Original Article/*Özgün Araştırma*

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Investigation of Apoptotic Effect of Propofol, Dexmedetomidine and Medetomidine on Oocyte Cumulus Granulosa Cells in Rats

Propofol, Deksmedetomidin ve Medetomidinin Sıçanlarda Oosit Kümülüs Granulosa Hücreleri Üzerine Apoptotik Etkilerinin Araştırılması

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ABSTRACT

Objective: We aimed to evaluate the potential apoptotic effects of propofol, dexmedetomidine and medetomidine that were used during oocyte retrieval on cumulus cells in rat ovulation induction model.

Methods: After the ovulation induction model was created, rats were received dexmedetomidine in Group D, propofol in Group P, medetomidine in Group M. Oocytes collection was performed 10 minutes after the administration of study drugs. For Caspase-3 immunohistochemical evaluation, the staining level was scored in five randomly selected areas and the areas with the highest score were determined. Immunohistochemical staining scoring for each section was performed using a scoring algorithm called H-score.

Results: Caspase-3 expression in cumulus cells was found to be lowest in Group D and highest in Group P. The mean caspase-3 H-score was lower in Group D than that of Group P and M ($p<0.001$), and in Group M than that of Group P ($p<0.001$).

Conclusion: Our results demonstrate that dexmedetomidine and medetomidine exhibit less apoptotic effects in terms of caspase-3 activity in oocyte cumulus cells than propofol in a rat ovulation induction model.

Keywords: Apoptosis, cumulus cells, dexmedetomidine, medetomidine, propofol, oocyte retrieval

ÖZ

Amaç: Oosit toplama işlemi sırasında kullanılan propofol, deksmedetomidin ve medetomidinin kümülüs hücreleri üzerindeki potansiyel apoptotik etkilerini rat ovulasyon indüksiyon modelinde değerlendirmeyi amaçladık.

Yöntem: Ovulasyon indüksiyon modeli oluşturulduktan sonra, sıçanlara Grup D'de deksmedetomidin, Grup P'de propofol, Grup M'de medetomidin verildi. Çalışma ilaçlarının uygulanmasından 10 dakika sonra oosit toplama işlemi yapıldı. Kaspaz-3 immunohistokimyasal değerlendirmede rastgele seçilen beş alanda boyanma düzeyi skorlandı ve en yüksek skora sahip alanlar belirlendi. Her bölüm için immünohistokimyasal boyama puanlaması, H-skoru adı verilen bir puanlama algoritması kullanılarak yapıldı.

Bulgular: Kümülüs hücrelerinde kaspaz-3 ekspresyonu Grup D'de en düşük, Grup P'de en yüksek bulundu. Ortalama kaspaz-3 H-skoru Grup D'de Grup P ve M'ye göre daha düşüktü ($p<0,001$) ve Grup M'de Grup P'den daha düşük bulundu ($p<0,001$).

Sonuç: Sonuçlarımız deksmedetomidin ve medetomidinin sıçan ovulasyon indüksiyon modelinde oosit kümülüs hücrelerinde kaspaz-3 aktivitesi açısından propofole göre daha az apoptotik etkisi olduğunu göstermektedir.

Anahtar sözcükler: Apoptozis, kümülüs hücreleri, deksmedetomidin, medetomidin, propofol, oosit toplama

INTRODUCTION

Transvaginal ultrasound-guided oocyte retrieval (TUGOR) is a painful procedure that is often performed under anesthesia. Different anesthetic techniques (conscious sedation, general anesthesia, regional anesthesia) are used to manage periprocedural pain. However, anesthetic drugs can enter follicular fluids and effect the embryonic development (1-3).

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Propofol is an intravenous anesthetic drug commonly used during TUGOR because of its favorable pharmacokinetic properties and safety profile (3). Another drug used for TUGOR is dexmedetomidine, a highly selective α-2 adrenoreceptor agonist with dose-dependent sedative, hypnotic, analgesic, and sympathetic-blocking properties, and without any significant respiration-inhibiting effects (4,5). On the other hand, medetomidine, another α-2 adrenoreceptor agonist, is used in veterinary medicine for its analgesic and sedative properties (6). While these drugs are potent anesthetic agents with an important role in TUGOR, so far, no studies have reported the effects of propofol or α-2 agonists on the cumulus cells found in the ovary.

Cumulus cells are critical in oocyte maturation and fertilization, as they release and mediate signals to oocytes. Consequently, the occurrence of apoptosis, in terms of caspase-3 determination, in cumulus cells can affect oocyte development (7). Therefore, we aimed to evaluate the potential apoptotic effects of propofol, dexmedetomidine, and medetomidine on cumulus cells during TUGOR in a rat ovulation induction model.

MATERIAL and METHODS

Study Design and Animal Model

This study was conducted at Sakarya University Animal Experiments Laboratory in 2020. The study protocol was approved by the Animal Research Committee of Sakarya University. All animals were maintained according to the recommendations of the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals (Ethical comitee date and number: 04.03.2020 / 18). Twenty one adult female Sprague Dawley rats (weighing 250–330 g) were housed in a 12-hour luminous–12 hour dark environment until the beginning of the research.

Superovulation Protocol

For ovulation induction, 150–300 internal units (IU) kg^{-1} pregnant mare serum gonadotropin (PMSG) (Chronogest®; Intervet, Istanbul, Turkey) were administered intraperitoneally to all rats. After a gap of 48 hours, 150–300 IU kg^{-1} human chorionic gonadotropin (hCG) (Gonatropin, Chorulon® Intervet, Istanbul, Turkey) was administered intraperitoneally. Approximately 17-19 hours after the hCG administration, 15 IU of PMSG were administered for final oocyte development (8). Afterwards, a ventral midline incision was made and oocytes were collected. After all of these procedures, the rats were sacrificed by cervical dislocation.

Anesthesia Protocol

The rats were randomly divided into three groups to receive anesthesia via intraperitoneal injection: Group D (n=7) – rats received 25 μg kg^{-1} dexmedetomidine (Precedex 200 μg mL^{-1}; Abbott, Istanbul, Turkey), Group P (n=7) – rats received 100 mg kg^{-1} propofol (Propofol 1% Fresenius; Fresenius Kabi, Istanbul, Turkey), and Group M (n=7) – rats received 0.4 mg kg^{-1} medetomidine (Domitor 1 mg mL^{-1}; Orion Pharma, Finland).

The tail vein was cannulated for hydration. The surgical procedure was performed with an abdominal incision 10 minutes after anesthetic administration. Since there was no particular guideline in the literature about how long to wait, a 10-minute waiting time was selected to ensure similarity between the experimental model and clinical practice.

Preparation of Granulosa Cells

To incubate the oocytes, a human tubal fluid (HTF) medium containing 4 mg mL^{-1} human serum albumin (HSA) additive was prepared the day before the experiment. The prepared HTF was cultured in an incubator at 37 °C - 5% CO_2 concentration. Culture drops were prepared on the culture tooth under mineral oil and the collected oocytes were incubated for at least 2 hours. They were then treated with Irvine ScientificTM Hyaluronidase solution to separate them from the cumulus cells. Spread preparations were prepared with two separate polylyzed slides of enzyme-purified cumulus cells. The smears were fixed in fixation solution at -20°C for 30 minutes and incubated at 4°C for approximately 1 week.

Immunohistochemical Evaluation

Preparations were washed with phosphate buffered saline (PBS) three times for 5 minutes. Next, Triton X-100 was dropped and kept for 10 minutes for permeabilization, followed by treatment with 3% H_2O_2 (hydrogen peroxide) solution for 20 minutes. The preparation was kept at 37°C for 1 hour, covered with primary antibody (Thermo Fisher Scientific; catalogue no: PA5-16335), and then washed with PBS three times for 5 minutes. Afterward, it was kept covered for another 10 minutes with secondary antibody, and then washed three times for 5 minutes with PBS. Next, streptavidin peroxidase was dropped and kept covered for 10 minutes, followed by washing three times with PBS for 5 minutes. Then, it was treated with DAB for 2 minutes and washed in running water.

Counter staining was done with hematoxylin for 1 minute, and the preparations were then washed in distilled water for 5 minutes. After drying, the mounting medium was dropped onto them and covered with a coverslip. An antibody diluent was dropped instead of the primary antibody for the negative control. The stained preparations were examined with an Olympus BX53 model light microscope and counted.

For caspase-3 immunohistochemical evaluation, the staining level was scored in five randomly selected areas to determine the areas with the highest score. At least 200 cells were counted at 400x magnification in different areas for all groups. Immunohistochemical staining scoring for each section was performed using a scoring algorithm called the H-score calculated by the formula = I × PC (I; degree of staining density, PC; percentage of cells stained at each grade) (9). The degree of staining in the preparations was used as the criterion as *low (+), intense (++), or high (+++).*

Statistical Analysis

We used SPSS version 22.0 (Statistical Package for Social Sciences, SPSS Inc., Chicago, IL, USA) for the statistical analysis. The results were expressed as the mean ± standard deviation (SD). The normality of the distribution of numerical variables was evaluated by the Shapiro-Wilks test. Repeated measurements of continuous variables with normal distribution were analyzed by the repeated-measures ANOVA test. Tukey's HSD test was used for post hoc analysis. A p value <0.05 was considered statistically significant.

RESULTS

Representative staining patterns are shown in Figure 1. The caspase-3 staining shows the damage caused by the anesthetic agent in the cumulus cells' nuclei and cytoplasm. The immunohistochemical evaluation showed low caspase-3 activity in the cumulus cells of Group D (**A**), intense caspase-3 activity in the cumulus cells of Group P (**B**), and high caspase-3 activity in the cumulus cells of group M (**C**). The lowest caspase-3 expression was seen in Group D and the highest was in group P; i.e., the maximum number of damaged cells was found in Group P. The damage caused by medetomidine to the cumulus cells was greater than that in Group D and less than in Group P.

Caspase-3 had a mean H-score of 21.31 ± 1.02 in Group D, 32.90 ± 1.62 in Group P, and 25.3 ± 1.85 in Group M. There was a statistically significant difference among the groups (p<0.01). The post hoc testing revealed that the mean caspase-3 H-score was lower in Group D than that of Groups P and M (p<0.001), and in Group M was lower than that of Group P (p<0.001) (Table I). The lowest H-score in cumulus cells was found in Group D, indicating the least damage caused by the anesthetic agent.

DISCUSSION

The present study evaluated the apoptotic effects of α-2 agonists (dexmedetomidine and medetomidine) and propofol on oocyte cumulus cells during TUGOR. Cumulus granulosa cells are important mediators in regulating oocyte development (10). Additionally, these cells release growth factors and proteins that are sequentially expressed during oocyte maturation, post-fertilization embryo development and growth (11).

Table I. Comparison of the Caspase-3 H-Score in Groups (Mean ± SD)

Group	Caspase-3 H-Score
Group D (n=7)	21.31 ± 1.02*
Group P (n=7)	32.90 ± 1.62
Group M (n=7)	25.30 ± 1.85**

***:** p<0.001 versus Group P and M; **:** p<0.001 versus Group P, **SD:** Standard deviation.

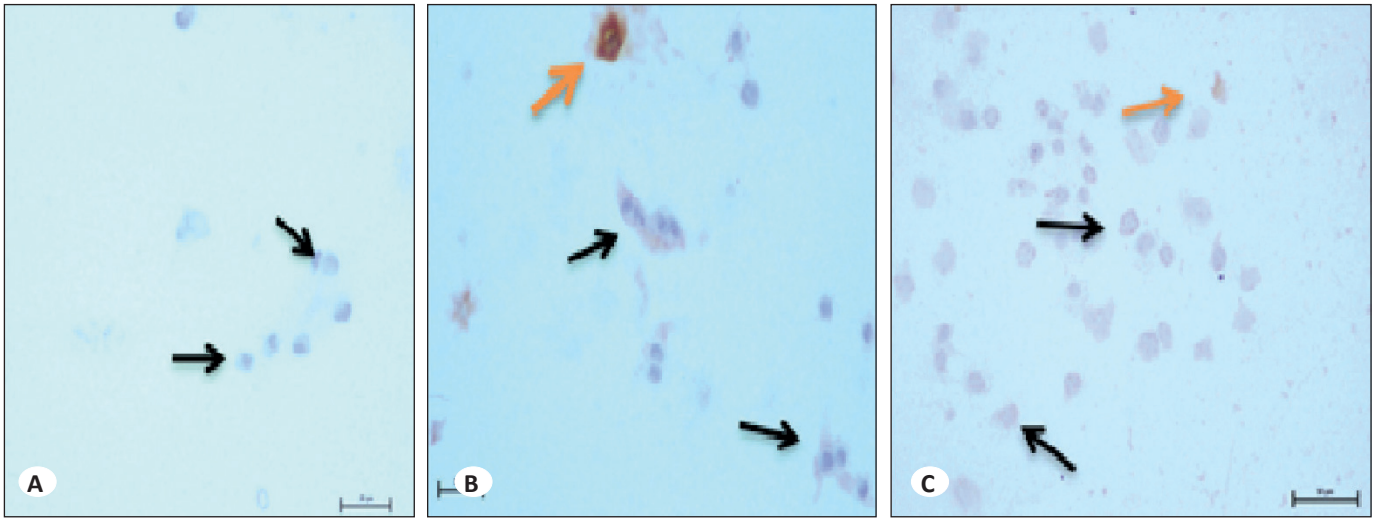

Figure 1. Immunohistochemical caspase-3 staining of cumulus cells, at 400x magnification, 20 scale bar. Caspase-3 activity indicates apoptosis of cumulus cells. **A)** Low caspase-3 activity of cumulus cells in Group D (black arrow). **B)** Intense caspase-3 activity of cumulus cells in Group P (black and orange arrows). **C)** High caspase-3 activity of cumulus cells of Group M (orange arrow).

Apoptosis of granulosa cells is considered as an indicator of the developmental potential of oocytes which is associated with egg maturation, fertilization, and the quality of resultant embryos (12-14).

Apoptosis is a form of programmed cell death that is indispensable to embryonic development and homeostasis and for monitoring cellular insults (15). Apoptosis is characterized shrinkage of the cell, fragmentation into membrane-bound apoptotic bodies, and rapid phagocytosis by neighboring cells (16). Caspases are a family of cysteine proteases which play essential role in apoptosis (17). The protein caspase-3 is considered an early marker of cell apoptosis (18). In ovarian tissue, caspase-3 positivity indicates the apoptosis of granulosa cells (19). Accordingly, we evaluated the apoptotic effects of propofol, dexmedetomidine, and medetomidine on cumulus granulosa cells by screening the caspase-3 activity levels.

The existing evidence on the relationship between propofol and apoptosis is debatable. Kang et al. reported that propofol induced apoptosis by activating caspases and mitogen-activated protein kinase pathways in Leyding tumor cells in mice (20,21). Similarly, in a recent study, Budak et al. demonstrated that increased exposure to propofol led to a decrease in the number and quality of embryos obtained and in the pup count (22). On the other hand, an in vitro animal study reported that different concentrations of propofol had no effect on oocyte culture, pronucleus formation, or early embryo development (23). In the current study, propofol was associated with intense caspase-3 activity in cumulus cells, which may negatively affect the number or quality of embryos.

It has been reported that dexmedetomidine suppresses oxidative stress-induced damage, inhibits the inflammatory response, prevents apoptosis, and reduces necrosis, thus protecting many organs from damage (24). Dexmedetomidine's inhibitory effect on neuronal apoptosis is mediated by activating the Sigma-1 receptor (Sig-1R) and inhibiting caspase-3, thereby attenuating brain damage after ischemia-reperfusion (25). It was also showed that dexmedetomidine has a protective effect on ovarian tissue against oxidative stress associated with pneumoperitoneum (26). We also found concurring results, as the caspase-3 activity of granulosa cells was lower when dexmedetomidine was used as an anesthetic agent, indicating that the drug has no adverse effects on cumulus cells.

Medetomidine is a racemic mixture of two optical stereoisomers: dexmedetomidine and levomedetomidine (27). It has been reported that dexmedetomidine is more viable than medetomidine in domestic cats subjected to semen collection by urethral catheterization (28). In the literature there is no study investigating the effect of medetomidine on caspase-3 activity. According to our study, dexmedetomidine appeared to be more reliable than medetomidine due to lower caspase-3 activity.

One of the limitations of this study was that the caspase-3 level could not be determined in the absence of any anesthetic/analgesic drugs as a control group; therefore, we could not show how caspase-3 levels changed after the use of the study drugs. Another limitation was that we could verify our results by using different determination techniques, such as polymerase chain reaction analysis for apoptotic assay or flow cytometry.

CONCLUSION

Our results demonstrate that dexmedetomidine and medetomidine exhibit less apoptotic effects in terms of caspase-3 activity in oocyte cumulus cells than propofol in a rat ovulation induction model. Alpha-2 agonists can be used more safely during TUGOR.

AUTHOR CONTRIBUTIONS

Conception or design of the work: ATT, DBG, MSB
Data collection: GD, HC
Data analysis and interpretation: HK, OB
Drafting the article: ATT, MK
Critical revision of the article: ATT, DBG
Other (study supervision, fundings, materials, etc): MSB
All authors (ATT, HK, GD, OB, MSB, HC, DBG) reviewed the results and approved the final version of the manuscript.

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