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Immunohistochemical Expression of Ki-67, cytokeratin-18 and BCL-2 in Wistar rats testes treated with *Rauwolfia vomitoria, Chlorpromazine* and Co-administration of Reserpine, Ascorbate and Zinc

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Abstract

Aim: The expression of Ki-67, cytokeratin-18, and BCL-2 proteins in Wistar rats testes was studied using *Rauwolfia vomitoria* RV extract, Chlorpromazine (CPZ), and combination of Reserpine, Ascorbate, and Zinc (RAZ).

Methods: Forty-five eight weeks old male Wistar rats (170-190 g) were selected into nine groups of five rats each. Group A was the control group, animals in groups B and C received 10 and 20 mg/kg of chlorpromazine respectively, animals in groups D and E received 2.5 and 5 mg/kg of reserpine respectively, animals in groups F and G received 150 and 300 mg/kg of *RV* leaf extract respectively while groups H and I animals received (2.5:5:100) mg/kg and (5:10:200) mg/kg of combination of RAZ respectively. All compounds were administered orally for 56 days.

Results: Chlorpromazine and reserpine treated rats showed weak immunoreactivity to ki-67 and strong positive immunoreactivity to cytokeratin and BCL-2 proteins while RVand combination of RAZ treated rats showed weak positive immunoreactivity to cytokeratin and BCL-2 and strong immunoreactivity to ki-67. Furthermore, slight significant increase in germ cell proliferation index was seen in RV *and* RAZ treated groups when compared with CPZ and RES treated groups while significant decrease in germ cell apoptotic index and immature sertoli cell index were seen in RV *and* RAZ treated groups when compared with CPZ and RES treated groups.

Conclusion: This research revealed the reproductive toxicity of synthetic antipsychotic drugs (CPZ and RES) and also unveiled the fertility potential of antipsychotic herb (RV) extract alongside RAZ by reducing the reproductive toxicity that is commonly associated with antipsychotic drugs.

Keywords: Antipsychotic compounds, cytokeratin, *Rauwolfia vomitaria*

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Immunohistochimique de ki-67, cytokératine-18 et bcl-2 dans les testicules de rats wistar traites avec*rauwolfiavomitoria, chlorpromazine* et co-administration de réserpine, ascorbate et zinc

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Résumé

Objectif de l'étude : L'expression des protéines Ki-67, cytokératine-18 et BCL-2 dans les testicules de rats Wistar a été étudiée à l'aide d'extrait de *Rauwolfiavomitoria* RV, de chlorpromazine (CPZ) et d'une combinaison de réserpine, d'ascorbate et de zinc (RAZ).

Méthode de l'étude : Quarante –cinq (45) rats Wistar mâles âgés de huit semaines (170-190 g) ont été sélectionnés en neuf groupes de cinq rats chacun. Le groupe A était le groupe témoin, les animaux des groupes B et C ont reçu respectivement 10 et 20 mg/kg de chlorpromazine, les animaux des groupes D et E ont reçu respectivement 2,5 et 5 mg/kg de réserpine, les animaux des groupes F et G ont reçu 150 et 300 mg/kg d'extrait de feuille de *RV* respectivement tandis que les animaux des groupes H et I recevaient respectivement (2,5:5:100) mg/kg et (5:10:200) mg/kg de combinaison de RAZ. Tous les composés ont été administrés par voie orale pendant 56 jours.

Résultat de l'étude : Les rats traités à la chlorpromazine et à la réserpine ont montré une faible immun réactivité au ki-67 et une forte immun réactivité positive à la cytokératine et aux protéines BCL-2 tandis que la combinaison RV et RAZ a montré une faible immunoréactivité positive à la cytokératine et au BCL-2 et une forte immunoréactivité au ki-67. En outre, une légère augmentation significative de l'indice de prolifération des cellules germinales a été observée dans les groupes traités par RV *et* RAZ par rapport aux groupes traités par CPZ et RES, tandis qu'une diminution significative de l'indice d'apoptose des cellules germinales et de l'indice des cellules de sertoli immatures a été observée dans les groupes traités par RV *et* RAZ par rapport aux groupes traités. Groupes traités CPZ et RES.

Conclusion : Cette recherche a révélé la toxicité reproductive des médicaments antipsychotiques synthétiques (CPZ et RES) et a également dévoilé le potentiel de fertilité de l'extrait d'herbe antipsychotique (RV) aux côtés de RAZ en réduisant la toxicité reproductive couramment associée aux médicaments antipsychotiques.

Mots-clés : Composés antipsychotiques, cytokératine , rauwolfiavomitaria

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INTRODUCTION

Synthetic antipsychotics, also known as neuroleptics, are chemically synthesized medications used to treat major mental illnesses including psychosis, as well as other emotional and mental disorders (1). Synthetic antipsychotic drugs can be divided into a number of subtypes, based around when they were first synthesized or their constitutive receptor occupancy. Some of these antipsychotic drugs are Chlorpromazine, Reserpine, Haloperidol, Fluphenazine, Molindone and Loxapine which are first generation antipsychotic drugs (1).

Chlorpromazine (CPZ) has been used to treat both acute and chronic psychoses, such as schizophrenia and manic-depressive disease, for many years. Thorazine, Largactil, and other trade names were used to sell CPZ, a phenothiazine derivative. It's most commonly used to treat psychotic diseases like schizophrenia (2). Treatment of bipolar illness, nausea and vomiting, anxiety before surgery, and hiccups that do not resolve after conventional interventions are among the other uses (3). CPZ antipsychotic activity is through blockage of dopamine (D2) receptors in the mesolimbic pathway of the brain, over activity of which is understood to be responsible for the symptoms of schizophrenia (2). CPZ is well absorbed through the mouth, with a maximum effect of around three hours and a long lasting effect. After 10 mg/kg body weight CPZ administration in rats, Williams *et al.* (4) found a large increase in serum prolactin and a significant drop in testosterone and Luteinizing Hormone (LH). In immature female rats, Ali and Bhagya (5) observed that CPZ causes follicular atresia and delays puberty onset.

Reserpine (RES) is an odorless white or pale buff to slightly yellowish crystalline powder that is a pure crystalline alkaloid of the *Rauwolfia vomitoria* (RV) plant. When exposed to light, it darkens slowly, but when in solution, it darkens more quickly. It is insoluble in water, freely soluble in acetic acid, chloroform, benzene, and in alcohol. Nur and Adam (6) reported that Reserpine's irreversible pharmacology potency makes it a better antipsychotic because its effect lasts longer than any other antipsychotic drug. Though RES only as an antipsychotic drug has been reported to pose some side effects which make its use to be discontinued in the United Kingdom for some years but find its way back into the market by combining with other chemical such has chlorthalidone, thiazide etc (6). Some anti-pyramidal side effects have been recorded with RES (6). Khazan *et al.* (7) found significant atrophy and decreased spermatogenesis in the testes of pigeons given reserpine, whereas Mosad *et al.* (8) found mild to severe degenerative alterations in the testes of rats given RES while the prevalence of hyper-prolactin has been estimated to be between 23 and 72 percent in treated male patients (9).

The use of herbal medicine in this present century cannot be overlook. Recent trends in research into African plant uses show that traditional medicine is commonly used to treat neurological disorders in the West African region. Use of these natural products cannot be overlooked in the treatment of mental and other ailments in Nigeria as well (10). *Rauwolfia vomitoria* (RV) is one of the natural products used traditionally for the treatment of mental disorders in Nigeria (10). The plant *RV* belongs to the family Apocynaceae. It is called Swizzle stick in English, Asofeyeje in Yoruba, Wadda in Hausa and utoenyin in Efik (***11***). Some of the phytochemicals present in *Rauwolfia vomitoria* leaf are Alkaloids, rauwolfine, rescinnamine, serpentine, ajmaline serpentinine, steroid-serposterol, saponin, and reserpine (active molecule used in the treatment of psychosis) (10) Furthermore, *Rauwolfia vomitoria* leaf extract has been found to contain significant levels of zinc and vitamin C, both of which are necessary for male fertility (12). Dieudonne *et al.* (13) reported that *RV* enhance testosterone production and protect Leydig cells against oxidative stress. Ajao *et al.* (14) also reported its beneficiary effects on male Wistar rat reproductive parameters after administration of 150 mg/kg and 300 mg/kg per body weight of aqueous leaves extract of *RV*.

From the literatures, it has been revealed that synthetic antipsychotic drugs such as Reserpine and Chlorpromazine induced reproductive toxicity while few researches have shown the beneficiary effects of the *Rauwolfia vomitoria* on reproductive parameters. To our knowledge, no research has looked at the effects of concurrent administration of Reserpine, Ascorbate, and Zinc (RAZ), the selected phytochemicals found in *Rauwolfia vomitoria* leaves, on male reproductive parameters. Thus, this work aimed at comparing the effects of *Rauwolfia vomitoria leaf crude extracts, Chlropromazine, Reserpine* and RAZ on thecytoarchitecture of Wistar rat testes.

MATERIALS AND METHOD

Sourcing and preparation of compounds

All of the substances utilized (Chlorpromazine, Reserpine, Zinc, and Vitamin C) were pure compounds obtained from Hefei TDJ Chemical Co. Ltd in China and authenticated by the University of Ilorin's Pharmacy Department. 100 mg of these chemicals were dissolved in 100 mL of distilled water, resulting in 1 mg of solvent per mL of solution (Stock solution). For proper dissolution, the solution was allowed to stand for a few minutes while being constantly stirred.

Ethanolic extraction and authentication of *Rauwolfia vomitoria* leaves

Rauwolfia vomitoria leaves were taken locally from a farmland in Osogbo and identified at the University of Ilorin's Division of Botany, Department of Biological Sciences. The leaves of *Rauwolfia vomitoria* were air dried, then pulverized using an electric blender (Blender/Miller III, model MS223, Taiwan, China), and the extraction technique has described by Adeleke et al. (15).

Experimental Design

The University of Ilorin ethical review committee granted approval with the approval number (UERC/ASN/2017/1067) (UERC). All procedures were carried out in accordance with international best practices and institutional norms for animal care and usage.

Animal Sacrifice and Samples Collection

The rats were sacrificed 24 hours after the last day of compound administration by euthanizing them with 80 mg/kg of ketamine and using the transcardial perfusion method by fixing the entire rat's body with 4% paraformaldehyde. Testicular tissues were excised for histological and immunohistochemical examinations.

Histological examination

The testes were fixed in bouin's fluid, dehydrated in increasing concentrations of alcohol, cleaned in xylene, immediately dipped in molten paraffin wax, and finally embedded in molten paraffin wax to make a paraffin block. The tissue was then sectioned at 4 um thickness from the paraffin block using the rotary microtome. The sections were transferred to a glass slide and stained with hematoxylin and eosin after floating in a water bath at 40°C.

Immunohistochemical examination

The slides were dried after mounting to remove any water that had become trapped beneath the portion. The slides were washed with TBS buffer once the section was brought down to distilled water. Endogenous peroxidase was then inhibited for 10 minutes with hydrogen peroxide and rinsed with buffer for 1 minute before being digested with proteinase K and rinsed for two minutes. The protein block was added for 10 minutes before being shaken off. Anti-Ki-67, cytokeratin-18, and BCL-2 were applied to each tissue and rinsed with buffer for 2 minutes, followed by antibody enhancer for 10 minutes and a one-minute rinse with buffer. Polymer was added for 15 minutes and rinsed for two minutes with buffer, whereas DAB was added for ten minutes and washed for two minutes with buffer. Hematoxylin was used as a counterstain, and the tissue was washed with buffer for two minutesand finally washed in water, dehydrated, cleared and mounted.

Germinal Cell Count

An Olympus binocular research microscope (Olympus, New Jersey, USA) was used to take immunohistochemical examinations of the testes, which was coupled to a 5.0 MP Amscope camera (AmscopeInc, USA). Germ cell proliferation index and apoptotic index were counted from captured testicular images (n=5 per group/per analysis) using physical fractionators techniques (stereological grid). The slides used for germ cell count were sectioned at the same level and for each section; the count was across the testicular profile (using cellular morphology and layer-dependent cell densities) for 8 different fields of view. A restricted artificial boundary was marked on the testicle limited to boundary of the testicle. On the field was placed a counting grid with a collection of evenly spaced points. All points that hit profiles were counted, regardless of their connection to the frame. 84 If a profile (including its boundary) covers the upper right corner of the cross where the two lines cross, the point is called a hit. The volume density was calculated as proportion of volume by simple percentile fraction; VD (profile counted/total specimen profile x 100). Positive cells were counted and recorded and their mean with standard error of mean was plotted on bar chart for each of the groups (16).

Statistical Analysis

For all statistical studies, GraphPad Prism version 8.03 was utilized. All results were

reported as Mean ± SEM, with one-way ANOVA used to examine differences between groups. Multiple comparisons were adjusted using Tukey's test. Statistical significance was defined as a *p* value of less than 0.05.

RESULTS

Histoarchitecture of Rat's Testes Stained with Hematoxylin and Eosin dye

Figure 1 showed photomicrograph of rat testes stained with H&E. The control group A animals revealed normal testicular architecture without any observable presentation of spermatogenic arrest and the lumen could also be observed with the presence of spermatozoa. The basement membrane is thin and the interstitial space contains Leydig cells. Groups B and C treated with CPZ (5 mg/kg and 10 mg/kg respectively) showed severe observable degenerative changes characterized by maturation arrest of the spermatogenic cell line in several seminiferous tubules, widened lumen that lack spermatozoa (black thin arrow), fragmented basement membrane and pyknoticLeydig cells while Groups D and E **treated with** RES (2.5 mg/kg and 5 mg/kg respectively) unveil degenerative changes characterized mainly by wide lumen with little spermatozoa in some seminiferous tubules was observed in Group D while severe degenerative changes characterized mainly by wide lumen with no spermatozoa in most of the semeiniferous tubules. Similar morphological presentations with similar cellular density were observed in groups F, G, H and I when compared with the control group. The testicular cytoarchitecture was well structured and characterized by seminiferous tubules having numerous Spermatogonia cells that have differentiated into numerous Spermatocytes, the presence of sertolic cells at the adlumina border, presence of Leydig cells in the interstitial spaces, and seminiferous tubule lumen filled with spermatozoa.

Immunohistochemical expression of Ki-67 in Rats' Testes

Figure 2 depicts immunohistochemical staining for Ki-67 in control group A with strong positive Ki-67 immunoreactivity which indicates normal proliferation and differentiation of spermatogenic cell lines. Both groups B and C showed weak Ki-67 immunoreactivity, presented with lightly stained dark brown granules which signal poor proliferation and arrest of differentiation in testicular germ cells while moderate Ki-67 immunoreactivity characterized by mild arrest of differentiation of spermatogenic cell lines were observed in groups D and E. Groups F, G, H and I expressed strong positive Ki-67 immunoreactivity, which presented deeply stained dark brown granules characterized by normal proliferation and differentiation of spermatogenic cell lines.

Immunohistochemical expression of Cytokeratin-18 in Rats' Testes

Figure 3 depicts immunohistochemical staining for cytokeratin-18 in control group A with negative immunoreactivity to cytokeratin-18 stain indicative of less immature sertoli cell index. The immunohistochemical staining appeared as deep dark-brown granules stained in the immature sertoli cells of groups B, C, D and E which is a sign of prevalent immature sertoli cells localized at the periphery of the seminiferous tubules. Group F expressed weak positive immunoreactivity to cytokeratin-18 stain with few immature sertoli cells localized at the periphery of the seminiferous tubules while group G expressed negative immunoreactivity to cytokeratin-18. Group H expressed weak positive immunoreactivity to cytokeratin-18 stain with few immature Sertoli cells localized at the periphery of the seminiferous tubules while group I expressed negative immunoreactivity to cytokeratin-18 stain.

Immunohistochemical expression of BCL-2 in Rats' Testes

Figure 4 depicts immunohistochemical staining for BCL-2 in control group A with negative BCL-2 immunoreactivity which indicates reduced number of germinal cells death in the testes. Groups B, C, D and E showed strong positive immunoreactivity, presented with deeply stained dark brown germinal and Leydig cells which is indicative of a high germinal cells apoptotic index in the testes while groups F, G, H and I expressed a weak positive immunoreactivity, presented with lightly stained dark brown Leydig cells to BCL-2 stain.

Germ Cell Proliferation Index

Figure 5 showed mean values of germ cell proliferation index among the groups after the administration of CPZ, RES, RV and Co-administration of RAZ. From the graph, significant decreased ($p<0.05$) in germ cell proliferation index was observed when CPZ and RES treated groups were compared with control group A. Furthermore, slight significant increased ($p<0.05$) in germ cell proliferation

index was seen in RV*and* co-administration of RES, Ascorbate and Zn treated groups when compared with CPZ and RES treated groups. No significant difference (p>0.05) when compare RV and co-administration of RES, Ascorbate and Zn treated groups with the control group A.

Immature Sertoli Cell Index

Figure 6 showed mean values of immature sertoli cell index among the groups after the administration of CPZ, RES, RV and Co-administration of RAZ. From the graph, significant increase (p<0.05) in immature sertoli cell index was observed in CPZ and RES treated groups when compared with the control group A. Furthermore, slight significant decreased (p<0.05) in immature sertoli cell index was seen in RV*and* co-administration of RES, Ascorbate and Zn treated groups when compared with CPZ and RES treated groups. No significant difference (p>0.05) when compare RV*and* co-administration of RES, Ascorbate and Zn treated groups with the control group A.

Germ Cell Apoptotic Index

Figure 7 showed mean values of germ cell apoptotic index among the groups after the administration of CPZ, RES, RV and Co-administration of RAZ. From the graph, significant increased (p<0.05) in germ cell apoptotic index was observed when CPZ and RES treated groups were compared with the control group A.
Furthermore, slight significant decreased in germ cell apoptotic index was seen in RV *and* co-administration of RES, Ascorbate and Zn treated groups when compared with CPZ and RES treated groups. No significant difference (p>0.05) when compare RV*and* co-administration of RAZ treated groups with the control group A.

DISCUSSION

Male infertility is a problem which has gained increased attention over the past several decades. While many factors may contribute to male infertility, some medications may also affect reproductive functioning and thus may have an impact on fertility. Synthetic antipsychotic drugs, such as CPZ and RES, have been linked to a variety of adverse effects on the body, including the reproductive system (17) while psychiatric patients treated with traditionally used antipsychotic herbs like RV showed no traces of reproductive toxicity (14). Thus, this work investigated the expression of Ki-67, cytokeratin-18 and BCL-2 proteins in Wistar rats testes treated with *RV, Chlorpromazine (CPZ)* and Co-administration of Reserpine, Ascorbate and Zinc (RAZ).

Spermatogenesis is a complicated process that involves mitotic, meiotic, and spermatogonial stem cell division and differentiation into mature spermatozoa. These activities involved activation and deactivation of some certain proteins such as Ki-67, cytokeratin-18 and BCL-2.For decades, the Ki-67 protein has been employed as a proliferation marker for human tumor cells. Ki-67 has a role in both interphase and mitotic cells, and its cellular distribution shifts substantially as chromosomes proceed through the cell cycle (18). Furthermore, the BCL-2 family of proteins regulates mitochondrial outer membrane permeability, which leads to the irreversible release of intermembrane space proteins, caspase activation, and apoptosis. The predominant interactions between anti-apoptotic and pro-apoptotic BCL-2 family proteins that regulate mitochondrial outer membrane permeability are dictated by the affinities and relative abundance of BCL-2 family proteins. (19). Cytokeratins are intermediate filaments that represent an excellent marker for epithelial differentiation because all epithelial cells, whether of ectodermal, mesodermal, or endodermal origin, contain cytokeratins; Cytokeratin-18 is typically expressed in the sertoli cell cytoplasm of the male fetus and additionally all through childhood (20).

Testicular cytoarchitecture has shown from this study revealed moderate to severe degeneration of seminiferous tubular epithelium in the rats treated with CPZ and RES. More also, testicular immuno-staining of the CPZ and RES treated groups showed positive immunoreactivity to BCL-2, cytokeratin-18 staining intensity and weak positive immunoreactivity to ki-67 staining intensity. According to Xiaoming and Paul (18), Ki-67 is necessary for appropriate cellular distribution of heterochromatin antigens and heterochromatin nucleolar interaction during interphase. Ki-67 is required for the creation of the perichromosomal layer, a ribonucleoprotein sheath that surrounds the condensed chromosomes during mitosis. Ki-67 prevents the clumping of mitotic chromosomes in this structure. Furthermore, Kruse *et al.* (21) found that Cytokeratin expression was found in adult Sertoli cells in testis with mixed atrophy and spermatogenic arrest at the spermatogonia level. This clinical disorder identified different patterns of

Cytokeratin-18 expression in Sertoli cells in adult males exhibiting various degrees of spermatogenic failure as well as carcinoma in situ cells in independent biopsies of individual testes with mixed tubular atrophy (22). Orth *et al.* (23) revealed that the first wave of spermatogenesis is accompanied by widespread death of pre-meiotic germ cells, which could indicate that the number of germ cells is adjusted to fit the capacity of the available supporting Sertoli cells. As a result, BCL-2 protein expression is expected to be strong at this time. While apoptosis occurs in germ cells during adult spermatogenesis, it is less common and may indicate the selective elimination of damaged cells in some circumstances (24).

Testicular histoarchitecture were improved in RV and co-administration of RAZ treated groups. Previous studies demonstrated by Colager *et al*. (25); Yamaguchi *et al*. (26) and Deborah and Haim (27) support these findings. Low concentration of Zn in the diet, according to Colager *et al.* (25) is a significant risk factor for low sperm quality and idiopathic male infertility (25). Moreover, Yamaguchi *et al.* reported that Zn deficiency impedes spermatogenesis which is a major reason for sperm abnormalities (26). Zinc is necessary for making the outer membrane and tail of the sperm and also important for sperm maturation. Zinc supplements have been shown to improve sperm count, motility, form, function, quality and fertilizing capacity (27).

Stereological evaluations from this study revealed significant increase in germ cell apoptotic and immature sertoli index was observed in CPZ and RES treated groups while germ cell proliferation index were significantly reduced. *Rauwolifia vomitoria* and co-administration of RAZ treated groups showed decrease in germ cell apoptotic and immature sertoli index and significant increase in germ cell proliferation. As reported by Croxford *et al.* (28) Zn plays an important function in spermatozoa physiology. Primary testicular failure is caused by Zn deficiency, which reduces the activity of the luteinizing hormone receptor, reduces steroid production, and damages Leydig cells due to oxidative stress (28).

Zinc ion and Ascorbate are present in *Rauwolifia vomitoria* and co-administration of RAZ treated groups. Zinc ion might have mitigated the reproductive toxicity effects of RES by binding to seminal plasma proteins and protecting sperm chromatin stability. This ion is involved in the formation of S-Zn-S type bonds in protamine structure, which helps to keep chromatin stable (29). Zinc is secreted in two forms: free and as part of protein complexes with a high molecular weight (30). Zinc is abundant in the tails of mature spermatozoa, where it is linked to sulfhydryl groups and disulfide linkages (29). By forming a specific number of SH-Zn-SH structures in the sperm nucleus, Zn controls disulfide cross-links (31). Zn is involved in the formation of coagulum, DNA stability control, antibacterial action and sperm movement inhibition. In addition to this, the observed effects of Ascorbic acid upon spermatogenesis cycle are linked to both antioxidant and non-antioxidant, the cellular enzyme activity of the vitamin. Ascorbic acid is essential to the production of sperm DNA content by being a coenzyme in DNA methylation. The role of healthy DNA is a healthy spermatozoon (32).

CONCLUSION

Healthy spermatozoa are parts of prerequisite to ascertain fertility in men, and changes in the expression of proteins involved in spermatogenesis may result in spermatozoa of poor quality. This research has revealed the reproductive toxicity of synthetic antipsychotic drugs (CPZ and RES), as well as the activity of compounds contained in the commonly used antipsychotic herb (RV) through crude extract administration and concurrent administration of RAZ to reduce the danger of reproductive toxicity associated with antipsychotic drugs.

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Table 1: Shown research experimental design

	Control	CPZ		RES		RV		RAZ	
Groups	A	B	C	D	E	F	G	H	I
Dosage	**N. Saline**	**5mg/kg**	**10mg/kg**	**2.5mg/kg**	**5mg/kg**	**150mg/kg**	**300mg/kg**	**(2.5:100:5) mg/kg**	**(5:200:10) mg/kg**
Number of rats	5	5	5	5	5	5	5	5	5
Duration of treatment (days)	56	56	56	56	56	56	56	56	56

Total number of animal used = 45; Mode of administration = orogastric

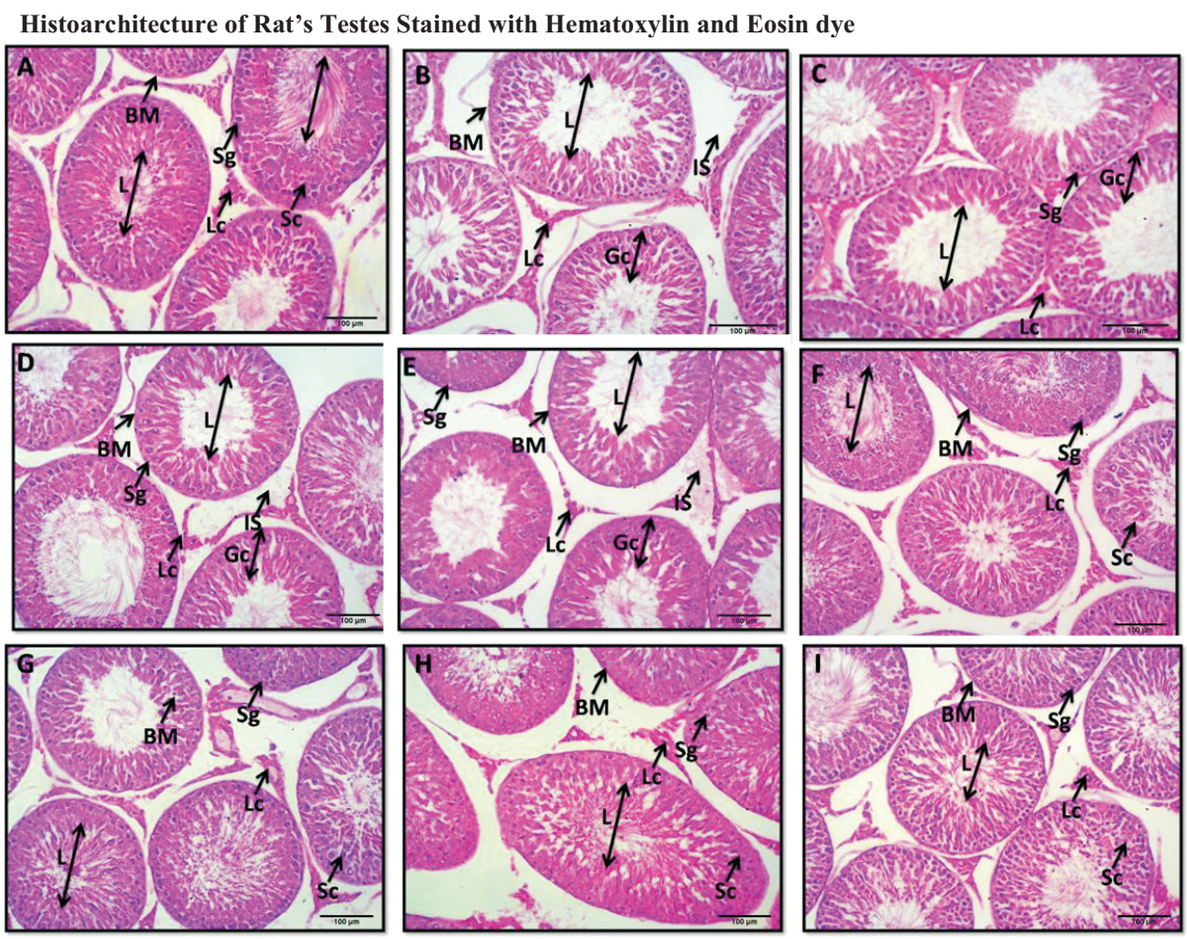

Figure 1: Cytoarchitectural presentations of the rat testes stained with H&E (Scale bar: 100 µm). Presented within and outside the seminiferous tubules are germinal cell (Gc), basement membrane (BM), lumen (L), Leydig cells (Lc), interstitial space (IS) and spermatogonia cells (Sg).

Immunohistochemical expression of Ki-67 in Rats' Testes

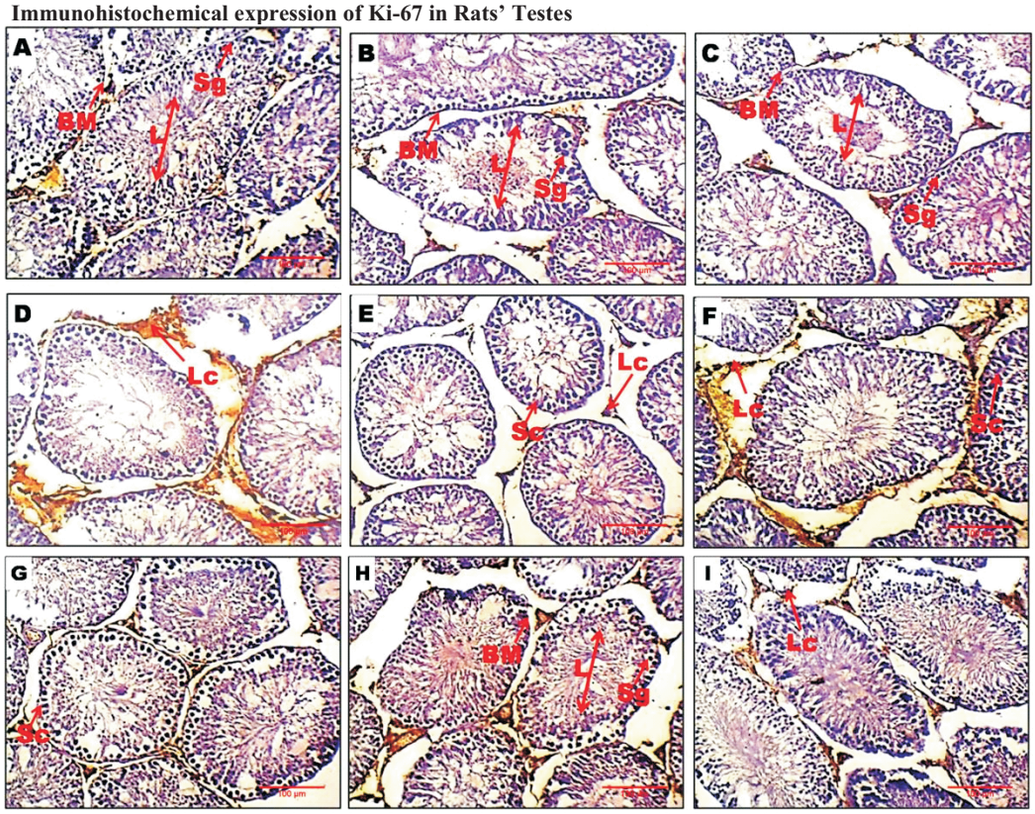

Figure 2: Immunohistochemical staining for Ki-67 in control group A(Scale bar: 100 µm). (Scale bar: 100 µm). Presented within and outside the seminiferous tubules are germinal cell (Gc), basement membrane (BM), lumen (L), Leydig cells (Lc), interstitial space (IS) and spermatogonia cells (Sg).

Immunohistochemical expression of Cytokeratin-18 in Rats' Testes

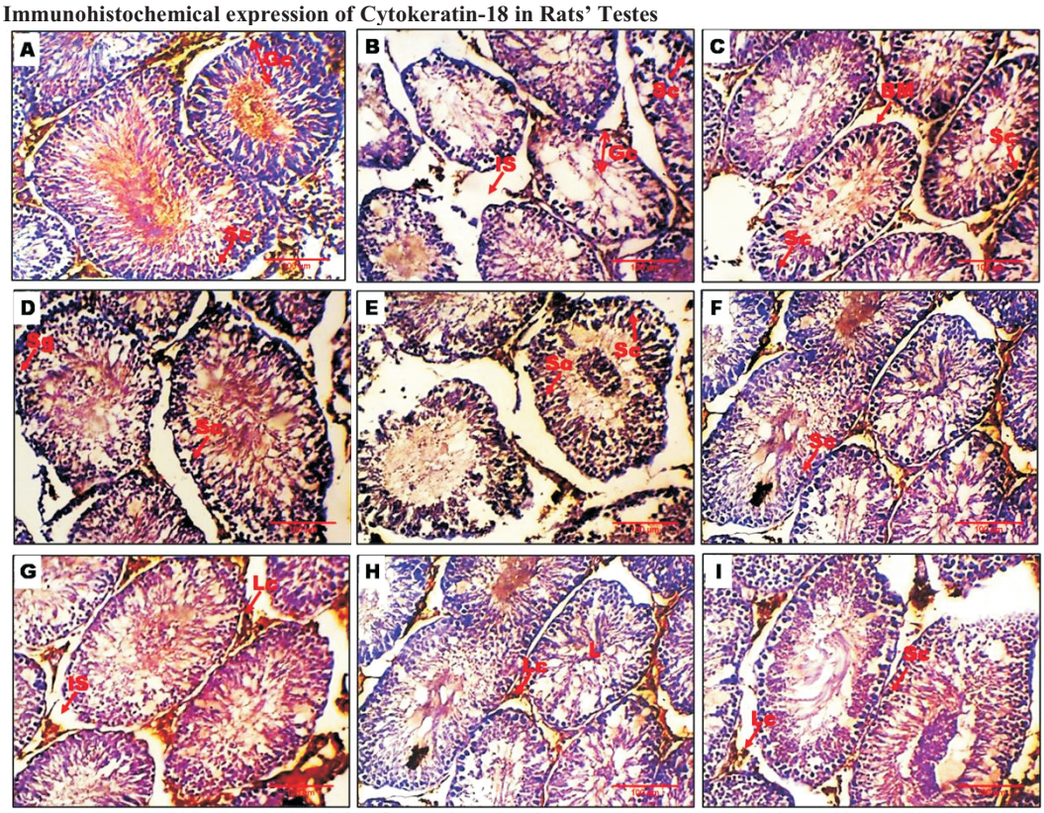

Figure 3: Immunohistochemical staining for cytokeratin-18 in control group A(Scale bar: 100 µm). (Scale bar: 100 µm). Presented within and outside the seminiferous tubules are germinal cell (Gc), basement membrane (BM), lumen (L), Leydig cells (Lc), interstitial space (IS) and spermatogonia cells (Sg).

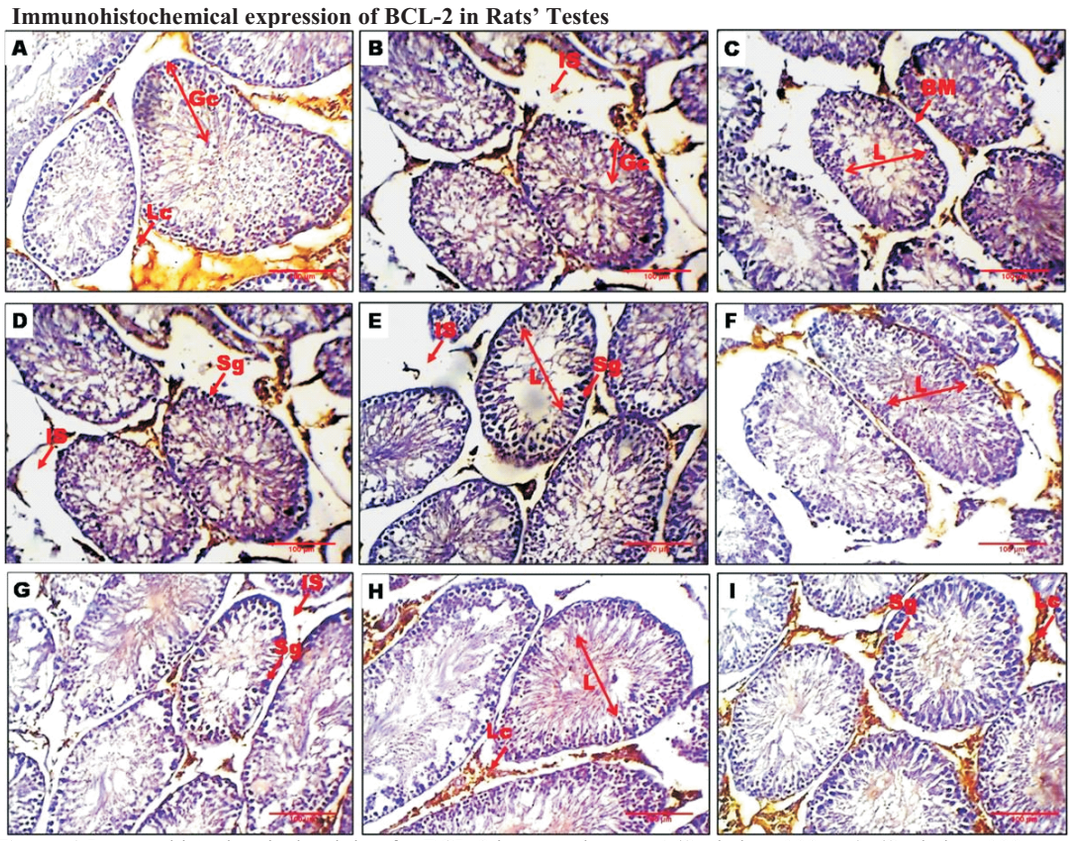

Figure 4: Immunohistochemical staining for BCL-2 in control group A(Scale bar: 100 µm). (Scale bar: 100 µm). Presented within and outside the seminiferous tubules are germinal cell (Gc), basement membrane (BM), lumen (L), Leydig cells (Lc), interstitial space (IS) and spermatogonia cells (Sg).

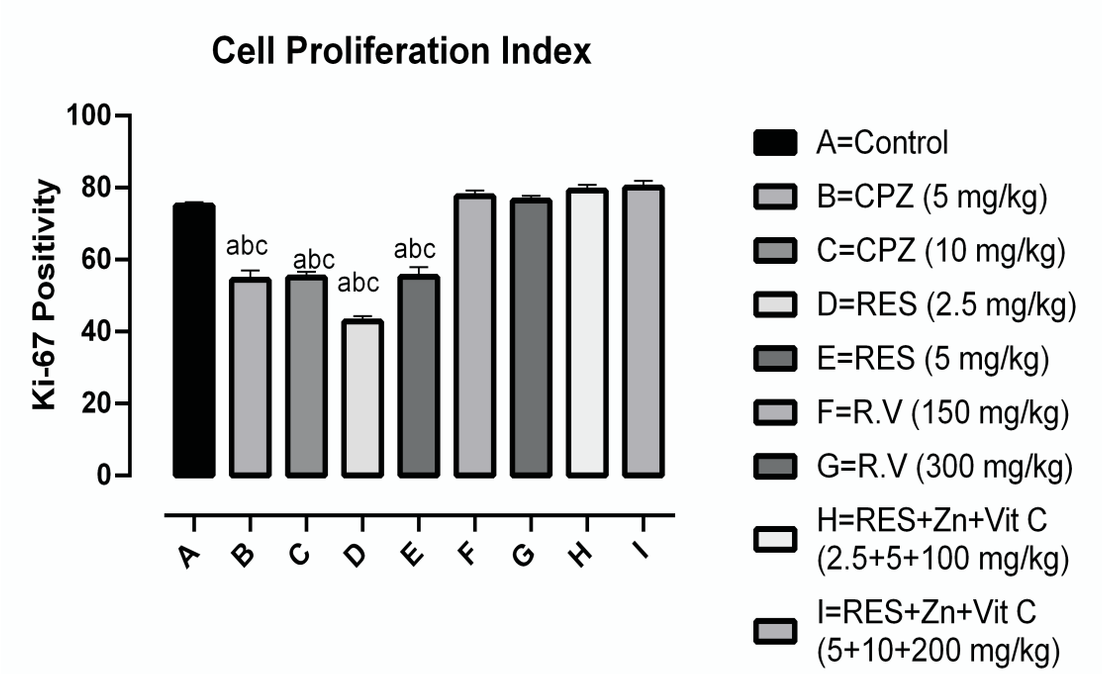

Figure 5: Showed comparison in germ cell proliferation index among the groups after the administration of CPZ, RES, RV and Co-administration of RAZ. **a** = Comparison with Control Group A; **b** = Comparison with Group H and **c** = Comparison with group I. * $p<0.05$ (n=5).

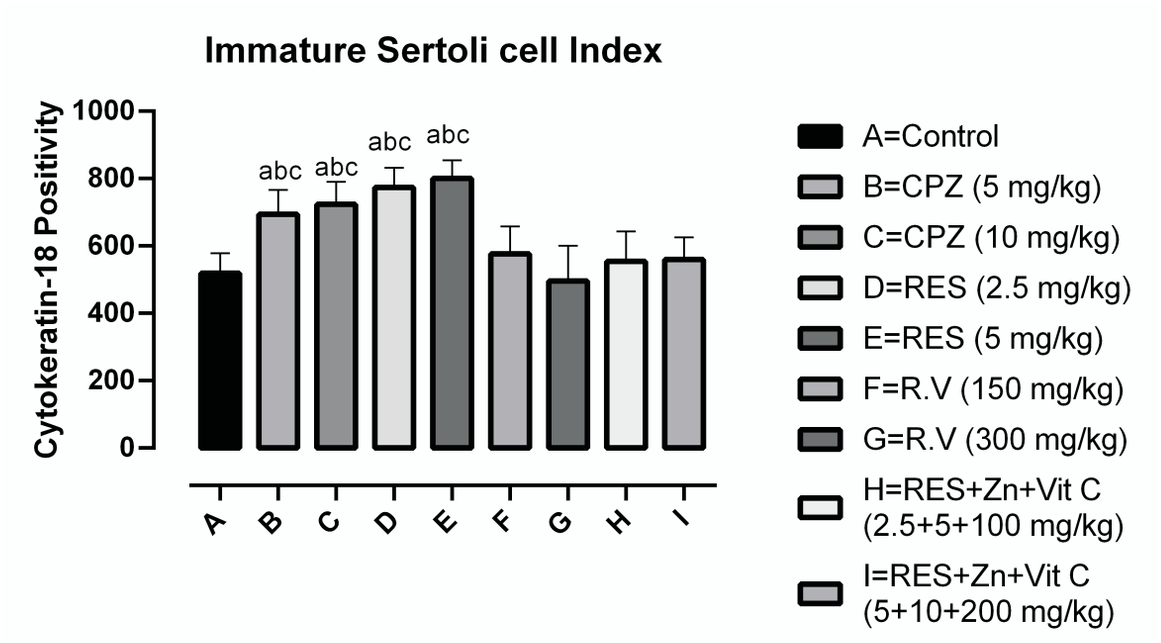

Figure 6: Showed comparison in immature sertolic cell index among the groups after the administration of CPZ, RES, RV and Co-administration of RAZ. **a** = Comparison with Control Group A; **b** = Comparison with Group H and **c** = Comparison with group I. **p*<0.05 (n=5).

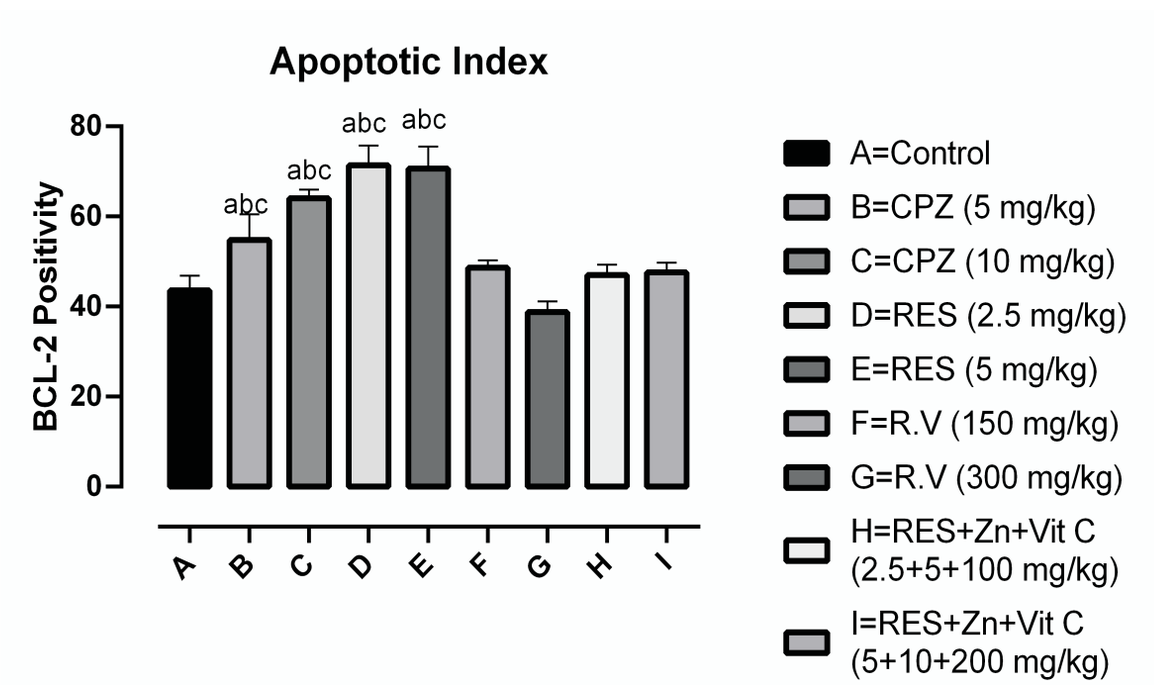

Figure 7: Showed comparison in germ cell apoptotic index among the groups after the administration of CPZ, RES, RV and Co-administration of RAZ. **a** = Comparison with Control Group A; **b** = Comparison with Group H and **c** = Comparison with group I. * *p*<0.05 (n=5).