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ORIGINAL ARTICLE

Role Of Reactive Oxygen, Nitrogen Intermediates, And Micro-Organisms In The Prevention Of Injuries Related To Copper Intrauterine Devices

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ABSTRACT

Objective: The aim was to study reactive oxygen intermediates (ROI) and reactive nitrogen intermediates (RNI) along with the microbiological flora in intrauterine device users before and after its insertion.
Study and design: Fifty three women seeking a copper intrauterine contraceptive device (Cu-IUD), were included in the study. Microbiological flora were studied from endocervical and uterine aspirates along with reactive oxygen and nitrogen intermediates, before insertion of Cu IUD, and subsequently at one, four, and 12 weeks, post insertion. Twenty subjects were also followed up at 24 weeks.
Results: There was a steady increase in reactive oxygen intermediates at 1, 4, and 12 weeks, though it was not statistically significant ($p>0.5$). On the other hand, there was a decrease in the reactive nitrogen intermediates and L-citrulline levels, which is statistically significant ($p<0.5$). The number of organisms grown at one week post insertion were more than those present during preinsertion, but there was a steady fall in the number observed at four and 12 weeks. The values of RNI and ROI were the same at 24 weeks follow-up as preinsertion in the 20 subjects.
Conclusion: The balance between reactive oxygen and nitrogen intermediates is perhaps responsible for the action of activated macrophages on different organisms. Presence of microorganisms introduced at the time of insertion of an IUD causes a rise in ROI levels and a fall in RNI levels. Presence of copper in copper IUDs perhaps enhances these reactions.
Key words: Intrauterine device, reactive nitrogen intermediates, microbiological flora.

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Introduction

In contrast to previous belief that intrauterine devices (IUD) were associated with an increase in pelvic inflammatory disease (PID)[1], copper IUD seems to carry less risk for PID. The copper in the IUD has been shown to have some bactericidal activity. Mishell et al[2] found a decrease in the incidence of *Gardnerella vaginalis* in copper IUD users. Fiscina et al[3] observed inhibition in the growth of *Neisseria gonorrhoea* with micromolar concentration of copper, and reported about the bactericidal activity of copper in vitro. Elhag et al[4] showed the inhibitory effect of copper on gram positive bacteria and lactobacilli. Kleinman et al[5] demonstrated that *Chlamydia trachomatis* growth could be inhibited in cultured human endometrial cells by copper ions, at concentrations of copper known to be released by the copper IUDs. However, the exact mechanism of antimicrobial action of copper IUD is not known.

The increase in the number of polymorphonuclear leukocytes in the endometrium following IUD insertion has been documented.[6] The cells are activated by various stimuli such as opsonized microorganisms, complement fragments, and by

N. formylated oligopeptides of bacterial origin that are actively secreted or released by the lysis of dead organisms.[7] These activated inflammatory cells release reactive oxygen intermediates (ROI) and reactive nitrogen intermediates (RNI).[8] It has been shown that metals like iron and copper act as catalysts in augmenting the toxicity of reactive oxygen intermediates. Gupta et al[9] observed that the copper levels in the uterine flushings were significantly higher at one week post insertion. Similarly, Nathan and Hibbs[10] have also shown the effect of RNI in the control of infection, and interaction of nitric oxide with microbial enzymes containing iron-sulphydryl proteins that could account for antimicrobial action. ROI and RNI levels have been studied separately [11], [12] and subsequently together[13] in copper IUD users, but, no microbiological studies which have been done in the present study were carried out, and the number of subjects studied is larger, with a longer follow-up.

Subjects and Methods

The subjects for the study included women seeking Cu IUD insertion in the family planning clinic of the department of Obstetrics and Gynaecology, Medical microbiology, Experimental Medicine and Biotechnology, Postgraduate Institute of Medical Education and Research, Chandigarh, India from February 2001 to February 2003 . After routine examination and excluding women with local infection and any contraindication for IUD insertion, swabs were taken from the endocervix. Then the cervix and vagina were cleansed with betadine, and the uterine aspirate was collected. The copper IUD was inserted in the immediate post menstrual phase. All the subjects recruited for the study were followed up at one, four and 12 weeks, and cervical swabs and uterine aspirate were collected on each visit again, in the post menstrual phase. Thus, each subject acted as her own control. A written informed consent was taken from each patient. The plan of work was approved by the Institute Ethics Committee. In twenty subjects, the endocervical swabs and uterine aspirate were collected at 24 weeks also.

Collection of cervical swab samples

After cleansing and draping before any vaginal examination was made, swabs were taken from the endocervix for smear examination and culture for aerobic and anaerobic organisms. Endocervical swabs were collected to screen for the following:

- *Chalamydia trachomatis*
- *Neisseria gonorrhoea*
- *Group B streptococcus*
- *Staphylococcus species*
- *Ureaplasma urealyticum*
- *Gardnerella vaginalis*
- *Aaerobic organisms*

The *Chlamydia trachomatis antigen* was detected in the endocervical epithelial cells collected with the swab by ELISA test, using Micro Track kit Syva company 3403 Verba Bnena Road, Sanjose CA-95-161-9013.

Out of three swabs from the endocervix, one swab was used for detection of pus cells, epithelial cells, aerobic and anaerobic organisms and yeast cells, after staining with Gram's stain. The second swab was inoculated in pleuro pneumonia – like organisms (PPLO) media for detecting genital mycoplasma. The third swab was inoculated on two plates of sheep blood agar for detecting aerobic and anaerobic organisms. New York City media and human blood agar were used for detecting *Neisseria gonorrhoea* and *Gardnerella vaginalis,* respectively. Different organisms were identified according to standard methods. [14]

Collection of uterine aspirate and isolation of cells

After taking the endocervical samples, the vagina and cervix were cleansed and 5ml of sterile normal saline was instilled into the uterine cavity through a polythene cannula. It was aspirated after one minute and collected in a siliconized centrifuge tube. This was followed by copper IUD insertion.

The aspirate was centrifuged at 500 rpm for 10min at 4°C, to separate any tissue debris. The supernatant containing mononuclear cells was separated by density gradient centrifugation, by using a Ficoll isopaque column. Cells in the interface i.e. monocytes and lymphocytes were taken in a separate tube, and suspended in 1 ml minimum essential medium (MEM) (pH 7.2). It was kept for 2 hours at 30°C in 5% CO_2 atmosphere, and viability of monocytes was checked by the trypan blue exclusion test[15]. Cells were identified by esterase staining.[16]

Determination of reactive nitrogen intermediates

Monocytes in the concentration of 1 x 10^6 cells/ml MEM, were centrifuged at 1000 rpm at 4°C for 10 min. The supernatant was collected, and nitrite determination was carried out by using Griess reagent following the methods of Green et al[17], and expressed as nmoles of RNI/10^6 cells.

Estimation of L-citrulline

L-citrulline in the uterine aspirate was determined by the colorimetric method of Boyde et al[18] and expressed as nmoles/10^6 cells.

Determination of reactive oxygen intermediates

Luminol was used as an amplifier in measuring luminol dependent chemiluminescence. To the cells in a 24 well sample plate [1450 – 402 Waller, Finland], 2 ml of fresh MEM was added. Total free oxygen radicals were measured by luminol dependent chemiluminescence.[19] Counting was done for 1 sec and 5 minutes, and basal response was measured after addition of 20μL Luminol. Twenty μL of latex was added to read peak response.

CL Index (chemiluminescence index) = $\frac{\text{Peak CI}}{\text{Basal CI}}$

The uterine aspirate was processed in the same manner as the endocervical specimen, for microbiological studies.

The results were analyzed by using Student's paired t-test and Mcnemar's Chi-square test.

Results

Of the 53 recruited subjects, 45 completed all 4 visits, and 20 subjects came for a 24 weeks of follow up also. Forty nine subject completed one week post insertion follow up. The online statistical programme (GraphPad Instat 3) was used for statistical analysis.

RNI values and L-citrulline in uterine aspirate

A statistically significant decrease in the RNI and L-citrulline values was observed at one week post insertion, as compared with preinsertion values ($p<0.05$). A similar declining trend was observed at four and 12 weeks, and the decrease at all times was statistically significant ($p<0.05$). However, in 20 subjects in whom samples were collected at 24 weeks, the values observed were similar to the preinsertion value (Table/Fig 1 and Table/Fig 2)

Table/Fig 1
RNI, L-citrulline and ROI values in uterine aspirate before and after insertion of Copper IUCD

Weeks	*RNI (Mean ± SD) with 95%CI.*	*L-citrulline (Mean ± SD) With 95%CI*	*ROI (Mean ± SD) With 95%CI*
0	155.35 ± 62.28* (138.59-172.11)	118.33 ± 48.56* (105.23-131.43)	2.9 ± 2.63 (2.2-3.6)
1	128.3 ± 42.61* (116.7-140.2)	104.3 ± 45.03* (91.7-116.9)	3.09 ± 2.79 (2.33-3.85)
4	112.2 ± 37.47* (101.23-123.17)	97.24 ± 46.40* (83.64-110.84)	3.62 ± 3.02 (2.74-4.5)
12	97.47 ± 30.96* (88.42-106.52)	80.80 ± 36.63* (70.1-91)	4.8 ± 4.05 (3.63-5.97)

SD – Standard Deviation, *P < 0.05

Table/Fig 2
RNI, L-citrulline and ROI values at 0 and 24 weeks

	RNI (Mean ± SD) with 95%CI	*L-citrulline(Mean±SD) with 95%CI*	*ROI (Mean ± SD) with 95%CI*
Pre insertion	155.35 ± 62.28 (138-172.11)	118.33 ± 48.56 (105.23-131.43)	2.9 ± 2.63 (2.2-3.6)
6 months	146.33 ± 32.09 (132.36-160.3)	103.1 ± 24.91 (92.27-113.93)	2.26 ± 2.58 (1.14-3.38)
P value	> 0.05 (N.S.)	> 0.05 (N.S.)	> 0.05 (N.S.)

N.S. – Not significant

ROI level in uterine aspirate

The CL Index at one week post insertion was higher than the preinsertion value. However, the difference was not statistically significant ($p>0.05$). A similar rising trend was observed at four and 12 weeks, but the difference was not statistically significant ($p>0.05$). These values at 24 weeks were similar to the values observed before insertion of the device (Table/Fig 1 and Table/Fig 2).

Microbiological studies

On all visits (both before and after CuT insertion), the organisms found in the cervical smear, as well as in the smear of the uterine aspirate, were gram positive and negative bacilli, mainly lacto bacilli, which is a normal commensal in the female genital tract. There were some gram negative and positive cocci also.

Six subjects had grown *Gardnerella vaginalis* on the first visit. One of these did not come for follow up, as she got the CuT removed the same day due to excessive bleeding. The other five were given treatment when they came one week after insertion. In three of these subjects, the endocervical swab was positive for *Gardnerella* on the 4 week visit also, but negative in the subsequent samples. Three had the growth at one week, two in the cervical swab, and one in both the cervical swab and uterine aspirate.

The total number of subjects having growth of organisms, increased at one week from 29 to 32 in the cervical swab, and from 13 to 15 in the uterine aspirate though most of these were normal commensals. The same subjects when followed up at a 4 week period, showed that only 13 had growth in cervical swab, and 3 subjects in uterine aspirate. A similar decrease was observed at 12 weeks, when 7 had the growth in cervical swab, and 2 in the uterine aspirate. However, the decrease in the number of subjects having growth of organisms when compared to those having no growth, was not found statistically significant by using Mcnmars Chi-squre test ($p<0.05$) in both cervical swab and uterine aspirate.

Many subjects who had no growth at preinsertion visit (24 had no growth in cervical swab and 40 had no growth in uterine aspirate), showed a growth of organisms in their subsequent cervical and uterine samples. However, the difference in the number of subjects with growth in their 1, 4 and 12 weekly visit was not statistically significant.
The total number of women having growth at each visit did not show any significant increasing trend (Table/Fig 3 and Table/ Fig 4).

Table/Fig 3
No. of subjects with growth of organisms in cervical swab

N – 53		*1 wk n = 49*	*4 wks n = 46*	*12 wks n = 45*	*24 wks n = 20*
29 subjects with growth at 0 wks	With growth	21	12	06	01
	No growth	08	15	18	08
24 subjects with no growth at 0 wks	With growth	11	01	01	02
	No growth	09	18	20	09

Table/Fig 4
No. of subjects with growth of organisms in uterine aspirate

N – 53		*1 wk n = 49*	*4 wks n = 46*	*12 wks n = 45*	*24 wks n = 20*
Subjects with growth at 0 wks (N = 13)	With growth	09	02	01	01
	No growth	04	09	09	08
Subject with no growth at 0 wks (N = 40)	With growth	06	01	01	01
	No growth	30	34	34	10

Discussion

Copper IUD is an effective, safe, and convenient contraceptive method, and is one of the popular methods of temporary contraception used today. [1] The association of PID with IUD use is still controversial. Moyer and Mishell showed that there is an increased number of polymorphonuclear cells in IUD users.[6] However, the local inflammation in the endometrium following IUD insertion may cause the inflammatory cells to release reactive oxygen intermediates and nitrogen intermediates, thereby causing oxidative stress.

In the present study, we observed a steady and significant increase in the levels of CL index at 4 and 12 weeks, indicating an increased production of ROI at this time. Our results are in accordance with those of Amla et al.[11] At the same time, a steady decline in RNI levels was observed at 1, 4 and 12 weeks, post insertion. Anjalika et al[13], who studied RNI levels in Cu IUD users, also reported similar results. The findings of the present study corroborate the observations made by these two groups of workers. However, it was also observed that CL index and RNI values at 24 weeks, were similar to the values observed preinsertion. This corroborates the findings of Moyer et al[6], who studied the endometrium at intervals up to 5 years after insertion, and observed that the changes reverted back to normal at 6 months.

The fall observed in the RNI levels may be due to an increased local inflammation in the endometrium due to Cu IUD insertion, as well as due to the increase in the number of organisms post insertion. It has been reported that the phagocytic respiratory burst with production of superoxide anion, is a critical component of host defense against infection.[20] The enzymatic pathways leading to synthesis of ROI and RNI are

distinct, and the end products can influence each other.[21] The superoxide anion then produced, may rapidly inactivate nitric oxide produced by the cells. The other manner could be by the activation of cAMP, which decreases cGMP, which forms the basis of nitric oxide activity.[22]

This observation, that synergistic antimicrobial activity results, perhaps, from the combination of the phagocytic respiratory burst and the nitric oxide synthase interaction in vivo, is further strengthened by our findings, that the increased growth of organisms, immediately following Cu IUD insertion, was attenuated significantly during the 12 week post insertion period. Timothy et al[23] and Mishell et al[2] reported that copper enhances the microbicidal activity of an IUD, but did not comment on the mechanism. In another study, Gupta et al[9] have shown that there is increase in copper levels in the uterine aspirate, which is associated with a rise in ROI levels, following Cu IUD insertion. Inorganic mediators like ROI and RNI released by the activated inflammatory cells, could be responsible for the microbicidal action of copper in the IUD.

The progressive fall in RNI levels, along with a decrease in the number of the growth of organisms, as well as a concomitant increase in ROI levels as observed in our study, indicate the control of infection due to activation of macrophages, thereby leading to a fall in RNI levels. The number of subjects whose cervical swabs/uterine aspirate showed growth of organisms, were found to be negative for all pathogenic organisms, except one subject who had persistently grown chlamydia at 12 and 24 weeks, and was given antibiotics. Also though 45.4% and 54.7% subjects had positive cultures for mycoplasma and ureaplasma urealyticum respectively, both these organisms were found in a considerable number of sexually active health adults, and all these subjects were also asymptomatic.

Hence, the hypothesis that the radicals NO and superoxide anion play an important role in biological regulation and superoxide anion thus produced, may give rise to other forms of ROI that may serve as mediators in regulatory process. In bacteria and mammalian cells, most of the redox-sensitive regulatory mechanisms serve to protect against oxidative stress, and to re-establish redox homeostasis.

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