Indian J Physiol Pharmacol 2008; 52 (3) : 267–273

# RELEASE OF COPPER FROM CuT 380A CO-INCUBATED WITH HUMAN SEMEN AND ITS EFFECT ON SPERM FUNCTION *IN VITRO*

M. M. MISRO*, S. P. CHAKI, M. CHANDRA, A. MAHESHWARI AND D. NANDAN

*Department of Reproductive Biomedicine,*
*National Institute of Health and Family Welfare,*
*Baba Gang Nath Marg, Munirka, New Delhi – 110 067*

**( Received on April 4, 2008 )**

**Abstract :** Release of copper and its effect on functional integrity of human sperms *in vitro* were assessed following co-incubation of semen with CuT 380A. After 30 min of incubation with semen, release of copper ions from CuT 380A was found to be 9.2 to 40 times higher compared to control incubations with PBS. Sperm function tests, when simultaneously performed following loss of motility in sperms (>95%) after 120 min of copper exposure, depicted a significant (P<0.001) reduction in sperm viability and hypo-osmotic swelling (HOS) response. However, the affected sperm populations revealed no significant alterations in other functional tests like acrosomal status or nuclear chromatin decondensation. It is therefore concluded that the high release of copper from CuT 380A drastically lowers sperm motility. viability and HOS response but only marginally affects the acrosome status or nuclear chromatin condensation in short term incubations.

**Key words :** CuT 380A copper sperm function tests motility viability acrosome status nuclear chromatin decondensation

## INTRODUCTION

Worldwide, an estimated 128 million women use intra uterine devices (IUDs). Copper containing IUDs (CuTs) are considered as one of the most effective reversible methods of birth control in females. The contraceptive efficacy is believed to be basically due to release of copper ions which enhance the inflammatory response that are not conducive to male or female gametes and also to the embryo (1). In addition, there are also direct harmful effects of copper on gamete survival (2). CuTs are reported to increase the copper content of the mucus (3). Migration through cervical mucus is the first obligatory step for the fertilizing sperm and its success is entirely dependant on sperm motility. Sperm motility is routinely examined in human semen as it forms one of the main functional evaluations of sperms *in vitro*. However, there are other sperm function parameters such as plasma membrane integrity, acrosome status and nuclear chromatin condensation which were never analysed

*Corresponding Author : Fax : 91-11-26101623; E-mail : mm_misro@yahoo.com

before with respect to copper exposure *in vitro* (4).

Though toxic effect of copper on motility is more or less established, it is not clear at this stage whether or not this leads to impairment of all other functional characteristics of sperms in short-term incubations. The present study was therefore initiated first, to assess the quantum of release of copper ions in semen co-incubated with CuT 380A and second, to find out the parallel effect on other sperm function parameters when motility is affected due to copper exposure *in vitro*. It is hard to simulate the *in vivo* uterine environment with IUD in situ and study the effect of copper on sperms. Therefore, co-incubation of semen with total copper from IUD 380A was tried *in vitro*. Copper release was assayed utilizing an atomic absorption spectrophotometer (AAS) which was more sensitive than the colorimetric assay reported earlier (2).

## MATERIAL AND METHODS

Semen samples, obtained by masturbation after 3–4 d of sexual abstinence, were collected from twenty-five proven fertile healthy volunteers. Samples were collected in sterile plastic containers and allowed to liquefy at 37°C for 30 min. The CuT 380A is a flexible polyethylene T-shaped body with a copper collar of approximately 67 mg (33 $mm^2$ solid copper sleeves) of copper on each of its transverse arms and 176 mg (314 $mm^2$ copper wire) of copper wire coiled around its vertical shaft. The whole copper (two solid sleeves and one wire) was separated from each CuT and incubated separately with 2 ml of liquefied semen in a standard microfuge tube. Proper care was taken to ensure that the whole copper lies submerged in the semen during the entire incubation period.

### Estimation of copper ions

Five semen samples were utilized for the purpose with PBS serving as control. Semen or PBS with or without CuT 380A was incubated for 30, 60, 90 and 120 mins at 37°C. Three aliquots of each incubation were diluted, filtered and estimated for copper release using an Atomic Absorption Spectrophotometer (AAS), AA6800 (Shimadzu, Japan). Standard solutions of copper was prepared as described in AAS (1983) manual (5).

### Semen preparation

Routine semen analysis was carried out as per World Health Organization (1999) criteria (6) in rest of the 20 samples. Sperm motility was assessed in every 30 min for 180 min at 37°C. Following 120 min of co-incubation with copper, when sperm motility was severely declined, tests for other sperm function parameters like viability, hypo-osmotic swelling, acrosomal status and nuclear chromatin decondensation were simultaneously carried out. In each case, an aliquot of semen sample without CuT was also maintained as control.

### Sperm motility

One drop of liquefied semen was taken on a glass slide (warm at 37°C), covered with a glass coverslip and observed under microscope at 40x objective as per WHO criteria (6). Motility was monitored in every 30 min for 180 min.

**Sperm viability**

Viability was assessed as per WHO criteria (6). In brief, 50 µl of semen was mixed with 100 µl of 1% Eosin Y for 30 seconds. Then 150 µl of 10% Nigrosin solution was added and mixed well. A drop of the semen-Eosin-Nigrosin mixture was placed on a clean glass slide, smeared, air-dried and examined under microscope for live (unstained) or dead (stained) sperms.

**Hypo-osmotic swelling test**

A modified hypo-osmotic swelling (HOS) test protocol was adopted as developed in our laboratory and described (7). In brief, 50 µl of semen was incubated in 500 µl of colored hypo-osmotic swelling solution for 5 min at room temperature. One drop of the incubation mixture was taken on a clean glass slide and examined under a microscope. Percentages of spermatozoa with tail coiling were recorded.

**Nuclear chromatin decondensation test**

Nuclear chromatin decondensation (NCD) test was performed as described earlier (8). In brief, liquefied semen sample was centrifuged and sperm pellet was washed in 0.05 M borate buffer. One volume of sample was incubated with nine volumes of EDTA (6 mM) and SDS (1%) mixture at 37°C for 60 min. The reaction was stopped by addition of an equal volume of 2.5% glutaraldehyde in borate buffer. One drop of the mixture was then taken on a clean glass slide, and examined under a microscope. Percentage of sperms with swollen chromatin was recorded.

**Acrosomal status test**

Acrosomal status (AS) test was carried out as described (4). In brief, Semen was diluted in PBS: D-Glucose (1:20) and equilibrated at 37°C for 30 min. A smear of the diluted mixture was gently made on a gelatin coated glass slide and excess water was evaporated. The slides were incubated at 37°C for 120 min in a humid chamber, air-dried and examined under the microscope. Percentage of spermatozoa with halos surrounding the sperm head was recorded.

**Statistical analysis**

Multiple two-tailed t-test was utilized to find out whether the differences of mean value of parameters between groups are significant using statistical program SPSS. Differences were considered significant when $P<0.05$.

## RESULTS

The routine semen analysis data from the 25 volunteers were shown in Table I. The first five samples were used for the study and estimation of copper release into the semen. The release of copper ions from CuT 380A was 9.2 to 40 times higher when incubated in semen than in PBS. The rate of release of copper from CuT was not uniform and varied with individual semen samples (Fig. 1). Sperms co-incubated with copper showed gradual decline in motility which was significant ($P<0.001$) by 60 min of exposure. More than 95% of sperms were found immotile after 120 min of incubation. The quality of motility in the remaining sperms was very poor and limited to only a

TABLE I : Semen characteristics of proven fertile volunteers.

| Subjects | Volume (ml) | Sperm characteristics | | |
|---|---|---|---|---|
| | | Concen-tration ($x10^6$/ml) | % Total motility | % Normal morphology |
| 1 | 3.2 | 60 | 70 | 40 |
| 2 | 3.5 | 80 | 60 | 45 |
| 3 | 3.0 | 80 | 70 | 50 |
| 4 | 3.5 | 70 | 60 | 44 |
| 5 | 3.0 | 60 | 50 | 40 |
| 6 | 3.5 | 65 | 60 | 47 |
| 7 | 3.7 | 80 | 70 | 50 |
| 8 | 4.0 | 55 | 50 | 44 |
| 9 | 3.0 | 70 | 60 | 40 |
| 10 | 2.5 | 70 | 60 | 48 |
| 11 | 3.5 | 80 | 50 | 40 |
| 12 | 4.1 | 100 | 60 | 45 |
| 13 | 4.2 | 80 | 50 | 40 |
| 14 | 4.0 | 90 | 70 | 50 |
| 15 | 3.5 | 70 | 70 | 53 |
| 16 | 4.5 | 60 | 50 | 42 |
| 17 | 5.0 | 80 | 70 | 50 |
| 18 | 3.5 | 60 | 55 | 40 |
| 19 | 4.0 | 85 | 70 | 40 |
| 20 | 3.1 | 95 | 60 | 40 |
| 21 | 2.5 | 90 | 70 | 43 |
| 22 | 3.4 | 70 | 65 | 47 |
| 23 | 3.5 | 80 | 70 | 45 |
| 24 | 4.0 | 90 | 60 | 45 |
| 25 | 3.7 | 85 | 75 | 40 |

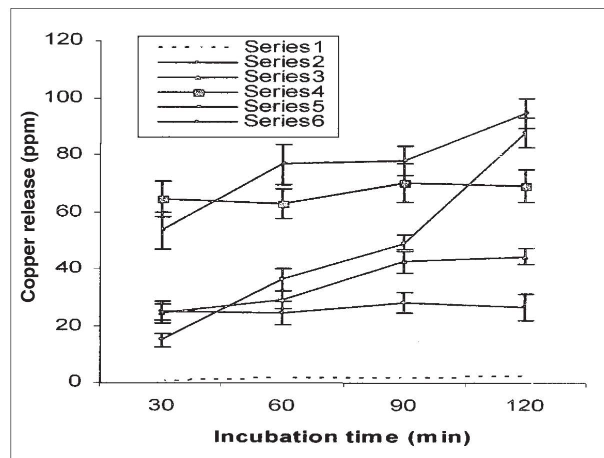


Fig. 1 : Release of copper (ppm) using Atomic absorption spectrophotometer in the five semen incubations (Series 2-6) compared with PBS (Series 1).

vibrating type. Forward progressive motility in all the sperms was completely inhibited. By 150 min, all the sperms were immotile (Fig. 2). The effect on motility was found irreversible following either withdrawal of the copper from semen or re-suspension of the treated sperms in freshly prepared medium, Ham's F-10 or normal untreated seminal plasma till 180 min. With significant reduction in motility (>95%) after 120 min, sperm viability (↓ 66%) and hypo-osmotic swelling response (↓ 59%) were also found significantly (P<0.001) declined (Fig. 3a).

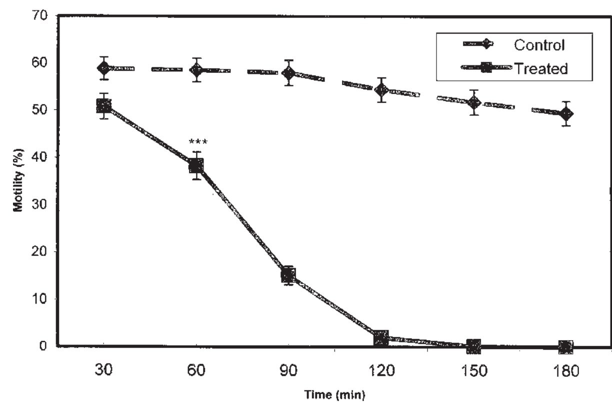


Fig. 2 : Effect of copper on human sperm motility in vitro. Sperm motility was drastically lowered after 120 min of co-incubation with CuT 380A. ***P<0.001.

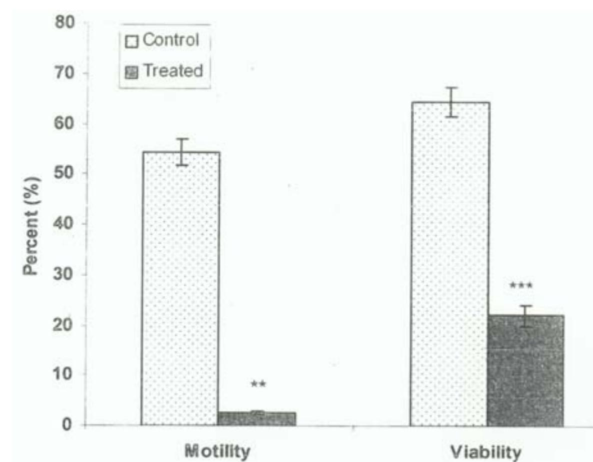


Fig. 3a : Sperm function test of motility and viability after 120 min of incubation of semen with CuT 380A. ***P<0.001.

However, in the same lot of sperms, the decline in the acrosomal status and nuclear chromatin decondensation was 16% and 11% respectively which was not statistically significant compared to controls (Fig. 3b).

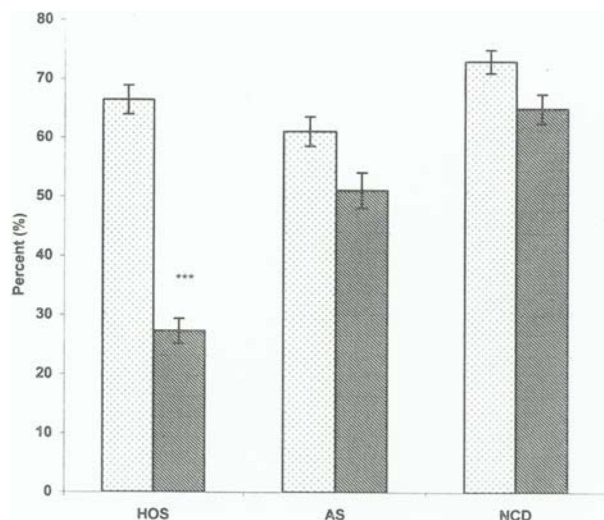


Fig. 3b : Sperm function test of hypoosmotic swelling (HOS), Acrosome status (AS) and Nuclear chromatin decondensation (NCD) after 120 min of incubation of semen with CuT 380A. Insert at the top of each section of the bar shows the type of sperm response (HOS-Tail coiling, AS-Halos surrounding the head and NCD-Chrornatin swelling) after the test. ***P<0.001.

## DISCUSSION

The above data have demonstrated that the release of copper ions from CuT 380A was much higher in semen incubations than in plain PBS. A differential decline in the effect of different sperm functional parameters following exposure to copper was observed. Sperm motility, viability and HOS response are found to be more sensitive and found to be affected in greater percentage of sperms than AS and NCD following 120 min of co-incubation with copper.

The release of copper ions in sperm incubations were reported earlier (2). The concentration of copper ion is directly dependent on the type of medium used for such incubations. In the present study, semen compared to PBS demonstrated 9.2 to 40 times higher release of copper ions (Fig. 1). The reason for this significantly higher release of copper ion is probably due to the higher protein content of the semen sample which increases the rate of dissolution of copper . Such high release of copper 43 μg per 100 mg of copper has been reported with respect to co-incubations of Cu-IUD for 5 h in cervical mucus (9). The wide variations in the rate of release of copper ions in the five semen samples in the present study may be due to individual variations of protein and sperm count in these samples (Fig. 1).

It has been shown that conventional semen analysis is unable to detect the functional deficiencies responsible for lack of fertility (10). Appropriate methods are reported in the literature to test for these deficiencies *in vitro* (4, 7). In the present study we utilized all these methods simultaneously to identify the most sensitive sperm function affected as a result of copper exposure (CuT 380A) with semen that induces significantly higher dissolution of copper ions into the medium.

It is reported that copper is toxic to sperms. Copper has substantial spermicidal properties, which can be enhanced by removing the seminal plasma (11). At the concentration of 100 μg/ml of copper in the BWW culture medium, sperm motility, viability and acrosome reaction and hamster oocyte penetration scores were significantly

affected after 5 h of incubation (12). The mean percentage of penetrated oocytes declined significantly with increasing concentrations of copper in the medium more with the preincubated sperms than with preincubated oocytes. When both gametes are exposed no penetration of oocytes occurred at all even with lowest concentration (10 ng) of copper exposure (12).

Inhibition of sperm motility in vitro by copper wire was also reported earlier (13). But, this is the first time that total copper from the CuT 380A was used to study not only sperm motility but also other functional parameters of sperms simultaneously. Roblero et al. (1996) reported a decline in the progressive sperm motility by 50% of the controls following 100 µg of copper exposure to sperms for 5 h. In our studies, inhibition of motility in more than 95 % of the sperms was seen only after 120 min of co-incubation with copper. Such a fast decline in motility may be attributed to the higher copper content of the CuT 380A and its release into semen, the effect of which on sperm function was never reported before.

A number of investigators have attempted to recover sperms from fallopian tubes in IUD users and non users in a bid to understand the mechanism of action of IUDs and its association with the process of sperm inactivation and subsequent migration through cervical mucus and endometrium (14, 15). Compared to subjects fitted with inert IUDs, no sperm were found near the site of fertilization in women fitted with Multiload-250 (16, 17). In some of the Cu-T users head–tail detachment was also observed in a large proportion of the sperms recovered. It was thus confirmed that there is a tendency for more definite impairment of sperm migration with Cu-T IUDs than with inert IUDs. As sperms in semen loose complete motility following 2 h exposure to CuT (Fig. 2), sperm migration is likely to be affected whether it is *in vitro* or *in vivo*.

The present data also proved the point that sperms those are non-motile may not be necessarily non-viable. A non-viable sperm is non-motile but not vice versa. A fairly good population of sperms (22%) from the motility affected pool continued to be viable by dye exclusion test and still a higher percentage (27%) depicted normal plasma membrane integrity following the HOS test. But the decline in the values for acrosome status and the nuclear chromatin decondensation test was marginal and not statistically significant (Fig. 3b). This drives home the point that acrosomal contents and the nuclear chromatin were only marginally affected by copper ions after a short-term exposure of 120 min. It is however, not clear at this stage, whether or not intra-cytoplasmic sperm injection of these sperms into oocytes will ensure normal fertilization leading to further embryonic growth and development. It was earlier reported that 100 µg $Cu^{2+}$/ml significantly increased the acrosome reaction in the sperms exposed to copper for 5 h (12). In the present work, the acrosome status (AS) or intactness of acrosome but not the acrosome reaction that was examined and the marginal effect of copper on AS, as obtained in the present study, cannot thus be considered as a deviation (12). Moreover, the duration of copper exposure in the present study was limited to only 2 h.

The copper induced immobilization of sperms is completely irreversible. In our case the suspension medium was seminal plasma. But, following immobilization and

replacing the seminal plasma with either medium Ham F-10 (pH 7.2) or fresh untreated seminal plasma did not help in reversing the effect. The effect on motility was reported to be much harsh if the sperms are suspended in any artificial medium (13). The anti-oxidative properties of seminal plasma usually provide added protection to sperms but probably to a certain extent only (18). Beyond that, the sperms are vulnerable first loosing the motility and probably with rest of the functions subsequently.

In conclusion, the present study proves that with the availability of very high concentrations of copper ions in the semen, sperms *in vitro* exhibit differential sensitivity in functional parameters. Sperm motility is the most sensitive functional parameter first to be affected followed by viability and plasma membrane integrity (HOS) following 120 min of copper exposure at 37°C. The other sperm function parameters marginally altered are acrosomal status and nuclear chromatin condensation.

## REFERENCES


1. Ortiz ME, Croxatto HB, Bardin CW. Mechanism of action of intrauterine devices. *Obstet Gynecol Surv* 51(12 Suppl) 1996; 42–51.
2. Holland MK, White IG. Heavy metals and human spermatozoa. III. The toxicity of copper ions for spermatozoa. *Contraception* 1988; 38: 685–695.
3. Hagenfeldt K. Intrauterine contraception with the copper T device. Effect on trace elements in the endometrium, cervical mucus and plasma. *Contraception* 1972; 6: 37–54.
4. Gopalkrishnan K. Standardized procedures in human sperm analysis. *Curr Sci* 1995; 68: 353–362.
5. AAS manual. Phillips Analytical AAS Data Book, Bear Press Limited, Cambridge, UK, 1983.
6. World Health Organization WHO laboratory manual for the examination of human semen and sperm-cervical mucus interaction, Cambridge University Press, Cambridge, UK, 1999.
7. Misro MM, Chaki SP. Development of a rapid, sensitive and reproducible laboratory test kit for the assessment of plasma membrane integrity of human sperms. *Fertil Steril* 2007. (Electronic version ahead of print).
8. Gopalkrishnan K, Hinduja IN, Kumar TC. *In vitro* decondensation of nuclear chromatin of human spermatozoa: assessing fertilizing potential. *Arch Androl* 1991; 27: 43–50.
9. Rush F, Elstein M. The effect of incubating a copper releasing intrauterine device on sperm penetration and spinnbarkheit of cervical mucus. *J Obstet Gynecol (Br. Common)* 1974; 81: 483–485.
10. Aitken RJ. Sperm function tests and fertility. *Int J Andro* 2006; 29: 69–75.
11. Holland MK, White IG. Heavy metals and human spermatozoa: II. The effect of seminal plasma on the toxicity of copper metal for spermatozoa. *Int J Fertil* 1982; 27: 95–99.
12. Roblero L, Guadarrama A, Lopez T, Zegers-Hochschild F. Effect of copper ion on the motility, viability, acrosome reaction and fertilizing capacity of human spermatozoa *in vitro*. *Reprod Fertil Dev* 19996; 8: 871–874.
13. Ullmann G, Hammerstein J. Inhibition of sperm motility *in vitro* by copper wire. *Contraception* 1972; 6: 71–76.
14. Oritz ME, Croxatto HB. Copper-T intrauterine device and levonorgestrel intrauterine system: biological bases of their mechanism of action. *Contraception* 2007; 75: S16–S30.
15. Stanford JB, Mikolajczyk RT. Mechanisms of action of intrauterine devices: Update and estimation of postfertilization effects. *Am J Obstet Gynecol* 2002; 187: 1699–1708.
16. Koch UJ. Sperm migration in the human female genital tract with and without intrauterine devices. *Acta Eur Fertil* 1980; 2: 33–60.
17. Aref I, Kandil O, El-tagi A, Morad MR. Effects of non-medicated and copper IUDs on sperm migration. *Contracept Deliv Syst* 1983; 4: 203–206.
18. Misro MM, Choudhury L, Upreti K, Gautam D, Chaki SP, Mahajan AS, Babbar R. Use of hydrogen peroxide to assess the sperm susceptibility to oxidative stress in subjects presenting a normal semen profile. *Intl J Androl* 2003; 26: 1–6.