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*Original Article*

# Arjunolic acid counteracts fluoxetine-induced reproductive neuroendocrine dysfunction through inhibition of chromosomal derangements and hypercortisolism

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## Abstract

Antidepression-related HPA-HPG alteration is gaining more attention in stress research on humans and animals with depression. Therefore, the search for therapeutic drugs such as Arjunolic acid (AA) might be a core value in the management of reproductive neuro-endocrine dysfuction in rats treated with FXT. In this context, this study aimed to determine the effects of AA on reproductive neuro-endocrine functions in fluoxetine (FXT)-induced HPA-HPG axis dysfunction in rats. The subjects were randomly divided into 6 groups with six (6) rats each after 14 days of acclimatization. Rats in group 1 received normal saline (10 mL/kg); groups 2 & 3 were respectively given AA (1.0 mg/100gm body weight) and AA (2.0 mg/100gm body weight), whereas rats in group 4 were given FXT (10 mg/kg/p.o./day), and groups 5 & 6 were respectively given a combination of FXT (10 mg/kg) + AA (1.0 mg/100g body weight) and of FXT (10 mg/kg) + AA (2.0 mg/100g body weight). The results revealed that FXT altered reproductive neuro-endocrine function as evidenced by increased corticosterone, tDFI, tCSA, and abnormal sperm morphology; with corresponding decreases in Kisspeptin, GnRH, LH, FSH, testosterone, HOST value, TP, Sialic acid, Johnson score, sperm count, motility, and viability. However, AA dose dependently significantly counteracted the FXT-elicited changes in corticosterone, tDFI, tCSA and abnormal sperm morphology as well as Kisspeptin, GnRH, LH, FSH, testosterone, HOST value, TP, Sialic acid, Johnson score, sperm count, motility, and viability; and improved the body and testicular weight in rats. In conclusion, AA attenuates fluoxetine-induced reproductive neuroendocrine dysfunction through inhibition of chromosomal derangements and hypercortisolism. However, co-administration of FXT with AA could be a better therapeutic option in the management of FXT-induced altered HPA-HPG-axis.

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**Keywords**: fluoxetine, arjunolic acid, spermatogenesis, hypercortisolism, reproductive hormone

## 1. Introduction

Preclinical and clinical studies have shown that major depressive and postpartum depressive disorders, as well as antidepressant drugs interfere with reproductive neuro-endocrine functions, thus affecting the secretion of pituitary hormones and levels of the peripheral sex steroid hormones necessary for spermatogenesis and fertility in males (Chávez-Castillo *et al.*, 2019; Dwyer *et al.*, 2020; Maguire, 2019; Nandam, Brazel, Zhou, & Jhaveri, 2020). In animal studies, long-term or chronic administration of fluoxetine (FXT) has been reported to decrease spermatogenesis, testosterone, follicle stimulating hormone (FSH) levels and weights of reproductive organs (Sakr, Mahran, & ElDeeb, 2013), while in human subjects' sexual disorders, abnormal DNA fragmentation and decreased semen have been reported in patients treated with antidepressants like paroxetine, duloxetine, and fluoxetine (Pharm *et al.*, 2022). More so, the timing of observed effects of the selective serotonin reuptake inhibitors (SSRIs) on spermatozoa suggests that sperm DNA damage may occur as a consequence of the slowing of sperm transport (Tanrikut & Schlegel, 2007). Previous studies have shown that high levels of sperm DNA fragmentation (SDF) are associated with lower fertilization rates, impaired implantation rates, and an increased incidence of miscarriages (Viktorin, Levine, Altemus, Reichenberg, & Sandin 2018). These sexual side effects can considerably affect a person's quality of life, and when this results in reduced compliance with medication, it may lead to less effective treatment of the primary psychiatric disorder (Durairajanayagam, 2018). Therefore, the search for therapeutic drugs such as Arjunolic acid (AA: 2,3,23-trihydroxyolean-12-en-28-oic acid), a natural product found in Terminalia chebula, Symplocos lancifolia, and Juglans sinensis (Ghosh, & Sil, 2013), might be a core value in the management of reproductive neuro-endocrine impairment in rats treated with FXT. In this context, it is therefore very important to determine the effects of AA on fluoxetine (FXT) therapy, which is frequently used in patients of reproductive age, during reproductive development and maturation processes.

## 2. Materials and Methods

### 2.1 Animals

A total of 36 Wistar rats weighing 150-250 g (6-8 weeks old) were used in the experiment. The animals were kept in a controlled environment of about 25 ± 2 ℃ in 12:12 h day and night cycle. The animals were acclimatized for 14 days with unrestricted access to food and water. The study protocols used in handling the animals were in line with the establishments by National institutes of Health (NIH) Guideline for the Care and Use of Laboratory Animals (Publication No. 85-23, revised).

### 2.2 Drugs and chemicals

Fluoxetine (FXT) and Arjunolic acid (AA) used in this study were bought from Tesi Pharmaceuticals, Ughelli, Delta State, Nigeria, and Sigma Aldrich, USA, respectively. FXT and AA were dissolved separately in 10 mL of saline immediately before use and administered orally. The doses and routes of Saline (Oyovwi *et al.*, 2022), FXT at 10 mg/kg (Sakr *et al.*, 2013) and AA at 1.0 and 2.0 mg/100gm body weight (Khatun, Chatterjee, & Chattopadhyay 2019) were selected based on previous dose-response effects and a preliminary investigation. Moreover, saline (10 mL/kg, p.o.) was administered as a normal control and vehicle to naïve rats in different groups. All administrations were given between 8 am and 9 am through oral route for 4 weeks.

### 2.3 Experimental procedures

After 14 days of acclimatization, the animals were randomly divided into 6 groups with six (6) rats each. Rats in group 1 received normal saline (10 mL/kg); groups 2 & 3 were given AA (1.0 mg/100gm body weight) and corn oil (2.0 mg/100gm body weight) respectively, whereas rats in group 4 were given FXT (10 mg/kg/day), and groups 5 & 6 were respectively given a combination of FXT (10 mg/kg) + AA (1.0 mg/100g body weight) and FXT (10 mg/kg) + AA (2.0 mg/100g body weight).

### 2.4 Sample collection

At the end of the experimental period, the animals were euthanized and samples of blood were obtained from each rat via the retro-orbital sinus into a plain bottle. After centrifugation, we obtained serum from clot and it was stored at 80°C prior to analysis of neuro-endocrine related factors and reproductive hormone assay using ELISA kit. Cleaned testicular tissues were weighed, homogenized and centrifuged at 14,000 g in a radioimmunoprecipitation buffer solution prior to determinations of testicular protein, sialic acid, DFI, sperm chromatin integrity and testes chromosome. For sperm parameters, each animal's cauda epididymis was used to determine count, motility, viability and morphology. The other testis was preserved in Bouin's fluid for histological examination.

### 2.5 Assays

#### 2.5.1 Serum hormone analysis

The enzyme linked immunosorbent assay technique was used for testosterone, GnRH, follicle stimulating hormone, luteinizing hormone, Kisspeptin, and corticosterone estimation, using kits (Calbiotech Inc. California). The ELISA kits, which contained the different EIA enzyme labels, substrate reagents, and quality control samples, were used. The procedures for the estimation of serum concentration of each hormone were carried out according to the kits' manuals.

### 2.5.2 Semen analysis

Epididymal total sperm count, Sperm motility, sperm viability as well as Sperm abnormalities were evaluated as described by Nwangwa (2012).

### 2.5.3 Assay of sperm DNA fragmentation

Assay of sperm DNA fragmentation was carried out as previously described (Cho and Agarwal, 2018) using aniline blue staining method.

### 2.5.4 Cytogenetic analysis for meiotic chromosomes

Chromosomal aberrations such as aneuploids, autosomal univalents, sex-univalents, polyploids and translocations, were screened using Giemsa solution (10 per cent) after chromosomal preparations were made by air-drying technique (Evans, Breckon, & Ford, 1964).

### 2.5.5 Examination of sperm membrane integrity using Hypo-osmotic swelling test (HOST)

This test evaluates the response of spermatozoa to hypo-osmotic stress. The sperms was exposed to hypo-osmotic medium and observed for coiled tails under the microscope and the percent of coiling was estimated by the method of Jeyendran, Van der Ven, & Zaneveld, (1992). In this method, a hypo-osmotic solution was prepared by adding 0.735g of sodium citrate dehydrate and 1.351g of fructose to 100 ml of distilled water. After this, 0.1 mL of epididymis sperm suspension was added to 1.0 mL of hypo-osmotic solution and incubated for 30–60 min at 37 degrees. After incubation, a drop of the mixture was placed on a glass slide and a cover slip was applied. The slide was then viewed with an X40 lens to observe swelling. Sperm with a swollen tail were regarded as host positive, while those without a swollen tail were regarded as host negative.

### 2.5.6 Evaluation of Sperm chromatin integrity using toluidine blue (TB)

Evaluation of Sperm chromatin integrity was carried out as previously described (Oyovwi *et al.*, 2022) using toluidine blue (TB).

### 2.5.7 Measurement of protein and sialic acid markers in rats' testes

Protein and sialic acid activities in testicular tissue homogenates were measured using ELISA kits (obtained from Sigma-Aldrich Chemical Co) as indicated by the protocol of manufacturer.

### 2.5.8 Examination of histopathology and spermatogenesis scoring

Cross-sections of testes were paraffin embedded, fixed in a 10% formaldehyde solution, stained using haematoxylin and eosin stain (H&E), mounted, and examined by a trained histopathologist using a microscope in a blinded manner, and scored as previously described (Johnsen, 1970).

### 2.6 Statistical analysis

Data were analyzed using GraphPad prism 8 Biostatistics software (GraphPad Software, Inc., La Jolla, USA, version 8.0). All data are presented as mean ± SEM. A one-way analysis of variance (ANOVA) was followed by a *post hoc* test (Bonferroni correction for multiple comparisons) and the level of significance for all tests was set at $p < 0.05$.

## 3. Results

### 3.1 Effect of Arjunolic acid on fluoxetine-induced alteration in serum hormonal parameters in rats

As presented in Figure 1a-c, one-way analysis of variance (ANOVA) followed by *post hoc* test revealed that fluoxetine significantly $(p < 0.05)$ decreased LH (Figure 1a), FSH (Figure 1b) and testosterone (Figure 1c) activities as compared to normal control group. In comparison to fluoxetine-treated rats, Arjunolic acid dose dependently and dramatically restored fluoxetine-induced changes in FSH, LH, and testosterone (Figure 1a-c). Notably, Arjunolic acid given at 2.0 mg/kg alone significantly increased LH and testosterone relative to control.

### 3.2 Effect of Arjunolic acid on fluoxetine-induced alteration in neuro-endocrine disruption in rats

As presented in Figure 2a-c, one-way analysis of variance (ANOVA) followed by the *post hoc* test revealed that fluoxetine significantly $(p < 0.05)$ decreased Kisspeptin (Figure 2a) and GnRH (Figure 2b) activities, as well as increased corticosterone level when compared to normal control group. In comparison to fluoxetine-treated rats, Arjunolic acid dose dependently and dramatically restored fluoxetine-induced changes in kisspeptin, GnRH and corticosterone level (Figure 2a-c). Notably, Arjunolic acid given at 2.0 mg/kg alone significantly increased kisspeptin and GnRH relative to control.

### 3.3 Effect of Arjunolic acid on fluoxetine-induced perturbation in sperm indices in rats' testes

In order to establish the therapeutic efficacy of gonado-protective impacts of Arjunolic acid against fluoxetine-induced perturbations in sperm indices, we evaluated the effects of Arjunolic acid on sperm indices in the testes of the rats. Based on this context, as shown in Figure 3a-d, one-way ANOVA followed by the *post hoc* test showed that fluoxetine significantly decreased sperm count (Figure 3a), sperm motility (Figure 3b), sperm viability (Figure 3c) and abnormal sperm morphology (Figure 3d) when compared with normal control group. Nevertheless, Arjunolic acid significantly attenuated fluoxetine-induced alterations in sperm indices relative to fluoxetine-treated rats (Figure 3a-d).

### 3.4 Effect of Arjunolic acid on fluoxetine-induced alteration in sperm membrane integrity in rats using HOST (Hypo-osmotic swelling test)

The effect of Arjunolic acid on fluoxetine-induced alteration in sperm membrane integrity using HOST is shown

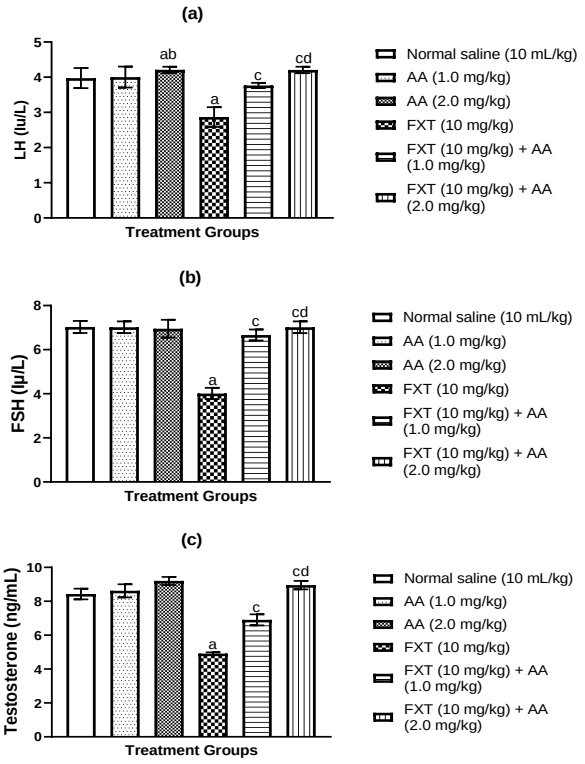


Figure 1. Effect of Arjunolic acid on fluoxetine-induced alteration in serum hormonal parameters in rats

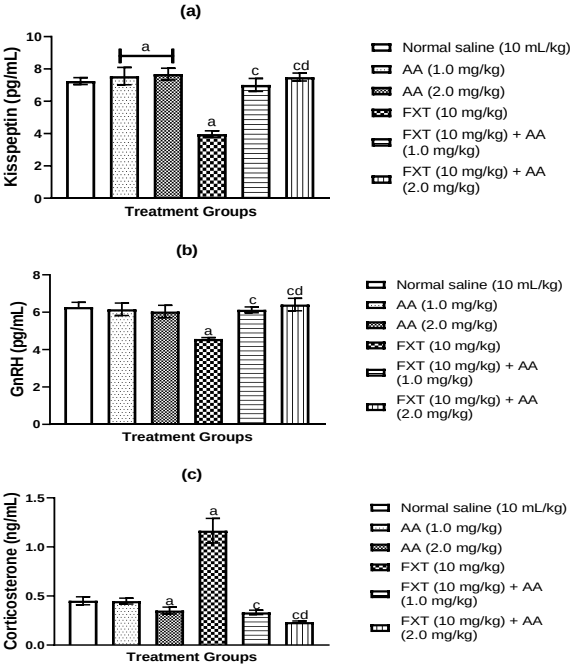


Figure 2. Effect of Arjunolic acid on fluoxetine-induced alteration in neuro-endocrine disruption in rats

in Figure 4. Fluoxetine produced a significant ($p < 0.05$) decrease in HOST values of rat's caudal epidymal spermatozoa. Arjunolic acid administered at different doses significantly ($p < 0.05$) abated fluoxetine-induced decrease in

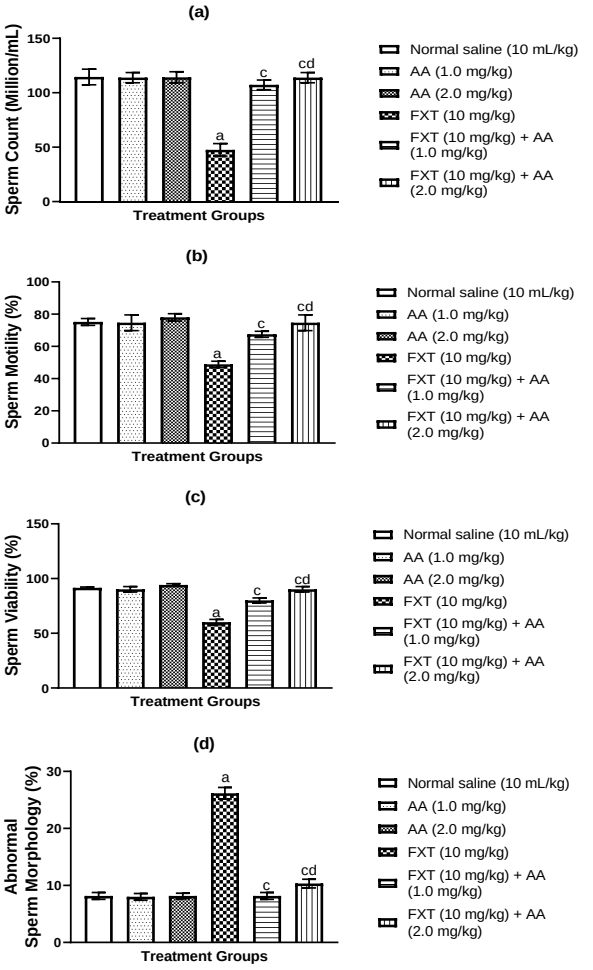


Figure 3. Effect of Arjunolic acid on fluoxetine-induced perturbation in sperm indices in rats' testes

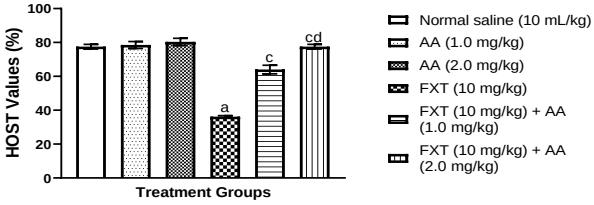


Figure 4. Effect of Arjunolic acid on fluoxetine-induced alteration in sperm membrane integrity in rats using HOST (Hypo-osmotic swelling test)

HOST values relative to the fluoxetine-treated rats. When Arjunolic acid alone was given at different doses, there was no significant difference as compared to control animals.

### 3.5 Effect of Arjunolic acid on fluoxetine-induced changes in testicular protein and sialic acid level in rat testes

Endosulfan (5 mg/kg, p.o.) mediated a marked ($p < 0.05$) decrease in testicular protein content (Figure 5a) and sialic acid content in rats (Figure 5b). When compared to fluoxetine-treated rats, Arjunolic acid at different doses (1.0

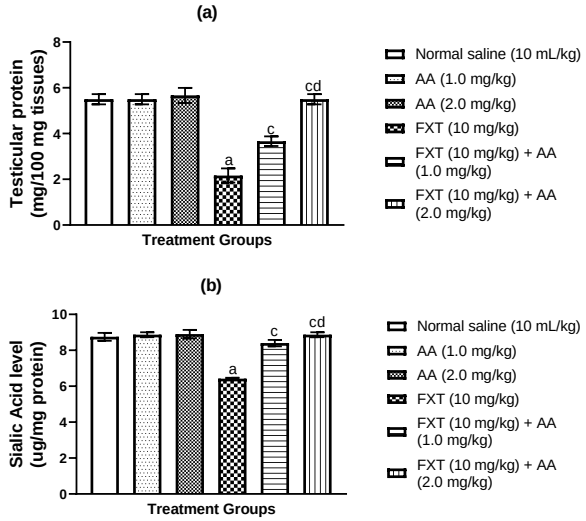


Figure 5. Effect of Arjunolic acid on fluoxetine-induced changes in testicular protein and sialic acid level in rat testes

mg/kg and 2.0 mg/kg, p.o.) significantly ($p< 0.05$) ameliorated fluoxetine-mediated decrease in testicular protein and sialic acid.

### 3.6 Effect of Arjunolic acid on fluoxetine-induced decrease in body and testicular weights of rats

The impacts of Arjunolic acid co-treatment on testicular and body weights of rats treated with fluoxetine are shown in Figure 6a-b. As illustrated in Figure 6, there was a significant decrease in the body (Figure 6a) and testicular (Figure 6b) weights in rats exposed to fluoxetine when compared to control group. Arjunolic acid co-treatment profoundly normalized the body and testicular weights in comparison to fluoxetine treated rats (Figure 6a-b).

### 3.7 Effect of Arjunolic acid on fluoxetine-induced alteration in testicular DNA fragmentation index (index of apoptosis related factor) and sperm chromatin integrity using toluidine blue staining (TBS) in rats

Figure 7a-b presents the changes in testicular DNA fragmentation index (tDFI) and epididymal sperm chromatin integrity, when subjected to fluoxetine treatment. Fluoxetine significantly ($p < 0.05$) decreased tDFI (Figure 7a) and sperm chromatin integrity (Figure 7b). However, this fluoxetine-induced decrease in tDFI activities was attenuated in rats co-treated with Arjunolic acid at different doses. The decrease in sperm chromatin integrity observed in fluoxetine-treated rats (Group IV) was evidenced by a significantly increased percentage ($p < 0.05$) of positive toluidine blue staining in the epididymal sperm. This increased percentage significantly differed from the related control. Arjunolic acid co-treatment significantly ($p < 0.05$) suppressed the alteration seen in chromatin quality induced by fluoxetine.

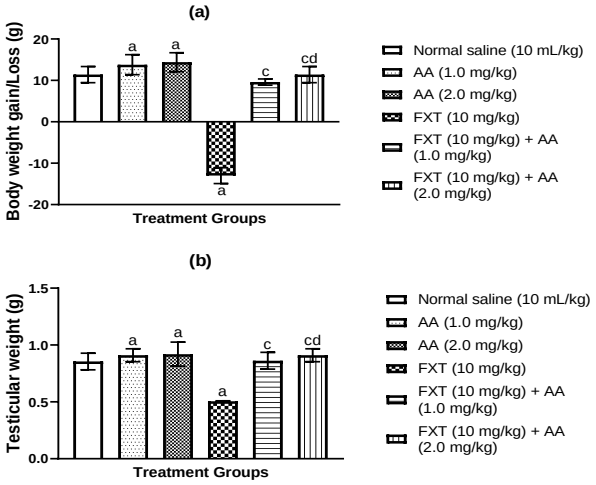


Figure 6. Effect of Arjunolic acid on fluoxetine-induced decrease in body and testicular weights of rats

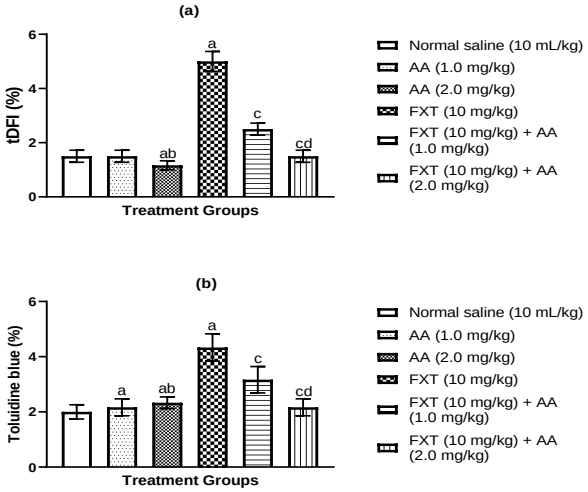


Figure 7. Effect of Arjunolic acid on fluoxetine-induced alteration in testicular DNA fragmentation index (index of apoptosis related factor) and sperm chromatin integrity using toluidine blue staining (TBS) in rats

### 3.8 Effect of Arjunolic acid on fluoxetine-induced aberrations in testes chromosome of rats

The impacts of Arjunolic acid co-treatment on testis chromosome of rats treated with fluoxetine are shown in Figure 8a-f. As illustrated in Figure 8, a significant ($p<0.05$) increase was noticed in the frequency of aneuploids, sex-univalents, and total percent chromosomal aberrations in rats exposed to fluoxetine as compared to the values observed in the control group. Arjunolic acid co-treatment profoundly abated the percent chromosomal aberrations obsereved in comparison to fluoxetine treated rats (Figure 8a-f). However, no significant changes were observed in the frequency of autosomal univalents and polyploids in rats treated with fluoxetine and Arjunolic acid.

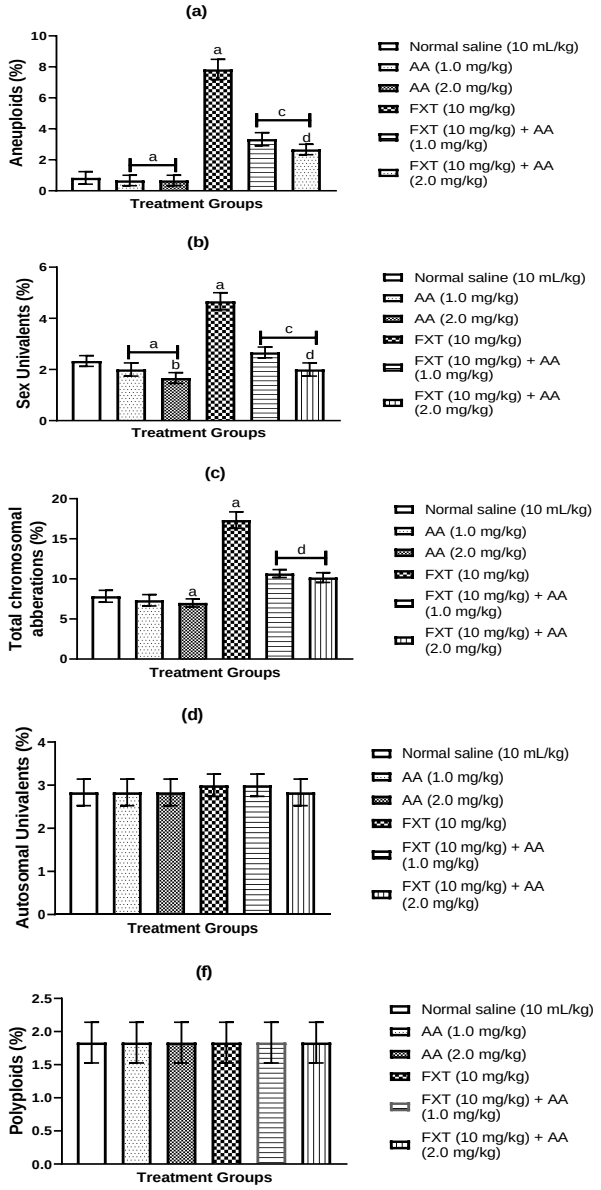


Figure 8. Effect of Arjunolic acid on fluoxetine-induced aberrations in testes chromosome of rats

## 3.9 Effect of Arjunolic (AA) acid on fluoxetine (FXT)-induced decreased spermatogenesis scores in rats' testes

Spermatogenesis scores differ significantly between treatment groups, according to one-way ANOVA. When compared to control group, FXT caused a significant decrease in spermatogenesis scores. In comparison to FXT, AA therapy dramatically reduced testicular enhanced spermatogenesis scores.

## 4. Discussion

Fluoxetine (FXT) induced male gonadal damage and sexual dysfunction have been reported previously

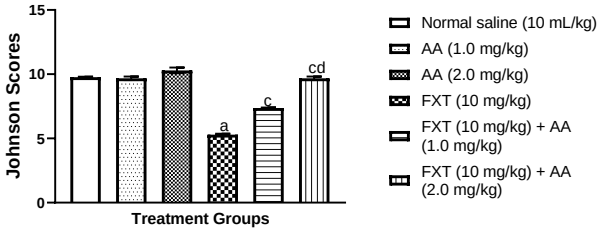


Figure 9. Effect of Arjunolic (AA) acid on fluoxetine (FXT)-induced decreased spermatogenesis scores in rats' testes

(Elsedawi, Hussein, Sabry & Aziz, 2021; Soliman, Mahmoud, Kefafy, Yassien & El-Roghy, 2017). However, our study focused on the therapeutic immunoprotective mechanism of Arjunolic acid (AA) as an anti-oxidant and anti-inflammatory agent against FXT-induced testicular toxicity.

Oral administration of FXT to male rats caused a significant reduction in testis weight, which can be attributed to the loss of germ cells and to testicular protein degradation (Solima *et al.*, 2017). Hence, testicular weight less than control values in these 28 days treated rats is probably due to the degeneration of germ cells, Sertoli cells, and decreased level of testosterone as these organs are androgen dependent (Oyovwi *et al.*, 2021b). In line with the previous observation, oral administration of FXT resulted in decreased testicular weight (Soliman *et al.*, 2017). In concordance to previous studies, highly significant reduction of body weight in FXT-treated rats was also observed in the current work (Hajizadeh, Soleimani Mehranjani, Najafi, Shariatzadeh, & Shalizar Jalali, 2016; Solima *et al.*, 2017). More interestingly, in the present study AA with FXT shows a marked significant improvement of body and testicular weights. These improvements could be attributed to its antioxidant effects.

In this study relative to previous studies, FXT treated rats show significantly decreased plasma levels of gonadotropins (FSH and LH) and steroidogenesis (testosterone) (Manna *et al.*, 2008). Spermatogenesis in rats requires a normal level of FSH and LH (Oyovwi *et al.*, 2021a). Notwithstanding, it has also been reported that the spermatogenic process is testosterone dependent and testosterone along with other testicular androgens can initiate and maintain spermatogenesis (Oyovwi *et al.*, 2021a). Therefore, the reduced testosterone level resulted in degeneration of germ cells and spermatogenic arrest.

Gonadal damage is a common adverse effect of many toxicants and chemotherapeutic drugs, affecting both the endocrine and exocrine functions of the testis (Emojevwe *et al.*, 2022; Oyovwi *et al.*, 2022). In the current study, FXT treatment resulted in reduced levels of kisspeptin and GnRH. This could be due to a shift in the hypothalamus nuclei that regulate these substances' releases. This may be the primary mechanism through which they cause reproductive disruption. Fortunately, AA was able to reverse FXT's neuro-endocrine pathway effects.

In agreement to previous findings (Beeder & Samplaski 2019; Tesi *et al.*, 2022), spermatotoxicity of FXT was evidenced by a decreasing percentage of sperm count, motility, viability and DNA fragmentation as well as increasing percentage of dead sperms and abnormal morphology of sperms in FXT treated rats with a concurrent

depletion in sperm count and motility. In contrast, AA therapeutic efficacy against spermatotoxicity was evaluated by the significant increase in testicular weight as earlier noticed, sperm count, viability, and motility. In supporting our present results, Manna *et al.* (2008) reported that AA attenuates testicular toxicity and spermatotoxicity induced by FXT exposure in male rats and this could be via its antioxidant and free radical scavenging effects.

Treatment with FXT leads to histopathological changes in the testes architecture (Hajizadeh *et al.*, 2016) as evidenced by a decreased Johnson score. However, co-treatment of FXT with AA was showed to cause a decline in the arrest of spermatogenesis and increased Johnson Score (JS). It could be suggested herein that AA improved the disrupted testicular structure through its antioxidant effects, thereby expressing positive impacts on spermatogenesis. FXT treatment caused an increase in frequency of chromosomal aberrations like aneuploids and sex-univalents in the testes. The total chromosomal aberrations were also significantly increased. However, AA co-treatment presents a reduction in chromosomal abnormalities.

In addition, the structure of both sperm membrane and acrosomal membrane integrity is dependent on HOST value and sialic acid concentration, so that a low concentration of sialic acid in seminal plasma may possibly negatively impact the structural integrity of both cell membrane and acrosome, thereby leading to changes in sperm motility and fertilizing ability (Yakubu, Akanji, & Oladiji, 2007). Meanwhile, in the present study, sialic acid level in the testis was altered in FXT-treated group. Changes in sialic acid concentration indicate altered androgen production and reduced spermatogenesis (Yakubu *et al.*, 2007). However, the significantly elevated level of testicular sialic acid was observed with AA treated rats in this study, clearly indicating that AA possesses the ability to reduce friction among the spermatozoa. It may also suggest enhancement in the structural sperm membrane integrity (as indexed by increased HOST value), which ultimately may stimulate the metabolism, motility and fertilizing capacity of spermatozoa (Oyovwi *et al.*, 2021b), thus demonstrating its possible protective function to maintain the structural integrity of the testes against testicular immuno-oxidative injury caused by AA.

Studies have shown that antidepressant drugs are more likely to cause some effects on the neuroendocrine system with manifestations of HPA-HPG axis dysfunction, such as hypercortisolism, decreased reproductive hormones and decreased spermatogenesis (Zhao, Zhang & Sun, 2021). In the present study, FXT was shown to cause hypercortisolism as evidenced by increased serum corticosterone. Furthermore, co-administration of FXT with AA at different doses for 28 days was able to reverse the effects of FXT on serum corticosterone level in rats. These findings suggest that the mechanisms involved in stress regulation can induce changes in neuroendocrine related factor expression. This may have consequences for neuronal survival, particularly in the hypothalamus and hippocampus, where both neuroendocrine related factors and corticosteroid receptors are respectively highly concentrated. In this context, it is of note that antidepressant FXT is reported to increase levels of serum corticosterone, which could be the result of the decreased neuroendocrine related factors (Kisspeptin and GnRH) and reproductive hormones.

## 5. Conclusions

In conclusion, co-administration of FXT with AA could contribute to the management of FXT-induced altered HPA-HPG-axis and hence possibly counteracts the stress-elicited decreased neuroendocrine related factors, reproductive hormones, spermatogenesis and testes chromosomal aberration, as well as increased tDFI and altered sperm chromatin integrity.

## Abbreviations

LH – luteinizing hormone
FSH- follicle stimulating hormone,
GnRH- gonadotropin releasing hormone,
tDFI- testicular DNA fragmentation index
tCSA- testicular chromosomal abberration,
HOST- hypo-osmotic swelling test,
TP- testicular protein,
HPA axis – hypothalamo-pituitary adrenal axis,
HPG axis – hypothalamo-pituitary gonadal axis

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