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Research Article

THE EFFECTS OF VITAMIN C ON GLYCIDAMIDE-INDUCED CELLULAR DAMAGE AND APOPTOSIS IN MOUSE LEYDIG CELLS

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Abstract: The aim of this study was to elucidate the role of vitamin C on glycidamide-induced cytotoxicity, oxidative damage and cell death in Leydig (TM3) cells. Leydig cells were exposed to glycidamide (1, 10, 100 and 1000 μM) and/or vitamin C (50 μM) for 24 h. After completion of the exposure time, cell viability, amount of lactate dehydrogenase enzyme, apoptosis-necrosis rates, levels of oxidative stress parameters such as hydroxyl radical, hydrogen peroxide and lipid peroxidation were determined in Leydig cells. The results showed that glycidamide administration decreased Leydig cell viability and increased cytotoxicity significantly at high concentration (1000 μM). In addition, glycidamide generated oxidative damage by significantly increasing the production of reactive oxygen species and lipid peroxidation. Exposure to glycidamide increased the formation of early apoptosis, apoptosis and necrosis in Leydig cells. Consequently, glycidamide has been shown to cause apoptosis due to lipid peroxidation and formation of reactive oxygen species in Leydig cells, and vitamin C has a therapeutic role against toxicity caused by glycidamide.

Key words: Glycidamide, cytotoxicity, apoptosis, oxidative stress, Vitamin C, Leydig cell.

Özet: Bu çalışmanın amacı, C vitamininin Leydig (TM3) hücrelerinde glisidamid kaynaklı sitotoksisite, oksidatif stres ve apoptoz üzerindeki rolünü ortaya çıkarmaktır. Leydig hücreleri 24 saat boyunca glisidamid (1, 10, 100 ve 1000 μM) ve / veya C vitamini (50 μM) ile muamele edilmiştir. Deney süresinin tamamlanmasından sonra, Leydig hücrelerinde hücre canlılığı, laktat dehidrogenaz enzimi miktarı, apoptoz-nekroz oranları, hidroksil radikali, hidrojen peroksit ve lipit peroksidasyonu gibi oksidatif stres parametrelerinin seviyeleri belirlendi. Sonuçlar glisidamid uygulamasının yüksek konsantrasyonda (1000 μM) önemli ölçüde Leydig hücre canlılığını azalttığını ve sitotoksisitenin arttığını gösterdi. Ayrıca, glisidamid reaktif oksijen türlerinin ve lipid peroksidasyonunun üretimini önemli ölçüde arttırarak oksidatif hasara yol açmıştır. Glisidamide maruz kalma, Leydig hücrelerinde erken apoptoz, apoptoz ve nekroz oluşumunu artırmıştır. Sonuç olarak, glisidamid lipit peroksidasyonuna ve Leydig hücrelerinde reaktif oksijen türlerinin oluşmasına bağlı apoptosise neden olmuş, C vitamininin ise glisidamidin neden olduğu toksisiteye karşı iyileştirici bir rolü olduğu gösterilmiştir.

Introduction

Acrylamide is produced when vegetal products with high carbohydrate and low protein content are exposed to elevated temperatures (e.g. 120°C and above) during thermal processes such as cooking (Mottram *et al*. 2002, Stadler *et al*. 2002). Food products are the leading sources of acrylamide, and the food with the highest acrylamide content are potato chips, fast food products (hamburgers, all fried sandwiches), fried potatoes, fried bread, biscuits, breakfast cereals, bakery products, instant soups and coffee (Friedman 2015). Acrylamide is used in the enhancement of drinking and wastewater, the production of adhesives in the cosmetic sector, as a filling substance in dentistry, and in the production of paper, photography film and polyester (Lingnert *et al*. 2002, Taeymans *et al*. 2004). Tobacco smoke also serves as a source of acrylamide (Vattem & Shetty 2003). Studies on various experimental organisms have led to acrylamide being classified as a chemical with a serious toxicity profile. The International Agency for Research on Cancer (IARC) classified acrylamide as a Group 2A compound, which refers to compounds with potential carcinogenic effects in humans (IARC 1994). The United Nations Food and Agriculture Organization (FAO) and the World Health Organization (WHO) also classify acrylamide as a toxic substance with potential carcinogenic and teratogenic effects on humans that can be absorbed through the skin and can cause harm in the nervous and reproductive systems (WHO 2002).

Acrylamide is metabolized in the body via the CYP2E1 enzyme and converted to glycidamide, an epoxide derivative. (Fennel *et al*. 2005). In terms of its chemical activity, glycidamide is a more reactive (70%) compound than acrylamide, showing a high level of interaction with hemoglobin, and particularly with DNA

molecule (Paulsson *et al*. 2002, Martins *et al*. 2006). Previous studies reported that glycidamide plays a leading role in the development of the effects of acrylamide on the reproductive system (Li *et al*. 2017, Yilmaz *et al*. 2017, Sun *et al*. 2018). Studies performed on males of various animal models suggested that acrylamide exposure caused an atrophy of the seminiferous tubules in the testes, multiple nuclei formation, vacuolization, apoptosis of cell, aberration of sperm chromosomes, decreased sperm viability and abnormal giant cell formation, as well as impairment in spermatogenesis (Wang *et al*. 2010, Hamdy *et al*. 2012, Sen *et al*. 2015, Wang *et al*. 2015). Although there are studies on the toxic mechanism of acrylamide on various cells in the testes and the reproductive system, studies on the glycidamide, an epoxide derivative known to be more toxic than acrylamide, are low in number. Therefore, the direct mechanism(s) by which glycidamide performs toxicity in the testes has not been fully understood.

Various antioxidants are required to minimize the negative health effects of exposure to different toxic compounds. Vitamin C is distributed around the body and has a strong antioxidant effect in the neutralization of free radicals (Baba *et al*. 2013). Vitamin C enters the body predominantly through diet, with natural vitamin C sources including citrus fruits, rosehip, green walnut, cabbage, tomato, red and green pepper and all green leaved vegetables (Levine *et al*. 1999). It has been reported that vitamin C has anti-oxidative, anti-proliferative and anti-inflammatory capacities (Padayatty *et al*. 2003). It also plays a protective role against oxidative stress, stimulates cell division and reproduction, protects sperm from harmful oxidative processes and improves fertility (Das *et al*. 2002, Chang *et al*. 2007, Orta & Erkan 2014).

Leydig cells, characterized by their high hormonal activity, are over-specialized somatic cells found in interstitial area of testes (Haider 2004). The main function of Leydig cells is to synthetize and release testosterone, and they are the target of toxic substances that affect the reproductive system. In the present study, Leydig cells were used as an *in vitro* model to investigate the direct mechanisms underlying the toxic effects of glycidamide in the testes. Therefore, we aimed to evaluate the toxicity caused by glycidamide on Leydig cells, focusing on the changes in cytotoxicity, oxidative damage and apoptosis/necrosis levels. We also investigated whether vitamin C played a protective role in this damage.

Materials and Methods

Cell Culture and Treatment

The TM3 Leydig cell line was obtained from the American Type Culture Collection (ATCC): The Global Bioresource Center and was grown *in vitro* conditions. A cell culture medium containing 50:50 DMEM-F12 (Dulbecco's Modified Eagle Medium, nutrient mix F12 Ham medium) supplemented with 5% horse serum, 2.5% fetal bovine serum, 2.5 mM l-glutamine, 0.5 mM sodium pyruvate, 1.2 g / L Sodium Bicarbonate, 15 mM HEPES and 1% penicillin-streptomycin-amphoterin mixture was used. Cells were incubated in sterile conditions at 37°C in an incubator with 5% CO_2 and 95% air. Glycidamide (GA, Sigma Chemical Company) was dissolved in medium containing 1% horse serum for administration to the cells to obtain a 10 mM stock solution. Different concentrations of glycidamide were prepared from this stock solution by diluting with medium containing 1% horse serum. In the experiments, the concentration of vitamin C was 50 μM, which is the average antioxidant concentration *in vitro*. The vitamin C (VitC) was dissolved in 1% horse serum and was prepared freshly.

Cytotoxicity

Cell Viability Assay

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay (Roche Diagnostics GmbH, Mannheim, Germany) was used to determine the viability of the cells. The viability of the control cells not exposed to the test substance GA was accepted as 100%. After 24 h of incubation with GA, maximal inhibitor concentrations of 50% (IC_{50}) for cell number reduction were calculated. Based on the calculations, GA concentrations of 1, 10, 100 and 1000 μM were selected for use in further experiments.

Lactate Dehydrogenase (LDH) Assay

The amount of lactate dehydrogenase enzyme was determined using the Cytotoxicity Detection Kit (Roche Diagnostics GmbH, Mannheim, Germany) from cell supernatants of cells exposed with GA. The kit procedure was applied individually, and the results were measured with ELISA reader device at 492 nm. The cytotoxicity of the control cells not exposed with the test substance was accepted as 100%.

Measurement of Lipid Peroxidation and Reactive Oxygen Species

For biochemical experiments, cells were seeded to ($1x10^6$) six-well plates per well. After 24 hours of application of GA and vitamin C, the cells were sonicated in Tris-HCl buffer (pH: 7.2) with an ultrasonicator. After the resulting cell suspension was centrifuged at 14,000 g in a refrigerated centrifuge, the supernatants were collected and the lipid peroxidation, hydroxyl radical and hydrogen peroxide (H_2O_2) measurements were performed.

The amount of lipid peroxidation was measured according to the method of Devasagayam & Tarachand (1987). As is the case in this method, the formation of malondialdehyde (MDA) in the presence of thiobarbituric acid was calculated by measuring the absorbance with the spectrophotometer at 532 nm.

Levels of hydroxyl radical was determined according the method of Puntarulo & Cederbaum (1988). This method is based on the formation of a formaldehyde by hydroxyl radical. Formaldehyde gives an absorbance at 570 nm with trichloroacetic acid (TCA).

Holland & Storey (1981) method was used for determination of amounts of H_2O_2. The amounts of H_2O_2 resulting from the oxidation of the acidified ferrocytochrome c were measured with a spectrophotometer at 550 nm.

Detection of apoptosis and necrosis

Hoechst 33342 (HO342) was used to determine apoptosis in cells and Propidium iodide (PI) fluorescence labeling method was used to determine necrosis. HO342 fluorescent dye marks apoptotic cells in purple color, while PI fluorescent dye passes through the weak cell membrane of necrotic cells and marks the cell in red color. The combination of these dyes makes it possible to distinguish viable, early apoptotic, apoptotic and necrotic cell populations with fluorescence microscopy. Cells were seeded in culture well-plates as 10^4 cells per well. After 24 hours of glycidamide and vitamin C administration, the cells were washed with phosphate-buffered solution (PBS) and incubated with PI (1 mg/ml PI) and HO342 (1 mg/ml Hoechst 33342) solutions for 20 min at 37°C. When the experiment was completed, the cells were washed with PBS and examined under fluorescent microscope (Olympus IX71, New York, USA) equipped with UV filter and photographed in equal intervals with a digital camera (Olympus DP72, New York, USA). For each group, the fluorescence-emitting cells were counted separately, and the mean percentage values were calculated. For each group, viable, early apoptotic, apoptotic and necrotic cells were counted by screening 10-15 sites and 1000 cells in 12 wells in three different experiments.

Statistical analysis

All experimental data were analyzed with one-way ANOVA, followed by Tukey post-hoc test using the software program GraphPad Prism Version 5.0 (GraphPad Software, San Diego, CA, USA). All values were expressed as mean ± standard error mean. Shapiro-Wilk test was used as the normality test and the significance was accepted as $p<0.001$, $p<0.01$ and $p<0.05$.

Results

Effects of GA and GA+VitC on Cytotoxicity

The MTT test was carried out to determine the effects of ten different glycidamide concentrations on 24-hour cell viability of Leydig cells. Figure 1 (one-way ANOVA; $F_{0.95}(10,66)=267.1$; $p<0.001$) shows cell viability rates (%) in the control and experimental groups. When the MTT values of the experimental groups were compared with that of the control group, significant reductions were noted in the groups exposed to glycidamide at concentrations of 5 μM and higher ($p<0.05$, $p<0.01$ and $p<0.001$). Fifty percent inhibition concentration (IC_{50}) of glycidamide was calculated according to MTT results for 24 h and the IC_{50} value for glycidamide in Leydig cells was found to be 872.4 μM.

Glycidamide concentrations that reduced cell viability to 94.61% (1 μM GA), 92.29% (10 μM GA), 78.30% (100 μM GA) and 42.34% (1000 μM GA) were selected and treated with vitamin C (50 μM). The MTT value of the control group was compared with four different glycidamide concentration groups selected and the concentration groups formed with the addition of vitamin C (Fig. 2; one-way ANOVA; $F_{0.95}(9,70)=24.43$; $p<0.0001$). The comparisons revealed significant reduction in cell viability in the groups exposed to glycidamide at concentrations of 10 μM and above, and in combined groups of 100 and 1000 μM concentrations treated with vitamin C ($p<0.05$ and $p<0.001$). When the groups treated with vitamin C were compared only with the glycidamide groups, vitamin C was found to significantly increase cell viability at a glycidamide concentration of 1000 μM ($p<0.05$).

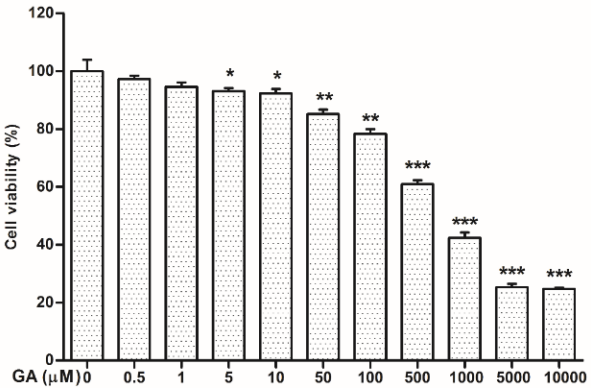

Fig. 1. The viability of TM3 Leydig cells exposed to various concentrations of GA. *p<0.05, **p<0.01 and ***p<0.001, (*) compared with control.

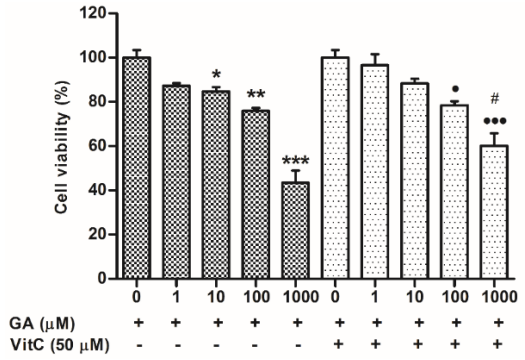

Fig. 2. Effects of glycidamide and vitamin C on cell viability of TM3 Leydig cells *in vitro*. *p<0.05, ** p<0.01 and ***p<0.001. (*) comparison with the control group, (•) comparison with Vit C combination and (#) comparison with glycidamide alone group.

Concentration-dependent changes in LDH levels in the control and experimental groups in TM3 Leydig cells are given in Fig. 3 (one-way ANOVA; $F_{0.95}(9,69)=22.21$; $p<0.0001$). When the LDH levels of the control group and the groups exposed to four different glycidamide concentrations were compared after 24 hours, a significant increase was noted at 1000 μM glycidamide concentration ($p<0.001$). On the other hand, the comparison of LDH levels of the control group with the groups formed with addition of Vitamin C revealed a

significant reduction only in the 1000 µM glycidamide concentration group ($p<0.001$).

Effects of GA and GA+VitC on Lipid Peroxidation and Reactive Oxygen Species

Figure 4A shows the lipid peroxidation values in TM3 Leydig cells of the control and experimental groups, as estimated after 24 hours through the use of spectrophotometric methods. The comparison of the MDA values of the control group with those of four different glycidamide concentration groups revealed significant increase for concentrations of 10 µM and above ($p<0.001$). In the groups treated with vitamin C, significant reductions were noted in glycidamide concentrations of 10 µM and above when compared to the groups not treated with vitamin C ($p<0.001$) (Fig 4A; one-way ANOVA; $F_{0.95}(9,69)=25.34$; $p<0.0001$).

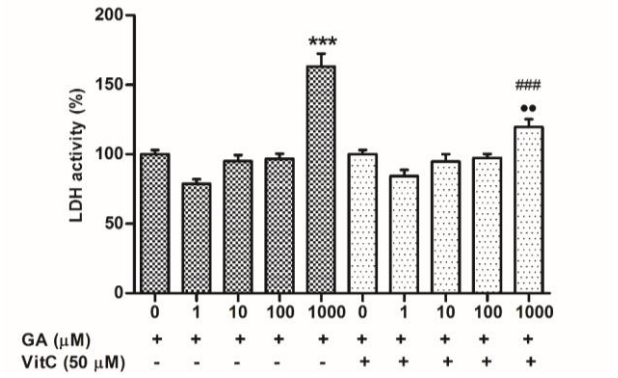

Fig. 3. Effects of glycidamide and vitamin C on lactate dehydrogenase activity of TM3 Leydig cells *in vitro*. *p<0.05, ***p<0.001. (*) comparison with the control group, (•) comparison with VitC addition and (#) comparison with glycidamide alone group.

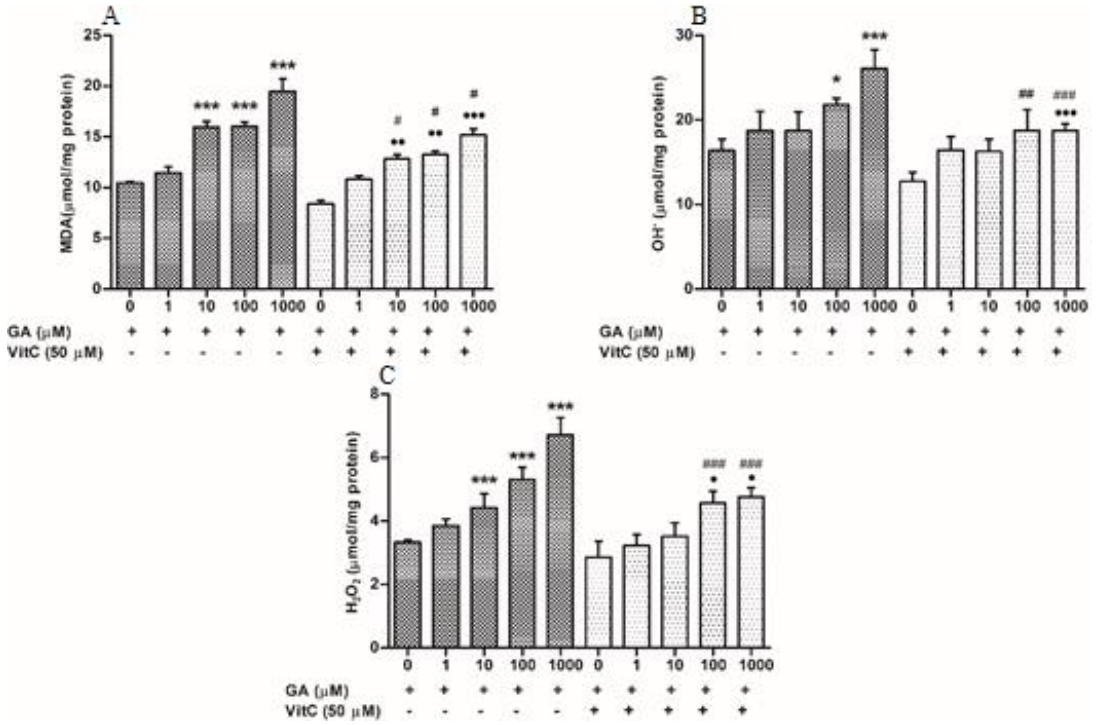

Fig. 4. Effects of glycidamide and vitamin C on lipid peroxidation (A), hydroxyl radical (B) and hydrogen peroxide (C) levels in TM3 Leydig cells *in vitro*. *p<0.05, **p<0.01 and ***p<0.001. (*) comparison with the control group, (•) comparison with VitC addition and (#) comparison with glycidamide alone group.

Table 1. Effects of GA and GA+VitC on viable cell, early apoptosis, apoptosis and necrosis of TM3 Leydig cells.

	Concentrations	Viable Cell	Early Apoptosis	Apoptosis	Necrosis
GA groups	**0**	97.4±0.4	1.6±0.3	0.7±0.1	0.3±0.01
	1 µM	90.3±0.7	8.3±0.6*	0.8±0.1	0.6±0.02
	10 µM	87.8±1.8**	10.3±1.5***	1.8±0.3***	0.1±0.3
	100 µM	75.9±0.4***	19.6±0.2***	4.2±0.1***	0.3±0.2
	1000 µM	42.3±2.0***	33.8±1.3***	10.2±0.5***	13.7±1.1***
GA+VitC groups	**0**	98.1±0.1	1.7±0.2	0.1±0.1	0.1±0.06
	1 µM	97.2±0.5 #	2.4±0.3 #	0.1±0.1	0.3±0.04
	10 µM	95.1±0.4 #	4.1±0.4• #	0.3±0.1#	0.5±0.06
	100 µM	84.3±1.1••• #	13.5±0.8••• #	1.8±0.3•• #	0.4±0.07
	1000 µM	64.5±2.0••• #	29.1±1.8••• #	4.1±1.0•• #	2.3±1.1• #

* p<0.05, **p<0.01, ***p<0.001. (*) comparison with the control group, (•) comparison with VitC addition and (#) comparison with glycidamide alone group. Viable cell (one-way ANOVA; $F_{0.95}(9,45)=58.31$; $p<0.0001$), early apoptosis (one-way ANOVA; $F_{0.95}(9,36)=15.33$; $p<0.0001$), apoptosis (one-way ANOVA; $F_{0.95}(9,45)=12.14$; $p<0.0001$) and necrosis (one-way ANOVA; $F_{0.95}(9,45)=8.822$; $p<0.0001$).

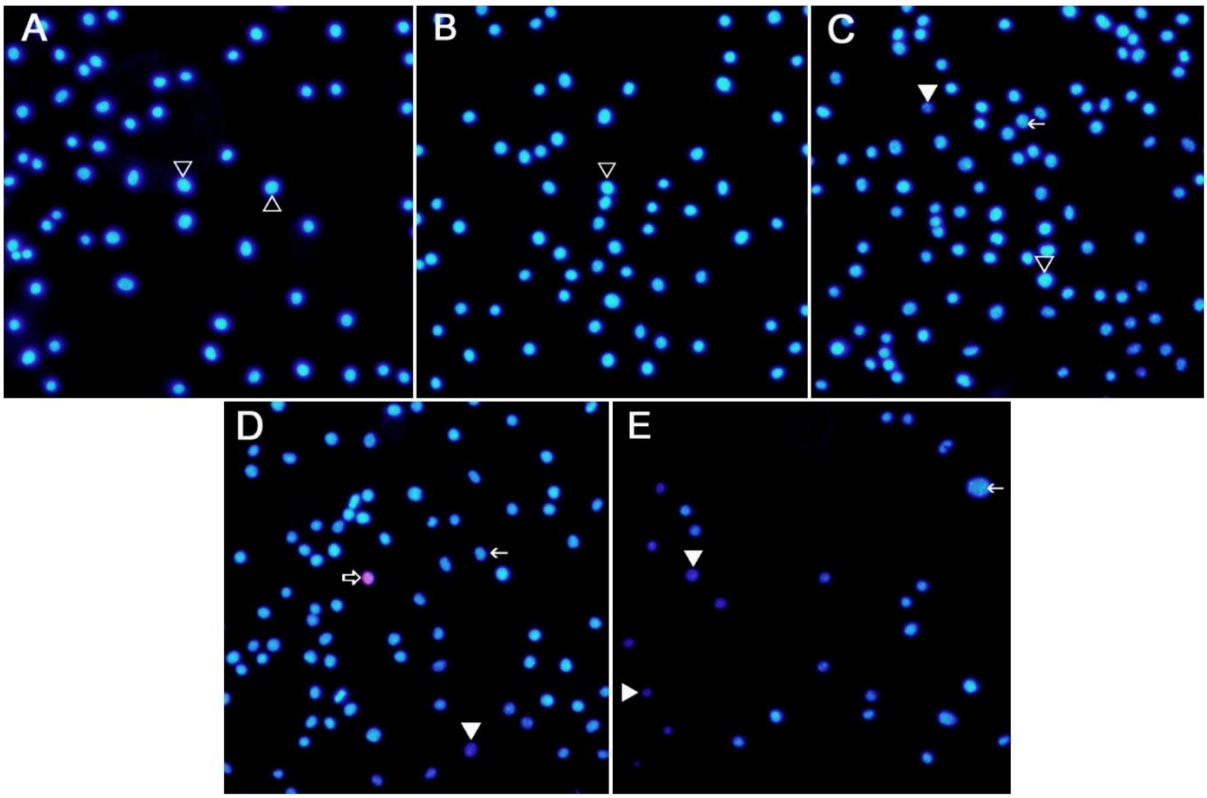

Fig. 5. Early apoptosis, apoptosis, necrosis findings in 24 h glycidamide exposure in TM3 Leydig cells. (A) Control, (B) 1 µM GA, (C) 10 µM GA, (D) 100 µM GA, (E) 1000 µM GA; ▽: viable cell, ←: early apoptosis, ▼: apoptosis, ⇨: necrosis. GA: Glycidamide. (10X magnification).

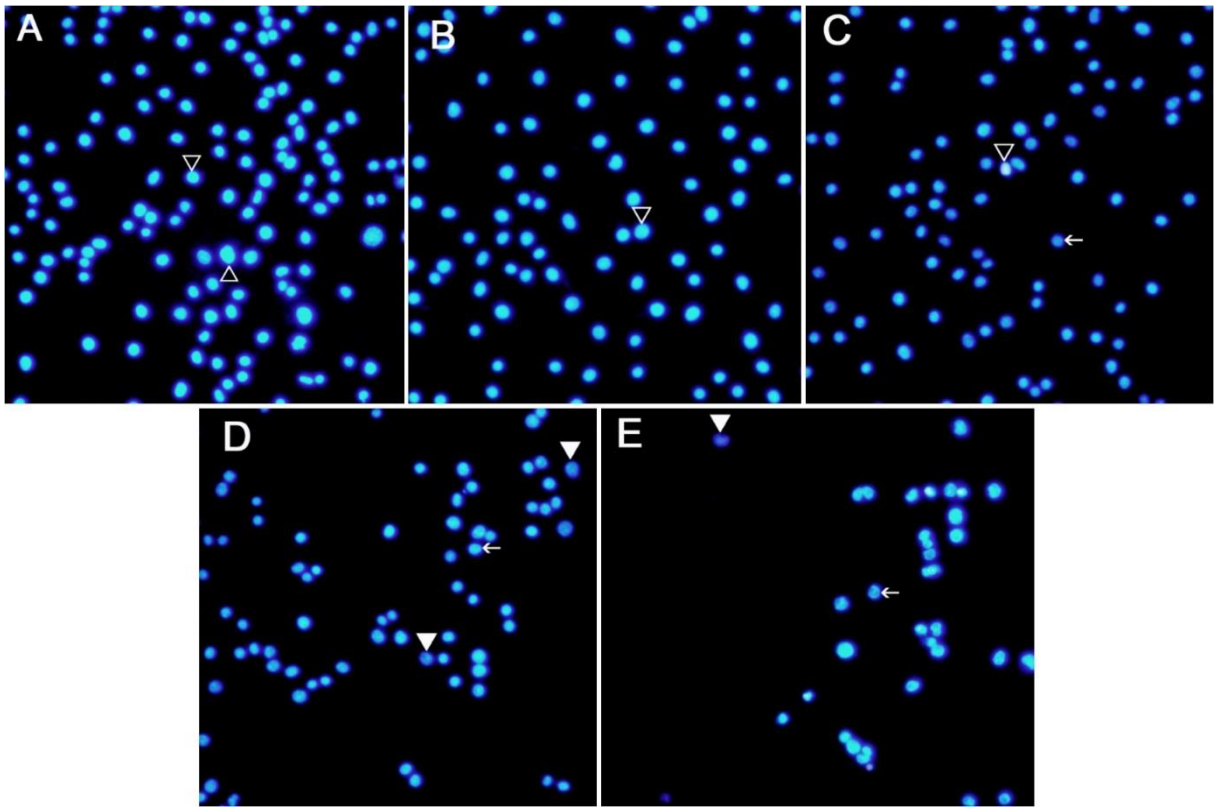

Fig. 6. Early apoptosis, apoptosis, necrosis findings in 24 h glycidamide and vitamin C exposure in TM3 Leydig cells. (A) Vitamin C, (B) 1 µM GA+VitC, (C) 10 µM GA+VitC, (D) 100 µM GA+VitC, (E) 1000 µM GA+VitC; ▽: viable cell, ←: early apoptosis, ▼: apoptosis, ⇨: necrosis. GA: Glycidamide, VitC: Vitamin C. (10X magnification).

When the levels of hydroxyl radicals in TM3 Leydig cells of the control and glycidamide groups were compared after 24 hours, significantly higher increases were noted in the glycidamide concentrations of 100 and 1000 μM ($p<0.05$ and $p<0.001$) (Fig.4B; one-way ANOVA; $F_{0.95}(9,81)=4.129$; $p<0.0002$). When the levels of hydroxyl radicals of the control group, four different glycidamide concentration groups and the groups treated with vitamin C were compared, significantly higher reductions were noted in the glycidamide concentrations of 100 and 1000 μM in vitamin C treated groups ($p<0.01$ and $p<0.001$).

Hydrogen peroxide levels in control and GA-exposed TM3 Leydig cells. were given in Fig. 4C. The comparison of hydrogen peroxide levels in the control and glycidamide groups after 24 hours showed significant increases in the concentration groups of 10 μM and above ($p<0.001$). When the hydrogen peroxide levels of the GA and GA+VitC groups were compared, significant decreases were detected in the 100 and 1000 μM glycidamide concentration groups treated with vitamin C ($p<0.001$) (Fig 4C; one-way ANOVA; $F_{0.95}(9,80)=9.125$; $p<0.0001$).

Apoptosis and necrosis rate in TM3 Leydig cells exposed to GA and GA+VitC

When the percentages of viable, early apoptotic, apoptotic and necrotic cells were compared, significant differences were observed in the glycidamide-treated groups when compared to the control groups (Table 1, Figs. 5-6). According to the results of fluorescence emission, the morphological changes observed in the experimental groups have the following characteristics: i) viable cells have highly organized nuclei and appear bright blue, ii) early apoptotic cells have nuclear condensation and appear blue, iii) apoptotic cells have highly nuclear condensation, fragmentation and appear dull blue to purple, iv) necrotic cells have no chromatin fragmentation and appear red.

Discussion

Studies on acrylamide showed that exposure is not solely related to diet, as it may also result from environmental sources. This has led to an increase in number of studies worldwide investigating acrylamide levels in both food and environmental sources. The direct or indirect intake of acrylamide into the body can cause various changes in cells which are known to be a highly dynamic system. Glycidamide, a major epoxide metabolite of acrylamide, possesses greater cytotoxicity and genotoxicity than acrylamide (Martins *et al*. 2006). Despite a limited number of studies conducted on acrylamide, there is still a need for more laboratory data concerning the toxicity of glycidamide. Thus, it has been demonstrated that glycidamide, a molecule that can enter the body via food, has a toxic effect on the functions of Leydig cells in the reproductive system, and this negative effect can be reduced with natural antioxidants.

Previous studies investigated the effects of acrylamide and glycidamide on viability of various cell lines. The administration of glycidamide on human breast epithelial cell line at concentrations of 1 mM, 2 mM, 3 mM and 4 mM for 24 hours was found to significantly reduce cell viability (Bandarra *et al*. 2013). Studies showed that acrylamide significantly reduced cell viability when applied to human astrocytoma cells and rat astrocyte cells at concentrations of 0.1 mM, 0.5 mM, 1 mM and 2 mM (Chen *et al*. 2013, Lee *et al*. 2014). Baum *et al.* (2005) conducted a study on V79 cells and human blood and determined that acrylamide was not cytotoxic at concentrations below 5000 μM, while glycidamide was cytotoxic at concentrations of 800 μM and above, with an LC_{50} concentration of 2000 μM for glycidamide. Another study investigating the toxic effects of glycidamide on RC2 Leydig cells found that glycidamide significantly reduced cell viability, with IC_{25}, IC_{50} and IC_{75} values being calculated as 0.635, 0.872 and 1.198 mM, respectively (Li *et al.* 2017). Many *in vivo* studies demonstrating the toxic effects of acrylamide on the male reproductive system also reported a significant reduction of Leydig cell viability as a result of administration of acrylamide on various laboratory animals (Yang *et al.* 2005, Ma *et al.* 2011, Camacho *et al.* 2012). Several antioxidants have been tested in studies to remove the toxic effects of acrylamide and glycidamide. In these studies, various antioxidants such as Vitamin C (Soliman *et al.* 2013), hesperetin (Shrivastava *et al.* 2018), L-carnitine (Zamani *et al.* 2017) and carnosic acid (Albalawi *et al.* 2017) were used to prevent acrylamide toxicity, and allicin (Wang *et al.* 2015) was used to prevent glycidamide toxicity. In the present study, the administration of glycidamide at concentrations of 5 μM and above significantly reduced Leydig cell viability, as in the case of acrylamide studies performed on other cell lines. However, glycidamide also reduced cell viability but at quite low concentrations in contrast to acrylamide. In addition, Vitamin C demonstrated a protective effect on cell viability at glycidamide concentrations as high as 1000 μM.

An increase in the cellular quantity of the lactate dehydrogenase enzyme, which is found in all tissues and cell cytoplasm, is an indication of cell cytotoxicity. Studies on various animals and cell lines/types showed that acrylamide and glycidamide exposure disrupted cell membrane integrity, causing a noticeable suppression of LDH activity (Chen *et al*. 2009, Zhang *et al.* 2013, Wang *et al.* 2015). In a recent study in which Leydig and Sertoli cells were exposed to 10 and 1000 μM acrylamide and 1 and 500 μM glycidamide, it was found that a significant increase was noted in lactate dehydrogenase levels in both cell lines for the applied concentrations (Yılmaz *et al.* 2017). The study of Yıldızbayrak and Erkan (2018) on Leydig cells revealed a significant increase in cell cytotoxicity at acrylamide concentrations of 100 μM and above. In a previous *in vivo* study measuring LDH levels in rats exposed to acrylamide, a significant increase was determined in enzyme levels when compared to the control group (Soliman 2013). The same study also administered Vitamin C to mitigate acrylamide-induced

cytotoxicity, resulting in a significant decrease in LDH levels (Soliman 2013). In another study with rats, glycidamide was found to increase LDH levels, while allicin, a natural antioxidant, was observed to reduce this cytotoxicity (Wang *et al.* 2015). In the light of these findings and given that the high glycidamide concentrations (1000 µM) applied in the present study led to a significant increase in LDH levels and that Vitamin C was found to reduce this form of cytotoxicity in former studies, it can be stated that excessive glycidamide exposure necessitates the use of antioxidants.

Reactive oxygen species (ROS) are metabolites that form naturally in cells during the conversion of nutrients into energy through the use of oxygen. Leydig cells can overcome the free radicals formed during normal energy metabolism by utilizing their antioxidant defense system (Sun *et al*. 2018), although an excess of reactive oxygen species that form in cells due to the presence of toxic substances can alter the oxidant-antioxidant balance, and the resulting reactive oxygen species may cause significant damage to nucleic acids, cell membranes and proteins (Zhang *et al.* 2010). Studies showed that acrylamide and glycidamide can cause toxicity, resulting in the formation, at a mitochondrial level, of excess ROS that are known to damage cellular components (Wang *et al.* 2015, Li *et al*. 2017, Sun *et al.* 2018). In the present study conducted with Leydig cells, glycidamide caused hydrogen peroxide formation at concentrations of 10 µM and above, and hydroxyl radical formation at concentrations of 100 µM and above. These findings are in parallel with studies suggesting that glycidamide triggers ROS formation *in vivo* and *in vitro* and demonstrate that glycidamide readily triggers oxidative stress in cells (Wang *et al*. 2015, Sun *et al*. 2018). The administration of Vitamin C in the present study reduced significantly, in a concentration-dependent manner, the formation of reactive oxygen species, indicating that Vitamin C may assume a protective role against glycidamide-induced oxidative stress. In addition, the release of these reactive oxygen species has an oxidizing effect on membrane lipids, disrupting membrane integrity through the formation of lipid peroxidation products (Lee *et al*. 2014). In the present study, glycidamide concentrations of 10 µM and above significantly increased levels of MDA – which is a final product of lipid peroxidation – when compared to the control, while groups administered with Vitamin C showed significantly lower MDA levels than the glycidamide-treated groups. These results are in parallel with studies demonstrating that acrylamide and glycidamide significantly increase MDA levels in various tissues, and that allicin (Wang *et al.* 2015), N-acetylcysteine (Alturfan *et al.* 2012) and garlic oil (Elghaffar *et al*. 2015), which are known as antioxidants, inhibit MDA levels.

Acrylamide and glycidamide were reported to generally trigger apoptotic signals and cause cell death through an increase in oxidative stress (Orta Yilmaz *et al.* 2017). This effect can be attributed to the increase in lipid peroxidation and mitochondrial membrane depolarization associated with oxidative stress (Lee *et al.* 2014). Acrylamide and glycidamide are known to trigger apoptosis in numerous cell lines and various laboratory animal tissues (Yousef & El-Demerdash 2006, Lee *et al*. 2014, Li *et al*. 2017, Kacar *et al.* 2018, Sun *et al*. 2018). In one such study, Li *et al.* (2017) applied glycidamide to R2C Leydig cells at concentrations of 0.635, 0.872 and 1.198 mM, and found, through a Comet test, that glycidamide induced cell apoptosis at an early phase. In a study performed on A549 human lung adenocarcinoma cell line, the ratio of apoptosis in cells exposed to 4.6 mM concentrations of acrylamide was reported as 64 percent (Kacar *et al.* 2018). Chen *et al.* (2013) found that acrylamide at 1 and 2 mM concentrations caused structural changes in the mitochondria of astrocytoma cells, along with mitochondria-dependent apoptosis. In their study with male rats, Yang *et al.* (2005) noted increased Leydig cell death in the testes of acrylamide-administered groups, as well as abnormal histopathological lesions containing apoptotic cells (Yang *et al.* 2005). In the present study, the glycidamide-administered groups of Leydig cells under *in vitro* conditions showed a concentration-dependent increase in apoptosis, accompanied by a significant increase in the ratio of cells in early apoptosis. It was also observed that Vitamin C suppressed apoptosis induced by glycidamide toxicity, and these results are supported by other *in vitro* studies conducted on various cell lines (Chen *et al.* 2013, Sun *et al*. 2018).

Conclusion

Since the mechanism of the effect of glycidamide on the male reproductive system has not yet been elucidated, this study can be relied upon as an *in vitro* model involving Leydig cells. Our findings showed that glycidamide has cytotoxic, anti-proliferative and apoptotic effects on Leydig cells, even at low concentrations. The results also revealed that Vitamin C could be a good antioxidant in preventing the harmful effects of glycidamide on male fertility.

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