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The impact of *Annona muricata* and metformin on semen quality and hormonal profile in Arsenic trioxide-induced testicular dysfunction in male Wistar rats

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Abstract

Male Infertility has been an issue of significant concern affecting an appraised 20-30% of couples worldwide. The study aims to access the hormonal and semen profile following metformin and ethanolic leave extract of *Annona muricata* (ELAM) on arsenic-induced toxicity in Male Wistar rats. Thirty-male Wistar rats were used in the study, weighing 150-170 grams, and were divided into six groups of five rats each. Group A received 50 mg/kg of arsenic trioxide (As2O3) only. Group B received feed and water ad libitum. Groups C and D received 50mg/kg of As2O3 and were immediately treated with 30 mg/kg of metformin and 500 mg/kg of ELAM. Group E received 500mg/kg of ELAM and treated immediately with As2O3, and group F received 30 mg/kg of metformin and treated immediately with As2O3. Data obtained were subjected to ANOVA followed by post Hoc LSD using SPSS version 25. Semen quality, Testosterone, FSH, LH levels, and gonadal-somatic index were analysed. Values were presented as Mean±SEM and considered significant at $p<0.05$. The gonadal-somatic index revealed a significant decrease in group D, while groups B, C, E, and F had no significant decrease compared to A. As2O3 treated rats showed impaired sperm motility, morphology, and total sperm count significantly in groups A to B, while groups C, D, E, and F improved sperm motility, morphology, and total sperm count. Testosterone, LH, and FSH showed a significant decline in group A compared to B, except for LH. Groups C, D, E, and F showed a significant increase in testosterone and LH, except group C in FSH. The study concluded that MET and ELAM improved semen quality and hormonal profile, which could be used in the reversing and prevention of male infecundity linked to arsenic trioxide toxicity caused by oxidative stress.

Keywords: Arsenic trioxide; Metformin; *Annona muricata*; Male infertility; Semen quality; Oxidative stress

1. Introduction

Arsenic and its different isoforms such as trioxide, arsenite, or arsenate are a poisonous metalloid found in the environment, which causes global health challenges because of its carcinogenic nature (1). Exposure to arsenic forms results in biological dysfunction processes that have resulted in cardiovascular diseases, diabetes, neurocognitive outcomes, and reproductive dysfunction (2,3) linked to oxidative stress. Arsenic-associated male infertility is caused by oxidative stress, which results to altered hormonal profiles, increased oxidative stress, DNA damage, and metabolic issues connected to spermatogenesis and steroidogeneses (4), which acts through the activation of the ERK/AKT/NF-

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kβ pathway been the primary molecular mechanisms in male infertility following arsenic trioxide exposure (5,6). Its exposure to humans is through drinking water, highly industrialized activities, cosmetics, smoking, and other air sources (7). Infertility in males has increased across the globe, which is linked to significant decline in semen quality (truncated sperm count and sperm motility, as well as abnormal morphological structures) in about 2% of men (8– 10), which results from environmental toxicants such as cadmium and arsenic compounds E.t.c (11) following oxidative stress processes resulting to generation of free oxygen radicals such as reactive oxygen species (ROS). A decrease in the functionality of male sex hormones linked to oxidative stress processes are responsible for the discontinuity of species, which accounts for below 3% of infertility cases in males (12,13), resulting from endocrine disruptors and hormone assay for males is observed for the identification of causative factors and obtaining prognostic information. However, oxidative stress has been associated with the etiology of male infertility, which resulted to deleterious changes in spermatogenesis, epididymal maturation, and sperm capacitation (14) and increase oxidation of proteins and lipid DNA of the testicular membrane (15).

Spermatogenesis is the production of sperm cells, by which the germ cells produce haploid spermatozoa. Sperm is produced within the seminiferous tubules, a convoluted cluster of tubes located within the testes (16). The hypothalamic-pituitary-gonadal axis (HPGA) is a crucial regulator of testosterone and gonadal activities (17), which potentiates spermatogenic processes and sustenance of male reproductive function. Male steroidogenesis involves the conversion of cholesterol moiety to the production of male steroid hormones through complex biochemical pathways and involves several enzymatic pathways (18,19). However, cholesterol homeostasis is a significant factor in the male reproductive function, which is involved in the normal sperm production (19). The gonadal trophic releasing hormone-(GnRH) regulates the secretion of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) through the anterior portal system. Luteinizing hormone mainly acts on the Leydig cells to increase testosterone production, with testosterone having a limit on its secretion via negative feedback (20). Gonadal steroids and pituitary gonadotropins play a significant physiological role in establishing homeostatic mechanisms that maintain male reproductive functions (21,22).

Metformin (MET) is a known biguanide-derivative antidiabetic drug most widely used as an oral agent in humans (23,24), and used in the treatment of polycystic ovarian syndrome. It is a reliable drug used in treating tumor cells, aging cells, cardioprotective effect, and neuroprotective effect (25). Despite its antidiabetic activities, reports have shown that metformin reduces antioxidant levels of glutathione, superoxide dismutase, catalase, glutathione peroxidase, and glutathione-S- transferase (26,27), and improves spermatic function through enhancement of the activity of adenosine monophosphate kinase (AMPK) pathway (28,29). Medicinal plants are an excellent source of traditional medicines from which different modern medicines are developed (30,31). *Annona muricata* L. (Magnoliales: Annonaceae) is a tropical plant species known for its edible fruit, which has some medicinal advantages and some toxicological effects, such as, cardioprotective, and neurotoxic effect. The pharmacological actions of *Annona muricata* includes, anxiolytic, anti-stress, anti-inflammatory, contraceptive, anti-tumoral, antiulcer, wound healing, hepato-protective, anti-icteric, and hypoglycemic activities in-vivo study of the crude extracts and isolated compounds of A. muricata (32,33). Despite therapeutic effects of metformin and *Annona muricata*, there is dearth to literature on the effects of *Annona muricata* and metformin on semen quality and hormonal profile in Arsenic trioxide-induced testicular dysfunction in Wistar rats, which the study investigates.

2. Material and methods

2.1. Area of Study

The study was carried out in the Animal House, Department of Human Physiology, Faculty of Basic Medical Sciences, College of Health Sciences, Nnamdi Azikiwe University, Nnewi Campus.

2.2. Ethical Approval

Ethical approval was obtained from the Faculty of Basic Medical Science, College of Health Science, Nnamdi Azikiwe University, Nnewi campus. Rats handling and treatments conform to the National Institute of Health guidelines for laboratory animal care and use (34). The ethical approval number is NAU/CHS/NC/FMBS/477.

2.3. Materials

Arsenic Trioxide (MAY & BAKER LTD, DAGENHAM ENGLAND), Metformin Hydrochloride (SKG, Pharm Limited Nigeria), Ethanol (JHD Chemicals, Guangdong China), *Annona muricata* (Soursop) leaves, and 30-Male Wistar rats. S. Pyrex Beakers (Techmel, USA), Measuring cylinder (MINGHE), 2ml hypodermic syringe, weighing balance (CAMRY LB11),

Electronic weighing balance (M-Metlar M311L; China), Oral cannula, Slide, Microscope (Olympus XSZ-107BN), and Neubauer Counting Chamber (England). Centrifuge 90(1) (Alpin Medical, England), Plain Blood tube (Fantastik, China), Filter paper (Whatman Qualitative Filter Paper No. 1, Sigma Aldrich WHA1001042), Distilled water, Standard Plastic Cages and water can, Cotton wool (KENS LINT, Benin City, Nigeria), Latex Medical Hand gloves (Supermax Gloves, Selangor, Malaysia), Chloroform (Guondghuo, China), Vital feed grower (JOS, Nigeria), Dissecting kits, and UV-VIS 752N Spectrophotometer (Shanghai, Yoke Instrument Co., Ltd. China). Automatic Water distiller (SZ-1 Search Tech Instrument), Nexus Refrigerator, Rotary evaporator (Digital) TT-52 (Techmel & Techmel, USA), and Thermostat Oven (DHG-9023A, PEC MEDICAL USA).

2.4. Plant Procurement, Identification, and Extraction

Samples of *Annona muricata* (Soursop) leaves was harvested from a local farm in Okofia Community, Nnewi, Otolo Anambra State. The Department of Botany, Nnamdi Azikiwe University, Awka, Anambra State, identified the leaves, and the herbarium number was deposited in the herbarium catalogue. The dried leaves of *Annona muricata* (Soursop) was milled into a coarsely powdered form using a local grinder. Two hundred and fifty-(250) gram of the dried leaf was macerated in 1000 mls of 95% absolute ethanol for 48hours. It was filtered using a clean white cloth and further filtration using Whatman No 1 filter paper. The filtrate was concentrated using a rotatory evaporator and dried further using a laboratory oven at 45ºC into a gel-like form. The extract was preserved in airtight container and kept in a refrigerator for further usage. The extraction method was done with modifications as described according to the method employed by Al-Attar and Abu Zeid (35).

2.5. Experimental Animals and Design

Thirty-(30) male Wistar rats weighing 150-170 gram was obtained from the Animal House, Department of Physiology, Faculty of Basic Medical Sciences, College of Health Sciences, Nnamdi Azikiwe University, Nnewi Campus. Animals were kept in standard cages at a room temperature of 27±2°C. The animals were maintained with normal laboratory chow and water ad libitium. The animals were acclimatized for two weeks and before administering the ethanolic leaf extract of *Annona muricata* (ELAM), metformin, and Arsenic trioxide. The animals were kept on 12hours light and dark cycles. The animals were divided into six-groups of five animals per group as follows: Group A served as negative control (received 50 mg/kg of Arsenic trioxide only). Group B served as positive control (Animals received only distilled water and laboratory chow). Group C received 50 mg/kg of Arsenic trioxide for two weeks and was treated immediately with 30 mg/kg of metformin for 4-weeks. Group D received 50 mg/kg of Arsenic trioxide for two weeks and was treated immediately with 500 mg/kg of ELAM for 4- weeks. Group E received 500 mg/kg of ELAM for 4-weeks and was treated immediately with 50 mg/kg of Arsenic trioxide two-weeks. Group F received 30 mg/kg of Metformin 4-weeks and was treated immediately with 50 mg/kg of Arsenic trioxide two-weeks. All experimental protocols were observed under strict supervision, the experiment lasted for six-weeks, and administration was done through oral gavage.

2.6. Stock solution and Dosage Determination

Arsenic trioxide stock solution was obtained by dissolving 2-gram of arsenic trioxide in 100 mls of distilled water to obtain 20 mg/ml as stock solution. The stock solution of MET was obtained by dissolving 1000 mg of metformin in 20 mls of distilled to obtain 50 mg/ml. Lastly, ELAM stock was obtained by weighing 1gram of the gel extract and dissolved in 10 mls of distilled water to obtain 100 mg/ml. Arsenic trioxide administration equivalents of 50 mg/kg of arsenic trioxide 0.4 mls in groups B, C, D, E, and F. In contrast, 30 mg/kg of MET in groups C and F equivalent was 0.1 ml and 500 mg/kg of ELAM in groups D and E was 0.9 mls. Dosage in mls= Average weight of Animal X dosage in mg/kg divided by the stock solution.

2.7. Sample Collection

At the end of the experiment, animals in the different groups were anesthetized using chloroform in an enclosed container after 24-hours of the last administered dose of the ethanolic leaf extract of *Annona muricata*, metformin, and arsenic trioxide. Blood was collected from the animals using a heparinized capillary tube through ocular puncture as described by Parasuraman et al. (36). Blood obtained was put in a plain bottle, and it was allowed to cool, and centrifuged for 10-minutes at 3000 revolution per minute (rpm), after which the serum was retrieved using a micropipette. The retrieved serum was used to assay for hormonal profile (testosterone, FSH, and LH). Semen was obtained from the caudal end epididymal gland immediately and fixed in a cleaned, labeled slide.

2.8. Sperm Motility

Sperm cells was obtained from the epididymis' caudal end, placed in a clean glass slide, and mixed with a physiological solution of 990 µL (paraformaldehyde and sodium citrate) in the ration of 1-20. About 5.0 µL of supernatant containing

the sperm was placed between the slide and coverslip and observed at 100 x in a negative phase contrast microscopy (XSZ-107BN). The evaluation of the movement of the sperm was held in three different fields, and motility was expressed from the middle of the fields in the percentage of motile sperm of the total sperm counted (37).

2.9. Total Sperm Count

Approximately 10 µL of the diluted contents was transferred to hemocytometer (Neubauer chamber) and taken in light microscopy at 400 x. The pelleted cells were counted on the surface of the chamber. The sperm concentration calculation was performed according to the number of counted cells and hemocytometer dimensions. The concentration was expressed in millions of sperm per mL (37).

2.10. Sperm Morphology

About 20 µL of the sperm suspension was placed on the slides and swiped with 95% v/v ethanol for proper fixation for 5-10 minutes and was allowed to air-dry. The smear was washed with sodium bicarbonate solution (reagent no. 72) to remove any mucus, which may be present. The smear was rinsed with several changes of distilled water, thereafter, it was allowed to air-dry for two minutes and was covered with carbon fuchsin (1 in 20). It was allowed for staining for 3 minutes and wash off with distilled water. After that, the counterstain was done by covering the smear with dilute Loeffler's methylene blue (1 in 20) for 2 minutes; thereafter, it was allowed to dry and wash off with distilled water (37,38).

2.11. Testosterone Test Procedure

The serum retrieved was used to assay for testosterone levels in the different groups using the ELISA Method described by the Manufacturer's manual using the commercial ELISA based kits Monobind Inc. USA.

2.12. Principle and Method

The normal range of the standard in the Kits was standardized and then 25 µl serum samples was put in the well plates. Hundred-(100) µl of enzyme conjugate was added in each well. After that, it was left for incubation at 37°C in an incubator for 1 hour. Then, the wells were washed with 300 µl-distilled water for at least 3-times and blotted. Then, 100 µl TMB solution was added as a substrate in each well plate and was left for the incubation for 15 minutes for the colour changes. Finally, a 100 µl stop solution was added in each well to stop the reaction. Reading was taken at 630nm through Merck ELISA reader in ng/ml value.

2.13. Luteinizing Hormone and Follicle Stimulating Hormone Test

Serum samples retrieved was used to assay luteinizing hormone and FSH using the enzyme immunoassay (EIA) technique as described by the Manufacturer's manual using the commercial ELISA based kits Monobind Inc. USA.

2.14. Principle and Method

This involves a simple 3-step procedure that includes the following: incubation of standards and samples with the pre-coated antibody plates for 2 hours at 37°C; incubation with the peroxidase-labeled antibody for 1 hours at room temperature; and (3) colour development with TMB substrate. A linear dose-response curve was obtained in the range 0–10 ng/ml ($r^2 > 0.99$). Both hormones were measured using the EIA technique (39).

2.15. Statistical Analysis

Data obtained from this study was analyzed using Statistical Package for Social Sciences (SPSS) version 25 (IBM, USA, 2018). Data obtained serum hormonal profile (testosterone, FSH, and LH), gonado-somatic index, and semen quality (total sperm count, motility, and morphology) was analyzed using ANOVA followed by post hoc Fisher's Least Square Difference (LSD). Data was considered significant at $p \leq 0.05$.

3. Results

Table 1 Effect of ethanolic leaf extract of *Anonna muricata* and Metformin on sperm motility and total sperm count following Arsenic trioxide toxicity

	Active motility (%)	**None-motile (%)**	**Total sperm count (X10^6/mls)**
N= 5	MEAN±SEM	MEAN±SEM	MEAN±SEM
Group A (50 mg/kg of Arsenic only)	23.75±6.88	76.25±6.88	237.50±20.96
Group B (positive control)	82.50±4.33*	17.50±4.33*	536.25±96.81*
Group C (Arsenic + 30 mg/kg of MET)	77.50±4.79*	22.50±4.78*	565.25±77.44*
Group D (Arsenic + 500 mg/kg of ELAM)	65.00±6.45*	35.00±6.45*	462.50±96.87*
Group E (500 mg/kg of ELAM + 50mg/kg of Arsenic)	85.00±2.04*	15.00±2.04*	691.25±39.07*
Group F (30 mg/kg of MET + 50 mg/kg of Arsenic)	82.50±1.44*	17.50±1.44*	643.50±25.96*
F-Ratio	24.14	24.14	5.69

Data was analyzed using ANOVA followed by post Hoc LSD and values were considered significant at $p<0.05$. SEM: Standard error of mean, MET: Metformin, ELAM: ethanolic leaf extract of Anonna muricata, significant (*) and not significant (a). N= number of rats used

Table 1 result showed a significant decrease in the active sperm motility in-group A compared to B, groups C, D, E, and F had a significant increase in the active sperm motility when compared to group A. The non-motile sperm result indicated a significant increase in the non-motile sperm in- group A compared to B, groups C, D, E, and F had a significant decrease in the non-motile sperm when compared to group A. The total sperm count results showed a significant decrease in-group A compared to B, groups C, D, E, and F had a significant increase when compared to group A.

Table 2 Effect of ethanolic leaf extract of Anonna muricata and Metformin on sperm morphology following Arsenic trioxide toxicity

	Normal sperm cells (%)	**Abnormal sperm cells (%)**
N= 5	**MEAN±SEM**	**MEAN±SEM**
Group A (50 mg/kg of Arsenic only)	41.25±8.26	58.75±8.26
Group B (positive control)	86.25±1.25*	13.75±1.25*
Group C (Arsenic + 30 mg/kg of MET)	83.75±2.39*	16.25±2.39*
Group D (Arsenic + 500 mg/kg of ELAM)	82.50±4.79*	17.50±4.79*
Group E (500 mg/kg of ELAM + 50 mg/kg of Arsenic)	82.50±3.23*	17.50±3.23*
Group F (30 mg/kg of MET + 50 mg/kg of Arsenic)	80.00±4.08*	20.00±4.08*
F-Ratio	14.08	14.08

Data was analyzed using ANOVA followed by post LSD and values were considered significant at $p<0.05$. SEM: Standard error of mean, MET: Metformin, ELAM: ethanolic leaf extract of Anonna muricata, significant (*) and not significant (a)

Table 2 result revealed a significant decrease in the normal sperm cells in-group A compared to B, groups C, D, E, and F had a significant increase when compared to group A. The abnormal sperm cells result demonstrated a significant increase in the normal sperm cells in-group A compared to B, groups C, D, E, and F had a significant decrease when compared to group A.

Table 3 Effect of ethanolic leaf extract of *Anonna muricata* and Metformin on testosterone, luteinizing hormone, and follicle stimulating hormone following Arsenic trioxide toxicity

	Testosterone level (ng/ml)	**Luteinizing Hormone (mlU/ml)**	**Follicle stimulating Hormone (mlU/ml)**
N= 5	**MEAN±SEM**	**MEAN±SEM**	**MEAN±SEM**
Group A (50 mg/kg of Arsenic only)	0.45±0.10	0.28±0.10	0.65±0.12
Group B (positive control)	1.30±0.14*	0.87±0.17a	3.50±0.65*
Group C (Arsenic + 30 mg/kg of MET)	0.98±0.07*	1.60±0.32*	1.10±0.11a
Group D (Arsenic + 500 mg/kg of ELAM)	0.85±0.21*	1.67±0.39*	2.38±0.28*
Group E (500 mg/kg of ELAM + 50 mg/kg of Arsenic)	1.15±0.05*	2.15±0.03*	2.05±0.26*
Group F (30 mg/kg of MET +50 mg/kg of Arsenic)	1.45±0.03*	1.55±0.26*	1.60±0.17*
F-Ratio	9.47	7.36	9.88

Data was analyzed using ANOVA followed by post Hoc LSD and values were considered significant at $p<0.05$. SEM: Standard error of mean, MET: Metformin, ELAM: ethanolic leaf extract of Anonna muricata, significant (*) and not significant (a)

Table 3 result revealed a significant decrease in testosterone level in-group A compared to B, groups C, D, E, and F had significant increase compared to group A. The luteinizing hormone level showed an insignificant decrease in-group A compared to group B, groups C, D, E, and F had a significant increase compared to group A. The follicle stimulating hormone revealed a significant decrease in-group A compared to B, groups D, E, and F had significant surge, and group C had an insignificant increase compared to group A.

Table 4 Effect of ethanolic leaf extract of *Anonna muricata* and Metformin on gonado-testicular weight following Arsenic trioxide toxicity

Gonad testicular weight (g)	
N= 5	**MEAN±SEM**
Group A (50 mg/kg of Arsenic only)	0.93±0.03
Group B (positive control)	0.68±0.03*
Group C (Arsenic + 30 mg/kg of MET)	0.78±0.05a
Group D (Arsenic + 500 mg/kg of ELAM)	0.71±0.06*
Group E (500 mg/kg of ELAM + 50 mg/kg of Arsenic)	0.82±0.08a
Group F (30 mg/kg of MET + 50 mg/kg of Arsenic)	0.81±0.03a
F-Ratio	2.56

Data was analyzed using ANOVA followed by post Hoc LSD and values were considered significant at $p<0.05$. SEM: Standard error of mean, MET: Metformin, ELAM: ethanolic leaf extract of Anonna muricata, significant (*) and not significant (a)

Table 4 result demonstrated a significant increase in the gonado-testicular weight in-group A when compared to B, group D had a significant decrease; in contrast groups C, E, and F had an insignificant decrease compared to group A.

4. Discussion

Male infecundity has been linked to environmental toxicant exposure, which has contributed immensely to infertility in animal and human studies (40–42) resulting from oxidative stress processes. However, exposure to environmental carcinogenic substances or endocrine disruptors has been significantly associated with infertility and, thus, has a substantial influence on hormones of reproductive function in males, which alters spermatogenesis and impairs

glycolytic pathways and impairment of sperm capacitation (4,41,43) resulting from oxidative stress. Medicinal plants have shown significance in the management of reproductive abnormality, which results from their secondary metabolites (44) such as polyphenols, tannins, flavonoids, quercetin, Etc. The study investigates the hormonal and semen profile following metformin and ethanolic leave extract of *Annona muricata* (ELAM) on arsenic trioxide-induced toxicity in Male Wistar rats. Male fertility is characterized by sperm motility, total sperm count, and sperm morphology (normal and abnormal sperm cells) along with hormonal levels and histology of the testes (45,46), having an incomplete profile of these parameters in shape and function is termed infertility (47).

The study findings demonstrated a significant decrease in the active sperm motility in-group A compared to B; groups C, D, E, and F had a significant increase in active sperm motility when compared to group A. The non-motile sperm result indicated a significant increase in the non- motile sperm in-group A compared to B; however, groups C, D, E, and F had a significant decrease in the non-motile sperm when compared to group A. The study showed a significant decrease in active sperm motility and a significant increase in non-motile sperm cells following ingestion of arsenic trioxide; howbeit, the physiology linked to the decline in sperm motility is lipid peroxidation following arsenic accumulation in the testicular membrane (48,49) resulting from oxidative stress. Thus, this promotes the generation of reactive oxygen species, which causes significant changes in spermatic DNA activities and thus binds with thiol-tissue proteins and impaired sperm motility (50–52). The study findings are similar to the reports of Pant et al. (53); Chang et al. (54); Li et al. (55); Huang et al. (56); Bashandy et al. (57); Frenedoso Da Silva et al. (58); Zubair et al. (59); Daramola et al. (60); Lima et al. (61); Renu et al. (4); Barsøe et al. (62); Han et al. (63); Ilieva et al. (64) indicating a decline in active sperm motility and increase in non- motile sperm. Also revealed in the study is the attenuated effects of MET and ELAM in the ameliorative phase as indicated in groups C and D. The attenuated effect of MET is attributed to its ability to combat lipid peroxidation in the testicular membrane and its tissues as well, and the physiological mechanism of action by ELAM revealing a significant increase in active sperm motility and decrease in non-motile sperm levels is attributed to the presence of flavonoids and saponins, which combats redox formation by arsenic trioxide in the testicular tissue and spermatogenic process. Reports showed that *Annona muricata* improves sperm motility and non- motile sperm in testicular dysfunction caused by toxins, which supports the study's findings (65– 69). However, MET administration showed improvement in sperm motility following testicular dysfunction in infertile studies (70–73), which is in line with the study's outcome. Further, the preventive phase showed that both MET, and ELAM prevented arsenic toxicity and documented a significant increase in sperm motility. Also, ELAM has a more protective role than MET treatment, which indicates the presence of flavonoids and saponins' action attributing to their protective function of sperm motility.

The study's finding demonstrated a significant decrease in total sperm count in group A compared to B. At the same time, groups C, D, E, and F had a significant increase when compared to group A. Arsenic toxicity has been shown to affect sperm count through oxidative stress and compromise of the testicular DNA, thus, affecting the volume of sperm which is vital in fertilization process (49) acting through the ERK/AKT/NF-κ B-dependent signalling pathway. Reports have shown that arsenic ingestion indicates low sperm count (53,54,74–77), which corroborates the study findings. Thus, Treatment with MET and ELAM in both ameliorative and preventive phases (groups C and D; groups E and F) demonstrated a significant increase in the total sperm count. The mechanism of action linked to ELAM improved sperm count is flavonoids and saponins, which tend to repair tissue proteins and combat ROS formation in the testicular plasma membrane. The study has accordance with the reports of the study (65,66,68,78,79), revealing improved sperm count in testicular insult following *Annona muricata* extract, but contradict the findings of Oladipo et al. (80) indicating significantly lowered levels of total sperm count following *Annona muricata* bark extracts. However, MET treatment significantly improved total sperm count, which is linked to the decline in oxidative stress and lipid peroxidation, enhancement of 5'-AMP-activated protein kinase activity, and restoration to the expected levels of pituitary-gonadal hormones (28). The study corroborates the report of Attia et al. (81), Nna et al. (82,83), Pourheydar et al. (84), and Morgante et al. (85), revealing a significant increase in sperm count following metformin treatment in testicular dysfunction. The report of Zaidi et al. (86) is inconsistent with the study findings showing a non-significant difference in sperm count following MET administration in testicular dysfunction. Adaramoye et al. (87) indicated a significant decline in sperm count with respect to MET administration, which refutes the study findings.

The study demonstrated a significant decrease in the normal sperm cells in-group A compared to B, and groups C, D, E, and F significantly increased compared to group A. The abnormal sperm cells result demonstrated a significant increase in the normal sperm cells in-group A compared to B, and groups C, D, E, and F significantly decreased when compared to group A. The study showed that arsenic trioxide depleted sperm morphology following its ingestion, which is linked to lipid peroxidation and ROS generation suppressing the hypothalamic Gonadal axis function (88–90) acting through the AKT signalling pathway. The study is in line with (56,58–60,91–94), indicating a significant increase in abnormal sperm cells and significant decline in normal sperm cells. However, treatments with MET and ELAM in the ameliorative

and protective phase improve sperm morphology. Thus, the physiology linked to MET ameliorative and protective phase is associated with the enhancement of 5'-AMP-activated protein kinase activity and restoring expected levels of pituitary-gonadal hormones (28). Bosman et al. (95), Banihani (28), and McPherson and Lane (96) had a similar report to the study findings showing an improved sperm morphology following MET in testicular insult. The study disagrees with the report of Calle- Guisado et al. (97) and Naglaa et al. (98), indicating a significant decline in sperm morphology following MET administration. However, ELAM improvement in sperm morphology is linked to flavonoids and saponins, which help combat ROS formation and reduces lipid peroxidative processes caused by arsenic trioxide through the suppression of the HPG-Axis function. Ekaluo et al. (67), Adeleye et al. (65), and Gugun et al. (68) demonstrated an improved sperm morphology following ELAM administration against testicular toxicity, which corroborates the study findings. Oladipo et al. (80) findings disagree with the study report, which reported significantly lowered levels of sperm morphology with respect to ELAM ingestion.

Gonadotrophs secreted by the brain oversee male fertility, and their regulations have significance in the continuity of species (99,100). However, LH and FSH hormones are determinants of testosterone function in steroidogenesis (101). The study findings showed a significant decrease in testosterone levels in-group A compared to B; groups C, D, E, and F significantly increased compared to group A. The luteinizing hormone level showed an insignificant decrease in group A compared to group B; groups C, D, E, and F showed a significant increase compared to group A. The follicle-stimulating hormone revealed a significant decrease in group A compared to B, and groups D, E, and F had a significant surge. Group C had an insignificant increase compared to group A. The mechanism of action following the significant decline in testosterone and FSH activity after arsenic trioxide exposure is associated with an inhibition of the hypothalamic-pituitary axis, which caused changes in LH and FSH plasma concentrations. Thus, the decline of plasma LH could impair Leydig cell function and result in a consequent reduction in testosterone production (1,102). However, the LH decline after arsenic trioxide exposure is poorly understood, but it suggests its impact on germ cells, which alters spermatogenic processes through ROS generation (59). The study agrees with the reports of Chiou et al. (91), Zubair et al. (59), Kumar et al. (103), Ali et al. (104), Huang et al. (56), Mehrzadi et al. (105), Guvvala et al. (49) demonstrating a significant decline in testosterone level after arsenic trioxide exposure. Further, the study indicated a significant increase in the testosterone level following treatment with MET and ELAM in ameliorative and preventive phases. Thus, the mechanism of action linked to MET is poorly understood. The study findings correspond with the works of Fernández-García et al. (106) and Nasrolahi et al. (107), revealing a significant increase in testosterone levels after MET treatment in testicular toxicity. Also, the report refutes the findings of Naglaa et al. (98) Cai et al. (108) Hu et al. (109), and Tseng (110), revealing a significant decline in testosterone levels following MET in testicular injury. Further, the physiology behind the significant increase in testosterone levels after ELAM administration is linked to the presence of flavonoids and saponins contents. Thus, the study is in line with the report of Cristopher et al. (111), Alsenosy et al. (112), and Anacletus et al. (113) revealing a significant upsurge in testosterone levels following a testicular injury after treatment with *Annona muricata* leaves. Furthermore, the study revealed that arsenic trioxide showed an insignificant decline in LH activity; but indicated a significant increase in the ameliorative and preventive phase following MET and ELAM administration. Thus, the study disagrees with the report of Ali et al. (104), who showed that arsenic caused a significant increase in LH hormone. Also, the result of Zhang and Tang (114), Jana et al. (102), Saberi-Sis and Zargari (115), and Zubair et al. (116) showed a significant decline in LH activity following arsenic exposure, which refutes the study. Administration of MET indicated a significant increase in LH activity in the ameliorative and protective phase; further, its physiology is linked reduction of lipid peroxidation, which alters the hypothalamic gonado axis regulating gonadotrophs production. The study corresponds to the findings of Safiah et al. (117), demonstrating a significant increase in LH activity following MET administration in testicular injury. Also, Krysiak et al. (118) showed a significant decline in the LH level, which disagree with the study findings following MET treatment in hypergonadotropic hypogonadism. However, ELAM revealed a significant increase in LH activity, which is linked to the presence of flavonoids, thus, combating ROS formation and reducing its impact on LH activity and favouring spermatogenesis.

The study indicates that the FSH hormone following arsenic trioxide intoxication showed a significant decline, which is linked to oxidative stress and releasing of ROS, which suppresses the HPA-gonadal axis in the hypothalamus. The study is in line with the report of Erkan et al. (41), Renu et al. (4), Zubair et al. (116), and Zubair et al. (119) following arsenic intoxication revealing a significant decline in FSH levels. However, Zhang and Tang (114) reported a non-significant decline in FSH level, which refutes the study findings. Also, Gunduzoz et al. (120) and Chen et al. (121) documented a significant increase in FSH levels following arsenic intoxication, which disagrees with the study outcome. The study showed that MET and ELAM increased FSH levels in both phases, but MET had an insignificant increase; however, an exception was seen in the ameliorative phase. The mechanism of action is not well understood. However, findings showed that MET administration indicates a significant decline in FSH levels, which disagrees with the study outcome (122). Jindal et al. (123) and Oride et al. (124) had similar findings to the study's outcome revealing a non-significant change in FSH level following MET. However, the preventive phase showed a significant increase in FSH levels, which is linked to the reduction of lipid peroxidation and improved HPA-axis regulating gonadotroph production. The study

disagrees with the report of Tosca et al. (125), indicating a significant reduction of FSH levels following MET. Furthermore, ELAM significantly increased the FSH levels in the ameliorative and protective phase, which has its physiology linked to combating ROS formation attached to the flavonoids and saponins. Onyegeme-Okerenta et al. (126) and Olowofolahan et al. (127) reported a significant decline in FSH level following *Annona muricata* in reproductive dysfunction, which disagree with the study outcome. The study corroborates the findings of Abd El-Monem and Elwakeel (128) and El-Sawi et al. (129), revealing a significantly higher level of FSH following *Annona muricata* extract against testicular toxicity.

Organ toxicity has been linked to the route of administration to different environmental toxins or endocrine disruptors, which is linked to male dysfunction leading to infertility characterized by other parameters of fertility (130,131) resulting from oxidative stress. The study findings showed a significant decrease in the gonado-testicular weight in-group A when compared to B. However, group D had a significant increase; in contrast, groups C, E, and F had a non-significant decrease compared to group A. The significant increase in the gonado-testicular weight is linked to an increase in lipid peroxidation, which resulted in the generation of reactive oxygen species (ROS) in association with arsenic trioxide (132). Also, it is directly linked to testicular function by binding to thiol proteins to elicit its action on the gonads, either having an increased weight or reduced weight of testes. Pant et al. (53), Jana et al. (102), Chang et al. (54), Zubair et al. (119), Frenedoso Da Silva et al. (58), Mamoun et al. (133), Liu et al. (134), Olfati and Tvrda, (135) showed disagreement with the study report, which arsenic indicated a significant reduction in testicular weight.

Further, the study inferred that ELAM showed a significant decline in the gonado-testicular weight in the ameliorative phase, and metformin had no significance. The physiology that is linked to improved gonado-testicular weight after arsenic trioxide intoxication following ELAM treatment results from the presence of flavonoids and saponins, which antagonizes ROS production. Ekaluo et al. (136) indicated an improved testicular weight significantly, as revealed by *Annona muricata* against caffeine toxicity, which is in line with the study results. Nweke and Akpuaka (137) findings disagree with the study outcome indicating a significant increase in testicular weight concerning *Annona muricata* extract. Ihejieto et al. (138) showed a significant decrease in testicular weight following *Annona muricata* in prostatic cancer rats, which agrees with the study findings. Liu et al. (139) report is in line with the study result following metformin administration in a high-fat diet indicating a non-significance difference in relative testicular weight. Yan et al. (70) showed no consistency with the study report indicating that metformin significantly increased testicular weight in obese rats. Wang et al. (73) indicated that MET had an insignificant increase in the testicular index in triptolide toxicity, which refutes the study outcome. Pourheydar et al. (84) showed a significant increase in testicular weight in MET treated diabetic model, which contradicts the study findings. The preventive phase demonstrated an insignificant decrease in the gonado testicular weight in groups E and F following treatment with metformin and ELAM, as well as arsenic trioxide. The physiology linked to the changes needs to be better understood, suggesting the presence of flavonoids and saponins in ELAM. However, the reason(s) for the metformin activity on the gonado-testicular weight is poorly understood.

5. Conclusion

The study showed that Metformin and ethanolic leaf extract of *Annona muricata* could be used as an anti-infertility agent in the management of testicular dysfunction caused by arsenic trioxide. Further, the study indicated an improved ameliorative and protective impact on semen quality and hormonal levels following metformin and ethanolic leaf extract of *Annona muricata* on arsenic trioxide testicular dysfunction.

Compliance with ethical standards

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Disclosure of conflict of interest

There was no conflict among the authors.

Statement of ethical approval

The ethical committee of Faculty of Basic Medical Sciences, Nnamdi Azikiwe University, Nnewi, Campus gave approval for the commencement after the authors fully met the requirement of the study, and was monitored to make sure all protocols were followed sequentially. An approval number for the commencement of the work was documented by the Head of the ethical committee for reference purposes as stated above in 2.2.

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