Evaluation of effect of silymarin on granulosa cell apoptosis and follicular development in patients undergoing *in vitro* fertilization

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تقييم أثر السيليمارين على استماتة الخلايا الحبيبية والتطور الجريبي في مرضى الإخصاب في المختبر

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الخلاصة: يهدف البحث إلى دراسة آثار السيليمارين على التطور الجريبي. وقد أدرج الباحثون في هذه الدراسة 40 امرأة ممن يجرين الإخصاب في المختبر ويتمتعن بصحة جيدة. فأجروا لهن تحريضاً للإباضة، وعيَّن بعضهن على أسس عشوائية ومعماة لتلقي السيليمارين (70 ميلي غرام ثلاث مرات يومياً) أو الدواء الغفل منذ بداية دورة التحريض. وقيّم الباحثون عدد وجودة الخلايا البيضية التي حصلوا عليها، ودرسوا استماتة الخلايا الحبيبية، فلم يجدوا فرقاً بين مجموعات كان في إحداها العدد الوسطي للجريبات يساوي أو يزيد على 18 ميلي متر (قوة الاحتمال $P = 0.131$)، والعدد الوسطي للخلايا البيضية التي حصلوا عليها (قوة الاحتمال $P = 0.209$)، وثخانة بطانة الرحم (قوة الاحتمال $P = 0.673$). إلا أن النسبة المئوية للاستماتة الإجمالية في المجموعة المدروسة كانت منخفضة بدرجة يُعتد بها إحصائياً عما هي عليه في المجموعة التي تلقت الدواء الغفل (قوة الاحتمال $P = 0.032$). وتشير هذه المعطيات إلى أن إعطاء السيليمارين لمريضات الإخصاب في المختبر بالتزامن مع موجِّهة الغدد التناسلية يؤدي إلى إنقاص استماتة الخلايا الحبيبية ولكنه لا تأثير له على تعزيز التطور الجريبي، وعلى الحصول على الخلايا البيضية، وعلى ثخانة بطانة الرحم.

ABSTRACT To investigate the effects of silymarin on follicular development, we enrolled 40 healthy women undergoing *in vitro* fertilization (IVF) due to male factor infertility in this trial. They underwent ovulation induction and on a random and blind basis, patients were assigned to receive silymarin (70 mg × 3/day) or placebo from the beginning of the induction cycle. The number and quality of oocytes retrieved were evaluated and apoptosis of granolusa cells was studied. There was no significant difference between the groups for mean number of follicles ≥ 18 mm ($P = 0.131$), mean number of oocytes retrieved ($P = 0.209$) or endometrial thickness ($P = 0.673$). However, the proportion of total apoptosis in the study group was significantly lower than in the placebo group ($P = 0.032$). 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.

Évaluation de l'effet de la silymarine sur l'apoptose des cellules de la granulosa et le développement folliculaire chez les patientes subissant une fécondation *in vitro*

RÉSUMÉ Pour étudier les effets de la silymarine sur le développement folliculaire, nous avons recruté 40 femmes en bonne santé subissant une fécondation *in vitro* (FIV) en raison d'une infertilité masculine. Ces patientes ont été soumises à une induction d'ovulation et ont reçu, sur la base d'une répartition aléatoire et en aveugle, de la silymarine (70 mg × 3 fois par jour) ou un placebo, dès le début du cycle d'induction. Le nombre et la qualité des ovocytes prélevés ont été évalués, et l'apoptose des cellules de la granulosa a été étudiée. Aucune différence significative n'a été observée entre les deux groupes en termes de nombre moyen de follicules ≥ 18 mm ($P = 0{,}131$), de nombre moyen d'ovocytes prélevés ($P = 0{,}209$) ou d'épaisseur de l'endomètre ($P = 0{,}673$). Toutefois, la proportion de l'apoptose totale dans le groupe d'expérimentation était significativement inférieure à celle observée dans le groupe sous placebo ($P = 0{,}032$). Ces données suggèrent que, chez les patientes qui subissent une FIV, l'administration concomitante de silymarine et de gonadotrophine induit une réduction de l'apoptose des cellules de la granulosa, mais n'a aucun effet sur la stimulation du développement folliculaire, sur le nombre d'ovocytes prélevés ni sur l'épaisseur de l'endomètre.

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Introduction

Normal maturation and growth of oocytes depends on adequate support from ovarian granolusa cells of the follicles [*1*]. Therefore, apoptosis of granolusa cells seems to have a negative effect on follicular development, oocyte quality and pregnancy rates [*2*].

Reactive oxygen species (ROS) serve not only as key signal molecules in physiological processes, but also have a role in pathological processes in female reproduction. ROS are involved in the modulation of an entire spectrum of physiological reproductive functions such as folliculogenesis, oocyte maturation, steroidogenesis, corpus luteal function and luteolysis [*3*].The role of ROS in gynaecological disease and assisted reproduction has been widely studied in recent years.

Follicular fluid microenvironment has a crucial role in determining the quality of the oocyte. There is a potent antioxidant enzymatic defence in human follicular fluid that protects oocytes against oxidative stress (OS) [*3,4*]. Changes in the antioxidant enzymatic pattern could impair ROS scavenging efficacy in the follicular environment and result in OS. It has been shown that increased ROS concentration in the follicular fluid has been associated with increased granolusa cell apoptosis and thus impaired follicular development [*5*].

Silymarin, which is a standardized extract (a mixture of 3 isomeric flavonolignans) from dried fruits of milk thistle, *Silybum marianum*, appears to function as an antioxidant by scavenging free radicals to increase glutathione levels and activate superoxide dismutase (SOD) and glutathione peroxides; it also inhibits the formation of damaging chemicals[*6,7*]. It has also been shown to act as a membrane stabilizer, preventing lipoperoxidation and associated cell damage in some experimental models [*8*].

In a study by Plíšková et al. silymarin and its components elicited partial or full estrogen receptor activation. Silybin B, one of the components of silymarin, has (probable) weak estrogen receptor (ER)-mediated activity. Silybin-A and other flavonolignans are inactive, and taxifolin, which is a minor constituent of silymarin, is a potent ER-agonist [*9*]. Considering the antioxidant effect and estrogen receptor activity, it is suggested that silymarin and its components can affect folliculogenesis, oocyte maturation, granulosa cell apoptosis and endometrial thickness.

This is the first attempt to evaluate the effects of silymarin on folliculogenesis and granolusa cell apoptosis. To study the actual effect of silymarin and to determine whether supplementation can increase the pregnancy rate in infertile patients undergoing assisted reproduction, we carried out an interventional study.

Methods

Forty healthy women undergoing *in vitro* fertilization (IVF) for male factor infertility were included from the patients attending the Infertility Clinic of Monasteries Hospital at Mashhad University of Medical Sciences between April 2006 and April 2007. Inclusion criteria were: the cause of infertility should be exclusively a male factor (total motile sperm count < 5 million per sample); women should undergo intracytoplasmic sperm injection cycles; age should be 18–35 years. Exclusion criteria were: cigarette smoking and taking vitamin C, vitamin E and other antioxidants recently or concurrently.

For all patients, demographic data, physical examination findings, hormonal and tubal assessment and probable concomitant disease were recorded.

Before the beginning of the cycles, patients were divided into 2 groups of 20 on a random and blinded basis. All the patients underwent a standard long protocol of IVF cycle. After pituitary down regulation with GnRh analogue and from the first day of ovarian stimulation, one group of patients received gonadotropin plus silymarin (70 mg × 3/day) and the other group gonadotropin plus placebo. Transvaginal ultrasound examination was performed to evaluate follicular development and endometrial thickness on the sixth day after human menopausal gonadotropin administration and then every other day according to the follicular size. When at least 3 follicles measured ≥ 18 mm, human chorionic gonadotropin (HCG) 10 000 U was administered intramuscularly. 34–36 h after HCG injection, egg recovery was performed under transvaginal ultrasonographic guidance.

The number of follicles and endometrial thickness were recorded and oocyte quality examined by an embryologist according to a comprehensive grading system, which included evaluation of oocyte maturational status, morphological quality, and fertilization capacity from grade I to grade IV [*10*].

Measurement of granulosa cell apoptosis was performed by flow cytometry. Apoptotic granolusa cells were detected using Annexin V and propidium iodide. For 10 women selected at random (due to economic constraints, we could not manage more) granulosa cells were isolated from each aspirated follicle using hyaluronidase. Analysis of phosphatidylserine exposure in granolusa cells was carried out by the following process: first granolusa cells were washed with calcium buffer and the cell concentration was adjusted to 1.5 × 10^6 cells/mL in calcium buffer. Then 10 µL annexin V-FITC was added to 100 µL cell suspension and incubated for 20 minutes on ice in the dark. After incubation, the cells were washed again with calcium buffer; 10 µL propidium iodide was added to the suspension and incubated at least 10 minutes on ice, then the cell suspension was ready to analyse by flow cytometer. Positive con-

trol was carried out by Dexamethasone incubation

The number of follicles of different sizes, endometrial thickness on day of HCG injection, the number, and quality of oocytes retrieved and the percentage of early and total apoptosis were analysed for the 2 groups. *P*-value < 0.05 was considered significant.

Results

All 40 women completed the study. The mean age of patients was 28.38 [standard deviation (SD) 4.59] years in the study group and 27.59 (SD 4.05) years in the placebo group, and was not significantly different. Other paraclinical parameters are listed in Table 1.

The mean number of follicles ≥ 18mm and 15–18 mm and also the total number of oocytes retrieved were not significantly greater in the therapeutic group ($P = 0.118$, 0.360 and 0.125 respectively) (Table 2). There was no significant difference between the 2 groups regarding endometrial thickness ($P = 0.673$).

The proportion of early apoptosis and total apoptosis in therapeutic group was significantly lower than in the control group ($P = 0.014$ and 0.027 respectively) while late apoptosis was reduced, but not significantly ($P = 0.086$) (Table 3).

Table 1 Baseline characteristics of the sylimarin group and the placebo group

Parameter	Therapeutic group Mean (SD)	Control group Mean (SD)	Overall Mean (SD)
Age (years)	28.38 (4.59)	27.59 (4.05)	27.97 (4.29)
LH (mIU/mL) on day 3	5.75 (3.20)	5.17 (2.20)	5.45 (2.72)
FSH (mIU/mL) on day 3	6.68 (2.16)	6.89 (3.10)	6.79 (2.65)
LH/FSH on day 3	0.94 (0.58)	0.91 (0.54)	0.92 (0.55)
TSH (mIU/mL)	1.84 (1.33)	2.09 (1.30)	1.97 (1.30)
Prolactin (mIU/mL)	218.71 (218.23)	237.22 (230.38)	228.18 (222.04)

SD = standard deviation.
LH = luteinizing hormone.
FSH = follicle stimulating hormone.
TSH = thyroid stimulating hormone.

Discussion

Granulosa cells are essential in the normal follicular maturation process since they produce steroidal hormones and growth factors and also they play a crucial role in follicular atresia. Apoptosis of granolusa cells seems to have a negative effect on follicular maturation. A higher incidence of apoptotic granulosa cell has been associated with fewer oocytes retrieved and poorer quality of oocytes and embryos [*2*].

Various pathological stimuli such as OS can initiate apoptosis in mammalian oocytes [*11*]. Intra-cellular accumulation of ROS, i.e. OS, can damage cells by causing nucleic acid strand breaks, lipid peroxidation, protein degradation and ultimately, cell death [*12*]. It has been suggested that steroidogenically active cells such as granulosa cells of antral follicles, require high levels of energy production and thus generate large amounts of ROS [*13*]. Therefore it is possible that OS is involved in the mechanisms that trigger apoptosis in healthy steroidogenic antral follicles [*2*].

An increasing number of published studies have pointed towards increased importance of the role of OS in female reproduction [*2,3*]. OS can be overcome by reducing generation of ROS or increasing the amounts of total antioxidant capacity.

Pradeep et al. indicated that silymarin exhibits good hepatoprotectivity and antioxidant potential against diethyl nitrosamine-induced hepatocellular damage in rats [*14*].

Table 2 Mean number of follicles and oocytes retrieved in the sylimarin group and the placebo group

Parameter	Therapeutic group Mean (SD)	Control group Mean (SD)	*P*-value[a]
No. of follicles ≥ 18 mm	7.76 (3.37)	6.14 (3.40)	0.118
No. of follicles 15–17 mm	3.28 (3.27)	4.61 (4.35)	0.360
No. of oocytes retrieved	10.09 (3.56)	8.52 (4.54)	0.125
Endometrial thickness (mm)	9.83 (1.79)	10.08 (2.11)	0.673

SD = standard deviation.
[a]Mann–Whitney U-test.

Table 3 Mean percentage of granulosa cell apoptosis in the sylimarin group and the placebo group

Apoptosis	Therapeutic group Mean (SD)	Control group Mean (SD)	*P*-value[a]
Early	2.70 (1.61)	13.77 (10.06)	0.014
Late	2.17 (2.65)	5.77 (2.33)	0.086
Total	4.88 (2.48)	19.54 (12.15)	0.027

SD = standard deviation.
[a]Mann–Whitney U-test.

Regarding the antioxidant effect of silymarin on fertility, Jancar et al. reported that an increased percentage of ROS-producing granulosa cells resulted in fewer oocytes retrieved and diminished implantation rate [*2*].

Tsani et al. evaluated opposing effects of glutathione depletion and follicle-stimulating hormone on reactive oxygen species and apoptosis in cultured pre-ovulatory follicles. They found that OS induced apoptosis in pre-ovulatory follicles and antiapoptotic effect of follicle stimulating hormone was mediated by stimulation of follicular glutathione synthesis and suppression of ROS production [*15*]. Our findings demonstrated that apoptosis of granulosa cells was reduced but follicular development was not increased significantly by silymarin administration in women undergoing intracytoplasmic sperm injection for male factor infertility.

In a study by Plíšková et al. silymarin elicited partial ER activation, with the silybin B component being probably responsible for a majority of the weak ER-mediated activity of silymarin [*9*]; silybin A and other flavonolignans were found to be inactive, and the potent ER-agonist toxifolin is only a minor constituent of silymarin. In the study by Kummer et al. uterotrophic effects of 30 days treatment with Silymarin were evident from increased heights of the luminal epithelium and endometrium of ovariectomized rats [*16*].

In our study the endometrial thickness on the day of HCG injection did not differ significantly in the treatment and control groups; this may be because of the short treatment period.

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