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Contents

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Contents

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Management of type 2 diabetes mellitus in youth

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Abstract

The rising rates of obesity in youth have concurrently led to an increase in the rates of type 2 diabetes mellitus (T2DM) in this age group. However, there are limited data on the efficacy of different antidiabetic agents in youth. In this context, the Treatment Options for Type 2 Diabetes in Adolescents and Youth trial recently reported that the majority of obese children and adolescents 10-17-years old with newly diagnosed T2DM (T2DM duration less than 2 years) could not achieve HbA1c levels < 8% for more than 1 year with metformin monotherapy, metformin plus rosiglitazone combination, or metformin and lifestyle changes. These findings suggest that, in the majority of youth with T2DM, tight long-term glycemic control with oral agents is an elusive goal and that most patients will require treatment with insulin within a few years of diagnosis to achieve HbA1c targets and reduce the risk of macro- and microvascular complications. Therefore, reducing the incidence of T2DM by preventing pediatric obesity through the implementation of lifestyle changes in the community should be the primary objective of healthcare systems.

Key words: Type 2 diabetes mellitus; Metformin; Rosiglitazone; Lifestyle changes; Insulin

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INVITED COMMENTARY ON HOT ARTICLES

Obesity is becoming increasingly prevalent in children and adolescents, putting considerable burden on public healthcare services[1,2]. According to the 2007-2010 National Health and Nutrition Examination Survey data, 16.9% of 6- to 19-year-old in the United States are obese[2]. These rising rates of obesity in youth have concurrently led to an increase in the rates of type 2 diabetes mellitus (T2DM) in this age group[3]. The overall prevalence of T2DM in youth is 0.22 cases per 1000[4] and it is estimated that T2DM accounts for 15% to 86% of newly diagnosed cases of diabetes mellitus in ages 10-19 years with the higher prevalence rates reported among ethnic minorities[5].

Despite the increasing rates of T2DM in youth, there are limited data on the efficacy of different antidiabetic agents in this age group. Furthermore, additional dif-

ficulties emerge during the treatment of this special population, including the psychological and emotional changes of adolescence as well as particularities of the specific familial and socioeconomic environment[6,7].

In this context, the Treatment Options for Type 2 Diabetes in Adolescents and Youth (TODAY) trial provides new insights on the management of this understudied group of patients[8]. This multicenter study included children and adolescents 10- to 17-year-old who had T2DM for less than 2 years (mean T2DM duration 7.8 mo) and body mass index (BMI) ⩾ the 85th percentile for age and sex. Of the 1211 subjects who were screened, 927 patients entered a run-in phase during which metformin was administered at a dose of 1000-2000 mg/d to achieve HbA1c levels < 8%. At the end of the run-in period, 699 patients were randomly assigned to continue metformin monotherapy at a dose of 1000 mg twice daily, to receive metformin and rosiglitazone 4 mg/d combination treatment, or to receive metformin and lifestyle intervention focusing on weight loss through family-based changes in eating and activity behaviors. The primary endpoint was treatment failure, defined as HbA1c levels persistently ⩾ 8% over a 6-mo period or persistent metabolic decompensation (i.e., inability to discontinue insulin within 3 mo after its initiation for decompensation or recurrent decompensation within 3 mo of stopping insulin). Patients were followed-up for a mean of 3.86 years.

Treatment failure occurred in 51.7% of patients in the metformin monotherapy group (95% CI 45.3-58.2), in 38.6% of patients treated with metformin plus rosiglitazone combination (95% CI 32.4-44.9), and in 46.6% of patients managed with metformin and lifestyle modification (95% CI 40.2-53.0)[8]. Metformin plus rosiglitazone treatment reduced the occurrence of treatment failure by 25.3% compared with metformin monotherapy (P = 0.006). Treatment failure rates did not differ significantly between patients treated with metformin combined with lifestyle intervention and patients treated with either metformin monotherapy or metformin combined with rosiglitazone. The median time to treatment failure was 11.5 mo (range, < 1 to 66 mo) and did not differ between the 3 groups. The BMI increased significantly more in patients treated with metformin plus rosiglitazone than in the other groups. The group that received metformin and lifestyle intervention exhibited less BMI increase than patients treated with metformin monotherapy. However, neither BMI at baseline nor BMI during treatment predicted treatment failure. Adherence to treatment was 57% at month 60 and did not differ between the 3 groups. Changes in blood pressure and lipids were also comparable in the 3 groups. Serious adverse events were reported by 18.1%, 14.6% and 24.8% of patients treated with metformin alone, metformin plus rosiglitazone, and metformin plus lifestyle intervention, respectively (P = 0.02). The most frequent adverse effects in all groups were infections, gastrointestinal symptoms, rash, muscle ache and elevation of liver enzymes.

Until now, metformin and glimepiride are the only oral agents approved by the Food and Drug Administration for the treatment of children with T2DM[9]. Although metformin is recommended as first-line treatment in this age group[10], the TODAY study showed that in children and adolescents who have T2DM for < 2 years, metformin maintains optimal glycemic status in < 50% of patients after 1 year. When metformin monotherapy does not achieve HbA1c targets, sulphonylureas are the most frequently added oral agents[10]. However, sulphonylureas are associated with weight gain and increase the risk for hypoglycemia[11]. Unfortunately, the TODAY study did not include a sulphonylurea arm and the benefit/risk ratio of metformin plus sulphonylurea combination in this age group remains unclear. Nevertheless, in adults with newly diagnosed T2DM, sulphonylurea monotherapy maintains HbA1c targets after 3 years in < 50% of patients[12]. On the other hand, in adults, rosiglitazone monotherapy appears to be associated with more sustained glycemic control than monotherapy with either metformin or sulphonylureas[13]. Nevertheless, rosiglitazone has been withdrawn from Europe and its use is restricted in the United States because it appears to increase the risk for myocardial infarction[14]. Pioglitazone, the other member of the thiazolidinediones class, does not appear to increase cardiovascular risk[15], but both agents are associated with weight gain, edema and increased risk for heart failure and fractures[11]. Moreover, pioglitazone was recently withdrawn from France because of increased risk for bladder cancer[16,17]. In addition to these safety concerns, almost 40% of patients treated with metformin plus rosiglitazone combination in the TODAY study could not maintain HbA1c levels < 8% after 1 year. Therefore, the efficacy of adding rosiglitazone in this age group also appears to be suboptimal. It should also be emphasized that treatment failure rates did not differ in the TODAY study between patients treated with metformin plus rosiglitazone and patients given metformin and lifestyle advice.

Overall, the findings of the TODAY study suggest that, in order to achieve optimal glycemic control, the majority of children and adolescents with T2DM will require treatment with insulin within a few years after diagnosis[8]. Even though insulin can achieve sustained normalization of HbA1c levels, it has the drawbacks of weight gain and elevated risk of hypoglycemic episodes[11,18]. In addition, the parenteral administration of insulin is an important barrier for the introduction of this treatment[19]. Moreover, the need in some cases for multiple daily injections to optimize glycemic control hampers the intensification of insulin treatment[19]. Common misperceptions of patients regarding insulin, including the belief that it represents failure of oral agents or a sign of uncontrolled diabetes with a higher risk for long-term complications, are additional obstacles for initiating insulin[20]. In addition, after the introduction of insulin, adherence is lower than those with oral

antidiabetic agents[19]. The issue of adherence to treatment is particularly pertinent to adolescents[18]. Indeed, in the TODAY study, only 57.6% of patients adhered to treatment with oral antidiabetic agents[8]. Moreover, satisfaction with antidiabetic treatment, which is directly correlated with adherence, is lower in patients treated with insulin than in those who receive oral agents[20].

In conclusion, the findings of the TODAY study suggest that, in the majority of youth with T2DM, tight glycemic control is an elusive goal with oral agents even in the context of a clinical trial involving presumably motivated patients. Therefore, achieving HbA1c goals will probably be even more difficult in everyday clinical practice. It remains to be established whether newer antidiabetic agents, particularly dipeptidyl-peptidase Ⅳ (DPP-Ⅳ) inhibitors and glucagon-like peptide 1 (GLP-1) analogues, will provide more sustained glycemic control in adolescents with T2DM. These agents have the advantage that they either not cause weight gain (the DPP-Ⅳ inhibitors) or induce weight loss (the GLP-1 analogues) and are considered second line treatment in adult diabetic patients who cannot achieve glycemic targets with metformin monotherapy[21]. However, they are not currently licensed for use in patients younger than 18 years. Accordingly, reducing the incidence of T2DM by preventing pediatric obesity through lifestyle changes should be the primary objective of healthcare systems. Randomized trials in adults showed that diet and exercise reduces the risk of T2DM in patients with impaired fasting glucose or impaired glucose tolerance[22,23]. However, long-term adherence to lifestyle changes is difficult to achieve, particularly in adolescents, as shown in the TODAY and other studies[8,24]. Therefore, implementing healthcare policies to address causes of low adherence to lifestyle modifications, including low socioeconomic and educational status, limited health care accessibility and family problems[25,26], are imperative to prevent the development of obesity and T2DM in children and adolescents.

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REVIEW

Smoking in diabetic nephropathy: sparks in the fuel tank?

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Abstract

Diabetic nephropathy is associated with high morbidity and mortality and the prevalence of this disease is continuously increasing worldwide. Long-term diabetes increases the likelihood of developing secondary complications like nephropathy, the most common cause of end stage renal disease. Usually, other factors like hypertension, alcoholism and smoking also partly contribute to the progression of diabetic nephropathy. Among this, cigarette smoking in diabetes has been repeatedly confirmed as an independent risk factor for the onset and progression of diabetic nephropathy. Various studies suggest that smoking is a major fuel in the development of high oxidative stress and subsequently hyperlipidemia, accumulation of advanced glycation end products, activation of the renin angiotensin system and Rho-kinase, which are observed to play a pathogenic role in the progression of diabetic nephropathy. Furthermore, cigarette smoking in diabetic patients with vascular complications produces a variety of pathological changes in the kidney, such as thickening of the glomerular basement membrane and mesangial expansion with progression in glomerulosclerosis and interstitial fibrosis, which ultimately results in end stage renal failure. Strong associations are consistently found between chronic cigarette smoking and diabetic microvascular complications. A diverse group of studies unveil potential mechanisms that may explain the role of cigarette smoking in the progression of diabetic nephropathy. Tremendous efforts are being made to control smoking mediated progression of diabetic nephropathy, but no promising therapy is yet available. The present review critically discusses the possible detrimental role of chronic cigarette smoking in the progression of diabetic nephropathy and various possible pharmacological interventions to attenuate the exacerbation of diabetic nephropathy.

Key words: Smoking; Nicotine; Oxidative stress; Hyperlipidemia; Diabetic nephropathy

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INTRODUCTION

Diabetic nephropathy is considered to be one of the major complications of diabetes mellitus and its prevalence is continuously progressing worldwide. Progression of this disease is further accelerated by the partial or complete contribution of various factors, such as hypertension, chewing tobacco, alcoholism and smoking. Predominantly, nicotine exposure via chronic cigarette smoking is an emerging cause that accelerates the microvascular complications in diabetes mellitus[1]. The pathophysiological mechanisms underlying the health effects of cigarette smoking in diabetes are complex.

Diabetic smokers are usually associated with glomerular hypertrophy, glomerulosclerosis, tubulointerstitial fibrosis and mesangial cell expansion, followed by albuminuria and reduction in the glomerular filtration rate[2,3]. Thus, establishing the causes of smoking mediated progression of diabetic nephropathy remains the key step towards the prevention and amelioration of this disease.

In diabetic microvasculature, a high level of glucose probably synergizes with superimposed stresses such as oxidative stress and hyperlipidemia to continue the stress on vascular cells, which is further increased by smoking. The potential risk factors involved in the progression of diabetic nephropathy remain unclear, although smoking mediated hastening of oxidative stress and hyperlipidemia play partial but key roles in this. Chronic and uncontrolled diabetes mellitus is often coupled with oxidative stress, a risk factor for diabetic microvascular complications. Furthermore, nicotine (an active constituent of a cigarette) exposure is noted to promote excessive oxidative stress in diabetes[3]. Together, diabetes and smoking mediated increased oxidative stress subsequently leads to vascular endothelial cell dysfunction (VED), which is one of the earliest metabolic consequence of chronic hyperglycemia[3-5]. In addition to this, both hyperglycemia and smoking (nicotine) downregulates endothelial nitric oxide synthase (eNOS), an enzyme involved in the generation of NO, decreases endothelium dependent vasodilation and results in VED, which is subsequently involved in the pathogenesis of diabetic nephropathy[5-7]. Evidently, diabetes mediated oxidative stress upregulates the expression of transforming growth factor-β (TGF-β), a prosclerotic and profibrogenic cytokine, which again is implicated in the pathogenesis of nephropathy[8,9]. Similarly, nicotine has also been noted to upregulate the expression of TGF-β and is involved in the pathogenesis of diabetic nephropathy[8]. Thus, TGF-β is considered to be another common pathway for diabetes and smoking to worsen the nephropathy.

Hyperlipidemia is considered another major risk factor implicated in the progression of diabetic nephropathy[10]. Dyslipidemia is a condition associated with hypertriglyceridemia, elevated low density lipoprotein (LDL) levels and decreased high density lipoprotein (HDL) levels. Diabetes mediated hyperlipidemia is noted to be responsible for the progression of nephropathy in rats[5]. The astonishing fact is that nicotine causes an impairment of lipoprotein lipase (LPL), an enzyme involved in the hydrolysis and clearance of triglyceride (TG) from the circulation, and thus causes hyperlipidemia[11,12]. Furthermore, smokers have higher serum concentrations of TG and LDL and lower serum concentrations of HDL compared with non-smokers[13]. This indicates that smoking independently contributes to hyperlipidemia and/or dyslipidemia-like conditions. Although the precise mechanism involved in diabetes-associated dyslipidemia is not clear, the insulin resistance in type Ⅱ diabetes mellitus could play a key role in elevating lipid levels[2,10]. In diabetic patients, high lipids could induce renal injury by stimulating TGF-β and thereby inducing the generation of reactive oxygen species (ROS) to damage the glomeruli, showing that diabetic hyperlipidemia accelerates reno-vascular complications[10]. Cigarette smoking associated hyperlipidemia has been identified as a progression factor in the development of diabetic reno-vascular complications[14]. A study illustrated that diabetes mellitus may mediate renal injury by increasing the renal expression of sterol regulatory element-binding protein-1 (SREBP-1), which is responsible for increasing the synthesis of TGs and cholesterol, that are further associated with upregulation of TGF-β and could play a pivotal role in the pathogenesis of glomerulosclerosis and tubulointerstitial fibrosis[15]. One potential explanation for supporting the intricate effects of nicotine is that smoking stimulates renal lipid accumulation by increasing expression of SREBP-1, which increases the synthesis of TGs and cholesterol[16]. In addition to this, diabetes mediated activation of Rho-kinase and advanced glycation end products (AGEs)-like factors also participate in the pathogenesis of nephropathy[17,18]. Taken together, it could be indubitably suggested that cigarette smoking could induce and worsen diabetic nephropathy. Therefore, initial interest focuses on strict glycemic control and subsequently smoking secession. Thus, identifying the major culprits and path of their involvement in the pathogenesis of diabetic nephropathy may open a vista in exploring novel therapeutic agents to ameliorate the induction and progression of this disease.

OPTIMISTIC CONTRIBUTION OF DIABETIC OXIDATIVE STRESS AND HYPERLIPIDEMIA IN THE DEVELOPMENT OF NEPHROPATHY

In diabetes, chronic hyperglycemia is the single most important factor in the generation of sustained oxidative stress. Under normal physiological conditions, a homeostatic balance exists between the formation of ROS and their removal by endogenous antioxidant compounds. However, oxidative stress occurs when this balance is disrupted by excessive production of ROS and decreasing endogenous antioxidants, probably due to chronic hyperglycemia[19,20]. ROS encompasses diverse chemical species, including superoxide or hydroxyl, are produced by oxygen metabolism and play a major part in cell signaling, aging and microvascular diseases[21]. ROS acts as intracellular messengers and integral glucose signaling molecules in the diabetic kidney. The metabolism of glucose through harmful alternate pathways, such as glycolysis, specific defects in the polyol pathway, nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, advanced glycation and uncoupling of nitric oxide

synthase, are mainly responsible to generate ROS[9]. In physiology, the unused glucose in the cytosol is diverted to the polyol pathway, where the aldose reductase reduces it to sorbitol by utilizing cofactor NADPH from the pentose phosphate pathway. In addition, chronic hyperglycemia causes excessive consumption of NADPH in the polyol pathway and the net effect is the generation of ROS[21].

Most estimates suggest that the excessive generation of ROS in diabetes precedes endothelial dysfunction by decreasing eNOS expression and decreasing NO production[5,19,22,23]. Thus, the impaired ability of endothelial cells to modulate the vascular tone is a result of low bioavailability of nitric oxide in the vascular lumen. Furthermore, in diabetes, low levels of nitric oxide in endothelial cells may potentially result in ineffective suppression of ROS and could indirectly lead to enhanced vasoconstriction. This contention was further confirmed in subsequent *in vitro* and *in vivo* studies, which explains the enhancement in superoxide generation that ultimately increases endothelin- I production in diabetic rat glomeruli[24]. These alterations of nitric oxide metabolism promote oxidative stress, particularly in the diabetic renal milieu (glomerular and tubulointerstitial cells)[25]. In support of these claims, oxidative stress coupled with chronic hyperglycemia may have an important role in the pathogenesis of glomerular and tubular functional and structural abnormalities [20].

Cavernous mechanism often pertains in the development of oxidative stress. In diabetes, intricate mechanisms are involved in the promotion of oxidative stress. NADPH oxidase is the major source for superoxide generation. NADPH oxidase is located in the plasma membrane of various cells, including renal endothelial cells, fibroblast mesangial cells, proximal tubular cells and vascular smooth muscle cells[26]. Furthermore, in the diabetic rat, the stimulation of expression of NADPH oxidase was noted to be increased in the kidney and NADPH oxidase dependent overproduction of ROS plays a key role in the induction of renal hypertrophy and nephropathy[23,27]. Intriguingly, activation of glomerular SREBP-1 increases NADPH oxidase-mediated ROS production, which further progresses diabetic nephropathy[28]. Thus, this evidence suggests a possible pathological role of NADPH in diabetic nephropathy. Enhanced oxidative stress has been shown to activate TGF-β, which regulates the extracellular matrix remodeling in the mesangial cells[29]. It is plausible that diabetes mediated oxidative stress activates TGF-β and could play a role in the development of the characters of diabetic nephropathy[20,30]. In the case of signaling kinases, protein kinase C (PKC) is considered to be the central culprit involved diversely to the pathogenesis of diabetic nephropathy[31]. Evidently, involvement of PKC is further confirmed by the treatment with ruboxistaurin, a specific PKC inhibitor, which prevented the development of diabetes-induced nephropathy by reducing the increased mRNA expression of TGF-β1 and fibronectin[32]. Therefore, in diabetes, NADPH oxidase acts as an engine for the generation of oxidative stress and oxidative stress mediated TGF-β initiates secondary microvascular complications.

Dyslipidemia is a condition associated with hypertriglyceridemia, elevated LDL levels and decreased HDL levels[10]. The association between hyperglycemia and lipid accumulation is a hall mark of diabetic nephropathy. Insulin resistance in diabetes is the initial step in the formation of dyslipidemia[33]. Furthermore, dyslipidemia has been suggested as an independent risk factor for the development and progression of diabetic nephropathy[34]. This indicates that insulin resistance/hyperinsulinemia is a primary cause of diabetic dyslipidemia. Thus, patients with diabetic nephropathy often have multiple lipoprotein abnormalities[35]. However, two key mechanisms explain the association between diabetes mellitus and hyperlipidemia. Firstly, insulin deficiency downregulates the LPL, an enzyme involved in the hydrolysis and clearance of TGs from the circulation[36]. Secondly, insulin has an inhibitory action on 3-hydroxy-3-methyl-glutaryl-Co-A (HMG-COA) reductase, a key rate limiting enzyme involved in the synthesis of cholesterol[37]. Jointly, it is possible that hypoinsulinemia during long term diabetes downregulates LPL and activates the HMG-COA reductase pathway and might play a role in excessive lipid accumulation during early stages of diabetic nephropathy. The strong correlation between diabetic-endothelial dysfunction and nephropathy has been demonstrated in various studies[5,38,39]. Worthy of note is that increased concentrations of free fatty acids impairs NO production by downregulating eNOS and decreases endothelial dependent vasodilation[40,41]. The diabetic hyperlipidemia-induced VED is characterized by reduced activation of eNOS, reduced generation and bioavailability of NO[5,39]. Moreover, the accumulation of renal lipid and generation of ROS is collectively involved in the pathogenesis of diabetic nephropathy[42]. Supporting this contention, Chen *et al*[43] observed that both native and oxidized LDL enhances superoxide generation in isolated diabetic rat glomeruli. Furthermore, a recent study certainly emphasized that, in diabetes, an excess amount of a variety of lipid progressively affects glomerular and tubular function[44]. Likewise, diabetic dyslipidemia is often associated with glomerular, mesangial and tubulointerstitial injury[43]. Additionally, it has been suggested that an increased expression of SREBP-1 in diabetic mice could play a central role in renal lipid accumulation, glomerulosclerosis and proteinuria[15]. Thus, growing evidence suggests that hyperlipidemia is considered a serious risk factor involved in the pathogenesis of diabetic renal diseases.

TGF-β PLAYS AN ABYSMAL ROLE IN PATHOGENESIS OF NEPHROPATHY: A PROFOUND LOOK

Although precise mechanisms involved in diabetes-asso-

ciated renal complications are not clear, oxidative stress and dyslipidemia could play a key role in elevating renal complications. As many factors contribute to the induction and progression of diabetic nephropathy, the association between plasma levels of TGF-β (pro-sclerotic cytokine) and diabetes is considered an independent and major determinant of the progression of renal disease in patients with diabetes mellitus[8]. Various studies strongly suggested that high lipids could induce renal injury by stimulating TGF-β. This contention is supported by the fact that TGF-β is a fibrogenic cytokine and seems to promote extracellular matrix accumulation, a cardinal structural feature of the kidney in diabetic mice[45]. Furthermore, TGF-β appears to be an important mediator in oxidized LDL-induced mesangial matrix expansion, which changes the architecture of the kidney[46]. The strong correlation between hyperglycemia and TGF-β has been demonstrated as high-content glucose medium increases TGF-β mRNA expression glomeruli[47] and diabetic smokers are noted to have increased serum concentration of TGF-β[8,48]. Additionally, TGF-β stimulates the expression of connective tissue growth factor, which promotes glomerulosclerosis, renal deposition of extracellular matrix and hypertrophy of mesangial cells[49]. Furthermore, abnormal regulation of the renin angiotensin system is directly involved in the pathogenesis of diabetic nephropathy. It has been noted that angiotensin-Ⅱ (Ang-Ⅱ) increases the expression of TGF-β, which stimulates the synthesis of the mesangial matrix[50]. Thus, it may be concluded that long-term hyperglycemia elevates Ang-Ⅱ, which increases the expression of TGF-β and plays a pathological role in the induction of diabetic nephropathy.

Ordinarily, in diabetes, AGEs are a heterogeneous compound formed non-enzymatically through an interaction of reducing sugar with an amino group of proteins and lipids[51]. Various studies have reported that the renal accumulation of AGEs is implicated in the pathogenesis of diabetic vascular complications[52]. As well, AGEs are noted to upregulate the expression of TGF-β and collagen, which accumulates particularly in glomerular and extracellular matrix, thus provoking glomerular hypertrophy in diabetes[53].

A further step towards the pathogenesis of diabetic nephropathy is involvement of Rho-kinase, a serine/threonine kinase noted to promote diabetic nephropathy[54]. This notation is further supported by the fact that the Rho-kinase plays a pivotal role in the pathogenesis of VED, which again complicates the condition of diabetic secondary complications[55]. Apparently, diabetes mediated activation of Rho-kinase contributes to the induction of glomerulosclerosis and upregulation of glomerular matrix deposition in rats[54,55]. This fact is further confirmed by treatment with fasudil, a selective Rho-kinase inhibitor, which markedly attenuated the development of diabetic nephropathy by inhibiting the renal upregulation of TGF-β, connective tissue growth factor and NADPH oxidase in rats[56]. Therefore, TGF-β is a marked contributor in the pathogenesis of diabetic nephropathy.

PARTICIPATION OF SMOKING MEDIATED OXIDATIVE STRESS IN THE DEVELOPMENT OF DIABETIC NEPHROPATHY

As there is no prior information about the role of nicotine as a powerful fierce molecule, many efforts entail a conceptual shift to understand the health hazards of nicotine. Smoking has been noted to develop large amount of free radicals and pro-oxidant molecules, exerting an adverse influence on endothelial cells through an inhibitory effect on components of the L-arginine-nitric oxide pathway[54,55]. Furthermore, nicotine plays a key role in the pathogenesis of endothelial dysfunction by decreasing the generation and bioavailability of NO and down-regulating the expression of eNOS[7,56]. Thus, smoking mediated high oxidative stress and low availability of eNOS may engender VED, which is the initial step in the pathogenesis of glomerulosclerosis in diabetic nephropathy. In recent years, the increasing prevalence of smoking has been pinpointed as a progression factor for diabetic nephropathy. The chronic administration of nicotine is noted to increase lipid peroxidation products (cell membrane phospholipids) in serum and various tissues of rat. The concentration of these products was found to be inversely proportional to activity of endogenous antioxidants like catalase and superoxide dismutase[57]. Accordingly, breakdown of membrane phospholipids by lipid peroxidation is expected to play an important role in the vascular pathogenesis. Recently, David *et al* tested the hypothesis that exposure to tobacco smoke (nicotine) in db/db mice worsens the progression of diabetic nephropathy by increasing the severity of ECM deposition and increasing the expression of the profibrotic cytokine TGF-β. The knowledge about the involvement of ROS in the pathogenesis of vascular disease should facilitate the development of therapies that directly target ROS production to prevent microvascular complications.

During smoking, nicotine is fiercely worked for the development of oxidative stress. Nicotine has been noted to increase generation of superoxide by activation of NADPH oxidase and PKC, which ultimately damages the kidney[23,31,58]. The destructive role of PKC is manifested as inhibition of PKC by calphostin C, a potent PKC inhibitor, which prevents nicotine-induced mesangial cell proliferation and fibronectin production. It suggests that nicotine-mediated growth promoting effects is through activation of NADPH oxidase and PKC, probably ROS as second messengers[3,59,60]. Thus, the inhibition of PKC halts the progression of nicotine-mediated renal damage and decreases NADPH oxidase mediated ROS generation. One unique mechanism explains that smoking stimulates lipid accumulation by

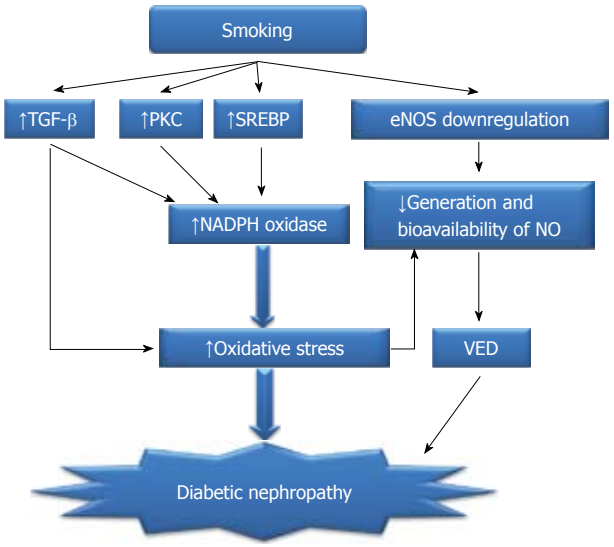

Figure 1 Possible mechanism involved in smoking-induced oxidative stress. TGF-β: Transforming growth factor β; PKC: Protein kinase C; eNOS: Endothelial nitric oxide synthase; NO: Nitric oxide; SREBP: Sterol regulating element binding protein; VED: Vascular endothelial dysfunction.

increasing expression of SREBP-1, which is responsible for increasing the synthesis of TGs and cholesterol, which again sidewise contributes in the pathogenesis of nephropathy[16]. The possible mechanisms involved in smoking-induced oxidative stress mediated renal damage in diabetes have been depicted in Figure 1.

PARTICIPATION OF SMOKING MEDIATED HYPERLIPIDEMIA IN DEVELOPMENT OF DIABETIC NEPHROPATHY

It is very difficult to understand smoking-mediated lipid wiggles because nicotine has intricate mechanisms; however, serial analysis may explore its role in the development of dyslipidemia in diabetes. Smoking has numerous atrocious effects, which initially pertain to the pathogenesis of microvascular and subsequently renal complications. Cigarette smoking is positively associated with higher serum concentrations of TGs and LDL and lower serum concentrations of HDL[13]. In fact, nicotine causes an impairment of LPL and increases plasma lipid concentration[11]. Elegant studies show that nicotine-mediated hyperlipidemia is considered to be an underlying mechanism involved in the nicotine-induced endothelial dysfunction[6,7]. Especially, nicotine plays a key role in induction of VED by decreasing the generation and bioavailability of NO and downregulating the expression of eNOS[7,34,59]. Nicotine is also noted to induce glomerulosclererosis and provokes reno-vascular pathogenesis[12]. Thus, smoking endorses dyslipidemia and worsens the severity of nephropathy in diabetes. After pondering over the above discussion, it can be said that the deleterious effect of cigarette smoking, such as renal damage, is through dyslipidemia. Taken together, through direct or indirect multiple mechanisms, smoking in diabetes has been regarded as a major risk factor in the induction and progression of oxidative stress and dyslipidemia mediated renal damage (Figure 2).

COMRADESHIP BETWEEN SMOKING AND DIABETIC NEPHROPATHY: EXACERBATION OF NEPHROPATHY?

Various epidemiological studies have explored potential mechanisms that could be responsible for smoking-mediated progression diabetes nephropathy. The main clinical associations that frequently precede diabetic nephropathy are hypertension and poor glycemic control. A recent gender-specific, dose-response relationships study demonstrated that smoking is a significant risk factor for future kidney failure[61]. Clinical hallmarks of diabetic nephropathy include a progressive increase in urinary albumin excretion and a decline in glomerular filtration rate. Diabetes and smoking concurrently activated cellular pathways uncompromisingly and participate in step wise progression of nephropathy[21,57]. The frequent microalbuminuria is more commonly noted in diabetic smokers than non smokers with nephropathy[4,60]. Similarly, the rate of loss of measured creatinine clearance was noted to be higher in smokers compared to non-smoking diabetic patients[61]. Intriguingly, smoking also increases the risk of subjects to develop type Ⅱ diabetes, possibly because it increases insulin resistance[62]. Furthermore, a growing body of evidence compared non-diabetic smokers and non-smokers and found that smokers were more insulin resistant and hyperinsulinemic[63]. Furthermore, smoking impaired insulin action, mainly due to a lowering of peripheral glucose uptake[64]; thus, smoking is a key risk factor for the subject to develop type Ⅱ diabetes and secondary complications. Preclinical studies further support the fact that smoking accelerates diabetic nephropathy. It is worth mentioning that administration of nicotine develops glomerular hypertrophy and mesangial expansion and increases high glucose mediated ROS generation through activation of NADPH oxidase and aggravates nephropathy in diabetic mice[3]. In addition, exposure to tobacco smoke in db/db mice significantly increases urinary albumin excretion, mesangial expansion and extracellular matrix deposition and worsens diabetic nephropathy[65].

Despite this, various endogenous molecules may be associated with smoking and could partially contribute to the pathogenesis of microvascular complications in diabetes, which are yet poorly understood. Importantly, it has been noted that smoking increases serum concentration of Ang-Ⅱ and TGF-β1 in diabetic patients[8]. Along with this, it has been noted that cessation of smoking downregulates TGF-β1 when compared with diabetic non-smokers[66]. Prominently, cigarette smoking enhances the accumulation of AGEs, which crosslink

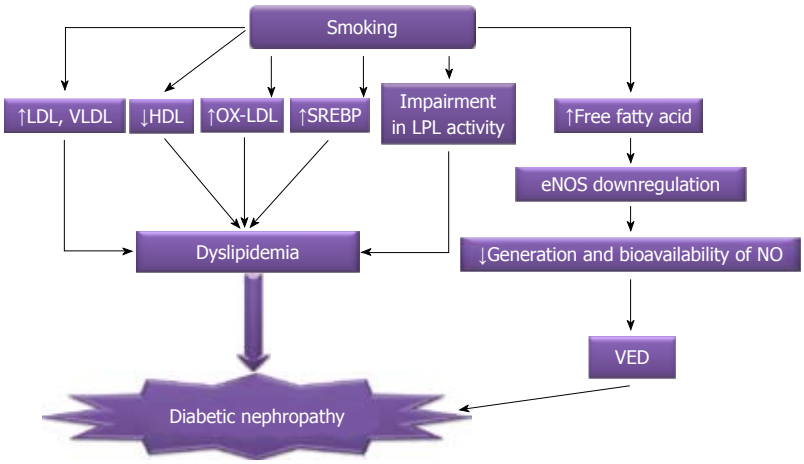

Figure 2 Possible mechanisms involved in smoking-induced dyslipidemia. eNOS: Endothelial nitric oxide synthase; NO: Nitric oxide; SREBP: Sterol regulating element binding protein; VED: Vascular endothelial dysfunction; LDL: Low density lipoprotein; VLDL: Very low density lipoprotein; HDL: High density lipoprotein; LPL: Lipoprotein lipase.

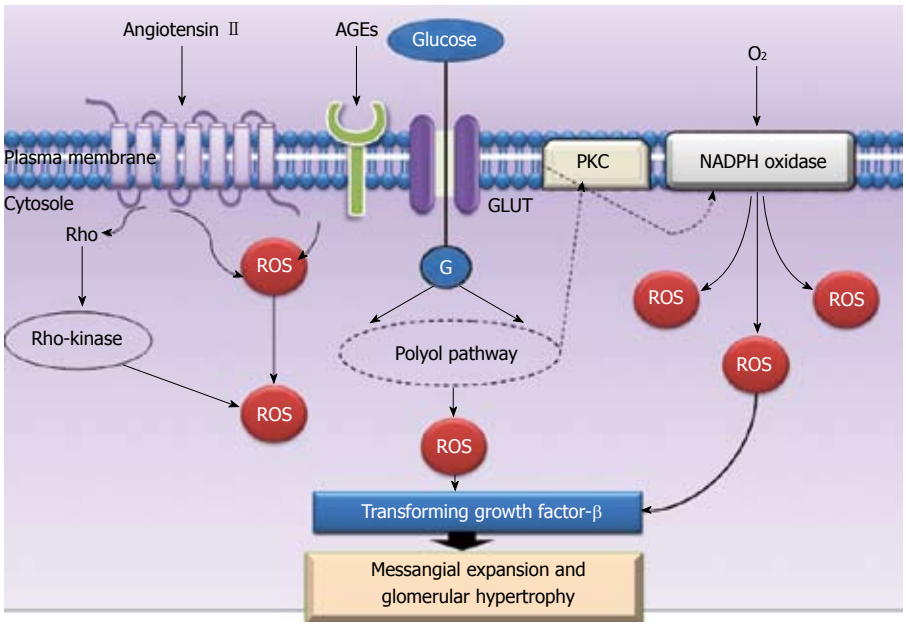

Figure 3 Various endogenous modulators contribute to renal complications. AGEs: Advance glycation end products; ROS: Reactive oxygen species; GLUT: Glucose transporters; PKC: Protein kinase C; NADPH oxidase: Nicotinamide adenine dinucleotide phosphate-oxidase.

with collagen to promote vascular complications[67] and also increases production of TGF-β in aortic endothelial cells[48], indicating the strong relationship between smoking, AGEs and TGF-β in the pathogenesis of vascular complications. As described above, accumulation of AGEs by diabetes and smoking is directly proportional to expression of TGF-β, which is finally involved in reno-vascular complications. Taken together, available evidence indicates that accumulation of AGEs, either by diabetes or by smoking, promotes an appalling role for TGF-β in the progression of nephropathy. Furthermore, crucial involvement of Rho-kinase provides a suitable target system to understand the basic pathways of smoking-mediated reno-vascular disease. It is interesting to note that treatment with fasudil, a Rho-kinase inhibitor, significantly prevented the smoking-induced impairment in endothelium-dependent vasodilation[68], which suggests the detrimental involvement of Rho-kinase in nicotine-induced endothelial dysfunction. The broad utility of these approaches is to target these endogenous molecules to attenuate smoking-mediated reno-vascular damage. The availability of this comprehensive data allows accelerating development of active compounds and strategies for intervention at various stages in the development of diabetic nephropathy. Thus, smoking mediated insulin resistance, frequent microalbuminuria, messangial expansion and glomerular hypertrophy indicate the detrimental effects of smoking in development and progression of diabetic nephropathy (Figure 3).

Therefore, from the above critical discussion and

pondering over accumulating evidence, it may be understood that oxidative stress and hyperlipidemia are key players in the pathogenesis of diabetic nephropathy. Furthermore, smoking provokes oxidative stress and dyslipidemia upregulates TGF-β in diabetes, which worsens the severity of nephropathy. Simultaneously, Ang-Ⅱ, AGEs and Rho/Rho-kinase upregulate TGF-β, which plays a pathogenic role in the induction of renal hypertrophy and progression of diabetic nephropathy. Thus, it may be concluded that chronic cigarette smoking may exacerbate diabetic nephropathy.

PHARMACOLOGICAL INTERVENTIONS TO TREAT DIABETIC SMOKERS WITH NEPHROPATHY

Over several decades, there have been extensive investigations concerning the development of novel target sites and thus numerous agents have been explored for therapeutic potential in treating diabetic nephropathy. Smoking is an additional factor directly involved in the progression of nephropathy. Thus, diabetic smokers are more complicated to treat than non smokers. Yet, a promising and effective therapy has not been deduced for these complicated diseases. Hyperglycemia and cigarette smoking mainly arouses oxidative stress and hyperlipidemia. Hence, strict glycemic control and smoking secession remains the cornerstone of the current standard therapeutic approaches and may help to ameliorate vascular complications.

The drugs currently used to treat diabetic nephropathy mainly target the hypertensive component, such as drugs that interrupt the renin-angiotensin system, angiotensin-converting enzyme (ACE) inhibitors and Ang-Ⅱ receptor antagonists, and are currently considered first-line treatments for diabetic nephropathy. In particular, the use of a different class of drugs, such as ACE inhibitors captopril, lisinopril, fosinopril, benazapril and quinapril, and AT-Ⅰ receptor blockers, such as losartan, olmesartan and irbesartan, have been observed in numerous experimental and clinical studies to have therapeutic potential in the treatment of diabetic nephropathy[69-77]. Captopril was the first drug approved by Food and Drug Administration in the nineties for the treatment of diabetic nephropathy[48].

Cigarette smoking is major fuel for the generation of oxidative stress. As mentioned in the preceding section, NADHP oxidase is a master enzyme involved in ROS formation. Therefore, a superior approach to ameliorate oxidative stress is to inhibit the culprit enzyme NADHP oxidase. This contention is further supported by the fact that apocynin restores renal function by decreasing the expression of collagen-1, mesangial expansion and albuminuria diabetic rats[78,79]. The evidence presented above provides a strong rationale for the use of pharmacological inhibitors of NADPH oxidase to combat oxidative stress and its associated vascular pathologies. The current research interest is to treat diabetic nephropathy by use of a fibrates class of interventions, such as fenofibrate, bezafibrate and gemfibrozil, well-known hypolipidemic agents[80-82]. Recently, in our laboratory we have shown that treatment with fenofibrate and concurrent administration of benfotiamine, a transketolase activator, prevented the development of diabetic nephropathy. This renoprotective effect of fenofibrate was associated with its actions on reducing the circulating lipids and oxidative stress[5]. Furthermore, fenofibrate was shown to ameliorate nicotine-induced endothelial dysfunction by reducing hyperlipidemia and oxidative stress in rats[7]. Thus, the available evidence says that the use of fenofibrate attenuates diabetes and nicotine-mediated hyperlipidemia and oxidative stress and ameliorates endothelial dysfunction and nephropathy. Therefore, use of fenofibrate is the most logical pharmacological intervention to treat smoking mediated progression of diabetic nephropathy, like killing two birds with one stone. From the above discussion, it may be concluded that fibrate really preserves kidney function in diabetes. On other hand, cyclohexenonic long-chain fatty alcohol (N-hexacosanol) was noted to reduce significantly TGF-β and concentrations of PKC, which ameliorate the diabetic-induced tubulointerstitial pathological changes[83]. Furthermore, N-hexacosanol was also noted to reduce diabetes-mediated alteration in eNOS, which attenuates glomerulosclerosis[84].

Taken together, these studies suggest that these drugs may provide supportive therapeutic advancement for treating diabetic smokers with nephropathy. However, further studies are needed to illuminate their therapeutic potential in treating diabetic smokers with vascular pathogenesis.

CONCLUSION

Smoking is ubiquitous in patients with diabetes mellitus. Smoking and hyperglycemia increase oxidative stress and lipid accumulation, which upregulates TGF-β, accumulates AGEs, decreases nitric oxide production, which leads to thickening of glomerular basement membrane and mesangial expansion, with progression in glomerulosclerosis and interstitial fibrosis, and results in nephropathy. It is emerging from above that smoking is a hitherto major fuel to aggravate diabetic nephropathy.

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MINIREVIEW

Frontiers in research on maternal diabetes-induced neural tube defects: Past, present and future

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Abstract

Diabetes mellitus rightly regarded as a silent-epidemic is continually on the rise and estimated to have a global prevalence of 6.4 % as of 2010. Diabetes during pregnancy is a well known risk factor for congenital anomalies in various organ systems that contribute to neonatal mortality, including cardiovascular, gastrointestinal, genitourinary and neurological systems, among which the neural tube defects are frequently reported. Over the last two to three decades, several groups around the world have focussed on identifying the molecular cues and cellular changes resulting in altered gene expression and the morphological defects and in diabetic pregnancy. In recent years, the focus has gradually shifted to looking at pre-programmed changes and activation of epigenetic mechanisms that cause altered gene expression. While several theories such as oxidative stress, hypoxia, and apoptosis triggered due to hyperglycemic conditions have been proposed and proven for being the cause for these defects, the exact mechanism or the link between how high glucose can alter gene expression/transcriptome and activate epigenetic mechanisms is largely unknown. Although preconceptual control of diabetes, (i.e., managing glucose levels during pregnancy), and in utero therapies has been proposed as an effective solution for managing diabetes during pregnancy, the impact that a fluctuating glycemic index can have on foetal development has not been evaluated in detail. A tight glycemic control started before pregnancy has shown to reduce the incidence of congenital abnormalities in diabetic mothers. On the other hand, a tight glycemic control after organogenesis and embryogenesis have begun may prove insufficient to prevent or reverse the onset of congenital defects. The importance of determining the extent to which glycemic levels in diabetic mothers should be regulated is critical as foetal hypoglycemia has also been shown to be teratogenic. Finally, the major question remaining is if this whole issue is negligible and not worthy of investigation as the efficient management of diabetes during pregnancy is well in place in many countries.

Key words: Maternal diabetes; Congenital anomalies; Neural tube defects; Hyperglycemia; Hypoxia; Oxidative stress; Neural stem cells; Epigenetics; Epigenome

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EPIDEMIOLOGY

The global incidence of diabetes mellitus is constantly on the rise and is estimated to be 6.4% as of 2010[1]. According to the World Health Organization statistics

2012 report, diabetes is prevalent in one in ten adults. The incidence of diabetes in pregnancy however, varies with the geographical and ethnic background of women. About 3%-5 % of pregnancies are reported to be complicated by diabetes mellitus which is the leading cause for mortality and morbidity[2,3].

Diabetes during pregnancy is of serious concern as it causes spontaneous abortions, still birth, foetal macrosomia, and congenital malformations[4-7]. Our understanding and management of diabetes over the years have reduced the risk for spontaneous abortion and still birth in maternal diabetics. However, even in recent years diabetes during pregnancy has been considered to have a teratogenic effect causing foetal anomalies[7-11]. Although all of the foetal tissues are susceptible to glucose induced anomalies, the developing heart and brain have shown maximum defects[12] and these defects are the most frequently reported birth defects in humans and mouse models of diabetic pregnancy[9,13-15], indicating that these tissues are more sensitive to glucose toxicity.

Indeed, initial studies have shown that the type of malformation varied with the maternal glucose concentration[6,16] and achieving a particular glucose threshold may be beneficial in reducing or eliminating the risk for a particular malformation. While a tight glycemic control started before pregnancy has shown to reduce the risk for congenital anomalies[5,10,17,18], the same after organogenesis (first 8 wk of pregnancy) has taken place may prove insufficient to reverse the damages already caused. While majority of the pregnancies are diagnosed only after 7-8 wk of conception, in a mother with undiagnosed and pre-existing diabetes, organogenesis in the fetus is definitely impaired and is irreversible. Neural tube defects (NTDs), the second most common birth defect caused by maternal diabetes has been long studied and characterised. In this review, we summarize some of the major findings of the past and present and propose future directions for research to alleviate NTDs.

MORPHOLOGICAL AND MOLECULAR CHARACTERISATION OF NTDS

Maternal diabetes has been shown to cause severe patterning defects in the brain of developing embryos. We have reported maternal diabetes-induced malformations in several parts of the brain including the forebrain (telencephalon), spinal neural tube, choroid plexus (CP) and ventricles in mouse embryos[19,20]. The impaired development of CP by maternal diabetes results in decreased production of cerebro-spinal fluid which is proposed to cause defective patterning and shaping of the brain during development in diabetic pregnancy[21-24]. In addition, the neural tissue with defective patterning showed altered expression of several signalling molecules and transcription factors such as Shh, Nkx2.1, bafilomycin-1, transforming growth factor-β and Pax3 which are critical in forebrain patterning and neural tube closure[25-28].

Recently, high throughput gene expression profiling using cDNA microarray revealed the altered expression of several genes in the cranial neural tubes of embryos from diabetic pregnancy[20,28-30]. Higher numbers of genes involved in metabolism and cellular process were found to be altered by maternal diabetes[20]. Microarray analysis on whole embryos from diabetic pregnancy has also revealed the altered expression of several genes involved in critical developmental pathways that could contribute to maternal diabetes-induced birth defects including genes known to cause NTDs[29]. These descriptive reports suggest that brain development is impaired due to the altered expression of developmental control genes caused by maternal diabetes-induced glucotoxicity.

CHARACTERISATION OF METABOLIC PATHWAYS IN NTDS

In recent years, the research on maternal diabetes- induced congenital malformations in fetus has moved on from morphological characterisation to identifying the etiology of birth defects. It has been widely shown that maternal diabetes induces hypoxia, oxidative stress and other metabolic disturbances in the embryo[31] and these changes alter several signalling pathways and molecules which have been proposed to be the major causative factors for the diabetes-induced malformations leading to embryopathy. For example, the oxidative stress caused by hyperglycemia has been shown to disrupt the expression of genes such as Pax3 that is involved in neural tube formation which explains in part that hyperglycemia-induced oxidative stress alters gene expression leading to NTD[32].

Hyperglycemia-induced birth defects are attributed to the excessive production of reactive oxygen species (ROS) which has been shown to cause oxidative stress and subsequently increase the risk for fetal malformations[33,34]. Administration of antioxidants such as vitamin E and overexpression of ROS scavenging enzymes such as superoxide dismutase have been shown to prevent or reduce the risk for diabetic malformations in several animal studies although the exact mechanism is unknown[35-40].

Development and patterning of normal brain depend on proliferation and differentiation of neural stem cells (NSCs) which are self renewing multipotent cells giving rise to neuronal and non-neuronal cells (glial cells such as astrocytes and oligodendrocytes) in the central nervous system. We have shown that NSCs are extremely sensitive to glucotoxicity, which alters the expression of genes involved in proliferation and lineage specification of NSCs[41]. Apoptosis in the neuroepithelium is the hallmark of maternal diabetes-induced NTDs and the balance between cell proliferation and cell death that is altered by hyperglycemia may contribute to the malformations seen in the developing neural tube. We have also shown that high glucose induces ROS production and intracellular oxidative stress in NSCs by increasing glucose reduction *via* the polyol pathway. These changes appeared to be mediated by aldose reductase (AR), the rate

limiting enzyme in the polyol pathway, since its expression was found to be increased in NSCs exposed to Hg *in vitro* and inhibition of AR using fidarestat, reversed the changes induced by Hg[42]. Overall, these studies indicate that the development of strategy to prevent oxidative stress during fetal development may alleviate the risk of congenital anomalies in embryos.

UNRAVELLING EPIGENETIC MECHANISMS CONTRIBUTING TO NTDS

It has been reported that maternal nutrition and metabolic disturbances during fetal development can alter epigenetic mechanisms such as histone modifications and DNA methylation in fetus, and such epigenetic changes may have long lasting effects on the offspring postnatally[43-46]. Study on pregnant mice fed with diets low in choline/methionine have resulted in decreased methylation in genes that control brain development[47,48], and altered memory and long-term potentiation[49,50] indicating that the developing embryo is influenced by maternal diet. In addition, diabetic rodents supplemented with folate (a methyl donor) prevented NTDs in the embryos[51,52] that define the role of maternal nutrition on fetal outcome.

It has been widely shown that diabetic complications are associated with epigenetic modifications. In recent years, several reports have shown that the onset of diabetes in adults is caused by DNA methylation at specific gene promoters or chromosomal regions[53-56] signifying epigenetic basis for onset of diabetes in adults. High glucose has also shown to cause persistent alterations in gene expression through histone modifications (by acetylation or methylation of lysine residues) during transient exposure of human aortic endothelial cells[57] and chronic exposure of human monocytic cell line[58] to high glucose.

Further, excess glucose has been shown to increase histone acetylation in mammalian cells[59] while excess dietary methyl donors increase DNA/histone methylation in offspring[60] providing evidence for the relationship between maternal hyperglycemia (or diet) and fetal epigenome. Recently, glucose responsive microRNAs such as miR-26a, miR107 and miR-16 that show increased expression in high glucose conditions have been identified[61] suggesting that epigenetic mechanisms could be activated by hyperglycemia. Further, epigenetic factors have been shown to regulate gene expression of developmental control genes and fate specification of NSCs[62,63]. It is possible that high glucose modifies epigenetic mechanisms which subsequently alter expression of genes involved in cell fate specification of NSCs thereby resulting in NTDs. Further investigation of epigenetics will be useful to understand the relationship between maternal diet and the fetal epigenome. It will be intriguing to know how high glucose/hyperglycemia activates epigenetic mechanisms that alter gene expression in the fetus resulting in birth defects.

CONCLUSION

Diabetes during pregnancy is a well known teratogen that causes congenital anomalies. Over the last 2-3 decades, the focus on this field of research has shifted from morphological and molecular characterisation of the etiology of maternal diabetes-induced malformation to understanding the mechanisms behind how maternal diabetes alters fetal development. While epigenetic mechanisms have been proposed to modify the expression of critical genes involved in development, the exact mechanism behind this still remains largely unidentified. It will be interesting to elucidate how epigenetic mechanisms such as DNA methylation, chromatin/histone modifications and microRNAs are activated in embryonic tissue by maternal diabetes. Identifying specific histone/DNA modifying enzymes involved in response to glucose prove as significant therapeutic targets, since epigenetic changes are reversible. Modulating fetal epigenetic programming in such metabolic syndromes may prove valuable to improve fetal outcomes or prevent onset of diabetes in offspring of diabetic mothers.

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BRIEF ARTICLE

Mechanistic studies of lifestyle interventions in type 2 diabetes

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Abstract

AIM: To investigate the effect of lifestyle interventions in the non-pharmacological management of type 2 diabetes *via* a mechanistic approach.

METHODS: A randomized controlled trial was carried out on 60 type 2 diabetic male and female volunteers that fulfilled the inclusion criteria, with their proper consent and permission of the International Electrotechnical Commission for 1 year. 30 patients were included in the test group and 30 patients in the control group. Demographic details, anthropometrical status, physical activity, food habits and blood glucose lipid profile of the volunteers were recorded at baseline, the test group was directed for lifestyle intervention and final blood glucose lipid data were collected at the end of one year of patient follow-up.

RESULTS: After 1 year, the test group who had a lifestyle intervention was found to show a significant improvement in blood glucose lipid profile. The fasting plasma glucose level (FPG), postprandial plasma glucose level (PPG), glycosylated hemoglobin (HbA1c) and body mass index (BMI) values of the test group were reduced significantly, up to 145 ± 2.52, 174 ± 2.59, 6.3 ± 0.32 and 25 ± 0.41 respectively at the end of the study period, in comparison to the control group where FPG, PPG, HbA1c and BMI values were 193 ± 3.36, 249 ± 4.24, 7.2 ± 0.42 and 26 ± 0.65 respectively. Improvement in the total cholesterol (TC), triglyceride (TG), high-density lipoproteins (HDL) and low-density lipoproteins (LDL) values of the test group was also remarkable in comparison to the control group. The TC, TG, HDL and LDL values of the test group were reduced significantly, up to 149 ± 3.32, 124 ± 2.16, 58 ± 0.62 and 118 ± 2.31, respectively.

CONCLUSION: The significant improvement in the blood glucose lipid profile of the test group after 1 year signifies the value of non-pharmacological management of type 2 diabetes *via* lifestyle intervention strategies.

Key words: Metabolic; Unconventional; Therapeutic; Interventions; Non-pharmacological; Sedentary; Lifestyle; Counseling

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INTRODUCTION

Diabetes is fast becoming the epidemic of the 21st century, with type 2 diabetes the most prevalent form. The International Diabetes Federation has estimated the number of diabetics in India to be 40.9 million, which is projected to be 60.9 million by 2025[1,2]. The epidemiol-

ogy of diabetes being very vast, the financial expenditures involved in the treatment of this disease, delayed diagnosis until micro and macrovascular complications arise, life-threatening complications like end stage renal disease, limb amputations, retinopathy leading to blindness and failure of the current therapies to restore normoglycemia, necessitates the adoption of preventive strategies in controlling type 2 diabetes[3-6]. In affluent societies, the disease is seen mostly in the age group of 55-74 years[7,8]. The results of several clinical trials have documented that therapeutic life style changes (TLC), an effective lifestyle therapy, helps to control the risk factors associated with type 2diabetes. It is an established fact that obesity, "westernized dietary pattern", sedentary lifestyle, smoking habits, alcoholism *etc.* are significant lifestyle risk factors associated with type 2 diabetes. Sleep deprivation, anxiety and depression are other contributing factors[9-13]. Type 2 diabetes in high risk individuals can be controlled by proper lifestyle modifications, like an increase in physical activities, dietary modifications, control of obesity *etc.* However, non-modifiable risk factors in type 2 diabetes, like genetic inheritance, prior gestational diabetes and glucose intolerance, cannot be controlled by lifestyle modifications. The lifestyle interventions mostly aim at increasing physical activities (2.5-4 h/wk) like brisk walking, jogging, some aerobic and stretching exercises, weight reduction to control obesity, dietary modifications like increased intake of non-starch polysaccharides, whole grains, dietary fibers, vegetables, fruits, oily fish and poultry products for polyunsaturated fatty acids and absolutely no or a very low intake of saturated and trans fats, refined cereals, sugar, red meat, high calorie foods like French fries, sweetened beverages *etc.*, cessation of smoking and abstinence from alcohol intake[14-20]. Reduction of anxiety and stress is another intervention parameter. Proper patient counseling is of vital importance in this matter. TLC is a comprehensive lifestyle approach that includes specific dietary recommendations, weight management and increased physical activity[21-24]. The TLC diet plan aims to provide a proper balance of carbohydrates, proteins, fats and other nutrients at a 2000 calories level. TLC recommends engaging in at least 30 min of moderate intensity physical activity; however, involvement in moderate to high intensity physical activity most days of the week should not exceed the calorie intake requirements. Results of randomized control trials after proper patient counseling have shown that changes in diet and increase in physical activity resulted in improvement in the blood glucose lipid profile of the individuals under trial. Hence, a therapeutic lifestyle change is a protective measure against Type 2 diabetes[22-24].

An in-depth insight into the mechanistic details of lifestyle risk factors and type 2 diabetes have shown that visceral obesity and a sedentary lifestyle has a deleterious effect on glucose homeostasis and a significant role in insulin resistance, either due to its high metabolic activity or being anatomically located just next to hepatic portal circulation which allows free entry of fatty acids from visceral fat to liver, leading to elevation in hepatic triglyceride (TG) synthesis which decreases liver insulin sensitivity, leading to increased hepatic glucose production[16,21,25-29]. Adipocytes in the visceral fat release a number of cytokines called adipokines, like the adiponectins, tumor necrosis factors (TNFs) and interleukin-6 (IL-6), which modify insulin signaling and development of insulin resistance, leading to type 2 diabetes. Obesity is found to be associated with endothelial dysfunction and impaired muscle microcirculation which can impair whole body insulin sensitivity by hindering the entry of insulin and glucose into skeletal muscle and decreasing their availability to muscle cells[16,30-34]. Diet has direct effects on insulin sensitivity. The 'Western diet' contributes to type 2 diabetes, whereas a diet rich in omega fatty acids, low glycemic index foods and exclusive breast feeding are regarded as protective against type 2 diabetes[16,35,36].

Inverse associations have been found between physical activity and the reduced risk of type 2 diabetes; regular physical activity decreases visceral and body fat and resistance exercises increase skeletal muscle mass, increasing muscle glucose uptake. Regular exercises increase the glycogen synthase (GS) content of the muscle which accelerates non-oxidative glucose disposal as glycogen and activates the glucose transporter subtype 4 (GLUT4) that facilitates the passive diffusion of circulating glucose down its concentration gradient into muscle cells since muscle glucose transport is closely associated with the GLUT4 content of the cells. Exercise potentiates insulin signaling by up-regulating the expression and activity of proteins involved in insulin signal transduction, improves the oxidative capacity of the skeletal muscles, decreases the free fatty acid concentration in the circulation, auto phosphorylation and increased expression of downstream signaling components of insulin[16,37-43].

Pro-inflammatory cytokines and C-reactive proteins play a significant role in the pathogenesis of insulin resistance and type 2 diabetes. Regular physical activity triggers the release of a number of anti-inflammatory cytokines, like the IL-6, IL-1 receptor antagonist, soluble TNF-α receptor and IL-10, and is thought to play a mechanistic role offering protection against TNF-α induced insulin resistance [16,31,32].

MATERIALS AND METHODS

A randomized controlled study was carried out over a period of one year in the medicine department of three different hospitals in West Midnapore district of Bengal. Both male and female patient volunteers aged over 40 years with reported type 2 diabetes were selected for the study with their proper written consent and permission from the Institutional Ethical Committee. As per inclusion criteria, newly diagnosed and known cases of type 2 diabetics with glycated hemoglobin in the range of 6.8%-10% were selected for the study. However, pregnant ladies, those with uncontrolled complications,

glycated hemoglobin above 10%, those subjected to a prior lifestyle intervention in the last year or those who planned to start insulin therapy during the intervention period were excluded from the study, as per the exclusion criteria. Out of the total of 72 patients willing to participate in the study, 12 patients were discontinued from the study as per exclusion criteria and 60 patients participated.

Study procedure

Individuals who met the inclusion criteria were enrolled for the study after giving their proper consent. These patients were divided into the test and control groups, with 30 patients in each group. Demographical details of the patient, like age, socio-economic status, family history, past and present medication history, anthropometrical details, dietary pattern through structured food frequency questionnaire, mental status relating to anxiety/depression, records of their daily physical activity and information on smoking habits or alcohol intake, were collected and recorded on the form (for both test and control patients). During each visit, the patient's random capillary blood glucose level was measured by using a standard glucometer. Patients in the test group received counseling and patient information leaflets but not the control group[21,44-47].

The test group patients, who received proper counseling, were advised to avoid a sedentary lifestyle, increase physical activity, like brisk walking, jogging, aerobic and stretching exercises, reduce anxiety and mental tension and find some spare time for light recreation. Details of their food habits were recorded from the structured food frequency questionnaire and they were advised to modify their dietary pattern by avoiding high calorie foods, sugar-containing items, red meat, saturated and trans fats and consume more whole grain foods, green vegetables, fruits, polyunsaturated fats, poultry products *etc*. Initially, glycosylated hemoglobin (HbA1c), fasting plasma glucose (FPG), postprandial plasma glucose (PPG), total cholesterol (TC), serum TG, high-density lipoproteins (HDL), low-density lipoproteins (LDL) and body mass index (BMI) were measured at baseline and at the end of the study. The FPG, postprandial plasma glucose, blood pressure and lipid profile were noted at each follow-up. Then, blood HbA1c, FPG, PPG, TC, TG, HDL, LDL level and BMI were measured in the test group as well as control group after the one year follow up. Data on patients' blood glucose lipid profile were statistically analysed [46,48-52].

Statistical analysis

Statistical analyses was carried out using SPSS version, the descriptive results were expressed as mean ± SD values and inter group differences were explored by *t*-test for independent samples. Model testing was done by partial least square (PLS) since it is a non-parametric method that makes no restrictive assumptions about the distribution of data and is ideally suited for a smaller sample size since, due to its partial nature, only one part of the model can be estimated at a time. For PLS analyses, list wise deletion of missing data was implemented and participants with all data points for all variables in the model included. mean ± SD values were tested for significance; baselines scores between tests and controls were compared and no significant differences were found[53-57].

RESULTS

Out of a total of 60 patients who participated in the study, 71.66% were male and 28.33% were female. The patients were divided into control and test groups with thirty patients in each group. Predominance of type 2 diabetes was noticed among patients aged over 50 years (74.99%), in contrast to those aged under 50 years (25%). Demographic details of patients have shown that the onset of diabetes is more frequent among patients about 10 years (73.33%) senior in age than those about 10 years (26.66%) younger. The results are demonstrated in Table 1.

At baseline, patients were interviewed to obtain their medical and medication history and the details were noted in a data collection form. All baseline parameters were also recorded and are given in Table 2.

The effect of counseling on FPG, PPG, HbA1c and BMI at the end of the study period on the control and test group showed that FPG, PPG, HbA1c and BMI values of the control group (without counseling) were 193 ± 3.36, 249 ± 4.24, 7.2 ± 0.42 and 26 ± 0.65 respectively. The FPG, PPG, HbA1c and BMI values of the test group (received counselling) were reduced significantly, up to 145 ± 2.52, 174 ± 2.59, 6.3 ± 0.32 and 25 ± 0.41 respectively at the end of the study period.

In order to study the effect of lifestyle modifications on the control and test group, at the end of the study (one year), the test group who received proper patient counseling showed significant improvements in TC, TG, HDL and LDL values. The TC, TG, HDL and LDL values of the control group without counseling were 167 ± 3.96, 152 ± 4.36, 49 ± 0.96 and 136 ± 3.55 respectively. In contrast, the TC, TG, HDL and LDL values of the test group were reduced significantly, up to 149 ± 3.32, 124 ± 2.16, 58 ± 0.62 and 118 ± 2.31 respectively at the end of one year of the study period.

DISCUSSION

Management of type 2 diabetes is mostly done by prescribing oral hypoglycemic drugs and other pharmacological regimens, but the results of several randomized controlled trials (RCTs) have shown that type 2 diabetes can be controlled in a non-pharmacological manner, like proper lifestyle interventions. Patient counseling plays a crucial role in this regard, making them aware about the modifiable lifestyle risk factors (obesity, physical inactivity, adoption of sedentary lifestyle and unhealthy dietary

Table 1 Demographic details of patients

Sl.No.	Variables	*n* (%)
1	Sex	
	Male	43 (71.66)
	Female	17 (28.33)
2	Age (yr)	
	40-50	15 (25)
	50-60	28 (46.66)
	> 60	17 (28.33)
3	Onset of diabetes (yr)	
	1-10	16 (26.66)
	10-20	20 (33.33)
	> 20	24 (40)

Table 2 Baseline blood glucose lipid of the enrolled patients

Sl.No.	Parameter	Baseline reading of enrolled patient
1	FPG (mg/dL)	223 ± 9.23
2	PPG (mg/dL)	295 ± 12.3
3	HbA1c (%)	8.3 ± 0.58
4	Total cholesterol (mg/dL)	186 ± 8.62
5	Serum triglyceride (mg/dL)	180 ± 10.36
6	HDL (mg/dL)	38 ± 2.12
7	LDL (mg/dL)	122 ± 7.58
8	BMI	27 ± 0.92

FPG: Plasma glucose level; PPG: Postprandial plasma glucose level; HbA1c: Glycosylated hemoglobin; HDL: High-density lipoprotein; LDL: Low-density lipoprotein; BMI: Body mass index.

pattern) strongly correlated with type 2 diabetes. BMI of 30 kg/m^2 exponentially increases the risk of type 2 diabetes among women by 3-fold and the risk increases to about 20 times when the BMI value is above 35 kg/m^2; for men the risk increases about 40 times with a BMI value above 35 kg/m^2[13,14,16,44]. Visceral obesity, physical inactivity, sedentary lifestyle, unhealthy dietary patterns like a high glycemic load in diet, low intake of dietary fibers and unsaturated fats, high intake of saturated fats, junk foods, French fries, sweetened beverages and smoking habits have deleterious effects on glucose homeostasis and greatly increases the risk of type 2 diabetes; however, intake of coffee and moderate consumption of alcohol are found to be protective[43,55-57]. From the mechanistic point of view, due to the high deposition of visceral fat (obesity), the adipocytes of these deposited fats release a number of circulating cytokines called adipokines that can cause insulin resistance in skeletal muscle. Moreover, high levels of circulating fatty acids can decrease muscle glucose uptake and increase fatty acid uptake; such imbalances in fatty acids and glucose uptake will cause an accumulation of intramyocellular lipid metabolites, thereby disrupting the insulin signaling cascade[16]. Obesity is found to be associated with endothelial dysfunction and impaired muscle microcirculation, which can impair whole body insulin sensitivity by hindering the entry of insulin and glucose into skeletal muscle and decreasing their availability to muscle cells. Chronic exposure to glucose and fatty acids causes beta cell apoptosis which is a triggering factor for the transition from an obese, insulin resistant state to full blown type 2 diabetes[16]. Therapeutic lifestyle changes or TLC, a comprehensive lifestyle approach, as recommended by different health associations like the American Diabetes Association, American Heart Association and The Obesity Society to combat the risk factors associated with type 2 diabetes, stresses specific dietary recommendations, weight management and increased physical activity[16]. TLC dietary recommendations emphasize reducing total fat intake below 25%-35% of total calories, saturated fat < 7% of total calories, polyunsaturated fat intake should be increased to 10% of total calories, total reduction in trans fat consumption, daily dietary fiber intake about 20-30 g per day, intake of complex carbohydrates, especially whole grain, fruits and vegetables, should be around 50%-60% of the total calories, protein intake should be around 15%-20% of the total calories, cholesterol intake should be reduced to less than < 200 mg/d and salt intake < 2300 mg/d[43,54-58]. Plant sterols, soluble fibers and fatty oily fish should be incorporated in the diet, the corresponding amounts being 2 g/d for plant sterols, soluble fibers around 5-10 g/d and an arrangement to consume oily fish at least once a week. The aim of a TLC diet plan is to provide a proper balance of carbohydrates, proteins, fats and other nutrients at a level of 2000 calories. Inverse associations have been found between physical activity and the reduced risk of type 2 diabetes; regular physical activity decreases visceral and body fat, resistance exercises increase skeletal muscle mass, increasing muscle glucose uptake. Skeletal muscle is the major site for insulin mediated glucose disposal and its capability for glucose uptake and utilization is greatly impaired in type 2 diabetics[16,57]. Regular exercises increase the GS content of the muscle which accelerates non-oxidative glucose disposal as glycogen and activates GLUT4, potentiates insulin signaling by up-regulating the expression and activity of proteins involved in insulin signal transduction and improves the oxidative capacity of the skeletal muscles which can prevent lipid mediated insulin resistance; with exercise induced improvement in lipid oxidation, there will be improvement in fatty acid turnover that will prevent accumulation of fatty acid metabolites in the muscle with an enhancement in insulin sensitivity. Exercise decreases the free fatty acid concentration in the circulation and helps to improve liver insulin sensitivity. Regular physical activity counteracts with the micro vascular dysfunctions in type 2 diabetics, dilates muscle capillaries *via* increased formation of nitric oxide and at the same time improves the ability of insulin to increase muscle capillary perfusion[16,58-63]. TLC recommends engaging in at least 30 minutes of moderate intensity physical activity, above usual activity at work or home on most days of the week and greater health benefits can be obtained by engaging in physical activity of more vigorous intensity or longer duration; however, involvement in moderate to high intensity physical activity most days of the week should

not exceed the calorie intake requirements. For sustained weight loss in adulthood, TLC recommends 60-90 minutes of daily moderate intensity physical activity while not exceeding calorie intake requirements[64-68].

In this present one year study, blood glucose lipid profile values, BMI and HbA1c values of the enrolled patients were recorded before and after counseling. The BMI baseline value was 27 ± 0.92; for the control group it was 26 ± 0.65 but in the test group it was 25 ± 0.41, showing a significant reduction in BMI after one year. There were also significant reductions in FPG and PPG found in the test group due to their positive impact of regular diet control and exercise. The cholesterol value was significantly reduced in the test group from 167 ± 3.96 to 149 ± 3.32 after one year. Reduced TG value was significant due to diet control for the test group after one year. The HDL values increased significantly in the test group. The value of LDL was significantly reduced in the test group compared to the control group. In the present study, the baseline value of HbA1c was 8.3 ± 0.58 and it reduced significantly in the test group to 6.3 ± 0.32, compared to the control group which was 7.2 ± 0.42 at the end of one year. Thus, significant reductions in the HbA1c level were observed in the test group in contrast to the control group at the end of one year as a positive impact of lifestyle interventions. From the results of this RCT carried out in West Bengal, it can be concluded that type 2 diabetes can be effectively controlled in a non-pharmacological manner through proper lifestyle interventions like obesity control, dietary modifications and increased physical activities. However, proper patient motivation for lifestyle interventions remains a challenging issue in this trial.

In conclusion, diabetes is a chronic endocrinological disorder with serious long term complications, like diabetic foot ulcers necessitating limb amputations, retinopathy leading to blindness, nephropathy leading to end stage renal disease, neuropathy *etc*., and hence requires an amalgamation of pharmacological and non-pharmacological measures for effective case management strategy to have an enhanced glycemic control. The pharmacological approach recommends the use of oral hypoglycemics or insulin therapy but each has one or more side effects/adverse reactions on prolonged use. A successful non-pharmacological approach in diabetes management is proper lifestyle modifications in order to counteract the modifiable risk factors of type 2 diabetes. The majority of type 2 diabetics are overweight, do not undertake the recommended levels of physical activity and do not pursue dietary guidelines for proper dietary intake of fats, fruits and vegetables or avoidance of high calorie junk foods. Hence, proper patient motivation is needed to encourage them in regular physical activity and to adopt TLC recommended dietary habits. This study provides confirmation that proper patient counseling regarding type 2 diabetes, complications and successful lifestyle interventions for patients can be successfully implemented in developing nations where diabetes is an important factor in morbidity and mortality. Thus apart from pharmacological approaches recommending use of several synthetic oral hypoglycemic as well as herbal remedies, a non-pharmacological approach *via* lifestyle interventions can be successfully implemented for proper management of type 2 diabetes.

COMMENTS

Background

Diabetes is a complex metabolic disorder with improper utilization of glucose and disturbances in protein and fat metabolism, causing a spillover of these substances in the urine, mostly due to insufficient insulin secretion by the β cells of the pancreas. Increments in the number of type 2 diabetics around the world are a matter of concern. Both conventional and unconventional therapeutic approaches have attempted to control the chronic disabling and progressive nature of the disease and severe complications associated with it. Apart from insulin insensitivity and genetic predisposition, this multi-factorial disease involves a number of risk factors, like obesity, imbalanced diet, physical inactivity *etc*., collectively depicted as an "unhealthy/sedentary lifestyle".

Research frontiers

In this research review, management of type 2 diabetes has been attempted in a non-pharmacological manner with mechanistic details by proper "lifestyle interventions".

Innovations and breakthroughs

The results of several randomized controlled trials (RCTs) support the fact that adoption of a "healthy lifestyle" has improved the blood glucose lipid profile of type 2 diabetic subjects. The data of an RCT carried out on type 2 diabetic subjects in West Midnapore District of Bengal showed significant improvements in blood glucose lipid profiles at the end of one year of patient follow-up with suitable "lifestyle interventions", suggesting that type 2 diabetes can be prevented, or at least the onset of the disease can be delayed, by proper lifestyle modification.

Peer review

This paper carried out several RCTs to discuss type 2 diabetic subjects. This was a well written paper.

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MEETING

Events Calendar 2012

January 15-17, 2012
ICADIT 2012: International conference on Advances in Diabetes and Insulin Therapy
Zurich, Switzerland

January 29-February 3, 2012
Genetic and Molecular Basis of Obesity and Body Weight Regulation
Santa Fe, NM, United States

February 3, 2012
The Future of Obesity Treatment
London, United Kingdom

February 8-11, 2012
5th International Conference on Advanced Technologies and Treatments for Diabetes
Barcelona, Spain

February 9-10, 2012
EC Conference on Diabetes and Obesity Research - Save the Date
Brussels, Belgium

February 21, 2012
Association of Children's Diabetes Clinicians 6th Annual Meeting
Coventry, United Kingdom

February 23, 2012
Diabetes and kidney disease: advances and controversies
Birmingham, United Kingdom

March 1-3, 2012
International conference on Nutrition and Growth
Paris, France

March 7-9, 2012
Diabetes UK Annual Professional Conference 2012
Glasgow, United Kingdom

March 15 -16, 2012
Monogenic Disorders of Insulin Secretion: Congenital Hyperinsulinism and Neonatal Diabetes
Philadelphia, PA, United States

March 15 -17, 2012
2012 DF Con - Diabetic Foot Global Conference
Hollywood, CA, United States

March 19-22, 2012
Society for Endocrinology BES 2012
Harrogate, United Kingdom

March 22-25, 2012
2nd Latin America Congress on Controversies to Consensus in Diabetes, Obesity and Hypertension
Rio de Janeiro, Brazil

March 29-31, 2012
The 4th International Conference on Advances in Diabetes and Insulin Therapy
Riga, Latvia

March 29-April 1, 2012
New Frontiers in Diabetes Management
Ocho Rios, Jamaica

April 2-6, 2012
6th Annual Primary Care Spring Conference: Session 1
Palm Coast, FL, United States

April 4-7, 2012
39th Panhellenic Congress of Endocrinology and Metabolism
Athens, Greece

April 11-13, 2012
ICDM 2012: International Conference on Diabetes and Metabolism
Venice, Italy

April 11-13, 2012
ICDHLSP 2012: International Conference on Diabetes, Hypertension, Lipids and Stroke Prevention
Venice, Italy

April 16-17, 2012
Paediatric and Adolescent Diabetes
Birmingham, United Kingdom

April 22-25, 2012
9th International Podocyte Conference
Miami, FL, United States

May 9-12, 2012
19th European Congress on Obesity
Lyon, France

May 23-27, 2012
AACE 21st Annual Scientific and Clinical Congress - American Association of Clinical Endocrinologists
Philadelphia, PA, United States

May 24-27, 2012
27th Annual Clinical Conference on Diabetes
Bonita Springs, FL, United States

June 8-12, 2012
American Diabetes Association's 72nd Scientific Sessions
Philadelphia, PA, United States

June 29-August 2, 2012
ESE Summer School on Endocrinology
Bregenz, Austria

August 1-4, 2012
AADE 39th Annual Meeting - American Association of Diabetes Educators
Indianapolis, IN, United States

September 13-16, 2012
EMBO-EMBL Symposium: Diabetes and Obesity
Heidelberg, Germany

October 1-5, 2012
48th European Association for the Study of Diabetes Annual Meeting
Berlin, Germany

November 7-9, 2012
40th Meeting of the British Society for Paediatric Endocrinology and Diabetes
Leeds, United Kingdom

November 8-11, 2012
The 4th World Congress on Controversies to Consensus in Diabetes, Obesity and Hypertension
Barcelona, Spain

December 4-6, 2012
1st American Diabetes Association Middle East Congress
Dubai, United Arab Emirates

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INSTRUCTIONS TO AUTHORS

GENERAL INFORMATION

World Journal of Diabetes (*World J Diabetes*, *WJD*, online ISSN 1948-9358, DOI: 10.4239), is a monthly, open-access (OA), pe er-reviewed journal supported by an editorial board of 323 experts in diabetes mellitus research from 38 countries.

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3 **Tian D**, Araki H, Stahl E, Bergelson J, Kreitman M. Signature of balancing selection in Arabidopsis. *Proc Natl Acad Sci USA* 2006; In press

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4 **Diabetes Prevention Program Research Group**. Hypertension, insulin, and proinsulin in participants with impaired glucose tolerance. *Hypertension* 2002; **40**: 679-686 [PMID: 12411462 PMCID:2516377 DOI:10.1161/01.HYP.0000035706.28494.09]

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6 21st century heart solution may have a sting in the tail. *BMJ* 2002; **325**: 184 [PMID: 12142303 DOI:10.1136/bmj.325.7357.184]

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9 Outreach: Bringing HIV-positive individuals into care. *HRSA Careaction* 2002; 1-6 [PMID: 12154804]

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10 **Sherlock S**, Dooley J. Diseases of the liver and billiary system. 9th ed. Oxford: Blackwell Sci Pub, 1993: 258-296

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13 **Harnden P**, Joffe JK, Jones WG, editors. Germ cell tumours V. Proceedings of the 5th Germ cell tumours Conference; 2001 Sep 13-15; Leeds, UK. New York: Springer, 2002: 30-56

Conference paper

14 **Christensen S**, Oppacher F. An analysis of Koza's computational effort statistic for genetic programming. In: Foster JA, Lutton E, Miller J, Ryan C, Tettamanzi AG, editors. Genetic programming. EuroGP 2002: Proceedings of the 5th European Conference on Genetic Programming; 2002 Apr 3-5; Kinsdale, Ireland. Berlin: Springer, 2002: 182-191

Electronic journal (list all authors)

15 Morse SS. Factors in the emergence of infectious diseases. Emerg Infect Dis serial online, 1995-01-03, cited 1996-06-05; 1(1): 24 screens. Available from: URL: http://www.cdc.gov/ncidod/eid/index.htm

Patent (list all authors)

16 **Pagedas AC**, inventor; Ancel Surgical R&D Inc., assignee. Flexible endoscopic grasping and cutting device and positioning tool assembly. United States patent US 20020103498. 2002 Aug 1

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