

# Biochemical Effects of Caffeine and Melatonin on RAT Embryo Brain

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**Abstract: Background:** Oxidative stress plays an important role in fetus brain development. The aim of this study was to investigate the protective role of melatonin against the effect of caffeine (given 30 mg/kg and 60 mg/kg) in embryo brain. **Material and Methods:** For this purpose, 35 adult female Sprague-Dawley rats were used in the study. Pregnant rats were detected by vaginal smear test and were divided into seven groups with five rats per group. Thirty (30) mg/kg and 60 mg/kg caffeine were administered to the experimental groups between the 1st and 20th days of pregnancy, while melatonin (10 mg/kg) was administered to the treatment groups in addition to caffeine. On the 20th day of pregnancy, fetuses were taken by cesarean section and their weights/lengths were measured. **Results:** Superoxide dismutase (SOD), glutathione (GSH), glutathione disulfide (GSSG), total antioxidant status (TAS), thiobarbituric acid reactive substances (TBARS), calcium (Ca) and vitamin D (Vit D) levels were measured by spectrophotometric methods. Oxidative stress index (OSI) and total glutathione (GSH/GSSG) levels were the markers used for oxidative stress and measured by TOS/TAS and GSH/GSSG, respectively. All data were statistically evaluated. **Conclusion:** In this study, it was observed that high dose caffeine reduces oxidative stress in brain tissues while increasing the antioxidant level.

**Key words:** Caffeine, fetus, brain, rat, melatonin.

## 1. Introduction

Caffeine is a bioactive compound consumed worldwide and is a natural methylxanthine. The behavioral effects are similar to those of classical psychostimulants, especially amphetamines with motor activation. In nature, coffee beans are found in the seeds of *Coffea arabia*, in plants belonging to the Sterculiaceae and in Theace family, enter the human food chain through plant nutrients. Caffeine is the most active ingredient in tea and coffee, as well as cola drinks, chocolate and several medicines. Since the late 1980s, the energy drink market has emerged as another source of caffeine and its consumption is increasing each year [1]. After consumption, caffeine

is first metabolized in the liver with cytochrome P450. In addition to the organism, factors such as caffeine consumption time, sex, physiological status and temperature also affect the pharmacokinetics of caffeine. In several experimental studies on animals, teratogenic and embryotoxic effects of high caffeine doses have been determined [2-4].

Caffeine is used for premature apnea, bronchopulmonary dysplasia and orthostatic hypotension. Caffeine and similar substances are also being investigated in various diseases such as Alzheimer's disease, asthma, cancer, diabetes and Parkinson disease [4]. The toxic dose was determined to be 10 grams per day in adults, and approximately 80 to 175 mg in 1 cup of coffee per day. Caffeine directly affects the central nervous system, especially the synapses of neural conduction, to keep the person

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awake. Basically, it is to ease the neural conduction by causing the breakdown of acetylcholine and adenosine causing fatigue and accumulates in the nerve cavities [5]. It has been reported that caffeine passes through the placenta and causes teratogenic and embryo toxic effects. Melatonin is one of these antioxidants and acts as a natural neurotransmitter. Melatonin hormone, which is known to affect several biological and physiological processes in the body, may directly affect various conditions, from sleep disorders to psychological disorders. Melatonin also acting as an antioxidant in the body, has been reported to be highly effective in protecting against infection, inflammation and immune system diseases [6]. Melatonin passes through the placental barrier easily and quickly into fetal circulation [7, 8].

Oxidative stress might be determined by measuring parameters that indicate the increase or decrease in concentration of enzymes and metabolites involved in the antioxidant defense mechanism, indicating an increase in free radical production. It may also be evaluated by measuring damaged tissues or metabolites in biological fluids in tissues. For this purpose, various methods have been developed for analysis of biological samples such as erythrocytes, serum, plasma and tissue samples. In addition to the difficulties of each of the available techniques, direct analysis is difficult due to the tendency of free radicals to react that is high and the reason of their short half-life. On the other hand, since the parameters determining oxidant and antioxidant levels are affected by various factors, it has been considered appropriate to use techniques for measuring total antioxidant capacity and total oxidant level [9, 10].

Melatonin and its metabolites could directly prevent oxidative stress by effectively removing reactive oxygen species (ROS) due to the presence of an electron-rich aromatic ring system. It was obtained that the effect of melatonin and caffeine interaction on caffeine-induced oxidative stress and sleep disorders

is having melatonin increased protein synthesis [11].

It was examined that the possible effects of caffeine on fetal brain development might be obtained. However, the protective effect of melatonin versus caffeine has never studied. In this study, it was assumed that melatonin may play a critical role in the normalization of oxidative and inflammatory parameters in fetal brain tissue due to the use of different doses of caffeine during pregnancy. Therefore, it was aimed to evaluate the effect of melatonin on brain tissue after low dose and high dose caffeine administration.

## 2. Material and Method

### 2.1 Animals

About 200-250 g female Sprague-Dawley rats used in the study were obtained from Erciyes University Experimental Animals and Clinical Research Center (DEKAM). The rats were caged at 5 p.m. for mating of two female and one male. The next morning at 7 a.m., female rats were subjected to vaginal smear test and females with smear test were accepted as pregnant for 0.5 days. The rats were kept in specially prepared and automatically air-conditioned rooms with constant temperatures of 19-21 °C and 12 hours of light/dark periods during the study. Rats were fed with pellet feed.

### 2.2 Experiment Groups

Pregnant rats were randomly allocated to seven groups ( $n=5$ ). Injections were given daily between the 1st and 20th days of pregnancy.

(1) Control group: serum physiological (SF) was administered as i.p. (1 mL/kg).

(2) Sham group: 0.1 mL hanks solution was administered as i.p.

(3) Low dose caffeine group: 30mg/kg caffeine was applied as gavage.

(4) Low dose caffeine + melatonin group: 30mg/kg dose caffeine applied as gavage accompanying with 10 mg/kg melatonin i.p.

(5) High dose caffeine: 60 mg/kg caffeine was administered as gavage.

(6) High dose caffeine + melatonin group: 60 mg/kg caffeine was applied as gavage accompanying with 10 mg/kg melatonin i.p.

(7) Melatonin group: 10 mg/kg melatonin was applied as i.p.

### 2.3 Preparation of Injections

Caffeine and melatonin powder 98% were obtained from Sigma Aldrich. Drinking water was used as the solvent solution to adjust the amount of caffeine to be given to the rats. In the process of dissolving melatonin in powder form, hanks solution was used. Both substances were prepared daily and no stock solution was made.

### 2.4 Manipulation of Rats and Obtaining of Fetus

Pregnant rats were anesthetized with ketamine (75 mg/kg) and xylazine (10 mg/kg) on the 20th day of pregnancy. The abdominal wall of the rats was cleaned with 70% alcohol and the anterior abdominal walls were removed with a transverse incision. The uterus and the fetuses were dissected individually with their placentas. The distance between occipito-frontal and bi-parietal measurements of the fetuses was measured by electronic caliper. The brains of the animals were collected in sterile plastic bags and stored at -80 °C until biochemical analysis.

### 2.5 Protein Isolation from Brain Tissues

After the tissues were homogenized for biochemical analysis, it was centrifuged at 14,000 rpm for 15 minutes at +4 °C. After centrifugation, the supernatants were collected and stored at -20 °C until use. Protein content of all samples was measured by Bradford method.

### 2.6 Biochemical Analysis

TAS, TOS, OSI, GSSG TBARS, SOD, Ca, Vitamin D and GSSG levels were measured by commercial Enzyme Linked Immunosorbent Sandwich Assay

(ELISA) method. Samples were added to the wells previously coated with the corresponding monoclonal antibodies and incubated. After incubation, biotin-labeled antibodies forming an immune complex with streptavidin-HRP were added. Subsequently, unbound enzymes were washed off and substrates A and B were added. Finally, the solution turned blue and yellow with the effect of acid. Levels of markers were determined according to the kit (Catalog No. CK-E10376, Hangzhou Eastbiopharm Co.Ltd., Shanghai, CHINA) procedure used.

GSH levels were determined by spectrophotometer according to the method reported by Sedlak and Lindsay [12]. Also, GSH-Px enzyme activities were determined by spectrophotometer according to the method improved by Lawrence and Burk [13].

### 2.7 Statistical Analysis

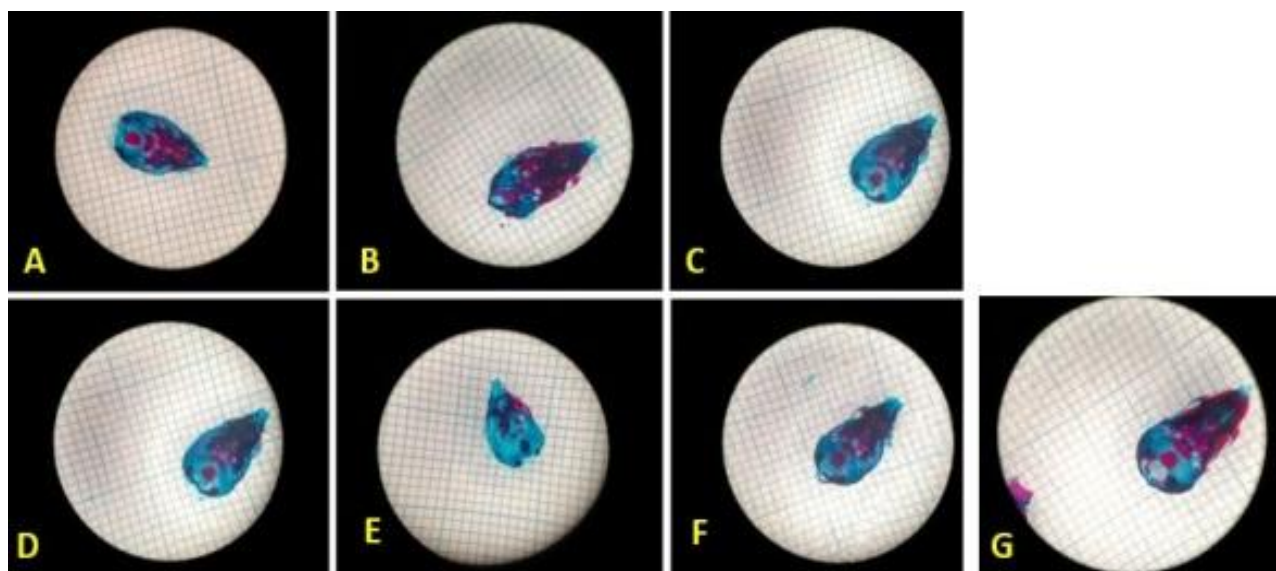
The results obtained from the experiments were expressed as “mean value  $\pm$  standard error” ( $x \pm$ SEM). The significance between the groups was determined using one-way ANOVA test and followed by Fisher’s post-hoc LSD (least significant differences). All statistical procedures were performed by the “IBM SPSS Statistics Version 20” statistical program and  $p < 0.01$  was considered as significant.

## 3. Results

The results of occipito-frontal and bi-parietal bones of fetuses are given in Table 1. According to the result, a statistically significant decrease in these parameters was found as 30 mg/kg caffeine was given ( $p < 0.05$ ). As the caffeine dose was increased to 60 mg/kg, the decreases in the parameter statistically increased ( $p < 0.05$ ). It was determined that the related parameters significantly increased while melatonin at a dose of 10 mg/kg was given as a preservative. Subsequently, the dual staining method of fetuses, it was determined morphologically that the bone cartilage ratios in the caffeine-treated group were lower than the control and melatonin-treated groups (Fig. 1).

**Table1** Measurements of fetus groups.

Groups	N	Occipito-frontal	Bi-parietal	p
Control	30	12.75±0.87	7.24±0.54	0.001
Melatonin	30	12.29±1.07	7.08±0.81	0.001
Sham	30	12.24±1.07	7.00±1.02	0.001
LDC+melatonin	30	10.58±0.94	6.74±0.92	0.001
LDC	30	10.37±1.17	6.51±0.90	0.001
HDC+melatonin	30	8.23±1.52	6.27±0.84	0.001
HDC	30	6.84±1.64	4.68±0.97	0.001

**Fig. 1** Dual bone staining of groups.

\* The red color indicates bone tissue and the blue color for cartilage tissue.

A: Melatonin group;

B:Control group;

C:LDC caffeine group

D:HDC + melatonin group

E:HDC group

F:LDC caffeine+ melatonin group

G: Sham group

### 3.1 Biochemical Findings

It was obtained that high dose caffeine reduces oxidative stress. In Fig. 2, TAS and OSI levels were found to be the lowest in the high-dose caffeine-treated group. As high-dose and low-dose caffeine jointly melatonin were administered, TAS and OSI levels were increased. In the melatonin group, there was no significant change in TAS and OSI levels. TBARS, GSSG and GSH levels were found to be minimal in high dose caffeine treated groups. All these levels increased significantly after melatonin

administration (Fig. 2).

SOD and Ca levels were the highest in the high dose caffeine group. Melatonin significantly reduced SOD and Ca levels and inhibited apoptosis mechanisms in cells. In addition, melatonin significantly decreased TOS and vitamin D levels compared to high-dose and low-dose caffeine-treated groups (Fig. 3). While GSH/GSSG ratio was higher in the control groups, the ratio decreased in the high-dose and low-dose caffeine-treated groups (Figs. 3 and 4).

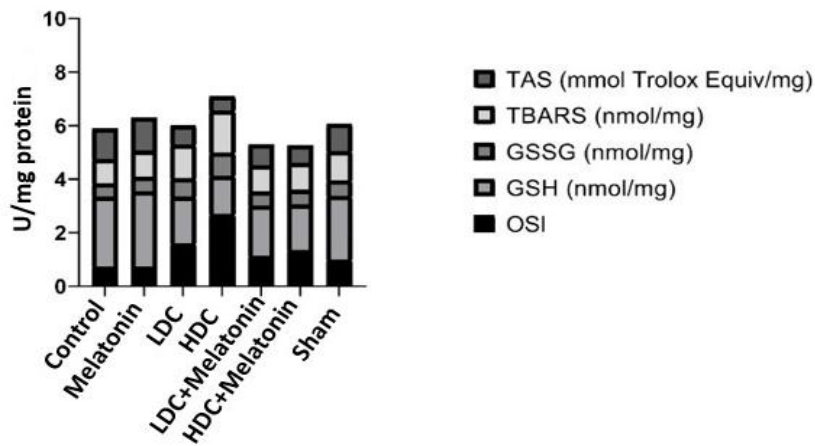


Fig. 2 TAS, TBARS, GSSG, GSH, OSI results. Results are given in units/1G protein.

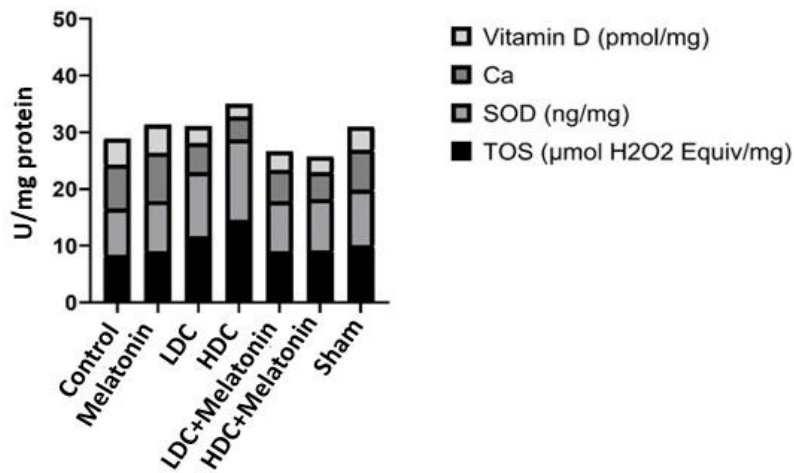


Fig. 3 Vitamin D, Ca, SOD and TOS results. Results are given in units/1G protein.

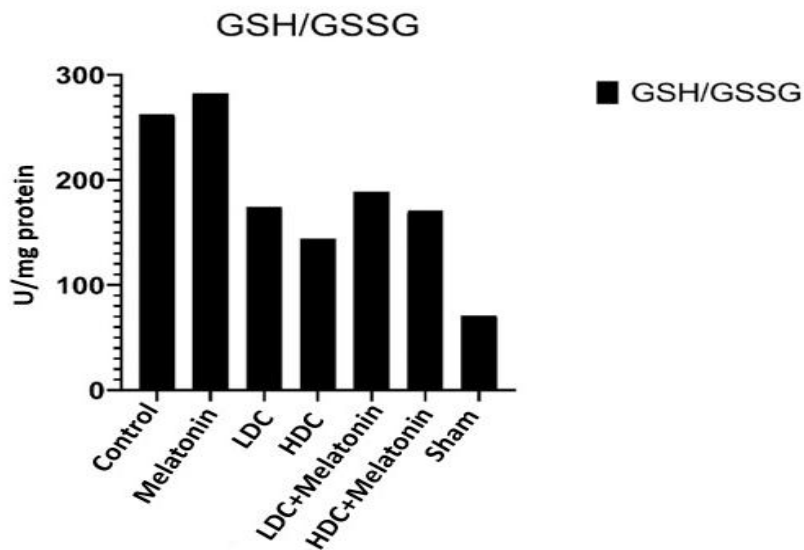


Fig. 4 GSH/GSSG results. Results are given in units/1 Gr protein.

#### 4. Discussion

Caffeine (1,3,7-trimethylxanthine) is a naturally occurring xanthine alkaloid [14]. It is known that approximately 87% of the world population consumes caffeine with an average intake of 193 mg/day [14, 15]. Psychotropic substances, including caffeine, are increasingly used by pregnant women over the years. Psycho-stimulating and anxiolytic effects were observed to determine the wide use of caffeine-containing drinks and mixtures [14]. The neurophysiological effects of caffeine depend mainly on the dose and also on the duration of administration. Caffeine acts as a central and peripheral nervous system stimulant in both animals and humans. The primary effects of acute caffeine consumption in children and adolescents include increased arousal, attention and locomotor behavior.

In children, caffeine toxicity causes severe emesis, tachycardia, central nervous system agitation and diuresis [16]. Although mild intake of caffeine is generally thought to have significant effects on human health, its effect on bone metabolism is controversial [11]. Caffeine during pregnancy might adversely affect the development of the fetus. It was reported that widespread abnormalities related to impaired mineralization of bone tissue and adverse effects of high-dose caffeine in bone tissue were observed [17].

Recently, *in vivo* experimental studies on the skeleton have indicated the detrimental effects of caffeine. However, the mechanisms underlying the effect of caffeine on the developing fetal brain have not been sufficiently studied [18, 19]. Antiapoptotic and anti-inflammatory effects of caffeine have been reported in recent studies [19-21]. Accordingly, an increase in TNF $\alpha$ , IFN $\gamma$  and IL-1 $\beta$  expression was detected in the caffeine-treated group [22].

Melatonin is lipophilic, so that the blood may quickly cross the brain barrier [23]. Melatonin has strong free radical scavenging and neuroprotection properties and also regulates the activity of

antioxidant enzymes [24, 25]. Melatonin stimulates gonadotrophin release hormone, estrogen levels and androgen responses. It may also inhibit ovarian function in women and may be useful as oral contraceptives. In addition, melatonin improves leukopenia and winter depression. It was reported that 10 mg/kg melatonin group had a significant improvement in behavior results compared to nonylphenol group [26]. In another study, both melatonin and vitamin E were found to reduce lipid peroxidation in the hippocampus and frontal cortex [27]. The effect of melatonin and caffeine interaction on caffeine-induced oxidative stress and sleep disorders was investigated and it was observed that melatonin and caffeine interaction increased protein synthesis and stimulated gonadotrophin release. However, total brain tryptophan levels were increased in the group of melatonin and caffeine given together [28].

In our study, TAS, TOS, TBARS, GSSG, GSH, Vitamin D, Ca and SOD values were examined as biochemical parameters. OSI and GSH/GSSG ratios were also determined. Oxidative stress markers have enabled to give information about the apoptotic status of the cell [29]. TBARS and TOS levels, which are oxidative stress markers, were increased in high dose and low dose of caffeine groups. Therefore, caffeine administered to subjects caused oxidative stress damage to cells and has been obtained with various studies [30, 31]. Summarily, melatonin has a therapeutic feature [32]. For our study, oxidative stress decreased and antioxidant levels increased in melatonin groups. Glutathione reductase (GR) converts GSSG to GSH by simultaneous oxidation of SS-nicotinamide adenine dinucleotide phosphate ( $\beta$ -NADPH<sub>2</sub>). In healthy cells, 90% of the total glutathione (GSH) pool is in reduced form.

As cells are exposed to increased levels of oxidative stress, GSSG accumulates and the ratio of GSH to GSSG increases. The decreasing ratio of GSSG-GSH to GSSG is an indicator of oxidative stress [33]. In our

study, high-dose and low-dose caffeine reduced the GSH/GSSG ratio. Increased GSH/GSSG ratio after melatonin administration resulted a therapeutic effect of melatonin [34]. SOD is one of the essential oxidative stress markers which are effective against free O<sub>2</sub> radicals. The rising SOD level indicates that the cell is defending against oxidative stress. In our study convenient results were obtained with this situation [35]. Vitamin D and Ca deficiency problems occur during brain development [36, 37]. In the study, it was observed that vitamin D and Ca levels significantly decreased. Vitamin D and Ca levels were significantly increased after melatonin administration. The data obtained are consistent with the study conducted by Berridge et al. [38, 39]. According to a study by Thibault et al., Ca values have a significant effect on brain damage [40]. In our study, it was thought that melatonin might be useful in preventing brain damage since Ca values approached the control group level after melatonin administration.

## 5. Conclusion

As a result of this study, occipito-frontal and biparietal bone development decreased in fetuses of pregnant rats exposed to caffeine. A decrease in the rate of ossification was also observed. The rats were given melatonin against different doses of caffeine, and values in the melatonin-treated groups were similar to those of the control group. As a result, melatonin treatment after administration of different doses of caffeine significantly improved anatomical and biochemical findings. According to the data, we suggest that melatonin may be used as a potential therapeutic agent to prevent adverse effects caused by caffeine, and our results will be useful in model studies on melatonin and caffeine.

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