Protective effects of *Camellia sinensis* on *Syzygium aromaticum*- or chlorpyrifos-induced reproductive toxicity in male Wistar rats

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Abstract

Background: The potential toxicity of clove, *Syzygium aromaticum*, notwithstanding its beneficial health effect to human health remains a critical issue.

Purpose: This study was designed to assess the effects of oil extracts of clove (*S. aromaticum)* on reproductive parameters in Wistar rats. The ameliorative effect due to co-administration with green tea, *Camellia sinensis*, was also determined.

Methods: Adult rats were exposed via oral gavage to mineral oil (negative control), 5% green tea (GT), 12.5 mg/kg/day chlorpyrifos (CHL, positive control), 360 mg/kg/day clove oil (CO), green tea + chlorpyrifos (GT+CHL), or green tea + clove oil (GT+CO) for 3 weeks, after which the animals were sacrificed and the following sperm parameters: total sperm count, sperm motile count, sperm progressive assessment, and sperm morphology were determined. The serum levels of reproductive hormones, testosterone, follicle stimulating hormone (FSH) and luteinizing hormone (LH), were determined. The histological sections of the testes were also performed.

Results: The results revealed that *S. aromaticum* treatment resulted in significant damage to the sperm morphology especially at the neck and tail regions with only a marginal change to the total sperm count, sperm motile count, and sperm progressive assessment. The levels of testosterone, FSH, and LH decreased significantly in rats treated with *S. aromaticum*. Histopathological analyses revealed significant disruption of normal testes structure in rats that were treated with either clove oil or chlorpyrifos.

Conclusion: Overall, the results of this study show that the co-administration with *C. sinensis* has the potential to ameliorate the clove-induced reproductive toxicity in rats.

Keywords: Plant-based pesticide, Chlorpyrifos, *Syzygium aromaticum*, Sperm parameters, Sex hormones, Histopathology

Background

In recent times, there is strong advocacy for the use of eco-friendly plant-based pesticides as alternatives to the non-eco-friendly synthetic pesticides (Dubey, Srivastava, & Kumar, 2008; Kedia, Prakash, Mishra, Singh, & Dubey, 2015). The literature is rich on the toxic effects of synthetic pesticides on man and other living organisms; these toxic effects include developmental impairments, DNA fragmentation, compromised immunity, organ toxicity, endocrine disruption, neuro-degeneration, oxidative stress, and reproductive abnormality (BenAbdallah, Slima, Dammak, Keskes-Ammar, & Mallek, 2009; Joshi, Mathur, gajraj, & Sharma, 2003; Sakr, & Al-Amoudi, 2012). Plant-based pesticides are considered as better alternatives to conventional synthetic pesticides for obvious reasons; they tend to have a broad-spectrum activity, they are humanly safe and biodegradable and are easy to process and use (Dinesh, Kimara, Kumar, & Das, 2014).

Several plant materials have been reported to possess insecticidal properties. Examples are *Azadirachta indica*,

Nicotiana sp, *Derris* sp, *Schoenocaulon officinale*, *Syzygium aromaticum Clerodendrum infortunatum*, *Dennettia tripetala*, *Anchomanes difformis*, etc. (Adedire & Akinkurolere, 2005; Dimetry, 2012; Dinesh et al., 2015; Mikami & Ventura 2008). In spite of the wide advocacy for their use as pesticides, little is currently known about the potentially toxic effects that may arise from their use. The treatment of albino rats with an aqueous solution of nicotine, a compound derived from *Nicotiana tabacum*, caused oxidative stress and damage to the liver (BenSaad et al., 2018; Chowanski et al., 2016). Recently, we showed that oil extracts of clove, *S. aromaticum* also caused oxidative stress and liver damage in male Wistar rats (Adeyemi et al., 2018). These and other studies are evidence of potential toxicity of plant-based pesticides to mammals.

The health benefits of drinking green tea are quite enormous; they include reduced risk of cardiovascular diseases, degenerative diseases, and cancer. Also, green tea has been shown to result in improvement of asthenia, diarrhea, bronchitis, asthma, hyperlipidemia, cellulitis, and abscesses as well as weight reduction (Kao, Chang, Lee, & Chen, 2006; Shula, 2007; Schönthal, 2011; Wolfram, 2007). More importantly, green tea has been shown to improve the reproductive health in humans (Ly, Yockell-Lelievre, Ferraro, Arnason, & Gruslin, 2015; Roshdy et al., 2013). The therapeutic ability of green tea has been closely linked to the presence of polyphenols such as epigallocatechin gallate (EGCG), epigallocatechin (EGC), epicatechin gallate (ECG), epicatechin (EC), catechin gallate, and and gallocatechin gallate (Chen et al., 2003; Harold & Graham 1992; Henning et al., 2003).

Reproductive impairments such as low sperm counts, damaged sperm and testes, and alteration in hormonal levels are often associated with exposure to synthetic chemical pesticides (Hu et al., 2013; Li, Pan, Hu, Li, & Xu, 2013), so the likelihood of plant-based pesticides to as well cause reproductive defects is worth investigating. This study is therefore designed to investigate the reproductive toxicity of oil extract of clove, *S. aromaticum*, in male Wistar rats. The ameliorative effect of green tea, *Camellia sinensis* was equally investigated.

Materials and methods

Extraction of essential oils from *S. aromaticum*

The essential oil was extracted from clove flower buds following the procedures of Ileke and Ogungbite (2015). Dried flower buds of *S. aromaticum* were obtained from a local market within Akure metropolis and pulverized into a fine powder using a blender. Acetone extracts of *S. aromaticum* were obtained using a cold extraction method. This was done by soaking 100 g of the powder in an extraction bottle containing 300 ml of acetone.

The mixture was stirred occasionally with a glass rod, and extraction was terminated after 72 h. The extract was filtered through Whatman filter paper (pore size; 0.7 μm). The extraction solvent was evaporated using a rotary evaporator set at 56 °C. The resulting extract was air-dried in order to remove traces of solvent.

Experimental animals

Adult male Wistar rats weighing approximately 200 g were obtained from a commercial farm within Akure metropolis and were placed individually in polypropylene cages, with laboratory grade pine shavings as bedding. Rats were allowed to acclimatize to experimental room conditions for 2 weeks prior to commencement of experiments. Rats were fed with rat chow and tap water ad libitum, throughout the period of the experiment.

Experimental design

The animals were randomly allocated into six groups (*n* = 5 per group) and were exposed through oral gavages to one of the following treatments: mineral oil (OIL, vehicle for extracted clove oil, thus serving as negative control), 5% green tea (GT), 12.5 mg/kg/day chlorpyrifos (CHL, positive control), 360 mg/kg/day extracted clove oil (CO), green tea + chlorpyrifos (GT+CHL), and green tea + extracted clove oil (GT+CO). Experimental treatment was done every day and lasted for 3 weeks. At the end of the third week of treatment, animals were sacrificed using cervical dislocation.

Total sperm count and motility assessment

The total sperm count and motility assessment were done following the procedure described by Yokoi, Uthus, and Nielsen (2003) with minor modification. The caudal epididymis was removed and minced with surgical blade in 1 ml of normal saline, placed in a rocker for 5–10 min, and incubated at 37 °C for about 2 min. The solution was diluted 100-folds with sodium bicarbonate/formalin solution. The total sperm count was done by placing 10 μl of the aliquot into the Neuber's counting chamber. The sample was allowed to stand for 5 min and then observed under the microscope at × 400 magnification. Sperm motility progressive assessment was assessed by placing a drop of the sperm aliquot on the slide, covered with coverslip and then observed under the microscope at × 400 objectives. A sperm was considered motile when it exhibited distinct flagella activity. Sperm motility was determined by assessing at least five microscopic fields to classify 100 spermatozoa. Motile sperms were further categorized into either fast or slow based on recommendations of the WHO (1999).

Sperm morphology

The morphology of the spermatozoa was determined on the same sperm dilution that was used for motility assessment using the procedures described by Rezvanfar et al. (2008). In detail, a drop of sperm suspension was added into an equal volume 1% eosin-y 5% nigrosin, which was then mixed together and smeared on pre-warmed clean glass slides and air-dried. A total of 200 sperm cells were examined at × 400. The morphology of the sperm cells was categorized based on the presence of one or more abnormal features into tail defects (short, irregular, coiled or multiple tail), neck and middle piece defects (distended, irregular, bent middle piece, abnormally thin middle piece), and head defects (round head, small or large size, double or detached head).

Histology of the testis

The excised testes from the rats were processed for hematoxylin and eosin staining as described by Bancroft and Stevens (1996). The testes were clearly dissected out, sliced, and fixed in neutral buffered 10% formalin solution for 48 h after which they were transferred into 70% ethanol for storage until further histological procedures were performed. Fixed tissues were dehydrated in ascending series of ethanol, cleared in methyl benzoate, and embedded in paraffin wax. About 7-μm-thick sections of the samples were cut with microtome and stained with hematoxylin and eosin dyes. Sections were later observed under light microscope and photomicrographs were taken.

Determination of serum hormonal levels

Blood samples were collected into plain bottles through cardiac puncture and were allowed to clot to get the sera. The sera were then pipetted into plain sample bottles and stored at – 20 ℃ until assayed for hormonal levels. The levels of testosterone, luteinizing hormone (LH), and follicle-stimulating hormone (FSH) were determined using enzyme-linked immunosorbent assay (ELISA) kits obtained from Cayman Chemical, USA. The assays were performed following the instructions of the manufacturer.

Statistical analysis

All statistical analyses were performed using Statistical Package for Social Sciences (SPSS version 15.0) software. Analysis of variance (ANOVA) and post hoc tests were used to analyze the data. Tukey's multiple comparisons was used to test for statistically significant difference between control and experimental groups. Results are presented as the mean ± standard error of mean (SEM). Results were considered significant at $p < 0.05$.

Results

Total sperm count

The total sperm counts are presented in Fig. 1. The total sperm counts differed significantly among the groups ($F_{5, 24} = 10.99$; $p < 0.0001$). The total sperm count was lowest in the positive control group (CHL) in which the sperm count was $45 \pm 4.47 \times 10^6$ while the group that was treated with green tea (GT) had the highest sperm count of $99.80 \pm 6.60 \times 10^6$. The animals that were treated with the extract of clove oil had a sperm count of $61.25 \pm 10.08 \times 10^6$. The co-administration with green tea resulted in increased total sperm count in the chlorpyrifos (CHL) and the clove oil-treated animals.

Motility assessment

The motile count differed significantly among the groups ($F_{5, 24} = 9.348$; $p = 0.0001$). The motile count was lowest in the positive control group (CHL) in which the motile count was $23 \pm 1.23 \times 10^6$ while the group that was treated green tea (GT) exhibited the highest motile count of $75.2 \pm 6.25 \times 10^6$. The animals that were treated with the extract of clove oil showed a motile count of $41.25 \pm 10.08 \times 10^6$. The co-administration with green tea resulted in increased sperm motility in the chlorpyrifos (CHL) and the clove oil-treated animals (Fig. 2).

Sperm progressive assessment

The sperm progressive assessment (slow and fast) is presented in Fig. 3. There was a significant difference in the percentage fast ($F_{5, 24} = 6.119$; $p = 0.0014$) and slow ($F_{5, 24} = 5.556$; $p = 0.0019$) sperms among the treatment groups. The percentage of slow sperm was significantly higher in both the positive control (CHL) and the
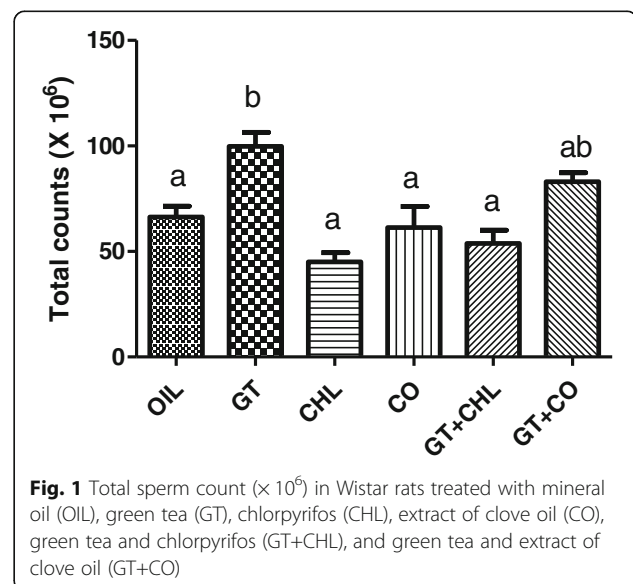

Fig. 1 Total sperm count (× 10⁶) in Wistar rats treated with mineral oil (OIL), green tea (GT), chlorpyrifos (CHL), extract of clove oil (CO), green tea and chlorpyrifos (GT+CHL), and green tea and extract of clove oil (GT+CO)

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Fig. 2 Sperm motile counts ($\times 10^6$) in Wistar rats treated with mineral oil (OIL), green tea (GT), chlorpyrifos (CHL), extract of clove oil (CO), green tea and chlorpyrifos (GT+CHL), and green tea and extract of clove oil (GT+CO)
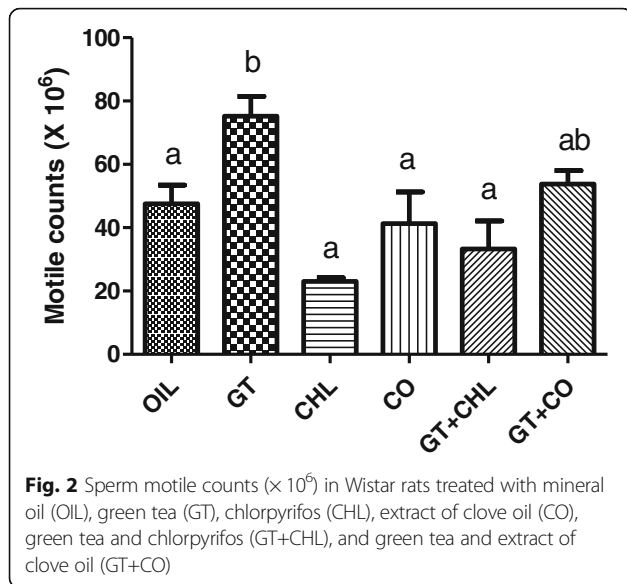
clove oil-treated animals. The percentage of slow-moving sperms for the positive control (CHL) and the clove oil-treated animals was 68.60 ± 11.67 and 53.00 ± 10.16, respectively. The values for the mineral oil- and green tea-treated groups were 44.20 ± 3.338 and 27.00 ± 2.49, respectively. The co-administration with green tea resulted in an increased number of fast-moving sperms in the chlorpyrifos (CHL) and the clove oil-treated

Fig. 3 Sperm progressive assessment (%) in Wistar rats treated with mineral oil (OIL), green tea (GT), chlorpyrifos (CHL), extract of clove oil (CO), green tea and chlorpyrifos (GT+CHL), and green tea and extract of clove oil (GT+CO)
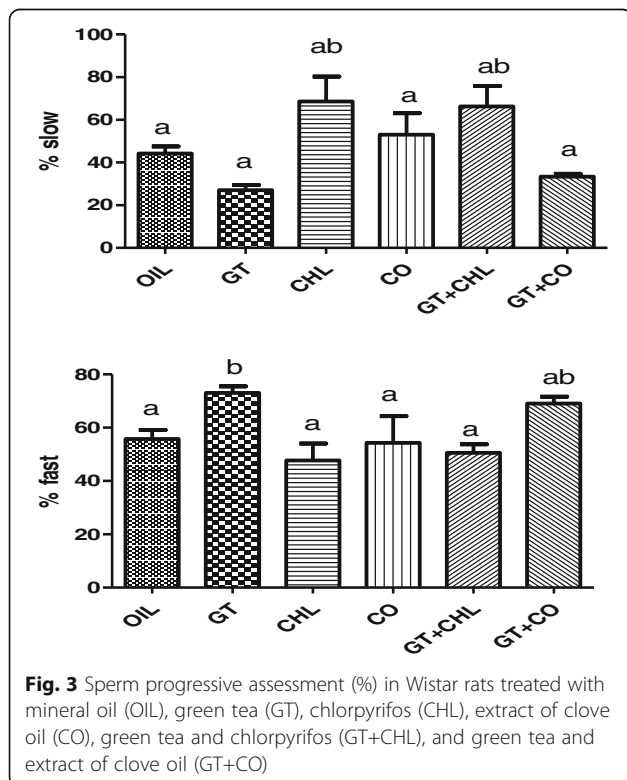
animals especially those that were treated with clove oil and green tea.

Sperm morphology

The data on sperm morphology were summarized in Fig. 4. The percentage of sperm with normal morphology was significantly higher ($p < 0.05$) in the green tea- and mineral oil-treated animals in comparison with those treated with clove oil or chlorpyrifos or those that received co-administration ($F_{5, 24} = 6.757$; $p = 0.0005$). The percentage of sperm with normal physiology in mineral oil- and green tea-treated groups was 56.20 ± 5.652 and 73.40 ± 2.676, respectively, while the percentage in those that were treated with clove oil and chlorpyrifos were 46.20 ± 9.378 and 27.20 ± 11.04, respectively.

The co-administration with green tea resulted in increased percentages of sperms with normal morphology in the chlorpyrifos (CHL) and the clove oil-treated animals. The percentage of sperm with tail deformity morphology was significantly ($p < 0.05$) lower in the green tea- and mineral oil-treated animals in comparison with those treated with clove oil or chlorpyrifos or those that received the co-administration treatment ($F_{5, 24} = 10.95$; $p < 0.0001$). The percentage of sperms with tail deformity was 9.60 ± 1.939, 17.67 ± 1.453, 22.25 ± 3.425, 32.25 ± 4.029, 26.25 ± 2.394, and 14.40 ± 1.122 for GT, OIL, CO, CHL, GT+CHL, and GT+CO, respectively.

The percentage of sperm with neck deformity was significantly lower in the green tea-and mineral oil-treated animals in comparison with those treated with clove oil or chlorpyrifos or those that received the co-administration treatment ($F_{5, 24} = 6.067$; $p = 0.0010$). The percentage of sperm with head deformity was significantly lower ($p < 0.05$) in the green tea- and mineral oil-treated animals in comparison with those treated with clove oil or chlorpyrifos or those that received the co-administration treatment ($F_{5, 24} = 4.336$; $p = 0.0063$).

Serum hormonal levels

The serum testosterone levels decreased significantly ($p < 0.05$) in rats that were treated with chlorpyrifos and clove oil in comparison to those treated with green tea, mineral oil, or the co-administration treatment ($F_{5, 24} = 6.442$; $p = 0.0010$). The levels of testosterone for the positive control (CHL) and the clove oil-treated animals were 0.0638 ± 0.0119 and 0.1194 ± 0.0222 ng/ml respectively. The values for the mineral oil- and green tea-treated groups were 0.1586 ± 0.0155 and 0.2005 ± 0.0178 ng/ml respectively. The co-administration with green tea increased the levels of testosterone in the chlorpyrifos (CHL) and the clove oil-treated animals. The levels of follicle stimulating hormone (FSH) differed significantly among the groups ($F_{5, 24} = 153$; $p < 0.0010$). The levels of follicle stimulating hormone (FSH) were
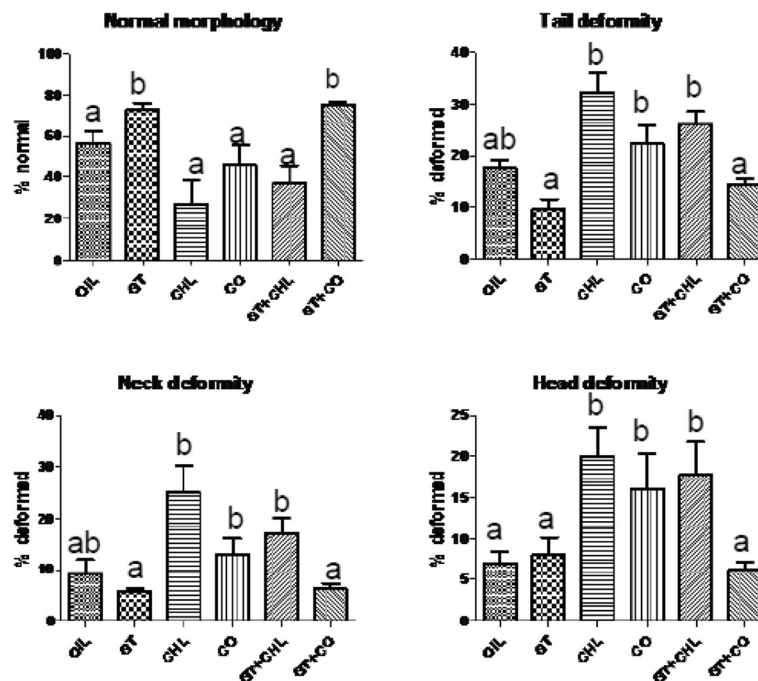
Fig. 4 Sperm deformity (%) in Wistar rats treated with mineral oil (OIL), green tea (GT), chlorpyrifos (CHL), extract of clove oil (CO), green tea and chlorpyrifos (GT+CHL), and green tea and extract of clove oil (GT+CO)

9.824 ± 0.560, 2.748 ± 0.328, 1.678 ± 0.1295, 1.018 ± 0.1245, 6.284 ± 0.1636, and 1.238 ± 0.0963 μg/ml for GT, OIL, CO, CHL, GT+CHL, and GT+CO, respectively. The levels of luteinizing hormone (LH) differed significantly among the groups ($F_{5, 24}$ = 98.31; $p < 0.0010$). The levels of luteinizing hormone (LH) were 7.586 ± 0.5614, 1.544 ± 0.08548, 1.404 ± 0.1463, 0.7560 ± 0.1282, 4.656 ± 0.2902, and 1.062 ± 0.0887 μg/ml for GT, OIL, CO, CHL, GT+CHL, and GT+CO, respectively (Fig. 5).
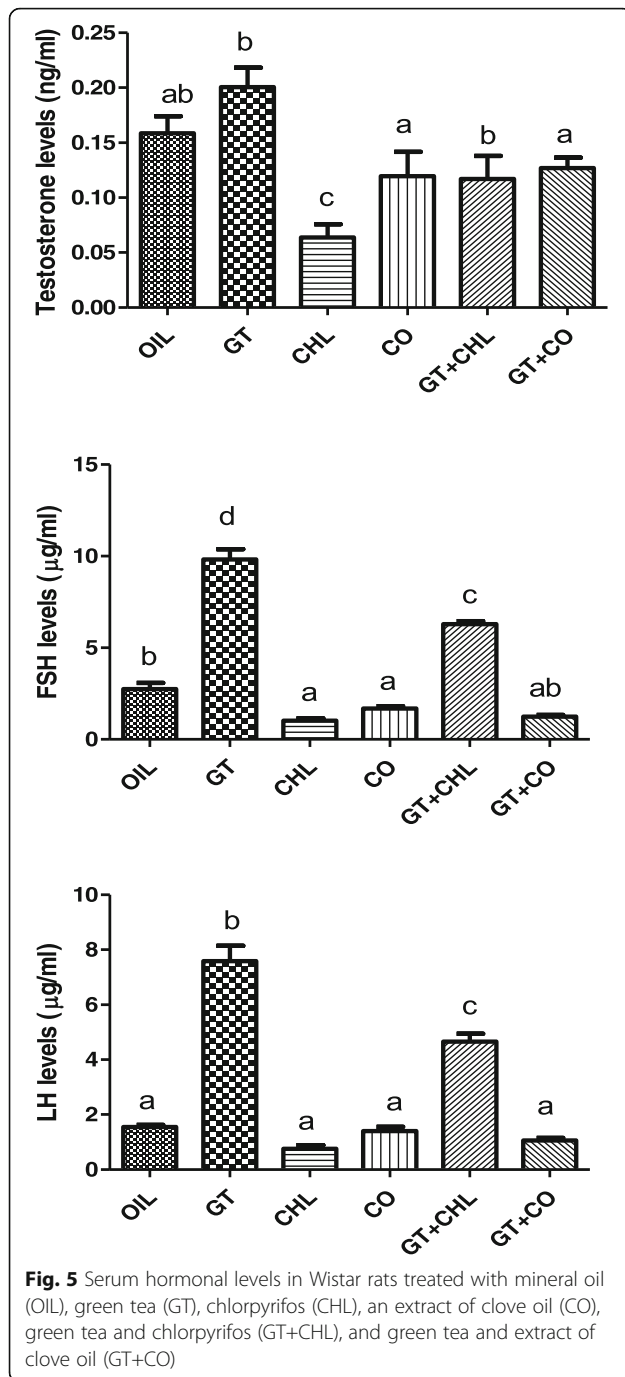
Histology of testes

The changes in the histological sections of the testes were summarized in Table 1 and shown in Plate 1. Overall, there was no obvious damage to the seminiferous tubules of the testes in rat groups that were treated with mineral oil, green tea, green tea + chlorpyrifos, and green tea + clove oil. However, the structural architecture of the testes was disrupted in rats that were treated with chlorpyrifos and extracts of clove oil. The observed histological changes included loss of both the cellular and tubular constituents of the seminiferous tubules, derangement of the seminiferous tubules, infiltration of fats, and outright loss of the seminiferous tubules.

Discussion

The present study investigated the potential spermicidal effects of oil extract of clove and chlorpyrifos on male Wistar rats and the ameliorating effect of the green tea. Results did show that both the clove oil and chlorpyrifos had adverse effects on certain sperm parameters like motility, total sperm count, and sperm progressive assessments. The decreased sperm count in rats that were treated with oil extract of *S. aromaticum* could be an indication that this plant material has the potential to alter the processes of spermatogenesis by evoking either hormonal imbalance or damage to important testicular cells such as Sertoli cells (Bretveld, Brouwers, Ebisch, & Roeleveld, 2007; Mehrpour, Karrari, Zamani, Tsatsakis, & Abdollahi, 2014). ATP is the energy currency of the cell and very important to cellular activities including motility. The decrease in sperm motility and viability in rats that were treated with extracts of clove oil and chlorpyrifos may be due to non-availability of adenosine triphosphate (ATP) for metabolism as a result of damage of sperm plasma membrane architecture by clove oil and chlorpyrifos (Chaki & Misro, 2002; Chaudhury, Bhattacharyya, & Guha, 2004). Similarly, the morphological analyses of sperm revealed that rats that were treated with clove oil or chlorpyrifos had a higher frequency of sperm abnormalities especially at the tail and neck regions. Although the mechanistic explanations for these abnormalities are conjectural, however, the abnormalities could have been due to abnormal chromosome, minor alteration in testicular DNA, or errors during the process of spermatogenesis (Giri, Prasad, Giri, & Sharma, 2002; Otitoloju, Obe, Adewale, Otubanjo, & Osunkalu, 2010).

Aside from sperm parameters, hormonal assays quantifying the levels of important hormones such as testosterone,

Fig. 5 Serum hormonal levels in Wistar rats treated with mineral oil (OIL), green tea (GT), chlorpyrifos (CHL), an extract of clove oil (CO), green tea and chlorpyrifos (GT+CHL), and green tea and extract of clove oil (GT+CO)

frequency of abnormal sperm in rats that were treated with clove oil and chlorpyrifos (Bretveld, Brouwers, Ebisch, & Roeleveld, 2007; Mehrpour, Karrari, Zamani, Tsatsakis, & Abdollahi, 2014).

The reproductive toxicity of clove oil and chlorpyrifos was further substantiated by histopathological analysis of the testes. The treatment of Wistar rats with clove oil and chlorpyrifos resulted in disruption of the normal architecture of the testes, e.g., loss of both the cellular and tubular constituents of the seminiferous tubules, derangement of the seminiferous tubules, fatty infiltration, and outright loss of the seminiferous tubules. The results of this study are consistent with the findings of Sebastian and Raghavan (2015) in which Wistar rats that were exposed to endosulfan, an organochlorine pesticide developed testicular atrophies. Similarly, Joshi, Mathur, and Gulati (2007) in another but similar study also reported that the treatment of Wistar rats with chlorpyrifos for 30 days at various dosage levels caused degenerated seminiferous tubule having decreased number of spermatogenic elements in exposed rats. The loss of cells of the seminiferous tubules in rats that were exposed to clove oil and chlorpyrifos could have been due to increased induction of reactive oxygen species leading to apoptosis in the testicular cells (Sebastian & Raghavan, 2015).

In this study, the potential of green tea to ameliorate the reproductive toxicity induced by clove oil and chlorpyrifos was investigated since there are reports that green tea has the potential to improve the fertility of male rats (Abdelrazek, Helmy, Elsayed, Ebaid, & Mohamed, 2016; Ghafurniyan, Azania, Nabiuni, & Karimzadeh, 2015). The data from the present study supported the assertion that treatment of rats with extracts of green tea has the potential to improve the reproductive fitness of rats. The rats treated with extracts of green tea either alone or in combination with clove oil or chlorpyrifos had higher total and motile sperm counts, the percentage of fast sperm was higher and the frequency of deformed sperms was low compared to the groups that were treated with clove oil or chlorpyrifos. Also, the serum levels of reproductive hormones were higher in rats that were treated with extracts of green tea either alone or in combination with clove oil or chlorpyrifos.

The mechanisms through which the green tea performs these functions are still speculatory but its antioxidant properties may be involved. One of the proposed mechanisms through which the sperm quality in stressed organisms could be compromised is oxidative stress through excessive production of reactive oxygen species (Sanocka & Kurpisz, 2004; Schulte, Ohl, Sigman, & Smith, 2010). Green tea possesses a good number of antioxidant compounds such as catechin which has the potential to inactivate free radicals (Al-Wafai, 2013; Burits and Bucar, 2000).

FSH, and LH are useful biomarkers in reproductive toxicology (Rockett & Kim, 2005). In the present study, the serum levels of testosterone, FSH, and LH were significantly lower in the rats that were treated with clove oil and chlorpyrifos in comparison to other treatment groups. Since these hormones play important roles in the process of spermatogenesis, rats with reduced level of these hormones are prone to developing abnormal sperm and also reduced sexual activity. This could explain the reason for a higher

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Table 1 Histological changes in testes of Wistar rats

Histological signs	Oil	GT	CHL	CO	GT+CHL	GT+CO
Normal seminiferous tubules	+	+	-	-	+	+
Loss of cellular constituents	-	-	+	+	-	-
Loss of tubular constituents	-	-	+	+	-	-
Derangement of seminiferous tubules	-	-	+	+	-	-
Loss of seminiferous tubules	-	-	+	-	-	-
Fatty infilteration	-	-	+	+	-	-
Legends: - No + Yes						

Conclusion

In conclusion, the data obtained from this study showed that *S. aromaticum* significantly affected the sperm morphology: a higher percentage of tail-, neck- and head-deformed sperms in rats while the effects on sperm total counts, sperm motile counts, and sperm progressive assessment were merely marginal. Also, hormonal assays showed that *S. aromaticum* has the tendency to cause hormonal imbalance in rats. The histopathological analyses of sections of the testes of stressed rats revealed apparent damage to the structure of the testes; loss of tubular and cellular constituents, fatty infiltration, derangement of seminiferous tubules, etc. The treatment of rats with *C. sinensis* alone or in combination with *S. aromaticum* resulted in improved sperm parameters: higher sperm total counts, sperm motile counts,
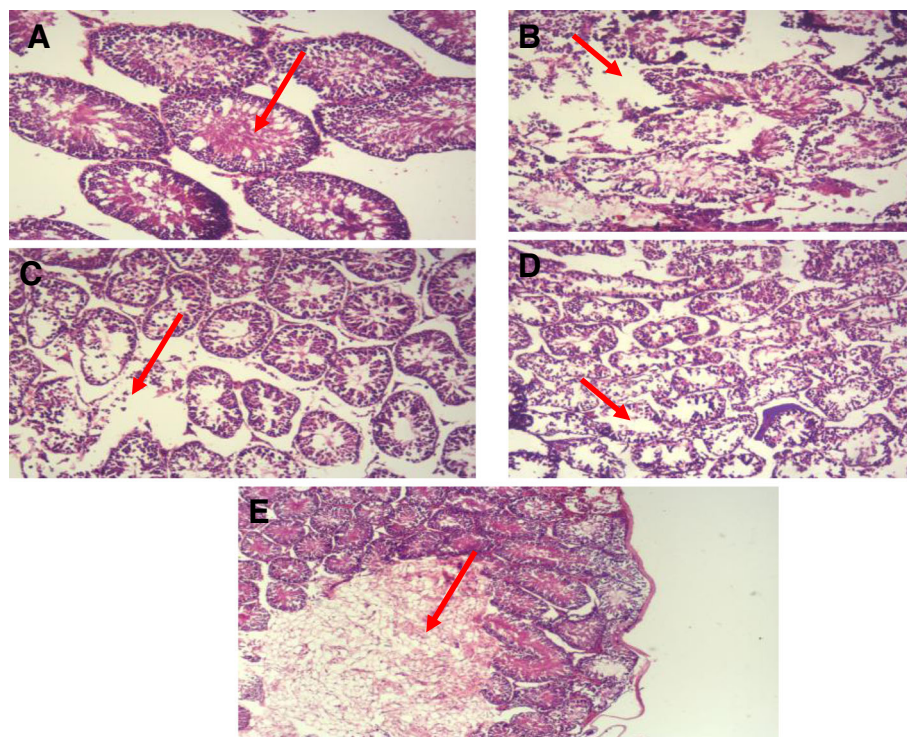

Plate 1 Representative micrograph of sections through the testes of Wistar rats showing A) normal structure of seminiferous tubules, B) loss of tubular constituents of seminiferous tubules, C) derangement of seminiferous tubules, D) loss of cellular constituents of seminiferous tubules, and E) fatty infiltration of seminiferous tubules

higher percentage fast sperm, less percentage deformed sperms, higher levels of reproductive hormones, and less damage to the testes. From the foregoing, *S. aromaticum* is potentially toxic to mammals; caution must, therefore, be applied to ensure that a low level of the plant material was used as a pesticide to avoid potential negative effects on mammals.

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