# Effects of Aldosterone Receptor Antagonist on Vascular Calcification and Bone Disorder in Streptozotocin-Induced Diabetic Rat

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**Abstract:** Background: Vascular calcification and bone disorders are increasingly recognized problems in patients with diabetes due to calcium dyshomeostasis is a major risk factor for cardiovascular morbidity and mortality. Diabetic osteoporosis seems to be dependent on qualitative and quantitative alterations of the bone, as well as microangiopathic complications of diabetes mellitus. Aim: We investigated calcium dyshomeostasis, and bone histological and metabolic abnormalities in Streptozotocin-induced Type 1 Diabetes Mellitus in rats. The possible role of the aldosterone receptor antagonist, spironolactone, in reversing these effects was assessed. Materials and Methods: Adult Female Wistar rats were divided into three groups: Control group, Streptozotocin-induced diabetic group (STZ-D), and Aldosterone-receptor antagonist-supplemented diabetic group (ARA-STZ). Diabetes was induced by a single intraperitoneal injection of streptozotocin, 40 mg/Kg BW. Spironolactone (aldosterone receptor antagonist) was given by oral gavage in a daily dose of 15 mg/kg BW for 4 weeks. At the end of the experiment, serum levels of calcium, phosphate, and alkaline phosphatase were evaluated. Histological examination of the tibia was performed, together with analysis of renal vascular calcification and Immunohistochemistry for inducible nitric oxide synthase (iNOS) in renal tissue specimens. Results: STZ-D rats showed normophosphatemia and significant hypercalcemia with significantly increased serum alkaline phosphatase compared to control group. Bone loss was also observed. Histological examination of the small renal blood vessels showed calcification in the walls, as well as, reduction in iNOS immunostaining. These metabolic and histological abnormalities in STZ-D rats were remarkably corrected by the administration of spironolactone. Conclusion: The current results underscore the important role of aldosterone in promoting vascular calcification and osteoporosis in diabetic rats and the potential role of aldosterone receptor antagonist, spironolactone, in correcting these clinical problems in diabetic rats.
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**Key words:** $Ca^{2+}$ homeostasis, osteoporosis, vascular calcification, DM, Aldosterone.

## 1. Introduction:

Diabetes mellitus (DM) is associated with increased cardiovascular mortality compared to the general population. The etiology of the increased mortality is multi-factorial, but recent data supports that vascular calcification may be a contributing factor (Al-Aly, 2007). In agreement, Chen *et al.* (2006) reported that vascular calcification is more common in patients with DM compared with the general population and is associated with increased mortality, stroke and amputations.

Studies on skeletal involvement in patients with DM have generated conflicting results, largely because of the pathogenetic complexity of the condition (Carnevale *et al.*, 2004). Cohort studies carried out on large samples indicate that diabetic patients (with both type 1 and type 2 disease) have a higher risk for fracture, in particular for hip fracture, the most dangerous osteoporotic complication. This seems to be dependent both on qualitative and quantitative alterations of the bone, as well as on extra-skeletal factors due to, for example, microangiopathic complications of the disease (Carnevale *et al.*, 2004).

Also, studies on bone mineral characteristics in children with type 1 DM have generated conflicting results. Many studies have shown that children with type 1 DM are at risk of having a decrease in bone mass (Valerio *et al.*, 2002, Heap *et al.*, 2004 and Moyer-Mileur *et al.*, 2004), whereas other studies found no bone mineralization abnormalities in diabetic children (Pascual *et al.*, 1998, De Schepper *et al.*, 1998 and Liu *et al.*, 2003). However, diabetic osteoporosis is increasingly recognized as a significant co-morbidity of type 1 DM (Thrailkill *et al.*, 2005).

Giachelli *et al.*, (2005) reported that local environmental cues such as disturbances in phosphate or calcium levels control vascular calcification that is associated with vascular smooth muscle cells (VSMCs) phenotyping modulation. VSMCs are implicated to have the capacity to undergo

modulation from a contractile to an osteochondrogenic phenotypic state.

The classic understanding of aldosterone as a hormone produced by the adrenal cortex and involved in the reabsorption of sodium and the secretion of potassium and protons in the collecting duct needs to be extended. Aldosterone is found to be generated in many other tissues besides the adrenal cortex; the most convincing evidence relates to the central nervous system. However, suggestions that aldosterone is produced in the heart remain controversial (Connell and Davies 2005). Recent studies have begun to explore the molecular mechanisms of the direct actions of aldosterone on the cardiovascular system. They have demonstrated the expression of functional mineralocorticoid receptors (MRs) in the heart, large arteries, VSMCs, and endothelial cells (Nishiyama *et al*., 2009). Recent human and animal studies suggest that activation of the MRs by aldosterone causes diabetic complications such as; microvascular damage, vascular inflammation and endothelial dysfunction (McFarlane and Sowers 2003). Aldosterone is capable of inducing cardiac fibrosis characterized by enhanced accumulation of collagen and increased fibroblast proliferation *in vivo*. In a previous study, we demonstrated that a low-dose MRs antagonist therapy (Spironolactone) markedly reduced cardiac fibrosis in diabetic rats (Saleh and Saleh, 2005). Moreover, recently, Aldosterone has been reported to play a relevant role in vascular calcification (De Solís *et al*., 2008). MRs in VSMCs, in response to aldosterone, modulates expression of osteogenic genes including alkaline phosphatase (ALP) stimulating vascular calcification (Jaffe *et al*., 2007).

The reported benefits of aldosterone receptor antagonists (ARA) in diabetic nephropathy, despite reported normal or low levels of plasma aldosterone, suggest that this hormone may be produced locally within the kidney (Xue and Siragy, 2005) and this local renal production of aldosterone was confirmed by Taira *et al*. (2008). The local renal aldosterone system is regulated by insulin-deficient diabetic hyperglycemia (Xue and Siragy, 2005). In animals treated with streptozotocin (STZ), a significant increase in renal aldosterone synthase mRNA was reported (Xue and Siragy, 2005).

From the above mentioned data we hypothesize that elevation of aldosterone in type 1 DM could play a role in $Ca^{2+}$ dyshomeostasis and vascular calcification. Thus, interruption of the system would be expected to produce parallel improvement in vascular and bone outcome. Many human and animal studies suggest that angiotensin converting enzyme (ACE) inhibitors and angiotensin receptor blockers (ARBs) are unable to provide long-term aldosterone suppression (Naruse *et al*., 2002 and Otani *et al*., 2008). Therefore, we hypothesize that aldosterone antagonism could be a rational therapy rather than ACE inhibitors.

So, this work was designed to clarify the possible role of aldosterone antagonist on $Ca^{2+}$ dyshomeostasis and vascular calcification in type 1 DM.

**2. Material and Methods:**

This work was performed on 17 female Wistar rats aged 12-14 months. The rats were maintained under standard conditions of boarding. The investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health. Rats were allocated into 3 groups: a) Control group, injected intraperitoneally (i.p) with citrate buffer (n=6). b) Streptozotocin-induced diabetic (STZ-D) group, (n=5). c) Aldosterone-receptor antagonist-supplemented diabetic group (ARA-STZ) (n=6); where diabetic rats were orally supplemented with spironolactone, an aldosterone receptor antagonist (15 mg/Kg BW), by gavage for 4 weeks starting 3 days from induction of diabetes, when these rats were proved to be diabetic.

Induction of diabetes:

Rats destined to be diabetic were given a single intraperitoneal injection of STZ (Sigma) in a dose of 40 mg/kg body weight, dissolved in 2 ml of 0.05 M citrate buffer according to Yoshino *et al*, (1992). Because of unstable nature of STZ, it was freshly dissolved 10 minutes just prior to injection. Citrate buffer was prepared by adding 47 ml of 0.05 M citric acid (Vabberlin - chemic) to 53 ml of 0.05 M trisodium citrate dehydrate (Adwic). Both were prepared according to Dawson *et al*. (1986). pH of citrate buffer was adjusted exactly at 4.5. Rats received 5% sucrose solution for the first 48 hours after STZ injection to minimize deaths from hypoglycemia. After 72 hours from STZ injection, tail blood samples were obtained by sharp cut and blood glucose concentration was determined by using glucose strips supplied from Boehringer Mannheim using Reflolux S-type 117 2115 6 V apparatus in order to ensure successful induction of diabetes in STZ-treated animals. Rats having blood glucose below 200 mg% were considered non-diabetic and were excluded from further study. STZ-induced diabetic rats had free access to food and water. Blood glucose was reestimated before sacrifice and only rats with blood glucose above 200 mg % were included in the study.

Experimental procedures:

On the day of sacrifice, overnight fasted rats were weighed and anaesthetized with thiopental sodium 40 mg/kg intraperitoneally (Sandoz GmbH, Kundl-Austria). Blood sugar was tested using glucose strips. The blood samples were collected from the abdominal aorta then centrifuged, and the serum was stored at -20°C for biochemical analysis.

Biochemical determinations:

Serum calcium, phosphate and alkaline phosphatase levels were measured using standard laboratory methods.

Histological examination of bone: Bone segments (proximal end of tibia) were removed. The tibias were dissected out, fixed in 10% buffered neutral paraformalehyde, and decalcified in EDTA solution for 2 weeks. Once decalcified, the specimens followed routine histological processing and paraffin embedding was done. Paraffin sections (5µm thick) from the metaphysis of the tibias were deparaffinized and stained by haematoxylin & eosin (H&E) (Bancroft and Gamble, 2002) for light microscopic examination.

Histochemical detection of calcium in small renal blood vessels:

Kidney specimens were fixed in 10% buffered neutral paraformaldehyde solution, embedded in paraffin, and deparaffinized by standard procedures. Thin sections (5 µm) were stained by Alizarin red S stain (Bancroft and Cook, 1994) for demonstration of calcium deposits.

Immunohistochemistry for iNOS:

Kidney specimens were stained with polyclonal anti-inducible NOS (iNOS) antibodies. Immunohistochemical localization of iNOS protein was performed based on the method previously described by Kawaguchi *et al*. (2001).

**3. Results:**

**Table 1: Serum levels of calcium and phosphate, and alkaline phosphatase (ALP) activity in: Control, Streptozotocin-induced diabetic rats (STZ-D), and Aldosterone receptor antagonist-supplemented diabetic rats (ARA-STZ rats).**

| Parameters | Control | STZ-D | ARA-STZ |
|---|---|---|---|
| **Calcium (mg/dl)** | 6.4±0.39 (6) | 9.8±0.89[a] (5) | 5.5±0.66[b] (6) |
| **Phosphate (mg/dl)** | 5.7±0.40 (6) | 5.5±0.25 (5) | 6.3±0.52 (6) |
| **ALP activity (IU/liter)** | 188.6±13.3 (6) | 402±69.8[a] (5) | 779.2±77.5[a,b] (6) |

Data are presented as means ± SEM.
In parenthesis is the number of observations
(a) Significance calculated by least significant difference (LSD) at < 0.05 from control group.
(b) Significance calculated by LSD at < 0.05 from STZ-D group.

$Ca^{2+}$ and phosphate serum levels and alkaline phosphatase activity (table 1):

The results of this study clearly demonstrated that the serum $Ca^{2+}$ level was significantly elevated 4 weeks after the induction of diabetes in STZ-D rats as compared to the control rats. This increase was significantly inhibited by administration of spironolactone to the ARA-STZ rats. Serum inorganic phosphate level was insignificantly changed among all studied groups. Serum alkaline phosphatase actvity was significantly increased in the STZ-D rats as compared to the control rats. Spironolactone administration resulted in a more significant rise of serum alkaline phosphatase activity in the ARA-STZ rats as compared to both the control and the STZ-D rats.

Histological examination of the bone:

Examination of sections from the control group revealed that the proximal metaphysis of the tibia was formed of an outer shell of compact bone (cortical bone) and inner trabeculae of cancellous bone. The compact bone consisted of outer, inner and interstitial bone lamellae as well as Haversian systems. Inbetween the lamellae osteocytes resided in their lacunae. The shell of compact bone was covered by periosteum and lined by endosteum.

Cancellous bone was formed of a network of bone trabeculae composed of irregular bone lamellae between which osteocytes resided in their lacunae. The endosteal surface of trabeculae was lined by osteoprogenitor cells, osteoblasts and osteoclasts. Bone marrow spaces were seen between the trabeculae (Fig. 1).

Bone sections from STZ-D rats revealed thinning of the outer cortical bone as well as the inner cancellous bone trabeculae as compared to the control

group. The cancellous bone trabeculae lost their normal architecture and appeared as discontinuous bony ossicles separated by widened bone marrow spaces (Fig. 2).

Bone sections in ARA-STZ rats revealed marked improvement as compared to those of the STZ-D rats. Cortical bone thickness was very similar to the control group. The cancellous bone trabeculae partially regained near normal structure and appeared more continuous, with less widened bone marrow spaces (Fig. 3).

Histological detection of calcium in small renal blood vessels:

Examination of the small blood vessels of kidneys of the control rats revealed no calcium deposits (Fig. 4). The kidneys of STZ-D rats revealed calcium deposits in the small blood vessels, detected as red lines in both cortex and medulla (Fig. 5). Group ARA-STZ revealed minimal calcium deposits in the small blood vessels of the kidneys (Fig. 6).

Immunostaining of the small renal blood vessels for iNOS:

In the control group, iNOS immunostaining was densely detected in the endothelium of the peritubular blood capillaries (Fig. 7). In the STZ-D rats, iNOS immunostaining was markedly reduced in the endothelium of the small blood vessels as compared to the control group (Fig. 8). In the ARA-STZ rats the iNOS immunostaining was increased as compared to the STZ-D group (Fig. 9).

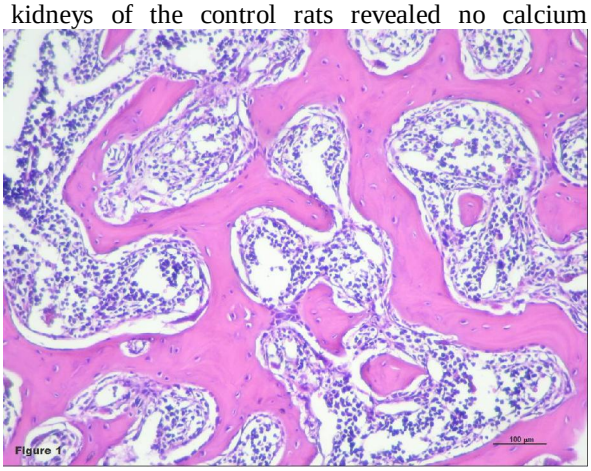


**Fig. (1):** Photomicrograph of proximal metaphysis of tibia of the control group showing well-formed network of trabeculae of cancellous bone. (H&E X250)

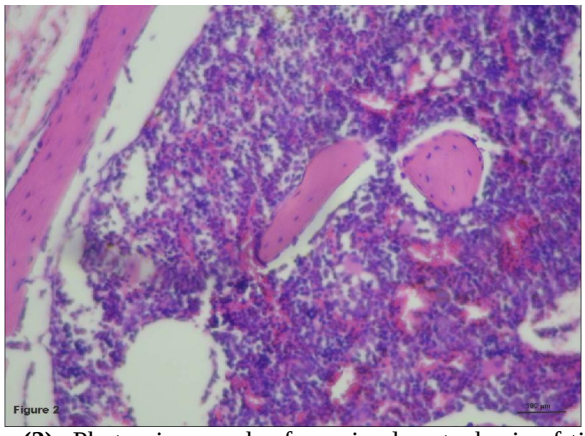


**Fig. (2):** Photomicrograph of proximal metaphysis of tibia of STZ-D group showing loss of the normal architecture of trabeculae of cancellous bone with widening of bone marrow spaces. (H&E X250)

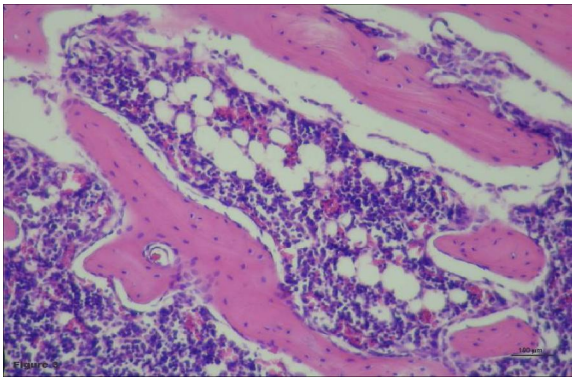


**Fig. (3)**: Photomicrograph of proximal metaphysis of tibia in ARA-STZ group showing preserved architecture of trabeculae in comparison to STZ-D group. Trabeculae appear more or less continuous with bone marrow spaces inbetween. (H&E X250)

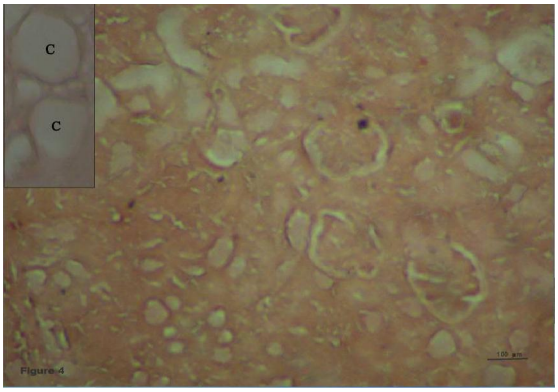
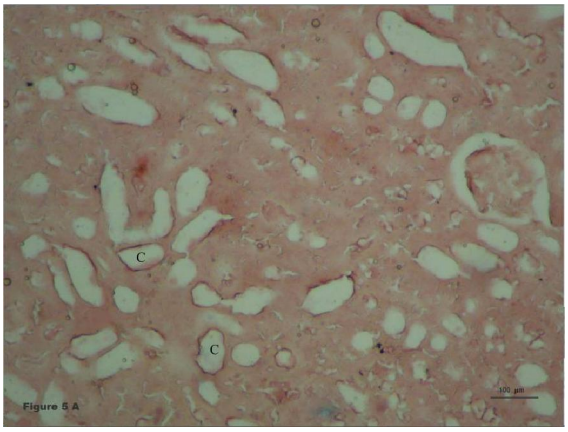
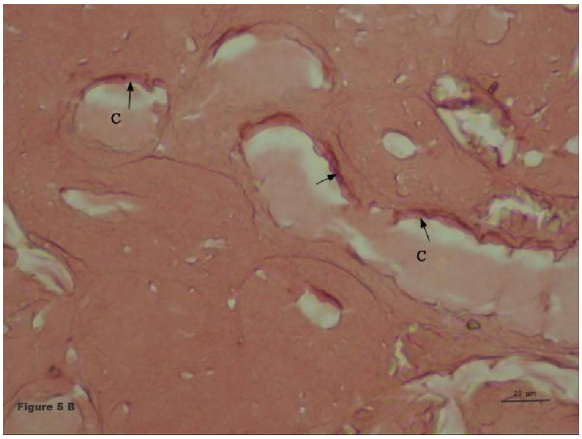



**Fig. (4):** Photomicrograph showing cortex of the kidney of the control group. No calcium deposits are detected in the peritubular capillaries. (Alizarin red S stain X250)
***Inset:*** Higher magnification showing two capillaries (C) with no calcium depostis (Alizarin red S stain X640)




A




B

**Fig. (5):** Photomicrograph showing cortex of the kidney of the STZ-D rats.
**A:** Calcium deposits can be detected in the wall of the small blood vessels (C) (Alizarin red S stain X250)
**B:** higher magnification showing calcium deposition (↑) in the wall of the renal blood vessels (C)(Alizarin red S stain X640)

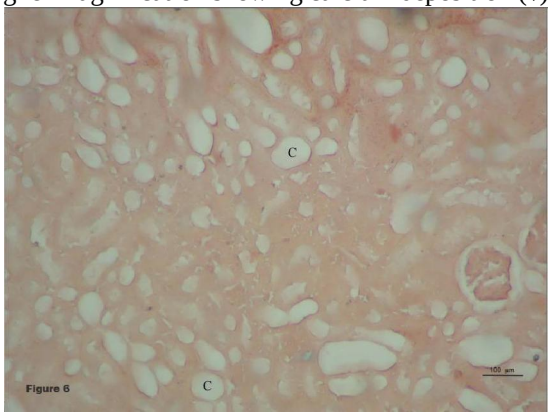



**Fig. (6):** Photomicrograph showing negligible calcium deposits in the small renal blood vessels (C) in ARA-STZ rats. (Alizarin red S stain X250).

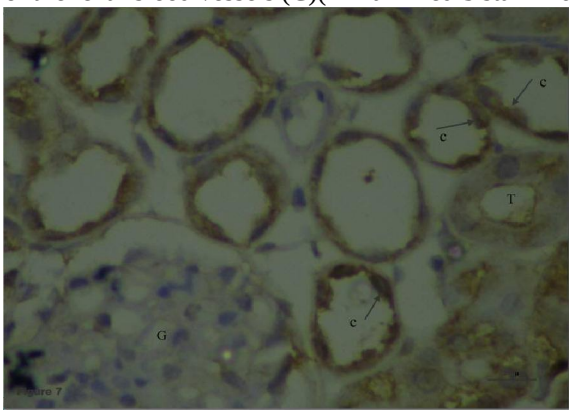



**Figure (7):** Photomicrograph of iNOS immunostaining of peritubular capillaries from control rats showing dark brown densely stained areas (↑) in endothelial cells (G: glomerulus; T: tubule; C: capillary). (iNOS X 640)

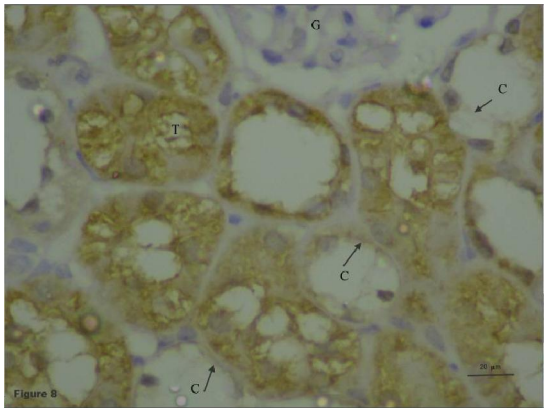



**Fig. (8):** Photomicrograph showing faintly stained endothelial cells (↑) in peritubular capillaries for iNOS in STZ-D rats (G: glomerulus; T: tubule; C: capillary). (iNOS X640)

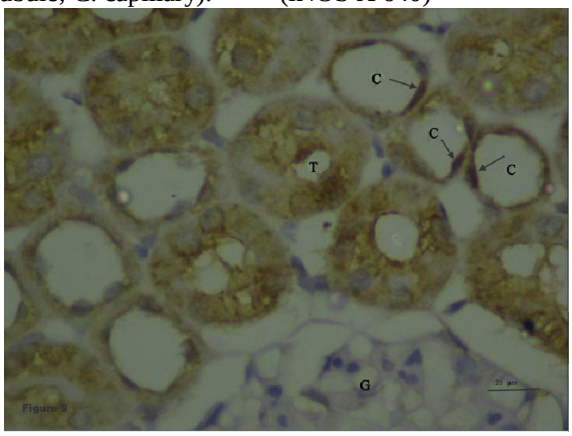



**Fig. (9):** Photomicrograph showing moderate iNOS-staining of endothelial cells (↑) in peritubular capillaries of ARA-STZ rats (G: glomerulus; T: tubule; C: capillar

**4. Discussion:**

The current study clearly demonstrated dysregulation of $Ca^{2+}$ metabolism in STZ-D rats. Serum $Ca^{2+}$ level was significantly elevated 4 weeks after induction of diabetes, histological examination of bone revealed osteoporotic changes, and histological examination of renal blood vessels revealed calcification of small renal blood vessels, together with reduced iNOS staining. Treatment with spironolactone, an aldosterone-receptor antagonist, reduced all the previously mentioned findings.

The increased serum $Ca^{2+}$ concentration in STZ-D rats could result from the release of $Ca^{2+}$ from bone tissues. Several mechanisms could underlie osteoporosis observed in STZ-D rats. Advanced glycation end products (AGEs) had been reported to contribute to poor bone strength, and increased receptors for AGEs (RAGEs) were manifested in chemically-induced diabetes in mice (Thrailkill *et al*., 2005). RAGE, a multiligand receptor, contributed to the pathogenesis of diabetic complications through regulating osteoclast maturation, function, and bone remodeling (Zhou, *et al*., 2006). Osteoclasts are multinucleated, terminally differentiated cells from hematopoietic monocyte/macrophage precursors, whose bone resorption activity is critical in regulating bone mass (Zhou, *et al*., 2006). The increase in oxidative stress (Johansen *et al*., 2005) together with the increase in proinflammatory cytokines reported in diabetes (Barakat *et al*., 2008) have been recognized as contributing factors to enhanced osteoclast activity (Sheweita and Khoshhal, 2007).

Insulinopenia in STZ-D rats could be a contributing factor to the osteoporosis observed in these rats. Several lines of evidence from in vitro bone cell cultures support the idea that insulin can exert direct anabolic effects on bone cells. The lack of insulin in patients with type 1 DM, may be disadvantageous for osteoblast number and activity and collagen formation (Botolin *et al*., 2005). In addition to the direct effects of insulin on bone cells, insulin was reported to exert synergistic effects with other anabolic agents in bone, such as insulin-like growth factor-1 (IGF-I) (Thrailkill *et al*., 2005).

Elevations in plasma aldosterone level were reported in STZ-diabetic animals (Xue and Siragy, 2005). Aldosterone was reported to be accompanied by a proinflammatory state, characterized by oxidative and nitrosative stress, immune cell activation, and bone loss (Vidal *et al*., 2006 and Chhokar *et al*., 2005). Thereby, hyperaldosteronism could be another contributing factor to osteoporosis observed in STZ-D rats.

The results of the current study demonstrated correction of the hypercalcemia and osteoporosis in ARA-STZ rats. Aldosterone antagonism, by attenuating oxidative stress (Nishiyama and Abe, 2006) and reducing inflammatory changes could provide a mechanism for attenuation of osteoporosis observed in the STZ-D rats.

The observed elevation of ALP in ARA-STZ rats could reflect increased osteoblast activity as histological examination of bone revealed apparent improvement in thickness and continuity of trabeculae.

At the same time, histological examination of blood vessels revealed calcification of small renal blood vessels in diabetic rats. Multiple factors could be suggested to play a role in the pathophysiology of vascular calcification in STZ-D rats. Chen *et al*., (2006) reported that high glucose could induce or modulate phenotypic transformation of VSMCs to osteoblast-like cells with subsequent mineralization in vitro.

In a previous study, we reported significant increase in tumor necrosis factor-alpha (TNF-alpha) in the STZ-D rats (Barakat *et al*., 2008). TNF-alpha was reported to increase calcium deposition, and induce the expression of osteogenic signals like ALP (Al-Aly *et al*., 2007). In agreement, we reported in the current study a significant increase in serum ALP in the STZ-D rats. At the same time, it was reported that abnormalities in mineral metabolism that enhance the calcium x phosphate product, as the hypercalcemia encountered in STZ-D rats in our results, could exacerbate vascular calcification (Giachelli *et al*., 2005).

Aldosterone was reported to regulate the expression of several genes that have been implicated in vascular calcification including the ALP gene (Jaffe *et al*., 2007). Aldosterone was found to promote mineralization of calcifying VSMCs in a MR-dependent manner. These observations suggest that one of the clinical effects of MR antagonists (as spironolactone) may be inhibition of this MR-mediated vascular calcification (Jaffe *et al*., 2007). This could explain the present findings of increased vascular calcification in the untreated diabetic group and its reduction with spironolactone treatment.

Moreover, Aldosterone induces the generation of reactive oxygen species (Rüster and Wolf, 2006). Studies showed that hydrogen peroxide enhanced bone markers expression in VSMCs implicated in osteoblastic differentiation (Sutra *et al*., 2008).

Jaffe *et al*., (2007) reported that endothelin-1 (ET-1) synthesis is elevated during aldosteronism. ET-1 induced oxidative and nitrosative stress and expression of adhesion molecules in the affected vasculature in rats with chronic stress of mineralocorticoid.

Nitric oxide (NO) is a messenger molecule produced by the NO synthase (NOS) isoforms. Inducible NOS (iNOS) isoform can be found in the vasculature, in VSMCs. iNOS was found to inhibit VSMC calcification. These data suggest that NO regulates vascular calcification (Kanno *et al*., 2008). Substantial evidence indicates that diabetic vascular dysfunction is associated with marked alterations of NO pathways. In the current study, iNOS-like immunostaining was decreased in the endothelium of small renal blood vessels in the STZ-D rats. This result is consistent with a previous finding in a study which reported that high glucose reduces cytokine-induced iNOS activity in VSMCs (Muniyappa *et al*., 1998). This decrease in vascular iNOS immunostaining was reflected on the increased vascular calcification in the untreated diabetic group.

Endogenous iNOS expression and activity have key function in increasing endothelial survival and maintaining function. Thus, suppression of iNOS was found to significantly contribute to endothelial dysfunctions during oxidative stress (Hemmrich *et al*., 2003).

Therefore, in the present study, increased inflammatory cytokines, diminished iNOS activity, elevated serum aldosterone, high blood glucose and high calcium levels could provide stimulatory signals for the observed vasculopathy in the STZ-D rats.

The vasculopathy observed in the STZ-D rats was clearly ameliorated in the ARA-STZ rats. In the current study, iNOS-like immunostaining was enhanced in the ARA-STZ rats. Induction of iNOS could have reduced pathological vascular calcification.

In the vascular system, MR could be localized in endothelial and VSMCs. Aldosterone induces oxidative stress in vascular cells through NADPH oxidase activation. MR antagonist decreases oxidative stress and increased NO bioavailability (Bauersachs and Fraccarollo, 2006).

Taken together, in the current study aldosterone antagonism could prohibit vasculopathy in ARA-STZ rats by its antioxidant effects, increasing NO bioavailabilty, decreasing expression of vascular osteogenic signals, as well as normalization of blood calcium levels.

From the aforementioned data, our findings draw attention to a significant role of the aldosterone hormone in the vascular calcification & osteoporosis; two significant complications that accompany DM type 1. In this study, we presented the evidence supporting the potential benefits of early blocking the actions of aldosterone in diabetes in lessening the burden of vascular and bone effects in this metabolic disorder.

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