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Hydrogen Gas Device has Therapeutic Effect on Skin Damage and Alleviates Dermatitis in Mice

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Introduction

Molecular hydrogen (H_2) emerged a decade ago as a potent therapeutic and is attracting more and more attention. The beneficial effects of H_2 are ascribed to its antioxidant and anti-inflammatory function, as well as its ability to induce defense responses and confer protective effects. Remarkably, H_2 has no known adverse effects on cell function.

Substantial H_2 is produced by the intestinal microflora under physiological conditions [1]. However, concentrations of endogenously produced H_2 is not sufficient to treat disease or restore normal physiology. Notable effects are observed only when exogenous H_2 is applied. H_2 is administered via inhalation, oral intake, or injection of H_2 saturated solution [2,3]. It can also be applied transdermally by applying H_2 saturated water or mixing chemicals together that react to form H_2 [2,3]. All these approaches achieve a transient increase in the concentration of H_2, along with beneficial effects on cell function [4-8]. Although administration of H_2 by inhalation or ingestion is the predominant method, application of H_2 transdermally provides targeted delivery to specific tissue, allowing for a local effect with a powerful H_2 concentration gradient.

When applied topically, H_2 not only acts on underlying skin and mucosal tissue, but is absorbed through the epidermis and delivered directly to damaged muscle and nerves. The aim of this study is to test a method for delivering H_2 to skin and subcutaneous tissue and demonstrate its effectiveness in treating skin lesions. For that purpose, we developed an H_2-producing device to treat a mouse model of oxazolone-induced dermatitis. Oxazolone is a chemical known to cause skin sensitization, allergy-like symptoms, and oxidative damage. More specifically, oxazolone is capable of causing skin inflammatory lesions similar to those in atopic dermatitis and contact dermatitis [9]. Since H_2 is known for its antioxidant and anti-inflammatory activity, we were able to demonstrate the efficacy of the developed method on dermatitis-like skin lesions.

Materials and methods

Animals

All experimental procedures and a humane euthanasia were performed according to the protocol approved by SMU Institutional Animal Care and Use Committee. Eight week old male mice strain SKH-1 (hairless) was purchased from the Charles River Laboratories (Wilmington, MA, USA). Mice were maintained at ambient temperature 25 ± 2ºC and 40-60% humidity under a 12 h light-dark cycle.

Induction of a dermatitis-like skin lesions

Mice were exposed to 4-ethoxymethylene-2-phenyl-2-oxazolin-5-one (oxazolone, Sigma-Aldrich), a chemical that causes atopic dermatitis-like skin lesions [10]. Oxazolone was repeatedly applied topically over a long period according to schemes [11-13]. Briefly, animals (n=10) were sensitized by applying 0.5% oxazolone dissolved in a mixture of acetone and olive oil (3:1). 200 µL of oxazolone solution was applied to an area of ~1 x 3 cm of dorsal skin three times per week for two weeks. Allergy symptoms developed based on the appearance of thick, scaly, red skin where oxazolone was applied (Figure S1) and animals were then randomly divided in two groups, experimental (n=5) and control (n=5). Sensitization with oxazolone continued for another two weeks followed by the therapeutic device treatment (Figure 1). During this period, both groups were treated with 200 µL of 0.2% oxazolone to dorsal skin three times per week.

The degree of skin lesions was evaluated based on visual assessment of skin redness and scaling (Figure S1). The intensity of skin redness was assessed based on scores none (0), mild (1), moderate (2), severe (3), and extremely severe (4) according to intensity of color (Figure S1) [14]. Degree of skin lesions were also assessed by measuring skin thickness in the middle of the back of mice using QUICKmini micrometer (Mitutoyo Corp., Japan).

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Design of the hydrogen-generating device

H_2 was delivered to the skin using a device made in the laboratory. The device consisted of a bag made of a KT-like tape (from a hydrophobic waterproof woven material of CVS brand) and a Mylar® film (DuPont) soldered together (Figure S2). Device contained aluminum foil inserts, calcium hydroxide, and filter paper. The chemical reaction to create H2 was initiated by adding a solution of sodium chloride (Figures S2-S3 for details). Bags were sealed and applied to damaged areas of the skin using adhesive plasters daily for 4 hours.

Device application

The experimental group was treated with a device that produced H_2 gas from a chemical reaction. The device resembled a plastic bag taped to the skin. The control group was treated with an inert device that did not produce H_2 because it lacked the catalyst, aluminum foil.

Skin lesions were assessed every day after start of therapeutic treatment. The degree of skin lesions, such as thickness, scaling, and redness, was determined as specified above. On the last day of the experiment (day 34), animals were sacrificed and H_2 diffusion into tissue was assessed using a special electrode. Skin and serum samples were taken for determination of oxidative damage and markers of inflammation.

Hydrogen diffusion into the tissue

Diffusion of H_2 into the skin was determined by using a hydrogen microsensor, a needle-like electrode (Unisense, Denmark) connected to a high-sensitivity picoammeter (Unisense Microsensor Monometer version 1.01), and corresponding software (SensorTraced SUITE v.3.1.5, Unisense, Denmark). Calibration and readings were conducted per the manual. Calibration curve was obtained using H_2O saturated with H_2 gas at room temperature. According to the literature, maximum concentrations of H_2 gas dissolved in water at ambient temperature and normal atmosphere pressure is ~ 800 μM. H_2 concentrations were calculated from the calibration curve using known levels of H_2-saturated water.

Oxidative damage

Dorsal skin samples were taken from oxazolone-exposed areas and stored at -80°C. Skin homogenates for determination of malondialdehyde (MDA) as a biomarker of lipid peroxidation were prepared in RIPA buffer, followed by determination of protein concentration by the Lowry method (Biorad). Homogenates were standardized for protein concentration. MDA levels were determined using the TBARS assay kit (Cayman Chemicals) per manufacturer protocol.

Markers of inflammation

Blood samples drawn from sacrificed animals were evaluated for cytokine interleukin-1β (IL-1β) as a marker of pro-inflammatory response. Collected blood was allowed to coagulate, followed by aspiration of cell-free serum. Serum IL-1β levels were measured using the Mouse IL-1β ELISA Ready-SET-Go! kit (eBioscience Inc.) per manufacturer instructions.

Statistical analysis: Data was analyzed using Microsoft Excel for Mac and values were expressed as mean ± SD. Group means were analyzed and compared using one-way ANOVA followed by multiple comparison tests, detailed in Figure legends, using Prism version 9.0 software packages (GraphPad Software Inc.).

Results

Hydrogen-releasing patches reduced skin lesions caused by oxazolone

H_2-producing packs were applied to the skin lesions of the experimental group. Inert packs were applied control group. H_2 packs and oxazolone treatments were applied according to scheme (Figure 1A).

In both experimental and control, animals were monitored daily for changes to skin condition. Repeated oxazolone applications provoked dermatitis-like skin lesions entailing scaling, redness, and increased skin thickness (Figure 1). Lesions appeared after 3-5 days of oxazolone use. Desired degree of sustained skin damage, a “4” on our “0 to 4” scale (Figure S1), was achieved by day 30.

Introduction of the H_2-producing device began once the mice established dermatitis-like symptoms from repeated oxazolone application (Figure 1). Effectiveness of the device was assessed visually and by measuring skin thickness as an indicator of inflammation/edema. Based on visual evaluation (Figure 1 and Figure S1), animals that received oxazolone without subsequent H_2 device administration exhibited severe skin scaling and redness. Scaling and redness were significantly reduced in experimental group receiving the H_2-releasing device (Figure 1B). Additionally, the experimental group experienced a slight but significant reduction in skin thickness.

After signs of recovery were established in the experimental group on day 4 post-treatment, mice were humanely euthanized, followed by tissue and blood sampling for analysis of cytokine levels and oxidative damage.

Effect of H_2-relasing device on oxidative damage

Oxazolone exposure has been shown to cause oxidative stress and inflammation in many studies [15]. Oxidative stress and reactive oxygen species (ROS) production lead to the generation of lipid peroxides. Beyond being a useful biomarker of oxidative stress, lipid peroxides are cytotoxic and involve in a number of biological processes. An extensive body of literature points to lipid peroxides having a role in signaling mechanisms. In particular, reactions are mediated by lipid peroxidation products, such as malondialdehyde (MDA), the accumulation of which can lead to the development of various pathological processes (reviewed in) [16].

MDA is a product of free radical attacks on membrane lipoproteins and polyunsaturated fatty acids. MDA levels reflect a local degree of lipid peroxidation and cellular damage. Since oxazolone has been reported to increase levels of lipid peroxidation [17-19], oxidative damages caused by oxazolone were assessed by measuring MDA levels.

MDA levels were determined in skin samples taken from different sites (lateral and dorsal) using the TBARS assay. As shown in Figure 2, MDA levels were more than 2-fold higher in skin exposed to oxazolone, the dorsal skin. Application of the H_2-releasing device significantly reduced MDA levels in the dorsal skin in the experimental group, but the lateral skin, which was not treated with oxazolone, did not experience changes in MDA levels. Ultimately, oxazolone caused local oxidative damage to the skin which was significantly reduced by H2 therapy, although no reduction to the levels of intact lateral skin was achieved.

Hydrogen-releasing device provided anti-inflammatory effects in mice

Levels of cytokines, key mediators of the inflammatory response, such as IL-1β, increase in response to oxazolone exposure [12,13,15]. Serum levels of cytokine IL-1β were determined in order to evaluate the efficacy of the H2 device as a therapy for dermatitis-like lesions. We found that H2-treated mice showed significantly decreased serum concentrations of IL-1β when compared to control group (Figure 3).

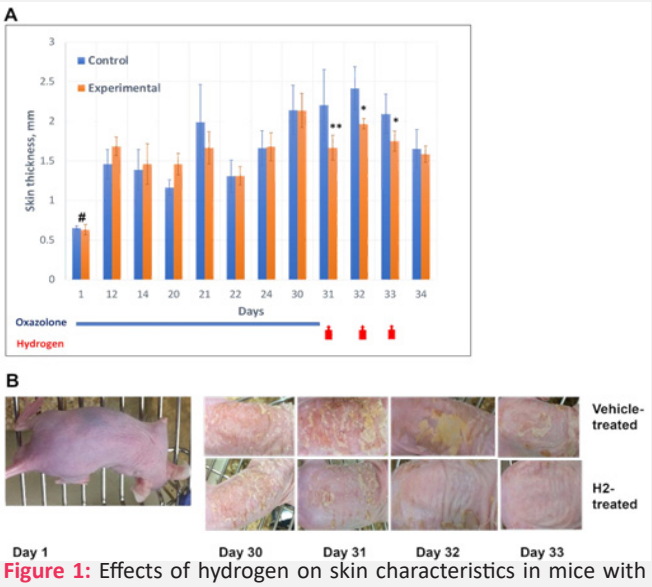

Figure 1: Effects of hydrogen on skin characteristics in mice with oxazolone-induced lesions.
A, Dorsal skin thickness of group treated with hydrogen and control group. Oxazolone was applied day 1 to 30. Hydrogen-releasing packs applied as shown in diagram by red arrows. Skin thickness values are mean ± SD (n=5). Significance was analyzed by 2-way ANOVA with Dunnett post-test. Significant difference (*p<0.05, **p<0.005) between inert vehicle-treated (Control) and hydrogen-treated (Experimental) animals is marked by asterisks. # marks a significant difference between time points. **B**, Representative skin images of mice with different treatments. Oxazolone has been applied through day 30. Hydrogen treatment started after day 30.

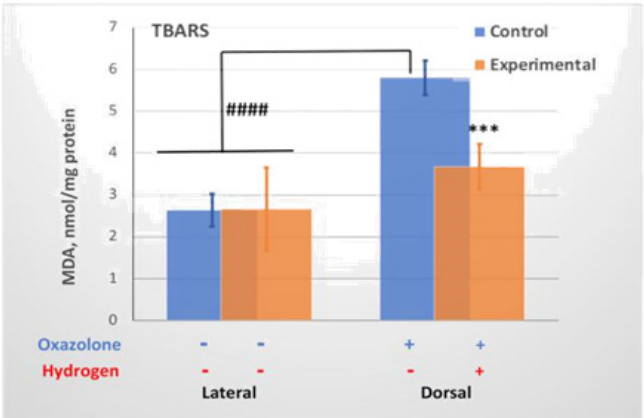

Figure 2: Malondialdehyde (MDA) levels in skin samples taken from dorsal (oxazolone-exposed, '+') and lateral (not exposed to oxazolone, '–') areas.
Control – mice not treated with hydrogen. Experimental – mice treated with H2. Values represent mean ± SD (n = 5). Data were analyzed by 2-way ANOVA and Tukey's post-test. A significant difference (***p<0.0005) between vehicle-treated (Control) and hydrogen-treated (Experimental) animals marked by asterisk. ####p<0.0001 marks a significant difference between lateral (no exposure to oxazolone) and dorsal (oxazolone treated) skin samples.

Concentration of H_2 under the skin

We tested three variants of devices containing different H_2-producing compositions (Figure S4 and Figure 4). Shown in Figure 4 is the variant of the device used in this study. The obtained data shows that the device effectively delivers H_2 to the targeted tissue, in this case the dorsal area. Device maintained a considerably high level of H_2 for about an hour. Moreover, H_2 did not return to baseline one hour after removing device.

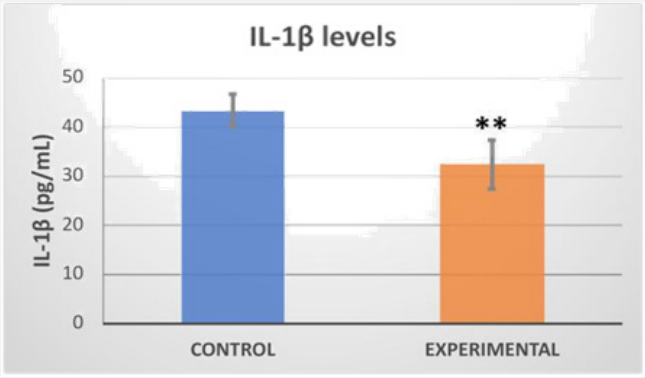

Figure 3: Effects of H2 on IL-1β levels.
Serum samples were collected on last day of experiments (day 34, Figure 1). Values represent mean±SD (n=5). Significant difference between control and experimental (hydrogen treated) groups determined by Student's t-test. ** P<0.01.

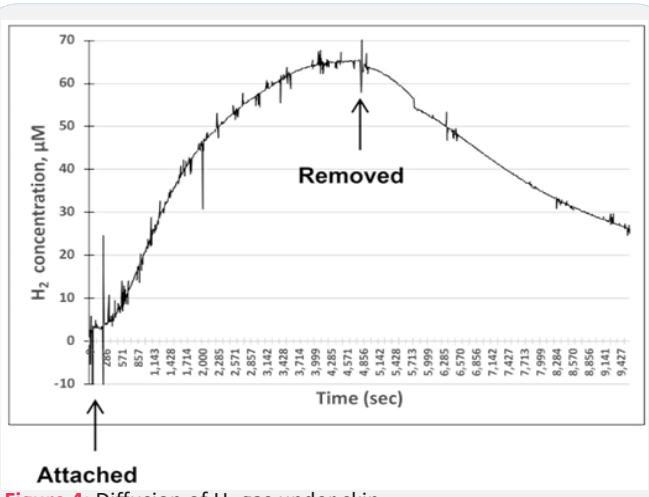

Figure 4: Diffusion of H_2 gas under skin.
Data obtained by using H_2 electrode (Unisense) inserted under skin (Figure S4). Hydrogen-producing device was securely attached to area of skin with inserted electrode. Representative graph shows increase in H_2 concentration for the variant of the H_2 producing device used in study (Figure S4A). The bag is made of KT + Mylar and H_2 is produced in a reaction detailed in Figure S2. Time points of device attachment and removal indicated by arrows. Maximum concentrations of H_2 were 60-70 µM. After removal of the bag, H2 concentration slowly decreased.

Discussion

Here we tested a device that produces H_2 gas by applying it topically to treat oxazolone-induced skin inflammation. For this purpose, we used the SKH-1 hairless mouse strain, ideal for studying skin conditions [20]. In particular, this model makes it possible to minimize the background inflammatory response caused by stress unrelated to the studied pathology, such as eliminating the need for epilation. Using this model, we were able to test the developed H_2 gas-releasing device and demon-

strate its therapeutic effect on mice with dermatitis-like skin lesions.

We chose oxazolone to induce the dermatitis-like lesions. This chemical acts as a hapten and capable of inducing immune system hypersensitivity reactions and allergy-like symptoms largely identical to those of atopic dermatitis and contact dermatitis in humans. These immunological abnormalities are associated with a pro-inflammatory response and increased production of ROS [10,12,13,15,21].

Using the approaches described in other studies [11,12,21], we achieved comparable oxazolone-provoked effects on the development of skin lesions, such as scaling and increased epidermal thickness (Figure 1), as well as an increase in tissue oxidative damage and pro-inflammatory cytokine levels (Figures 2,3).

Application of the H_2-releasing device alleviated the severity of oxazolone-induced atopic dermatitis in SKH-1 mice, resulting in a significant reduction in skin damage. This was evident from reduced epidermal thickness and scaling (Figure 1). In a different study, similar effects in the treatment of skin lesions caused by UV radiation in hairless mice was achieved by bathing animals in electrolyzed reduced water, which is characterized by significant changes in redox potential [22]. Although H2 concentrations were not measured in that study, it is known that electrolysis of water produces H_2. It is likely that the positive effects of such treatment were due to H_2.

H_2 is known for its antioxidant and anti-inflammatory activity [23,24]. The role of H_2 in attenuating the hapten-induced inflammatory response is supported by finding that the increase in serum IL-1β was reduced by administration of the H_2 releasing device. Moreover, our data showed that H_2 had a systemic anti-inflammatory effect, as a decrease in IL-1β was detected in the blood (Figure 3). This suggests that application of the H_2-releasing device protects against acute chemical injury by suppressing the skin's inflammatory response.

The results of the H_2-releasing device in this study are consistent with the anti-inflammatory effects using hydrogen-rich water in another studies. For example, hydrogen-rich water alleviated the severity of atopic dermatitis in NC/Nga mice by suppressing skin inflammation evident by decreased IL-1β [25,26]. Similar anti-inflammatory effects were observed in rats when hydrogen-rich water was used to reduce radiation-caused dermatitis and UVB injury [27,28].

Lower levels of cytokine IL-1β correspond to reduced levels of lipid peroxidation (Figures 2,3). Lipid peroxidation is a marker of inflammation and oxidative damage and plays an important role in dermatitis development. There is ample evidence that allergic and inflammatory skin diseases are mediated by oxidative stress (reviewed in Corsini) [29]. For example, in rats with skin injury caused by ischemia/reperfusion, both anti-oxidant and anti-inflammatory effects of hydrogen-rich saline were noted [30]. In another study, an increase in pro-inflammatory cytokines was associated with the release of ROS [12]. Thus, our data showing an increase in lipid peroxidation (Figure 2) points to a role for oxidative stress in hapten-evoked, atopic dermatitis pathology.

Thus, we successfully used the dermatitis model and found the H_2-releasing device has a therapeutic effect on skin damage and can improve dermatitis-like symptoms in hairless mice. However, further research needs to be extended to longer-term use of oxazolone because we did not achieve the profound immunological changes associated with chronic dermatitis. This was clear from the rapid recovery of the animals after cessation of oxazolone irritation. The rapid recovery suggests we induced an acute rather than chronic condition, even though we followed protocols and schemes proposed for induction of experimental dermatitis in SKH-1 mice and other mouse strains [11-13,15].

The advantage of the transdermal device is its convenience and high degree of H_2 delivery to target sites. The device delivers comparable levels of H_2 as bathing the skin in hydrogen-rich water. Only, bathing in hydrogen-rich water requires massive volumes and laborious prolonged skin exposure [3]. Treatment of skin conditions can also be achieved by injections of hydrogen-saturated saline, but this method suffers from systemic side effects because the administration method is invasive [30]. In that study, H_2 concentrations in tissues were evaluated using the hydrogen microelectrode. Remarkably, our method of H_2 administration using the developed device on skin achieved levels comparable to injection. Thus, we have achieved efficient, non-invasive delivery of H_2 to tissue with minimal side effect.

In some ways, human studies will be easier and may have greater success than using the mouse model because the mice tend to remove the device, which had to be tightly secured. This hurdle will be minimal in the treatment of humans.

Declarations

Author contributions: VK conceived, designed and conducted experiments and analyzed data. DC conceived study and participated in preparation of the Animal Use Protocol and manuscript. SR conceived, designed and conducted experiments; analyzed data; and wrote manuscript. All authors approved final version.

Conflict of interest: The authors confirm that this article content has no conflict of interest.

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