

Study of prenatal exposure to 4-tetra octyl phenol on lipid peroxidation and activity levels of anti-oxidant enzymes in the testis of mature rats

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ABSTRACT

4-tert-Octylphenol (OP) is major degradation products of alkylphenol ethoxylates. These chemicals are found in the waste water and sludge of sewage treatment works and river sediments. Alkylphenols are also found in drinking water. Thus, alkylphenols are widely dispersed in the environment. Several alkylphenols, including 4-tert-octylphenol have included altered sex hormone levels and hypothalamic-pituitary outcomes, impaired spermatogenesis 4-tert-octylphenol is not considered directly genotoxic. The present study aims at evaluating the effects of prenatal exposure to octylphenol (4-tert OP) in the pregnant rats. The rats were randomly allocated into two groups of 10 rats/groups. Control rats were treated same as the experimental group but received intra-peritoneal injection of corn oil in a 20 μ l volume. The rats in octylphenol group were injected with 50mg octylphenol/kg body weight on 1st, 7th and 14th day of pregnancy. Rats were allowed to deliver pups and weaned. The F1 generation male rats were maintained up to postnatal day. The rats were sacrificed to assess the testicular and epididymal toxicity and reproductive ability. This results stating that, significant increase in the levels of lipid peroxidation with a significant reduction in the activity levels of superoxide dismutase and catalase in testis was observed in rats exposed to 4-tert-Octylphenol during embryonic development. The present data demonstrate that exposed to 4-tert-Octylphenol can produce adverse effects on fertility, reproductive performance, and sperm parameters in adult male rats. Specifically, transplacental exposure to 4-tert-Octylphenol altered the growth of the testis, epididymis, and prostate gland as well as the structure of testes, suggesting that it had adverse on the reproductive organs.

Key Words: 4-tert-Octylphenol, Lipid Peroxidation, SOD, CAT, Testis, Mature Rat.

INTRODUCTION

4-tert-Octylphenol, an alkylphenol, is used to manufacture alkylphenol ethoxylates, which are anionic surfactants used in detergent, industrial cleaners and emulsifiers. Commercial formulations of alkylphenol ethoxylates usually contain a mixture of oligomers and

isomers, and the polythoxy chain may consist of up to 50s ethoxy units. Less frequently, the various alkylphenols have also been used as emulsifiers and modifiers in paints, pesticides, textiles, and some personal care products like cosmetics (Weinberger P,

Rea MS. 1982; Rantuccio E Sinisi D, *et al.*, 1984). The primary alkyl groups are branched octyl or nonyl chains positioned opposite the para-substituted ethoxylate chain. 4-tert-Octylphenol (OP) and 4-nonylphenol (NP) are major degradation products of alkylphenol ethoxylates. These chemicals are found in the waste water and sludge of sewage treatment works and river sediments. Alkylphenols are also found in drinking water. Thus, alkylphenols are widely dispersed in the environment. Several alkylphenols, including 4-tert-octylphenol have included altered sex hormone levels and hypothalamic-pituitary outcomes, impaired steroidogenesis, altered estrus cycles and reproductive outcomes; impaired spermatogenesis 4-tert-octylphenol is not considered directly genotoxic (Sharpe and Skakkebaek, 1993).

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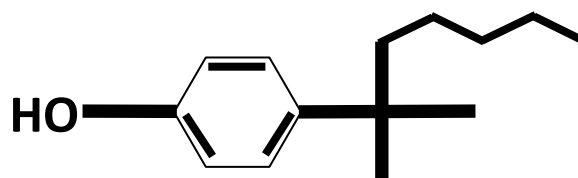
Recent studies of Kim *et al.*, (2007) suggested that 4-tert-OP alters the expression of cholesterol transport gene, steroidogenic acute regulatory protein, and affects steroidogenesis. Whereas exposure to 4-tert-OP during neonatal period results in altered testosterone and FSH levels and caused prolonged impairment of male reproductive tract with poor sperm numbers (Yoshida *et al.*, 2001). 4-tert-OP is soluble and bioaccumulation in fat deposits. The body fat is mobilized in the starvation especially in wildlife; in this period, 4-tert-OP is released from fat, and then passes through blood flow. In a short time, this estrogenic substance may reach high levels and affect male reproductive tract. In many studies, it has been shown that 4-tert-OP escapes coupling with the steroid binding proteins of serum, and freely crosses the placental barrier (Tan and Mohd, 2003). In particular, using fat deposits throughout pregnancy and lactation may cause to pass these substances to male fetus and pups, and affect sexual development of the male progeny (Heavisto *et al.*, 2003). 4-tert-OP is an anionic substance and widely used in preparation of vaginal contraceptives, detergents, personal care products, cosmetics, plastics, pesticide formulations and industrial products (Blackburn and Walldock, 1995; Ying *et al.*, 2002) and thus is ubiquitous low-level environmental contaminant. It is speculated that approximately 50,000 tons of octylphenol was produced annually worldwide and studies of Isobe *et al.* (2001) reported that the levels of 4-tert-OP in sediments and surface waters reached up to $670 \mu\text{g}/\text{kg}^{-1}$ and $0.18 \mu\text{g}/\text{l}^{-1}$ respectively and thus, exposure of wildlife and humans to these levels is therefore possible. OP possesses two important properties like relative stability and lipophilic nature; which pose them to persist in the environment and tend to bioaccumulate in fat deposits of organisms (Tsuda *et al.*, 2001; Ying *et al.*, 2002; Quiros *et al.*, 2005). Exposure to 4-tert-OP is a matter of concern because it has been shown to be both toxic and estrogenic (White *et al.*, 1994; Hossaini *et al.*, 2003).

The mechanism of action of 4-tert-OP on the production of ROS remains unclear. Phenolic compounds have been shown to accumulate in the fatty tissues and is metabolized to hydroxyl-phenols by cytochrome P450-dependent oxidases and further converted to phenol-quinones (Atkinson and Roy, 1995). Cytochrome P450 activate, inactivate, and facilitate the excretion of most xenobiotics, thus modulating the duration and intensity of their toxicity. Cytochrome P450 has been shown to induce ROS that permanently impairs sperm function thereby resulting in decline of sperm counts in men and experimental animals (Aitken *et al.*, 1989). ROS, such as hydrogen peroxide, appears to be a key agent causing cytotoxic effects in spermatozoa (Griveau and Lannou, 1997).

Recently, it was reported that exposure of adult male rats to 4-tert-OP can cause shrinkage of the testes; epididymis and male accessory sex glands and alter normal histological structure (Boockfor *et al.*, 1997). It was also presented that consumption of 4-tert-OP in drinking water at concentrations approximately 50-fold

lower than that which has been measured in water in the environment caused an increased incidence of tail abnormalities in epididymal sperm (Blake *et al.*, 2004). In addition to this, 4-tert-OP has been reported to alter the secretions of testosterone, luteinizing hormone (LH), follicle stimulating hormone (FSH), and prolactin (Blake and Boockfor, 1997). It is being considered that the abnormal development of reproductive organs and increases in the frequencies of reproductive disorders in both human and wildlife stems from the growing usage of environmental chemicals in recent years (Carlsen *et al.*, 1992). 4-tert-OP is relatively persistent, bioaccumulates in environment and lipids of living organisms, and has toxic and estrogenic effects on mammalian cells. In particular, it was suggested that excess exposure of estrogen at fetal period when reproductive organs are highly susceptible to hormonal exposures is a key factor (Toppari *et al.*, 2002). Abnormal development of the testes in fetal or neonatal life can have life-time consequences on all aspects of reproductive functions in adulthood, including sperm counts (Sharpe, 2001). For these reasons, a study of the potential toxic effects of prenatal exposure to 4-tert-OP on the reproductive tract of male rats in adult life was performed.

In this background, the author is intended to study the fertilization capacity of sperm produced in 4-tert-OP exposed rats. Male rats exposed to octylphenol were able to impregnate the control female and the rate of pregnancy was observed to be comparatively lower as compared to unexposed male rats. The production of fertile spermatozoa and eggs is the most important starting point for successful reproduction of animals, including human. Administration of octylphenol (Yoshida *et al.*, 2001; Herath *et al.*, 2004) resulted in a steep suppression of circulatory testosterone levels. Further, administration of octylphenol (Aydogan and Barlas, 2006; Sainath *et al.*, 2011) resulted in reduction in daily sperm production, epididymal spermatozoon concentration and deterioration in sperm quality. Thus, the present study was aimed to investigate the effects of exposure to octylphenol on male reproductive health in rats.



4-tert-Octylphenol

MATERIALS AND METHODS

Experimental Design

The pregnant rats were randomly allocated into two groups of 10 rats/groups. Control rats were treated same as the experimental group but received intra-peritoneal injection of corn oil in a $20\mu\text{l}$ volume. The

rats in octylphenol group were injected with 50mg octylphenol/kg body weight on 1st, 7th and 14th day of pregnancy. Rats were allowed to deliver pups and weaned. The F1 generation male rats were maintained up to postnatal day. The rats were sacrificed to assess the testicular and epididymal toxicity and reproductive ability of rats.

The Rat were sacrificed by cervical dislocation. Each rat was opened, cauda epididymis was identified and isolated. Isolated cauda epididymis was kept in petriplated having physiological saline. By squeezing cauda epididymis, immediately immersed in liquid nitrogen and stored at -800C for biochemical analysis and enzymatic assays. Before assay, the tissues were thawed, sliced and homogenized under ice-cold conditions. Selected parameters were estimated by employing standard methods.

Biochemical Investigations:

Lipid Peroxides:

The lipid peroxides were determined by the TBA method of Hiroshi *et al.* Briefly, the testes were homogenized (10% W/V) in 1.15% potassium chloride solution. 0.5 ml of saline (0.9% sodium chloride), 1.0 ml of (20% W/V) trichloroacetic acid (TCA) were added to 2.5 ml of homogenate. Samples were centrifuged for 20 minutes on a refrigerated centrifuge at 4000 x g. 0.25 ml of TBA reagent was added to 1.0 ml of supernatant, and samples were then incubated at 95°C for 1 h. One ml of n-butanol was added to it. After thorough mixing, the contents were centrifuged for 15 minutes at 4000 x g in a refrigerated centrifuge. The organic layer was transferred into a clear tube and its absorbance was measured at 532 nm. The rate of lipid peroxidation was expressed as μ moles of malondialdehyde formed/gram wet weight of tissue.

Superoxide dismutase (EC 1.15.1.1):

Superoxide dismutase was assayed by the method of Misra and Fridovich. (1972). Briefly, the testes were homogenized (10% W/V) in 50 mM ice-cold sodium phosphate buffer (pH 7.0) containing 0.1 mM EDTA. The homogenate was centrifuged at 105,000 x g for 60 min. The supernatant (cytosol) fraction was used for the assay of the enzyme activity. The reaction mixture in a final volume of 2.0 ml contained: 0.05 M carbonate buffer (pH 10.2), 30 mM epinephrine (freshly prepared) and the enzyme extract. Changes in absorbance were recorded at 480 nm, measured at 10 seconds intervals for 1 minute in a UVVIS spectrophotometer (Hitachi model: U-2001). The protein content in the enzyme source was determined by the method of Lowry *et al.* (1951) using bovinclozoline serum albumin as standard. The enzyme activity was expressed as Units/mg protein/min.

Catalase (EC. 1.11.1.6):

Catalase was assayed by the method of Chance and Machly (1955). The reaction mixture in a final volume of

2.5 ml contained: 0.05 M phosphate buffer (pH 7.0), and appropriate amount of enzyme protein. The reaction was initiated by the addition of 19 mM hydrogen peroxide (H₂O₂). The decomposition of H₂O₂ was tracked directly by measuring the decrease in absorbance at 240 nm, at 10 seconds intervals for 1 minute in a UV-VIS spectrophotometer (Hitachi model: U-2001). The activity of the enzyme was expressed as μ moles of H₂O₂ metabolized/mg protein/min.

Somatic and reproductive tissue index:

The body weight of the rat was taken before decapitation and the tissues like testis and reproductive organs were isolated. The organs were weighed to a nearest milligram using Shimadzur electronic balance. The tissue somatic indices were calculated by using the following formula.

$$\text{Tissue somatic index} = \frac{\text{Weight of tissue (g)}}{\text{Weight of body (g)}} \times 100$$

Statistical analysis:

Values are expressed in mean \pm S.D. For comparison of results between the control and experimental group, were compared using independent sample t-test (Petrie & Watson, 1999) was utilized. Differences were considered significant when $p < 0.05$.

RESULTS AND DISCUSSION

Lipid peroxidation:

In the present study the levels of lipid peroxidation products (malondialdehyde) are presented in (Table 1). A significant ($p < 0.05$) increase in malondialdehyde content in the testes of 4-tert-Octylphenol treated rats (129.06) was observed when compared with testis of control rats.

To our data, this study examined Lipid peroxidation is an oxidative process in which lipid molecules undergo a series of chemical alterations initiated by free radical and oxygen (Kappus, 1991). In this process in which molecular oxygen interacts with unsaturated membrane lipids (Cholesterol, Phospholipids) through a series of complex multistage reaction. For lipid peroxidation to occur activation of lipid molecules and pro-oxidants are both necessary. This process can become autocatalytic after initiation and will yield lipid peroxide, lipid alcohol and aldehyde by products. In the present study in testis lipid peroxidation was increased in the 4-tetra-octylphenol treated rats. MDA concentration of testis in this group which means a significant reduction in lipid peroxidation within the testis. (Bhavanarayana and Reddy, 2013). MDA is considered an index of lipid peroxidation is a reactive end product of lipid peroxidation. MDA level can reflect the degree of damage of testicular tissues induced by ROS (reactive oxygen species, e.g., O₂⁻ and OH[•]). (Hand JW, *et al.*, 1979). The ROS-induced injury normally causes increase of MDA level in testicular tissues. This study showed that after negative control was exposed to 4-tetra-octylphenol the MDA level significantly increased

in the testicular tissues ($P < 0.05$) (Table 1). These observations in the 4-tetra- octylphenol treated rats led to significant increase in the MDA, an index of lipid peroxidation, in testes and spermatozoa of rats in the present study. Increased sperm membrane lipid peroxidation has been shown to impede sperm progress motility and increase percentage total sperm abnormalities as well as cause a dramatic loss in the fertilizing potential of sperm (El-Demerdash *et al.*, 2004).

Reactive oxygen metabolites such as superoxide, hydroxyl radical, singlet oxygen and hydrogen peroxide are generally considered cytotoxic agents because of their ability to induce lipid peroxidation in tissues. Several antioxidant defense systems, namely, SOD, Catalase and Glutathione peroxidase are known to operate in tissues. SOD generally dismutates the superoxide radical into hydrogen peroxide which is further degraded by catalase. The reduction in the activity levels of SOD and catalase in the testis and different regions of epididymis after exposure to 4-tert-OP reflects the inability of tissues to eliminate the radicals generated after the exposure to 4-tert-OP. Increased lipid peroxidation may indicate an increased oxygen free radical generation and elevated oxygen radicals has been associated with decreased sperm count and deteriorated sperm quality (Thiele *et al.*, 1995).

Superoxide Dismutase (SOD)

In the present study the activity levels of superoxide dismutase (SOD) was decreased significantly (-45.34) in the testes of rats exposed to 4-tert-Octylphenol during embryonic development when compared to control rats (Table-1).

Table.1: Effect of prenatal exposure to 4-tetra octyl phenol on lipid peroxidation and Activity levels of catalase and superoxide dismutase in the testis of Mature rats.

Parameter	Control	50 mg/kg BW
Lipid peroxidation (μ moles of malondialdehyde formed/g wet wt.)	7.19 \pm 2.87	16.47 \pm 2.52 (129.06)
Superoxide dismutase (Units/mg protein/min.)	0.86 \pm 0.46	0.47 \pm 0.28 (-45.34)
Catalase (μ moles of H ₂ O ₂ metabolised/mg protein/min)	0.32 \pm 0.66	0.14 \pm 0.28 (-56.25)

Values are mean \pm S.D. of 6 individuals.

Values in parentheses are percent change from control.

Mean values in a row that do not share the same superscript differ significantly at $p < 0.05$.

The superoxide dismutase (SOD) catalyzes the dismutation of two superoxide radicals (O_2^-) into hydrogen peroxide (H_2O_2) and oxygen. These enzymes obey first order reaction kinetics and the forward rate

constants are almost diffusion limited. This results in steady state concentration of superoxide radicals in tissues that may vary directly with the rate of superoxide generation and inversely with the tissue concentration of scavenging enzymes (Enghild *et al.*, 1999; Fattman *et al.*, 2003). It is well known that SOD is involved in destroying the superoxide radical and exists in several isoforms different in both cellular location and the metal co-factor bound to its active site. (Powers and Lennon 1999; Enghild *et al.*, 1999; Kao *et al.*, 2002). Similar studies have been reported by several authors. The depletion of SOD activity may be due to dispose of the free radical, produced by the nicotine toxicity. Helen *et al.*, (2000) reported the decreased SOD activity in brain tissue of rat due to nicotine toxicity. El-Sokkary *et al.*, (2007) reported chronic administration of nicotine the SOD activity was decreased the rat liver and lung. Chattopadhyay and Chattopadhyay (2008) reported due to nicotine treatment the SOD activity was decreased in Ovary tissue. Similar changes in SOD activity was reported in various toxic conditions. Mahendran and Syamala Devi, (2001) reported decrease in SOD activity with 18% ethanol treatment in the hepatic tissue. Somani and Husain, (1997b) reported significant decrease in plasma and hepatic SOD activity with 20% of chronic ethanol treatment.

Similar results were reported by Bindhumol *et al.*, 2003; Chitra *et al.*, 2003; and Kabuto *et al.*, (2003). Enzymatic scavengers like SOD and CAT protect the cell system from deleterious effects of ROS. In this study, decreased levels of antioxidant enzymes, resulting in oxidative cell damage. SOD is the first enzyme to respond against oxygen radicals and is the one that offers the greatest response to oxidative stress. SOD protects cells from oxidative stress and damage by catalyzing the conversion of $O_2^{\bullet-}$ to H_2O_2 , a more stable reactive oxygen species. Catalase catalyzes the dismutation of the superoxide anion (O_2^-) into hydrogen peroxide (H_2O_2) which then converts hydrogen peroxide to water, in this manner, providing protection against reactive oxygen species. Hence, this enzyme protects cells from highly reactive hydroxyl radical (OH), derived from H_2O_2 . (Halliwell, G, 1965).

It is known that some xenobiotics may inhibit the activity of antioxidant enzymes system, which probably may lead to intracellular accumulation of reactive species with subsequent development of tissue injury. These reactive species may cause decline in the activity of cellular antioxidants, in particular SOD, CAT and GSH (Prakash *et al.*, 1997). SOD is the first line of defence against the deleterious effects of oxygen radicals in cells, and it scavenges reactive species by catalyzing the dismutation of superoxide radicals to hydrogen peroxide (Okado- Matsumoto and Fridovich, 2001). The inhibition of SOD activity during OP treatment may result in an increased flux of superoxide radicals in the animals, which explain, in part, the increased LPO in tissues of treated rats. CAT acts as a preventive antioxidant in cells. Both CAT and SOD are co-regulated

in tissues in response to toxic assaults (Lew and Quintanilha, 1991). The enzyme substrate interaction of the mitochondrial form does not appear to involve autoinactivation mechanism (Karuzina and Archakov, 1994) indicating that mitochondrial SOD levels are maintained for longer period of time compared to the cytosolic form. Increased levels of SOD are generally taken as indirect evidence of an increased oxidant milieu. Since this is a sulfhydryl-containing enzyme, decrease of its tissue level can also reflect oxidative denaturation. As with GSH biphasic fluxes are common and change in either direction may relate to the presence of excess of ROS (Bondy and Naderi, 1994; Kodavanti, 1999).

Catalase (CAT):

In the present study the activity levels of catalase (CAT) was decreased significantly (-56.25) in the testis of rats exposed to 4-tert-Octylphenol during embryonic development when compared to control rats (Table-1).

Catalase is one of the most important antioxidant enzyme, which can function either in the catabolism of hydrogen peroxide (H_2O_2) or in the peroxidative oxidation of small substances such as ethanol or methanol. Catalase has four subunits and each subunit contains a heme group. Heme consists of a protoporphyrin ring and a central iron (Fe) atom. The iron can either be in the ferrous (Fe^{2+}) or the ferric (Fe^{3+}) oxidative state. This heme group is responsible for carrying out catalase activity. To maintain catalytic activity CAT requires Fe^{2+} as a co-factor (Powers and Lennon, 1999; Temel *et al.*, 2002). Catalase is widely distributed in the body compartments, tissues and cell. In many cases the enzyme is located in subcellular organelles such as, peroxisomes and cytosol of liver (Atalay and Laaksonen, 2002; Lesiuk *et al.*, 2003). Catalase plays an important role in ROS metabolism and in adaptation to oxidant stress (Mates *et al.*, 1999; Vaziri *et al.*, 2003).

In the present study the animals treated with 4-tert-OP showed decreased activities of anti-oxidant enzymes Catalase in the testis and different regions of epididymis. Spermatozoa have been considered to be highly susceptible to the damage induced by reactive oxygen species (ROS) because of their high content of polyunsaturated fatty acids. To counteract the effects of ROS, testes are equipped with antioxidant defense systems, which prevent cellular damage. A balance between the benefits and risks from ROS and antioxidants appears to be necessary for survival and normal functioning of spermatozoa in the testes (Aitken and Roman, 2008). Impaired antioxidant defense mechanism may induce testicular damage, low sperm counts, and infertility (Makker *et al.*, 2009). High levels of reactive oxygen species have been correlated with reduced sperm motility (Armstrong *et al.*, 1999; Wang *et al.*, 2004).

Similar changes in CAT activity was reported in various toxic conditions by various authors. Bindu *et al.*, (2002) reported the decrease in CAT activity with 4g/kg body weight alcohol treatment for a period of 50 days in

Sprague Dawley albino rats. Recently Oluwatosin Adaramoye *et al.*, (2012) reported decrease in the activities of testicular superoxide dismutase (SOD) and Catalase (CAT) with MET (metformin hydrochloride) at doses of 5 and 30 mg/kg, respectively. Das and Vasudevan, (2005b) reported a significant decrease in CAT activity with 2g /kg body weight ethanol treatment for a period of 4 weeks in hepatic tissue of Wistar strain male albino rats. This ethanol induced decrease in CAT activity may be due to enzyme protein oxidation as a result of accumulation of H_2O_2 and other cytotoxic radicals (Somani *et al.*, 1996). We found that the administration of 4-tert-Octylphenol observed the decrease in CAT activity in the testis of mature rat. CAT has been reported in the testicular cells in this study. It has been also indicated that the activity of CAT is low in the different testicular cells, in which the activity of CAT in the rat testis is very low compared with the level in the liver (Peltola *et al.*, 1992). A similar low testicular activity of CAT has been reported in the rabbit (Ihrig *et al.*, 1974). CAT and SOD are considered to be indispensable for the survival of the cell against deleterious effects of hydroperoxides. The combination of SOD and CAT provide an efficient mechanism for removal of free radicals from the cell (Husain *et al.*, 1996; Bhaskar Reddy, 2002). The exposure to combination of stresses induced testicular oxidative stress as evidenced by increased levels of lipid peroxidation and decreased activities of SOD and catalase in testes and epididymis. In general, SOD is the first line of defense against oxidative stress (Hassan and Schellhorn, 1988) and play a pivotal role in dismutation of superoxide anions to hydrogen peroxide and catalase neutralizes hydrogen peroxides to molecular oxygen and water (Inal *et al.*, 2001). The decrease in these enzymes in experimental rats clearly postulates improper dismutation of superoxides and improper decomposition of H_2O_2 . Increase in the oxidative stress with decreased activity of antioxidants in the experimental rats indicates that the pro- and antioxidant balance is disturbed.

CONCLUSION

In conclusion, The present study demonstrates that 4-tert-OP induced oxidative stress in rat testis by decreasing the activities of antioxidant enzymes, this work provides the novel evidence that Octylphenol can also cause oxidative stress and damage rat testes (Aydogan *et al.*, 2010). 4-tert-OP can cause apoptosis of testicular germ cells (Zhou *et al.*, 2001; Kim *et al.*, 2007) and Sertoli cells (Qian *et al.*, 2006). It has been shown to be both estrogenic and toxic to mammalian cells (Kuiper *et al.*, 1998; Lee, 1998; Safe *et al.*, 2000). This finding confirms previous reports concerning in prenatal 4-tert-OP exposed rats might be due to induced oxidative stress in testis of rats.

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Conflict of Interests

Authors declare that there is no conflict of interests regarding the publication of this paper.

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