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Histomorphometric study of the protective role of purslane seed extract on bone development of rat fetuses maternally treated with titanium dioxide nanoparticles.

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Abstract: Titanium dioxide nanoparticles (TiO2NPs) are widely used in many commercial products. Early developmental stages of rats are sensitive to TiO2NPs especially during bone development. Purslane (P) seeds are used as natural source of antioxidants. The present study was designed to evaluate the possible protective role of P seed extract on the structure of femur tissue of 20-day old fetuses maternally treated with TiO2NPs during pregnancy. Thirty-two pregnant rats were randomly divided into four groups. Group (C): untreated control. Group (P): rats were treated with 10 ml/kg/day P extract. Group (T): rats received 0.5mg/kg/day TiO2NPs. Group (P+T): rats received P extract, followed by TiO2NPs at the same previous dose. All doses were orally administered from the 6th to the 15th day and rats were sacrificed on the 20th day of gestation. The fetuses were exposed and fetal hind limbs were cut and fixed in formol then decalcified and stained for histological study. Titanium dioxide nanoparticles induced alterations in the fetal femur tissue as incomplete chondrification of mesenchymal cells, few cartilaginous cells in resting zone, decreased width of proliferation zone, decreased size of chondrocytes in the hypertrophic zone as well as degenerated chondrocytes in calcification zone. However, P improved the destructive changes caused by TiO2NPs. The length and width of femur and tibia bones of P treated group as well as distribution of collagen fibers was improved. Therefore, it is recommended to be very careful while dealing with nanomaterials. It is preferred for eating leafy green vegetables rich in antioxidants during pregnancy.

keywords: Nanoparticles – Titanium dioxide –Fetus – Bone -Purslane.

1.Introduction

The use of nanotechnology and the production of nanoparticles (NPs) have created new hope for solving human problems (1). Modern human industry widely used titanium dioxide nanoparticles (TiO_2NPs), mainly anatase form in many products including food, cosmetics, sunscreen and medicine **(**2**)**. Titanium dioxide NPs can cause oxidative DNA damage, impairment of anti-oxidative capacity and increased production of reactive oxygen species (ROS) through lipid peroxidation and increased hydrogen peroxide and nitric acid production **(**3**)**. The toxicity of TiO_2 also can induce damage to the liver, lung, kidney, spleen, heart, brain, testis and ovary of mice or rats **(**4**)**.

Early developmental stages of rats are particularly sensitive to TiO_2NPs. Administration of TiO_2NPs to pregnant mice was transferred to the offspring and affected various developmental processes during the embryonic period, which results in a reduction of offspring quality **(**5**)**.

Bone is a complex living organ that is made up of many cells, protein, fibers and minerals. The skeleton acts as a scaffold by providing support and protection for the soft tissues, also provides attachment points for muscles to allow movements at the joints **(**6**)**.

Bone is a specialized connective tissue, which classify either as compact (dense) or spongy (cancellous). The compact, which is

dense layer, the sponge like meshwork bone consists of trabeculae localized in the inner side of the bone. The spaces within the meshwork are continuous and in a living bone are occupied by marrow and blood vessels **(**6**)**.

The long bone consists of three major components diaphysis, epiphysis at each end of the bone and epiphyseal growth plate (GP). The diaphysis is composed of compact bone. The epiphysis is composed of cancellous or spongy bone, which has many spaces or cavities within the bone matrix. The GP is the site of major bone elongation and when bone growth stops, the GP becomes ossified and is called the epiphyseal line **(**7**)**.

Most bones develop from an initial cartilage model. Once the cartilage model has been formed, the osteoblasts gradually replace the cartilage with bone matrix through a process known as endochondral ossification (8**)**.

The cartilage model once formed is invaded first at its centre and later at each end by a mixture of cells that establish the primary and secondary centers of ossification **(**6**).**

Ossification of the cartilage model is preceded by hypertrophy of the chondrocytes in the prospective mid-shaft of the bone and deposition of a periosteal bone collar by recently differentiated osteoblasts surrounding the mid-shaft. Blood vessels, osteoclasts (cartilage- and bone-resorbing cells), as well as bone marrow and osteoblast precursors then invade the model from the bone collar and proceed to form the primary ossification center. The primary center expands towards the ends of the cartilage model, as the osteoclasts remove cartilage extra cellular matrix (ECM) and osteoblasts deposit bone on cartilage remnants **(**9**)**.

The GP is divided in to five recognizable zones of resting, proliferation, hypertrophy, calcification and ossification zone. In the resting zone, the chondrocytes are distributed randomly throughout the matrix and are mitotically active. In the proliferative zone, the chondrocytes form rows of cells that parallel the direction of bone growth. Zone of maturation and hypertrophy appear with mature chondrocytes which undergo apoptosis and die. In zone of calcification, the lacunae become confluent, hypertrophied chondrocytes die and cartilage matrix becomes calcified. The last ossification zone in which osteoprogenitor cells invade the area and differentiate into osteoblasts which elaborate matrix on the surface of calcified cartilage **(**10**)**.

Portulaca oleracea L. (purslane) is extensively used not only as an edible plant but also as a medicinal plant **(**11**)**. The seeds have muscle relaxant, anticonvulsive, analgesic **(**12**),** anti-oxidative **(**13**),** anti-inflammatory and anti-cancer properties **(**14**)**. Purslane is a rich source of omega-3-fatty acids, α-tocopherols, ascorbic acid, β-carotene and glutathione; the seeds also contain a high percentage of α-linolenic acid **(**15,16**)**.

The aim of this study was to evaluate the effect of water extract of purslane seeds on the structure of the growing femur bone of 20-day old fetuses maternally treated with TiO_2NPs during pregnancy.

2. Materials and methods

Ten mature fertile male and 50 virgin female albino rats (12 weeks old, 180 ± 200 g) were purchased from the Laboratory Animal Colony, Helwan. The males were kept separated from females until mating. All rats were kept under strict care and hygienic conditions of temperature, relative humidity and a 12-hr. light/dark cycle. They fed on food pellets from the Factory of Oil and Soap Company, Cairo, Egypt, as well as some vegetables as a source of vitamins were available *ad libitum* with drinking tap water. The rats were acclimated to the laboratory environment for one week. Finally, female rats were mated overnight (each male rat was mated with 2 females). Vaginal smears were checked daily for finding a vaginal plug and that day was considered as gestational day (GD) one.

Titanium dioxide nanoparticles

Titanium dioxide NPs used in this study were a kind of nano powder, anatase, with a particle size of <25 nm, purity 99.7% trace metals basis (SIGMA-ALDRICH). The TiO_2NPs were suspended in ultrapure water (Promega, Madison, WI, USA) at a concentration of 20 mg/ml as a stock solution. The stock solution was dispersed by

an ultrasonic vibrator for 30 min., after which the suspension was diluted in 1× Holt buffer (60 mmol/L NaCl, 0.67 mmol/L KCl, 0.3 mmol/L $NaHCO_3$, 0.9 mmol/L $CaCl_2$, pH 7.2) to a working concentration of 0.5 mg/L (17). The dose for rats was calculated according to **Paget and Barnes** formula **(**18**)**. Titanium dioxide NPs were orally administered at a dose of 0.5 mg/kg/day from day 6 to day 15 of gestation.

Preparation of purslane seeds extract

Purslane seeds were purchased from the seed seller, Cairo, Egypt. It was authenticated by the botanists (Botany Department, Faculty of Science, Al-Azhar University, Cairo, Egypt). One liter of boiled distilled H_2O was added to 100 g of grinded purslane seeds, cooled and filtered. The concentrated yield extract was diluted with distilled H_2O for the desired volume (1:10 wt/vol.) **(**19**)**. Purslane seed extract was administered at a dose of 10 mL/kg b.w./day by oral gavage from day 6 to day 15 of gestation.

Experimental design

Thirty-two pregnant females were randomly divided into four groups (8 animals each) as follows:

- **Group (C):** Untreated control in which pregnant rats were fed on normal diet.
- **Group (P):** Rats were treated orally with aqueous purslane seed extract at dose of 10 mL/kg/day.
- **Group (T):** Animals received orally 0.5mg/kg b.w./day TiO_2NPs.
- **Group (P+T):** Rats received orally purslane seed extract followed by TiO_2NPs at the same pervious dose.

All doses were given from the 6^{th} to 15^{th} day and the rats were sacrificed on the 20^{th} day of gestation.

Histological study:

On the 20^{th} day of gestation, all pregnant rats were sacrificed after being anaesthetized with ether inhalation. The intact uteri were removed and the fetuses were detached. The hind limbs of fetal rats of all four groups were dissected. The femur bones were trimmed from muscle tissue and fixed in neutral buffer formol then decalcified. The specimens of decalcified femur bones were dehydrated in ascending grades of alcohol, cleared in xylene, embedded in paraffin wax, sectioned at 7 µ thicknesses then stained with Haematoxylene and Eosin **(**20**)** for general structure and Mallory triple stain **(**21**)** for demonstrating collagen fibers. Carl Zeiss light microscope and its digital camera were used for examining and capturing images from the stained histological sections. Images were saved as jpg at 10, 20 and 40X objective magnifications for morphological evaluation at the Faculty of Medicine, Al-Azhar University for Girls.

Morphometric study:

Femur length was measured from the proximal end of the shaft to lateral condyle. Tibia length was measured from the proximal end to the distal end. The width of both femur and tibia was measured at the medial diaphyses. **(**22**)**. The length and width of the fetal femur and tibia bones stained with alizarin red S and alcian blue double staining were measured on 8 jpg photos at 10x. The density of collagen fibers of bone tissue was measured on 5 serial sections stained with Mallory triple from 8 slides at 100x. The length, width and density of collagen fibers were measured using IPWIN32 image analysis software.

Statistical analysis

Results were expressed as mean ± standard error (SE). The obtained data were analyzed using the one-way analysis of variance (ANOVA) **(**23**)** followed by Post HOC tests (LSD) analysis to compare various groups with each other.

Results

Longitudinal sections of control fetal femur bone stained with H&E showed that the GP is divided to five zones. The first zone was the resting zone consisted of hyaline cartilage with small inactive and irregularly arranged chondrocytes in their lacunae. The chondrocytes were embedded in transparent basophilic matrix. The second proliferation zone appeared with increased number of chondrocytes arranged in parallel rows and separated by bars of matrix **(Plate 1A)**. Many mitotic figures were demonstrated in proliferation zone. The third zone was hypertrophy which showed enlarged, vacuolated chondrocyte separated by minimal

amount of matrix. The calcification zone showed various degrees of chondrocyte apoptosis due to calcification of the cartilaginous matrix. The chondrocytes became small in size with small pyknotic nuclei and vacuolated cytoplasm. The chondrocyte lacunae fused with each other forming empty spaces. After invasion of these empty spaces by vascular tissue rich in osteogenic cells and blood capillaries, ossification started. The last zone was ossification zone which appeared with well-developed periosteum and sub periosteal bone collar. Many osteogenic cells and blood capillaries appeared in well-developed spongy bone trabeculae separated by bone marrow spaces (Plate 2A). The anastomosed bone trabeculae were composed of osteocytes which appeared as oval cells housed in lacunae and embedded in acidophilic bone matrix. The osteoblasts appeared side by side, in a way that resembles simple epithelium covered the bone trabeculae. The medullary cavity was filled with red marrow formed of precursors of blood cells (Plate 3A).

These all zones did not show obvious difference in histological findings in P extract treated group (Plate 1B, 2B&3B).

Examination of fetal femur of TiO_2NPs treated group showed irregularity and shortening of the femur as well as incomplete chondrification of mesenchymal cells. In the resting and proliferation zones, the cartilaginous cells became scattered few (Plate 1C). The proliferation zone showed few, small and irregularly arranged chondrocytes. The femur of fetal rats showed few, small and degenerated cells in the hypertrophic zone with the absence of mitotic figures. The calcification zone resulted in very few empty lacunae (Plate 2C). The ossification zone appeared with failure in formation of periosteum and sub periosteal bone collar. The irregular thin bone trabeculae were separated by small bone marrow spaces with a complete failure of ossification. The osteocytes were abnormal small cells with shrunken nucleus. The osteoblasts were absent or rarely seen on the surface of bone trabeculae (Plate 3C).

Fetal rats maternally treated with P+T exhibited moderate improvement in the process of bone ossification. The resting zone and proliferation zone appeared more or less similar to that of control. In comparison with T treated group, the thickness of resting and proliferation zones was improved (Plate 1D). The number of chondrocytes was increased. Moderate improvement in calcification zone and zone of ossification was detected with the presence of thick and confluent trabeculae enclosing haemopoietic cells in bone marrow cavity. The ossification zone appeared with well-developed periosteum and sub periosteal bone collar (Plate 2D). The bone trabeculae recovered their confluence with the appearance of haemopoietic cells in bone marrow cavity. The osteocytes were more or less normal and well-arranged osteoblasts were clearly seen (Plate 3D).

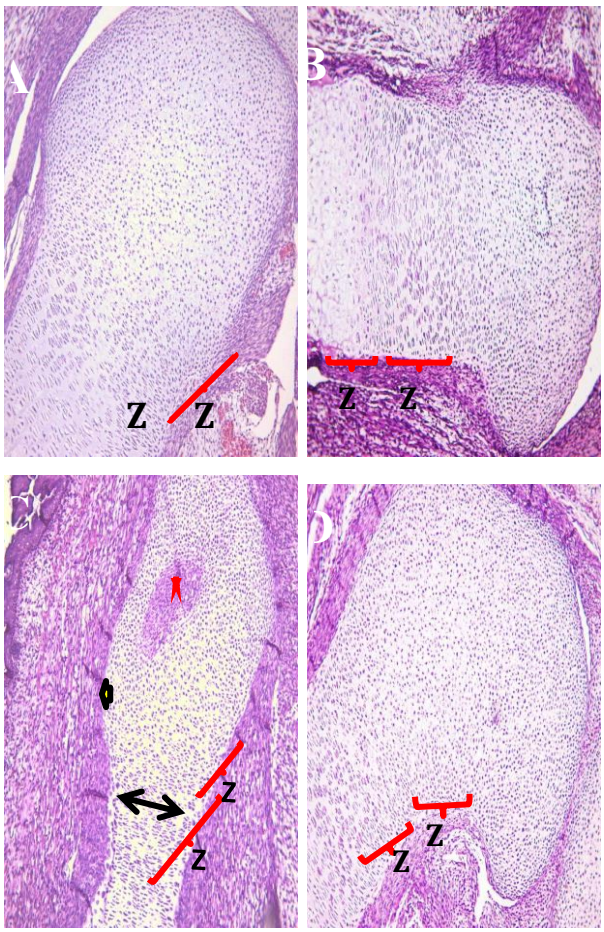


Plate 1: Photomicrographs of longitudinal sections of fetal femur of 20-day old fetuses showing: A and B (Control and P extract groups): zone of resting (ZR) and zone of proliferation (ZP). C; Titanium group: incomplete chondrification of mesenchymal cells (red star), irregular shape of diaphysis (arrow head), scattered few cartilaginous cells in zone of resting (ZR) as well as decreased width (double headed arrow) of the

proliferation zone (ZP). **D; P+T group:** moderate improvement in resting zone (ZR) proliferation zone (ZP) **(H & E X100)**.

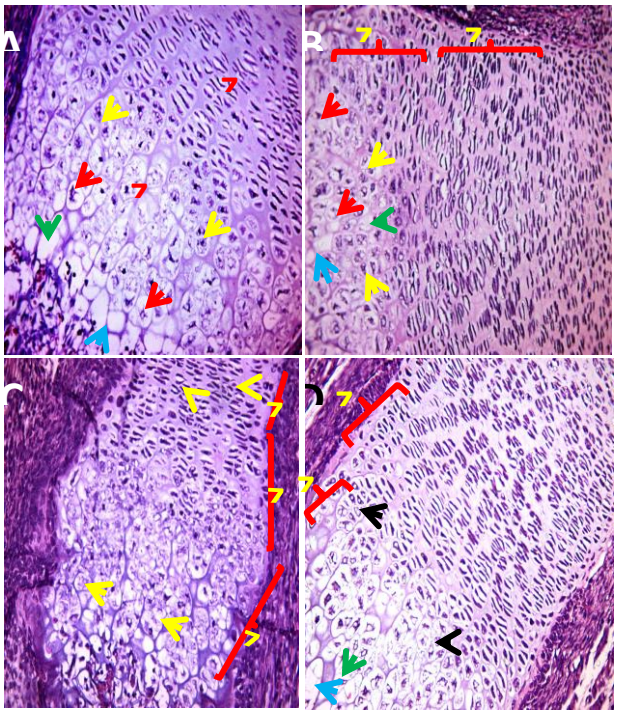


Plate 2: Photomicrographs of longitudinal sections of fetal femur of 20-day old fetuses showing: A and B (Control and P extract groups): regular arrangement of chondrocytes in the proliferating zone (ZP), increased size of chondrocytes in the hypertrophic zone (ZH), dividing cells (yellow arrows). Degenerated chondrocytes (red arrows), complete apoptosis of chondrocytes (green arrow) and fusion of lacunae (blue arrow).C; Titanium group: few ,small and irregularly arranged chondrocytes (arrow heads) in the proliferating zone (ZP), small degenerated cells in the hypertrophic zone (ZH) as well as few empty lacunae (yellow arrows) in calcification zone (ZC) D; P+T group: well organized chondrocytes in the (ZP), increased size of chondrocytes (black arrows) in (ZH), apoptosis of chondrocyte (green arrow) forming empty lacuna (blue arrow) (H&E, X200).

Plate 3: Photomicrographs of longitudinal sections of fetal femur of 20-day old fetuses showing A and B (Control and P extract groups); bone trabeculae (yellow stars), haemopoietic tissue (H), osteocytes (blue arrows), osteoblasts (red arrows), well developed periosteum (P) and sub periosteal bone collar (SP). C; Titanium group: thin and irregular bone trabeculae (yellow stars), complete failure in ossification (red star), abnormal and small osteocytes (blue arrow), abnormal osteoblasts (red arrow). D; P+T group: thickened bone trabeculae (yellow stars), haemopoietic cells in bone marrow cavity (blue star), more or less normal osteocyte (blue arrow) as well as well-arranged osteoblast (red arrow). Notice moderately well-developed periosteum (P) and sub periosteal bone collar (SP) (H&E, X200).

Staining of the fetal femur tissue by Mallory stain in control group showed normal distribution of collagen fibers in ossification zones (Plate 4A). Minimal collagen fibers also appeared in the calcification zone in bone trabeculae and around the haemopoietic tissue (Plate 5A). More or less normal distribution of collagen fibers was seen in sections of P extract treated group (Plate 4&5B). While T treated group exhibited decreased amount of collagen fibers in the resting, proliferating and maturation and hypertrophy zones (Plate 4C). Decreased amount of the collagen fibers was observed in the zone of calcification, in the bone trabeculae and around the haemopoietic tissue (Plate 5C). The amount of collagen fibers increased in P+T treated group in resting, proliferating and hypertrophy zones (Plate 4D), also increased in calcification zone especially in the bone trabeculae, around the haemopoietic tissue in addition to periosteal bone collar compared to TiO_2NPs treated group (Plate 5D).

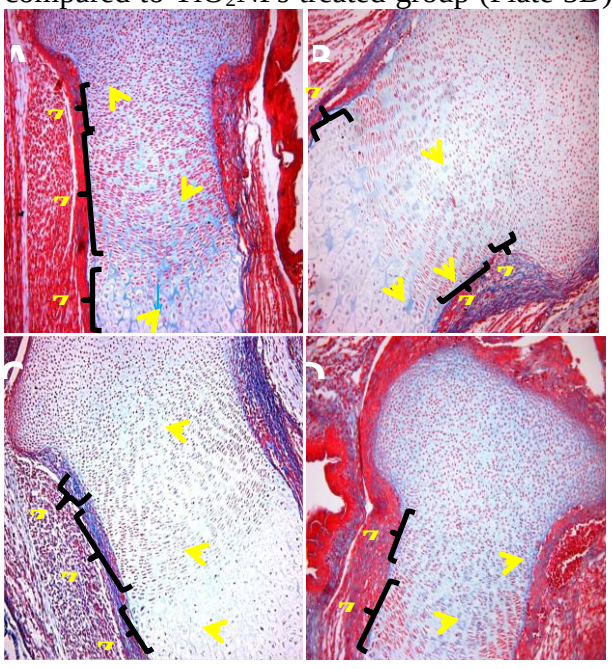


Plate 4: Photomicrographs of longitudinal sections of fetal femur of 20-day old fetuses

showing: A and B (Control and P extract groups); normal distribution of collagen fibers (yellow arrows) in resting zone (ZR), proliferation zone (ZP) and hypertrophy zone (ZH). C; Titanium group: decreased amount of collagen fibers (yellow arrows) in (ZR), (ZP) and (ZH). D; P+T group: increasing the amount of collagen fibers (yellow arrows) in

(ZR), (ZP) and (ZH) (Mallory triple, X100).

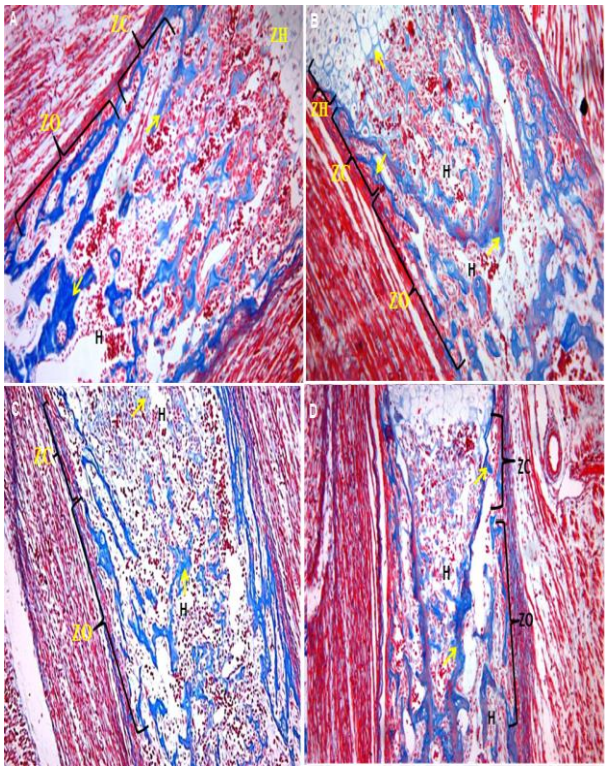


Plate 5: Photomicrographs of longitudinal sections of fetal femur of 20-day old fetuses showing**:** A and B (Control and P extract

groups): normal distribution of collagen fibers (yellow arrows) in hypertrophy zone (ZH),calcification zone (ZC) and ossification zone (ZO), also in bone trabeculae and around the haemopoietic tissue (H) C; Titanium group: decreased amount of collagen fibers (yellow arrows) in (ZC),(ZO), and around haemopoietic tissue (H) D; P+T group: increase in the amount of collagen fibers (yellow arrows) in (ZC), (ZO) and around haemopoietic tissue (H)(Mallory triple, X100).

The femur tissue of rat fetuses maternally treated with P extract showed non-statistically significant difference in the density of collagen fibers as compared to control group (P≤0.05). In contrast, a significant decrease in

the density of collagen fibers was observed in TiO_2NPs treated group in comparison with the control (P≤0.05). However, the density of collagen fibers was non-significantly increased in P+T treated group in comparison with TiO_2NPs treated group (Table 1 and Histogram 1).

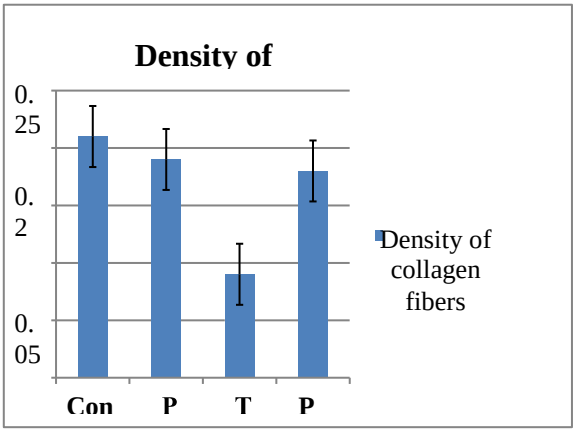
Table 1: Density of collagen fibers in the fetal femur bone of control and different treated rat groups:

Groups	**Density of collagen fibers**
	Mean ± S.E
Control	0.21±0.03
P	0.19±0.02
T	0.09±0.01[ab]
P+T	0.18±0.01
Significance between groups	P≤0.01
F- value	6.1

All results represent Mean ± SE of 8 animals.

[a]Significant when compared to control group (P ≤ 0.05).

[b]Significant when compared to treated group (P ≤ 0.05). Means, which have the superscript symbol (N.S.), are not significantly different. Where: P= Portulaca. T= Titanium. P+T= Portulaca +titanium.



Histogram 1: The mean values of the density of collagen fibers in the fetal femur bone of control and different treated rat groups.

In the present study, the length of femur and tibia of fetal rats from control and different treated groups were measured.

There was no significant change in the mean values of femur length of P extract group compared to control group. Fetal rats maternally treated with TiO_2NPs from day 6 to day 15 of gestation exhibited a significant

decrease in the mean value of femur length (P≤0.05). The mean values of fetal femur length showed no significant change in P+T treated group compared to control group.

The tibia length was non significantly increased in P extract group than that of T or P+T treated groups compared to control group (Table 2 and Histogram 2).

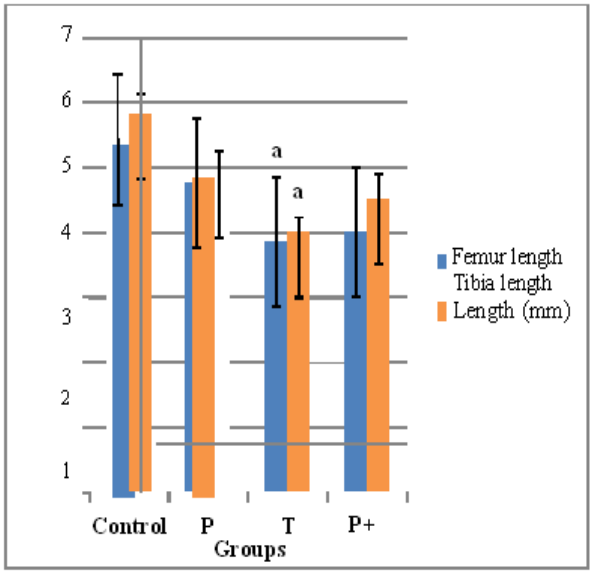
Table 2: Effect of TiO_2NPs on the length of femur and tibia of control and different fetal rat groups:

Groups	Length (mm)	
	Femur length	Tibia length
	Mean ± S.E	Mean ± S.E
Control	5.43±0.32	5.82±0.31
P	4.76±0.19	4.92±0.34
T	3.86±0.09 [a]	4.00±0.23 [a]
P+T	4.01±0.20	4.51±0.39
Significance betweengroups	P≤0.001	P≤0.01
F- value	11.3	5.7

All results represent Mean ± SE of 8 animals.

[a]Significant when compared to control group (P ≤ 0.05).

Means, which have the superscript symbol (N.S.), are not significantly different. Where: P= Portulaca. T= Titanium. P+T= Portulaca +titanium.



Histogram 2: The length of femur and tibia of control and different fetal rat groups.

The width of femur and tibia bones of fetal rats of control and different treated groups were measured. Fetal rats maternally treated with P extract detected non-significant change in the width of femur and tibia bones compared to control. On the other hand, a significant decrease in the width of femur and tibia bones was demonstrated in TiO_2NPs treated group. The mean values of femur and tibia width detected non-significant change in P+T treated group in comparison with the **control (Table 3 and Histogram 3).**

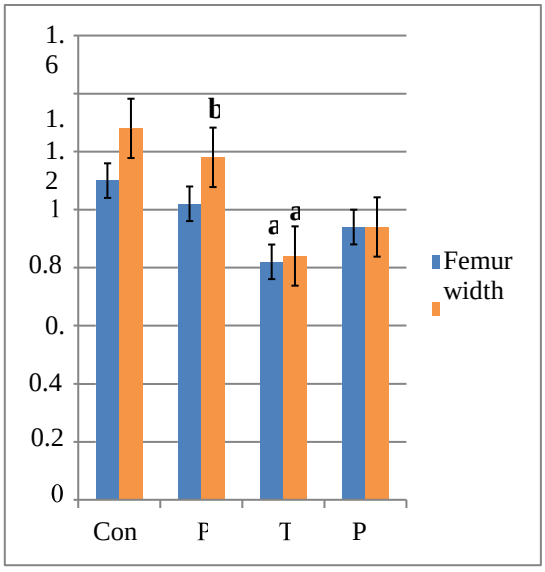
Table 3: Effect of TiO_2NPs on the width of femur and tibia of control and different fetal rat groups:

Groups	Width (mm)	
	Femur width	Tibia width
	Mean ± S.E	Mean ± S.E
Control	1.10±0.10	1.28±0.11
P	1.02±0.08	1.18±0.09[b]
T	0.82±0.09[a]	0.84±0.08[a]
P+T	0.94±0.09	0.94±0.06
Significance between groups	N.S	P≤0.01
F- value	1.7	5.7

All results represent Mean ± SE of 8 animals.

[a]Significant when compared to control group (P ≤ 0.05).

[b]Significant when compared to treated group (P ≤ 0.05). Means, which have the superscript symbol (N.S.), are not significantly different. Where: P= Portulaca. T= Titanium. P+T= Portulaca +titanium.



Histogram 3: The width of femur and tibia of control and different fetal rat groups.

Discussion:

Recently, the applications of nanotechnology can be observed in various fields as medicine, pharmacy, environmental protection, agriculture etc. With increasing scope of NPs applications, the exposure of humans to them is inevitable. Many studies revealed that, after inhalation or oral exposure, NPs accumulate in many organs, lungs, alimentary tract, liver, heart, spleen, kidneys and cardiac muscle. In a wide group of NPs currently used, TiO_2NPs are particularly popular **(**4**)**.

Bones are the site of attachment for tendons and ligaments, providing a skeletal framework that can produce movement through the coordinated use of levers, muscles, tendons and ligaments **(**24**)**

Plants are natural source of antioxidant. Purslane has high nutritional value and its seeds have been used in the traditional medicine **(**12**)**.

Therefore, the present study aimed to study the protective role of purslane seed extract on the development of fetal bones maternally treated with TiO_2NPs during pregnancy.

In human, bones begin to form *in utero* in the first eight weeks following fertilization, while development and ossification in rodents and rabbits occurs in the perinatal period **(**25**)**.

In the present study, treatment with TiO_2NPs from 6^{th} to the 15^{th} day of gestation revealed severe destructive changes in the femur bone structure. Gestational exposure to TiO_2NPs resulted in incomplete chondrification of mesenchymal cells. Resting and proliferation zones appeared with apparent decrease in the number of small and irregularly arranged chondrocytes. The femur of fetal rats showed few, small and degenerated cells in the hypertrophic zone with the absence of mitotic figures. The calcification zone resulted in very few empty lacunae. The ossification zone appeared with failure in ossification of periosteum and sub periosteal bone collar.

Bone trabeculae were irregular, thin and separated by small bone marrow spaces with decreased haemopoietic tissue in the medullary cavity. The osteocytes were abnormal small cells with shrunken nucleus and the osteoblasts were absent or rarely seen on the surface of bone trabeculae. These results may be attributed to reduction of Ca levels after exposure to TiO_2NPs in fetuses which may be an important aspect of TiO_2NPs induced toxicity

These results are in agreement with the study of Hong *et al*. **(**26**)** who reported that, exposure to TiO_2NPs may decrease Ca absorption and increase excretion leading to a negative Ca balance and a subsequent reduction in Ca levels in the embryo. Also, maternal TiO_2NPs exposure interfered with metabolism in embryos and thus affected ossification in the fetus. Nano-TiO_2 may bind to proteins on the cell membrane, thereby changing membrane function and signal transduction resulting in toxicity.

Nanoparticles interact with bone cells and tissue depending on their composition, size and shape **(**27**)**. With respect to NP size, titania NPs of up to 40 nm diameter decreased osteoblastic cell proliferation and viability **(**28**)**. Xu *et al*. **(**29**)** added that, the inhibition of bone Ca deposition may lead to the formation of a barrier in the ossification center during bone development. On the other hand, damage to osteoblasts and chondrocytes as well as increased osteoclast activity may result in an increase in bone Ca dissolution. Also, Xie *et al*. **(**30**)** declared that, silver nanoparticles (AgNPs) could adhere to cell membrane easily. This may be due to high surface activity and then interact with membrane proteins which lead to the damage of cell membrane.

The current results showed moderate improvement in the histological structure of the femur of rat fetuses maternally treated with P extract followed by T. The improvement may be due to the antioxidant effect of P that ameliorates the adverse effects caused by TiO_2NPs. This result is in agreement with the result obtained by Kim *et al.* **(**31**)** who proved that, P decreased bone resorption activity of mature osteoclasts, which was accompanied by a rapid disruption of the actin ring structure in mature osteoclasts *via* the regulation of osteoclast-specific genes. They also detected that; P may be useful in preventing or treating various destructive bone diseases. Moreover, Rahimi *et al*. **(**14**)** reported that, purslane seed oil (PSO) showed superior anti-oxidant activity

due to its high omega-3 fatty acid content. Thus, it is a good candidate as both a healthy food and cosmetic ingredient.

The current study demonstrated that, gestational exposure to TiO_2NPs exhibited decrease in the amount of collagen fibers in the fetal femur stained with Mallory compared to control. This may be due to diffusion of NPs in the ECM which consists of collagen fibers. Lieleg *et al.* **(**32**)** have shown decrease in the diffusion coefficient of positively and negatively charged NPs in reconstituted ECM hydrogels due to electrostatic attraction and binding. The same authors detected that, the ECM presents an effective electrostatic bandpass, suppressing the diffusive motion of both positively and negatively charged objects. This mechanism allows uncharged particles to easily diffuse through the matrix, while charged particles are effectively trapped.

Rat fetuses maternally treated with P extract revealed more or less normal distribution of collagen fibers compared to control. This may be due to various active components of P including alkaloids, fatty acids, flavonoids, polysaccharides and terpenoids (33,34**)**. The antioxidant effect of P may improve the distribution of collagen fibers.

Fetuses maternally treated with TiO_2NPs recorded significant decrease in both length and width of femur and tibia in comparison to control. This may be due to the sensitivity of the developing skeleton, particularly growth of cartilage, to TiO_2NPs. Charuta *et al.* (35) indicated that bone length is related to sexual dimorphism. In contrast, Van Wyhe *et al.* (36) suggested that, increase in bone length would be expected to correlate with the bone width indicating the overall bone size.

Treatment with P extract detected non-significant change in the mean values of length and width of femur and tibia bones of fetuses compared to control. This may be due to the antioxidant effect of P on the developing skeleton. Moreover, **Ko *et al.* (**37) found that, water extract from *Portulaca oleracea* L. can stimulate longitudinal bone growth in weanling male rats and the proliferative and hypertrophic zones of the GP were greater in the legs of P treated rats. They also concluded that, P is potential to promote the proliferation of the leg GP.

Conclusion:

Treatment with TiO_2NPs during the period of organogenesis causes relevant histopathological alterations in the structure of the growing fetal femur. However, treatment with P followed by T exhibited moderate improvement in the histological structure of femur. Therefore, it is recommended to be very careful while dealing with nanomaterials and it is preferred to eat leafy green vegetables rich in antioxidants during pregnancy

4. References

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