# The histobiochemical effects of melatonin on ischemia reperfusion-related injuries in vascular trauma of lower limbs

*Ann. Ital. Chir., 2012 83: 49-54*

Rohollah Sobhani*, Hassan Masoudpour**, Mojtaba Akbari***, Hamid Reza Suzangar****, Samira AleSaeidi°, Shahriar Adibi°°, Sayed Amir Hossein Khademi°°°, Elham Fatemeh Khademi°°°°, Fahimeh Sobhani°°°°°

**Department of Surgery, Isfahan University of Medical Sciences Gonabad University of Medical Sciences, Isfahan Gonabad, Iran*
***Isfahan University of Medical Sciences, Isfahan, Department of Surgery, Abarkouh Faculty of Paramedical Science, Shahid Sadoughi University of Medical Sciences, Yazd, Iran*
****Isfahan University of Medical Sciences, Isfahan, Iran*
*****Department of Pathology, Alzahra Hospital, Isfahan University of Medical Sciences, Isfahan, Iran*
*°Department of Internal Medicine, Alzahra Hospital, Isfahan University of Medical Sciences, Isfahan, Gonahad University of Medical Sciences, Gonabad, Iran*
*°°Veterinary Department, Isfahan Torabinejad Research Center, Isfahan University of Medical Sciences, Isfahan, Iran*
*°°°Canadian Medical Association, Vancouver, Canada, Master of Science in Clinical Research (MSCR), Mount Sinai School of Medicine, New York, USA*
*°°°°Isfahan University of Technology, Isfahan, Iran*
*°°°°°Azad University of Medical Sciences, Mashhad, Iran*

**The histobiochemical effects of melatonin on ischemia reperfusion-related injuries in vascular trauma of lower limbs**

BACKGROUND*: The aim of this study was to evaluate the protective effect of melatonin on ischemia-reperfusion syndrome.*
METHODS: *Thirty-three adult male Wistar albino rats were randomized into three experimental groups of 11: Group C – control group with no ischemia or reperfusion. Groups I/R and I/R + M had 2.5 hours of ischemia and of two hours of reperfusion by means of clamping of the common femoral artery. All the animals received maintenance fluid with intraperitoneal (i.p) normal saline following general anesthesia. The animals of Group I/R + M were treated with i.p Melatonin (10 mg/kg) five minutes before reperfusion. At the end of reperfusion, samples were taken for histological evaluation and biochemical analysis. Parameters studied were biopsies of the soleus muscle, level of lactate, creatine phosphokinase (CPK), lactate dehydrogenase (LDH), sodium, potassium (K), calcium (Ca) and arterial blood gasometry.*
RESULTS: *In I/R group, the levels of K, CPK increased dramatically contrast with groups C and IR+M ($P<0.05$). A significant decrease in HCO3 was found in I/R Group in comparison with Group IR+M and Group C ($P<0.001$). In Group IR+M, lactate level decreased dramatically compared to other groups ($P<0.001$). Histological injury in I/R + M was found to be less than in I/R group ($P<0.05$). There was no significant difference in PO2, pH, carbon dioxide, partial pressure of oxygen, Na, LDH, Ca and P in three groups ($P>0.05$). Histological change in the group C and group M didn't differ significantly, but the difference in group I/R was significant compared to group C and IR+M ($P<0.05$).*
CONCLUSION: *We suggest that melatonin has protective effect against I/R syndrome in blood and skeletal muscle and may reduce the morbidity following revascularization surgery in vascular trauma.*

KEY WORDS: Antioxidants, Ischemic-Reperfusion injuries, Melatonin, Reperfusion syndrome, Vascular Trauma.

## Introduction

Peripheral vascular injuries are responsible for about 80% of all cases of vascular trauma which involve most commonly the lower extremities [1]. One of the important factors of outcome is the period immediately following revascularization that called ischemia reperfusion (I/R) syndrome. Releasing toxic oxygen-derived free radicals during reperfusion syndrome overwhelm defensive enzyme-scavenging systems resulting in cell damage and even death [2]. By means of experimentation, some scavengers of oxygen free radicals such as superoxide dismutase, catalase [3], mannitol [4], and allopurinol [5] were reported to protect against systemic and local manifes-

*Pervenuto in Redazione Dicembre 2010. Accettato per la pubblicazione Febbraio 2011.*
*Correspondence to: Hassan Masoudpour, Department of Surgery, Saint Alzahra Hospital, Isfahan University of Medical Sciences, Isfahan, Iran. (E-mail: Masodupour@yahoo.com).*

tations of reperfusion syndrome in animals pretreated with these agents. The pineal secretary product melatonin (5-methoxy-N-acetyl-tryptamine) is released in a circadian rhythm that regulates various physiological and neuroendocrine functions. Melatonin is a potent direct free radical scavenger that also induces antioxidative enzymes [6]. Moreover, melatonin may inhibit nitric oxide synthesis as a pro-oxidative enzyme [7] and prevent production of leukocyte adhesion molecule that overstates tissue destruction [8]. Some of melatonin's scavenging actions may actually be due to its metabolites, N-acetyl-N-formyl-5-methoxykynuramine (AFMK) and N1-acetyl-5-methoxykynuramine (AMK), which are also scavengers [9-11]..In this study, we evaluate the effect of melatonin in preventing I/R injury of rats investigated.

## Materials and Methods

### Animal preparation

This study was carried out on 33 young male Wistar-Albino rats. They were housed in a controlled room (temperature, 20 to 25°C humidity, 65% to 75%) in which a 12:12 h light: dark cycle was maintained. During experimentation, the rats were fed with standard rat chow and tap water. Studies on all groups were made during the same hours..All rats were handled according to the Ethical Principles for Animal Experiments of the International Council for Animal Protection, and Law no. 663 of May 8, 1979. All the experimental procedures were approved by the Research Ethics Committee of Isfahan University of Medical Science as per research protocol no. 287209. The rats were divided randomly into three groups of eleven animals and they received the same volume of melatonin or placebo solution intraperitonellay (i.p). Melatonin (Sigma-Aldrich Chemical, St Louis, MO) was dissolved in pure ethanol and later was diluted with normal saline summing up to a final concentration of 1% ethanol.

### Experimental Design

The anesthesia was done using inhalational anesthesia with ether and ketamine injection (intramuscular) to increase depth of anesthesia. All the animals were received drugs using intramuscular or intraperitoneal injections without insertion of venous cannulae. The femoral artery in the groin was exposed in all animals. In the groups M and I/R, unilateral lower limb ischemia were performed by clamping the common femoral artery with fine vascular clamp (Fig. 1) and the occlusion were verified helping LifeDop™ (SummitDoppler) Hand-Held Doppler (L250AC) device. In the group C, no occlusion of femoral artery was done. The control group (Group C, n=11) was received only the placebo solution (1% ethanol-containing saline) i.p without ischemic-reperfusion (I/R) period. The ischemic reperfusion group (Group I/R) underwent 2.5 hours of ischemia with

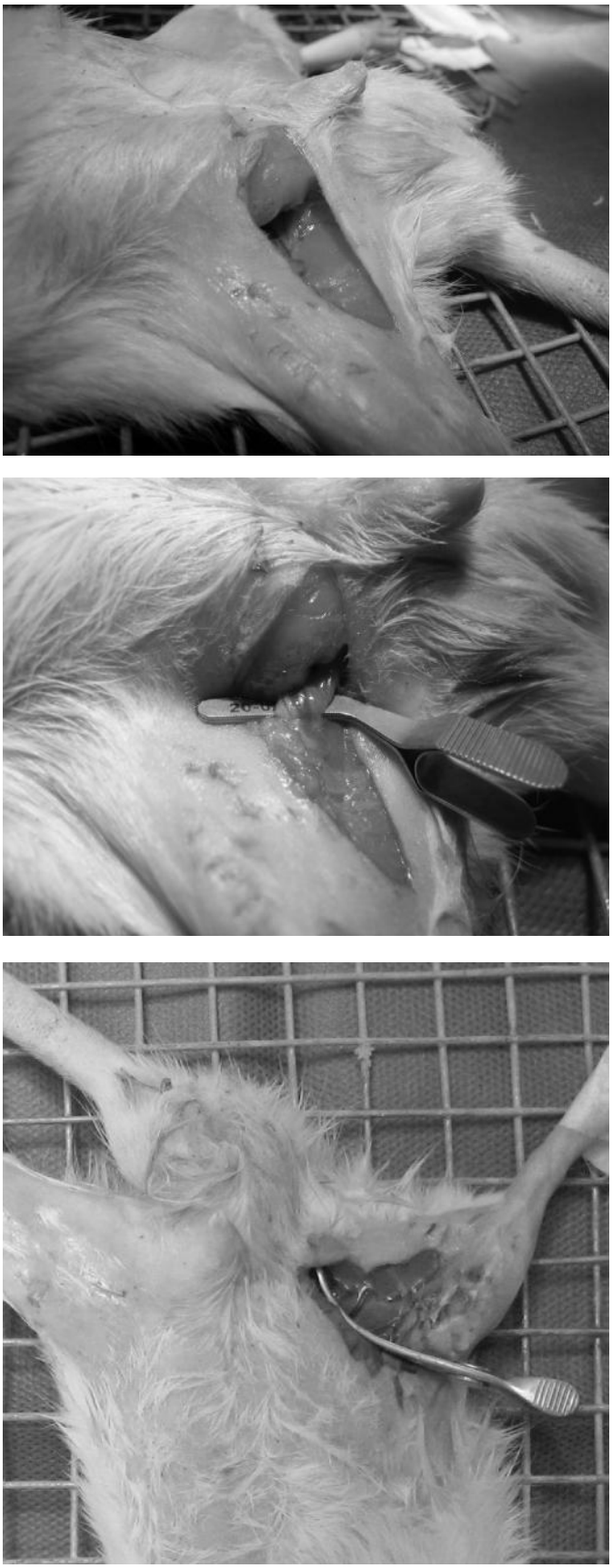

Fig. 1: Exploring the femoral artery in the groin and inducing limb ischemia using fine vascular clamp.

clamping the femoral artery followed by two hours of reperfusion. The animals in this group received only the placebo solution i.p 5 minutes before reperfusion. The melatonin group (Group M) received melatonin solution (10 mg/kg) 5 minutes before reperfusion instead of placebo solution in the group I/R. Heparin were administrated i.p 20 min before ischemia to prevent thrombosis in the clamped femoral artery. The reperfusion was confirmed by color change of the sole and detection of flow helping color Doppler device. At the end of reperfusion period, biopsy of soleus muscle for histological analysis and arterial blood sample from aorta for biochemistry were taken.

### Serum biochemical assay

For biochemistry evaluation of reperfusion syndrome, the following values are determined: Creatine phosphokinase (CPK), Lactate dehydrogenase (LDH), potassium (K), calcium (Ca), arterial blood gas (PO2, PCO2, PH and bicarbonate), lactate.

### Histologic Evaluation

The histological evaluation of muscle specimen was carried out at the department of pathology of Saint Alzahra Hospital. Following placing of soleus muscle specimen in 10% formaldehyde and fixing in paraffin, the five microm-

Table I - *Comparison of biochemical values in three groups*

| Lab | Groups | Mean | Std. Deviation | P Value |
|---|---|---|---|---|
| PH | Control Group | 7.2409 | .05804 | 0.177 |
| | IR Group | 7.2291 | .08938 | |
| | Melatonin Group | 7.2827 | .05405 | |
| $paCO_2$ (mmHg) | Control Group | 45.3400 | 4.38342 | 0.256 |
| | IR Group | 43.2400 | 5.49549 | |
| | Melatonin Group | 41.9300 | 4.35363 | |
| $HCO_3$ (meq/L) | Control Group | 19.8200 | 2.80314 | 0.00014 |
| | IR Group | 14.3555 | 1.98200 | |
| | Melatonin Group | 18.9800 | 3.43796 | |
| $PaO_2$ (mmHg) | Control Group | 60.2700 | 12.28292 | 0.970 |
| | IR Group | 59.2500 | 11.83641 | |
| | Melatonin Group | 60.3900 | 11.56853 | |
| Na (mg/d) | Control Group | 144.2500 | 6.35551 | 0.995 |
| | IR Group | 144.5600 | 7.86793 | |
| | Melatonin Group | 144.4900 | 8.59365 | |
| K (mg/d) | Control Group | 6.1982 | .92027 | 0.0399 |
| | IR Group | 7.4636 | 1.47535 | |
| | Melatonin Group | 6.3936 | 1.11426 | |
| Ca (mg/d) | Control Group | 9.8800 | 1.07126 | 0.420 |
| | IR Group | 9.1900 | 1.29108 | |
| | Melatonin Group | 9.4236 | 1.31757 | |
| P (mg/d) | Control Group | 9.4709 | 1.84921 | 0.696 |
| | IR Group | 10.0909 | 2.05794 | |
| | Melatonin Group | 9.3500 | 2.55783 | |
| CPK | Control Group | 2655.4000 | 1361.08135 | 0.00016 |
| | IR Group | 5177.2000 | 1322.93475 | |
| | Melatonin Group | 3172.5000 | 1158.96594 | |
| LDH | Control Group | 2531.7000 | 897.97617 | 0.910 |
| | IR Group | 2505.8000 | 1535.77478 | |
| | Melatonin Group | 2332.3000 | 976.30057 | |
| Lactate | Control Group | 23.4000 | 2.28910 | < 0.0001 |
| | IR Group | 34.3000 | 5.36749 | |
| | Melatonin Group | 20.9000 | 3.30000 | |

eters sections were stained with hematoxylin and eosin (H&E) and evaluated under Olympus optical microscope..Histological score was performed as follows: disorganization and degeneration of the muscle fibers (0: Normal, 1: Mild, 2: Moderate, 3: Severe); Infiltration of inflammatory cells (0: Normal, 1: Mild, 2: Moderate, 3: Severe).

### Statistical analysis

The data of three groups was evaluated using SPSS software V.16. All data were compared using
Kruskal Wallis test (ANOVA) and chi-square test. Values of $P < 0.05$ were considered as significant.

## Results

The levels of potassium and CPK increased significantly in the Group I/R in comparison with to Group M and Group C ($P<0.05$). A dramatic decrease in the HCO3 and increase in the lactate was determined in I/R Group in comparison with to Group M and Group C ($P<0.001$). There was no significant difference in PO2, PH, CO2, Na, LDH, Ca and P in three groups ($P>0.05$) (Table I, II). Evaluating the histological variables, we found mild perivascular infiltration of lymphocytes and edema of muscle fibers in six rats of I/R group (Fig. 2). In one rat of the I/R group, muscle fiber necrosis accom-

Table II - *The statistical result of significant biochemical changes ($HCO_3$,.CPK, LDH, K) in the rats.*

| Group | | N | Subset for alpha = .05 1 | 2 |
|---|---|---|---|---|
| | | **HCO3** | | |
| Tukey HSD[a] | IR Group | 11 | 14.3555 | |
| | Melatonin Group | 11 | | 18.9800 |
| | Control Group | 11 | | 19.8200 |
| | Sig. | | 1.000 | .764 |
| Duncan[a] | IR Group | 11 | 14.3555 | |
| | Melatonin Group | 11 | | 18.9800 |
| | Control Group | 11 | | 19.8200 |
| | Sig. | | 1.000 | .488 |
| | | **CPK** | | |
| Tukey HSD[a] | Control Group | 11 | 2655.4000 | |
| | Melatonin Group | 11 | 3172.5000 | |
| | IR Group | 11 | | 5177.2000 |
| | Sig. | | .617 | 1.000 |
| Duncan[a] | Control Group | 11 | 2655.4000 | |
| | Melatonin Group | 11 | 3172.5000 | |
| | IR Group | 11 | | 5177.2000 |
| | Sig. | | .352 | 1.000 |
| | | **Lactate** | | |
| Tukey HSD[a] | Melatonin Group | 11 | 20.9000 | |
| | Control Group | 11 | 23.4000 | |
| | IR Group | 11 | | 34.3000 |
| | Sig. | | .298 | 1.000 |
| Duncan[a] | Melatonin Group | 11 | 20.9000 | |
| | Control Group | 11 | 23.4000 | |
| | IR Group | 11 | | 34.3000 |
| | Sig | | .140 | 1.000 |
| | | **K** | | |
| Tukey HSD[a] | Control Group | 11 | 6.1982 | |
| | Melatonin Group | 11 | 6,3936 | 6.3936 |
| | IR Group | 11 | | 7.4636 |
| | Sig. | | .922 | .106 |
| Duncan[a] | Melatonin Group | 11 | 6.1982 | |
| | Melatonin Group | 11 | 6.3936 | |
| | IR Group | 11 | | 7.4636 |
| | Sig. | | .703 | 1.000 |

Means for groups in homogeneous subsets are displayed.
[a] Uses Harmonic Mean Sample Size = 11.000.

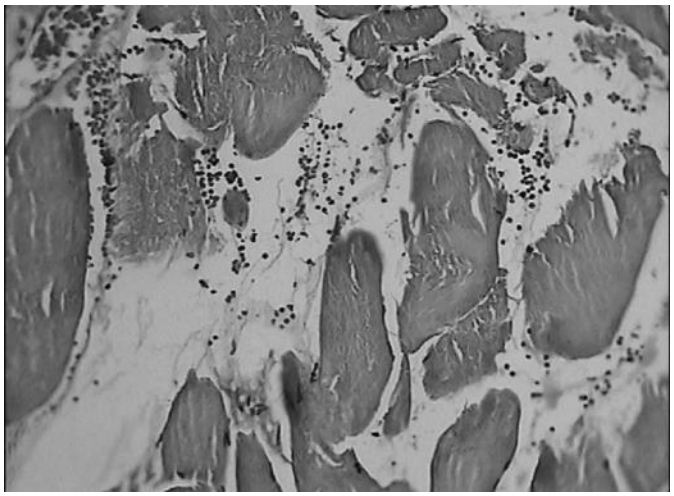

Fig. 2: Vascular congestion, edema, mild lymphocytic infiltration and hemorrhage between muscle fibers in rats of Group I/R (Olympus Co., Tokyo, Japan * 400).

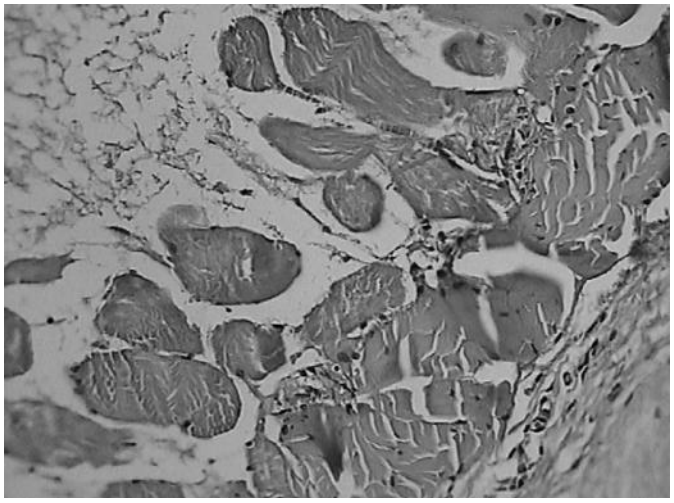

Fig. 3: Muscle fiber necrosis with macrophages infiltrating the necrotic muscle in one rat of Group I/R. Edema and mild lymphocyte infiltration between muscle fibers is also seen (Olympus Co., Tokyo, Japan * 400).

panied with macrophages infiltrating the necrotic muscle,.edema and mild lymphocyte infiltration between.muscle.fibers is also seen (Fig. 3). There was no histological change in the group C and group M, but the difference in histological changes between group I/R and group C and M was significant (P< 0.05).

## Discussion

Following many vascular and crash injuries of lower extremity, ischemic-reperfusion injury in skeletal muscle is inevitable in numerous vascular and crash injuries. It can result in significant complications including muscle necrosis. These injuries dramatically increased during a variety of time-consuming vascular surgeries [9].

Particularly in underdeveloped countries, the unimproved transportation system of patients with lower extremity vascular trauma causing prolonged ischemia and resulting in compartment syndrome and reperfusion injury follows vascular repair surgery. To get rid of complications of releasing toxic oxygen-derived free radicals during reperfusion, antioxidants extensively investigated. Several studies have shown that melatonin dramatically decreased I/R injury in the some organs such as heart, brain, peripheral nerve, kidney, liver, intestine, groin flap and skeletal muscle [9-19]. Hence, the antioxidative impact of melatonin such as scavenging of oxygen based reactants and nitrogen-derived species, is more effective than other intracellular antioxidants [10]. Some features of melatonin such as amphiphilic nature and small size, promote its ability to reach all cellular Unlike superoxide dismutase, melatonin is able to easily reach all cellular cavities in in contrast to superoxide dismutase.[16.] Furthermore, serious side effects had not reported in people who use it for prolonged periods of time [17]. In this research, we show that melatonin prevent ischemic-reperfusion syndrome biochemically and histologically. Histological damage scores in the skeletal muscle specimens of Group I/R were significantly higher than Group M and C. Similar results in the field of histology were reported by a Erkanli K and his colleges [18.] In the field of biochemistry of reperfusion syndrome, a dramatic decrease in HCO3 and lactate and significant increase in K, CPK shows the effect of melatonin in attenuating the morbid reperfusion syndrome following vascular surgery of traumatic injuries of lower extremities.
Hence, melatonin reduces reperfusion syndrome and skeletal damage following surgical initiation of blood flow in rats. In agreement with beneficial effect of melatonin and nontoxic effects, we suggest that administration of melatonin before reperfusion period would prevent ischemic-reperfusion syndrome and prevent probable morbidity and mortality following revascularization surgery.


## Riassunto

Lo scopo di questo studio è quello di valutare l'effetto protettivo della melatonina sulla sindrome da riperfusione dopo ischemia.
Allo scopo sono stati randomizzati 33 ratti Wistar albini adulti e di sesso maschile in tre differenti gruppi sperimentali ciascuno di 11 individui: Gruppo C è il gruppo di controllo, senza ischemia né riperfusione.
Gli individui dei Gruppi I/R e I/R + M sono stati sottoposti per 2,5 ore ad ischemia e successivamente a due ore di riperfusione per mezzo del clampaggio dell'arteria femorale comune. Tutti gli animali hanno ricevuto soluzione fisiologica di mantenimento per via intraperitoneale (i.p.) durante l'anestesia generale.
Agli animali del gruppo I/R + M sono stati somministrati per via intraperitoneale 10 mg/kg di Melatonina

cinque minuti prima della riperfusione. Al termine della riperfasione sono stati raccolti campioni per analisi biochimiche ed istologiche. I parametri studiati sono stati biopsie del muscolo soleo, il livello dei lattati, della creatinfosfochinasi (CPK), della lattico deidrogenasi (LDH), del sodio, del potassio, del calcio ed una emogasanalisi del sangue arterioso.
Negli animali del gruppo I/R si è avuto un drammatico incremento del K e del CPK, in contrasto con il Gruppo C e con il Gruppo I/R + M ($P<0.05$). Una significativa diminuzione dei bicarbonati è stata rilevata nel Gruppo I/R in paragone col Gruppo I/R+M e Gruppo C ($P<0,001$). Nel gruppo FR + M il livello dei lattati è diminuito drammaticamente in paragone con gli altri gruppi ($P<0,001$).
Il danno istologico è risultato inferiore nel Gruppo I/R + M che non nel Gruppo I/R ($P<0,05$). Non si è rilevata nessuna differenza significativa nella $PO_2$, pH, $CO_2$, $pO_2$, Na, LDH, Ca e P nei tre gruppi ($P>0,05$). Le alterazioni istologiche nei Gruppi C ed M non sono stati differenti in modo significativo, ma le differenze nel Gruppo I/R sono state significative rispetto al Gruppi C e I/R+M ($P<0,05$).
In conclusione è suggestivo che la melatonina esercita un effetto protettivo nei confronti della sindrome da ischemia/riperfusione sia a livello ematico che nel muscoli scheletrici e può ridurre la morbilità conseguente alla chirurgia di riperfusione nei traumi vascolari.

## References


1. Frykberg ER:. *Advances in the diagnosis and treatment of extremity vascular trauma*. Surg Clin North Am, 1995; 75:207-23.

2. Odeh M: *Mechanisms of disease: The role of reperfusion-induced injury in the pathogenesis of the crush syndrome.* N Engl J Med, 1991; 324:1417.

3. Gianello P, Saliez A, Bufkens X, Pettinger R, Misseleyn D, Hori S, Malfroy B: *EUK-134, a synthetic superoxide dismutase and catalase mimetic, protects rat kidneys from ischemia-reperfusion-induced damage*.Transplantation, 1996; 62(11):1664-66.

4. Shah DM, Bock DE, Darling RC 3rd, Chang BB, Kupinski AM, Leather RP: *Beneficial effects of hypertonic mannitol in acute ischemia. Reperfusion injuries in humans*. Cardiovasc Surg, 1996; 4(1):97-100.

5. Ferrari RP, Battiston B, Brunelli G, Casella A, Caimi L:. *The role of allopurinol in preventing oxygen free radical injury to skeletal muscle and endothelial cells after ischemia-reperfusion.* J Reconstr Microsurg, 1996; 12(7):447-50.

6. Reiter RJ, Tan DX, Castroviejo AD et al.: *Melatonin: mechanism and actions a antioxidant.* Curr Top Biophys, 2000; 24:171-83.

7. Pozo D, Reiter RJ, Calvo JR et al.: *Physiological concentrations of melatonin inhibits nitric oxide synthase in rat cerebellum.* Life Sci, 1994; 55:455-60.

8. Gurlek A, Celik M, Parlakpinar H, Aydogan H, Bay-Karabulut A: *The protective effect of melatonin on ischemia. Reperfusion injury in the groin (inferior epigastric) flap model in rats.* J Pineal Res, 2006; 40:312-17.

9. Blaisdell FW: *The pathophysiology of skeletal muscle ischemia and the reperfusion syndrome: A review*. Cardiovasc Surg, 2002; 10:620-30.

10. Giacomo CG, Antonio M: *Melatonin in cardiac ischemia/reperfusion-induced mitochondrial adaptive changes*. Cardiovasc Hematol Disord Drug Targets, 2007; 7(3):163-69.

11. Tan DX, Manchester LC, Reiter RJ, Qi WB, Karbownik M, Calvo JR: *Significance of melatonin in antioxidative defense system: Reactions and products*.Biol Signals Recept, 2000; 9(3-4):137-59.

12. Lee EJ, Lee MY, Chen HY et al.: *Melatonin attenuates gray and white matter damage in a mouse model of transient focal cerebral ischemia*. J Pineal Res, 2005; 38:42-52.

13. Sayan H, Ozacmak VH, Ozen OA et al.: *Beneficial effects of melatonin on reperfusion injury in rat sciatic nerve*. J Pineal Res, 2004; 37:143-48.

14. Wang WZ, Fang XH, Stephenson LL, Baynosa RC, Khiabani KT, Zamboni WA: *Microcirculatory effects of melatonin in rat skeletal muscle after prolonged ischemia.* J Pineal Res, 2005; 39(1):57-65.

15. Kazez A, Demirba€ M, Ustünda, Ozercan IH, Sa€lam M: *The role of melatonin in prevention of intestinal ischemia-reperfusion injury in rats*.J Pediatr Surg, 2000; 35(10):1444-48.

16. Colak C, Parlakpinar H, Ozer MK, Sahna E, Cigremis Y, Acet A: *Investigating the protective effect of melatonin on liver injury related to myocardial ischemia-reperfusion.* Med Sci Monit, 2007; 13(11):BR251-54.

17. Gurlek A, Celik M, Parlakpinar H, Aydogan H, Bay-Karabulut A: *The protective effect of melatonin on ischemia-reperfusion injury in the groin (inferior epigastric) flap model in rats*. J Pineal Res, 2006; 40(4):312-17.

18. Cheung RTF: *The utility of melatonin in reducing cerebral damage resulting from ischemia and reperfusion.* J Pineal Res, 2003; 34:153-60.

19. BandyOpadhyay D, Chattopadhyay A, Ghosh G et al.: *Oxidative stress-induced ischemic heart disease: Protection by antioxidants.* Curr Med Chem, 2004; 11:369-87.