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Antioxidant effects of piperine on steroid-induced hepatotoxicity

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Abstract. – OBJECTIVE: Glucocorticoids (GCs) are steroids that play an essential role in physiological processes and are valuable therapeutic agents against various diseases. The aim of our study was to evaluate the antioxidant effects of piperine (PIP) on steroid-induced oxidative stress in liver tissue.

MATERIALS AND METHODS: We used 36 fertilized specific-pathogen-free (SPF) chicken eggs that were divided into the following 6 groups: group 1 (n=6), phosphate buffered saline (PBS) (pH 7.4 saline solution [0.9%] isotonic); group 2 (n=6), 0.50 μmol hydrocortisone succinate sodium (HC); group 3 (n=6), 0.50 μmol HC and 100 mg/kg piperine (PIP); group 4 (n=6), 0.50 μmol HC and 50 mg/kg PIP; group 5 (n=6), 0.50 μmol HC and 25 mg/kg PIP; and group 6 (n=6), 0.50 μmol HC and 10 mg/kg PIP. Chick embryos were removed from the eggs and the livers dissected from the embryos. The total antioxidant status (TAS), total oxidant status (TOS), reduced glutathione (GSH), and lipid peroxidation (malondialdehyde [MDA]) levels were measured.

RESULTS: The highest levels of GSH and TAS in the liver tissues were observed in group 3, with a significant difference from those in group 2 (p <0.001 and p =0.006, respectively). The lowest levels of MDA and TOS in the liver tissues were observed in group 3, with a significant difference from those in group 2 (p <0.001 and p =0.021, respectively).

CONCLUSIONS: The antioxidant and hepatoprotective properties of PIP were observed only at high doses.

Key Words:
Chick embryo, Liver, Oxidative stress, Piperine, Steroid.

Introduction

The liver is a vital organ that detoxifies substances that are harmful to the body. It regulates many metabolic functions and contributes to the maintenance of homeostasis[1]. Liver cells that are overexposed to drugs and toxins must expend a great amount of energy during the detoxification process. Reactive oxygen species (ROS) are known to formed due to this increase, and these ROS can damage cells such as proteins and lipids and molecules such as nucleic acids and membrane lipids, which disrupt cell function and can result in cell death if not eliminated. This oxidative stress is believed to contribute to the onset and progression of many different diseases, such as hepatic inflammation, hypercholesterolemia, diabetes, and hepatic cirrhosis[1-4]. Corticosteroids are used in the treatment of many liver diseases with beneficial therapeutic efficacy; however, they are known to cause serious health problems[5].

Glucocorticoids (GCs) are compounds that play a physiologically important role and are widely used in the treatment of various diseases[6,7]. They are essential in the body's response to oxidative stress and participate in the recovery of homeostasis by regulating the immune system, metabolism, and body fluids. Synthetic GCs, such as dexamethasone (DEX) and hydrocortisone, have been widely used to treat various hematological malignancies and as anti-inflammatory and immunosuppressive agents[6,8,9].

Increased ROS destruction plays a role in the pathogenesis of various diseases[8]. GCs have also been reported to contribute to the growth of products such as superoxide, hydrogen peroxide, and hydroxyl radicals, also known as ROS[8-10]. Studies have reported that exogenous high GC administration is associated with deoxyribonucleic acid (DNA) damage in various tissues (e.g., liver and heart)[7].

Black pepper (Piper negrum) is widely used in India and surrounding regions as a seasoning; however, this naturally occurring alkaloid has also

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been used for centuries to treat diseases, such as migraines, fevers, and flu, in several Asian countries, especially in India and China[11,12]. Piperine (PIP; 1-piperoyl piperidine) is a piperidine derivative present in the upper layer of pepper fruits (Piperis nigri)[13]. PIP increases the production of gastric juices and promotes proper digestion, stimulates the secretion of pancreatic and intestinal juices, increases appetite, and has diuretic and cleansing properties. PIP also facilitates the absorption of group B vitamins, beta-carotene, selenium, coenzyme Q10, and resveratrol[14].

The biological properties of PIP have shown that it has antioxidant, anti-inflammatory, and antiulcer effects, and that it also has the potential to modulate certain immune responses and exhibits hepatoprotective activity[15]. In addition, this compound promotes the absorption of certain drugs, thereby decreasing their metabolism and cholesterol levels in the blood[12,16-18].

In this study, it was aimed to apply PIP treatment for the toxic effects of GC on the liver. Since the antioxidant and liver protective efficacy of PIP is known, a study plan was developed to evaluate the effects of PIP against liver damage caused by GC. The aim of this study was to investigate the antioxidant activity of PIP against steroid-induced liver oxidants.

Materials and Methods

PIP and hydrocortisone succinate sodium (HC) were provided by Sigma-Aldrich Corp. (St. Louis, MO, USA). Thirty-six fertilized specific-pathogen-free (SPF) chicken eggs were used. Each egg weighed from 53 to 62 g (mean, 58 g). SPF eggs were obtained from Izmir Bornova Veterinary Control Institute (Bornova, Izmir, Turkey). Permission for the study was obtained from the Local Ethics Committee of Afyon Kocatepe University Animal Experiments (Date: 06/20/2019, Number: 49533702/74).

The eggs were placed in a horizontal position and incubated at 37°C and relative humidity of 70% for 17 days, after which they were randomly divided into the following 6 groups (Table I): group 1 (n=6), PBS (pH 7.4 saline solution [0.9%] isotonic); group 2 (n=6), 0.50 µmol HC; group 3 (n=6), 0.50 µmol HC and 100 mg/kg PIP; group 4 (n=6), 0.50 µmol HC and 50 mg/kg PIP; group 5 (n=6), 0.50 µmol HC and 25 mg/kg PIP; and group 6 (n=6), 0.50 µmol HC and 10 mg/kg PIP.

Injection was performed on SPF eggs on the 15th day of incubation. SPF eggs were removed from the incubator and cleaned with 70% ethanol, after which the air sac in each egg was injected with HC, phosphate buffered saline (PBS), or PIP or combinations of HC and PIP, using insulin injectors (AS, inner shell membrane intact). After injection, the puncture was sealed with sterile cellophane tape, and the eggs were further incubated for 48 h in the same incubator[19,20]. SPF eggs were removed from the incubator on the 17th day to take tissue samples and terminate the experiment protocol. For the stock solution, HC was dissolved in distilled water at a concentration of 50 µmol. PIP solution was added to the fertilized chick egg 3 h after the injection of HC in groups 3, 4, 5, and 6 (Table I).

Table I. Materials and procedures.

Group	Agent	Dosage	Method of administration	Volume	Delivery frequency	Effect duration (d)
1. PBS (n = 6)	PBS	PBS (pH 7.4)	AS	0.2 mL	Once	15–17
2. HC (n = 6)	HC	0.50 µmol	AS	0.2 mL	Once	15–17
3. HC + PIP (n = 6)	HC PIP	0.50 µmol 100 mg/kg	AS	0.1 mL 0.1 mL	Once	15–17
4. HC + PIP (n = 6)	HC PIP	0.50 µmol 50 mg/kg	AS	0.1 mL 0.1 mL	Once	15–17
5. HC + PIP (n = 6)	HC PIP	0.50 µmol 25 mg/kg	AS	0.1 mL	Once	15–17
6. HC + PIP (n = 6)	HC PIP	0.50 µmol 10 mg/kg	AS	0.1 mL 0.1 mL	Once	15–17

Preparation of Liver Tissue Samples

Samples of liver tissue were carefully dissected out of the embryos, after which 0.1- to 0.2 g samples were weighed out. Then, 1-2 mL 0.1 M phosphate buffer (pH 7.4) was added to reach a total volume of 10 times the tissue weight, immediately after which the solutions were homogenized in an ice-containing vessel using Ultra Turrax (IKA Works, Wilmington, North Carolina, NC, USA) for 1 min at 24000 rpm. The resulting homogenate was then sonicated for 1 min at 20000 cycles/s using the Hielscher (Hielscher Ultrasonics, Teltow, Germany) sonicator for better homogenization. The prepared liver tissue homogenates were immediately centrifuged at 10000 g for 15 min, and the supernatant layer was transferred into a separate tube. Supernatant samples were stored at -80°C until needed for assessing the biochemical parameters.

Glutathione Levels

Reduced glutathione (GSH) levels were determined using a glutathione assay kit (Chromsystems Diagnostics, Munich, Germany) with the Thermo Scientific Ultimate 3000 high-performance liquid chromatography (HPLC) device and a fluorescence detector (Ex: 385 nm; Em: 515 nm). GSH levels were expressed as µmol/g wet tissue.

Total Oxidants and Antioxidants

The total antioxidant status (TAS) and total oxidant status (TOS) in liver tissues were evaluated using the colorimetric method with a commercial kit (Mega Tıp, Gaziantep, Turkey) according to the manufacturer's protocols. The ChemWell 2910 microanalyzer was used for the absorbance assays (Awareness Technology Inc., Palm City, Florida, FL, USA). The results from the tissue analyses are expressed as follows: TAS, TOS; mmol Trolox, µmol H_2O_2 equiv/L, equiv/L, µmol Trolox equiv/wet tissue, and µmol H_2O_2 equiv/g wet tissue.

Malondialdehyde Levels

The levels of malondialdehyde (MDA) in the tissues were determined using an enzyme-linked immunosorbent assay kit (ELISA; Cayman Chemical, Ann Arbor, Michigan, MI, USA) according to the manufacturer's protocols. The ChemWell 2910 microanalyzer was used to conduct the absorbance assays (Awareness Technology Inc., Palm City, Florida, USA). The MDA levels in the wet tissue are expressed in µmol/g.

Statistical Analysis

Statistical Package for the Social Sciences (SPSS 18.0, SPSS Inc., Chicago, IL, USA) software program was used for statistical analysis of the data. One-Way ANOVA test was used for comparisons between groups. Tukey's HSD (honestly significant difference) test was used to determine which groups were different. *p*- values which are smaller than 0.05 were considered statistically significant. Mean values are presented as Mean±Standard Deviation (m±sd).

Results

As shown in Figure 1, the GSH levels in the liver tissues from the chick embryos were significantly lower in the HC groups than in group 1 (Figure 1a; p =0.02), significantly higher in groups 3 and 4 than in group 2 (Figure 1b and 1c; p =0.000 and p =0.006, respectively), and significantly lower in group 6 than in group 3 (Figure 1d; p =0.006).

The TAS levels in the liver tissues, an additional antioxidant marker, are shown in Figure 2. These levels were significantly higher in groups 3 and 4 than in group 2 (Figure 2a, Figure 2b; p =0.006 and p =0.006, respectively) and were significantly lower in group 6 than in group 3 (Figure 2c, Figure 2d; p =0.021 and p =0.044, respectively).

TOS and MDA are liver tissue oxidant destruction markers. The levels of these markers are shown in Figure 3 and Figure 4, respectively. The TOS levels were significantly higher in group

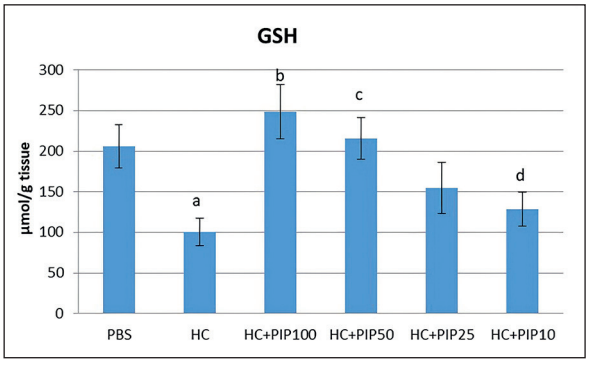

Figure 1. Reduced glutathione (GSH) levels in the chick embryo liver tissue 2 d after the hydrocortisone succinate sodium (HC) and piperine (PIP) treatment, on day 17. (a) Compared with group 1 (p =0.020). (b) Compared with group 2 (p =0.000). (c) Compared with group 3 (p =0.006). (d) Compared with group 4 (p =0.006).

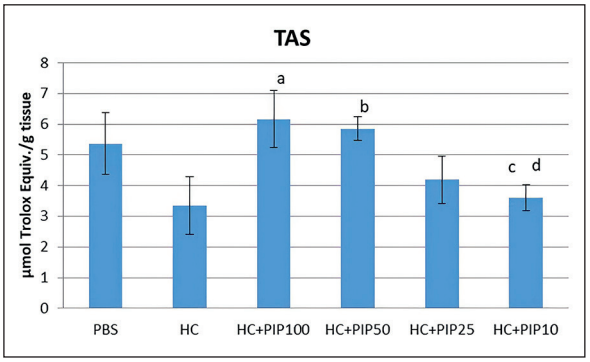

Figure 2. Total antioxidant status (TAS) levels in the chick embryo liver 2 d after the hydrocortisone succinate sodium (HC) and piperine (PIP) treatment, on day 17. (a) Compared with group 1 (p =0.006). (b) Compared with group 2 (p =0.013). (c) Compared with group 3 (p =0.021). (d) Compared with group 4 (p =0.044).

2 than in group 1 (Figure 3a; p =0.022), significantly lower in groups 3 and 4 than in group 2 (Figure 3b, Figure 3c; p =0.000 and p =0.004, respectively), and significantly higher in group 6 than in group 3 (Figure 3d; p =0.006). A significant difference was found in MDA levels in the liver tissue between only groups 2 and 3 (Figure 4, p =0.021).

Discussion

GCs are widely used for treating various hematological malignancies, particularly acute or chronic lymphoblastic leukemia and multiple myeloma. The detrimental effects of a chronically

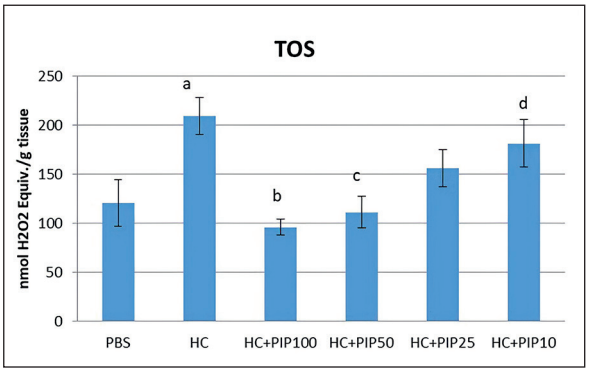

Figure 3. Total oxidant status (TOS) levels in the chick embryo liver 2 d after the hydrocortisone succinate sodium (HC) and piperine (PIP) treatment, on day 17. (a) Compared with group 1 (p =0.022). (b) Compared with group 2 (p =0.000). (c) Compared with group 3 (p =0.004). (d) Compared with group 4 (p =0.006).

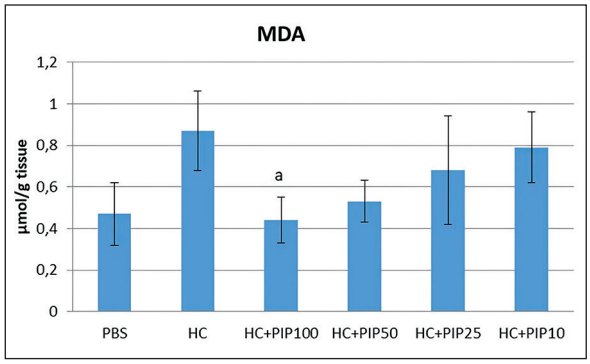

Figure 4. Malondialdehyde (MDA) levels in the chick embryo liver 2 d after the hydrocortisone succinate sodium (HC) and piperine (PIP) treatment, on day 17. (a) Compared with group 2 (p =0.021).

elevated GC level from prolonged stress or exogenous hormone administration are most likely linked with an increase in the formation of ROS or reactive nitrogen species. The efficacy of this approach depends on the ability of the GCs to produce ROS, resulting in oxidative stress that causes cellular damage. These findings suggest that the effect of GCs on the transcription of mitochondrial DNA stimulates excess ROS production in the mitochondrial electron transport chain[10].

GCs are known to exert harmful effects both *in vivo* and *in vitro*. These include inducing oxidative damage to various macromolecules, such as lipids, proteins, and DNA[10,21].

Some studies[22,23] have linked fetal exposure to high GC levels with fetal and adult neurogenesis and behavioral changes have reported that prenatal exposure to synthetic GCs may cause life-long behavioral and emotional problems in children, including attention deficit hyperactivity disorder (ADHD), which may increase the risk of subsequent development. The cellular damage caused by excessive oxidative stress also plays an important role in the onset of various diseases[22,23].

Black pepper contains various alkaloids, volatile oils, carbohydrates, starches, and proteins. Some studies[11,12,17,24] on the biological properties of PIP have shown that it has antioxidant, anti-inflammatory, neuroprotective, anxiolytic, cognitive-enhancing, and antimicrobial effects; it also has the potential to modulate some immune responses. PIP has been shown to reduce the level of thiobarbituric acid reagents, such as catalase (CAT), GSH, glutathione peroxidase (GPx), and superoxide dismutase (SOD). It may

also improve the activity of biotransformation enzymes in the liver in a dose-dependent manner[11,12,17,24].

In addition to its varied pharmacological activities, such as antifungal, anticancer, hepatoprotective, and antioxidative effects, PIP has been reported to increase the bioavailability of various drugs both structurally and therapeutically, and promote the absorption of certain drugs to reduce blood metabolism and cholesterol levels[12,17,24].

Many of the physiological effects of black pepper extracts, or of PIP, its main component, have been reported in recent years. Damage from oxidative stress and lipid peroxidation have been proposed as major causes of atherosclerosis, cancer, and aging. PIP, which has hepatoprotective properties, has been shown *in vitro* to protect against oxidative stress damage by inhibiting or quenching free radicals, ROS, and lipid peroxidation and was reported to act as a hydroxyl radical scavenger at low concentrations. At higher concentrations, it triggers the Fenton reaction, which results in the increased production of hydroxyl radicals. PIP also acts as a strong superoxide scavenger with a half maximal inhibitory concentration (IC50) value of 1.82 mM; whereas, 52% of the inhibition of lipid peroxidation was observed at a dose of 1.4 mM with an IC50 value of 1.23 mM. PIP is also an effective antioxidant that protects against the oxidation of human low-density lipoprotein[11,15,25-27].

The results obtained from analyzing the liver tissue from chick embryos treated with HC revealed that the lowest levels of antioxidant GSH were observed in group 2, which was treated with only HC (Figure 1); however, the highest GSH levels were observed in group 3, which was treated with 100 mg/kg PIP and HC; these results were significant. The GSH levels were significantly higher in group 4, which was treated with a lower dose of PIP, than in group 2, and were also higher in groups 5 and 6, but the differences were not significant compared with group 2 levels. The results of the present study were consistent with those of previous findings that reported the oxidant effects of steroids[10,21].

The lowest TAS levels were observed in group 2; whereas, the highest levels were observed in group 3, which is like the results obtained for GSH levels (Figure 2). The high TAS levels observed in group 3 were significantly higher than those in group 2.

The results of assessing the TOS levels provided information about the oxidant status and showed that the highest levels were observed in group 2 and the lowest in group 3 (Figure 3), a significant difference (Figure 3b; p =0.000). The MDA levels, another oxidant status marker, were highest in group 2 and lowest in group 3 (Figure 4a; p =0.021). Although the TOS levels in group 4 were also significantly low (Figure 3c; p =0.004), a significant low MDA level was observed in group 3 (Figure 4).

Vijayakumar et al[28] (2004) have investigated the effect of PIP, a nitrogenous alkaloid, on the lipid peroxidation of tissue and enzymatic and nonenzymatic antioxidants in rats fed a high-fat diet. PIP was found to reduce the levels of reactive substances, such as acid and conjugated dienes. Moreover, GSH, GPx, CAT, and SOD levels were maintained, with values close to those in the control rats; therefore, the results suggested that PIP reduces the high levels of oxidative stress caused by a high-fat diet[28].

Rathee et al[29] (2018) have investigated the effects of Aegle marmelos and PIP after inducing liver damage using carbon tetrachloride in Wistar rats. They have reported high levels of SOD, GSH, and CAT, all antioxidant markers, and low levels of MDA, an oxidant status marker, in the liver tissues in the group treated with PIP. The findings of their study were consistent with those of the present study[29].

Mao et al[30] (2017) have investigated the antioxidant effects of PIP on brain tissue in a pilocarpine-induced rat epilepsy model. They reported high levels of SOD and CAT and low levels of MDA. The antioxidant properties of PIP in this study were consistent with the findings of the present study[30].

Sethiya et al[15] (2015) have also investigated the antioxidant effects of PIP on brain tissue in a pilocarpine-induced rat epilepsy model. They reported high levels of SOD, GSH, and CAT and low levels of MDA in the PIP group. The results of their study were also consistent with the findings of the present study[15].

According to the results of studies conducted with different harmful oxidant agents, PIP has been reported to be successful in alleviating the effects of ROS and has also been reported to have hepatoprotective properties[28,31-35].

When a general evaluation is made, this study will give a different perspective to the literature in terms of experimental model. There are a few limitations of our study. One of them is that our results cannot be presented in the light of histopathological findings. Another is that the chosen experimental model cannot directly reflect the environment and conditions of a developing human

embryo. Therefore, it is not possible to simply disseminate and apply the results observed in chick embryos to humans. However, the chick embryo model has the advantage of allowing potentially hazardous, toxic and curative agents/substances to be investigated directly on the embryo. The fact that the study was conducted on chick embryos enabled the subject to be addressed with a new methodological approach. These are also the strengths of the study. In addition to all these, we think that it would be beneficial to conduct studies with larger samples in which technical materials and special test kits will be used to obtain more comprehensive results. Considering the limitations and strengths of our study, we believe that it will contribute greatly to the literature.

Conclusions

High ROS levels were observed in steroid-induced liver damage in a chick embryo model. PIP was used to inhibit and/or reduce the harmful effects of ROS, and high doses of PIP (100 mg/kg) were found to be more successful in reducing these effects. Additional large-sample studies that test different tissues should be conducted to better demonstrate PIP's protective effects.

Conflict of Interest

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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Authors' Contribution

All authors participated in the design, interpretation of the studies, analysis of the data, wrote the manuscript and review of the manuscript.

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