The intergenerational effects of oligomeric proanthocyanidins on expression of Sult2a2, Sult2al1 and Sult2a1 in Bisphenol A-exposed male rats

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ABSTRACT

Infertility is among the diverse pathological conditions that beset the human society. Bisphenol A (BPA) is an endocrine-disrupting agent that was used as a monomer in the production of hard plastics. It can mimic the hormones of the body, and it can interfere with natural hormone production, absorption, transportation, action, function, and elimination. Oligomeric proanthocyanidins (OPC) is made up of bioflavonoids and polyphenols that act as free radical scavengers in the body. They can be found naturally in the bark, seeds, fruits and leaves of a vast range of plants in the plant kingdom including grapes, coffee and apple. The aim of the present research is to map the intergenerational effects of BPA and OPC intervention on the expression of Sult2a2, Sult2al1 and Sult2a1 genes. In this experimental study, 36 adult male Sprague Dawley rats weighing approximately 280 ± 20 grams aged 10 weeks old were randomly divided into 6 groups, namely negative control (NEC), BPA 200 mg/kg bwt (POC), low dose 10 μg/kg bwt OPC (OPC10), high dose 20 μg/kg bwt OPC (OPC20), BPA plus low dose OPC (BPA+OPC10) and BPA plus high dose OPC (BPA+OPC20) (n = 6 per group). Treatment and supplementation of BPA and OPC were conducted for 21 consecutive days before the male rats were mated with female rats at a ratio of 1:1. After the F1 generation were 10 weeks old, the same procedure and analyses were conducted. The expressions of Sult2a2, Sult2al1 and Sult2a1 in the blood were determined by oligonucleotide array-based comparative genomic hybridization (CGH). All data were analyzed by one-way analysis of variance (ANOVA) using SPSS software, version 21. The data were assessed at α = 0.05. There was significantly lower expression of Sult2a2, Sult2al1 and Sult2a1 genes in the POC group compared to the NEC group (p < 0.001) when compared both intragenerationally and intergenerationally. The number of gene deletion increased significantly from -0.85 in the P generation to -1.37 in the F1 generation. In contrast, the number of deleted genes in rats of the OPC10 group significantly decreased from -0.37 in the P generation to -0.14 in the F1 generation (p < 0.001). Similarly, rats that were treated with 200 mg/kg bwt BPA and then supplemented with 10 μg/kg bwt (BPA+OPC10) showed a significant decrease in terms of gene deletion, from -0.59 in the P generation to -0.46 in the F1 generation (p < 0.001). The remaining groups showed no significant difference (p > 0.05). OPC specifically at a dosage of 10 μg/kg bwt is able to alleviate the intergenerational detrimental effects of BPA on the expressions of Sult2a2, Sult2al1 and Sult2a1 genes.

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INTRODUCTION

Male infertility specifically refers to the incapability of a male to conceive a fertile female usually due to low sperm count and poor sperm quality. Infertility is among the diverse pathological conditions that beset the human society. Birth rates in developing countries have decreased over the years and there has been a dramatic rise in medical cases diagnosed with male infertility [1]. Cooper et al. (2010) reported low semen quality among young men aged 18-21 years [2]. A recent study by Virtanen et al. (2017) showed that the major factor causing this problem is influenced by the man’s lifestyle; men who smoke and drink alcohol are more prone to have low sperm count and poor sperm quality [1]. The exposure to chemicals in tobacco leads to endocrine disruption which then results in infertility. Oxidative stress which results in the DNA damage of spermatozoa is closely related to poor sperm function and infertility in male [3]. However, of all the factors contributing to human infertility, approximately half of all cases reported is mainly caused by the male [4]. Hence, thorough research regarding the potency and ability of antioxidant therapies must be conducted in order to deal with this consequential issue. Various types of traditional herbs and natural antioxidants such as oligomeric proanthocyanidins, Phyllanthus gomphocarpus, Zingiber zerumbet etc. can be used as alternatives to drugs and medicines to overcome this problem.

Over the years, the usage and continuous release of Bisphenol A (BPA) and other toxicants into the environment has caught great attention worldwide due to their harmful effects and toxicity. BPA is a type of chemical that is used as a monomer in the manufacture of hard plastics, and can act in the human body in a similar way to estrogen and other hormones. It is one of the organic synthetic compounds with the chemical formula $\text{(CH}_3\text{)}_2\text{C(C}_6\text{H}_4\text{OH)}_2$ that belongs to the group of diphenylmethane derivatives and bisphenols, with two hydroxyphenyl groups. BPA is used globally especially in the production of consumer products such as food and beverage cans, dental sealants, eyeglass lenses, medical devices and as an additive in other plastics. According to data issued and reported by Merchant Research and Consulting (2008), one of the highest volumes of chemicals produced globally is BPA with a capacity of 2.2 million metric tons in 2003 with an increase in demand of 6% to 10% every year [5]. Moreover, Rykowska and Wasiak (2006) also reported that between the years of 1957 to 2001, the production of BPA at the global scale in the United States of America is approximately calculated to be as much as 2.5 million tons [6].

Numerous studies have been conducted to show the harmful effects of Bisphenol A, not only on humans, but also on other mammals and plants. BPA is an example of chemicals that interfere with endocrine processes because they have low estrogenic, antiandrogenic and antithyroid activity. BPA can accumulate in various tissues, given its rapid metabolism. Several studies have shown BPA’s effects on human growth, metabolism, and reproductive system. Relatively high doses of estrogenic endocrine-disrupting chemicals (EEDCs) can help in boosting puberty of rodents that are developing which in turn, leads to a modified function of the reproductive system. Low dosage of EEDCs can also have an impact on rodents such as an elevation of genetic abnormality in males and advanced puberty in females [7]. Chronic BPA exposure may have adverse effects and can reduce levels of testosterone in human and animal blood [8]. The effects of BPA on males were faster and easier to detect than the effects on females, because males have very low level of estrogen. Indeed, several studies have been conducted to show mammals that are repeatedly exposed to BPA would have shrinking rates of testosterone, which causes sexual dysfunction [9-13].

Antioxidants are one of the important components for couples with infertility problem. In any fertility plan, it is crucial to protect sperm cells from free radical damage as it is capable of causing spermatogenic DNA damage. One can increase the antioxidants in the body simply by practicing diets that are high in fruits and vegetables on a daily basis besides taking antioxidant supplementation. Oligomeric proanthocyanidins (OPC) is made up of bioflavonoids and polyphenols that act as free radical scavengers in the body. They can be found naturally in the bark, seeds, fruits and leaves of a vast range of plants in the plant kingdom including...
grapes, coffee and apple. According to Dixon et al. (2004), OPC has an antioxidant property that is 50 times more than vitamin E and 20 times more than vitamin C besides being able to neutralize free radicals, protect cells from DNA damage and prevent mutations of DNA [14].

MATERIALS AND METHODS

Animals and protocols

A total number of 72 Sprague Dawley rats, with an equal number of 36 male and 36 female rats were used in this study. Sexually matured male and female rats aged about 10 weeks with body weight approximately 280 grams to 350 grams and 180 grams to 230 grams, respectively were purchased from A-Sapphire Sdn. Bhd., Taman Universiti Indah, Seri Kembangan, Selangor. All rats received the same set of a standard diet of pellets which contains 21% crude protein, 13% moisture, 8% ash, 5% crude fiber, 3% crude fat, 0.8% calcium and 0.4% phosphorus and similar amount of food ad libitum. Water was also supplied using a bottle and was made available as necessary throughout the study period. The rats were housed in standard shoebox cages without filter tops placed in the Animal Holding Room of the Faculty of Health Sciences, UiTM Puncak Alam with central air-conditioning. They were also provided with sawdust animal bedding in the shoebox cages. The room was maintained at 30% to 70% of humidity and a temperature of 18 °C to 26 °C. A 12 hour light and dark cycle was also maintained in the room every day to mimic natural conditions.

A total of 36 male rats (n = 36) were randomly divided into six groups, whereby each group were allocated with six randomly picked rats (n = 6). The division of groups is as shown in Table 1.

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Negative control (NEC) : 10 ml/kg body weight distilled water (n = 6)</td>
</tr>
<tr>
<td>2</td>
<td>Positive control (POC) : 200 mg/kg body weight BPA (n = 6)</td>
</tr>
<tr>
<td>3</td>
<td>OPC low dose 10 μg/kg body weight (OPC10) (n = 6)</td>
</tr>
<tr>
<td>4</td>
<td>OPC high dose 20 μg/kg body weight (OPC20) (n = 6)</td>
</tr>
<tr>
<td>5</td>
<td>BPA 200 mg/kg body weight + OPC 10 μg/kg body weight (BPA+OPC10) (n=6)</td>
</tr>
<tr>
<td>6</td>
<td>BPA 200 mg/kg body weight + OPC 20 μg/kg body weight (BPA+OPC20) (n=6)</td>
</tr>
</tbody>
</table>

Before the initiation of treatment, the animals were fasted overnight. After 21 days of treatment, rats of the P generation in all six groups (n = 36) were mated with female rats at a ratio of 1:1. Mating of rats was done by housing the male rats together with the female rats in shoebox cages for 7 days. On the 8th day, pregnant females were identified by making vaginal smears and also by checking the presence of copulation plugs. Pregnant female rats were immediately separated from the males and those that were still not pregnant were left together with the male rats for another 7 days. Following 21 days of treatment, the rats were euthanized by decapitation using guillotine. After the F1 generation were 10 weeks old, the same procedure and analyses were conducted.

Blood sample preparation

Approximately 5 ml of blood was collected in an ethylenediaminetetraacetic acid (EDTA) tube immediately after decapitation and stored at -80 °C before being used for array-based comparative genomic hybridization (CGH) analysis.

Oligonucleotide array-based comparative genomic hybridization (CGH)

Oligonucleotide array-based comparative genomic hybridization (CGH) is one of the molecular cytogenetic techniques to analyze any chromosomal copy number alterations in a genome which involves enzymatically labelling of DNA from blood using Cyanine 3 (Cy3) and Cyanine 5 (Cy5) dyes. Briefly, this analysis requires (i) DNA isolation, (ii) DNA sample preparation, (iii) DNA sample labelling and (iv) microarray processing.

DNA isolation

The isolation of DNA was divided into two major steps which include genomic DNA (gDNA)
extraction and gDNA quantitation and quality analysis. For the extraction of gDNA from the collected blood, DNeasy Blood and Tissue Kit was used. The instrument used for the quantitation and quality analysis of gDNA was NanoDrop ND-1000 UV-VIS Spectrophotometer, whereby the concentration and purity of gDNA was evaluated. Firstly, 1.5 μl of Buffer AE was used to blank the instrument. Then, 1.5 μl of each gDNA sample was used to measure the concentration of DNA. The concentration, in ng/μl for each sample was recorded and the yield was calculated using the formula:

\[
\text{Yield (μg)} = \frac{\text{DNA Concentration (ng/μl)} \times \text{Same Volume (μl)}}{1000 \text{ ng/μg}}
\]

The ratio values of \(A_{260}/A_{280}\) and \(A_{260}/A_{230}\) were recorded for each sample. According to Agilent Technologies, high quality genomic DNA samples should have an \(A_{260}/A_{280}\) ratio ranging from 1.8 to 2.0 which indicates the absence of contaminating proteins. The ideal ratio value of \(A_{260}/A_{230}\) for pure DNA should be more than 1.0.

**DNA sample preparation**

Samples were prepared by the direct method whereby restriction digestion with the SureTag Complete DNA Labelling Kit was used. Prior to preparation, 10X Restriction Enzyme Buffer and bovine serum albumin (BSA) were first thawed before being spun in a microcentrifuge. For each reaction, gDNA was added to the appropriate nuclease-free tube or well in the polymerase chain reaction (PCR) plate. The digested gDNA were then stored at -20 °C before proceeding with the next step which was DNA sample labelling.

**DNA sample labelling**

Sample labelling comprised of two major steps which were fluorescent labeling of gDNA and purification of labelled gDNA. In this study, the experimental samples were labelled with one dye which was cyanine 5 (Cy5) while the reference samples were labelled with a different dye which was cyanine 3 (Cy3). According to Wang et al. (2010), cyanine 3-dUTP and cyanine 5-dUTP are very sensitive to light and prone to undergo photobleaching, besides being easily degraded by multiple freeze thaw cycles. Hence, light exposure was kept to the minimum level throughout the whole labelling procedure. The samples were then stored at -20 °C in the dark. The second phase in sample labelling was purification of labelled gDNA. In this section, labelled gDNA was purified using the reaction Purification Column provided in the SureTag Complete DNA Labelling Kit. Labelled DNA was then stored at -20 °C in the dark.

**Microarray processing**

Microarray processing consisted of three steps which include hybridization, washing and scanning. Firstly, the 10X blocking agent was prepared by adding 1350 μl of DNase/RNase-free distilled water to the vial containing lyophilized 10X aCGH blocking agent. The vial was then left at room temperature for 1 hour before mixing thoroughly with a vortex mixer to recompose the sample. Then, to label gDNA for hybridization, the components in Table 2 were first mixed to prepare the hybridization master mix.

Next, the appropriate volume of the hybridization master mix was added to the 1.5 ml RNase-free Microfuge Tube, 200 μl Thin-Wall Tube, and 96-well PCR plate well containing the labelled gDNA to make the total volume of 110 μl. The sample was then mixed by pipetting up and down before being spun immediately in a centrifuge to drive the contents to the bottom of the reaction tubes. All tubes were then transferred to a thermal cycler for 3 minutes exactly at 98 °C before reducing the temperature to 37 °C for another 30 minutes before being spun at 6000 x g for 60 seconds in a centrifuge to collect the sample at the bottom of the tube. For the hybridization step, each assembled chamber was loaded into the oven rotator rack starting from the center of the rack at 20 rpm before hybridizing at 67 °C overnight.

The next step was microarray washing. Firstly, the volume of Oligo aCGH Wash Buffer 2 required...
Expression of Sult2a2, Sult2al1 and Sult2a1 in male rats

The number of Sult2a2, Sult2al1 and Sult2a1 gene deletion from the POC group increased significantly when compared intergenerationally from P generation (-0.85) to F1 generation (-1.37) (p < 0.05). However, the value of sulfotransferase gene deletion in the OPC20 group decreased significantly from P generation (-0.78) to F1 generation (-0.65) (p < 0.05). Similarly, rats that were first treated with 200 mg/kg bwt BPA and then supplemented with 10 μg/kg bwt OPC showed a significant decrease across generations, from P generation (-0.59) to F1 generation (-0.46) (p < 0.05). Table 4 shows the selected genes present in both P and F1 generations.

Table 2. Hybridization master mix (for 4-pack microarray).

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume (μl) per hybridization</th>
<th>X 8 reactions (μl) (including excess)</th>
<th>X 24 reactions (μl) (including excess)</th>
<th>X 48 reactions (μl) (including excess)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cot-1 DNA (1.0 mg/ml)</td>
<td>5</td>
<td>42.5</td>
<td>125</td>
<td>250</td>
</tr>
<tr>
<td>10X aCGH Blocking Agent</td>
<td>11</td>
<td>93.5</td>
<td>275</td>
<td>550</td>
</tr>
<tr>
<td>2X HI-RPM Hybridization Buffer</td>
<td>55</td>
<td>467.5</td>
<td>1375</td>
<td>2750</td>
</tr>
<tr>
<td>Final Volume of Hybridization Master Mix</td>
<td>71</td>
<td>603.5</td>
<td>1775</td>
<td>3550</td>
</tr>
</tbody>
</table>

Table 3. Wash conditions.

<table>
<thead>
<tr>
<th>Dish</th>
<th>Wash buffer</th>
<th>Temperature (°C)</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Disassembly</td>
<td>Agilent Oligo aCGH Wash Buffer 1</td>
<td>Room temperature</td>
<td>-</td>
</tr>
<tr>
<td>1st wash</td>
<td>Agilent Oligo aCGH Wash Buffer 1</td>
<td>Room temperature</td>
<td>5 minutes</td>
</tr>
<tr>
<td>2nd wash</td>
<td>Agilent Oligo aCGH Wash Buffer 2</td>
<td>37</td>
<td>1 minute</td>
</tr>
</tbody>
</table>

was added to a sterile storage bottle and warmed at 37 °C for 24 hours in an incubator. Other than that, a slide-staining dish with a lid, a 1.5 ml glass dish and 2L of Milli-Q ultrapure water were also pre-warmed in an incubator at 37 °C prior to washing procedure. The slide-staining dishes, slide racks and stir bars were washed with plentiful amounts of Milli-Q ultrapure water. The water collected in the dishes was emptied out at least five times. These steps were repeated until all materials were free from any contaminants. This was then followed by washing microarrays by referring to Table 3.

After preparing all three dishes, one hybridization chamber was removed from the incubator while resuming rotation of the others. The formation of bubbles during hybridization was recorded. Then, the hybridization chamber disassembly was prepared before prying the sandwich open from the barcode ensuring that the sandwich was completely submerged in Oligo aCGH Wash Buffer 1. These steps were repeated for up to four additional slides in the group. When all slides in the group were placed into the slide rack in slide-staining dish #2, the slides were then stirred at 350 rpm for 5 minutes. Next, the slides were washed in Oligo aCGH Wash Buffer 2. Upon completion, the slide rack was slowly removed to minimize droplets on the slides. Then, used Oligo aCGH Wash Buffer 1 and 2 were discarded.

The last step in microarray washing was to put the slides in a slide holder. The end of the slide without the barcode label was carefully placed on the slide ledge. The slide of the microarray was then gently lowered into the slide holder to ensure that the active microarray surface faces up to the slide cover. Then, the plastic slide cover was closed; the tab end was pushed until a “click” sound was heard. Finally, the microarray slides were scanned and analyzed using Agilent SureScan Microarray Scanner.

RESULTS

The number of Sult2a2, Sult2al1 and Sult2a1 gene deletion from the POC group increased significantly when compared intergenerationally from P generation (-0.85) to F1 generation (-1.37) (p < 0.05). However, the value of sulfotransferase gene deletion in the OPC20 group decreased significantly from P generation (-0.78) to F1 generation (-0.65) (p < 0.05). Similarly, rats that were first treated with 200 mg/kg bwt BPA and then supplemented with 10 μg/kg bwt OPC showed a significant decrease across generations, from P generation (-0.59) to F1 generation (-0.46) (p < 0.05). Table 4 shows the selected genes present in both P and F1 generations.
In reference to a study by Sanderson (2006), endocrine-disrupting toxicants like BPA targets the enzymes involved in the biosynthetic pathway of steroid hormone such as cytochrome P450 enzymes encoded by Cyp17a1 gene [17]. As depicted in Figure 5, cytochrome P450 enzyme 17α-hydroxylase assists the hydroxylation of pregnenolone to 17α-hydroxypregnenolone which

Table 4. Selected genes present in both P and F1 generations.

<table>
<thead>
<tr>
<th>Group (Generation)</th>
<th>Chromosome number</th>
<th>Cytoband</th>
<th>Start</th>
<th>Stop</th>
<th>Amp/ Del</th>
<th>Gene Names</th>
</tr>
</thead>
<tbody>
<tr>
<td>POC (P)</td>
<td>chr1</td>
<td>q21</td>
<td>74535461</td>
<td>75757245</td>
<td>-0.85</td>
<td>Sult2a2, Sult2a11, Sult2a1</td>
</tr>
<tr>
<td>POC (F1)</td>
<td></td>
<td></td>
<td>74586373</td>
<td>75757245</td>
<td>-1.37</td>
<td></td>
</tr>
<tr>
<td>OPC20 (P)</td>
<td>chr1</td>
<td>q21</td>
<td>74501144</td>
<td>76069783</td>
<td>-0.78</td>
<td>Sult2a2, Sult2a11, Sult2a1</td>
</tr>
<tr>
<td>OPC20 (F1)</td>
<td></td>
<td></td>
<td>74501144</td>
<td>75966914</td>
<td>-0.65</td>
<td></td>
</tr>
<tr>
<td>BPA+OPC10 (P)</td>
<td>chr1</td>
<td>q21</td>
<td>74495971</td>
<td>75822222</td>
<td>-0.59</td>
<td>Sult2a2, Sult2a11, Sult2a1</td>
</tr>
<tr>
<td>BPA+OPC10 (F1)</td>
<td></td>
<td></td>
<td>74570870</td>
<td>75844510</td>
<td>-0.46</td>
<td></td>
</tr>
</tbody>
</table>

Figure 1 shows the chromosome view of P and F1 generations in the BPA+OPC10 group which illustrates the exact locus at which the Sult2a2, Sult2a11 and Sult2a1 genes were deleted (chromosome 1; cytoband q21). In the figure, three dots in circle A indicate gene deletion and one dot in circle B indicates the amplification of genes. As depicted in Table 4, the number of deletion of sulfotransferase family genes decreased from -0.59 in P generation to -0.46 in F1 generation, which is illustrated by the dots within circles in Figure 1. Figure 2 shows the gene view of sulfotransferase gene deletion in POC group in both generations. Figure 3 shows the gene view of sulfotransferase gene deletion in OPC20 group in both generations and Figure 4 shows the gene view of sulfotransferase gene deletion in BPA+OPC10 group in both generations.

DISCUSSION

According to the Rat Genome Database (2017), sulfotransferase family 2A genes symbolized as Sult2a2, Sult2a11 and Sult2a1 on the q21 arm of chromosome 1 code for a protein that is involved in steroid sulfotransferase activity [15]. Sulfotransferase enzymes are of major importance in many biosynthetic pathways, one of them is steroid hormone pathway in which these enzymes are involved in the production of dehydroepiandrosterone (DHEA) [16]. DHEA, also known as androstenolone, is the most plentiful circulating steroid hormone that is manufactured naturally by the adrenal glands. It is a synthetic hormone used to transform cholesterol into androgens and estrogens including testosterone and estradiol.

In reference to a study by Sanderson (2006), endocrine-disrupting toxicants like BPA targets the enzymes involved in the biosynthetic pathway of steroid hormone such as cytochrome P450 enzymes encoded by Cyp17a1 gene [17]. As depicted in Figure 5, cytochrome P450 enzyme 17α-hydroxylase assists the hydroxylation of pregnenolone to 17α-hydroxypregnenolone which
Figure 2. Gene view of the P generation Sprague Dawley rats of NEC and POC (within left box) and F₁ generation of NEC and POC (within right box) groups. Arrow labelled with different alphabets indicate A=Deletion of Sult2a2, Sult2a11, Sult2a1 genes in P generation; B=Deletion of Sult2a2, Sult2a11, Sult2a1 genes in F₁ generation.

Figure 3. Gene view of the P generation Sprague Dawley rats of NEC and OPC20 (within left box) and F₁ generation of NEC and OPC20 (within right box) groups. Arrow labelled with different alphabets indicate A = Deletion of Sult2a2, Sult2a11, Sult2a1 genes in P generation; B=Deletion of Sult2a2, Sult2a11, Sult2a1 genes in F₁ generation.
Figure 4. Gene view of the P generation Sprague Dawley rats of NEC and BPA+OPC10 (within left box) and F1 generation of NEC and BPA+OPC10 (within right box) groups. Arrow labelled with different alphabets indicate A = Deletion of Sult2a2, Sult2a11, Sult2a1 genes in P generation; B = Deletion of Sult2a2, Sult2a11, Sult2a1 genes in F1 generation.

Figure 5. Steroid hormone biosynthetic pathway (Delić et al., 2010) [18].
is then converted to DHEA. Eventually, DHEA undergoes multiple complicated steps to get converted to testosterone and estradiol, hormones that play important roles in the reproductive system. This clearly explains the increase in the deletion of sulfotransferase gene family across generations from -0.85 (P generation) to -1.37 (F1 generation) with a significant value of p < 0.05 in rats treated with BPA alone which can be correlated with other parameters. As shown in the results section, rats that were exposed to BPA and then supplemented with 10 μg OPC have lesser Sult2a2, Sult2a1 and Sult2a1 gene deletion when compared intragenerationally in both generations as well as intergenerationally across generations which is from -0.59 (P generation) to -0.46 (F1 generation). This condition shows that OPC, being a powerful antioxidant, is able to alleviate the detrimental effects of BPA and hence, improving the male reproductive system.

CONCLUSION
The current study findings indicate that BPA-exposed rats are linked to decreased Sult2a2, Sult2a1 and Sult2a1 gene expression. However, these detrimental effects were alleviated by the supplementation of 10 μg/kg body weight of OPC as seen in the results section showing increased expression of these genes in the BPA+OPC10 group. Despite the promising results obtained in this present study, we only measured one gene family; hence, future studies could survey other genes considered vital in the male reproductive system of Sprague Dawley rats. This investigation has provided a new way to cure infertility amongst men.

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AUTHOR’S CONTRIBUTIONS
RD, FNZ, NKH and MAM were involved in designing the experimental works and treatment.

All members contributed in writing the draft, revision of the manuscript and research management. SAZ, FNO, NHI and MHM were involved in the data collection and performed the statistical analysis and contributed to the discussion of the results. RD is the Principal Investigator of the study.

CONFLICT OF INTEREST STATEMENT
The authors declare that they have no competing interests.

REFERENCES