Investigating the Effect of Pistachio Pericarp Extract on the Level of Oxidative Stress and Structural Changes of the Uterus of Female Mice Exposed to Titanium Dioxide Nanoparticles

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Abstract

Background and Objectives: This study investigates the potential of pistachio pericarp extract to mitigate TiO2-induced uterine damage and oxidative stress in mice, addressing concerns arising from public exposure to titanium dioxide in food and cosmetics.

Materials and Methods: Forty-eight female mice were divided into six groups: a control, a TiO2 group (10 mg/kg/day), three TiO2-exposed groups treated with pistachio pericarp extract (20, 80, and 160 mg/kg/day), and a pistachio pericarp extract group (80 mg/kg/day). On day 28, uteri were dissected, weighed, and fixed for histomorphometry. Hematoxylin-eosin staining was performed to assess uterine thickness and diameter. A portion of the uterine tissue was used to evaluate oxidative stress parameters and apoptotic cells.

Results: Compared to the control group, the TiO2 group exhibited a significant increase in malondialdehyde ($p<0.001$), a significant decrease in superoxide dismutase ($p<0.001$), and a reduction in endometrial thickness ($p<0.001$). Uterine diameter was also significantly reduced in the TiO2 group compared to both the control and pistachio groups ($p<0.001$).

Conclusion: TiO2 can impair fertility by causing oxidative stress and damaging uterine tissue. Treatment with pistachio pericarp extract in mice exposed to TiO2 has been shown to improve uterine tissue and oxidative stress parameters.

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1. Introduction

The significance of fertility and its associated issues as the primary factor influencing population size has resulted in a growing body of research on fertility and related topics [1]. Infertility is a major concern in medical science, impacting approximately 15-20% of individuals in society [2]. Among infertility cases, 40% are attributed to men, 40% to women, 10% to both genders, and in 10% of couples, the cause remains unknown [3].

Evidence suggests that high levels of oxidative stress can lead to pathological processes such as inflammation in uterine tissue, unexplained infertility, and hormonal disorders [4, 5]. Oxidative stress may also disrupt sex hormone production, with an imbalance of hormone levels contributing to female infertility. However, managing oxidative stress can positively influence physiological functions associated with fertility, especially in women [6].

Titanium dioxide (TiO2) nanoparticles are extensively used across various industries, including cosmetics (such as sunscreens and skin bleaches), food, paint, ceramics, and in the purification of water and air. This broad application makes TiO2 a compelling subject for research into its biological functions [7]. The findings indicated that titanium dioxide negatively impacts the reproductive potential of female mice [8, 9].

Herbal medicine has been a longstanding focus for pain relief and the treatment of various ailments [10]. In recent years, substantial research has explored the effects of herbal remedies on different health conditions. One plant that has garnered attention is the pistachio, a member of the Anacardiaceae family, known for its medicinal properties since ancient times. Pistachios are abundant in beneficial compounds, including unsaturated fatty acids, beta-carotene, alpha-tocopherol, flavonoids, and lutein, and they exhibit notable antioxidant and anti-inflammatory properties. Traditionally, various parts of the pistachio plant-such as the resin, leaves, pericarp, and fruit, have been used for medicinal purposes [11].

The pistachio pericarp is rich in various compounds, including saturated fatty acids such as myristic, palmitic, and stearic acids, along with unsaturated fatty acids like linoleic and oleic acids. It also contains plant sterols and essential minerals, including selenium, zinc, calcium, potassium, iron, and magnesium [12]. Previous studies suggest that compounds in pistachio extract may inhibit the production of nitric oxide (NO), a compound that influences steroidogenesis [13, 14]. Consequently, pistachios and their oil have traditionally been used as remedies for sexual health-related conditions [15].

Given the established antioxidant properties of pistachio pericarp extract and its beneficial effects on the reproductive system, this research aims to explore the impact of pistachio pericarp extract on tissue changes, apoptosis in the uterus, and oxidative stress levels in female mice exposed to titanium dioxide nanoparticles.

2. Material and Methods

Animals

Forty-eight female mice, each weighing 25 ± 5 grams, were obtained from the Laboratory Animal Center of Rafsanjan University of Medical Sciences. They were housed in polypropylene cages under controlled conditions, including a 12-hour light/dark cycle,

a room temperature of 22 ± 3 °C, and relative humidity of 40–50%.

The mice had free access to water and standard pelleted mouse food.

Synchronization of the sexual cycle

At the start of this study, the animals were assessed for the regularity of their sexual cycle stages and their weight. To evaluate the regularity of the sexual cycle, vaginal secretions were sampled, and the cells in the smear were examined under a light microscope. In laboratory mice, the estrus period lasts four to five days and consists of four main stages: 1) Proestrus, 2) Estrus, 3) Metestrus, and 4) Diestrus.

To induce the sexual cycle, 100 micrograms of estradiol valerate were dissolved in 0.2 ml of olive oil and injected intramuscularly using an insulin syringe. After 42 hours, each animal received an additional intramuscular injection of 50 micrograms of progesterone. To confirm the sexual cycle, vaginal smears were collected from the mice six hours after the injections. The presence of epithelial cells, leukocytes, and horn cells in the smears from all animals across different groups indicated that all mice were at the same stage of the estrous cycle [16].

TIO2 preparation

After purchasing TIO2 powder (anatase/rutile, 99%, 20 nm, Nanosony Co, Iran) to prepare TIO2 nanoparticles for a study, the powder was dissolved in distilled water. The resulting suspension, with a concentration of 10 mg/kg, was then homogenized using a sonicator for 10 minutes [17].

Pistachio pericarp extraction

To extract the pistachio pericarp, fresh fruit from the pistachio tree (Pistacia vera) was collected and verified by an expert from the Department of Botany at Rafsanjan Valiasr University, Iran (genetic code: M30). An aqueous extract was prepared by shaking 50 grams of ground and sifted pistachio pericarp in 200 mL of water for 48 hours at room temperature. The mixture was then filtered to separate the pistachio pericarp from the water, and the resulting extract was evaporated using a rotary evaporator at 40 °C [18].

Grouping

Female mice were randomly assigned to six groups, each containing eight mice:

I) Control group: received an equal volume of water via gavage.

II) TiO2 exposure group: received TiO2 at a dosage of 10 mg/kg via gavage for 28 days (TiO2),

III) TiO2 and low-dose Pistachio pericarp extract group: received TIO2 at 10 mg/kg and Pistachio pericarp extract at 20 mg/kg via gavage for 28 days (TiO2 + Pis Ext 20),

IV) TiO2 and medium-dose Pistachio pericarp extract group: received TIO2 at 10 mg/kg and Pistachio pericarp extract at 80 mg/kg via gavage for 28 days (TiO2 + Pis Ext 80),

V) TiO2 and high-dose Pistachio pericarp extract group: received TIO2 at 10 mg/kg and Pistachio pericarp extract at 160 mg/kg via gavage for 28 days (TiO2 + Pis Ext 160),

VI) Pistachio pericarp extract group: received Pistachio pericarp extract at 80 mg/kg via gavage for 28 days (Pis Ext 80).

Sampling and Tests

At the end of the 28-day experimental period, all six groups of mice were euthanized through cervical dislocation. The uteri were then removed and placed in Bowen's fixative solution. Hematoxylin and eosin staining was performed on the tissues for histological examination.

Uterus Morphometric Assessment

After hematoxylin-eosin staining, the diameter of the uterus was measured by analyzing ten consecutive longitudinal sections with a light microscope (Olympus BX51, Japan) at 40X magnification. Endometrial thickness was determined from the basal lamina to the highest point of the cells, also using the Olympus BX51 microscope at 40X magnification [19].

Determination of Malondialdehyde (MDA)

MDA serves as an indicator of lipid peroxidation. To assess the level of lipid peroxidation, we measured MDA production in uterine tissue using the thiobarbituric acid reaction method. This process involves mixing the uterine tissue with 2 cc of MDA solution and heating the mixture in a boiler at 100 degrees Celsius for 15 minutes. After heating, the mixture is centrifuged at 10,000 rpm for 10 minutes. Once the microtubes have cooled on ice, we measured the absorbance of the supernatant at a wavelength of 550 nm using an ELISA device (Biottech-ELX-808iu). Finally, we calculated the amount of malondialdehyde and expressed it in nanomoles per milligram of protein, using the Bradford protein assay method to determine protein levels in the tissue [20].

Determination of SOD

To evaluate the superoxide dismutase (SOD) enzyme, we utilized the Zell Bio GmbH kit (Product of Zell Bio, Germany, Cat No: Z185-S) according to the manufacturer's instructions. This kit quantifies the conversion of superoxide anions into hydrogen peroxide and oxygen. SOD activity is measured through the production of superoxide radicals in the xanthine and xanthine oxidase system, which react with Nitro blue tetrazolium (NBT) to generate a purple formazan compound. In this assay, superoxide radicals convert Nitro blue tetrazoliuminto purple NBTH2. When serum is introduced to the test medium, SOD inhibits the formation of the purple color. By measuring the color change at a wavelength of 560 nm, we determine SOD activity as the percentage inhibition of NBTH2 production, with the reaction rate expressed in millimoles per minute [21].

Determination of GSH

Glutathione (GSH) concentration in the uterus (10% homogenate in phosphate-buffered saline, pH 8.0) was measured using the method described by Sedlak and Lindsay (1968). The homogenates were deproteinized with 5-sulfosalicylic acid, and non-protein thiol groups were quantified by reacting with Ellman's reagent. The resulting yellow color was measured spectrophotometrically at 412 nm. Concurrently, a calibration curve was generated using reduced GSH as the standard. GSH concentration was reported as micromoles per gram of tissue (µmol/g tissue) [22].

Determination of CAT

We measured catalase activity using the CAT 240 colorimetric assay kit for catalase activity (Applied Bioanalytical Labs). According to the kit protocol, we first homogenized the uterine tissue and then centrifuged it. CAT activity in the supernatant of the uterine homogenate was assessed using the same CAT 240 colorimetric assay kit, following the manufacturer's instructions. A Hitachi U-2900 spectrophotometer was used for spectrophotometric analysis, and CAT activity was expressed as U/mg of protein. Protein concentrations in the supernatants were determined using the method of Lowry et al. (1951), with bovine serum albumin as the standard [21].

TUNEL assay

To evaluate apoptotic cells in uterine tissue, the sections were first deparaffinized and dehydrated using xylene and a series of ethanol

concentrations (70%, 80%, 90%, and 100%). The slides were then incubated in PBS for 30 minutes, followed by a 15-minute incubation in 3% H2O2 mixed with methanol, and a 20-minute incubation in 0.1% Triton X-100 at a temperature of 2-8 degrees Celsius. After each incubation step, the samples were washed three times with PBS. Next, the slides were treated with TUNEL for 60 minutes at 37 degrees Celsius, followed by a 5-minute incubation with DAB. Finally, the sections were stained with hematoxylin for 5 minutes to visualize the nuclei. Observations were made using a light microscope (Olympus BX51, Japan) at 40X magnification, where normal cells appeared blue and apoptotic cells appeared dark brown [23].

Ethical approval

All procedures involving mice were conducted in accordance with the Ethical Committee of Rafsanjan University of Medical Sciences (Code: IR.RUMS.REC.1402.023).

Statistical analysis

Data analysis was conducted using SPSS software version 20. The Kolmogorov-Smirnov test was used to assess the normality of the quantitative variables, while the Chi-square test was applied to categorical variables. To compare the mean of oxidative stress and apoptotic cell counts across different doses of pistachio pericarp, one-way ANOVA was conducted. A significance level of 0.05 was established for all statistical tests.

3. Results

The results of the oxidative stress parameter, MDA, among the studied groups indicated that the group receiving TIO2 exhibited a higher level of MDA compared to the other groups. This increase was statistically significant when compared to both the control group and the group receiving pistachio 80 (P=0.001). Additionally, the groups receiving TIO2+Pis20, TIO2+Pis 80, and TIO2+Pis 160 also showed a significant increase in MDA levels compared to the control group and the Pis 80 group (P=0.001). (Table1, Figure1)

The statistical analysis of SOD, CAT, and GSH levels across the studied groups revealed a significant decrease in these oxidative stress parameters for the TIO2+Pis20, TIO2+Pis80, and TIO2 groups compared to both the control group and the Pis 80 groups (P=0.001). However, there was no statistically significant difference in superoxide dismutase levels in the TIO2+Pis160 group when compared to the control and Pis 80 group. (Table1, Figure1)

The examination and comparison of endometrial thickness across the studied groups revealed that the groups receiving TIO2+Pis20, TIO2+Pis80, and TIO2 alone exhibited significantly lower thickness compared to the control group and the Pis 80 group (P=0.001). In contrast, the mean endometrial thickness in the TIO2+Pis160 group did not show a statistically significant difference when compared to the control and Pis 80 groups. (Table2, Figure2 and 3)

The measurements of uterine diameter among the studied groups revealed statistically significant reductions in the TIO2+Pis20, TIO2+Pis80, and TiO2 groups compared to the control and Pis80 groups (P=0.001). Additionally, the uterine diameter was significantly lower in the TIO2+Pis20 and TIO2 groups compared to the Pis80 group (P=0.001). (Table2, Figure2 and3)

The analysis of apoptotic cells in the uterine tissue revealed that the group treated with TiO2 exhibited the highest count of apoptotic cells compared to the other groups. A comparison of apoptosis levels indicated that the increases in apoptotic cells in the TIO2+Pis20 group and the

TIO2 group were statistically significant when compared to the control and Pis 80 groups (P=0.001). No other groups showed a statistically significant difference in the number of apoptotic cells relative to the control group. (Figures 4 and 5)

Table1. stress oxidative parameters in all experimental groups.

Groups / OS	Control	Pis Ext 80	TiO2 10	TiO2 + Pis Ext 20	TiO2 + Pis Ext 80	TiO2 + Pis Ext 160
MDA	3.60±0.27	3.31±0.27	6.20±0.43	5.22±0.44	5.08±0.34	4.29±0.27
SOD	6.11±0.33	6.32±0.80	5.00±0.22	5.12±0.38	5.55±0.14	5.77±0.12
CAT	491.60±42.21	479.00±39.64	343.20±43.28	353.80±29.29	430.20±20.81	466.40±19.94
GSH	0.26±0.01	0.28±0.01	0.25±0.01	0.25±0.01	0.26±0.01	0.27±0.01

Data preseted by mean±SD. One Way ANOVA was used for data analysis.OS: Oxidative stress; Pis Ext: Pistachio pericarp extract; TiO2: Titanium di oxide; MDA: malondialdehyde; SOD: superoxide dismutase; CAT: Catalase; GSH: Glutathione

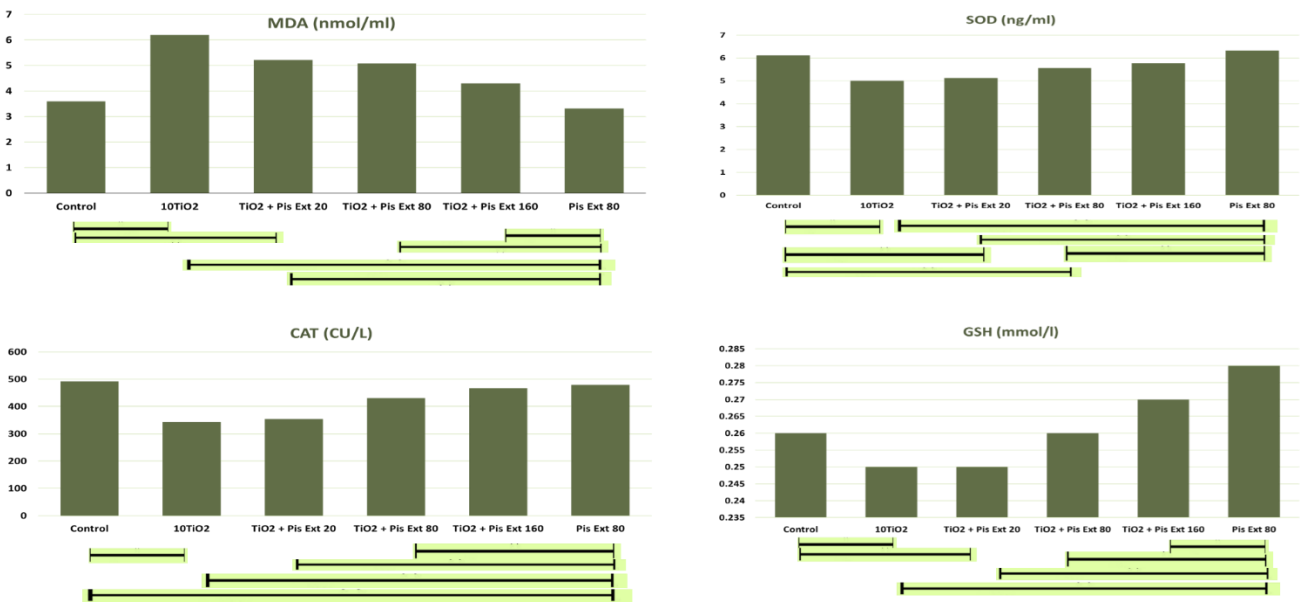

Fig 1. Comparison of oxidative parameters among the experimental groups was conducted using One-Way ANOVA for data analysis, with a significance level set at P≤0.05. OS: Oxidative stress; Pis Ext: Pistachio pericarp extract; TiO2: Titanium di oxide; MDA: malondialdehyde; SOD: superoxide dismutase; CAT: Catalase; GSH: Glutathione

Table 2. Uterus parameters in all experimental groups.

Groups Factors	Control	Pis Ext 80	TiO2 10	TiO2 + Pis Ext 20	TiO2 + Pis Ext 80	TiO2 + Pis Ext 160
Endometrial thickness (µm)	1572.02± 111.59	1525.52± 100.62	921.31± 108.09	1015.08± 110.21	1003.39± 71.71	1444.08± 97.78
Uterus diameter (µm)	8828.41± 214.73	8023.85± 438.64	5756.56± 585.43	5697.43± 128.40	6873.77± 297.85	7332.10± 130.57

Data presented by mean±SD. One-way ANOVA was used for data analysis. Pis Ext: Pistachio pericarp extract; TiO2: Titanium dioxide

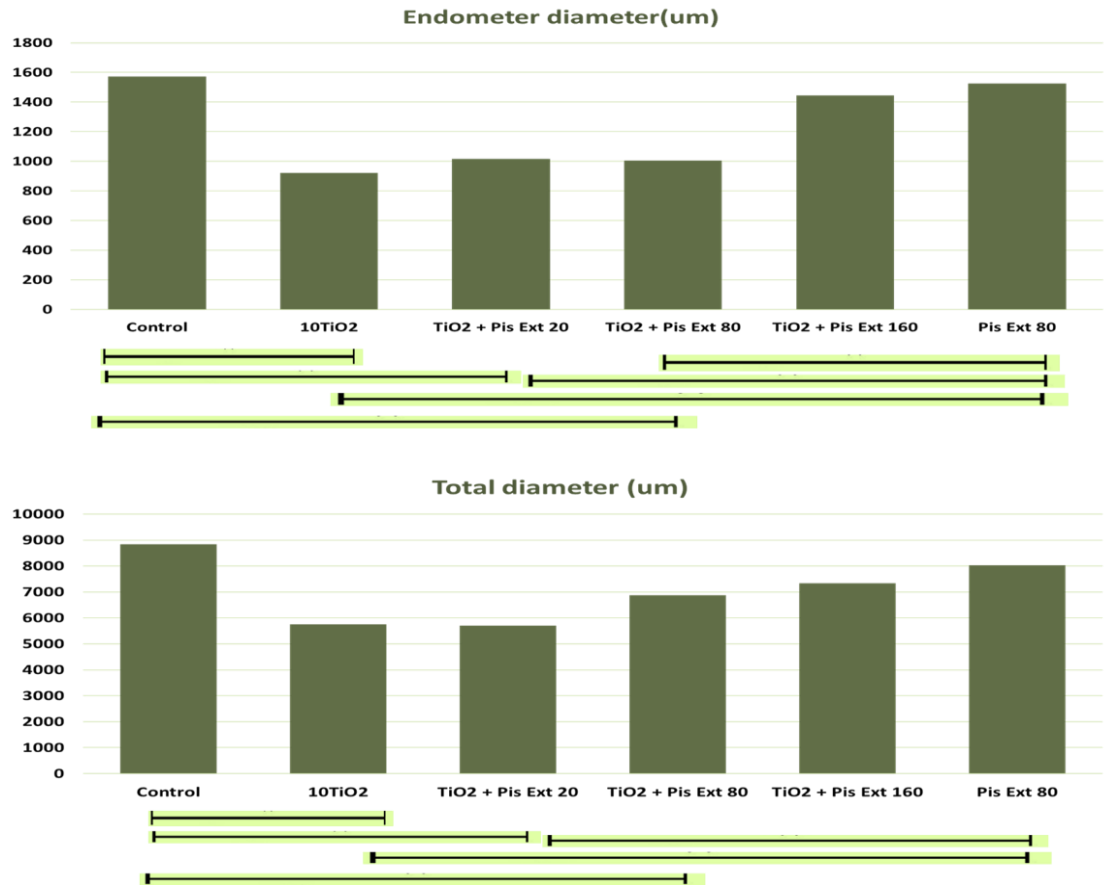

Fig 2. Comparison of Uterus parameters among the experimental groups was conducted using One-Way ANOVA for data analysis, with a significance level set at P≤0.05. Pis Ext: Pistachio pericarp extract; TiO2: Titanium di oxide

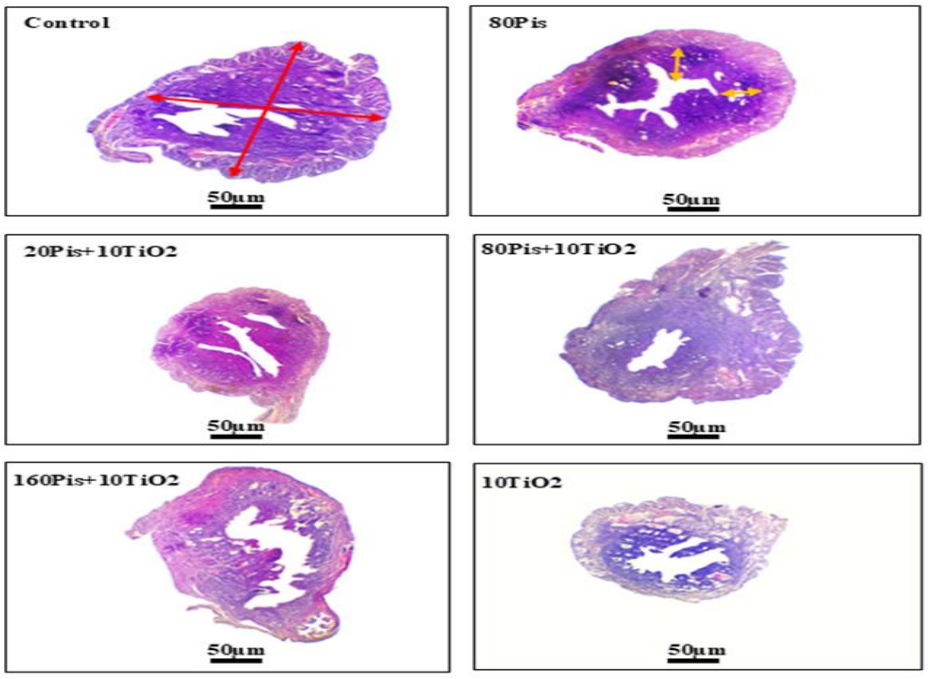

Fig 3. Histochemical H&E assay: Uterus diameter is indicated by the red arrows, while endometrial thickness is shown by the yellow arrow. Pis: Pistachio pericarp extract; TiO2: Titanium di oxide

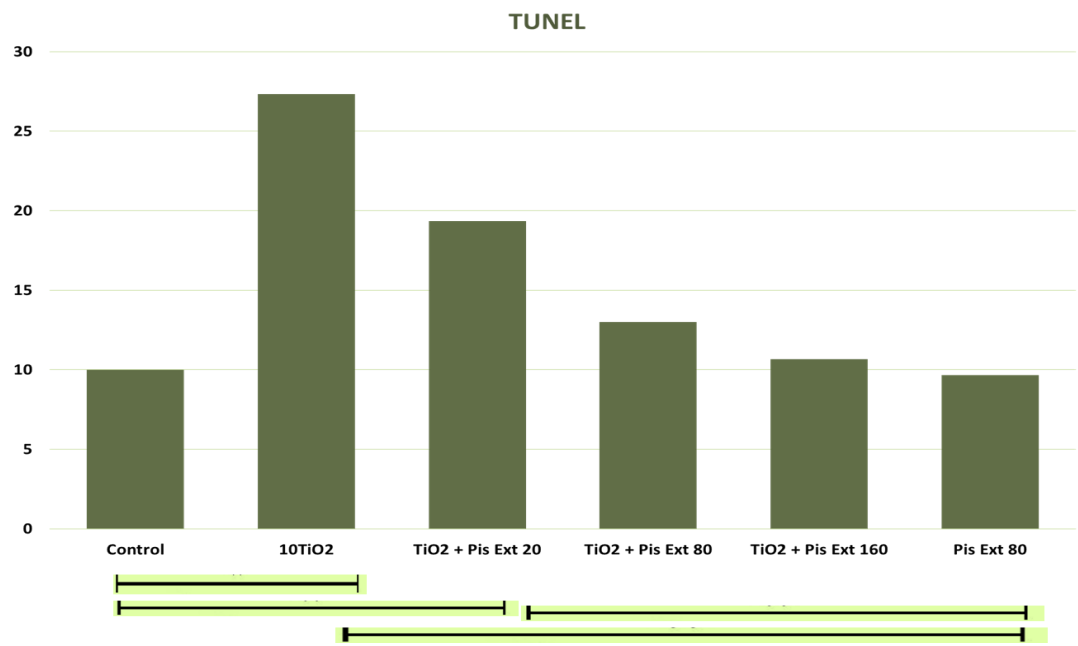

Fig 4. Comparison of TUNEL assay among the experimental groups was conducted using One-Way ANOVA for data analysis, with a significance level set at P≤0.05. Pis Ext: Pistachio pericarp extract; TiO2: Titanium di oxide

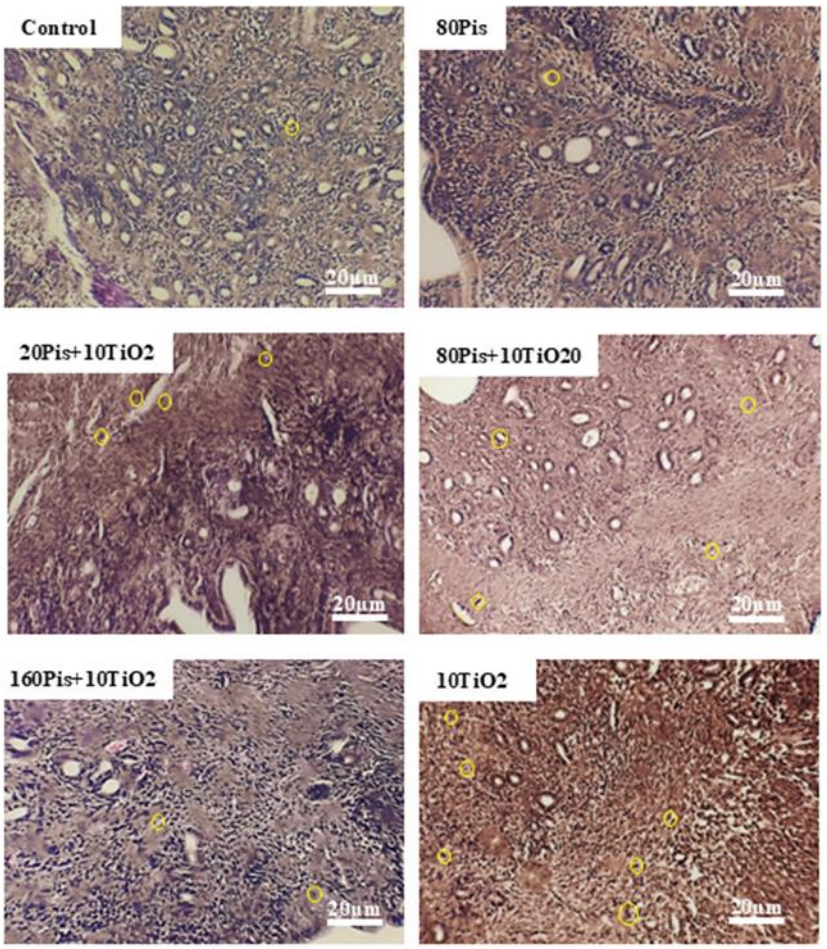

Fig 5. TUNEL assay. Apoptotic cells were shown by yellow circles. Pis: Pistachio pericarp extract; TiO2: Titanium di oxide

4. Discussion

Our findings indicate that chronic exposure to titanium dioxide led to significant changes in oxidative stress parameters, aligning with previous studies. Treatment with pistachio pericarp extract notably reduced these changes, suggesting its antioxidant properties. Mice exposed to titanium dioxide exhibited a significant increase in malonaldehyde levels. Prior research has demonstrated that titanium dioxide elevates reactive oxygen species levels in mice. [24, 25]. Malonaldehyde is produced when reactive oxygen species react with lipids, and its levels rise following an increase in reactive oxygen species [26]. Consequently, malonaldehyde is commonly used as a marker for reactive oxygen species. In our study, analysis of tissue malonaldehyde levels after 4 weeks revealed that the titanium dioxide group had higher levels compared to the control group, consistent with previous findings.

The toxic effects of titanium dioxide exposure in the body can occur through both direct and indirect pathways [27]. Directly, titanium dioxide can enter the bloodstream

following oral contact and be distributed to various tissues, including the liver, spleen, kidneys, lungs, ovaries, and uterus [27]. In a separate study utilizing scanning electron microscopy and transmission electron microscopy, researchers found that short-term (5 days) oral exposure to titanium dioxide nanoparticles in rats resulted in significant accumulation of these nanoparticles in ovarian and uterine tissues [28].

In this study, the accumulation of this nanoparticle in the uterus appears to have caused tissue toxicity, as evidenced by oxidative stress and histological changes. Titanium dioxide indirectly affects uterine tissue by increasing free radical levels and stimulating inflammatory responses. Previous studies have demonstrated that the oral administration of titanium dioxide results in increased levels of inflammatory cytokines, such as tumor necrosis factor alpha, interleukin-6, and interleukin-8, in the blood serum of mice. This suggests a significant inflammatory response [29, 30]. As noted earlier, we observed a significant rise in malondialdehyde levels and a decrease in superoxide dismutase levels, findings consistent with prior research. Thus, oxidative stress induced by titanium dioxide exposure is likely the cause of tissue damage in the female reproductive system. Additionally, treatment with pistachio pericarp extract was effective in modulating oxidative stress levels and preventing further tissue damage in the uterus.

We believe that the antioxidant properties of the pistachio pericarp are due to their active ingredients, particularly quercetin. Quercetin, a flavonoid with notable biological properties, is a key component of pistachio extract. Research has demonstrated that this compound possesses anticancer, anti-inflammatory, antiviral, and antioxidant effects. A 2017 study revealed that quercetin significantly increased both the number and diameter of primary follicles [31]. Li et al. reported that quercetin inhibits prostaglandin production by reducing the activity of the enzymes cyclooxygenase and lipoxygenase, thereby alleviating tissue inflammation [32].

In the present study, the examination of uterine endometrial thickness revealed that exposure to titanium dioxide reduced endometrial thickness, while treatment with pistachio pericarp extract improved it. The observed reduction in endometrial thickness may be linked to an imbalance in sex hormone concentrations.

There is a well-established relationship between endometrial thickness and sex hormone levels [33]. In women, estrogen is crucial for the development of the uterine endometrium, while progesterone is essential for ovulation, implantation, and pregnancy [33]. Additionally, LH and FSH are key hormones in the hypothalamic-pituitary-gonadal axis that regulate gamete production and fertility [33]. Consequently, the decrease in endometrial thickness associated with titanium dioxide exposure may result from impaired secretion of sex hormones such as estrogen, FSH, LH, and progesterone [9, 34].

Gao et al. conducted a study in which female mice were exposed to titanium dioxide nanoparticles at doses of 2.5, 5, and 10 mg/kg via intragastric injection for 90 consecutive days. Following this exposure, they noted significant reductions in body weight, as well as relative ovarian and uterine weights. There was also a decrease in implantation rates and overall

fertility. The researchers found that titanium dioxide accumulated in the ovaries and uterus, leading to altered blood parameters. Furthermore, they observed an increase in serum parameters, sex hormone levels, and the number of atretic follicles, along with signs of inflammation and necrosis in ovarian and uterine tissues. Their findings indicated that changes in the expression of cytokines linked to inflammation and follicular atresia were associated with decreased fertility and tissue damage in the ovaries and uterus of the mice [35]. Our results corroborate these findings.

Intravenous injection of titanium dioxide nanoparticles at a dose of 0.8 mg in pregnant mice resulted in decreased uterine weight, reduced endometrial thickness, and increased implantation loss. This suggests that titanium dioxide can cross the placental barrier, potentially leading to pregnancy complications [36].

Our investigation into apoptosis rates revealed that titanium dioxide nanoparticles elevated apoptosis in uterine tissue. However, treatment with pistachio peel extract was found to mitigate the adverse effects of titanium dioxide and decrease the apoptosis rate in the uterine tissue.

It is well-established that elevated free radical levels induce oxidative stress, initiating a cascade of cellular events that culminate in apoptosis. Increased oxidative stress is also linked to heightened inflammatory activity, cell membrane damage, DNA damage, and other pathological processes that contribute to apoptosis [37]. Consequently, the elevated apoptosis rate observed in the titanium dioxide groups may be attributed to increased oxidative stress levels. Additionally, titanium dioxide seems to induce lipid peroxidation and oxidative damage to cell membranes, leading to alterations in membrane fluidity, membrane potential, permeability, and receptor function. These changes can exacerbate tissue damage and further increase the rate of apoptosis.

5. Conclusion

The female population is particularly vulnerable and deserves special attention, as exposure to toxins can negatively impact reproductive development and fetal growth. However, there is a lack of studies investigating the potential toxic effects of titanium dioxide nanoparticles on the female reproductive system. Pistachio pericarp extract may provide protective benefits against the effects of titanium dioxide in the female uterus, likely due to its high content of various antioxidant components with therapeutic properties. Nevertheless, further research is essential to clarify the specific cellular and molecular signaling pathways that contribute to this protection in females.

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Conflict of Interest

The Authors declare no conflict of interest.

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