

Chemical hypermethioninemia in young mice: oxidative damage and reduction of antioxidant enzyme activity in brain, kidney, and liver

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Research Article

Keywords: methionine, methionine sulfoxide, catalase, superoxide dismutase, oxidative stress, aminoacidopathy

Posted Date: April 11th, 2022

DOI: <https://doi.org/10.21203/rs.3.rs-1523315/v1>

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Abstract

High levels of methionine (Met) and its metabolites, such as methionine sulfoxide (MetO), found in hypermethioninemia, can be detrimental to the body; however, the underlying mechanisms are still uncertain. Using a recently standardized protocol, the aim of this study was to investigate the effects of chronic administration of Met and/or MetO on parameters of oxidative damage in the total brain, liver, and kidney of young mice. Swiss male mice were subcutaneously injected with Met and MetO at concentrations of 0.35–1.2 g/kg body weight and 0.09–0.3 g/kg body weight, respectively, from the 10th – 38th day post-birth, while the control group was treated with saline solution. Results showed that Met and/or MetO caused an increase in reactive oxygen species (ROS) and lipoperoxidation, along with a reduction of superoxide dismutase (SOD) and catalase (CAT) activities in the brain. In the liver, Met and/or MetO enhanced ROS and nitrite levels, and reduced SOD, CAT, and delta aminolevulinic dehydratase activities. The effects on the kidney were an increase in ROS production and SOD activity, and a reduction in thiol content and CAT activity. These data demonstrated the contribution of redox imbalance to the systemic changes found in patients with hypermethioninemia. In conclusion, our findings may help future studies to better understand the pathophysiological mechanisms of hypermethioninemia as well as contribute to the search for new therapeutic agents for this pathology.

1. Introduction

In recent years, several clinical studies have demonstrated the harmful effects of hypermethioninemia, especially associated with the brain (Ismayilova et al., 2019; Allen et al., 2019; Kido et al., 2019; Nashabat et al., 2018; Zhang et al., 2020). Concomitant to this, preclinical studies of hypermethioninemia using experimental models *in vitro* (Soares et al., 2020a; Dos Santos et al., 2017) and *in vivo* (Franceschi et al., 2020; Soares et al., 2020; Figueiró et al., 2019), have helped in understanding the pathophysiological mechanisms involved in this condition. However, knowledge of the comprehensive implications of high tissue and plasma concentrations of methionine (Met) are still limited.

The main cause of hypermethioninemia is the deficiency of the methionine adenosyltransferase enzyme. The major clinical characterization for this pathology is high plasma concentrations of Met, and the symptoms are reported to be associated with changes in the liver and brain. Currently, the treatment for hypermethioninemia is based on the absence of Met-rich foods in association with supplementation by S-adenosylmethionine (Mudd et al., 2011; Schweinberger and Wyse, 2016).

The hepatic symptoms of hypermethioninemia include cirrhosis and steatosis (Lu & Mato, 2008; Lu et al., 2001; Mudd, 2011). In addition, studies using animals have reported that high concentrations of Met and its metabolites, such as methionine sulfoxide (MetO), similar to those found in hypermethioninemic patients, can cause inflammatory cell infiltration (Stefanello et al., 2009), histological changes (Stefanello et al., 2009), and oxidative damage (Stefanello et al., 2009; Soares et al., 2017). These changes are likely related to the fact that the liver is the main metabolizer of amino acids, and Met and MetO are included in

this group. Thus, high concentrations of these amino acids can cause liver overload with consequent alteration of homeostasis, followed by liver damage with impaired physiological functions (Mudd, 2011).

In addition, neurological symptoms have been reported both in patients and experimental models. In animals, it has recently been reported that hypermethioninemia induced by the administration of Met and/or MetO causes oxidative damage, reduces the activity of the enzymes AChE and $\text{Na}^+ / \text{K}^+ \text{-ATPase}$, reduces BDNF levels, and decreases neuron numbers (Soares et al., 2020b), causing cell death by apoptosis (Soares et al., 2017). Furthermore, it has been shown that exposure to Met and/or MetO invokes memory deficit in young rats, and changes in the homeostasis of rat astrocytes (Soares et al., 2020b). However, animal studies to date have only evaluated the effects of hypermethioninemia on isolated brain structures, rather than on the brain as a whole.

Although several effects have already been demonstrated, the understanding behind these is still unknown. For this reason, it is extremely important that new studies continue to be carried out in order to expand the knowledge base around the pathophysiological mechanisms involved in the evolution of hypermethioninemia. In this sense, the objective of the present study was to investigate, using a recently standardized protocol, the effects of the chronic administration of Met and/or MetO on parameters of oxidative damage in the total brain, liver, and kidney of young mice.

2. Materials And Methods

2.1. Chemicals

Methionine and methionine sulfoxide were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

2.2. Animals and ethical procedures

The Ethics Committee of Animal Experimentation from the Federal University of Pelotas approved the experimental design and procedures used in this study (CEEA 9221 – 2013). Swiss male mice were requested and made available by the Central Animal House of the Federal University of Pelotas, Pelotas, RS, Brazil. During all the experiments, the mice were placed in a room with constant temperature ($22 \pm 1^\circ\text{C}$) on a 12 h light/dark cycle and with free access to food and water.

2.3. Chemical hypermethioninemia protocol

The protocol used in this study was developed according to Franceschi et al. (2020). First, twenty four male Swiss mice were divided into four groups: Group I – Control, group II – Met, group III – MetO, and group IV – Met + MetO. The mice were treated for twenty eight days, from the 10th to the 38th day of life. In the first week of treatment, 0.35 g/kg of Met and 0.09 g/kg of MetO were given. In the second week, the doses administered were 0.5 g/kg of Met and 0.14 g/kg of MetO. In the third week, the doses were 0.8 g/kg of Met and 0.2 g/kg of MetO. Finally, in the fourth week of the experiment, the mice were given 1.2 g/kg of Met and 0.3 g/kg of body weight of MetO. The animals of group IV received a combination of Met and MetO, whereas control mice received saline solution with the same volumes. The amino acids were

administered to mice subcutaneously twice a day (Fig. 1). Twelve hours after the last injection, the mice were euthanized and the brain, liver, and kidney were dissected for analysis.

2.4. Preparation of homogenized tissues and protein determination

Total tissues were homogenized in 10 volumes (1:10 w/v) of 20 mM sodium phosphate buffer, pH 7.4, containing 140 mM KCl. Homogenates were centrifuged at $800 \times g$ for 10 min at 4°C , and the supernatant was obtained from biochemical analysis. Protein levels were measured by the method of Lowry et al. (1951), using bovine serum albumin as a standard.

2.5. Characterization of redox status

2.5.1. Reactive oxygen species (ROS)

For determination of ROS production, oxidation of 2',7'-dichlorofluorescein diacetate (DCFH-DA) to fluorescent 2',7'-dichlorofluorescein (DCF) was measured according to the method of Ali et al. (1992). This parameter was reported as μmol DCF per mg of protein.

2.5.2. Nitrite levels

The nitrite level was determined using 1% sulfanilamide and 0.3% N-1-naphthylethylenediamine dihydrochloride as reagents in the Griess reaction, following the method of Stuehr and Nathan (1989). Results are expressed as μM nitrite per mg of protein.

2.5.3. Total sulfhydryl content (SH)

The SH content assay was realized by the reduction of 5, 5'-dithiobis-(2-nitrobenzoate) (DTNB) by thiols, which in turn became oxidized (disulfide production), generating a yellow derivative (TNB) (Aksenov and Markesbery, 2001). Results are reported as nmol TNB per mg of protein.

2.5.4. Thiobarbituric acid reactive substances (TBARS)

TBARS content was measured using 10% trichloroacetic acid and 0.67% thiobarbituric acid, and heated in a boiling water bath for 25 min according to Esterbauer and Cheeseman (1990). Results are reported as nmol of TBARS per mg of protein.

2.5.5. Superoxide dismutase activity (SOD)

This enzyme was quantified based on the inhibition of superoxide-dependent adrenaline auto-oxidation using the method described by Misra and Fridovich (1972). The specific activity of SOD is reported as units per mg of protein.

2.5.6. Catalase activity (CAT)

CAT activity was measured by depletion monitoring of H_2O_2 for 90 s according to the method of Aebi (1984). The results are reported as units per mg of protein.

2.5.7. Delta aminolevulinic dehydratase (ALA-D)

ALA-D activity was assayed according to the method of Sassa (1982) by measuring the rate of porphobilinogen (PBG) formation, except that in all enzyme assays, the final concentration of ALA was 2.2 mM. The reaction product was determined using modified Ehrlich's reagent. ALA-D activity was expressed as nmol porphobilinogen (PBG)/ mg of protein/h.

2.6. Statistical analysis

Data were analyzed by one-way analysis of variance followed by Tukey's post hoc test for mean comparison using GraphPad Prism version 5.0 software (Intuitive Software for Science, São Diego, CA). A *P*-value of less than or equal to 0.05 was considered statistically significant. All data are expressed as the mean \pm standard error of mean (SEM).

3. Results

3.1. Redox status in brains of young mice subjected to protocol of chemical hypermethioninemia

In the brain, there was an increase in ROS production ($F_{(3,16)} = 5.45$, $P < 0.05$) in the Met, MetO, and Met + MetO groups. There was also an increase in lipid peroxidation in the Met + MetO ($F_{(3,16)} = 20.77$, $P < 0.001$) treated mice compared with the control group. No significant changes were found in the levels of nitrites ($F_{(3,17)} = 0.71$, $P > 0.05$) and total SH ($F_{(3,16)} = 2.38$, $P < 0.05$) in the brain (Fig. 2).

As for the antioxidant enzymes, there was a significant reduction in the activity of the SOD ($F_{(3,17)} = 5.53$, $P < 0.05$) and CAT ($F_{(3,17)} = 12.27$, $P < 0.01$) enzymes in the Met, MetO, and Met + MetO groups than in the control group. As for ALA-D activity, no significant differences were found in any experimental group ($F_{(3,17)} = 1.10$, $P > 0.05$) (Fig. 3).

3.2. Redox status in livers of young mice subjected to protocol of chemical hypermethioninemia

There was an increase in ROS production in the MetO and Met + MetO groups ($F_{(3,18)} = 4.63$, $P < 0.05$). We also observed an escalated level of nitrites in the Met + MetO group ($F_{(3,14)} = 5.77$, $P < 0.05$). No significant changes were found in the levels of total SH ($F_{(3,18)} = 0.64$, $P > 0.05$) and TBARS ($F_{(3,17)} = 0.99$, $P > 0.05$) in any experimental group (Fig. 4).

In terms of antioxidant enzymes, there was a significant reduction in the activity of SOD ($F_{(3,18)} = 11.69$, $P < 0.05$), CAT ($F_{(3,16)} = 6.07$, $P < 0.05$) and ALA-D ($F_{(3,17)} = 10.43$, $P < 0.05$) in the Met, MetO and Met +

MetO groups when compared to the control group (Fig. 6).

3.3. Redox status in kidneys of young mice subjected to protocol of chemical hypermethioninemia

We observed a significant increase in the production of ROS in the Met, MetO and Met + MetO groups ($F_{(3,16)} = 8.88, P < 0.01$). In contrast, the total SH levels ($F_{(3,14)} = 17.94, P < 0.01$) were reduced by Met, MetO, and Met + MetO when compared to the control group. No changes were observed in the nitrite ($F_{(3,18)} = 2.47, P > 0.05$) and TBARS ($F_{(3,16)} = 1.12, P > 0.05$) levels in any experimental group (Fig. 5).

The SOD ($F_{(3,17)} = 4.14, P < 0.05$) activity was increased by MetO and Met + MetO, while CAT ($F_{(3,17)} = 13.16, P < 0.01$) was reduced by Met, MetO, and Met + MetO compared to the control group. No significant changes were found in the activity of the ALA-D enzyme ($F_{(3,17)} = 1.01, P > 0.05$) (Fig. 6).

4. Discussion

Epidemiological data on hypermethioninemia are extremely limited; however, Villani et

al. (2019) demonstrated in a ten-year study that two of seventy-seven thousand newborns were diagnosed with hypermethioninemia: one with classic homocystinuria and the other with MAT I/III deficiency (Villani et al., 2019). Martins et al. (2012) demonstrated an incidence of one out of twenty-six thousand newborns with the condition on an island in Portugal (Martins et al., 2012), while Marcão et al. (2015) demonstrated a frequency of approximately one out of twenty-seven thousand four hundred cases of MAT I/III deficiency (Marcão et al., 2015).

It is known that the dominant form is typically benign, but the recessive form of the disease can cause several serious neurological and hepatic alterations, in addition to changes in clinical signs and symptoms, as demonstrated in several studies (Lu & Mato, 2008; Lu et al., 2001; Allen et al., 2019; Kido et al., 2019; Nashabat et al., 2018; Zhang et al., 2020; Sem et al., 2019). However, the pathophysiological mechanisms involved in these symptoms are poorly understood. We investigated the possible involvement of changes in redox status in the brain, liver, and kidney in a recently standardized model of hypermethioninemia in young mice. The plasma concentrations of Met, 1 h after administration of Met, MetO, and Met + MetO, were similar in the mice to those found in patients with isolated and persistent hypermethioninemia (Franceschi et al., 2020).

We demonstrated that there was an increase in the production of ROS in the brain in all experimental groups, which could be caused by a change in mitochondrial activity, associated with a reduction in the activity of the detoxifying enzymes SOD and CAT. Accordingly, Soares et al. (2017) demonstrated that in an acute protocol, Met and MetO reduce the mitochondrial electrochemical potential. A decrease in antioxidant enzyme activity has also been reported previously in macrophages of mice *in vivo* and *in vitro* (Franceschi et al., 2020; Dos Santos et al., 2017).

An increase in ROS levels can cause a series of deleterious events in the central nervous system. Oxidative damage in the brain may have occurred due to the increased production of ROS, reduced antioxidant activity, and the high levels of membrane polyunsaturated lipids, which can be easily oxidized, leading to lipid peroxidation, which is assessed by TBARS levels. Altogether, these changes can modify cell homeostasis and lead to cell death, as reported by Soares et al. (2017). Thus, it can be suggested that oxidative damage may be involved in the pathophysiology of neurological damage found in patients with hypermethioninemia, since this condition can lead to damage to other important biomolecules such as proteins and DNA.

Considering that the liver and kidneys are very important for the metabolism of amino acids and that liver changes have already been reported in patients with hypermethioninemia (Lu & Mato, 2008; Lu et al., 2001), it is worthwhile to investigate the effects of chronic administration of Met and/or MetO on oxidative markers in these tissues.

In the present study, Met and/or MetO increased the liver production of ROS and nitrite. In addition to mitochondria, the endoplasmic reticulum can also be a significant source of ROS via cytochrome P450 enzymes. Additionally, when compared to the control, the reduction of activity of SOD and CAT, observed in all experimental groups, can contribute significantly to the accumulation of reactive species. It is important to note that reducing SOD activity concomitantly with increasing nitrite level may favor the production of peroxynitrite, which is a reactive species that is very harmful to biomolecules. However, as the liver is a central organ of metabolism, it is rich in enzymatic and non-enzymatic antioxidant defenses (Czaja, 2007; Cichoż-Lach and Michalak, 2014); therefore, an imbalance that is capable of altering liver redox homeostasis should be considered with caution.

In general, chronic diseases of hepatic origin are characterized by the exacerbated presence of oxidative stress, regardless of the cause of the disease (Czaja, 2007; Cichoż-Lach and Michalak, 2014). In this context, some studies have reported that the expression of certain proteins can be modulated by free radicals, and that this modulation can occur in relation to the activation of redox-sensitive transcription factors, such as Egr-1, NF-kappaB and AP-1, as well as G proteins. Cellular kinases are a mitogen-activated protein kinase family that also plays an essential role (Czaja, 2007; Cichoż-Lach and Michalak, 2014). Considering the power of oxidative stress in modulating important signaling pathways for cellular homeostasis, the identification of this condition in hypermethioninemia is extremely relevant, as it may contribute significantly to the liver damage caused by cirrhosis and steatosis, already reported in hypermethioninemic patients (Lu & Mato, 2008; Lu et al., 2001).

A reduction in ALA-D enzyme activity in the liver was also found in groups treated with Met and/or MetO. The ALA-D enzyme catalyzes the second reaction in the heme biosynthesis pathway and is expressed in all tissues, but is more common in the liver. In contrast to the results of Soares et al. (2017), a reduction in the activity of ALA-D was observed, and this can lead to the development of signs and symptoms of typical hepatic porphyria, a condition that is detected in other inborn errors of metabolism such as type 1 tyrosinemia (Fujita et al., 1995; Kelada et al., 2001). ALA-D activity can be reduced in the presence of

oxidizing agents, such as free radicals, and this reduction can lead to pathological consequences with damage to the heme biosynthesis route, accumulation of its substrate in the blood, and overproduction of ROS (Fujita et al., 1995; Kelada et al., 2001).

Finally, we also demonstrated that Met and/or MetO induces an increase in the production of ROS, a reduction in the levels of total thiol content, and a reduction in CAT activity. In contrast, SOD activity was significantly increased in the MetO and Met + MetO groups. The increase in SOD activity can be a compensatory mechanism for the high ROS production. However, it can be postulated that even with increased SOD enzyme activity, damage to the SH groups still occurs. Furthermore, knowing that SOD is responsible for the dismutation of the superoxide anion to hydrogen peroxide, another possible conclusion is that oxidative damage in the kidney is caused by the accumulation of hydrogen peroxide, since there is an increased production of this substance, and a reduction in detoxification caused by CAT inhibition.

The data found in the present study corroborate and complement the previous findings of the literature demonstrating that high doses of Met and/or MetO, similar to those found in patients with hypermethioninemia, can be extremely harmful to several organs, particularly the brain, liver, and kidney (Soares 2020b; Soares et al., 2017; Stefanello et al., 2009). In addition, it should be noted that one of the possible intensifiers of this damage is the presence of MetO, since this metabolite can not only cause damage itself, but is able to form other metabolites with even greater toxic potential, such as methionine sulfone and homocysteic acid. Another interesting point is that the oxidative damage caused by experimental hypermethioninemia in isolated brain structures remains when the total brain is evaluated. Finally, the importance of investigating the possible pathophysiological mechanisms of hypermethioninemia in several experimental models is highlighted, with the purposes of expanding our knowledge on this pathology and determining how high doses of amino acids act in different conditions and biological models.

5. Conclusion

In conclusion, using a recent standard protocol, our data showed that young mice exposed to chronic doses of Met and/or MetO, present redox imbalance in the total brain, liver, and kidney (Fig. 7). These findings contribute to a better understanding of the systemic changes found in biological models of chemically induced hypermethioninemia, and may provide new perspectives for future studies.

Declarations

Animal ethics declarations: The Ethics Committee of Animal Experimentation from the Federal University of Pelotas approved the experimental design and procedures used in this study (CEEA 9221-2013). Swiss male mice were requested and made available by the Central Animal House of the Federal University of Pelotas, Pelotas, RS, Brazil.

Author contributions: Meine BM, Franceschi TS, Bona NP, Spohr L and Pedra NS performed experiments. Soares MSA, Spanevello RS, and Stefanello FM supervised experimental work and analysed data. Meine BM and Franceschi TS wrote the manuscript.

Conflict of interest statement: The authors declare that there are no conflicts of interest.

Acknowledgments: This research was supported by the Conselho Nacional de Desenvolvimento Científico e Tecnológico and Fundação de Amparo à Pesquisa do Rio Grande do Sul (FAPERGS). This study was financed in part by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior- Brasil (CAPES) – Finance code 001.

Availability of data and material Datasets generated in the current study are available from the corresponding author on reasonable request.

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Figures

Experimental design

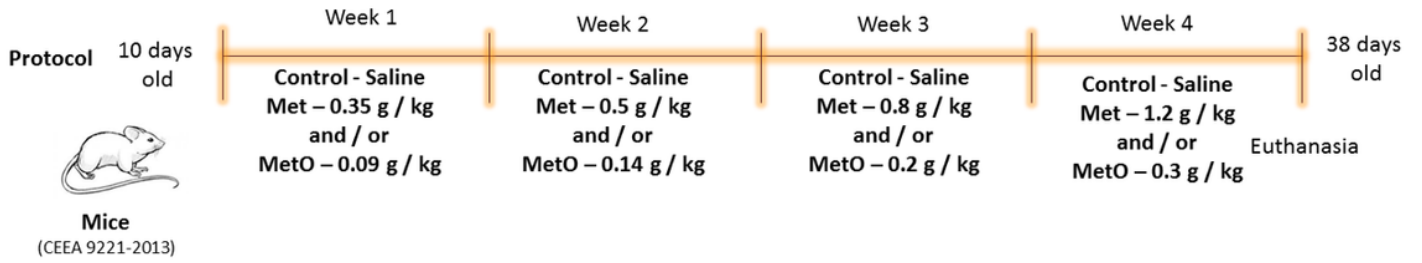


Figure 1

Experimental design for chemical hypermethioninemia model in mice.

Brain

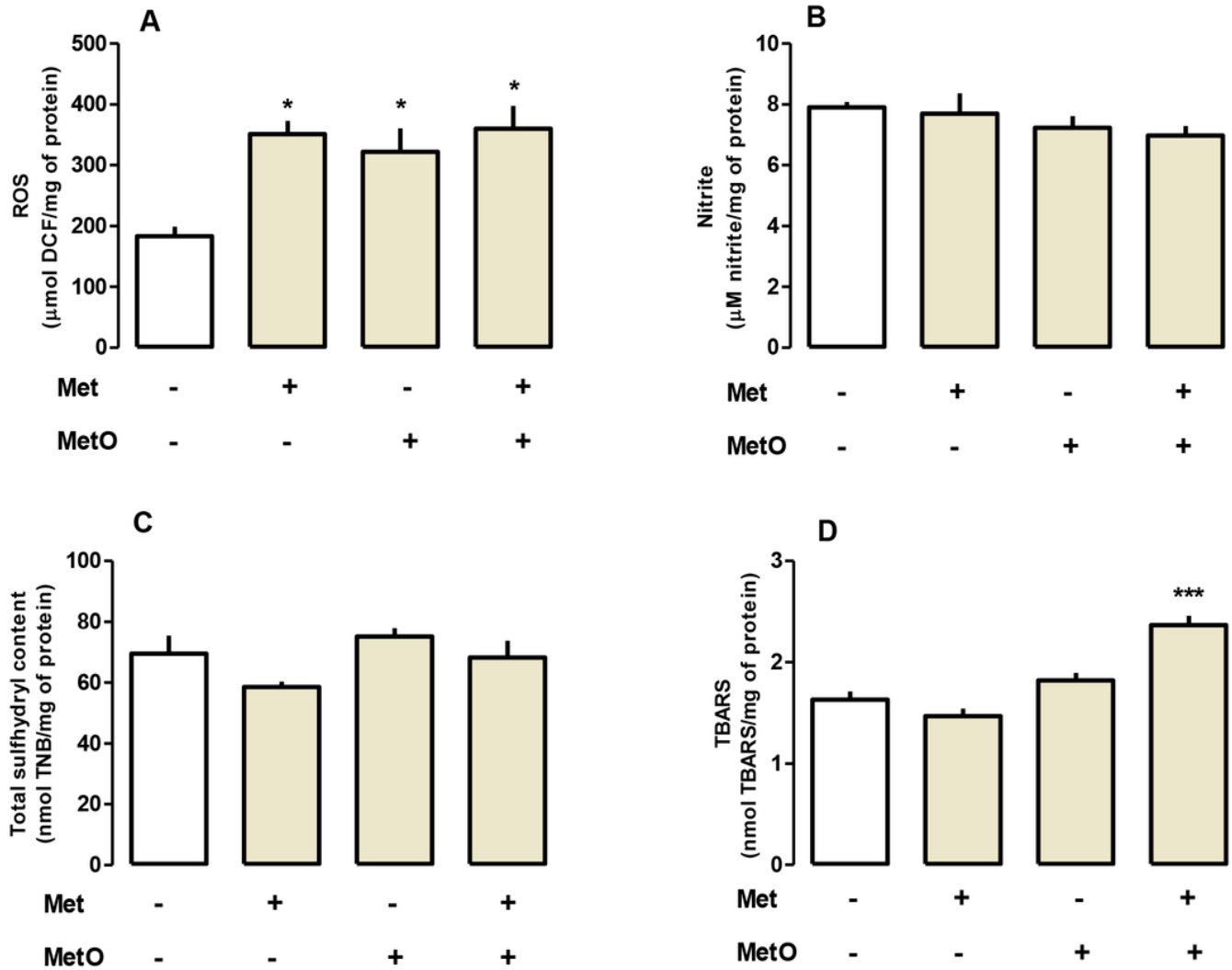


Figure 2

Reactive oxygen species – ROS (A), nitrite (B) levels, total sulfhydryl content - SH (C), and thiobarbituric acid reactive substances – TBARS (D) in brain of young mice subjected to chronic hypermethioninemia protocol. ROS levels were expressed as $\mu\text{mol DCF/mg}$ of protein, TBARS levels were reported as $\text{nmol TBARS/mg protein}$, total sulfhydryl content as $\text{nmol TNB/mg of protein}$ and nitrite levels were expressed as $\mu\text{M nitrite/mg of protein}$. Data were expressed as $\text{mean(s)} \pm \text{S.E.M.}$ (*) Denotes $P < 0.05$ and (***) $P < 0.001$ as compared to the control group (One-way ANOVA followed by Tukey test, $n=4-6$). Met - Methionine, MetO - Methionine sulfoxide.

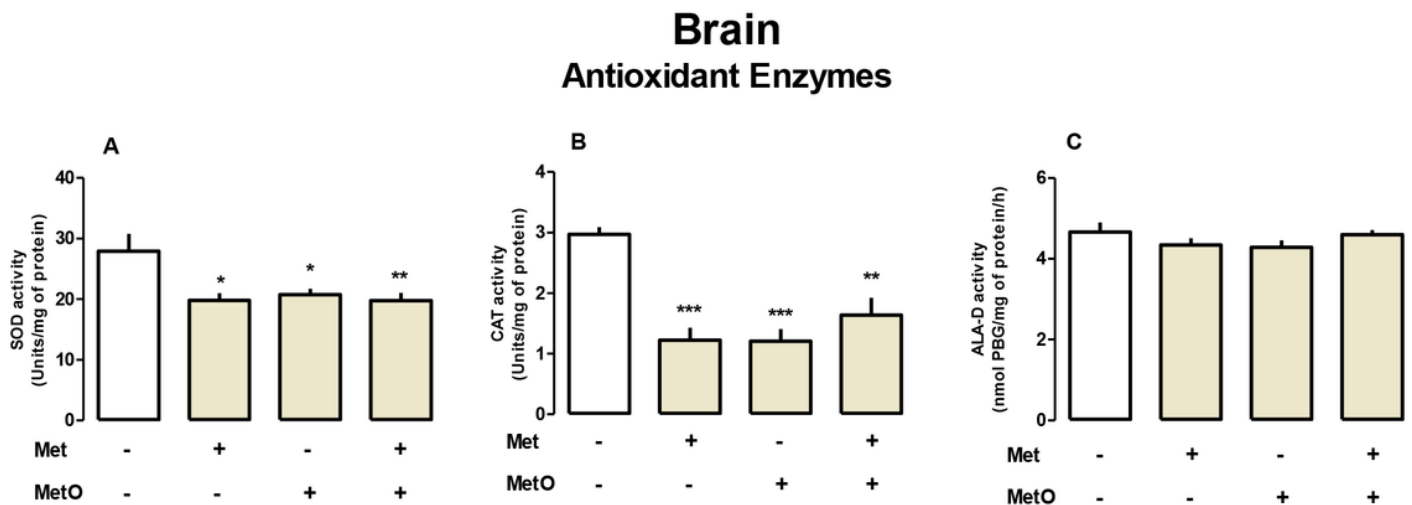


Figure 3

Superoxide dismutase - SOD (A), catalase – CAT (B), and delta aminolevulinic dehydratase (ALA-D) (C) in brains of young mice subjected to chronic hypermethioninemia protocol. SOD and CAT were expressed as $\text{units/mg of protein}$ and ALA-D as $\text{nmol porphobilinogen (PBG) / mg of protein / h}$. Data were expressed as $\text{mean(s)} \pm \text{S.E.M.}$ (*) Denotes $P < 0.05$, (**) $P < 0.01$ and (***) $P < 0.001$ as compared to the control group (One-way ANOVA followed by Tukey test, $n=4-6$). Met - Methionine, MetO – Methionine sulfoxide.

Liver

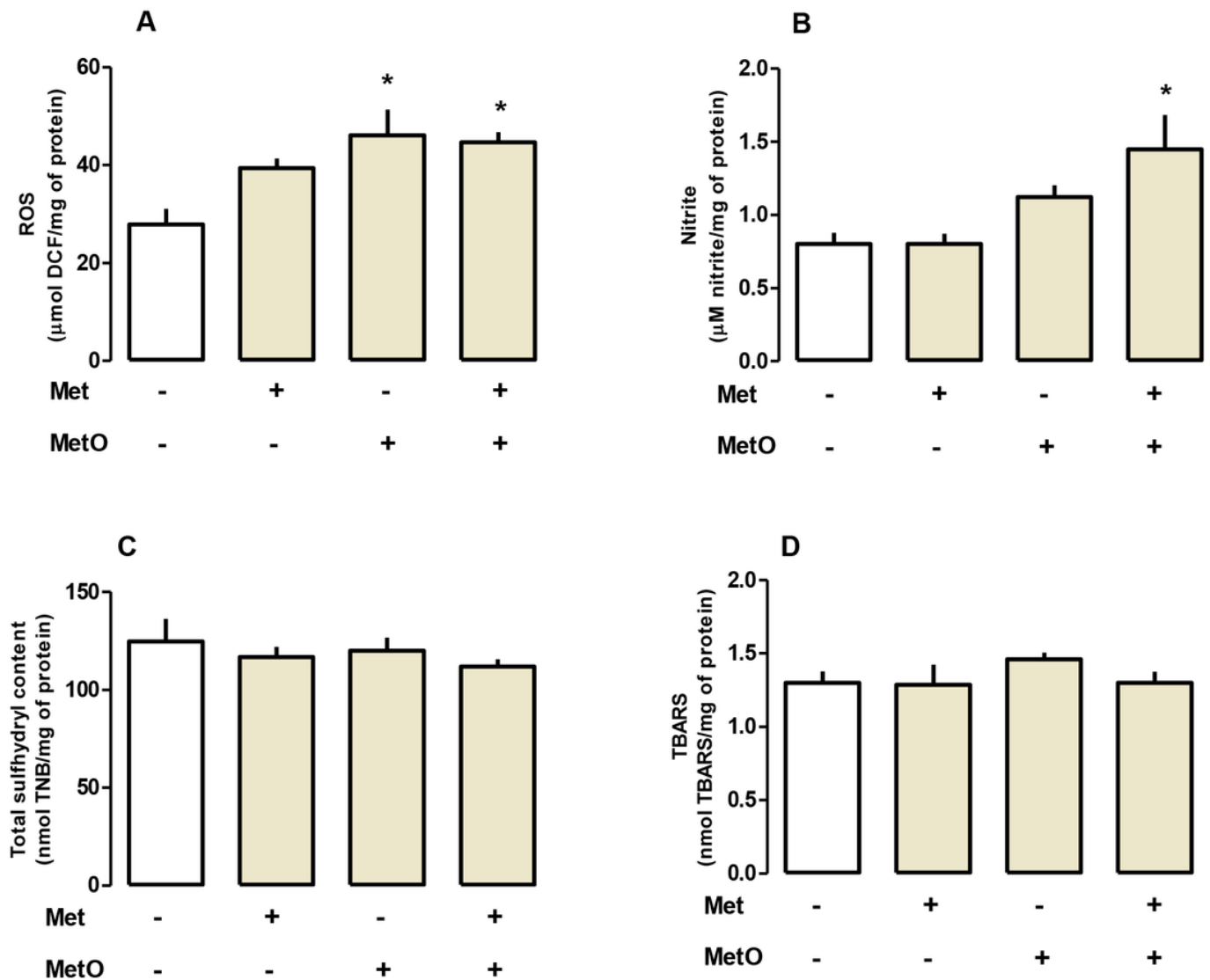


Figure 4

Reactive oxygen species – ROS (A), nitrite (B) levels, total sulfhydryl content - SH (C), thiobarbituric acid reactive substances – TBARS (D) in liver of young mice subjected to chronic hypermethioninemia protocol. ROS levels were expressed as $\mu\text{mol DCF/mg}$ of protein, TBARS levels were reported as nmol TBARS/mg of protein, sulfhydryl content as nmol TNB/mg of protein and nitrite levels were expressed as $\mu\text{M nitrite/mg}$ of protein. Data were expressed as $\text{mean}(s) \pm \text{S.E.M.}$ (*) Denotes $P < 0.05$ as compared to the control group (One-way ANOVA followed by Tukey test, $n=4-6$). Met - Methionine, MetO - Methionine sulfoxide.

Kidney

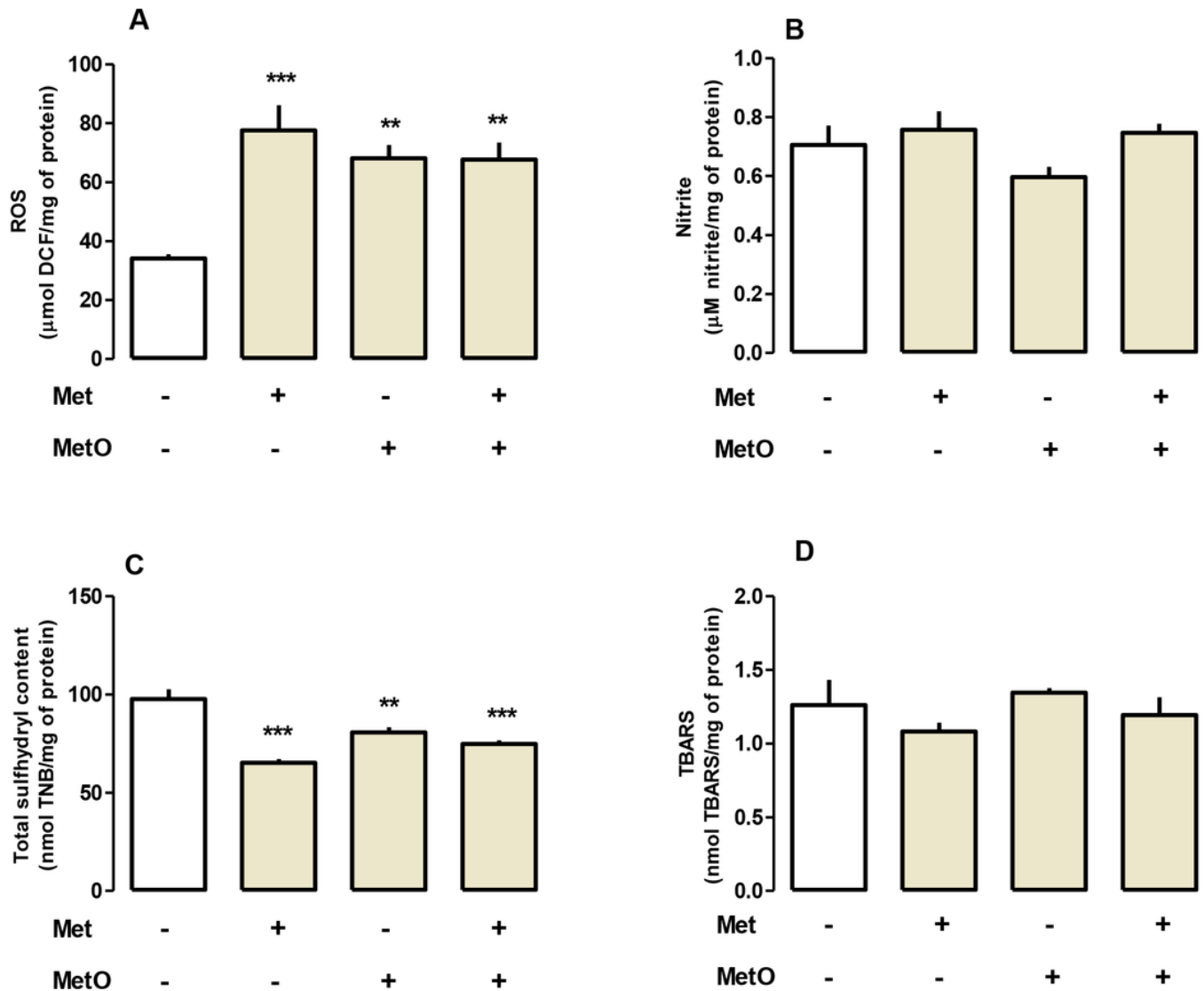


Figure 5

Reactive oxygen species – ROS (A), nitrite (B) levels, total sulfhydryl content - SH (C), thiobarbituric acid reactive substances – TBARS (D) in kidney of young mice subjected to chronic hypermethioninemia protocol. ROS levels were expressed as $\mu\text{mol DCF/mg of protein}$, TBARS levels were reported as $\text{nmol TBARS/mg of protein}$, SH content as $\text{nmol TNB/mg of protein}$ and nitrite levels were expressed as $\mu\text{M nitrite/mg of protein}$. Data were expressed as mean(s) \pm S.E.M. (**) $P < 0.01$ and (***) $P < 0.001$ as compared to the control group (One-way ANOVA followed by Tukey test, $n = 4-6$). Met - Methionine, MetO - Methionine sulfoxide.

Antioxidant enzymes

Liver

Kidney

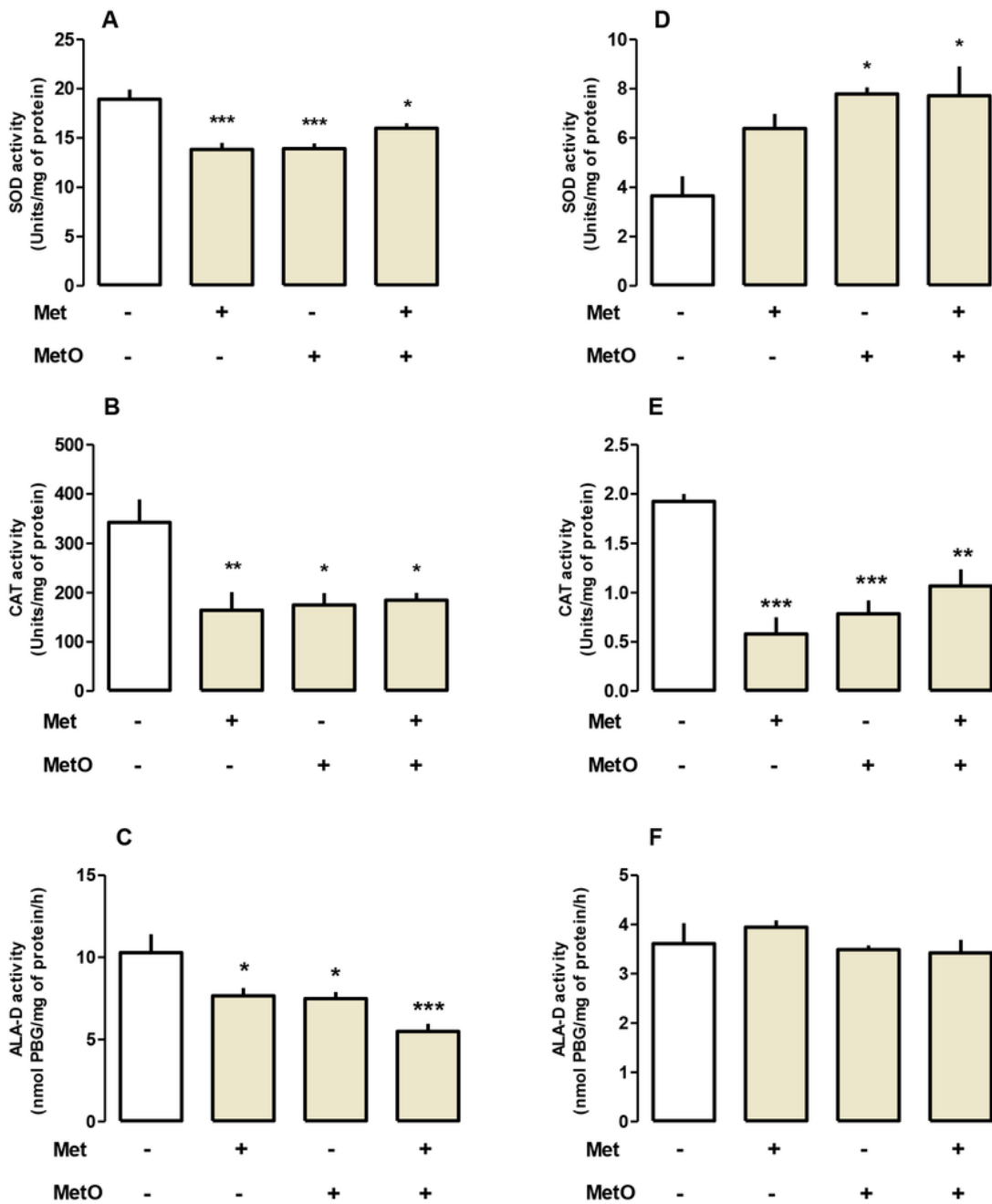


Figure 6

Superoxide dismutase - SOD (A-D), catalase - CAT (B-E) and delta aminolevulinic dehydratase - ALA-D (C-F) in liver and kidney of young mice after chronic hypermethioninemia protocol. SOD and CAT are expressed as units/mg of protein and ALA-D as nmol porphobilinogen (PBG) / mg of protein / h. Data were expressed as mean(s) \pm S.E.M. (*) Denotes $P < 0.05$, (**) $P < 0.01$ and (***) $P < 0.001$ as compared to

the control group (One-way ANOVA followed by Tukey test, n=4-6). Met - Methionine, MetO – Methionine sulfoxide.

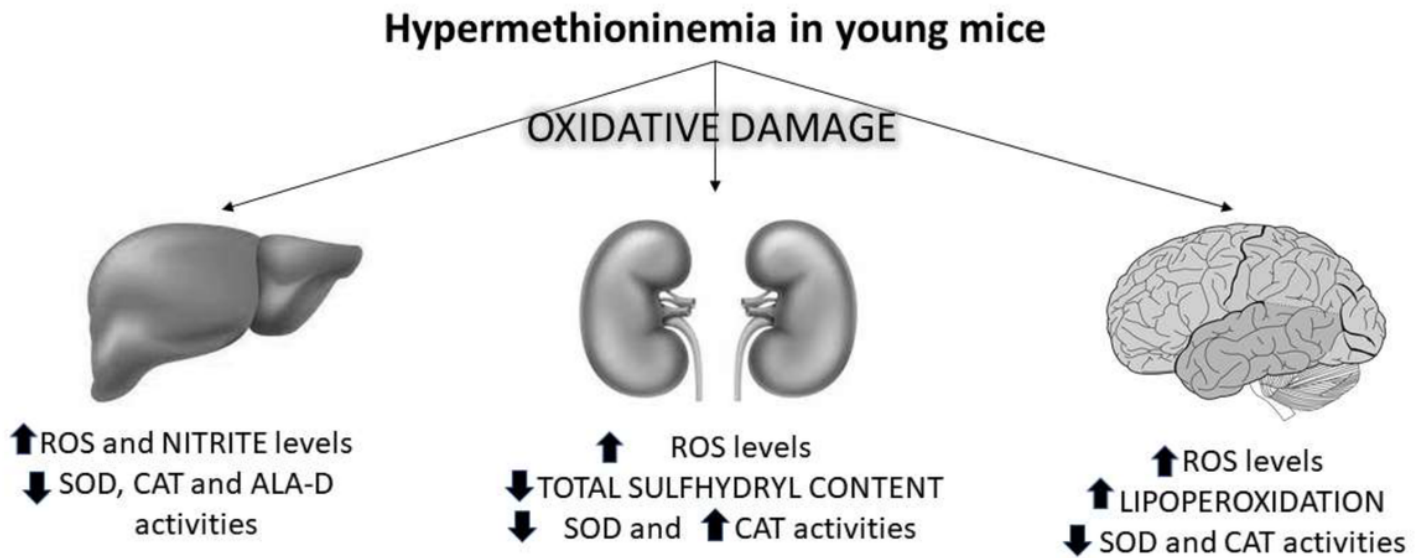


Figure 7

Effects of chronic chemical-induced hypermethioninemia in young mice.