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Full-Length Research Article

Age-Dependent Effects of Copper Toxicity on Connective Tissue Structural Stability in Wistar Rats Skin

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Summary: Over the last three decades, there has been increasing global concern over the public health impacts attributed to direct and indirect environmental pollution, in particular, the global burden of disease. The World Health Organization estimates that, about a quarter of the diseases facing mankind today occur due to prolonged exposure to environmental pollution; the health of 200 million people in lower-income countries is at risk from toxins such as lead, copper, mercury, and that, nearly a quarter of deaths in developing countries including Nigeria and Ghana, are linked to pollution. The purpose of study was to investigate the effects of ingestion of large dose of copper on the structural stability of collagen molecules, as well as reveal age-dependent differences in the phenomena. The content of de novo synthesized collagen was determined by hydroxyproline concentration using Stegmann-Staeder's method as modified by Utevskaya and Persky; the nature of intra- and inter-molecular covalent cross-links in collagen matrix was estimated by electrophoretic separation of products of partial thermal denaturation of collagen in polyacrylamide gel. There was intensification of synthesis over degradation in young rats, and that administration of copper led to a decrease in collagen solubility. This study concluded that, effects of copper toxicity on the structural stability of collagen appeared mostly in young rats.

Keywords*: Age, Collagen, Copper, Hydroxyproline, Thermo-Stability, Structural Stability.*

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INTRODUCTION

Most environment-related diseases are not easily detected but may be acquired during childhood and manifested in later ontogenesis (UN Report, 2017). One type of pollution that bears a pronounced technological character is that of salts of heavy metals: lead, mercury, copper, arsenic, to mention a few, the level of pollution of these compounds is increasing every year.

Copper (Cu) is an essential trace element for humans and animals because it is a key constituent of the respiratory enzyme cytochrome coxidase (MIC, 2019, Muriel *et al*., 2016). In the human body, copper shifts between the cuprous (Cu^{1+}) and cupric (Cu^{2+}) forms, though the majority of the body's copper is in the latter form. The ability of copper to easily accept and donate electrons explains its significance in oxidation-reduction (redox) reactions, involving copper-containing oxidases; and in scavenging free radicals (Peter, 2018). Copper is a critical functional component of several essential enzymes known as cupro-enzymes (Ludmila *et al*., 2018, Peter, 2018, Prohaska, 2011). A cupro-enzyme, lysyl oxidase, is required for the cross-linking of collagen and elastin, which are essential for the formation of strong and flexible connective tissue. The action of lysyl oxidase helps maintain the integrity of connective tissue in the heart and blood vessels and also plays a role in bone formation (Prohaska *et al*., 2012).

The oxidative potential of copper may be responsible for some of its toxicity in excess ingestion cases (Turnlund, 2006). At high concentrations, copper is known to produce oxidative damage to biological systems, including peroxidation of lipids or other macromolecules. It is therefore important to study the impact of this toxic metal on living organisms. The commonest type of tissue in the human body is the connective tissue and therefore, the effect of copper pollution on its various components must be the commonest in the body as a whole.

One of the most important functional and structural components of the connective tissue is collagen (Elena *et al*., 2013). Unlike most other proteins, the turnover of collagen is extremely slow and can therefore, reflect age-related changes (Bailey and Paul, 1999). There is no doubt that, a change in collagen matrix under the influence of various toxic substances will have a significant impact on both the connective tissue and organs containing it. The stabilisation of collagen fibers during development through growth to maturation is now fairly documented; it is a carefully controlled enzymatic process, which produces inter-molecular cross-links at specific locations (Muriel *et al*., 2016, NPIC, 2014).

Collagen concentration in tissues; the degree and types of protein's cross covalent bonds; as well as content of hydroxyproline in individual collagen molecules, are the basic parameters of structural stability of matrix sub-molecular collagen complexes.

The biomedical potential of natural collagen is limited by its poor mechanical strength, thermal stability, and enzyme resistance, but exogenous physico-chemical or biological cross-links have been used to modify the molecular structure of the protein to minimize degradation and enhance mechanical stability. By way of thermal stability of collagen in sub-molecular complexes of the extracellular matrix, structural stability of the skin could be assessed (Altug and Bumsoo, 2016, Selestina and Vanja, 2011, Tsereteli *et al*., 1996).

Changes in collagen physical properties that occur towards old age are stochastic and involve oxidative reactions that result in the formation of glucose-mediated cross-links. This excessive and random cross-linking leads to deterioration in the functional properties of the connective tissue at old age, i.e. leads to a devastating loss of tissue functionality of vital organs (Chan-Sik *et al*., 2017, Jess and Alfonso, 2014, NPIC, 2014, Elena *et al*., 2013, Yamauchi and Sricholpech, 2012 Turnlund, 2006, Waller and Maibach, 2006). In addition, specific residues involved in cell-matrix interactions may become chemically modified; and this can affect the expression of cells, as well as lead to the formation of inappropriate collagen matrix during its slower turnover in ontogenesis (UN Report, 2017). This is exemplified in the ubiquitous disorders, osteoporosis and osteoarthritis, as well as age-related diseases in which gene-regulated changes in the collagen are deposited. At the same time, post-translational changes such as over-hydration of lysine residues were noted (Chan-Sik *et al*., 2017, Jess and Alfonso, 2014, NPIC, 2014, David *et al*., 2019); both effects can have a profound deleterious impact on the functioning of the matrix tissue. In view of this, the study of the influence of copper heavy metal on collagen structural stability is therefore important.

Hydroxyproline (4-OHPr), is a non-proteinogenic amino acid formed by the post-translational hydroxylation of proline, and is a major component of collagen, where it serves to stabilize the helical structure; it accounts for 13-14% of collagen's total amino acid content (Ivan *et al*., 2018, Paietta, *et al*., 2013, Richard and Rosemary, 2007). Endogenous hydroxyproline reflects rate of degradation of collagen, which in turn, is related at least in part, to the rate of synthesis of collagen; due to its highly restricted distribution in collagen, the measurement of OHPr levels can be used as an indicator of both the presence and metabolism of collagen (Carmen, *et al*., 2016, Bishnu. and Eric, 2014, Kondo, *et al*., 2014, Huije, 2013, Corrine *et al*, 2011, El-ta'alu *et al*., 2010, Deyl and Miksik, 2000). We estimated this de novo synthesized collagen content using oxidation and condensation methods earlier proposed by Stegmann and Staeder (1967) as modified by Utevskaya and Persky (1982). The method is based on condensation reaction of products obtained from the interaction of hydroxyproline oxidation (pyrole) with para-di-methyl-amine-benzo-aldehyde (DABA). Separation of products of partial thermal collagen denaturation was carried out by electrophoresis in polyacrylamide gel (Carmen, *et al*., 2016, Deyl and Miksik, 2000, Corrine *et al*., 1979).

The purpose of this study was to investigate copper toxicity on the structural stability of collagen molecules, as well as reveal age-depended differences in the phenomena. At present however, there are practically no reports that focus on age-related effects of copper toxicity on integrated structural stability of collagen structures. It is therefore, expedient to deeply study the aforementioned phenomenon, as well as the consequential impact on the structural stability of collagenous structures. To achieve this aim, the following tasks were set as objectives: 1. Determine the concentration of hydroxyproline in the skin and based on this, the content of de novo synthesized collagen in the organ. 2. Estimate the nature of intra- and inter-molecular covalent cross-links in collagen matrix based on analysis of electrophoretic separation of products of partial thermal denaturation of collagen in polyacrylamide gel. 3. Evaluate the stability of collagen cross-links through determination of the degree of collagen solubility in neutral salt solution and determination of the degree of thermal stability of collagen samples.

MATERIALS AND METHODS

Subjects/Materials and Methods: The study was partly carried out in A.M Gorky Kharkov State University, Ukraine, and partly in the Physiology Research Laboratory of the Department of Human Physiology, Faculty of Basic Medical Sciences, Bayero University, Kano-Nigeria. Experimental animals were handled in accordance with international principles of the European Convention «On protection of vertebrate animals used for experimental and other scientific purposes» (European Convention, 1985), as well as norms of biomedical ethics in accordance with the Law of Ukraine «On protecting animals from wicked handling.

A total of 40 male Wistar rats comprising of eleven 3-months old (young) and nine 20-months old (adults) animals weighing between 150-250 grams were studied. Rats that corresponded with the same age-groups were used as control. Before decapitation, animals received large doses of copper, three (3) times after every 48 hours by intravenous injection of 1mg of $CuSO_4$/100g of body weight (NPIC, 2014).

In order to study the trend of the effect of copper on collagen protein, loose connective tissue of dorsal skin (skin of the spine region) of young and adult Wistar rats was chosen (Zaki, 2015). This allowed the tracing of not only the general pattern of copper pollution, but also revealed the age-related characteristic features of its effects. Briefly after obtaining informed consent, Wistar rats were lulled to sleep by intravenous injection of 0.5cm^3 of 0.4% solution of sodium thiopental (Stephen, 2002), and then decapitated. Dorsal skin samples (20 × 5) mm were cleaned from subcutaneous fat and hair.

Data were expressed as Mean±SEM. Chi-square method and Student's Test were used to determine differences in hydroxyproline concentration between normal and psoriatic patients. All analyses were carried out using IBM-SPSS version 20.0 statistical software. $P < 0.05$ was set as level of significance.

Skin Incubation: Samples of skin were incubated in Ringer-Kreb's medium for warm-blooded animals, which contained 9.075g of tris HCl; 8.2998g NaCl; 0.5206g KC1;

2222g $MgCl_2$; 0.4333g K_2HSO_4 and 5.4g of $C_6H_2O_6$ in 1 litre of distilled water (El-ta'alu *et al.*, 2010). Incubation was carried out for 6 hours, at 37°C.

Isolation of Type I Collagen: Incubated samples were defatted using acetone and diethyl ether for 24 hours in each case, weighed, and ground to powder form in liquid nitrogen. Type I collagen was extracted from the powder using 1M solution of NaCl; and freshly synthesized collagen isolated from the extract was obtained by dialysis against phosphate buffer solution (pH 8.0) for 10 days with daily change of buffer (El-ta'alu *et al.*, 2010).

Determination of the Content of *de novo* Synthesized Collagen: This was carried out by hydroxyproline concentration using Stegmann-Staeder's method as modified by Utevskaya and Persky;

Determination of the Nature of Intra- and Inter-molecular Covalent Cross-links in Collagen Matrix: This was estimated by electrophoretic separation of products of partial thermal denaturation of collagen in polyacrylamide gel (Laurent and Marianne, 2011, Corrine *et al.*, 1979).

Evaluation of Stability of Collagen Cross-links: To determine the extent and nature of cross-links in collagen of studied samples, the degree of protein's solubility in neutral salt solution, before and after heating, was studied (Laurent and Marianne, 2011, Marta and Krystyna, 2012).

Determination of Degree of Collagen Solubility in Neutral Salt Solution : About $5cm^3$ of 1M solution of NaCl were added to 100mg of fresh skin samples. Extraction was carried out for 3 days in the cold, and collagen released into solution was precipitated by adding dry NaCl up to a concentration of 3.5M. Solubility of collagen was evaluated by the ratio of hydroxyproline released into solution during collagen extraction to the total content of hydroxyproline in the skin before extraction, and the resulting value was expressed in percentage.

Determination of the Degree of Collagen Thermal Stability: 100mg of fresh skin samples were heated in $3cm^3$ of distilled water for 20 minutes at 50°C. The degree of thermal stability of collagen was evaluated by the relative ratio of hydroxyproline concentration released from collagen into solution to its total content before heating. Obtained result was expressed in percentage.

RESULTS

Results on age-related effect of copper on hydroxyproline concentration in the skin of rats, as well as changes in total collagen degradation in age groups (Table 1; Figure 1) showed an intensification of synthesis over degradation in young rats.

Tables 2 and 3; Figures 2 and 3 showed that, administration of copper led to a decrease in collagen solubility in 1M solution of NaCl before and after heating in both age groups; these significant changes in solubility were observed only in young rats.

Table 1:
Age-dependent Effect of Copper Administration on Hydroxyproline Concentration in the Skin of Rats

Age of Rats (Months)	Condition of Investigation	Hydroxyproline Concentration (mg/100mg of Fresh Skin)
3	Control	1.81±0.09
	Experiment	1.94±0.08
20	Control	2.80±0.11
	Experiment	2.63±0.09

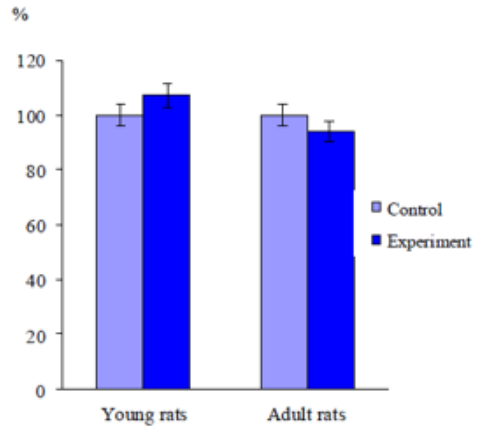

Figure 1:
Age-dependent Effect of Copper Over-dose on Hydroxyproline Concentration in the Skin of Wistar Rats (% of Control)

Table 2: Age-dependent Effect of Copper Administration on Collagen Solubility before Heating in 1M Solution of NaCl

Age of Rats (Months)	Condition of Investigation	Content of Collagen in Solution (% of Total Collagen Concentration)
3	Control	2.31±o.08
	Experiment	2.05±0.11*
20	Control	1.50±0.08
	Experiment	1.41±0.08

* Significant compared to control (P<0.05)

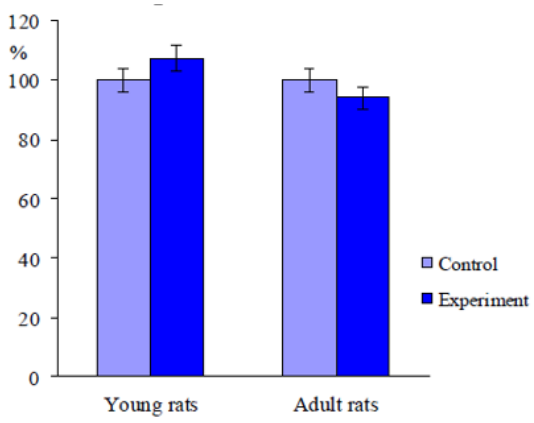

Figure 2:
Age-dependent Effect of Copper on Skin Collagen Solubility in 1M NaCl Solution (% of Control)

Table 3:
Age-dependent Effect of Copper Administration on Collagen Solubility after Heating in 1M Solution of NaCl

Age of Rats (Months)	Condition of Investigation	Content of Collagen in Solution (% of Total Collagen Concentration)
3	Control	22.1±1.0
	Experiment	19.6±0.9*
20	Control	9.0±0.3
	Experiment	8.1±0.4

* Significant compared to control (P<0.05)

Analyses of cross-links in collagen of control and experimental rats by electrophoretic separation of collagen that was released into solution after heating (thermo-labile collagen - Table 4; figures 4 and 5) showed a marked increase in collagen structures in both study groups. This effect was manifested in the decrease of relative content of α-particles, corresponding to free unbound cross-linked α-chains. At the same time, the relative content of α-linked chains increased. It should be noted that, such an increase was expressed in both young and adult rats (3- and 20-months old, respectively. An increase in the stiffness in the linking of α-chains in both young and adult rats were different: intensification of cross-linking in 3-months old rats was accompanied not only by a rise in the number of β-particles but also by an increase in the content of γ-particles; in 20-months old rats, an increase was observed only in the content of β-particles (Table 5).

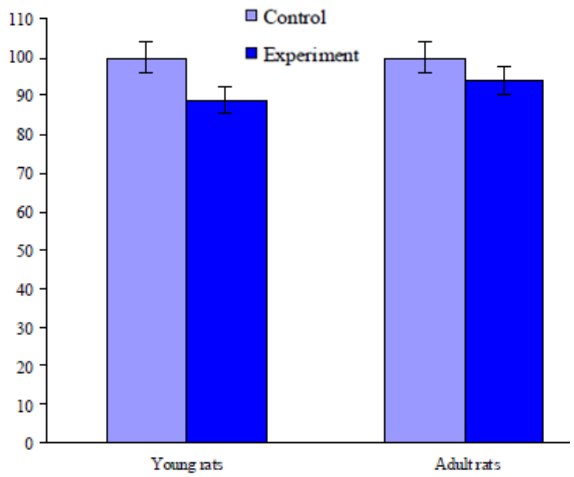

Figure 3:
Age-dependent Effect of Copper Over-dose on Collagen Solubility After Heating (% of Control)

Table 4:
Age-dependent Effect of Copper Administration on Relative Contents of α-chains of Varying Degrees of Polymerization in Skim Collagen after heating (% of Heat-liable Collagen)

Age of Rats (Months)	Condition of Investigation	Content of α-chains of Different Degrees of Bonding (%)		
		Free α-chains	α-chains Bound (in twos)	α-chains Bound (in threes and above)
3	Control	62.1±0.7	29.9±0.3	8.0±0.1
	Experiment	53.9±0.6*	34.0±0.3*	12.1±0.1*
20	Control	47.1±0.5	32.5±0.2	20.4±0.1
	Experiment	41.7±0.6*	36.4±0.3*	21.9±0.2

** Significant compared to control (P<0.05)*

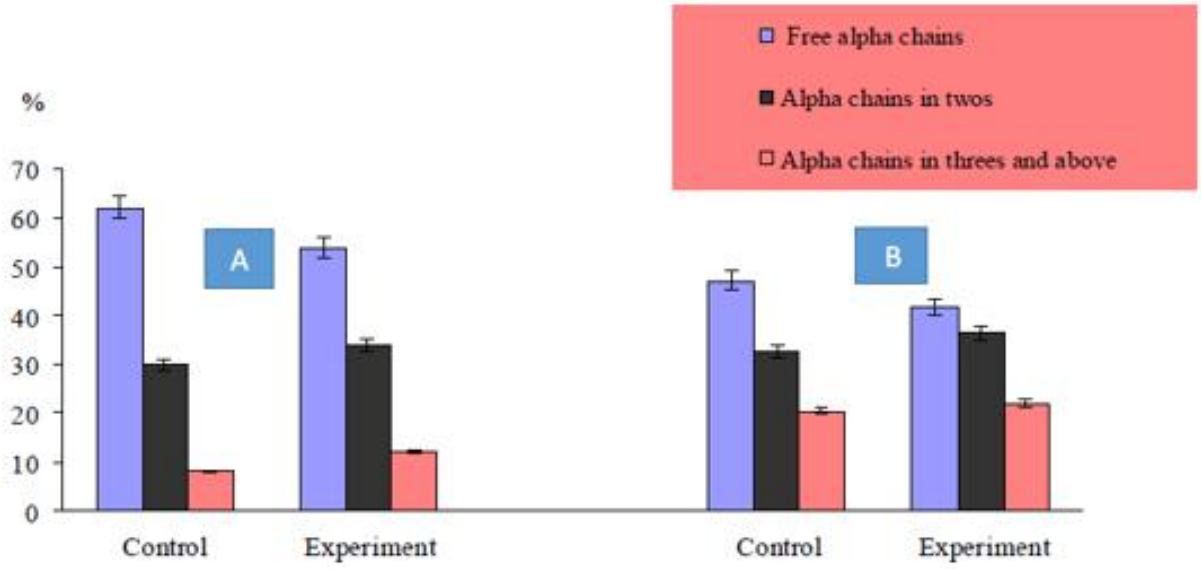

Figure 4:
Effect of Copper Over-dose on the Degree of Polymerization of α-chains of Thermo-liable Collagen in 3-months (A) and 20-months old (B) Wistar Rats (%).

Table 5:
Age-dependent Effect of Copper Administration on the Ratio β-particles:γ-particles in Heat-liable Collagen of the Skin

Age of Animals (Months)	Control	Experiment
3	3.74	2.80
20	1.59	1.66

DISCUSSION

Intensification of synthesis over degradation of collagen in young rats. These changes subsequently became balanced. Thus, changes in collagen concentration due to copper toxicity are reflections of different sensitivities of synthetic and catabolic processes to the toxic effects of copper in the skin of both young and adult Wistar rats. In the skin, where damaging effects are less; the degree of cross-linking, assessed based on the solubility of collagen in neutral salt solutions before and after heating varied depending on the age of rats. This agrees with findings by El-ta'alu *et al* (2010).

It should be noted that, thermal destruction of the collagen matrix in the skin was less pronounced than in neutral salt solution; the degree of the decrease in solubility for 1M solution of NaCl and temperature during heating were about the same (about 10%). In the skin of experimental rats, exit of collagen into solution after heating, and after treatment with salt, was also about the same as in young rats but remained below the reference value (in controls - about 5-7%). As reported by Laurent and Marianne, 2011, as a result of this, the difference in reliability from the control, for solubility in salt solution was only 90%.

It has been well documented by Marta and Krystyna (2012) that the different types of cross-covalent bonds in collagen have different sensitivities to thermal treatment. Cross-links formed in the initial stages of the process of collagen cross-linking through Aldole condensation, were more readily destroyed by heat than aldimine bonds that were formed later (Laurent and Marianne, 2011). Modified as a result of further post-synthetic modifications of cross-links, as a result of for example (1) changes in Schiff's bases to form ketoamines, and (2) formation of poly-functional cross-links, with the formation of pyridine cross-links, etc., cross-links were less destroyed by heat (Laurent and Marianne, 2011). This led to the fact that, heat initially destroyed labile cross-links, and then thermo-stable ones and as a result, aggregates of α-chains of varying degrees of bonding found their way into solution depending on the number of the bonds themselves and their chemical nature.

Analyses of cross-links in collagen of control and experimental rats by electrophoretic separation of collagen that was released into solution after heating (thermo-labile collagen) showed a marked increase in collagen structures in both study groups was manifested in the decrease of relative content of α-particles, corresponding to free unbound cross-linked α-chains. At the same time, the relative content of α-linked chains increased. It should be noted that, such an increase was expressed in both young and adult rats (3- and 20-months old, respectively. It is interesting to note that, an increase in the stiffness in the linking of α-chains in both young and adult rats were different: intensification of cross-linking in 3-months old rats was accompanied not only by a rise in the number of β-particles but also by an increase in the content of γ-particles; in 20-months old rats, an increase was observed only in the content of β-particles. This also agrees with the findings of El-ta'alu *et al* (2010).

This study concluded that effects of copper appeared mostly in young rats; copper toxicity led to stiffness and hardening of collagen structures of the skin in 3- and 20-months old Wistar rats and this was a result of increased intra- and inter-molecular bonding; and in adult rats mainly due to only intra-molecular bonding.; the toxicity also led to a decrease in the concentration, and an increase in the degradation of collagen; there was a decrease in the solubility of collagen after heating in 1M solution of NaCl; cross-linking of collagen increased in 3- and 20-months old Wistar rats.

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