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RESEARCH ARTICLE

Changes of oxidative stress indices and antioxidant system in the liver tissue on the administration of some coordination compound of copper, derivatives of thiosemicarbazide

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What is not yet known on the issue addressed in the submitted manuscript

The biochemical action mechanisms of a some new copper coordination compound, derivatives of thiosemicarbazide (CC), in particular, on the peroxidative processes and the level of antioxidant system in liver tissue have not yet been studied.

The research hypothesis

CC could be exerting a significant influence on the metabolic processes in the liver tissue, in particular on free radical oxidative processes and on the antioxidant system.

The novelty added by manuscript to the already published scientific literature

The influence of new CC on free radical oxidation processes and antioxidant system has been elucidated in liver tissue, estimate, and select the most informational biomarkers for the assessment of oxidative stress in liver tissue and which can be used to determine the effectiveness of new local remedies.

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Abstract

Introduction. Identification, study and testing of new remedies for treatment approaches of diseases, resulting from imbalance between oxidants and antioxidants in favor of oxidants, with potentially destructive potential and pathogenesis in liver disorders is of particular interest due to the increase in incidence and severity of these pathologies.

Material and methods. The action of novel local copper coordination compounds, thiosemicarbazide derivatives - CMD-4, CMJ-33 and CMT-67, was evaluated in experiments on white rats after subcutaneous administration in two different doses (0.1 and 1.0 μM/kg) 3 times a week for 30 days. The main indices of oxidative stress were evaluated: the level of malonic dialdehyde (*MDA*), nitric oxide derivatives (*NO*), S-nitrosothiols, advanced glycation end products (*AGEs*), advanced oxidation protein products (*AOPP*) and ischemia-modified proteins (*IMP*), and antioxidant system: - superoxidismutase (*SOD*) and catalase activity *(CAT)*, the level of histidine (*His*) and total antioxidant activity (*TAA*) in liver tissue of white rats.

Results. The administration of CC resulted in the reduction of oxidative stress indices - *MDA*, *AGEs* and *S-nitrosothiols*, which denotes the antioxidant effect of the studied compounds. The level of *NO* and *AOPP* derivatives does not change substantially. When administering CMD-4 (1 μM/kg), *SOD* activity and catalase function decreased markedly. Changes in the content of *His* and *TAA* have been shown to be inconclusive, maintaining within the limits of the values recorded in the control group.

Conclusions. The elucidation of the modifications of the free radicals processes in liver tissues, which are the basis of the *CC* action, broadens the theoretical knowledge about the biological properties of a number of chemical compounds; as well provide new possibilities to explore perspective objects in order to obtain new efficient drug preparations.

Keywords. Oxidative stress, antioxidant system, liver tissue, coordination compounds of copper.

Introduction

Studies in recent years have brought more and more evidence, such as that damage to cells by reactive oxygen species *(ROS)* and reactive nitrogen species *(RNS)* are the most important factors leading to aging and degenerative diseases, such as cancer, cardiovascular and liver diseases, cataracts, chronic inflammatory processes, renal insufficiency and so on [1].

Identification, study and testing of new remedies for treatment approaches of diseases, resulting from imbalance

between oxidants and antioxidants in favor of oxidants, with potentially destructive potential and pathogenesis in liver disorders is of particular interest due to the increase in incidence and severity of these pathologies.

Thus, it is certain the need to develop new compounds, which could serve as a basis for the development of drug preparations for the prevention and treatment of the above mentioned diseases, including liver diseases.

In this respect, derivatives of thiosemicarbazides are of particular interest, which could have a significant influence on metabolic processes.

Research carried out over the last decade has shown their therapeutic efficiency and perspective of valorification as raw material for obtaining medicinal remedies [2-5].

At the same time, their biochemical action mechanisms, in particular, on the peroxidative processes in the liver tissue, are not known in detail.

The aim of this study is to research the influence of new local copper coordination compounds, derivatives of thiosemicarbazides on free radical oxidation processes and antioxidant system in liver tissue, estimation, and selection of the most informative biomarkers for assessing the level of oxidative stress and which can be used to determine the effectiveness of new local remedies.

Material and methods

Study design

The study is preclinical experimental. The research was approved by the Research Ethics Committee of the *Nicolae Testemitanu* State University of Medicine and Pharmacy (favorable minute no. 73 from 26.04.2017).

Animals included in the study

In this study have been used new local copper coordination compounds, derivatives of thiosemicarbazides (CC) – 4 – ethyl – 2 - [phenyl (pyridine – 2 - yl) methylidene] hydrazine – 1 - carbothioamide (CMD-4) , chloro - {4 - (3 - methoxyphenyl) – 2 - [1 - (pyridine – 2 - yl) ethylidene] hydrazine – 1 - carbothioamide} copper (CMJ-33) and nitrato - {N – phenyl – N' - (pyridine - 2 - ylmethylidene) carbamohydrazonothioato} copper (CMT-67) synthesized in the Laboratory of Advanced Materials in Biopharmaceuticals and Technics at Moldova State University [5]. The autochthonous *CC* action on the liver tissue has been evaluated in experiments on a sample of 46 Wistar line male rats with 180 – 250 g mass, divided into 6 groups of 7-8 animals in each.

The first group – control, was made up of 8 intact animals, maintained at a normal diet of vivarium and whom was administered subcutaneous three times per week for 30 days physiological solution. Animals on the experimental groups 2 - 6 were administered the subcutaneous *CC* 3 times a week for 30 days during 30 days in the following sequence: 2nd group - CMD-4 (0.1 μM/kg body weight), 3rd group - CMD-4 (1.0 μM/kg body weight), 4th group - CMJ-33 (0.1 μM/kg body weight), 5th group - CMJ-33 (1.0 μM/kg body weight) and 6th group CMT-67 (0.1 μM/kg body weight).

Method of processing liver tissue

24 hours after the last administration of local *CC*, the animals were sacrificed under mild narcosis with sulfuric ether and the liver was collected. All operations were performed in glacial environment. The preparation of the material for the determination of the biochemical indices has been carried out as follows. The phosphate buffer solution 0.1 M (pH 7.4) containing 1 mm EDTA has been used as the dispersion medium, so that the final dilution of the homogenate constitutes 1:10. For complete destruction of the cell membranes the homogenate was processed with triton X-100 in the final concentration 0.1%. Subsequently, the tissue homogenates were subjected to centrifugation for 15 minutes at 5000 rpm, and the supernatant was transferred to clean tubes and until examined kept in the freezer at minus 40°C. The entire process of preparing tissue homogenates is performed under regulatory conditions for the assessment of biochemical parameters.

Tests of oxidative stress and antioxidant system tested

The intensity of oxidative stress was evaluated by determining the following laboratory parameters: malonic dialdehyde *(MDA)*, nitric oxide *(NO)* derivatives, S-nitrosothiols *(RSNO)*, advanced glycation end products *(AGEs),* advanced oxidation protein products *(AOPP)* and ischemia-modified proteins *(IMP)*.

The changes in the antioxidant protection indices were evaluated by determination of the activity of superoxidismutase *(SOD),* catalase *(CAT)*, histidine *(His)*, as well as the level of total antioxidant activity that is based on the antioxidants inhibition of the absorption of the 2.2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) radical cation *(ABTS).*

The assessment of oxidative stress and antioxidant system was performed according to the procedures described previously [6].

Statistical processing method

The statistical evaluation of the obtained data performed with use of the computer program StatsDirect. The arithmetic mean ± error of the mean (M ± m) was calculated. The nonparametric statistical test „U Mann-Whitney" and the significance threshold „p" ($p < 0.05$) were used to test the significant difference between the studied indices of the compared groups.

Results

The evaluation results of lipid peroxide indices: *MDA, NO, AGEs, IMP, AOPP,* and *RSNO* in liver tissue when administering local *CC* are reflected in the statistical data of Table 1.

The study shows that *MDA* decreases statistical suggestive in the liver tissue by 14% - 40% under the influence of most of the studied preparations, except only the CMJ-33 compound (0.1 μM/kg), which shows a discrete tendency to decrease. Changes in the level of *NO* derivatives in liver tissue under the action of tested *CC* proved to be inconclusive. Administration of the tested *CC* results in a discrete reduction in the level of S-nitrosothiols in the liver, with

Table 1. *Influence of local CC on the lipid peroxidation indices in the liver tissue.*

Study groups	MDA nM/g.tissue	NO µM/g.tissue	RSNO nM/g.prot.	AGEs µg/g.prot.	AOPP nM/g.prot.	IMP µM/g.prot.
Control	112.5±5.5 (100%)	4.6±0.4 (100%)	73.7±4.6 (100%)	81.6±5.3 (100%)	60.4±4.2 (100%)	182.6±8.0 (100%)
CMD-4 0.1 µM/kg	95.8±5.6* (85%)	3.8±0,2 (83%)	68.6±4.6 (93%)	70.5±9.0 (86%)	65.2±6.4 (108%)	131.3±15.0* (72%)
CMD-4 1 µM/kg	96.6±4.5* (86%)	4.0±0.3 (88%)	61.8±2.0* (84%)	60.2±4.5** (74%)	63.6±6.0 (105%)	156.4±14.5 (86%)
CMJ-33 0.1 µM/kg	89.9±7.8 (80%)	4.4±0.4 (95%)	62.4±4.7 (85%)	67.7±4.1 (83%)	66.9±7.1 (111%)	159.5±14.2 (87%)
CMJ-33 1 µM/kg	90.4±4.0* (80%)	4.3±0.2 (93%)	58.7±2.8* (80%)	65.5±5.6* (80%)	66.1±5.0 (109%)	166.4±6.2 (91%)
CMT-67 0.1µM/kg	67.2±4.6** (60%)	3.8±0.3 (81%)	63.5±2.8 (86%)	71.9±6.7 (88%)	64.8±10.0 (107%)	190.5±23.5 (104%)

*Note:** - statistically significant difference with the control group (*-p<0.05; ** - p<0.01; *** - p<0.001); local CC - copper coordination compounds, derivatives of thiosemicarbazides; CMD-4 - 4- ethyl – 2 - [phenyl (pyridine – 2 - yl) methylidene] hydrazine – 1 - carbothioamide; CMJ-33 - chloro - {4 - (3 - methoxyphenyl) – 2 - [1 - (pyridine – 2 - yl) ethylidene] hydrazine – 1 - carbothioamide}copper; CMT-67 – nitrato - {N - phenyl – N '- (pyridine – 2 - ylmethylidene) carbamohydrazonothioato} copper; MDA - malonic dialdehyde ; NO - nitric oxide derivatives; RSNO - S-nitrosothiols; AGEs - advanced glycation end products ; AOPP - advanced oxidation protein products; IMP - ischemia-modified proteins.

the exception of the compounds CMD-4 and CMJ-33, which at maximum doses (1.0 µM/kg) have the property to veraciously decrease the level of *S- nitrosothiols* by 16% and 20% ($p < 0.05$) respectively compared to the control group. There was a discrete tendency to decrease the intensity of the *AGEs* product formation processes in the liver of white rats after the administration of the tested bioactive compounds. At the same time, compounds CMD-4 and CMJ-33 at the maximum dose of 1.0 µM/kg statistically significantly reduce the level of *AGEs* by 20% and 26% compared to the values attested in the control group.

Changes in the values of advanced oxidation protein products — *AOPP* in the liver were found to be without statistical relevance, these values remaining at the level of the reference parameters.

The results of the study show that the tested *CC* did not induce statistical relevance changes in the *IMP* content in the liver, except for the compound CMD-4 (0.1 µM/kg), which reduces this index by 28% compared to the control values.

The evaluation results of the antioxidant protection indexes: *SOD, CAT, His* and *TAA* in hepatic tissue on the action of local *CC* are reflected in the statistical data of Table 2.

Table 2. *Influence of local CC on the antioxidant protection indices in the liver tissue.*

Study groups	SOD u/g.prot.	CAT µM/s.g.prot.	His µM/g.prot	TAA nM/g.prot.
Control	23.0±0.9 (100%)	66.9±5.2 (100%)	6.72±0.4 (100%)	2.36±0.04 (100%)
CMD-4 0.1 µM/kg	21.1±1.03 (92%)	48.0±9.1 (72%)	7.46±0.2 (111%)	2.38±0.03 (101%)
CMD-4 1 µM/kg	17.6±0.8* (76%)	62.8±3.4 (94%)	7.14±0.1 (106%)	2.23±0.02 (94%)
CMJ-33 0.1 µM/kg	26.5±2.2 (115%)	60.2±3.4 (90%)	6.49±0.2 (97%)	2.29±0.03 (97%)
CMJ-33 1 µM/kg	26.7±1.15 (116%)	51,.5±2.1* (77%)	6.98±0.1 (104%)	2.35±0.04 (100%)
CMT-67 0.1 µM/kg	24.5±2.4 (106%)	53.4±3.1 (80%)	7.28±0.3 (108%)	2.37±0.04 (100%)

*Note:** - statistically significant difference with the control group (*-p<0.05; ** - p<0.01; *** - p<0.001); local CC - copper coordination compounds, derivatives of thiosemicarbazides; CMD-4 – 4 - ethyl – 2 - [phenyl (pyridine – 2 - yl) methylidene] hydrazine – 1 - carbothioamide; CMJ-33 - chloro - {4 - (3 - methoxyphenyl) – 2 - [1 - (pyridine - 2 - yl) ethylidene] hydrazine – 1 - carbothioamide} copper; CMT-67 - nitrato - {N – phenyl - N' - (pyridine – 2 - ylmethylidene) carbamohydrazonothioato} copper; SOD - superoxidismutase; CAT - catalase activity; His - histidine; TAA - total antioxidant activity.

The study shows that *SOD* decreases statistically conclusively by 24% ($p < 0.05$) when administering CMD-4 (1.0 µM/kg), and the catalase function in this case is veracious decreasing by 23%. The changes in the content of *His* and *TAA* proved to be inconclusive, keeping them within the limits of the values recorded in the control.

Discussion

In this study, the level of oxidative stress in the liver tissue of the laboratory animals subjected to the action of local CC, derivatives of thiosemicarbazide, was analyzed. It is evident the selective action of the studied compounds on the indices of oxidative stress and antioxidant system, which depends

on the degree of their employment at different stages of the metabolic processes that occur in the liver tissue.

As free radicals have very short half-life periods, the *in vivo* assessment of oxidative stress is based on the measurement of different stable oxidized products of biomolecules in the cells and tissues of living organisms. Oxidative stress assessment methods are often referred to as fingerprinting methods, by which end products resulting from the interaction of *ROS* and *RNS* with various biomolecules such as membrane lipids, proteins and amino acids, carbohydrates, nitrogenous bases, etc., are measured [7].

Primary products of the peroxidation of polyunsaturated fatty acids result in the formation of peroxyl and alkoxyl radicals, which are very reactive and have a short life, being further subjected to other reactions with the formation of different aldehydes, such as *MDA*, 4-hydroxyalkenals and acrolein. These aldehydes, because of their electrophilic nature, have a very high damage potential. The most abundant aldehyde resulting from the lipids peroxidation is the *MDA* and its accumulation may cause alterations in proteins, nucleic acids and many other biomolecules.

In this study we obtained significantly lower levels of the end product of lipid peroxidation - *MDA* in liver tissue under the influence of most of the preparations studied, except only CMJ-33 (0,1 μM / kg), which shows a discreet tendency to decrease. Reducing the level of *MDA* under the influence of most of the tested *CC* in liver tissue, possibly, is an expression of several factors: the intensity of the formation of the primary products of lipid peroxidation, the biosynthesis of *MDA*, the speed of the metabolic processes in the tissue and the ability of the body to eliminate this final product, as well as the level of substances with antioxidant role. We can admit that the tested compounds, due to their property of lowering the level of *MDA* by various mechanisms, can increase the efficiency of cellular protection against various peroxidants and cytotoxic agents.

Therefore, the hepatic level of *MDA* could be used as a test to assess the efficacy of tissue protection against lipid peroxidation by free radicals when testing different *CC*.

NO is a free radical that plays an important role in various physiological processes, and *RSNO* represent the natural reservoir (deposit) and form of transport of nitric oxide. Nitric oxide due to its high reactivity can cause S-nitrosylation of cysteine residues from proteins or peptides, which is an important redox signaling mechanism that regulates a wide range of biological, physiological and cellular functions in various tissues, including liver [8].

The tendency to decrease *NO* concentration, recorded in our research, even if it did not reach the statistical significance limit, is probably due to the inhibition by tested compounds of nitric oxide synthase (enzyme that increases the level of *NO* in tissues) and could be explained by the antitumor activity of these compounds [4-5]. These findings are consistent with the data of some authors, who found that nitric oxide synthase inhibitors that reduce *NO* production might have a therapeutic role in certain cancers due to their property of reducing angiogenesis, proliferation, and causes suppression of tumor invasion [9].

The decrease in the level of *RSNO* in the liver when administering the maximum doses (1.0 μM / kg) of the compounds CMD-4 and CMJ-33, recorded in our experiences is probably due to their intense decomposition by the transition metal ions, such as copper (II) ions, which are part of the tested compounds, and by various redox active species, including reactive oxygen species [10], or by catalytic denitrosylation by specific enzymes, such as thioredoxin (Trx) and S-nitrozoglutation reductase *(GSNOR*) - enzymes that eliminate *NO* from nitrosylated proteins / peptides [11].

This could create a deficiency of *NO* signaling, which would decrease the proliferation intensity of the liver progenitor cells and cause the reduction of the processes of restoration in the hepatic lesions and, it is not excluded, could cause manifestations of liver toxicity. Indeed, according to some researchers, the increased *NO* and *RSNO* levels, in contrast to the low levels, induce the proliferation of liver progenitor cells and improve the liver restoration from partial hepatectomy [12].

In view of these aspects, it is important for the research of new bioactive compounds to evaluate the NO/RSNO system in order to identify potential changes of this system and to provide a better understanding of the mechanisms of action of these compounds, which will facilitate not only the discovery of new targets for the action of bioactive compounds, but also the development of new therapeutic agents.

Studies carried out over the last decades have brought more and more evidence that during the glycation process, namely the interaction of carbohydrates with the free amine groups of proteins, the early glycation products are formed first, and they are subsequently rearranged into final structures, called advanced glycation end products - *AGEs* through a series of complex chemical reactions. *AGEs* are involved in the pathogenesis of atherosclerosis, chronic inflammatory processes, aging, cancer, neurodegenerative diseases, such as Alzheimer's disease, etc. [13].

The obtained results indicated the discrete tendency to decrease the intensity of the *AGEs* product formation processes, and the compounds CMD-4 and CMJ-33 at the maximum dose of 1 μM / kg significantly decrease the level of AGEs in the liver tissue, compared to the values attested in the control group. This fact indicates the anti-*AGEs* activity, and capacity to prevent the formation of *AGEs*, exercised by studied *CC*, and can be qualified as a positive moment, because, *AGEs* can interact with the specific receptors of the cell surface - *RAGE* (receptor for advanced glycation end products), which in the liver are expressed in hepatic stellate cells and myofibroblasts - cells involved in the fibrogenesis of liver disease, and therefore, they may alter intracellular cell signaling, gene expression, enhance *ROS* production and activation of several inflammatory pathways, including the release of pro-inflammatory cytokines, growth factors and adhesion molecules by activating the *NF*-κB (nuclear factor κappa-light-chain-enhancer of activated B cells) pathway [14].

Therefore, the level of *AGEs* can serve as a valuable indicator when evaluating the action of new *CC* on cells and tissues, including the liver.

As is known, proteins by their complex structure, present numerous points where they can suffer oxidative attacks, making them one of the first targets of free radicals.

The oxidative changes of proteins, called advanced oxidation protein products - *AOPP*, are protein products that contain dityrosine cross-linking bonds which can lead to various important functional consequences such as inactivation of enzymes, increased susceptibility to aggregation and resistance upon proteolysis, increased formation of free radicals, activation of the nuclear factor *NF*-κB and release of proinflammatory cytokines, adhesion molecules and growth factors [15, 16].

For that reason, it is considered that *AOPP* may be a more accurate biomarker of oxidative stress than lipid peroxidation products [16-18].

In this study the changes in the values of the advanced oxidation protein products - *AOPP* in the liver, proved to be statistically unsuggestive, they keeping within the limits of the reference parameters, so the studied compounds do not cause excessive synthesis of FR with prooxidative character at the local tissue level and, it is not excluded that they, on the contrary, annihilate the *FR* action due to their antioxidant properties. This fact is in agreement with the data of researchers who have detected antioxidant properties characteristic of thiosemicarbazonic compounds [19].

As is known in hypoxic and ischemic conditions in blood serum increases the concentration of ischemia modified albumin *(IMA)* increases, characterized by a significant change in the ability to bind the transition metals, especially cobalt [20]. The *IMA* level may increase in certain circumstances, such as myocardial ischemia, malignant neoformations, chronic diseases, and inflammatory processes [21, 22].

In view of this, we investigated the influence of *CC* on the content of *IMP* in liver tissue. The results of the study show that the tested *CC* did not induce statistically significant changes in the *IMP* content in the liver tissue, except for the CMD-4 (0.1 μM/kg) compound, which statistical truthful reduces this index to the control values, suggesting that this compound has the property of diminishing tissue hypoxia.

Thus, the results obtained demonstrate that the tested *CC* does not cause oxidative damage to the proteins of the liver tissue, which is manifested by the discrete tendency to decrease the intensity of the *AGEs* product formation processes under the influence of the majority of the investigated *CC* and to maintain the *PPOA* values within the normal values.

The conclusive reduction of the content of *AGEs* and *IMP* by the compound CMD-4 and *AGEs* by the CMJ-33 in the maximum dose of 1 μM/kg denotes their antioxidant effect.

Under the action of local *CC* studied the indices of antioxidant protection: *SOD, catalase, His* and *TAA* in the liver tissue of laboratory animals undergo changes of different intensity. Decrease of the activity of antioxidant enzymatic links *SOD* - enzymes that destroy superoxide anion O_2^- with the formation of O_2 and hydrogen peroxide), catalase (which inactivates H_2O_2) in the liver after administration of compounds CMD-4, CMJ-33 and CMT-67 can result with weakening of the cells enzymatic protection against the oxidation reactions with free radicals under conditions of a pronounced activation of this process. Not coincidentally, these two enzymes have the highest reaction rates (about 2 x 10^9 M-1 s-1), which allows them not only maximum efficiency, but also the possibility to act independently by providing substrates or coenzymes [23].

On the other hand, changes in the content of *His* (which has relevant antioxidant effects [24-25] and *TAA* (which includes various antioxidants, both enzymatic and non-enzymatic) have been shown to be inconclusive, maintaining them within the limits of the control values. This demonstrates that the studied compounds possess the ability to effectively remove free radicals (*FR)* and maintain the redox balance at local liver tissue level.

The antioxidant system, whereby aggressive compounds formed by the action of *FR* are converted into non-active compounds, play a decisive role in cell defending against the harmful action of *FR*. This system comprises a large number of elements, being as diverse as free radicals, cells containing a variety of scavenging-capacity substances for multiple radical species precisely to ensure maximum protection [26]. The high efficiency of antioxidants is explained by their wide diversity, the location in different cell compartments, the synergistic nature of their action, the summation of their combined action, each acting according to different mechanisms and varying levels of the chain of *FR* evolution in the body. Under normal conditions, antioxidants eliminate pro-oxidants, but under oxidative conditions, pro-oxidants predominate over antioxidants, which can lead to many inflammatory diseases, including cancer [27-28].

For these reasons, indices of antioxidant protection may be useful as diagnostic markers or therapeutic targets.

Because excessive *FR* formation can cause multiple cellular and tissue damage, their effects can be analyzed by local tissue markers of oxidative stress and antioxidant system. Further studies are needed to confirm the therapeutic utility of these bioactive tested compounds.

Conclusions

The most informative biomarkers of functionality of the prooxidant and antioxidant system have been estimated and selected to assess the level of oxidative stress in liver tissue to experimental exposure through the administration of local *CC* to laboratory animals and which can be used to determine the efficiency of new local drug preparations.

The reduction of oxidative stress indices (*MDA, AGEs, S-nitrosothiols, IMP*) when using local *CC* denotes their antioxidant effect.

The elucidation of molecular mechanisms who stay on the basis of the *CC* action expand the theoretical knowledge of the biological properties of a range of chemical compounds and also offers new possibilities to explore prospective objects for the purpose of obtaining new efficient drug preparations.

Declaration of conflicting interests

The authors declare no conflicts of interest.

Authors` contribution

The authors have equally contributed to the manuscript drafting, design and paper editing. All authors approved the final version of manuscript.

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