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A Novel Modulatory Role of Vitamin D₃ in Exercise-Induced Apoptosis of Rat Skeletal Muscle

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Abstract: The present study has been designed to investigate the possible modulating role of vitamin D₃ on chronic exercise-induced apoptosis in skeletal (gastrocnemius) muscle of rats. Rats were run for 8 weeks on a treadmill at a speed of 36 m min⁻¹ to induce apoptosis in their skeletal muscle. Vitamin D₃ (0.03 µg/100 g and 0.06 µg/100 g, s.c.) treatment, thrice weekly, was started three days prior to subjecting the animals to chronic exercise and was continued for 8 weeks of the experimental protocol. Oxidative stress was assessed by measuring the levels of TBARS, super oxide anion generation and reduced glutathione in gastrocnemius muscle. Further, serum level of corticosterone (glucocorticoid), a potent indicator of stress, was measured. Moreover, the apoptotic cell death was assessed using DNA gel electrophoresis. Chronic treadmill exercise produced profound DNA smearing. The levels of TBARS, super oxide anion generation and serum corticosterone were noted to be increased and the level of GSH was noted to be decreased in rats subjected to chronic treadmill exercise. However, treatment with vitamin D₃ markedly reduced DNA smearing. Moreover, the chronic exercise-induced increases in TBARS level, superoxide anion generation and corticosterone levels were significantly attenuated by treatment with vitamin D₃. In addition, vitamin D₃ markedly increased the exercise-induced decrease in GSH level. It suggests that antioxidant potential of vitamin D₃ and its novel efficacy in modulating exercise-induced corticosterone levels in rats may contribute to its anti-apoptotic effect.

Key words: Vitamin D₃, apoptosis, skeletal muscle, stress, antioxidant

INTRODUCTION

The term apoptosis is derived from a Greek word meaning falling off leaves from tree and is described as programmed cell death (PCD) (Geske and Gerschenson, 2001). It is a genetically controlled, biochemical and morphological response of cells to commit suicide (Steller, 1995) evoked through internal (Taniike *et al.*, 2008) as well as external pathological or physiological signals (Hets, 1998). It plays an integral role in a variety of biological events, including morphogenesis, tissue homeostasis and removal of unwanted or harmful cells (Schultz and Harrington, 2003). The morphological features of apoptosis include changes in plasma membrane asymmetry and attachment, condensation of cytoplasm and nucleus and inter-nucleosomal cleavage of DNA (Ameisen, 2002).

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Exercise has been reported to induce apoptosis in the skeletal muscle of mice and rats (Sandri *et al.*, 1995). Strenuous exercise stimulates a variety of signals, such as, increase in reactive oxygen species (ROS), glucocorticoids (Yuan *et al.*, 2003), which in turn have been suggested to be the possible reason for increased apoptosis after acute intense exercise.

Vitamin D₃, formed in the skin during the exposure of 7-dehydrocholesterol to sunlight, undergoes a hydroxylation on C-25 in the liver and a subsequent hydroxylation in the kidney to form 1,25-dihydroxyvitamin D₃ [1,25(OH)₂D₃], which is the biologically active form of vitamin D (DeLuca, 1974). Until the 1980s, the function of vitamin D was considered largely in the area of calcium, phosphorus and bone metabolism. Continued pursuit has resulted in the understanding that vitamin D₃ is a multi-potential vitamin having influence on numerous target cells. Its pro-differentiating, anti-proliferating and immuno-modulatory roles have instigated extensive work in the field of cancer, hypertension, diabetes (Holick, 2004), psoriasis (Morimoto, 1986), arthritis (Cantorna *et al.*, 1998) and autoimmune disorders (Cantorna *et al.*, 2004).

Few reports propose vitamin D₃ as a pro-apoptotic agent (Park *et al.*, 2000; Narvaez and Welsh, 2001), while others describe a protective role of vitamin D₃ against apoptosis (Manggau *et al.*, 2001; Riachy *et al.*, 2002). The effect of 1,25(OH)₂D₃ is undoubtedly dose dependent, apparent even in nano-molar concentration and time dependent, maximal after a 24 h pre-incubation (Ravid *et al.*, 2002). Physiological concentration of 1,25(OH)₂D₃ do not induce apoptosis in primary melanocytes despite a cell growth inhibitory effect (Sauer *et al.*, 2003). Proposed mechanisms of the anti-apoptotic effect of 1,25(OH)₂D₃ involve reduction in the release of mitochondrial cytochrome-c (DeHaes *et al.*, 2003) and formation of Sphingolipid breakdown product sphingosine-1-phosphate (S1P) (Manggau *et al.*, 2001), resulting in a changed Bcl-2/Bax ratio (Sauer *et al.*, 2005) and by the up-regulation of Bcl-2 expression (Wang *et al.*, 1999). Pretreatment with vitamin D₃ has been reported to inhibit efficiently but not completely UVB-induced activation of poly (ADP-ribose) polymerase (PARP) (DeHaes *et al.*, 2003). PARP, a DNA repairing enzyme enhances the survival of cells (Burkle, 2001). 1,25(OH)₂D₃ has been reported to inhibit caspase-3 activation and protects the cells from caspase-independent cell death-induced by hyper-osmotic and oxidative stress (Diker-Cohen *et al.*, 2003). 1,25(OH)₂D₃ regulates the expression of death receptors and protects cancer cells from apoptosis induced by death ligands (Zhang *et al.*, 2005). Pretreatment of hippocampal cells with vitamin D₃ (close to physiological level) *in vitro* has been reported to inhibit cell death (Obradovic *et al.*, 2006).

It is reported that the anti-oxidant properties of vitamin D₃ *in vivo* (Sardar *et al.*, 1996). Its efficacy as an effective anti-neoplastic agent and its anti-oxidant potential in murine lymphoma was also subsequently reported by us (Mukhopadhyay *et al.*, 2000). Vitamin D at physiological concentration has been found to protect cell proteins and membranes against oxidative damage (Ravid and Koren, 2003) and there is evidence that it may stabilize chromosomal structure and enhance repair of double strand breaks (Chatterjee, 2001). Active form of vitamin D₃ has been recognized as an anti-oxidative agent through their induction of metallothionein, a potent free radical-scavenging protein (Hanada *et al.*, 1995).

The present study therefore aims to investigate the possible role of Vitamin D₃ in chronic treadmill exercise-induced apoptosis in skeletal muscle (gastrocnemius) of rats. This is also an effort to elucidate whether vitamin D₃'s antioxidant potential can possibly contribute to its anti-apoptotic efficacy. Vitamin D₃ has been reported to decrease glucocorticoid levels *in vitro* (Obradovic *et al.*, 2006). We have attempted to understand if a cross-talk exists between exercise-induced glucocorticoid levels and vitamin D₃ *in vivo* and can this contribute to its possible effect on apoptosis.

MATERIALS AND METHODS

The experimental protocols used in the present study were approved by institutional animal ethical committee. Male Wister albino rats weighing about 200-225 g were maintained on the standard

rat food (Kissan Feed Ltd. Chandigarh, India) and water *ad libitum*. They were raised in departmental animal house and regulated for temperature ($25\pm 0.5^\circ\text{C}$), humidity (50-60%) and were exposed to 12 h cycle of light and dark.

Experimental Protocol

In the present study, 5 groups were employed and each group comprised of 8 animals.

Group 1 (Normal Control, n = 8)

The rats were not allowed to treadmill exercise but were handled daily to the noise of the treadmill.

Group 2 (Chronic Exercise Control, n = 8)

The rats were subjected to chronic treadmill exercise for 8 weeks.

Group 3 (Chronic Exercise + Vehicle Control, n = 8)

The rats were treated with propylene glycol (10%, used as vehicle, s.c.), thrice weekly, starting three days prior to subjecting the animals to chronic treadmill exercise and this was continued throughout the duration of the exercise.

Group 4 (Chronic Exercise + Vitamin D₃, 0.03 µg/100 g, n = 8)

The rats were treated with vitamin D₃ (0.03 µg/100 g, dissolved in 10% propylene glycol, s.c.), thrice weekly, starting three days prior to subjecting the animals to chronic treadmill exercise and was continued throughout the duration of exercise.

Group 5 (Chronic Exercise + Vitamin D₃, 0.06 µg/100 g, n = 8)

The rats were treated with vitamin D₃ (0.06 µg/100 g, dissolved in 10% propylene glycol, s.c.), thrice weekly, starting three days prior to subjecting the animals to chronic treadmill exercise and was continued throughout the duration of exercise.

Animals of Groups 2, 3, 4 and 5 ran on a level motorized rodent treadmill (Patiala Sports Industries, Patiala, India) 5 days weekly for 8 weeks. During the 1st 4 weeks, the speed of the treadmill and duration of the running sessions were gradually increased from a speed of 10 m min^{-1} for 10 min, to a running speed of 36 m min^{-1} for 60 min by the end of the 4th week. For the next 4 weeks, a 5 min warm up session at a speed of 20 m min^{-1} was followed by the 60 min running session at a speed of 36 m min^{-1} . Animals of the normal control group (Group 1) were handled daily and subjected to the noise of the running treadmill by placing their cages next to the treadmill when their other partners ran on the treadmill. This procedure was designed to minimize any possible confounding effect of external factors (e.g., handling, treadmill noise) on the dependent variables. At the end of 8th week, exercised animals as well as normal control animals were sacrificed and gastrocnemius tissues were quickly removed. Blood was collected prior to their sacrifice by retro orbital bleeding for serum preparation.

Gastrocnemius Muscle DNA Extraction and Gel Electrophoresis

The concentration of DNA was determined spectrophotometrically (DU 640 Spectrophotometer, Beckman Coulter Inc. CA, USA) at 260 nm. Protein contamination of DNA was assessed by determining the ratio of absorbance at 260 and 280 nm, which should be more than 1.75. To detect the inter-nucleosomal cleavage, 10-12 µg of extracted DNA was added to equal volume of loading dye and

it was loaded in the well. Electrophoresis was carried out using 1.8% agarose gel in 1x TBE buffer for 1.15 h at 400 mA, 50 V and 3 W in submarine electrophoresis unit (Pharmacia Biotech, Freiburg, Germany). Ethidium bromide ($0.5 \mu\text{g mL}^{-1}$) was added to the gel for DNA detection.

Estimation of Tissue Thiobarbituric Acid Reactive Substances (TBARS) Level

The whole gastrocnemius tissue TBARS level was measured by the method of Ohkawa *et al.* (1979). Tissue was homogenized in Tris buffer (pH-8.3) and the homogenate was mixed with sodium dodecyl sulphate (SDS), acetic acid (pH-3.5) and thiobarbituric acid (TBA). The test tubes were incubated at 95°C for 60 min and then cooled. n-Butanol: pyridine (15:1 v/v) mixture was added to the test tubes and centrifuged at the 4,000 g for 10 min. The absorbance of developed colour in organic layer was measured spectrophotometrically at 532 nm. The absorbance was extrapolated from standard curve generated using tetra-methoxy propane as standard. Results were expressed as $\mu\text{mole mg}^{-1}$ of protein and protein content was estimated by the method of Lowry *et al.* (1951).

Estimation of Tissue Superoxide Anion Generation Level

The whole gastrocnemius tissue super oxide anion level was measured by the method of Wang *et al.* (1998). Weighed amount of tissue was placed for 1.5 h in buffer at 37°C containing 100 mM L^{-1} of nitrobluetetrazolium (NBT). NBT reduction was stopped by addition of 0.5 N HCl. The tissue was minced and homogenized in a mixture of 0.1 N NaOH and 0.1% SDS in water containing 40 mg L^{-1} diethylene triamine penta acetic acid. The mixture was centrifuged at 20,000 g for 20 min and the resultant pellet was re-suspended in 1.5 mL of pyridine and kept at 80°C for 1.5 h to extract formazan, an adduct formed after reaction of NBT with super oxide anions. The mixture was centrifuged at 10,000 g for 10 min and the absorbance of the formazan was determined spectrophotometrically at 540 nm. The amount of reduced NBT was calculated using the formula: Amount of reduced NBT = $A \times V / T \times Wt \times \epsilon \times l$, where A is the absorbance, V is the volume, T is the time for which the tissue was incubated with NBT, Wt is the blotted wet weight of the tissue, ϵ is the extinction coefficient (0.72 L/mM/mm) and l is the length of the light path. Results were expressed as Reduced NBT picomole/minute per mg.

Estimation of Reduced Glutathione (GSH) Level

The whole gastrocnemius tissue GSH level was measured by the method of Beutler *et al.* (1963). Tissue homogenate was taken and the proteins were precipitated with 10% w/v chilled trichloroacetic acid. Samples were kept in ice bath and were centrifuged after 30 min at 1000 g for 10 min at 4°C . GSH levels were measured in the supernatant, which was mixed with 0.3 M disodium hydrogen phosphate solution. Freshly prepared DTNB (5,5'-dithiobis-2-nitrobenzoic acid) solution ($40 \text{ mg}/100 \text{ mL}$ in 1% w/v sodium citrate) was added just before measuring the absorbance spectrophotometrically at 412 nm. Different concentrations of GSH standard was also used to prepare a standard curve simultaneously. Results were expressed as micro mole/mg of protein

Estimation of Serum Corticosterone Level

The estimation of corticosterone in serum was carried out by the method of Silber *et al.* (1958). Serum was treated with freshly prepared chloroform: methanol mixture (2:1, v/v), followed by chloroform. The samples were vortexed for 30 sec and centrifuged at 2000 rpm for 10 min. The chloroform layer was carefully removed with the help of a syringe and was transferred to a fresh tube. The chloroform extract was then treated with 0.1 N NaOH by vortexing rapidly and NaOH layer was immediately removed. The samples were then treated with 30 N H_2SO_4 by vortexing vigorously. After phase separation, chloroform layer on the top was removed using the syringe and discarded. The tubes containing H_2SO_4 layer were kept in dark for 30-60 min and thereafter, fluorescence measurements

were carried out in a spectrofluorometer (ELCO ® SL-174) with excitation and emission wavelengths set at 472 and 532.2 nm, respectively. Blank was prepared without adding sample. Different concentration of corticosterone standard was used to prepare standard curve. Results were expressed as micro gram/deciliter.

Statistical Analysis

All data was expressed as mean±SEM. Statistical significance was calculated using one-way ANOVA. Tukey's multiple range test was employed as post hoc test for multiple comparison. Statistical significance was indicated if $p < 0.05$.

Drugs and Chemicals

Vitamin D₃ was obtained as a gift sample from Ind-Swift Ltd. Parwanoo (HP), India. DNA, proteinase K, RNAase, Agarose gel, BSA, glutathione, 5,5'-dithiobis-2-nitrobenzoic acid were purchased from SRL Pvt. Ltd., Mumbai, India. Corticosterone was purchased from Sigma, St. Louis, MO, USA. All other chemicals were obtained from SD. Fine Chem Ltd., Mumbai, India and were of analytical grade.

RESULTS

Effect of Vitamin D₃ on Gastrocnemius Muscle DNA Smearing

There was apparently no DNA smearing in the gastrocnemius muscle of the Normal Control Group (Group 1), as is evident from Fig. 1. Chronic treadmill exercise produced significant gastrocnemius muscle DNA smearing in Chronic Exercise Control Group (Group 2), as compared to Normal Control Group (Fig. 2). The administration of vehicle did not alter the chronic treadmill exercise-induced DNA smearing in Vehicle Control Group (Group 3), as compared to Chronic Exercise Control Group (Group 2). However, the administration of Vitamin D₃ reduced chronic exercise-induced DNA smearing significantly. Interestingly, this attenuating effect of the vitamin was more pronounced in the group receiving 0.06 µg/100 g dose (Group 5), than in the one receiving the lower dose of 0.03 µg/100 g, (Group 4), thus exhibiting dose dependency of the vitamin, as is evident from Fig. 2.

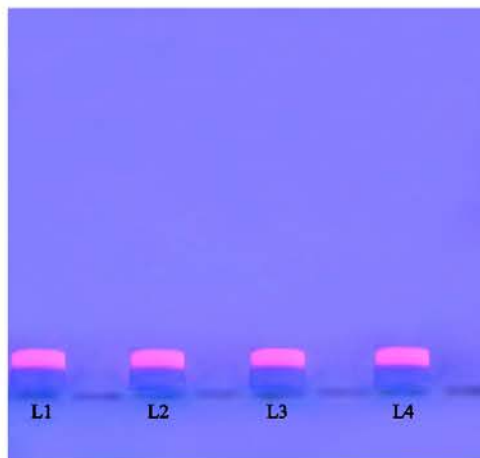


Fig. 1: Electrophoretic pattern of DNA extracted from gastrocnemius muscle of Normal Control Group rats. L1, L2, L3 and L4 represent lanes. No DNA smearing was observed in Group 1. Wells were loaded with 12 µg of DNA

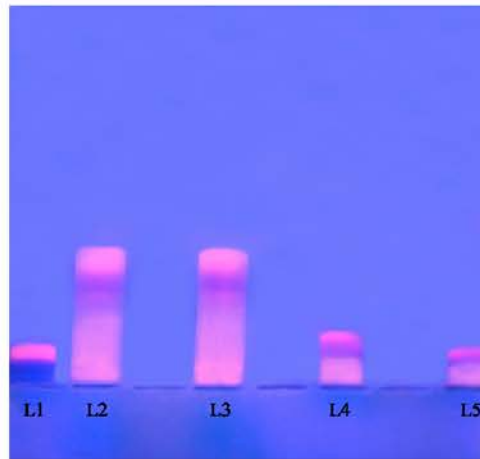


Fig. 2: Electrophoretic pattern of DNA extracted from gastrocnemius muscle of rats. L1, L2, L3, L4 and L5 represent DNA extracted from gastrocnemius muscle of Group 1, 2, 3, 4 and 5 rats respectively. Chronic exercise produce DNA smearing in Chronic Exercise Control Group as compare to Normal Control Group and vehicle has no modulating effect on DNA smearing. However, Vitamin D₃ treatment reduces DNA smearing significantly in a dose dependant manner and higher dose exhibiting more efficacies. Wells were loaded with 12 µg of DNA

Table 1: Effect of vitamin D₃ on TBARS level, superoxide anion (Reduced NBT) generation, GSH level and corticosterone level

Parameters	Normal control group (Group 1)	Chronic exercise control group (Group 2)	Chronic exercise+ vehicle control group (Group 3)	Chronic exercise+ vitamin D ₃ (0.03 µg) treated group (Group 4)	Chronic exercise+ vitamin D ₃ (0.06 µg) treated group (Group 5)
TBARS (µmol mg ⁻¹ of protein)	5.520±0.4864	10.400±0.927 ^a	10.220±1.02	8.400±0.96 ^b	5.500±0.82 ^{b,c}
Superoxide anion (Reduced NBT) (µmol/min/mg)	13.900±1.46	31.400±2.43 ^a	31.670±3.24	21.900±1.93 ^b	14.600±1.62 ^{b,c}
GSH (µmol mg ⁻¹ of protein)	1.300±0.21	0.510±0.36 ^a	0.540±0.22	0.970±0.27 ^b	1.250±0.74 ^{b,c}
Corticosterone (mg dL ⁻¹)	0.034±0.001	0.082±0.005 ^a	0.079±0.003	0.052±0.005 ^b	0.033±0.004 ^{b,c}

Results are expressed as mean±SEM (n = 8). ^ap<0.05 vs normal control group (Group 1); ^bp<0.05 vs chronic exercise control group (Group 2); ^cp<0.05 vs chronic exercise + vitamin D₃ (0.03 µg/100 g) treated group (Group 4)

Effect of Vitamin D₃ on Gastrocnemius Muscle TBARS Level

Chronic treadmill running causes significant increase in gastrocnemius muscle TBARS level in Chronic Exercise Control Group (Group 2), as compared to Normal Control Group (Group 1), as can be seen in Table 1. TBARS level in the Vehicle Control Group (Group 3), exhibited no difference from that of Groups 2 animals. However, treatment of Vitamin D₃ reduces chronic exercise-induced increase in TBARS level significantly in a dose dependent manner (Table 1).

Effect of Vitamin D₃ on Gastrocnemius Muscle Super Oxide Anion Generation Level

Table 1 shows the gastrocnemius muscle superoxide anion generation in the different experimental groups. As is evident from the Table 1, chronic treadmill running causes significant increase in super oxide anion production (Group 2), compared to that of the Normal Control Group (Group 1).

Administration of the vehicle had no effect on this exercise-induced increase in super oxide anion production. However, administration of Vitamin D₃ reduces chronic exercise-induced increase in super oxide anion production significantly, the higher dose being more effective than the lower.

Effect of Vitamin D₃ on Gastrocnemius Muscle Reduced Glutathione (GSH) Level

Chronic treadmill running causes significant decrease in gastrocnemius muscle GSH level in Chronic Exercise Control Group (Group 2), in comparison to the Normal Control Group (Group 1), as can be observed in Table 1. Administration of the vehicle did not alter chronic treadmill running-induced decrease in GSH level in Group 3 as compared to Group 2 animals. However, administrations of Vitamin D₃ increase the chronic exercise-induced decrease in GSH level significantly, again in a dose dependent manner (Table 1).

Effect of Vitamin D₃ on Serum Corticosterone Level

Chronic treadmill running causes significant increase in serum corticosterone level, as was seen in Group 2 animals (Table 1). Administration of the vehicle did not modulate chronic treadmill running-induced increase in corticosterone level in the Vehicle Control Group (Group 3), as compared to the Chronic Exercise Control Group (Group 2). However, both groups of vitamin D₃ treated animals demonstrated marked significant reduction in chronic exercise induce-increase corticosterone production. Here also the higher dose of the vitamin was more effective in attenuating the elevated serum corticosterone level, as is evident from Table 1.

DISCUSSION

The active gene directed process of cell death, apoptosis, is characterized by fragmentation of chromosomal DNA (Steller, 1995). A characteristic pattern of this is DNA ladder formation (Wyllie, 1980) and can be recognized by electrophoresis on an agarose gel. Some reports have investigated the influence of acute intense exercise on apoptosis in skeletal muscles of both young normal and dystrophin-deficient (mdx) mice and have measured the apoptotic changes by DNA gel electrophoresis (Sandri *et al.*, 1995). Chronic treadmill running at a speed of 36 m min⁻¹, starting from 10 m min⁻¹, for 8 week, has been used to induce apoptosis in the skeletal (gastrocnemius) muscle of rats in this study. The DNA smearing noted in the Exercise Control Group (Group 2) may thus reflect apoptosis-induced-necrotic cell death, as observed in other similar studies (Gold *et al.*, 1994).

Two different doses of vitamin D₃ were employed in the present study, keeping in view the abundance of reports on both pro (Park *et al.*, 2000; Narvaez and Welsh, 2001) and anti-apoptotic role of the vitamin D₃ (Manggau *et al.*, 2001; Riachy *et al.*, 2002). However, since most studies report calcium-mediated toxicity of high doses of vitamin D in rats (Kitagawa *et al.*, 1992), 0.03 µg/ 100 g and 0.06 µg/100 g, thrice weekly doses were chosen for the present study. Studies with such doses have also been reported to have beneficial effect on apoptosis (Manggau *et al.*, 2001; Obradovic *et al.*, 2006). This is of utmost importance because the hypercalcaemic action of vitamin D₃ is considered a major limitation in its therapeutic use (Studzinski *et al.*, 1993). The antiapoptotic effect of Vitamin D by upregulating antiapoptotic protein Bcl-2 has been demonstrated (Duque *et al.*, 2004). The complete checking of DNA smear formation in Group 5 and the near-normal pattern in Group 4 animals in this study definitely speak of the potential of vitamin D₃ as an anti-apoptotic agent.

Strenuous exercise has been reported to stimulate a variety of signals, such as, increase in ROS and glucocorticoids secretion, which in turn can potentially induce apoptosis (Bejma and Ji, 1999). Therefore, estimation of parameters such as DNA gel electrophoresis and TBARS, ROS, GSH and Corticosterone (Glucocorticoids) in this study stand justified.

Free radicals are toxic agents created as by-products of metabolic activity. Under aerobic conditions, the participation of oxygen in redox reactions is unavoidable and ROS such as super oxide anion, hydroxyl radical, hydrogen peroxide, lipid peroxides and nitric oxide are produced (Sjodin *et al.*, 1990). It has been documented that exercise can increase the generation of ROS in biological tissues like skeletal muscles and myocardium and also increase metabolic rate and oxygen consumption (Reid *et al.*, 1992; Bejma *et al.*, 2000). Increased production of ROS during intense exercise has thus been suggested to be the possible reason for increased apoptosis after exercise (Phaneuf and Leeuwenburgh, 2001). Rats exercising on treadmill have been reported to show an increase in ROS above resting levels (Bejma and Ji, 1999). One of the predominant processes resulting from oxidative stress includes oxidative lipid peroxidation, measured as TBARS (Carlson and Sawada, 1995). Study results corroborate with the above mentioned studies and show that chronic treadmill exercise leads to a considerable increase in TBARS concentration, an index of lipid peroxidation, in the gastrocnemius muscle of rats.

In this study, vitamin D₃ administration significantly attenuated the exercise-induced lipid peroxidation (TBARS) and this effect was significantly more pronounced in the higher dose of Vitamin D₃ treated animals (Group 5). It has been suggested that ROS influences apoptosis mainly through the modulation of the mitochondrial mediated pathway. It has also been hypothesized that a high oxidative stress level destabilizes the mitochondrial membrane homeostasis and therefore induces the formation of mitochondrial membrane permeability pores and release of pro-apoptotic factors such as cytochrome-c (Pollack and Leeuwenburgh, 2001). Vitamin D₃, a highly lipophilic compound, may accumulate in cell membranes, stabilize the membranes, decrease membrane fluidity and thus decrease lipid peroxidation (Mukhopadhyay *et al.*, 2000). Thus, one of the possible underlying mechanisms of the anti-apoptotic effect of the vitamin could be its inhibitory effect on lipid peroxidation.

The generation of super oxide anion (ROS) in the gastrocnemius muscle can be successfully quantified by measuring the amount of NBT reduction (Wang *et al.*, 1998). This indicator has been used in the present study as NBT reduction is very sensitive to even low levels of super oxide anion as a result of the accumulation of blue formazan in the tissue over time. This study reveals that chronic treadmill exercise drastically increases the production of super oxide anion in the gastrocnemius muscle and vitamin D₃ treatment reduces this exercise-induced increase in super oxide anion level to near-normal values, the higher dose exhibiting a greater efficacy.

The thiol system and the antioxidant enzymes are a major cell defense against acute oxygen toxicity. GSH is known to maintain the intra-cellular redox balance in mammalian cells (Uhlrig and Wendel, 1992) and produce an ameliorative effect on cellular injury (Meister, 1988). GSH can react with reactive oxygen metabolites (ROMs) in a number of ways. It reduces H₂O₂, one of the major ROS, to form water, itself getting oxidized to form glutathione disulphide, GSSG (Ross *et al.*, 1985).

The observed significant decrease in GSH level in the chronic exercise control group thus, is in support of the above mentioned reports. Also, GSH has been reported to prevent DNA damage caused by certain free radicals (Sarkar *et al.*, 1994). In addition, exercise-induced decreased GSH levels, can trigger mitochondria to release caspase-activating proteins, such as cytochrome-c and AIF (Apoptosis Inducing Factor) into the cytosol, marking early apoptotic event (Green and Reed, 1998). Thus, decrease in GSH, along with heightened pro-oxidant status, may contribute to the DNA damage observed in exercise-induced apoptosis in gastrocnemius muscle of rat.

Treatment of vitamin D₃ causes significant increase in reduced glutathione (GSH) levels, the higher dose again showing greater ability in elevating GSH levels. Dietary vitamin D₃ has been reported to possess more antioxidant efficacy than vitamin E. It effectively modulates GSH and enzymes of the cellular antioxidant defense system, i.e. superoxide dismutase, glutathione peroxidase, glutathione reductase and glucose 6-phosphate dehydrogenase (Sardar *et al.*, 1996). It has also been shown that

high levels of oxidative stress, induced by dietary selenium and vitamin E deficiencies, increase caspase-like activity and DNA fragmentation (as measured by DNA gel electrophoresis) in chick skeletal myocytes (Nunes *et al.*, 2003). Thus, decreased lipid peroxidation and super oxide anion, in association with elevated GSH levels, undoubtedly speaks of the controlled pro-oxidant status and improved antioxidant defense brought about by vitamin D₃ treatment in this study.

Increased secretion of glucocorticoids during intense exercise could also be suggested as one of the positive factors for increased apoptosis after acute intense exercise (Phaneuf and Leeuwenburgh, 2001). Corticosterone (Glucocorticoids) overload in rats has been reported to result in the impairment of mitochondrial oxidative energy metabolism in brain (Katyare *et al.*, 2003). The present study recorded significantly higher level of corticosterone following chronic treadmill exercise, which is well in agreement with the above mentioned studies.

Vitamin D₃ has been reported to inhibit *in vitro* dexamethasone (specific agonist of glucocorticoid receptor) induced apoptosis in hippocampal cells (Obradovic *et al.*, 2006). In the present study, vitamin D₃ effectively decreased the level of exercise-induced serum corticosterone. Therefore, the protective effect of vitamin D₃ in exercise-induced apoptosis of gastrocnemius muscle may additionally be due to the vitamin's inhibitory effect on glucocorticoids.

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