ORIGINAL ARTICLE

D-Ribose-L-Cysteine Regimen Corrects Formaldehyde-Induced Testicular Perturbed Biochemical Redox, Hormonal Deficits and Histomorphological Alterations in Swiss Albino Adult Male Mice

RUNNING TITLE: Riboceine attenuate testicular toxicity

Abstract

Reports of declining sperm counts over the past 50 years and other disturbing trends alerted scientists to the possibility that exposure to chemicals in the environment may damage male reproductive health. The aim of this study was is to investigate the possible hazard effects of formaldehyde (FA) and the possible therapeutic potential of D-Ribose-L-Cysteine on key reproductive indices and cytoarchitecture of male mice testes.

Twenty eight (28) adult male Swiss albino mice weighing between 70-80g were assigned into four groups; A, B, C and D with each group containing seven mice. Group A animals served as control, animals in group B were administered with 5 mg/kg body weight of formaldehyde for 14 days; animals in group C received 5 mg/kg of formaldehyde and 30 mg/kg body weight of riboceine (*Ribcys*) for 14 days; while group D animals were treated with riboceine (30mg/kg b.w) for 14.

This study showed that formaldehyde treatment altered key semen parameters (sperm count, motility, and morphology) and caused testicular damage mainly by increasing ROS generation and promoting oxidative stress in the testes. Results from this study demonstrate a significant depletion of the collagen content of the testes following formaldehyde treatment. *Ribcys* treatment maintained the levels of key antioxidant markers and semen quality. Also, serum testosterone level in mice treated with *Ribcys* increased significantly ($p<0.05$) in comparison to formaldehyde treated mice. In addition, the cytoarchitecture of the testes of *Ribcys* treated animals appeared better structured and similar to that of the control group with little or no degenerative changes when compared to the formaldehyde group.

D-Ribose-L-Cysteine has potent antioxidant properties and could provide therapeutic defense

Keywords: Formaldehyde, D-ribose-L-cysteine, testes, oxidative stress

Introduction

Infertility is one of the major health problems of reproductive age couples (Radford *et al*., 1999) with increasing incidence rates in males (Oyeyipo *et al.* 2014). It is reported that sperm quality has decreased during the 20th century (Jørgensen *et al.* 2012).The cause of this is unknown but is theorized to be due to the increase in concentrations of endocrine disruptors and number of chemical pollutants in the environment air (Dindyal, 2004). Several studies have reported the effects of exposure to occupational chemicals on semen quality (Vaziri *et al.* 2011).

Formaldehyde (FA, H_2CO), one of the simplest organic molecules, is an important chemical that has a wide range of use in science, households, and industry (Pala *et al.* 2008; Gules and Eren, 2010). The harmful effects of FA are well documented for the respiratory and hematological systems (Coins, 2004; Arican *et al.* 2009). Formaldehyde (FA) is a colorless and highly water soluble aldehyde. Its intake occurs through the topical, oral, injection and mostly via the respiratory system. It can be inhaled with smokes due to the combustion of burning fossil fuels and in the fumes of paints and in cigarette smoke (Nazaroff, 2004). It can be ingested in fresh water, food and drugs. In food, it can occur naturally or through contamination as it can be added as a preservative or disinfectant agent. It can also result from cooking or smoking of foods (Yang *et al.* 2007). Even in infancy, the babies might be exposed by injection to FA present in diphtheria, polio and tetanus vaccine preparations as a result of the manufacturing process (Thaysen-Andersen *et al.* 2007). Several malignancy treating drugs are formulated with FA which is required for drug activation (Evison *et al.* 2008). Some cosmetics especially hair smoothing products containing FA or methylene glycol which require the use of heat that leads to volatilization of both FA gas as well as methylene glycol vapors. This increases the potential for hair stylist and consumer exposure to FA from methylene glycol formulated in keratin products (Golden and Valentini, 2014). Formaldehyde (FA) is an organic carbon compound which receives an increasing attention as pollutants with potential adverse health effects (Reuss *et al.* 2002). It induces oxidative stress which has been reported to be the mechanism of FA toxicity in multiple tissues of the exposed animals, in liver, lymphocytes, heart, brain, lung and gonads (Lino-dos-Santos-Franco *et al.* 2011).

Nowadays, antioxidants are widely used to break the oxidative chain reaction (Chen *et al.* 2006; Bansal and Bilaspuri, 2008). The ability to utilize oxygen has provided humans as well as animals with the benefit of metabolizing fats, proteins, and carbohydrates for energy; however, it does not come without cost. Oxygen is a highly reactive atom that is capable of becoming part of potentially damaging molecules commonly called “free radicals.” Antioxidants are capable of stabilizing, or deactivating, free radicals before they attack cells. Antioxidants are absolutely critical for maintaining optimal cellular and systemic health and well-being.

D-Ribose-L-Cysteine is a cysteine analogue designed to increase the synthesis of glutathione (GSH) (Kader *et al.* 2014). D-ribose and L-cysteine contains anti-oxidant nutrients that are naturally occurring in human, to more effectively deliver cysteine directly to the body cells.

Therefore, this present study is aimed to investigate the possible FA-induced changes in some biochemical, androgenical and histomorphological parameters in the testis of adult male mice. Since there is always need for a successful therapeutic approach that might inhibit the initiation and progression of diseases, the present study also evaluates the potential haematological effect of D-Ribose-L-Cysteine in ameliorating these possible alterations.

Materials and Methods

Experimental Animals and Care

All protocols and treatment procedures were done according to the Institutional Animal Care and Use Committee (IACUC) guidelines and as approved by the Faculty of Basic Medical Sciences Ethics Review Committee, Osun State University, Osogbo, Nigeria.

Twenty eight (28) male Swiss albino mice with an average weight of 80g±10g were purchased from a private animal holding in Osogbo and used for this research. These mice were kept in the animal house of the Faculty of Basic Medical Sciences, Osun State University. The mice were on a daily basis fed with mice feed and had access to tap water *ad libitum*.

Chemicals and Supplement

Riboceine was purchased as supplement from Max International LLC Salt Lake City Utah, USA. While formaldehyde was obtained from Anatomy Department in the College of Health Sciences, Osun State University.

Experimental Design and Procedure

3.3.1 Grouping

Animals were grouped into four as evidenced in the table below:

Table 1: Showing the grouping of the animals with their corresponding treatments, dosages, duration of administration and number of animals in each set.

Group A	Distilled water	mg/kg body weight	14 days	7
Group B	Formaldehyde	5 mg/kg body weight	14 days	7
Group C	Riboceine + Formaldehyde	30 mg/kg body weight/ 5 mg/kg body weight	14 days	7
Group D	Riboceine	30 mg/kg body weight	14 days	7

Formaldehyde was administered to the experimental animals via intraperitoneal route while *Ribcy* was dispensed to the experimental animals with a calibrated syringe fitted with a beaded oral cannula.

Sacrifice

Twenty-four hours after 14th day of treatment, mice for histological analysis were euthanized using 20 mg/kg of ketamine (intraperitoneal). Blood samples were collected via cardiac puncture into lithium-heparin bottles for serum levels of Testosterone, Follicle stimulating hormone (FSH) and Luteinizing hormone (LH) analysis while Caudal epididymis were excised from the testes for sperm count, motility and morphology analysis. The animals were later fixed by transcardial perfusion method using 4% paraformaldehyde as fixative agent and the testes were fixed in Bouin's fluid. Mice for enzymatic assays were sacrificed by separating the head from the trunk to avoid the interference of ketamine with biochemical redox; testes were then excised, rinsed in 0.25 M sucrose 3 times for 5 minutes each and placed in 30 % sucrose in which they were stored at 4°C.

Sperm count assay

The spermatozoa were counted by hemocytometer using the improved Neubauer (Deep 1/10 mm, LABART, Germany) chamber as described by Rouge and Bowen (Rouge and Bowen, 2002).

Sperm morphology and motility assay

Sperm live/dead ratio and motility were determined using 1% Eosin and 5% Nigrosin in 3% sodium-citrate dehydrate solution according to the method described by Rouge and Bowen (Rouge and Bowen, 2002).

Hormone Measuring Assay

Blood were collected by cardiac puncture into a heparinized bottle and serum were immediately collected by centrifugation (4000 rpm at 4°C) and stored at -20°C for further analysis. Serum levels of testosterone were determined using ELISA kits according to the manufacturer's instructions and all samples were tested in triplicate. ELISA Kit was obtained from Monobind Inc. Lake forest, CA, USA (Testosterone Cat #: 3725-300).

Histological Examination

Histological processing using Hematoxylin and Eosin and Masson Trichrome staining methods were carried out. The testes were fixed in Bouin's fluid, dehydrated in ascending grades of alcohol, cleared in xylene, and infiltrated in molten paraffin wax before finally embedded in molten paraffin wax to form block. The paraffin block containing the tissue was then sectioned by the rotary microtome at 4 μm thickness. The sections were then floated in water bath at 40°C and transferred to a glass slide and stained with the appropriate stains. The slides were then viewed under light microscope and photomicrographs were taken in at x100 and x400 magnifications.

Determination of Antioxidant Parameters

Determination of Catalase (CAT), Glutathione peroxidase (GPx), and Malondialdehyde (MDA) activities were carried out on the testes of treated mice using spectrophotometric technique. Each of the assay kits were procured from Bio Legend Inc., San Diego, CA, USA. Testes (in sucrose at 4° C) from mice across groups were weighed and pulverized in 0.25 M sucrose (Sigma) with the aid of an automated homogenizer at 4°C. Lysates from the testes were centrifuged for 10 minutes in a microfuge at 12,000 rpm to obtain the supernatant containing organelle fragments and synaptosomes. The supernatants were aspirated into plain labelled glass cuvette placed in ice. CAT, GPx and MDA activities were assayed according to the manufacturer's instruction in the assay kit pack.

Statistical Analysis

GraphPad Prism version 7.0 was used for all statistical analyses. All data were expressed as Mean±SEM and differences among the groups were analyzed by one-way ANOVA. Turkey's correction was used to adjust for multiple comparisons while *p* value at <0.05 was considered to be statistically significant.

Results

Semen Quality

The sperm count, sperm motility, and sperm morphology was analyzed after the caudal epididymis of the rats were excised. The data obtained was subjected to statistical analysis and the data obtained is presented in tables and graphs below.

Result from table 2 show that animals administered with FA had a significant reduction in the total sperm count when compared with the control group and the FA+Ribcys group ($p<0.05$). Sperm count values in animals treated with riboceine only (group D) were at par with the control group.

Sperm motility analysis mean value (table 3) revealed a significant decreased in motile sperm and significant increase in non-motile sperm in animals exposed to FA (group B) when compared with the control group. Riboceine treated mice showed high level of motile sperm which were at par with the control group.

In addition, sperm morphology mean values as shown in table 4 revealed significant increase ($p<0.05$) in spermatozoa abnormalities ranging from defects in head, tail, acrosomal percentile, in the FA only (group B) in comparison with the control group. No significant difference was seen in sperm morphology between the control and riboceine treated animals.

Table 2: Showing total sperm count across the experimental groups. Data were presented as mean and standard error of mean (Mean ± SEM) *($P<0.05$) – statistical significant difference when compared across groups; $^{+}$($P<0.05$) – when compared with group B (Formaldehyde only).

TOTAL SPERM COUNT ($x10^6$/ml)	
GROUPS	**MEAN±SEM**
Control (group A)	103.0 ± 2.950

Formaldehyde (group B)	77.60 ± 1.806*
Formaldehyde+Riboceine (group C)	97.00 ± 2.608^{+}
Riboceine (group D)	102.6 ± 3.415

Table 3: Showing mean values of sperm motility across the experimental groups. Data were presented as mean and standard error of mean (Mean ± SEM) *($P<0.05$) – statistical significant difference when compared across groups; $^{+}$($P<0.05$) – when compared with group B (Formaldehyde only).

SPERM MOTILITY (%)

GROUPS	MOTILE	NON-MOTILE
Control (group A)	90.00 ± 2.03	10.00± 2.21
Formaldehyde (group B)	62.50 ± 0.53*	37.50 ± 0.62*
Formaldehyde+Riboceine (group C)	87.45 ± 1.74^{+}	13.55 ± 1.49^{+}
Riboceine (group D)	91.91 ± 2.07^{+}	9.09± 2.01^{+}

Table 4: Showing sperm morphology across the experimental groups. Data were presented as mean and standard error of mean (Mean ± SEM) *($P<0.05$) – statistical significant difference when compared across groups; $^{+}$($P<0.05$) – when compared with group B (Formaldehyde only).

SPERM MORPHOLOGY (%)

GROUPS	NORMAL	ABNORMAL
Control (group A)	94.50 ± 2.03	5.50± 2.21

Formaldehyde (group B)	66.70 ± 0.53*	33.30 ± 0.62*
Formaldehyde+riboceine (group C)	93.00 ± 3.74^{+}	7.00 ± 4.49^{+}
Riboceine (group D)	95.00 ± 1.17^{+}	5.00± 1.01^{+}

Hormonal Analysis

Serum Testosterone: The serum testosterone levels were estimated following biochemical assay. Result revealed a decrease in serum testosterone level of mice treated with FA (group B) and this decrease was significant in comparison with control mice (group A) and riboceine treated mice (groups C and D).

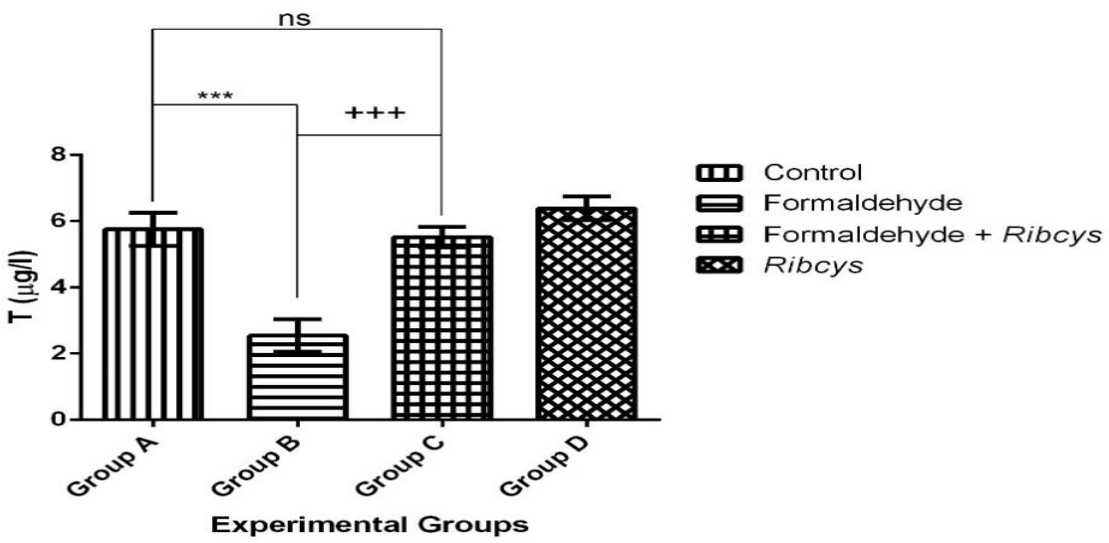

Figure 1: Chart showing serum testosterone levels across the different experimental groups. Data were presented as mean and standard error of mean (Mean ± SEM) *(p<0.05) – statistical significant difference when compared with the control; $^{+}$(p<0.05) – when compared with group B (Formaldehyde only). **ns** means not significant.

Oxidative Stress Markers

GPx Profiles Decreases Following FA Administration

Results from spectrophotometric assay (figure 2) showed significant reduction (p<0.05) in glutathione peroxidase activity in formaldehyde treated mice when compared to the control animals (group A). There was a slight significant decrease in the GPx profile of animals in the FA+*Ribcys* group. However, there was no significant difference in the GPx levels of mice treated with *Ribcys* only when compared with the control group.

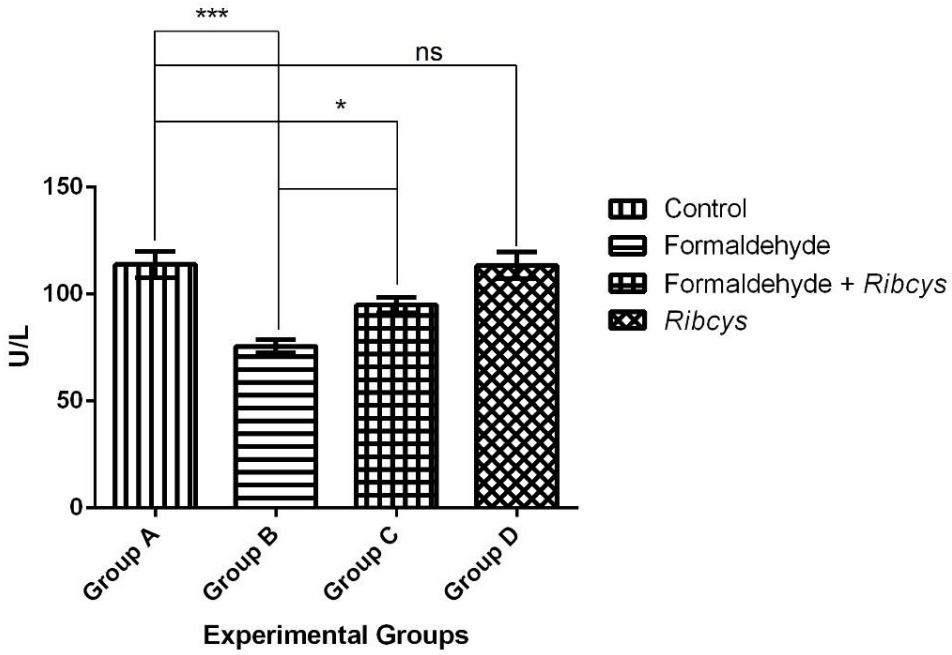

Figure 2: Chart showing levels GPx activity across the different experimental groups. Data were presented as mean and standard error of mean (Mean ± SEM) *(p<0.05) – statistical significant difference when compared with the control; $^{+}$(p<0.05) – when compared with group B (Formaldehyde only).**ns** means not significant.

Ribcys prevented Testicular Lipid Peroxidation

Malondialdehyde (MDA) level significantly increased in the testes of mice treated with formaldehyde when compared with the control group. Rats treated with *Ribcys* had significantly lower MDA level when compared with formaldehyde group. Ribcys offered protection against formaldehyde-induced increased MDA levels in the testes of treated mice.

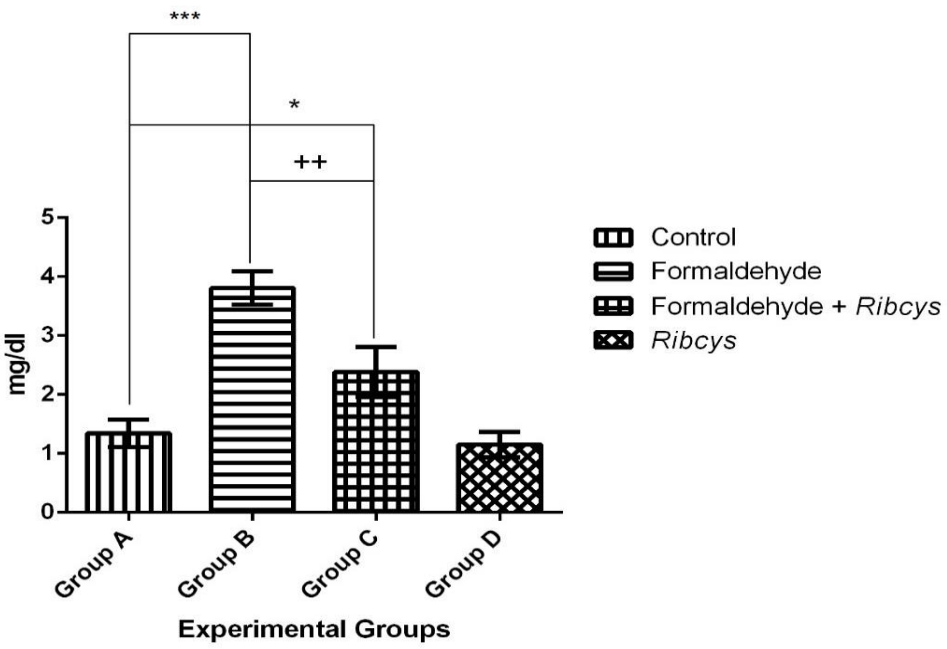

Figure 3: Chart showing levels of lipid peroxidation across the different experimental groups. Data were presented as mean and standard error of mean (Mean ± SEM) *(p<0.05) – statistical significant difference when compared with the control; $^{+}$(p<0.05) – when compared with group B (Formaldehyde only). **ns** means not significant.

Ribcys maintained the integrity of Cellular Catalase (CAT) Activity

Results from spectrophotometric assay showed that the level of Catalase activity (CAT) in the testes of formaldehyde treated mice were significantly decreased in comparison to the control group and *Ribcys* treated groups. CAT level in the formaldehyde+*Ribcys* treated mice increased significantly when compared with the formaldehyde group.

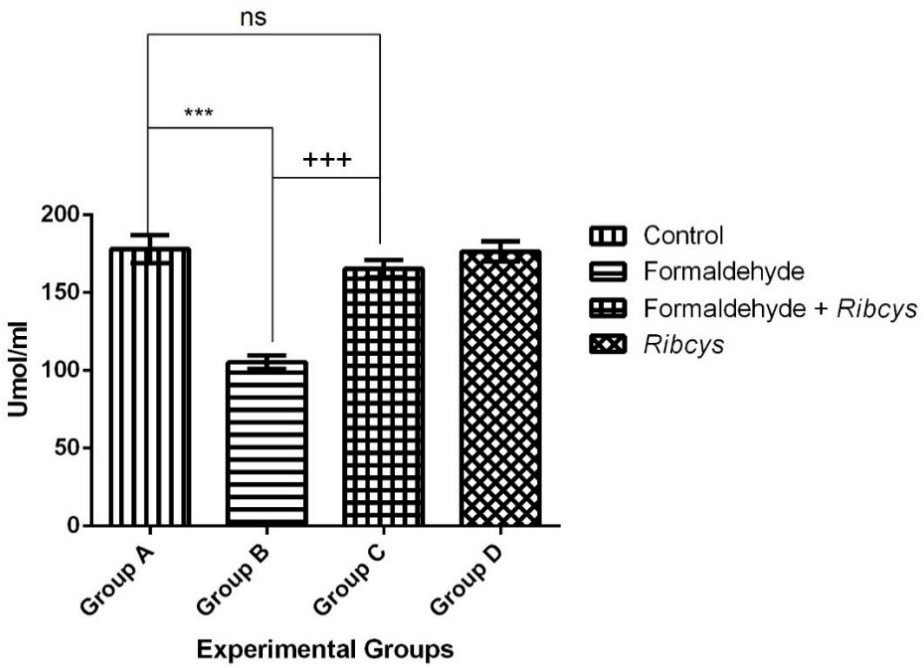

Figure 4: Chart showing levels of CAT activity across the different experimental groups.Data were presented as mean and standard error of mean (Mean ± SEM) *(p<0.05) – statistical significant difference when compared with the control; $^{+}$(p<0.05) – when compared with group B (Formaldehyde only). **n**s means not significant

Histological/Histochemical Investigation

Hematoxylin and Eosin Stain

Histo-architecture of the groups A, B, C, and D of mice testes stained with Hematoxylin and Eosin as shown in figure 4 and 6 show a normal presentation in the control and *Ribcys* treatments characterized with concentric seminiferous tubules (ST) with intact basal membrane and lumen condensed with mature sperm cells. Also, the interstitial spaces (IS) are well delineated with no signs of testicular degeneration. There are however mild distortions with a relatively few seminiferous tubules in the formaldehyde treatment with little to no mature spermatozoa in the lumen. Formaldehyde treatment with *Ribcys* show a mild presentation in the general cytoarchitecture similar to the control and *Ribcys* treated groups.

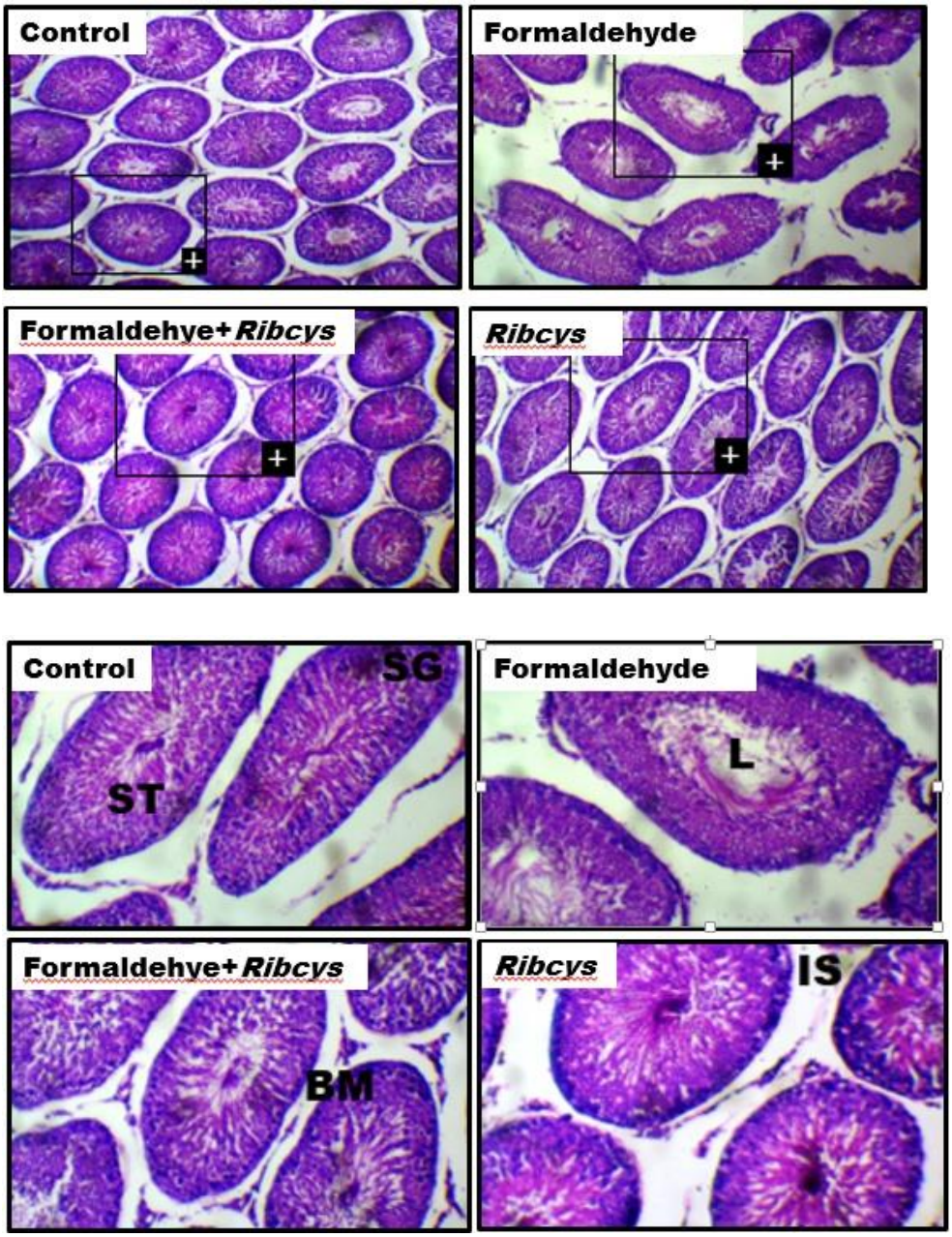

Fig. 5 & 6: Representative photomicrograph showing the testicular histoarchitecture of mice in experimental groups. H&E stain at x100 and x400 magnification.

Masson Trichrome Stain

The connective tissue components of the control and *Ribcys* treated groups appeared intact and were characterized by organized connective tissue septa, robust interstitial tissue with well distributed blood vessels and lydig cells.The formaldehyde treated group show sparse interstitial tissue with poorly stained CT septae. The lydig cells appear chromatolysed. The testicular

cytoarchitecture of formaldehyde+*Ribcys* treated mice appear normal and is characterized by well-stained connective tissue stroma intersperse with blood vessels and lydig cells.

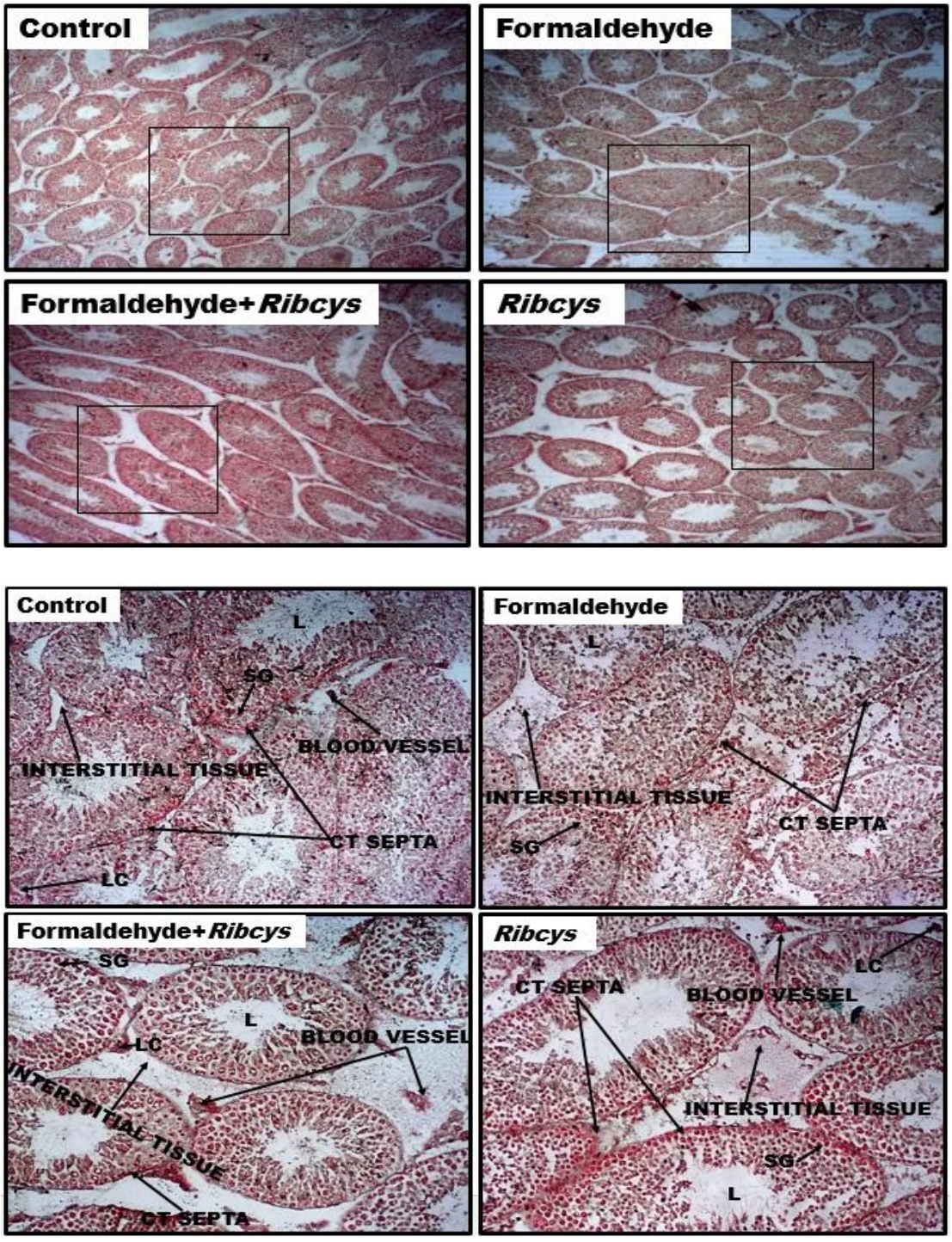

Figure: 7 & 8: Photomicrographs cytoarchitectural presentations of testicular general morphology Male albino Mice. Masson Trichrome stain, x100 and x400.

Discussion

In many studies investigating the toxicity of formaldehyde (FA), tissue malondialdehyde (MDA) levels were significantly higher in the FA group compared with the control group (Gurel *et al*. 2005). In this study, the levels of tissue MDA were also higher, and this increase was statistically significant (p>0.05). This situation was evaluated to be a result of the acute effect of FA. The increase in tissue MDA level in the FA group shows that FA has an effect on lipid peroxidation. In the literature there is no study investigating the effect of Riboceine (*Ribcys*) against FA toxicity in testicular tissue. In this study, it was observed that tissue MDA levels were lower in the FA + *Ribcys* group compared to the FA group. This result seems to be important in terms of disclosing the antioxidant property of *Ribcys* against FA toxicity in testicular tissue. In studies with different chemical substances and experimental models, it has been demonstrated that *Ribcys* had positive effects on the level of tissue MDA.

Glutathione peroxidase (GPx) is the general name of an enzyme family with peroxidase activity whose main biological role is to protect the organism from oxidative damage. The biochemical function of glutathione peroxidase is to reduce lipid hydroperoxide to their corresponding alcohols and to reduce free hydrogen peroxide to water. Catalase (CAT) is a major antioxidant enzyme having heme as the prosthetic group (Xia *et al.* 2010) and converts hydrogen peroxide into water (Uzun *et al.* 2010). Evaluation of the activities of these antioxidant systems in this present study show a significant decrease in animals exposed to FA. Mice treated with *Ribcys* had increased levels of GPx and CAT.

Oxidative stress and a failure of antioxidant defense system cause several sperm abnormalities and result in infertility. Thus, an imbalance between the ROS generation and scavenging system might be one of the reasons for FA-induced male reproductive toxicity where decreased antioxidant activity due to FA exposure could result in rapid generation of ROS far more than the mechanism which produces endogenous antioxidants that should eliminate FA (Zhang *et al.* 2010). As well as functioning as a prodrug for cysteine, administration of effective amounts of *Ribcys* can deliver good amounts of ribose to ATP-depleted tissues that stimulate the in vivo synthesis of ATP and that also can stimulate the synthesis of NADPH (nicotinamide adenine dinucleotide phosphate, reduced). This coenzyme supplies the electrons to glutathione reductase, which in turn recycles oxidized GSH via GSSG, to free GSH, which resumes its protective role as a cofactor for antioxidant enzymes in the cell (Roberts and Francetic, 1991).

In this current study, serum testosterone levels were significantly reduced in FA-treated mice compared to normal controls. Cao *et al.* (2004) indicated that excessive oxidative stress reduced levels of key enzymatic and non-enzymatic antioxidants in Leydig cells, and resulted in decline in testosterone secretion (Cao *et al.* 2004). Accordingly, the reduced serum testosterone level in FA-treated mice in this study could be attributed to the impairment of Leydig cells.

The administration of riboceine (30 mg/kg) caused a significant increase in serum testosterone level of FA treated mice. These results are in agreement with our earlier findings (Falana *et al.* 2017) indicating that riboceine increases the plasma levels of testosterone. The results of the present study indicated that sperm count, progressive motility, and normal morphology reduced significantly in the formaldehyde treated group. The reduction in epididymal sperm motility and count in FA treated mice could be connected to the reduction in the serum testosterone level. Gong and Han in their report explained that lowering of epididymal sperm motility and count suggested an undersupply of testosterone to the epididymis (Gong and Han, 2006). Besides hormonal alteration, the spermatogenic inhibition may also be due to the generation of ROS by FA in the testicular tissue and the consequential elimination of sperm cells at different stages of development. Results showed that all the sperm parameters were maintained with treatment with riboceine (30mg/kg).

The improvement that is observed in spermatogenesis among riboceine treated mice may be associated with the antioxidant properties of riboceine. This result may suggest that riboceine could have an effect on spermiogenesis process and it could thus favor normal sperm production. Additionally, testosterone deficiency (like that observed in the FA-treated mice) produces more immature sperm by early sloughing of spermatids from the Sertoli cells (O'Donnell *et al.* 1999).

With respect to the findings of this study, exposure to formaldehyde can lead to histological changes in the seminiferous tubules and Leydig cells. Due to FA toxicity, an increase in gaps between the germ cells in testicular tissue, a decrease in the number of germinal cells, basal membrane damage, and vacuolization occurring in the interstitial area have been reported (Golalipour *et al.* 2007; Han *et al.* 2015). These findings put forward by researchers are compatible with results from this study. In this study, intracellular vacuolization, basement membrane damage, impaired germinal epithelium cell layout, an increase in the volume of germinal epithelium cells, and epithelium cells being thrown together with spermatids into the lumen, were the degenerative changes observed. In this study, in which the therapeutic effects of

Ribcys with known antioxidant activity in different body tissue is being put forward, degenerative changes caused by FA were repaired by Ribcys administration as seen in the FA + *Ribcys* group. The structure and numbers of Leydig cells, whose basic functions are to produce testosterone, are very important in the development of germinal epithelium cells.

The structure of the extracellular matrix affects the proliferation and testosterone synthesis of Leydig cells (Diaz *et al.* 2002). In terms of providing the adhesions between Sertoli cells, the germinal epithelium cells, and the basement membrane, the components of the extracellular matrix are very important (Siu and Cheng, 2004). The basement membrane has a very important role in the integrity of the germinal epithelium and the development of cells. It is known that an abnormal basement membrane structure is observed in infertile men. Basement membrane damage observed in the seminiferous tubules and Leydig cells is important first for the cells and then for the integrity of the tissue. In this study, testicular tissue damage in the basement membrane emerged depending on formaldehyde toxicity. The present study evaluated the framework of these findings; the toxic effect of formaldehyde was set out clearly by Masson Trichrome histological stain. With reference to the connective tissue content in the current study, mason trichrome examination revealed thickening and irregularity. *Ribcys* treatment may have stimulated myoid cells to produce more collagen and extracellular matrix (Shokri *et al.* 2012). Increased production of glycosaminoglycans and proteoglycans has been considered as a defense reaction against the damaging effect of free radicals (Jedlinska *et al.* 2005).

Conclusion

In conclusion, this study shows the effect of riboceine against formaldehyde toxicity in mice testes at the biochemical and light microscopic levels. The present study observed that D-ribose L-Cystein has protective effects against FA-induced swiss albino mice testis toxicity. Thus, the use of riboceine is suggested as a pretreatment agent in FA toxicity. This study will shed light on further studies that could be conducted on both antioxidant capacity and the immunohistochemical determination of adhesion molecules.

Conflict of Interest

The authors declare no actual or potential conflict of interests including any financial or personal relationships with other people or organizations from the onset of this work that could inappropriately influence, or be perceived to influence the study.

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