Nanomed. J. 5(4): 192-198, Autumn 2018

RESEARCH PAPER

Evaluation of epigenetic changes of liver tissue induced by oral administration of titanium dioxide nanoparticles and possible protective role of Nigella Sativa oil in adult male albino rats

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ABSTRACT

Objective (s): Titanium dioxide nanoparticles (TiO_2 NPs) have been recognized as biologically inert material and have been used in a multitude of applications. Nevertheless, the negative impact on the human health is not yet well understood. The study attempted to evaluate the epigenetic changes of the genome, in the form of DNA methylation in liver tissue samples, resulting from oral administration of TiO_2 NPs (mixed rutile and anatase) in adult male albino rats. Furthermore, the preventive activity of the *Nigella sativa* oil (NSO) on the toxic effects of TiO_2 NPs was investigated.

Materials and Methods: Thirty-two adult male albino rats were divided into four groups. (I) control, (II) nigella sativa oil, (III) TiO_2 NPs and (IV) TiO_2 NPs + *Nigella sativa* oil. The impact of TiO_2 NPs on the global DNA methylation and the oxidative status were assessed.

Results: Among the study groups, TiO_2 NPs exposure provoked oxidative stress; increased blood levels of MDA and decreased reduced glutathione (GSH) level. The global DNA methylation levels decreased after exposure to titanium nanoparticles. Significant differences were recorded between the control group and the group receiving TiO_2 NPs. Marked improvement was noticed after supplementation of *Nigella sativa* oil in terms of DNA methylation and oxidative stress markers.

Conclusion: Oral administration of TiO_2 NPs caused global DNA hypo-methylation in liver tissue samples. The epigenetic damage raises the concern about the safety associated with applications of the TiO_2 NPs. The maintenance of DNA methylation patterns by *Nigella sativa* oil has a role in protection against genomic instability.

Keywords: *Epigenetic, Nanotoxicity, Titanium dioxide, Nigella sativa*

How to cite this article

Mohamed Magdy Badr El Dine F, Samie Mohamed Hussein HA, Abdelmawla Ghazala R. Evaluation of epigenetic changes of liver tissue induced by oral administration of Titanium dioxide nanoparticles and possible protective role of Nigella Sativa oil, in adult male albino rats. Nanomed J. 2018; 5(4): 192-198. DOI: 10.22038/nmj.2018.05.00002

INTRODUCTION

The exponential growth of nanotechnology has had major socioeconomic impacts. It has many useful applications in medicine, industry and military field. Among those are metal oxide nanoparticles. Considering it as a photocatalyst, titanium dioxide nanoparticles (TiO_2 NPs) are widely used in manufacturing of sunscreens and cosmetics. Moreover, it is also used as a food coloring and additive because of its white color. TiO_2 NPs have many mineral forms but the most common are rutile and anatase which have different toxicity potentials [1].

Up till now, there is controversial evidence about the genotoxic potential of titanium dioxide [2, 3] and whether the oxidative stress has an incidental influence in the genetic as well as the epigenetic damage induced by it [4].

Epigenetics entails changes in the expression of genes as a result of changes in the epigenome without affecting the DNA sequence. At the same time, epigenetic changes may be considered as a non-genotoxic mechanism of carcinogenicity [5].

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Note. This manuscript was submitted on July 30, 2018; approved on September 5, 2018

On the other hand, *Nigella sativa* oil (NSO) which is the main ingredient of *Nigella sativa* plant, has been used for centuries throughout the world as a natural remedy for a broad spectrum of therapeutic purposes. It has been reported to possess a protective antioxidant activity against toxicities triggered by oxidative stress generating agents [6, 7].

The current study aimed to evaluate and compare the epigenetic changes of the genome, in the form of DNA methylation in liver tissue samples, resulting from oral administration of TiO_2 NPs (mixed rutile and anatase) in male adult albino rats. Furthermore, potential protecting effect of *Nigella sativa* oil was also evaluated in the current work.

MATERIALS AND METHODS

Chemicals

The NS oil was purchased from an herbal drug store, Alexandria, Egypt.

Commercial kits

- QIAamp DNA Mini Kit Qiagen Sciences, Maryland, USA.Cat No./ID: 51104.
- Quantification of global DNA methylation by Colorimetric method using MethylFlash Kit (Epigentek)

Animal experimental design

Thirty two male Albino rats were used in this study with an average weight of 200-230 g. They were free of pathogens. Five days before the experiment, animals were acclimatized and had standard water and nutrition. Environmental conditions were standardized for all animals. The duration of the experiment extended to 6 weeks. Guidelines of the international ethics committee on animal welfare for animal handling were considered in the present study procedures. The study protocol was accepted by the Ethics Committee of Alexandria Faculty of Medicine.

Animals were classified equally into four different groups:

1. Group I: Control group where orogastric tube was used to give saline 0.9% to this group of rats.
2. Group II: This group of rats had Nigella Sativa oil orally via orogastric tube (2 ml/kg bwt) [8].
3. Group III (TiO_2 NPs-treated group): Rats received TiO_2 NPs suspension (100 mg/kg of body weight) through orogastric tube for 6 weeks [9].
4. Group IV (Protected group): This group of rats received TiO_2 NPs suspension 100 mg/kg of body weight and for the same period as in-group III, with concomitant oral administration of *Nigella sativa* oil (2 ml/kg).

Rats were sacrificed at the end of the study under anesthesia. Three ml of blood from animals were collected in EDTA-coated tubes for determination of the oxidative stress markers in plasma. Liver tissue samples were collected for determination of 5-methylcytosine content in genomic DNA. Both plasma and tissue samples were stored at -20 °C till further analysis.

Nanomaterials characterization and preparation of treated suspension

Titanium dioxide nanoparticles (TiO_2 NPs) were obtained from Sigma-Aldrich. TiO_2 NPs were a mixture of rutile and anatase Nano powder, white in color, particle size of 21 nm and the purity of the particles was 99.5% based on trace metals. Titanium nanoparticles were suspended in 0.9% saline and that suspension was sonicated for 10 min before use.

Spectrophotometric determination of malonaldehyde (MDA) and reduced glutathione (GSH) levels in plasma

Malonaldehyde level assessment

MDA is an oxidative stress marker measuring lipid peroxidation. Pink chromogen was formed after MDA reaction with thiobarbituric acid. MDA was measured by a method described by Qhwa *et al.* [10]. The absorbance was read at 532 nm and concentration was expressed in nmol/ml [10].

Reduced glutathione (GSH) measurement

A spectrophotometric method for reduced glutathione (GSH) was used involving oxidation of GSH by 5,5'-dithio-bis(2-nitrobenzoic acid) (DTNB) to form the yellow chromogen. The absorbance was read at 405nm and concentration was expressed in mg\dl [11].

Nucleic acid isolation and quantification of the 5-methylcytosine content in genomic DNA from liver tissue

The weight of liver tissue samples ranged between 20-25 mg. Tissues were sliced and placed in clean microcentrifuge tubes (1.5 ml) with 180 µl buffer for liver lysis provided in the extraction kit.

DNA purification from hepatic tissue was performed using Spin Protocol QIAamp DNA

Mini Kit, (Qiagen) according to manufacturer's instruction. The concentration of total DNA was quantified by a Nanodrop 2000 (Nanodrop, USA). The range of the results was from50-200 ng/µl. An optimal amount is 150 ng per reaction was optimized. Tris EDTA (TE) buffer was used for extracted DNA elution and storage at -20 °C till global methylation measurement.

Global DNA methylation assessment

Enzyme-linked immunosorbent method was utilized to assess extracted DNA cytosine methylation in animals liver using MethylFlash Kit (Epigentek). Thirty µL of binding buffers was used to dilute the DNA samples and they were incubated at 60°C. Capture and detection antibodies were used and optical densities were read at 450 nm. DNA methylation was quantified by calculation of 5-methylcytosine percentage which was expressed relative to methylation in positive control provided within the kit. The positive control is a polynucleotide containing 50% of 5-methylcytosine. The amount of DNA input in positive control was 5 ng/µl.

Methylcytosine percentage calculation

Global DNA methylation analysis was carried out by dividing the difference between the sample and the negative control optical densities by the samples DNA input concentration in ng.

The negative control is a polynucleotide containing 50% of unmethylated cytosine. Then the obtained values were further divided by the optical densities difference between positive and negative controls provided [12].

Statistical analysis

Statistical Analyses Package for Social Sciences Software (SPSS) (Armonk, NY: IBM Corp) version 20 was used to analyze data .

Studied groups were compared to one another and F-test (ANOVA) and Post Hoc (Scheffe) were used for analysis. P-value considered statistically significant if less than 0.001. To determine relationship between different variables Spearman's rank correlation coefficient r was used.

RESULTS

Malonaldehyde (MDA) level assessment (lipid peroxidation assessment)

The current work revealed that the level of MDA was greatest in group III, followed by group II then group IV with values of 14.59±2.15, 12.60 ±0.02 and 8.47±0.85 respectively. The least level was found in group IV (receiving TiO2NPs+NS). Significant difference was noticed between the control group and each of group II and III with p<0.001. In contrast, insignificant difference was observed between the protected and the control groups with p=0.993 (Table 1).

Measurement of reduced glutathione (GSH) level

The level of reduced GSH was highest (0.44 ± 0.03) in the protected group and was least (0.16 ±0.04) in the TiO2 treated group. Significant difference was identified between the control group and each of group III and IV with p <0.001. Oppositely, insignificant difference was found between group II (received Nigella Sativa oil) and the control group with p=0.972. Moreover, significant difference was detected between groups II, III and IV (Table 2). Moreover, significant

Table 1. Comparison between the studied groups according to the level of MDA

MDA (nmol/ml)	Control group (n = 8)	Control group (n = 8)	TiO2NPs-treated group (n = 8)	TiO2NPs+NS group (n = 8)	F	p
Min. – Max.	8.53 – 8.71	12.57 – 12.63	12.52 – 17.21	7.52 – 9.40		
Mean ± SD.	8.63 ±0.06	12.60 ±0.02	14.59 ±2.15	8.47 ±0.85	54.911*	<0.001*
Median	8.63	12.61	13.41	8.46		
p control		<0.001*	<0.001*	0.993		
Significance between groups	p1=0.009*, p2<0.001*, p3<0.001*					

Normally distributed data was presented in mean ± SD and was compared using F test (ANOVA).
Significance between groups were done using Post Hoc Test (Tukey).
P1:p-value for comparing between (*Nigella sativa* oil) and (group received titanium dioxide nanoparticle).
P2:p-value for comparing between (*Nigella sativa* oil) and (group received titanium dioxide nanoparticle + *Nigella sativa*).
P3:p-value for comparing between (group received titanium nanoparticles) and (group received titanium dioxide nanoparticles + *Nigella sativa*).
*: Statistically significant at p≤0.001

Table 2. Comparison between the studied groups according to the level of reduced GSH

GSH (mg\dl)	Control group (n = 8)	Control group (n = 8)	TiO2NPs-treated group (n = 8)	TiO2NPs+NS group (n = 8)	F	p
Min. – Max.	0.28 – 0.35	0.29 – 0.35	0.12 – 0.21	0.41 – 0.48		
Mean ± SD.	0.32 ±0.02	0.32 ±0.02	0.16 ±0.04	0.44 ±0.03	135.959*	<0.001*
Median	0.32	0.33	0.13	0.45		
p control		0.972	<0.001*	<0.001*		
Significance between groups	p_1<0.001*, p_2<0.001*, p_3<0.001*					

Normally distributed data was presented in mean±SD and was compared using F test (ANOVA).
Significance between groups were done using Post Hoc Test (Tukey).
P1:p-value for comparing between (*Nigella sativa* oil) and (group received titanium dioxide nanoparticle).
P2:p-value for comparing between (*Nigella sativa* oil) and (group received titanium dioxide nanoparticle + *Nigella sativa*).
P3:p-value for comparing between (titanium nanoparticle treated group) and (group received titanium dioxide nanoparticle+ *Nigella sativa*).
r: Pearson coefficient
*: Statistically significant at p ≤ 0.001

negative correlation was observed between DNA methylation and level of reduced glutathione in the group of rats exposed to titanium dioxide nanoparticles (Table 3).

DNA methylation in liver tissue samples

In the current work, the highest level of DNA methylation (3.18±0.05) was observed in group II (received *Nigella Sativa* oil), while the least level (1.53±0.35) was shown in group III (TiO_2 NPs-treated group).

Table 3. Correlation between DNA methylation in liver tissue samples and markers of oxidative stress

		Tissue DNA methylation			
		Control (n = 8)	NigellaSativa oil, (n = 8)	TiO2 (n = 8)	TiO2+NS (n = 8)
MDA (nmol\ml)	r	-0.487	0.238	0.082	0.985*
	p	0.221	0.571	0.848	<0.001*
GSH(mg\dl)	r	0.020	-0.287	-0.909*	0.943*
	p	0.962	0.491	0.002*	<0.001*

Significant difference was noted between the control group (group I) and each of group II (received *Nigella Sativa* oil NS) and group III (TiO_2 NPs-treated group) with p=0.013 and 0.001 respectively. Oppositely, non-significant difference was noticed between group IV (received (TiO_2+NS) and the control group with p= 0.530. Furthermore, significant difference was detected between groups II, III, IV (Table 4).

Significant negative correlation between DNA methylation in liver tissue samples and GSH level was observed. On the other hand, significant positive correlation was observed between DNA methylation in liver tissue samples and level of MDA (Table 3).

DISCUSSION

Nowadays, concerns are increasing about possible health consequences of oral exposure to TiO_2 nanoparticles. They are used in manufacturing of toothpaste, tablets as medicine or food supplements and as a color food additive in candies and chewing gums, processed nuts,

Table 4. Comparison between the studied groups according to the level of DNA methylation in liver tissue samples

Tissue methylation	Control group (n = 8)	Control group (n = 8)	TiO2NPs-treated group (n = 8)	TiO2NPs+NS group (n = 8)	F	p
Min. – Max.	2.71 – 2.93	3.11 – 3.24	1.13 – 1.97	2.65 – 2.82		
Mean ± SD.	2.87 ±0.07	3.18 ±0.05	1.53 ±0.35	2.74 ±0.08	119.929*	<0.001*
Median	2.89	3.18	1.63	2.74		
p control		0.013*	<0.001*	0.530		
Significance between groups	p_1<0.001*, p_2<0.001*, p_3<0.001*					

Normally distributed data was presented in mean±SD and was compared using F test (ANOVA).
Significance between groups was done using Post Hoc Test (Tukey).
P1: p-value for comparing between (*Nigella sativa* oil) and (group received titanium dioxide nanoparticle).
P2: p-value for comparing between (*Nigella sativa* oil) and (group received titanium dioxide nanoparticle+*Nigella sativa*).
P3: p-value for comparing between (group received titanium nanoparticle) and (group received titanium dioxide nanoparticle+*Nigella sativa*).
*: Statistically significant at p≤0.001

coffee creamers, sauces and fine bakery products. Although considered safe and non-absorbable from gastrointestinal tract, TiO_2 NPs were found to infiltrate and accumulate in the liver which is the main detoxification tissue for exogenous chemicals [13- 15].

Furthermore, it was shown that nanoparticles could induce epigenetic alterations which may encourage malignancy. "Nano-epigenetics" which is the field of nanoscience that studies the epigenetic effects of nanoparticles, would help in the production of nanoparticles of less adverse effects and toxicity [16].

The present work evaluated the influence of oral intake of TiO_2 NPs on the epigenome, in liver tissue of male adult albino rats, in the form of global DNA methylation. Moreover, the potential protecting effect of *Nigella sativa* oil was also assessed in the current work.

The dose selected in the present study (100 mg/kg of body weight) was determined according to the report of World Health Organization in 1969, which showed that LD50 of TiO_2 is more than 12,000 mg/kg body weight, for rats, following oral intake. This was in accordance to Vasantharaja D *et al.* (2015), who concluded that this dose could induce biochemical markers alteration of liver and kidneys [9].

At the same time, the ultrafine size of nanoparticles (21 nm), used in the current study, might result in their long-time retention *in vivo* with its difficult clearance and consequently induced liver affection [17].

In their study, Wang *et al.* (2007) reported obvious changes in the liver function (total bilirubin level, ALT/AST and LDH) and distortion of architecture following oral intake of titanium dioxide nanoparticles [17].

In the present study, oral exposure to TiO_2 NPs was found to cause global DNA hypomethylation, in liver tissue samples, which was significantly lesser than its level in the control group. This coincided with Patil NA (2016) [18]. Patil NA *et al.* (2016) showed that TiO_2 NPs, even at low concentrations that cause no noticeable cytotoxicity or change in the oxidative status, can still cause DNA hypomethylation. Moreover, Bin *et al.* (2017) [19] reported that TiO_2 NPs could cause global hypomethylation in neural cell lines (PC12 cells). In addition, exposure to TiO_2 NPs triggers genomic hypomethylation and altered expression of methylation-related genes in human respiratory cells [20].

In fact, epigenetic errors have been implicated in uncontrollable proliferation and cancers [21]. Moreover, oncogene expression, genomic instability as well as loss of imprinting are all considered as consequences of global DNA hypomethylation [22, 23].

At the same time, DNA hypo-methylation can be considered as an important signal for the early recognition of malignancy and many other diseases such as obesity, diabetes and stress [24, 25].

The current study revealed that TiO_2 NPs induced low level of glutathione together with high level of MDA, significant negative correlation between DNA methylation in liver tissue samples and GSH level was noticed. On the other hand, significant positive correlation was observed between DNA methylation in liver tissue samples and level of MDA. These results may explain the mechanism by which TiO_2 NPs produce their epigenetic changes through causing imbalance between reactive oxygen species production and the antioxidant activity of the cells, producing oxidative stress. This coincides with Saquib Q *et al.* (2012) [26].

Several studies have found that cellular toxic effects of nanoparticles may be due to direct or indirect interactions with the molecules inside the cell. The main mechanism of which is the disturbance of cellular redox homeostasis which consecutively causes oxidative damage of cellular lipids, nucleic acids and proteins [27-29].

As a result, reactive oxygen species (ROS) produced inside the cell and DNA damage caused by them can influence the interaction between methyltransferases and DNA resulting in DNA hypo-methylation. Consequently, it can modify the expression of genes regulated by this methylated DNA [30, 31].

Malondialdehyde (MDA) is one of the end products of peroxidation of polyunsaturated fatty acids inside the cell. Increase in the levels of free radicals results in increase in MDA produced. Furthermore, MDA level is considered an indicator for cellular oxidative stress and lipid peroxidation. It can alter cell membrane physical structure and is involved, in an indirect way, in the synthesis of proteins, DNA and RNA. Moreover, it can induce mutagenesis and carcinogenicity [32].

On the other hand, the advantageous properties of *Nigella sativa* oil were demonstrated in the present study, where liver tissue samples

exposed to *Nigella Sativa* oil showed global DNA hyper-methylation with significant increase compared to the control group. Furthermore, rats receiving both TiO_2 NPs and NS oil showed non-significant difference from that in the control group. These results reflect the beneficial influence of *Nigella Sativa* oil that antagonizes the DNA hypo-methylation produced by TiO_2 NPs in liver tissue samples.

Al-Okbi SY *et al.* (2018) concluded that oral administration of *Nigella Sativa* oil helped the regeneration of hepatic tissue as well as protection of renal tissue. It decreased the injurious effects, on liver and kidney of rat model, of CCl_4 by antagonism of the inflammatory and its oxidant effects [33].

Nigella sativa (NS) is a promising plant that has health improving ability as a result of its beneficial chemical constituents. The most important one is thymoquinone which is a powerful free radicals scavenger against hydroxyl radicals and superoxide anions [34].

CONCLUSION

In conclusion, based upon the data presented herein, oral intake of TiO_2 NPs has a relevant negative impact on hepatic global DNA methylation in the form DNA hypo-methylation. This was explained by depletion of reduced glutathione levels denoting reductive stress and increased lipid peroxidation with high levels of MDA. *Nigella sativa* oil corrected the reported changes induced by titanium dioxide nanoparticles.

RECOMMENDATIONS

Future researches should entail possible toxicity following ingestion of titanium dioxide nanoparticles of different sizes and doses over variable duration. Every probable molecular mechanism of TiO_2 NPs-induced hepatotoxicity must be investigated expansively. This study only assessed the global DNA methylation, and further studies are required for the local DNA methylation.

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