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Effect of repeated inhalations of gaseous hypochlorous acid on the antioxidant properties of rat blood plasma

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ABSTRACT

The development of disinfection systems based on the introduction of a gaseous antimicrobial agent into indoor air represents an effective strategy to prevent airborne infections. Gaseous hypochlorous acid HOCl(g) is an attractive candidate for this purpose. However, its high oxidizing capacity necessitates a thorough investigation of its inhalation toxicity. In this study, Wistar Han rats (n = 6 per group per time point) were exposed to daily 5-hour inhalations of 0.32±0.03 ppm HOCl(g) for 25 days, followed by a 7-day recovery period. To assess oxidative stress, the activities of catalase (CAT) and superoxide dismutase (SOD), total antioxidant capacity (TAC), and malondialdehyde (MDA) levels were measured in blood plasma on days 2, 7, 14, 25 and 32. A sharp increase in MDA was observed 24 h after the first inhalation, indicating intense lipid peroxidation. Plasma antioxidant capacity increased from day 1, reaching a maximum on day 7, substantially exceeding control values: CAT by 1.9-fold, SOD by 2.3-fold, and TAC by 4-fold. This allowed MDA to return to control levels by day 14. After day 7, antioxidant enzyme activities and TAC gradually declined, and by day 32 (one week post-exposure), all parameters were indistinguishable from controls. These findings suggest that repeated HOCl(g) inhalations do not cause severe oxidative stress but rather induce a hormetic response that stimulates the antioxidant system. This may be exploitable not only for airborne disinfection but also for therapeutic preconditioning against oxidative damage. Further research is required to establish an HOCl(g) concentration with an optimal safety/antimicrobial efficacy ratio.

Introduction

The growing threat of infectious disease epidemics underscores an urgent need for the development of effective disinfection systems that can simultaneously disrupt contact and airborne transmission mechanisms in crowded places [1,2]. One promising technology for achieving this is filling indoor spaces with a gaseous agent that, while exhibiting rapid microbiocidal action, would have sufficiently low toxicity for use in the presence of people [3,4]. A classic example is ozonation, which has long been used to disinfect medical facilities [5]. However, the high toxicity and carcinogenicity of ozone, as well as the difficulty of controlling its air concentration, impose certain limitations on its use in non-specialized public places [6]. An effective, although more technologically complex, method is the treatment of rooms with gaseous hydrogen peroxide, which is generated by vacuum evaporation from solutions (so-called "VHP biodecontamination") [7,8]. This chemical agent is less toxic, odorless, stable in the gas phase, and exhibits good results against microbial aerosols. However, the working solutions at the required concentrations may be difficult to obtain when global supply chains are disrupted, as was the case during the COVID-19 pandemic. Several studies have also focused on the gas-phase disinfection with chlorine dioxide [9,10]. The latter has more pronounced antimicrobial properties, allowing rapid neutralization of viruses and bacteria using very low concentrations, which are safe for human inhalation. However, the instability of this gas during storage means that its use is largerly limited to on-site synthesis from sodium chlorite [11], making it unsuitable for treating large objects. Therefore, the search for new gaseous, low-toxicity chemicals is both relevant and promising.

In this context, hypochlorous acid HOCl remains very attractive candidate [12–14]. Being one of the most effective biocide of all active chlorine compounds, it can easily be converted into a gaseous state from sodium hypochlorite (NaOCl) solutions [15,16], which, in turn, can be

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obtained on-site by electrolysis of sodium chloride [17–19]. The widespread availability of raw materials and the simplicity of the technology are an undeniable advantage in conditions of limited resources caused by emergencies or military conflicts. Moreover, the use of gaseous hypochlorous acid HOCl(g), generated in various types of evaporators [20–22], eliminates the risk of corrosion of structural materials and short-circuiting of electrical appliances, which are significant during the aerosolization of HOCl solutions, which is also actively studied for disease prevention [23,24]. The antimicrobial properties of HOCl(g) have been the subject of a number of recent publications. It has been shown that at concentrations of 0.15–0.25 ppm, the inhibition rate of various types of airborne microorganisms reaches over 50%/min [25]. In a real-world experiment in a 480 m^3 room, a significant reduction in viable microorganisms was observed both in the air and on wet agar plates at 25–52 ppb HOCl(g) [26]. Furthermore, the development of highly sensitive HOCl(g) sensors enable precise monitoring the concentration and automating its maintenance, which, theoretically, allows creating safe and easy-to-manage disinfection systems.

At the same time, when discussing using HOCl(g)-based disinfection systems in the presence of people, the issue of toxicity comes to the fore. Unlike dermal [27], inhalation toxicity of HOCl(g) remains largely unexplored. It is known that HOCl is synthesized in the body during phagocytosis and is an important component of the immune response to intoxications of various origins, neutralizing microorganisms, their metabolites, and many other toxins due to its high oxidizing and chlorinating properties [28]. However, high reactivity make it a powerful factor in oxidative stress: excess HOCl itself, as well as other reactive oxygen species (ROS) formed with its participation, can inactivate many enzymes and disrupt the metabolism of healthy cells and tissues [29,30]. Therefore, when using HOCl in any form, it is essential to determine the optimal balance between pharmacological effect and toxicity. Biochemical and toxicological studies of HOCl(g) are extremely scarce. Thus, Fukuzaki and colleagues demonstrated that HOCl(g) is safe for respiratory tract cells at concentrations of up to 0.5 ppm [31]. We previously showed that single 4-hour inhalations of HOCl(g) at concentrations of up to 1.5 ppm affected the activities of antioxidant enzymes and cysteine cathepsins in rats [32,33]. The most significant finding was that these inhalations significantly increased the activities of antioxidant enzymes, and this effect was dose-dependent, with no critical or irreversible changes observed. This allowed us to interpret the elicited changes as the induction of an adaptive antioxidant defense mechanism, which could fundamentally shift the application of HOCl(g) from a purely disinfectant toward a therapeutic agent. Therefore, building upon our previous findings on acute exposure, the present study was designed to evaluate the effects of repeated HOCl(g) inhalations on selected components of the rat antioxidant system, aiming for a more comprehensive assessment of its pharmacological potential and its activity-to-toxicity profile.

Materials and methods

Working solutions for obtaining HOCl(g)

To transfer HOCl into the gas phase, we used working solutions of NaOCl at a concentration of approximately 1.0 g/L and an initial pH of 9.5–9.7. These solutions are commercially available under the brand name "SEKOBREN" and are approved by the Ministry of Health of Ukraine as a disinfectant and antiseptic. They are produced using the previously described technology by electrolysis of NaCl solutions in specially designed flow electrochemical cells [17,18,34]. These solutions are highly stable, contain negligible amounts of sodium chlorate and low free alkali content, and therefore are the most suitable for our study. The active chlorine concentrations in the working solution were measured by iodometric titration immediately before each filling into the evaporator [35,36]. The acidity of the solutions was monitored with an ADWA AD-131 pH meter (ADWA Instruments, Hungary).

Installation for inhalation exposure to animals

The previously described installation was used to conduct the inhalation exposure [37]. Its key element is a specially designed evaporator connected to a 0.568 m^3 inhalation chamber. In brief, its operating principle is that HEPA-filtered air is brought into contact with a foamed working NaOCl solution, enriched with its volatile component HOCl(g), and then forced into the inhalation chamber by a fan. This system ensures the supply of air with a constant HOCl(g) content, humidity, and CO_2 level, prevents aerosol droplets ingress, allows regulation of the airborne concentration of the antimicrobial agent without interrupting the operation, and does not require constant replacement of the working solution, making it highly convenient for long-term experiments. The HOCl(g) concentration, as well as temperature and humidity in the air within the chamber, were continuously monitored using a Dräger X-node 1 HOCl detector (Dräger MSI GmbH, Germany) with an electrochemical sensor, and its corresponding software. Additionally, the chamber was equipped with a Walcom GD-09A NH3 detector (Walcom International Industry Limited, China) to monitor ammonia levels, an AZ 7530 (AZ Instrument Corp., Taiwan) to monitor CO_2 content, and a Walcom HT-9600 (Walcom International Industry Limited, China) to track the dispersed particles.

Laboratory animals and conditions of their maintenance

The design of the experiment has been approved and treatment of animals has been supervised by Commission on the Ethics of Dnipro State Medical University (protocol №30 from 17.09.2025 of the meeting of the Ethics Commission of Dnipro State Medical University) in accordance with international recommendations of the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes. The study involved Wistar Han rats aged 12 weeks old at the beginning of the experiment. Only female rats were selected for this study because of their higher sensitivity to inhalation exposure, their use in our previous investigations, allowing direct comparison of results, and the advantage of lower inter-individual variability compared to mixed-sex cohorts. The animals were obtained from the SPF vivarium of Dnipro State Medical University, examined by a veterinarian, and placed in a room with limited access, equipped with forced-air ventilation and artificial lighting (12 h of darkness, 12 h of light). The room temperature (20–24 °C) and relative humidity (50–60%) were continuously monitored and maintained within a specified range using a Cole-Palmer Digi-Sens Temperature/RH recorder (Cole-Palmer, China). The animals were housed in standard M4 polycarbonate 40 × 30 × 15 cm cages with removable metal bars on top, 5 animals per cage. The bedding made of sterilized, non-chlorinated food paper was changed every 5 days, simultaneously with washing and disinfecting the cage. The rats had *ad libitum* access to pelleted autoclaved food (Altromin, Germany) and reverse-osmosis-purified water. The animals were acclimatized for 10 days before being transferred to the experiment. After acclimatization they were randomly assigned into 2 groups of 30 animals each, each with identification tags. The first (experimental) group was exposed to inhalation of HOCl(g) generated from a working NaOCl solution, while the second (control) group was exposed under the same conditions using a 0.9% NaCl solution in an evaporator. The baseline characteristics of the animal groups are presented in Table 1.

Table 1
The baseline characteristics of the animal groups.

Group number	Group character	Number of animals	Working solution used for inhalations	Average weight of animals, g
1	Control	30	0.9% NaCl	165.6 ± 12.5
2	Experiment	30	0.1% NaOCl	168.3 ± 14.9

Organization of inhalation exposure

Washed and disinfected cages filled with fresh bedding were placed in the inhalation chamber before the experiment and positioned symmetrically on either side of the air inlet. Initial amount of 5.0 liters of NaOCl solution with a pre-measured concentration and pH were added to the evaporator. The necessary pH was calculated based on previously obtained patterns [37,38] so that the air concentration of HOCl(g) at the evaporator outlet was in the range of 0.30–0.35 ppm. The installation was started with an air supply rate of approximately 50 m^3/h through the evaporator into the chamber, which ensured an air exchange in the chamber of approximately 90 vol per hour. The installation was operated until a stable, predetermined level of HOCl(g) in the air was established. If necessary, the latter was adjusted by adding the minimal required amounts of hydrochloric acid or sodium hydroxide into the working solution.

After the required air parameters in the chamber were reached, the animals were placed in cages inside it. The treatment was performed in the "whole body" mode. The exposure duration was 5 h per day. Air parameters in the chamber were continuously monitored. Animal behavior was also constantly observed, with particular attention to signs of anxiety, salivation, aggression, etc. Control animals were treated simultaneously under identical conditions using 5.0 l of 0.9% NaCl solution. After 5 h, the animals were removed from the chambers, placed in cages with fresh bedding, and transferred to the permanent holding room. The cages in the chamber were washed and disinfected. The chambers themselves were disconnected from the evaporator, thoroughly ventilated, washed, and disinfected with a UV lamp. The following day, the working solution parameters in the evaporator were determined and adjusted, if necessary, to ensure a target HOCl(g) level of 0.30–0.35 ppm in the air. The installation was operated until the required air parameters were stable in the chamber, then the animals were placed inside, and the entire procedure was repeated. The working solution in the evaporators was completely replaced when it evaporated below 3.0 liters. If it was necessary to adjust the HOCl(g) air concentration directly during animal inhalation due to the tendency of the working solution to become alkaline during operation [37], this was accomplished without interrupting the process by adding the minimal required calculated amount of hydrochloric acid into the bottom of the evaporator.

The procedure was repeated as described for 25 consecutive days. On days 2, 7, 14, and 25, the animals were weighed, and clinical samples were collected from 6 rats in each group. For this, the narcotized animal was decapitated, and blood was collected into prepared tubes with EDTA as an anticoagulant for subsequent biochemical analysis. After 25 days, the remaining animals were transferred to the permanent holding room and kept for 7 days without further exposure to inhalation to study the recovery of biochemical parameters. Their clinical samples were then (day 32) collected in a similar manner. The choice of $n = 6$ animals per time point (rather than $n = 10$ recommended by "OECD Test Guideline 413: Subchronic Inhalation Toxicity" for terminal studies) was deliberately made to balance statistical robustness, ethical compliance, and experimental feasibility. Since our study involved repeated euthanasia at 5 time points, using 30 animals per group instead of 50 reduced the total number of animals by 40 %, fully adhering to the 3R principle. Moreover, this sample size was based on a power analysis using data from our previous studies [32,33], which indicated that experiments with $n = 4$–5 lacked statistical power for reliable intergroup comparisons. Thus, $n = 6$ represents the optimal compromise between scientific validity and animal welfare.

Determination of the studied biochemical parameters in rat blood plasma

Selected biochemical parameters were measured colorimetrically using corresponding commercially available assay kits from ZellBio GmbH (Germany) and a Multiskan FC microplate photometer (Thermo Fisher Scientific, China). All procedures were performed in strict accordance with the manufacturer's instructions for plasma samples. A brief description of each assay is provided below.

Assay for determining catalase (CAT) activity is based on the catalase-like activity of horseradish peroxidase, which uses hydrogen peroxide H_2O_2 to oxidize a colorless substrate to a pink-colored product measured at 560 nm. The presence of CAT in the plasma sample reduces the H_2O_2 concentration and, consequently, the optical density of the test sample.

The superoxide dismutase (SOD) assay is based on the use of xanthine oxidase, which, by generating superoxide in the presence of oxygen, promotes oxidation of the substrate, forming a yellow-colored product measured at 450 nm. The presence of SOD in the sample reduces the superoxide concentration and, consequently, the intensity of the corresponding peak in the spectrum.

The determination of the concentration of malondialdehyde (MDA) is based on the ability of the latter to conjugate with thiobarbituric acid to form a pink adduct, quantified by absorbance at 535 nm (standard TBARS assay).

To determine total antioxidant activity (TAC), the widely accepted Ferric Reducing Antioxidant Power (FRAP) assay was used. This assay is based on the ability of small-molecule antioxidants and specialized enzymes to reduce the ferric-tripyridyltriazine complex to its ferrous form. The reaction generates a blue-colored product which is measured at 560 nm, proportional to TAC.

To avoid bias, all biochemical analyses were performed by researchers blinded to the group allocation.

Statistical analysis of the results was carried out using the STATISTICA 6.1 (Stat Soft Inc., serial number AGAR909E415822FA). Mathematical processing included calculations of arithmetic means (M) and standard deviations (M±SD). The normality of the data distribution was initially tested using the Shapiro-Wilk test. Since the distribution of most parameters deviated from normal, which was also observed in our previous studies [32,33], we further employed non-parametric statistics: Mann-Whitney *U test* was used to determine the degree and nature of the relationship between the tested parameters at the thresholds of reliability $p < 0.01$, $p < 0.05$.

Results

Air composition in the inhalation chamber

As in our previous study, the use of a specially designed installation allowed maintenance of the HOCl(g) in the air at a constant level of 0.32 ±0.03 ppm, significantly exceeding the uniformity of exposure described in studies using aerosols [39]. The acidity of the NaOCl working solution required adjustment no more than twice a day, and during this adjustment, the HOCl(g) concentration in the chamber never exceeded the specified limits. In both chambers, relative humidity never exceeded 60% at any time, and CO_2 levels varied between 450 and 550 ppm, which are physiologically comfortable for animals. Ammonia was absent in both chambers. No droplets of the working solution or condensation of air components were detected by the detector of dispersed particles.

Survival of animals and their behavior during the experiment

Not a single animal died during the entire experiment. Under inhalation, the behavior of animals in the experimental group did not differ from that of the control group. The rats remained active, and no signs of anxiety or salivation were observed. Water and food consumption, as well as appearance and behavior, were identical in both groups throughout the observation period. The weight gain rate of the experimental animals also did not statistically differ from that of the controls.

Effect of repeated inhalations of HOCl(g) on studied biochemical indicators

Changes in CAT and SOD activities, as well as plasma TAC and MDA concentration, are summarized in Table 2 and illustrated in Fig. 1.

As shown in Table 2, all biochemical parameters in the control group showed no significant changes throughout the experiment, indicating the stable condition of the animals, the appropriate age chosen, and the adequacy of the analytical methods used. As early as the next day after the start of the experiment, HOCl(g) inhalations significantly affected all studied biomarkers, leading to their increase compared to the control: CAT - by 20%, SOD - by 41%, TAC - by 27%, and, most noticeably, MDA - by 2.2 times. After 7 days of inhalations, the MDA concentration in the plasma of experimental animals decreased by almost half, while remaining 45% higher than in the control group. All other parameters continued to increase. Compared to the day 2, CAT activity increased by 45%, SOD - by 70%. The most pronounced changes were observed in TAC, which increased 3-fold over the same period and exceeded control values 4-fold. All parameters then began to decline. Within a week between days 7 and 14, SOD activity and TAC decreased by half, while MDA levels returned to normal. On day 25 (the last day of inhalation), MDA and SOD levels in the control and experimental groups were not statistically different, while CAT activity and TAC in experimental animals remained 40% and 90% higher, respectively. On day 32 of the experiment, *i.e.* one week after the end of inhalation, all parameters returned to baseline.

Discussion

The primary objective of this study was to obtain a general understanding of the effect of repeated HOCl(g) inhalations on the antioxidant activity of the blood plasma of animals, which is one of the markers of oxidative stress and allows judging its intensity [40]. This is particularly important, since when evaluating the prospects for the biomedical application of HOCl, it is always necessary to take into account the inevitable oxidative processes in the contacting cells and tissues, which are a major component of its biological activity. It is known that *in vitro* HOCl rapidly inhibits numerous enzymes, primarily through the oxidation of thiol fragments of cysteine and methionine [41]. HOCl also oxidizes small antioxidant molecules such as glutathione, ascorbic acid, etc. [42]. Under its influence, phospholipids and low-density lipoproteins are oxidized, forming chlorohydrins and chlorinated lipids, which have pro-inflammatory and cytotoxic properties and are associated with many pathological conditions, in particular, atherosclerosis and ischemia [43,44]. In addition to its own oxidative activity, HOCl can generate conjugated ROS: for example, upon reaction with H_2O_2 or lipid hydroperoxides, singlet oxygen is formed, and during the reaction with superoxide anion, a hydroxyl radical is formed [45,46]. Although all the listed processes are important for the activation of numerous signaling pathways [47], as well as for the neutralization of pathogens, excess HOCl is a powerful factor in oxidative distress and, from this point of view, has rather negative consequences. However, most of the mentioned data were obtained *in vitro* and cannot be fully transferred to a living organism. Thus, studies show that HOCl solutions can be tissue-compatible at concentrations of up to 1300 mg/l [48]. At concentrations of 50–1000 mg/l, HOCl solutions are widely used in medicine for treating skin, mucous membranes, including eyes, wound surfaces, for rinsing cavities during surgeries, etc. [49]. There are also clinical studies in which such solutions were administered intravenously and by inhalations without significant deterioration in the biochemical and hematological indicators in patients [50]. The high resistance of the body to HOCl is due to several factors. Firstly, upon contact with tissues, HOCl reacts rapidly with amines and amides, forming the corresponding N-chloro derivatives, which have a lower redox potential and, accordingly, are less cytotoxic [28]. In addition, the constant formation of HOCl during phagocytosis has led to the appearance of numerous scavengers that neutralize its excess [51]. For example, taurine, being the most abundant non-proteinogenic amino acid, reacts with HOCl to form N-chlorotaurine, a stable and non-toxic compound of great biochemical and pharmacological significance [52,53]. Thus, oxidative stress induced by HOCl(g) inhalation and, accordingly, the "toxicity-pharmacological effect" balance can hardly be predicted and requires experimental *in vivo* determination.

It is obvious that the main factor determining the degree of oxidative stress during inhalation is the air HOCl(g) concentration. Its choice in this work is due to several factors related to the potential practical application of the HOCl(g) for disinfection. Thus, as was shown in work [37], it is the level of 0.3 ppm HOCl(g) at the outlet of this type of evaporator that is most easily achieved and maintained when using approx. 1.0 g/L NaOCl as the working solution. Such solutions, in turn, can be obtained via electrolysis of low-concentration NaCl solutions using accessible electrodes, and this process can be technically organized directly inside the evaporator [34–36]. This would make it possible to create a universal, compact, ergonomic and easy-to-use device combining HOCl solution production and its transfer to the gas phase. Therefore, studying the toxicological properties of air treated in it is important for optimizing its operating parameters. Importantly, we consider the use of HOCl(g) not only as a method for air and surfaces decontamination, but also as a basis for medical technologies, for example, for the treatment of respiratory tract diseases and gas therapy of infected wounds [13,54]. Therefore, the HOCl(g) concentration must be sufficient to exhibit pronounced antimicrobial properties against both airborne microorganisms and those settled on various surfaces, including living tissue. An analysis of the literature has shown that against microbial aerosols, even 0.15 ppm HOCl(g) is quite effective, suppressing *Escherichia coli, S. aureus*, and *P. aeruginosa* at a high rate [25]. However, in the case of bacteria applied to a nutrient medium, the effect manifests more slowly [26], is dose-dependent, and will decrease in the presence of an organic load. It was also necessary to consider that as the gas passes through the respiratory tract, some of the active agent will be inactivated upon contact with mucous membranes, before reaching the lungs, where it could cause the most pronounced systemic effects. Therefore, given previously obtained data on the acute inhalation toxicity of HOCl(g), a concentration of approximately 0.3 ppm was deemed appropriate.

Table 2
Dynamics of changes in rats antioxidant system parameters during repeated inhalations of HOCl(g).

Day	CAT activity, U/ml		SOD activity, U/ml		TAC, μM		MDA concentration, μM	
	Experiment	Control	Experiment	Control	Experiment	Control	Experiment	Control
2	0.30±0.02*	0.25±0.01	76.73±2.45*	54.27±3.34	659±24*	521±30	62.92±5.16*	28.65±1.13
7	0.44±0.04*	0.24±0.04	129.19±12.14*	56.35±4.11	1978±65*	503±23	33.63±4.82*	22.62±2.74
14	0.36±0.03*	0.24±0.02	62.26±7.12*	52.82±3.74	1045±46*	511±26	26.24±1.13	25.14±3.14
25	0.34±0.04*	0.24±0.03	49.26±6.45	52.94±3.23	992±48*	519±34	22.19±2.15	24.39±3.21
32	0.25±0.02	0.25±0.01	55.85±4.46	53.71±3.86	571±21	560±31	24.39±3.11	21.36±2.23

* $p < 0.05$ compared to the control group at the same time point (Mann-Whitney *U* test).

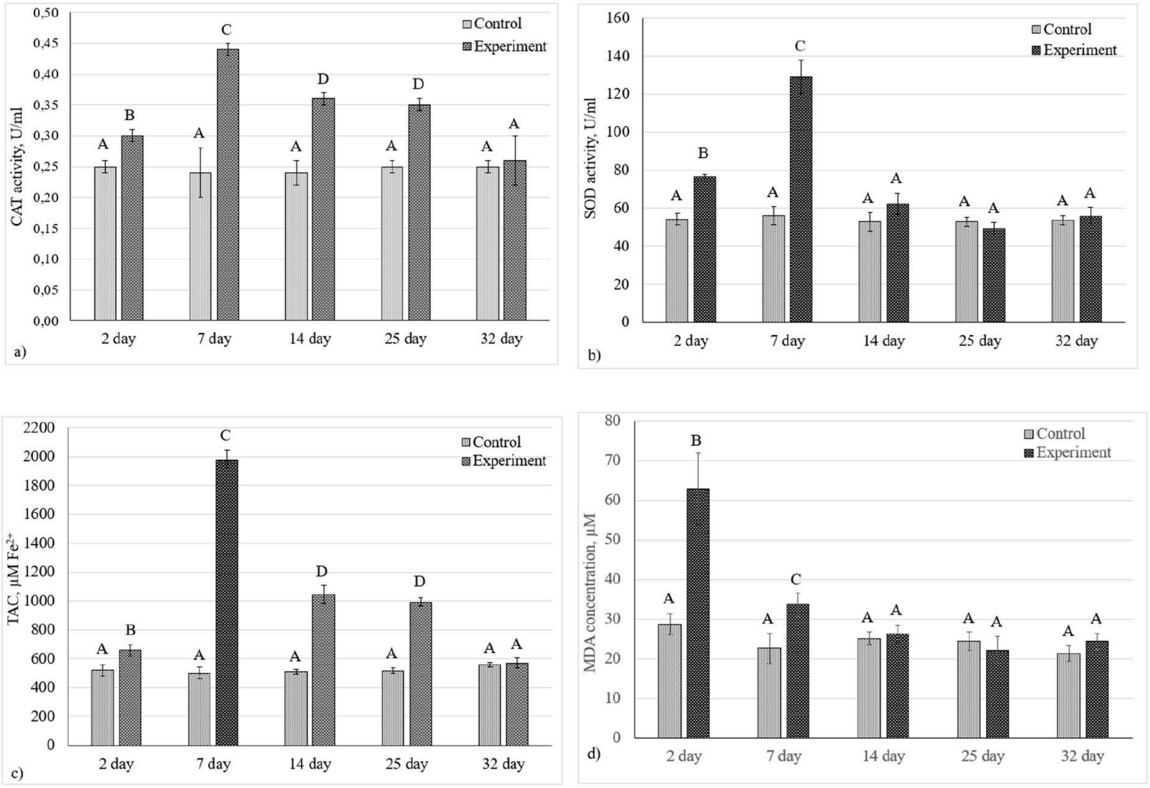


Fig. 1. Effect of repeated inhalations of HOCl(g) on the studied biomarkers in the rat blood plasma: a) catalase activity, b) superoxide dismutase activity, c) total antioxidant capacity, d) malondialdehyde concentration. Bars with different letters are significantly different ($p < 0.05$, Mann-Whitney *U test*).

Changes in antioxidant properties of rat blood plasma during repeated inhalations of HOCl(g)

Oxidative distress, that is, an imbalance between the generation of free radicals and their neutralization by antioxidants, is one of the prominent risks of introducing additional oxidants, including HOCl, into the body [55]. It can contribute to the development of many pathologies (cancer, neurodegenerative and cardiovascular diseases, etc.), and *vice versa*, many processes, such as chronic inflammation, are accompanied by oxidative distress. At the same time, temporary mild-to-moderate oxidative stress, which occurs, for example, during physical training, acts as a signaling mechanism for hormesis, boosting antioxidant defenses and improving long-term resilience [56]. A convenient approach for assessing the degree of oxidative stress is to measure the antioxidant properties of cells and tissues, which are determined by the activity of antioxidant defense enzymes and the concentration of non-enzymatic antioxidants, and most quickly provide a response to changes in redox homeostasis of the system.

Catalase is one of the main antioxidant enzymes, catalyzing the decomposition of H_2O_2 to oxygen and water [57]. It acts primarily at the subcellular level, being localized in peroxisomes of eukaryotic cells. However, it is also present in blood plasma, often used as a marker of oxidative distress. Numerous *in vitro* studies have shown that HOCl inhibits CAT via heme damage, the formation of protein radicals and carbonyls, with subsequent aggregation [58]. There are no *in vivo* studies in this area. At the same time, it is known that there are multiple mechanisms regulating CAT expression both at the transcriptional and post-translational levels, and its activity can increase to compensate for the increase in ROS concentration under conditions of mild oxidative stress [59]. However, in the case of a severe or prolonged increase in ROS, for example, in COPD, cancer, or smoking, CAT activity decreases [60,61]. The results obtained (Table 2) indicate that HOCl(g) inhalation rapidly, within 24 h, causes an increase in CAT activity in animals. These data are consistent with our previous study [33] and confirm the pronounced dose-dependent nature of this effect: with a single inhalation of approximately 0.75 ppm HOCl(g) in animals of the same age, CAT activity in their plasma doubled within 24 h, and in the current experiment, at a concentration of 0.3 ppm, the increase was 20%. Elevated CAT levels were observed throughout the entire inhalation period, reaching maximum values after 7 days and then demonstrating a downward trend. The absence of CAT inhibition indirectly indicates that repeated inhalations do not lead to plasma supersaturation with various ROS, which could chemically inhibit the enzyme. The rapid return of CAT to normal levels after cessation of inhalation can also be considered positive. Interestingly, in our previous study with aerosolized NaOCl solution [39], we did not observe a significant increase in plasma CAT activity despite a 3-fold higher total chlorine content in the air. This discrepancy may be explained by the lower systemic bioavailability of HOCl delivered via aerosol compared to the gaseous form. Apparently, unlike HOCl(g) molecules, which reaches the alveoli, aerosol particles are predominantly deposited in the upper respiratory tract and are less efficiently absorbed. Thus, only inhalation of HOCl(g) provides a sufficient concentration to induce a systemic antioxidant response, highlighting the importance of delivery route selection in the development of HOCl(g)-based strategies.

Superoxide dismutase is the second key enzyme in antioxidant defense, converting highly reactive superoxide into less active oxygen and hydrogen peroxide [62]. Unlike catalase, the concentration of this enzyme in normal plasma is high, and in many pathologies, its increase is observed as one of the compensatory mechanisms for combating oxidative damage and inflammation [63,64]. In our study, this indicator was of particular interest, since SOD is known to be much less sensitive to the action of HOCl than CAT [65]. Accordingly, by observing the dynamics of its activity, it is possible to more accurately assess the actual level of its hyperexpression and, consequently, the degree of oxidative stress provoked by inhalations, since direct inhibition by HOCl is less

likely in this case. As seen from Table 2, SOD activity increased already the next day after the first inhalation, and its growth was more pronounced than that of CAT: 41% *versus* 20%, respectively. After 7 days of inhalation, SOD activity, as with CAT, reached its maximum level, exceeding the control group's levels by 2.3 times, which was also more significant than for CAT, where the excess was 1.8 times. In the following days, SOD levels declined, but this was much more significant than for CAT: on the 25th day, the activity of this enzyme decreased by 2.6 times and was statistically indistinguishable from the control group, unlike CAT, which remained elevated until the end of the experiment. However, this decline is unlikely to be explained by the direct effect of HOCl(g), given the greater resistance of SOD to oxidation, the unchanged HOCl(g) concentration in the chamber, and the persistently elevated CAT level, which would primarily be inhibited by excess active chlorine in the plasma.

The total antioxidant capacity of plasma is a simple and at the same time informative marker of its ability to resist oxidative stress [66]. The FRAP assay used by us in this study evaluates the level of non-enzymatic antioxidants, both endogenous (bilirubin, albumin, uric acid, etc.) and exogenous (ascorbic acid, tocopherol, carotenoids, etc.) [67]. Measuring TAC by this method in this study was important, since the body does not have enzymes directly acting to neutralize hypochlorites, so it could be assumed that small chain-breaking substances would quickly react with HOCl with an increase in its concentration in plasma. Changes in TAC are observed in many pathologies [66,68]. In general, in chronic or severe inflammation of various etiologies, TAC decreases due to the excess of antioxidant consumption for the neutralization of ROS and other radicals over their biosynthesis/intake. However, in the early stages of inflammation or with mild oxidative stress, an increase in TAC is often observed as part of the body's adaptive response. As seen from Table 2, the dynamics of TAC changes mirror those of antioxidant enzymes: beginning to rise from the first day of inhalation, it reaches a maximum by day 7, and then begins to decline. At its peak, its value exceeds the control group value by a much greater margin, reaching 4-fold. By day 25 of the experiment, when SOD activity has already returned to normal and CAT exceeds normal levels by 40%, TAC remained elevated by almost 2-fold. Since all animals were fed the same diet, these results cannot be due to different amounts of exogenous antioxidants consumed. Presumably, the sharp increase in antioxidant enzyme activity allows for the utilization of a greater amount of ROS, which protects non-enzymatic scavengers from oxidation. Another possible explanation is the compensatory increase in the biosynthesis of these scavengers in the liver.

In general, all studied antioxidant components exhibited similar dynamics: a rapid increase immediately after the first inhalation, peaking on day 7, and subsequently declining. This pattern reflects an enhanced plasma capacity to counteract ROS and is consistent with hormesis - a biphasic dose-response phenomenon where low-dose stimulation triggers beneficial adaptation, whereas high doses cause inhibition or toxicity [69]. Hormesis is a well-established biological principle, observed not only in response to chemical stressors but also to physical stimuli such as exercise, which induces a transient oxidative stress followed by upregulation of antioxidant defenses and improved resilience [70]. The molecular basis for the hormetic response apparently observed in our study is strongly supported by the works of Pi et al. They demonstrated that HOCl exposure increases nuclear Nrf2 protein levels in murine macrophages and upregulates the expression of CAT, SOD, and heme oxygenase-1 [71]. An initial decrease in intracellular glutathione within the first hour of HOCl exposure, followed by its marked rebound that exceeded baseline levels by up to 4-fold was also shown. Genome-wide profiling of HOCl-treated macrophages identified that the Nrf2-mediated antioxidant response is the most sensitive, activated already at concentrations within 0.15–0.35 mM [72]. Moderate (0.35–1.4 mM) HOCl concentrations caused maximal activation of the Nrf2 pathway, whereas higher (2.8–3.5 mM) concentrations, oppositely, led to a suppression of Nrf2-target genes and activation of inflammatory and stress-related pathways. The authors proposed that such phenomenon arises from the interplay between adaptive stress responses and the energetic costs of maintaining homeostasis [73]. According to their model, mild oxidative stress activates Nrf2-mediated adaptive pathways that require significant energy expenditure for the synthesis of protective enzymes, leading to non-monotonic dose-response curves for certain endpoints. They described discrete cellular states - normal, adaptive, stressed, and toxic - as a function of exposure concentration, with the adaptive state being characterized by reversible upregulation of cytoprotective genes and restoration of homeostasis. While all these insights were derived from *in vitro* models, our study appears to be the first to confirm the hormetic response to HOCl *in vivo* at the systemic level. Together, these data suggest that mild, repeated HOCl(g) exposure may precondition the organism against subsequent oxidative challenges, offering a potential therapeutic strategy for inflammatory conditions of various etiologies. Definitive confirmation of this connection would require analysis of Nrf2 protein levels in the blood and tissues of HOCl (g)-treated animals, which will be the subject of our further investigations.

Another important aspect concerning HOCl metabolism is the complex interaction between CAT and SOD with myeloperoxidase (MPO), an enzyme of neutrophils that synthesizes HOCl from H_2O_2 and chloride anion [74]. Both antioxidant enzymes are MPO antagonists. Thus, CAT competes with MPO for one substrate - H_2O_2, thus regulating the amount of HOCl formed and protecting the cell and MPO itself from its excess [75]. At the same time, SOD scavenges superoxide, which is necessary to maintain MPO activity by converting it into oxymyeloperoxidase [76]. So, an increase in the activity of CAT and SOD probably reduces the synthesis of endogenous HOCl, the overproduction of which is a serious problem in chronic inflammation, associated with atherosclerosis, arthritis, neurological diseases, etc. [28].

Changes in malondialdehyde levels during HOCl(g) inhalations

Malondialdehyde level is an indicative marker of oxidative stress and inflammation [77]. This compound is formed directly as an end product of the ROS interaction with unsaturated fatty acids and is also one of the metabolites in the synthesis of prostaglandins. Being a highly reactive compound, it has high toxicity, including due to the ability to form adducts with DNA and proteins, and exhibits mutagenic, atherogenic, and carcinogenic properties. Elevated MDA levels are strongly linked to respiratory tract disorders, including chronic obstructive pulmonary disease and pneumonia of various origins [78]. In addition to intrinsic toxicity, MDA is positively correlated with increased expression of Toll-like receptor 4 (TLR4) and factor nuclear kappa B (NF-κB)-signaling pathways involved in lung diseases [79]. There are also studies indicating that it acts as a messenger that activates the expression of IL-6 and other inflammatory cytokines, which also has rather negative clinical consequences [80]. Therefore, this indicator is especially important in studying the inhalation toxicity of chemicals. Our data indicate that the first inhalation contact with HOCl(g) leads to intense lipid peroxidation, which is expressed in a noticeable, more than 2-fold, increase in the plasma MDA level. Such an increase can be regarded as evidence of an oxidative stress and/or inflammatory process. Nevertheless, by the 7th day of inhalation, the MDA concentration decreases significantly, which correlates with the achievement of maximum activity of CAT and SOD. By the 14th day and until the end of the experiment, MDA in the plasma of experimental and control animals do not differ statistically. Thus, stimulation of the antioxidant system allows for a fairly rapid neutralization of the undesirable process of lipid oxidation during HOCl(g) inhalation. At the same time, the MDA jump observed at the first stage dictates the need to revise the applied HOCl(g) concentrations towards a decrease.

In summary, it can be concluded that the first stage of inhalation of 0.32±0.03 ppm HOCl(g) is accompanied by oxidative stress with pronounced lipid peroxidation, as evidenced by an increase in MDA

concentrations in the blood plasma of experimental animals. However, the absence of inhibition of plasma CAT and SOD suggests that antioxidant defenses are not overcome, permitting this stress to be classified as mild or moderate. Consequently, an adaptive hormetic response is triggered, manifested by pronounced upregulation in both enzymatic and non-enzymatic antioxidant components in plasma, which persists throughout the experiment. The cause of the trend toward a decrease in plasma antioxidant activity after reaching its peak on the 7th day of inhalation at a constant HOCl(g) concentration remains unclear. This is likely due to increased synthesis of low-molecular antioxidants in liver, the expression of antioxidant enzymes not studied in this work, or the inhibition of MPO. In any case, the consistently normal MDA levels from day 14 throughout the remainder of the experiment, together with the absence of any decline in CAT, SOD, and TAC below control values, strongly suggest that the observed trend reflects homeostatic rebalancing rather than depletion of antioxidant reserves.

Thus, HOCl(g) inhalation can be considered a way to stimulate the antioxidant system while simultaneously providing an antimicrobial effect, making HOCl(g)-based disinfection systems particularly promising for use in healthcare facilities. However, further research is needed to determine the optimal air HOCl(g) concentration.

Being the first *in vivo* study in the area, this work has several limitations. The lack of estrogen cycle monitoring is the first of them. But, its potential confounding effect is mitigated by the presence of a parallel control group of female rats subjected to identical conditions, sufficient sample size (n = 6 per time point) and the longitudinal design with repeated measurements over 25 days, altogether allowing us to attribute the observed differences specifically to HOCl(g) exposure rather than to cycle-related fluctuations. The dynamics of changes in selected biochemical parameters in plasma may differ significantly from processes occurring in blood cells, particularly erythrocytes, where many antioxidant enzymes are localized. Complementary studies are needed for a more comprehensive assessment of oxidative stress. However, plasma parameters provide a reliable indication of oxidative stress degree and are more convenient for rapid analysis. Another drawback is the lack of data on changes in the activity of glutathione peroxidase, which is the third first-line antioxidant enzyme along with CAT and SOD, and MPO, which is responsible for the biosynthesis of endogenous HOCl. This is due to the insufficient amount of clinical material obtained from a single animal to determine all parameters, and the limited volume of the inhalation chamber, which does not allow for the proper accommodation of a larger number of animals. Valuable insights could be obtained by studying these biomarkers in lung tissue as a target organ, especially in conjunction with data from their histology and immunohistochemistry. These aspects will be addressed in our future investigations.

Conclusions

Repeated inhalations of 0.32±0.03 ppm HOCl(g) rapidly, within the first day, induced significant changes in the antioxidant properties of rat blood plasma, manifested by increased activity of CAT, SOD, and TAC. A sharp increase in MDA concentrations was also observed initially, indicating pronounced lipid peroxidation due to interaction with HOCl itself and associated ROS. The activity of enzymes and low-molecular HOCl scavengers peaked within 7 days, and this compensatory mechanism returned MDA levels to normal by day 14. After day 7 of inhalation, a gradual decrease in plasma antioxidant activity was observed, likely due to an increase in intraerythrocytic antioxidant enzyme concentrations and a suppression of endogenous HOCl production. One week after the end of inhalation exposure, all studied parameters in the experimental animals were no different from those in the control group. The absence of CAT and SOD inhibition in plasma and the reversibility of all observed processes provide indirect evidence that inhalation-induced oxidative stress is mild-to-moderate. Moreover, the observed changes can be considered as an example of hormetic response, so such a procedure, along with the antimicrobial effect, potentially can be used to stimulate the antioxidant system, which is beneficial in anti-inflammatory therapeutic strategies. However, additional studies are needed to more fully define the toxicological profile of HOCl(g) and determine the optimal concentration for its use in the prevention and treatment of infectious diseases.

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CRediT authorship contribution statement

Bohdan Murashevych: Writing – review & editing, Writing – original draft, Project administration, Methodology, Investigation, Funding acquisition, Conceptualization. **Olha Abraimova:** Visualization, Methodology, Investigation, Formal analysis, Data curation. **Olha Netronina:** Methodology, Investigation, Data curation. **Dmitry Girenko:** Supervision, Methodology, Conceptualization. **Tetiana Herhel:** Visualization, Data curation. **Hanna Maslak:** Writing – original draft, Supervision, Methodology, Conceptualization.

Declaration of competing interest

none.

Data availability

Data will be made available on request.

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