Evaluation of sex steroid hormones and reproductive irregularities in diethyl phthalate exposed premature mice: modulatory effect of raw honey against potential anomalies

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Abstract

Phthalates, plasticizing chemicals are top-rated environmental contaminants. Diethyl phthalate (DEP), a chief member of this family was declared a potent endocrine-disruptor and carcinogen in animals and humans. The current study was designed to explore the probable reproductive damage induced by DEP and the therapeutic efficacy of raw honey in male albino mice. Four weeks old 50 male mice, were randomized equally in five groups, as control (C) received 0.1 ml distilled water; vehicle control (VC) received 0.1 ml corn oil; DEP (3mg/g/BW) dissolved in corn oil; Honey control (HC) administered 0.2 mg/g/day); P + H administered with DEP and honey (3mg and 0.2 mg/g/BW/day respectively). Mice were treated through oral gavage for 54 days routinely, acclimatized for 6 days and dissected. In the first instance, the antioxidant potential and total phenolic contents of honey were analyzed through Ferric reducing antioxidant power assay and Folin-Ciocalteu assay to confirm the antioxidant capacity of honey. The morphological, morphometric, histological, micrometric, sperm count, hormonal analyses, and antioxidant capacity test in tissue homogenates were conducted by using tissues (testis, epididymis) and blood samples of mice. Mice exposed to DEP have a significant increase in body weight, LH level, seminiferous tubule lumen diameter and decrease in the gonado-somatic index, testosterone level, sperm count, and seminiferous tubule diameter. Additionally, histopathology of testes showed interstitial spaces dilations, exfoliations, Leydig cells atrophy, germ cell degenerations and spermatid retention in DEP exposed testes sections. However, concomitant use of honey and DEP had shown a significant improvement in histopathological lesions, steroid hormone levels, and healthy sperm count. By these results, it is concluded that honey possessed antioxidant potential that can efficiently protect DEP-induced anomalies in male mice.

Introduction

In the era of urbanization and industrialization, the general population is unavoidably exposed to various contaminants from diversified sources (Hernandez et al., 2019; Docea et al., 2018). It is highlighted by recent epidemiological and biomonitoring studies that exposure to these contaminants is associated with public health-hazardous that leads to nephrotoxicity, cardiotoxicity, hepatotoxicity, immunotoxicity, nephrotoxicity, as well as endocrine disruption (Andjelkovic et al., 2019; Buha et al., 2013; Docea et al., 2019; Tsatsakis et al., 2017). Some of the already proven and well-established chemicals involved in endocrine-disruption are, polybrominated diphenyl ethers, polychlorinated biphenyls, heavy metals, microplastics, pesticides, bisphenols as well as phthalates (Buha et al., 2018; Djordjevic et al., 2019; Gore et al., 2015). Recently, phthalates are considered top-listed classified endocrine disruptors that disrupt the hormonal balance on excessive exposure through diet, environment and daily household stuff (Tanner et al., 2020).

Phthalates are recognizable environmental pollutants that are frequently used as additives to enhance polymer versatility and malleability in the production of plastics. These are synthetic, colorless, odorless, and lipophilic compounds (Shaha and Pandit, 2020). In 1920, phthalates were first time used for commercial applications (Rahman et al., 2004). The six most familiar phthalates such as di (2-ethyl

hexyl) phthalate, diethyl phthalate, dibutyl phthalate, dibenzyl phthalate, di-isopropanol phthalate were declared as environmental pollutants by the United States Environmental Protection Agency (Gani and Kazmi, 2020; Li et al., 2020).

Diethyl phthalate (DEP) is a widely used co-contaminant of microplastic and proved as an endocrine disruptor. It is structurally composed of alkyl chains with colorless, odorless and oily liquid nature soluble in the organic solvent (Zhou et al., 2020; Jeong et al. 2020). DEP is commonly used in pharmaceuticals, medical bags, shampoo, perfumes, toys, and food packaging (Radke et al. 2020).

Although DEP has non-covalent bonding with plastic but makes it easy to leach in food products as well as the environment (NRC, 2009). When humans are exposed to DEP, it is metabolized into mono-ethyl phthalate (MEP) within 3 to 28 h (half-life) and eliminated from the body through urine (Radke et al., 2020). DEP compared to other phthalate detected more in the urine sample of the US population. Due to its ubiquity, its enormous and continuous exposure is associated with impaired fertility, shorter anogenital distance, increased oxidative damage to sperm cells and carcinogenesis (Hauser et al., 2007, Gopalakrishnan et al., 2020). Hence, animal studies are a prerequisite to understanding the potential link between diethyl phthalate exposure and adverse outcomes. These investigations may provide an understanding of the dose-related characteristics of phthalates. Therefore, it is inevitable to evaluate the noxious effects of diethyl phthalate on prepubertal mice's reproductive parameters.

On the other hand, honey is a natural sweetener and utilized enormously for health benefits (Selvaraju et al., 2019). It is made up as a result of regurgitation and evaporation of floral nectar by honeybees (Pipicelli et al., 2009). Honey consists of flavonoids, antioxidants, organic acid, minerals, protein, vitamins and carbohydrates such as fructose, sucrose, raffinose and glucose (Mosavat et al., 2019). These chemical constituents had made honey, a well-known antibacterial, antimicrobial and antifungal agent (Aswin and Neelusree, 2019). Ancient people used honey both for nutritional purposes and for its medicinal aims (Adebolu, 2005). Experimental studies support its usage due to its anti-inflammatory, antiviral, antibacterial, antioxidant and other bioactivities (Murosak et al., 2002). The therapeutic efficacy of honey is acknowledged in various major diseases, Diabetes, cancer, cardiovascular and degenerative diseases (Hossen et al, 2017). Its positive role as a nephroprotective agent in chemical-induced toxicity is also well-established (Ibrahim et al., 2016). Honey supplementation involved *in-vitro* maturation and improvement of sheep oocytes (Kaabi et al., 2020). Additionally, honey was also reported as an effective remedy to improve fertility status in males and females but results are still inconclusive (Meo et al., 2017). The present study aims to explore the therapeutic potential of honey against DEP-induced probable testicular lesions and reproductive disruptions in albino mice.

Materials And Methods

Ethical statement

All animal trials were executed according to local and worldwide procedures. The nearby way is the Wet op de dierproeven (article 9) of Dutch law (international) and an associated rule planned via the Bureau

of Animal Research Licensing, Local University as detailed in our earlier papers (Ali et al. 2020; Hussain et al. 2020; Ali et al. 2020; Ara et al. 2020; Ali et al. 2020; Khan et al. 2019; Ali et al. 2019; Mumtaz et al. 2019; Mughal et al. 2019; Dar et al. 2019). The rearing and use of mice were carried out using NIH Publication "Guide for the Care and Use of Laboratory Animals" (NRC 2004) and with the approval vide No. D/681/UZ dated 04-04-2019 by the local bioethical committee of the University on animal experimentation.

Chemicals

Diethyl Phthalate 99.5% purity, MW: 222.24 were purchased by Sigma Aldrich. Honey used in this research was multiflora honey collected in April from *Apis mellifera* colonies taken from Honeybee Research Farm, University of the Punjab, Lahore, Pakistan. Corn oil with 99.9% purity was purchased from the Akbari market, Lahore. Hormones Diagnostic Kits were BioVision Company, distributed by Lab Science, Pakistan. Ethanol, hematoxylin, eosin, hydrochloric acid, ferric chloride and ferrous sulphate were obtained from Merck & Co., Inc. TPTZ (2,4,6-Tris(2-pyridyl)-s-triazine) was acquired from Sigma-Aldrich Company. All chemicals used in the present research were of analytical grade.

Animals Rearing

Swiss Webster strain of albino mice *Mus musculus* was used in the experiment. Mice were raised employing steel cages with well-managed conditions of 12 hours' light/dark cycle at 26 ± 2 °C and 45-55% relative humidity in the animal house of the Institute of Zoology, University of the Punjab, Lahore, Pakistan. Mice were fed with commercially available feed (national feed No. 14 by National Feeds industries, Lahore, Pakistan) pellets and water *ad libitum*.

Experimental Design

Four-week-old 50 male mice with body weight (B.W.) 13 ± 2 g were randomly classified into five groups (n=10). Group 1: control (C) provided 0.1 ml distilled water; group 2: vehicle control (VC) administered 0.1 ml corn oil; group 3: DEP treated with 3 mg/g B.W. DEP, dissolved in corn oil in such a way that 0.1 ml contained required concentrations of DEP; group 4: DEP (3 mg/g B.W) and honey (0.2 mg/g); Group 5: honey control (HC) exposed to honey (0.2 mg/g B.W). Treatments were given through oral gavage for 54 days daily, once a day. Before treatment, antioxidant potential and total phenolic content (TPC) were also assessed separately for raw honey used in this study.

General observations

Behavioral and other physical changes in mice of all groups were noted and recorded twice a day regularly. The body weight of each animal in each group was measured on weekly basis and dose concentrations were adjusted considering their weights accordingly.

Samples recovery

After 54 days, the animals were acclimatized for 6 days for self-restoration and were euthanized after isoflurane inhalation. Testes and epididymis were successfully recovered for morphometric, histopathologic, micrometric analyses, sperm count, and antioxidant capacity test (FRAP). The blood samples were collected by cardiac perfusion for hormonal assays under deep anesthesia.

Morphometric Analysis

Mice body weight and wet weight of the testes were measured with analytical balance (Ax120 SHIMADZU, JAPAN). Furthermore, the size (length/width) of testes was recorded using a digital Vernier caliper.

Histopathology

Testicular tissues were fixed in Bouin's fixative for 40 hrs at room temperature (33°C), dehydrated with graded ethanol, cleared with xylene and embedded in paraffin blocks. The thick sections (5µm) were prepared by rotary microtome and stained with eosin and hematoxylin following established protocols (Bancroft and Layton, 2013). The sections were observed and digital photographs were captured under a camera-fitted microscope to highlight histopathological defects and micrometric data.

Micrometry

For micrometric measurements, from photomicrographs taken at 40X, twenty nearly round randomly chosen seminiferous tubules were traced. Seminiferous tubule and lumen diameters were measured through bisecting lines drawn at the circumference of the tubule using ImageJ software at 400 magnifications (Montoto et al. 2012). The cross-sectional area of the seminiferous tubule (STA) was calculated following a geometric constant equation (Mustafa et al. 2019)

$$STA = \pi r^2$$

Where r is the tubule radius.

The area of the tubular lumen (LA) was calculated by the equation:

$$LA = \pi Lr^2$$

Where Lr is the luminal radius.

The epithelium area (EA) was obtained by subtracting LA from STA. Results were expressed as square millimeters (mm²).

Smear preparations for sperm count and morphology

Testes with attached epididymis recovered in saline solution, give a midline incision, gently crushed on clean glass with a glass rod and curdy material used for the sperm count and sperm morphological

analysis through the chamber counting method. The sperm with complete head and tail counted once in 4 big 1 mm² quadrates under a light microscope (Kirkman-Brown et al., 2009).

Hormonal Analysis

Hormonal analysis for testosterone level and Luteinizing hormone were assessed in the mice blood samples by kits (BioVision). Hormones were assayed following kit instructions in triplicates.

Antioxidant Capacity Test

Tissue homogenate preparation

Testis tissues were preserved in 1.15% ice-cold KCl at -80⁰C for few hours. Fresh testes were weighed and homogenates were prepared using a glass homogenizer with ice-cold 1.15% KCl (10% w/v) to record the antioxidant effect of honey within tissues. Then homogenates were centrifuged at 15000 rpm for 10 min at 4⁰C. The supernatants were collected for further processing (Katalinica et al., 2005).

Ferric Reducing Antioxidant Power in mice tissues and honey

The antioxidant power in tissues and honey was measured following the ferric reducing antioxidant power (FRAP) assay (Katalinica et al., 2005). The antioxidants present in the supernatant were evaluated as a reducer of Fe⁺³ to Fe⁺², which is chelated by 2,4,6-Tris(2-pyridyl)-s-triazine (TPTZ) to form the complex and evaluated using the maximal absorption at 593 nm. The results were presented in µM equivalent ascorbic acid/mg sample.

Total Phenolic Content (TPC)

Honey was assessed for the presence of phenolic contents following Valverde et al. (2015) protocol. Briefly, 1.0 mL of the diluted honey samples were transferred in separate tubes containing 5.0 mL of 1/10 dilution of Folin-Ciocalteu's reagent in water. Then, 4 mL of a sodium carbonate solution (7.5% w/v) was added. The tubes were allowed to stand at room temperature for 60 min before absorbance at 765 nm was measured. The unit was expressed in a Gallic acid equivalent g/100 g sample.

Statistical analysis

Statistical analysis was carried out using SPSS software (IBM, version 21.0). The data were expressed in terms of mean ± SEM and mean differences between all experimental groups were measured by one-way ANOVA followed by Post hoc Tukey and Duncan's multiple range tests. All the tabulated data presented as mean ± standard error of means and value p < 0.05 was considered significant.

Results

Biometric Parameters

The morphological and morphometric analyses included mice body weight, gonado-somatic index (GSI), testes size (width and length) in all groups is presented in Figure 1 and Table 1. Animals in group C, VC, and P+H showed normal morphology and morphometric results. However, the DEP group showed a significant ($P \leq 0.05$) increase (33.29 ± 3.30 g) and the HC group showed a remarkable decrease (28.34 ± 1.73 g) in body weight gain against the control (30.80 ± 0.34 g). Contrary to these findings, a decrease ($P \leq 0.05$) in the testes weight and gonado-somatic index (2.6 ± 0.0033 g) in the DEP group as compared to control (0.8 ± 0.0040 g) was noted. On the other hand, P+H (DEP co-administered with honey) showed significant improvement in the above-mentioned parameters.

Micrometry and sperm count observations

Micrometric observations in which post hoc analysis of means among groups showed significant variations ($p < 0.05$) in seminiferous tubule (ST) diameter and luminal diameter of the seminiferous tubule (Figure 2; Table 1). The group DEP showed significantly ($p < 0.01$) reduced ST diameter, 204.8 ± 1.92 μm and increased ($p < 0.001$) lumen diameter, 92.0 ± 3.5 μm as compared to control (243.3 ± 0.77 and 67.1 ± 1.3 μm). Contrary to this, the P+H group showed less change ($p < 0.05$) in diameter in comparison with the control. The cross-sectional area of seminiferous tubules (STA), decreases (0.033 ± 2.71 mm^2) remarkably ($p < 0.05$) in the DEP group contrary to the luminal area (LA), 0.0066 ± 1.27 mm^2 that increased significantly in the same group against controls (0.046 ± 1.40 and 0.0035 ± 1.05 mm^2 , respectively). The results depicted a decline in mature sperms in the said group. STA and LA also showed variations in other groups as compared to control but the difference is less significant (Figure 2; Table 1). The above results are further cleared through sperm count, which decreased in a significant number ($p < 0.001$) in the DEP group (24.7 ± 10.5 million/ml) as compared to the control (C) group (41.8 ± 2.11 million/ml) (Table 2). The normal to abnormal sperm ratio is also comparable to the above results. A novelty found in the HC group (given honey solely), data about micrometric studies as well as sperm count showed more positive results even from the control group (Figure 2; Table 1-2).

Hormonal Analysis

The results of serum testosterone and luteinizing hormone quantification in all groups are presented in Figure 3. There was a statistically significant decrease ($p < 0.001$) in testosterone level (0.10 ± 2.05 ng/ml), alternatively increased LH level (2.02 ± 4.15 mIU/ml) was observed in the DEP group as compared to control, in which testosterone and LH levels were 0.24 ± 3.22 ng/ml and 1.66 ± 7.38 mIU/ml, respectively. At the same time, the P+H group showed comparable results with the C and VC groups with 0.23 ± 1.26 ng/ml, testosterone and 1.57 ± 9.03 mIU/ml, respectively. On the other hand, the serum testosterone levels in the HC group again showed a positive impact of honey on steroidogenesis.

Antioxidant Capacity Test findings in Honey and testicular tissues

There was a significant difference in reducing ability in terms of FRAP value among testicular tissues of various groups. FRAP value in DEP exposed testes tissues (112.54 ± 2.3 μM ascorbic acid equi/ kg) was significantly decreased ($p < 0.001$) as compared to C (247.65 ± 2.1) and VC (250.67 ± 1.1 μM ascorbic acid

equi/ kg). Moreover, FRAP indicated the successful enhancement of antioxidant capacity in testes tissues (241.6 ± 1.4) in the P+H group compared to the DEP group (112.54 ± 2.3). On another side, testis of the animals who received honey solely (HC group) showed significantly higher FRAP value ($282.9 \pm 1.9 \mu\text{M}$ ascorbic acid equi/ kg) even than C group, which has a FRAP value of 247.65 ± 2.1 (Table 3).

The findings of honey antioxidant capacity along with the total phenolic content were presented in Table 3. Results indicate a strong correlation ($R^2=0.9109$) between TPC and antioxidant capacity (FRAP values) of honey (Figure 4).

Histopathology

Histopathological sections of testes from the control group (C) showed a regular pattern of seminiferous tubules surrounded by well-defined interstitial tissues. Primordial germ cells, spermatogonia are arranged in concentric layers on the intact basement membrane followed by layers of spermatocytes. The lumina of seminiferous tubules were filled with mature sperms (Figure 5 A1&A2) Almost similar anatomical layouts were observed in VC and HC groups and P+H group to a great extent (Figure 5 D, E and C1&C2 respectively). However, the DEP group showed severe kinds of pathological alterations including germ cell declination and degenerations, presence of residual bodies, Amyloids, degeneration of seminiferous tubules, exfoliations, Pyknotic nuclei, disrupted spermiation and Leydig cell autolysis. Even in the mitotic zone, spermatogonial cells were scattered and randomized. Wide luminal spaces were a clear indication for the declination of mature sperms (Figure 5 B1 and B2). However, the group P+H showed the presence of healthy seminiferous tubules well occupied with germ and supporting cells (Sertoli cells) as compared to the DEP group (Figure 5 C1&C2 and Table 4).

Discussion

Repeated exposure to DEP interferes with steroidogenesis as well as other reproductive parameters in developing mice. In this study, the mice's body weight gain was increased and they showed sluggish behavior after DEP treatment. Similarly, in another study, multiple phthalate types present in the urine samples resulted in an increased body mass index (BMI) of 5 to 12 years old children compared to unexposed individuals (Harley et al. 2017). Probably, the mechanism behind this was metabolites of DEP showed more affinity for PPAR γ receptors. It is strongly associated with commencing adipogenesis (Hurst and Waxman, 2003, Rodríguez-Carmona, 2019). Honey supplementation in the P+H group successfully maintained the bodyweight like that of the control group and acted sometimes as a lipolytic compound. Another study on honey corroborated our findings, which stated that intake of 20% mono-floral honey reduced the body mass of rats (Nemoseck et al., 2011; Terzo et al.2020).

The testes morphometry of the DEP group in present data showed testicular hypoplasia resulted in a decrease in the testes weight, testes width and even gonado-somatic index. However, the length was similar to the control group. Several other researchers reported decreased gonadal size (Barakat et al., 2020). Where male mice were prenatally exposed with DEP mixture in 20 $\mu\text{g/kg}$ and 500 mg/kg showed

decreased gonadal weight in a dose-dependent way. In the P+H group, testes showed improved morphological and morphometric parameters as compared to the DEP group.

Histological sections showed histopathological defects in DEP exposed testes like Leydig cell atrophy, degenerations, declination of germ cells, exfoliations, spacious lumen and disrupted spermiation. These findings are correlated with previous evidence in which DEP exposure causes degenerations of seminiferous tubules, thin basement membrane of testes and azoospermia (Mondal et al., 2019). In the P+H group, the testes histology showed much improvement in interstitial tissues, healthy spermatozoa with more spermatogonia and Sertoli cells lining the basement membrane.

The micrometric observations revealed a significant reduction in seminiferous tubular diameter and a profound increase in the lumen diameter in the DEP group as compared to the control group. One obvious reason for the increase in luminal diameter is less production or degeneration of germ cells. Besides, honey supplementation increased seminiferous tubule diameter and decreased lumen diameter. Likewise, in another study, the utilization of honey enhanced the number of Leydig cells, seminiferous tubule diameter and decreased lumen size against cigarette smoke-induced toxicity (Mohamed et al., 2011). The protection against DEP instigated histopathological lesions seems that raw honey contains handsome amounts of flavonoids and other polyphenols which may function as antioxidants and the consumption of antioxidants along with the protection against various pathologies, has a lot of potential health benefits (Yeung, et al., 2019; Blassa et al., 2006).

There was a significant increase observed in luteinizing hormones and an alternatively decrease in testosterone levels in the DEP group. These results are justified by another study that an obvious decrease in gonadal size was correlated with decreased testosterone levels (Wang et al., 2004). The mechanistic approach of the present study is in line with other researchers (Ozcan-Sezer et al., 2019). The possible cause of impaired fertility in males was the increased number of adipose tissues and simultaneously P450 aromatase enzymes which convert the testosterone to estrogen hormone (Ozcan-Sezer et al., 2019). Similar research reported where DEP and other phthalates exposure resulted in a significant decrease in testosterone level, mice body weight, and testes weight in human fetal testes (Sedha et al., 2021). Furthermore, the sperm count and morphology were also significantly affected in our observations, probably due to the hormonal imbalance by DEP. But another research contradicts aforementioned studies, it states that DEP exposure may induce androgen-independent male reproductive toxicity (James et al., 2020).

The sperm number and percentage of normal sperm also decreased in the DEP group, simultaneously abnormal sperm percentage significantly higher than the control group. In previously reported data, DEP exposure caused a reduction in sperm motility and sperm density while sperm count has not exhibited any change among treatment and control groups (Mondal et al., 2019). All these findings were a clear indication of diethyl phthalate caused a decrease or reduce fertility rate. Our data indicates in animals exposed to honey, testosterone level increased above average, while the LH level has decreased in the same mice. Various researches proved that honey maintained the level of testosterone (Banihani, 2019).

Less adipose tissue and more Leydig cell stimulated the testosterone balance in the body of male mice. Thus, honey is also loaded with a chrysin molecule which acts as the inhibitor of the aromatase enzyme in adipose tissues. The inhibition of the aromatase enzyme resulted in decreased conversion of testosterone into estrogen, hence testosterone level maintained (Jeong et al., 1999). In contrast to our results, honey administration raised the LH level in the blood serum (Kolawole, 2015). Similar to our outcomes, honey supplemented to rats in diabetic conditions maintained the LH level in blood serum (Nasrolahi et al., 2013).

Our results clearly showed the direct and strong correlation between antioxidant capacity and total phenolic compounds. The increase in phenolic contents ultimately increased the antioxidant capacity. Another research reported that there was a positive correlation between antioxidant capacity and total phenolic contents of honey analyzed through FRAP and TPC assay, respectively. Honey was enriched with antioxidant compounds and stabilized ROS by enhancing the activity of catalase and superoxide dismutase enzymes (SOD) (Mohamed et al., 2011). Thus, treatment with honey has shown clear histopathologic, morphometric, steroidogenic and micrometric recoveries against DEP instigated reproductive deteriorations in mice.

Conclusion

The present research showed that total phenols present in honey act as free radical scavengers and significantly prevent diethyl phthalate-induced reproductive damages in male mice. Multiflora honey from *Apis mellifera* can efficiently protect testicular lesions and hormonal deviations during the biological development of mice. Our observations revealed the remedial efficacy of honey on the reducing ability of testes, hormone production, and reclamation of germ cells and micrometric dimensions of seminiferous tubules. Based on the current findings we suggest honey consumption revitalize spermatogenesis and steroidogenesis in mice exposed to environmental contaminants like DEP. Further studies are needed relating oxidative response and mechanism of action of honey against DEP in the mammalian model.

Declarations

Acknowledgments:

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Consent to Participate:

The rearing and use of mice was following NIH Publication "Guide for the Care and Use of Laboratory Animals" (NRC 2004) and with the approval by the local ethical committee of University of the Punjab, Lahore, Pakistan on animal experimentation **(can provide a certificate on demand)**

*Statement about this also added in manuscript.

Ethical statement:

All animal trials were executed according to local and worldwide procedures. The nearby way is the Wet op de dierproeven (article 9) of Dutch law (international) and an associated rule planned via the Bureau of Animal Research Licensing, Local University as detailed in our earlier papers (Ali et al. 2020; Hussain et al. 2020; Ali et al. 2020; Ara et al. 2020; Ali et al. 2020; Khan et al. 2019; Ali et al. 2019; Mumtaz et al. 2019; Mughal et al. 2019; Dar et al. 2019). The rearing and use of mice were carried out using NIH Publication "Guide for the Care and Use of Laboratory Animals" (NRC 2004) and with the approval vide No. D/681/UZ dated 04-04-2019 by the local bioethical committee of the University on animal experimentation.

Consent to Publish:

"Not applicable"

Authors Contributions:

Conceptualization: Chaman Ara, Asmatullah: **Data Curation:** Chaman Ara, Asmatullah, Faiza Yaseen, Shaukat Ali, Nagina Ramzan, Hafiz Abdullah Shakir, Shagufta Andleeb, Muhammad Khan: **Formal Analysis:** Chaman Ara, Asmatullah, Faiza Yaseen, Shaukat Ali, Nagina Ramzan: **Investigation:** Chaman Ara, Asmatullah, Shaukat Ali, Hafiz Abdullah Shakir, Muhammad Khan: **Methodology:** Chaman Ara, Asmatullah, Faiza Yaseen, Shagufta Andleeb, Shaukat Ali, Nagina Ramzan, Hafiz Abdullah Shakir, Muhammad Khan: **Histopathology:** Faiza Yaseen, Nagina Ramzan, Muhammad Khan: **Software:** Shaukat Ali, Hafiz Abdullah Shakir: **Supervision:** Chaman Ara, Asmatullah: **Writing Original draft:** Faiza Yaseen, Nagina Ramzan, Hafiz Abdullah Shakir, Shaukat Ali, Muhammad Khan, Shagufta Andleeb: **Review and Editing:** Chaman Ara, Asmatullah, Shagufta Andleeb, Shaukat Ali, Hafiz Abdullah Shakir

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Availability of data and materials: Most of the data generated during this study are included in this article. However, raw data sheets and histopathological Figures are available from the corresponding as well as co-authors upon reasonable request.

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Tables

Table 1. Bodyweight gain gonado-somatic index and seminiferous tubules micrometry of DEP exposed mice with and without honey against control.

Dose Groups / Parameters	C (N=10)	VC (N=10)	DEP (N=10)	HC (N=10)	P+H (N=10)	P Value
Initial body weight of mice(gm)	13.84a±0.22	13.50a±0.33	13.54a±0.27	13.72a±0.27	13.78a±0.26	0.875
Final body weight of mice (gm)	30.80b±0.34	30.58b±0.44	33.29a±0.30	28.34c±0.73	30.57b±0.43	0.000
Test weight (mg)	124.1 ^b ± 1.58	128.2 ^b ± 0.8	110.0 ^a ± 2.1	123.8 ^b ± 0.8	128.7 ^b ± 0.6	0.000
Gonado somatic index (GSI)	0.40b±0.008	0.42b ±0.007	0.33a±0.006	0.43b±0.001	0.42b± 0.007	0.000
Mean Seminiferous tubular diameter (µm)	243.3b± 0.77	218.9d ± 0.69	204.8e± 0.92	253.1a± 0.52	225.9c± 0.68	0.000
Mean Luminal Diameter (µm)	67.1c ± 0.3	77.3b ± 0.7	92.0a ± 0.5	63.0d ± 0.7	65.0cd ± 0.7	0.000
Seminiferous tubule area (mm ²)	0.046a ±0.41	0.037 b±0.07	0.033 c ± 0.009	0.050 a ± 0.06	0.041 b±0.059	0.000
Luminal Area(mm ²)	0.0035b± 0.002	0.0047c ± 0.004	0.0066 a± 0.0001	0.0031 b± 0.0002	0.0034 b± 0.0002	0.000
Epithelial Area (mm ²)	0.0425 ^b ±0.001	0.0323 ^C ± 0.038	0.0264 ^a ± 0.001	0.0469 ^b ± 0.006	0.0367 ^C ± 0.007	0.000

Note: Values are expressed as **mean±SEM**; **N** = Number of samples analyzed; **C**, untreated; **VC**, corn oil treated **DEP**, diethyl phthalate exposed; **P+H**, diethyl phthalate + honey; **HC**, honey, In rows different alphabets showed significant difference among groups analyzed through one-way ANOVA followed Tukey's post hoc at level minimally $P \leq 0.05$.

Table 2. Comparison of Hormonal variations, sperm count, percentages of normal to abnormal sperms among DEP treated groups with and without honey against controls.

Dose Groups / Parameters	C (N=10)	VC (N=10)	DEP (N=10)	HC (N=10)	P+H (N=10)	P Value
Testosterone level (ng/ml)	0.24b ± 0.22	0.22b ± 0.02	0.10c ± 0.005	0.48a ± 0.16	0.23b ± 0.26	0.000
Luteinizing hormone (mIU/ml)	1.66b± 0.38	1.56b ± 0.08	2.02a ± 0.53	0.49c ± 0.09	1.57b ± 0.036	0.000
Sperm count (million/ml)	41.8b ± 1.1	41.8b ± 1.0	24.7c ± 0.5	49.4a ± 2.2	40.9b ± 1.3	0.000
Normal sperms (%)	67.5b ± 1.2	69.3b ± 1.2	59.6a ± 0.8	79.2c ± 1.2	70.0b ± 1.6	0.000
Abnormal sperms (%)	32.5b ± 1.2	30.7b ± 1.2	40.4a ± 0.8	20.8c ± 1.2	30.0b ± 1.6	0.000
FRAP value (µM ascorbic acid equi/ mg)	247.65c± 0.0005	250.67b ± 0.0005	112.54e± 0.0005	282.9a ± 0.0005	241.6d ± 0.0005	0.000

Note: Values are expressed as **mean± SEM**; **N** = Number of samples analyzed; **C**, untreated; **VC**, corn oil treated **DEP**, diethyl phthalate exposed; **P+H**, diethyl phthalate + honey; **HC**, honey treated; FRAP, ferric reducing antioxidant power in testes homogenates. In rows different alphabets showed significant difference among groups analyzed through one-way ANOVA followed Tukey's post hoc at level minimally $P \leq 0.05$.

Table 3- Histopathological lesions scoring in testes of mice in different treatment categories.

Groups	C	VC	DEP	P+H	HC	Chi	P value
(Total sections examined) Testis lesions	(10)	(10)	(10)	(10)	(10)		
Vacuulations	0 (0%)	▼4(40%)	7 (70%)	2 (20%)	1 (10%)	14.099	.004
Exfoliations	1 (10%)	1 (10%)	6 (60%)	3 (30%)	0 (0%)	11.306	.012
Lydig cells hypoplasia	0 (0%)	1 (10%)	9 (90%)	2 (20%)	0 (0%)	26.075	.000
Degenerative germ cells	0 (0%)	0 (0%)	10(100%)	4 (40%)	0 (0%)	35.893	.000
Degeneration of seminiferous tubules	0 (0%)	0 (0%)	6 (60%)	0 (0%)	0 (0%)	16.508	.000
Sloughing of spermatogenic cells	0 (0%)	1 (10%)	8 (80%)	2 (20%)	0 (0%)	20.954	.000
Pyknotic Nuclei	0 (0%)	2 (20%)	6 (60%)	1 (10%)	0 (0%)	13.071	.003
Amyloid	0 (0%)	0 (0%)	4 (40%)	0 (0%)	0 (0%)	9.484	.005
Dystrophic Mineralization	0 (0%)	0 (0%)	9 (90%)	0 (0%)	0 (0%)	31.379	.000
Sperm retention	0 (0%)	0 (0%)	0 (0%)	4 (40%)	0 (0%)	9.484	.005

Note: Sign▼indicates number of sections in which this particular defect seen

Figures

Figure 1

Macro photographs of testes and Bar graphs of morphometric measurements of Paired testes represented as). A: Control group, B: Vehicle Control, C: DEP group, D: DEP co-administered with Honey, E: Honey Control, Bar Graph showed a statistical Comparison of testes a) length and b) width. Different alphabets on bars of the same graph are statistically significant at $P \leq 0.05$.

Figure 2

Photomicrographs of magnified seminiferous tubules calibrated with image J software. A: Control group, B: Vehicle Control, C: DEP group, D: DEP co-administered with Honey, E: Honey Control, Bar Graph showed a statistical Comparison of a) Seminiferous tubule diameter (μm) b) seminiferous lumen diameter (μm) between five groups received different treatments. Bar Graph showed a statistical c) comparison for Antioxidant capacity (μM / mg) in Testes Tissue between five groups received different treatments expressed in mean $\pm$ S.E.M followed by Tukey's Post Hoc Test. (H & E stain; magnification 40X; scale bar

50µm). Different alphabets on bars of the same graph are showing significant differences among groups minimally $P \leq 0.05$.

Figure 3

Bar Graph showed a statistical Comparison of Luteinizing Hormone and Testosterone Level in Plasma (mIU/ml) between five groups received different treatments expressed in mean $\pm$ S.E followed by Tukey's Post Hoc Test.

Total Antioxidant Capacity of Honey checked by TPC and FRAP Assay.

Sample	Parameters	Antioxidant Capacity (mean $\pm$ S.E.M)
Honey	Total Phenolic Contents	217.40 $\pm$ 4.62(μ g gallic acid equiv/ kg Honey)
	FRAP	319.77 $\pm$ 9.4 (equiv μ M ascorbic acid/kg Honey)

Figure 4

Correlation between FRAP and Total Phenolic Contents of Honey for Total Antioxidant Capacity.

Figure 5

Photomicrographs of histological sections of testes. A1 and A2: Control group, B1 and B2: DEP group, C1 and C2: DEP co-administered with Honey, D: Vehicle Control Group E: Honey Control. Labeled as SG: spermatogonia, SpC: spermatocyte, Spt: spermatid, S: sperm, Ld: Leydig cells, L: lumen, SC: Sertoli cells, SM: smooth muscle cells, IS: Interstitials space Exf: exfoliation, RS: residual bodies, DeG: degenerative germ cells, DGE: Degenerative germinal epithelium, Am: Amyloid, solid triangle: dystrophic mineralization,

PN: pyknotic nuclei, SpR: spermatid retention, triangle: vacuolation, double headed arrow: disrupted spermiation, Ldh: leydig cell hypoplasia (H & E stain ;A1, B1, C1 at 10X; A2,B2,C2,D & E at 40X; scale bar 50µm).