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# The Toxic and Genetic Effects of Zinc Oxide Nanoparticles on Thyroid Gland and Ovary Structures and Function in Female Rats

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بِسْمِ اللَّهِ الرَّحْمَٰنِ الرَّحِيمِ

﴿ يَرْفَعِ اللَّهُ الَّذِينَ آمَنُوا مِنْكُمْ وَالَّذِينَ أُوتُوا الْعِلْمَ دَرَجَاتٍ وَاللَّهُ بِمَا تَعْمَلُونَ خَبِيرٌ ﴾

صدق الله العظيم

سورة المجادلة الاية (11)

# Supervisor Certification

We certify that this thesis entitled **"The Toxic and Genetic Effects of Zinc Oxide Nanoparticles on Thyroid Gland and Ovary Structures and Function in Female Rats"** was prepared by **"Faisal Ghazi Lazim"** under our supervision at College of Science/ Mustansiriyah University as partial fulfillment of the requirements for the degree of Master of Science in Biology/Zoology.

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# *Dedication*

To my father for his massive compassion and support.

To tenderness in all its sense, my beloved mother.

To my lovely brother and sisters, without them life would be meaningless.

To all my dear friends, especially for those who support and stood beside me during this study.

*Faisal Ghazi*

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# Summary

Zinc oxide nanoparticles are one of the most important nanoparticles commonly used in various fields due to the unique physic-chemical properties and low cost of production. The products that contain zinc oxide, may be dissolved and give rise to the nanoparticles themselves or produce Zn ions to the environments and may transfer into the bloodstream of human through different routes and then accumulate in organs. Therefore, since the numerous used of ZnO NPs in large quantities, it is necessary to find out the toxicity and harmful effects of these particles on growth, proliferation, and viability in cells and organisms. For this purpose, the aim of this study was to determine the effect of ZnO NPs on the structure and function of the thyroid gland and ovary as well as to estimation the level of gene expression of TSHR that present in thyroid gland and THRβ that present in the ovary. Fifty-four adults female rats have been randomly divided into three main groups according to the periods of exposure (1, 2 and 4) weeks, then these groups also subdivided into three subgroups, each group consist of 6 rats, one of them used as a control group and injected with (1ml) of distilled water, while the others used as treated groups and injected with (1 ml) of ZnO NPs at low and high concentrations (50 and 200) mg/kg respectively, an average of three injections per week via intra-peritoneal route. After end of each treatment period, the animals have been weighed, then are followed by collected 4ml of blood to estimation the levels of (T3, T4, TSH, LH, FSH, E2, and P) hormones. After that, the animals are dissected and the thyroid glands and ovaries are removed and weighed as well, then are kept in fixative solutions for histological and molecular study. The statistical analysis of obtained results were as the following:

* High significant decrease ($P \leq 0.01$) in the weights of body and ovaries of treated animals with ZnO NPs at doses (50 and 200) mg/kg in different durations (1, 2 and 4) weeks, when compared with the control groups.

* High significant increase ($P \leq 0.01$) in the weights of thyroid glands of treated animals during (1, 2 and 4) weeks at different doses (50 and 200) mg/kg when compared with the control groups.
* Non-significant decrease ($P \leq 0.05$) in the levels of TSH hormone after (1and 2) weeks of treatment with ZnO NPs at doses (50 and 200) mg/kg, whereas there was a significant decrease ($P \leq 0.05$) for duration 4 weeks at doses (50 and 200) mg/kg when compared with the control groups.
* High significant increase ($P \leq 0.01$) in the serum levels of T3 and T4 hormones in all treated animals with both doses (50 and 200) mg/kg of ZnO NPs in duration (1, 2 and 4) weeks, when compared with control groups.
* Non-significant decrease ($P \leq 0.05$) in the serum levels of progesterone, LH and FSH hormones after 1 week of treatment with (50 and 200) mg/kg of ZnO NPs, while, during (2 and 4) weeks the results were demonstrated high significant decrease ($P \leq 0.01$) in the levels of these hormones in animals that treated with both doses (50 and 200) mg/kg when compared with the control groups.
* High significant decrease ($P \leq 0.01$) in the serum levels of estrogen for all treated animals through different periods compared with the control groups.
* Histological changes in the studied organs in all treated animals with ZnO NPs at different periods of exposure, and these alterations increase gradually with increase the doses and time of treatment in all examined organs (ovary and thyroid glands) when compared with the control groups.
* An obvious decline in the level of TSHR gene in the tissue of thyroid gland in all treated rats at different duration when compared with the control animals.
* An uneven decrement in the level of THRβ gene in the tissue of the ovary in all treated rats with ZnO NPs at different periods of exposure when compared with the control animals.

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# List of Abbreviations

| Abbreviation | The Term |
|---|---|
| **aa** | Amino acids |
| **Ag NPs** | Silver Nanoparticles |
| **ANOVA** | Analysis of Variation |
| **CT** | Cycle threshold |
| **DIO1** | Iodothyronine deiodinase type 1 |
| **DIO2** | Iodothyronine deiodinase type 2 |
| **E2** | Estrogen |
| **FSH** | Follicle-stimulating hormone |
| **GnRH** | Gonadotropin-releasing hormone |
| **H&E** | Hematoxylin and Eosin |
| **kb** | Kilobytes |
| **LH** | Luteinizing hormone |
| **LRR** | Lucine-rich repeats |
| **LSD** | Least significant difference |
| **$MoO_3$ NPs** | Molybdenum oxide nanoparticles |
| **NCBI** | National Center for Biotechnology Information |
| **NCDCR** | National Center for Drug Control and Research |
| **Ni(II)** | Nickel (2+) |
| **Nm** | Nano meter |
| **NMs** | Nanomaterials |
| **NPs** | Nanoparticles |

| | |
|---|---|
| **P** | Progesterone |
| **ppm** | Part per million |
| **PFOS** | Perfluorooctane sulfonate |
| **qRT-PCR** | Quantitative Real Time-Polymerase Chain Reaction |
| **ROS** | Reactive oxygen species |
| **rpm** | Revolutions per minute |
| **SAS** | Statistical Analysis System |
| **T3** | Triiodothyronine |
| **T4** | Thyroxine |
| **TF** | Transcription factors |
| **THRβ** | Thyroid hormone receptor beta |
| **THs** | Thyroid hormones |
| **$TiO_2$-NPs** | Titanium dioxide nanoparticles |
| **TMD** | Transmembrane domains |
| **TRH** | Thyrotropin-releasing hormone |
| **TRs** | Thyroid Receptors |
| **TSH** | Thyroid stimulated hormone |
| **TSHR** | Thyroid stimulated hormone receptor |
| **YWHAZ** | Housekeeping gene (tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta) |
| **ZnO NPs** | Zinc Oxide Nanoparticles |

# Chapter One

# Introduction and Literature Review

# Chapter One

## 1. Introduction and Literature Review

### 1.1. Introduction

Nanoparticles (NPs) are defined as small materials have at least one dimension and their size ranging from 1 to 100 nanometers compared with particles in micro-scale, these particles have different degrees of biological effects due to their small size and large specific surface area (Esmaeillou *et al.*, 2013). As well they are used in a wide range of applications such as cosmetics, medical, energy and environmental technologies (Heera and Shanmugam, 2015).

Depending on these applications it is very important to focus on their possible toxic and harmful effects on growth, viability, and proliferation in cells and organisms (Omidi *et al.*, 2015). The small size of nanoparticles (NPs) allows them to penetrate the cytoplasmic membranes and interfere with the cellular functions (Radhi and Al-Bairuty, 2019). These particles may enter the human bodies through different routes including inhalation, dermal penetration, ingestion, and injection (Shen *et al.*, 2019). As one of the most important metal oxide nanoparticles is zinc oxide nanoparticles (ZnO NPs), which is popularly used in various fields due to their particular physical and chemical properties (Jiang *et al.*, 2018). ZnO NPs is the third most widely used nanoparticles, with an annual production range of 550 to 33,400 tons (Connolly *et al.*, 2016). It is exist as a mineral zincite in the earth's crust, while most of it can be produced commercially by synthetic methods (Prasad, 2019).

In this research, two organs were studied, namely thyroid gland and the ovary. The Thyroid gland plays important 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). As well, it plays a major role in the development of the reproductive tract (Choksi *et al.*, 2003). In mammals, the hypothalamus gland secretes the thyrotropin-releasing hormone (TRH) to stimulate the secretion and regulation of the synthesis of thyroid stimulated hormone (TSH) (De Groef *et al.*, 2006). Then the thyroid-stimulating hormone (TSH) binding on the thyroid gland to its receptor (TSHR), to stimulate synthesis and release of thyroid hormones (Rose and Chuang, 2017). The major hormones that secreted by the thyroid gland are triiodothyronine (T3) and thyroxine (T4), which have a wide range of biological effects in development, growth, and metabolism (Shi *et al.*, 2009).

The ovaries are paired pelvic organs located on either side of the uterus, near the pelvic sidewall and anterior to the rectum (Weidner *et al*., 2009). The function of ovary is controlled by two hormones, luteinizing hormone (LH) and follicle-stimulating hormone (FSH), which are secreted from the anterior-pituitary gland under the control of gonadotropin-releasing hormone (GnRH) that secreted from the hypothalamus (Richards and Pangas, 2010). These hormones work on the ovary and play a key role in gonadal functions, follicular development and growth (Raju *et al.*, 2013). The ovary is responsible for developing and releasing of oocytes and producing a variety of steroid hormones, especially estrogen and progesterone, which affect other organs throughout the body (Vidal and Dixon, 2018). On the other hand, the Ovarian surface cells express multiple receptor genes for nuclear hormones, including those that encode thyroid hormones (TR) (Rae *et al.*, 2007).

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). Some studies on the reproductive function indicate that exposure to some nanoparticles (NPs) may disrupt the endocrine functions such as regulation levels of sexual hormones (Iavicoli *et al.*, 2013).

(Zhao *et al.*, 2015), observed that exposure to ZnO NPs alters the regulatory expression of some genes, especially in the female reproductive system.

## Aims of Study :

The present study aims to evaluate the effect of ZnO NPs on the structure and function of thyroid gland and ovary through the following:

- Body weight.
- Thyroid gland and ovary weight.
- Function of thyroid gland {Thyroid stimulating hormone (TSH), T3 and T4}
- Function of ovary by estimating the level of hormones {Estrogen and Progesterone hormones}.
- Estimating the gonadotropin hormones {Luteinizing hormone (LH) and follicle-stimulating hormone (FSH)}.
- Histological changes in the ovary and thyroid gland.
- Gene expression of thyroid hormone receptor beta (THRβ) in ovary and thyroid stimulate hormone receptor (TSHR) in thyroid gland.

## 1.2. Literature Review

### 1.2.1. Overview

Nanoscience is the study of nanoscale structures and molecules, and the technology that used in practical applications called nanotechnology, which is capable of converting nanoscience theory into useful applications by observing, measuring, assembling, manipulating, controlling, and manufacturing matter on a nanometer scale (Bayda *et al.*, 2020).

Due to the wide applications of nanomaterials in multiple domains such as in industry, agriculture, medicine, and public health, nanotechnology has gained much public interest ( Pasupuleti *et al*., 2012; Karthik *et al*., 2020). The size of nanoparticles ranges between 1 to 100 nanometres, and they are made up of metal, carbon, metal oxides, and organic matter ( Ealias and Saravanakumar, 2017). The major aim of designing the nanoparticles as a delivery system is to control the particle size, surface properties, and release of pharmacologically active agents to achieve the drug's site-specific action at the therapeutically optimal rate and dose regime *(*Kadam *et al.*, 2014). According to their size, the nanoparticles can easily penetrate the human body and reach the organs including the heart, kidneys, and liver via the bloodstream (Simkó *et al.*, 2010). High surface area and other nanoparticular characteristics cause it to be very reactive and toxic and can harm human and animal cells by raising the process of oxidative stress (Mohammadi Fartkhooni *et al.*, 2013). These nanoparticles (NPs) usually have different Physico-chemical and electronic properties including significantly higher specific surface area, high surface reactivity, and increased quantum effects, depending on the size of the corresponding microparticles, as well the NPs have enhanced reactivity and greater capacity to penetrate tissues and cell membranes (Kumari *et al.*, 2014).

### 1.2.2. Classification of Nanoparticles

Nanoparticles can be synthesized by various physical or chemical methods from many materials, with particles differing in their elementary composition, shape, size, and chemical or physical properties (Kango *et al.*, 2013; Iravani *et al.*, 2014).

**1.** Nanoparticles can be classified based on their structure into two broadly groups, namely organic and inorganic nanoparticles:

Organic nanoparticles, also known as polymeric nanoparticles, are usually biodegradable materials, which are including dendrimers, nanoparticles of ferritin and hollow spheres such as micelles and liposomes (Teleanu *et al.*, 2018). These include nanomaterials (NMs) that made mainly from organic matter, the use of non-covalent (weak) interactions or molecular self-assembly and design helps turn organic NMs into desired structures such as dendrimers, micelles, liposomes, and polymer NPs (Jeevanandam *et al*., 2018). Organic nanoparticles are widely used in the biomedical field, for example, a drug delivery system because they are effective and can also be injected into certain parts of the body which are also known as targeted drug delivery (Ealias and Saravanakumar, 2017).

The second group is inorganic nanoparticles include magnetic nanoparticles, noble metal nanoparticles (such as gold and silver), and semiconductor nanoparticles (such as titanium dioxide and zinc oxide) (Prathna *et al.*, 2010). Because of its size and advantages over current chemical imaging drugs and medicines, inorganic nanoparticles were explored as potential tools for medical imaging as well as disease treatment, inorganic nanomaterials were widely used for cellular delivery due to their versatile features such as wide distribution, rich functionality, strong biocompatibility, the capability of targeted drug delivery, and controlled release of drugs (Xu *et al.*, 2006). Inorganic nanoparticles include a

range of nanoparticles from metal and a metal oxide, and almost all metal materials can be synthesized into their nanoparticles (Ama and Ray, 2020).

**2.** The nanoparticles also can be classified according to their composition into natural and synthetic nanoparticles:

Natural nanoparticles can be found outside the field of life, e.g. nanoscopic ash or soot particles result from volcanic activity, fires or other combustion types, these particles composed primarily from silicate and iron compounds, they are readily diffusion in the air and can cause severe respiratory disorders when inhaled (Griffin *et al*., 2018). These particles categorized into various types including, metal nanoparticles (such as gold and silver nanoparticles), metal oxide nanoparticles (such as zinc oxide), carbon nanoparticles, and quantum dots (such as cadmium selenide) (Fulekar and Pathak, 2017).

Whereas, the synthetic nanoparticles can be obtained by a variety of methods including physical, chemical, biological, and hybrid techniques (Li *et al*., 2011; Patra and Baek, 2014). They have Physico-chemical properties and a unique reaction, because of their small size and high surface area (Elshama *et al*., 2018). As a particle decreases in size, a larger proportion of atoms are found on the surface compared to the inside (Pidgeon *et al.,* 2004*)*. These factors give the synthetic nanoparticles higher surface interaction than their counterparts of the same material (Handy *et al.*, 2008).

### 1.2.3. Zinc Oxide Nanoparticles (ZnO NPs)

Zinc oxide (ZnO) is a chemical compound usually in the form of a white powder that is insoluble in water, this powder is mostly using as an additive in many materials and products (Alrahabi, 2014). Often used in several filed, due to the low cost of production and unique physic-chemical properties (Mishra *et al*., 2017). The widespread use of zinc oxide nanoparticles (ZnO NPs) in the global consumer market makes people more vulnerable to exposure to ZnO nanoparticles

and their harmful effects (Sharma *et al*., 2012). Products that containing ZnO-NPs may degrade and give rise to the nanoparticles itself or Zn ions that may pass into the circulation of human blood and accumulate in organs that may lead to toxicity (Choi *et al*., 2015; Mir *et al.*, 2020). These particles can enter the body through different routes include inhalation, skin, and digestive (Ajdary *et al.*, 2018). Then accumulate in multiple organs, such as the liver, kidney, spleen, lungs, heart and reproductive system via circulation, and may produce an adverse effect, such as edema and degeneration of hepatocytes, as well as inflammation and damage of organs and tissues (Singh, 2019).

Recently, in vivo cell experiments showed that exposure to nano-ZnO induced significant cytotoxicity, inflammation, and oxidative DNA damage in various cells (Fadeel *et al.*, 2017).

### 1.2.3.1. ZnO NPs Applications and Uses

Nanoparticles include zinc oxide (ZnO) whether simple or complex are used in different applications in physics, biology, and biomedical medications (Nikalje, 2015). Several researches in recent years have used nanoparticles, especially ZnO, in treatment of several diseases (Elshama *et al*., 2018; Jiang *et al.*, 2018). It was used as a conductor for drug and this feature was derived from two main properties, the first one is due to their smaller size of these particles can easily pass through smaller capillaries and may enter the cells, allowing an efficient accumulation of drugs at the target sites. Secondly, use of biodegradable materials to prepare the nanoparticles allows the prolonged discharge of drugs within the target site over a period of days or even weeks (Kalpana and Devi Rajeswari, 2018). Others, applied the Zinc to maintain the tumor suppressor activity of gene p53 that has a role in regulating apoptosis activity (Ng *et al.*, 2011).

In contrast, ZnO nanoparticles are suggested to be an effective cancer treatment factor, as they show preferential toxicity to cancer cells compared to normal cells (Hanley *et al.*, 2008; Bisht and Rayamajhi, 2016). In addition, the Zinc oxide nanoparticles play a major role in various metabolic pathways including the metabolism of glucose, promote hepatic glycogenesis through insulin pathways, and thus improves the use of glucose (Bayrami *et al.*, 2018). As for the industrial aspect, they are used in different fields such as cosmetics, coatings, pigments, catalysts, and electronic devices (Raisi Dehkourdi *et al.*, 2017). As well used widely in cotton fabric, rubber, and food packaging industries (Kadhim *et al.*, 2016).

A further feature is the ability of nanomaterials to reflect ultraviolet irradiation (Morabito *et al.*, 2009). Thus making ZnO-NPs an important physical ultraviolet filter in sunscreens, ZnO-NPs have a very high value to protect against ultraviolet, in this way many industries prefer ZnO-NPs to cosmetic products compared to larger particles (Smijs and Pavel, 2011).

### 1.2.3.2. Exposure Routes of Zinc Oxide Nanoparticles

1. **Dermal exposure** : The skin is the outer barrier of the total body and an entry route for many foreign materials including NPS (Ryu *et al.*, 2014). Zinc oxide nanoparticles (NPs) are widely using in many topical skin care products, such as ointments and sunscreen products to protect from skin damage caused by UV rays (Zvyagin *et al.*, 2008). According to the broad application of nanoparticles in cosmetics, therefore the skin is the main exposure route to ZnO-NP for consumers (Rossner Jr *et al.*, 2019), and due to the size of these particles makes the process of penetration of the skin easy through the stratum corneum then reach the underlying viable epidermis and finally rest in all of the body (OSE, 2013).

2. **Inhalation exposure:** Airway exposure to the particles through inhalation are a dominant means in workers that have direct dealing with chemical, cosmetic, as well as paint industries, these particles may have the ability to reach peripheral airway regions, such as the bronchiolar and alveolar sites (Baskoutas, 2018). Exposure to ZnO NPs via inhalation seems to present a significant hazard, and there is an urgent need to estimate the risks in this context (Vandebriel and De Jong, 2012). The nanoparticles (NPs) may effect on alveolar cells and cause toxic, and genotoxic, as well as inflammatory effects (Osmond and Mccall, 2010).

   The previous study that presented by Elshama *et al*., (2018) reported that the inhalation of ZnO NPs induced inflammation and fibrosis in tissues of alveolar and tracheobronchial. Likewise, the acidic lung fluid dissolves the ZnO nanoparticles that entered into the lung by inhalation, thus increasing its concentration resulting in pulmonary toxicity (Cho *et al.*, 2011).

3. **Ingestion exposure:** Nowadays, many consumer products contain nanoscale materials in their ingredients to improve their properties, quality of production, and ease of use, thus, the nanoparticles that present in these items may enter to the body by oral, as well as can be ingested the nanoparticles through food additives and cosmetics (such as toothpaste) (Fröhlich and Roblegg, 2012). In addition, water and soil contain concentrations of ZnO that may reach approximately 0.093 g / kg (Gottschalk *et al*., 2009). It is possible that the ZnO NPs may be enter to the cell as form of intact particles or zinc ions (Paek *et al.*, 2013).

   Consequently, the zinc oxide nanoparticles that pass into the gastrointestinal tract can affect the microvilli in the cells of the intestine, thereby reducing the surface area available for nutrient absorption (Moreno-Olivas *et al.*, 2018). Then these particles may diffusion through the intestinal layers and reach the blood or

lymphatic system and may accumulation in most organs of the body, Figure(1-1) (Molina *et al.*, 2019).

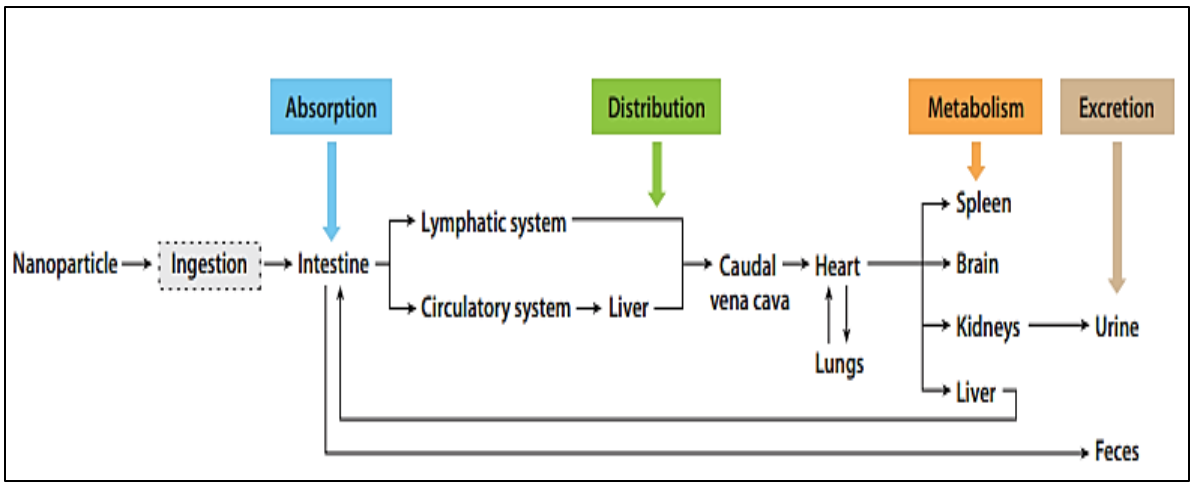



**Figure (1-1):** A flowchart describing the pathway of nanoparticles across the body from ingestion excretion, and ingestion-diffusion through the body (Borel and Sabliov, 2014)

### 1.2.3.3. Toxicity of ZnO NPs

Due to the increasing use of nanoparticles and their release into the environment as products or industrial waste, it is necessary to study these materials and assess their effect and toxicity. There are many previous reports about the toxicity of ZnO nanoparticles. The toxic effect of ZnO nanoparticles is due to their solubility, therefore the ZnO nanoparticles dissolve in extracellular region, which in turn increases the intracellular $Zn^{2+}$ level (Pandurangan and Kim, 2015; Siddiqi *et al*., 2018). Thereafter, release of $Zn^{2+}$ is the intrinsic reason for the high toxicity of ZnO NPs (Wang *et al.*, 2014).

Fukui *et al.*, (2012) indicated that the maximum release of $Zn^{2+}$ from ZnO nanoparticles is higher than those of Zn, and ZnO microparticles. Cross nanoparticles the cellular membranes and release $Zn^{2+}$ in a higher quantity that leads to producing reactive oxygen species (ROS) (Vandebriel and De Jong, 2012; Kwon *et al.*, 2014). The generation of ROS caused by ZnO nanoparticles is a

major indication of the toxicity of ZnO nanoparticles (Jalal *et al.*, 2010; Tang *et al.*, 2018). At high levels of ROS, can be lead to impaired physiological function through cellular damage to DNA, fats, proteins, and other large molecules, which can leads to certain pathologies and disorders in the human body (Rowe *et al.*, 2008). As well as it lead to altered in the regulation of gene expression (Preedy, 2020)

At this point, when ZnO nanoparticles enter into the cell, the guard of cell induced and the cellular defense mechanism begin to create ROS as an antioxidant protective for the cells, so when the generation of ROS exceeds the normal of production which leads to prompting inflammation, Figure (1-2) (Khanna *et al.*, 2015). On the other hand, the ZnO-NPs enter the lungs and via systemic circulation, it reaches to other parts of the body to induce hazardous effects the nature of acidic lung fluids assist in dissolve of particle that responses to induces inflammatory in lungs (Adamcakova-Dodd *et al.*, 2014).

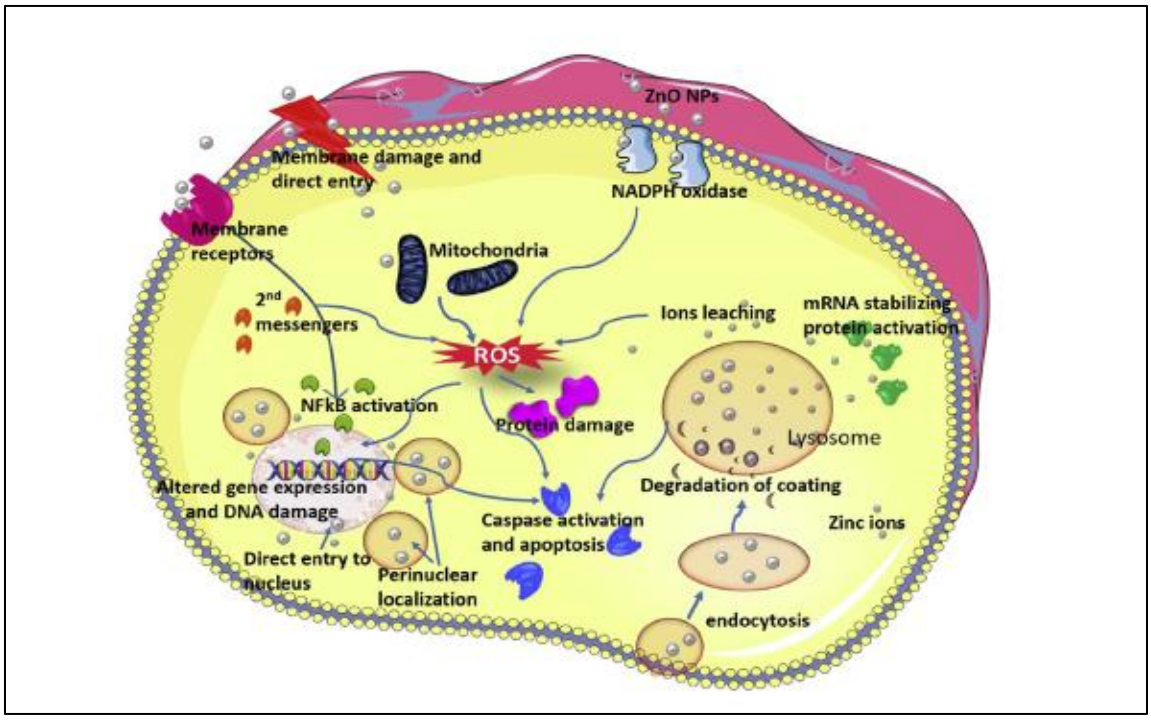



**Figure (1-2):** The mechanism of toxicity of ZnO-NPs inside the cell (Sruthi *et al.*, 2018).

### 1.2.4. Thyroid Gland

The thyroid gland is the earliest endocrine structure that appears during the development of human, and that their hormones are necessary for the proper development of organisms (Vitale *et al.*, 2017). It is one of the largest endocrine glands in the human body, weighing approximately between (10 to 20 g) in adult humans, and it is larger in men than women and increases in size with age and body weight (Llahana *et al.*, 2019).

The thyroid gland is a butterfly-shaped organ located in the lower part of the neck in front of the trachea at the level of the second and third rings of the trachea (Stathatos, 2012). Structurally, the mammalian gland consists of two lobes, each lobe formed by follicles and interfollicular spaces, the follicles are surrounded by thyrocytes that synthesize the thyroid hormones, triiodothyronine (T3) and tetraiodothyronine (T4), while the interfollicular spaces secrete calcitonin from C cells (Grimm, 2017). The thyroid hormone ( T4 and T3) play a significant role in reproduction, differentiation, and migration of cells during embryonic stages (Eroschenko, 2008).

The thyroid gland consists of three distinct regions as shown in Figure (1-3):

- The isthmus, that lies above the second and third rings of the trachea.
- The lateral lobes, each one extending from the side of the thyroid cartilage down to the sixth ring of tracheal.
- The pyramidal lobe, it is the inconstant portion which projects from the isthmus to upward usually on left side and it represents the remains of the embryological descent of thyroid (Ellis, 2007).

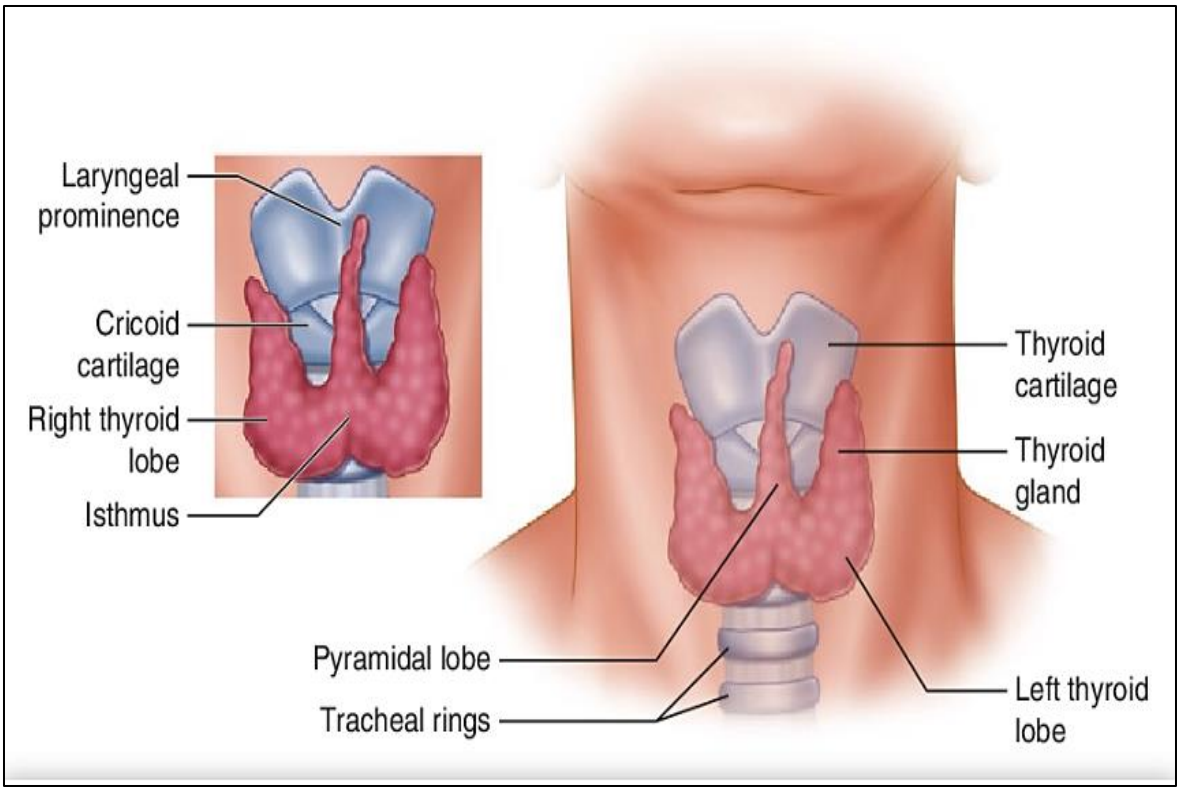



**Figure (1-3):** Thyroid gland overview (Parsa and Gharib, 2018).

### 1.2.4.1. Histological of Thyroid Gland

The thyroid gland of human made up of many spherical structures called follicles, and it is considered the functional unit for the thyroid gland, these follicles consist of follicular cells that form a simple cuboidal epithelial, Figure (1-4) (Lee *et al*., 2016). The size and shape of thyroid follicles as well as the height of the follicular epithelium vary, depending on the functional activity of the thyroid gland (Lee *et al.*, 2016). These follicles are also surrounded by loose connective tissue and consist of a single layer of epithelial cells surrounding a central cavity filled with glycoprotein called thyroglobulin (Zoeller, 2007).

In general, the thyroid gland is enveloped by the deep cervical fascia layers and is anteriorly covered by the strap muscles and laterally by the sternocleidomastoid

muscle, the thyroid capsule adherent tightly to the gland and continues into parenchyma to formation the fibrous septa which are separating the gland into several lobules, thereafter the middle layer of the deep cervical fascia intensify to form the berry posterior suspensory ligament which has a function that connects the lobes of the thyroid gland to the cricoid cartilage and the first two tracheal rings as well (Fancy *et al*., 2010).

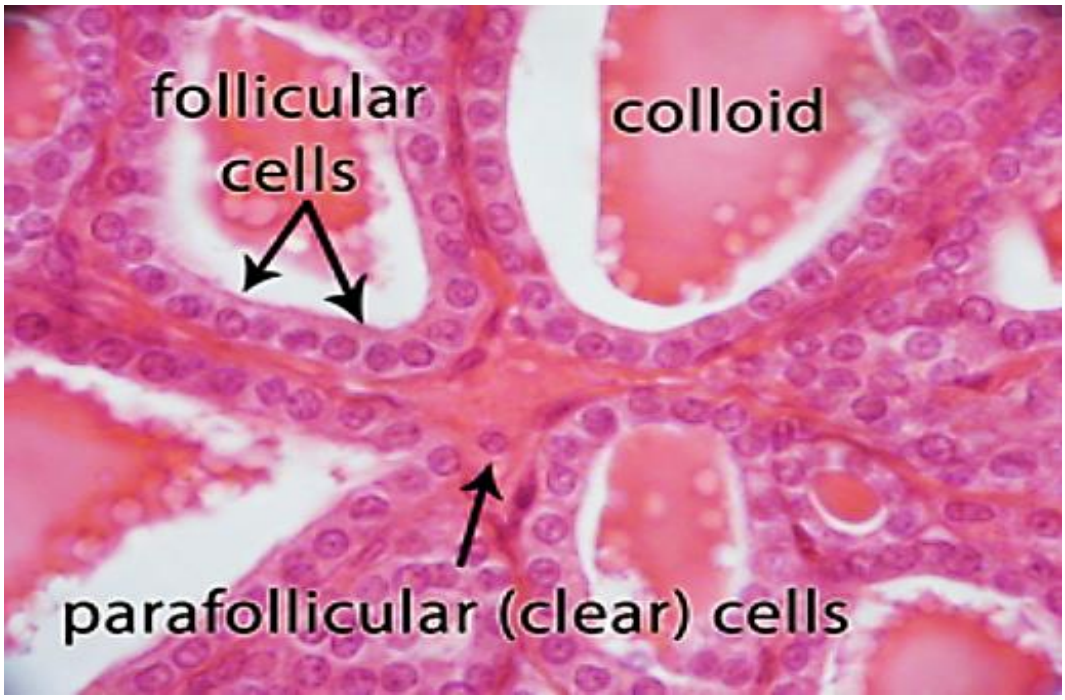



**Figure (1-4):** The histological structure of thyroid gland (Yalcin and Ozan , 2006).

### 1.2.4.2. Physiological and Regulation of Thyroid Hormone Secretion

The thyroid gland plays an essential role in the regulation of multiple body functions such as metabolic rate, energy expenditure and function of organs (Cicatiello *et al.*, 2018; Stathatos, 2019). There are three main components that play a key role in regulating the production of thyroid hormones; the first component is the thyroid gland itself with their functional units (thyroid follicle), which represents the site of synthesis and release of T3 and T4 hormones into the

circulation, the other two components are the hypothalamus that responsible for the secretion of thyroid releasing hormone (TRH) that trigger the anterior pituitary gland to secreted thyroid-stimulating hormone (TSH), which stimulates the thyroid gland to secrete its hormones, Figure (1-5) (Stathatos, 2012). In contrast, the thyroid gland also have opposite feedback on the hypothalamus-pituitary-thyroid axis to suppress or decrease the TRH and TSH hormones (Rajab *et al*., 2017).

The thyroid hormones are very important during growth and development of the mammalian body (Rhoades and Bell, 2012), skeletal (Bassett and Williams, 2003), brain (Moog *et al*., 2017), neural system (Bernal, 2007), as well as the reproductive system (Choksi *et al*., 2003). Production and secretion of thyroid hormones depend on the presence of the iodine and tyrosine and on the maturation of the hypothalamic-pituitary-thyroid system (Kirsten, 2000). The thyroid gland secretes two iodine hormones, which are thyroxine (T4) and triiodothyronine (T3), both of them are derived from the amino acid tyrosine (Light and Cooley, 2009). The thyroxin (T4) consider as pro-hormone that secreted from the thyroid gland predominantly, while the presence of triiodothyronine (T3) hormone in the circulation is a result of the conversion of T4 hormone by type 2 of iodothyronine deiodinase (DIO2) and type 1 of iodothyronine deiodinase (DIO1) (Williams and Duncan Bassett, 2011).

All concentration of the T4 hormone that presence in circulation produced by the thyroid gland, whereas only 20 percent of the T3 hormone synthesized by the thyroid gland and the remaining (80 percent) produced in the periphery from the conversion of T4 to T3 (Zoeller, 2007). The T4 hormone is a quantitatively dominant hormone, but the T3 biologically is more active (Boelaert and Franklyn, 2005).

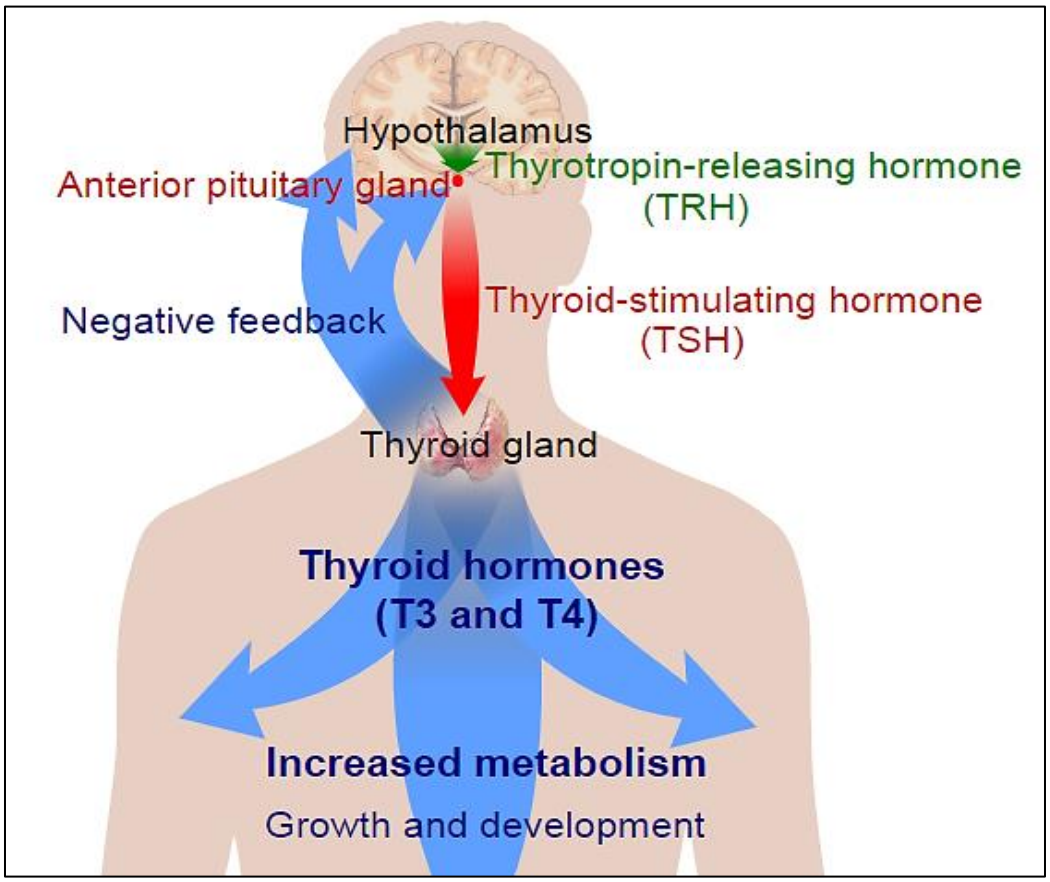



**Figure (1-5):** Regulation of thyroid hormones through the hypothalamic-pituitary-thyroid axis (Molnar and Gair, 2013).

### 1.2.4.3. Thyroid Stimulate Hormones Receptor (TSH-R)

The thyroid stimulates hormones receptor gene (TSHR gene) provides instructions for making a protein, known as a receptor (NCBI, 2020). TSH receptors expressed on the cell surface of thyroid follicular membrane and it plays an essential role in the regulation growth and function of the thyroid gland (Borel and Sabliov, 2014). As well expressed widely in a variety of extra-thyroidal tissues including; anterior pituitary, hypothalamus, ovary, testis, kidney, bone marrow, and adipose tissue (Williams, 2011). TSHR protein 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,

Figure (1-6) (Musa *et al.*, 2007). While in the Rat, it is located on chromosome 6q31(NCBI, 2020). Structurally and functionally composed of a large extracellular domain called Lucine-rich repeats (LRR) with the binding site of thyroid-stimulating hormone (TSH) which is represent the α subunit, 7-transmembrane domains (TMD), and a small intracellular domain (intracytoplasmic tail) and both of them represent β subunit, Figure (1-7) (Rapoport and McLachlan, 2016).

TSH receptor is a key regulator of thyroid hormone function, 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 (T4) and 3,3,5-triiodothyronine (T3) hormones, as well as cell proliferation and survival (Morshed *et al.*, 2009). Therefore, the defect in the TSH receptor is accompanied dysfunctions in the thyroid gland, thus it loses their activities (Kleinau and Vassart, 2017).

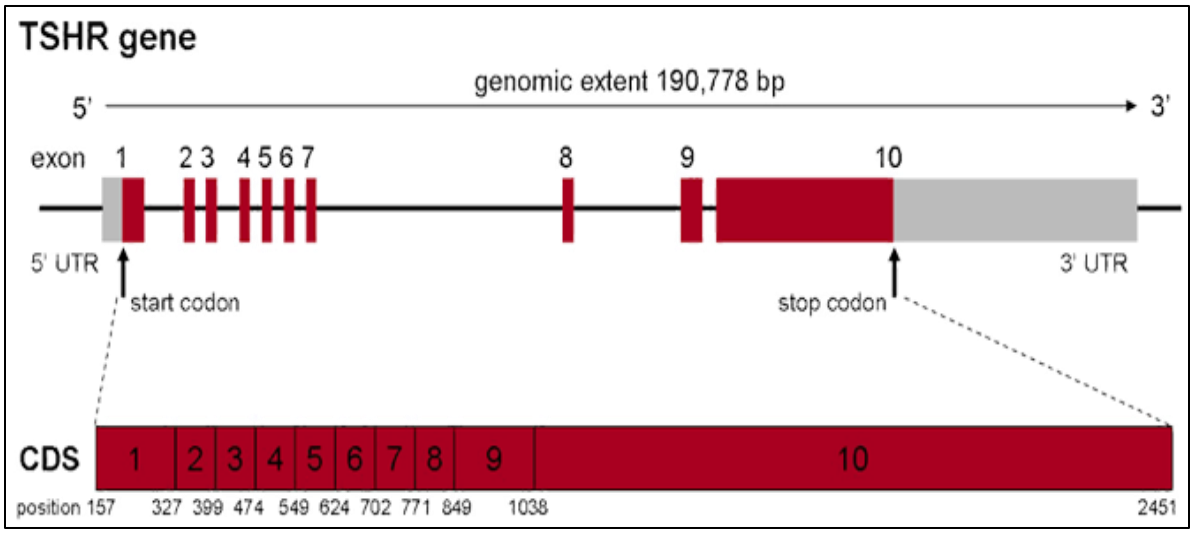



**Figure (1-6):** Structure of the TSHR gene and coding sequence (CDS), boxes represent exons numbered 1 to 10 and proportional to length, red representing the coding sequence, grey representing untranslated regions (UTR); the horizontal line joining exons represents introns, shrunk to minimal length. Positions below the CDS are numbered relative to the transcription start (Iosco and Rhoden, 2010).

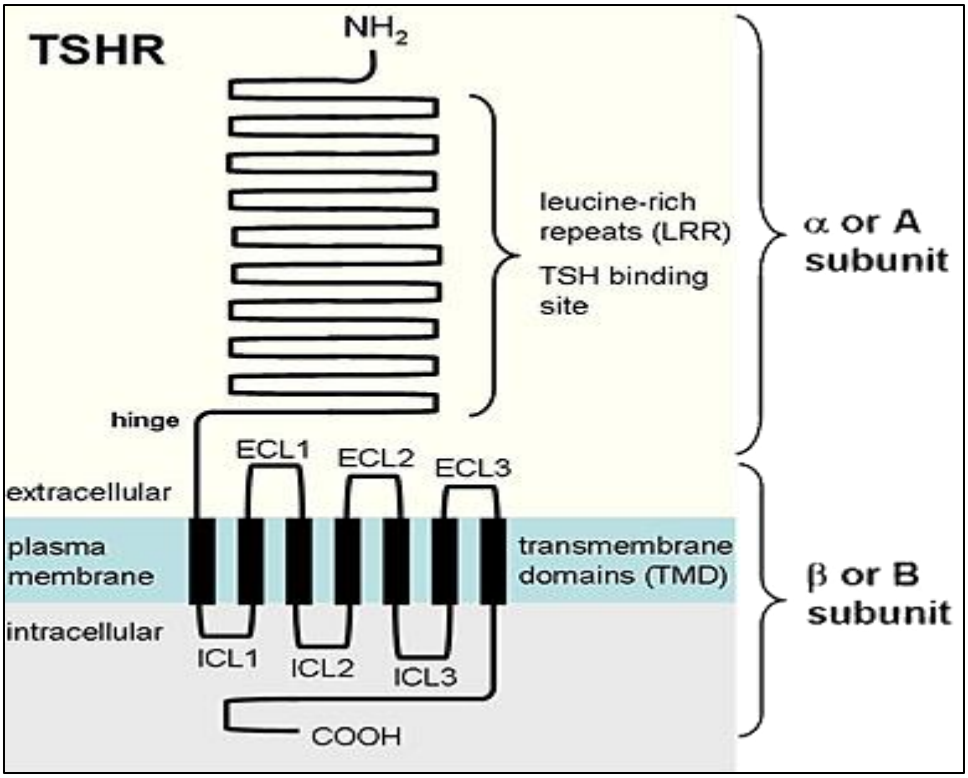



**Figure (1-7):** Thyroid stimulate hormone receptor (TSHR) protein structure, showing α subunit that composed of leucine-rich repeats (LRR) with binding site of TSH, and the β subunit that composed of 7-transmembrane domains (TMD) with intracellular loop (Iosco and Rhoden, 2010).

## 1.2.5. The Ovary

The ovaries represent the female gonads that responsible for the generation of female gametes (oocytes), and synthesis and secretion of hormones which are necessary for the regulation of reproductive functions (Rojas *et al.*, 2015; Bahr, 2018). It is an oval almond-shaped structure that vary considerably in size from a person to another depending on age, hormonal status and stage of the menstrual cycle (Saksouk and Johnson, 2004). Usually located in posterior to the fallopian tube on the pelvic sidewall and connects to the fallopian tube via small muscular complex, known the fimbria ovarica (Sokkary and Dietrich, 2018).

In a neonate, typically the size of the ovary is 1 cm in diameter and weighs between 250-350 mg (Sokkary and Dietrich; 2018). While in the adult female the ovary measure approximately (3- 5) cm in length, (1.5-3) cm in width and (0.5–1.5) cm thickness (Forstner *et al.*, 2018). In the female reproductive-aged, the growth of ovarian follicular occurs in response to secretion of hypothalamic-pituitary hormones (Ledbetter and Johnson, 2018).

### 1.2.5.1. Physiology of Ovary

The ovary has major physiological responsibilities that are summarized by; First, releasing of oocytes, second is production and secretion of the steroid hormones which are include estrogen and progesterone hormone (Sokkary and Dietrich, 2018). Steroid hormones have been known to play a major role in regulating ovarian function, besides that it plays an important central role in communication between the ovary and pituitary gland, steroids are also thought to serve as intra-ovarian regulators, although their functions can vary from one species to another (Juengel *et al.*, 2006).

The primary signal that begin from the central nervous system is a gonadotropin-releasing hormone (GnRH), which in turn act on the anterior pituitary to modulates regulating synthesis and secretion of two hormones, the follicle-stimulating hormone (FSH) and luteinizing hormone (LH) that releasing them into the circulation (Christensen *et al.*, 2012). These signals act on the ovaries to secreting the estrogen and progesterone hormone, as well as to regulation the menstrual cycle (Singh and Loscalzo, 2017).

In contrast, the ovary has an adverse feedback on the hypothalamic-pituitary-gonadal axis, when altering their hormone levels (estrogen and progesterone) in circulation (Bates and Bowling, 2013).

### 1.2.5.2. Histological of Ovary

The ovary is an organ that have high tissue plasticity (Spanel-Borowski, 2012). The ovary is consists of an outer region called the cortex, the next regain is the inner that called medulla which is separated from the cortex Figure (1-8) , both of regions (cortex and medulla) are composed of connective tissue which is called ovarian stroma (Van Blerkom and Motta, 2012). The surface of the ovaries are covered with a germinal epithelium, which is a simple cubic epithelium that rests on the basal lamina, below the germinal epithelium directly there is a thin layer of dense connective tissue which is called the tunica albuginea (Krstic, 2013). Generally, the cortex contains many ovarian follicles, and each follicle contains the oocyte and surrounding by cells that called follicular cells (Szmelskyj *et al.*, 2015). The ovarian follicles pass through their growing and mature to several stages; primordial follicle, primary follicle, secondary follicle then mature to the Graafian follicle, Figure (1-9) (Williams and Erickson, 2012).

The granulosa cells and theca cells surround with the mature follicles and they have the main function by produce steroid hormones that needed for follicular development, and reproductive control (Guyton and Hall, 2006). Whereas, the medullary region contains many prominent blood vessels, lymphatic's, connective tissue stroma and accumulation of pale-staining from polygonal interstitial cell (Spanel-Borowski, 2012).

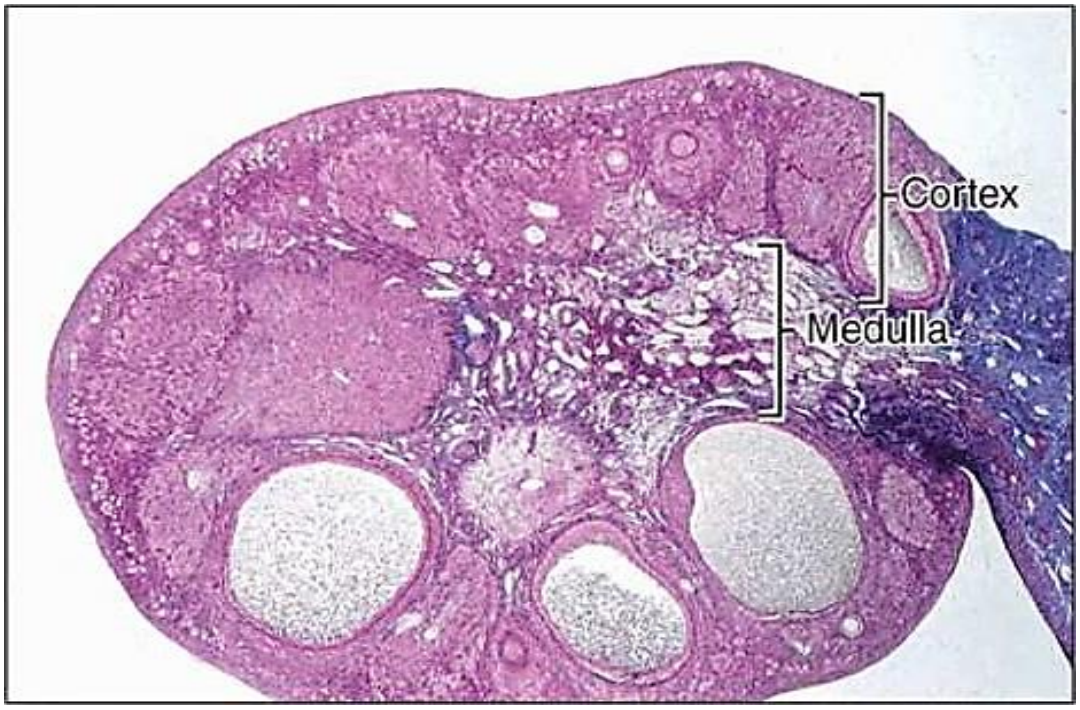



**Figure (1-8):** Sectioned of ovary, indicating the medulla and cortex, with follicles of several different sizes in the cortex (Mescher, 2018)

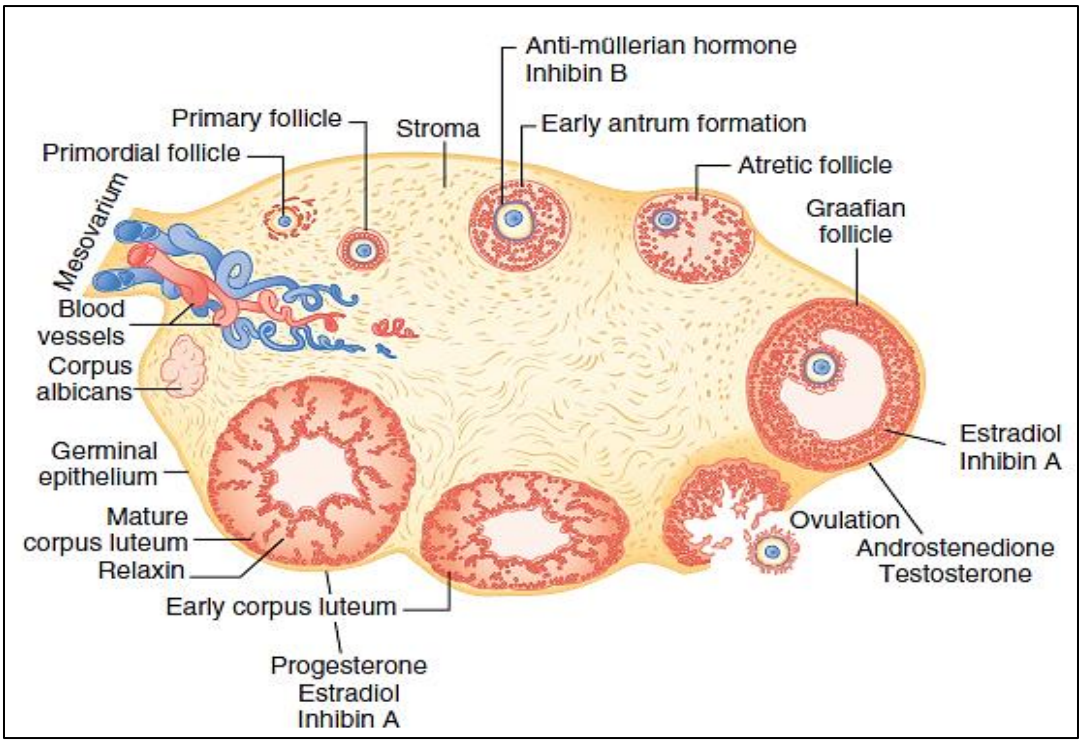



**Figure (1-9):** The follicular maturation cycle of the human ovary and main secretory products (Strauss and Williams, 2019).

### 1.2.5.3. Luteinizing (LH) and Follicle-Stimulating Hormone (FSH)

Ovarian follicle development and regulation their hormones is controlled by anterior pituitary hormones include FSH and LH hormones (Robker *et al.*, 2009). The Luteinizing hormone (LH) and follicle-stimulating hormone (FSH) are also called gonadotropins, according to their action that stimulates the gonads which include testes and ovaries (Parhar, 2002). These two hormones are secreted from cells present in the anterior pituitary gland known as gonadotrophs (Sehgal, 2004). The synthesis and secretion of gonadotropin hormones controlled by the gonadotropin-releasing hormone (GnRH) which is released by the hypothalamus gland in the brain, as well as by complex steroid hormone feedback mechanisms (Mateos *et al.*, 2002).

In mice, previous studies suggested that the gonadotropins may participate in the initiation of follicular growth at days 5 to 7 of animal life (Leung and Adashi, 2004). Both The Luteinizing hormone (LH) and follicle-stimulating hormone (FSH) are hetero-dimer glycoproteins consist of two peptide chains alpha (α) and beta (β) chain (Hua *et al.*, 2013). FSH stimulates granule cells to increase expression of the cytochrome P450 enzyme and aromatase, while LH promotes the production of androgens in the theca cells, these androgens then spread into granulosa cells where they are converted to estrogen through the activity of the aromatase enzyme (Howles, 2000). The release of the pituitary gland from LH is maintained by a complex balance of positive and negative feedback mechanisms, estrogen in high levels turn in positive feedback effect on LH, in contrast, the luteal phase of the progesterone cycle exerts a negative feedback effect on the secretion of LH, the biological activity of Luteinizing hormone (LH) is mediated by binding to a specific membrane receptors on the theca cells (Shoham, 2002).

In general, the Luteinizing hormone (LH) has three functions in the ovary; the first one is stimulating androgen synthesis during the follicular phase, second is

stimulating ovulation and the third function is the differentiation of the thecal and granulosa cell of the ruptured follicle into luteinized cells, the luteal cells constitute the steroidogenic cells of the corpus luteum and transform these cells from predominantly estrogen-producing cells, to progesterone-producing cells, these structures and their products needful to preparing the female reproductive tract to the fertilization, implantation and pregnancy (Casarini *et al.*, 2018).

### 1.2.5.4. Ovarian Hormones (Estrogen and Progesterone)

The ovary contains many steroid-producing cells, including stromal cells, theca cells, granule cells, and lutein cell, each cell contains all the enzymes are required to synthesis hormones, (Gupta and Chia, 2013). The main hormones produced and secreted by the ovaries are estrogens (E2) and progesterone (P), these hormones belong to steroids hormones and are derived from the cholesterol through a series of reactions (Jiang *et al.*, 2018). Estrogen and progesterone hormones play an essential role in the regulation of mammalian reproduction, and it's one of the primary action for these hormones to regulate development and function of the uterus (Demayo *et al*., 2002).

There is substantial biologic evidence to support that the estrogen and progesterone have another effected such as growth, differentiation, maturation, and function of various tissues throughout the body (Zlotnik *et al.*, 2011).

The major components that control the regulating of secretion of the ovarian hormones are the hypothalamus gland which is responsible for secretion gonadotropin-releasing hormone (GnRH), this hormone effected on the pituitary gland to trigger synthesis and secretion of gonadotropins hormones include luteinizing hormone (LH) and follicle-stimulating hormone (FSH), Figure (1-10) (Ferin, 2000). In contrast, the hypothalamic and pituitary glands activities are strictly controlled by adverse feedback loops of the ovarian hormone (Sherwood,

2015). The estrogen (E2) plays a key role during the follicular phase of the cycle, while the progesterone (P) contribute to control the secretion of LH and FSH (Messinis, 2006). The major physiological roles of estrogens are essential in the development of the female phenotype, maturation of germ cells and maintenance of pregnancy (Rochira *et al*., 2016). In addition to its effect on the reproductive system, as well the estrogens has many other effects on the non-reproductive system such as a metabolism, bone, skeletal system, nervous system and endothelial response (Falcone and Hurd, 2017).

At puberty, estrogen stimulates breast growth, enlargement, and maturation of the ovaries, uterus and vagina (Huether and McCance, 2015). Whereas, in the adult female it plays a vital role in maintaining the menstrual cycle (Hurd and Falcone, 2007).

On the other hand, the progesterone hormone has major physiological roles in the uterus and ovary of mammals, represented by release of mature oocytes, Facilitate of implantation, in addition to the maintenance of pregnancy by promoting uterine growth and suppressing of the myometrium contractility (Graham and Clarke, 1997). Progesterone also plays an essential role in many other tissues out of the reproductive system, including the mammary gland to prepare it for breastfeeding, cardiovascular system, nervous system, and bones (Taraborrelli, 2015).

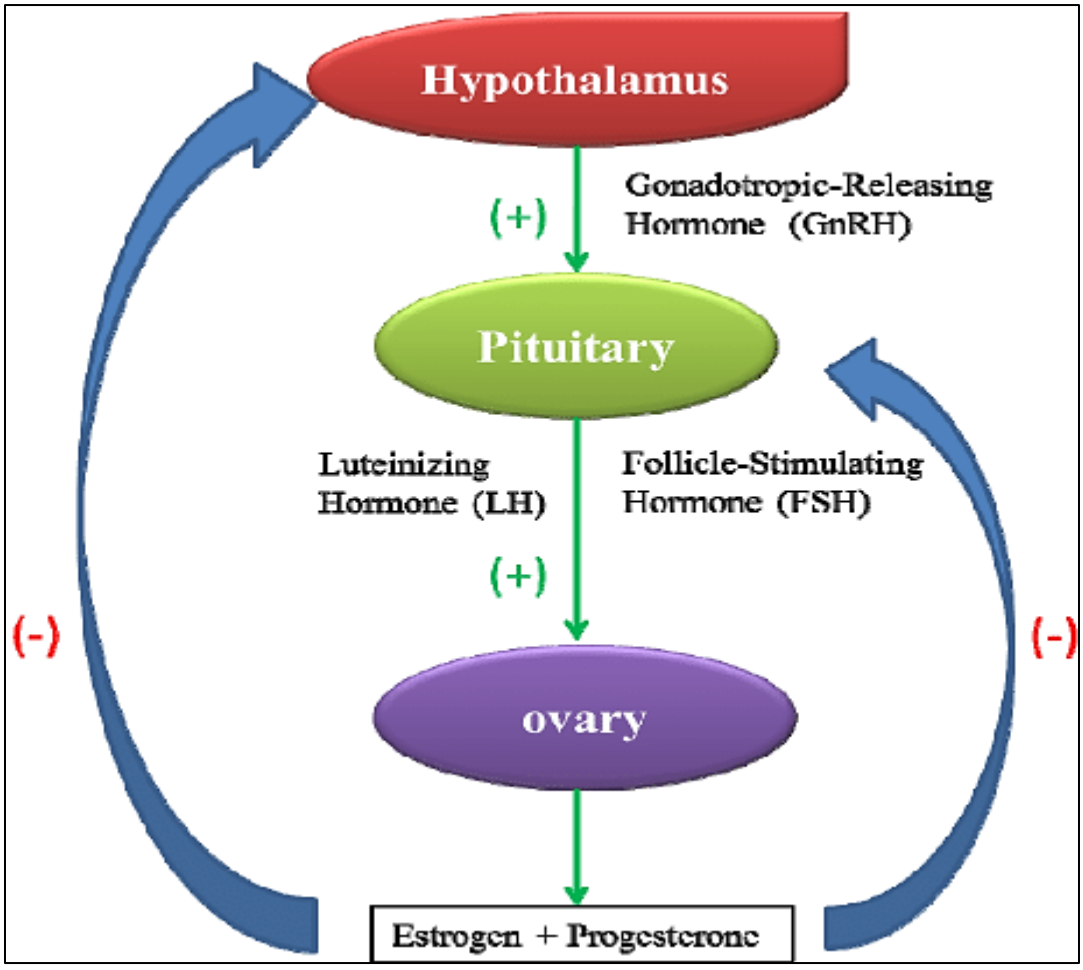



**Figure (1-10):** Regulation of ovarian hormone secretion through the mechanism of hypothalamic-pituitary-ovarian axis of the female reproductive (Sun *et al.*, 2013).

## 1.2.5.5. Thyroid Hormones Receptors of Ovary (TRs)

The thyroid hormone physiologically effects on most of the organ system in the body including the ovary, Figure (1-11) (Shahid *et al.*, 2020). Thus, the mediation action between the body organisms and the thyroid gland through binding to specific receptor is called thyroid hormone receptor (THR) (Huang *et al.*, 2008). So this receptor is widely expressed on every organ and tissue have shared the action with thyroid hormone (Kanaho *et al.*, 2006).

Human ovarian epithelium expressed on their surface multiple nuclear receptor genes, which are important in regulation the signaling that reception and synthesis of steroid hormones (Rae *et al.*, 2004). Among the proteins that the expression has been presented to be influenced by the ovarian function and their steroid hormones are receptors for the thyroid hormone (TRs), (Aghajanova *et al.*, 2011). This receptor (TRs) exists in granulosa cells, primordial and primary and secondary follicles (Stavreus-Evers, 2012). Thyroid hormone receptors (TR) have two subunits including TRα and TRβ, and these genes generate four thyroid hormone-binding receptors through alternative splicing, namely TRα1, TRα2, TRβ1, and TRβ2 (Keijzer *et al.*, 2007).

Thyroid hormone receptor beta (TR-β) is a member of the nuclear receptor superfamily that mediate the action of thyroid hormone signals in various tissues to regulate essential physiological and developmental processes (Anyetei-Anum *et al*., 2018). It is encoded by the THR- gene that located on the chromosomes 3 of human (Gonçalves *et al*., 2015). Whereas, it is located on chromosome 15 of Rat (Master and Nauman, 2014). This receptor (TR-β) is considered as a mediator between the thyroid gland and the ovary, and therefore it is binding to the thyroid hormones especially T3 hormone to regulate their activity on the ovary (Kowalik *et al.*, 2010). Thus, thyroid gland dysfunction in hyperthyroidism or hypothyroidism cause an adverse effect on the reproductive female health, by occurring changes in the ovarian structure, as well as abnormalities in synthesis and production of sexual hormones (Abd-El Fattah and El-Deeb, 2011).

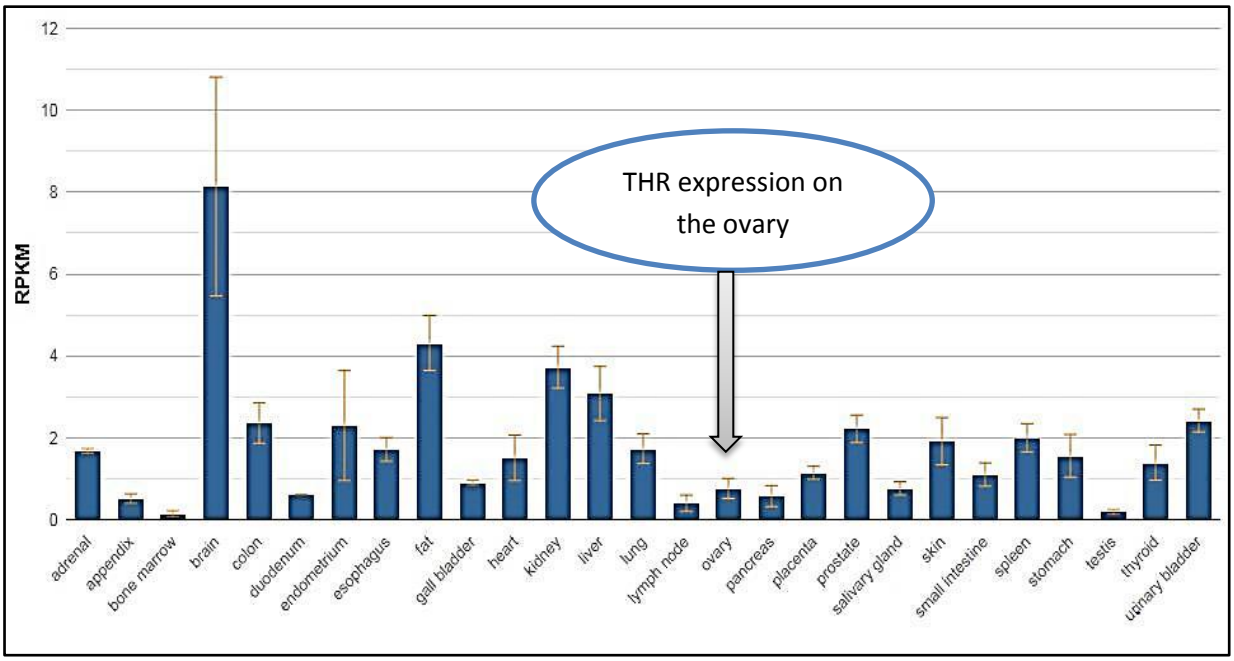



**Figure (1-11):** Expression of THR in some organs of human body include the ovary in (NCBI, 2018).

## 1.2.6. Thyroid Dysfunction and Female Reproduction

Thyroid hormones act on almost every cell in the body, whereas the thyroid gland continuously interacts with the ovaries, and their hormones are participate in almost all reproductive stages (Cho, 2015). Plasma levels of the thyroid hormone (THs) in women and animals have been known to influence molecular mechanisms including menstrual, estrous cycle control, sexual maturation, and behavior, maternal ability, ovulation, pregnancy maintenance, postnatal and fetal growth (Silva *et al.*, 2018).

Therefore, any defect in the thyroid hormones lead to adverse effects on the reproductive system (Alahmar *et al.*, 2019). Thyroid hormone disorder is mainly divided into type, hyperthyroidism, and hypothyroidism and in these two type there is an adverse impact on the female reproductive system health (Jefferys *et al.*, 2015). In the same path, Abdelsamad *et al.*, (2018) observed both hypothyroidism

and hyperthyroidism have been associated with ovulatory dysfunction and menstrual disturbances. Thyroid hormone dysfunction can impact on oocyte maturation and ovulation by altering prolactin levels, and may altering in GnRH secretion which leading to delayed LH response and insufficient corpus luteum function (Jefferys *et al*., 2015).

As well Akram, (2019) reported in both increase or decrease the thyroid hormone have adversely effected on the ovary lead to disturbances or irregularities of the menstrual cycle such as menorrhagia, amenorrhea, oligomenorrhea, anovulation, and reduced fertility. Others observed that the increased in thyroid hormone reduced the expression of gonads aromatase in addition to all three types of estrogen receptors (Sharma, 2012).

# Chapter Two

# Materials and Methods

# Chapter two

## 2. Materials and Methods

## 2.1. Materials

### 2.1.1. Apparatus

Apparatus used in this study are listed in the table (2-1) with their manufacturer and origin.

**Table (2-1) :** Apparatus utilized in the present study

| Apparatus | Company | Origin |
|---|---|---|
| Centrifuge | Universal hittich | Germany |
| Cobase e411 analyser | Roche | Germany |
| Electric Oven | Memmert | Germany |
| Electronic Digital Balance (gm) | OHAUS | Switzerland |
| Light Microscope with Camera | Micros | Austria |
| Mic qPCR Cycler | Bio Molecular System | Australia |
| Micro spin Centrifuge | My Fugene | China |
| Quantus Florometer | Promega | USA |
| Refrigerator | Concord | Lebanon |
| Rotary microtome | Kedee | China |
| Sensitive Balance (four digit) | Sartorius | Germany |
| Vortex | Griffin | UK |
| Water Bath | Tafesa | Germany |

### 2.1.2. Tools

Tools used in this study are listed in the table (2-2) with their manufacturer and origin.

**Table (2-2) :** Tools used in the present study

| Tools | Company | Origin |
|---|---|---|
| 1.5ml, 0.5ml and 0.2ml Tube | JET BIOFIL | Singapore |
| Disposable tips | Esplf | Germany |
| Dissecting set | MMK | China |
| Eppendorf tubes | Esplf | Germany |
| Filter papers | Zelpa | Belgium |
| Gel tubes | Esplf | Germany |
| Gloves | Broche | Malaysia |
| Mic Tube | Bio Molecular System | Australia |
| Micropipette | Human | Germany |
| Microscope slides | Sail brand | China |
| Pipette | Kamble | USA |
| Plane tube | Afco | Jordan |
| Slide covers | Marienfeld | Germany |

### 2.1.3. Chemicals

Chemical materials and solutions used in this study are listed in the table (2-3) with their manufacturer and origin.

**Table (2-3) :** Chemicals used in the present study

| Chemicals | Manufacturer | Origin |
|---|---|---|
| 70% Ethanol | ROMIL pure chemistry | UK |
| Absolute Ethanol Alcohol | Scharlau | Spain |
| Canada Balsam | Afco | Jordan |
| Chloroform | LiChrosolv | Germany |
| Diethyl Ether | United Horizon | UK |
| Eosin Stain | Syrbio | Syria |
| Formaldehyde 40% | Edutek | India |
| Glycerin | Media | Iraq |
| Hematoxylin Stain | Syrbio | Syria |
| Isopropanol | ROMIL pure chemistry | UK |
| MgCL2 | Promega | USA |
| Normal Saline | Pioneer | IRAQ |
| Nuclease Free Water | Promega | USA |
| Paraffin Wax | Koltek | Italy |
| TRIzol Reagent | Thermo Scientific | USA |
| Xylene | Scharlau | Spain |
| Zinc Oxide Nanoparticles | SkySpring Nanomaterials | USA |

### 2.1.4. Kits

Kits used in this study are listed in the table (2-4) with their manufacturer and origin.

**Table (2-4) :** Kits used in the present study

| Kits | Company | Origin |
|---|---|---|
| Estrogen (E2) Test | Cobas | JAPAN |
| FSH Test | Cobas | JAPAN |
| GoTaq® 1-Step RT-qPCR System | Promega | USA |
| LH Test | Cobas | JAPAN |
| Primers | Macrogen | Korea |
| Progesterone Test | Cobas | JAPAN |
| Quantiflor RNA System | Promega | USA |
| T3 Test | Cobas | JAPAN |
| T4 Test | Cobas | JAPAN |
| TSH Test | Cobas | JAPAN |

## 2.2. Methods

### 2.2.1. Preparation of Solutions

#### 2.2.1.1. Preparation of ZnO-NP Suspension

The ZnO NPs which is used in this study was obtained from skyspring nanomaterials, these particles have a white appearance, purity is 99.8% and their sizes range from 10-30 nm.

The preparation of an injected suspension of different concentrations from ZnO NPs that were used in this study prepared by dissolving the powder of ZnO nanoparticles in distilled water, after that mixed by vortex for 10 minutes. Two concentrations of ZnO NPs solution were prepared as follows:- Concerning the low dose (50 mg/kg) was prepared by dissolving (0.5 g) of ZnO NPs in 10 ml of distilled water. Whereas, the high dose (200 mg/kg) was prepared by dissolving (2 g) of ZnO NPs in 10 ml of distilled water.

#### 2.2.1.2. Preparation of 10% Formalin (Suvarna *et al.*, 2019)

- Formalin (40% formaldehyde solution) . . . . . 100 ml
- Tap water . . . . . 900 ml

The solution was prepared by mixing these contents together.

#### 2.2.1.3. Preparation of Mayer's Egg Albumin (Suvarna *et al.*, 2019)

- White albumin of egg . . . . . 50 ml.
- Glycerin . . . . . 50 ml.

The solution was prepared by mixing these contents together.

### 2.2.1.4. Preparation of Ehrlich's Haematoxylin Stain (Suvarna *et al.*, 2019)

- Hematoxylin . . . . . 2 g
- Glycerin . . . . . .100 ml
- Glacial acetic acid . . . . . 10 ml
- Potassium alum . . . . . .10 g
- Absolute ethanol . . . . . 100 ml
- Distilled water . . . . . 100 ml

The solution was prepared by mixing these contents together.

### 2.2.1.5. Preparation of Eosin Stain (Suvarna *et al.*, 2019)

- Distilled water . . . . . 100 ml
- Eosin . . . . . 1 g

The solution was prepared by mixing these contents together.

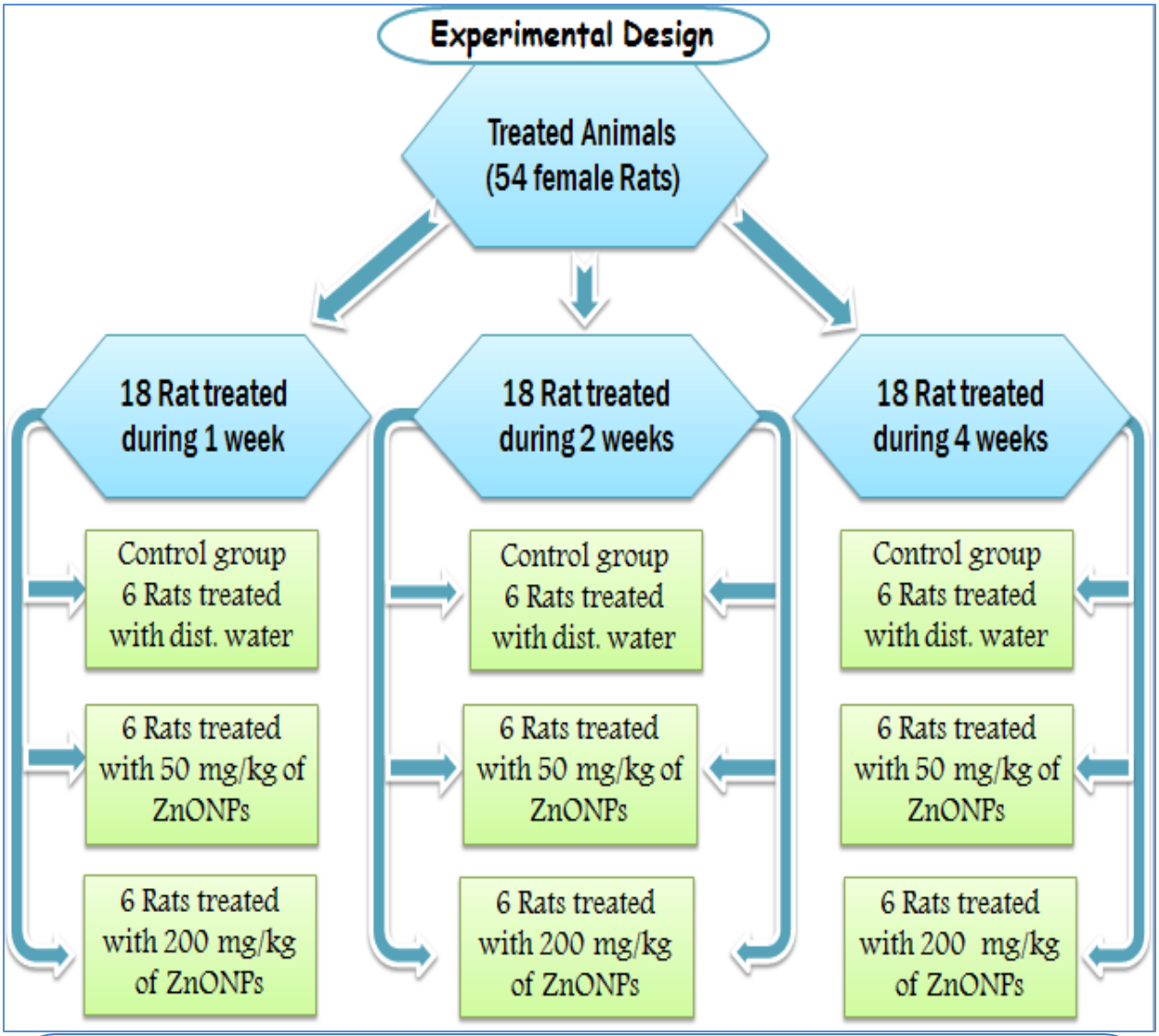
### 2.2.2. Animal Care

Fifty-four adult female Sprague-Dawley albino rats weighing 220-240 gm at age of 8-10 weeks were purchased from the National Center for Drug Control and Research (NCDCR), Iraqi ministry of health. These animals were transferred to animal house laboratory in the College of Science, Mustansyriah University. They were kept for 10 days for adaptation before starting the treatment under the controlled temperature conditions (25 °C). Animals were provided with rat pellets and tap water for feeding and drinking.

### 2.2.3. Experimental Design

A total of 54 healthy adult female rats were assigned randomly to three main groups according to the periods of exposure, which they were (1, 2 and 4) weeks. Then, each group was subdivided into three subgroups and every one has same

number of animals (6 Rats), one of them used as control group and the others used as treated groups. The treated groups injected with two different doses of ZnO NPs (50 and 200) mg/kg, while, the control group injected with distilled water, at a rate of three doses per week via intraperitoneal route, as showing in the below diagram.




## Parameters carried in this study:

- **Weights of experimental animals and specific organs (thyroid and ovaries).**
- **Hormonal study for TSH, T3, T4, LH, FSH, E2 and progesterone.**
- **Histological study for thyroid glands and ovaries .**
- **Molecular study for gene expression of thyroid stimulate hormone receptor (TSHR) and thyroid receptor on ovary (THRβ).**

### 2.2.4. Collection of Blood Samples and Tissue Specimen

After the experiment has been ended, the rats are weighed and then they were completely 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 for the hormonal study, then the serum was separated into several equal parts in Eppendorf tubes and kept at -20ºC. The rats were dissected and the ovary and thyroid glands for all the animals were excised and washed with normal saline in concentration (0.9% NaCl) to remove the blood, then blotted by filter paper weighed and some of them preserved immediately in 10% formalin solution for histological study and the others preserved in triazole preservation solution for molecular study.

### 2.2.5. Body and Organs Weights Measurements

All animals for each group were weighed before and after the exposure periods (1, 2, and 4) weeks, In addition to the weight of isolated organs (thyroid gland and ovary).

### 2.2.6. Hormonal Analysis

Reproductive hormones (LH, FSH, E2, and progesterone) and thyroid hormones (T3, T4, and TSH) were evaluated in collected serum by Cobas e411 analyzer. This device was designed and manufactured by Roche/Hitachi company (Germany origin). It is a fully automated analyzer that uses a patented ElectroChemiLuminescence (ECL) technology for immunoassay analysis, this device needs a shortest time ranging between 9-18 minutes to obtain the final result of test, in addition to, it needs small quantities of serum up to 25 µl.

### 2.2.7. Histological and Morphological Study

The Preparation for histological sections was performed according to the method of paraffin sections technique (Suvarna *et al*., 2019). After fixation of isolated organs specimens (thyroid gland and ovary) in 10% formalin solution, they were dehydrated by exposing them gradually to different concentrations of ethanol (50 %, 70%, 80 %, 90 %) for one hour in each concentration respectively, then the specimens left in absolute ethanol (100 %) for an overnight. Clearing of specimens were performed by using two changes of xylene for 30 minutes per each change. This was followed by embedding the specimens in a mold containing pure melted paraffin wax and heated in an electric oven to 60-65 ºC, then mold paraffin left to solidify and after that kept in the refrigerator at 4-8 ºC. The paraffin blocks were sectioned at 4-5µm in thickness by a rotary microtome. The tissue sections were floated in the water bath then placed on glass slides and afterwards they were stained with hematoxylin and eosin stain, then mounted with Canada balsam and left at room temperature to dry and finally examined under a light microscope.

### 2.2.8. Molecular Study

#### 2.2.8.1. Primers Design

The cDNA sequences of (THRβ, TSHR and YWHAZ) genes were obtained from the NCBI GenBank database. RT-qPCR primers were designed using Primer Premier 3 plus software with melting temperature between $60^0$C, primer length between 21 to 22 nucleotides, PCR amplicon length of THRβ is 103 and TSHR is108 base pairs.

**Table (2-5):** Primers with their sequences and annealing temperature

| Primer Name | Sequence | Annealing Temp.(C) |
|---|---|---|
| THRβ- Forward | 5`-GACTGGAAGCTGGTAGGAATG-3` | 60 |
| THRβ-Reveres | 5`-GGATGAGGTGTGAGGATGTTT-3` | |
| TSHR- Forward | 5`-CAGCACCCAGACTCTCTATCTA-3` | |
| TSHR- Reveres | 5`-GGACATCTGAGAACCAGGAATC-3` | |
| YWHAZ- Forward | 5`-GAT GAA GCC ATT GCT GAA CTT G-3` | |
| YWHAZ- Reveres | 5`-GTC TCC TTG GGT ATC CGA TGT C-3` | |

### 2.2.8.2. Primers 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/µl as a stock solution. A working solution of these primers was prepared by adding 10µl of primer stock solution (stored at freezer -20 C) to 90µl of nuclease-free water to obtain a working primer solution of 10pmol/µl.

**Table (2-6):** Concentrations of Primers

| Primer Name | Vol. of nuclease free water (µl) | Concentration (pmol/µl) |
|---|---|---|
| THRβ-Forward | 300 | 100 |
| THRβ-Reveres | 300 | 100 |
| TSHR-Forward | 300 | 100 |
| TSHR-Reveres | 300 | 100 |
| YWHAZ-Forward | 300 | 100 |
| YWHAZ-Reveres | 300 | 100 |

### 2.2.8.3. RNA Isolation and Purification

After the animals were sacrificed, Fifty-four tissue samples of the ovary and thyroid gland were taken from twenty-seven animals in equal numbers, and these samples were kept in Eppendorf tubes containing 1 mL of triazole preservation solution and stored them at (- 4C ) for 24-48 hours, the RNA was isolated from sample according to the protocol of TRIzol™ Reagent (Rio *et al*., 2010), as the following steps:-

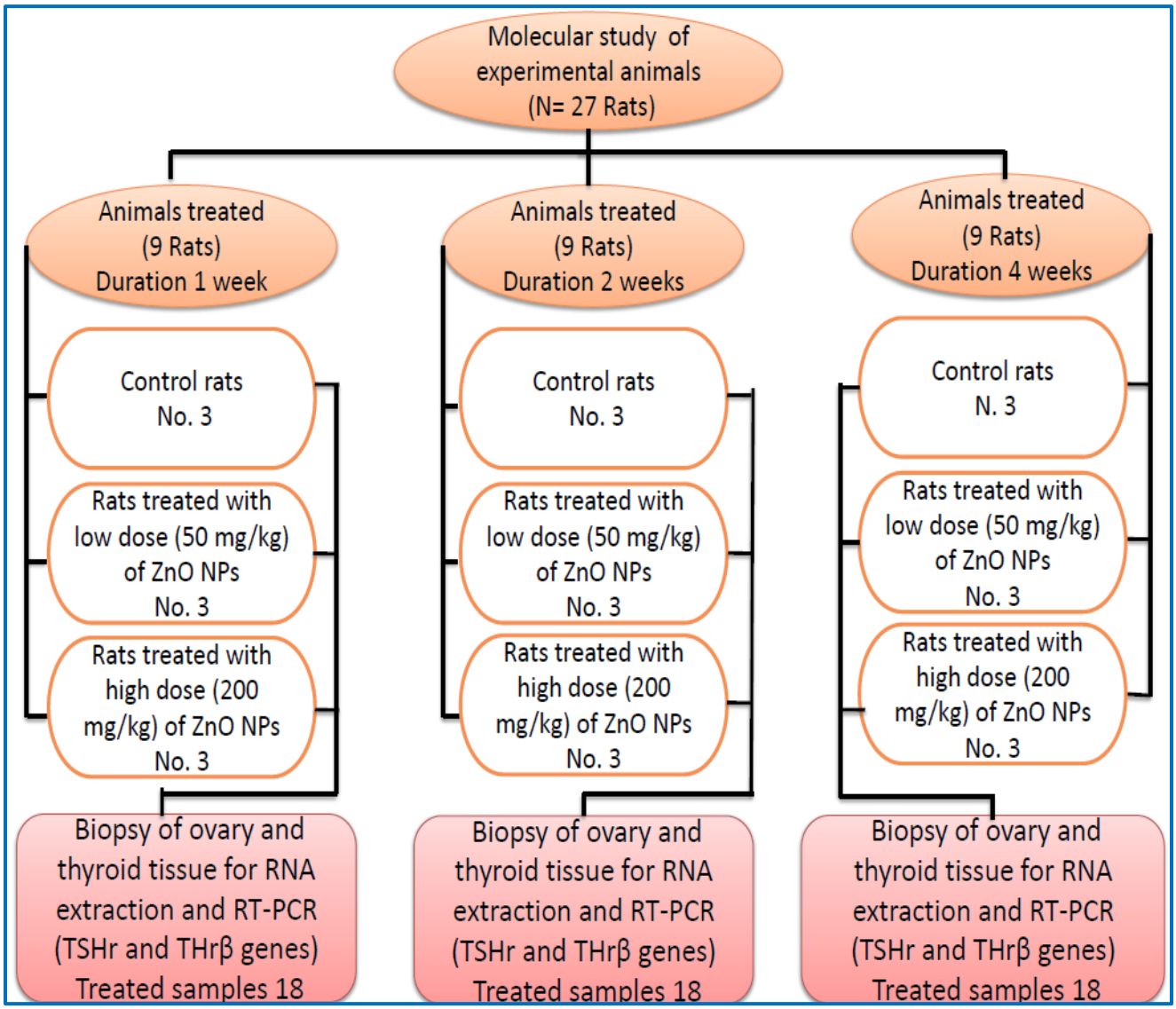



Diagram explains rats' total collection and their RNA extraction from biopsies of the ovary and thyroid tissue for RT-PCR Technique (TSHR and THR-β genes).

## 1. Tissue Lysis

One mL of TRIzol™ Reagent was added to each tube that contains an isolated organ, whether thyroid gland or ovary, and gently mixed with a vortex for 3-4 seconds.

## 2. Phase Separation

For each tube, 0.2 mL of chloroform was added to the lysate and then the tube cap secured, the samples were vortexed for 3-4 seconds, incubated for 2 to 3 minutes at room temperature, then the samples were centrifuged for 10 minutes at 12,000 rpm. The mixture separated into three phases; a lower organic phase, interphase and a colorless upper aqueous phase, the aqueous phase containing the RNA that has been transferred to a new tube.

## 3. RNA Precipitation

The precipitation of the RNA from the aqueous phase has been done by adding 0.5 mL of isopropanol and after that incubated the samples at 15 $^{0}$C for 10 minutes, then centrifuged for 10 minutes at 12,000 rpm. Often, RNA is invisible before the centrifugation but after precipitation, it formed a white gel-like pellet at the bottom of the tube.

## 4. RNA Washing

The RNA pellet was washed once by added 0.5mL of 70% ethanol for each tube, then the samples was mixed by vortex briefly and after that centrifuged for 5 minutes at 10000 rpm. Thereafter, the tubes left open at room temperature to ensure the evaporation of the ethanol.

## 5. Solubilize the RNA

Finally, The pellet was rehydrated by adding 100 µl of Nuclease Free Water, then the samples left overnight at 4 $^{0}$C.

### 2.2.8.4. Determine RNA Yield

Fluorescence method was applied to detect the concentration of extracted RNA by using the Quantus Fluorometer in order to detect the goodness of samples for downstream applications, 1 µl of RNA was mixed with 199 µl of diluted quantiFlour Dye. After 5 minutes of incubation at room temperature in a dark place, RNA concentration values were detected.

### 2.2.8.5. PCR Master Mixed

The PCR master mix is a premixed concentrated solution that was prepared from the components summarized in the table (2-7) these components needful for a real-time PCR reaction.

**Table (2-7):** PCR component and master mixed volume

| Master mix components | Stock Con. | Final Con. | Volume (1 sample) |
|---|---|---|---|
| qPCR Master Mix | 2 X | 1 X | 5 µl |
| RT mix | 50 X | 1 X | 0.25 µl |
| MgCl2 | - | - | 0.25 µl |
| Forward primer | 10 µM | 1 µM | 0. 5 µl |
| Reverse primer | 10 µM | 1 µM | 0. 5 µl |
| Nuclease Free Water | - | - | 2. 5 µl |
| RNA | - | - | 1ng/µl |
| **Total Volume** | | | 10 µl |
| Aliquot per single rxn: 9µl of Master mix per tube and add 1µl of Template | | | |

### 2.2.8.6. Reaction Conditions of qRT-PCR Steps

There are several steps that make up each cycle in a real-time PCR reaction, two of these reactions are generally run for 1 cycle and the others run for 40 cycles as shown in Table (2-8).

**Table (2-8):** Reaction conditions of qRT-PCR steps

| Steps | $^0$C | m:s | Cycle |
|---|---|---|---|
| RT. Enzyme Activation | 37 | 15:00 | 1 |
| Initial Denaturation | 95 | 10:00 | |
| Denaturation | 95 | 00:20 | 40 |
| Annealing | 60 | 00:20 | |
| Extension | 72 | 00:20 | |
| Melt on Green | Melt from 72$^0$C to 95$^0$C at 0.3 $^0$C/s | | |

### 2.2.8.7. 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, Relative quantification is based on the expression levels of a target gene versus a reference gene. To calculate the expression of a target gene in relation to an adequate reference gene various mathematical models are established, one of these method by determination the cycle of threshold values (Ct) at a constant level of fluorescence (Pfaffl, 2004), as shown by the following equations:

$$\Delta CT = CT_{\text{gene}} - CT_{\text{reference gene}}$$

$$\Delta\Delta CT = \Delta CT_{\text{Treated}} - \Delta CT_{\text{Control}}$$

$$\text{Folding} = 2^{\wedge -\Delta\Delta CT}$$

### 2.2.9. Statistical Analysis

The Statistical Analysis System- SAS; (2012) program was used to find out the effect of different factors on all parameters used in this study. as well the least significant difference –LSD test (Analysis of Variation-ANOVA) was applied to significantly compare between the means.

# Chapter Three

# Results and Discussion

# Chapter Three

## 3. Results and Discussion

### 3.1. Body Weight of Animals

The results of statistical analysis in this study presented in Figure (3-1), these data showed that there is a gradual decrease in the body weight of animals that were exposed to ZnO NPs compared to control groups. Animals that were exposed to two different concentrations of ZnO NPs (50 and 200) mg/kg during 1 week demonstrated a highly significant decrease ($P \leq 0.01$) in average body weight (221.50±1.43) and (217.66±1.93) gm respectively when compared with the control groups (241.83 ±2.13) gm. Also, there was a highly significant decrease ($P \leq 0.01$) in average body weight for animals that were exposed to the two different concentrations of ZnO NPs for 2 weeks (201.50±1.69) and (181.33±1.73) gm respectively when compared with the control groups (247.17±2.74) gm. Regarding the period of 4 weeks, as well there was a highly significant decrease ($P \leq 0.01$) in average body weight for animals that were exposed to these two different concentrations of ZnO NPs (175.33±1.68) and (162.17±1.83) gm respectively when compared with the control groups (253.33±2.89) gm.

According to the above-obtained results, the bodyweight loss increases depending on the concentration of the injected material and the exposure time, whenever the increase of the concentration of ZnO NPs through prolonged periods leads to more reduction in body weight. The results of the current study were identical with the previous study conducted by Radhi and Al-Bairuty, (2019) which showed a significant decrease in the body weight of mice when treated orally with ZnO NPs by two different doses (100 and 200) mg/kg after 7 and 14 days, which is supporting the current study.

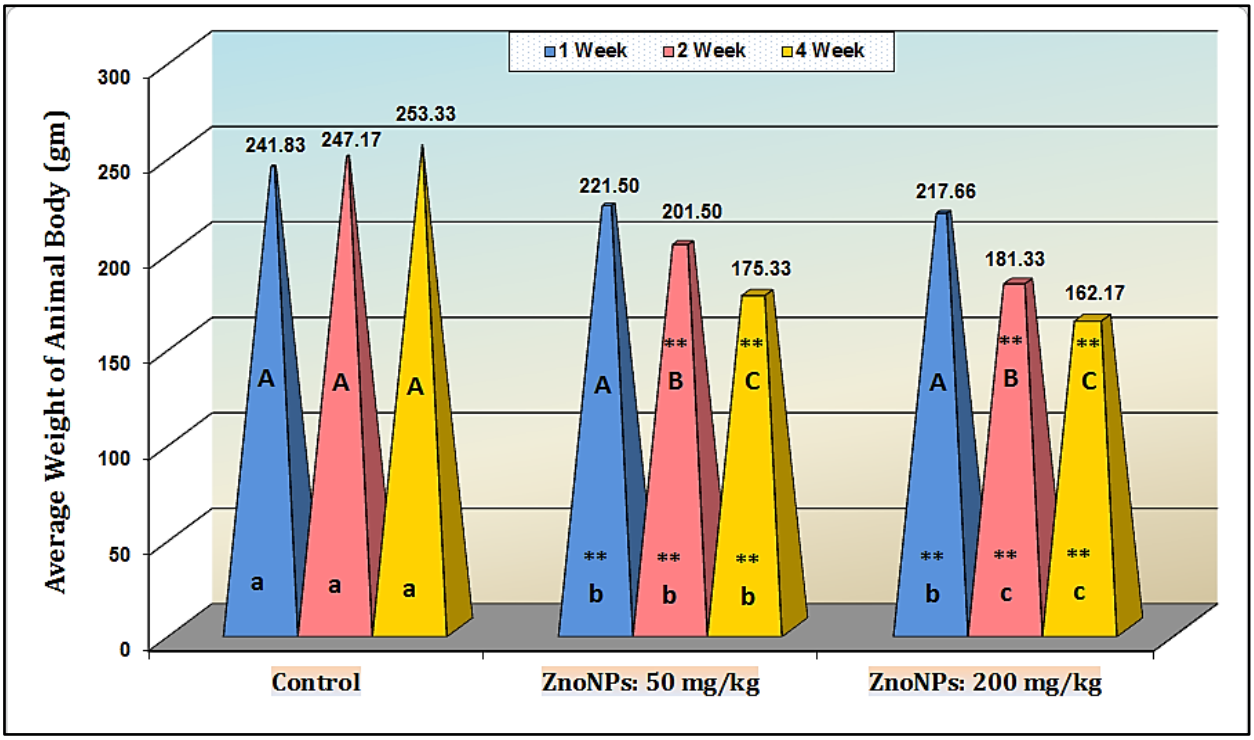



**Figure (3-1):** Effect of ZnO NPs in different concentrations (50 and 200 mg/kg) during different exposure periods on body weight of Rats.

- (**) Mean high significant variation ($p \leq 0.01$).
- **(A, B, C)** Represent the significant difference between groups when time is variable factor and concentration as a fixed factor.
- **(a, b, c)** Represent the significant difference between groups when time is a fixed factor and concentration as a variable factor.

Furthermore, when Wang *et al.*, (2016) added different concentrations of ZnO NPs (0, 50, 500 and 5000) mg/kg into the basal diet, the results showed significant decrease in the body weight of male mice at dose 5000 mg/kg of ZnO NPs within 4 weeks, while at the (50 and 500) mg/kg demonstrated significant increase in body weight when compared with control group. In the same direction Srivastav *et al.*, (2016) detected the acute oral toxicity of ZnO NPS in female Wistar rats at two doses (300 and 2000) mg/kg, where the obtained results showed significant decrease in the body weight of rats that treated with high dose (2000) mg/kg of ZnO NPS after 7 and 15 days in comparison with the control group.

A study presented by Ko *et al*., (2015) showed a significant decrease in the weight of rats after treatment with zinc oxide nanoparticles orally at doses (1000 and 2000) mg/kg for 2 weeks. As well as in previous studies which support the present study reported when exposed the male mice to three different doses (25, 50 and 100 mg/kg) of ZnO NPs by intraperitoneally injected for 2 and 4 weeks, the results were highly significant decrease in body weight in all treated groups over injection time comparing with the control group (Razooki and Rabee, 2020). Also, Hong *et al*., (2014) have found out that the animals that treated through gavage with high dose (400 mg/kg) of ZnO NPs had significantly decreased in body weight of animals when compared with the control group. Chen and his group in (2020), observed that the ZnO NPs administration orally to pregnant mice with different doses (20, 60, 180 and 540) mg/kg demonstrated a significant reduction in the bodyweight of those exposed to (180 and 540) mg/kg of ZnO NPs, whereas none observed any changes in body weight of animals that exposed to (20 and 60) mg/kg of ZnO NPs.

The body weight clearly is a sensitive indicator affected by toxic chemicals (Asadi *et al*., 2019). A previous study suggested that oral exposure to nanoparticles caused digestive disturbances, especially effective on the mucous membrane which leads to appetite loss (anorexia), consider one of the reasons for the high toxicity that eventually decreases the body weight of animals (Zhang *et al*., 2010).

Furthermore, thyroid hormones play essential roles in controlling energy homeostasis and can effect on the growth and composition of the body (Kwon *et al*., 2018). So, thyroid dysfunction is responsible for changes in body weight and composition (Biondi, 2010). Thyroid hormones act as a regulator of multiple processes in the body, stimulating several activities such as metabolism changes (carbohydrate, lipids, and protein), heat production, affecting cell proliferation and development, modulate the regulation of hormones, which lead to an increase or

decrease in energy expenditure, as well as the thermogenesis in adipose tissue (Radhi and Al-Bairuty, 2019). Excessive thyroid hormone levels in the blood (Hyperthyroidism), promotes hypermetabolic status to increase the resting energy expenditure, weight loss, reduced levels of cholesterol, increased lipolysis and gluconeogenesis (Mullur *et al*., 2014). For this reason, Cinar and Gurlek, (2013) suggested that the disturbances in the thyroid function lead to a change in body weight, fat tissue, and muscle mass.

## 3.2. Thyroid gland

### 3.2.1. Thyroid Gland Weights and Function

In the present study, thyroid gland weights and their hormone levels T3 and T4 in addition to TSH were evaluated and statistical analysis performed for all animals exposed to ZnO NPs. The results in figure (3-2) shows that there are changes in the weights of the thyroid gland in experimentally treated animals that were exposed to ZnO NPs when compared to control groups as the follows:

The weights of thyroid gland demonstrated high significant increase ($P \leq 0.01$) in the rats that were exposure to different doses (50 and 200) mg/kg of ZnO NPs (0.0292±0.0002) and (0.0316±0.0001) gm respectively, for 1 week when compared with the control group (0.0279 ±0.0001). Also, the rats exposed for 2 weeks with a low and high dose of ZnO NPs (50 and 200) mg/kg demonstrated a highly significant increase ($P \leq 0.01$) in the thyroid weight of animals (0.0321±0.0002) and (0.0339 ±0.0002) gm respectively, when compared with the control group (0.0278 ±0.0002) gm. As well the rats that were exposure for 4 weeks observed a highly significant increase ($P \leq 0.01$) in thyroid weight (0.0369±0.0002) and (0.0410±0.0003) gm when exposed to ZnO NPs at doses (50 and 200) mg/kg respectively, when compared with the control group (0.0277±0.0001) gm.

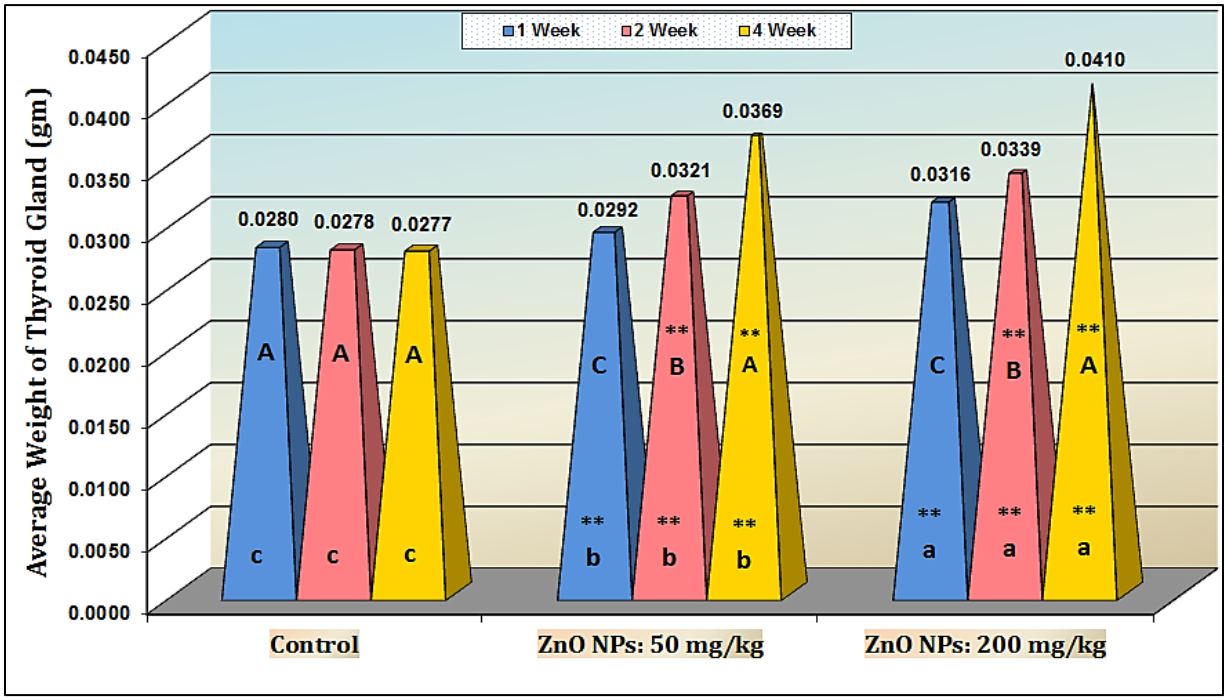



**Figure (3-2):** Effect of ZnO NPs in different concentrations (50 and 200 mg/kg) during different exposure periods on thyroid weight of Rats.

- (**) Mean high significant variation ($p \leq 0.01$).
- **(A, B, C)** Represent the significant difference between groups when time is variable factor and concentration as a fixed factor.
- **(a, b, c)** Represent the significant difference between groups when time is a fixed factor and concentration as a variable factor.

- The results in figure (3-3) showed no significant change ($p \leq 0.05$) in the TSH value (µlU/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±0.012) 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 ($p \leq 0.05$) in the TSH level (0.172±0.002) and (0.172±0.02) 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).

- The value of T3 hormone (Nmol/L) demonstrated a highly significant increase ($p \leq 0.01$) 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 ($p \leq 0.01$) 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 ($p \leq 0.01$) 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 figure (3-4).

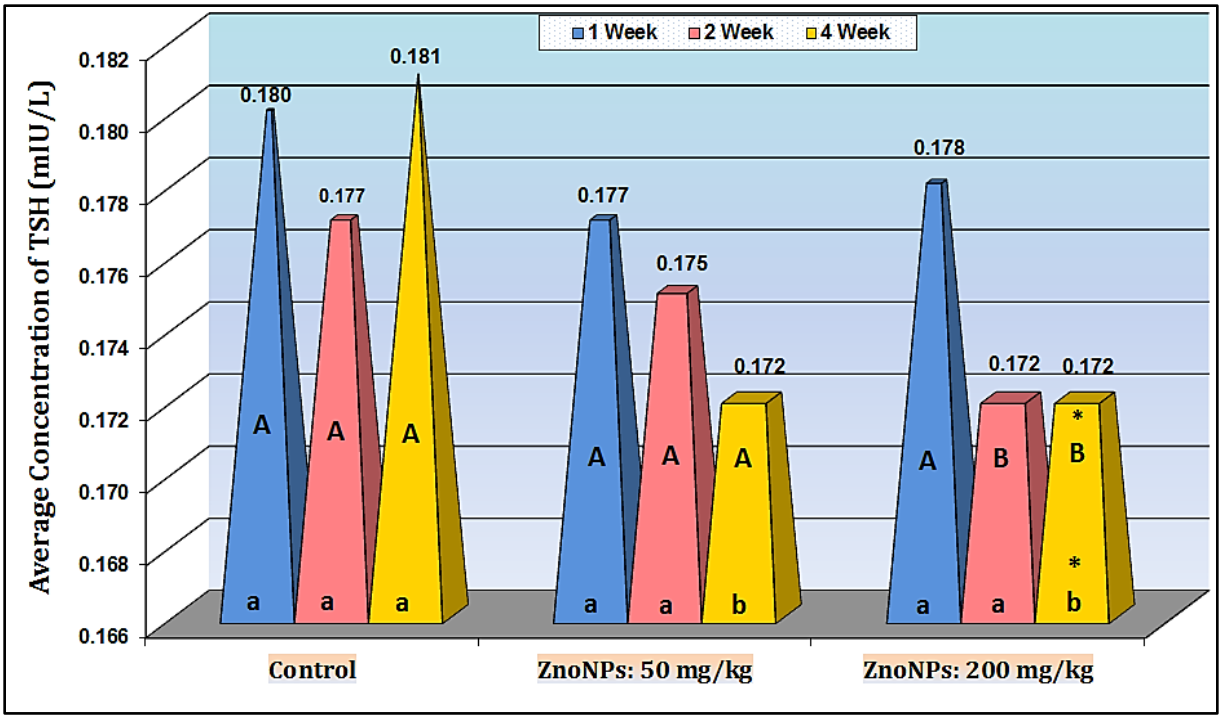



**Figure (3-3):** Effect of ZnO NPs in different concentrations (50 and 200 mg/kg) during different exposure periods on TSH level (mIU/L).

- **(*)** Mean high significant variation ($p \leq 0.05$).
- **(A, B, C)** Represent the significant difference between groups when time is variable factor and concentration as a fixed factor.
- **(a, b, c)** Represent the significant difference between groups when time is a fixed factor and concentration as a variable factor.

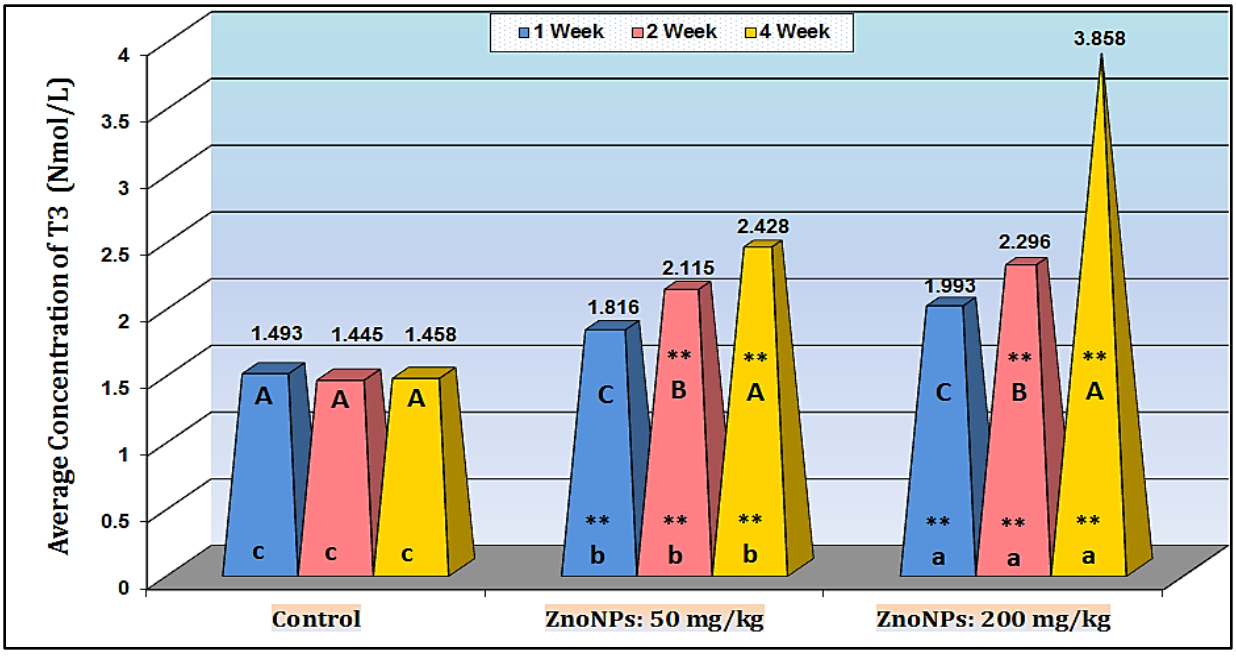



**Figure (3-4):** Effect of ZnO NPs in different concentrations (50 and 200 mg/kg) during different exposure periods on T3 level (Nmol/L).

- **(**)** Mean high significant variation ($p \leq 0.01$).
- **(A, B, C)** Represent the significant difference between groups when time is variable factor and concentration as a fixed factor.
- **(a, b, c)** Represent the significant difference between groups when time is a fixed factor and concentration as a variable factor.

- Concerning the results of the T4 hormone (Nmol/L), Figure (3-5) shows a highly significant increase ($p \leq 0.01$) for the animals that exposed to ZnO NPs at two doses (50 and 200 ) mg/kg (71.25±1.27) 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 ($p \leq 0.01$) in both treated animals with low and high doses (50 and 200) mg/kg of ZnO NPs (109.68±1.43) 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 ($p \leq 0.01$) 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).

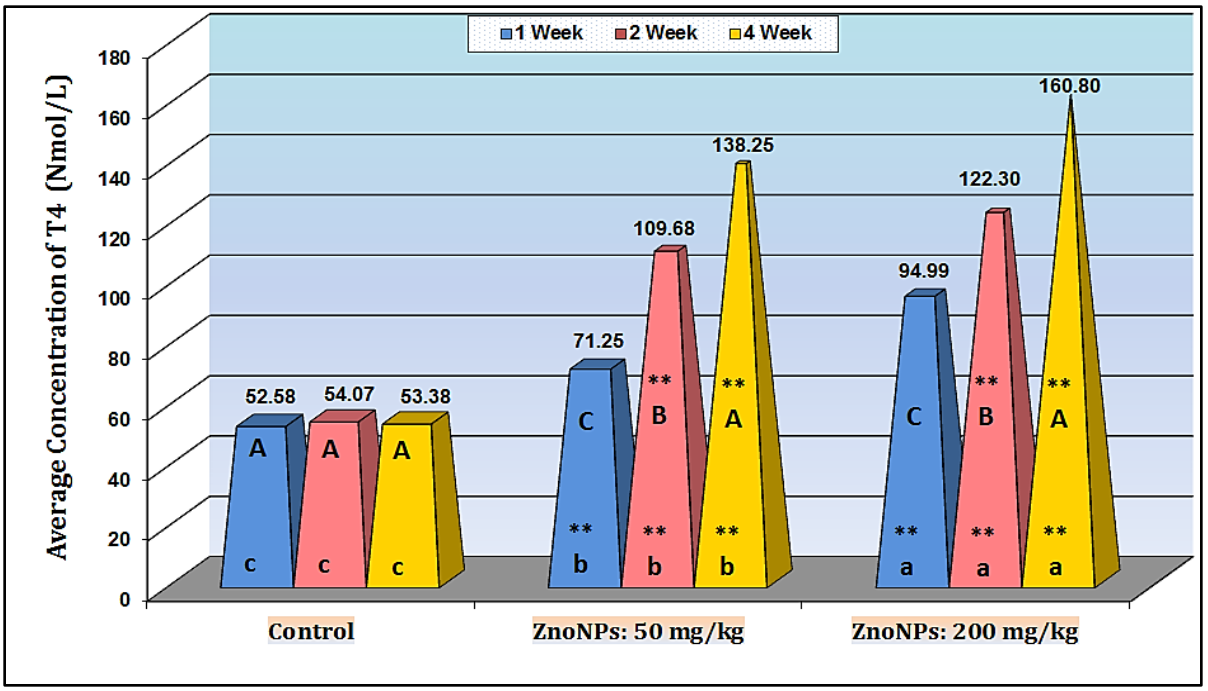



**Figure (3-5):** Effect of ZnO NPs in different concentrations (50 and 200 mg/kg) during different exposure periods on T4 level (Nmol/L).

- (**) Mean high significant variation ($p \leq 0.01$).
- **(A, B, C)** Represent the significant difference between groups when time is variable factor and concentration as a fixed factor.
- **(a, b, c)** Represent the significant difference between groups when time is a fixed factor and concentration as a variable factor.

The results of the present study display increase in the weight of thyroid gland that approach to results from a previous study that was reported by Sulaiman *et al.*, (2018) which showed a highly significant increase ($p<0.01$) in the thyroid weight in animals that exposed to Ag NPs at a dose (50 mg/kg) when compared with control groups. Increased thyroid weight associated with a progressive hypertrophy and hyperplasia of the thyrocytes (Authority *et al.*, 2019). The current study showed that the zinc oxide nanoparticles have a significant effect on the weight of the thyroid gland, therefore, these particles with an increase in their concentration and long exposure period lead to an increment in the weight of the thyroid gland.

The regulation and control of the thyroid hormone function is starting from the hypothalamus gland by secreting thyroid releasing hormone (TRH) that effects on the anterior pituitary gland to trigger secreting thyroid-stimulating hormone (TSH), in the end, this hormone is responsible for the synthesis and secretion of T3 and T4 hormones (Shahid *et al*., 2020). In turn, these hormones have negative feedback effecting on the hypothalamus-pituitary axis to regulate production of TSH hormone (Uduak *et al*., 2014).

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 *et al.*, (2019) who reported that the T3 and T4 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 contrast, 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 rabbits at dose 30 mg/kg of body weight during 2 months at the rate of one injection in a week, the results showed a significant increase in both T3 and T4 concentrations when compared to the control group (Eid *et al*., 2019).

Mahdieh *et al*, (2015) 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.

In contrast, the present study conflicted in results with a previous study which presented by Valipour and Rafieirad, (2015) that reported a significant decrease in the level of T4 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 T3 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, these data may indicate that a decrease in the secretion of thyroid hormones may be due to a reduced regulatory effect of the pituitary gland on the thyroid gland.

Shirband *et al*., (2013) reported that the nanoparticles at different doses have toxic effects on the thyroid gland and cause an imbalance in their activities. Yousef *et al.*, (2019) indicated that the increase in thyroid hormones may be due to the increase the levels of free radicals and nitric oxide and reduction in the antioxidants. 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 and Rafieirad, 2015).

In this study, the results of thyroid hormones are identical to the obtained results of measurements of body weights, increase the thyroid hormones is the main reason to decrease the body weights. This result was supported by the previous study who approved that the alteration in the levels of hormone caused adverse effects on the metabolism and development of the body (Mullur *et al*., 2014).

### 3.2.2. Histological Changes of Thyroid Gland

The microscopic examination assessment of the thyroid tissue sections for the control groups (untreated animals) showed normal structural appearance in their tissue sections by having thyroid follicles in normal size that filled with the colloid materials, these follicles lined by thyroid follicular cells (cuboidal epithelial cells), Figure (3-6).

Whereas there were different histological changes in the thyroid tissue sections in all treated groups that exposed to ZnO NPs at different period times of exposure. The section of thyroid gland tissue after 1 week treated with ZnO NPS showed follicles with colloid, in addition to the presence aggregation of follicular cells with the beginning formation of small follicles (Folliculogenesis), as shown in Figure

(3-7). While the section of the thyroid gland tissue after 2 weeks treated with ZnO NPS showed the formation of new follicles (Neo-Folliculogenesis), they were large and small follicles filled with the colloid materials, with engulfment of colloid material by the follicular cells which lead to voluminous cytoplasmic appearance, shown in Figure (3-8).

As well cross section of thyroid gland tissue after 4 weeks treated with ZnO NPS showed presence of follicles with the same size filled with colloid materials in different color due to an existent of different amount of protein material, some of these follicles are surrounded by follicular cells that became inactivate and flat in shape (Cuboidal form), while other cells remain active (synthesis and produce of hormone in high quantities) that were columnar in an appearance, Figure (3-9).

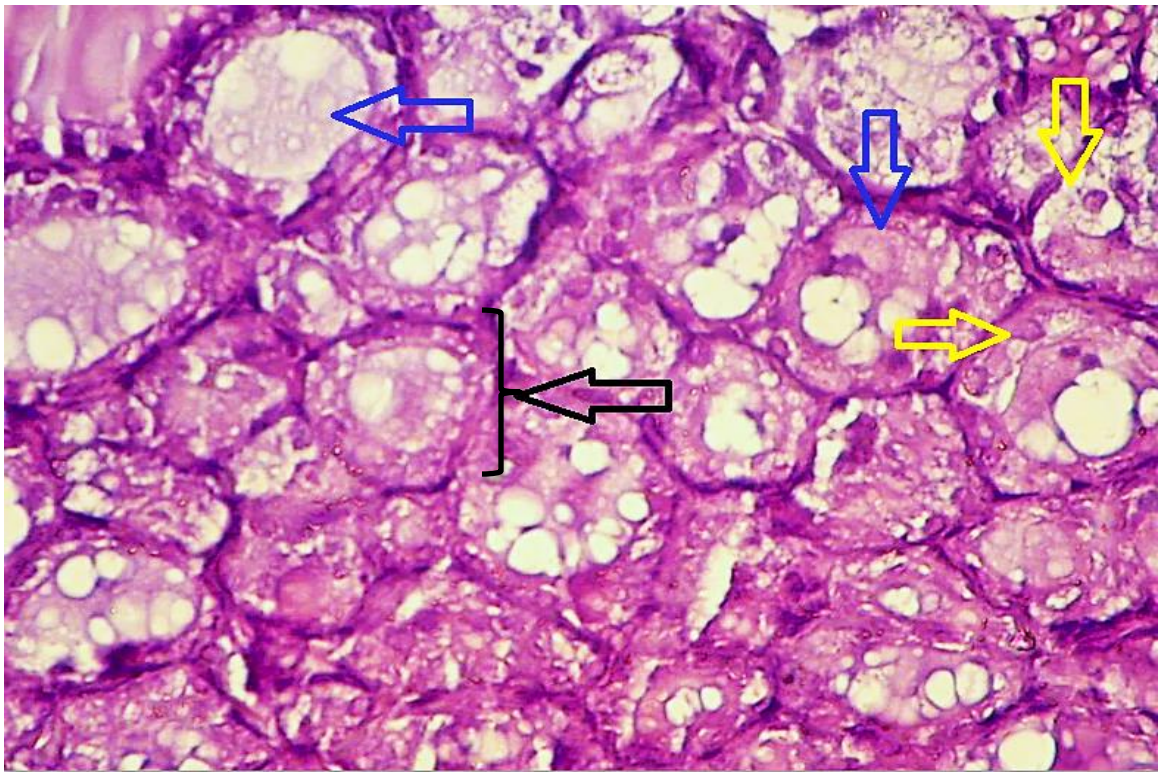


**Figure (3-6):** Section of thyroid gland of control group showing normal structural of the thyroid follicles which contain colloid materials that lined by cuboidal epithelium cells, (H&E) 10x

**Thyroid follicles** **Colloid materials** **Thyroid follicular cells**

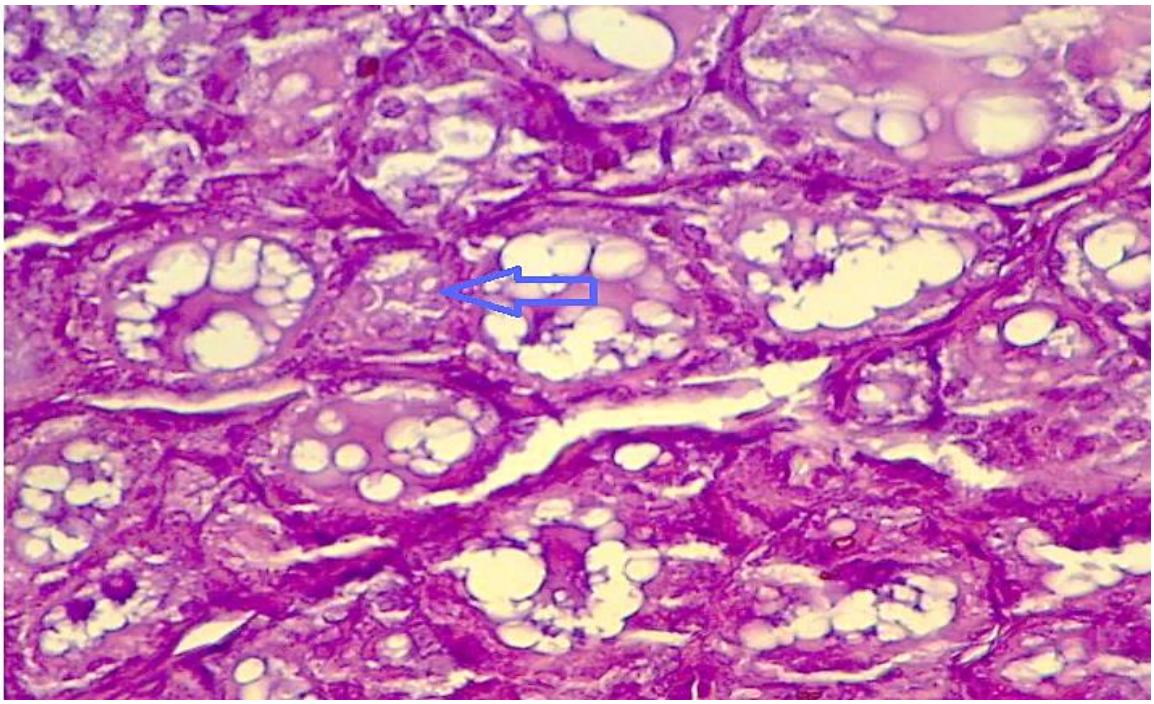


**Figure (3-7):** Section of thyroid gland of rat group treated with 200 mg/kg of ZnO NPs for 1 week, showing beginning formation of small follicles (Folliculogenesis), (H&E) 10x.

⇨ **Small follicles under formation**

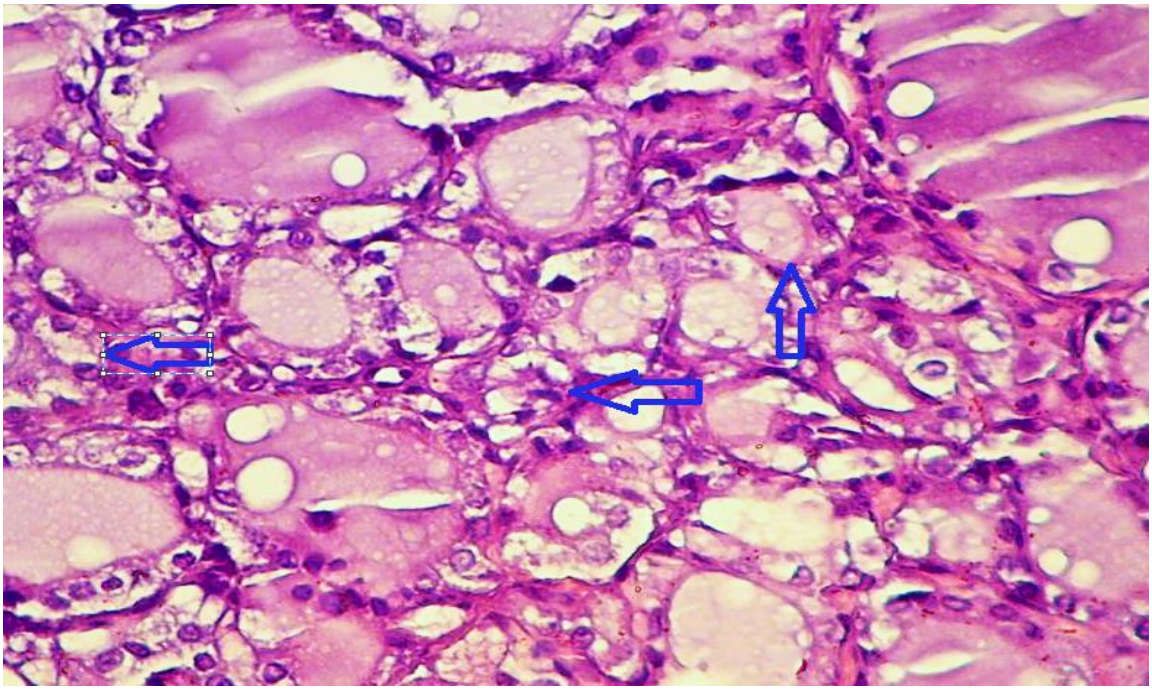


**Figure (3-8):** Section of thyroid gland of rat group treated with 200 mg/kg of ZnO NPs for 2 weeks, showing formation of new follicles (Neo-Folliculogenesis), (H&E) 10x.

⇨ **New thyroid follicles (Neo-Folliculogenesis)**

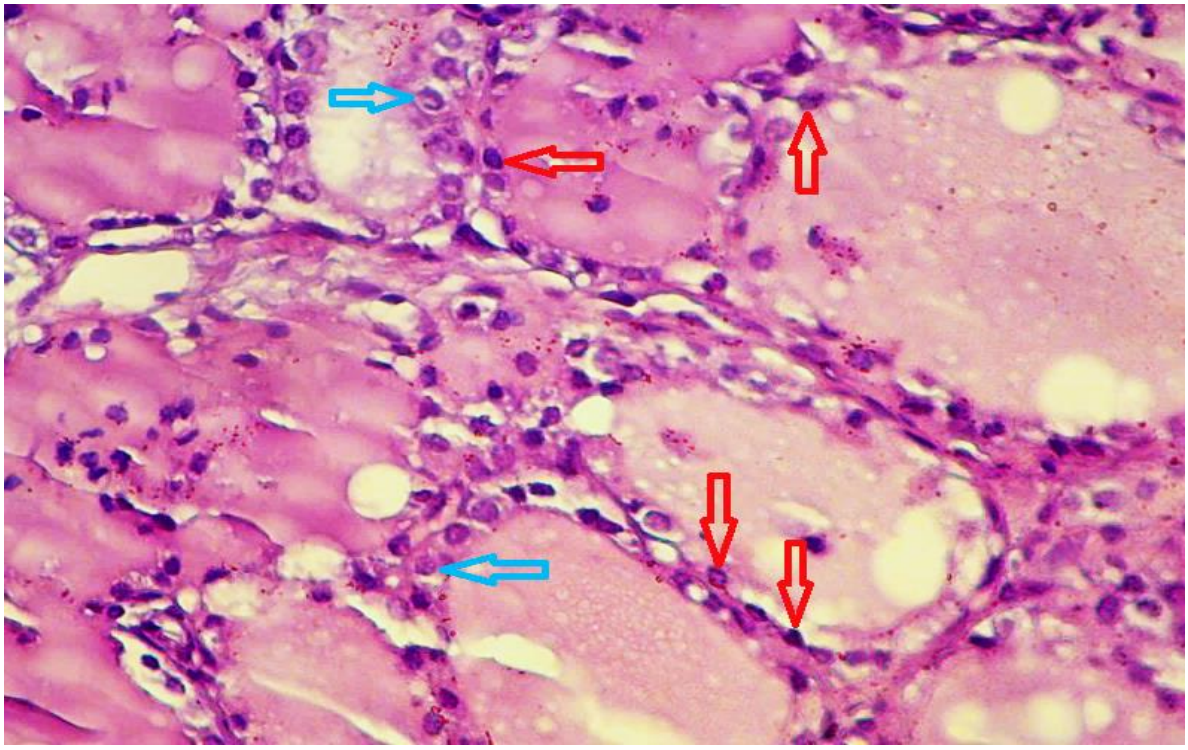


**Figure (3-9):** Section of thyroid gland of rat group treated with 200 mg/kg of ZnO NPs for 4 weeks, showing follicles which filled with different amount of colloid materials that lined with active and inactive follicular cells, (H&E) 10x.

⇨ **Active follicular cells** ⇨ **Inactive follicular cells**

In this study, the changes in the thyroid tissue were covenant to the results that obtained for thyroid gland functions which were showed alteration in the thyroid hormones levels (hyperthyroidism).

Nanoparticles can enter the body orally or by injection, and cross most of the body organs to speared and accumulation in their tissues including the thyroid gland (Assadi *et al*., 2016). Aggregation of nanoparticles (NPs) principally metal-oxide in the tissues regardless of their size are able to cross through the membranes and enter into the blood stream, then the lymphatic system and finally accumulate in the tissues (Canli and Canli, 2019). For that several studies suggested that the nanoparticles (NPs) aggregates causing some adverse effects, like histological

changes, blocking capillaries or changing membrane permeability resulting in potential health risks (Boudreau *et al*., 2016; Wu and Tang, 2018). As well, these materials after entering into the organ and tissue, it will uptake by body defenses such as macrophages and may cause releasing of cytokines from the macrophages which lead to histological abnormalities, and/or functional damages (Nishimori *et al*., 2009). The histological changes and cytotoxicity cells induced by Zinc Oxide NPs were dependent on the size and dose of exposed materials and time of exposure (Al-Suhaibani and El-Morshedi, 2014). Therefore a previous study reported that the smaller particles are more toxic than the larger ones (Abdelhalim and Jarrar, 2012). Zinc oxide NPs can stimulate reactive oxygen species ROS that disruptor the intracellular metabolic activities, and the antioxidant system (Najim, 2015; Singh *et al.*, 2020).

## 3.3. The Ovary

### 3.3.1. Ovary Weights

Statistical analysis results of the present study for the effect of ZnO NPs on the right and left ovary explained in Figure (3-10) and (3-11) respectively, the results showed high significant decrease ($p \leq 0.01$) in the weight of right ovary (0.156±0.003) and (0.154 ±0.001) gm in all treated animals with different doses (50 and 200) mg/kg respectively, for 1 week when compared to control group (0.163±0.001) gm, on the other hand, demonstrated no significant change ($P \leq 0.05$) in weights of the left ovary at a low dose (50 mg/kg) of ZnO NPs (0.159 ±0.002) gm, while there was high significant decrease ($p \leq 0.01$) at high dose (200 mg/kg) of ZnO NPs (0.155 ±0.001) gm for 1 week when compared to control group (0.161 ±0.001). As well outcomes demonstrated high significant decrease ($p \leq 0.01$) in the right ovary (0.123±0.001) and (0.108±0.001) gm more than the left part (0.129 ±0.001) and (0.116 ±0.001) gm at doses (50 and 200) mg/kg of ZnO NPs

respectively, for 2 weeks when compared to right (0.162 ±0.001) gm and left ovary (0.161±0.001) gm of control groups. Also, the rats that exposed to ZnO NPs in the long term (4 weeks) at low and high dose (50 and 200) mg/kg showed high significant decrease (P≤ 0.01) in the weights of right ovaries (0.101±0.001) and (0.087±0.001) gm and left ovaries (0.104 ±0.002) and (0.094 ±0.001) respectively, when compared to the right (0.161 ±0.001) gm and left ovary (0.160 ±0.001) gm of control groups.

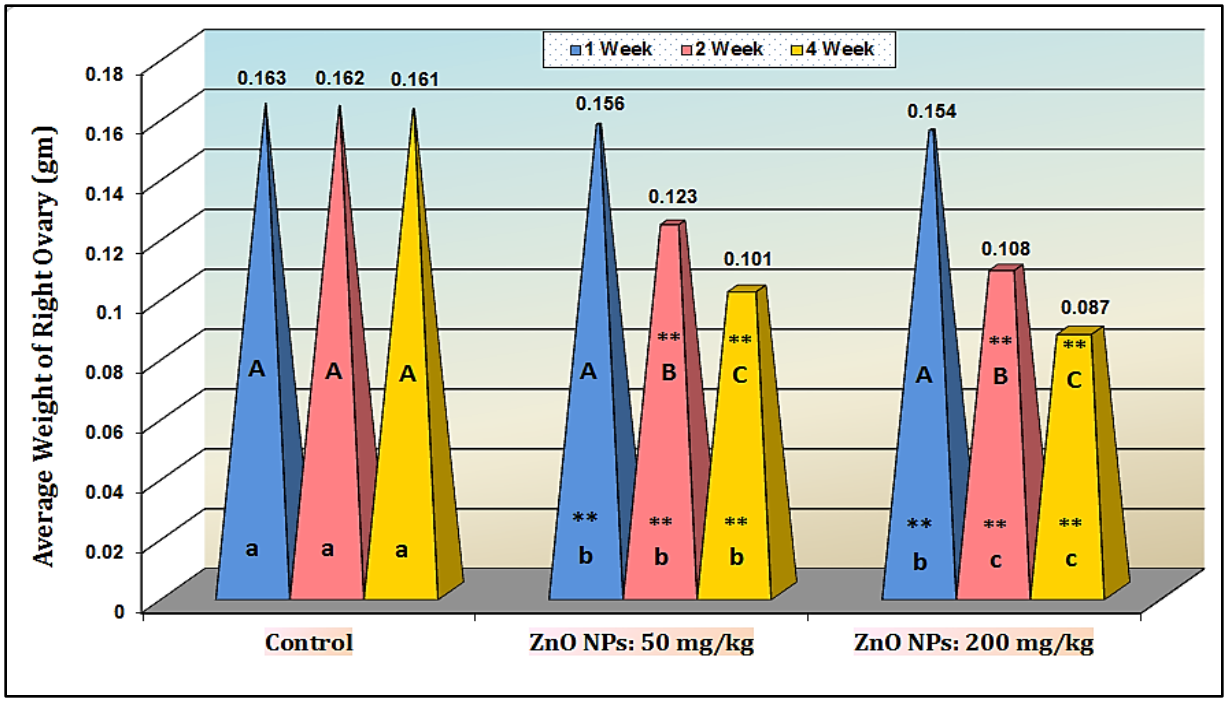



**Figure (3-10):** Effect of ZnO NPs in different concentrations (50 and 200 mg/kg) during different exposure periods on right ovary of Rats.

- **(**)** Mean highly significant variation (p≤0.01).
- **(A, B, C)** Represent the significant difference between groups when time is variable factor and concentration as a fixed factor.
- **(a, b, c)** Represent the significant difference between groups when time is a fixed factor and concentration as a variable factor.

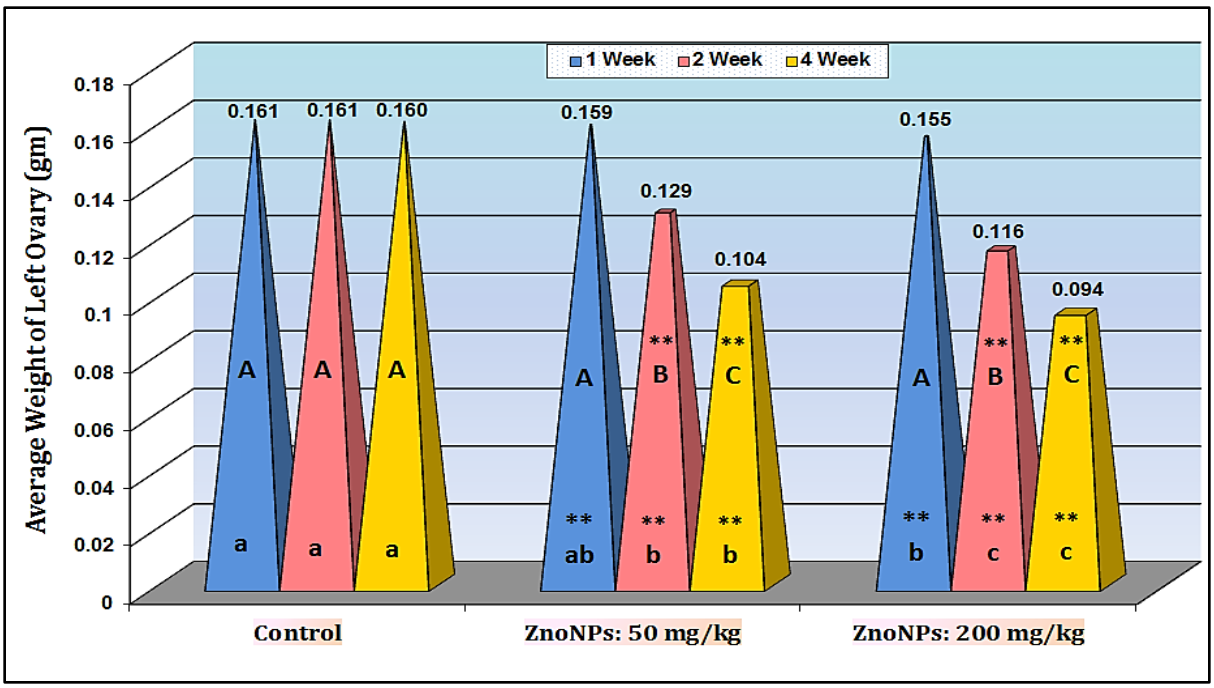



**Figure (3-11)**: Effect of ZnO NPs in different concentrations (50 and 200 mg/kg) during different exposure periods on left ovary of Rats.

- **(**)** Mean highly significant variation ($p \leq 0.01$).
- **(A, B, C)** Represent the significant difference between groups when time is variable factor and concentration as a fixed factor.
- **(a, b, c)** Represent the significant difference between groups when time is a fixed factor and concentration as a variable factor.

There are several studies that have evaluated the toxic effect of NPs on the female reproductive system, especially on the ovary. Previous study that conducted by Zhai *et al*., (2018) who exposed the pregnant female mice to ZnO NPs at dose 16 mg/kg of body weight by intravenously injected on two consecutive days, then analyzed the ovaries of offspring after 3 and 21 days post-partum (dpp), the results showed decrease in the oocyte numbers and impaired primordial follicle in the ovaries of the offspring, they suggested that the zinc oxide nanoparticles accumulated through the cytoplasm of oocyte and caused DNA damage and apoptosis for their cells. Another study conducted by Poormoosavi *et al*., (2018),

noted after injection a group of female rats with (15 mg/kg) of iron oxide and three groups with different doses (5, 15, and 45 mg/kg) of iron oxide nanoparticles for 16 days via intraperitoneal route, they observed a significant decrease in the weight of left and right ovaries in all treated groups when compared to control group, so they suggested that the nanoparticles including the iron oxide can accumulate in the reproductive organs and causing an increase in cell death. As well as Asadi *et al*., (2019), demonstrated significant reduction in the weight of right ovary in treated rats with molybdenum trioxide nanoparticles ($MoO_3$ NPs) at a dose (5 mg/kg) via intraperitoneal injection for 28 days every other day, whereas the weight of left ovary showed non-significant change in compared with the control group. On the other hand, the effect of ZnO NPs was observed in a previous study that was conducted by Radhi and Al-Bairuty, (2019) who treated the ZnO NP to the male albino mice through oral gavage, the statistical analysis results recorded a significant decrease ($P \leq 0.05$) in the testicular weights of mice that exposed to ZnO NPs at two different concentrations (100 and 200) mg/kg, during 1 and 2 weeks when compared with the control group.

It has been noted the accumulation of the nanoparticles, especially ZnO NPs in the female reproductive system including the ovary of animals that exposure to these particles and these aggregation has an adverse effect on the structure of the reproductive organs and the rate of perturbation depends on dosage and time of exposure to these nanoparticles (Brohi *et al*., 2017). In addition to the direct effect of nanoparticles on the structure of the ovary, the thyroid gland also has an effect on metabolic and function of ovary, therefore, the excessive secretion of the thyroid gland (hyperthyroidism) leads to decrease in the size of the ovary due to the increase in the metabolic processes (Muderris *et al*., 2011; Wei *et al*., 2018).

### 3.3.2. Ovarian Hormones (P and E2)

Statistical analysis of results from the present study for the ovarian hormones (P and E2) levels in the serum of animals that treated with ZnO NPs demonstrated in Figure (3-12), (3-13), the data showed:

- High significant decrease ($p \leq 0.01$) was observed in the levels of estrogen (E2) (Pg/ml) in all animals that exposed to doses (50 and 200) mg/kg of ZnO NPs (49.48±0.19) and (47.18±0.26) respectively, for 1 week when compared to the control group (51.20±0.40), likewise, demonstrated high significant decrease ($p \leq 0.01$) in the levels of estrogen in the experimental group that treatment with the low and high doses (50 and 200) mg/kg of ZnO NPs (34.59 ±0.39) and (29.99 ±0.28) respectively, for 2 weeks when compared to control group (52.63±0.21). On the other hand, during 4 weeks period of time, the results were showed high significant decrease ($p \leq 0.01$) in the levels of estrogen (26.65 ±0.22) and (19.39 ±0.18) at these doses (50 and 200) mg/kg of ZnO NPs respectively when compared to the control group (51.87 ±0.36). As demonstrated in Figure (3-12).
- For the levels of progesterone (P) (ng/ml) as shown in Figure (3-13), the results showed no significant changes ($p \leq 0.05$) in the levels of progesterone hormone in the groups that exposed to two different doses (50 and 200 mg/kg) of ZnO NPs (12.36±0.22) and (11.96±0.14) respectively, in short term (1 week) when compared to the control group (12.28±0.13). Whereas, there was high significant decrease ($p \leq 0.01$) were observed in the experimental treated group with doses (50 and 200 mg/kg) of ZnO NPS (11.67±0.24) and (11.46±0.14) respectively, during 2 weeks when compared to control group (12.62±0.13). The results also demonstrated high significant decrease ($p \leq 0.01$) in all Rats that exposed to these doses of ZnO NPs (11.23±0.16) and (8.96±0.15) respectively, in long term (4 weeks) when compared to control group. (12.52±0.09).

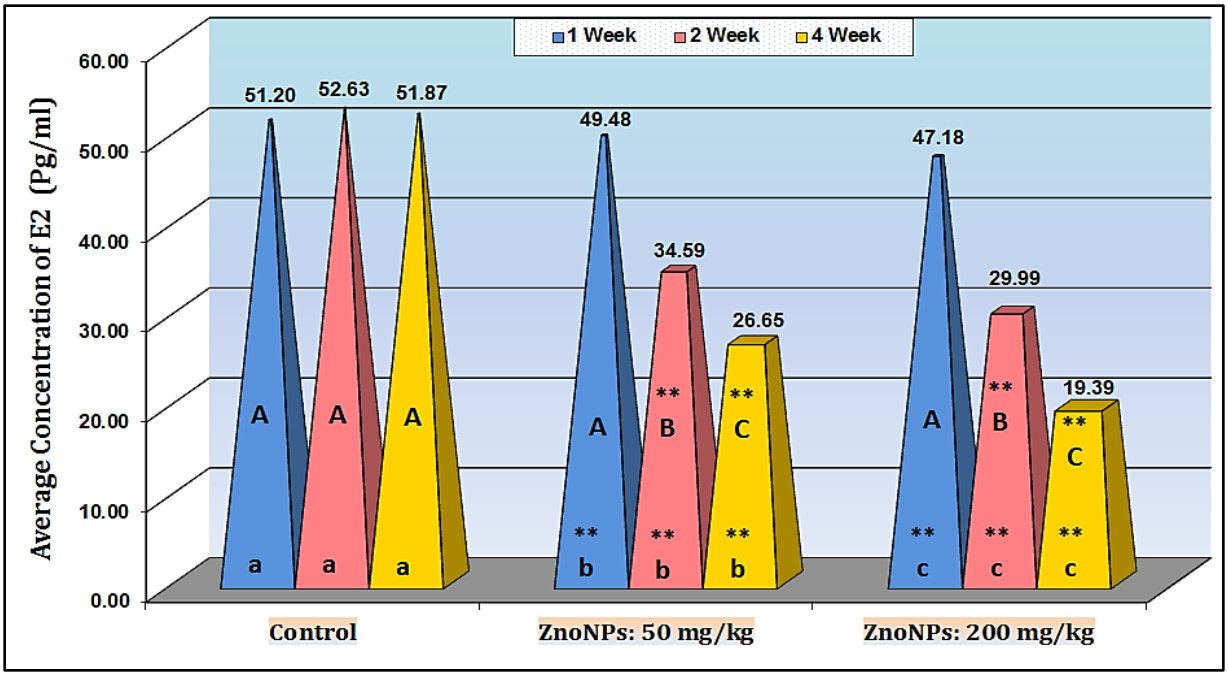



**Figure (3-12):** Effect of ZnO NPs in different concentrations (50 and 200 mg/kg) during different exposure periods on E2 level (Pg/ml).

- **(**)** Mean highly significant variation ($p \leq 0.01$).
- **(A, B, C)** Represent the significant difference between groups when time is variable factor and concentration as a fixed factor.
- **(a, b, c)** Represent the significant difference between groups when time is a fixed factor and concentration as a variable factor.

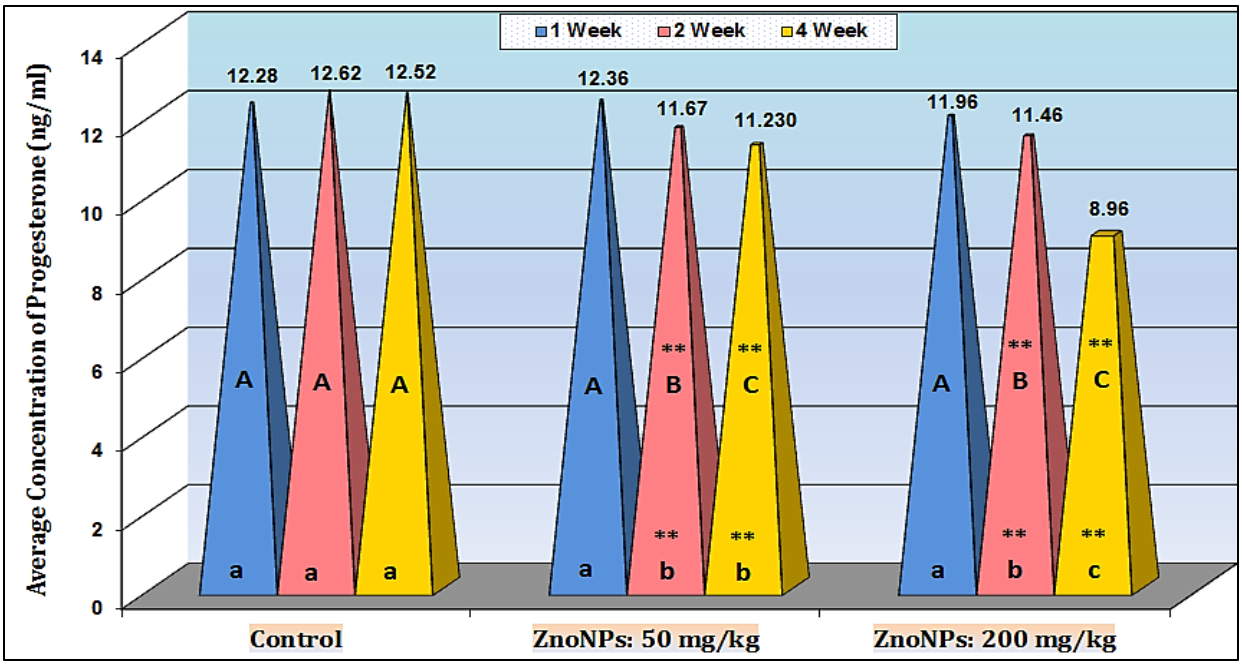



**Figure (3-13):** Effect of ZnO NPs in different concentrations (50 and 200 mg/kg) during different exposure periods on progesterone level (ng/ml).

- **(**)** Mean highly significant variation ($p \leq 0.01$).
- **(A, B, C)** Represent the significant difference between groups when time is variable factor and concentration as a fixed factor.
- **(a, b, c)** Represent the significant difference between groups when time is a fixed factor and concentration as a variable factor.

The present study showed that the ZnO NPs had effects on the level of the ovarian hormones. A previous study have been reported changes in the sex hormone levels, after exposure to some NPs (Wang et al., 2018). The previous study that conducted by Hosseini et al., (2019), where injected the female Rats via intraperitoneal route with different doses of ZnO NPs (4, 8, 25, 50, 100, and 200) mg/kg twice a week for 4 weeks, the results showed that the exposure to the ZnO nanoparticle dose-dependent manner increases the estrogen hormone level at doses (4, 8, 25, and 50) mg/kg while decreased at doses (100 and 200) mg/kg, on the other hand, the progesterone level increased after exposure to doses (4, 8, 25)

mg/kg while decreased at doses (100 and 200) mg/kg when compared to the control group.

Yoosefi *et al*., (2015), mentioned after injecting the female mice orally via gavage with titanium dioxide nanoparticles with a different doses (10 and 100 ppm) for 2 weeks, the results demonstrated significant decrease ($P<0.05$) in the levels of estrogen and progesterone hormones in all treated experimental groups that exposed to these two doses (10 and 100 ppm) of $TiO_2$ NPs when compared to the control group, they concluded that the $TiO_2$ nanoparticles can effect on reproductive activities via decreasing pituitary gonadal-axis and has adverse effects on the reproductive potential of female sex hormones.

From a previous study, it was suggested that the exposure to the nanoparticles (NPs) might causes a change in the rate of sex hormone levels in the blood through an indirect effect on the hypothalamic-pituitary-gonadal axis, or direct effect on the stimulation of secretory cells such as granule cells, theca cells, follicle cells and the corpus luteum (Gifford *et al*., 2017). For that, Reza *et al*., (2014) confirmed that by exposing the animals to ZnO NPs, they observed reduced fertility potential in the reproductive of male Rats. In other words, due to the size of these particles can be accumulated in the secretory cells and can affect directly on synthesis and secretion of hormones in the ovary (Hou and Zhu, 2017). Other study conducted by Hosseini *et al*., (2019) was reported that the accumulation of heavy metals in the reproductive organs of female Rats lead to reduce the levels of progesterone and estradiol hormones in the serum.

In summary, the effects of nanoparticles on the secretion of ovarian hormone and hypothalamic-pituitary-gonadal axis can be in two ways; the first one occurs by pass of the nanoparticles (NPs) through the blood-brain barrier into the hypothalamus, and secretory cells of the pituitary which lead to altering secretion of GnRH, LH, and FSH, therefore undermining the normal mechanism of positive

and negative feedback of the hypothalamic-pituitary-gonadal axis which causes the abnormal secretion of ovarian estrogen and progesterone hormones, the second way is that the nanoparticles may enter the ovaries via the circulation system and accumulate in granulosa cells and theca cells which affects steroid genesis (Hou and Zhu, 2017). On the other hand, the surface of the ovary has a receptor for the thyroid hormone, thus these hormones have a direct effect on the ovarian cells (Kong *et al*., 2015). The dysfunction of the thyroid hormone has an adverse effect on the hormones of female ovarian (Liu *et al*., 2018). Which is support the current study.

Therefore, there are several previous studies showed that both hypothyroidism and hyperthyroidism have effect on the metabolism of sexual steroids and ovarian function in women and associated with a wide range of reproductive disorders from abnormal sexual development to menstrual disorder as well as infertility (Aghajanova *et al*., 2011).

### 3.3.3. Gonadotropin Hormones (LH and FSH)

Statistical analysis results of the present study for the gonadotropin hormones (LH and FSH) levels in the serum of animals that treated with ZnO NPs demonstrated in Figure (3-14) and (3-15) respectively, the data showed:

- The LH hormone (mIU/ml) evaluated in the animals that exposed to ZnO NPs at different doses (50 and 200) mg/kg during 1 week showed non- significant changes (0.308 ±0.003) and (0.306 ±0.002) respectively, when compared to the control group (0.312 ±0.002). Whereas, there was high significant decrease ($p \leq 0.01$) in the value of LH hormone for animals that exposed to low and high doses (50 and 200) mg/kg of ZnO NPs (0.197 ±0.002) and (0.172 ±0.002) respectively, for 2 weeks period of time when compared to the control group

(0.307 ±0.002). likewise, the results showed high significant decrease ($p \leq 0.01$) in experimental animals that exposed to both doses (0.139 ±0.003) and (0.099 ±0.002) respectively, during the long term (4 weeks) when compared to the control group (0.314 ±0.003). As shown in Figure (3-14).

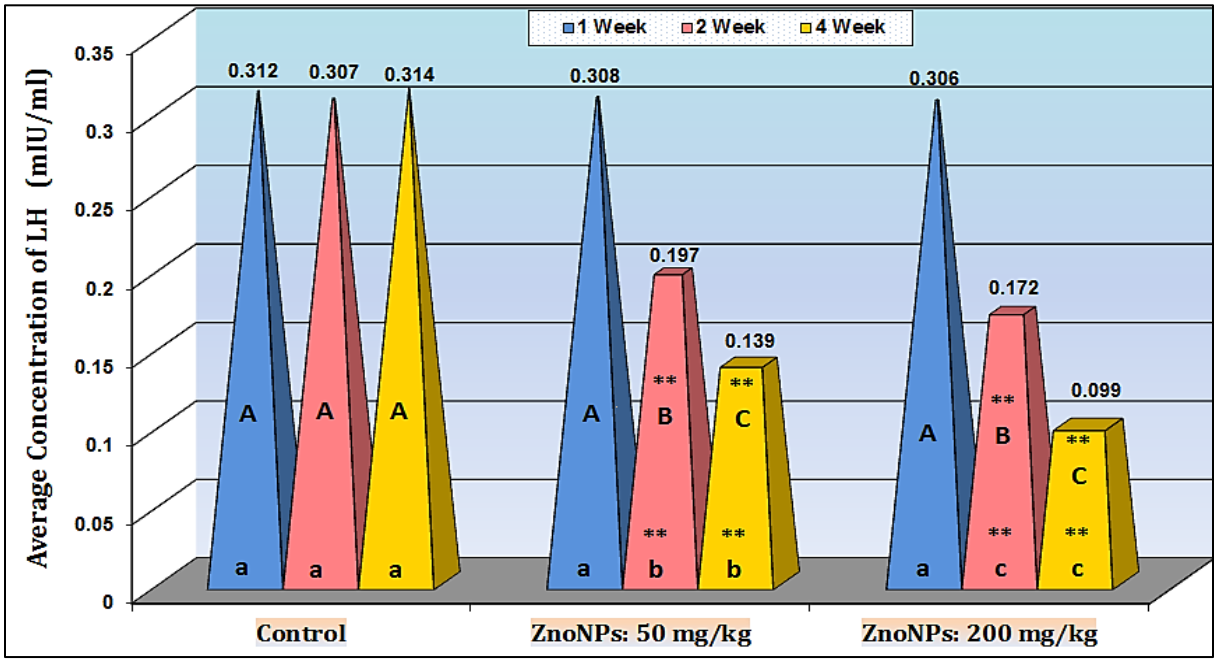



**Figure (3-14):** Effect of ZnO NPs in different concentrations (50 and 200 mg/kg) during different exposure periods on LH level (mIU/ml).

- **(**)** Mean highly significant variation ($p \leq 0.01$).
- **(A, B, C)** Represent the significant difference between groups when time is variable factor and concentration as a fixed factor.
- **(a, b, c)** Represent the significant difference between groups when time is a fixed factor and concentration as a variable factor.

- With regard to the value of FSH hormone as illustrated in Figure (3-15), the results showed no significant change ($p \leq 0.05$) in FSH level in serum of experimental animals that exposed to ZnO NPs (0.208 ±0.002) and (0.205 ±0.001) in short term period (1 week) at doses (50 and 200) mg/kg respectively, when compared to the control group (0.209±0.001). While, the results showed high significant decrease ($p \leq 0.01$) in animals that exposed to both doses (50 and 200) mg/kg of ZnO NPs (0.173 ±0.002) and (0.143 ±0.002) respectively, for 2 weeks when compared to the control group (0.215 ±0.002). As well as, the animals that exposure for 4 weeks showed high significant decrease ($p \leq 0.01$) in FSH level at both doses (50 and 200) mg/kg of ZnO NPs (0.130 ±0.002) and (0.100±0.002) respectively, when compared to the control group (0.211 ±0.003).

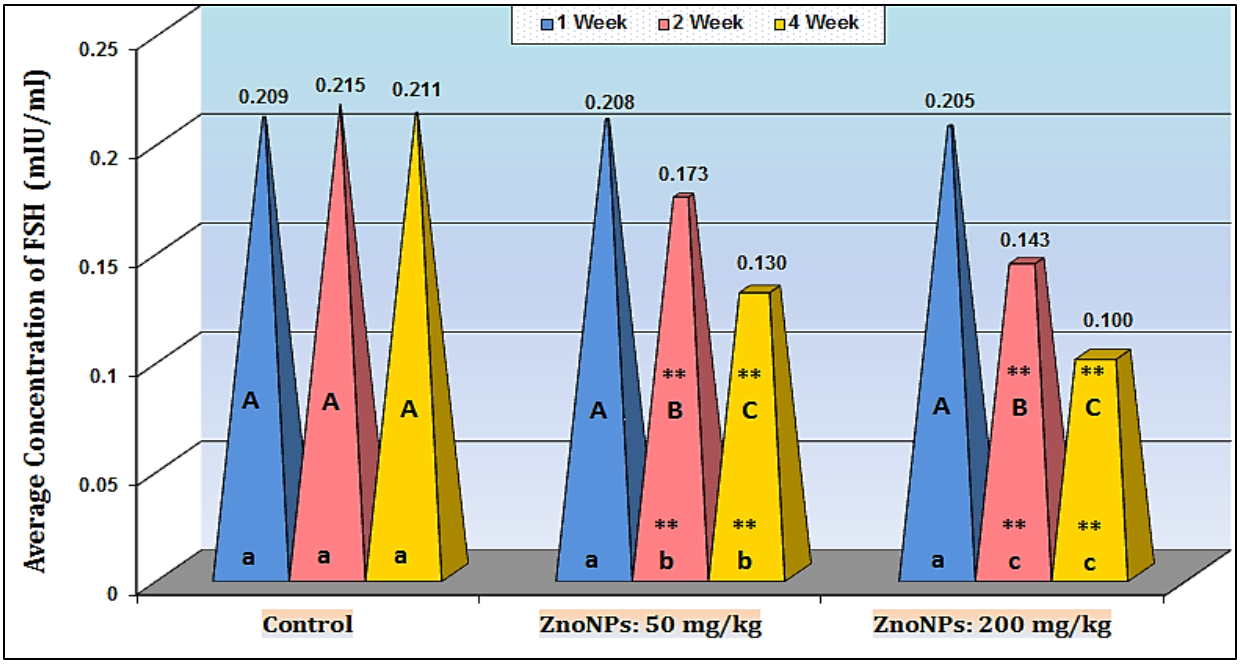



**Figure (3-15):** Effect of ZnO NPs in different concentrations (50 and 200 mg/kg) during different exposure periods on FSH level (mIU/ml).

- (**) Mean highly significant variation ($p \leq 0.01$).
- **(A, B, C)** Represent the significant difference between groups when time is variable factor and concentration as a fixed factor.
- **(a, b, c)** Represent the significant difference between groups when time is a fixed factor and concentration as a variable factor.

Normally, the secretion of gonadotropin hormones (LH and FSH) from the anterior pituitary gland in women is under ovarian control by negative and positive feedback mechanisms (Messinis, 2006). So, the defect in the ovarian functions (Klein, 2003), or disrupt the hypothalamic-pituitary-gonadal axis causes altering the levels of GnRH, LH, and FSH (Dougher and Pillai, 2015).

For that, the statistical analysis results of this study was on the same path with the previous study which presented by Espanani *et al.*, ( 2013), who reported a significant decrease in FSH level when exposed the male Rats by intraberitoneally injection with the high dose (40 mg/kg) of ZnO NPs every day through 21 days, whereas no changes observed in the groups that exposed to other different dose (5, 10 and 20 mg/kg) of ZnO NPs when compared with the control group, in contrast, there are non-significant changes in the level of LH hormone in all treated experimental groups with different concentrations (5, 10, 20, and 40 mg/kg) of ZnO NPs for 21 days when compared to the control group, they suggested that the nanoparticles might inhibit the function of the endocrine system by blocking the pituitary hypothalamus axis, It may also be due to a reduction in the level of GnRH. Other studies used fifty adult male Rats were randomly divided into five groups each group has ten animals, one of them groups consider as a control group and other four groups were received (orally) different dose of ZnO NPs (5, 10, 20, and 40 mg/kg) respectively for 56 days, the blood samples were collected via cardiac puncture in (Zero, 28 and 56 days of the experiment), the results showed a significant ($P<0.05$) decrement in LH hormones levels in all treated groups at 56 days when compared to the control group and other period time, while there was significant ($P<0.05$) increase in FSH hormone levels in animals that treated with (20 and 40 mg/kg) of ZnO NPs after 56 days, while there were no changes observed in other period times (zero and 28 days) when compared to the control group (Husain *et al.*, 2019).

In another study presented by Omidi *et al*., (2015) who reported that the level of LH hormone got a significant decrease in the female rats that exposed to high dose (400 mg/kg) of zirconium oxide nanoparticles for 10 days, while no changes observed in the level of LH at doses (100 and 200) mg/kg of zirconium oxide nanoparticles for the same period when compared to the control group, with regard to the FSH hormone, they noted there was no significant change in the FSH level in the rats that exposed to (100 and 200) mg/kg of zirconium oxide nanoparticles for 10 days, whereas, the results demonstrated a significant decrease in the level of FSH at a dose (400 mg/kg) in the same period when compared to the control group, they reported that the changes occur as a result of the inhibition of the cells by the nanoparticles that are responsible for the production of LH and FSH hormone. As well, the previous study that supports the current study reported by Yoosefi *et al*., (2015), demonstrated that the levels of LH and FSH hormones have a significant decrease in the female mice that exposed via gavage to $TiO_2$ nanoparticle at concentrations (10 and 100 ppm) in duration 14 days compared to the control group, according to their results, they clarified that the titanium dioxide can affect reproductive activities by decreasing-pituitary gonadal axis and has negative effects on the reproductive potential of the female sex.

In contrast, this study disagree with the previous study conducted by Mozaffari *et al*., (2020) indicated that the zinc oxide nanoparticles at dose 250 mg/kg caused a significant increase in FSH level of male rats compared to the control group, however, with other doses (500 and 700) mg/kg of ZnO NPs did not show significant change in FSH hormone levels, also, there was a non-significant change in the LH levels at different doses of ZnO NPs (250, 500, and 700 mg/kg) comparison to control group.

### 3.3.4. Histological Changes of Ovary

The microscopic examination assessment of the ovarian tissue sections for the control groups (untreated animals) showed normal appearance in their histological structure, where they have a primordial and primary follicles as well as observed the formation of corpus lutium, as shown in Figure (3-16).

In contrast, the experimental treatment groups with ZnO NPs in different doses and period of times showed different histological changes in their ovarian tissues. The section of the ovarian tissue for animals that exposure to ZnO NPs for 1 week showed the beginning formation of a corpus luteal cyst surrounded by fibrous tissue reaction (fibrosis), as shown in Figure (3-17). While, after 2 weeks of treatment, the section of the ovarian tissue showed necrosis of the lining cells of corpus luteum with the formation of the lumen of the cyst, as in Figure (3-18). Whereas the section of the ovarian tissue for animals groups that exposure to ZnO NPs for ling time of period (4 weeks) showed noticeable advance necrosis of luteinizing follicular cells surrounded with abundant of fibrous tissue reaction (increase in fibrosis), in Figure (3-19).

Therefore, this result is consistent with a obtained results regarding the levels of sex hormones, which showed decrease in the levels of progesterone and estrogen hormones, as well as the necrosis and fibrosis that formed in the ovarian cells may lead the granulosa cell losing its cellular function by the decrease in regulation of gene expression especially thyroid hormone receptor gene

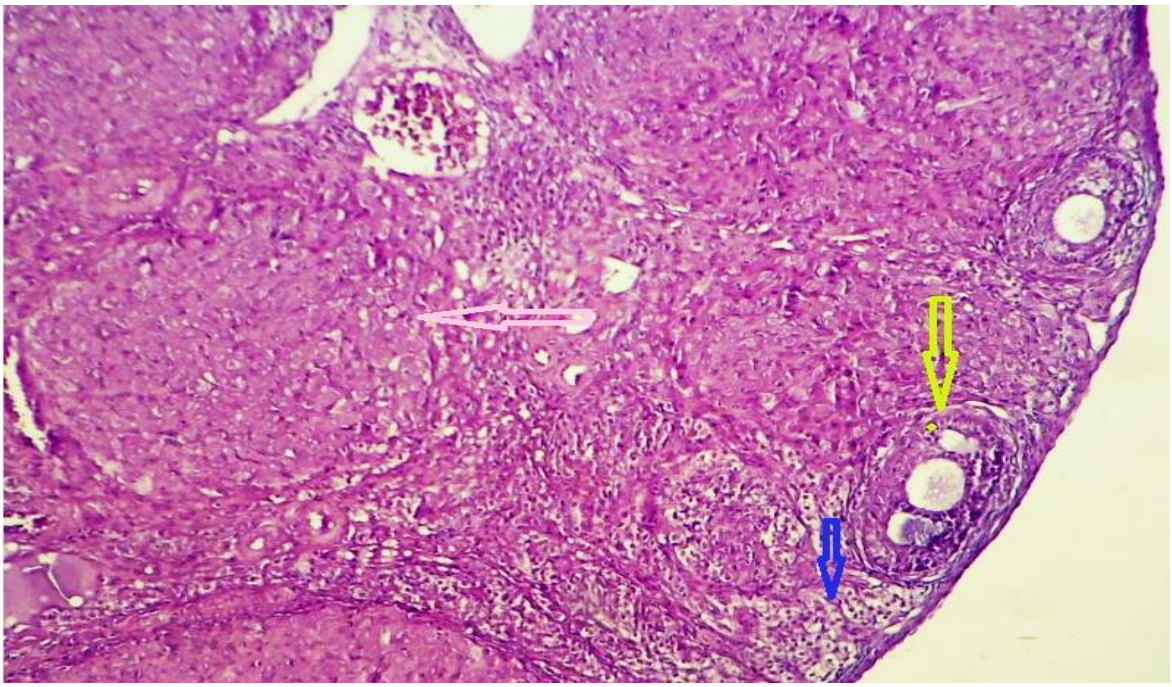


**Figure (3-16):** Section of ovary for control group showing primordial and primary follicles with corpus luteal, (H&E) 10x.

⇨ **Primordial Follicle** ⇨ **Primary Follicle** ⇨ **Corpus Luteum**

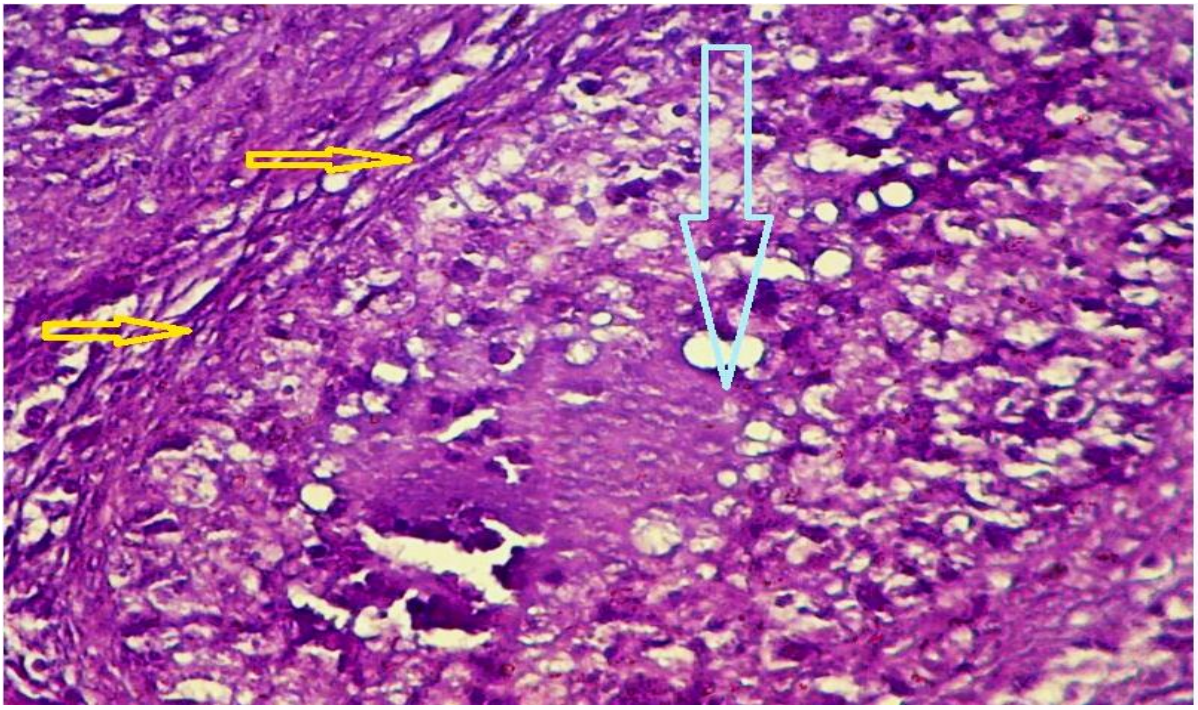


**Figure (3-17):** Section of ovary of rat group treated with 200 mg/kg of ZnO NPs for 1 week, showing beginning formation of corpus luteal cyst surrounded by fibrous tissue reaction, (H&E) 40x.

⇨ **Corpus Luteal Cyst** ⇨ **Fibrous Tissue Reaction (Fibrosis)**

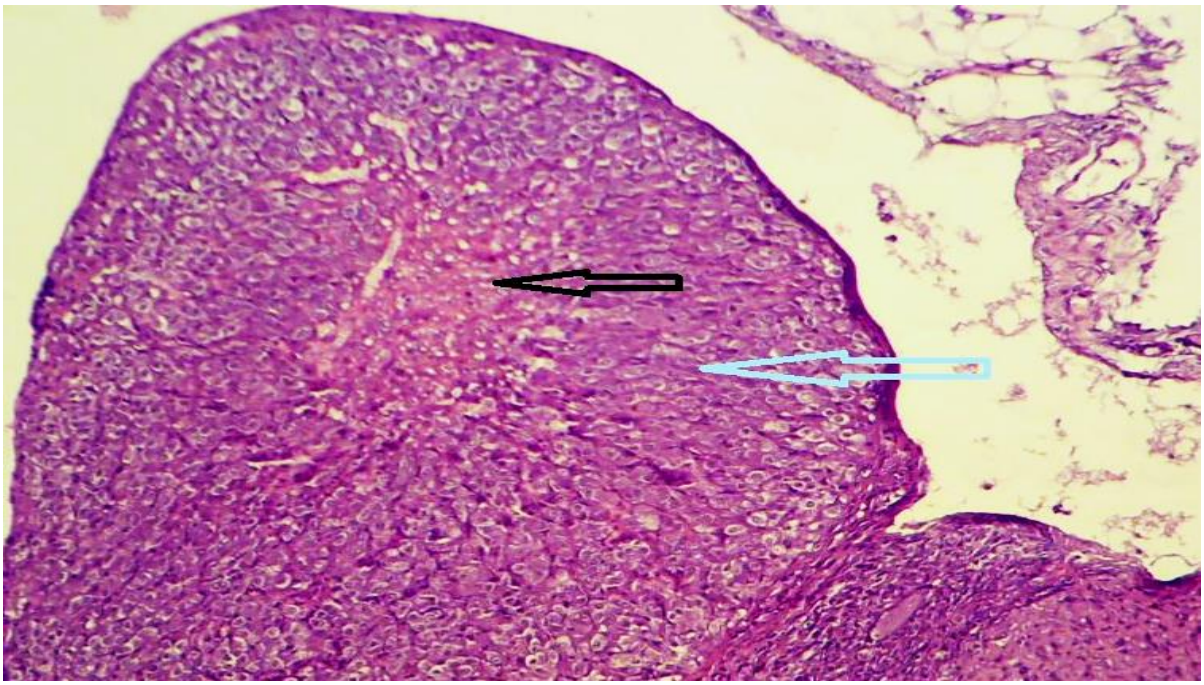


**Figure (3-18):** Section of ovary of rat group treated with 200 mg/kg of ZnO NPs for 2 weeks showing necrosis of lining cells of corpus luteum with formation of the lumen of cyst, (H&E) 40x.

⇨ **Necrosis of Corpus Luteum** ⇨ **Lumen of Cyst**

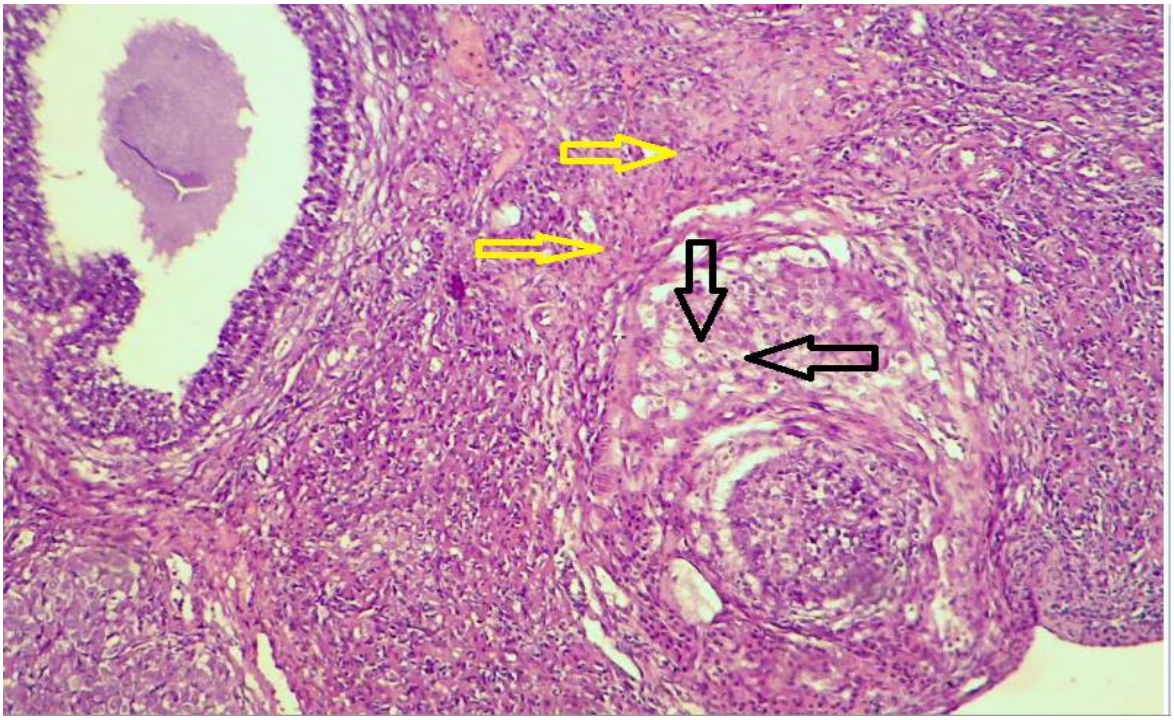


**Figure (3-19):** Section of ovary of rat group treated with 200 mg/kg of ZnO NPs for 2 weeks showing advance necrosis of luteinizing follicular cells surrounded with abundant of fibrous tissue reaction (fibrosis), (H&E) 40x.

⇨ **Necrosis in Corpus Luteum** ⇨ **Fibrous Tissue Reaction (Fibrosis)**

The small sizes of the nanoparticles (NPs) have the ability to enter the organs of the body by penetrating the physiological barriers by traveling through circulatory systems (Salman, 2018). One of these organs that are affected by nanoparticles is the ovary (Mahdieh *et al*., 2016; Asadi *et al*., 2017). Destructive effects on various organs and tissue can be caused by exposure through short and long terms with heavy metals such-as zinc, it was observed that after injecting the female rats with ZnO NPs intraperitoneally, the light microscopic section showed changes in the tissue of the ovary (Hosseini *et al*., 2019). In the same path, Hou and Zhu in (2017) who observed that the zinc oxide nanoparticles (ZnO NPs) accumulated in several organs including the ovary and cause a disturbance in their tissues which lead to ovarian damage and generated oxidative stress after repeated application of ZnO NPs in female rats, the nanoparticles (NPs) can disturb the developing of the oocyte by invading the protective barrier of granulosa cells, theca cell layers, and zona pellucida, which lead to effect on the sex hormone levels in blood.

Another study by Zhao *et al*., (2016) observed that ZnO-NPs inhibited the growth of ovarian granulosa cells after 24-hour of treatment. Mahdi and Al-Nakeeb, (2018) demonstrated that the result of histological sections of treated groups with (1ppm) of silver nanoparticles during 7 days, indicated some of the histopathological changes such as shrinkage in the oocyte in growing follicle, fat degeneration, pyknosis in all follicle cells in granulosa layer of growing follicle, furthermore occurred precipitation of amyloid protein in follicle cells.

The accumulation of NPs in organ tissues along with the multiplication of many defense cells such as phagocytic cells to formation of reactive oxygen species (ROS) and antioxidant defenses, which makes these organs targets to oxidative stress (Sharifi *et al*., 2012).

On the other hand, the thyroid hormones (THs) play a critical role in the development of ovarian cells (Kong *et al*., 2015). It has been reported that the hyperthyroidism is associated with dysregulation of the hypothalamic-pituitary axis and suppressed growth and function of ovarian follicular (Liu *et al*., 2018). Which is supported by Fedail *et al*., (2014) that observed the ovarian histologic sections of rats when the gland is in hyperthyroidism status there are significantly reduce in numbers of healthy follicles (primordial, primary, secondary), and the number of Graafian follicle were considerably much lower than in control rats.

## 3.4. Gene Expression of THR-β and TSHR

The obtained results in this study showed the effected of ZnO NPs on the regulation of gene expression represented by the thyroid-stimulating hormone receptor (TSHR gene) that expression on the thyroid gland, as shown in table (3-1), and thyroid hormone receptor beta (THRβ gene) that expression on the ovary, revealed by table (3-2). Where these results indicated that there is a decrement in the expression of genes (TSHR and THRβ gene) in treated animals compared with control groups, and this alteration in the levels of gene expression varies from group to other according to different doses and exposure periods with ZnO NPs as the following:

**Table (3-1):** RT-PCR results of Thyroid stimulate hormone receptor gene (TSHR gene)

| Groups | Samples | TSHR- gene (ng/µl) | House Keeping (Reference) gene (ng/µl) | ΔCT | ΔΔCT | Folding | Mean |
|---|---|---|---|---|---|---|---|
| **1 WEEK** | | | | | | | |
| Control Group | 1 | 27.59 | 24.79 | 2.81 | 0.50 | 0.70 | 1.380 |
| | 2 | 27.87 | 27.12 | 0.75 | -1.56 | 2.95 | |
| | 3 | 27.73 | 24.34 | 3.39 | 1.08 | 0.47 | |
| Rat Treated Groups with Low Dose (50 mg/kg) | 4 | 28.07 | 26.19 | 1.89 | -0.42 | 1.34 | 0.819 |
| | 5 | 26.21 | 22.52 | 3.68 | 1.37 | 0.38 | |
| | 6 | 26.00 | 23.24 | 2.76 | 0.45 | 0.72 | |
| Rat Treated Groups with High Dose (200 mg/kg) | 7 | 34.75 | 23.38 | 11.37 | 9.06 | 0.002 | 0.002 |
| | 8 | 33.67 | 23.09 | 10.58 | 8.27 | 0.003 | |
| | 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 | 0.10 | |

$\Delta CT = CT_{\text{gene}} - CT_{\text{House Keeping gene}}$

$\Delta\Delta CT = \Delta CT_{\text{Treated}} - \Delta CT_{\text{Control}}$

$\text{Folding} = 2^{\wedge -\Delta\Delta CT}$

**Table (3-2):** RT-PCR Results of Thyroid Hormone Receptor Beta Gene (THRβ-gene)

| Groups | Samples | THRβ- gene (ng/µl) | House Keeping (Reference) gene (ng/µl) | ΔCT | ΔΔCT | Folding | Mean |
|---|---|---|---|---|---|---|---|
| **1 WEEK** | | | | | | | |
| Control Group | 1 | 27.02 | 21.18 | 5.84 | 0.49 | 0.71 | 1.031 |
| | 2 | 24.15 | 19.17 | 4.97 | -0.38 | 1.30 | |
| | 3 | 25.25 | 20.01 | 5.24 | -0.11 | 1.08 | |
| Rat Treated Groups with Low Dose (50 mg/kg) | 4 | 37.78 | 18.39 | 19.39 | 14.04 | 0.00 | 0.001 |
| | 5 | 37.27 | 21.63 | 15.63 | 10.28 | 0.00 | |
| | 6 | 33.92 | 18.89 | 15.03 | 9.68 | 0.00 | |
| Rat Treated Groups with High Dose (200 mg/kg) | 7 | 26.55 | 18.87 | 7.67 | 2.32 | 0.20 | 0.918 |
| | 8 | 26.05 | 19.48 | 6.57 | 1.22 | 0.43 | |
| | 9 | 26.23 | 21.96 | 4.26 | -1.09 | 2.12 | |
| **2 WEEKS** | | | | | | | |
| Control Group | 10 | 25.63 | 20.37 | 5.27 | -0.24 | 1.18 | 1.026 |
| | 11 | 26.47 | 20.48 | 5.99 | 0.48 | 0.72 | |
| | 12 | 26.58 | 21.31 | 5.27 | -0.24 | 1.18 | |
| Rat Treated Groups with Low Dose (50 mg/kg) | 13 | 26.07 | 21.15 | 4.92 | -0.59 | 1.51 | 0.842 |
| | 14 | 27.11 | 20.06 | 7.05 | 1.54 | 0.34 | |
| | 15 | 27.25 | 21.17 | 6.08 | 0.57 | 0.67 | |
| Rat Treated Groups with High Dose (200 mg/kg) | 16 | 27.69 | 20.62 | 7.07 | 1.56 | 0.34 | 0.509 |
| | 17 | 26.03 | 20.17 | 5.87 | 0.36 | 0.78 | |
| | 18 | 26.13 | 19.32 | 6.81 | 1.30 | 0.41 | |
| **4 WEEKS** | | | | | | | |
| Control Group | 19 | 25.63 | 20.37 | 5.27 | -0.24 | 1.18 | 1.026 |
| | 20 | 26.47 | 20.48 | 5.99 | 0.48 | 0.72 | |
| | 21 | 26.58 | 21.31 | 5.27 | -0.24 | 1.18 | |
| Rat Treated Groups with Low Dose (50 mg/kg) | 22 | 27.84 | 24.11 | 3.73 | -1.78 | 3.44 | 1.419 |
| | 23 | 27.06 | 20.51 | 6.55 | 1.04 | 0.49 | |
| | 24 | 26.04 | 18.92 | 7.12 | 1.61 | 0.33 | |
| Rat Treated Groups with High Dose (200 mg/kg) | 25 | 26.86 | 20.25 | 6.61 | 1.10 | 0.47 | 0.440 |
| | 26 | 26.67 | 19.85 | 6.82 | 1.31 | 0.40 | |
| | 27 | 26.27 | 19.61 | 6.66 | 1.15 | 0.45 | |

$\Delta CT = CT_{\text{gene}} - CT_{\text{House Keeping gene}}$

$\Delta\Delta CT = \Delta CT_{\text{Treated}} - \Delta CT_{\text{Control}}$

$\text{Folding} = 2^{\wedge -\Delta\Delta CT}$

- The results showed a gradual decrease in expression of TSH receptor (ng/µl) in all animals groups that exposed to ZnO NPs at doses (50 and 200) mg/kg in different duration (1, 2, and 4 weeks) in comparison with the control group. As shown in Figure (3-20).

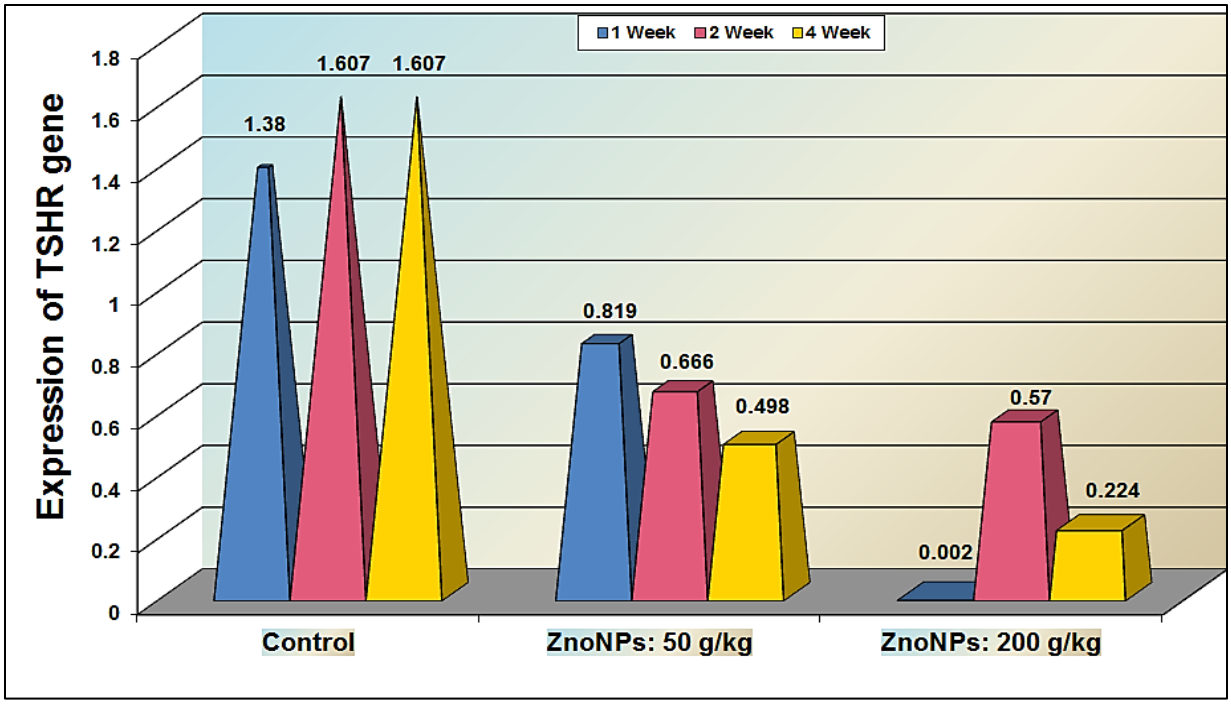



**Figure (3-20):** Effect of ZnO NPs in different concentrations (50 and 200 mg/kg) during different exposure periods on expression of TSHR gene on thyroid glands of rats.

- Regarding to the levels of TRβ gene (ng/µl), the results showed noticeable reduction in the expression of TRβ gene in all treated group that exposed to ZnO NPs at a low and high doses (50 and 200 mg/kg) during different durations (1, 2, and 4) weeks, except for the experimental group that injected with a low dose (50 mg/kg) of ZnO NPs for a long time (4 weeks), the result demonstrated an increase in the level of TRβ gene when compared with the control groups. As shown in Figure (3-21).

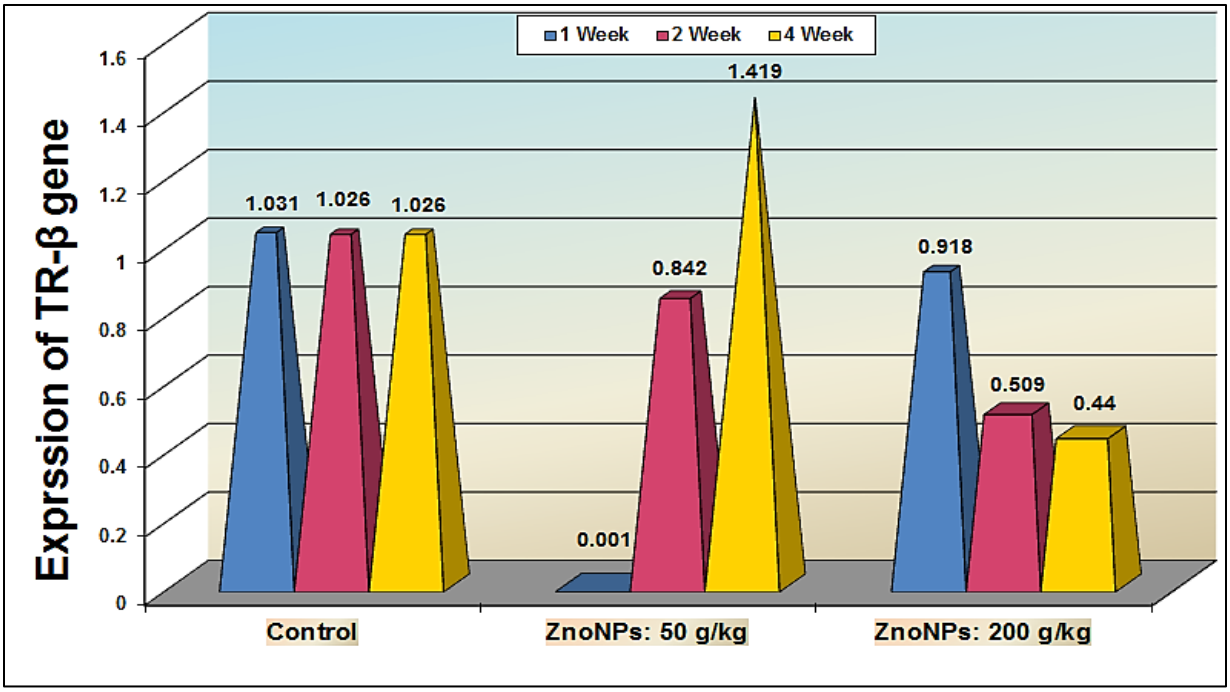



**Figure (3-21):** Effect of ZnO NPs in different concentrations (50 and 200 mg/kg) during different exposure periods on expression of TRβ on ovaries of rats.

The 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).

Several studies reported that there are many exogenous influence factors such as, environmental and industrial chemicals that can alter the levels of gene expression (Wittkopp, 2007; Baccarelli and Bollati, 2009). One of these chemical agents that effected 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 data were found to be consistent approximately with the previous study conducted by Du *et al*., (2016) who reported down-regulation in the expression of TSHR gene in zebrafish larvae after co-exposure to Perfluorooctane sulfonate (PFOS) and ZnO NPs in comparison to control group. In the same path, Parang and Moghadamnia, (2018) demonstrated decrement in the level of beta-receptor of thyroid hormones (TRβ) in the adult male Rats that exposed to silver nanoparticles in different doses (25 and 100 mg/kg) at range of size 5-10 nm via intraperitoneally route for 14 days. Another study observed, the female mice that exposed to titanium dioxide nanoparticles at dose (10 mg/kg) for long-term (90 consecutive days) caused ovarian dysfunction and alteration in their genes expression including, 223 gene function was up-regulated ($P< 0.05$), while 65 genes were down-regulated ($P< 0.05$), when compared to the control group (Gao *et al*., 2012). 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 down regulation 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. In another previous study presented by Wei *et al*., (2018) who demonstrated, in the case of hyperthyroidism or hypothyroidism the expressions of thyroid hormone receptor on the ovary were significantly decreased.

So through several molecular studies and hypothesis related with this current study and affected about these results of gene expression may have shown that the exposure to ZnO NPs lead to production of reactive oxygen species (ROS) in tissues and cells, by the interaction of nanoparticles with biological molecules

including DNA (Sharma *et al*., 2012; 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 decrease in the number of mRNA transcripts as well as decrease in expression of protein (Nishino *et al*., 2011). While, it have been 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 mediates 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 generated of ROS, the direct action of $Zn^{2+}$ ions on DNA causes damage to this DNA (Heim *et al*., 2015). Even when exposed to the low dose of ZnO NPs can causes damage in the DNA (Ma *et al*., 2016).

Furthermore, DNA methylation represented one of major factors that affected on the processing and mediating of the gene expression by regulating the chromatin structure which either causes transcriptional activation or suppression of genes (Mahmood and Rabbani, 2019). Also, several studies have established a correlation between environmental metals and DNA methylation (Baccarelli and Bollati, 2009). Therefore, Sierra *et al*., (2016) reported changes in DNA methylation in response to exposure to nanoparticles. As well, another previous study was observed that the DNA methylation and gene expression levels alteration by titanium dioxide nanoparticles when treated the human cell with (100 mg/mL) of TiO2 for 24 or 72 hours (Pogribna *et al*., 2020).

According to the scientific literature that we have available, the obtained results in this study regarding the following parameters in the rats that exposed to ZnO NPs in different doses and periods of exposure were found out for the first time :

1. Alterations of the structures and functions of the thyroid glands and gained significantly increase in their weights.
2. Reduction in the gene expression of thyroid stimulates hormone receptor gene (TSHR-gene) that present in the tissue of the thyroid gland.
3. Uneven changed in the gene expression of the thyroid hormone receptor gene (THR-gene) that present in the tissue of the ovary.

Overall, according to the obtained results which showed, in addition to the direct effect of ZnO NPs on the structure and function of the studied organs in current research, there is an indirect effect that caused by changes in some parameters which had adverse effect on the others. Whereas, the changes that occurred in the weight and composition of the thyroid gland were identical to the obtained results of thyroid hormones, as the increase in the number of follicles caused an increase in the secretion of thyroid hormones and since the thyroid gland has a major role in the developmental and metabolic processes in every cell of the body. This excessive increase has led to:

- A decrease in average body weights due to an increase in the metabolic processes of the body.
- Also, this increase adversely affected on secretion of the TSH hormone and caused a decline in their level in blood. Consequently, high levels of T3 and T4 hormones and the associated decrease in hormone levels reduced the gene expression of TSHR that present in the tissues of the thyroid gland.

- On the other hand, the excessive secretion of thyroid hormones had a significant effect on the histological and functional of the ovary in addition to their weight, as the effect of thyroid hormones in above-normal quantities caused damage to the ovarian tissue, especially the granulosa cells, which in turn reduced the ability of the ovaries to produce estrogen and progesterone hormones.

Concerning the decrement in the gene expression of THRβ may because of the damage of ovarian tissue which becomes unable to produce the THRβ gene or may be affected by abnormal secretion of thyroid hormones (hyperthyroidism). In contrast, it is possible that the damage in the ovarian structure as well as the decrease in the levels of sex hormones may reduce or inhibit the secretion of LH and FSH hormones as shown in the obtained results.

# Conclusions and Recommendations

# Conclusions

According to the obtained results of this study, we conclude the following:

1. Exposure to ZnO NPs at different concentrations caused structure and functions disorders (time dependent) in the thyroid gland, characterized by enlargement of the gland and increase T3 and T4 hormone levels.
2. Increase T3 and T4 hormones (Hyperthyroidism) inhibit TSH levels in blood as well as caused reduction in the level of gene that responsible for formation of TSHR on the tissue of thyroid gland.
3. Hyperthyroidism causes decrease in the body weight of animals due to administration of ZnO NPs.
4. A significant decrease in the weight of ovary in addition to damage their tissue, which caused a decrease in the production of progesterone and estrogen hormones, this disorder also caused downregulation in the gene expression that responsible for formation of THRβ on the tissue of ovary.
5. High and low dose treatment of ZnO NPs at different periods of time caused reduction in the levels of LH and FSH in blood.

# Recommendations

Depending on the obtained results in the present study, our recommendations required for future studies include the following:

1. genetic study to reveal the extent of the effect of ZnO NPs on the DNA sequence and the problems that inherit to the successive generations from parents that exposure to these nanoparticles.
2. Study the effects of ZnO NPs on the cellular and humoral immunity.
3. Detect the structural and functional changes in other organs.
4. More studies are needed to find out the effect of ZnO NPs on the gene expression of other genes present in the ovarian tissue that are responsible for the formation of receptors such as FSH receptors and LH receptors.
5. Because of the changes that got in organs and their functions that were studied in this experiment, it is possible that these particles cause carcinogenic diseases, therefore a study in this aspect is required.
6. Study the ultrastructure changes of the ovary and thyroid gland caused by ZnO NPs in the cell by an electron microscope.

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# الخلاصة

تعتبر جزيئات اوكسيد الزنك النانوي (ZnO NPs) واحدة من أهم الجسيمات النانوية المستخدمة بشكل واسع في مختلف المجالات بسبب ما تمتلكه من خصائص فيزيائية وكيميائية فريدة وكذلك تكلفة انتاجه المنخفضة. قد تتحلل المنتجات التي تحتوي على أوكسيد الزنك وينتج عنها الجسيمات النانوية نفسها أو تنتج أيونات الزنك الى البيئات وقد تنتقل إلى مجرى دم الإنسان من خلال طرق مختلفة ثم تتراكم في الأعضاء. لذلك، نظرًا لاستخدام هذه الجزيئات بكميات كبيرة ، من الضروري معرفة مدى التاثيرات السامة والضارة لهذه الجزيئات على نمو، تكاثر وحيوية الخلايا والاعضاء. ان الهدف من هذه الدراسة هو تحديد تأثيراوكسيد الزنك على بنية ووظيفة الغدة الدرقية والمبيض بالإضافة إلى تقدير مستوى التعبير الجيني لمستقبلات (TSHR) الموجود في انسجة الغدة الدرقية وكذلك (THRβ) الموجود في انسجة المبيض.

أستخدم ٥٤ من اناث الجرذان البالغة وقسمت بشكل عشوائي إلى ثلاث مجاميع رئيسية وفقًا لفترات التعرض (١, ٢, ٤) اسابيع. كل مجموعة من هذه المجاميع ايضا قسمت إلى ثلاث مجاميع فرعية كل واحدة منها مكونة من ٦ حيوانات. احدهم استخدمت كمجموعة سيطرة وحقنت بـ (١ مل) من الماء المقطر، بينما المجوعتين الاخرى حقنت بـ (١ مل) من محلول اوكسيد الزنك النانوي وبتركيزين مختلفين (٥٠ , ٢٠٠) ملغم/كغم على التوالي ، بمعدل ثلاث جرعات في الأسبوع عن طريق التجويف البريتوني.

بعد انتهاء فترة الحقن وزنت الحيوانات , بعدها تم جمع 4 مل من الدم لاستخدامه في قياس كل من ( TSH, T3, T4, LH, FSH, P, E2) , شرحت الحيوانات وعزلت الغدة الدرقية والمبيض ووضعت بمحلول حفظ لغرض الدراسة النسيجية والجزيئية. كان التحليل الإحصائي للنتائج التي تم الحصول عليها على النحو التالي:

* حصول انخفاض معنوي عالي ($p\leq 0.01$) في وزن الجسم والمبايض لجميع الحيوانات المعاملة بكلا التركيزين من دقائق اوكسيد الزنك النانوي (٥٠ , ٢٠٠) ملغم/كغم في جميع الفترات الزمنية (١, ٢, ٤) اسابيع بالمقارنة مع حيوانات السيطرة.

* حصول زيادة معنوية عالية ($p\leq 0.01$) في وزن الغدة الدرقية في الحيوانات المعاملة خلال الفترات الزمنية (١, ٢, ٤) اسابيع عند التجريع بكلا التركيزين (٥٠ , ٢٠٠) ملغم/كغم بالمقارنة مع حيوانات السيطرة.

* حصول انخفاض غير معنوي ($p\leq 0.05$) في مستويات هرمون (TSH) في الحيوانات المعاملة بكلا التركيزين (٥٠ , ٢٠٠) ملغم/كغم وبفترات زمنية (١, ٢) اسابيع , بينما يوجد هنالك نقصان معنوي ( $p\leq 0.05$) عند المعاملة خلال الفترة الزمنية الطويلة (٤) اسابيع بكلا التركيزين عند المقارنة بحيوانات السيطرة.

* حصول زيادة معنوية عالية ($p\leq 0.01$) في مستويات هرمونات (T3, T4) في جميع الحيوانات المعالجة بكلا التركيزين (٥٠ , ٢٠٠) ملغم/كغم خلال الفترات الزمنية المختلفة (١, ٢, ٤) اسابيع بالمقارنة مع حيوانات السيطرة.

* حصول انخفاض غير معنوي ($p\leq 0.05$) في مستويات هرمونات (LH, FSH, Progesterone) في الحيوانات المعاملة خلال الفترة الزمنية القصيرة (١اسبوع) بدقائق الزنك النانوي بالتركيزين (٥٠ , ٢٠٠) ملغم/كغم , بينما يوجد هنالك نقصان معنوي عالي ($p\leq 0.01$) في مستويات هذه الهرمونات عند التجريع بكلا التركيزين بالفترات الزمنية (٢, ٤) اسابيع بالمقارنة مع حيوانات السيطرة.

* حصول انخفاض معنوي عالي ($p\leq 0.01$) في مستويات هرمون الاستروجين في جميع الحيوانات المعالجة بدقائق اوكسيد الزنك النانوي خلال الفترات الزمنية المختلفة المستخدمة في هذه الدراسة بالمقارنة مع حيوانات السيطرة.

* ظهورتغيرات نسيجية في الاعضاء المدروسة لجميع الحيوانات المعاملة خلال فترات التعرض المختلفة، وهذه التغيرات تزداد تدريجياً مع زيادة تركيز المادة النانوية وفترة التجريع في جميع انسجة الأعضاء التي تم فحصها (المبيض والغدة الدرقية).

* انخفاض واضح في مستويات التعبير الجيني المسؤول عن انتاج (TSHR) الموجود في انسجة الغدة الدرقية في جميع الحيوانات المعالجة خلال الفترات الزمنية المختلفة بالمقارنة مع حيوانات السيطرة.

* انخفاض متفاوت في مستويات التعبير الجيني المسؤول عن انتاج (THRβ) الموجود في انسجة المبيض لجميع الحيوانات المعالجة بدقائق اوكسيد الزنك النانوي خلال الفترات الزمنية المختلفة بالمقارنة مع حيوانات السيطرة.

جمهورية العراق
وزارة التعليم العالي والبحث العلمي
الجامعة المستنصرية
كلية العلوم قسم علوم الحياة

# التأثيرات السمية والوراثية لأوكسيد الزنك النانوي على تركيب ووظائف الغدة الدرقية والمبيض في أناث الجرذان

رسالة مقدمة الى
مجلس كلية العلوم الجامعة المستنصرية
وهي جزء من متطلبات نيل درجة الماجستير في علوم الحياة / فرع الحيوان

من قبل

**فيصل غازي لازم**

بكلوريوس علوم حياة (٢٠١٠-٢٠١١م)
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١٤٤٢هـ ٢٠٢٠م