**Effect of Honey on Testicular Functions in Rats Exposed to Cigarette Smoke**

Mohamed Mahaneem1*, Siti Amrah Sulaiman2, Hasnan Jaafar3, Kuttulebbai Nainamohamed Salam Sirajudeen4, Zul Izhar Mohd Ismail5 and Mohammed Nazrul Islam6

1 Department of Physiology, School of Medical Sciences, Universiti Sains Malaysia, Kelantan, Malaysia
2 Department of Pharmacology, School of Medical Sciences, Universiti Sains Malaysia, Kelantan, Malaysia
3 Department of Pathology, School of Medical Sciences, Universiti Sains Malaysia, Kelantan, Malaysia
4 Department of Chemical Pathology, School of Medical Sciences, Universiti Sains Malaysia, Kelantan, Malaysia
5 Department of Anatomy, School of Medical Sciences, Universiti Sains Malaysia, Kelantan, Malaysia
6 Islami Bank Medical College, Rajshahi, Bangladesh

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*Corresponding author: Email: mahaneem@kck.usm.my

Summary

Honey is traditionally consumed by the local Malaysian population as a nutrient, as well as for the enhancement of fertility. The decline in male reproductive health and fertility in the last 30 years has been linked to environmental toxicants including cigarette smoke (CS). In human and experimental studies, CS exposure has been associated with decreased plasma testosterone level, lower sperm count and increased percentage of abnormal sperm. The aim of this study, therefore, was to determine the possible protective role of honey against the toxic effects of CS on testicular functions in rats. Thirty-two adult Sprague-Dawley rats were randomly divided into 4 groups (8 rats per group) i.e. control group, honey-treated group (H), cigarette smoke-exposed group (CS) and honey-treated plus CS-exposed group (H+CS). Rats in control and CS groups received oral administration of distilled water daily while rats in H and H+CS groups received honey (1.2 g/kg body weight) orally by gavage daily. Rats in CS and H+CS groups were also exposed to CS for 8 min (3 times/day). After 13 weeks of treatment, each rat was sacrificed for reproductive parameters analysis. Rats in CS group had significantly lower sperm count, daily sperm production, percentage of motile sperm and testosterone level as well as a higher percentage of abnormal sperm compared to control and H groups. However, supplementation of honey significantly improved all these parameters in H+CS group. Administration of honey significantly attenuated the toxic effects of CS on spermatogenesis and testosterone level in rats. This study suggests that honey might have a protective effect against CS-induced impaired testicular functions in rats.

Keywords: Honey, cigarette smoke, sperm, testis, testosterone.

Introduction

Honey is a natural product of bees formed from nectar collected from flowering vegetation. It has been reported that honey contains moisture, sugars such as glucose and fructose, enzymes such as catalase and glutathione reductase, trace essential elements such as iron, copper, zinc and calcium, vitamins such as vitamin A, C and E as well as some flavonoids and phenolic acids (Al-Waili, 2003; Yao et al., 2004; Michalkiewicz et al., 2008; Gheldof et al., 2002). In addition, it possesses some biological properties such as antioxidant (Perez et al., 2006), antimicrobial (Estevinho et al., 2008), anti-inflammatory (Prakash et al., 2008) and immunomodulatory effects (Timm et al., 2008). Traditionally, honey is frequently consumed by the local Malaysian population as a nutrient, as well as for the enhancement of fertility and vitality. Recently, a higher sperm count was observed in our study following the oral administration of Malaysian honey at a dose of 1.2 g/kg body weight/day for 28 days in rats (Mahaneem et al., 2007). A significantly higher epididymal sperm count was also found in adult rats following the daily treatment of 5% Palestinian honey for 20 days (Abdul-Ghani et al., 2008).

The decline in male reproductive health and fertility for the past 30 years has been linked to environmental toxicants and xenobiotics (Sikka and Wang, 2008). One of the toxicants that have
detrimental effects on male reproductive function is cigarette smoke (CS). Cigarette smoking has been reported to be associated with abnormalities in male reproductive function such as decreased sperm count (Richthoff et al., 2008) and motility (Gaur et al., 2007), increased percentage of abnormal sperms (Evans et al., 1981) and sperm chromatin damage (Potts et al., 1999). In experimental studies, rodents that were exposed to CS were also associated with a decreased plasma testosterone level (Yardimci et al., 1997), reduced sperm count and motility (Yamamoto et al., 1998), presence of degenerated and lower number of Leydig cells (Yardimci et al., 1997) as well as reduced sperm fertilizing capacity and embryonic development (Kapawa et al., 2004). To date, whether honey has any protective effect against the toxic effects of CS on testicular functions is yet to be reported. Therefore, this study was undertaken to investigate the effect of honey on testicular functions in adult rats exposed to CS.

Materials and methods

Chemicals

Sodium chloride and eosin Y were purchased from Sigma-Aldrich (USA) and Ted Pella Inc. (CA), respectively. Milli-Q® water system (Millipore, USA) was used for all preparations. Commercially available cigarettes (Benson & Hedges, British American Tobacco Bhd., Malaysia) containing about 1.4 mg nicotine and 15 mg tar per cigarette were used for all CS exposures.

Honey sample

Honey used in this study was kindly supplied by Federal Agricultural Marketing Authority (FAMA), Malaysia. It is a wild multifloral honey collected form beehives built on a tall tree, Koompassia excelsa (locally named as ‘Tualang’ tree) that grows in the Rain Forest of Kedah, Malaysia. This honey which is also locally known as Tualang honey was collected in March 2007. It was filtered to remove solid particles and concentrated (20% w/v water) by oven drying at 40°C by the supplier.

Animals

A total of thirty-two adult male Sprague Dawley rats, aged 10 weeks (270-320g) were obtained from the Laboratory Animal Research Unit, Health Campus, Universiti Sains Malaysia. Rats were individually housed in a polycarbonate cage and maintained on a 12-h light/dark cycle at 20-24°C. They were provided a standard pellet diet and water ad libitum and acclimatized to the environment for 2 weeks prior to experimental use. This study protocol was approved by the Animal Ethics Committee, Universiti Sains Malaysia (PPSG/07 (A)/080) and conducted in accordance to the Code of Practice for the Care and Use of Animals for Scientific Purposes, USM Health Campus (2002).

Experimental study

Animals were randomly divided into 4 groups (8 rats per group) i.e. control group, honey-treated group (H), CS-exposed group (CS) and honey-treated plus CS-exposed group (H+CS). Rats in control and CS groups received oral administration of distilled water (0.5 mL) while rats in H and H+CS groups received freshly prepared honey orally by gavage between 08:00 h to 08:30 h daily. The honey at a dose of 1.2 g/kg body weight was freshly diluted using distilled water (as a vehicle) to a volume of 0.5 mL of solution. Based on the body surface area normalization method (Reagan-Shaw et al., 2007), this dose was calculated relative to the local human consumption of honey which is 0.2 g/kg body weight daily. Animals in CS and H+CS groups were exposed to CS for 3 times daily using a whole body smoke exposure chamber (45 x 25 x 20 cm with 2 compartments). For each exposure, ten cigarettes were burnt in one compartment and the smoke produced was continuously ventilated by 2 air pumps to another compartment where the rats were placed and exposed to the smoke for 8 min (Cesar-Neto et al., 2003). In our preliminary study, this method of exposure produced serum cotinine levels ranging from 1.58 to 3.73 ng/mL in male rats (unpublished observation). These levels correspond to the serum cotinine level in human who smoke more than 20 cigarettes per day (more than 0.26 ng/mL) (Barua et al., 2002). The rats in the control and H groups were subjected to similar conditions but exposed to room air and not cigarette smoke.

Body weight and food intake of each rat were monitored weekly and total food intake and weight gain were calculated. After 13 weeks of treatment, laparotomy was performed on each rat under diethyl ether anaesthesia. Vas deferens was quickly removed and trunk blood was collected. Animals were then sacrificed by an overdose of anesthesia. The blood was allowed to clot, centrifuged at 4000 rpm and the collected serum was kept frozen at -80°C until assayed for the levels of reproductive hormones. Other reproductive organs including testis and epididymis were carefully dissected out for elongated spermatid count and sperm analysis, respectively. Absolute weight of testis was recorded and its relative weight (in comparison to body weight in percentage) was calculated.

Elongated spermatid count and daily sperm production

Elongated spermatid count was done as reported earlier (Faqi et al., 2004) with some modifications. Briefly, the testis was cleaned from adhering connective tissue and weighed. A portion of the testicular tissue was taken, weighed and homogenized with 2% eosin in normal saline for 1 min at a medium speed (IKA Labortechnik, Germany) followed by filtration through a nylon mesh. The suspension was then used for the estimation of homogenization-resistant elongated spermatid (Stage 17-19) count using Neubauer chamber (Assistant, Germany). Counts for the spermatid in eight
haemocytometer chambers (except the central erythrocyte chamber) were averaged and expressed as the number of spermatid count per testis. Daily sperm production was then calculated as the number of spermatid divided by 6.1.

**Sperm motility**

The content of the vas deferens was squeezed into 1 mL of normal saline at room temperature. One drop of the sperm suspension was charged into Markler’s counting chamber (Sefi-Medical Instruments, Israel) and the number of motile and non-motile sperm was counted in ten random fields. The number of motile sperm was then expressed as a percentage of the total number of sperm (Kaur and Bansal, 2004).

**Sperm count**

Sperm count was performed as reported earlier (Narayana et al., 2005) with minor modifications. Briefly, cauda epididymis was carefully separated from the testis and minced in 2 mL of normal saline followed by filtration through a nylon mesh. The suspension was then stained with 2% eosin in normal saline and sperm heads were counted using a Neubauer haemocytometer chamber. Counts for the sperm head in eight haemocytometer chambers (except the central erythrocyte chamber) were averaged and expressed as the number of sperm per cauda epididymis.

**Sperm morphology**

A drop of stained sperm suspension (which was prepared for sperm count) was smeared on a glass slide, air-dried and visualized microscopically at a magnification of 400x. For each rat, 200 sperms were screened and the percentage of total abnormalities of heads (such as microcephalus, detached head, flattened head, doubled head and bent neck) and/or tails (such as coiled tail, bent tail and doubled tail) was determined (Narayana et al., 2005).

**Reproductive hormones measurement**

Testosterone, luteinizing hormone and follicle-stimulating hormone in rat serum were measured by enzyme immunoassays using commercially available kits form Endocrine Technologies (USA), Usclife (China) and Biocode-hycel (Belgium), respectively. Samples were run in the same assay to avoid inter-assay variations.

**Statistical analysis**

Data were analyzed using the Statistical Package for Social Science (SPSS) version 12.0.1. and presented as median (interquartile range). Kruskal-Wallis test was used to assess the differences among groups. If the results were statistically significant, the differences between two groups were assessed by Mann-Whitney U test. A value of p < 0.05 was considered as significant.

**Results**

There were significant differences for total food intake and body weight gain among the experimental groups (Table 1). Rats in H group had significantly lower food intake but similar body weight gain as compared to control group. However, rats in CS and H+CS groups had significantly lower total food intake and body weight gain compared to control and H groups. Body weight was increased in all groups by 54%, 54%, 13% and 9% for control, H, CS and H+CS groups, respectively. Furthermore, no significant differences were found for total food intake and body weight gain between CS and H+CS groups.

Following 13 weeks of treatment, no significant differences were found between control and H groups for all the parameters such as testicular weight, spermatid count, daily sperm production (DSP) and sperm parameters (Table 2). However, rats in CS and H+CS groups significantly had lower absolute testicular weight and higher relative testicular weight as compared to control and H groups. No significant differences were found for the testicular weight between CS and H+CS groups.

Epididymal sperm and spermatid counts as well as DSP in rats from CS group were significantly lower than control and H groups (Table 2). However, these parameters in H+CS group were significantly higher than CS group and similar with control and H groups.

**Table 1.** Total food intake and body weight in all experimental groups.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control (g)</th>
<th>H (g)</th>
<th>CS (g)</th>
<th>H+CS (g)</th>
<th>p (KW test)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total food intake</td>
<td>2601(450)</td>
<td>2270(159) a</td>
<td>1753(111) b, a</td>
<td>1774(217) a, b</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Initial BW (g)</td>
<td>296(23)</td>
<td>287(18)</td>
<td>293(9)</td>
<td>307(15)</td>
<td>-</td>
</tr>
<tr>
<td>Final BW (g)</td>
<td>453(78)</td>
<td>447(65)</td>
<td>332(33)</td>
<td>335(35)</td>
<td>-</td>
</tr>
<tr>
<td>BW gain (g)</td>
<td>159(48)</td>
<td>156(53)</td>
<td>38(38) b</td>
<td>28(22) a, b</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Data are presented as median (interquartile range), (n=8). BW = body weight; H = honey; CS = cigarette smoke; H+CS = honey plus cigarette smoke; KW = Kruskal Wallis; a p < 0.05 compared to control group and b p < 0.05 compared to H group by Mann-Whitney U test.
groups. Rats in CS and H+CS groups had a significantly higher percentage of abnormal sperm and a lower percentage of motile sperm than rats in control and H groups (Table 2). However, when compared to CS group, rats in H+CS group had a significantly lower percentage of abnormal sperm and a higher percentage of motile sperm.

There were no significant differences for the serum levels of luteinizing and follicle-stimulating hormones among the groups (Table 3). The levels of these reproductive hormones were also similar between control and H groups. However, rats in CS group had significantly lower testosterone level compared to control and H groups. In H+CS group, the testosterone level was significantly higher than CS group but similar with control and H groups.

## Discussion

In this study we examined the effect of honey supplementation on spermatid count, DSP, sperm parameters and reproductive hormones following 13-week whole body cigarette smoke exposure in adult Sprague-Dawley rats. In rats from H group, the lower food intake but similar body weight gain compared to control and H groups (Table 2). However, when compared to CS group, rats in H+CS group had a significantly lower percentage of abnormal sperm and a higher percentage of motile sperm.

Data are presented as median (interquartile range) (n=8). TW, testicular weight; DSP, daily sperm production; H, honey; CS, cigarette smoke; H+CS, honey plus cigarette smoke; KW, Kruskal Wallis; AW, absolute weight; RW, relative weight. *p < 0.05 compared to control group, **p < 0.05 compared to H group and ***p < 0.05 compared to CS group by Mann-Whitney U test.

### Table 2. Testicular weight, spermatid count, daily sperm production and sperm parameters in all experimental groups

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control</th>
<th>H</th>
<th>CS</th>
<th>H+CS</th>
<th>p (KW test)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Absolute TW (g)</td>
<td>1.62(0.21)</td>
<td>1.57(0.08)</td>
<td>1.33(0.18)</td>
<td>1.38(0.09)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Relative TW (%)</td>
<td>0.35(0.06)</td>
<td>0.35(0.03)</td>
<td>0.40(0.06)</td>
<td>0.41(0.05)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Spermatid count (x10^6/testis)</td>
<td>39.93(12.21)</td>
<td>37.10(5.48)</td>
<td>26.03(10.65)</td>
<td>39.93(12.29)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>DSP (x10^6/testis/day)</td>
<td>6.55(2.00)</td>
<td>6.08(0.90)</td>
<td>4.27(1.75)</td>
<td>6.55(2.01)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Sperm count (x10^6/epididymis)</td>
<td>37.15(14.10)</td>
<td>28.25(10.63)</td>
<td>15.23(6.71)</td>
<td>32.88(1.19)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Abnormal sperm (%)</td>
<td>7.00(3.00)</td>
<td>8.00(3.00)</td>
<td>21.50(5.00)</td>
<td>13.50(9.00)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Motile sperm (%)</td>
<td>79(13)</td>
<td>71(25)</td>
<td>25(13)</td>
<td>53(39)</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

### Table 3. Serum reproductive hormones in all experimental groups

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>H</th>
<th>CS</th>
<th>H+CS</th>
<th>p (KW test)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Testosterone (ng/mL)</td>
<td>9.00(8.27)</td>
<td>12.05(4.36)</td>
<td>3.10(4.18)</td>
<td>11.69(9.94)</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>LH (mIU/mL)</td>
<td>0.96(1.51)</td>
<td>1.42(1.21)</td>
<td>0.99(1.17)</td>
<td>1.22(0.52)</td>
<td>NS</td>
</tr>
<tr>
<td>FSH (ng/mL)</td>
<td>4.07(0.99)</td>
<td>4.02(0.82)</td>
<td>4.61(2.76)</td>
<td>5.78(2.97)</td>
<td>NS</td>
</tr>
</tbody>
</table>

Data are presented as median (interquartile range) (n=8). H = honey; CS = cigarette smoke; H+CS = honey plus cigarette smoke; KW = Kruskal Wallis; LH = luteinizing hormone; FSH, follicle-stimulating hormone; NS, not significant. *p< 0.05 compared to control group, **p < 0.05 compared to H group and ***p < 0.05 compared to CS group by Mann-Whitney U test.
this study indicate that honey acts locally at the testicular level by possibly restoring or improving the function of Leydig cell, but this effect needs further study.

The higher percentage of abnormal sperm and the lower percentage of motile sperm following the exposure to CS as found in CS group compared to the control group might also suggest the direct toxic effect of CS on germ cells. However, these parameters were improved with the administration of honey in rats from H+CS group. So honey may also have some protective effect against the direct toxic effect of CS on germ cell structure and function in testis and/or in the post-testicular organs such as epididymis and vas deferens.

Oxidative stress has been postulated as one of the mechanisms leading to testicular damage following exposure to CS (Rajpurkar et al., 2000). Honey has been reported to have some antioxidants such as vitamins A and E (Al-Waili, 2003), catalase (Gheldof et al., 2002) and flavonoids (Yao et al., 2004). Recently, it was reported that this Malaysian honey contained antioxidant such as phenols and possess anti-radical and antioxidant properties (Mohamed et al., 2010). Therefore, it is plausible to suggest that the effect of honey in attenuating the CS-induced impaired testicular functions in this study could be partly mediated by its counteraction on oxidative stress within rat reproductive organs via its antioxidant properties. Thus, further study is needed to determine the levels of oxidative stress markers in the rat testis to support this hypothesis.

Apart from that, the impaired testicular functions following the exposure to CS could also be due to hypoxia, which in turn might decrease blood flow and inhibit vasmotion in the testis (Koskinen et al., 2000). Phenolic compounds in plants, termed phytoestrogens that possess oestrogenic activity (Qosoki and Kennely, 2003), are also found in honey (Gheldof et al., 2002, Mohamed et al., 2010). Moreover, there is a strong correlation between plasma oestrogen level and testicular blood flow in male horses suggesting that oestrogen may play a role in testicular perfusion (Bollwein et al., 2008). Indeed, the presence of oestrogen receptors and aromatase, an enzyme that transforms androgens into oestrogens, in germ cells of the testis might suggest that locally produced oestrogen may also involved in spermatogenesis (Carreau et al., 2007). Consequently, it is also possible that honey could ameliorate the toxic effect of CS on testicular function partly by improving testicular blood flow and spermatogenesis via the oestrogenic activity of its phenolic compounds and this requires further study.

In conclusion, administration of honey significantly attenuated the detrimental effect of CS on the spermatogenesis and testosterone level in rats. This study indicates that honey has a protective effect against CS-induced impaired testicular functions in rats, but further research to elucidate its exact mechanism of action is essential.

Acknowledgements

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