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Research Article

Journal of Applied Toxicology

Received 25 April 2010, Revised 24 August 2010, Accepted 25 August 2010, Published online in Wiley Online Library

(wileyonlinelibrary.com) DOI 10.1002/jat.1600

NADPH oxidase participates in the oxidative damage caused by fluoride in rat spermatozoa. Protective role of α-tocopherol

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ABSTRACT: Fluorosis, caused by drinking water contaminated with inorganic fluoride, is a public health problem in many areas around the world. The aim of this study was to evaluate oxidative stress in spermatozoa caused by fluoride and NADPH oxidase in relationship to fluoride. Four experimental groups of male Wistar rats were administered with deionized water, NaF, at a dose equivalent to 5 mg fluoride kg^{-1} per 24 h, NaF plus 20 mg kg^{-1} per 24 h α-tocopherol, or α-tocopherol alone for 60 days. We evaluated several spermatozoa parameters in the four groups: standard quality analysis, superoxide dismutase (SOD) activity, the generation of reactive oxygen species (ROS), NADPH oxidase activity, TBARS formation, ultrastructural analyses of spermatozoa using transmission electron microscopy and *in vitro* fertilization (IVF) capacity. After 60 days of treatment, urinary excretion of fluoride was not modified by α-tocopherol. Spermatozoa from fluoride-treated rats exhibited a significant increase in the generation of ROS, accompanied by a significant increase in NADPH oxidase activity. The increase in ROS generation was significantly diminished by diphenylene iodonium, an inhibitor of NADPH oxidase activity. In contrast, a decrease in the generation of ROS, an increase in SOD activity and the prevention of TBARS formation process were observed in spermatozoa of rats exposed to fluoride plus α-tocopherol. Finally, α-tocopherol treatment prevented the IVF incapacity observed in the spermatozoa from fluoride-treated rats. These results suggest that NADPH oxidase participates in the oxidative stress damage caused by subchronic exposure to fluoride. Copyright © 2010 John Wiley & Sons, Ltd.

Keywords: α-tocopherol; fluoride; *in vitro* fertilization; NADPH oxidase; oxidative stress

INTRODUCTION

Fluoride is an environmental pollutant. Humans are exposed to fluoride via dental products, food and pesticides. Drinking water contaminated with fluoride from subsoil constitutes the greatest source of fluoride exposure for most people (National Research Council, 2006). The natural concentration of fluoride in groundwater depends on the geology, chemical conditions and physical characteristics of the water-bearing, the soil porosity and acidity, the bedrock, temperature and the depth of extraction wells (Pauwels and Ahmed, 2007). High fluoride concentrations in groundwater have been reported in India, China, Spain and Mexico, where levels are higher than 1.5 mg l^{-1} (Armienta and Segovia, 2008; Del Razo *et al.*, 1993; Gupta *et al.*, 1993; Hardisson *et al.*, 2001; Wang *et al.*, 2007).

The most significant risks of increased fluoride exposure effects are on bone cells that can lead to the development of skeletal fluorosis. However, fluoride also affects cells from soft tissues, i.e. renal, endothelial, neurological and gonadal. Fluoride induces reproductive defects, affecting the fertility capacity. Freni (1994) showed an inverse correlation between human fertility and fluoride levels in drinking water. Epidemiological data have also indicated that fluoride may adversely affect the reproductive systems of men living in fluorosis endemic areas (Ortiz-Perez *et al.*, 2003).

A variety of mechanisms have been proposed to explain fluoride-induced toxicity, including oxidative stress. Oxidative stress has been observed in soft tissues such as the liver, kidney, brain and testes in animals exposed to fluoride (Ghosh *et al.*, 2002; Guo *et al.*, 2003; Shanthakumari *et al.*, 2004) and people living in areas of endemic fluorosis (Shivashankara *et al.*, 2000). Exposure to fluoride decreases glutathione levels and can inhibit the activity of antioxidant enzymes such as superoxide dismutase (SOD), glutathione peroxidase, and catalase (Chlubek and Poland 2003). Many studies have shown that fluoride induces the production of reactive oxygen species (ROS) (Chouhan and Flora 2008; Izquierdo-Vega *et al.*, 2008; Wang *et al.*, 1997).

The main sources of ROS in spermatozoa are the mitochondria and other spermatozoa-specific enzymes. It has been proposed that NADPH oxidases (NOX) could have an important contribution to ROS generation (Aitken *et al.*, 1992, 1997). In humans, the NOX family consists of seven members, NOX1, NOX2, NOX3, NOX4, NOX5 and dual oxidases (DUOX1 and DUOX2). The catalytic core of NOX is a membrane-integrated glycoprotein with an apparent molecular mass of about 91 kDa ($gp91^{phox}$). It contains

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two hemes in the N-terminal transmembrane region and NADPH-binding and FAD-binding domains in the C-terminal cytoplasmic region, forming a complete apparatus that transports electrons from NADPH via FAD and two hemes to molecular oxygen. Despite their similar structure and enzymatic function, NOX family enzymes differ in their mechanism of activation (Bedard and Krause 2007).

ROS are implicated as important pathologic mediators in many disorders. Various studies have investigated whether oxidative stress is involved in the adverse reproductive effects caused by fluorosis (Ghosh *et al*., 2002; Izquierdo-Vega *et al*., 2008). α-Tocopherol protects testes and male accessory sex organs from oxidative stress caused by fluoride exposure (Sarkar *et al*., 2006), and it is a well-known antioxidant that protects cell membranes against peroxidative damage (Bourges-Rodríguez, 2008). It also has functions that cannot be only attributed to its antioxidant properties, such as the negative modulation of PKC-related signaling and the impairment of NADPH oxidase assembly (Cachia *et al*., 1998; Varga *et al*., 2008). Here, we investigated whether NADPH oxidase participates in the oxidative stress caused by fluoride and the protective role of α-tocopherol.

MATERIALS AND METHODS

Chemicals

Acrylamide, bovine serum albumin fraction V (BSA), butylated hydroxytoluene (BHT), deferroxamine (DFA), dimethyl sulfoxide (DMSO), diphenyleneiodonium chloride (DPI), sodium fluoride (NaF), formaldehyde, glycerol, hyaluronidase, hoechst 33342, human chorionic gonadotropin (hCG), β-reduced nicotinamide adenine dinucleotide phosphate (NADPH), Nonidet P-40, phenylmethanesulfonyl fluoride (PMSF), α-tocopherol, sodium dodecyl sulfate (SDS), thiobarbituric acid (TBA), triton X-100, glutaraldehyde, tween-20, osmium tetroxide, trypan blue and sodium orthovanadate were purchased from Sigma-Aldrich (St Louis, MO, USA). Spurr's resine was from Electron Microscope Sciences (Fort Washington, PA, USA). Pregnant mare's serum gonadotropin (PMSG; Folligon) was purchased from Intervet International B.V. (Boxmeer, The Netherlands). Complete mini protease inhibitor cocktail tablets were purchased from Roche Diagnostics (Mannheim, Germany), and the RANSOD Assay kit from Randox Laboratories Ltd (Crumlin, UK). Dihydroethidium (DHE), SYTOX green was purchased from Molecular Probes, Invitrogen (Mount Waverley, Australia), and a protein assay kit was purchased from Bio-Rad (Hercules, CA, USA). All other chemicals used were of the highest purity commercially available.

Animals and Experimental Design

Male Wistar rats (75–99 g) and immature (5 weeks old) female Wistar rats were obtained from Harlan (Mexico). Animals were maintained according to the Institutional (CINVESTAV-IPN), Animal Care and Use Committee, in compliance with Guidelines for Use and Care of Laboratory Animals. Animals were maintained in groups of six per cage, on a 12–12 h light/dark cycle at constant temperature (22 ± 2 °C) and humidity (50%), with food (LabDiet® 5013, PMI Nutrition International, St Louis, MO, USA) and water freely available in their home cages.

The animals were distributed randomly into four experimental groups. Six male rats in each group were administered deionized water for the control group, NaF at a dose equivalent to 5 mg fluoride kg^{-1} per 24 h, NaF plus 20 mg kg^{-1} per 24 h α-tocopherol, or α-tocopherol. In all treated groups, the doses were given by oral gavage once a day for 60 days. In order to avoid a possible interference in the kinetic processes of both xenobiotics given by gavage (fluoride and α-tocopherol), the α-tocopherol treatment was given to rats 5 h after fluoride administration. The duration of the treatment was 60 days, since one spermatogenic cycle in the rat is 50 ± 2 days, and thus we ensured that the fluoride exposure occurred during at least one complete period of spermatogenesis in the rat.

Urinary Fluoride Concentration

Every 15 days urine was collected from each individual in each treatment group in order to quantify the fluoride concentration by a potentiometric method using an ion selective electrode (Orion 9609; Del Razo *et al*., 1993).

Spermatozoa Isolation and Capacitation

After 60 days of treatment, rats were euthanized by cervical dislocation, the testes-epididymis–vas deferens complexes were dissected, and spermatozoa were isolated by flushing the vas deferens and cauda epididymis lumens with 1 ml of phosphate buffered saline (PBS, pH 7.4). Spermatozoa counts were determined using a Neubauer chamber. To induce capacitation, 10×10^6 spermatozoa ml^{-1} in enriched Krebs–Ringer bicarbonate (EKRB) supplemented with 3 mg ml^{-1} BSA were incubated for 4 h at 37 °C in a high-humidity incubator under 5% CO_2 (Bendahmane *et al*., 2002).

Spermatozoa Quality

Sperm parameters, including concentration, viability and progressive motility, were evaluated according to WHO (2001) guidelines. Spermatozoa motility (the percentage of cells that were motile), was assessed by microscopic examination of 10 random fields. Spermatozoa viability was determined by trypan blue exclusion assay. Spermatozoa concentrations were determined using a hemocytometer. Two aliquots (100–200 cells each), were separately counted for each animal. Although epididymal spermatozoa are not at this point subjected to peroxidative damage caused by leukocytes, the absence of these cells was evaluated by optical microscopy in all samples.

SOD Activity in Spermatozoa

SOD was extracted from 10×10^6 spermatozoa, treated 1 : 1 with 0.1% Triton X-100–PBS, and incubated at 4 °C for 15 min. Samples were then centrifuged at 600 *g* for 8 min at 4 °C, and supernatants were removed for measurement of SOD using the RANSOD Assay kit. This method uses xanthine and xanthine oxidase to generate superoxide radicals, which react with 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride to form a red formazan dye. SOD activity was measured by the degree of inhibition of chromogen formation at 505 nm using a spectrophotometer (VitaLab ECLIPSE Merck, Darmstadt, Germany). SOD activity was calculated using a standard graph, according to the manufacturer's instructions. The unit of activity of the assay was defined as

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the amount of SOD that inhibited the rate of formazan dye formation by 50%. The results are presented as units per milligram of protein.

Measurement of ROS Generation

Levels of ROS were measured by flow cytometry using DHE and SYTOX Green, which is a vitality stain, as previously described (De Iuliis *et al*., 2006). DHE is a poorly fluorescent product of the two-electron reduction of ethidium that, upon oxidation, produces DNA-sensitive fluorochromes that generate a red nuclear fluorescence when excited at a wavelength of 510 nm. For the assay, 2×10^6 spermatozoa in EKRB medium with BSA were incubated with 3 μM DHE and 0.25 μM SYTOX Green in the dark at 37 °C for 1 h.

To evaluate the participation of NADPH oxidase in the generation of ROS in spermatozoa, 2×10^6 spermatozoa in EKRB-BSA medium were incubated with 1 μM DPI (dissolved in 10% DMSO), 10 min before staining. Fluorescence was then measured for 10 000 cells using a flow cytometer (FACSCalibur system, Becton Dickinson; Franklin Lakes, NJ, USA).

NADPH Oxidase Activity Assay

NADPH oxidase activity in spermatozoa was measured by inducing ROS generation with the addition of NADPH as previously described (Aitken *et al*., 1997). For the assay, 2×10^6 spermatozoa in EKRB medium with BSA were incubated with 150 μM of NADPH and stained with 3 μM DHE and 0.25 μM SYTOX Green in the dark at 37 °C for 1 h. Fluorescence was then measured for 10 000 cells using a flow cytometer (FACSCalibur system, Becton Dickinson; Franklin Lakes, NJ, USA).

Protein Extraction and Separation

Proteins were extracted from spermatozoa of control and fluoride-treated rats. Spermatozoa samples were pooled by treatment group. The samples were washed twice with PBS and lysed with 1% Nonidet P-40 in PBS, pH 7.4, containing 1 mM PMSF, 1 mM orthovanadate and protease inhibitors cocktail. After 30 min of incubation at 4 °C, spermatozoa were centrifuged at 10 000 × g for 10 min at 4 °C, and the supernatant was collected. Protein concentration was determined with the Bio-Rad (Hercules, CA, USA) protein assay reagent, using bovine serum albumin as a standard. Equal quantities of each sample were separated by electrophoresis on 12.5% SDS-polyacrylamide gels with pre-stained protein.

Western Blotting

After electrophoresis, proteins were transferred to nitrocellulose membranes (Bio-Rad Laboratories, Richmond, CA, USA), which were then blocked with 5% nonfat dry milk in 0.05% Tween-20 and reacted with antibodies against $p47^{phox}$ (1 : 125), or actin (1 : 12000; Santa Cruz Biotechnologies, Santa Cruz, CA, USA). Inmunodetection was followed by incubation with horseradish peroxidase coupled to secondary antibodies against $p47^{phox}$ (1 : 125), and IgG-HRP (1 : 12 000; Santa Cruz Biotechnologies, Santa Cruz, CA, USA). Antigen–antibody complexes were visualized using chemiluminescent ECL reagents (Amersham Pharmacia Biotech UK Limited, UK). All experiments were repeated three times. The intensity of the bands was subjected to quantitative analysis, digital images were analyzed with *Image J* software (NIEH, RTP, NC), and the results were averaged.

TBARS Concentration in Spermatozoa

Thiobarbituric acid reactive substances (TBARS) were determined according to Buege and Aust (1978). Briefly, 1 ml of 0.5% TBA, 5 μl of 3.75% BHT in methanol, and 5 μl of 1.5 mM DFA were added to 1 ml of spermatozoa suspension (2×10^6 cells). Samples were then heated in a boiling water bath for 20 min, cooled, and the absorbance was measured at 532 nm using a spectrophotometer (UV–vis Lambda-2S Perkin-Elmer). Measurements are expressed as nmol TBARS/2×10^6 spermatozoa.

Transmission Electron Microscopy

Spermatozoa samples from control and fluoride-treated rats were fixed with 3% (v/v) glutaraldehyde in PBS buffer for 1 h at room temperature. Samples were then postfixed in 1% (v/v) osmium tetroxide in PBS for 1 h. The cells were rinsed in PBS, dehydrated through a grade ethanol series, and embedded in Spurr's resin. Resin blocks were ultra-thin sectioned and double-stained with uranyl acetate and lead nitrate. The samples were examined using a JEM-1200 EXII transmission electron microscope at 60 keV (Jeol Ltd; Tokyo, Japan). Ten ultra-thin sections from each sample were separately analyzed.

In Vitro Fertilization Assay

Egg recovery

Five-week-old female Wistar rats were superovulated by intraperitoneal injection of 20 IU PMSG, followed by 20 IU hCG 48 h later. Animals were euthanized 14–16 h after hCG injection by cervical dislocation. Uterine ovary–salpinge–horn complexes were dissected, ampullae punctured and cumulus–egg complexes were extruded and placed in 0.1% (w/v) hyaluronidase/EKRB medium to remove cumulus cells. Cumulus-free eggs were pooled and washed with EKRB medium and then incubated at 37 °C under 5% CO_2 until use. Approximately 40 eggs were obtained from each female. Only eggs with polar bodies and with intact zonae pelucidae were used for fertilization assays.

Insemination of zone-intact eggs

To assess spermatozoa fertility, 40 eggs were suspended in 200 μl of EKRB medium in a glass slide with two polished spherical depressions of approximately 0.5–0.8 mm depth (VWR International), inseminated with 10 μl of capacitated spermatozoa (1×10^5 cells), from control or treated rats (fluoride, α-tocopherol and fluoride plus α-tocopherol), and incubated for 4 h at 37 °C in a high-humidity incubator under 5% CO_2. After gamete co-incubation, eggs were fixed in 3% formaldehyde in PBS and stained with 20 μM Hoechst 33342 for 20 min. Samples were then washed three times in PBS, mounted on a glass slide with 50% glycerol-PBS and examined by fluorescence microscopy to assess fertilization. Eggs were considered fertilized when decondensed spermatozoa heads were detected within the egg cytoplasm.

Statistical Analysis

Results comparing two samples are expressed as means ± standard deviation (SD) of at least three individual experiments. Statistical analysis was carried using ANOVA followed by Tukey's test, and a P-value < 0.05 was considered significant. All analyses were performed using the statistical software Stata 8.0 (Stata Corp., College Station, TX, USA).

RESULTS

Water intake and food intake in the fluoride-exposed group were similar to the control group during the exposure time. Consequently, no significant differences were observed in the body weight of exposed and control rats (data not shown).

α-Tocopherol Does Not Modify Urinary Fluoride Concentration

Urinary fluoride concentrations were measured in all groups during treatment. The level of fluoride increased significantly in the fluoride group compared with the control group. There were no statistical differences in the levels of fluoride between the fluoride plus α-tocopherol group and the fluoride group (Fig. 1).

α-Tocopherol Protects Spermatozoa Motility Affected by Fluoride Exposure

Next, we analyzed overall quality of spermatozoa according to several parameters, summarized in Table 1. The spermatozoa motility was affected only in the fluoride-exposed rats. α-Tocopherol prevented the reduction in spermatozoa motility caused by the exposure to fluoride.

α-Tocopherol Protects Against Oxidative Stress and Oxidative Damage in Spermatozoa Caused by Subchronic Fluoride Exposure

To evaluate oxidative stress, the functional activity of SOD and generation of ROS were assessed. As shown in Fig. 2(a), fluoride exposure led to a significant decrease in total SOD activity,

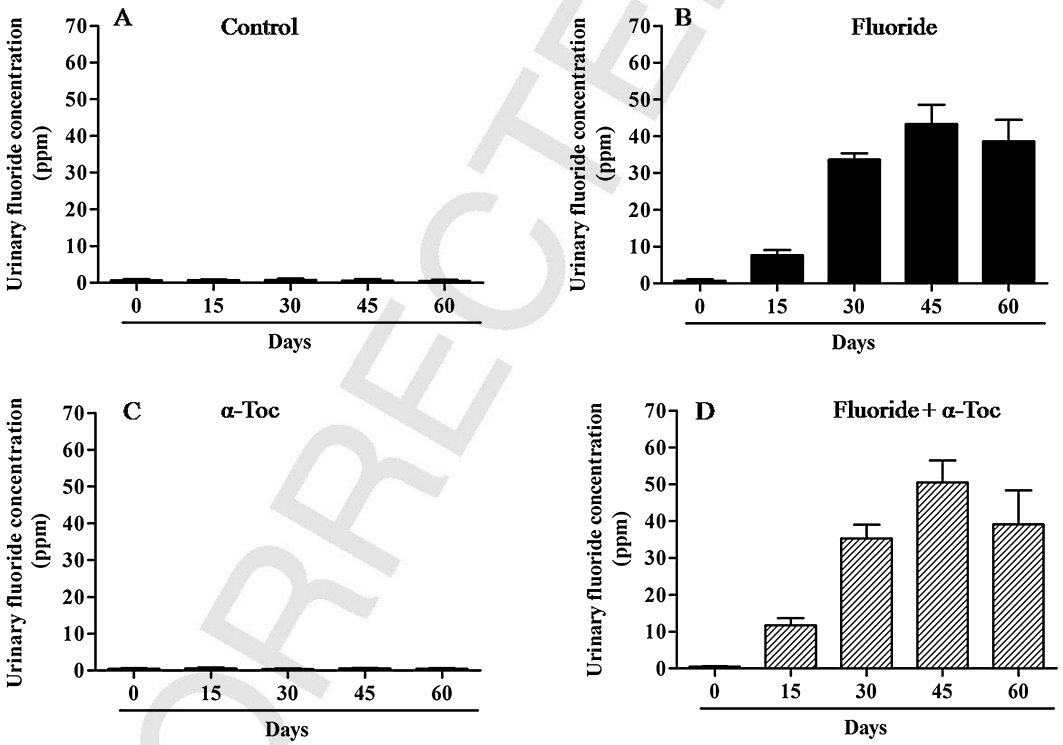

Figure 1.

Table 1. Assessment of spermatozoa parameters

Sperm parameters	Control	Fluoride	α-Tocopherol	Fluoride + α-tocopherol
Motility (%)	92 ± 5.6	81 ± 6.8*	90 ± 2.1	88 ± 5.7
Sperm concentration (10^6 ml^{-1})	41.1 ± 14.2	30.6 ± 13.5	40.9 ± 5.4	39.4 ± 3.2
Viability (%)	97 ± 0.95	95 ± 1.5	90 ± 5.4	90 ± 4.8

Values are means ± SD. *P < 0.05 vs control group.

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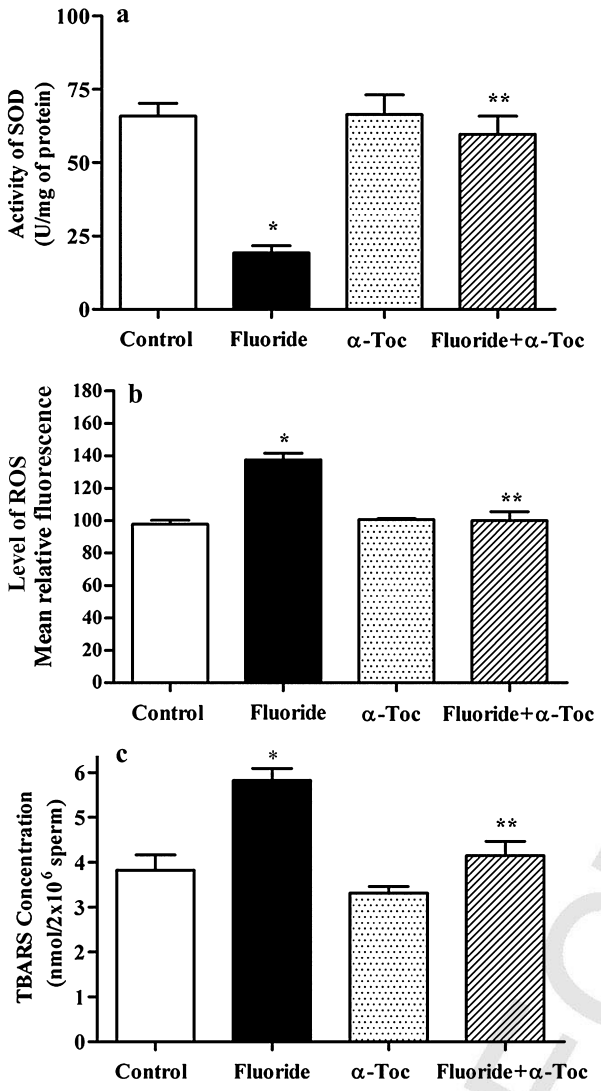

Figure 2.

which was 3.28-fold lower than in the control group ($P < 0.001$). The co-administration of fluoride plus α-tocopherol prevented the diminution of SOD activity caused by fluoride exposure ($P < 0.001$). In spermatozoa from rats exposed to fluoride, DHE fluorescence was 1.4-fold greater than in the control group ($P < 0.001$). In spermatozoa co-treated with fluoride plus α-tocopherol, the DHE fluorescence was found at the same level as in the control and α-tocopherol groups, indicating that α-tocopherol prevents the increase of ROS levels in spermatozoa from fluoride-treated rats (Fig. 2b). We also examined TBARS formation, a marker of oxidative damage (Fig. 2c). TBARS levels were increased 1.5-fold in spermatozoa from fluoride-treated rats compared with the control group ($P < 0.001$). After co-treatment with fluoride plus α-tocopherol, the TBARS formation was 1.4-fold lower with respect to the fluoride-exposed group. There was no significant difference between the control group and fluoride plus α-tocopherol group. These results shown that α-tocopherol protects from the oxidative damage caused by fluoride exposure.

NADPH Oxidase Participates in the Oxidative Stress Caused by Fluoride in Spermatozoa

To evaluate the importance of spermatozoa NADPH oxidase in creating the oxidative stress caused by fluoride exposure, the generation of ROS in spermatozoa with and without the presence of DPI and the functional activity of NADPH oxidase were evaluated. Spermatozoa from fluoride-treated rats exhibited a significant increase in the generation of ROS, which were significantly diminished 1.8-fold by DPI ($P < 0.001$). The generation of ROS in spermatozoa of the control group was diminished only 1.2-fold by DPI ($P < 0.001$) (Fig. 3a). In contrast, the generation of ROS was prevented in spermatozoa from α-tocopherol-treated rats and was not inhibited by DPI (Fig. 3b). In order to corroborate the participation of NADPH oxidase in spermatozoa, its activity was evaluated. The activity of NADPH oxidase was increased 1.2-fold in spermatozoa from fluoride-treated rats compared with the control group ($P < 0.001$), while the activity of NADPH oxidase in both groups treated with α-tocopherol was not different from the activity in the control group (Fig. 4). These results indicate that NADPH oxidase participates in the generation of ROS observed in the fluoride exposure and that NADPH oxidase activity is negatively modulated by α-tocopherol.

In addition, we determined $p47^{phox}$, a subunit NADPH oxidase by western blotting. The amount of $p47^{phox}$ protein in spermatozoa was not modified by fluoride exposure as compared with the control group (Fig. 5). These results suggest that ROS generation by NADPH oxidase in the fluoride-exposed rats is due to a positive modulation of this enzyme activity.

α-Tocopherol Protects Against the Oxidative Damage Caused by Subchronic Exposure to Fluoride in Spermatozoa as Shown by Transmission Electron Microscopy

Ultrastructural evaluations of spermatozoa from all groups were performed via transmission electron microscopy (TEM), and representative images are shown in Fig. 6. The control and α-tocopherol-exposed spermatozoa exhibited an intact plasma membrane around the cell (Fig. 6a and d). This normal appearance was visibly altered in the plasma membrane along the sperm head spermatozoa from fluoride-treated rats (Fig. 6b). The co-exposure to fluoride plus α-tocopherol showed a structural protection against fluoride-induced plasma membrane damage (Fig. 6c).

α-Tocopherol Prevented the *in Vitro* Fertilization Incapacity Observed in the Spermatozoa from Fluoride-treated Rats

Next, we examined the ability of spermatozoa co-treated with fluoride plus α-tocopherol to fertilize zona-intact eggs by *in vitro* fertilization (IVF). As shown in Figs 7 and 8, spermatozoa from rats exposed to fluoride exhibited a significantly lower ability to fertilize eggs compared with the control group (13 ± 5.10 vs 72 ± 4.69), while the co-treatment with fluoride plus α-tocopherol caused a significant increase in the ability expressed in percentage of spermatozoa to fertilize eggs compared with fluoride exposure.

DISCUSSION

In the present study, we evaluated whether NADPH oxidase participates in the oxidative stress caused by fluoride and examined

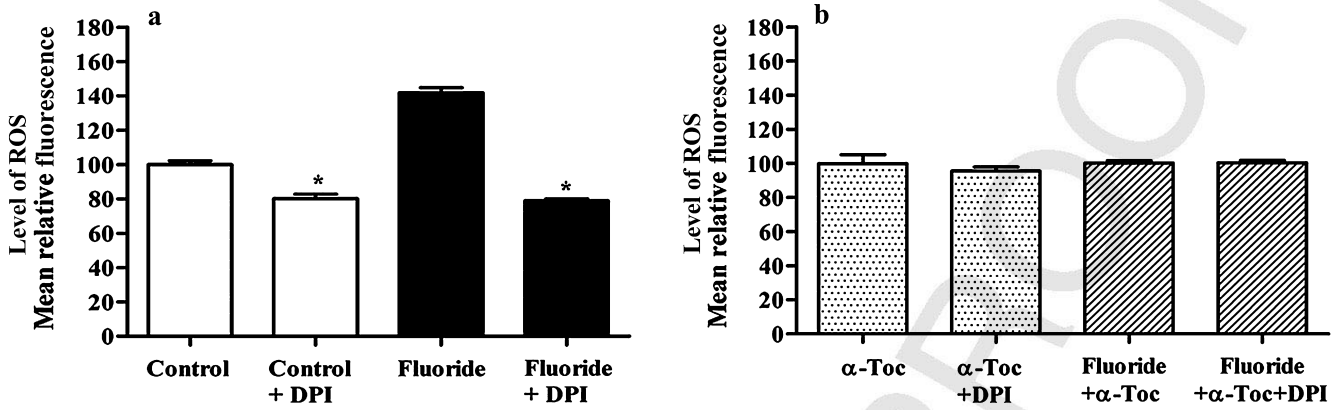

Figure 3.

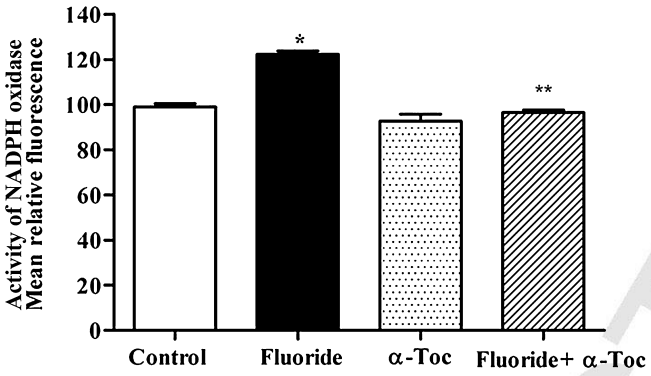

Figure 4.

the protective role of α-tocopherol. Oxidative stress is involved in the etiology of male infertility (Aitken and Baker, 2006). Male reproductive defects in humans (Ortiz-Pérez *et al*., 2003) and experimental models have been associated with oxidative stress as a result of fluoride exposure (Ghosh *et al*., 2002), which produces a significant reduction in the IVF capacity of spermatozoa (Izquierdo-Vega *et al*., 2008). Moreover, α-tocopherol protects testes and male accessory sex organs from oxidative stress caused by fluoride exposure (Sarkar *et al*., 2006).

We have previously reported that fluoride increased ROS production in the spermatozoa (Izquierdo-Vega *et al*., 2008). However, it is clear from the results presented in this study that NADPH oxidase participates in the oxidative stress caused by fluoride because the generation of ROS in spermatozoa was blocked by DPI (Fig. 3a). This was accompanied by a significant increase in the catalytic activity of NADPH oxidase (Fig. 4), without a modification in the amount of $p47^{phox}$ protein in spermatozoa from fluoride-exposed rats (Fig. 5b). Many studies have previously evaluated the activity of NADPH oxidase in human, equine and rat spermatozoa through generation of ROS induced by the addition of exogenous calcium ionophore and NADPH and inhibited by DPI (Aitken *et al*., 1992; 1997; Sabeur and Ball, 2007; Vernet *et al*., 2001). In addition to mitochondrial sources, an enzymatic system for ROS generation located in the spermatozoa plasma membrane was identified as NOX. All subunits of NOX ($gp91^{phox}$, $p67^{phox}$, $p40^{phox}$ and $p47^{phox}$) with the exception of $p22^{phox}$ were detected by western blot and confocal laser scan microscopy in rodent spermatozoa (Shukla *et al*., 2005); interestingly NOX5 does not require $p22^{phox}$ association in its activation (Kawahara *et al*., 2005). Previously, it was only known that mRNA of NOX5 is expressed in spermatocytes from human testes (Bánfi *et al*., 2001). Recently, proteins identified in human spermatozoa using LC-MS/MS analysis revealed the presence of DUOX2 in mature spermatozoa (Baker *et al*., 2007). Kawahara *et al*. (2007), showed the presence of genes DUOX1 and DUOX2 but not the presence of the gene NOX5 in rodents. NOX5 and DUOX2 are activated by calcium and do not appear to require subunits for its activation (Bedard and Krause, 2007). In addition, it has been reported that fluoride increases the concentration of intracellular calcium [Ca_i^{2+}], in spermatozoa (Chinoy *et al*., 1995). Recently, it has been shown that hydrogen peroxide positively modulates NOX5 activation by c-Abl through a calcium-mediated, redox-dependent signaling pathway (El Jamali *et al*., 2008). Consistent with this observation, the generation of ROS is an important mediator of fluoride-induced toxicity (Chouhan and Flora, 2008; Izquierdo-Vega *et al*., 2008; Sarkar *et al*., 2006).

In the present study, we evaluated the protective role of α-tocopherol against oxidative stress caused by fluoride exposure. SOD acts as an important line of defense against ROS by catalyzing the dismutation of $O_2^{\cdot-}$ into oxygen and hydrogen peroxide. We found that the SOD activity was significantly reduced as a result of fluoride exposure, while the co-administration of fluoride plus α-tocopherol prevented the diminution in SOD activity observed in fluoride-exposed rats (Fig. 2a). Additionally, the co-treatment with fluoride plus α-tocopherol prevented the increase in the generation of ROS observed in spermatozoa from fluoride-exposed rats (Fig. 2b). In support of these results, it has been previously demonstrated that α-tocopherol inhibits the production of ROS in the spermatozoa of rats exposed to polychlorinated biphenyls (Krishnamoorthy *et al*., 2007). It has also been observed in experimental models that α-tocopherol treatment significantly prevents the inhibition of SOD by fluoride exposure in rats (Guney *et al*., 2007; Sarkar *et al*., 2006). In addition, the generation of ROS in spermatozoa from α-tocopherol-exposed rats was not inhibited by DPI (Fig. 3b), indicating that the activity of NADPH oxidase could be modulated by α-tocopherol.

Oxidative damage is considered the main indicator of oxidative stress-induced loss of cellular function (Storey, 1996). Results of the present study showed an increase in TBARS concentration of spermatozoa from rats exposed to fluoride, while the co-administration of fluoride plus α-tocopherol protected against the oxidative damage caused by fluoride-exposure

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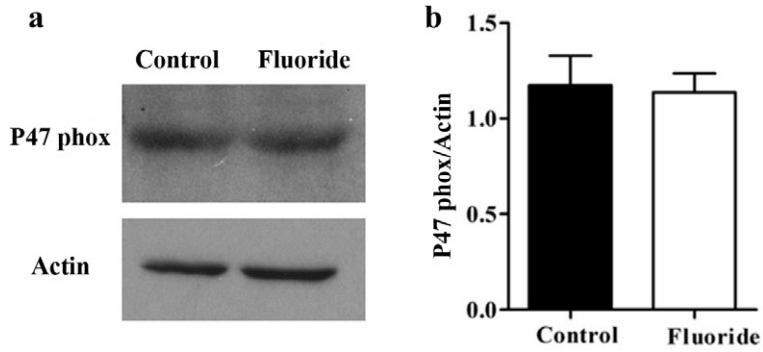

Figure 5.

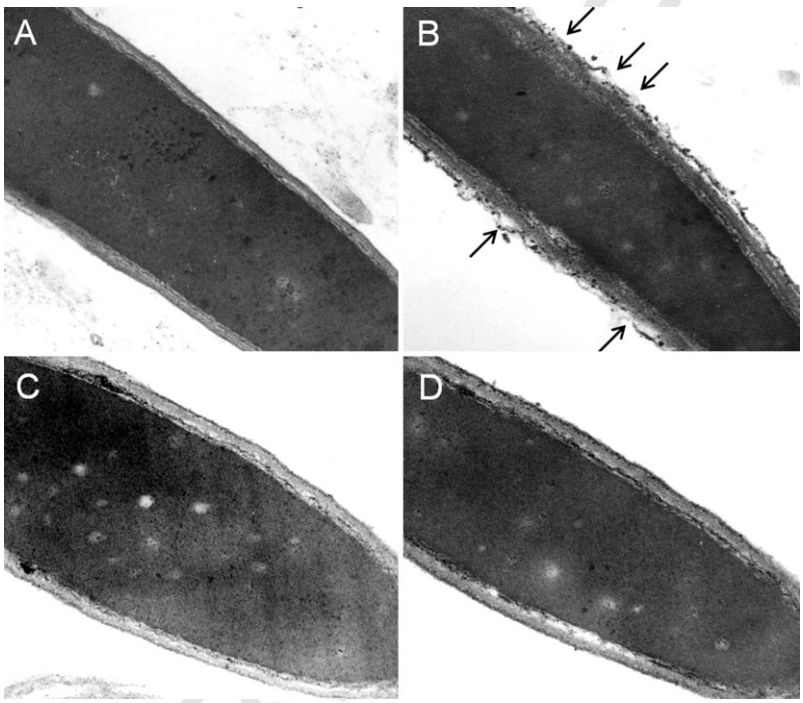

Figure 6.

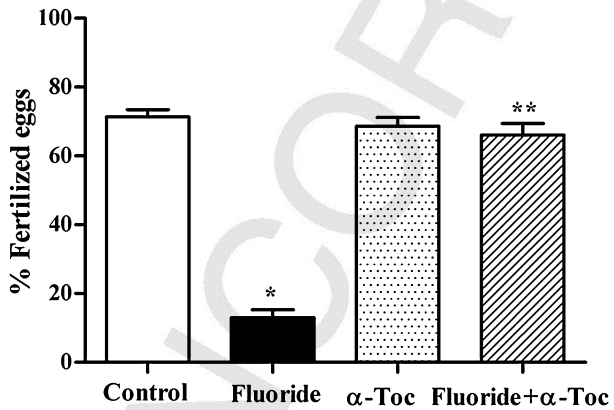

Figure 7.

(Fig. 2c). Here, oxidative damage to spermatozoa membranes and the protective role of α-tocopherol were evidenced by electron microscopy (Fig. 6). Consistent with this observation, a positive correlation has been observed between the concentrations of fluoride and TBARS in several tissues, including the testes (Krechniak and Inkielewicz, 2005). Also, chronic fluorosis causes oxidative damage in the testes of first- and second-generation rats (Oncü *et al*., 2007). Evidence reveals that the peroxidation process results in the formation of cholesterol domains in model membranes that increase in a time-dependent manner in parallel with lipid peroxide accumulation, which is inhibited by α-tocopherol (Jacob and Masson, 2005). Additionally, an inverse correlation between oxidative damage and human spermatozoa motility has been shown, which was prevented by α-tocopherol. In human spermatozoa, the TBARS formation occurs as a result of mitochondrial disruption in complex I, suggesting that this peroxidative damage is induced once intra-mitochondrial antioxidant defenses have been lowered (Koppers *et al*., 2008). We

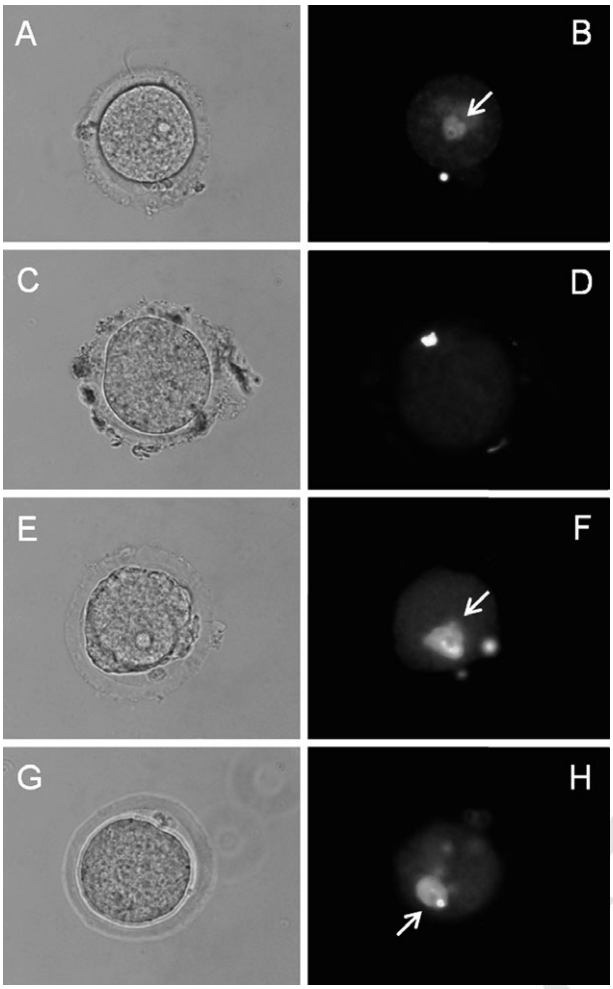

Figure 8.

observed that the diminution in SOD activity was accompanied by an increase in the generation of ROS in the spermatozoa of fluoride-exposed rats.

In this study, the α-tocopherol treatment did not modify the urinary fluoride concentration (Fig. 1). Similarly, Guney *et al.*, (2007) observed that the plasma fluoride concentration was not modified by α-tocopherol, suggesting that the pharmacokinetic of fluoride are not altered by this antioxidant. Unfortunately, we did not evaluate fluoride concentration in spermatozoon. However, Inkielewicz and Krechniak (2003) showed that the testes concentration of fluoride in rats that drank water with 25 ppm of fluoride for 3 months was 4.93-fold greater than in the control group. Interestingly levels of fluoride in rat testes were similar to rat urine fluoride levels. The accumulation of fluoride in testes suggests that fluoride is in contact with spermatozoon.

Spermatozoa quality is a major factor in successful IVF. Here, we observed that spermatozoa motility was significantly reduced as a result of fluoride exposure, while spermatozoa motility in the co-administration with fluoride plus α-tocopherol was not different compared with the control (Table 1), suggesting the participation of ROS in spermatozoa motility. It has been shown that ROS affect spermatozoa motility and ATP concentration through a mechanism independent of oxidative phosphorylation (Armstrong *et al.*, 1999), exerting a direct action on flagella (de Lamirande and Gagnon, 1992). High levels of ROS are associated with reduced motility and fertilization potential (Aitken *et al.*, 1998).

We previously found that fluoride exposure caused a decrease in spermatozoa IVF capability (Izquierdo-Vega *et al.*, 2008). Here, the importance of the regulation of oxidative stress in fertilization was evaluated with the co-exposure of fluoride plus α-tocopherol. α-Tocopherol prevented the oxidative stress and oxidative damage and also the diminution of IVF (Figs 7 and 8). Supporting these results, α-tocopherol has been shown to improve sperm motility and fertility in men (Suleiman *et al.*, 1996).

In conclusion, the results of this study indicate that NADPH oxidase participates in the oxidative damage caused by fluoride exposure in rat spermatozoa. α-Tocopherol protects against oxidative damage in spermatozoa caused by fluoride exposure. Further studies are required to elucidate the presence of NOXs isoforms present in spermatozoa that could participate as a mediator of ROS production spermatozoa by fluoride exposure.

Acknowledgment

The authors are thankful to Angel Barrera-Hernández from CINVESTAV Mexico for assistance in the animal care and Víctor H. Rosales-García for helping with flow cytometry. The authors also thank Rodolfo Paredes-Díaz from Electronic Microscopic Unit of Cellular Physiology of UNAM, Mexico for helping with electron microscopic analysis. J.A.I.V. was recipient of a CONACYT Mexico scholarship (170210).

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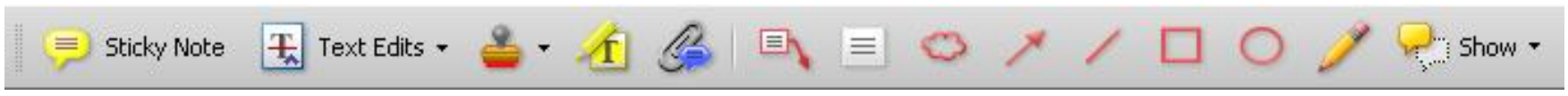

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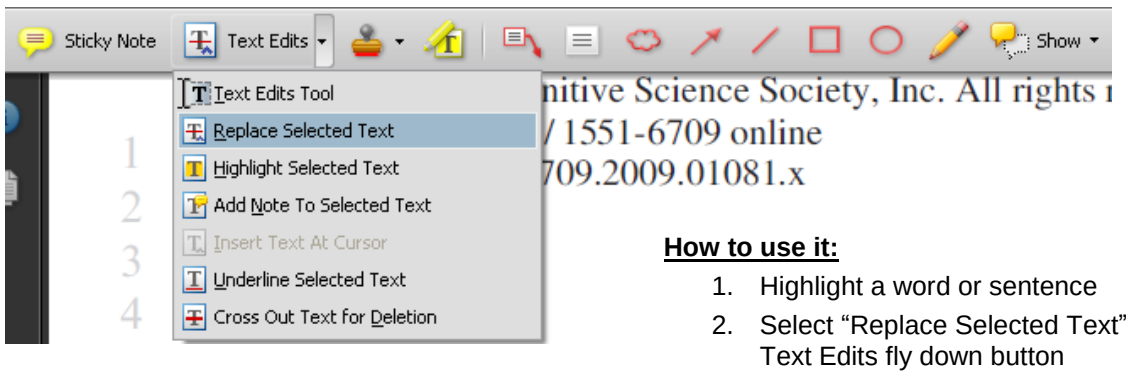

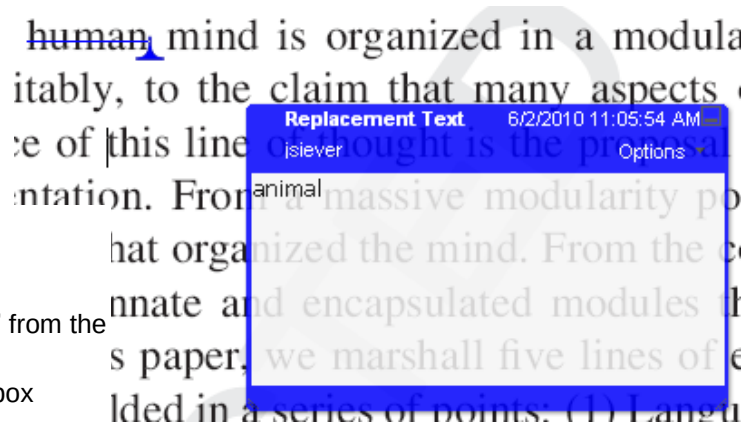

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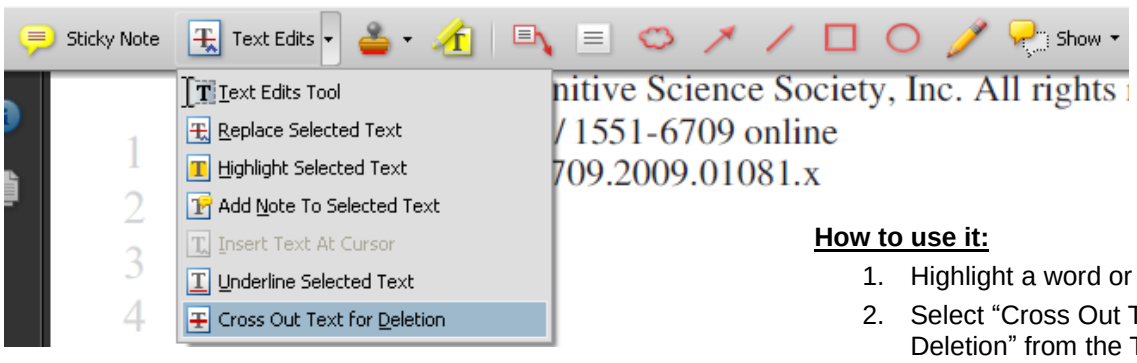

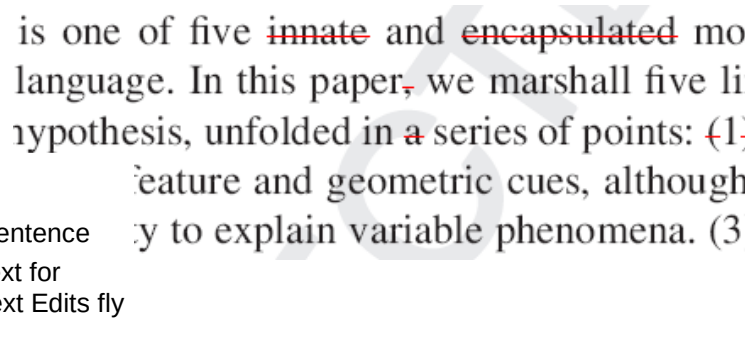

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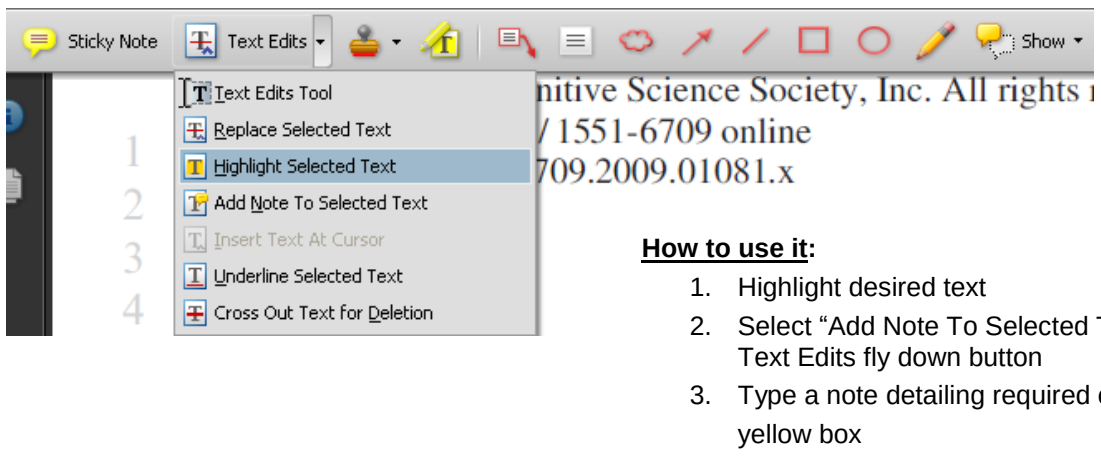

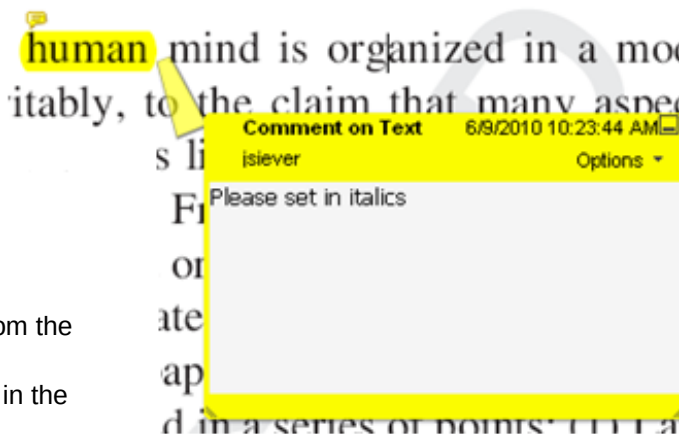

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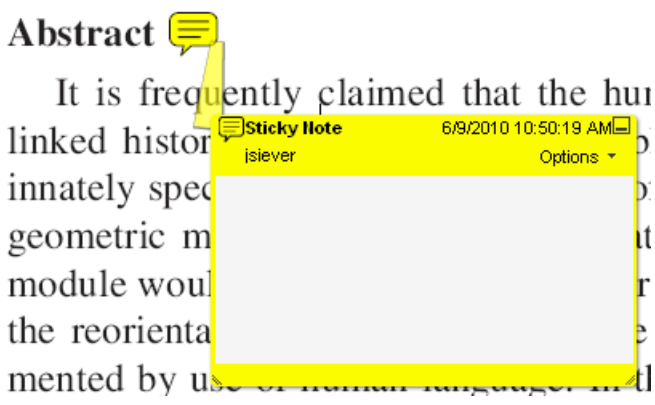

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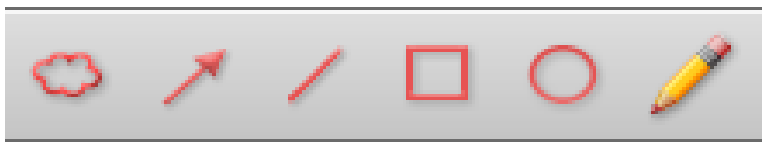

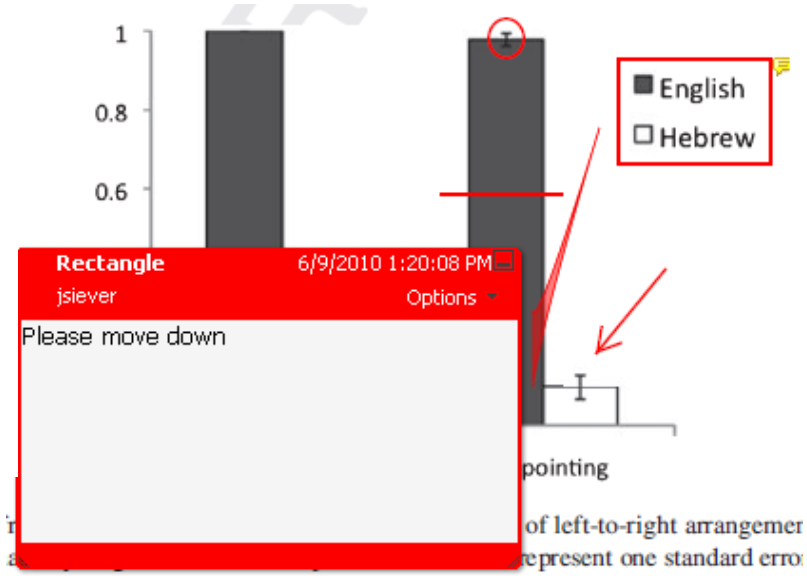

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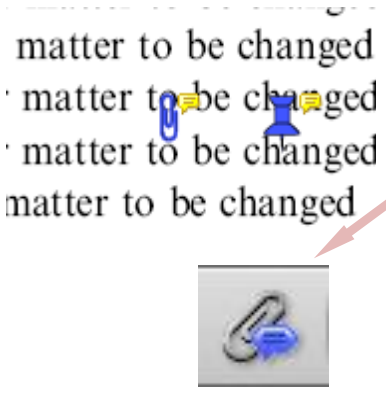

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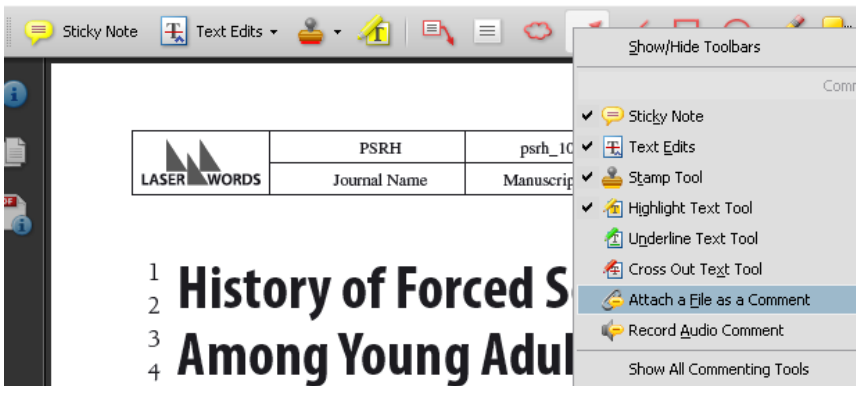
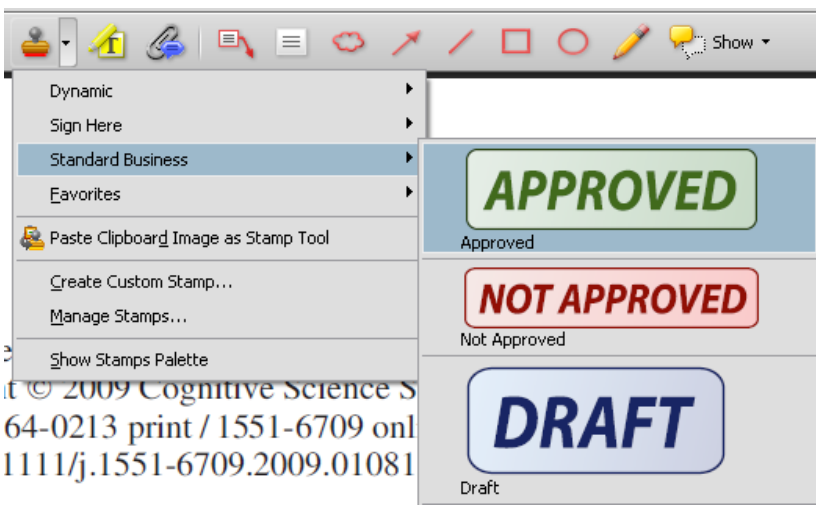
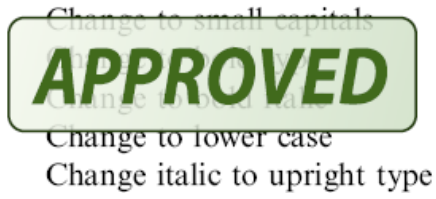


7. Approved Tool (Stamp) — For approving a proof if no corrections are required.



How to use it:

1. Click on the Stamp Tool in the toolbar
2. Select the Approved rubber stamp from the ‘standard business’ selection
3. Click on the text where you want to rubber stamp to appear (usually first page)



Help

For further information on how to annotate proofs click on the Help button to activate a list of instructions: