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Original article

Effect of treatment with vitamin D plus calcium on oxidative stress in streptozotocin-induced diabetic rats

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ABSTRACT

Background: In diabetes mellitus, uncontrolled hyperglycemia has been reported to induce oxidative stress, which may lead to health complications. Vitamin D, however, acts as a non-enzymatic antioxidant to protect cells against oxidative stress and damage.
Objective: To investigate the antioxidative effect of vitamin D combined with calcium in streptozotocin (STZ)-induced diabetic rats.
Methods: Rats were divided into four groups (ten rats in each group). The first group (control) received a normal diet and water. The second group, including STZ-induced diabetic rats (diabetic controls), received a normal diet and water. The third group, also including STZ-induced diabetic rats, received vitamin D (2000 IU/day) with calcium (500 mg/kg/day) orally for 28 consecutive days. The fourth group consisted of STZ-induced diabetic rats that received insulin treatment for 28 consecutive days. Activities of superoxide dismutase (SOD), glutathione peroxidase (GPO) and catalase were measured in the liver tissues. The level of malonaldehyde (MDA) was measured in the plasma.
Results: Diabetic rats showed a significant decrease in the activities of SOD, GPO and catalase compared to normal rats. Oral administration of vitamin D with calcium to diabetic rats caused a significant increase in the activities of SOD, GPO and catalase compared with the untreated group. Furthermore, the plasma level of MDA was significantly elevated in diabetic rats compared to normal rats. Diabetic rats treated with vitamin D and calcium had a significantly reduced level of MDA, suggesting that vitamin D with calcium played a vital role in the protection of tissues from damage by free radicals.
Conclusion: Oral supplementation with vitamin D and calcium may be a useful treatment for diabetic patients to reduce/prevent the pathological complications of diabetes.

1. Introduction

Diabetes mellitus is a metabolic disorder characterized by chronic hyperglycemia (American Diabetes Association, 2013). There are two major types of diabetes: insulin-dependent diabetes mellitus (type 1), which results from impaired insulin production by islet cells, and non-insulin-dependent diabetes mellitus (type 2), which results from insulin resistance (American Diabetes Association, 2013).

In diabetes, long-term uncontrolled hyperglycemia has been reported, which may cause health complications including cardiovascular disease and damage to organs such as the nerves, kidneys, eyes, and feet (English and Williams, 2004; Hunt et al., 1988; Jaganjac et al., 2013; Paneni et al., 2013). Furthermore, chronic hyperglycemia can also induce oxidative stress and stimulate free radical formation in humans as well as in animal models (Evans et al., 2002; Low and Nickander, 1991; Singh et al., 2001).

Oxidative stress is a condition that results from excessive production of reactive oxygen species (ROS) and a diminished antioxidant system (Halliwell and Gutteridge, 2007), which leads to

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many pathological conditions, such as cancer, cardiovascular disease, and diabetes (Al-Abrash et al., 2000; Matés et al., 1999).

It is believed that oxidative stress is the main factor that induces secondary complications of diabetes mellitus, such as injury and foot ulceration (Baynes and Thorpe, 1996; Sivitz and Yorek, 2010). In cell membranes, polyunsaturated fatty acids are the primary targets of ROS, which can interact with the cell membrane lipid bilayer and cause lipid peroxidation (Cade, 2008; Halliwell and Chirico, 1993). One of the stable products of lipid peroxidation is malonaldehyde (MDA), which serves as a biomarker for the peroxidation of polyunsaturated fatty acids and oxidative stress (Halliwell and Chirico, 1993). MDA is a highly reactive aldehyde and has mutagenic effects (Esterbauer et al., 1990). As a result, excessive production of MDA can lead to inactivation of many cellular proteins and receptors (Esterbauer et al., 1990; Marnett, 1999; Siu and Draper, 1982).

Recently, vitamin D has drawn more attention as a non-enzymatic antioxidant compound. At physiological concentrations, vitamin D plays a very important role in protecting cells from oxidative stress and cellular damage through a mechanism involved in reducing the plasma level of MDA and increasing the total antioxidant capacity in the plasma (Bhat and Ismail, 2015; Foroozanfard et al., 2015).

Vitamin D deficiency has been reported to induce oxidative stress in both *in vivo* and *in vitro* studies (Foroozanfard et al., 2015; Ke et al., 2016; Lin et al., 2005). In rat muscle, vitamin D deficiency was associated with reductions in the activities of superoxide dismutase (SOD) and catalase enzymes, causing mild oxidative stress in the muscle (Ke et al., 2016). On the other hand, administration of vitamin D caused a significant increase in the activity of SOD in rat muscle and a reduction in oxidative stress (Ke et al., 2016). In comparison to other non-enzymatic antioxidants, vitamin D was shown to have a significantly higher antioxidant effect compared to vitamin E, melatonin and β-estradiol, as seen in an *in vitro* study (Lin et al., 2005).

The ability of vitamin D to control free radical generation and reduce oxidative stress may be exerted either through binding to the vitamin D receptor (VDR) in the nucleus or through the hydrophobic parts of vitamin D, depending on the type of cell. In mature erythrocytes, vitamin D has shown a potential protective role against oxidative stress and lipid peroxidation, even in cells that lack a nucleus, supporting the idea that the antioxidant effect of vitamin D on cell membranes directly occurs through its hydrophobic parts (Wiseman, 1993). In contrast, vitamin D plays an important role in controlling free radical generation in the liver cells of diabetic mice through binding to the VDR in the cell nucleus (Labudzynskyi et al., 2015).

Little information is available regarding the effect of vitamin D on reducing oxidative stress in diabetes mellitus. A few studies have shown that vitamin D alone or in combination with calcium may play an important role in reducing the incidence of oxidative stress in diabetes (Foroozanfard et al., 2015; Nikooyeh et al., 2014). A study by Foroozanfard et al. (2015) showed that supplementation of vitamin D combined with calcium caused a greater reduction in the plasma level of MDA and increased the total antioxidant capacity in the plasma compared with either vitamin D or calcium separately.

The present study aimed to investigate the effect of oral supplementation of vitamin D combined with calcium on lipid peroxidation and the activities of antioxidant enzymes in streptozotocin (STZ)-induced diabetic rats to assess whether vitamin D with calcium can reduce the oxidative stress caused by diabetes. Furthermore, this study sought to examine whether supplementation of vitamin D combined with calcium has similar effects to those of insulin.

2. Materials and methods

2.1. Reagents

Reagents and chemicals were purchased from Sigma-Aldrich (MO, USA), unless otherwise stated.

The solutions used in this experiment were prepared as follows:

Na-citrate (0.1 M) was prepared by dissolving 14.71 g of Na-citrate in 200 ml water. Citric acid (0.1 M) was prepared by dissolving 20.1 g of citric acid in 200 ml water. Na-citrate buffer was prepared by mixing 0.1 M Na-citrate and 0.1 M citric acid, followed by adjusting the pH to 4.5 with 0.1 M citric acid. STZ solution was prepared by dissolving 675 mg of STZ in 15 ml citrate buffer.

2.2. Animals

Male Wistar rats (n = 40) aged 8–12 weeks and weighing 120 g were provided for use by the King Fahad Research Centre (Jeddah, Saudi Arabia). Rats were housed in groups of three rats/cage at 22–24 °C and exposed to alternating cycles of 12 h of light and darkness with access to water and food provided *ad libitum*. The rats were kept for two weeks in a laboratory for habituation. The study has been approved by the Research Ethics Committee of Tabuk University. All experiments were performed under ethyl carbamate anesthesia, and we ensured that the animals did not suffer at any stage of the experiments.

2.3. STZ induction of diabetes

Forty adult Wistar rats were used in this study. Ten rats were used as controls, while thirty rats were used for inducing diabetes. Diabetes was induced in the rats via intraperitoneal injection of a freshly prepared solution of STZ (45 mg/kg body weight) in 0.1 M citrate buffer at pH 4.5 after overnight fasting. Blood glucose levels were examined 72 h after STZ injection by obtaining blood from the lateral saphenous vein and measuring the glucose level with a glucometer. Rats with blood glucose levels greater than 300 mg/dl were considered diabetic.

2.4. Treatment

Rats were grouped randomly into four groups (ten rats in each group). The first group (control) received a normal diet and water. The second group, including STZ-induced diabetic rats (diabetic control), received a normal diet and water. The third group, also including STZ-induced diabetic rats, received a single daily oral dose of 2,000 IU of vitamin D3 with 500 mg/kg body weight of calcium for 28 consecutive days. The fourth group contained STZ-induced diabetic rats that received subcutaneous initial doses of 4–8 units insulin followed by 1–2 units daily to obtain euglycemia for 28 consecutive days. All groups of rats were maintained in standard environmental conditions and fed a standard diet and water. Body weight was measured weekly during this study.

During the study, four of the ten STZ-induced diabetic rats (diabetic control) died after 21 days of diabetic induction. One of the ten STZ-induced diabetic rats treated with insulin died two days after diabetic induction.

2.5. Sample preparation

Blood samples were collected from each animal by puncturing the sub-lingual vein and then placed in a clean and dry test tube. The samples were left for 10 min to clot, followed by centrifugation at 5000 rpm for 15 min. The serum was collected and stored at −80 °C until use to measure the level of MDA.

2.6. Preparation of tissue homogenate

The liver tissues were accurately weighed (0.5 g m from each liver) and homogenized in 5 ml phosphate buffer (pH 7.4) using an electrical homogenizer to prepare 10% (w/v) clear tissue homogenate for determination of the glutathione peroxidase, catalase and SOD activities.

2.7. Evaluation of antioxidant enzymes

1. **SOD** activity in the supernatant was measured according to the method suggested by Nishikimi and Appaji (Nishikimi et al., 1972). Results were defined as units per milligram protein (U/mg protein).
2. **Catalase** activity in the supernatant was determined according to the method described by Aebi (1984) Results were reported as the rate constant per second per milligram protein (k/mg protein). Measurements of the decomposition of hydrogen peroxide (H_2O_2) were performed spectrophotometrically according to the method suggested by Aebi (1984) in the UV area of the light spectrum (240 nm, reading every 0.1 sec for 65 sec) using Lambda Software for data computation.
3. **Glutathione peroxidase** activity in the supernatant was measured according to the method described by Griffith (1980). Results were defined as the units per milligram protein (U/mg protein).

2.8. MDA determination

Estimation of MDA was performed in the plasma using a thiobarbituric acid-reactive substance assay according to the method described by Ohkawa et al. (1979).

2.9. Statistical analysis

All data were statistically analyzed using a commercially available software package (InStat, GraphPad Software, San Diego, California, USA). Differences between the groups were measured by one-way analysis of variance (ANOVA) followed by Dunnett's posttest. Results are expressed as the mean ± standard error of the mean (SEM). P values ≤ 0.05 were considered significant.

3. Results

Blood glucose levels were examined at 3, 10, 17, 24, and 31 days post-injection. Elevated blood glucose levels (337 ± 4.3) were observed at the third day post-injection. Untreated diabetic rats showed a significant increase in glucose levels (409.5 ± 1.7, $P < 0.001$) compared with those of normal rats (data not shown).

Activities of catalase, glutathione peroxidase, and SOD were measured in the liver tissues of all groups of rats, and the level of MDA was measured in the plasma of all groups of rats. Data are presented as the mean ± SEM (Table 1).

Table 1
Effects of oral supplementation of vitamin D combined with calcium on the biomarkers of oxidative stress in STZ-induced diabetic rats after 28 days of treatment. Data are shown as the mean ± standard error of mean (SEM).

Parameters	Control	STZ-induced diabetic rat groups		
		Untreated	Treated with vitamin D & Ca	Treated with insulin
Catalase				
Mean ± SEM	18.23 ± 0.08	2.79 ± 0.11	10.8 ± 0.1	15.89 ± 0.1
%Improvement			43.94%	71.86%
%Change		−84.7 [a]%	−40.76[a]%	−12.84 [a]%
			287[b]%	469.5[b]%
Significance		P < 0.001[a]	P < 0.001[a]	P < 0.01[a]
(ANOVA, P)			P < 0.001[b]	P < 0.001[b]
Glutathione peroxidase				
Mean ± SEM	12.96 ± 0.07	1.98 ± 0.04	8.54 ± 0.06	9.03 ± 0.05
% Improvement			50.61%	54.39%
%Change		−84.72[a]%	−34.1[a]%	−30.3[a] %
			331[b]%	356[b]%
Significance		P < 0.001[a]	P < 0.01[a]	P < 0.01[a]
(ANOVA, P)			P < 0.001[b]	P < 0.001[b]
Superoxide dismutase (SOD)				
Mean ± SEM	653.43 ± 7.2	129.3 ± 2.1	526.2 ± 2.7	463.1 ± 3.7
%Improvement			60.74%	51.1%
%Change		−80.2[a]%	−19.5[a]%	−29[a]%
			307[b]%	258[b]%
Significance		P < 0.001[a]	P < 0.05[a]	P < 0.001[a]
(ANOVA, P)			P < 0.001[b]	P < 0.001[b]
Malonaldehyde (MDA)				
Mean ± SEM	16.23 ± 0.09	58.76 ± 1.7	29.54 ± 0.3	20.55 ± 0.19
%Improvement			−180%	−235.4%
%Change		262%	82[a]%	26.62[a]%
			−49.7[b]%	−65[b]%
Significance		P < 0.001[a]	P < 0.001[a]	P < 0.001[a]
(ANOVA, P)			P < 0.001[b]	P < 0.001[b]

%Improvement = mean treated-mean untreated/mean control × 100.
%Change (a) = mean treated-mean control/mean control × 100.
%Change (b) = mean treated-mean untreated/mean untreated × 100.
P (a) = ANOVA significance in comparison with control.
P (b) = ANOVA significance in comparison with untreated.

The untreated group of STZ-induced diabetic rats showed a significant decrease ($P < 0.001$) in the activities of catalase (by −84.7%, 2.79 ± 0.11), glutathione peroxidase (by −84.72%, 1.98 ± 0.04), and SOD (by −80.2%, 129.3 ± 2.1) compared with those of normal rats. On the other hand, diabetic rats treated with vitamin D combined with calcium showed a significant increase ($P < 0.001$) in the levels of catalase (by 287%), glutathione peroxidase (by 331%), and SOD (by 307%) compared with those of the untreated diabetic rats. Diabetic rats treated with insulin showed a significant increase ($P < 0.001$) in the levels of catalase (by 469.5%), glutathione peroxidase (by 356%), and SOD (by 258%) compared with those of the untreated diabetic rats.

The level of MDA was significantly increased ($P < 0.001$) in the untreated group of STZ-induced diabetic rats (by 262%, 58.76 ± 1.7) compared with the normal rats (16.23 ± 0.09). Both diabetic rats treated with vitamin D (2000 IU) combined with calcium (500 mg/kg body weight) and those treated with insulin showed a significant reduction ($P < 0.001$) in the level of MDA (29.54 ± 0.3 and 20.55 ± 0.19, respectively) in comparison with untreated diabetic rats.

4. Discussion

A growing body of evidence has reported the elevation of oxidative stress in diabetes mellitus (Halliwell and Gutteridge, 2007; Low and Nickander, 1991; Singh et al., 2001). This increase in oxidative stress in diabetes may result from impairment of the antioxidant system. For example, Sindhu et al. observed that the expression of antioxidant enzymes were decreased in diabetic rats compared to controls (Sindhu et al., 2004). Furthermore, a study carried out by Ramakrishna and Jailkhani showed a significant reduction in the level of non-enzymatic antioxidants (vitamin C and E), as well as antioxidant enzymes, in diabetes patients (Ramakrishna and Jailkhani, 2008). Antioxidant therapy and control of the glucose level by insulin therapy has been reported to alleviate oxidative stress (Ramakrishna and Jailkhani, 2008; Sindhu et al., 2004). A few studies have observed that vitamin D alone or in combination with calcium may play a role in reducing the incidence of oxidative stress in diabetes (Foroozanfard et al., 2015; Nikooyeh et al., 2014).

The current study was designed to investigate the effect of oral supplementation with vitamin D combined with calcium on lipid peroxidation and the activities of antioxidant enzymes in STZ-induced diabetic rats and to determine whether vitamin D with calcium can reduce the oxidative stress caused by diabetes. Furthermore, this study attempted to examine whether supplementation with vitamin D combined with calcium is able to produce effects similar to those of insulin.

To investigate the effects of vitamin D and calcium on the activities of antioxidant enzymes in STZ-induced diabetic rats, the activities of SOD, catalase and glutathione peroxidase were estimated in the liver tissues of all groups. These enzymes act complementarily to each other to eliminate the overproduction of ROS. Subsequently, any defect in their activity or synthesis causes oxidative stress (Chelikani et al., 2004; Finkel and Holbrook, 2000; Fridovich, 1986; Jang et al., 2000).

SOD is the first enzyme that encounters superoxide radicals and converts them into less reactive compounds, namely, hydrogen peroxide (H_2O_2) (Fridovich, 1986). Catalase and glutathione peroxidase enzymes convert hydrogen peroxide (H_2O_2) into oxygen and water molecules (Chelikani et al., 2004; Finkel and Holbrook, 2000). Therefore, the decreases in the activity of SOD causes accumulation of free radicals and leading to oxidative stress, tissue destruction, and metabolic defects.

In STZ-induced diabetic rats, the activity of SOD is not fully understood. Some studies have reported elevation of the activity of SOD in diabetic rats (Jang et al., 2000), whereas other studies have reported a reduction in the activity of SOD in diabetic animals (Aragno et al., 1999; Maritim et al., 1999). This decrease in the SOD activity in diabetic animals could be combined with a reduction in the level of glutathione (Koo et al., 2002), which plays an essential role in stimulating SOD synthesis (Bravenboer et al., 1992).

Our results reported a significant decrease in the activity of SOD (by −80.2%) in diabetic rats compared with normal rats. On the other hand, diabetic rats treated either with vitamin D combined with calcium or with insulin showed a significant increase in the activity of SOD (by 307% and 258%, respectively) compared with untreated diabetic rats.

Catalase and glutathione peroxidase constitute the second line of defense in the antioxidant system. Catalase is expressed in all living cells that require oxygen for normal metabolism. Catalase is a metalloenzyme that contains iron as a cofactor, and this enzyme catalyzes the decomposition of H_2O_2 molecules into water (H_2O) and oxygen (O_2) (Chelikani et al., 2004; Tiwari et al., 2013). Catalase plays a vital role in protecting pancreatic β-cells from damage by free radicals. Pancreatic β-cells are rich in mitochondria, and catalase deficiency in these cells leads to excessive production of ROS, inducing the oxidative stress and cellular dysfunction seen in diabetes mellitus (Góth and Eaton, 2000).

Glutathione peroxidase is a selenium-containing enzyme that causes the decomposition of H_2O_2 into water and oxygen using glutathione as a hydrogen donor. When the level of oxidative stress is low, glutathione peroxidase is the major enzyme that protects cells from damage caused by ROS (Birben et al., 2012; Tiwari et al., 2013).

In the current study, we reported that the activities of catalase and glutathione peroxidase were significantly decreased in diabetic rats (by −84.7% and by −84.72%, respectively) compared with those in normal rats. However, daily oral supplementation of vitamin D combined with calcium resulted in a significant increase in the activities of catalase (by 287%) and glutathione peroxidase (by 331%) in diabetic rats compared with untreated diabetic rats. A similar effect has been reported when insulin was administered to diabetic rats. Insulin caused a significant elevation of the activities of catalase (by 469.5%) and glutathione peroxidase (by 356%) compared to those in the untreated diabetic group.

The reduction of the activities of the antioxidant enzymes in diabetic rats reported in our study lends further support to the hypothesis that oxidative stress in diabetes mellitus results from an impaired antioxidant system (Ramakrishna and Jailkhani, 2008; Sindhu et al., 2004). However, vitamin D in combination with calcium increased the activity of antioxidant enzymes, providing additional evidence for the role of vitamin D and calcium in reducing oxidative stress and protecting cell membranes from damage caused by ROS. The mechanism by which vitamin D and calcium can activate antioxidant enzymes remains understudied. One possible explanation is that vitamin D stimulates the synthesis of metal ion protein carriers, such as Zn and Cu (Claro da Silva et al., 2016), leading to increases in the bioavailability of these ions in the cell. These metal ions act as cofactors required for the activity of several enzymes, including antioxidant enzymes.

On the other hand, our results show that treating diabetic rats with insulin caused a significant increase in catalase activity compared with the effect of treatment with vitamin D combined with calcium. Insulin may influence the activity of catalase in different ways. First, insulin is responsible for stimulating iron uptake by cells and increasing the synthesis of ferritin (stored iron), causing an increase in the body storage of iron, which is required as a cofactor of catalase (Davis et al., 1986; Yokomori et al., 1991).

Second, insulin has been reported to upregulate the expression of catalase in diabetic rats (Sindhu et al., 2004).

It is well known that MDA is the stable end product of lipid oxidation and serves as a biomarker of lipid damage and oxidative stress (Halliwell and Chirico, 1993). In diabetes mellitus, hyperglycemia was reported to induce excessive ROS production (Evans et al., 2002; Low and Nickander, 1991; Singh et al., 2001). These ROS interact with unsaturated lipids within the cell membrane, causing lipid peroxidation. As a result, the level of MDA becomes markedly increased, leading to inactivation of many cellular proteins and receptors and inducing cell membrane damage (Esterbauer et al., 1990; Marnett, 1999; Siu and Draper, 1982).

To evaluate lipid peroxidation in STZ-induced diabetic rats, the serum levels of MDA were measured. The results of our study showed that the serum level of MDA was significantly elevated (by 262%, 58.76 ± 1.7) in diabetic rats compared with controls. These results confirm previous reports that also showed an elevated level of MDA in diabetes mellitus in both human and animal studies (Ramakrishna and Jailkhani, 2008; Sindhu et al., 2004).

On the other hand, diabetic rats treated with vitamin D (2000 IU) combined with calcium (500 mg/kg body weight) showed a significant reduction in the level of MDA compared with that in untreated diabetic rats. This finding provides further support for the hypothesis that vitamin D has an antioxidant effect and potentially protects tissues from the damage induced by lipid oxidation. Furthermore, consumption of vitamin D as an antioxidant may explain why the vitamin D level is significantly decreased in diabetic patients (Mathieu et al., 2005; Song et al., 2013).

In contrast, diabetic rats treated with insulin showed a significant reduction in the level of MDA (by −65%) in comparison to untreated diabetic rats. These results agree with previous results showing that insulin therapy normalized the level of MDA in diabetic rats (Sindhu et al., 2004). Taken together, our results show that vitamin D with calcium may mimic the effect of insulin, in reducing the MDA level and the level of lipid peroxidation.

In conclusion, the results of the present study show the beneficial effects of vitamin D combined with calcium in reducing lipid peroxidation and oxidative stress, as observed in STZ-induced diabetic rats by the reduction of the MDA level and improvement of the activities of antioxidant enzymes, such as SOD, catalase, and glutathione peroxidase. Our results suggest that complications in the pathogenesis of diabetes mellitus result from oxidative stress and could be prevented or limited by oral supplementation of vitamin D combined with calcium.

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Conflicts of interest

The authors have no conflicts of interest to declare.

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