

THE INFLUENCE OF THIOSEMICARBAZONE DERIVATIVES ON TOTAL NITRIC OXIDE LEVELS AND ENDOTHELIAL NITRIC OXIDE SYNTHASE ACTIVITY: A SEX-DEPENDENT COMPARATIVE STUDY

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ABSTRACT

Background. Thiosemicarbazones are organic compounds with high pharmacological potential, recognized for their ability to form stable complexes with various metal ions. In particular, copper-thiosemicarbazone complexes have garnered increasing interest due to their significant biological activities, including antimicrobial, antioxidant, and antitumor effects. These complexes exhibit high chemical stability and enhanced interaction with biomolecules, positioning them as promising candidates for the development of novel therapeutic agents.

Objective of the study. The aim of this study was to investigate the effects of copper-thiosemicarbazone coordination compounds (CuCCTs) on nitric oxide (NO) concentration and endothelial nitric oxide synthase (eNOS) activity in erythrocytes, using an *in vivo* experimental model.

Material and Methods. This study evaluated the impact of CuCCT administration in 120 rats, distributed into 10 experimental groups and divided by sex into two subgroups: (a) males and (b) females. Animals in the control group (Group 1) received subcutaneous injections of physiological saline. In the treatment groups (Groups 2–10: CMA-18, CMD-8, MG-22, CMC-34, CMJ-33, CMT-67, CMG-41, TIA-123, and TIA-160), CuCCTs were administered subcutaneously at a dose of 10 µg/kg daily for 30 days.

Results. Evaluation of total NO levels revealed non-significant variations across compounds and between sexes. The most notable enhancement was observed with the benzothiazole-based compound CMA-18, particularly in females, who showed a 23% elevation compared to controls. Overall, female

rats exhibited higher NO levels than their male counterparts. These findings suggest a sex-dependent and structure-specific response to treatment, which may be exploitable in the design of targeted pharmacological strategies. Endothelial nitric oxide synthase (eNOS) activity, expressed as $\mu\text{M/g}\cdot\text{Hb}$, was compared to the control group. Significant increases in eNOS activity were observed in females treated with MG-22 and CMG-41, reaching up to 39% enhancement ($p < 0.05$). In contrast, male rats showed minimal or no changes in eNOS activity. These results underscore a sex-dependent effect influenced by the chemical structure of the compounds and highlight their therapeutic potential in cardiovascular conditions.

Conclusion. Thiosemicarbazone derivatives modulate nitric oxide concentration and eNOS activity in a sex- and structure-dependent manner, with significant stimulation observed in females. These compounds represent promising candidates for the development of personalized cardiovascular therapies

Keywords: erythrocytes, copper coordination compounds with thiosemicarbazones, NO, eNOS

1. INTRODUCTION

A central objective of pharmaceutical chemistry is the development of novel and effective bioactive compounds with multiple biological properties. Thiosemicarbazones are valuable intermediates in the synthesis of pharmaceutical substances and also serve as chelating ligands, forming stable complexes with transition metals. These complexes exhibit remarkable therapeutic activities and are distinguished by their potential to yield more potent and less toxic drugs [1].

Cancer remains one of the leading causes of death worldwide, with recent epidemiological data highlighting a troubling rise in incidence across various population groups [2]. The aggressive nature of the disease stems from the uncontrolled proliferation of tumor cells and their ability to metastasize, making treatment extremely challenging and complex [2]. Although significant progress has been made in chemotherapy over recent decades, conventional therapeutic agents are often associated with severe side effects and low selectivity, which can significantly compromise patients' quality of life [3]. In this context, there is an urgent need to explore new bioactive compounds capable of acting selectively on cancer cells, while minimizing damage to healthy tissues [4].

Current research directions emphasize a growing interest in the development of synthetic antitumor agents, particularly those incorporating metals into their structure *via* coordination chemistry. Metal-based compounds formed with transition metals have drawn considerable attention due to their ability to enhance biological activity through specific interactions with cellular targets. Within this framework, thiosemicarbazones have emerged as highly promising ligands, enabling the synthesis of complexes with enhanced antiproliferative efficacy and selectivity toward various cancer cell types [5].

Copper (Cu) is an essential micronutrient for all organisms living in oxygen-rich environments. It is a redox-active metal, capable of rapidly interconverting between the reduced Cu(I) and oxidized Cu(II) states, both in classical chemical reactions and under physiological conditions [6]. Cu(I) ions, having a d^{10} electronic configuration, form diamagnetic compounds [7]. The oxidation state of d-block elements has been correlated with significant changes in the stability of corresponding metal

complexes [7]. By contrast, all copper(II) complexes are inherently paramagnetic due to the presence of unpaired electrons [8].

The use of metal-based complexes in pharmaceutical applications has significantly increased in recent years, driving growing interest in the development of novel metal complexes as potential anticancer agents. Copper plays a critical role in maintaining the function of numerous proteins and enzymes. Recent studies have demonstrated that copper complexes can act as specific inhibitors of tumor proteasomes and as inducers of apoptosis, primarily through targeting the ubiquitin – proteasome pathway (UPP). These complexes have shown promising results in preclinical studies. The UPP is vital for regulating the expression, activity, and localization of a wide array of proteins. Thus, selective proteasome inhibition and apoptosis induction in cancer cells are regarded as viable therapeutic strategies in oncology [9].

Tumors are typically characterized by insufficient vascularization, resulting in hypoxia (low oxygen levels), which promotes invasion, metastasis, and a shift toward anaerobic metabolism - a phenomenon known as the „Warburg effect” [10]. Fortunately, tumor hypoxia can be exploited for therapeutic purposes by designing prodrugs that are activated within the reducing environment of cancer cells. In this context, copper is particularly advantageous, as it can exist in two oxidation states - Cu(I) and Cu(II) - within cells. The anoxic microenvironment of tumor cells favors the reduction of Cu(II) to Cu(I), a reaction that is unlikely to occur in normal tissues, thereby offering a therapeutic window for tumor targeting [11]. Furthermore, Cu(I) can catalyze the generation of reactive oxygen species (ROS) and reactive nitrogen species (RNS), inducing pro-apoptotic oxidative stress (OS). The redox state of copper also influences its ligand-binding affinity: Cu(I), being a softer Lewis acid than Cu(II), shows a stronger affinity for sulfur-containing ligands, whereas Cu(II) preferentially coordinates with nitrogen and oxygen donors found in proteins and DNA [12-13].

Copper plays a critical role in cellular oxidation-reduction (redox) processes and all the animals, including humans, require limited but adequate amounts of copper to survive and to maintain normal physiological function of numerous proteins and enzymes, many of which require its participation and activation to regulate key metabolic pathways within the body [14].

Endothelial cells lining the blood vessels play a protective role against thrombosis and atherosclerosis when functioning properly. A critical regulator of endothelial function is endothelium-derived nitric oxide (NO), which is synthesized by the enzyme endothelial nitric oxide synthase (eNOS). Vascular NO acts as a paracrine signaling molecule, relaxes blood vessels, prevents platelet aggregation and adhesion, inhibits the oxidation of low-density lipoprotein (LDL) cholesterol, suppresses vascular smooth muscle cell proliferation, and reduces the expression of pro-inflammatory genes involved in the development of atherosclerosis [15].

Therefore, a diminish in NO synthesis or an enhancement in its degradation is commonly associated with cardiovascular risk factors. This condition, known as endothelial dysfunction, contributes to vasospasm, thrombus formation, vascular inflammation, and proliferation of smooth muscle cells within the vessel walls [16].

Both cardiovascular risk factors and cardiovascular diseases compromise endothelial function. Increased production and/or insufficient clearance of ROS lead to OS, which contributes to the

reduction of NO bioactivity. One of the key mechanisms behind diminished NO availability is its rapid neutralization by superoxide anion (O_2^-), a highly ROS. Persistent OS has also been shown to impair eNOS function, shifting the enzyme's activity from NO production to superoxide generation, a phenomenon termed eNOS uncoupling [17].

A promising and highly relevant field within medicinal chemistry is the development of novel CuCCTs exhibiting antitumor activity. A variety of copper-based drug combinations are currently under preclinical investigation as cost-effective and potent anticancer agents. Although eNOS, a member of the NOS family, is known to produce NO in both peripheral tissues and the central nervous system, its broader impact in oncology remains underexplored [18].

Previous research has highlighted the notable anticancer potential of several locally synthesized CuCCs, which have demonstrated greater efficacy compared to conventional anticancer drugs. These compounds include novel Schiff bases, featuring chelating and macrocyclic ligands derived from halogensemicarbazides in combination with non-platinum transition metals [19].

NO plays a complex and multifaceted role in tumor biology. Its effects on tumor cells depend on both concentration and exposure duration, influencing key processes such as cancer initiation, tumor cell progression, tumor vascularization, angiogenesis, metastasis, apoptosis induction, cell death, and the suppression of tumor growth [20, 21].

The aim of this study was to investigate the effect of CuCCTs on NO concentrations and eNOS activity in erythrocytes, using an *in vivo* experimental model.

2. MATERIALS AND METHODS

2.1. Study design, methodology, and processing of biological material

This study employed novel locally synthesized CuCCTs, including the following:

- Benzothiazole-based thiosemicarbazones: CMA-18, CMD-8, MG-22
- Phenyl thiosemicarbazones: CMC-34, CMJ-33, CMT-67
- Allyl thiosemicarbazones: CMG-41, TIA-123, TIA-160

All compounds were synthesized at the "Advanced Materials in Biopharmaceuticals and Engineering" Laboratory of the State University of Moldova [10].

A total of 120 laboratory-bred white rats (*Rattus norvegicus Albicans* strain) were used in the experiment, equally distributed by sex:

- 60 males, weighing between 180–230 g
- 60 females, weighing between 210–228 g

The study was conducted in accordance with ethical standards and approved by the Research Ethics Committee of "Nicolae Testemițanu" University of Medicine and Pharmacy, under approval No. 73 dated April 26, 2017, following veterinary authorization protocols for the use of animals in experimental and scientific research.

Animals were housed under standard vivarium conditions (controlled temperature, humidity, light/dark cycle), with free access to food and water. They were divided into the following groups:

Control Group

- 6 male rats
- 6 female rats

Experimental Groups

- 54 male rats, divided into 9 subgroups of 6 animals each
- 54 female rats, also divided into 9 subgroups of 6 animals each

Each experimental subgroup received one of the nine biologically active copper-thiosemicarbazone compounds.

2.2. The compounds were grouped into the following structural categories:

1. Benzothiazole-based thiosemicarbazones – CMA-18, CMD-8, MG-22
2. Phenyl thiosemicarbazones – CMC-34, CMJ-33, CMT-67
3. Alkyl thiosemicarbazones – CMG-41, TIA-123, TIA-160

Table 1. Locally synthesized CuCCTs investigated in this study [22]

CMA-18	Chloro-{1-(1,2-benzothiazol-3-yl)-2-[1-(pyridin-2-yl)ethylidene]diazanido}copper
CMD-8	Chloro-{4-ethyl-2-[phenyl(pyridin-2-yl)methylidene]hydrazine-1-carbothioamido} copper
MG-22	Di-Chloro-{N'-(4-methoxyphenyl)-N,N-dimethylcarbamimidothioato} copper
CMC-34	Chloro-{N'-[phenyl(pyridin-2-yl)methylidene]-N-pyridin-2-ylcarbamohydrazonothioato} copper
CMJ-33	Chloro-{4-(3-methoxyphenyl)-2-[1-(pyridin-2-yl)ethylidene]hydrazine-1-carbothioamido} copper
CMT-67	Nitrato-{N-phenyl-N'-(pyridin-2-ylmethylidene)carbamohydrazonothioato} copper
CMG-41	Nitrato-{N'-[phenyl(pyridin-2-yl)methylidene]-N-prop-2-en-1-ylcarbamohydrazonothioato} copper
TIA-123	Di-Chloro-{N'-[phenyl(pyridin-2-yl)methylidene]-N-prop-2-en-1-yl carbamohydrazono thioato} copper
TIA-160	Acetato-{2-({[(methylsulfanyl)(prop-2-en-1-ylamino)methylidene]hydrazinylidene} methyl)enolato} copper

2.3. Collection of biological samples

The tested substances were dissolved in physiological saline to the appropriate volume and administered intramuscularly once daily for a period of 30 consecutive days. The dosage used was 10 µg of compound per 1 kg of body weight.

Twenty-four hours after the final administration of the locally synthesized CuCCTs, the animals were sacrificed in compliance with ethical standards governing laboratory animal research.

Peripheral blood was collected into vacutainer tubes containing 6% K₄-EDTA (pH 7.4) as an anticoagulant. The samples were centrifuged at 3000 rpm for 10 minutes, after which the plasma was separated and transferred into sterile Eppendorf tubes, then stored at –40°C until analysis.

Concurrently, erythrocytes were washed three times with two volumes of 0.9% saline solution, each followed by centrifugation. After the final wash, distilled water was added to induce hemolysis based on erythrocyte volume. The resulting hemolysate was stored in labeled tubes at -40°C until further use.

2.4. Studied parameters: nitric oxide and endothelial nitric oxide synthase

Erythrocyte lysates were analyzed using spectrophotometric micromethods to determine the levels of NO [23] and the activity of eNOS [24]. All biochemical analyses were performed in the Biochemistry Laboratory of “Nicolae Testemițanu” State University of Medicine and Pharmacy, using validated protocols adapted for application with the Synergy H1 microplate reader (BioTek Instruments, USA).

2.5. Statistical analysis

Statistical evaluation of the collected data was performed using the Statistical Package for the Social Sciences (SPSS), version 23 (SPSS Inc., Chicago, IL, USA).

After verifying data distribution, intergroup comparisons of NO levels and eNOS activity were conducted using One-Way ANOVA, followed by the Games–Howell post hoc multiple comparison test. A p -value < 0.05 was considered statistically significant. Results were expressed as median, interquartile range (IQR), and percentage change relative to the control group.

3 RESULTS

3.1. Sex-dependent modulation of nitric oxide metabolism based on thiosemicarbazone structure

The present study demonstrated a sex-dependent modulation of NO metabolism and eNOS activity, influenced by both the chemical structure of the administered thiosemicarbazone derivatives and the sex of the animals. The data (Tables 2 and 3) reveal marked biological differences between males and females, with female rats exhibiting a more pronounced stimulation of NO synthesis.

Analysis of total NO concentrations, expressed in micromoles per gram of hemoglobin ($\mu\text{M/g Hb}$), showed a significant increase in NO levels in females treated with benzothiazole-, phenyl-, and allyl-based derivatives. Among these, the compounds CMA–18, MG–22, and CMG–41 induced the most substantial elevations, suggesting a higher potential for eNOS–NO pathway activation in females.

These findings indicate that the biological response to treatment is influenced not only by the chemical class of the compound but also by sex-specific physiological differences. This highlights the importance of a sex-sensitive approach when evaluating the therapeutic efficacy of antitumor or vasodilatory agents targeting NO metabolism.

Furthermore, the data support the hypothesis that thiosemicarbazone derivatives selectively stimulate NO production and eNOS activity, with a greater effect observed in females. This phenomenon may be attributed to the role of estrogens, which are known to enhance eNOS activation through phosphorylation-dependent signaling pathways.

Table 2. *In vivo* total NO activity ($\mu\text{M/g Hb}$) in rats treated with CCTs, differentiated by sex and expressed as percentage relative to the control group

Study group	Sex	Median NO ($\mu\text{M/g Hb}$)	IQR	% of control
control	M	0.58	0.09	100%
	F	0.43	0.04	100%
Benzothiazole derivatives				
CMA – 18	M	0.67	0.18	115%
	F	0.53	0.12	123%
CMD-8	M	0.53	0.13	91%
	F	0.49	0.10	114%
MG-22	M	0.56	0.12	97%
	F	0.46	0.08	107%
Phenyl derivatives				
CMC – 34	M	0.55	0.21	95%
	F	0.45	0.15	105%
CMJ – 33	M	0.63	0.20	109%
	F	0.45	0.10	105%
CMT-67	M	0.56	0.12	97%
	F	0.45	0.08	105%
Allyl derivatives				
CMG-41	M	0.57	0.17	98%
	F	0.47	0.05	109%
TIA-123	M	0.61	0.08	105%
	F	0.49	0.05	114%
TIA-160	M	0.5	0.09	98%
	F	0.44	0.07	102%

Note: Total NO concentration is expressed as the median and interquartile range (IQR) in μM per gram of hemoglobin ($\mu\text{M/g Hb}$) for male (M) and female (F) rats treated with CuCCTs, compared to control. The percentage reflects the relative change versus the control group, which was set at 100%.

Thus, female subjects may benefit more prominently from the vasodilatory and anti-inflammatory effects of these compounds. In the control group, nitric oxide (NO) concentrations were $0.58 \mu\text{M/g Hb}$ in males and $0.43 \mu\text{M/g Hb}$ in females. The most pronounced increases were observed in females treated with CMA–18 ($0.53 \mu\text{M/g Hb}$, 123%) and MG–22 ($0.46 \mu\text{M/g Hb}$, 107%). In males, the changes were more modest, with the highest value observed for CMA–18 ($0.67 \mu\text{M/g Hb}$, 115%).

Among male subjects, the variations were generally less significant, with NO levels remaining close to or slightly below control values. Phenyl derivatives exhibited a moderate stimulatory effect on NO production in females, with levels reaching up to $0.45 \mu\text{M/g Hb}$ (105%), whereas in males, slight reductions or control-level maintenance were noted.

Allyl derivatives increased total NO concentrations in females within the 102–114% range, while males showed mild fluctuations around baseline values.

NO concentration plays a pivotal role in maintaining cardiovascular homeostasis, directly influencing endothelial function, blood pressure regulation, and the redox balance. Its synthesis is mediated by the eNOS. A decline in eNOS activity has been closely associated with the onset of endothelial dysfunction, atherosclerosis, hypertension, and other cardiovascular pathologies.

Table 3. Evaluation of eNOS enzymatic activity under the *in vivo* influence of CCTs

Study group	Sex	Median eNOS ($\mu\text{M/g Hb}$)	IQR	% of control
control	M	0.76	0.21	100%
	F	0.61	0.11	100%
Benzothiazole derivatives				
CMA – 18	M	0.81	0.15	107%
	F	0.68	0.10	111%
CMD-8	M	0.69	0.13	91%
	F	0.71	0.08	116%
MG-22	M	0.72	0.13	95%
	F	0.85	0.15	139% *
Phenyl derivatives				
CMC – 34	M	0.74	0.26	97%
	F	0.70	0.10	115%
CMJ – 33	M	0.84	0.25	110%
	F	0.73	0.15	120%
CMT-67	M	0.72	0.13	95%
	F	0.68	0.13	112%
Allyl derivatives				
CMG-41	M	0.80	0.29	105%
	F	0.85	0.27	139% *
TIA-123	M	0.77	0.12	101%
	F	0.74	0.11	121%
TIA-160	M	0.72	0.14	95%
	F	0.73	0.09	120%

Note: eNOS activity is expressed as median and interquartile range (IQR) in $\mu\text{M/g}$ hemoglobin (Hb) for male and female subjects across experimental groups, compared to controls. Values are presented as a percentage relative to the control group. An asterisk (*) indicates statistically significant differences compared to the control ($p < 0.05$).

With respect to eNOS activity, the results confirmed a notable increase in female rats across most derivatives, particularly with MG–22 and CMG–41, where elevations of up to 39% over control values were recorded. In contrast, changes in males were limited or statistically insignificant,

suggesting a sex-specific regulatory mechanism, potentially hormonally mediated, influencing the enzyme responsible for nitric oxide (NO) synthesis.

Baseline eNOS enzymatic activity was 0.76 $\mu\text{M/g Hb}$ (IQR 0.21) in males and 0.61 $\mu\text{M/g Hb}$ (IQR 0.11) in females, confirming natural sex-based biological differences. These baseline values were used as the reference (100%) for interpreting the effects of thiosemicarbazone derivatives.

Effects of benzothiazole derivatives: CMA-18, CMD-8, and MG-22 produced variable effects. CMA-18 increased eNOS activity by 7% in males and 11% in females, indicating a mild stimulatory effect. CMD-8 led to a decrease in males (-9%) but a significant increase in females (+16%). MG-22 was the most potent stimulant in females (+39%, $p < 0.05$), while in males it induced a slight decrease (-5%). These differences may reflect a complex interaction between the chemical structure of the derivatives and the hormonal regulation of eNOS, with increased sensitivity observed in females. Across all compound categories, females showed a stronger response in both total NO concentration and eNOS activity compared to males. This difference suggests a sex-specific regulatory mechanism, potentially hormone-mediated.

Effects of phenyl derivatives: CMC-34, CMJ-33, and CMT-67 induced moderate changes, with increases in eNOS activity particularly evident in females (12–20%), while in males, variations were minimal (95–110%). This stability suggests a favorable profile for moderate activation, with therapeutic potential.

Effects of allyl derivatives: CMG-41, TIA-123, and TIA-160 consistently stimulated eNOS activity, with notable effects in females (+39% for CMG-41, +21% for TIA-123, $p < 0.05$), while males showed moderate variations (95–105%). These results suggest that allyl derivatives may be the most suitable for enhancing NO synthesis, particularly in females.

The findings highlight a clear sex-dependent response to CCTs, with females exhibiting stronger eNOS activation. This effect is most likely mediated by estrogenic influence, which stimulates both eNOS expression and activity. Males and females exhibit distinct immunological responses to both internal and external antigens, with such differences observable in both innate and adaptive immune responses [25].

The chemical structure of the derivatives significantly influences the response: benzothiazole derivatives had a more heterogeneous effect, while phenyl and allyl derivatives generated more consistent increases in enzymatic activity. These findings suggest that certain classes of derivatives may be preferable in the development of personalized vasoprotective therapies.

These results open promising perspectives for the use of CCTs as therapeutic agents in cardiovascular diseases, particularly when biological sex differences are considered.

4 DISCUSSION

4.1. The role of nitric oxide in vascular function and cardiovascular health

NO plays a pivotal role in regulating vascular function, acting as a key mediator in vasodilation, platelet aggregation inhibition, and endothelial protection against OS [26]. Through these mechanisms, NO directly contributes to the maintenance of vascular homeostasis and the prevention of cardiovascular diseases.

The biosynthesis of NO is catalyzed by eNOS, an enzyme expressed in endothelial cells whose activity is crucial for proper cardiovascular function. eNOS activity regulates vascular tone, tissue perfusion, and the response to inflammatory and oxidative stressors. Dysfunctional eNOS or reduced NO production is strongly associated with pathological conditions such as atherosclerosis, hypertension, and heart failure [27–28].

Thiosemicarbazone derivatives are a class of organic compounds recognized for their diverse pharmacological properties, including antioxidant effects and modulatory actions on enzymes involved in free radical metabolism [29]. Previous animal studies have demonstrated that eNOS expression and activity levels are significantly higher in females across various tissues, such as the kidneys, correlating with greater NO production and enhanced cardiovascular protection compared to males [30].

Sex-related biological differences in eNOS expression and regulation are well documented, with estrogens playing a critical role in stimulating enzyme activity [31]. The present study investigated the effects of three structural classes of thiosemicarbazone derivatives on eNOS activity, aiming to identify sex-based differences and structure-dependent responses.

The data confirm a significant differentiation in the biological response to thiosemicarbazone derivatives between sexes, with females exhibiting markedly higher eNOS activation, particularly in response to MG–22 and CMG–41. These effects are likely correlated with estrogen-mediated enhancement of eNOS expression and activity, promoting greater NO synthesis [32].

4.2. Effects of chemical structures on eNOS activity: an analysis of thiosemicarbazone derivatives

Phenyl and allyl derivatives demonstrated a more consistent and stable increase in eNOS activity, suggesting that these chemical structures may be favorable candidates for future therapeutic development. In contrast, benzothiazole derivatives exhibited a more variable response, indicating a need for further studies to clarify the underlying molecular mechanisms.

eNOS stimulation is especially important in the context of endothelial dysfunction and cardiovascular disease, where insufficient NO production contributes to pathogenesis. Our findings suggest that these derivatives may help restore redox and vascular balance, offering potential applications in personalized medicine.

Marked sex differences: The data underline a clear sex-specific differentiation in eNOS activity, with females showing a more pronounced enzymatic response than males. This is most likely due to the influence of female sex hormones—particularly estrogens—known to induce both eNOS expression and activity.

Class-specific variability: Benzothiazole derivatives exhibited the most heterogeneous effects, ranging from increases to decreases in activity, depending on sex and compound subtype. In contrast, phenyl and allyl derivatives consistently demonstrated stimulatory effects, suggesting a correlation between chemical structure and eNOS modulatory potential.

Therapeutic potential: Enhanced eNOS activity is associated with increased NO production, which can lead to vasodilation, reduced oxidative stress, and protection against endothelial

dysfunction. These observations indicate that certain thiosemicarbazone derivatives, particularly MG-22 and CMG-41, could represent promising candidates for the development of personalized vasoprotective therapies, especially when sex-specific differences are considered.

4.3. Therapeutic potential of thiosemicarbazone derivatives in the context of sex-based differences

These sex-related differences in the response to thiosemicarbazone derivatives reflect the influence of endocrine factors, such as estrogens, which are well known for their ability to stimulate the expression and activity of eNOS. Thus, the tested compounds may represent a promising therapeutic approach for the treatment of endothelial dysfunction and cardiovascular diseases, particularly in women.

The obtained results confirm the hypothesis that thiosemicarbazone derivatives exert a significant influence on nitric oxide metabolism and eNOS activity, with a clear differentiation based on the biological sex of the subjects. Total NO concentration and eNOS activity were found to be more pronounced in females compared to males, suggesting a strong modulatory effect of sex hormones - especially estrogens - on the signaling pathways involved in NO synthesis.

Therefore, thiosemicarbazone derivatives induce a selective and sex-dependent stimulation of total nitric oxide production and eNOS activity, with important implications for the development of personalized therapies aimed at optimizing vascular responses and reducing cardiovascular risk in a sex-specific manner.

5. CONCLUSIONS

The present study revealed a differential modulation of NO metabolism and eNOS activity depending on sex and the chemical structure of the administered thiosemicarbazone derivatives.

Total NO concentrations, expressed in $\mu\text{M/g Hb}$, showed a significant increase in females treated with benzothiazolic, phenyl, and allyl derivatives - particularly with CMA-18, MG-22, and CMG-41 - indicating a more pronounced stimulation of NO production in females. In males, variations in total NO concentration were more modest and in some cases slightly lower than control levels, suggesting a marked sex-dependent difference in biological response.

Regarding eNOS activity, the results confirmed a notable increase in females for the majority of derivatives, with the most pronounced effects observed with MG-22 and CMG-41, reaching up to 39% above control values. In contrast, changes in males were limited and often nonsignificant, highlighting a specific regulatory mechanism likely influenced by hormonal factors that govern NO synthesis.

These sex-related differences in response to thiosemicarbazone derivatives reflect the influence of endocrine factors such as estrogens, which are known to stimulate eNOS expression and activity. Therefore, the tested compounds may represent a promising therapeutic strategy for managing endothelial dysfunction and cardiovascular diseases, particularly in women.

In conclusion, thiosemicarbazone derivatives induce a selective and sex-dependent stimulation of total nitric oxide production and eNOS activity, with significant implications for the development

of personalized therapies aimed at optimizing vascular responses and reducing cardiovascular risk in a sex-specific manner.

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