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Research Article

Ultrastructural effects of (4S)-2-(4-Hydroxy-3-Methoxyphenyl) Thiazolidine-4-carboxylic acid on zebrafish testicular tissue

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Abstract

Thiazolidinones are heterocyclic organic medicines and the antifungal, antibacterial, and antiproliferative effects were shown in the studies. The aim of the study was to investigate the possible negative effects of (4S)-2-(4-hydroxy-3-methoxyphenyl)thiazolidine-4-carboxylic acid in zebrafish testicular tissue on an ultrastructural level. Experimental groups were exposed to different doses (0.2mM, 0.4mM, and 0.6mM) of thiazolidinone for 5 days. Further, no treatment was given to the control group. The testicular tissues were fixed and embedded in epoxy resin. The pathological sections were evaluated with transmission electron microscope. The sertoli, leydig, and spermatogenic cells of the control group showed intact morphology. In experimental groups, mitochondrial swelling, intense cristae loss, and autophagic vacuoles were observed in the Sertoli cells. Dilated perinuclear space, mitochondrial degeneration, and dilated smooth endoplasmic reticulum (sER) were detected in spermatocytes. The spermatids showed separation between genetic material and nuclear membrane. The condensation of genetic material was irregular. Mitochondrial degeneration and dilatation of sER tubules were detected. The Leydig cells showed perinuclear space dilatation, mitochondria degeneration, and dilatation in sER tubules. Our findings suggest that thiazolidinone has degenerative effects on zebrafish testis and this chemical should be carefully used in the pharmaceutical industry.

Introduction

Five-membered nitrogen-containing heterocyclics are found in many natural products and pharmaceutical structures. In addition, many have been used as synthetic intermediates, co-reagents, ligands or asymmetric synthesis catalysts (Sriramurthy *et al.,* 2007). One of these heterocyclic organic compounds is thiazolidines are used in the pharmaceutical industry due to their minimal side effects. They can be synthesized in vitro as a result of the condensation of aldehyde or ketone with thiol (Singh *et al.,* 1981; Sriramurthy *et al.,* 2007). Derivatives of thiazolidine ring systems show a broad spectrum of bioactivities. Studies have shown the antifungal, antibacterial, antiproliferative, and anticarcinogenic effects of these substances (Andres *et al.*, 2000; Gududuru *et al.,* 2004; Shanmugapandiyan *et al.,* 2010; Zhang *et al*., 2010). Shanmugapandiyan *et al.* (2010) reported anti-inflammatory activity of 4-(azetidine-2-one)-3-chloro-4-phenyl]-1H-Phenylbenzimidazoles and 2-(thiazolidine-4-one)-phenyl]-1H-phenylbenzimidazoles on various types of bacteria. 4-thiazolidinones also showed antibacterial effects by the inhibition of bacterial enzyme MurB (Andres *et al.,* 2000). Anticancer effects of thiazolidines were well-known (Zhang *et al.,* 2010). In addition, antiproliferative activity of 2-aryl-4-oxo-thiazolidin-3-yl-amides in human prostate cell lines were reported (Gududuru *et al.,* 2004).

Among thiazolidines, thiazolidine-4-carboxylic acid (TCA), a proline analog, is a highly studied compound with a wide range of uses (Bilgiçli *et al.,* 2021). TCA is a cyclic sulfur amino acid whose chemical structure has a thiazolidine ring and a carboxylic acid group attached to the fourth carbon (Weber *et al.,* 1982). These heterocyclic derivatives have various applications in pharmaceutical and chemical research. TCA derivatives are used to study various processes in biological systems, such as the analysis of enzymatic reactions or studies of cellular metabolism (Wlodek *et al.,* 1993; Iwamoto *et al.,* 2004; Thalamuthu *et al.,* 2013; Jeelani *et al.,* 2014). These derivatives are also the subject of research in drug development, such as antioxidants (Jagtap and Pardeshi, 2014; Önen-Bayram *et al*., 2016), anticancers (Önen-Bayram *et al.,* 2015; Bilgiçli *et al.,* 2021), and antiviral (Yang *et al.,* 2021). They have the potential for use in the field of agricultural chemicals, as they may have plant growth-promoting or protective properties against plant diseases (Hota *et al.,* 2017; Nanjappanavar *et al.,* 2017). They are used in polymer or materials science to contribute to the synthesis of new materials (Goodman and Su, 1972; Kothakota *et al.,* 1995; Deng *et al.,* 2011).

The synthesis process of thiazolidines is reversible. Thiazolidines decompose into aldehydes and thiols in water (Singh *et al.,* 1981). Various studies have shown that aldehydes produced in the environment can cause various diseases by interacting with endogenous aldehydes formed during oxidative stress (LoPachin and Gavin, 2014).

Zebrafish are suitable vertebrate models for breeding and developmental studies. They provide an advantage in scientific studies with their genomes having

significant homology with the human genome, their small size and high reproductive potential (Dai *et al.*, 2014).

Thiazolidinone derivatives are widely scattered in agricultural and aquatic environments. Although there were some studies about their effects on rodents there is limited knowledge about the effects on aquatic organisms. Above all, there is no ultrastructural study in existing literature. Therefore it was aimed to investigate the potential ultrastructural effects of (4S)-2-(4-hydroxy-3-methoxyphenyl)thiazolidine-4-carboxylic acid a new type of thiazolidinone on the Sertoli cells, Leydig cells, and spermatogenic lineage cells in zebrafish (*Danio rerio*) testis tissue in our study.

Materials and methods

Synthesis of the chemical

4-hydroxy-3-methoxybenzaldehyde (2.55 g, 10 mmol) was dissolved in ethanol (5 ml). L-cysteine hydrochloride (1.57 g, 10 mmol) and sodium acetate (0.98 g, 12 mmol) were dissolved in water (5ml) and added to the solution. After stirring at room temperature for 24 hours, the precipitate was separated by filtration and washed several times with ethanol to give the white solid product in 72% yield (Fig. 1) (Liu *et al.*, 2011; Bilgicli *et al.*, 2021).

Experimental process

In this study, we used adult male zebrafish that were obtained from Sakarya University Aquaculture Lab., Esentepe, Turkey. They were kept in dechlorinated tap water in a photoperiod of 15 hours light/10 hours dark, a temperature of 28.5±1 °C, a pH of 7.0±0.5, and a humidity of 61%. They were fed with Artemia sp. and TetraMin Hauptfutter (Tetra Werke, Germany) twice a day. After a one-week adaptation period, zebrafish were divided into 4 groups (n=10). While no treatment was given to the control group, the experimental groups were exposed to different doses (0.2, 0.4, 0.6mM) of (4S)-2-(4-hydroxy-3-methoxyphenyl) thiazolidine-4-carboxylic acid (TIA) for 5 days. The fish were anesthesized and testis tissues were dissected after the 5th day of exposure.

Electron Microscopy

Testicular tissues obtained at the end of the experiment were fixed with 2.5% glutaraldehyde and secondary fixation was made with osmium tetroxide. After washing with phosphate buffer, the tissues were kept in 1% uranyl acetate solution at +4°C for 10 minutes for contrast enhancement. After washing with phosphate buffer, they were transferred through the increasing alcohol series as 30% and 50% alcohol for 10 minutes at +4°C and then in 70%, 90% and 100% alcohol at room temperature for 10 minutes. Tissues were kept in propylene oxide twice for 10 minutes at room temperature and then were incubated in propylene oxide: epon (1:1), propylene oxide: epon (1:3) and pure epon at room temperature for 1 hour, respectively. Then, they were embedded in capsules containing pure upon. The capsules were placed in an incubator at 60°C and polymerized for 18 hours. Thin sections were taken on copper grids with an ultramicrotome. After contrasting the thin sections with uranyl acetate and lead nitrate, they were evaluated and photographed with the Jeol Jem 1011

transmission electron microscope (Karabay-Akgül and Ekiz-Yılmaz, 2023).

Results

Histological examination of the control group revealed normal and intact morphology in all cell types of interest, including the Sertoli cells, Leydig cells, and cells of the spermatogenic lineage (Fig. 2a-f). Sertoli cells displayed characteristic triangular nuclei and intact organelles (Fig. 2a).

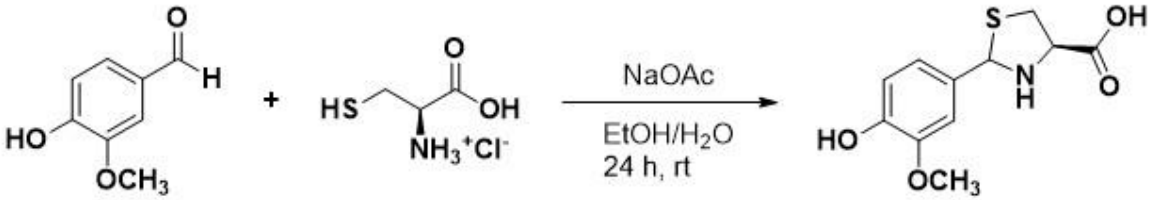

Figure 1: Synthesis of (4S)-2-(4-hydroxy-3-methoxyphenyl)thiazolidine-4-carboxylic acid.

In 0.2 mM TIA treated group, Sertoli cells showed mitochondria degeneration due to swelling and intense cristae loss. Autophagic vacuoles were seen only in the cytoplasm of the Sertoli cells (Fig 3a). Cristae loss of mitochondria was also detected in spermatogonia cells (Fig. 3b). In spermatocytes, dilatations of endoplasmic reticulum cisterns were observed alongside the mitochondrial degeneration (Fig. 3c). In spermatids, there was excessive invagination of the nuclear membrane. Gaps were seen between the genetic material and the nuclear membrane and also between the nuclear membrane and cytoplasm. In addition, the condensation of genetic material of some spermatids was irregular (Fig. 3d). Swelling of mitochondria and cristae loss were visible and several decondensed areas in the genetic material of spermatozoa were detected (Fig. 3e). At the interstitial area, Leydig cells showed dilatation of the perinuclear space, degeneration of mitochondria due to the loss of cristae and swelling, and dilated sER tubules (Fig. 3f).

Sertoli cells of the 0.4 mM TIA treated group had loosed cytoplasm with degenerated mitochondria and excess accumulation of autophagic vacuoles (Fig. 4a). The mitochondria of spermatogonia cells were swollen and had vacuoles in their matrix (Fig. 4b). The interactions between spermatocytes were loose, cristae loss of mitochondria were also seen in their cytoplasm (Fig. 4c). Several decondensed areas were seen in the genetic material of spermatids (Fig. 4d). We have detected spermatids with loose genetic material in the spermatozoa region of the seminiferous tubules (Fig. 4e). There were excess dilatations of sER tubules in the Leydig cells. Dilated perinuclear space was also seen (Fig. 4f).

In 0.6 mM TIA treated group, Sertoli cells showed cytoplasmic degeneration with autophagic vacuoles. Excess mitochondrial swelling and complete criastae loss were detected in their cytoplasm (Figs. 5a, and 5b). Mitochondrial degeneration was also seen in spermatogonium and spermatocyte cells (Fig. 5a and 5b). Spermatid cells were seen

between spermatozoa cells. In their cytoplasm cristae loss and vacuoles were visible in the mitochondria, decondensed areas were detected in the genetic material of these cells (Fig. 5c, 5d, and 5e). Spermatozoa cells had degenerated mitochondria (Fig. 5e). In the Leydig cells, perinuclear space dilatations were detected.

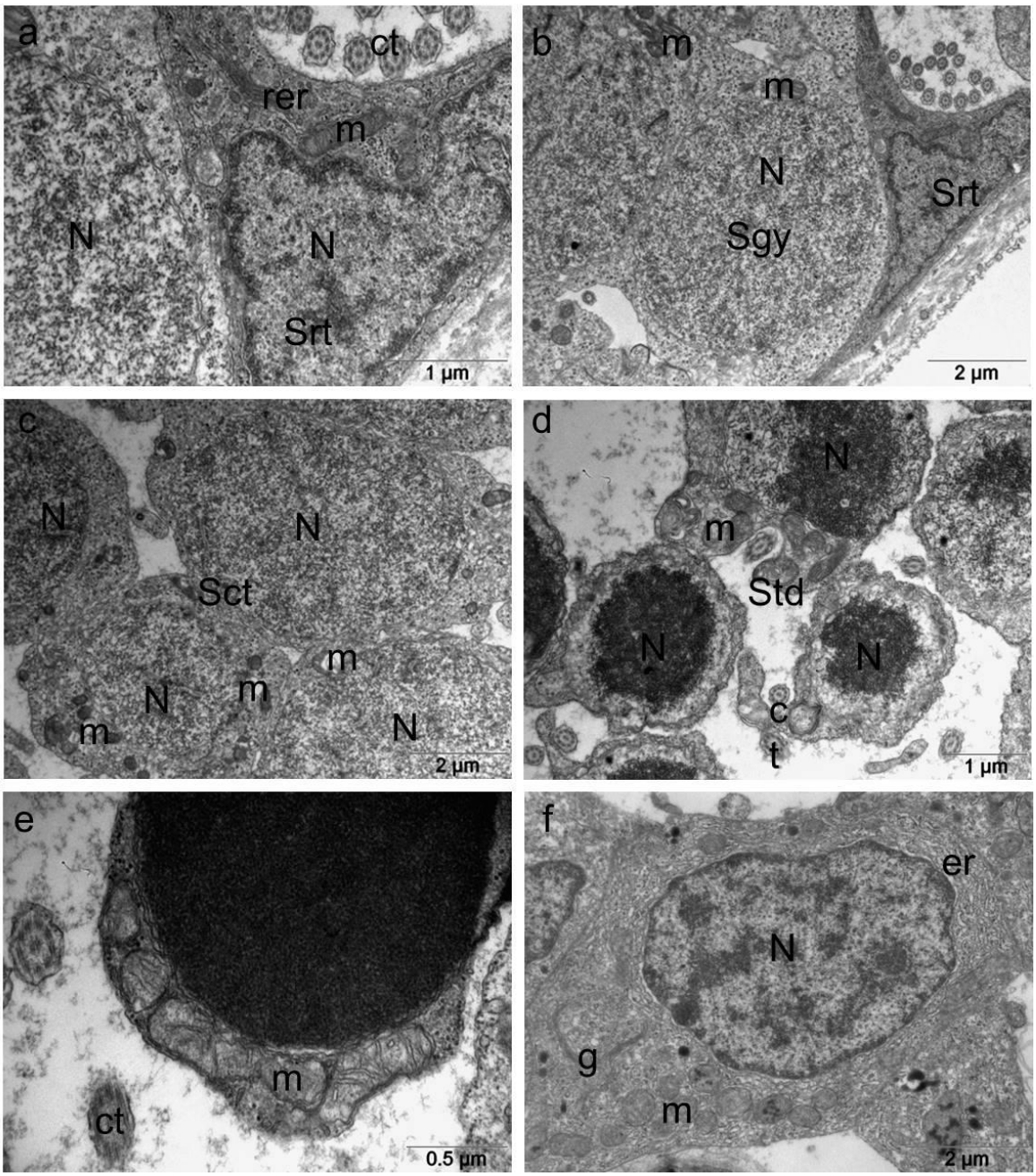

Figure 2: Ultrastructure of the cells in the control group; a) Sertoli cell with its intact mitochondria and other cytoplasmic organelles; b) Intact spermatogonium cell morphology; c) Spermatocyte cell with normal mitochondrial morphology; d) Spermatid cell with condensed genetic material; e) Spermatozoa with mitochondria with clear cristae; f) Leydig cell with clear Golgi apparatus, endoplasmic reticulum cisterns and mitochondria with intact morphology. N: nucleus, m: mitochondria, rer: rough endoplasmic reticulum, ct: centriol, Srt: Sertoli cell, Sgy: Spermatogonium, Sct: Spermatocyte, Std: Spermatid, g: Golgi apparatus, er: endoplasmic reticulum.

Mitochondrial degeneration was visible as swelling of mitochondria, cristae loss and vacuolization of the mitochondrial matrix (Fig. 5f).

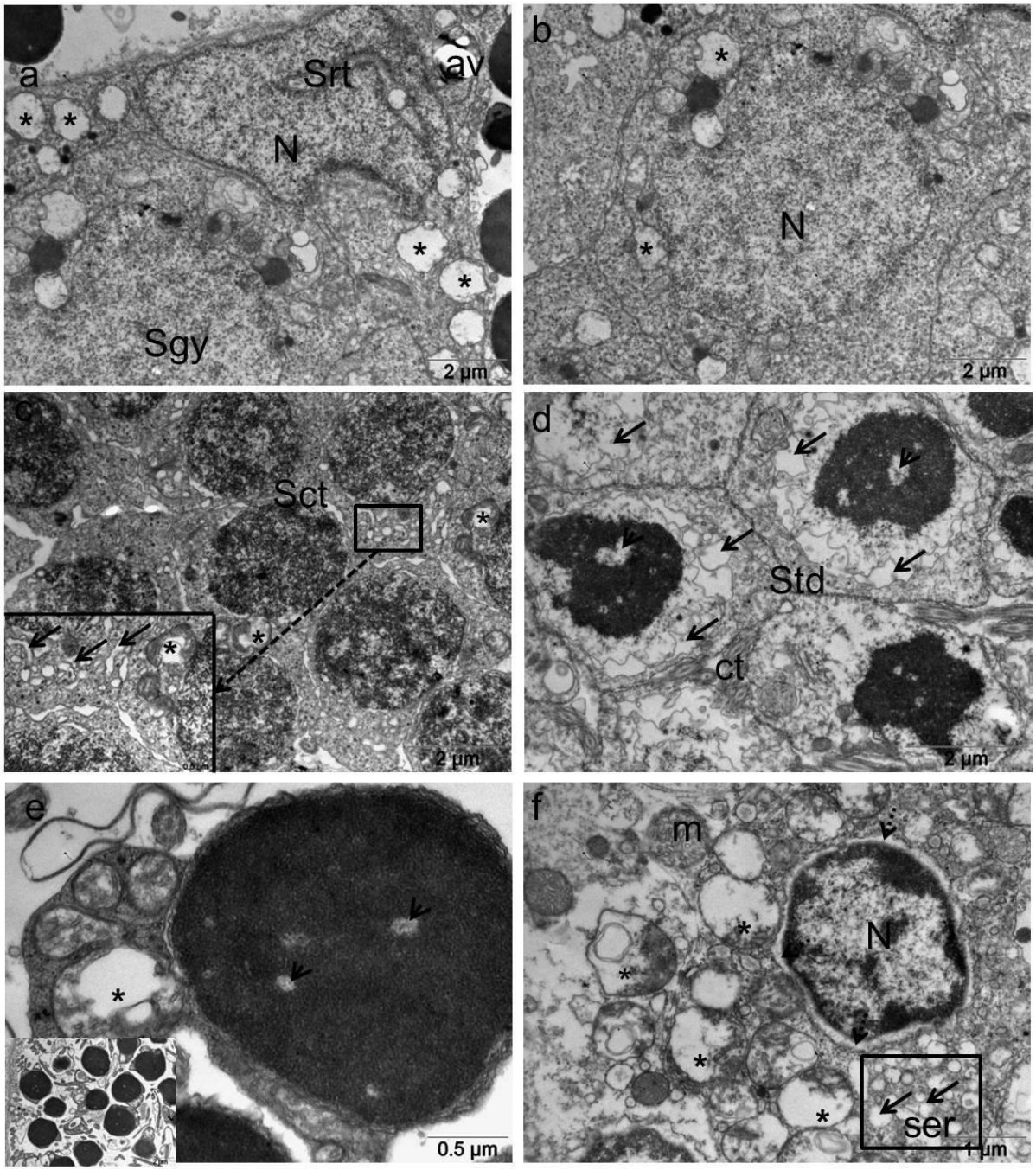

Figure 3: 0,2mM TIA treated group; a) Sertoli cell showed autophagic vacuole (av) and degenerated, swelled mitochondria (asterisk); b) Spermatogonium showed loss of mitochondrial cristae (asterisk); c) Spermatocyte with degenerated mitochondria (asterisk) and dilated endoplasmic reticulum (rectangle), inset: bigger magnification of dilated endoplasmic reticulum cisterns (arrows); d) Spermatids with gaps between nuclear membrane and cytoplasm (arrows) and areas of decondensed genetic material (arrowhead); e) Spermatozoa showed areas of decondensed genetic material (arrowhead), degenerated mitochondria (asterisk), inset: general morphology; f) Leydig cell showed dilated perinuclear space (dotted arrow), degenerated mitochondria and dilated smooth endoplasmic reticulum tubules (arrows). N: nucleus, av: autophagic vacuole, ct: centriole, m: mitochondria, Srt: Sertoli cell, Sct: Spermatocyte, Std: Spermatid.

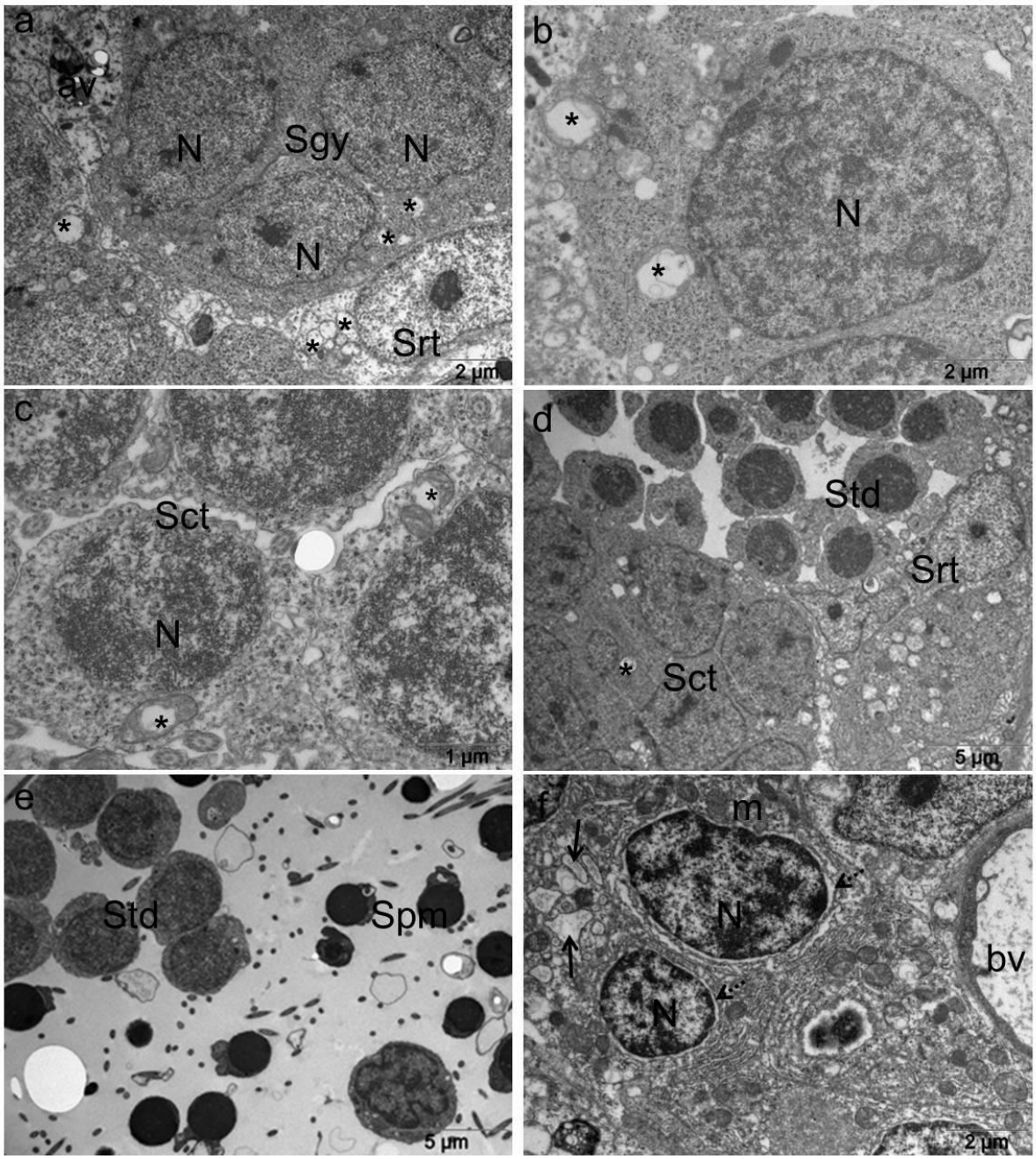

Figure 4: 0,4mM TIA treated group; a) Sertoli cells with degenerated mitochondria (asterisk) and autophagic vacuoles (av); Mitochondria with loss of cristae were seen in b) Spermatogonium, c) Spermatocytes and d) Spermatids; e) Spermatids were detected in the spermatozoa region; f) Leydig cells showed dilated perinuclear space, dilated smooth endoplasmic reticulum tubules. N: nucleus, av: autophagic vacuole, bv: blood vessel, Srt: Sertoli cell, Sgy: Spermatogonium, Sct: Spermatocyte, Std: Spermatid, Spm: Spermatozoa.

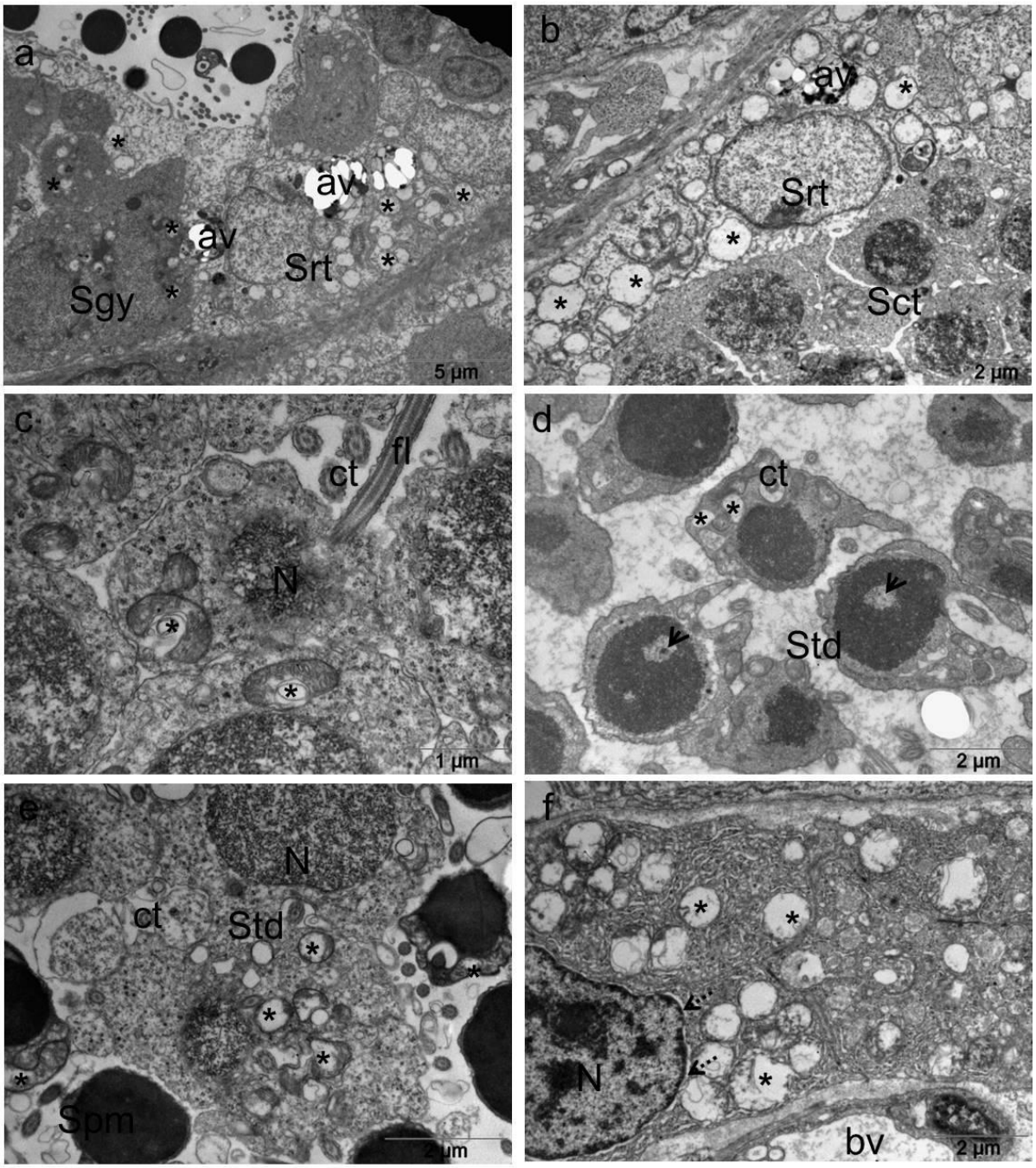

Figure 5: 0,6mM TIA treated group; a) Sertoli cells with autophagic vacuoles (av) and degenerated mitochondria and spermatogonium with irregular plasma membrane; b) Sertoli cells showed swelling mitochondria; c, d, and e) Spermatids showed vacuolized mitochondria (asterisk), decondensed genetic material areas (arrowheads); f) Leydig cell with dilated perinuclear space (dotted arrows), mitochondria with swelling and loss of cristae. av: autophagic vacuole, ct: centriole, N: nucleus, f: flagellum, bv: blood vessel, Srt: Sertoli cell, Sgy: Spermatogonium, Sct: Spermatocyte, Std: Spermatid.

Discussion

The effects of thiazolidinones on different vertebrates as well as sea creatures have been investigated. They show antifungal and antibacterial effects on unicellular organisms, in addition were used as an effective oral antidiabetic drug. Types of thiazolidinones such as Troglitazone, Rosiglitazone and Pioglitazone were used to treat type 2 diabetes. These drugs inhibit insulin resistance and induce insulin sensitivity (Zhang *et al.,* 2022; Eggleton and Jialal, 2023). Owing to their pharmacological effects, thiazolidinones are widely used in medicine therefore long term use of thiazolidinones can cause various side effects such as edema, weight gain, and hepatotoxicity. They can also affect steroid hormone production and cause reproductive problems. It has stimulatory effects on female steroid hormones while it has inhibitory effects on male steroid hormones (Eggleton and Jialal, 2023). Some researchers reported that thiazolidinones derivatives exhibit side effects on the male reproductive system by affecting steroidogenesis and androgenic activity. Couto *et al*. (2010) showed that Rosiglitazone, a type of thiazolidinone, caused degenerative changes such as swelling and vacuolization in mitochondria and sER vesiculation in the Leydig cells of Wistar albino rats, resulting a decrease in testosterone production. Equally important, they can show toxic impacts on aquatic organisms due to their decomposition in the aquatic environment. Akbulut *et al.* (2017) investigated the possible toxic effects of (4S)-2- (4-hydroxy-3-methoxyphenyl) thiazolidine-4-carboxylic acid on zebrafish embryo development and concentration related pericardial edema, tail malformations, decrease of the apoptotic rate and increase in the death rate has been reported. It has also been detected that (4S)-2- (4-hydroxy-3-methoxyphenyl) thiazolidine-4-carboxylic acid causes vacuolization and degeneration both in hepatocyte cells and Kupffer cells and damage of sinusoidal capillaries in zebrafish liver tissue (Yon *et al.,* 2017).

In our study, we have observed negative impacts of thiazolidinone on different cell types of zebrafish testis. As such degeneration of mitochondria due to loss of cristae, swelling and vacuolization were detected in the leydig cells. We have also seen morphological changes such as enlargement of the sER tubules. These findings are consistent with the existing literature. Leydig cells synthesize testosteron and structural alterations of mitochondria and sER tubules that are responsible for steroid synthesis lead to hormonal disruptions. It has been reported that production of testosterone and dihydrotestosterone decreased in healthy male individuals who were treated with rosiglitazone (Vierhapper *et al.,* 2003). Researchers have reported in studies that thizaolidinones cause suppression of enzymes that play a role in steroid hormone synthesis and secretion *in vitro* (Arlt *et al*., 2001; Kempná *et al*., 2007). Morphological abnormalities seen in the Leydig cells in our study suggest that there may be a decrease in testosterone production due to ultrastructural abnormalities, which have been reported in recent studies.

It has also shown that thiazolidines cause functional pressure on Sertoli cells, that is one of the most important cells in the

seminiferous tubules. A study by Zhang *et al*. (2022) in which they examined the effects of thiazolidinone on chicken Sertoli cells, reported decreased cellular viability, cell proliferation and metabolic activity while increased production of reactive oxygen species (ROS).

The findings of our study showed that thiazolidinone triggered mitochondrial damage and autophagy, especially in the Sertoli cells. Although autophagy is a survival mechanism that cells enter under stressful conditions to protect cell viability, in case of excess stress, the cells may die. We have detected autophagic vacuoles in the cytoplasm of the Sertoli cells by ultrastructural evaluation however, no dose-related necrotic or apoptotic death was observed in either Sertoli cells or spermatogenic cells. One of the reasons that triggers autophagy is oxidative stress which occurs with the increase of ROS (Filomeni *et al.,* 2015). In a study on hepatocytes, researchers reported that thiazolidinedione triggered an increase in ROS level, causing mitochondrial respiratory disorders, decrease in ATP level and deterioration in mitochondrial structure (Hu *et al.,* 2015). Rachek *et al.* (2009) reported that troglitazone causes mitochondrial damage and cell death due to the increase of ROS production in hepatocytes. In light of our findings, we suggest that the doses of the thiazolidine that we used and exposure time might triggered oxidative stress by increasing the ROS level although the threshold of the doses and exposure time were insufficient to cause cell death.

Sertoli cells are the cells in the blood-testis barrier and provide mechanical and metabolic support to all spermatogenic cells. Damage and dysfunction of these cells may also cause adverse effects in the development of spermatogenic cells. Mitochodrial swelling and loss of cristae, endoplasmic reticulum dilatations were observed in all types of the spermatogenic cells of the experimental groups. Mitochondria of spermatozoa have an important role in male gamete motility and damage of these organelles may adversely affect fertility. Also, genetic material decondensations that are a kind of developmental abnormality were also seen in spermatids and spermatozoa of the experimental groups. In addition, spermatids were seen in the lumen of the tubule with released spermatozoa. These findings suggest that (4S)-2-(4-Hydroxy-3-Methoxyphenyl) Thiazolidine-4-carboxylic acid may cause developmental delay of spermatogenic cells in the experimental groups. There is no recent study that shows the effects of thiazodinones on spermatogenic cells. So this is a new and important finding of our study.

Our investigation has revealed the ultrastructural degenerative effects of (4S)-2-(4-hydroxy-3-methoxyphenyl) thiazolidine-4-carboxylic acid on zebrafish testicular cells. This chemical is an important thiazolidine derivate that might be used in the pharmaceutic industry in the future. Our findings suggest that the use of this chemical should be carefully applied.

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Conflicts of interest

The authors declare no conflict of interest.

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