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Xiu-Xia Song, Vice Director
*World Journal of Diabetes*
Room 903, Building D, Ocean International Center,
No. 62 Dongsihuan Zhonglu, Chaoyang District,
Beijing 100025, China
Telephone: +86-10-85381891
Fax: +86-10-85381893
E-mail: editorialoffice@wjgnet.com
Help Desk: http://www.wjgnet.com/esps/helpdesk.aspx
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PUBLISHER
Baishideng Publishing Group Inc
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Telephone: +1-925-223-8242
Fax: +1-925-223-8243
E-mail: bpgoffice@wjgnet.com
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REVIEW

# Role of bile acids in the regulation of the metabolic pathways

Hiroki Taoka, Yoko Yokoyama, Kohkichi Morimoto, Naho Kitamura, Tatsuya Tanigaki, Yoko Takashina, Kazuo Tsubota, Mitsuhiro Watanabe

Hiroki Taoka, Tatsuya Tanigaki, Department of Environmental Information, Keio University, Fujisawa, Kanagawa 252-0882, Japan

Yoko Yokoyama, Naho Kitamura, Graduate School of Media and Governance, Keio University, Fujisawa, Kanagawa 252-0882, Japan

Kohkichi Morimoto, Division of Endocrinology, Metabolism and Nephrology, Department of Internal Medicine, Keio University School of Medicine, Shinjuku, Tokyo 160-8582, Japan

Yoko Takashina, Research Institute of SFC, Keio University, Fujisawa, Kanagawa 252-0882, Japan

Kazuo Tsubota, Department of Ophthalmology, Keio University School of Medicine, Shinjuku, Tokyo 160-8582, Japan

Mitsuhiro Watanabe, Graduate School of Media and Governance, Department of Environment and Information Studies, Keio University, Fujisawa, Kanagawa 252-0882, Japan

Mitsuhiro Watanabe, Department of Internal Medicine, Keio University School of Medicine, Shinjuku, Tokyo 160-8582, Japan

Author contributions: Taoka H, Watanabe M devised the study concept and design; Taoka H, Yokoyama Y, Morimoto K, Watanabe M searched the literature; Taoka H, Yokoyama Y, Morimoto K, Kitamura N, Watanabe M drafted the article; all authors revised the article for important intellectual content; Watanabe M gave final approval for the article.

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Correspondence to: Mitsuhiro Watanabe, PhD, Professor of Graduate School of Media and Governance, Professor of Faculty of Environment and Information Studies, Graduate School of Media and Governance, Department of Environment and Information Studies, Keio University, tau42, 5322 Endo, Fujisawa, Kanagawa 252-0882, Japan. wmitsu@sfc.keio.ac.jp
Telephone: +81-466-493516
Fax: +81-466-493516

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## Abstract

Recent studies have revealed that bile acids (BAs) are not only facilitators of dietary lipid absorption but also important signaling molecules exerting multiple physiological functions. Some major signaling pathways involving the nuclear BAs receptor farnesoid X receptor and the G protein-coupled BAs receptor TGR5/M-BAR have been identified to be the targets of BAs. BAs regulate their own homeostasis *via* signaling pathways. BAs also affect diverse metabolic pathways including glucose metabolism, lipid metabolism and energy expenditure. This paper suggests the mechanism of controlling metabolism *via* BA signaling and demonstrates that BA signaling is an attractive therapeutic target of the metabolic syndrome.

**Key words:** Bile acids; TGR5/M-BAR; Farnesoid X receptor; Glucose metabolism; Energy metabolism; Lipid metabolism; Bariatric surgery; Microbiota; Incretin; Bile acid binding resin

**Core tip:** Bile acids (BAs) are important molecules that participate in various metabolic pathways. BA signaling mechanisms are attractive therapeutic targets of the metabolic syndrome. In this review, we show the mechanisms of controlling glucose, lipid and energy metabolism *via* BA signaling. Furthermore, the authors also describe how those basic scientific studies have been applied to the clinical setting. Particularly, bile acid binding resin (BABR) originally used to treat hypercholesterolemia also stimulates incretin secretion and improves glucose metabolism. In addition to BABR, the clinical application of farnesoid X receptor and TGR5/M-BAR agonists are ongoing for the treatment of metabolic syndrome. The effects of bariatric surgery on glycemic control are also associated with BA metabolism.

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## INTRODUCTION

Bile acids (BAs) are the main constituents of bile and amphipathic molecules, containing both hydrophilic and hydrophobic regions. BAs are synthesized from cholesterol in the liver, stored in the gall bladder, and flow into the small intestine after meal ingestion. Intestinal BAs facilitate digestion and absorption of lipids and fat-soluble vitamins[1].

Recent reports suggest that BAs are responsible not only for the absorption of lipids but also for signal transduction. Some major signaling mechanisms have been identified, including the MAPK pathways, nuclear hormone receptor farnesoid X receptor (FXR)-mediated pathway and G protein-coupled receptor TGR5/M-BAR (also named GPR131)-mediated pathway[2-5]. BAs have been demonstrated to be natural ligands of FXR. The main role of the FXR signaling pathway is regulating both enterohepatic circulation and BA biosynthesis to maintain the homeostasis of BA[6]. In addition, FXR signaling has been known to regulate lipogenesis gene expression and improve hepatic steatosis[7]. Moreover, recent studies have shown that BAs and FXR signaling are associated with the beneficial glycemic effects of bariatric surgery and regulation of autophagy[8-10]. BAs also activate TGR5/M-BAR. The TGR5/M-BAR signaling pathway stimulates energy expenditure in both brown adipose tissue (BAT) as well as skeletal muscle[11]. Furthermore, TGR5/M-BAR plays a role in hepatic microcirculation as well as cytokine release from macrophages[12]. Taken together, BAs not only participate in the digestion and absorption of lipids but also in various metabolic pathways. BA signaling participates in various diseases such as cancer, immune disorders, and metabolic syndrome[13-15]. In this review, we summarize the current knowledge of the metabolic regulation mechanisms of BAs and propose BA signaling pathways as a therapeutic target of the metabolic syndrome.

## BILE ACIDS METABOLISM

The majority of synthesized BAs are secreted into the bile and kept in the gallbladder. When food enters the gastrointestinal tract, bile flows into the small intestine, and are efficiently absorbed by active transport and passive diffusion in the terminal ileum. BAs are then transported again to the liver through the portal vein and re-uptaken at the sinusoidal membranes of hepatocytes. These BAs are then secreted into the bile again; each BA molecule can complete 4-12 cycles of circulation per day[16].

BA synthesis has two differential pathways: The "classic (or neutral) pathway" and the "alternative (or acidic) pathway". In the classic pathway, the enzyme cholesterol-7α-hydroxylase (CYP7A1) hydroxylates the C7α position during the first step. In the alternate pathway, the enzyme sterol-27α-hydroxylase (CYP27A1) first hydroxylates the C27 position. The classic pathway seems more important than the alternative pathway because the classic pathway is responsible for maintaining cholesterol homeostasis by controlling BA synthesis[17]. The rate-limiting enzyme CYP7A1 converts cholesterol to 7α-hydroxycholesterol, and other enzymes including sterol-12α-hydroxylase (CYP8B1), 25-hydroxycholesterol-7α-hydroxylase (CYP7B1) and CYP27A1 convert 7α-hydroxycholesterol to primary BAs, including cholic acid (CA) and chenodeoxycholic acid (CDCA)[18]. CYP8B1 controls the production of CA, and CA regulates the CA/CDCA ratio in humans or the CA/MCA ratio in mice by mediating feedback regulation[19]. Regulation of this ratio is important because previous studies demonstrated that the ratio of CA/CDCA is associated with liver diseases in humans[20]. For example, this ratio is decreased in patients with liver cirrhosis and hepatic cancer but is increased in cholestasis. Most of the BAs are conjugated to glycine or taurine, and the ratio of BAs conjugated to taurine and glycine differ depending on the animal species. In humans, the ratio of BAs conjugated to taurine and glycine are approximately 1:2, and most BAs are conjugated with taurine in mice. BAs inhibit the expression of CYP7A1 and CYP8B1 in liver through several pathways, which are mainly FXR-dependent. BAs activate FXR, leading to the upregulation of a small heterodimer partner (SHP; NR0B2), which suppresses the activity of hepatocyte nuclear factor-4α (HNF-4α; NR2A1), liver X receptor (LXR; Nr1h3) and liver receptor homolog-1 (LRH-1; NR5A2), which are both required for transcriptional induction of BA synthesis enzymes *via* binding to BA-response elements in promoters[21-23]. Additionally, the intestinal activation of FXR by BAs causes an increased expression of fibroblast growth

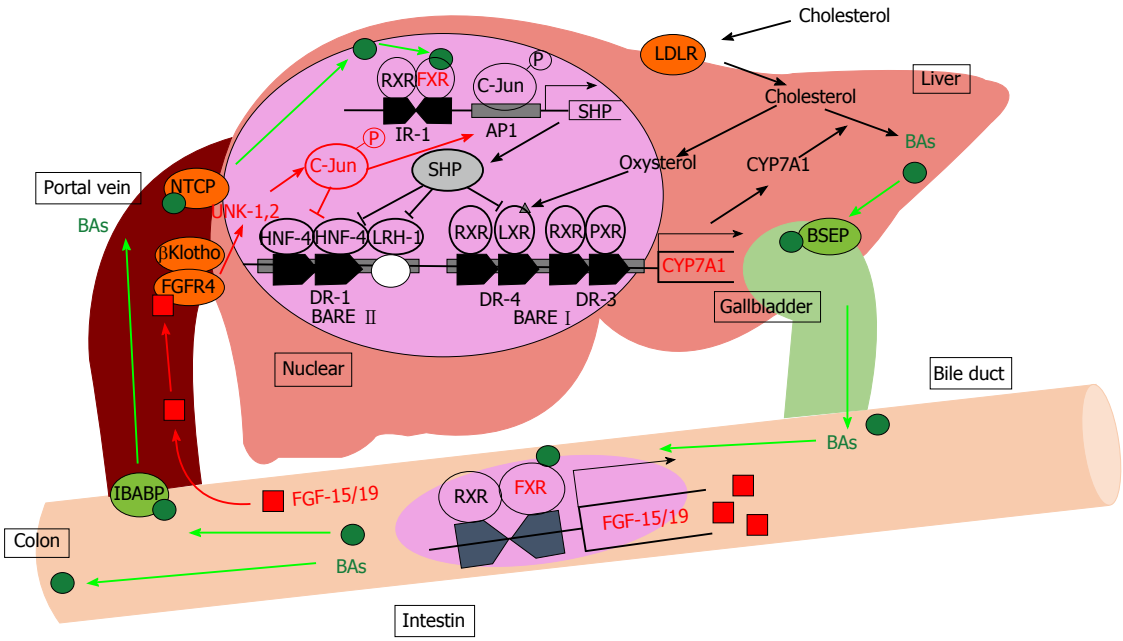


**Figure 1 Bile acid metabolism in the liver.** BAs induce the FXR-SHP-mediated pathway and repress BA synthesis enzyme gene expression such as CYP7A1 and CYP8B1. Synthesized BAs increase the expression of FGF-15/19 in the small intestine. FGF-15/19 signaling induces JNK pathway activation resulting in the repression of CYP7A1 transcription. AP1: Activator protein 1; BAs: Bile acids; BARE: Bile acid response element; BSEP: Bile salt export pump; CYP7A1: Cholesterol-7α-hydroxylase; DR: Direct repeat element; FGF-15/19: Fibroblast growth factor-15/19; FGFR4: Fibroblast growth factor receptor 4; FXR: Farnesoid X receptor; HNF-4: Hepatocyte nuclear factor; IBABP: Intestinal bile acid-binding protein; IR-1: Inverted repeat element-1; JNK: Jun-N-terminal kinase; LDLR: Low-density lipoprotein receptor; LRH-1: Liver receptor homolog-1; LXR: Liver X receptor; NTCP: Sodium-taurocholate cotransporting polypeptide; PXR: Pregnane X receptor; RXR: Retinoid X receptor; SHP: Small heterodimer partner.

factor (FGF)-15 in rodents and FGF-19 in humans. BAs absorbed in the terminal ileum activate intestinal FXR and induce enterocytic production of FGF-15/19. This FGF-15/19 is passed from the portal vein to the hepatocytes and couples with a receptor, FGF receptor 4 (FGFR4). These signaling pathways *via* FGF-15/19 and FGFR4 induce receptor dimerization, autophosphorylation, and c-Jun N-terminal kinase pathway activation resulting in the repression of CYP7A1 transcription (Figure 1)[24,25]. A second BA receptor, TGR5/M-BAR, also contributes to regulation of BA homeostasis. TGR5/M-BAR knockout mice present with a decrease in the BA pool size and the impaired suppression of CYP7A1 expression upon BA administration[26,27]. Vitamin D also regulates BAs synthesis. Vitamin D receptor activation induces the expression of FGF-15/19, and BA synthesis is decreased by reducing CYP7A1 expression[28,29]. BAs regulate BA homeostasis *via* FXR, TGR5/M-BAR and other signaling pathways primarily by maintaining gene expression of the rate-limiting enzymes CYP7A1 and CYP8B1.

## BILE ACIDS IN GLUCOSE METABOLISM

Previous studies have clarified that BAs affect glucose metabolism. Glucose induces the expression of FXR and CYP7A1, and insulin reduces their expression *in vitro*[30]. Further studies have shown that BAs seem to regulate gluconeogenesis, but the mechanisms remain poorly understood. Some studies have indicated that the expression of phosphoenolpyruvate carboxykinase (PEPCK), which is the rate-limiting enzyme of gluconeogenesis, is suppressed by BAs in human liver cancer cells (HepG2 cells) and the mouse liver[31-33]. Additionally, enzymes such as glucose 6-phosphatase and fructose 1,6-bisphosphatase 1 which also participate in gluconeogenesis are repressed by BAs[31]. These effects are decreased in FXR and SHP knockout mice, which supports the idea that BAs suppress gluconeogenesis in a FXR-SHP-dependent manner[33]; however, others have reported that FXR-dependent signaling induces PEPCK expression and increases gluconeogenesis in primary hepatocytes and rat hepatoma cell lines[34]. In terms of glycogen synthesis, BAs increase hepatic glycogen synthesis and storage, resulting in decreased blood glucose levels in an FXR-dependent manner (Figure 2B)[35]. A previous study demonstrated that long-term FXR activation (3 mo) with a synthetic FXR agonist, GW4064, worsened glucose intolerance and insulin resistance in high-fat fed C57BL/6J mice[33,36]. The mechanism behind the effects of GW4064 is lowering the BA pool size following FXR activation. Some reports have suggested that short-term (10 d) FXR activation by the synthetic FXR agonist GW4064 reduced glycolytic gene expression and improved insulin resistance in *ob/ob* or *db/db* mice[35,37]. In contrast, the difference of the GW4064 administration period may lead to the opposite result. Long-term administration of BAs, the endogenous natural ligands of FXR, did not decrease the BA pool size and subsequently improved glucose intolerance and insulin resistance[36].

BA administration improved metabolism including glucose tolerance and insulin resistance. The beneficial

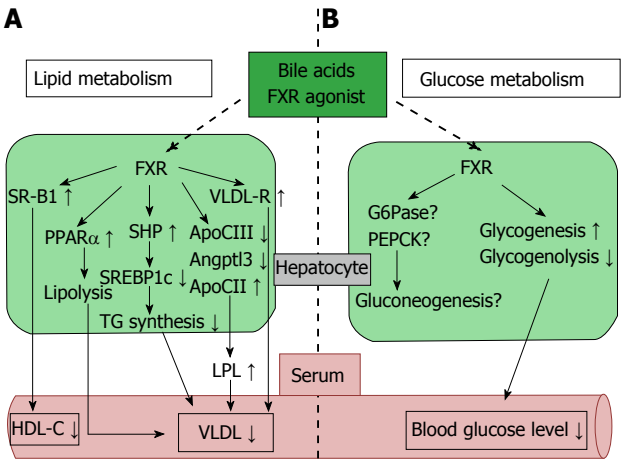


**Figure 2 Farnesoid X receptor-dependent metabolic regulation in the liver.** Hepatic FXR signaling regulates lipid and glucose metabolism. A: FXR signaling reduces lipogenesis (SREBP1c) and induces fatty acid β oxidation (PPARα) and plasma TG clearance (LPL and VLDL-R), resulting in decreased plasma VLDL levels. Plasma HDL-C uptake is also increased by FXR and SRB1 activity; B: FXR signaling up-regulates glycogenesis, down-regulates glycogenolysis, and reduces blood glucose levels. Hepatic FXR signaling is also associated with gluconeogenesis, but the controlling mechanism is still unclear. Angptl3: Angiopoietin-like protein 3; ApoCⅡ/CⅢ: Apolipoprotein-CⅡ/CⅢ; FXR: Farnesoid X receptor; G6Pase: Glucose-6-phosphatase; HDL-C: High density lipoprotein-cholesterol; LPL: Lipoprotein lipase; PEPCK: Phosphoenolpyruvate carboxykinase; PPARα: Peroxisome proliferator-activated receptor α; SR-B1: Scavenger receptor-B1; SREBP1c: Sterol regulatory element-binding protein 1c; TG: Triglyceride; VLDL-R: Very low density lipoprotein-receptor.

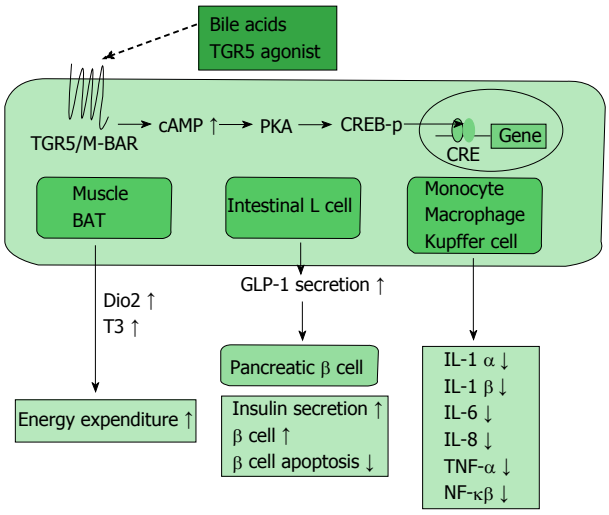


**Figure 3 TGR5/M-BAR-dependent metabolic regulation.** TGR5/M-BAR activation leads to increased intracellular cAMP levels, the activation of PKA and induction of CREB phosphorylation. This series of signaling activity induces the expression of genes bearing CRE and exists in various tissues. TGR5/M-BAR signaling induces energy expenditure in the muscle and BAT, increases GLP-1 secretion in the intestinal L cell, and reduces inflammatory cytokine release in immune cells. CREB-p: cAMP response element-binding protein phosphorylation; Dio2: Deiodinase iodothyronine type Ⅱ; T3: Tri-iodothyronine; BAT: Brown adipose tissue; GLP-1: Glucagon-like peptide-1.

effects of BAs, such as a decrease in gluconeogenesis and increase in glycogen synthesis, seem to occur not only through FXR signaling but also through a number of other signaling molecules, such as TGR5/M-BAR. BAs stimulate incretins, such as glucagon-like peptide-1 (GLP-1; Figure 3). GLP-1 is secreted by dietary stimulation from enteric L cells and promotes insulin secretion by binding to the GLP-1 receptor in the pancreatic β cell. Further, GLP-1 maintains pancreatic function, and GLP-1 receptor agonists have been developed for the treatment of diabetes[38]. TGR5/M-BAR signaling causes GLP-1 secretion in mouse enteroendocrine STC-1 cells[39]. Moreover, 6-ethyl-23(S)-methylcholic acid (6EMCA or INT-777[40]), a semisynthetic TGR5/M-BAR agonist, stimulates the secretion of GLP-1 in both mouse and human enteroendocrine cells. In the present study, knock-down of TGR5/M-BAR by shRNA decreased 6EMCA-induced secretion of GLP-1 in STC-1 cells[41]. The natural TGR5/M-BAR agonist oleanolic acid also improves the metabolism of glucose[42]. This evidence indicates the importance of TGR5/M-BAR in GLP-1 secretion. An *in vivo* study with TGR5/M-BAR transgenic and TGR5/M-BAR knockout mice strongly supports the relationship between TGR5/M-BAR and GLP-1 secretion[43]. Considering the current mechanism, TGR5/M-BAR activation increases cAMP levels and the ATP/ADP ratio, which then leads to depolarization of the plasma membrane as well as $Ca^{2+}$ mobilization, resulting in increased GLP-1 release[41]. Additionally, a human genetic study revealed an association between a single nucleotide polymorphism, rs3731859, of the *TGR5/M-BAR* gene and various metabolic indexes including BMI, waist circumference, intramyocellular lipid, and fasting serum GLP-1 levels[44]. Hence, these findings suggested that GLP-1 secretion was stimulated by TGR5/M-BAR signaling *in vivo.* BAs and TGR5/M-BAR could become therapeutic targets of diabetes.

## BILE ACIDS IN LIPID METABOLISM

BAs are important in regulating triglyceride (TG) metabolism as well as cholesterol metabolism. The relationship between BAs and TG was first reported in the treatment of gallstones with CDCA. CDCA treatment decreased the serum TG level in patients with gallstones[45]. In fact, BAs or a synthetic FXR agonist affected TG metabolism *via* several mechanisms including the FXR-mediated pathway. The target of FXR, SHP, suppressed up-regulation of sterol regulatory element-binding protein-1c (SREBP-1c), the master regulator of fatty acid and TG synthesis, to reduce the expression of the lipogenic genes such as acetyl CoA synthetase, acetyl CoA carboxylase, stearoyl CoA desaturase 1, and fatty acid synthase[7,46]. In addition, the TG-lowering effects were attenuated in SHP knockout mice, indicating that lipogenesis mediated by SREBP-1c is suppressed in an FXR-SHP-dependent manner[7]. Additionally, FXR activation by BAs increases expression of apolipoprotein (Apo) CⅡ. Apo CⅡ activates lipoprotein lipase, which in turn stimulates TG hydrolysis in very low density lipoprotein (VLDL) and chylomicrons, and also facilitates the clearance of TG from the serum[47]. The expression of ApoCⅢ and angiopoietin-like protein 3, which inhibits

the activity of lipoprotein lipase, were repressed by FXR stimulation with BAs[48-50]. In addition, FXR induces the expression of the VLDL receptor, which acts to clear plasma TG (Figure 2A)[51].

BAs also represses the expression of microsomal triglyceride transfer protein (MTP) and ApoB in an FXR-independent manner to suppress the formation of VLDL and chylomicrons[52]. Not only VLDL but also high density lipoprotein (HDL) clearance are suggested to be subject to modulation by BAs. Expression of scavenger receptor B1 (SRB1), a molecule in charge of hepatic uptake of HDL, is decreased, and HDL-C (HDL-cholesterol) is elevated in FXR knockout mice[53]. In addition, the administration of an FXR ligand increases hepatic SRB1 expression and decreases HDL-C levels (Figure 2A)[54].

BAs control other major regulators of lipid metabolism such as proliferator-activated receptor α (PPARα) and pyruvate dehydrogenase kinase-4 (PDK4). The nuclear receptor PPARα, which is activated by free fatty acids (FFA), decreases serum TG levels and exerts an important role for controlling enzymes participating in fatty acid β oxidation (Figure 2A)[55]. A study suggested that BAs directly regulate PPARα through FXR in humans, but not in mice[56]. PDK4 is also up regulated by BAs in an FXR-dependent manner, resulting in inactivation of pyruvate dehydrogenase, decreased glycolysis and increased oxidation of fatty acid β[57]. BAs are also associated with atherosclerosis[58,59]. Treatment with TGR5/M-BAR agonist INT-777 represses the activation of inflammatory cytokines such as NF-κB and inhibits foam cell formation and subsequent atherosclerotic plaques. In addition, INT-777 does not inhibit atherosclerosis in TGR5/M-BAR knockout mice, supporting the anti-atherosclerotic effect of TGR5/M-BAR (Figure 3)[58].

## BILE ACIDS IN ENERGY METABOLISM

BAs have been reported to stimulate adaptive thermogenesis and energy expenditure *via* TGR5/M-BAR (Figure 3)[11]. TGR5/M-BAR activation leads to increased intracellular cAMP levels, activation of PKA and induction of CREB phosphorylation. This series of signaling activity induces the expression of genes bearing a cAMP responsive element and exists in various tissues[60,61].

In the BAT, TGR5/M-BAR stimulation increases the intracellular cAMP level and induces cAMP-dependent iodothyronine deiodinase type 2 (*Dio2*) expression, which converts inactive thyroxine (T4) to active 3,5,3'-triiodothyronine (T3) to evoke increased energy expenditure[11]. *Dio2* increases the nuclear T3 level without various unwanted side effects caused by increased blood T3 levels. Only 20% of nuclear T3 is produced and secreted from the human thyroid gland, and the remaining nuclear T3 is supplemented from other tissues. *Dio2* supplies approximately 50% of the T3 in the nucleus including the BAT[62]. The BAT is one of the most important targets of BAs to increase energy expenditure. Although BAT had been regarded as a tissue only in newborn infants, recent studies with FDG-PET revealed the existence of BAT in the shoulders and neck in adult humans, especially with brief cold exposure[63-65]. Furthermore, several groups have shown the importance of BAT in adult humans. In healthy patients, the amount of BAT is large and its activity is high but are reduced in obese patients[66-68]. In addition, TGR5/M-BAR and *Dio2* are co-expressed in skeletal muscle in humans, which suggests the presence of a thermogenic mechanism in humans[11]. Moreover, a recent study found another type of adipocyte termed "beige" cells which are derived from white adipose tissue. These adipocytes also respond to cyclic AMP stimulation with high uncoupling protein (UCP) 1 expression and respiration rates similar to BAT cells[69,70]. These accumulating findings suggest a therapeutic approach to improve obesity and metabolic syndrome by increasing energy expenditure through TGR5/M-BAR stimulation.

## BILE ACIDS IN AUTOPHAGY

Autophagy is an evolutionarily conserved catabolic system that maintains energy homeostasis by recycling nutrients in the fasted state. Recent studies have revealed that FXR stimulation suppresses autophagy in the liver. FXR and peroxisome PPARα competitively bind to the promoter regions of autophagic genes, and these receptors show conflicting effects on transcription[8]. In the liver, PPARα activation under fasted conditions promotes autophagic lipolysis, while FXR activation under fed conditions suppresses autophagy. That is, PPARα and FXR competitively regulate autophagy based on the nutritional condition (Figure 4A). Another study also revealed that FXR and cAMP response element-binding protein (CREB), which is a transcriptional activator under starvation, competitively regulate autophagy in the liver[9]. In the fasted condition, CREB binding to its coactivator CREB regulated the transcription coactivator 2 (CRTC2) to induce CRTC2 activity and subsequent autophagic-related gene expression. Additionally, FXR stimulation caused by feeding disrupts the functional CREB–CRTC2 complex and downregulates autophagy (Figure 4B). In any case, there is no doubt that FXR acts as a suppresser of autophagy.

## ROLES OF BILE ACIDS IN THE GASTROINTESTINAL TRACT

Intestinal FXR has been recently identified as a possible target for improving metabolic syndrome. Intestinal FXR activation induces the expression of FGF-15/19, and several studies have demonstrated that FGF-15/19 affects glucose and energy homeostasis. FGF-19 transgenic mice showed increased hepatic β oxidation, reduced adipose tissue weight, and improved glucose tolerance and insulin sensitivity[71]. In mice, hepatic acetyl-CoA carboxylase 2 (ACC2) mRNA was decreased, and the mass of the BAT was increased. ACC2 exists at the mitochondrial membrane and converts acetyl-CoA

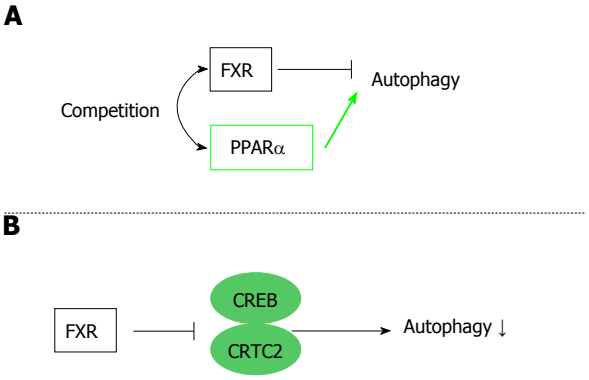


**Figure 4 Autophagy regulation by the farnesoid X receptor.** FXR is associated with regulation of autophagy. Two different mechanisms are reported. A: FXR and PPARα competitively bind to the promoter regions of autophagic genes, and FXR activation suppresses autophagy; B: FXR stimulation disrupts the functional CREB–CRTC2 complex and suppresses autophagy. FXR: Farnesoid X receptor; PPARα: Peroxisome proliferator-activated receptor α; CREB: cAMP response element-binding protein; CRTC2: CREB regulated transcription coactivator 2.

to malonyl-CoA. ACC2 activation results in an elevation of malonyl-CoA levels, which inhibit carnitine palmitoyl transferase-1 (CPT-1) activation[72]. CPT-1 transfers FFA from the cytoplasm to the mitochondria and induces fatty acid β oxidation. Thus, the overexpression of FGF-19 suppresses ACC2 mRNA levels, decreases malonyl-CoA levels, activates CPT-1, and thereby increases β-oxidation in the liver. In addition, hyperglycemia is improved upon administration of FGF-19 protein in obese mice[73]. Furthermore, activation of intestinal FXR by administration of fexaramine, an FXR agonist, improved obesity and insulin resistance by inducing FGF-15, changing the serum BA composition and stimulating systemic TGR5/M-BAR[74]. These results suggest the possibility that metabolic disease is improved through the intestinal FXR-FGF-15/19 signaling pathway (Figure 5B).

The primary BAs excreted into the intestine become deconjugated BAs and are converted into various secondary BAs by microbial enzymes[75]. In germ-free (GF) mice, a decrease in the gut microbiota that facilitate BA deconjugation leads to increased tauro-beta-muricholic acid (T-β-MCA). In comparison to conventionally raised mice, FXR-dependent BA synthesis is reduced in GF mice. Therefore, T-β-MCA is an FXR antagonist, and the microbiota affect bile acid homeostasis *via* the inhibition of intestinal FXR signaling by change in the BA composition[76]. In contrast to previous reports, recent studies have noted that alteration of the BA composition by microbiota and inhibition of intestinal FXR activity improved lipid and glucose metabolism. Increased T-β-MCA reduced intestinal FXR activation and decreased serum ceramide levels through repression of ceramide synthesis. Decreased ceramide downregulated expression of hepatic SREBP-1c and resulted in an improvement of obesity and nonalcoholic fatty liver disease (NAFLD)[77-79]. Additionally, intestinal FXR deactivation may also improve glucose metabolism as well as lipid metabolism. FXR activation in L cells decreased glycolysis, proglucagon expression and cAMP levels. Thus, GLP-1 production and secretion were inhibited (Figure 5A)[80]. Conflicting opinions suggest that microbiota regulation of BA homeostasis and intestinal FXR activation are involved in controlling hepatic lipid accumulation and glucose metabolism. Further studies are needed to clarify the roles of intestinal FXR signaling for improving metabolic diseases.

Bariatric surgery provides another clue to identifying the link between BAs and glucose homeostasis. Bariatric surgery, particularly gastric bypass surgery, is an established modality for obesity and type 2 diabetes mellitus, albeit that the mechanism of its effectiveness remains unclear. Interestingly, an improvement in glycemic control is seen soon after the surgery, when the body weight remains unchanged. Therefore, some of the anti-metabolic syndrome effects of this surgical intervention appear to be independent of body weight reduction. One recent study suggested that BAs might participate in this immediate effect of bariatric surgery. Following gastric bypass, the bile flow is changed, which leads to an increase in plasma BA level and incretin secretion[81]. Hormonal factors and the gut microbiota might also be involved in the effects of this surgery. The gut microbiota is responsible for the enteral BA metabolism, and the normal spectrum of gut microbiota is impacted by gastrointestinal surgery. As one example, the predominant presence of *Firmicutes* was reportedly diminished, and other species, such as methanogens and *Prevotellacaea,* were also inhibited after bariatric surgery[82]. In addition to these studies, recent research has revealed that FXR is associated with the effect of bariatric surgery[83]. Interestingly, in FXR knockout mice, metabolic improvements such as weight loss and improved glucose tolerance were reduced after bariatric surgery. Furthermore, the surgery changed the gut microbial communities differently between wild type and FXR knockout mice. This study suggested that BAs may affect glucose homeostasis *via* FXR signaling and alterations of the gut microbiota after bariatric surgery. Further investigations are expected.

Bile acid binding resin (BABR) is an effective drug for the treatment of hypercholesterolemia by lowering LDL-cholesterol. BABR absorbs BAs in the intestine, thereby preventing their uptake in the ileum, interrupting their enterohepatic circulation, and facilitating their excretion in the feces. The inhibition of the enterohepatic circulation leads to a reduction of the BA pool size, repression of FXR-SHP and FGF-15/19 signaling, and induction of CYP7A1 expression and synthesis of BAs from the cholesterol to maintain the BA pool size. A decrease in intrahepatic cholesterol levels activates SREBP-2, which induces the expression of the LDL receptor (LDLR) to enhance cholesterol uptake, reducing serum cholesterol levels. In addition to lowering the serum cholesterol effect, there is interaction between BABR and glucose

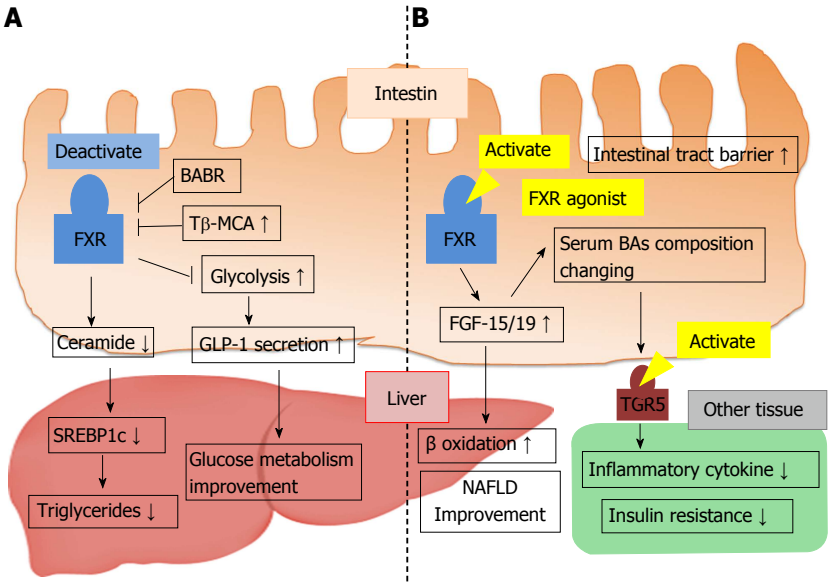


**Figure 5 Conflicting mechanisms of metabolic regulation *via* intestinal farnesoid X receptor activity.** A: FXR activation decreases hepatic TG levels and improves glucose metabolism; B: Intestinal FXR activation of FXR agonist leads to FGF-15/19 production and improves nonalcoholic fatty liver disease. Synthesized FGF-15/19 changes BA metabolism and serum BA composition, which causes TGR5/M-BAR activation, reduced inflammatory cytokine release, and improved insulin resistance. BABR: Bile acid binding resin; FGF-15/19: Fibroblast growth factor-15/19; FXR: Farnesoid X receptor; NAFLD: Nonalcoholic fatty liver disease; SREBP1c: Sterol regulatory element-binding protein 1c; Tβ-MCA: Tauro-β-muricholic acid; GLP-1: Glucagon-like peptide-1; TG: Triglyceride.

metabolism[84]. In a diet-induced obesity rat model, BABR decreased serum glucose and improved glucose tolerance[85,86]. In a clinical trial, cholestyramine, a first generation BABR, improved glycemia by 13% in patients with type 2 diabetes[87]. In addition, a second generation BABR also improved glucose clearance and increased serum GIP and GLP-1 levels in patients with type 2 diabetes mellitus[88]. These studies clarified that BABR is not absorbed in the body and there are few unwanted side effects. Furthermore, BABR can decrease blood glucose levels only in high glucose situations. As a result, in January 2008, this drug was approved as a therapeutic drug for diabetes by the Food and Drug Administration (FDA) in the United States[87,89-92].

Although how BABR improves diabetes remains unknown, several possible mechanisms have been proposed. BABR-mediated improvement of hepatic insulin sensitivity depends on downregulating the hepatic cholesterol-LXR-IRS2 pathway[93]. In addition, BABR induces GLP-1 secretion *via* the activation of TGR5/M-BAR or GPR40, each being activated by BAs binding with BABR or unabsorbed long-chain fatty acids[39,94,95]. Further, BABR affects the make-up of the BA pool and peripheral BAs, which results in the induction of peripheral energy expenditure and improved glucose tolerance[84]. The BABR effects of improving diabetes may be explained by the inhibition of intestinal FXR as well as TGR5/M-BAR signaling[80]. BABR colesevelam inhibits intestinal FXR activation and improves glucose metabolism by increasing proglucagon gene expression and inducing GLP-1 secretion in *ob/ob* mice[80]. These findings suggest that inhibiting FXR in the L cell *via* BABR could be a new target for diabetes.

## CLINICAL APPLICATION IN BA SIGNALING

Currently, BABR has been approved by the FDA and has been clinically used as a diabetes treatment drug. The association between bariatric surgery and BA homeostasis was confirmed. In addition to BABR and bariatric surgery, other clinical applications based on the mechanism of metabolic control *via* BA signaling are ongoing. For instance, INT-747 (also named 6-ethyl-CDCA), which is a synthetic FXR agonist, exerts a hepatoprotective effect in patients of primary biliary cirrhosis (PBC)[96-98], and a phase Ⅲ clinical study has already been completed and confirmed the effect of PBC. In addition to medicine, INT-747 has also entered into a study for NAFLD treatment. A phase Ⅱ clinical trial for NAFLD has been completed, and an improvement was observed in type 2 diabetes mellitus patients with NAFLD. Clinical trials with TGR5 agonists, such as INT-777, are ongoing, and future studies are expected[40,41,99].

Altogether, these clinical applications will elucidate the BA signaling mechanisms that will lead to the improvement of metabolic disorders including obesity and diabetes.

## CONCLUSION

Today, BAs have become important molecules to control metabolic homeostasis. In this review we discussed the relationship between BA metabolism and signal transmission, such as the FXR and TGR5/M-BAR pathways and the possibility that BAs may improve metabolic diseases. Current evidence shows that BAs regulate lipid,

glucose, and energy metabolism *via* FXR or TGR/M-BAR-mediated pathways. Furthermore, the clinical application of FXR and TGR/M-BAR agonists are ongoing.

Recent studies have focused on intestinal FXR signaling; however, conflicting data have been reported regarding the metabolic regulation of intestinal FXR activity. Further studies are necessary to determine intestinal FXR signaling taking into consideration various factors such as microbiota regulation, BA pool size, and BA composition.

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ORIGINAL ARTICLE

Basic Study

# Preliminary study on overproduction of reactive oxygen species by neutrophils in diabetes mellitus

Noridzzaida Ridzuan, Cini Mathew John, Pratheep Sandrasaigaran, Maryam Maqbool, Lee Chuen Liew, Jonathan Lim, Rajesh Ramasamy

Noridzzaida Ridzuan, Cini Mathew John, Pratheep Sandrasaigaran, Maryam Maqbool, Lee Chuen Liew, Rajesh Ramasamy, Immunology Laboratory, Department of Pathology, Faculty of Medicine and Health Sciences, Universiti Putra Malaysia, Serdang 43400, Selangor, Malaysia

Noridzzaida Ridzuan, Maryam Maqbool, Rajesh Ramasamy, Stem Cell Research Laboratory, Genetic and Regenerative Medicine Research Center, Faculty of Medicine and Health Sciences, Universiti Putra Malaysia, Serdang 43400, Selangor, Malaysia

Cini Mathew John, Department of Physiology and Pharmacology, Faculty of Medicine, University of Calgary, Calgary, AB T2N 4N1, Canada

Jonathan Lim, Department of Medicine, Faculty of Medicine and Health Sciences, Universiti Putra Malaysia, Serdang 43400, Selangor, Malaysia

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Informed consent statement: Informed consent was obtained from the voluntarily recruited patients. They were briefed on the purpose of the study and its implication prior to donating 10 mL of peripheral blood. History of medication and management of diabetes was collected with permission of the relevant patients.

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Correspondence to: Rajesh Ramasamy, PhD, Immunology Laboratory, Department of Pathology, Faculty of Medicine and Health Sciences, Universiti Putra Malaysia, Serdang Hospital Street, Serdang 43400, Selangor, Malaysia. rajesh@upm.edu.my
Telephone: +603-89-472377
Fax: +603-89-412787

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## Abstract

**AIM:** To assess the amount and pattern of reactive oxygen species (ROS) production in diabetic patient-derived neutrophils.

**METHODS:** Blood samples from type 2 diabetes mellitus (DM) patients and volunteers (controls) were subjected to neutrophil isolation and the assessment of neutrophil oxidative burst using chemiluminescence assay. Neutrophils were activated by using phorbol myristate acetate (PMA) and neutrophils without activation were kept as a negative control. The chemilu-

minescence readings were obtained by transferring cell suspension into a 1.5 mL Eppendorf tube, with PMA and luminol. Reaction mixtures were gently vortexed and placed inside luminometer for a duration of 5 min.

**RESULTS:** Our results showed that in the resting condition, the secretion of ROS in normal non-diabetic individuals was relatively low compared to diabetic patients. However, the time scale observation revealed that the secreted ROS declined accordingly with time in non-diabetic individuals, yet such a reduction was not detected in diabetic patients where at all the time points, the secretion of ROS was maintained at similar magnitudes. This preliminary study demonstrated that ROS production was significantly higher in patients with DM compared to non-diabetic subjects in both resting and activated conditions.

**CONCLUSION:** The respiratory burst activity of neutrophils could be affected by DM and the elevation of ROS production might be an aggravating factor in diabetic-related complications.

**Key words:** Neutrophils; Diabetes mellitus; Reactive oxygen species; Chemiluminescence



**Core tip:** This is a preliminary study that investigates the activation status of peripheral blood-derived neutrophils in type 2 diabetes. This study clearly indicated that the neutrophils from type 2 diabetic patients are highly activated upon *in vitro* stimulation and hence produce greater amounts of reactive oxygen species (ROS) compared to a normal individual. Release of a greater volume of ROS could serve as an additional risk for end organ injury in type 2 diabetes mellitus.

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## INTRODUCTION

Neutrophils are a crucial first line cellular host defence against infections as they are potent mediators of inflammation[1]. Elimination of pathogens by neutrophils follows a sequence of events such as adherence, chemotaxis, phagocytosis, microbial killing and apoptosis. The microbial killing by the formation of reactive oxygen species (ROS) and reactive nitrogen species (RNS) *via* activation of respiratory burst cascades plays a vital role in removing phagocytosed microbes[2]. ROS and RNS produced during the respiratory burst provide an important neutrophil-mediated defence system, yet the overproduction ROS can trigger vascular damage in chronic diseases such as hypertension and diabetes mellitus (DM)[3].

In recent years, the understanding of DM has changed from the perception of a chronic metabolic disease to an immune-mediated disease. Many expert reports advocate that DM may be a paradox of immune reactivity, which results in the development of the diabetic state and may lead to severe complications. The alteration of an innate immune response due to hyperglycemia has been recognised as the major factor resulting in the development of DM. Both type 1 and 2 DM are afflicted by the host immune system. Autoimmune T cells and autoantibody production against pancreatic beta cells are responsible for the development of type 1 DM, whereas the chronic low-grade inflammation and activation of the innate immune system are closely related to the pathogenesis of type 2 DM[4]. The elevated levels of inflammatory markers such as tumor necrosis factor-$\alpha$, interleukin-6 and C-reactive protein were seen in subjects with diabetes[5].

In diabetic patients, ROS is produced *via* glucose autoxidation and non-enzymatic protein glycation in various tissues such as neural cells, lens crystalline and recently reported in pancreatic $\beta$-cells[4,6]. Since the activity of antioxidant enzymes in the pancreas is relatively low compared to other tissues, the pancreas is one of the organs sensitive to an oxygen radical attack[7]. Apart from that, the uncontrolled release of free radical nitric oxide (NO) from endothelium also possesses a toxic effect on microvasculature[8,9]. The excessive and ill-controlled NO and ROS can react to form peroxynitrite anion, a highly reactive and toxic compound which rapidly decomposes to hydroxyl anion and nitrogen dioxide[10]. This possibly leads to cytotoxic and cytostatic effects on parenchymal cells and heads to irreversible pathologies[11,12]. Overall, ROS formation is considered a direct consequence of hyperglycemia. Hence the glycation process and the subsequent oxidative stress pave a way to the detrimental effects of DM[13].

The prime effector function of a neutrophil relies on its ability to generate ROS within the phagolysosome for the degradation of engulfed pathogens. However, the excessive or inappropriate production of these reactive compounds may lead to detrimental effects such as hypertension, atherosclerosis and DM. The elevated oxidative stress which results from superoxide release by neutrophils in the diabetic condition is well documented[14-17]. The assessment of neutrophil-mediated respiratory burst activity from Hispanic diabetic individuals demonstrated a significant rise in ROS outburst compared to the normal group. Interestingly, upon treatment with PKC inhibitors and azithromycin, the magnitude of the respiratory burst response was substantially reduced[14]. Similarly, the high levels of glucose and AGE also induced neutrophil activation and subsequently escalated the oxidative stress *via* the RAGE-ERK1/2 pathway[15]. It is clear that the harmful effect of ROS is

**Table 1 Demographics and characteristics of the patients with type 2 diabetes mellitus**

| Patient | Age | Duration (yr) | HbA1c (%) | Family history | Types of medication |
|---|---|---|---|---|---|
| 1 | 65 | 12 | 6.2 | Father and mother | Metformin, atorvastatin and multivitamins |
| 2 | 72 | 17 | 6.6 | Mother | Glibenclamide, insulin (humapen), ecospirin and viatril S |
| 3 | 82 | 28 | 6.1 | - | Metformin, insulin (mixtard), cardipirin, prostin and lovastatin |
| 4 | 69 | 11 | 6.3 | Father | Diamicron MR, simvastatin |
| 5 | 65 | 13 | 6.2 | Mother | Herbal medication, glibenclamide and metoprolol |
| 6 | 64 | 14 | 7.2 | Father | Insulin, metformin, simvastatin and captopril |

very much linked with the augmented production of the advanced glycated end-products (AGE) and their cognate receptors (RAGE). The ligation between AGE and RAGE potentially increases the cytosolic ROS and facilitates mitochondrial superoxide production in the hyperglycemic condition[18]. Although the actual mechanism that governs the production and release of ROS in diabetic patients' neutrophils is still elusive, it does not negate the possible role of damaged mitochondria to generate an excess amount of superoxide which is fuelled by a sustained supply of NADH[19].

Despite growing data which show the role of oxidative stress in the etiology and pathophysiology of DM, there are no consistent results of ROS overproduction in diabetic patients[4,6-9]. Therefore, the current pilot study was designed to assess the pattern of ROS production in type 2 DM. The outcome of this present study showed that neutrophils from patients with DM constitutively secrete a significantly higher volume of ROS in both resting and activated conditions.

## MATERIALS AND METHODS

### Subjects

This is an experimental study where a total of 6 type 2 DM patients (duration of disease 11-28 years) aged 64-82 years were included in this study. The patients and normal subjects were voluntarily recruited, briefed on the purpose of the study and verbal consent was obtained. Patients were selected based on the inclusion criteria of type 2 DM for more than 10 years duration and the age range of 60-82 years. The exclusion criteria were patients undergoing dialysis, inflicted with anemia, polycythemia and gout or had a history of severe immunological, hepatic, cardiac, renal, hematological or other organ impairment. The details of HbA1c and anti-diabetic treatment of each patient were extracted from the latest laboratory screening. The particulars of the patients are shown in Table 1. Samples of the non-diabetic control group were obtained from 3 volunteers aged between 30-50 years old.

### Blood sampling

Ten millilitres of a peripheral venous blood sample from diabetic patients and non-diabetic individuals were collected by a certified phlebotomist. Whole blood was collected in two 9 mL vacutainers with sodium heparin as anticoagulant (Greiner bio-one, Australia). Peripheral blood samples were processed immediately after the collection.

### Neutrophil isolation

Neutrophil isolation and verification by morphology were conducted as per our established laboratory procedures[3,20]. Briefly, 10 mL of peripheral blood was collected and diluted in 1 × Hank's balanced salt solution (HBSS Gibco, United Kingdom) medium at 1:1 ratio. Ten millilitres (10 mL) diluted blood was then layered over 5 mL Ficoll-Paque solution (GE Health care, life sciences, Sweden) and centrifuged at 1800 rpm for 30 min at room temperature. Once the unwanted mononuclear cells and plasma were decanted, the red cell pellet which contains the polymorphonuclear cells (PMN) was suspended in 5 mL HBSS, layered on 3% Dextran (Fisher Scientific, NJ, United States) and sedimented at room temperature for 45-60 min. The sedimented supernatant was further subjected to the RBC lysing procedure to obtain uncontaminated PMN. Leishman staining was performed to confirm the neutrophil morphology. Briefly, a few drops of the neutrophil suspension were spread on a glass slide and covered with Leishman solution (Merck, Germany) for 1 min. Subsequently, the smear was immersed in phosphate buffer solution for 15 min. The slide was rinsed off with tap water, dried and examined under the light microscope at 20 × and 40 × magnifications. The viability of cells was determined by trypan blue exclusion during the manual cell counting process.

### Assessment of neutrophil oxidative burst

Oxidative burst by human neutrophils was measured by production of ROS through chemiluminescence assay. Freshly isolated neutrophils ($0.5 \times 10^5$ cells/well) were seeded in complete Roswell Park Memorial Institute medium without phenol red (Gibco, United Kingdom) and were concurrently activated with 500 nmol/L phorbol myristate acetate (PMA) (Sigma, Germany). Neutrophils without activation were kept as a negative control. Cells and the tested compound were maintained at 37 ℃ in a water bath prior to adding the stimulants. Readings were obtained by transferring the cell suspension into a 1.5 mL Eppendorf tube, with 100 μL PMA and 100 μL luminol (500 μmol/L). Reaction mixtures containing a final volume of 1000 μL were gently vortexed and placed inside Glomax luminometer 20/20 (Promega) for a duration of 5 min.

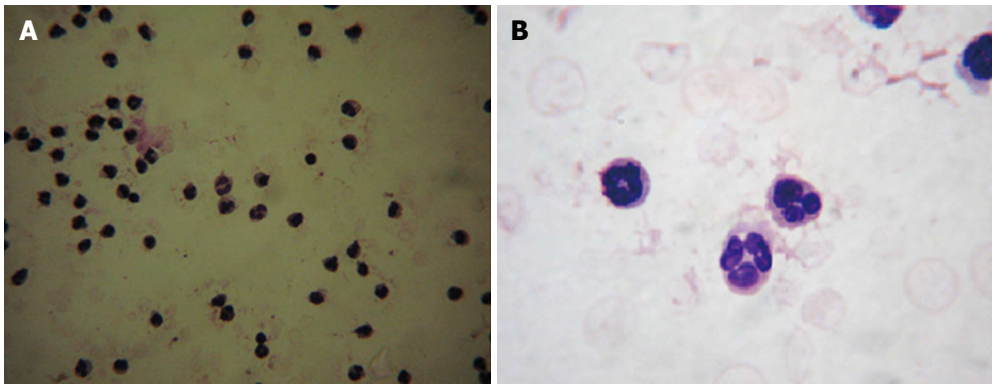


**Figure 1 Morphological analysis of isolated neutrophils.** Isolated neutrophils were smeared on a glass slide and labeled with Leishman stain. Under the 20 × magnification, cells appeared homogeneous (A) and 40 × magnification exhibited the multi-lobulated nucleus (B).

### *Statistical analysis*

Data are expressed as mean ± SD[21]. Differences were considered significant at $P \leqslant 0.001$. Statistical analyses were conducted using 2-way ANOVA using Microsoft Office 2007 (Excel).

## RESULTS

### *Isolation and confirmation of neutrophils*

Employing density gradient centrifugation followed by a dextran sedimentation procedure, peripheral blood from non-diabetic individuals and diabetic patients were fractioned into several layers. The layer enriched with neutrophils was further subjected to the hypotonic red blood lysis to obtain a pure population of neutrophils. Isolated neutrophils were labeled with Leishman staining to confirm the purity of neutrophils. The isolation process yielded more than 85% of the pure neutrophil population. Examination under the light microscope with low magnification revealed that isolated cells were uniform and free from RBC and mononuclear cell contamination (Figure 1A). The morphology of neutrophils was further confirmed by microscopical examination with higher magnification where cells displaying a 3-5 lobulated nucleus were confirmed as neutrophils (Figure 1B).

### *Neutrophils from diabetic patients constitutively produced a higher amount of ROS in a resting condition*

In a resting condition, both neutrophils that were isolated from non-diabetic individuals and diabetic patients showed a basal amount of ROS production. It was noticed that neutrophils from non-diabetic individuals secreted ROS approximately 8000 chemiluminescence counts (CC) at the initial 10 s and the secretion declined with time when the lowest 2000 CC was noticed at 50 s. However, neutrophils from diabetic patients exhibited a statistically significant increase in ROS secretion (13000-14000 CC) ($P \leqslant 0.001$) and the amount was maintained throughout the measurement points up to 50 s (Figure 2). In order to determine whether a similar ROS production pattern could be observed when stimulated, a potent microbial agent, PMA was used to induce the neutrophil's ROS secretion. As expected, PMA profoundly increased ROS secretion at all measured time points regardless of the source of neutrophils. The secretion of ROS from neutrophils in non-diabetic and diabetic subjects was elevated at approximately 13000 CC and 21000 CC, respectively. The amount of ROS secreted from activated neutrophils of non-diabetic and diabetic subjects was maintained throughout the measurement points. Besides that, neutrophils from both non-diabetic and diabetic subjects displayed a maximal amount of secretion at the initial 10 s of the measurement period.

### *Neutrophils from diabetic patients secreted higher ROS in both resting and activated conditions compared to the non-diabetic subjects*

The maximal production of ROS by neutrophils of non-diabetic and diabetic subjects during any time point of the measured period was recorded. In a resting condition, the maximal amount of ROS produced by neutrophils of non-diabetic and diabetic subjects were 5000 CC and 13000 CC, respectively, whereas, PMA activated neutrophils of non-diabetic and diabetic subjects secreted 14000 CC and 20000 CC, respectively. In both resting and activated conditions, neutrophils from diabetic patients showed a statistically significant elevation of ROS production. However, a radical escalation index of ROS was noticed in the resting condition where ROS secreted from neutrophils of diabetic patients was approximately 2.8 fold higher than non-diabetic controls (Figure 3).

## DISCUSSION

Neutrophils play a major role in innate immunity by executing acute inflammation due to infectious agents. Unlike other immune cells, neutrophils exist in peripheral blood in a larger quantity and the loss of neutrophils due to inflammation is rapidly substituted by its production in bone marrow. Due to its important role in the early inflammatory process, neutrophils are able to migrate towards the site of inflammation through the tight epithelial junction. The most critical effector functions of

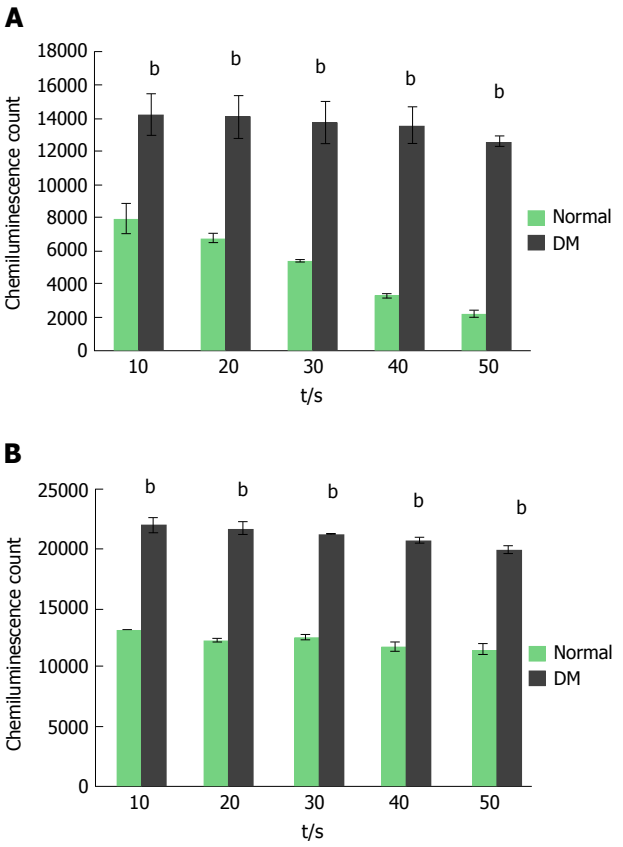


**Figure 2 Temporal analysis of reactive oxygen species secreted by neutrophils of non-diabetic and diabetic subjects.** Isolated neutrophils were measured for ROS secretion *via* chemiluminescence method at every 10 s over a period of 50 s. Neutrophils isolated from diabetic patients showed a higher and consistent secretion of ROS in resting condition (A). PMA stimulation elevated the ROS secretion from neutrophils of both non-diabetic and diabetic subjects. Neutrophils from diabetic patients produced a higher amount of ROS compared to non-diabetic subjects. ROS production was determined at every 10 s beginning from the administration of PMA to the time point where the ROS production reached a maximal level and started to decline (B). Data expressed as mean ± SD and the statistical significance was determined at $^{b}P$ value ⩽ 0.001. ROS: Reactive oxygen species; PMA: Phorbol myristate acetate.

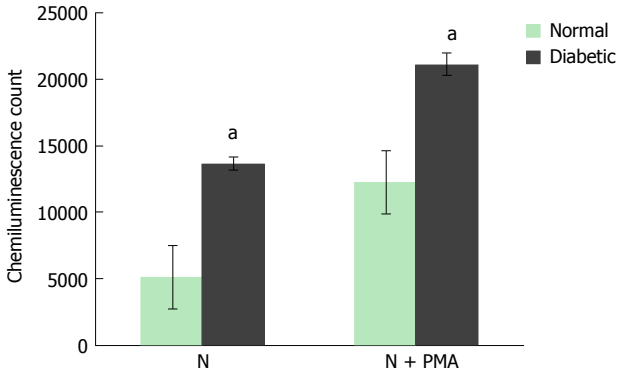


**Figure 3 Total reactive oxygen species produced by neutrophils of non-diabetic and diabetic subjects.** Neutrophils isolated from non-diabetic individuals and diabetic patients subjected for ROS secretion at resting condition and upon stimulation with PMA. The maximal production of ROS at any time point over a period of 50 s was recorded and compared. Neutrophils from diabetic patients showed a higher production of ROS at resting and activated conditions. Data expressed as mean ± SD and the statistical significance was determined at $^{a}P$ value ⩽ 0.05. ROS: Reactive oxygen species; PMA: Phorbol myristate acetate.

neutrophils are phagocytosis and killing the invading bacteria through the activation of the respiratory burst. Respiratory or oxidative burst in neutrophils starts with a consequent formation of ROS, such as superoxide radicals ($O_2$-) and hydrogen peroxide ($H_2O_2$), and RNS, such as NO and peroxynitrate anion (ONOO-)[22]. Besides inflammation induced by pathogens, neutrophils can be activated to produce free oxygen radicals and other superoxide derivatives with a variety of stimuli, such as the chemotactic peptide N-formyl methyl leucyl phenylalanine, the anaphylatoxin C5a, platelet-activating factor, leukotriene B, PMA and calcium ionophores[23-29].

Oxidative stress resulting from a raised ROS level has become a common reflection of many chronic illnesses[3]. The present preliminary study showed that neutrophils from type 2 DM produced a significantly higher amount of ROS compared to non-diabetic individuals in both resting and activated conditions. The current result is in line with Houstis *et al*[30] (2006) who demonstrated the involvement of increased ROS production in insulin resistance in type 2 DM using a cell culture model and murine models. Conversely, Alba-Loureiro *et al*[31] (2007) reported the secretion of ROS in DM was indeed reduced compared with normal controls. This observation was based on the assessment of neutrophil activities, such as chemotaxis, phagocytosis, ROS production and microbial killing, where these activities consume a substantial amount of ATP. Since diabetes affects energy metabolism, it could also result in down-regulation or a decrease in neutrophil activities. The pattern of ROS production in patients with DM is not consistent and may be due to many reasons. The inclusion of patients undergoing a specific regime of drugs that lower the overall oxidative stress might have confounded the experimental outcomes. Besides that, utilization of different techniques to isolate neutrophils and detect ROS might possibly affect the final volume of ROS secreted. The laboratory procedures such as hypotonic lysis of RBC for isolating neutrophils from peripheral blood and the long duration of sample processing may potentially affect the viability of neutrophils, thus reflected as reduced ROS secretion. In the current study, the isolation of neutrophils was conducted within 2-3 h of peripheral blood withdrawal and a chemiluminescence technique was utilised for detecting ROS over a period of time. This technique is highly sensitive as it utilizes luminol which allows detection of intracellular and extracellular ROS and RNS, such as $O_2$-, $H_2O_2$, hydroxyl radical (HO), hypochlorous acid (HOCl), NO and ONOO-. Furthermore, morphological observation of isolated neutrophils displayed a healthier appearance and high viability.

This present study also revealed that as the time increased during ROS measurement over a total of 50 s, the ROS production in neutrophils of resting non-diabetic subjects constantly reduced, whereas the level of ROS from neutrophils of diabetic patients was maintained throughout the measurement period. The constant

and elevated secretion of ROS at all the time points by neutrophils of diabetic patients could be a consequence of the raised oxidative burst in DM. However, this could be a serious phenomenon where neutrophils of diabetic patients may have lost the ability to switch off or tame down the respiratory burst activity, hence predisposing the patients to microvascular injuries. The continuous suboptimal activation of the respiratory burst in neutrophils of diabetic patients may also deplete ROS or exhaust the mechanism that generates ROS, rendering neutrophils inefficient and thus increasing susceptibility to microbial infection. This could also be a potential cause for slower wound healing and occurrence of gangrene in diabetic patients. When neutrophils were stimulated with a robust infectious agent-derived substance, PMA, neutrophils of both non-diabetic and diabetic subjects secreted a tremendous amount of ROS. Our results demonstrated that neutrophils responded well to PMA stimulation, as reported by Ramasamy *et al*[3] in 2010. Although the current study did not decipher the potential signaling pathway that might be involved in PMA stimulation, human neutrophils showed that the activation of the protein kinase C (PKC) signaling cascade serves as an inducer of rapid ROS synthesis. Notably, the 2.8 fold increase of secreted ROS between non-diabetic and diabetic subjects in a resting condition was much higher compared to the induced activated condition. This supports our notion that neutrophils from diabetic patients are in a state of auto ROS secretion, which explains the possible contribution to microvascular injuries in DM.

The overproduction of ROS by the neutrophil-mediated respiratory burst can be controlled either *via* inhibition of ROS-generating enzymes, NADPH oxidase or through the direct ROS-scavenging effect. In the physiological condition, the activation of NADPH oxidase in generating ROS in neutrophils is beneficial for host defence. In this case, overproduction of free radicals and proteolytic enzymes used as defences against infections can be highly toxic to the surrounding cells and tissues[32]. Nevertheless, it can be deleterious to the host if the enzyme cascade is inappropriately activated or loses its control. Hence, drugs such pyrazolones and its derivatives such as aminopyrine and dipyrone can be used in the management of DM. The study conducted by Costa *et al*[32] (2006) showed that these drugs not only normalize the glucose level but scavenge the overproduced neutrophil reactive species. Besides that, plasma glucose level should also be strictly controlled as hyperglycemia promotes the production of ROS by affecting the first-phase of glucose-induced insulin secretion through the suppression of GAPDH activity[33]. However, the outcomes of this study should be further evaluated with a larger sample size as the current study was conducted with a very limited number of diabetic patients. Although the overall pattern of ROS secretion in other chronic metabolic diseases is similar to the current study, a stringent sample selection with a statistically required sample size will add value to future studies. Moreover, this study was conducted with a conventional yet highly specific and sensitive technique which is laborious in nature due to two critical processes, namely the neutrophil isolation and measurement of ROS. Opting for a much-advanced technique such as flow cytometer using unfractionated peripheral blood will potentially cut down the tedious laboratory procedures and allow investigation of a larger sample size. Nonetheless, harnessing the semi-activated neutrophils in DM could serve as an auxiliary therapy that maintains the oxidative/anti-oxidative balance and integrity of the immune system.

Our pilot study employed a sensitive and specific method, a chemiluminescence technique to measure the ROS production by neutrophils from non-diabetic individuals and diabetic patients. Neutrophils from diabetic patients showed a constitutive and elevated level of respiratory burst compared to non-diabetic individuals in resting and activated conditions. The data from this preliminary study revealed an inherent disability of diabetic-derived neutrophils in regulating ROS secretion; however, such a pathological condition should be verified by a larger sample size and well-designed research study.

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## COMMENTS

### Background

Diabetes mellitus is a metabolic disorder that is often associated with vital organ failure if untreated. The excessive production and release of reactive oxygen species (ROS) are known as key factors that contribute to diabetic complications. Particularly, neutrophils are much more susceptible to the hyperglycemic condition and significantly contribute to the severity of diabetic complications by spontaneously releasing an abundant amount of ROS.

### Research frontiers

Controlling the excessive release of ROS by neutrophils could serve as a promising tool in managing or preventing diabetic complications. Inhibiting a relevant signaling pathway that governs the release or production of excessive ROS can be exploited therapeutically.

### Innovations and breakthroughs

The current preliminary study strengthens the existing laboratory and clinical data where the vulnerability of diabetic patients' derived neutrophils to release an excessive amount of ROS at both resting and activated conditions was noted.

### Applications

Reduction of ROS release in neutrophils could serve as an axillary therapy in managing diabetic complications.

### Terminology

ROS: Reactive oxygen species.

### Peer-review

This research article is very well written, clearly presenting augmented ROS production in type 2 diabetes mellitus patients' neutrophils for the first time.

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