Int. J. Nanosci. Nanotechnol., Vol. 19, No. 2, June 2023, pp. 77-84
DOI: 10.22034/ijnn.2023.557252.2236

Screening Assessment of the Potential Hazard of Nanomaterials using a Bull Sperm

O. Demetska[1,*], O. Beliuha[2], V. Movchan[2], M. Didenko[2], V. Riabovol[3], T. Patyka[2], V. Tsapko[2] and A. Balia[4]

[1]Shupyk National Healthcare University of Ukraine, Kyiv, Ukraine
[2]State Institution «Kundiiev Institute of Occupational Health of the National Academy of Medical Sciences of Ukraine», Kyiv, Ukraine
[3]Bogomolets National Medical University, Kyiv, Ukraine
[4]Graduate School of Pharmaceutical Sciences, Nagoya City University, Nagoya, Japan

(*) Corresponding author: dalexandra@ukr.net
(Received: 6 July 2022 and Accepted: 25 March 2023)

Abstract

This paper studied a potential hazard of titanium dioxide nanomaterials in the primary sreening methods on the sample of bull sperm. A method for stabilizing metal nanopowders and their derivatives for biomedical studies is proposed. It was found that the complex of titanium dioxide doped with silver (Ag-TiO2) and nanopowder of titanium dioxide (TiO2) at concentrations of 3 mg/ml initiate pathological changes in the bull sperm: the release of phospholipids as a result of the destruction of membranes and morphological abnormalities (abnormalities of the head, middle part, and tail, as well as the absence of acrosome, etc). The pathological effect of Ag-TiO2 nanocomposite was more pronounced. Doping with silver can increase the toxicity of nanotitanium dioxide, which requires further in-depth experimental studies using different concentrations by in vivo and in vitro methods.

Keywords: *Bull Sperm, Nanomaterials, Screening, Titanium dioxide.*

1. INRODUCTION

Nano-titanium dioxide (TiO2) is one of the most commonly used materials being synthesized for use as one of the top five nanoparticles [1]. The applications of nano-structured TiO_2 can be now found in a wide range of areas including electronic materials, energy, environment, health & medicine, catalysts, etc because of its special photovoltaic and photocatalytic activities [2, 3, 4]. Due to the extensive application of TiO_2 nanoparticles and their inclusion in many commercial products, increased exposure of human beings to nanoparticles is possible. Therefore, the assessment of potential hazards associated with new technologies and related products has become a new area for health risk assessment [5, 6]. Screening evaluation of nanomaterials in vitro allows to obtain additional information on the possible danger and is also appropriate from the standpoint of bioethics [7, 8, 9]. The main advantage of most in vitro studies is the ability to test a large array of objects, as well as the ability to assess the direct toxic effect on the cell (target). It should be noted that the toxicity of nanoparticles is primarily due to the development of OS, and peroxidation of the membrane with a subsequent increase in their permeability, dysfunction, and destruction [10, 11]. In the body, the substrate of the lipid peroxidation reaction is lipophilic compounds localized in membranes and other lipid structures, and the initiators are hydrophilic reactive oxygen species (ROS) [12, 13].

The choice of sperm as a test object is conditioned by the fact that despite the short period of life, their biological features (plasma membrane and acrosome, which are lipoprotein and glycoprotein formations, packing density of proteins and nucleic acids in the nucleus, low content of

low level of metabolism in a stationary state) cause great resistance to external influences [14, 15, 16]. Sperm are more sensitive to OS than other cells due to a large number of polyunsaturated fatty acids and lipids found in their cell membranes, which are easily peroxidized; the low amount of cytoplasm with low concentrations of DNA repair systems and antioxidant enzymes, which are unable to protect the cell membrane and acrosome [17]. Accumulations of peroxides in tissues are accompanied by the destruction of the molecular structure of cellular lipids, particularly phospholipids [18, 19].

Thus, the work aimed to evaluate the expediency of using a combination of indicators (phospholipid release and morphological abnormalities) that reflect the potentially dangerous impact of nanomaterials for their primary screening.

2. MATERIALS AND METHODS

2.1. Materials

The study used a complex of titanium dioxide doped with a silver (nanocomposite Ag-TiO2, mass fraction of Ag~4%), which was obtained by ultrasonic dispersion of TiO2 + AgNO3 [20] (Fig. 1). and nanopowder of titanium dioxide (TiO2) synthesized by thermal decomposition [21] (Fig. 2).

Frozen bull sperm (granules) was used as a test object.

2.2. Methods

A. Determination of Hydrodynamic Diameter of Nanomaterials

The particle size was determined by the method of dynamic light scattering using the device Analysette 12 DynaSizer (Fritsch, Germany).

B. Sperm Sample Preparation and Phospholipid Extraction

As a screening method for determining the toxicity and damaging effects of nanomaterials in vitro using bull sperm as a test object, the method of extracting phospholipids from a rat brain was adapted and used [22]. Instead of a rat brain, frozen bull semen (granules) was used, which was thawed in a glucose-citrate buffer (ratio of glucose and sodium citrate 4:1) in a thermostat at a temperature of 37 ^{0}C for 90 minutes. To the test solution at the beginning of thawing was added 3 mg of nanopowder in 1 ml of glucose-citrate buffer. The phospholipid extract of the experimental and control samples was examined on a ULAB 101 UV spectrophotometer (HLR, China) at a wavelength of 540 nm.

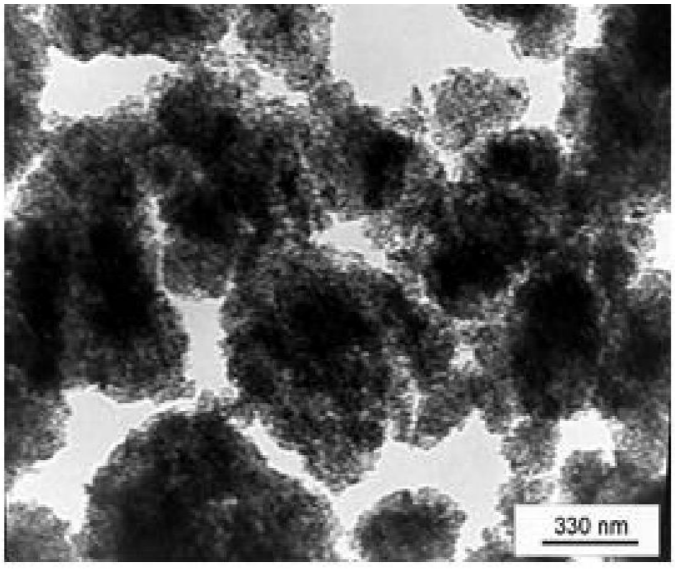

Figure 1. *Electron microscopy of TiO2 (JEM-1400, JEOL, Japan).*

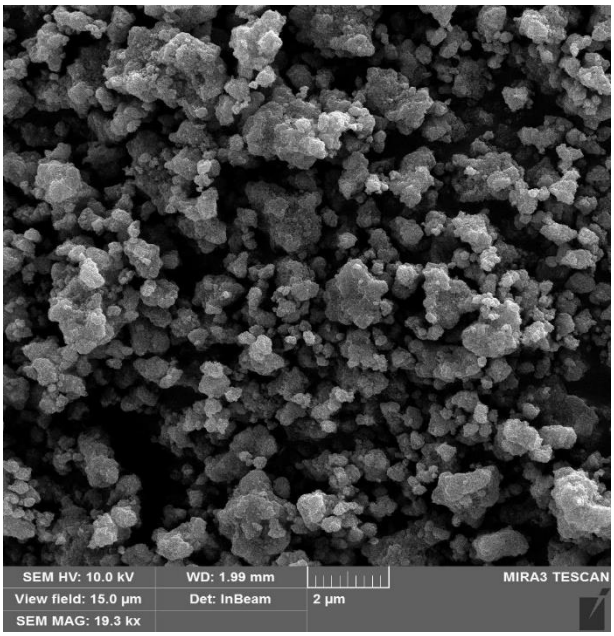

Figure 2. *Electron microscopy of Ag-TiO2 (JEM-1400, JEOL, Japan).*

C. Morphological Studies of the Test Object

For morphological studies of sperm defects under the action of TiO_2 nanopowder and Ag-TiO_2 composite, ejaculate smears were prepared by evenly distributing a drop of bioliquid on a glass slide, air-dried, and fixed with ethanol for one minute. Staining of smears was performed according to the method of Lefler (methylene blue), fixed by Main-Grunwald with staining by Romanovsky diluted (1/3) and undiluted paint [23]. Stained specimens were analyzed by immersion under an x1000 lens using a Charles Zeiss microscope (Germany).

D. Data Analysis

The obtained research results were statistically processed by the methods of variational statistics using the Microsoft Excel 2007 with StatPlus add-in. Data are represented by the mean and standard deviation, and the Mann–Whitney U test [24] was used for comparison between groups.

3. RESULTS AND DISCUSSIONS

To study the potentially damaging effects of nanomaterials, it was necessary to develop a method of stabilizing metal nanopowders and their derivatives in aqueous solutions for subsequent use in biomedical studies in vitro and in vivo. It is known for the stabilization of nanomaterials in solutions using organic and inorganic compounds that provide colloidal solutions of varying degrees of stability. The toxicity of the composition is determined not only by the toxicity of the active substance (eg, metal nanoparticles) but also by stabilizing or other auxiliary components that may affect the biological activity of the resulting solution. A key aspect was the search for a stabilizer that would not increase the toxicity of the source nanomaterial and would also provide a «comfortable» environment for bovine sperm.

As a stabilizer, a glucose-citrate buffer (glucose (4 g), sodium citrate (1 g) in 100 ml of distilled water) is proposed, which is usually used for thawing sperm, as well as as a control solution [25]. Thus, the proposed stabilizer allows for obtaining relatively stable hydrosols of metal nanopowders, while avoiding undesirable effects on the toxicity of the source nanomaterial (Table 1, Fig. 3, 4).

Table 1. *Dispersion of Ag-TiO_2 nanocomposite (4%) in glucose citrate buffer over time.*

Glucose: sodium citrate, correlation	Day	Hydrodynamic diameter, nm
4:1	I	48,65±1,08 nm
4:1	II	53,37±1,43 nm
4:1	III	52,12±1,37 nm

Determination of the optical density of the obtained phospholipid extracts indicates favor of the destruction of the molecular structure of sperm membranes that were exposed to nanomaterials and the release of phospholipids (Table 2).

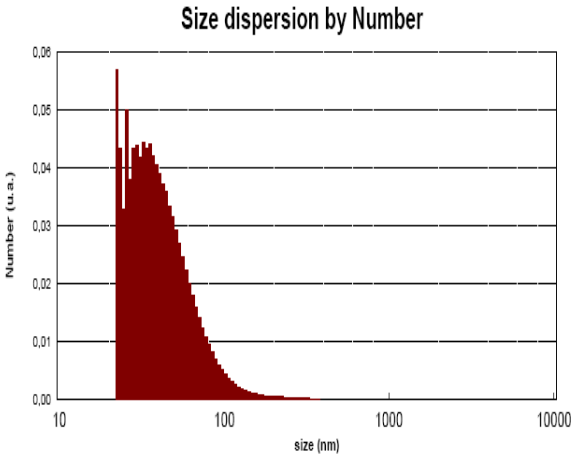

Figure 3. *Distribution by the number of particles in the sample of nanocomposite Ag-TiO2 (4%) in glucose and sodium citrate 4:1 (1st day).*

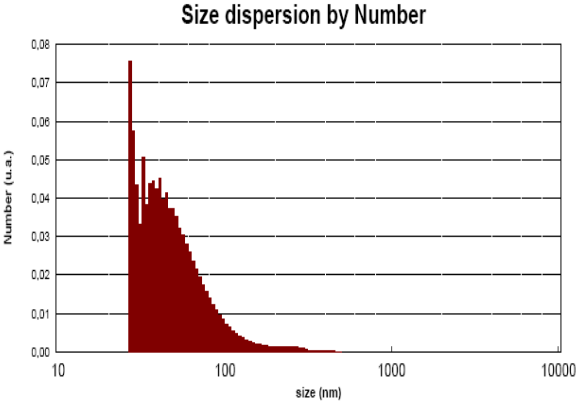

Figure 4. *Distribution by the number of particles in the sample of nanocomposite Ag-TiO2 (4%) in from the ratio of glucose and sodium citrate 4: 1 (2nd day).*

The application of the Mann-Whitney U-test showed that the differences in the sample levels are significant (U1 (0.05)=23, U2c(0.01)=16, U=0,), which indicates the existence of a correlation between the effect of the investigated nanomaterials and the increase in optical density.

The obtained results correspond to the data of morphological analysis of sperm anomalies exposed by the studied nanomaterials: the share of their defects was significantly higher in the experimental samples (61% under the influence of Ag-TiO2 nanocomposite and 39% under the influence of TiO2) compared with control (20%) (Table 3).

Table 2. *Optical density and transmittance of experimental and control extracts of phospholipids (λ = 540 nm).*

Sample	Transmittance%	Optical density	P*
Control (n=10)	98,6±0,8	0,0085±0,001	<0,0005
TiO_2 (n=10)	50,1±0,02	0,204±0,003	
Ag-TiO_2 (n=10)	57,65±0,07	0,243±0,004	

* P values were calculated from Mann–Whitney U test

When evaluating the sperm head, its edema and absence of the acrosomal region were noted, which was more often detected in all observations compared to the control (Figs. 5, 6, 7). Sperm tail defects were characterized by shortening, twisting, and swelling, which indicates their low motility.

In addition, the aggregation of sperm was determined, which further indicates their pathological condition.

Table 3. *Morphological abnormalities of sperm exposed to nanomaterials.*

Morphological abnormalities	Control, % n=10	TiO_2, % n=10	Ag-TiO_2, % n=10
Defects of the head	3±0,47	6±1,05	11±3,06
Lack of acrosome	12±1,94	21±2,71	23±2,21
Defects of the middle part	-	5±1,15	9±3,27
Tail defects	5±1,41	7±1,56	18±3,33
The share of anomalies	20±1,27	39±1,62	61±2,97

After exposure of sperm to Ag-TiO_2 nanocomposite, a significant number of residual cells were detected as a result of cell death. These changes, along with the detected abnormalities of sperm, indicate in favor of a more pronounced pathological effect of nanocomposite Ag-TiO_2: the percentage of morphological abnormalities of sperm caused by exposure Ag-TiO_2 was 3.05 times higher than by TiO_2 and 1.5 times higher than control ($P<0,0005$). In particular, Ag-TiO_2 caused 1.8 times more head defects compared to TiO_2 and 3.7 times more than the control, as well as 2.6 times and 3.6 times more tail defects, respectively.

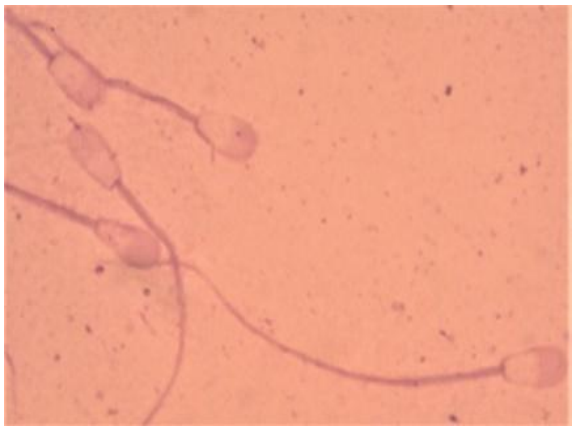

Figure 5. *Bull sperm (control).*

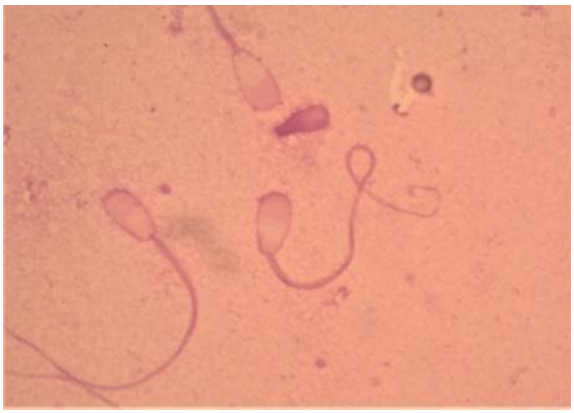

Figure 6. *Bull sperm exposed to Ag-TiO_2 (double defects: head-edema, tilt, reduction of the acrosomal region; tail-twisting of the middle section and loop in the end).*

Thus, Ag-TiO_2 nanocomposite (mass fraction of Ag ~ 4%) and TiO_2 nanopowder in concentrations of 3 mg/ml initiate pathological changes in bull sperm, which are markers of oxidative stress, anomalies of the head, middle part, and tail, as well as the absence of acrosomes, etc.), while the pathological effect of the nanocomposite Ag-TiO_2 is more pronounced.

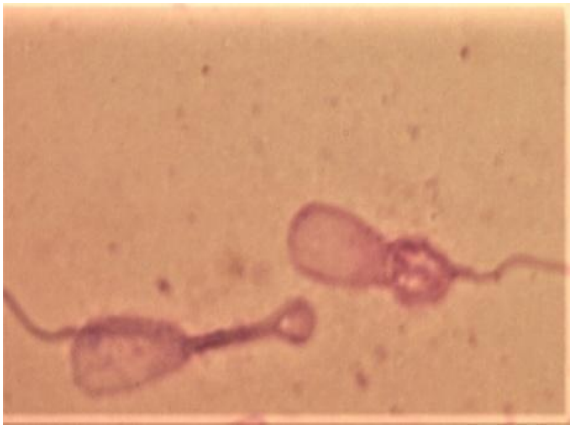

Figure 7. *Bull sperm exposed to Ag-TiO2 (defects of the middle part - edema).*

It should be noted that the functionalization of nanoparticles by the components of the environment can be excluded because sodium citrate and glucose are usually used for thawing human and animal spermatozoa. They contribute to the prevention of a negative impact on mobility and other morpho-

functional indicators [26]. The proposed approach to the express evaluation of nanomaterials was also used in the evaluation of the potential danger of nano-sized fractions of the solid component of welding aerosols. The data on the content of membrane phospholipids and morphological abnormalities of bull sperm corresponded to the results of our experimental studies in vivo and in vitro [27]. It should be noted that the presented data correspond to the results obtained during the exposure of titanium-containing nanomaterials to other test objects. In particular, the skin exposed to ultraviolet light can be a significant target for photosensitized damage to commercial nanoproducts TiO_2 in concentrations of 0.2-3.0 mg/ml [28]. In turn, in the study of the effect of nanofractions of food TiO_2-containing nanofractions on individual pathogenic and opportunistic intestinal bacteria, the concentration of 0.3 mg/ml was recognized as the minimum effective antibacterial concentration [29]. Other experimental studies in vitro have shown that under the influence of TiO2 at a concentration of 30 μg/ml on peripheral blood mononuclear cells, we observe a statistically significant increase in IL-1 production. In contrast, Ag-TiO_2 at concentrations of 30 μg/ml can increase the functional activity of peripheral blood mononuclear cells by producing pro-inflammatory cytokines IL-1, IL-6, TNF-α and IL-4 production in volunteer donors, indicating a potential effect on the formation of chronic inflammation and allergic reactions in the relevant occupational groups [30].

Other studies have also shown that Ag-TiO_2 nanoparticles are more toxic than TiO_2 nanoparticles. The toxicity intensity of TiO_2 nanoparticles increased with increasing amounts of Ag doping. In addition, it was found that Ag-doped TiO_2 nanoparticles provoke the formation of ROS and the depletion of antioxidants [31]. When loading Ag nanoparticles in TiO_2, it increases the toxic effect of Ag-TiO_2 due to synergistic effects.

Thus, it is quite likely that doping nano titanium dioxide with silver contributes to increased toxicity, which requires further in-depth experimental studies using different concentrations in vivo and in vitro methods.

4. CONCLUSION

A simple, informative, inexpensive, and acceptable approach from the point of view of bioethics to the assessment of the potential hazard of nanomaterials with a bull sperm as a test object is proposed. It can be used as a primary screening in the study of nanomaterials in vitro. It was found that the complex of titanium dioxide doped with a silver (mass fraction of Ag 4%) and nanopowder of titanium dioxide in concentration 3 mg/ml caused the release of phospholipids as a result of the destruction of membranes and morphological abnormalities of the bull sperm (abnormalities of the head, middle part, and tail, as well as the absence of acrosome, etc). Doping with silver can increase the toxicity of nanotitanium dioxide, which requires further in-depth experimental studies using different concentrations by in vivo and in vitro methods.

ACKNOWLEDGEMENTS

The authors would like to acknowledge the Frantsevich Institute of Materials Science Problems of National Academy of Sciences of Ukraine for the provided nanomaterials and electron microscopy studies of it.

CONFLICT OF INTERESTS

On behalf of all authors, the corresponding author states that there is no conflict of interest.

REFERENCES

1. Shakeel, M., Jabeen, F., Shabbir, S., Asghar, M. S., Khan, M. S., Chaudhry A. S., "Toxicity of Nano-Titanium Dioxide (TiO_2-NP) Through Various Routes of Exposure: a Review", *Biol. Trace. Elem. Res*., 172(1) (2016) 1-36.
2. Arjomandi Rad, F., Talat Mehrabad, J., "Structural and Optical Properties Study of Ag and Mg co-Doped TiO_2 by Comparison between DFT Calculation with Experimental Results", *International Journal of Nanoscience and Nanotechnology*, 19(1) (2023) 43-50.
3. Ziental, D., Czarczynska-Goslinska, B., Mlynarczyk, D. T., Glowacka-Sobotta, A., Stanisz, B., Goslinski, T., Sobotta, L., "Titanium Dioxide Nanoparticles: Prospects and Applications in Medicine", *Nanomaterials (Basel)*, 10(2) (2020) 387-391.
4. Ama, O. M., Khoele, K., Ray, S. S., "TiO_2/Ag_2O-Exfoliated Graphite as Visible Responsive -Light Nanostructure for Improved Photoelectrochemical Degradation of BPA", *International Journal of Nanoscience and Nanotechnology*, 17(1) (2021) 1-10.
5. Warheit, D. B., "Hazard and risk assessment strategies for nanoparticle exposures: how far have we come in the past 10 years?", *F1000Res.*, 7 (2018) 376.
6. Savage, D. T., Hilt, J. Z., Dziubla, T. D., "In Vitro Methods for Assessing Nanoparticle Toxicity", *Methods Mol. Biol*., 1894 (2019) 1-29.
7. Joris, F., Manshian, B. B., Peynshaert, K., De Smedt, S. C., Braeckmans, K., Soenen, S. J., "Assessing nanoparticle toxicity in cell-based assays: influence of cell culture parameters and optimized models for bridging the in vitro-in vivo gap", *Chem. Soc. Rev.*, 42(21) (210) 8339-8359.
8. Endes, C., Schmid, O., Kinnear, C., Mueller, S., Camarero-Espinosa, S., Vanhecke, D., Foster, E. J., Petri-Fink, A., Rothen-Rutishauser, B., Weder, C., Clift, M. J., "An in vitro testing strategy towards mimicking the inhalation of high aspect ratio nanoparticles", *Part Fibre Toxicol*., 11(40) (2014) 1-12.
9. Guadagnini, R., Halamoda, B., Kenzaoui B., Walker, L., Pojana, G., Magdolenova, Z., Bilanicova, D., Boland, S., "Toxicity screenings of nanomaterials: challenges due to interference with assay processes and components of classic in vitro tests", *Nanotoxicology*, 1 (2015) 13-24.
10. Horie, M., Tabei, Y., "Role of oxidative stress in nanoparticles toxicity", *Free Radic. Res.*, 18 (2020) 1-12.
11. Wu, W. T., Jung, W. T., Lee, H. L., "Lipid peroxidation metabolites associated with biomarkers of inflammation and oxidation stress in workers handling carbon nanotubes and metal oxide nanoparticles", *Nanotoxicology*, 15(5) (2021) 577-587.
12. Ayala, A., Muñoz, M. F., Argüelles, S., "Lipid peroxidation: production, metabolism, and signaling mechanisms of malondialdehyde and 4-hydroxy-2-nonenal", *Oxid Med. Cell Longev*., 5 (2014) 1-31.
13. Gaschler, M. M., Stockwell, B. R., "Lipid peroxidation in cell death", *Biochem. Biophys. Res. Commun*., 482(3) (2017) 419-425.
14. Palacin, I., Santolaria, P., Alquezar-Baeta, C., Soler, C., Silvestre, M., Yániz, Y., "Relationship of sperm plasma membrane and acrosomal integrities with sperm morphometry in *Bos taurus*", *Asian journal of andrology*, 22(6) (2020) 578-582.
15. Alahmar, A.T., "Role of Oxidative Stress in Male Infertility: An Updated Review", *J. Hum. Reprod. Sci.*, 12(1) (2019) 4-18.
16. Mustafa, M., Dar, S. A., Azmi, S., Haque, S., "The Role of Environmental Toxicant-Induced Oxidative Stress in Male Infertility", *Adv. Exp. Med. Biol.*, 1391 (2022) 17-32.
17. Agarwal, A., Virk, G., Ong, C., du Plessis, S. S., "Effect of oxidative stress on male reproduction", *World J. Mens Health*, 32(1) (2014) 1-17.
18. Hosseinzadeh, C. A., Karimi, F., Jorsaraei, S. G., "Correlation of sperm parameters with semen lipid peroxidation and total antioxidants levels in astheno- and oligoceno-teratospermia men", *Iran Red. Crescent Med. J.*, 15(9) (2013) 780-785.
19. Pawar, K., Kaul, G., "Toxicity of titanium oxide nanoparticles causes functionality and DNA damage in buffalo (*Bubalus bubalis*) sperm *in vitro*", *Toxicology and Industrial Health*, 30(6) (2014) 520-533.
20. Taurozzi, J. S., Hackley, V. A., "Preparation of Nanoparticle Dispersions from Powdered Material Using Ultrasonic Disruption", *National Institute of Standards and Technology Special Publication*, 1200(2) (2012) 1-14.
21. Viana, M. M., Soares, V. F., Mohallem, N. D. S.,"Synthesis and characterization of TiO2 nanoparticles", *Ceramics International*, 36(7) (2010) 2047-2053.
22. Folch, J., Lees, M., "A simple method for the isolation and purification of total lipids from animal tissues", *J. Biol. Chem.*, 226(1) (1957) 497-509.
23. Sehr, G., "Improvement in the first-insemination rate after insemination with bull sperm frozen in pellet form and the use of a sodium citrate-glucose solution for thawing. Short report from the practice", *Dtsch Tierarztl Wochenschr.*, 82(11) (1975) 441-442.
24. Banaszewska, D., Andraszek, K., Czubaszek, M., Biesiada-Drzazga, B., "The effect of selected staining techniques on bull sperm morphometry", *Anim. Reprod. Sci*., 8 (2015) 17-24.

25. Jung, J. W, Koch, G. G. “Multivariate non-parametric methods for Mann-Whitney statistics to analyze cross-over studies with two treatment sequences”, *Stat. Med.,* 18(8) (1999) 989-1017.
26. Nema, S. P., Dhami, A. J., Kavani, F. S., “Evaluation of egg.yolk-glycerol combination in Tris, citrate and phosphate extenders for freezing of ram semen”, *Indian Journal of Animal Reproduction,* 32(1) (2011) 20-23.
27. Demetska, O. V., Movchan, V. O., Beliuha, O. G., Didenko, M. M. , Balia, A. G., Andrusyshyna, I. M., “Approaches to express potential hazard assessment of nanosized fractions of welding fumes”, *Ukrainian Journal of Occupational Health*, 18(2) (2022) 130–137.
28. Lu, N., Zhu, Z., Zhao, X., Tao, R., Yang, X., Gao, Z., “Nano titanium dioxide photocatalytic protein tyrosine nitration: a potential hazard of TiO_2 on skin”, *Biochem. Biophys. Res.Commun.,* 370(4) (2008) 675-80.
29. Baranowska-Wójcik, E., Szwajgier, D., Gustaw, K., “Effect of TiO_2 on Selected Pathogenic and Opportunistic Intestinal Bacteria”, *Biol. Trace Elem. Res*., 200(5) (2022) 2468-2474.
30. Riabovol, V., Kurchenko, A., Yavorovskyi, A., Savchenko, V., Taran, N. “A study of the influence of photoactive titanium composite nanoparticles of the functional activity of cytokine-producing mononuclear blood cells in vitro”, *Immunology and allergology: science and practice,* 3 (2021) 23-20.
31. Ahamed, M., Khan, M. A. M., Akhtar, M. J., Alhadlaq, H. A., Alshamsan, A., “Ag-doping regulates the cytotoxicity of TiO_2 nanoparticles via oxidative stress in human cancer cells”, *Sci. Rep.,* 7(1) (2017) 17662.