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The protective effect of cilostazol on experimental ischemia/reperfusion injury in rats ovaries on in vitro fertilization outcomes

Silostazolün sıçan yumurtalığında deneysel iskemi/reperfüzyon hasarına karşı in vitro fertilizasyon sonuçlarına koruyucu etkisi

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Abstract

Objective: Ovarian torsion decreases ovarian reserve because of ischemic and reperfusion damage it causes. In this study, we investigated the protective effect of cilostazol (CIL) on experimental ischemia (I) and ischemic-reperfusion (I/R) damage in rat ovaries with in vitro fertilization (IVF) results.

Materials and Methods: Forty-eight adult female Sprague-Dawley albino rats were randomly assigned to 6 groups with 8 animals in each group: Sham (S), I, I/R, S + CIL, I + CIL and I/R + CIL. The I groups were subjected to bilateral adnexal torsion for 3 h, while the I/R and I/R + CIL groups received subsequent detorsion for 3 h. Twenty-two mg/kg of CIL was given via oral gavage 30 min before surgery on the I (I+ CIL) or reperfusion (I/R + CIL) groups. Oocytes were collected before the IVF procedure and after ovulation induction with 150-300 IU/kg pregnant mare serum gonadotropin.

Results: The metaphase oocytes reached their highest value of 4.73±0.96 in the S+ CIL group and reached their lowest value of 0.51±0.55 in the I/R group. There were statistically significant differences in the number of second-day embryos among the I, I+ CIL, and I/R and I/R+ CIL groups (p=0.000). When the groups were compared in terms of Anti-Müllerian hormone change, the highest decrease was observed in the I and I/R groups.

Conclusion: CIL pretreatment before surgery has a protective effect against I and I/R in rats with ovarian torsion.

Keywords: Reperfusion injuries, ovarian torsion, in vitro fertilization, cilostazol

Öz

Amaç: Over torsiyonu, neden olduğu iskemik ve reperfüzyon hasarı sonucunda over rezervinde azalmaya neden olur. Bu çalışmada, rat overlerinde oluşturulan deneysel iskemi (I) ve iskemik-reperfüzyon (I/R) hasarına karşı silostazolün (CIL) koruyucu etkisinin in vitro fertilizasyon (IVF) sonuçları ile araştırılması amaçlanmıştır.

Gereç ve Yöntemler: Kırk sekiz yetişkin dişi Sprague-Dawley albino ratı, her grupta 8 hayvan bulunan 6 gruba rastgele atandı: Sham (S), I, I/R, S + CIL, I + CIL ve I/R + CIL. I grupları 3 saat boyunca bilateral adneksiyal torsiyona maruz kalırken, I/R ve I/R + CIL grupları 3 saat boyunca detorsiyon alındı. I (I + CIL) veya reperfüzyon (I / R + CIL) gruplarında ameliyattan 30 dakika önce oral gavage yoluyla 22 mg/kg CIL verildi. IVF işlemi öncesi ile 150-300 IU/kg gebe kısrak serum gonadotropin ile ovülasyon indüksiyonu yapıldıktan sonra oositler toplandı.

PRECIS: The use of cilastazole has a protective effect against ovarian torsion, which is one of the important causes of infertility by causing a decrease in ovarian reserve.

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Bulgular: Metafaz oositleri S+ CIL grubunda 4,73±0,96 ile en düşük değerlerine ulaşırken I/R grubunda 0,51±0,55 ile en düşük değerlerine ulaştılar. I, I+ CIL ve I/R ve I/R+ CIL grupları arasında ikinci gün embriyo sayısında istatistiksel olarak anlamlı farklılıklar vardı (p=0,000). Gruplar kendi içlerinde Anti-Müllerian hormon değişimi açısından karşılaştırıldığında, en yüksek düşüşün I ve I/R gruplarında olduğu gözlenmiştir.

Sonuç: Over torsiyonunda, ratlarda cerrahi uygulama öncesi CIL ön tedavisinin I ve I/R'ye karşı koruyucu etkisi vardır.

Anahtar Kelimeler: Reperfüzyon hasarları, over torsiyonları, in vitro fertilizasyon, silostazol

Introduction

Adnexal torsion is defined as twisting the ovary on its own or with the fallopian tube in the axis between the infundibulopelvic ligament and the utero-ovarian ligament[1]. Ovarian torsion, which is an important gynecological emergency, is most common in the reproductive years, with annual prevalence of approximately 2-6%[2]. Although there are many risk factors for this condition, the most common ones are ovulation induction, ovarian cysts, endometriosis, and hyperlaxity of the ovarian ligaments[3]. If not diagnosed and treated promptly, ovarian torsion causes ovarian necrosis and irreversible damage to the tissue. The duration of torsion is significant for tissue damage (loss of ovarian function) and the resulting decrease in infertility. While I injury is a possible cause of tissue damage because of torsion, post-detorsion ischemic-reperfusion (I/R) injury also contributes to tissue damage due to overproduction of reactive oxygen species (ROS)[4].

Cilostazol (CIL) is a phosphodiesterase III inhibitor that increases cyclic adenosine monophosphate levels, leading to protective effects, antioxidant activity and anti-apoptosis effects in endothelial cells[5]. It has been shown that CIL reduces the damage caused by I and R in various tissues such as the myocardium and kidney[6,7].

When the literature is evaluated, it is seen that there are studies with histopathological or biochemical markers evaluating the protective effects of many agents in preventing I- and I/R-related damage in the ovarian tissue because of torsion[8-10]. It is seen that these studies give an idea about the future results of adnexal torsion on variables such as ovarian morphology and follicular numbers, and they do not provide sufficient information about the expected follicular development in the future, the number of oocytes that can be obtained and the embryo formed after fertilization.

Therefore, in this study, which is supported by the comparison of serum Anti-Müllerian hormone (AMH) levels, which is a reliable ovarian reserve test method, it was planned to obtain oocytes after ovulation induction in rats, to evaluate fertilization and embryo quality by in vitro fertilization (IVF).

In this experimental study, it was aimed to evaluate the protective effect of CIL on the formation of I and I/R damage in the ovaries by using IVF results and serum AMH levels.

Materials and Methods

Ethics and Animals

The study was conducted out in the Sakarya University SUDETAM laboratory with the authorization and approval of the Experimental Animals Ethics Committee of Sakarya University, based on the European Commission Directive 86/609/ECC guide protocol, with the decision dated 05/05/2021 and numbered 27.

The study comprised 48 virgin Sprague-Dawley albino rats (weighing from 220 to 260 g). For one week before the study, the animals were maintained under appropriate humidity (50±5%) and heat regulation (22±2 °C) over a 12-hour light/dark cycle.

Surgical Procedures and Experimental Protocol

The rats were randomly divided into six groups of 8 animals each: S operation, I (3 h), I/R (3 h ischemia plus 3 h reperfusion), S, S + CIL, I + CIL and I/R + CIL (3 h ischemia and 3 h reperfusion). Rats in the CIL groups (S + CIL, I + C, I/R + CIL) were given 12 mg/kg of cilostazol via oral gavage 30 min before surgery. A blood sample (AMH1) was drawn from each rat to measure the serum AMH level before operations began.

Before surgery, rats were anesthetized with ketamine hydrochloride (60 mg/kg of Ketalar; Eczacibasi, Istanbul, Turkey) and xylazine hydrochloride (7 mg/kg of Rompun; Bayer Türk Ilaç Ltd., Istanbul, Turkey) under sterile conditions. During surgery, the rats were covered with a sterile drape in the dorsal recumbent position. Uterine horns and adnexa were observed after entering the abdomen by making a longitudinal 2 cm long midline incision in the lower abdomen of the rats. In the S group, the abdominal folds were closed again with 3/0 silk sutures after 2 min of observation. In the ischemia group, the ovarian pedicles on both sides were rotated 360 ° clockwise and fixed to the abdominal wall with 5/0 silk sutures, and the abdominal folds were closed with 3/0 silk sutures. In the I/R group, after the above-mentioned 3-hour ischemia period, after the abdominal layers were reopened, bilateral adnexal detorsion was performed by removing the torsion sutures. In the S + CIL group, the S operation was performed as described. In the I + CIL group, adnexal torsion was performed. In the I/R + CIL group, sequential bilateral adnexal torsion and detorsion were performed.

Ovulation induction was performed in rats that had undergone at least three consecutive estrous cycles as determined by daily vaginal smear. All rats with ovulation induction were sacrificed to collect oocytes. To compare meiotic progression in classifying oocytes according to germinal vesicle (GV), metaphase I (MI), and metaphase (MII) stages, the mean time for each stage of nuclear progression Sirard et al.[11] the previously described method was used.

HTF (Human tubal fluid) medium (Cat. no. 90166, Irvine Scientific, USA) was used for sperm preincubation, fertilization

and embryo transfer. Embryos were washed by passing through a 35-mm culture dish (Nunc, Cat. No.63754, Denmark) covered with liquid paraffin oil (Cat. No. 9305, Irvine Scientific, USA), and maintained at 37 °C under 5% CO_2 in humidified air overnight.

Following administration of 150 to 300 µIU/kg [Chronogest/ pregnant mare serum gonadotropin (PMSG), Intervet, Istanbul, Turkey] using an intraperitoneal (i.p.) injection as an ovulation stimulation protocol, 150-300 µIU/kg human chorionic gonadotropin after approximately 48 h; (Gonatropin, Chorulon® Intervet, Istanbul, Turkey) was applied. PMSG was administered at a dose of 15 IU 17 to 19 h after administration[12]. Before anesthesia, all rats were weighed, and intramuscular administered of 50 µmg/kg ketamine hydrochloride (Ketalar; Eczacıbaşı Warner Lambert İlaç Sanayi, Levent, Istanbul, Turkey) and 7 µmg/kg xylazine hydrochloride (Rompun, Bayer Şişli, Istanbul, Turkey) were used for the procedure.

After the anesthetized rats were placed in suitable conditions, second blood samples were collected to measure the serum AMH2 level. After the ovaries were properly dissected and removed, oocytes were collected. Oocytes were cultured for one day before being placed in an incubator in HTF medium supplemented with human serum albumin 4 mg/mL, 37 °C, and 5% CO_2. After transferring oocytes and capacitive sperm (approximately 1x106 mL-1) to fertilization droplets, fertilization was controlled and the resulting embryos were followed up to the two-cell stage[12].

A male rat was sacrificed with appropriate anesthesia ketamine hydrochloride (60 mg/kg Ketalar; Eczacıbaşı, Istanbul, Turkey) and xylazine hydrochloride (7 mg/kg Rompun; Bayer Türk Ilaç Ltd., Istanbul, Turkey) just before oocyte retrieval. A vertical incision was made in the abdomen of this rat and the abdomen was entered and the male reproductive system was observed. The bilateral epididymis was separated from the testicles with appropriate dissection and transferred to HTF medium. The obtained epididymis was stripped by appropriate dissection and the obtained sperm was transferred to Petri dishes and incubated at 37 °C for 30 min before IVF procedure[13].

The collected oocytes were cultured as described above after washing three times with HTF medium before insemination. Seven to eight hours after insemination of oocytes, evaluation for sperm penetration or pronuclear formation was performed under an inverted microscope to identify polyspermic fertilization or parthenogenetic embryos (approximately 6.5% of total). Following this process, the culture was continued for another 20 h and the embryos at the 2 cell stage were counted[14].

Enzyme-linked immunosorbent assay (ELISA) was used in the evaluation of serum concentrations of AMH in accordance with the application guidelines of the manufacturer (BT LAB Biotech Co. Ltd., Shanghai Cat. No: E0456Ra). The sensitivity of the kit used in the study for the AMH value was reported as 0.1 ng/mL to 40 ng/mL by the company.

Statistical Analysis

The Kolmogorov-Smirnov test was used for the normal distribution of the data, while the Kruskal-Wallis test was used to compare more than two variables that did not show normal distribution. Mann-Whitney U test was used for pairwise comparisons between groups for differing parameters. Since AMH values showed a normal distribution, dependent groups were compared using the paired sample test. After all, results were evaluated as mean ± standard deviation, the results with a $p<0.05$ value were considered statistically significant.

Results

Total Oocyte Count

The number of oocytes collected from the rats showed statistically significant differences between the groups (p=0.000). The group with the highest oocyte collection, with an average of 8.75±1.35, was the S group, while the lowest number of oocytes was seen in the I/R group with 2.37±1.32. When the groups were compared after cilostazol application, the number of oocytes collected did not differ between the S and S+CIL groups (p>0.05).

However, it was observed that the number of oocytes collected in the I and I+CIL (p=0.021) and I/R and I/R+CIL (p=0.003) groups increased with the effect of cilostazol (Figure 1), so cilostazol increased the number of oocytes collected.

MII, MI and GV Oocyte Counts

Statistically significant differences in MII, MI and GV oocytes were also seen between the groups (p=0.000 for all three parameters). It was observed that cilostazol administration did not create a statistically significant difference between the S and S+CIL groups for MII oocyte counts (p>0.05). It was observed that cilostazol administration had a positive effect on the increase of MII oocyte numbers in groups I and I/R. Statistically significant differences were observed between I and I+CIL (p=0.001) and I/R and I/R+CIL (p=0.000) groups. It was also observed that CIL application did not create a difference between the study groups in terms of M1 oocyte numbers. It was seen that the number of GVs collected decreased significantly after CIL application. There was a statistically significant difference between the S and S+CIL (p=0.011), the I and I+CIL (p=0.003), and the I/R and I/R+CIL (p=0.036) groups (Figure 1).

Second Day Embryo Counts

When the groups were compared in terms of the number of embryos obtained on the second day, statistically significant differences were found (p=0.000). While there was no statistically significant difference in the number of embryos obtained on the second day between the S and S+CIL groups (p>0.05), it was observed that the number of embryos on the second day was higher in the I+CIL and I/R+CIL groups compared to the groups without CIL. When the groups were compared among

themselves, there was a statistically significant difference in p values between I and I+CIL groups, with p=0.001, p=0.000 between I/R and I/R+CIL groups (Figure 1).

AMH Concentrations

There was no statistically significant difference in AMH1 concentrations between the study groups (p>0.05). To compare the effects of CIL application on AMH, the correlation between AMH values of S+CIL, I+CIL and I/R+CIL groups were examined. For this, the paired-samples t-test was applied for the correlation of binary groups. It was observed that CIL application had no effect on the AMH1 and AMH2 values of the S+CIL group (p>0.05), while it was observed that CIL application had positive effects on the AMH concentration in the I+CIL and I/R+CIL groups. A high degree of correlation was observed when comparing AMH values in the I+CIL and I/R+CIL groups (p=0.000 for both groups) (Figure 2).

Discussion

In this study, the aim was to evaluate the efficacy of cilostazol and detorsion in preserving ovarian reserves and structure. Pretreatment with CIL is effective in preserving ovarian reserves after post-torsion injury. This is the first study to evaluate IVF outcomes in predicting ovarian reserve in rat ovaries and provide evidence of the protective effect of CIL on I and I/R injury. To apply a detorsion procedure to preserve ovarian reserves in cases of torsion. Misdiagnosis or delay in treatment in these patients affects fertility in the long term by causing serious losses in the ovarian reserve.

Various physiopathological mechanisms explain the causes of tissue damage due to ovarian torsion and detorsion[15,16]. I/R injury usually occurs due of increased ROS production due to activated complement proteins and other inflammatory components around the inflammation site[17]. It was observed

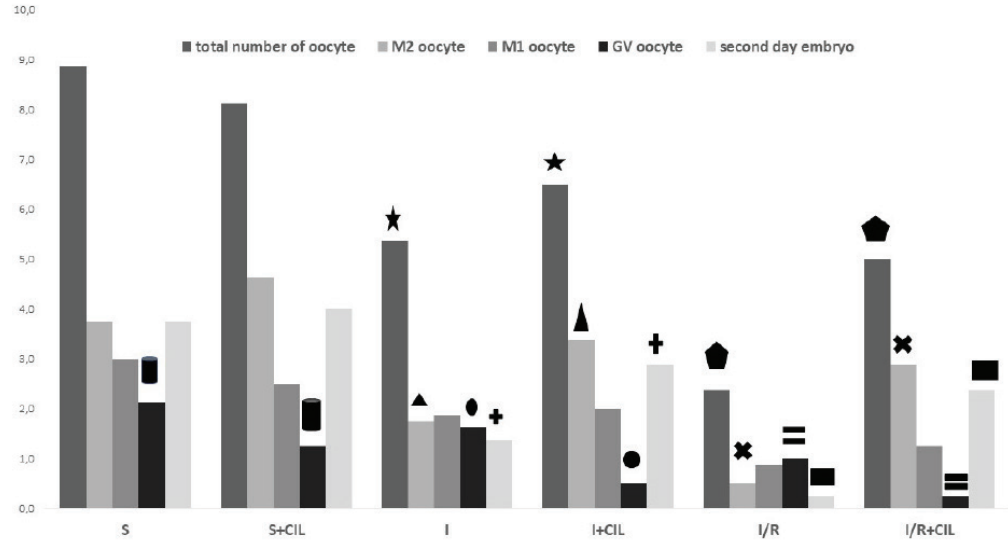

Figure 1. A comparison of the study groups' total oocyte count, germinal vesicle (GV), metaphase I (MI), metaphase II (MII) oocyte counts, and second-day embryo counts. Statistical analysis between all groups was performed with a Kruskal-Wallis test. Pairwise comparisons were made with the Mann-Whitney test. The black cylinder indicates the statistically significant difference between the sham (S) and S+ (cilostazol) CIL groups. There was a statistically significant difference between the black star, triangle, circle and plus sign ischemia (I) and I+CIL groups. There was a statistically significant difference between the black pentegon, cross, equalitiy and rectangle ischemia reperfusion (I/R) and I/R+CIL groups

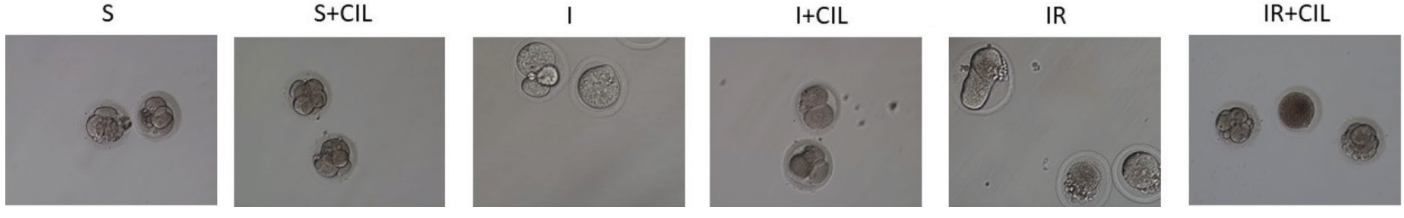

Figure 2. Comparison of second day embryos between groups. Second-day embryos of the groups are seen at 100x magnification. The second-day embryo counts and embryo quality in the sham (S) and S+ cilostazol (CIL) groups were quite good compared to the other groups. It is seen that the quality and number of embryos on the second day are significantly better in the Ischemia (I) + CIL and ischemia reperfusion (I/R) + CIL groups compared to the I and I/R groups, respectively

that ovarian injury was more severe in the detorsion group than in the torsion group, which followed reports in published literature[8,18]. For this reason, it is important not only to prevent I in cases of ovarian torsion but also to prevent I/R damage that will occur because of detorsion applied in the treatment.

CIL, a phosphodiesterase inhibitor that suppresses platelet aggregation and has a vasodilator effect, plays an important role in modulating the oxidant-antioxidant system to reduce I/R damage[19]. Because of these properties, CIL are widely used in treating chronic peripheral artery disease and for treating ischemic coronary artery disease[20]. CIL pretreatment significantly reduces IL-6 levels in long-term use, reducing lipid peroxidation and ROS in I/R injuries[21].

CIL reduces drug-induced nephrotoxicity with antioxidant and anti-apoptotic activities[19]. CIL effectively repairs tissue damage caused by I/R and reduces oxidant stress in heart tissue[22]. Experimental studies have demonstrated that CIL produced a protective effect against I injury in animal models when used for skeletal muscle[6,23].

In published literature, it has been shown with histopathological and serum biochemical markers that many treatment agents have a protective effect against I and I/R in cases of ovarian torsion[8,24,25]. In terms of ovarian reserve, it is seen that studies are evaluating the number of different types of follicles that affect ovarian reserves[26]. These results cannot give a clear idea about the number of oocytes that can be obtained in the later reproductive period and the number and quality of embryos formed after fertilization of these oocytes. Additionally, these results do not fully reflect the long-term effectiveness of the protective effect of these treatment agents in terms of reproductive health.

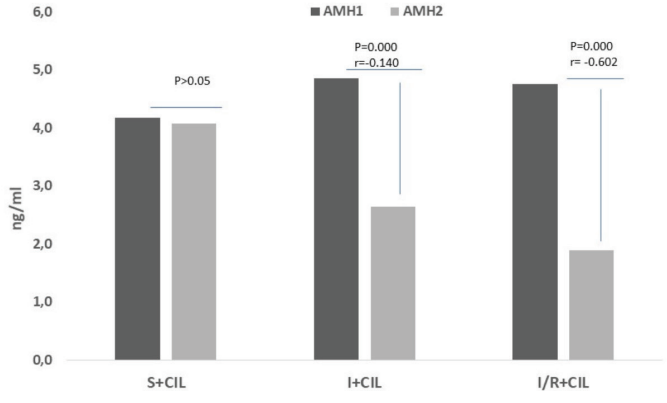

Figure 3. Comparison of Anti-Müllerian hormone (AMH) levels after cilostazol administration between groups via an AMH1 and AMH2 correlation plot between the sham (S)+ cilostazol (CIL), Ischemia (I) +CIL and Ischemia reperfusion (I/R) +CIL groups (mean and 95% confidence interval). There was no correlation between AMH1 and AMH2 values in the S+CIL group (P>0.05). A high level of correlation was observed between AMH1 and AMH2 values in the I+SI and I/R+SI groups. Analysis was done with a paired sample test. P<0.05 was considered statistically significant

In this study, while the results obtained with ovulation induction in the cycle after the treatment process show the long-term effects of the protective effect on the follicles constituting the ovarian reserve more significantly, the evaluation of the fertilization of the obtained oocytes and the comparison of the obtained two-cell embryo numbers will be important in estimating the clinical results.

In this study, the total number of oocytes obtained in the I and I/R groups that underwent CIL, and especially the MII oocyte counts, were higher than those in the I and I/R groups (Figure 1). While these results showed the protective effect of CIL on the follicles, similar results were obtained in the number of two-cell embryos, and the high number of embryos after fertilization suggests a protective effect that also affects the oocyte quality (Figure 1, 2). When the groups were evaluated in terms of total oocyte counts, the protective effect of CIL was seen in the I+CIL and I/R+CIL groups compared with the I and I/R groups. Since the results of embryos obtained by ovulation induction and subsequent IVF were evaluated in this study, the main limitation of the study is that the natural cycle results are not known.

To support IVF results, AMH levels, a current and reliable marker of ovarian reserve, were measured and compared in the preoperative and postoperative periods in our study. Supporting the difference in oocyte numbers, the decrease in AMH values was significantly higher in the I and I/R groups compared with the I+CIL and I/R+CIL groups (Figure 3).

Study Limitations

Other limitations of the study are that it is not a human study and there are no results about embryo implantation, the pregnancy rate obtained and live birth after pregnancy, which are other parameters that provide information about reproductive status. We think that this study is the first experimental rat study in which the effects of CIL on ovarian torsion and detorsion-related damage were investigated by evaluating IVF results, and it showed that it was effective in reducing ischemic and detorsion-induced ovarian damage.

Conclusion

In a rat ovarian torsion model of ovarian ischemia, cilostazol pretreatment was associated with viable oocytes and successful embryo implantation. Further studies including human subjects will are required to confirm these findings.

Ethics

Ethics Committee Approval: The study was conducted out in the Sakarya University SUDETAM laboratory with the authorization and approval of the Experimental Animals Ethics Committee of Sakarya University, based on the European Commission Directive 86/609/ECC guide protocol, with the decision dated 05/05/2021 and numbered 27.

Informed Consent: Not necessary.

Peer-review: Externally and internally peer-reviewed.

Authorship Contributions

Concept: Ö.B., O.K., Ö.D., M.S.B., H.Ç., E.Ç., Design: H.Ç., Ö.B., M.S.B., O.K., Ö.D., E.Ç., Data Collection or Processing: Ö.B., M.S.B., O.K., H.Ç., Ö.D., E.Ç., Analysis or Interpretation: E.Ç., Ö.B., M.S.B., O.K., H.Ç., Ö.D., Literature Search: M.S.B., Ö.B., O.K., H.Ç., Ö.D., E.Ç., Writing: M.S.B., Ö.B., O.K., H.Ç., Ö.D., E.Ç.

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