ORIGINAL ARTICLE

STUDY EFFECTS OF SILYMARIN ON REPRODUCTIVE VARIABLES IN MALE WISTAR RATS WITH CARBON TETRACHLORIDE (CCl_4) - INDUCED LIVER FIBROSIS

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ABSTRACT

Background: Hepatic fibrosis is an effusive wound healing process in which excessive connective tissue builds up in the liver. Carbon tetrachloride (CCl_4)-induced liver damage is a well characterized experimental model for studying liver fibrosis, and silymarin is a drug made up of mixture of flavonoids extracted from seeds of milk thistle plant (*Silybum marianum*), which is well known for its hepato-protective effect. **Objectives:** To study the effect of silymarin on reproductive variables in experimental liver fibrosis induced by CCl_4 in male Wistar rats. **Method:** Twenty Wistar were randomized into four groups (Group A, B, C and D) of 5 rats each group, receiving 0.25ml/100g body weight of normal saline, 0.25ml/100g body weight of olive oil, 0.25ml/100g body weight of 40% CCl_4 and 0.25ml/100g body weight of 40% CCl_4 with 6mg/kg silymarin treatment respectively. CCl_4 and Olive oil were given by subcutaneous injection three time a week, while normal saline and silymarin were given orally daily. At end of the seventh week, the animals were sacrificed; Liver enzymes and reproductive variables were quantified, testicular, liver and general body weights were measured, and histological studies of the testes and liver were also assessed. **Results:** The plasma levels of ALT, AST and ALP were significantly ($P<0.05$) increased, while the sperm count, motility, viability, morphology and change body weight were significantly ($P<0.05$) decreased in the group receiving CCl_4 alone. There was increase in liver weight and decrease in testicular weight which were not significant. Microscopic examination of liver and testes sections from rats treated with CCl_4 showed an abnormal cytoarchitecture. Some of the abnormalities of liver fibrosis were reversible with administration of silymarin, except for some of the reproductive variables, where by only sperm count and motility were significantly ($P<0.05$) increased. The results also showed decrease in plasma levels of FSH, LH, testosterone, the decrease were not significant. Also there was no significant difference in morphology and viability in the rat receiving CCl_4 with silymarin treatment when compared with group D (CCl_4 alone). **Conclusions:** There was improvements found in reproductive variables in silymarin treated groups, though, were not enough to reach the values of the control group receiving normal saline. The results also suggest that not only the fibrosis alone, but also the CCl_4 on its own affect the reproductive function of the testis.

***Keywords**: liver fibrosis, Liver enzymes, Silymarin, Histology, Testes.*

1. INTRODUCTION

Hepatic fibrosis is an effusive wound healing process in which excessive connective tissue builds up in the liver. Carbon tetrachloride (CCl_4)-induced liver damage is a well characterized experimental model for studying liver fibrosis. Without effective treatments, reversible liver fibrosis at an early stage leads to irreversible cirrhosis. Chronic liver injury leads to a progressive wound healing response that eventually results in liver fibrosis characterized by both quantity and quality alteration of hepatic extracellular matrix, ECM [1]. Moreover, liver fibrosis represents the response of the liver to diverse chronic insults such as parasitic disease, chronic viral infection (hepatitis B and C), immunologic attack (autoimmune hepatitis), hereditary metal overload, and toxic damage e.t.c. Because of the worldwide prevalence of these insults, liver fibrosis is common and is associated with significant morbidity and mortality [2]. Hypogonadism is characterized by low testosterone levels and relative hyperestrogenism, loss of libido, sexual impotence and feminine body features in men. This is a common complication of advanced liver cirrhosis [3]. There are reports of a prevalence of hypogonadism in 70 to 80% of cirrhotic patients [4] even 89% in individuals with liver cirrhosis of different etiologies [5]. The production of testosterone by cirrhotic individuals is, on average, 25% of that found in normal individuals [6]. Rats with advanced cirrhosis caused by CCl_4 showed reduced testicular size and severe histopathological testicular abnormalities, loss of the germinal line [7] and spermatogenesis [8].

Silymarin (SM) is a drug made up of mixture of flavonoids extracted from seeds of milk thistle plant (*Silybum marianum*), which is well known for its hepato-protective effect. The action of silymarin resulted from the strong antioxidant activity by scavenging and inhibition of free radicals generation, inhibition of lipid peroxidation in cell membranes, stimulation of RNA

polymerase and biosynthesis of cell proteins, and in a strong inhibition of enzymes catalyzing the production of leukotrienes and prostaglandins such as 5-lipoxygenase and cyclooxygenase [9,10].

Most literature have repute that consequence of liver cirrhosis affect male fertility, but there is no substantial evidences to clearly differentiate as to whether it is the factor causing these liver diseases or the liver diseases themselves or consequence of liver diseases that cause the reproductive problem in males. Therefore, the objectives of current study are to determine whether the consequence of liver fibrosis or the fibrosis themselves can cause reproductive problems in rats.

2. MATERIALS AND METHODS

2.1 Animals

Male Wistar rats (140–200g) were procured from University college hospital (UCH), Ibadan, Nigeria, and used throughout the study. They were housed in plastic boxes and acclimatized for two weeks in a controlled environment (temperature 25±2 °C and 12 h dark/light cycle) with standard laboratory diet and water *ad libitum*.

2.2 Experimental protocol

Twenty male Wistar rats weighing 140-200g each were randomly divided into four groups of 5 animals in each group in this study. The first group (A) which is the control received nothing but 0.25ml/100g body weight of normal saline, Group (B) received 0.25ml/100g body weight of olive oil alone, Group (C) received 0.25ml/100g body weight of 40% of CCl_4 solution (mixture of CCl_4 and Olive at ratio 2:3) as described by LI et al. (2011), and the fourth group (D) received 0.25ml/100g of 40% CCl_4 and 6mg/kg body weight of silymarin [11]. Administration of the olive oil in (B) and CCl_4 in (C) and (D) were by subcutaneous injection three times in a week, while normal saline in group (A) and silymarin in group (D) were given orally daily for seven weeks using a modified method used by Hyeonjin et al. (2008) and by Atef (2012) [12,13]. At end of the seventh week, the animals were sacrificed by cervical dislocation for investigation of liver fibrosis and some reproduction variables.

2.3 Semen Analysis

Orchidectomy was performed by open castration method. The testicle was exposed by incising the *tunica vaginalis* and the *cauda epididymis* were harvested. The *cauda epididymis* of rats in each of the experimental group was removed and minced thoroughly in a specimen bottle containing normal saline for few minutes to allow the sperms to become motile and swim out from the *cauda epididymis* [14,15].

2.4.1 Motility

The semen was then taken with 1ml pipette and dropped on a clean slide, and covered with cover slips. The slides were examined under light microscope for sperm motility according to [14,15].

2.4.2 Livability

Smear was prepared from the collected epididymal samples and stained with Eosin and Nigrosin stain. This was followed immediately by examination under the microscope as previously described by Zemjanis (1977) [16].

2.4.3 Sperm count

The spermatozoa were counted under the light microscope, with the aid of the improved Neubauer hemocytometer (Deep1/10mm LABART, Germany) counting chamber as described by Pant and Srivastava [17]. Counting was done in five thoma chambers.

2.4.5 Morphological characteristics

Smear was prepared from the collected sperm cells and stained with Wells and Awa stain. Evaluation under the microscope was done as described by Zemjanis (1977) [16].

2.5 Liver Biochemical Analyses

To establish the case of liver fibrosis in all the groups, blood samples of the animals in all of the groups were collected through orbital venous plexus [18] using the method approved by Institutional Animal Care & Use Committee in year 2011 [19], placed in Lithium heparinized sample tubes and centrifuged at 3000 revolution per minute. Serum analysis for the presence of liver cell enzymes i.e., aspartate amino transferase (AST) alanine amino transferase (ALT), and alkaline phosphatase (ALP) were measured according to the reported methods by Atef (2012) [13].

2.6 Hormonal analysis

Blood samples of the animals in all of the groups were also collected into non heparinised test tubes, through orbital venous plexus [18] using the method approved by Institutional Animal Care & Use Committee in year 2011 [19]. Centrifugation was done at 3000 revolution per minute to get the serum for analysis. The concentration of Follicle stimulating hormone (FSH), luteinizing hormone (LH) and testosterone were measured by enzyme–linked immunosorbent assays (ELISA) method. The hormonal kits used for the assay was a product of Dialab, USA.

2.7 Histopathological studies

The livers and testes of all the rats were fixed in 10% formalin and processed by the usual method for paraffin embedding at University College Hospital (UCH), Ibadan, Nigeria. Section of 4-5 µm thickness by microtome was taken, stained with hematoxylin and eosin stain for histopathological examination through light microscope [20].

2.8 Statistical analysis

Student t-test and one way analysis of variance (ANOVA) were used to analyze the data. The results were expressed as mean ± standard error of the mean (SEM). The difference of the means was considered significant at $p < 0.05$.

3. RESULTS

Liver enzymes

The plasma levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST) and alkaline phosphatase (ALP) were significantly increased in the group receiving CCl_4 alone when compared with the control receiving normal saline, while in the group receiving CCl_4 with silymarin (SM) treatment, there was no significant difference in plasma levels of ALT, AST and ALP when compared with the control.

Semen Analysis

Sperm count, motility, viability and morphology were significantly decreased in the group receiving CCl_4 alone. In the group receiving CCl_4 with silymarin treatment, there was significant increase in sperm count and motility and morphology, but only increase in sperm count and motility were significant and viability remain the same when compared with the group receiving CCl_4 alone, those increase observed where not up to the values of the control receiving normal saline except for the sperm count.

Change in body and organ weight

In the group receiving CCl_4 alone, there was increase in liver weight and decrease in testicular weight which were not significant. While in the group receiving CCl_4 with silymarin treatment, there was further decrease in the weight of the testes which was still not significant.

Hormonal analysis

The results also showed decrease in plasma levels of FSH, LH, testosterone, the decrease were not significant in both groups receiving CCl_4 alone and CCl_4 with silymarin treatment, except for testosterone in the group receiving CCl_4 with silymarin treatment that shows significant decrease.

Table 1: Showing testosterone, follicle stimulating hormone (FSH) and Leutinizing Hormone (LH) level.

Groups	*Testosterone Level(nmol/l)*	*FSH level (mIU/ml)*	*LH level (mIU/ml)*
A(N/saline)	12.14±2.30	1.18±0.72	0.92±0.92
B(Olive oil)	23.62±5.59	0.84±0.74	1.5±1.09
C(CCl_4	9.90 ± 2.49	0.78±0.68	5.08±1.65
D(CCl_4+SM	5.68±0.96*	0.70±0.46	3.36±1.05

*P Value<0.05 significant

Table 2: Showing Change in body and organ weight.

Groups	*Change in weight(g)*	*Liver weight (g)*	*Testes weight (g)*
A(N/saline)	79.6 ± 6.2	9.88±0.95	1.22± 0.08
B(Olive oil)	83.4 ± 9.1	8.14±0.24	1.22± 0.03
C(CCl_4)	4.4 ± 6.5*	11.32±1.01	1.03± 0.11
D(CCl_4+SM	5 .0± 3.3*	12.30±0.47	1.00± 0.12

*P Value<0.05 significant

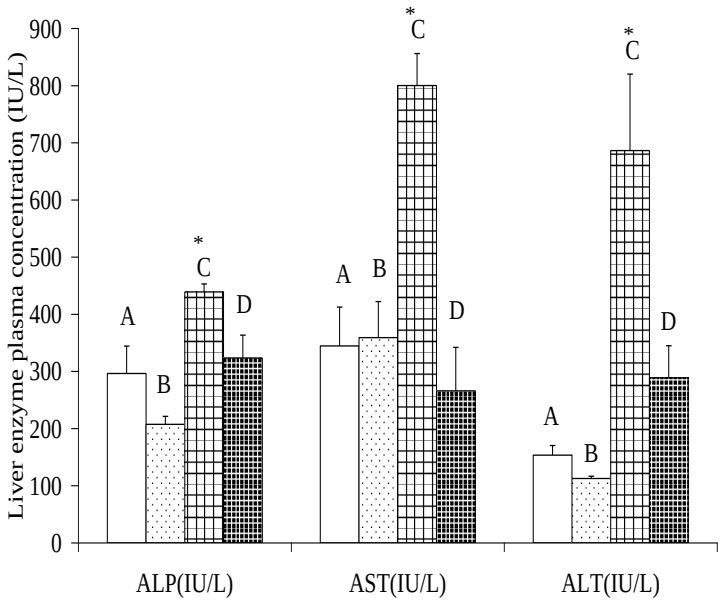


Figure 1: plasma levels of ALT, AST and ALP.

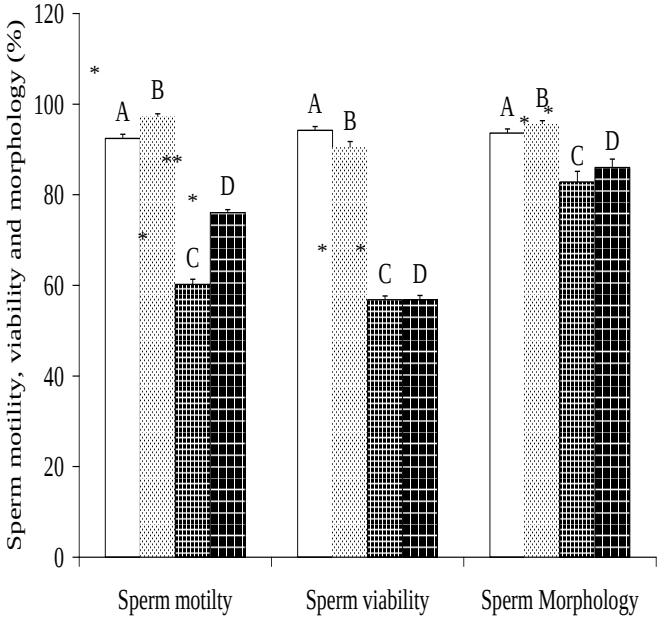
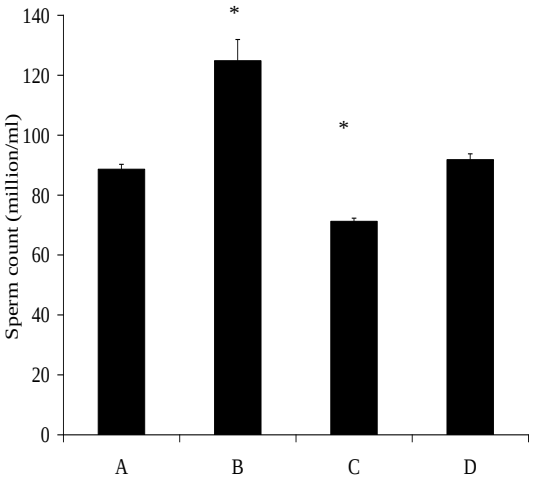
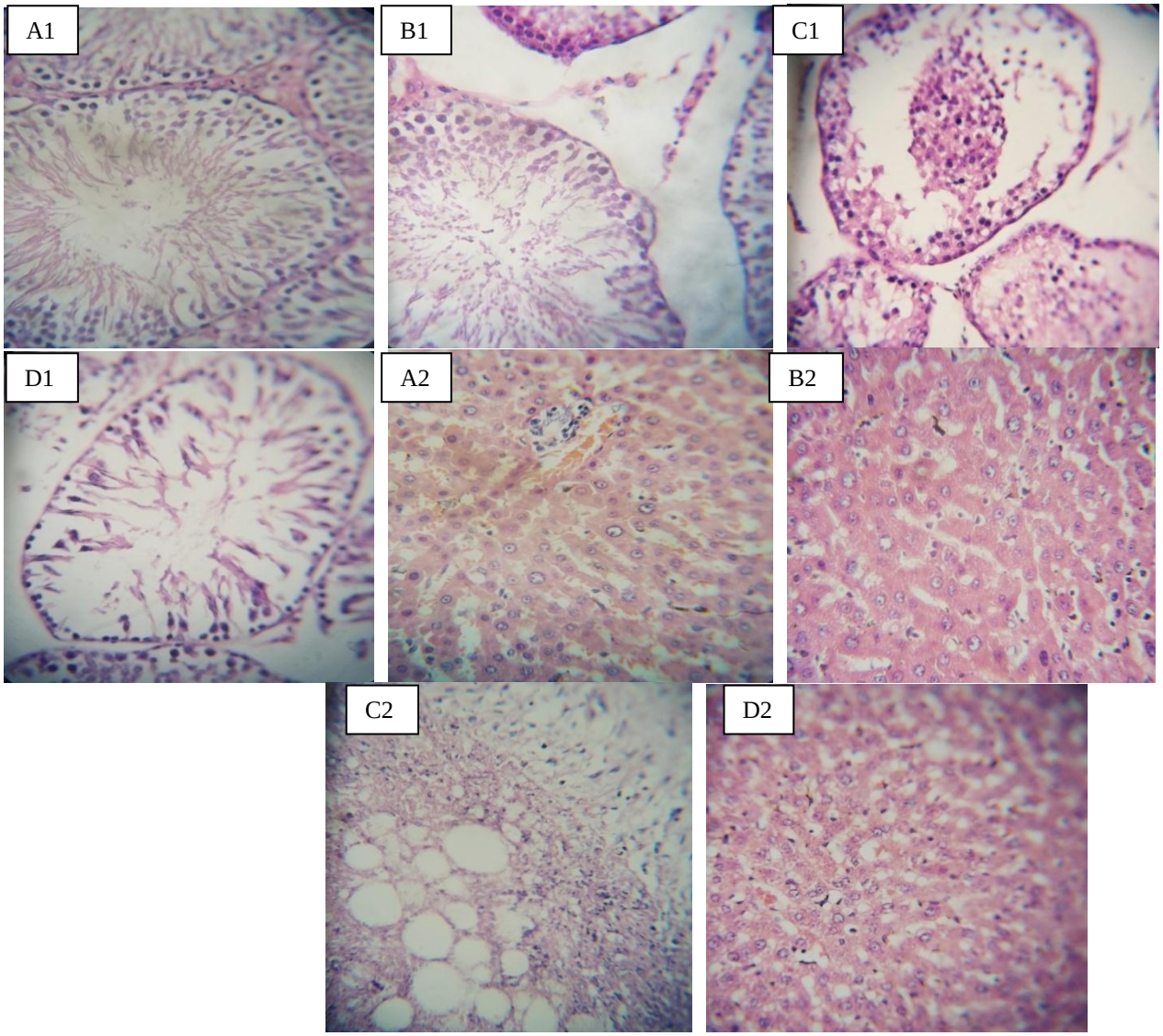


Figure 2: Sperm motility, viability and morphology.



In figure 1, 2 and 3, *P Value<0.05 significant when comparing with control and **P Value<0.05 significant when comparing with CCl_4 group
A – Control group receiving only normal saline
B –Group receiving only olive oil
C –Group receiving carbon tetrachloride (CCl_4)
D –Group receiving carbon tetrachloride (CCl_4) with silymarin treatment
A1, B1, C1 and D1 are testes histology, while A1, B1, C1 and D1 are the liver histology

Figure 3: Sperm count.



The histology of the testes in the group receiving normal saline (fig A1) and olive oil alone (fig. B1) appears normal; those in olive oil group alone only show highest level of increase in the spermatogenic activities. In the group receiving carbon tetrachloride alone (CCl_4)(fig. C1), the seminiferous tubes are seen with loss of some germ cell, including spermatogonial cells, there is maturation arrest, with germ cells sloughed into the lumen these leads to decrease in the spermatogenic activity. However, in group reciving CCl_4 with silymarin treatment (fig. D1), the interstitial cells and the lumen appear

normal with contents of spermatozoa, with loss of some germ cell but the loss is as much as that seen in the CCl_4 - induced fibrosis group. The histology of the liver in the group receiving normal saline (fig A2) and olive oil (fig. B2) appears normal, only in olive oil group alone, shows mild infiltration of inflammatory cells with few hepatocytes seen with mild deposits of fat in their cytoplasm. While in the group receiving carbon tetra chloride alone (CCl_4) (fig. C2), the liver section showing macro and microvesicular steatosis with infiltration of inflammatory cells. The hepatocytes are seen with deposits of fat in their cytoplasm. There are focal area of necrosis and fat lobules (short arrow). However, liver section in group reciving CCl_4 with silymarin treatment (fig. D2), shows presence of moderate microvesicular steatosis with mild infiltration of inflammatory cells.

4. DISCUSSION

The results of this study show that, in the groups receiving CCl_4 alone, there is appearance of liver fibrosis, evidenced by the increasing activities of liver enzymes, ALT, AST and ALP. This finding is in agreement with the work of Mir Asif et al. (2010), where they study curative role of *Solanum nigrum* in carbon tetrachloride (CCl_4) - induced hepatotoxicity in rats [21]. Animals in this group appeared ill looking with decrease appetite for feeding. This observations support the report of van Thiel et al. (1981) on the common complication of advanced liver disease [3]. There is increased liver weight due fat accumulation and extracellular fibrotic tissue which in agreement with [22]. Histopathological studies show the presence of vesicular steatosis, and infiltration of inflammatory cells. The hepatocytes are seen with heavy deposits of fat in their cytoplasm, presences necrosis and there is bridging fibrosis (figure C2). The histopathological finding in liver correlate with the biochemical values in the group. There is evidence of significant decrease in sperm motility, morphology, viability and count. Histology of the testes show the presence of tubular disorganization, seminiferous tubes with loss of some germ cells and decrease in the amount of mature spermatozoa (figure C1). This testicular degeneration is evidence by decrease in the testicular weight (table 2). According to Gluud (1988), the decreasing liver function may lead to low or subnormal plasma concentrations of non-protein bound and non-steroid hormones binding globulin (non-SHBG) bound testosterone [23]. This may explain the increased prevalence of testicular atrophy. There is decrease in the concentration of testosterone, follicle stimulating hormone (FSH) and leutinizing hormone (LH), when CCl_4 group compared with control group receiving normal saline. This is in agreement with the work of Yoshitsugu and Ihori (1997) who worked on Endocrine disturbances in liver cirrhosis [24]. FSH acts as a mitogen for postnatal Sertoli cell proliferation and is required for establishing normal Sertoli cell numbers [25], and the Sertoli cell number determines spermatogenic output in adulthood. Follicle stimulating hormone stimulates both the production of androgen binding protein which is essential to concentrating testosterone in levels high enough to initiate and maintain spermatogenesis. The primary stimulus for the initiation of spermatogenesis is the LH-induced rise in testosterone [26]. However, all these decrease in the andrological variables may not be due to consequences of liver fibrosis alone. It may be due toxic effect of CCl_4 that can generate free radicals leading to oxidative stress that affect testicular germ line, decreasing the testicular weight or even affect the gonadotropins (FSH and LH) from the brain. Sometimes it can also be due to prevention of binding of the androgens to their receptor site. Within the testis, androgen receptor (AR) is expressed in Sertoli cells, peritubular myoid cells, Leydig cells and vascular endothelial cells [27]. In terms of androgen action on spermatogenesis, AR expression in Sertoli cells is essential, as no sperm are produced in mice with targeted deletion of Sertoli cell AR expression [28].

In the group receiving silymarin at the same time with CCl_4, the activities of ALT, AST and ALP significantly decreases toward the normal value of control group receiving normal saline, confirming the hepatoprotective property of silymarin when compared with the fibrotic group receiving CCl_4 alone. This result is supported by histopathological studies done in the liver of animal in this group showing moderate microvesicular steatosis, mild infiltration of inflammatory cells (figure D2). The hepatocytes are seen with mild deposits of fat in their cytoplasm without fibrosis. This reveels an improved hepatoprotection when compared to the group receiving CCl_4 alone. There is significant improvement in the concentration of some of the reproductive variables when compared to the group receiving only CCl_4. Sperm count and motility significantly increase, while viability remains the same as the fibrotic group receiving CCl_4 alone and non-significant increase in morphology (figure 2 - 3). The observed reduction in fibrosis formation and improvement in some of the reproductive variables are in agreement with the report of Morimoto (1994), which explained that the abnormalities in liver cirrhosis (advance form of liver fibrosis) are reversible [29]. The improvements seen in some of the reproductive variables were not significant enough to reach the values of the control group receiving normal saline. When these results were compared to the control group receiving normal saline, the sperm count increase non-significantly, while motility, morphology and viability significantly decrease. There was no significant difference between the plasma concentration of FSH, LH, testosterone level and even change in their testicular and body weight gain, when compared with the CCl_4 group. However, when this treatment group compared with the control group receiving normal saline, all the androgenital hormones decrease except testosterone which significantly decreased. The improvement in reproductive variables when silymarin treated group compared to the toxicity group, might be due to regenerating and synthetic ability of silymarin as reported by Valenzuela and Garrido (1994) [9]. These might have stimulated the regeneration and production more

receptors for the testosterone, FSH and LH in the testes which enhance the utilization of the remaining hormones in the blood for the improvement in the processes of spermatogenesis. Thereby, causing marked decrease in the concentration of some of these andrological hormones in the group receiving silymarin treatment.The study was only done using samples from the liver, testes and blood, thus, may be considered as limitations. More studies would have done on the histology of the pituitary gland in the brain to give us more information regarding the pituitary gonadotropin (FSH and LH) production mention in the work.

5. CONCLUSION

The present study shows that CCl_4 - induced liver fibrosis decrease some reproductive variabes in male Wistar rats. This effect occurs as a result of hypogonadism due to decrease in testicular spermatogenic, steroidogenic functions, and decrease in the plasma level of pituitary gonadotropin (FSH and LH). This is further evidenced from the marked decrease in distortion of the cytoarchitecture of the testes section of the CCl_4 group treated with treated with silymarin. However, it was also found that not only the fibrosis alone, but also the CCl_4 on its own affect the reproductive function of the testis. This is evidenced from decrease in the testicular weight, with no change seen in the andrological hormones when comparing the CCl_4 group treated with silymarin, with fibrotic group (CCl_4 group).

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