Title: Cytotoxic Effect of Nanosilver Particles on Testicular Tissue; Evidence for biochemical Stress and Hsp70-2 Protein Expression

Author: Zari Rezazadeh-Reyhani Mazdak Razi Hassan Malekinejad Rajabali Sadrkhanlou

PII: S1382-6689(15)30068-5
DOI: http://dx.doi.org/doi:10.1016/j.etap.2015.08.024
Reference: ENVTOX 2335

To appear in: *Environmental Toxicology and Pharmacology*

Received date: 20-6-2015
Revised date: 19-8-2015
Accepted date: 24-8-2015

Please cite this article as: Rezazadeh-Reyhani, Z., Razi, M., Malekinejad, H., Sadrkhanlou, R.,Cytotoxic Effect of Nanosilver Particles on Testicular Tissue; Evidence for biochemical Stress and Hsp70-2 Protein Expression, *Environmental Toxicology and Pharmacology* (2015), http://dx.doi.org/10.1016/j.etap.2015.08.024

Cytotoxic Effect of Nanosilver Particles on Testicular Tissue; Evidence for biochemical Stress and Hsp70-2 Protein Expression

Zari, Rezazadeh-Reyhani[1];

Mazdak, Razi[1*];

Hassan, Malekinejad[2];

Rajabali, Sadrkhanlou[1];

1- Department of Comparative Histology, Faculty of Veterinary Medicine, P.O.BOX: 1177, Urmia University, Urmia, Iran.

2- Department of Pharmacology & Toxicology, Faculty of Veterinary Medicine, P.O.BOX: 1177, Urmia University, Urmia, Iran.

[*]**Corresponding Email:** Mazdak.razi@gmail.com

TEL: +989144462762

Key words: Oxidative Stress, RNA damage, Spermatogenesis

Abstract:

Lastly, there are growing evidences that nanosilver (NS) particles highly induce cytotoxic impacts *in vitro* and *in vivo*. Here, we analyzed the dose dependent effect of NS on histological changes, biochemical alterations and endocrine statuses, sperm parameters as well as chaperone Hsp70-2 expression. NS particles (50-60nm) were administrated in 3 doses of 0.5, 1 and 5 mg/kg, intraperitoneally, for 35 days. The 0.3 mL normal saline was administrated in control-sham group. Histological alterations, sperm parameters, serum levels of LH, FSH and testosterone were evaluated. Germinal and Leydig cells RNA damage, Leydig cells steroidogenic foci, the testicular and sperm total antioxidant capacity (TAC), malondialdehyde (MDA), nitric oxide (NO) levels, immunohistochemical (IHC) expression and mRNA level of Hsp70-2 were analyzed. The NS, dose dependently, resulted in enhanced germinal cells degeneration, necrosis, seminiferous tubules atrophy and decreased serum levels of LH, FSH and testosterone. Elevated germinal and Leydig cells RNA damage associated with increased sperm abnormalities were observed in NS-treated groups. Expression of Hsp70-2 was up-regulated in 0.5mg/kg, while its expression was decreased in 1 and 5 mg/kg NS-treated groups. Testicular and sperm TAC levels reduced. However, the MDA and NO levels significantly ($P<0.05$) increased in all NS-treated groups. No histological and biochemical changes were detected in control-sham group. In conclusion, the NS particles exert their pathological impact via affecting testicular antioxidant

and endocrine statuses, which in turn lead to diminished expression of Hsp70-2. Ultimately, by this mechanism NS particles adversely impact the cellular RNA, DNA and protein contents.

Key words: Nanosilver, Testis, RNA damage, Hsp70-2, Oxidative stress, Sperm

Introduction:

The nanosilver (NS) is used commonly in a combination of the pure-de-ionized water with superfine silver nanoparticles (5-50nm) in suspension (Alt et al. 2004). Lastly, there are increasing hazards about elevated industrial wastes for NS particles in water and soil (Alt et al. 2004; Oberdorster et al. 2005). Due to small sizes of NS particles, the silver can entry into human body via ingestion, inhalation (Phalen and Morrow, 1973; Shi and Ye, 2012), from skin by contacting with jewelry and cosmetic (Furchner et al. 1968; Drake et al. 2005) and through different medical interventions such as the use of acupuncture needles (Sato et al. 1999). Lastly, it has been illustrated that, the NS particles enter through the vascular system and accumulate in different tissues such as: lungs, kidneys, liver and nervous system (Reddy et al. 2008). Previous observations showed that NS accumulation in lungs results in severe damages in alveolar region via producing surface radicals and reactive oxygen species (ROS) (Limbach et al. 2007). In other study, the prolonged exposure to NS resulted in cytotoxic effects in nervous tissue especially in cerebra (Aaseth et al. 1981). Other report showed that the mechanisms, which the NS exerts its derangements, are involving in disruption of mitochondrial respiratory chain leading to severe production of reactive oxygen species (ROS) as well as interruption of ATP synthesis (Arora et al. 2008; Foldbjerg et al. 2009).

Previously, it has been established that the defected endocrine system both at hypophysis and testicular levels is able to adversely impact the spermatogenesis. Accordingly, the luteinizing (LH) and follicle stimulating hormones (FSH) stimulate the testicular endocrine activities (Shan et al. 1995; Akkoyunlu et al. 2007). As a result for damaged endocrine interactions, testicular tissue undergoes to ROS and reactive nitrogen species (RNS)-dependent stresses (Sharma et al. 1999; Forlenza and Miller, 2006; Agarwal et al. 2008). In this line, the relation between generation of ROS/RNS and infertility has been established well. The abnormal spermatozoa, damaged germinal epithelium and infiltration of immune cells are reported for producing intensive amounts of ROS in male gonads (Forlenza and Miller, 2006). On the other hand, the high levels of ROS are responsible for oxidating the NO into peroxynitrate, which is known for its cytotoxic impacts (Agarwal et al., 2008). Moreover, the high generation of ROS/RNS results in remarkable damages in sperm DNA integrity, motility and plasma membrane fluidity (Sharma et al. 1999; Forlenza and Miller, 2006).

Under oxidative and nitrosative stress conditions, the heat shock proteins 70 (Hsp70) family are expressed highly in various cell types of the germinal epithelium (Eddy, 1995; Khosravanian et al., 2014; Vaziri et al., 1998). The Hsp70-2 gene is expressed during early meiosis and mitosis for presenting, assembling, folding and refolding of the proteins during different cell cycles (Eddy, 1995; Dix et al., 1997). Moreover, the Hsp70-2 proteins are involved in recovering the DNA and RNA damages in germinal epithelium via improving the DNA integrity as well as enhancing the RNA binding proteins stability in haploid cells (Eddy, 1995; Lamb et al., 2010). Various previous studies generally analyzed the adverse impacts of NS particles at different doses and concentrations on reproductive potential. In the line with this issue, Mirshokraei et al. (2011) showed that, incubating the ram sperms with different concentrations of NS particles for

30, 60, 120 and 180 min results in a significant reduction in sperm kinematic parameters (Mirshokraei et al., 2011). It has been shown that, 25, 50, 100 and 200 mg/kg of NS particles (70 nm) remarkably reduced primary spermatocytes, spermatids and spermatozoa number and significantly decreased acrosomal reaction in Wistar rats (Miresmaeili et al., 2013). Moreover, previous reports illustrated that NS particles in different doses results in hormonal imbalance in male rats and significantly affect the reproductive organs maturation process as well as sperm parameters (Baki et al., 2014; Sleiman et al., 2013). However, the possible roles of molecular alteration(s) as well as biochemical mechanism(s) involving in NS particles-induced detrimental effects remained unknown. Therefore, present study was conducted to analyze the role of Hsp70-2, as a protein required for normal spermatogenesis, following the NS-induced oxidative and nitrostaive stress conditions. In order to investigate this hypothesis, the mice were exposed into different doses of NS and the histological and biochemical analyses were performed. The IHC analyses and semi-quantitative RT-PCR were assessed in order to estimating the protein and mRNA expression of Hsp70-2, respectively. In order to evaluate the correlation between altered Hsp70-2 expression and RNA stability, the RNA damage ratio was assessed at germinal and Leydig cells levels. In addition, we tried to uncover testicular endocrine activity by assessing the Leydig cells steroid foci, serum level of testosterone. Finally, the NS particles-induced changes in sperm parameters were evaluated.

Materials and Methods:

Chemicals:

The 50-60 nm nanosilver combination was obtained from Nano Nasb Pars Co. (Tehran, Iran). The acridine-orange was purchased from sigma chemical Co. (St. Louis, MO, USA). The FITC-conjugated 1-anilinonaphthalene-8-sulfonate was obtained from AYANDEH Lab Co. (Urmia, Iran). The TCM 199 cell culture medium was purchased from Minitube Co. (Smythesdale, Australia). The rabbit anti-mouse primary antibody for Hsp70-2 was purchased from Histo-line laboratories (Istanbul, Turkey). The DAB chromogen was from Agilent technologies Co. (Istanbul, Turkey). Mounting medium for immunohistochemical analysis (Vectashield) was from Vector Laboratories (Burlingame, USA). All other chemicals, which were used, were commercial products of analytic grade.

Animals:

Twenty four laboratory albino mice (10-12 weeks old), with body weight 20-30gm, were used. They were housed under controlled condition of illumination (12 h light/12 h darkness) and temperature 20^{o}C-25^{o}C, throughout the experiment period. Standard pellet diet (Mahabad pellet, Iran) and water were provided ad libitum. In this study all experiments which conducted on animals were in accordance with the guidance of ethical committee for research on laboratory animals of Urmia University. Both the international guidelines for the animals' welfare and the compatible local regulations for experimenting were respected during the study.

Chemical administration and grouping:

After one week adaptation the animals were divided into four groups as control-sham and test (N=6 in each group). The animals in control-sham group received 0.3 mL normal saline, intraperitoneally per day. The test group subdivided in three test groups as;

a- Low dose NS-treated group: animals in this group received 0.5mg/kg^{-1}b.wt. from NS .

b- Medium dose NS-treated group: animals in this group received 1mg/kg^{-1}b.wt. from NS.

c- High dose NS-treated group: animals in this group received 5mg/kg^{-1}b.wt. from NS.

In order to analyze the mechanism(s), which NS particles exert pathological impacts the NS particles (50nm-60nm) were administrated intraperitoneally (Shin and Ye, 2012). Animals received 0.5mg/kg were exposed to an environmentally relevant concentration (0.00047 mg/kg/day, oral dose and 0.000027 mg/kg/day, dermal dose), animals received 1mg/kg were exposed to an environmentally relevant concentration (0.00094 mg/kg, oral dose and 0.00056 mg/kg/day) and finally the animals received 5 mg/kg were exposed to 10 times higher concentration. All groups received the chemicals for 35 days. Before and after the experiment, the animals were weighed individually in order to evaluate any changes in total body weight gain related to treatment. The testicular weights relative to body weights were evaluated after 35 days.

Serum and tissue samples preparation:

Following 35 days, blood samples were obtained directly from heart under light anesthesia (provided by using diethyl ether). After 15 min at room temperature, the samples were centrifuged at 3000g for 10 min to obtain the serum. Then the prepared serum samples were stored at −70 ^{0}C for further analyses.

Immediately after blood sampling, the animals were anesthetized by using CO_2 gas in special CO_2 device (Urum ADACO, Iran). The testicular specimens were immediately removed and rinsed with chilled normal saline. One of the testis samples from each individual mouse was snap frozen in liquid nitrogen and then kept in −70 ^{0}C until further biochemical analyses and other side testes were fixed in Bouin's solution for histological examinations.

Histological Analyses:

Previously fixed testicular samples were paraffin embedded and cut (5-6µm) by rotary microtome (Microm, Germany). The sections (5-6 µm) were stained with Iron-Weigert (Pajohesh Asia, Iran) for germinal epithelium's nuclei detection. The prepared slides were analyzed under light microscope by multiple magnifications (400× and 1000×). The Leydig cells number per one mm^2 of interstitial connective tissue were numerated. The percentage of seminiferous tubules with more than 3-4 germinal layers and percentage of tubules with normal spermiogenesis were considered as tubules with positive tubular differentiation index (TDI) and positive spermiogenesis index (SPI), respectively. The percentage of tubules with positive repopulation index (RI), as ratio of active spermatogonia (spermatogonia type B with light nuclei in Iron-Weigert staining technique) to inactive spermatogonia (spermatogonia type A with dark nuclei in Iron-Weigert staining) was calculated.

Determination of NS in testicular tissue:

In order to visualize the NS particles in testicular tissue, the UV/VIS proton spectrophotometery (Borders, USA) was used. In short; 0.3-0.4 g of testicular tissue was homogenized in ice-cold PBS medium and used for UV/VIS spectrophotometry. The absorbance rates for NS in 558 nm (special for NS) in different samples and blank solution (containing the PBS) were evaluated. The absorbance was estimated based on reaction of electrons bands which was evaluated based on $A=log(I_0/I)=\varepsilon c$.

Assessment of serum levels of testosterone, LH and FSH:

Testosterone was assessed by using competitive chemiluminescent immunoassay kit (DRG, Germany). The serum concentrations of LH and FSH were determined by using Radioimmunoassay kit (MP Biomedicine., SimulTRAC, LH/FSH, Hungary).

Immunohistochemical analyze for Hsp70-2:

Tissue section slides were heated at 60°C for approximately 25 min in a hot air oven (Venticell, Germany). The tissue sections were de-paraffinized in xylene and rehydrated using alcohol gradients. The antigen retrieval process was performed in 10 mM sodium citrate buffer. Briefly; endogenous peroxidase was blocked in aperoxidase blocking solution (0.03% hydrogen peroxide containing 0.05% (w/v) sodium azide) for 5 min. Tissue sections were washed gently with washing buffer and subsequently incubated with Hsp70 (1:500) biotinylated primary antibodies for 18 Hrs at -4^{o}C. The sections were rinsed gently with washing buffer and placed in a buffer bath. The slides were then placed in a humidified chamber with a sufficient amount of

streptavidin–HRP (streptavidin conjugated to horseradish peroxidase in phosphate-buffered saline (PBS) containing an anti-microbial agent). The slides were incubated for 15 min. Subsequently, the tissue sections were rinsed gently in washing buffer and placed in a buffer bath. A DAB chromogen was added to the tissue sections and incubated for 5 min, followed by washing and counter staining with hematoxylin for 5 sec. The sections were then dipped in weak ammonia (0.037 M/L) 10 times, rinsed with distilled water and cover slipped. Positive immunohistochemical staining was observed as brown stains under a light microscope. The pixel value intensity for brown chromogen was evaluated by using image pro insight software (Media Cybernetics, USA) for Pixel value histogram.

Assessment of RNA damage:

The RNA damage was assessed using the acridine-orange NO dye according to Darzynkiewicz method (Darzynkiewicz, 1999). In brief, the testes were washed out with ether-alcohol and cut by cryostat (8 μm). The prepared sections were fixed by different degrees of ethanol alcohol for 15 min. Then the sections briefly were rinsed in acetic acid, 1% aqueous, followed by washing in distilled water. The specimens then were stained in acridine-orange for 3 min and distained in phosphate buffer. Subsequently, the slides were followed for fluorescent colors differentiation in calcium chloride. The degenerated/necrotic cells were characterized by loss of RNA and/or with faint red stained RNA. The normal cells were marked with bright red RNA at the apex of the nuclei. In order to reduce the bias problems for staining density, 20 sections for each sample

were investigated. The percentage of tubules and number of Leydig cells (per one mm^2 of the connective tissue) with RNA damage reported for all groups.

RNA isolation and semiquantitative RT-PCR:

Total RNA was isolated from the testicular tissues according to the standard TRIZOL method (Scieglińska et al., 1997). DNA was removed from RNA samples by digestion with RNase-free DNase I. The RNA amount was determined by spectrophotometric analyses (260 nm and A260/280 = 1.8-2.0).

RT-PCR assays were performed essentially according to Shibata e*t al*. (1999). At the reverse transcription step the reaction mixture (final volume 50 µL) contained RNA (0.5–1 µg), dNTPs (0.2 mm each), primers (0.4 µm each), RNase Inhibitor (40 U/µl, Fermentas), MMLV reverse transcriptase (50 U/µl, Gibco BRL) and Taq polymerase (2 U/µl, Fermentas). The reverse transcription step was performed for 10 min at 50 °C and after inactivation of reverse transcriptase at 94 °C for 4 min, 20–35 cycles of the PCR were performed (94 °C for 30 s; 55 °C for 30 s; 72 °C for 45 s). Products were analyzed on 2% agarose gels stained with ethidium bromide. The specific primers for mice Hsp70-2 (370 bp) and GAPDH (380 bp) were designed and manufactured by Cinna Gen (Cinna Gen , Iran).

Assessment of steroidogenic foci (unsaturated fatty acid accumulation) in Leydig cells:

For this purpose, the commercially available kit for fluorescent assay of Leydig cells intracytoplasmic steroid droplets (AYANDEH Lab, Iran) was used. In brief; the frozen section prepared slides were dehydrated and stained with hematoxylin. The slides were washed with running water for 3-5 min and were stained in special fluorescent dye (FITC-conjugated 1-

anilinonaphthalene-8-sulfonate) for steroids and rinsed in distilled water. Then, the slides were dehydrated in 95 and absolute isopropanol and mounted with fluorescent mountant.

Sperm preparation and DNA damage assessment:

Epididymal tissues were separated carefully from the testicle under a 10 time magnification provided by Stereo Zoom Microscope (TL2, Olympus Co., Tokyo). The left epididymis was divided into three segment; caput, corpus and cauda. The epididymal cauda was trimmed and minced in 5 ml TCM199 medium for 30 min, 6% CO2, 36.5 ^{o}C in CO2 culture device (LEEC, England). After centrifugation the sperm pellet was re-suspended in 0.5 mL of TCM199 medium. A small aliquot (20 μl) of sperm suspension was glass smeared. The slides were air dried and then fixed overnight in Carnoy's solution (methanol/acetic acid, 3: 1). Once rinsed and air dried, the slides were stained for 5 min with freshly prepared acridine-orange dye (AO). After washing and drying, the slides were examined using a fluorescent microscope (Leitz, Germany; excitation of 450–490 nm). After AO staining, the samples were immediately observed. Each field was observed for some seconds under the fluorescence microscope. On each slide an average of 100 spermatozoa were counted. The percentage of spermatozoa with single-stranded DNA was calculated (Tejada et al., 1984).

Sperm motility, chromatin condensation and viability:

The sperm motility was examined based on the standard WHO method for manual examination of sperm motility (WHO, 1999). Briefly, the sperm samples were diluted 1:8 in TCM199 before examination. A 20 µl of sperm sample was placed on sperm examination area and examined

under 10× magnification. Only the motile sperm with forward progression counted within 10 boxes and recorded. Finally, motility was evaluated based on the following equation:

[Motility (%) = motile sperm/motile+ non-motile sperm] × 100.

The Eosin-nigrosin staining was performed for sperm viability assay. In brief, 50 µl of epididymal sperm was mixed with 20 µl of Eosin in sterile test tube. After 5 s 50 µl of Nigrosin was added and mixed thoroughly. The mixture of stained sperm was smeared on the slide and examined under bright field microscope (1,000× magnification, Olympus, Germany). The colorless sperm were considered as live and stained sperm were marked as dead spermatozoa. The sperm count was performed according to standard hemocytometric method as described previously by Pant and Srivastava, (2003). In addition, the aniline-blue staining was performed in order to analyze chromatin condensation in sperms. The sperms with dark stained nucleus were detected as sperms with decondensed chromatin and the sperms with light blue stained nucleus were marked as sperms with condensed chromatin (Pant and Srivastava, 2003). The sperm viability, motility, and chromatin condensation were reported in percentage.

Assessment of total antioxidant capacity (TAC) malondialdehyde (MDA):

0.3-0.4 gram of the testicular tissue was homogenized in ice-cold KCL (150mM) and then the mixture was centrifuged at 3000 *g* for 10 min. The supernatants were used for evaluating TAC and MDA. The assessment of TAC was carried out based on ferric reduction antioxidant power (FRAP) assay as previously reported (Benzie and Strain, 1999). To determine the lipid peroxidation rate, MDA content of the collected testis samples was measured using the thiobarbituric acid (TBA) reaction as described previously (Niehaus and Samuelsson, 1951). The amount of MDA was expressed as nMol per mg protein of the samples. The protein content of

the samples was measured according to the Lowry's method (Lowry et al., 1951). 500 µl from collected sperms from epididymal cauda were used for evaluating TAC and MDA levels with the same method mentioned for tissue.

Testicular and sperm NO analyzes:

The total NO content of the testis was measured according to the Griess reaction (Green et al., 1982). In Griess reaction nitric oxide rapidly converted into the more stable nitrite, which in an acidic environment nitrite is converted to HNO_2. In reaction with sulphanilamide, HNO_2 forms a diazonium salt, which reacts with N-(1-naphthyl) ethylenediamine.2HCL to form an azo dye that can be detected by absorbance at a wavelength of 540 nm. The NO content of the examined tissues were expressed as nMol per mg protein of the samples. 500 µl from collected sperms from epididymal cauda were used for evaluating NO level with the same method mentioned for tissue.

Statistical analyses:

For the measured parameters, mean and standard deviations were calculated. Results were analyzed using SPSS software (version 16.00, California, USA). The comparisons between groups were made by analysis of variance (two way ANOVA) followed by Bonferroni post-hoc test. A P value < 0.05 was considered significant. The correlations between data were evaluated using an Indigo-2 O_2 work station (Silicon Graphics, Mountain View, CA) using Matlab (Math Works, Inc., Natick, MA).

Results:

General findings:

After 35 days, the total body weight gain did not change (P>0.05) in all groups. The testicular weight relative to body weight was decreased (P<0.05) in medium and high dose NS-treated groups (Fig 1.A, 1.B). Moreover, the NS in a dose dependent manner, significantly (P<0.05) reduced the serum levels of LH, FSH and testosterone (Table 1).

NS accumulation in testicular tissue elevated depending on dose:

UV/VIS proton spectrophotometery showed that, the NS accumulation in testicular tissue elevated, dose dependently. Accordingly, the animals in high dose NS-treated group exhibited the highest NS accumulation (Fig2.A, 2.B).

Histological alterations:

Severe seminiferous tubules atrophy and remarkable edema in connective tissue were revealed in NS-treated animals. Moreover, the animals in NS-treated groups exhibited increased percentage of seminiferous tubules with negative TDI and SPI versus control-sham group. These impairments developed depending on dose. In a dose dependent manner, the animals in NS-treated group showed a remarkable (P<0.05) decrease in germinal epithelium height and the seminiferous tubules diameter (Fig 3.A, 3.B, 3.C, 3.D, 3.E, 3.F, 3.G, 3.H). No histopathological changes were observed in control-sham group. Data for histomorphometric analyses are presented in table 2.

NS resulted in severe damages at Leydig cells level:

Animals in NS-treated group exhibited a remarkable ($P<0.05$) reduction in Leydig cells distribution per one mm^2 of the interstitial connective. NS, dose dependently, increased the percentage of hypertrophied Leydig cells ($P<0.05$). Moreover, NS administration resulted in remarkable ($P<0.05$) RNA damage in Leydig cells, which was more intensive in high dose NS-treated (5mg/kg) group. The intracytoplasmic steroid foci of Leydig cells were significantly decreased in NS-treated animals (Fig 4.A, 4.B, 4.C). Accordingly, the animals in high dose NS-treated (5mg/kg) group showed lowest percentage of Leydig cells with intracytoplasmic steroid content (Table 3).

NS increased RNA damage and declined Hsp70-2 expression at germinal epithelium level:

The high dose NS-treated (5mg/kg) groups showed the highest percentage of tubules with damaged RNA in germinal epithelium. IHC analyses were performed in order to clarify HSp70-2 expression in different cellular layers of germinal epithelium. Observations demonstrated that, biosynthesis of Hsp70-2 increased in low dose NS-treated (0.5mg/kg) animals (especially at spermatocytes and spermatids cells lineages) versus control-sham group (Fig 5A, 5.B, 5.C, 5.D, 5.E, 5.F, 5.G, 5.H). Meanwhile, it was significantly decreased in medium (1mg/kg) and high (5mg/kg) dose NS-treated groups. The plot profile for reactive sites of IHC positive cells decreased in 100 µm of the tissue, dose dependently (Fig 6.A, 6.B, 6.C, 6.D). Semiquantitative RT-PCR analyze confirmed the IHC results. The animals in low dose (0.5mg/kg) NS-treated group exhibited a significant ($P<0.05$) elevation at mRNA level of Hsp70-2. However, the mRNA levels of Hsp70-2 were decreased in medium (1mg/kg) and high (5mg/kg) dose NS-treated groups (Fig 7.A, 7.B). Administration of NS, dose dependently, resulted in a significant ($P<0.05$) reduction in total RNA content and total protein levels of the testicular tissue.

Assessing the correlation between total RNA and protein levels showed that there was a positive correlation between reduced RNA content and decreased total protein content of testis in NS-treated animals (Fig 8.A, 8.B, 8.C).

NS reduced sperm quality:

In order to evaluate the dose dependent effects of NS on sperm parameters, the sperm count, motility, chromatin condensation and DNA damage were assessed. The NS-treated animals showed a significant ($P<0.05$) decline in sperm count in comparison to control-sham group. Fluorescent staining for DNA damage indicated a remarkable ($P<0.05$) enhancement in DNA damage in NS-treated groups. No changes in sperm count and DNA integrity were observed in control-sham groups. Moreover, sperm chromatin condensation as well as sperm motility were significantly ($P<0.05$) decreased in NS-treated groups (Fig 9.A, 9.B, 9.C, 9.D). The data for sperm parameters are presented in table 4.

NS reduced testicular and sperm TAC levels and increased MDA contents:

Testicular and sperm antioxidant statuses were significantly ($P<0.05$) decreased in NS-treated groups. Accordingly, the animals in high dose NS-treated (5mg/kg) group exhibited the lowest level of TAC versus low (0.5mg/kg) and medium (1mg/kg) dose-treated groups. The Moreover NS, dose dependently, resulted in a remarkable ($P<0.05$) elevation in testicular MDA No biochemical alterations were detected for control-sham animals (Fig 10.A, 10.B).

NS elevated the NO level in testicles:

Biochemical analyses for NO showed that NS, dose dependently, increased the testicular and sperm NO contents. Accordingly, the animals in high dose NS-treated (5mg/kg) group showed the highest NO content versus to other test and control-sham groups (Fig 11).

Discussion:

Many previous studies showed that NS particles exert degenerative impacts on liver, kidney, nervous tissue (Reddy et al., 2008; Limbach et al., 2007) and even respiratory system (Aaseth et al., 1981). In present study, we extended the evidences to show NS-treated adverse impact at testicles and sperm levels. The NS-treated animals exhibited reduced percentage of TDI, RI, SPI, elevated germinal cells RNA damage and lowered sperm quality. To understand involved mechanism(s), we analyzed the testicular endocrine activity by assessing the Leydig cells histological features, steroidogenic activity as well as RNA damage. On the other hand, we focused on biosynthesis of chaperone Hsp70-2 at different layers of the germinal epithelium as well as Leydig cells. Moreover, the mRNA level of Hsp70-2 was assessed in all groups. Observations revealed that NS, dose dependently, increased RNA damage both at Leydig and germinal cell levels and reduced Leydig cells distribution as well as steroidogenic activity. The NS-treated animals exhibited significantly lowered Hsp70-2 expression at higher doses. Finally, our data showed that administrated NS down-regulated antioxidant status and elevated MDA and NO contents both at testicular and sperm levels.

As a primordial finding, our analyses showed that the NS directly impacted the testicular tissue. Accordingly, the total body weight of the NS-treated animals did not significantly differ with those in control-sham group. Meanwhile, the testicular weight relative to body weight was

remarkably decreased versus control-sham animals. These alterations may be attributed to degenerative effect of NS particles on testicular tissue.

It is well known that the gonadotropins LH and FSH are the major endocrine regulators of spermatogenesis. The LH stimulates the Leydig cells in order to synthesis androgens (mainly testosterone) and FSH targets the Sertoli cells to regulate the spermatogenesis (Shan et al., 1995). Significant reduction at serum levels of LH and FSH suggests that NS affects the testicular endocrine potential via systemically influencing the pituitary-gonadal axis. This hypothesis supported by diminished steroid foci in Leydig cells and reduced testosterone level. On the other hand, our histological analyses showed that NS, in a dose dependent manner, decreased Leydig cells number per one mm^2 of the connective tissue, enhanced hypertrophied Leydig cells distribution and resulted in severe RNA damage in these cells. Therefore, we can conclude that further to NS-induced derangement in pituitary-gonadal axis, it was able to reduce the testicular endocrine status by directly influencing the Leydig cells.

In order to understand the mechanism governing the NS impact on testicular endocrine status, one should note that there is an appositional correlation between androgen evacuation and increasing oxidative stress. As a result for androgen deficiency-induced oxidative stress, the apoptosis rate increases in germinal cells (Lue et al., 1990; Agarwal et al., 1994; Dierich et al., 1998). On the other hand, the pathologically produced oxidative stress affects all damaged and intact cellular DNA, RNA, and protein contents and eventually results in protein and lipids peroxidation (Agarwal et al., 1994; Agarwal et al., 2008). In order to show these impairments, we analyzed testicular and sperm TAC levels. Our biochemical analyses showed that, NS down-regulated the TAC both at testicular and sperm levels. Previous researched have shown that pathologically increased reactive oxygen and nitrogen species (ROS/RNS) including;

superoxides, peroxides and nitric oxide (NO), result in cellular apoptosis/necrosis via inducing structural and biochemical changes (Calabrese et al., 2000; Forlenza and Miller, 2006). To confirm these findings, the NO content of the testicular tissue assessed and observations demonstrated that NS, dose dependently, enhanced the NO level. Therefore, we can come close to this fact that, the elevated NO content and reduced TAC level coordinated with endocrine deficiency-induced damages, and provoked cellular degeneration in NS-treated groups. Increased percentage of tubules with reduced TDI, RI and SPI indices confirmed this hypothesis.

Although in physiological condition the Hsp70-2 involves in intacytoplasmic proteins assembling, folding and refolding processes (Matsumoto M, Fujimoto, 1990; Shonhai et al., 2007), under different stress conditions this chaperone plays an essential role in homeostasis (Shonhai et al., 2007). Accordingly, biosynthesis of the Hsp70-2 protein in testicular tissue alters depending on free radicals generation ratio (Zhu et al., 1997) and indirectly changes depending on androgens withdrawal (Ricci et al., 2004; Khosravanian et al., 2014). Our IHC and semi-quantitative RT-PCR analyses showed that in low dose NS-exposed animals (0.5mg/kg) the expression of Hsp70-2 increased versus control-sham group. However, medium (1mg/kg) and high (5mg/kg) dose NS-treated animals exhibited significantly reduced expression of the Hsp70-2 both at IHC and mRNA levels. Considering these finding, we can suggest that under lower stress condition (low dose NS-induced NOS/ROS stress and androgen evacuation), the Hs70-2 overexpression was occurred in testicular tissue in order to control the stress-induced derangements. In contrast, at higher dose levels, the NS acted via different mechanism. Indeed, different stimulant agents, including superoxide, free radicals and NO adversely impact the cellular protein structures even the Hsp70-2 (Kaur and Bansal, 2003). Beside this fact, it should be noted that the NS particles are capable to highly bind to proteins, lipids and DNA and

consequently result in remarkable peroxidation at these levels (Lamb et al., 2010). Thus, we can hypothesis that, in high dose NS-treated animals the NS alone-induced damages in association with ROS/NOS-induced impairments leaded to remarkable damage at protein and RNA levels of Hsp70-2. Decreased biosynthesis and mRNA levels of Hsp70-2 associated with significant reduction at total RNA and protein levels confirmed this hypothesis.

It has been reported that the spermatocytes, with lack of Hsp70-2 cannot complete the meiosis and are subsequently deleted by apoptosis (Tesarik, 1998). Therefore we can suggest that, the NS resulted in severe damages at spermatocyte cell levels (Marked with diminished TDI and SPI indices) via affecting the expression and/or biosynthesis of Hsp70-2. Moreover, function and expression of the Hsp70-2 changes during late stages of spermiogenesis process and becomes specifically associated with spermatid-specific-DNA-packing proteins (Govin et al., 2006). In other words, the synthesis of DNA-packing transition proteins 1 and 2, and protamines 1 and 2 largely depend on Hsp70-2 chaperones expression (Zhu et al., 1997; Govin et al., 2006). Our analyses for sperm nuclear maturation showed that the percentage of sperms with chromatin condensation decreased depending on administrated doses of NS. Thus, it can be concluded that, the NS reduced protamination partly by down-regulating the Hsp70-2 expression. In this regard, the sperms with impaired packing of DNA are highly susceptible against oxidative and nitrosative stresses (Malekinejad et al., 2012; Razi et al., 2011). In the line with this issue, the percentage of sperms with DNA damage was increased in NS-treated animals.

Intensive arrangement of unsaturated fatty acids, plasmalogenes and sphingomyelins in cell membrane of the sperms are responsible to make these cells susceptible against free radicals-induced lipid peroxidation (Sanocka and Kurpisz, 2004). In order to identify the effect of NS-induced oxidative stress on lipid peroxidation at sperm level, we analyzed the epididymal sperm

viability and the MDA content. Observations revealed that the sperm viability decreased in NS-treated animals and the MDA level increased depending on administrated doses. More analyses for sperm TAC showed a remarkable decrease in TAC level of sperm samples from NS-treated groups. Therefore, it would be more logic to hypothesis that, the NS-induced lipid peroxidation associated with NS-caused oxidative stress resulted in loss of the sperm viability that consequently leaded to loss of sperm motility (note Fig 12). Current hypothesis has been illustrated with other studies about the effect of free radicals on sperm viability and motility (Sanocka and Kurpisz, 2004; Aticken and Sawyer, 2003).

Conclusion:

Our data showed that NS exerts its detrimental impact by a- reducing endocrine status at both pituitary and testicular levels, b- increasing oxidative and nitrosative stresses, c- down-regulating chaperone Hsp70-2 expression/biosynthesis, d- enhancing RNA damage in testicular cell lineages and e- decreasing sperm quality. In continue, decreased antioxidant capacity and Hsp70-2 expression associated with increased nitrostaive stress result in severe damages at DNA, RNA and protein contents of testicular cells as well as sperms.

Acknowledgment:

Authors wish to thank Faculty of Veterinary Medicine, Urmia University for laboratory and scientific helps.

References:

1- Aaseth, J., Olsen, A., Halse, J., Hovig, T., 1981. Argyria-tissue deposition of silver as selenide. Scand. J. Clin. Lab. Invest. 41, 247-251.

2- Akkoyunlu, G., Erdogru, T., Seval, Y., Ustunel, I., Koksal, T., Usta, M.F., 2007. Immunolocalization of glial cell-derived neurotrophic factor (GDNF) and its receptor GFR-alpha1 in varicocele induced rat testis. Acta. Histochem. 109, 130-137.

3- Agarwal, A., Ikemoto, I., Loughlin, K.R., 1994. Relationship of sperm parameters to levels of reactive oxygen species in semen specimens. J. Urol. 152,107-110.

4- Agarwal, A., Makker, K., Sharma, R., 2008. Clinical relevance of oxidative stress in male factor infertility: An update. Am. J. Reprod. Immunol. 59, 2-1.

5- Alt, V., Bechert, T., Steinru Cke, P., Wegener, M., Seidel, P., Dingeldein, E., et al., 2004. An In-vitro assessment of the antibacterial properties and cytotoxicity of nanopatriculate silver bone cement. Bimaterial.25, 4383-4391.

6- Arora, S., Jain, J., Rajwade, J.M., Paknikar, K.M., 2008. Cellular responses induced by silver nanoparticles: In vitro studies. Toxicol. Lett. 179, 93-100.

7- Aticken, R.J., Sawyer, D., 2003. The human spermatozoa-not waving but drawing. Adv. Exp. Med. Biol. 518, 85-98.

8- Baki, M.E., Miresmaili, S.M., Pourentezar, M., Amraii, E., Yousefi, V., Spenani, H.R., Talebi, A.R., Anvari, M., Fazilati, M., Fallah, A.A.,. Mangoli, E., 2014. Effects of silver nano-particles on sperm parameters, number of Leydig cells and sex hormones in rats. Iran, J. Reprod. Med, 12, 139-144.

9- Benzie, I.F., Strain, J.J., 1999. Ferric reducing/antioxidant power assay: Direct measure of total antioxidant activity of biological fluids and modified version for simultaneous

measurement of total antioxidant power and ascorbic acid concentration. Methods. Enzymol. 299, 15-27.

10- Calabrese, V., Bates, T.E., Giuffrida, Stella, A.M., 2000. NO synthase and NO-dependent signal pathways in brain aging and neurodegenerative disorders: the role of oxidant/antioxidant balance. Neurochem. Res. 65, 1315-1341.

11- Chomczynski, P., Sacchi, N., 2006. The single-step method of RNA isolation by acid guanidiniumthiocyanate-phenol-chloroform extraction: Twenty-something years on. Nat. Protoc. 1, 581-585.

12- Darzynkiewicz, Z., 1990. Differential staining of DNA and RNA in intact cells and isolated cell nuclei with acridine-orange. Methods. Cell. Biology. 33, 285-298.

13- Dierich, A., Sairam, M.R., Monaco, L., Fimia, G.M., Gansmuller, A., LeMeur, M., et al., 1998. Impairing follicle-stimulating hormone (FSH) signaling in vivo: Targeted disruption of the FSH receptor leads to aberrant gametogenesis and hormonal imbalance. Proc. Natl. Acad. Sci. U.S.A. 95, 13612-13617.

14- Dix, D.J., Allen, J.W., Collins, B.W., Poorman-Allen, P., Mori, C., Blizard, D.R., et al., 1997. HSP70-2 is requiredfordesynapsis of synaptonemal complexes during meiotic prophasein juvenile and adult mouse spermatocytes. Development. 124, 4595-4603.

15- Drake, P.L., Hazelwood, K.J., 2005. Exposure-related health effects of silver and silver compounds. Ann. Occup. Hyg. 49, 575-585.

16- Eddy, E.M., 1995. Chauvinist genes of male germ cells: gene expression duringmouse spermatogenesis. Reprod. Fertility. Develop. 7, 695-704.

17- Foldbjerg, R., 2009. PVP-coated silver nanoparticles and silver ions induce reactive oxygen species, apoptosis and necrosis in THP-1 monocytes. Toxicol. Lett. 190, 156-162.

18- Forlenza, M.J., Miller, G.E., 2006. Increased serum levels of 8-hydroxy-2'-deoxyguanosine in clinical depression. Psychosom. Med. 68, 1-7.

19- Furchner, J.E., Richmond, C.R., Drake, G.A., 1968. Comparative metabolism of radionuclides in mammals-IV- Retention of silver 110m in the mouse, rat, monkey, dog. Health. Phys15, 505-514.

20- Govin, J., Caron, C., Escoffier, E., Ferro, M., Kuhn, L., Rousseaux, Set al., 2006. Post-meiotic Shifts in HSPA2/HSP70.2 Chaperone Activity during Mouse Spermatogenesis. J. Biol. Chem. 281, 37888-37892.

21- Green, L.C., Wagner, D.A., Glogowski, J., Skipper, P.L., Wishnok, J.S., Tannenbaum, S.R., 1982. Analysis of nitrate, nitrite and nitrate in biological fluids. Anal. Biochem.126, 131-138.

22- Khosravanian, N., Razi, M., Farokhi, F., Khosravanian, H., 2014. Testosterone and Vitamin E administration up-regulated varicocele-reduced Hsp70-2 protein expression and ameliorated biochemical latrations. J. Assist. Reprod. Genet. 31, 341-354.

23- Kaur, P., Bansal, Mp., 2003. Effect of oxidative stress on spermatogenesis process and Hsp70 expressions in mice testis. Indian J. Biochem. Biophys. 40, 246-251.

24- Lamb, J.G., Hathaway, L.B., Munger, M.A., Raucy, J.L., Franklin, M.R., 2010. Nanosilver particles effect on drug metabolism in vitro. Drug. Metab. Dispus. 38, 2246-2251.

25- Limbach, L.K., Wick, P., Manser, P., Grass, R.N., Bruinink, A., et al., 2007. Exposure of engineered nanoparticles to human lung epithelial cells: Influence of chemical composition and catalytic activity on oxidative stress. Environ. Sci. Technol. 41, 4158-4163.

26- Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J., 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193, 265-275.

27- Lue, Y.H., Sinha-Hikim, A.P., Swerdloff, R.S., 1990. Single exposure to heat induces stage-specific germ cell apoptosis in rats: Role of intratesticular testosterone on stage specificity. Endocrinology 140, 1709-1717.

28- Malekinejad, H., Janbaz-Acyabar, H., Razi, M.,Varasteh, S., 2012. Preventive and protective effects of silymarin on doxorubicin-induced testicular damages correlate with changes in c-myc gene expression. Phytomedicine. 19, 1077-1084.

29- Matsumoto, M., Fujimoto, H., 1990. Cloning of a hsp70-related gene expressed in mouse spermatids. Biochem. Biophys. Res. Commun. 166, 43-49.

30- Miresmaeili, S.M., Halvaei, I., Fesahat, F., Fallah, A., Nikonahad, N., Taherinejad, M., 2013. Evaluating the role of silver nanoparticles on acrosomal reaction and spermatogenic cells in rat. Iran, J. Reprod. Med, 11, 423-430.

31- Mirshokraei, P., Hassanpour, H., Akhavan Taheri, M., Riyahi, M., Shams-Esfandabadi, N., 2011. The *in vitro* effects of nanosilver colloid on kinematic parameters of ram spermatozoa. Iran. J. Vet. Res. 12, 317-323.

32- Niehaus, W.G., Samuelsson, J.R.B., 1968. Formation of malonaldehyde from phospholipid arachidonate during microsomal lipid peroxidation. Eur. J. Biochem. 6, 126-130.

33- Oberdorster, G., Oberdorster, E., Oberdorster, J., 2005. Nanotoxicology: An emerging discipline evolving from studies of ultrafine particles. Environ. Health. Perspect. 113, 823-839.

34- Pant, N., Srivastava, S.P., 2003. Testicular and spermatotoxic effect of quinaphos in rats. J. Appl. Toxicol. 23, 271-274.

35- Phalen, R.F., Morrow, P.E., 1973. Experimental inhalation of metal silver. Health. Phys. 24, 509-518.

36- Razi, M., Sadrkhanloo, R.A., Malekinejad, H., 2011. Varicocele Time-dependently Affects DNAIntegrity of Sperm Cells: Evidence for Lower In vitro Fertilization Rate in Varicocele-positive Rats. Inter. J. Fertil. Steril. 5, 174-185.

37- Reddy, Pn., Pena-Mendez, E.M., Havel, J., 2008. Silver or silver nanoparticles: A hazardous threat to the environment and human?. J. Applied. Biomed. 6, 177-179.

38- Ricci, J.E., Munoz-Pinedo, C., Fitzgerald, P., 2004. Disruption of mitochondrial function duringapoptosis is mediated by caspase cleavage of the p75 subunit of complex I of the electrontransport chain. Cell. 117, 773-786.

39- Sanocka, D., Kurpisz, M., 2004. Reactive oxygen species and sperm cells. Reprod. Biol. Endocrinol. 2,12.

40- Sato, S., Sueki, H., Nishijima, A., 1999. Two unusual causes of argyria: The application of an improved tissue processing for X-ray microanalysis of selenium and sulfur in silver-laden granules. Br. J. Dermatol. 140, 158-163.

41- Scieglińska, D., Widłak, W., Rusin, M., Markkula, M., Krawczyk, Z., 1997. Expression of the testis-specific HSP70-related gene (hst70 gene) in somatic non-testicular rat tissues revealed by RT-PCR and transgenic mice analysis. Cell. Biol. Int. 21, 813–821.

42- Shan, L., Hardy, D.O., Catterall, J.F., Hardy, M.P., 1995. Effects of luteinizing hormone (LH) and androgen on steady state levels of messenger ribonucleic acid for LH receptors, androgen receptors, and steroidogenic enzymes in rat Leydig cell progenitors in vivo. Endocrinology. 136, 1686-1693.

43- Sharma, R.K., Pasqualotto, F.F., Nelson, D.R., 1999. The reactive oxygen species-total antioxidant capacity score is a new measure of oxidatives tress to predict male infertility. Hum. Reprod.14, 2801-2807.

44- Shibata, M., Hariya, T., Hatao, M., Ashikaga, T., Ichikawa, H., 1999. Quantitative polymerase chain reaction using an external control mRNA for determination of gene expression in a heterogeneous cell population. Toxicol. Sci. 49, 290-296.

45- Shin, S.H., Ye, M.M.K., 2012. The Effect of Nano-Silver on Allergic Rhinitis Model in Mice. Clin.Experiment. Otorhinol. 5, 222-227.

46- Shonhai, A., Boshoff, A., Blatch, G., 2007. The structural and functional diversity of Hsp70 proteins from Plasmodium falciparum. Pro. Science. 16, 1803-1818.

47- Sleiman, H.K., Romano, r.M., Alvarenga de Oliveira, C., Romano, M.A., 2013. Effects of Prepubertal Exposure to Silver Nanoparticles on Reproductive Parameters in Adult Male Wistar Rats. J.Toxicol Environ Health, Part A, 76:1023–1032.

48- Tejada, R.I., Mitchel, J.C., Norman, A., 1984. A test for the practical evaluation of male infertility by acridine orange (AO) fluorescence. Fertil. Steril. 42, 87-91.

49- Tesarik, J., Greco, E., Cohen-Bacrie, P., Mendoza, C., 1998. Germ cell apoptosis in men with complete and incomplete spermiogenesis failure. Mol. Hum. Reprod. 4, 757-762.

50- Vaziri, N.D., wang, X.Q., Oveisi, F., Zhou, X.G.J., 1998. Down regulation of nitric oxide synthesis in chronic renal insufficiency: Role of excess PTH. A. M. J. Physiol. 274, 642-649.

51- W.H.O., 1999. WHO laboratory manual for the examination of human semen and sperm cervical mucus interaction. Cambridge University Press, Cambridge, UK.

52- Zhu, D., Dix, D.J., Eddy, E.M., 1997. HSP70-2 is required for CDC2 kinase activity in meiosis I of mouse spermatocytes. Development. 124, 3007-3014.

Fig. 1: Effect of different doses of NS on total body weight (**A**) and on testicular weight to body weight ratio (**B**). All data are presented in Mean±SD.
[a,b,c] presented the significant differences (P<0.05) between test groups with each other and with control-sham group. **Note: Con:** Control-sham, **LD:** Low dose (0.5 mg/kg), **MD:** Medium dose (1 mg/kg), **HD:** High dose (5 mg/kg).

Fig. 2: (**A**) UV/VIS proton spectrophotometery for NS accumulation in testicular tissue, representing increased NS accumulation depending on administrated dose of NS. The control-sham group shows 100 T%, illustrating zero accumulation of NS. (**B**) The quantitative data for NS accumulation in testicular tissue. All data are presented in Mean±SD. Note increased NS accumulation depending on dose. [a,b,c,d] presented the significant differences (P<0.05) between test groups with each other and with control-sham group. **Note: Con:** Control-sham, **LD:** Low dose (0.5 mg/kg), **MD:** Medium dose (1 mg/kg), **HD:** High dose (5 mg/kg).

Fig. 3: Cross section from testis, the magnified sections (inserts) are presented in section 2 of each figure. (**A**) control-sham group; intact seminiferous tubules with normal spermatogenesis

are presented in figure **A-1**, note the magnified section, which exhibits positive TDI (**A-2**). (**B**) 0.5 mg/kg NS-treated group; see mild edema in interstitial connective tissue and tubule with negative TDI (*non-continuous line*) in figure **B-2**. (**C**) 1 mg/kg NS-treated group; Note increased percentage of tubules with negative TDI and germinal epithelium dissociation (*tubule in circle line*). (**D**) 5 mg/kg NS-treated group; note atrophied tubules with negative TDI, SPI. See depleted tubules with degenerated cells (*circle line*). Figures **E**, **F**, **G** and **H** are representing normal spermiogenesis, arrested spermiogenesis, negative TDI and edema associated with intensive immune cells infiltration, respectively. *Iron-Weigert staining (**A-1, B-1, C-1, D-1:** 100×; **A-2, B-2, C-2, D-2:** 400×; **E, F, G, H:** 800×).*

Fig. 4: Cross section from testis: (**A-1,2,3,4**) Iron-Weigert's staining for Leydig cells; Note the intact cells (*head arrow*) in control-sham testis, which are presented hypertrophied (*arrows*) in 0.5 mg/kg (**A-2**), 1 mg/kg (**A-3**) and 5 mg/kg (**A-4**) NS-treated groups. (**B-1,2,3,4**) Epi-fluorescent architecture for RNA in Leydig cells; the Leydig cells with normal RNA content are presented with dense red fluorescent reaction (*head arrow*), while the cells with damaged RNA are exhibiting yellowish fluorescent reaction (*arrows*) in 0.5 mg/kg (**B-2**), 1 mg/kg (**B-3**) and 5 mg/kg (**B-4**) NS-treated groups. (**C-1,2,3,4**) fluorescent staining for steroid foci; The damaged Leydig cells (*arrows*) in 0.5 mg/kg (**C-2**), 1 mg/kg (**C-3**) and 5 mg/kg (**C-4**) NS-treated animals showed significantly decreased Steroid accumulation (marked with green fluorescent reaction) in cytoplasm versus the intact cells (head arrows) in control-sham group (**C-1**), *600*×.

Fig. 5: Cross section from testis: (**A**) control-sham, (**B**) 0.5 mg/kg NS-treated, (**C**) 1 mg/kg NS-treated and (**D**) 5 mg/kg NS-treated group; the normal DNA content of the spermatozoa are presented with green fluorescent reaction (*arrow*) and the germinal epithelium with normal RNA content are exhibited with dense red fluorescent sites (*non-continuous line*). Meanwhile, the germinal epitheliums of the NS-treated testes are exhibited with severe RNA damage (*head arrows*). (**E**) Normal Hsp70-2 expression in two cells legions of spermatogenesis (*no filled arrow*) and spermiogenesis (black arrow), which is elevated in all cellular layers of 0.5 mg/kg NS-treated group and significantly decreased in 1 (G) and 5 mg/kg (H) NS-treated groups, respectively, (***A, B, C, D, F:*** *400× and* ***E, F, G, H:*** *600*×).

Fig. 6: Plot profile for Hsp70-2 immunohistochemical positive cells per 100 µm in (**A**) control-sham group, (**B**) 0.5 mg/kg NS-treated group, (**C**) 1 mg/kg NS-treated group and (**D**) 5 mg/kg NS-treated group. The profile for brown reactive sites (Hsp70-2 immunohistochemical expression) decreased depending on dose. (E) The HS70-2-positive cells number per 100 cell in

different groups, all data are presented in Mean±SD. [a,b,c,d] presented the significant differences (P<0.05) between test groups with each other and with control-sham group. **Note: Con:** Control-sham, **LD:** Low dose (0.5 mg/kg), **MD:** Medium dose (1 mg/kg), **HD:** High dose (5 mg/kg).

Fig. 7: Effect of NS on mRNA levels of Hsp70-2 in different groups; (**A**) the mRNA levels of Hsp70-2 and GAPDH were evaluated by using semi-quantitative RT-PCR. (**B**) Represents the density of Hsp70-2 mRNA levels in testicular tissue that were measured by densitometry and normalized to GAPDH mRNA expression level. Results are expressed as integrate intensity value (IDV) of Hsp70-2 mRNA level. [a,b,c,d] represent significant (P<0.05) differences between groups. **Note: Con:** Control-sham, **LD:** Low dose (0.5 mg/kg), **MD:** Medium dose (1 mg/kg), **HD:** High dose (5 mg/kg).

Fig. 8: (**A**) Total protein, (**B**) total RNA in different groups. All data are presented in Mean±SD, [a,b,c] presented the significant differences (P<0.05) between test groups with each other and with control-sham group. (**C**) Correlation between decreased total protein and reduced total protein content in different control-sham and test groups. r=0.875, P<0.05.

Fig. 9: (**A**) fluorescent staining for DNA integrity: the normal sperms are presented with light green DNA (*arrow*) and the sperms with damages DNA are marked with light red stained DNA (*tick arrow*), (**B**) Aniline-Blue staining for chromatin condensation: sperm with condensed chromatin is presented with light stained head piece (*arrow*) and the sperm with de-condensed chromatin is marked with dense blue stained head (tick arrow), (**C and D**) Eosin-nigrosin staining for sperm viability; death sperms are presented with stained cytoplasm (*tickarrows*) and the normal sperms are presented with unstained cytoplasm (*arrow*), (*1000*×).

Fig. 10: (**A**) tissue and sperm total antioxidant capacity (TAC) and (**B**) malondialdehyde (MDA) content in testis and sperm of the NS-treated and control-sham groups. All data are presented in Mean±SD. [a,b,c,d] are indicated significant (P<0.05) differences between marked groups. **Note: Con:** Control-sham, **LD:** Low dose (0.5 mg/kg), **MD:** Medium dose (1 mg/kg), **HD:** High dose (5 mg/kg).

Fig. 11: Mean alterations of testicular and sperm NO level. All data are presented in Mean±SD. NS enhanced the NO levels both at testicular and sperm levels. [a,b,c] presented the significant differences (P<0.05) between test groups with each other and with control-sham group. **Note: Con:** Control-sham, **LD:** Low dose (0.5 mg/kg), **MD:** Medium dose (1 mg/kg), **HD:** High dose (5 mg/kg).

Fig. 12: NS particles affect the testicular tissue via different pathways; a- the NS particles impact the gonadotropins LH and FSH secretion that can inhibit the testicular endocrine status. b- Through another mechanism, NS particles affect the Leydig cells directly, which in turn results in remarkable reduction in testosterone level. These two different pathways can influence the Sertoli cells physiologic activities, which promotes oxidative stress via enhancing the cellular apoptosis. c- On the other hand, the NS particles are capable to damage the cellular protein, DNA, RNA and homeostasis contents such as chaperones that in turn provoke severe oxidative stress. The sperm abnormalities increase as result for induced oxidative stress.

Table 1: Effect of NS administration on serum levels of LH, FSH and testosterone, all data are presented in Mean±SD.

Groups	**LH (ng/ml)**	**FSH (ng/ml)**	**Testosterone (ng/ml)**
Control-sham	31.55±0.40[a]	3.27±0.20[a]	7.24±0.43[a]

Low Dose	2.30±0.17[b]	2.34±0.31[b]	6.30±0.37[b]
Medium Dose	1.86±0.24[c]	1.53±0.27[c]	5.42±0.42[c]
High Dose	0.91±0.006[d]	0.89±0.17[d]	2.06±0.70[d]

Note: Low Dose: 0.5mg/kg NS-treated, **Medium Dose**: 1 mg/kg NS-treated, **High Dose**: 5 mg/kg NS-treated.

[a, b, c, d] indicate the significant differences between data in the same column. $P<0.05$ was considered as significant difference.

Table 2: Histomorphometric data for different test and control-sham groups, all data are presented in Mean±SD.

Parameters	Control-sham	Low Dose	Medium Dose	High Dose
Tubular Diameter (µm)	181.04±2.14[a]	167.66±5.52[b]	159.64±3.71[b]	150.75±1.64[c]
G.E. Height (µm)	70.03±6.55[a]	56.21±4.27[b]	40.09±3.21[c]	36.44±1.80[c]
TDI (%)	4.12±0.60[a]	8.00±2.94[b]	34.75±4.11[c]	43.50±2.64[d]
SPI (%)	9.75±1.70[a]	25.00±3.63[b]	70.33±5.27[c]	87.50±6.68[d]
E. Dissociation (µm)	0.50±0.01[a]	34.25±2.88[b]	42.31±4.41[c]	60.05±4.32[d]
T.RNA Damage (%)	4.25± 0.41[a]	21.37±1.28[b]	39.15±3.07[c]	46.28±5.11[d]

Note: Low Dose: 0.5mg/kg NS-treated, **Medium Dose**: 1 mg/kg NS-treated, **High Dose**: 5 mg/kg NS-treated, **G.E. Height:** Germinal Epithelium Height, **TDI:** Tubular differentiation index, **SPI:** Spermiogenesis Index, **E. Dissociation**: Epithelial Dissociation, **T.RNA Damage**: Tubules with RNA damage.

[a, b, c, d] indicate the significant differences between data in the same row. $P<0.05$ was considered as significant difference.

Table 3: Effect of NS on Leydig cells, all data are presented in Mean±SD.

Parameters	Control-sham	Low Dose	Medium Dose	High Dose
T.Leydig Cells (NO/mm^2)	35.25±2.50[a]	26.74±2.21[b]	19.00±1.03[c]	10.50±1.29[d]
H.Leydig Cells (%)	5.11±1.01[a]	9.60±2.59[b]	12.31±1.37[b]	18.79±1.82[c]
S.P.Leydig Cells (%)	86.25±2.62[a]	76.77±4.23[b]	53.08±6.57[c]	43.61±7.28[c]
RNA Damage (%)	3.11±1.08[a]	25.22± 3.21[b] 39.18±2.08[c]	41.28±4.22[c]	

Note: Low Dose: 0.5mg/kg NS-treated, **Medium Dose**: 1 mg/kg NS-treated, **High Dose**: 5 mg/kg NS-treated, **T.Leydig Cell:** Total Leydig Cells, **H.Leydig Cells:** Hypertrophied Leydig Cells, **S.P. Leydig Cells:** Steroid Positive Leydig Cells, **RNA Damage:** Leydig Cells with RNA Damage

[a, b, c, d] indicate the significant differences between data in the same row. $P<0.05$ was considered as significant difference.

Table 4: Impact of NS on sperm parameters in NS-treated and control-sham group, all data are presented in Mean±SD.

Parameter	**Control-sham**	**Low Dose**	**Medium Dose**	**High Dose**
Count ($\times 10^{6)}$	54.95±6.34[a]	39.75±4.28[b]	30.41±3.52[c]	21.59±3.08[d]
Motility (%)	90.12±6.75[a]	73.03±6.11[b]	68.30±5.41[b]	50.11±6.74[c]
Chromatin				
Condensation (%)	10.50±1.29[a]	27.41±5.56[b]	44.32±50.3[c]	71.00±6.78[d]
DNA damage (%)	8.75±1.11[a]	31.61±2.23[b]	44.27±2.96[c]	56.49±1.53[d]

Note: Low Dose: 0.5mg/kg NS-treated, **Medium Dose**: 1 mg/kg NS-treated, **High Dose**: 5 mg/kg NS-treated.

[a, b, c, d] indicate the significant differences between data in the same row. $P<0.05$ was considered as significant difference.

- Nanosilver changed gonadotropins biosynthesis.

- Nanosilver reduced steroidogenesis, affects testicular endocrine and antioxidant statuses.
- Nanosilver enhanced RNA damage and diminished Hsp70-2 expression.
- Nanosilver-induced oxidative and biochemical stress influenced spermatogenesis.
- Defected spermatogenesis and spermiogenesis adversely affected sperm quality.