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Research Article

The Toxicological Impact of Copper in Uterine Leiomyomas and its Influence on the Hormonal Activity in Indian Women

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ABSTRACT

Copper (Cu) is an essential trace metal that is necessary for most animals including humans. Cu is closely related to estrogen metabolism, and Cu toxicity in women is often found to be related to estrogen dominance. The aim of the present research work is to investigate the contribution of Cu in the initiation and progression of uterine leiomyomas (UL) and its influence on the altering hormonal activity. The Cu levels in 148 uterine tissues which includes 98 leiomyoma samples (intramural and subserosol leiomyoma) and 50 control samples of premenopausal women aged 20-50 years were analysed by Inductively Coupled Plasma – Atomic Emission Spectroscopy (ICP-AES) method. The blood samples drawn from the above subjects were analyzed by Chemiluminescent Microparticle Immunoassay (CMIA) technology to estimate E2 level. The patient categorisation was done on BMI basis which showed a positive relationship with occurrence of disease. The results of analysed samples show significantly higher levels of Cu in the leiomyoma tissues than in normal samples. The statistical results obtained revealed that obese women showed higher Cu and E2 concentration than their counterparts. A positive correlation was found between the E2 level of the subjects and the Cu concentration in UL and control samples**.** A case – control study was conducted to further evaluate the sociodemographic data obtained from patients. The elevated Cu levels may suggest an independent, positive association with risk for clinically detected uterine leiomyomas.

Keywords: Uterine Leiomyomas, Copper, Estrogen, ICP-AES, CMIA.

INTRODUCTION

Copper Toxicity is a condition that is increasingly common in this day and age, due to the widespread occurrence of Cu in our food, hot water pipes, along with the common nutritional deficiencies in zinc, manganese and other trace minerals that keep levels of Cu from getting too high. Although of paramount importance in normal homeostasis, especially with regard to haemoglobin, Cu is necessary only in minute amounts in comparison with other minerals such as iron and zinc. Although the role of Cu in tumor formation has yet to be adequately explained, elevated Cu is related to cancer, either as a possible cause or as a sign of malignancy. A physiological feature of many tumor tissues and cells is the tendency to accumulate high concentrations of Cu. Researchers detected a significant increase in serum Cu in patients suffering from certain cancers[1].

Estrogen and Cu are succinctly related. Cu tends to raise estrogen in the body, and estrogen tends to cause Cu to rise. Both Cu and estrogen tend to feed one another. The use of birth control pills increases a woman's risk of having a Cu toxicity condition due to estrogen's effect of increasing Cu retention in the kidneys[2]. Estrogen overstimulates Aldosterone receptors in the kidneys, increasing sodium, copper and water retention. Both estrogen and Cu tend to raise the blood pressure by increasing water retention, raising the blood volume. Cu builds up first in the liver and disrupts the liver's ability to detoxify the blood in general. This Cu toxicity in the liver therefore disrupts the Liver's ability to detoxify excess estrogen and other toxic heavy metals from the body by blocking zinc in the binding sites of metallothionein and other zinc dependent Liver enzymes.

Other sources of chemicals which mimic estrogen, known as xenoestrogens, may also increase the retention of Cu. These include pesticides, Volatile Organic Compounds (VOC's), growth hormones used on animals, and all petrochemical waste products used in the manufacturing of plastic, gasoline and other petrochemical derivatives. One of the effects of Xenoestrogens is to reduce the excretion rate of Cu from the body. All estrogens cause Cu accumulation, xenoestrogens look like estrogens and hence also cause Cu retention. This leads to very high Cu levels in the body. Copper Intra Uterine Devices (IUDs) also contribute to excess Cu in the body. Copper IUDs work by releasing small but significant amount of Cu into the uterus. This Cu enters into the blood causing all sorts of problems. Consistently high levels of Cu have been found in many types of human cancers, including breast, prostate, colon, lung and brain[3,4].

There are long term consequences of both Cu toxicity and excess estrogen. Estrogenic steroids have been shown to increase Ceruloplasmin which is a Cu carrying protein in the blood of women taking oral contraceptives[5]. Side effects of these elevated Cu levels are common, with hypertension being the most frequent manifestation. Research by Horwitt and his co-workers[6] revealed the rise in Ceruloplasmin and blood pressure exhibited by oral contraceptive users is directly related to the potency of the estrogen contained in the contraceptive.

Recent results obtained indicate that trace levels of Cu enhances the biological damage caused by O_2[7]. Accumulation of Cu traces in a given organ may cause intracellular damage, ultimately expressing itself in cancerous lesions. In order to be able to shed light on the role of Cu in the progression of UL which is a benign tumour of the uterus, it was sought to investigate its association in the formation of the tumour. It is critical to identify the roles of the potentially carcinogenic metals in the etiology of UL, since it remains the leading cause of hysterectomy (removal of the entire uterus) for women.

The current paper aims at investigating the contribution of Cu in the initiation and progression of UL and its possible association with hormonal alterations. Given the relation between Cu and estrogen, we hypothesized that Cu would be positively associated with an increased odds of UL.

MATERIALS AND METHODS

Study population, cases and controls

The study population consisted of premenopausal women under the care of gynaecologists at Beams Fibroid centre, Bandra. All the uterine tissue samples were collected during the period of June 2009 till April 2010. Out of 250 patients a total of 148 (59%) were eligible for the research study, 98 (66%) being leiomyoma samples and 50 (34%) control samples.

Cases were first diagnosed with UL on the basis of symptoms or abnormal pelvic examination or by MRI scan. The patients considered were of 20-40 years of age who were undergoing Myomectomy for removal of fibroids. In order to focus on a population most likely to develop uterine leiomyoma, postmenopausal women were excluded. Patients were considered to be postmenopausal if the date of the last menstrual period was more than six months and the records did not mention any situations or conditions that induce suppression of menses. The other category of patients excluded were those who were pregnant, suffering from diseases like diabetes, hypertension, thyroid, etc. The women who had uterine cancer were also excluded from the current study population. Controls were women of above age 40 who was undergoing hysterectomy for removal of entire uterus. In addition these patient's medical records had to have no mention of confirmed or suspected uterine leiomyoma. The blood samples of all the above mentioned 148 subjects were collected and analysed for the estimation of estradiol (E2).

A structured questionnaire was collected regarding the sociodemographic data like date of birth, marital status, medical history, menstrual patterns, use of oral contraceptives, family history of hysterectomy due to UL, smoking history, use of cosmetics, height and body weight. A survey regarding the diet of the patients was also conducted.

The study protocol and the questionnaire were approved by the hospital and institution's Ethics Committee. The patient consent form was obtained before collecting the samples.

Sample Collection and storage

The uterine tissue samples of controls and the UL were collected directly from the operation table of Beams Fibroid centre, Mumbai, India. These samples were stored in labelled, pre-treated and steam sterilized vials. These vials were stored at -10°C to -20°C in a deep freeze. The tissue samples were dried by Lyophilisation method to remove moisture at KEM Hospital, Mumbai. The dried samples were ground to fine powder using mortar and pestle. The powdered tissue samples were pretreated for analysis of Cu by using conc. HNO_3 and conc. $HClO_4$ in 1:1 ratio. This solution was diluted upto 25 mL. The diluted solution of samples was used for analysis. The concentration of Cu was determined by using ICP-AES technique.

The blood samples of controls and the leiomyoma patients were collected before the surgery. This was allowed to clot for 1 hour at room temperature. The clotted blood samples were centrifuged at 1000 rpm for 10 mins. The serum was removed using a micropipette and stored in sterilized and labeled vials at 5°C. These serum samples were subjected to E2 level detection. The Architect i2000 SR was used as a Chemiluminescent Microparticle Immunoassay (CMIA) technique for quantitative determination of E2 level in the serum samples.

Instrumentation

Estimation of Copper

The concentration of Copper was determined using ICP-AES technique. Among the vast analytical techniques available for analysis of metals, Inductively Coupled Plasma Atomic Emission Spectroscopy (ICP-AES) has enhanced advantage over other methods. It is an emission spectrophotometric technique, exploiting the fact that excited electrons emit energy at a given wavelength as they return to ground state after excitation by high temperature Argon Plasma. It is a multielement analysis technique with highly effective multi-element detection source. Furthermore, it is beneficial for all types of watery and solid samples. The instrument has detection limits of elements up to ppb level. The instrument used for the current project was ARCOS Spectro (Germany) at IIT, Mumbai. Commercially available standards (MERCK multielemental standard) were used to calibrate the instrument. The sample solutions were analysed in triplicate series. The operating parameters of ARCOS Spectro which were followed during the experimental procedure have been given in Table 1.

Estimation of estradiol (E2)

The serum samples were analysed for the concentration of Estradiol (E2) at Microlabs, Mumbai. The *ARCHITECT Estradiol* assay is a delayed one step immunoassay used for the quantitative determination of estradiol in human serum and plasma using Chemiluminescent Microparticle Immunoassay (CMIA) technology. It provides immunoassay testing with increased sample and reagent capacity. It has the best precision and accuracy of the assays evaluated for measurement of serum estradiol concentrations **[8]**. The Instrument used for the estimation of serum estradiol in the present study was ARCHITECT i2000 SR (Abbott) at Microlabs Ltd, Mumbai.

Patient Characterisation

Epidemiologic studies show mixed results with respect to the association between BMI and uterine leiomyoma[9,10]. In the present research work, the patients were categorized as Underweight, Normal, Overweight and Obese patients as illustrated in Table 2. The calculated data suggests that higher BMI might be associated with the higher occurrence of the disease **($P < 0.05$).**

Statistical Analysis

In the current research work statistical evaluation were carried out by using GraphPad Prism (version 5). Data from the experiment were analysed using unpaired t-test. The groups categorised on the basis of BMI were investigated for its significance by Analysis of Variance. Statistical significance was set at 95% confidence level. Also Spearman correlation was used to compare the levels of Cu as well as E2 in controls and diseased samples. A model with all potential confounders included was built by calculating the odds ratio (OR) and adjusted to 95% confidence interval (CI) to evaluate the related risk factor of sociodemographic data and the incidence of UL.

RESULTS

The Mean ±SD of the Cu and E2 concentration in the leiomyoma samples and control samples as per the BMI categorisation have been illustrated in Table 3 and Table 4 respectively.

The total mean concentration of Cu observed in control samples which include Underweight, Normal, Overweight and Obese women were 1.83 ± 0.87. The mean concentration of the leiomyoma samples which comprises of both intramural leiomyoma and subserosol leiomyoma samples of all the four categories of women were 6.29 ± 2.37. It has been observed from the results that the Cu concentration was significantly higher in UL samples than in the controls.

The concentration of Cu (Mean ± SD) when calculated individually for intramural and subserosol leiomyoma samples showed that the average concentration of Cu in subserosol leiomyoma (8.20 ± 1.07) was much higher as compared to intramural leiomyoma (4.13 ± 1.36) samples. The result reveals that the average concentration of Cu in the diseased tissue was higher ($P < 0.05$) than the control samples. The Cu concentration in the leiomyoma samples was found to be in the order of Underweight (5.07 ± 2.27) < Normal (5.68 ± 2.24) < Overweight (6.28 ± 2.24) < Obese (7.89 ± 1.84).

The Mean ± SD calculated for the E2 levels in the blood samples of the women revealed that the average E2 concentration in controls were of the order of Underweight < Normal < Overweight > Obese. The total mean concentration of E2 levels in the controls of all the four categories was 193.82 ± 61.57. The average E2 levels in the leiomyoma patients which includes women with intramural and subserosol leiomyomas was found to be 543.68 ± 127.55. When calculated individually, the average concentration of E2 in intramural leiomyoma patients and subserosol leiomyoma patients was 429.56 ± 71.88 and 644.63 ± 65.17 respectively. The above mentioned results show an increased level of E2 in leiomyoma patients than in controls. The results portray an increased level of E2 in women with subserosol leiomyoma patients than in intramural leiomyoma patients. The results calculated showed that the E2 concentration in the leiomyoma patients were in the order of Underweight (474.63 ± 101.66) < Normal (489.40 ± 126.66) < Overweight (569.68 ± 98.95) < Obese (633.03 ± 111.52).

The Influence of BMI on the Cu levels and E2 levels in control and leiomyoma patients (Intramural leiomyoma and subserosol leiomyoma) has been shown in Figure 1 and Figure 2 respectively.

Finally Spearman correlation test showed a significant relationship between Cu concentration and E2 levels in women with UL ($r = 0.984$, $P < 0.0001$) and controls ($r = 0.866$, $P < 0.0001$). Table 5 illustrates the results of the correlation of Cu with E2 in Underweight, Normal, Overweight and Obese group of patients. The data shown is indicative that the E2 level has positive correlation with the concentration of Cu. The data of the altering distributions of Cu concentration and E2 concentration in normal and diseased tissues have been graphically represented in Figure 3, Figure 4 and Figure 5. A positive correlation was observed between the Cu concentration and E2 level in the controls as well as cases with intramural and subserosol leiomyomas.

Table 6 demonstrates the association of UL with the sociodemographic characteristics obtained from the leiomyoma patients and controls. According to the sociodemographic survey it was observed that married women have a low risk factor of suffering from UL when compared to unmarried females (OR = 0.42. 95% CI = 0.17 - 1.06). It was observed that women with irregular menstrual pattern (84%) showed greater incidences of UL than controls (OR =29.05. 95% CI = 11.40 - 73.98) and 53% of the cases had a history of maternal hysterectomy due to UL (OR = 4.52. 95% CI = 2.03- 10.04).

From the diet survey report obtained from the subjects (Table 7), it was found that 71.4% of the patients were Vegetarians and had higher risk of suffering from UL (OR = 4.07. 95% CI = 1.98 – 8.37). 56 % of the cases used copper cookwares and were found to have an increased risk factor when compared to controls (OR = 1.50. 95% CI = 0.75- 2.97). Hence the results show that the source of Cu bioaccumulation in the tissues might be due to the higher intake of vegetarian diets and using copper cookwares.

DISCUSSION

Elevated Cu level produces a number of symptoms. The current environmental and social climate suggests that the incidences of Cu precipitated disease are on rise. Cu ions are a general stimulant of nervous tissue, as well as of most tissues of the body. Cu imbalance impairs the immune system. Elevated Cu on a hair mineral analysis is often related to a tendency for infections and even cancer. It has been discovered that Cu is the cause of Histapenia, a schizophrenia-like disorder characterized by high serum copper and low serum histamine levels[11]. The current study provides data on the altering Cu levels in human uterine tissue samples. It was observed that in the diseased tissue, which includes intramural and subserosol leiomyoma samples, the mean concentration of Cu was significantly higher as compared to the control tissues.

Obesity and overweight are major contributors to the global burden of chronic diseases and are associated with diabetes, hyper-insulinemia, insulin resistance, coronary heart disease, high blood pressure, stroke, gout, liver disease, asthma and pulmonary problems, gall bladder disease, kidney disease, reproductive problems, osteoarthritis, and some forms of cancer[12,13]**.** High fat volume is associated with elevated levels of serum estrogen [14]. It was observed in the current research paper that there was an increased UL risk among women in the upper quartile of the BMI. Overall findings for BMI and risk of UL showed an inverse J-shaped pattern for all categories of BMI above 18.4 kg/m^2 and a peak incidence associated with a BMI category of more than 25 kg/m^2. These results are consistent with previous studies that found a positive association of UL with the higher BMI[15].

The levels of estrogen and Cu have a direct relationship. This is one reason many women are estrogen dominant. This imbalance is related to tumour formation because estrogen is a potent carcinogen. The present study has examined the association of Cu and its correlation with the increased E2 level in the patients, finding that there was a positive correlation between the two. The present study indicates possible association of Cu and its incidence in the formation of UL. As UL have elevated estrogen levels[16], and as Cu also increases estrogen levels, we hypothesised that the increased concentration of Cu in the diseased samples might be a reason for the leiomyoma formation. The small difference in the concentration of Cu can alter the hormonal level which initiates the leiomyoma formation. The result shows that the concentration of Cu is significantly higher in subserosol leiomyomas as compared to intramural leiomyomas. This might indicate that women with higher level of tissue Cu deposition are more prone to suffer from subserosol leiomyomas.

Foods high in Cu are nuts, liver, mushrooms, oysters, and wheat germ. Other sources of copper are copper cookware, dental materials, vitamin pills, fungicides and pesticide residue on food. Deficiencies of manganese, iron, B-vitamins and vitamin C can cause Cu to accumulate. In the current research paper, when a case – control study was conducted it was found that leiomyoma patients were consuming more vegetarian diets as compared to controls. Also, the percentage of patients using copper cookwares was also higher. The above mentioned source is indicative of higher Cu concentration in the cases when compared to controls. Women on oral contraceptives have considerably increased serum copper concentrations[17] and accumulated more Cu, which curiously is likely to be beneficial in the event of pregnancy. Release of Cu from intra uterine contraceptive devices containing Cu has been shown to be significant, amounting to about 10 mg Cu per year[18]**.** In the present work, the survey conducted revealed a slight significant difference between patients using intra uterine devices and oral contraceptives than patients not using it.

The involvement of Cu ions in biological damage may be proved since most of the Cu found in tissues is specifically bound, comprising an integral part of various enzymes. In the present study, we observed an increase in the total tissue copper content which may represent a considerable increase in the nonspecific complexed Cu that is responsible for the biological damage.

Limitations to be considered for the present study as the sample size may have been too small to provide adequate statistical power to detect meaningful differences between the values compared. The results cannot be extrapolated to general population as this was a hospital based study.

CONCLUSION

Although Cu is an essential trace mineral, a serious health threat is posed by the excess intake of the metal. This study confirmed the existence of a substantially higher risk of UL in the presence of the heavy metal Cu. The results may imply that Cu, by way of accumulation in the uterus and metalloestrogenic activity contribute to the generation of hormone-dependent UL. While existing data are very limited, it is encouraging that Cu identified in this study is important in distinguishing between normal and diseased tissues. The amount of Cu in UL is augmented when compared to controls specimens and it proves that the incidences of elevated Cu act as a potential carcinogen in the etiology of UL. Its influence in uterine tissue is very much evident in causing intramural and subserosol leiomyomas. The interpretation of results could help to understand the potential role of Cu toxicity in elevating the levels of E2 in patients diagonised with UL. Also a significant relationship was found between increased BMI and formation of UL. From the above mentioned studies, it provides evidence for a possible link between Cu and incidence of UL, and identifies priority areas that should motivate further studies.

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Table 1: ICP-AES instrument characteristics and operating parameters

Parameters	Setting
RF Generator	1000 watts
Power Required	220±10 V
Flame Temperature	11000 K
Plasma	Argon
Spectra Range	189-800 nm
Coolant Flow	12 L/min
Auxillary Flow	1 L/min
Nebulizer	0.8L/min
Sensitivity	ppb level of detection

Table 2: Categorization of the samples on the basis of BMI criteria of the patients

Category	BMI (Kg/m^2)	Cases		Controls	
		No.	%	No.	%
Underweight	>18.4	22	22.44	12	24
Normal	18.5 – 22.9	27	27.55	13	26
Overweight	23 – 24.9	22	22.44	11	22
Obese	<25	27	27.55	14	28
Total		98	99.98	50	100

Table 3: The Concentration of Cu (Mean ±SD) of the samples on the basis of BMI criteria of the patients

Category	BMI(kg/m²)	Leiomyoma Samples		Controls (n = 50)
		Intramural (n = 46)	Subserosol (n = 52)	
Underweight	>18.4	3.00 ± 0.80	7.15 ± 0.88	0.98 ± 0.60
Normal	18.5 – 22.9	3.51 ± 0.77	7.69 ± 0.63	1.21 ± 0.42
Overweight	23 – 24.9	3.99 ± 0.78	8.19 ± 0.60	2.34 ± 0.45
Obese	< 25	5.95 ± 0.65	9.45 ± 0.39	2.73 ± 0.29

Table 4: The Concentration of E2 (Mean ±SD) of the samples on the basis of BMI criteria of the patients

Category	BMI(kg/m²)	Leiomyoma Samples		Controls (n = 50)
		Intramural (n = 46)	Subserosol (n = 52)	
Underweight	>18.4	376.27 ± 12.59	573 ± 13.68	111.25± 4.69
Normal	18.5 – 22.9	364.38 ± 40.07	605.5 ± 21.52	166.84 ± 29.19
Overweight	23 – 24.9	471.10 ± 41.33	651.83± 33.15	258.27 ± 28.38
Obese	< 25	514.41± 22.17	727.93 ± 31.65	239 ± 11.42

Table 5: Correlation coefficient (r) of Cu and E2 on the basis of BMI criteria of the patients

Patient Groups	Underweight (< 18.4)	Normal (18.5 – 22.9)	Overweight (23 – 24.9)	Obese (> 25)
Controls	r = 0.996 P < 0.0001	r = 0.994 P < 0.0001	r = 0.998 P < 0.0001	r = 0.989 P < 0.0001
Intramural leiomyoma	r = 0.993 P < 0.0001	r = 0.999 P < 0.0001	r = 1.000 P < 0.0001	r = 1.000 P < 0.0001
Subserosol leiomyoma	r = 0.998 P < 0.0001	r = 0.997 P < 0.0001	r = 0.998 P < 0.0001	r = 0.999 P < 0.0001

Table 6: Study characteristics of cases with UL and controls according to the sociodemographic survey

Sociodemographic Data	Inference	Cases (n = 98)		Controls (n = 50)		Odds Ratio (95% CI)
		No.	%	No.	%	
Marital status	Married Never married	71 27	72.4 27.5	43 7	86 14	0.42 (0.17 - 1.06)
Smoking status	Yes No	24 74	24.4 75.5	14 36	28 72	0.83 (0.38 - 1.80)
Alcohol use	Yes No	29 69	29.5 70.4	21 29	42 58	0.58 (0.28 - 1.18)
Menstrual Irregularity	Yes No	83 15	84 15.3	8 42	16 84	29.05 (11.40-73.98)
Use of birth control pills	Yes No	40 58	40.8 59.1	25 25	50 50	0.68 (0.34 - 1.36)
Use of Cosmetics	Yes No	50 48	51 49	26 24	52 48	0.96 (0.48 - 1.90)
Maternal hysterectomy due to uterine leiomyoma	Yes No	52 46	53 47	10 40	20 80	4.52 (2.03- 10.04)

Table 7: Study characteristics of cases with UL and controls according to diet survey

Diet Survey	Inference	Cases (n = 98)		Controls (n = 50)		Odds Ratio (95% CI)
		No.	%	No.	%	
Type of Diet	Vegetarian Non Vegetarian	70 28	71.4 28.5	19 31	38 31.6	4.07 (1.98 - 8.37)
Use copper cookwares	Yes No	55 43	56.1 43.8	23 27	46 27.5	1.50 (0.75- 2.97)
Eat Chocolates frequently	Yes No	83 15	84.6 15.3	36 14	72 14.2	2.15 (0.94- 4.91)
Use Tap Water	Yes No	21 77	21.4 78.5	15 35	15.3 35.7	0.63 (0.29- 1.37)
Use Intra Uterine Devices	Yes No	45 53	45.9 54	20 30	40 60	1.27 (0.63- 2.54)
Use Oral Contraceptives	Yes No	49 49	50 50	24 26	48 52	1.08 (0.54- 2.14)

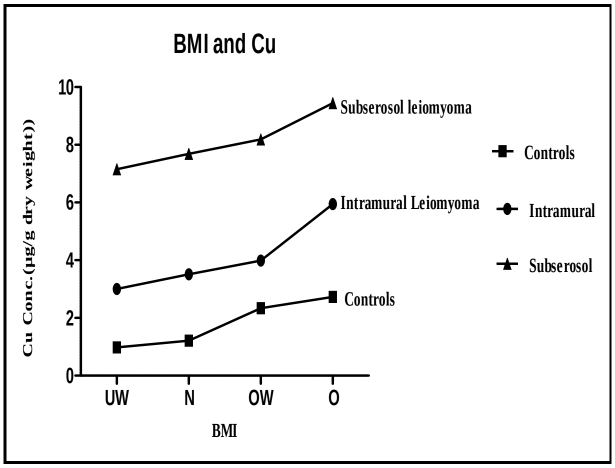

Fig. 1: Influence of BMI on the concentration of Cu in the leiomyoma and control samples

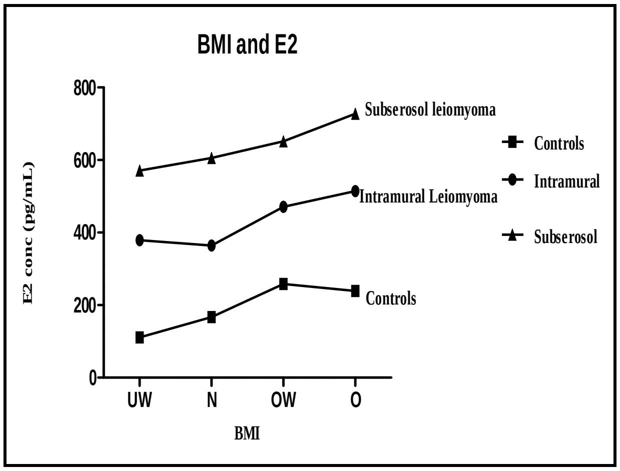

Fig. 2: Influence of BMI on the E2 levels in the leiomyoma patients and controls

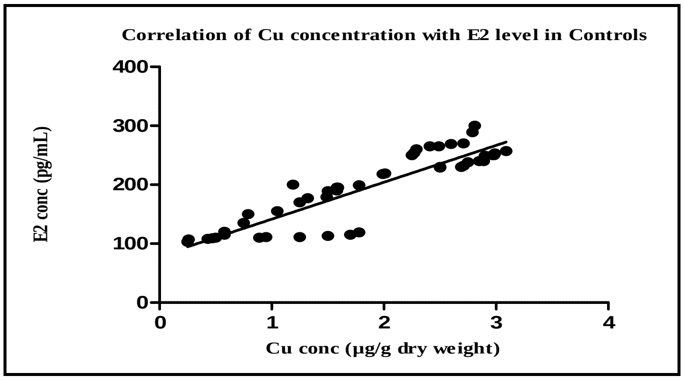

Fig. 3: Correlation of Cu concentration with E2 levels in control

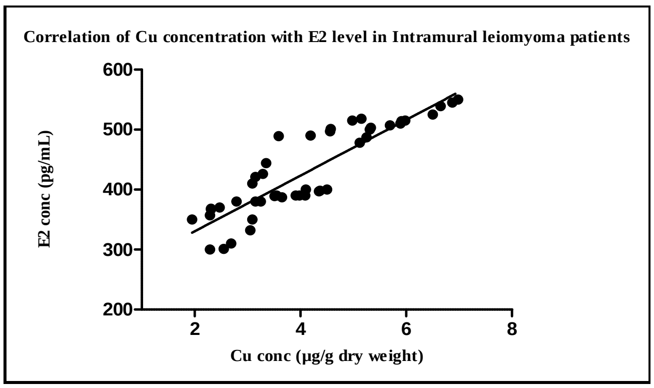

Fig. 4: Correlation of Cu concentration with E2 levels in Intramural leiomyoma Patients

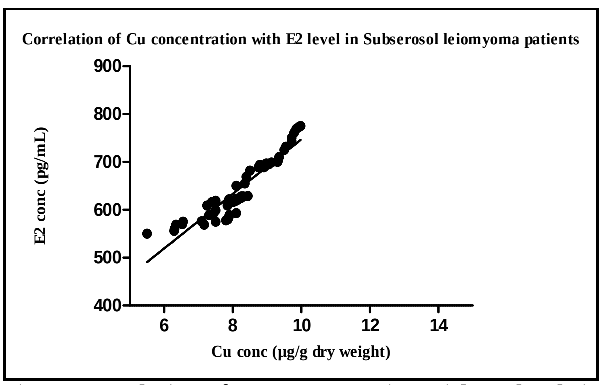

Fig. 5: Correlation of Cu concentration with E2 levels in Subserosol leiomyoma Patients

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