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The protective effect of caffeine and melatonin on antioxidant enzymes in rat fetal lung tissues

Kafein ve melatoninin sıçan fetus akciğer dokularındaki antioksidan enzimler üzerine koruyucu etkisi

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Abstract

Aim: Teratogenic substances such as nicotine, alcohol, caffeine, and derivatives, which pregnant women may get exposed to unconsciously or use consciously can harm the mother and directly or indirectly damage embryonal and fetal tissues. Melatonin has been shown to exert direct free radical trapping and indirect antioxidant effects in different organs and tissues. In this study, we aimed to biochemically evaluate the effects of melatonin, a powerful antioxidant battling the oxidative effects of high and low doses of caffeine administered to pregnant rats in fetal lung tissues.

Methods: In our study, 35 pregnant adult female Sprague-Dawley rats were used. Pregnant rats were divided into 7 groups with 5 rats in each. Caffeine and melatonin were administered for 20 days during the pregnancy. The gestational period lasted 21 days in average. The offspring were sacrificed, and lung tissues were removed. Superoxide dismutase (SOD), glutathione (GSH), glutathione disulfide (GSSG), total oxidant status (TOS), total antioxidant status (TAS), calcium (Ca) and vitamin D (Vit D) were measured by spectrophotometric assay. The oxidative stress index (OSI) and total glutathione (GSH/GSSG) were determinants of oxidative stress and were calculated as TOS/TAS and GSH/GSSG ratios, respectively.

Results: The highest TAS value was obtained in the Melatonin group (M) group. GSH and GSH/GSSG was highest in the control group, whereas GSSG was the highest in the high-dose caffeine group (HDC) group. HDC group had the highest SOD value compared to the other groups (P<0.05).

Conclusions: According to these data, it was determined that caffeine used during pregnancy delayed the development of lung, and melatonin, which is a strong antioxidant, minimized the delay.

Keywords: Rat, Caffeine, Melatonin, Lung, Fetus

Öz

Amaç: Hamilelik sırasında bilinçli olarak kullanılan veya bilinçsizce maruz kalınan nikotin, alkol, kafein ve türevler gibi teratojenik maddelerin embriyonal ve fetal dokulara doğrudan veya dolaylı olarak zarar vermesinin yanı sıra anneye de zarar verebilir. Melatoninin farklı organ ve dokularda doğrudan serbest radikal toplayıcısı ve dolaylı antioksidan etkileri olduğu gösterilmiştir. Bu çalışmada, fetus akciğer dokularında gebe sıçanlara uygulanan yüksek ve düşük dozdaki kafeinin etkisine karşı güçlü bir antioksidan olan melatoninin değerlendirilmesi amaçlanmıştır.

Yöntemler: Çalışmamızda 35 adet yetişkin dişi Sprague-Dawley sıçan kullanıldı. Gebe sıçanlar, her birinde 5 sıçan olacak şekilde 7 gruba ayrıldı. Gebelere 20 gün boyunca invaziv işlemler uygulandı. Deney grubuna kafein, kafein yanı sıra tedavi gruplarına melatonin uygulandı. Yavrular sakrifiye edildi ve akciğer dokuları çıkarıldı. Süperoksit dismutaz (SOD), Glutatyon (GSH), Glutatyon disülfür (GSSG) toplam oksidan durumu (TOS), toplam antioksidan durumu (TAS), Kalsiyum (Ca) ve D vitamini (Vit D)) spektrofotometrik analiz ile ölçüldü. Oksidatif stres indeksi (OSI) ve Total glutation (GSH/ GSSG), oksidatif stres dokularının yararlı göstergeleridir ve sırasıyla TOS/TAS ve GSH/ GSSG oranı olarak hesaplandı.

Bulgular: M grubunda elde edilen en yüksek TAS değeri, GSH ve GSH / GSSG kontrol grubunda en yüksek, GSSG ise HDC grubunda en yüksek değere sahipti. HDC grubunda SOD diğer gruplara göre en yüksek değere sahipti (P<0,05).

Sonuçlar: Bu verilere göre, gebelikte kullanılan kafeinin akciğer gelişimini geciktirdiği ve güçlü bir antioksidan olan melatonin gecikmeyi en aza indirdiği gözlendi.

Anahtar kelimeler: Sıçan, Kefein, Melatonin, Akciğer, Fetüs

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Introduction

Caffeine, an important stimulant, has been associated with many positive and negative effects on health. Teratogenic substances such as nicotine, alcohol, caffeine, and derivatives that pregnant women use consciously or get exposed to unconsciously can harm the mother and directly or indirectly damage embryonal and fetal tissues. The immature detoxification enzymes of the fetus and the failure of fetal renal excretion of these substances, which can easily pass through the placenta, are the two main reasons why the embryo is affected by these teratogenic substances [1].

Caffeine is naturally found in the seeds, leaves and fruits of many plants, including coffee and cocoa beans, tea leaves and cola seeds [2,3].

Caffeine [1,3,7-trimethylxanthine] is an alkaloid naturally found in coffee beans [4]. The caffeine content of coffee varies according to the preparation method and type. After caffeine is orally ingested, it is rapidly absorbed from the stomach and the small intestine, reaching its peak level in the blood. As a result of its metabolism in the liver, many metabolites such as paraxanthine, theobromine and theophylline are released [5-7]. Since there is no barrier for caffeine, it readily diffuses into the brain, testes, and other tissues throughout the body along with the placenta into the fetus [5]. It has also been reported to adversely affect bone health and increase the risk of post-menopausal osteoporosis, as high-dose caffeine increases the excretion of calcium and magnesium from the urine, leading to the development of osteoporosis [8].

Melatonin (5-methoxy-N-acetyl-tryptamine) is synthesized and secreted mainly from the pineal gland, ovary, lens and bone marrow cells and bile and gastrointestinal system in mammals [9]. Melatonin is synthesized from tryptophan in the pineal gland, secreted in the dark and binds to plasma proteins. It is metabolized in the liver and has been shown to have direct free radical trapping and indirect antioxidant effects in different organs and tissues [10-12].

To the best of our knowledge, there are no studies on the protective effect of melatonin against the oxidative stress caused by caffeine on fetal lung tissue in the literature. In this study, we aimed to biochemically evaluate the effects of melatonin, a powerful antioxidant battling the oxidative effects of high and low doses of caffeine administered to pregnant rats in fetal lung tissues.

Materials and methods

Animal selection and breeding

Sprague-Dawley female rats weighing between 200-250 g, obtained from Experimental Animals and Clinical Research Center of Erciyes University (DEKAM), were used in this study. Ethics approval was obtained from the Local Ethics Committee of Animal Experiments of Erciyes University on 13/09/2017 (no: 17/086), and rules set forth by this committee were adhered to. One male and two female rats were put into each cage at 17.00 pm on the day of mating. The following morning at 07.00, vaginal smear was obtained from the female rats. Those in which sperm was observed were considered 0.5 day pregnant. During the study, rats were kept in DEKAM at a constant temperature of 22ºC with 12 hours of light/dark cycles. All rats were fed pellets containing 21% crude protein and tap water ad-libitum.

Preparation of injections

Caffeine and Melatonin powder (CAS Number 73-31-4) were obtained from Sigma Aldrich. Drinking water was used as the solvent to adjust the amount of caffeine to be administered to the rats. Hanks Balanced Salt Solution (Hanks) was used for dissolving powdered melatonin. Both substances were prepared daily, and no stock solution was made.

Formation of experimental groups

Pregnant rats were randomly divided into 7 groups with 5 rats in each group, as listed below:

1.Control group (C): The rats were administered physiological saline solution (SF) (1 ml/kg/day) intraperitoneally (i.p.) at 17.00 for 20 consecutive gestational days.

2.Sham group (S): The rats were administered 0.1 ml Hanks i.p. at 17.00 for 20 consecutive gestational days.

3.Melatonin group (M): 10 mg/kg melatonin was administered i.p. for 20 consecutive gestational days.

4.Low-dose caffeine group (LDC): The rats were administered 30 mg/kg caffeine by gavage for 20 consecutive gestational days.

5.High-dose caffeine group (HDC): The rats were administered 60 mg/kg caffeine by gavage for 20 consecutive gestational days.

6.Low-dose caffeine+Melatonin group (LDC+M): The rats were administered 30 mg/kg caffeine by gavage and 10 mg/kg melatonin i.p for 20 consecutive gestational days.

7.High dose caffeine+Melatonin group (HDC+M): The rats were administered 60 mg/kg caffeine by gavage and 10 mg/kg melatonin i.p for 20 consecutive gestational days.

Obtaining fetuses from rats

Pregnant rats were anesthetized with ketamine (75 mg/kg) + xylazine (10 mg/kg) on the 20th day of pregnancy. The abdominal skins of the rats were cleaned with 70% alcohol and the anterior abdominal walls were removed by a 'V' shaped incision extending from the pubis to the rib cage. The uterus and fetuses were removed together with their placenta and dissected individually. The lung tissues of the fetuses were stored at -80ºC until biochemical parameters were evaluated.

Biochemical methods

Tissue preparation and protein quantification

Lung tissue used for analysis was placed into microcentrifuge tubes, washed 3 times with 1mL 100 mM PBS and aspirated. Stainless steel beads (1.6 mm blend) were used for homogenization with 100 mM PBS. After homogenization, homogenates were centrifuged at 10.000 RPM for 30 minutes at +4°C. Supernatants were used as protein samples. BioRAD DC Protein Assay Kit (BioRAD, 5000116) was used to assay the protein content.

Oxidative stress parameters

The parameters of oxidative stress (TAS, TOS and SOD levels) were determined with the spectrophotometric method. The TAS and TOS tissue levels were calculated based on Erel [13]. To compute antioxidant levels in samples, the antioxidant of a known amount (1.65 mmol/l) was used. TAS level is presented as mmolTrolox equivalent/l (mmolTrolox equiv./l). For determination of the TOS level, the assay was calibrated

with a standard hydrogen peroxide solution (39.16 µmol/l). The results are presented as µmol H_2O_2 equivalent/l (µmol H_2O_2 equiv./l). Other oxidative stress parameters such as GSH (Cat. No: E-BC-K030-M), GSSG (Cat. No: 703002, Cayman, USA) and SOD (Cat. E-BC-K020, Elabscience, USA) were calculated with a spectrophotometer (Multiskan, Thermo Fisher) according to the manufacturer's instructions. Commercial ELISA kits (EIA 5396 DRG, Germany) were utilized for Vitamin D analysis.

Ca^{+2} assay

Total Ca^{+2} was evaluated with the calcium colorimetric assay kit (ab102505; Abcam) according to the manufacturer's datasheet. 25 µL of standard solution and 25 µL of supernatant extracted from tissue (diluted 1:10) were mixed with 45 µL of chromogenic reagent and 30 µL assay buffer. The mixture was incubated at room temperature for 15 minutes in the dark. The signal was screened at 575 nm (ThermoVarioscan). The concentration of calcium in the samples was calculated according to the technique described by Sen et al. in 2018.

Statistical analysis

SPSS version 23.0 (IBM Co., NY, USA) was used for all analyses. Data were presented as mean (standard deviation). Differences among the groups were analyzed with one-way analysis of variance (ANOVA) and Post hoc Tukey's tests for continuous variables and parametric data, respectively. The Kruskal-Wallis and the Post hoc Dunn's tests were used for nonparametric data. *P*-value <0.05 was considered statistically significant.

Results

Assessment of oxidative stress parameters

The highest TAS and TOS values were obtained in the M and control groups, respectively. Melatonin administration increased antioxidant status significantly in a dose dependent manner when compared with the caffeine group. Dose-dependent melatonin exerted a more powerful antioxidant effect when used without caffeine. TOS values showed that the highest oxidant effect was observed in the HDC group. Control group had the lowest value. Oxidative stress index was calculated by the ratio of TOS/TAS. OSI was considerably higher in the HDC group and lower in the melatonin-administrated groups (Table 1).

GSH and GSH/GSSG were the highest in the control group, whereas GSSG was highest in the HDC group. Melatonin increased GSH and GSH/GSSG values while decreasing GSSG (Table 1).

SOD was highest in the SDC group. The intracellular calcium and vitamin D values of the M group were higher than all the other groups. According to these results, melatonin markedly decreased oxidative stress by stimulating the antioxidant system.

Discussion

In case of a disturbance in embryological and fetal lung development, congenital lung anomalies and malformations may be encountered [14-16]. It is emphasized by many researchers that cytotoxic agents adversely affect the development of organs during embryo-fetal development [17-22]. These studies evaluate the physiological, biochemical, and pathological changes related to anatomical structure and function of the lungs in the prenatal and postnatal periods [23-25]. In the literature, it is emphasized that information on the fetal development of lungs is of great significance in terms of early diagnosis and treatment of lung anomalies [14-16].

Lipids are the most sensitive to the toxic effects of reactive oxygen products in biological structures. Polyunsaturated fatty acids in the cell membrane readily react with free oxygen radicals and lipid peroxidation occurs. As a result of the damage of these unsaturated fatty acids, membrane fluidity decreases. MDA is the end-product of lipid peroxidation that develops as a result of oxidative stress and is an important indicator of lipid damage [26].

There are some studies about melatonin for its decreasing effect on lipid peroxidation. However, antioxidant enzyme activity shows various results with the use of melatonin [26,27]. Maarman et al. [26] found a decrease in the activity of plasma and lipoperoxidation of SOD and CAT enzymes in animals with Pulmonary Hypertension treated with Melatonin. Taşlıdere et al. [27] associated melatonin use with increased activity of CAT and Glutathione (GSH) enzymes in rat lung tissue after decreased lipoperoxidation and cirrhosis induced by carbon tetrachloride (CCl4). Borges et al. [23] showed that the use of melatonin reduced muscle lipoperoxidation induced by vigorous exercise and increased SOD activity, but there was no notable change in CAT and Glutathione Peroxidase (GPx) activity. Similarly, Rosa et al. [24] showed that melatonin reduced lipoperoxidation and increased SOD activity in the livers of animals with experimental Sleep Apnea models.

Oxidative stress is reportedly important in the pathogenesis of neonatal diseases. Giuffre et al. [29] reported that glutathione, lipid hydroperoxides and heat shock protein chaperone 60 may have functional and diagnostic importance for oxidative stress in newborns.

In the study of Taban et al. [30] the effects of lipid peroxidation product MDA, oxidative stress markers TAS, TOS and OSI, and the effect of bosentan were evaluated in rats by creating a hyperoxic lung injury model.

Table 1: The biochemical parameters of fetus lung

Value	Control	Sham group	M group	LDC group	HDC group	LDC+M group	HDC+M group	*P*-value
TAS	1.25(0.05)	1.12(0.06)[a]	1.36(0.07)[b]	0.80(0.06)[c]	0.59(0.03)[d]	0.88(0.08)[e]	0.74(0.03)[f]	<0.001
TOS	5.06(0.82)	6.09(0.49)[a]	5.46(0.22)[b]	7.03(0.44)[c]	8.81(0.42)[d]	5.45(0.66)[e]	5.57(0.36)[f]	<0.001
OSI	0.40(0.05)	0.54(0.07)[a]	0.40(0.02)[b]	0.88(0.10)[c]	1.47(0.13)[d]	0.62(0.10)[e]	0.74(0.04)[f]	<0.001
GSH	2.81(0.11)	2.56(0.14)[a]	3.13(0.16)[b]	1.86(0.13)[c]	1.38(0.06)[d]	2.01(0.18)[e]	1.70(0.07)[f]	<0.001
GSSG	0.39(0.06)	0.48(0.03)[a]	0.42(0.01)[b]	0.55(0.03)[c]	0.70(0.03)[d]	0.43(0.05)[e]	0.44(0.02)[f]	<0.001
GSH/GSSG	281.12(11.82)	61.38(2.91)[a]	313.96(16.20)[b]	186.50(13.40)[c]	138.53(6.58)[d]	201.15(18.99)[e]	170.42(7.13)[f]	<0.001
TBARS	0.68(0.11)	0.82(0.06)[a]	0.73(0.02)[b]	0.94(0.05)[c]	1.19(0.05)[d]	0.73(0.08)[e]	0.75(0.04)[f]	<0.001
SOD	8.56(1.38)	10.29(0.84)[a]	9.23(0.37)[b]	11.88(0.75)[c]	14.91(0.71)[d]	9.21(1.11)[e]	9.41(0.60)[f]	<0.001
Ca	8.23(0.35)	7.40(0.43)[a]	8.99(0.47)[b]	5.35(0.40)[c]	4.05(0.21)[d]	5.85(0.57)[e]	4.96(0.21)[f]	<0.001
VİT D	4.90(0.20)	4.39(0.26)[a]	5.35(0.28)[b]	3.13(0.24)[c]	2.32(0.13)[d]	3.46(0.34)[e]	2.91(0.12)[f]	<0.001

a, b, c, d, e, and f represent comparisons with the control group. a: Sham group vs Control group b: M group vs Control group c: LDC group vs Control group d: HDC group vs Control group e: LDC+M group vs Control group f: HDC+M group vs Control group

TAS levels were found to be significantly lower in the group with the highest room air + bosentan group and in groups with hyperoxic lung injury. TOS level was higher in the room air + bosentan group than the late bosentan group. There was no significant difference between the other groups. There were no significant differences between the groups in terms of OSI and MDA levels. With these results, they reported that bosentan reduced IL-6 and TNF-α in BPD-induced rats by a hyperoxic lung injury model and provided histopathological improvement with its anti-inflammatory effect.

Durceylan et al. [31] investigated the protective effects of melatonin with proven antioxidant activity on lung injury after TAV in rats. The biochemical analysis of lung tissue samples taken during and at the end of the experiment revealed that SOD levels were significantly higher in the melatonin-treated group after TAV and there was a significant decrease in MDA. It was concluded that preoperative melatonin use may reduce postoperative lung injury in patients with high tissue oxidation and inflammatory potentials.

Recent studies have emphasized the antioxidant properties of melatonin, and melatonin hormone has been shown to have a protective effect against lung damage due to oxidative stress [32-34]. Arslan et al. [32] reported that melatonin hormone prevents bleomycin-induced pulmonary fibrosis in the rat lung. Topal et al. [33] showed that melatonin administration has protective effects against oxidative stress in rat lung caused by hyperbaric oxygen exposure.

Harmless et al. [34] reported that melatonin prevented damage to lung tissue due to formaldehyde (FA) exposure. Showing that MDA, XO and SOD enzyme levels decreased and reached that of the control group in melatonin-treated rats with FA exposure, they explained that the increase in SOD enzyme activity due to FA exposure decreased with melatonin treatment. Crossley et al. [35] investigated the effects of acute caffeine administration on renal and pulmonary functions in preterm lambs. They found that caffeine did not affect blood flow to the pulmonary artery, patent ductus arteriosus or other renal, respiratory or cardiovascular parameters examined. However, they reported that caffeine increased neonatal heart rate and urine output.

In the current study, HDC group had the lowest GSH activity. After caffeine treatment following melatonin administration GSH increased in HDC+M group.

TAS, TOS, SOD and Vitamin D are crucial biomarkers to evaluate oxidative damage. In this study we detected that melatonin decreased TAS while increasing TOS levels in the HDC group. The highest value of SOD was observed in HDC group and the lowest value was recorded in the control group. Caffeine significantly increased SOD by stimulating the reactive oxygen system, and melatonin slightly decreased the effect of caffeine. We conclude that melatonin may exert its effect by reducing the production of superoxide radicals and suggest that melatonin is effective in reducing oxidative stress and impaired development of the lung induced by caffeine in rats.

Limitations

Melatonin dosage was not investigated in this study, and nor were its effect on different tissues, particularly liver and kidneys. Further studies on these aspects will illuminate the antioxidant properties of melatonin.

Conclusion

Melatonin therapy after caffeine administration markedly improved biochemical findings and prohibited oxidative stress and inflammation. According to these data we propose that melatonin at the dose of 10mg/kg may be used as a potential therapeutic agent to prevent the impaired development of the lung by caffeine. We believe that our results will be beneficial in model studies conducted on melatonin and caffeine.

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