

The effect of proanthocyanidin on formaldehyde-induced toxicity in rat testes

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Background/aim: This study investigated the effect of proanthocyanidin (PA) against formaldehyde (FA)-induced lipid peroxidation damage and morphological changes in rat testes.

Materials and methods: Twenty-one Wistar albino rats were randomized into 3 groups: control, FA, and FA + PA groups. Plasma and tissue malondialdehyde (MDA) and total sialic acid (TSA) levels were measured. Testes tissues were observed by light and electron microscopy.

Results: TSA (plasma and tissue) levels decreased and MDA (plasma) significantly increased ($P < 0.05$) in rats treated with FA compared to the controls. Tissue MDA levels were not significantly different. Several necrotic changes were observed in testes tissues by light and electron microscopy. Disordering in epithelia of seminiferous tubules, vacuolization between germinal epithelium cells, and separated basement membranes were observed by light microscope. Immunopositivity in Leydig cells decreased in the FA group ($P < 0.05$). In the FA + PA group there were more immune Leydig cells reacting immune-positively than in the FA group ($P < 0.05$). Ultrastructurally, FA also caused disorganization and loss of mitochondrial cristae, and dilatation in endoplasmic reticulum in testes.

Conclusion: The results suggest that PA has a protective effect on FA toxicity in testes.

Key words: Formaldehyde toxicity, malondialdehyde, proanthocyanidin, sialic acid, testis

1. Introduction

Proanthocyanidins (PAs) are natural antioxidants whose polyphenolic properties are known and are commonly found in fruits and vegetables. PAs are intensively found in grape seeds and nuts and it has been demonstrated that they prevent the formation of free radicals released due to oxidative stress in both in vivo and in vitro studies (1–5). In addition, it has been reported that they have antiinflammatory, antiallergic, anticarcinogenic, antibacterial, antiviral, neuroprotective, cardioprotective, vasodilator, renoprotective, and immune system-stimulating effects (4,6,7). Moreover, it has been stated that PA prevents platelet aggregation and capillary permeability and organizes the activation of some enzyme systems such as cyclooxygenase and lipoxygenase (3,8).

Formaldehyde (FA) is an aldehyde that is easily soluble in water, colorless, an irritant, pungent in pure form, and very commonly used. It is intensively used in the food industry as a preservative (E240) as well being in general usage in the medical field. Therefore, it can enter the human body, where it has harmful effects. In the human

body, it is metabolized in the liver and erythrocytes are converted to methanol and formic acid. According to numerous studies, depending on the exposure to FA, it has been demonstrated that a variety of symptoms such as sensory irritation, salivation, dyspnea, headaches, insomnia, seizures, behavioral disorders, and abnormal sperm production occur (9–13).

FA toxicity-induced free radicals affect important components of cells, such as lipids, proteins, carbohydrates, and DNA (14). As a result, all these effects of free radicals, together with the autocatalytic effect, may lead to lipid oxidation and membrane damage (14–16). Malondialdehyde (MDA) is the final product of lipid peroxidation and is a marker of oxidative stress (17,18). It has been reported that MDA level was shown to increase as a result of oxidative stress developed by FA toxicity in experimental models, and its level decreases depending on the use of antioxidants (10,19). The cell surface components are also affected by this membrane damage. Sialic acids (SAs), derived from neuraminic acid, have a negative electric charge, are important components

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of the cell surface, and have been found to be present in some micro- and higher organisms (20–23). The external positions of SAs, the negative electrical charge that they have, and their placement on the outer surface of the cell membrane are extremely important for cell biology. Increases, decreases, or changes in the molecular properties of SAs in the cell surface create different effects in cells and tissues (24). In addition, in recent years, plasma total sialic acid (TSA) levels have been shown to be a risk factor for cardiovascular diseases (25) and are considered to be a potential tumor marker (26).

The protective effect of PA against FA toxicity in testis tissue has not been investigated in previous studies. The aim of this study is to reveal the possible effects of PA against FA toxicity in rat testes at biochemical and light and electron microscopic levels.

2. Materials and methods

2.1. Animals

Twenty-one male Wistar albino rats (200–250 g in weight, 10–12 weeks old) were provided by the Experimental Animal Unit of Trakya University. Our study was performed after approval from the Trakya University Local Ethics Committee for Animal Experiments. All animals were fed with rat pellets (21% crude protein, Purina) and tap water on a daily basis and they were kept under appropriate laboratory conditions (22 ± 10 °C and a 12-h light/dark cycle) during the whole experiment.

2.2. Chemicals

PA was obtained from General Nutrition Centers, Inc. (Pittsburgh, PA, USA). FA solution was purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.3. Experimental protocol

The experiment was performed with three groups including one control and two experimental groups. The following procedures were performed on the animals in the experimental groups after considering the doses mentioned in the literature: saline solution was given to the control group ($n = 7$) every other day for 14 days intraperitoneally (i.p.). Saline solution and 1/10 diluted 10 mg/kg doses of FA were given to the FA group ($n = 7$) every other day for 14 days i.p. (12,27,28). Saline solution and 1/10 diluted 10 mg/kg doses of FA were given i.p. every other day and daily intragastric 100 mg/kg (5) doses of PA were given to the FA + PA group ($n = 7$) for 14 days.

At the end of the experimental period, intramuscular xylazine (10 mg/kg) and ketamine (50 mg/kg) were given to the animals and heart blood and testes were taken under anesthesia. The tissues were stored at -80 °C until the time of analysis.

2.4. Determinations of biochemical parameters

Testicular tissue samples were homogenized using a glass-glass homogenizer (Glass-Col) in 10% KCl (1:10 w/v) for the determination of MDA; 8.1% SDS, 0.82% thiobarbituric acid, and acetate buffer (3 M, pH 3.5) were added to the plasma and homogenated samples; and the reaction was measured at a wavelength of 532 nm spectrophotometrically (Shimadzu, Japan) (29). Plasma and tissue MDA levels were measured as $\mu\text{mol/L}$ and $\mu\text{mol/g}$ tissue, respectively, using an MDA standard graphic.

The amount of TSA in plasma and testicular tissue homogenates was measured spectrophotometrically at 525 nm. In this method, tissue homogenates and plasma samples were incubated in perchloric acid (0.2 mL of sample + 1.5 mL of 5% perchloric acid) at 100 °C for 5 min. After centrifugation at $2500 \times g$ for 4 min, 0.2 mL of Ehrlich's reagent was added to the supernatant and it was incubated at 100 °C for 15 min and absorbance was measured at 525 nm. The levels of plasma and tissue TSA were measured in mg/mL using a TSA standard graph.

2.5. Light microscopy and immunohistochemical analysis

The tissues, fixed in formalin for 24 h, were embedded in paraffin after dehydration and a clearing process. They were stained with hematoxylin and eosin (H&E) after obtaining sections of 4–5 μm thick and examined by light microscope (Nikon E-100).

The tissue samples taken for microscopic examination were subjected to routine histological processing and were embedded in paraffin. Sections of 5 μm thick obtained from paraffin blocks were put on slides coated with poly-L-lysine. These sections were subjected to the avidin-biotin peroxidase complex (ABC) method and the presence of testosterone was demonstrated by microscopy (30,31). For testosterone, rabbit polyclonal IgG AR (N-20) (Santa Cruz, sc-816) was used. The positive immunopositive cells were scored as weak (0–1 points), moderate (2–3 points), and strong (4–5 points). This analysis was performed in all groups in at least 10 interstitial areas and in 2 sections from each animal.

2.6. Electron microscopic analysis

The tissues taken for the electron microscopic examination were fixed in 4% glutaraldehyde for 2 h, washed with buffer (0.01 M, pH 7.2–7.4), and, after that, subjected to postfixation with 1% osmic acid. The tissues were passed into acetone and propylene oxide in series and embedded in a mixture of Epon 812/Araldite (32). The thin sections that were taken by a Leica EM UC 6 ultramicrotome (serial no: 522637) were examined by an FEI Tecnai™ G2 Spirit/Biotwin (serial no: 12TN47B/1043) transmission electron microscope.

2.7. Statistical analysis

For statistical analysis, SPSS 19 was used at the Trakya University Department of Biostatistics. Results were expressed as a mean ± standard deviation (SD). The suitability of the data for normal distribution was determined by one-sample Kolmogorov–Smirnov test. The one-way analysis of variance test for the variables matching normal distribution and the Tukey post hoc test for multiple comparisons between the variables were performed. The Kruskal–Wallis test was performed for the variables that did not match normal distribution. All statistics were considered significant at P < 0.05.

3. Results

3.1. Plasma and tissue MDA and TSA findings

Plasma MDA levels were higher in the FA group than in the control group and this increase was statistically significant (P < 0.05). The plasma MDA level in the FA + PA group was higher than in the control group (P < 0.05). In terms of MDA levels in the FA group compared with the FA + PA group, there was a significant decrease (P < 0.05) (Table 1).

Tissue MDA levels in the FA group were higher than in the control group, but this difference was not significant (P > 0.05). When the MDA levels of the FA + PA group were compared to the control group, the difference between MDA levels was also not significant (P > 0.05) (Table 1).

While plasma TSA levels in the FA + PA group were significantly lower than in the control group (P < 0.05), those in the FA group were not significantly lower than in the control group (P > 0.05). There was no significant difference in plasma TSA levels between the FA group and the FA + PA group. Tissue TSA levels in the FA group were lower than in the control group (P < 0.05). Tissue TSA level in the FA + PA group was significantly higher than in the FA group (P < 0.05) (Table 1).

3.2. Light microscopic findings

When the FA group was compared with the control group (Figures 1A and 1B), it was observed that cell sequences in the germ cells were impaired. The spermatogonia were

separated from the basal membrane and vacuolization had occurred between the germ cells. Hypertrophy of spermatogonia was noted. In the FA + PA group, vacuolization between the cells was observed, but to a lesser extent than in the FA group (Figures 1B and 1C).

3.3. Immunohistochemical findings

In the interstitial Leydig cells of the control group a strong immunopositivity to the testosterone was identified. It was observed that there was decreased immunopositivity in Leydig cells in the FA group (P < 0.05) (Figures 2A and 2B). The immunopositivity in the Leydig cells of the FA + PA group decreased compared to the control group but was higher than in the FA group (P < 0.05) (Figures 2A and 2C). Table 2 shows testosterone expression in Leydig cells.

3.4. Electron microscopic findings

It was observed that the structures of mitochondrial cristae in cells were disrupted, the smooth endoplasmic reticulum (SER) sacs were extended, and there was intracellular vacuolization in the FA group when compared with the control group (Figures 3A and 3B). In the FA + PA group, less mitochondrial cristae degeneration was observed when compared with the FA group (Figures 3B and 3C).

4. Discussion

In many studies investigating the toxicity of FA, tissue MDA levels were significantly higher in the FA group compared with the control group (11,13,33). In our study, the levels of tissue MDA were also higher, although this increase was not statistically significant. This situation was evaluated to be a result of the acute effect of FA. The increase in tissue MDA level in the FA group shows that FA has an effect on lipid peroxidation. In the literature there is no study investigating the effect of PA against FA toxicity in testicular tissue. In our study, it was observed that tissue MDA levels were lower in the FA + PA group compared to the FA group. This result seems to be important in terms of disclosing the antioxidant property of PA against FA toxicity in testicular tissue. In studies with different

Table 1. Plasma and tissue TSA and MDA levels.

	Control	FA	FA + PA
Plasma MDA (µmol/L)	1.631 ± 0.366	2.776 ± 0.313 *	2.132 ± 0.376 * †
Tissue MDA (µmol/g tissue)	16.610 ± 7.905	28.040 ± 13.300	16.151 ± 2.632
Plasma TSA (mg/mL)	8.818 ± 0.735	8.132 ± 0.762	7.646 ± 0.696 *
Tissue TSA (mg/mL)	0.271 ± 0.042	0.031 ± 0.012 *	0.174 ± 0.039 * †

Values are expressed as means ± SD; n = 7 for each treatment group.

* P < 0.05: Comparison to the control group, † P < 0.05: Comparison to the FA group.

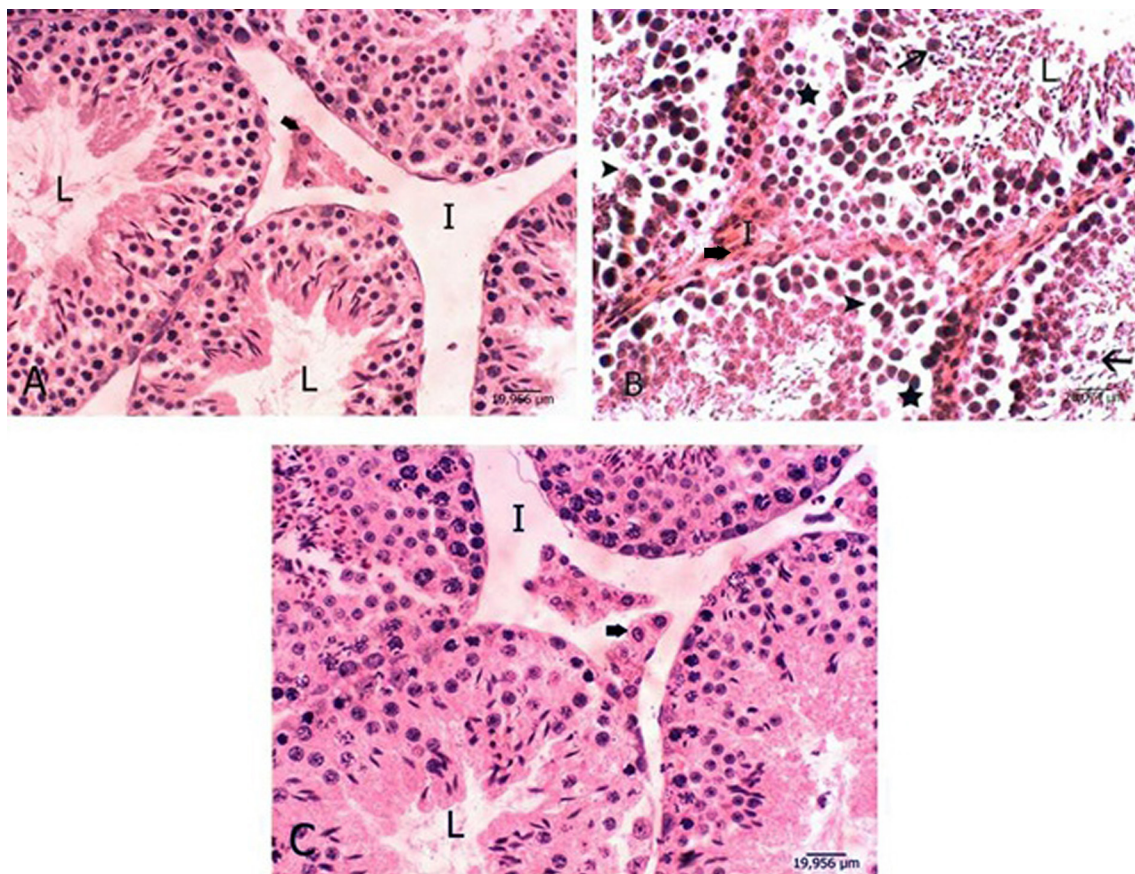


Figure 1. Light microscopic findings. A) Control group (400×, H&E). L: lumen, I: interstitial area, Leydig cell (short thick arrow). B) FA group (400×, H&E). L: lumen, I: interstitial area, damaged cell array at germinal epithelium, basement membrane damage (asterisk), hypertrophy (thin arrow), vacuolization between cells (triangular arrow head), Leydig cell (short thick arrow). C) FA + PA group (400×, H&E). L: lumen, I: interstitial area, Leydig cell (short thick arrow).

Table 2. Testosterone-positive immunoreactivity in the testis tissue cell score.

	Control	FA	FA + PA
Score	3.8 ± 0.3	1.3 ± 0.1*	2.8 ± 0.3 * †

Values are presented as the mean ± SD; n = 7 for all groups.

*: Compared with the control group, P < 0.05, †: Compared with the FA group, P < 0.05.

chemical substances and experimental models, it has been demonstrated that PA had positive effects on the level of tissue MDA (5,34–38).

In our study, plasma MDA levels were significantly higher in the FA group than in the control group. It was evaluated that other tissues may be affected by acute FA toxicity. In the FA + PA group, plasma MDA levels were found to be different from the FA group, and this was interpreted as the positive healing effect of PA in terms of plasma MDA levels. Lipid peroxidation, resulting from the oxidation of the membrane lipids, is a very important

event for the cell; it causes the start of the events that result in cell death. These degenerative changes in testicular tissue are thought to be related to lipid peroxidation.

Today, it is known that SAs play an important role in the diagnosis of many diseases (39–41). Increases in tissue and plasma TSA levels are associated with a variety of different types of cancer (41–43). Increases, decreases, or changes in the amount of TSA in the cell surface create different effects on the molecular properties of cells and tissues (25). A decrease in the TSA levels may be related to deterioration in TSA biosynthesis occurring due to

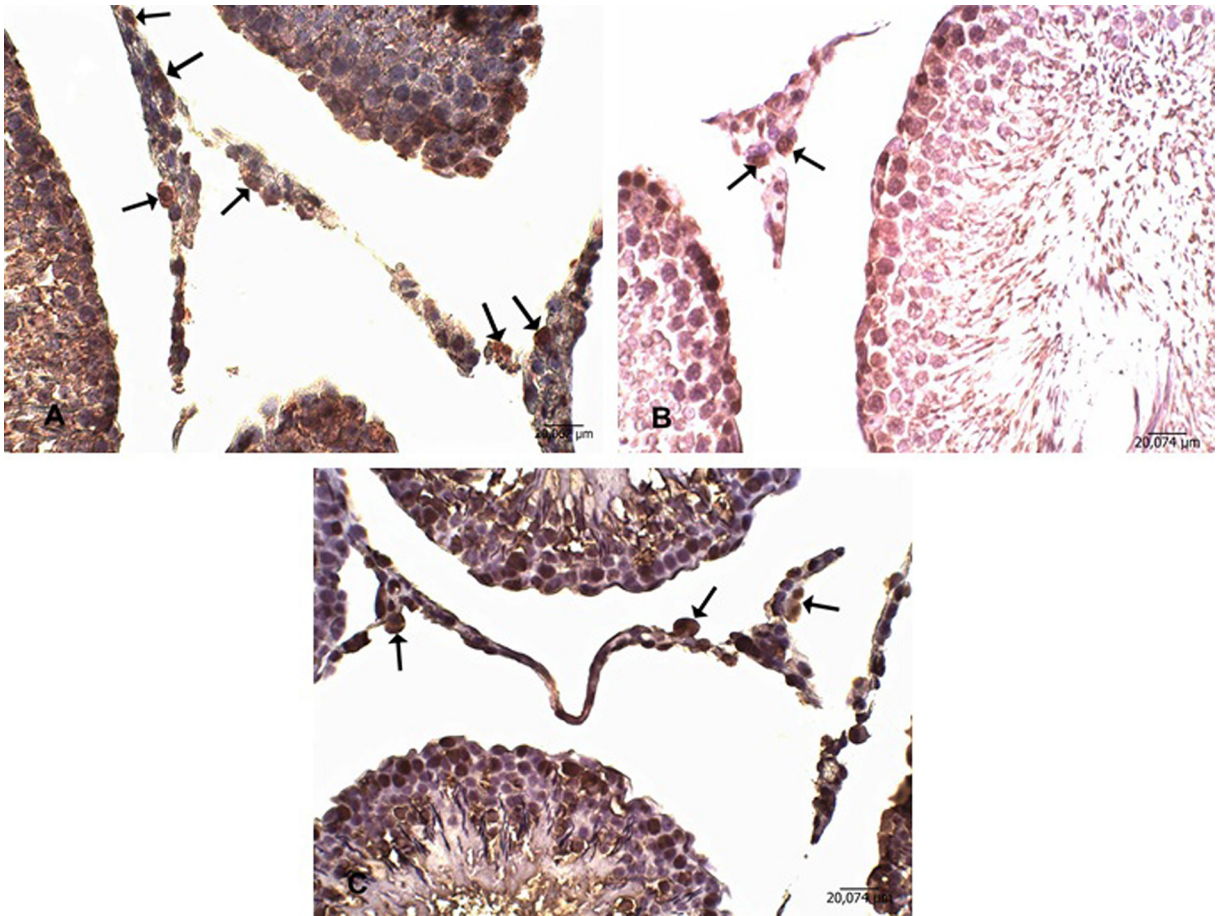


Figure 2. Immunohistochemical findings. A) Control group: immunohistochemical staining of testosterone showing strong immunopositive reaction in the cytoplasm of Leydig cells in interstitial area (thin arrow). Magnification: 400×. B) FA group: immunohistochemical staining of testosterone showing low immunopositive reaction in the cytoplasm of Leydig cells in interstitial area (thin arrow). Magnification: 400×. C) FA + PA group: immunohistochemical staining of testosterone showing moderate immunopositive reaction in the cytoplasm of Leydig cells in interstitial area (thin arrow). Magnification: 400×.

tissue damage. In our study the levels of TSA in testicular tissue decreased depending on the toxicity of FA. In the literature there are some studies demonstrating that the levels of TSA in testicular tissue decreased depending on the different toxic substances. It has been revealed that pesticides reduced the levels of TSA in testicular tissue (44,45). It is obvious that the decrease occurring in the tissue and plasma levels of sialic acids, which are known to be very important in cell adhesion, has a negative effect on the cell adhesion. These findings are compatible with our findings. The tissue levels of the TSA in the FA + PA group got close to the levels of the control group. This change is thought to be due to the positive effects of using antioxidants on the biosynthesis of TSA.

It is known that FA causes adverse effects on the respiratory system, the gastrointestinal system, the central nervous system, and testicular tissue (13,33,46). Due to FA toxicity, an increase in gaps between the germ cells in

testicular tissue, a decrease in the number of germinal cells, basal membrane damage (47), and vacuolization occurring in the interstitial area were observed (48). These findings put forward by researchers are compatible with our results. In our study, intracellular vacuolization, basement membrane damage, impaired germinal epithelium cell layout, an increase in the volume of germinal epithelium cells, epithelium cells being thrown together with spermatids into the lumen, and edema in interstitial area spermatids were the degenerative changes observed. In a previously conducted study, edema in the interstitial area was recorded as a degenerative change observed in testicular tissue (13). In our study, in which we wanted to put forward the effect of PA, whose antioxidant activity in intestinal and renal ischemia-reperfusion injury is known (5,37), on testicular tissue, the positive effects of PA were observed. In the FA + PA group, degenerative changes, which were known to be caused by FA, were repaired in some areas.

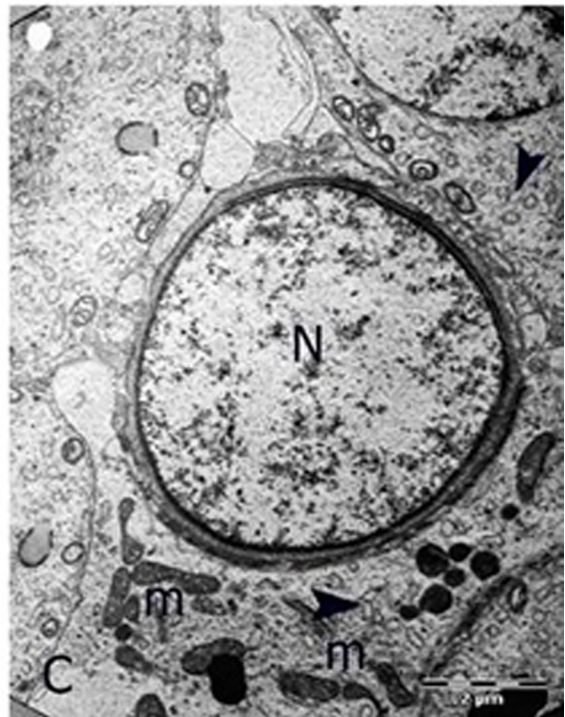
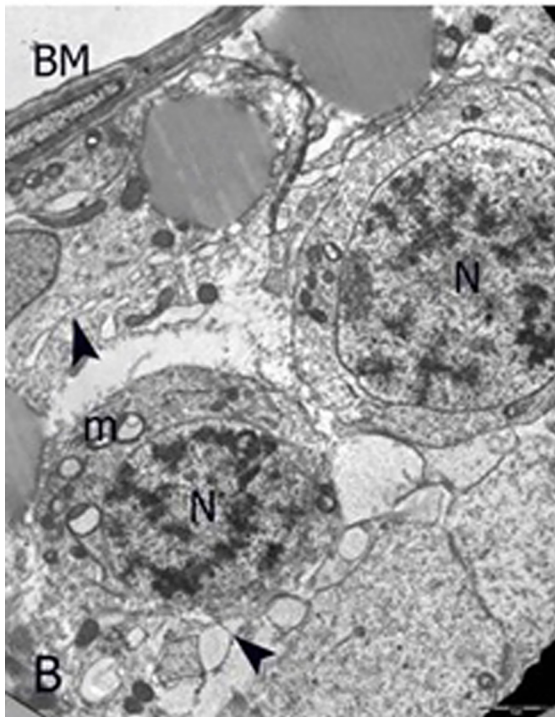
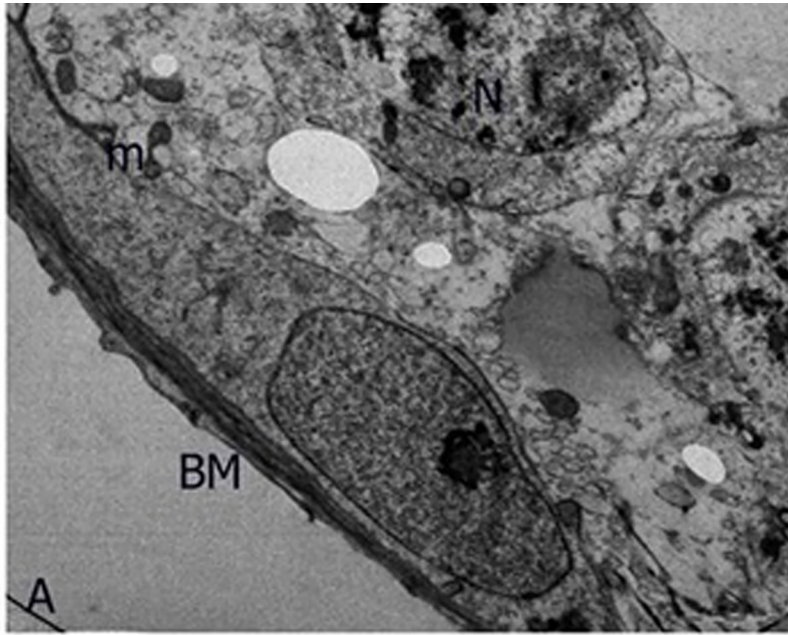


Figure 3. Electron microscopic findings. A) Control group. N: nucleus, BM: basement membrane, m: mitochondria (bar = 2 μ m). B) FA group. m: mitochondria, BM: basement membrane, smooth endoplasmic reticulum (arrow head) (bar = 2 μ m). C) FA + PA group. N: nucleus, m: mitochondria, smooth endoplasmic reticulum (arrow head) (bar = 2 μ m).

The structure and numbers of Leydig cells, whose basic functions are to produce testosterone, are very important in the development of germinal epithelium cells. In our study, testosterone-positive immunoreactivity in Leydig cells was observed to be weaker in the FA group compared to the control group and the immunoreactivity of the FA

+ PA group was observed to be close to that of the control group.

As a result of electron microscopic examination, degenerative changes observed in mitochondrial cristae in the FA group were considered to be the most important factor to negatively affect the oxidative metabolism of

the cell. In studies that used different toxic substances in testicular tissue, it was reported that degenerative changes occurred in mitochondria and the ER in testicular tissue (49). It should be considered that both the degeneration of mitochondria and the expansions observed in ER sacs had negative effects on protein synthesis. In particular, the expansions observed in SER sacs had a negative effect on the biosynthesis of lipid molecules. At the same time, disruption revealed in lipid biosynthesis had a negative effect on the synthesis of steroid hormones.

In the germinal epithelium and Sertoli cells, depending on the protein synthesis defects that may occur, ER expansion will negatively affect the structure of the cell membrane. These proteins are quite significant for the connection complexes formed by germinal epithelium cells with each other and with Sertoli cells. In our study, the gap observed between spermatogonia is thought to be the result of a loss of adhesion in the developing spermatogonium. At the same time, the ER is very important in the biosynthesis of protein structures of extracellular matrix components consisting mainly of factors such as glycoproteins and polysaccharides (50). The extracellular matrix has a vital importance in the development of spermatogonia and Sertoli cells (51). The structure of the extracellular matrix affects the proliferation and testosterone synthesis of Leydig cells (52). In terms of providing the adhesions between Sertoli cells, the germinal epithelium cells, and the basement membrane, the components of the extracellular matrix are very important (53).

The basement membrane has a very important role in the integrity of the germinal epithelium and the development of cells. It is known that an abnormal basement membrane structure is observed in infertile men (54,55). Basement membrane damage observed in the seminiferous tubules and Leydig cells is important first for the cells and then for the integrity of the tissue. In our study, testicular tissue damage in the basement membrane emerged depending on formaldehyde toxicity. The present study evaluated the framework of these findings; the toxic effect of formaldehyde was set out clearly by electron microscope. In the PA group, the recoveries observed in the cristae of mitochondria and ER sacs are important for both the synthesis of the basement membrane and the components of the extracellular matrix.

In conclusion, this study shows the effect of PA against formaldehyde toxicity in rat testes at the biochemical and light and electron microscopic levels. The present study observed that PA has protective effects against FA-induced testis toxicity. The use of PA is suggested as a pretreatment agent in FA toxicity. We think that our study will shed light on further studies that could be conducted on both antioxidant capacity and the immunohistochemical determination of adhesion molecules.

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