HYDROGEN-RICH PBS PROTECTS CULTURED HUMAN CELLS FROM IONIZING RADIATION-INDUCED CELLULAR DAMAGE

by

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Hydroxyl radicals play an important role in ionizing radiation-induced cellular damage, while hydrogen can selectively reduce hydroxyl radicals *in vitro*. This study was designed to test the hypothesis that hydrogen-rich PBS may be an effective radioprotective agent *in vitro*. Compared to cells pretreated without hydrogen, we demonstrated that treating cells with hydrogen-rich PBS before irradiation could significantly inhibit IR-induced apoptosis, increase viability of human intestinal crypt cells, significantly increase endogenous antioxidant, and decrease malondialdehyde and 8-hydroxydeoxyguanosine concentrations of human lymphocyte AHH-1 cells. It is concluded that hydrogen has a potential as an effective and safe radioprotective agent.

Key words: hydrogen, ionizing radiation, radioprotection, apoptosis, reactive oxygen species

INTRODUCTION

Hydrogen is the most abundant chemical element. It is a colorless, odorless, non-metallic, tasteless, highly flammable diatomic gas which was considered as a physiological inert gas. Hydrogen is seldom regarded as an important agent in medical usage. Howerver, Ohsawa *et al*. [1] found that molecular hydrogen could selectively reduce cytotoxic reactive oxygen species, such as OH and ONOO– *in vitro* and exert therapeutic antioxidant activity in a rat middle cerebral artery occlusion model.

Reactive oxygen species (ROS) or reactive nitrogen species (RNS), such as hydroxyl radical (OH), superoxide anion (O_2-), hydrogen dioxide (H_2O_2), nitric oxide (NO), peroxynitrite (ONOO-), appear to play a critical role in cerebral, myocardial and hepatic ischemia-reperfusion injuries, transplantation injuries, and other injuries [2-4]. It has also been demonstrated that H_2 is effective in the prevention of these injuries. However, the potential effect of hydrogen gas on another damage type in which free radicals play an important role is largely ignored. That type is the damage induced by irradiation.

Approximately 65% of the DNA damage is caused by the indirect effect of free radicals, such as hydroxyl radicals (OH), that are formed from the radiolysis of surrounding water molecules and that successively attack DNA [5]. Lipid peroxidation (LPO) is also considered as a critical event during ionizing radiation induced damage [6]. Apart from genetic damage and lipid peroxidation, ROS can also alter the balance of endogenous protective systems such as glutathione and enzymic antioxidant defence systems [7] . The endogenous antioxidant defences are inadequate to reduce the radiation-induced free radical changes. Appropriate antioxidant intervention seems to inhibit or reduce free radical toxicity and thus offers protection against radiation.

Therefore, we reasoned that hydrogen might be protective against detrimental effects of radiation. However, the application of H_2 gas inhalation is not convenient and may be dangerous because the gas is inflammable and explosive. On the other hand, H_2 gas saturated PBS, which is called hydrogen-rich PBS, is easy to apply and safe. In the current study, we demonstrated that hydrogen-rich PBS treatment can protect human cells from γ-radiation *in vitro*.

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MATERIALS AND METHODS

Preparation of hydrogen-rich PBS

Hydrogen was being dissolved in PBS for 6 hours under high pressure (0.4 MPa) in order to reach a supersaturated level by using hydrogen-rich water-producing apparatus which was produced by our department. The saturated hydrogen PBS was stored under the atmospheric pressure at 4 °C in an aluminum bag with no dead volume. Hydrogen-rich PBS was freshly prepared every week, which ensured that a concentration of more than 0.6 mmol/L was maintained. Gas chromatography was used to confirm the content of hydrogen in PBS by the method described by Ohsawa, *et al.* [1].

Hydrogen treatment of cultured cells

Human lymphocyte AHH-1 cells and intestinal crypt HIEC cells were maintained in RPMI 1640 (Invitrogen) with 10% fetal bovine serum and 1% penicillin-streptomycin-glutamine at 37 °C in a 5% CO_2 humidified chamber. For radioprotective studies, cells were treated with different volume of hydrogen-rich PBS and accordingly we added different volume of PBS in order to obtain the desired concentration of H_2 and make the final volume of the medium the same. Then, the treated cells were immediately irradiated with different doses of γ-ray, depending on the requirement of the present study.

Irradiation

Cobalt-60 gamma rays in irradiation center (Faculty of Naval Medicine, Second Military Medical University, China) were used for the irradiation purpose. Cells (with or without hydrogen pre-treatment) were exposed to different doses of radiation, depending on the requirement of the present study.

Clonogenic survival

Colony-forming assay was performed as previously described [8]. Briefly, calculated numbers of cells were plated to enable normalization for plating efficiencies. Pretreated cells were then irradiated with 0, 2, 4, 6, or 8 Gy. After incubating for 7 days, the plates were fixed with 70 % EtOH and stained with 1% methylene blue. Colonies consisting of >50 cells were counted under microscope. The survival fractions were calculated as (number of colonies / number of cells plated) / (number of colonies for corresponding control / number of cells plated).

Lactate dehydrogenase (LDH) leakage assay

LDH leakage assay was carried out using LDH cytotoxicity detection kit (Nanjing KeyGen Biotech. Co. Ltd.) according to the protocol in the user's manual. Cells were pretreated with hydrogen-rich PBS and the final concentration of H_2 was maintained above 0.3 mmol/L. The cells were immediately exposed to gamma radiation and then transported to an ice bucket. After 4 hour time period we analysed the content of LDH in cell suspension.

Apoptosis assays for cultured cells

Apoptosis was determined by Annexin V-APC and propidium iodide staining using apoptosis detection kit (Bipec Biopharma). Treated cells were incubated with Annexin V-APC for 15 minutes at 4 °C and propidium iodide for 5 minutes at room temperature. The cells were then analyzed by flow cytometry. Alternatively, apoptosis was determined by Hochest33258, flourescein diacetate (FDA) and propidium iodide staining. The treated cells were washed with PBS twice, and then stained with 40 mg/L flourescein diacetate, 20 mg/L Hoechst33258 at room temperature for 15 minutes, and stained with 20 mg/L propidine iodine at room temperature for 5 minutes. The cellular morphology was observed using Olympus BX60 fluorescent microscope equipped with Retiga 2000R digital camera. The average percentage of apoptotic cells was calculated in 5-7 randomly selected high power fields (HPF).

Determination of malondialdehyde (MDA) superoxide (SOD) glutathione (GSH)

MDA is a breakdown product of the oxidative degradation of cell membrane lipids and is generally considered an indicator of lipid peroxidation. SOD is a scavenger of superoxide, and GSH is an important cellular non-enzymatic antioxidant. In the present study, 4 hours after transporting the irradiated cells to an ice bucket, the concentrations of MDA, SOD, and GSH were measured, respectively, by using the MDA, SOD, GSH assay kit (Nanjing KeyGen Biotech. Co. Ltd.) according to the protocols in the user's manual.

Determination of 8-OHdG concentration

Half an hour after the irradiation, the 8-OHdG concentration was measured by using human 8-OHdG elisa kit (Nanjing KeyGen Biotech. Co. Ltd.) according to the protocol in the user's manual. Briefly after that, the treated cells were lysed by a cell lysis buffer. After the centrifuge, we added suspension to plate

wells coated with human 8-OHdG antibody, and sequentially treated them with biotinylated anti-lgG and streptavidin-HRP. After that, we added TMB substrate solution, and TMB substrate changed color into blue at HRP enzyme-catalyzed. At the effect of acid, the color finally become yellow. The intensity of this colored product is directly proportional to the concentration of 8-OHdG. Measuring the optical density (OD) at 450 nm with a microtiter plate reader, we calculated human 8-OHdG concentration by the standard curve.

STATISTICAL ANALYSIS

Data are expressed as means S. E. M. (standard error of the mean) for each experiment. The number of samples is indicated in the description of each experiment. Statistical analysis was performed by using one way analysis of variance. Between groups, variance was determined using the Student-Newman-Keuls post hoc test. A P value of less than 0.05 was considered to be statistically significant.

RESULTS

Hydrogen-rich PBS increases clonogenic survival of irradiated HIEC cells

To study radioprotective effects of H_2 in a cell culture, we examined the viability of the irradiated human intestinal crypt (HIEC) cells using a clonogenic survival assay. Pretreatment of HIEC cells with 0.1-0.4 mmol/L H_2 before irradiation significantly increased cell survival as compared to the cells treated with radiation alone at all examined doses (up to 8 Gy), fig. 1(a).

Hydrogen-rich PBS decrease cellular lactate dehydrogenase (LDH) leakage of irradiated HIEC cells

Beside the cell viability, we also determined LDH activities to estimate cellular LDH leakage from damaged cells. The result indicated that pretreatment with 0.3 mM H_2 before irradiation significantly decreased LDH leakage of HIEC cells which were exposed to different doses of γ-radiation, fig. 1(b). This result too was consistent with the result obtained by cell viability observation.

Hydrogen-rich PBS attenuates apoptosis in irradiated HIEC cells

To determine the radiation-induced apoptosis of the irradiated HIEC cells, we analyzed the treated cells by using Annexin V-APC and propidium iodide staining in flow cytometry assay. The early apoptotic cells decreased when pretreated with hydrogen-rich PBS as compared to the cells pretreated with PBS, fig. 2(a) and (b), 10.2% *vs*. 21.5%, respectively). We further evaluated the morphology of dying cells using Hochest33258, flourescein diacetate and propidium iodide staining. The irradiated HIEC cells pretreated with hydrogen-rich PBS demonstrated a protective effect with the reduced number of apoptotic cells to 26.1% as compared to 49.3% in PBS-pretreated irradiated cells, fig. 2(c) and (d). These data suggest that H_2 can attenuate apoptosis in irradiated HIEC cells.

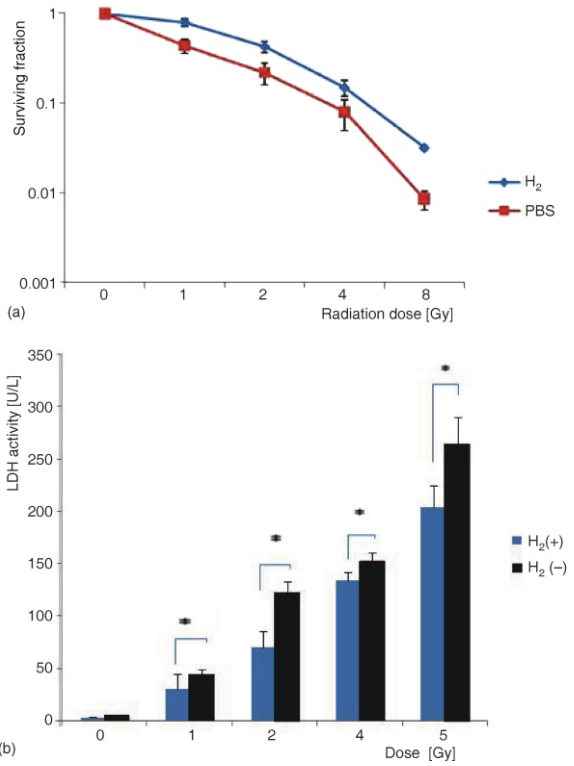

Figure 1. The treated HIEC cells were irradiated with 0, 1, 2, 4, and 8 Gy and plated for clonogenic survival assay and for LDH leakage assay; the surviving fractions (a) and changes in the levels of LDH in normal, γ-irradiated and H_2 pretreated lymphocytes from three experiments (b) are shown; values are given as mean S. E. M. *P < 0.05

Treating AHH-1 cells with hydrogen before irradiation could increase endogenous antioxidant status

The levels of enzymatic antioxidants (SOD) and the activities of non-enzymatic antioxidant (GSH) are shown in fig. 3. Gamma irradiated lymphocytes showed a significant decrease in the levels of both enzymatic and non-enzymatic antioxidant status when compared to hydrogen-rich PBS pretreated groups. The results indicated that pretreatment with H_2 could restore the antioxidant status.

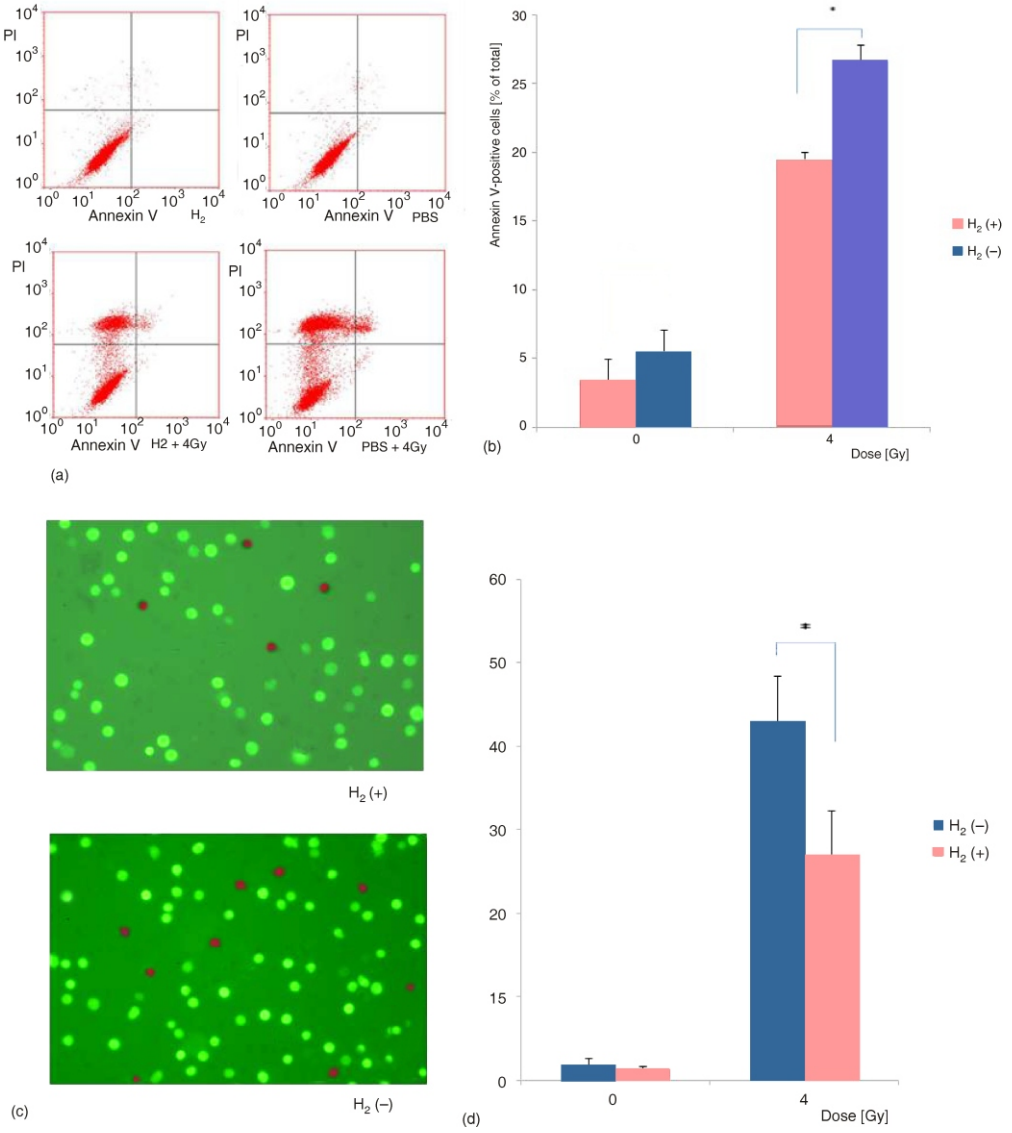

Figure 2. Hydrogen-rich PBS attenuates radiation-induced apoptosis in HIEC cells. The treated cells were collected 24 hours after the irradiation, stained with Annexin V-APC and propidium iodide and analyzed by flow cytometry. The representative diagrams of distribution of the stained cells (a) and a bar graph of apoptotic cells expressed as a percent of total cells are shown. Values are given as mean ±S. E. M. ($n = 4$); *P < 0.01 (b); cells were stained with FDA, Hoechst33258 and PI 24 hours after irradiation and apoptotic cells were counted in multiple randomly selected fields. The representative micrographs (c) and a bar graph of apoptotic cells expressed as a percent of total cells are shown; values are given as mean S. E. M. ($n = 4$); *P < 0.01 (d)

Hydrogen-rich PBS protects lipids and nuclear DNA of AHH-1cells from peroxidation induced by radiation

As shown in fig. 4(a), cellular MDA concentration at 4 hours after the irradiation in the H_2 group was significantly lower than that of the control group. This result indicated that H_2 could protect lipids from peroxidation induced by radiation. As shown in fig. 4(b), H_2 comparably decreased the concentration of 8-OHdG relative to the control group, indicating that H_2 can protect DNA from peroxidation induced by radiation.

DISCUSSION

This study shows that hydrogen can significantly protect human cells from ionizing radiation. Inhalation of H_2 was reported to protect cerebral [1], myocardial [2], and hepatic [9] I/R injury in animal models in several recent studies. Also, it is reported that hydrogen inhalation ameliorates oxidative stress in transplantation induced intestinal graft injury [3].

Since most of the ionizing radiation-induced cellular damage is caused by hydroxyl radicals, we speculate that the radioprotective effect may result from its radical oxygen species (ROS) scavenging effect. It was reported

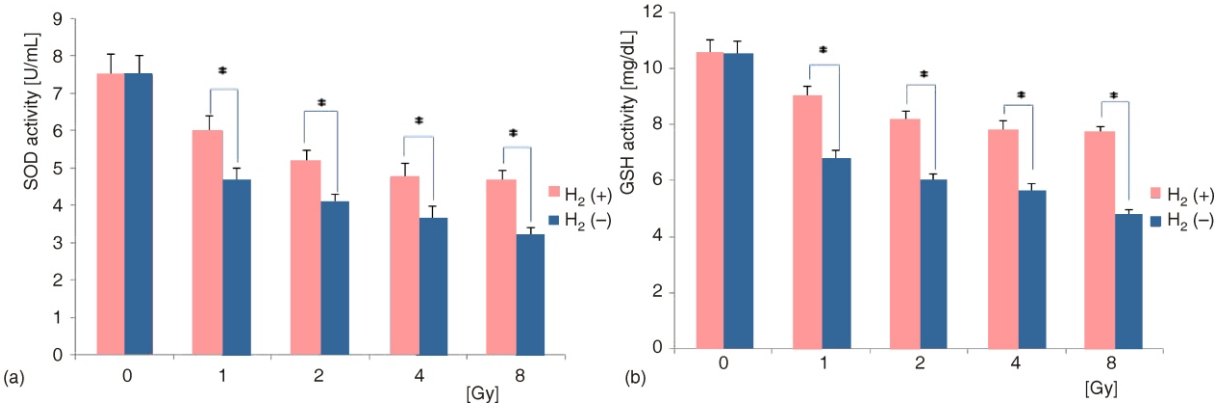

Figure 3. Changes in the activities of SOD and GSH in normal, γ-irradiated, and H_2 pretreated lymphocytes; values are given as mean S. E. M. (n = 4); *P < 0.01.

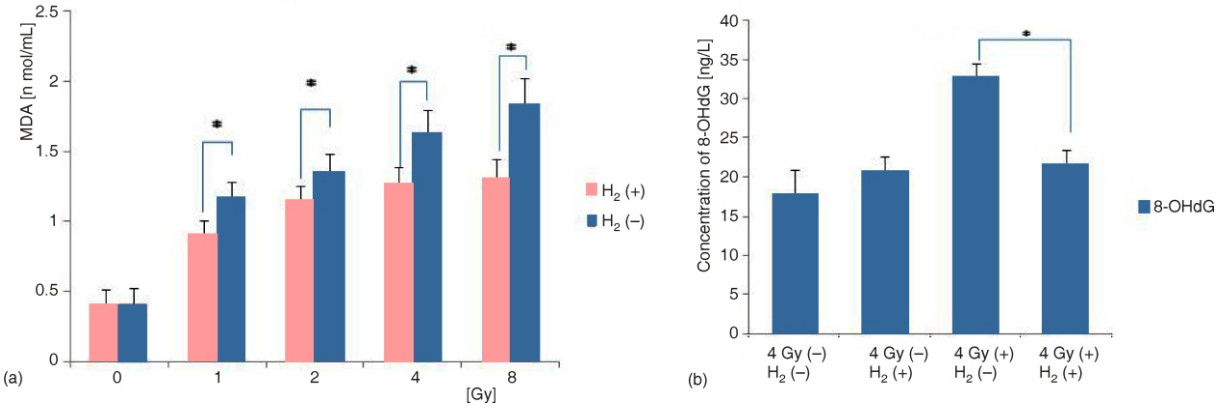

Figure 4. Hydrogen-rich PBS significantly decreased the levels of MDA, a marker of oxidative stress (a), and oxidative DNA damage assessed by 8-OHdG immunoreactivity. 8-OHdG concentration in normal, -irradiated, and H_2 pretreated groups (b) half an hour after the irradiation are shown; relative to the control group, H_2 significantly decreased the concentration of 8-OHdG; values are mean S. E. M. (n = 6); *P < 0.01.

that the effect of free radical scavengers could ameliorate the oxidative injuries due to ionizing radiation [10, 11]. The sulfhydryl compound amifostine (WR-2721), which is the only radioprotectant registered in use for humans, has shown good radioprotective effects [12]. However, when it was administered by injection, it caused many negative effects such as vomiting, hypertension, nausea, and other side effects caused by the toxicity [13, 14]. Some other radioprotectors, such as natural antioxidants, vitamin E, flavonoids and others, have fewer toxic side effects but also a lower degree of protection compared to thiol agents [13], and cytokines and immunomodulators should be used with low radiation doses or in combination with radical scavengers and antioxidants [15]. However, it is physiologically safe for humans to inhale hydrogen at a relatively low concentration, because hydrogen is continuously produced by colonic bacteria in the body and normally circulates in the blood [16]. It is a highly diffusible gas which could eliminate hydroxyl radical [17]. Dissolving H_2 in solvents such as PBS makes this explosive gas more safe for clinical use.

Radical oxygen species O_2^- and H_2O_2 are detoxified by antioxidant defense enzymes, unlike OH and $ONOO^-$, which so far could not be detoxified by any antioxidant defense enzyme. Hydrogen gas selectively reduces these two detrimental ROS [1]. A hydroxyl radical is the most reactive product of reactive oxygen species generated in cells. Cellular macromolecules, such as DNA, proteins, and lipids, can easily react with hydroxyl radicals to exert a cytotoxic effect.

Antioxidant enzymes (SOD) are important in providing protection from radiation exposure [18] and glutathione (GSH) participates non-enzymatically in protection against radiation damage [19]. Endogenous antioxidants are a group of substances which could significantly inhibit or delay oxidative processes [20]. A number of harms can result from a reduction of the activity of these substances. DNA is one of the major

targets of free radicals, and 8-OHdG is formed from deoxyguanosine in DNA by hydroxyl free radicals [21]. Also, membrane lipids are the major targets of free radicals [22]. The increase in the levels of lipid peroxidation products such as malondialdehyde and TBARs is the indication of membrane lipid damage [23]. In our study, we found that the pretreatment of hydrogen-rich PBS prior to radiation exposure increased the antioxidant status at both enzymic and non-enzymic levels and decreased the levels of MDA and 8-OHdG compared with the cells pretreated without hydrogen-rich PBS. We may conclude that the increases of the antioxidant status have further decreased the attack of free radicals on biomolecules including DNA and membrane lipids and thereby decreased the deleterious effects of radiation on cells.

In conclusion, hydrogen-rich PBS could protect human cells from radiation. This radioprotective effect may result from its radical oxygen species scavenging effect. Dissolving hydrogen in solution (PBS, water, saline) makes it safer and more convenient to use in clinic. We believe that hydrogen gas, especially hydrogen-rich solution, may give us more hope for greater protection from irradiation.

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PBS ОБОГАЋЕН ВОДОНИКОМ ШТИТИ ЧОВЕЧИЈЕ ЋЕЛИЈЕ У КУЛТУРИ ОД ЗРАЧЕЊЕМ НАНЕТИХ ОЗЛЕДА

Водонични радикали имају значајну улогу у индукцији ћелијских озледа дејством јонизујућег зрачења. Молекуларни водоник може селективно да смањи концентрацију хидроксилних радикала *in vitro*. Такве особине чине га потенцијалним радиопротектором. У овом раду тестирана су радиопротективна својства водоника *in vitro* коришћењем PBS обогаћеног водоником. Третирање ћелија с PBS обогаћеним водоником, пре озрачивања, значајно смањује апоптозу индуковану јонизујућим зрачењем, повећава виталност и преживљавање озрачених криптичних ћелија танког црева (HIEC), повећава концентрацију ендогених антиоксиданата и смањује концентрацију 8-хидроксидеоксигуанозин-малондиалдехида у лимфоцитима (AHH-1 ћелије). Из добијених резултата може се закључити да водоник поседује значајну особину радиопротектора: ефикасан је и нема нежељених својстава.

Кључне речи: водоник, јонизујуће зрачење, заштита од зрачења, апоптоза, реактивне кисеоничне врсте