Ameliorative potentials of *Annona muricata* (Linn) on Sodium Fluoride-induced Toxicity on Haematology indices and Fecundity of Adult Male Wistar Rats

Abstract

Aim: Ameliorative potentials of *Annona muricata* (Linn) on Sodium fluoride-induced toxicity on haematology indices and fecundity of adult male Wistar rats. **Methods:** Eighty-five (85) adult male Wistar rats were divided into 17 groups of 5 rats each. NaF (10 mg/kg) + fruit juice, ethanol stem bark, and leaf extracts of *A. muricata* at five different doses of 500, 1000, 1500, 2000, and 2500 mg/kg body weight were administered to the rats for 6 weeks. Blood samples were taken after 6 weeks through the ocular puncture and the sera were used for testosterone, follicle stimulating hormone (FSH), luteinizing hormone (LH) Triiodothyronine (T3), Thyroxine (T4), and Thyroid Stimulating Hormone (TSH) tests, while whole blood was used for haematological parameters such as haemoglobin (Hb), packed cell volume (PCV), total white blood cell, platelets count, lymphocytes % and neutrophils%. The testes and epididymis of the rats were harvested for histological studies and sperm analysis such as sperm motility, viability, count and sperm head abnormality. **Results:** Administration of NaF fruit juice, NaF + Stem bark, and NaF + leaf extracts caused an increase ($p<0.05$) in epididymal sperm count, sperm motility, and live spermatozoa along with a simultaneous decrease in dead spermatozoa as compared to the rats of the group treated with NaF alone. Result also showed a non-significant increase in haemoglobin, platelet count, lymphocyte count and decrease in neutrophil count, total white blood cell count except for the group treated with NaF and leaf extract that showed a non-significant increase, while treatment with stem bark and leaf extracts exhibited a varied effect on the packed cell volume.

Histological examination showed that NaF treatment brought about severe testicular damage while treatment with the extracts ameliorated this effect. **Conclusion:** *A. muricata* fruit juice and extracts were found to increase testosterone concentration, thus validating its ameliorative potential in NaF-induced toxicity.

Keywords: Sodium fluoride, *Annona muricata* (Linn), fecundity, haematology, hormones, histology.

Introduction

Several clinical investigations and animal experiments suggest that fluoride has adverse
impacts on male reproductive function producing structural and functional defects in spermatozoa, a decrease in sperm count, disturbances in the levels of reproductive
hormones and reduced fertility [1, 2]. Spermatozoa undergo various processes to ultimately fertilize an oocyte, including spermatogenesis,

capacitation, and the acrosome reaction.
Fluoride has been shown to impair all three of these processes [3]. *In vitro* fluoride exposure at high concentrations affected certain signal pathways, such as inhibition of the cell cycle, apoptosis and proliferation [4]. Thyroid hormone disruption caused by fluoride results in abnormal function and development of testes, lowering libido, reducing sex hormones, interferes directly and indirectly with spermatogenesis, influencing steroid hormone receptors, inducing oxidative stress in testes. However, the most important mechanism by which fluoride reduces the level of testosterone is interference with steroidogenesis in the Leydig cells. This interference has been demonstrated in several studies in which activity levels of testicular steroidogenic marker enzymes 3β-hydroxysteroid dehydrogenase (3β HSD) and 17β-hydroxysteroid dehydrogenase (17-HSD) decreased significantly in NaF- treated rats [5].
Annona muricata fruit juice has been shown to possess antibacterial, antifungal, anticancerous, antimalarial, antidiabetic, hepatoprotective, anti-inflammatory, hypotensive and immune enhancing effect [6]. Phytochemical screening of *A. muricata* leaf ethanolic extract shows the presence of saponins, triterpenoids, flavonoids, tannins, alkaloids, and cardiac glycosides [7]. *A. muricata* leaf extract is believed to stabilize blood sugar level in a normal range that is very useful for diabetic management [8]. Several types of research have shown that *A. muricata* leaf has hypoglycemic activity and revealed regeneration of pancreatic islet [9, 10, 11]. The ethanol leaf extract of *A. muricata* also is known to reduce serum uric acid level [12], contain essential oils with parasiticidal, antibacterial, antidiarrheal, rheumatological and antineuralgic properties [13, 14, 15]. The extract from *A. muricata* induced necrosis of pancreatic cancer cells by inhibiting cellular metabolism [16]. *A. muricata* leaf extract may possess anticancer properties by enhancing caspase-3 activity which is a pro-apoptosis marker [17]. The use of different parts of *A. muricata* for the treatment of these pathological disorders suggests it may possess anti-toxic properties and stimulated our interest to study its ameliorative effect on NaF- induced toxicity on haematology indices and fecundity of adult male Wistar rats.

Materials and Methods

2.1 Animals

Male adult albino rats (150-250 g) were obtained from the animal house of College of Medicine, University of Nigeria, Enugu Campus. The animals were housed in steel cages within the Laboratory Animals Facility of Brain-Phosphorylationship Scientific Solution Services, No9. Ogui Road Enugu, Enugu State, 5th Floor, Right Wing, maintained and given standard feed and clean drinking water *ad libitum.* They were allowed to acclimatize for a period of four weeks before use. All animal experiments were in compliance with the National Institute of Health Guide for care and use of laboratory animal.

Collection and Extraction of Plant Materials and Fruit Juice.

Fresh stem bark, leaf, and fruits of *Annona muricata* were collected from Abua, Rivers State, in March, 2017. The stem bark and the leaf were cut to pieces, dried under room temperature, ground and pulverized to a coarse powder using a Hammer mill (Gallenkamp, U.S.A.). The plant materials were identified and authenticated by Mr. Alfred Ozioko of International Centre for Ethnomedicines and Drug Development Nsukka, Nigeria and deposited in herbarium with Voucher Number: Intercedd/16091. Known quantities (1.851kg) of the dried stem bark powder and 1.016 kg of the dried leaf powder were extracted with analytical grade ethanol using maceration method for 48 hours. The mixture was a vacuum- filtered through Whatman No 1 filter paper and concentrated using a vacuum rotary evaporator (Eyla N-1000, Japan) to afford 97.352 g (5.257 % w/w) for stem bark extract and 126.312 g (12.432 % w/w) for leaf extract. The extractive yield was calculated using the relation: Yield (%) = [Weight of extract (g)/Weight of plant material (g)]*100.The fruit juices were used raw without concentrating it. The epicarps and the seeds of the ripen fruits were removed with hand and the mesocarps were sliced with a knife into small sizes and ground with and an electric grinder into paste form. This was further sieved with a muslin cloth to remove the fibres. The filtrate was transferred into clean glass container, sealed and preserved in refrigerator at -10ºC until use.

Experimental Design

Eighty five sexually matured male adult albino rats (150-250 g) were divided into 17 groups
of 5 rats each, according to their average weight, and received daily oral dose of the
treatment as follows:

Group 1: Normal feed and water (positive control)
Group 2: NaF (10mg/kg) (negative control)
Group 3: NaF (10mg/kg) + Fruit Juice Extract (500mg/kg)
Group 4: NaF (10mg/kg) + Fruit Juice Extract (1000mg/kg)
Group 5: NaF (10mg/kg) + Fruit Juice Extract (1500mg/kg)
Group 6: NaF (10mg/kg) + Fruit Juice Extract (2000mg/kg)
Group 7: NaF (10mg/kg) + Fruit Juice Extract (2500mg/kg)
Group 8: NaF (10mg/kg) + Leaf Extract (500mg/kg)
Group 9: NaF (10mg/kg) + Leaf Extract (1000mg/kg)
Group 10: NaF (10mg/kg) + Leaf Extract (1500mg/kg)
Group 11: NaF (10mg/kg) + Leaf Extract (2000mg/kg)
Group 12: NaF (10mg/kg) + Leaf Extract (2500mg/kg)
Group 13: NaF (10mg/kg) + Stem Bark Extract (500mg/kg)
Group 14: NaF (10mg/kg) + Stem Bark Extract (1000mg/kg)
Group 15: NaF (10mg/kg) + Stem Bark Extract (1500mg/kg)
Group 16: NaF (10mg/kg) + Stem Bark Extract (2000mg/kg)
Group 17: NaF (10mg/kg) + Stem Bark Extract (2500mg/kg)

Blood was taken after the 6th week of administration through the ocular puncture. Two ml of the blood samples from each group (n=4) were

collected in test tubes and put into centrifuge tubes, spun at 3000 rpm for 10 min and the serum collected for hormonal assays which include: testosterone, follicle stimulating hormone (FSH), luteinizing hormone (LH), Triiodothyronine (T3), Thyroxine (T4) hormone and Thyroid stimulating hormone. Whole bloods (2 ml) for haematological studies were placed in EDTA tubes and assayed for full blood count. The rats were sacrificed under chloroform anaesthesia after collection of blood samples. The testes and epididymis, were dissected out and rapidly fixed in buffered neutral formalin (10%) for histological studies. The epididymis was processed for epididymal sperm motility, viability, count and sperm head abnormality.

Histopathological examination

The tissues were subjected to standard routine histological procedures [18]. The slides were viewed using the light microscope and histopathological changes were observed and recorded at x400 magnification identifying both the normal and atrophied seminiferous tubules and spermatocytes.

Haematological studies

Determination of haematological parameters

Determination of haematological parameters such as haemoglobin concentration (Hb), packed cell volume (PCV), total white blood cell count (TWBC), platelet count, neutrophils and lymphocytes) were done using standard operative procedures[19].

Hormonal Assay

Plasma Testosterone, Follicle-stimulating and Luteinizing hormones were determined by fluorescence immunoassay (FIA) methods with commercial kits (Boditech Med Incorporated, Republic of Korea), using the ichroma machine (Boditech: BOD13303, Korea).

Sperm Analyses

Semen pH and sperm motility

Immediately after dissection, a puncture was made in the epididymis with a sterile pin. The semen smeared on the pin was rubbed on a pH paper of range 1.0-10.0. The colour change corresponds to the pH and was read from the paper. The dissected epididymis was measured and sliced into small pieces with a sterilized surgical blade and finally introduced into a beaker. The epididymal sperm samples were obtained by macerating known weight (100 mg) of cauda epididymis in physiological saline in the ratio of 1:10 weight by volume.

After vigorous shaking, two drops of sperm suspension was put on a microscope slide and a coverslip was placed. The numbers of progressively motile sperm cells were counted under

$$\textbf{\% Motility} = \frac{\text{No of motile spermatozoa}}{\text{Total no of spermatozoa counted}} \times 100$$

Percentage of dead sperm cells

The percentage of dead sperm cells was determined using Eosin-Nigrosin

one-step staining technique" [20]. A portion of the sperm suspension was mixed with equal volume of Eosin- Nigrosin stain and two (2) air-dried smears were prepared on glass slides for each sample. Dead sperm cells took up the stain and appeared pinkish. Percentage of dead sperm cells were calculated based on the number of dead sperm cells out of the total number of sperm cells observed.

Sperm viability

The sperm viability test was determined using "Eosin-Nigrosin one-step staining technique" [20]. A portion of the sperm suspension was mixed with equal volume of Eosin-Nigrosin stain and two (2) air-dried smears were prepared on glass slides for each sample. The slides were examined for percentage viability. Normal live sperm cells excluded the stain and appeared whitish, whereas dead sperm cells took up the stain and appeared pinkish. Percentage viability was calculated based on the number of live sperm cells out of the total number of sperm cells counted.

Sperm viability count

$$\frac{\text{Live cells(viable cels)} \times 100}{\text{Total cells (both dead \& alive)}}$$

Sperm count

The dissected epididymis was measured and sliced into small pieces with a sterilized surgical blade and finally introduced into a beaker. The epididymal sperm samples were obtained by macerating this known weight of cauda epididymis in physiological saline in the ratio of 1:10 weight by volume. After vigorous shaking, two drops of sperm suspension was put on a microscope slide and a coverslip was placed. Epididymal sperm count was obtained by cytometry using the improved Neubauer cytometer and was expressed as million/ml of suspension [21].

Sperm head abnormality test

A known volume of the sperm suspension was mixed with 1% eosin solution (10:1) for 30 min and air-dried smears were prepared on glass slides for each sample. The percentage of sperm head abnormality was calculated accordingly [21]

Statistical analysis

The data were analyzed by (SPSS version 17.5, SPSS Inc.). Significant differences between means were determined by One-way ANOVA and regarded significant at $p < 0.05$. Results were presented as Mean ± Standard Deviation

Results

Haemoglobin Concentration, Packed Cell Volume, and Platelets Count

Effects of fruit juice, ethanol extracts of stem bark and leaf of *A. muricata* on haemoglobin concentration of NaF-induced toxicity and fecundity profile of adult male Wistar rats is shown in Table 3.1. It was observed that NaF at a dose of 10 mg/kg caused a significant decrease ($p<0.05$) in Hb concentration, percentage PCV and platelets count in the adult male rats

when compared with the control. However, treatment with doses above 500 mg/kg body weight of NaF + fruit juice, NaF + stem bark and leaf extracts produced significant increases in Hb concentration and percentage PCV values when compared with group 2 rats. Similarly, groups treated with NaF + 2000 mg/kg of fruit juice and NaF + 1000 mg/kg of stem bark, and leaf extracts, showed a significant increase ($p<0.05$) in platelet count in comparison with both the group treated with NaF alone and control group.

Discussion

The recent findings that fluoride exposure leads to iochemical/histological alterations in male reproductive system through multiple pathways indicates that both assessment/prophylasis of chronic fluoride exposures in human populations is urgently required. Observation from this research work also indicates that sodium fluoride at a dose of 10 mg/kg caused non-significant decrease in haemoglobin concentration, platelet count, packed cell volume, and non-significant increase in neutrophil count, total white blood cell, and lymphocytes count. However, combined administration of NaF + the fruit juice and ethanol extracts of stem bark and leaf produced non-significant increase in the haemoglobin, packed cell volume and lymphocytes. The fruit juice at the concentrations of 1000 – 2500 mg/kg, and the groups treated with 500 and 1000 mg/kg of leaf extract, and 1000 and 2500 mg/kg of stem bark extract exhibited significant increase in platelet count. Reduction in haemoglobin and packed cell volume is an indication of either the destruction of red blood cells or the decreased production, which may lead to anaemia. On the contrary an increase in the count of red blood cell, haemoglobin and packed cell volume is suggestive ofpolycythaemia and positive erythropoiesis [22, 23]. Hence a non-significant increase or activation on haemoglobin and packed cell volume in fruit juice, stem bark and leaf extracts treated animals in comparison with the normal control is indicative of the ameliorative potential of these extracts against NaF induced toxicity. Therefore, an increased count of white blood cells and lymphocytes in NaF treated group, as observed in the present study, suggests that NaF might have compromised the immune system. This report is in agreement with [24], who reported a non-significant decrease in haemoglobin concentration of rats treated with NaF alone in comparison with the control group. [25, 26] reported that reduced blood platelets affect the viscosity of blood, which is correlated positively to blood pressure. Concomitant administration of NaF and *A. muricata* extracts for 30 days adversely affected the count of blood platelets which may produce a positive effect on the viscosity of blood. Probably prolonged duration of the treatment may ameliorate the toxic effect of NaF [27]. Reduction in platelet count in experimental animals has been reported to indicate an adverse effect on the oxygen carrying capacity of the blood as well as thrombopoietin. Both significant and non-significant increase in platelets counts observed

from the results of this study suggests that the administration of *A. muricata* fruit juice, leaf and stem bark extracts may ameliorate the disruption in the oxygen-carrying capacity of the blood caused by NaF.

The most important biochemical mechanism by which fluoride decreases the level of testosterone is its interference with steroidogenesis in Leydig cells. According to earlier research, this interference has been demonstrated, in which activity levels of testicular steroidogenic marker enzymes 3β-hydroxysteroid dehydrogenase (3β-HSD) and 17β-hydroxysteroid dehydrogenase (17β-HSD) decreased significantly in NaF-treated rats [5, 28]. Since testicular steroidogenesis is controlled by these two rate-limiting enzymes, a decline in their activities in Leydig cells significantly decreases the production and therefore the level of testosterone. Known mechanisms by which fluoride decrease testosterone levels are; inducing changes in both structures and enzyme activities in Leydig cells and interfering with hypothalamus-hypophysis-testis axis [29]. Leydig cells require normal expression and function of epidermal growth factor receptor (EGFR), androgen receptor (AR) and G-proteins in order to synthesize testosterone. Fluoride exposure has been shown to reduce both EGFR and AR expression [30] and to interfere with G-proteins in Leydig cells. However, fluoride has been found to interfere with hypothalamus-hypophysis-testis axis [31]. The non-significant decrease in testosterone level in NaF treated group in relation to the control group reported in this study is consistent with so many previous research works which had demonstrated that the NaF toxicity leads to a decrease in testosterone, a key hormone in spermatogenesis [2, 32]. The result further reveals that concomitant administration of 10 mg/kg of NaF and extracts on testosterone levels of all the stem bark and leaf extracts treated groups exhibited concentration-dependent significant increases while groups treated with NaF and fruit juices exhibited no obvious changes. This observed increase could be attributed to the interference of their phytochemical constituent(s) on the inhibitory action of fluoride ion on steroidogenesis in Leydig cells or their antioxidant effect (properties) on free radical generation by fluoride.

A lower concentration of 500 mg/kg of stem bark extract and 1000 mg/kg of fruit juice produced a significant increase in FSH and LH concentrations respectively. This suggests that stem bark extract and fruit juice at lower doses, with its antioxidant properties ameliorated the toxicity effects of NaF on Gonadotropin hormones. Gonadotropins are luteinizing hormone and follicle stimulating hormone from the pituitary gland. Testosterone in males secreted by Leydig interstitial cells is increased under the influence of luteinizinghormone. FSH regulates the development, growth, pubertal maturation and reproductive processes of the body. Diminished secretion of FSH can result in hypogonadism. This condition is typically manifested in males as a failure in the production of normal numbers of sperm. Serum levels of FSH are decreased in anterior pituitary hypofunction, hypothalamic disorders. Serum levels of LH are decreased in pituitary

hypothalamic impairment.
Gonadotropin-releasing hormone stimulates the production and release of follicle stimulate hormone (FSH) and luteinizing hormone (LH) from the pituitary gland [33].
Studies have reported that fluoride affects the synthesis of thyroid hormones, which inversely impair the normal function of the male fecundity. Fluoride has been shown to increase thyroid stimulating hormone (TSH) and reduce triiodothyronine (T_3) and thyroxine (T_4) [34]. Fluoride is considered to interfere with thyroid hormone levels mainly through three mechanisms; impairing normal structures of the thyroid gland, disrupting iodine metabolism in thyroid glands and interfering with the tissue-specific metabolism of thyroid hormones. Clinch in her review pointed out that fluoride interferes with the activity of Na/K-ATPase and the sodium-iodide symporter. Since iodide uptake is facilitated by the combined actions of the Na/k-ATPase and the sodium/iodide symporter [35], a decrease in the activities of these enzymes caused by fluoride would reduce the uptake of iodide in the thyroid gland and subsequent production of thyroid hormones. High fluoride intake has also been shown to inhibit the activity of thyroid peroxidase [36]. Since thyroid peroxidase is an enzyme which is essential for the production of thyroid hormones, decreased activity of thyroid peroxidase caused by fluoride would also lead to reduced thyroid hormone synthesis [35].
Hypothyroidism is known to be associated with impotence and decreased libido since thyroid hormone affect brain chemistry involved in sexual arousal, which in turn stimulates the autonomic nervous system and affects many other hormones necessary for energy [37].
There is a correlation between hypothyroidism and low serum testosterone concentration.
Also, type 2 iodothyronine deiodinase which regulates the tissue-specific conversion of T_4 to the genomically active T_3 is predominantly expressed in elongated spermatids, suggesting that thyroid hormone might have a direct effect on spermatogenesis [38, 39]. It is an established fact that T_3 regulates the maturation and growth of testis, controlling Sertoli cell and Leydig cell proliferation and differentiation during testicular development in rats and other mammal species [40]. However, our observations on the effect of NaF on thyroid hormone agree with the previous research that indicated that fluoride increases TSH but reduces T_3 and T_4 [41]. Fluoride is considered to interfere with thyroid hormone levels mainly through three mechanisms; impairing normal structures of the thyroid gland, disruptive iodine metabolism in thyroid glands and interfering with the tissue-specific metabolism of thyroid hormones [42]. Several studies reveal that fluoride can directly damage the structures of thyroid follicles, resulting in the following abnormalities; flattened follicle epithelial cells, reduced cytoplasm [43]. These structural disruptions by fluoride will disrupt the synthesis of thyroid hormones in the

thyroid follicles [44].

Once fluoride crosses blood-testis membrane barriers that protect spermatogenesis, after a prolonged exposure, it causes lack of maturation and differentiation of spermatocytes, fragmentation of spermatozoa in the epididymis, and even cessation of spermatogenesis [45]. The present investigation was carried out to explore the effects of fluoride (10mg/kg NaF) and the possible ameliorative role of concomitant administration of fruit juice, leaf and stem bark ethanol extract on the seminal characteristic of adult male Wistar rats. The sodium fluoride treatment caused a substantial significant decrease in epididymal sperm motility, progressive sperm motility, sperm concentration and live spermatozoa (%) along with a simultaneous increase in dead spermatozoa (%) as compared to the rats of the control group. Findings from this research work agree with [46, 47] who reported that exposure to high concentrations of NaF leads to decreased sperm count, sperm motility, sperm survival and increase in sperm abnormalities. The most important consequence of these fluoride exposures is changes in the structure and functional behaviour of spermatozoa, disruption of spermatogenesis and disturbance of multiple hormone systems that impact male fecundity.

Conclusion

The histopathologic findings in the present study justify the finding reported from cauda epididymal spermatozoa analysis. It might be concluded that NaF at 10 mg/kg caused potential reproductive cytotoxicities leading to significant alterations in testicular tissue, altered semen characteristics, various morphological abnormalities in spermatozoa, haematological parameters, nephrons and hepatocytes membrane permeabilities.

Concomitant administration of the fruit juice, ethanol stem bark and leaf extracts of *A. muricata* for a period of 6 weeks resulted in significant prophylactic amelioration in all parameters altered. Therefore, fruit juice, ethanol extracts of stem bark and leaf of *A. muricata* therapy could be beneficial for the amelioration of fluoride-induced toxicity in male reproductive system and fertility in general.