Supplementation with hydrogen-producing composition confers beneficial effects on physiology and life span in Drosophila

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ABSTRACT

Recently, molecular hydrogen (H₂) has become known as a new class of antioxidants and redox-modulating interventions. Effects of H₂ have been documented for many acute and chronic pathological conditions. The present study was aimed at determining the effect of hydrogen on the physiology and longevity of Drosophila. The flies were given a patented food supplement consisting of a mixture of inert salts with metallic magnesium, which reacted with acidic aqueous solutions, thereby releasing hydrogen gas. The supplementation with hydrogen-rich food prolonged the life span of the wild-type strain. To gain insights into the effect of hydrogen, we used previously generated mutant under-expressing redox-regulating enzymes, peroxiredoxins, in mitochondria. The hydrogen-releasing material lessened the severe shortening of life span of the mutant. Hydrogen also delayed the development of intestinal dysfunction caused by under-expression of peroxiredoxins in the intestinal epithelium. Hydrogen also averted a significant decrease in the mobility of mutant flies that under-expressed peroxiredoxins globally or in specific tissues. Together, the results showed that the introduction of hydrogen to aging or short-lived flies could increase their survival, delay the development of the intestinal barrier dysfunction and significantly improve physical activity.

1. Introduction

Over the last two decades, previously considered as an inert gas, molecular hydrogen (H₂) has emerged as a novel agent with profound biological effects on cell function and physiology. The effects of hydrogen on the treatment of various pathological conditions and diseases have been demonstrated in numerous studies with animal models and clinical studies (reviewed in [1, 2, 3]). The beneficial effects of hydrogen are attributed to its ability to function as a selective antioxidant, which unlike other antioxidants is highly effective in scavenging strictly harmful reactive oxygen species (ROS), such as hydroxyl radicals and peroxy-nitrite [4], while retaining the activity of functionally important ROS, such as H₂O₂ and NO [4, 5]. H₂ can also act as a signaling molecule and induce various defense responses [6, 7, 8]. The most pronounced therapeutic effects of H₂ have been documented in diseases associated with oxidative stress [1, 5, 9, 10].

Another advantage of molecular hydrogen as a therapeutic agent is its mobility and permeability. A small uncharged H₂ molecule can easily diffuse through cell membranes and penetrate into organelles, such as mitochondria, one of the main source of ROS production, and protect this organelle from damage by excessive concentrations of ROS, thereby preventing the development of diseases associated with mitochondrial dysfunction and oxidative stress.

There are many obstacles that impede the development of H₂-based therapy. The introduction of hydrogen by inhalation, intake or injection of H₂-rich solutions suffers from many drawbacks, such as safety problems or difficulties in controlling the dosage [11]. An alternative is to produce hydrogen in the reaction of metals with acidic solutions.

In the presented study, we used magnesium (Mg)-containing food supplement, MagH₂ and tested its biological effects on fruit flies. Although molecular hydrogen is now recognized as a promising therapeutic option for the treatment of various diseases associated with oxidative stress and mitochondrial dysfunction, studies showing hydrogen influence on aging are virtually absent. Here we used the Drosophila model, well known in the aging studies, to determine the effect of H₂ on aging and physiology at the organismal level.
2. Materials and methods

2.1. Fly material

The y w reference strain has been maintained in this laboratory for >23 years. The Da-GAL4 and D42-GAL4 driver lines were kindly supplied by Blanka Rogina (University of Connecticut Health Science Center), and NP1-GAL4 driver line was a kind gift of Heinrich Jasper (The Buck Institute for Research on Aging). The dprx3,dprx5 double mutant (DM) under-expressing both mitochondrial peroxiredoxins (Prxs) is described in previous publications [12, 13, 14]. Briefly, global or tissue-specific under-expression of Prxs has been achieved by expressing the RNAI-dPrx3 hairpin construct, using the global Da-GAL4 or tissue-specific D42-GAL4 (motor neurons) and NP1-GAL4 (midgut) drivers in the dprx5−/− mutant background.

2.2. Preparation and evaluation of hydrogen-enriched fly food

The yeast-sugar broth was made of the 0.5% yeast extract and 5% sugar and methylparaben added to the final concentration 0.19%. A mix of propionic, acetic acid and o-phosphoric acid (31.5%, 10% and 3.5% respectively) was used to adjust the pH to 4.5–4.8. The broth has been applied to cotton balls or sponges inserted in glass vials.

To prepare hydrogen-rich broth, we used a patented composition named MagH2, kindly provided by Dr. Miljikovic (Nano H2 Minus, Inc., US patents 8,852,660 B2 and 9,144,581 B2). The composition contains metallic magnesium microparticles mixed with basic magnesium carbonate and is able to release molecular hydrogen when added to acidic aqueous solutions. MagH2 was added to the acidic yeast-sugar broth at different concentrations immediately before experiments. pH of the solution has been adjusted to 4.5–4.8 using the same mix of acids used for the broth preparation. Under these conditions, the metallic magnesium instantly produces molecular hydrogen in a reaction: Mg + 2H+ Mg2+ + H2.

To determine potential toxicity of the MagH2 component and to exclude the possibility that the effects of MagH2 were exclusively due to molecular hydrogen and not associated with other components in the mixture, MagH2 solutions were boiled for three times to remove the generated hydrogen gas. The anti-oxidant capacity of fly food has been evaluated by measuring the oxidation-reduction potential (ORP) using Beckman - 350 pH Temp/mV meter (Beckman Coulter).

2.3. Procedures

For experimental studies, male and female flies were collected within 1–2 days after hatching and reared on a standard sucrose-cornmeal medium at 25 °C or transferred to food containing MagH2. Survivorship studies were performed as described in previous publications [12]. In each experiment, approximately 50–60 flies were used for each fly line.

Negative geotaxis was evaluated as described [15, 16] with some modifications. Briefly, a number of flies that are able to climb or jump ~4 cm distance and to reach the top of a vial was counted at different time intervals. The flies were gently tapped down to the bottom of the vial and allowed to climb for 30 seconds. The assay was repeat for the same group three times, allowing for 10 minute rest period between each trial. The geotaxis was expressed as a number of climbers/jumpers to the total number of flies with observations performed for 30 seconds for each vial.

Intestinal barrier dysfunction was tested using the “smurf” phenotype assay [17]. Briefly, flies were transferred to vials with food containing 2.5% (wt./vol.) of Blue dye no. 1. Flies with normal intestinal function had the blue stain restricted to the intestinal lumen. When integrity of the intestinal epithelial barrier was impaired, the blue dye produced a broader staining throughout the body, which was documented by microscopy examination.

2.4. Statistical analysis

All statistics were calculated using Excel and Prism for Macintosh version 6.0b software (GraphPad Software, Inc. San Diego, CA). Differences in negative geotaxis were compared between groups by analysis of variance. The mean survivorship time and statistical significance of differences between survival curves were assessed by the log-rank test. Differences in survivorship at 10% mortality and the development of the “smurf” phenotype were determined by two-way analysis of variance.

3. Results

3.1. MagH2 reduced the oxidation-reduction potentials

Previous in vitro studies have shown that when dissolved in water, MagH2 forms H2 nanobubbles, thereby maintaining high amounts of dissolved H2 over a relatively long period of time [18]. It also led to a stable negative oxidation-reduction potential at relatively high pH values [18]. Here we have determined that MagH2 is also capable of increasing negative ORP values in fly food under acidic conditions that are comparable to those in the gastric space, which approximates the conditions to those in vivo.

Addition of different concentrations of MagH2 to the yeast-sugar broth (pH 4.4–4.8) caused a significant reduction in the oxidation-reduction potential. Thus, ORP rapidly dropped from +185 mV (regular food) to (-160) to (-320) mV (0.1 mg/ml MagH2), (-330) mV (0.3 mg/ml MagH2), and to (-410) to (-470) mV (1 and 3 mg/ml MagH2) and lasted at negative values for at least 4 h. Thus, the observed effects of MagH2 on fly food were comparable to those observed in water solutions [18], suggesting the existence of molecular hydrogen in the form of nanobubbles and relative stability of its concentrations in the fly food. We also determined that 5 minutes of boiling was sufficient to remove hydrogen gas from solutions, since the ORP values ranged from +180 to +190 mV and were comparable to the ORP values in a regular fly food.

3.2. MagH2 extended life span

To determine the effects of MagH2 on longevity, male and female flies of the long-lived y w strain of Drosophila received food mixed with MagH2 in the range of concentrations of 0.1, 0.3, 1 and 10 mg/ml. MagH2 has been given starting at 42 days of age, or at ~50% of their respective life span and just before the onset of rapid increase in mortality, to avoid potential adverse effects in young healthy organisms. The beneficial effects were observed when males were fed food with 0.1–0.3 mg/ml of MagH2 and females with 0.3–1 mg/ml, while higher concentrations, 3 and 10 mg/ml were toxic and decreased survivorship (Fig. 1 and unpublished data). The addition of MagH2 in the optimal range of concentrations led to an increase in median (50% death) by ~15% in males and ~25% in females and mean (average) age, but a small increase in maximum (90% death) age (Fig. 1). Thus, MagH2 only moderately prolonged the longevity, but significantly improved the age conditions, or life span of flies. The removal of H2 by heating solutions prior to the introduction into flies completely abolished the positive effects of the optimal doses of MagH2. On the other hand, the observed toxicity of high MagH2 content (3–10 mg/ml) was due to other components of MagH2, rather than to H2, since the longevity of flies was significantly reduced, regardless of the removal of H2 (unpublished observations).

3.3. MagH2 delayed premature aging in mutants underexpressing mitochondrial peroxiredoxins

To get insights into the mechanisms of H2 effects, we used flies with impaired mitochondrial function. The importance of mitochondria in the regulation of aging is well recognized [19]. Normal mitochondrial function and integrity is maintained by many redox-related factors,
including thiol-related peroxidases, peroxiredoxins, which are known to play an important role in longevity [12, 13]. Previously, we showed that the deficit in activity of mitochondria-localized Prxs, dPrx3 and dPrx5, resulted in a 80% decrease in mean life span, accompanied by age-associated increase in tissue-specific apoptosis, changes in cellular redox, as well as altered transcriptional profiling of the genes involved in stress responses and mitochondrial maintenance [12, 13].

Although the normal life span of the mutant underexpressing Prxs globally with Da-GAL4 driver was not completely restored, the intake of MagH2 positively influenced the survival of these flies. Like in the old flies, the effects were dose-dependent with optimal concentrations of MagH2 0.1 mg/ml for males and 0.3 mg/ml for females (Fig. 2). Similarly to control y w flies, concentrations of MagH2 three times higher than optimal were toxic and led to increased mortality of the mutant, irrespective of removal of H2 by heating (data not shown).

3.4. MagH2 has positive effects on the intestinal barrier function

It is reported that the intake of hydrogen can ameliorate intestinal inflammatory disorders by reducing injuries and breakdown of the epithelial barrier (reviewed in [8]). Normal functioning of the gastrointestinal tract is essential for a healthy aging and longevity. Decline in intestinal barrier function is a characteristic of old age and can be measured by the negative geotaxis test (Fig. 4). Negative geotaxis (startle-induced vertical locomotion) is frequently used to measure locomotor ability and track the age-related locomotor impairment in Drosophila [20].

To extend the studies and pinpoint critical tissues for which MagH2 might have a particularly beneficial effects, we under-expressed peroxiredoxins in motor neurons using the D42-GAL4 driver. The flies fed MagH2 showed a more than 50-fold increase in negative geotaxis (Fig. 4). Surprisingly, flies under-expressing Prx in other tissues with the NP1-GAL4 driver or globally (Da-GAL4) also showed a dramatic...
improvement in fitness when feeding MagH₂ (Fig. 4), suggesting a global positive effect of hydrogen, regardless of in which tissues Prxs were underexpressed. Although not fully restored, the activity of mutants treated with MagH₂ was comparable to the activity levels of y w controls of the same chronological age (Fig. 4). Comparable improvements in activity due to MagH₂ feeding were also observed in old flies (data not shown). Thus, the results suggest that MagH₂ can be particularly beneficial for maintaining proper motor activity in older population, as well as in diseased flies with impaired motorneuronal and/or neuromuscular function.

4. Discussion

Aging is a complex process due to the involvement of many factors, such as chronic inflammation of the intestinal epithelium, oxidative stress, slowing of mobility and dysfunction of mitochondria. The main finding of this study is that the introduction of molecular hydrogen by feeding fruit flies with a H₂-producing supplement, MagH₂, extends the life span of Drosophila, and also favorably affects the physiology of mutants with impaired mitochondrial function. To our knowledge, this study is the first attempt to investigate the effect of hydrogen on longevity of wild-type animals. So far, the only report related to the effect of hydrogen on life span was on a partial restoration of the life span of mice shortened by a high-fat diet [21].

Table 1

| Treatment | Males | % vs. Control | Males | % vs. Control |
|-----------|-------|---------------|-------|---------------|
| Control   | 100*  |                | 100*  |                |
| MagH₂ 0.1 |       | 133*; 114*    |       | 125*; 115*    |
| MagH₂ 0.3 |       | 115*; 114*    |       | 124*; 127*    |
| MagH₂ 0.1 heat |       | 124*; 127*    |       | 124*; 127*    |
| MagH₂ 0.3 heat |       | 124*; 127*    |       | 124*; 127*    |

The studies conducted on the mutant with impaired redox and mitochondrial dysfunction also showed beneficial effects of hydrogen on survivorship (Figs. 2 and 3). Mitochondria is a major source of production of ROS, which tends to increase during aging. Mitochondrial dysfunction can induce oxidative stress; conversely, oxidative stress may worsen mitochondria-related pathological conditions. Since conventional antioxidants have limited therapeutic effects because they are not effectively taken up by mitochondria, hydrogen can be particularly useful for controlling the mitochondrial disorders. A recent insight into the mechanisms of exogenous H₂ has revealed prominent effects on mitochondria, as was determined in cultured neuroblastoma cells [22]. Mitochondrial function and physiological parameters were improved in patients taking H₂-generating minerals [23]. Metabolism- and mitochondrial function-related lactic acidosis have been reduced in the
models, including mitochondrial myopathy and neuromuscular disease changes observed in the DM [12] can lead to accumulation of acidic thus counteracting potential metabolic acidosis in the DM. The metabolic mitochondrial metabolism or alter mitochondrial function [27, 28].

observed in the mutants that affects regulation of intracellular sugar/-metabolites and alterations in pH homeostasis, similarly to those metabolic disturbance and acidosis due to lactate accumulation were could be due to a shift to more alkalizing conditions caused by MagH2, stress and apoptosis, which led to improved locomotor function [26]. Another possibility is that H2 could act by increasing energy production and in the midgut (NP1-DM), as well as expressing mitochondrial Prxs globally (Da-DM), in motor neurons (D42-DM) function.

physiology of that molecular hydrogen has had a dramatic positive effect on the treatment of a patient by drinking H2 water [24]. Since the phenotype characteristics of the DM [12] resembled those of mitochondrial disease models, including mitochondrial myopathy and neuromuscular disease [25], positive effects of H2 could be due to improved mitochondrial function.

Aging and mitochondrial diseases compromise many physiological functions including mobility. The most remarkable finding of this study is that molecular hydrogen has had a dramatic positive effect on the physiology of flies by maintaining physical vigor. Documented as negative geotaxis (Fig. 4), the motor capability was significantly improved in flies fed H2-enriched food. One possibility is that hydrogen may restore the neuromuscular functional deficits observed in the DM by counteracting oxidative damages and changes in the cellular redox [13]. For instance, administration of an H2-rich saline solution reduced oxidative stress and apoptosis, which led to improved locomotor function [26]. Another possibility is that H2 could act by increasing energy production and consumption of O2 by mitochondria.

Alternatively, the effects of hydrogen in maintaining physical vigor could be due to a shift to more alkalinizing conditions caused by MagH2, thus counteracting potential metabolic acidosis in the DM. The metabolic changes observed in the DM [12] can lead to accumulation of acidic metabolites and alterations in pH homeostasis, similarly to those observed in the mutants that affects regulation of intracelluar sugar/-mitochondrial metabolism or alter mitochondrial function [27, 28]. Studies conducted in humans have shown that the exercise-induced metabolic disturbance and acidosis due to lactate accumulation were mitigated by the administration of alkalinizing H2-rich water [29, 30].

We also found the protective effect of H2 on the integrity of the intestinal epithelium, usually affected by aging and pro-inflammatory conditions. In the DM with a deficiency in mitochondrial function in the midgut, the development of the “smurf” phenotype appears to have occurred just before the onset of mortality, since almost all dead flies had a “smurf” phenotype. This means that their death coincided with the loss of intestinal integrity.

Our data demonstrate that hydrogen has the ability to delay the development of the “smurf” phenotype and, therefore, has the potential for improving the survivability of the mutants with impaired mitochondrial function in the guts. The positive effects of H2-rich solutions by reducing oxidative damage and inflammation have also been reported in other intestinal injury models [31, 32], and gaseous hydrogen released by intestinal bacteria has been linked to a decrease in the symptoms of inflammatory bowel disease [33]. In another study, H2 administration down-regulated inflammatory mediators in the intestinal tissue and prevented intestinal barrier dysfunction [34].

Our study also revealed the dose-dependent effects of MagH2. As stated in studies conducted on human and animal models, the introduction of hydrogen by inhalation or consumption of H2-rich water usually does not adversely affect the cellular function and physiology of the organism (reviewed in [2]), although there are limited data on the hydrogen-associated toxicity [35, 36, 37, 38]. However, the likelihood of adverse effects of hydrogen is indeed low, although such an assumption was made on the grounds that hydrogen, acting as an antioxidant, does not react with functionally important ROS as H2O2 [4]. However, the toxicity of the high doses of MagH2 observed in our study was most likely due to high concentrations of magnesium that are harmful to Drosophila, rather than hydrogen itself.

Taken together, the data showed that the introduction of molecular hydrogen orally by consuming H2-rich food leads to the preservation of intestinal integrity and improved physical activity in flies with impaired mitochondrial function. Consumption of H2-rich food also extended healthy aging.

Declarations

Author contribution statement

Vladimir I. Klichko: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data.

Vladimir L. Safonov: Conceived and designed the experiments; Contributed reagents, materials, analysis tools or data.

Marina Yu. Safonov: Contributed reagents, materials, analysis tools or data.

Svetlana N. Radyuk: Conceived and designed the experiments; Analyzed and interpreted the data; Wrote the paper.

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Competing interest statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

References

[1] K. Ohno, M. Ito, M. Ichihara, M. Ito, Molecular hydrogen as an emerging therapeutic medical gas for neurodegenerative and other diseases, Oxid. Med. Cell Longev. 2012 (2012) 353152.
A.L. Han, S.H. Park, M.S. Park, Hydrogen treatment protects against cell death and S. Ohta, Recent progress toward hydrogen medicine: potential of molecular S.N. Radyuk, K. Michalak, V.I. Klichko, J. Benes, I. Rebrin, R.S. Sohal, W.C. Orr, Molecular hydrogen affects body composition, metabolic profiles, and mitochondrial function in middle-aged overweight women, Ir. J. Med. Sci. 187 (2018) 85–89. S. Ohta, A. Nakao, K. Ohno, The 2011 medical molecular hydrogen symposium: an inaugural symposium of the journal medical gas research, Med. Gas. Res. 1 (2011) 10. D.J. Fernandez-Ayala, S. Chen, E. Kemppainen, K.M. O’Dell, H.T. Jacobs, Gene expression in a Drosophila model of mitochondrial disease, PLoS One 5 (2010), e8549. C. Chen, Q. Chen, Y. Mao, S. Xu, C. Xia, X. Shi, J.H. Zhang, H. Yuan, X. Sun, Hydrogen-rich saline protects against spinal cord injury in rats, Neurochem. Res. 35 (2010) 1111–1116. D.K. Bricker, E.B. Taylor, J.C. Schell, T. Orsak, A. Bouton, Y.C. Chen, J.E. Cox, C.M. Cardon, J.G. Van Vranken, N. Dopheide, C. Sourd, B. Drolina, S.P. Gygi, M. Brivet, C.S. Thummel, J. Rutter, A mitochondrial pyruvate carrier required for pyruvate uptake in yeast, Drosophila, and humans, Science 337 (2012) 96–100. A.C. Ghosh, M.B. O’Connor, Systemic Activin signaling independently regulates sugar homeostasis, cellular metabolism, and pH balance in Drosophila melanogaster, Proc. Natl. Acad. Sci. U. S. A. 111 (2014) 5729–5734. S.M. Ostojic, M.D. Stejanovic, Hydrogen-rich water affected blood alkalinity in physically active men, Res. Sports Med. 22 (2014) 49–60. K. Aoki, A. Nakao, T. Adachi, Y. Matsui, S. Miyakawa, Pilot study: effects of drinking hydrogen-rich water on muscle fatigue caused by acute exercise in elite athletes, Med. Gas. Res. 2 (2012) 12. X. Zheng, Y. Mao, J. Cai, Y. Li, W. Liu, P. Sun, J.H. Zhang, X. Sun, H. Yuan, Hydrogen-rich saline protects against intestinal ischemia/reperfusion injury in rats, Free Radic. Res. 43 (2009) 478–484. B.M. Buchholz, D.J. Kaczorowski, R. Sugimoto, R. Yang, Y. Wang, T.R. Billiar, R.C. Gianani, K.R. McCarry, A.J. Bauer, A. Nakao, Hydrogen inhalation ameliorates oxidative stress in transplantation induced intestinal graft injury, Am. J. Transplant. 8 (2008) 2015–2024. M. Pimentel, E.J. Chow, H.C. Lin, Edzardic of small intestinal bacterial overgrowth reduces symptoms of irritable bowel syndrome, Am. J. Gastroenterol. 95 (2000) 3505–3506. M. Ikekda, K. Shimizu, H. Ogura, T. Kurakawa, E. Umemoto, D. Motooka, S. Nakamura, N. Ichimaru, T. Takahara, S.I. Hirano, T. Shimazu, Hydrogen-rich saline regulates intestinal barrier dysfunction, dysbiosis and bacterial translocation in a murine model of sepsis, Shock (2017). A. Nakao, Y. Toyoeda, P. Sharma, M. Evans, N. Guthrie, Effectiveness of hydrogen rich water on antioxidant status of subjects with potential metabolic syndrome-an open label pilot study, J. Clin. Biochem. Nutr. 46 (2010) 140–149. S.M. Ostojic, B. Bukmanovic, J. Calleja-Gonzalez, J.R. Hoffman, Effectiveness of oral and topical hydrogen for sports-related soft tissue injuries, Postgrad. Med. 126 (2014) 187–195. M. Ito, T. Ibi, K. Sahashi, M. Ichihara, M. Ito, K. Ohno, Open-label trial and randomized, double-blind, placebo-controlled, crossover trial of hydrogen-enriched water for mitochondrial and inflammatory myopathies, Med. Gas. Res. 1 (2011) 24. T. Ishibashi, B. Sato, M. Rikitake, T. Seo, R. Kurokawa, Y. Hara, Y. Narisomi, H. Hara, T. Nagao, Consumption of water containing a high concentration of molecular hydrogen reduces oxidative stress and disease activity in patients with rheumatoid arthritis: an open-label pilot study, Med. Gas. Res. 2 (2012) 27.