Effect of vitamin D on isoprenaline induced myocardial infarction in rats; possible role of Peroxisome Proliferator Activated Receptor-γ (PPAR-γ)

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Ola Ahmed El-Gohary and Mona Maher Allam

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Running title: Effect of vitamin D on isoprenaline induced myocardial infarction in rats and role of PPAR-γ.

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Effect of vitamin D on isoprenaline induced myocardial infarction in rats; possible role of Peroxisome Proliferator Activated Receptor-γ (PPAR-γ)

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Abstract:
Infarct-like lesion induced by isoprenaline is a well-known model to study myocardial infarction (MI). Vitamin D has been shown to have anti-inflammatory and antioxidant effects. Recent studies highlighted cross talk between vitamin D and peroxisome proliferator-activated receptor gamma (PPAR-γ). The present study was designed to investigate the effect of pretreatment with vitamin D on the isoprenaline-induced infarct-like lesion in rats and the role of PPAR-γ as a novel mechanism in vitamin D-mediated cardio protective effect. Markers chosen to assess cardiac damage included serum level of creatine kinase (CPK), lactate dehydrogenase (LDH), tumor necrosis factor-alpha (TNF-α), and interleukin-6 (IL-6). Cardiac contents of malondialdehyde (MDA), superoxide dismutase (SOD) and glutathione peroxidase (GSH) have been also assessed. Furthermore, ECG monitoring and measurement of injury extension were carried out. Isoprenaline increased the level of cardiac enzymes as well as inflammatory and oxidative stress biomarkers. In addition, it produced ST-segment elevation. Pretreatment with vitamin D significantly improved previous parameters. The prior treatment with PPAR-γ antagonist; bisphenol A diglycidyl ether; (BADGE) significantly attenuated the protective effect of vitamin D. In conclusion, vitamin D can be demonstrated as a
promising cardio-protective agent in MI and PPAR-γ significantly contributes toward vitamin D-mediated protection.

*Key words*: vitamin D, isoprenaline, myocardial infarction, Peroxisome Proliferator Activated Receptor-γ, oxidative stress.
1. Introduction:

Despite great efforts during the last decades, cardiovascular diseases (CVDs) remain the major cause of death worldwide, increasing their prevalence every year (Basak et al. 2015). Myocardial infarction (MI) is a cardiovascular disease that occurs when the blood supply to a part of the heart is interrupted, causing death to the heart tissue. It is a complex phenomenon affecting the mechanical, electrical, structural, and biochemical properties of the heart (Bharti et al. 2010; Upaganlawar et al. 2010).

Isoprenaline (ISO), a β adrenergic agonist, has been reported to induce infarct-like lesions in rats and other animal species (Zhang et al. 2008). It has been shown to exhibit many metabolic and morphological aberrations in the heart tissue of experimental animals similar to those seen in humans with myocardial infarction (Panda and Naik 2009). Injected isoprenaline undergoes auto-oxidation resulting in the generation of free radicals that stimulate lipid peroxidation which causes destruction and damage to the myocardial cell membrane (Loh et al. 2007; Upaganlawar et al. 2010). Inflammation is a key process involved in mediating myocardial tissue damage through the release of proteolytic enzymes (Jordan et al. 1999).

Vitamin D plays a classical hormonal role in skeletal structures by regulating calcium and phosphorus metabolism. Recently, much research has focused on the cardio protective effects of vitamin D (Brandenburg et al. 2012; Mancuso et al. 2008) and on its anti-inflammatory and anti-oxidant actions (Shab-Bidar et al. 2014; Zittermann and Koerfer 2008), which may play a role during acute infarction. It has been shown that insufficient vitamin D levels are linked to nonskeletal major chronic diseases, especially cardiovascular diseases (Wang et al. 2013). Vitamin D deficiency has been found in a
high proportion of patients with myocardial infarction (Goleniewska et al. 2014; Hlaing et al. 2014) and has been associated with coronary heart diseases and myocardial infarctions (Lee et al. 2011).

Vitamin D receptors have been found in all major cardiovascular cell types including cardiomyocytes, arterial wall cells, and immune cells. It has been established that they are closely related to the Peroxisome Proliferator Activated Receptor-γ (PPAR-γ) (Al Mheid et al. 2013). Both vitamin D receptors and PPAR-γ are ligand-activated nuclear receptors. Recently, a few in vitro studies suggested cross talk between these two nuclear receptors with involvement of PPAR-γ in vitamin D mediated biological responses (Dai et al. 2008).

In spite of the accumulating data about the cardiovascular effect of vitamin D, the role of vitamin D supplementation in the management of cardiovascular diseases remains to be established. Hence, the present study was designed to investigate the effect of vitamin D on the outcome of ISO- induced myocardial infarct-like lesions in rats, and also to outline the role of (PPAR-γ) as a novel mechanism for this effect if found. Parameters chosen to assess the myocardial damage and the protective effect of vitamin D included; serum cardiac enzymes, inflammatory markers and oxidative stress parameters in addition to ECG monitoring and measurement of injury extension.

2. Material and Methods

2.1. Animals:

This study was conducted on 50 adult Wistar albino male rats, 6-8 weeks old and weighing between 200 and 250 g. Animals were housed in the animal laboratory at the
Medical Research Center of Benha Faculty of Medicine. They were housed at room temperature (25°C) and 12h/12h light/dark cycle. All Rats were fed a standard diet and water. The study was carried out according to the guidelines of the Ethics Committee, Faculty of Medicine, Benha University.

2.2. Experimental design:

The rats were randomly divided into five groups (n:10). **Group I (Control group):** Received no medication and was given free access to food and water. **Group II (Vit. D group):** Received Vitamin D (0.5 µg/kg; i.p.) once daily for 7 days (Akanksha et al. 2013). **Group III (ISO group):** Infarct-like lesion was induced in the rats by subcutaneous injection of 100mg/ kg isoprenaline hydrochloride dissolved in saline once daily for two successive days (Kumaran and Prince 2010). **Group IV (ISO+Vit. D group):** Received Vitamin D (0.5 µg/kg; i.p.) once daily for 7 days. For induction of infarct-like lesion, rats received isoprenaline (100mg/kg; s.c.) after 1 h of vitamin D administration on the last 2 days of the treatment period. **Group V (ISO+Vit. D+ BADGE group):** This group was treated with PPAR-γ antagonist; Bisphenol A diglycidyl ether (BADGE); It was dissolved in minimal volume of ethanol, diluted with saline and injected intraperitoneally at a dose of (30 mg/kg) given 30 min before vitamin D injection (0.5 µg/kg; i.p.) once daily for 7 days (Akanksha et al. 2013). For induction of infarct-like lesion, rats received isoprenaline (100mg/kg; s.c.) after 1 h of vitamin D administration on the last 2 days of the treatment period. Isoprenaline hydrochloride, vitamin D and Bisphenol A diglycidyl ether (BADGE) were purchased from Sigma–Aldrich (USA).
24 hours after the last treatment, overnight-fasted rats were anaesthetized with urethane (1.5 g/kg; i.p.) for ECG monitoring (Abood and Elshal 2015; Zaafan et al. 2013). Thereafter, blood samples were collected via cardiac puncture for serum separation and estimation of cardiac marker enzymes such as serum level of creatine kinase (CPK) and lactate dehydrogenase (LDH) as well as inflammatory markers such as tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6). The rats were then sacrificed by decapitation and their hearts were rapidly isolated. The injury extension was measured in the excised hearts. Portions of the heart tissues were used to determine the oxidative stress as malondialdehyde (MDA) and antioxidant enzymes such as superoxide dismutase (SOD) and glutathione peroxidase (GSH).

2.3. ECG monitoring:
The anesthetized rats were placed in the supine position on a board and ECG was recorded continuously with standard artifact free lead II (right forelimb to left hind limb). Needle electrodes were inserted subcutaneously into the paw pads of each rat, and connected to Biocare ECG 101 (Shenzhen Biocare Electronics Co., Ltd., China). The ECG was measured to determine the duration and amplitude of the P wave, the QRS complex, and the ST segment alterations.

2.4. Measurement of extension of cardiac injury:
The excised beating heart was submerged in cold (8°C) 30 mmol KCl to achieve diastolic arrest. The right ventricle and both atria were excised to isolate the left ventricle (the septum and free wall). The left ventricle was then sectioned by a sharp surgical scissor into transverse slices, each of about 1.5mm thick. The slices were stained in a 1.5% triphenyltetrazolium chloride (TTC) (MP biomedical, France) in phosphate buffer, PH
7.4, for 10-15 minutes at 37ºC. The TTC stain formed red color precipitates in the presence of an intact dehydrogenase enzyme system. Areas of necrosis lost dehydrogenase activity and therefore failed to stain (Sharma and Singh 2000; Vivaldi et al. 1985). The slices were washed with saline and then clear glass plates were placed over both sides of each slice. Epicardial and endocardial outlines as well as the TTC stained and non-stained areas were traced on clear plastic sheets. The plastic sheets were then fixed on an E.C.G paper and the small squares occupying the stained and non-stained areas were counted giving each in mm$^2$. The sum of the stained and the non-stained areas give the surface area of the whole heart slices. The surface area of the whole left ventricle was calculated by adding the surface areas of all cardiac sections measured on E.C.G paper. The surface area of the injured tissue of the whole heart was obtained by adding the surface area of the injured tissue in all cardiac slices and the injury extension was calculated as percentage of the sum of the injured areas to the sum of surface areas of all the slices (Evans et al. 1985).

2.5. Biochemical analysis:

The serum was separated by centrifugation (5000 rpm for 5 min) and used for biochemical analysis. Cardiac marker enzymes such as CPK and LDH were detected using Stanbio CK-MB diagnostic kit (USA). In addition inflammatory markers such as TNF-α and IL-6 were determined by the ELISA technique using standard kits (Ray Biotech, Inc., USA). The sensitivities of the methods as stated in the instructions of enzyme immunoassay kits are 12 pg/ml for IL-6 and 11.2 pg/ml for TNFα. The intra-assay coefficient of variation for the measurements was <5%.
Portions of the heart tissue were homogenized in a saline solution (0.9%) and centrifuged at 3000 rpm for 15 min; the supernatant was kept at –20°C and used to determine the oxidative stress as level of MDA (Uchiyama and Miha 1978) and antioxidant enzymes such as SOD (Das et al. 2000) and GSH (Moron et al. 1979).

2.6. Statistical analysis:
All analyses were performed using the program "Statistical Package for Social Sciences (SPSS) version 16" (SPSS Inc, Chicago, IL, USA). The data are presented as the mean ± standard deviation (SD). Comparisons among groups, in all studied parameters, were analyzed by using one-way analysis of variance (ANOVA) test and Post-Hoc multiple comparisons (LSD). Probability of chance (P value) < 0.05 was considered statistically significant.

3. Results
3.1. Effect of vitamin D on ECG parameters and extension of cardiac injury in isoprenaline-induced infarct-like lesion in rats and the role of PPAR-γ (Fig. 1, Table 2):
Isoprenaline injection induced infarct-like lesion represented by ST segment elevation, and a decrease in R wave amplitude as compared to the normal group. Pretreatment with vitamin D resulted in a reduction in the ST segment elevation with an increase in R wave amplitude as compared to the isoprenaline group. The BADGE pretreatment abolished the protective effect of vitamin D in rats subjected to infarct-like lesion (Fig. 1).

As regards the extension of cardiac injury, vitamin D treatment significantly decreased the injury extension (P<0.05) as compared to the isoprenaline group. Moreover, BADGE
pretreatment in rats receiving vitamin D and undergone infarct-like lesion significantly increased the injury extension ($P<0.05$) that was still reduced ($P<0.05$) as compared to isoprenaline group. (Table 2).

### 3.2. Effect of vitamin D on the biochemical changes associated with isoprenaline-induced infarct-like lesion in rats and the role of PPAR-γ (Table 1, 2):

The levels of cardiac enzymes (CPK and LDH) in the plasma showed a significant rise ($P<0.05$) in the ISO group, ISO + Vit.D group and ISO + Vit. D + BADGE group compared to the control group. Pretreatment with vitamin D significantly reduced the cardiac enzymes to near normal values. On the other hand, BADGE pretreatment in the rats that received vitamin D and subjected to infarct-like lesion significantly increased ($P<0.05$) the plasma levels of cardiac enzymes that still showed significant reduction ($P<0.05$) as compared to the isoprenaline group. (Table 1).

As regards pro-inflammatory cytokines, TNF-α and IL-6 increased significantly ($P<0.05$) in the ISO group, ISO + Vit.D group and ISO + Vit. D + BADGE group as compared with the control group. However, these cytokines showed significant reduction in the ISO-injected rats pretreated with vitamin D ($P<0.05$) compared to the ISO group. Furthermore, the pretreatment with BADGE in the rats that received vitamin D and subjected to infarct-like lesion produced significant elevation ($P<0.05$) of pro-inflammatory cytokines that still revealed significant reduction ($P<0.05$) as compared to isoprenaline group. (Table 1).

Similarly, cardiac MDA significantly increased ($P<0.05$) with a parallel significant decrease in SOD and GSH content ($P<0.05$) in the ISO group, ISO + Vit.D group and
ISO + Vit. D + BADGE group as compared to the normal rats. Pretreatment with vitamin D resulted in a significant decrease in MDA ($P<0.05$) and a significant increase in SOD and GSH content ($P<0.05$) as compared to the isoprenaline group. Similar to other parameters, the BADGE pretreatment abolished the protective effect of vitamin D in rats subjected to infarct-like lesion. There was a significant decrease ($P<0.05$) in cardiac MDA while there was a significant increase ($P<0.05$) in SOD content and a non-significant increase ($P>0.05$) in GSH content comparing the ISO + Vit. D + BADGE group to the ISO group. (Table 2).

4. Discussion

MI continues to be a major health problem worldwide and contributes significantly to mortality statistics (Gao et al. 2001). The term MI is thought to reflect death of cardiac myocytes due to prolonged ischemia (Patel et al. 2011). In the current study, infarct-like lesion was induced in rats by intraperitoneal injection of ISO. It has been reported that isoprenaline administration in high doses to animals produces ‘infarct like’ lesions in the heart (Vennila et al. 2010). The mechanisms proposed to explain isoprenaline-induced cardiac damage include increases in heart rate, myocardial contractility, hypoxia due to myocardial hyperactivity, depletion in energy reserve, calcium overload, excessive production of highly cytotoxic free radicals, and mitochondrial injury or dysfunction (Mohanty et al. 2004; Rathore et al. 2000).

In the present study, isoproterenol injection was seen to result in significant alterations in ECG patterns such as ST segment elevation coupled with marked decrease in R wave amplitude that reflect isoprenaline-induced infarct-like lesion. ECG pattern alterations by isoprenaline were previously demonstrated by other investigators (Prince and Sathya
In addition to the measurement of injury extension, ECG abnormalities were the main criteria used for the diagnosis of infarct-like lesion. These alterations could be due to the consecutive loss of cell membrane potential in the injured myocardium as a result of oxidative stress (Peacock et al. 2007). It was reported that ST elevation correlates well with the leak of creatine kinase from the myocardium (Mohan et al. 2010).

Cardiac enzymes, as a marker for acute myocardial infarction were all significantly elevated in our model. Since these enzymes are released from necrotic cells to the extracellular fluid upon the incidence of infarction, their high levels give another evidence for necrosis and infarction (Wang et al. 2009). Our findings are in agreement with those of Kurian et al. (2005) and Rajadurai et al. (2007), who reported that these cardio specific marker enzymes were released from the heart into the blood during myocardial damage.

Inflammation is a key process involved in mediating myocardial tissue damage after an ischemic event. In the current study, isoprenaline produced an increase in the serum level of pro-inflammatory cytokine TNF-α and IL-6, an effect that is in accordance with the work of other investigators (Cusack et al. 2002; Tawfik et al. 2010). The injured region undergoes local necrosis and myocyte apoptosis resulting in complement activation, free radicals generation, and accumulation of cellular debris. Phagocytosis of the resultant cellular debris by macrophages and neutrophils triggers the inflammatory cytokines as TNF-α (Frangogiannis et al. 2002).

The increase in the cardiac MDA content (an indicator of lipid peroxidation) and the
decrease in the cardiac content of SOD and GSH (cardiac antioxidants) in isoprenaline group could be expected. Isoprenaline causes oxidative stress, which results from a serious imbalance between the generation of reactive oxygen species (ROS) and their clearance by the body’s endogenous antioxidative defenses. Our findings are in agreement with those of Shikalgar and Naikwade (2010) and Upaganlawar et al. (2010).

Administration of vitamin D for 7 days before induction of infarct-like lesion resulted in amelioration of cardiac injury. Specifically, there was improvement in ECG parameters, cardiac injury extension and cardiac enzymes of ISO-injected rats pretreated with vitamin D. These findings are in line with a recent study of Abood and Elshal (2015), who reported a cardio-protective effect against MI through vitamin D receptor (VDR) stimulation based on improvement of ECG criteria in addition to return of cardiac enzyme parameters to near normal.

To explain the protective effect of vitamin D on isoprenaline-induced infarct-like lesion we measured plasma TNF-α and IL-6, as markers for inflammation, together with cardiac MDA, SOD and GSH content, as markers for the oxidative state. The down regulation of inflammatory cytokines demonstrated by vitamin D administration in ISO-injected rats denotes that vitamin D exerts its protective effect, at least in part, by an anti-inflammatory action. The reduction of inflammatory cytokines as evidenced in our study has previously been reported by Arnson et al. (2013). Contrarily, Witham et al (2013) showed no reduction of inflammatory cytokines after 8 weeks of vitamin D supplementation. This contradiction is probably due to genetic polymorphism that may modulate the response to vitamin D supplementation (Gagnon et al. 2014). In addition, pretreatment with vitamin D in ISO-injected rats was found to be associated with a
significant reduction in lipid peroxidation products resulting in amelioration of the oxidative stress. This conforms to the recent data published by Cavalcante and colleagues (2015) regarding the antioxidant effect of vitamin D.

We revealed that vitamin D treatment reduced ISO-induced infarct-like lesion through PPAR-γ activation. The PPAR-γ agonists are well documented to protect against IRI in various tissues through down-regulation of molecular pathways like nuclear factor kappa beta, thromboxane synthase, monocyte chemoattractant protein-1, inducible nitric oxide synthase, and fibronectin (Chatterjee et al. 2004; Panchapakesan et al. 2005). Moreover, the PPAR-γ agonists are proposed to inhibit Jun N-terminal kinase phosphorylation that belongs to mitogen-activated protein kinase and production of endothelin-1 that are produced in response to stress stimuli including cytokines and are responsible for cellular apoptosis and damage (Delerive et al. 1999). There are previous studies that reported the protective action of vitamin D on the heart (Abood and Elshal 2015; Creighton et al. 2012; Schleithoff et al. 2006). To the best of our knowledge, our study reports for the first time that vitamin D essentially involves PPAR-γ activation for its cardio protective effect, as pharmacologic inhibition of PPAR-γ using BADGE abolished vitamin D-mediated cardio protection, supporting the involvement of PPAR-γ in protective action of vitamin D.

**Conclusion:**

In conclusion, the present study revealed that vitamin D pretreatment could improve the outcome of isoprenaline-induced infarct-like lesion in rats. This protective effect could be attributed to the strong anti-inflammatory and antioxidant effects of vitamin D. The study also demonstrated for the first time that PPAR-γ significantly contributes toward vitamin
D mediated cardio protection.

Declaration of interest statement:
The authors declare that they have no conflict of interest.

References:


Goleniewska, B., Kacprzak, M., and Zielińska, M. 2014. Vitamin D level and extent of coronary


Table 1: Changes in serum cardiac enzymes and inflammatory markers in different experimental groups.

<table>
<thead>
<tr>
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<th>control group</th>
<th>Vit. D group</th>
<th>ISO group</th>
<th>ISO + Vit. D group</th>
<th>ISO + Vit. D + BADGE group</th>
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<tr>
<td>CPK (U/l)</td>
<td>681.4 ± 2.9</td>
<td>679.5 ± 3.2</td>
<td>1011.3 ± 7.3*</td>
<td>743.3 ± 9.3*†</td>
<td>983.1 ± 4.0*†‡</td>
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<tr>
<td>LDH (U/l)</td>
<td>2156 ± 5.2</td>
<td>2160 ± 7.1</td>
<td>8234 ± 3.9*</td>
<td>3642 ± 4.6*†</td>
<td>7454 ± 1.9*†‡</td>
</tr>
<tr>
<td>TNF-α (pg/ml)</td>
<td>80.4 ± 0.43</td>
<td>79.6 ± 0.52</td>
<td>132.0 ± 0.12*</td>
<td>91.1 ± 0.90*†</td>
<td>124.5 ± 0.41*†‡</td>
</tr>
<tr>
<td>IL-6 (pg/ml)</td>
<td>41.5 ± 0.57</td>
<td>39.8 ± 0.78</td>
<td>126.4 ± 0.61*</td>
<td>57.0 ± 0.24*†</td>
<td>119.2 ± 0.74*†‡</td>
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CPK, creatine kinase; LDH, lactate dehydrogenase; TNF-α, tumor necrosis factor-α; IL-6, interleukin-6.

Data is expressed as mean ± standard deviation (n = 10 per group). P < 0.05 is significant tested by using One-way analysis of variance (ANOVA) and Post Hoc multiple comparisons (LSD). * P < 0.05 vs. control group; † P < 0.05 vs. ISO group; ‡ P < 0.05 vs. ISO+Vit. D group.
Table 2: Changes in cardiac oxidative stress, cardiac antioxidant status and size of infarction in different experimental groups.

<table>
<thead>
<tr>
<th></th>
<th>control group</th>
<th>Vit. D group</th>
<th>ISO group</th>
<th>ISO + Vit. D group</th>
<th>ISO + Vit. D + BADGE group</th>
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</thead>
<tbody>
<tr>
<td>MDA (nmol/g tissue)</td>
<td>115.1 ± 1.3</td>
<td>113.8 ± 3.9</td>
<td>157.8 ± 6.5*</td>
<td>126.5 ± 7.1*†</td>
<td>149.9 ± 5.2*†‡</td>
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<tr>
<td>SOD (IU/mg protein)</td>
<td>7.4 ± 0.93</td>
<td>7.0 ± 0.49</td>
<td>3.1 ± 0.12*</td>
<td>6.2 ± 0.61*†</td>
<td>3.6 ± 0.40*†‡</td>
</tr>
<tr>
<td>GSH (IU/mg protein)</td>
<td>4.6 ± 0.82</td>
<td>4.8 ± 0.63</td>
<td>1.2 ± 0.60*</td>
<td>3.5 ± 0.34*†</td>
<td>1.7 ± 0.57*</td>
</tr>
<tr>
<td>Infarction size (% LV)</td>
<td>0.0</td>
<td>0.0</td>
<td>36.4 ± 2.7*</td>
<td>19.3 ± 3.1*†</td>
<td>28.8 ± 4.3*†‡</td>
</tr>
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</table>

MDA, malondialdehyde; SOD, superoxide dismutase; GSH, glutathione peroxidase.

Data is expressed as mean ± standard deviation (n = 10 per group). P < 0.05 is significant tested by using One-way analysis of variance (ANOVA) and Post Hoc multiple comparisons (LSD). * P < 0.05 vs. control group; † P < 0.05 vs. ISO group; ‡ P < 0.05 vs. ISO+Vit. D group.
Figure Caption:

Fig. 1 (A,B,C,D,E): Lead II trace showing ECG changes in different experimental groups

(A) Normal control group
(B) Vitamin D-treated group
(C) Isoprenaline group
(D) Isoprenaline group pretreated with vitamin D
(E) isoprenaline group pretreated with vitamin D received BADGE injection