTCOME AND FOLLOW-UP

After undergoing transtibial amputation of the lower limb and knee prosthesis surgery as part of therapy for Charcot's neuroarthropathy, our patient received photocoagulation treatment for retinopathy and thrice-weekly hemodialysis for end-stage renal failure. The patient is currently registered on the French national waiting list for a double kidney-

pancreas transplant.

DISCUSSION

The link between chronic glycemic imbalance and vascular complications is already well known and proven among people living with type 1 and type 2 diabetes[2,3]. The management of people living with diabetes should aim to prevent these complications by achieving long-term glycemic stability[11]. Nevertheless, the intense and rapid correction of glucose levels after a long period of hyperglycemia can produce or aggravate numerous macrovascular complications such as myocardial infarction or the risk of cardiovascular mortality[4]. Microvascular complications also seem prone to worsen with the rapid correction of chronic hyperglycemia[12]. Nevertheless, few studies have evaluated the impact of the rapid correction of HbA1c levels on microvascular complications among individuals living with diabetes. Gibbons *et al*[7] reported this impact with the well-known treatment-induced neuropathy in diabetes (TIND). The authors described a small number of cases in which preexisting minimal retinopathy deteriorated in patients who underwent the rapid correction of chronic hyperglycemia[7]. The degradation of renal function has previously been linked to the deterioration of retinopathy[15].

Our case report focuses on the rare presentation of numerous microvascular complications involving multiple joints and organs, probably induced by the rapid correction of HbA1c levels. Following the chronological order of the complications, the patient first presented acute Charcot neuroarthropathy that affected two joints[9]. The onset of acute Charcot neuroarthropathy can sometimes accompany the rapid correction of hyperglycemia, although it has been described in multiple sites in recent cohorts[16,17]. Regarding gastroparesis, very few descriptions associate this complication with the rapid correction of HbA1c levels. Data have also shown that the change is caused by the degree of HbA1c decrease rather than the type of therapy (insulin, oral hypoglycemic drugs, or diet control).

TIND is an acute neuropathy affecting the autonomic and small somatosensory nerve fibres that occurs subsequent to rapid improvements in glucose control. Gibbons *et al*[7] demonstrates that: (1) There is an unexpectedly high proportion of individuals with TIND in their tertiary referral diabetic clinic; (2) the risk of developing TIND is associated with the magnitude and rate of change in HbA1c; (3) the severity of neuropathic pain and autonomic dysfunction is correlated with the magnitude of change in HbA1c; (4) patients with type 1 diabetes and a history of eating disorders are at a high risk of developing TIND; and (5) TIND can occur following use of insulin or oral hypoglycemic agents[7]. Data from Gibbons *et al*[7] also provide indications for the prevention of TIND. As its incidence significantly increases when HbA1c decreases by 42% over 3 mo, this suggests that limiting treatment goals to an HbA1c reduction over 3 mo is a reasonable threshold. Patients with higher baseline HbA1c or a history of diabetic anorexia or weight loss may be at higher risk of TIND; special attention is therefore warranted in the case of intensive glycemic management in these patients[7].

Diabetes-related gastroparesis is a challenging complication of diabetes, which often results in intractable vomiting and recurrent hospitalizations. It is defined as delayed gastric emptying in the absence of mechanical obstruction. It is estimated that 30%-50% of patients with diabetes have delayed gastric emptying. Gastroparesis is most often diagnosed with type 1 diabetes and can occur as early as adolescence, although it can also occur in patients with type 2 diabetes, usually several years after diagnosis. The onset is insidious, with the disease course characterized by phases of exacerbation and remission[18]. In the literature, gastroparesis has been described during pregnancy and not 1-year postpartum as in the case of our patient[18]. The pathogenesis of diabetic gastroparesis is distinctly multifactorial. People living with diabetes often suffer from other physiological problems of the digestive tract that affect motor, absorptive, secretory, and sensorial systems. Gastropathy renders diabetic control very difficult, and poor diabetic control in turn worsens gastric emptying, thus making life difficult for people with gastropathy. It is vital that patients and their healthcare providers reach an agreement about a control goal that will allow patients to feel better and avoid further complications, while preventing severe hypoglycemia[18]. In the work of Gibbons, certain cases of gastroparesis were described along with TIDN[7].

Our case report also details the appearance of severe and debilitating diabetic retinopathy, which was not present before the rapid correction of HbA1c, as the three fundus examinations performed during pregnancy formally excluded the presence of retinopathy, even at the early stages. One potential explanation for this deterioration may be the intensity of insulin therapy, as the serum concentration probably increased following the correction of hyperglycemia, thus leading to a reduction in the availability of cellular energy substrates. A reduction in retinal vascular auto-regulation and an increase in growth factors trigger the neovascularization responsible for severe retinopathy[8]. Urinary retention leading to incontinence in our patient is a little-known mechanism linked to urinary disturbances in autonomic neuropathy[19]. Regarding the patient's renal failure, she presented a very rapid decline in renal function, corresponding to an annual decrease in the glomerular filtration rate of more than 5 mL/min/1.73 m²[20]. The time to end-stage renal failure was only 3 years, which was much faster than described in the literature[21].

The association between the HbA1c variability score and the estimated glomerular filtration rate has been evaluated in people living with type 2 diabetes[22,23], although very few clinical trials have evaluated the same effect in patients living with type 1 diabetes.

One limitation of this case report is its retrospective nature, which may be an obstacle to the interpretation of the medical elements. Another limitation is the lack of data such as the presence or absence of glycemic variability, which prevented accurate HbA1c measurements. Unfortunately, the patient systematically refused to provide real-time glycemic data by either capillary blood glucose monitoring or continuous glucose measurements.

Our patient presented multiple rare conditions following the appearance of microvascular complications and their fast deterioration after the rapid correction of chronic hyperglycemia Our case description supports the use of personalized

algorithms such as the hybrid closed-loop systems with automated insulin delivery to allow for more precise adjustments when high insulin doses are used in subjects living with chronic glycemic instability.

CONCLUSION

The association between the HbA1c variability score and the estimated glomerular filtration rate has been evaluated in people living with type 2 diabetes[24,25], although very few clinical trials have evaluated the same effect in patients living with type 1 diabetes. One limitation of this case report is its retrospective nature, which may be an obstacle to the interpretation of the medical elements. Another limitation is the lack of data such as the presence or absence of glycemic variability, which prevented accurate HbA1c measurements. Unfortunately, the patient systematically refused to provide real-time glycemic data by either capillary blood glucose monitoring or continuous glucose measurements.

FOOTNOTES

Author contributions: Dardari D and Huret P conceived paper; Huret P developed the theory and performed the computations; Huret P, Lopes P, and Thomas C verified the analytical methods; Dardari D encouraged Huret P to investigate supervised the findings of this work; and all authors discussed the results and contributed to the final manuscript.

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