



Effect of Silymarin on Oxidative Stress Biomarker, Lipid Peroxidation and Reproductive Function of Diabetic Albino Rats

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Abstract:

The present study was carried out in the animal house and laboratories of the College of Veterinary Medicine/ University of Basrah to investigate the oral administration of silymarin on oxidative stress biomarker, lipid peroxidation and reproductive function in alloxan induced diabetic of albino rats. After the period of acclimation, 32 male rats were divided into 4 equal groups with 8 animals in each as the following: Control group: Animals of this group administered distilled water, Silymarin group: administered silymarin (20mg/kg) dissolved in distill water orally by gavages. Alloxan treated (DM) group : were given alloxan intraperitoneal injection 150mg/BW, as single dose for induced diabetic mellitus and Alloxan + silmarin treated group : were injection alloxan intraperitoneally and then after 3 days, oral administered of (20 mg/kg bw) silymarin . The experimental was continued for 45 day. At the end of the experiment, animals of each group were sacrificed, and blood samples were obtained for oxidative stress biomarker, Lipid Peroxidation and hormonal assessment and the caudal epididymis was then separated from the testicles for the evaluation of epididymal sperm characteristics, while the testes were kept in 10% neutral buffer-formalin for histopathological study. The results showed a significant decrease ($P \leq 0.05$) in serum superoxide dismutase (SOD) , Glutathione Peroxidase (GP_x) , follicle stimulating hormone (FSH), and luteinizing hormone (LH) and testosterone, concentrations of DM group. On the other hand a significant decrease ($P \leq 0.05$) in epididymal sperm concentration, individual sperm motility, live sperm percentage and a significant increase in malondialdehyde (MDA) and sperm abnormalities percentage were recorded in DM group. Histopathological examination of the testes revealed marked suppression of spermatogenesis with absence of mature spermatocytes of DM group. Moreover DM groups treated with Silymarine showed normal oxidative biomarkers, hormonal concentration, epididymal sperm characteristics, and normal testicular tissues compare with control group.

key words: Silymarin, Oxidative stress ,Lipid peroxidation, Reproductive efficiency
تأثير السيليمارين في الضادات الحيوية و اكسدة الدهون والوظيفة التناسلية لذكور الجرذان المصابة
بالسكري

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الخلاصة :

أجريت الدراسة الحالية في البيت الحيواني والمختبرات التابعة الى كلية الطب البيطري / جامعة البصرة لدراسة دور
التجريع الفموي للسيليمارين على انزيمات مضادات الاكسدة ,العلامات الحيوية , اكسده الدهون والوظيفة التناسلية في

ذكور الجرذان المصابة بالسكري بواسطة الألوكان. بعد فترة التأقلم ، تم تقسيم 32 جرذ إلى 4 مجاميع متساوية بواقع 8 جرذ لكل مجموعة وعلى النحو التالي: مجموعة السيطرة: جرعت حيوانات هذه المجموعة بالماء مقطر ، مجموعة السيليمارين: جرعت 20 مجم / كجم سيليمارين مذاب في الماء المقطر. مجموعة الألوكان (مجموعة السكري): حقنت بـ 150 ملغم / كجم الألوكان داخل البريتون ، جرعة مفردة لاستحداث داء السكري والمجموعة الأخيرة (مجموعة السكري المجرعة بالسلمارين): استحدثت السكري ومن ثم بعد 3 أيام جرعت بـ 20 ملغم / كجم من وزن الجسم سيليمارين. استمرت التجربة لمدة 45 يوما. في النهاية ، تم التضحية بالحيوانات للحصول على عينات دم من أجل قياس مستوى العلامات الحيوية ، مضادات التأكسد ، والتقييم الهرموني . وبعد ذلك تم فصل البربخ الذيلي عن الخصيتين لتقييم خصائص الحيوانات المنوية ، في حين تم حفظ الخصيتين في 10 ٪ من الفورمالين لدراسة التشريح المرضي. أظهرت النتائج انخفاضاً معنوياً ($P \leq 0.05$) في مستوى انزيمات مضادات الأكسدة (GPX, SOD)، وهرمون الشحمون الخصوي والهرمون المحفز للجريبات وهرمون التبويض في الحيوانات المصابة بداء السكري في حين تم تسجيل انخفاض معنوياً ($P \leq 0.05$) في تركيز الحيوانات المنوية ، وحركة الحيوانات المنوية الفردية ، ونسبة الحيوانات المنوية الحية وزيادة كبيرة في المالنو داي الديهايد (MDA) ونسبة التشوهات للحيوانات المنوية في مجموعة السكري مقارنة مع مجموعة السيطرة. أما الفحص النسيجي للخصيتين فقد أظهر انخفاض ملحوظ في الحيوانات المنوية مع عدم وجود الخلايا المنوية ناضجة للجرذان المصابة بداء السكري. أما الحيوانات المصابة بداء السكري والمعاملة بالسيليمارين فقد أظهرت تحسن معنوي في العلامات حيوية ومستويات مضادات التأكسد الطبيعية ، وتركيز هرمون الشحمون الخصوي ، وخصائص الحيوانات المنوية ، وأنسجة الخصية مقارنة مع مجموعة السيطرة .

Introduction:

Diabetes mellitus (DM) refers to a group of multifactorial metabolic disorders characterized by elevated blood glucose levels that result from defects in the body's ability to produce and/or insufficiency of insulin action (1). Without enough insulin, the cells of the body cannot absorb sufficient glucose from the blood; hence blood glucose levels increase, which is termed as hyperglycemia. If the glucose level in the blood remains high over a long period of time, this can result in long term damage to organs, such as the kidneys, liver, eyes, nerves, heart and blood vessels(2). The increased extra- and intracellular glucose concentrations result in oxidative stress, which seems to be due mainly to increased production of reactive oxygen species (ROS) and free radicals with a sharp reduction in antioxidant body defenses(3). Generation of reactive oxygen species and lipid peroxidation are associated with diabetes mellitus (4). Free radicals are continuously produced during normal physiologic processes and attack macromolecules including proteins, lipids, and DNA, so causing tissue injury. It has been widely

accepted that oxidative stress plays a key role in the onset and development of diabetes complications, notably nephropathy(5).

Silymarin, a flavanolignan extracted from the seeds of 'milk thistle', has been widely used from ancient times because of its excellent hepatoprotective action. Silymarin is a mixture of silybin (silibinin), isosilybin, silychristin and silydianin. Flavanolignans belong to the family of the benzo gamma-pyrone. It usually possesses good antioxidant activity (6). This flavonoid has a phenolic structure, which allows electron donation to free radicals and reactive oxygen species (ROS) in order to stabilize them and prevents lipid peroxidation by interaction with intracellular glutathione (7,8). Silymarin acts mainly as a hepatoprotective (9) and anticancer agent (10). The present study aimed to evaluate the oral administration of silymarin on oxidative stress biomarker, lipid peroxidation and reproductive function in alloxan induced diabetic of albino rats.

Materials and Methods:

Study design:

The experiment animals was conducted at the animal house of the veterinary medicine college University of Basrah. Thirty two healthy males rats sexually mature, 12 weeks old , and (200-250 gm) grams

of body weight were used. The experiment conditions were the same for all animals, where the room temperature was set between 20-25°C by the use of an air conditioner, and the daily light Period was 12 hours by use of two fluorescent lamp, the rate of humidity was about 50%. All nutritional requirements were provided.

After the period of acclimation, 32 male rats were divided into 4 equal groups with 8 animals in each as the following:

- 1- Control group: Animals of this group administered distilled water (1 ml /kg bw)/day orally by gavages.
- 2- Silymarin group: Animals of this group administered silymarin (20mg/kg) dissolved in distilled water (1 ml /kg bw)/day orally by gavages.
- 3- Alloxan treated group: Animals of this group were given alloxan intraperitoneal injection 150mg/BW, as single dose for induced diabetic mellitus.
- 4- Alloxan + silymarin treated group: Animals of this group were given Alloxan intraperitoneal injection and then after 3 days, administered silymarin (20 mg/kg bw) day orally by gavages.

The experimental was continued for 45 day.

At the end of the experiment, animals of each group were anaesthetized by chloroform and sacrificed. Blood sample were collected from the heart via the cardiac puncture by using 5cc sterile syringe and dropped in plain without anticoagulant tubes and serum samples were isolated from blood by centrifugation at 3000rpm for 15 min, and used for measurement of MDA, SOD GPX, testosterone, FSH, and LH hormones concentration. The testes were removed from the animals of each treated group along with its epididymis. The caudal epididymis was then separated from the testicles

for the evaluation of epididymal sperm characteristics, while the testes were kept in 10% neutral buffer-formalin for histopathological study.

Studied parameters:

Measurement of Sperm Characteristics:

The caudal epididymis placed in a clean Petri dish, containing 5ml of physiological saline then minced by a surgical scalpel blade according to (11). Sperm concentration was determined by using the new improved Neubauer hemocytometer. Sperm Motility percentage was determined visually under light microscope, according to (12). Sperm livability (percentage of live and dead spermatozoa) was done by staining one drop of semen and one drop of warm Eosin-Nigrosin stain on a warm slide. Sperm abnormalities percentage was calculated by using the same slide used for determination of sperm viability according to Evan's and Maxwell(12). The slides were examined under the microscope under oil immersion with x100 objective.

Measurements of Some oxidative biomarker :

Measurements of Malondialdehyde (MDA): The main end product of lipid peroxidation is Malondialdehyde, will be carried out in serum according to Yagi method (13).

Measurements of Superoxide Dismutase (SOD) and Glutathione Peroxidase (GPX): The serum Superoxide Dismutase (SOD) is determined by ELISA kit Elabscience Biotechnology Inc. China

Hormonal Analysis:

Measurement of Luteinizing Hormone (LH) Concentration (ng/ml): Serum LH was measured by ELISA kit supplied (Calbiotech Inc., USA). Catalog No. :LH231F. (14).

Measurement of Follicle Stimulating Hormone (FSH)

Concentration ($\mu\text{IU/ml}$) : Serum FSH was measured by ELISA kit supplied by (Foresight, ACON Laboratories, Inc. CA 92121 . USA). (15).

Testosterone ELISA kit: The quantitative determination of total testosterone concentration in serum or plasma by a microplate enzyme immunoassay. Kit was used by bioactiva diagnostica GmbH (Germany). (16).

The Histological Study: Testes samples were dissected and fixed in formalin, dehydrated and imbedded in paraffin wax. Then, the sections were stained by haematoxylin and eosin (H and E). Each slide contained two sections and examined for histopathological changes under light microscope. based on (17).

Statistical Analysis: The data were subjected to analysis of variance and the significance differences at ($p \leq 0.05$) which were determined by analysis of variance (ANOVA), one-way by using the statistical

soft ware's sigma statistical (18).

Result:

Effect of Silymarin on Serum MDA, SOD and GPX in diabetic Male Rats : The obtained from the present study revealed a significant increase ($P \leq 0.05$) in serum MDA level and a significant decrease ($P \leq 0.05$) in serum SOD and GPx enzymes level in DM group compared with control groups ,Table (1). On the other hand no significant changes in serum MDA levels was recorded in silymarin groups compare with control group but remained significantly lower than those of DM group.

A significant decrease ($P \leq 0.05$) in serum level of SOD enzyme was observed in DM groups compared with control and silymarin group but no significant changes between DM + Silymarin and control group.

Finally a significant decrease ($P \leq 0.05$) in level of GPx enzyme was recorded in DM groups compared with other groups while no significantly change between DM + Silymarin and control group.

Table (1): Effect of Silymarin on Serum MDA, SOD and GPX in diabetic Male Rats.

Group	Parameter	MDA (U/l)	SOD (U/l)	GPx (U/l)
Control group		3.95 $\pm$ 1.24 b	68.81 $\pm$ 4.84 a	465.46 $\pm$ 16.6 a
Silmarine group		3.22 $\pm$ 2.93 b	68.99 $\pm$ 3.78 a	445.76 $\pm$ 17.9 a
DM group		13.40 $\pm$ 1.18 a	55.12 $\pm$ 2.78 b	137.19 $\pm$ 18.75 b
DM+ Silmarine		4.92 $\pm$ 0.87 b	64.19 $\pm$ 1.60 a	396.78 $\pm$ 28.19 a
LSD		1.72	4.7	58.37

- Values expressed as Mean $\pm$ SD (n=8)
- Different small letters denote significant defferences ($P \leq 0.05$) between experimental groups.

Effect of Silymarin on Serum Reproductive Hormones Concentration in Diabetic Male Rats: The results of the treatment with alloxan on the reproductive hormones in adult male rats revealed a significant decrease ($P \leq 0.05$) in serum

concentration of LH, FSH, and testosterone(T) hormones Table (2). However a significant degrees of improvement ($P \leq 0.05$) were noted in male reproductive hormones concentration of DM male rats treated with Silymarine while , no significant

differences were recorded in serum hormones between silymarine group concentration of LH, FSH and T and control group.

Table (2): Effect of Silymarin on Serum Reproductive Hormones Concentration in Diabetic Male Rats.

Parameters Groups	LH ng/ml	FSH ng/ml	Testosterone ng/ml
Control group	4.11 ± 1.89 a	2.40 ± 0.92 a	7.02 ± 0.09 a
Silmarine group	4.33 ± 1.22 a	2.34 ± 0.48 a	6.94 ± 1.03 a
DM group	0.91 ± 1.45 b	0.92 ± 0.99 b	2.52 ± 0.88 b
DM+ Silmarine	3.12 ± 0.64 b	2.59 ± 0.41 b	6.34 ± 1.32 b
LSD	1.11	0.87	1.08

- Values expressed as Mean ±SD (n=8)
- Different small letters denote significant differences ($P \leq 0.05$) between experimental groups.

Effect of Silymarin on Epididymal Sperm Characteristics in Diabetic Male Rats: Results illustrated in table (3) showed a significant decrease ($P \leq 0.05$) in sperm concentration, sperm motility percentage, live sperm percentage and a significant increase in abnormal spermatozoa percentage

in DM group compared with control groups. On the other hand no significant difference in sperms concentration, sperms motility, live sperms percentages and abnormal spermatozoa percentage in DM+ Silymarin group compared with control groups

Table (3): Effect of Silymarin on Epididymal Sperm Characteristics in Diabetic Male Rats .

Parameter s Groups	Spermss Concentration ($\times 10^6$/ ml)	Sperms Motility (%)	Live sperms (%)	Dead sperms (%)	normal sperms (%)
Control group	81.49 ± 11.33 a	81.00 ± 6.14 a	80.02 ± 6.52 a	19.98 ± 6.52 b	11.00 ± 4.11 a
Silmarine group	82.21 ± 5.37 a	77.75 ± 9.21 a	79.00 ± 5.19 a	21.00 ± 5.19 b	12.37 ± 4.55 a
DM group	46.04 ± 6.14 b	44.22 ± 5.17 b	40.42 ± 2.09 b	59.58 ± 2.09 a	20.57 ± 3.41 a
DM+ Silmarine	81.46 ± 3.12 a	80.87 ± 6.89 a	79.50 ± 3.42 a	20.50 ± 3.41 b	15.11 ± 2.92 a
LSD	24.72	30.17	28.44	11.97	9.08

- Values expressed as Mean $\pm$ SD (n=8)
- Different small letters denote significant differences ($P \leq 0.05$) between experimental groups.

Histological study:**Testis:**

The section of testis in the control and Silymarin groups revealed the normal appearance of seminiferous tubules with normal spermatogenesis figure (1, 2). While histopathological section in testis of adult male rats

treated with alloxan (DM group) figure (3) revealed marked suppression of spermatogenesis with with absence of mature spermatocytes. Figure (4) testis of DM+ Silymarin groups, showed impairment of spermatogenesis with marked restoration of spermatocytes.

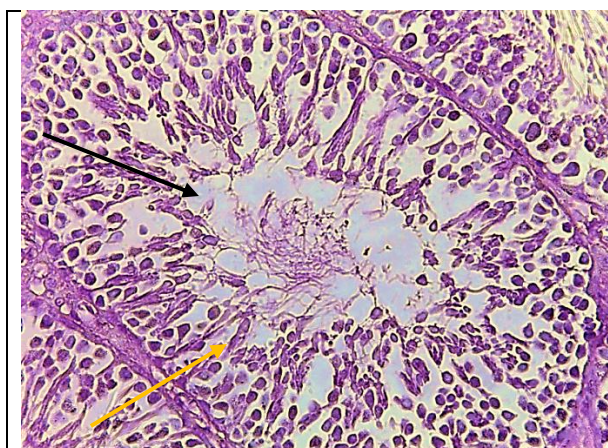


Fig (1) (section of testis) control group reveal the normal appearance of seminiferous tubules (→) with normal spermatogenesis (→) H&E 500X

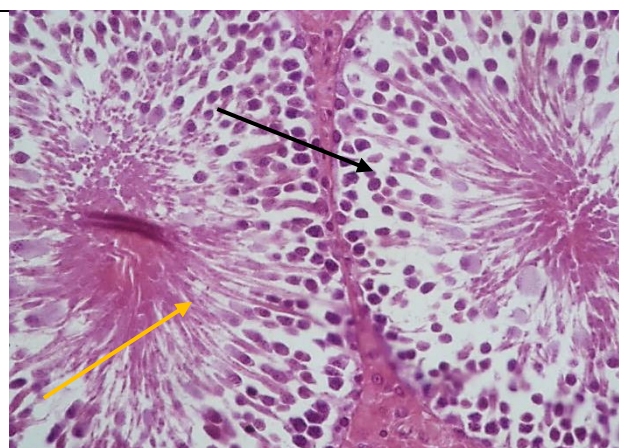


Fig (2) (section of testis) Silymarin group reveal the normal appearance of seminiferous tubules (→) with normal spermatogenesis (→) H&E 500X

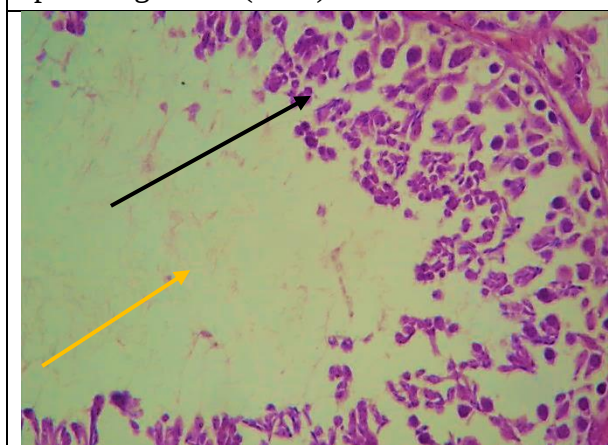


Fig (3) (section of testis) DM group reveal moderate spermatogenesis (→) with absence of mature spermatocytes (→) H&E 500X

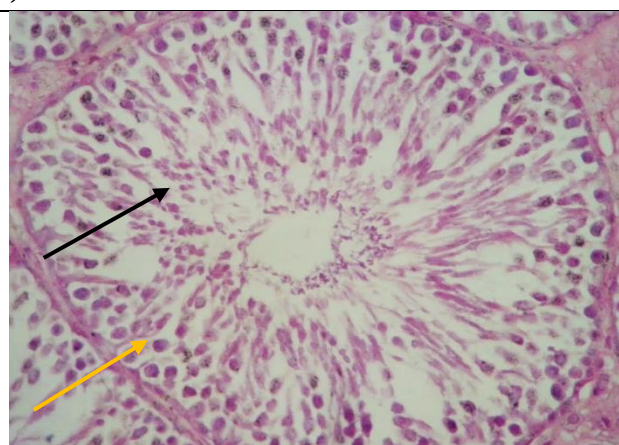


Fig (4) (section of testis) DM+ Silymarin group reveal normal spermatogenesis (→) with marked restoration of spermatocytes (→) H&E 500X

Discussion:

The results indicated a significant increase in serum MDA level and a significant decrease in serum SOD and GP_X levels in alloxan treated group compared with silymarine alone and control groups. These results agreed with the previous (19,20,21 and 22)

Diabetes is a serious and growing disorder which is associated with severe acute and chronic complications. Different sources of evidence indicate that oxidative stress has been implicated in diabetic

(23,24). In other words, ROS have been defined as an autocatalytic mechanism that can lead to apoptosis and free radical damage which is one of the possible mechanisms in the progress of diabetic (25). Free radicals generated lead to lipid peroxidation which in turn releases a large amount of MDA (26). In fact, MDA together with catalase and hydrogen peroxide is used as a reliable oxidative stress marker in diabetes (27). Therefore, regulation of oxidative stress could be a promising therapeutic strategy used

to prevent or delay diabetes complications. Decreased activity SOD may result from excessive utilization for neutralizing superoxide and ROS generated by alloxan toxicants, ROS are continuously produced during normal physiologic events, and removed by antioxidant defence mechanism. In pathological conditions, ROS are over produced and result in lipid peroxidation and oxidative damage. The imbalance between ROS and antioxidant defence mechanisms leads to oxidative modification in the cellular membrane or intracellular molecules (28).

Administration of silymarin combination to DM experimental rats showed recover in serum MDA, SOD and GPX. This finding, also is in line with some studies (29, 30), Some studies have shown that Silymarin protective effect was also reflected on preserving the integrity of the plasma membrane and it seems Silymarin can affect Ca^{2+} modulation as an essential role in hepatoprotection produced by Silymarin (31,32). Silymarin eliminates oxygen free radicals such as hydroxyl anions, phenoxy radicals, and hypochlorous acid in various model systems such as platelets, fibroblasts, liver microsomes and mitochondria and inhibits oxidative stress (33).

The present results revealed a significant decrease in serum LH, FSH and testosterone concentrations in DM group. These results are in consistent with (34, 35). The reduction of LH, FSH and testosterone may be resulted from the oxidative damage to the hypothalamus which responsible for the secretion of gonadotrophin-releasing hormone (GnRH) that stimulates LH and FSH release by the pituitary gland (36) which in turn inhibit testosterone biosynthesis in the testes of rat (37). Oral administration of silymarin for DM rats, induced renormalization of male sex hormone. Silymarin has also been

reported to have antioxidant, anti-inflammatory and immunomodulatory effects (38). The results were matched with the results obtained by (39) that found, Oral concomitant treatment with silymarin was effective in attenuating testosterone-induced testicular damage in adult male albino rats.

In present study, Silymarin caused recovery from decrease in sperm concentration, motility, viability and decrease sperm abnormalities that induced by alloxan. Testosterone, FSH and LH plays a crucial role in initiation, maintenance and maturation of the Spermatogonia. These improvement in sperm quality and quantity could be attributed with the increase of serum testosterone, FSH and LH hormones as found in table (2). The Antioxidant and Antiapoptotic properties of Silymarin may be predicted to lend a helping hand in delivering a healthy and completely matured sperm (40). The Hypothesis what we have derived from the above results is; silymarin as a natural antioxidant will obviously have a protective effect on the mitochondrial membrane which usually gets disturbed in the apoptosis (40).

The microscopic examination of the testes of the male rats treated with alloxan showed a marked suppression of spermatogenesis with with absence of mature spermatocytes while, showed marked restoration of spermatocytes and impairment of spermatogenesis of DM+ Silymarin groups. These results are correlated with those reported by Vijay *et al.*, (40) who revealed that an ameliorating effect of Silymarin on the doxorubicin induced decrease in testicular spermatid head concentrations and the daily sperm production. Similarly (39) reported that a significant decreases in testicular weights in testosterone treated rats compared with the controls whereas the mean testis weight in testosterone and silymarin treated rats showed nonsignificant difference compared with the controls.

Conclusion: The results of the present study demonstrate that silymarin is able to control oxidative stress, lipid peroxidation and renormalization of reproductive function and

thus may be useful in delaying complicated effects of diabetes.

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