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# INFLUENCE OF CERTAIN D-METALS ON FORMATION OF ADVANCED GLYCATION END PRODUCTS, AGGREGATION AND AMYLOID TRANSFORMATION OF ALBUMIN IN GLYCATION REACTION

**R.A. Litvinov[1,2], A.V. Gontareva[1], L.E. Usmiyanova[1], D.R. Klimenko[1]**

[1] Volgograd State Medical University
1, Pavshikh Bortsov Sq., Volgograd, Russia, 400131
[2] Volgograd Medical Research Center,
1, Pavshikh Bortsov Sq., Volgograd, Russia, 400131

E-mail: litvinov.volggmu@mail.ru

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**The aim** of the research is to investigate the influence of the factor of the glycation behavior of bovine serum albumin (BSA) by glucose, and the factor of d-metal cations (nickel (II), cobalt (II), iron (II), iron (III), copper (II) or zinc (II)) presence, on the process of aggregation and the amyloid transformation of BSA and, therefore, to establish the effect of these cations on the rate of the formation of advanced glycation end products (AGEs), and the intensity of fluorescence of the amino acids tyrosine and tryptophan.

**Materials and methods.** Reagents in the glycation are: glucose (at the final concentration of 0.36 M), BSA (at the final concentration of 1 mg/ml), deionized water, one of the d-metal cations, i. e. nickel (II), cobalt (II), iron (II), iron (III), copper (II) or zinc (II) (in the form of chloride, sulfate or nitrate salts, at the final concentration of 40 μM). The conditions for the glycation reaction are the incubation for 24 hours at the temperature of 60°C. The influence of two factors (the factor of the glycation reaction and the factor of a d-metal ion presence in the reaction medium) on the concentration of glycation end products (AGEs) formed during the glycation reaction, on the fluorescence intensity of the amino acids tryptophan and tyrosine, on the aggregation of BSA, and on the ability of BSA to the amyloid transformation under the described conditions, have been studied.

**Results.** It was found out that the studied factors have a statistically significant effect on the considered parameters. The highest activity was found for the copper ion (II), which intensifies the formation of the AGEs in the samples where glycation occurs, reduces the fluorescence intensity of the amino acids' tryptophan and tyrosine (independently and increasing the effect against the background of glycation). Besides, it independently causes the aggregation of BSA hereby intensifying the effect against the background of glycation, it independently causes the amyloid transformation of BSA enhancing the effect against the background of glycation. The above-listed effects were the least pronounced in the reaction media with the addition of nickel (II) or cobalt (II). These cations reduce the rate of the AGEs formation, do not cause the formation of protein aggregates. In the presence of glucose, nickel (II) weakly suppresses the fluorescence intensity of tryptophan and tyrosine, and slightly enhances the amyloid transformation of BSA. Cobalt (II) slightly inhibits the amyloid transformation of BSA. In terms of the severity and nature of the effects, the iron (II), iron (III) and zinc (II) cations occupy an intermediate position between copper (II), on the one hand, and nickel (II) and cobalt (II), on the other hand, combining the influence on the AGEs formation, the intensity of fluorescence of tryptophan and tyrosine, the aggregation and amyloid transformation of BSA. In the absence of glucose, the ability of zinc (II) to induce the formation of protein aggregates turned out to be the highest, and its ability to stimulate the amyloid transformation of BSA corresponded to that of copper (II).

**Conclusion.** The presence of d-metal cations affects the rate of the AGEs formation in the glycation reaction, affects the rate of the BSA amyloid transformation and the protein aggregates formation. Among such ions as nickel (II), cobalt (II), iron (II), iron (III), copper (II) and zinc (II), copper (II) ions turned out to be the most active in their ability to accelerate the AGEs formation, suppress the fluorescence of tryptophan and tyrosine, enhance the aggregation and amyloid transformation of BSA in the glycation reaction. The least manifestation of these properties is observed for nickel (II) and cobalt (II) ions.

**Keywords:** advanced glycation end products; glycation; protein aggregation; amyloid transformation, d-metal

**Abbreviations:** AGEs – advanced glycation end products; BSA – bovine serum albumin; RAGEs – Receptor for advanced glycation end products; ThT – thioflavine T

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# ВЛИЯНИЕ НЕКОТОРЫХ D-МЕТАЛЛОВ НА ОБРАЗОВАНИЕ КОНЕЧНЫХ ПРОДУКТОВ ГЛИКИРОВАНИЯ, АГРЕГАЦИЮ И АМИЛОИДНУЮ ТРАНСФОРМАЦИЮ АЛЬБУМИНА В РЕАКЦИИ ГЛИКИРОВАНИЯ

**Р.А. Литвинов[1,2], А.В. Гонтарева[1], Л.Э. Усмиянова[1], Д.Р. Клименко[1]**

[1] Федеральное государственное бюджетное образовательное учреждение высшего образования «Волгоградский государственный медицинский университет» Министерства здравоохранения Российской Федерации
400131, Россия, г. Волгоград, площадь Павших Борцов, д. 1
[2] Государственное бюджетное учреждение «Волгоградский медицинский научный центр»
400131, Россия, г. Волгоград, площадь Павших Борцов, д. 1

E-mail: litvinov.volggmu@mail.ru

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**Цель.** Исследование влияния фактора протекания реакции гликирования бычьего сывороточного альбумина (БСА) глюкозой и фактора присутствия в среде реакции гликирования катионов d-металлов (никель (II), кобальт (II), железо (II), железо (III), медь (II) или цинк (II)) на процесс агрегации и амилоидной трансформации БСА. Установление влияния указанных катионов на интенсивность образования конечных продуктов реакции гликирования (КПГ) и интенсивность флуоресценции аминокислот тирозин и триптофан.

**Материалы и методы.** Реагенты в реакции гликирования: глюкоза (в конечной концентрации 0,36 М), БСА (в конечной концентрации 1 мг/мл), деионизированная вода, один из катионов d-металлов, а именно никель (II), кобальт (II), железо (II), железо (III), медь (II) или цинк (II) (в виде соли хлорида, сульфата или нитрата, в конечной концентрации 40 мкМ). Условия протекания реакции гликирования: инкубация 24 ч при температуре 60°С. Исследовано влияние двух факторов (фактор протекания гликирования и фактор присутствия иона d-металла в реакционной среде) на концентрацию КПГ, образуемых в ходе реакции гликирования, на интенсивность флуоресценции аминокислот триптофан и тирозин, на агрегацию БСА и на способность БСА к амилоидной трансформации в описанных условиях.

**Результаты.** Установлено, что исследуемые факторы статистически значимо влияют на рассматриваемые параметры. Наивысшая активность установлена для иона меди (II), который интенсифицирует образование КПГ в пробах, где протекает гликирование, снижает интенсивность флуоресценции аминокислот триптофан и тирозин (самостоятельно и усиливая эффект на фоне гликирования), вызывает агрегацию БСА (самостоятельно и усиливая эффект на фоне гликирования), вызывает амилоидную трансформацию БСА (самостоятельно и усиливая эффект на фоне гликирования). Наименее выражены перечисленные эффекты были в реакционных средах с добавлением никеля (II) или кобальта (II). Данные катионы снижают интенсивность образования КПГ, не вызывают образования белковых агрегатов. В присутствии глюкозы никель (II) слабо подавляет интенсивность флуоресценции триптофана и тирозина, незначительно усиливает амилоидную трансформацию БСА. Кобальт (II) незначительно подавляет амилоидную трансформацию БСА. Катионы железа (II), железа (III) и цинка (II) по выраженности и характеру эффектов занимают промежуточное положение между медью (II) с одной стороны, и никелем (II) и кобальтом (II) с другой стороны, в разной степени сочетая влияние на образование КПГ, интенсивность флуоресценции триптофана и тирозина, агрегацию и амилоидную трансформацию БСА. В отсутствии глюкозы способность цинка (II) вызывать образование белковых агрегатов оказалась наивысшей, а его способность стимулировать амилоидную трансформацию БСА соответствовала таковой у меди (II).

**Заключение.** Присутствие катионов d-металлов влияет на интенсивность образования КПГ в реакции гликирования, влияет на интенсивность амилоидной трансформации БСА и на образование агрегатов белка. В ряду таких ионов, как никель (II), кобальт (II), железо (II), железо (III), медь (II) и цинк (II), ионы меди (II) оказались наиболее активными по способности ускорять образование КПГ, подавлять флуоресценцию триптофана и тирозина, усиливать агрегацию и амилоидную трансформацию БСА в реакции гликирования. Наименьшая выраженность указанных свойств отмечается для ионов никеля (II) и кобальта (II).

**Ключевые слова:** конечные продукты гликирования; гликирование; агрегация белка; амилоидная трансформация; d-металл

**Список сокращений:** КПГ – конечные продукты гликирования; БСА – бычий сывороточный альбумин; РКПГ – рецептор к конечным продуктам гликирования; ThT – тиофлавин Т

## INTRODUCTION

Non-enzymatic glycation is the source of toxic advanced glycation end products (AGEs). AGEs are a group of more than 20 molecules [1, 2], differing in their properties, including the ability to autofluorescence and the crosslinks formation. AGEs are important pathogenetic factors in the development of cognitive impairments (diabetic encephalopathy, conformational brain diseases) [3–6]. The structures of some AGEs are shown in Fig. 1.

AGEs are involved in the pathogenesis of cognitive impairment through various mechanisms. In the culture of hippocampal neurons, AGEs induced apoptosis, increased the production of pro-apoptotic Bax protein and acetylcholinesterase, decreased the level of antiapoptotic protein Bcl-2, glutathione peroxidase, superoxide dismutase and

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choline acetyltransferase, increased the concentration of malondialdehyde, etc. [7] AGEs are able of activating the AGEs receptor (RAGEs). It is assumed that the RAGEs links the pathogenesis of diabetes mellitus and Alzheimer's disease [8]. The RAGEs can be activated by Aβ protein [9] and is involved in its intraneuronal transport from the blood [10]. The RAGEs activation can lead to the development of the neuronal oxidative stress [11]. Under these conditions, if amyloid peptides are glycated, their RAGEs-mediated action can be enhanced [12]. For these reasons, it is advisable to consider the amyloid transformation and protein glycation as pathogenetically related processes.

In addition to the receptor-mediated relationship, AGEs are able to directly influence the amyloid transformation of proteins. By modifying the lateral chains of amino acids and the N-terminal residue of some proteins, AGEs can cause a change in the surface charge of the protein, a change in its hydrophobic properties and, as a consequence, lead to the amyloid transformation [13]. It is assumed that glycation contributes to the stabilization of protofibrillar structures, and the ability of AGEs to cross-link proteins to make the formation of larger conglomerates from amyloid aggregates possible [13, 14]. Under certain conditions, glycation slows down the formation of mature amyloid fibrils; however, this is associated with an extension of the life span of cytotoxic oligomeric forms [15, 16]. Amyloid oligomeric forms are able of destroying cell membranes, leading to a calcium imbalance, causing a mitochondrial dysfunction, and directly interacting with membrane proteins, leading to a change in their native state [14]. Thus, against the background of glycation, both acceleration and deceleration of the amyloid transformation have negative consequences, which makes the task of studying the effect of glycation on this process urgent.

The glycation reaction depends on many factors, in particular, on the presence of d-metal ions in trace concentrations (including transition metals and those close to them in properties), the presence of reactive oxygen intermediates, etc. [17, 18]. Transition metals include chemical elements the atoms of which have a partially filled d-sublevel or are able of forming cations with an incompletely filled d-sublevel (IUPAC[1]). The zinc subgroup and zinc itself belong to the metals close in properties to the transitional ones (some of them are called post-transition at times). Many properties of zinc are identical to those of transition metals, but its d-orbitals are filled[2]. To a great extent, a biological role of transition metals is due to the presence of an incomplete *d*-sublevel and how this sublevel is filled with electrons, since this, in turn, determines the tendency of a particular transition metal to form certain chemical bonds and their stability. In addition, the electronic configuration of the d-sublevel determines the presence of specific stable oxidation states and, as a consequence, the redox properties of the metal itself and its ions [19]. It was found out that the ability to change the oxidation state is an important property due to which some transition metals are able to stimulate glycation [20, 21]. Thus, in glycation reactions, copper (II) is reduced to copper (I), and oxygen is converted into superoxide anion with the participation of hydrogen peroxide, the source of which is some stages of glycation. After that, copper (I) ions are oxidized to copper (II), catalyzing the decomposition of hydrogen peroxide to a hydroxyl radical [20]. It should be noted that d-metals (zinc, iron, copper, etc.) are involved in the pathogenesis of cerebral conformational diseases by the mechanisms independent of glycation, associated with both the direct action of the ion on the protein (cross-linking of tyrosine residues, etc.) and indirectly, through the influence on the activity of enzymes (secretase, etc.) [22–25, 10]. Thus, the glycation reaction, the amyloid transformation of proteins, and the activity of d-metal ions, which can influence both of these processes, are pathogenetically related.

The amyloid protein transformation can be modeled using bovine serum albumin (BSA) [26, 27]. BSA is prone to the enhanced formation of β-sheets and the amyloid transformation under the physicochemical action (eg, heating), which causes its frequent use as a model protein in the study of amyloid transformation processes [28]. At the temperature that is borderline for the initiation of BSA amyloid aggregation, it becomes possible to assess the ability of the studied factor to accelerate or slow down the course of aggregation and amyloid transformation.

A comparative study of the d-metal ions ability to influence the rate of the AGEs formation in the glycation reaction, as well as their ability to stimulate or suppress the formation of amyloid and non-amyloid protein aggregates against the background of the glycation reaction and independently of it, are of interest.

**THE AIM** of the research is to evaluate the combined and independent influence of the factor of the BSA glycation reaction and the factor of the presence of d-metal cations (nickel (II), cobalt (II), iron (II), iron (III), copper (II) or zinc (II)) in the reaction medium, on the process of the BSA transformation into aggregates of the amyloid and non-amyloid nature. Besides, the influence of these factors on the fluorescence intensity of the amino acids' tryptophan and tyrosine and the ability of the indicated cations of d-metals to influence the intensity of the AGEs formation in the glycation reaction, are to be assessed.

## MATERIALS AND METHODS

### Modeling of glycation reaction

Glycation substrate is BSA (fraction V, 1 mg/ml, Himmed, Russia); glycating agent – glucose (0.36 M, Vekton, Russia); the reaction medium was deionized water (pH 6.2, deionizer Milli-Q, Germany) with the addition of one of the transition metal cations, i.e. nickel (II), cobalt (II), iron (II), iron (III), copper (II) or zinc (II) at the final concentration of 40 μM in the form of salts $NiSO_4 \cdot 7H_2O$, $Co(NO_3)_2 \cdot 6H_2O$, $FeSO_4$, $FeCl_3 \cdot 6H_2O$, $CuSO_4 \cdot 5H_2O$ or Zn-

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$SO_4$•7$H_2O$, respectively (or without them). The studied concentration of metal salts was selected on the basis of the previously adjusted model of BSA glyoxidation by glucose in the presence of $CuSO_4$•5$H_2O$, with modifications [29, 30]. The interest in a comparative study of the activities of d-metals, determines the need to research them in equal concentrations. In the body, the concentrations of the studied metals are variable. Thus, the physiological concentrations of copper and zinc in the serum of healthy people are ~1 mg/L (~15.7 μM and ~15.3 μM, respectively) [31, 32]. At the same time, in Alzheimer's disease, a significantly higher concentration of copper in amyloid plaques (~400 μM) has been reported [10, 25]. During the neuronal activity, zinc is released into the synaptic cleft and can reach the concentration of 300 μM [25]. For these reasons, the selected concentration (40 μM) is a compromise with respect to a wide range of d-metal concentrations in the body under normal and pathological conditions, and is more related to the previously established activity of the copper (II) cation.

The reaction conditions are: the temperature is 60°C, the incubation duration is 24 hours. These conditions were selected on the basis of the literature data [33], indicating that the effect of the selected temperature on BSA (the author used a Tris buffer containing NaCl) is favorable for the transformation of protein α-helices into β-sheets (which may not occur at lower temperatures), while the transformation process is intensified at higher temperatures. In another study it was shown that the BSA aggregation does not occur when exposed to the given temperature (for BSA in a Na-phosphate buffer solution) [34].

### Determination of glycation end products and fluorescence intensity of amino acids tryptophan and tyrosine

After the incubation, the aliquots of the samples (200 μL) were added to a 96-well flat-bottom black plate, the fluorescence intensity samples was determined at the excitation/emission wavelengths specific for the following AGEs: pentosidine (335/385 nm), vesperlysine C (345/405 nm), vesperlysines A and B (366/442 nm), crossline (379/463 nm) [35]. The fluorescence intensity at the excitation wavelengths exceeding 400 nm (440/520 nm), was also measured [36]. There is evidence that the product (or products) fluorescent at the excitation/emission wavelengths of 440/520 nm, belongs to the AGEs and is/are able of forming protein cross-links [37]. In addition to AGEs, the fluorescence intensity of the amino acids tryptophan and tyrosine was determined at the excitation/emission wavelengths of 295/335 nm. These wavelenghts are more specific for tryptophan, and 270/330 nm are characteristic of both amino acids (Infinite M200 Pro spectrofluorimeter, TECAN, Austria) [38–40]. The evaluation of the glycation effect and the d-metal ions action on the fluorescence of these amino acids is informative in view of the sensitivity of their fluorescence intensity to changes in the protein conformation and glycation of their amino acid environment [41, 42]. In addition, copper (II) cations promote the formation of dityrosine crosslinks, which is important for the pathogenesis of Alzheimer's disease [43], and, as a result, the fluorescent characteristics of tyrosine change. The possibility of a change in fluorescence upon oxidation of tryptophan and/or tyrosine, as well as an activation of tyrosine and its direct interaction with the reaction products upon glycation of its environment, cannot be ruled out [44, 42]. This makes the determination of the fluorescence of these amino acids relevant for the present study, and makes it possible to indirectly note changes in the course of both glycation and protein aggregation.

### Investigation of aggregation and amyloid transformation of proteins

In the study using spectrophotometry, the intensity of the protein aggregates formation was assessed, and the spectrofluorimetric determination of their amyloid affiliation in the reaction with thioflavin T (ThT) was carried out. A spectrophotometric detection of BSA aggregates was carried out by the increase in the optical density at the wavelength of 405 nm [45] in aliquots of 200 μL in a 96-well flat-bottom transparent plate (Infinite M200 Pro spectrofluorimeter, TECAN, Austria). The study [45] showed that the optical density at the given wavelength is directly proportional to the aggregation degree. The confirmation of the amyloid belonging of the aggregates, was carried out in the reaction with ThT (Sigma Aldrich, USA), at the final ThT concentration of 20 μM [46], by determining the fluorescence intensity of the samples at the excitation/emission wavelength's of 450/482 nm (Infinite M200 Pro spectrofluorimeter, TECAN, Austria).

### Statistical data analysis

In order to determine the contribution of each factor (both the factor of the glycation reaction and the factor of a metal ion presence), statistical data processing was carried out using a two-way analysis of variance, followed by a multiple comparison of data groups "all with all" according to Tukey test, at the significance level of $p \leq 0,05$ (GraphPad Prism 9). The correlation analysis was carried out using the Spearman rank correlation method (GraphPad Prism 9). The results are presented as mean with a standard error (M ± SEM).

## RESULTS

### Analysis of fluorescence intensity of tryptophan and tyrosine

As a result of the influence analysis of the investigated factors on the intensity of tryptophan and tyrosine fluorescence, it was found out that metal cations make a more significant contribution to the change in this parameter. For the cations of copper (II), iron (II), iron (III) and zinc (II), the ability to exert an effect was noted regardless of the glucose presence (a decrease in the fluorescence intensity of the amino acids tryptophan and tyrosine was noted not only in glycated, but also in glucose-free samples) (Table 1). At the same time,

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no differences were revealed when comparing the fluorescence intensity of tryptophan and tyrosine in the glycated samples that do not contain metals with the fluorescence intensity of the corresponding non-glycated samples (in which only BSA is present). Despite the absence of an intrinsic effect of the glycation reaction on the fluorescence intensity of amino acids, for some metals it was found out that they enhance their ability to suppress the fluorescence of amino acids in the presence of glucose, which indicates the importance of the glycation factor. Thus, a statistically significant decrease in the fluorescence intensity of tryptophan and tyrosine in glucose-containing samples (in comparison with the corresponding samples without glucose) was observed for the cases of glycation in the presence of copper (II) cations (at the excitation/emission wavelengths of 270/330 nm and 295/335 nm), iron (II) (at the excitation/emission wavelengths of 270/330 nm) and zinc (II) (at the excitation/emission wavelengths of 270/330 nm). Thus, these cations were able to enhance their action in the presence of glucose. With respect to cobalt (II), the effect of reducing the fluorescence of tyrosine and tryptophan in the presence of glucose was not observed when compared with the glucose-free samples. In the presence of nickel (II), this effect was weak (there were differences in the fluorescence intensity of the glycated samples containing nickel (II) and the glycated samples without metals, but there were no statistically significant differences in signals from the glycated and glucose-free samples containing nickel (II)).

### Influence of d-metals on the rate of AGEs formation in glycation reactions

The intensity of fluorescence at the wavelength's characteristic of various AGEs, reflects the intensity of the glycation reaction. As expected, in all cases, glucose-containing samples showed an increase in the intensity of AGEs fluorescence in comparison with the corresponding glucose-free samples (Table 1). However, the attention is drawn to the differences in the nature and degree of d-metal cations influence on the glycation reaction. When comparing the AGEs fluorescence intensities of the metal-containing samples in which glycation occurred, with the indices of the corresponding samples without metals, the following was found out. Only copper (II) cations (at all the excitation/emission wavelengths except 335/385 nm) and zinc (II) (the excitation/emission wavelengths of 440/520 nm) had the ability to enhance the formation of AGEs. The rest of the metals, on the contrary, or with statistical significance, prevented an increase in fluorescence at the wavelength's characteristic of AGEs, or did not change the values of the indicator. At the wavelengths characteristic of pentosidine (335/385 nm), no metal was able to intensify the formation of AGEs in comparison with the samples without metals. At the same time, nickel (II), cobalt (II), iron (II), and iron (III) were able to reduce the intensity of the signal detected at these wavelengths. The established activity makes it possible to isolate copper (II) and zinc (II) into the category of d-metals capable of accelerating the glycation under the described conditions. The cumulative results are shown in Table 1.

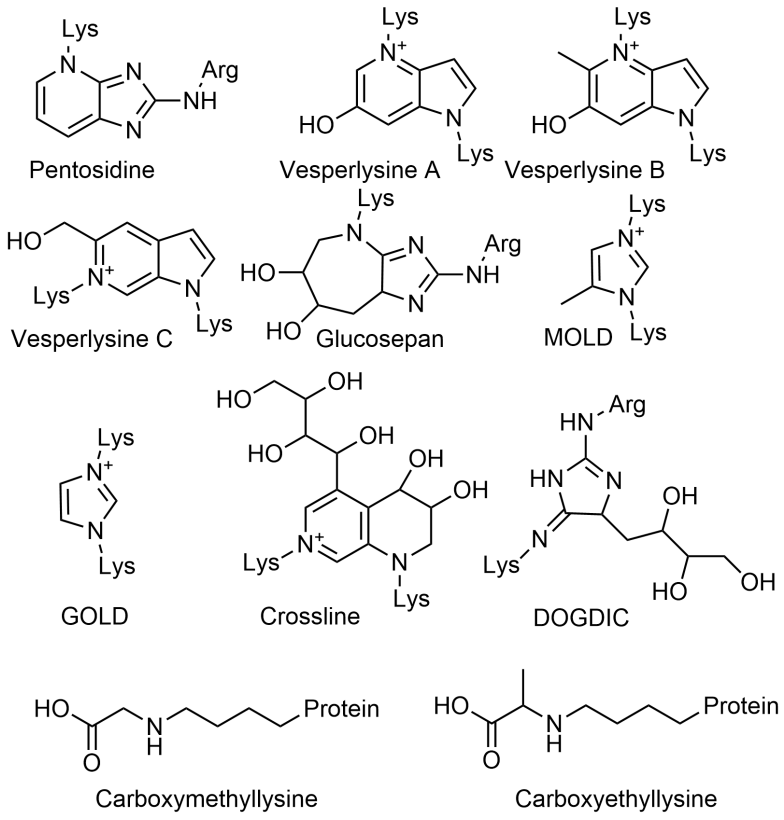


**Figure 1 – Structures of various advanced glycation end products**

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**Table 1 – Influence of investigated factors on the intensity of fluorescence emission characteristic of various AGEs, as well as amino acids tryptophan and tyrosine; absolute values of fluorescence (M ± SEM)**

| Glucose factor | Fluorescence parameters (λexc/λtest), nm and corresponding product | | Metal cation factor | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | None | Nickel (II) | Cobalt (II) | Iron (II) | Copper (II) | Iron (III) | Zinc (II) |
| Glucose | 270/330 | Tryptophan and tyrosine | 83147.2±1561.7 | 75475.8±1415.6 ** | 78131.2±1441.9 | 68838.8±1290.9 ****## | 30211.8±1680.2 ****#### | 63999.6±1399.6 **** | 70210.8±1417.6 *****# |
| Glucose-free | | | 83063.0±1000.5 | 80763.0±664.4 | 78733.8±926.2 | 76121.0±279.1 * | 56691.4±1055.4 **** | 62589.2±1609.8 **** | 76616.8±837.1 * |
| Glucose | 295/335 | Tryptophan (predominantly) | 41994.3±1290.6 | 37012.8±1205.5 * | 39814.8±1459.9 | 34861.6±1056.5 *** | 14637.2±587.9 ****#### | 31014.8±845.1 **** | 33909.6±707.4 **** |
| Glucose-free | | | 43126.5±303.6 | 40918.8±757.4 | 38680.0±881.2 * | 36587.8±813.1 ** | 27971.5±785.8 **** | 30535.0±1180.7 **** | 37617.0±456.1 ** |
| Glucose | 335/385 | Pentosidine | 752.8±13.4 #### | 538.6±15.8 ****#### | 580.6±17.2 ****#### | 490.2±15.0 ****#### | 757.0±29.5 #### | 395.0±11.9 ****#### | 748.8±17.7 #### |
| Glucose-free | | | 284.5±6.9 | 278.0±4.0 | 268.5±3.1 | 244.3±4.2 | 250.5±6.5 | 202.8±6.3 ** | 302.5±12.7 |
| Glucose | 345/405 | Argpyrimidine | 812.4±14.6 #### | 578.8±15.3 ****#### | 609.4±14.5 ****#### | 594.0±10.2 ****#### | 1058.2±19.1 ****#### | 452.4±7.3 ****#### | 861.8±8.6 #### |
| Glucose-free | | | 313.0±14.1 | 291.3±6.8 | 313.0±12.1 | 273.3±4.5 | 302.3±11.1 | 229.0±11.1 ** | 391.3±30.2 ** |
| Glucose | 366/442 | Vesperlysines A and B | 921.0±22.8 #### | 744.0±17.0 **#### | 728.2±13.6 **# | 874.0±61.5 #### | 2009.8±37.0 ****#### | 666.0±13.2 ****#### | 977.0±5.6 #### |
| Glucose-free | | | 491.0±11.0 | 477.2±9.2 | 561.2±33.9 | 504.8±49.4 | 594.8±21.5 | 390.0±27.6 | 661.4±40.8 * |
| Glucose | 379/463 | Crossline | 826.6±21.7 #### | 675.4±18.7 **#### | 644.2±10.9 ****## | 786.6±47.2 #### | 1917.8±34.9 ****#### | 618.8±12.1 ****#### | 828.6±7.6 #### |
| Glucose-free | | | 477.8±11.2 | 471.6±9.8 | 500.0±23.9 | 439.3±5.9 | 594.4±22.9 * | 365.2±20.7 * | 603.6±32.9 * |
| Glucose | 440/520 | Cross-links | 451.6±8.1 #### | 355.4±9.2 ****#### | 348.8±9.3 ****#### | 416.2±9.9 #### | 727.4±9.3 ****#### | 372.4±8.2 ****#### | 496.0±8.0 *#### |
| Glucose-free | | | 323.4±4.9 | 282.6±6.3 | 300.0±4.7 | 291.2±6.3 | 310.0±4.9 | 233.8±6.7 **** | 375.0±15.0 ** |

Note: significance level p when compared with the corresponding metal-free samples (**** corresponds to p<0.0001; *** corresponds to p<0.001; ** corresponds to p<0.01; * corresponds to p<0.05). Significance level p when compared with corresponding glucose-free samples (#### corresponds to p<0.0001; ### corresponds to p<0.001; ## corresponds to p<0.01; # corresponds to p<0.05 (two-way ANOVA, Tukey's post-test).

**Table 2 – Influence of investigated factors on formation of BSA aggregates, optical density of samples at wavelength of 405 nm; absolute values (M ± SEM)**

| Glucose factor | Metal cation factor | | | | | | |
|---|---|---|---|---|---|---|---|
| | None | Nickel (II) | Cobalt (II) | Iron (II) | Copper (II) | Iron (III) | Zinc (II) |
| Glucose | 0.06±0.0004 | 0.06±0.0006 | 0.06±0.0020 | 0.19±0.0021 ****#### | 1.08±0.0122 ****#### | 0.37±0.0188 **** | 0.65±0.0236 ****#### |
| Glucose-free | 0.05±0.0004 | 0.06±0.0007 | 0.05±0.0007 | 0.06±0.0007 | 0.18±0.0036 **** | 0.4±0.0090 **** | 1.02±0.0180 **** |

Note: significance level p when compared with corresponding metal-free samples (**** corresponds to p<0.0001; *** corresponds to p<0.001; ** corresponds to p<0.01; * corresponds to p<0.05). Significance level p when compared with corresponding glucose-free samples: #### corresponds to p<0.0001; ### matches p<0.001; ## matches p<0.01; # corresponds to p<0.05 (two-way ANOVA, Tukey's post-test

**Table 3 – Influence of the studied factors on amyloid BSA transformation, intensity of fluorescent ThT emission at excitation/emission wavelengths of 450/482 nm; absolute values (M ± SEM)**

| Glucose factor | Metal cation factor | | | | | | |
|---|---|---|---|---|---|---|---|
| | None | Nickel (II) | Cobalt (II) | Iron (II) | Copper (II) | Iron (III) | Zinc (II) |
| Glucose | 6051.0±320.2 #### | 7954.2±391.5 *#### | 5740.8±283.3 #### | 6083.4±241.7 #### | 18277.3±792.1 ****# | 2704.8±190.4 ****#### | 13415.2±238.7 ****#### |
| Glucose-free | 11693.8±280.2 | 13841.6±109.3 ** | 9695.4±132.0 ** | 10887.6±157.8 | 16304.8±335.2 **** | 7266.0±257.3 **** | 17900.0±672.5 **** |

Note: significance level p when compared with the corresponding metal-free samples (**** corresponds to p<0.0001; *** corresponds to p<0.001; ** corresponds to p<0.01; * corresponds to p<0.05). Significance level p when compared with corresponding glucose-free samples (#### corresponds to p<0.0001; ### corresponds to p<0.001; ## corresponds to p<0.01; # corresponds to p<0.05 (two-way ANOVA, Tukey's post-test)

In the absence of glucose, a slight increase in fluorescence was observed under the action of copper (II) cations (at all the excitation/emission wavelengths of 379/463 nm) and zinc (II) (at all the excitation/emission wavelengths of 345/405 nm, 366/442 nm, 379/463 nm and 440/520 nm, respectively). A partially similar effect, as well as the ability of iron (III) to reduce the fluorescence intensity at the excitation/emission wavelength's characteristic of AGEs, can be associated with the effect on residual amounts of glucose, the presence of which is probably explained by the technology of albumin production. At the same time, it was shown that, in the identical test system, the presence of zinc cations also leads to the appearance of fluorescence at the wavelengths of 320/438 nm, due to the coordination (or complexation) of zinc cations with amino acid residues at different sites of albumin [47]. In this case it can suggest the formation of coordination structures similar to those previously described by Wu F.-Y. et al. [47]. In the case of copper, the possibility of its interaction with the lysine residue resulting in the formation of a product, fluorescent at the wavelengths of 370/440 nm, was considered in the work by Zhang M. et al. [48]. The above-mentioned information does not exclude the possibility of the formation of other products associated with the specific effect of the metal ion on the protein, and significant for the pathogenesis analized in the article.

### Effect of d-metals on BSA aggregation and amyloid transformation

It is known from the literature data that cations of transition metals (copper (II), iron (III)) are able to stimulate the amyloid transformation of proteins [10, 49]. The research of the glycation reaction influence on this metal's property is of great interest. Studying the optical density of the reaction medium at the wavelength of 405 nm is used to assess the kinetics of the protein aggregates formation in the process of the amyloid transformation [45]. The result obtained in the course of this study (during the glycation reaction and/or independently), confirms the ability of some of the studied d-metals to stimulate the BSA aggregation. The results are shown in Table 2.

Considering the intrinsic (independent of glycation) ability of the studied d-metals to cause the BSA aggregation, it should be noted that the activity of metals can be arranged in the ascending order: nickel (II) (inactive) = cobalt (II) (inactive) = iron (II) (inactive) < copper (II) < iron (III) < zinc (II) (Table 2).

The glycation reaction in a metals-free medium did not lead to any statistically significant aggregation of BSA. In this case, glycation affected the ability of some cations to induce the BSA aggregation. Nickel (II) and cobalt (II) cations did not cause any BSA aggregation either independently or in the presence of glucose.

The ability to enhance the BSA aggregation only in the presence of glucose and not without it, has been established for iron (II) cations. For copper (II), iron (III), and zinc (II), the BSA aggregation was observed both in the absence of glucose (due to its own activity) and under the action of cations against the background of the glycation reaction. In case of copper (II), the intensity of the aggregation was higher in the samples with glucose, for iron (III), the result is the same for glucose-containing and glucose-free samples, and in case of zinc (II), the in-

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tensity of the aggregation was higher in the glucose-free samples.

For the studied metal cations, there is a mismatch between the ability to influence the formation of AGEs in the glycation reaction and the ability to stimulate the BSA aggregation. Thus, in the presence of glucose, iron (II) and iron (III), causing the aggregation (Table 2), do not stimulate the AGEs formation (Table 1). The ability of these cations to aggregate BSA does not depend on the AGEs formation, and in case of iron (II), it does not depend on the AGEs formation, but is obviously associated with the presence of glucose. At the same time, when conducting a correlation analysis according to Spearman, a statistically significant inverse correlation between the ability of metal ions to aggregate BSA in the glycated samples and the fluorescence intensity of the amino acids tyrosine and tryptophan at the wavelengths of 270/330 nm specific for both amino acids ($r = –0, 85$, $p = 0.03$), and 295/335 nm, more specific for tryptophan ($r = –0.93$, $p = 0.01$), has been established.

Alongside with the study of the BSA aggregation, the samples were examined for the amyloid belonging of the aggregates in the reaction with thioflavin T (ThT), an amyloid-specific agent. A more pronounced amyloid BSA transformation in the glucose-free samples compared with the glucose-containing samples in all cases, except for the reaction in the presence of copper (II), is noteworthy.

In the presence of glucose, copper (II) stimulates the amyloid BSA transformation more intensively than without it (Table 3). Regarding the other metals and the reaction medium without the ones, the equally increased intensity of ThT fluorescence in the glucose-free samples in comparison with the corresponding glucose-containing samples, indicates that under the described experimental conditions, glycation prevents the amyloid BSA transformation. At the same time, in the samples glycated in the presence of copper (II) cations, a statistically significant higher intensity of ThT fluorescence indicates a mutual reinforcing effect of glucose and copper factors on the intensity of the amyloid transformation (Table 3). It follows from the result that copper showed both the ability to stimulate the amyloid BSA transformation, independent of glycation, and leveled the ability of the ongoing glycation reaction to slow it down.

When assessing the influence of the factor of each cation presence on the amyloid BSA transformation, it was found out that in both glucose and glucose-free samples, this process is enhanced by the cations of copper (II) and zinc (II) (to the maximum extent) as well as nickel (II) (least of all). Cobalt (II) cations slightly suppress the reaction (statistically significant only in the absence of glucose), while iron (II) cations were found to be inactive. Iron (III) was found out to be able to reduce ThT fluorescence in both glucose-containing and glucose-free samples. The ability of copper and zinc to transform proteins into the amyloid form has been previously described more than once. The authors' unexpected results regarding a weak activity of nickel, are consistent with the recently established role of nickel in the formation of human β-amyloid [24].

## DISCUSSION

As a result of the study, it was found out that in the absence of metals, the glycation reaction does not affect the fluorescence intensity of tyrosine and tryptophan (the fluorescence intensity of glycated and non-glycated samples is the same). However, glycation can enhance the ability of metals to suppress the fluorescence of these amino acids. In the case of tyrosine, a possible mechanism for decreasing its fluorescence has been described for the reaction of collagen with ribose-5-phosphate [41]. It may be associated with such events as glycation of amino acids located in the spatial proximity to tyrosine (which leads to quenching of its fluorescence); a change in the spatial organization of the protein (which leads to quenching of the fluorescence of tyrosine), the reaction of tyrosine itself. Herewith tyrosine is not expected to directly interact with the glycating agent, but the participation of nearby lysine or arginine residues in the reaction can lead to the activation of tyrosine and its subsequent glycation, or oxidation.

At the same time, as for tryptophan, its residue can be oxidized during glycation [44], and this process can be enhanced in the presence of copper (II) [42]. Thus, a decrease in the intensity of tryptophan fluorescence during the glycation reaction in the presence of copper (II) is due to both the oxidation of the amino acid residue and a change in the protein conformation [42]. Summing up what has been said, it can be assumed that d-metals, which are able of suppressing the fluorescence of tyrosine and/or tryptophan during glycation more intensely, are active, influencing these mechanisms.

It is known that the ability of some d-metals to accelerate the AGEs formation in the glycation reaction is associated with their stimulation of oxidative reactions. This property is characteristic of metals such as copper, and the reaction that occurs with their participation is called the glyoxidation reaction [20]. Thus, the role of copper (II) in the course of glyoxidation is presumably in the catalysis of the electron transfer from enediols formed from reduced monosaccharides or during the fragmentation of Schiff bases and Amadori products. That leads to the formation of reactive oxygen species and dicarbonyl compounds. In these reactions, copper (II) is probably reduced to copper (I), and oxygen is converted to superoxide anion with the participation of hydrogen peroxide. Its source of some glycation stages. Then copper (I) ions are oxidized to copper (II), catalyzing the decomposition of hydrogen peroxide to a hydroxyl radical. Considering this, it can be concluded that the intensification of the glycation reaction in the presence of some transition metals under study can occur according to the described mechanism of glyoxidation.

The analysis of the zinc activity as a non-transition but d-metal, is of particular interest. According to the concept of the glyoxidation mechanism with the participation of copper (II), for the reaction to proceed, it is necessary for the metal ion to be able of changing the oxidation degree. A zinc ion has a constant oxidation degree, but it was able to slightly increase the fluorescence intensity at some excitation/emission wavelengths characteristic of AGEs. According to the literature data [50], zinc can exhibit antiglycating, antioxidant, and antiapoptotic properties, and its deficiency can contribute to the accelerated AGEs formation. However, according to the studies conducted by Zhuang X. et al. [51], in the *in vitro* glycation reaction, zinc (II) is able to enhance the formation of fluorescent AGEs, which is consistent with the results of the research. According to the same study [51], the transition metal manganese exhibits the ability to suppress the AGEs formation in the manner similar to that described for nickel, cobalt and iron, which are also transition metals. All these data indicate that belonging of the metal to transitional ones and the ability of its ion to change the oxidation degree is probably not a necessary and sufficient condition for accelerating a glycation reaction. This also indicates the uniqueness of the zinc properties among the studied metals.

Summarizing the investigation results of the d-metals effect on the fluorescence of the amino acids tyrosine and tryptophan, and the effect of the d-metals on the glycation reaction, it can be assumed that the mechanisms of the metals influence on both processes differ (at least, there are activity components associated with the effect on only one process). The results make it possible to assume the following: there is an additional mechanism of damage to tyrosine and/or tryptophan caused by the glycation factor (not limited by the intrinsic action of the metal ion), which manifests itself during the course of the reaction in the presence of copper (II), iron (II), zinc (II) cations, and, possibly nickel (II). Herewith, an increase in the fluorescence intensity at the wavelengths characteristic of AGEs, regarded as an intensification of the glycation reaction, was noted only when glycation proceeded in the presence of copper (II) cations (to a greater extent) and zinc (II) (to a lesser extent).

As for the results of albumin aggregation studies, it should be noted that, according to the literature data [43], one of the factors of the pathogenetic action of copper (II) cations in Alzheimer's disease, is its participation in the formation of amyloid dimers linked by dityrosine cross-links. This is consistent with the intense suppression of amino acid fluorescence observed in this study in the presence of copper, as well as with the presence of a statistically significant inverse correlation between the fluorescence intensity of these amino acids and the degree of BSA aggregation for the studied cations. Thus, a decrease in the fluorescence intensity of the amino acids tryptophan and tyrosine in the presence of d-metal ions, obviously accompanies the BSA aggregation process. The most intense aggregation of BSA proceeded in the presence of ions that showed the ability to enhance the formation of AGEs (copper, zinc).

As mentioned above, the amyloid transformation of a protein during glycation, depends on many factors, and in particular on the type of protein. Thus, according to the literature data, an increase in the amyloid transformation is observed in the process of albumin glycation (bovine and human), Aβ-protein, β2 microglobulin, etc. In contrast, the ability of α-synuclein to form amyloid fibrils after the glycation by methylglyoxal was reduced, and the resulting aggregates had the character of a molten glo

bule [52]. According to the results obtained, glycation under the described experimental conditions prevents the formation of ThT-sensitive amyloid forms. A similar result is observed when glycation occurs in the presence of nickel (II), cobalt (II), iron (II), iron (III), and zinc (II), but not copper (II), which has shown the ability to enhance the amyloid transformation and the BSA aggregation. Banerjee S. describes a decrease in the intensity of the amyloid transformation of chicken lysozyme after the exposure to the protein with methylglyoxal. Based on this result, the author puts forward a controversial assumption that carbonyl compounds can be used for pharmacological purposes [53]. Despite the doubtfulness of the therapeutic use of carbonyl compounds, this study and the similar ones confirm the fact that glycation can not only potentiate the amyloid transformation [13], but also prevent it [13, 53]. At the same time, it is known that the retardation of the amyloid transformation under the glycation action can be associated with the prolongation of the amyloid residence time in the oligomeric form – the form with the highest cytotoxicity. The latter negates the potential utility of glycation-induced slowing down of the amyloid transformation. According to the literature data [54, 55], ThT, in contrast to other forms (monomers, fibrils), may not detect the formation of amyloid oligomeric forms. It can be suggested that a comparatively lower intensity of ThT fluorescence in the samples glycated in the presence of all d-metal ions with the exception of copper (II), is related to the possibility of slowing down the reaction at the stage of the oligomer formation. However, that will be the subject of a further research. This property has not been found for the glycation in the presence of copper (II).

When evaluating the results obtained, one should obviously take into account all the conditions of the experiment (not only the factors of glycation and the presence of metal, but also heating, the content and pH of the reaction medium, etc.). It can be assumed that unaccounted for factors, can also affect the ability of albumin to undergo the amyloid transformation during glycation, and under other conditions, the course of the reaction can lead to a different result. Thus, for a comprehensive assessment of the glycation ability to influence the amyloid transformation, one should take into account the ability of a protein to transform into an amyloid form

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under various experimental conditions (different pH values, temperatures, ionic strength of the buffer solution, etc.), including conducting studies under the conditions close to physiological ones.

## CONCLUSION

As a result of the research, it was shown that the course of the glycation reaction affects the amyloid transformation of BSA, and in the presence of d-metal ions it affects the ability of some of them to cause aggregation and the amyloid transformation. Under the described experimental conditions, copper (II) cations were the only ones able of enhancing the formation of AGEs, reducing the fluorescence intensity of the amino acids tryptophan and tyrosine in the glucose-mediated and glucose-independent ways, causing aggregation and the amyloid transformation of BSA. Ions of other metals showed these effects only partially, in various combinations. This makes it possible to suggest that in the series of d-metal ions such as nickel (II), cobalt (II), iron (II), iron (III), copper (II) and zinc (II), only copper ions (II) are probably the most significant factors in enhancing the amyloid transformation and BSA aggregation, and are the most active catalysts for the formation of AGEs in the glycation reaction. Thus, we believe that this element is a promising target for the development of methods for a pharmacological control of pathological conditions associated with all the processes considered – glycation, aggregation, and amyloid transformation of proteins (long term complications of diabetes mellitus, including diabetic encephalopathy; conformational brain diseases, etc.).

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## CONFLICT OF INTEREST

The authors declare no conflict of interest.

## AUTHORS' CONTRIBUTION

Roman A. Litvinov – development of the research idea, planning, preparation and writing of the publication text, organization and control of the research at all its stages; Arina V. Gontareva – setting up and carrying out the glycation reaction, preparation of reagents, obtaining primary data; Lyudmila E. Usmiyanova – setting and carrying out the glycation reaction, preparation of reagents, obtaining primary data; Daria R. Klimenko – preparation of reagents, statistical processing of primary data, correction of the publication text at the stage of its preparation, work with literature sources.

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## AUTHORS

**Roman A. Litvinov –** Candidate of Sciences (Medicine), Senior Researcher, Laboratory of Metabotropic Medicines, Department of Pharmacology and Bioinformatics, Scientific Center for Innovative Medicines with Pilot Production, Volgograd State Medical University; Researcher, Laboratory of Experimental Pharmacology, Volgograd Medical Research Center. ORCID ID: 0000-0002-0162-0653. E-mail: litvinov.volggmu@mail.ru

**Arina V. Gontareva –** 6th year student, specialty "Medical Biochemistry", Volgograd State Medical University, co-executor of the grant of the President of the Russian Federation to support young scientists, candidates of sciences MK-1887.2020.7. E-mail: arinaarinag@gmail.com

**Lyudmila E. Usmiyanova –** 6th year student, specialty "Medical Biochemistry", Volgograd State Medical University, co-executor of the grant of the President of the Russian Federation to support young scientists, candidates of sciences MK-1887.2020.7. E-mail: mila.getmanets@gmail.com

**Daria R. Klimenko –** 4th year student, specialty "Pharmacy", Volgograd State Medical University. E-mail: vip.klimenko.darya@mail.ru