International Journal of Clinical and Experimental Medicine
Home Editorial Board Info for Authors Submission

Int J Clin Exp Med. 2015; 8(4): 5565–5570.
Published online 2015 Apr 15.

PMCID: PMC4483942
PMID: 26131139

Effect of hydrogen injected subcutaneously on testicular tissues of rats exposed to cigarette smoke

Song Chen[1,2,*] and Wei Jiang[2,*]

Abstract

Smoking is one of the most common reasons inducing reactive oxygen species in semen. High concentration of active oxygen will cause decrease of sperm density and viability and induce oxidative injury of sperm DNA which has become the hot spot in male infertility. Although hydrogen was found to be an effective remover of active oxygen in liver, heart, kidney and brain, the same effect has not been discussed in reproductive system. The aim of this study was to investigate the protective effect of hydrogen against cigarette smoke-induced damage in rat reproductive system. Adult male Wistar rats were randomly divided into four groups to conduct this experiment, results showed that rats in SK+HSI group (passive smoking and hydrogen subcutaneous injection group) exhibited larger amount of sperm count, smaller sperm deformation rate, higher levels of testosterone and SOD in serum and testis, lower levels of MDA in testis and less morphologic abnormalities compared to SK+NSI group (passive smoking and nitrogen subcutaneous injection group). As a consequence, we concluded that injected subcutaneously exerted protective effects on reproductive system injury of male rats exposed to cigarette smoke through inhibiting oxidative damage.

Keywords: Hydrogen, testicles, cigarette smoke

Introduction

Infertility is a worldwide problem, affecting about 10% to 15% childbearing couples in a global scope [1]. According to China Infertility Investigation Report in 2009, number of patients with infertility in China had been over 40,000,000, close to the level of developed countries. Among all infertility patients, male infertility accounted for about 30%~50%, which seriously affected life quality of young couples, therefore diagnosis and treatment of male infertility is an urgent task in the

biomedical field [2]. Although there are many causes for male infertility, infertility caused by sperm DNA damage has been paid more and more attention. The mechanism of sperm DNA damage hasn't been entirely clear, but some studies indicated that high concentration of active oxygen in semen caused decrease of sperm density and viability and induced oxidative injury of DNA [3]. It was reported that production of reactive oxygen species (ROS) could be induced by many factors, of which smoking was one of the most common reasons. Excessive generation of reactive oxygen species such as superoxide free radical, oxygen ions could break oxidant-antioxidant balance in the local environment, thus causing oxidative damage to body tissues [4]. A large number of studies showed that smoking could cause DNA damage of germ cells, reduction of semen quality and quantity and imbalance of the level of sex hormone [5]. Although total antioxidant levels decreased in smokers' body, quality of sperm could be improved by oral administration of antioxidants.

Hydrogen is colorless, odorless, tasteless gas which is not active at room temperature. Most biologists in the past thought that hydrogen belonged to physiological inert gas and deserved no attention in higher organisms. There were still a few people who believed hydrogen had antioxidant effects in vivo. In July 2007, Ohsawa et al. reported from Nippon Medical University in Nature Medicine that animals inhaling of 2% hydrogen could be effectively removed free radicals and improved cerebral ischemia reperfusion injury on base of selective antioxidation function of hydrogen in vivo [6], this study quickly attracted wide attention and raised research upsurge of hydrogen treatment for diseases. Sun et al. from Shanghai Second Military Medical University demonstrated through a large number of animal experiments that 2% hydrogen inhalation could treat neonatal hypoxic-ischemic brain damage; the result was quite similar to that of Japanese scholars [7]. The aim of this study was to investigate whether hydrogen injected subcutaneously had the protective effect on the reproductive system injury of male rats exposed to smoke through effectively scavenging ROS, and to provide experimental and theoretical basis for further treatment of male infertility by applying new antioxidants.

Methods and materials

Animals

Forty adult male Wistar rats (6 to 8 weeks old) weighing 180 ± 20 g were used for this study. All experimental procedures were conducted in accordance with the Guiding Principle in the Care and Use of Animals approved by the Experimental Animal Center of Chengdu University of Traditional Chinese Medicine.

Hydrogen and nitrogen preparation

For hydrogen preparation, purified H_2 was stored under atmospheric pressure at 20°C in hydrogen gas bag. For Nitrogen preparation, purified N_2 was stored under atmospheric pressure at 20°C in nitrogen gas bag. Gas flow meter was used to ensure the accuracy of gas consumption.

Protocol

Smoke was obtained from burning cigarettes. Rats were randomly divided into four groups as follows: nitrogen subcutaneous injection group (control group, n = 10); smoking and nitrogen subcutaneous injection group (SK+NSI group, n = 10); smoking and hydrogen subcutaneous injection group (SK+HSI group, n = 10); hydrogen subcutaneous injection group (HSI group, n = 10). The rats in control group accepted subcutaneous injection with nitrogen (0.2 ml/kg) and HSI group hydrogen (0.2 ml/kg). The rats in SK+NSI group and SK+HSI group were exposed to cigarette smoke (four times a day, thirty minutes per time) first. Then the rats in SK+NSI group accepted subcutaneous injection with nitrogen (0.2 ml/kg) and SK+HSI group hydrogen (0.2 ml/kg). After artificial feeding for 8 weeks, animals were killed by cervical dislocation and followed by later procedures.

Total sperm count

Immediately after cervical dislocation, the caudal epididymides were collected and weighed, then put into a 37°C preheated beaker containing 2 mL Hank's liquid. The tissue was cut into pieces, laid for 5 minutes, and filtered through single layer muslin. 20 μL of the collected sperm solution was taken into 1980 μL 0.5% formalin saline to make semen diluted, 8 μL of above diluted solution was dripped on counting chamber. Detail counts of sperms in four 1 × 1 mm grids were recorded under 200 times light microscope, data was then summed and averaged. The sperm was included if its head lay either in the left lane or at the top, and was excluded if its head lay either in the right lane or at the bottom.

Sperm count = [The mean value of sperms in four 1 × 1 mm grids] $\times 10^4$ × 100 (dilution multiple) × 2.0 (the amount of sperm concentrate)/the weight of caudal epididymides.

Measurement of abnormal sperm morphology

20 μL of the collected sperm solution was dripped 3 drops into the middle of the glass slide, then wiped with a tip from the front to the end of the slide. The wipe was performed only once. After natural drying, the slide was stained by multiple staining, and fixed for measurement of 300 abnormal sperm morphology.

Measurement of testosterone and superoxide dismutase (SOD)

Testicular testosterone levels were determined using an enzyme-linked immunosorbent assay (ELISA) kit according to the operation manual (KeMin biological technology Co., Ltd., Shanghai). The activity of SOD as an indicator for cellular antioxidative process was measured at 560 nm using an SOD determination kit according to the manufacturer's instructions. SOD activity was expressed as U/mg, using an SOD standard.

Testis malondialdehyde (MDA) measurement

Testis MDA levels were determined using an MDA Assay kit according to the operation manual. Briefly, frozen testis tissues were homogenized, then added to 500 mL enzyme, 0.5% TBA water and mixed them intensively. After bath boiling at 100°C for 15 min, rapid cooling and centrifugation at 10,000 rpm for 10 min, free MDA in the supernatant was measured at 532 nm using an MDA determination kit according to the manufacturer's instructions. MDA activity was expressed as nmol/mL, using an MDA standard.

H&E staining

After animals were killed by cervical dislocation, the rat bilateral testis tissues were fixed with 10% poly Formaldehyde Solution for 24 h, and then were paraffin embedded, paraffin sections of 4 μm thickness were cut and placed on glass microscope slides. 60°C oven for 2 h, xylene dewaxing for two times, anhydrous ethanol, 95% ethanol, 85% ethanol, distilled water, hematoxylin stained nuclei for 15 min, washing, 5% hydrochloric acid alcohol differentiation, 0.1% ammonia water washing, 15% eosin dyeing, all levels of alcohol dehydration, baking, xylene, gum resin sheet, and finally observed under microscope.

Statistical analysis

The data was statistically analyzed using software SPSS 13.0. A level of $P < 0.01$ was considered statistically significant.

Results

Sperm count and abnormal sperm morphology

After being exposed to cigarette smoke for 8 weeks, the rats in both SK+NSI and SK+HSI groups had reduction in sperm count. However, the amount of reduction was significantly greater in SK+NSI group (47.12×10^6) than that in SK+HSI group (59.1×10^6, $P < 0.01$) (Figure 1). The sperm deformation rate in SK+NSI group was significantly higher than that in the control and HSI groups (4, 5, $P < 0.01$). In the SK+HSI group, the sperm deformation rate also increased [8], but it was significantly less than that in SK+NSI group (Figure 2) ($P < 0.01$).

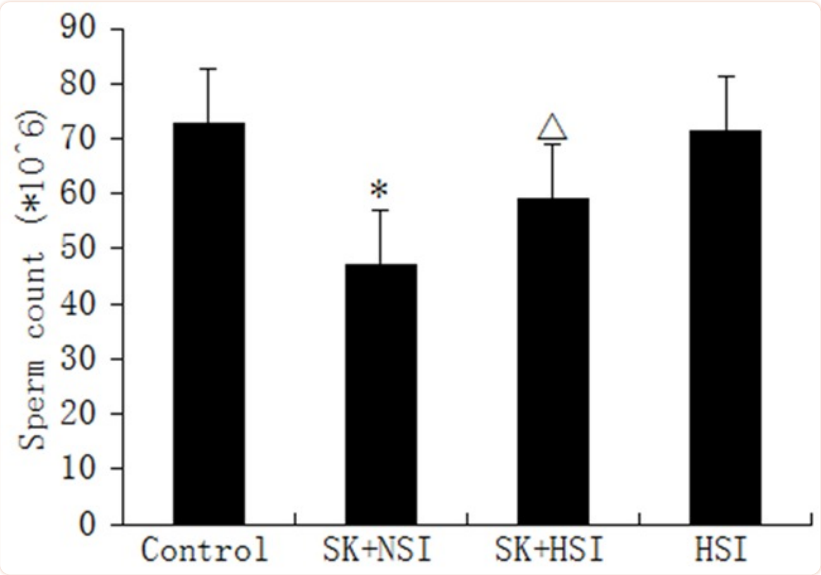

Figure 1

Effects of hydrogen subcutaneous injection on sperm count. The rats in SK+NSI group exhibited a decrease in the amount of sperm count at the end of 8 weeks, whereas in SK+HSI group this decrease was significantly smaller (*P < 0.01 compared to control; ΔP < 0.01 compared to SK+NSI group).

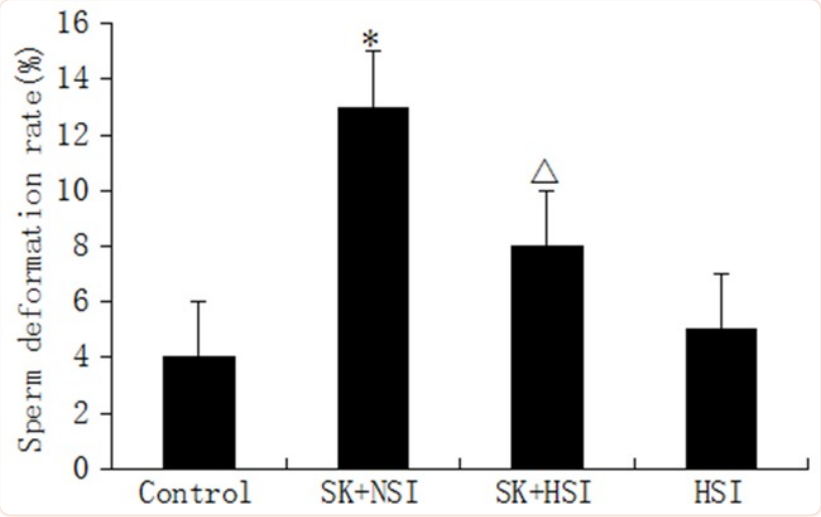

Figure 2

Effects of hydrogen subcutaneous injection on abnormal sperm morphology. The sperm deformation rate in SK+NSI group was significantly higher than that of control and HSI groups (P < 0.01). Compared to the SK+NSI group, the sperm deformation rate in SK+HSI group was significantly decreased (*P < 0.01 compared to control; ΔP < 0.01 compared to SK+NSI group).

Testosterone and SOD in serum and testis

Rats exposed to cigarette smoke exhibited significant decrease in the testosterone level in serum compared to rats in the control group ($P < 0.01$). However, the decrease in SK+NSI (1.3 ng/mL) was significantly larger than that in SK+HSI group (1.5 ng/mL, $P < 0.01$). Similarly, SOD both in serum and testis decreased in SK groups compared to control and HSI groups ($P < 0.01$), but the decrease in SK+NSI was significantly larger than that in SK+HSI ($P < 0.01$) (Table 1).

Table 1

Effects of hydrogen subcutaneous injection on testosterone and SOD in serum and testis

Groups	Serum T (ng/ml)	Serum SOD (U/mg)	Testis SOD (U/mg)
Control	1.7 ± 0.6	135.5 ± 11.5	131.4 ± 15.7
SK+NSI	1.3 ± 0.2*	91.7 ± 8.4*	89.6 ± 12.7*
SK+HSI	1.5 ± 0.4*,Δ	120.9 ± 1.5*,Δ	123.5 ± 13.0*,Δ
HSI	1.7 ± 0.5	137.0 ± 11.7	134.4 ± 15.7

Rats exposed to cigarette smoke exhibited significant decrease in the testosterone level in serum compared to rats in control and HSI groups, whereas a significant decrease of testosterone in serum was observed in SK+HSI group compared to that in SK+NSI group. Our results showed SOD levels both in serum and testis were significantly decreased in SK+NSI group compared to control and HSI groups ($P < 0.01$). In contrast, rats in SK+HSI group demonstrated a significant lesser decrease in SOD levels

* $P < 0.01$ compared to control;

Δ $P < 0.01$ compared to SK+NSI group

.

MDA in testis tissue

Testis MDA levels increased both in SK+NSI group (11.7 nmol/mL) and SK+HSI group (8.6 nmol/mL) in comparison to control and HSI groups (7.5 nmol/mL, 7.4 nmol/mL, $P < 0.01$). The increase in SK+NSI, however, was significantly larger than in SK+HSI group (Figure 3).

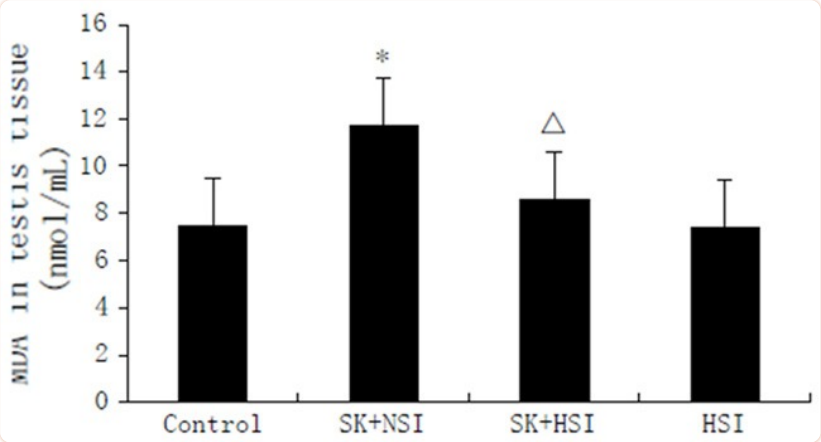

Figure 3

MDA in testis tissue. Our results demonstrated that cigarette smoke increased testis MDA levels. In contrast, MDA levels were lesser in SK+HSI group (*$P < 0.01$ compared to control; Δ$P < 0.01$ compared to SK+NSI group).

Histopathological examination of the testis tissue using H&E staining

Histopathological analyses in testis slices in the four groups were shown in Figure 4. Striking differences were observed in testis histology between the two experimental groups. Marked testicular epithelial damage, sperm gathering were observed in rats suffering from passive smoking alone. In contrast, in SK+HSI group, similar changes were found but in lesser degree, suggesting an alleviation of testis damage.

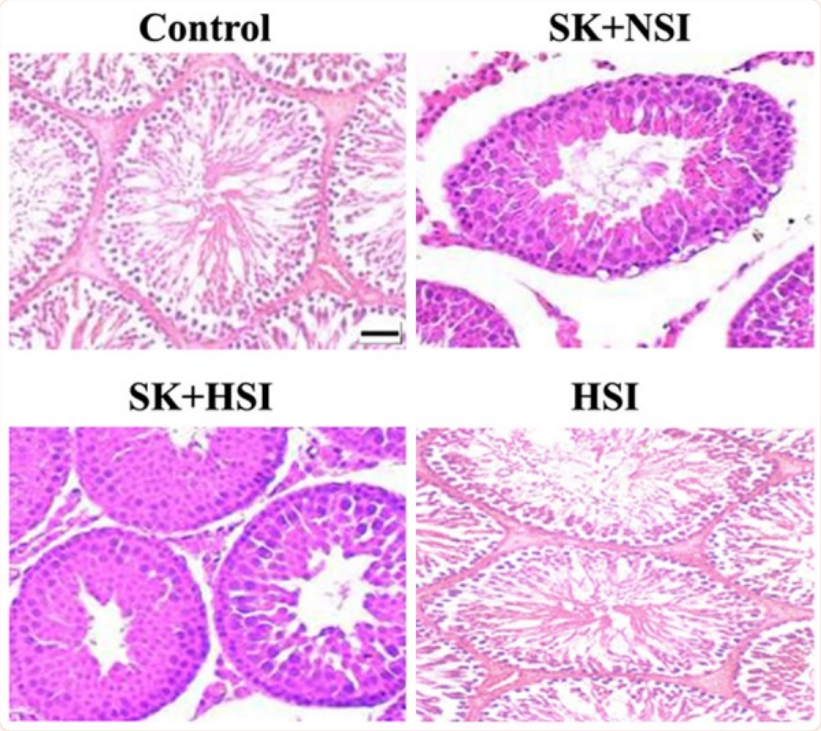

Figure 4

Histopathological examination by H&E staining. Striking differences were observed in testis histology between the two experimental groups. Marked testicular epithelial damage, sperm gathering were observed in rats suffering from passive smoking alone. In contrast, in SK+HSI group, the testicular epithelial damage was alleviated, suggesting hydrogen injected subcutaneously protected smoke-induced testis injury. Scale bar = 5 μm.

Discussion

Since oxidative damage is known as the key issue in the harm of cigarette smoke, several antioxidants have been studied to ameliorate smoke-induced testis injuries. It was suggested that superoxide dismutase and vitamin might be effective in male infertility [8]. The protective effects on oxidative testis injury by other antioxidants were also studied, but the results turned out to be quite disappointing, e.g., the effect of Vitamin C was limited due to its inability to cross biological membranes and the effect of Vitamin E was limited due to its inability to stay in the cytosol. Thus a quest for a suitable and effective treatment for smoke-induced testis injury is still going on.

The present work was undertaken to determine the putative protective effect of hydrogen subcutaneous injection on smoke-induced testis injury. We chose the hydrogen subcutaneous injection for treatment, the effectiveness of which had been validated as same as hydrogen water intraperitoneal injection. Both the results of the biochemical assays and the histopathological findings demonstrated that consumption of subcutaneous hydrogen reduces the severity of smoke-induced testis injury and oxidative stress in rats.

The rats in SK+HSI group exhibited significantly lesser degree of testis injury, as manifested by larger amount of sperm count, smaller sperm deformation rate and less morphologic abnormalities compared to SK+NSI group. Testosterone and SOD in Serum and Testis in SK+HSI group were also significantly higher than those in the SK+NSI group, demonstrating an ameliorated sperm damage. The levels of MDA in testis tissue known to be produced by peroxidation of cellular lipid and reliable indicators of oxidative damage were less in rats treated with hydrogen subcutaneous injection, suggesting that hydrogen alleviated oxidative damage. The prevention of oxidative damage was the probable mechanism of the protection of testes in rats exposed to cigarette smoke and treated with hydrogen subcutaneous injection.

Previous studies have shown that testicular epithelial damage is an important event in oxidative injury of testis [9]. The damage resistant effect of hydrogen water intraperitoneal injection was shown in neonatal hypoxia-ischemia rat model. To determine whether hydrogen subcutaneous injection exhibited the same inhibition of damage in cigarette smoke induced rat testes injury, we examined testicular epithelial damage by H&E staining. We found a significant inhibition of cell damage, consistent with previous studies.

Compared to traditional antioxidants, hydrogen, the newly explored antioxidant, offers a number of advantages. At first, modern researches have shown that hydrogen can selectively scavenge destructive free radical, especially hydrogen subcutaneous injection can reduce excessive O_2^-, H_2O_2 and OH^-, and protect DNA [10,11]. Second, due to its small molecular weight, water-solubility and lipid-solubility, hydrogen can easily penetrate biomembranes and diffuse into the cytosol, mitochondria, and nucleus [12]. Third, as hydrogen presents weak reductive, other important ROS (e.g., H_2O_2 and O_2^-) involved in cell signaling don't decreased, so the metabolic oxidation-reduction reactions are not disturbed. In addition, the application of hydrogen subcutaneous injection has unique benefits. On one hand, the latest study has confirmed that the effectiveness of hydrogen subcutaneous injection is the same as that of hydrogen water intraperitoneal injection. On the other hand, the manufacture of hydrogen-saturated saline is much more difficult due to H_2 low solubility, and it is harder to control the dose. All of these properties of hydrogen make hydrogen subcutaneous injection a promising treatment for a developing smoke-induced testis injury.

To conclude, our study confirmed that hydrogen subcutaneous injection reduced the smoke-induced testis injury. Following these encouraging results, further studies should be performed to clarify these protective effects and to elucidate the exact mechanisms of this protection.

Acknowledgements

This study is supported by the Second Affiliated Hospital of Chengdu University of TCM. The authors thank Dr. Yu Xujun in the laboratory for critically revising the manuscript.

Disclosure of conflict of interests

None.

References

1. Ombelet W, Cooke I, Dyer S, Serour G, Devroey P. Infertility and the provision of infertility medical services in developing countries. *Hum Reprod Update.* 2008;14:605–621. [PMC free article] [PubMed] [Google Scholar]

2. Hammarberg K, Baker HW, Fisher JR. Men's experiences of infertility and infertility treatment 5 years after diagnosis of male factor infertility: a retrospective cohort study. *Hum Reprod.* 2010;25:2815–2820. [PubMed] [Google Scholar]

3. Tunc O, Tremellen K. Oxidative DNA damage impairs global sperm DNA methylation in infertile men. *J Assist Reprod Genet.* 2009;26:537–544. [PMC free article] [PubMed] [Google Scholar]

4. Rahal A, Kumar A, Singh V Yadav B, Tiwari R, Chakraborty S, Dhama K. Oxidative stress, prooxidants, and antioxidants: the interplay. *Biomed Res Int.* 2014;2014:761264. [PMC free article] [PubMed] [Google Scholar]

5. Esakky P, Hansen DA, Drury AM, Moley KH. Cigarette smoke condensate induces aryl hydrocarbon receptor-dependent changes in gene expression in spermatocytes. *Reprod Toxicol.* 2012;34:665–676. [PubMed] [Google Scholar]

6. Ohsawa I, Ishikawa M, Takahashi K Watanabe M, Nishimaki K, Yamagata K, Katsura K, Katayama Y, Asoh S, Ohta S. Hydrogen acts as a therapeutic antioxidant by selectively reducing cytotoxic oxygen radicals. *Nat Med.* 2007;13:688–694. [PubMed] [Google Scholar]

7. Zheng XF, Sun XJ, Xia ZF. Hydrogen resuscitation, a new cytoprotective approach. *Clin Exp Pharmacol Physiol.* 2011;38:155–163. [PubMed] [Google Scholar]

8. Li Y, Fang J, Zhou K, Wang C, Mu D, Hua Y. Evaluation of oxidative stress in placenta of fetal cardiac dysfunction rat model and antioxidant defenses of maternal vitamin C supplementation with the impacts on P-glycoprotein. *J Obstet Gynaecol Res.* 2014;40:1623–1642. [PubMed] [Google Scholar]

9. Yuluğ E, Türedi S, Alver A, Türedi S, Kahraman C. Effects of resveratrol on methotrexate-induced testicular damage in rats. *ScientificWorldJournal.* 2013;2013:489659. [PMC free article] [PubMed] [Google Scholar]

10. Xiang L, Tan JW, Huang LJ Jia L, Liu YQ, Zhao YQ, Wang K, Dong JH. Inhalation of hydrogen gas reduces liver injury during major hepatotectomy in swine. *World J Gastroenterol.* 2012;18:5197–5204. [PMC free article] [PubMed] [Google Scholar]

11. Saitoh Y, Okayasu H, Xiao L, Harata Y, Miwa N. Neutral pH hydrogen-enriched electrolyzed water achieves tumor-preferential clonal growth inhibition overnormal cells and tumor invasion inhibition concurrently with intracellular oxidant repression. *Oncol Res.* 2008;17:247–55. [PubMed] [Google Scholar]

12. Ohta S. Recent progress toward hydrogen medicine: potential of molecular hydrogen for preventive and therapeutic applications. *Curr Pharm Des.* 2011;17:2241–2252. [PMC free article] [PubMed] [Google Scholar]