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Quantitative analysis of Copper in arthritic joint effusion and its correlation with Allopathy and Ayurvedic medicinal system.

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Copper being an essential element in human biology, its relevance in joint effusion has been served to be a non-invasive diagnostic tool for the characterization of joint diseases. The main objective of present work was to establish the correlation between the levels of copper in the synovial fluids (SF) of patients consuming Antiarthritic Ayurvedic drugs (AAD) and Allopathy medicine. Arthrocentesis was adopted to acquire the samples of joint effusion from proved cases of Osteoarthritis (OA), Rheumatoid arthritis (RA) and Bursitis patients. Patient's population was divided into two categories based on the intake of Allopathy and Ayurvedic Drugs. The control comprised of healthy adult volunteers. The concentrations of copper in joint fluid of patients were assessed by Inductively Coupled Plasma Atomic Emission Spectroscopy (ICP-AES) analytical technique. It was observed that the patients under the therapy of Antiarthritic Ayurvedic drugs formulation had significantly altering level of mean copper concentration than those under Allopathy drug therapy. Furthermore, the post treatment effects of Antiarthritic Ayurvedic drugs on the amount of copper in joint fluid of patients were fairly analogous to controls. Thus the current study implicates an unequivocal positive alteration of copper in joint fluid of arthritis patients under treatment of traditional medicinal system, thus elucidating its antidote effect for potent joint health. Moreover, it demarcates the two medicinal systems pertaining to its possible elemental variation in joint effusion.

Keywords: Synovial fluid, Osteoarthritis, Rheumatoid Arthritis, Bursitis, Copper, Antiarthritic Ayurvedic drugs

Introduction

According to Ayurveda, vata dosha causes body ache and is basically an air disease. When ama, a toxin produced by a poor digestive system, accumulates within the body it aggravates vata. The ama then circulates in the body and most often gets deposited in the joint areas. The deposited ama causes arthritis in the affected joints. Among the most diagnosed musculoskeleton disorders are Osteoarthritis, Rheumatoid arthritis and Bursitis. The major site of pathology in arthritis is synovium. The synovial fluid is an ultrafiltrate of blood plasma present in between the synovial joints. Also, during inflammation or injury bursitis arises due to accumulation of bursal fluid in the bursae. In arthritic joints, the chemical components of cartilage and synovial fluid degrade, reducing their ability to retain water and limiting their protective properties. Besides, the biomarkers originating from bony tissue changes and cartilage are released in increased amounts into synovial fluid (SF) and thereafter into the blood. Also, the composition of the fluid can reflect either a transudative or exudative etiology; hence the analysis of synovial fluid becomes crucial in assessment of disease.

Cu is one of the essential mineral which helps in maintaining the connective tissue integrity, thus it plays an essential role in joint health[1]. Though the changes in copper concentrations in body fluids and tissues have been reported in literature but its role in most of them is not completely clarified [2].

Cu has been implicated in many musculoskeletal disorders; as long ago as 1938 hypercupreamia[3] was observed in RA, but it was only in 1951 that it was established that Cu complexes can be effective in

treating arthritis. Studies have shown a priority role for Cu in bone formation, mineralization and growth plate integrity. It is also well established that dietary deficiency of Cu correlates with enhanced acute inflammatory responses[4,5]. Osteoporosis has been linked with both copper deficiency and copper excess and has been categorized as type I or type II osteoporosis respectively[6]. In contrast to above, in inflammatory conditions, such as RA and OA, there is an arc alteration in blood, plasma and synovial fluid concentrations of the Cu micronutrients[7].

Ayurvedic medicines can be treated as source for the bioavailable form of trace elements; it serves as a supplier of essential minerals like Cu, Zn, and Fe. The use of Ayurvedic medication system was based on the assumption that there is at least a functional role of Cu in treatment of Arthritis. The aim of present work was to recognize the alteration in concentration of Cu in arthritic joint effusions and to establish a possible association with the therapeutic effect of Allopathy and Ayurvedic medicine.

Moreover, previous studies on Nonsteroidal Anti- inflammatory drug (NSAID) and steroids drugs proved no significant difference on serum Cu levels when compared with patients not receiving any treatment. It was further suggested that these drugs on their own do not exhibit any effect on metal levels[8]. The estimation of Cu in joint fluid samples was performed, using Inductively Coupled Plasma-Atomic emission Spectroscopy (ICP-AES), as the technique is highly sensitive and has better efficiency in analysis of elements in biological samples.

Therefore the current work heaves light on the comparative study of the effect of variation in the concentration of Cu in arthritic joint effusion of patients under the treatment of Allopathy and Ayurvedic drug. Also it evaluates the relevance of Cu and its possible impact in arthritic joint effusions.

Materials And Methods

Reagents and Standard solution

All the reagents used were of analytical grade obtained from Lennetech Laboratory without further purification. Elemental standard solution was of ultrapure quality obtained from Merck (Germany). The standard solution for standardizing the calibration curves were prepared by diluting the 1000 ug/mL stock solutions. Nitric acid (HNO_3) and Perchloric acid ($HClO_4$) used for sample mineralization were of Suprapur grade (Merck).

Study population

Arthritis patients who attended the outpatient department of (B.Y.L Nair, Navneet and Jeevan Rekha) hospital were considered for study. The criteria of American College of Rheumatology (ACRC)[9] were followed by expert physician while diagnosing the patients with three musculoskeletal disorders i.e. OA, RA and Bursitis. Only cases of proved knee Bursitis, RA and OA were included in the study. The Declaration of Helsinki (1964) as a statement of ethical principles was followed by the physicians during the research procedure[10]. Patients included were male and female of age group ranging from 18 to 90 years. Patients excluded from the study were pregnant females, HIV patients and subjects suffering from chronic disease such as cancer. The patient population was categorized into three set;

a) Patients intaking allopathy medicines,(n=135, NSAID)

A waiver of authorization from the ethics committee of respective Hospitals was obtained to acquire the sample of SF from 44 patients of OA and 50 patients of RA and 41 Bursitis.

b) Patients under the treatment of Antiarthritic Ayurvedic drugs, (n=142, AAD)

The OA patients under the treatment of formulated poly herb Relistif were designated as AYOA, whereas RA patients under the treatment of Rumaquit (supplied by Jeevanrekha ayurved chikitsalaya & Research Center) were represented as AYRA and Bursitis patients under the treatment of Triphala (Baidynath) were labeled as AYB. The patients under the treatment of AAD considered for SF tapping were 42 AYOA, 58 AYRA and 42 AYB patients

c) The Controls comprised of SF of healthy volunteers (n=46).

In addition to that every patient and control was imparted with complete information and written consent was also taken. The general data, major disease history, and current medication and lifestyle patterns of all the patients was obtained.

Sample aspiration

Synovial fluid samples were aspirated by arthrocentesis. This is a safe and relatively simple procedure that involves introduction of needle into the joint space to remove synovial fluid. Strict aseptic techniques were used when SF was aspirated from knee joints of patients. SF samples were stored in autoclave sterile vials. These containers were pre-cleaned by keeping them in contact with 5% HNO_3 overnight and then they were rinsed with high purity water. Samples were centrifuged to precipitate cells and particles. A 2mL aliquot of the clear fluid was transferred to an Eppendorf capsule and stored in sterile vials kept at $-5^{o}C$ until further analysis.

Instrumentation

The alteration in the fluid biochemistry is reflected as spectral changes. This is the principle behind several diagnostic tools that are based on spectroscopic methods. The samples for analysis were digested by conc HNO_3 and conc $HClO_4$ in 1:1 ratio. The estimation of Cu in SF/BF samples was performed using ARCOS ICP-AES device (M/s. Spectro, Germany) in Indian Institute of Technology, Sophisticated Analytical Instrument Facility (SAIF), and Mumbai. The standard and sample solutions were analyzed in triplicate series. The sample solution was analyzed against calibration curve. The presence of Cu is identified by wavelength of emitted radiation (Cu = 324.75nm) and the concentration was calculated by intensity of radiation. The Instrumental characteristics and operating parameters are given in Table 1.

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

Statistical analysis

Statistical analyses were carried out by using Graph pad prism software. Data were expressed as Mean±SD. One way Anova was used to perform multiple comparison among the groups. Also Wilcoxon's test was used to evaluate the significance between matched pair groups. Significance was considered at 95% confidence interval for all the sets of samples.

Results

The patients were categorized in various groups I, II, III and IV based on gender and age. The mean concentration of Cu in all three categories of patients is represented in the Table 2.

Table 2: The levels of Cu (Mean±SD) in Joint fluid of Controls and patients under the Treatment of Allopathy drug with its Pre and Post intervention.

Groups	Cu in Joint fluid of patients under Allopathy Treatment*						Controls
	AL-OA		AL-RA		AL-Bursitis		
	Pre	Post	Pre	Post	Pre	Post	
I	0.956±0.03	0.897±0.22	1.339±0.04	1.159±0.12	0.415±0.04	0.425±0.05	0.638±0.05
II	1.128±0.07	0.974±0.08	1.525±0.024	1.326±0.02	0.344±0.01	0.394±0.05	0.741±0.05
III	1.077±0.02	0.855±0.01	1.433±0.07	1.240±0.02	0.460±0.01	0.475±0.01	0.683±0.06
IV	1.153±0.01	1.081±0.01	1.636±0.02	1.348±0.19	0.395±0.02	0.409±0.01	0.782±0.08

All the categories of patients at the baseline showed alteration in mean amount of Cu when compared to controls. Subsequent association with the different disease states illustrate that the Cu concentration was higher in OA and RA whereas in Bursitis patients it was in detoriated amount than controls. The pre and post intervention of patients under the treatment of Allopathy drugs demonstrated significant differences ($P<0.05$) in total OA (Figure 1), RA (Figure 2) and Bursitis (Figure 3) subjects after the medication although insignificant difference was observed in some of the subgroups.

Figure 1: Variation in the levels of Cu in Joint fluid of Controls and AL-OA patients under Allopathy Treatment.

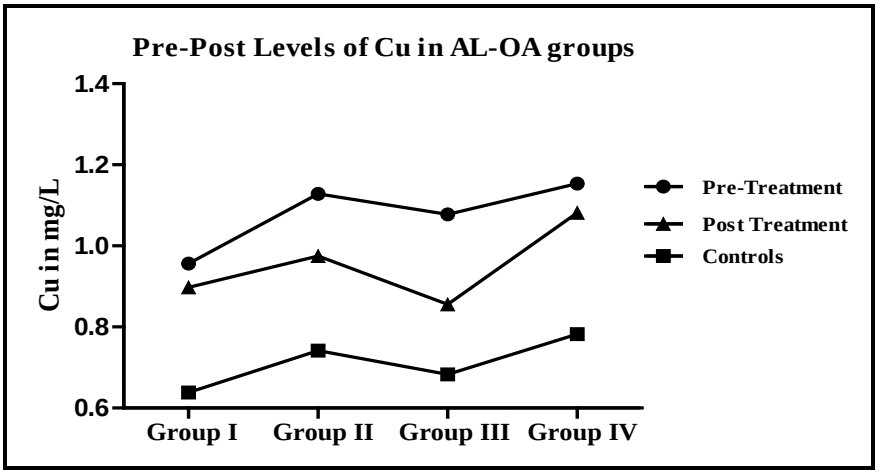


Figure 2: Variation in the levels of Cu in joint fluid of Controls and AL-RA patients under Allopathy Treatment.

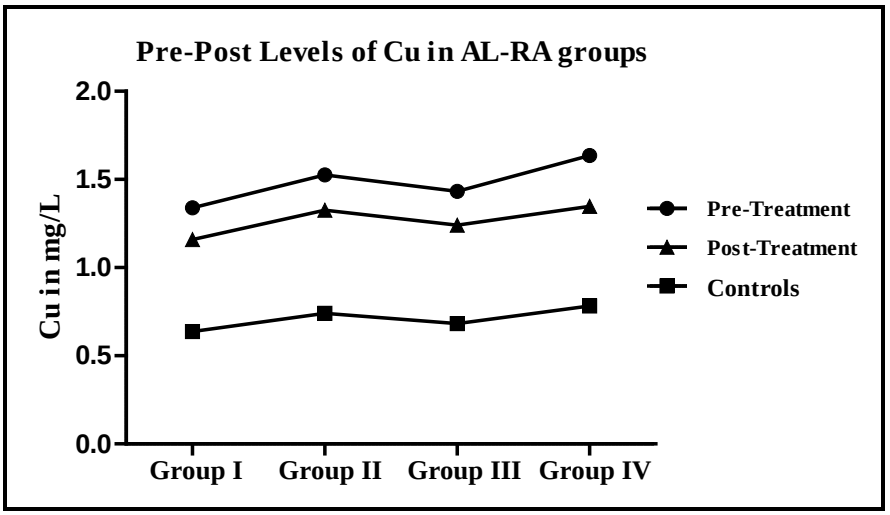
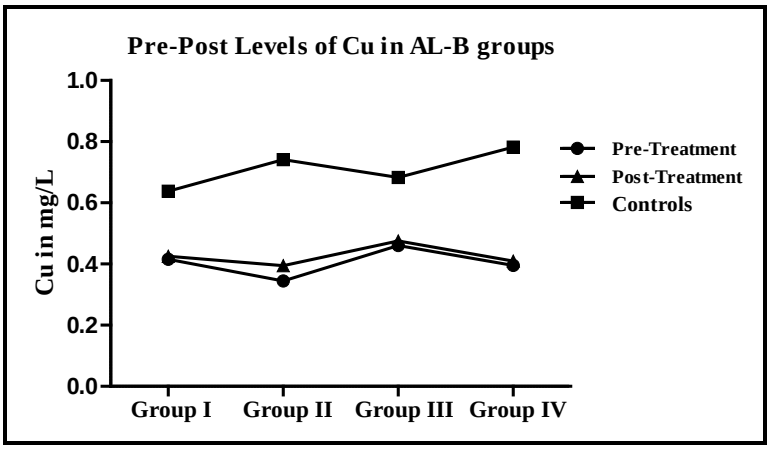


Figure 3: Variation in the levels of Cu in joint fluid of Controls and AL-B patients under Allopathy Treatment.



In case of bursitis, it is the first ever attempt made in the path of detection of trace elements in bursal effusion which required further investigation for strong scientific evidence. Whereas the reports on Cu status in SF of RA and OA patients are supported on the basis of research presented by Yazar et.al[11].

The patients under the Ayurvedic medication therapy were AY-OA being treated by Relistif, AY-RA consuming Rumaquit and AY-B Bursitis patient's intaking Triphala powder from last 12 weeks illustrated notable discrepancy in the status of Cu in joint fluid. **(Table 3**)

Table 3: The levels of Cu (Mean±SD) in Joint fluid of Controls and patients under the Treatment of Ayurvedic drug with its Pre and Post intervention.

Groups	Cu in Joint fluid of patients under Ayurvedic drug treatment*						
	AY-OA		AY-RA		AY-B		Controls
	Pre	Post	Pre	Post	Pre	Post	
I	0.956±0.02	0.691±0.13	1.222±0.01	0.940±0.03	0.418±0.07	0.658±0.02	538±0.05
II	1.103±0.03	0.844±0.02	1.443±0.03	0.975±0.01	0.372±0.02	0.560±0.02	741±0.05
III	1.018±0.02	0.816±0.14	1.112±0.01	0.919±0.01	0.488±0.05	0.648±0.06	583±0.06
IV	1.139±0.01	0.944±0.32	1.312±0.01	0.949±0.02	0.380±0.01	0.563±0.03	782±0.08

**Mean±SD (mg/L)*

The mean concentration of Cu after 12 weeks treatment with Ayurvedic drug established remarkable alteration in post treatment groups when compared to pre treatment groups. Such variation observed in case of AY-RA ($P<0.05$), AY-OA ($P<0.05$) and AY-B ($P<0.05$) **(Figure 4, 5 & 6)**

Figure 4: Variation in the levels of Cu in Joint fluid of Controls and AY-OA patients under Ayurvedic drug Treatment.

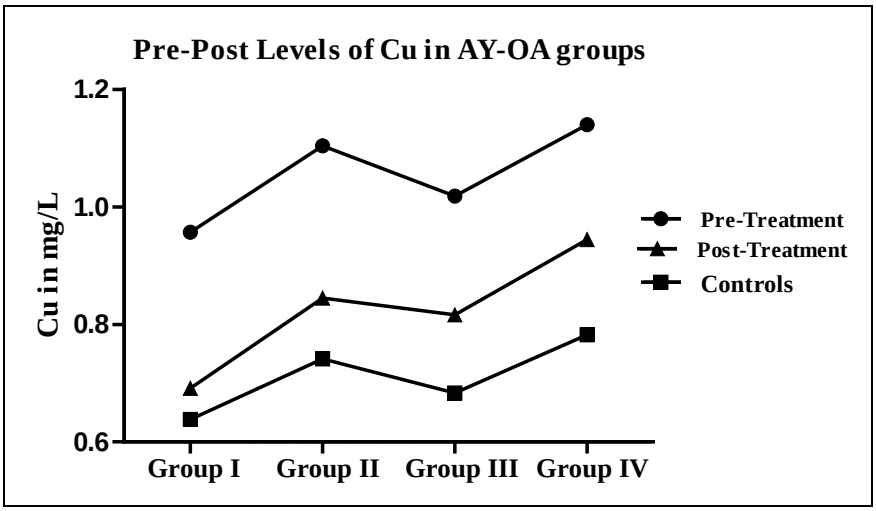


Figure 5: Variation in the levels of Cu in joint fluid of Controls and AY-RA patients under Ayurvedic drug Treatment.

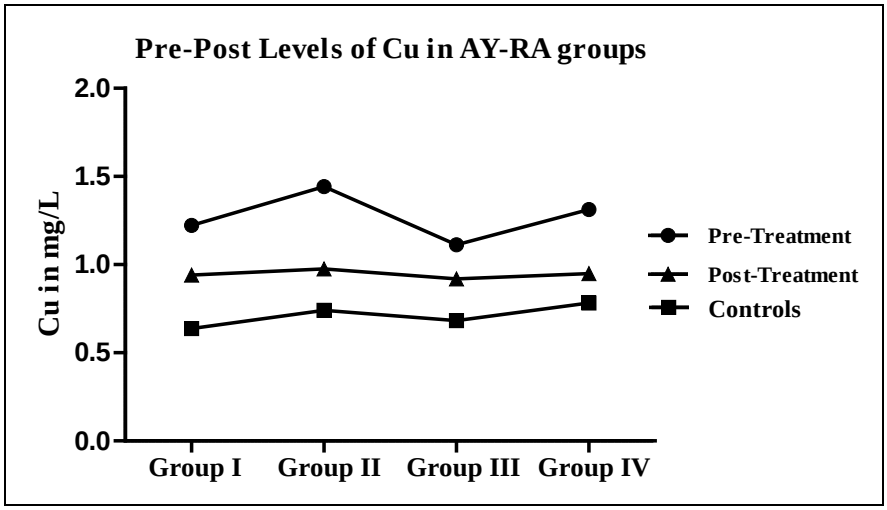
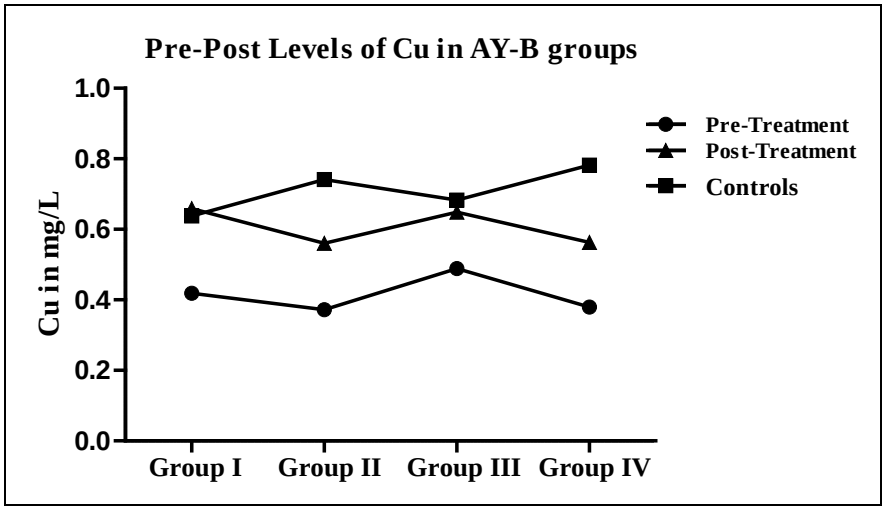


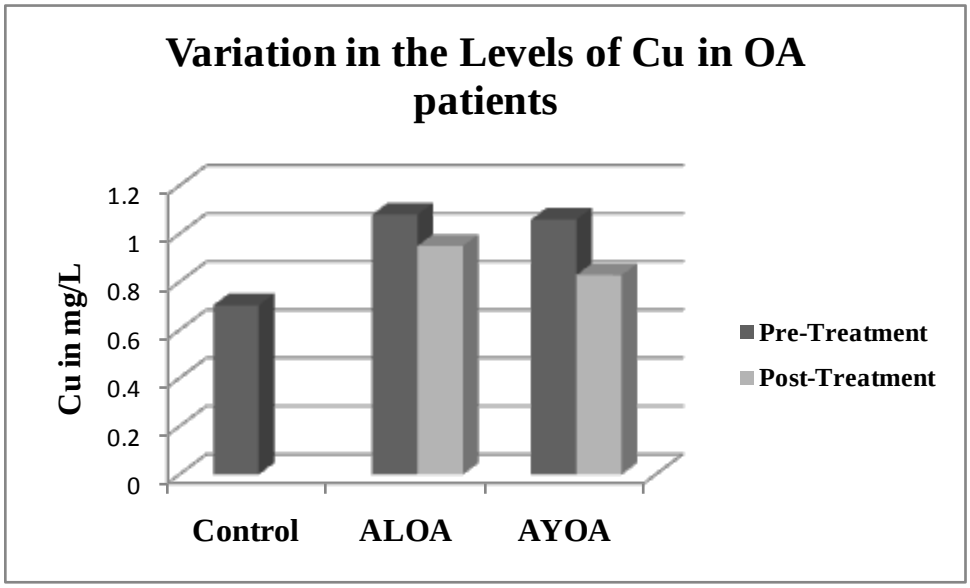
Figure 6: Variation in the levels of Cu in joint fluid of Controls and AY- B patients under Ayurvedic drug Treatment.



may be due to higher activity of disease which may require more time span to display an accurate recuperation impact.

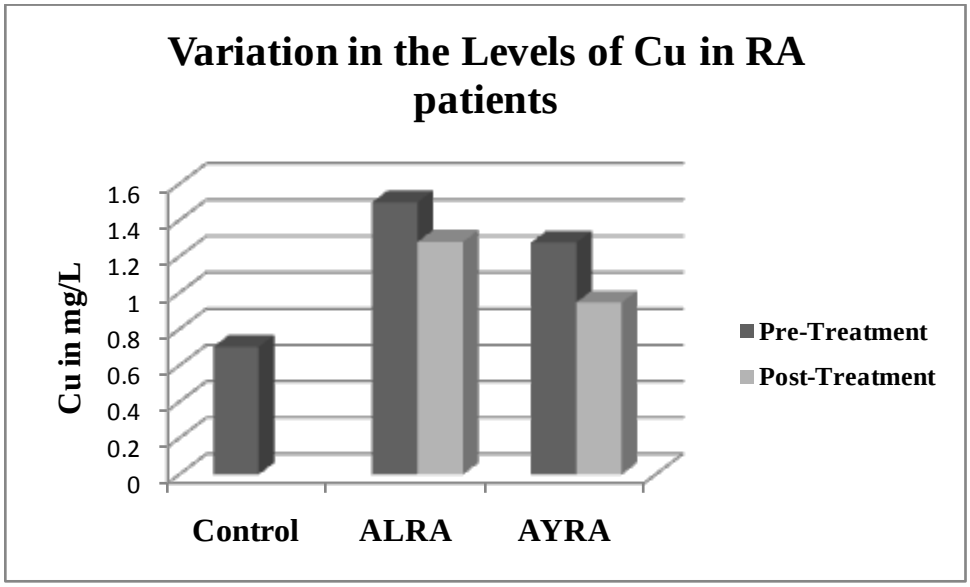
In order to assess the comparative efficacy of Allopathy and Ayurvedic treatment system percent differences were evaluated for pre and post intervention. It was observed that **(Figure 7)**

Figure 7: Variation in the levels of Cu in joint fluid of Controls and OA patients under Allopathy and Ayurvedic drug Treatment.



AL-OA groups had 13% detoriation in Cu levels whereas AY-OA had 23.09% deprivation in Cu concentration post treatment by Ayurvedic medication. Similarly in RA patients **(Figure 8)**,

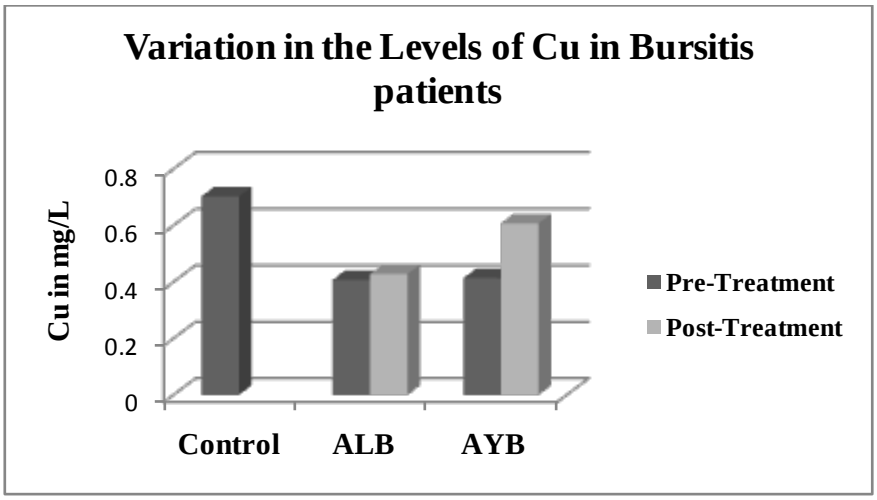
Figure 8: Variation in the levels of Cu in joint fluid of Controls and RA patients under Allopathy and Ayurvedic drug Treatment



the amount of Cu after the treatment decreased by 21.9% in allopathy groups and 32.94% in ayurvedic groups.This clearly implicates the superior restoration competence of ayurvedic medicine in the regaining

appropriate levels of copper as compared to allopathy drugs. However in case of bursitis the amount of Cu was increased by 2.13% in AL-B subjects **(Figure 9)**

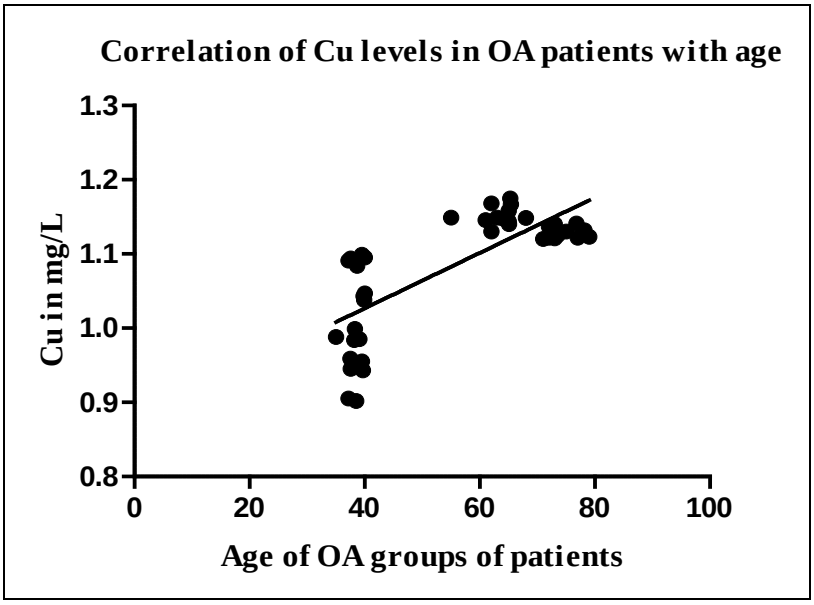
Figure 9: Variation in the levels of Cu in joint fluid of Controls and Bursitis patients under Allopathy and Ayurvedic drug Treatment



whereas in AYB it augmented by 19.4%. Since ayurveda is a holistic science of medicine, its efficacy is very well evident in present work; due to its potential of retrieving the appropriate levels of essential mineral in different disease state.

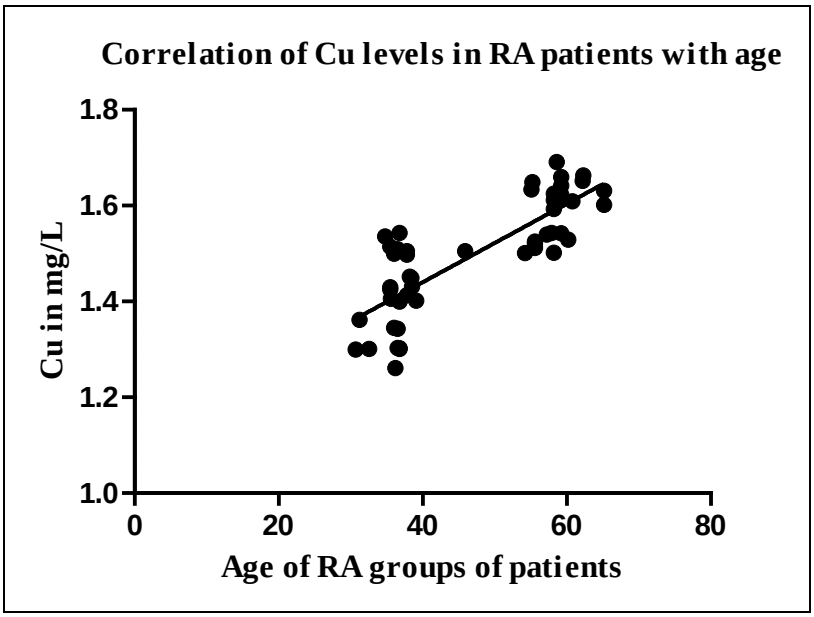
A positive correlation in OA ($r=0.6820$, $P<0.05$)

(Figure 10) Figure 10: Correlation in levels of Cu in OA patients with age



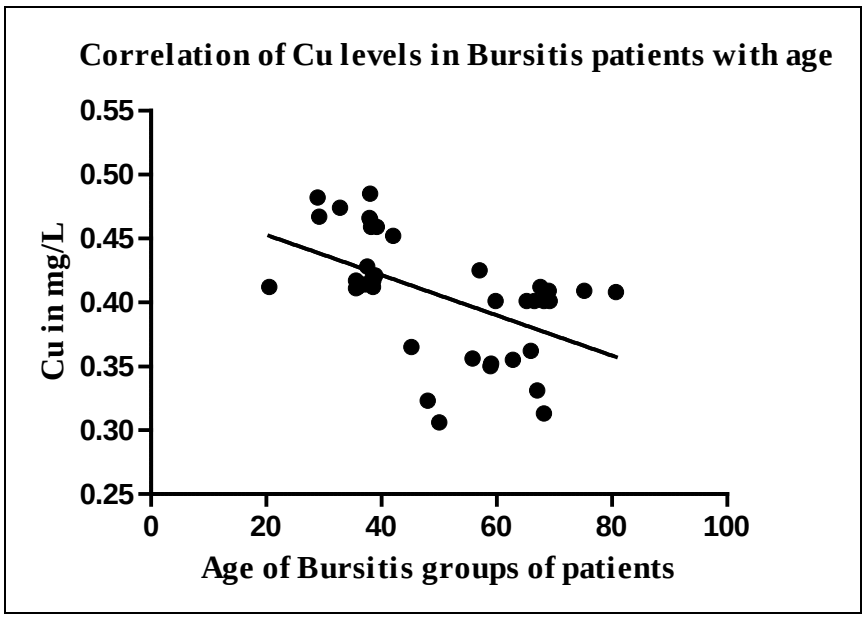
and RA patients (r=0.7846, P<0.05) was observed between levels of Cu and age indicating Cu toxicity is more prevalent in aged patients

Figure 11: Correlation in levels of Cu in RA patients with age



whereas a negative correlation was observed in Bursitis (r=-0.6502, P < 0.05)

Figure 12: Correlation in levels of Cu in Bursitis patients with age



implicating increase in Cu deficiency with age**.**

Discussion

The data on OA and RA patients illustrates direct proportionality with the levels of Cu and age of patients. Perhaps the data on Bursitis subject establish Cu deficiency in older age groups of patients. Although arthritic risk factors certainly increase with age, it is afflicted to individuals of every age, race and gender. More than one half of the people suffering from arthritis are under 65 years of age, with greater prevalence seen among women than men[12, 13].

Although, the results is contradictory for Bursitis but is in good agreement in case of OA and RA patients where the level of coppe r was higher in women than men. Studies by Bajpayee[14] have illustrated the role of oestrogens, copper, and caeruloplasmin in causing exacerbation of RA symptoms. However, it shows that the specificity of oestrogens in raising caeruloplasmin levels in female RA patients is more prevalent.

Moreover, as Cu is known to be an essential element in many of the metabolic processes, involving articular tissues and immune system function. In human synovial fluid, copper is suggested to be loosely bound to albumin or to amino acids such as histidine and able to catalyse the free-radical-mediated reactions which have been implicated as an important part of the destructive process that occurs within the arthritic joints [15]**.**

In inflammatory conditions, such as Rheumatoid arthritis (RA), there are arc alterations in body fluid concentrations of various micronutrients. The present work illustrates higher levels of Cu in SF of RA & OA than controls. The increase in SF copper may be due to increased levels of cuproenzyme and ceruloplasmin[16-19] which was observed in various specific and non specific pathological conditions and was attributed to biological damage caused by superoxide, a radical found in all living tissues. According to one of the theory, superoxide radical or other reducing agents, such as ascorbate, reduces the copper complexes to the cuprous state. In turn, these complexes react with hydrogen peroxidase to form hydroxyl radicals that damage proteins, RNA and most important DNA. Repetitive formation of OH radicals at a specific location-where the copper ions are found is probably the mechanism of this process. These radicals may cause double

strand break in the cellular DNA that are not repairable by cellular mechanisms[20]. Thus damaging the articular cartilage and degradation of SF leads to severe arthritic pain in the joints. These data are supported by the previous findings of Scudder et.al[13], Yazar et.al[11], Niedermeier [21] and Krachler [12]. The evidences presented by Niedermeier showed that a raised synovial fluid caeruloplasmin is characteristic of rheumatoid, as distinct from osteoarthritic effusions. In osteoarthritic fluid the copper/caeruloplasmin ratio was suggested to be marginally reduced compared to serum whereas in rheumatoid fluid caeruloplasmin is known to increase disproportionately in relation to other plasma proteins present in rheumatoid effusions.

Bursits being of diverse type, the exact pathological features are still unclear. Bacterial infection in case of septic and inflammatory condition or injury in aseptic bursitis is asserted to be the primitive cause of Bursitis. The result describes diminished levels of Cu in BF than control effusions. The Literature survey reveals so far no work has been cited regarding the possible mechanism of Cu in BF

Copper is a cofactor in synthesis of collagen and elastin fiber in connective tissues. During low copper concentration, the connective tissues are not properly crosslinked and there is a fragmentation of elastin lamina. Biochemically elastin precursor fails to polymerise into fibrous elastic sheath. As a consequence focal lesion develops where the stress is greatest. These lesions lead to ballonlike swelling in the knees of Bursitis patients[22]. This tissue mineral pattern is strongly indicative of a chronic infection. This gives a possible mechanism of reduced BF Cu content during bursitis.

Moreover permeability of synovial membrane to plasma proteins is poorly understood. It is a highly selective process which probably depends on molecular size, molecular weight, and metabolic changes in the joint as well as on the plasma concentration of different proteins. Destruction of the membrane or its damage will result in loss of its ability to selectively limit or exclude certain plasma proteins. The changes induced by acute or subacute inflammation may be superimposed on this pattern.

In the present investigation few formulated polyherbal drugs were studied for their antiarthritic properties, as the above observation speculated the possible curative property of essential elements present in AAD. Human studies using these herbs have shown relief of muscle discomfort and pain, reduced morning stiffness, and reduced joint swelling comparable to patients receiving anti-inflammatory drugs[23, 24].

The post treatment effect of AAD therapy was assessed after a period of 3 months by evaluating the SF copper levels of arthritis patients under treatment of each drug listed for respective disorder. It was observed that AAD therapy was very fruitful for balancing the levels of Cu in OA and Bursitis patients. However, SF Cu levels, which are known to vary quite widely in patients with AYRA, may stabilise probably by higher dose of AAD drug therapies and after longer duration of treatment.

At the dosage of 0.5g of Relistif, changes exhibited by it were remarkable on AYOA patients. Also the questionnaire obtained from patients indicated pain relief effect after the treatment by AAD. Relistif contains herbs that have a dual action of inhibiting leukotrines and prostaglandin imparting a smooth analgesic effect[25]. It was known that Glutathiones (GSH) markedly diminished the inhibitory effect of prostagladins and leukotrines. Since copper ions are powerful promoters of free-radical damage, accelerating lipid peroxidation and causing formation of hydroxyl radical, Polyherbs provide a complex system of proteins to ensure that the essential metals are rarely allowed to be "free" to produce any scavenging effect [26].

It may be indicated that GSH inhibits free radical formation by copper ions in the presence of H_2O_2, ascorbate, and DNA. The protective effect of GSH is attributed to its stabilization of copper in the +1 oxidation state, thereby compromising its ability to participate in free radical generating reactions[27]

The amount of Cu in Rumaquit is lower compared to Relistif and Triphala. The polyherbs in Rumaquit are known to have synergistic effect in boosting the potent anti-inflammatory activity of the drug. Since augmented Cu levels results in formation of free radicals in SF. It is reported that curcumin in humans is known to enhance Immunoglobulin G (IgG) level and prevents lipid peroxidation in a significant higher degree than the commonly used antioxidant. Moreover it intervenes in free radical propagation by quenching pre formed free radicals [28].

Thus, the free copper concentration and caeruloplasmin increases in inflammatory state causing it to aggregate or create reactive oxygen species. Curcumin might affect activity of many proteins by binding their associated metal ions[29].

Besides Triphala powder was observed to be effective in case of bursitis .It has been reported that Triphala inhibit matrix metalloproteinase (MMP) in inflammatory disease state. Philips et al has reported that the lower copper concentrations stimulate activity of MMPs whereas the higher concentrations stimulate the expression of MMPs in fibroblasts[30].

Since much is not known about the molecular and biochemical pathophysiology of bursitis. There is possibility of increase in the levels of MMP during disease state but the probable inhibition of MMP activity occurs after the treatment of Triphala. The results obtained clearly indicate the fact that the Indian Ayurvedic herbal formulation Triphala has promising therapeutic effect for Bursitis patients.

Therefore, the present study investigated the efficacy of two medicinal systems and highlights the prevalence of copper levels in joint fluids of different musculoskeletal disorders and its possible association with the therapautic effect of medicinal system.

Conclusions

Despite the fact that the action of Cu in humans has been intensively studied, the clinical picture the status of Cu in ayurvedic medicines is not always so straightforward, and less is known about the role of Cu in traditional medicines and its potential effect in joint disorder. Yet it is evident from present research that ayurvedic system can serve as a very important medicinal procedure, and its role has been clearly implicated as potential antiarthritic therapy. Furthermore, the experimental and clinical studies in present work aid at unraveling the prominent activities of Antiarthritis Ayurvedic Drug on the Cu levels in arthritis patients, thus discovering Cu as biomarkers in assessment of the disease. The present therapy offers superior reconciliation of health in OA and Bursitis patients whereas the treatment of RA requires more influential therapy. Also, the detection of Cu in musculosketon disorders has generated new options for early intervention in Cu-related joint defects. Thus the distinct level of Cu in diverse arthritic condition caters in distinguishing discrete joint ailments. In addition to that the clinical importance of these natural inhibitors may prove to be better antiarthritic therapeutics for the efficient treatment of the musculoskeleton disorders. Further clinical trials can provide better scientific evidences in evaluating the most promising therapeutic treatment for joint diseases.

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