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EFFECT OF ZnO NPs ON THYROID FUNCTION AND EVALUATION OF THE LEVELS OF TSH RECEPTOR GENE IN THYROID TISSUE OF FEMALE RAT

Faisal G. Lazim*, Noori M. Luaibi and Haidar J. Muhammed

Department of Biology, College of Science, Mustansiriyah University, Iraq.
*e-mail: f.bio1987@gmail.com

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ABSTRACT : Nanoparticles are an increasingly used material at present, due to their unique properties that enable them to increase their effectiveness. one of these materials used in many fields, especially medical and cosmetic products is ZnO NPs. The frequent use of these particles makes them in direct contact with human bodies. The thyroid gland is one of the most important organs responsible for all activities that take place inside the body. The thyroid gland expresses on its surface receptor called thyroid-stimulating hormone receptor (TSHR). This receptor is an important aspect of the thyroid gland that interaction with the thyroid stimulates hormone on the follicular cells of the thyroid gland, through which it regulating the function of the gland. Consequently, TSHR is a part of the system that responsible for the development and metabolism processes in the human body. Aim of study: the purpose of this study was to find out the effect of the ZnO NP on the levels of T_3, T_4 and TSH hormones in addition to estimate the expression of TSHR-gene that present in the thyroid gland tissues of female rats. Fifty-four adult female rats at randomly classified into three main groups, one of them used as a control and the others as treatment groups, each group was subdivided into three subgroups, and every one consist of 6 rats. The control groups had been injected with 1ml of distilled water, while the treatment groups injected with 1 ml of ZnO NPs at two different doses (50 and 200) mg/kg, in duration (1, 2 and 4) weeks at a rate of three doses per week through intra-peritoneal route. Statistical analysis: The data were calculated to compare between the means of treatment and control groups. The results of this study indicated a highly significant increase in the levels of T_3 and T_4 hormones in all treated animals at different period of times, in contrast, demonstrated a significant decrease in the serum level of TSH hormone in animals that received high and low doses in duration 4 weeks, the results as well indicate clearly reduction in the expression level of TSHR genes in treated animals at different periods of time in comparison with the control groups.

Key words : ZnO NPs, thyroid gland, TSHR gene, T_3, T_4, TSH.

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INTRODUCTION

Nanoparticles (NPS) are a wide class of materials that present on a nanometre scale, which have at least one dimension with a size less than 100 nm (Laurent *et al*, 2008). They are known to have completely different Physico-chemical properties from those of larger particles due to their relatively larger surface area (Ryu *et al*, 2014). Due to the wide application of nanomaterials in different fields and products, these particles have gained a great deal of public interest (Pasupuleti *et al*, 2012). Zinc oxide nanoparticles (ZnO NPs) consider the important metal oxide nanoparticles commonly used in various fields, like industry products and foods due to their distinctive physical and chemical properties (Jiang *et al*, 2018). In addition to its strong UV absorbing properties, so the ZnO is increasingly used in personal care products, such as cosmetics and sunscreen (Newman *et al*, 2009).

The thyroid gland is a section of the endocrine system which consists of cells that produce, storage, and release hormones into the bloodstream (Hiller-Sturmhöfel and Bartke, 1998). The thyroid gland and its hormones play multiple roles in the development of the body and their organs and in the homeostatic regulation of basic physiological processes such as body growth and energy expenditure in the vertebrates (Nilsson and Fagman, 2017). There are receptors expressed on the cell surface of the thyroid follicular membrane that plays an essential role in the regulation growth and function of the thyroid gland (Borel and Sabliov, 2014). It is consists of 764 amino acids

including 21 aa signal peptide (Nagayama, 2017). Their gene spans at least 60 kb, divided into 10 exons and 9 introns and It is located on human chromosome 14q (Musa *et al*, 2007). TSH receptor is a key regulator of thyroid hormone function (Davies *et al*, 2010). By interacting with TSH on follicular cells of the thyroid gland (Liu *et al*, 2019). TSH receptor binding contributes to the activation of several signaling pathways responsible for the synthesis and secretion of thyroxine (T_4) and 3,3,5-triiodothyronine (T_3) hormones, as well as cell proliferation and survival (Morshed *et al*, 2009).

The human skin, intestinal system, and lungs are constantly in direct contact with the environment, so these particles can enter the human body in various ways including inhalation, swallowing, and penetration of the skin (Hoet *et al*, 2004). And because of the properties that they possess, allows them to penetrate cytoplasmic membranes and interfere with important cellular functions, several reports have shown that NPs are more toxic than other larger materials because of their high reaction potential (Radhi and Al-Bairuty, 2019). There are several mechanisms within a cell to regulate gene expression during cellular metabolism, growth, and differentiation, If these cells do not work properly, they will die or develop abnormally and even develop into tumors in some cases (Chun *et al*, 2018).

MATERIALS AND METHODS

Preparation of ZnO NPs solution

Zinc oxide nanoparticles which are used in this study was obtained from skyspring nanomaterials, the injection solution of ZnO NPs at different concentrations that were used in this study prepared by dissolving the powder of zinc oxide nanoparticles in an amount of distilled water, after that mixed by vortex for 10 minutes, by this process two concentrations of ZnO NPs solutions were prepared as follows:

Concerning the low dose (50 mg/kg) was prepared by dissolving (0.5 mg) of ZnO NPs in 10 ml of distilled water. Whereas, the high dose (200 mg/kg) was prepared by dissolving (2 mg) of ZnO NPs in 10 ml of distilled water.

Animal care

Fifty-four adult female Sprague-Dawley albino rats weighing 220-250 gm at age of 8-10 weeks were bought from the National Center for Drug Control and Research (NCDCR) belong to the Iraqi ministry of health, then these rats transmitted to animals residence laboratory under suitable environmental conditions for10 days for adaptation before starting the treatment. The laboratory temperature was (25°C) and the dark cycle period was 12 h light/12 h.

Experiment design

A total of 54 healthy adult female Wister rats were assigned in randomly to three experimental groups including the treatment and control groups according to the injection periods which they were 1, 2 and 4 weeks. An average of three doses per week. These groups were divided into nine subgroups, each subgroup consists of six rats as shown in the below details:

Group 1, 2 and 3 (Control animals) : All the animals in the groups received an intra-peritoneal injection of distilled water three times per week during various periods, which are 1, 2 and 4 weeks respectively.

Group 4, 5 and 6 (treated animals with a low dose of ZnO NPs) : All the animals in these groups received an intra-peritoneal injection with a low dose (50 mg/Kg of body weight) of ZnO NPs suspension three times per week during various periods, which are 1, 2 and 4 weeks, respectively.

Group 7, 8 and 9 (treated animals with a high dose of ZnO NPs) : All the animals in these groups received an intra-peritoneal injection with a high dose (200 mg/Kg of body weight) of ZnO NPs suspension three times per week during various periods, which are 1, 2 and 4 weeks, respectively.

Collection of blood samples

After the experiment has been ended, the rats are anesthetized by diethyl ether for several minutes. 4ml of blood samples were collected from the heart puncture and put in the clot activator gel tubes, serum was separated by centrifugation of blood at (3000) rpm for (15) min kept at -20ºC to be ready for the analyzer.

Hormonal analysis

Thyroid hormones (T_3, T_4) in addition to the TSH hormone were evaluated from collected serum by Cobas e411 automated analyzer.

Primers design

The cDNA sequences of TSHR genes were obtained from the NCBI GenBank database. RT-qPCR primers were designed using Primer Premier 3 software with melting temperature between 58 to 62^{0}C, primer length between 18 to 23 nucleotides and PCR amplicon length within 75 to 150 base pairs as shown in Table 1.

Primer preparation

These primers were supplied by Macrogen Company in a lyophilized form. Lyophilized primers were dissolved in nuclease-free water to give a final concentration of 100 pmol/ìl as a stock solution. A working solution of

Table 1 : Primers with their sequences and annealing temperature.

Primer name	Seq.	Annealing Temp.(°C)
TSHR-R	5'-GGACATCTGAGAACCA GGAATC-3'	60
TSHR-F	5'-CAGCACCCAGACTCTCTA TCTA-3'	
YWHAZ-F	5'-GAT GAA GCC ATT GCT GAA CTT G-3'	
YWHAZ-R	5'-GTC TCC TTG GGT ATC CGA TGT C-3'	

these primers was prepared by adding 10ìl of primer stock solution (stored at freezer -20^{0}C) to 90ìl of nuclease-free water to obtain a working primer solution of 10pmol/ìl.

RNA isolation and purification

After the animals were sacrificed, twenty-seven tissue samples of the thyroid gland were isolated from the animals an average three different samples from each group and these samples were kept in Eppendorf tubes containing 1 ml of triazole preservation solution and stored them at (-4ºC) for 24-48 hours, the RNA was isolated from sample according to the protocol of TRIzol™ Reagent.

Analysis Gene expression

The data analysis results of gene expression were calculated by the relative real-time RT-PCR analysis applying the Pfaffl analysis method:

ΔCT =CT gene - CT House Keeping gene

ΔΔCT = ΔCT Treated - ΔCT control

Folding = 2^-ΔΔCT

Statistical analysis

The Statistical Analysis System application (SAS, 2012) was used to find out the effects of different factors on some parameters that used in this study, as well the least significant difference-LSD test (ANOVA) was used to significantly compare among the means.

RESULTS AND DISCUSSION

Thyroid gland functions

In the present study, thyroid hormone levels T_3 and T_4 in addition to TSH were evaluated and statistical analysis performed for all animals exposed to ZnO NPs.

The value of T_3 hormone (Nmol/L) demonstrated a highly significant increase ($p<0.01$) for the animals that exposed to ZnO NPs at both doses (50 and 200) mg/kg (1.816 ±0.02) and (1.993 ±0.02) respectively, during 1 week when compared with the control group (1.493 ±0.02). As well there was a highly significant increase ($p<0.01$) in treated animals with these two-doses (2.115 ±0.02) and (2.296 ±0.01) respectively, during 2 weeks when compared with the control group (1.445 ±0.02). The results also showed a highly significant increase ($p<0.01$) in both treated animals by ZnO NPs at doses (50 and 200) mg/kg (2.428±0.03) and (3.858±0.04) respectively, for 4 weeks when compared with the control group (1.458 ±0.02), as demonstrated in Table 2.

Concerning the results of the T_4 hormone (Nmol/L), Table 3 shows a highly significant increase ($p<0.01$) for the animals that exposed to ZnO NPs at two doses (50 and 200) mg/kg (71.25±0.27) and (94.99±0.78) respectively, for 1 week when compared with the control group (52.58±0.35). Also, there was a highly significant increase ($p<0.01$) in both treated animals with low and high doses (50 and 200) mg/kg of ZnO NPs (109.68±0.43) and (122.30±0.56) respectively, for 2 weeks compared with the control group (54.07±0.36). Finally, there was a highly significant increase ($p<0.01$) in this hormone for animals exposed with both doses of ZnO NPs (138.25 ±0.52) and (160.80±0.68) respectively at 4 weeks when compared with the control group (53.38±0.19).

The results in Table 4 showed no significant change ($p>0.05$) in the TSH value (ìlU/ml) for all animals exposed to different doses (50 and 200) mg/kg of ZnO NPs in duration periods 1 week (0.177 ±0.002) and (0.178 ±0.002) compared with the control group (0.180 ±0.002) and during 2 weeks as well (0.175 ±0.002) and (0.172 ±0.002) respectively, compared with the control groups (0.177 ±0.003). Whereas, there was a significant decrease ($p<0.05$) in the TSH level (0.172 ±0.002) and (0.172 ±0.002) in experimental groups that exposed to ZnO NPs at low and high dose (50 and 200 mg/kg) respectively, during 4 weeks when compared with the control group (0.181±0.002).

Hyperthyroidism or thyrotoxicosis is a clinical status that results from hyper-secretion of thyroid hormones (Torlak *et al*, 2007). The results were obtained in this study may be in the line of other studies conducted by (Yousef, 2019), who reported that the T_3 and T_4 hormones levels significantly increased in rats that were exposed to ZnO NPs at a dose (100 mg/kg) administered orally every day for 75 days, in the same path (Eid *et al*, 2019) there was a significant decrease in TSH level in rats that exposed to the same dose of ZnO NPS (100 mg/kg) during 75 days compared to the control group. Others administered the Nano-Selenium to male rats at a dose 30 mg/kg of body weight for 2 months at the rate of one injection in a week, the results showed a significant increase in both T_3 and T_4 concentrations when compared to the control group. As well as (Mahdieh *et al,* 2016)

Table 2 : Effect of ZnO NPs on T_3 hormone during three periods.

Groups	Mean ± SE of T_3 Hormone (Nmol/L)			LSD value
	1st Week	2nd Week	4th Week	
Control	1.493 ±0.02A c	1.445 ±0.02A c	1.458 ±0.02A c	0.0506 NS
ZnoNPs: 50 g/kg	1.816 ±0.02C b	2.115 ±0.02B b	2.428 ±0.03A b	0.0643 **
ZnoNPs: 200 g/kg	1.993 ±0.02C a	2.296 ±0.01B a	3.858 ±0.04A a	0.0852 **
LSD value	0.0584 **	0.0517 **	0.088 **	—

** ($p \leq 0.01$) represent high significant alteration
(A,B,C) expresses the significant difference among groups with concentrations as a fixed element and weeks as an alterable element.
(a,b,c) expresses the significant difference among groups with weeks as a fixed element and concentrations as an alterable element.

Table 3 : Effect of ZnO NPs on T_4 hormone during three periods.

Groups	Mean ± SE of T_4 Hormone (Nmol/L)			LSD value
	1st Week	2nd Week	4th Week	
Control	52.58±0.35A c	54.07±0.36A c	53.38±0.19A c	0.929 NS
ZnoNPs: 50 g/kg	71.25±0.27C b	109.68±0.43B b	138.25±0.52A b	1.273 **
ZnoNPs: 200 g/kg	94.99±0.78C a	122.30±0.56B a	160.80±0.68A a	2.040 **
LSD value	1.557**	1.377**	1.524**	—

** ($p \leq 0.01$) represent high significant alteration
(A,B,C) expresses the significant difference among groups with concentrations as a fixed element and weeks as an alterable element.
(a,b,c) expresses the significant difference among groups with weeks as a fixed element and concentrations as an alterable element.

Table 4 : Effect of ZnO NPs on TSH hormone during three periods.

Groups	Mean ± SE of TSH Hormone (mIU/L)			LSD value
	1st Week	2nd Week	4th Week	
Control	0.180±0.002A a	0.177 ±0.003A a	0.181 ±0.002A a	0.0066 NS
ZnoNPs: 50 g/kg	0.177±0.002A a	0.175 ±0.002A a	0.172 ±0.002A b	0.0064 NS
ZnoNPs: 200 g/kg	0.178±0.002A a	0.172 ±0.002B a	0.172 ±0.002B b	0.0056 *
LSD value	0.005 NS	0.0074 NS	0.0061*	—

* ($p \leq 0.05$) represent significant alteration
(A,B,C) expresses the significant difference among groups with concentrations as a fixed element and weeks as an alterable element.
(a,b,c) expresses the significant difference among groups with weeks as a fixed element and concentrations as an alterable element.

observed the TSH level in the serum of mice gained a significant decrease ($P<0.05$) in all experimental groups that received 10 and 100 ppm of titanium dioxide at duration 14 days when compared with the control group. Another study by Espanani *et al* (2013) showed after injecting ZnO NPs with four different doses (5, 10, 20, and 40) mg/kg in adult male rat intraperitoneally during 21 days, the results showed that no significant change in TSH level at doses (5, 10 and 20) mg/kg, whereas there was a significant increase in TSH level in Rat treated with high dose (40 mg/kg) of ZnO NPs compared with the control group.

In contrast, the present study conflicted in results with a previous study which presented by Valipour (2015) reported a significant decrease in the level of T_4 hormone in adult male rats that received 2.5 and 5 mg/kg of ZnO NPs on 3 and 14 days when compared with the control group, and also there was a significant decrease in the level of T_3 hormone in the male rats that received 1.25, 2.5 and 5 mg/kg of ZnO NPs at 3 days when compared with the control group. Razooki and Rabee (2020) demonstrated alteration in the level of TSH in rats that were exposed to ZnO NPs, they suggested that the zinc oxide nanoparticles, especially in high quantity, can change the production and secretion processes of thyroid hormones. Boas *et al* (2012) have reported that there are many chemical compounds that cause an underactive thyroid gland by interfering with the TSH receptor, as TSH stimulates all steps to produce thyroid hormones. Shirband *et al* (2013) concluded that the nanoparticles at different doses have toxic effects on the thyroid gland and inhibit its activities. It can be concluded that the ZnO NPs even in small quantities can have negative effects on thyroid activity and disrupt the secretion of their hormones (Valipour, 2015).

According to the obtained results in this study, showed the ZnO NPs have been effected on the thyroid function which is caused hyperthyroidism, in turn, this increase in

the thyroid hormones have opposite feedback on the pituitary gland which caused inhibition and reduction in the secretion of TSH.

Evaluation of TSHR gene

The obtained results in this study showed the regulation of gene expression affected by exposure the animals with ZnO NPs, the results in Table 5, showed an obvious gradual decrease in the expression of the TSH receptor gene in all tissue of animals that exposed to ZnO NPs at both doses (50 and 200) mg/kg in different durations (1, 2 and 4 weeks) in comparison with the control groups, the alteration in the gene expression be rise with increasing the dose and duration of exposure.

Gene expression is the process in which a cell's genetic information leads to generating a functional gene product to performing specific functions of the cell (Ghorbani *et al*, 2018). It plays an important role in converting information encoded in a gene into a functional product (Mitsis *et al*, 2020). There are many exogenous influence factors such as, environmental chemicals that can alter the levels of gene expression (Baccarelli and Bollati, 2009). One of these chemical agents that effected

Table 5 : RT-PCR results of Thyroid stimulate hormone receptor gene (TSHR gene).

1 Week							
Groups	**Samples**	**TSHR-gene**	**House keeping (or Reference) gene**	**ΔCT**	**ΔΔCT**	**Folding**	**Mean**
Control Group	1	27.59	24.79	2.81	0.50	0.7090	1.380
	2	27.87	27.12	0.75	-1.56	2.9563	
	3	27.73	24.34	3.39	1.08	0.4743	
Rat treated groups with low dose (50 mg/kg)	4	28.07	26.19	1.89	-0.42	1.3425	0.819
	5	26.21	22.52	3.68	1.37	0.3859	
	6	26.00	23.24	2.76	0.45	0.7298	
Rat treated groups with high dose (200 mg/kg)	7	34.75	23.38	11.37	9.06	0.0019	0.002
	8	33.67	23.09	10.58	8.27	0.0032	
	9	35.89	21.76	14.13	11.82	0.0003	
2 Weeks							
Control Group	10	32.38	22.90	9.48	-1.68	3.20	1.607
	11	34.03	23.35	10.68	-0.48	1.40	
	12	36.25	22.95	13.31	2.15	0.23	
Rat treated groups with low dose (50 mg/kg)	13	35.93	24.11	11.82	0.66	0.63	0.666
	14	33.57	22.26	11.31	0.15	0.90	
	15	35.41	23.13	12.28	1.12	0.46	
Rat treated groups with high dose (200 mg/kg)	16	40.68	22.65	18.03	6.87	0.01	0.570
	17	33.67	23.10	10.57	-0.59	1.51	
	18	35.86	22.35	13.51	2.35	0.20	
4 Weeks							
Control Group	19	32.38	22.90	9.48	-1.68	3.20	1.607
	20	34.03	23.35	10.68	-0.48	1.40	
	21	36.25	22.95	13.31	2.15	0.23	
Rat treated groups with low dose (50 mg/kg)	22	35.12	23.09	12.04	0.88	0.55	0.498
	23	35.26	23.53	11.73	0.57	0.67	
	24	36.35	23.31	13.03	1.87	0.27	
Rat treated groups with high dose (200 mg/kg)	25	34.74	22.66	12.09	0.93	0.53	0.224
	26	39.11	24.10	15.01	3.85	0.07	
	27	39.42	24.53	14.89	3.73	0.1	

on expression regulation is the nanoparticles (Sierra *et al*, 2016). These substances can cause epigenetic changes at levels of DNA, RNA as well as protein (Babele *et al*, 2019). The ability of the nanoparticles to change in gene expression due to their Physico-chemical properties, represented in the size and shape of these particles (Chun *et al*, 2018).

The obtained results of the TSHR gene were found to be consistent with the previous study conducted by Du *et al* (2016), who reported down-regulation in the expression of the TSHR gene in zebrafish larvae after co-exposure to Perfluorooctane sulfonate (PFOS) and ZnO NPs in comparison to the control group. Also, Lü *et al* (2009) investigated from the cytotoxic effects of Ni(II) on the level of gene expression of mouse fibroblast cells, then the expression of the gene was detected after the cells were cultured in the medium with 200 mM Ni(II) for 24, 48 and 72 h, the obtained resulted were 20 up-regulated genes and 19 down-regulated genes in all three-culture periods, they suggested that the downregulation in gene expression may be caused by Ni(II) particles which could inhibit cell proliferation, reduce cell adhesion, and influence the cell morphogenesis and cell migration. Several molecular studies have shown that exposure to ZnO NPs results in the production of reactive oxygen species (ROS) in tissues and cells, by the interaction of nanoparticles with biological molecules including DNA (Zhao *et al*, 2016).

The reactive oxygen species (ROS) is one of the most reasons that play a role in induced DNA damage, apoptosis, and cell death (Kocyigit and Guler, 2017). The increasing DNA damage leads to a decrease in the number of mRNA transcripts as well as a decrease in expression protein (Nishino *et al*, 2011). While others have suggested that most nanoparticles including ZnO NPs are rabidly dissolved to form hydrated Zn^{2+}, then these dissolved Zn^{2+} ions accumulate particularly in the nucleus of cells and mediate the observed adverse cellular responses, the amount of DNA damage depends on the cellular intake of dissolved Zn^{2+} ions rather than of solid ZnO NPs. Moreover, they observed in addition to the generated ROS, the direct action of Zn^{2+} ions on DNA causes damage to this DNA (Heim *et al*, 2015). As it's known, the transcription process depends on several steps associate with many factors that are necessary for the synthesis mRNA strand, including the transcription factors. It is well established that the eukaryotic transcriptional factors have modular activator or repressor sequences of corresponding nucleotides that attach to specific promoters that regulate gene expression (Gottesfeld *et al*, 2001). Since the nanoparticles have the ability to dissolve inside the cell and release an ion Zn^{+2}. In contrast, the DNA molecule has an intrinsic negative charge at its backbone of the double helix (Zhang *et al*, 2020).

It can be concluded that the decreased expression of the TSHR gene may be due to the direct effect of nanoparticles on the process of gene production or by an adverse response to the excess secretion of thyroid hormones (hyperthyroidism), which negatively effects on the production of a gene that responds to formation this receptor.

CONCLUSION

The data of this study clearly showed a negative relationship between the zinc oxide nanoparticles and the thyroid gland functions, which is caused a significant increase in the T_3 and T_4 hormones, therefore these abnormal increases have an adverse effect on the synthesis and release of TSH hormone in the blood, as well as caused reduction in the gene expression that responsible for the production of TSH receptor.

For this, it concludes that the small size of the nanoparticle enables it to penetrate the tissue and cellular barrier to reach in any site inside the body especially the thyroid gland.

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