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OXIDATIVE STRESS MARKERS IN GnRH AGONIST AND ANTAGONIST PROTOCOLS IN IVF

MARKERI OKSIDATIVNOG STRESA U PROTOKOLIMA STIMULACIJE SA GnRH AGONISTIMA I ANTAGONISTIMA U IVF CIKLUSIMA

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Summary

Background: Our aim was to study the effect of GnRH agonist and antagonist protocols of ovarian stimulation on oxidative stress parameters in serum and the influence of oxidative stress parameters change on the outcome of IVF cycles.
Methods: This prospective study included 82 patients who underwent IVF procedures. We determined SOD, MDA and SH groups in serum. Serum samples were obtained between the second and fourth day of the cycle and on the day of HCG administration during ovarian stimulation.
Results: Patients were divided into two groups depending on the protocol of stimulation. The mean total and mature oocytes number and number of fertilized oocytes were higher in GnRH agonist group. There was no significant difference in biochemical pregnancy, miscarriage and live –birth rate in both groups. Mean serum SOD was significantly lower, while mean serum MDA and SH groups were significantly higher after ovarian stimulation. Delivery rate was higher in patients without OS while miscarriage rate was higher in patients with OS.
Conclusions: Our study confirmed that there is a difference in the concentration of oxidative stress parameters before and after ovarian stimulation. IVF outcome is better in patients without OS after ovarian stimulation. However, the protocol of ovarian stimulation is neither associated with a change in oxidative stress parameters nor with the outcome of ART procedures.

Keywords: stimulation protocols, oxidative stress, IVF outcome

Kratak sadržaj

Uvod: Cilj studije bio je da se ispita uticaj protokola stimulacije sa GnRH agonistima i antagonistima na parametre oksidativnog stresa u serumu, kao i uticaj poremećaja parametara oksidativnog stresa na ishod IVF ciklusa.
Metode: Istraživanje je obuhvatilo 82 pacijentkinje koje su bile uključene u postupak vantelesnog oplođenja (VTO). Određivali smo sledeće markere oksidativnog stresa u serumu: aktivnost SOD, koncentraciju MDA i SH grupe. Uzorci su bili uzimani između drugog i četvrtog dana ciklusa i na dan administracije hCG u toku stimulacije.
Rezultati: Pacijenti su bili podeljeni u dve grupe zavisno od primenjenog protokola stimulacije sa GnRH agonistima i GnRH antagonistima. Prosečan broj jajnih ćelija, broj zrelih jajnih ćelija i broj fertilisanih jajnih ćelija bili su veći u grupi sa GnRH agonistima. Nije bilo značajne razlike u stopama biohemijskih trudnoća, pobačaja i stopama živorođenosti između dve grupe. Prosečne vrednosti aktivnosti SOD u serumu bile su značajno niže, dok su prosečne vrednosti koncentracija MDA i SH grupa bile značajno više posle završene ovarijalne stimulacije. Stope porođaja bile su veće kod pacijentkinja bez oksidativnog stresa, dok su stope pobačaja bile više kod pacijentkinja sa prisutnim oksidativnim stresom posle stimulacije.
Zaključak: Naša studija je potvrdila da postoji razlika u koncentracijama markera oksidativnog stresa u serumu pre i posle stimulacije, nezavisno od protokola stimulacije. Ishod IVF postupka je bolji kod pacijentkinja kod kojih oksidativni stres nije aktiviran posle ovarijalne stimulacije. Protokol stimulacije nije uticao na promenu parametara oksidativnog stresa u serumu, niti na ishod IVF postupka.

Ključne reči: protokoli stimulacije, oksidativni stres, ishod IVF

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List of abbreviations: GnRH – Gonadotropin-releasing hormone; FSH – follicle-stimulating hormone; LH – luteinizing hormone; IVF – In Vitro Fertilization; ICSI – intracytoplasmatic sperm injection; ART – Assisted Reproductive Technology.

Introduction

The outcome of ART procedures is affected by several factors including the protocol of ovarian stimulation and oxidative stress. Controlled ovarian stimulation is part of assisted reproductive procedures and several protocols are designed with the combination of GnRH agonists and antagonists and gonadotropins.

Long-acting GnRH agonists were introduced in the late 1980s to ovarian stimulation in ART to downregulate endogenous pituitary gonadotropin secretion and prevent premature LH surge during exogenous gonadotropin stimulation. In the typical »long protocol«, GnRH agonist application begins in the midluteal phase with an acute reaction and release of stored pituitary gonadotropins in the response known as »Flare effect«, and then suppresses endogenous gonadotropin secretion during the next 10 days and longer while occupying the receptor. When agonist treatment begins in the luteal phase, gonadotropin stimulation yields more follicles and oocytes, while the ovarian stimulation itself is longer (1). Contrary to the long-acting agonists, the GnRH antagonists block the GnRH receptor in a competitive, dose-dependent manner, and with no flare effect. The effect is almost immediate and the administration of GnRH antagonist is needed on a daily basis. The only purpose of antagonist use is to prevent premature LH surge. Antagonists suppress endogenous gonadotropin secretion more completely than agonists. The duration of stimulation is shorter, and the total dose of exogenous gonadotropins is decreased (2).

The choice of protocol is usually based on the clinical characteristics of the patient, which include the age, BMI, cause of infertility, the outcome of previous IVF attempts, as well as the parameters of ovarian reserve – basal FSH, estradiol, AMH and antral follicle count (AFC).

Ovarian stimulation may have a direct impact on oxidative stress markers and it is associated with the production of Reactive Oxygen Species (ROS) and perturbation of oxidant-antioxidant balance (3). Although oxidative stress has been suggested as an important factor that negatively affects the outcome of *in vitro* fertilization, there is only a small number of studies that investigated the effects of oxidative stress parameters disbalance on the outcome of ART procedures in women (4–6).

Physiological levels of ROS in the female reproductive tract play a role in folliculogenesis, ovulation (7, 8), sperm-oocyte interaction, fertilization (9), implantation and early embryo development. However, the increased production of ROS can adversely affect the microenvironment in the reproductive tract and the normal physiological process (10), which eventually affects the course and outcome of pregnancy (11).

The aim of this study was to investigate the influence of long GnRH agonist and short GnRH antagonist protocols of ovarian stimulation on the oxidative stress parameters in serum as well as the influence of change in oxidative stress parameters on IVF outcome: number of received oocytes, number of mature oocytes, embryo quality, fertilization and pregnancy rate.

Materials and Methods

Study subjects

This prospective clinical study was conducted at the Clinic of Gynecology and Obstetrics, Clinical Center of Serbia. We recruited 82 patients admitted for fertility treatment. All investigated patients agreed to participate in the study and signed an informed consent for all the undertaken procedures. The study was approved by the Ethics Committee of the Faculty of Medicine, University of Belgrade. Including criteria were: age 18–40 years, BMI from 18 to 30 kg/m^2, regular menstrual cycles from 25–32 days and without any medical disease or endometriosis stage III and IV. For all patients, age, body mass index (BMI), years of treating infertility, smoking status (smoker/nonsmoker) and the cause of infertility were determined. Infertility cause was categorized as male, tubal, ovarian, unknown or combined. The protocols of stimulation were determined individually. Patients were submitted to short GnRH antagonist protocols (n=58) and long GnRH agonist protocols (n=24), depending on age, ovarian reserve and previous IVF cycle.

Controlled ovarian stimulation protocols

Patients on a long agonist protocol received Triptoreline (Diphereline, Ipsen Pharma Biotech, France) at a dose of 0.1 mg daily starting in the midluteal phase of the previous cycle. After suppression of the pituitary, recombinant FSH – follitropin α (Gonal-F, Serono, Switzerland) was commenced at a dose of 150–300 IU/day. Patients on a short GnRH antagonist protocol received the same gonadotropin stimulation starting on cycle day two or three, after the proper ultrasound findings and basal hormone levels. GnRH Antagonists – Cetrorelix (Cetrotide) at a dose of up to 0.25 mg per day were added when the leading follicle reached a diameter of 14 mm and were administered by the day of HCG.

When there was a consistent rise in the concentration of estradiol followed by the presence of two or more follicles > 18 mm, human chorionic gonadotropin (Pregnyl, Organon, the Netherlands) was administered at a dose of 5000 IU, 34–36 hours before oocyte retrieval. Methods of insemination were IVF, ICSI, or a combined method. In assessing the quality of embryos, the Istanbul consensus clinical embryologists criteria were used as the reference

frame (12). Embryo transfer was performed transcervically on day 2 or 3 after the oocyte retrieval, controlled by transabdominal ultrasonography. Patients received Utrogestan (micronized progesterone) at a dose of 200 mg, three times daily, for luteal phase support, and continued until the 12th week of gestation. Pregnancy was diagnosed by positive serum β-hCG and then confirmed as clinical pregnancy by ultrasound findings: gestational sac with a vital embryo and the 6-week gestation. Finally, we registered whether patients had a term delivery or a miscarriage.

Sample collection and determination of oxidative stress parameters

Serum levels of estradiol (E2 – pg/mL), progesterone (PROG – ng/mL), follicle-stimulating hormone (FSH – mIU/mL), luteinizing hormone (LH – mIU/mL) and anti-Mullerian hormone (AMH – ng/L) were measured between the 2nd and 4th cycle day prior to stimulation commencement (basal levels). Blood samples were taken by Vacutainer tubes (BD Vacutainer Systems) and centrifuged according to the manufacturer's instructions for the preparation of serum samples. AMH value (Gen II ELISA ref. No. A79765; Beckman Coulter) in serum was measured by ELISA (enzyme-linked immunosorbent assay, 1 ng/mL). FSH, LH, estradiol and progesterone were analyzed by chemiluminescent immunoassay (Access 2 immunoassay system, Beckman Coulter) (13, 14).

Serum samples for oxidative stress parameters were obtained from each patient when the basal hormonal status was determined. The second time point when serum samples were obtained was on the day of HCG administration. The following parameters of oxidative stress were determined in serum: activity of SOD and concentrations of MDA and SH groups.

After separation, the serum/plasma was frozen and stored at a temperature of –70 °C. After incomplete defrosting, by a quick procedure on ice (0–4 °C), preparation of the serum for the analysis was carried out by homogenization and centrifugation. After preparing a serum sample, OS parameters, malondialdehyde (MDA), the activity of total superoxide dismutase (SOD) and the concentration of total sulfhydryl (SH) groups were determined. Proteins were determined by the Bradford method, based on colors for the binding of a protein molecule, wherein there is a shift of the absorption maximum, as compared to the color of the absorption maximum of the free form (15). Determination of MDA in serum was based on the reaction of MDA with thiobarbituratic acid (TBA) in an acidic medium for 15 minutes at 95 °C in a water bath. The intensity of light yellow to violet was measured at wavelengths of 523 nm and 600 nm, in order to remove interference. The activity of superoxide dismutase (SOD, EC 1.15.1.1.) in the serum was determined by the method of Misra and Fridovich, at a wavelength of 480 nm, by a kinetic method in an alkaline environment. The total concentration of SH groups was determined by Ellman's method (16). The absorbance of the yellow color was measured at 412 nm, and was based on the reaction with 2,2-dinitro-5,5-dithio-benzoic acid (DTNB) with an aliphatic thiol compound in an alkaline medium (pH = 9.0), thereby forming a p-nitrophenyl anion.

Statistical analysis

For statistical analysis of the obtained data, the Statistical Package for the Social Sciences (SPSS) 22 was used and differences were considered statistically significant at a probability level less than 0.05 for all tests. Results were presented as arithmetic mean ± standard deviation for variables with a normal distribution and as median and interquartile range for variables whose distribution was not normal. Categorical variables are presented as relative or absolute frequency. Testing of distribution was carried out by Kolmogorov-Smirnov analysis. Comparison of the mean values of independent groups of data was performed by Student t-test and ANOVA analysis with Tukey's *post hoc* test for differences between subgroups. For parameters without normal distribution, test of significance between groups was performed using the Mann Whitney test or Kruskal-Wallis test with *post hoc* Mann Whitney test. Comparison of two dependent populations was performed by Wilcoxon signed-rank test for data without normal distribution. Analysis of categorical values was performed using the Chi-square test.

Results

The general characteristics and clinical data of the study population are given in *Table I*. There is a difference in age between GnRH antagonist (GnRHant) and GnRH agonist (GnRHa) protocols (35.5 vs. 32.2; p=0.002). In the group with GnRHant protocol, AMH values were significantly lower compared to the group with GnRHa (0.95 vs. 2.3; p=0.014), while FSH values were higher (7.87 vs. 6.65; p=0.041). However, basal values of LH, estradiol and progesterone were similar in both groups. Patients with a GnRH agonist protocol had a significantly higher number of oocytes (12 vs. 4; p<0.001) as well as of mature oocytes (9 vs. 4; p<0.001). Similarly, the number of fertilized oocytes was higher in the GnRHa group (5 vs. 2; p=0.009). However, fertilization rate was similar in both groups (p=0.398). The most frequent infertility cause was male in the GnRHa protocol (40%) while in the GnRHant protocol it was ovarian (32%). There was no significant difference in the cause of infertility among patients with different protcols of ovarian stimulation (p=0.079; χ^2 test).

Table I General characteristics, basal hormonal status, number and quality of oocytes, number of fertilized oocytes and fertilization rate of the study population.

Parameters	GnRH antagonists	GnRH agonists	*P*
Age	35.5±4.0*	32.2±2.9	0.002a
BMI, kg/m^2	22.13±2.52	22.21±3.52	0.592a
Smoker, %	28.8	20.0	0.587
Infertility, years	5 (4–6)	4 (3–5)	0.250b
FSH, mIU/mL	7.87±2.69	6.65±1.84	0.041a
AMH, ng/mL	0.95 (0.42–2.79)*	2.30 (1.28–4.08)	0.014b
E2, pg/mL	44.88±20.95	42.9±18.13	0.755a
LH, mIU/mL	5.18 ±1.95	4.23±0.95	0.252a
P4, ng/mL	1.26±0.46	1.1±0.36	0.725a
GT dose, IU	2209.25±577.61	2354.78±641.68	0.625a
Oocyte No	4 (1–10)*	12(6–14)	<0.001b
Mature oocyte No	4 (2–8) *	9 (5–12)	0.001b
No of fertilized oocytes	2 (1–5)*	5 (3–8)	0.009b
Fertilization rate, %	50.00 (36.4–75.0)	47.9 (24.1–78.16)	0.398b

Arithmetic mean values ± SD for normally distributed variables or median (inter-quartile range) for variables that do not have a normal distribution are shown. According to a ANOVA test; according to b Kruskal-Wallis test. * Significant difference compared to the long protocol.

Table II Comparison of serum concentrations of OS parameters before and after ovarian stimulation in the group with GnRH antagonist and the agonist group.

Parameters	Before stimulation	After stimulation	*P*
GnRH antagonists			
SOD, U/L	17.56 (15.57–19.61)	14.5 (12.97–16.03)	<0.001
MDA, μmol/L	1.40 (1.29–1.50)	1.71 (1.49–1.93)	<0.001
SH groups, mmol/L	0.24 (0.19–0.29)	0.47 (0.34–0.57)	<0.001
GnRH agonists			
SOD, U/L	17.37 (16.58–19.99)	14.08 (12.59–15.22)	<0.001
MDA, μmol/L	1.38 (1.26–1.46)	1.78 (1.45–1.96)	0.003
SH groups, mmol/L	0.24 (0.17– 0.32)	0.51 (0.42–0.57)	<0.001

The median (25th and 75th percentile) values are shown. The comparison was made using Wilcoxon signed-rank test (comparison of two dependent populations).

Table III Comparison of serum concentrations of OS parameters after ovarian stimulation between GnRH antagonist group and GnRH agonist group.

Parameters	GnRH antagonists	GnRH agonists	P
SOD, U/L	14.5 (12.97–16.03)	14.08 (12.59–15.22)	0.289
MDA, μmol/L	1.71 (1.49–1.93)	1.78 (1.45–1.96)	0.561
SH groups, mmol/L	0.47 (0.34–0.57)	0.51 (0.42–0.57)	0.207

The median (25th and 75th percentile) values are shown. Comparison of values after stimulation was performed by Kruskal Wallis test.

Table IV Pregnancy outcome in patients with and without OS after ovarian stimulation.

Pregnancy outcome	Without OS	With OS	P
Delivery rate %	81.8	68.2	0.347
Miscarriage rate %	0	31.8	0.040
Biochemical pregnancy %	18.2	0	0.104

The embryo quality was evaluated in women with different stimulation protocols. Independently of the stimulation protocol, embryos class A prevailed in women with 44% of women stimulated by short protocols and 57% of women stimulated by long protocols. The quality of the embryo in relation to this criterion was not significantly different between the groups ($p=0.684$; χ^2 test).

Concerning the outcome of IVF, there were no significant differences in live–birth rate in both groups ($p=0.828$; χ^2 test). Miscarriage rate ($p=0.894$) as well as the rate of biochemical pregnancies ($p=0.449$) were similar in both groups. The effect of different protocols of ovarian stimulation on oxidative stress parameters in serum was shown in *Table II*. In 40 women with short GnRH antagonist protocols SOD activity was significantly lower while MDA and SH-groups levels were higher after ovarian stimulation. In long agonist protocols the results were similar. In GnRHant group, mean serum SOD was significantly lower (17.56 vs. 14.5 U/L, $p<0.001$), while mean serum MDA value (1.40 vs. 1.71 micromole/L, $p<0.001$) and SH-groups content were significantly higher (0.24 vs. 0.47 mmol/L, $p<0.001$) after ovarian stimulation (*Table II*). In GnRHa group the results were similar, the mean serum SOD was significantly lower (17.37 vs. 14.08 U/L, $p<0.001$), while mean serum MDA (1.38 vs. 1.78 μmol/L, $p=0.003$) and SH groups (0.24 vs. 0.51 mmol/L, $p<0.001$) were significantly higher after ovarian stimulation (*Table II*). We compared differences in oxidative stress parameters after ovarian stimulation between GnRHant and GnRHa groups. There were no significant differences in concentrations of SOD (14.5 vs. 14.08 U/L, $p=0.289$), MDA (1.71 vs. 1.78 μmol/L, $p=0.561$) and SH groups (0.47 vs. 0.51 mmol/L, $p=0.207$) between the two groups after ovarian stimulation (*Table III*).

As there was a difference in the oxidative stress parameters after ovarian stimulation, we examined its influence on the IVF outcome. All patients with SOD activity less than the 25th percentile after and MDA and SH concentrations higher than the 75th percentile after ovarian stimulation were in the group with OS after ovarian stimulation (62.7%). Other patients were in the group without induced OS after ovarian stimulation ($p<0.021$; χ^2 test). There was no difference in the number and quality of oocytes, embryo quality, fertilization and pregnancy rate between patients with and without OS after ovarian stimulation. However, patients without oxidative stress after ovarian stimulation had a delivery rate of 82% and no miscarriage, while patients with OS had a delivery rate of 68% ($p=0.347$) and a miscarriage rate of 31.8% ($p=0.040$) (*Table IV*).

Discussion

The success of ART procedures depends on several factors, including the protocol of controlled ovarian stimulation applied. Which protocol will be applied depends on several factors, among which are: age, basal hormonal status, number of antral follicles, previous IVF and others.

Stimulation protocols with GnRH antagonists have a number of advantages in comparison to protocols with GnRH agonists, such as the significantly shorter duration of treatment and the lower total dose of gonadotropin used in stimulation (17, 18). Another advantage is a lower risk of developing the ovarian hyperstimulation syndrome (19). However, the impact on the outcome of IVF and pregnancy rates is controversial (20, 21). Therefore, we investigated the impact of different stimulation protocols on the outcome of IVF. In our study, stimulation protocols were determined individually. The results showed that the fertilization rate, quality of embryos as well as pregnancy and live–birth rates were similar in GnRH antagonist and GnRH agonist protocols. This finding is consistent with the meta-analysis that included five randomized trials comparing the GnRHa and GnRGant GnRH protocols and showed similar rates of implantation and pregnancies per cycle in both protocols (22). Other studies had similar results (19, 20). It was also shown that there was no detrimental effect on the embryos when large doses of GnRH antagonists were administrated and no differences in delivery rates in GnRH agonist as compared to GnRH antagonist cycles (21). However, the number of mature oocytes as well as the number of fertilized oocytes were higher in the GnRHa group, which could be explained by the higher number of obtained oocytes and younger age.

Values of basal FSH (24) and E2 (23) are widely used as a prognostic test for ovarian reserve. The first study to link the value of FSH and E2 with the outcome of assisted reproduction suggested that the measurement of basal values of estradiol and FSH may provide a more accurate prediction of the reproductive potential than individual FSH levels combined with age (25). Also, it has been demonstrated that elevated estradiol values in the early follicular phase have a worse prognosis, with a higher rate of cycle cancelation and lower pregnancy rate after IVF, regardless of FSH levels (26).

The development of immunoassays for protein hormones such as AMH has led to its growing use in the prediction of ovarian reserve (27). AMH is a dimeric glycoprotein produced by the granulosa cells of preantral and small antral follicles. The size of small antral follicle pool is related to the size of the pool of primordial follicles which is the true measure of ovarian reserve. The small antral follicle pool number decreases with age; AMH production diminishes and it is undetectable in menopause. Unlike other biochemical markers, it can be measured at any day of the cycle (28). It is a marker of ovarian reserve and the predictor of quantitative reaction to exogenous ovarian stimulation in ART procedures. The various thresholds of 0.2–1.26 ng/mL are markers of poor ovarian response with 80–87% sensitivity and 64–93% specificity. However, values > 5.0 ng/mL are related to the polycystic ovary syndrome (PCOS). Serum levels of AMH are not good predictors for pregnancy (29, 30). In assisted reproductive cycles, women with a poor response had lower values of AMH and a smaller number of antral follicles before the start of ovarian stimulation than women with a good response to exogenous gonadotropins (31).

A higher level of FSH in the follicular phase is associated with poor ovarian response (32), while a higher value of AMH is associated with good ovarian response (33). Concerning the basal hormonal status, statistically significant differences in the values of FSH ($p=0.044$) and AMH ($p<0.007$) were observed, which contribute to the number of obtained oocytes. AMH values were significantly lower in the GnRHant group, while FSH values were significantly higher. These data suggest a decreased ovarian reserve as the possible common cause of infertility in this group, and it has been shown that in 32.2% of cases it was a disorder of ovarian function, which in our work was the most common cause of infertility in this group. However, higher values of AMH in the GnRH agonist group can be explained by the younger patients submitted to long protocols. Still, a lower number of mature ooytes in the GnRH antagonist group needs further assessment.

In combination with genetic predisposition and lifestyle habits, the outcome of IVF can be significantly altered by stress and natural defense mechanisms necessary to overcome it (34). Different studies investigated the correlation between administration of gonadotropins and IVF outcome (35). Controlled ovarian stimulation can lead to disruption of oxidative-antioxidative balance. In our study, we showed that ovarian stimulation in IVF induces oxidative stress, which can be detected in serum. We also showed that patients without OS after ovarian stimulation had a better IVF outcome. When we compared the effects of different stimulation protocols on the parameters of oxidative status, SOD activity was decreased after stimulation, while the content of MDA and SH groups were increased after stimulation. However, the value of SOD, MDA and SH groups did not differ significantly among patients with different stimulation protocols. Similar results were reported by Aurrekoerxea et al. (3). Other studies have also shown that ovarian stimulation has an impact on the activity of SOD, GPx and IL-6 level (36). Oral et al. (37) found that the levels of MDA were lower in the group that was pregnant, suggesting that the MDA level could be used as a predictive marker of the success of IVF. Pallini et al. (38) found no difference in the values of antioxidants concentration after suppression of the pituitary gland when comparing with the values before treatment. However, it has been shown that stimulation leads to a decrease in antioxidant concentrations in plasma and reduction of protection against oxidation in the serum, when the concentration of antioxidants in serum after gonadotropin stimulation is compared in women undergoing IVF with that in women in the follicular phase of the natural cycle (3). The reduction of antioxidants after ovarian stimulation is consistent with the results obtained in our study and may be due to the administration of GT.

In summary, ovarian stimulation causes a difference in SOD activity, MDA and SH groups concentrations in serum in both GnRHa and GnRHant protocols, but the change in oxidative stress parameters is similar in both protocols. Protocol of stimulation does not affect IVF outcome; namely, the quality of oocytes and embryos, fertilization, pregnancy and miscarriage rates are similar in both protocols. However, the change in oxidative stress parameters affects IVF outcome; patients with induced oxidative stress after ovarian stimulation had a lower delivery rate and a higher miscarriage rate compared to patients with normal oxidative status.

Conflict of interest statement

The authors stated that they have no conflicts of interest regarding the publication of this article.

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