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RESEARCH ARTICLE

Protective Effects of Astaxanthin Against Formaldehyde Induced Apoptosis of Testicular Tissue in Rats

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A B S T R A C T

This study was aimed to investigate the protective effects of astaxanthin (AST) against formaldehyde (FA) induced tissue injury on the rat testes. 10 weeks old and weighing 280 (±20) g 32 male Wistar Albino rats were used. The rats were randomly divided into 4 groups. In the Control group, 1 ml of physiological saline solution was injected intraperitoneally, and 1 ml of drinking water given orally. 10 mg/kg 10% FA was applied intraperitoneally to Group II (FA group), Group III (FA+AST16), and Group IV (FA+AST32), and respectively 1 ml drinking water, 16 mg/kg AST and 32 mg/kg AST were given orally to rats in these groups. Testes were stained immunohistochemically PCNA (proliferating cell nuclear antigen), and the TUNEL technique for apoptosis and examined histopathologically for alterations. Total testosterone levels were evaluated by competitive ELISA. Apoptosis and PCNA index calculations and histopathological changes in testicular tissue indicate that FA is the cause of alterations on the testicular tissue. In addition, it has been demonstrated that the AST administration positively affects the prevention of toxic and oxidative damage due to FA in testicular tissue. This study, which was experimented on rats with ELISA testosterone levels and, immunohistochemical alterations in testis tissue, suggested that FA exposure caused the testicular tissue injury. It has been shown that FA administration causes apoptosis and decreases the PCNA index in spermatogenic cells in seminiferous tubules and Leydig cells responsible for testosterone production.

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INTRODUCTION

Formaldehyde (FA) is a widely exposed chemical agent that has both acute and chronic effects on humans and is also found in the natural structure of the organism. Cigarette smoke, cosmetics, paints, plastics, building materials, textile and paper products, building materials and wood furnitures contain FA and these may cause FA exposure (Salthammer, 2013). FA exposure related negative effects like as cytotoxicty, hematotoxicity, immunotoxicty, genotoxicty (Costa *et al*., 2019), increase in apoptosis and reactive oxygen species (ROS) levels (Thrasher and Kilburn, 2001), have been shown on the skin, eyes, menstrual cycle, nerves, reproductive organs, digestion, and respiratory system (Bernardini *et al*., 2020). FA with a minimum concentration of 0.05 ppm in respiratory air causes environmental pollution and is considered harmful to the organism (Bernardini *et al*., 2020). FA damages Leydig cells and germ cells in seminiferous tubules and causes primary and secondary infertility in the female and male reproductive systems (Han *et al*., 2015).

Astaxanthin (AST) (3-3 dihydroxy β-β carotene 4-4 dione) is an antioxidant compound of the xanthophyll class of carotenoids found in microalgae such as *Haematococcuspluvialis*, *Chlorella zofingiensis* and aquatic animals (Capelli *et al*., 2013). AST is a radical scavenger that terminates the chain reaction of reactive

oxygen and nitrogen species. This property gives it an antioxidant feature that supplied a protective effect against oxidative stress, atherosclerosis, cardiovascular disease, inflammation, diabetes, cancer and several diseases (Ambati *et al*., 2013). Numerous studies have shown that AST provides a wide range of physiological benefits to animals, such as survival, growth performance, reproductive capacity, stress tolerance, and disease resistance (Lim *et al*., 2018).

In this study, the protective effects of AST against FA-related testicular tissue toxicity and spermatogenesis mechanism failure was investigated by histopathologically and immunohistochemically and testosterone levels analyzed by ELISA in rats.

MATERIALS AND METHODS

Experimental Animals, Ethic and Design: This study was conducted in Bingöl University Experimental Research Center with the approval of Bingöl University Animal Experiments Local Ethics Commission (20/02/2017 -2017/02, 02-04). 10 weeks old and weighing 280 (±20) g 32 male Wistar albino rats were used in this study. The rats were randomly divided into 4 groups with 8 rats (n = 8) in each and kept in a room with 22-24 ºC and the relative humidity was set 55±5% and applied 12 hours light-dark cycle. Rats fed ad libitum. Astaxanthin was prepared as an active ingredient (Astaxanthin (natural extract), ≥97% (HPLC), SML0982, Sigma-Aldrich, Germany) in emulsion with drinking water.

The experiment lasted for 14 days and the following applications were made during this time. In Group I (Control group), 1 ml of physiological saline solution was injected intraperitoneally (i.p.) every other day (7 days), and 1 ml of drinking water given daily by gavage orally. At a dose of 10 mg/kg 10% FA (Han *et al*., 2015) was applied i.p. to Group II (FA group), Group III (FA+AST16), and Group IV (FA+AST32) every other day (7 days), and respectively 1 ml drinking water, 16 mg/kg AST and 32 mg/kg AST (Mortazavi *et al*., 2014) were given orally to rats daily by gavage in these groups.

Anesthesia, Necropsy, Processing of Blood and Tissue Samples: At the end of the study, all rats were anesthetized using Sevoflurane at a dose of 230 ppm. Blood samples was taken by intracardiac route and then euthanasia applied. Testes of all animals were removed and weighed during systemic necropsy and placed in 10% buffered formaldehyde for fixation before the histopathology was performed. Paraffin-embedded samples were cut with a microtome (RM 2155, Leica, Germany) to a thickness of 5 micrometers (µm). Slides were stained with Masson Trichrome (MT) stain using the Masson Trichrome staining kit (04-010802, Bio-Optica Milano S.p.A., Italy) and evaluated using light microscopy (DM2500, Leica, Germany) (Bancroft and Gamble, 2008).

Calculation of PCNA and Apoptosis Index: PCNA was determined by the immunohistochemically SABC-POD method with using PCNA Polyclonal Antibody (PA5-27214, Thermo Fisher Scientific, USA). TUNEL method was used for demonstration of apoptosis within situ Apoptosis Detection Kit (CA 92590, EMD Millipore, USA) and applied according to the manufacturer's application manual (Abe *et al*., 2017; Janardhan *et al*., 2018). PCNA index was calculated according to the immunopositive-PCNA stained spermatogonia located in a circular manner near the basal membrane of the seminiferous tubules were evaluated as a percentage (D'Andrea *et al*., 2008). Apoptosis index was evaluated of immunopositive stained apoptotic cells in 10 different seminiferous tubules randomly selected from different regions of testicular tissue (Zhang *et al*., 2012). The presence of apoptosis in the slides was examined by the researchers under the light microscope by the evaluated "blind analysis" technique.

Serum Total Testosterone Levels Measurement with ELISA: Testosterone values of the samples were evaluated by competitive ELISA method using Testosterone ELISA Kit (ab108666, Abcam, UK). ELISA kit protocol was followed when preparing samples and reagents. Standard solutions and detection curves for each administration were previously made available in the ELISA reader device (SpectraMax Plus 384, USA). Total testosterone OD values in the samples were automatically measured at 450 nanometers (nm) using ELISA reader (Ozgecer *et al*., 2017).

Statistical Evaluation: SPSS 18.0.0 for Windows (Release 18.0.0, Copyright© SPSS Inc, The Apache Software Foundation, 1989-2009) used for statistical analyses. For parametric data, One-way analysis of variance (ANOVA) followed by post hoc Tukey test performed to determine differences between the groups. For non-parametric data, Kruskal Wallis followed by Mann Whitney-U test performed to determine differences between the groups (Ozdamar, 2004) The $P<0.05$ value was considered statistically significant.

RESULTS

Clinical and Macroscopic Findings: No statistically significant difference was found in terms of testicular weights belonging to the experimental groups, and testicular weight averages are presented in Fig. 1. In Group II, yellowing of the furs was observed after the 5th day of experiment and gray-white colored areas (local necrosis) were defined in the testes at the necropsy. No clinical and macroscopic findings were observed in rats in the Group I, Group III and, Group IV.

Histopathological Findings: Histopathological findings in testicular tissues of rats in experimental groups are given in Fig. 2. In addition, relatively shrinkage in tubular diameters; diffusely decrease of germinative cells; apoptotic changes like as cytoplasmic decrease, picnosis, and karyorrhexis of most of the germinative cells in seminiferous tubules without necrosis were observed (Fig. 2b). It was determined that the lesions were suppressed in testicular tissues of the Group III compared to the Group II (Fig. 2c). On the other hand, lesions were significantly decreased in the testicular tissues of the Group IV compared to Group II and III, and apoptotic findings were very limited in the cells (Fig. 2d).

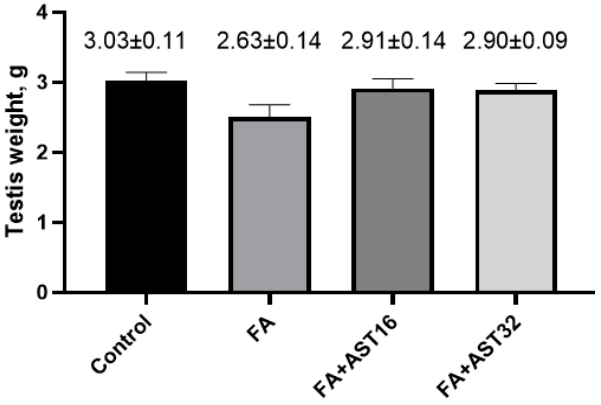

Fig. 1: Effects of AST administration on left testis weight. Error bars demonstrate the standard error of the mean. For the comparisons of the testis weight among the groups, one-way ANOVA was used followed by the Tukey post hoc test.

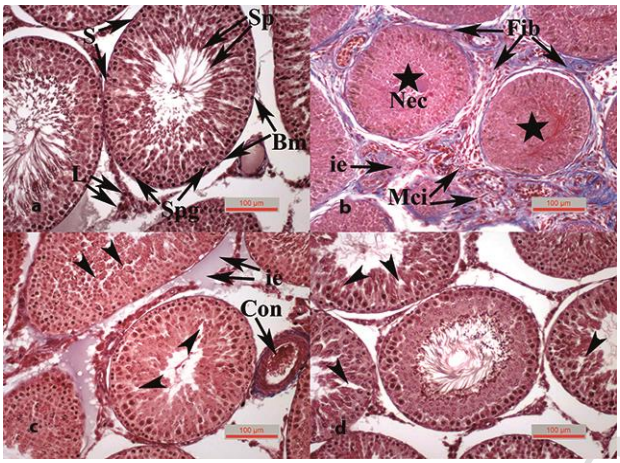

Fig. 2: Histopathology of testicular tissues (MT staining, X200 magnification). The control group (a), normal histomorphology. Histopathological alterations in the FA group (b), FA+AST16 group (c), and FA + AST32 group (d). Sp: spermatid cell; Spg: spermatogonia cell; S: Sertoli cell; L: Leydig cell; Bm: Seminifer tubular basement membrane; Nec; Necrosis (stars); Fib: Fibrosis; Mci: Mononuclear cell infiltrations; ie: interstitial edema; Con: Congestion; Loss of germinative cells (Arrowheads).

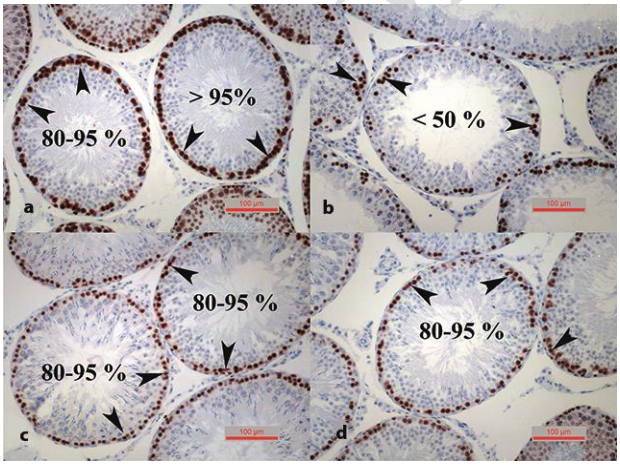

Fig. 3: Immunohistochemical PCNA staining of testicular tissues of experimental groups (X200 magnifications). The control group (a), seminiferous tubules containing multiple PCNA-positive stained cells (arrowheads). FA group (b), seminiferous tubules containing a small number of PCNA positive stained cells (arrowheads). FA+AST16 group (c) and FA+AST32 group (d), seminiferous tubules containing numerous PCNA positive stained cells (arrowheads).

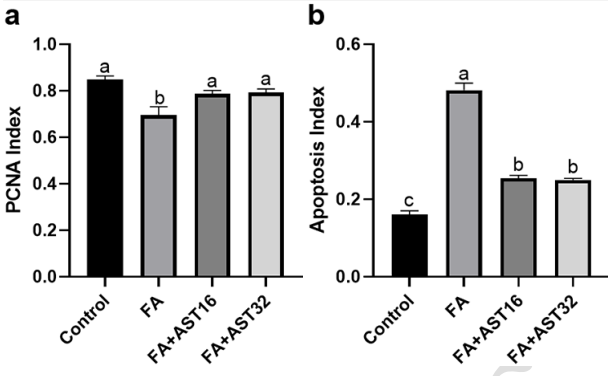

Fig. 4: Effects of AST administration on PCNA (a) and apoptosis (b) indexes. Error bars demonstrate the standard error of the mean. Lowercase letters above the groups (a-c) show differences between the groups. For the comparisons of the PCNA index among the groups, one-way ANOVA was used followed by the Tukey post hoc test (P<0.01). For the comparisons apoptosis index among the groups, Kruskall Wallis was used followed by the Mann Whitney-U test (P<0.01).

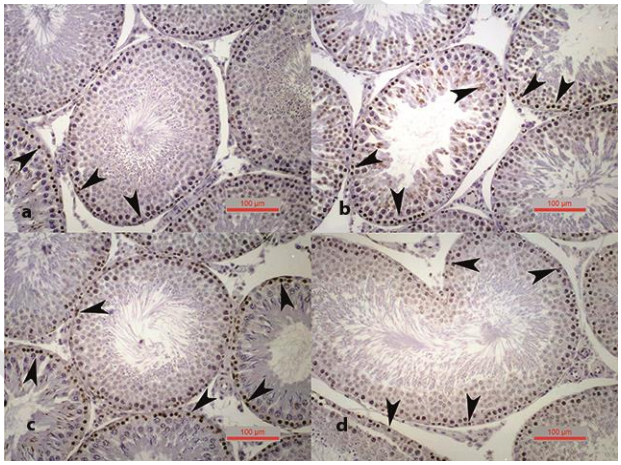

Fig. 5: Immunohistochemical TUNEL staining of testicular tissues of experimental groups (X200 magnifications). The control group (a), seminiferous tubules containing a small number of apoptotic cells (arrowheads). FA group (b) and FA+AST16 group (c), seminiferous tubules containing multiple apoptotic cells (arrowheads). FA+AST32 group (d), seminiferous tubules containing a small number of apoptotic cells (arrowheads).

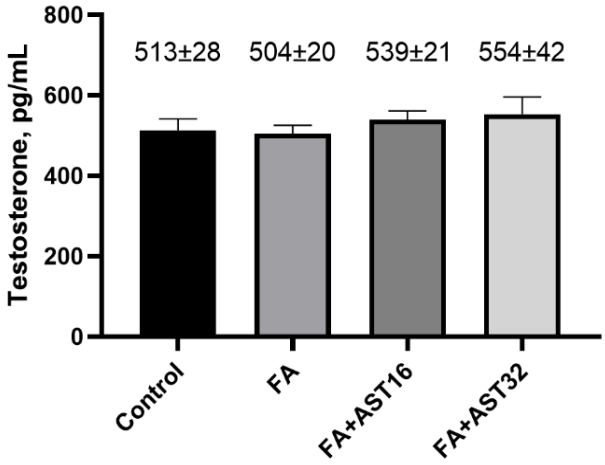

Fig. 6: Effects of AST administration on serum total testosterone level. Error bars demonstrate standard error of mean. For the comparisons of the serum total testosterone level among the groups, one-way ANOVA was used followed by the Tukey post hoc test.

PCNA and Apoptosis Findings: PCNA-positively stained cells findings of the testicular tissues in experimental groups are given in Fig. 3. Statistically, it was revealed that there was significant difference related to PCNA scores ($P<0.05$) between the Group II and all other groups (Fig. 4a). In addition, there were statistically significant differences ($P<0.05$) in Group III and IV compared with Group I and II (Fig. 4b). Apoptosis findings related to TUNEL technique stained cells of the testicular tissues of experimental groups were shown in the Fig. 5. Apoptosis was also detected in Sertoli and Leydig cells in the Group II.

ELISA Findings: The data of the mean values of serum total testosterone levels in the experimental groups were given in Fig. 6. No statistically significant difference was found between the groups as a result of statistical evaluation of serum total testosterone values.

DISCUSSION

FA is a widely exposed substance that has both acute and chronic adverse effects on the various organs and physiological mechanisms of humans and animals (Bernardini *et al*., 2020) and it has adverse effects on reproductive functions, damages germ cells and causes fertility problems (Thrasher and Kilburn, 2001). It was observed that FA caused histopathological changes in testicular morphology and Leydig cells and, provide to decreasing sperm amount, testicular weight, seminiferous tubule diameters, superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) activities in rats (Chowdhury *et al*.,1992; Zhou *et al*., 2006; Kose *et al*., 2010). The relation between Sertoli cells and germinal cells are found to be damaged related to FA and it has been disappeared of spermatozoids (Golalipour *et al*., 2007). Although different studies have reported that FA exposures at different doses and durations cause shrinkage of seminiferous tubule diameters and reduction in germ cells, different findings have been revealed about whether this finding is statistically significant or not (Golalipour *et al*., 2007; Kose *et al*., 2010). In our study, interstitial fibrosis, edema, and perivascular mononuclear cell infiltrations, necrosis in seminiferous tubules, relative decreasing in the number of germinative cells were observed in testicular tissue in FA group.

As a clinical finding, rats treated with FA were reported to have yellowing hairs after the 10th day of administration (Kose *et al*., 2010). We observed the presence of yellowing in the furs of the animals in the group that we applied FA alone from the 5th day. The presence of local necrosis foci observed macroscopically in testes only in FA group was also a remarkable finding. The fact that these findings appear in a short time may be due to intraperitoneal injection of FA.

FA exposure-related ROS production caused oxidative stress in the testis and has negative effects on sperm function. This mechanism is a common factor in many of the underlying causes of male infertility (Turner and Lysiak, 2008). Due to the cytotoxic effects of FA, apoptosis in testicular tissue is associated with DNA and chromosomal damage in cells. The tissue showing a high rate of apoptosis in vertebrates is testis where 75 percent of all male germ cells produced are excreted through the apoptosis process (Shaha *et al*., 2010; Thrasher and Kilburn, 2001). Various antioxidants such as AST, Vitamins A, E, and C have been shown to reduce the increased level of apoptosis in rat testicular tissue due to the effects of some toxic substances (Mortazavi *et al*., 2014) and increase the proliferation of Leydig cells with seminiferous tubular epithelial cells (Sonmez *et al*., 2016; Wang *et al*., 2015). AST has a positive effect on gonadal development and oocyte maturation, while also preventing apoptosis (Jang *et al*., 2010). It has been determined that the administration of 16 mg of AST for 3 months in humans provides a protective effect against infertility problems and increases the pregnancy rate (Martin-Hidalgo *et al*., 2019). AST can increase the expression of anti-apoptotic Bcl-2 and antioxidant genes (Jang *et al*., 2010).

In our study, the apoptosis indexes determined by TUNEL technique in testicular tissues were increased related to FA administration in the experimental groups. While a high apoptosis index was shown in the FA group, it was observed that 16 mg/kg dose of AST had a limited protective effect on germ cells against the harmful effect of FA; AST treatment at a dose of 32 mg/kg decreased apoptosis index more successfully than other groups. While the decrease in PCNA index was defined in germ cells due to FA exposure, it was observed that, the administration of AST at a dose of 16 mg/kg showed limited protective effects for germ cells against the FA; 32 mg/kg AST treatment was more successful in protecting germ cells against the harmful effects of FA and, at a higher rate compared to other groups.

Some antioxidants such as vitamin E, C and AST have been identified as ROS cleaners that preserve steroidogenesis and/or sperm quality in the testis by inhibiting infertility due to low testosterone production due to high ROS (Cocuzza *et al*., 2007). The percentage of motile sperm in the semen sample was correlated with the sperm antioxidant content and, AST help to protect the functional adequacy of sperm exposed to oxidative attacks (Dokumacioglu *et al*., 2018). The induction of any serious germ cell apoptosis increases the need for Sertoli cells to include a large number of dying germ cells. Faced with an increase in testicular germ cell apoptosis, how Sertoli cells response to the demand for increased retention and phagocytosis of germ cells is an undiscovered aspect of testicular oxidative stress (Turner and Lysiak, 2008; Dokumacioglu *et al*., 2018). We found that the increased apoptosis rates in Group II and the presence of apoptosis in both Leydig cells and Sertoli cells are in parallel with the literature findings.

It has been reported that AST produced a significant improvement in cyclophosphamide-related testicular injury caused by changes in sperm count, morphology, motility, seminiferous tubules diameters, and germ cell count (Heidari Kohei *et al*., 2019; Jeminiwa *et al*., 2020; Sonmez *et al*., 2016). In our study, the number of germ cells in the AST group was higher than the FA group and the apoptosis index was lower; the higher PCNA index also indicates that AST may prevent apoptosis-related germ cell loss due to FA. In addition to the evidence that AST has protective effects on germ cell damage and increases fertility rate, it is clear that larger studies are

needed for the use of this antioxidant in the treatment of infertility.

Biochemical studies have reported a decrease in blood testosterone levels as a result of FA exposure. It has been shown that seminiferous tubular atrophy occurs in rats by inhalation FA for 2 weeks (Zou *et al*., 2006). Although the findings of serum total testosterone levels in our study did not show statistical significance, a significant decrease in total testosterone levels in the only FA administered group supported this data. In addition, the increase in testosterone levels in the groups given AST indicates the protective effect of AST. The differences in serum total testosterone levels between the experimental groups were also similar in terms of a decrease in apoptosis index and an increase in the PCNA index.

This study, which we conducted serologically and immunohistochemically on rats, suggests that FA exposure damage the normal histomorphology of testicular tissue. In addition, it has been shown that FA administration increases the apoptosis index and decreases the PCNA index in spermatogenic cells. Apoptotic changes due to FA exposure have been shown to be suppressed by AST administration. AST administration positively affects the prevention of toxic testicular tissue damage due to FA. FA and AST administration related serum total testosterone levels have similarities to apoptosis and PCNA findings in experimental groups.

Conclusions: As a result, FA exposure has shown negative effects on testicular tissue and spermatogenesis. However, it has been found that oral administration of AST, which has antioxidant properties, has a successful protective effect against the negative effects of FA-induced testicular tissue damage in rats. Main limitations of this study are using a lower number of male rats related to ethic permission, are not counting spermatozoa number, and can't application of fertilization trial. It is needed studies with greater numbers of test subject, including large control groups to address the effects of oral AST consumption on reproductive outcome. The future of antioxidant therapy to improve AST-related testicular tissue repair, there need to development of fertilization and sperm motility research. Consequently, the findings suggest that FA seems to damage the male fertility, and germ cells in the testes through increasing the apoptosis in one hand, and AST administration can improve the male fertility by preventing FA-related apoptosis induced fertility disorders, on the other. Further studies are thus needed to investigate the molecular mechanism of AST.

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Author Contribution: AU and HY designed the study, ES and SY performed animal experiments, MFB, AU and SY performed laboratory analysis, ES analysed the data, AU wrote the manuscript.

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