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Investigation of the toxic effect of titanium dioxide nanoparticles and the possible recovery after 4 weeks withdrawal on the submandibular salivary gland of albino rats: An in vivo study

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Abstract

This study aimed to investigate the toxic effect of titanium dioxide nanoparticles (TiO₂NPs) on the submandibular salivary glands (SMGs) of albino rats histologically and immunohistochemically and assess the possible recovery after withdrawal for 4 weeks. Twenty-eight male albino rats weighing between 80 mg and 120 mg were divided into three main groups: the control group (CI) which was subdivided into Group IA and IB each contained 7 rats that received distilled water by intragastric (IG) administration daily for 30 days and group IB was left for a recovery period 4 weeks resembling the experimental group; the TiO₂NPs group (Group II) contained 7 rats that received 50 mg/kg (body weight (bw)) IG TiO₂NPs daily for 30 days; and the recovery group (Group III) contained 7 rats received 50 mg/kg (body weight (bw)) IG TiO₂NPs daily for 30 days and were left for recovery for 4 weeks. Histological examination of Group II showed that acinar cells demonstrated some histological alterations including pyknotic, hyperchromatic and crescent shaped nuclei with variable sized cytoplasmic vacuolations. The ducts showed some signs of degeneration with loss of their normal cellular outlines. However, there was some improvement in Group III but was not very close to the control group or the normal state. Apoptotic changes expressed by anti-active caspase 3 were highest in Group II and statistical results showed statistical significance between studied groups. The use of TiO₂NP leads to histological alterations in submandibular salivary glands of rats by inducing apoptotic effect on their cells.

Conclusion: oral administration of TiO₂NP for 30 days caused histological alterations in SMGs and 4 weeks recovery succeeded to some extent to reduce these alterations.

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Introduction:

The importance of major salivary glands and saliva in our everyday activities and the medicinal properties it possesses are often taken for granted. However, when disruptions in the quality or quantity of saliva do occur in an individual, it is likely that oral and systemic health will be harmfully affected (Tiwari, 2011).

Titanium dioxide (TiO₂) nanoparticles (NPs) are among the most manufactured nanomaterials in the industry, and are used in food production, dietary supplements, food packaging materials, medicine, toothpastes, cosmetics and waste water treatment. Consequently, the constant use of TiO₂ nanoparticle-containing products increases the possibility of chronic exposure and accumulation in the internal organs of humans which has put TiO₂ NPs under toxicological inspection (Lee, et al., 2019).

Other in vitro and in vivo studies have reported on the toxicity of TiO₂ NPs (Cui et al., 2012). Deposition of Nano-TiO₂ on different body organs leads to oxidative damage and inflammatory cell infiltration, atherosclerosis (Yu et al., 2014), myocardial injury, alveoli haemorrhaging, angiectasis and hyperaemia of the liver (Hong et al., 2014). Another experiment also found that when the body is exposed to long-term/low-dose and short-term/high-dose of nano-TiO₂, vital organs are harmed such as heart, liver, spleen, lung, kidney, brain, hippocampal neurons, and testes (Hong and Zhang, 2016).

MATERIALS & METHODS

Animals

Twenty-eight male albino rats of two months' age were used in this study (weighing 100 gms +/- 50 gms) each. The animals were housed into sterile, controlled environment (temperature \pm 2 C° and 12 hours dark/light cycles) and fed with commercial pellet diet and water ad libitum. A license for animal testing was obtained by the ethical committee of Faculty of dentistry Ain Shams University. Rats were obtained

and housed in the animal research center of Ain Shams University, Cairo, Egypt.

Rats were randomly divided into 3 main groups with 2 subgroups in the control group (Table 1)

After the experimental period, animals were killed by overdose of anesthesia. The submandibular salivary gland was excised and half the specimens were examined by light microscope (Faculty of Dentistry, The British University in Egypt, Cairo, Egypt) and the other half were prepared and stained for immunohistochemical examination by active caspase 3 staining for detection of apoptosis (Faculty of Medicine, Al Azhar University, Cairo, Egypt).

Group	No. of rats	Dose	Duration Recovery period
Subgroup IA	7	Distilled water IG once daily	30 days
Subgroup IB	7	Distilled water IG once daily	30 days + 4 weeks
Group II	7	50 mg/kg/day b.w of TiO ₂ NPs I.G	30 days
Group III	7	50 mg/kg/day b.w of TiO ₂ NPs I.G	30 days + 4 weeks

Table (1): showing summary of the control and experimental group.

Statistical analysis

The obtained data were collected and tabulated. The numerical data were explored for normality by checking the data distribution, calculating the mean and median values, evaluating histograms and normality curves and using Kolmogorov-Smirnova and Shapiro-Wilk tests. One-way ANOVA test was used to compare between groups regarding surface area of positive reaction to Anti-active caspase 3 followed by Tukey post hock test. The significance level was set at P=0.05 Statistical analysis was performed with IBM*SPSS* statistics version 20 for windows.

Results

Histological results:

- **Group I (control group):**

The control group was subdivided into two subgroups A and B both received distilled water orally for 30 days and IB was left for 4 weeks corresponding to the experimental group. All the examined Hematoxylin and Eosin Stained sections specimen showed almost the same histological results as follows:

Examination of the H&E stained sections revealed submandibular salivary gland with normal histologic architecture.

The serous acini appeared more or less spherical in shape and consisted of pyramidal cells surrounding a narrow lumen. The cells possessed granular, moderately basophilic cytoplasm and rounded basally situated nuclei while the ductal linings appeared more acidophilic. (**Figure 1**)

The intercalated ducts were hardly recognized as they were compressed between the acini. They appeared small with cuboidal cell lining, centrally situated nuclei and little cytoplasm. (**Figure 1**)

The granular convoluted tubules (GCTs) appeared large, mostly rounded and lined with tall columnar cells with large rounded basally situated nuclei, and apical eosinophilic granules. (**Figure 1**)

The striated ducts were lined by columnar cells with centrally placed nuclei and intensely eosinophilic cytoplasm with basal striations. The ducts were occasionally accompanied with blood vessels. (**Figure 1**)

- **Group II:**

Examination of the H&E stained sections of the submandibular salivary glands of group II revealed faintly stained cytoplasmic basophilia of the acini with deeply stained nuclei. (**Figure 2**).

Some acini showed ill-defined lining cell outline. Some cell nuclei were found pyknotic, hyperchromatic or crescent shaped. Different size of cytoplasmic vacuolations were detected (**figure 2**). Some areas showed spacing in between acini and surrounding the ducts. Some acini remnants were observed (**figure 2**). The

intercalated ducts were occasionally found compressed between acini.

The GCTs showed ill-defined cell outline. Some lining cells' nuclei appeared hyperchromatic or crescent. The cells appeared with loss of apical acidophilic granules. Some cytoplasmic vacuolations were detected. Few GCT lining cells showed signs of degeneration, while few cells remnants were observed in some areas (**figure 2**).

The striated ducts showed ill-defined lining cells' outline. Cells appeared with cytoplasmic vacuolations and loss of basal striations (**figure 2**).

- **Group III:**

Examination of H&E stained sections of submandibular salivary gland of rats of group III showed deep basophilic staining of most of acinar cell nuclei. The cells appeared with granular deeply stained basophilic cytoplasm and few cytoplasmic vacuolations were observed (**Figure 3**). Intercalated duct -if observed- appeared with same histological picture of those of previous groups.

Some sections showed GCTs with almost normal histological picture. Cell lining was found regular in arrangement with little vacuolation and apical eosinophilic granules was seen (**Figure 3**).

Some striated duct cells appeared lined with columnar cells eosinophilic stained with basal striations, other showed loss of basal striations and few cytoplasmic vacuolations. The ducts lumen was found without stagnation (**Figure 3**)

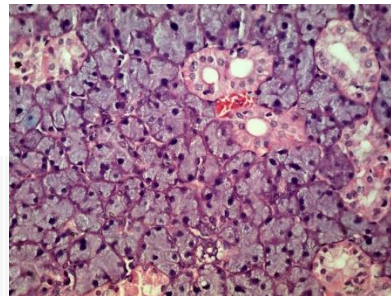


Figure 1: A photomicrograph of rat submandibular salivary gland of the control group (Group I) showing normal secretory serous acini, intercalated duct GCTs with apical eosinophilic granules is seen Striated ducts with

basal striations neighbored with BV. (H&E, orig. mag. X400).

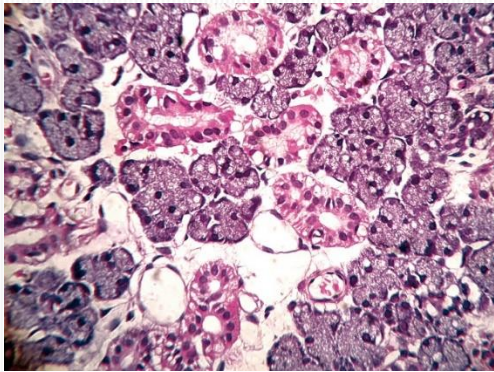


Figure 2: A photomicrograph of rat submandibular salivary gland of Group II showing ill-defined lining acinar cell outline. Different size of acinar cytoplasmic vacuolations were detected. GCT showed ill-defined cell. Loss of apical acidophilic granules and cytoplasmic vacuolations were detected. Striated duct showing ill-defined cell outline with cytoplasmic vacuolations and loss of basal striations. (H&E, orig. mag. X400).

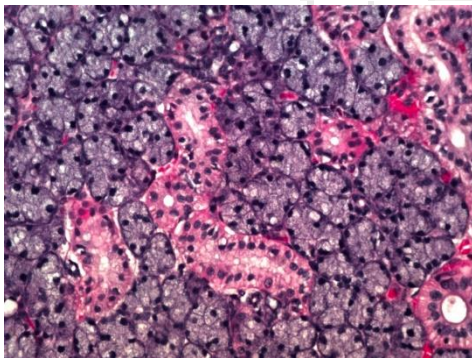


Figure 3: A photomicrograph of rat submandibular salivary gland of Group III showing deep basophilic staining of acinar cytoplasm and nuclei. Few cytoplasmic vacuolations in acinar cells were observed. GCTs cells showing little cytoplasmic vacuolations and acidophilic apical granules can be seen. (H&E, orig. mag. X400).

Immunohistochemical results:

Caspase-3 apoptotic marker staining results

Group I (control group):

Immunohistochemical staining of submandibular salivary gland sections of control group showed almost negative staining reaction in both acinar and ductal cells according to

image analysis results. Few acinar cells showed mild positive cytoplasmic staining reaction to anti active caspase 3. Similarly, most of the GCTs and ducts were negatively stained, few cells demonstrated mild positive reaction to anti active caspase 3. (Figure 4)

Group II:

Examination of submandibular salivary gland sections of this group revealed wide areas of positive and stained cells (nuclear and cytoplasmic positive reaction) of both acini and ducts to active caspase. (Figure 5)

Group III:

Examination of immunostained section of this group revealed apparently few positive staining reaction cells to active caspase 3 in both acinar and ductal cells. (Figure 6)

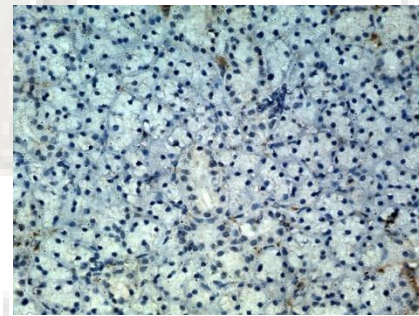


Figure 4: An immunostained photomicrograph of control group showing minimal positively stained acinar and ductal cells with caspase 3 (anti active caspase 3 orig. mag. X400)

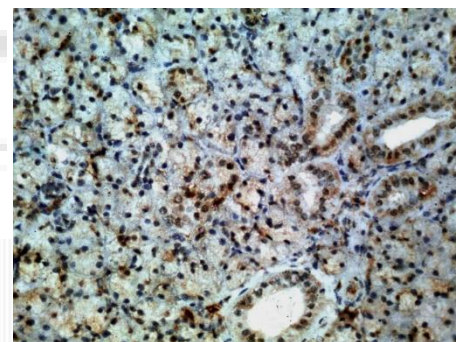


Figure 5: An immunostained photomicrograph of group II showing cells with dominant positive

staining reaction to caspase 3 (anti active caspase 3 orig. mag. X400)

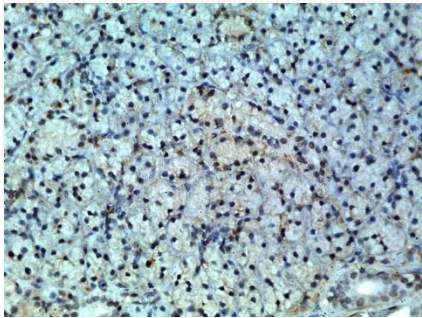


Figure 6: An immunostained photomicrograph of group III showing few positive staining reaction to caspase 3 (anti active caspase 3 orig. mag. X400)

Statistical Analysis

Caspase 3 Surface Area ($\times 10^4$) in submandibular salivary gland cells:

Group II with mean value \pm standard deviation (SD) (287.4 ± 19.42), group III with mean value \pm SD (225.9 ± 14.59) showed significant increase in the caspase 3 surface area compared to that in control group (172.1 ± 37.36)

	Control	Group II	Group III	ANOVA p value
Mean \pm SD	172.1 \pm 37.36	287.4 \pm 19.42	225.9 \pm 14.59	<0.001*
Posthoc		P1=<0.001*	P1=<0.001* P2=<0.001*	

($p < 0.001$).

Group III (225.9 ± 14.59) showed significant decrease in the caspase 3 surface area compared to that in group II (287.4 ± 19.42) ($p < 0.001$).

(Table 2) (Figure 7)

Table (2): Showing comparison of surface area ($\times 10^4$) among different studied groups

Data expressed either as mean \pm SD
SD: standard deviation P: Probability *: significance <0.05
Test used: One way ANOVA followed by post-hoc tukey
P1: significance relative to Control Group
P2: significance relative to Group II

Data was analyzed using Statistical Package for Social Science software computer program version 23 (SPSS, Inc., Chicago, IL, USA). Data were presented in mean and standard deviation. One way Analysis of variance (ANOVA) and tukey were used for comparing data. P value less

than 0.05 was considered statistically significant.

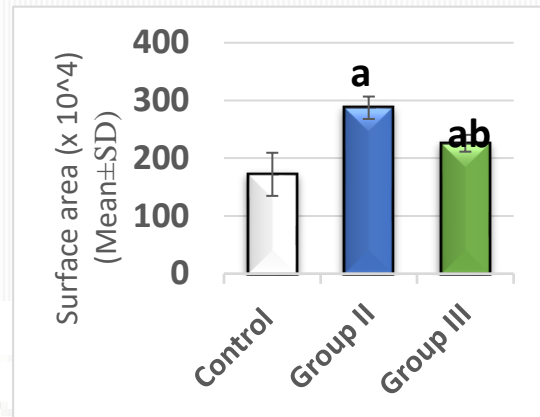


Figure 7: Bar chart representing mean caspase 3 surface area in submandibular salivary gland cells of the three groups (a: group II statistical significance compared to the control group, ab: group III statistical significance compared to control and group II).

Discussion

Industrially, titanium dioxide (TiO₂) white powder is famous for its low production costs, photostability in solution, anticorrosive properties, high stability and redox selectivity. Their nanoparticles are highly preferred due to their superior properties in terms of stability and resistance to corrosion. (Gupta and Tripathi, 2011).

TiO₂ NPs was the material of choice in this study because it is the most widely used white pigment in food industry due to its brightness and very high refractive index (Ortlieb, 2010). Oral administration was the route of choice in the current study because the highest exposure to TiO₂ NP in children is through foods specially sweets that has high TiO₂ content when compared to other food products (Weir, et al., 2012).

In the current study, 4 weeks recovery period was chosen in parallel with a study made by Fabian, et al., (2008) who reported that there was a decrease in TiO₂ NPs level after single intravenous injections with 5 mg/kg body weight in spleen after 14 and 28 days, and a return to control levels in kidney and lung after 14 days. In the same manner, the recovery of submandibular salivary glands was monitored from possible toxicity of TiO₂ NP for 2, 4 and 6 weeks. Moreover, it was stated by a recent study that TiO₂ nanoparticles' half-life in the body is about 10 days (Elgrabli, et al 2015).

As TiO₂ NP was reported to induce apoptosis (Jin, et al., 2013), active caspase staining was used for investigating and detection of apoptosis in this research. Anti active caspase 3 was preferred as an anti-apoptotic marker to detect the possible apoptotic changes that could be caused by TiO₂ NP in the submandibular salivary glands. Moreover, TiO₂ NP causes mitochondrial dysfunction, decrease in ATP levels and upregulation of caspase 3 enzyme activity, accordingly anti-active caspase was the antiapoptotic marker of choice in this study (Wang, J. et al., 2019).

In the present study, histological examination of the submandibular salivary glands of group II showed marked degeneration in the parenchymal elements of the gland, suggesting that TiO₂ NPs have a potential to induce cytotoxic effects on the acinar cells as well as the duct system. These were revealed by the variable degrees of degenerative changes involving most of the acinar and ductal cells. The serous acini and the intercalated ducts showed apparent shrinkage and degeneration and some acini were lost leaving cell remnants. Acinar cells of group II and some of the specimens of recovery groups showed pyknotic and crescent shaped nuclei with numerous cytoplasmic vacuolations which might indicate DNA damage and genotoxic effect of TiO₂ NPs. These histological changes coincide with Wang, et al., (2013), where young rats specimens showed perilobular cell swelling, cytoplasmic vacuolization and hydropic degeneration in liver cells following oral exposure of TiO₂ NP at doses of 0, 10, 50, 200 mg kg⁻¹ body weight per day for 30 days. Schins and Knaapen (2007) reported that reactive oxygen species (ROS) play a major role in the genotoxicity of nanoparticles, due to their surface properties, the presence of transition metals and lipid peroxidation. Moreover, Jin, et al., (2013) stated that nano-anatase TiO₂ particles, the same phase used in this study, can directly bind to DNA molecules causing DNA damage and interfere with DNA repair and replication, altering normal biological/cellular functions and ultimately leading to cell death.

In Group II specimens, striated duct cells also showed loss of basal striation in histological study, which might be related to mitochondrial injury. Our results are also supported by Tang, et al., (2013) who reported that nano-TiO₂ is related to the generation of intracellular (ROS), which injured mitochondria and prevented the synthesis of ATP.

In this research, the blood vessels in between the parenchymal elements and around excretory ducts of group II and III showed dilatation and engorgement with RBCs. This goes in accordance to Mohammadi, et al., (2015) who reported hemorrhage in alveoli wall and alveolar space of rats after I.P injection of TiO₂ NP at different doses (15, 30, 60 and 70 mg/kg). This may be due to the damage resulting from ROS that attach to the epithelial cells in vessel wall and cause blood vessels injuries (Mohammadi, et al., 2015).

In this research, findings of active caspase 3 staining revealed that the least positive reaction was in control group as apoptosis takes place in normal tissues as well (Elmore, S., 2007). The marked area fraction of immunopositive reaction with anti-active caspase 3 and statistically significant highest mean area percentage was in the experimental Group II that received TiO₂ NP for 30 days in comparison with the control group. And this confirms the apoptotic action of TiO₂ NP. This coincided with the findings of Jin. et al., 2013 who found that TiO₂ upregulated the expression of caspase 3.

The SMGs of rats of groups III showed less positive immunostained areas in comparison to group II and this was statistically confirmed. This confirms that the apoptotic activity and elevated caspase 3 level caused by TiO₂ NPs was decreased after its cessation and the regenerative powers of salivary glands that was previously discussed in this research (Emmerson et al. 2018).

Conclusion

From the previous study, the following could be concluded:

Oral administration of TiO₂ NPs to albino rats for 30 days caused serious alteration in the submandibular salivary glands as revealed histologically and immunohistochemically.

Recovery periods of four weeks succeeded to some extent were not enough to restore normal gland architecture after cessation of the drug as well as reduce apoptotic changes.

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