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Study of wound-healing properties of humic substance – zinc complexes in the aseptic wound model *in vivo*

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ABSTRACT

The aim was to investigate wound-healing properties of zinc-containing biocomposites based on humic ligands (humic substance (HS) – Zn) in the *in vivo* experiment on the aseptic wound model and to evaluate their resorptive properties.

Materials and methods. The objects of the study were 5 samples of HS-Zn in the form of complex salts comprising fine black powders synthesized in the Laboratory for Natural Humic Systems of the Faculty of Chemistry at Moscow State University. The wound-healing effect of the substances was studied on 70 male Wistar rats using a traumatic model of an excisional aseptic skin wound. The degree of affected skin healing was evaluated during 21 days by the planimetric method. The resorptive properties of the HS-Zn samples were studied by inductively coupled plasma mass spectrometry (ICP-MS) in the biomaterial (blood serum, fur, skin from the wound surface).

Results. It was found that course application of zinc-containing HS-Zn biocomposites to the wound surface led to a decrease in the wound area in comparison with $ZnSO_4$ with the equivalent concentration of elemental Zn (1.67 mg / ml). Two samples FA-Zn and Peat1-Zn showed the most pronounced regenerating effect. We noted an increase in Zn level in the tested skin samples from the wound area, in fur, and in the blood serum, which indicates the resorptive effect of zinc-containing HS-Zn biocomposites during course application; however, the parameters did not exceed limiting permissible concentrations. The correlation between the tested samples was not equal, which indicates a significant impact of the initial HS matrix on the Zn bioavailability.

Conclusion. The observed reparative effect of zinc and HS complexes in the context of their low toxicity is of interest for further study to develop effective wound-healing preparations.

Keywords: zinc, humic substances, ligands, wound healing

Conflict of interest. The authors declare the absence of obvious and potential conflicts of interest related to the publication of this article.

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Исследование ранозаживляющих свойств комплексов цинка с гуминовыми веществами в эксперименте *in vivo* на модели асептической раны

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РЕЗЮМЕ

Цель – исследовать ранозаживляющие свойства цинксодержащих биокомпозиций на основе гуминовых лигандов (ГВ-Zn) в эксперименте *in vivo* на модели асептической раны и оценить их резорбтивные свойства.

Материалы и методы. Объекты исследования – пять образцов ГВ-Zn в форме комплексных солей, синтезированные в лаборатории природных гуминовых систем химического факультета МГУ, представляющие собой мелкодисперсные порошки черного цвета. Ранозаживляющее действие исследуемых веществ было изучено на 70 самцах крыс линии Wistar с использованием травматической модели плоскостной асептической кожной раны. Степень заживления пораженного участка кожи оценивали в течение 21 сут планиметрическим методом. Изучение резорбтивных свойств образцов ГВ-Zn проводилось методом масс-спектрометрии с индуктивно-связанной плазмой в биоматериале (сыворотка крови, шерсть, кожа с раневой поверхности).

Результаты. Установлено, что курсовое нанесение на раневую поверхность цинксодержащих биокомпозиций ГВ-Zn приводит к уменьшению площади раны в сравнении с площадью раны при нанесении $ZnSO_4$ с эквивалентной концентрацией элементарного Zn (1,67 мг/мл). Наиболее выраженный регенерирующий эффект проявили два образца: FA-Zn и Peat1-Zn. Отмечено увеличение уровня Zn в опытных участках кожи раневой поверхности, в шерсти и сыворотке крови, что указывает на резорбтивное действие цинксодержащих биокомпозиций ГВ-Zn при их курсовом применении, но показатели не превышали уровня допустимых предельных концентраций. Также отмечена неодинаковая зависимость между тестируемыми образцами, что свидетельствует о значительном влиянии исходной матрицы ГВ на биодоступность Zn.

Заключение. Обнаруженный репарационный эффект композиций цинка и гуминовых лигандов на фоне их низкой токсичности представляет интерес для дальнейшего изучения с целью разработки на их основе эффективных ранозаживляющих препаратов.

Ключевые слова: цинк, гуминовые вещества, лиганды, ранозаживление

Конфликт интересов. Авторы декларируют отсутствие явных и потенциальных конфликтов интересов, связанных с публикацией настоящей статьи.

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INTRODUCTION

Traumatism, and in particular, injuries of various etiologies are a priority and relevant issue in modern medicine, despite the introduction of state-of-the-art advances in high technology. Aseptic, infected, and purulent lesions of soft tissues are very likely to be obtained in domestic and industrial conditions, during the occurrence and elimination of the consequences of natural and man-made disasters [1]. Moreover, the aggravation of the issue is facilitated by a decrease in the activity of the body natural resistance systems and rapid changes in the morphophysiological organization of the wound area microflora [1–3]. As a result, people get temporary or lifelong disability, working capacity is significantly reduced, and a multiple increase in government spending is observed [4]. In view of this, the search for substances that have a positive effect on the main stages of the repair process and are more accessible in comparison with device-based treatment is relevant.

Advances in molecular biology, medical elementology, and a number of other natural sciences have proven that normal wound healing occurs with the participation of biometals, in particular zinc (Zn) [5–7]. Zinc is a cofactor of more than 300 matrix metalloproteinases, which have anti-inflammatory, antioxidant, immunomodulatory, and antibacterial effects and inhibit the proliferation and differentiation of keratinocytes by directly inhibiting / activating enzymes and influencing gene expression [6–8]. Today, in the form of oxide and salts (sulfate, acetate, gluconate, etc.), zinc is included in topical medications for the treatment of wounds, diaper rash, and skin defects [6, 9–11].

It is known that the ionic form of Zn^{2+} has low bioavailability, therefore, to achieve the optimal concentration locally, long-term administration is required, which is associated with the development of both local and systemic side effects [5, 12]. Recently, it has become possible to simultaneously reduce toxicity and increase biological activity, and sometimes achieve new types of pharmacological action in Zn (as well as in a number of other metals) and its derivatives, which are absent in the ionic form due to the formation of complexes whose ligands are polymers [3, 5, 13, 14].

Humic substances (HS), products of the transformation of plant matter under the influence of biotic and abiotic factors, are promising high-molecular compounds. Having a wide variety of oxygen- and nitrogen-containing functional groups in their structure, HS can interact with various compounds of living cells, forming bonds with them through exchange, donor – acceptor, and other mechanisms. As a result, it becomes possible to affect the activity of cells in many organs and systems and, thereby, realize pleiotropic biological effects: immunomodulatory, anti-inflammatory, antioxidant, antihypoxic, etc.

The effect of hepatitis B on cells of the immune system is one of the most studied types of their activity. It was proven that they increase the humoral immune response in mice, enhance the synthesis of tumor necrosis factor (TNF)α, interleukin (IL)-1β, IL-12 by animal peritoneal macrophages and the production of interferon (IFN)γ and TNFα by peripheral blood mononuclear cells of healthy donors [15]. Due to a common type of pharmacological action in zinc and HS, but a different mechanism for its implementation, it can be assumed that the creation of a complex coordination zinc compound containing HS as a ligand will allow to achieve synergism, which will make it possible to heal wound rapidly without developing complications. Therefore, the aim of this work was to investigate the wound-healing properties of zinc-containing biocomposites based on humic ligands (HS – Zn) in an *in vivo* experiment on an aseptic wound model and to evaluate their resorptive properties.

MATERIALS AND METHODS

The objects of research were 5 samples of zinc-containing biocomposites based on humic ligands (HS – Zn), synthesized in the Laboratory for Natural Humic Systems of the Faculty of Chemistry of Moscow State University. Their characteristics are presented in Table 1. In order to synthesize HS – Zn samples, a HS solution with a concentration of 15 g / l was prepared, which was centrifuged at 5,000 rpm to separate the ballast. Next, this solution was mixed with the prepared solution of zinc nitrate with a concentration of 4.84 g / l in a ratio of 1:5 ($Zn(NO_3)_2$:humate). The concentration of humates in the solution was recalculated taking into account the mass of the separated ballast to maintain equal ratios of humate to zinc nitrate. The synthesis was carried out for 4 hours without heating while maintaining pH = 9, using sodium hydroxide. The samples were then freeze-dried. The samples were frozen at –50 °C and placed in the Scientz-18ND Top-Press multi-manifolds freeze-drying system. Drying was carried out in external flasks for 2 days. The HS – Zn samples are complex salts comprising fine black powders.

Table 1

Experimental samples of zinc-containing biocomposites based on humic ligands (HS – Zn)

HS sample code (base ligand)	Description of HS samples	HS – Zn sample code
CHP	Humic acids of Powhumus coal (Humintech, Germany)	CHP-Zn
FA	Peat fulvic acids Fulvagra (Humintech, Germany)	FA-Zn
CHS	Coal humic substances (Sakhalin humates, Russia)	CHS-Zn
Peat1	Humic acids of high Angustifolium peat (Tomsk, Russia)	Peat1-Zn
Peat2	Humic acids of high-moor sphagnum-hollow peat (Tomsk, Russia)	Peat2-Zn

The wound-healing effect of the studied substances was studied on 70 male Wistar rats weighing 250–300 g. All manipulations and euthanasia were carried out in mandatory compliance with the rules of the "European Convention for the Protection of Vertebrate Animals used for Experimental or for other Scientific Purposes." The keeping of animals and the design of the experiment complied with the ethical standards and principles of biomedical research and were approved by the committee for control of the care and use of laboratory animals ((IACUC of the Center for Preclinical Research of Siberian State Medical University (Report No. 02/21 of 02.02.21)).

Before the experiment began, the animals were randomly divided into 7 groups (*n* = 10): group 1 – intact, saline solution (SS) was injected into the wounds; group 2 – control, the wounds were treated with a reference listed drug, $ZnSO_4$ solution; group 3 – the wound surface was treated with CHP-Zn solution; in group 4, FA-Zn was used; in group 5, CHS-Zn was used; in group 6, Peat1-Zn was applied, and in group 7, Peat2-Zn was used. To reproduce the traumatic model of an excisional skin wound, each rat was anesthetized by the complex administration of Zoletil-100 and XilaVeta under aseptic conditions, the area of the shoulder blades was depilated, followed by the formation of a round wound (*d* = 20 mm) through excision of the skin and a subcutaneous tissue layer. The operative field was treated once with 70 % ethyl alcohol. All substances (SS, $ZnSO_4$ solution, HS – Zn) were applied to the wounds daily for 21 days in a volume of 0.5 ml (Zn concentration in terms of elemental Zn 1.67 mg / ml). The condition of the animals involved in the experiment was assessed daily. A comprehensive assessment of the course of the wound process was conducted using the planimetric analysis of wounds on day 3, 5, 7, 9, 11, 13, 15, 17, 19, and 21 with a digital camera under the same conditions, followed by image analysis using the ImageJ software.

The study of the resorptive properties of the HS – Zn samples was carried out using inductively coupled plasma mass spectrometry (ICP-MS) in order to establish the ability of zinc to overcome cellular barriers and accumulate in tissues and biological substrates. After the intravital course application of HS – Zn to the wounds, blood was taken from the animals, then serum was obtained. Then the rats were euthanized by CO_2 asphyxia and necropsy with the collection of biomaterial for subsequent determination of the Zn content in animal tissues, in particular, fur and skin were taken from the wound surface. To carry out ICP-MS, the studied samples were dried and subject to incineration at 500 °C for 2 hours. The resulting ash residues were converted into solution. Conversion into solution was carried out using pre-purified concentrated nitric acid (special purity), hydrogen peroxide, and the Milestone Start D microwave digestion system (200 °C, 700 W). After that, the samples were dried at 100–110 °C to the state of wet salts, then quantitatively transferred into disposable 50-ml polypropylene tubes using a background solution, i.e. 15% nitric acid with traces of hydrofluoric acid.

A blank experiment was prepared along with the samples. Before the analysis, an internal standard, an indium solution, was added to each test tube with samples and a blank sample. After that, all samples were diluted to the same volume. The calculation of the final results included taking into account the dilution factor, the internal standard, and the blank experiment. The analysis was performed on the low-resolution inductively coupled plasma mass spectrometer Agilent 7500cx (Agilent Technologies, USA).

Statistical analysis of the data obtained during *in vivo* experiments was carried out using the Statistica 8.0 program. The Shapiro – Wilk test was used to check the data for normality of distribution, followed by the assessment of equality of variances using Levene's test. If the distribution in the experimental groups was normal and intergroup equality of variances was observed, further data processing was carried out using the analysis of variance (ANOVA, parametric method) followed by post-hoc comparison using the Bonferroni method and the Dunnett's test. When the distribution was different from normal and the intergroup equality of variances was not observed, the Kruskal – Wallis and Friedman tests were used (nonparametric statistics).

All results were presented as the mean and the error of the mean ($M \pm SE$). In the study of resorptive properties, statistical data was processed using the Statistica 8.0 and GraphPad Prism 8.0 programs. The level of statistical significance of differences between the samples was analyzed using the Kruskal – Wallis test. The differences were considered statistically significant at $p < 0.05$.

RESULTS AND DISCUSSION

The results of a planimetric study on the effect of the zinc-containing biocomposites based on HS when applied daily to aseptic wounds are presented in Tables 2 and 3. Starting from the 5th day of applying SS, as well as samples of CHS-Zn, Peat1-Zn and Peat2-Zn, a statistically significant decrease in the proportion of the initial area of the wound surface was observed. A statistically significant decrease in the proportion of the initial wound surface area when applying CHP-Zn and FA-Zn samples was observed later (from day 7) ($p < 0.05$). It is important to note that when Peat1-Zn (on day 5, 7 and 11), FA-Zn (on day 7, 11 and 17) and CHP-Zn (on day 21) were administered there was a statistically significant decrease in the proportion of the initial wound surface area in comparison with the comparison group $ZnSO_4$ ($p < 0.05$).

Table 2

Effect of zinc-containing biocomposites (HS – Zn) on the healing of aseptic wounds, $M \pm SE$

Group	Proportion of the initial wound surface area, %									
	day 3	day 5	day 7	day 9	day 11	day 13	day 15	day 17	day 19	day 21
SS	94.02±4.97	90.81±5.61^	69.43±1.74^	42.00±5.33	27.83±1.93^	19.48±3.05^	12.42±1.83^	6.94±1.03^	3.09±0.69^	1.63±0.63^
$ZnSO_4$	95.14±4.70	91.75±5.19	74.59±4.09^	46.57±6.76^	28.25±2.32^	19.48±1.82^	14.17±1.65^	7.31±0.97^	3.13±0.81^	2.04±0.35^
CHP-Zn	93.00±4.09	85.17±3.84	65.23±3.19^	39.24±3.65^	20.44±1.27^	15.12±1.76^	7.59±1.32^	3.22±1.09^	0.15±0.15^	0.00±0.00#^
FA-Zn	86.37±4.58	79.56±4.71	59.66±2.46#^	34.03±2.06^	17.05±1.47#^	11.50±1.03^	6.91±0.94^	2.68±0.79#*^	0.56±0.28^	0.27±0.17^
CHS-Zn	108.05±4.02	95.07±3.01^	81.13±3.47^	48.66±5.13^	24.73±1.76^	22.85±2.14^	11.53±1.63^	8.37±1.10^	2.55±0.94^	1.56±0.65^
Peat1-Zn	95.50±6.45	77.29±5.51#^	58.06±4.53#^	33.09±2.86^	16.05±1.90#^	12.69±1.65^	8.14±1.40^	3.55±0.65^	1.61±1.01^	0.56±0.56^
Peat2-Zn	104.69±6.17	90.25±3.40^	68.06±4.34^	41.82±2.68^	22.31±1.35^	17.42±1.59^	9.82±0.76^	3.63±0.73^	1.25±0.53^	0.46±0.33^

The differences are statistically significant: * with the SS group, $p < 0.05$; # with the $ZnSO_4$ group, $p < 0.05$; ^ with the Day 3 group, $p < 0.05$.

Table 3

Changes in excisional wound healing in rats under the influence of zinc-containing biocomposites (HS – Zn)

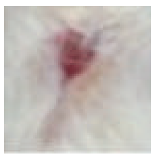
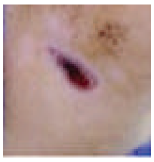
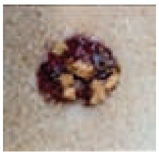
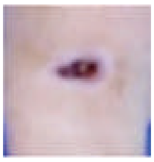
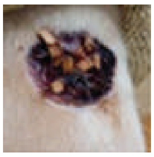
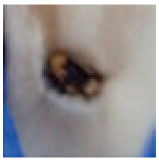
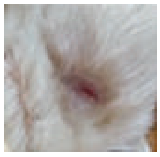
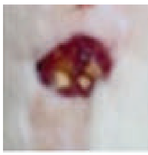
Group	Injected substance	Duration of treatment, days			
		3	7	13	21
1	SS				

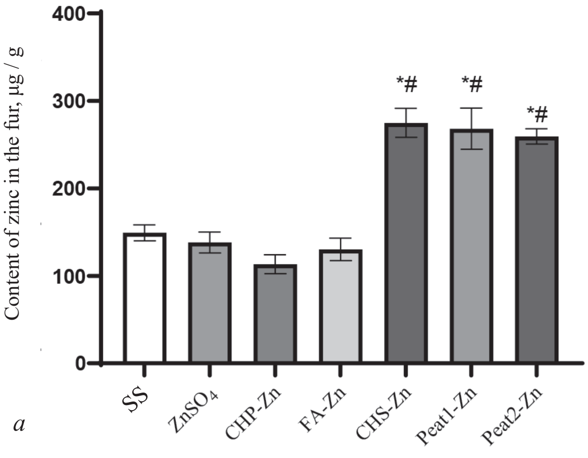
Table 3 (continued)

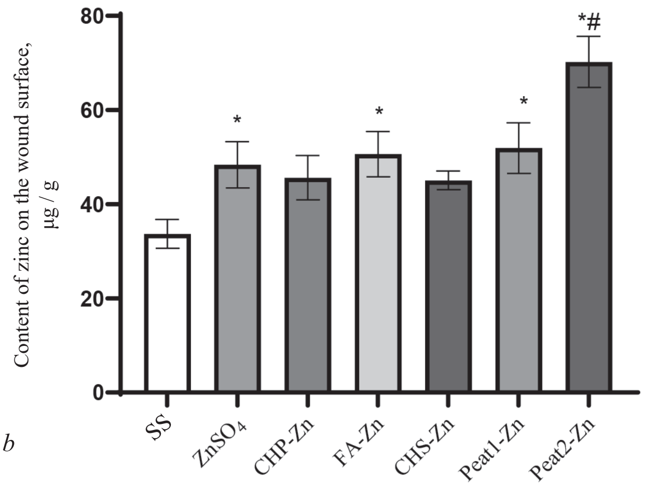
Group	Injected substance	Duration of treatment, days			
		3	7	13	21
2	$ZnSO_4$				
3	CHP-Zn				
4	FA-Zn				
5	CHS-Zn				
6	Peat1-Zn				
7	Peat2-Zn				

The results of assessing Zn content in the wound surface area of the skin, fur, serum, and in intact areas when zinc-containing samples (HS-Zn) were applied externally to the wound surface are presented in Figure 1. It was established that Zn content in the fur of rats whose wound surface areas were treated with SS was comparable with the literature data: 149.23 ± 9.04 μg / ml [16]. In rats treated with the reference listed drug $ZnSO_4$ (the studied samples CHP-Zn and FA-Zn), no significant differences from the control group were observed in terms of zinc content in fur (Fig. 1, *a*).

When studying CHS-Zn, Peat1-Zn, and Peat2-Zn, there was a significant increase in Zn content in rat fur by 82, 78, and 73%, respectively, compared to the controls, which may indicate the presence of a resorptive effect when they were applied to the area treated. Zn content in the wound surface of the skin is consistent with the physiological indices of Zn in the skin [17]. It was shown that all skin samples to which HS – Zn was applied had a higher Zn concentration compared to the control group (Fig. 1, *b*).

The maximum Zn concentration in the skin was noted for the Peat2-Zn sample (70.2 ± 5.4 μg / g), in the remaining samples CHP-Zn, FA-Zn, CHS-Zn, Peat1-Zn, including the reference listed drug $ZnSO_4$, there was an increase in Zn concentration compared to the controls by 36, 51, 36, 54, and 45%, respectively, but no significant differences were found between the samples. Based on the results of assessing Zn content in the blood serum of rats, it was shown that an increase in Zn concentration was observed only in the CHS-Zn, Peat1-Zn, and Peat2-Zn samples compared to the control group (3.2 ± 0.37; 2.9 ± 0.2, and 3.1 ± 0.3 μg / g). No significant differences were





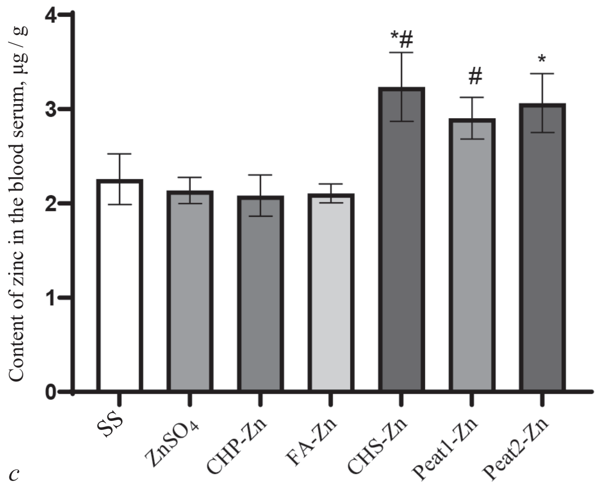


Fig. 1. Effect of daily course application of HS – Zn on Zn content in the fur (*a*), samples obtained from the wound surface (*b*), and blood serum (*c*) of experimental rats: SS – sodium saline. The differences are statistically significant: * compared to the SS group; # compared to the ZnSO4 group

noted in the remaining $ZnSO_4$, CHP-Zn, and FA-Zn samples compared to the control group.

CONCLUSION

The course application of zinc-containing biocomposites FA-Zn and Peat1-Zn, containing elemental zinc at a concentration of 1.67 mg / ml, accelerates the healing of aseptic wounds, as evidenced by a decrease in the wound surface area in comparison with the wound area when applying $ZnSO_4$ with an equivalent concentration of elemental zinc. It was established that course application of zinc-containing HS – Zn biocomposites provides a resorptive effect, but Zn content in the biomaterial does not exceed the level of limiting permissible concentrations.

It was also noted that the base ligands of HS affect the bioavailability of Zn. Thus, the CHP-Zn and FA-Zn samples do not affect the bioavailability of Zn, while the CHS-Zn, Peat1-Zn, and Peat2-Zn samples increase the bioavailability of Zn compared to the control group. The discovered reparative effect of HS – Zn biocomposites, in addition to their low toxicity, is of interest for further study in order to develop effective wound-healing drugs.

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Authors' contribution

Zykova M.V., Perminova I.V., Ivanov V.V. – conception and design, interpretation of the data, critical revision of the manuscript for important intellectual content. Larionov K.S. – synthesis of zinc-containing biocomposites based on humic ligands. Bratishko K.A., Azarkina L.A., Ufandeev A.A., Rabtsevich E.S., Mikhalev D.A., Kopnov I.S. – studies of biological activity and resorptive action, analysis of the data. Azarkina L.A. – drafting of the manuscript. Perminova I.V., Belousov M.V. – fundraising, final approval of the manuscript for publication.

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