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Original Paper

Protective role of vitamin B_{12} in oxidative stress-mediated testicular dysfunction in fluoride intoxicated rats

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Abstract

Long-term fluoride intake is known to cause development of oxidative stress and fluoride-induced oxidative damage is regarded as a key contributing factor of testicular dysfunction. Vitamin B_{12} is an essential nutrient and has been reported to be a potent antioxidant and fertility improving agent. In the present study, the protective effect of vitamin B_{12} against sodium fluoride (NaF) induced oxidative damage in testes was investigated in rats. Twenty four adult Wister rats were divided into four groups, with six rats in each group. Group 1 was a control and received distilled water, NaF (100 mg L^{-1}) with drinking water was given to group 2, rats in group 3 were given vitamin B_{12} (0.63 μg kg^{-1} body weight) orally, and group 4 were administered with NaF with vitamin B_{12} for 21 consecutive days. Selected reproductive parameters, serum testosterone level, testicular histology and biomarkers of oxidative damage were determined. Degenerative changes in testicular tissue, significant reduction in sperm count, sperm motility, sperm viability, semen volume, low testosterone level, enhanced testicular malodialdehyde and NO level along with significant reduction in superoxide dismutase and catalase activity and reduced glutathion level were observed following NaF treatment, while vitamin B_{12} supplementation had ameliorative effect against these adverse changes. The results suggest that vitamin B_{12} plays a protective role against NaF-induced oxidative damage in testis and suggests the possibility of this vitamin as a potential nutritional strategy in the treatment of fluoride-induced testicular dysfunction.

Key words: antioxidant, oxidative stress, oxidative damage, reactive oxygen species, sodium fluoride, vitamin B_{12}, testis.
Abbreviations: CAT, catalase; GSH, reduced glutathione; MDA, malondialdehyde; NaF, sodium fluoride; NO, nitrogen oxide; SOD, superoxide dismutase; ROS, reactive oxygen species

Introduction

The impact of fluoride on the growing rate of infertility, both in females and males, is a matter of concern and has now become a serious health problem around the world. In India, one out of four couples is facing infertility (Shah 2017). Furthermore, the prevalence of infertility was reported in the regions of India with high fluoride concentration in drinking water (Neelam et al. 1987). Growing evidence shows that the long-term intake of fluoride leads to inhibition of spermatogenesis, changes sperm numbers and function, and lowers testosterone secretion (Ortaa, Erkan 2014). However, despite numerous investigations, the precise mechanism of fluoride toxicity in the male reproductive system is still not clear.

A number of studies on laboratory animals demonstrated that fluoride-induced oxidative stress is a possible and potential underlying mode of action of fluoride toxicity in the male reproductive system (Ghosh et al. 2002; Ortaa, Erkann 2014). It has been reported that fluoride can easily cross the blood-testis barrier and develops oxidative stress in testis by causing imbalance of the testicular oxidative and antioxidative status (Ghosh et al. 2002). Further, fluoride is a pro-inflammatory factor and increases the formation of reactive oxygen species (ROS; Flora et al. 2011). Oxidative stress adversely affects male reproductive function because of a higher level of unsaturated fatty acid, high level of cell division and high mitochondrial O_2 consumption in testes, and less ability of testes to detoxify ROS than the other tissues (Asadi etal. 2017). Ample studies can be found concerning fluoride-induced oxidative damage in testis, explaining how fluoride incites oxidative stress (Sarkar et al. 2006), damages Leydig cells (Ortaa, Erkan 2014), and Sertoli cell (Gupta et al. 2007) and reduces testosterone secretion (Kim et al. 2015). The reported toxic effects of fluoride-induced oxidative stress are diverse, ranging from inhibition of spermatogenesis (Pushpalatha et al. 2005) to apoptosis of germ cells (Tian et al. 2017). As a result of the toxic effect, there is a decline in sperm quality, capacitation, and reproductive ability (Wan et al. 2006; Reddy et al. 2007).

In this regard, previous studies reported that

concomitant/concurrent intake of various plant extracts, antioxidants, amino acids, and vitamins can reduce the toxic effects of fluoride. Vitamin B_{12} is a water-soluble vitamin and has been used in traditional medicine as a nutritional agent for the improvement of male fertility (Chaterjee et al. 2006). Both animal and human studies have demonstrated that vitamin B_{12} supplementation helps to maintain the normal structure and function of testes (Kawata et al. 1997) and also improves/restores semen quality (Sinclair 2000). Studies on both fertile and infertile humans have shown that there exists a positive correlation between vitamin B_{12} and semen parameters (e.g. sperm count, sperm motility, sperm DNA) and this finding confirms the essential role of vitamin B_{12} in spermatogenesis and also for maintenance of normal sperm function and structure (Chen et al. 2001; Boxmeer et al. 2007; Gual et al. 2015). Furthermore, vitamin B_{12} is a potent antioxidant (Abad et al. 2013; Boyum et al. 2014) and supplementation of it successfully reduces oxidative stress and enhances semen quality in bovine (Cai et al. 2004; Hu et al. 2011). Recently we have shown that combined supplementation of vitamin B_{12} and folic acid successfully counteracted nicotine-induced oxidative stress in testes and restored their normal function and structure (Ray et al. 2019).

To date, the protective role of vitamin B_{12} on fluoride-induced reproductive toxicity is not much studied. Based on the above fertility-enhancing property and other beneficial properties of vitamin B_{12}, the objective of the present study was to investigate the detrimental effects of sodium fluoride (NaF) and the protective role of vitamin B_{12} on reproductive parameters and testicular structure.

Materials and methods

Chemicals and reagents

Sodium fluoride (NaF) and vitamin B_{12} were purchased from Sigma-Aldrich. Sulfanilamide, phosphoric acid, naphthyl ethylene diamine dihydrochloride, thiobarbituric acid, trichloroacetic acid, xanthine, bovine serum albumin, nitroblue tetrazolium, xanthine oxidase, and 5,5 dithiobsis-2-nitrobenzoic acid were purchased from Merck (Darmstadt, Germany). All other reagents and chemicals were purchased commercially and were of analytical grade.

Experimental animals and study design

In the present study, male Wister rats weighing 110 to 125 g were used. The animals were housed in plastic cages and acclimatized under standard environmental conditions at temperature 23 ± 2 °C, light (12-h light / dark cycle), and 10% relative humidity for 14 days before performing the experiments. The animals were fed standard pellet diets with purified water available ad libitum. All animal experiments were performed in accordance with the Committee for Control and Supervision of Experiments on Animals guidelines under the direct supervision of the Institutional Animal Ethical Committee (IEAC) of Serampore College, Serampore, West Bengal, India, [Registration Number 1946/PO/Re/18/CPCSEA]. The study was approved by the IEAC, Serampore College with the approval No. 02/P/S/Sc/IAEC/2017.

The animals were randomly divided into four groups of six rats in each. Group 1 was a control and received distilled water, NaF (100 mg L^{-1}) with drinking water was given to group 2, rats in group 3 were given vitamin B_{12} (0.63 µg kg^{-1} body weight) orally, and group 4 was administered with NaF with vitamin B_{12} for 21 consecutive days.

The dose and route of NaF were selected based on a previous study (Atmaca et al. 2014) whereas the dose of vitamin B_{12} was chosen according to our earlier report (Ray et al. 2019).

Collection of epididymal sperm, blood, serum and testis

At the end of the treatment period, all rats were euthanized using cervical dislocation. After exposing the reproductive tract, the right caudal epididymis was attentively isolated and excised with scissors in 1 mL of physiological buffered saline (pH 7.4) to release the sperm. Semen samples were incubated at 37 °C for 20 to 25 min and sperm quality was evaluated as described previously (Wang et al. 2016).

Blood samples were collected and serum was prepared by incubating the collected blood in 37 °C for 30 min followed by centrifugation at 3000 g_n for 30 min. The obtained serum was stored at –80 °C for performing various biochemical assays.

Testes were removed aseptically and were fixed in 10% buffered formalin for histological examination.

Assay of serum testosterone concentration

Serum testosterone level was analyzed using the ELISA kit obtained from DRG Diagnostics, Germany. The sample was read against a blank at 450 nm within 30 min in an ELISA Reader (Merck).

Preparation of testicular tissue extract

The testes were homogenized using a glass homogenizer either in 100 mM phosphate buffer (pH 7.4, for catalase and reduced glutathione measurement) or in 100 mM Tris-HCl buffer (for determination of malondialdehyde, nitric oxide and superoxide dismutase) and centrifuged at 12000 g_n for 30 min at 4 C. The supernatant was collected and used for estimation of oxidative stress markers.

Estimation of nitric oxide production and lipid peroxidation

The role of nitric oxide synthase was indirectly analyzed by measuring the amount of nitric oxide (NO) produced. NO decomposes rapidly in aerated solutions to form stable nitrite/nitrate products. In this study, nitrite accumulation was estimated by the Griess reaction (Raso et al. 1999) and was used as an indicator of NO production. The amount of

nitrite in the sample (micromolar unit) was calculated from a sodium nitrite standard curve.

Intensity of lipid peroxidation was measured as concentration of thiobarbituric acid-reactive substances (TBARS) by a spectrophotometric method at 532 nm (Wills 1987). The level of lipid peroxidation in testicular homogenate was based on the formation of TBARS. The experiment was carried out by reaction of TBA with the experimental samples. The level of TBARS formed acts as an index of lipid peroxidation and is measured spectrophotometrically at 532 nm. Since 99% of thiobarbituric acid-reactive substances exist as malondialdehyde (MDA), the results were reported as amount of MDA per milligram of protein using the molar extinction coefficient (1.56×10^5 cm^{-1} $mmol^{-1}$).

Analysis of enzyme activity, protein and glutathione concentration

Superoxide dismutase (SOD) activity was determined based on the inhibition of superoxide-dependent nitro blue tetrazolium reduction by SOD (Sun et al. 1988). The relative absorbance was then converted into a unit of SOD activity per mL or mg protein. In this assay, 1 unit of SOD activity was equivalent to the quantity of SOD that caused a 50% reduction in the background rate of nitro blue tetrazolium reduction.

In the ultraviolet range, H_2O_2 shows a continual rise in absorption with decreasing wavelength, and decomposition of H_2O_2 was followed by monitoring the decrease in absorbance in a spectrophotometer at 240 nm at 25 °C (Aebi 1984). The alteration in the rate of absorbance was used as a measure of CAT activity. The values were expressed as U mg^{-1} protein.

Reduced glutathione (GSH) concentration was measured using 5,5-dithiobis-2-nitrobenzoic acid. The absorbance of reduced chromogen was followed spectrophotometrically at 412 nm. The GSH level was then determined using a standard curve and expressed as mmol mg^{-1} protein (Ellman 1979).

The total protein content was measured by the Lowry (1951) method using bovine serum albumin as a standard (Lowry 1951).

Histological examinations of testis

Immediately after removal, the testes were fixed with Bouin's fluid at room temperature for 24 hours and embedded in paraffin wax. Thin sections of 5 μm were prepared from the mid-portion of each testis with a rotary microtome and then stained with haematoxylin and eosin protocol. The pathophysiological changes were observed under light microscopy (Carl Zeiss, Germany).

Statistical analysis

Results were expressed as mean ± standard deviation. The Kruskal–Wallis nonparametric ANOVA test was first performed to test for any differences between the mean values of experimental groups. To test for significant differences between groups, the Mann-Whitney U multiple comparison test was performed. A value of $p < 0.05$ was considered as statistically significant.

Results

Sperm count (46.53%), sperm motility (61.92%), live sperm count (19.79%) and semen volume (8.63%) were significantly ($p < 0.05$) decreased in the NaF-treated group (group 2) compared to the control group. Animals from group 3 receiving B_{12} showed no significant change in these sperm parameters. However, co-administration of vitamin B12 with NaF significantly ($p < 0.05$) restored these sperm characteristics towards normal levels (Table 1).

Further, data presented in the Fig. 1 show that repeated exposure of sodium fluoride significantly ($p < 0.01$) decreased the level of serum testosterone in comparison with the control, indicating the inhibitory effect of NaF on androgenesis. Treatment of vitamin B_{12} for 21 days along with NaF showed a significant ability to recover the serum level of testosterone ($p < 0.05$; Fig. 1).

The level of MDA and NO are indicators of lipid peroxidation and inflammation respectively. NaF treatment for 21 days showed significant ($p < 0.01$) elevation in testicular content of MDA with a concurrent increase in NO generation in testis ($p < 0.01$), indicating that NaF treatment can induce lipid peroxidation and ROS generation in testis. In contrast, vitamin B_{12} co-treatment with NaF significantly ($p < 0.01$) prevented NaF-induced ROS generation towards the level of the control (Fig. 2 A and B).

Fig. 3 illustrates the activities of SOD and CAT in testicular tissue after the treatment of NaF and vitamin B_{12} for 21 days. An extreme inhibitory response on the testicular antioxidant status was noted after NaF exposure. Superoxide dismutase (SOD) and catalase (CAT) activity

Table 1. Effect of vitamin B_{12} on reproductive parameters of Wister rats. Values are expressed as mean ± SD; $n = 6$ in each group. a, indicates control vs. NaF ($Pa^* < 0.05$); b, NaF vs. B_{12} ($Pb^* < 0.05$); c, NaF vs. NaF + B12 ($Pc^* < 0.05$). NaF, sodium fluoride

Parameter	Control	NaF	Vitamin B_{12}	NaF + vitamin B_{12}
Sperm count (10^6 mL^{-1})	97.83 ± 0.60	52.30 ± 0.43 a*	100.50 ± 0.76 b	73.16 ± 1.01 c*
Sperm motility (%)	82.33 ± 0.66	31.55 ±0.47a*	92.13 ± 0.80 b	79.66 ± 0.73 c*
Live/dead ratio (%)	94.75 ± 0.77	76.00 ± 0.68a*	94.83 ± 0.31 b	88.50 ± 1.11 c*
Semen volume (μL)	4.98 ± 0.03	4.55 ± 0.022a*	5.10 ± 0.25	4.85 ± 0.02 c*

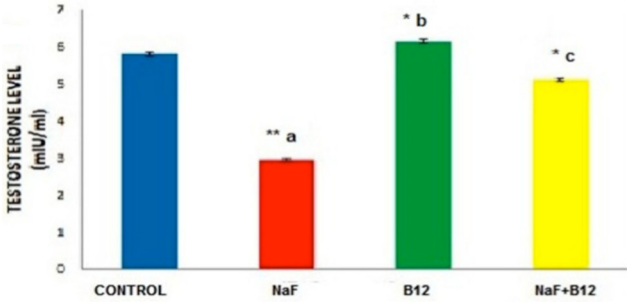

Fig. 1. Comparison of the mean concentration of testosterone. Values are expressed as mean ± SD of six observations. Significant based on Kruskal Wallis nonparametric ANOVA ($p < 0.001$). Significance level based on Mann-Whitney U multiple comparison tests: 'a' indicates control versus NaF (** $p < 0.01$); 'b' control versus vitamin B_{12} (* $p < 0.05$); 'c' NaF versus NaF + vitamin B_{12} (*$p < 0.05$). NaF, sodium fluoride; B_{12}, vitamin B_{12}.

significantly ($p < 0.01$) decreased in the NaF-treated group (group 2) compared to the control (Fig. 3 A and B). On the other hand, vitamin B_{12} treatment was able to significantly ($p < 0.01$) increase the activities of these enzymes (Fig. 3 A and B). In testicular tissue, the GSH level also significantly decreased compared to the level in the control group. After treatment with vitamin B_{12}, the GSH concentration increased (Fig. 3 C). However, there was no significant difference only between the control and vitamin B_{12} groups (Fig. 3 A to C).

The impact of NaF and vitamin B_{12} on the histology of rats is presented in Fig. 4. Histological study showed that testicular histoarchitecture was clear and intact, without any degenerative changes in the testicular tissue of control and vitamin B_{12} treated rats (Fig. 4 A and C, respectively), indicating lack of toxic side effect of vitamin B_{12} itself. After 21 days of NaF treatment, the NaF group (group 2) exhibited drastic degenerative changes in the seminiferous tubules. The majority of the tubules were wrinkled and distorted with disappearance of testicular cells like Sertoli cells, and sloughing of centrally located spermatozoa. In the interstitial space, loss of Leydig cells and edema was observed (Fig. 4 B). On the other hand, cosupplementation of vitamin B_{12} with NaF caused almost complete restoration of seminiferous tubular structure along with moderate reestablishment of interstitial histological arrangement (Fig. 4 D).

Discussion

Fluoride-induced overproduction of free radicals and lipid peroxidation have been proposed as the main underlying mechanisms involved in male reproductive dysfunction (Cheng et al. 2013; Ortaa, Erkan 2014). The findings of this investigation suggested that the treatment of sodium fluoride had adverse effects on testicular histology, sperm characteristics, and reduction in testosterone secretion as well as imbalance between oxidant and antioxidant status

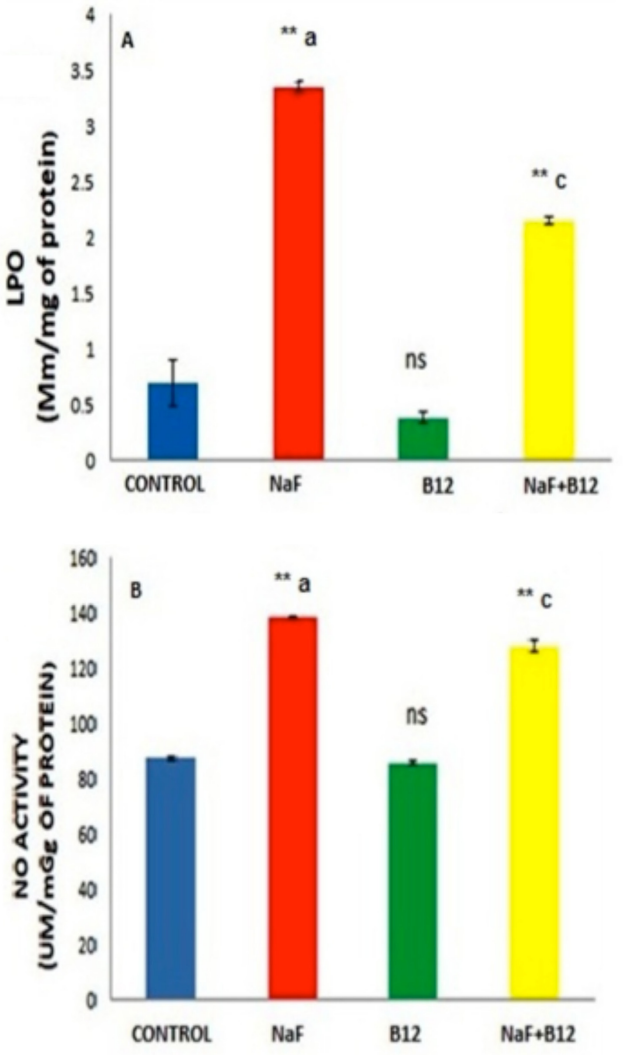

Fig. 2. Comparison of the status of free radical generation among the treatment groups. A, lipid peroxidation measured as MDA concentration; B, NO concentration. Data expressed as mean ± SD of six measurements. Significance level based on Kruskal Wallis nonparametric ANOVA test ($p < 0.0001$). Significance based on Mann-Whitney U multiple comparison test: 'a' indicates control versus NaF (**$p < 0.01$); 'b' control versus B_{12} (ns); 'c' NaF versus NaF + B_{12} (** $p < 0.01$.). NaF, sodium fluoride; B_{12}, vitamin B_{12}; ns, non significant.

in testis. In contrast, co-administration of vitamin B_{12} as an antioxidant and spermatogenic nutrient successfully antagonized/alleviated the diverse toxic effects of fluoride on male reproductive parameters.

Sperm quality is an important indicator of male reproductive function, which inevitably depends on various sperm indices (e.g. sperm count, motility, and viability). The decreased sperm count, motility, and viability of rats observed in the present study agree with previous reports demonstrating that NaF impairs sperm numbers and qualities (Zhu et al. 2000). The significant reduction in sperm quality, induced by NaF, may be associated with

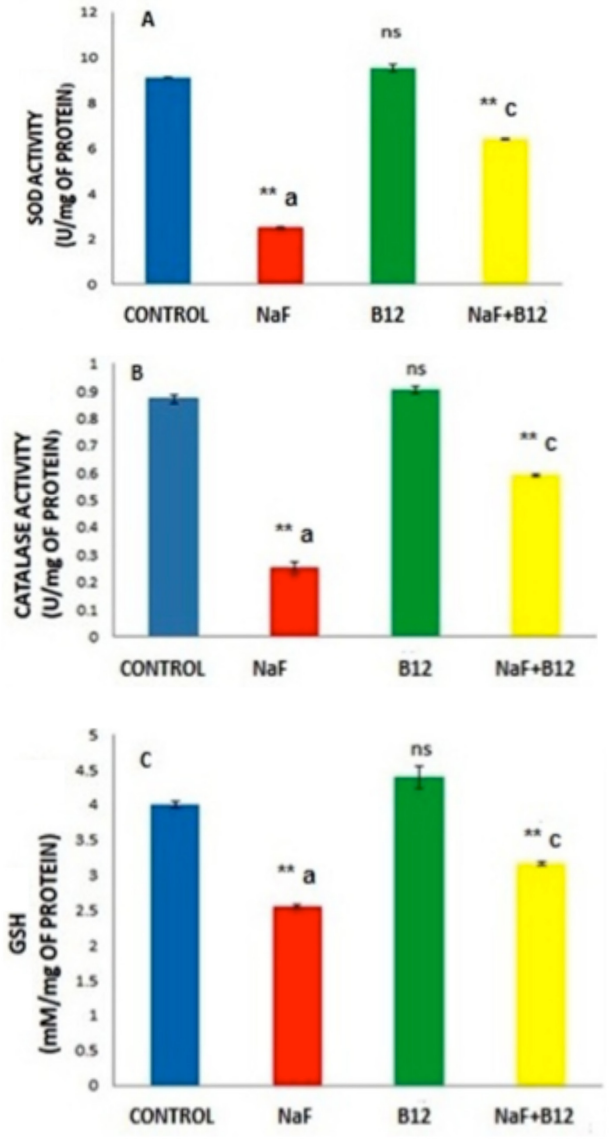

Fig. 3. Comparison of the endogenous antioxidants of testicular tissue among the treatment groups. A, SOD activity; B, CAT activity; C, GSH concentration. Values expressed as mean ± SD of six measurements. Significance level based on Kruskal Wallis nonparametric ANOVA test ($p < 0.0001$). Significance based on Mann-Whitney U multiple comparison test: 'a' control versus NaF (** $p < 0.01$); 'b' control versus B_{12} (ns); 'c' NaF versus NaF + B_{12} (** $p < 0.01$). NaF, sodium fluoride; B_{12}, vitamin B_{12}; ns, non significant.

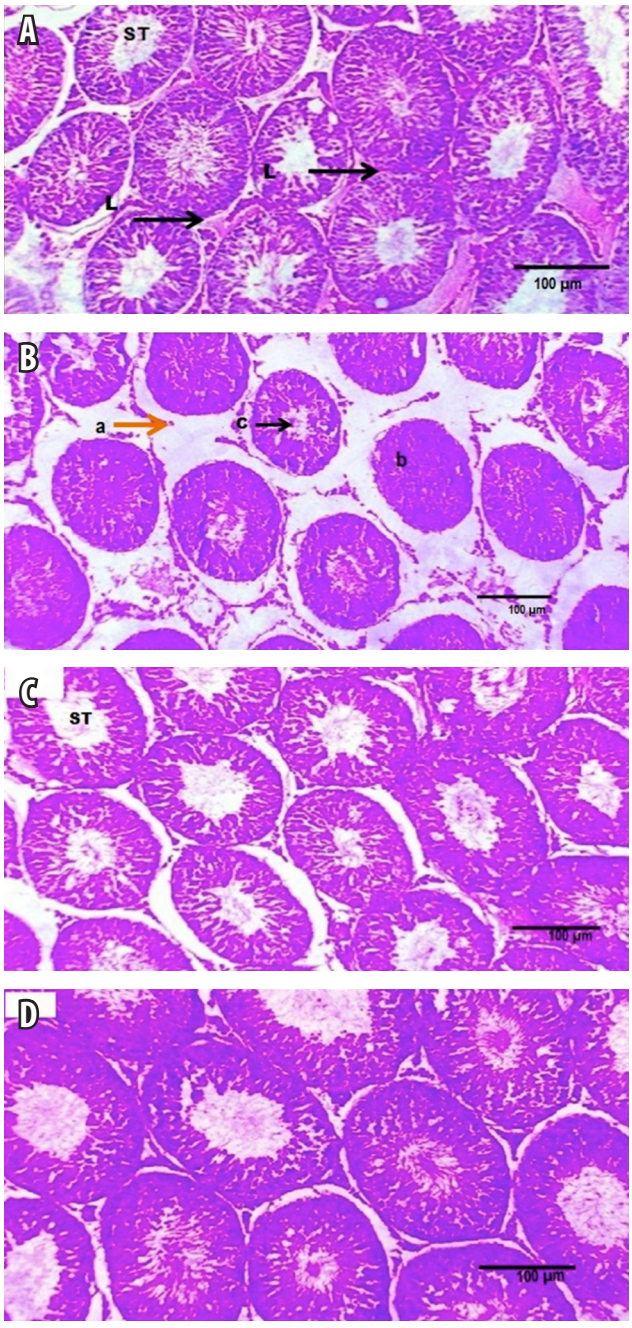

Fig. 4. Representative photomicrographs of hematoxylin and eosin-stained section (100 ×) showing the morphology of testicular tissue of control (A) with no pathological changes seen, normal structural features of seminiferous tubules and interstitial tissues. Sodium fluoride-treated (B) with signs of degenerative changes. Vitamin B_{12} (C) with normal cellular features like that of control. Sodium fluoride-treated and vitamin B_{12} supplemented rats (D) showing marked restoration of the sodium fluoride mediated disruption in the testicular histoarchitecture. ST, seminiferous tubules; L, Leydig cell; a, absence of Leydig cells and edema; b, shrinkage of seminiferous tubule; c, lack of luminal spermatozoa.

impairment of spermatogenesis (Ortiz-Perez et al. 2003; Smith, Walker 2014). Fluoride may inhibit spermatogenesis either by inhibiting ACE or by lowering Zn concentration in testes (Fatma et.el. 2009). Co-administration of NaF and vitamin B_{12} counteracted the adverse impacts of fluoride on sperm parameters and maintained normal semen quality. This protective effect of vitamin B_{12} is probably its essential role in the maintenance of cell cycle progression and tissue growth (Bohnsack, Hirschi 2004). Moreover, studies have demonstrated that cobalamin also plays a crucial role in the maturation of human spermatozoa and therefore has

been recognized as a useful nutrient in maintaining normal fertility (Watson 1962; Moriyama et al. 1987).

The normal structure of testis and epididymis is essentially required, since they provide a suitable microenvironment for spermatogenesis and maturation of sperm and also their storage. The histological section of testes revealed that fluoride treatment significantly damaged the structure of testis by degenerating seminiferous tubules. This finding is also corroborated with previous findings related to the effects of NaF on testicular structure (Feng et al. 2015; Yang et al. 2015). The disruption of testicular structure further leads to inhibition of spermatogenesis. The normal function of testis and epididymis as well as normal progression of spermatogenesis are regulated by testosterone. Previous studies have proven that fluoride can lower the level testosterone either by directly destroying Leydig cells (Ma et al. 2008) or by reducing the activities of steroidogenic enzymes such as 3β-hydrosteroid dehydrogenase and 17β-hydrosteroid dehydrogenase (Ghosh et al. 2004), indicating that degeneration of testicular structure and decreased sperm count and motility may be related to a reduced level of testosterone. The present results show that the level of testosterone declined in NaF-treated rats. Vitamin B_{12} supplementation prevented fluoride-induced degeneration of testis and restored epididymal sperm counts and motility. However, the way by which testosterone level and number and function of sperm and other cells are improved by this vitamin is not yet clear.

Fluoride treatment was shown earlier to increase ROS generation in testicular tissue. When electronegative fluoride (F^-) ions attack molecular O_2, various free radicals such as superoxide anion, H_2O_2, and peroxynitrite radicals, etc, collectively known as ROS, are produced (Rao, Bhatt 2012). ROS show a wide range of pathogenic properties and their uncontrolled overproduction plays a central role in the development of organ pathophysiology (Yamagishi et al. 2001). Excess production of ROS induces lipid peroxidation, which is considered as the main contributing factor of ROS-induced testicular dysfunction and impairment of normal structure and function of spermatozoa (Peltola et al. 1994). In the present study, testicular tissue of NaF treated rats showed a significantly higher level of MDA and NO, confirming that fluoride induces ROS generation. The decrease in sperm count, increase in sperm deformity, impairment of testicular structure, and low testosterone level with a concomitant increase of ROS, observed in the present study, agree with previous reports (Wang et al. 2009; Rao, Bhatt 2012. Sperm seems to more susceptible to ROS attacks, because it loses a large amount of cytoplasm and thus has relatively less antioxidant. When ROS production exceeds the intracellular antioxidant defense capacity, a pathophysiological state develops called oxidative stress, which causes cellular and macromolecular damage. In normal conditions, the cellular damage caused by ROS can be counteracted by endogenous antioxidants viz. SOD, CAT and GSH that together form a frontline defense against oxidative damage. The superoxide anions (O_2^-) are detoxified by the enzyme SOD into H_2O_2, which may be further converted into inactive forms by Fenton's reaction or by other enzymes such as CAT. The decrease in activity of SOD, CAT in NaF-exposed rat testicular tissue may further cause overproduction of ROS and lipid peroxidation, as described in previous studies (Ghosh et al. 2002). Glutathione (GSH) is a tripeptide and is a natural antioxidant that scavenges free radicals. It also protects proteins by preserving their SH group. Fluoride ions bind with GSH and inactivate it (Anuradha et al. 2001). According to the present study, there was a significant reduction in GSH levels in NaF treatment groups. This is in agreement with previous observations (Ghosh et al. 2008) showing that fluoride can trigger a particular mechanism liked to NaF-induced oxidative stress that causes all of the adverse effects observed. The increased level of lipid peroxidation and NO with a simultaneous decline in antioxidants in testicular tissue are in harmony with this conception.

Information is available regarding the use of antioxidants targeted as a possible natural preventive agent in NaF-mediated testicular dysfunction. Recently, increased attention has been focused on dietary factors because the toxicity of environmental chemicals can be modified by dietary factors (Trautner, Einwag 1989). Vitamin B_{12} is a water-soluble vitamin and thus safe. Additionally, it is abundantly present in natural foods like fish, meat, and milk and milk products. It has been established that vitamin B_{12} possesses antioxidant properties (Hu et al. 2011; Boyum et al. 2014). Furthermore, some *in vivo* and *in vitro* studies have reported that vitamin B_{12} acts as a scavenger of superoxide anions, as administration of it significantly reduces the generation of superoxide ions in aortic cells and in the ganglion cells of the retina (Moreira et al. 2011; Chan et al.2017) .

In the present study, it was found that vitamin B_{12} supplementation significantly prevented NaF-induced testicular dysfunction in spermatogenesis, testosterone formation, and oxidant-antioxidant status. SOD and CAT activity in the testis of vitamin B_{12} supplemented NaF-treated group were shown to be higher than in the NaF-treated group, indicating that vitamin B_{12} may have protective action against ROS-mediated fluoride toxicity. This is in agreement with previous studies that revealed that vitamin B_{12} protected the aortic cell and ganglion cells by scavenging O_2^- ions. The beneficial effect of vitamin B_{12} as an antioxidant in sperm motility and viability has been attributed to its ability to protect the sperm membrane and scavenging ROS (Hu et al.2011). Further, vitamin B_{12} is known to preserve GSH, which explains the increased GSH level in the vitamin supplemented rats in the present study. Thus, in accordance with previous studies, the results of the present study showed that vitamin B_{12} supplementation normalized the increased level of

oxidative stress by scavenging harmful ROS and also by restoring the antioxidant system in the testis. Also, fluoride is believed to induce hyperhomocysteinemia (Mehdi et al. 1990). Homocysteine, because of its auto-oxidation to H_2O_2, accelerates secondary ROS production. Additionally, homocysteine has been reported to block the activity of SOD and glutathione peroxidase, leading to oxidative stress. Hyperhomocysteinemia-induced oxidative stress has been liked to various health disorders including reproductive problems like reduced sperm numbers and counts (Crha et al. 2016). Vitamin B_{12} can lower the homocysteine level by metabolizing it to methionine. Consequently, supplementation of vitamin B_{12} in the present study further improved testicular dysfunction indirectly by decreasing the homocysteine level. Thus, based on the above observations, vitamin B_{12} can be proposed to play a protective role in testicular dysfunction by its direct as well as in indirect antioxidant activity.

It can be concluded that concurrent administration of vitamin B_{12} to NaF-treated rats prevents NaF-induced decline in sperm count and movement, improves the testosterone level and testicular morphology, and attenuates oxidative stress by improving antioxidant status and also by scavenging free radicals. The present study thus suggests that supplementation of vitamin B_{12} or vitamin B_{12} based compounds may have protective action against fluoride-induced reproductive toxicity in male rats.

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