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# The protective role of melatonin against the effects of different doses of caffeine on the fetus

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**Abstract:** Skeletal system and some organs development changes in rat fetuses with 30 and 60 mg/kg caffeine and melatonin's (10 mg/kg) protective role against rat fetuses were investigated. Groups (n = 4) were formed as Control, LDC, HDC, LDC+melatonin, HDC+melatonin and melatonin. Fetuses were taken by cesarean section and stained using dual skeletal staining method and FESEM. TRAP and AP immune-reactivity concentrations were calculated. Oxidative stress and inflammatory markers were also measured by liver, bone and placenta samples. TNF-α, IL-1ß, IL-6, VEGF-A, SOST and Fetuin-A levels were measured in tissue by using ELISA. TBARS, SOD, GSH, GSSG, TOS, TAS, measured by spectrophotometric assay method. The mRNA levels of *Agtr2* gene expressed in placental tissues of control rats and in placental tissues of rats exposed to HDC, LDC, MEL, HDC+MEL, LDC+MEL were analyzed by Real-time PCR. The gene expressions of *Agtr2* were significantly upregulated in the placentas exposed to HDC, MEL, HDC+MEL and LDC+MEL (P<0,001). No significant difference in samples of the LDC group (P>0,05). According to these data, caffeine used during pregnancy delayed ossification; melatonin, a powerful antioxidant, was found to eliminate this effect. Besides, changes in angiotensin receptor expression observed in response to a caffeine and melatonin exposure result from high dose and join effect.

***Key words:*** Rat; Caffeine; Ossification; Oxidative stress; Pregnancy.

## Introduction

Caffeine originates from coffee, cocoa beans, nuts, or tea leaves occurring alkaloid found plant parameters. It is known that caffeine has high impact on the pregnancy and the development of the fetus due to the maternal intake and possibly on the metabolism rate of mother. Circulation of caffeine-half life is about 3-5 hours while it affects respiratory parameters or oral contraceptive usage during pregnancy (1-3). It is said that different doses of caffeine applied to rats during pregnancy cause significant fetal and postnatal growth reterdation and therefore smaller adults occur (3). Epidemiological and clinical studies have shown that caffeine induced embryo toxicity and maternal caffeine intake causes intrauterine growth retardation. Placental metabolic functions are very important in first stages of pregnancy, feeding offsprings and placenta and meeting their energy needs.

It was showed that caffeine may pass through the placenta and may cause possible growth retardation in the fetus (4, 5). Accordingly, it was reported that caffeine could reduce the development of fetal rats and might cause adverse pregnancy effects (6-8). Also, it was speculated that high caffeine doses may accepted as risk-increasing for osteoporosis in adults (9). In addition, to cause mineralization abnormalities in carpal and metatarsal bones, skull, pelvic belt, sternum, and spine bone tissue were reported (10, 11). Researchers are extensively focusing on the mechanism-based effects of antioxidants in different diseases (12, 13). Melatonin (N-acetyl 5-methoxy tryptamine) is released by pineal gland, particularly during night. Apart from adjusting the basic rhythm of the biological, it also takes part in other essential tasks such as cell regeneration, strengthening of the immune system and regulation of body temperature. Melatonin is one of the most powerful antioxidants known because of its lipophilic structure (14). The overall antioxidant effect of melatonin is increased as it works with other antioxidants. Finally, melatonin has been shown to be twice as active as vitamin E, and amphiphilicity has been reported to distinguish melato-

nin from other classical antioxidants such as vitamin C and vitamin E (15). It is known that high doses of caffeine promote bone loss and bone mechanical strength loss in rats. Furthermore, it has been shown that caffeine is a risk factor for osteoporosis (6, 7). For treatment, melatonin reduces oxidative stress in the liver and other tissues while it has been involved in regulation of embryonic ossification in subjects treated with caffeine and melatonin (16-18). Kuczkowski (2009), reported that that caffeine intake might cause negative outcomes on reproduction together with fetal development, increase premature delivery and spontaneous abortion risk, and intrauterine growth retardation (19). Stimulation of the renin-angiotensin system helps to maintain the blood pressure during pregnancy (20). Agtr2 is renin-angiotensin signaling (RAS) pathway that has been thoroughly studied for its role in blood-pressure regulation (21). Beside, Cook et al. (2011), suggested that melatonin alters vascular blood flow through a combination of mechanisms (22). Recently, superoxide dismutase (SOD), glutathione disulfide (GSSG), total antioxidant status (TAS), thiobarbituric acid reactive substances (TBARS) levels are tried as oxidative stress parameters. Also, interleukin 1β (IL-1β), tumor necrosis factor alpha (TNF-α) and Interleukin 6 (IL-6) parameters are used to adjust the inflammation-associated mechanisms (23). Therefore, it was found appropriate to investigate these parameters in the study. It has been examined the effects of caffeine on fetal bone development. In our study we investigated the protective effects of melatonin against caffeine. We also scientifically analyzed how use of caffeine in different doses during pregnancy induced bone damage. We evaluated biochemical parameters, field-emission scanning electron microscope (FESEM), tartrate resistant acid phosphatase (TRAP) and alkaline phosphatase (AP), gene expression of Agtr2, in placenta, bone and liver.

## Materials and Methods

### Animals

Twenty-four Wistar Albino rats (180-220 gr) were used. These rats taken from clinical research center (DEKAM) of Kayseri Erciyes University. For the research, ethics committee permission of Kayseri Erciyes University local ethics committee dated 11.01.2017 and numbered 17/003 was obtained. The rats were rested in cages at 5 pm for mating, with two female and one male. After the smear test, female rats were considered pregnant for 0.5 days. our experimental groups were kept in rooms with automatic air-conditioning at constant temperature of 19-21ºC and 12 hours of light/dark periods during the study. The rats were fed with normal pellet feed.

### Experimental groups

Control: Serum physiological (SF) 1 ml/kg intraperitoneally (i.p).
Low Dose Caffeine (LDC): 30 mg/kg caffeine (gavage).
LDC + Melatonin: 30 mg/kg (gavage) and 10 mg/kg (i.p).
High Dose Caffeine (HDC): 60 mg/kg (gavage).
HDC + Melatonin: 60 mg/kg (gavage) and 10 mg/kg melatonin (i.p).
Melatonin: 10 mg/kg melatonin (i.p.)

### Preparation of injections

Sigma Aldrich provided the caffeine powder and melatonin, and both substances were prepared daily with no stock solution.

### Manipulation of rats and obtaining fetuses

Anesthesia was applied to pregnant rats by using 75 mg/kg ketamine and 10 mg/kg xylazine in 20 th day. Uterus and fetuses were dissected with placentas (18).

### Skeletal staining in fetuses

Ethyl alcohol (70% ) was used to keep fetuses for 4-7 days, and the water contained was withdrawn. After this process, pure acetone was applied for 1-3 days and degreased. Fetuses extracted from acetone were taken into solution that contained Alizarin red S (100 mg) and Alcian Blue (300 mg). The tissues were stained by incubating for 7 days, and the fetuses were then washed with water for 2 hours and allowed to react with water. Then, 1% potasyum hidroksit (KOH) was started to be transparent. The solutions were then transferred to 20%, 50%, and 80% glycerin respectively, followed by pure glycerin. The extremities of fetuses were photographed with stereomicroscope (Nikon E5700 camera) for morphometric measurements. Then, the obtained photos were transferred to computer. The Image J Program was employed for length-area measurements of bones (10, 18, 24).

### Immunohistochemistry

Femoral tissues of the fetuses were fixed in 48 hours with 10% formaldehyde for histological examination. In femoral tissues, avidin to determine TRAP (1/100, bs-6434R; Bioss, Boston, USA) and AP (1/200, sc-16626; Santa Cruz Biotechnology, USA) biotin-peroxidase method and immunohistochemistry staining method were used. The immunohistochemistry staining kit of the Large Volume Detection System (TP-125-HL) was used for the next steps. Ultra V block was applied to tissues washed with PBS for 5 minutes at room temperature to ensure that the regions outside the antigenic areas were closed. Immediately thereafter, TRAP and AP were applied to the sections and incubated for 1 night at +4 °C. As negative control, PBS was used instead of primary antibody, analyse under Olympus BX51 microscope by sealing with capping solution (Entellan®, Merck). For x40 magnification, 5 different areas as random sections from femoral tissues were included in the measurement. The TRAP and AP immune-reactivity concentrations were calculated for each femur section using the Image J software program (25, 26).

### FESEM method

After removal of bone tissues according to ethical rules, pH was determining 7.2-7.4 range in 0.1 M phosphate buffered glutaraldehyde solution in 24-48 hours. Then, the soft tissues were removed at 37 °C for 48 hours in 1% detergent (sodium dodecyl sulphate) was kept. After washing 3 times with 5 minutes distilled water, the bones passed through the acetone series (50-70-80-90-100-100-100) for 15 minutes were taken to the

ether (24 hours) and degreased. The bone tissues were dried for 48 hours. In order to get a scanning electron microscope, it was first coated with gold palladium using a Sputter coater and imaging was performed using a FEIQuanta450FEG scanning electron microscope. Trabecular thickness was measured using ImageJ program. Calcium analyzes were performed in the bone tissues obtained by EDX analysis (26).

**Biochemical analysis**

Colorimetric kits were used for TAS, TOS, TNF-α levels, each sample was examined in duplicate. Glutathione level was calculated using formula GSH = T− GSH - (2 −GSSG). The levels of GSH was calculated by the formula: GSH = GSHt−2 × GSSG. The results of GSHt, GSH, and GSSG were expressed as nmol of GSH or GSSG per mg of protein (nmol GSH/mg protein or nmol GSSG/mg protein), of all samples was measured (27). Protein samples were thawed, and commercial ELISA kits were used for the quantitative measurement of TNF-α (E-EL-R0019, Elabscience USA), IL-1ß (E-EL-R0012, Elabscience USA), IL-6 (E-EL-R0015, Elabscience USA) levels, according to the manufacturer's instructions. Results are expressed in milligram per milliliter of proteins. ELISA was performed with protein samples extracted from tissues, according to the manufacturer's instructions. Total $Ca^{+2}$ concentration was evaluated by using calcium colorimetric assay kit (ab102505; Abcam). Vitamin D analysis was determined using the commercial ELISA kit (EIA 5396 DRG, Germany). Vascular endothelial growth factor (VEGF-A), Sclerostin (SOST) and Fetuin-A levels were measured by ELISA method using rat fetus liver samples according to the manufacturer's protocol. VEGF-A (Elabscience Rat VEGF-A kit catalog No: E-EL-R2603, Elabscience (Wuhan)), SOST (Elabscience Rat SOST kit catalog No: E-EL-R2427, Elabscience (Wuhan) and Fetuin-A (Elabscience Rat Fetuin A kit catalog No: E-EL-R2451, Elabscience (Wuhan)) kits were used. Absorbance measurements were measured using a Multiskan GO plate reader (Thermo Scientific, USA) and calibrated according to the standard curve. Results were calculated per unit (28).

**Placental gene expression of Agtr2**

Total RNA from the placental tissues was extracted with PureZole reagent. (Biorad, USA, Cat. No: 732-6890) according to the manufacturer's protocol. Then, RNA amount and RNA purity were quantified for each RNA sample by Nanodrop ND-1000 spectrophotometer V3.7. All the RNA samples were reverse transcribed into cDNA from 1 µg of total RNA (iScript Reverse Trancription Supermix Biorad, USA, Cat. No: 170884) under the following conditions: One cycle at 25°C for 5 minutes, 46°C for 20 minutes and 95°C for 1 minute. We prepared 1:10 fold serial dilutions of cDNA in six tube. For assessing the relative efficiencies of the target gene Agtr2 and the reference gene Gapdh amplification is achieved by running standart curves for each amplicon using the same sample**.** The mean Ct values and the concentrations entered the REST 2009 software. Agtr2 mRNA expression analysis performed by Rotor Gene-Q (Qiagen, Hilden, Germany). Appropriate dilued cDNA's were added to a master mixture according to the manufacturer's protocol (SsoAdvanced Universal Inhibitor-Tolerant SYBR Green Supermix, Biorad, USA, Cat. No: 172-50-16). Oligonucleotide primers were designed by Oligomere (Ankara, TURKEY) based on following primer sequences: Rat-Agtr2-F: 5'-TAACTGCTCACACAAACCGG-3', Rat-Agtr2-R: 5'-CAAGACTTGGTCACGGGTAA-3' [Rattus norvegicus angiotensin II receptor, type 2 (Agtr2).

NCBI Reference Sequence: NM_012494.3]; Gapdh-F5'-CTCTCTGCTCCTCCCTGTTC-3', Gapdh-R5'-GCCAAATCCGTTCACACCG-3' [Rattus norvegicus glyceraldehyde-3-phosphate dehydrogenase (Gapdh), NCBI Reference Sequence: NM_017008.4]. We used the following RT-PCR protocol: 98°C for 3 minutes initial denaturation followed by 40 cycles of 98°C for 15 seconds and 61°C for 30 minutes. Melting curve analysis was performed for confirmation of single product amplification at the end of the PCR. 65-95°C, 0,5°C increments at 5 sec/step. Each run has been performed triplicate (29). Calculation of relative fold-changes in mRNA levels, the efficiency of amplification value and mean Ct (Threshold cycle) values of each gene were examined into the REST 2009 software program.

**Statistical analysis**

SPSS 22 program used for analysis. One-way ANOVA test and Pearson Chi-Square Test were used to evaluate the fetus growth parameters, bone total length and area showing ossification (bone length, ossification percentage). For biochemical parameters, the results are presented as mean±standard deviation of the three replicates. Data in all experiments were analyzed for statistical significance using variance analysis (One-way ANOVA). Post hoc analyzes were performed to compare the parameters of the different groups. Statistical analysis related genetics were performed using REST 2009 V2.0.13 Software. $p < 0.05$ was taken as statistical significance level.

## Results

**Effects on growth parameters**

Table I shows the fore and aft length-weight of fetuses by groups. A statistically significant decrease was observed in these parameters as caffeine was given 30 mg/kg. As the caffeine dose increased to 60 mg/kg, the decreases in this parameter statistically increased. After melatonin at a dose of 10 mg/kg was given as a preservative, it was found that the growth parameters increased at significant levels and the result of it approached the control group (Table I).

**Effects on upper and lower limb bones**

In our study, long bones of upper and lower extremities were evaluated. As 30 mg/kg caffeine was administered, significant decrease was detected in ossification and length of the ossicles ($P < 0.05$). After the caffeine dose was increased to 60 mg/kg, ossification was found to be significantly reduced more drastically than 30 mg/kg caffeine group ($P < 0.05$). With additional melatonin was given to caffeine, it was calculated that ossification increased and its results approached the control group (Figure 1, 2) (Table II, III).

**Table 1**. Findings of fetus length and weight.

| | | Head-Stern Length (mm) | | | Placenta (gr) | | | Fetus Weight (gr) | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | N | Max | Min | Med | Max | Min | Med | Max | Min | Med |
| **CONTROL** | 15 | 30,63 | 25,47 | 28,42±1,66 | 0,90 | 0,52 | 0,70±0,09 | 2,40 | 1,87 | 2,10±0,16* |
| **LDC** | 15 | 31,69 | 25,50 | 29,07±1,64 | 1,08 | 0,39 | 0,56±0,10 | 2,59 | 1,91 | 2,23±0,19* |
| **LDC+MEL.** | 15 | 32,03 | 27,06 | 28,65±1,57 | 0,97 | 0,50 | 0,66±0,11 | 2,73 | 1,68 | 2,16±0,29* |
| **HDC** | 15 | 29,90 | 24,95 | 27,86±1,49 | 0,68 | 0,32 | 0,53±0,09 | 2,16 | 1,44 | 1,80±0,20 |
| **HDC+MEL.** | 15 | 30,67 | 27,80 | 29,36±0,95 | 0,76 | 0,34 | 0,57±0,12 | 2,39 | 1,98 | 2,18±0,12* |
| **MEL.** | 15 | 30,35 | 27,48 | 28,59±0,96 | 0,79 | 0,38 | 0,54±0,11 | 2,34 | 1,87 | 2,11±0,21* |

* It is significantly heavier compared to high dose caffeine group. (One-Way Anova $p<0.05$). (LDC: Low Dose Caffeine, HDC: High Dose Caffeine. MEL: Melatonin).

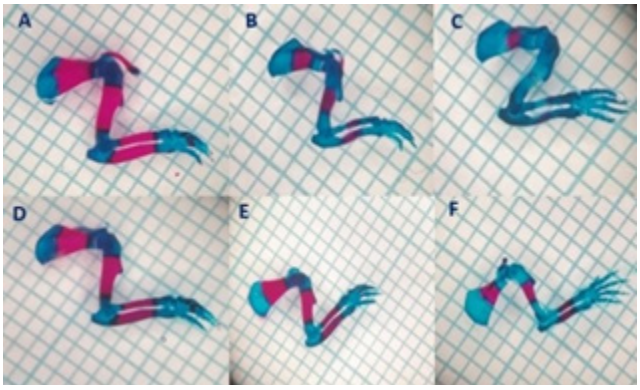


**Figure 1.** Images of upper extremities. A: Control Group, B: LDC Group, C: HDC Group , D: Melatonin Group, E: LDC + Melatonin, F: HDC + Melatonin Group.

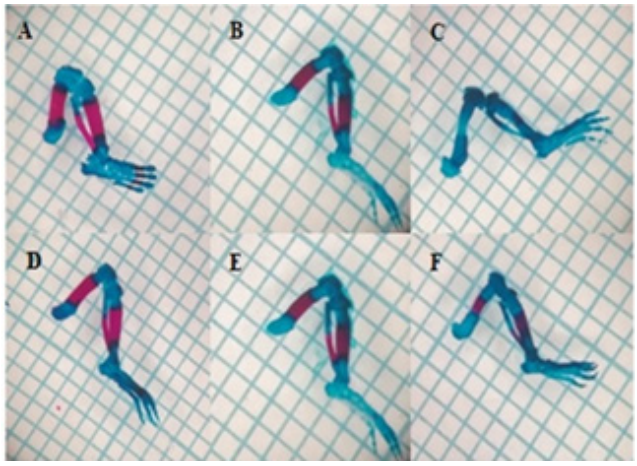


**Figure 2.** Images of the lower extremities. A: Control Group, B: LDC Group, C: HDC Group , D: Melatonin Group, E: LDC + Melatonin, F: HDC + Melatonin Group

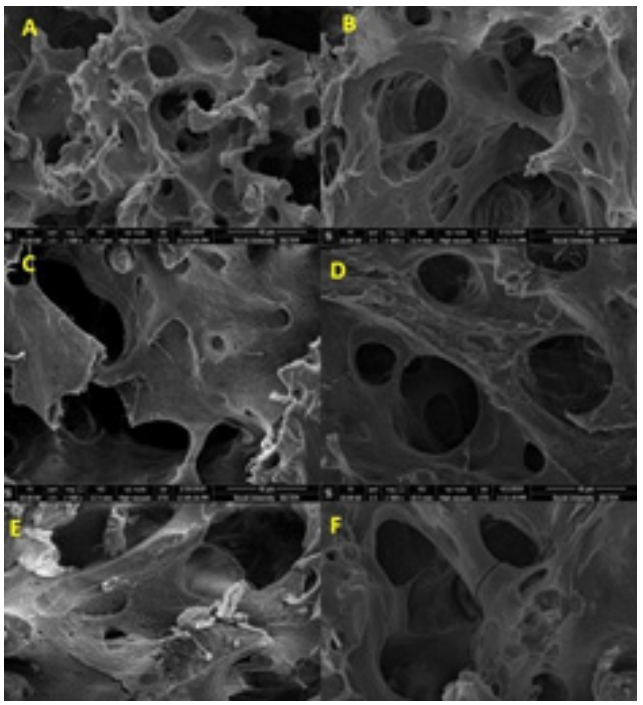


**Figure 3.** Trabecular structures all groups. A. Trabecular structures of the control group B. Trabecular structures of the Melatonin group. C. Trabecular structures of the LDC group. D. Trabecular structures of the LDC + Melatonin group. E. Trabecular structures of HDC F. Trabecular structures of HDC + Melatonin group.

## Histopathological findings

As FESEM images and measurement results were obtained by using ImageJ program, it was observed that caffeine had an adverse effect on bone tissue during pregnancy. It was also determined that LDC and HDC administration during pregnancy decreased the amount of calcium. In all groups, trabecular structures locating in branched form are selected. Haversian canals are found in the thick areas of the trabeculae (Figure 3).

A significant difference between the experimental groups in level of calcium averages was observed. The significance is due to the difference between the control group and the groups exposed to HDC administration. In terms of mean calcium values, it was found that there was a decrease in calcium value as a result of both low and high dose caffeine administration. There was an increase in the mean calcium values as a result of melatonin application although it was not statistically significant (Table IV).

A significant difference between the groups in terms of data distribution was obtained. The difference was determined by "Siegel castellan test" and the data obtained are shown in (Table V).

The mean trabecular thickness was lower in both melatonin and caffeine treated groups compared to control groups. It was observed that melatonin applying for protective purposes, increased trabecular thickness in groups exposed to low dose caffeine. In the groups exposed to high doses of caffeine, melatonin was not found to have a significant effect on mean trabecular thickness.

## Intensity of tartrate resistant acid phosphatase intensity

In immunohistochemical staining, TRAP immunereactivity intensity was determined in sections taken from tissues belonging to each group. When the preparations of the control group were examined, it was determined that TRAP expression was intense in the

**Table 2.** Findings of upper extremity bones.

| | | Humerus | | | Ulna | | | Radius | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | **N** | Total field | Bone Field | Ossification percentage | Total field | Bone field | Ossification percentage | Total field | Bone field | Ossification percentage |
| CONTROL | 30 | 4.05±0.31 | 1.78±0.16 | 43.98±2.91 | 1.99±0.29 | 0.97±0.13 | 48.70±2.52 | 1.51±0.17 | 0.64±0.09 | 42.50±3.30 |
| LDC | 30 | 3.54±0.49[a,b,d] | 1.19±0.12[a,b,d] | 33.61±3.8[a,b,d] | 1.85±0.25[a,b,d] | 0.67±0.09[a,b,d] | 36.21±4.61[a,b,d] | 1.33±0.19 | 0.44±0.08 | 33.08±3.11[a,b,d] |
| LDC+MEL. | 30 | 3.94±0.66 | 1.57±0.37 | 39.92±5.11 | 2.02±0.31 | 0.92±0.11 | 42.85±5.48 | 1.63±0.28 | 0.64±0.08 | 39.70±4.70 |
| HDC | 30 | 2.98±0.17[a,b,c,d,e] | 0.94±0.09[a,b,c,d,e] | 31.67±4.31[a,b,c,d,e] | 1.70±0.22[a,b,c,d,e] | 0.48±0.07[a,b,c,d,e] | 28.62±2.38[a,b,c,d,e] | 1.53±0.29[a,b,c,d,e] | 0.33±0.05[a,b,c,d,e] | 29.52±2.62[a,b,c,d,e] |
| HDC+MEL. | 30 | 3.33±0.24[a,d] | 1.21±0.13[a,d] | 36.35±2.15[a,d] | 1.97±0.27[a,d] | 0.83±0.12[a,d] | 42.40±3.93[a,d] | 1.29±0.21[a,d] | 0.52±0.09[a,d] | 40.90±4.72[a,d] |
| MEL. | 30 | 4.08±0.44 | 1.81±0.18 | 44.27±3.05 | 2.04±0.31 | 0.99±0.14 | 48.52±4.63 | 1.57±0.19 | 0.68±0.11 | 43.31±3.72 |

In the ANOVA test, $p<0.05$ was considered statistically significant. a) Significantly smaller compared to the control group. b) Significantly smaller compared to the Low Dose Caffeine + Melatonin group. c) Significantly smaller compared to the High Dose Caffeine + Melatonin group. d) Significantly smaller compared to the melatonin group. e) Significantly smaller compared with the Low Dose Caffeine group.

**Table 3.** Findings of lower extremity bones.

| | | Femur | | | Tibia | | | Fibula | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | N | Total field | Bone field | Ossification percentage | Total field | Bone field | Ossification percentage | Total field | Bone field | Ossification percentage |
| CONTROL | 30 | 2.74±0.29 | 0.84±0.11 | 30.78±2.44 | 2.28±0.22 | 0.90±0.13 | 39.42±2.77 | 1.03±0.12 | 0.37±0.05 | 35.92±4.52 |
| LDC | 30 | 2.31±038[a,b,d] | 0.52±0.08[a,b,d] | 22.51±3.04[a,b,d] | 1.86±0.26[a,b,d] | 0.51±0.08[a,b,d] | 27.41±4.01[a,b,d] | 0.84±0.12[a,b,d] | 0.26±0.04[a,b,d] | 30.95±4.32[a,b,d] |
| LDC+MEL | 30 | 2.62±0.31 | 0.81±0.13 | 29.77±3.11 | 2.11±0.31 | 0.80±0.12 | 37.91±4.92 | 1.05±0.11 | 0.36±0.05 | 34.28±4.62 |
| HDC | 30 | 2.10±0.25[a,b,c,d,e] | 0.32±0.06[a,b,c,d,e] | 15.33±1.01[a,b,c,d,e] | 1.64±0.21[a,b,c,d,e] | 0.38±0.06[a,b,c,d,e] | 23.51±3.08[a,b,c,d,e] | 0.76±0.11[a,b,c,d,e] | 0.16±0.03[a,b,c,d,e] | 21.05±3.06[a,b,c,d,e] |
| HDC+MEL | 30 | 2.40±0.26[a,d] | 0.61±0.09[a,d] | 25.52±2.68[a,d] | 1.89±027[a,d] | 0.67±0.10[a,d] | 35.71±3.73[a,d] | 0.96±0.14[a,d] | 0.31±0.06[a,d] | 32.29±4.34[a,d] |
| MEL. | 30 | 2.76±0.31 | 0.87±0.13 | 31.52±3.07 | 2.31±0.32 | 0.93±0.12 | 40.25±4.27 | 1.05±0.13 | 0.40±0.06 | 38.09±4.17 |

$p<0.05$ was considered statistically significant in ANOVA test. a) Significantly smaller compared to the control group. b) Significantly smaller compared to the Low Dose Caffeine + Melatonin group. c) Significantly smaller compared to the High Dose Caffeine + Melatonin group. d) Significantly smaller compared to the melatonin group. e) Significantly smaller compared to the Low Dose Caffeine group.

**Table 4.** Mean calcium values of control and experimental groups.

| | Control | Melatonin | LDC | LDC+ Melatonin | HDC | HDC + Melatonin | p |
|---|---|---|---|---|---|---|---|
| Calcium | 25,02±1,97[a] | 21,86±1,07[ab] | 22,32±2,46[ab] | 23,44±5,01[ab] | 17,78±1,51[b] | 22,23±3,2[ab] | 0,022 |

The data are expressed as mean ± standard deviation. The same letters on the same line refer to the similarities between the groups, and the different letters refer to the differences between the groups.

**Table 5.** Comparison of trabecular thickness between groups.

| Control (n=104) | Melatonin (n=117) | LDC (n=206) | LDC +Melatonin (n=181) | HDC (n=145) | HDC +Melatonin (n=227) | p |
|---|---|---|---|---|---|---|
| 4,1 (1,4-2,1)[a] | 2,9 (2,2-3,4)[b] | 1,7 (1,4-2,1)[c] | 2,4(2,0-2,9)[b] | 0,6 (0,5-0,8)[d] | 1,0 (0,7-1,4)[d] | <0.001 |

The data are expressed as median (1st quarter-3rd quarter). The same letters on the same line indicate similarity between the groups and the different letters indicate the difference between the groups.

**Table 6.** TRAP expression intensity of all groups.

| Groups (n=50) | Med+Std. Error | *p** |
|---|---|---|
| **Control** | 72.76 ± 5.93[a] | 0.001 |
| **MEL** | 73.20 ±5.20[b] | |
| **LDC** | 76.27 ±5.67[bc] | |
| **LDC+MEL** | 73.84 ±6.19[ab] | |
| **HDC** | 77.27 ±5.27[c] | |
| **HDC+MEL** | 70.98 ±6.20[a] | |

* One-Way ANOVA, p <0.05 was considered statistically significant. a. There was no statistically significant difference between Control, LDC + Melatonin, HDC + MEL groups. b. There was no statistically significant difference between Melatonin, LDC, LDC + Melatonin groups. c. There was no statistically significant difference between LDC and HDC groups.

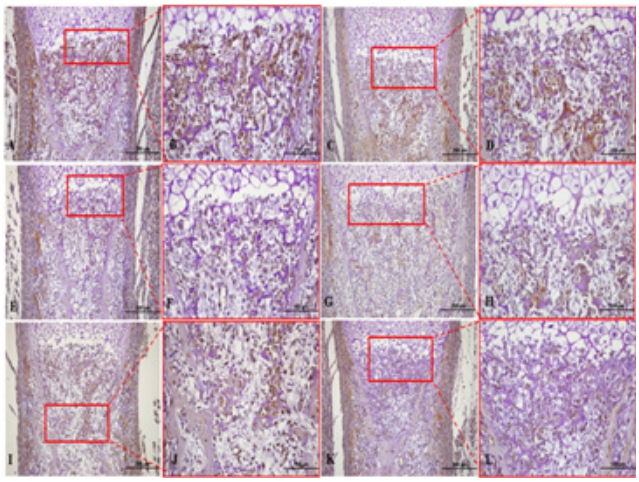

**Figure 4.** TRAP expression in the ossification area of all groups with immunohistochemical staining (→). A (20X) - B(40X): Control Group, C (20X)-D (40X): Melatonin Group, E (20X) -F (40X): LDC Group, G (20X)-H (40X): LDC+Melatonin Group, I (20X)-J (40X): HDC Group, K (20X)-L (40X): HDC+Melatonin Group.

ossification region. (Figure 4) and TRAP was 72.76 ± 5.93 in this group. The concentration of TRAP immunereactivity in the ossified region is thought to be the result of osteoblast-osteoclast balance. Because the AP immune reactivity concentration of the control group, 72.90 ± 5.73 TRAP was found to be close to the immune reactivity intensity. A significant difference in TRAP expression intensity between control group and melatonin, LDC and HDC groups were obtained (P < 0.05) (Table VI).

## Density of alkaline phosphatase

It was shown that AP expression intensity was observable in the region of the ossification (Figure 5). The control group was examined, a significant difference between the other groups were observed (P < 0.05) (Table VII). AP expression was observed in the ossification area but the expression intensity was not as high as the control group. In the LDC group, the mean expression intensity of the group was 69.38 ± 5.69. However, microscopic examination showed that AP expression was weak staining. A statistically significant difference was found between the data of LDC group and the control

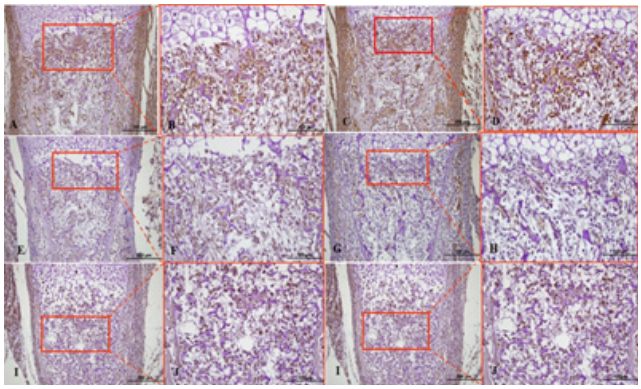

**Figure 5.** Expression of AP in the ossification region in immunochemical staining preparations of all groups (→). A (20X)-B (40X): Control group, C (20X)-D (40X): Melatonin group, E (20X)-F (40X): LDC group, G (20X)-H (40X): LDC+melatonin group, I (20X)-J (40X): HDC group, K (20X)-L (40X): HDC+melatonin group.

**Table 7.** AP expression intensity of all groups.

| Groups (n=50) | Mean+Std. Dev. | P* |
|---|---|---|
| **Control** | 72.90 ±5.73[a] | 0.001 |
| **MEL** | 73.99 ±4.44[a] | |
| **LDC** | 69.38 ±5.69[b] | |
| **LDC+MEL** | 72.69 ±6.14[a] | |
| **HDC** | 69.53 ±4.80[bc] | |
| **HDC+MEL** | 1.97 ±5.67[a] | |

* One-Way ANOVA, p <0.05 was considered statistically significant. a. There was no statistically significant difference between the control, Melatonin, LDC + Melatonin and HDC + Melatonin groups. b. There was no statistically significant difference between LDC and HDC groups. c. There is a statistically significant difference between HDC group and other groups.

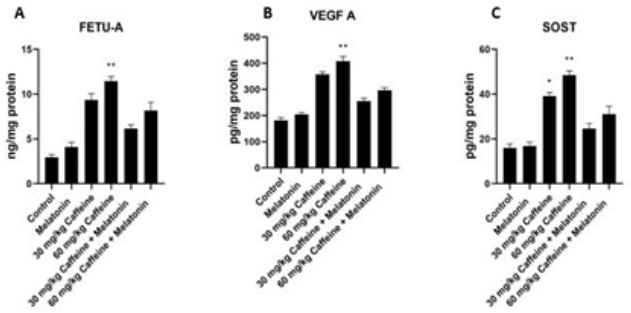


**Figure 6.** Measurements in the liver. A. FETU-A, B. VEGF-A, C. SOST. The data are expressed as mean ± standard deviation (*P < 0.05, ** P < 0.01. One-Way ANOVA, post hoc Tukey test).

group (P < 0.05). Expression intensity was calculated as 72.69 ± 6.14 in HDC + melatonin group, and no significant difference was detected between the control group and this group. Also, melatonin was found to have a positive effect on expression. In the HDC group, the data for ImageJ program used for AP density was calculated as 69.53 ± 4.80. Statistical similarity was found between this group and only LDC group. A statistically significant difference between control, melatonin, LDC+ melatonin and HDC+melatonin groups were obtained (P < 0.05).

**Biochemical findings**

In the study, fetuin-A level was highest in animals in the group given 60 mg / kg caffeine, and it was determined that this value decreased in groups given melatonin in addition to caffeine (Figure 6-A). According to the results obtained in our study (Figure 6-B), highest levels of VEGF-A were found in the group treated with high dose of caffeine (60 mg/kg). VEGF-A levels were found to be close to the control group in melatonin addition group. According to the results of our study, SOST value had the lowest value in the control and melatonin group, while it showed the highest value in the 30 mg/kg caffeine and 60 mg/kg caffeine groups (Figure 6-C).

It was performed with liver, bone and placenta samples, and oxidative stress parameters measurement results were obtained consistent with previous the data. Liver, TAS, TOS, GSH, TBARS, GSSG, SOD and intracellular calcium levels were found to be the highest and significant in the group containing 60 mg/kg caffeine and the lowest in the control group. It might be concluded that melatonin is a good source of antioxidants. Inflammatory markers indicate the condition of the damaged cell and increased inflammatory levels (IL-1ß, IL-6, TNF-α) provided clear evidence that high doses of caffeine caused damage to liver cells. However, expectedly, melatonin exerted repressive effects on inflammatory levels, while the best effect was seen in the group containing 30 mg/kg caffeine +melatonin. Considering vitamin D levels, it was found that this level decreased in the group receiving high doses of caffeine in the liver and bone and increased in the groups given melatonin in addition to caffeine. Caffeine effectively inhibited adipogenic differentiation by downregulation of adipogenesis-related genes and cytokines in bone and placenta. In our results, inflammatory markers showed the highest value in 60 mg/kg caffeine groups (Figure 7-9).

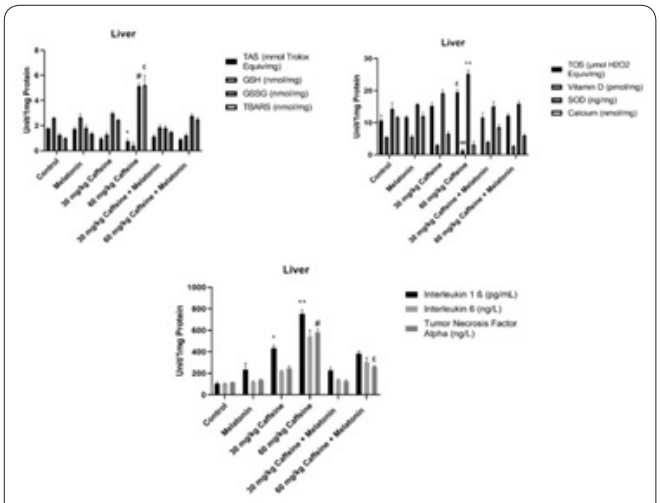


**Figure 7.** Measurements of biochemical markers in the liver. (*P < 0.05,**P < 0.01 #P < 0.001, ƐP < 0.005 vs. caffeine group. One-Way ANOVA, post hoc Tukey test).

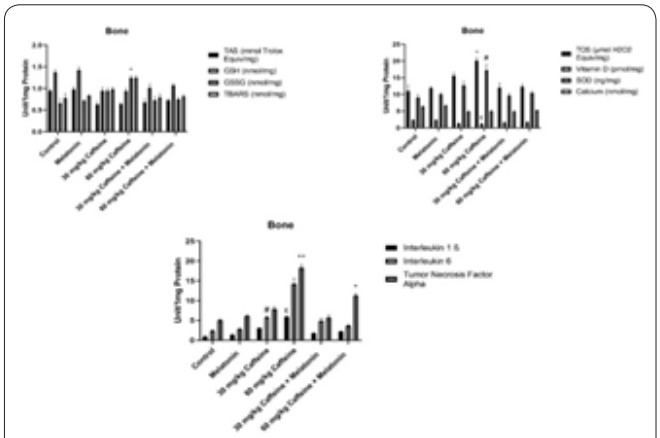


**Figure 8.** Measurements of biochemical markers in bone tissue. (*P < 0.05, **P < 0.01 #P < 0.001, ƐP < 0.005 vs. caffeine group. One-Way ANOVA, post hoc Tukey test).

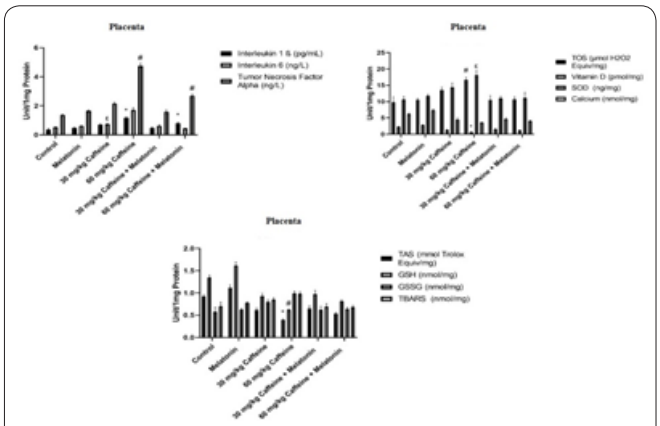


**Figure 9.** Results of biochemical markers in placenta. (*P < 0.05, **P < 0.01, #P < 0.001, ƐP < 0.005 vs. caffeine group. One-Way ANOVA, post hoc Tukey test).

**Findings of placental gene expression of Agtr2**

The mRNA levels of *Agtr2* gene expressed in placental tissues of control rats and in placental tissues of rats exposed to HDC, LDC, MEL, HDC+MEL, LDC+MEL were analyzed by Real-time PCR. Changes in mRNA levels of *Agtr2* gene expressed in placental tissues of each application group rats were determined according to the mRNA levels of *Agtr2* gene expressed in placental tissues of the control rats. *Gapdh* is refer-

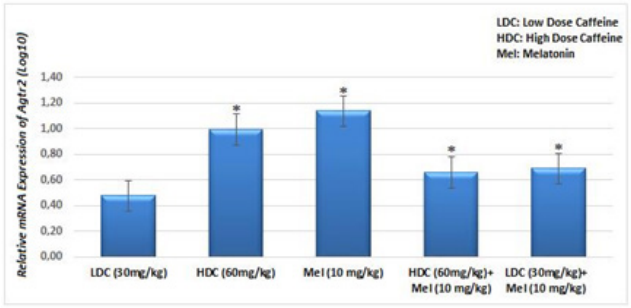


**Figure 10.** Relative mRNA expression of Agtr2 in rat placentas. HDC+melatonin and LDC+melatonin. *P < 0.001.

ence gene for normalization. The gene expressions of *Agtr2* were significantly upregulated in the placentas exposed to HDC, MEL, HDC+MEL and LDC+MEL (P<0,001). No significant differences were observed in LDC group samples (P>0,05). The upregulation of *Agtr2* gene in placental tissues of rats were given as fold regulation levels. The fold regulation values were, 2,99 for LDC (P>0,05), 9.89 for HDC, 13.64 for MEL, 4.55 for HDC+MEL and 4.89 for LDC+MEL (P<0,001) (Figure 10). In order to calculate relative fold changes in mRNA levels of target gene between application and control group rats, the amplification efficiency value and mean Ct (Treshold cycle) values of each gene were entered into the REST 2009 software program.

## Discussion

When a living organism consumes caffeine, it increases the excretion of calcium in the urine, and also has the effect of reducing total calcium, osteocalcin and alkaline phosphatase levels in plasma, inhibiting the apoptosis induction in osteoblasts. The uncontrolled pathological process is manifested by decreased bone mineral density and leads to the development of osteoporosis with increased risk of bone fracture in the future (7). The half-life of caffeine is longer in pregnant women, however, maternal half-life returns to normal within the first week after birth (30).

Given that caffeine passes through the placenta barrier, it might disrupt the development of various tissues, including bone tissue (31, 32). One study reported that the weight of newborns in caffeine-treated groups was lower than in the control group (33). Caffeine during pregnancy could disturb development. In another study, it was reported that the bone density of tibia, fibula and humerus bones of newborns decreased as 100 mg/kg caffeine was given to pregnant rats (34). In a study conducted in 2019, 68 ± 51 mg / day caffeine was given to pregnant women and it was stated that there was no relationship between the weight, length and head and chest circumference measurements of newborns after caffeine intake (35).

Learning and behavioral effects of long-term prenatal caffeine administration were investigated. As a result, long-term caffeine intake of pregnant rats during pregnancy has been found to change the locomotor activity of the offspring (36). For therapeutic purposes, the protective effects of melatonin and nicotine on the bones were investigated and compared to the control group (43.71 ± 2.33), 6 mg/kg nicotine group (31.42 ± 10.33) showed a lower percentage of ossification in bones of both extremity. In the study, it was stated that the results in the melatonin group were similar to those in the control group (18). In the 2019 study, it was reported that dual skeletal staining method is one of the methods used to determine bone cartilage ratio (24). The effects of fetal bone development and immunohistochemistry were investigated and dual skeletal staining method was used in the study. The highest ossification rates in bones of upper extremity were reported to be lower than in the control group. In TRAP, differences were detected between the control group and the experimental group, between AP expression of the femur with immunohistochemical staining, and also reported an increase in the rates in groups receiving melatonin (37). Fetuin-A is a serum glycoprotein weighing about 60 kDa. Similar to serum albumin, Fetuin-A is also pre-dominant in liver origin. Non-liver Fetuin-A might be synthesized in the kidney, choroid plexus and organs during fetal development. Serum concentration is between 0.4 and 1.0 g/L. Fetuin-A is defined as a powerful inhibitor of hydroxyapatite formation in a saturated solution containing calcium, phosphate (38-40). VEGF-A exhibits significant activity with vascular and endothelial cells through its interactions with VEGFR1 and R2 receptors, which are prominent on the endothelial cell membrane. However, it has effects on several other cell types In vitro, VEGF-A is also a vasodilator, increases microvascular permeability, is called vascular permeability factor. Sclerostin is a glycoprotein with a C-terminal cysteine node-like (CTCK) domain that resembles a bone morphogenetic protein (BMP) antagonist in the DAN (gene selected from anorative differential screening in neuroblastoma). Sclerostin is produced primarily by the osteocyte but is also expressed in other tissues and has anti-anabolic effects on bone formation (41). Guanabens et al. found that SOST expression in the liver increased 2.7-fold in primary biliary cirrhosis (PBC). In PBC patients, it has been suggested that higher serum sclerostin is inversely proportional to markers of bone metabolism, and that high sclerostin contributes to low bone formation in these patients. Immunohistochemical study revealed sclerostin protein in seven of eleven PBC liver biopsies, mainly due to its location in bile duct cholangocytes (42). In our study, Fetuin-A, VEGF-A and SOST values were the highest in the group given 60 mg/kg caffeine.

Renin's angiotensin system (RAS) was initially considered as a system that regulates sodium fluid balance and hemodynamics (43). Agtr2 is an important part of the renin angiotensin signaling (RAS) pathway, which is studied for regulation of blood pressure (21). Stimulation of the renin-angiotensin system is a response that helps regulate blood pressure against increased vasodilation with pregnancy. (20). Paulis (2007) reported in their study that melatonin increased the placental antioxidant capacity and vasodilation by vascular network receptors. Suggested that melatonin alters vascular blood flow through a combination of mechanisms (44). Goyal (2010) reported that, placental insufficiency is associated with increased expression of many RAS genes. It is also reported that Agtr2 is re-expressed or upregulated after vascular damage, myocardial infarction, heart failure or wound healing, and reflects the reactivation of the fetal genetic program (45). In our study, the upregu-

lation of Atgr2 mRNA expression was detected in all study groups. The results demonstrated that significant upregulation of mRNA of Agtr2 receptor from placentas which exposed to HDC, melatonin and join effect of high dose caffeine and melatonin and join effect of LDC and melatonin. But, the effects of high dose caffeine and melatonin each alone were the most effective on the upregulation Agtr2 mRNA expression. From these findings, high dose caffeine and melatonin may give rise to pathological circumstances on the rat placentas. Even in the low dose caffeine group, there was an up regulation compared to the control, but non-significant. Menk (2015) reported that cytokine response modulation may be beneficial in inflammatory conditions thanks to AT2 receptor activation (46). Oxidation of GSH to GSSG and then a decrease in GSH / GSSG ratio are known in relation to oxidative stress. Therefore, GSH / GSSG is an indicator of cellular redox status (47). There are studies in the literature reporting that caffeine intake can decrease $Ca^{+2}$ absorption and the level of vitamin D in the blood, depending on the dose (48,49). In this study, GSH, $Ca^{+2}$ and vitamin D values increased in melatonin groups. This values decreased in the caffeine groups.

Inflammatory responses are observed after necrotic cell death. Inflammatory markers are commonly used to show inflammatory conditions such as IL-6, IL-1β and TNF-α (50). In this study, melatonin showed an anti-inflammatory effect in contrast to caffeine in all melatonin treatment groups by reducing levels of IL-6, IL-1β and TNF-α.

In this study, low calcium levels were determined by immune-histological/biochemical parameters and EDX and Agtr2 mRNA expression levels results in dose-dependent caffeine groups. Calcium levels slightly increased as melatonin was administered following caffeine administration while low calcium levels were obtained in the highest dose caffeine-treated groups. Fetuin-A, VEGF-A and SOST levels were increased in the high dose caffeine group. Angiotensin receptor expression observed in response to a caffeine-melatonin exposure result from high dose or join effect. Melatonin has been shown to be effective in reducing caffeine induced oxidative stress and bone loss in rats' fetus.

## Conflict of Interest

The authors declare that there is no conflict of interest

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## Author Contributions

S. Yılmaz, AH. Yay, ES. Arıkan designed the study. E. Akyüz, A. Tokpınar, M. Nisari, H Yılmaz collected the data. AH. Yay, B. Yalçın immunohistochemical studies and staining worked. AY. Göçmen did biochemical study. S. Yılmaz, M. Nisari, E. Unur and AH. Yay analyzed the data. All authors contributed to the data interpretation, review and revision of the manuscript.

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