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The impact of hydrogen inhalation therapy on blood reactive oxygen species levels: A randomized controlled study

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ABSTRACT

Reactive Oxygen Species (ROS) play a key role in physiological processes. However, the imbalance between ROS and antioxidants in favor of the former causes oxidative stress linked to numerous pathologies. Due to its unique attributes, including distinguished permeability and selective antioxidant capability, molecular hydrogen (H_2) has become an essential therapeutic agent. Hydrogen Inhalation Therapy (HIT) has come to light as a promising strategy to counteract oxidative stress.

In this randomized controlled study, we aimed to evaluate the effectiveness of HIT in reducing blood ROS levels. 37 participants with elevated ROS levels (d-ROMs value > 350 U.CARR) were enrolled in the study. Participants were divided into test and control groups. The test group participants received HIT, and then their blood ROS levels were measured immediately post-treatment and after 24 h. Their results were compared to those of the control group participants who did not undergo HIT. The test group demonstrated a significant reduction in blood ROS levels after the treatment. These findings suggested the efficacy of HIT in reducing oxidative stress.

1. Introduction

Hydrogen, the most abundant element in the universe [1,2], has garnered significant attention as an appealing option for therapeutic purposes due to its distinct characteristics [3]. As the smallest and lightest molecule [4], hydrogen (H_2) demonstrates remarkable permeability, effortlessly penetrating cell membranes to access subcellular compartments such as the mitochondria and nucleus [5]. Its high diffusivity enables the exertion of therapeutic effects throughout various tissues and organs [6].

Reactive oxygen species (ROS) are highly reactive oxygen-containing compounds formed as natural byproducts of cellular metabolism. ROS include molecules such as superoxide anion radical ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($^{\bullet}OH$) [7,8].

ROS play a pivotal role in many biological processes [9,10]. Our body tries to counteract ROS by consuming antioxidants, aiming to maintain only the physiologically required amounts of ROS [10]. In oxidative stress conditions, an imbalance between ROS and antioxidants occurs, which is considered the root cause of many pathologies, including asthma, cardiovascular disease, inflammation, and certain mental health disorders [8,11]. Prolonged oxidative stress can induce cell and DNA damage, potentially leading to organ dysfunction and disease progression, and ultimately resulting in cell death [12]. The derivatives of reactive oxygen metabolites (d-ROMs) test is increasingly recognized as the preferred method for assessing oxidative stress markers [13], particularly plasma organic peroxides.

Hydrogen is considered as a selective antioxidant, capable of scavenging harmful ROS [14,15]. Hydrogen could be administered by several routes, such as hydrogen gas inhalation, oral intake by drinking hydrogen-rich water, intravenous injection of hydrogen-rich saline, hydrogen water bath, or hydrogen ophthalmic drops [16–18]. Among the previous administration routes, inhalation was found to be an effective and rapid method, particularly against acute oxidative stress [17–19]. Therefore, Hydrogen Inhalation Therapy (HIT) is quickly becoming a novel and effective treatment to counter the effects of oxidative stress [16,18,19]. The impact of hydrogen gas can continue

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longer after gas has been removed from the system [17]. There is no known enzyme that effectively neutralizes $^{\bullet}OH$. Consequently, $^{\bullet}OH$ reacts non-selectively with biological molecules [9]. H_2 acts as a reductant that can penetrate the cell membrane and neutralize harmful ROS that damage the body such as $^{\bullet}OH$ and peroxynitrite ($ONOO^-$) within the cellular structure, while it has almost no effects on $O_2^{\bullet-}$ and H_2O_2, thereby maintaining their physiological functions and internal environment stability [20]. A literature review revealed the potential of hydrogen therapy in overcoming oxidative stress [21,22]. Additionally, the effects of HIT have been demonstrated in many disease models, including neurological disorders [23,24], ocular diseases [25], pulmonary diseases [26,27], and cancer [28]. However, these studies focused on assessing the impact of HIT on various parameters other than blood ROS levels.

Therefore, the aim of this study was to evaluate the effect of HIT on blood ROS levels. Participants underwent screening to determine their ROS levels, with only those who exhibited high levels of ROS (d-ROMs value greater than 350 U.CARR) being included in the study.

2. Materials and methods

2.1. Participants and study design

In this randomized controlled study, a total of 67 candidates were assessed to determine their ROS levels. Out of these candidates, 30 individuals with d-ROMs values below (350 U.CARR) were excluded from the study. Consequently, the remaining 37 participants with d-ROMs values exceeding (350 U.CARR) were included in the study and randomly assigned to two groups: 18 participants formed the study group (H_2 group), while 19 participants comprised the control group.

The study wasn't executed under full blinding protocols due to the absence of a placebo device. Nevertheless, data gathering and analysis were conducted under blinded conditions. Group names of participants were withheld from laboratory technicians and additional staff involved in the process to preserve blinding throughout these procedures.

The study was conducted in accordance with the principles of the Declaration of Helsinki and received ethical approval from Cyprus International University Ethics Review Board (Approval Number: EKK23-24/008/11).

2.2. Hydrogen inhalation treatment

Participants assigned to the H_2 group underwent a 1-h session of hydrogen inhalation treatment using the Molecular Hydrogen Inhalation Device H-2000 (manufactured by Hue Light Co. Ltd., South Korea). According to the manufacturer's specifications, this device produces 1447 cc of molecular Hydrogen and 723 cc of Oxygen per minute, with a purity exceeding 99.99 % and a pressure of 1 bar. The device operates via a polyelectrolyte system, utilizing a Proton Exchange Membrane (PEM) to separate the sterile distilled water and generate hydrogen. Inhalation was conducted using a new sterile nasal cannula for each participant.

Conversely, the control group did not undergo any hydrogen inhalation treatment.

2.3. ROS levels assessment

Participants in the H_2 group had their ROS levels measured at three time points: before the 1-h hydrogen inhalation session, immediately after the 1-h hydrogen inhalation session, and 24 h after the hydrogen inhalation session. Control group participants, who did not undergo hydrogen inhalation treatment, were similarly assessed for ROS levels initially, after 1 h, and after 24 h.

ROS levels were assessed using the FRAS5 – Free Radical Analytical System and d-ROMs kit (H&D srl., Italy), following the manufacturer's instructions. Approximately 200 μL blood samples were collected via finger pricks, and plasma peroxide levels were measured.

The d-ROMs test operates based on Fenton's reaction [29], which involves a two-step process. Initially, hydroperoxides (ROOH) present in the plasma sample react with iron ions (Fe^{2+}, Fe^{3+}) present in the reaction medium within an acidic buffer, generating alkoxyl ($RO^{\bullet}$) and peroxyl ($ROO^{\bullet}$) radicals [30]. Subsequently, these radicals oxidize the alkyl-substituted aromatic amine present in the reaction medium, resulting in the formation of the N,N-diethyl-paraphenyldiamine radical cation [29,30], which is characterized by a pink coloration. The absorbance of this radical cation is measured at 505 nm [31], and it is directly proportional to the concentration of ROMs in the sample.

The d-ROMs result is expressed in Carratelli Units (U.CARR), where 1 U.CARR corresponds to 0.08 mg of H_2O_2/100 mL [32]. The d-ROMs test is recognized for its simplicity, rapidity, cost-effectiveness, practicality, and ease of setup [29,31,33,34].

2.4. Statistical analysis

All statistical analyses were performed using R software (version 4.3.1), an open-source environment for statistical computing and graphics. This software is developed by the R Foundation for Statistical Computing.

3. Results

3.1. Participants

The average age of the participants in the H_2 group was 33 years, with an age range from 25 to 45 years. In contrast, the average age of individuals in the control group was 35.8 years, ranging from 22 to 48 years. These differences were not statistically significant as the independent *t*-test yielded a p-value of 0.247 which exceeds the 0.05 threshold. The H_2 group comprised 14 males and 4 females, while the control group included 9 males and 10 females. These sex variations are not expected to affect the d-ROMs results [29].

3.2. Results of the d-ROMs test

The results of the d-ROMs test for the H_2 group and control group are presented in Figs. 1 and 2, respectively. The data clearly demonstrate a significant decrease in d-ROMs values among participants in the H_2 group immediately after the 1-h hydrogen inhalation session (T1) and 24 h after the session (T24), compared to measurements taken immediately before the inhalation session (T0). In contrast, no significant changes in d-ROMs values were observed in the control group after 1 h or 24 h.

Fig. 3 illustrates the percentage reduction of d-ROMs for the H_2 group participants, immediately after the 1-h hydrogen inhalation session (R1) and 24 h after treatment (R24), compared to baseline d-ROMs levels measured just before the start of hydrogen inhalation treatment. The average reduction recorded after the 1-h hydrogen inhalation session was 15.0 %, ranging from −8.8 % to 39.7 %, whereas the average reduction recorded after 24 h was 23.3 %, ranging from 4.5 % to 43.8 %. The observed reduction after 24 h indicates that hydrogen may exert a prolonged effect on reducing ROS levels in the participants, even after the cessation of gas exposure.

3.3. Statistical analysis

Fig. 4 illustrates the interactive boxplot of d-ROMs values for the participants in the H_2 group before and after hydrogen inhalation treatment at T0, T1, and T24 time points.

The paired sample *t*-test was carried out to compare d-ROMs values for the participants in the H_2 group at (T0 vs T1) and (T0 vs T24) and the results were (t-statistic: 3.79, p-value: 0.001458) and (t-statistic: 6.49, p-value: 0.000006), respectively. Both p-values were well below the 0.05

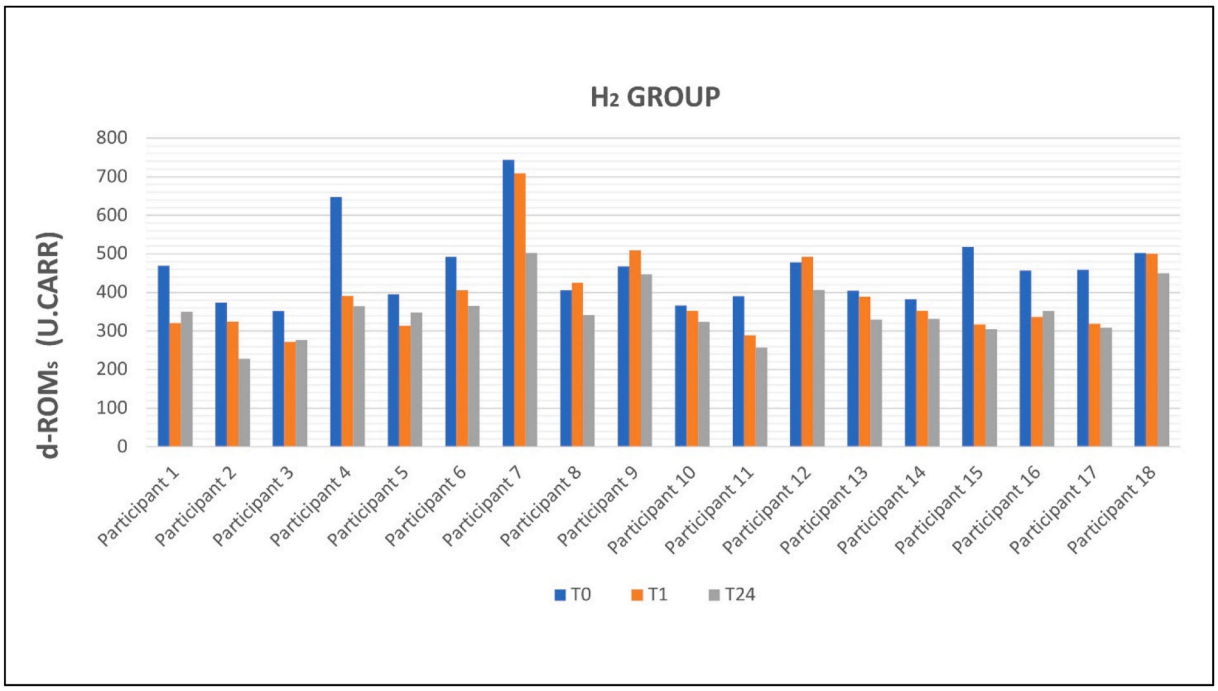

Fig. 1. The results of the d-ROMs test for the H_2 group before and after HIT.

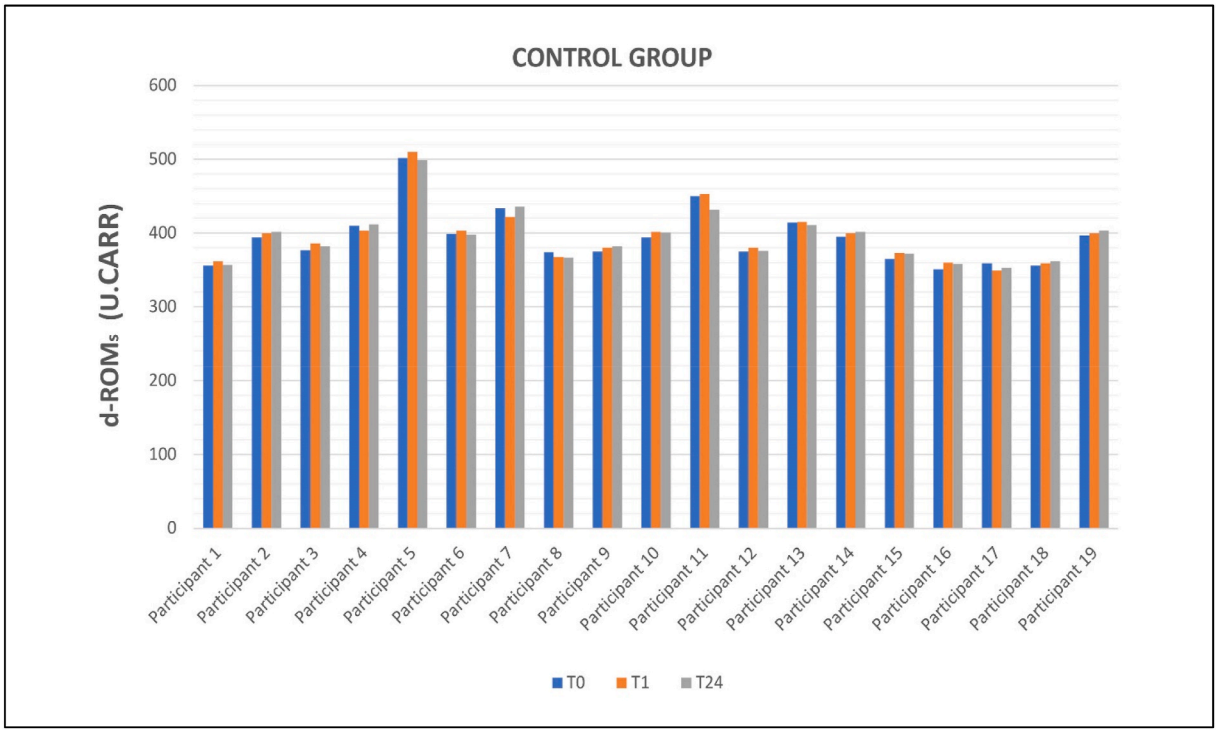

Fig. 2. The results of the d-ROMs test for the control group.

threshold. These findings demonstrate statistically significant reductions in ROS levels after hydrogen inhalation treatment, with the reduction being more pronounced at the 24-h point compared to the 1-h point. Thus, it suggests that hydrogen inhalation treatment may exert a significant and prolonged effect on reducing ROS levels in H_2 participants.

The ANOVA tests were conducted to compare the d-ROMs values between the H_2 group and the control group at each time point ($T0_{H2}$ vs $T0_{Control}$), ($T1_{H2}$ vs $T1_{Control}$), and ($T24_{H2}$ vs $T24_{Control}$) and the results were (F-statistic = 7.61, p-value = 0.0092), (F-statistic = 0.05, p-value = 0.8196), and (F-statistic = 6.57, p-value = 0.0149), respectively. These findings indicate a statistically significant difference in d-ROMs values between the H_2 group and the control group before H_2 treatment and 24 h post-treatment. However, no significant difference was observed after 1 h of H_2 treatment. This suggests that the impact of H_2 treatment on ROS levels becomes significant after 24 h.

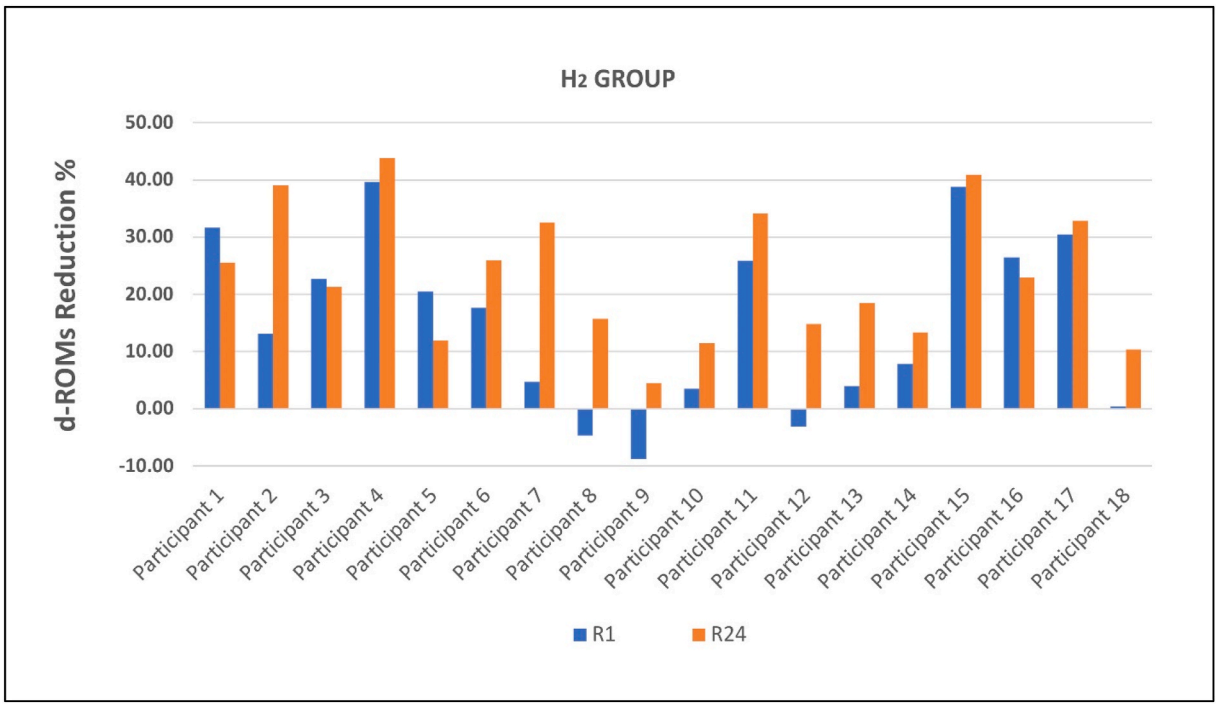

Fig. 3. The percentage reduction of d-ROMs in the H_2 group.

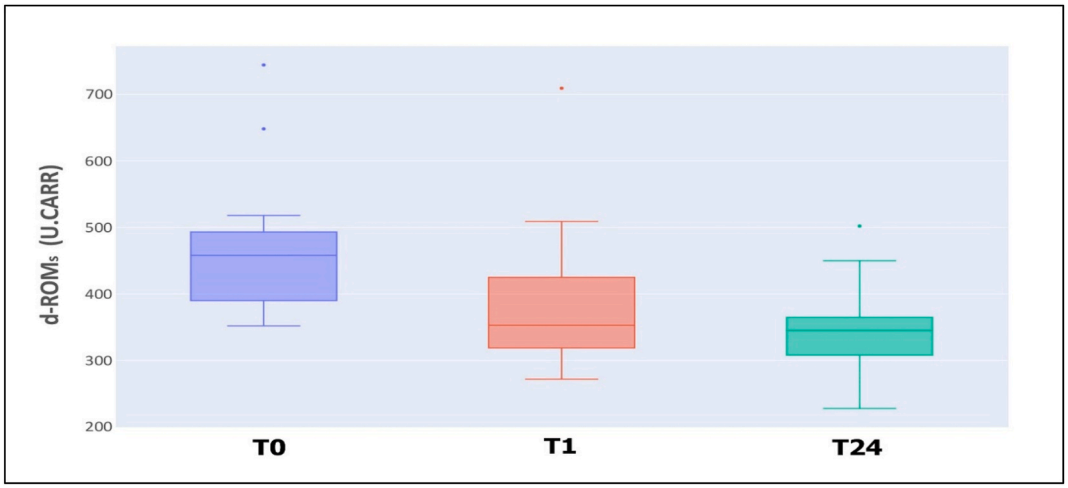

Fig. 4. The interactive boxplot of d-ROMs values for the participants in the H_2 group.

4. Discussion

In this study, we demonstrated the efficacy of H_2 in lowering blood ROS levels. The mechanism by which H_2 reduces ROS involves multiple processes. H_2 can permeate the cell membrane and reach the cytosol, nucleus, and mitochondria through gaseous diffusion, neutralizing harmful ROS such as $^{\bullet}OH$ and $ONOO^{-}$ within cellular structures [35–37]. H_2 acts as an electron donor for harmful ROS molecules. For example, it scavenges $^{\bullet}OH$ according to the following chemical reactions [4]:

$$H_2 + {}^{\bullet}OH \rightarrow H_2O + {}^{\bullet}H$$

$$^{\bullet}H + O_2^{-} \rightarrow HO_2^{-}$$

The reaction rate between H_2 and $^{\bullet}OH$ is significantly low due to the high activation energy [38]. Despite this, Jin et al. noted that the Fe-porphyrin in hemoglobin catalyzes H_2-related reactions in the body [38]. It has also been suggested that H_2 selectively scavenges $^{\bullet}OH$ rather than other ROS, attributed to the smaller size of $^{\bullet}OH$ compared to other ROS. The smaller size facilitates both $^{\bullet}OH$ and H_2 in penetrating the porous channels of hemoglobin proteins and reacting with Fe-porphyrin therein [38]. On the other hand, Liu et al. proposed that H_2 selectively interacts with the most potent oxidants, such as $^{\bullet}OH$ and $ONOO^{-}$, owing to their heightened reactivity compared to other ROS [35].

Our study demonstrated that the effect of H_2 on ROS levels became significant 24 h after gas exposure cessation. This sustained effect was also observed with the administration of high H_2 water, where the influence of H_2 on oxidative stress persisted even during the washout period when patients did not drink high H_2 water for 4 weeks [36,39]. Moreover, Sano et al. reported that H_2 could still be detected in venous blood 1 h after a single inhalation of H_2 in pigs [40]. The extended presence of H_2 in the system following gas exposure cessation could lead to more pronounced cumulative effects at 24 h compared to earlier time

point.

Some limitations affected this study. The number of participants was limited to 37, and extending the study to a larger sample size would provide more comprehensive data. Another constraint was the absence of a placebo device; while an air respiration device would have been ideal, a similar device with the same shape as the hydrogen inhalation device was not accessible. Additionally, the number of hydrogen inhalation sessions was relatively small due to cost constraints. Despite these limitations, the study still achieved statistically significant results. However, future research should consider conducting studies with a larger participant pool, integrating a placebo device, and increasing the number of inhalation sessions.

5. Conclusion

This randomized controlled study showed that HIT was effective in mitigating ROS levels among H_2 group participants. Results demonstrated a significant decrease in ROS levels 24 hours after completing the treatment. These findings suggest that HIT would be a promising therapeutic choice for counteracting oxidative stress and the pathological conditions linked to it.

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Disclosure of using AI-assisted technologies

During the preparation of this work the authors used ChatGPT version 3.5 solely for identifying and correcting language mistakes to improve language and readability. After using this tool, the authors reviewed and edited the content as needed and take full responsibility for the content of the publication.

CRediT authorship contribution statement

Mohamed Chair: Investigation. **Hashem AlAani:** Writing – review & editing, Writing – original draft, Visualization. **Sevda Lafci Fahrioglu:** Supervision. **Cherif Ben Hamda:** Data curation. **Umut Fahrioglu:** Writing – review & editing, Methodology. **Tamer Degheidy:** Supervision, Methodology, Conceptualization.

Declaration of competing interest

The authors declare that they do not possess any identifiable conflicting financial interests or personal relationships that may have seemed to impact the work reported in this paper.

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