Objective: This study was aimed to investigate the protective effect of hydrogen on cigarette smoke-induced damage in rat reproductive system.

Methods: Adult male rats which suffered smoke were randomly divided into four groups, then total sperm count was counted, testosterone, superoxide dismutase (SOD), malondialdehyde (MDA) of serum and testis were determined. H&E straining of the testis tissues was also performed.

Results: Our results showed that rats in SK+HSI group (passive smoking and hydrogen 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 injection group).

Conclusion: We concluded that hydrogen injected exerted protective effects on rats’ reproductive system exposed to cigarette smoke through inhibiting oxidative damage.

Keywords: Cigarette smoke, hydrogen injected subcutaneously, protective effect

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INTRODUCTION

Infertility is a worldwide problem, affecting about 10-15% childbearing couples (1-3). 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 (4). 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 (5). Sperm DNA damage is one of the most important reasons for male infertility, and its mechanism 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 (6). It was reported that production of reactive oxygen species (ROS) could be induced by many factors, of which smoking was a common reason. 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 (7). 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 (8).

Previous reports indicated that hydrogen had antioxidant effects in vivo. It’s reported that animals inhaling of 2% hydrogen could effectively remove free radicals and improve cerebral ischemia reperfusion injury (9), and this study quickly attracted wide attention and raised research upsurge of hydrogen treatment for diseases. Sun et al. demonstrated that 2% hydrogen inhalation could treat neonatal hypoxic-ischemic brain damage (10). The aim of this study was to investigate whether hydrogen injected subcutaneously had protective effect
on reproductive system exposed to smoke via ROS scavenging, and provide an experimental and theoretical basis for treatment of male infertility.

METHODS AND MATERIALS

Animals
Forty adult male rats (6-8 weeks old, weighing 180 ± 20 g) were used. 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 flowmeter was used to ensure the accuracy of gas consumption.

Protocol
Smoke was obtained by burning cigarettes. Rats were randomly divided into four groups: nitrogen subcutaneous injection group (control, $n =10$); smoking and nitrogen subcutaneous injection group (SK+NSI, $n =10$); smoking and hydrogen subcutaneous injection group (SK+HSI, $n =10$); hydrogen subcutaneous injection group (HSI, $n =10$). 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 and SK+HSI group were exposed to smoke (4 times/day, each time lasts 30 min). Then rats in SK+NSI and SK+HSI group accepted subcutaneous nitrogen and hydrogen injection (0.2 mL/kg), respectively. After feeding for 8 weeks, animals were sacrificed and followed by later procedures.
Protective Effect of Hydrogen Injection on Rats’ Testicular Tissues

Total sperm count

The cauda 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 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] ×10⁴ ×100 (dilution multiple) × 2.0 (the amount of sperm concentrate) / the weight of cauda epididymides.

Measurement of abnormal sperm morphology

The 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 ELISA kit according to the operation manual (KeMin biological technology, Shanghai, China). The activity of SOD as an indicator for cellular anti-oxidative process was measured at 560nm using an SOD determination kit. 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 500mL enzyme, 0.5% TBA water and mixed them intensively. After bath boiling at 100°C for 15min, rapid cooling and centrifugation at 10,000 rpm for 10 min, free MDA in the supernatant was measured at 532nm 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 rats bilateral testis tissues were fixed with 10% poly Formaldehyde Solution for 24h, then were paraffin embedded, paraffin sections of 4 um 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 (11), but it was significantly less than that in SK+NSI group (Figure 2) (P <0.01).

**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).

**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).

**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.

**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 (12). It was suggested that superoxide dismutase and vitamin might be effective in male infertility (11). 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,
Protective Effect of Hydrogen Injection on Rats’ Testicular Tissues

demonstrating the 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 (13). 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 (14). 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 (15). At first, modern researches have shown that hydrogen can selectively scavenge destructive free radical, especially hydrogen subcutaneous injection can reduce excessive O2\(^-\), H\(_2\)O\(_2\) and OH\(^-\), and protect DNA (16,17). 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 (18). Third, as hydrogen presents weak reductive, other important ROS (e.g., H\(_2\)O\(_2\) and O2\(^-\)) 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 H2 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.

CONCLUSION
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.

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AUTHORS’ NOTE
The authors declare that there is no conflict of interests.
REFERENCES


Table 1. Effects of hydrogen subcutaneous injection on testosterone and SOD in serum and testis.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Serum T (ng/ml)</th>
<th>Serum SOD (U/mg)</th>
<th>Testis SOD (U/mg)</th>
</tr>
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<tbody>
<tr>
<td>Control</td>
<td>1.7 ± 0.6</td>
<td>135.5 ± 11.5</td>
<td>131.4 ± 15.7</td>
</tr>
<tr>
<td>SK+NSI</td>
<td>1.3 ± 0.2 *</td>
<td>91.7 ± 8.4 *</td>
<td>89.6 ± 12.7 *</td>
</tr>
<tr>
<td>SK+HSI</td>
<td>1.5 ± 0.4 *Δ</td>
<td>120.9 ± 1.5 *Δ</td>
<td>123.5 ± 13.0 *Δ</td>
</tr>
<tr>
<td>HSI</td>
<td>1.7 ± 0.5</td>
<td>137.0 ± 11.7</td>
<td>134.4 ± 15.7</td>
</tr>
</tbody>
</table>
Fig 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).

Fig 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).
Protective Effect of Hydrogen Injection on Rats’ Testicular Tissues

Fig 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; ^p <0.01 compared to SK+NSI group).

Fig 4. Histopathological examination by H&E staining. A-D represented the groups of Control, SK+NSI, SK+HIS, and HIS, respectively. 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 = 5μm.