

The Protective Effect of Melatonin vs. Vitamin E in the Ischemic/Reperfused Skeletal Muscle in the Adult Male Rat Model

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Abstract

Aim: The present study was designed to evaluate the role vitamin E vs. melatonin in the prevention of the harmful effect of ischemia/reperfusion on the skeletal muscle of the adult male albino rat.

Materials and methods: forty-four adult male albino rats were used in this study. The rats were divided into four groups; each group consisted of 6 rats. Group I (control group), group II (ischemic/reperfusion group): ischemia was induced by clamping the right femoral arteries for two hours then the clamps were removed for 2 hours to induce reperfusion, group III (Vit E-treated group): the rats received Vitamin E injection one hour prior to reperfusion and group VI (Melatonin-treated group): the rats received melatonin injection one hour prior to reperfusion. At the end of the experiment, rats were sacrificed and samples from the right quadriceps muscles were subjected to biochemical, light and electron microscopic studies. The oxidative markers malondialdehyde (MDA) and glutathione (GSH) were measured in the muscular tissue.

Results: The skeletal muscle was markedly affected after induction of ischemia/reperfusion. The skeletal myocytes showed fragmentation, cytoplasmic lysis and degeneration. The nuclei were pyknotic and central. There were intercellular edema and exudation. The mitochondria were damaged and there was vascular congestion. The mean value of MDA increased, while that of the GSH decreased. The administration of vitamin E or melatonin showed marked improvement in the biochemical profile as well as the histological architecture of the skeletal muscle. However, the improvement was more obvious in the melatonin-treated group.

Conclusion: The protective effect of melatonin is superior to vitamin E in the protection of the skeletal muscle against the harmful effect of I/R.

Keywords: Skeletal muscle; Ischemia; Vitamin E; Melatonin; Electron microscopy

Introduction

Ischemia/reperfusion (I/R) injury in skeletal muscle results from many vascular and muscular traumas, diseases or surgical procedures such as infrarenal aortic reconstruction and muscular flap [1]. The authors reported that I/R injury results in damage of the endothelial and the parenchymal cells. They added that I/R accelerate necrosis and apoptosis of skeletal muscle cells. Toledo-Pereyra et al. [2] pointed out that I/R causes significant mitochondrial damage that could be the mechanism of I/R – induced cell death. In addition, I/R can activate macrophages causing release of cytokines which influence leucocytic activation, transmigration and target cell adhesion. The enormous number of leucocytes that enter the circulation after reperfusion release large amount of reactive oxygen species (ROS) overwhelming the cell antioxidant defenses and cause damage to lipids, proteins and nucleic acids [3].

Vitamin E (α -Tocopherol) is one of the known antioxidants that prevents the propagation of free radical reaction [4]. In addition, vitamin E has an anti-inflammatory [5] and angiogenic [6] properties. It was found that plasma level of vitamin E decreased significantly one hour after reperfusion, suggesting an increase requirement of this vitamin to protect against I/R injury [7].

Melatonin is a highly effective scavenger of ROS through activation of enzymatic antioxidants. It also acts synergistically with other antioxidant such as vitamin C [8]. In addition, it inhibits directly the process generating ROS, protects nuclear DNA, membrane lipids and cytosolic proteins from oxidative stress-induced damage [9].

The present study was designed to evaluate the protective role of

vitamin E vs. Melatonin against I/R injury in the skeletal muscle in an animal model.

Materials and Methods

Chemicals

Vitamin E (α -Tocopherol) (Sigma-Aldrich; St. Louis, Mo. USA) was obtained in the form of one ml ampoules in a concentration of one mg/ml. It was given in a dose of 10 mg/kg body weight by intraperitoneal injection (I.P.) [1].

Melatonin (Sigma-Aldrich; St. Louis, Mo. USA) was obtained in the form of one gram vial. It was dissolved in sterile physiological saline and was given in a dose of 10 mg/kg body weight by I.P. injection [8].

Animal and experimental design

Twenty-four adult male albino rats weighing 180-200 g were used in the current study. They were obtained from the Animal House, Faculty of Medicine, Cairo University. Every three rats were

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housed in a separate cage and received tap water and fed ad libitum. All procedures were done according to the recommendations of the animal experimental committee.

All surgical procedures were performed under anesthesia with I.P. injection of Ketamine hydrochloride (Ketame, Egyptian Int. Co. E.P.I.C.O.) in a dose of 60 mg/kg body weight and 10 mg/kg body weight xylazine cocktail (Xyla Ject, Egyptian Co. for chemicals and pharmaceuticals) [1]. Anesthesia was maintained by an additional half dose every 90 minutes. In all animals, the right femoral artery was exposed via transverse groin incision and the artery was identified. The rats were divided into four groups, six rats in each group as follow:

Group I: (control group): divided as follow: three rats did not receive any injection and three rats received normal isotonic saline (2 ml) by intraperitoneal injection.

Group II (I/R group): The right femoral arteries were ligated by atraumatic microvascular clamps for two hours to induce ischemia then the clamps were removed for 2 hours to induce reperfusion.

Group III (I/R+ vitamin E-treated group): Vitamin E was injected I.P. one hour prior to reperfusion.

Group IV (I/R+Melatonin-treated group): Melatonin was injected I.P. one hour prior to reperfusion.

After 2 hours of induction of ischemia, confirmed by color changes of the sole of the foot and by palpation the femoral artery, the clamps were removed for reperfusion, confirmed by restoration of hind limb color. After 2 hours of reperfusion, the rats were sacrificed by Ketamine hydrochloride (100 mg/kg) [10]. Muscle samples were taken from the quadriceps muscles. The central portion was taken for sampling while the edges of the muscle flap were not included to avoid the complication of surgical trauma.

Biochemical investigations

Muscle samples were immediately stored at -30°C for the assessment of glutathione (GSH) as an indicator of oxidative stress and the results were expressed as µg/g tissue and malondialdehyde (MDA) to evaluate the degree of oxidative damage and results were estimated as nanomole/g tissue.

Histological evaluation

Light microscopic study: The muscle samples of all groups were fixed in 10% formol solution and kept for 24 hours then dehydrated in ascending grades of alcohol and cleared in xylene then embedded into paraffin wax. Serial sections (5 µm thickness) were prepared. The sections were stained with Haematoxylin and Eosin (H&E) [11].

Electron microscopic study: Small pieces of the muscles were fixed in 3% glutaraldehyde (PH 7.4) in phosphate buffer for 24 hours and post fixed in 2% osmium tetroxide in phosphate buffer for one hour. One micron-thick sections were cut and stained with toluidine blue. Ultrathin sections (80-90 nm) were stained with uranyl acetate and lead citrate [12]. Tissue sections were evaluated using a JEOL transmission electron microscope JEM-1200. Ex, Japan.

Statistical analysis: Statistical analysis was achieved by using SPSS (Statistical Package for Social Sciences), windows version 20, Chicago, USA. Comparison between different groups was made using one way analysis of variance (ANOVA). Results were considered statistically significant with the value of $P < 0.05$ and the data were tabulated [13].

Results

Biochemical analysis

The mean concentrations of the MDA in the muscle tissue were significantly increased, while that of the GSH was significantly decreased in group II as compared to that of the control group. In group III (vitamin E-treated group) and group IV (melatonin-treated group) the mean values of MDA were significantly decreased as compared to that of group II. The mean values of GSH were significantly increased in groups III and IV as compared to that of group II. However, the mean values of MDA and GSH showed significant difference between groups III and IV (Table 1).

Light microscopic results

The sections of the skeletal muscle, in the control group, showed long cylindrical muscle fibers with multiple, peripheral and long nuclei. Delicate connective tissue (endomysium) was present between the muscle fibers (Figures 1 and 2).

In ischemic rats (group II), the skeletal muscle fibers showed degenerative changes including fragmentation, disruption of the muscle fibers, intercellular edema (Figures 3-6) and pyknotic central nuclei (Figures 6 and 7). Hyalinization of the sarcoplasm (Figure 5) was noticed. Some sections showed congested blood vessels and cellular infiltration (Figure 7).

In group III (vitamin E-treated), the skeletal muscle sections showed relatively normal histological architecture with narrower intercellular spaces except for few central nuclei (Figures 8 and 9).

In group IV (Melatonin-treated), the skeletal muscle sections showed relatively normal histological architecture and intercellular spaces (Figures 10 and 11).

	Control group	I/R group	Vitamin E-treated group	Melatonin-treated group
GSH	2.15 ± 0.17	0.5 ± 0.3*	1.6 ± 0.14*	2.01 ± 0.11**
MDA	15.5 ± 2	40.7 ± 4.6*	24.4 ± 3.9*	18.4 ± 2.8*

*Significantly different from the corresponding value of the control group.

**Significantly different from the corresponding value of the I/R group.

*Significantly different from the corresponding value of vitamin E-treated group.

Table 1: Mean values of tissue biochemical parameters in the studied groups.

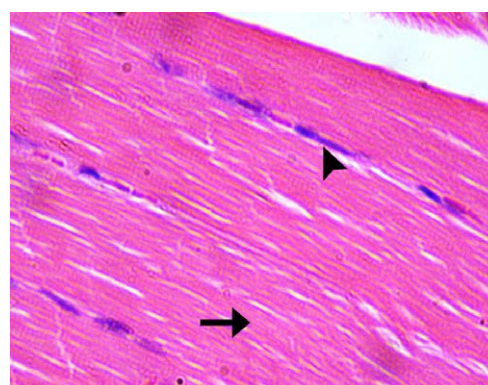


Figure 1: Photomicrograph of a section in the skeletal muscle of a control rat (group I) showing: Normal histological architecture of the skeletal muscle fibers which appear longitudinally cut (arrow) with multiple, long and peripheral nuclei (arrow head). Hx&E 400x.

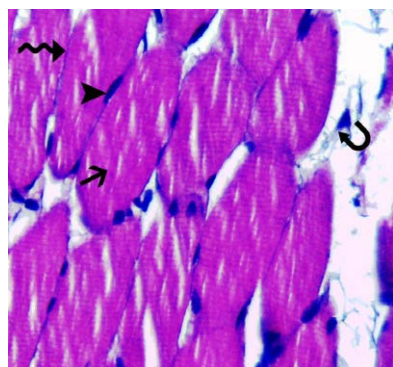


Figure 2: Photomicrograph of a section in the skeletal muscle of a control rat (group I) showing: Normal histological architecture of the skeletal muscle fibers which appear transversely cut (arrow) with multiple, long and peripheral nuclei (arrowhead). Note the connective tissue endomysium separating the muscle fibers (wavy arrow) and the connective tissue perimysium separating the muscle bundles (curved arrow). Hx&E 400x.

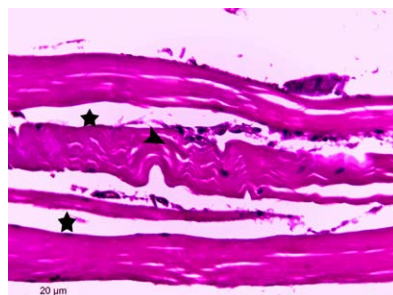


Figure 3: Photomicrograph of a section in skeletal muscle of a rat of group II showing: wide separation of the skeletal myocytes (stars) and fragmentation (arrowhead) of the skeletal muscle fibers. Hx&E 400x.

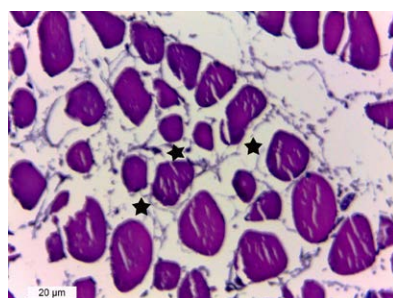


Figure 4: Photomicrograph of a section in the skeletal muscle of a rat of group II showing: wide separation of the skeletal myocytes (stars). Hx&E 400x.

Electron microscopic results

The skeletal muscle tissue of the control group showed normal pattern. The myofibrils showed regular arrangement (Figures 12-14). The nuclei were euchromatic (Figure 12). Globular mitochondria were seen between the myofibrils (Figures 13 and 14).

In group II, ischemia induced deleterious effects on the architecture of the skeletal muscle tissue in the form of fragmented and degenerated myofibrils (Figures 15-19), cytoplasmic lysis (Figure 18), increased intermyofibrillar space and damaged nuclei (Figure 15) electron dense mitochondria (Figures 15 and 16) and ballooning of the mitochondria with rupture of its membrane (Figures 17 and 19). There were

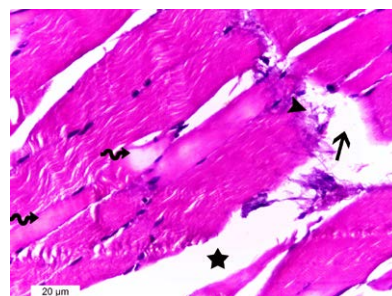


Figure 5: Photomicrograph of a section in skeletal muscle of a rat of group II showing: wide separation of the skeletal myocytes (star), fragmentation (arrowhead) and marked disruption (arrow) of the skeletal muscle fibers. Some muscle fibers showed areas of hyalinization of the sarcoplasm (wavy arrows). Hx&E 400x.

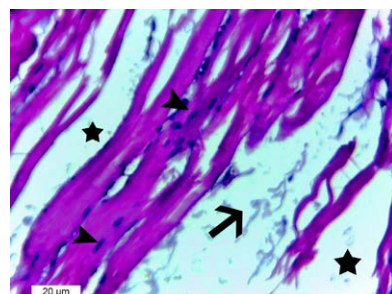


Figure 6: Photomicrograph of a section in skeletal muscle of a rat of group II showing: wide separation of the skeletal myocytes (stars) and marked disruption (arrow) of the skeletal muscle fibers. Note central nuclei (arrowheads) Hx&E 4x.

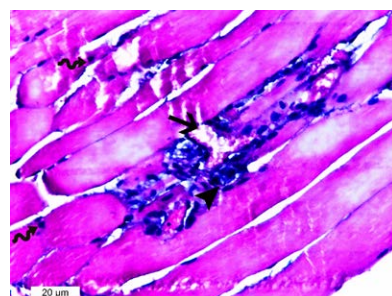


Figure 7: Photomicrograph of a section in skeletal muscle of a rat of group II showing: cellular infiltration of the skeletal muscle fibers (arrowhead). Note the congested blood vessels (arrow) and pyknotic nuclei (wavy arrows). Hx&E 400x.

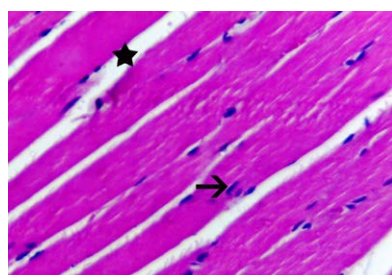


Figure 8: Photomicrograph of a section in skeletal muscle of a rat of group III (vitamin E-treated) showing: narrower intercellular spaces (star) between the skeletal muscle fibers. Note: central nucleus (arrow). Hx&E 400x.

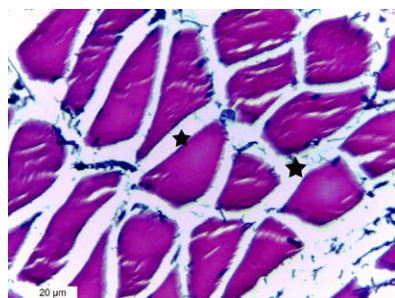


Figure 9: photomicrograph of a section in the skeletal muscle of a rat of group III showing: relatively narrow intercellular space between the skeletal myocytes (stars). Hx&E 400x.

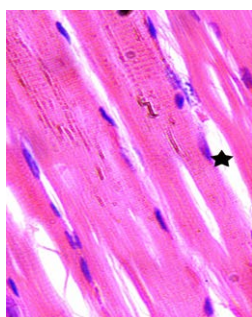


Figure 10: photomicrograph of a section in skeletal muscle of a rat of group IV (Melatonin-treated) showing: narrower intercellular spaces (star) between the skeletal muscle fibers which appear relatively normal compared to the control group. H&E 400x.

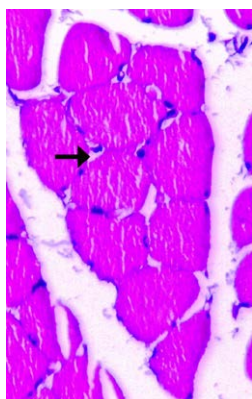


Figure 11: photomicrograph of a section in the skeletal muscle of a rat of group IV showing: normal intercellular space between the skeletal myocytes (arrow). Hx&E 400x.

congested blood vessels with damaged endothelial cell nuclei (Figure 16) and intermyofibrillar exudate (Figure 18).

In the vitamin E-treated group, the skeletal muscle tissue showed improvement of the architecture. The myofibrils had apparently normal pattern of arrangement except for focal areas of degeneration (Figures 20 and 21) and few electron-dense (Figure 21).

In the Melatonin-treated group, the architecture of the skeletal muscle was apparently normal as compared to the control group with regularly arranged myofibrils (Figures 22 and 23) and normal mitochondrial pattern (Figure 23).

Discussion

Restoration of blood flow after induction of ischemia causes severe skeletal muscle damage [9]. In the present study, ischemia caused deleterious damage to the skeletal myocytes in the form of vacuolization, fragmentation and hyalinization of the skeletal muscle fibers, pyknotic central nuclei and damaged mitochondria. Similar findings were reported by Gian et al. [14]. It was reported that ischemia induced the production and the accumulation of xanthine oxidase enzyme leading to the production of unstable oxygen molecules. Secondary chemical reactions, using these oxygen molecules, produced reactive oxygen species (ROS) which interact with the cell membrane

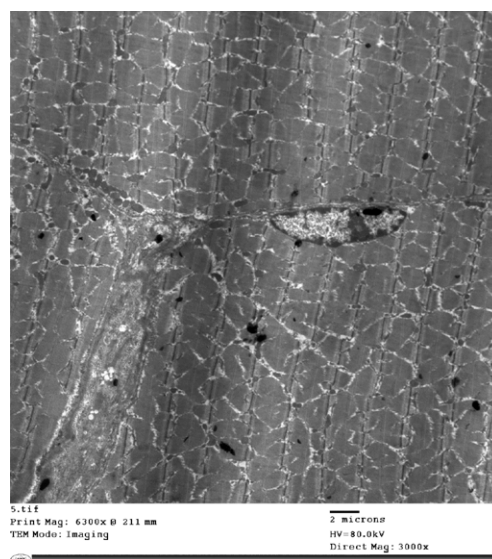


Figure 12: Electron micrograph of a skeletal muscle of a control rat showing regular arrangement of the myofibrils and euchromatic peripheral flattened nucleus (N) with prominent nucleolus (NO). (X3000).

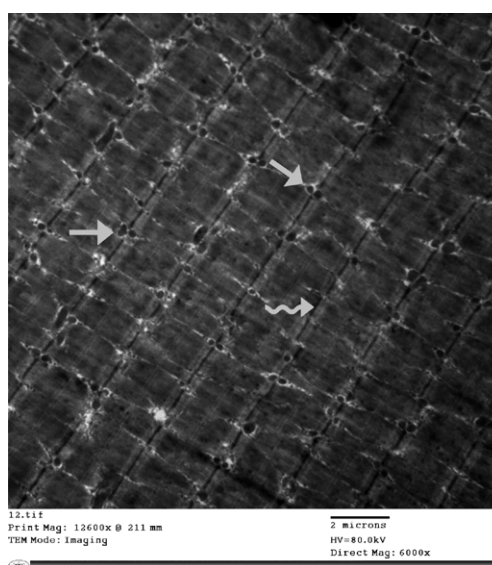


Figure 13: Electron micrograph of a skeletal muscle of a control rat showing regular arrangement of the myofibrils. Note the z line (wavy arrow) and the mitochondria (arrow). (X6000).

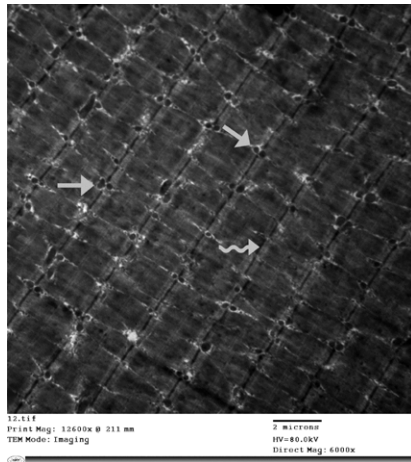


Figure 14: Electron micrograph of a skeletal muscle of a control rat showing the Z lines (arrow) in the center of the I bands (I). Note the arrangement of the mitochondria (arrowhead) x12000.

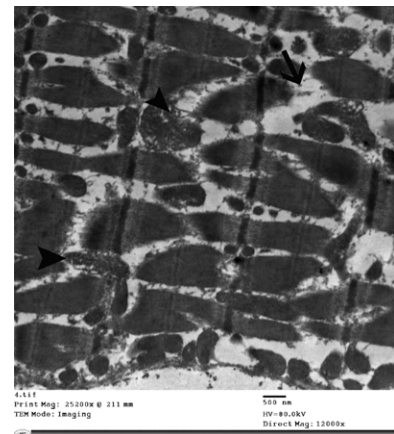


Figure 17: Electron micrograph of a skeletal muscle of a rat of group II showing fragmentation of the myofibrils (arrow) and ballooning of the mitochondria (arrowheads). X12000.

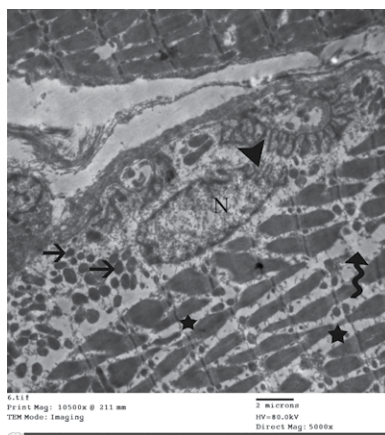


Figure 15: Electron micrograph of a skeletal muscle of a rat of group II showing loss of regular arrangement of the myofibrils, fragmentation of the myofibrils (wavy arrow) with increased inter myofibrillar space (star), electron-dense mitochondria (arrows) and irregular outline of the nuclear membrane (arrowhead). X5000.

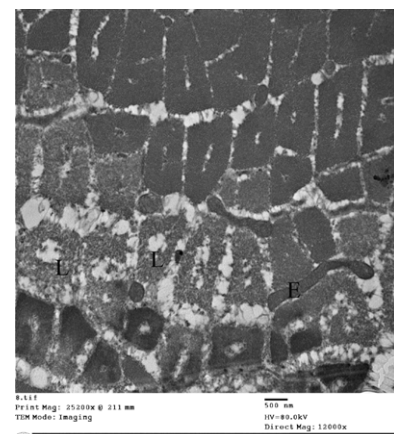


Figure 18: Electron micrograph of a skeletal muscle of an ischemic rat showing transversely oriented myofibrils exhibiting cytoplasmic lysis (L) with complete loss the normal architecture. Note the presence of intermyofibrillar exudate (E). (X12000).

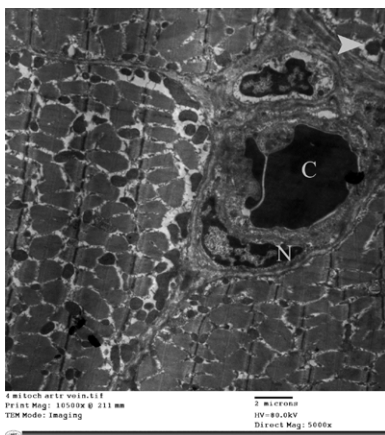


Figure 16: Electron micrograph of a skeletal muscle of an ischemic rat showing congested blood vessel (C) and heterochromatic nucleus of the endothelial cell (N). NOTE: electron-dense mitochondria (arrow head) (X5000).

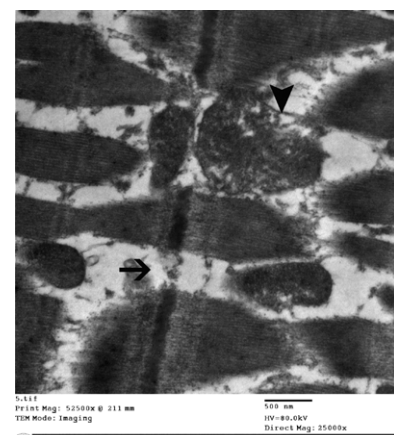


Figure 19: higher magnification of the previous figure showing ruptured mitochondrial membrane (arrow head) and interrupted z line (arrow). (X25000).

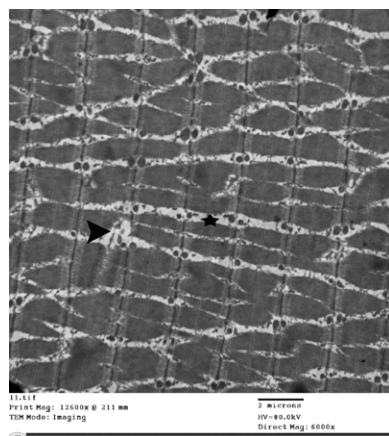


Figure 20: Electron micrograph of a skeletal muscle of a vitamin E-treated rat with relatively normal pattern and arrangement of the myofibrils and decreased intermyofibrillar space (star). Note: focal area of degenerated myofibrils (arrow head). X60000.

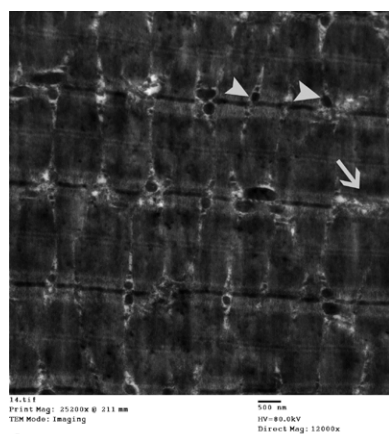


Figure 21: Electron micrograph of a skeletal muscle of a vitamin E-treated rat with relatively normal pattern and arrangement of the myofibrils and normal mitochondria. Note interrupted Z line and few electron dense mitochondria (arrow head). X12000.

phospholipids, proteins and nucleic acids causing lipid peroxidation and damage of the cell membrane integrity [1]. Dianat et al. [15] reported that, in addition to ROS production, ischemia/reperfusion decreased the cellular ATP production thus lead to morphological, biochemical and physiological cell damage.

Wang et al. [16] reported that reperfusion causes calcium ions overload, increase in PH and production of high levels of ROS leading to opening of nonspecific pores in the inner mitochondrial membrane causing its depolarization and ATP depletion, rupture of the mitochondrial membrane and release of the pre-apoptotic protein, cytochrome c, into the cytoplasm leading to cell apoptosis. In the present study, the mean value of GSH was significantly decreased as compared to that of the control group. This finding was in agreement with Gulden et al. [1]. The authors reported that the tissue damage caused by ROS production could stimulate many defense mechanisms under normal condition including antioxidant production such as glutathione (GSH), which is one of the most important antioxidants

against oxidative stress. However, during reperfusion these defense mechanisms were overcome.

In the current study, the mean value of MDA was significantly increased in group II as compared to that of the control group. This finding was in agreement with Gian el al. [14] who reported that reperfusion cause increase in the level of Malondialdehyde (MDA) in the muscle culture which is an indicator of peroxidative burst causing destruction of the unsaturated fatty acid of the cell membranes and cellular damage.

In the current study, there was intermyofiber, intermyofibrillar edema, exudation and vascular congestion. Similar findings were documented by Gulden et al. [1]. The authors found that ROS production cause endothelial cell damage and increased microvascular permeability to proteins. They added that reperfusion resulted in short term vasodilatation followed by gradual vasoconstriction. Wei et al. [8] found that the endothelial cells form protrusions occluding the capillary lumen in I/R and that endothelial cell damage is a major contributor to I/R-induced muscle cell apoptosis.

Tran et al. [17] reported that I/R induced reduction in the

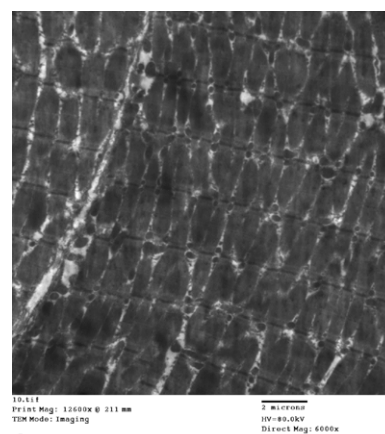


Figure 22: Electron micrograph of a skeletal muscle of a rat of group IV showing apparently normal arrangement of most of the myofibrils. X6000.

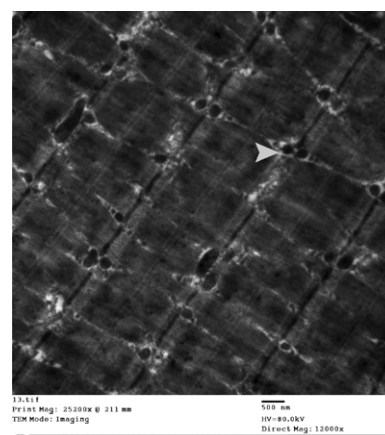


Figure 23: Electron micrograph of a skeletal muscle of a melatonin-treated rat with relatively normal pattern and arrangement of the myofibrils and normal mitochondria. Note electron dense mitochondria (arrow head). X12000.

mitochondrial respiratory function and increased the superoxide production thus mediating I/R-induced cellular damage in skeletal muscles.

Moreover, the circulating pro-inflammatory cytokines such as tumor necrosis factor- α and interleukin-1 increased in I/R [1], thus might provide another explanation for I/R-induced damage of the skeletal muscles.

In the current study, it was found that I/R induced apoptotic changes in the skeletal myocytes such as pyknotic nuclei. This was considered as typical features of apoptosis according to Hayakawa et al. [18].

In the present study, administration of vitamin E ameliorated the histological architecture of the skeletal muscle. These findings were in agreement with Estevez and Heinonen [19].

They reported that vitamin E inhibited the oxidation of myofibrillar proteins induced by I/R as proven by decrease of protein oxidation activities. They also added that vitamin E administration prevented ROS-induced lipid peroxidation as proven by decrease of MDA levels which was in agreement with the current study.

Dianat et al. [15] reported that vitamin E administration increased the antioxidant enzyme levels such as catalase (CAT) and glutathione peroxidase (GSH) which converted ROS into less reactive species which correlated with the current study. In addition, Koga et al. [20] found that vitamin E administration prior to induction of I/R decreased the level of pro-inflammatory cytokines and caspase-3 expression which is an apoptotic protein marker.

Moreover, Zinqq et al. [6] mentioned that vitamin E stimulated angiogenesis through stimulating the expression of Vascular Endothelial Growth Factor (VEGF) and thus having a beneficial role in prevention the I/R-induced damage.

In the present study, Melatonin administration proved to be beneficial in protecting the skeletal muscles from the injurious effects of I/R. These findings were in agreement with Korhan et al. [9] and Wei et al. [8].

It was reported that melatonin could reach all cellular compartments due to its small size and its amphiphilic properties [8]. The authors reported that administration of melatonin prior to I/R prevented the mitochondrial membrane depolarization and the release of the harmful cytochrome C protein into the cytoplasm induced by I/R. They added that melatonin, in addition to its direct ROS scavenging properties, decreased the superoxide generation in the arterial wall thus decreasing the endothelial cell dysfunction. In addition, Parlaktas et al. [21] documented that melatonin could increase the levels of antioxidant enzymes such as glutathione peroxidase. Moreover, it could decrease the tissue level of MDA, protein carbonyl and nitric oxide, which was in agreement with the present study. Takhtfooladi et al. [22] reported that preventing I/R injury depend, to an extent, on preventing the disturbance of nitric oxide metabolism and the generation of nitrogen-derived species. The authors found that melatonin could prevent the generation of these harmful nitrogen-based reactants.

Li et al. [23] found that melatonin administration in I/R was capable of diminishing the increase of NOX2 and NOX4 (belong to NO-protein family which is the major source of ROS) suggesting that melatonin exerted its antioxidant and antiapoptotic effects through decreasing the expressions of these proteins.

Another protective mechanism of melatonin against I/R-induced damage was reported by Kang et al. [24] who declared that melatonin down regulated autophagy stimulated by I/R. The authors mentioned that autophagy is a self-digestion system responsible for maintenance of cellular homeostasis and that it reacts with ROS produced by I/R.

From the present study, it could be concluded that prior administration of vitamin E or Melatonin had prophylactic role against I/R-induced injury of skeletal muscles. However, melatonin was proved to be more effective than vitamin E and that it produced more protective effect on the biochemical and the histological structure of the skeletal muscle.

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