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Alcohol-induced male infertility: Is sperm DNA fragmentation a causative?

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Abstract:

BACKGROUND: There is a passionate desire for couples to own their own biological children. Unfortunately, infertility index has been increasing with about 50% attributed to male factor infertility. Sperm DNA fragmentation (SDF) has been suggested as one of the causes of infertility in men; however, there have been controversies as regards its relationship with the successful management of infertility.

AIM: This study is aimed at determining the impact of SDF on fertility potentials in a rat model.

MATERIALS AND METHODS: Twenty adult male Sprague–Dawley (SD) rats were randomly divided into four groups of five rats each. Groups A_1 (distilled water) and B_1 (2 g/kg of 30% v/v ethanol) lasted for 4 weeks while Groups A_2 (control; distilled water) and B_2 (2 g/kg of 30% v/v ethanol) lasted for 8 weeks. At the end of each treatment, the animals were introduced to female SD rats on the proestrous day of their cycle. The testis was harvested and tested for oxidative stress while the cauda epididymis was harvested to test for epididymal sperm parameters and SDF.

RESULTS: The sperm count, sperm motility, and the number of fetuses sired by the animals that received alcohol decreased significantly ($P < 0.05$). There was also a significant increase in malondialdehyde and SDF and a concomitant decrease in testicular superoxide dismutase and reduced glutathione levels in animals that received alcohol compared to controls.

CONCLUSION: Alcohol Increased oxidative stress and SDF altering the ability of spermatozoa to fertilize oocytes.

Keywords:

Alcohol, DNA fragmentation, infertility, oocyte, oxidative stress, sperm

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Introduction

There has been a passionate desire to have one's own biological children regardless of the current trend of adoption. Worldwide, it has been generally reported that 8%–12% of couples are infertile (Inhorn, 2003). However, this incidence varies from one region to the other with the highest reports in the infertility belt of Africa which includes Nigeria (Araoye, 2003; Okonofua, 2003; Abarikwu, 2013). Unfortunately, about 50% of these infertility cases are male factor related (Onyeka *et al*., 2012). This staggering figure may become increasingly worrisome if adequate measures are not taken to prevent its causative factors.

A definite diagnosis of male infertility is usually obtained by conventional semen analysis (Esbert *et al*., 2011). Nonetheless, in recent times, most clinicians and scientists have pointed out limitations in the conventional semen analysis (Robinson *et al*., 2012; Osman *et al*., 2015). It has also been reported that 25% of infertile couples are diagnosed with idiopathic infertility as a result of the limitations of the conventional semen analysis (Lewis, 2015). More so, couples with idiopathic infertility have poor results in *in vitro* fertilization (IVF)

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compared to couples with detectable causes of infertility added to which a significant number of men have high sperm DNA damage (Oleszczuk *et al*., 2013; Simon *et al*., 2013).

Oleszczuk *et al*. (2013) reported that about 25% of unexplained infertility cases are associated with sperm DNA fragmentation (SDF), and oxidative stress (OS) is a major culprit (Zalata *et al*., 2004; Celino *et al*., 2012). OS occurs when the production of reactive oxygen species (ROS) exceeds the capacity of the antioxidant defenses to neutralize these highly reactive compounds creating an imbalance between pro- and anti-oxidative compounds which result in the accumulation of oxidative damage (Zimmer and Spencer, 2015). Increased OS impedes spermatogenesis by damaging the polyunsaturated fatty acids (PUFAs) that make up the plasma membranes of the spermatozoa (Aitken *et al*., 2014).

Alcohol has been reported as one of the causative factors of male infertility. Clinically, the most important endocrine consequences of long-term alcohol use are its effects on the gonads as it affects the synthesis of testosterone, consequently reducing sperm parameters (Martinez *et al*., 2009) and nuclear maturity as well as DNA integrity of spermatozoa (Talebi *et al*., 2011). Most alarming is a report by Chia *et al*. (2000) that over 42% of men with infertility consume alcohol (Chia *et al*., 2000). This may pose a big problem in the management of male factor infertility as alcohol consumption is addictive and most patients keep relapsing (Vengeliene *et al*., 2008).

Male alcoholics and heavy consumers also frequently report problems such as erectile dysfunction and lowered fertility (Pasqualotto *et al*., 2004; Costa *et al*., 2014). Muthusami and Chinnaswamy (2005) reported that alcohol has a direct toxic effect on the testis which leads to decreased seminiferous tubular function. It is reported to increase OS by producing free radicals known as ROS which overwhelm the antioxidant status of the testis impeding spermatogenesis (Oremosu and Akang, 2015). More so, spermatozoa are particularly susceptible to OS-induced damage because their plasma membranes contain large quantities of PUFAs (Alvarez and Storey, 1995) and their cytoplasm contains low concentrations of scavenging enzymes (Sharma and Agarwal, 1996; Aitken *et al*., 2014). In humans, OS also damages the sperm nuclear DNA (Zalata *et al*., 2004; Celino *et al*., 2012).

Sperm DNA damage has been associated with several infertility phenotypes, including idiopathic infertility, repeated intrauterine and IVF failure, and recurrent miscarriage (Saleh *et al*., 2003; Feijo and Esteves, 2014). Studies suggest that ROS attack the sperm nucleus by causing base modifications, DNA double and single strand breaks, and chromatin cross-linking (Said *et al*., 2005). The sperm cell has limited defense mechanisms against an oxidative attack on their DNA mainly due to the complex packaging arrangement of DNA. *In vivo*, such damage may not be a cause for concern because the collective peroxidative damage to the sperm membrane ensures that spermatozoa susceptible to OS are unable to fertilize the oocyte. However, these safeguards are circumvented during the course of intracytoplasmic sperm injection (ICSI) and some spermatozoa with significant DNA fragmentation may be used leading to adverse unfavorable results (Lewis, 2015). Consequently, the assessment of sperm DNA integrity has emerged as an important biomarker for male infertility. Therefore, this study is aimed at investigating the effects of alcohol-induced OS on SDF and fertility potential in rats.

Materials and Methods

Chemicals

Thirty percent ethanol prepared from absolute ethanol (99.86% v/v) with substance identification number 1170 manufactured by James Burrough (F.A.D. Ltd., UK) was used for the study.

Animal experiments

A total of 20 adult male Sprague–Dawley (SD) rats weighing between 200 and 220 g were randomly selected from the Laboratory Animal Center of the College of Medicine, University of Lagos (CMUL), and authenticated in the Department of Zoology, University of Lagos. Likewise, for the fertility test, 20 sexually mature female SD rats were used. The rats were fed with standard rat chow (Pfizer Nig Ltd.,). They had access to water *ad libitum*. The animals were housed in the Laboratory Animal Center, CMUL. The animal house was well ventilated with a temperature range of 28°C–32°C under day/night 12–12 h photoperiodicity.

Ethanol was orally administered by gastric gavage. Group A_1 received distilled water for 4 weeks, Group B_1 received 2 g/kg of 30% v/v of ethanol for 4 weeks, Group A_2 received distilled water for 8 weeks, and Group B_2 received 2 g/kg of 30% v/v of ethanol for 8 weeks (Dosumu *et al*., 2012).

Analysis of sperm parameters

The cauda epididymides of the rats were incised and a drop of epididymal fluid delivered onto a glass slide at 36°C, covered by a 22 mm × 22 mm cover slip and examined under the light microscope at a magnification of ×100 while evaluating different fields (World Health Organization, 1999). For the purpose of this study, motility was classified as either motile or nonmotile (Osinubi *et al*., 2007). After assessing different

microscopic fields, the relative percentage of motile sperm was estimated using the subjective determination of motility (Keel and Webster, 1990). The sperm count was determined using the Neubauer-improved hemocytometer. The epididymal fluid ratio of 1:20 was prepared by adding 0.1 ml of fluid to 1.9 ml of normal saline. The dilution was mixed thoroughly and both sides of the counting chamber were scored and the average was taken. Spermatozoa within five of the red blood cell squares including those which lie across the outermost lines at the top and right sides were counted, while those at the bottom and left sides were left out. The number of spermatozoa counted was expressed in millions/milliliter (Keel and Webster, 1990).

Biochemical estimations

Estimation of malondialdehyde

Lipid peroxidation as evidenced by the formation of TBARS was measured by the method of Niehaus and Samuelson (1968). In brief, 0.1 ml of tissue homogenate (Tris-HCl buffer, pH 7.5)/serum was treated with 2 ml of (1:1:1 ratio) TBA-TCA-HCI reagent (thiobarbituric acid 0.37%, 0.25N HCI, and 15% TCA) and placed in water bath for 15 min, cooled, and centrifuged at room temperature for 10 min at 1000 rpm. The absorbance of the clear supernatant was measured against reference blank at 535 nm.

Estimation of reduced glutathione

Reduced glutathione (GSH) was determined by the method of Ellman (1959). To the homogenate was added 10% TCA and centrifuged. 1.0 ml of supernatant was treated with 0.5 ml of Ellman's reagent ([19.8 mg of 5, 5'-dithiobis(2-nitrobenzoic acid) in 100 ml of 0.1% sodium nitrate] in 100 ml of 0.1% sodium nitrate) and 3.0 ml of phosphate buffer (0.2M, pH 8.0). The absorbance was read at 412 nm.

Determination of superoxide dismutase

Superoxide dismutase (SOD) was assayed utilizing the technique of Kakkar *et al*. (1984). A single unit of enzyme was expressed as 50% inhibition of nitroblue tetrazolium reduction/min/mg protein.

Assessment of sperm DNA fragmentation

DNA fragmentation was assessed using an alkaline single-cell gel electrophor0esis (comet) assay (Hughes *et al*., 1997; Donnelly *et al*., 1999). Briefly, the epididymal fluid ratio of 1:20 was prepared by adding 0.1 ml of fluid to 1.9 ml of PureSperm® wash (Nidacon International AB, Mölndal, Sweden). With the aid of a Neubauer-improved hemocytometer, aliquots of neat epididymal sperm were adjusted to give a sperm concentration of 6×10^6/ml. All subsequent steps were carried out in a climate-controlled room (20°C) under yellow light, to prevent induced DNA damage. This was done with modifications of the methods used by Agbaje *et al*. (2007). Frosted microscope slides were heated gently, coated with 100 µl of 0.5% normal melting point agarose in phosphate-buffered saline, kept at 45°C, and immediately covered with a glass coverslip (22 mm × 50 mm). Slides were left at ambient temperature (18°C) to allow the agarose to solidify. The coverslips were removed, and 10 µl of diluted epididymal sperm (6×10^6 ml^{-1}) was mixed with 75 µl of 0.5% low-melting-point agarose at 37°C. This cell suspension was pipetted over the first layer of gel, covered with a glass coverslip, and allowed to solidify at ambient temperature. The coverslips were removed and the slides immersed in a Coplin jar containing 22.5 ml of fresh lysis solution (2.5 M NaCl, 100 mM EDTA, and 10 mM Tris (pH 10), with 1% Triton X-100 added just before use), for 1 h at 4°C. Subsequently, 2.5 mL of 0.1 M dithiothreitol was added to achieve a final concentration of 10 mM for a further 30 min at 4°C. This was followed by 2.5 mL of 40 mM lithium diiodosalicylate to achieve a final concentration of 4 mM which was then incubated at ambient temperature for 90 min.

Slides were removed from the lysis solution and drained of any residual fluid. Fresh alkaline buffer (300 mM NaOH/1 mM EDTA) was prepared and poured into a horizontal gel electrophoresis tank at 24V/300 mA for 30 min allowing the exposed DNA to unwind.

The slides were removed from the tank, drained and flooded with three changes of neutralization buffer (0.4 M Tris; pH 7.5), removing any residual alkali or detergents that may interfere with staining. Slides were stained with PicoGreen dye, covered with a glass coverslip and stored in a humidified container in darkness at 4°C overnight, until analysis. Comet images were captured on 3 slides per group using Leica DM fluorescence microscope and CCD camera. Images were analyzed using Cometscore™ software (TriTek Corp., Sumerduck, VA).

Determination of cyclicity

The estrous cycle of each animal was characterized for 2 weeks, using vaginal lavage obtained between 8:00 a.m. and 9:00 a.m. before the commencement of the experiment. Cyclicity was determined by the modification of the method described by Ucheya and Biose (2010). Briefly, fresh normal saline was drawn into a fresh plastic pasteur pipette which was inserted into the vaginal canal 1 mm deep and irrigated. The lavage was then smeared on a microscopic slide and viewed under a microscope, before it dried. The presence of large nucleated cells with a few leucocytes on the slide was marked the preestrous day of the cycle. On the preestrous day of each rat's cycle, a marked male from a known group for male rats was introduced into a marked female cage in a 1:1 ratio. These paired animals were left together overnight. Vaginal lavage was taken

on the morning (estrous day of the cycle) following pairing between 8:00 a.m. and 9:00 a.m. The presence of spermatozoa in the lavage was marked as day 1 of pregnancy (Ratnasooriya and Dharmasiri, 2000).

Collection of fetuses

On the 20th day of gestation, fetuses were removed from pregnant rats by ventral laparotomy and examined. The number of live fetuses was recorded. Live fetuses were removed from the uterus, weighed and examined for gross malformations. The crown-rump length was also measured using methods described by Oderinde *et al.* (2002).

Statistical analysis

The differences between means in all the parameters tested were compared using the independent sample *t*-test of IBM SPSS statistics 23 (Saalu *et al*., 2002).

Results

Effect of alcohol on sperm parameter

There was a significant decrease in sperm count and sperm motility of animals administered alcohol compared to control after 4 and 8 weeks of administration [Tables 1 and 2].

Effect of alcohol on testicular malondialdehyde levels and testicular antioxidant enzymes

Testicular malondialdehyde (MDA) level was increased significantly ($P < 0.05$) in animals administered alcohol compared to controls after 4 and 8 weeks of administration. There was also a significant decrease in SOD levels after 4 weeks and GSH levels after 8 weeks of administration compared to controls. After 8 weeks of administration, there was an observable decrease in SOD levels howbeit, this decrease had no significant difference with control ($P > 0.05$) [Tables 1 and 2].

Effect of alcohol on sperm DNA fragmentation

There was an increase in tail length, tail moment and % DNA in tail after 4 weeks of administration and an observable significant increase in tail length and % DNA in tail after 8 weeks of administration compared to controls ($P < 0.05$) as seen in Tables 1 and 2.

Effect of alcohol on fertility

There were no observable malformations on the fetuses but there was a decrease in the weight and crown-rump length of the fetuses of dams that were mated with male animals administered alcohol, though this difference was not statistically significant ($P > 0.05$). There was a significant decrease ($P < 0.05$) in the number of fetuses of dams that were mated with male animals administered alcohol for 4 and 8 weeks of administration compared to control [Table 1 and 2].

Table 1: Effect of alcohol on testicular malondialdehyde, antioxidant enzymes, sperm parameters, sperm DNA fragmentation and fertility after 4 weeks of administration

	Group A_1 (control) *n*=5	Group B_1 (Alcohol treated) *n*=5
MDA (nmol/ml)	11.34±0.91	18.42±3.11*
SOD (min/mg/protein)	30.24±0.85	19.34±1.89*
GSH (µmol/ml)	0.19±0.01	0.21±0.22
Sperm Count (10^6/ml)	60.80±4.75	29.70±2.27*
Sperm motility (%)	85.5±0.85	30.50±4.27*
Tail Length (px)	2.67±0.90	18.20±1.96*
Tail Moment	0.06±0.02	1.19±0.33
%DNA in Tail	7.85±0.08	13.80±1.29
Fetal number	8.67±1.15	4.33±3.79*
Fetal weight (g)	3.20±1.41	3.18±0.29
Crown-rump Length (cm)	4.40±0.11	4.28±0.29

Values are reported as mean ± standard deviation. *$P<0.05$; *n*=5. MDA - Malondialdehyde; SOD - Superoxide dismutase; GSH: reduced glutathione

Table 2: Effect of alcohol on testicular malondialdehyde, antioxidant enzymes, sperm parameters, sperm DNA fragmentation and fertility after 8 weeks of administration

	Group A_2 (control) *n*=5	Group B_2 (Alcohol treated) *n*=5
MDA (nmol/ml)	8.42±0.95	20.14±2.34*
SOD (min/mg/protein)	28.96±0.54	20.38±3.40
GSH (µmol/ml)	0.43±0.44	0.12±0.02*
Sperm Count (10^6/ml)	65.50±3.44	21.20±1.27*
Sperm motility (%)	80.50±5.4	28.50±1.99*
Tail Length (px)	3.20±0.72	25.27±2.34*
Tail Moment	0.26±0.09	11.70±0.81*
%DNA in Tail	10.80±0.11	71.10±5.20*
Fetal number	8.40±1.41	4.00±1.29*
Fetal weight (g)	3.20±1.41	3.00±1.15
Crown-rump Length (cm)	4.45±0.41	4.15±0.11

Values are reported as mean ± standard deviation. *$P<0.05$. MDA - Malondialdehyde; SOD - Superoxide dismutase; GSH - Reduced glutathione

Discussion

Alcohol has been reported to be rapidly absorbed into the reproductive tract (Asher *et al*., 1979). This study showed that testicular MDA level of animals that received alcohol was inversely proportional to their testicular antioxidant enzyme level. This reflects an ongoing OS action which is in concert with reports by (Quintans *et al*., 2005; Siervo *et al*., 2015). One of the by-products of lipid peroxidation is MDA (Saleh and Agarwal, 2002). Therefore, the increased MDA level as observed in this study proves an increased peroxidation of lipids which has a negative effect on both sperm motility and the competence of these cells for fertilization (Alvarez *et al*., 1987; Tremellen, 2008; Aitken and Curry, 2011). This is because plasma membrane of the mammalian spermatozoa are particularly susceptible to peroxidative damage because they are well endowed with PUFAs that are highly vulnerable to ROS attack (Aitken *et al*., 2012).

The link between ROS and reduced motility may be due to a cascade of events that result in a decrease in axonemal protein phosphorylation and sperm immobilization. Both of which are associated with a reduction in membrane fluidity that is necessary for sperm-oocyte fusion (De Lamirande and Gagnon, 1995). More so, increased production of ROS leads to an error in spermiogenesis. This involves the release of spermatozoa with abnormal retention of cytoplasm (Sanocka and Kurpisz, 2004) and a reduction in the fluidity of the plasma membrane of the spermatozoa (Bansal and Bilaspuri, 2011). These also explain the decreased number of fetuses sired from animals that received alcohol in this study because, a decrease in fluidity of plasma membrane of spermatozoa impairs capacitation and impedes its oocyte penetrating ability (Saleh and Agarwal, 2002; Aitken *et al*., 2013).

Our findings posit that the toxic effects of alcohol go beyond the decrease in sperm quality, it also damages sperm DNA by increasing its fragmentation. There are three main causes of sperm DNA damage, these include abnormal chromatin packaging during spermiogenesis, abortive apoptosis and excessive production of ROS (Tavalaee *et al*., 2008). From this study, alcohol increased the production of ROS resulting in increased lipid peroxidation as evident in the increased MDA level. This is in concordance with the report by Tremellen (2008) that peroxides affect DNA bases causing modifications and breakages. It is also in consonance with reports from Cocuzza *et al*. (2007), Agarwal and Shekhon (2011) and Aitken *et al*. (2013) that OS in the reproductive tract has potentially harmful effects because high levels of ROS is detrimental to sperm number, motility, quality, and function including the integrity of sperm nuclear DNA.

OS increases with apoptosis (Kannan and Jain, 2000). Chronic alcohol intake leads to germ cell apoptosis and reduction in fertility potential via the up-regulation of the Fas system and the activation of caspases (Eid *et al*., 2002). Although this study did not include the relationship between sperm cell apoptosis and ethanol consumption, there are a few studies that support it: Koh and Kim (2006), demonstrated that ethanol administration reduces cell proliferation and spermatogenesis and also enhances cell death in testes. Another study demonstrated that chronic alcoholic men have increased rates of germ cell apoptosis that leads to testicular atrophy (Zhu *et al*., 2000). Increased ROS is positively correlated with apoptotic sperm (Cheema *et al*., 2009). There is also a report that during intrinsic apoptotic cascade, the only product generated that can induce DNA damage in the sperm nucleus is the hydrogen peroxide released from the sperm mitochondria which, because of its small size and lack of charge, can readily move from the midpiece to the sperm head and penetrate the nucleus (De Iuliis *et al*., 2009). Much later in the apoptotic process, the sperm DNA begins to fragment (Mitchell *et al*., 2011). From our findings and these literatures, it is suggestive that at least one of the mechanisms by which alcohol causes SDF is by ROS-induced apoptosis.

A relationship between high SDF and male infertility is well documented in the literature (Aitken and Curry, 2011; Simon *et al*., 2011; and Evgeni *et al*., 2014). This study reveals a negative relationship between alcohol and fertilizing ability of spermatozoa in rats. This may be either due to the fact that increased peroxidation had hampered the process of capacitation making it impossible for sperm cells to penetrate the zona pellucida or it may be due to the fact that increased DNA fragmentation had impaired the spermatozoon's ability to trigger the cascade of ooplasmic events that results in embryonic development and capacity for implantation. Our findings are in agreement with the report by Dimitriadis *et al*. (2009) but at variance with postulations that fertilization can be achieved even in the presence of elevated SDF rates because DNA damage can be balanced by reparative ability of the oocyte (Benchaib *et al*., 2007; Collins *et al*., 2008; and Lewis *et al*., 2008). Even in IVF cycles, damaged sperm DNA integrity results in repeated failure (Simon *et al*., 2013; Evgeni *et al*., 2014) and increased miscarriages in ICSI (Carrell *et al*., 2003).

Conclusion

This study demonstrated that alcohol increases SDF by increasing the activity of ROS. It also demonstrates that increased SDF affects sperm quality and ultimately, the fertility potential of the spermatozoon. It further demonstrates that alcohol incapacitates the sperm ability to trigger the fertilized oocyte development for implantation. Therefore, it is imperative that SDF is ascertained in the management of male factor infertility. Further studies should be done on the fertility of offspring sired by animals administered alcohol.

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Conflicts of interest
There are no conflicts of interest.

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