MANSOURA JOURNAL OF BIOLOGY
Official Journal of Faculty of Science, Mansoura University, Egypt
E-mail: scimag@mans.edu.eg ISSN: 2974-492X

Histological and immunohistochemical studies of the effect of nano-zinc on harderian gland of mother rats and their offspring treated with lipo polysaccharides.

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Received:5/9/2022
Accepted: 17/10/2022

Abstract: The present study focused on the therapeutic effect of zinc oxide nanoparticles (ZnO NPs) on the Harderian gland of mother rats and their offspring at 21 day-old affected by lipopolysaccharides. Eight male and forty virgin female rats (*Rattus norvegicus*) were used in this study. After mating, pregnant rats divided into four groups; control, ZnO NPs-treated, lipopolysaccharides (LPS)-treated, and LPS & ZnO NPs-treated groups. ZnO NPs with or without LPS were injected four times on the 14^{th}, 17^{th}, and 20^{th} days of gestation, plus a dose after birth. LPS-treated group was injected two times on the 7^{th} and 9^{th} days of gestation. The mothers and their 21-day-old offspring were sacrificed and the Harderian glands were removed for histological and Proliferating Cell Nuclear Antigen (PCNA) immunohistochemistry examination. The therapeutic role of ZnO NPs in LPS and ZnO NPs-treated rats appeared in the histological improvement and reducing immunostaining of the mothers and offspring Harderian glands affected by LPS.

keywords: Lipopolysacccharides, zinc oxide nanoparticles, Harderian gland, mother, offspring, PCNA immunohistochemistry.

1.Introduction

The Harderian gland occupies the majority of the orbit and is located posterior and medial to the eyeball as shown in fig. (1). This gland, which is only seen in animals with *a membrana nictitans*, were discovered in 1864 by famous Swiss scientist called Johann Jacob Harder [1]. It is present in many terrestrial vertebrates as rat, mouse and hamster. It is responsible for conjunctiva lubrication, pheromones secretion, protection of the retina from UV light, making and secreting porphyrins, acting as a photoreceptor for the pineal gland, and producing growth factors [2]. It is lobulated and has three clefts where the optic nerve and extraocular muscles are coiled. A single excretory duct runs from the medial side of the eye to the nictitating membrane, where it opens onto the anterior surface [3].

Infectious disease is associated with elevated body temperature and increased bacterial toxin in the blood stream. This may cause inflammation of the eye regions and consequently damaging the Harderian gland. Lipopolysaccharide (LPS) is a toxic component of cell walls in Gram-negative bacteria as shown in fig. (2). LPS increases oxidative stress by increasing reactive oxygen species in many organs. Maternal exposure to LPS during utero life led to perinatal mortalities and abortion [4]. Endotoxic LPS is a potent systemic inflammatory mediator and septic shock inducer. The potential for endotoxicity of LPS is variable. Macromolecules of intact bacterial lipopolysaccharides are made up of three structural components; a hydrophobic lipid section known as lipid A that is responsible for the molecule's toxic properties if it does not include C14-2OH fatty acids, LPS does not generate a significant inflammatory response in the host., a hydrophilic core polysaccharide chain, and a repeating hydrophilic O-antigenic oligosaccharide side chain [5]. Only a few amounts of LPS from infecting pathogens lead to potent innate immune responses occurring

after recognition of it by many LPS receptors and accessory proteins like CD14/TLR4/MD2 receptor complex which stimulate the production of proinflammatory cytokines including tumor necrosis factor-α and interleukin-1 [6]. The immune system is then stimulated against infection. If the LPS response is not controlled it may cause fatal septic shock syndrome [7]. Gram-negative bacteria's membranes contain LPS, which protects them from bile salts and lipophilic antibiotics [8].

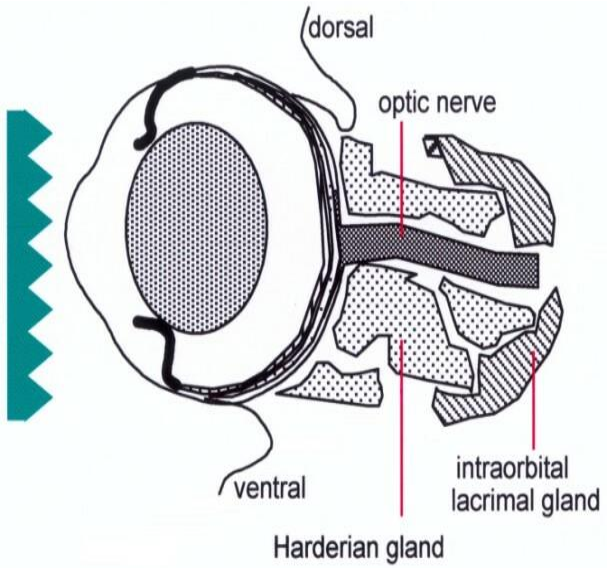

Fig. 1. Diagram showing Harderian gland.

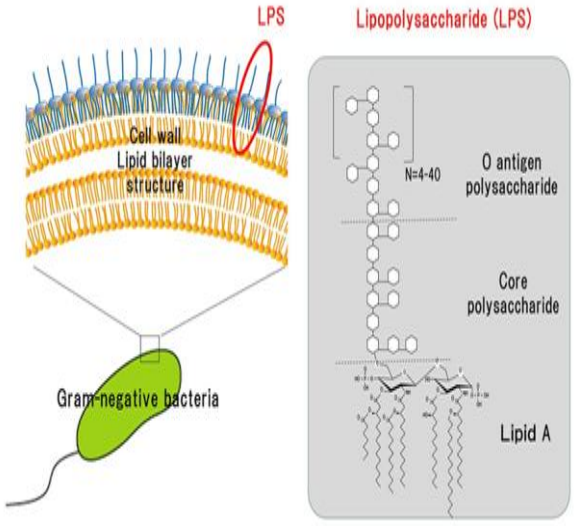

Fig. 2. Illustrating the structure of LPS

It has been shown that PCNA immunohistochemical staining is more reliable and efficient. It is overexpressed in case of inflammation and tissue damage. The less differentiated cells had a better propensity to multiply, hence improving tumor malignancy. Cell proliferation generally contributed significantly to the degree of tumor malignancy [9] and a proper balance between cell proliferation, differentiation, and cell death is essential to maintaining homeostasis.

Nanotechnology has an essential and virtual role in a wide range of applications, from innovative fabric compounds to food processing and agricultural production to sophisticated medical treatments [10]. Commercial applications of nanoscale minerals in fields such as medical, food, biotechnology, and pharmaceuticals have been revolutionized by nanotechnology. Due to their antibacterial characteristics, bioavailability, bio-distribution, elimination, immune system response, and interactions with host cells or food and feed matrix, ZnO NPs are the third most manufactured nano-metals in the world [11]. As well as ZnO NPs are a novel form of low-toxicity and low-cost nanomaterial so they used in a variety of biomedical applications, including anticancer, antioxidant, anti-inflammatory, and antidiabetic activities, as well as bioimaging and drug delivery [12]. Zinc oxide (ZnO) is a source of zinc, which is an important micronutrient which plays a role in human and animal growth and development [13]. The Food and Drug Administration (FDA) has approved ZnO NPs as a new and effective anticancer therapy [14]. Due to their great biocompatibility and unique physical and chemical characteristics, modified zinc nanoparticles such as oxides, silanes, or sulphide have been intensively explored for the treatment and diagnosis of cancer or tumors. ZnO NPs come in a variety of forms, including ZnO, the most common type, zinc selenide, zinc ferrite, zinc phosphide, zinc telluride, and zinc sulphide. These particles are used in a variety of fields, including biomedicine, agriculture, and many other industries [11].

The current study proved that ZnO NPs can reduce toxic effect of LPS on the Harderian gland that was associated with increasing immunohistochemical staining of PCNA.

2.Materials and methods:

2.1.Ethical approval :

The ethical Committee for Animal Experimentation at Faculty of Science,

Mansoura University, Egypt gave its approval to the study.

2.1.Chemicals:

2.1.1.Lipopolysaccharides:

LPS were purchased from Sigma-Aldrich (chemical, Life Science, and Biotechnology Company). 1.8 mg were dissolved in 30 ml distilled water. Two doses (150 µg/kg) were intraperitoneally (IP) injected on the 7th and 9th days of gestation.

2.1.1. ZnO NPs:

ZnO NPs are in the form of a white fine powder that was also purchased from Sigma-Aldrich. 400 mg were dissolved in 50 ml distilled water. Four doses (20 mg/kg) were IP injected on the 14th, 17th, and 20th days of gestation, plus a dose after birth.

2.3. Experimental work:

Eight male and forty virgin female rats (*Rattus norvegicus*), weighing 120-150g, were obtained from Helwan Breeding Farm, the Ministry of Health and Population, Egypt. After mating, pregnant rats were divided into four categories (n=10); control, ZnO NPs-, LPS-, and LPS & ZnO NPs-treated groups. On the 21st post-partum day, the mother rats and their offspring in all studied groups were slaughtered by cervical dislocation after anesthesia with phenobarbitone. The Harderian glands were separated and fixed in 10% phosphate-buffered formalin (pH 7.4).

2.4.Histology and Immunohistochemistry:

The formalin-fixed maternal and offspring Harderian glands were paraffin embedded, serially sectioned at 5µm thickness, and counter-stained with hematoxylin and eosin for histological examination. For immunohistochemical investigation, some paraffin sections were mounted on polylysine-coated glass slides and stored at room temperature. They were treated with antibodies against PCNA. All sections were examined by the light microscope.

2.5. Statistical analysis:

The data is presented as means ± standard error (SE). One way ANOVA test followed by *t*-test test were used for all studied groups. The results were considered statistically significant at $p \leq 0.05$. All statistical calculations were carried out by SPSS (version 13).

3. Results and Discussion

Characterization of ZnO NPs was determined by using TEM technique that showed its spherical shape and its size that is ranged from 7 nm to 15 nm (Fig. 3).

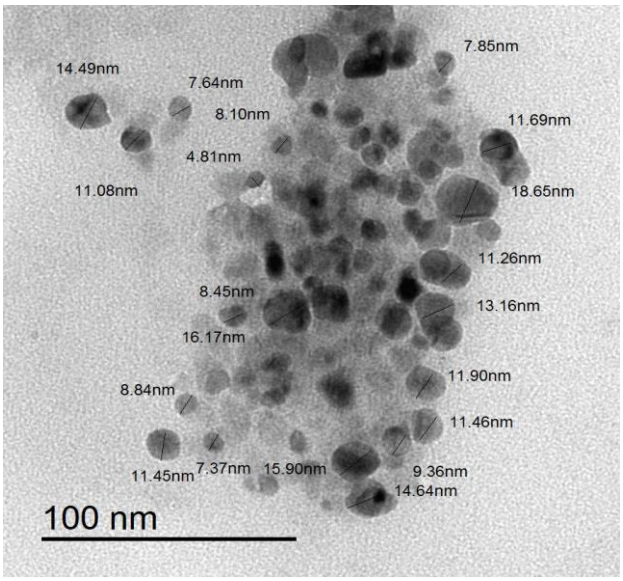

Fig. 3. Transmission electron micrographs of ZnO NPs showing its shape and size.

The Harderian glands of mother rats and their offspring treated with LPS showed leukocyte infiltration, atrophied alveoli, irregular secretory cells and emigrated cells (Fig.4 C&C1). On the other hand, Harderian gland of the control and ZnO NPs-treated groups exhibited normal histological structure. It is a multilobar tubuloalveolar gland mainly consists of secretory alveoli lined with cuboidal-columnar epithelium, which is supported by a basement membrane (Fig. 4 A&A1-B&B1).

Additionally, LPS-treated mother rats and their offspring showed overexpression of PCNA in their Harderian glands, which resulted in cell proliferation (Fig. 5 C&C1) when compared with the control and ZnO NPs-treated groups that exhibited low immunohistochemical reaction (Fig.5 A&A1-B&B1, respectively).

On the other hand, the Harderian gland, showed improved histological image in mother rats and their offspring treated with LPS and ZnO NPs (Fig.4 D&D1) with reduced PCNA immunohistochemically but was still higher than the control (Fig.5 D&D1).

In comparison with the other experimental groups, image analysis showed that LPS-treated mother rats and their offspring had higher levels of PCNA in their Harderian glands (Fig.6).

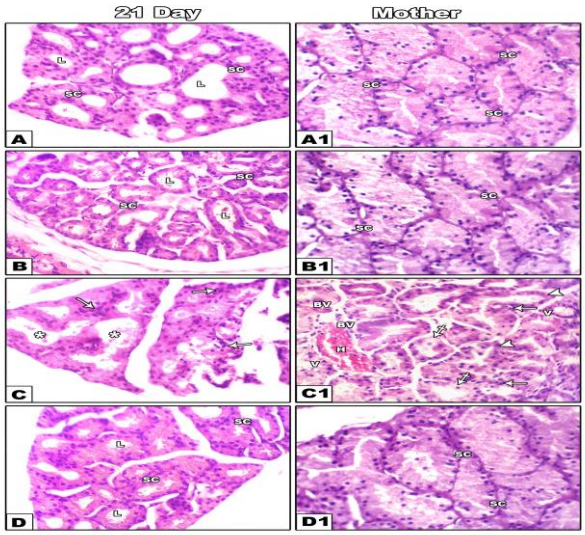

Fig. 4. Photomicrographs of cross histological sections of Hardeian gland of 21-day-old offspring (A-D) and mother (A1-D1) rats; Control (A&A1) and ZnO NPs treated group (B&B1) showing normal histological structure; 21-day old offspring rats and their mothers treated with LPS showing irregular secretory cells, leukocyte infiltration, atrophied alveoli, and emigrated cells (C&C1); 21-day old offspring and their mother rats treated with LPS and ZnO NPs showing histological improvement (D&D1). H&E. Abbreviations: Asterisk indicating atrophied alveoli; BV-blood vessel; H- hemorrhage; L-lumen; SC-secretory cells; V-vacuole; Arrow indicating infiltration; Arrow head indicating irregular cells; Arrow with plus on tail indicating emigrated cells.

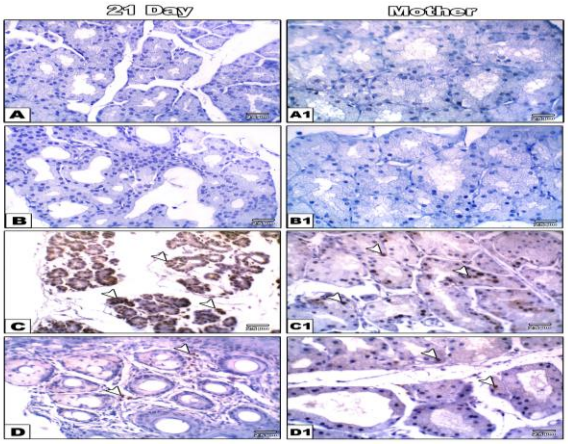

Fig. 5. Photomicrographs of cross histological sections of Harderian gland immunohistochemical stained with PCNA of 21-day-old offspring (A-D) and mother (A1-D1) rat. Control (A-A1) and zinc oxide nanoparticles treated group (B-B1) showing missing immunohistochemical reaction of PCNA. 21-day old offspring and mother rats treated with LPS showing increase dark brown immunohistochemical reaction of PCNA (C&C1). 21-day old offspring and mother rats treated with LPS plus ZnO NPs showing decrease immunohistochemical reaction of PCNA (D&D1). Arrowhead indicates to the PCNA reaction.

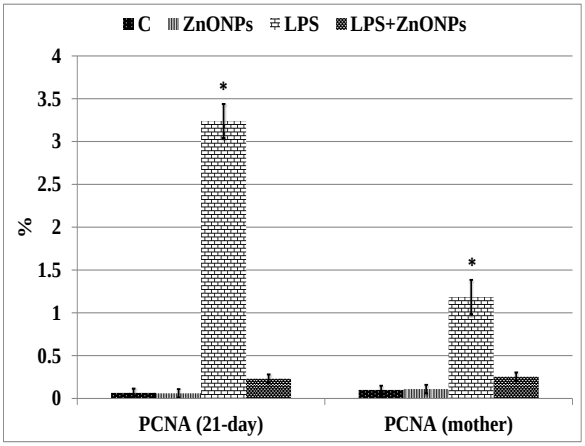

Fig. (6). The percentages of immunohisto-chemical reaction of PCNA in the Harderian gland of 21-day-old offspring and their mother rats. Star means significant at $P \leq 0.05$.

The present study showing presence of infiltration of leukocytes, irregular secretory cells, atrophied alveoli, and emigrated cells that occurs as a result to breakdown the wall of the alveoli and epithelial cells emigrated into the Harderian gland lumen of the offspring and their mother rats that treated with LPS during gestation. There are previous studies agree with the present results that proved toxicological effects of LPS on the rat's Harderian gland. This expressed as several morphological alterations as well as lipid peroxidation that appeared in increasing the levels of lipid peroxidation product (MDA). Hyperchromasia, vesicular degeneration, necrosis, and infiltration of macrophages, monocytes, and neutrophils, are all characteristics of this pathological condition [15]. LPS can pass through the placental barrier and interact with functional TLR4 on fetal/placental tissues, which can raise the levels of cytokines in the amniotic fluid and have a detrimental effect on the embryo development and even result in fetal brain injury [16]. This study showed

overexpression of PCNA in the tissue of the Harderian gland of mother rats and their offspring treated with LPS that is associated with high levels of oxidative stress and proliferation. This is because PCNA possesses anti-apoptotic functions and stimulates tumor cell growth as well as it is a nuclear protein necessary for cell replication, so it serves as an indicator for cell proliferation [17]. High oxidative stress levels are the main factor that contributes Harderian gland physiology and morphology alternation by changing typical structure producing a disorganised tubulo-alveolar tissue [18]. It was observed the histological improvement that is appeared in the spherical regular arrangement of the epithelial cells with lumen and reduction of the immunohistochemical reaction of PCNA in the Harderian gland of mother rats and their offspring treated with LPS and ZnO NPs are small so they can penetrate blood arteries and reach the interstitial spaces, where they can affect different cells in various tissues [19] and it can pass through the placental barrier and effect on fetus [20]. Our findings indicated that ZnO NPs have antioxidant properties and therapeutic role against LPS that is consistent with another study reported that ZnO NPs synthesized by *Lactobacillus reuteri* E81 have significant anti-inflammatory and antioxidant effects [21]. There are numerous antioxidant properties of zinc. These include, for instance, zinc (Zn), which acts as a cofactor of the Cu/Zn superoxide dismutase, which hydrolyzes the dismutation of the superoxide radical (O_2) into the less hazardous O_2 and H_2O_2, which are then detoxified by catalase and glutathione peroxidase. Additionally, Zn inhibits NADPH oxidases, which diminishes the production of reactive oxygen species (ROS) [22]. Zinc's potential to decline oxidative stress and glutathione loss has been demonstrated in various other researches. ZnO NPs elevate antioxidant enzyme levels while lowering MDA levels, protecting cell membrane integrity from oxidative stress [23]. ZnO NPs suppress angiogenesis via triggering death in endothelial cells and reducing the expression of VEGF and VEGFR genes [24]. Angiogenesis is involved in the growth, metastasis, and rheumatoid arthritis of tumors, as well as physiological processes such as the development and proliferation of cells [25]. This study proved that ZnO NPs also have anti-inflammatory properties so they could be used as anti-cancer and anti-inflammatory therapeutic candidate [26]. Nagajyothi et al. used ZnO NPs generated by *Polygala tenuifolia* root extract due to their moderate antioxidant activity and excellent anti-inflammatory activity by inhibiting both mRNA and protein expressions of iNOS, COX-2, IL-1β, IL-6, and TNF-α [27]. Other studies used ZnO NPs as anti-cancer by promoting disequilibrium of zinc-dependent protein activity and the formation of reactive oxygen species, ZnO NPs exert a specific cytotoxic effect on cancer cells [28]. This anti-cancer effects of ZnO NPs are mediated by the activation of DNA repair and the inhibition of both apoptosis and cancer cell growth [29].

4. Conclusion

ZnO NPs have therapeutic effects on the Harderian gland of mother rats and their offspring treated by LPS due to their antioxidant activities. The improvement appeared in histological image and reduction of PCNA immunostaining.

5. References

1. G.Y. Kennedy, A.H. Jackson, G.W. Kenner, C.J. Suckling," (1970).Isolation, structure and synthesis of a tricarboxylic porphyrin from the harderian glands of the rat," FEBS Lett, vol. **6**, no. 1, pp. 9-12,
2. G. Chieffi, G.C.Baccari, L.D. Matteo, S. d'Istria M,Minucci, B.Varriale, ,(1996). "Cell Biology of the Harderian gland," Int. Rev. Cytol., vol.**168**,pp. 1-80
3. Y. Djeridane, (1994)."The harderian gland and its excretory duct in the Wistar rat. A histological and ultrastructural study*," J. Anat.*, vol.**184**, pp. 553-566,
4. T.M. Leazer, , B. Barbee, M. Ebron-McCoy, G. A. Henry-Sam, J. M. Rogers, (2002)"Role of the maternal acute phase response and tumor necrosis factor alphain the developmental toxicity of lipopolysaccharide in the CD-1 mouse,"Reprod.Toxicol.,vol.**16**, pp. 173–179,
5. R.F. Maldonado RF, I. Sá-Correia, M.A. Valvano, (2016). "Lipopolysaccharide modification in Gram-negative bacteria

during chronic infection," FEMS Microbiol. Rev., vol. **40**, no.4, pp. 480–493,
6. M. Muroi, K. Tanamoto, (2002) The Polysaccharide Portion Plays an Indispensable Role in Salmonella Lipopolysaccharide-Induced Activation of NF- kappaB through Human Toll-Like Receptor 4," Infect. Immun., vol. **70**, no. 11, pp. 6043-6047.
7. B.S. Park, J.O. Lee," (2013) Recognition of lipopolysaccharide pattern by TLR4 complexes," Exp. Mol. Med. Vol. **45**, no. 12, pp. e66,
8. H. Mayer, R. Tharanathan, J. Weckesser," 6 (1985).Analysis of Lipopolysaccharides of Gram-Negative Bacteria. Methods Microbiol.," vol. **18**, pp.157-207,
9. D. Wang, J.Q. Shi, F.X. Liu," (1997) Immunohistochemical detection of proliferating cell nuclear antigen in hepatocellular carcinoma," World *J. Gastroenterol.*, vol. **3**, no.2, pp.101-103,.
10. D.T. Tandra, T. Lakshmi," (2020).Application of zinc oxide nanoparticles in dentistry," IJSD, vol. **5**, no. 3,pp. 42-49,
11. S. Rani, P. Kumar, P. Dahiya, A.S. Dang, P.Suneja," (2022).Biogenic Synthesis of Zinc Nanoparticles, Their Applications, and Toxicity Prospects," Front. Microbiol., Vol. **13**, pp. 824427,
12. J.A. Ruszkiewicz, A . Pinkas, B. Ferrer, T.V. Peres, A . Tsatsakis, A. Aschner , (2017). Neurotoxic effect of active ingredients in sunscreen products, a contemporary review," Toxicol. Rep., vol. **4**, pp. 245-259,
13. L. Shi, J. Zhou, S. Gunasekaran, (2008.) " Low temperature fabrication of ZnO—whey protein isolate nanocomposite," Mater. Lett., vol. **62**, no. 28, pp. 4383–4385,
14. C. Shen, S.A. James, M.D. de Jonge, T.W. Turney, P.F. Wright, B.N. Feltis, (2013) "Relating cytotoxicity, zinc ions, and reactive oxygen in ZnO nanoparticle-exposed human immune cells," Toxicol. Sci., vol. **136**, no. 1, pp. 120-130,
15. G.G. Ortiz, A. Coto-Montes, O.K. Bitzer-Quintero, M.A. Falcón-Franco, L. Ruiz-Rizo, A. Bravo-Cuellar, R.J. Reiter, A. Feria-Velasco, (1999)." Effects of melatonin on the Harderian gland of lipopolysaccharide-treated rats: morphological observations," Biomed. Pharmacother., vol. **53**, no. 9, pp. 432-437
16. A.G. Brown, M.E. Maubert, L. Anton, L.M. Heiser, M.A. Elovitz," (2019.)The tracking of lipopolysaccharide through the feto-maternal compartment and the involvement of maternal TLR4 in inflammation-induced fetal brain injury," Am. *J. Reprod. Immunol.*, vol.**82**, no. 6, pp. e13189,
17. K.E. Tachibana, M.A. Gonzalez, N. Coleman," (2005). Cell-cycle-dependent regulation of DNA replication and its relevance to cancer pathology," *J. Pathol*., vol**. 205**, no. 2, pp. 123-129,
18. A. Coto-Montes, M. García-Macía, B. Caballero, V. Sierra, M.J. Rodríguez-Colunga, R.J. Reiter, I. Vega-Naredo," (2013). Analysis of constant tissue remodeling in Syrian hamster Harderian gland: intra-tubular and inter-tubular syncytial masses," *J. Anat*., vol. **222**,no. 5, pp. 558-569,
19. H.S. Choi, Y. Ashitate, J.H. Lee, S.H. Kim, A. Matsui, N. Insin, A.Tsuda," (2010). Rapid translocation of nanoparticles from the lung airspaces to the body," Nat. Biotechnol., vol. **28**, no. 12, pp. 1300-1303,
20. B. Chen, W. Hong, P. Yang, Y. Tang, Y. Zhao, Z.P. Aguilar, H. Xu," (2020.) Nano Zinc Oxide Induced Fetal Mice Growth Restriction, Based on Oxide Stress and Endoplasmic Reticulum Stress," Nanomaterials (Basel), vol.**10**, no. 2, pp. 259,
21. N,Yücel, H. İspirli, E. Mercan, Ü. Erdoğan, E. Dertli," (2021). Synthesis of alternan-stabilized zinc nanoparticles: morphological, thermal, antioxidant and antimicrobial characterization," Prep. Biochem. Biotechnol., vol. **51**, no.4, pp. 331-339,
22. AS. Prasad, (2014.) " Zinc: an antioxidant and anti-inflammatory agent: role of zinc in degenerative disorders of aging," *J. Trace Elem. Med. Biol.*, vol. **28**, no. 4, pp. 364-367,

23. A.I. Dawei, W. Zhisheng, Z. Angu," (2009) Protective effects of Nano-ZnO on the primary culture mice intestinal epithelial cells in in vitro against oxidative injury," *Int. J. Nanotechnol*., vol. **3**, pp. 1-6,.
24. S. Tada-Oikawa, G. Ichihara, Y. Suzuki, K. Izuoka, W. Wu, Y. Yamada, S. Ichihara, "Zn (II) (2015).released from zinc oxide nano/micro particles suppresses vasculogenesis in human endothelial colony-forming cells," Toxicol. Rep. vol. **2,** pp. 692-701,
25. C. Ruhrberg, (2001). "Endogenous inhibitors of angiogenesis," *J. Cell Sci*., vol. **114**, no. 18, pp. 3215–3216,
26. G.R.K.S. Mohammad, M.H. Tabrizi, T. Ardalan, S. Yadamani, E. Safavi, (2019.) "Green synthesis of zinc oxide nanoparticles and evaluation of anti-angiogenesis, anti-inflammatory and cytotoxicity properties. *J. Biosci*. Vol. **44**, no. 2, pp. 30,
27. P.C. Nagajyothi, S.J. Cha, I.J. Yang, T.V.M. Sreekanth, K.J. Kim, H.M Shin," (2015).Antioxidant and anti-inflammatory activities of zinc oxide nanoparticles synthesized using Polygala tenuifolia root extract", *J. Photochem. Photobiol.B.* vol. **146**, pp. 10-17,
28. M.P. Vinardell, M. Mitjans, (2015). "Antitumor activities of metal oxide nanoparticles," Nanomaterials (Basel), vol. **5**, no. 2, pp. 1004-1021,
29. J.W. Rasmussen, E. Martinez, P. Louka, D.G. Wingett, (2010.) "Zinc oxide nanoparticles for selective destruction of tumor cells and potential for drug delivery applications," Expert. Opin. Drug Deliv., vol. **7,** no. 9, pp. 1063-1077,