

Protective Effect of N-Acetylcysteine on Testicular Oxidative Damage, Spermatological Parameters and DNA Damage in Glyphosate-Based Herbicide-Exposed Rats

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ABSTRACT

The aim of this study was to examine the protective effect of N-acetylcysteine (NAC) on testicular oxidative damage, spermatological parameters and DNA damage caused by Glyphosate (GLF) in rats. In total, twenty-eight Wistar male rats were evaluated by being separated into four groups in an equal way. Rats in group I, which represented the control group, were fed normal diet without GLF or NAC, group II received normal feed containing 160 mg/kg/daily NAC, group III received normal feed containing 375 mg/kg/daily GLF, and group IV received normal feed containing 160 mg/kg/daily NAC + 375 mg/kg/daily GLF. GLF administration decreased sperm motility, abnormal sperm rate, sperm plasma membrane integrity, glutathione level and superoxide dismutase in the rats' testicular tissue. On the other hand, high malondialdehyde level and DNA damage were detected in the group administered with GLF. Besides, in histopathological terms, a decrease in sperm concentration and degeneration of sertoli cells were determined in the testicular tissue. NAC and NAC+GLF administration reversed lipid peroxidation and DNA damage induced by GLF, the activity of antioxidant enzymes and cell integrity in rats' testis. The above-mentioned findings indicate that NAC reduces lipid peroxidation caused by GLF, improves the antioxidant defense mechanism and regenerates tissue damage in rats' testis.

Keywords: DNA damage, Glyphosate, N-acetylcysteine, Rat sperm

Glifosat Bazlı Herbisite Maruz Kalan Sıçanlarda N-Asetilsisteinin Testis Oksidatif Hasarı, Spermatolojik Parametreler ve DNA Hasarı Üzerindeki Koruyucu Etkisi

ÖZ

Bu çalışmanın amacı, N-asetilsisteinin (NAC) sıçanlarda Glifosat (GLF) 'nin neden olduğu testiküler oksidatif hasar, spermatolojik parametreler ve DNA hasarı üzerindeki koruyucu etkisini incelemektir. Toplam yirmi sekiz Wistar erkek sıçan, eşit bir şekilde dört gruba ayrılarak değerlendirildi. Kontrol grubunu olan grup I'deki sıçanlar, GLF veya NAC olmaksızın normal diyetle beslendiler, grup II'deki hayvanlara 160 mg / kg / günlük NAC içeren normal rat diyeti uygulandı, grup III'deki hayvanlara 375 mg / kg / günlük GLF içeren normal rat diyeti verildi ve grup IV'deki hayvanlara 160 mg / kg / günlük NAC + 375 mg / kg / günlük GLF içeren normal rat diyeti uygulandı. GLF uygulaması sıçanların testis dokusunda spermatozoon motilitesini, anormal spermatozoon oranını, spermatozoon plazma membran bütünlüğünü, glutatyon ve süperoksit dismutaz düzeyini azaltmıştır. Diğer taraftan GLF uygulanan grupta yüksek malondialdehid düzeyi ve DNA hasarı saptandı. Ayrıca histopatolojik olarak testis dokusunda spermatozoon konsantrasyonunda azalma ve sertoli hücrelerin dejenerasyonu belirlendi. NAC ve NAC + GLF uygulaması, lipid peroksidasyonunu ve GLF tarafından indüklenen DNA hasarını, sıçanların testislerinde antioksidan enzimlerin aktivitesini ve hücre bütünlüğünü tersine çevirdi. Yukarıda belirtilen bulgular, NAC'nin GLF'nin neden olduğu lipid peroksidasyonunu azalttığını, antioksidan savunma mekanizmasını geliştirdiğini ve sıçanların testislerinde doku hasarını rejenere ettiğini göstermektedir.

Anahtar Kelimeler: DNA hasarı, Glifosat, N-Asetilsistein, Rat spermatozoon

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INTRODUCTION

Herbicides are only one of many pesticide types. These are chemical substances that farmers use to weed. When people are exposed to pesticides in food and plastic, industrial pollutants and synthetic chemicals, these substances are thought to affect the endogenous reproductive hormone function adversely. This can lead to a variety of reproductive anomalies (Dallegrave et al., 2007). In the studies conducted, it has been demonstrated that the above-mentioned compounds and their metabolites represent the main pollutants of surface waters and they usually continue to exist in agricultural products, which constitutes a significant threat to the health of humans and animals (Feron et al., 2002; Bohm et al., 2008; Clair et al., 2012). Glyphosate (GLF)-based herbicides are the first herbicides started to be utilized around the world. It is expected that GLF is specific on plant metabolism, and it has been claimed to have adverse impacts on animals and humans (Takahashi et al., 2001). GLF is a broad-spectrum herbicide commonly utilized for killing undesired plants in lands used for agricultural as well as nonagricultural purposes. It has been determined that GLF alters the cellular antioxidant status considerably, which induces glutathione depletion, enzymatic disorders and an increase in lipid peroxidation in keratinocytes (Gehin et al., 2006). GLF may have an endocrine disruptor effect or affect the reproductive system of males since it may cause changes in aromatase activity and expression, genes regulating estrogen and testosterone levels (Richard et al., 2005; Romano et al., 2010; Clair et al., 2012).

N-acetylcysteine (NAC), which was developed in the 1960s, represents the N-acetyl derivative of the amino acid L-cysteine that occurs naturally (Aitken et al., 1993). It is thiol-based antioxidant, takes a significant part both in protecting the components of cells against oxidative damage and in detoxifying numerous electrophiles (Martha et al., 1998). NAC has a wide area of use in the clinic as a mucolytic agent and an antidote for acetaminophen overdose. Moreover, NAC can play a role in regulating gene expression related to oxidative stress, therefore, it has an antagonistic impact on oxidative damage (Meister, 1991). NAC is almost nontoxic, and it is commonly utilized for decreasing the elasticity and viscosity of mucus due to its capacity to reduce disulfide bonds. NAC can enter into direct interaction with oxidants and different thiols, including glutathione, in addition to being a perfect scavenger of hydroxyl radicals. Along with the scavenger function of NAC, numerous pieces of evidence have demonstrated that it stimulates

cellular glutathione production as well. Hence, it is possible that oxidant-mediated damage in cell cultures or animals is decreased or inhibited by NAC (Ciftci et al., 2009).

Thus, the aim of this study was to examine the protective effect of NAC on testicular oxidative damage, spermatological parameters and DNA damage caused by GLF in rats.

MATERIALS and METHODS

Chemicals

Knockdown 48 SL which was a commercial preparation used as a GLF source, (Hektaş, Kocaeli, Turkey) and N-acetylcysteine (600 mg/20 tablet) was used (Basel, İstanbul, Turkey). Ketalar (Ketamin HCl 50 mg/ml; Pfizer, İstanbul, Turkey) and xylazine (20 mg/ml; Bayer, İstanbul, Turkey) were used for anesthesia purpose and euthanasia.

Animals and Experimental Design

In this study, 28 male Wistar Albino rats aged 12 weeks on average and weighed 290-350 g were used at Afyon Kocatepe University Experimental Animal Research Center. Approval for conducting experiments was obtained from the Animal Care and Use Committee (2017-49533702 / 26) at Afyon Kocatepe University, while the National Institutes of Health performed the care and use of laboratory animals. The animals were randomized into four groups ($n = 7$) and housed in a controlled environment (22 ° C, 12 h light-dark cycle), and free access to food and water was ensured. Group I (the control group) was fed a normal diet without GLF or NAC, group II received normal feed with 160 mg / kg / day NAC, group III received normal feed with 375 mg/kg/daily GLF, and group IV received normal feed containing 160 mg/kg/daily NAC + 375 mg/kg/daily GLF. The 8-week period of administration is required to identify the impact of these substances on the production of sperm since the exact spermatogenic cycle in rats, consisting of spermatocytogenesis, meiosis and spermiogenesis, requires 40-50 days.

Epididymal Sperm Assessment

The percentage of progressive sperm motility was evaluated in accordance with the study of Sönmez et al. (2005) using a phase contrast microscope with a heated stage. Briefly, a heated slide was put on a phase contrast microscope, which was heated to the temperature of 37 ° C, and afterwards a few drops of Tris buffer solution [0.3m Tris (hydroxymethyl) aminomethane, 0.027m glucose, 0.1m citric acid], a very small drop of liquid obtained from the epididymis of the left cauda using a pipette was put into the Tris buffer

solution, and its mixture with a cover-slip was ensured. The visual assessment of the ratio of forward progressive sperm motility was performed at 200 x and 400 x magnification. The estimation of motility was carried out in three various areas in every specimen. Three different forecast averages were utilized as the final motility score.

The Hypo-osmotic Eosin stain test (HE-test) was used in the semen samples in which the ratio of dead-live spermatozoa and the hypo-osmotic swelling test were applied together (Ducci et al., 2002; Fukui et al., 2004; Mansour, 2009).

The estimation of sperm cells abnormal in morphological terms was performed on a wet mount slide by using 2 - 3 semen drops thinned in Hancock's solution (Hancock, 1952) under a phase contrast microscope (Olympus CX 31, Olympus Optical Co., Ltd., Japan), and spermatozoa ratios were recorded.

Homogenate preparation

Ice cold 0.9% NaCl was used to wash testicular tissues obtained from the rats. Foreign materials were flushed out of the tissues. Cold 0.15 M Tris-HCl buffer (pH 7.4) was used to rinse them, and the homogenization of the tissues in buffer was performed for the purpose of obtaining 10% (w/v) homogenate. Afterwards, they were subjected to centrifugation for 10 minutes at 2100 g and kept in a deep freeze prior to the use (Kucukkurt et al., 2008).

MDA, GSH, SOD and CAT measurement in tissue homogenates

The technique described by Ohkawa et al. in 1979 was employed for the determination of malondialdehyde (MDA), and the technique described by Beutler et al. (1963) was used in order to measure GSH concentration in the tissue homogenates. The methods described by Sun et al. (1988) and Aebi (1984) were employed in order to measure SOD and CAT antioxidant enzyme activity, respectively, in the tissue samples. The colourimetric method described by Lowry et al. (1951) was employed for the measurement of the protein concentration in the tissue. A Shimadzu 1601 UV-VIS spectrophotometer (Tokyo, Japan) was utilized in the spectrophotometric measurements.

DNA Integrity

Sperm DNA damage was investigated using the single cell gel electrophoresis (comet) assay, which is generally performed under high alkaline conditions (pH \geq 13). The evaluation was performed by the visual scoring method. DNAs

with no damage were scored as 0, and the damaged DNAs were scored from 1 to 4 according to the degree of damage. The results were evaluated as arbitrary unit (AU) (Gündoğan et al., 2010).

Histologic Examination

The collection of testicular tissues from all animals and their fixation in Bouin's solution were performed, following which they were embedded in paraffin wax and cut into sections 5 μ m in thickness. Mayer's hematoxylin and eosin (H&E) were utilized for staining. The assessment of the tissues was performed using a light microscope (Olympus Bx51 model) having a camera (Olympus DP20).

Statistical Analysis

The data were presented as mean \pm standard error of means (SEM). The value of $P < 0.05$ was accepted to be the level of significance. The differences between the groups in relation to all the sperm properties, histological results and biochemical parameters were detected by employing the one-way analysis of variance (ANOVA) and post hoc Duncan test. The SPSS/PC (Version 10.0; SPSS, Chicago, IL) package program was utilized for performing all the analyses.

RESULTS

Table 1 contains information on the epididymal sperm motility and abnormal sperm rate, while Table 2 contains information on plasma membrane integrity. A significant increase in the sperm motility ($P < 0.05$) was observed in the NAC administration group. The abnormal sperm rate was significantly decreased in both NAC and NAC+GLF administration groups in comparison with the control and GLF groups. A significant increase in plasma membrane integrity (HE test) ($P < 0.05$) was detected in the NAC administration group in comparison with the remaining groups. A considerable increase in the testis MDA levels was determined in the GLF group when compared to the control group ($P < 0.05$). Furthermore, the GSH level decreased in the GLF group in comparison with the control group. As can be seen from Table 3, the activities of antioxidant enzymes, CAT and SOD were detected in the rats' testis tissue. The lower SOD activity was detected in the testis tissues ($P < 0.05$) in the GLF group when compared to the control group. At the same time, the GLF-induced change in MDA, GSH and SOD levels was reversed as a result of NAC administration. As can be seen from Fig. 1, damage to DNA was identified in the rats' sperm cells. The

high levels of DNA damage (94.8 ± 3.79 AU) were detected in the GLF group in comparison with the control group (38.8 ± 2.04 AU) ($P < 0.05$). Moreover, DNA damage was determined to be at 25.8 ± 2.34 AU in the NAC group. The above-mentioned findings indicated that administering NAC inhibited the change in DNA damage caused by GLF in sperm cells ($P < 0.05$). The detailed description of histopathological alterations in the testis of the experimental group is presented in Fig.

2. In the GLF group, a decrease in sperm concentration and degeneration of Sertoli cells in the testis were detected (Fig. 2 A3). In the NAC groups, insignificant histopathological alterations were observed in the rats' testis tissues (Fig. 2 A2). Beside, no considerable histopathological alterations were detected in the rats' testis tissues in the control group (Fig. 2 A1).

Table 1. Mean (\pm SEM) spermatological parameters in epididymal rat semen.

GROUPS	Motility %	Head %	Mid-Piece %	Tail %	Total %
CONTROL	72.8 ± 2.86^b	2.3 ± 0.14^b	0.6 ± 0.13^{ab}	7.8 ± 0.51^b	10.7 ± 0.53^b
NAC	85.7 ± 2.02^a	1.9 ± 0.17^b	0.1 ± 0.09^c	3.3 ± 0.24^d	5.3 ± 0.40^d
GLF	35.7 ± 2.02^c	6.2 ± 0.70^a	0.9 ± 0.07^a	11.2 ± 0.42^a	18.3 ± 0.92^a
NAC +GLF	72.8 ± 2.75^b	2.2 ± 0.21^b	0.3 ± 0.17^{bc}	5.5 ± 0.43^c	8.1 ± 0.50^c

Values (Mean \pm S.E.M.) with different superscripts (a and c) within the same column showed significant differences ($P < 0.05$).

Table 2. Mean (\pm SEM) HE test parameters in epididymal rat semen.

GROUPS	H+/E- %	H-/E- %	H+/E+ %	H-/E+ %
CONTROL	63.7 ± 1.53^b	29.2 ± 1.35^a	3.4 ± 0.57^c	3.5 ± 0.81^b
NAC	70.0 ± 0.88^a	23.1 ± 2.38^b	3.2 ± 1.12^c	3.57 ± 0.99^b
GLF	18.4 ± 1.63^c	7.8 ± 0.96^c	44.1 ± 1.33^a	29.5 ± 2.69^a
NAC +GLF	62.3 ± 2.36^b	22.0 ± 1.48^b	7.5 ± 1.68^b	8.1 ± 0.73^b

Values (Mean \pm S.E.M.) with different superscripts (a and c) within the same column showed significant differences ($P < 0.05$).

Table 3. Mean (\pm SEM) oxidative stress parameters in epididymal rat semen.

GROUPS	MDA (nmol/ml)	GSH (nmol/g doku)	SOD (U/ μ g protein)	CAT (k/ μ g protein)
CONTROL	3.6 ± 0.09^b	11.3 ± 0.23^a	2.8 ± 0.06^b	0.02 ± 0.03
NAC	3.5 ± 0.05^b	11.7 ± 0.32^a	3.2 ± 0.04^a	0.01 ± 0.02
GLF	5.3 ± 0.56^a	9.5 ± 0.36^b	2.6 ± 0.06^c	0.02 ± 0.04
NAC +GLF	3.7 ± 0.13^b	10.9 ± 0.33^a	2.8 ± 0.04^{bc}	0.02 ± 0.02

Values (Mean \pm S.E.M.) with different superscripts (a and c) within the same column showed significant differences ($P < 0.05$).

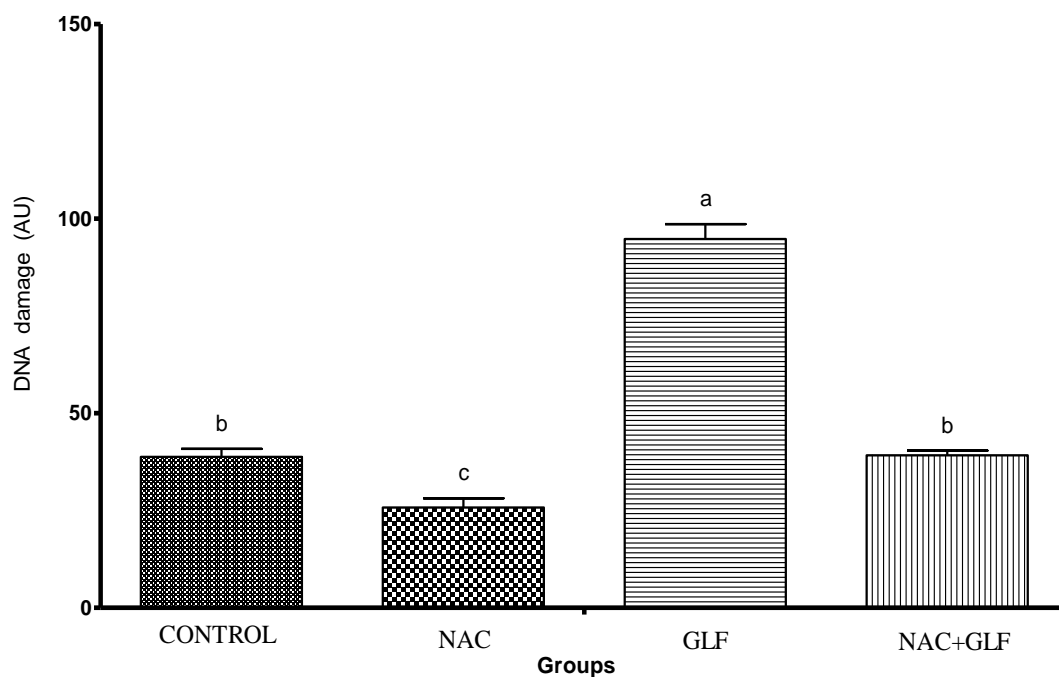


Fig. 1 The effect of GLF alone and treated with NAC on DNA damage in rats. Results are expressed as mean + SEM of seven rats. a,b,c; different letters show statistically significant differences ($p < 0.05$).

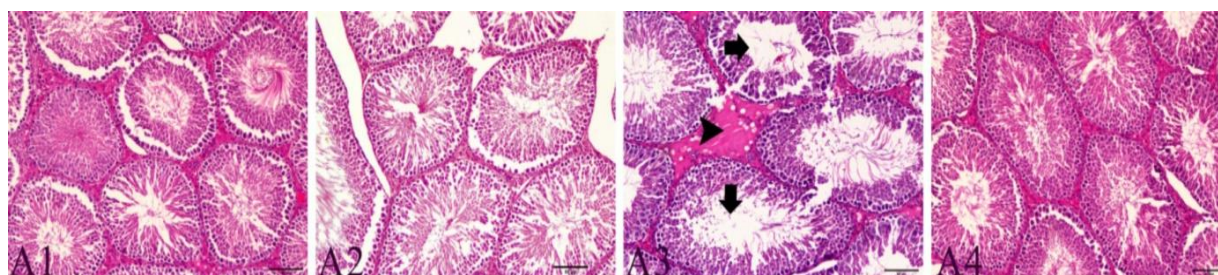


Fig 2. The effect of N-acetylcysteine (NAC) on GLF-induced damage in testis of rats. Representative figures were stained with H&E. Arrows and arrow heads indicate decreases sperm concentration and degeneration of Sertoli cells (Fig. 2 A3). Control group (A1), animals treated with NAC (A2) animals treated with GLF (A3), animals treated with GLF+ NAC (A4).

DISCUSSION

GLF has influenced the rats' reproductive system and has caused a decrease in the epididymal sperm motility and morphology. In the present study, GLF caused a reduction in motility, plasma membrane integrity of spermatozoa and an increase in abnormal sperm rate. This finding is in agreement with the findings of many studies conducted on different animal species. Reduced serum testosterone levels, decreased daily sperm count, increased ratio of abnormal sperm in adult male rats exposed to glyphosate-Roundup were indicated in the study of Dallegrave et al. (2007). Lopes et al. (2014) studied zebrafish (*Danio rerio*) and they reported that glyphosate could have negative impacts on reproductive parameters, including a reduction in sperm motility and the motility period and the alteration in question would decrease the fertility rate of the above-mentioned animals. In contrast, the study performed by Bhide

(1988) reported no negative impact on the reproductive system of rats that were exposed to glyphosate in the maximum dose of 15 mg/kg/day. NAC effect has been shown to increase the body's major antioxidant glutathione levels. Glutathione takes a significant part in the removal of toxic substances and free radicals, therefore, it has a protective effect on the cells. In this study, GLF-associated sperm morphology was prevented by NAC and it caused an increase in the epididymal sperm motility and improved the capacity of sperm plasma membrane integrity. Similar results are also reported in some studies conducted on different kinds of animals. Michael et al. (2009) reported that the addition of tris-based diluent 1.5 mM NAC improved the sperm motility of frozen-thawed canine sperm. Partyka et al. reported that NAC (15 mM) improved the parameters of chicken sperm during storing liquid at the temperature of 5 °C for 24 and 48 h. Oeda et al. (1997) reported that the semen specimens of humans containing and not

containing NAC (1.0 mg/mL) were incubated at room temperature and the researchers determined that NAC enhanced the total sperm motility and considerably decreased ROS, indicating that the function of impaired sperm could be enhanced by NAC. Çiftçi et al. (2009) reported that the study group consisting of 60 males was given NAC in the dose of 600 mg/d orally for three months and the control group also consisting of 60 males was given a placebo. NAC considerably improved the motility, volume, and viscosity of semen. The assessment of ROS formation and sperm function prior to and following the administration of NAC was performed in the study of Akiyama (1999) and the researcher observed no improvement in sperm density and sperm motility, however, there was a tendency for sperm function to get better, and a significant decrease in the ROS level in human sperm was determined following the administration of NAC. A possible toxic impact of 5 and 10 mM NAC doses on ram semen in the process of cryopreservation was reported in the study of Yıldız et al. (2015). Besides same researchers identified the harmful impacts of higher NAC doses on sperm motility. Contrary to the results of the above-mentioned studies, an improvement in sperm motility following the administration of NAC was determined in this study.

In this study, the MDA content of the testis considerably increased as a result of treatment with GLF. Increased MDA can demonstrate an increase in ROS production, which may damage sperm and other cytoplasmic organelle membrane structures as a result of lipid, protein and nucleotide peroxidation, therefore, leading to a change in sperm motility. A physiological impact of glutathione (GSH) is known in the repair of cellular oxidative damage through the formation of disulfide via the action of glutathione peroxidase (Raina et al., 2009a). In this study, a reduction in GSH was observed in GLF exposure. Superoxide is the enzyme that catalyzes the dismutation of superoxide, H_2O_2 , the first step of advocating the dismutase antioxidant defense. A reduction in the testicular SOD activity was determined in GLF exposed group when compared to the control group. The oxidative stress induced by GLF exposure may have depleted the cellular SOD level (Raina et al., 2009b). Oxidative stress and multiple stress-response pathways were caused by GLF, which induced a death of Sertoli cells in the testis of prepubertal rats. A Ca^{2+} overload and a cell signaling misregulation were stimulated by glyphosate. The cellular stress response and/or the decreased antioxidant defenses might have an impact on the disruption of Sertoli cells. Therefore, they might affect spermatogenesis and the fertility of males (de Liz Oliveira Cavalli et al., 2013). Jasper et al. (2012) observed that glyphosate caused ROS

generation in the exposed rats' testes, therefore, leading to polyunsaturated fatty acid peroxidation in the membrane of the testes that caused MDA, which represents one of the by-products of lipid peroxidation, to form. Upon conducting in vitro studies, a noticeable scavenging impact of NAC against ROS was confirmed. The structure and chemical reaction of NAC, which bears a similarity to that of glutathione, explain the above-mentioned antioxidant features. It is considered to function primarily as a pre-indicator of intracellular cysteine and glutathione and as a stimulator of cytosolic enzymes taking part in glutathione metabolism. Furthermore, NAC can function by entering into direct chemical reaction with radical species and/or ROS-dependent by-products (Cocco et al., 2005). Reddy et al. (2011) reported that the administration of intraperitoneal NAC in the dose of 75 mg/kg/day to mice induced with 4 ppm sodium arsenide for drinking water for 35 days reduced the oxidative damage of NAC. Farombi et al. (2008) reported that NAC considerably alleviated the toxic impact of tetracycline on the parameters of sperm, the negative histopathologic alterations caused by antibiotic were not enhanced by the antioxidants, and NAC considerably decreased the toxic impacts of tetracycline on the antioxidant and testicular marker enzymes along with oxidative stress markers. The contribution of oxidative stress to defective spermatogenesis as a result of decreasing the antioxidant level in the testes and therefore causing the infertility of males was reported in the study of Nithya and Elango (2015). Baker et al. (1996) indicated that the protection of spermatozoa against the damaging impact of leucocyte derived ROS on sperm movement in humans was ensured by the high concentrations (10 mM) of antioxidants, including glutathione and NAC. Ari et al. (2016) reported that NAC in medium doses, for example, 0.5 mM and below, added in skim milk based extenders may be utilized for the protection of ram sperm cells against oxidative stress. This study showed that NAC inhibited ROS-related oxidative stress (MDA, GSH and SOD) in animals exposed to GLF as a result of its antioxidant activity. The findings in question are consistent with the studies reported above.

Cells might possess higher sensitivity to chemical agents, which are capable of binding DNA, leading to damage, as a result of which single-strand DNA breaks may occur (Kumaravel and Jha, 2006). The adverse impacts of glyphosate on reproductive parameters in zebrafish *Danio rerio*, including damage to sperm DNA, decreasing the mitochondrial membrane integrity and functionality, were reported in the study of Lopes et al. (2014). In the present study, GLF damaged sperm DNA and caused a more significant DNA damage in comparison with the control and other

groups but NAC alleviated DNA damage, and the reason for this may be the pharmacological impacts of NAC particularly on antioxidant activities. Erkkila et al. (1998) reported that NAC considerably prevented apoptosis in the testicular germ cells of humans in vitro, therefore demonstrating the important role of antioxidative mechanisms for the survival of germ cells in the seminiferous tubules in spermatogenesis. Li Ji et al. (2013) reported the protective effect of NAC from germ cell apoptosis caused by cadmium as a result of its preventing endoplasmic reticulum stress in the CD-1 mice testes. Whitker et al. (2012) showed that adding of 1.0 mM NAC enhanced the usability of frozen-thawed boar sperm in IVF since it causes a decrease in the DNA fragmentation and lipid peroxidation of the sperm.

The degenerative changes observed in the seminiferous tubule and interstitial cells of the testes of rats signify that GLF is toxic to the reproductive system of male rats. Romano et al. (2010) detected a decrease in epithelium depth and bigger diameter from the lumen in the seminiferous tubule of rats to which GLF was administered. This result is consistent with the present study, GLF caused a decrease in sperm concentration in the lumen and degeneration of Sertoli cells in the testis. It is possible to inhibit the GLF toxicity and preserve the normality of the testicular architecture as a result of treatment with NAC.

As a result of the current study, it has also been determined that administering NAC decreases oxidative stress and DNA damage caused by GLF through reducing lipid peroxidation and causing antioxidant enzyme activation in the testis. Therefore, it improves suppressed reproduction in rats which has been induced by GLF.

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