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EDITORIAL

Diabetic ketoacidosis: Treatment in the intensive care unit or general medical/surgical ward?

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Abstract

Diabetic ketoacidosis (DKA) is defined as an acute metabolic disorder, which is characterized by an increased presence of circulating ketones, and the development of ketoacidosis in the presence of hyperglycemia. This syndrome occurs as a result of insulin deficiency. Patients can be dramatically ill, however, with aggressive treatment, most patients recover rapidly. Despite being a low-risk condition, the development of acidosis, is one of the admission criteria to the intensive care unit (ICU) for these patients, in order to provide close monitoring, and recognize complications that could result from the use of aggressive therapy, such as continuous infusions if insulin. In some institutions, DKA is treated in the emergency department and general medical/surgical wards to avoid ICU overcrowding.

Key words: Diabetic ketoacidosis; Diabetes; Hyperosmolar non-ketotic state; Clinical outcomes; Serum ketones

Core tip: Diabetic ketoacidosis is a complication for some patients with insulin-dependent diabetes mellitus as well as for non-insulin dependent. It is treated commonly in the intensive care unit (ICU), even though clinical data from many studies support management in regular (medical/surgical) wards, avoiding expensive critical care unit costs and preventing bed crisis in these higher level of care units for sicker patients. Once the patient is treated, adequate follow up and education is mandatory. Noncompliance remains the primary concern for repeated admissions.

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INTRODUCTION

Patients with diabetes mellitus (DM) have health care costs 2.3 times higher than others without this diagnosis[1]. In a prevalence-based study, by the American Diabetes Association, in the United States in 2012, the total cost for diagnosed DM was $245 billion United States dollars, and of it, $176 billion was used for direct medical care costs[1]. In addition, and even more concerning, is the fact that hospitalizations for patients with DM have being increasing[2]. The National Surveillance of Diabetes Public Health Resources, reported that diabetic ketoacidosis (DKA) admissions increased from 80000/year in 1988 to 140000/year in 2009[2].

DKA causes an acute metabolic disorder, which is primarily characterized by an increased presence of circulating ketone bodies, and the development of severe ketoacidosis in the presence of prolonged uncontrolled hyperglycemia, usually due to insulin deficiency[3]. It is more commonly seen in patients with insulin-dependent diabetes mellitus (IDDM), especially among children and young adults. Occasionally, patients with insulin resistant DM can present this complication; especially those that are noncompliant with insulin therapy or who present severe infection[3]. DKA has arbitrarily been classified by some as mild, moderate and severe, according to the initial diagnostic criteria (which includes plasma glucose, arterial pH, serum bicarbonate, urine and serum ketones, serum osmolality and anion gap; and the alteration in the mental status)[4].

EPIDEMIOLOGY

In 2012, 29.1 million Americans or 9.3% of the population were estimated to suffer from DM, according to the American Diabetes Association and the Center for Disease Control and Prevention[2]. Of them, approximately 1.25 million American children and adults have IDDM. This clinical condition has a cumulative incidence of 1.4 million Americans per year and it remains the 7th leading cause of death in the United States since 2010[2]. As noted above, the number of cases of DKA has steadily increased over the past 2 decades[2,3]. In one study in the United States, DKA presentations to the emergency department (ED) increased 35% from 1996 to 2006[3]. When compared to other countries like England, Austria and Germany, the United States has the highest rates of DKA in children with IDDM[5]. Mortality rates for patients with hyperglycemic syndromes (DKA and hyperosmolar non-ketotic states) have been reported as 0.02% in patients with diabetes who are 45 years or younger, and 0.014% among older adults[6]. In some studies, the average length of stay in the hospital for patients with DKA has decreased from 5.7 to 3.4 d, being longer for patients categorized in the "severe" group[2,7]. In the authors' experience, some patients can even be discharged within 23 h of hospital admission despite an initial severe acidemia.

IS DKA A CRITERION FOR ICU ADMISSION?

In many institutions, and for decades, DKA has been routinely treated in ICU environments, including recommendations by the American Diabetes Association guidelines for DKA treatment[3,4,7-9]. The primary reason for these level of care requirements, has been the presence of severe metabolic acidosis, even if patients are grouped as mild or moderate in severity[10]. Frequent blood glucose monitoring, the need for intravenous insulin infusions, and the requirement of frequent vital signs is cited as the hospital structural requirements for this ICU level of care[11]. However, several studies have shown that DKA can be safely treated in the ED or even in medical wards (Table 1)[12-17]. By taking this lower level of care approach, we can potentially avoid ICU hospitalization rate and higher costs, bed overcrowding and reserving the beds for patients who present complications such as hypotension, coma, acute myocardial ischemia, or those with several comorbidities (*i.e*., end-stage renal disease, congestive heart failure) and anyone categorized as suffering severe DKA[12,18,19]. In some observational studies DKA patients admitted to the ICU have a shorter length of stay when compared to non-diabetic mellitus ICU patients[20,21]. A recent retrospective cohort study of 156, 842 hospitalizations among 94 acute-care hospitals, analyzed the adjusted cost of hospitalizations in lower and higher ICU utilizations groups, and concluded that the overuse of ICU only increases the cost and the utilization of invasive procedures but with no improvement in hospital mortality[22].

In a prospective, randomized clinical trial in India, Karoli and coworkers reported that once the DKA patient is evaluated in the ED, and categorized in the severity score, direct admission to a regular ward provided no additional mortality and the only complication noted was hypoglycemia. Other groups have used other classifications to allocate resources for patients with DKA[15]. In a retrospective study, Marinac and Mesa, using laboratory criteria (serum bicarbonate, anion gap, base excess and serum osmolality), and diastolic blood pressure, patients were grouped in 5 grades (Grade 0 - Ⅳ)[19]. ICU admission was recommended only for those who had grade Ⅳ DKA[19] (Table 2).

TREATMENT OPTIONS IN THE ED OR ICU

The treatment of acute DKA includes restoration of fluid

Table 1 Clinical trials comparing care in the intensive care unit *vs* the emergency department or medical ward for patients with diabetic ketoacidosis

Ref.	Country	Patients enrolled	Site of management	Therapy used	Outcome	Length of stay
Dunbar *et al*[12] Retrospective study (January 1994 - March 1995)	United States	61	15: ICU 46: Regular floor	Not mentioned	Mortality due to sepsis in only 1 patient with initial pH < 7.00	ICU: 2 d Regular floor: Not mentioned
Umpierrez *et al*[14] Prospective randomized open trial	United States	45	15: ICU 30: ED	ICU: Intravenous insulin drip ED: 15 subcutaneous insulin aspart Q1H ED: 15 subcutaneous insulin aspart Q2H	Hypoglycemic event presented in each group in only 1 patient per group. No complications, no recurrence of ketoacidosis and no mortality	ICU: 4.5 ± 3 d ED with SC Q1H: 3.4 ± 3 d ED with SC Q2H 3.9 ± 3 d
Karoli *et al*[15] Prospective randomized open trial (January 2009 - June 2010)	India	50	25: ICU 25: ED	ICU: 25 intravenous regular insulin ED: 25 subcutaneous insulin lispro	Hypoglycemic event presented, 2 patients in the ICU group and 1 patient in the ED group. No complications, no recurrence of ketoacidosis and no mortality	ICU: 6.6 ± 1.5 d ED: 6.0 ± 1.2 d
Ersöz *et al*[16] Prospective randomized open trial	Turkey	20	20: ICU	ICU: 10 intravenous regular insulin ICU: 10 subcutaneous insulin lispro	No need to switch to IV regular insulin, no hypoglycemic events, no complications, no recurrence of ketoacidosis and no mortality	Not mentioned
Umpierrez *et al*[18] Prospective randomized open trial	United States	20	10: ICU 10: MW	ICU: 20 intravenous regular insulin IMU: 10 subcutaneous insulin lispro Regular floor: 10 subcutaneous insulin lispro	Hypoglycemic event presented in each group in only 1 patient per group, no complications, np recurrence of ketoacidosis and no mortality	IMU and Regular floor: 4 ± 2 d ICU: 4 ± 1 d
Sotiropoulos *et al*[25] Prospective study (June 2007 - May 31 2008)	Greece	21	21: ED	ED: 21 intravenous regular insulin	Myocardial infarction in only 1 patient - Mortality 4.7%	Not mentioned
Della Manna *et al*[26] Controlled clinical trial (June 2001 - June 2003)	Brazil	60	3: ICU 57: ED	ICU: 3 intravenous regular insulin ED: 27 intravenous regular insulin ED: 30 subcutaneous insulin lispro	Hypoglycemic event on 10 patients, 6 patients due to regular insulin and 4 due to lispro; no complications, no recurrence of ketoacidosis and no mortality	Not mentioned

IMU: Intermediate care unit; SC: Subcutaneous; Q1H: Every hour; Q2H: Every two hours; ICU: Intensive care unit; ED: Emergency department; MW: Medical ward; DKA: Diabetic ketoacidosis.

Table 2 List of conditions requiring admission of patients with diabetic ketoacidosis in the intensive care unit

Myocardial infarction
Congestive heart failure
Acute renal failure
Acute respiratory failure
Altered mental status
Coma
Shock
Hypothermia
Sepsis
Pancreatitis
Gastrointestinal bleeding
Uncontrolled hypertension
End stage renal disease
Hyperkalemia

deficits in the first 24 to 36 h, electrolyte replacement and insulin therapy, which is administered slowly to decreased plasma glucose[23,24]. As noted above, a few randomized, open label trials have proved good outcome and non-inferiority for patients who are managed on regular medical/surgical wards while using with rapid acting insulin, aspart or lispro[13,15,17,25-29].

By establishing a rapid diagnosis and starting treatment in the ED, clinicians can help patients to decrease their costs and hospital stay.

The primary issue in patients with DKA remains the need for repeated hospital admissions. Non-compliance in these patients makes the outcome and prognosis worst. Indeed, medical non-compliance and adherence to the outpatient treatment is the most common precipitating factor leading to the development of moderate-to-severe DKA, requiring ICU admission secondary to complications (*i.e.*, cerebral edema, sepsis) and making the management in the ED and/or ICU very complex[21,25,30]. Life-support care, such

as mechanical ventilation, vasopressors, intravenous antibiotic therapy and mortality rates are higher in these patients, when compared to patients not requiring these interventions[30].

CONCLUSION

The benefit of ICU level of care for patients with DKA rather than regular medical/surgical wards is not well established for patients with mild-to-moderate DKA. Many studies suggest the utilization of the ED or the regular (medical/surgical) wards in the management of these patients. There is significant cost-benefit in managing DKA in the ED and regular wards instead of the ICU, where only patients that require life-supportive intervention should go. Once patients are discharged from the hospital adequate follow up is necessary to avoid readmissions and assure compliance.

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REVIEW

Integrating insulin-like growth factor 1 and sex hormones into neuroprotection: Implications for diabetes

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Abstract

Brain integrity and cognitive aptitude are often impaired in patients with diabetes mellitus, presumably a result of the metabolic complications inherent to the disease. However, an increasing body of evidence has demonstrated the central role of insulin-like growth factor 1 (IGF1) and its relation to sex hormones in many neuroprotective processes. Both male and female patients with diabetes display abnormal IGF1 and sex-hormone levels but the comparison of these fluctuations is seldom a topic of interest. It is interesting to note that both IGF1 and sex hormones have the ability to regulate phosphoinositide 3-kinase-Akt and mitogen-activated protein kinases-extracellular signal-related kinase signaling cascades in animal and cell culture models of neuroprotection. Additionally, there is considerable evidence demonstrating the neuroprotective coupling of IGF1 and estrogen. Androgens have also been implicated in many neuroprotective processes that operate on similar signaling cascades as the estrogen-IGF1 relation. Yet, androgens have not been directly linked to the brain IGF1 system and neuroprotection. Despite the sex-specific variations in brain integrity and hormone levels observed in diabetic patients, the IGF1-sex hormone relation in neuroprotection has yet to be fully substantiated in experimental models of diabetes. Taken together, there is a clear need for the comprehensive analysis of sex differences on brain integrity of diabetic patients and the relationship between IGF1 and sex hormones that may influence brain-health outcomes. As such, this review will briefly outline the basic relation of diabetes and IGF1 and its role in neuroprotection. We will also consider the findings on sex hormones and diabetes as a basis for separately analyzing males and females to identify possible hormone-induced brain abnormalities. Finally, we will introduce the neuroprotective interplay of IGF1 and estrogen and how androgen-derived neuroprotection operates through similar signaling cascades. Future research on both neuroprotection and diabetes should include androgens into the interplay of IGF1 and sex hormones.

Key words: Diabetes; Androgens; Estrogen; Insulin; Insulin-like growth factor 1; Neuroprotection; Brain

integrity; Cognition

Core tip: Insulin-like growth factor 1 (IGF1), estrogen, and androgens are known to have neuroprotective properties. Fluctuations in these hormones is observed in patients with diabetes, varies with sex, and may contribute to abnormalities in brain integrity and cognitive impairment typical of the disease. While the neuroprotective coupling of estrogen and IGF1 has been studied extensively, little research has focused similarly on androgens. Furthermore, research investigating the IGF1-sex hormones relation to diabetes and brain-health outcomes is minimal. One avenue of approach to extend this literature may be to examine sex differences by comparison of these hormone levels, brain integrity, and cognitive aptitude.

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INTRODUCTION

Diabetes mellitus is a metabolic syndrome known for impaired insulin production. This condition is associated with an abundance of sequelae including cardiovascular disease[1,2], brain atrophy[3,4], and more recently, Alzheimer's disease[5-7]. Over the past thirty years, researchers have established strong evidence supporting a link between patients with diabetes and subsequent cognitive impairments and abnormalities in brain integrity.

While meta-analyses have found inconsistencies in the specifics of the literature[8-10], general trends point to cognitive impairments and abnormalities in related structural and functional brain areas. For example, patients with type 1 diabetes (T1D) are frequently found to have decreased psychomotor speed, mental flexibility, and IQ scores[8,11-13]. T1D patients also often show reductions in the volume of regional gray matter in areas such as the prefrontal cortex, hippocampus, and thalamus[12,14,15]. On the other hand, affected skills in type 2 diabetes (T2D) are largely executive function, memory, and information processing[16,17]. Neuroimaging studies done on T2D patients indicate global brain atrophy and microstructural changes[4,7,9,18], while findings regarding white matter hyperintensities are mixed[3].

In both T1D and T2D these decrements are considered mild across most age groups[8,11,19]. The severity of cognitive impairments and brain abnormalities are correlated with age of onset in T1D[11] and duration of the disease in T2D[20,21]. Age is also a risk factor as deficits in learning and memory have been reported to worsen considerably in T2D patients above 65 years of age[22]. Findings suggest the decreased brain volume in patients with T2D is correlated with increased insulin resistance[23], and both brain atrophy and microstructural changes are associated with impaired cognitive performance[18,20].

These data lend support to the idea that brain integrity is compromised in patients with both T1D and T2D, but also emphasize the need to integrate peripheral biomarkers associated with neuroprotection into diabetes research in humans. Various hormones altered as a result of diabetes have been recognized as neuroprotective, including insulin-like growth factor 1 (IGF1) and sex hormones. Research has revealed differences in the serum levels of IGF1 and gonadal hormones in diabetic patients[24-27], with clear sex differences in the effects of androgens and estrogens on the brain in animal models[28].

There is currently a movement in biomedical research to incorporate analyses of sex differences into studies[29-31]; however, studies on brain integrity of diabetic patients often fail to examine men and women separately. This is despite findings of sex-specific differences in regional brain volume between men and women[32-34]. For instance, DTI scans have also reported white matter hyperintensities are different in men and women diabetics[35]. Others have shown that, by combining the data of men and women, T2D patients had smaller gray matter volume with larger ventricular volume and white matter lesions compared to healthy controls. However, when the sexes were analyzed separately, the data for men failed to reach statistical significance[36].

Because sex hormones can act on similar molecular pathways as IGF1, and IGF1 is functionally related to insulin and diabetes, there is a need to further investigate how these hormones interact in the brains of diabetic patients. The relationship between estrogen and IGF1 is the most extensively studied in the neuroprotection literature[37-39], but it has yet to expand experimentally into diabetes research. Furthermore, little attention has been paid to androgen-IGF1 interactions, even in the animal literature, despite the similar mechanisms underlying estrogenic and androgenic neuroprotection.

DIABETES AND IGF1 RELATION

IGF1 has a hypoglycemic response similar to insulin and, in some circumstances, is capable of modulating insulin receptor (IR) activities. Research has demonstrated that low IGF1 is associated with T1D and T2D[40-42]. Moreover, genetic studies suggest decreased IGF1, due to a genetic polymorphism in the promoter region of the IGF1 gene, increases the risk of glucose intolerance and T2D[43].

On the other hand, T2D has also been correlated

with excessively high levels of IGF1. For example, people with acromegaly - a condition known for its overproduction of pituitary growth hormone - have both high levels of IGF1 and a greater risk of developing T2D[44]. These findings were corroborated by two large studies from Denmark (n = 3354) and Germany (n = 7777) which found U-shaped associations between IGF1 levels and the likelihood of developing insulin resistance and T2D[24,25]. Moreover, treatment with IGF1 can improve glycemic control in patients with T1D and T2D[45,46], which may suggest an optimal range of IGF1 for normal glycemic control.

Although IGF1 is synthesized in the brain, peripheral values cannot be used to accurately infer brain levels of IGF1 in humans as local synthesis of IGF1 in the brain appears not to correlate with the quantity of IGF1 receptors (IGF1R)[47-49]. Evidence from animal models suggest that brain atrophy and loss of DNA are prevented following injection of insulin and IGF1, but not insulin alone, into cerebrospinal fluid of mice[50]. Thus, proper systemic levels of IGF1 and its transport from the periphery into the brain is likely necessary for the maintenance of various cognitive processes[51].

Collectively, these data support the involvement of IGF1 in diabetes but also point to an "optimal range" of IGF1. Future research should examine the significance of an optimal peripheral range in the development and maintenance of diabetes and cognitive decline. Moreover, there is a need for data on the role of central *vs* peripheral IGF1 levels and the subsequent impact on cognitive impairment and brain atrophy.

THE IGF1 SYSTEM

Transportation

IGF1 is a polypeptide, structurally similar to insulin, that is released in response to growth hormones secreted by the anterior pituitary[52]. While synthesized predominantly by hepatocytes in the liver and released into general circulation, both paracrine and autocrine functions contribute through local tissue synthesis of IGF1. The concentration of IGF1 is greatest during perinatal development and decreases markedly into adulthood. IGF1R are expressed in nearly all neural cells of the CNS, being most highly expressed in the cortex, hippocampus, cerebellum, brainstem, hypothalamus, and spinal cord[53].

The blood brain barrier and blood-cerebrospinal fluid barrier are the two primary routes involved with transporting systemic IGF1 into the brain. Both barriers utilize lipoprotein receptor-related proteins along with IGF1R as transporters to enter the brain[54,55]. However, the bioavailability of IGF1 is largely determined by the amount of hormone bound to IGF binding proteins (IGFBPs). Most circulating IGF is bound by IGFBPs, which are proteins that control the distribution and functional capabilities of IGF1 throughout the body. Six different IGFBPs modulate the activity of IGFs *via* binding affinities exceeding that of its respective receptor and, thus, help regulate the amount of IGF1 that enters the brain[56].

Signaling pathways

The role of IGF1 is dependent on its binding to insulin-like peptide receptors. The three most important include the IGF1R, IR, and a hybrid receptor formed from heterodimer α-β IR and IGF1R subunits[53,57]. These receptors are important to the functional efficacy of IGF1 and have defined downstream molecular pathways. As part of the tyrosine kinase receptor family, activation of IGF1R leads to the signaling of either the mitogen-activated protein kinases-extracellular signal-related kinase (MAPK-ERK) or phosphoinositide 3-kinase (PI3K)-Akt pathways[53,57]. These pathways are involved in several important cellular processes including the regulation of gene transcription, apoptosis, oxidative stress, and cellular proliferation and differentiation.

The affinity of IGF1 varies among the three receptors with the highest affinity for IGF1R. Activation of the IGF1R is capable of directly stimulating the RAS-ERK pathway, leading to the modulation of gene transcription by way of activating ETS-like transcription factor, ELK1[57]. The capacity of insulin-like peptide receptors to initiate downstream molecular activity is modified in part by the recruitment of insulin receptor substrate (IRS) scaffolding proteins[57-59]. This scaffolding helps adjust pathway choice following receptor phosphorylation. The result is activation of PI3K-Akt and subsequent expression of downstream effectors, including glycogen synthase 3 kinase (GSK3β) and mammalian target of rapamycin[53,57,60].

Relationship to the insulin system

IGF1 acts primarily through binding to the IGF1R, but also shares with insulin the capacity to bind the IR and hybrid receptor[53,56,57]. Insulin is produced exclusively by β-cells of the pancreas and, hence, is strictly transported in the systemic circulation. The amount of insulin capable of entering the brain varies considerably[54,55]. Unlike IGF1, insulin appears not to be locally synthesized in adult brain cells[53,56]. Similar to IGF1, IR located on endothelial and epithelial cell membranes allow insulin to be transported into the brain from systemic circulation. IRs are concentrated mostly in the olfactory bulb, cerebral cortex, hypothalamus, hippocampus, and cerebellum[55]. The movement of systemic insulin into the brain is not controlled by binding proteins.

Both insulin and IGF1 produced in the periphery contribute to varied physiological processes. Proper peripheral IGF1 activation is necessary for insulin secretion from the pancreas and, hence, is implicated in many facets of diabetes[61]. However, their functions differ once entering the brain. IGF1R are expressed at notably higher rates in the brain than the rate IGF1 is synthesized. This differential suggests that active transport of IGF1 into the brain is required to furnish sufficient IGF1 for proper neuronal function[47-49]. For example, peripheral IGF1 supplies the brain with

information regarding body mass, is related to neural plasticity and cognitive processes, and attenuates cognitive impairment induced by diabetes[51,62,63]. Deficiency of IGF1 can also lead to hippocampal atrophy and impaired learning[64]. Indeed, IGF1 in the brain is required for proper tissue growth in both the brain and periphery, as well as sufficient glucose regulation and insulin sensitivity[65,66].

Insulin in the periphery is well-known for its role in glucose regulation and communication with the brain to maintain energy homeostasis. Similar to IGF1, insulin is involved in modifying BBB permeability in the brain[55] with T2D patients showing greater permeability of the BBB[67]. Insulin also acts on the PI3K and MAPK signaling cascades to enhance neuronal survival, plasticity, and subsequent cognitive processes[55,68,69]. With that said, insulin does not necessarily regulate glucose activity in neuronal cells after entering the brain. Rather, insulin modulates energy homeostasis through its actions at the level of the hypothalamus[70].

INTEGRATING SEX HORMONES INTO DIABETES AND IGF1

Diabetes is associated with imbalances in sex steroid hormone levels. This is not surprising as androgens and estrogens are known to play an important role in body composition[71] while maintaining glucose and lipid homeostasis[72,73]. Research into these imbalances suggests a complex relation between estradiol (E2) and insulin insensitivity. Several studies have reported that postmenopausal women with T2D have increased levels of circulating E2[27,74,75]. Elevated E2 has been correlated with the development of insulin resistance and T2D in these women[76,77]. Nevertheless, there are at least two studies that have shown inconsistencies between E2 levels and the development of diabetes in postmenopausal women[78,79].

There is also a link between high levels of E2 and diabetes in men. Diabetic men have shown relatively high basal levels of E2[27,78], while men with higher levels of circulating E2 have an increased risk of developing T2D[80]. Although this may simply be a product of higher body fat content as adrenal androgens are readily converted to E2 in adipose tissue[81-83], two studies reported E2 results in men were independent of obesity[78,80].

Findings with animal models suggest an opposite conclusion for E2 and diabetes, at least during reproductive ages. Male mice with streptozotocin-induced insulin insensitivity are more likely to develop diabetes than their female cohorts. This increased risk of diabetes in the males can be attenuated with E2 supplements[84]. Also, mice lacking the alpha subtype of estrogen receptor (ERα) have been reported to develop insulin insensitivity[85]. In contrast, these data in animals mirror those from postmenopausal women in which glucose homeostasis was positively impacted with estrogen therapy in the short term[86].

Sex differences in androgen-diabetes relations have also been reported. Postmenopausal women with diabetes displayed elevated circulating testosterone (TS) levels[27,75]. Reports suggest that premenopausal women with higher levels of TS[76,79], as well as female mice administered the androgen[84], had a greater risk of developing diabetes. Another example is the link between T2D development and hyperandrogenism experienced by patients with polycystic ovarian syndrome[87]. Still, much like E2, there are also studies that dispute these reports, particularly in postmenopausal women[77,78].

A clear sex difference is also indicated in that diabetic men tend to have either lower total, free, or bioavailable TS than healthy men[27,88,89]. Indeed, men with the highest levels of TS were at the lowest risk and men with lowest levels of TS were at highest risk for developing T2D[78,79,90]. Moreover, men undergoing androgen deprivation treatments for prostatic cancer had a greatly increased risk of developing T2D[91]. Yet again, these reports are not without contradiction[92] and some studies found this relationship to be dependent on obesity[80,93].

Taken together, there are clear inconsistencies in the findings on sex hormones and diabetes. There is also an apparent lack of research focusing on sex hormones in premenopausal diabetic women that should be addressed[26]. It is again important to note that many studies fail to acknowledge the possible relation of sex hormones to the IGF1 system. Findings with serum E2 data are consistent with findings from meta-analyses examining IGF1[24,25]. Their proposed U-shaped association of IGF1 and T2D fits into the well-defined mechanistic relationship between E2 and IGF1, described in more detail below. The relation between sex hormones and IGF1 suggests that a delicate hormonal balance is likely an important facet of diabetes-induced brain and cognitive impairment.

NEUROPROTECTION: SEX HORMONES AND IGF1

Estrogen and IGF1

An intriguing feature of neuroactive hormones is their ability to protect the CNS from damage, especially in regards to estrogen. ER activation is implicated in the maintenance of various metabolic processes that are also associated with diabetes, including glucose homeostasis and obesity[94,95]. Only recently has research with animal models focused on neuroprotection from IGF1-E2 interactions. Evidence suggests that neuroprotective properties of E2 are directly related to receptor activities of insulin-like peptide receptors, mainly IGF1R. E2 and IGF1 work in tandem to reciprocally modulate and facilitate ER and IGFIR activation of the PI3K-Akt and MAPK-ERK signaling cascades[96-100].

IGF1 shows differential sensitivities to the two

estrogen receptor subtypes with ERα being more sensitive than ERβ[97,101]. Selective inhibition of IGF1R, for instance, downregulates ERα expression in the hypothalamus, hippocampus, and cerebral cortex, with the only significant changes of ERβ occurring in the cerebellum[38]. Many glial and neuronal cells in the brain express IGF1R and both ER subtypes[102]. In particular, ERα is uniquely capable of increasing IGF1R activity of downstream PI3K-Akt signaling in rodent models[103,104]. ERα activation also increases the binding of p85 and IRS-1 regulatory subunits of PI3K and, thus, may be one mechanism assisting in Akt pro-survival signaling through the IGF1R[39,97] (Figure 1).

Administration of E2 to mice increased IGF1R and ERα activity in the brain, enabling activation of IGF1R and downstream PI3K-Akt pathway signaling[97]. Similarly, IGF1 and insulin modulated ER effects on gene transcription and the PI3K-Akt-GSK3β signaling cascade[38,98,103,105,106]. GSK3β is a protein kinase known particularly well for its role in glycogen synthesis. However, as reviewed by Jacobs *et al*[60], recent attention has turned to the dual pro- and anti-apoptosis capabilities of GSK3β regulated through multiple different pathways. Indeed, the neuroprotective effects of IGF1 may be consequent to Akt-derived inhibition of GSK3β in a hypoxic state[107] (Figure 1).

Activation of the MAPK pathway is another important signal transduction pathway involved with regulating gene transcription and cellular proliferation and differentiation, particularly in cancer[108]. However, multiple studies have demonstrated that the neuroprotective properties of estrogen are also derived from its ability to regulate MAPK signaling in the brain[38]. Both estrogen and IGF1 can facilitate MAPK signaling through the IGF1R, with IGF1 increasing ERα activities in the presence of E2[104]. Akt inhibitors are capable of nullifying the neuroprotective effects of IGF1 and E2 regardless of MAPK signaling[99,104], while ERK suppression increases PI3K-Akt activity *via* ER and IGF1R heterodimers[39]. Thus, it appears the PI3K-Akt pro-survival signaling cascade is the most involved with the neuroprotective coupling of E2 and IGF1[39].

It is important to note that IGF1 and E2 have a remarkable reciprocity. Inhibition of ER activity can downregulate IGF1R expression in the hippocampus[109], a brain region known to atrophy in patients with diabetes and glucose intolerance[110-112]. Similarly, IGF1 has the capacity to upregulate ERα in the hippocampus and is impaired following administration of IGF1R antagonists[109]. Agonists or antagonists of either hormone can respectively facilitate or inhibit the neuroprotective and memory enhancing properties of the other[96,109,113-116]. This has led some to suggest that cooperation between IGF1R and ER is required for many E2-induced neuroprotective processes. The present section does not, however, do justice to the complexity of the relation between estrogen and IGF1 receptors. A fuller explanation can be found in one of several reviews[37-39,101,109,117].

Androgens and IGF1

Far less research has examined a functional link between IGF1 and androgens in the brain. This is an unfortunate but common trend in neuroendocrinology. Estrogens are the most intensely studied gonadal hormone, despite estrogens and androgens sharing metabolic pathways and functional properties. Much of the current literature on IGF1-androgen relations are directed at the periphery, particularly prostate cancer and motor systems, for which there are a number of recent reviews[118,119]. Few studies have examined IGF1-androgen interactions in neuroprotection[120,121] and none, to our knowledge, have empirically examined this interaction in diabetes. Therefore, we have relied on peripheral data, often from *in vitro* experiments, to extrapolate the androgen receptor (AR) brain discussion.

There is evidence that the two main androgens, TS and dihydrotestosterone (DHT), are capable of neuroprotection through binding the AR[122-126]. Similar to ERα, androgen activation of the AR in mouse vas deferens epithelial cells can modulate the p85 regulatory subunits of PI3K and subsequently trigger Akt expression (Figure 1). Inhibiting the AR prevents these signaling effects[127]. Phosphorylation of MAPK and Akt can also increase AR activation in low androgen and estrogen concentrations, as well as increase the neuroprotective activities of ERα and AR[128]. Recent findings showed that DHT, which has a higher affinity than TS for the AR, prevents apoptosis in a C6 glial cell line through the PI3K-Akt signaling cascade[129]. These effects were also impaired by inhibition of PI3K and suggest a functional relationship between apoptosis and AR activities.

Interestingly, studies have demonstrated that binding of DHT to the transmembrane AR impairs MAPK and PI3K signaling and subsequent neuroprotection from DHT or E2[130-132]. This suggests that nuclear activation of the AR by DHT is likely one mechanism behind DHT's neuroprotective properties[130]. DHT may also interact with effectors downstream of ER and IGF1R signaling. Both TS and DHT can activate the MAPK-ERK signaling cascade[132] which has been shown to induce ribosomal S6 kinase (Rsk) expression. Rsk signaling can lead to the inhibition of the pro-apoptosis Bad protein and the activation of downstream effectors including the ER, GSK3β and ELK1[133] (Figure 1).

One possible explanation for the neuroprotective role of androgens is the conversion in the steroid metabolic cascade of TS into E2 by the enzyme aromatase. That is, TS may be involved in neuroprotection only to the extent that TS is a precursor for E2, which is capable of activating MAPK or PI3K signaling through the ER and IGF1R. The aromatization of TS into E2, as well as the aromatase enzyme, have been suggested to play an important role in neuroprotection[134-139].

The ratio of endogenous TS to E2, and subsequent influences of aromatized TS, is indeed a topic of recent interest[26]. Increased local synthesis of E2 from elevated aromatase expression is seen in models

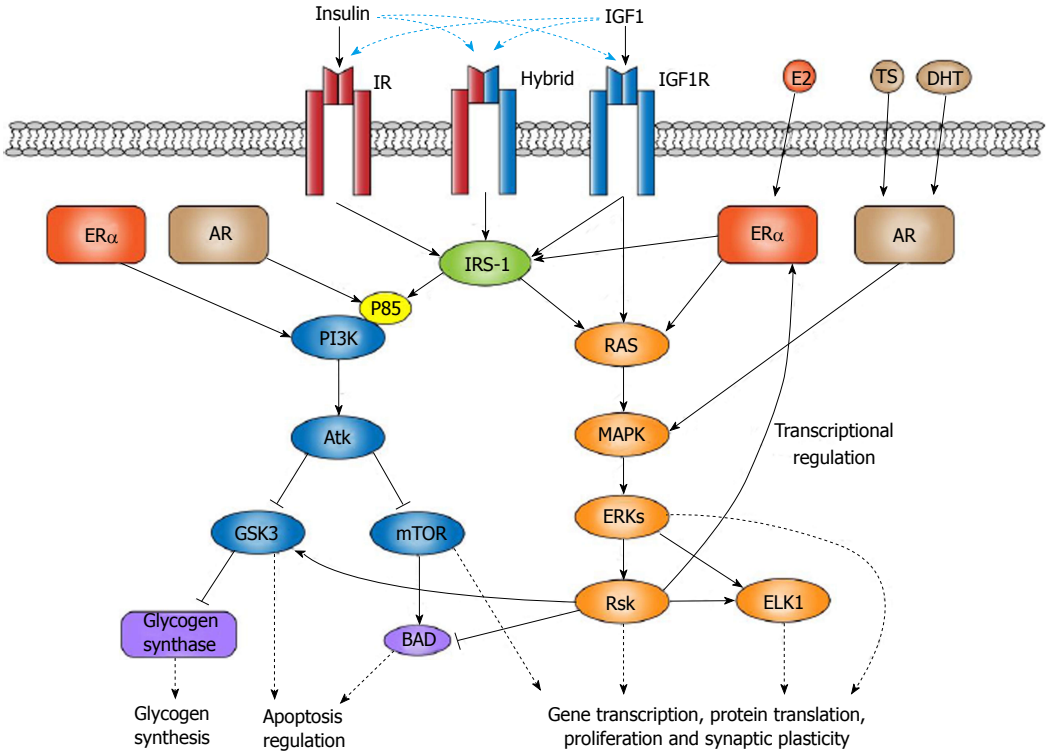

Figure 1 Similar signaling cascades involved with neuroprotection for insulin-like peptides and sex hormones. The insulin receptor (IR), insulin-like growth factor 1 receptor (IGF1R), and insulin-IGF1 hybrid receptor enact their neuroprotection through the mitogen-activated protein kinases-extracellular signal-related kinase (MAPK-ERK) or phosphoinositide 3-kinase (PI3K)-Akt pathways signaling cascades. Although IGF1R can directly activate the RAS-ERK pathway, both the insulin-like peptide receptors and the estrogen receptor alpha (ERα) firstly interact with insulin receptor substrate 1 (IRS-1) scaffolding proteins. ERα and the androgen receptor (AR) can also directly modulate PI3K-Akt and MAPK-ERK signaling. Both IRS-1 and p85 binding of PI3K are increased with ERα activation, leading to downstream Akt-derived inhibition of glycogen synthase kinase 3 (GSK3) and mammalian target of rapamycin (mTOR). GSK3, specifically, is involved with glycogen synthesis, while both effectors are involved in apoptosis. A similar effect may occur with AR's ability to modulate p85 binding to PI3K. AR-induced MAPK-ERK signaling also results in ribosomal S6 kinase (Rsk) expression that can inhibit the pro-apoptosis bcl-2-associated death promoter protein, as well as effects on the ER, GSK3, and the ETS-like transcription factor, ELK1. Solid black arrows indicate downstream interaction. Dashed black arrows represent the influence of kinases or proteins on the cellular environment. Dashed blue arrows represent the binding capabilities of IGF1 and insulin across all three receptor types.

of neuroprotection from other brain disorders, *e.g.*, stroke[140]. More pertinent to this review, streptozotocin-induced diabetes causes a considerable reduction in aromatase synthesis in female and male reproductive systems[141]. Notably, inhibition of aromatase decreases E2 and impairs insulin sensitivity and peripheral glucose disposal in healthy males[142], although the influence this may have on brain integrity and cognitive outcomes remains debated[143].

Another explanation places greater emphasis on the other pathway in the steroid metabolic cascade leading to DHT. Metabolites of DHT, 3α-Diol and 3β-Diol, are also bioactive and may bind the ER or insulin-like peptide receptors to initiate MAPK or PI3K signaling cascades. Indeed, research shows that 3α-Diol stimulated PI3K-Akt signaling enhances cell survival in the prostate[144]. Similarly, DHT metabolites may influence transcriptional activities of nuclear ER by modulating ER-induced MAPK or PI3K signaling cascades.

Few *in vivo* studies examining these sex steroid metabolites have focused on MAPK or PI3K signal cascades in the brain. There is, however, evidence that 3α-Diol inhibits protein kinase A expression in the rat hippocampus[145]. Others have reported that streptozotocin-induced diabetic mice had lower levels of TS and 3α-Diol in the cerebral cortex, and lower levels of DHT and 3α-Diol in the spinal cord[146]. It is still unclear, though, whether 3α-Diol and 3β-Diol interact with or initiate the MAPK or PI3K signaling cascades following activation of the ER, AR, or, possibly, IGF1R.

None of these explanations clarify fully the ability of the AR to directly trigger these signaling cascades. We do not aim to discount the neuroprotective mechanisms of ER and AR, or the clear link between E2 and IGF1 processes in neuroprotection. Rather, we simply suggest that androgen-derived neuroprotection may be intertwined with IGF1, the activation of insulin-like peptide receptors, and/or the IGF1R and ER coupling. Given the common signaling pathways between these hormones, we suggest future research should aim to include androgens and AR activities into the ER-IGF1R neuroprotective coupling, as well as serum comparisons in brain-health outcomes of diabetic patients.

CONCLUSION

The reciprocity of IGF1 and estrogen in neuroprotective processes is well-established in cell cultures and

animal models[38]. Interactions between androgens and IGF1 may also play an important role in the E2-IGF1 neuroprotective coupling. Both estrogens and androgens enact their neuroprotection through similar, but not identical, signal transduction pathways. Recognition of this has led us to consider the possibility that these sex hormones may work together with IGF1 and insulin-like peptide receptors to modulate MAPK and PI3K signaling and their neuroprotective properties.

Regulation of MAPK and PI3K activity may also be a driving force behind the structural changes, atrophy of brain regions, or functional changes, often observed in diabetic patients. Drawing conclusions from imaging data in humans to those found in animal models is indeed difficult. Nevertheless, there is a need for a clearer mechanistic explanation grounding the cognitive decline and brain abnormalities observed in diabetic patients.

Future studies in human research on diabetic brain integrity should integrate hormone titer measures to help substantiate sex differences in brain-health outcomes of diabetic patients. This approach may also assist in identifying region-specific brain abnormalities resulting from fluctuations in IGF1 and sex hormones between men and women. Moreover, animal models examining the E2-IGF1 coupling in neuroprotection should employ streptozotocin-induced diabetes, as well as the possible role of androgens and AR activities. These conclusions warrant further examination of the variability present in cognitive and brain-health outcomes for patients with diabetes as a result of sex hormone relations to IGF1, insulin, and the insulin-like peptide receptors.

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ORIGINAL ARTICLE

Basic Study

High fat diet dysregulates microRNA-17-5p and triggers retinal inflammation: Role of endoplasmic-reticulum-stress

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Abstract

AIM

To elucidate how high diet-induced endoplasmic reticulum-stress upregulates thioredoxin interacting protein expression in Müller cells leading to retinal inflammation.

METHODS

Male C57Bl/J mice were fed either normal diet or 60% high fat diet for 4-8 wk. During the 4 wk study, mice received phenyl-butyric acid (PBA); endoplasmic reticulum-stress inhibitor; for 2 wk. Insulin resistance was assessed by oral glucose tolerance. Effects of palmitate-bovine serum albumin (BSA) (400 μmol/L) were examined in retinal Müller glial cell line and primary Müller cells isolated from wild type and thioredoxin interacting protein knock-out mice. Expression of thioredoxin interacting protein, endoplasmic reticulum-stress markers, miR-17-5p mRNA, as well as nucleotide-binding oligomerization domain-like receptor protein (NLRP3) and IL1β protein was determined.

RESULTS

High fat diet for 8 wk induced obesity and insulin resistance evident by increases in body weight and impaired glucose tolerance. By performing quantitative real-time polymerase chain reaction, we found that high fat diet triggered the expression of retinal endoplasmic reticulum-stress markers ($P < 0.05$). These effects were associated with increased thioredoxin interacting protein and decreased miR-17-5p expression, which

were restored by inhibiting endoplasmic reticulum-stress with PBA ($P < 0.05$). *In vitro*, palmitate-BSA triggered endoplasmic reticulum-stress markers, which was accompanied with reduced miR-17-5p and induced thioredoxin interacting protein mRNA in retinal Müller glial cell line ($P < 0.05$). Palmitate upregulated NLRP3 and IL1β expression in primary Müller cells isolated from wild type. However, using primary Müller cells isolated from thioredoxin interacting protein knock-out mice abolished palmitate-mediated increase in NLRP3 and IL1β.

CONCLUSION

Our work suggests that targeting endoplasmic reticulum-stress or thioredoxin interacting protein are potential therapeutic strategies for early intervention of obesity-induced retinal inflammation.

Key words: High fat diet; Palmitate; Endoplasmic-reticulum-stress; Inflammation; Thioredoxin-interacting protein; Micro-RNA 17-5p

Core tip: We previously showed that high fat diets (HFD) induced retinal inflammation and vascular dysfunction. These results were associated with an increase in thioredoxin interacting protein (TXNIP) at the mRNA and protein level. Here, we examined the mechanisms by which HFD triggers retinal TXNIP. Interestingly, we found that HFD/palmitate triggers ER-stress mediators including the inositol requiring enzyme 1, an RNAse that can degrade number of mRNAs including the microRNA; miR-17-5p and sustains TXNIP expression. Inhibiting ER-stress prevented the increase in TXNIP *in vivo* and in Müller cells, the main glia in the retina. Deletion of TXNIP blunted NLRP-3 inflammasome and IL-1β release in Müller cells.

Coucha M, Mohamed IN, Elshaer SL, Mbata O, Bartasis ML, El-Remessy AB. High fat diet dysregulates microRNA-17-5p and triggers retinal inflammation: Role of endoplasmic-reticulum-stress. *World J Diabetes* 2017; 8(2): 56-65 Available from: URL: http://www.wjgnet.com/1948-9358/full/v8/i2/56.htm DOI: http://dx.doi.org/10.4239/wjd.v8.i2.56

INTRODUCTION

Obesity, recently upgraded from a mere risk factor to a disease state, is affecting one third of United States population[1]. Clinical evidence showed that obesity not only can accelerate developing type-2 diabetes and cardiovascular complications, but also induce retinal microvascular abnormalities, which eventually leads to visual impairments[2,3]. High fat diets (HFD) together with the improper physical activity are the culprit in the obesity-induced pre-diabetes. Therefore, there is an urgent need to unravel the mechanisms involved in HFD-mediated neurovascular abnormalities. Our lab has previously shown that consumption of high caloric diet saturated fatty acids induced retinal inflammation and microvascular dysfunction *via* upregulating the expression of thioredoxin interacting protein (TXNIP); a regulator of the antioxidant thioredoxin; and activating NOD (NOD)-like receptor protein (NLRP3)-inflammasome[4]. Similar observations showed the contribution of TXNIP/NLRP3-inflammasome signaling pathway to the development of various disorders in other organs[5-7]. However, molecular mechanisms by which HFD triggers early TXNIP expression in the retina are still unclear.

MicroRNAs are small non-coding RNAs that control the translation and transcription of various genes *via* annealing to the complementary sequences in the 3′ untranslated region of their target gene[8]. To date, several miR classes have been identified to be involved in development of obesity, diabetes and diabetic complications[9]. Bioinformatic analysis of the TXNIP 3′ UTR identified 11 possible miRNAs that can regulate its expression including miR-130/301, miR-128, miR-148/152, miR-135, miR-106/302, miR-17-5p/20/93.mr/106/519.d, miR-128, miR-15/16/195/424/497, miR-106/302, miR-148/152. Nevertheless, levels of miR-17-5p have been reported to rapidly decline under stress condition resulting in enhancing TXNIP expression[10,11].

Unfolded protein response (UPR) is an adaptive response, which prevents the accumulation of misfolded proteins in the lumen of the endoplasmic reticulum (ER). The UPR is transduced by three major ER-resident stress sensors, namely Protein Kinase RNA-like ER kinase (PERK), activating transcription factor 6 (ATF6), and inositol requiring enzyme 1 (IRE1). However, when protein misfolding exceeds the capacity of the UPR an ER-stress will result that triggers programmed cell death. So far, ER-stress has been shown to play a critical role in the pathogenic progression of various chronic diseases including diabetic retinopathy (reviewed in[12-14]). Among UPR pathways, IRE1α, an ER bifunctional kinase/RNase has been shown to destabilize number of RNA and microRNA including miR-17-5p in pancreatic beta-cells[10,11]. Several studies reported the impact of HFD and its related metabolite such as free fatty acid in inducing ER-stress[15-17]. In the current study we were trying to decipher the underlying mechanisms that link HFD-mediated ER-stress to retinal inflammation. Here, we tested the hypothesis that HFD-mediated ER-stress upregulates TXNIP mRNA expression *via* dysregulating miR-17-5p resulting in retinal inflammation.

MATERIALS AND METHODS

Animals

All animal experiments were conducted in agreement with Association for Research in Vision and Ophthalmology

Table 1 The sequence of the polymerase chain reaction primers used in the experiments

Gene	Forward	Reverse
18 S	CGCGGTTCTATTTTGTTGGT	AGTCGGCATCGTTTATGGTC
XBP1	ACACGCTTGGGAATGGACAC	CCATGGGAAGATGTTCTGGG
XBP1-SPLICED	GAGTCCGCAGCAGGTG	GTGTCAGAGTCCATGGGA
PERK	AGTCCCTGCTCGAATCTTCCT	TCCCAAGGCAGAACAGATATACC
IRE1α	GGGTTGCTGTCGTGCCTCGAG	TGGGGGCCTTCCAGCAAAGGA
ATF6	TGCCTTGGGAGTCAGACCTAT	GCTGAGTTGAAGAACACGAGTC
CHOP	CTGGAAGCCTGGTATGAGGAT	CAGGGTCAAGAGTAGTGAAGGT
TXNIP	AAGCTGTCCTCAGTCAGAGGCAAT	ATGACTTTCTTGGAGCCAGGGACA

statement for use of animals in ophthalmic and vision research, and Charlie Norwood VA Medical Center Animal Care and Use Committee (ACORP#15-04-080). 6-8 wk old male C57BL6/J mice (Stock 000664, Jackson Laboratory, ME, United States) were used in the *in vivo* studies. For the long term study, mice were fed ad libitum with normal rat chow (7% fat) or HFD [36 g %, 251 kJ (60 kcal) %fat] (F2685 Bioserv, Frenchtown, NJ, United States) for 8 wk. For the short term study, mice were fed either normal diet (ND) or 60% HFD for 2 wk. Mice were then kept on HFD for additional 2 wk while receiving an ER-stress inhibitor [Phenyl-butyric acid (PBA), 100 mg/kg] or vehicle. PBA was dissolved in DMSO/PBS and administered *via* oral gavage 5 d/wk. Mice were weighed weekly to track the increase in the body weight.

Intra-peritoneal glucose tolerance test

Mice went overnight fasting, and their fasting plasma blood glucose was measured as the baseline. Then all mice received an intraperitoneal injection of glucose (2 g/kg). Blood glucose levels were measured at different time points till 120 min after the glucose injection using a glucometer.

In-vitro studies

The rat retinal Müller glial cell line (rMC-1) was obtained originally from V. Sarthy (Department of Ophthalmology, Northwestern University, Chicago, IL, United States)[18]. Primary mouse Müller Cells from WT and TKO mice were isolated and cultured as described previously[19]. Cells were grown to confluency in complete media (DMEM, 10% vol/vol. FBS, 1% vol/vol. penicillin/streptomycin). Sodium palmitate (Cat.# P9767; Sigma-Aldrich, St. Louis,MO, United States) was dissolved in 50% ethyl alcohol, then added drop-wise to preheated 10% endotoxin- and fatty acid-free BSA (Cat.# 22070017; Bioworld, Dublin, OH) in DMEM at 50 ℃ to create an intermediate stock solution of palmitate coupled to BSA (Pal-BSA). Confluent cells were switched to serum-free medium for overnight then were treated for 6 h with Pal-BSA solutions (400 μmol/L final concentration). Equal volumes of 50% ethyl alcohol with BSA alone served as control. In another set of rMC-1, cells were serum starved for 4 h then treated with PBA (1 mmol/L, Cat.#P21005, Sigma-Aldrich) or IRE1α inhibitor (STF-083010, 50 μmol/L) for 2 h then palmitate was added and kept overnight.

Quantitative real-time PCR

A one-step quantitative RT-PCR kit (Invitrogen) was used to amplify 10 ng retinal mRNA as described previously[4]. PCR primers (Table 1) were obtained from Integrated DNA Technologies (Coralville, IA, United States). Quantitative PCR was conducted using StepOnePlus qPCR system (Applied BioSystems, Life Technologies). The percent expression of various genes was normalized to 18S.

Micro-RNA detection

MirVana PARIS kit (Cat.# AM1556, Invitrogen) was used for miRNA isolation according to manufacturer's protocol. Reverse transcriptase reactions; including samples and no-template controls; were run using TaqMan® MicroRNA Reverse Transcription Kit (Cat.# 4366596, Applied Biosystems) as described previously[20]. PCR amplification was performed using TaqMan® Universal PCR Master Mix (Cat.# 4324018, Applied Biosystems) according to manufacturer's protocol. The percent expression of miR-17-5p was normalized to U6.

Western blot analysis

Retinas were isolated and homogenized in cell disruption buffer as described previously[21]. Müller cells were harvested by scraping thoroughly with cell scraper after the addition of cell disruption buffer. Samples (25 μg protein) were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane. Membranes were probed with the primary antibodies; anti-TXNIP (Cat.# K0205-3 MBL Abacus ALS Australia and Cat.# 403700, Invitrogen, Grand Island, NY), anti-NLRP-3 (Cat.# LS-B4321, LifeSpan Biosciences, Inc, Seatle, WA), anti-IL1β (Cat.# ab9722, Abcam, Cambridge, MA, United States) then reprobed with housekeeping gene; anti-GAPDH (Cat.# 5174, Cell Signaling, Danvers, MA, United States), anti-tubulin (Cat.# ab4074, Abcam, Cambridge, MA, United States) or anti-actin (Cat.# a5060, Sigma-Aldrich) to confirm equal loading. The primary antibody was detected using a horseradish peroxidase (HRP) and enhanced chemiluminescence. The films were scanned and the band intensity was quantified using densitometry software version 6.0.0 Software from alphaEaseFC (Santa Clara, CA) and expressed as relative

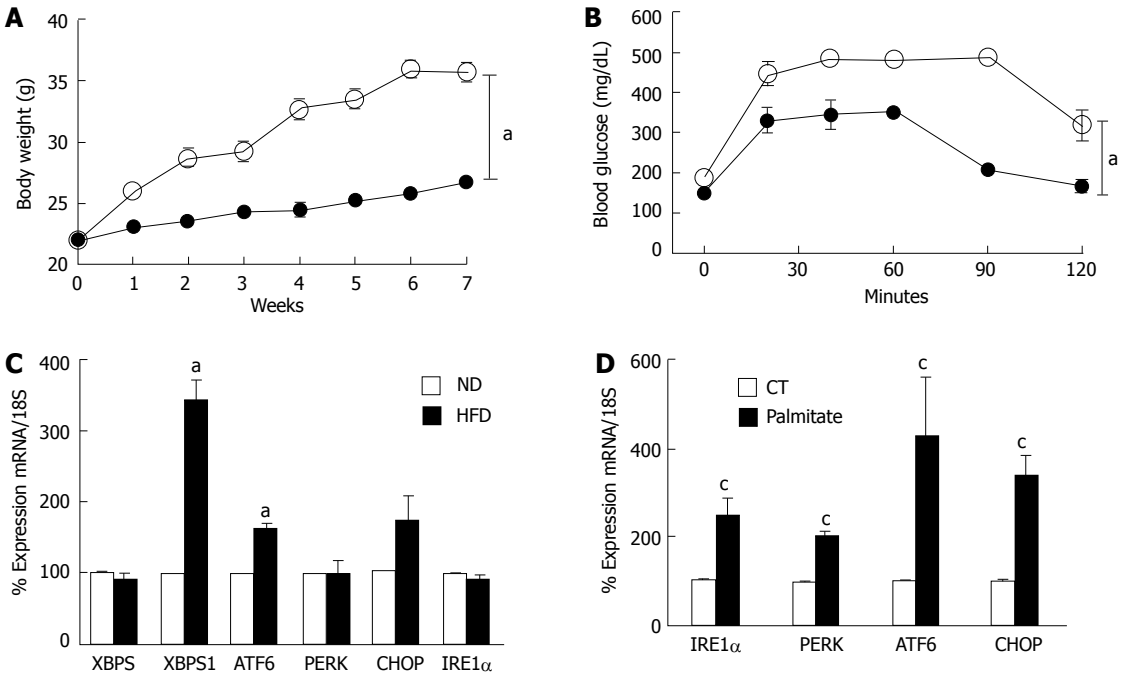

Figure 1 High fat diet/palmitate triggered endoplasmic-reticulum-stress markers in retina and Müller cells. A: Total body weight (grams) recorded weekly was significantly higher in mice fed with HFD for 8 wk compared to ND; B: Glucose tolerance was impaired after 8 wk of HFD compared to ND; C: Realtime PCR showing increases in mRNA levels of XBP1S and ATF6, while no change in XBP1, PERK, CHOP and IRE1α mRNA in retina after 8 wk of HFD compared to ND; D: Realtime PCR showing significant increases in IRE1α, PERK, ATF6 and CHOP mRNA levels in rMC1 treated with palmitate compared to control (CT) ($^{a}P < 0.05$ *vs* ND, $n = 3$-4 and $^{c}P < 0.05$ *vs* CT, area under the curve across all the time points was calculated, $n = 3$-4). HFD: High fat diet; PERK: Protein Kinase RNA-like endoplasmic-reticulum kinase; XBP: Xbox binding protein; ATF6: Activating transcription factor 6; CHOP: CCAAT-enhancer-binding protein homologous protein; IRE1: Inositol requiring enzyme 1.

optical density (OD).

Statistical analysis

All the data are expressed as mean ± SD or SEM. Differences between ND *vs* HFD and control *vs* palmitate were tested using two-sample *t* tests. One-way ANOVA followed by Bonferroni post-hoc multiple comparisons to assess significant differences between 3 or more groups (Graphpad-Ver.6). For body weight and blood glucose measurements, area under the curve (AUC) across all the time points was calculated. A series of 2 gene (WT *vs* KO) × 2 treatment (TRT) (no *vs* yes) ANOVAs with interaction were used to determine the effect of palmitate on NLRP3 and IL1β. A Bonferroni post-hoc multiple comparison test was used for significant interactions. Significance for all tests was determined at alpha = 0.05.

RESULTS

HFD/palmitate triggered ER-stress markers in retina and Müller cells

Several studies showed that HFD or palmitate triggers ER-stress in different organs and cell types[17,22-24]. Therefore, we checked the levels of various ER-stress markers in the retina isolated from mice fed with HFD, and rMC1 treated with palmitate. HFD for 8 wk induced obesity and impaired glucose tolerance indicated by an increase in body weights (Figure 1A) and glucose levels (Figure 1B) across the different time points compared to ND. We also found that HFD induced an increase in XBPS1 and ATF6 mRNA levels only, while, there was no change in XBP1, PERK, CHOP and IRE1α (Figure 1C). In order to study the role of Müller cells in HFD-induced inflammation, rMC-1 were treated with 400 μmol/L palmitate coupled to bovine serum albumin (Pal-BSA) for 6hr. Palmitate; a saturated fatty acid that is increased in plasma following a HFD[25]; significantly upregulated IRE1α, PERK, ATF6 and CHOP (Figure 1D).

HFD/palmitate induced TXNIP upregulation and miR-17-5p dysregulation in retina and Müller cells

Our lab has previously reported that HFD and palmitate can induce TXNIP mRNA expression in whole retina and retina endothelial cells respectively[4]. However, the upstream events by which HFD/palmitate trigger TXNIP expression are still unclear. In agreement with the previous study, we found that 8 wk of HFD and palmitate led to an upregulation of TXNIP mRNA levels in whole retina and Müller cells (Figure 2). These results were associated with miR-17-5p dysregulation in both whole retina and Müller cells (Figure 2).

PBA mitigated HFD-mediated ER-stress

To verify the role of ER-stress in HFD-induced TXNIP upregulation, mice were fed either ND or HFD for 2 wk. Then mice were kept on HFD for additional 2 wk while receiving PBA; an ER-stress inhibitor. Body weights were not changed by the HFD or PBA treatment (Figure 3A). However, blood glucose tolerance was significantly less in mice fed with HFD compared to ND after intra-peritoneal glucose tolerance test (Figure 3B). HFD-induced insulin

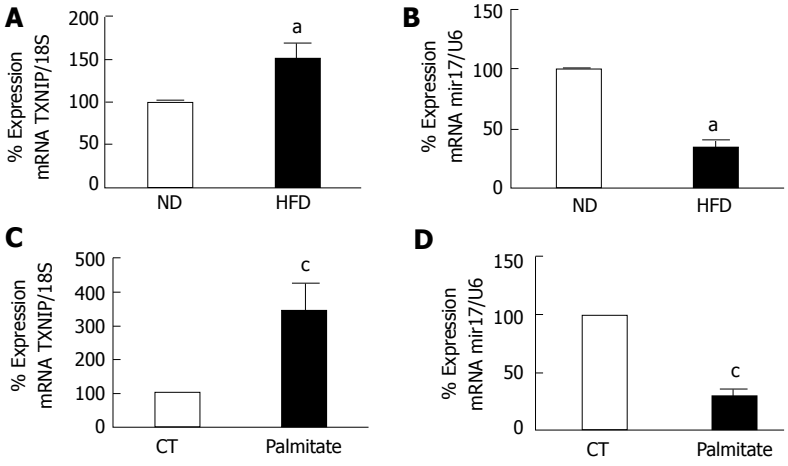

Figure 2 Realtime polymerase chain reaction. It shows significant (A) increase in TXNIP mRNA and (B) miR-17-5p dysregulation in retina after 8 wk of HFD compared to ND. Realtime PCR showing significant (C) increase in TXNIP mRNA levels (D) reduction in miR-17-5p in rMC1 treated with palmitate compared to control (CT) ($^{a}P < 0.05$ *vs* ND, $n = 3$-4 and $^{c}P < 0.05$ *vs* CT, $n = 3$). ND: Normal diet; HFD: High fat diet.

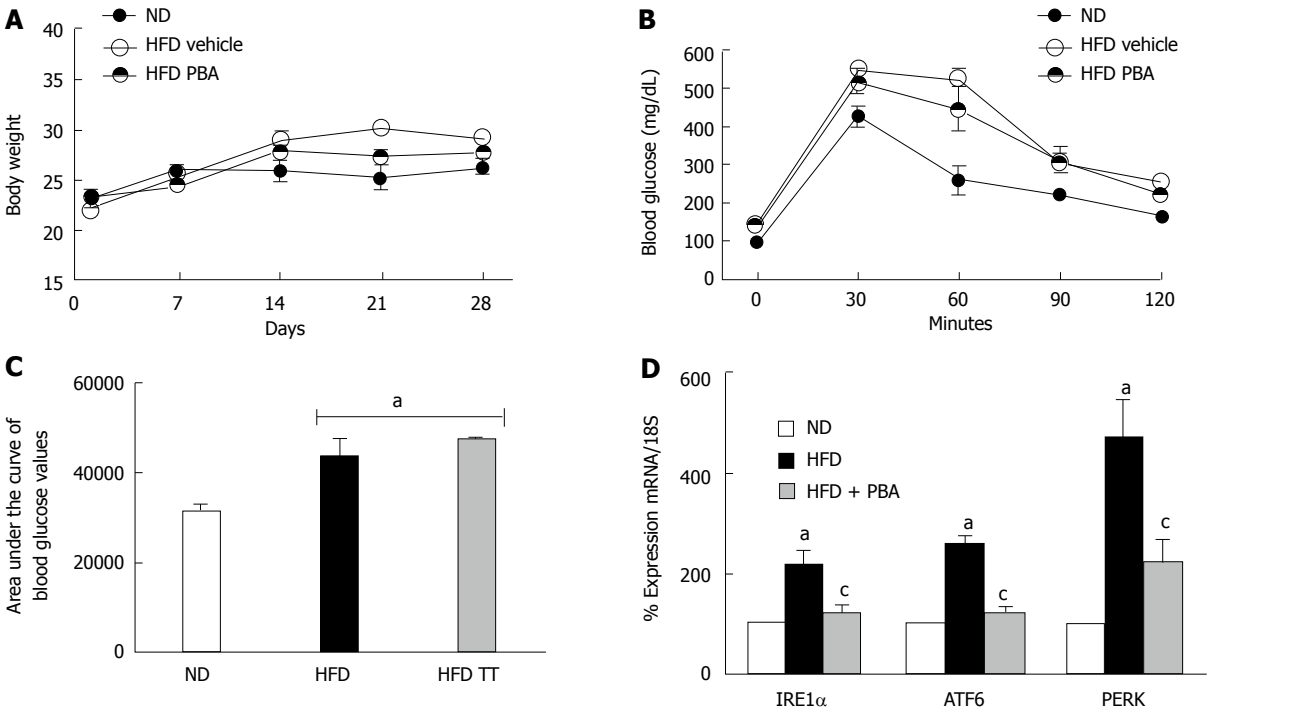

Figure 3 PBA mitigated high fat diet-mediated endoplasmic-reticulum-stress. A: Total body weight (g) recorded weekly for 4 wk was not changed among the different groups; B: Glucose tolerance was impaired after 4 wk of HFD compared to ND, and was not restored with PBA treatment; C: Statistical analysis of area under the curve showing an increase in blood glucose levels in HFD compared to ND, which was not reversed by the treatment; D: Realtime PCR showing significant increases in IRE1α, ATF6, and PERK mRNA levels in mice kept on HFD for 4 wk compared to ND, which were nullified with PBA treatment ($^{a}P < 0.05$ *vs* ND, $^{c}P < 0.05$ *vs* HFD, $n = 3$-4). ND: Normal diet; HFD: High fat diet; PBA: Phenyl-butyric acid; PERK: Protein Kinase RNA-like endoplasmic-reticulum kinase; IRE: Inositol requiring enzyme.

resistance suggested by marked increase in the area under the curve remained unaffected by inhibiting ER-stress with PBA (Figure 3C). HFD for 4 wk induced expression of retinal ER-stress markers mRNA including the RNAse IRE1α, ATF6 and PERK which were restored by PBA treatment to control level (Figure 3D).

ER-stress inhibition prevented HFD-induced TXNIP upregulation and miR-17-5p dysregulation

To establish a causal relationship of the role of ER-stress miR-17-5p and TXNIP expression, we assessed their expression in animals that were treated with ER-stress inhibitor PBA. As shown in Figure 4A, intervention with PBA treatment in HFD partially but significantly increased retinal miR-17-5p compared to untreated HFD. HFD triggered TXNIP mRNA and protein expression compared to ND, which were significantly inhibited in HFD-animals treated with PBA (Figure 4B-D). To establish a causal relationship of the role of ER-stress and activation of IRE1α in palmitate-induced TXNIP expression, rMC1 were treated for 2 h with PBA or IRE1α inhibitor prior to the addition of palmitate. As shown in Figure 4, inhibiting ER-stress or IRE1α markedly reduced the increase in TXNIP protein expression in palmitate-treated cells.

Knocking out TXNIP abolished palmitate induced inflammation in Müller cells

We recently showed that HFD induced expression

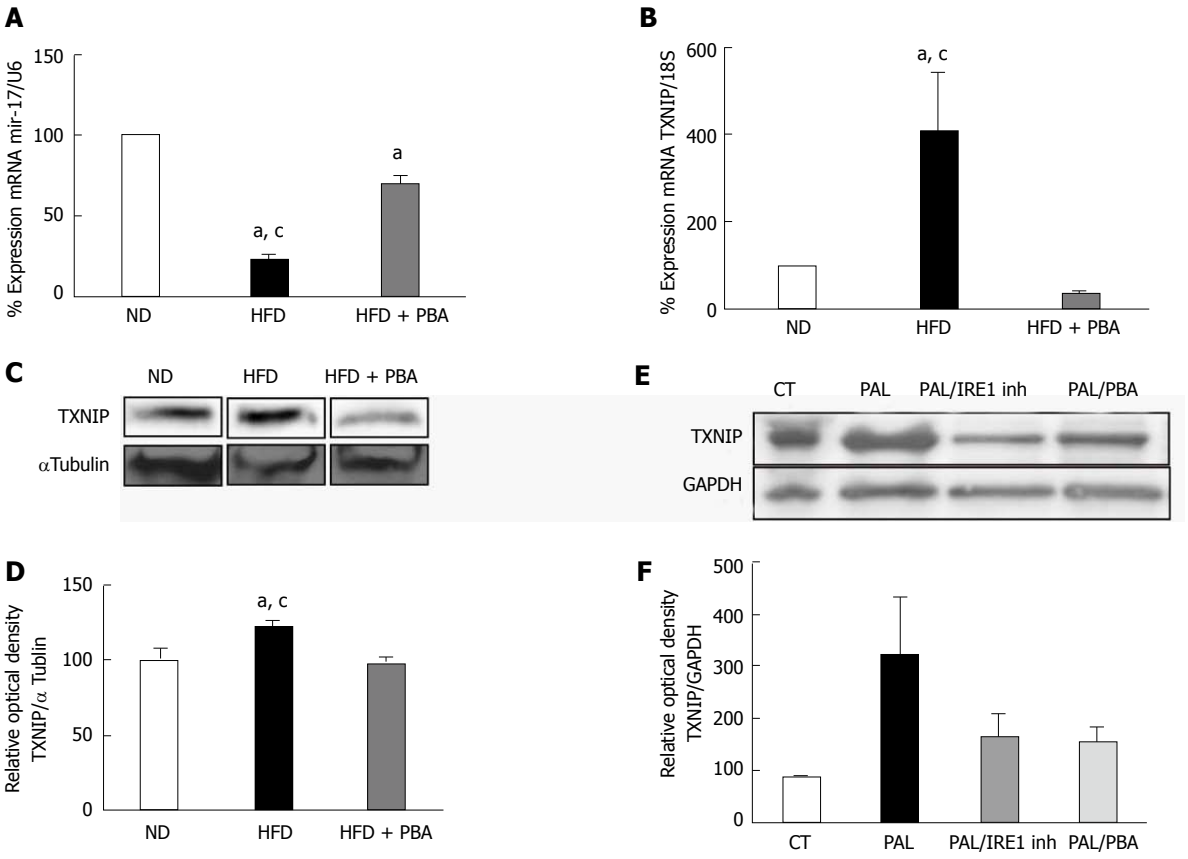

Figure 4 Dysregulation of realtime polymerase chain reaction. It shows significant (A) reduction in miR-17-5p and (B) increase in TXNIP mRNA levels in mice kept on HFD for 4 wk compared to ND, which were reversed with PBA treatment (n = 3-4); C: Representative western blot were cut from the same membrane for TXNIP and αtubulin from retina (D) Statistical analysis showed an upregulation in TXNIP expression in HFD mice compared to ND, PBA treatment nullified this effect (n = 4-5) ($^{a}P < 0.05$ *vs* ND, $^{\circ}P < 0.05$ *vs* HFD + PBA) (E) Representative western blot of TXNIP and GAPDH from rMC1 treated with palmitate (pal), after the addition of IRE inhibitor or PBA; D: Statistical analysis showed a trend increase in TXNIP expression, which is reversed by IRE inhibitor or PBA ($P = 0.076$, n = 3). HFD: High fat diet; ND: Normal diet; PBA: Phenyl-butyric acid; TXNIP: Thioredoxin-interacting protein; CT: Control; PAL: Palmitate; PAL/IRE1: Palmitate + inositol requiring enzyme 1; PAL/PBA: Palmitate + phenyl-butyric acid.

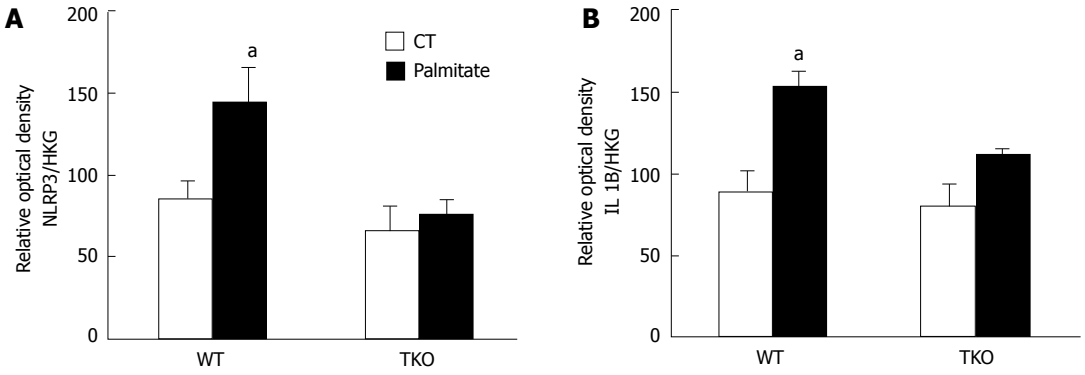

Figure 5 Statistical analysis showed an increase in. (A) NLRP3 and (B) IL1β expression following palmitate treatment in primary Müller cells isolated from WT but no effect on TKO. Actin and α-tubulin were used as housekeeping genes (HKG), to which NLRP3 and IL1β expression was normalized ($^{a}P < 0.05$ *vs* ND, n = 3).

of TXNIP in Müller cells, which was associated with increased TXNIP-NLRP3 inflammasome interaction as well as the expression of cleaved caspase-1 and IL-1β[4]. Therefore, to dissect the role of TXNIP in palmitate-mediated inflammation in Müller cells, primary Müller cells from both WT and TKO mice were used. Primary Müller cells were serum starved overnight then treated with 400 μmol/L palmitate coupled to bovine serum albumin (Pal-BSA) for 6 h. We found that palmitate led to an increase in NLRP3 and IL1β protein expression in cells isolated from WT but has no effect on cells isolated from TKO mice (Figure 5).

DISCUSSION

Central obesity and insulin resistance are hallmarks

of metabolic syndrome that comprises dyslipidemia, hypertriglyceridemia, hyperinsulinemia, hypertension, and reduced HDL cholesterol. Changes in lipid profile and accumulation of free fatty acids are highly significant in all forms of diabetes pointing to its possible link with inflammation and vascular complications (reviewed in[26]). Several studies showed the role of free fatty acids mainly palmitate in inducing pro-inflammatory response[27,28]. It should be noted that thorough understanding of the interaction between vascular and non-vascular cells is crucial for the management of retinal dysfunction. Müller cells are the principal glial cell found in the retina, which span the entire retinal layers and considered as resident innate immune cells (reviewed in[29]). Because of their unique morphology, Müller cells are considered a signaling hub that senses minute changes in retinal milieu, connecting retinal neuronal with retinal endothelial cells. In the current study we were interested in unraveling the mechanisms through which HFD leads to retinal inflammation. We also highlighted the critical role of Müller cells after the insult with the free fatty acid palmitate, which hasn't been reported so far. The main findings of this study are that (1) HFD or palmitate induced ER-stress dysregulates miR-17-5p in retina and Müller cells; (2) ER-stress triggers TXNIP expression in retina and Müller cells and (3) amplified TXNIP levels activate NLRP3, which contributes to inflammation.

Müller cells are considered major sources of inflammatory mediators, which become activated in response to various insults[19,30-32]. We and others have shown the increase of TXNIP expression in glial Müller cells due to chronic hyperglycemia[33-35] or HFD[4]. TXNIP is a physiological inhibitor of the thioredoxin system, which is one of the main antioxidant defense mechanisms in our body. TXNIP acts *via* binding to thioredoxin, making it unable to bind with other proteins (reviewed in[36]). In addition to the ability of TXNIP in inducing inflammatory cytokines *via* activating nuclear factor κB, it can act as a direct activator of NOD-like receptor protein (NLRP3)[34,37]. NLRP3-inflammasome is a component of the innate immune system responsible for initiating obesity-induced inflammation[38]. TXNIP-NLRP3 interaction results in NLRP3 complex assembly and auto-activation of caspase-1, which eventually processes pro-IL1β into its mature form leading to inflammation[38,39]. Recent studies showed that HFD and palmitate trigger ER-stress in various organs and cell types[17,22-24]. However, the link between HFD/palmitate-induced ER-stress and TXNIP expression in Müller cells is yet to be determined. Here, we observed significant activation of the unfolded protein response ER-stress chaperons in retinas from 8-wk HFD mice (Figure 1). We also observed no difference in mRNA level of IRE1α an ER-stress marker and a bifunctional kinase/Rnase in HFD. However, there was an increase in the splicing of XBP1; IRE1α downstream target; evident by 3.5-fold increase in spliced XBP-1 in HFD compared to ND, which suggests IRE1α activation. Interestingly, treatment of Müller cells with palmitate; one of the most abundant saturated fatty acids in plasma that is significantly increased following HFD[25]; led to an increase in all ER-stress markers at the mRNA level including IRE1α (Figure 1). Among UPR pathways, IRE1α has been shown to degrade key cell regulators such as the neuronal cue, netrin in the retina[39,40] and miR-17-5p in pancreatic beta-cells[10,11]. MiR-17-5p is a small noncoding RNAs that binds predominantly to the 3′-UTR of TXNIP leading to down-regulation of its expression[10]. Indeed, HFD and palmitate resulted in a significant decrease in miR-17-5p in the total retina and Müller cells, respectively, an effect that coincided with TXNIP upregulation (Figure 2). These findings support the link between HFD, ER-stress and TXNIP upregulation in Müller cells.

Epidemiological studies showed a significant reduction in miR-17-5p in omental fat and blood from obese non-diabetic subjects compared to lean subjects[41,42]. In the current study, we showed that HFD or palmitate dysregulated miR-17-5p in retina and Müller cells (Figure 2). Interestingly, retinal miR-17-5p expression is not affected by hyperglycemia or diabetes compared to normal glycemic controls (data not shown). In agreement, Lerner *et al*[10] reported similar insensitivity of miR-17-5p to high glucose treatment in pancreatic beta cells. These findings shed light on the selective sensitivity of miR-17-5p to degrade in response to HFD and palmitate. Taken together, our findings suggest that HFD-induced ER-stress uniquely triggers TXNIP expression *via* dysregulating miR-17-5p.

To dissect the role of ER-stress in regulating TXNIP expression, PBA was added to cultured rMC1 prior to palmitate treatment. PBA is an FDA approved drug for the clinical management of urea cycle disorder. PBA is a chemical chaperone that stabilizes protein conformation and in turns ER-folding (reviewed in[43,44]). Indeed, treating the cells with PBA a general ER-stress inhibitor showed a trend decrease in TXNIP expression. Similar findings were obtained by the use of a selective IRE1α inhibitor (Figure 4). However, the observed reduction didn't reach significance, which could be due to the small sample size. We overcame this limitation, by treating mice kept on HFD with PBA for 2 wk. We showed that inhibiting ER-stress significantly blunted the increase in TXNIP observed in HFD group (Figure 4), without altering insulin resistance (Figure 3). Next step we tried to verify the role of TXNIP in inflammatory response in Müller cells. Building on our previous findings that silencing TXNIP reversed palmitate-induced IL1β release and eventually cell death in endothelial cells[4], we isolated primary Müller cells from WT and TKO mice then exposed them to palmitate. We demonstrated that palmitate led to an increase in NLRP3 and IL1β expression in WT and has no effect on TKO (Figure 5), which indicates that TXNIP is responsible for inflammation in Müller cells. These results lend further support to prior findings that manifest the critical role of IL1β in mediating vascular injury in the pathogenesis of diabetic retinopathy. Kowluru *et al*[45] showed that injecting IL-1β

into the vitreous of normal rats increased cell apoptosis similar to what is observed in diabetes. Deletion of IL1β receptor prevented autocrine loop of inflammation[46] and protected retinas from diabetes-induced development of acellular capillaries[47].

In summary, clinical and experimental studies have repeatedly reported the contribution of inflammation to the pathogenesis of diabetic retinopathy (reviewed in[48,49]). Similarly, suppression of inflammation has shown protective effects *via* decreasing leukostasis, blood-retinal barrier breakdown and the acellular capillaries formation[50]. Here, we provide preliminary evidence that exposure to high fat diet and palmitate trigger retinal ER-stress and glial TXNIP expression and render the retina vulnerable to inflammation. Early intervention of ER-stress or TXNIP presents potential therapeutic strategy in obesity-induced inflammation in diabetic retinopathy.

COMMENTS

Background

The authors have previously shown that high fat diet (HFD) induced retinal inflammation and vascular dysfunction. These results were associated with an increase in the thioredoxin interacting protein (TXNIP) at the mRNA and protein level. Here, they examined the mechanisms by which HFD triggers retinal TXNIP and regulates inflammation.

Research frontiers

Currently, there is a great interest to understand how microRNA, the endogenous regulators of transcription can contribute to metabolic disorders. Here, they examined the impact of HFD or the free fatty acid palmitate on microRNA; miR-17-5p as it has been shown to regulate TXNIP mRNA expression. This study demonstrates the effect of HFD-induced obesity on degradation of miR-17-5p *via* activation of the ER-stress mediators including the inositol requiring enzyme 1α (IRE1α). The authors also demonstrate that inhibiting ER-stress can restore miR-17-5p and TXNIP levels and hence inflammation back to comparable levels seen in normal controls.

Innovations and breakthroughs

The results of their study delineate the contribution of Müller cells, main glia in the retina in palmitate-mediated retinal inflammation. They identify ER-stress as new therapeutic target that is involved in obesity-induced inflammation in pre-diabetic retinopathy.

Applications

Their results suggest that inhibitors of ER-stress reversed the increase in TXNIP *in vivo* and in Müller cells, the main glia in the retina. The findings of their short-term study support the interventional use of the ER-Stress inhibitor PBA, FDA approved drug with high safety profile. This report should open the door for its future studies in diseases associated with TXNIP-NLRP3 inflammation.

Terminology

MicroRNAs are small non-coding RNAs that contribute to the post-transcriptional regulation of various genes expressions. Inflammasome is a multiprotein oligomer responsible for the induction of inflammatory process. Unfolded protein response (UPR) is unfolded protein response, an adaptive mechanism to resolve and slow down protein processing. ER-stress is when the endoplasmic reticulum capacity to deal with UPR is overwhelmed then stress markers such as ATF6, PERK and IRE1α are expressed.

Peer-review

The paper is interesting.

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ORIGINAL ARTICLE

Case Control Study

Association of *NFKB1* gene polymorphism (rs28362491) with levels of inflammatory biomarkers and susceptibility to diabetic nephropathy in Asian Indians

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Author contributions: Gautam A and Gupta S contributed equally to this article; Gautam A, Gupta S, Sharma M and Singh K performed sample collection, majority of experiments, analyzed data and wrote manuscript; Mehndiratta M, Kalra OP, Agarwal S and Gambhir JK contributed to conceptual and study design, data analysis and interpretation, as well as manuscript writing.

Institutional review board statement: The protocol of the present study was reviewed and approved by Institutional Ethics Committee for Human Research, Delhi University, and UCMS and GTB Hospital, Delhi.

Informed consent statement: Informed consent was obtained from the all recruited subjects. They were briefed on the purpose of the study and its implication prior to donating peripheral blood.

Conflict-of-interest statement: No conflict of interest exists for this study.

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Abstract

AIM

To investigate the association of *NFKB1* gene -94 ATTG insertion/deletion (rs28362491) polymorphism with inflammatory markers and risk of diabetic nephropathy in Asian Indians.

METHODS

A total of 300 subjects were recruited (100 each), normoglycemic, (NG); type 2 diabetes mellitus (T2DM) without any complications (DM) and T2DM with diabetic nephropathy [DM-chronic renal disease (CRD)]. Analysis was carried out by polymerase chain reaction-restriction fragment length polymorphism and ELISA. Pearson's correlation, analysis of variance and logistic regression were

used for statistical analysis.

RESULTS

The allelic frequencies of -94 ATTG insertion/deletion were 0.655/0.345 (NG), 0.62/0.38 (DM) and 0.775/0.225 (DM-CRD). The -94 ATTG ins allele was associated with significantly increased levels of urinary monocyte chemoattractant protein-1 (uMCP-1); uMCP-1 (P = 0.026) and plasma tumor necrosis factor-alpha (TNF-α); TNF-α (P = 0.030) and almost doubled the risk of diabetic nephropathy (OR = 1.91, 95%CI: 1.080-3.386, P = 0.025).

CONCLUSION

-94 ATTG ins/ins polymorphism might be associated with increased risk of developing nephropathy in Asian Indian subjects with diabetes mellitus.

Key words: Diabetic nephropathy; Inflammation; *NFKB1* -94 ATTG ins/del polymorphism; Urinary monocyte chemoattractant protein-1; Tumor necrosis factor-alpha

Core tip: Type 2 diabetes mellitus (T2DM) is considered as long standing inflammatory disease. Diabetic nephropathy (DN) is the most common micro-vascular complication of T2DM. Pro-inflammatory cytokines like Monocyte chemoattractant protein-1 (MCP-1) and tumor necrosis factor-alpha (TNF-α) plays a crucial role in the pathogenesis of DN. Therefore we investigated -94 ins/del ATTG polymorphism in *NFKB1* gene and its association with the risk of DN in Asian Indians. -94 ins/del ATTG single nucleotide polymorphism was found to increase the urinary MCP-1 and plasma TNF-α levels. Our findings open a new area of research to explore that -94 ins/del ATTG may be considered as genetic markers for early detection of diabetic patients who are at greater risk of development of nephropathy.

Gautam A, Gupta S, Mehndiratta M, Sharma M, Singh K, Kalra OP, Agarwal S, Gambhir JK. Association of *NFKB1* gene polymorphism (rs28362491) with levels of inflammatory biomarkers and susceptibility to diabetic nephropathy in Asian Indians. *World J Diabetes* 2017; 8(2): 66-73 Available from: URL: http://www.wjgnet.com/1948-9358/full/v8/i2/66.htm DOI: http://dx.doi.org/10.4239/wjd.v8.i2.66

INTRODUCTION

Chronic renal disease (CRD) is an intricate pathological process, often leading to end stage renal disease. The causes of CRD are quite multi-factorial ranging from infections to heredity, but type 2 diabetes mellitus (T2DM) is the major culprit amongst them[1]. In spite of the improvement in our knowledge about the etiopathogenesis of diabetic nephropathy (DN), the intricate mechanisms leading to the development of renal injury from chronic hyperglycemia are not yet fully understood. DN has been considered a micro-vascular complication of hyperglycemia, but various clinical and experimental studies have observed that there is a close link between hyperglycemia, inflammation and oxidative stress (OS)[2]. OS may also be involved in promoting a low grade systemic inflammation in patients with T2DM and vice versa[3]. Nuclear factor-kappa B (NF-κB) activation through hyperglycemia induced OS may lead to increased concentration of inflammatory cytokines[4].

NF-κB was identified as a transcription factor which controls the expression of numerous genes affecting immune response, inflammation, cell-growth control, apoptosis and therefore, is an emerging candidate for studies on the pathogenesis of inflammatory diseases including DN. There are five members of the NF-κB family in mammals: NF-κB1: p105/p50, NF-κB2: p52/p100, RelA: p65, RelB, and c-Rel. The chief form of NF-κB is a hetero-dimer of the p50 and p65/RelA subunits, encoded by the *NFKB1* and *RelA* gene. Normally, inactive NF-κB is found in the cytoplasm bound to IkBs, which are specific inhibitor proteins in cytoplasm. Cell when exposed to a variety of proinflammatory stimuli leads to the quick phosphorylation followed by ubiquitinylation, and finally proteolytic breakdown of I-κB. This causes transfer of NF-κB in nucleus and thus leading to increased transcription of gene[5]. NF-κB transcriptionally regulates many downstream proinflammatory genes, mainly including monocyte chemoattractant protein-1 (*MCP-1*), tumor necrosis factor-alpha (*TNF-α*)[6].

MCP-1 is an important proinflammatory chemokine which affects the recruitment and function of monocyte[7]. MCP-1 is synthesized in response to a various proinflammatory stimuli by kidney cell[8]. A study done by Wada *et al*[9] in 2000 has shown that expression of MCP-1 increases in inflammation induced kidney diseases including DN. Urinary MCP-1 (uMCP-1) is a potential biomarker for renal damage[10]. Hyperglycemia induced secretion of abundant MCP-1 from renal parenchymal cells, attract monocytes into the kidney stimulating myofibroblast-like properties in mesangial cells. Kidney macrophages when exposed to MCP-1 in diabetic milieu promotes activation of macrophage. Thus, leading to release of reactive oxygen species (ROS), various pro-inflammatory cytokines and profibrotic growth factors[11,12]. Thus, resulting in exaggerated inflammation that leads to renal injury through proliferation of myofibroblast, augmented production of extracellular matrix by mesangial cells and fibroblasts.

TNF-α is a well known proinflammatory cytokine associated with systemic inflammation[13,14]. It is produced predominantly by macrophages and monocytes[13,14]. TNF-α acts *via* NF-κB signaling and mediates the transcription of various cytokines performing roles in cell survival, proliferation, inflammatory responses, cell adhesion and inflammation[15]. A study has shown that

there is upregulation of TNF-α expression in glomeruli of diabetic rats[16]. TNF-α is well acknowledged to cause damage to renal cells by enhancing renal hypertrophy, hemodynamic imbalance, albumin permeability[17]. The harmful effects of these responses lead to the development of renal disease in patients with T2DM, hence resulting in the progression of renal failure.

In addition to poor glycemic control, OS and inflammation; genetic factors seem to be main determinants of DN in terms of both occurrence and severity[18]; however the genetic mechanism causing DN is still unexplored. In our knowledge, there is no study available regarding the polymorphisms of *NFKB1* and their correlation with levels of uMCP-1 and plasma TNF-α. We have reported[19] increased uMCP-1, plasma TNF-α levels in subjects with DN when compared to subjects with T2DM without nephropathy and observed a positive correlation between uMCP-1 and plasma TNF-α[20]. We have also highlighted that DN is associated with *TNFA* gene single nucleotide polymorphism (SNP)[20]. In recent times, a new functional *NFKB1* promoter SNP consisting of a insertion/deletion (-94ins/del ATTG) (rs28362491) has been identified which can elicit a regulatory effect on the *NFKB1* gene[21]. Since above mentioned polymorphism has been associated with various inflammatory diseases, autoimmune diseases and cancers[22], therefore, it is worthwhile to further investigate the association of -94 ins/del ATTG *NFKB1* gene SNP with levels of uMCP-1, plasma TNF-α and nephropathy risk in subjects with T2DM.

MATERIALS AND METHODS

Study design

The present study comprises of total 300 subjects visiting Nephrology Outpatient Clinic and Medicine OPD at University College of Medical Sciences and Guru Teg Bahadur Hospital, Delhi. Subjects were divided into three groups of 100 each namely; Group 1: Normoglycemic (NG), Group 2: Subjects with T2DM for ⩾ 10 years without nephropathy (DM), Group 3: Subjects with T2DM for ⩾ 5 years with nephropathy (DM-CRD). T2DM was diagnosed according to revised ADA criteria[23]. Detailed clinical history and physical examination were recorded. Blood pressure (BP) of subjects was estimated using sphygmomanometer in the sitting position after a resting period of 10 min. The estimated glomerular filtration rate (eGFR) was measured by Modification of Diet in Renal Disease Abbreviated Equation (MDRD)[24].

The presence of micro-albuminuria in T2DM subjects was detected by Urine Test 11 MAU dipstick (Piramal Diagnostic, sensitivity: 10-15 mg/dL), and all participants having proteinuria and micro-albuminuria were clubbed in Group 3. All participants with nephropathy were in pre-dialysis stage. Normoglycemic (Group 1) subjects were recruited from employees of UCMS and GTB Hospital with the following criteria: (1) they did not have of diabetes mellitus (fasting plasma glucose < 100 mg% or postprandial glucose < 140 mg% or HbA1c < 5.7%) according to ADA criteria; (2) there was no presence of diabetes in their first or second degree relatives; and (3) they had normal BP, with systolic and diastolic BP not > 120 mmHg and 80 mmHg[25].

To circumvent any possible confounding factors, patients having renal disorders (hypertensive nephropathy, chronic glomerular nephritis, chronic interstitial disease, ischemic nephropathy, obstructive nephropathy), acute and chronic infections, congestive heart failure, malignancy and liver disorder were not included into the study. All subjects in Group 3 had retinopathy; but participants with macro-vascular complications like coronary artery disease and stroke were not included into the study. Patients taking renin-angiotension aldosterone system inhibitors, aspirin and vitamin D analogues were advised to discontinue these drugs for a period of a week before inclusion in the study since they have been found to influence the synthesis of uMCP-1 and TNF-α. However, patients were prescribed beta-blockers to control BP in that duration of one week. The Institutional Ethics Committee for Human Research approved the protocol of this study (approval number-UCMS/IEC-HR/2010/10). Prior to the inclusion into the present study, informed written consent was taken from all participants.

Biochemical parameters

Under aseptic conditions fasting venous blood samples were withdrawn and collected into EDTA and fluoride vials. For glycosylated hemoglobin (HbA1c) 200 μL whole blood was preserved at 4 ℃-8 ℃ and processed within one week of collection. Blood samples collected in EDTA vial was subjected to centrifugation at 3000 rpm for 10 min in order to separate the plasma. Early morning first mid-stream urine sample was collected and stored in aliquots at -20 ℃ for estimation of MCP-1, albumin and creatinine.

Routine investigations such as fasting and post-prandial plasma glucose, urea, creatinine and uric acid were carried out using commercially available kits on autoanalyser (Olympus AU-400). HbA1c was estimated by ion-exchange resin chromatography using commercially available kits (Fortress, United Kingdom). Urinary protein excretion was expressed as albumin to creatinine ratio.

Markers of inflammation

uMCP-1 (Weldon, California; sensitivity less than 7.8 pg/mL) and plasma TNF-α (Diaclone, France; sensitivity less than 8 pg/mL) were estimated by commercially available ELISA kit.

DNA extraction and polymorphism genotyping

Cellular DNA of every individual was extracted from 200 μL EDTA-anticoagulated peripheral blood sample by means of DNA isolation kit (Zymo research, United States). The polymerase chain reaction was carried out in Thermocycler (Eppendorf Mastercycler Gradient-5331). In brief, 0.1 μg of DNA was amplified in a reaction mixture

Table 1 The baseline demographic and biochemical parameters in various study groups

Variables	NG (n = 100)	DM (n = 100)	DM-CRD (n = 100)
Age (yr)	46.0 ± 4.0	56.40 ± 3.5	55.7 ± 4.2
Sex ratio (male/female)	52/48	54/46	52/48
Duration of DM (yr)	-	12.7 ± 1.5	8.1 ± 2.3[d]
BMI (kg/m^2)	20.1 ± 1.7	21.1 ± 2.1	21.6 ± 3.4
SBP (mmHg)	118.1 ± 0.5	138.0 ± 2.1[b]	137.7 ± 2.8[b]
DBP (mmHg)	75.2 ± 1.0	81.6 ± 1.9[b]	82.8 ± 0.0[b]
Fasting glucose (mg/dL)	82.4 ± 3.1	153.5 ± 3.5[b]	184.3 ± 9.2[b,d]
Postprandial glucose (mg/dL)	118.2 ± 2.4	201.7 ± 10.1[b]	261.1 ± 12.2[b,d]
HbA1c (%)	5.11 ± 0.46	7.10 ± 0.25[b]	9.16 ± 0.16[b,d]
Urea (mg/dL)	31.5 ± 5.5	30.7 ± 5.8	93.2 ± 4.8[b,d]
Creatinine (mg/dL)	0.83 ± 0.23	0.90 ± 0.20	3.7 ± 1.5[b,d]
Uric acid (mg/dL)	4.2 ± 0.8	4.9 ± 0.6	9.1 ± 0.8[b,d]
eGFR (mL/min per 1.73 m^2)	99.1 ± 0.7	96.4 ± 0.6	51.2 ± 0.9[d]
Urinary albumin/creatinine	-	-	0.42 ± 0.35

[b]Significantly different from Normoglycemic at P < 0.001; [d]Significantly different from diabetic patients without nephropathy at P < 0.001. Data are expressed as mean + SD. NG: Normoglycemic; DM: Diabetes mellitus without nephropathy; DM-CRD: Diabetic nephropathy; BMI: Body mass index; SBP and DBP: Systolic and diastolic blood pressure; eGFR: Estimated glomerular filtration rate.

of 20 μL containing 0.5 μmol/L each of the following primer pairs (Forward 5'-TGGGCACAAGTCGTTTATGA-3' and Reverse 5'CTGGAGCCGGTAGGGAAG-3'). The reaction mixture also contained 0.5 mmol/L (dNTP mix), 2 μL (10 × PCR buffer) and 2.0 units Taq DNA polymerase, 2 mmol/L $MgCl_2$. The PCR protocol consist an initial temperature of 94 ℃ (5 min) followed by 35 cycles of amplification (30 s at 94 ℃, 45 s at 59 ℃, and extension for 1 min at 72 ℃). Final extension step was carried out for 2-min at 72 ℃[22].

For the study of the -94 insertion/deletion ATTG SNP in *NFKB1*, PCR product (281/285 bp) was subjected to fast digestion with restriction enzyme *PfIMI*. PCR products was treated with enzyme *PfIMI* in at 37 ℃ for 1 h and inactivated at 65 ℃ for 20 min. The insertion allele (ins) was cut down into two fragments of 45 bp and 240 bp by *PfIMI* restriction enzyme. But, there was no cleavage at the deletion allele (del) that has only one ATTG at its promoter[22]. The bands of digested products were visualized in 2% agarose gel electrophoresis stained with ethidium bromide.

Statistical analysis

Demographic profiles and routine investigation was compared by χ^2 and Student's t test and one-way ANOVA was used. To associate all the study groups with genotype two-way ANOVA followed by *post-hoc* Tukey's test was used. For association of genotypes with uMCP-1 and plasma TNF-α levels, analysis of variance was used. Logistic regressions was used to evaluate the risk of development of DN at the single SNP level. Power of sample size keeping 5% significance level and 80% power was calculated by genetic power calculator. A P value < 0.05 was considered statistically significant (two-tailed). All statistical tests were performed using SPSS version 20.

Table 2 The genotype and allele frequencies of *NFKB1* gene for -94 insertion/deletion ATTG polymorphism in different study groups

	NG (n = 100) n (%)	DM (n = 100) n (%)	DM-CRD (n = 100) n (%)
ins/ins	41 (41)	38 (38)	61 (61)
ins/del	49 (49)	48 (48)	33 (33)
del/del	10 (10)	14 (14)	06[b] (06)
ins allele	131 (65.5)	124 (62)	155 (77.5)
del allele	69 (34.5)	76 (38)	45 (22.5)

[b]Significantly different from diabetic patients without nephropathy at P < 0.001. NG: Normoglycemic; DM: Diabetes mellitus without nephropathy; DM-CRD: Diabetic nephropathy.

RESULTS

Characteristics of the study population

Biochemical and demographic parameters of the various study groups are shown in Table 1. There was no difference in sex distribution and BMI within all the three study groups. The subjects of Group 2 (DM) and Group 3 (DM-CRD) were older than Group 1 (NG) subjects; however the period of diabetes was more in Group 2 (DM) than Group 3 (DM-CRD) which was as per our selection criteria. Incidence of hypertension was significantly higher in Group 2 (DM) and Group 3 (DM-CRD) participants as suggested by raised SBP and DBP (P < 0.001) when compared to NG. Poor glucose control was observed in DM-CRD as compared to DM as suggested by significantly higher (P < 0.001) fasting, postprandial plasma glucose and HbA1c. Renal function tests suggested that blood urea, plasma creatinine, and uric acid were significantly higher (P < 0.001) and eGFR was decreased (P < 0.001) in Group 3 (DM-CRD) as compared to Group 2 (DM).

Distribution of ins/del in study population

The allele frequencies and genotype of the *NFKB1* gene for -94 insertion/deletion ATTG SNP in various study groups are shown in Table 2. The distribution percentage of ins/ins, ins/del, del/del genotypes in Group 1 (NG), Group 2 (DM) and Group 3 (DM-CRD) (expressed in percentage) were 41%, 49% and 10%; 38%, 48% and 14%; and 61%, 33% and 6% respectively. The frequency of del/del genotype was significantly lower (P < 0.001) in Group 3 (DM-CRD) as compared to Group 2 (DM). However, allele frequencies of -94 insertion/deletion ATTG were 65.5%/34.5% in Group 1 (NG), 62%/38% in Group 2 (DM) and 77.5%/22.5% in Group 3 (DM-CRD).

Relationship between the -94 ins/del AGGT SNP with inflammatory markers and disease risk

Correlation of -94 ins/del AGGT SNP with levels of

Table 3 Interaction analysis of -94 ins/del ATTG polymorphism with inflammatory markers

Inflammatory marker	Groups	NG (*n* = 100)	DM (*n* = 100)	DM-CRD (*n* = 100)	*P* value
uMCP-1 (pg/mg creatinine)	Total	130.00 ± 42.22	271.00 ± 120.01	5632.70 ± 1007.20[a,b]	
	del/del	85.1 ± 9.2	200.6 ± 66.5	4609.9 ± 900.6	*P* =
	ins/del	110.9 ± 15.6	278.9 ± 105.9	5879.9 ± 1016.3	0.026
	ins/ins	166.8 ± 26.8	302.2 ± 100.1	6405.1 ± 1550.6	
Plasma TNF-α (pg/mL)	Total	15.55 ± 2.22	16.51 ± 3.75	21.38 ± 3.67[a,b]	
	del/del	8.27 ± 1.06	10.21 ± 1.32	17.31 ± 1.17	*P* =
	ins/del	11.55 ± 0.05	14.05 ± 0.18	19.31 ± 0.44	0.030
	ins/ins	15.08 ± 1.15	16.36 ± 1.20	23.12 ± 0.70	

[a]Significantly different from Normoglycemic at $P < 0.001$; [b]Significantly different from diabetic patients without nephropathy at $P < 0.001$. uMCP-1 levels, plasma TNF-α levels are expressed as mean + SD. NG: Normoglycemic; DM: Diabetes mellitus without nephropathy; DM-CRD: Diabetic nephropathy.

uMCP-1 and plasma TNF-α have been studied and the results are shown in Table 3. The -94 ins allele were associated with increased levels of uMCP-1 ($P = 0.026$) and plasma TNF-α ($P = 0.030$) in the disease study groups, *i.e.*, Group 2 (DM), Group 3 (DM-CRD).

The associations at the level of genotype is shown in Table 4. Highly significant association was observed for -94 ins/del AGGT polymorphism in subjects with Group 3 (DM-CRD) in comparison to Group 1 (NG); $P = 0.022$. In our present study, -94 ins SNP was found to increase risk for the development of DN by 1.91-fold in subjects with diabetes (OR = 1.91, 95%CI: 1.080-3.386, $P = 0.025$).

Table 4 Association between -94 ins/del ATTG polymorphism in the *NFKB1* gene and diabetic nephropathy at the genotype level

Genotype	OR	95%CI	*P* value
DM *vs* NG ref	1.04	0.607-4.987	0.887
DM-CRD *vs* NG ref	1.95	1.101-3.467	0.022
DM-CRD *vs* DM ref	1.91	1.080-3.386	0.025

Ref: Referencegroup.

DISCUSSION

Polymorphism in the NFKB1 promoter region at position -94 ins/del AGGT has been correlated with many long standing inflammatory diseases like autoimmune diseases such as rheumatoid arthritis, asthma, AIDS, cancers and various diabetic complications[26,27]. Our study is the first to report the association of above mentioned polymorphism with DN in North Indian population. In the current study, we observed that the frequency distribution of ins/del is maximum in NG and DM subjects followed by ins/ins, with least distribution of del/del in the same. However the trend was different in DM-CRD subjects with respect to ins/del genotype which was less as compared to ins/ins this group. The frequency of different genotypes observed in the present study were in accordance with studies on NFKB1 polymorphism in healthy volunteer in different ethnic population like Turkish[22], Caucasians[28], English[29], Polish[30]. But our results were not in agreement with healthy Chinese population[28]. When our findings were compared with studies on inflammatory diseases like cancer, they are in accordance with a studies conducted in Asian by Huo *et al*[31] and Zhou *et al*[32]. However our results were in contrast with a genomic study on cancer conducted by Yang *et al*[28] in 2014. The dissimilarity of results could be due to diverse geographical distribution and ethnicity between our study and theirs was different, which could result in diverse genetic background.

Latest evidence has shown that the production of MCP-1 by kidney affected by diabetes along with TNF-α is a major cause of inflammation, renal injury and fibrosis in DN[10,17]. The present study is the foremost one to document the correlation of -94 ins/del AGGT SNP with levels of inflammatory markers namely uMCP-1 and plasma TNF-α in DN from North Indian patients. In our previous study, we have observed that plasma TNF-α and uMCP-1levels were significantly raised in patients with T2DM and so more in patients with DN[19]. To explicate the role of NFKB1 gene SNP in the development of DN, -94 ins/del AGGT SNP were analyzed in various study, *i.e.*, Group 2 (DM) and Group 3 (DM-CRD) and further correlated with measured inflammatory markers like uMCP-1 and plasma TNF-α levels. Interestingly, this study has also shown that ins allele was significantly associated with increased urinary MCP-1 and plasma TNF-α levels in NG as well as patient groups. However, there is no report in literature to compare our results.

A recent study has shown that TNF-α stimulates the MCP-1 production *via* NF-κB signalling pathway in rat astrocyte cultures[33]. TNF-α was found to increase p65 and phosphorylated p65 levels in nuclear extracts of rat astrocytes, hence augmenting MCP-1 levels[33]. This supports our finding that increased levels of TNF-α are associated with increased levels of uMCP-1.

Genetic variations are known to play a vital role in determining risk of DN. A number of studies have investigated the relationship of ins allele of -94 ins/del AGGT polymorphism with various inflammatory diseases. Till date not a single study has tried to evaluate the association between this polymorphism and DN risk. Our study is first to document that patients with T2DM having ins/ins genotype were found to have increased risk of developing nephropathy. Latest studies have reported that p50 null mice have a significantly reduced inflammatory response in various models of inflammation such as asthma[34], arthritis[35], and autoimmune encephalomyelitis[36]. A similar study conducted in sporadic colorectal cancer (CRC)[37] and epithelial ovarian cancer (EOC)[31] has supported

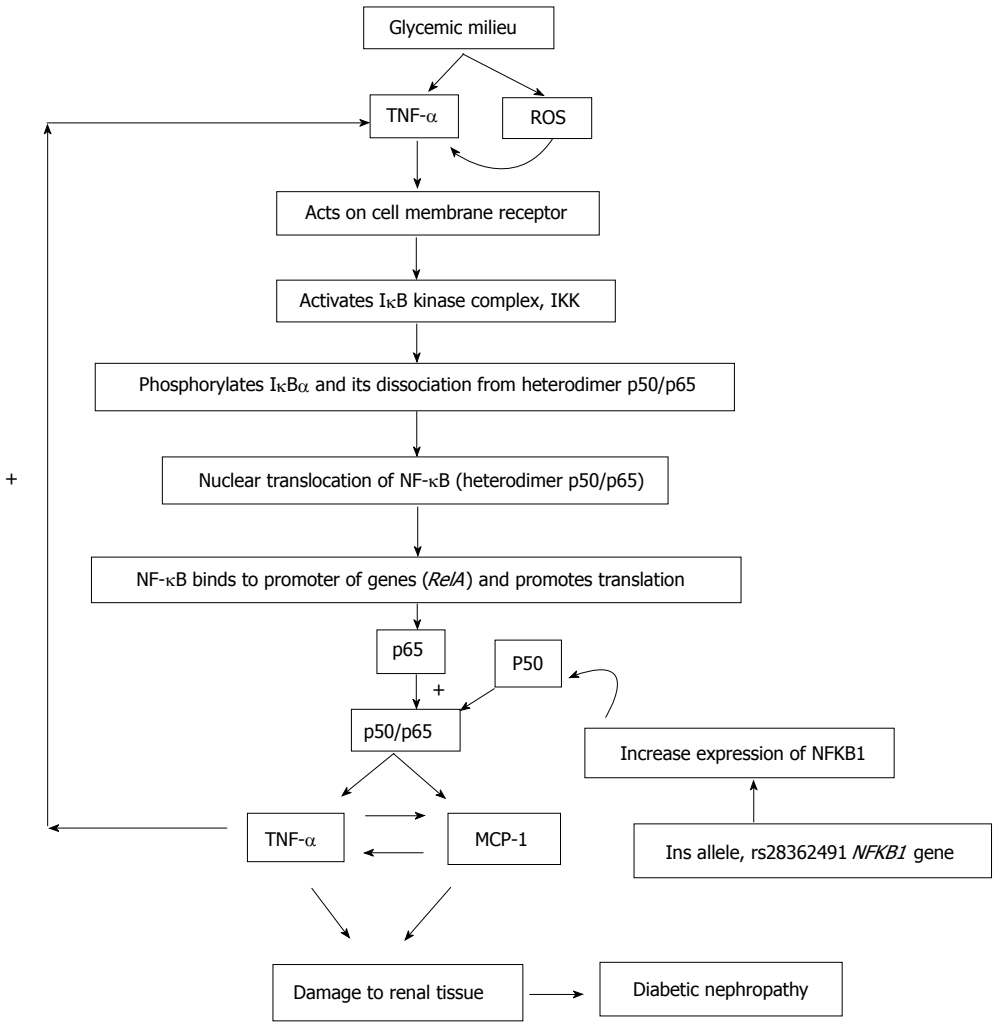

Figure 1 NFKB1 gene and inflammatory markers: Probable mechanisms in the pathogenesis of diabetic nephropathy. Hypoglycemia induced ROS and TNF-α leads to activation of IKK. IKK causes phosphorylation of IκBα bound to p50/p65. Phosphorylated IκBα dissociate from p50/p65 leading to nuclear translocation of unbound heterodimer p50/p65 (NF-κB). Binding of NF-κB to promoter gene causes translation of p65. Ins allele, rs28362491 *NFKB1* gene, if present, causes increase expression of p50. Hence there is increased production of p50/p65 heterodimer complex. This heterodimer acts on its downstream proinflammatory targets viz: MCP-1 and TNF-α leading to its synthesis. MCP-1 is a positive regulator of TNF-α and vice versa. Both MCP-1 and TNF-α causes renal damage leading to development of Diabetic nephropathy. ROS: Reactive oxygen species; TNF-α: Tumor necrosis factor-alpha; IKK: IκB kinase complex; MCP-1: Monocyte chemoattractant protein-1.

our findings which suggested that ins/ins genotype contribute to significantly increased risk of CRC and EOC. The probable mechanism of -94 ins/del AGGT polymorphism leading to increased risk of developing DN is explained in Figure 1. In almost all cell types, NF-κB complexes are typically localized in the cytoplasm where they bind to IKB inhibitory proteins. However, stimulation with hyperglycemia induced ROS and TNF-α leads to rapid phosphorylation of IKB *via* I-κB kinases complex which is then degraded by ubiquitin-proteosome pathway. On the other hand, simultaneously -94 ins/del AGGT polymorphism might lead to increased synthesis of p50 mRNA. Hence there will be increased production of p50/p65 hetrodimer complex which is a well known proinflammatory molecule, since p50/p65 hetrodimer acts on its downstream proinflammatory targets *viz*: MCP-1 and TNF-α, leading to over production of MCP-1 and TNF-α. Thus, there occurs a viscous cycle, *i.e*., MCP-1 is a positive regulator of TNF-α and vice versa. The above mentioned probable hypothesis might lead to increased risk of developing renal damage in T2DM. However results of a recent study from China[38] in bladder cancer is in contradiction to our findings which could be due to ethnic and geographical differences. Furthermore, the sample size of our study was fairly small than aforementioned bladder cancer study.

The results of the current study suggest that the NFKB1 promoter -94 ins/del AGGT SNP is associated with increased possibility of developing nephropathy in patients with diabetes. This SNP may be considered as genetic markers for susceptibility to develop nephropathy in patients with T2DM. The limitation of the study is the small sample size. Therefore, further evaluation is necessary in big sample size to look for the possibility of this polymorphisms as potential genetic markers in the near future. This would help to identify patients with type 2 diabetics who may be at higher risk of developing nephropathy.

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COMMENTS

Background

Type 2 diabetes mellitus (T2DM) is considered as a long standing inflammatory disease. Nuclear factor-kappa B (NF-κB) controls the expression of numerous genes affecting inflammation, immune response. Immunogenic and inflammatory cytokines like monocyte chemoattractant protein-1 (MCP-1) and tumor necrosis factor-alpha (TNF-α) plays a crucial role in the pathogenesis of micro-vascular complication of T2DM, *i.e.*, diabetic nephropathy (DN) and clinical outcome.

Research frontiers

In spite of the present advances in our knowledge about the etiopathogenesis of DN, the intricate mechanisms leading to the development of renal injury from chronic hyperglycemia are not yet fully understood. *NFKB1* promoter polymorphism -94 ins/del ATTG has been associated with inflammatory diseases, autoimmune diseases and cancers. However, its role in the development of T2DM and DN has not been explored till date. The authors hypothesized that the -94 ins/del ATTG polymorphism would affect the levels of urinary MCP-1 and plasma TNF-α and therefore might be culprit in developing DN.

Innovations and breakthroughs

The authors have recently reported that -94 ATTG ins allele was associated with significantly increased levels of urinary MCP-1, plasma TNF-α and was found to increase risk for the development of DN by 1.91-fold in subjects with diabetes.

Applications

-94 ins/del AGGT polymorphisms can be considered as genetic marker for identifying those more susceptible and provide suitable interventions to delay the progression of DN. This study provides a ground for the development of newer anti-inflammatory therapeutic agents that may have potential to affect primary mechanisms contributing to the pathogenesis of DN.

Terminology

DN: Diabetic nephropathy; NF-κB: Nuclear factor-kappa B; *NFKB1*: Nuclear factor-kappa B1 gene; T2DM: Type 2 diabetes mellitus; TNF-α: Tumor necrosis factor-alpha; uMCP-1: Urinary Monocyte chemoattractant protein-1.

Peer-review

The manuscript is well informative.

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ORIGINAL ARTICLE

Case Control Study

Exercise-induced albuminuria *vs* circadian variations in blood pressure in type 1 diabetes

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Informed consent statement: All participants and their parents or guardians (since many were adolescents) provided informed written concern prior to study enrollment.

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Data sharing statement: Data are available from the corresponding author upon request at sobngwieugene@yahoo.fr.

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Abstract

AIM

To investigated the relationship between exercise-induced ambulatory blood pressure measurement (ABPM) abnormalities in type 1 diabetes mellitus (T1DM) adolescents.

METHODS

We conducted a case-control at the National Obesity Center of the Yaoundé Central Hospital, Cameroon. We compared 24 h ABPM and urinary albumin-to-creatinine ratio (ACR) at rest and after a standardized treadmill exercise between 20 Cameroonian T1DM patients and 20 matched controls. T1DM adolescents were aged 12-18 years, with diabetes for at least one year, without proteinuria, with normal office blood pressure (BP) and renal function according to the general reference

population. Non-diabetic controls were adolescents of general population matched for sex, age and BMI.

RESULTS

Mean duration of diabetes was 4.2 ± 2.8 years. The mean 24 h systolic blood pressure (SBP) and diastolic blood pressure (DBP) were respectively 116 ± 9 mmHg in the diabetic group *vs* 111 ± 8 mmHg in the non-diabetic (P = 0.06), and 69 ± 7 mm Hg *vs* 66 ± 5 mm Hg (P = 0.19). There was no difference in the diurnal pattern of BP in diabetes patients and non-diabetic controls (SBP: 118 ± 10 mmHg *vs* 114 ± 10 mmHg, P = 0.11; DBP: 71 ± 7 mmHg *vs* 68 ± 6 mmHg, P = 0.22). Nighttime BP was higher in the diabetic group with respect to SBP (112 ± 11 mmHg *vs* 106 ± 7 mmHg, P = 0.06) and to the mean arterial pressure (MAP) (89 ± 9 mmHg *vs* 81 ± 6 mmHg, P = 0.06). ACR at rest was similar in both groups (5.5 mg/g *vs* 5.5 mg/g, P = 0.74), but significantly higher in diabetes patients after exercise (10.5 mg/g *vs* 5.5 mg/g, P = 0.03). SBP was higher in patients having exercise-induced albuminuria (116 ± 10 mmHg *vs* 108 ± 10 mmHg, P = 0.09).

CONCLUSION

Exercise-induced albuminuria could be useful for early diagnosis of kidney damage in adolescents with T1DM.

Key words: Albuminuria; Blood pressure; Ambulatory blood pressure measurement; Exercise; Type 1 diabetes

Core tip: Diabetic nephropathy (DN) is a major complication of type 1 diabetes mellitus (T1DM). Therefore, strategies for early detection are of critical importance. Ambulatory blood pressure measurement is useful for detection of precocious abnormalities in the occurrence of DN and exercise-induced albuminuria has been proposed as a potential predictor of DN. Our study therefore aimed to investigate the relationship between exercise-induced albuminuria and ambulatory blood pressure measurement abnormalities in T1DM Cameroonian adolescents. We found that T1DM patients had higher nocturnal and 24 h blood pressure figures than non-diabetics suggesting that exercise-induced albuminuria could be useful early detection of diabetes kidney injuries in T1DM.

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INTRODUCTION

Diabetes nephropathy is the major life-threatening complication of type 1 diabetes mellitus (T1DM)[1,2]. Abnormal albumin excretion has been shown to predict the development of clinically significant nephropathy in T1DM. Indeed, persistent minimal elevation of albuminuria at rest predicts the development of more severe proteinuria and clinical diabetic nephropathy, which frequently progresses to renal failure[3]. In T1DM, nephropathy develops in 30% to 40% of cases and impaired renal function or end-stage kidney disease affect up to a third of patients[4]. Thus, strategies for early detection and for preventative interventions are of critical importance since interventions at these late stages of disease may only slow but not completely arrest the inexorable progression towards renal failure[5,6]. In this direction, it has been shown that physical exercise can stimulate albuminuria in diabetes patients and can be a useful provocative test to detect early renal abnormalities[7]. However, there is still limited evidence on its value for early detection of renal disease in T1DM.

Previous studies has proven that during exercise, urinary albumin excretion rate is more increased in long term T1DM patients thus, at risk of developing diabetes nephropathy than in general population[8]. In the contrary, some evidence suggest that the level of albumin excretion during exercise is related to the quality of metabolic control; for example, exercise-induced microalbuminuria is more pronounced in newly diagnosed patients, and this abnormality is reversed by insulin treatment. Exercise-induced microalbuminuria generally is not well correlated with the duration of disease and does not predict clinical nephropathy[9]. On the other hand, the contribution of night-time blood pressure (BP) on the onset of nephropathy in diabetic patients is now established[10]. Therefore ambulatory blood pressure monitoring (ABPM) could be proposed as an useful tool for early detection of diabetic nephropathy[11,12]. This study aimed to investigate the relationship between exercise-induced albuminuria and ABPM abnormalities in early detection of diabetic nephropathy in adolescents with T1DM from Cameroon.

MATERIALS AND METHODS

Study subjects

This case-control study was carried out at the National Obesity Center of the Yaoundé Central Hospital, the reference diabetes center in the town. Our population was made of two groups, T1DM adolescents and non-diabetic controls. T1DM patients were aged 12-18 years, with diabetes for at least one year; without proteinuria, with normal office BP and renal function according to the general reference population. Non-diabetic controls were adolescents of general population matched for sex, age and BMI. We excluded patients and controls with an important night activity, those receiving drugs for hypertension or any other drugs able to modify albuminuria, those with contra-indication to exercise or presenting signs of urinary tract infection as well as those having fever and pregnant women.

Procedure and investigations

The procedure was made of an inclusion visit and two exploration visits. Within 2 wk following an information visit, for all eligible participants, we performed a careful clinical exam including BP measurement and a urinary dipstick. We enrolled 40 participants, 20 in each group.

All exploration visits were conducted in the morning between 8:00 and 10:00. After arrival, participants were invited to stay in sitting position for at least five minutes. Then, clinical measurement of BP was done three time using an automated sphygmomanometer Omron HEM-705 CP (Omron Corporation, Tokyo, Japan) placed on the left arm raised itself at the heart level. The average of three measures was considered for analysis. Weight and height were respectively valued to the nearest 0.5 unit using a mechanical scale and a measuring rod and body mass index (BMI in kg/m²) calculated as *weight* (cm)/[*height* (m) × *height* (m)]. A dipstick was done to assess proteinuria and considered positive for at least 1+.

ABPM was carried out on twenty four hours using an automatic portable, light weight monitor device the i-MAPA® CE 004 1.1 TM (High-tech Medical St Louis, Paris) which performs measurements every 15 min during daytime (07:00 to 22:00) and twice an hour during night time defined from 22:00 to 07:00. Device was activated and the two first measures performed in the laboratory to ensure functionality. Detailed information on the operation and use of the device were then given to the participant who then returned to his daily activities. At least 70% of valid measurements were considered for interpretation.

The exercise protocol was developed according to the Hierarchy of individual calibration levels for heart rate and accelerometry to measure physical activity[13]. It was made of 1 km race on a treadmill at 5.8 km/h and was divided in two phases. The first phase was made of a 3 min gathering speed up to 3.2 km/h, followed by an acceleration of 0.33 km/h every 6 min. The second phase was a walking step between 5.2-5.8 km/h on the treadmill.

Albuminuria was calculated using albumin-to-creatinine ratio in order to avoid effect of exercise on urinary concentration and expressed in mg/g. First void urine collection was used for rest albuminuria and a random sample urine was collected within the 20 min following physical exercise to measure exercise-induced albuminuria. Albuminuria or exercise-induced albuminuria was diagnosed on the basis of a urinary albumin excretion rate greater than 20 but less than 200 mg/g[14]. Adverse events such as hypoglycemia during physical exercise or exercise intolerance, were closely monitored.

Statistical analysis

Data acquisition was done by Epi-data 3.1 software and statistical analysis was performed using Stata 12.0 software. Continuous variables are expressed as means with standard deviation (SD) where appropriate, and categorical variables as count (percentage). The Spearman rank coefficient was used to test correlations. The χ^2 test and Mann-Whitney rank sum test were used to test associations between qualitative variables and difference between two respectively. A P value $\leqslant 0.05$ was considered statistically significant. The statistical methods of this study were reviewed by Mr. Sontsa.

Table 1 Ambulatory blood pressure measurement of the diabetes and non-diabetes patients

Variables	Type 1 diabetic patients (n = 20)	Non-diabetic patients (n = 20)	P value
24 h BP			
SBP	116 ± 9	111 ± 8	0.06
DBP	69 ± 7	66 ± 5	0.19
PP	48 ± 8	45 ± 5	0.11
Diurnal BP			
SBP	118 ± 10	114 ± 10	0.11
MAP	92 ± 7	89 ± 7	0.15
DBP	71 ± 7	68 ± 6	0.22
Nocturnal BP			
SBP	112 ± 11	106 ± 7	0.06
MAP	85 ± 9	81 ± 6	0.06
DBP	64 ± 9	60 ± 6	0.11

SBP: Systolic blood pressure; DBP: Diastolic blood pressure; MAP: Mean arterial blood pressure; PP: Pulse pressure.

RESULTS

General characteristics

We enrolled 40 participants, 24 males, average age of 16 ± 2 years. The mean BMI of diabetes patients was 22.6 ± 2.9 kg/m² *vs* 22.7 ± 3.3 kg/m² for non-diabetic. Average duration of diabetes was 4.2 ± 2.8 years with mean glycated hemoglobin of 9.9 ± 2.8. Nine diabetes patients had a family history of hypertension *vs* six in the non-diabetic group.

ABPM measurement of study population

Diabetes participants had lightly higher BP values compared to non-diabetic on every component (Table 1). Thus, 24 h SBP measurement in the diabetic group was 116 ± 9 mmHg *vs* 111 ± 8 mmHg for non-diabetics at borderline of significance (P = 0.06) while difference in DBP of two groups was non-significant (69 ± 7 mmHg *vs* 66 ± 5 mmHg; P = 0.19). In keeping with that, diurnal BP figures were slightly higher in the diabetic group but with a non-significant difference (SBP: 118 ± 10 mmHg *vs* 114 ± 10 mmHg, P = 0.11; DBP: 71 ± 7 mmHg *vs* 68 ± 6 mmHg; P = 0.22). One important finding was the elevated night time BP in diabetes adolescents with a borderline significance for SBP (112 ± 11 mmHg *vs* 106 ± 7 mmHg, P = 0.06) and MAP (85 ± 9 mmHg *vs* 81 ± 6 mmHg, P = 0.06).

Urinary albumin excretion of study population

In adolescents with diabetes, 06/20 (30%) developed abnormal exercise-induced albuminuria but none in the group of adolescents without diabetes. Urinary albumin

Table 2 Comparison of blood pressure values for albuminurics and non albuminurics patients

	UAE < 20 mg/g	UAE > 20 mg/g	*P* value
24 h BP			
SBP	113 ± 9	119 ± 10	0.14
DBP	67 ± 6	70 ± 8	0.51
Diurnal BP			
SBP	116 ± 10	120 ± 10	0.32
DBP	70 ± 5	72 ± 8	0.51
Nocturnal BP			
SBP	108 ± 10	116 ± 10	0.09
DBP	61 ± 8	66 ± 9	0.17

SBP: Systolic blood pressure; DBP: Diastolic blood pressure; UAE: Urinary albumin excretion.

excretion at rest was similar in both groups (5.5 mg/g *vs* 5.5 mg/g, $P = 0.74$). After exercise, we found a significant increase in urinary albumin excretion in diabetes patients as compared to non-diabetics (10.5 mg/g *vs* 5.5 mg/g, $P = 0.03$).

Relation between BP profile and albuminuria at rest and after exercise

We compared diabetes adolescents presenting exercise-induced albuminuria after exercise to those without albuminuria (Table 2). We found that diabetes patients with exercise-induced albuminuria had higher but non-significant nighttime SBP figures than those exercise-induced albuminuria (116 mmHg *vs* 108 mmHg, $P = 0.09$) while DBP were similar. In contrast, 24 h SBP and DBP were similar in both as well as diurnal SBP and DBP.

DISCUSSION

This study aimed to investigate the relationship between exercise-induced albuminuria and circadian BP abnormalities revealed by ABPM in non proteinuric T1DM adolescents. In order to achieve this objective, we compared young T1DM patients to non-diabetic matched controls. We found that nocturnal SBP of diabetic patients was slightly higher than that of non-diabetics as well as 24 h SBP with borderline significance. Most T1DM studies on albuminuria disease have been done in Caucasians[14-17]. This study confirms these findings in Africans. This increase in nocturnal SBP values and 24 h SBP already found by others studies suggest the existence in this group of probable subclinical kidney injuries. Indeed, it was demonstrated that diabetes patients with kidney injury or subclinical diabetic nephropathy had a tendency to higher BP than the general population[14-18]. Similarly, diabetes patients in our study have a tendency to increased nocturnal BP figures in comparison to non-diabetics leading to a reduction in the difference of day-night BP evaluated by dipping[19,20]. This anomaly is found more frequently in diabetes patients compared than in the general population and is attributed to the presence of kidney damage, still subclinical, but already leading to an increase in renal and cardiovascular risk[18]. Thus, the studies comparing individuals with impaired nocturnal decline in BP and those with normal nocturnal BP have revealed that individuals with insufficient decrease of BP and therefore higher values of BP during the night will present in future monitoring a more rapid degradation of renal function marked by a significant decrease in creatinine clearance[21]. In the same sense, these studies did not find any difference between daytime BP as well as diastolic BP which was also to be the case in our study where daytime BP were similar in both groups of participants[18,20]. However, unlike these studies, we found 24 h BP figures slightly higher in diabetes individuals but still of borderline significance. This could be attributed to the impact of nighttime BP on the 24 h BP and would be a reflection of the nocturnal difference since for similar diurnal BP, if the nocturnal BP is elevated in one group, then it becomes logical that the 24 h BP which is the average daytime and nighttime BP appears to be also more elevated.

Secondly, our study showed that for similar or even identical values of albuminuria at rest, diabetes patients having an increase in nocturnal BP and therefore probable subclinical kidney injuries had a significantly increase in exercise-induced albuminuria in comparison to non-diabetic individuals. This suggests that exercise-induced albuminuria increases with the existence of renal alterations revealed by abnormal nocturnal BP and therefore could be used to detect patients with these abnormalities. This finding support the assumption that exercise-induced albuminuria could serve as a marker of early diabetic renal injuries and allow detection or at least help to suspect the existence of subclinical diabetic nephropathy still undetectable by albuminuria at rest. This had been suggested in 1995 by O'Brien who found during a prospective follow-up on a half-decade that patients having abnormal exercise-induced albuminuria were those who would develop a clinical albuminuria at rest and therefore faster diabetic nephropathy[22-25]. But to the best of our knowledge, nobody has so far studied the relationship between exercise-induced albuminuria and nocturnal abnormalities of BP in type 1 diabetes patients. This first finding then proves very encouraging since it opens the way to new opportunities and show new research fields to explore.

Finally, we compared the diurnal and nocturnal BP values of patients who developed exercise-induced albuminuria to those of other participants without this abnormality. We found that patients with exercise-induced albuminuria had higher non-significant figures of BP during the night than those without this abnormality. These data support the hypothesis emitted above that exercise induced-albuminuria could be used to identify T1DM patients with abnormal nocturnal BP and therefore at risk of developing diabetic nephropathy or already presenting subclinical damage due to diabetic nephropathy. However, these findings casually refer to other studies on the subject with larger population study

and ideally with a prospective follow-up in order to clearly establish the link between exercise-induced albuminuria and renal prognosis and cardiovascular evaluated by circadian BP on ABPM and especially nocturnal BP abnormalities in T1DM[26-28].

In summary, T1DM patients having an increase in nocturnal BP exhibit an increase exercise-induced albuminuria and patients developing abnormal exercise-induced albuminuria have higher figures of nocturnal BP than others. These findings strongly suggest that exercise-induced albuminuria could to be use identify diabetes patients with subclinical renal damage, therefore it would be useful in the early diagnosis of nephropathy in T1DM.

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COMMENTS

Background

Nocturnal abnormalities of blood pressure are correlated with incipient diabetes nephropathy in type 1 diabetes adolescents, but relation with exercised induced-albuminuria has not been investigated yet. Few studies have been conducted on diabetic nephropathy in Africans adolescents.

Research frontiers

Studies on diabetic nephropathy in Africans adolescents are scarce. These data are important to determine the tie between exercise-induced albuminuria and nocturnal blood pressure abnormalities in type 1 diabetes adolescents and the possibility to use it as an earlier marker for diabetes nephropathy.

Innovations and breakthroughs

The authors confirm data of Caucasians studies suggesting that most type 1 diabetes adolescents developed diabetes nephropathy after five years. This study was the first investigating the relationship between exercise-induced albuminuria and ambulatory blood pressure measurement measurements in type 1 diabetes adolescents in the search of early markers of diabetic nephropathy.

Applications

This study shows that there is a relation between exercised-induced albuminuria and nocturnal abnormalities of circadian blood pressure suggesting that exercised-induced albuminuria could be useful as clinical marker for blunted nighttime in type 1 diabetes adolescents.

Peer-review

This is a nice study and well done, the topic is clear and the conclusion is novel.

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EVIDENCE-BASED MEDICINE

Fuzzy expert system for diagnosing diabetic neuropathy

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Abstract

AIM

To design a fuzzy expert system to help detect and diagnose the severity of diabetic neuropathy.

METHODS

The research was completed in 2014 and consisted of two main phases. In the first phase, the diagnostic parameters were determined based on the literature review and by investigating specialists' perspectives (n = 8). In the second phase, 244 medical records related to the patients who were visited in an endocrinology and metabolism research centre during the first six months of 2014 and were primarily diagnosed with diabetic neuropathy, were used to test the sensitivity, specificity, and accuracy of the fuzzy expert system.

RESULTS

The final diagnostic parameters included the duration of diabetes, the score of a symptom examination based on the Michigan questionnaire, the score of a sign examination based on the Michigan questionnaire, the glycolysis haemoglobin level, fasting blood sugar, blood creatinine, and albuminuria. The output variable was the severity of diabetic neuropathy which was shown as a number between zero and 10, had been divided into four categories: absence of the disease, (the degree of severity) mild, moderate, and severe. The interface of the system was designed by ASP.Net (Active Server Pages Network Enabled Technology) and the system function was tested in terms of sensitivity (true positive rate) (89%), specificity (true negative rate) (98%), and accuracy (a proportion of true results, both positive and negative) (93%).

CONCLUSION

The system designed in this study can help specialists

and general practitioners to diagnose the disease more quickly to improve the quality of care for patients.

Key words: Expert systems; Fuzzy logic; Artificial intelligence; Diabetes mellitus; Diabetes complications; Diabetic neuropathies

Core tip: In this study, an expert system was designed for diagnosing diabetic neuropathy. This system can help specialists to diagnose the disease more quickly by using the most common diagnostic parameters. Even general practitioners can use this system in remote areas to improve the quality of care for patients with diabetes. With it, patients will no longer need to undertake complex procedures, and the care plan can be applied at the right time.

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INTRODUCTION

One of the biggest challenges currently experienced by healthcare organizations is the increasing burden of chronic diseases posing serious threats to public health in developing countries[1]. Diabetes is one of the world's most common and costly chronic diseases, and the number of patients suffering from diabetes has been showing an increasing trend in many countries[2]. This can be attributed to population growth, aging, urbanization, prevalence of obesity, and a sedentary lifestyle[2,3]. Long-term complications of diabetes develop gradually and might be disabling or life-threatening - for example, vascular and tissue injuries caused by the progression of diabetes can lead to serious complications, such as retinopathy, nephropathy, cardiovascular disease, cerebrovascular disease, peripheral vascular disease, metabolic disease, and diabetic foot ulcer[4,5]. However, the most common complication of diabetes is impairment of the peripheral neural system, which is known as diabetic neuropathy and a major problem with different signs and symptoms. Compared with other diabetes complications, it is one of the first reasons for hospitalizing patients with diabetes[6]. The severity of pain, decreased or lack of sensation, increased risk of foot ulceration, and amputation are the consequences of diabetic neuropathy[7].

Diabetic peripheral neuropathy is usually seen in more than 10% of patients with type Ⅱ diabetes. Early diagnosis and treatment is the first step to reduce the incidence of foot ulcers and amputations[8]. The main cost of this disease is related to organ amputation. The risk of lower extremity amputation in patients is significantly high in case of this disease. Nevertheless, almost 85% of amputations are preventable by early detection of the disease, early intervention, good control of diabetes, and patient education[9]. Moreover, several studies show that neuropathy may negatively affect the quality of life for patients with diabetes[10,11].

Owing to the high prevalence of neuropathy among patients with diabetes, it is necessary to conduct annual screening and further evaluation as well as to devise a plan for managing the disease. However, one of the major problems associated with the diagnosis of diabetic neuropathy is the lack of a reliable clinical scale for grading the severity of the disease[12]. A variety of methods are used to detect peripheral neuropathy. These include the nerve conduction velocity test, the vibration perception threshold, the monofilament test, the clinical neuropathy examination, the Toronto clinical scoring system, and the Michigan neuropathy screening instrument (MNSI)[13]. Other than clinical examination, laboratory tests, such as haemoglobin A1c level, fasting blood sugar, and oral glucose tolerance test, along with risk factors like age, sex, renal disease, and smoking need to be considered[14].

It is notable that the boundary between illness and health is not clear in diabetic neuropathy, and it is difficult to express clinical diagnosis as the lack of or the existence of the disease. Since the disease develops on a continuous basis, two-valued logic cannot be used to express this continuity anymore[6]. Therefore, new methods for diagnosing the disease have been considered[15]. Among these methods, special attention has been paid to the development of information technology applications, decision support systems, and fuzzy expert systems[16,17]. The fuzzy expert system is a new version of expert systems that uses fuzzy logic for data processing. In a fuzzy expert system, the inference is conducted by a set of membership functions and fuzzy rules rather than by the rules of two-valued logic[18]. The Fuzzy expert systems are used to describe uncertain phenomena because real-world phenomena are much more complex than an exact and absolute description[19,20]. The ability to implement human science through specific linguistic concepts and fuzzy rules, non-linearity, adaptability of these systems, and the level of accuracy are the most important features of these systems[21]. Although fuzzy expert systems have been designed for different purposes in the healthcare setting, only a few studies have focused on the use of these systems with regard to the diagnosis of diabetic neuropathy[22].

MATERIALS AND METHODS

Objective

To design a fuzzy expert system to categorize the severity of diabetic neuropathy based on clinical exa-

minations and results of laboratory tests.

Setting, design, and sample size

This study was completed in 2014. The study consisted of two main phases. In the first phase, the parameters required for the diagnosis of diabetic neuropathy were determined on the basis of the literature review[23,24]. These parameters formed a questionnaire to investigate specialists' views about the importance of each of them. In the second phase, the system was tested by using real data. In the first phase, eight endocrinologists participated in the study. Owing to the limited number of specialists, no sampling method was applied in this phase. In the second phase, 244 medical records were identified from a database located in an endocrinology and metabolism research centre. These records were related to those patients who visited the centre during the first six months of 2014 and who were primarily diagnosed with diabetic neuropathy.

Methods for data collection and distribution

The questionnaire was distributed among the specialists by one the researchers (MRK), and their views on the importance of the diagnostic parameters were investigated. In second phase, a form was used to extract the required data from the medical records.

Development of the questionnaire

As noted before, the questionnaire was designed based on the literature review[23,24]. It comprised two parts: The first part included the specialists' demographic information, such as age, gender, and work experience; the second part was designed based on a five-point Likert scale (5 = very important, 4 = important, 3 = relatively important, 2 = less important, 1 = unimportant) and consisted of 15 questions to identify the degree of importance of each diagnostic parameter. The face and content validity of the questionnaire was approved by experts in the field of endocrinology. Its reliability was confirmed by using the test-retest method ($\alpha = 0.9$).

Statistical analysis

A data analysis was performed by using SPSS (version 20.0) software, and parameters with a mean score of less than three were excluded to facilitate the process of writing fuzzy rules. To test the system, the sensitivity, specificity, and accuracy of the fuzzy expert system were measured and compared with the final diagnosis recorded in the database. Cohen's kappa coefficient and the receiver operating characteristic (ROC) curve were used to report data.

Participants and recruitment

Before conducting the research, the approval of an institutional review board was obtained. In the first phase, the target population comprised endocrinologists working in an endocrinology and metabolism research centre. They were contacted by one of the researchers (MRK) and the research facilitator (MM), and were invited to take part in the study. Their participation in the research was completely voluntary. Regarding the medical records, patient identities were excluded and only the required data was extracted so that it can be used in the process of evaluation.

RESULTS

Participants

As noted before, the first part of the questionnaire included the participants' demographic information. According to the results, most of the participants were men (n = 5, 62.5%) aged between 30-50 years. The highest frequency (n = 3, 37.5%) was related to the age group of 46-50 years and the specialists with more than 16 years of work experience.

Diagnostic parameters for diagnosing diabetic neuropathy

The second part of the questionnaire was related to the diagnostic parameters required for diagnosing diabetic neuropathy. This part included the duration of diabetes, the symptom assessment based on MNSI, the sign examination based on MNSI, and the related laboratory tests. Table 1 presents the specialists' views in relation to the importance of the aforementioned diagnostic parameters.

As Table 1 shows, from the specialists' point of view, the most important diagnostic parameters were the duration of diabetes (4.88 ± 0.35), the glycolysis haemoglobin level (4.50 ± 0.75), and the score of the sign examination based on the Michigan questionnaire (4.38 ± 0.51). The lowest degree of importance (2.13 ± 0.83) was related to the amount of phosphorus in blood. After determining the diagnostic parameters of diabetic neuropathy, the semantic network of the expert system was drawn (Figure 1).

Designing a fuzzy expert system

As can be seen in the above figure, the ultimate goal, namely diagnosing diabetic neuropathy, is shown in the centre, and the diagnostic parameters are in the leaf nodes. In order to design the fuzzy expert system, all input variables were fuzzified based on membership functions. The system had seven input variables: The duration of diabetes, the score of the symptom examination based on the Michigan questionnaire, the score of the sign examination based on the Michigan questionnaire, the glycolysis haemoglobin level, fasting blood sugar, blood creatinine, and albuminuria. The system also had one output variable, which was the severity of diabetic neuropathy. The rules of the expert system were written based on the semantic network, consulting a specialist, and giving the same weight to all rules. The inference engine of the system was designed by using the Mamdani inference method. Figure 2 provides

Table 1 The degree of importance of the diagnostic parameters for diagnosing diabetic neuropathy from the specialists' perspectives

Degree of importance	Unimportant (1)	Less important (2)	Relatively important (3)	Important (4)	Very important (5)	Mean ± SD
Duration of diabetes	0	0	0	1 (12.5%)	7 (87.5%)	4.88 ± 0.35
Symptom assessment based on MNSI	0	0	1 (12.5%)	5 (62.5%)	2 (25%)	4.13 ± 0.64
Sign examination based on MNSI	0	0	0	5 (62.5%)	3 (37.5%)	4.38 ± 0.51
HbA1c	0	0	1 (12.5%)	2 (25%)	5 (62.5%)	4.50 ± 0.75
CBC	1 (12.5%)	3 (37.5%)	4 (50%)	0	0	2.38 ± 0.74
FBS	0	0	0	6 (75%)	2 (25%)	4.25 ± 046
ESR	1 (12.5%)	3 (37.5%)	3 (37.5%)	1 (12.5%)	0	2.52 ± 092
Oral GTT	1 (12.5%)	4 (50%)	1 (12.5%)	2 (25%)	0	2.50 ± 1.06
Albuminuria	0	1 (12.5%)	1 (12.5%)	4 (50%)	2 (25%)	3.88 ± 0.99
TSH	2 (25%)	1 (12.5%)	3 (37.5%)	2 (25%)	0	2.63 ± 1.18
B12 Vitamin	2 (25%)	1 (12.5%)	1 (12.5%)	4 (50%)	0	2.88 ± 1.35
BUN	1 (12.5%)	3 (37.5%)	3 (37.5%)	1 (12.5%)	0	2.38 ± 0.91
BCr	0	1 (12.5%)	2 (25%)	5 (62.5%)	0	3.50 ± 0.75
Calcium	2 (25%)	1 (12.5%)	4 (50%)	1 (12.5%)	0	2.50 ± 1.06
Phosphorus	2 (25%)	3 (37.5%)	3 (37.5%)	0	0	2.13 ± 0.83

BCr: Blood Creatinine; BUN: Blood urea nitrogen; TSH: Thyroid-stimulating hormone; GTT: Glucose tolerance test; ESR: Erythrocyte sedimentation rate; MNSI: Michigan Neuropathy Screening Instrument; HbA1c: Hemoglobin A1c; CBC: Complete blood count; FBS: Fasting blood sugar.

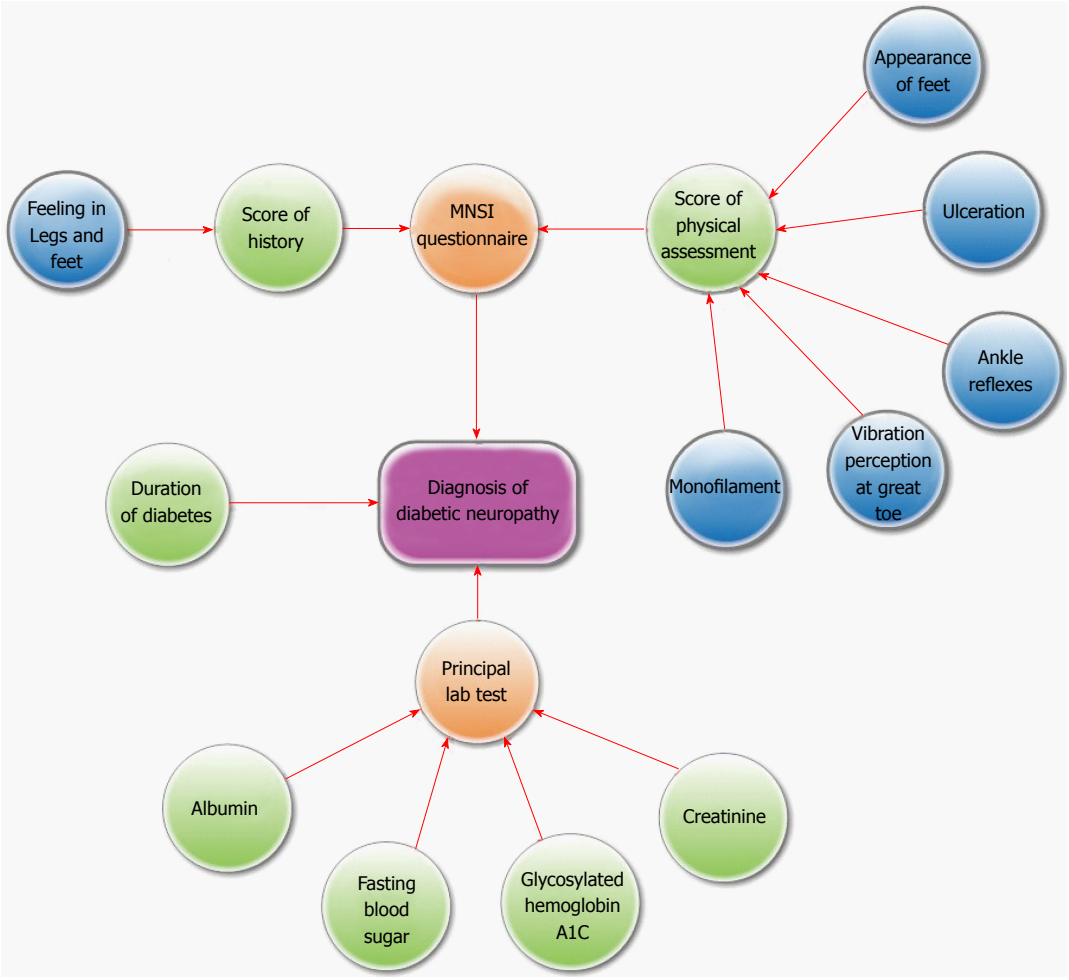

Figure 1 The semantic network of the expert system. MNSI: Michigan Neuropathy Screening Instrument.

an overview of the fuzzy inference architecture of the system.

Finally, the graphical user interface of the expert system was designed by using Active Server Page. Network Enabled Technology (ASP.NET). It is an open-source server-side web application framework designed for web development to produce dynamic web pages (Figure 3). The input variables, such as the duration

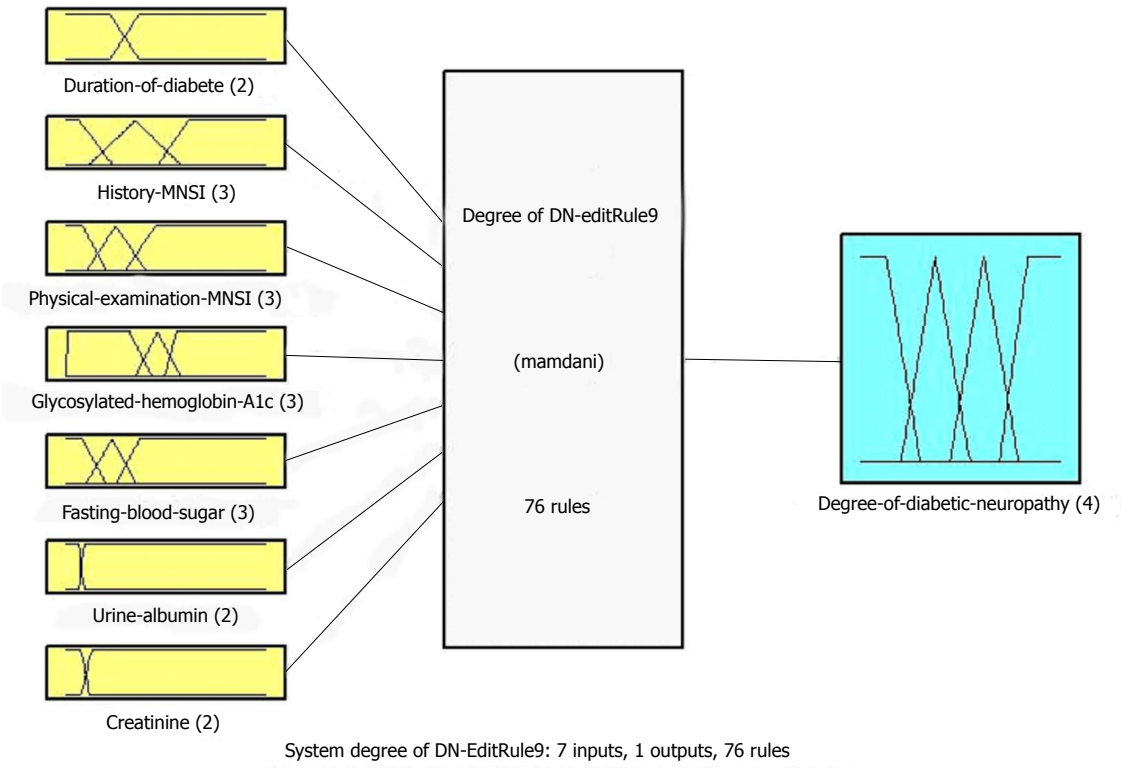

Figure 2 An overview of the fuzzy inference architecture of the system.

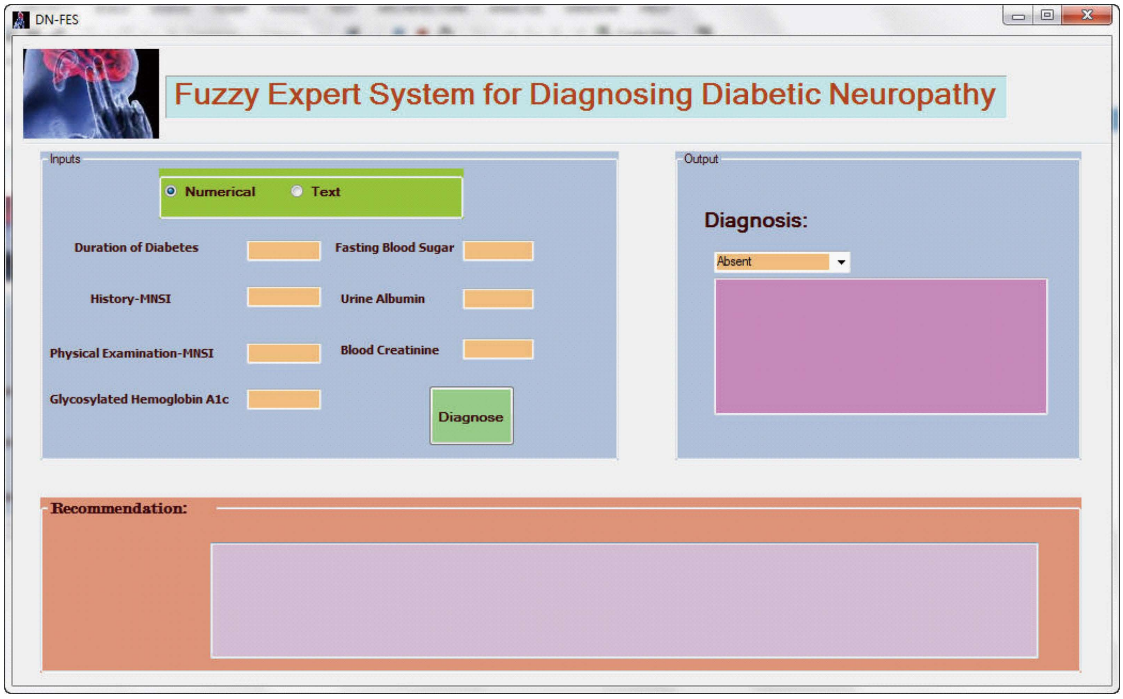

Figure 3 The graphical user interface of the fuzzy expert system.

of diabetes, the results of laboratory tests, and scores obtained from the Michigan questionnaire, could be entered into the system manually either in the textual or in the numerical format based on the user's choice. The output variable, namely the severity of the disease, which was shown as a number between zero and 10, had been divided into four categories: absence of the disease, (the degree of severity) mild, moderate, and severe. Figure 4 shows the risk of diabetic neuropathy based on the scores obtained from the Michigan questionnaire.

According to Figure 4, by increasing the scores

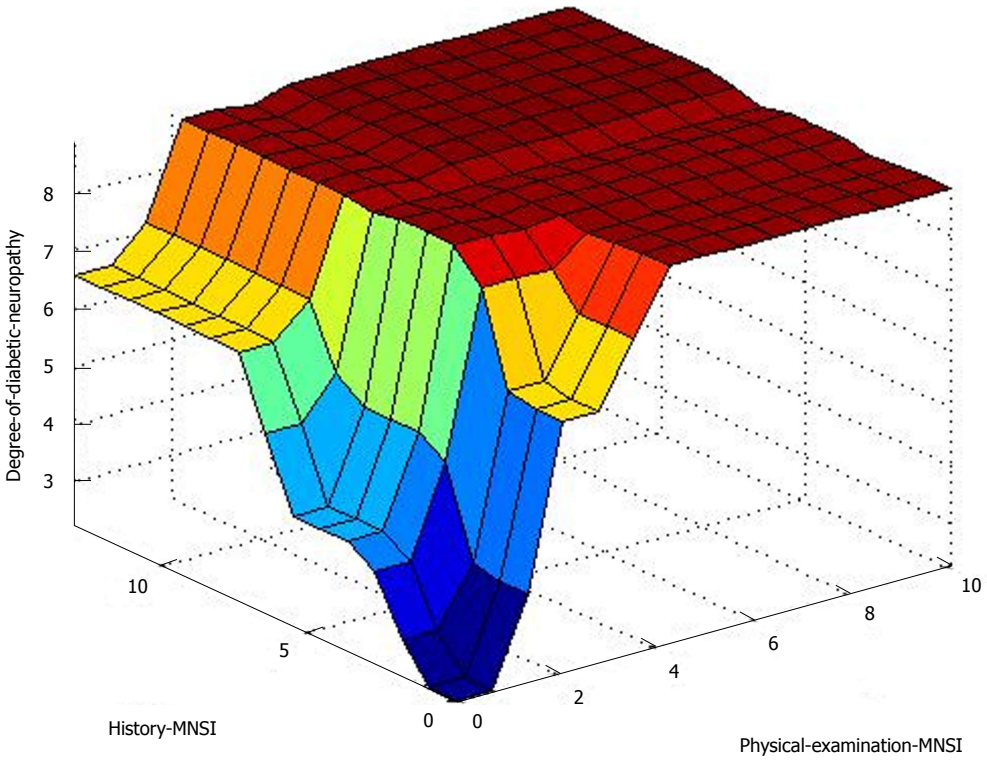

Figure 4 The risk of diabetic neuropathy based on the scores of the Michigan Neuropathy Screening Instrument questionnaire. MNSI: Michigan Neuropathy Screening Instrument.

obtained from the Michigan questionnaire, the severity of diabetic neuropathy will increase accordingly.

System function evaluation

The system was tested by using real data. In total, the records of 244 patients with diabetic neuropathy were identified. However, 31 records were excluded due to the incompleteness of clinical data. The remaining records (n = 213) included 118 patients who were diagnosed with diabetic neuropathy, while diagnosis was ruled out for the rest (n = 95). The system function was tested in terms of sensitivity (true positive rate), specificity (true negative rate), and accuracy (proportion of the true results, both positive and negative), which were 89%, 98%, and 93%, respectively.

Finally, the system's output was compared with the final diagnoses made by the specialists and recorded in the patients' records. These diagnoses were made by using the nerve conduction velocity test, the vibration perception threshold, the monofilament test, and the clinical neuropathy examination. The comparison was conducted by using the Kappa coefficient and the K value was 0.6. According to Landis and Koch, a Kappa value between 0.4 and 0.75 shows a fair to good agreement[25]. Therefore, the system designed in this study showed a fair to good level of similarity between the system's function and the specialists' diagnoses. The ROC curve presents the results of testing the system (Figure 5).

As can be seen in the above figure, the ROC curve is ideal. It is close to the high point of the square that represents an appropriate function of the system.

DISCUSSION

As mentioned before, one of the most common long-term complications of diabetes mellitus is diabetic neuropathy. In order to control this complication, it is important to diagnose it both accurately and timely[10]. Although there are a variety of methods to detect the disease, it is difficult to diagnose it at the very early stage[13]. Therefore, the use of IT applications, such as fuzzy expert systems, is suggested.

In the present study, seven diagnostic parameters-the duration of diabetes, the symptom assessment, the sign examination based on the MNSI, the glycolysis haemoglobin level, fasting blood sugar, blood creatinine, and albuminuria-were considered as input variables, and the severity of diabetic neuropathy was considered as an output variable. These variables were selected based on the specialists' perspectives and the literature review. Similarly, the knowledge and experience of four experts in the field of diabetic neuropathy was investigated in the study conducted by Picon *et al*[22] to determine the diagnostic parameters and to design a knowledge-based system. In their research, four inputs variables included symptom, the sign assessment based on the Michigan questionnaire, the glycolysis haemoglobin level, and the duration of diabetes. The output of the system classified the severity of diabetic neuropathy in three categories: Mild, moderate, and severe. In contrast with the study of Picon *et al*[22] the number of input variables increased

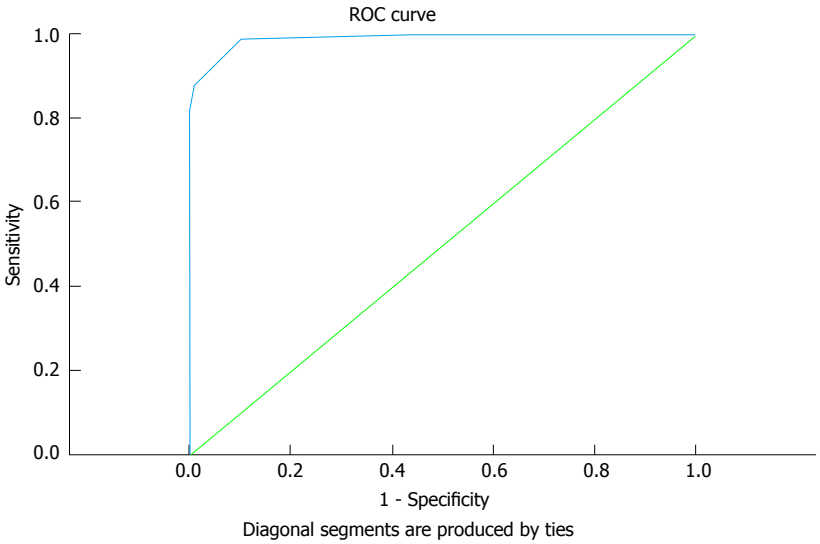

Figure 5 The receiver operating characteristic curve.

in the current research and laboratory test results were included to improve the accuracy of diagnosis. Similarly, Neshat *et al*[26]'s study considered six input variables and one output variable to diagnose liver disorders. To diagnose heart ailments, Adeli *et al*[27] used 12 input variables and considered the diagnosis of heart diseases as the output variable.

In the present study, values between zero and 10 were considered for the output variable, which was the severity of diabetic neuropathy. An increase in the value of output variable showed the level of severity for diabetic neuropathy.

In the current study, the fuzzy sets and membership functions for each of the seven input variables and the output variable were finalized after consulting a specialist. This approach can help eliminate the rules that could be covered by other rules, and finally, 76 rules were used to design the system. Similarly, DoostHoseini *et al*[28] consulted doctors to reduce the number of rules to an appropriate number. In another study, Zolnoori *et al*[29] developed a fuzzy expert system for diagnosing asthma. Given that the patients' records were incomplete, an indirect approach was used to develop the system's knowledge base. In this approach, the researchers reviewed books and scientific papers, and also conducted structured and unstructured interviews with doctors and patients. Having analysed the data, the most important variables useful for diagnosing asthma were identified.

In the present study, the system interface was designed by using ASP.NET rather than matrix laboratory (MATLAB). In fact, web-based applications have more flexibility and can be used by multiple users at the same time. Ease of use is another feature of these systems, which, in turn, can increase the work efficiency.

In this study, the output of the system was divided into four categories: The absence of the disease, mild, moderate, and severe. In contrast, Picon *et al*[22] classified the severity of neuropathy into three categories: Mild, moderate, and severe. Moreover, the specificity and sensitivity of the system were not reported in their study. In the current study, the specificity of the system was 98%, which shows a high level of system performance. Also, there was a relatively good agreement between the system's function and the diagnoses recorded by the specialists. Although other methods of diagnosis were not considered in the current study, the specificity and sensitivity of the system highly suggested that such a system could help physicians to diagnose the disease more quickly by using parameters like results of laboratory tests.

In the current study, the main aim was to develop an expert system for diagnosing diabetic neuropathy. Therefore, the clinical effectiveness of the system was not evaluated due to resource restrictions. Conducting evaluation studies after implementing the system in the actual healthcare setting would help determine the impact of the system on the health status of patients.

In conclusion, an expert system was designed for diagnosing diabetic neuropathy in this study. As diabetic neuropathy is a chronic disease that may have serious consequences, early diagnosis of the disease is important to control it. The system designed in the current study could help specialists to diagnose the disease more quickly by using the most common diagnostic parameters. General practitioners can use such a system in remote areas to improve the quality of care for patients with diabetes. With it, the disease can be diagnosed more easily and quickly. There is no need to undertake complex procedures, and the care plan can be applied at the right time. Further research is suggested to increase the number of variables to improve the accuracy, sensitivity, and specificity of the system. Moreover, the feasibility of using this method in daily clinical practice and its impact on the efficiency and

cost-effectiveness compared to those of other methods need to be investigated in future studies.

COMMENTS

Background

One of the major problems associated with the diagnosis of diabetic neuropathy is the lack of reliable clinical scale for grading the severity of the disease. A variety of methods, such as the nerve conduction velocity test, the vibration perception threshold, and the monofilament test, are used to detect the peripheral neuropathy. In addition to clinical examination, laboratory tests and risk factors of the disease such as age, sex, renal disease, and smoking need to be considered.

Research frontiers

Since the disease usually develops on a continuous basis, two-valued logic cannot be used to express this continuity any more. Therefore, new methods for diagnosing the disease have been considered. Among these methods, the development of information technology applications, decision support systems, and fuzzy expert systems have received special attention.

Innovations and breakthroughs

In order to diagnose diabetic neuropathy, clinical examinations as well as results of laboratory tests like the haemoglobin A1c level, fasting blood sugar, and the oral glucose tolerance test should be considered. In this study, information technology was used to design a fuzzy expert system to diagnose the severity of diabetic neuropathy based on clinical examinations and laboratory tests.

Applications

The system designed in the current study can help specialists to diagnose the disease more quickly by using the most common diagnostic parameters. General practitioners, too, can use it in remote areas to improve the quality of care for patients with diabetes. With it, the disease can be diagnosed more easily and quickly. There is no need to undertake complex procedures, and the care plan can be applied at the right time.

Terminology

The fuzzy expert system is a new version of expert systems that uses fuzzy logic for data processing. A fuzzy expert system is used to describe uncertain phenomena because the real-world phenomena are much more complex than an exact and absolute description. The most common complication of diabetes is impairment of the peripheral neural system, which is known as diabetic neuropathy.

Peer-review

This is interesting and important paper for diagnosis of diabetic complications. The paper is well-written and focused.

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