INVOLVEMENT OF ZINC-BASED MECHANISM IN THE AMELIORATION OF PSYCHOLOGICAL STRESS-INDUCED TESTICULAR REDOX IMBALANCE BY *MASSULARIA ACUMINATA*

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Abstract

Testicular redox balance is critical to spermatogenesis, and testicular steroidogenesis and evidence suggest that psychological may offset this balance leading to impaired testicular function. This study was designed to evaluate whether *Massularia acuminata* has the potential to maintain testicular redox balance and by what mechanism. In a series of three experiments, aqueous stem extract of *M. acuminata* (*ASEMA*), at a dose of 50mg/Kg body weight and Zn at a dose of 10mg/Kg body, was administered to rats at the baseline, under psychological stress, and to rat treated with dexamethasone (DX) either for 7 or 14 days. Following euthanasia, the testes and epididymides were recovered, cleaned of fat, and weighed. From each rat, 0.5g was cut from the left testis and placed in ice-cold 0.25 M sucrose solution where it was homogenized immediately and centrifuged at 2000 ×g for 15 minutes. The supernatant obtained was stored at -20^{o}C until assayed within two weeks for markers of redox status. Analysis of data obtained shows that *ASEMA* does not have significant effect on testicular redox status at the baseline. During stress, however, animals treated with *ASEMA* and Zn exhibited significantly higher ($p > 0.05$) level of SOD, GSH and CAT compared with animals that received only saline. In addition, while animals treated with DX and saline had significantly lower level of testicular Zn concentration, ASEMA-treated group significantly higher testicular Zn concentration compared with baseline saline group. Therefore, it can be concluded that ASEMA has the potential to maintain testicular redox balance during exposure to psychology stress by maintaining Zn homeostasis during stress.

Keywords: Testis, Redox balance, Zinc, Anti-oxidants, Psychological stress

Introduction

Psychological stress has become one of the recognized etiologies of male reproductive dysfunction (Pook *et al*., 2004). With accumulating data, most reproductive epidemiologists now agree that psychosocial stress is relevant to the etiology of mammalian (and particularly male) reproductive dysfunction (Monga *et al*., 2004). Incidentally, it has been discovered that infertility and psychosocial stress are mutually inductive (Pook *et al*., 2004; Monga *et al*., 2004; Levy *et al*., 2006) This explains why infertile couples that attend fertility clinics report elevated level of stress, which is believed to consequently reduce their chances of conception (Pook *et al*., 2004).

There is an ongoing concern as well as controversies about the global decrease in male reproductive capacity evidenced by the deteriorating human semen quality (Jozkow and Mędras 2012). But regardless of lack of consensus on how and why male reproductive function is being compromised, the human spermatozoon is suspected to be in an oxidative stress-induced crisis (Aitken 1999) and it is now known that psychological stress is a risk factor for oxidative stress (Teague *et al*., 2007).

Earlier studies in humans have shown that psychosocial stress similar to predator stress has been observed in troops and civilians in war-torn regions of the world (Kobeissi *et al*., 2008). This observation was inferred to have resulted from constant threats of attack, sleep deprivation, and physical exertions. The use of herbs in the treatment of male infertility remains a common practice in many parts of the developing world; and while some of the results can hardly be denied, the mechanisms by which some of the phytotherapies produce their effects remain poorly understood. For instance, the use of aqueous stem extract of *Massularia acuminata* (ASEMA) for enhancement of male fertility is common in Southwestern Nigeria. However, there are few scientific studies (Yakubu *et al*., 2008) that have confirmed the androgenic potentials of ASEMA but they come short of definitive mechanisms by which ASEMA effects its action on the male reproductive function. The putative beneficial effect *ASEMA* on testicular biology during stress is therefore investigated in the present study.

Materials and Methods

Plant Material and Extraction

Fresh stems of *M. acuminata* were obtained from a vendor in Ilorin and were botanically identified at the herbarium of the Department of Plant Biology, University of Ilorin Aqueous stem extract of *M. acuminata* (ASEMA) was made as described by Yakubu *et al*.,(2008) with slight modifications. Precisely, tiny flakes

were made from the stem with the aid of a grater. The flakes were then ground using a blender to obtain a thoroughly pulverized material weighing 1.74 kg that was then soaked in 1L of distilled water for 72 hours. Following the 72 hours of soaking, the mixture was triple-filtered with pure white cotton. The filtrate was then oven-dried at 40^{o}C to obtain a brownish substance, which is the aqueous stem extract of *M. acuminata* (*ASEMA*). The extract was stored air-tight at room temperature until used. Three experiments comprising three groups each were performed in this and throughout the study a group of animal comprises of six (6) rats and where applicable *ASEMA* was orally administered with the aid of oral cannula at a dose of 50 mg/kg body weight and Zn at 10mg/Kg body weight (Yakubu *et al*., 2008).

Experiment I: The Baseline Studies

Experiment I which was described as the baseline studies involved studying the effect of administration of *ASEMA* and Zn on pro- and ant-oxidants status of the testis. There were three groups in this experiment. The first group received 1ml/kg body weight of saline for 7-14 days and thus served as the control. The second group identified as the *ASEMA* baseline group received 50 mg/kg body weight of *ASEMA* for 7- 14 days while the third group, known as the Zn baseline group received 10 mg/kg body weight of zinc sulphate for 7- 14 days.

Experiment II: The Stress Studies

Experiment II, also known as the stress studies, was designed to determine whether Experiment I can modulate the effect of psychological stress on male rat reproductive biology by layering exposure to psychological stress on Experiment I. To do this, rats were randomly assigned to three groups as in Experiment I and treated in like manner but further subjected to psychological stress. In details, the first group in this experiment, which served as the control for Experiment II was stress-matched with the other group within Experiment II but received only saline and it is thus referred to as the saline stress group. The second group designated as the *ASEMA* stress group was orally pretreated with 50 mg/Kg body weight of *ASEMA* 12 hours before exposure to stress. The third group, the Zn stress group, was given 10 mg Zn /kg body weight 12 hours before being subjected to stress.

Experiment III: The Glucocorticoid Studies

Experiment III, also known as glucocorticoid studies, was set up to directly manipulate the level of glucocorticoid in the rats to ascertain whether the effects observed in experiment II were due solely to stress-induced elevated glucocorticoid. This became necessary to validate whether Zn reduction is secondary to increase in glucocorticoid or independent of it during exposure to

psychological stress. To accomplish this, stress exposure was subtracted from Experiment II and replaced with (DX) loading at 10 mg of dexamethasone/Kg body weight according to Yazawa *et at* (2001). Hence, animals in Experiment III were randomly divided into three groups. The control for Experiment III was the group called saline DX group and was given saline at 12 hours before glucocorticoid administration. The second group called *ASEMA* DX group was given *ASEMA* 12 hours before treatment with DX at 0900 GMT while the third group, the DX Zn group received Zn 12 hours before treatment with DX.

Stress Induction

The method used for induction of psychological stress in rat has been previously and variously described. However, the procedure used in this study is as described by Figueiredo *et al.* (2003). To induce psychological stress in rats, the cage containing the rats was placed inside the cat cage and the cat left in the cage in a closet throughout the stress session. For the purpose of this investigation, a stress session consisted of a procedure for inducing psychological stress in rats by exposure of rats to cat for 60 minutes per day. Efforts were made to ensure that all rats had the same handling and cage experience to eliminate the effect of handling, cage, and novel environment on differences between groups.

Euthanasia and Collection of Biological Samples

All animals were euthanized by cervical dislocation and, on each one; dissection was immediately made through the anterior thoracic wall to sufficiently expose the heart from where blood was collected through left ventricular cardiac puncture. The left testes were harvested and used for oxidative stress evaluation. Precisely, each harvested testis was quickly weighed and. And 0.5g was cut and placed in ice-cold 0.25 M sucrose solution where it was homogenized immediately and centrifuged at 2000 ×g for 15 minutes. The supernatant obtained was stored at -20^{o}C until assayed within two weeks.

Quantitative Determination of Corticosterone MDA, SOD, CAT, GSH, and Zn

Malondialdehyde (MDA) is a reliable proxy index for lipid peroxidation according to Draper (1990). The method of Mihara and Uchiyama (1978) was employed in this study for the determination of MDA. Specifically, 1.0 ml of each testicular sample was combined with 2.0 ml of solution composed of 15% TCA, 0.4% thiobarbituric acid (TBA), and 2.5% HCL and heated for 15 minutes in a water bath at 40^{o}C. After cooling, the flocculent precipitate was removed by centrifugation at 10000 rpm for 10 minutes, the absorbance of the sample was determined at 535 nm. The level of activity of the superoxide dismutase (SOD) enzyme was determined

as previously described (Sun and Zigman 1978). The method was based on the ability of SOD to inhibit the auto-oxidation of epinephrine as determined by the increase in absorbance at 320 nm. The level of activity of catalase (CAT) was determined as described elsewhere (Aebi 1984). Catalase degrades hydrogen peroxide to water and oxygen, which was measured directly by the decrease in the absorbance at 240 nm. GSH was determined using 5, 5'-dithiobis (2-nitrobenzoic acid). The concentration of zinc in seminal plasma was determined calorimetrically as previous described (Makino *et al*., 1982). Corticosterone was determined with aid of ELISA kit (Monobind, California, USA) according to the manufacturer instructions.

2.4.2 Quantitative Determination of Elements in *ASEMA*

Determination of minerals/elements in *ASEMA* was according to the procedure in Official Methods of Analysis of AOAC (1990) using 2 g of *ASEMA*. The principle of this method involves dissolving organic matrix by dry ash in muffle furnace and the analyte determined by atomic absorption spectrophometry. Briefly, 2 g of *ASEMA* was placed in clean Vycor evaporating dish and aliquot dried in microwave oven programmed over 30 minutes. The ash obtained was dissolved in 5 mL of 1M HNO_3 and warmed over steam hot plate for about 3 minutes to form a solution. The solution was added to 50 mL volumetric flask and repeated with additional portion of 1M HNO_3. Standard solution for each of the elements was then added to the final solution for the determination of each element.

2.6 Statistical Analysis

Statistical analysis was done with GraphPad Prism 5 for Windows (GraphPad Software, San Diego California USA). Statistical significance was set at $P<0.05$ following two-way analysis of variance with Bonferroni's post test. Results are presented as mean ± standard error mean (SEM).

3. Results

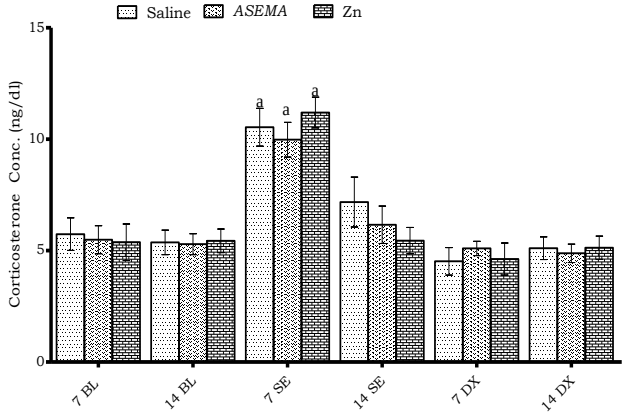

Figure 1: Serum corticosterone levels in the baseline (BL), stress exposed (SE) and DX treated (DX) rats. Only bars with 'a' are significantly different from all other bars with no alphabets at P< 0.05.

In Experiment I (baselines studies), the circulating level of corticosterone did not differ statistically (P<0.05) in saline-, *ASEMA*- and Zn-pretreated rats (Figure 1). In Experiment II (stress studies), circulating levels of corticosterone in animals that had 7 stress exposures was significantly (P<0.05) higher than those that had 14 stress exposures. After seven stress exposures, corticosterone level in *ASEMA*-pretreated rats was the lowest compared with Saline-pretreated and Zn-pretreated) rats. In animals that had fourteen stress exposures, following pretreatment with saline, *ASEMA* and Zn, the levels of corticosterone were not significantly different (at P<0.05). Corticosterone level was not elevated in all the three groups of Experiment III.

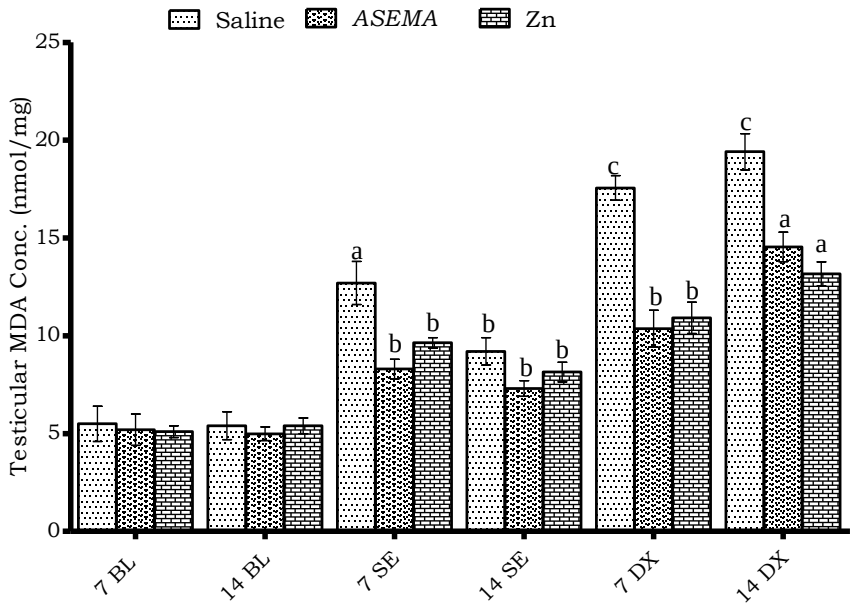

Figure 2: Testicular MDA in the baseline (BL), stress exposed (SE) and DX treated (DX) rats. Bars with different alphabets are significantly different from each other and from bars with no alphabets at P< 0.05.

As shown in figure 2, in Experiment I (baseline studies), testicular MDA was insignificantly (P>0.05) lower in *ASEMA*-pretreated and Zn-pretreated groups compared with saline pretreated group (Figure 3). In Experiment II (stress studies), after seven stress sessions, MDA concentration in the testis was significantly (P<0.05) higher in both the saline-pretreated (12.70±1.10ng/dl) and Zn-pretreated rats compared with *ASEMA*-pretreated rats. The Level of MDA concentration in Zn-pretreated rats was, however, significant lower compared with saline-pretreated rats. In Experiment III (glucocorticoid studies), level of MDA concentrations in animals that received either 7 or 14 administrations of DX followed the same pattern. In the groups that received 7 DX administrations, testicular MDA level were only significantly higher in saline-pretreated group compared with *ASEMA*- and Zn-pretreated groups. Similarly, after 14 DX administrations, MDA level was only significantly (P<0.05) higher in saline-pretreated group compared with *ASEMA*- and Zn-pretreated groups. Inter-experimental comparison shows that testicular MDA concentrations was significantly (P>0.05) higher in the groups of animals in Experiment III compared with Experiment II and I.

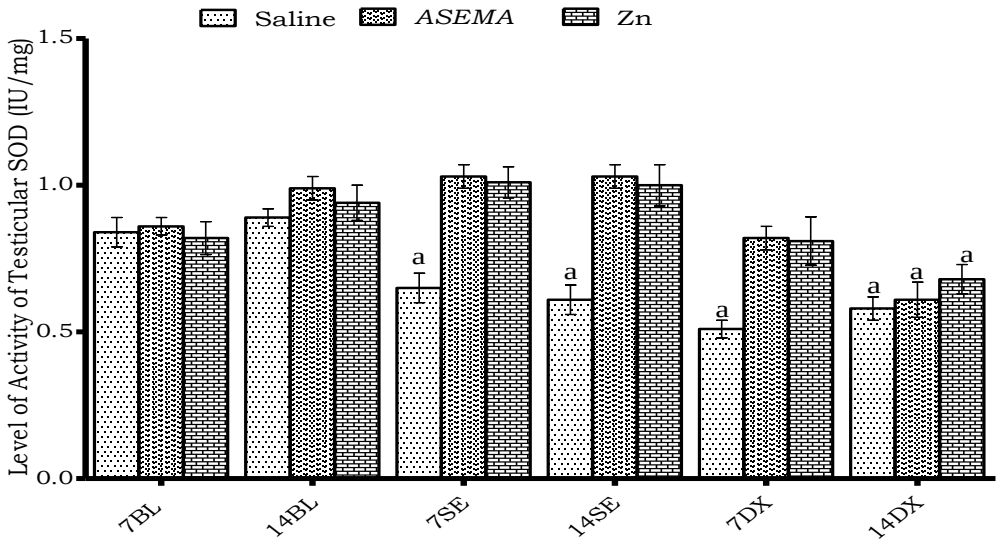

Figure 3: Testicular SOD level of activity in the baseline (BL), stress exposed (SE) and DX treated (DX) rats. Bars with different alphabet 'a' are significantly different from other from bars with no alphabets at P< 0.05.

As shown figure 3, in Experiment I (baseline studies), testicular SOD activity was significantly (P<0.05) higher in *ASEMA*-pretreated compared with saline-pretreated and Zn-pretreated groups. In Experiment II (stress studies) SOD activity also followed the same pattern irrespective of the number of stress exposures. After seven stress exposures, testicular SOD activity was significantly (P<0.05) lower in saline-pretreated animals compared with *ASEMA*- and Zn-pretreated animals. Similarly, in animals that had fourteen stress exposures, SOD activity in the testis was significantly (P<0.05) depressed in saline-pretreated animals compared with *ASEMA*- and Zn-pretreated animals. In Experiment III (glucocorticoid studies), the pattern of SOD activity was similar irrespective of the number of DX administrations. After seven DX administrations, testicular SOD activity was significantly (P<0.05) lower in both saline-pretreated and *ASEMA*-pretreated animals compared with Zn-pretreated animals. Similarly, after fourteen DX administrations, SOD activity in the testis was significantly (P<0.05) lower in both saline-pretreated and *ASEMA*-pretreated animals compared with Zn-pretreated animals. Inter-Experimental comparison revealed that SOD activity in the testis was significantly (P<0.05) depressed in the groups of animals in Experiment III compared with Experiment II and the baselines (Experiment I).

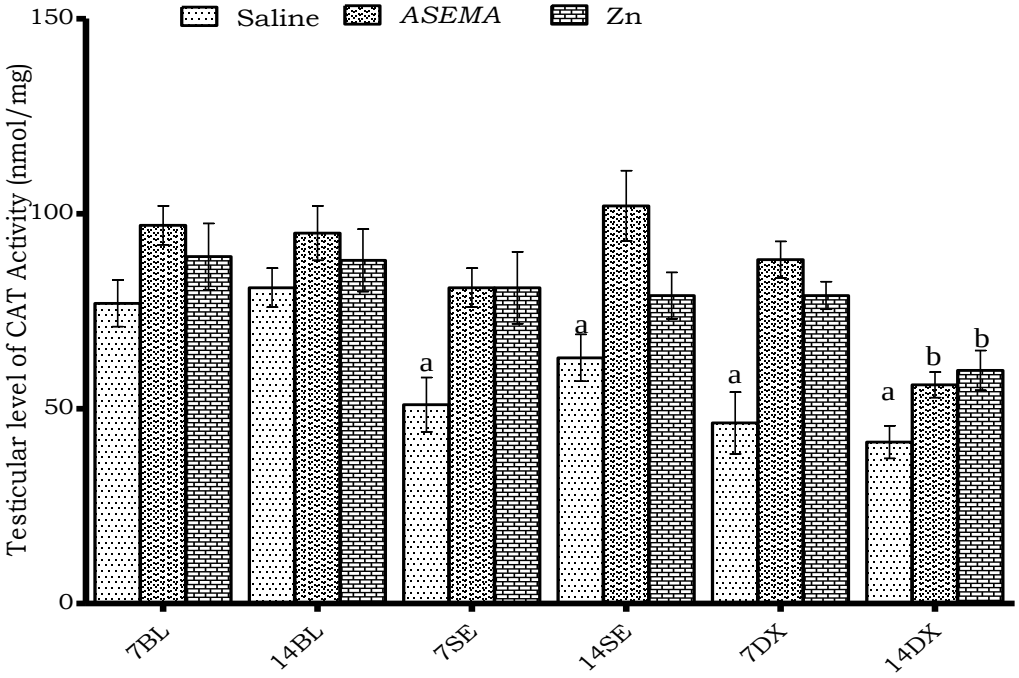

Figure 4: Testicular CAT level of activity in the baseline (BL), stress exposed (SE) and DX treated (DX) rats. Bars with different alphabets are significantly different from each other and from bars with no alphabets at P< 0.05.

As shown in figure 4, the level of testicular CAT activity following chronic exposure to either psychological stress or DX treatment. In Experiment I (baseline studies), testicular CAT activity was significantly (P<0.05) higher in *ASEMA*-pretreated compared with saline-pretreated and Zn-pretreated groups. In Experiment II (stress studies), after a single stress exposure, CAT activity in the testis was significantly (P<0.05) depressed in saline-pretreated and Zn-pretreated (81.00±6.90nmol/mg) groups compared to *ASEMA*-pretreated group. Similarly, in the groups of animals that had three stress exposures, testicular CAT was significantly (P<0.05) depressed in saline-pretreated and Zn-pretreated groups compared to *ASEMA*-pretreated group. Compared with those that had a single stress exposure, testicular CAT activity was significantly depressed in those that had three stress exposures. In Experiment III (glucocorticoid studies), CAT activity in the testis was significantly (P<0.05) depressed in saline-pretreated animals compared with *ASEMA*- and Zn-pretreated animals after a single administration with DX. CAT activity in the testis of animals pretreated with *ASEMA* was also significantly (P>0.05) lower than Zn-pretreated animals. But after three administrations with

DX, testicular CAT activity was only significantly (P<0.05) depressed in saline-pretreated animals compared with *ASEMA*-) and Zn-pretreated animals.

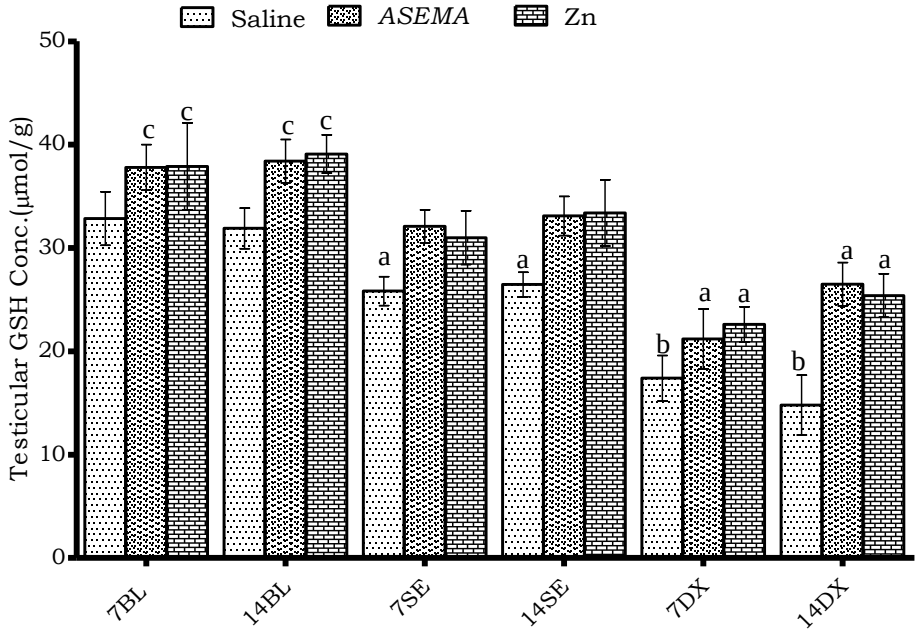

Figure 5: Testicular GSH level of activity in the baseline (BL), stress exposed (SE) and DX treated (DX) rats. Only bars with different alphabets are significantly different from each other and from bars with no alphabets at P< 0.05.

As shown in figure 5, in Experiment I (baseline studies), testicular GSH concentration is not significantly (P>0.05) different in saline-pretreated, *ASEMA*-pretreated and Zn-pretreated groups irrespective of whether they received seven or fourteen doses of normal saline respectively. In Experiment II (stress studies), after seven stress exposure, GSH concentration in the testis was significantly (P<0.05) lower in saline-pretreated group compared with *ASEMA*-pretreated and Zn-pretreated groups. Similarly, in the groups of animals that had fourteen stress exposures, testicular GSH concentration was significantly (P<0.05) lower in saline-pretreated group compared with *ASEMA*-pretreated and Zn-pretreated groups. In Experiment III (glucocorticoid studies), concentration of glutathione in the testis was significantly (P<0.05) depressed in saline-pretreated animals compared with *ASEMA*- and Zn-pretreated animals after seven administration with DX. However, after fourteen administrations with DX, testicular GSH concentration was not significantly (P>0.05) different from each other in saline-pretreated, *ASEMA*-pretreated and Zn-pretreated animals.

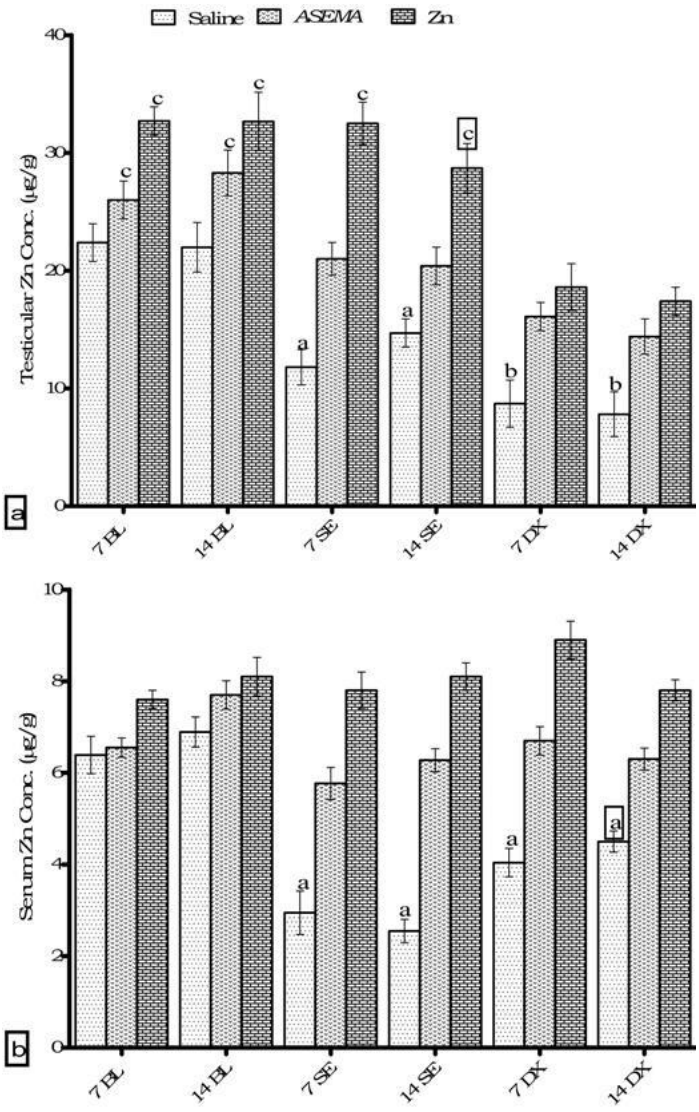

Figure 6: Serum and testicular Zn concentration the baseline (BL), stress exposed (SE) and DX treated (DX) rats. Only bars with different alphabets are significantly different from each other and from bars with no alphabets at P< 0.05.

As shown in 6, in Experiment I, testicular Zn concentration is not significantly (P>0.05) different in saline-pretreated, *ASEMA*-pretreated and Zn-pretreated groups irrespective of whether they received seven or fourteen doses of normal saline respectively (Figure 4). In Experiment II (stress studies), after seven stress exposure, testicular Zn concentration was significantly (P<0.05) lower in saline-pretreated group compared with *ASEMA*-pretreated and Zn-pretreated groups.

Similarly, in the groups of animals that had fourteen stress exposures, testicular Zn concentration was significantly ($P<0.05$) lower in saline-pretreated group compared with *ASEMA*-pretreated and Zn-pretreated groups. In Experiment III (glucocorticoid studies), testicular Zn concentration was significantly ($P<0.05$) lower in saline-pretreated animals compared with *ASEMA*- and Zn-pretreated animals after seven administration with DX. Similarly, after fourteen administrations with DX, testicular Zn concentration was significantly ($P<0.05$) lower in saline-pretreated animals compared with *ASEMA*- and Zn-pretreated animals. However, testicular Zn concentration in Zn-pretreated animals, though significantly ($P<0.05$) higher than *ASEMA*-pretreated animals, was significantly ($P<0.05$) lower than Zn-pretreated animals in the baseline studies Inter-Experimental comparison revealed that serum Zn concentration was only significantly ($P<0.05$) lower in the saline-pretreated groups of animals in Experiment III and Experiment II compared with the baselines (Experiment I). Serum Zn concentrations as shown in figure 6b follows a similar pattern to testicular Zn concentration.

Table 1: Quantity of Elements in *ASEMA*

Elements	Conc. (mg/g $\times 10^{-3}$)
Ca	212.45±10.73
Fe	127.03±5.91
Mg	77.03±6.45
K	42.28±3.02
Zn	40.28±3.76
Na	17.03±1.49
Mn	9.45±1.02
Cu	8.40±0.05
Cr	2.50±0.01
Ni	1.00±0.01

Table 1 reveals 10 elements present in ASEMA as well as their concentrations. Calcium (Ca) is the most abundant elements followed by iron, (Fe), Magnesium (Mg) potassium (K), and zinc (Zn). Sodium (Na), and manganese (Mn), copper (Cu), chromium (Cr) and nickel (Ni) are also present in ASEMA.

4. Discussion and Conclusion

4.1 Discussion

Beginning from baseline studies in Experiment I, it was observed that corticosterone levels in the *ASEMA* baseline group and Zn baseline group remained

statistically similar with corticosterone level in the saline baseline group. This is taken as evidence that neither *ASEMA* nor Zn administration, under normal conditions, possesses any significant influence on the HPA axis (Fig 1). The establishment of this point in the baseline studies of Experiment I is crucial to the laboratory interventional application of *ASEMA* or Zn in the stress groups of Experiment II. Physiologically intact HPA axis is critical to the maintenance of functional male reproductive biology (Whirledge and Cidlowski 2010); and since neither *ASEMA* nor Zn essentially has any affect on this intactness in unstressed animals, the justification for using them in the stressed groups as is thus established. An initial and interesting observation from Experiment II is the fact that the groups of animals pretreated with either *ASEMA* or Zn exhibited statistically similar level of stress reactivity compared with stress-matched saline pretreated animals (Fig 1). This observation is significant in that it demonstrates that: One, animals under the current stress paradigm continued to produce hormonal signal for stress which indicates lack of capacity to become desensitized to the unwanted and unwarranted presence of predator. Two, this observation, shows that pretreatment with either *ASEMA* or Zn does not necessarily prevent rats from exhibiting glucocorticoid stress response during the seven days of coupling stress exposure with the pretreatment with the extract or Zn. This evidently suggest the possibility that whatever modulatory effects produced by pretreatment with *ASEMA* or Zn involve mechanism(s) that could proceed in spite of stress-induced elevated glucocorticoids. Deciphering the mechanisms— in its entirety— by which this is brought about will require future investigations.

Meanwhile, at the end of the 14th stress exposure, there was a drastic downward review of HPA axis response in all the three groups of rats in Experiment II. Although corticosterone is elevated compared to the level recorded Experiment I, it is much more lower compared with the levels observed following single, three and seven stress exposures in Experiment II. This obviously is an indication of desensitization of the rats in Experiment II to the stress paradigm used in this study. Therefore, it can be stated that the data obtained following the 14th stress exposure provide evidence for possible habituation to the paradigm of psychological stress used in this study.

Evidence from current investigation, as shown in Fig. 2, strongly support the theory that exposure to psychological stress is associated with oxidative stress This observation support reports that have hypothesized that psychological stress disrupts the morphofunctional integrity of biological structures by escalating ROS production resulting in net imbalance in ROS level and the capacity of anti oxidant defense system (Bouayed *et al*., 2009; Aitken *et al*., 2014). The current study

includes an empirical scrutiny of this hypothesis by measuring indices of oxidative stress and anti-oxidant defense system in testis under various conditions of three experimental set up. The levels of testicular MDA in saline stress group, *ASEMA* stress group, and Zn stress group became significantly higher compared with those of corresponding baseline groups following subjection to 7 or 14 psychological stress sessions. Whereas this significantly higher level of MDA varies across time, that is, in relation to the number of times that the rats were subjected to psychological stress session, the interesting observation is that it peaked following the 7th stress exposure and declined by the 14th. Meanwhile, the level of lipid peroxidation was much higher in Experiment III suggesting that glucocorticoid is the mastermind of excessive ROS generation seen in Experiment II. Interestingly, however, the significantly lower level of MDA seen in *ASEMA* and Zn pre-treated groups in Experiment II and III suggests that *ASEMA* posses the capacity to blunt or slow down glucocorticoid-induced elevated production of ROS in the rodent testis.

Precisely, the capacity of the anti-oxidant defense system in stress-exposed rats (Experiment II) was not affected to the same extent that the pro-oxidant (ROS) was (Fig 2-5). Nonetheless, repeated exposure to psychological stress did indeed negatively reflect on testicular anti-oxidant capacity as all the parameters measured declined by end of the 7th stress exposure sessions (Figure 2). However, it is interesting to observe that both pre-treatment with *ASEMA* and Zn produce significant improvement on testicular anti-oxidants in stress-exposed rats. Under homeostasis, there is a balance between ROS level and anti-oxidants so that the anti-oxidant defense system can effectively prevent ROS from causing any significant damage that or any cellular damage that is genetically programmed into the cell.

In Experiment III, DX treatments significantly depress testicular anti-oxidant defense capacity than what was observed in Experiment II but much less in *ASEMA*- and Zn-pre-treated groups in this Experiment. This suggests that DX is probably more *oxidatively* injurious than endogenous glucocorticoids. Over all, exposure to psychological stress does not induce proportional changes in anti-oxidant defense system that corresponds negatively with changes in ROS level. The excessively elevated ROS level, rather than depressed anti-oxidants levels seems to be one of the causal mechanism by which stress negatively interferes with testicular biology in the rat (Fig 2). This imbalance is believed to create oxidative stress that possibly masterminds further testicular deteriorations (Aitken *et al*., 2014).

While it is not yet absolutely clear how *ASEMA* accomplish most of the observations made in this study, a working hypothesis may be built based on existing body of data so far generated; and this particularly involve its effect on Zn (Fig 6). Exposure to psychological stress severely depleted Zn levels in both the serum and testis of saline-pretreated rats. But pretreatment with *ASEMA* substantially prevent this loss (Fig 6).

The potential role of Zn deficiency in male infertility is yet to receive deserved attention, and as such it will take future investigations to elucidate the total dimension of testicular Zn depletion secondary to many conditions such as malnutrition and chronic stress and depression. Nevertheless, it is important to point out that evidence abound for the beneficial effects of Zn supplementation in certain metallic intoxication of rodent testis (Chandra *et al*., 2007).

The fact that stress of different paradigms is known to induce loss of micronutrients points to the need to widen the search for the etiologies of male reproductive dysfunction— especially when hormonal indices appear normal. The current study has succeeded in showing that Zn concentration in chronically stressed rats remained significantly low compared to controls even when the animals have levels of HPA axis response that could be interpreted as normal.

Furthermore, this investigation provided data that indicate that the capacity of *ASEMA* to subdue the impact of psychological stress on male reproductive function is connected to its ability to hold serum and testicular Zn concentration close to baseline during stress, suggesting that a Zn-based supplementation approach may be useful in designing an effective therapeutic regimen for stress-induced male reproductive dysfunction.

Most importantly, the fact that *ASEMA* (see Table 1) like many other plant-base alternative medicine contain more than one active substance suggests the possibility that it could act at different levels, on different cell types and/or receptors, modifying actions of multiple molecules and consequently producing two or more independent and/or inter-dependent effects. In the light of the current study, it is important to stress again that while the possibility of multiple actions is held, none of such action produced detectable negative effect. Thus the absence of detectable toxic effect of *ASEMA* may justify the tradition notion about its consumption safety. Part of the purpose of experiment III was to determine if Zn dyshomeostasis in Experiment II was due to elevated glucocorticoid. From the available data in Experiment III, it can be established that Zn loss was secondary to elevated corticosterone (See Fig 1-5). This was validated by the fact administration of DX

did indeed induce significant Zn loss that paralleled Zn loss observed in Experiment II (Fig 6). While the mechanism for Zn loss in psychologically stressed rat is not totally understood, Experiment III provide solid base for a possible glucocorticoid-mediated mechanism. Association between elevated cortisol and low Zn level is reported to characterize depression (Cowen 2002; Siwek *et al*., 2010; Johnson *et al*., 2008).

It is thus inferred that in the current study, stress-induced Zn loss is through a mechanism that is dependent on elevated glucocorticoid. However, this explanation seems to be valid for half of the observation. In experiment II, it was observed that although stress-induced glucocorticoid response in saline stress group had abated by the end of the 14th stress session; both serum and testicular Zn levels remained significantly lower compared with saline baseline group. This suggests that although elevated glucocorticoid may initiate Zn loss, the recovery of normal Zn status takes place much later after the blunting of stress-induced elevated glucocorticoid resulting from desensitization. This may explain, in part, why testicular

Conclusion

Psychological stress accentuate testicular redox imbalance through HPA stress reactivity in the form increased glucocorticoid secretion. However, administration Zn and Zn-rich *ASEMA* can significantly improve testicular anti-oxidant defense system through a mechanism though that does not change HPA stress reactivity.

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