Influence of Copper Coordination Compounds and Cyanobacterian Remedy BioR on Bone Collagen and Hydroxyproline Concentrations (ontogenetic view)

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***Abstract* – The aim of our research was to assess the influence of copper coordination compounds CMT-28 and CMT-67 and cyanobacterian remedy BioR on bone collagen and hydroxyproline (HYP) concentrations at different stages of postnatal ontogenesis in normal physiological conditions and experimental osteopathy (EO). The survey was conducted on a sample of 301 white laboratory rats of both sexes, which were divided depending on their stage of postnatal ontogenetic development and experimental model: control; animal with experimental osteopathy (EO); animals with EO+CMT-28 1 mg/kg body weight; animals with EO+CMT-67 1 mg/kg body weight; animals with EO+BioR 1mg/kg body weight; animals with EO+BioR 2 mg/kg body weight; animals with EO+CMT-28+BioR (1 mg/kg body weight each); animals with EO+BioR+CMT-67 (1 mg/kg body weight each).**

In physiological conditions the ontogenetic modifications of the bone collagen and HYP content are statistically conclusive and of the same orientation, the maximum concentration was identified in adult animals and the minimum at advanced stages of postnatal ontogenesis regardless of sex.

Experimental osteopathy is characterized by age and sex dependent changes of the collagen and hydroxyproline concentrations in bone, the most significant changes being revealed in young females.

Copper coordination compounds, BioR and their combinations increase the turnover of the collagen in bone in EO that is accompanied by decrease of collagen and increase of the HYP concentration that is probably a compensatory mechanism oriented to the replacement of the damaged collagen and the restoration of its normal nanostructure with the recovery of the bone.

I. INTRODUCTION

Nanofibers are defined as fibers with diameters less than 1000 nanometers. Nature has created a variety of biological nanofibers with outstanding properties that are used in different ways by the living organisms. As classic examples of natural nanofibers serve elastin, collagen, keratins, fibrin, etc.

Collagen is the most abundant protein in the human body, comprising about 25% of their total [1]. Collagen constitutes 90-95% of the extracellular matrix (ECM) of the bone, represented predominantly by type I. Types V, XI and XII and *FACIT* forms (*F*ibrils *A*ssociated *C*ollagens with *I*nterrupted *T*riple helices) that are associated with large-diameter collagen fibers, constitute a minor quantity, but are essential for bone morphogenesis.

Regardless of the collagen type the molecules are an indubitable example of a hierarchical biological nanomaterial [2]. Fibers consist of tropocollagen molecules with diameter of 1,5 nm and length of 300 nm, which are linked by covalent cross-links and pyridinolinic links in the non-helical N-and C-terminal regions to form collagen [3].

Collagen synthesis and degradation are continuous phasic processes that are vital for maintaining normal concentrations of components and specific nanostructure of the extracellular matrix of the bone and its mineralization. Major steps of the collagen nanofibers formation are hydroxylation of proline and lysine radicals and the tropocollagen cross- through oxidized lysine radicals [4].

Prolyl-4-hydroxylase and prolyl-3-hydroxilase are responsible for proline hydroxylation and formation of hydroxyproline (HYP) [4]. The lysine oxidation is catalyzed by lysyl oxidase that uses copper ions as coenzyme [5]. The necessity for copper in the formation of bone and maintenance of its structure is well established. There are also a lot of studies that show that the lack of this mineral will lead to abnormal bone formation and fractures in newborns, infants, and sometimes even in adults [6, 7].

Thus, the aim of our research was to assess the influence of copper coordination compounds and cyanobacterian remedy on bone collagen and hydroxyproline concentrations at different stages of postnatal ontogenesis in normal physiological conditions and experimental osteopathy.

II. MATERIALS AND METHODS

The survey was conducted on a sample of 301 white laboratory rats of both sexes without pedigree. The animals were divided into the following experimental groups depending on ontogenetic stage of postnatal development and experimental model.

- Group I - 90 young rats before sexual maturity (two months);

- Group II - 78 adult rats in the reproductive period (six months);
- Group III - 54 old, postmenopausal rats (18 months);
- Group IV - 33 senile rats (24 months).

Animals from groups I-III were divided into the following subgroups: control (intact animals), animals with EO induced by administration of 0,1 ml of 50% CCl_4 in olive oil per 100 g body weight twice a week for 8 weeks; animals EO+BioR 1mg/kg body weight; animals with EO+BioR 2 mg/kg body weight, animals with EO+CMT-28 1 mg/kg body weight; animals with OE+CMT-67 1 mg/kg body weight; animals with OE+BioR+CMT-28 (each 1 mg/kg body weight); animals with EO+BioR+CMT-67 (each 1 mg/kg body weight).

The remedy BioR obtained from the biomass of cyanobacterium *Spirulina platensis* was provided by Valeriu Rudic, professor, director of the Institute of Microbiology and Biotechnology of the Academy of Sciences of Modova and copper coordination compounds (CC) CMT-28 and CMT-67 - by Aurelian Gulea, professor, Head of the Inorganic Chemistry Department of MSU.

BioR was diluted with sterile 0,9% NaCl until the final concentration of 1 mg/ml and was injected intramuscularly each 0,1 ml/100 g body weight (1 mg/kg body weight) and 0,2 ml/100 g body weight (2 mg/kg body weight) for 10 consecutive days. Coordination copper compounds CMT-28 and CMT-67 were diluted in 10% sol. of propilenglicol until the final concentration of 1 mg/ml and injected subcutaneously each 0,1 ml/100 g body weight (1 mg/kg body weight) for 10 consecutive days.

At 24 hours after the last administration the animals were euthanized under light narcosis with sulfuric ether. The femoral bones were removed, cleaned from adjacent tissues and the bone marrow removed by repeated washings with ice solution of 0,9% NaCl. Femoral bones were then frozen in liquid nitrogen, grinded to the state of powder and weighed on torsion balance.

Determination of collagen content was performed after the dissolution in 0,43 M trichloroacetic acid of a sample of bone tissue triturated in liquid nitrogen until the state of powder (20 mg). Determination of collagen in bone tissue was performed according to the procedure described [9]. The method is based on the preliminary transformation of the native bone collagen in soluble gelatin by processing the biological material with a heated to 90°C solution of trichloroacetic acid (ATA), sedimentation of non-collagenic proteins by centrifugation and determination of the gelatin concentration by classical Lowry assay. The amount of collagen was expressed in milligrams of collagen per gram of bone (mg/g).

Hydroxiproline content in bone was determined by Шараев П.Н. (1981) method in modification of [9]. The method is based on the oxidation of HYP to pyrol which interacts in acidic environment with p-dimetilaminobenzaldehyde (DMBA) and form a colored product that is assayed spectrophotometrically. The amount of HYP is expressed in mmol/g tissue.

For testing the significance of the differences between the studied groups the non-parametric statistical test Mann-Whitney were applied. Statistical evaluation of data obtained was performed with computer program Statistical Software "StatsDirect" (2001)

III. RESULTS AND DISCUSSION

The results of the study show the same orientation of the ontogenetic changes of free HYP and collagen content in the bone tissue in the postnatal period (Table 1).

TABLE 1.ONTOGENETIC CHANGES OF HYDROXYPROLINE (HYP) AND COLLAGEN CONCENTRATIONS IN BONE TISSUE IN NORMAL PHYSIOLOGICAL CONDITIONS AND EO

POS	Sex	Group	HYP	Colagen
Young	M	Control	110,46±5,03	2,45±0,13
		EO	105,99±4,32	2,74±0,33
	F	Control	104,66±5,48	$3,59±0,17^{\#\#\#\#}$
		EO	$126,46±5,63^{\S\#}$	$2,98±0,18^{\S}$
Adult	M	Control	$135,46±5,90^{***T}$	$4,44±0,23^{****T}$
		EO	$137,27±7,34^{*T}$	$5,02±0,32^{**T}$
	F	Control	128,67±5,78	$4,83±0,18^{****T}$
		EO	$116,07±4,45^{\#}$	$4,59±0,16^{****T}$
Old	M	Control	$54,80±1,46^{***A}$	$2,82±0,17^{****A}$
		EO	$52,94±6,54^{**A}$	$2,71±0,21^{**A}$
	F	Control	$57,69±5,17^{****A}$	$2,79±0,17^{****A}$
		EO	$51,54±3,87^{***A}$	$2,96±0,11^{****A}$
Senile	M	Control	$85,14±2,78^{****B}$	$2,04±0,06^{****B}$
	F	Control	$75,54±2,40^{***B\#}$	2,73±0,08

a) Each value represents $\bar{X} \pm m$

b) POS – postnatal ontogenetic stages

c) Statistical significance between different ontogenetic stages according to *U Mann-Whitney*: * p<0,05;** p<0,01; *** p<0,001; **** p<0,0001

d) Statistical significance between male and female rats according to *U Mann-Whitney*: $^{\#}$ p<0,05; $^{\#\#}$ p<0,01

e) Statistical significance compared with control group according to *U Mann-Whitney*: § – p<0,05; §§ – p<0,01; §§§ – p<0,001.

The research has established that the concentration of collagen is significantly higher in adult animals compared with all other groups studied, the amount of the collagen peaks at this stage of ontogenetic development, regardless of animal sex. Thus, collagen concentration is higher in adult males with 81% (p<0,001) and females - 36% (p<0,001) compared with the young rats. At later stages collagen levels gradually decreased. In old males the collagen content is 42% (p<0,001) lower than in adults, and in senile with 27% (p<0,001) lower than in the old one.

In females it was established the same trend of decreasing the collagen content, but statistically reliable were only changes of collagen concentration in old rats compared to adult animals (reduction by 39%, p<0,001).

It was established that the content of the free HYP increased in young males versus adult (23%, p<0,005), decreased in the old compared to young (50%, p<0,001) and adult (45%, p<0,005), and then again increased in the senile versus old (55%, p<0,001). Similar changes have been identified in females, but statistically significant in this case were only the differences between the free HYP content in adult and old animals (reduction by 55%, p<0,001), and the

old and senile one (31%, p<0,005). The maximum concentration of free HYP was identified in adult animals, and the minimum in old animals regardless of sex.

Statistically reliable sex differences of the concentration of free HYP were recorded only in senile rats, the level of the compound is lower for females (11%, p<0,05) compared to males.

It was found that EO induced by CCl_4 administration caused statistically reliable changes in the level of collagen only in young females, in which an 17% decrease (p<0,05) compared with controls were depicted (Table 1).

Ontogenetic dynamics of the level of collagen in bone tissue of animals with the EO is similar to that determined in control animals. Both in males and females with EO the concentration of collagen increases in adult animals compared with young by 83% (p<0,01), respectively, 54% (p<0,001), and then decreases in the old compared to the adult by 46% (p<0,01) and 35% (p<0,001), respectively. There were no sex-dependent differences in the level of collagen in animals with EO, regardless of the ontogenetic stage of development.

It was established that in EO the concentration of free HYP in the bone of young males did not change statistically conclusive. In young females, by contrast, it was established a 21% increase (p<0,05) compared to the control group. In males was identified an ontogenetic dynamics of the free HYP concentration similar to that specific to control animals. Thus, the HYP concentration in adult rats were by 30% (p<0,05) higher than in young rats and hydroxyproline content in the old animals were by 42% (p<0,01) lower than in the adults. In females with EO were established a progressive decrease in bone level of free HYP: HYP content of adult animals show a tendency to decrease by 8% compared to young animals and in those old values were by 56% (p<0,001) lower compared with those specific for the adult animals.

Gender differences had been established in young and adult animals with EO – in young males HYP concentration was by 19% (p<0,05) lower than in females, and in adults by 15% (p<0,05) higher than the values in females.

It was founded that the medication with copper coordination compounds CMT-28 and CMT-67 does not induce statistically conclusive changes in the concentration of collagen in bone tissue of experimental animals with EO. Only in adult animals copper coordination compound CMT-28 reduced collagen concentration compared with controls (24%, p<0,001), as well as with EO (20%, p<0,001) (Table 2).

Copper coordination compounds CMT-28 and CMT-67 did not induce statistically conclusive changes in the concentration of free HYP in bone compared with EO group level, with the exception of adult ones. In this group CMT-28 increases the level of free HYP by 24% (p<0,05) compared to EO animals.

Thus, in adult animals the copper coordination compound CMT-28 is decreasing the collagen content and at the same time increasing the amount of free HYP in bone. This can be an evidence of increased breakdown of damaged collagen in the bone of adult animals with EO. This process is a stage of the restoration of the collagen content and structure in the bone that precedes the biosynthesis of normal collagen fibers.

TABLE 2IINFLUENCE OF COPPER COORDINATION COMPOUNDS CMT-28 AND CMT-67 ON THE CONCENTRATION OF HYDROXYPROLINE (HYP) AND COLLAGEN IN THE BONE OF ANIMALS WITH EO AT POSTNATAL ONTOGENETIC STAGES

POS	Group	HYP	Collagen
Young	Control	104,66±5,48	3,59±0,17
	EO	126,46±5,63§	2,98±0,18§
	EO+CMT-28	123,46±5,54§	2,89±0,29
	EO+CMT-67	140,77±13,41§	3,34±0,29
Adult	Control	128,67±5,78	4,83±0,18
	EO	116,07±4,45	4,59±0,16
	EO+CMT-28	$^{\#}$144,16±8,91	$^{\#\#}$3,65±0,16§§§§
	EO+CMT-67	115,09±6,91	4,44±0,26
Old	Control	57,69±5,17	2,79±0,17
	EO	51,54±3,87	2,96±0,11
	EO+CMT-28	65,91±7,46	3,12±0,18
	EO+CMT-67	72,27±11,28	3,25±0,18

a) Each value represents $\overline{X} \pm m$

b) POS – postnatal ontogenetic stages

c) Statistical significance between different ontogenetic stages according to *U Mann-Whitney*: * p<0,05; ** p<0,01; *** p<0,001; **** p<0,0001;

d) Statistical significance compared with EO group according to *U Mann-Whitney*: $^{\#}$ – p<0,05; $^{\#\#}$ – p<0,01; $^{\#\#\#}$ – p<0,005, $^{\#\#\#\#}$ – p<0,001;

e) Statistical significance compared with control group according to *U Mann-Whitney*: § – p<0,05; §§ – p<0,01; §§§ – p<0,001.

The results of the research show that cyanobacterial remedy BioR in both doses did not alter the concentration of free HYP in the bone of rats with EO regardless of ontogenetic development stage (Table 3).

Only a slight tendency of increase of the HYP content were revealed in young animals compared with the control and EO specific levels after the administration of 2 mg/kg BioR, in adult animals after the administration of BioR in both doses and in old one after the administration of 1 mg/kg BioR.

Collagen levels were not statistically significant changed after the administration of BioR regardless of dose or age of the animals. Nevertheless, administration of 1 mg/kg of BioR induced a trend of increase in old animals by 17% compared with animals with EO. This amount of collagen was higher then the control levels by 12%.

This data revealed that BioR is conserving the collagen content in the bone of the animals with EO. Possible the high content of active compounds in the cyanobacterial remedy BioR (aminoacids, oligopeptides, polysaccharides, microelements etc.) create favorable environment for optimization of bone metabolism and adaptation to pathological conditions in EO.

The combination BioR+CMT-28 reduced the concentration of collagen in young (18%, p<0,05) and adult (19%, p<0,01) animals compared with the control, and in the adult (15%, p<0,01) and old (12%, p<0,05) animals compared EO group. The combination BioR+CMT-67 changed statistically conclusive the collagen concentration only in adult animals (decrease by 19%, p<0,05) compared with the group with EO (Fig. 1 and 2).

Administration of the cyanobacterial remedy BioR in combination with the copper coordination compounds CMT-28 and CMT-67 produced significant changes in the concentration of free HYP in young animals with EO.

TABLE 3.IINFLUENCE OF CYANOBACTERIAN REMEDY BIOR ON THE CONCENTRATION OF HYDROXYPROLINE (HYP) AND COLLAGEN IN THE BONE OF ANIMALS WITH EO AT POSTNATAL ONTOGENETIC STAGES

POS	Group	HYP	Collagen
Young	Control	110,46±5,03	2,45±0,13
	EO	105,99±4,32	2,74±0,33
	BioR 1mg/kg	105,99±4,32	2,76±0,14
	BioR 2 mg/kg	120,67±10,15	2,74±0,17
Adult	Control	135,46±5,90***^{T}	4,44±0,23****^{T}
	EO	137,27±7,34^{*T}	5,02±0,32^{**T}
	BioR 1 mg/kg	146,67±4,76	5,02±0,22
	BioR 2 mg/kg	149,05±9,20	4,55±0,06
Old	Control	54,80±1,46***^{A}	2,82±0,17****^{A}
	EO	52,94±6,54^{**A}	2,71±0,21^{**A}
	BioR 1 mg/kg	63,29±3,92	3,16±0,08
	BioR 2 mg/kg	54,03±7,40	2,85±0,24

a) Each value represents $\overline{X} \pm m$

b) POS – postnatal ontogenetic stages

c) Statistical significance between different ontogenetic stages according to *U Mann-Whitney*: * p<0,05; ** p<0,01; *** p<0,001; **** p<0,0001,

d) Statistical significance compared with EO group according to *U Mann-Whitney*: $^{\#}$ – p<0,05; $^{\#\#}$ – p<0,01; $^{\#\#\#}$ – p<0,001,

e) Statistical significance compared with control group according to *U Mann-Whitney*: § – p<0,05; §§ – p<0,01; §§§ – p<0,001.

The combined administration to the young animals of BioR+CMT-28 increased the bone content of free HYP compared with control values by 35% (p<0,05) and BioR+ CMT-67 – by 30% (p<0,05), while compared with animals from the EO group the free HYP content was modified only by the combination BioR+CMT-67 (35%, p<0,05).

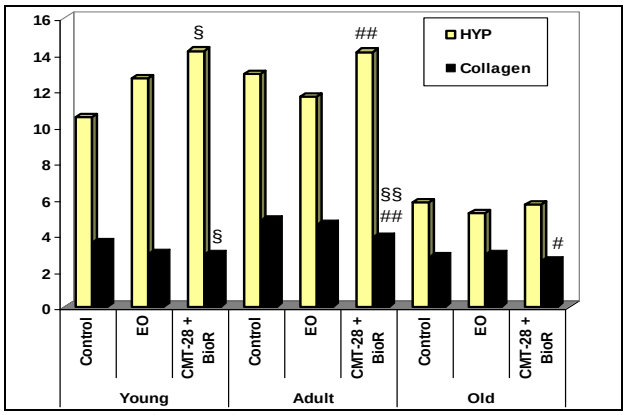

Figure 1. Iinfluence of cyanobacterial remedy BioR and copper coordination compound CMT-28 on the concentrations of hydroxyproline (HYP) and collagen in the bone of animals with experimental osteopathy (EO) at postnatal ontogenetic stages

a) Statistical significance compared with EO group according to *U Mann-Whitney*: $^{\#}$ – p<0,05; $^{\#\#}$ – p<0,01; $^{\#\#\#}$ – p<0,001,

b) Statistical significance compared with control group according to *U Mann-Whitney*: § – p<0,05; §§ – p<0,01; §§§ – p<0,001.

In adult animals the combination BioR+CMT-28 increased the concentration of free HYP by 22% (p<0,01) compared with the values observed in animals with EO, bringing it to 10% higher values (p>0,05) than the control.

The combination BioR+CMT-67 also enhanced the content of HYP over control values and those specific for the EO animals, but changes are not statistical reliable. None of the studied combinations produced statistically reliable changes in old animals with EO.

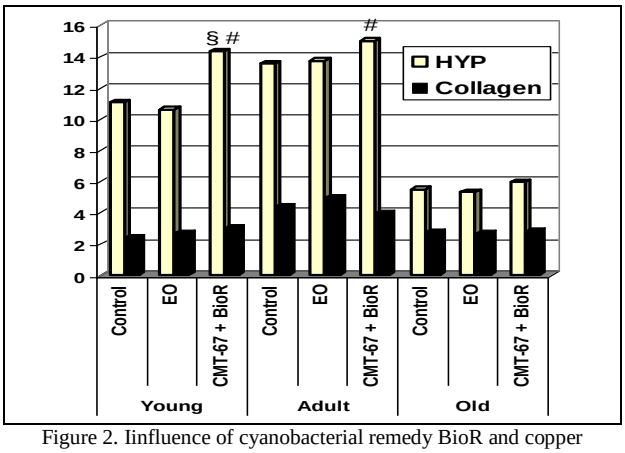

Figure 2. Iinfluence of cyanobacterial remedy BioR and copper coordination compound CMT-28 on the concentrations of hydroxyproline (HYP) and collagen in the bone of animals with experimental osteopathy (EO) at postnatal ontogenetic stages.

a) Statistical significance compared with EO group according to *U Mann-Whitney*: $^{\#}$ – p<0,05; $^{\#\#}$ – p<0,01; $^{\#\#\#}$ – p<0,001,

b) Statistical significance compared with control group according to *U Mann-Whitney*: § – p<0,05; §§ – p<0,01; §§§ – p<0,001.

Combined administration of the copper coordination compounds CMT-28 and CMT-67 with the cyanobacterial remedy BioR is characterized by potentiation of the drug influence on bone metabolism.

The increased content of HYP that is correlated with a decreased amount of collagen that we reveal in our study can be a sign of the enhanced collagen turnover in EO. Prolonged administration of CCl_4 triggered free radical production and oxidative damage of cellular and extracellular compounds [9]. Damaged proteins, including collagen, are removed from the tissues and replaced with normal one. Possible, the decrease of collagen concentration and increase of HYP – marker of bone resorption, is reflecting the efforts of the tissue to compensate the deterioration caused by the EO by degrading the abnormal collagen and replacing it with proper fibers that will restore the normal structure on the extracellular network.

Previous studies revealed that the properties of collagen are scale-dependent and that the strength of the tropocollagen molecules differs from the strength of the collagen fibril. The late depends on the number of the cross-links in the fibril, on the fibril length, etc. Thus, the nanostructure of collagen may provide either a strong bone or a brittle one [2].

Our results prove the ability of the copper coordination compounds CMT-28 and CMT-67 and cyanobacterial remedy BioR to intervene in the metabolism of collagen nanofibers at the essential stages. The magnitude of the tissue response is depending on the postnatal ontogenetic stage. Copper coordination compounds and cyanobacterial remedy can induce the correction of the damage caused by the CCl_4 to the collagen fibers and provide the restoration of its physiological nanostructure with the recovery of the bone structure and properties.

IV. CONCLUSIONS

1. In physiological conditions the ontogenetic modifications of the content of bone collagen and HYP are statistically conclusive and of the same orientation,

the maximum concentration of collagen and free HYP was identified in adult animals and the minimum at advanced stages of postnatal ontogenesis regardless of sex.

2. Experimental osteopathy is characterized by age and sex dependent changes of the collagen and hydroxyproline concentrations in bone, the most significant changes being revealed in young females.
3. Copper coordination compounds (CMT-28 and CMT-67), cyanobacterial remedy BioR and their combinations increase the turnover of the collagen in bone in experimental osteopathy, that is accompanied by decrease of collagen concentration and increase of the hydroxyproline amount.
4. The increase of the turnover of the collagen in bone in experimental osteopathy induced by copper coordination compounds (CMT-28 and CMT-67), cyanobacterial remedy BioR and their combinations is probably a compensatory mechanism oriented to the replacement of the abnormal collagen and the restoration of its normal nanostructure with the recovery of the bone.

REFERENCES

[1] M.K. Gordon, R.A. Hahn, *Collagens*, Cell Tissue Res., vol. 339, no. 1, 2010, pp. 247-257.

[2] M.J.Buehler, *Nature designs tough collagen: Explaining the nanostructure of collagen fibrils*. PNAS, vol. 103, no. 33, 2006, pp. 12285-12290.

[3] П.Г. Роби, *Биохимия кости*. В: Остеопороз. Этиология, диагностика, лечение. Под ред. Риггз Б.Л., Мелтон III Л.Дж.. СПб, "Издательство БИНОМ", "Невский диалект", 2000, стр. 57-84

[4] L. Koláčná, J. Bakešova , F. Varga et al. *Biochemical and Biophysical Aspects of Collagen Nanostructure in the Extracellular Matrix*. Physiol. Res., no. 56, suppl. 1, 2007, pp. S52-S60.

[5] R.B. Rucker, J. Murray, *Cross-linking amino acids in collagen and elastin*. American Journal of Clinical Nutrition, no. 31, 1978, pp. 1221-1236.

[6] Y.L. Lai, M. Yamaguchi. *Effects of Copper on Bone Component in the Femoral Tissues of Rats: Anabolic Effect of Zinc Is Weakened by Copper*. Biol. Pharm. Bull., vol. 28, no. 12, 2005, pp. 2296-2301.

[7] D. Conlan, R. Korula, D. Tallentire, *Serum copper levels in elderly patients with femoral-neck fractures*. Age and Ageing, vol. 19, 1990, pp. 212-214.

[8] V. Gudumac, O. Tagadiuc, V. Rîvneac et al. *Investigaţii biochimice*. Elaborare metodică. Micrometode. Vol.II. Ch.: Elena V. I. SRL, 2010. 104 p.

[9] M.I. Díaz Gómez, J.A. Castro, *Covalent binding of carbon tetrachloride metabolites to liver nuclear DNA, protein and lipids*. Toxicol Appl Pharmacol, no. 56, 1980, pp. 199-206.

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