Bulletin of Pure and Applied Sciences
Zoology, Vol.44A, No.1,
January-June 2025: P.45-56

Print version ISSN 0970 0765
Online version ISSN 2320 3188

Original Article
Content Available online at: https://www.bpasjournals.com/

Impact of Dietary Copper Deficiency on Wistar Rat Sperm Morphology and Sperm Kinetics

[1,2]Aastha Saini*, [1]Ankita Rajendra Kurup, and [1]Neena Nair

Author's Affiliation:
[1]Cell & Molecular Biology Laboratory, Department of Zoology, Centre for Advanced Studies, University of Rajasthan, Jaipur-302004, Rajasthan, India.
[2]Government College Dholpur, Dholpur-328001, Rajasthan, India.

***Corresponding author:**
Dr. Aastha Saini,
Assistant Professor, Department of Zoology, Government College Dholpur, Dholpur-328001, Rajasthan, India.
E-mail: aasthasaini2502@gmail.com

Received on 22.01.2025
Revised on 19.03.2025
Accepted on 23.05.2025

ABSTRACT:
Copper a transition element is essential for innumerable physiological functions. Copper deficiency has now been recognized and is global problem-affecting person of all age groups leading to untold problems. The study determines the effect of copper deficiency on caudal sperm density, and viability as well as sperm vas deferens morphology of male Wistar rats. Pre-pubertal male Wistar rats (35-50 g) were divided into four groups: Negative control (NC), copper control (CC) and pair fed (PF) group [126 nmol Cu/g of diet] as well as copper deficient (CD) group [6.3 nmol Cu/g of diet]. Duration of experiments was 2-, 4- and 6- weeks. Caudal epididymal sperm density and viability were recorded while vas deferens sperm morphology was analysed. Studies revealed significant ($P<0.05$) decrease in sperm density as well as viability in the copper deficient group animals. Sperm morphology showed various abnormalities in dietary copper deficient group animals, which were more pronounced after 6 weeks. Copper under nutrition starting from prepubertal period negatively affected sperm density and viability as well as morphology as evident by insufficient acrosome, abnormal head and tail cytoplasmic integrity – all of which could probably be due to enhanced oxidative stress and other protein factors which could be one of the causative factors leading to infertility if the duration of copper deficit enhances.
Keywords: Copper deficiency, sperm morphology, sperm density and viability

How to cite this article: Saini A., Kurup A.R., and Nair N. (2025). Impact of Dietary Copper Deficiency on Wistar Rat Sperm Morphology and Sperm Kinetics. *Bulletin of Pure and Applied Sciences-Zoology*, 44A (1), 45-56

INTRODUCTION

Infertility has become a global problem and it is anticipated that 48 million couples and 186 million persons worldwide are infertile (Ombelet, 2020). Impaired spermatogenesis and sperm function are the most common cause of male infertility (Elbashir et al, 2021). Microelements are crucial for the maintenance of various critical physiological as well as biochemical processes due to their high activity at molecular level (Saini and Nair, 2022; Tvrdá et al, 2012). The involvement of copper in one - electron transfer reaction defines the activity and function of numerous enzymatic and non-

enzymatic proteins, which play a significant role in homeostatic process. Homeostasis of copper is achieved by copper transporters, chaperone proteins and metallothioneins (Chen et al, 2022; Ruiz et al, 2021; Herman et al, 2020; Kaplan and Maryon 2016). The occurrence of cupric (Cu^{2+}) ions in epididymal lumen secreted by its epithelial cells has been reported (Roy et al, 2014). In addition to being present in the somatic cells of the testis and epididymis, copper-dependent enzymes such as ceruloplasmin, superoxide dismutase SOD1 and SOD3, group of metallothionein and cytochrome c oxidase are present at all phases of gametogenesis (Ogórek et al, 2017a). Copper has a role in sperm volume, vitality and motility (Kňažická et al, 2013; Eidi et al, 2010) as Slc31a1 (copper importer) was located in mouse spermatozoa/sperm isolated from epididymis (caput, corpus and cauda) and vas deferens (Ogórek et al, 2019). Further, CCS/SOD1 has also been detected in spermatozoa (Ogórek et al, 2019; Park et al, 2012). Significant quantities of copper are also present in fluids connected to sperm in the prostate and epididymis (Ogórek et al, 2017a). Abnormal concentrations of calcium and magnesium as well as trace metals, including zinc and copper, can have an impact on spermatogenesis affecting the spermatozoa's ability to fertilize and mature (Chow et al, 2023; Skandhan 1992).

Mammalian spermatozoa / sperm DNA is susceptible to damage during spermiogenesis and also at later stages (Kuchakulla et al, 2021; González-Marín et al, 2012). Oxidative stress caused by an excessive formation of reactive oxygen species (ROS) can have a severe negative impact on the sperm plasma membrane and its functional integrity (Walke et al, 2023). Sabeti et al, (2016) reported generation of ROS from immature and abnormal spermatozoa. Defense against oxidative stress in shielding spermatozoa/ sperms is achieved by secretions in epididymis and seminal plasma on account of antioxidant enzymes as well as glutathione (Fouchecourt et al, 2000; Tramer et al, 1998). Copper has redox activity and hence can generate hydroxyl radicals through Fenton reaction (Van den Berghe and Klomp, 2010) which may cause oxidative damage to proteins, lipids and nucleic acids. Hence, an attempt was made to study the consequence of dietary copper deficiency on prepubertal Wistar rat sperm morphology and kinetics.

MATERIALS AND METHODS

The basal diets were prepared by using ICN Research Diet Protocol (1999). The diet (gm / kg diet) was composed of: Egg white/ albumin- 180 gm, Corn oil- 100 gm, Corn starch- 443 gm, Sucrose- 200 gm, Cellulose -30 gm, Choline chloride- 2 gm, DL- methionine- 7 gm, AIN- 76 salt mixture- 35 gm, AIN- 76C vitamin-antibiotic mixture- 10 gm. Copper contents of basal diet for each group were estimated at 324.8 nm in air acetylene flame on GBC 902 atomic absorption spectrophotometer. Copper concentrations were adjusted to 126 nmol/gm and 6.3 nmol/gm using copper sulfate for control group and deficient group respectively.

Experimental design: Male Wistar rats (30-40 days- prepubertal period) of 35-50 gms wt. were divided into four groups (n=10 each): Group 1: Negative control (NC): fed commercial (Ashirwad Ltd.,) diet; Group 2: Copper control (CC): Fed with diet containing 126 nmol Cu/gm. Tap water was provided *ad libitum*; Group 3: Pair-fed group (PF): Given 126 nmol Cu /gm diet but the amount of feed given was equal to the feed consumed by copper deficient group the previous day to account for starvation and stress effect. Tap water was provided *ad libitum*; Group 4: Copper deficient (CD): Given 6.3 nmol Cu / gm diet and demineralized water were provided *ad libitum*. Animals were kept in isolation in polypropylene cages with stainless steel grills. Cages and water bottles were washed with detergent solution, demineralized water and finally rinsed in 1% EDTA solution prepared in demineralized water for elimination of copper traces.

Ethical approval: Experiments were conducted for 2-, 4- and 6-weeks and approved by Department Research Ethics Committee as well as Committee for the purpose of Control and Supervision of Experiments on Animals (CPSEA -1678/GO/Re/S/12).

Parameters

- **Caudal sperm density and viability:** Sperm density and sperm viability were assessed by method given in Sigma bulletin (1998). Briefly, cauda epididymis was cut into small pieces in normal saline. Sperm collected was centrifuged at 225-x g for 10 min. The pellet was re-suspended in 2.0 ml normal physiological saline solution. Sperm counting and sperm viability observation was carried using standard Neubauer hemocytometer. Trypan blue – dye exclusion procedure was used to study sperm density and viability.
- **Vas deferens sperm morphology:** For evaluation of sperm morphology staining technique procedure as given by Unnithan (1976) was followed.

Statistical analysis

Data expressed as Mean ± SEM. One way Analysis of Variance (ANOVA) was carried out separately of 2-, 4- and 6- week experimental groups followed by post hoc test (Tukey's Multiple Comparison test) if the difference was found to be significant. Data were analyzed using GraphPad Prism Version 7.0e. $P < 0.05$ was considered to be significant.

RESULTS

Caudal sperm density recorded significant decrease ($P<0.05$) in deficient groups (2.244±0.067, 2 week; 1.697±0.237, 4 week and 0.907±0.053, 6 week) when compared with their respective controls (NC and CC) and PF groups. Decline was also evident when NC and CC groups were compared with respective PF groups (Table 1).

Table 1: Wistar rat cauda epididymal sperm kinetics after 2-, 4- and 6-weeks of dietary copper deficient diet (Mean ± SEM)

Groups	Sperm density (10^6/ml)	Sperm viability (%)
2NC	5.773±0.036	47.693±0.406
2CC	5.772±0.091	47.967±0.320
2PF	4.290±0.194 b*d*	37.172±0.208 b*d*
2CD	2.244±0.067 c*e*f*	21.510±1.363 c*e*f*
4NC	5.937±0.204	58.147±0.470
4CC	5.910±0.184	58.115±0.222
4PF	3.703±0.254 b*d*	35.697±0.166 b*d*
4CD	1.697±0.237 c*e*f*	15.073±0.169 c*e*f*
6NC	8.328±0.045	68.111±0.85
6CC	8.320±0.054	67.908±0.95
6PF	3.452±0.190 b*d*	22.058±0.63 b*d*
6CD	0.907±0.053 c*e*f*	8.11±0.52 c*e*f*

*** P<0.05 Significant**
Where: a= NC Vs CC,b = NC Vs PF, c= NC Vs CD, d=CC Vs PF,e =CC Vs CD, f =PF Vs CD.
Multiple comparison procedures were performed for 2-, 4- and 6 weeks experimental groups.

Caudal sperm viability reduced significantly ($P<0.05$) in deficient groups (21.510 ± 1.363, 2 week; 15.073 ± 0.169, 4 week and 8.11 ± 0.52, 6 week) on comparison with their respective NC, CC and PF groups. Assessment of NC and CC with respective PF groups also recorded significant decrease (Table 1).

Sperms of vas deferens in negative control (2 weeks – Fig 1a; 4 weeks – Fig.2a; 6 weeks Fig. 3a) and copper control 2 weeks – Fig. 1b; 4 weeks- Fig. 2b; 6 weeks – 3b) showed normal morphology. Dietary copper deficiency revealed thinning of principal piece (2 weeks; Figs 1d) followed by addition of abnormality such as detached head and coiled terminal tail (4 weeks; Fig. 2d) with further enhancement of occurrence of sperm abnormality after 6 weeks of deficiency as evident by deformed head (amorphous), abnormal/ lack of complete acrosome and head,

detached head and principal piece thinning with cytoplasmic droplet. Detached mid piece with proximal piece of tail was also observed (Figs.3 d–f). Coiled tail was also evident in pair fed group of 4 (Fig. 2c) and 6 weeks (Fig. 3c) although the number of coiled tails was more compared to 4 weeks PF group.

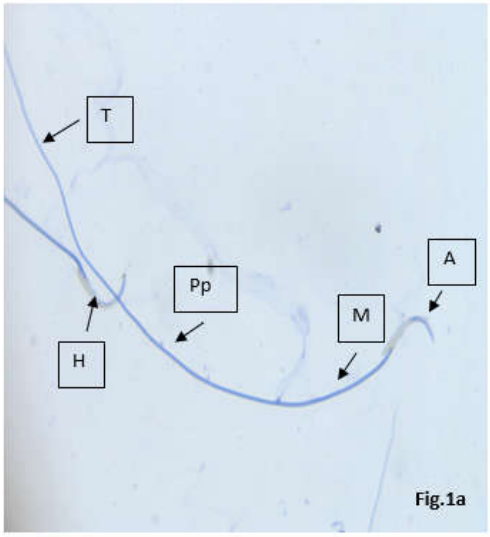

Fig. 1a: Microphotograph of sperm of 2 weeks Negative Control (2NC) group showing the normal architecture: acrosome (A), hooked head (H),mid piece (M), principal piece (Pp) and terminal tail (T).Triple stain,1000X.

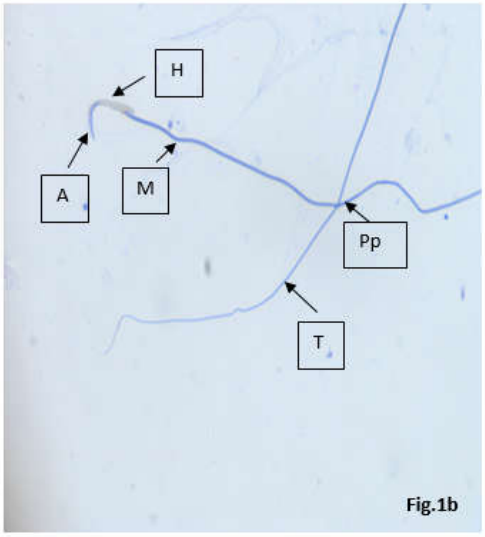

Fig. 1b: Microphotograph of sperm 2 weeks Copper Control (2CC) group showing the normal architecture: acrosome (A), hooked head (H), mid piece (M), principal piece (Pp) and terminal tail (T).Triple stain,1000X.

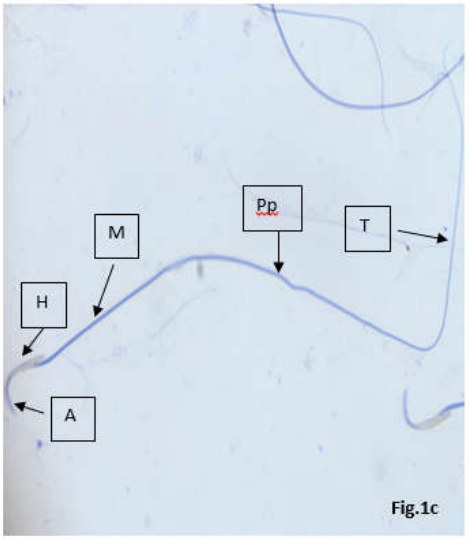

Fig. 1c: Microphotograph of sperm of 2 weeks Pair Fed (2PF) group showing the normal architecture: acrosome (A), hooked head (H), mid piece (M) and terminal tail (T).Triple stain, 1000X.

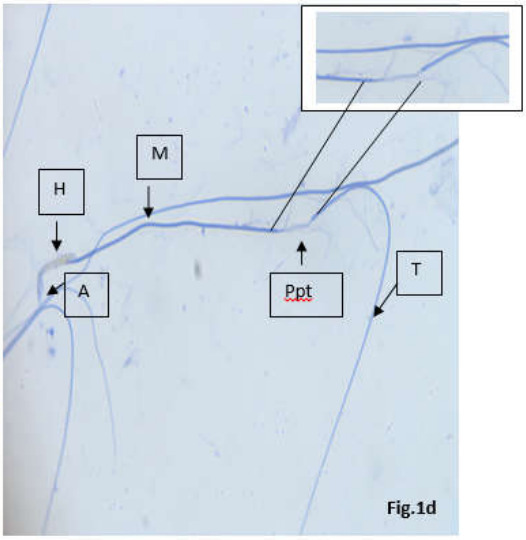

Fig. 1d: Microphotograph of sperm of 2 weeks Copper Deficient (2CD) group exhibiting acrosome (A), hooked head (H) ,mid piece (M) and thinning of principal piece at one region (Ppt) and terminal tail region (T). Inset showing thinning region.Triple stain, 1000X.

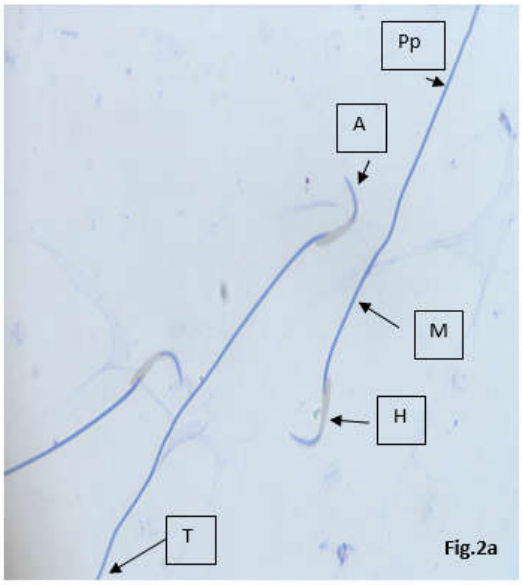

Fig. 2a: Microphotograph of sperm of 4 weeks Negative Control (4NC) group showing the normal architecture: acrosome (A), hooked head (H),mid piece (M) principal piece (Pp) and terminal tail (T). Triple stain, 1000X.

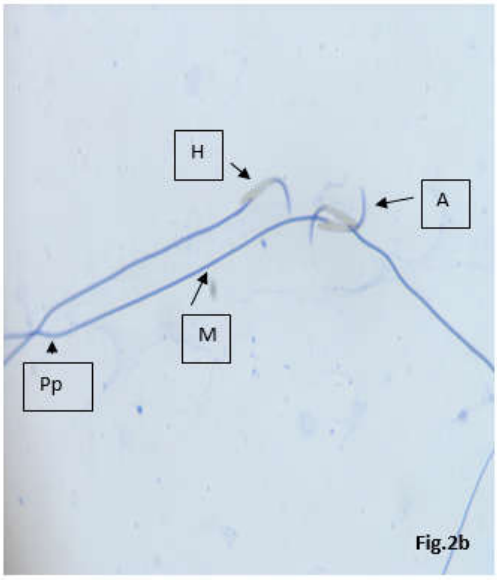

Fig. 2b: Microphotograph of sperm of 4 weeks Copper Control (4CC) group showing the normal architecture of acrosome (A), hooked head (H), mid piece (M) and principal piece (Pp). Triple stain, 1000X.

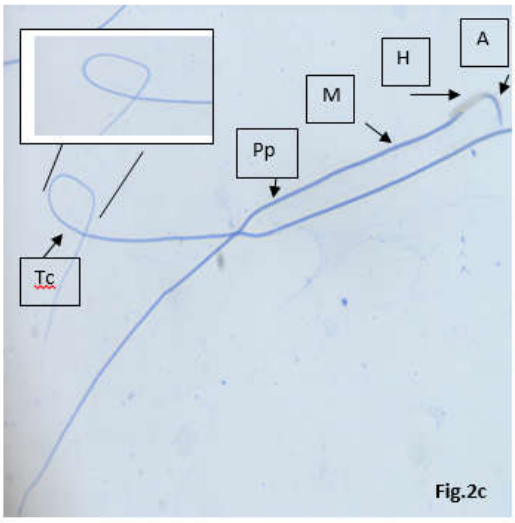

Fig. 2c: Microphotograph of sperm of 4 weeks Pair Fed (4PF) group showing overall normal architecture: acrosome (A), hooked head (H),mid piece (M) except for coiling of terminal tail (Tc) Inset showing coiled tail. Triple stain, 1000X

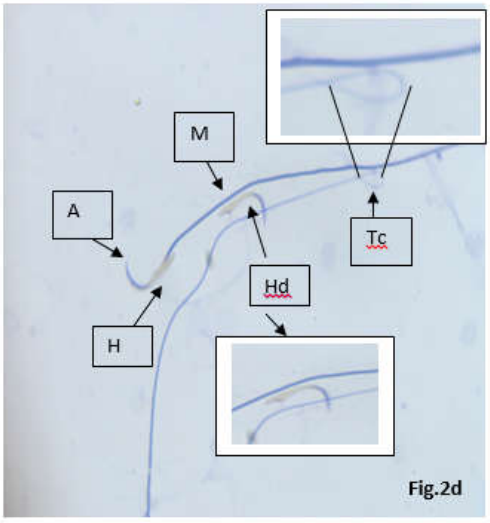

Fig. 2d: Microphotograph of sperm of 4 weeks Copper deficient (4CD) group showing acrosome (A), head (H) ,detached head (Hd), mid piece (m) and coiling of terminal tail (Tc). Inset exhibiting detached head and coiled terminal tail. Triple stain, 1000X.

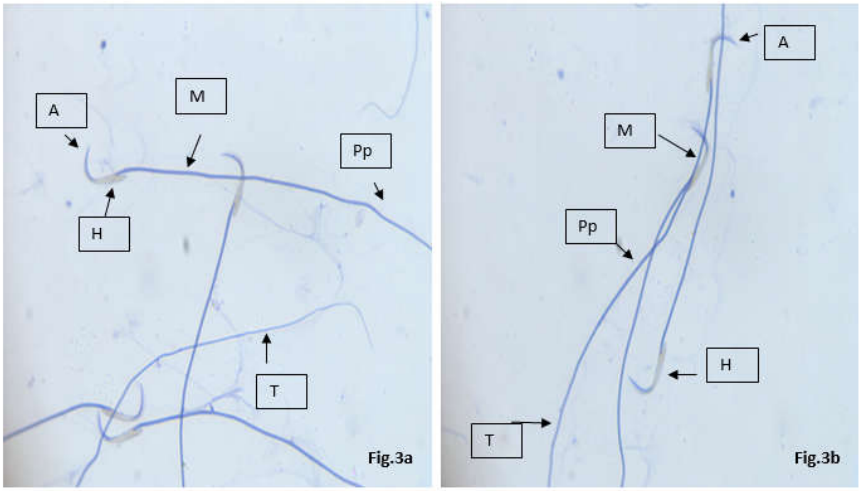

Fig. 3a & 3b: Microphotograph of sperm of 6 weeks Negative Control (6NC) group showing acrosome (A), hooked head (H), mid piece (M) and terminal tail (T). Triple stain,1000X.

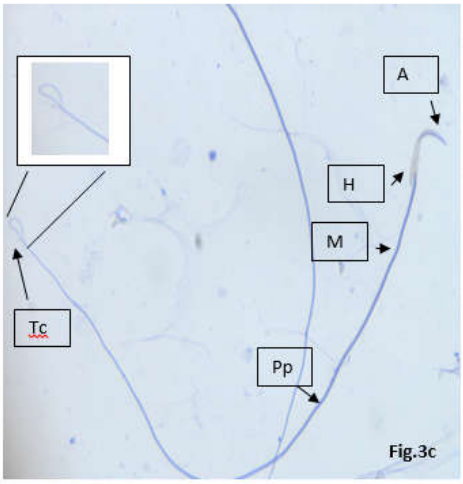

Fig. 3c: Microphotograph of sperm of 6 weeks Pair fed Control (6PF) group showing acrosome (A), hooked head (H), mid piece (M) and coiling of tail (T).Inset showing coiled tail. Triple stain,1000X.

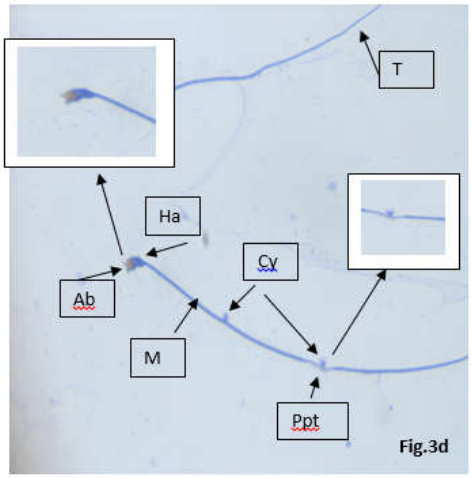

Fig. 3d: Microphotograph of sperm of 6 weeks Copper deficient (6CD) group showing abnormal acrosome (Ab), deformed head (Amorphous) (Ha), mid piece (M), retention of cytoplasmic droplets (Cy) in mid piece region and proximal piece, proximal piece thinning (Ppt) and terminal tail (T). Insets showing abnormal acrosome and head and another showing principal piece thinning with cytoplasmic droplets. Triple stain, 1000X.

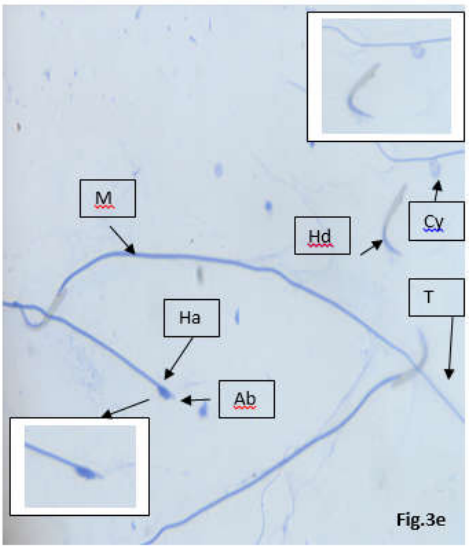

Fig. 3e: Microphotograph of sperm of 6 weeks Copper Deficient (6CD) group exhibiting abnormal acrosome (Aa), deformed head (Amorphous) (Ha), detached head (Hd), mid piece (M), retention of cytoplasmic droplet (Cy) and terminal tail (T). Triple stain, 1000X.

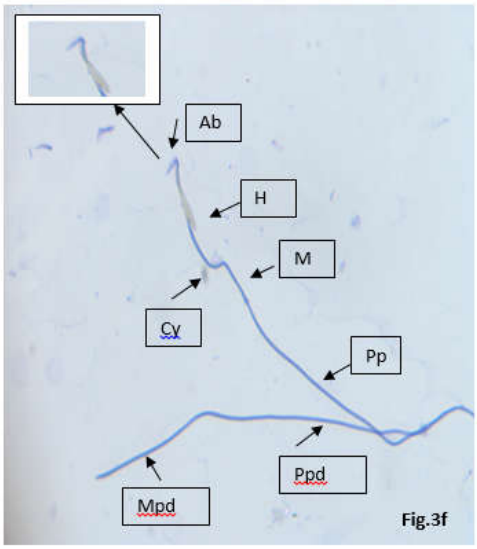

Fig. 3f: Microphotograph of sperm of 6 weeks Copper Deficient (6CD) group exhibiting abnormal /lack of complete hook/acrosome (Aa) (also in inset) ,head (H) ,mid piece (M), principal piece (Pp), detached mid piece (Mpd) with proximal piece of tail (Ppd) and retention of cytoplasmic droplet (Cy). Triple stain, 1000X.

DISCUSSION

Copper has a role in spermatogenesis / sperm owing to the fact that copper transporters are expressed in testes, epididymis and vas deferens (Ogórek et al, 2017b; 2019; Roy et al, 2014; Beaudoin et al, 2012). Study revealed diminution in caudal sperm density in deficient groups. Although effect of copper on sperm is uncertain, yet it has been implicated in spermatozoan motility and may potentially have an impact on the pituitary receptors that regulate LH release (Skandhan 1992). Hussain et al, (2011) reported significant decline of mean copper concentration in azoospermia (0.044 ± 0.010), asthenozoospermia (0.025 ± 0.004) and oligozoospermia (0.051 ± 0.008) males compared to controls (fertile; 157.593 ± 11.785). Contrary to this, increased copper /zinc ratio with low zinc was recorded in asthenozoospermia (López-Botella et al, 2021). Assessment was carried out in infertile Sudanese males wherein decreased copper concentration was observed in azoospermic as well as oligozoospermic patients (Hassan et al, 2020). Authors (Wong et al, 2001) reported that in fertile and infertile men there exists a correlation between copper concentration in seminal fluid and spermatozoa count, motility and morphology.

The study recorded decline in caudal sperm viability in deficient groups as well as pair fed groups. Increased ROS (i) alters the sperm movement effecting calcium channels due to lipid peroxidation of the outer membrane with consequent depletion of ATP stores (Sharma et al, 2023) (ii) decreased / abnormal sperm mitochondrial membrane potential which can also be connected to weak DNA integrity- a feature correlated with decreased motility (Zini et al, 2001;Raad et al, 2024). Kowal et al, (2010) studied mutant mice (ms/- males) and observed low sperm DCm (sperm mitochondrial transmembrane potential) and high ssDNA compared to +/- males with presence of cytoplasmic droplets on spermatozoa flagellum accounting for decrease in motility Several authors (Bisht et al, 2017; Gosálvez et al, 2017) reported two primary sources of superoxide

radical generation in mitochondria (found abundantly in mid piece of spermatozoa):
(i) Inner mitochondrial membrane NADH-dependent oxidoreductase and (ii) plasma membrane NAD (P) H–oxidase. Decline in sperm density and viability can be related to testicular decline in glutathione concentration and Cu-Zn SOD activity after dietary copper deficiency with concomitant increase in total superoxide dismutase, Manganese SOD and catalase activities indicative of generation of reactive oxygen species (Saini et al, 2022). In contrast to normozoospermic males, patients with asthenozoospermia, asthenoteratozoospermia and oligo-asthenoteratozoospermia had significantly low catalase activity which was related to low sperm quality (Khosrowbeygi et al, 2004). Copper deficit affected seminiferous tubules in animals which was related to DNA damage due to enhanced reactive oxygen species as both metallothionein as well as glutathione levels were altered leading to necrozoospermia, asthenospermia and sperm DNA fragmentation (Tvrda et al, 2015; Picco et al, 2001; Van Niekerk et al, 1989). In the current study, decline in density and viability was evident which was duration dependent after dietary copper deficiency and can be correlated to altered antioxidant levels with enhanced occurrence of abnormal sperms.

Sperms have high level of plasma membrane polyunsaturated fatty acids and low antioxidant defense enzymes (catalase, superoxide dismutase), which makes it more prone to peroxidative damage. ROS cause a cascade of peroxidation cycles that lead to the oxidation of membrane lipids and the fragmentation of nucleic acids – all causative factors for sperm dysfunction Alvarez and Aitken, 2012). Authors (Xu et al, 2013) accorded progressive motility to the presence of cytoplasmic droplets indicating its essentiality during sperm epididymal maturation coupled with unwarranted ROS production. Sperm cytoplasmic droplets are removed by clear cells of epididymal epithelium. Oxidative stress has an effect on sperm fertilizing ability, genomic integrity, with sperm possessing cytoplasmic residual droplets in the proximal region being more prone to DNA damage (Hussain et al, 2023; Aydemir et al, 2006). Males with MS/- may experience reduced motility and/or altered mitochondrial activity as a result of delayed residual droplet migration along the sperm tail because peroxidation impacts lipids, particularly in the mid-piece (Shamsi et al, 2008; Gavella et al, 1995). Dietary copper deficiency led to alteration/abnormality in the sperm morphology. Different concentrations of ROS were found to be produced in the subsets of ejaculated human spermatozoa using density gradient centrifugation, with fraction 2 having undeveloped spermatozoa (abnormal head morphology as well as cytoplasmic droplet) and fraction 4 having mature spermatozoa and immature testicular germ cells having the highest concentration of ROS (Cavallini, 2006). The primary sperm tail thinness observed is responsible for the axoneme's structural assembly being disrupted which would impair motility. Depletion of many genes, such as Spag6, SPAG16L isoform MEIG1, typically results in a complete loss of progressive motility due to annulus and connecting piece abnormalities. Further, detachment of mid piece – region would alter the outer dense fibers and cause abnormality in mitochondria (Lehti and Sironen, 2017; Zhang et al, 2006, 2009; Sapiro et al, 2002). According to Zhang *et al.,* (2012) mitochondrial sheath deformities caused by kinesin light chain 3 (KLC3)'s failure to bind outer dense fibers result in modifications to progressive motility and sub-fertility. Sperm chromatin deficiencies/ abnormality are one of the most important causes of fertility failure in humans (Chemes and Alvarez, 2012). Leung et al, (2023) detected >60 sperm DMTs proteins of which minimum 15 were sperm associated and 16 linked to infertility. Further, the authors also reported that protein tektins located within the axoneme has a role in sperm motility and male fertility. Tetkins-2,--3,-4 and- 5 have been found to affect sperm motility and fertility with genetic variants of tetkins-2 and the sperm-specific Tektin-5 related to human male infertility(Wyrwoll et al, 2022; Zhang et al, 2016; Roy et al, 2007, 2009). Expression of copper proteins (CTR1, ATP7A, ATP7B, Superoxide dismutase 1, ceruloplasmin) for maintaining copper homeostasis in testicular cell types including germ cells and somatic cells have been reported (Herman et al, 2020; Ogórek et al, 2017b) which would have an impact on sperm volume, motility, and fertilization ability (Tvrda et al, 2015; Roy et al, 2014). It is unknown

how copper functions in sperms, however it seems to affect their motility and could potentially interact with pituitary receptors to regulate LH secretion (Slivkova et al, 2009).The present study detected low sperm concentration with abnormal sperm morphology which can also be related to altered sperm proteins, antioxidants and hormonal level.

Dyshomeostasis of copper concentration affects male fertility in several ways. The abnormal morphological features, decreased motility and viability observed after dietary copper deficiency is indicative of disruption of structural assembly and mitochondria, retention of cytoplasmic droplet as well as DNA damage probably due to excessive ROS generation leading to enhanced oxidative stress. The data indicates that dietary copper deficiency from pre-pubertal stage onwards has a detrimental effect on sperms, which may be one of the causative factors for infertility and reflects the essentiality of copper for normal functioning of sperms.

Acknowledgement: Dr. Aastha Saini thanks University Grants Commission for the award of Junior Research Fellowship (No. 22/06/2014 (1) EU-V) and Senior Fellowship. Ms Ankita Rajendra Kurup thanks University Grants Commission, New Delhi for award of non NET fellowship. The authors gratefully acknowledge Centre for Advanced Studies (CAS), Department of Zoology, University of Rajasthan and Jaipur, India for providing necessary facilities.

Conflicts of interest: There is no conflict between the authors.

REFERENCES

Alvarez, J. G. and Aitken, R. J. (2012). Lipid peroxidation in human spermatozoa. In: Agarwal, A., Aitken, R.J. Alvarez J G (Eds), Studies on men's health and fertility. *Humana Totowa, NJ.* pp 119—130.

Aydemir, B., Kiziler, A.R., Onaran, I., Alici, B., Ozkara, H., & Akyolcu, M.C.(2006) Impact of Cu and Fe concentrations on oxidative damage in male infertility. *Biological Trace Element Research,* 112(3), 193–203.

Beaudoin ,J., Ioannoni, R.,and Labb´e, S.(2012) Mfc1 is a novel copper transporter during meiosis. *Communicative & Integrative Biology*, 5,118–121.

Bisht, S., Faiq, M., Tolahunase, M., & Dada, R. (2017) Oxidative stress and male infertility. *Nature Reviews Urology*, 14(8), 470-485.

Cavallini, G. (2006). Male idiopathic oligoasthenoteratozoospermia. Asian Journal of Andrology, 8,143–157.

Chao, H.H., Zhang, Y., Dong, P.Y., Gurunathan, S., & Zhang, X.F. (2023). Comprehensive review on the positive and negative effects of various important regulators on male spermatogenesis and fertility. *Frontiers in Nutrition*, 9, 063510. doi: 10.3389/fnut.2022.1063510.

Chemes, H.E. and Alvarez, S.C. (2012) Tales of the tail and sperm headaches: Changing concepts on the prognostic significance of sperm pathologies affecting the head, neck and tail. *Asian Journal of Andrology*, 14(1), 14-23.

Chen, L., Min, J. & Wang, F. (2022) Copper homeostasis and cuproptosis in health and disease. *Signal Transduction and Targeted Therapy,* **7**, 378. https://doi.org/ 10.1038/ s 41392-022-01229-y.

Eidi, M., Eidi, A., Pouyan, O., Shahmohammadi, P., Fazaeli, R., & Bahar, M.(2010) Seminal plasma levels of copper and its relationship with seminal parameters. *Iranian Journal of Reproductive Medicine*, 8(2), 60-65.

Elbashir, S., Magdi, Y., Rashed, A., Henkel, R., & Agarwal, A.(2021). Epididymal contribution to male infertility: An overlooked problem. *Andrologia,* 53(1), e13721. doi: 10.1111/and.13721.

Fouchecourt, S., Metayer, S., Locatelli, A., Dacheux, F.,& Dacheux, J-L.(2000). Stallion epididymal fluid proteome: qualitative and quantitative characterization: Secretion and dynamic changes of major proteins. *Biology of Reproduction*, 62,1790–1803.

Gavella, M., Lipovac, V.,& Sverko, V.(1995). Superoxide anion production and some sperm-specific enzyme activities in infertile men. *Andrologia,* 27:7–12.

González-Marín, C., Gosálvez, J., & Roy, R.(2012).

Types, causes, detection and repair of DNA fragmentation in animal and human sperm cells. *International Journal of Molecular Sciences,* 13(11), 14026-14052.

Gosálvez,, J., Coppola, L., Fernández, J.L., López-Fernández, C., Góngora, A., Faundez, R., Kim, J., Sayme, N., de la Casa, M., Santiso, R., & Harrison, K.(2017). Multi-centre assessment of nitrobluetetrazolium reactivity in human semen as a potential marker of oxidative stress. *Reproductive Biomedicine Online,* 34(5), 513- 521.

Hassan, M.I., Ahmed, A.M.S., Hamad, M.N.M., & Elimairy, G.M.(2020). Assessment of seminal plasma trace elements among infertile Sudanese males in Khartoum state, 2019. *Saudi Journal of Biomedical Research*, 5(1), 9-13.

Herman, S., Lipiński, P., Ogórek, M., Starzyński, R., Grzmi,l P., Bednarz, A.,& Lenartowicz, M.(2020). Molecular regulation of copper homeostasis in the male gonad during the process of spermatogenesis. *International Journal of Molecular Sciences*, 21(23), 9053. doi: 10.3390/ijms21239053.

Hussain, N.K., Rzoqi, S. S., Numan, A. W.,& Ali, D.T. (2011). A comparative study of fructose, zinc and copper levels in seminal plasma in fertile and infertile men. *Iraqi Journal of Medical Sciences*, 9(1), 48-54.

Hussain, T., Kandeel, M., Metwally, E., Murtaza, G., Kalhoro, D..H, Yin, Y., Tan, B., Chughtai, M.I., Yaseen, A., Afzal, A.,& Kalhoro, M.S. (2023) Unraveling the harmful effect of oxidative stress on male fertility: A mechanistic insight. *Frontiers in Endocrinology,* 14, 1070692. doi: 10.3389/fendo.2023.1070692

ICN Nutritional Biochemical. Life Sciences Group (1999) Research diets. P.O Box 88050, Cleveland, Ohio.

Kaplan, J. H. and Maryon, E. B. (2016). How mammalian cells acquire copper: An essential but potentially toxic metal. *Biophysical Journal*, 110, 7-13.

Khosrowbeygi, A., Zarghami, N., & Deldar, Y.(2004). Correlation between sperm quality parameters and seminal plasma antioxidants status. *Iranian Journal of Reproductive Medicine*, 2, 58-64.

Kňažická, Z., Lukáčová, J., Greń, A., Formicki, G., Massány,i P., & Lukáč, N.(2013). Relationship between level of copper in bovine seminal plasma and spermatozoa motility. *Journal of Microbiology Biotechnology and Food Sciences*, 2 (Special issue 1), 1351-1362.

Kowal, M., Lenartowicz, M., Pecio, A., Gołas, A., Błaszkiewicz, T., & Styrna, J. (2010). Copper metabolism disorders affect testes structure and gamete quality in male mice. *Systems Biology in Reproductive Medicine*, 56,431–444.

Kuchakulla, M., Narasimman, M., Khodamoradi, K., Khosravizadeh, Z., & Ramasamy, R.(2021). How defective spermatogenesis affects sperm DNA integrity. *Andrologia,* 53(1), e13615. doi: 10.1111/and.13615.

Lehti, M.S. and Sironen, A.(2017).Formation and function of sperm tail structures in association with sperm motility defects, *Biology of Reproduction*, 97(4),522-536.

Leung, M.R., Zeng, J., Wang, X., Roelofs, M.C., Huang, W., Chiozzi, R. Z., Hevler, J. F., Heck, A. J.R., Dutcher, S. K., Brown, A., Zhang, R., & Zeev-Ben-Mordehai, T. (2023). Structural specializations of the sperm tail. *Cell,* 186(13), 2880-2896.

López-Botella, A., Velasco, I., Acién, M., Sáez-Espinosa, P., Todolí-Torró, J.L., Sánchez-Romero, R., & Gómez-Torres, M.J. (2021). Impact of heavy metals on human male fertility-An overview. *Antioxidants (Basel*), 10(9), 1473. doi: 10.3390/ antiox 10091473.

Ogórek, M., Gąsior, Ł., Pierzchała, O., Daszkiewicz, R., & Lenartowicz, M.(2017a). Role of copper in the process of spermatogenesis. *Advances in Hygiene and Experimental Medicine,* 71, 662-680.

Ogórek, M., Herman, S., Pierzchała, O., Bednarz, A., Rajfur, Z., Baster, Z., Grzmil, P., Starzynski, R.R., Szudzik, M., Jonczy, A., Lipinski, P.,& Lenartowicz, M.(2019). Molecular machinery providing copper bioavailability for spermatozoa along the epididymial tubule in mouse. *Biology of Reproduction*, 100(6), 1505 – 1520.

Ogórek, M., Lenartowicz, M., Starzyński, R., Jończy, A., Staroń, R., Doniec, A., Krzeptowski, W., Bednarz, A., Pierzchała, O., Lipiński, P., Rajfur, Z., Baster, ´ Z., Gibas-Tybur, P., & Grzmil, P.(2017b). Atp7a and Atp7b regulate copper homeostasis in developing male germ cells in mice. *Metallomics*, 9, 1288–1303.

Ombelet, W.(2020). WHO fact sheet on infertility gives hope to millions of infertile couples worldwide. *Facts Views & Vision in ObGyn,* 12, 249–251.

Park, K., Jeon, S., Song, Y.J., & Yi, L.S.(2012). Proteomic analysis of boar spermatozoa and quantity changes of superoxide dismutase 1, glutathione peroxidase, and peroxiredoxin 5 during epididymal maturation. *Animal Reproduction Science,* 135, 53–61.

Picco, S., De Luca, J., Mattioli, G., & Dulout, F.(2001). DNA damage induced by copper deficiency in cattle assessed by the Comet assay. *Mutation Research*, 498(1-2), 1–6.

Raad, M. V., Firouzabadi, A.M., Niaki, M. T., Henkel, R., & Fesahat, F. (2024). The impact of mitochondrial impairments on sperm function and male fertility: A systematic review. *Reproductive Biology and Endocrinology*, 22(1), 83. doi: 10.1186/s12958-024-01252-4.

Roy, A., Lin, Y.N., Agno, J.E., DeMayo, F.J., & Matzuk, M.M.(2007). Absence of tektin 4 causes asthenozoospermia and subfertility in male mice. *FASEB Journal,* 21, 1013–1025.

Roy, A., Lin, Y.N., Agno, J.E., Demayo, F.J.,& Matzuk, M.M.(2009). Tektin 3 is required for progressive sperm motility in mice. *Molecular Reproduction & Development,* 76, 453–459.

Roy, D., Dey, S., Majumder, G.C., & Bhattacharyya,D.(2014). Copper: A biphasic regulator of caprine sperm forward progression. *System Biology in Reprodctive Medicine*, 60, 52–57.

Ruiz, L.M., Libedinsky, A., & Elorza, A.A.(2021). Role of copper on mitochondrial function and metabolism. *Frontiers in Molecular Biosciences,* 8, 711227. doi: 10.3389/ fmolb.2021.711227.

Sabeti, P., Pourmasumi, S., Rahiminia, T., Akyash, F., & Talebi, A.R. (2016). Etiologies of sperm oxidative stress. *International Journal of Reproductive BioMedicine*, 14(4), 231-240.

Saini, A., Kurup, A.R.,& Nair, N.(2022). Glutathione and antioxidant study in testes of Wistar rats after dietary copper deficiency. *International Journal of Pharmaceutical Sciences and Research*, 13(4), 1747-1754.

Saini, A. and Nair, N.(2022). Trace element copper in male and female reproduction: A review. *Bulletin of Environment of Pharmacology and Life Sciences*, 11 (9), 15-22.

Sapiro, R., Kostetskii, I., Olds-Clarke, P., Gerton, G.L., Radice, G.L., & Strauss, J.F. III.(2002). Male infertility, impaired sperm motility, and hydrocephalus in mice deficient in sperm-associated antigen 6. *Molecular and Cellular Biology*, 22, 6298– 6305.

Shamsi, M. B., Kumar, R., & Dada, R.(2008). Evaluation of nuclear DNA damage in human spermatozoa in men opting for assisted reproduction. *Indian Journal of Medical Research*, 127, 115–123.

Sharma, P., Kaushal, N., Saleth, L. R., Ghavami, S., Dhingra, S., & Kaur, P. (2023). Oxidative stress-induced apoptosis and autophagy: Balancing the contrary forces in spermatogenesis. *Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease,* 1869(6), 166742, https://doi.org/10.1016/ j.bbadis.2023.166742.

Sigma bulletin.(1998). Use of trypan blue stain and the hemocytometer to determine total cell counts and viable cell number. Product Nos 7154, T6146 and Z35962, 1844-1845.

Skandhan, K.P.(1992). Review on copper in male reproduction and contraception. *Revue Francaise de Gynécolgie et d' Obstétriqu*e , 7, 594-598.

Slivkova, J., Popelkova, M., Massanyi, P., Toporcerova, S., Stawarz, R., Formicki, G., Lukac, N., Putała, A., & Guzik, M.(2009). Concentration of trace elements in human semen and relation to spermatozoa quality. *Journal of*

Environmental Science and Health, Part A: Toxic/Hazardous Substances and Environmental Engineering, 44(4), 370-375.

Tramer, F., Rocco,F., Micali, F., Sandri, G., & Panfili, E.(1998). Antioxidant systems in rat epididymal spermatozoa. *Biology of Reprodction,* 59,753 - 758.

Tvrdá, E., Kňažická, Z., Lukáčová, J., Schneidgenová, M., Massányi, P., Goc, Z., Stawarz, R., & Lukáč, N.(2012). Relationships between iron and copper content, motility characteristics and antioxidant status in bovine seminal plasma. *Journal of Microbiology, Biotechnology and Food Sciences*, 2 (2), 536-547.

Tvrda, E., Peer, R., Sikka, S.C., & Agarwal, A.(2015). Iron and copper in male reproduction: A double-edged sword. *Journal of Assisted Reproduction and Genetics*, 32,3–16.

Unnithan, RR,(1976). A rapid single step stain for mammalian spermatozoa.Indian Journal of Experimental Biology, 14, 611-613.

Van den Berghe, P.V.E. and Klomp, L.W.J.(2010). Post translational regulation of copper transporters. *Journal of Biological and Inorganic Chemistry*, 15, 37-46.

Van Niekerk, F.E. and Van Niekerk, C.H.(1989). The influence of experimentally induced copper deficiency on the fertility of rams. II. Macro- and microscopic changes in the testes. *Journal of the South African Veterinary Association*, 60, 32– 35.

Walke, G., Gaurkar, S.S., Prasad, R., Lohakare, T., & Wanjari, M.(2023). The impact of oxidative stress on male reproductive function: Exploring the role of antioxidant supplementation. *Cureus,* 15(7), e42583. doi: 10.7759/cureus.42583.

Wong, W.Y., Flik, G., Groenen, P.M., Swinkels, D.W., Thomas, C.M., Copius-Peereboom, J.H., Merkus, H.M., & Steegers-Theunissen, R.P.(2001). The impact of calcium, magnesium, zinc, and copper in blood and seminal plasma on semen parameters in men. *Reproductive Toxicology*, 15,131 - 136.

Wyrwoll, M.J., Wabschke, R., Röpke, A., Wöste, M., Ruckert, C., Perrey, S., Rotte, N., Hardy, J., Astica, L., Lupiáñez, D.G., Wistuba, J., Westernströer, B., Schlatt, S., Berman, A.J., Müller, A.M., Kliesch, S., Yatsenko, A.N., Tüttelmann, F., & Friedrich, C.(2022). Analysis of copy number variation in men with non-obstructive azoospermia. *Andrology,* 10, 1593–1604.

Xu, H., Yuan, S.Q., Zheng, Z.H., & Yan, W. (2013).The cytoplasmic droplet may be indicative of sperm motility and normal spermiogenesis. *Asian Journal of Andrology,* 15(6), 799-805.

Zhang, S.H., Chen, H., Ding, X.P., Zhang, S., Chen, H.H.,& Jing, Y.L.(Association of polymorphisms in tektin-t gene with idiopathic asthenozoospermia in Sichuan, China. Journal of Assisted Reproduction and Genetics, 33, 181 - 187.

Zhang, Y., Ou, Y., Cheng, M., Saadi, H.S., Thundathil, J.C., & van der Hoorn, F.A.(2012). KLC3 is involved in sperm tail mid piece formation and sperm function. *Developmental Biology*, 366, 101– 110.

Zhang, Z., Kostetskii, I., Tang, W., Haig-Ladewig, L., Sapiro, R., Wei, Z., Patel, A.M., Bennett, J., Gerton, G.L., Moss, S.B., Radice, G.L., & Strauss, J.F. 3rd.(2006). Deficiency of SPAG16L causes male infertility associated with impaired sperm motility. *Biology of Reproduction*, 74,751-759.

Zhang, Z., Shen, X., Gude, D.R., Wilkinson, B.M., Justice, M.J., Flickinger, C.J., Herr, J.C., Eddy, E.M., & Strauss, J.F 3rd (2009). MEIG1 is essential for spermiogenesis in mice. *Proceedings of the National Academy of Sciences of the United States of America*, 106, 17055-17060.

Zini, A., Bielecki, R., Phang, D., & Zenzes, M. T, (2001). Correlation between two markers of sperm DNA integrity, DNA denaturation and DNA fragmentation, in fertile and infertile men. *Fertility and Sterility,* 75, 674 - 677.

****