The effect of melatonin on rats gastrocnemius muscle applied with carbon tetrachloride (CCl4)

Duygu Fevziye Vural, Suna Cebesoy, Dilşad Ozerkan and Hatice Mutlu Eyison

Abstract
In this study, it is purposed that the protective effect of the melatonin (MEL) is examined against the toxic effect of the CCl4 to the gastrocnemius muscle. The rats were separated into 4 groups that were control groups (Group 1, Group 2), CCl4 (Group 3) and CCl4 + MEL (Group 4). The tissues were processed the routine preparation processing for the light microscope. The tissues were stained respectively with hematoxylin-eosin, Masson-trichrome and Periodic Acid- Schiff. When the tissues were stained with histological stains whole of the control groups was regular. When the tissues were stained with hematoxylin-eosin; and in the group (group 3), in the muscle fibers there were fusion in places, hypertrophy and orientation disorder. In the group (Group 4) was observed that the muscles were close to the control group. When the tissues were stained with the Masson-trichrome, in the group 3, it was observed that the collagen fibers were increased in the connective tissue. In the CCl4 group which was applied the melatnine, it was observed that in the collagen fibers there were decreased. In addition to these, in PAS staining all groups were seen PAS positive.

Keywords: Gastrocnemius, fibrosis, histological stains, melatonin, carbon tetrachloride

1. Introduction
CCl4 (carbon tetrachloride) is a colorless, non-inflammable, volatile, aromatic and intensive liquid. Although the CCl4 is naturally available, it is not produced as chemical. Due to the stable form of the CCl4, it disintegrates too slowly also its atmospheric half-life is approximately 30-100 years [1].

CCl4 and other liquid halogenated hydrocarbons are used in paint and solvents, extinguishers, also used as oil repellents (detergent) as well as dry cleaning for long times [2, 3]. In addition to this, it is applied for the parasitary struggle in the veterinary medicine against the helmintes [4, 5]. The carbon tetrachloride (CCl4) which can be taken via respiratory tract, gastrointestinal tract and trancutanely in the body, separates to the tissues like notably river, brain, kidney, muscle, lungs and testis [6, 7]. It is indicated that this effect of it is ridden on free oxygen radicals [8]. Xenobiotic which can form the cellular damage with CCl4 free radical production, can cause to hepatotoxicity for human and animals [9-11]. CCl4 causes to increase the lipid peroxidation production and to decrease the protective enzymes against these products. These effects are resulted in that CCl4 is changed into the trichloromethyl and the trichloromethyl peroxide (CCl3/CCl4O2) or N-acetyl-p-benzoquinone (NAPQI) free radicals which they are most toxic with cytochrome P450 (CYP) enzyme [12]. The effects of the antioxidants are quite a few for being minimized the damage which the free radicals form in the body [13].

The melatonin which is the hormone of the pineal gland [14], its basic cell is pinealosin [15], is a strong antioxidant and sweeps up the superoxide radicals, other radical oxygen sorts (ROS) and radical nitrogen sorts. In the APUD (precursor uptake and decarboxylation) cells which are accepted that they are in the diffuse neuroendocrine, it was seen that the melatnine is synthesized. These cells take place in the retina, lachrymal glands, the other parts of brain; and bronchus, river, kidney, adrenal glands, gastrointestinal system, thymus, placenta, over, testis and endometrium. In addition to this, the mast cell synthesizes the melatonin in the leucocytes and natural killer cells [16]. Likewise, the melatnine supports the expression of the antioxidantive enzyme genes in a roundabout way; and inhibits the expression of the prooxidant gene [17]. The melatnine, also, has an impact on the activation of the antioxidantive enzymes in a roundabout way, like superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase [18, 19].

E-ISSN: 2320-7078
P-ISSN: 2349-6900
JEZS 2017; 5(3): 441-447
© 2017 JEZS
Received: 05-03-2017
Accepted: 06-04-2017

Duygu Fevziye Vural
Ankara University, Faculty of Science, Department of Biology, Tandogan, Ankara, Turkey
Suna Cebesoy
Ankara University, Faculty of Science, Department of Biology, Tandogan, Ankara, Turkey
Dilşad Ozerkan
Kastamonu University, Faculty of Engineering and Architecture, Department of Genetics and Bioengineering, 37100 Kastamonu, Turkey
Hatice Mutlu Eyison
Ankara University, Faculty of Science, Department of Biology, Tandogan, Ankara, Turkey

Correspondence
Duygu Fevziye Vural
Ankara University, Faculty of Science, Department of Biology, Tandogan, Ankara, Turkey
This activation is taken shape in a result of synthesizing the antioxidative enzymes mRNA and finally by being stimulating the enzyme [20-22].

The melatonin has an amphophilic feature higher up; also, it quickly mixes with blood and body fluids without being stored in the body [23, 24]. Because of its feature, it can reach to all organelles of the cell including the nucleus; and for this reason, it more effective than the vitamin and mineral antioxidants [17]; as a result, this situation overtops to the melatonin for protecting the DNA and the cells against the oxidative damage [25-27].

Skeletal muscles include the soleus, gastrocnemius and extensor digitorum longus. The skeletal muscles can be classified as fast Convulsion fibers and slow Convulsion fibers, based on energy metabolisms and the characters of the fibers' spasm [28]. The gastrocnemius muscle is a thick muscle [29] which takes place in the backside of the leg that has both the fast and slow Convulsive fibers [30]. The skeletal muscle fibrils of the vertebrate are three types when basing on the morphological, histochemical and functional situations; these are red, white and recess fibrils [31, 32]. The red fibrils are described as type I and slow oxidative; the white fibrils are called type IIB and fast glycolytic; the recess fibrils are called type IIA and fast oxidative [28]. The type I (red muscle) carries myoglobin which colors pink to the muscle. When the type I muscle is cramping, its speed is 17 mm per a second; and the cramping of the type II is 42 mm per a second [33]. In the type I, the blood vessels are more than the type II and they activate longer time than the type II. They are thinner than the type II and they carry mitochondria a lot. The type II fibrils cramp fast and they have bigger diameter than the others but they get tired quickly. The glycolytic enzymes of the type II are more and they carry plenty of glycogen. Because of the fact that in the type II fibrils, the oxidative metabolism is back seat, there is less mitochondria. In addition this, the type I fibrils have a great number of mitochondria in order to support the higher up oxidative metabolism [34]. Soleus has the fibers which cramp slow; the gastrocnemius has cramped fibers which are both slow and fast [35].

Fibroblasts play a significant role for healing by secreting the extracellular matrix proteins which include the collagen and growth factor for repairing when the tissues are damaged [35-37]. The skeletal muscle fibrosis is that the collagen and the other extracellular matrix proteins are produced by the muscle fibroblasts and are stored in the tissues [38]. In these researches which were studied with the carbon tetrachloride and the melatonin, it was used more river, kidney and lung. It was not histologically reached the researches about the CCl4 and the melatonin. In this study, it was histologically examined not only the negative effect of the CCl4 to the gastrocnemius muscle but the healing and the protective effect of the melatonin.

2. Material and Method
2.1 Laboratory Animals
In our study, 24 Wistar albino male rats taken from Gazi University Laboratory Animals Research Center and weigh in between 200-220 gr were used. The experiments were carried out at GUDAM and experiments were done from January 2013 to January 2015. Therats were fed with the Standard pellet feed in their Standard rat cages under optimal conditions. The lights were adjusted in a way to allow that there was 12 hours day and 12 hours night. Therats were weighed before starting the ten-week long study and 24 rats of 200-220 gr were randomly divided into 4 groups involving 6 in each group.

1. Group 1 (Control 1, Corn oil) The corn oil which is CCl4 solvent was applied to the rats, in this group, for 1,5ml/kg during 4 weeks subcutaneously two times per a week for 10 weeks.
2. Group 2 (Control group, ethanol+PBS) The ethanol which is the melatonine solvent was given subcutaneously every day to this group for 1% ethanol+PBS during 10 weeks.
3. Group 3 (CCl4) 1,5ml/kg melatonine was subcutaneously given two times per a week from the sterile CCl4 injection, which was dissolved in the corn oil at the rate of 1:1.
4. Group 4 (CCl4+Melatonine) since starting the CCl4 injection, 10 mg/kg dose of the melatone was subcutaneously given every day.

At the end of tenth week, from the all experiment groups the muscle tissues were taken out by taking intracardial blood under ketamine/rompun anesthesia.

2.2 Histochemical examination
Some part of the striated muscle tissue was determined by being put into 10% of formol. After the determined tissues were processed the routine histological operations, they were embedded to the paraffin and the thicknesses of 5μm sections were taken out. For the histological examination the tissues were stained with the hematoxylin-eosin (HE). The fibrosis (Type I collagen) formation was shown by the Masson-Trikrom staining and the glycocen particles were shown by Periodic Acid Shift (PAS) staining.

3. Results
3.1 Hematoxylin & Eosin
When the gastrocnemius muscle tissue sections of the control group (Group 1, 2) rats were examined, it was seen that the morphological structure of the muscle cells were regular (Fig. 1a, Fig. 1b).

![Fig 1a: The histological structure of the gastrocnemius muscle of the control group (corn oil) rat. H&E, b. The histological structure of the gastrocnemius muscle of the control group (PBS+ ethanol) rat. H&E](image-url)
When the gastrocnemius muscle of the CCl₄ group’s rats was examined, it was seen that in the muscle fibrils there was melting in places and protein loss, in the muscle tissue there was malformation and in the muscle cells there was hypertrophy (Fig. 2a, Fig. 2b, Fig. 2c).

![Fig 2a: Hypertrophy in the gastrocnemius muscle of the CCl₄ group rats and the melting in the fibrils. H&E. b. Melting (star) in the gastrocnemius muscle cells of the CCl₄ group rats and disruption in the morphological character. H&E. c. Hypertrophy in the gastrocnemius muscle cells of the CCl₄ group rats. H&E](image)

When the CCl₄ groups which were applied the melatonin were examined, it was determined that the tissue integrity was partly maintained according to the CCl₄ group, and the morphological structure of the cells was near normal (Fig. 3).

![Fig 3: The gastrocnemius muscle cells of the applied melatonin to the CCl₄ group rats follow an outlook near normal.](image)

### 3.2 Masson-Trikrom

The muscle cell nucleus was black, which was stained with the Masson-Trikrom, the collagen fibers were stained with blue. In the control group, the collagen fibers were at the normal level (Fig. 4a, Fig. 4b).

![Fig 4 a: Connective tissue in the gastrocnemius muscle of the control group (corn oil) rat. Masson-Trikrom, b. Connective tissue in the gastrocnemius muscle of the control group (PBS+ ethanol) rat. Masson-Trikrom](image)
It was seen that in the group which was applied the CCl₄, the fibrosis was around the nerve plexus, in the muscle cells there were orientation disorder and the hypertrophy (Fig. 5a, Fig. 5b).

![Fig 5a: The fibrosis around the nerve plexus in the gastrocnemius muscle of the CCl₄ group rat, the orientation disorder and the contractile protein loss (star). Masso-Trikrom b. The fibrosis around the nerve plexus in the gastrocnemius muscle of the CCl₄ group rat, the orientation disorder and the melting in the fibrils. Masso-Trikrom](image)

When the CCl₄ groups which were applied the melatonin, were examined, it was determined that the tissue integrity was partly maintained according to the CCl₄ group, and decreasing in the collagen fiber and decreasing around the blood vessel in the fibrosis (Fig. 6a, Fig. 6b).

![Fig 6a: Gastrocnemius muscle belonging to CCl₄+melatonin. Masson – Trikrom b. Decreasing of the collagen fiber in the CCl₄ group rat’s gastrocnemius muscle in the applied melatonin and the view of tissue near normal. Masson–Trikrom](image)

### 3.3 Periodic Acid-Schiff (PAS)

It was observed that all groups reacted PAS positive when the gastrocnemius muscle tissue of the rats were examined in the control groups (Group 1, 2). In the control groups, it was seen that the connective tissue was at normal level; the cores were more than one and in near; the cells were at normal size and the tissue integrity occurred (Fig. 7a, Fig. 7b).

![Fig 7a: Control group (corn oil) rat’s gastrocnemius muscle is PAS positive. PAS b. Control group (PBS+ ethanol) rat’s gastrocnemius muscle is PAS positive. PAS](image)

When the groups which were applied the CCl₄ were examined it reacted PAS positive; nevertheless it was observed that in the muscle fibrils there were melting in places and hypertrophy (Fig. 8a, Fig. 8b).
When the CCl₄ groups which were applied to the melatonin were examined, they were reacted Pas positive. It was observed the decreasing in the collagen fiber and the near normal scene in the muscle fibrils as far as the group with the CCl₄ (Fig. (17), Fig. (18)).

4. Discussion

CCl₄, which is frequently used in the industry and agriculture, is a chemical that is well known its hepatotoxicity and nephrotoxicity [6]. The reactive oxygen products, which are appeared during “oxidative stress” cause that the cellular proteins release and these proteins escape to the vein surface by making way the deterioration, degeneration, necrosis of the muscle fibrils’ spasm and organization; the vascular endothelium cell swelling; and the increasing of the micro vascular permeability [39, 40]. Cetin and Cetin [41] did oxidative damage to the rats with CCl₄ in their brains and kidneys; and they observed the increasing of MDA and NO levels in these tissues, and the decreasing in the SOD and CAT activities; in addition to these, they did not observe any noticeable change in the GPx activity.

Miyazaki et al., [42] applied the CCl₄ to the rats during 10 weeks and took blood tissue, brain tissue, liver tissue and skeletal muscle tissue of these rats; and researched the effect of the CCl₄. They also observed that CCl₄ created the fibrosis in these tissues. Differently from our study, they showed the muscle analyses as biochemical.

Vural et al., [43] applied the CCl₄ to the Wistar albino rats during 12 weeks and took their soleus muscles at the end of 12 weeks and they histologically examined these. As a result of these, they observed the hypertrophy in the muscles, the orientation disorder and the fibrosis in the muscles cells. In analogy to these studies, in comparison to the control groups, it was observed the hypertrophy in the muscle, the orientation disorder in the muscle cells and the increasing of the collagen fibers around the nerve plexus in our study. Moreover, it was seen the melting in the fibrils.

The melatonin which is a pineal gland hormone that is a neuroendocrine organ, [44] is a strong and effective endogen radical collector. Kus and his friends reported that the damage was decreased which occurred in the kidney and the liver after the intoxication created from CCl₄ as a result of the studies with the melatonin. Erdem et al., [45] histologically and biologically researched the antioxidant protective effect of the melatonin on the skeletal muscles of the Wistar albino rats which created the acut ischemia-reperfusion (I/R) injury. As a consequence, they emphasized the protective effect of the melatonin.

Karaca et al., [46], in their study with Wistar-Albino major male rats, applied the CCl₄ to this group and applied the CCl₄+melatonin to the other group during one month. Consequently, it was seen an intensive coloration which showed the heat shock protein 70 (hsp70) immunoreactions in the liver as a result of CCl₄ toxicity. The researchers determined that the detrimental effect, occurred in the liver as a result of the CCl₄ exposition was prevented by the
melatonin hormone thereby being confirmed the minimal hsp70 coloration in the river tissue sections belonging to the rats injected the melatonin with the CCl₄ exposition. Vural et al., [43] applied the CCl₄ and the melatonin to the Wistar albino rats during 12 weeks and at the end of 12 weeks, took the soleus muscles and histologically examined these muscles. As a consequence, in comparison to the applied the CCl₄ group, it was observed that the tissue integrity was maintained and the collagen fibers around the blood vessel were decreased. The researchers emphasized the damage inhibitor effect as well the protective effect of the melatonin. In comparably our study, in the group which was applied the melatonin, it was observed the decrease in the collagen fibers and the tissue integrity near the control group. It was succeeded a scene which was near the normal with decreasing in melting occurred in the muscle fibrils. Vural et al., [43] studied the CCl₄ effect to the soleus muscle. As is known, the soleus is a slow-twitch muscle. If it is compared to this study, we can say that the gastrocnemius muscle is more affected from the CCl₄ than soleus muscle. Accordingly, it can be said that the healing effect of the melatonin in the soleus is more than the gastrocnemius.

5. Declarations
Acknowledgements
We would like to thank Prof. Dr.K.Gonca AKBULUT and Doç. Dr. Şevin Güney Department of physiology Faculty of Science-Gazi University, for experimenting this study.

Competing interests
The authors declare that they have no competing interests.

Ethics approval and consent to participate
This study was carried out at Gazi University Faculty of Medicine Department of Physiology with the decision of ethical committee dated 17.01.2012 and coded G.Ü ET-12.010.

Funding
The authors declare that there is no funding for this research.

6. References
24. Reiter RJ. Melatonin. The chemical expression of