Original Article

Effect of N-acetylcysteine on the sperm biochemical parameters and expression of HSPA70 and caspase-3 genes in asthenoteratospermic infertile males undergoing cryopreservation

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Abstract

Protecting sperm against oxidative reactions during cryopreservation is one of the supportive measures to enhance sperm quality and thus the success of ART. The present study aimed to investigate the effect of N-acetylcysteine (NAC) on the functional parameters of human sperm and to evaluate the apoptosis and expression of HSP70 and Caspase3 genes after cryopreservation. In this in vitro prospective study, 40 men referred to infertility center of ACECR, Qom were used. They were divided into two groups: the first group of men with normal WHO criteria and the second group of people with asthenoteratospermia, and was cryopreserved with sperm freeze supplemented with NAC at concentrations of 0.01, 0.02, 0.05, 0.1 and 0.25 mM. Semen samples were evaluated for sperm parameters including concentration, morphology, motility, viability, reactive oxygen species (ROS), apoptosis and expression of HSP70 and caspase 3 genes. Findings showed that NAC 0.02 and 0.05 mM significantly increased sperm motility, however it was decreased by NAC 0.025 mM compared to control group ($P<0.05$). Except for NAC 0.25 mM, all doses of NAC especially 0.05 mM increased sperm viability ($P<0.05$). Interestingly, the same results were observed about normal sperm morphology, concentrations and production of reactive oxygen species (ROS). Also, all doses of NAC especially 0.02 mM and 0.05 mM significantly reduced the apoptosis and expression of HSP70 and caspase3 genes. Overall, it is determined that NAC supplementation prior to cryopreservation dramatically enhances the quality of sperm and decreases the freezing-shock side effects.

Keywords: Cryopreservation, N-acetylcysteine, Human semen, Asthenoteratospermia, Apoptosis

Introduction

One of the important techniques of fertility management, semen cryopreservation is proposed for obtaining functional spermatozoa in individuals who are undergoing assisted reproduction technologies [1]. During cryopreservation, semen is exposed to cold shock and osmotic pressure and as a result, the membrane oxidation rate increases due to the higher amount of oxidative reactions, which ultimately reduces the sperm motility and viability. Researches have shown that fertilization rates after cryopreservation of spermatozoa are significantly reduced due to improper membrane function [2].

One of the mechanisms proposed in this area which could be to increase the activity of heat shock proteins (HSPs) and are found in the cells of all living organisms, and they increase in response to stress [3]. One of the most important HSPs effective in male's fertility is HSPA2 protein. During stress, cells are encountered by adverse conditions considering protein folding, and the HSP70 family is produced in large quantities [4]. The high production of these chaperons leads to the recovery of stress-induced degraded proteins and to the synthesis of new proteins to replace with not repairable proteins. Furthermore, it has been shown that changes in HSP70 levels are a good value for the diagnosis of human disease [5]. On the other hand, various studies have shown that

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infertile men have a higher histone/protamine ratio than fertile men. The defect in the histone/protamine ratio leads to a defect in sperm chromatin accumulation, which makes the sperm DNA susceptible to external shock damage and consequently reduced fertilization rates in this kind of patient [6, 7].

Cell death is mainly caused by the mechanism of apoptosis, which is a physiological phenomenon associated with a series of cascade events that could lead to the reduction of germ cells to the number supported by Sertoli cells and the selective removal of abnormal sperm [8]. During this process, many dysfunctional cells are removed, including old, immature, or damaged cells. The consequence of apoptosis is the occurrence of several events, including cell membrane disruption, disruption of the cell skeleton, nucleation of the nucleus, and cleavage of the DNA structure [9]. Besides, several specific proteins called caspases play an essential role in regulating apoptosis rates. These proteinases are first secreted as pro-enzymes into the epithelium of the seminal tubes and then lead to other cascade activities that induce pro-apoptotic signals. Cell death receptors in the spermatozoa, such as FAS and TNFα receptors, are among the factors activated by the caspase-like enzyme and leading to cell death [10, 11].

In recent researches, various antioxidants have been tested to protect the plasma membrane of sperm against oxidative reactions and other damages caused by cryopreservation [12-16]. Antioxidants alter cellular conditions by reducing oxygen free radical formation to maintain sperm motility [17-19]. N-acetylcysteine (NAC) is a thiol compound with potent antioxidant properties. Being a precursor of L-cysteine and reduced glutathione, it is also a free radical scavenger because it interacts with reactive oxygen species (ROS) [20]. Glutathione is one of the primary antioxidants of the body that deactivates various toxic substances such as xenobiotics, peroxide compounds, and other molecules that create free radicals and thus play an active role in cell protection [21]. During oxidative stress, glutathione concentration decreases but, consumption of NAC eliminates this deficiency, with NAC acting as an antioxidant by increasing glutathione [20]. Since oxygen free radicals are involved in a wide range of diseases, it is, therefore, to be expected that NAC will be useful in the treatment of a variety of conditions. On the other hand, NAC facilitates artery dilation by promoting the production and function of nitric oxide, and therefore may have therapeutic effects [22, 23].

Although several studies have investigated the antioxidant effect of NAC to alleviate the damage caused by cryopreservation of sperm in animal samples, and they have achieved some positive results, the findings are somewhat controversial [24-26]. Furthermore, because of possible limitations in the field of human resources, including the preparation and storage of semen samples and repeated courses of IVF treatment, to the best of our information, there is no comprehensive data on the effect of NAC antioxidant on the preservation of human sperm parameters after freezing-thawing procedure, and there is a lot to vague points in this case. Therefore, the present study aimed to investigate the effect of this antioxidant on the quality of functional parameters of sperm in men with asthenoteratospermia and to evaluate the apoptosis status and expression of related genes after cryopreservation.

Methods and Materials

Study design

In this in vitro prospective study, 40 men referred to the infertility center of the Academic Center for Education, Culture and Research (ACECR), Qom/Iran, were used. The study group included infertile and fertile men. The infertile group comprised 20 asthenoteratospermia infertile men (29.8 ± 1.4 years), who were diagnosed after at least two successive semen analyses fulfilled the World Health Organization's (WHO) infertility criteria. The first group consisted of people with asthenoteratospermia who had no children after two years of marriage, and the second group consisted of men who were normal for sperm criteria. Sperm concentration, motility rate, and morphology analysis were evaluated according to the WHO recommendations [27].

This study was approved by the Ethics Committee of Islamic Azad University, Qom (approval No.

IR.IAU.QOM.REC.1398.002). All patients signed a written consent form before participation in the study. The exclusion criteria were the presence of azoospermia, severe oligospermia or leukocytospermia, use of medication/antioxidants, exposure to chemotherapy or radiation, or a varicocele. Fertile men were excluded if they had a history of vasovasostomy or varicocelectomy.

After evaluating the different parameters in fresh semen, the remaining volume of each sample was separated into four aliquots: without N-acetylcysteine (NAC; Sigma-Aldrich Corp., St. Louis, MO, USA) and with at concentrations of 0.01 0.02 ،0.05 ،0.1 and 0.25 mM [28].

Assessment of sperm parameters

All men had been asked to abstain from ejaculation for at least 72 hours before semen was collected. The samples were placed in CO_2 incubation at 37 ℃ for 30 minutes so that seminal liquefaction could be performed before evaluating sperm parameters. Semen samples were then evaluated for various parameters, including sperm concentration, morphology, and motility [27]. Sperm motility was assessed at 3 degrees: Grade A: In situ, rotational and low speed, Grade B: Low speed linear, Grade C: Linear, progressive, and fast.

An examination of sperm viability was performed using histological smears stained with eosin–nigrosin (4% eosin solution and 8% nigrosin solution in sodium citrate) [29].

Cryopreservation procedure

Each of the semen samples was separated into four aliquots: without NAC (control) and with 0.01 0.02 ،0.05 ،0.1 and 0.25 mM of NAC and incubated for 1 hour at 37 °C. The semen was then diluted with a cryoprotective medium in a 1: 1 ratio with Life Global (Life global® Group, USA) and incubated at 4°C for one hour. They were then frozen at -20 °C for 10 minutes and suspended in vapor-phase nitrogen for 1 hour before being stored in liquid nitrogen at -196 °C for 7 days [30]. Frozen specimens were extracted from the liquid nitrogen tank for thawing, and after 20 seconds, equilibration in laboratory air, were immersed in 37 °C for complete melting. Then, Ham's F-10 (Life Technologies Inc., Grand Island, NY) with 10% albumin was added to the medium and centrifuged at 300 g for 5 minutes to remove the antifreeze. The samples were then analyzed for sperm parameters.

Measurement of ROS and MDA

The thiobarbituric acid reactive species (TBARS) were determined as a measure of the concentration of malondialdehyde (MDA) in sperm, the end product of lipid peroxidation (LPO), by MDA ELISA Kit (Cat. NO. ZB-11371C-H9648, ZellBio Company Germany) according to the supplier's instructions. MDA levels at 532 nm were reported as nmol/ml [31].

ROS Level in sperm samples was evaluated using (5-(and-6)-chloromethyl-2′,7′-dichlorofluorescein diacetate and acetyl ester (CM-H2DCFDA) by ROS Assay Kit (Cat. NO. ab-113851, Abcam Company, USA), according to the supplier's instructions. The method is based on the ROS-dependent oxidation of 2′,7′-dichlorofluorescin diacetate to fluorescent dichlorofluorescein (DCF). DCF is a highly fluorescent compound which can be detected by fluorescence spectroscopy at 535 nm. Spermatozoa (5×10^6/mL) were treated with 10 μM CM-H2DCFDA for 30 minutes at 37 °C in the dark.

RNA extraction and real-time PCR

The mRNA extraction was performed by triazole, and the samples were stored at -80°C until cDNA production. The RNA concentration was measured by absorbance readings of the samples at 260 nm using the NanoDrop Spectrophotometer (Thermo Fisher Scientific, Inc., Waltham, MA, USA). As the absorbance ratio of 260/280 was above 1.8, the samples were used for cDNA synthesis. cDNA synthesis was performed using the cDNA Synthesis Kit (Takara Bio, Otsu, Japan). Primer design for quantitative polymerase chain reaction for the genomic regions targeted was performed by Perl Primer (version 0.1.1). After selecting a suitable primer pair, to ensure its correct design, the selected primer was examined by Gene Runner (Hastings Software, Hudson, NY; version 6.5.47) and the UCSC and Blast NCBI sites (Table 1).

Step-One Plus real-time PCR system (Applied Biosystems, Foster City, CA, USA) was used for real-time PCR. Reactions were made in a final volume of 10 μl of SYBR® Green Real-time PCR Master Mixes (Thermo Fisher Scientific, Waltham, MA, USA). The PCR temperature program was performed according to Table 2.

Comparison of ΔΔ (CT) method was used to study gene expression changes in this study, and this ratio was calculated as internal control compared to 18s gene expression. In the relative comparison method, the relative difference between the test sample and the control sample was compared with CT ΔΔ 2 formula.

Determination of Apoptosis

For each sample sperm, DNA fragmentation is evaluated by the TUNEL assay at the end of each condition, using the In-Situ Cell Death Detection Kit (Roche, Mannheim, Germany). Sperm smears are prepared onto adhesive microscope slides and fixed in a 4 % paraformaldehyde (Merck) in PBS solution for 1 hour at RT. Slides were then washed in PBS and permeabilized with a 0,1 % Triton-X in 0,1 % sodium citrate (Sigma) solution for 2 minutes at 4 °C. Once again, slides were washed in PBS and then incubated for one hour in a dark, humid chamber at 37 ºC with 50 μl of TUNEL mixture.

Subsequently, the slides were washed in PBS and counterstained with mounting medium containing DAPI (Burlingame, CA, USA). On each slide, a minimum of 200 morphologically normal sperm were blindly was evaluated on a Leitz DMRBE fluorescence microscope (Leica, Wetzlar, Germany). The number of spermatozoa emitting green fluorescence (TUNEL-positive) was recorded as a percentage of the total number of sperm counted (DAPI stained) [32].

Statistical analysis

The normality of continuous variables was confirmed using the Kolmogorov-Smirnov test, and data was reported as means ± SEM. Data analysis were performed using one-way ANOVA and Tukey's test for post-hoc. Means were considered significantly different at $P<0.05$. All data were analyzed with the statistical software SPSS (version20 for windows, Chicago, IL, USA).

Results

Sperm motility

Based on Figure 1, NAC significantly increased mean sperm motility in concentrations 0.01, 0.02, and 0.05 mM compared to control group (14.25±4.06, 20±2.81 and 27.25±3.02 vs. 11.25±3.58, respectively. $P<0.05$). However, NAC 0.1 mM insignificantly raised this parameter and sperm motility was considerably decreased by NAC 0.25 mM compared to control (7±2.51 vs 11.25±3.58, $P<0.05$).

Also, mean grade B was significantly increased by NAC 0.02 and 0.05 mM compared to control group (11.5±2.35 and 15.75±4.06 vs. 6±2.61, $P<0.05$, Fig.1). While, NAC 0.25 mM considerably diminished grade B compared to control (2±2.51 vs. 6±2.61, $P<0.05$, Fig.1).

As Figure 1 displays, NAC at doses of 0.02 and 0.05 mM significantly increased the mean grade C compared to the control group (8.5±3.28 and 10.75±2.93 vs. 5.25±2.55, P<0.05). However, there were no significant differences between the other concentrations of NAC and the control group.

Sperm viability, morphology and concentration

As figure 2 shows, approximately all doses of NAC especially 0.05 mM significantly increased sperm viability compared to control group, except 0.25 mM (0.01 =29.55±3.74, 0.02 mM =44.75±6.71, 0.05 mM =60.45±3.5 and, 0.01 mM =35.15±5.56 vs. control=15.5±4.56, P<0.05).

Our findings revealed that the mean normal morphology of sperms increased by all NAC concentrations; however, it was just significantly higher in NAC 0.05 mM group compared to the control group (1.75±0.55 vs. 1.2±0.41, P<0.05, Fig.2).

Moreover, according to Figure 2, sperm concentrations were significantly increased by most NAC doses compared to control group (0.01 mM =11.7±2.29, 0.02 mM =13.3±2.22, 0.05 mM =20.35±1.56, 0.1 mM =11.4±2.18 vs. control=9.35±3.13, P<0.05). Interestingly, in this case, also the NAC 0.05 mM had the highest efficacy (20.35±1.56) while NAC 0.25 mM decreased the sperm concentration (8.85±2.79) compared to the control group, although it wasn't considerable.

Reactive oxygen species and MDA

According to Figure 3, our findings show that except the dose of 0.25 mM (0.76±0.06), all other concentrations of NAC especially 0.05 mM significantly reduced the production of ROS compared to control group (0.01 mM =0.75±0.04, 0.02 mM =0.68±0.05, 0.05 mM =0.34±0.04 and 0.1 mM =0.72±0.05 vs. control=0.81±0.66, P<0.05).

Based on Figure 3, NAC 0.02 and especially NAC 0.05 mM significantly reduced the MDA compared to control group (0.04±0.001 and 0.03±0.001 vs. 0.05±0.001, P<0.05).

Apoptosis and Expression of HSPA70 and Caspase3 genes

Findings revealed that the most expression of HSPA70 gene occurred in control group and all doses of NAC especially 0.02 and 0.05 mM significantly reduced the expression compared to the control group (0.01 mM =23.69, 0.02 mM =14.11, 0.05 mM =18.65, 0.1 mM =27.23, 0.25 mM =30.89 vs. control=36.55, P<0.05, Fig.4).

Interestingly, the same results were observed about the expression of caspase3 gene (0.01 mM =18.46, 0.02 mM =11.56, 0.05 mM =13.56, 0.1 mM =22.46, 0.25 mM =20.45 vs. control=23.59, P<0.05, Fig.4).

Furthermore, Figure 4 confirmed that all concentrations of NAC reduced the apoptosis in comparison to the control group (0.01 mM =41.6±2.37 0.02 mM =34.75±2.51, 0.05 mM =23.25±1.61, 0.1 mM =38.2±2.11, 0.25 mM =42.95±1.73 vs. control=48.5±2.6, P<0.05). As shown in Fig. 4, the apoptosis decreased by up to NAC 0.5 mM, and the lowest apoptosis occurred at this dose. But then apoptosis also increased with increasing NAC dose, although it was still lower than the control group.

Discussion

Sperm cryopreservation through cold shock results in reduced sperm motility and disruption of membrane integrity, disruption of acrosome reaction, and mitochondrial function [33]. Research results show that water-soluble antioxidants such as vitamin C and fat-soluble antioxidants such as vitamin E reduce the free radicals [34]. Since sperm parameters influence fertilization, it seems that antioxidants are of clinical importance in ART techniques by reducing free radicals and reducing the adverse effects of oxidative stress as well as improving sperm parameters. Although few studies have investigated the antioxidant effect of NAC on reducing the side-effects of sperm cryopreservation in different animal species [24-26, 35], there is no report on the impact of this antioxidant on the quality of human sperm parameters.

The results of the present study showed that the addition of N-acetylcysteine, especially doses of 0.02 and 0.05 mM, significantly increases the motility and especially the progressive motility of sperm (Figure 1). In fact, antioxidants, like enzymes Glutathione peroxidase, Superoxide dismutase, by decomposing superoxide anions, hydrogen peroxide, peroxide, prevent damage to the sperm membrane and its functional parameters. Partyka et al. (2013) and Michael et al. (2007) presented that consumption of 5 mM and 1.5 mM NAC increased sperm motility in chicken and canine samples, respectively [27, 35]. Whereas, in contrast, Mata et al. (2012) concluded that NAC reduced the motility by 1 mM NAC [24]. Similarly, in the present study, the decrease in A and B motility began at the dose of 0.1 mM, and the highest significant decrease was seen at the highest NAC dose 0.25 mM, (Figure 1). This may suggest the dose-dependent origin of NAC and its adverse effects at doses above 0.05 mM.

Also, the findings of our study showed that almost all doses of NAC increased viability (Figure 2). Again, this increase was more pronounced at doses of 0.02 and 0.05, which confirms the effect of antioxidant levels on its efficacy, so doses of 0.02 mM and 0.05 mM can be considered as optimal doses. The most significant effects on sperm morphology and concentration were at NAC 0.05 and 0.02 mM. Interestingly, the decreasing trend of these indices was also significantly reversed with increasing NAC doses (Figure 2). Michael et al. (2007) showed that consumption of NAC 1.5 increased viability in canine sperm after freezing protection [25]. However, Perez et al. (2015) concluded that NAC did not affect viability [26].

Besides, damage to sperm DNA can lead to decreased fertilization ability, impaired fetal development, non-pregnancy, and birth defects. The oxidative stress has been implicated in several injuries to male infertility, including varicocele, leukocytospermia, oligozoospermia, teratozoospermia, and idiopathic infertility [8]. As shown in

Figure 3, ROS levels decreased at all doses of NAC except 0.25 mM. But the most significant decrease occurred at a dose of 0.02 mM, and in particular, 0.05 mM, which may confirm the dose-dependent hypothesis of NAC and consider the 0.05 mM as the optimal dose for human samples. These findings are consistent with the results of studies by Perez et al. (2015) and Mata et al. (2012) that used doses of 1 mM and 1.5 mM for bovine and red deer sperm samples, respectively [24, 26]. But Michael et al. (2007) showed that 1.5 mM NAC did not affect ROS levels [25]. Also, Mata et al. (2012) demonstrated that NAC 1mM reduced DNA fragmentation [24], although Perez et al. (2015) did not report similar results [26].

Teratozoospermic individuals or those whose abnormal sperm morphology is higher than threshold levels (96%) may produce endogenous ROS due to having excess cytoplasm [36]. Increased levels of oxidative stress in infertile individuals decrease sperm telomere length, and given that telomeres have a specific function of maintaining the integrity of chromosomes, this could have adverse consequences on fertilization and fertility [36]. Because at high levels of oxidative stress, sperm chromatin begins to fragment and eventually leads to sperm DNA damage, which can contribute to the rate of telomere degradation and shorten telomere length in the germ cell lines. Thus, these short telomeres may lead to increased apoptosis of cells and a decrease in the number of spermatozoa [9, 10].

Numerous studies have suggested that HSP70 plays a role in embryo development in addition to fertilization [37]. Neur et al. (1998) have shown that the presence of HSP70 antibodies decreases the rate of blastocyte development in mice [38]. Several studies have also suggested that HSP70 is an inhibitor of apoptosis in early embryonic development, and inhibition of HSP70 decreases blastocyte development and may be associated with increased cell death and male infertility [39]. The findings of our study showed that HSP70 gene expression decreased with all NAC doses. In this case, doses of 0.02 and 0.05 mM had the greatest effect, too. The presence of this protein is related to the maturation and fertility of sperm. A decrease or lack of expression of this protein in meiotic stages will be associated with meiotic defects, such as aneuploidy, oligozoospermia, and azoospermia [39]. Any defect in the expression of this protein in the final phase of spermiogenesis leads to DNA damage, and due to lack of cytoplasmic deletion, abnormal morphology, and inappropriate substitution of this protein, the oocyte binding will also fail [37]. This protein also acts as a calcium-binding protein and a proper collector of sperm-specific membrane proteins in the capacitance process [40].

On the other hand, the results of the present study indicated that NAC consumption, especially at doses of 0.02 and 0.05 mM, significantly reduced apoptosis (Figure 4). In terms of the decreasing and increasing trend, previous results were repeated, confirming the efficacy of specific doses of this antioxidant. Concerning the effect of NAC antioxidant on Caspase gene expression, the results were quite similar to the findings regarding the HSP70 gene and apoptosis status [41]. Several mechanisms have been proposed to justify the effects of caspase-3 gene expression, part of which is related to enzyme activity, inhibition of cell proliferation, alteration of glutathione levels, and elimination of free radicals [42]. Caspase 9 and 3 genes play important roles in inhibiting the apoptosis of cancer cells [43]. Khori et al. (2015) showed that there is a close relationship between bcl2 and caspase-3 and that caspase-3 is the final effector in apoptosis [44]. Caspase 9 is an initiator caspase in the mitochondrial pathway of apoptosis and activates caspase 3, the downstream executive caspase, which are important factors in the apoptosis process [39, 45].

Overall, our study concluded that NAC supplementation as a survival agent before cryopreservation had an active role in reducing the complications of this procedure and could, therefore, lead to increased rates of ART success. This antioxidant dramatically enhances the quality of sperm parameters and decreases the level of reactive oxygen species and reduces apoptosis and expression of HSP70 and Caspase3 genes. Doses of 0.02 and 0.05 mM, as optimum doses, appear to be most useful and effective in ART. However, given the scarcity of human specimen studies and the lack of curvature of the specimens, further studies focusing on precise NAC dosage and attention to relevant functional mechanisms may be necessary.

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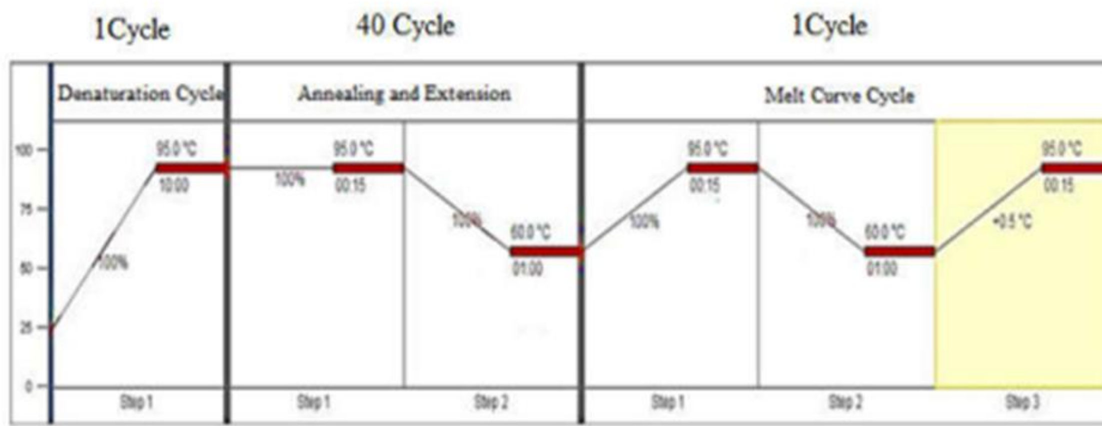
Table 1- Sequence of Caspase3, HSP70 and GAPDH gene primers.

Gene name		Sequence	Length	Melting temperature	CG%
Caspase3	F	AAGCGAATCAATGGACTCTGG	134	59.4	47.62
	R	CTGTACCAGACCGAGATGTC		60.5	55
HSP70	F	CATCGACTTCTACACGTCCA	20		50
	R	CAAAGTCCTTGAGTCCCAAC			50
GAPDH	F	CCATGAGAAGTATGACAAC	161	53	42.11
	R	GAGTCCTTCCACGATACC		56.1	55.56

Table 2- Real-time PCR temperature program.



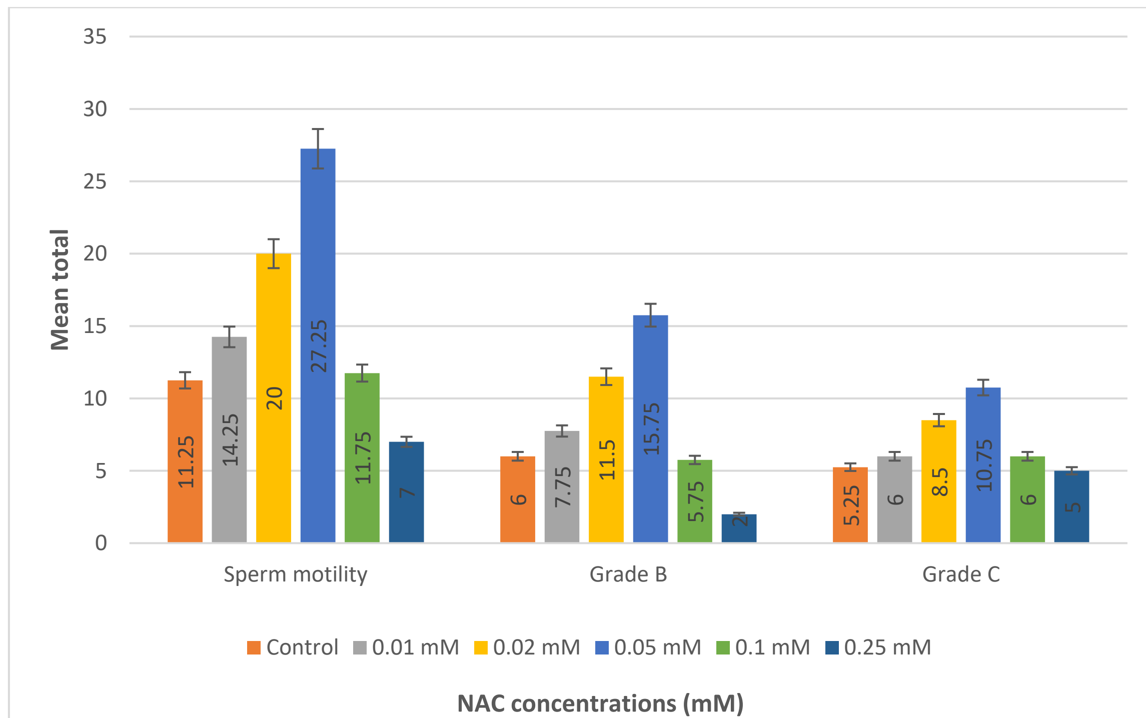


Figure 1. Effect of different concentrations of N-acetylcysteine on the sperm motility; Grade B; and Grade C. Columns with the unalike letter do differ significantly from each other (one-way ANOVA, Games-Howell, P<0.05).

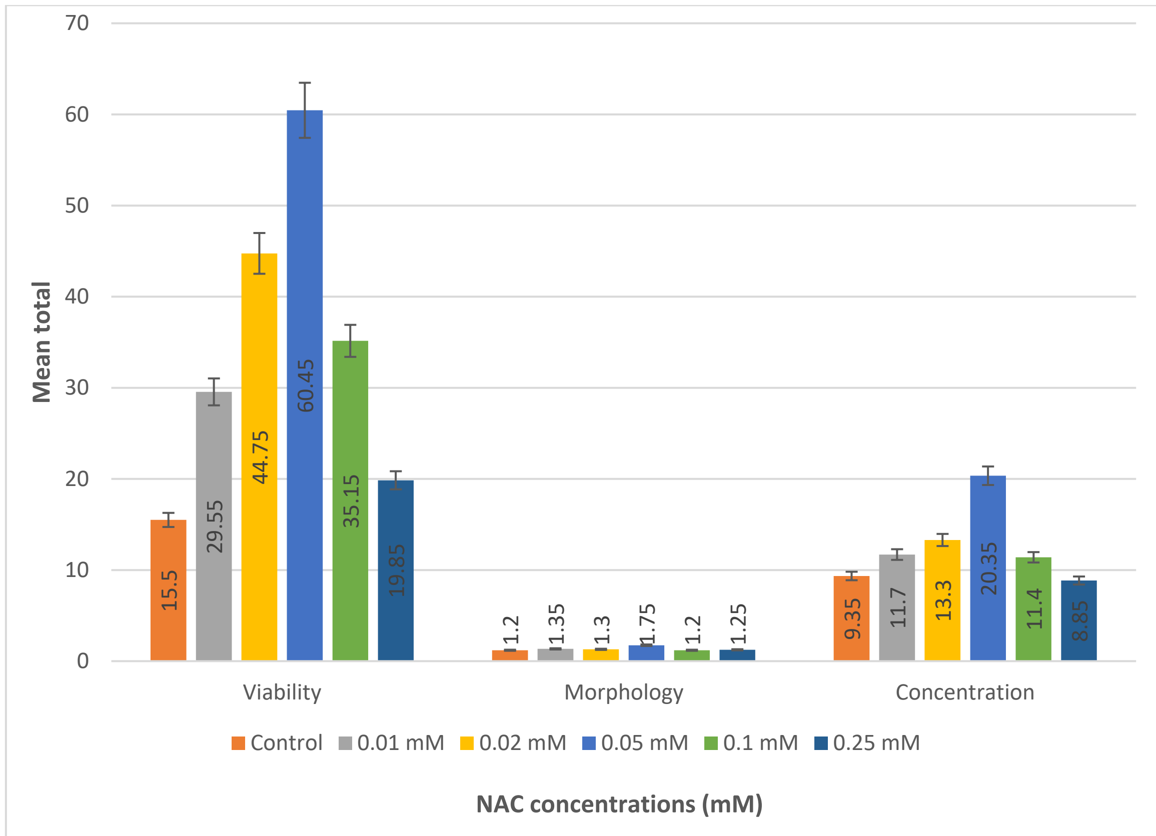


Figure 2. Effect of different concentrations of N-acetylcysteine on the sperm viability; morphology; and concentration. Columns with the unalike letter do differ significantly from each other (One-way ANOVA, Games-Howell, P<0.05).

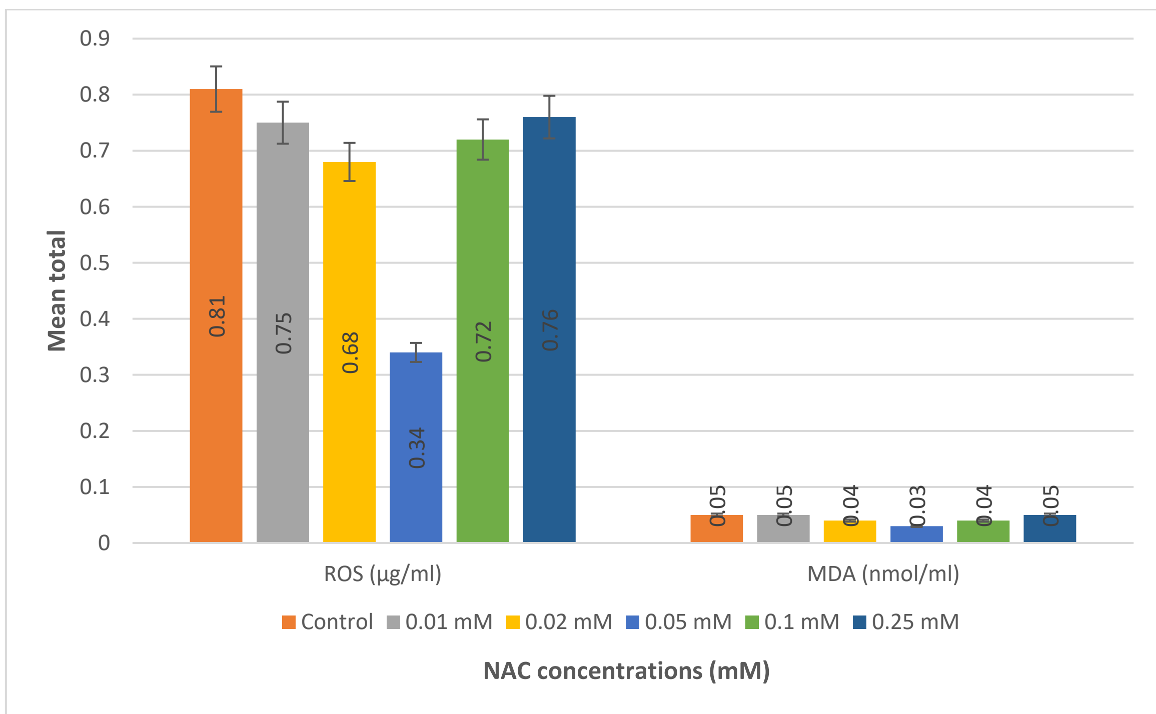


Figure 3. Effect of different concentrations of N-acetylcysteine on the production of ROS; and MDA. Columns with the unalike letter do differ significantly from each other (one-way ANOVA, Games-Howell, P<0.05).