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International Journal of Current Research
Vol. 5, Issue, 12, pp.4113-4117, December, 2013

INTERNATIONAL JOURNAL OF CURRENT RESEARCH

ISSN: 0975-833X

RESEARCH ARTICLE

THE ROLE OF SOME NATURAL ANTIOXIDANTS IN AMELIORATING THE TOXIC EFFECTS OF NANO TITANIUM DIOXIDE ON BONE COMPLICATIONS IN RATS

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ARTICLE INFO

Article History:
Received 28th September, 2013
Received in revised form
10th October, 2013
Accepted 19th November, 2013
Published online 25th December, 2013

Key words:
nTiO2,
Idebenone,
Carnosine,
Melatonin, IL-6,
TNF-α,
B-ALP and CTx

ABSTRACT

Several potential health hazards are associated with the wide use of titanium dioxide nanoparticles (e.g., n-TiO_2). The primary aim of the current study was to detect the toxic effects of 800 mg/kg 50 nm n-TiO_2 on bone metabolism. Comparative studies were conducted to determine whether natural antioxidants, such as idebenone, carnosine and melatonin, could ameliorate the hazardous effects of n-TiO_2 on down regulation of bone. The results of this study revealed that n-TiO_2 treatment produced bone complications, which were confirmed by alteration of serum tumor necrosis factor-α (TNF-α, inflammatory cytokine) level and bone alkaline phosphatase (B-ALP; bone formation marker), and C-terminal peptide of type I collagen (CTx; bone resorption marker) in treated rats compared with controls. The administration of the previously mentioned antioxidants along with n-TiO_2 treatment significantly modulated the alterations in most the inflammatory cytokines and biomarkers. It was concluded that treatment with idebenone, carnosine and melatonin protects against n-TiO_2 oxidative stress-induced bone complications.

INTRODUCTION

Nanoparticles with a diameter between 1 and 100 nm in at least one direction may provide enhanced or novel properties that are useful for the generation of new industrial materials, medicines and consumer products. Such nanoparticles may also have toxicological properties that are different from their parental compounds (Karin *et al*., 2009). The wide use of nanomaterials is accompanied by the production of wastes containing nanoparticles, which could contaminate the environment and may cause health problems in human beings (Zhanga *et al*., 2010). Titanium dioxide, a new type of photocatalyst, anti-ultraviolet light agent and photoelectric effect agent, is used in several commercial contexts, e.g., white pigment synthesis, anti-aging research, water purification techniques, and cosmetics applications (Nohynek, 2007). Thus, there are multiple different sources from which these materials may be released into the environment, ecosystem, water and food supplies and other routes of non-voluntary entry into the human body (Gurr *et al*., 2005), and there is an urgent need for studies on the acute toxicity of TiO_2 nanoparticles (Zhanga *et al*., 2010). The toxicity of TiO_2 in cosmetics is controversial (Nohynek *et al*., 2007).

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TiO_2 absorbs a substantial amount of UV radiation; however, in aqueous media, TiO_2 produces reactive oxygen species, including superoxide anion radicals, hydrogen peroxide, free hydroxyl radicals, and singlet oxygen. These reactive oxygen species can cause substantial damage to DNA (Gurr *et al*., 2005). Recently, the effects of several natural products that directly cause inflammatory responses and oxidative damage (Woo *et al*., 2011 and Chana *et al*., 2011). L-carnosine has a number of antioxidant properties that may have beneficial effects. It has been proven to scavenge reactive oxygen species (ROS) and alpha-beta unsaturated aldehydes formed from the peroxidation of cell membrane fatty acids during oxidative stress. L-carnosine can inhibit glycation (Rashid *et al*., 2007, Hipkiss 2006) and chelate divalent metal ions (Janssen *et al*., 2005). Melatonin is a naturally occurring compound that acts as an antioxidant by scavenging ROS and inhibiting Lipid Peroxidation (LP). In addition, it counteracts mitochondrial oxidative stress and the cyanide-induced inhibition of ATP by increasing ATP synthesis (Leone *et al*., 2004, Martin *et al*., 2002). There is evidence indicating the complexity of melatonin's role in modulating a diverse number of physiological processes, including the control of seasonal reproduction, retinal physiology, blood pressure control, regulation of the immune system and tumor growth (Ohta *et al*., 2002). Idebenone, a synthetic analogue of coenzyme Q_{10}, is a vital cell membrane antioxidant and an essential constituent of the ATP-producing mitochondrial electron

transport chain (ETC). It is a potent antioxidant radical, and research into its role in diseases has opened new areas of research into the use of antioxidants as disease prevention agents (Nagy, 1990). Because of its ability to inhibit lipid preoxidation, it protects the cell membrane and mitochondria from oxidative damage (Geromel *et al*., 2002). The aim of this work was to study the potential protective beneficial activities of the antioxidants such as idebenone, carnosine and melatonin against changes in bone turnover induced by n-TiO_2.

MATERIALS AND METHODS

Chemicals

The 50nm n-TiO_2 powder was purchased from Sigma Co. (USA). All of the chemicals used were of high analytical grade and were purchased from Sigma or Merck.

Experimental animals

Sixty Wistar albino rats weighing 170-200 g were used. The rats were obtained from the Experimental Animal Care Center, College of Pharmacy, King Saud University. The animals were kept in special cages and maintained under a constant 12-h light/12-h dark cycle at temperatures ranging from 20–22°C and 60% humidity. Rats were fed a standard rat pellet chow and had free access to tap water ad libitum for one week for acclimatization before the experiment. All of the animal protocols were performed in accordance with the guidelines provided by the Experimental Animal Laboratory and approved by the Animal Care and Use Committee of King Saud University, College of Pharmacy. After one week of acclimation, the rats were fasted overnight before treatment and randomly divided into 5 groups of ten rats each: G1, normal healthy animals; G2, animals administered 800 mg/ K body weight/day n-Tio_2 for FIVE consecutive days (Wang *et al*. 2008); G3, n-TiO_2-intoxicated animals co-administered L-carnosine (200 mg/Kg) daily (R); G4, n-TiO_2-intoxicated animals co-administered idebenone (200 mg/Kg) daily; and G5, n-TiO_2-intoxicated animals co-administered melatonin. All of the antioxidants were orally administered daily for three consecutive weeks from the beginning of the experiment. Three weeks later, 24 hours after the last dose administration, rats were fasted overnight and euthanized, and blood samples were collected. Serum was separated after clotting by centrifugation at 3000 rpm for 10 minutes and kept at -80°C until biochemical analysis.

Biochemical analysis

Serum B-ALP, CTX and TNF-α levels were measured using enzyme-linked immunosorbent assay (ELISA) kits (B-ALP (AviBion, Orgenium Laboratories Division, Vantaa, Finland); TNF-α (Ani Biotech Oy, Orgenium Laboratories Division, Vantaa, Finland)). The reading was carried out using an ELISA microplate reader (VERSA Max, Molecular Devices Corporation, MN, USA). Serum total calcium and phosphorus were estimated colorimetrically by the method of Weather burn *et al.,* 1982 as cited in United Diagnostic Industry. Serum magnesium was estimated according to the method of Bohuon *et al.,* 1962 as cited in United Diagnostic Industry.

Statistical analysis

SPSS (Statistical Package for the Social Sciences) version 7.0 was used for all statistical analyses. All of the numeric data were expressed as the mean ± S.E. Data were analyzed using ANOVA. Unpaired t-tests were used to compare the data before and after treatment. Differences of $p < 0.05$ were considered to be significant.

RESULTS

Comparison the activity of B- ALK in n-TiO2 rats with antioxidants

In the present study, when comparing rats exposed to n-TiO2 and normal groups. The activity of serum B-ALP (Bone formation marker) in n-TiO2 group was found to be statistically inhibited (Figure 1). However, administration of idebenone, L-carnosine and melatonin were significantly increase when compared with n-TiO2 group after 3-wks treatment (Figure 1). Idebenone and L-carnosine were more effective compared to melatonin (Figure 1).

Comparison CTx **level** *in n-TiO2 rats with antioxidants*

Rats exposed to n-TiO2 had a significantly increase of serum CTx (bone resorption marker) compared to normal group (Figure 2). Treatment with different antioxidants revealed a significantly decrease of serum CTx in idebenone, L-carnosine or melatonin groups compared with n-TiO2 group. However, idebenone and melatonin groups were more effective compared to melatonin group.

Comparison the **proinflammatory cytokine level** *in n-TiO2 rats with antioxidants*

Serum TNF- α (proinflammatory cytokines, marker of bone resorption factor) was increased significantly in n-TiO2 group compared with normal group. When comparing the antioxidants and n-TiO2 groups, serum TNF- α was found to be statistical decreased after oral administration of idebenone, L-carnosine or melatonin ($p< 0.01$). These data suggest that treatment with those antioxidants have beneficial effect on bone loss for rats exposed to n-TiO2 via inhibition of proinflammatory cytokines (Figure 3).

Comparison **serum Ca, P or Mg levels** *in n-TiO2 rats with antioxidants*

There was no significant difference in serum Ca, P or Mg levels among the different antioxidant groups of the study compared with n-TiO_2 group (Table 1).

DISCUSSION

TiO_2 particles (150–500 nm) ingested orally can translocate to the blood and accumulate in the spleen and liver (Borm *et al*., 2006). The liver, spleen and bone marrow are the main organs of the reticulo-endothelial system (RES). RES cells have the capacity to take up nanoparticles, and the consequences of nano particles on macrophages are not yet known. It is important to develop new strategies to ameliorate the

consequences of n-TiO_2 on bone because a number of disease-modifying nanoparticle drugs often have side effects at high doses and/or during long-term administration. IL-6 and TNF-α are closely related to inflammation and particular damage in several osteoporosis and arthritis models, and it is therefore generally accepted that IL-6 and TNF-α have pivotal roles in the pathophysiology of bone loss and rheumatoid arthritis. In the present study, there was a significant increase in the levels of TNF-α, IL-6 and CTx after n-TiO_2 administration (Table 1).

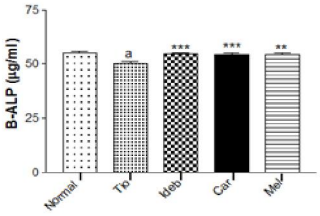
Table 1. The effects of different antioxidants on rats exposed to nano titanium dioxide (n-TiO_2)

	S. Ca (mg/dl)	S. P (mg/dl)	S. Mg (mg/dl)
Untreated controls	10.80	5.52	3.47
	±.252	±.158	±.175
N-TiO2	10.33	5.90	3.75
	±.433	±.073	.156
P=	.468	.104	.06
Idebenone	9.33	5.57	3.607
	±.518	±.2591	.074
P=	.280	.135	.293
L-Carnitine	10.30	5.47	3.63
	±.322	±.076	.083
P=	.969	.06	.370
Melatonin	9.2667	5.47	3.76
	±.433	±.304	.156
P=	.133	.245	.946

The mechanism *of action of* n-TiO_2 *on cells is mediated by the stimulation of* macrophages via reactive oxygen species generation. In turn, oxidative stress and inflammation are the major causes of DNA damage and cytogenetic effects (Reeves *et al*., 2008 and Trouiller *et al*., 2009). Cytogenetic toxicity, which can lead to dysplasia, gives rise to clastogenic damage in erythroblasts in the bone marrow (Westbrook *et al*., 2009) and in turn enhances bone resorption and the expression of pro-inflammatory cytokines that affect on bone. Reactive oxygen species and free radicals are involved in osteoclastogenesis, the apoptosis of osteoblasts, osteocytes and, consequently, bone resorption (Almeida *et al*., 2007 and Jilka *et al*., 2007). Free radicals may affect bone remodeling by regulating osteoclast activity under either physiological or pathological conditions. Therefore, it seems that reactive oxygen species enhance osteoclastogenesis and bone resorption. These findings suggest that there is an urgent need for natural antioxidants that can protect bone metabolism against oxidative stress (Muthusami *et al.*, 2005; Valko, *et al.*, 2007; Almeida *et al.,* 2007 and Ozgocmen *et al.,* 2007).

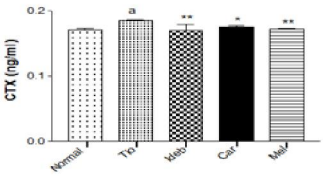
In the present work, L-carnosine caused a significant decrease in the levels of TNF-α, IL-6 and CTX in response to n-TiO_2 treatment, whereas a significant increase in the activity of B-ALP was observed (Figures 1, 2, 3, and 4). The role of L-carnosine in the cytosol is to scavenge oxidated mediators (metals and oxygen radicals). Due to its biological function of scavenging active oxygen radicals, it has an antioxidant property. It acts as a scavenger of hydroxyl and superoxide radicals and, most effectively, the singlet oxygen molecule. (Boldyrev *et al*., 2004; Fouad *et al*., 2007; Fujii *et al*., 2003 and 2005). L-carnosine, which is abundant in skeletal muscle, has been suggested to possess antioxidant and anti-aging properties. It increases the intracellular Ca^{2+} concentration in the absence of extracellular Ca^{2+}. These results indicate that L-carnosine mobilizes Ca^{2+} from intracellular Ca^{2+} stores, leading to increased intracellular Ca^{2+} levels and thereby increasing bone strength by decreasing osteoclastogenesis via inhibiting the release of cytokines and increasing the activity of B-ALP (Baykaraa *et al*., 2009). Administration of melatonin (200 mg/day) in our results had an inhibitory effect on pro-inflammatory cytokines (TNF-α, IL-6) and bone resorption (CTx), whereas it stimulated the B-ALP activity induced by n-TiO_2 (Figures 1, 2, 3, and 4).

Figure 1. The effect of different antioxidants on bone alkaline phosphatase in rats exposed to nano titanium dioxide (n-TiO_2)



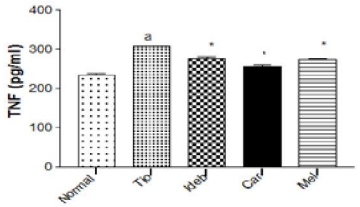
The data are presented as the mean ± S.E.M. *a* $P \leq 0.01$ compared with untreated controls *$P \leq 0.05$, **$P \leq 0.01$ and ***$P \leq 0.001$ compared with the TiO group. Comparisons were conducted using ANOVA followed by

Figure 2. The effect of different antioxidants on C-terminal peptide of type I collagen (CTx) in rats exposed to nano titanium dioxide (n-TiO_2)



The data are presented as the mean ± S.E.M. *a* $P \leq 0.01$ compared with untreated controls, **$P \leq 0.01$ and *$P \leq 0.05$ compared with the TiO group. Comparisons were conducted using ANOVA followed by Tukey's test.

Figure 3. The effect of different antioxidants on tumor necrosis factor-α (TNF-α) in rats exposed to nano titanium dioxide (n-TiO_2)



The data are presented as the mean ± S.E.M. *a* $P \leq 0.001$ compared with untreated controls, *$P \leq 0.05$, **$P \leq 0.01$ and ***$P \leq 0.001$ compared with the n-TiO_2 group. Comparisons were conducted using ANOVA followed by Tukey's test.

The bone remodeling process is controlled by growth factors and cytokines that are produced in the bone marrow microenvironment and by the action of systemic hormones, such as parathyroid hormone, estradiol or Growth Hormone (GH) (Cardinali *et al*., 2003). One candidate for the hormonal modulation of osteoblast and osteoclast formation is melatonin. Melatonin could act as an autacoid in bone cells because it is present in high quantities in bone marrow, in which bone cell precursors are located. Melatonin dose-dependently augmented proteins that are incorporated into the bone matrix, such as procollagen type I c-peptide. In vitro, melatonin can augment osteoprotegerin, an osteoblastic protein that inhibits the differentiation of osteoclasts. Another possible melatonin target cell is the osteoclast, which degrades bone partly by generating free radicals. Melatonin, through its free

radical scavenger and antioxidant properties, may impair osteoclast activity and bone resorption. One study reported that melatonin inhibited both osteoclastic and osteoblastic cells. Therefore, the bone-protecting effect of melatonin in ovariectomized rats could depend in part on the free radical-scavenging properties of melatonin. Thus, melatonin can be used as a novel model of therapy for augmenting bone mass. The administration of idebenone produced anti-inflammatory effects by inhibiting the release of proinflammatory cytokines (TNF-α, IL-6) and CTx, whereas it significantly enhanced the decrease in B-ALP induced by n-TiO_2 (Figures 1, 2, 3, and 4). Inflammation may contribute to bone loss by affecting the bone remodeling process by increasing the bone resorption activity by osteoclasts relative to the bone formation activity by osteoblasts (Moschen *et al*., 2005). Idebenone inhibits lipid peroxidation and consequently protects cell membranes and mitochondria from oxidative damage (Zs-Nagy, 1990 and Schulz *et al*., 2000). Due to its antioxidative capabilities, it blocks pro-inflammatory cytokines and consequently is able to inhibit osteoclastogenesis and bone resorption via decreasing the CTx level.

Conclusions

The results of this study showed that the antioxidants L-carnosine, melatonin and idebenone have beneficial effects in suppressing bone resorption cytokines and stimulating the activity of B-ALP. These effects may be attributed to their potent antioxidant properties. The antioxidant status of these compounds were also maintained within the rat bone. Thus, the antiosteoporotic effect of these antioxidants ameliorated the changes in bone metabolism induced by n-TiO_2.

Acknowledgments

The authors extend their appreciation to the Deanship of Scientific Research at King Saud University for funding this work through research group

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