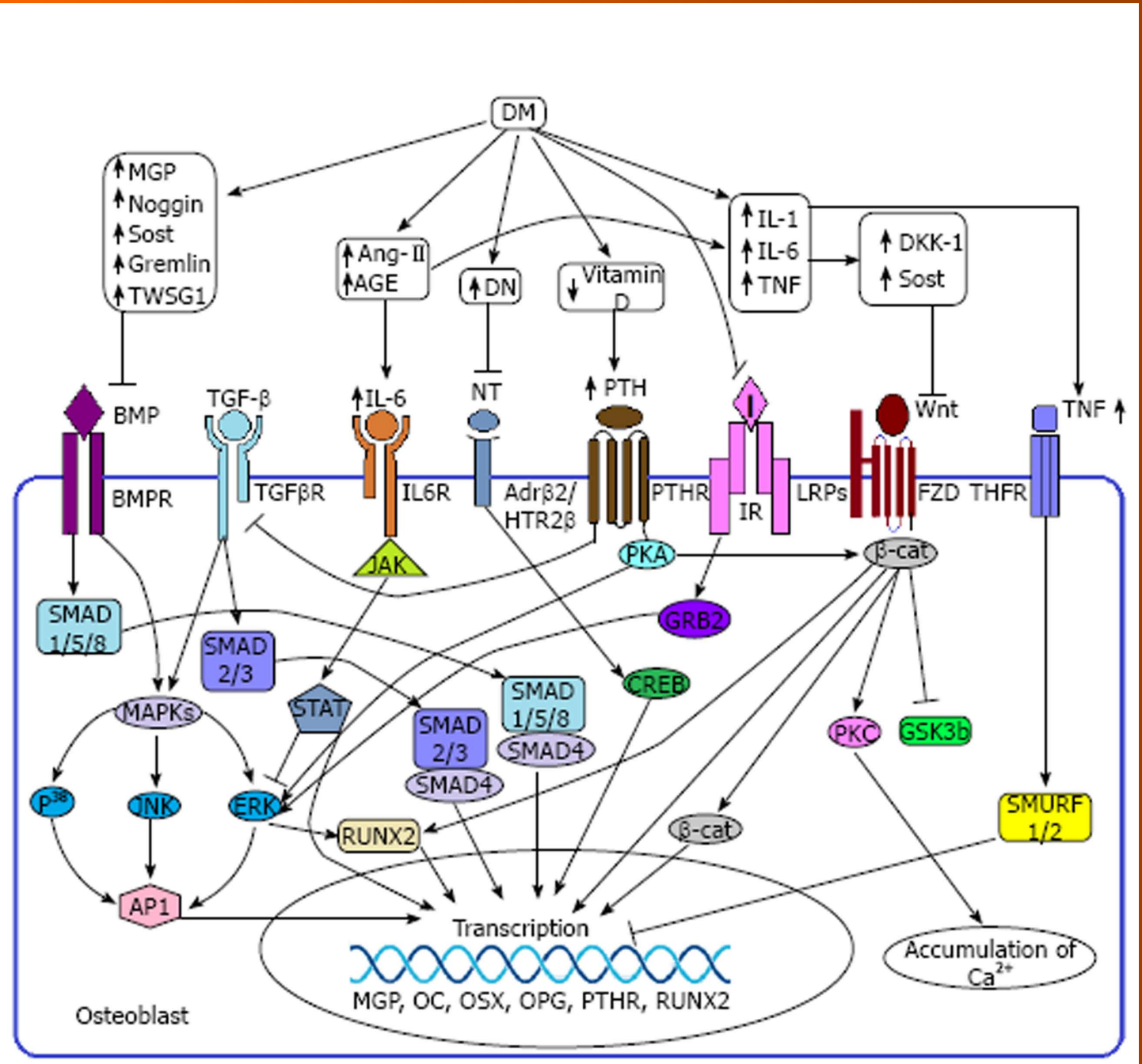
ISSN 1948-9358 (online)

World Journal of *Diabetes*

World J Diabetes 2013 August 15; 4(4): 88-161



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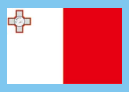

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World Journal of Diabetes

Contents

Bimonthly Volume 4 Number 4 August 15, 2013

AIM AND SCOPE

World Journal of Diabetes (*World J Diabetes*, *WJD*, online ISSN 1948-9358, DOI: 10.4239), is a peer-reviewed open access academic journal that aims to guide clinical practice and improve diagnostic and therapeutic skills of clinicians.

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INDEXING/ ABSTRACTING

World Journal of Diabetes is now indexed in PubMed Central, PubMed, Digital Object Identifier, and Directory of Open Access Journals.

NAME OF JOURNAL
World Journal of Diabetes

ISSN
ISSN 1948-9358 (online)

LAUNCH DATE
April 15, 2010

FREQUENCY
Bimonthly

EDITORIAL OFFICE
Jin-Lei Wang, Director
Xiu-Xia Song, Vice Director
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No. 62 Dongsihuan Zhonglu, Chaoyang District,
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http://www.wjgnet.com

PUBLISHER
Baishideng Publishing Group Co., Limited
Flat C, 23/F., Lucky Plaza,
315-321 Lockhart Road, Wan Chai, Hong Kong, China
Fax: +852-65557188
Telephone: +852-31779906
E-mail: bpgoffice@wjgnet.com
http://www.wjgnet.com

PUBLICATION DATE
August 15, 2013

INSTRUCTIONS TO AUTHORS
Full instructions are available online at http://www.wjgnet.com/1948-9358/g_info_20100107165233.htm

ONLINE SUBMISSION
http://www.wjgnet.com/esps/

World Journal of Diabetes

Online Submissions: http://www.wjgnet.com/esps/
wjd@wjgnet.com
doi:10.4239/wjd.v4.i4.88

World J Diabetes 2013 August 15; 4(4): 88-91
ISSN 1948-9358 (online)

Status of autoimmune diabetes 20-years after generation of BDC2.5-TCR transgenic non-obese diabetic mouse

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Received: April 9, 2013 Revised: May 22, 2013
Accepted: June 8, 2013
Published online: August 15, 2013

Abstract

Type 1 diabetes (T1D) is an autoimmune disease that results from the destruction of insulin-producing β cells by autoreactive T cells, leading to lifelong dependency on insulin therapy and increased risk of long-term cardiovascular complications. Here we take the opportunity of the 20th anniversary of the generation of the BDC2.5 TCR transgenic non-obese diabetic (NOD) mouse model, to provide a brief overview of the significant progress that has been made in understanding the role of T cells in the disease pathogenesis period. This included development of hundreds of reagents that block or even reverse new-onset disease by directly or indirectly controlling T cells. We also reflect on the sobering fact that none of these strategies has shown significant efficacy in clinical trials and discuss potential reasons hindering translation of the preclinical findings into successful therapeutic strategies and potential ways forward.

Key words: Autoimmune diabetes; Immunotherapy; T cells; BDC2.5 T cells; Anti-CD3; Immunosuppression

Core tip: Our understanding of type 1 diabetes pathogenesis has significantly improved over the last three decades. We went from not knowing very little to acquisition of significant details about the role of the immune system and different T cell subsets in the disease process. The non-obese diabetic mouse model contributed and continues to contribute to our understanding of the disease process. This article pays tributes to the major role T-cells bearing -cell - speci fic T-cell receptors transgenic mouse played in shaping of our understanding of the disease process. We also divulge to briefly discuss current challenges facing development of a safe immunotherapy for the disease.

Ramirez L, Hamad ARA. Status of autoimmune diabetes 20-years after generation of BDC2.5-TCR transgenic non-obese diabetic mouse. *World J Diabetes* 2013; 4(4): 88-91 Available from: URL: http://www.wjgnet.com/1948-9358/full/v4/i4/88.htm DOI: http://dx.doi.org/10.4239/wjd.v4.i4.88

COMMENTARY ON HOT TOPICS

Diabetes is a heterogeneous metabolic disease caused by glucose intolerance and manifested clinically as hyperglycemia. Based on the underlying cause of the hyperglycemia, diabetes is divided into type 1 (T1D) and 2 (T2D). T1D is autoimmune in nature and results from the destruction of insulin-producing β cells by autoreactive T cells, leading to insulin deficiency and dependency on exogenous insulin to maintain glucose homeostasis. In contrast, T2D is a complex metabolic disorder associated with insulin resistance in peripheral tissues. Currently, there is no cure for either type of diabetes. In the interim, T1D is managed by multiple daily injections of insulin, whereas T2D is controlled by medications that improve insulin sensitivity and/or reduce glucose production by

the liver. Maintenance of glucose homeostasis, however, is challenging and most patients eventually develop fatal cardiovascular complications. Intensive efforts are therefore being directed toward development of cure or prevention strategies. Small animal models play profoundly important roles in these efforts, particularly in T1D research.

Small animal research in T1D began in earnest with the development and use of spontaneous and induced disease models in 1970s and 1980s. Among several T1D models, the non-obese diabetic (NOD) mouse became the most commonly used and favorite model soon after its development about 33 years ago[1]. The value of the NOD mouse in understanding the disease mechanism increased exponentially in the late 80s and early 90s following development of technologies that allowed engineering of the genome to generate mice bearing particular transgenes or lacking specific molecules to interrogate their roles in the disease process[2]. Consequently, more than 250 different genetically modified NOD mice were produced and characterized (http://jaxmice.jax.org/findmice/index.html). Results of these efforts uncovered a wealth of information about the roles of various cell types and molecules in modulating T cells and established key cellular and molecular events in the disease process.

Of interest is that uncovering the role of T cells in autoimmune diabetes traversed several key steps that culminated in the generation of the NOD mouse bearing TCR transgenic T cells [reviewed in detail in by Haskins[3]. Considerable evidence accumulated in the early 1990s indicating a central role for T cells in mediating T1D in mice. These included demonstration that the disease development can be prevented by immunosuppressive agents that target T cells[4], and by anti-CD4 and anti-CD8 antibody treatments[5,6]. Furthermore, the disease was shown to be transferrable to neonatal NOD mice and immunodeficient NOD-severe combined immunodeficiency mice (NOD-SCID) by adoptive transfer of T cells from spontaneously diabetic NOD donors[7]. A clearer picture of the role of T cells began to emerge with the generation of islet antigen-specific T cell clones. Several groups independently generated islet antigen-specific T cell clones capable of transferring the disease to susceptible recipients[4]. It was found that different T cell clones expressed different T-cell receptors (TCRs), suggesting for the first time that islet-specific T cells recognize several different islet antigens and pointing to the complexity of the disease. Among the well-characterized clones is the BDC2.5 clone, the TCR that was later used to generate the T-cells bearing-cell-specific T-cell receptors (BDC 2.5 TCR) transgenic (tg) mouse in 1993[8]. Thus, generation of T cell clones was crucial in cementing the role of T cell in the disease pathogenesis and the existence of diabetogenic T cells in autoimmune-prone hosts. Yet clones have limited value in providing details regarding the nature and *in vivo* action mechanisms of diabetogenic T cells. Among the pressing questions (some of which are still incompletely understood) are how autoreactive T cells escape negative selection, where they reside in the periphery, what triggers them to become diabetogenic, and how they cause the disease. Diabetogenic T cells among the peripheral T cell repertoire are rare and the lack of appropriate reagents that permit their identification *in vivo* precluded addressing these questions directly *in vivo* in unmanipulated NOD mice. To overcome this problem, researchers generated TCR tg mice by using TCRs derived from generated clones. Among the widely used TCR transgenic mice in autoimmune diabetes is the BDC2.5 TCR tg mouse generated in 1993 by Katz *et al*[8], in which all T cells express the TCRα (Vα1) and β (Vβ4) chain genes from the BDC2.5 TCR CD4 T cell clone[9]. Unlike in wild type NOD mice, which harbor a diverse repertoire where autoreactive T cells are very rare and are difficult to track *in vivo*, all T cells in BDC2.5 tg mice recognize and respond uniformly to an elusive islet autoantigen [It was recently reported by two groups[10,11] that BDC2.5 T cells recognize peptides from chromogranin A (ChgA)]. Therefore, by studying T cells in BDC2.5 tg mice, the authors were able to track the behavior and fate of diabetogenic T cells *in vivo* and test hypotheses pertaining to roles of thymic selection, site of priming and peripheral activation of diabetogenic T cells, trafficking, and timing of response to islet autoantigens. Results showed that diabetogenic TCR can be produced in a large proportion of thymocytes in the TCR αβ tg mice, are positively selected without undergoing massive clonal deletion, and migrate to the periphery where they constitute the majority of the T cell repertoire. The model is still providing an important platform for *in vivo* dissecting of diabetogenic T cells, including roles of various molecules and cell types in modulating their pathogenicity. It has not only resulted in a wealth of information regarding pathogenesis of autoimmune diabetes, but also shed light on the immune system and autoimmunity.

Tracking disease development in BDC2.5 TCR tg mice showed that initiation of the disease is highly regulated with two important checkpoints controlling the diabetogenic process. These two checkpoints are especially evident and synchronous in BDC2.5 tg mice. The autoreactive T cells appear to ignore the β cells for the first 2 wk of life. Soon after, BDC2.5 T cells abruptly invade the pancreatic islets resulting in insulitis that progresses rapidly, with almost all islets heavily infiltrated by the age of 3 to 4 wk. Surprising at the time, however, was the observation that insulitis in most BDC2.5 tg mice never progresses to full-blown diabetes. But when the BDC2.5 transgene is introduced into NOD-Rag-1 knockout mice, they do develop aggressive disease at a very early age. Failure of BDC2.5 TCR tg mice to develop full-blown disease in Rag-1-sufficient background was due to incomplete allelic exclusion of endogenous TCRβ chains, resulting in developing thymocytes that differentiate into regulatory T cells that oppose the pathogenic effect of diabetogenic T cells leading to standstill insulitis. On the other hand, in the absence of the *Rag-1* gene all developing T cells bear the BDC2.5 TCR transgene, resulting in a pathogenic repertoire devoid of regulatory cells, inducing

a rapid onset of aggressive disease. The results provide critical hints of a major role for regulatory T cells in opposing the disease development. The synchronous development of the disease in BDC2.5 mice combined with other studies, including adoptive transfer of BDC2.5 T cells, led to the concept that immunoregulatory mechanisms exist at two check points, at the pancreatic draining lymph nodes and the islet itself, respectively. Breach of these checkpoints by diabetogenic T cells is clearly visualized in NOD mice by using adoptive transfer of BDC2.5 in appropriate hosts[12,13]. This paradigm is depicted in Figure 1. Subsequent studies revealed critical roles for regulatory T and B cells and various molecules involved in controlling the major checkpoints, and prevention and cure of the disease in the NOD mouse. Over the last two decades, vast numbers of molecules necessary for maintaining immunoregulatory mechanisms and others that facilitate their subversion have been identified. Targeting these molecules identified more than 250 interventions capable of preventing the disease in the NOD mouse. Some, like treatment with anti-CD3[14] and anti-CD20[15] reversed the disease in as many as 30%-50% of new-onset cases, raising hope of developing strategies to reverse disease in newly diabetic patients. Consequently, in the last few years, clinical trials have been conducted to test efficacy of several molecules including anti-CD3 and anti-CD20.

Sobering reality facing translation of preclinical data into effective immunotherapeutics and ways forward

Translating immunotherapies found effective in preclinical studies into human therapies is proving challenging[16], at least for now. Several high profile clinical trials including phase Ⅲ have failed to demonstrate significant efficacy for all those tested[17,18]. The disappointing results in the clinic are forcing a retreat to drawing boards and generating second thoughts about whether the NOD mouse has surpassed its life expectancy as a research model and even the value of NOD mice in predicting and evaluating immunotherapy for T1D. It is easy to lay the blame on biologic differences between humans and mice, accentuated by more than 60 million years since their divergence into two species that differ in size, lifespan, and lifestyle (habitat/environment). The immune system in humans and mice, however, are generally quite similar, and with few notable exceptions, most paradigms translate well between them. Thus, the intangible efficacy of modalities such as anti-CD3 in humans is not entirely justified by biologic differences between the two species.

We argue that environmental factors play a dominant, if not the dominant role, in subverting therapeutic efficacy of modulators acting alone or in synergy with genetic factors[19,20]. This is acutely evident in the NOD mouse itself. For instance, the variability of anti-CD3 efficacy in reversing new-onset hyperglycemia ranges from about 30%-80% in newly diabetic NOD mice housed in the same facility[14,21] and mostly likely mice in the same cage responded differently. The low efficacy in NOD mice

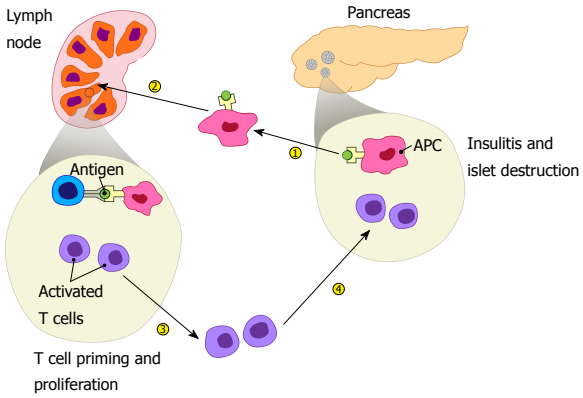

Figure 1 Pathogenesis of type 1 diabetes includes four major steps: islet autoantigens are picked up by antigen presenting cells from the pancreas, which then migrate to draining lymph nodes and present the autoantigens to autoreactive T cells, leading to their priming. Activated autoreactive T cells undergo proliferation, differentiation, and acquire homing molecules that direct them to the pancreas where they infiltrate the islets resulting in insulitis and β cell destruction.

given the extremely small variations in their genetic make-up and exogenous influence of the environment suggests that treating the same mice under virtually identical conditions, the treatment would be successful only once out of at least two attempts. Applying the comparison to patients with markedly different genetic backgrounds, types of food, environment, and microbiota, the odds of success would be extremely low. Therefore, there is still much to be learned in the NOD mouse to uncover causes of variability on rate of disease onset, timing and response to treatment. In addition, understanding why females are more susceptible to disease than males[22-24] and why NOD mice housed in conventional facilities do not develop disease remains unclear[25]. It will also be important to understand why inactivation of molecules such as Fas death receptor or its ligand prevents disease in NOD mice[13,16,26-29]. Understanding mechanisms underlying these observations would provide important clues that could potentially facilitate the development of therapeutic strategies with high efficacy rates that are effective in both mice and men.

ACKNOWLEDGMENTS

We apologize for not having the opportunity to cite all seminal work describing islet reactive clones and transgenic mice expressing islet antigens. We thank Catherine E. Kiefe, MLA, at The Department of Art as Applied to Medicine at Johns Hopkins University for illustration of Figure 1.

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World Journal of Diabetes

Online Submissions: http://www.wjgnet.com/esps/
wjd@wjgnet.com
doi:10.4239/wjd.v4.i4.92

World J Diabetes 2013 August 15; 4(4): 92-100
ISSN 1948-9358 (online)

REVIEW

Trace elements in diabetic cardiomyopathy: An electrophysiological overview

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Received: June 6, 2013 Revised: July 12, 2013
Accepted: July 18, 2013
Published online: August 15, 2013

Abstract

There is a growing body of evidence that Diabetes Mellitus leads to a specific cardiomyopathy apart from vascular disease and bring about high morbidity and mortality throughout the world. Recent clinical and experimental studies have extensively demonstrated that this cardiomyopathy causes impaired cardiac performance manifested by early diastolic and late systolic dysfunction. This impaired cardiac performance most probably have emerged upon the expression and activity of regulatory proteins such as Na^+/Ca^{2+} exchanger, sarcoplasmic reticulum Ca^{2+}-ATPase, ryanodine receptor and phospholamban. Over years many therapeutic strategies have been recommended for treatment of diabetic cardiomyopathy. Lately, inorganic elements have been suggested to have anti-diabetic effects due to their suggested ability to regulate glucose homeostasis, reduce oxidative stress or suppress phosphatases. Recent findings have shown that trace elements exert many biological effects including insulin-mimetic or antioxidant activity and in this manner they have been recommended as potential candidates for treatment of diabetes-induced cardiac complications, an effect based on their modes of action. Some of these trace elements are known to play an essential role as component of enzymes and thus modulate the organ function in physiological and pathological conditions. Besides, they can also manipulate redox state of the channels *via* antioxidant properties and thus contribute to the regulation of $[Ca^{2+}]_i$ homeostasis and cardiac ion channels. On account of little information about some trace elements, we discussed the effect of vanadium, selenium, zinc and tungstate on diabetic heart complications.

Key words: Diabetic cardiomyopathy; Electrophysiology; Trace elements; Insulin-mimetic; Antioxidant

Core tip: Diabetic cardiomyopathy is one of the major causes of mortality in diabetic patients. Common cellular defects underlying the progressive cardiac complications of diabetes are reduction in the rate of contraction, low myosin ATPase activity, dysregulation of $[Ca^{2+}]_i$ homeostasis and altered ionic currents. Accordingly, it is of critical importance to develop therapeutic strategies that will effectively inhibit diabetes induced fatal complications. In last decade, several trace elements have been suggested to improve performance of diabetic heart based due to their potential anti-diabetic and/or antioxidant activity. In this article the effects of trace elements on electrophysiological alterations of diabetic heart were discussed in detail.

Ozturk N, Olgar Y, Ozdemir S. Trace elements in diabetic cardiomyopathy: An electrophysiological overview. *World J Diabetes* 2013; 4(4): 92-100 Available from: URL: http://www.wjgnet.com/1948-9358/full/v4/i4/92.htm DOI: http://dx.doi.org/10.4239/wjd.v4.i4.92

INTRODUCTION

Cardiomyopathy, which develops independent of any major vascular disease, is one of the main complications

of diabetes resulting in a high percentage of morbidity and mortality. Although atherosclerotic vascular diseases occur frequently in diabetic conditions, a specific type of cardiomyopathy that results in impaired cardiac performance has been widely described in clinical and experimental studies[1-7]. In clinical aspect, diabetic cardiomyopathy is a disease which manifests itself particularly by early diastolic and late systolic dysfunction. As a matter of fact, elevated end-diastolic left ventricular (LV) pressure, reduced end-diastolic LV volume, impaired LV function in response to physiological stress and reduced LV filling rates in diabetic humans and animals are well-characterized[7-9]. These functional abnormalities of diabetic heart are likely to stem from multiple cellular defects such as reduction in the rate of contraction and relaxation, low myosin ATPase activity, myosin isoforms' shift from V1 (fast) to V3 (slow), deterioration of sarcoplasmic reticulum (SR) calcium uptake and reduction in glucose carrier (GLUT-4)[10,11]. Consistent with this, our reports and other studies have demonstrated prolonged periods of contraction and relaxation and in turn reduced tensile strength of rat papillary muscle in type 1 diabetes[12,13]. However, unchanged tensile strength despite slow left ventricular papillary muscle contraction and relaxation has been also suggested in experimental diabetes model in rats[3,4,14]. At cellular level prolongation of action potential (AP) duration has been consistently shown in diabetic hearts[3,4,15,16]. Significant alterations in the ionic currents that constitute AP configuration have been proposed as the main culprit of this prolongation, and indeed reduced transient outward (I_{to}) along with smaller steady-state K^+ currents (or I_{ss}) have been reported[3-6,15,16], despite unchanged Ca^{2+} currents[5,6]. Additionally, inward rectifier K^+ current (I_{K1}) has not been stated to have changed, but the delayed rectifier current (I_K), thought to modulate late repolarization of AP, has decreased in diabetic ventricular cells[6,16].

On the other hand, regulation of intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) is very critical for myocardium and has overriding impact on the contraction of heart. Therefore, diabetes induced abnormalities in cardiac contractility have been correlated with the intracellular $[Ca^{2+}]_i$ changes[5, 6,12,14,17]. However, despite the plenty of data about dysregulated $[Ca^{2+}]_i$ in diabetic myocardium, current findings are somehow controversial particularly in terms of the direction of change[14,18,19]. Nevertheless, amplitudes of Ca^{2+} transients recorded under electrical stimuli have been reported to be smaller, while their time to peak and decay were mostly longer[5,6,19-22]. Therefore, it is most likely that diabetic cardiac dysfunction arises due to changes in expression and/or activity of cellular mechanisms that regulate $[Ca^{2+}]_i$ during cardiac cycle. This possibility has been widely studied over years and indeed a significant decrease has been found in expression of regulatory proteins such as Na^+/Ca^{2+} exchanger NCX (NCX), sarcoplasmic reticulum Ca^{2+} ATPase (SERCA), ryanodine receptor (RyR) and phospholamban (PLB), along with reduced activity of NCX and SERCA[11,18,20,22-25].

However diabetes is characterized by complexity; it likely involves activation of different pathways leading to abnormal $[Ca^{2+}]_i$ homeostasis and thus contractile dysfunction. For example, currently it is clearly evident that reactive oxygen species (ROS) and resultant oxidative stress is involved in the pathogenesis of diabetic cardiomyopathy. Hyperglycemia leads to generation of superoxide radicals from both mitochondrial (*via* oxidation of glucose) and non-mitochondrial sources (xanthine oxidase, nitric oxide synthase and NADPH-oxidase)[26].

DIABETES AND TRACE ELEMENTS

In recent years many inorganic elements have been recommended as dietary supplement to alleviate the impaired insulin metabolism in diabetic patients[5,26-30]. Being essential or not, trace elements have been identified for long time as potential candidates for treatment or to mitigate severity of complications of some metabolic disorders including diabetes (Figure 1). Activation of insulin receptor signaling, antioxidant properties or inhibition of phosphatases have been depicted as potential ways of action in modulating glucose homeostasis and preventing organ damage[26,29,31]. On the other hand, cardiac complications have been progressively becoming the main cause of death among diabetics due to the improvements in the treatment of diabetic complications with non-cardiac origin. Accordingly, it is of critical importance to develop therapeutic strategies that will effectively inhibit diabetes induced fatal cardiac disorders. Consistently, trace elements, some of which are involved in metabolism as essential components of enzymes, have also been suggested to improve the reduced cardiac performance in diabetic heart due to their presumed insulin-mimetic or antioxidant activity[3,5,28,32]. Furthermore, recent studies have demonstrated that the underlying mechanism of this improvement is due most probably to restoration of abnormal $[Ca^{2+}]_i$ homeostasis and cardiac ion channels. Despite the limited number of studies, it is evident that either insulin-mimetic or antioxidant, trace elements are capable of modulating expression and/or redox status of ion channels and $[Ca^{2+}]_i$ regulating proteins[33-36]. Of the inorganic or trace elements currently known; vanadium, selenium, zinc and tungstate were discussed in this review, since the effects of other inorganic elements on diabetic cardiac complications have not been well-documented yet.

Selenium

Selenium was first discovered by Berzelius in 1818. This Swedish chemist named that new chemical element after Selene, the Greek goddess of the moon. Selenium is an essential trace element in man and animals, since it is an integral part of selenium dependent glutathione peroxidase[36]. In humans and experimental studies, selenium deficiency has been suggested to result in increased risk of various pathologies including cardiovascular diseases[37]. Particularly, selenium deficiency results in Keshan disease, which is a special type of cardiomyopathy caused by di-

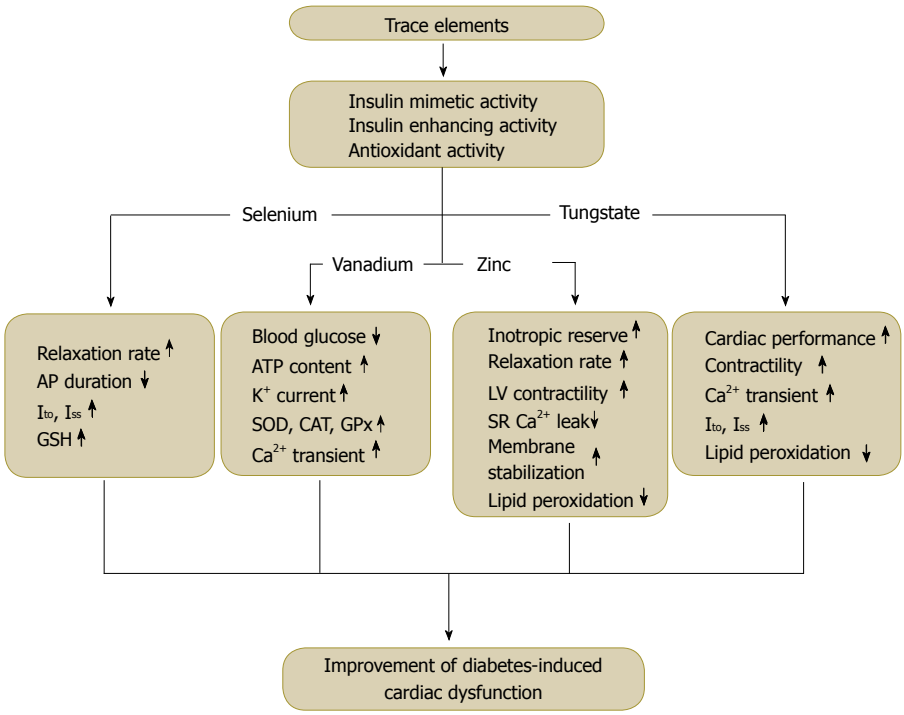

Figure 1 The summarized effects of trace elements on diabetes-induced cardiac complications. AP: Action potential; LV: Left ventricle; SR: Sarcoplasmic reticulum; SOD: Superoxide dismutase; CAT: Catalase; GPx: Glutathione peroxidase; GSH: Glutathione.

etary inadequacy of selenium and responds to treatment with sodium selenite[38]. Furthermore, adequate selenium intake is required for optimal activity of some key antioxidant enzymes, including glutathione peroxidases and thioredoxin reductases, which act to prevent free radical damage to various cells[39,40]. As a result of its protective role against oxidative stress, selenium raised considerable expectations for the prevention of cardiovascular diseases including diabetic cardiomyopathy. It appears to have insulin-like effects when administered *in vivo*[41]. In fact, several reports have suggested that plasma glucose levels were significantly though not completely improved in the diabetic rats treated with selenite in different ways either orally or *via* injection[3,42-44]. Interestingly, similar to vanadium, selenite decreases plasma glucose levels in hypoinsulinemic rats without an accompanying correction of the insulin levels[3,42]. Nevertheless, the reduced cardiac performance characteristics of diabetic rats such as left ventricular developed pressure (LVDP), positive dP/dt (+ dP/dt) and negative dP/dt (- dP/dt) have been found to be reversed with selenite treatment[42,45].

The effects of sodium selenite treatment on mechanical and electrical properties of diabetic heart have been also studied in detail. Ayaz *et al*[3] demonstrated that selenium supplementation for 5 wk was capable of reducing the prolonged peak time and relaxation of electrically stimulated papillary muscle twitch in diabetic rats, although no change was reported between peak tension of the experimental groups. Additionally, the prolonged AP duration, which is a typical characteristic of diabetic heart, was shown to be restored after the treatment. In the same study, the major repolarizing currents of AP, I_{to} and I_{ss}, were lower in untreated diabetic cardiomyocytes while selenium achieved an apparent increase in treated-diabetics. However, plasma insulin levels didn't increase significantly despite the long-term administration of selenium[3]. Although the precise mechanism of this beneficial effect is not known currently, it is likely that oxidative stress, which has been suggested to involve in the etiology of diabetes-induced downregulation of ion channels, is balanced through selenite-mediated augmentation of glutathione levels, and resultant enhancement of endogenous antioxidant defense mechanisms[3,5,28,46]. Additionally, oxidative species have been recognized to modulate K^+ channels and cellular Ca^{2+} regulation, notably *via* redox modifications of key amino acid residues involved in the function of ion channels and transporters[33-35,47]. Therefore, it is most likely that selenium may achieve recovery of impaired cardiac performance and altered K^+ currents of diabetic cardiomyocytes *via* restoration of the oxidized groups of ion channel proteins.

Vanadium

Vanadium is a trace element that exists naturally in water and soil and found in different physiologically active oxidation states[29]. Although the exact physiological actions of vanadium are not known yet, it is supposed to be necessary for the body as a trace element since its deficiency has been suggested to result in a variety of side-effects[29,48]. In addition to reproductive problems and skeletal abnormalities observed in case of deficiency, vanadium is likely to have a significant role in thyroid, iron, glucose and lipid metabolism[29,49]. The total vanadium content of the body has been estimated to be approximately 200 μg[29,50]. The

beneficial effects of vanadium have been widely studied in diabetic conditions and speculated to exert insulin-mimetic activity through a specific tyrosine kinase receptor or to annihilate free radicals due to its antioxidant activity[51-55]. Therefore, the potential use of vanadium in the treatment of diabetic complications including cardiomyopathy has been assessed and indeed its hypoglycemic effect along with reversal of functional abnormalities has been clearly demonstrated by several studies[28,56-59].

In the last decade, the effect of vanadate compounds on impaired performance of diabetic heart has been investigated in a large number of studies that have shown significant improvement in diabetes with vanadate treatment[28,56]. Ozcelikay *et al*[60] reported that vanadate treatment was capable of normalizing blood glucose and serum thyroid hormone levels, despite the fact that serum insulin level of diabetic animals was not corrected significantly. Moreover, vanadate treatment resulted in normalization of mechanical alterations and reversed the decreased responsiveness of diabetic atria to isoprenaline in spontaneously-beating preparations from diabetic rats. Similarly Heyliger *et al*[56] assessed the impact of vanadate on cardiac performance in diabetic female rats and found that vanadate was capable of restoring blood glucose but not insulin levels when administered for a 4-wk period to the diabetic rats. In the same study, vanadate treatment prevented the decline in cardiac performance due to diabetes. Organic vanadium complex, bis (maltolato) oxovanadium (Ⅳ) was also reported to correct working heart parameters such as LVDP and ± dP/dT values in streptozotocin-induced diabetic rats, which indicated the protective effect of vanadium derivatives against heart dysfunction associated with type 1 diabetes in rats[61]. Consistently, decreased peak ± dP/dt and reduced cardiac efficiency of diabetic hearts were fully restored while myocardial ATP content significantly increased by vanadate administration[62]. These results, thus, indicate that the normalizing effect of vanadate on diabetes can contribute to the prevention of cardiac changes observed at the early and late stages of diabetes.

Taking the central role that Ca^{2+} plays in cardiac electrical and mechanical activity, it is likely to suggest that the beneficial effects of vanadate entail modulation of Ca^{2+} regulation in diabetic cardiomyocyte. In fact Clark *et al*[28,29] demonstrated that tea-vanadate treatment had normalized the contractile response of diabetic cardiomyocytes and ameliorated the Ca^{2+} transients to an extent equal to or better than that of insulin treated diabetic animals. It is an effect that were attributed to the alleviated glycemic status because tea/vanadate decoction has been shown to restore glycemic status effectively in rodent models of both Type Ⅰ and Type Ⅱ diabetes mellitus[63,64]. Interestingly, tea/vanadate decoction exhibited vastly improved glycemic status that could persist beyond treatment period[63] and relieved diabetic animals from non-specific side-effects of vanadate or its analogues to other organs in the body[65,66]. Vanadate also mimics the enhancing effect of insulin on cardiac K^+ currents (particularly I_{to}) in sucrose-fed rats with 3-4 wk treatment or 5-6 h incubation of myocytes, an effect suggested to arise due probably to synthesis of new channels[67]. Hence, although we don't have such data, it is tempting to speculate that vanadate is likely to shorten AP duration in diabetic myocardium and thereby modulate ventricular repolarization and dispersion of repolarization that have been shown to be a major cause of cardiac arrhythmias in diabetes mellitus[68].

Vanadate is thought to act *via* insulin-mimetic and/or insulin-enhancing action[69] or through activation of lipid signaling mechanisms like the phosphatidylinositol pathway[54]. It can also scavenge free radicals[55], and accordingly, vanadate administration has been reported to decrease oxidative damage remarkably in the diabetic heart[70]. Therefore, the beneficial effect of vanadate on diabetes-induced cardiac dysfunction may stem from its ability to serve as a scavenger of free radicals[27,54,71]. With vanadium treatment, glutathione peroxidase, catalase and superoxide dismutase levels have been corrected to near normal values in diabetic rats[54,55]. However, one another study attributed some of these effects to vanadate's ability to prevent diabetic hypothyroidism[60]. In conclusion, despite the plenty of findings that provide evidences for improving effect of vanadium on diabetic heart dysfunction due most probably to its insulin-mimetic and/or antioxidant action, further studies are needed to fully elucidate the molecular mechanism of these beneficial effects.

Zinc

Zinc is an essential trace element that is critical in maintaining cellular functions since it is the cofactor of numerous enzymes and transcription factors[26,72]. In normal cellular physiology, much of the intracellular zinc is found in protein bound form and participates in phosphorylation/dephosphorylation cascades. Besides, it acts as a second messenger in the signaling system[73] and affects the redox status of the cell. Thus, in particular conditions zinc can either enhance the cell's antioxidant capacity or trigger the production of reactive oxygen species[26,74]. Consistent with this, Zn deficiency has been suggested to result in increased oxidative damage in multiple organs including the heart[75-77] due to the decreased cardiac antioxidant capacity[76,78].

It has been demonstrated that Zn deficiency induced by low concentrations of Zn in drinking water[79] and by Zn chelators increases the likelihood of diabetes in humans and animals[80]. Therefore, it is likely that Zn deficiency can be a risk factor for the development of diabetes, and in reciprocal manner, diabetes itself can dysregulate Zn homeostasis. Indeed, systemic Zn deficiency has been associated with the high incidence of diabetic cardiovascular complications[72,79,81,82]. The potential role of zinc in the protection of diabetic patients from coronary heart disease has been investigated in a recent clinical trial in which serum zinc level was inversely proportional to cardiovascular complications[83]. Measurements of cardiac function have demonstrated that Zn is

capable of improving left ventricular systolic and diastolic function. Moreover, inotropic reserve of left ventricle was enhanced in the heart of the diabetic mice treated with Zn compared to that without Zn, which implicates alleviated cardiac function with Zn supplementation[30]. Wang *et al*[84] observed lower ± dP/dtmax, suggesting reduced LV contractility along with slowing of relaxation in the diabetic mice, which both improved following Zn supplementation to near control levels. Furthermore, Zn ameliorated the diabetes-induced catecholamine desensitization markedly, which was quantified by measure of augmentation of dP/dtmax after β-adrenergic stimulation. Thus, they concluded that zinc is capable of improving both basal and stimulated LV function as well as inotropic reserve in diabetic hearts.

On the other hand, incomplete relaxation and reduced contractile function which were more prominent as pacing frequency increased has been reported in diabetic cardiomyocytes, but these changes were significantly restored by extracellular Zn exposure[8]. These findings provide evidences that suggest zinc administration could be a possible long term management regimen for incomplete relaxation and diastolic dysfunction associated with diabetic cardiomyopathy. In addition, extracellular zinc ion has been proposed to compete with Ca^{2+} for the cardiomyocyte L-type Ca^{2+} channel and, the release of SR Zn through the RyR also appears to be regulated similarly to that of SR Ca^{2+}[85-87]. Moreover, extracellular Zn exposure could lower the open probability of RyR and presumably reduces SR Ca^{2+} leak through the RyR, which has been shown to be elevated in hyperglycemic conditions[88,89]. Given these results, it is likely that Zn exerts a competitive effect on Ca^{2+} regulatory mechanisms and modulates cardiomyocyte function.

Although the cellular and molecular mechanisms responsible for zinc-induced protection against diabetic cardiomyopathy has not been fully understood yet, zinc-binding protein metallothionein (MT) has been proposed to play a role in cellular defence against oxidative stress associated with diabetic cardiomyopathy[72,84,90]. Indeed, Zn supplementation provides significant protection of the heart from oxidative stress. Zn has been demonstrated to act as an antioxidant through participation in SOD and thioredoxin enzymatic and chelator activities, stabilizing cell membranes, and inhibiting lipid peroxidation[26,74,91]. Additionally, the relationship between Zn and diabetes appears to be complex. Several complications of diabetes have been supposed to be related to increased intracellular oxidants and free radicals associated with decreases in intracellular Zn and in Zn-dependent antioxidant enzymes[92]. Moreover, Zn is suggested to be important for the normal conformation, secretion and function of insulin[26,30].

All these observations strongly support the notion that Zn deficiency occurs in diabetic subjects[82] and Zn supplementation may improve cardiac dysfunction or damage in these patients due to its systemic antioxidant capacity or modulation of the cellular ionic mechanisms. However, the understanding of molecular mechanisms that involve in Zn related changes in diabetic heart deserves further investigation.

Tungstate

Over the past decade, sodium tungstate (Na_2WO_4), which chemically resembles vanadium has become a molecule of interest, since it has a relatively low toxicity and it has been suggested to have antidiabetic activity in experimental studies[93-96]. Although numerous studies have demonstrated the efficacy of tungstate as an antidiabetic agent in various models of experimental diabetes, only few of them have investigated whether it can improve cardiac performance of diabetic heart as well. One of these studies performed by Nagareddy *et al*[31] has assessed cardiac function by measuring left ventricular pressure, the rate of contraction and the rate of relaxation. An apparent cardiac dysfunction has been shown in untreated diabetic rat hearts, which exhibited an inability to respond to the increase in left atrial filling pressure. However, the treatment of diabetic rats with tungstate has improved LVP, + dP/dt, and - dP/dt, particularly at higher filling pressures.

On the other hand, recently we have studied the cellular mechanism of that beneficial effect of sodium tungstate on diabetic myocardium at cellular level. We demonstrated that long-term sodium tungstate treatment was capable of ameliorating the amplitude of shortening and associated Ca^{2+} transients of diabetic cardiomyocytes, although it didn't improve the rate of relaxation in either traces. Moreover, we showed depressed I_{to} and I_{ss} in diabetic cardiomyocytes which were recovered significantly by tungstate administration that might be accomplished due to its antioxidant property[5]. This finding is important because diminished potassium currents and thus prolonged action potential in ventricular cells have been suggested to increase the likelihood of arrhythmia in diabetic patients[4,33,67,97]. Hence, tungstate administration is likely to reduce this propensity in diabetic patients.

The underlying mechanism of these beneficial effects has been mostly attributed to antioxidant or insulin-like activity of tungstate. Because hyperglycemia leads to abnormal increase of ROS production[5,11,35] that have been recognized to be capable of modulating K^+ channels and $[Ca^{2+}]_i$ regulation due to redox modifications of key amino acid residues involved in the function of intracellular and plasma membrane ion channels and transporters[33,35]. In fact, tungstate treatment was associated with significant reduction of lipid and protein oxidation levels in treated-diabetic rats, a finding that further supports this hypothesis. Insulin-mimetic or insulin-enhancing activity of tungstate is less likely since we didn't observe a remarkable change either in insulin or blood glucose levels after supplementation[5]. Contrary to this, some investigators have reported increased insulin and/or decreased glucose levels that might arise from very high level of tungstate they administered, which may cause side effects[98].

CONCLUSION

Diabetic cardiomyopathy, one of the major causes of mortality in diabetic patients, is associated with progressive contractile dysfunction. Therefore, it is crucial to develop therapeutic strategies that will effectively inhibit diabetes-induced fatal complications of the heart. Among the various therapeutic strategies, the restoration of glycemic status by insulin-enhancing or insulin-mimetic agents can be useful in the prevention of cardiomyopathy in diabetic patients.

In the last decade, several inorganic compounds such as selenium, vanadium, zinc and tungstate have been suggested to improve cardiac performance in diabetic heart based on its potential anti-diabetic and/or antioxidant activity. Some of these trace elements are known to play an essential role as components of enzymes and thus modulate the organ function in physiological and pathological conditions. Current findings clearly demonstrate that diabetic cardiomyopathy leads to ventricular dysfunction due to altered ionic homeostasis in myocytes which results in defective excitation-contraction coupling of myocardium and, trace element supplementation can prevent these changes and thus ameliorate the diminished cardiac function. Therefore, they may have a potential therapeutic use in preventing diabetic cardiomyopathy, although further investigations and substantial efforts are needed to elucidate the underlying mechanism of their beneficial effect. Furthermore, prior to clinical trials, the question whether they have side effects or not should be addressed unequivocally.

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P- Reviewers Cai L, Kumar R **S- Editor** Wen LL **L- Editor** A **E- Editor** Lu YJ

World Journal of Diabetes

Online Submissions: http://www.wjgnet.com/esps/
wjd@wjgnet.com
doi:10.4239/wjd.v4.i4.101

World J Diabetes 2013 August 15; 4(4): 101-113
ISSN 1948-9358 (online)

REVIEW

Biomolecular basis of the role of diabetes mellitus in osteoporosis and bone fractures

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Received: May 4, 2013 Revised: June 27, 2013
Accepted: July 17, 2013
Published online: August 15, 2013

Abstract

Osteoporosis has become a serious health problem throughout the world which is associated with an increased risk of bone fractures and mortality among the people of middle to old ages. Diabetes is also a major health problem among the people of all age ranges and the sufferers due to this abnormality increasing day by day. The aim of this review is to summarize the possible mechanisms through which diabetes may induce osteoporosis. Diabetes mellitus generally exerts its effect on different parts of the body including bone cells specially the osteoblast and osteoclast, muscles, retina of the eyes, adipose tissue, endocrine system specially parathyroid hormone (PTH) and estrogen, cytokines, nervous system and digestive system. Diabetes negatively regulates osteoblast differentiation and function while positively regulates osteoclast differentiation and function through the regulation of different intermediate factors and thereby decreases bone formation while increases bone resorption. Some factors such as diabetic neuropathy, reactive oxygen species, Vitamin D, PTH have their effects on muscle cells. Diabetes decreases the muscle strength through regulating these factors in various ways and ultimately increases the risk of fall that may cause bone fractures.

Key words: Diabetes; Osteoporosis; Diabetic neuropathy; Muscle atrophy; Insulin; Receptor activator for nuclear factor κ-B ligand; Interleukin 6; Angiotensin Ⅱ; Tumor necrosis factor; Advanced glycation end product

Core tip: The physical complications due to diabetes mellitus are not limited since there have been going research to elucidate the relation of other diseases with diabetes mellitus (DM). Osteoporosis is one of the complicated diseases of human that may be linked with DM through different networks in the body. In this review a precise relationship has been made between DM and osteoporosis through a broad range of biophysical pathways.

Roy B. Biomolecular basis of the role of diabetes mellitus in osteoporosis and bone fractures. *World J Diabetes* 2013; 4(4): 101-113 Available from: URL: http://www.wjgnet.com/1948-9358/full/v4/i4/101.htm DOI: http://dx.doi.org/10.4239/wjd.v4.i4.101

INTRODUCTION

Osteoporosis (OP) has become an alarming health problem through the entire world and about 200 million people in the world are under the threat of this deleterious health problem[1]. Although OP is often described as a silent disease because it is typically asymptomatic until a fracture occurs, the disease negatively and significantly impacts morbidity and mortality as it can lead to severe pain, deformity, disability, and death[2]. The signs of OP are deterioration of the microstructure of bone specifically at trabecular sites including vertebrae, ribs and hips, culmination in fragility fractures, pain and disability[2,3]. The occurrence of OP is prevalent among the aging women than the aging men although corticosteroid treatment, intake of excessive alcohol, cigarette smoking, low calcium intake and hypogonadism may be the secondary cause[1,2].

Like osteoporosis, diabetes mellitus is a pandemic and a chronic metabolic disorder with substantial morbid-

ity and mortality, characterized by the presence of high blood glucose[2,4,5]. According to the report (September 2012) of the World Health Organization (WHO) about 374 million people in the world are under the threat of this deleterious health problem[6]. Under chronic condition DM adversely affects the different parts of the body including bone, nerve, muscles, retina of the eyes, cardiovascular system and nephron of kidney[4]. The effects of DM on bone cell are very complex and several investigations have been conducted to explore the exact mechanisms through which DM induces osteoporosis and bone fractures and all the investigations have come to the end with few findings[6]. The exact mechanism of diabetes mellitus (DM) induced osteoporosis is almost unknown but it is plausible that, patients with DM have increased rate of osteoporosis and bone fractures[3,7-10]. Hyperglycemia may induce osteoporosis and bone fractures through exerting its effects on bone cells and muscle cells through different possible pathways. This review has explained the possible molecular mechanisms through which DM may induce osteoporosis and bone fractures.

EFFECT OF DIABETES MELLITUS ON BONE CELLS

The bone mainly comprise of three basic types of cells osteoblast, osteocyte and osteoclast[11]. Osteoblasts commonly called bone-forming cells which derived from the osteoblast progenitor cells, participate in mineralization and are unable to multiply[11]. Osteocytes are mature osteoblast which no longer secretes matrix, participates in nutrient/waste exchange *via* blood and unable to divide. Osteoclasts are cells that derive from the macrophage-monocyte cell lineage and participate in bone resorption[1,11].

Osteoblast

Osteoblast originates from the mesodermal progenitor cell and among the three basic types of bone cells it plays a crucial role in bone formation. Binding of different types of growth factors and hormones including bone morphogenetic protein (BMP), Wnt, transforming growth factor-β (TGF-β), parathyroid hormone (PTH), platelet derived growth factors (PDGFs), fibroblast growth factors (FGF) with their receptors expressed on the cell surface of mesodermal progenitor cells (also known as mesenchymal stem cells) induce the activation of different types of transcription factors responsible for osteoblast differentiation, maturation and survival[1,12].

BMPs are the members of TGF-β superfamily and known to be a potent inducer of osteoblast formation and thereby increase collagen synthesis and decrease collagenase-3 production[1,13]. There are several types of BMP proteins and among them BMP-2, BMP-4, BMP-5, BMP-6 and BMP-7 have strong capacity in osteogenesis[14]. BMP-2 and BMP-6 induce osteoblast formation and chondrocyte proliferation[14,15]. BMP-4 could participate in endochondralossification[16,17] and BMP-7 induces the expression of markers including ALP activity and accelerated calcium mineralization which are required for osteoblast differentiation[14]. But BMP-3 has adverse effect on osteoblastogenesis[14]. BMP signaling has been identified as the major signaling molecules in the pre osteoblast because the binding of BMPs to its receptors (BMPRs) induce phosphorylation of SMADs proteins specially SMAD-1, SMAD-5 and SMAD-8 (Figure 1). SMADs then in turn directly activate the SMAD binding element (SBE) through the SMAD depended pathway and thereby induces the transcription of corresponding genes. On the non SMAD depended pathway BMPRs directly activate MAPK and then in turn activate the particular genes through inducing runt related transcription factor 2 (RUNX2) or activator protein 1 (AP-1)[14,18,19].

Wnt is the member of highly conserved secreted glycoprotein family, rich in cystein residue and are divided into two classes: canonical Wnts (wnt1, wnt3a) and non-canonical Wnts (wnt5a). Binding of canonical Wnts with frizzled (FZD) and LDL receptor related proteins (LRPs) promotes: the phosphorylation and inactivation of *glycogen synthase kinase 3 beta* (GSK3b), prevents the degradation of β-catenin (β-cat) as well as subsequent translocation of β-cat in the nucleus for binding with the target genes (Figure 1). Binding of non-canonical wnts with FZD receptor promote the activation of heterotrimeric G proteins in order to enhance the deposition of intracellular calcium ion (Ca^{2+}) through protein kinase C (PKC) mediated pathway or induce the formation of the cytoskeleton *via* Rho/c-Jun N-terminal kinase dependent mechanism[18,19].

TGF-β signaling is important for the regulation, proliferation and commitment to the osteoblastic lineage of MSC. Binding of TGF-β with its receptor TGFβR regulates the expression of target genes through two possible pathways: canonical and non-canonical. In the canonical or smad dependent pathway activated TGFβR promotes the phosphorylation of R-SMADs (SMAD-2, 3) and thereby activate the target genes through SMAD-4 m = ediated signal transduction (Figure 1). In the non-canonical or non smad dependent pathway activated TGFβR promotes the expression of responsive genes through MAPK, P38, ERK mediated signal transduction pathway[12,14].

Immunohistochemical analysis revealed that the periosteum and bone are linked with the sympathetic, sensory and the glutaminergic nervous system specifically the growth plate and the metaphysic of long bones are more exposed to the neural network. Close contact of the nervous system with the bone cells, strongly implying a physiological role of neural signal on bone health[20,21]. In addition, osteoblast has been reported to express β-2 adrenergic receptors (β2AR) and 5-hydroxytryptamine receptor (5HTR) for several neurotransmitters including serotonin and norepinephrine[21,22]. An *in vivo* experiment showed that 5HTR 2β facilitate osteoblast recruitment and proliferation and the absence of this receptor leads to osteopenia[22]. Binding of neurotransmitter with particular receptors activates the transcription factor CREB and ultimately induces the gene for osteoblast prolifera-

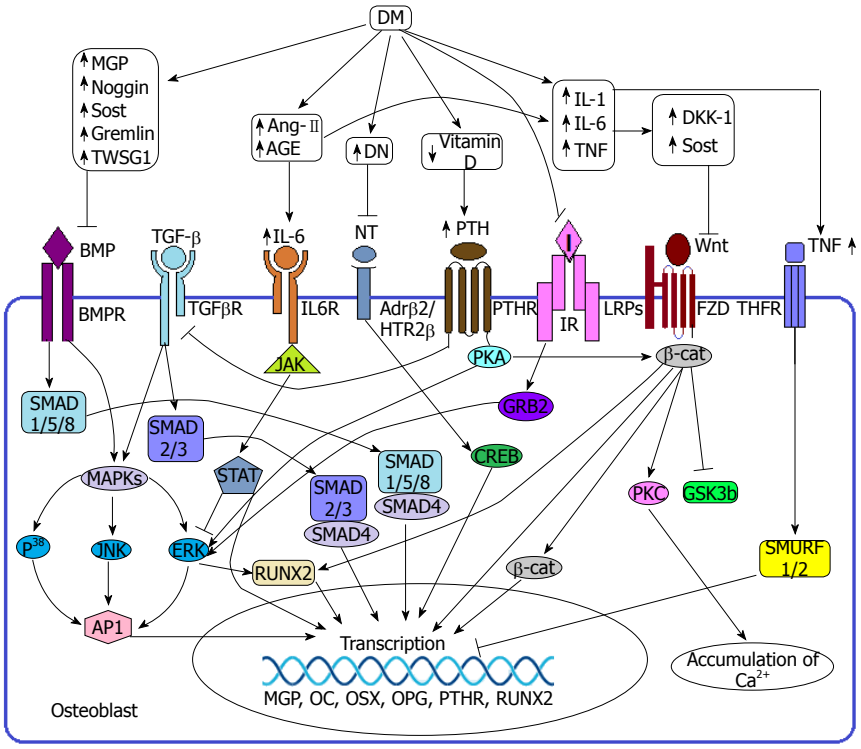

Figure 1 Diabetes mellitus induced regulation of osteoblast. Duringhealthy condition bone morphogenetic protein (BMP), transforming growth factor β (TGF-β), Wnt, insulin and neurotransmitter signaling are mandatory to the osteoblast for its normal functioning and survival. Binding of BMP with its receptor (BMPR) activates the corresponding gene through (a) Smad depended pathway: which requires the SMADs protein (SMAD 1/5/8) or (b) non smad depended pathway: in which activates RUNX2 or AP-1 through MAPK-ERK mediated pathway. Wnt-Frizzled pathway positively regulates gene expression through β-catenin or RUNX2 mediated pathway and Calcium accumulation through PKC mediated pathway. TGF-β is also a positive regulator of osteoblast function and exerts its effect on the respective gene through SMAD 2/3 depended pathway or MAPK-ERK mediated pathway. Peripheral nerve exposure to the osteoblast signals through the adrenergic receptor 2 β (Adrβ2) or 5HTR induced pathway. Binding of neurotransmitters on the Adrβ2 or 5HTR receptor activates ERK or CREB to induce the expression of osteoblastic gene. Insulin is a beneficial factor of bone formation and it exerts its effect through GRB2-ERK mediated pathway. During diabetes mellitus (DM), hyperglycemia may induce the expression of several BMP inhibitors including MGP, Noggin, Sost, Gremlin, TWSG1 as well as several Wnt inhibitors including DKK-1, Sost. DM also induces the production of different proinflammatory cytokines including interleukin 6 (IL-6), IL-1, AT-2 and TNF which negatively regulates osteoblast functioning. Binding of IL-6 with its receptor IL-6 receptor (IL6R) sequesters ERK pathway as well as induce the gene to transcribe several inhibitors including MGP, OPG OSX. DM induced DN limits the nerve signaling through damaging the peripheral nerves. TNF binding with TNFR induce SMURF1/2 and thereby inhibit the transcription process. DM also reduces the production of vitamin D which in turn induces the secretion of parathyroid hormone (PTH). PTH binding with PTH receptor (PTHR) inhibits TGF-β signaling through inhibiting TGFβ receptor (TGFβR) although PTHR activates β-Cat and ERK pathways. DM induced IR (type-2DM) or insulin deficiency (Type-1DM) also limits insulin mediated bone formation. TNT: Neurotransmitter; HTR2β: 5-hydroxytryptamine receptor 2 β; I: Insulin; IR: Insulin receptor; LRP: Low density lipoprotein receptor related protein; FZD: Frizzled; TNF: Tumor necrosis factor; TNFR: TNF receptor; JAK: Janus kinase; STAT: Signal transducers and activators of transcription; AP-1: Activator protein 1; ERK: Extracellular signal regulated kinase; MAPK: Mitogen activated protein kinase; RUNX2: Runt related transcription factor 2; PKA: Protein kinase A; PKA: Protein kinase C; β-cat: β catenin; GSK3b: Glycogen synthase kinase 3b; SMURF: SMAD ubiquitylation regulatory factor; MGP: Matrix gla protein; OC: Osteocalcin; OSX: Osterix; OPG: Osteoprotegerin; DKK-1: Dickkopf related protein 1; Sost: Sclerostin; TWSG1: Twisted gremlin; Ang-Ⅱ: Angiotensin-Ⅱ; AGE: Advance glycation end product; GRB2: Growth factor receptor bound protein.

tion through AP1 activation[18](Figure 1).

Elevated secretion of PTH has been reported to sequester osteoblast differentiation and activation. Attachment of PTH with its receptor PTHR activates protein kinase A (PKA) and extracellular signal regulated kinase (ERK) and ultimately induces the expression of matrix gla protein (MGP) on osteoblast which is a potent inhibitor of BMP signaling[1,14]. PTH binding also drives internalization of PTHR-TGFβR complex, which attenuates TGF-β signaling in bone development[14].

Several extracellular, intracellular and transcriptional BMP inhibitors such as matrix gla protein (MGP), Noggin, dickkopf-related protein 1 (DKK-1), Sclerostin, Gremlin, Ski, Smurf-1, Smurf-2, twisted gastrulation (Twsg1), Interleukin 6 (IL-6) and TNFs have been identified in the down regulation of BMP and TGF-β signaling pathways and ultimately suppress osteoblast function[1,13,14,23,24]. MGP is the member of mineral binding γ-carboxyglutamic acid containing protein family that directly and indirectly sequesters mineralization of bone cells. In the direct effect it acts as a part of a complex with α-2-HS glycoprotein and in the indirect effect it inhibits the binding of BMP-2 with its receptor expressed on the osteoblast precursors[1].

Diabetes mellitus (DM) not only induces the overexpression of DKK-1[25,26] Sclerostin[27,28] Gremlin[29,30] PTH[31] angiotensin Ⅱ (Ang-Ⅱ)[32] IL-6[33] and TNFs[33-35] but also sequesters the over expression of Vitamin D and neurotransmitters required for the normal growth of osteoblast. DM induced diabetic neuropathy is the com-

monest complication of non-traumatic lower limb amputations in diabetic patients. Although the exact pathogenesis of diabetic neuropathy remains unclear, there are emerging data from *in-vitro* and *in-vivo* clinical studies suggesting that hyperglycemia induced formation of advanced glycation end products (AGEs) may play a key role in the pathogenesis of diabetic neuropathy[36,37]. Under hyperglycemic conditions, concentrations of methylglyoxal, 3-deoxyglucosone and glyceraldehyde increase rapidly due to the increased breakdown of glucose. Elevated levels of methylglyoxal, 3-deoxyglucosone and glyceraldehyde lead to the formation of advance AGEs which in turn modify nerve cell components as well as signal through the receptor for advance glycation end product (RAGE) expressed on the nerve cells in order to produce different types of cytokines which may have roles on nerve damage[36-38]. AGEs have deleterious effect on nerve cells because they modify neuronal proteins including tubulin, neurofilament, laminin and actin through glycation and thereby sequester the nerve function (Figure 2)[36,37].

Beyond the damage of peripheral nerve cells on osteoblast through diabetic neuropathy, DM induced AGEs and angiotensin-Ⅱ also upregulate the expression of IL-6 that regulates osteoblastic genes required for their survival, differentiation and function[32,39,40](Figure 1).

Reduced vitamin D levels in the body have been identified as a potential risk factor of osteoporosis and bone fractures. Deficiency of Vitamin D in the serum sequesters the intestine to absorb Ca^{2+} from diet and thereby signals the parathyroid gland to secrete elevated levels of PTH. Hyper secretion of PTH induces bone resorption and inhibit osteoblastogenesis in order to maintain the optimal level of calcium and phosphorus in the blood required for metabolic process and neuromuscular functions[41,42]. Through binding of PTH with its receptor PTH-1 expressed on osteoblast triggers intracellular signaling molecules such as PKA, mitogen activated protein kinase A (MAPK), cyclic AMP-responsive element binding protein, AP1 and RUNX2 and thereby induce the expression of the MGP responsive element[1](Figure 1).

Bone marrow derived endothelial progenitor cells (EPCs) may have roles in angiogenesis during bone healing[3,43]. DM down regulates the expression of EPCs through different mechanisms and hereby decreases the rate of angiogenesis required for bone formation in the fracture sites[3,44,45]. Mesenchymal stem cells (MSC) derived from bone marrow act as a precursor of osteoblast formation[46-48]. Several labs based trials have come to the decision that, DM is responsible for the upregulation of peroxisome proliferator-activated receptor-γ (PPAR-γ), adipocyte fatty acid binding protein (aP2), TNF-α and consequently decrease the availability of MSC for osteoblast formation but increase the availability of MSC for adipocyte formation[3,4,34,35,48,49]. So it is intuitive that, in addition to direct interference with osteoblast formation DM also responsible for the deposition of lipid in the bone marrow and thereby leading to the expansion of marrow cavity as well as decreases the rate of blood flows to the bone which is required for the transfer of nutrients[3,5]. The transformation of osteoblast to adipocyte makes the reduction of osteoblast number available for bone formation[3,50]. Advanced glycation end products (AGEs) have been identified as a biomarker for the increased risk of fractures because it decreases the synthesis of type Ⅰ collagen and thereby decreases the bone strength. It is now well researched that DM is responsible for the over expression of AGE and have roles in bone rigidity[51-53].

Several experimental studies implicated that, insulin has an anabolic effect on osteoblast development and it is intuitive that, insulin may exert its effect on osteoblast through IR-GRB2-ERK mediated pathway[4,54](Figure 1). Beyond the synthesis of insulin pancreatic β cells also produce other osteoporotic factors including amylin and preptin. Amylin induces bone formation and sequesters bone resorption, preptin induces osteoblast differentiation and mineralization as well as reducing the apoptosis of osteoblast[4]. Osteocalcin is a peptide which positively regulates osteogenesis. DM limits the production of osteocalcin through the negative regulation of osteoblast by decreased synthesis of insulin, amylin and preptin. Testosterone is also an important factor of osteogenesis and it is obvious that, limited production of osteocalcin reduces the production of testosterone from the testes[4].

Osteoclast

Osteoclasts are cells that derived from the monocyte-macrophage cell lineage and strongly participate in osteoclastogenesis. It is well documented that different types of mediators such as nuclear factor κ-B (NF-κB), receptor activator for nuclear factor κ-B ligand (RANKL), osteopontin (OPN), parathyroid hormone (PTH), macrophage colony stimulating factor (M-CSF), and angiotensin-Ⅱ (AT-Ⅱ) have prominent roles to induce osteoclastogenesis[1,13].

In general osteoclast exerts its effects in osteoclastogenesis through three possible pathways (1) RANKL mediated; (2) M-CSF mediated; and (3) immunoreceptor tyrosine-based activation motifs (ITAMs). But in inflammatory condition osteoclastogenesis may take place through other pathways like MCP mediated, TNF mediated and IL-6 mediated[12,55].

RANKL is a key factor derived from osteoblast and stromal cells, binds with the receptor expressed on the cell surface of monocyte-macrophage cell lineage and thereby triggers the differentiation of pre osteoclast to osteoclast through activating NF-κB and NFATc1[56]. RANKL inhibits the apoptosis of osteoclast through inducing the anti-apoptotic enzyme protein kinase B (PKB) (Figure 3). RANKL also responsible for the production of reactive oxygen species (ROS) including free radicals, oxygen ions and peroxides which are potent inducer of osteoclastogenesis[1,12,56-59].

Binding of RANKL with its receptor RANK activates signal transduction pathways involving the adaptor protein TNF receptor-associated factor 6. Subsequently, several kinases such as p38 MAPK and JUN N-terminal

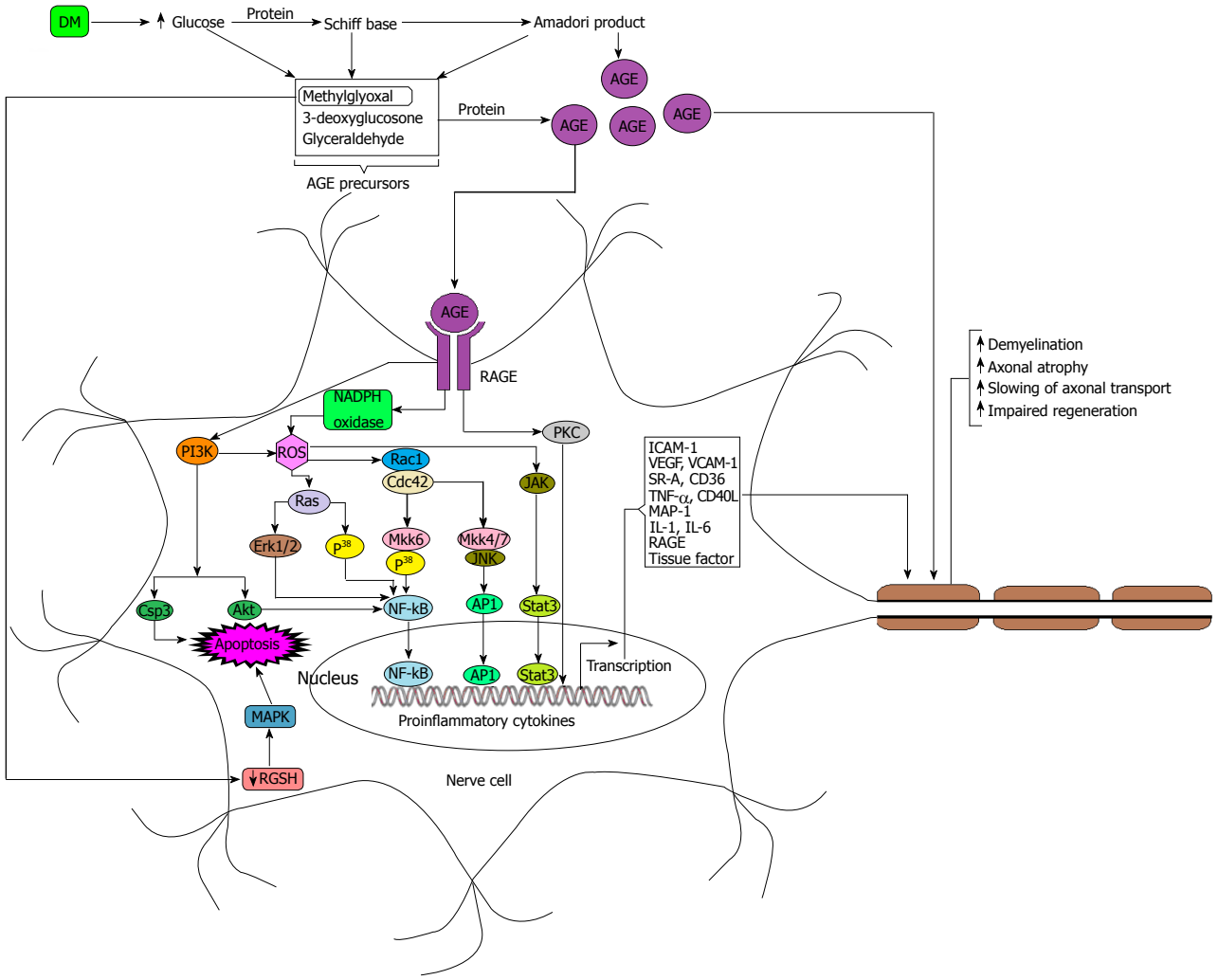

Figure 2 Diabetes mellitus induced Peripheral nerve damage. During hyperglycemic condition concentrations of methylglyoxal, 3-deoxyglucosone and glyceraldehyde increase rapidly due to the increased breakdown of glucose. Elevated levels of methylglyoxal, 3-deoxyglucosone and glyceraldehyde lead to the formation of advance advance glycation end products (AGEs) which in turn modify nerve cell components as well as signal through the receptor for advance glycation end product (RAGE) expressed on the nerve cells in order to produce different types of cytokines which may have roles on nerve damage. RAGE induced nicotinamide adenine diphosphate hydrogen (NADPH) oxidase is the major source of reactive oxygen species (ROS) and ROS plays a crucial role to activate nuclear factor kappa B (NF-κB) through Ras-Erk, Rac1-Mkk6 depended pathway. ROS also activates AP-1 and Stat-3 through Rac1-Mkk4/7, JAK-Stat mediated pathway respectively. RAGE may induce apoptosis through PI3K-Csp3 depended pathway as well as activates NF-κB through PI3-Akt mediated pathway although PI3K may participates in ROS production. Diabetes mellitus (DM) induced methylglyoxal may directly participates in apoptosis through MAPK mediated pathway. Activated NF-κB, AP-1 and Stat3 act congruously to transcribe the genes of proinflammatory cytokines and other factors which are responsible for the destruction of peripheral nerve cells. AGE also participate directly on the modification of axon and thereby reduce the potentiality of signal transduction. DM: Diabetes mellitus; AGE: Advance glycation end product; JAK: Janus kinase; RAGE: Receptor advance glycation end product; ROS: Reactive oxygen species.

kinase 1 are activated, which in turn induce the transcription *via* the various hetero and homodimers of the AP1 family of proteins including FOS, FOSB, FOS-related antigen 1 (FRA1), FRA2, JUN, JUNB and JUND (Figure 3). AP1 regulates the differentiation, proliferation and apoptosis, of various cell types[12].

RANKL is necessary for osteoclastogenesis but an experiment conducted on mouse model showed that, M-CSF acts as a positive catalyst in RANKL activation because the addition of M-CSF requires less time to do a particular resorption process than the RANKL alone[60]. Osteoprotegerin (OPG) is a prominent factor for osteoclast activation because the affinity of OPG for RANKL prevents the binding of RANKL with its receptor RANK and thereby decrease the RANKL-RANK mediated pathway of octeoclast multiplication, survival and bone resorption[1].

According to the immunoreceptor tyrosine-based activation motifs (ITAMs), binding of immune complex like immunoglobulin G (IgG) with its receptor FcγR activates spleen tyrosine kinase (SYK), which in turn induces NFATC1 through the activation of phospholipase Cγ (PLCγ) (Figure 3). NFATC1 is an important transcription factor that transcribes the genes that encode calcitonin receptor, tartrate-resistant acid phosphatase, matrix metalloproteinase 13 and cathepsin K. All these factors enable the acidification and degradation of the bony matrix[12]. DM is thought to be a potent inducer of IgG because an experiment conducted on mouse model showed that non-obese diabetic mice spontaneously produce natural

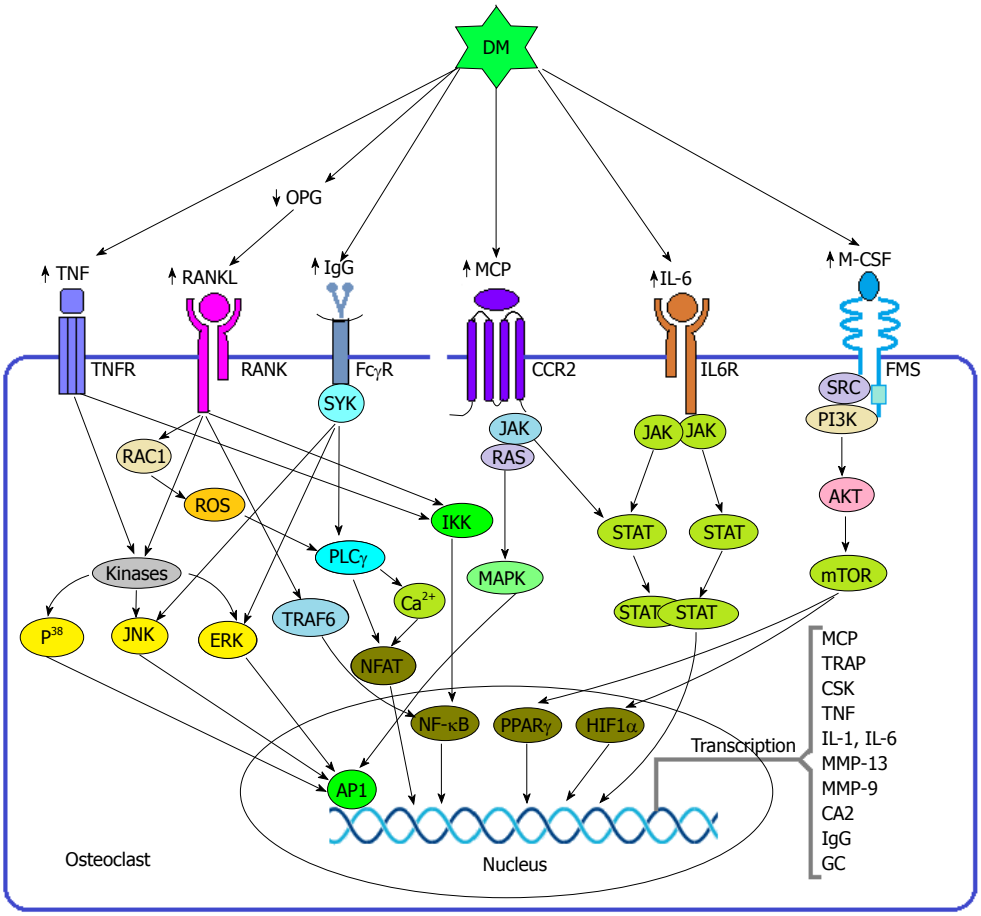

Figure 3 Diabetes mellitus induced regulation of osteoclast. During normal physiology several osteoblastogenic modulators including RANKL, M-CSF, monocyte chemoattractant protein (MCP), and immunoglobulin G (IgG) binds with their receptors expressed on osteoclast and activates different signal transduction pathway to transcribe the particular gene. Binding of RANKL with RANK triggers several possible pathways to induce the corresponding element. It may induce transcription factor NF-κB through TRAF or IkB kinase (IKK) mediated pathway as well as induces nuclear factor of activated T cells (NFAT) through reactive oxygen species (ROS)-phospholipase Cγ (PLCγ) mediated pathway. RANK also may induce AP-1 through triggering the kinase enzymes. Macrophage colony stimulating factor (M-CSF) activates transcription factors peroxisome proliferator activated receptor γ (PPARγ) and hypoxia inducible factor 1 α (HIF1α) through PI3K-AKT mediated pathway. MCP activates AP-1 signaling through RAS-MAPK mediated pathway which requires the assistance of JAK. IgG also signals through the Fc receptor γ chain (FcγR) to activate NFAT *via* the induction of PLCγ as well as activates AP-1 through the kinase enzyme systems and both of the pathways require the activation of SYK. \During the state of DM, it induces the upregulation of osteoclastogenic factors stated above and thereby induce the differentiation and activity of osteoclast. In addition to the above factors, DM also induces the synthesis of some proinflammatory cytokines which also favor the bone resorption by osteoclast. interleukin 6 (IL-6) exerts its effect through JAK-STAT mediated pathway although MCP activated JAK may contribute to the activation of STAT to some extent. TNF also activates NF-κB and AP-1 through IKK and Kinase system respectively. CCR2: CC chemokine receptor 2; mTOR: Mammalian target of rapamycin; OPG: Osteoprotegerin; ERK: Extracellular signal regulated kinase; JNK: JUN N terminal kinase; TRAP: Tartrate resistant acid phosphatase; CSK: Cathepsin K; MMP: Matrix metalloproteinase; CA2: Carbonic anhydrase 2; GC: Glucocorticoid.

IgG autoantibodies[61].

Beyond the roles of RANKL and M-CSF in osteoclastogenesis, on the state of hyperglycemia a group of proinflammatory cytokines is activated including TNF, IL-1 and IL-6 and these cytokines have profound effects on the differentiation and activation of osteoclast[12,62-64]. Although osteoclast differentiation and activation is primarily dependent on the presence of M-CSF and RANKL, osteoclastogenesis is enhanced in the presence of TNF, IL-1 or IL-6. This is partly a consequence of the induction of RANKL in target cells, but these pro-inflammatory cytokines also responsible for the differentiation and activation of osteoclasts from the preosteoclast. In addition, under normal concentrations of RANKL, TNF can induce the differentiation of monocytes and macrophages to preosteoclasts. The osteoclastogenic activity of TNF is mediated by p55 TNF receptor and may be partly counteracted by the activation of the p75 TNF receptor[12].

IL-6 is thought to be the most abundant and effective cytokines in blood because: (1) the concentration of IL-6 and IL-6 receptor (IL-6R) is higher than the other cytokines; (2) IL-6 mediates the production of other cytokines related to osteoclastogenesis like glucocorticoid (Figure 3); and (3) Estrogen deficiency exerts its effects in osteoclastogenesis *via* IL-6 mediated pathway as well as IL-6 is a potent inducer of IgG production[12,61,65].

DM not only induces the overexpression of RANKL[3,66] M-CSF[3,66] NF-κB[67] and OPN[34,68] but also stimuli the over expression of several proinflammatory stimulus such as IL-6, MCP, IgG and TNFs which are so important for the maturation and activation of osteoclast. The

DM may induce monocyte to secrete IL-6 through ROS, PKC, MAPK, and NF-κB mediated pathways[69,70].

Estrogen deficiency stimulates osteoclast formation not only by decreasing the OPG production but also by increasing the production of TNF-α, RANKL and osteoclast precursors through stimulating the T cells[71,72]. There is striking evidence on behalf of this regard that, estrogen levels are significantly lower in DM patients[73]. Adiponectin is another factor secreted by the adipose tissue and there has been increasing evidence suggest that, adiponectin stimulates the differentiation and mineralization of osteoblast but directly inhibits osteoclast activity and bone resorption[74]. Some *in situ* studies have shown that adiponectin percentage is lower in individuals with DM than the individuals without DM[75].

Intracellular ROS mediated oxidative stress plays a crucial role in bone health because ROS promotes RANKL mediated osteoclast differentiation and function. Patients with type 2 DM have shown elevated level of mitochondrial ROS and thus supporting the point that, DM may have another role in ROS mediated osteolysis and bone fractures[76,77]. As mentioned before, diabetic neuropathy is a cause of increased production of IL-6, TNF and some other factors, so it is intuitive that, diabetic neuropathy may have a positive role in osteoclast functioning[12,38,64].

EFFECT OF DIABETES MELLITUS ON MUSCLE CELLS

Muscle atrophy is a physiological condition which associated with the depression of protein synthesis as well as an increase in protein degradation[78]. There are some other evidences showed that, DM is associated with diabetic neuropathy mediated muscle atrophy or directly triggers muscle atrophy through TNF-α, NF-κB mediated pathway and thereby induces muscle weakness[68,79-81]. Weakness of the muscle is a risk factor of bone fractures because an individual with weak muscles is more likely to fall down than a normal individual. In addition to muscle weakness, diabetic polyneuropathy also induces bone resorption through osteolysis[82,83].

DM is directly associated with muscle atrophy through an increased activity of the ubiquitin proteosome system (UPS) although other pathways may involve in this process[78]. There are several inducers of UPS including glucose[33] TNF-α[84,85] Ang-Ⅱ[86] IL-6, Glucocorticoid (GC)[85] and most of them exert their effects on myogenesis responsive gene through NF-κB mediated pathway[68,78].

High extracellular glucose concentrations is a potential precursor of AGE formation and several evidences have shown that, AGE may induce the formation of ROS through NADPH oxidase and PI3K/Akt mediated pathway and ultimately activates the transcription factor NF-κB[37,38,78]. AGE may induce PKR through caspase-3 mediated pathway and activated PKR then in turn induces NF-κB through P^{38} MAPK mediated pathway as well as activates eIF2α which would depress protein synthesis by decreasing translational efficiency[78](Figure 4).

Several studies have implicated that TNF-α is a prominent cytokine in cachexia induced muscle atrophy[84] as well as a potent inducer of insulin resistance[87]. Binding of TNF-α with its receptor expressed on myocyte activates nuclear transcription factor NF-κB through P^{38}MAPK or IKK mediated pathway and activated NF-κB then in turn induces the transcription of inducible nitric oxide synthase (iNOS) as well as transcribes the gene MuRF-1 responsible for muscle wasting[84](Figure 4).

Ang-Ⅱ which is the major peptide of the renin-angiotensin system has been implicated as a modulator of muscle wasting[88]. Ang-Ⅱ exerts its effect on muscle atrophy not only through the generation of ROS but also through the activation of IL-6 and Glucocorticoid as well as through disrupting insulin signaling in muscle cells. It is experimentally determined that, ROS has a significant role in the reduction of muscle strength[89,90]. There are two sources of Ang-Ⅱ induced ROS production (1) NADPH oxidase; and (2) Mitochondria, but NADPH oxidase is thought to be prominent between the two sources. ROS may contribute to muscle wasting activity through three mechanisms (1) by increasing the absorption of Ca^{2+} in order to activate calcium-activated proteases; (2) by stimulating the UPS through activating caspase-3; and (3) by up-regulating atrogin-1 and MuRF-1 in muscle to activate the proteasome system through transcribing E3 ligases[86](Figure 4).

Ang-Ⅱ induced glucocorticoid (GC) plays an important role in muscle wasting because several *in-vivo* and *in-vitro* studies have shown that, addition of different types of GC antagonist of experimental model reduce the rate of muscle wasting[86,91,92]. GC exerts its effect on muscle through two ways (1) through sequestering the anabolic action, and (2) through inducing the catabolic action[91]. On behalf of the anti-anabolic action firstly, GC inhibits the transport of amino acids into the muscle and thereby limits the protein synthesis[91]. Secondly, GC sequesters the stimulatory effects of insulin and insulin like growth factor 1 (IGF-1)[91,92]. Thirdly, GC negatively regulates the synthesis of MyoD, an important transcription factor that regulates the differentiation and development of muscle cells as well as required for regeneration and self-renewal of skeletal muscle cells[92]. Fourthly, mechanistic target of rapamycin (mTOR) is a kinase protein which regulates the translation of muscle protein. GC inhibits the activity of mTOR through enhancing the transcription of REDD1, a repressor of mTOR function[91]. Finally, GC inhibits myogenesis through the downregulation of myogenin, an important a transcription factor required for differentiation of satellite cells into myofibrils[91,92]. On behalf of the catabolic activity firstly, GC stimulates the synthesis of several components (*e.g.,* E3) required for UPS through the upregulation of the respective genes including MuRF-1 and atrogin-1[91,92]. Secondly, GC induces the overexpression of myostatin a growth regulator which inhibits the development of muscle mass through

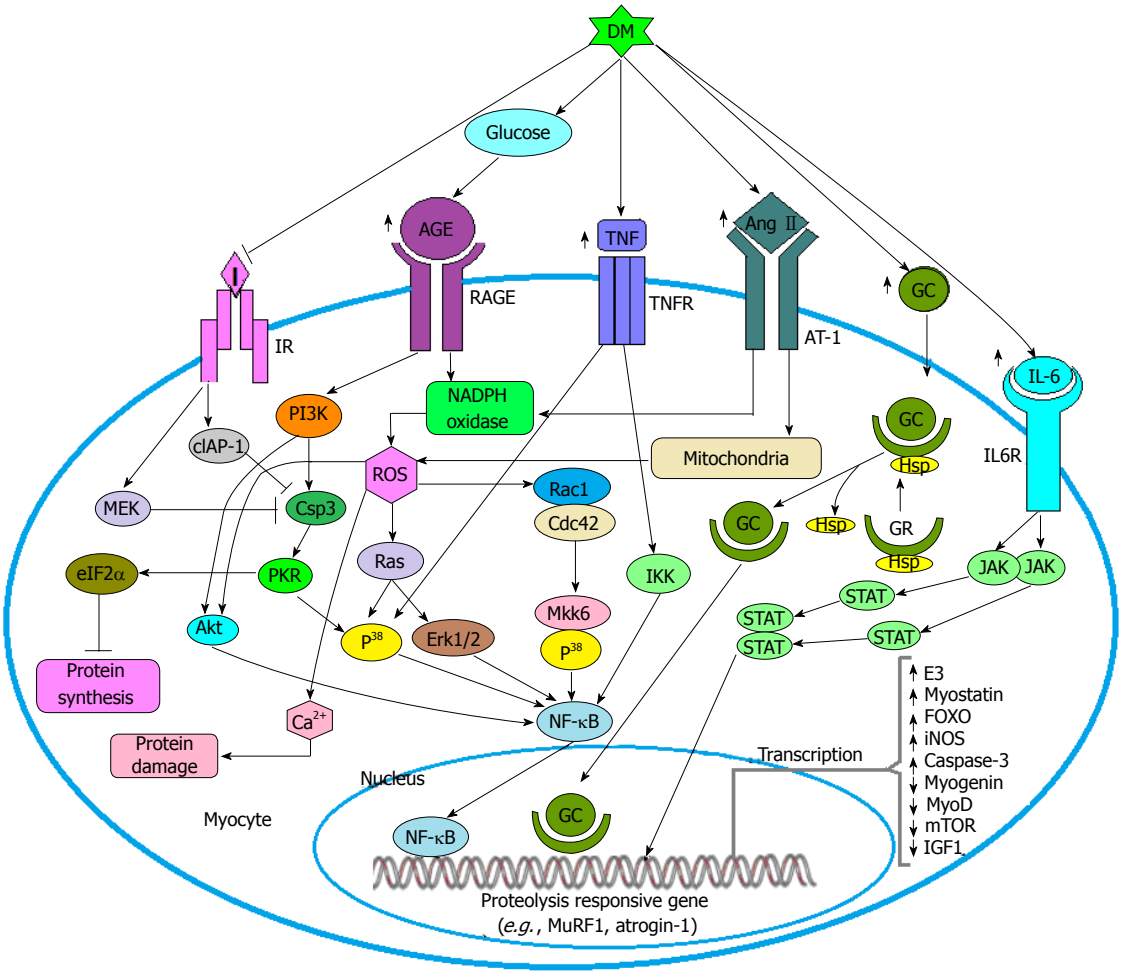

Figure 4 Diabetes mellitus induced regulation of skeletal muscle. Diabetes mellitus (DM) induced elevated blood glucose is the major source of advance advance glycation end product (AGE) which binds with its receptor advance glycation end product (RAGE) to activate the signal cascade into myocyte. RAGE activation enhances the generation of reactive oxygen species (ROS) through the activation of nicotinamide adenine diphosphate hydrogen (NADPH) oxidase. Ang-II also induce the production ROS not only by activating NADPH oxidase but also by inducing the mitochondria. ROS may exert its effects on nuclear factor kappa B (NF-κB) through Rac1-Mkk6 and Ras mediated pathway or accelerate the damage of muscle protein through Ca^{2+} depended pathway. Beyond the generation of ROS, RAGE also activates PI3K which in turn activates NF-κB through Csp3- PKR and Akt mediated pathway. Activated PKR may induce the activation of eIF2α that inhibits protein synthesis. DM induced Proinflammatory cytokines interleukin 6 (IL-6) activates the gene through JAK-STAT signaling pathway and TNF activates the factor NF-κB *via* IKK or MAPKP38 induced pathway. Ang-Ⅱ induced GC also have role in muscle atrophy and GC exerts its effect through GC-GCR complex mediated pathway. Insulin signaling is also important for muscle growth because it sequesters the activity of Csp3 through inducing the production of cIAP-1 and MEK which are potential inhibitor of Cap3. Type 1 DM reduces the production of insulin and type 2 DM makes the cell insulin resistance, so due to the deficiency of insulin it limits the functioning of cIAP-1 and MEK.

downregulating the proliferation and differentiation of satellite cells[91,93,94]. Thirdly, GC induces the breakdown of myofibrillar protein through the upregulation of caspase-3[89]. Finally, Forkhead Box O-1 (FOXO-1) is a transcription factor that induces UPS through the upregulation of genes including atrogin-1/MAFbx and MuRF1. Several lab based experiments have come to the decision that, GC induces the production of FOXO-1 through stimulating the respective genes[91,92](Figure 4).

IL-6 is a proinflammatory cytokine which has been implicated as a potential factor of muscle atrophy[86,95,96]. Ang-Ⅱ induced IL-6 upregulates the transcription of serum amyloid A (SAA) and both of the factors (IL-6 and SAA) act synergistically to trigger muscle atrophy[93]. An *in-vitro* study has shown that, IL-6 exerts its effect on muscle wasting through JAK/STAT mediated pathway[97](Figure 4).

Insulin deficiency (ID) and insulin resistance (IR) are the hallmark of type-1 and type-2 DM respectively. IR has been implicated as a potential inducer of overall protein degradation as well as caspase-3 mediated actin cleavage. Elevated level of intracellular insulin inhibits caspase-3 protein through MEK and cIAP-1 mediated pathway but during IR or ID condition insufficiency of insulin cannot exert its inhibitory effect on caspase-3[98](Figure 4).

DM induced diabetic retinopathy may be another risk factor of bone fractures because diabetic retinopathy is a leading cause of vision loss and blindness and consequently augments the rate of stumble mediated bone fractures[99]. Abnormal movement caused by polyneuropathy and heart failure caused by diabetic cardiovascular complications also promotes the rate of fall[4,5].

Beyond the role of vitamin D in osteolysis, it is intui-

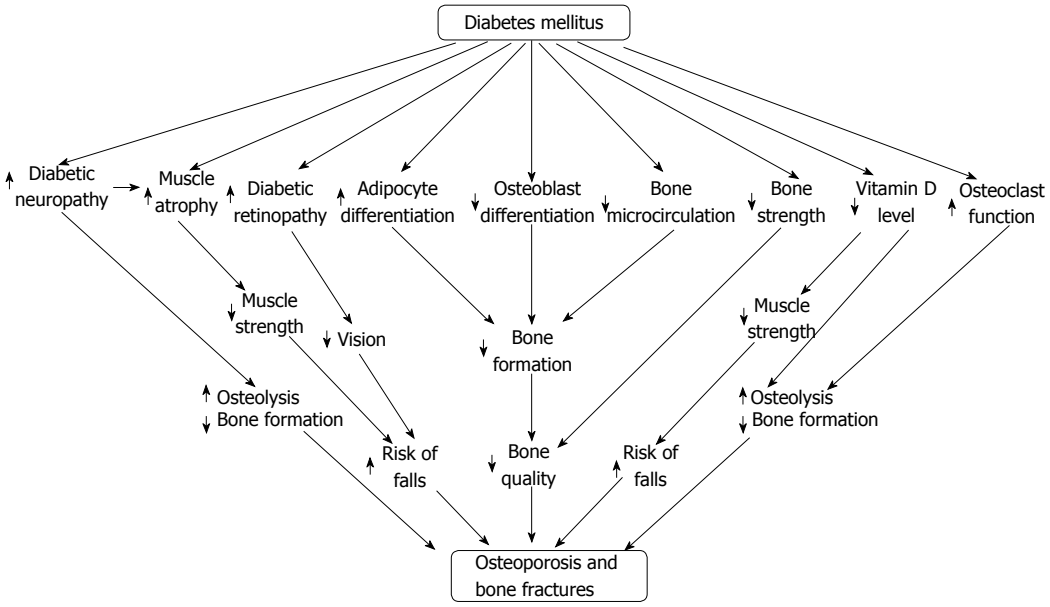

Figure 5 Possible pathways of diabetes mellitus induced osteoporosis.

tive that, vitamin D exerts a range of effects in skeletal muscle cells. Muscle activity specially the power stroke is a Ca^{2+} depended process and due to the lack of Ca^{2+} the system will be shut down. An inadequacy of vitamin D can turn down the availability of calcium and phosphorus and thereby postpones the activity of muscles[100]. Some *in vitro* and *in vivo* trial have shown that, vitamin D levels are significantly lower in patients with DM[101,102].

DISCUSSION

Diabetes mellitus may be an obvious cause of osteoporosis and bone fractures due to its broad range of effects on different mediators of the human body. It mainly regulates the bone cells (specifically osteoblast and osteoclast) and the muscles to exert its effects to facilitate osteoporosis as well as reduction of muscle strength[3,98](Figure 5). DM negatively regulates the normal functioning of osteoblast but positively regulates the osteoclast functioning in order to facilitate the process of osteoporosis[1,98]. DM reduces the availability of MSC to produce osteoblast but simultaneously increases the availability of MSC for adipocyte formation[1]. Due to the continual differentiation and deposition of adipocytes into the bone marrow increase the bone marrow cavity to make the bone fragile as well as decrease the bone microcirculation[1,99]. The limitation of osteoblast functioning, over production of adipocytes and fluent functioning of osteoclasts all these effects negatively regulate the bone formation but positively regulate the bone resorption and ultimately cause osteoporosis. DM induced diabetic neuropathy acts as a prominent factor in osteoporosis and muscle atrophy. Diabetic neuropathy inhibits bone formation through sequestering osteoblast formation and function but facilitates osteolysis through inducing the osteoclast generation and function as well as reducing muscle strength through inducing the expression of different cytokines and ROS[75,98]. DM directly causes muscle atrophy through different mediators or indirectly causes muscle atrophy through diabetic neuropathy. DM induced diabetic retinopathy is another risk factor that may cause bone fractures through reducing eye sight[98]. Reduced muscle strength as well as reduced eye sight may cause elevated rate of fall down or stumble that may cause bone fractures. Vitamin D is an essential factor of bone and muscle activities because deficiency of vitamin D stimulates the production of PTH which is a negative regulator of osteoblast functioning but a positive regulator of osteoclast functioning which then in turn reduce bone formation and increase bone resorption respectively[53,54]. DM induced Vitamin D deficiency also causes the reduction of muscle strength because it lowers the rate of Ca^{2+} absorption by the intestine and thereby reduces the activity of muscle which may be a risk factor of bone fractures through increasing the rate of fall[55,98].

CONCLUSION

Diabetes mellitus exerts its diabolical effects on bone, neuron and muscle cells through a broad spectrum of mechanisms. It declines the production of various stimuli required for normal homeostasis of the above cells and accelerates the synthesis of several cytokines and other factors which may directly destroy the target cells or indirectly antagonize the signaling pathways of the stimulus. As human body is a network of different pathways so any imbalance on any part of the pathway may tends the body vulnerable to different threats. DM is the potent source of excess glucose in the blood which is the principal key to create a lot of abnormalities in the body including Osteoporosis and bone fractures, cardiovascular disease, diabetic nephropathy, diabetic neuropathy, Dia-

betic retinopathy and muscle atrophy. Other fatal diseases like HIV and cancer may be linked with hyperglycemia and several investigations have been running to elucidate the mystery of DM induced mechanism. Although several drugs are available to treat osteoporosis, regular physical exercise would be a better way to get rid of from this type of life threatening disease.

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P- Reviewers Scuteri A, Wong WTJ **S- Editor** Wen LL
L- Editor A **E- Editor** Lu YJ

World Journal of Diabetes

Online Submissions: http://www.wjgnet.com/esps/
wjd@wjgnet.com
doi:10.4239/wjd.v4.i4.114

World J Diabetes 2013 August 15; 4(4): 114-123
ISSN 1948-9358 (online)

MINIREVIEWS

Genetics of type 2 diabetes

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Received: March 3, 2013 Revised: June 1, 2013
Accepted: July 18, 2013
Published online: August 15, 2013

Abstract

Type 2 diabetes (T2D) is the result of interaction between environmental factors and a strong hereditary component. We review the heritability of T2D as well as the history of genetic and genomic research in this area. Very few T2D risk genes were identified using candidate gene and linkage-based studies, but the advent of genome-wide association studies has led to the identification of multiple genes, including several that were not previously known to play any role in T2D. Highly replicated genes, for example TCF7L2, KCNQ1 and KCNJ11, are discussed in greater detail. Taken together, the genetic loci discovered to date explain only a small proportion of the observed heritability. We discuss possible explanations for this "missing heritability", including the role of rare variants, gene-environment interactions and epigenetics. The clinical utility of current findings and avenues of future research are also discussed.

Key words: Type 2 diabetes; Genetics; TCF7L2; Genome-wide association studies; Heritability

Core tip: We review the history and the current state of knowledge regarding the genetic component of type 2 diabetes risk. Genes like TCF7L2 that have been replicated in multiple studies are discussed in detail. The significance of these findings is discussed and gaps in our knowledge are identified, as are avenues for future research.

Ali O. Genetics of type 2 diabetes. *World J Diabetes* 2013; 4(4): 114-123 Available from: URL: http://www.wjgnet.com/1948-9358/full/v4/i4/114.htm DOI: http://dx.doi.org/10.4239/wjd.v4.i4.114

INTRODUCTION

Diabetes has been recognized as a distinct disease for over 2000 years[1] but it was not until 1935 that Hinsworth established that there were two district types of diabetes[2]. While both common types of diabetes are characterized by sustained elevations of plasma glucose levels, type 1 diabetes is an autoimmune disease that results in complete loss of the insulin-producing β-cells in the pancreatic islets, while type 2 diabetes (T2D) typically results when insulin secretion from the islets fails to keep pace with increasing insensitivity to the action of circulating insulin on its target tissues (particularly muscle, liver, and fat).

The development of T2D is the result of interaction between environmental factors and a strong hereditary component. Environmental risks factors known to impact the development of T2D include obesity, sedentary lifestyle, small or large birth weight and stress. Other nutritional factors and toxins may also play a role[3]. These environmental factors clearly play a major role in the development of diabetes, but they do not impact everyone in the same way. Even with the same environmental exposures, some people are more susceptible to developing diabetes than others, and this increased risk appears to be inherited. But while hereditary factors clearly play a role in the development of diabetes, the actual genetic variants involved in this inherited risk were completely unknown prior to the advent of modern genetic technologies. The advance of human genetic studies in the 1980s finally made it possible to try and identify genetic loci that underlie this hereditary component. Here, we will re-

view the heritability of T2D and the various genetic loci identified to date as contributing to this heritability.

HERITABILITY OF T2D

Estimates for the heritability of T2DM range from 20%-80% and evidence for heritability comes from a variety of population, family, and twin-based studies[4,5]. The lifetime risk of developing T2D is 40% for individuals who have one parent with T2D and 70% if both parents are affected[6]. First degree relatives of individuals with T2D are about 3 times more likely to develop the disease than individuals without a positive family history of the disease[7]. The concordance rate in monozygotic twins is about 70% whereas the concordance in dizygotic twins has been observed to be only 20%-30%[8]. The observed familial risk is higher when studies are restricted to parents in the 35-60 year age range, indicating the greater role played by environmental factors in those who develop diabetes late in life[9]. It should be noted that a significant proportion of this heritability reflects heritability of obesity rather than diabetes, obesity being a major driver of T2D in every population.

This familial clustering of T2DM risk found in various family studies is not entirely due to genetic factors. Epigenetic processes can produce inherited risk over one or several generations, intrauterine and pregnancy related factors can impact the risk of siblings, and shared environment can be hard to control for in many such studies. Thus the genetic component of T2D may turn out to be less than what was estimated in older studies.

GENETIC ARCHITECTURE OF T2DM DISEASE RISK

The detailed genetic architecture of T2D risk has not yet been precisely defined. A relatively small percentage (5% or less) of non-autoimmune diabetes is due to monogenic causes and is classified as monogenic diabetes of the young or MODY (previously referred to as maturity onset diabetes of the young). These cases are understood to be caused by single genes of high penetrance, of which mutations in the Hepatocyte nuclear factor-1A (HNF1A) and the glucokinase (GCK) gene are the most common[10]. These forms of diabetes are sometimes misdiagnosed as T2D but clinically they are distinct diseases. They will not be considered further in this review but it should be kept in mind that the boundaries between polygenic and monogenic forms are not always sharply defined at the genetic level. Polymorphisms in genes involved in monogenic forms of diabetes also play a role in polygenic T2D[11].

T2D itself is thought to be a polygenic disorder that develops due to complex interaction between multiple genes and environmental factors. How these genes interact with each other and with the environment to produce T2D is still poorly understood. Unlike T1D, where the genetic risk is mostly concentrated in the HLA region, the genetic component of T2D risk is not concentrated in one region and appears to be the result of the interaction of multiple genes scattered all across the genome. It is possible; even likely, that the genetic component of T2D is due to multiple common genetic variants of small effect (common disease common variant hypothesis) but this is by no means certain and it may turn out that the effect is due to multiple rare variants or even a few rare variants of large effect[12-14].

IDENTIFICATION OF DIABETES RISK GENES

Linkage studies

Linkage is the tendency for genes and other genetic markers to be inherited together because of their location near one another on the same chromosome. While linkage analysis is simple in principle, it has relatively poor resolution as only a few hundred markers were usually genotyped across the genome, and the regions identified by linkage could include millions of base pairs and hundreds of genes. While these methods were quite successful in detecting rare variants of large effect (*e.g.*, classical single gene disorders), they proved relatively unsuccessful in identifying genes that are involved in complex polygenic disorders. These studies only revealed two genes, calpain 10 (*CAPN10*) and transcription factor 7-like 2 (T-cell specific, HMG-box) (*TCF7L2*) that were reliably identified as being associated with T2D.

***CAPN10*:** *CAPN10* encodes a cysteine protease that is part of the calpain family, a large family of ubiquitously expressed genes that play multiple roles in intracellular remodeling, post-receptor signaling and other intracellular functions. It became the first *T2D* gene to be discovered by linkage analysis when a locus on chromosome 2 was associated with T2D in 1996[15]. Initially the locus was labeled NIDDM1 but the gene (or genes) involved were not identified. In 2000 the causative gene was finally identified as *CAPN10*[16]. Subsequent studies did not always confirm this finding but larger meta-analyses have shown that variants in *CAPN10* are likely to be truly associated with T2D[17]. At this time the function of this gene in glucose metabolism remains unknown and its link to T2D, while confirmed in several populations, is not always consistent[18-20].

***TCF7L2*:** *TCF7L2* was discovered as a T2D susceptibility gene after a strong linkage signal was mapped to chromosome 10q in a Mexican-American population[21]. This region was later fine-mapped in the Icelandic population and confirmed in United States and Danish cohorts, where the risk locus was found to be located in intron 3 of the *TCF7L2* gene[22]. The association between T2D and a number of single-nucleotide polymorphisms (SNPs) in the *TCF7L2* gene has since been strongly confirmed in multiple Genome-wide association studies (GWAS) in different ethnic groups and this gene remains the most

replicated and most strongly associated T2D risk gene at this time[23]. We will discuss this gene further in the GWAS section of this review.

Candidate gene studies

In candidate gene studies, genes already suspected of playing a role in the pathogenesis of T2D were studied through focused sequencing efforts. The usual strategy was to focus on genes already known to be involved in glucose metabolism, insulin secretion, insulin receptors, post-receptor signaling and lipid metabolism. Somewhat to the surprise of investigators, most of the genes known to be involved in insulin secretion and action were not found to be associated with T2D in the population. The relatively few genes that were found to be associated with T2D include peroxisome proliferator-activated receptor gamma (*PPARG*), insulin receptor substrate 1 (*IRS1*) and *IRS-2*, potassium inwardly-rectifying channel, subfamily J, member 11 (*KCNJ11*), Wolfram syndrome 1 (wolframin) (*WFS1*), HNF1 homeobox A (*HNF1A*), HNF1 homeobox B (*HNF1B*) and *HNF4A*. Other genes including *RAPGEF1* and *TP53* were identified using an algorithm that prioritizes candidate genes for complex human traits based on trait-relevant functional annotation but have not been consistently replicated in later studies[24].

PPARG: *PPARG* gene was an attractive candidate gene for T2D because it encodes the molecular target of thiazolidenediones, a commonly used class of anti-diabetic medications. It was found that a proline to arginine change at position 12 in the *PPARG* gene led to a 20% increase in the risk of diabetes. This finding has since been confirmed in some other populations and other polymorphisms in this gene have been found to play a role in some cases of diabetes[25]. Even so, the significance of these mutations was not replicated in all populations and the contribution of these polymorphisms to the worldwide prevalence of diabetes remains low[26,27].

IRS1* and *IRS-2: Insulin receptor substrate *IRS-1* and *IRS-2* genes encode peptides that play an important role in insulin signal transduction. Polymorphisms in these genes were found to be associated with decreased insulin sensitivity in some populations[28,29] but as with other candidate genes, the role played by these polymorphisms in the global burden of diabetes and related insulin-resistance disorders like PCOS remains small.

KCNJ11: *KCNJ11* gene encodes the Kir6.2 ATP-sensitive potassium channel that plays an important role in the regulation of insulin secretion by beta cells. Activating mutations in this gene are a well-established cause of neonatal diabetes. A missense polymorphism in KCNJ11 was found to be associated with T2D and confirmed in subsequent studies[30]. The odds ratio of developing T2D is about 1.2 in carriers of the risk allele and this allele was also found to be associated with decreased insulin secretion in different populations[31-33].

WFS-1: *WFS-1* gene encodes Wolframin, a protein that is defective in individuals suffering from the Wolfram syndrome (characterized by diabetes insipidus, juvenile diabetes, optic atrophy, and deafness). *WFS1* gene appears to be involved in beta cell function and 2 SNPs in WFS-1 were found to be significantly associated with T2D in a large case-control study involving about 24000 samples[34]. This was subsequently confirmed in other studies in different populations[35]. These studies provided evidence that beta cell dysfunction plays a critical role in the development of T2D and pointed out novel genes that play a previously unknown role in beta cell survival and function, but their role in the global burden of diabetes remains minor.

HNF1A*, *HNF1B* and *HNF4A: *HNF1A*, *HNF1B* and *HNF4A* are all known MODY genes (*i.e.,* genes that harbor rare high penetrance mutations that cause monogenic diabetes of the young). These genes play a role in the development of the liver, in the regulation of hepatic metabolic functions, and in the development and functioning of beta cells. Variants in these genes that do not lead to MODY have been found to be associated with decreased insulin secretion and an increase in the risk of T2D in various populations, but as with other candidate genes, their role in worldwide diabetes prevalence appears to be relatively small[36-38].

Genome wide association studies

Candidate gene studies and linkage analysis identified a few T2D risk genes, but their overall contribution to the observed heritability of T2D remained small and it was clear that other techniques were needed to look for variants that were not easily identified by these methods. With the development of high-throughput SNP genotyping technology and the availability of Hapmap data, it became possible to scan hundreds of thousands of SNPs that were in linkage disequilibrium with millions of SNPs across the genome. TCF7L2, already identified *via* linkage studies, was the most significant and most replicated signal found in GWAS studies, but these studies also helped to identify scores of other genetic loci that appear to be linked to T2D[39]. Over the last 6 years, the number of known T2D variants has risen to over 60; including confirmation of variants identified earlier by candidate gene and linkage studies. While most studies have focused on Europid populations, this is being rectified as more studies of Asian, African and other populations become available.

Since obesity is a major contributor to the development of T2D, genes that increase the risk of obesity also show up in GWAS for T2D. These include some frequently replicated genes include like FTO and MC4R; these genes seem to primarily impact obesity risk and effect T2D risk mostly *via* their effect on obesity (though FTO may have a small but detectable influence on T2D risk independent of the risk of obesity). Here we will focus on genes that specifically increase the risk of T2D,

independent of obesity. The most important of these include

TCF7L2: This remains the most significant and consistently replicated gene linked to T2D. It was initially discovered by linkage studies, then confirmed in the very first large-scale GWAS study conducted in a French population by Sladek *et al*[40]. This publication was followed in quick succession by several other major GWAS paper, including the landmark Welcome Trust study that genotyped 2000 individuals with T2D along with 3000 controls and found that TCF7L2 was the most robust T2D signal, with an odds ratio of 1.36 for carriers heterozygous for the risk allele[41]. This finding was then replicated in almost every human population studied[42-48] and remains the most robust T2D risk gene identified to date. Carriers of the various identified risk alleles have an OR of 1.4[49] and homozygotes may have an OR of 2.5.

TCF7L2 encodes a transcription factor that is a member of the Wnt signaling pathway and is known to be active in the beta cells. Studies in multiple ethnicities indicate that the risk allele is present in intron 3 of the *TCF7L2* gene. An early investigation by Lyssenko *et al*[50] revealed that the risk alleles increased the level of TCF7L2 protein in beta cells and was associated with impaired insulin secretion, incretin effects and enhanced rate of hepatic glucose production. *TCF7L2* expression in human islets was increased 5-fold in T2D, particularly in homozygotes and overexpression of *TCF7L2* in human islets reduced glucose-stimulated insulin secretion. These findings were replicated in several subsequent studies, indicating that TCF7L2 probably plays a role in causation of T2D by decreasing insulin secretion from beta cells, perhaps by altering the action of incretins that modulate the insulin response to meals[51,52]. Other studies indicate that alternative splicing of this gene can lead to the production of different isoforms in different tissues and the presence of specific isoforms in adipose tissue may be related to insulin sensitivity in that tissue[53,54]. It is also possible that T2D risk is conferred by multiple mechanisms, including decreased beta cell insulin response and decreased insulin sensitivity in target tissues like adipose tissue. A recent murine study shows that, at least in mice, when *TCF7L2* is knocked out in liver cells it leads to hypoglycemia and when it is overexpressed it causes hyperglycemia, but there is no effect when it is knocked out in the beta cells[55]. This indicates that the liver may also be an important site where *TCF7L2* variants influence glucose metabolism. Finally, there are indications that this gene may play a role in cancer as well as in diabetes[56,57]. Thus, the discovery of its association with diabetes has opened up several new avenues of research and should eventually lead to the characterization of previously unknown physiological mechanisms that play a role in both diabetes and cancer.

HHEX: hematopoietically expressed homeobox (*HHEX*) While TCF7L2 remains the strongest T2D signal in GWAS studies from across the globe, several other genes have been repeatedly identified in different populations as being associated with T2D. *HHEX* was identified as one such gene in multiple studies in both Caucasian and Asian populations[58]. Located on chromosome 10q, this gene is also a member of the homeobox family and encodes a transcription factor involved in Wnt signaling. Risk alleles appear to confer an OR of developing T2D of 1.5. The mechanism by which this gene confers diabetes risk remains poorly understood.

SLC30A8: Solute carrier family 30 (zinc transporter), member 8 (*SLC30A8*). This gene encodes for a protein that is involved in the storage and secretion of insulin granules and that is expressed at a high level only in the pancreas, particularly in the islets of Langerhans[59]. This provides an obvious mechanism by which it may be involved in conferring T2D risk and this association has been replicated in multiple studies in different populations[60-62]. Interestingly, this gene has also been found to be associated with the development and progression of type 1 diabetes[63] though this has not been confirmed in all studies[64].

CDKN2A/B: Cyclin-dependent kinase inhibitor 2A/B (*CDKN2A/B*). SNPs located upstream of the *CDKN2A2B* genes have been associated with the risk of T2D in multiple large GWAS. These genes are located on chromosome 9p21 and generate several transcript variants. At least three alternatively spliced variants of *CDKN2A* encoding distinct proteins have been reported, two of which are known to function as inhibitors of *CDK4* kinase. *CDKN2B* is also located in the same region and generates at least 2 splice variants. Both genes are important cell cycle regulators with a role in tumor suppression. This region was found to be associated with T2D in multiple GWAS studies in different populations and it is estimated that the risk alleles confer an odds ratio for development of T2D of between 1.2 and 1.5[65]. How variations in these genes alter diabetes risk remains unclear but recent research points to a role in insulin secretion rather than insulin action[66]. These variants also show up in GWAS for cardiovascular disease, in particular for atherosclerosis, but the mechanism underlying this association remains unknown[67].

IGF2BP2: insulin-like growth factor 2 mRNA binding protein 2 (*IGF2BP2*) encodes a protein that binds to the 5' UTR of the insulin-like growth factor 2 (IGF2) mRNA and thereby regulates IGF2 translation. Alternate transcriptional splice variants, encoding different isoforms, have been characterized[68]. This gene has been found to be associated with T2D risk in multiple GWAS[69,70]. As with other variants like HHEX and CDKN2A/B, it may play a role in beta cell function[65] but the mechanism by which it influences T2D risk remains largely unknown.

Other genes linked to T2D risk include *CDKAL1* (CDK5 regulatory subunit associated protein 1-like 1),

Table 1 Thirty-eight genetic variants associated with type 2 diabetes at genome-wide significance

Locus	Chr	Risk allele frequency	OR (95%CI)
NOTCH2	1	0.11	1.13 (1.08-1.17)
PROX1	1	0.5	1.07 (1.05-1.09)
IRS1	2	0.61	1.19 (1.13-1.25)
THADA	2	0.92	1.15 (1.10-1.20)
RBMS1/ITGB6	2	0.57	1.11 (1.08-1.16)
BCL11A	2	0.46	1.08 (1.06-1.10)
GCKR	2	0.62	1.06 (1.04-1.08)
IGF2BP2	3	0.29	1.17 (1.10-1.25)
PPARG	3	0.92	1.14 (1.08-1.20)
ADCY5	3	0.78	1.12 (1.09-1.15)
ADAMTS9	3	0.81	1.09 (1.06-1.12)
WFS1	4	0.27	1.13 (1.07-1.18)
ZBED3	5	0.26	1.08 (1.06-1.11)
CDKAL1	6	0.31	1.12 (1.08-1.16)
JAZF1	7	0.52	1.10 (1.07-1.13)
GCK	7	0.2	1.07 (1.05-1.10)
KLF14	7	0.55	1.07 (1.05-1.10)
DGKB/TMEM195	7	0.47	1.06 (1.04-1.08)
SLC30A8	8	0.75	1.12 (1.07-1.16)
TP53INP1	8	0.48	1.06 (1.04-1.09)
CDKN2A/B	9	0.79	1.20 (1.14-1.25)
TLE4	9	0.93	1.11 (1.07-1.15)
TCF7L2	10	0.25	1.37 (1.28-1.47)
HHEX	10	0.56	1.13 (1.08-1.17)
CDC123/CAMK1D	10	0.23	1.11 (1.07-1.14)
KCNQ1	11	0.61	1.40 (1.34-1.47)
KCNJ11/ABCC8	11	0.5	1.15 (1.09-1.21)
CENTD2	11	0.88	1.14 (1.11-1.17)
MTNR1B	11	0.3	1.09 (1.06-1.12)
KCNQ1	11	0.52	1.08 (1.06-1.10)
HMGA2	12	0.1	1.10 (1.07-1.14)
TSPAN8/LGR5	12	0.23	1.09 (1.06-1.12)
OASL/HNF1A	12	0.85	1.07 (1.05-1.10)
PRC1	15	0.22	1.07 (1.05-1.09)
ZFAND6	15	0.56	1.06 (1.04-1.08)
FTO	16	0.45	1.15 (1.09-1.22)
HNF1B	17	0.43	1.12 (1.07-1.18)
DUSP9	X	0.12	1.27 (1.18-1.37)

Modified from Florez *et al*[71].

HMGA2 (high mobility group AT-hook 2), *KCNQ11* (potassium voltage gated channel, KQT like subfamily, member 1) and *NOTCH2-ADAM30* (Notch 2-ADAM metallopeptidase domain 30). Their exact role in the pathophysiology of T2D remains mostly unknown. A list of these and other variants is given below in Table 1.

As can be seen in Table 1, the odds ratios for individual risk alleles are generally less than 1.3 (TCF7L2 and KCNQ1 being the most prominent exceptions) and it has been estimated that all the risk alleles identified to date can only explain about 10% of the observed heritability of T2D. Thus these alleles cannot be used to estimate the genetic risk of developing T2D in an individual patient with any degree of certainty since a simple family history will be much more informative than a detailed genotype at this point. But the discovery of these genes has opened entirely new avenues in our quest to understand the regulation of glucose metabolism and the development of T2D. For example, prior to these genetic studies, no one could have predicted that *TCF7L2* plays any role in glucose regulation. But initially *via* linkage studies, and then in multiple GWAS, it has been shown to be the single most significantly associated diabetes risk gene in the world. This has led to intensive investigation of its physiological role and though those investigations are at an early stage, it is hoped that they will eventually yield a new and more complete understanding of the mechanisms that regulate insulin secretion and action and whose alteration may lead to an increased risk for T2D. That in turn may lead to the identification of new drug targets, diagnostic tests, and targeted therapies (pharmacogenomics).

What do these genes do?

The fact that many of these genes are active in beta cells or may be involved in insulin secretion support the notion that beta cell dysfunction is a crucial final step on the path to diabetes[72,73]. Very few of these genes seem to play a role in insulin sensitivity (though that may change as more information becomes available) and genes involved in the insulin signaling pathway rarely show up in T2D GWAS studies. When indices of beta-cell function (HOMA-B) and insulin sensitivity (HOMA-IR) derived from paired fasting glucose and insulin measures from 37000 individuals were used to try and identify the function most affected by various T2D risk genes, it was found that risk alleles at ten loci (*MTNR1B*, *SLC30A8*, *THADA*, *TCF7L2*, *KCNQ1*, *CAMK1D*, *CDKAL1*, *IGF2BP2*, *HNF1B* and *CENTD2*) were associated ($P < 0.05$) with reduced beta-cell function, and only three loci (*PPARG*, *FTO* and *KLF14*) were associated with reduced insulin sensitivity[74].

It is possible that this may be because rare variants have a greater impact on insulin sensitivity or because environmental factors play a greater role in altering insulin sensitivity and thus swamp underlying genetic variation in risk. Still, this finding was not expected when candidate gene studies were initiated and shows how agnostic high throughput methods like GWAS can help to generate novel hypotheses and illuminate new aspects of biology. Some of the genes found to be associated with T2D also appear to be linked to dyslipidemia, atherosclerotic heart disease and cancer and it is possible that as we learn more about the role of these genes, we may be able to understand more about the relationship between T2D and other components of the metabolic syndrome as well as cancer[71].

Gene-environment interactions: It is abundantly clear that the risk of developing T2D is heavily influenced by environmental factors. Since our genetic code does not change significantly in one or two generations, the recent secular trend in diabetes must be due mostly to changes in the environment. Increased adiposity is the single most significant factor in the development of T2D and the epidemics of obesity and T2D largely parallel one another. The increasing prevalence of obesity is thought

to be related primarily to changes in dietary habits and our increasingly sedentary lifestyle, though other factors (including toxins and infectious agents) may play a role. Genes may influence the risk of diabetes not only by directly altering insulin action or secretion, but also by altering how any given individual interacts with these environmental factors. Even within the same broad environment, individuals vary greatly in their adoption of unhealthy lifestyles and their willingness to change such lifestyles. By influencing who adopts a more unhealthy diet (this includes genetic influence on taste and food preferences), who exhibits greater willingness to change unhealthy behaviors[75], who burns more calories at rest, who exhibits greater activity levels when not actively exercising, what kind of microbiome an individual carries, and who opts for a more sedentary lifestyle, genetic factors can play a role in determining who becomes obese or develops diabetes in any given environment[76]. These gene-environment interactions may be extremely complex and may be one reason why such a small proportion of the heritability of T2D has been explained at this time[77].

Epigenetics

Epigenetics refers to heritable changes in gene function that occur without a change in nucleotide sequence. Mechanisms like DNA-methylation, histone acetylation and non-coding RNAs are used by the cell to regulate gene expression in response to environmental cues and can persist for an individual's lifetime and can be passed on over 2-3 generations[78]. It is well known that the maternal environment and early infancy can alter the lifelong risk of chronic diseases. For example, infants who are born small for gestational age are at an increased risk for the development of obesity and T2D as adults. Some or most of this risk may be due to epigenetic changes in critical genes and animal experiments[79] and initial human studies suggest that such mechanisms may indeed explain the impact of intrauterine nutrition and birth weight of future risk of diabetes, obesity and metabolic syndrome[80]. It is thus possible that some of the observed heritability of T2D is due to epigenetic changes during intra-uterine life that are the result of maternal environmental influences, rather than inherited variations in the DNA sequence. As our understanding of epigenetics advances and as the ability to profile genome-wide DNA methylation and other epigenetic mechanisms becomes more widely used, we are likely to see important discoveries regarding the epigenetic changes that alter the risk of T2D. Epigenetic profiling may also help to identify novel genes that play a role in the pathogenesis of T2D just as GWAS led to the identification of multiple genes that were previously unsuspected of having a role in diabetes.

Risk prediction based on genetic information

While we know that a person's future risk of developing T2D has a significant heritable component and believe that most of this inherited risk is associated with particular genotypic features (in most cases, multiple variants of small effect?), and have identified several risk variants in genome-wide association studies, these variants still explain a relatively small proportion of the observed heritability. Several studies have found that a risk score based on traditional risk factors (BMI, family history, age, sex, HDL, triglycerides, *etc.*) consistently outperforms any set of genetic markers and the addition of known genetic markers does not significantly improve prediction based on traditional risk factors[81-83].

This indicates that our current state of knowledge regarding specific genetic markers is still incomplete and fails to explain most of the inherited risk. But as more data becomes available and better statistical techniques are applied to analyze gene-gene and gene-environment interactions, this predictive ability is likely to improve[84]. Even before that happens, these genetic discoveries have already provided important new insights into the pathophysiology of T2D and as the physiologic role of these genes in glucose regulation becomes clearer, these discoveries can be expected to lead to better diagnostic and therapeutic tools. Potential applications are not limited to better risk prediction, new drug targets and better targeted drug therapy; some time in the future when our technologies have improved far beyond current levels, they may include the ability to alter the risk of diabetes using gene-therapy or epigenetic reprogramming.

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P- Reviewers Guerrero-Romero F, Wong WTJ
S- Editor Zhai HH **L- Editor** A **E- Editor** Lu YJ

World Journal of Diabetes

Online Submissions: http://www.wjgnet.com/esps/
wjd@wjgnet.com
doi:10.4239/wjd.v4.i4.124

World J Diabetes 2013 August 15; 4(4): 124-129
ISSN 1948-9358 (online)

MINIREVIEWS

Diabetic nephropathy: Treatment with phosphodiesterase type 5 inhibitors

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Received: April 11, 2013 Revised: June 3, 2013
Accepted: June 19, 2013
Published online: August 15, 2013

Abstract

The importance of nitric oxide (NO) in vascular physiology is irrefutable; it stimulates the intracellular production of cyclic guanosine monophosphate (cGMP), initiating vascular smooth muscle relaxation. This biochemical process increases the diameter of small arteries, regulating blood flow distribution between arterioles and the microvasculature. The kidney is no exception, since NO predominantly dilates the glomerular afferent arterioles. It is now evident that the vascular production of cGMP can be augmented by inhibitors of phosphodiesterase type 5 (PDE 5), the enzyme which breakdowns this cyclic nucleotide. This has clinical relevance, since diabetic nephropathy (DN) a major microvascular complication of diabetes mellitus and the most common cause of end-stage renal disease, increases intraglomerular capillary pressure, leading to glomerular hypertension. PDE 5 inhibitors may have, therefore, the potential to reduce glomerular hypertension. This review describes the use of PDE 5 inhibitors to improve the metabolic, haemodynamic and inflammatory pathways/responses, all of which are dysfunctional in DN.

Key words: Diabetic nephropathy; Phosphodiesterase type 5; Glomerular filtration rate; Inflammation; Angiotensin Ⅱ

Core tip: Diabetic nephropathy a leading cause of end-stage renal disease, is characterized by dysfunctional metabolic, haemodynamic and inflammatory pathways leading to glomerular hypertension. These pathways were normalized following treatment with phosphodiesterase type 5 inhibitors, which initiated renal vascular smooth muscle relaxation. This therapeutic option for treating diabetic nephropathy may negate the need for costly renal dialysis or transplantation.

Thompson CS. Diabetic nephropathy: Treatment with phosphodiesterase type 5 inhibitors. *World J Diabetes* 2013; 4(4): 124-129 Available from: URL: http://www.wjgnet.com/1948-9358/full/v4/i4/124.htm DOI: http://dx.doi.org/10.4239/wjd.v4.i4.124

INTRODUCTION

Chronic kidney disease is increasing worldwide at an annual rate of 8%, with the prevalence higher in developing countries[1]. Diabetic nephropathy (DN) is a common underlying cause[1]; indeed in many countries it is the main cause of end-stage renal disease (ESRD) and is associated with a high morbidity and mortality[2-5]. DN develops due to a complex interaction between metabolic and haemodynamic pathophysiological factors, which lead to renal damage[6,7]. It can affect 20%-30% of the diabetic population, who present with an increase in urinary albumin excretion (microalbuminuria) in the earliest stage[4]. This may progress to macroalbuminuria and later renal insufficiency and ESRD. There is also evidence of an increase in systemic and vascular markers of inflammation[8,9] as the size of the kidney progressively increases[10]. Accompanying these changes are abnormalities in the blood biochemical indices of renal function, which precede renal failure[11].

Treatment of DN has focused on the integrated control of dyslipidaemia, glycaemia and blood pressure to reduce microalbuminuria[3,12-14]. Nevertheless, although, current treatment strategies may slow the progression of DN to ESRD, it does not fully arrest this process[9,15]. Inevitably, some patients require renal replacement therapy (RRT), at an average cost of €40000-50000 per patient per year[5]. Not surprisingly, the increasing number of diabetic patients on RRT[9,16], is placing a financial strain on health care systems worldwide[2,17]. It has also been established that the cardiovascular morbidity and mortality is higher in diabetic ESRD patients on dialysis compared to those without diabetes[9]. Slowing the decline of renal function, from DN to ESRD is of paramount importance and hence there is a clear need for new strategies to treat DN.

One feasible option for treating the DN-induced enhancement of intraglomerular pressure[7] and the resultant glomerular hypertension[18] is to alter the renal cyclic guanosine monophosphate (cGMP)-NO pathway. This is because NO dilates vessels in the kidney, including the glomerular afferents[19], by stimulating intracellular production of cGMP[20,21], which in turn initiates renal vascular smooth muscle relaxation. It is now evident that the vascular cGMP-NO pathway can be augmented by inhibitors of phosphodiesterase type 5 (PDE 5), the enzyme which breakdowns cGMP[22]. These inhibitors (sildenafil, tadalafil and vardenafil) allow cGMP to accumulate and are increasingly used to treat penile erectile dysfunction[23-25], as they cause a significant relaxation of the corpus cavernosum[24,26] leading to erection. They may also have a therapeutic role in DN, since PDE 5 expression/activity is abundant in the kidney[27] and may contribute towards glomerular hypertension. In this scenario selective inhibition of PDE 5 enzymatic activity would provide renoprotection.

This review describes the use of PDE 5 inhibitors to improve metabolic and haemodynamic pathophysiological factors, as well as inflammatory pathways, all of which are dysfunctional in DN.

METABOLIC CHANGES

Strict glycaemic control is desirable for DN patients, especially as polyol and hexosamine pathways, the accumulation of [advanced glycation end products (AGEs) formed by glucosylation of proteins, lipids and nucleic acid] and activation of protein kinase C are all thought to play a role in disease progression[7]. However, there is little evidence that PDE 5 inhibition reduces diabetic hyperglycaemia, since sildenafil had no effect in streptozotocin-induced diabetic rats[28] or in a model of non-insulin-dependent diabetes mellitus, the Otsuka Long-Evans Tokushima Fatty (OLETF) rats[29]. Even so, the effect of PDE 5 inhibition on other renal metabolic abnormalities needs to be considered.

Kidney weight, histology and electron microscopy

Kidney size and weight are typically increased in diabetes mellitus, primarily due to glomerular and tubular hypertrophy. An increase in the number of glomerular cells (mainly mesangial and endothelial), in extracellular matrix and in capillary number and size all contribute to this hypertrophy[30]. This increase is also evident when kidney weight is corrected for body surface area[31], or in diabetic rats, when expressed as kidney: body-weight, a ratio lowered following treatment with sildenafil[28]. In terms of renal histology, glomerular lesions characterized by hypertrophy, mesangial matrix expansion and sclerotic lesions were evident in the OLETF rat kidney and were significantly reduced by sildenafil[29], demonstrating drug-induced amelioration of NIDDM nephropathy.

The reduction in kidney weight and improved histology afforded by treatment with sildenafil, suggests that PDE 5 inhibition may prove to be an important and effective therapeutic option for DN-induced hypertrophy. This is supported by the finding that renal morphological changes induced in spontaneously hypertensive rats (SHR) by cyclosporin A, a potent nephrotoxic immunosuppressant were also improved with a PDE 5 inhibitor (FR226807, Fujisawa Pharmaceutical, Japan)[32].

The earliest ultrastructural abnormality in DN relates to the diffuse thickening of the glomerular basement membrane, which increases as the disease advances. As previously mentioned, several biochemical changes contribute to this process, notably an increase in collagen type Ⅳ deposition and impairment of excess extracellular matrix degradation, mesangial expansion by extracellular matrix deposition and increased mesangial cellularity. There are also changes in glomerular epithelial cells (podocytes), including a decrease in number and/or density, with a reduced podocyte per glomerulus ratio, podocyte foot process broadening and effacement, glomerulosclerosis and tubulointerstitial fibrosis[18,33]. It would be interesting to establish whether PDE 5 inhibitors can prevent or reverse these DN-induced ultrastructural changes observed under electron microscopy.

Serum creatinine

Creatinine is a by-product of muscle-derived creatine. In the early stages of DN, kidney compensatory mechanisms maintain serum creatinine levels, but as the disease progresses this compensation fails due to the marked and continuous damage to functioning nephrons. It seems, therefore, that the increased serum creatinine seen in DN indicates the severity of the clinical renal damage. Interestingly, in the first study to treat DN with a PDE 5 inhibitor (vardenafil given orally for one month to alloxan-induced diabetic rabbits) the elevated serum creatinine level was restored to normal[34].

The notion that PDE 5 inhibition can ameliorate the progression of renal damage has been examined in other animal models. Sildenafil reduced the elevated serum creatinine concentration in rats following 5/6 nephrectomy[35], while another PDE 5 inhibitor normalized the level in SHR treated with cyclosporin A[32]. These finding demonstrate the beneficial effect PDE 5 inhibitors have on impaired renal function.

HAEMODYNAMIC FACTORS

Glomerular filtration rate/creatinine clearance

Glomerular filtration rate (GFR) and creatinine clearance (CrCl), a good index of GFR[36], are routinely used to check kidney function. Specifically, they estimate blood flow per minute through the glomeruli, and measure how well the kidneys filter the DN-induced build up of blood creatinine. The early stages of DN are characterized by an increase in glomerular hyperfiltration, which elevates GFR and contributes to renal impairment[37]. However, as the disease progresses, renal function deteriorates and there can be a relentless decline in GFR[16,38,39]. This functional change develops as a consequence of structural abnormalities, including an increase in kidney size[29,31], together with poor metabolic control[40]. Lau *et al*[34] found CrCl was reduced in diabetic rabbits and restored by vardenafil, suggesting that PDE 5 inhibitors can improve diabetes-induced renal impairment.

Cyclosporin A-induced nephrotoxicity and renal damage caused by 5/6 nephrectomy, provide indirect support for this concept; both are characterized by a decrease in CrCl and improved by treatment with a PDE5 inhibitor[32,35].

The beneficial effect of the PDE 5 inhibitors is likely to be due to NO-cGMP accumulation[20,21] causing dilatation of glomerular afferent blood vessels[19]. In this regard, it is proposed that glomerular hyperfiltration depends upon an increase in NO activity in the early phase of DN[41], whereas in the later phase when the GFR starts to fall, a concomitant reduction in NO activity would lead to glomerular hypertension. The diabetes-induced reduction in NO activity could be due to defective synthesis or quenching through the production of superoxide radicals and AGEs[42,43]. Therefore, the beneficial action of PDE 5 inhibitors is to increase renal NO-cGMP activity and restore GFR/CrCl. An increase in kidney cGMP content, rather than blood pressure reduction, was also thought responsible for the improved renal function in rats with cyclosporin A-induced nephrotoxicity following PDE 5 inhibitor treatment[32].

Hypertension is twice as frequent in diabetic patients and a major reason why most develop cardiovascular disease[44]. It also plays a significant role in the progression of DN[7,45]. Consequently, lowering blood pressure has to be an important consideration in the management of DN. Kuno *et al*[29] noted that sildenafil treatment for 8 wk significantly reduced systolic and diastolic blood pressure in OLETF rats, providing further evidence of the haemodynamic benefits that can be achieved by treatment with PDE 5 inhibitors.

Urinary albumin excretion and total protein/creatinine ratio

The progressive increase in urinary albumin excretion, commonly termed proteinuria is another clinical hallmark of DN and also a predictor of cardiovascular disease. Such DN-induced increase in proteinuria is part of a series of clinical events, which includes elevated blood pressure and a progressive decline in GFR. Moreover, proteinuria does not diminish as DN progresses. Proteinuria is, therefore, a consequence of the glomerular damage in diabetes mellitus and a cause of further damage, since it leads to inflammation and fibrosis in the renal tubules and a loss of functional nephrons. Urinary albumin excretion and total protein/creatinine ratio can be used to monitor proteinuria[46,47]. Both markers were elevated in six months diabetic rabbits, as well as diabetic and OLETF rats and were normalized by vardenafil and sildenafil treatment[28,29,34]. Sildenafil also reduced the elevated proteinuria in 5/6 nephrectomized rats[35]. Taken together, these findings imply that PDE 5 inhibitors reduce proteinuria and improve the renal status in DN.

Inflammation and fibrosis

Diabetes-induced kidney fibrosis results from prolonged renal injury initiated by excessive extracellular matrix deposition. Inflammation is central to the development and progression of this complication. It is characterized by glomerular and tubulointerstital migration of activated inflammatory cells (neutrophils, macrophages, T lymphocytes, and mast cells) and fibroblasts in the kidney, regardless of the initial insult. At the injury site, inflammatory cells synthesize reactive oxygen species plus fibrogenic cytokines and growth factors, which exacerbate the renal damage. This leads to excessive and poorly ordered matrix deposition and fibrosis; in turn this affects normal-tissue architecture and ultimately can disable proper functioning of the kidney[9,48]. Jeong *et al*[28] found that sildenafil treatment significantly attenuated the diabetes-induced increase in renal cortex 8-OHdG content and its elevated excretion (measures of oxidative stress and DNA damage), probably by inhibiting the accumulation of oxidized DNA in the kidney. DN-induced macrophage infiltration into the glomeruli and tubulointerstitium of diabetic rats, an indication of inflammation, was also ameliorated by sildenafil. It seems likely that controlling excessive inflammation and generation of reactive oxygen species with PDE 5 inhibitors will have therapeutic potential in inhibiting diabetes-induced kidney fibrosis. Interestingly, drugs with antiinflammatory activity have been found to slow or reverse DN[9].

Angiotensin Ⅱ

Angiotensin Ⅱ (Ang Ⅱ), is an octapeptide trophic hormone and a powerful vasoconstrictor widely recognised as a regulator of blood pressure, fluid and electrolyte homeostasis[7]. It has a central role in the pathogenesis of DN where increased Ang Ⅱ levels cause a preferential constriction of the efferent glomerular arterioles, an increased glomerular permeability to proteins and enhanced formation of AGEs[33]. It regulates, therefore, systemic and glomerular haemodynamics, as well as glomerular hypertrophy and sclerosis[7]. In mesangial cells it triggers the production and release of cytokines, chemokines and growth factors, with the net effect of mediating and/or amplifying renal damage[18]. The physiological actions of Ang Ⅱ in the kidney are due to activation of angiotensin Ⅱ receptor type 1

(AT1) receptors. These receptors are mainly expressed in smooth muscle cells where they induce vasoconstriction, proliferation and inflammation[21].

The regulation of Ang Ⅱ is governed by interplay with NO[21]. NO antagonizes the vasoconstrictive and pro-atherosclerotic effect of Ang Ⅱ, as well as directly modulating angiotensin-converting enzyme (ACE) activity. Conversely, Ang Ⅱ decreases NO bioavailability by promoting oxidative stress[21,49]. Ang Ⅱ has been reported to upregulate super oxide production in endothelial and vascular smooth muscle cells, which is thought to directly contribute to Ang Ⅱ-induced contraction of vascular smooth muscle[50].

It is possible that the beneficial effect of PDE 5 inhibitors in DN is due to their regulatory action on the renal Ang Ⅱ response. There is clinical evidence that inhibition of ACE and AT1 receptor blockade suppress development of DN[51-53]. Whether the beneficial effects of PDE 5 inhibitors in DN also include regulatory actions on the renal Ang Ⅱ response merits future investigation.

CONCLUSION

Over the last few years the cellular and molecular events underlying the renal structural and functional abnormalities of DN have been the subject of intense study. The role played by metabolic and haemodynamic stimuli in disease progression should not be underestimated. It is likely that diabetic hyperglycaemia and oxidative stress increase the formation of AGEs, cytokines and growth factors, which are important in the development of glomerulosclerosis and tubulointerstitial fibrosis, by stimulating the production of extracellular matrix and inhibiting its degradation. Ang Ⅱ also plays an important role in DN, since it increases mesangial proliferation and matrix accumulation and induces proinflammatory and fibrogenic processes. These events lead to the progressive decline of nephron function and their destruction.

The evidence outlined in this review suggests that PDE 5 inhibition may provide an additional therapeutic option to treat this debilitating disorder. Ultimately, it may be necessary to use PDE5 inhibitors in conjunction with other treatment modes; for example, hypoglycaemic drugs and antihypertensives, such as ACE inhibitors and AT1 receptor blockers. In addition, cardiovascular risk factors, such as hyperlipidaemia and smoking should be reduced. These life style changes, supported by appropriate drug therapy, could ultimately reduce the number of patients with DN who require costly renal dialysis or transplantation.

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P- Reviewers Lehtonen SH, Ortiz A **S- Editor** Zhai HH
L- Editor A **E- Editor** Lu YJ

World Journal of Diabetes

Online Submissions: http://www.wjgnet.com/esps/
wjd@wjgnet.com
doi:10.4239/wjd.v4.i4.130

World J Diabetes 2013 August 15; 4(4): 130-134
ISSN 1948-9358 (online)

MINIREVIEWS

Type 1 diabetes and celiac disease: The effects of gluten free diet on metabolic control

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Received: April 8, 2013 Revised: June 13, 2013
Accepted: July 18, 2013
Published online: August 15, 2013

Abstract

Type 1 diabetes mellitus is associated with celiac disease, with a prevalence that varies between 0.6% and 16.4%, according to different studies. After a diagnosis of celiac disease is confirmed by small bowel biopsy, patients are advised to commence a gluten-free diet (GFD). This dietary restriction may be particularly difficult for the child with diabetes, but in Europe (and in Italy) many food stores have targeted this section of the market with better labeling of products and more availability of specific GFD products. Treatment with a GFD in symptomatic patients has been shown to improve the symptoms, signs and complications of celiac disease. However, the effects of a GFD on diabetic control are less well established. Initial reports of improved hypoglycemic control were based on children who were diagnosed with celiac disease associated with malabsorption, but there have subsequently been reports of improvement in patients with type 1 diabetes with subclinical celiac disease. There are other studies reporting no effect, improved control and an improvement of hypoglycemic episodes. Moreover, in this review we wish to focus on low glycemic index foods, often suggested in people with type 1 diabetes, since they might reduce postprandial glycemic excursion and enhance long-term glycemic control. In contrast, GFD may be rich in high glycemic index foods that can increase the risk of obesity, insulin resistance and cardiovascular disease, worsening the metabolic control of the child with diabetes. Hence, it is important to evaluate the impact of a GFD on metabolic control, growth and nutritional status in children with type 1 diabetes.

Key words: Adolescents; Celiac disease; Children; Glycemic control; Type 1 diabetes

Core tip: It is important to evaluate the impact of a gluten-free diet (GFD) on metabolic control, growth and nutritional status in children with type 1 diabetes and celiac disease. Since compliance with a strict GFD and a safe choice of food for diabetes is not easy, these patients require extra education and dietary intervention. A specialized follow-up and dietary counseling are essential in the management of patients affected by both type 1 diabetes and celiac disease.

Scaramuzza AE, Mantegazza C, Bosetti A, Zuccotti GV. Type 1 diabetes and celiac disease: The effects of gluten free diet on metabolic control. *World J Diabetes* 2013; 4(4): 130-134 Available from: URL: http://www.wjgnet.com/1948-9358/full/v4/i4/130.htm DOI: http://dx.doi.org/10.4239/wjd.v4.i4.130

INTRODUCTION

Type 1 diabetes is an immune-mediated disorder characterized by a deficit or absence of insulin resulting from T cell-mediated destruction of beta cells of the pancreas[1].

Children with type 1 diabetes have an increased risk of developing other autoimmune disorders like Hashi-

moto's thyroiditis, Addison disease, vitiligo and celiac disease[2]. The relation between type 1 diabetes and these pathologies is a common genetic background. All of these diseases are associated with organ-specific autoantibodies that can be detected before the development of clinical diseases; consequently, patients affected by type 1 diabetes usually undergo a scheduled (usually once a year) screening for these pathologies.

Celiac disease is one of the most common autoimmune disease-based disorders; it is elicited by a failure of oral tolerance towards wheat, gluten and related cereals, which results in a multisystem inflammation of the intestinal tract. It usually develops in HLA-DQ2/8 positive individuals. The first association between type 1 diabetes and celiac disease was suggested in 1969[3]. The genetic risk factors associated with both diseases include human leukocyte antigen (*HLA*) genes and non-*HLA* genes.

The increased prevalence of celiac disease in patients with type 1 diabetes is due to an overlap in the genetic susceptibility to both diseases conferred by the *HLA-DR3/DQ2*[4]. This haplotype is present in over 90% of patients with celiac disease and 55% of those with type 1 diabetes, compared with only 20%-25% of the general population of European ancestry. HLA-DQ8 also confers a risk of type 1 diabetes[4].

Celiac disease affects at least 10% of patients with type 1 diabetes at some point in their lives[5], with a prevalence that varies between 0.6%-16.4%, according to different studies[6-8]. The prevalence of celiac disease among children with type 1 diabetes is significantly higher than in non-diabetic children (in Western countries celiac disease affects around 1%-2% of the non-diabetic population).

In Italy, the prevalence of celiac disease in children with type 1 diabetes is around 7%[9,10], 3.6% of which at type 1 diabetes onset[9], at a younger age[10] and in boys[10]; moreover, according to a study by Salardi *et al*[11], the prevalence of celiac disease has significantly increased since 1994 (10.6% *vs* 6.6%, $P = 0.015$), probably due to changes in environmental factors, namely, eating habits and viral infections.

Less than 10% of patients with type 1 diabetes who develop celiac disease show gastrointestinal symptoms, while most of the children are either asymptomatic or only mildly symptomatic. Therefore, children affected by type 1 diabetes undergo screening for celiac disease. Usually, celiac autoantibodies are tested at the time of diabetes onset and yearly during follow-up, but debate exists about timing and frequency for screening[12,13]. When celiac antibodies are detected (ideally confirmed at least twice), it is mandatory to perform esophagogastroduodenoscopy with bowel biopsies to confirm diagnosis[14].

TREATMENT OF CELIAC DISEASE FOR PATIENTS WITH TYPE 1 DIABETES

The presence of mucosal atrophy is an indication to start a gluten-free diet (GFD), which is the standard therapy for celiac disease, avoiding all foods containing wheat, rye, barley and oats.

Patients affected by celiac disease must follow a strict GFD for their entire life to prevent acute (malabsorption, diarrhea, folate deficiency, failure to thrive, iron deficiency) and chronic (intestinal lymphoma, osteoporosis, autoimmune diseases, infertility, mortality) complications[12,15,16].

Gluten restriction added to a diabetic dietary regimen imposes practical limitations and leads to considerable restrictions in the lifestyle of a child or adolescent. Unfortunately, as a result, non-adherence to GFD among patients with type 1 diabetes and celiac disease is very common. A study by Valerio *et al*[17] found that only 59% of patients with type 1 diabetes and celiac disease were compliant to a strict GFD, while compliance in patients with celiac disease only is around 78%[18]. This is an important factor to consider when treating a child or adolescent with type 1 diabetes. It is well established that an accurate diet is one of the cornerstone of the management in patients with type 1 diabetes[19]. Combining a GFD may raise major challenges and even some doubts. Dietary intervention aims to achieve and maintain blood glucose and blood pressure in the normal range, to attain normal lipid profile, to achieve normal body weight[19]. Preserving a steady glycemic control is essential to reduce both micro and macrovascular complications of type 1 diabetes[20]. For this reason, it is important to give patients advice on carbohydrate amount, type and distribution throughout the day, and to educate them about carbohydrate counting. In this context, the choice of low glycemic index food may be important[21]. In this respect, a GFD could be an obstacle as many of the gluten-free foods have a high glycemic index. This might influence glycemic values, HbA1c, insulin requirement, lipid profile, and possibly the development of long-term diabetic complications. Moreover, GFD could modify both anthropometric measures, such as height, weight, body mass index (BMI), growth velocity, even if not all researchers agree on the final effects of GFD.

BODY MASS INDEX IN CHILDREN WITH TYPE 1 DIABETES AND CELIAC DISEASE

While, in patients with celiac disease alone, concern has been raised about gaining weight when on a GFD[22], recent data show normal growth patterns in children and adolescents with type 1 diabetes and celiac disease[23], with body mass index and height standard deviation scores only marginally but not significantly higher in the control (non-celiac) than the study group, and similar to subjects with celiac disease with good or fair/poor adherence to a GFD throughout the follow-up period. Among the reasons for increased BMI, the macronutrient composition of gluten-free foods, a high percentage of saturated fat and carbohydrates with high glycemic index, and a low percentage of proteins and fiber can be included.

After clearing gluten, as villous atrophy resolves, intestinal absorption is certainly improved, but an excessive weight gain may increase the risk of morbidity and may

lead to higher risk of cardiovascular disease[24] especially in type 1 diabetes patients. However, data on weight gain (and BMI increasing) in patients with celiac disease are inconsistent. Dickey *et al*[22] showed that nearly 80% of patients gained some weight after 2 years on GFD, and about 51% were even overweight or obese. On the contrary, a recent study reported a weight loss in obese or overweight patients while on GFD[25], with a similar improvement in screen- and symptom-detected celiac disease patients on a GFD.

GLYCEMIC CONTROL IN CHILDREN WITH TYPE 1 DIABETES AND CELIAC DISEASE

Regarding patients with type 1 diabetes and celiac disease, the most recent data show no difference between patients with and without celiac disease[23]. However, a link between a change in body mass index and a possible improvement of metabolic control remains controversial. Acerini *et al*[26] observed an improvement both in body mass index and in HbA1c, while Nóvoa Medina *et al*[27], who studied only type 1 diabetes patients with symptomatic celiac disease, did not find any effects on metabolic control or on height or weight.

Other studies evaluated the influence of a GFD on metabolic parameters, such as insulin dose, HbA1c, glucose excretion and hypoglycemic episodes. Saadah *et al*[28] observed that a GFD resulted in a significant improvement of growth and influenced diabetic control (more insulin in celiac disease patients when compared to baseline). Other authors[26,29] did not find any significant difference in insulin dose, HbA1c, 24 h urinary glucose excretion, or number of hypoglycemic episodes. Similar findings have been observed in adult patients with type 1 diabetes and celiac disease[30]. Abid *et al*[31] documented in type 1 diabetes children with celiac disease that a GFD showed short-term benefits by reducing gastrointestinal symptoms and, in particular, episodes of severe hypoglycemia, while there was no change in standard deviation score for height, weight, and BMI or the mean HbA1c before and after GFD. The mean insulin requirement significantly increased. More refined indexes of an altered or better metabolic control, like continuous glucose monitoring, glycemic variability indexes, and frequency of insulin dose changes are usually difficult to measure.

TYPE 1 DIABETES, CELIAC DISEASE AND MICRO OR MACROANGIOPATHIC COMPLICATIONS

Few studies have been published about this topic and almost all involved adult patients with type 1 and celiac disease. Bakker *et al*[32] collected HbA1c before celiac disease diagnosis, at diagnosis and the most recent together with the presence of nephropathy and retinopathy. An interesting finding was that diabetes patients with celiac disease had a lower prevalence of retinopathy when compared to controls (diabetes patients without celiac disease), whereas no difference in the prevalence of nephropathy was found, suggesting that a GFD possibly favorably affects the development of vascular complications in diabetes patients.

Similar findings have also been observed about macrovascular complications. Picarelli *et al*[33], evaluated whether the presence of celiac disease in a group of type 1 diabetes patients is associated with different expression of some hemostatic factors and with a different manifestation/progression of complications. The authors claim a potential protective role of celiac disease in the prothrombotic state of type 1 diabetes (celiac disease patients had significantly lower HbA1c, total cholesterol, triglycerides, factor Ⅷ antigen, factor Ⅶ coagulant activity, and prothrombin degradation fragments). In contrast, Pitocco *et al*[34] found that in type 1 diabetes patients with long duration of celiac disease, the carotid intima-media layer was thicker compared to diabetes patients without celiac disease.

However, if GFD seems to have a protective role in the appearance of micro- and macroangiopathic complications, the misdiagnosis of celiac disease in adult patients with type 1 diabetes is associated with a higher prevalence of retinopathy, nephropathy and peripheral neuropathy[35]. These findings raise the issue of regular celiac disease screening in order to detect type 1 diabetes patients at risk of developing celiac disease in a timely manner.

In this context, the case reported by Sildorf *et al*[36] of a 6-year-old boy who, after type 1 diagnosis, even without celiac disease, was started on a GFD, gradually suspending insulin therapy and remaining free of exogenous insulin after 20 mo seems very interesting. The GFD was reported to be safe and without side effects, and it is believed that the GFD acted to prolong the remission phase of diabetes.

TYPE 1 DIABETES, CELIAC DISEASE AND GLYCEMIC INDEX

As stated above, the most difficult factor to handle for a child/adolescent with type 1 diabetes and celiac disease is that most GFD foods have a high glycemic index. Indeed, in 2002 the American Society for Clinical Nutrition compared many foods regarding their glycemic index. What they discovered was that gluten-free foods have a higher glycemic index than gluten-containing equivalents. Since glycemic index represents a direct measure of carbohydrate absorption, it is obvious that high glycemic index foods determine a rise in rapid blood glucose values. Hyperglycemia causes an increase in free fatty acids that induce oxidative stress and promote atherosclerosis[37]. On the other hand, the subsequent rapid fall in glucose removal is associated with a sensation of hunger and excessive caloric intake[38]. Thus, a diet with low glycemic index is suggested either because of a lack of normal insulin response to high glycemic index foods in diabetes patients, or because of the aim of reducing micro and macrovascular complications[21,39]. Indeed, we have seen that GFD seems to have a protective role rather than a

deteriorating one[32,33], even in pediatric age[40]. The means by which the presence of celiac disease might prevent micro- and macrovascular complications of diabetes asks further investigations. Hypothetically, a greater dietary vigilance, an increased awareness of food intake and several consultations by a skilled dietitian might result in a better controlled carbohydrate intake and could lead to healthier eating habits. Finally, gluten free foods have a reduced content of many micronutrients: B and D vitamins, calcium, iron, magnesium and zinc. In particular, calcium content in a GFD should be appropriate, since an impairment of bone metabolism and structure has been found both in type 1 diabetes and celiac disease.

CONCLUSION

Hence, it is important to evaluate the impact of a GFD on metabolic control, growth and nutritional status in children with type 1 diabetes and celiac disease.

Since compliance with a strict GFD and a safe choice of food for diabetes is not easy, these patients require extra education and dietary intervention.

A specialized follow-up and dietary counseling are essential in the management of patients affected by both type 1 diabetes and celiac disease.

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P- Reviewer Bortell R **S- Editor** Zhai HH **L- Editor** A
E- Editor Lu YJ

World Journal of Diabetes

Online Submissions: http://www.wjgnet.com/esps/
wjd@wjgnet.com
doi:10.4239/wjd.v4.i4.135

World J Diabetes 2013 August 15; 4(4): 135-144
ISSN 1948-9358 (online)

ORIGINAL ARTICLE

Association of comorbidities with increasing severity of peripheral neuropathy in diabetes mellitus

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Received: January 02, 2013 Revised: June 04, 2013
Accepted: June 19, 2013
Published online: August 15, 2013

Abstract

AIM: To analyze a large population of patients with diabetes and peripheral neuropathy (PN) to determine other meaningful comorbid etiologies for PN.

METHODS: Peripheral Neuropathy is a common complication of type 1 and 2 diabetes mellitus; however, other potential causes for PN may be co-existing in patients with diabetes. A prospective cohort study was performed to assess patients with diabetes and PN. We compared patients having PN due solely to diabetes with patients possessing co-existing comorbidities, performing clinical (Toronto Clinical Scoring System and the Utah Early Neuropathy Scale), laboratory and electrophysiological assessments in all patients.

RESULTS: Patients with either type 1 or 2 diabetes mellitus and co-existing comorbidities did not have more severe clinical or electrophysiological PN phenotypes overall. However, in patients with type 1 diabetes, presence of a lipid disorder was associated with greater PN severity. In type 2 diabetes patients, both a lipid disorder and cobalamin deficiency were associated with greater PN severity. There was no additive effect upon PN severity with presence of three or more comorbid etiologies.

CONCLUSION: The presence of specific, and not general, comorbidities in patients with type 1 or 2 diabetes corresponds with greater PN severity.

Key words: Diabetic peripheral neuropathy; Comorbidities; Lipidemia; Cobalamin; Methylmalonic acid

Core tip: The cause of diabetic peripheral neuropathy (DPN) has remained elusive. Comorbid conditions may contribute to the severity of DPN. We studied patients with type 1 or 2 diabetes and concurrent DPN in order to identify potential comorbid conditions associated with greater neuropathic deficit. Concurrent lipidemia was associated with worse DPN in either type 1 or 2 diabetes. A concurrent vitamin B12 deficiency increased severity of DPN in type 2 diabetes. Although our results were potentially confounded by higher HbA1C values in patients with comorbid conditions, vigilance should occur for other causes of PN when diabetes is present.

Sachedina S, Toth C. Association of comorbidities with increasing severity of peripheral neuropathy in diabetes mellitus. *World J Diabetes* 2013; 4(4): 135-144 Available from: URL: http://www.wjgnet.com/1948-9358/full/v4/i4/135.htm DOI: http://dx.doi.org/10.4239/wjd.v4.i4.135

INTRODUCTION

Peripheral neuropathy (PN) is a prevalent condition in the general population[1]. While the most common cause of PN is diabetes mellitus, of both type 1 and type 2

forms, there are many other proven etiologies and forms of PN. Patients with diabetes are subject to comorbid conditions, either by association or coincidence. As such, patients with PN due to diabetes [termed diabetic peripheral neuropathy (DPN)] may manifest other conditions capable of exacerbating or initiating PN. Although different etiologies of PN possess various pathophysiologies, the presence of PN and its increasing severity greatly reduces quality of life[2]. Clinically, patients presenting with symptoms of PN in the presence of already or newly diagnosed diabetes are often subsequently concluded to have only DPN without further laboratory investigations performed. This may preclude investigations to determine other potential, and sometimes treatable, causes of PN. The aim of this study was to identify those patients with the presence of multiple conditions capable of causing PN other than diabetes to determine if multiple comorbidities increases PN severity. We hypothesized that the presence of comorbidity capable of leading to PN occurring in conjunction with either type 1 or 2 diabetes would lead to an increase in severity of PN. Further, we hypothesized that the presence of multiple comorbidities would have an additive effect upon the severity of PN.

Particular comorbidities have shown relationship to greater severity of DPN, and have included elevated triglycerides, smoking, hypertension, and obesity[3]. Hyperlipidemia[4] and statin medication use[5,6] are both exceedingly common in patients with diabetes, and may also be implicated as causative for PN. Another recent association is that of metformin use, which was associated with elevation of fasting methylmalonic acid levels and greater presence of DPN[7]; this association may relate to a resulting vitamin B12 (cobalamin) deficiency. At present, we are not aware of other potential comorbidities important in the assessment of DPN patients. Therefore, in the current study, we sought for any additional comorbidities capable of contributing to the greater impact and severity of DPN.

We designed this prospective study to examine our hypotheses and to detect any clinically meaningful synergistic effects of comorbid conditions in patient populations with diabetes mellitus. We assessed for presence of both general and specific comorbidities, including alcoholism, thyroid disease, monoclonal gammopathy of uncertain significance, autoimmune antibody presence, uremia, and cobalamin or other vitamin deficiencies with or without associated high fasting methylmalonic acid levels. We concurrently examined hypercholesterolemia and hyperlipidemia (grouped as a lipid disorder), and hypertension, all of which are potential risk factors for the development of DPN[3].

MATERIALS AND METHODS

Subject recruitment

This study was ethically approved by the University of Calgary Centre for Advancement of Health. Recruitment of subjects occurred from December 2008 until July 2010 at the Neuromuscular and Neuropathic Pain Clinics at the University of Calgary. Subjects were recruited prospectively upon initial evaluation at the tertiary care clinics. Inclusion criteria consisted of the following: (1) all subjects provided informed written consent prior to involvement; and (2) a diagnosis of pre-existing type 1 or 2 diabetes was provided based upon laboratory testing-two prior fasting glucose results of $\geqslant$ 7.1 mmol/L (126 mg/dL) [or random glucose of $\geqslant$ 11.1 mmol/L (200 mg/dL) with symptoms of hyperglycemia for type 1 diabetes] or two oral glucose tolerance tests leading to a 2 h serum glucose of $\geqslant$ 11.1 mmol/L (200 mg/dL) (based on Canadian Diabetes Association guidelines). Exclusion criteria included: (1) subjects with impaired fasting glucose or impaired glucose tolerance; and (2) absence of discernible PN or presence of questionable PN (see below). The age of diagnosis of diabetes and the duration of symptoms of PN were recorded.There was no specific sample size calculation performed and no pre-specified cohort patient number was determined for this study.

Clinical assessment of peripheral neuropathy

Clinically, each patient was examined for PN and scored using the Toronto Clinical Scoring System (TCSS)[8] and the Utah Early Neuropathy Scale (UENS)[9]. The TCSS is a validated method for evaluation of PN with higher scores correlated with greater sural nerve pathology on biopsy[8]. The TCSS has greater emphasis upon sensory deficits related to PN as compared with other comparable scales. All clinical examination was performed prior to knowledge of blood testing results. The UENS has greater applicability to determining clinical progression of PN than TCSS, and also places emphasis upon sensory abnormalities. After clinical scales were completed, subjects with TCSS $\leqslant$ 5 and UENS $\leqslant$ 6 were excluded due to uncertainty regarding the presence of PN. Subjects receiving known neurotoxic medications or chemotherapy, or with a history of carcinoma were excluded. Although laboratory testing was performed after clinical evaluation, the evaluator was not blinded to the form of diabetes mellitus or the presence of previously diagnosed comorbid conditions.

Laboratory assessment of peripheral neuropathy

Laboratory testing (Calgary Laboratory Services) was performed after clinical evaluation, and consisted tests listed in Table 1. When abnormalities were identified with blood testing, those particular abnormal tests were repeated using new blood samples for verification. Past or present alcoholism was also taken into account and diagnosed based upon Diagnostic and Statistical Manual of Mental Disorders (DSM)-Ⅳ criteria. Seated blood pressure measurements were performed twice-hypertension was based upon two measurements $\geqslant$ 130/80 using the Canadian Diabetes Association criteria or based upon pre-existing diagnosis made prior to evaluation.

Diagnosis of comorbidity was provided based upon an identified laboratory abnormality or previously identified

Table 1 Blood testing performed for identification of comorbidities

Blood test	Normative range
Red blood cell counts	
Women	4.0-5.6 × 10^{12}/L
Men	4.5-6.0 × 10^{12}/L
White blood cell counts	4.0-11.0 × 10^{9}/L
Platelet counts	150-400 × 10^{9}/L
Electrolytes (mmol/L)	
Sodium	133-145
Potassium	3.3-5.1
Chloride	98-111
Bicarbonate	21-31
Calcium	2.10-2.55
Magnesium	0.65-1.05
Urea (mmol/L)	3.0-8.5
Creatinine (μmol/L)	50-120
Aspartate transaminase (U/L)	8-40
Alanine transaminase (U/L)	1-60
Gamma glutamyl-transferase (U/L)	8-40
Albumin (g/L)	33-48
Total bilirubin (μmol/L)	0-20
Total cholesterol (mmol/L)	3.8-5.2
Low density lipoproteins (mmol/L)	2.2-3.4
High density lipoproteins (mmol/L)	> 0.9
Triglycerides (mmol/L)	0.6-2.3
Cobalamin (measured by immunoassay), (pmol/L)	> 155
Thiamine (μg/L)	33-110
Thyroid stimulating hormone (mU/L)	0.2-6.0
Total thyroxine (nmol/L)	59-154
Antinuclear autoantibody detection	⩽ 1:80
Extractable nuclear antigens	Absent
Serum protein electrophoresis	The sensitivity for detection of gammopathy was 2 g/L with serum protein electrophoresis, and samples with peaks of 2-4 g/L were subjected to immunofixation for verification
Serum copper (μmol/L)	11-24
Fasting methylmalonic acid (measured using high performance liquid chromatography), (μmol/L)	< 0.15
Hemoglobin A1C	4.3%-6.1%

Normative data was supplied by the laboratory that performed all related testing-Calgary Laboratory Services in Calgary, Alberta, Canada. Normative data is presented as a range based upon 95%CI.

comorbidity diagnosed prior to this evaluation. Previously diagnosed comorbidities were verified using electronic or paper patient chart information. All abnormalities were determined using the population normal values as determined by Calgary Laboratory Services.

Once the presence of underlying comorbidities was determined, subjects were categorized into four groups: patients solely with type 1 diabetes (DM1 only); patients with type 1 diabetes and an existing comorbidity or comorbidities (DM1 plus comorbidity); patients solely with type 2 diabetes (DM2 only); and patients with type 2 diabetes and an existing comorbidity or comorbidities (DM2 plus comorbidity). Sub-categorization based upon individual comorbidities and number of comorbidities was performed subsequently. The following comorbidities were used for categorization: lipid disorder (elevated low density lipoprotein or triglycerides or both), cobalamin deficiency (depressed cobalamin level or elevated fasting MMA level or both), monoclonal gammopathy of uncertain significance (MGUS), thyroid disorder, renal dysfunction (elevated creatinine), autoimmune disorder [prior diagnosis or detection of positive extractable nuclear antigen (ENA) status of significance], alcoholism, or hypertension. We defined an autoimmune disorder as the presence of an inappropriate immune response with detectable auto-antibodies having potential for leading to a neurological disease including rheumatoid arthritis, sjogren syndrome, systemic lupus erythematosus, or systemic vasculitis. Other potential comorbidities were recorded for consideration of potential impact.

Electrophysiological assessment of peripheral neuropathy

All patients received electrophysiological evaluation for PN severity after clinical evaluation and prior to receipt of laboratory testing results. Cadwell Sierra Wave (Cadwell Laboratories, Kennewick, WA) electromyography machines were used. Both motor and sensory testing was performed on the non-dominant upper and lower limb; in the case of ambidextrous patients, the left upper and lower limbs were studied. Motor nerve conduction studies (NCS) were performed using stimulation of the median nerve (wrist, elbow), ulnar nerve (wrist, below elbow, above elbow), peroneal (ankle, below fibular head and above fibular head locations) and tibial (ankle, popliteal fossa locations) nerves. For each motor nerve, distal motor latencies, compound motor action potentials, and conduction velocities were obtained or calculated. F wave latencies were obtained from median, ulnar, peroneal and tibial nerves. Sensory antidromic NCS were performed using the median (digits 2 and 4), ulnar (digits 4 and 5), superficial radial, superficial peroneal and sural nerves, with sensory nerve action potentials (SNAP), onset latency, and conduction velocity obtained or calculated. Temperatures were maintained at ⩾ 32 ℃ for the upper extremities, and ⩾ 30 ℃ for the lower extremities during NCS testing. Absent electrophysiological responses were used to calculate amplitude values, but latency and velocity values were not entered in the analysis for absent responses in order to not obscure data analysis. Patients were excluded if they refused electrophysiological testing or laboratory testing.

Statistical analysis

Our primary objective was to determine the impact of presence of any comorbidities associated with development of PN upon the severity of PN in patients with either type 1 or 2 diabetes. Secondarily, we also analyzed specific individual comorbidities, and presence of multiple comorbidities for determination of impact upon PN severity. Analysis was performed for the subject categorizations described above. Group equivalence for age, duration of diabetes, duration of PN symptoms, A1C,

and alcohol exposure were compared by independent samples *t*-test; gender was compared by chi-square testing. In all cases, type 1 and 2 diabetes were considered separately and the two forms of diabetes were not directly compared. Other elements of the past medical history not specified above were not statistically compared due to their heterogeneity. Our primary outcome measures were clinical neuropathy severity (TCSS, UENS) and electrophysiological markers of neuropathy; for the latter we chose to test sensory NCS of the lower extremity (conduction velocity and SNAP for superficial peroneal and sural nerves) as we hypothesized these would most likely to demonstrate exacerbation due to progression of PN. Secondary outcome variables included the other sensorimotor electrophysiological parameters for motor responses of the lower limbs and sensorimotor studies of the upper limbs. We determined that these data did not follow a normal distribution (performed with Shapiro-Wilk testing) so comparisons were made using Mann-Whitney *U* test. Bivariate correlations of primary outcomes and numbers of comorbidities were calculated using Spearman rho test. In addition, we performed a post-hoc linear regression analysis for determination of any potential associations with worsening diabetic status (using HbA1C). We used HbA1C scores as the dependent variable, while explaining variables were chosen to be fasting Methylmalonic acid (MMA) levels, triglycerides, total cholesterol levels, low density cholesterol, and high density cholesterol. Furthermore, a post-hoc linear regression analysis was performed for the type 2 diabetes patient cohort to determine any potential association between cobalamin and fasting MMA levels with greater severity of PN-for this, we used TCSS and UENS scores as the dependent variables, while explaining variables were chosen to be fasting MMA levels. Lastly, a linear regression analysis was performed using TCSS amd UENS total scores as the dependent variable and age, duration of diabetes, A1C and presence of comorbidities and number of comorbities as explaining variables. We set α to be 0.05, and we utilized Bonferroni corrections for analysis of secondary outcome measures, applied whenever multiple comparisons for the same cohorts were performed. Values are presented as mean ± SE throughout.

RESULTS

Subject demographics

Demographics and individual comorbidities for each cohort are presented in Table 2. We prospectively enrolled a total of 369 patients. A total of 32 patients (3 type 1 diabetes, 29 type 2 diabetes) declined participation based upon personal choice. DM1 only and DM1 plus comorbidity cohorts were similar with respect to age, gender, duration of diabetes, and HbA1C. However, DM2 plus comorbidity cohorts had longer durations of diabetes and higher HbA1C levels as compared to the DM2 only cohort. We excluded a total of 10 patients for unwillingness to perform testing. Another 17 patients were excluded due to presence of impaired fasting glucose or impaired glucose tolerance rather than strict diabetes.

Type 1 diabetes and comorbidities

The presence of an identified comorbidity (Table 2) in patients with type 1 diabetes did not increase the TCSS (P = NS, F = 3.1) or UENS (P = NS, F = 1.4) scores (Figure 1). In addition, primary electrophysiological outcomes for sensory electrophysiological testing of the lower limbs were also not different between DM1 only and DM1 plus comorbidity cohorts (P = NS, F = 0.00-1.2).

For secondary outcome measures, after Bonferroni corrections were applied. Analysis showed DM1 plus comorbidity subjects had increased onset latency for the sensory conduction study at the ulnar nerve at digits 4 and 5 (3.3 ± 0.1 ms *vs* 3.6 ± 0.1 ms, $P < 0.001$, F = 8.9 and 3.2 ± 0.1 ms *vs* 3.6 ± 0.1 ms, $P < 0.001$, F = 10.6 respectively).

For individual comorbidities, type 1 diabetes patients[6,10] with presence of triglyceridemia or lipid disorder had greater TCSS (ANOVA, $P < 0.007$, F = 8.4) and UENS (ANOVA, $P < 0.007$, F = 13.7) scores (Figure 1) than type 1 diabetes patients without comorbidities. Other individual comorbidities did not impact upon severity of PN. Finally, the presence of multiple (≥ 3) comorbidities in type 1 diabetes patients did not have a compounding effect for the severity of PN (P = NS, F = 1.2).

Type 2 diabetes and comorbidities

The presence of an acknowledged comorbidity in patients with Type 2 diabetes did not increase the TCSS (P = NS, F = 2.3) or UENS (P = NS, F = 2.2) scores (Figure 2). Similarly, there were no electrophysiological differences between DM2 only and DM2 plus comorbidity for sural and superficial peroneal parameters (P = NS, F = 0.0-0.9).

For secondary outcome measures, there were significant differences for other electrophysiological parameters. DM2 plus comorbidity subjects did have prolonged onset latency for the median motor studies (5.4 ± 0.1 *vs* 4.2 ± 0.1, $P < 0.001$, F = 4.6), peroneal motor studies (5.5 ± 0.1 *vs* 5.0 ± 0.1, $P < 0.001$, F = 7.6), and tibial motor studies (5.5 ± 0.2 *vs* 4.9 ± 0.1, $P < 0.001$, F = 6.2) when compared to the DM2 only cohort. In addition, there was greater slowing of conduction velocities for the median nerve across the forearm (44.3 ± 0.7 *vs* 50.0 ± 0.5, $P < 0.001$, F = 11.5), for the ulnar nerve across the forearm (49.3 ± 1.3 *vs* 54.2 ± 0.8, $P < 0.001$, F = 7.7), and for the peroneal nerve across the lower leg (35.3 ± 1.1 *vs* 39.3 ± 0.6, $P < 0.001$, F = 5.5) as compared to the DM2 only cohort.

Examination of individual comorbidities again identified the presence of a lipid disorder to contribute to greater PN severity based upon TCSS (ANOVA, $P < 0.007$, F = 5.7) and UENS (ANOVA, $P < 0.007$, F = 2.5) scores (Figure 2) in type 2 diabetes subjects. In addition, the presence of cobalamin deficiency or elevated fasting MMA levels were associated with higher TCSS (ANOVA, $P < 0.007$,

Table 2 Demographics for cohorts with diabetes mellitus *n* (%)

	Type 1 diabetes only	Type 1 diabetes plus comorbidity	Type 2 diabetes only	Type 2 diabetes plus comorbidity
Patients	31	19	228	91
Duration of disease (mo)	316 ± 26	310 ± 25	107 ± 7	150 ± 12[a]
HbA1c level (%)	11.2 ± 1.4	11.7 ± 1.6	9.8 ± 0.2	11.2 ± 0.3[a]
Age (yr)	50 ± 7	54 ± 8	62.0 ± 1.8	61.0 ± 1.4
Sex (male)	17 (55)	12 (63)	91 (40)	36 (40)
	Nature of comorbidities	15 (79)		63 (69)
	lipid disorder			
	Low cobalamin/elevated MMA	9 (47)		36 (40)
	Monoclonal gammopathy	2 (11)		4 (4)
	Thyroid disease	5 (26)		11 (12)
	Uremia	7 (37)		13 (14)
	Autoimmune diseases	4 (21)		8 (9)
	Alcoholism	3 (16)		10 (11)
	Hypertension	13 (68)		74 (81)
Medications				
Insulin	31 (100)	19 (100)	55 (23)	24 (26)
Metformin			198 (86)	80 (88)
Glyburide			155 (68)	63 (69)
Gliclazide			41 (18)	11 (12)
Statins/Ezetemide	21 (68)	9 (47)	146 (64)	48 (53)
Blood pressure medications	22 (71)	14 (74)	118 (52)	57 (62)
Thyroid replacement	12 (39)	5 (26)	38 (17)	14 (15)
SSRIs	4 (13)	2 (11)	28 (12)	16 (18)
Anxiolytics/Sedatives	8 (26)	3 (16)	48 (21)	13 (14)
NSAIDs	12 (39)	9 (47)	104 (46)	55 (60)
Acetaminophen	6 (19)	3 (16)	37 (16)	17 (19)
Gabapentin	5 (16)	4 (21)	41 (18)	15 (16)
Pregabalin	4 (13)	3 (16)	36 (16)	11 (12)
Codeine	3 (10)	4 (21)	31 (13)	8 (9)
Amitriptyline	3 (10)	3 (16)	25 (11)	6 (7)
Oxycocet	1 (3)	2 (11)	18 (8)	4 (4)
Nortriptyline	1 (3)	1 (5)	12 (5)	4 (4)
Duloxetine	2 (6)	3 (16)	22 (10)	5 (5)
Venlafaxine	3 (10)	2 (11)	25 (11)	4 (4)
Fentanyl	1 (3)	2 (11)	6 (3)	3 (3)
Tramadol	1 (3)	0 (0)	8 (3)	2 (2)
Morphine	0 (0)	2 (11)	7 (3)	3 (3)

[a]$P < 0.05$ *vs* type 2 diabetes only and type 2 diabetes plus comorbidity cohort. MMA: Methylmalonic acid; SSRIs: Selective serotonin reuptake inhibitors; NSAIDs: Nonsteroidal antiinflammatory drugs.

$F = 3.9$) and UENS (ANOVA, $P < 0.007$, F = 3.3) scores in type 2 diabetes subjects as compared to DM2 only subjects. Finally, the presence of alcoholism was associated with a higher TCSS (ANOVA, $P < 0.007$, $F = 2.1$) but not UENS (ANOVA, P = NS, $F = 1.6$) score in type 2 diabetes cohorts relative to DM2 only subjects.

As with type 1 diabetes, the presence of multiple comorbidities in type 2 diabetes patients did not have an additive effect upon severity of PN.

Multiple comorbidities

We used linear regression to determine the impact of multiple comorbidities upon severity of PN. In type 1 diabetes, there was no significant linear relationship between multiple comorbidities and severity of PN using TCSS ($R^2 = 0.21$) or UENS ($R^2 = 0.33$) scores. Likewise, no additive effect upon PN severity could be shown for type 2 diabetes subjects for multiple comorbidities using TCSS ($R^2 = 0.04$) or UENS ($R^2 = 0.02$) scores.

Potential associations

We examined for association of potentially important comorbid factors with severity of diabetes mellitus using HbA1C levels. There were no significant associations between HbA1C for either of type 1 or 2 diabetes with any of fasting MMA, triglycerides, total cholesterol, low density cholesterol, or high density cholesterol levels ($R^2 = 0.08$-0.26). However, it should be noted that statin medication use was very common in both type 1 and type 2 diabetic cohorts.

In type 2 diabetes patients, there was a significant association between TCSS and UENS scores with fasting MMA levels ($R^2 = 0.48$ and $R^2 = 0.52$ respectively, $P < 0.025$), and a less robust, but still significant association with cobalamin levels ($R^2 = 0.42$ and $R^2 = 0.44$ respectively, $P < 0.025$). Greater elevation of MMA levels and greater depression of cobalamin levels were associated with greater severity of PN.

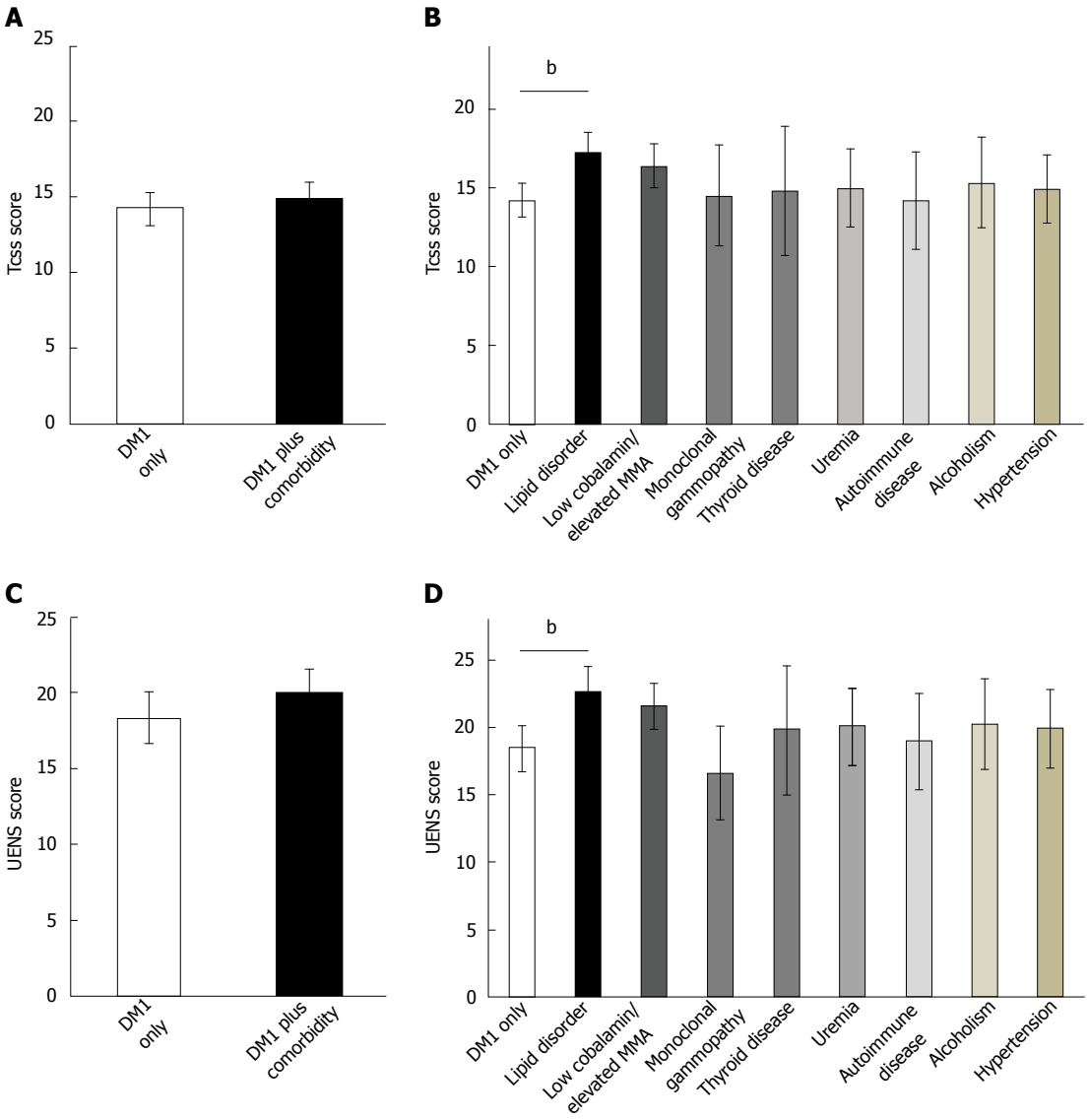

Figure 1 In subjects with type 1 diabetes. A, B: The level of peripheral neuropathy (PN) severity was measured using the Toronto Clinical Scoring System (TCSS); C, D: The Utah Early Neuropathy Scale (UENS) for patients without (DM1 only) and with (DM1 Plus Comorbidity) comorbidities important for the assessment of PN. There was no significant difference between cohorts for either TCSS (A) or UENS (C) values. However, when examined by specific comorbidity, patients with type 1 diabetes having a concomitant lipid disorder had greater TCSS (B) and UENS (D) values. Other specific comorbidities were not associated with greater severity of PN when compared to DM1 only subjects. The presence of a significant difference between the subcohort with lipid disorder and DM1 only cohort is indicated with ($^{b}P <$ 0.007 *vs* lipid disorder, ANOVA).

DISCUSSION

Although the presence of a coexisting comorbidity did not increase the severity of PN overall in patient cohorts with type 1 or 2 diabetes, particular comorbidities were associated with a more severe phenotype of PN. The presence of a lipid disorder in either type 1 or 2 diabetes was associated with greater neuropathy severity. The presence of cobalamin deficiency and/or elevated fasting MMA levels was correlated with greater presence of neuropathy in type 2 diabetes subjects. While these associations are not necessarily causative, they suggest that greater attention should be afforded to potentially correctable comorbid lipid disorders and for cobalamin deficiencies and/or elevated fasting MMA levels in patients with diabetes. In our patient populations, we have initiated management of cobalamin deficiencies and elevated fasting MMA levels with continuous monthly intramuscular cobalamin therapy; patients with lipid disorders have simultaneously started on appropriate management. Follow-up data for these interventions is not yet available. The worsening of PN was detected by clinical scoring and not electrophysiological measures for peripheral nerves in the lower extremities, suggesting that worsening of PN may relate to additional small fibre dysfunction, or dorsal column dysfunction in case of cobalamin deficiency, that is undetectable with nerve conduction studies. Another interesting and unexpected finding was the presence of electrophysiological prolongation of latencies and slowing of conduction velocities in DM2 plus comorbidity subjects.

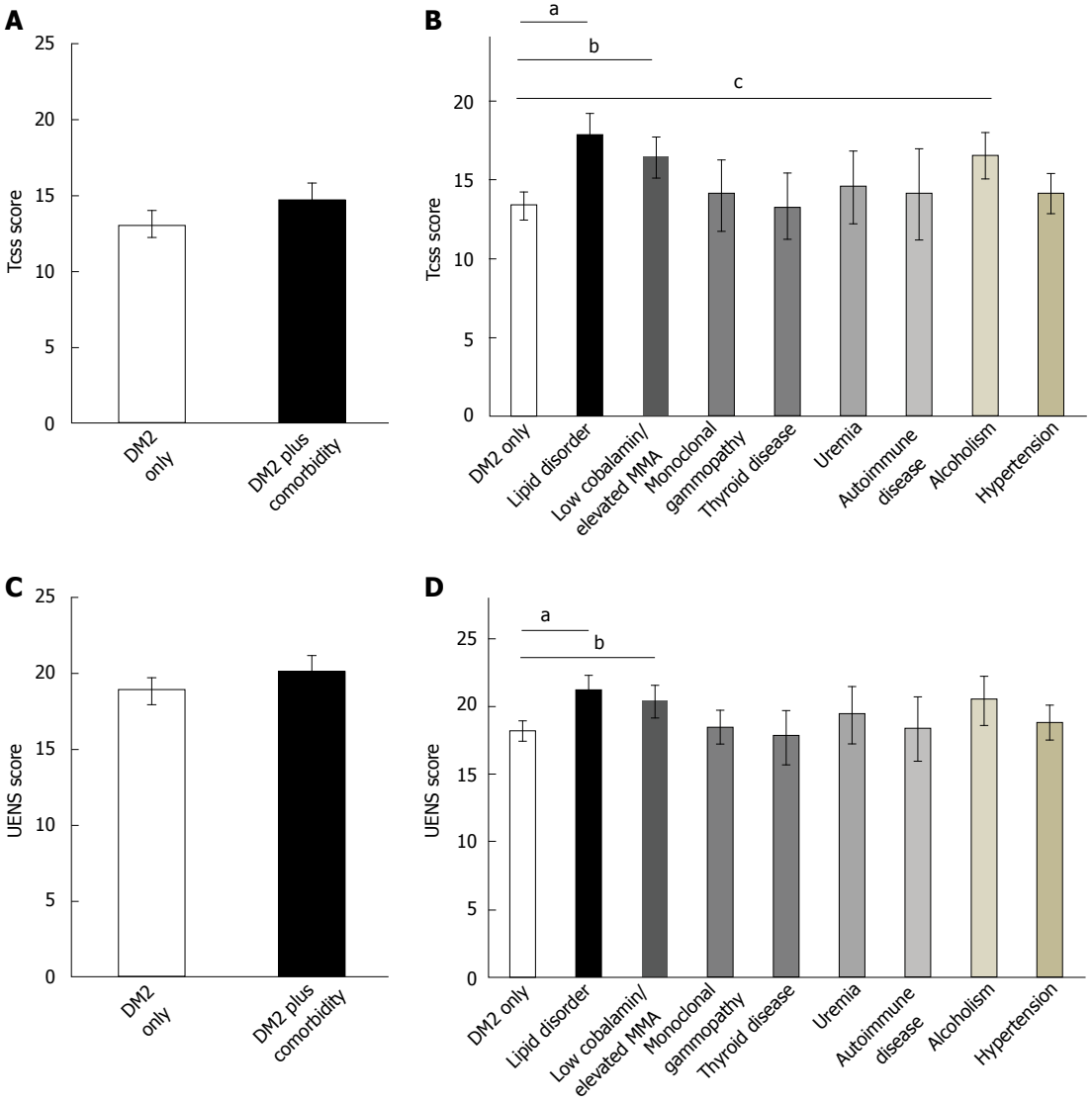

Figure 2 In type 2 diabetes subjects. A, B: Peripheral neuropathy (PN) severity was assessed using the Toronto Clinical Scoring System (TCSS); C, D: The Utah Early Neuropathy Scale (UENS) for patients without (DM2 Only) and with (DM2 Plus Comorbidity) comorbidities capable of contributing to PN. As with type 1 diabetes, there was no significant difference between cohorts for either TCSS (A) or UENS (C) values. When subcategorized by specific comorbidity, however, patients with type 2 diabetes having a concomitant lipid disorder, cobalamin deficiency or elevated fasting methylmalonic acid level, or alcoholism had greater TCSS (B) scores when compared to DM2 only subjects. Using UENS values (D), presence of a lipid disorder or cobalamin deficiency/elevated fasting methylmalonic acid level was associated with greater PN severity. Other examined comorbidities were not associated with greater severity of PN when compared to DM2 only subjects. The presence of significant differences between the subcohorts with comorbidities and DM2 only cohort are indicated with($^{a}P < 0.007$ *vs* lipid disorder, ANOVA), ($^{b}P < 0.007$ *vs* low cobalamin or elevated fasting methylmalonic acid level, ANOVA), and ($^{c}P < 0.007$ *vs* alcoholism, ANOVA).

Progression and severity of DPN has been reported to depend upon a number of factors including elevated triglycerides, smoking, hypertension, and obesity[3]. Hyperlipidemia may contribute to oxidative stress at the dorsal root ganglia, contributing to greater diabetes-induced neurodegeneration[11]. This may also relate to the presence of oxidized low density lipoprotein and its receptor, the lectin-like oxLDL (LOX-1) receptor[12]. Possibly toxic to dorsal root ganglia on its own, oxLDL presence, not quantified in this study, is known to be elevated in patients with diabetes and may contribute to other diabetic complications, including retinopathy[13]. Previously, there has been speculation about hyperlipidemia leading to peripheral neuropathy irrespective to diabetic status[4], but its occurrence in idiopathic peripheral neuropathy does not appear to be different from that of control subjects[14]. However, hypertriglyceridemia is more common in patients with PN due to diabetes, impaired glucose tolerance, or alcoholism[14] so its co-existence is not unexpected. As effective treatments do exist for the management of lipid disorders, future research should assess the potential for intervention in patient populations with DPN. The role of co-existing treatments of lipid modulating drugs, such as statins[6], in the presence of concurrent diabetes requires further investigation as well. Although controversially implicated in peripheral neuropathy[5,6], the role of statins is unclear but they may have played a confounding role in the present study. Lastly, it is possible that patients with concurrent lipid disorders have less rigorous care of their diabetes-patients with DM2 plus comorbidities had a

greater duration of diabetes as well as a higher HbA1C value.

Recently, the presence of elevated MMA levels in patients with DPN has been related to metformin use contributing to greater presence of DPN[7]. Moreover, while higher MMA levels are generally related to vitamin B12 (cobalamin) deficiency, this may result from renal dysfunction or elderly age as well[15]. Although cobalamin deficiency is most classically associated with subacute combined degeneration, an exclusive peripheral neuropathy (PN) presentation occurs, typically manifesting as an axonal polyneuropathy with additional small fiber dysfunction[16-18]. As accumulating evidence suggests that the cobalamin-deficiency-associated metabolite MMA is more sensitive and specific than serum cobalamin itself[19], its use for detection of potential cobalamin deficiency has been recommended as an investigations with high diagnostic yield in patients with distal symmetric polyneuropathy[20]. This concurrent deficiency was found in patients with type 2 diabetes, many of whom were taking metformin, with more severe neuropathy phenotypes. In this work, higher levels of fasting MMA or lower levels of cobalamin were also associated with greater severity of PN, as we showed previously in a separate cohort of type 2 diabetes patients[7]. However, our findings support the additional and often overlooked assessment for the concurrent presence of cobalamin deficiency, a potentially treatable contribution, in patients with DPN and type 2 diabetes.

Alcoholic polyneuropathy is another form or PN which can be associated with concomitant neuropathic pain. This may be due to the direct toxic effects of ethanol or its metabolites upon peripheral nerve fibers[21] or may be related to a subsequent thiamine deficiency[22]. Its potential for worsening existing DPN was possibly a factor in the type 2 diabetes population (using the TCSS but not the UENS scale). The presence of alcoholism in type 1 diabetes influences the presence of PN with a U-shaped associative curve[23]. This may be true in the type 2 diabetic patient also[24]. There were a number of other comorbidities that were not associated with greater severity of PN in type 1 or type 2 diabetes patients. Peripheral neuropathy associated with MGUS[25] was rarely coincidental in DPN patients. Thyroid disorders (both hyperthyroidism[26] and hypothyroidism)[27], frequently treated early after discovery, were not associated with further worsening of PN. Somewhat surprisingly, renal dysfunction, a potent cause of peripheral neuropathy[28] with potential relationship to diabetes[29] was not additive for DPN severity. Autoimmune disorders may have been too uncommon to contribute significantly to exacerbation of PN severity. Although speculated to impact upon DPN[3,30], hypertension was not a significant contributor to DPN severity in our cohorts of type 1 or 2 diabetes patients. We did not examine other potential factors implicated in progression of DPN, such as smoking or body mass index due to incomplete data acquisition during assessments. It is possible that some of the above comorbidities were not associated with greater severities of PN due to insufficient sample sizes.

We present these findings with limitations. Although we identified patients prospectively, they were not randomly selected from a population with type 1 or 2 diabetes with or without DPN; instead, these patients were referred for tertiary care. Our sample size was not based upon a pre-determined power analysis. We did not identify a separate cohort of patients with asymptomatic DPN. Higher HbA1C levels and longer durations of diabetes in patients with type 2 diabetes and comorbidities would certainly be anticipated to contribute to greater PN severity and may have impacted upon presented findings. Investigators were blinded to the laboratory results until clinical and electrophysiological studies were completed, but were not blinded to presence of type of diabetes or presence of comorbidities previously diagnosed. We did not use an established comorbidity burden tool to assess the studied comorbidities studied. All of our results were based upon TCSS and UENS scores-these are clinically relevant scales easily performed at the bedside, but have subjective components, and may not have the sensitivity of epidermal nerve fiber densities with skin biopsy[31] or confocal corneal microscopy[32]. Patients were on numerous medications for diabetes and other conditions; the heterogeneity of these medications and the conditions they were intended to treat made their individual assessment for contribution impossible. As a result, we did not exclude patients based upon any medication used, and acknowledge that this may have impacted upon clinical and electrophysiological assessments. Finally, this study was conducted at a single centre by a single assessor which could introduce biases due to referral patterns and assessment protocols used.

This study identified potential contributing comorbidities in approximately 30% of patients with diabetes and PN. Although we identified two potentially treatable specific comorbidities (cobalamin deficiency and lipid disorder), we do not yet know if management of these conditions will slow progression of PN, as may occur with procedures such as pancreatic islet transplantation[33] for treatment of type 1 diabetes. However, the use of simple, routine laboratory testing by primary physicians can identify factors for potential intervention in the future for DPN patients which may prevent clinical progression of PN. Lastly, the identification of these comorbidities, should not be viewed as a replacement for symptomatic relief, but as a potentially identifiable and modifiable component of an already diagnosed PN in patients with diabetes.

COMMENTS

Background

The pathophysiology of diabetic peripheral neuropathy remains uncertain and complex. Studies of comorbid conditions capable of causing peripheral neuropathy may assist in determination of causation of diabetic peripheral neuropathy and assist the clinician in managing patients with multiple conditions capable of causing peripheral neuropathy.

Research frontiers

The presence of multiple potentially causative conditions is not uncommon in patients with diabetic peripheral neuropathy. Those comorbid conditions capable of worsening diabetic peripheral neuropathy may be subject to intervention, slowing the progression of peripheral neuropathy in patients with diabetes mellitus. Future studies should address the management of lipid disorders and vitamin B12 deficiency in populations with diabetic peripheral neuropathy.

Innovations and breakthroughs

Although its isolated relationship to peripheral neuropathy is controversial, a lipid disorder was associated with greater severity of peripheral neuropathy in our patient populations with type 1 or 2 diabetes mellitus. However, lipidemia may have an additive effect when present with hyperglycemia present in diabetes mellitus. Also, prior studies have shown that metformin therapy is associated with impairment of vitamin B12 levels, a condition also associated with peripheral neuropathy, its management in patients with type 2 diabetes may also slow progression of diabetic peripheral neuropathy.

Applications

Greater vigilance for other comorbidities in patients with diabetic peripheral neuropathy may reveal potentially manageable conditions that may be contributing to worsening of peripheral neuropathy over time.

Peer review

The authors examined the severity of peripheral neuropathy in patients with diabetes mellitus with and without comorbid conditions capable of causing peripheral neuropathy. In particular, two conditions (lipid disorder and vitamin B12 deficiency) were detected and associated with greater neuropathic deficit. The results suggest that greater vigilance for these conditions may help patients with diabetic peripheral neuropathy by slowing the process of peripheral neurodegeneration.

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P- Reviewers Neumiller JJ, Uehara Y **S- Editor** Huang XZ
L- Editor A **E- Editor** Lu YJ

World Journal of Diabetes

Online Submissions: http://www.wjgnet.com/esps/
wjd@wjgnet.com
doi:10.4239/wjd.v4.i4.145

World J Diabetes 2013 August 15; 4(4): 145-150
ISSN 1948-9358 (online)

BRIEF ARTICLE

Diabetes-related impairment in bone strength is established early in the life course

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Supported by R00DK083333 (KC); T32DK007545 (LJH); P60DK079626 UAB Diabetes Research Center Pilot/Feasibility Grant
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Received: March 6, 2013 Revised: April 24, 2013
Accepted: June 18, 2013
Published online: August 15, 2013

Abstract

AIM: To evaluate properties of bone quantity/quality using young non-obese Type 1 (T1D)-diabetic (NOD) prone and syngenic non-diabetic (NOD.*scid*) mice.

METHODS: Quantitative bone assessment of tibia was conducted using dual-energy X-ray absorptiometry (DXA) for the evaluation of body mass, bone mineral content, body fat mass and lean mass. Qualitative assessment was accomplished by three-point breakage for assessment of force to failure and micro-computed tomography for evaluation of trabecular and cortical properties of bone. In addition, fasting blood was evaluated prior to sacrifice at week eleven and fifteen to evaluate and compare glucose homeostasis between the strains of mice.

RESULTS: Our findings support a perturbation in the relationship between bone quantity, quality, and subsequently, the association between structure and strength. There were no differences in DXA-assessed body composition (body fat, % fat mass and lean mass) and bone composition (bone mineral content and bone mineral density) between strains. However, relative to NOD.*scid*, NOD mice had lower trabecular bone volume, relative trabecular bone volume, trabecular number and trabecular total material density ($P < 0.05$). Conversely, NOD mice had greater cortical total mean volume ($P < 0.05$). General linear models analysis adjusted for body weight revealed a significant contribution of T1D to bone health as early as 5 wk.

CONCLUSION: It is well-established that diabetes is a significant risk factor for increased fractures, although the underlying mechanisms are not fully understood. Investigation of bone parameters encompassing strength and structure early in the life course will facilitate the elucidation of the pathogenesis of impaired bone integrity.

Key words: Diabetes; Bone strength; Skeletal integrity; Development and growth; Non-obese diabetic

Core tip: Diabetes-related impairment in bone microarchitectural properties and parameters of quality was apparent as early as 5 wk.

Casazza K, Hanks LJ, Clines GA, Tse HM, Eberhardt AW. Diabetes-related impairment in bone strength is established early in the life course. *World J Diabetes* 2013; 4(4): 145-150 Available from: URL: http://www.wjgnet.com/1948-9358/full/v4/i4/145.

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INTRODUCTION

Apart from the classical complications of diabetes, adverse effects associated with bone health are becoming increasingly apparent. Individuals with diabetes have higher incidence of fracture and greater prevalence of osteoporosis. Many[1,2], but not all[3-7] investigations report low bone mineral content (BMC) and density (BMD). Fracture healing is also compromised in diabetics with as high as 87% recovery delay relative to "healthy" counterparts[8-10]. While the skeletal manifestations of dysregulated glucose metabolism have been primarily considered in terms of bone quantity (*i.e.,* low bone mass), bone strength, the most obvious characteristic of bone structure/health is dependent upon various qualitative aspects.

A variety of animal models have been developed and used to examine the mechanisms of diabetes-related complications. Autoimmune-prone non-obese diabetic (NOD) mice are a widely studied model of spontaneous type 1 diabetes (T1D)[11-13]. In contrast to the pharmacologic streptozotocin (STZ)-induced T1D model, NOD mice become spontaneously diabetic secondary to a progressive diminished capacity of insulin-producing pancreatic beta islet cell function due to autoimmune destruction of the islet beta-cells. The earliest signs of autoimmune pathology in the NOD mouse occur at approximately 4 to 5 wk of age with leukocytes beginning to accumulate around the pancreatic islets, progressively intensifying and eventually leading to destruction of the insulin-producing beta cells at about 12 wk of age[14]. Whereas some studies have investigated bone phenotypes in adult NOD mice, the skeletal effects at disease initiation, to our knowledge have not been investigated. As a comparator strain, syngenic autoimmune-deficient NOD.*scid* mice lack functional lymphocytes, precluding the autoimmune destruction of beta cells and unlike NOD mice, do not develop T1D[11,13-16].

While insulin stimulates not only osteoblastic cell differentiation, but also osteoblastogenesis and thus plays a pivotal role in bone metabolism[17,18], an impairment in insulin regulation compromises bone processes giving rise to an altered phenotype. Accordingly, disease states related to insulin homeostasis/glucose handling might be expected to elicit profound physiologic alterations, particularly during growth[19]. The objective of the study was to evaluate properties of bone quantity (*via* DXA) and quality (*via* microCT, three point breakage) using young NOD *vs* NOD.*scid* mice, two different models in terms of insulin response to glucose. We hypothesized that as diabetes progressed in the NOD mice, the strength-structure impairments would manifest as illustrated by decreased force required to break, as well as trabecular and cortical bone measures.

MATERIALS AND METHODS

Mice

Five-to-seven week-old female NOD (n = 24) and NOD.*scid* mice (n = 23) were bred and housed at the Research Support Building of the University of Alabama at Birmingham, under pathogen-free conditions and observing IACUC approved mouse protocols. Mice were kept under a normal diurnal cycle in a temperature-controlled room and were fed with standard chow (NIH316 formula, Purina item #5K52) from age of weaning.

Dual-energy X-ray absorptiometry

For assessment of bone quantity by dual-energy X-ray absorptiometry (DXA) analysis, animals were briefly anesthetized with isoflurane (2%) and placed in a prostrate position on the imaging plate. BMD, bone mineral content (BMC) was assessed *in vivo* (GE-Lunar PIXImus, software version 1.45, GE-Lunar). Additionally, lean mass and fat mass as well as animal area was obtained at 5, 8, 11 and 15 wk.

Three-point breakage analysis and micro-computed tomography

For qualitative assessment, three point breakage and micro-computed tomography was used. Tibia strength was assessed by three-point breakage analysis using an MTS 858 MiniBionix (MTS Systems, Eden Prairie, MN) with a 100 N load cell. The span was 9 mm and the bones were loaded at a rate of 0.1 mm/s. For the determination of the 3D architecture of the trabecular and cortical bone, mouse femurs were scanned using the Scanco μCT40 desktop cone-beam micro-CT scanner (Scanco Medical AG, Brüttisellen, Switzerland). Femurs were placed vertically, but inverted (distal femur at the top) in 12 μm diameter scanning holders and scanned twice: one for cortical and one for trabecular bone. Scans were performed at the following settings: 12 mm resolution, 55 kvp, 145 μA with an integration time of 200 ms. Scans were automatically reconstructed into 2-D slices, and the region of interest was outlined in each slice using the micro-computed tomography (μCT) Evaluation Program (v5.0A, Scanco Medical). Cortical bone was determined at the mid-shaft of the femur with a scan of 25 slices. The region of interest (ROI) tool was used to outline the outside edge of the cortical bone. Cortical bone was identified and separated from the marrow by using a threshold value of 294. A 3D reconstruction was performed on the ROI consisting of everything within the outer cortical surface. Data was obtained for bone volume, (BV), bone density, total volume (TV) (bone plus marrow), bone volume fraction (BV/TV), trabecular thickness (Tb. Tk.), number (Tb. N) and cortical thickness. The scan of the trabecular bone was performed at the distal femur below the growth plate (on the inverted bone). Each scan consisted of 209 slices of which 100 were used for the analysis. ROI's were drawn on each of the 100 slices just inside

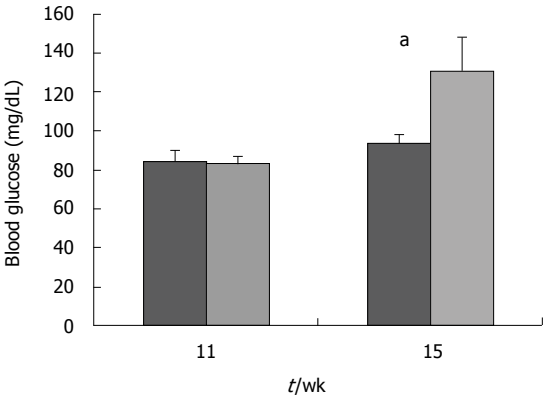

Figure 1 Comparison of blood glucose (mg/dL) in non-obese diabetic (dark gray bars) and syngenic non-diabetic (light gray bars) mice at 11 and 15 wk demonstrates initiation of the type 1 diabetic phenotype. Error bars represent SEM. At five, eight (data not shown) and eleven there was no difference in blood glucose levels between strains of mice. [a]However at 15 wk, non-obese diabetic mice had significantly greater blood glucose than NOD.*scid* mice and concentration was indicative of diabetes (< 120 mg/dL).

the cortical bone, to include only the trabecular bone and marrow. Trabecular bone threshold was set at 226 HU, to distinguish it from the marrow. The 3D reconstruction was performed on the ROI which only contained trabecular bone; no cortical bone was present in these ROI's.

Statistical analysis

Analysis of variance (ANOVA) was used to determine differences in bone properties between strains. If an aging effect was apparent for a given parameter, a post hoc comparison was performed. Statistical significance was set at $\alpha < 0.05$. In order to determine whether the strength-structure relationship was different, general linear models in which group, weight and body area were the covariates were conducted. All analyses were conducted using SAS (Institute Inc., Cary NC).

RESULTS

Serum glucose was collected from 11 and 15 wk old NOD and NOD.*scid* mice. As expected, NOD mice displayed increased glucose beginning at 15 wk (Figure 1) and is consistent with the phenotype of this autoimmune T1D mouse model[11-13]. The growth characteristics were then determined between NOD and NOD.*scid* mice. At 5 wk, NOD mice were smaller than NOD.*scid* as represented by significantly less total mass, lean mass, body weight. However, by 8 wk size and weight did not differ and the similarity in body dimensions. At 15 wk NOD mice were significantly heavier (Figure 2).

We next analyzed the bone mechanical properties of tibiae harvested from 5 and 8 wk old NOD and NOD.*scid* mice. In a three-point bending test, NOD mice demonstrated less mechanical strength than NOD.*scid* mice at 5 wk but not at 8 wk of age (Figure 3).

Body composition and bone parameters of 5 and 8 wk old NOD and NOD.*scid* mice were next examined (Table 1). The weight of the NOD and NOD.*scid* mice were similar (Table 1). There were no differences in body composition (body fat, fat mass and lean mass) and bone composition (bone mineral content and bone mineral density) were detected properties in NOD compared to NOD.*scid* mice (Table 1).

Table 1 Pooled data from all mice ages 5 to 15 wk

Parameter	mean ± SEM NOD	NOD.*scid*
Body composition properties (DXA)		
Body weight	19.7 ± 0.6	19.9 ± 0.4
BMC	0.37 ± 0.40	0.34 ± 0.30
BMD	0.04 ± 0.04	0.040 ± 0.001
Body fat	2.8 ± 0.1	2.9 ± 0.2
Lean mass	14.7 ± 0.4	14.8 ± 0.2
% Fat	15.8 ± 0.7	16.2 ± 0.7
Trabecular bone properties (microCT)		
TV	1.5 ± 0.1	1.5 ± 0.1
BV	0.10 ± 0.01	0.15 ± 0.01[1]
BV/TV	0.06 ± 0.01	0.10 ± 0.01[1]
Tb. Th	0.050 ± 0.002	0.050 ± 0.002
Tb. N	2.3 ± 0.2	2.80 ± 0.11
Tb. MBV	931.9 ± 20.1	922.9 ± 20.7
Tb. TMD	92.2 ± 8.8	124.5 ± 9.7[1]
Cortical bone properties (microCT)		
TV	0.19 ± 0.10	0.21 ± 0.01
BV	0.12 ± 0.02	0.120 ± 0.004
BV/TV	0.60 ± 0.01	0.60 ± 0.01
Ct. TMV	801.8 ± 22.5	770.4 ± 17.5[1]
Ct. TMD	1288.5 ± 15.9	1291.6 ± 16.3

[1]Indicates significant difference between groups $P < 0.05$. BMC: Bone mineral content; BMD: Bone mineral density; BV: Bone volume; TV: Total volume (bone plus marrow); BV/TV: Bone volume fraction; Tb. Tk.: Trabecular thickness; Tb. N: Trabecular number; Tb. TMV: Trabecular material volume; Tb. TMD: Trabecular material density; Ct. TMV: Total cortical material volume; Ct. TMD: Total cortical material density; NOD: Non-obese diabetic; NOD.*scid*: Syngenic non-diabetic.

Because DXA can be an insensitive bone research technique, we performed microCT analyses to dissect the differences in trabecular and cortical bone. Tibiae were harvested from 5 and 8 wk old NOD and NOD.*scid* mice. Including all mice, there was not a significant difference in bending strength between NOD and NOD.*scid* mice. However, relative to NOD.*scid*, NOD mice had lower trabecular bone volume, relative trabecular bone volume, Tb.N, and trabecular total material density ($P < 0.05$) (Table 1). Conversely, NOD mice had greater cortical total mean volume ($P < 0.05$). We next performed a general linear models analysis that adjusted for body weight that revealed a significant contribution of age (Table 2). Accordingly, analysis was conducted by age. Table 2 presents the bone quality measures using microCT assessed at 5 and 8 wk. Diabetes-related impairment in bone microarchitectural properties and parameters of quality was apparent as early as 5 wk.

DISCUSSION

There is a paucity of mechanistic information on how disease initiation and progression affect bone. While low bone mass in diabetes is often reported, material proper-

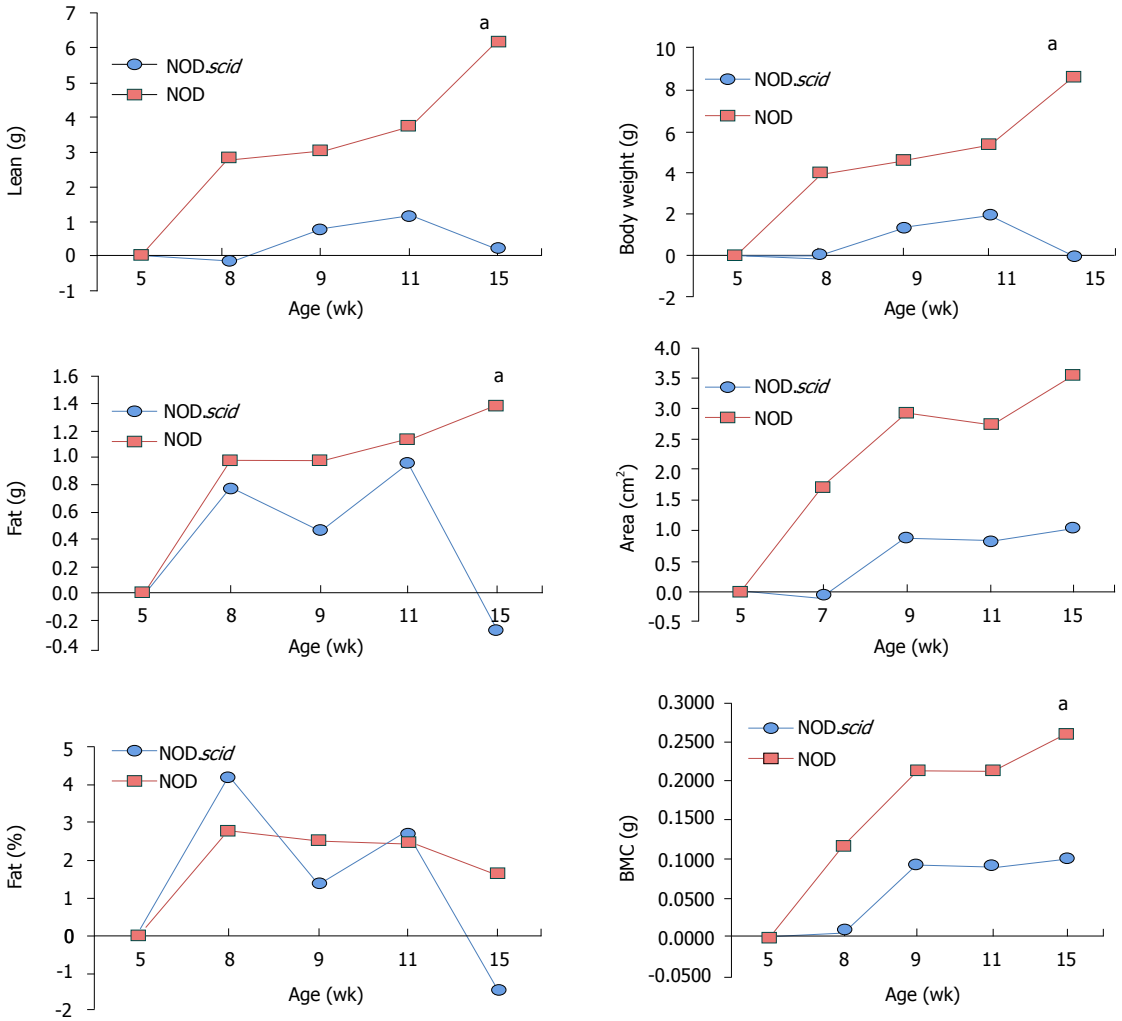

Figure 2 Comparison of changes in body composition parameters as assessed by dual-energy X-ray absorptiometry at 5, 8, 11 and 15 wk. [a]Non-obese diabetic mice were significantly heavier.

ties of bone, specifically those addressing initiation of impairment in material properties is lacking. The present study provides support of impaired bone structure/architecture with diabetes on bone *via* impairment of bone structure/architecture early in the life course.

Pre-diabetic NOD mice had lower trabecular properties, but greater cortical volume, suggesting compositional differences exist in tissue properties prior to disease progression. Nyman and colleagues recently reported a time-dependent alteration in matrix organization beginning approximately ten weeks after STZ injection, in line with consistent reports[20]. The mice in the study were 11 wk old when injected. While the authors noted a decrement in mineralization, they subsequently observed an increase in non-enzymatic collagen cross-linking[21]. It is possible that injections in close proximity or prior to rapid skeletal growth may lead to accelerated change in the strength-structure relationship. Further, a later-induced diabetic phenotype provokes skeletal phenotypes *via* different pathways and several conditions in both models may have indirect effects on the reported properties of bone. Notably, beyond those initiated with diabetic onset further changes in strength-structure relationship were not observed by Nyman and colleagues[20]. The increased Ct.MTV and Ct.TMD in NOD mice at 8 wk which did not translate into increased mechanical strength was surprising. Speculatively, a compensatory increase in insulin early in T1D prior to insulinitis may enhance anabolic properties at the outer surface. However, assessment of the strength-structure relationship requires evaluation of both outer and inner surfaces as well as the intrinsic properties within the bone (Ego Seeman, personal communication).

Particularly relevant during rapid skeletal growth, insulin has direct anabolic effects on periosteal apposition[17,18,21]. This would explain why diabetes did not affect the BMC or BMD, despite lower trabecular microarchitecture. In the context of humans, while it was recently reported that as adolescents with T1D attained reproductive maturation, had "normal" cortical cross sectional area[19], fracture risk remains greater among this population. It is important to note that it was recently reported that while mechanical properties of bone in humans with diabetes were impaired relative to non-diabetic controls, strength was not differ-

Table 2 Presents the bone quality measures using micro computed tomography assessed at 5 and 8 wk

	5 wk		8 wk	
	NOD (n = 4)	NOD.*scid* (n = 4)	NOD (n = 6)	NOD.*scid* (n = 4)
Body weight	14.9 ± 0.2	19.2 ± 0.4[1]	19.2 ± 0.5	19.9 ± 0.6
BMC	0.19 ± 0.01	0.30 ± 0.01[1]	0.30 ± 0.02	0.30 ± 0.01
BMD	0.030 ± 0.001	0.040 ± 0 .002	0.040 ± 0.001	0.040 ± 0.001
Tibia strength	4.5 ± 0.2	10.0 ± 0.8	8.4 ± 0.7	8.2 ± 0.8
Trabecular bone properties				
TV	1.4 ± 0.2	1.6 ± 0.1	1.50 ± 0.06	1.40 ± 0.04
BV	0.08 ± 0.02	0.14 ± 0.01	0.11 ± 0.02	0.16 ± 0.05
BV/TV	0.05 ± 0.02[1]	0.09 ± 0.00	0.07 ± 0.01	0.11 ± 0.04[1]
Tb. Tk	0.05 ± 0.01	0.05 ± 0.00	0.05 ± 0.00	0.05 ± 0.01
Tb. N	2.0 ± 0.3[1]	2.8 ± 0.3	2.40 ± 0.09	2.90 ± 0.58
Tb. TMV	63.8 ± 4.9	108.9 ± 7.4[1]	104.3 ± 8.9	137.0 ± 14.6
Tb. TMD	872.7 ± 42.5	930.5 ± 17.6	891.8 ± 21.3	948.8 ± 65.2
Cortical bone properties				
TV	0.18 ± 0.02[1]	0.23 ± 0.02	0.20 ± 0.02	0.20 ± 0.02
BV	0.10 ± 0.02	0.14 ± 0.02	0.12 ± 0.00	0.11 ± 0.01
BV/TV	0.60 ± 0.03	0.60 ± 0.01	0.63 ± 0.01[1]	0.58 ± 0.01
Ct. TMV	743.5 ± 56.9	787.4 ± 21.5	831.0 ± 13.2	753.0 ± 16.2[1]
Ct. TMD	1262.9 ± 18.9	1324.1 ± 20.8	1304.3 ± 11.0	1258.6 ± 13.4

[1]Indicates significant difference between groups $P < 0.05$. BMC: Bone mineral content; BMD: Bone mineral density; BV: Bone volume; TV: Total volume (bone plus marrow); BV/TV: Bone volume fraction; Tb. Tk: Trabecular thickness; Tb. N: Trabecular number; Tb. TMV: Trabecular material volume; Tb. TMD: Trabecular material density; Ct. TMV: Total cortical material volume; Ct. TMD: Total cortical material density; NOD: Non-obese diabetic; NOD.*scid*: Syngenic non-diabetic.

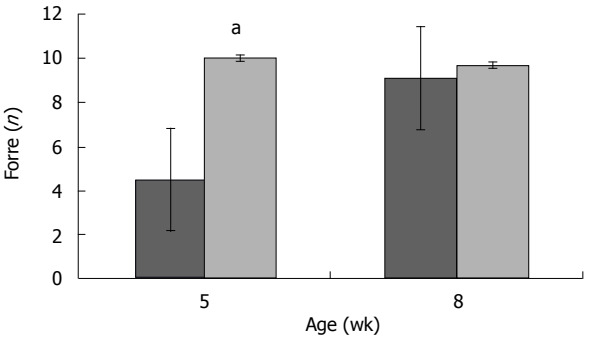

Figure 3 Tibia strength by 3-point breakage analysis using the NTS 85MTS Minibionix8 with a 100 N load cell. The span was 9 mm and the bones were loaded with a rate of 0.1 mm/s to evaluate maximum load to failure in 5 and 8 wk in non-obese diabetic (dark gray bars) and syngenic non-diabetic (light gray bars). Error bars represent SEM. [a]Non-obese diabetic mice demonstrated less mechanical strength than syngenic non-diabetic mice at 5 wk.

ent between middle-aged and older adults with diabetes[22], supporting our findings of the deleterious effects on bone integrity initiated early in disease progression.

While NOD mice are used to examine spontaneous T1D progression, immune-deficient NOD.*scid* mice may incur indirect effects on bone properties, which may explain the reported differences in the literature. Lacking mature B and T cells, NOD.*scid* mice are both insulitis- and diabetes-free throughout life. However, because of a high incidence of thymic lymphomas, the mean lifespan is relatively short[13,23,24]. Accordingly, while the unique immune defects provide an excellent *in vivo* environment for hematopoietic investigation extending to effects within the marrow compartment, this model may not be suitable for assessing bone material properties. The long-term tumorigenic effects provoked an unanticipated effect on growth in NOD.*scid* mice that likely affected strength-structure relationship. Further investigation in a comparable strain with optimal growth conditions [*e.g.*, non-obese diabetes resistant (NOR)] are needed to confirm our findings.

In conclusion, the T1D mouse model revealed complex changes early in the developmental process. There was diminished trabecular microarchitecture which manifested into weakened bone strength relative to non-diabetic mice, independent of bone mass. Our findings support a perturbation in the relationship between bone structure and strength and a need for intervention efforts to promote bone parameters during growth and development.

ACKNOWLEDGMENTS

The authors wish to thank Sasanka Ramanadham, Jake Fletcher, William Hancock and Maria Johnson for their invaluable contribution and assistance in bone parameter and body composition assessment.

COMMENTS

Background

While the skeletal manifestations of dysregulated glucose metabolism have been primarily considered in terms of bone quantity (*i.e.*, low bone mass), bone strength, the most obvious characteristic of bone structure/health is dependent upon various qualitative aspects.

Research frontiers

Diabetes-related impairments in insulin/glucose handling alter growth processes including those associated with bone development. However, the precise mechanisms accounting for perturbed bone accretion processes are not known and may differ, at least in part, by degree of glucose control.

Innovations and breakthroughs

Maintenance of glucose in circulation within a "normal" range, as well as the standard of "normality" throughout dynamic growth processes, varies, and has particular relevance to body composition trajectories during critical periods of development.

Applications

Peak bone mass, a major determinant of adult bone health is largely achieved by the end of sexual and skeletal maturity. Thus, an emerging area of investigation is the contribution of insulin/glucose homeostasis to bone (re)modeling, with considerable interest in understanding influential factors serving to maximize bone mass accrual in childhood, and therefore optimize bone phenotype throughout life.

Peer review

A potential explanation for observed differences in bone parameters in adults may be related to impairment in the remodeling-associated bone-resorption/formation coupling, which is maximally operational during the rapid skeletal development phase in childhood. Although the process of bone remodeling is complex, accumulating evidence supports glucose homeostasis as an integral part of bone formation (quantity) and micro-architecture (quality). Consistent with this, individuals exhibiting impaired glucose handling are at risk for compromised bone mass and integrity. In particular, type 1 diabetes mellitus is associated with increased bone mass loss (*e.g.*, osteopenia, osteoporosis), increased risk of fragility fracture, and poor bone healing following injury. Investigation early in the life course is highly relevant.

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P- Reviewers Robinson MK, Yin YW **S- Editor** Wen LL
L- Editor A **E- Editor** Lu YJ

World Journal of Diabetes

Online Submissions: http://www.wjgnet.com/esps/
wjd@wjgnet.com
doi:10.4239/wjd.v4.i4.151

World J Diabetes 2013 August 15; 4(4): 151-156
ISSN 1948-9358 (online)

BRIEF ARTICLE

Vildagliptin-insulin combination improves glycemic control in Asians with type 2 diabetes

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Supported by Novartis Pharma AG
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Received: March 28, 2013 Revised: May 11, 2013
Accepted: June 18, 2013
Published online: August 15, 2013

Abstract

AIM: To assess the efficacy and safety of vildagliptin 50 mg *bid* as add-on therapy to insulin in Asian patients with type 2 diabetes mellitus (T2DM).

METHODS: This was a post hoc analysis of a subgroup of Asian patients from a multicenter, randomized, double-blind, placebo-controlled, parallel-group study in T2DM patients inadequately controlled by stable insulin therapy, with or without metformin. A total of 173 patients were randomized 1:1 to receive treatment with vildagliptin 50 mg *bid* (n = 87) or placebo (n = 86) for 24 wk. Changes in HbA1c and fasting plasma glucose (FPG), from baseline to study endpoint, were analyzed using an analysis of covariance model. Change from baseline to endpoint in body weight was summarized by treatment. Safety and tolerability of vildagliptin was also evaluated.

RESULTS: After 24 wk, the difference in adjusted mean change in HbA1c between vildagliptin and placebo was 0.82% (8.96 mmol/mol; $P < 0.001$) in Asian subgroup, 0.85% (9.29 mmol/mol; $P < 0.001$) in patients also receiving metformin, and 0.73% (7.98 mmol/mol; $P < 0.001$) in patients without metformin, all in favor of vildagliptin. There was no significant difference in the change in FPG between treatments. Weight was stable in both treatment groups (+ 0.3 kg and -0.2 kg, for vildagliptin and placebo, respectively). Overall, vildagliptin was safe and well tolerated with similarly low incidences of hypoglycemia (8.0% *vs* 8.1%) and no severe hypoglycemic events were experienced in either group.

CONCLUSION: In Asian patients inadequately controlled with insulin (with or without concomitant metformin), insulin-vildagliptin combination treatment significantly reduced HbA1c compared with placebo, without an increase in risk of hypoglycemia or weight gain.

Key words: Asian; DPP-4 inhibitor; Hypoglycemia; Insulin; Oral antidiabetic drug; Type 2 diabetes; Vildagliptin

Core tip: In Asian patients, vildagliptin added to stable dose of insulin, with or without concomitant metformin, significantly improves glycemic control without increase in weight and hypoglycemia incidence.

Kozlovski P, Foley J, Shao Q, Lukashevich V, Kothny W. Vildagliptin-insulin combination improves glycemic control in Asians with type 2 diabetes. *World J Diabetes* 2013; 4(4): 151-156 Available from: URL: http://www.wjgnet.com/1948-9358/full/v4/i4/151.htm DOI: http://dx.doi.org/10.4239/wjd.v4.i4.151

INTRODUCTION

The unfolding diabetes epidemic is projected to affect more than 550 million people worldwide by the year 2030 with approximately 60% of patients coming from Asia[1]. Despite available antidiabetic treatments, glycemic control in most Asian countries is unsatisfactory[2,3]. The progressive nature of type 2 diabetes requires continuous treatment intensification with a combination of antidiabetic agents having different mechanisms of action, and initiation of insulin therapy when beta cell function significantly deteriorates. However, delay in insulin initiation and intensification is a major problem across the world. In Asia, the mean HbA1c at the time of insulin intensification exceeds 9%[4], with fear of hypoglycemia and concern of weight gain identified as the main barriers for early and optimal insulin use[5]. Therefore, antidiabetic agents that can significantly improve glycemic control without increasing the risk of hypoglycemia and weight gain when used in combination with insulin are needed. While the use of insulin in combination with oral antidiabetic drugs (OADs) is increasing in Asia[5], there is little data from randomized controlled trials investigating the efficacy and safety of OAD-insulin combination in Asian patients with type 2 diabetes mellitus (T2DM).

Vildagliptin is a potent and selective inhibitor of dipeptidyl peptidase-4 (DPP-4), which extends the physiological effects of glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic peptide (GIP) resulting in improvement of glycemic control in a glucose-sensitive manner[6-8]. In Asian patients with T2DM, vildagliptin showed significant improvements in HbA1c with low incidence of hypoglycemia when used as monotherapy[9], in combination with metformin[10], or in combination with a sulfonylurea[11].

We recently reported that vildagliptin added to insulin therapy resulted in a robust improvement in glycemic control without increasing the risks of hypoglycemia and weight gain[12]. This study included about 40% patients from Asia (n = 173) allowing for a meaningful analysis of the efficacy and safety data in this population. Asian patients with T2DM could be more susceptible to hypoglycemia than Caucasians due to their lower body weight and increased sensitivity to insulin[13,14] and there is a general lack of data in Asians as discussed above. We, therefore, analyzed the subgroup of Asian patients in this study to characterize the response to vildagliptin when combined with insulin in this growing patient population.

MATERIALS AND METHODS

Study design and patients

This was a post hoc analysis of a subgroup of Asian patients from a 24 wk, multicenter, randomized, double-blind, placebo-controlled, parallel-group study. Eligible patients included 89 men and 84 women aged 18-80 years with T2DM, HbA1c ≥ 7.5% (≥ 58.5 mmol/mol) and ≤ 11.0% (≤ 96.7 mmol/mol), and fasting plasma glucose levels (FPG) < 15 mmol/L, who were being treated with stable insulin doses ≤ 1 U/kg per day (long-acting, intermediate-acting, or premixed, once or twice daily) with or without stable concomitant metformin treatment (≥ 1500 mg or maximally tolerated dose) for at least 12 wk.

After a 2 wk screening period, patients were randomized in a 1:1 ratio to treatment with vildagliptin 50 mg *bid* or placebo. Randomization was stratified by metformin use and type of insulin used (long-acting *vs* intermediate acting/premixed). Further details of the study design were reported by Kothny *et al*[12].

Study assessments and endpoints

HbA1c, FPG, and body weight were assessed at every visit, scheduled at 4 wk intervals. The efficacy endpoints were the change in HbA1c, FPG, and body weight from baseline to 24 wk or to the final visit. Safety assessments included monitoring and recording of treatment emergent adverse events (AEs), serious AEs (SAEs), biochemistry and hematology laboratory test results, electrocardiogram (ECG) findings, and vital signs.

Hypoglycemia was defined by symptoms suggestive of hypoglycemia and a self-monitored plasma glucose measurement < 3.1 mmol/L. Severe hypoglycemia was defined as an episode that required assistance of another person or hospitalization with or without a plasma glucose measurement < 3.1 mmol/L.

Statistical analysis

The changes in HbA1c from baseline to week 24 were compared between vildagliptin and placebo using an analysis of covariance with treatment, region, metformin use, and insulin type as classification variables and baseline HbA1c as covariate. This comparison was performed for the overall Asian population and for patients with/without concomitant metformin. Change in FPG was analyzed using the same model as for HbA1c. In addition, responder rates [percentage of patients achieving endpoint HbA1c < 7.0% (53.0 mmol/mol)] were compared between treatments using a chi-squared test. The efficacy analyses were performed on the full analysis set population consisting of all randomized patients who received at least one dose of the study drug and had at least one post-baseline assessment of any efficacy variable.

Efficacy data used in analyses were censored at the start of major changes in insulin background therapy. Major changes in insulin therapy were defined as changes occurring ≥ 7 d in any 30-d period or ≥ 5 d consecutively, including changes in insulin frequency and/or type and/or a ≥ 10% dose increase either as rescue medication or for any other reasons. The last observation carried forward (LOCF) method was used to handle missing data because of early discontinuation or data censoring.

Change in body weight from baseline to endpoint was summarized descriptively. The safety data (AEs, SAEs, including hypoglycemia) were summarized descriptively by treatment on all available data.

Ethical considerations

This trial was conducted in accordance with the Declara-

Table 1 Baseline patient demographic and background characteristics (Asian population)

	Vildagliptin 50 mg *bid*	Placebo
	n = 87	*n* = 86
Age, years	54.0 ± 8.4	54.9 ± 10.5
⩾ 65, *n* (%)	8 (9.2)	13 (15.1)
Gender, female, *n* (%)	45 (51.7)	39 (45.3)
Race, *n* (%)		
Indian (Indian subcontinent)	62 (71.3)	61 (70.9)
Chinese	24 (27.6)	24 (27.9)
Other	1 (1.1)	1 (1.2)
BMI, kg/m^2	26.2 ± 3.0	26.7 ± 3.7
Body weight, kg	67.5 ± 9.5	68.8 ± 12.1
HbA1c, % (mmol/mol)	8.9 ± 1.0	9.0 ± 1.0
	(73.7 ± 10.9)	(74.8 ± 10.9)
FPG, mmol/L	9.1 ± 2.6	8.6 ± 2.5
T2DM duration, years	11.1 ± 6.4	12.1 ± 7.6
GFR, mL/min per 1.73 m^2, *n* (%)		
Normal, > 80	43 (49.4)	52 (60.5)
Mild, ⩾ 50 to ⩽ 80	42 (48.3)	33 (38.4)
Moderate, ⩾ 30 to < 50	2 (2.3)	1 (1.2)
Background antidiabetic therapy		
Insulin use at screening, *n* (%)		
Intermediate-acting	21 (24.1)	20 (23.3)
Long-acting	14 (16.1)	8 (9.3)
Pre-mixed	52 (59.8)	58 (67.4)
Duration of insulin use, years	3.3 ± 2.9	3.9 ± 4.3
Daily dose of insulin, *U*	39.5 ± 15.8	39.5 ± 15.3
Daily number of insulin injections	1.9 ± 0.4	1.8 ± 0.4
Metformin use at screening, *n* (%)		
Yes	52 (59.8)	52 (60.5)
No	35 (40.2)	34 (39.5)

Values are mean ± SD unless indicated otherwise. BMI: Body mass index; GFR: Glomerular filtration rate; FPG: Fasting plasma glucose; HbA1c: Hemoglobin A1c; T2DM: Type 2 diabetes mellitus.

tion of Helsinki. An independent ethics committee or institutional review board at each research site reviewed the study protocol. Each patient gave written informed consent before randomization.

RESULTS

Patient disposition and baseline characteristics

A total of 173 Asian patients (38.5% of the overall study population) were randomized: 87 patients to vildagliptin 50 mg *bid* and 86 patients to placebo. The demographic and baseline characteristics of the randomized patients are summarized in Table 1. The groups were well balanced for all the baseline characteristics. Most patients were from the Indian subcontinent (71%) followed by patients of Chinese ethnicity (27.7%) with a mean age of 54.5 years; just over 12% of patients were ⩾ 65 years of age. Mean baseline values of HbA1c and FPG were 8.9% (73.8 mmol/mol) and 8.8 mmol/L, respectively. Mean BMI was 26.4 kg/m^2 and the majority (86%) had body mass index (BMI) < 30 kg/m^2. The mean duration of T2DM was 11.6 years. Mean duration of insulin usage was 3.6 years, mean daily insulin dose at screening was 39.5 units, and pre-mixed insulin was the most frequent type of insulin used. Overall, 60.1% of patients were treated with metformin. The mean metformin dose at the time of randomization was approximately 2000 mg for both treatment groups.

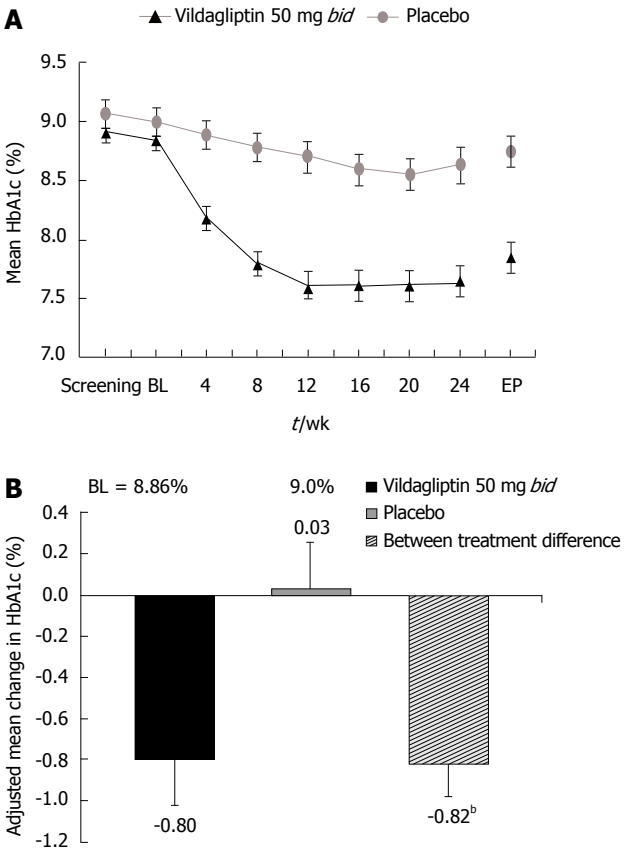

Figure 1 Mean change. A: Mean change in hemoglobin A1c over time; B: Adjusted mean change in HbA1c from baseline to endpoint. BL: Baseline; EP: End point; [b]*P* < 0.001.

Efficacy

In this Asian population, vildagliptin demonstrated consistent reductions in HbA1c from baseline to week 24 endpoint (Figure 1A). After 24 wk of treatment, HbA1c had decreased by 0.8 ± 0.2% (8.74 ± 2.2 mmol/mol) in patients receiving vildagliptin (*n* = 85) and HbA1c increased by 0.03 ± 0.2% (0.32 ± 2.2 mmol/mol) in patients receiving placebo (*n* = 84). The adjusted between-treatment difference (vildagliptin 50 mg *bid*-placebo) in HbA1c of 0.82 ± 0.1% (8.96 ± 1.1 mmol/mol) was statistically significant (*P* < 0.001) in favor of vildagliptin (Figure 1B).

Vildagliptin significantly reduced HbA1c in both patients with and without concomitant metformin therapy, with adjusted mean differences *vs* placebo of 0.85% (9.29 mmol/mol) and 0.73% (7.98 mmol/mol) (*P* < 0.001 for both groups), respectively, in favor of vildagliptin. In subgroups by ethnicity, reductions in HbA1c from baseline were 0.99% (10.82 mmol/mol) and 1.17% (12.78 mmol/mol) with vildagliptin, and 0.31% (3.38 mmol/mol) and 0.08% (0.87 mmol/mol) with placebo, in Indian and Chinese patients, respectively.

In a responder analysis, significantly more patients receiving vildagliptin achieved the HbA1c target of < 7.0%

(53.0 mmol/mol) than those receiving placebo (22.4% and 4.8%, respectively; $P = 0.001$). FPG did not change significantly in the vildagliptin group ($n = 85$) with a 0.2 mmol/L increase at week 24 from baseline of 9.6 mmol/L; a more pronounced change was seen in the placebo group ($n = 84$) with a 0.7 mmol/L increase at week 24 from baseline of 9.0 mmol/L; mean placebo-subtracted difference was 0.5 mmol/L ($P = 0.335$) in favor of vildagliptin.

The mean insulin dose at baseline was 39.5 units in both vildagliptin and placebo groups. The mean changes from baseline to study end were reductions of 1.39 and 1.48 units in the vildagliptin group and the placebo group, respectively. Overall, the small changes in the insulin dose in both treatment groups are consistent with the protocol requirement for a stable insulin dose during the study.

Safety

Vildagliptin 50 mg *bid* added to intermediate-acting, long-acting, or premixed insulin, with or without metformin, was generally safe and well tolerated.

The overall incidence of AEs was numerically higher with vildagliptin (62.1%) than with placebo (53.5%). This difference was driven by gastrointestinal disorders (14.9% *vs* 7.0%), blurred vision (9.2% *vs* 0%), and upper respiratory tract infections (13.8% *vs* 8.1%). The latter were assessed by investigators as mild or moderate and not related to study drug. Diarrhea and gastritis were more frequently reported in the vildagliptin group; however, the drug was not discontinued in any of the cases.

The proportion of patients who experienced hypoglycemic events was low and similar in both treatment groups (8.0% and 8.1% in the vildagliptin and placebo groups, respectively). No patient in either treatment group experienced a severe hypoglycemic event. Similar number of patients in the vildagliptin and placebo groups reported hyperhidrosis, dizziness, tremors, and palpitations, which may be symptoms of hypoglycemia. Blurred vision was reported by 8 patients (9.2%). For three of them, blurred vision was identified as hypoglycemia and included in the hypoglycemic events summary since they had accompanying glucose measurements < 3.1 mmol/L. Of the remaining five, four reported, together with the blurred vision, one or more other symptoms suggestive of hypoglycemia (dizziness, weakness, palpitations, tremor or hyperhidrosis); however no blood glucose measurement had been performed to confirm a hypoglycemic event. Six of these eight patients experienced considerable reduction in HbA1c of 1.4% (15.3 mmol/mol) or more during the study; another one had a smaller HbA1c reduction, but reached HbA1c of 6.5% (47.5 mmol/mol). These events of blurred vision could be symptoms of hypoglycemia, or in some cases a reflection of rapidly improving glucose levels.

The rate of serious AEs was very low in this subgroup with only one serious AE reported. This was a case of liver enzyme elevation reported in one vildagliptin-treated patient with history of non-alcoholic steato-hepatitis. This event was associated with respiratory infection and the adjudication committee concluded that it was unrelated to study drug.

Body weight remained stable during the study with an increase of 0.3 kg in the vildagliptin group and a decrease of 0.2 kg in the placebo group. Overall, the safety profile of vildagliptin in the Asian subgroup was consistent with the safety profile in the overall patient population[12], without any clinically relevant differences between treatments.

DISCUSSION

In Asian patients, the addition of vildagliptin 50 mg *bid* to stable therapy with basal or pre-mixed insulin, with or without concomitant metformin, demonstrated a robust reduction in HbA1c *vs* placebo after 24 wk of treatment. Vildagliptin was efficacious in patients both from Indian and Chinese ethnicity with clinically relevant reductions in HbA1c from baseline of about 1.0% (10.93 mmol/mol). Importantly, the addition of vildagliptin to insulin was not associated with an increased risk for hypoglycemia or weight gain. These findings are consistent with the results from the overall study population[12]. Mean baseline HbA1c was similar in both the overall population [8.8% (72.7 mmol/mol)] and in the Asian population [8.9% (73.8 mmol/mol)] and so was the reduction in HbA1c *vs* placebo after 24 wk of treatment [0.7% (7.6 mmol/mol) and 0.8% (8.7 mmol/mol), respectively].

Asian patients had lower BMI than patients in the overall study population (26.4 kg/m^2 *vs* 28.9 kg/m^2, respectively) which could make them more sensitive to insulin and, thus, place them at a higher risk of hypoglycemia[15]. However, the incidence of hypoglycemia was similar for vildagliptin and placebo in spite of better glycemic control with vildagliptin indicating that vildagliptin exerts its protective effect against hypoglycemia also in Asian patients. Vildagliptin has demonstrated a protective effect against hypoglycemia at all stages of type 2 diabetes[16] resulting from its ability to increase glucagon levels during hypoglycemia[6].

In this study, adding vildagliptin to a stable insulin dose was weight neutral, which is consistent with the known vildagliptin weight profile when used as monotherapy or in combination with other OADs[17-21]. The weight neutrality of vildagliptin likely results in part from its intrinsically low risk for hypoglycemia and avoidance of "defensive eating" characteristic for antidiabetic agents associated with increased hypoglycemia risk. Other potential mechanisms may include possible inhibition of intestinal fat extraction and fatty acid mobilization and oxidation in the postprandial state, in conjunction with increased sympathetic stimulation[21].

Multinational studies with DPP-4 inhibitors added to insulin included small numbers of Asian patients[22-24] and, therefore, meaningful analysis might have not been possible. However, in a Korean study, addition of sitagliptin to a stable insulin dose resulted in reduction in HbA1c of 0.6% (6.5 mmol/mol) from baseline of 9.2% (77.1 mmol/mol), with one patient experiencing severe hypoglycemia[25]. This is consistent with the find-

ings from a multinational study with sitagliptin, which showed improvement in glycemic control at the expense of increased hypoglycemia incidence compared with placebo[24]. In contrast, addition of vildagliptin to insulin in this study was not associated with an increased risk of hypoglycemia in Asian patients and no events of severe hypoglycemia were reported. This difference between vildagliptin and sitagliptin could be due to vildagliptin's ability to maintain elevated GIP levels into periods where hypoglycemia is likely to occur, thus resulting in improved glucagon counter-regulation[26].

In conclusion, the presented efficacy and safety data in Asian patients inadequately controlled with a stable insulin dose with or without concomitant metformin showed that vildagliptin can be a suitable add-on treatment leading to improved glycemic control without increased risk of hypoglycemia or weight gain. Despite some differences in diabetes phenotype between Asians and Caucasians as well as potential differences in the pathophysiology of T2DM in these populations, the beneficial effects of vildagliptin when added to insulin are maintained in an Asian population.

ACKNOWLEDGMENTS

The authors gratefully acknowledge the support of all the investigators and medical staff at the participating centers. The authors also thank Sanchika Agarwal for editorial assistance.

COMMENTS

Background

The increasing diabetes epidemic by 2030 with majority of patients from Asia is of major concern. The progressive nature of disease requires intensified treatment with multiple antidiabetic agents, and insulin initiation when beta cell function deteriorates. Therefore, agents which improve glycemic control without hypoglycemia and weight gain when used with insulin are needed. However, there is little data from randomized controlled trials investigating the efficacy and safety of oral antidiabetic drugs-insulin combination in Asian patients with type 2 diabetes. The authors recently reported that vildagliptin added to insulin therapy resulted in a robust improvement in glycemic control without increasing the risks of hypoglycemia and weight gain. This study included about 40% patients from Asia and thus the authors analyzed the subgroup of Asian patients to characterize the response to vildagliptin when combined with insulin in a patient population in which diabetes is a growing concern.

Research frontiers

Vildagliptin is a selective inhibitor of dipeptidyl peptidase-4 (DPP-4) enzyme, improves glycemic control by increasing the availability of incretins. Considering that Asian patients with type 2 diabetes could be more susceptible to hypoglycemia than Caucasians due to their lower body weight and increased sensitivity to insulin, and due to the general lack of data in Asians, the efficacy and safety of vildagliptin-insulin combination in this population was assessed.

Innovations and breakthroughs

This is the first double-blind placebo controlled study that reports the efficacy and safety of a DPP-4 inhibitor (vildagliptin) as add-on to insulin in an Asian population.

Applications

This study demonstrates that vildagliptin in combination with insulin is a safe and efficacious antidiabetic treatment by significantly reducing HbA1c without an increased incidence of hypoglycemia or weight gain.

Terminology

DPP-4 inhibitors: Dipeptidyl peptidase-4 inhibitors are a class of oral antihyperglycemic agents that inhibit the enzyme DPP-4. They are used to treat type 2 diabetes mellitus. HbA1c: Glycated hemoglobin is a form of hemoglobin that is measured primarily to identify the average plasma glucose concentration over prolonged periods of time. It is formed in a non-enzymatic glycation pathway by hemoglobin's exposure to plasma glucose. The 2010 American Diabetes Association Standards of Medical Care in Diabetes added the HbA1c $\geqslant$ 48 mmol/mol ($\geqslant$ 6.5%) as another criterion for the diagnosis of diabetes.

Peer review

This manuscript presents an analysis of the Asian subgroup of a recently published study. It addresses new findings regarding the effects of vildagliptin as add-on therapy to insulin in Asian patients with type 2 diabetes mellitus. The present work contains interesting data and appears timely.

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P- Reviewers GeorgescuA, Sourij H **S- Editor** Wen LL
L- Editor A **E- Editor** Lu YJ

World Journal of Diabetes

Online Submissions: http://www.wjgnet.com/esps/
wjd@wjgnet.com
doi:10.4239/wjd.v4.i4.157

World J Diabetes 2013 August 15; 4(4): 157-161
ISSN 1948-9358 (online)

BRIEF ARTICLE

Effect of treatment of overt hypothyroidism on insulin resistance

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Telephone: +966-56-8089574
Received: March 30, 2013 Revised: July 2, 2013
Accepted: July 9, 2013
Published online: August 15, 2013

Abstract

AIM: To investigate the impact of hypothyroidism and thyroxine therapy on insulin sensitivity in patients with overt hypothyroidism.

METHODS: The study included twenty seven overtly hypothyroid and fifteen healthy euthyroid South Western Asian females. Both groups had matching age and body mass index. Physiological and pathological conditions as well as medications that may alter thyroid function, glucose homeostasis or serum lipids were ruled out. Serum thyrotropin (TSH), free tetraiodothyronine (FT4), free triiodothyronine (FT3), fasting insulin (FI), fasting plasma glucose (FPG), total cholesterol and triglycerides were measured before and six months after initiating thyroxine therapy for hypothyroid patients and once for the control group. Insulin resistance (IR) was estimated using homeostasis model assessment (HOMA-IR) and Body mass index (BMI) was calculated.

RESULTS: Both study groups, hypothyroid patients and euthyroid control subjects, had matching age and body mass index (*P*-value 0.444, 0.607 respectively). No significant difference was found between the hypothyroid patients and the euthyroid control group regarding fasting plasma glucose, fasting insulin, insulin resistance, total cholesterol and triglycerides (*P*-values 0.432, 0.621, 0.883, 0.586, 0.05 respectively). In the hypothyroid patients, triglycerides showed direct correlation to TSH and inverse correlation to FT3. Similarly total cholesterol inversely correlated to FT3 but its direct correlation to TSH did not reach statistical significance. After thyroxine replacement and reaching an euthyroid state as confirmed by clinical and laboratory data, there was no significant change in fasting plasma glucose, insulin resistance or triglyceride level (*P*-value 0.216, 0.204, 0.175 respectively) while total cholesterol significantly decreased (*P*-value 0.043) and fasting insulin significantly increased (*P*-value 0.047).

CONCLUSION: Hypothyroidism has no impact on insulin sensitivity. Correction of hypothyroidism is not associated with a significant change of insulin sensitivity or triglycerides, but with a significant reduction of total cholesterol.

Key words: Hypothyroidism; Female; Thyroxine; Insulin resistance; Triglycerides; Cholesterol

Core tip: Thyroid dysfunction is the second most common endocrine disorder after diabetes mellitus. Both diseases are strong associated. Hypothyroidism is claimed to cause insulin resistance. Some available reports are in agreement and others are against this suggestion. In our study, we did not find a significant effect of hypothyroidism or thyroxine replacement on insulin resistance as calculated by insulin resistance was estimated using homeostasis model assessment. Thyroxine therapy leads to a significant reduction of total cholesterol but it does not change triglycerides. This may partially explain the association between hypothyroidism and dyslipidaemia as well as cardiovascular risk.

Nada AM, Effect of treatment of overt hypothyroidism on insulin resistance. *World J Diabetes* 2013; 4(4): 157-161 Available from: URL: http://www.wjgnet.com/1948-9358/full/v4/i4/157.htm DOI: http://dx.doi.org/10.4239/wjd.v4.i4.157

INTRODUCTION

Thyroid dysfunction and diabetes mellitus (DM) are the two most common endocrine disorders. Both disorders appear to be closely linked[1]. A recent meta-analysis that was conducted on available data in 10920 patients with DM revealed a mean frequency of thyroid disease of 11% with no difference between type 1 DM and type 2 DM. The prevalence in women was consistently more than two-folds that in men[2].

It has also been postulated that insulin secretion is regulated by the thyroid hormone[3,4] and diabetes risk is related to thyroid hormone levels[5,6]. The initial event of glucose-stimulated insulin secretion is glucose sensing. The glucose transporter 2 (GLUT2) and glucokinase (GK) are key molecules which affect various processes of glucose sensing in pancreatic β-cells[7]. Impairment in glucose sensing contributes to pancreatic β-cell dysfunction. Therefore, it is necessary to maintain adequate expression levels of GLUT2 and GK to ensure normal β-cell function[8]. Triiodothyronine (T_3) can modulate the expression of GLUT2 and GK mRNAs and proteins in pancreatic islets[9] and liver[10].

To date, only a few studies have investigated the effect of hypothyroidism and its recovery by thyroid hormone treatment on glucose metabolism and lipid profile, and the results have been controversial. Some researchers elucidated lower insulin sensitivity in patients with overt hypothyroidism which improved after thyroxine treatment[11,12]. Subclinical hypothyroidism was also encountered as a cause of insulin resistance and its related dyslipidaemia in patients with rheumatoid arthritis[13]. Contrary to that, Brenta *et al*[14] did not find significant differences in insulin sensitivity or lipid profile before and after thyroxine replacement in subclinical hypothyroidism.

In the light of existing data, we decided to study the impact of hypothyroidism on insulin sensitivity in overtly hypothyroid patients and to investigate the possible effect of thyroxine replacement on insulin sensitivity, triglycerides and total cholesterol in those populations.

MATERIALS AND METHODS

This study was approved by the Research and Ethics Committee of Asir Central Hospital and written informed consents were acquired from all participants.

Forty-two South Western Asian females were recruited from the endocrine clinic in a tertiary care hospital in southern region of Saudi Arabia, during January 2010 and December 2011. They included twenty seven patients with overt hypothyroidism and fifteen healthy euthyroid control women with matching age and body mass index (BMI). Full history taking and clinical examination were done for all participants. The inclusion criteria were: adult, premenopausal females, who were newly diagnosed with overt hypothyroidism. Exclusion criteria were diabetes, polycystic ovarian disease, liver disorders, renal disorders, congestive cardiac failure or any other systemic illness. In addition, pregnancy and lactation, intake of oral contraceptive pills, statins and other medications that may alter thyroid functions, glucose homeostasis or serum lipids also accounted for exclusion from the study.

After an overnight fasting, blood samples were collected from all participants for measuring biochemical parameters. Thyroid profile (TSH, FT4 and FT3), fasting insulin, fasting plasma glucose, total cholesterol and triglycerides were measured, before and six months after initiating thyroxine therapy and reaching an euthyroid state for hypothyroid patients. These parameters were measured once for the euthyroid control group.

Insulin resistance (IR) was estimated using HOMA-IR, IR = FPG in milli-gram per deciliter × FI in micro-international unit per milli-litre/405[15,16]. BMI was calculated by dividing weight of the patient in kilograms by square the height of the patient in meters[17].

Thyroid profile and insulin level were estimated by Advia centaur auto-analyzer Siemens using chemiluminescent technology. Fasting plasma glucose and triglycerides were measured by bichromatic technique while cholesterol was measured by polychromatic technique. Normal ranges for all parameters: TSH: 0.27-4.2 μIU/mL, FT4: 12-22 pmol/L, FT3: 3.9-6.8 pmol/L, FI: 2.6-37.6 μIU/mL, total cholesterol: 50-200 mg/dL, triglycerides: 30-150 mg/dL[18-22].

Statistical analysis

Collected data were analyzed using the Statistical Package for Social Sciences (SPSS ver. 19). Descriptive statistics (*i.e.*, mean and standard deviation) were applied. Pearson's Correlation Coefficients (r) between study variables were calculated. Significant P-values were considered at < 0.05.

RESULTS

Our study population consisted of 42 females; 27 patients with overt hypothyroidism and 15 euthyroid healthy participants. The two groups had matching age and body mass index (33.12 ± 10.4 *vs* 35.67 ± 9.1, $P = 0.44$, 31.11 ± 6.78 *vs* 32.24 ± 6.68, $P = 0.61$ respectively). Fasting insulin, FPG, IR, total cholesterol and triglycerides did not show significant difference in hypothyroid patients as compared to the euthyroid group (P-values 0.432, 0.621, 0.883, 0.586, 0.05 respectively) as shown in Table 1.

In the hypothyroid state, Triglycerides directly correlated to TSH and inversely to FT3 (P-value 0.009, 0.001 respectively). Total cholesterol inversely correlated to FT3 (P-value 0.029) and was directly proportionate to TSH although this relation did not reach statistical significance (P-value = 0.327) as shown in Table 2.

Table 1 Laboratory and anthropometric parameters in hypothyroid patients versus euthyroid subjects

Parameter	Hypothyroid (mean ± SD)	Euthyroid (mean ± SD)	*P*-value
Age (yr)	33.2 ± 10.4	35.7 ± 9.1	0.444
BMI	31.1 ± 6.8	32.2 ± 6.7	0.607
TSH	22.4 ± 36.2	2.9 ± 1.5	0.010
FT4	11.2 ± 4.0	13.7 ± 2.1	0.013
FT3	4.4 ± 1.0	4.5 ± 0.5	0.557
FPG	93.5 ± 14.7	89.8 ± 13.9	0.432
FI	10.6 ± 8.1	11.8 ± 6.3	0.621
IR	2.5 ± 2.1	2.6 ± 1.5	0.883
TG	144.8 ± 85.4	97.9 ± 36.1	0.050
TCH	195.0 ± 37.9	189.0 ± 29.9	0.586

BMI: Body mass index; TSH: Thyrotropin; FPG: Fasting plasma glucose; FI: Fasting insulin; IR: Insulin resistance; FT4: Free tetraiodothyronine; FT3: Free triiodothyronine; TG: Triglycerides; TCH: Total cholesterol.

Table 2 Correlation between different variables before thyroxine replacement

		TSH	FT4	FT3
TG	*r*	0.496	-0.321	-0.585
	P	0.009	0.102	0.001
TCH	*r*	0.196	-0.176	-0.420
	P	0.327	0.380	0.029

TSH: Thyrotropin; FT4: Free tetraiodothyronine; FT3: Free triiodothyronine; TG: Triglycerides; TCH: Total cholesterol; R: Relative coefficient.

After thyroxine replacement and attaining euthyroid state, there was no significant change in FPG or IR as compared to that before starting treatment (*P*-value = 0.216, 0.204 respectively) while FI significantly increased (P = 0.047). There was no significant change in triglycerides (*P*-value 0.175) meanwhile total cholesterol significantly decreased (*P*-value 0.043) as shown in Table 3.

DISCUSSION

The association between hypothyroidism and diabetes mellitus had raised great interest in studying the mechanism of this association. Many studies targeted the influence of hypothyroidism on insulin sensitivity as the main underlying pathophysiology of this relation. Despite the many studies, results are conflicting with several studies reporting that hypothyroidism is a state of increased insulin resistance[11,23].

In our study, there was no significant difference between the hypothyroid patients and the euthyroid healthy group regarding fasting insulin, FPG and insulin resistance. This is consistent with results of a study conducted by Giménez-Palop *et al*[24] on 17 hypothyroid women compared to 20 euthyroid control women.

Similarly, Owecki *et al*[25] did not find a significant difference in insulin sensitivity between hypothyroid patients and euthyroid participants.

Neither FPG nor insulin resistance as calculated by HOMA-IR significantly changed after thyroxine replacement and reaching an euthyroid state as per clinical and laboratory evidence. There was a significant increase in the fasting insulin as compared to the pretreatment level but this was not statistically significant when compared to the euthyroid control (13.55 ± 7.25 *vs* 11.82 ± 6.31, P = 0.445) and it did not affect the overall calculated insulin resistance. This is again in agreement with results demonstrated by Giménez-Palop *et al*[26] although the increase in insulin levels in his study did not reach a statistical significance.

Table 3 Comparison between different variables before and after thyroxine replacement

Variable	Before treatment (mean ± SD)	After treatment (mean ± SD)	*P*-value
BMI	31.1 ± 6.8	31.4 ± 7.2	0.485
TSH	22.4 ± 36.2	3.0 ± 1.9	0.010
FT4	11.2 ± 4.0	14.5 ± 2.6	0.001
FT3	4.4 ± 1.0	4.7 ± 0.7	0.037
FPG	93.5 ± 14.7	90.2 ± 12.2	0.216
FI	10.6 ± 8.1	13.6 ± 7.3	0.047
IR	2.5 ± 2.1	3.0 ± 1.9	0.204
TG	144.8 ± 85.4	128.1 ± 64.8	0.175

BMI: Body mass index; TSH: Thyrotropin; FPG: Fasting plasma glucose; FI: Fasting insulin; IR: Insulin resistance; FT4: Free tetraiodothyronine; FT3: Free triiodothyronine; TG: Triglycerides; TCH: total cholesterol.

Referring to our study and studies in agreement with our findings, we can say that the association between hypothyroidism and T2DM may be attributed to a complex interplay[26]. It may depend on the severity of hypothyroidism[27]. There may be direct genetic links between thyroid diseases and T2DM as suggested by few studies. These studies suggest that homozygosity of polymorphism of the deiodinase type 2 (DIO2) gene, Thr92Ala is associated with an increased risk of T2DM[28]. Thyroid hormones may also affect glucose and lipid homeostasis *via* central effects at the level of the hypothalamus[29].

Hypothyroidism is known to be associated with normal or high levels of triglycerides[30-32]. In our study, triglycerides in the hypothyroid patients did not differ significantly from the euthyroid control with direct proportion to TSH and inverse proportion to FT3[33]. Triglycerides did not significantly change after thyroxine replacement. This is in agreement with reports of several studies, which showed that triglycerides might be normalized or remain unchanged after treatment, suggesting a more complex cause of dyslipidaemia in hypothyroidism[34-43].

Total cholesterol inversely correlated to FT3 in the hypothyroid patients with a significant decrease after thyroxine therapy. This is consistent with results obtained by Melpomeni *et al* who found that restoration of an euthyroid state in hypothyroid patients was associated with a significant reduction in total cholesterol[33,43]. Our findings are also consistent with those demonstrated in several other studies[35,37-41,44].

The presence of some variations among different studies regarding the association between hypothyroidism and disturbed lipid profile may be explained by the variable effects of hypothyroidism on lipids according to the

severity of hypothyroidism in the studied groups of patients as evidenced by Sunanda *et al*[45]. Sunanda *et al*[45] studied the lipid profile in hypothyroid patients with different degrees of hypothyroidism and concluded that the effect of hypothyroidism on the serum lipids is more marked in patients with higher TSH levels.

So, the association between hypothyroidism and cardiovascular risk[45,46] may be attributed to the dyslipidaemic effect of hypothyroidism, underlying genetic factor or there may be another complex underlying mechanism that deserves further studies.

In conclusion, our study suggests that hypothyroidism has no impact on insulin sensitivity in overtly hypothyroid females of South Western Asian ethnicity. Thyroxine therapy does not cause significant change in insulin sensitivity in this ethnic group. So, other mechanisms that may explain the strong association between hypothyroidism and T2DM may exist. Although total cholesterol and triglycerides are not significantly higher in hypothyroid patients, thyroxine treatment leads to a significant reduction in total cholesterol without a significant effect on triglycerides. This may partially explain the association between hypothyroidism and cardiovascular risk.

COMMENTS

Background

Diabetes mellitus and hypothyroidism are the most common endocrine disorders. A strong association between both conditions exists. It was claimed that hypothyroidism increases the risk of developing diabetes mellitus through increased insulin resistance but studies in this field demonstrated conflicting data.

Research frontiers

Recent evidences suggest that hypothyroidism is associated with dyslipidaemia and increased cardiovascular risk.

Innovations and breakthroughs

The results presented herein show that in South Western Asian females, neither overt hypothyroidism nor thyroxine replacement has an effect on insulin resistance. Thyroxine therapy leads to a significant reduction in total cholesterol.

Applications

The authors study indicates that the increased risk of diabetes mellitus in hypothyroid patients cannot be attributed to increased insulin resistance. So, investigating other mechanisms that may be involved is highly encouraged. Thyroxine therapy leads to a significant reduction in total cholesterol but it does not affect triglycerides. This partially explains the association between hypothyroidism and increased cardiovascular risk.

Peer review

This is an interesting article about the effect of overt hypothyroidism and thyroxine therapy on insulin resistance and lipid profile in a specific ethnic population.

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World Journal of Diabetes

Online Submissions: http://www.wjgnet.com/esps/
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World J Diabetes 2013 August 15; 4(4): I-V
ISSN 1948-9358 (online)

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Name of journal

World Journal of Diabetes

ISSN

ISSN 1948-9358 (online)

Frequency

Bimonthly

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Editorial office
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World Journal of Diabetes
Editorial Department: Room 903, Building D,
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No. 62 Dongsihuan Zhonglu,
Chaoyang District, Beijing 100025, China
E-mail: wjd@wjgnet.com
http://www.wjgnet.com
Telephone: +86-10-85381891
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Publisher
Baishideng Publishing Group Co., Limited
Flat C, 23/F., Lucky Plaza,
315-321 Lockhart Road, Wan Chai, Hong Kong, China
Fax: +852-65557188
Telephone: +852-31779906
E-mail: bpgoffice@wjgnet.com
http://www.wjgnet.com

Production center
Beijing Baishideng BioMed Scientific Co., Limited
Room 903, Building D, Ocean International Center,
No. 62 Dongsihuan Zhonglu, Chaoyang District,
Beijing 100025, China
Telephone: +86-10-85381892
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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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5 **Vallancien G**, Emberton M, Harving N, van Moorselaar RJ; Alf-One Study Group. Sexual dysfunction in 1, 274 European men suffering from lower urinary tract symptoms. *J Urol* 2003; **169**: 2257-2261 [PMID: 12771764 DOI:10.1097/01.ju.0000067940.76090.73]

No author given

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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11 **Lam SK**. Academic investigator's perspectives of medical treatment for peptic ulcer. In: Swabb EA, Azabo S. Ulcer disease: investigation and basis for therapy. New York: Marcel Dekker, 1991: 431-450

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12 **Breedlove GK**, Schorfheide AM. Adolescent pregnancy. 2nd ed. Wieczorek RR, editor. White Plains (NY): March of Dimes Education Services, 2001: 20-34

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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

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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

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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 200201 03498. 2002 Aug 1

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