EXPERIMENTAL STUDY

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The effects of melatonin and caffeic acid phenethyl ester (CAPE) on fracture healing under ischemic conditions

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Objective: The aim of this study was to investigate the effects of antioxidant molecules Melatonin and Caffeic Acid Phenethyl Ester (CAPE) on fracture healing under ischemic conditions.

Methods: A right tibia fracture was created and fixed with an intramedullary pin in forty four male Wistar-albino rats. The rats were then randomly allocated to fracture, fracture-ischemia, fracture-ischemia-melatonin, and fracture-ischemia-CAPE groups. Ischemia was created by clamping femoral arteries four and a half hours. Animals were killed and radiographic, histological and biomechanical evaluation was performed sixth week after surgery.

Results: The radiological and histological scores of the fracture-ischemia-CAPE group were significantly better than the fracture- ischemia group at 6th week follow-up. Complete radiographical and histological healing of all fractures was detected in all groups. There was a significant difference between the maximum fracture force between the groups (fracture-ischemia<fracture-ischemia-melatonin<fracture<fracture-ischemia-CAPE) ($p<0.005$). Although difference was not statistically significant between fracture and fracture-ischemia-CAPE groups, all other groups revealed statistically significant difference with respect to toughness (N/mm). Fracture-ischemia group revealed the lowest toughness.

Conclusion: Ischemia adversely affects the fracture healing of rat tibias. Melatonin and CAPE eradicate adverse effects of ischemia. Possible adverse effects of ischemia on fracture healing can be eradicated with melatonin and CAPE in patients with tibia fractures associated with vascular injury or compartment syndrome.

Key words: Ischemia; fracture; melatonin; caffeic acid phenethyl ester; free oxygen radicals.

Fractures with vascular injury, compartment syndrome and extended tourniquet application expose the extremities to ischemia. Reperfusion exposes the ischemic tissue to oxygenized blood with excessive production of free oxygen radicals (FOR) such as hydroxyl (HO), superoxide anion (O_2-), singlet oxygen (O_2), hydrogen per-

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oxide (H_2O_2) and nitric oxide (NO). FORs lead to cell damage in the ischemic extremity.[1,2] During fracture healing, FOR reach the maximum level on the 15th day negatively affecting both the formation and inflammation phases.[3-5]

To prevent cellular damage of FOR, antioxidant defense system activates with the production of antioxidant enzymes and melatonin.[6-8] Melatonin, which is the main product of the pineal gland, affects the circadian and seasonal rhythms, retinal physiology, the immune system and reproductive functions.[7,9] In addition, melatonin has been shown to have a counteractive effect on FOR in various ischemia/reperfusion models.[7,9-11] CAPE is another important antioxidant molecule, obtained from propolis produced by honeybees and is also counteractive to FOR.[8,12]

The aim of this study was to investigate the effects of antioxidant molecules Melatonin and Caffeic Acid Phenethyl Ester (CAPE) on fracture healing under ischemic conditions.

Materials and methods

A total of 44 adult male Wistar-albino rats with a mean weight of 377 g (range; 334-422) were used in this study. Approval for the study was granted by Gaziosmanpasa University Medical Faculty Experimental Animals Ethics Committee. The study was conducted in the university experimental animal research laboratory. The 44 rats were then randomly assigned to 4 groups of 11 in each. The groups were named as fracture only, ischemia-fracture, ischemia-fracture-melatonin and ischemia-fracture-CAPE.

Cefazolin sodium 20 mg/kg was administered intramuscularly 30 minutes preoperatively as antibiotic prophylaxis and an additional dose was administered 8 hours postoperatively. General anesthesia of ketamine (90 mg/kg) and xylazine (10 mg/kg) was administered intramuscularly. After surgical dissection of the right lower extremity of the rats under aseptic conditions, the femoral nerve, vein and artery were dissected free of the distal inguinal ligament.

A microvascular clamp was readily available to prevent femoral arterial blood flow. Then a 1 cm medial parapatellar incision was made and the patella was dislocated laterally. The medullary canal of tibia was entered with a 21-gauge needle just above the tuberositas tibia and reamed. A 0.8 mm stainless steel wire (TST, Kurtkoy, Istanbul, Turkey) was placed in the medullary canal. The proximal part of the wire was bent and cut, the patella was reduced and the incision was closed. A closed, transverse mid-diaphyseal tibial fracture was created as described by An et al.[13] (Fig. 1).

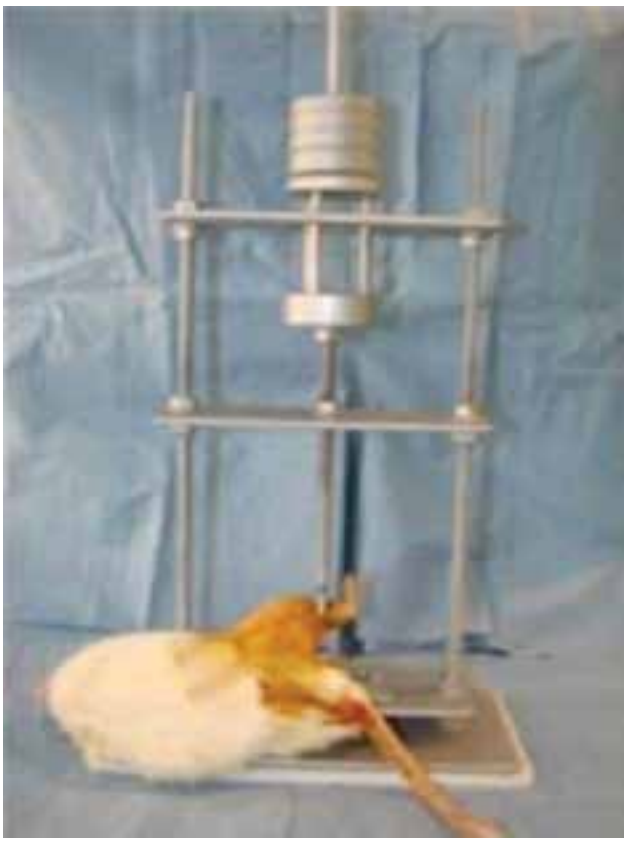

Fig. 1. Image showing the technique used to create closed, mid-diaphyseal fracture in rat tibia. [Color figure can be viewed in the online issue, which is available at www.aott.org.tr]

Immediately after the fracture, to create ischemic condition, femoral artery of the rats in the ischemia groups was ligated as described by Skjeldal et al.[14] After 4.5 hours, the clips were opened ending the ischemia, the femoral artery flow was observed and the groin area incision was closed. During and after the process of ischemia, the animals were kept at a room temperature of 24° C in a supine position inside a warming blanket. To prevent dehydration in the animals undergoing ischemia, 4 ml 0.9% NaCl isotonic solution was administered intraperitoneally during ischemia. Intramuscular narcotics were used to control postoperative pain. A single dose of melatonin (25 mg/kg, 10% ethanol per 1 ml) (Sigma, St Louis, MO, USA) was administered daily for 14 days to the ischemia-melatonin group. The ischemia–CAPE group received a single daily dose of CAPE (10 μmol/kg, 10% ethanol per 1 ml) (Sigma, St Louis, MO, USA) for 14 days. The doses were based on the study of Histing et al.[15] The experiment was terminated at the end of the 6th week by sacrificing the animals with a fatal dose of

Table 1. Radiological evaluation score of the new bone formation in the fracture zone.

	Radiological evaluation system	Points
Bone formation	Bone formation, none	0
	Bone formation, filling 25% of the defect	1
	Bone formation, filling 50% of the defect	2
	Bone formation, filling 75% of the defect	3
	Bone formation, filling 100% of the defect	4
Healing	Non-union	0
	Union starting	1
	Complete radiological union	2
Remodeling	No remodeling	0
	Formation of intramedullar canal	1
	Formation of cortex	2
	Maximum total points	10
	Bone formation	4
	Proximal union	2
	Distal union	2
	Remodeling	2

intravenous sodium phenobarbital. The tibia bones were dissected and kept at -20° C until the biomechanical examination. Radiographs were taken in all the groups at 0 and 6 weeks after the fracture. On these radiographs, the bone formation in the fracture zone was classified according to the Lane and Sandhu radiological classification system (Table 1).[16]

The biomechanical examination was made using the 3-point Bending Test machine (Hounsfield H50KM Surrey, England). After removing the intramedullary wire, the tibia samples were placed on the machine and force was applied to the fracture healing area at a fixed speed of 10 mm/min, to create a refracture. The force creating refracture was recorded as Newton units. Samples for histopathological examination were taken from the tibia of the sacrificed animals. After fixation in neutral buffered formalin for 48 hours, they were kept for 12 hours in 10% formic acid in 0.1-M citrate for decalcification. After the decalcification process, lengthwise sections were taken from the tibias. For histopathological examination of callus tissue, paraffin sections of 5 micrometer thickness were prepared and stained with hematoxylin-eosin (HE). A light microscope was used in the evaluation. Histological evaluation was made according to the Huddlestone et al. histological evaluation system.[17]

PASW v18 statistical software (SPSS Inc, Chicago, IL, USA) was used in the statistical analysis. One-way variance analysis (ANOVA) was used for the comparison between the 4 groups which showed normal distri-

Fig. 2. Radiological images of the four groups at the end of six weeks: **(a)** fracture-ischemia, **(b)** fracture only, **(c)** ischemia-melatonin, **(d)** ischemia-CAPE.

Table 2. Evaluation of the radiological scores and 6th week histological scores of the groups.

Groups	Radiological score Mean±SD	6th week histological score Mean±SD
Fracture	9.10±0.74	8.90±0.74
Fracture-ischemia	8.60±0.52	7.50±0.97
Fracture-ischemia +Melatonin	9.00±0.67	8.60±0.69
Fracture-ischemia+CAPE	9.40±0.52	9.30±0.48
P	0.048*	0.001**
One–way ANOVA test	*p<0.05	**p<0.01

Table 3. Biomechanical test results of the groups.

Group	Fracture no	Breaking force (N)	Stiffness (N/mm)
Fracture	10	52.6±16.6	91.7±33.9
Ischemia-fracture	10	19.1±9.3	35.5±13.2
Melatonin-ischemia-fracture	10	37.2±14.5	67.4±20.0
CAPE-ischemia-fracture	10	71.1±20.0	106.6±16.4
P		<0.001*	<0.001**

bution of the constant variables used in the study according to the Kolmogorov-Smirnov test. The Scheffe and Tamhane tests were used according to the variance homogenity in the paired comparisons between the groups. Constant variables were presented as mean and standard deviation (SD). A p value less than 0.05 was considered statistically significant.

Results

In each group 1 rat died as a result of infection or diarrhea, leaving a total of 40 rats for the evaluation. The radiological assessment was made by 2 blinded observer at the end of 6 weeks according to the Lane and Sandhu classification system (Fig. 2a-d).[16]

There was radiological evidence of healing in all rats. There was a significant difference between the mean radiological scores of the groups ($p<0.05$). Scheffe test showed that the mean score of the ischemia-CAPE group was significantly higher than that of the ischemia-fracture group ($p<0.05$). No statistically significant differences were found between the other groups ($p>0.05$) (Table 1).

There was also a statistically significant difference between the histological scores of the groups ($p<0.05$). Scheffe test showed mean scores of the fracture group, the ischemia-melatonin group and the ischemia-CAPE group to be significantly higher than that of the fracture-ischemia group ($p=0.002$, $p=0.021$, $p=0.001$) (Table 2). The histological evaluation was made according to the Huddlestone et al evaluation system.[17] In the histological evaluation, the appearance of the fracture only group, the ischemia-melatonin group and the ischemia-CAPE group was found to be rich in osteoblasts in the fracture line, and rich in mature compact bone islands with ample neovascularization.

In the fracture-ischemia group, the fracture zone was rich of healing tissue with hyaline cartilage and areas of mature compact bone. Osteoblastic activity was more intense particularly in the fracture only group and the ischemia-CAPE group. In the fracture ischemia group, osteoblastic activity was weaker and compact bone healing was observed (Fig. 3a-d).

In the comparison of the biomechanical strength, the lowest strength was observed to be in the fracture-ischemia group and the highest in the ischemia-CAPE group. From lowest to highest biomechanical strength, the groups were ranked as fracture-ischemia<ischemia-melatonin<fracture only<ischemia-CAPE and the differences between them were found to be statistically significant ($p<0.05$). In the comparison of the stiffness at the fracture zone, the ischemia-melatonin and ischemia-CAPE groups had significantly higher values than that of the fracture-ischemia group ($p<0.001$). The lowest value of stiffness was found in the fracture-ischemia group (Table 3).

Discussion

In fractures accompanied by vascular injuries and compartment syndrome, the extremity may be exposed to ischemia and ensuing reperfusion injury.[18] The majority of tissue damage following ischemia is caused during reperfusion.[19]

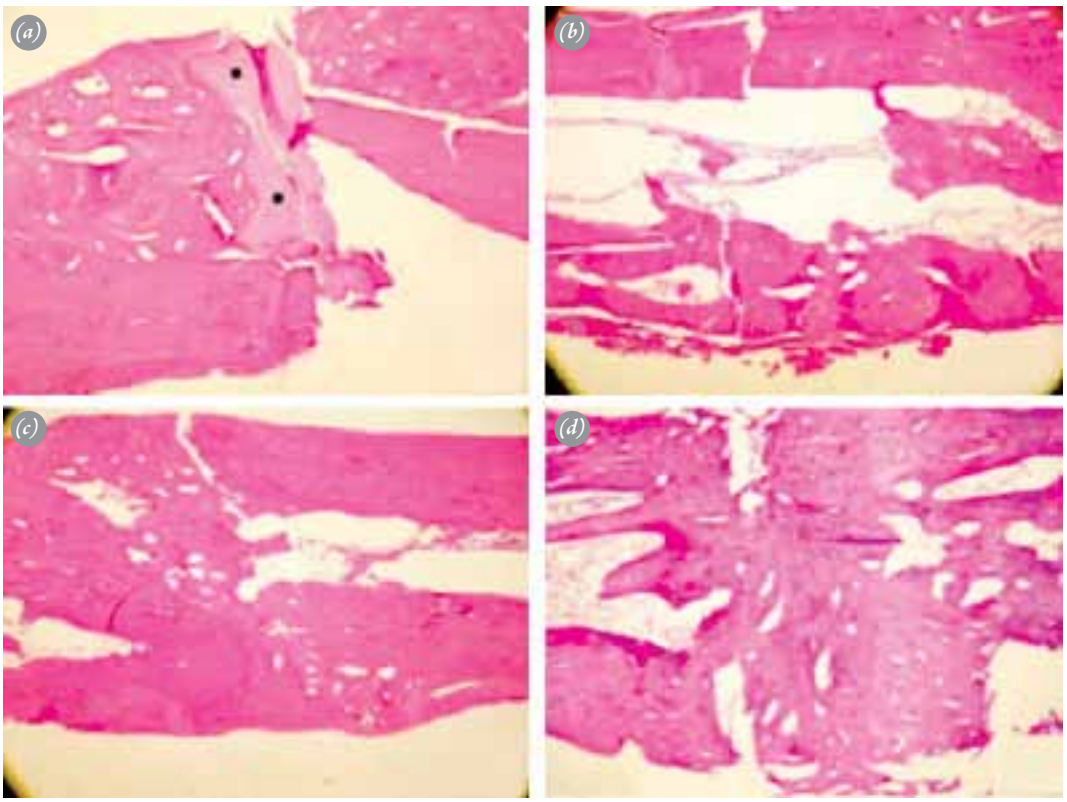

Fig. 3. Histopathological examination at the end of six weeks **(a)** fracture-ischemia group. Note the healing with intense hyaline cartilage in the fracture line; areas of mature compact bone tissue adjacent to hyaline cartilage (HE, x80), very few osteoblasts is observed **(b)** Fracture only group. Mature bone tissue in a large number of osteoblasts (HEx80) **(c)** Ischemia-melatonin group. Mature bone healing of few osteoblasts (HE, ×80) **(d)** Ischemia-CAPE group. Osteoblasts rich in mature lamella and compact bone healing tissue (HE, ×80). [Color figure can be viewed in the online issue, which is available at www.aott.org.tr]

The accumulation of neutrophils in ischemic tissues and increased xanthine oxidase activity in endothelial cells caused by reperfusion leads to rapid production of superoxide anion (O_2-), hydrogen peroxide (H_2O_2) and hydroxyl radical (OH), which are known as FORs. These free radicals have a toxic effect on skeletal muscle, endothelial and other cells as has been shown in ischemia / reperfusion models.[11,18-20]

Several researchers conducted *in vitro* and *in vivo* studies using different chemical agents to accelerate bone healing. In an experimental study on rats by Histing et al., the frequency of sildenafil was shown to accelerate bone healing by inhibiting guanosin monophosphate phosphodiesterase 5.[21] Spiro et al. demonstrated that Raloxifene, as a selective oestrogen modulator, made a contribution to bone healing in the early phase in an experimental rat study.[22] Bukato reported that teriparatide accelerated bone callus formation and bone healing.[23] In studies which have researched the effects of electromagnetic fields on bone resistance and bone healing, the negative effect of radiation has been shown.[24]

It has been shown that FORs are increased in the inflammation phase of bone healing.[3,4] The increase in FORs has a negative effect on bone healing.[5] Osteoclasts produce superoxide anion during bone resorption, and superoxide anion has been shown to make a significant contribution to bone destruction.[25] The number of FORs has been shown to be extremely high in the inflammation phase of healing in tibia fractures accompanied by temporary ischemia and reperfusion.[26]

In the current study, the biomechanical strength in the fracture-ischemia group was found to be significantly lower than that of the control group (fracture only). Therefore, long-term ischemia may negatively affect bone healing. This is thought to be due to the effect of the high level of FOR as reported by Çetinus et al.

Melatonin is released by the pineal gland and regulates body heat, circadian and seasonal rhythms and cleans FORs.[27,28] It has also been shown to have a stimulating effect on osteoblast differentiation and matrix mineralization in cell cultures.[27,28] Elimination of FORs has been shown to promote bone healing through

the antioxidant effect in the fracture zone.[29] Melatonin has also been shown to reduce tissue injury by reperfusion following ischemia.[6,7,9]

In the current study, the biomechanical strength of the fracture-melatonin group, although lower than the control group, was significantly higher than that of the fracture-ischemia group. We believe that melatonin had a positive effect on the healing of tibial fractures under ischemic conditions. This is probably due to the inhibition of FORs.

The histopathological, radiological and biomechanical results of the melatonin-ischemia group were lower than those of the control group but not statistically significant. Therefore, the use of melatonin in orthopaedic practice as an agent to eliminate the negative effect created by ischemia may be considered useful. Various experimental studies have shown the positive effects of melatonin on ischemia.[6,10,11]

CAPE is one of molecules found in propolis, which is produced by honeybees. CAPE is a powerful immunomodulator, anticarcinogenic, anti-inflammatory and anti-oxidant.[30] It has been introduced in medical treatments in recent years. The effect of antioxidant effect of CAPE has been demonstrated in ischemia-reperfusion models.[12,31,32] In an experimental study by Çiçek et al., CAPE was shown to increase the biomechanical strength in rat femurs.[24]

In an experimental study by Ang et al. CAPE was shown to inhibit osteoclast differentiation and activation by inhibiting NF-Kb which is associated with Receptor activator of nuclear factor kappa-b ligand (RANKL).[33] In the current study, the biomechanical strength, radiological and histopathological scores of the ischemia-CAPE group were higher than those of the other three groups. While the biomechanical strength of the melatonin group was not higher than the control group, the CAPE group did exceed the control group although this was not statistically significant. Thus it was observed that CAPE was superior to melatonin in making a positive contribution to bone healing in the tibial fracture model that was complicated with ischemia.

This effect of CAPE is thought to be due not only to its antioxidant effect, but also to the prevention of osteoclast activation through the inhibition of NF-Kb which is associated with RANKL. In the current study, better results were obtained in the ischemia-CAPE group than in the ischemia-melatonin group. Histing et al. reported that melatonin promotes bone healing by suppressing osteoclasts associated with RANKL.[15] Our results support this finding.

Despite these positive findings, the increase of cartilage tissue in the callus, and delayed bone remodeling may be the negative effects of melatonin. In an experimental study by Histing et al., a significant increase in callus tissue was observed in the rats that were treated with melatonin compared to the control group. The authors report an eventually delayed bone remodeling.[15]

A limitation of our study is the lack of measurement for FOR levels in the pre and postoperative periods.

The results of this experimental study showed a negative effect of ischemia on bone healing in a tibial fracture complicated by ischemia and subsequent reperfusion. Melatonin and CAPE may eliminate this negative effect. In orthopaedic practice, the use of melatonin and CAPE may remove the potential negative effects of ischemia on bone healing in cases of tibial fracture complicated by vascular injuries or compartment syndrome.

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Conflicts of Interest: No conflicts declared.

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