STUDIA UNIVERSITATIS BABEŞ-BOLYAI BIOLOGIA, 69, 2, 2024 (p. 109-123)
doi:10.24193/subbbiol.2024.2.07

Reprotoxicity of zinc oxide nanoparticles synthesized with *Crataegus monogyna* leaves extract: testis and sperm function

F. Remita[1]✉, A. Talbi[2], C. Abdennour[2], K. Khelili[2], F. Duman[3], Z. Bouslama[2], R. Rouabhi[4]✉ and M. R. Djebar[2]

[1]Laboratory of Animal Ecophysiology, Department of Biology, Faculty of Sciences, University of Badji Mokhtar. 23000 Annaba, Algeria; [2]Environmental Research Center, Alzon, Annaba, 23002 Annaba, Algeria; [3]Department of Biology, Faculty of Science, University of Erciyes, 38039 Kayseri, Turkey; [4]Toxicology and ecosystem pathologies laboratory, University of Tebessa, 12000, Algeria.
✉***Corresponding author, E-mail: r_rouabhi@univ-tebessa.dz***

Article history: Received 27 March 2024; Revised 29 November 2024; Accepted 01 December 2024; Available online 10 December 2024

©2024 Studia UBB Biologia. Published by Babeş-Bolyai University.

This work is licensed under a Creative Commons Attribution-NonCommercial-NoDerivatives 4.0 International License.

Abstract. This work examines the effect of three doses of zinc oxide nanoparticles (ZnONPs), synthetized with *Crataegus monogyna* leaves using zinc acetate, on the sperm quality of Wistar rats. Animals were divided into 4 groups; the control group maintained without treatment, while ZNP1, ZNP2, and ZNP3 received respectively 10 mg ZNP/kgbw, 50 mg ZNP/kgbw, and 100 mg ZNP/kgbw by gavage for 15 days. Epididymis sperm was collected for sperm parameters: concentration, live sperm, motility, velocity (VCL, VSL, and VAP), linearity (LIN), amplitude lateral head (ALH), and beat cross frequency (BCF). DNA fragmentation was measured in three samples selected from control, ZNP1, and ZNP2. Testicular and epididymis malondialdehyde (MDA), glutathione (GSH), and glutathione peroxidase (GPx) were evaluated. Compared to the control, ZNP1 has a significant reduction of testicular and epididymis weights, sperm concentration, live sperm, motility, VCL, VSL, VAP, LIN, and BCF, with a significant increase of MDA and a significant decrease of GSH levels. The ZNP2 group demonstrated a significant increase in epididymis weight, a raise in sperm parameters (concentration, motility, VCL VSL, VAP, LIN, ALH, and BCF), and an

augmentation in GSH and GPx levels. However, ZNP3 has a significant increase in VSL and ALH while ZNP1 and ZNP2 showed no effect on spermatozoa DNA. interestedly, we found that the lower dose of ZNP1 acted as toxic to testicular and epididymis parameters, while the higher ones may help to improve sperm quality and reduced oxidative stress.

Keywords: zinc oxide; nanoparticles; *Crataegus monogyna*; sperm quality.

Introduction

In recent decades, there has been a remarkable expansion in the prevalence of several diseases including diabetes, cardiovascular disorders, and fertility issues (Iavicoli and Bergamaschi, 2009; Bergman *et al.,* 2013). According to the World Health Organization (2001), approximately 90% of male infertility investigated cases are linked to sperm anomalies. Sperm concentration, vitality, and motility are considered as important markers of male fertility, along with DNA fragmentation, which has become a crucial tool in diagnosing genetic abnormalities. Reactive oxygen species (ROS), as stated by Iommiello *et al.,* (2015), can adversely affect sperm membrane and DNA integrity, leading to strand breaks and chromatin cross-linking (Agarwal and Sengupta, 2020).

The World Health Organization estimated that 80% of pharmaceutical drugs based on medicinal plants showed therapeutic efficacy (Prairna *et al.,* 2020). This opens up a new area of biomedical nanotechnology that focuses on the study of nanostructure properties derived from plants known for their therapeutic effects. By exploring and understanding these properties, researchers aim to detect and target molecules associated with various diseases (Logothetidis, 2012). Many recent investigations showed the uses of plants as nanomaterials' producers by different methods (Zong *et al.,* 2014; Ramesh and Viruthagiri, 2015). Biosynthesis of nanostructures is an eco-friendly and a non-toxic process used as antimicrobial agents, drug delivery, biosensors, imaging contrast agent, transfection vectors (Vishwakarma, 2013), cosmetics, environment protection (Hussein *et al.,* 2018; Rasmussen *et al.,* 2010), imaging and identification of cells, destruction of viruses and cancer cells, and repairing damaged cells (Abo Alhasan, 2014; Hartung and Mansoori, 2015).

Zinc is known as reparative element to DNA and has a protective effect against cellular necrosis (Ho, 2004). It stabilizes the suppression gene P53 activity as an apoptosis regulator (Ng *et al.,* 2011). In addition, it plays an important role in the formation of DNA and RNA synthesis (Chvapil, 1973).

Many previous findings showed its effect on male reproduction as an antioxidant, testosterone booster, and spermatogenesis activator (Roy *et al.,* 2013), in which there was a significant positive correlation between zinc and semen quality (Vásquez and Arango, 2003). The addition of zinc acetate to plants could produce zinc oxide nanoparticles (ZnONPs) that are considered as safe and biocompatible (Raghupathi and Manna, 2011; Rosi and Mirkin, 2005). Thus, humans can be exposed to this synthetic ZnONPs via skin, inhalation, oral, and intravenous routes (Elshama and Abdel-Karim, 2018), which easily enter through cell membrane and could be either cytoprotective or cytotoxic (Espanani *et al.,* 2013). Moreover, ZnONPs are considered as powerful antioxidants (Shen *et al.,* 2013); as well they could increase antioxidant enzymes, also it may decrease malondialdehyde (Elshama and Abdel-Karim, 2018).

Among 280 species of hawthorn (Arya and Thakur, 2012), *Crataegus monogyna* is known for its medicinal properties, because it was used to treat certain diseases as cardiovascular disorder (Chang *et al.,* 2002), heart failure, and some nervous system troubles (Bechkri *et al.,* 2017). *C. monogyna* was used also as food (Gürsoy and Yıldız 2019), as a source of vitamins and phenolic compounds (Bernatoniene *et al.,* 2009), and oils that make hawthorn as one of the powerful antioxidant plants (Bahorun *et al.,* 1994). In addition, *C. monogyna* was reported to have a protective activity on male fertility against the toxicity of cyclophosphamide (Shalizar and Malekinejad, 2011) and doxorubicin (Shalizar and Hasanzadeh, 2013).

Therefore, the objective of this study is to investigate the effect of different doses of ZnONPs, synthesized from *C. monogyna* leaves' extract, on the sperm quality, sperm DNA fragmentation, and testicular and epididymis oxidative stress in male Wistar rats.

Materials and methods

Plant preparation

C. monogyna was harvested in November from Annaba area, Northeast Algeria. Fresh leaves were washed, shade dried, and then 10 ml of distilled water was added to 10 g of the powdered leaves, mixed in a hitting magnetic stirrer for 1h at 60°C, and the filtered extract was obtained.

Nanoparticles extraction

5 g of zinc acetate was added to 50 ml of the filtered leaves' extract, and then the mixture was stirred for 2h at 95°C. Afterward, the mixture was incubated in an oven for 2h at 80°C in which a powder of (ZNP) was formed.

Experimental design

24 male Wistar rats were reared in the animal house at standard conditions and given water and standard diet *ad libitum*. Animals were divided into 4 groups; the control group maintained without treatment, the ZNP1 group received 10 mg ZNP/kgbw, the ZNP2 group administrated with 50 mg ZNP/kgbw, and the ZNP3 group given 100 mg ZNP/kgbw. After 15 days of treatment by gavage, animals were sacrificed by decapitation. The sperm was immediately collected from the epididymis for sperm quality test, while testis and epididymis were weighed and stored for further uses. Animals' treatments were authorized by the Ethical Committee of Animal Sciences at the University of Badji Mokhtar-Annaba, before starting the experimental work.

Semen analysis

Semen analysis was performed with the Computer-Assisted Sperm Analysis Method (CASA) using Sperm Class Analysis (SCA®, Microptic, Barcelona, Spain). The epididymal semen was obtained and then, a drop of semen (about 1 µl) was diluted with a physiological solution of NaCl 0.09%. Afterward, 5 µl of the mixture was placed in an empty chamber slide (GoldCyto model). The slide was then placed on a Nikon Eclipse (Nikon E200-LED) microscope using the phase objective (x4). The sperm markers of concentration, vitality, motility, linearity (VCL and VSL), velocity (VAP), the amplitude of lateral head displacement (ALH), and the beat cross frequency (BCF) were automatically calculated.

Vitality analysis

Sperm vitality was assessed by SCA® CASA. Vitality is determined using BrightVit, a solution derived from nigrosine-eosin (NE), when the entry of eosin (Vital dye) into cells with dead or compromised membranes, causing them to appear pink. Meanwhile, live cells retain their natural white coloration. The inclusion of nigrosin as a background stain enhances the contrast for better distinction. BrightVit solution was made up in a hypo-osmotic medium and accordingly intact cells/cell membranes will swell, but cells with burst cell membranes will show thin straight tails and no signs of swelling.

DNA fragmentation test

The GoldCyto Sperm Kit (Goldcyto Biotech Corp) is a simple test that allows assessment of sperm DNA fragmentation in animals. The DNA fragmentation exanimated in three groups selected according to the results of epididymis semen analysis using the CASA, three samples was chosen from the control, ZNP1 and ZNP2 groups. The method is based on the Sperm Chromatin Dispersion (SCD)

(Fernández *et al.*, 2005). Intact unfixed spermatozoa are immersed in an inert agarose microgel on a pretreated slide. An initial lysing treatment removes most of the nuclear proteins, and in the presence of massive DNA loops, emerging from a nucleoid from spermatozoa with central core. However, the nucleoids from spermatozoa with intact DNA either do not show a dispersion halo or the halo is minimal. Results based on statistic methods are shown using mederate plus high DNA fragmentation proportion. According to Evenson and Jost, (2002), less than 15% represents a high fertility statue, 15-30% represents a resemblance to a fertility statue, and more than 30% signifies a significant lack of fertility potential.

Measurement of oxidative stress parameters

Frozen stored testis was thawed, then 100 mg of each sample was transferred to test tubes for the determination of glutathione (GSH) using the method of Weckbecker and Cory (1988). The principle of this assay is based on the measurement of the optical density of the acid 2-nitro-5-mercapturic. The latter results from the reduction of 5,5'-dithio-bis-2- acid nitrobenzoïque (Ellman's reagent, DTNB) by groups (-SH) of glutathione. Deproteinization of the homogenate is essential in order to keep only specific thiol groups of glutathione.

The testicular total proteins were quantified according to the colorimetric method of Bradford (1976) by using the Coomassie Brilliant Blue (BBC) as a reagent and the bovine serum albumin as a standard. The BBC reacts with the protein amino groups ($-NH_2$) to form a complex of blue color. Color intensity reflects the concentration of protein which is measured at 595 nm.

Malondialdehyde (MDA) was estimated by using the method of Ohkawa and Yagi, (1979). The dosage is based on the formation of a colored pigment absorbing at 530 nm after a reaction between MDA and thiobarbituric acid in an acidic and hot environment (100°C).

The measurement of glutathione peroxidase (GPx) was realized by the method of Flohe and Günzler (1984). This method is based on the reduction of hydrogen peroxide (H_2O_2) in the presence of reduced glutathione (GSH); the latter is transformed into (GSSG) under the influence of the GPx.

Nanoparticles size

ZnONPs were measured using ZEISS GeminiSEM 500 in the Nanoteknoloji Uygulama ve Araştırma Merkezi (ERNAM) in the University of Erciyes, Kayseri, Turkey.

Statistical analysis

Statistics was realized using (MINITAB 18 Software ANOVA Tukey). Results are expressed as mean ±standard deviation. The significant test was considered at $p<0.05$.

Results

Results in Table 1 showed the effect of ZnONPs extracted from *C. monogyna* using zinc acetate with diameter less then 200nm (Fig. 1) on testis and epididymis weights. ZNP1 showed a significant decrease ($p<0.05$) in the testicular absolute weights compared to the control while ZNP2 and ZNP3 have maintained the same levels as that of the control. Compared to the control, epididymis absolute weights have decreased significantly in ZNP1, while it showed a significant increase in ZNP2, whilst that of ZNP3 has maintained almost the same level of the control.

Table 1. Absolute testicular and epididymis weights (Mean ± SD) in Wistar rats after 15 days of treatment with ZnONPs synthesized from *C. monogyna* leaves.

	Control	**ZNP1**	**ZNP2**	**ZNP3**
Testis (g)	1.758±0.013[a]	1.372±0.056[b]	1.756±0.004[a]	1.754±0.006[a]
Epididymis (g)	0.572±0.002[b]	0.518±0.039[c]	0.676±0.009[a]	0.572±0.002[b]

Means that do not share the same letter are significantly different, according to one-way ANOVA, followed by Tukey test.

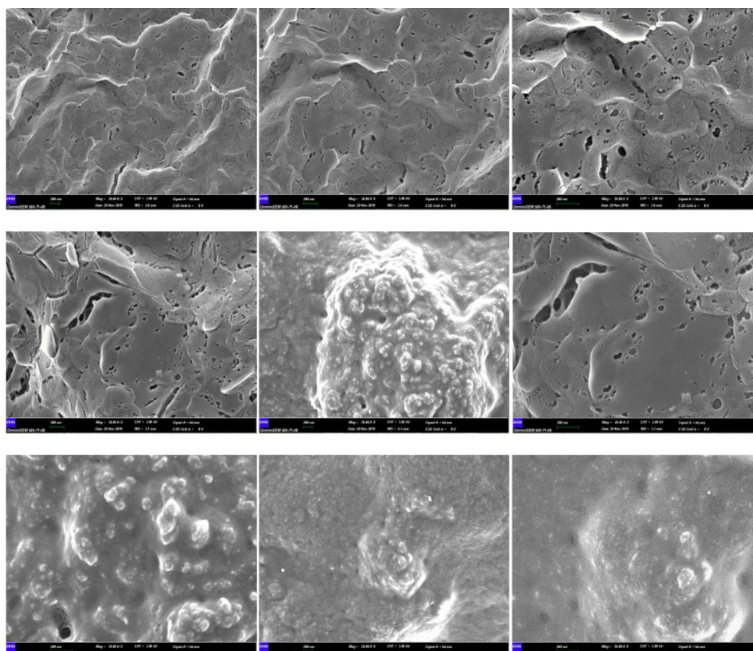

Figure 1. SEM image of zinc nanoparticles in the aqueous extract of *Crataegus monogyna* and their particles size of 200 nanometers.

Concerning the epididymis sperm parameters, a significant decrease of concentration, live sperm VCL, VCL, VAP, LIN, ALH, and BCF was observed in the ZNP1 compared to the control while in the ZNP2, the sperm concentration, VSL, LIN, ALH, and BCF have increased significantly (Table 2). Contrary, VSL and ALH levels were higher in rats of ZNP3 group compared to the control. In ZNP2 and ZNP3, both live and dead sperm have maintained the same percentages as that of the control.

The MDA concentration (Table 3) was significantly higher in ZNP1 compared to all groups. Contrary, a significant decrease in ZNP2 and ZNP3 compared to the control and ZNP1 group.

GSH showed a significant decrease in ZNP1 compared to the control while ZNP2 has demonstrated an elevation in the GSH level. In ZNP3, the level of GSH has similar as that of the control.

GPx activity illustrated a significant increase in ZNP2 group compared to the control. Though ZNP1 and ZNP3 groups have remained as that of the control.

Table 2. Epididymical semen parameters (Mean ± SD) in Wistar rats after 15 days of treatment with ZnONPs synthesized from C. monogyna leaves.

Parameter	Control	ZNP1	ZNP2	ZNP3
Concentration (Millions/ml)	82.05±1.31[b]	26.08±0.82[c]	88.80±0.67[a]	82.25±1.23[b]
Dead sperm (%)	26±0.89[b]	31.33±1.21[a]	24.66±1.21[b]	26±0.89[b]
Live sperm (%)	78.33±0.81[a]	71.16±0.75[b]	78.16±0.75[a]	77.83±0.75[a]
Motility (%)	77.14±0.88[b]	70.74±0.45[c]	81.23±1.009[a]	76.342±1.51[b]
VCL (μm/s)	88.84±0.70[b]	74.97±0.08[c]	92.85±0.63[a]	88.48±0.72[b]
VSL (μm/s)	16.27±0.16[b]	11.03±0.45[c]	19.47±0.60[a]	19.13±0.78[a]
VAP (μm/s)	39.002±0.46[b]	30.14±0.21[c]	45.84±0.16[a]	39.10±0.49[b]
LIN	29.93±0.36[b]	16.64±0.66[c]	31.77±0.60[a]	29.80±0.26[b]
ALH (μm)	4.35±0.09[c]	4.03±0.086[d]	4.93±0.04[a]	4.59±0.11[b]
BCF (Hz)	4.28±0.04[b]	2.60±0.178[c]	4.59±0.27[a]	4.26±0.08[b]

Means that do not share the same letter are significantly different, according to one-way ANOVA, followed by Tukey test.

Results in Table 4 showed that spermatozoa DNA fragmentation percentages of ZNP1 and ZNP2 are less than 15% which may indicate no effect of the doses used in our study (10 mg/kgbw and 50 mg/kgbw of ZnONPs extracted from *C. monogyna* leaves) on sperm DNA.

Table 3. Oxidative stress markers (Mean ± SD) in Wistar rats after 15 days of treatment with ZnONPs synthesized from C. monogyna leaves.

Parameter	Control	ZNP1	ZNP2	ZNP3
MDA (nmol/g tissue)	0.017±0. 00008[b]	0.018±0. 00004[a]	0.017±0. 00003[b]	0.017±0. 00001[b]
GSH (nmol/mg proteins)	0.447±0.011[b]	0.232±0.008[c]	0.549±0.011[a]	0.433±0.009[b]
GPx (µmol GSH/mg proteins)	0.055±0.002[b]	0.055±0.002[b]	0.080±0.003[a]	0.058±0.001[b]

Means that do not share the same letter are significantly different, according to one-way ANOVA, followed by Tukey test.

Table 4. Spermatozoa DNA fragmentation (%) in Wistar rats after 15 days of treatment with ZnONPs synthesized from *C. monogyna* leaves.

Parameter	Control	ZNP1	ZNP2
None fragmented DNA (%)	93	94	96.77
Fragmented DNA (%)	7	9	3.23

Discussion

In this study, the addition of zinc acetate to the *C. monogyna* leaves' extract has led to the synthesis of ZnONPs with a size of 200 nm. It is suggested that plant antioxidant compounds can reduce metals such as zinc acetate into nanoparticles (Mutukwa and Khotseng, 2022). Besides, *C. monogyna* has many active compounds such as flavones, rutin, catechin, and caffeic acid (Muradoğlu and Yıldız, 2019; Cosmulescu and Nour, 2017), which act as reducing agent and may be responsible in reducing zinc ions to ZnONPs (Shi and Jiang, 2013). Therefore, nanostructures are used in diagnosing, evaluating, and treating many health disorders (Paluszkiewicz *et al.*, 2021) by targeting organs, tissues, and specific cells (Prairna *et al.*, 2020). Furthermore, the absorption, distribution, and elimination of nanomaterials raise their ability to pass through cell barriers easily (Bleeker *et al.*, 2013). It was believed that nanoparticles exhibit better biomedical activity than the chemically synthesized ones (Agarwal *et al.*, 2018).

Results of treating rats using 10 mg/kgbw of ZnO synthesized nanoparticles using *C. monogyna* for 15 days showed a decline in sperm quality and an augmentation in oxidative stress reflected a higher level of MDA and GSH depletion. This dose has also no effect on sperm DNA fragmentation, despite it has not passed the threshold reported by Evenson and Jost (2002), who

postulated that fragmented DNA of less than 15% represent high fertility rate. It was confirmed that MWCNTs and other carbon-based nanoparticles injected to mice (5 g/kg) for 15 days didn't change the total sperm concentration, motility, and percentage of abnormal semen (Bai *et al*., 2010).

On the other hand, the administration of 50 and 300 mg/kgbw of ZnNPs during 35 days has affected sperm biology of mice (Rachid *et al*., 2008; Talebi and Moridian, 2013). Zinc nanoparticles have probably provoked testicular injuries (Boekelheide *et al*., 2000), and other nanoparticles may affect testicular tissue architecture (Iavicoli *et al*., 2013). Moreover, supplementation of 500 µL Au-NPs solution to human semen could reduce sperm motility and induce sperm DNA fragmentation (Wiwanitkit and Rojanathanes, 2009). In addition, Ag-NPs (nominal diameter of 20 ± 5 nm) may provoke a decrease in sperm concentration, and DNA damage when Wistar rats were treated with intravenous single dose of 5 mg/kg or 10 mg/kg (Gromadzka *et al*., 2012). This decline may be explained the effect of a varieties of nanoparticles on testosterone regulation (Iavicoli *et al*., 2013), via generating ROS such as superoxide ions, hydroxyl ions, singlet oxygen species, and peroxide molecules (Isık and Horzum, 2019), leading to poor sperm quality. As a result, ROS may induce apoptosis, lipid peroxidation, and DNA damage (Bardaweel *et al*., 2018) of Sertoli cells and disturb the spermatogenesis (Silva *et al.* 2022). In addition to modifying the sperm physiological function (Agarwal and Sengupta, 2020). Furthermore, sub-acute oral exposures to ZnNPs (300 mg/kg) for 14 consecutive days were stated to enhance ROS formation (Sharma *et al*., 2012), MDA augmentation, and GSH depletion. ZnONPs demonstrated effectiveness in combating various forms of cancer through the promotion of oxidative stress, enhancement of calcium influx into cancer cells, ultimately resulting in their demise (Bai *et al*., 2017).

In our finding, the rats of ZNP2 treated with 50 mg/kgbw have an increased fertility and a weaken oxidative stress, with a lower percentage of sperm DNA fragmentation while those of ZNP3 has kept sperm quality as that of the control. In addition, to having more protection of DNA structure. Likewise, NPs were suggested to have a role in activating proteins and inhibiting cell toxicity (Ali *et al*., 2009; Abbasi and Hano, 2017), while Kulandaivelu and Gothandam (2016) have found in an *in-vitro* study that silver nanoparticles at 100 µg/mL may inhibit cancerous cells through the activation of caspase-3. Similarly, ZnONPs of 1 mg/ml was not toxic and may prevent against oxidative stress (Nagajyothi *et al*., 2015).It was proven that ZnONPs using *Aloe vera* have capacity in scavenging free radicals and anticancer effect (Mahendiran *et al*., 2017). Accordingly, the ZnONPs *in vitro* (0.4, 1.6, and 6.4 µg ml) could increase the antioxidant enzymes in mice intestinal epithelium cell (Dawei, Zhisheng, and Anguo 2010) and could protect cells from ROS stress (Elshama and Abdel-Karim, 2018).

Nano-selenium supplementation (0.3 mg/kg) has proved to enhance sperm quality of goats after 90 days exposure (Shi *et al*., 2011). It was stated that the addition of zinc acetate to a plant extract may boost metal ions adsorption (Isık and Horzum, 2019). It could be argued in this circumstances that testis received ZnONPs has activated Sertoli cells for a better spermatogenesis. Also, zinc was proven to play an important role in improving spermatogenesis (Roy *et al.* 2013) and *C. monogyna* was effective in protecting against oxidative stress (Shalizar-Jalali and Malekinejad, 2011). *C. monogyna* components such as polyphenols (Muradoğlu and Yıldız, 2019), linoleic acid, oleic acid, oxalic acid bis (trimethylsilyl) ester, palmitic acid, and tetracosamethyl-cyclododecasiloxane (Bechkri *et al.,* 2017) may act as source of antioxidants and energy for better spermatozoa quality.

Conclusions

The effect of 200 nm ZnONPs synthesized from *C. monogyna* leaves' extract on sperm quality in Wistar rats was evaluated. At a dosage of 10 mg/kgbw for 15 days, fertility parameters were reduced, oxidative stress increased, and sperm DNA damage was observed. In contrast, a dosage of 50 mg/kgbw significantly improved sperm parameters, as well as levels of GSH and GPx activity. The dosage of 100 mg/kgbw had minimal impact on the parameters investigated.

Acknowledgements. All my thanks and gratitudes to MESRS and scientific research center of Annaba.

References

Abbasi, B.H., Anjum, S. & Hano, C. (2017). Differential effects of in vitro cultures of *Linum usitatissimum* L. (flax) on biosynthesis, stability, antibacterial and antileishmanial activities of zinc oxide nanoparticles: a mechanistic approach. *RSC Adv. 7*(26), 15931–15943. https://doi.org/10.1039/C7RA02070H.

Abo Alhasan, A.A. (2014). Rapid induced aggregation of gold nanoparticles by diolefinic dyes. *J. Nanomater. Mol. Nanotechnol. 3*(2). https://doi.org/10.4172/2324-8777.1000142.

Agarwal, A. & Sengupta, P. (2020). Oxidative stress and its association with male infertility. In: Parekattil, S.J., Esteves, S.C. & Agarwal, A. (eds.), Male infertility. *Springer International Publishing, Cham.* pp. 57–68. https://doi.org/10.1007/978-3-030-32300-4_6.

Agarwal, A., Barbăroșie, C., Ambar, R. & Finelli, R. (2020). The impact of single- and double-strand DNA breaks in human spermatozoa on assisted reproduction. *Int. J. Mol. Sci. 21* (11), 3882. https://doi.org/10.3390/ijms21113882.

Agarwal, H., Menon, S., Kumar, S.V. & Rajeshkumar, S. (2018). Mechanistic study on antibacterial action of zinc oxide nanoparticles synthesized using green route. *Chem. Biol. Interact. 286*, 60–70. https://doi.org/10.1016/j.cbi.2018.03.008.

Ali, C., Réda, D.M., Rachid, R. & Houria, B. (2009). Cadmium induced changes in metabolic function of mitochondrial isolated from potato tissue (*Solanum tuberosum* L.). *Am. J. Biochem. Biotech. 5*(1), 35-39.

Arya, V. & Thakur, N. (2012). Standardisation of haw fruits. *Am.-Eurasian J. Sci. Res. 7*(1), 16–22. https://doi.org/10.5829/idosi.aejsr.2012.7.1.62173.

Bahorun, T., Trotin, F., Pommery, J., Vasseur, J. & Pinkas, M. (1994). Antioxidant activities of *Crataegus monogyna* extracts. *Planta Med. 60*(4), 323–328. https://doi.org/10.1055/s-2006-959493.

Bai, D.-P., Zhang, X.-F., Zhang, G.-L., Huang, Y.-F. & Gurunathan, S. (2017). Zinc oxide nanoparticles induce apoptosis and autophagy in human ovarian cancer cells. *Int. J. Nanomed. 12,* 6521–6535. https://doi.org/10.2147/IJN.S140071.

Bai, Y., Zhang, Y., Zhang, J., Mu, Q., Zhang, W., Butch, E.R., Snyder, S.E. & Yan, B. (2010). Repeated administrations of carbon nanotubes in male mice cause reversible testis damage without affecting fertility. *Nat. Nanotechnol. 5,* 683–689. https://doi.org/10.1038/nnano.2010.153.

Bardaweel, S.K., Gul, M., Alzweiri, M., Ishaqat, A., ALSalamat, H.A. & Bashatwah, R.M. (2018). Reactive oxygen species: The dual role in physiological and pathological conditions of the human body. *Eurasian J. Med. 50,* 193–201. https://doi.org/10.5152/eurasianjmed.2018.17397.

Bechkri, S., Berrehal, D., Semra, Z., Bachari, K., Kabouche, A. & Kabouche, Z. (2017). Composition and biological activities of seeds oils of two *Crataegus* species growing in Algeria. *J. Mater. Environ. Sci. 8,* 1023–1028.

Bergman, Å., Heindel, J.J., Jobling, S., Kidd, K.A. & Zoeller, T.R. (eds.) (2013). Evidence for endocrine disruption in humans and wildlife. In: State of the science of endocrine disrupting chemicals - 2012, 23–237. Geneva, Switzerland: World Health Organization.

Bernatoniene, J., Trumbeckaite, S., Majiene, D., Baniene, R., Baliutyte, G., Savickas, A. & Toleikis, A. (2009). The effect of *Crataegus* fruit extract and some of its flavonoids on mitochondrial oxidative phosphorylation in the heart. *Phytother. Res. 23*(12), 1701–1707. https://doi.org/10.1002/ptr.2815

Bleeker, E.A.J., De Jong, W.H., Geertsma, R.E., Groenewold, M., Heugens, E.H.W., Koers-Jacquemijns, M., Van De Meent, D., *et al.* (2013). Considerations on the EU definition of a nanomaterial: Science to support policy making. *Regul. Toxicol. Pharmacol. 65*(1), 119–125. https://doi.org/10.1016/j.yrtph.2012.11.007

Boekelheide, K., Fleming, S.L., Johnson, K.J., Patel, S.R. & Schoenfeld, H.A. (2000). Role of Sertoli cells in injury-associated testicular germ cell apoptosis. *Proc. Soc. Exp. Biol. Med. 225*(2), 105–115. https://doi.org/10.1046/j.1525-1373.2000.22513.x

Bradford, M.M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem. 72*(1–2), 248–254. https://doi.org/10.1016/0003-2697(76)90527-3

Chang, Q., Zuo, Z., Harrison, F. & Chow, M.S.S. (2002). Hawthorn. *J. Clin. Pharmacol. 42*(6), 605–612. https://doi.org/10.1177/00970002042006003

Chvapil, M. (1973). New aspects in the biological role of zinc: A stabilizer of macromolecules and biological membranes. *Life Sci. 13*(8), 1041–1049. https://doi.org/10.1016/0024-3205(73)90372-X

Cosmulescu, S., Trandafir, I. & Nour, V. (2017). Phenolic acids and flavonoids profiles of extracts from edible wild fruits and their antioxidant properties. *Int. J. Food Prop. 20*(12), 3124–3134. https://doi.org/10.1080/10942912.2016.1274906

Ai, D., Wang, Z. & Zhou, A. (2010). Protective effects of nano-ZnO on the primary culture mice intestinal epithelial cells in vitro against oxidative injury. *World J. Agric. Sci. 6*(2), 149–153. doi:javaa.2009.1964.1967

Elshama, S.S., Abdallah, M.E. & Abdel-Karim, R.I. (2018). Zinc oxide nanoparticles: Therapeutic benefits and toxicological hazards. *Open Nanomed. J. 5*(1), 16–22. doi:10.2174/1875933501805010016.

Espanani, H.R., Fazilati, M., Sadeghi, L., Babadi Vahid Yousefi, B., Bakhshiani, S. & Amraie, E. (2013). Investigation the zinc oxide nanoparticle's effect on sex hormones and cholesterol in rat. *Int. Res. J. Biol. Sci. 2*(8), 54–58. http://www.isca.me/IJBS/Archive/v2/i8/11.ISCA-IRJBS-2013-124.php

Evenson, D.P., Larson, K.L. & Jost, L.K. (2002). Sperm chromatin structure assay: Its clinical use for detecting sperm DNA fragmentation in male infertility and comparisons with other techniques. *J. Androl. 23*(1), 25–43. doi:10.1002/j.1939-4640.2002.tb02599.x.

Fernández, J.L., Muriel, L., Goyanes, V., Segrelles, E., Gosálvez, J., Enciso, M., LaFromboise, M. & De Jonge, C. (2005). Simple determination of human sperm DNA fragmentation with an improved sperm chromatin dispersion test. *Fertility Sterility 84*(4), 833–842. doi:10.1016/j.fertnstert.2004.11.089

Flohé, L. & Günzler, W.A. (1984). Assays of glutathione peroxidase. *Method. Enzymol. 105,* 114–120. doi:10.1016/S0076-6879(84)05015-1

Gromadzka-Ostrowska, J., Dziendzikowska, K., Lankoff, A., Dobrzyńska, M., Instanes, C., Brunborg, G., Gajowik, A., Radzikowska, J., Wojewódzka, M. & Kruszewski, M. (2012). Silver nanoparticles effects on epididymal sperm in rats. *Toxicol. Lett. 214*(3), 251–258. doi:10.1016/j.toxlet.2012.08.028

Hartung, G.A. & Mansoori, G.A. (2015). In vivo general trends, filtration and toxicity of nanoparticles. *J. Nanomater. Mol. Nanotechnol. 2*(3). doi:10.4172/2324-8777.1000113

Ho, E. (2004). Zinc deficiency, DNA damage and cancer risk. *J. Nutr. Biochem. 15*(10), 572–578. doi:10.1016/j.jnutbio.2004.07.005

Hussein, J., El-Banna, M., Abdel Razik, T. & El-Naggar, M.E. (2018). Biocompatible zinc oxide nanocrystals stabilized via hydroxyethyl cellulose for mitigation of diabetic complications. *Int. J. Biol. Macromol. 107*, 748–754. doi:10.1016/j.ijbiomac.2017.09.056

Iavicoli, I., Fontana, L. & Bergamaschi, A. (2009). The effects of metals as endocrine disruptors. *J. Toxicol. Environ. Health, Part B 12*(3), 206–223. doi:10.1080/10937400902902062

Iavicoli, I., Fontana, L., Leso, V. & Bergamaschi, A. (2013). The effects of nanomaterials as endocrine disruptors. *Int. J. Mol. Sci. 14*(8), 16732–16801. doi:10.3390/ijms140816732

Iommiello, V.M., Albani, E., Di Rosa, A., Marras, A., Menduni, F., Morreale, G., Levi, S.L., Pisano, B. & Levi-Setti, P.E. (2015). Ejaculate oxidative stress is related with sperm DNA fragmentation and round cells. *Int. J. Endocrinol*. 1–6. doi:10.1155/2015/321901

Isık, T., Elhousseini Hilal, M. & Horzum, N. (2019). Green synthesis of zinc oxide nanostructures. In: Nahhas, A.M. (ed.), Zinc oxide based nano materials and devices. *IntechOpen*. doi:10.5772/intechopen.83338

Kulandaivelu, B. & Gothandam, K.M. (2016). Cytotoxic effect on cancerous cell lines by biologically synthesized silver nanoparticles. *Braz. Arch. Biol. Technol. 59, e16150529.* doi:10.1590/1678-4324-2016150529

Logothetidis, S. (2012). Nanomedicine: The medicine of tomorrow. In: Nanomedicine and nanobiotechnology, Logothetidis, S. (ed.), Springer Berlin Heidelberg, Berlin, Heidelberg, pp. 1–26. doi:10.1007/978-3-642-24181-9_1

Mahendiran, D., Subash, G., Arumai Selvan, D., Rehana, D., Senthil Kumar, R. & Kalilur Rahiman, A. (2017). Biosynthesis of zinc oxide nanoparticles using plant extracts of *Aloe vera* and *Hibiscus sabdariffa*: Phytochemical, antibacterial, antioxidant and anti-proliferative studies. *BioNanoSci. 7*(3), 530–545. doi:10.1007/s12668-017-0418-y

Muradoğlu, F., Gürsoy, S. & Yıldız, K. (2019). Quantification analysis of biochemical and phenolic composition in hawthorn (*Crataegus* spp.) fruits. *Erwerbs-Obstbau 61*(2), 189–194. doi:10.1007/s10341-018-00415-z

Mutukwa, D., Taziwa, R. & Khotseng, L.E. (2022). A review of the green synthesis of ZnO nanoparticles utilising Southern African indigenous medicinal plants. *Nanomaterials 12*(19), 3456. doi:10.3390/nano12193456

Nagajyothi, P.C., Cha, S.J., Yang, I.J., Sreekanth, T.V.M., Kim, K.J. & Shin, H.M. (2015). Antioxidant and anti-inflammatory activities of zinc oxide nanoparticles synthesized using *Polygala tenuifolia* root extract. *J. Photochem. Photobiol. B: Biol. 146,* 10–17. doi:10.1016/j.jphotobiol.2015.02.008

Ng, K.W., Khoo, S.P.K., Heng, B.C., Setyawati, M.I., Tan, E.C., Zhao, X., Xiong, S., Fang, W., Leong, D.T. & Loo, J.S.C. (2011). The role of the tumor suppressor p53 pathway in the cellular DNA damage response to zinc oxide nanoparticles. *Biomaterials 32*(32), 8218–8225. doi:10.1016/j.biomaterials.2011.07.036

Ohkawa, H., Ohishi, N. & Yagi, K. (1979). Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. *Anal. Biochem. 95*(2), 351–358. doi:10.1016/0003-2697(79)90738-3

Paluszkiewicz, P., Martuszewski, A., Zaręba, N., Wala, K., Banasik, M. & Kepinska, M. (2021). The application of nanoparticles in diagnosis and treatment of kidney diseases. *Int. J. Mol. Sci. 23*(1), 131. doi:10.3390/ijms23010131

Prairna, M., Paramanya, A., Sharma, S., Bahtiyarca Bagdat, R. & Ali, A. (2020). Recent practices of medicinal and aromatic plants in nanotechnology. In: Husen, A. & Jawaid, M. (eds.), Nanomaterials for agriculture and forestry applications, Elsevier, Amsterdam; Cambridge, MA, pp. 435–467. doi:10.1016/B978-0-12-817852-2.00018-4

Raghupathi, K.R., Koodali, R.T. & Manna, A.C. (2011). Size-dependent bacterial growth inhibition and mechanism of antibacterial activity of zinc oxide nanoparticles. *Langmuir 27*(7), 4020–4028. doi:10.1021/la104825u

Rachid, R., Houria D.B., Mohammed-Réda, D. (2008). Impact of Flufenoxuron, an IGR pesticide on *Gallus domesticus* embryonic development *in ovo*. J. *Cell & An. Biol. 2*(3), 87-91.

Ramesh, M., Anbuvannan, M. & Viruthagiri, G. (2015). Green synthesis of ZnO nanoparticles using *Solanum nigrum* leaf extract and their antibacterial activity. Spectrochimica Acta Part A: *Mol. Biomol. Spectro. 136,* 864–870. doi:10.1016/j.saa.2014.09.105

Rasmussen, J.W., Martinez, E., Louka, P. & Wingett, D.G. (2010). Zinc oxide nanoparticles for selective destruction of tumor cells and potential for drug delivery applications. *Expert Opin. Drug Deliv. 7*(9), 1063–1077. doi:10.1517/17425247.2010.502560

Rosi, N.L. & Mirkin, C.A. (2005). Nanostructures in biodiagnostics. *Chem. Rev. 105*(4), 1547–1562. doi:10.1021/cr030067f

Roy, B., Baghel, R.P.S., Mohanty, T.K. & Mondal, G. (2013). Zinc and male reproduction in domestic animals: A review*. Indian J. Anim. Nutr. 30*(4), 339–350.

Shalizar-Jalali, A. & Hasanzadeh, S. (2013). *Crataegus monogyna* fruit aqueous extract as a protective agent against doxorubicin-induced reproductive toxicity in male rats. *Avicenna J. Phytomed. 3*(2), 159–170. PMC4075703

Shalizar-Jalali, A., Hasanzadeh, S. & Malekinejad, H. (2011). Chemoprotective effect of *Crataegus monogyna* aqueous extract against cyclophosphamide-induced reproductive toxicity. *Vet. Res.* 266–273.

Sharma, V., Singh, P., Pandey, A.K. & Dhawan, A. (2012). Induction of oxidative stress, DNA damage and apoptosis in mouse liver after sub-acute oral exposure to zinc oxide nanoparticles*. Mutat. Res./Genet. Toxicol. Environ. Mutagen, 745*(1–2), 84–91. doi:10.1016/j.mrgentox.2011.12.009.

Shen, C., James, S.A., De Jonge, M.D., Turney, T.W., Wright, P.F.A. & Feltis, B.N. (2013). Relating cytotoxicity, zinc ions, and reactive oxygen in ZnO nanoparticle–exposed human immune cells. *Toxicol. Sci. 136*(1), 120–130. doi:10.1093/toxsci/kft187.

Shi, L., Xun, W., Yue, W., Zhang, C., Ren, Y., Shi, L., Wang, Q., Yang, R. & Lei, F. (2011). Effect of sodium selenite, Se-yeast and nano-elemental selenium on growth performance, Se concentration and antioxidant status in growing male goats. *Small Rumin. Res. 96*(1), 49–52. doi:10.1016/j.smallrumres.2010.11.005.

Shi, T. & Jiang, Y. (2013). Isolation of flavonoids from the aerial parts of *Polygala tenuifolia* Willd. and their antioxidant activities. *J. Chin. Pharm. Sci. 22*(1). doi:10.5246/jcps.2013.01.004.

Silva, R., Carrageta, D.F., Alves, M.G., Silva, B.M. & Oliveira, P.F. (2022). Antioxidants and male infertility. *Antioxidants 11*(6), 1152. doi:10.3390/antiox11061152.

Talebi, A. R., Khorsandi, L. & Moridian, M. (2013). The effect of zinc oxide nanoparticles on mouse spermatogenesis. *J. Assist. Reprod. Genet. 30*(9), 1203–1209. doi:10.1007/s10815-013-0078-y.

Vásquez, L., Vera, O. & Arango, J. (2003). Testicular growth and semen quality in peripuberal Brahman bulls. *Livest. Res. Rural Dev. 15*(10). Retrieved from http://www.lrrd.org/lrrd15/10/vasq1510.htm

Vishwakarma, K. (2013). Green synthesis of ZnO nanoparticles using *Abrus precatorius* seeds extract and their characterization. MSc thesis, National Institute of Technology, Rourkela, India. Retrieved from http://ethesis.nitrkl.ac.in/5012/.

Weckbecker, G. & Cory, J. G. (1988). Ribonucleotide reductase activity and growth of glutathione-depleted mouse leukemia L1210 cells in vitro. *Cancer Lett. 40*(3), 257–264. doi:10.1016/0304-3835(88)90084-5.

Wiwanitkit, V., Sereemaspun, A. & Rojanathanes, R. (2009). Effect of gold nanoparticles on spermatozoa: The first world report. *Fertility Sterility 91*(1), e7–e8. doi:10.1016/j.fertnstert.2007.08.021.

World Health Organization. (2001). Laboratory manual of the WHO for the examination of human semen and sperm-cervical mucus interaction. *Ann. Ist. Super. Sanità* 37(1), I–XII, 1–123.

Zong, Y., Li, Z., Wang, X., Ma, J. & Men, Y. (2014). Synthesis and high photocatalytic activity of Eu-doped ZnO nanoparticles. *Ceram. Int. 40*(7), 10375–10382. doi:10.1016/j.ceramint.2014.02.123.