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VITAMIN C AND HYDROXYPROLINE AS MARKERS OF RADIATION-INDUCED CHANGES IN THE EXTRACELLULAR MATRIX

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Introduction. Despite significant scientific interest in connective tissue, the effects of radiation on its components remain insufficiently studied. Existing research primarily focuses on the effects of ionizing radiation at radiotherapeutic doses, while only a limited number of studies address changes in the connective tissue matrix under radiation exposure.

Materials and methods. An experimental study was conducted on 40 sexually mature rats exposed to radiation doses of 1.0, 3.0, and 5.82 Gy to determine the role of vitamin C and hydroxyproline in the development of connective tissue disorders following exposure to different doses of ionizing radiation.

Results. It was established that total γ-irradiation leads to a dose-dependent decrease in ascorbic acid levels, which in turn disrupts the hydroxylation of proline to hydroxyproline.

Conclusions. The impairment of hydroxylation processes with increasing radiation doses results in disturbances in the post-translational modification of collagen. This is accompanied by a decrease in total and protein-bound hydroxyproline levels, indicating a predominance of collagen degradation over biosynthetic processes, an increase in free hydroxyproline, reflecting collagen degradation processes, a rise in peptide-bound hydroxyproline, suggesting incomplete collagen breakdown and an inability to be reutilized in secondary synthesis.

Keywords: total gamma irradiation, connective tissue, ascorbic acid, fractions of hydroxyproline.

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ВІТАМІН С ТА ОКСИПРОЛІН ЯК МАРКЕРИ РАДІАЦІЙНО-ІНДУКОВАНИХ ЗМІН У ЕКСТРАЦЕЛЮЛЯРНОМУ МАТРИКСІ
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В експериментальному дослідженні на статевозрілих щурах, опромінених дозами 1,0, 3,0 та 5,82 Гр, було визначено роль вітаміну С (аскорбінової кислоти) та оксипроліну у формуванні порушень сполучної тканини у тварин після опромінення різними дозами іонізуючого випромінювання. Встановлено, що тотальне γ-опромінення призводить до дозозалежного зниження вмісту аскорбінової кислоти, що обумовлює порушення процесів гідроксилювання проліну до оксипроліну. Послаблення процесів гідроксилювання із зростанням дози опромінення тварин призводить до порушення пострансляційної модифікації колагену і до зменшення рівня загального та білковозв'язаного оксипроліну, що свідчить про перевагу розпаду колагену над процесами біосинтезу, підвищенням вільного оксипроліну, що відображає процеси деградації колагену та пептиднозв'язаного оксипроліну, що свідчить про неповний розпад колагену й неможливість його залучення у вторинний синтез.

Ключові слова: тотальне γ-опромінення, сполучна тканина, аскорбінова кислота, фракції оксипроліну.

Introduction. Connective tissue, despite the apparent simplicity of its structure – predominantly consisting of specific amino acids and mechanisms of their hydroxylation – is characterized by a significant diversity in the extracellular matrix, collagenous, and elastic fibers. It is important to note that certain types of these structures are genetically determined, whereas others are formed under the influence of epigenetic factors. This underscores the complexity and multifaceted organization of connective tissue at both the molecular and cellular levels [1, 2].

The extracellular matrix of connective tissue is primarily composed of collagen proteins, represented by 20 different types. Some of these are true collagens, while others contain only collagen-like domains. Vitamin C (ascorbic acid) plays a crucial role in the post-translational formation of mature collagen structure; its deficiency impairs collagen synthesis, resulting in less stable and mechanically weaker collagen [3, 4].

Collagen is the only protein that contains hydroxyproline – a specific amino acid [5] which serves as a biomarker for collagen metabolism in the body [6]. Hydroxyproline levels are measured in biological substrates to assess the state of collagen metabolism. In diseases associated with connective tissue damage, an increased excretion of hydroxyproline is observed, which is due to active collagen degradation. This makes hydroxyproline a valuable indica-

tor for diagnosing and monitoring pathological processes that affect connective tissue [7, 8].

Despite considerable scientific interest in connective tissue [9], the impact of radiation on its components remains insufficiently studied. Existing research mainly focuses on the effects of ionizing radiation in therapeutic doses, with only a few studies addressing changes in the connective tissue matrix under radiation exposure. This complicates the formation of a comprehensive understanding of the patterns of radiation response in connective tissue and highlights the need for further in-depth investigation, particularly to elucidate the mechanisms of damage and possible means of correction [10–11].

Objective. To investigate the changes in ascorbic acid and hydroxyproline levels in the blood and urine of animals following exposure to varying doses of ionizing radiation.

Materials and Methods. The study was conducted on 40 sexually mature male Wistar rats weighing 180–220 g, maintained on a standard vivarium diet. For the experiment, the animals were subjected to total-body single-dose gamma irradiation using Co^{60} in special organic glass chambers, performed in the morning on an empty stomach using the "Agat" teletherapy unit. The distance to the absorption source was 75 cm, with a dose rate of 0.54 Gy/min. The absorbed doses administered were: 1.0 Gy (Group I), 3.0 Gy (Group II), and 5.82 Gy (Group III), with 10 animals irradiated in each group. A control group consisted of 10 intact (non-irradiated) animals.

Animal housing, handling, and experimental procedures were carried out in accordance with the "General Ethical Principles for Animal Experiments" adopted by the Fifth National Congress on Bioethics (Kyiv, 2013), the recommendations of the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes (Strasbourg, 1985), the guidelines of the State Expert Center of the Ministry of Health of Ukraine "Preclinical Drug Studies" (2001), and the regulations on humane treatment of laboratory animals approved by the Bioethics Committee of Odesa National Medical University (Protocol No. 32D dated 17.03.2016).

The animals' biostatus was assessed based on changes in locomotor activity, feeding behavior, grooming reflex, condition of the coat, mucous membranes, and gastrointestinal function.

To determine the levels of ascorbic acid and hydroxyproline (HP) fractions in blood serum, blood samples were collected from the tail vein, and urine was collected using metabolic cages 24 hours after irradiation [12].

Ascorbic acid content was measured colorimetrically using 2,4-dinitrophenylhydrazine: in blood serum (μmol/L) and in urine (nmol/day) [13].

Connective tissue metabolism was assessed by determining the content of total hydroxyproline (THP), free hydroxyproline (FHP), peptide-bound hydroxyproline (PHP), and protein-bound hydroxyproline (PBHP). The method is based on measuring the optical density of the red chromogen formed by the oxidation of hydroxyproline molecules with chloramine B and the condensation of the oxidation products with p-dimethylaminobenzaldehyde. Reagents of analytical grade (chemically pure and for analytical use) were employed [14]. Calibration solutions of hydroxyproline were prepared using Pierce reagents (The Netherlands).

The obtained data were subjected to statistical analysis using the Student's *t*-test and chi-squared (χ^2) test, as well as relevant statistical software. Statistical significance was accepted at $p < 0.05$.

Results and Discussion. The results of the study demonstrated that the concentration of ascorbic acid in the blood serum of animals in Group I (dose of 1.0 Gy) was 68.6 μmol/L, compared to 54.8 μmol/L in the control group (Table 1). These data indicate a non-significant increase in ascorbic acid concentration by 25.2%. Additionally, urinary excretion of ascorbic acid in Group I animals slightly exceeded that of the control group by 27.62%.

In the blood serum of animals in Group II (dose of 3.0 Gy), the ascorbic acid content significantly decreased by approximately 1.4 times compared to intact animals, accompanied by a 19.5% reduction in urinary excretion, which was not statistically significant relative to the control. Exposure of rats in Group III (dose of 5.82 Gy) resulted in the most pronounced changes in ascorbic acid concentration, with the lowest serum level observed – more than 2.3 times lower – alongside a significant decrease in urinary excretion by 34.2% compared to intact animals. Considering that urinary excretion of ascorbic acid decreased with increasing radiation dose (Groups II and III), it can be inferred that irradiated animals either exhibit reduced endogenous synthesis of this metabolite or impaired absorption of ascorbic acid in the gastrointestinal tract. Consequently, it can be assumed that the post-translational hydroxylation of proline in collagen synthesis is weakened. The reduction in ascorbic acid levels was associated with changes in the content of individual hydroxyproline fractions in the experimental animals, depending on the radiation dose (Table 2).

Table 1

Ascorbic acid content in biological fluids of experimental animals 24 hours after irradiation

Animal groups (n=40)	Ascorbic Acid	
	blood serum, μmol/L	urine, nmol/day
Control group	54.8 ± 4.2	144.8 ± 12.8
I group	68.6 ± 5.2	184.8 ± 13.6
II group	39.5 ± 3.5*	116.7 ± 11.8
III group	23.8 ± 2.4*	95.4 ± 10.1*

Note:

* – $p < 0.05$ denotes a statistically significant difference in the studied parameters compared to those in the intact control group.

Table 2

Serum collagen metabolic indicators 24 hours after irradiation

Animal groups (n = 40)	Total hydroxyproline (THP), μmol/L	Free hydroxyproline (FHP), μmol/L	Peptide-bound hydroxyproline (PHP), μmol/L	Protein-bound hydroxyproline (PBHP), μmol/L	Peptide-bound / free hydroxyproline (PHP/FHP)
Control group	119.6 ± 5.82	11.8 ± 0.31*	8.9 ± 0.22*	98.9 ± 3.02*	0.75 ± 0.03
I group	130.1 ± 6.38	13.6 ± 0.54**	11.2 ± 0.53**	105.3 ± 4.82	0.79 ± 0.06*
II group	119.4 ± 5.81	16.2±0.52**	24.6±1.24**	78.6±2.43**	1.52 ± 0.12**
III group	111.9 ± 5.23	16.8 ± 1.32**	31.7 ± 1.68**	63.2 ± 1.96**	1.89 ± 0.14**

Note:

* – $p < 0.05$ denotes a statistically significant difference in the studied parameters compared to those in the intact control group.

** – $p < 0.05$ – statistically significant differences in the studied parameters compared to the total hydroxyproline levels in intact animals.

It should be noted that the hydroxyproline content in the blood mainly reflects the metabolism of bone collagen [15]. Our study found that the total hydroxyproline content in intact animals significantly exceeded the level of protein-bound hydroxyproline by 21%, and was more than 10 times higher than that of free hydroxyproline. Meanwhile, the ratio of peptide-bound to free hydroxyproline was significantly lower, reaching 0.75.

After exposure to a dose of 1.0 Gy, a slight increase in all hydroxyproline fractions was observed, along with a slight rise in the ratio of peptide-bound to free hydroxyproline compared to control animals.

In Group II animals, the level of total hydroxyproline did not differ significantly from that of intact rats. However, a redistribution of values among the hydroxyproline fractions occurred. The content of protein-bound hydroxyproline was more than 20% lower than in the control animals, while free hydroxyproline increased sharply, exceeding the control value by 37%. The level of peptide-bound hydroxyproline increased nearly 2.8-fold. Notably, the ratio of peptide-bound to free hydroxyproline changed significantly – doubling compared to the control. This increase in the ratio was mainly due to the sharp rise in peptide-bound hydroxyproline, more than 2.7 times higher than in intact animals. The low level of protein-bound and high level of peptide-bound hydroxyproline indicate a predominance of collagen degradation over biosynthesis.

The most significant changes in hydroxyproline fractions were observed in Group III animals compared to the control group. A sharp increase in peptide-bound hydroxyproline (more than 3.5 times) was recorded, reflecting the degree of collagen breakdown. At the same time, the free hydroxyproline level increased by more than 1.4 times. Since the free hydroxyproline content in blood serum reflects collagen degradation processes [16], its elevation indicates the dominance of collagen fiber degradation.

A significant decrease in protein-bound hydroxyproline compared to control (by nearly 1.6 times) was observed against a background of an insignificant reduction in total hydroxyproline (by only 6.5%). The reduction in protein-bound hydroxyproline suggests impaired collagen biosynthesis.

A striking finding was the sharp increase in the ratio of protein-bound to free hydroxyproline, which rose 2.5 times compared to intact animals.

The detected changes in hydroxyproline fractions under different doses of ionizing radiation reflect damage to the extracellular matrix. It has been shown that increased levels of peptide-bound hydroxyproline in blood serum serve as a marker of incomplete collagen degradation, making it unavailable for reuse in collagen resynthesis [17].

An elevated concentration of peptide-bound hydroxyproline may indicate disruption of homeostasis at various regulatory levels, particularly in intercellular interactions. This, in turn, leads to impaired dynamics of reparative regeneration, rendering the process maladaptive and resulting in changes to collagen ultrastructure.

All of the above contributes to the disruption of biopolymer degradation, which is directly involved in connective tissue formation.

Thus, total γ-irradiation leads to a reduction in ascorbic acid content, which impairs the hydroxylation of proline to hydroxyproline. As radiation dose increases, the weakening of hydroxylation processes leads to depletion of collagen synthesis reserves and is accompanied by an insignificant decrease in total hydroxyproline levels alongside a significant decrease in protein-bound hydroxyproline in Groups II and III. This indicates a predominance of collagen breakdown over biosynthesis, an increase in free hydroxyproline reflecting collagen degradation processes, and an increase in peptide-bound hydroxyproline indicating incomplete collagen breakdown and its unavailability for secondary synthesis.

Hence, this dynamic of collagen metabolite changes reflects the development of structural, metabolic, and functional disturbances in connective tissue, the intensity of which correlates with the degree of collagen degradation.

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