Sodium tungstate alleviates biomechanical properties of diabetic rat femur via modulation of oxidative stress

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Abstract. Diabetes mellitus leads to bone disorders such as osteopenia and osteoporosis that can increase fracture risk. On the other hand, sodium tungstate is an inorganic compound which exerts anti-diabetic activity in experimental studies due to its suggested insulin-mimetic or antioxidant activity. Therefore this study was designed to investigate the effect of tungstate on bone quality in diabetic rat femurs. The rats were divided into four groups: Control (C), tungstate-treated control (C+Tung), diabetes (STZ-D) and tungstate-treated diabetes (STZ-D+Tung). Diabetes mellitus was induced by single injection of streptozotocin (50 mg/kg). The treated rats received 150 mg/kg/day of sodium tungstate for 12 weeks. Sodium tungstate achieved a little (17%) but significant reduction on blood glucose levels, while it didn’t recover the reduced body weights of diabetic rats. In addition, impaired bone mechanical quality was reversed, despite the unchanged mineral density. Sodium tungstate administration significantly lowered the 2-thiobarbituric acid reactive substances and restored the activity of tissue antioxidant enzymes such as glutathione peroxidase, catalase and superoxide dismutase in diabetic rats. On the other hand, glutathione levels didn’t change in either case. These findings indicate that tungstate can improve the reduced mechanical quality of diabetic rat femurs due probably to reduction of reactive oxygen species and modulation of antioxidant enzymes as well as reduction in blood glucose levels.

Key words: Diabetic osteopenia — Sodium tungstate — Oxidative stress — Antioxidants — Bone quality — Mechanical strength

Introduction

Diabetes mellitus is a systemic disease which causes many complications such as nephropathy, neuropathy, retinopathy and osteopenia (Hamada et al. 2007). Diabetic osteopenia is manifested by increase in bone fracture (Bouillon 1991; Forsen et al. 1999) and a delay in healing of fractures (Cozen 1972; Herskind et al. 1992) and thus affects the quality of life. Although many human and experimental studies on the complications of diabetes mellitus have demonstrated extensive alterations in bone and mineral metabolism (Seino and Ishida 1995), the mechanisms responsible for diabetic osteopenia have not been clearly identified.

Many defects associated with diabetes, such as oxidative stress, hyperglycemia and body weight loss are suggested to be involved in pathogenesis of diabetic osteopenia. Among them, oxidative stress may be the prominent one, since it is known to induce cellular dysfunction in a wide variety of cell types including osteoblasts and osteoclasts (Hamada et al. 2007). Concerning bone metabolism, many studies have reported that oxidative stress inhibits osteoblastic differentiation (Mody et al. 2001; Bai et al. 2004; Hamada et al. 2007) and leads to osteoblast insults and apoptotic cell death (Chen et al. 2005; Fatokun et al.
et al. 2008). Of these elements, sodium tungstate (Na$_2$WO$_4$) which chemically resembles vanadium was shown to have antidiabetic activity in experimental studies based on its suggested insulinomimetic or antioxidant activity (Barbera et al. 1994, 2001; Munoz et al. 2001; Aydemir et al. 2012). Oral administration of sodium tungstate has been reported to normalize glycemia in many animal models of diabetes (Barbera et al. 1994, 1997, 2001; Munoz et al. 2001). Furthermore, it also increases the total amount and translocation of GLUT4 transporter in muscle (Giron et al. 2001). Furthermore, it also increases the total amount and translocation of GLUT4 transporter in muscle (Giron et al. 2001) and restores the hepatic metabolism of glucose in streptozotocine(STZ)-treated rats (Rodriguez-Hernandez et al. 2012). Stimulation of insulin secretion (Rodriguez-Gallardo et al. 2000) and regeneration of β-cell population (Barbera et al. 1997; Rodriguez-Hernandez et al. 2012) following tungstate administration was also reported in STZ-induced diabetes. Similar to vanadium, accumulation of tungstate was mainly recognized in bone tissue of mice after long-term oral administration (Guandalini et al. 2011). However, despite the abundance of studies that have been investigated the effects of vanadate compounds on bone tissue (Barrio et al. 1997; Etcheverry et al. 1997; Facchini et al. 2006) the impact of tungstate administration either in healthy or diseased state has not been defined yet.

Growing evidence and our previous results suggest that reactive oxygen species (ROS) play an important role in diabetes-induced abnormalities in various tissues and tungstate can reduce the upregulated oxidative stress parameters probably via restoration of antioxidant defense mechanisms (Hamada et al. 2007, 2009; Nakhaee et al. 2010; Blakytny et al. 2011; Aydemir et al. 2012). However, despite the above studies reporting the influence of tungstate administration on various tissues, there is no information on the effect of tungstate on bone biomechanical properties in diabetic rats. Therefore, we focused on antioxidant capacity and beneficial effects of tungstate in bone tissue of diabetic rats. In the present study, we investigated the effects of tungstate on bone mechanics and evaluated its potential antioxidant activity in prevention of diabetic osteopenia. Our results showed that tungstate can improve impaired bone properties in diabetic rats via reduction of oxidative stress and restoration of the activity of the endogenous antioxidant enzymes including glutathione peroxidase (GPx), superoxide dismutase (SOD) and catalase (CAT).

**Materials and Methods**

**Animals**

Eighty male Wistar rats, 90 days old and 250–300 g body weight (Akdeniz University, Faculty of Medicine, Animal Laboratory, Antalya, Turkey) were used for the present study. They were maintained in a well-ventilated controlled room on a 12-h light and 12-h dark cycle at 22°C room temperature. The animals had access to standard laboratory chow and water ad libitum. Experimental procedures were approved and carried out in accordance with Akdeniz University Animal Care and Use Committee’s guidelines. They were divided into four groups: Control (C; n = 20), tungstate-treated control (C+Tung; n = 20), diabetes (STZ-D; n = 20) and tungstate-treated diabetes (STZ-D+Tung; n = 20).

Diabetes was induced by intraperitoneal injection of STZ (Applichem GmbH, Germany) at the dose of 50 mg/kg body weight (STZ-D and STZ-D+Tung). After injection, blood glucose levels were monitored one week later by Accu-check glucometer (Roche Diagnostic, Turkey). Only rats with blood glucose levels higher than 300 mg/dl were enrolled in the study to ensure diabetic state.

Tungstate was dissolved in water and administered via oral gavages (150 mg/kg/day) during 12 weeks (C+Tung and STZ-D+Tung). The same amount of vehicle was administered to the age-matched control groups (C and STZ-D groups) for the same period. All animals were sacrificed by overdose of urethane anesthesia at the end of 12th week. Femurs were collected for biomechanical evaluations, bone mineral density (BMD) measurements and oxidative stress measurement.

**Measurement of BMD**

BMD of the excised femurs was determined by DEXA (Hologic Discovery QDR, 2009, USA) using a dedicated small animal software. Before the measurements, the instrument was calibrated by means of a Hologic phantom. After BMD measurements were performed on all bones, they were separated into two parts for biomechanical and biochemical experiments, respectively.

**Preparation of bone for biomechanical test**

The biomechanical properties of bone were measured using tensile test and ten femurs were used for each group. Test was performed with a computerized Lloyd testing machine (Lloyd LR5K, West Sussex, UK) and analyzed by Lloyd software. The biomechanical test was conducted by a 5-kN load cell and at a crosshead speed of 2 mm/min at room temperature. Following removal of the soft tissues around
the femurs, they were mounted vertically in the machine with the use of acrylic cement. During test, isotonic solution was regularly applied to prevent drying of the bones. Tensile test was applied until fracture of bones. The bones that dislodged from the acrylic cement during the mechanical test excluded from the experiments.

Calculation of biomechanical data

Typical force-displacement and stress-strain curves obtained from the tensile test were analyzed as described before (Donmez et al. 2012). By this way ultimate tensile load, ultimate tensile strength, strain, stiffness, tensile modulus (Young’s modulus) was measured. After fracture, the thinnest region of the femoral shaft was cut horizontally and pictured. Cortical surface areas were calculated from the pictures as square millimeter by using Image-J Software (U. S. National Institutes of Health, 2008, Bethesda, Maryland, USA).

Stiffness ($K$, N/mm) is determined from the slope of the elastic region of the load-displacement curve which represents the extrinsic stiffness by means of the following equation; $K = \Delta P/\Delta u$. The ultimate stress was calculated from the equation: $\sigma = P/A$ where $\sigma$ is the ultimate stress (MPa), $P$ is the failure load (N) and $A$ is the cortical area ($\text{mm}^2$).

The Tensile or Young’s modulus is a measure of the intrinsic stiffness of the material. The tensile modulus ($E$) can be simply calculated from the slope of stress-strain curve within the elastic region. Furthermore the tensile modulus (MPa) was then calculated as follows: $E = \Delta \sigma/\Delta \epsilon$ where $\epsilon$ is the strain. The strain (% displacement) was obtained by the equation: $\epsilon = \Delta L/L_0$, where $\Delta L$ is the change in the length and $L_0$ is the original length (Donmez et al. 2012).

Biochemical assay

One of each femora pair was used for mechanical test while the other one were pulverized in liquid nitrogen for biochemical assays. The weighed powdery of bones transferred equally into appropriate assay buffer (0.2 mg/ml) and homogenized by PRO200 Homogenizer (PRO Scientific Inc., Connecticut, USA).

2-thiobarbituric acid reactive substances (TBARS)

TBARS levels were measured by fluorometric analysis using 1,1,3,3-tetramethoxypropane as a standard (Wasowicz et al. 1993). Briefly, tissue samples (50 μl) were placed into a tube containing 1 ml of distilled water. One milliliter of a solution containing 29 mmol/l 2-thiobarbituric acid (TBA) in acetic acid (8.75 mol/l) was added to the samples then placed in a water bath at 95–100°C for 1 h. After the samples cooled, 25 μl of 5 mol/l hydrochloric acid (HCl) was added to the mixture. Reaction product was extracted from the samples with 3.5 ml of n-butanol, vortexed, than centrifuged at 3000 × g for 10 min. The butanol phase containing the extracted reaction product was separated and fluorescence was measured by spectrophotometry (Perkin Elmer Luminescence spectrometer, LS50B) using wavelengths of 525 nm for excitation and 547 nm for emission.

SOD Activity

The femora were homogenized in cold 20 mM HEPES buffer, pH 7.2, containing 1 mM EDTA. Then samples were centrifuged at 1,500 × g for 5 min at 4°C and supernatants were kept at –80°C until assayed. Superoxide activity was measured using a SOD activity assay kit (Cayman-706002) (Malstrom et al. 1975). One unit of SOD activity was defined as the amount of enzyme needed to exhibit 50% dismutation of superoxide radical.

CAT activity

The femora were homogenized in cold 50 mM potassium phosphate, pH 7.0, containing 1 mM EDTA. Tissue homogenates were centrifuged at 10,000 × g for 15 min at 4°C. CAT activity was measured using a CAT activity assay kit (Cayman-707002) (Johansson and Borg 1988). The method is based on the reaction of the enzyme with methanol in the presence of H$_2$O$_2$. The formaldehyde produced is measured spectrophotometrically with purpald (4-amino-3-hydrazino-5-marcapto-1,2,4-triazole) as the chromogen. One unit of enzyme activity was defined as the amount of enzyme that caused the formation of 1 μmol formaldehyde per minute at 25°C.

GPx activity

The femora were homogenized in cold 50 mM Tris-HCl buffer at pH 7.5, containing 1 mM EDTA. Tissue homogenates were centrifuged at 10,000 × g for 15 min at 4°C. GPx was measured using a GPx activity assay kit (Cayman-707002) (Johansson and Borg 1988). The GPx activity assay kit measures enzyme activity indirectly by a coupled reaction with glutathione reductase (GR). The oxidation of NADPH to NADP is accompanied by a decrease in absorbance at 340 nm. Absorbance kinetics was assessed spectrophotometrically at 340 nm by using the NADPH extinction coefficient of 0.00622 μM$^{-1}$·cm$^{-1}$. One unit of enzyme activity was defined as the amount of enzyme that caused the oxidation of μmol NADPH to NADP per minute at 25°C.

Glutathione (GSH) content

GSH was measured by a commercially available GSH assay kit (Cayman Chemical Ann Arbor, MI). Supernatants were
deproteinated in 10% metaphosphoric acid (Sigma Aldrich, Steinheim, Switzerland). The GSSG was reduced to GSH by GSH reductase in the assay cocktail of the kit containing 5,5’-dithiobis-2-nitrobenzoic acid (DTNB), glucose-6-phosphate de-hydrogenase, GSH reductase, nicotinamide adenine dinucleotide phosphate (NADP$^+$) and glucose-6-phosphate. The sulfhydryl group of GSH reacts with DTNB to give a yellow colored 5-thio-2- nitrobenzoic acid (TNB) which is measured at 405 nm wavelength.

**Statistical analysis**

One-way ANOVA was used for the first step analysis of the data. Then, multiple comparisons of the relevant groups were performed by posthoc Tukey test. The difference was accepted significant when $p < 0.05$.

**Results**

**Body weight and blood glucose levels**

The blood glucose level of group STZ-D was significantly higher when compared to group C at the end of the third month ($p < 0.05$). Despite a significant reduction in blood glucose levels of STZ-D+Tung group after treatment, they were higher than control levels ($p < 0.01$). However, the body weight of group STZ-D was significantly decreased with respect to group C rats at the end of the experimental period ($p < 0.05$) and tungstate did not prevent the weight loss of diabetic rats ($p > 0.05$) (Table 1).

**Biomechanical findings and BMD**

According to our results, mineral density was decreased in STZ-D group with respect to control values ($p < 0.05$). However, tungstate did not reveal any change in STZ-D+Tung group values (Figure 1). Tensile strength and strain values of group STZ-D was lower than group C, but despite a striking and significant restoration of tensile strength was achieved in STZ-D+Tung group femurs ($p < 0.05$), the reversal of strain values didn’t reach a significant level (Figure 1). A lower maximum load was detected in group STZ-D femurs and tungstate administration reversed this reduction. In conclusion, tungstate administration to the diabetic rats reversed the decreased femur strength, increased the maximum load and improved the strain values. However, Young modulus and stiffness were not significantly changed between experimental groups (Figure 1). Additionally, there were no significant differences in mechanical properties and BMD values of treated control animals with respect to untreated controls.

**Biochemical parameters of bone**

Diabetes induction revealed a highly significant increase in TBARS values of rat bones and administration of tungstate reversed this increment ($p < 0.05$). However, GPx activity was decreased markedly in group STZ-D femurs with respect to control values, despite a significant elevation in CAT activity. Activities of both enzymes were significantly reversed in STZ-D+Tung group ($p < 0.05$) bones. Neither diabetes-induction nor tungstate administration elicited significant alteration in measured activities of GSH and SOD enzymes ($p > 0.05$) (Figure 2). Unexpectedly, increased TBARS levels and reduced GPx activity were detected in normal rats after tungstate administration, although it didn’t induce any change in the other parameters.

**Discussion**

In this study we demonstrated that tungstate improved the biomechanical properties of diabetic rat bone significantly. In addition, increased oxidative stress and altered antioxidant enzymatic activities of bone tissue were restored after tungstate administration. Therefore this is the first study to show tungstate exerts beneficial effect on diabetic osteopenia due to its antioxidant activity and suppression of oxidative stress. These findings render the possible use

<table>
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<tr>
<th>Groups</th>
<th>Body weight (g)</th>
<th>Glucose levels (mmol/l)</th>
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<tr>
<td></td>
<td>Start</td>
<td>Final</td>
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<tr>
<td>C</td>
<td>323 ± 30</td>
<td>387 ± 37</td>
</tr>
<tr>
<td>C+Tung</td>
<td>321 ± 26</td>
<td>379 ± 44</td>
</tr>
<tr>
<td>STZ-D</td>
<td>312 ± 25</td>
<td>258 ± 44*</td>
</tr>
<tr>
<td>STZ-D+Tung</td>
<td>319 ± 35</td>
<td>244 ± 62**</td>
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All values are given as the mean ± standard deviation (SD). * $p < 0.05$ initial vs. final values. ** $p < 0.05$ vs. C group. † $p < 0.05$ vs. STZ-D group ($n = 20$ for all groups). C, control; C+Tung, tungstate-treated control; STZ-D, diabetes; STZ-D+Tung, tungstate-treated diabetes.
Figure 1. Effects of tungstate on biomechanical parameters in diabetic rat femur. A. BMD was decreased in streptozotocin-induced diabetes (STZ-D) group with respect to control (C) group ($p < 0.05$) but tungstate treatment did not reveal any change in STZ-D+Tung group values ($p > 0.05$). B, C, D. Tungstate treatment significantly improved the maximum load and the femur strength in diabetic rats' femur. E, F. Young modulus and stiffness of STZ-D group were not different between groups ($p > 0.05$). Data are represented as mean ± SEM. * $p < 0.05$ vs. C; # $p < 0.05$ vs. STZ-D ($n = 14–19$).
of this compound as a therapeutic agent for alleviation of diabetic osteopenia.

Diabetes mellitus leads to musculoskeletal abnormalities such as diminished bone formation, delayed bone healing, osteopenia and osteoporosis (Bouillon 1991; Herskind et al. 1992; Hamada et al. 2007). Lowered bone turnover associated with alteration in bone mass and biomechanical integrity of bone has been shown in experimental animal models of diabetes (Reddy et al. 2001; Follak et al. 2004, 2005; Facchini et al. 2006; Kidder et al. 2009). As a result of this decreased bone strength, energy absorption capacity and mineral content have been characterized in diabetic rats’ femur (Verhaeghe et al. 1994). During mechanical test such as bending, torsion and compression diabetes caused a decrease in bending strength, compression strength and tensile strength value of bone (Reddy et al. 2001; Facchini et al. 2006; Silva et al. 2009). Consistent with previous studies diabetic osteopenia was successfully induced by single dose injection of STZ and characterized by a sharp increase in blood glucose levels, reduced BMD and decreased bone biomechanical quality (Reddy et al. 2001; Facchini et al. 2006; Liang et al. 2011). In the light of our biomechanical results, bones from type 1 diabetic animals were less stiff and strong than control animals. Despite the value of tensile strength and strain were decreased in STZ-D group with respect to C group animals, tungstate administration improved these properties of diabetic rats’ femurs, even though it didn’t reverse the reduced BMD. This may be due to the fact that bone strength is relevant not only to bone mineralization but also to bone quality which is dependent primarily on bone turnover. Consistently, previous reports and the findings of the present study infer lowered bone turnover and thus impaired bone remodeling in diabetic osteopenia (Hamada et al. 2007, 2009; Blakytny et al. 2011). Therefore it is likely to suggest that this improved quality of femurs in tungstate-treated diabetic rats may stem from the alleviated bone turnover rather than augmentation of mineral content.

Tungstate administration has been shown to exert anti-diabetic effects in many experimental studies. Furthermore, it is proposed as an efficient oral anti-diabetic agent since it potentiates insulin effects in adipocytes (Goto and Kida 1995; Li 1995), stimulates insulin release in pancreas (Silves-
which are produced at low levels even in physiological conditions (Ozdemir et al. 2012). ROS comprises hydrogen peroxide, nitric oxide (NO) and peroxynitrite which are produced at low levels even in physiological conditions and scavenged by endogenous antioxidant systems such as GPx, GSH, SOD, and CAT. But when the balance between these two systems shifted due to increased free radical generation and/or downregulated defence mechanisms ROS can cause severe damage to DNA, proteins, and lipids (Hamada et al. 2009). We also measured a striking increase in TBARS levels of diabetic rat femurs which were restored by tungstate and thereby proved the potential antioxidant impact of this agent.

The altered activities of endogenous antioxidants in the diabetic rats reinforce our hypothesis that suggests oxidative stress is one of the major factors behind the low bone quality in these animals. Interestingly, we measured a striking decrease in GPx along with a significant increase in CAT. Up to date divergent observations have been seen for antioxidant enzymes in diabetes and the reasons for these discrepancies have not been addressed unequivocally. The tissue type under consideration, the expression level of the studied enzyme and the temporal changes in enzyme’s activity may be the possible reasons underlying these variations. Consistent with this the expression of CAT in bone tissue was found to be extremely low with respect to GPx (Lean et al. 2005). On the other hand, the preferential enzymatic pathway undertaken by cells for detoxification of ROS can be an alternative explanation. Under normal conditions, H$_2$O$_2$ is derived by dismutation of superoxide radical and detoxified by either CAT and/or GPx. At low concentration of H$_2$O$_2$ GPx is the predominant pathway whereas catalase appears to be the preferred pathway for detoxification at higher amounts of H$_2$O$_2$ (Pieper et al. 1995; Baud et al. 2004). This is due probably to extremely high Km of CAT than the Km of GPx which means CAT is particularly important when the clearance of H$_2$O$_2$ in high concentrations is required (Baud et al. 2004). In summary at early stages of diabetes GPx can be the scavenger enzyme preferentially, but lately when oxidative stress increased progressively it might inhibit GPx and activate CAT.

The increased TBARS levels along with decreased GPx activity in control animals after tungstate administration imply a pro-oxidant activity for tungstate in normal conditions as suggested by Sachdeva and Flora (2014). This infers that the effect of tungstate in diseased and normal conditions can be different, although we don’t know the reason of this discrepancy explicitly. However human and animal studies revealed that diabetes causes significant decrease in selenium which is located at the catalytic site of the enzyme GPx and its deficiency is associated mainly with decreased GPx activity (Kljai and Runje 2001; Gür 2004). Therefore it is likely to speculate that tungstate may substitute selenium and restores GPx function in diabetic rats while it facilitate free radical production in normal condition due to its excessive level. The demonstration of the exact mechanism of side-effects of tungstate particularly in healthy animals is an important point and further studies should unveil the cover of this issue.

Diabetes induced only a partial and insignificant decrease in SOD and GSH activities and tungstate administration reversed these decrements back to the control levels. Despite the contradictory results that present decreased or unchanged SOD activities (Pieper et al. 1995; Liang et al. 2011), the lack of significant difference can be attributed to the greater rate constant of SOD for dismutase of superoxide radical to form H$_2$O$_2$ than the subsequent reaction which
degrades H$_2$O$_2$ (Pieper et al. 1995). Moreover the tissue used for assessment of oxidative stress can be the reason of these differences in enzymes’ activity. It is important to note that although the assessment of systemic oxidative stress products and antioxidant enzymes’ status is so common in previous reports (Hamada et al. 2007, 2009; Liang et al. 2011) the local measurements of those parameters such as TBARS, GSH, GPx, SOD and CAT are rarely performed. In this manner, our study provides direct evidence about the intensity of oxidative stress in bone tissue specifically and led to suggest a causal relationship between increased ROS production and etiology of diabetic osteopenia.

This study clearly shows that tungstate is capable of increasing quality of bone and its resistance to fracture in diabetic rats due probably to mitigated oxidative stress and partially improved glycemic status. Hence, tungstate exerts beneficial effect in diabetic osteopenia owing to its combined antioxidant activity and blood glucose improving capacity.

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Conflict of interest: The authors have no conflicts.

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