Effect of *Silybum marianum* (L.) Geartn extract on *in vitro* fertilization in mice

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تاثير مستخلص نبات الكلغان على بعض معايير النطف والاخصاب خارج الجسم في الفئران

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المستخلص

مستخلص الكلغان يكون غني بالفلافونايدات السلمارين التي تمتلك خاصية كمضاد للاكسدة. الهدف من هذا البحث , دراسة تاثير مستخلص نبات الكلغان على الاخصاب الخارجي ومراحل تطور الجنين في الفئران . استخدم للدراسة 15 ذكر وانثى بالغة باعمار (8-9 اسابيع) من الفئران قسمت الى ثلاثة مجاميع , كل مجموعة تضم 5 فئران , عوملت مجموعتان بتركيزي 25 و 50 ملغم من مستخلص نبات الكلغان /كغم من وزن الجسم عن طريق الحقن داخل الخلب البريتوني . وعوملت المجموعة الثالثة بالمحلول الملحي الفسيولوجي كمجموعة سيطرة . قتلت الحيوانات (الذكور والاناث) بعد 35 يوم من المعاملة عن طريق فصل الرقبة . عزلت النطف من ذيل البربخ وتم اجراء الفحوصات التالية : حركة النطف , نسبة النطف الحية والميتة وتشوهات النطف . وجمع المصل لاجراء تحليل هرمون الشحمون الخصوي كذلك تم عزل البويضات من قناة البيض لاناث الفئران بعد سحقها في 500 مايكرولتر من الوسط الزرعي النسيجي المحور 199 وجمع المصل لاجراء تحليل الهرمون المحفز لنمو الجريبات والهرمون اللوتيني . حضنت النطف والبويضات الناضجة في الوسط الزرعي النسيجي المحور 199 لغرض التلقيح . وتمت متابعة تطورات الجنين بعد 24 ساعة من التلقيح . اظهرت النتائج وجود زيادة معنوية في وزن الجسم والخصى لمجموعتي الحيوانات المعاملة بالمستخلص وزيادة معنوية في حركة النطف وانخفاض في نسبة النطف الميتة والتشوهات في مجموعتي الحيوانات المعاملة بالكلغان . واظهرت النتائج زيادة معنوية في هرمون الشحمون الخصوي والهرمون المحفز للجريبات والهرمون اللوتيني في مجموعتي الحيوانات المعاملة بالكلغان. اما دراسة الاخصاب الخارجي فقد اظهرت زيادة معنوية في نسبة الانتاج للبويضات , والاخصاب ومراحل تطور الاجنة (2-4 خلية) . استنتجنا من هذه الدراسة بان السلمارين له تاثيرات نافعة على الاخصاب الخارجي وعلى تطور مراحل انقسام الاجنة عند حقنها قبل اجراء عملية الاخصاب الخارجي في الذكور والاناث.

الكلمات المفتاحية: الكلغان, الحيامن, الاخصاب الخارجي

Abstract

Composition of *Silybum marianum* (L.) Geartn extract rich with flavonoid *sylimarin* which have antioxidant properties. Aim of this project study the effects of *sylimarin* on *In vitro* fertilization (IVF) and cleavage stages of embryos in mice. Fifteen adult males and females mice were divided into three groups , each group containing 5 mice , first and second groups were treated with (25 and 50 mg/kg body weight) of *Silybum marianum* (L.) Geartn extract (IP) while the third group treated with normal saline as control group . After 35 days of treatment the animals (male and female) were sacrificed and sperms isolated from tail of epididymis were used for the following tests: Sperm motility, dead / live sperm, sperm abnormalities and serum was collected to assay testosterone. Isolation of oocyte from oviduct and serum blood to hormonal assay (FSH and LH) . Sperm and matured ova were incubated in TCM-199 media for insemination. The results showed increase in body weight and testes weight in groups treated with *Silybum marianum* (L.) Geartn. Significant increase in sperm activity and decrease in

percentage of dead sperm and abnormalities in groups treated with *Silybum marianum* (L.) Geartn. The male testosterone and female FSH, LH increased significantly in groups treated with *Silybum marianum* (L.) Geartn. IVF study showed significant increase in percentage of maturation, fertilization and cleavage (2-4 cells). In conclusion *sylimarin* have benefits effect on IVF and cleavage stages of embryo development when injected before IVF by improve fertilization of males and females.

Key words: *Silybum marianum* (L.) Geartn, sperms, *in vitro* fertilization.

Introduction

Silymarin is a mixture of flavonoids extracted from seeds of milk thistle *Silybum marianum* (L.) Geartn, and it have been used to treated many diseases especially liver disease for more than 200 decade (1).

As it's known, the flavonoids plant origin and contain large group of polycyclic phenols, the flavonoids have estrogenic effects (phytoestrogen) it binds with receptor of estrogen and can modulates activity, this exchange hormonal balance of individual. The intake of feeds contain phytoestrogens for long time may cause transitory or persistent infertility (2).

In Egypt the *Silybum marianum* (L.) Geartn leave used traditionally as antinfertility effects (1).

Many studies suggested that the silymarin action resulted from the strong antioxidant activity (inhibition of generation and scavenging of free radicals), inhibition of lipid peroxid-ation in cell membranes , in the 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 (3,4).

Humphrey, (5) reported that intake of high levels of phytoestrogens can cause inverse effects on reproductive at end, including fertility in several animal species. Also they added high doses of dietary phytoestrogens may be correlated with the increased happening of reproductive problems in men.

Kumer, (6) was suggested that silymarin displayed estrogen effects in ovariectomized rats. Khalil, (7) was reported the contraceptive effects of hot water extract of Silybum marianum leaves in female albino rats.

Materials and methods

Extract preparing and doses

Leaves of *Silybum marianum* (L.) Geartn were obtained from Baghdad local markets and identified by the Iraqi National Herbarium, clean, dried leaves and finally grind to obtained powder. The *Silybum marianum* (L.) Geartn was extracted by using ethanol alcohol, dosage of *Silybum marianum* (L.) Geartn used was 25 and 50 mg/kg B.W (8).

Animals and experimental design

Males and females weighted 30-35 gm obtained from Biotechnology Research Center and keeping on a 14 hour light dark in the animal house, and the feed and water provided ad libitum. Mice (males and females) were randomly divided into 3 groups, each composed of 5 mice. First and second groups were treated with (25,50 gm/kg B.W) respectively of *Silybum marianum* (L.) Geartn intraperitoneally while administered for 35 days and the third group was given normal saline as a control group. The animals in each group were sacrificed by dislocation of cervical vertebrate. Sperms were obtained from the two tails of epididymides by mincing in 500 µl TCM-199, and maintained at 37°C in 5% CO2 incubator. Sperms maintained prior treatment to capacitation. (sperms motility , percentage of dead/live sperms and abnormalities of sperm were recorded).

As well as , in females the ovaries and oviduct were removed and placed in a sterile disposable petridish containing 1ml TCM-199 medium, then the oviducts were isolated, ova were obtained from the two oviduct by mincing in 500 µl of TCM-199 media, and maintained at 37°c in 5% Co_2 incubator.

Hormonal assay

Testosterone levels in serum of males were measured by using ELISA kit from (Accu-Bind Inc. USA) and calculation of results by using standard curve equation according leaflet of kit.

Females (FSH and LH) levels were measured by using ELISA kit from (Accu-Bind Inc. USA) and calculation of results by using standard curve equation according leaflet of kit.

Microscopic examination

Sperms parameters were assessed according to WHO Laboratory manual (9) for Motility, percentage of dead/live and abnormalities sperms. Ova were examined to isolate matured ova by obtained first polar body.

***In vitro* fertilization (IVF)**

Sperm and matured ova were co-incubated in TCM-media-199 having (20mg/ml B.S.A. and heparin) for insemination. Ova were observed for cleavage after 24h.of inseminate under phase contrast microscope.

Statistical evaluation

Data were analyzed by one-way analysis of variance (ANOVA- test), by using SPSS version 13 data are presented as means ± SE. The level of significance was $P < .05$.

Results and discussion

The results show significant increase in body weight and testes weight in groups treated with *Silybum marianum* (L.) Geartn extract compared with control group, (32.30±5.49; 35.88±4.02) (29.27±4.51) respectively, table 1.

Statistical analysis show higher significant in sperm motility (%) in groups treated with *Silybum marianum* (L.) Geartn extract (75.94±10.12; 81.02±11.04) compared with control group(68.33±5.06). While Lower significant differences in dead sperm (%), (21.83±5.73; 20.85±3.75) and sperm abnormalities (24.72±5.81; 22.11±3.46) in groups treated with

Silybum marianum (L.) Geartn extract (28.29±4.06; 30.71±4.92) respectively compared with control group, table 2**.**

Table (1): Effect of *Silybum marianum* (L.) Geartn extract on body weight and testes weight

Treatment groups	Final Body Weight gm(μ±SE)	Testes weight mg/100gm(μ±SE)
Control	29.27±4.51	0.22±0.014
***Silybum marianum* (L.) Geartn (25gm/kg B.W)**	32.30±5.49	0.34±0.088
***Silybum marianum* (L.) Geartn (50gm/kg B.W)**	35.88±4.02	0.35±1.064

n=(5) numbers of animals in each group

Table (2): Effect of *Silybum marianum* (L.) Geartn extract on sperm motility , dead sperm and abnormalities of sperm %

Treatment groups	Sperm motility % (μ±SE)	Dead sperm %(μ±SE)	Abnormalities sperm(μ±SE)
Control	A 68.33±5.06	A 28.29±4.06	A 30.71±4.92
***Silybum marianum* (L.) Geartn (25gm/kg B.W)**	B 75.94±10.12	B 21.83±5.73	B 24.72±5.81
***Silybum marianum* (L.) Geartn (50gm/kg B.W)**	B 81.02±11.04	B 20.85±3.75	B 22.11±3.46

Differences A, B, C are significant (P<0.05) to compared rows

Silymarin have benefits effect to maintains female rat pregnancy and may be cause changes in histology of uterus and ovary. And play important role on male to improve fertility (10).

Silybum marianum (L.) Geartn extract contain Flavonoid *silymarin* which have wide variety of phytotherapeutic applications, and it is the most biologically active, where it acts as an antioxidant (11).Wasfi *et al*., (12) suggested that a higher number of damaged spermatozoa may reduce sperm kinetic characteristics and probably fertilizing capacity by triggering specific morphological damages to the head and/or by inhibiting motility, and when treated with *Silybum marianum* (L.) Geartn extract improve fertility.Data obtained from this study revealed that (25 and 50mg/kg) *silymarin* injected IP to mice has a significant positive correlation of testosterone level in correlation with the diameter of Leydig cell and this may be due to the direct effect of *silymarin* on Leydig cell by enhancing the ability of Leydig cells for testosterone production (12).

Silymarin have significant effect on quality of sperm and oocyte to improve fertilization due to it have antioxidant property where it inhibits radical formation, binds some radical species, interferes with lipid peroxidation of membranes, and increases the intracellular content of scavengers (13).Membrane of spermatozoa in mammalian rich with polyunsaturated fatty acids,this makes them very sensitive to oxygen-induced damage, which is mediated by lipid peroxidation. Normally the antioxidant mechanisms support the reproductive tissues are likely to quench these reactive oxygen species (ROS) and protect gonadal cells and mature spermatozoa from oxidative damage (14). Increase accumulation of reactive oxygen species (ROSs) during spermatogenesis epididymal sperm maturation lead to Oxidative stress (OS) as well as from exposure of toxic chemicals, environmental pollutants etc… ROSs change lipid/protein ratio of membranes by affecting polyunsaturated fatty acids and lipid peroxidation causes functional irregularities of several cellular organelles (15,16).The results show elevation of testosterone level in groups treated with extract (2.33±0.73; 2.39±0.92) compared with control group (1.12±0.34), two groups treated with *silymarin* extract lead to increase testosterone level by the effective on pituitary-hypothalamus axes or on leydig cells directlyor on aromatase inhibitor peripherally or by interfere with testosterone synthesis.

The treatment of female with *silymarin* extract lead to increase FSH (0.62±0.073; 0.75±0.094) and LH (0.91±0.071; 1.32±0.094) levels compared with control group (0.53±0.089; 0.37±0.074), table 3.

Table (3): Effect of *Silybum marianum* (L.) Geartn extract on reproduction hormones (male testosterone, female FSH and LH) after 35 days treatment in mice

Treatment groups	Male testosterone ng/ml (µ±SE)	Female FSH IU/ml(µ±SE)	Female LH IU/ml(µ±SE)
Control	A 1.12±0.34	A 0.53±0.089	A 0.37±0.074
***Silybum marianum* (L.) Geartn (25gm/kg B.W)**	B 2.33±0.73	B 0.62±0.073	B 0.91±0.071
***Silybum marianum* (L.) Geartn (50gm/kg B.W)**	B 2.39±0.92	C 0.75±0.094	C 1.32±0.094

Differences A, B, C are significant (P<0.05) to compared rows

In male rats,a significant elevated of serum testosterone and LH levels while estradiol did not change by silymarin treatment at one month. Oliveira *et al*., (17) proven that estrogen is required for normal function of the efferent

ductules and is essential for maintain fertility in male rodent. Robertson *et al.,* (18) insisted that low levels of dietary phytoestrogen have a biological effect in the testis. Vermeulen *et al*.,(19) states that estrogen play an important role in the regulation of the gonadotrobin feedback , several brain functions, bone maturation , regulation of bone resorption , they affect skin metabolism and an important factor determing sex interest in man .

Hodek *et al*. (2002)(2) noticed that flavones and flavonone have higher aromatase inhibitory activity and it has been observed that *silymarin* has as an aromatase inhibitor property (7) and this could explain the increasing in testosterone level.This indicates that *silymarin* may improve the testicular cell function and spermatogenesis through prevention of the oxidative stress (20) and/or due to its anti-inflammatory effect (21).Elevated the percentage of *in vitro* fertilization (% incubated ova, maturated ova , fertilized ova cleavage ova (2-4 cells) after 35 days of treatment with *Silybum marianum* (L.) Geartn extract compared with control group, table.4.

Table (4):Effect of *Silybum marianum* (L.) Geartn extract on *in vitro* fertilization (% incubated ova, maturated ova, fertilized ova cleavage ova (2-4 cells) after 35 days treatment in mice

Treatment groups	% of incubated ova	% of maturated ova	% of fertilized ova	% ova cleavage (2-4 cells)	% ova cleavage (4 cells)
Control	28	57.14	56.25	66.66	66.66
***Silybum marianum* (L.) Geartn (25gm/kg B.W)**	26	73.076	68.42	69.23	66.66
***Silybum marianum* (L.) Geartn (50gm/kg B.W)**	21	76.19	66.66	71.42	70.00

These data suggest that administration of *silymarin* in IVF patients concomitantly with gonadotropin results in reduction of granolusa cell apoptosis but does not have any effect in promotion of follicular development, oocyte retrieval or endometrial thickness (6).The antioxidative effects of *silymarin* were examined against nitric oxide-induced oxidative stress on cell characteristics of bovine oviduct epithelial cell (BOEC) and developmental rates of bovine in vitro fertilisation (IVF) embryos. These results suggest that *silymarin* has positive effects on cell characteristics such as viability, morphology and lipid peroxidase (LPO) of BOEC, and the increase of bovine IVF embryo development rate might be through antioxidative and anti-apoptotic actions (22).

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