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Research Article

Effects of Calcium Supplementation on the Histology of the Cardiac Myocytes of Albino Wistar Rats

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Abstract

We studied the effects of calcium supplementation on cardiac myocytes of albino wistar rats. A total of 20 albino wistar rats were purchased and acclimatized for 2 weeks and were served regular rat food and drank tap water *ad libitum*. They were then randomly assigned to four groups of five animals each. Group A (control) received a placebo; Group B received a low dose (7.69 mg/kg) of calcium supplements; Group C received a moderate dose (15.38 mg/kg) of calcium supplements; Group D received a high dose (23.07 mg/kg) of calcium supplements. Calcium supplements were administered for 28 consecutive days after which the animals were euthanized under chloroform anesthesia and heart was removed for histological processing and microscopic examination. Blood samples were also collected for estimation of serum calcium concentration. Results obtained showed no difference in the serum calcium concentrations amongst the groups. The histology of the heart showed normal histology in control and low dose groups and degenerative changes in the moderate and high dose groups. It was thus concluded that calcium supplementation in moderate and high doses may lead to degeneration of the cardiac myocytes.

Keywords: *Calcium Supplementation; Cardiac Myocytes; Degeneration; Myocardial Infarction; Apoptosis; Histology*

Abbreviations

CICR: Calcium Induced Calcium Release; EC: Endocardium; PC: Pericardium; MC: Myocardium; DC: Degenerative Changes; Na^+/K^+: Sodium/Potassium Ion Ratio; Ca: Calcium; PTH: Parathyroid Hormone

Introduction

Background of study

The most common divalent mineral in the human body is calcium. The average adult body contains about 1 kg, with 99 percent stored in the skeleton as calcium phosphate salts. There are approximately 22 mmol in the extracellular fluid (ECF), with about 9 mmol in the plasma [1]. Over the course of twenty-four hours, about 10 mmol of calcium is shared between bone and the ECF [1]. Calcium ions are more than 7,000 times less concentrated within cells (in intracellular fluid) than in blood plasma (0.0002 mmol/L compared with 1.4

mmol/L in plasma). Calcium can be gotten both from dietary and supplementary sources and play many important roles in the body including roles in blood clotting, release of neurotransmitters, causing muscle contractions etc [2]. Calcium also plays a key role in the electrical conduction and subsequent contraction in cardiac myocytes [3]. Calcium supplementation is common amongst post-menopausal women, old and young people, those at the risk of osteoporosis and the healthy population. It has been shown to have effects on the body weight, blood pressure, serum lipid concentration, blood glucose [4,5].

Overview of cardiac myocytes

Cardiac muscle is a striated, involuntary muscle found in the walls and histological base of the heart, especially the myocardium. It is one of three major muscle types, the others being skeletal and smooth muscle. Cardiomyocytes or myocardiocytes, the cells that make up cardiac muscle, usually have only one nucleus, though populations of two to four nuclei do exist [3]. The myocardium is the heart's muscle tissue, and it is located in a thick middle layer between the outer epicardium and the inner endocardium layers. Through synchronized contractions of cardiac muscle cells in the heart, blood is pumped out of the atria and ventricles into the systemic and pulmonary circulatory systems. Unlike other tissues in the body, cardiovascular muscle cells rely on a healthy blood supply and nerve stimulation to provide oxygen and nutrients while also removing waste products like carbon dioxide [3].

The cardiac syncytium is a network of cardiomyocytes linked by intercalated discs that allow electrical impulses to move quickly through the network, enabling the syncytium to conduct synchronized myocardial contractions [6]. Intercalated disks have a very low electrical resistance, allowing for free ion diffusion. Owing to the ease with which ions migrate along the axis of cardiac muscle fibers, action potentials can quickly pass from one cardiac muscle cell to the next. The rule of all or none applies to each syncytium [7].

Cardiac muscle, unlike skeletal muscle, relies on extracellular calcium ions to contract. The entry of sodium ions through the sarcolemma initiates and ends the action potential in ventricular cardiomyocytes, in a regenerative process similar to skeletal muscle [8]. A longer-lasting depolarization of cardiac muscle cells is sustained by an inward flux of extracellular calcium ions through L-type calcium channels. The process of calcium-induced calcium release (CICR) from the sarcoplasmic reticulum, which must happen during normal excitation-contraction coupling to elicit contraction, induces calcium dependence. Calcium ions bind to the protein troponin as intracellular calcium concentration rises, allowing myosin to bind to actin and contraction to occur [7].

Organ structure and function are inextricably linked in both healthy and diseased states. As a result, a comprehensive understanding of the structure and function of the myocardium is needed for the treatment of patients with congenital and acquired heart diseases [7]. In cardiovascular medicine, there are several unanswered questions, which may be attributable to a lack of translational biomedical research [9]. Understanding the precise mechanisms of cardiac functions necessitates a thorough understanding of the architecture of the left ventricle. Not only for mechanical contraction, but also for electrical conduction and energy metabolism, the orientation of cardiac myofibers is important [10]. The musculature of the heart is arranged on the basis of a transformed blood vessel, rather than in the manner of a skeletal muscle, which has distinct origin and insertion [11].

Calcium supplementation

Calcium supplementation is common amongst people of various age groups as many individuals (especially in the developing countries) can barely meet up with the recommended daily intake from diet alone [2]. The older population, especially post-menopausal women, frequently take these supplements for the prevention and management of osteoporosis [12]. Calcium supplements have been linked to an increased risk of cardiovascular disease in healthy people, according to epidemiological evidence [13]. These effects could have a variety of pathophysiological mechanisms, including effects on vascular calcification, vascular cell function and blood coagulation. Some of these effects may be mediated by calcium-sensing receptors [14].

Calcium metabolism

The daily calcium intake of the average adult is about 25 mmol. The body consumes just around 5 mmol per day [15]. Calcium reaches the brush boundary membrane of the intestinal epithelial cell and is immediately bound to calbindin, a vitamin D-dependent calcium-binding protein. Calbindin transports calcium directly into the endoplasmic reticulum of epithelial cells, where it is passed to the cells opposite side's basal membrane without reaching the cytosol. From there, calcium is continuously pumped into the bloodstream [16].

Calcium absorption

Calcitriol is a hormone that regulates active calcium absorption from the stomach. It is also known as 1,25 dihydroxycholecalciferol or 1,25 dihydroxyvitamin D3. Under the influence of the parathyroid hormone PTH, which works on the epithelial cells (enterocytes) lining the small intestine to increase the rate of calcium absorption from the intestinal contents, the kidneys convert cholecalciferol into the active hormone, 1,25 dihydroxycholecalciferol. Humans absorb about 30% of the calcium in foods, although this varies depending on the type of food eaten [17]. Calcium absorption is affected by the amount of calcium consumed, the individual's age and life cycle, vitamin D intake, and other food components [2,17,18].

Elimination of calcium from the body

Any calcium in the body is excreted by urine, feces, and sweat. The amount of Calcium eliminated is affected by sodium and protein intake [19,20], caffeine intake [21], alcohol and phosphorus intake [22] and metabolic acids given by high-protein and cereal-grain diets [23]. Low blood parathyroid hormone levels (which occur physiologically when plasma ionized calcium levels are high) prevent cholecalciferol from being converted to calcitriol, which prevents calcium absorption from the gut. When plasma ionized calcium levels are low, the opposite occurs [21]. Since plasma calcitriol levels (which are essentially determined by plasma calcium levels) control how much biliary calcium is reabsorbed from the intestinal contents, the majority of excess calcium is excreted in the bile and feces [24]. 100 - 250 mg of calcium (15 - 20 mmol) is expected in an average adult urine sample collected over 24 hours [25].

Regulation of calcium metabolism

The concentration of ionized calcium in the blood is tightly regulated, hovering around 1.3 - 1.5 mmol/L [26]. This tight regulation is achieved by both the parafollicular cells of the thyroid gland and the parathyroid glands which constantly sense the concentration of calcium ions in the blood flowing through them. When the Ca^{2+} concentration rises, the parafollicular cells of the thyroid gland increase their secretion of calcitonin into the blood. At the same time the parathyroid glands reduce their rate of PTH secretion into the blood [27]. The resulting high levels of calcitonin in the blood stimulate the skeleton to remove calcium from the blood plasma and deposit it as bone. The reduced levels of PTH inhibit removal of calcium from the skeleton and have several other effects like increasing the loss of calcium in the urine and inhibiting the formation of calcitriol (1,25 dihydroxyvitamin D3) from cholecalciferol (vitamin D3) by the kidneys [27]. When blood calcitriol levels are low, the epithelial cells of the duodenum (enterocytes) are unable to absorb calcium from the intestinal contents [1,28]. Calcitriol deficiency has an effect on bone health because it causes osteoclasts to release less calcium ions into the bloodstream [27]. The reverse occurs when the plasma ionized calcium level is low or falls.

Experimental animals

Twenty Wistar rats were purchased from the department of Anatomy (University of Benin) and were maintained in well aerated plastic cages at 28°C, 50 - 70% humidity, on a 12-hour period of light/dark. Food and water were made available to them. Animal handling procedures were carried out in compliance with the National Institutes of Health's Guide for the Care and Use of Laboratory Animals [29].

Preparation of supplements to be administered

Calcium lactate pentahydrate (Accord- UK Ltd) were presented in tablet form with each tablet containing 300 mg of the calcium. The tablets were then grinded using a mashing mortar and pestle. The resulting powder was then dissolved in distilled water appropriately to form a stock solution of 2 mg/ml.

Experimental design

The animals were randomly assigned to four groups each containing five animals:

- Group A (control) - Were placed on a placebo and fed with normal diet.
- Group B - These were fed normal diet and a low dose of calcium supplements (7.69 mg/kg of body weight).
- Group C - These were fed normal diet and a moderate dose of calcium supplements (15.4 mg/kg of body weight).
- Group D - These were fed normal diet plus a high dose of calcium supplements. Specifically (23.1 mg/kg of body weight).

These doses were given in line with recommendations from the Committee to Review Dietary Reference Intakes for Vitamin D and Calcium of the Food and Nutrition Board [17].

Experimental procedure

Administration

This was done once a day for 28 consecutive days using the orogastric tube. At the end of the 28 days of administration, the animals were euthanized using chloroform anaesthesia after which a midline incision was made through the ventral wall of the thorax lateral to the sternum. The heart was excised and fixed in 10% buffered formalin for histological processing and microscopic examination.

Histological preparation/ tissue processing

- Cutting: A transverse section was made through the apex of the hearts and these were placed in tissue cassettes and then placed under running water to wash off the fixative (buffered formalin).
- Processing: The tissues were then dehydrated in ascending grades of alcohol (70%, 90%, 96% and 100% -absolute) after which they were cleared in xylene. After clearing in xylene they were impregnated with liquid paraffin.
- Embedding: This was done with the use of the automatic embedding machine®. This involves the use of liquid paraffin for orienting and casting in order to get a tissue block. The tissue and paraffin were then left to cool for some time on a frozen surface in order to cool and solidify (deblocking).
- Sectioning: This involved using a cutting machine to section the tissue blocks into thin portions about 5 µM thin for easy staining.
- Staining techniques used include haematoxylin and eosin (H and E) because they are majorly used for highlighting the morphology and structural characteristics of tissues. The slides were then examined and read under the light microscope at magnifications of 40 and 100.

Blood sample collection/electrolyte determination

Four milliliters of blood were collected from the abdominal aorta using a 5 ml butterfly syringe and placed in plain sample bottles allowed to clot before extraction of serum (using a Pasteur pipette) for determination of serum calcium concentration using the ion specific electrode machine®. We also determined the concentration of some important electrolytes like bicarbonate ion, potassium ion, sodium ion and chloride ion using the ion specific electrode machine®.

Statistical analysis

Data were expressed as means ± S.E.M. of 6 rats per group; statistical differences were calculated using Student's t-test, one-way and two-way ANOVA with repeated measures followed by Bonferroni post hoc test. Significance was set at $p < 0.05$. All statistical tests were performed using Graphpad Prism (v. 6.0 Graphpad software).

Results and Discussions

There were no significant changes in the serum calcium concentration across groups. The serum calcium concentration across the four groups showed no significant difference ($P > 0.05$) as seen in figure 1. There are several possible mechanisms for this including the fact that the plasma concentration of calcium is tightly regulated by the thyroid gland (producing calcitonin) and the parathyroid gland (producing parathormone). These two hormones monitor the plasma concentration of calcium closely and maintain it within a limit of 1.3 - 1.5 mmol/L [26]. The body uses bone tissue as a calcium reservoir and source to keep calcium levels in blood, muscle, and intercellular fluids constant [17]. Also, the doses administered are within therapeutic range and can be handled well as long as the thyroid and parathyroid glands are functioning properly.

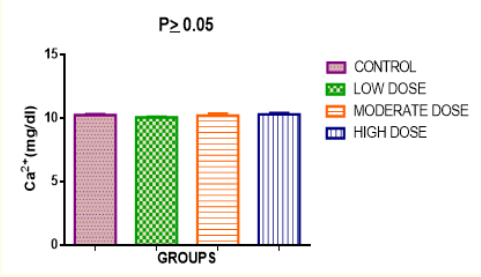

Figure 1: *Bar chart showing the serum calcium ion concentration following the administration of calcium. There were no significant changes across the groups administered different doses (P > 0.05).*

Ion concentration (mg/dl)	Group 1	Group 2	Group 3	Group 4
Calcium ion	10.25 ± 0.065	10.05 ± 0.065	10.20 ± 0.173	10.30 ± 0.115
Sodium ion	138.8 ± 0.629	139.3 ± 1.797	137.7 ± 1.202	137.3 ± 1.202
Potassium ion	5.325 ± 0.132	5.125 ± 0.111	5.233 ± 0.088	5.233 ± 0.088
Bicarbonate ion	22.25 ± 0.250	21.25 ± 0.478	22.00 ± 0.577	21.67 ± 0.667
Chloride ion	107.3 ± 0.478	108.0 ± 1.414	105.7 ± 0.333	106.0 ± 1.155
Na^+/K^+	26.10 ± 0.591	27.20 ± 0.542	26.32 ± 0.480	26.25 ± 0.355

Table 1: *Comparing the mean values of some plasma electrolytes in wistar rats following the administration calcium.*

Normal histology was observed in control group (Group A) and low dose group (Group B). The cardiac myocytes of the control and low dose group showed normal histology as seen in plates 3.2.1 through 3.2.4. These were comparable with results in a study by Gouda., *et al*. [8] who showed the various histologic architectures across different sections of the cardiac myofibers of normal rats.

Group A - Control group

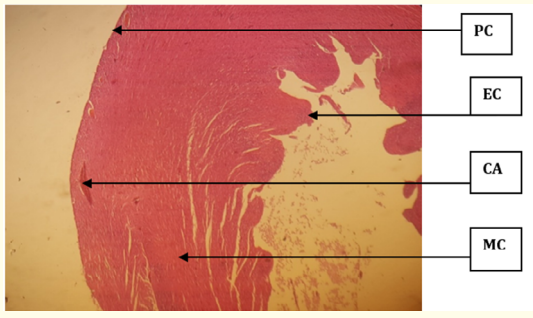

Plate 1: *Photomicrograph of control rats showing normal histological features: Pericardium (PC); Endocardium (EC); Coronary artery (CA); Syncytial arrangement of the Myocardium (MC) H&E x 40.*

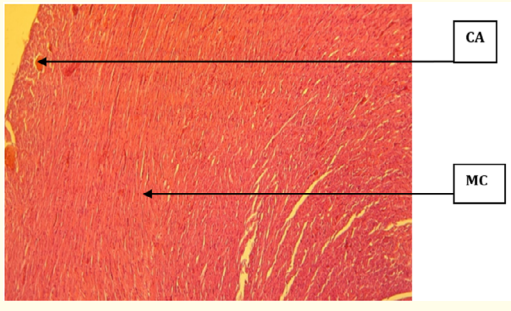

Plate 2: *Photomicrograph of control rats showing normal histological features: Coronary artery (CA); Syncytial arrangement of the myocardium (MC) H&E x 100.*

Group B - Administered a low dose (7.69 mg/kg) of calcium supplements for 28 days

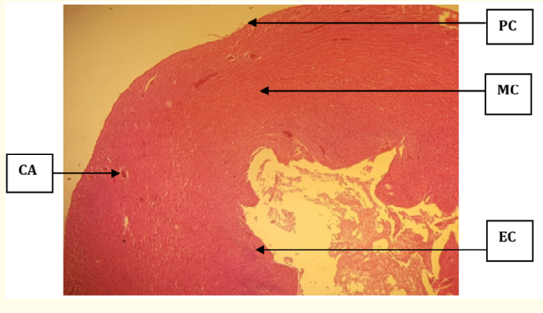

Plate 3: *Photomicrograph of rats treated with 7.69 mg/kg body weight of calcium supplements showing normal histological features: Pericardium (PC); Endocardium (EC); Coronary artery (CA); Syncytial arrangement of the Myocardium (MC) H&E x 40.*

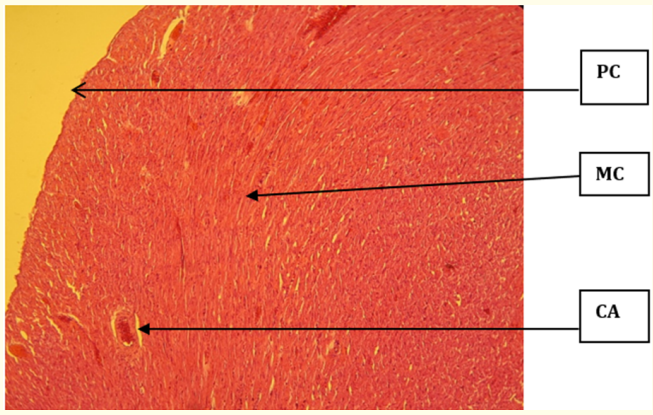

Plate 4: *Photomicrograph of rats treated with 7.69 mg/kg body weight of calcium supplements showing normal histological features: Coronary artery (CA); Pericardium (PC); Syncytial arrangement of the Myocardium (MC) H&E x 100.*

Disruption of the histology of cardiac myocytes were observed in moderate dose group (Group C) and high dose group (Group D). The cardiac myocytes of the moderate and high dose groups on the other hand showed disrupted morphology, vacuolations, and degenerative changes (DC) as seen in plate 6 and 8. The structural changes seen were similar to the findings of Ajibade., *et al.* [30] who administered experimental artesunate to adult Wister rats to investigate the acute, therapeutic and chronic effects of artesunate treatment on the heart. The results were also comparable with the experimental models of myocardial infarction proposed by Filho., *et al* [31].

Group C -Administered moderate dose (15.38 mg/kg) of calcium supplements

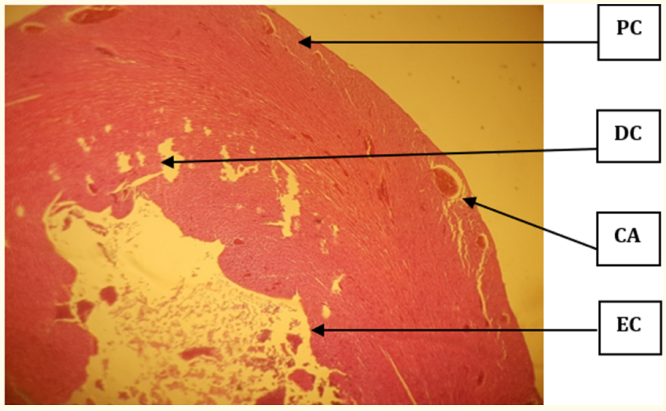

Plate 5: *Photomicrograph of rats treated with 15.38 mg/kg body weight of calcium supplements showing disrupted histology: Pericardium (PC) appear normal; Endocardium (EC) appear normal; Congested Coronary artery (CA); degenerative changes in the myocardium (DC) H&E x 40.*

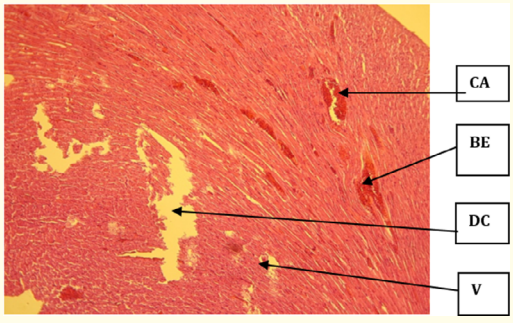

Plate 6: *Photomicrograph of rats treated with 15.38 mg/kg body weight of calcium supplements showing disrupted histology: Congested Coronary artery (CA); Blood extravasate in the myocardial interstices (BE); degenerative changes in the myocardium (DC); Vacuoles (V) H&E x 100.*

Group D -Administered a high dose (23.07 mg/kg) of calcium supplements

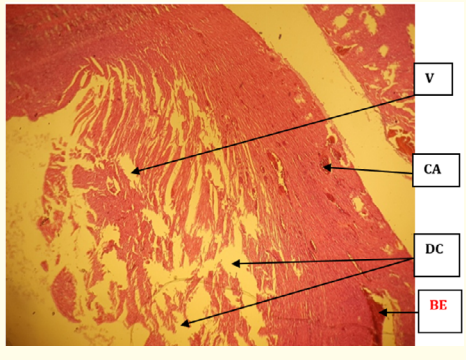

Plate 7: *Photomicrograph of rats treated with 23.07 mg/kg body weight of calcium supplements showing disrupted histology: Congested Coronary artery (CA); blood extravasate in the myocardial interstices (BE); degenerative changes in the myocardium (DC) H&E x 40.*

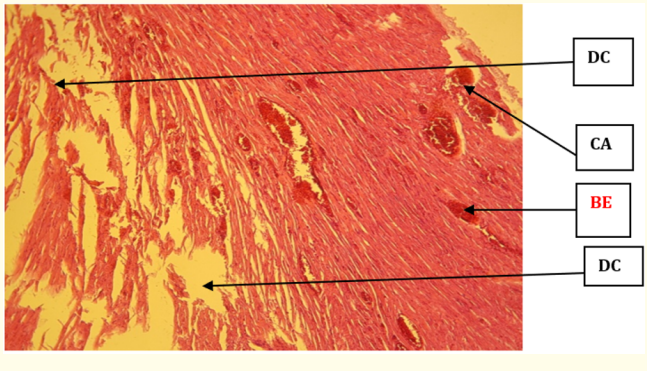

Plate 8: *Photomicrograph of rats treated with 23.07 mg/kg body weight of calcium supplements showing disrupted histology: Congested Coronary artery (CA); blood extravasate in the myocardial interstices (BE); degenerative changes in the myocardium (DC) H&E x 100.*

Discussion

Calcium loading caused by supplements may cause soft tissue or ectopic calcification, especially in the coronary arteries [13]. Calcium can play a role in cardiovascular disease pathogenesis through a variety of mechanisms, including lipid metabolism, insulin secretion and sensitivity, inflammation, thrombosis, body weight regulation, and vascular calcification [32]. Excess calcium from the diet and supplements is thought to accumulate in vascular tissues, forming plaques that eventually narrow the lumen of these blood vessels, resulting in poor blood flow and ischemia of the affected region rather than promoting bone health. Pathological modifications, most likely caused by atheromas, transform smooth muscle cells to bone forming cells, or osteoblasts [33]. Calcium ions are thought to be transferred from bone to arteries and other soft tissue locations in these situations. Instead of increasing skeletal mass, excessive calcium consumption can lead to cardiovascular calcification, especially smooth muscle calcification [34,35].

Filho., *et al.* [31] suggested several mechanisms to understand isoproterenol-induced myocardial infarction, one of which was that there is a mismatch between oxygen supply to cardiomyocytes and myocardial hyperfunction due to increased chronotropism and inotropism, as well as hypotension in the coronary bed [36]. Another proposed mechanism is an increase in Ca^{2+} overcharge within the cell [37]. Ca^{2+} is also related to the activation of the adenylate cyclase enzyme, which leads to the degradation of ATP levels over time [38]. There is an increase in oxidative stress and the formation of free radicals as a result of several metabolic products obtained from isoproterenol [39]. One of the limitations of this study was our inability to assess electrocardiographic, echocardiographic, and hemodynamic factors, which would have given more information about the myocardial injury's consequences.

Apoptosis has been observed in a variety of cardiac pathologies, including ischaemic and reperfusion damage, myocardial infarction and heart failure, according to some reports [40]. The pathophysiology of ischaemic heart disease and congestive heart failure includes contractile dysfunction due to altered calcium handling, impaired excitation-contraction coupling, electrical instability and myocyte degradation, altered neurohumoral equilibrium, calcium homeostasis and extracellular matrix composition, and cardiomyocyte apoptosis [41]. Almost all cell death in myocardial infarction is believed to be caused by apoptosis [41]. Apoptosis has been found in the center and boundary regions of the ischaemic environment, as well as in viable myocardium [42]. Apoptosis is thought to be linked to ventricular dysfunction in heart failure [41].

Conclusion

Calcium supplementation in low doses did not cause any significant changes in the cardiac myocyte structure. In moderate (15.38 mg/kg) and high (23.07 mg/kg) doses, it caused degeneration of cardiac myocytes and may pose an impeding danger of myocardial infarction and heart failure. These changes were however not linked to changes in serum calcium concentrations as there were no significant changes in the serum calcium concentrations amongst the groups due to the tightly regulated calcium homeostasis by the thyroid and parathyroid glands.

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