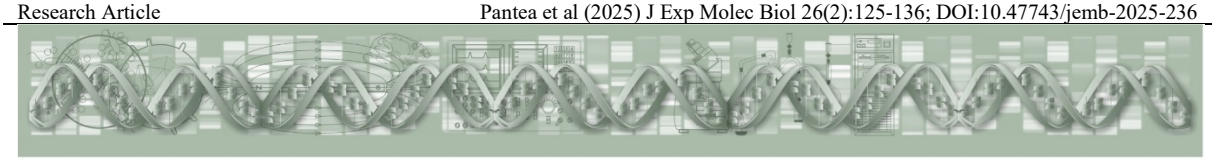

# INVESTIGATING THE *IN VIVO* EFFECTS OF COPPER COORDINATION COMPOUNDS WITH THIOSEMICARBAZONES ON ERYTHROCYTE REDOX BALANCE

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## Abstract

Thiol-disulfide homeostasis plays a vital role in cellular and systemic functions, regulating biosynthetic reactions, growth, transport, repair, and redox signaling through the dynamic interplay between thiol (-SH) and disulfide (-S-S-) states. This study evaluated the effects of copper coordination compounds with thiosemicarbazones (CCTs) on thiol-disulfide metabolism in 120 rats (*Rattus norvegicus Albicans*). The animals were divided into 10 groups by sex, with the control group receiving saline and experimental groups (Groups 2–10) administered specific CCTs (10 μg/kg, subcutaneously) for 30 days. CCTs, known for their medicinal potential, particularly as anticancer agents, enhanced antioxidant defenses by increasing total and reduced glutathione (tGSH, rGSH) and decreasing oxidized glutathione (GSSG). These findings underscore the potential of CCTs in modulating redox balance and their promise in therapeutic applications, including cancer treatment.
**Keywords:** thiol-disulfide metabolism, erythrocytes, copper coordination compounds with thiosemicarbazones

## Introduction

The tripeptide glutathione is a central component of an integrated antioxidant system that safeguards cells and tissues from oxidative stress (OS). Redox (oxidation-reduction) reactions are essential to cellular metabolism and homeostasis, producing electrophilic byproducts that are unavoidable. Cells expend significant energy synthesizing numerous proteins and processing both endogenous and dietary antioxidants to maintain redox equilibrium and protect critical cellular macromolecules from oxidative damage (Ribeiro et al. 2023).
Among these antioxidants, reduced glutathione (GSH) is particularly versatile. Composed of glutamate, cysteine, and glycine, GSH plays a key role in reduction and conjugation reactions, primarily through the sulfhydryl (-SH) group of cysteine. These reactions are crucial for neutralizing peroxides and detoxifying various xenobiotic compounds. Additionally, GSH is involved in regulating the cell cycle (Ribeiro et al. 2023). It is the most abundant cellular thiol,

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with concentrations reaching 1 to 10 mM in many cell types (Wu 2004, Lapenna et al. 2023, Vázquez-Meza et al. 2023).

Glutathione is recognized as a critical cellular redox regulator, influencing key cell fate decisions, such as proliferation and apoptosis (Jones 2002, Watson et al. 2003, Lu 2020). Its abundance allows GSH to play a vital role in shielding cells from toxicity caused by excessive endogenous and exogenous substances (Ballatori 2009). Notably, GSH is the primary defense against various toxic heavy metal ions (Arthur, 2000). Additionally, GSH acts as a cofactor for the GSH peroxidase (GPX) enzyme family, which neutralizes hydrogen peroxide ($H_2O_2$) and lipid peroxides (Rinaldi et al. 2002). Through the activity of the glutathione S-transferase (GST) enzyme family, GSH also conjugates with diverse endogenous electrophiles and xenobiotics, ensuring their safe and efficient elimination (Rinaldi et al. 2002).

Human life relies on oxygen and aerobic processes, yet a consequence of these processes is the production of reactive oxygen species (ROS), which can be harmful to cellular components (Yi et al. 2016). ROS, including superoxide anion radicals ($O^{2-}$), hydrogen peroxide ($H_2O_2$), and hydroxyl radicals ($^{-}OH$), are typically generated during essential metabolic activities, such as protein synthesis and mitochondrial respiration (Valgimigli et al. 2023).

The oxidative effects of ROS are neutralized by the antioxidant capacity of cells, and this battle against OS maintains homeostasis (Krylatov et al. 2007). Within the cell, redox couples are specifically controlled, particularly in the mitochondria, endoplasmic reticulum, and nucleus (Jones et al. 2004). Additionally, extracellular compartments provide defensive barriers against external oxidants. Cysteine (Cys) and its disulfide form, cystine (CySS), constitute the principal low-molecular-weight thiol/disulfide couple in human plasma. The Cys/CySS pool serves as a central redox control point in biological signaling (Jones et al. 2002).

Organic compounds containing the sulfhydryl group are called thiols (-SH), composed of sulfur and hydrogen atoms. Thiol groups are highly susceptible to oxidation due to their -SH nature. Disulfides (-S-S-) represent the most important class of dynamic, redox-sensitive covalent bonds formed between two thiol groups. Dynamic thiol-disulfide homeostasis (TDH) involves the reversible oxidation of thiols in proteins and reflects the levels of thiols and disulfides. This parameter is crucially associated with various biochemical processes, including the regulation of protein function, stabilization of protein structures, protection of proteins against irreversible oxidation of cysteine residues, chaperone function, and the regulation of enzymatic activities and transcription (Brülisauer et al. 2014, Ellgaard et al. 2018, Schmidt et al. 2023).

GSH acts as an essential cellular antioxidant with diverse protective functions. Maintaining normal GSH levels is therefore important for shielding cells against endogenous oxidants and low oxidative exposure.

ROS and nitrogen species (RNS) serve as significant signaling molecules, and changes in GSH levels can shift the threshold for this signaling. Research from various authors suggests that decreased GSH levels modify endothelial nitric oxide (eNO) signaling, affecting cellular responses (Sánchez-Rodríguez et al. 2019).

Thiosemicarbazones (TSCs) are a class of strong metal ion ligands currently being researched for various activities, including but not limited to anticancer treatment. In addition to these ligands, which exert their activity through interaction with metal ions, preformed metal-TSC complexes, particularly with essential metal ions such as iron, copper, and zinc, are also widely studied. Currently, it is unclear which are the active species, which complexes are present, and what their biological targets are. In this context, we have studied copper thiosemicarbazone complexes in preclinical studies regarding their activity in peripheral blood *in vivo*, focusing on thiol-disulfide metabolism.

The aim of the study was to investigate the influence of copper coordination compounds with thiosemicarbazones on thiol-disulfide metabolism in erythrocytes following subcutaneous administration *in vivo*.

## Materials and Methods

### Study Design

Novel local copper coordination compounds with thiosemicarbazones (CCTs), have been used in the study, including benzothiazole thiosemicarbazones (CMA-18, CMD-8, MG-22), phenyl thiosemicarbazones (CMC-34, CMJ-33, CMT-67), and allyl thiosemicarbazones (CMG-41, TIA-123, TIA-160). They were synthesized at the State University of Moldova, Republic of Moldova, in the "Advanced Materials in Biopharmaceutics and Technology" Laboratory (Gulea et al. 2008).

### Experimental Design

This study utilized **120 laboratory white rats (*Rattus norvegicus Albicans*)**, comprising **60 males** (weight: 180–230 g) and **60 females** (weight: 210–228 g).

All experiments were conducted in accordance with ethical standards and were approved by the **Research Ethics Committee** of the "Nicolae Testemițanu" State University of Medicine and Pharmacy, Chisinau, Republic of Moldova (**Approval No. 73, dated 26.04.2017**).

### Animal Maintenance

The animals were housed under **standard vivarium conditions** (temperature: 22 ± 2°C, humidity: 55–60%, 12-hour light/dark cycle) with *ad libitum* access to standard laboratory chow and water.

The rats were randomly divided into the following groups:

**Control Group (12 animals)**: 6 males and 6 females

**Experimental Groups (108 animals)**:

- **54 males**: Subdivided into 9 groups of 6 animals each.
- **54 females**: Subdivided into 9 groups of 6 animals each.

Each experimental group received one of **9 biologically active copper coordination compounds with thiosemicarbazones**.

### Screening and Preparations

The compounds tested were classified into three distinct groups based on their chemical structure:

1. **Benzothiazole derivatives of thiosemicarbazone:** CMA-18; CMD-8; MG-22;
2. **Phenyl thiosemicarbazone derivatives:** CMC-34; CMJ-33; CMT-67;
3. **Allyl thiosemicarbazone derivatives:** CMG-41; TIA-123; TIA-160;

Each compound was administered to the respective experimental group to assess its biological activity.

**Table 1.** Newly Studied Native Copper Coordination Compounds with Thiosemicarbazones

| | |
|---|---|
| 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-ylcarbamohydrazonothioato} copper |
| TIA-160 | Acetato-{2-({[(methylsulfanyl)(prop-2-en-1-ylamino) methylidene]hydrazinylidene}methyl)enolato} copper |

The test substances were dissolved in saline solution to the required volume and administered subcutaneously to the animals daily for 30 days at a dosage of 10 µg per kg of body weight. Twenty-four hours after the final administration of the local CCTs, the animals were euthanized in compliance with ethical standards and guidelines for laboratory animal care. Peripheral blood, the material for study, was collected in tubes containing a 6% K4-EDTA solution with a pH of 7.4 as an anticoagulant. The peripheral blood was centrifuged at 3000 rpm for 10 minutes. After centrifugation, the plasma was transferred to clean disposable Eppendorf tubes and stored in a refrigerator at -40°C until analysis. Simultaneously, the erythrocytes were washed three times with two volumes of 0.9% saline solution, followed by centrifugation. Subsequently, $H_2O$ was added to lyse the erythrocytes according to their volume. The lysed erythrocytes were then transferred to tubes and stored at -40°C until use.

**Assessment of the Action of CCT on Thiol-Disulfide Metabolism in Erythrocytes**

Thiol-disulfide metabolism indices were measured using techniques adapted for application on the Synergy H1 microplate spectrophotometer (Hybrid Reader) (BioTek Instruments, USA).

The activity of thiol-disulfide metabolism indices was evaluated by determining the following laboratory parameters: total glutathione (tGSH), reduced glutathione (rGSH), oxidized glutathione (GSSG), protein thiol (SH) groups, total thiol groups and free thiol groups.

**Statistical Analysis**

The statistical evaluation of the obtained data was performed using the Statistical Package for the Social Sciences (SPSS) software, version 23 (SPSS Inc., Chicago, IL, USA). After verifying the obtained data, the post-hoc Games-Howell multiple comparison test following One-Way ANOVA was used to highlight significant differences in thiol-disulfide metabolism parameters between the compared groups, with a significance threshold of $p < 0.05$. The median, interquartile range, and median percentage compared to the control group were calculated.

## Results and discussions

**Description of the action of CCT on glutathione metabolism**

The study evaluated the impact of various biologically active copper coordination compounds with thiosemicarbazones (CCTs) on the glutathione enzyme activity in erythrocytes of healthy rats, revealing notable sex-based differences. Figure 1 presents graphically the statistical data reflecting these biochemical shifts.

Analysis of the effects of benzothiazole derivatives, particularly CMA-18, on glutathione enzyme activity in male rats indicated a significant increase in total glutathione (tGSH) levels by 33% ($p < 0.05$) and reduced glutathione (rGSH) by 73% ($p < 0.01$) compared to control values. Both CMA-18 and CMD-8 led to a marked decrease in oxidized glutathione (GSSG) levels by 52% and 54%, respectively (CMA-18, $p < 0.05$; CMD-8, $p < 0.001$).

In the phenyl derivative group, all administered compounds showed a non-significant increase in tGSH and rGSH levels across both sexes. However, GSSG levels in male rats were significantly reduced with CMC-34 and CMJ-33 by 31% and 40%, respectively ($p < 0.01$).

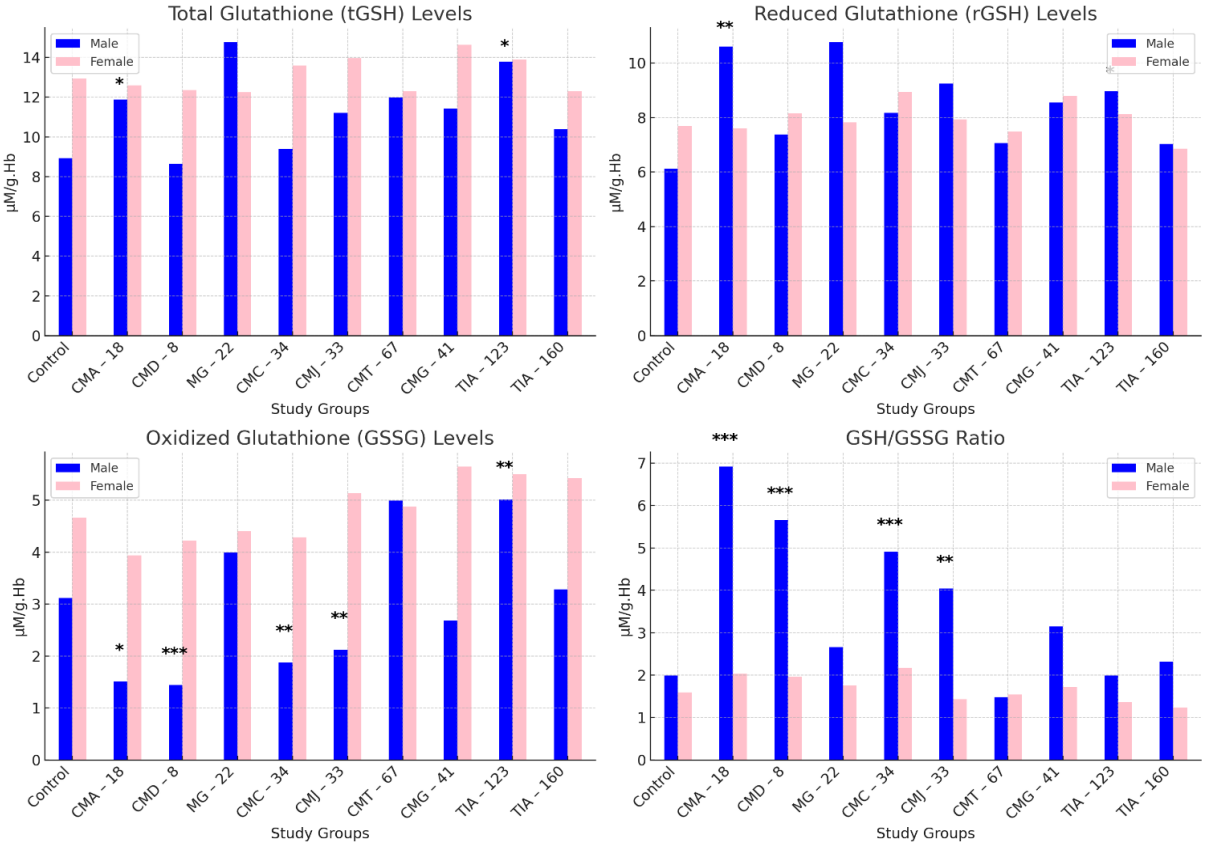


**Figure 1.** Changes in the activity of glutathione metabolism enzymes under the action of copper coordination compounds with thiosemicarbazones in erythrocytes *in vivo*
*Note:* Statistical significance compared to the control group: * – $p < 0.05$; ** – $p < 0.01$; *** – $p < 0.001$; **tGSH –** Total Glutathione**, rGSH –** Reduced Glutathione**, GSSG –** Oxidized Glutathione

Within the allyl group, significant enhancements were noted only with TIA-123 in male rats, showing a 54% increase in tGSH ($p < 0.05$) and a 47% rise in rGSH ($p < 0.05$), alongside a 61% elevation in GSSG levels ($p < 0.01$) compared to controls.

## Effects of CCT on thiol-disulfide metabolism

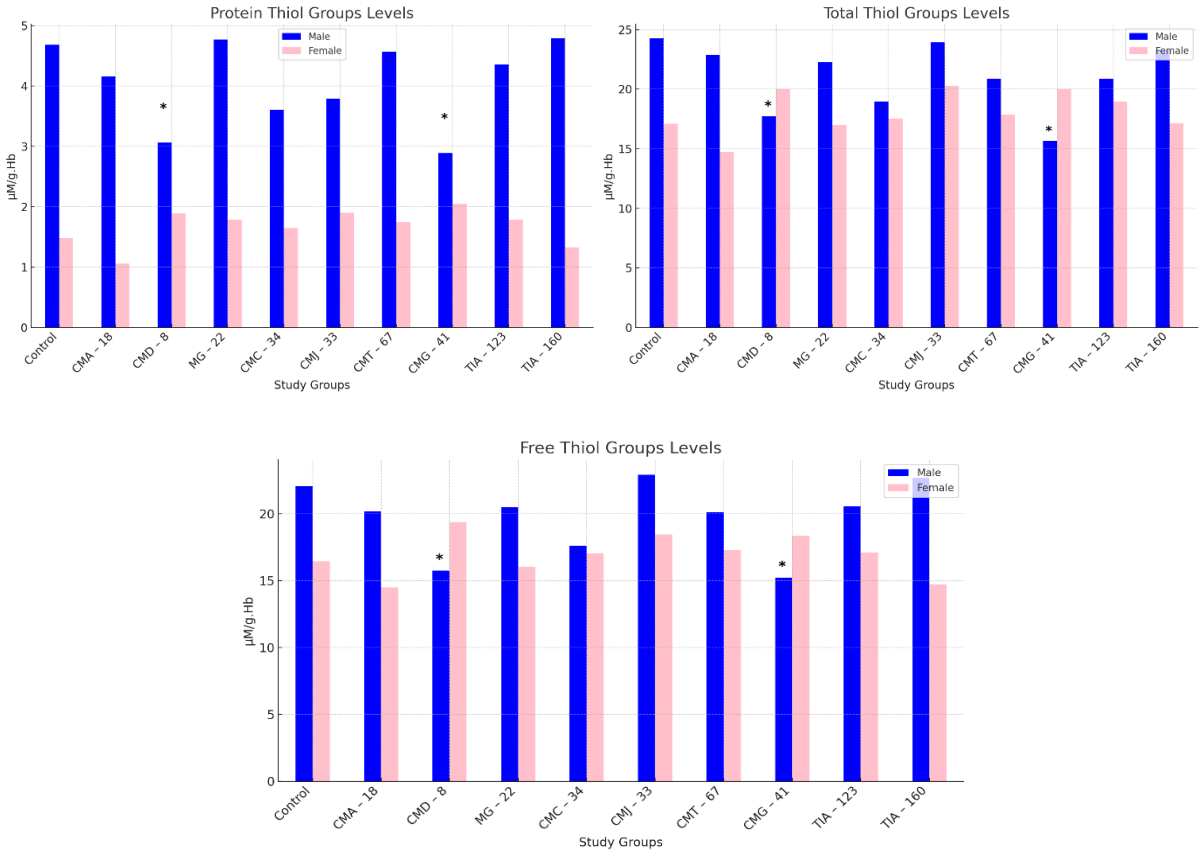


**Figure 2.** Evaluation of thiol groups level in rat erythrocytes following administration of copper coordination compounds with thiosemicarbazones
*Note:* Statistical significance compared to the control group: * − $p < 0.05$

The study also measured changes in thiol group levels in erythrocytes following administration of various CCTs (Figure 2), which highlighted both reductions and increases in thiol levels across different compounds and sexes. Significant reductions in male rats were observed with the benzothiazole derivative CMD-8, showing a 27-29% decrease in protein thiol, total thiol, and free thiol content ($p < 0.05$), and the allyl derivative CMG-41, which led to a 31-36% decrease ($p < 0.05$). In contrast, the remaining compounds demonstrated non-significant decreases ranging from 6-23% in thiol content relative to the control group.
In female rats, most compounds caused a non-significant increase in thiol levels, with enhancements ranging from 2-28% ($p > 0.05$). Notably, CMA-18 and TIA-160 led to slight reductions in thiol content in females, decreasing by 10-28% ($p > 0.05$).
These results underscore that specific CCTs modulate glutathione metabolism distinctly in male and female rats, likely due to underlying physiological differences between the sexes. GSH primarily exerts its antioxidant role through the action of GPx, which reduces hydrogen peroxide and lipid peroxides to maintain a balanced redox state by converting GSH into GSSG. This oxidized GSSG can be recycled back to GSH by glutathione reductase, a process essential for cellular redox homeostasis (Lu 2009). GPx and GST also facilitate organic peroxide reduction.
While catalase also breaks down hydrogen peroxide, it is confined to peroxisomes, rendering GSH crucial for protecting mitochondria from both physiological and pathological oxidative stress (Garcia-Ruiz et al. 2006, Chen et al. 2024). As the GSH/GSSG ratio dictates cellular redox potential, high OS prompts GSSG export from the cell or conjugation with protein

sulfhydryl groups, resulting in mixed disulfides. This mechanism prevents significant redox imbalances, as elevated OS can otherwise deplete cellular GSH (Lu 2009).

The modulation observed in this study supports the importance of the GSH/GSSG ratio in cellular redox signaling, with a higher GSH/GSSG ratio indicating a reduced state conducive to antioxidative processes. The ability of certain CCTs to enhance both tGSH and rGSH levels suggests that these compounds aid in maintaining redox homeostasis under oxidative conditions. Given that GSH is integral to the cell's antioxidant network, its upregulation by CCTs could enhance redox resilience during OS or inflammation. Conversely, elevated GSSG, particularly in male rats treated with TIA-123, may imply a compensatory response to high oxidative turnover, aligning with prior studies where increased GSH/GSSG ratios promoted cellular proliferation, while diminished ratios were associated with apoptotic pathways. This pro-oxidative redox environment could, therefore, influence cellular pathways that govern stress regulation (Anashkina et al. 2020, Ribeiro 2023).

The findings on thiol levels in Figure 2 also reveal CCT-induced changes in thiol-disulfide homeostasis, with notable thiol reductions in male rats for compounds CMD-8 and CMG-41, implying heightened oxidative activity. Such reductions might increase disulfide bond formation, suggesting a more oxidative cellular environment. Conversely, females displayed trends of boosted thiol levels, indicating an enhanced antioxidative response. This highlights a sex-specific ability to preserve thiol concentrations and underscores the relevance of thiol-disulfide balance in erythrocyte functionality and oxidative defense. These observations also suggest that males and females may differ in their response to CCT-induced oxidative stimuli, with implications for conditions relying on precise redox regulation.

A similar redox regulatory function of GSH has been previously documented, as it modulates cellular signaling via cysteine oxidation (Anashkina et al. 2020, Ribeiro 2023). Glutathionylation, where GSH binds reversibly to protein cysteine residues, creates glutathionylated proteins (Prot-SSG), which can alter protein activity, thereby protecting protein thiols from irreversible oxidation and helping conserve GSH under OS. Deglutathionylation, mediated by enzymes like glutaredoxin and sulfiredoxin, restores protein function using GSH as a reducing agent (Liu et al. 2010). The capacity for ROS and RNS to regulate proteins and signaling molecules through cysteine oxidation is well-documented, with GSH as a critical modulator in this process (Garcia-Ruiz et al. 2006, Liu et al. 2010, Anashkina et al. 2020, Lu 2020, Ribeiro 2023, Chen et al. 2024).

In the γ-glutamyl cycle, GSH is a sustained source of cysteine, essential given cysteine's instability and tendency to oxidize into cystine, which could produce harmful radicals. This cycle's involvement in GSH synthesis underscores its role in redox balance (Wu et al. 2004). Dysregulated GSH synthesis has been associated with aging, metabolic, and liver disorders (Wu et al. 2004).

The data affirm glutathione's effectiveness in maintaining oxidative balance. Thiols and disulfides participate in key biological functions, such as protein structure, redox homeostasis, and polymer secondary structure formation (Leichner et al. 2019). As an adaptive response to ROS, disulfide bonds formed within proteins contribute to self-repairing materials due to their reversible nature (Jin et al. 2013). Various studies confirm that altering the thiol-disulfide ratio modulates cellular processes: increased GSH/GSSG ratios promote proliferation, while lower ratios signal apoptosis (Nkabyo et al. 2002, Berndt et al. 2014).

Given ROS's multifaceted role in cellular regulation and cytotoxicity, targeted therapies against ROS must balance tumoricidal effects with normal cell protection. Future research should consider this complex regulatory network to develop redox-targeting treatments with precision (Yang et al. 2013).

The results herein reflect the selective biological effects of CCTs. Copper coordination compounds can directly affect the redox balance in erythrocytes by cycling between Cu(I) and

Cu(II) states, thereby influencing the formation and detoxification of reactive oxygen species (ROS). Specifically, these complexes may:

- Catalyze redox reactions: The copper ion can participate in Fenton-like or Haber-Weiss reactions, leading to ROS formation. Under controlled conditions, such activity may help regulate redox signaling (Malarz et al. 2018).
- Scavenge superoxide: Certain Cu-thiosemicarbazone complexes can mimic superoxide dismutase (SOD) activity, thereby reducing superoxide to hydrogen peroxide and mitigating oxidative stress (Menezes et al. 2024).
- Interact with GSH: Copper coordination compounds bind to glutathione, influencing its oxidation state. While this can transiently enhance OS, it also upregulates antioxidant defense pathways and maintains GSH/GSSG homeostasis (Hancock et al. 2011).
- Modulate protein sulfhydryls: By altering cysteine residues on hemoglobin and other proteins, these compounds can foster reversible glutathionylation, thus protecting protein thiols under oxidative conditions and stabilizing the redox environment in erythrocytes (Ramek 2021).

Through these mechanisms, copper coordination compounds with thiosemicarbazones display both prooxidant (facilitating ROS formation for signaling or cytotoxic effects) and antioxidant (SOD-like activity, GSH regeneration) properties, ultimately fine-tuning erythrocyte redox balance.

These results support the potential of CCTs as agents in redox modulation and warrant further exploration into their applications in oxidative stress-related pathologies as seen in other publications as described previously (Pantea et al. 2022, Pantea et al. 2023, Pantea 2023).

## Conclusions

CCTs serve as metal ion ligands with promising therapeutic applications, particularly in anticancer treatments. The biological effects of copper coordination compounds with thiosemicarbazones are highly selective and varied, attributed to their complex actions across multiple cellular targets. Specifically, TSC metal complexes can induce toxicity by generating reactive oxygen species through the activation of molecular oxygen *via* metal ions, leading to the formation of radicals that potentially reduce cellular thiol content. These points to the necessity of understanding the complex interplay of physiological and pathological pathways involved in cellular redox balance.

*In vivo* studies demonstrate that CCTs selectively preserve thiol-disulfide homeostasis in erythrocytes by enhancing total and reduced glutathione levels and reducing oxidized glutathione, underscoring their antioxidant properties.

These findings suggest that CCTs hold therapeutic promise in cancer treatment by modulating cellular redox status through both antioxidant and prooxidant effects. Consequently, this research has the potential to contribute significantly to the development of novel therapeutic insights. The redox activity and biocompatibility of copper ions, the stability of copper coordination compounds in the bloodstream, and promising therapeutic outcomes *in vivo* collectively support the potential for clinical application of copper coordination compounds in cancer and redox-related pathologies.

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