Excessive apoptosis and defective autophagy contribute to developmental testicular toxicity induced by fluoride

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Abstract
Fluoride, a ubiquitous environmental contaminant, is known to impair testicular functions and fertility; however, the underlying mechanisms remain obscure. In this study, we used a rat model to mimic human exposure and sought to investigate the roles of apoptosis and autophagy in testicular toxicity of fluoride. Sprague–Dawley rats were developmentally exposed to 25, 50, or 100 mg/L sodium fluoride (NaF) via drinking water from pre-pregnancy to post-puberty, and then the testes of offspring were excised on postnatal day 56. Our results demonstrated that developmental NaF exposure induced an enhanced testicular apoptosis, as manifested by a series of hallmarks such as caspase-3 activation, chromatin condensation and DNA fragmentation. Further study revealed that fluoride exposure elicited significant elevations in the levels of cell surface death receptor Fas with a parallel increase in cytoplasmic cytochrome c, indicating the involvement of both extrinsic and intrinsic apoptotic pathways. Intriguingly, fluoride treatment also simultaneously increased the number of autophagosomes and the levels of autophagy marker LC3-II but not Beclin1. Unexpectedly, the expression of p62, a substrate that is degraded by autophagy, was also significantly elevated, suggesting that the accumulated autophagosomes resulted from impaired autophagy degradation rather than increased formation. Importantly, these were associated with marked histopathological lesions including spermatogenic failure and germ cell loss, along with severe ultrastructural abnormalities in testes. Taken together, our findings provide deeper insights into roles of excessive apoptosis and defective autophagy in the aggravation of testicular damage, which could contribute to a better understanding of fluoride-induced male reproductive toxicity.

1. Introduction
Fluoride is a ubiquitous environmental pollutant of increasing worldwide concern. Naturally occurring high fluoride concentration in drinking water is the major cause of fluorosis in humans. It is estimated that over 200 million people throughout the globe consume water with fluoride ion concentration above 1.5 mg/L (the safe limit set by the World Health Organization), which remains a major public health challenge particularly in developing countries (Ayoob and Gupta, 2006; Amini et al., 2008). Cumulative evidence suggests that in addition to teeth and skeleton, excessive fluoride intake can also cause damage to soft tissues, such as the liver, kidney, brain, thyroid gland and testis (Barbier et al., 2010). Of note, the male reproductive toxicity of fluoride has received much attention in recent years. A number of epidemiological investigations have established a close relationship between increasing environmental fluoride levels and decreasing male fertility as well as birth rates of people living in areas of endemic fluorosis (Freni, 1994; Long et al., 2009). Animal studies further demonstrated that fluoride is a testicular toxicant that can cross the blood-testis barrier, resulting in disruption of spermatogenesis, structural and functional defects in spermatozoa, and disturbances of multiple reproductive hormones (Long et al., 2009; Zhang et al., 2013; Lu et al., 2014). Although it is well documented that excessive fluoride exposure has an adverse effect on testicular function, the underlying mechanisms are still not fully elucidated. Several lines of evidence support a role for apoptosis in the...
toxicity of fluoride (Barbier et al., 2010). Apoptosis is a highly regulated cell death program that can be triggered via the extrinsic pathway, which implicates activation of cell surface death receptors, or the intrinsic pathway, which involves mitochondrial outer membrane permeabilization (MOMP), or the apoptotic signaling evoked by endoplasmic reticulum (ER) stress (Tait and Green, 2010; Tabas and Ron, 2011). Now it is known that apoptosis plays a crucial role in sperm maturation; however, abnormal apoptosis has also been shown to adversely affect spermatogenesis and sperm number (Shaha et al., 2010). Previous studies have shown that fluoride exposure can induce death receptor Fas pathway activation in human neuroblastoma SH-SY5Y cells (Xu et al., 2011) and rat livers (Miao et al., 2013), and that the mitochondrial pathway was also involved in apoptosis of germ cells following various testicular insults (Shaha et al., 2010) and of liver cells after fluoride exposure (Cao et al., 2013). Recently, we have revealed that the ER stress-mediated signaling is involved in fluoride-induced testicular cell damage and death (Zhang et al., 2013), whether the extrinsic and intrinsic apoptotic pathways are also activated in this scenario remains unclear.

Distinct from apoptosis, autophagy is an evolutionarily conserved lysosomal catabolic mechanism in which damaged organelles and long-lived proteins are sequestered into autophagosomes and delivered to lysosomes for degradation. This bulk degradation not only provides nutrients to sustain cellular functions, but also prevents the toxic accumulation of damaged or dysfunctional components (Levine and Kroemer, 2008). Autophagy plays an essential role in cellular differentiation, development, homeostasis, cell survival and death (Yang and Klionsky, 2010; Wirawan et al., 2012), however, dysregulation of autophagy contributes to several pathological processes, including cancer, infections, muscular and degenerative diseases (Schneider and Cuervo, 2014). The function of autophagy in testes is currently under investigation and it has been shown to be associated with spermatogenesis, germ cell death, sperm function and testosterone secretion (Li et al., 2011; Zhang et al., 2012; Wang et al., 2014; Herpin et al., 2015). Although fluoride has been reported to induce excessive autophagy in exocrine pancreas cells (Ito et al., 2009), little is known about how fluoride influences the autophagy process in testis.

Therefore, the aim of the present study was to assess the possible effect of fluoride on testicular development and focus on the roles of apoptosis and autophagy in testicular injury. To this end, Sprague–Dawley rats were exposed daily before breeding, in utero and after birth to sodium fluoride at environmentally relevant concentrations to mimic human exposure occurring during the critical maternal, perinatal, and pubertal phases. We found that developmental exposure to fluoride induces excessive apoptosis and defective autophagy contributing to spermatogenic arrest and germ cell loss in male offspring.

2. Materials and methods

2.1. Chemicals

Sodium fluoride (NaF) was purchased from Shanghai Chemical Reagent Corp (Shanghai, China). All other chemical reagents were of analytical grade from standard commercial suppliers unless otherwise indicated in the specified methods.

2.2. Animals and treatments

Adult female and male Sprague–Dawley rats, aged 2–3 months, were purchased from the Laboratory Animal Center of Huazhong University of Science and Technology. Animals were maintained at constant temperature (22–26 °C) and humidity (55–60%) in a holding facility under a 12 h light/dark cycle, with free access to food and water. All experimental procedures were performed in compliance with Guidelines for animal studies and approved by the Institutional Animal Care and Use Committee of Tongji Medical College, Huazhong University of Science and Technology.

After acclimation, animals within each sex were weight-ranked and randomly divided into four groups as follows: tap water control group (fluoride ion concentration less than 1.0 mg/L), and three NaF-treated groups (NaF was administered at 25, 50 and 100 mg/L via drinking water). Animals were exposed to NaF via drinking water to mimic human exposure. The dosages were chosen based on previous studies concerning reproductive and other toxicities of fluoride, the environmental fluoride levels as well as the difference of clearance between humans and rats (see Discussion) (Collins et al., 2001; Long et al., 2009; Barbier et al., 2010). After 10 days of exposure, animals were randomly taken from each group (male: female 1:1) for mating. The pregnant rats were placed in separate cage and following continuous exposure throughout gestation and subsequent weaning of pups. The male pups were then re-caged according to treatment condition and with litter mates when possible, and given the same levels of NaF as the adult rats did until postnatal day 56 (Fig. 1). After treatment, all rats were sacrificed within 24 h and testes were immediately isolated. One part of the right testis was fixed in Bouin’s solution for histopathological and immunohistochemical analysis or TUNEL staining, another part was fixed in 25% glutaraldehyde phosphate for ultrastructural analysis, while the left testis was frozen immediately in liquid nitrogen and stored at −80 °C for protein extraction.

Somatic development and reproductive outcomes using this dosing paradigm have been reported previously (jiang et al., 2014). There were no signs of dysfunction over the experimental period, nor were there any significant deviations in survival rate, sex ratio, or organ coefficient of testis across treatment groups.

2.3. Histology

Routine histology was performed to evaluate the status of spermatogenesis in rat testis. In brief, the fixed testes were embedded in paraffin blocks and sectioned perpendicular to the longest axis of the testes at a 4 μm thickness. After deparaffinization and rehydration, the sections were stained with hematoxylin and eosin (H&E) using standard histological techniques. The stained sections were mounted and examined under a light microscopy. Three sections per sample were examined.

2.4. TUNEL assay

Testicular tissue processing for apoptosis-related DNA fragmentation was evaluated by TUNEL Assay using the In situ Cell Death Detection kit (Roche Diagnostics, Shanghai, China) as described by the manufacturer. Briefly, the 4 μm paraffin tissue sections were deparaffinized, hydrated and treated with proteinase K for 15 min at 37 °C after blocking endogenous peroxidase activity with 3% hydrogen peroxide for 15 min at room temperature (RT). After washing with 0.01 M phosphate-buffered saline, sections were incubated with TUNEL reaction mixture (mixture between enzyme solution and labeling solution in a ratio of 1:9) for 1 h at 37 °C in a humidified atmosphere in the dark. The sections were then incubated with converter-POD (anti-fluorescein conjugated with horse-radish peroxidase) for 30 min at 37 °C, and color was developed using 3, 3’-diaminobenzidine (DAB). The slides were counterstained with hematoxylin, dehydrated and mounted for.
light microscopy (Olympus, Japan). Cells exhibiting brown nuclear staining were considered to be positive for apoptosis-associated nuclear DNA fragmentation. Three sections were analyzed for each sample.

2.5. Transmission electron microscopy

For ultrastructural studies, tests were cut into 1 mm³ blocks, fixed immediately in 2.5% glutaraldehyde phosphate for 24 h at room temperature, and post-fixed in 1% osmium tetroxide for 2 h. The blocks were then dehydrated with an ascending graded alcohol and acetone series, immersed in acetone and embedded in epoxy resin at 80 °C for 10 h. Serial ultrathin sections (50 nm) were collected on copper grids and stained with 1% uranyl acetate and Lead Citrate for 10 min. The resulting micrographs were examined using a transmission electron microscope (TEM, Tecnai G212, Philips, Netherlands). Three rats were randomly selected from each group for ultrastructure evaluation.

2.6. Western blotting analysis

Testicular tissues were homogenized in RIPA buffer (Beyotime Institute of Biotechnology, Nanjing, China) followed by centrifugation at 12, 000 × g for 15 min at 4 °C and collection of the supernatant. The total protein concentrations were measured with the BCA Protein Assay Kit (Boster Biological Technology, Wuhan, China). The supernatant was mixed in loading buffer followed by boiling for 10 min. Proteins were resolved on a denaturing 10–12% SDS-PAGE gel and subsequently transferred to polyvinylidene fluoride membranes (PVDF; Roche Applied Science, Germany). The membranes were first blocked with 5% (w/v) powdered skimmed milk in tris-buffered saline (TBS) containing 0.1% Tween-20 (TBST) for 1 h at RT, and then treated overnight at 4 °C with the primary antibodies against Fas (1:1000; Bioworld Technology, CA, USA), cytochrome c (1:1000; Bioworld Technology, CA, USA), active caspase-3 (1:1000; Cell Signaling, Beverly, MA, USA), Beclin1 (1:1000; Proteintech Group, Chicago, IL, USA), LC3 (1:1000; Proteintech Group, Chicago, IL, USA), and P62 (1:1000; Abcam, Cambridge, MA, USA). The sections were then incubated with biotinylated secondary antibody and followed by peroxidase conjugated streptavidin. Staining was visualized by adding 3, 3′-diaminobenzidine (DAB Substrate, Roche, Germany) with subsequent counterstaining using hematoxylin. Immunohistochemical micrographs were viewed under a microscope (Olympus, Japan). Three sections were analyzed for each sample.

2.7. Immunohistochemistry

Paraffin-embedded tissue specimens were serially sectioned and analyzed by immunohistochemistry using a standard method as previously described (Zhang et al., 2013). Briefly, the 4 μm thick sections were dewaxed and rehydrated, followed by antigen retrieval. Endogenous peroxidase activity was quenched with 0.3% hydrogen peroxide/methanol solution, and then sections were blocked with 5% (v/v) bovine normal serum in phosphate-buffered saline. Thereafter, the sections were incubated overnight at 4 °C with the specific primary antibodies against Fas (1:100; Bioworld Technology, CA, USA), cytochrome c (1:1000; Bioworld Technology, CA, USA), LC3 (1:100; Proteintech Group, Chicago, IL, USA), and P62 (1:100; Abcam, Cambridge, MA, USA). The sections were then incubated with biotinylated secondary antibody and followed by peroxidase conjugated streptavidin. Staining was visualized by adding 3, 3′-diaminobenzidine (DAB Substrate, Roche, Germany) with subsequent counterstaining using hematoxylin. Immunohistochemical micrographs were viewed under a microscope (Olympus, Japan). Three sections were analyzed for each sample.

2.8. Statistical analysis

All data were presented as means ± standard deviation (S.D.) for four animals per group and analyzed by one-way analysis of variance (ANOVA) followed by the least significant difference (LSD) test. A value of P < 0.05 was considered statistically significant.

3. Results

3.1. Developmental exposure to NaF induced histological lesions in rat testes

Control testes demonstrated the normal testicular architecture and regular seminiferous tubular morphology, including the outermost layer of Sertoli cells and spermatogonia, the middle layer of spermatocytes, and the innermost layer of sperm cells, indicative of normal spermatogenesis (Fig. 2A). However, in the testes of NaF-treated rats, the size of the seminiferous tubules was obviously reduced and the morphology of the epithelium was severely damaged. Vacuole-like changes appeared in the spermatogenic epithelium and, in particular, this alteration was more significant. Collectively, the atrophy and disruption of the seminiferous epithelium suggest that developmental exposure of rats to NaF impaired spermatogenesis and caused germ cell loss in male offspring.
3.2. Developmental exposure to NaF induced ultrastructural abnormalities in rat testes

Transmission electron microscopy revealed normal testicular ultrastructure in control rats (Fig. 3A). In contrast, NaF exposure caused extensive abnormalities in testicular tissue. Consistent with the results of histology, vacuolation of the seminiferous epithelium was common in affected tubules, especially in the cytoplasm of Sertoli cells (Fig. 3B). In addition, growing spermatogonia and spermatocytes, together with occasionally round spermatids, displayed morphological characteristics of apoptosis, including condensation and margination of nuclear chromatin, as well as swollen mitochondria with the degeneration or loss of cristae (Fig. 3C). Furthermore, numerous autophagosomes-like structures (some containing mitochondria and other cytoplasmic contents) were obviously present in testes of NaF-treated rats, relative to controls (Fig. 3D and F).

3.3. Developmental exposure to NaF activated extrinsic and intrinsic apoptotic pathways in rat testes

To further confirm the findings obtained above, we also used in situ TUNEL assay to determine apoptosis-related DNA fragmentation in testes. As showed in Fig. 4A, TUNEL-positive cells were occasionally observed in the testes of control rats, whereas the number of positive cells was markedly increased in spermatogenic cells in the cytoplasm of Sertoli cells (Fig. 3B–F). In addition, growing spermatogonia and spermatocytes, together with occasionally round spermatids, displayed morphological characteristics of apoptosis, including condensation and margination of nuclear chromatin, as well as swollen mitochondria with the degeneration or loss of cristae (Fig. 3C–E). Furthermore, numerous autophagosomes-like structures (some containing mitochondria and other cytoplasmic contents) were obviously present in testes of NaF-treated rats, relative to controls (Fig. 3D and F).

3.4. Developmental exposure to NaF inhibited autophagic degradation in rat testes

The increase in the number of autophagosomes reflects either increased autophagosome formation due to enhanced autophagic activity or an accumulation of autophagosomes due to impaired autophagic degradation. We then tested whether autophagy was induced in testes by analyzing commonly used autophagy markers including Beclin-1, LC3 and p62. As shown in Fig. 5A-B, the protein expression levels of Beclin-1, which is required for the initiation of autophagosome formation, were not obviously influenced following NaF exposure (P > 0.05). Conversely, the levels of lipidated LC3-II, which has been extensively used as a reliable protein marker for mature autophagosomes, were remarkably increased in testes of NaF-treated rats (P < 0.05) (Fig. 5A), along with enhanced LC3 puncta formation in testicular cells in comparison to controls (Fig. 5E). Unexpectedly, the levels of p62, a protein that is degraded by autophagy, were also significantly elevated in rat testes after developmental NaF exposure (Fig. 5A and D) (P < 0.05). This is also accompanied by a strong staining of p62 in testicular cells of NaF-treated rats (Fig. 5E), suggestive of compromised autophagic degradation.
Fig. 3. Developmental exposure to NaF induced ultrastructural abnormalities in rat testes. Representative transmission electron microscopy images of testicular tissues from control (A), 25 mg/L (B), 50 mg/L (C and D) and 100 mg/L NaF-treated rats (E and F). Severe impairment of seminiferous tubules, such as vacuolation, mitochondrial swelling and degeneration, apoptosis and autophagosomes accumulation were observed in sections from NaF-treated rats compared with those from control rats. The asterisks indicate focal vacuolation; the double arrowheads indicate typical characteristics of apoptosis, such as condensation and margination of nuclear chromatin; the fold arrows indicate swollen mitochondria with the degeneration or loss of cristae; the thin arrows indicate autophagosomes (some containing mitochondria and other cytoplasmic cargos). Three rats were randomly selected from each group for ultrastructure evaluation.

Fig. 4. Developmental exposure to NaF activated extrinsic and intrinsic apoptosis pathways in rat testes. (A) Representative images of TUNEL-, Fas-, and cytochrome c-stained testicular cross sections from control and 100 mg/L NaF-treated rats (×400). (B) Representative images of western blotting for apoptotic markers Fas, cytochrome c and active caspase-3 in protein extracts from testes of the control and NaF-treated rats. (C–E) Quantitative analyses of the Fas, cytochrome c and active caspase-3 expression levels normalized to the internal control β-actin. Data are expressed as the means ± S.D. for four rats per group. Quantified band intensities are presented as fold of control. *P < 0.05 as compared to control rats. Three sections were analyzed for each sample.
Fluorosis, a disease caused by chronic exposure to excessive fluoride principally via drinking contaminated groundwater, is a serious public health issue particularly in developing countries including China, India and eastern Africa. In these affected areas, fluoride ion concentrations in groundwater have been reported to be higher than 0.5 mg/L and up to 48 mg/L (Muntaz et al., 2015). The fluoride doses used in this study (25, 50 and 100 mg/L NaF corresponding to 11.3, 22.6 and 45.2 mg/L fluoride ion, respectively) are equal to environmental levels. However, it should be noted that rodents are more effective in clearing fluoride from their bodies as compared with humans (Angmar-Mansson and Whitford, 1984). Especially, rats exposed to 50 mg/L NaF in drinking water have serum fluoride levels similar to humans ingesting 2–5 mg/L fluoride ion (Lyaruu et al., 2008), which is much close to and slightly above the drinking water safe limit set by WHO. Hence, the doses used in the current study are supposed to be environmentally relevant. In such a situation, the animals were developmentally exposed to fluoride daily from pre-pregnancy to post-puberty, which covers the critical maternal, perinatal, and pubertal periods. That is to say, our rat model actually mimicked the real human exposure in areas of endemic fluorosis. More importantly, the results of the present study demonstrated that developmental exposure of rats to fluoride resulted in evident testicular damage, as manifested by histopathological changes and ultrastructural abnormalities of testicular cells. These findings are consistent with previous observations (Long et al., 2009; Barbier et al., 2010), suggesting that our rat model for testicular toxicity following developmental fluoride exposure was successfully constructed.

In this study, we provided convincing evidence that developmental exposure of rats to fluoride strongly enhanced germ cell apoptosis in testes, as demonstrated by a series of hallmarks such as caspase-3 activation, chromatin condensation and DNA fragmentation. We also found that the apoptotic cells were chiefly spermatogonia and spermatocytes, together with few early spermatids, seemed to be more sensitive to fluoride. This was accompanied by a marked suppression of spermatogenesis and an overt decrease in germ cells, further confirming the crucial role of apoptosis in germ cell loss. Our result is consistent with a previous report (Krasowska et al., 2004), in which fluoride has been shown to induce testicular apoptosis in the bank vole.

The present study also extended our recent findings (Zhang et al., 2013) by showing that apart from ER stress signaling, both Fas/FasL (extrinsic) and mitochondrial (intrinsic) apoptotic pathways were also implicated in fluoride-elicited testicular cell death. The Fas/FasL system has been proposed as a key regulator for damage-induced germ cell apoptosis (Lee et al., 1997). In the current study, fluoride exposure resulted in apoptosis of germ cells, associated with upregulation of Fas expression in the rat testes. Moreover, the expression of Fas was localized in the same cell types as apoptosis occurred, further corroborating the central role of Fas-mediated pathway in germ cell apoptosis. Our results are in line with the studies of other ubiquitous environmental toxicants such as bisphenol A and fenvalerate in mice testes (Li et al., 2009; Zhao et al., 2011). Interestingly, we also found that the increased apoptosis was accompanied by the elevation of cytochrome c, a key mitochondrial proapoptotic protein that is released following MOMP and activates caspase cascade subsequently leading to apoptosis (Tait and Green, 2010). Correspondingly, the expression and distribution of cytochrome c, to a large extent, was in coincidence with those of the apoptotic germ cells, suggestive of the crucial role of mitochondrial-dependent pathway in germ cell death. In support of this, abnormal mitochondrial architecture was clearly observed in those affected cells. Similar observations were recently made by Sun et al. (2011) on mice sperm following in vivo fluoride exposure, although, in contrast to the present work, the effects were found to occur at a much higher concentration (150 mg/L NaF), perhaps due to differences in species of animals used, time windows of exposure and dosing regimen. Collectively, our findings are in agreement with the report of Saradha et al. (2009), who also demonstrated that lindane induces testicular apoptosis in adult Wistar rats through the Fas- and mitochondria-dependent pathways.

Another major novel finding of the current study is that developmental exposure of rats to fluoride impaired autophagic degradation in testes. In the present study, we observed an increased number of autophagosomes in the NaF-treated testes compared...
with the controls. However, the increase in autophagosomes may result from either an enhancement of autophagosome formation or inhibition of autophagosome degradation (Mizushima et al., 2010). We then determined the effect of fluoride on autophagic activity. We found that the protein levels of Beclin-1, a critical regulator of autophagy, are increased in fluoride-treated rat testis, which is supposed to be compromised autophagic and is efficiently degraded in lysosomes after autophagosomes fuse with lysosomes, therefore, its accumulation always suggests a block in autophagic degradation (Bjorkoy et al., 2005; Settembre et al., 2008). Taken together, these data indicate that fluoride induces autophagy impairment via inhibiting autophagy degradation resulting in autophagosome accumulation in testes. Our results are akin to the previous reports of formaldehyde treatment and heat exposure in testes (Zhang et al., 2012; Han et al., 2013). However, both studies simply showed increased autophagosomes with elevated expression of LC3-II, they did not evaluate whether this effect is due to increased formation or decreased degradation, making the results explanation confused and complicated.

Despite the functional roles of autophagy in developmental and pathological aspects of spermatogenesis remain largely unknown, emerging evidence has revealed that impaired or deficient autophagy adversely affects the later stages of spermatogenesis and causes defective acrosome biogenesis subsequently leading to infertility in mice (Wang et al., 2014). Most recently, defective autophagy has also been shown to impair selectively spermatogenesis and reduce mitochondrial clearance during the process of germ cell specification (Herpin et al., 2015). Coincidentally, our present work demonstrated that testicular autophagy impairment was accompanied with spermatogenic failure, germ cell apoptosis, and retention of abnormal mitochondrial. Additionally, we also found many mitochondria-containing autophagosomes appeared in testes, which is supposed to be compromised autophagic clearance of damaged and unhealthy mitochondria. This mitochon-drial autophagic degradation failure would lead to accumulation of dysfunctional mitochondria generating more reactive oxygen species and aggravating the injury. These, together with previous reports showing that fluoride is an oxidative stress inducer (Barbier et al., 2010; Zhang et al., 2013) and causes infertility in human beings and experimental animals (Long et al., 2009), suggest a potential role of defective autophagy in testicular toxicity following developmental fluoride exposure. Our results are consistent with the findings in smokers’ alveolar macrophages (Monick et al., 2010).

5. Conclusions

In summary, we provide in vivo evidence showing that both excessive apoptosis and defective autophagy are implicated in testicular damage and toxicity of rats after developmental exposure to the environmental toxicant fluoride. In particular, we have uncovered that the abnormal apoptosis is mediated by both Fas and mitochondrial pathways, while the autophagy defect is caused by impaired degradation. Our findings provide deeper insights into the roles of apoptosis and autophagy in the aggravation of testicular damage, which could contribute to a better understanding of fluoride-induced male reproductive toxicity, and possibly novel potential target for preventive and/or therapeutic treatments.

Conflicts of interest

None.

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