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AUTOLOGUS PLATELET RICH PLASMA (PRP) IN VITRO FERTILIZATION ANTAGONIST PROTOCOL CAUSED HOMEOBOX A 10 (HOXA10) EXPRESSION IN THE ENDOMETRIUM OF WISTAR STRAINED RATS HIGHER THAN WITHOUT AUTOLOGOUS PRP

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Keywords:
HOXA10; Autologous PRP; Ovarian Stimulation; In Vitro Fertilization.

ABSTRACT
Endometrial receptivity is highly correlated with the implantation rate, whereby in Indonesia, the implantation rate among in vitro fertilisation programs is still low, around 19.3%. Antagonist ovarian stimulation protocol can disrupt physiological endocrine milieu by the presence of supra-physiological levels of estrogen and progesterone, both of which are thought to indirectly affect the expression of adhesion molecule cells. Homeobox A 10 (HOXA10) is among one of the biomarkers used to assess endometrial receptivity. The role of HOXA10 is critical during the stage of endometrial development and embryo implantation, both in mice and human. This molecule is thought to work optimally by administering autologous platelet rich plasma (PRP) to the damaged endometrium. The aim of this study was to prove that administration of PRP in an antagonist protocol HOXA10 expression in the endometrium of wistar strain rats higher than without PRP. This research was an experimental study. The design for this study was randomized posttest only controlled group, using female wistar strain rats. A total of 40 wistar strain rats whose ovary were stimulated by the GnRH antagonist protocol were divided into 2 groups, 20 rats as intervention group in which PRP administration was done and 20 rats as control group. It was found that during the study, there was no significant difference between intervention and control group regarding age and body weight of the wistar strain rats, both at the start and at the end of the study. The results of HOXA10 H-score in the intervention group was 3.15 (2.86-3.52) and in the control group was 1.68 (1.43-2.18) ($p < 0.05$). Administration of PRP to ovarian stimulation with an antagonist protocol showed significantly higher expression of endometrial HOXA10 in wistar strain rats than without PRP administration.
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INTRODUCTION

The endometrium is the innermost layer of the uterus and the place where the embryo attaches. The endometrium plays a role in preparing for the implantation process which involves the interaction between competent endometrial and blastocyst receptivity. Therefore, this organ is important as a determining factor for the success of a pregnancy related to the blastocyst implantation process. However, it is not uncommon for this endometrium to experience damage either caused by an action trauma, infection, or hormonal, causing disruption of anatomical and physiological structures that adversely affect endometrial receptivity.

Globally, the prevalence rate of female infertility increased by 14.962% from 1,366.85 per 100,000 in 1990 to 1,571.35 per 100,000 in 2017 (Sun et al., 2019). Naturally, the probability of fertilization per cycle is relatively low at around 30%, with two-thirds of infertility occurring due to implantation failure. Assisted Reproductive Technology (ART) is one of the treatments for infertility patients used to achieve pregnancy in infertile couples who cannot be addressed the cause of infertility conventionally. Assisted reproductive technology consists of various techniques, one of which is in vitro fertilization (IVF) (Kim & Kim, 2017).

In the past decade, the success of IVF has been low, especially in terms of implantation rate (IR). Endometrial receptivity is highly correlated with IR, in addition to embryonic quality. The average IR of the IVF program at 36 IVF service centers in Indonesia is 19.3%. Low IR is clinical evidence of impaired endometrial receptivity thought to be associated with failure of the apposition, adhesion and invasion stages in the early implantation and placentation processes. About 3% of IVF program cycles are postponed due to non-optimal endometrial conditions, which are feared to interfere with embryo implantation (Nazari et al., 2019).

The IVF method includes highly coordinated steps, starting with controlled ovarian stimulation with exogenous gonadotropins, oocyte and sperm collection, fertilization, then embryo transfer into the uterus. In the IVF program, ovarian stimulation was carried out with two types of protocols, namely antagonist and agonist. Antagonist protocols are preferred by clinicians because they are cheaper, easier to perform, and require a shorter time, making them more comfortable for patients than agonist protocols. Meanwhile, stimulation of the ovaries with both types of protocols will disrupt the physiological endocrine milieu which will result in disruption of the regulation of estrogen and progesterone receptors in stroma and endometrial epithelial cells. Supraphysiological levels of the hormones estrogen and progesterone in the early luteal phase are thought to indirectly affect the expression of cell adhesion molecules, especially homeobox A 10 (HOXA10) in endometrial epithelial cells. This can reduce endometrial receptivity, resulting in lower implantation rates.

Endometrial receptivity is an important predictor of pregnancy after IVF and embryo transfer. Endometrial thickness is used as an indicator for endometrial receptivity. Endometrial thickness was measured on the day of administration of the hormone chorionic

gonadothrophin (hCG) and measured using transvaginal ultrasound in the mid-sagittal plane, where this method is considered non-traumatic and simple. The effect of endometrial thickness on IVF success has been evaluated by many investigators. A recent meta-analysis study demonstrated that the probability of clinical pregnancy in patients with an endometrial thickness of <7 mm was significantly lower than in patients with an endometrial thickness of >7 mm (Wu et al., 2014).

Various attempts have been made to improve receptivity in the endometrium, such as antibiotic therapy, curettage and therapeutic biopsy of polyposis, myomectomy and other appropriate therapies. In addition, corrective efforts have been made to endometrial receptivity with prolongation of estrogen administration, gonadotropin therapy, low-dose administration of hCG, aspirin, sildenafil, acupuncture and stem cell therapy, but the results have not been satisfactory (Zadehmodarres et al., 2017); (Eftekhar et al., 2018).

At the molecular level, endometrial damage is thought to be related to the role of adhesion and inflammation molecules, one of which is homeobox A 10 (HOXA10) which is a family of GATA transcription factors. HOXA10 is one of the genes that is normally up regulated in the endometrium during the implantation window period and its levels increase dramatically during the secretion phase midway of the menstrual cycle. β3 integrins are adhesion molecules to the endometrium that are locally responsible for the presence of pinopods, where β3 integrins are directly regulated by HOXA10 (Lessey & Young, 2019). HOXA10 is thought to work optimally in the administration of autologous platelet rich plasma in damaged endometrium.

Platelet rich plasma (PRP) is an autologous blood product that carries platelet rich products three to five times the normal amount. The content of PRP consists of growth factors, chemokines, cytokines, nutritional hormones, stabilizing proteins, such as albumin and other products that can function for cell growth and homeostasis (Zadehmodarres et al., 2017). PRP contains many α-granules that play an important role in the storage of intracellular growth factors, such as platelet-derived growth factor (PDGF), transforming growth factor (TGF)-ß and insulin-like growth factor (IGF)-1 (Dhurat & Sukesh, 2014). PRP is known to have proliferative and anti-fibrotic effects on damaged endometrium, where PRP administration can also trigger angiogenesis and cell migration associated with increased endometrial thickness. Thus, administration of PRP can increase endometrial receptivity associated with increased IR. Thus, the success of IR in IVF with pathological endometrial conditions or PRP-exerted endometrial damage is thought to be related to the role of HOXA10 (Du & Taylor, 2016); (Jang et al., 2017).

Research shows that the addition of PRP to endometrial preparation of IVF with Frozen Embryo Transfer (FET) was found to significantly increase endometrial thickness where endometrial thickness correlates with good endometrial receptivity, thus increasing the IR number in the cycle. Research also proves that the administration of PRP to the endometrium with a history of previous disruption also experienced anatomical and histological improvements through a complex process of growth hormone. However, the question is whether PRP administration will also increase endometrial HOXA10 expression in the IVF antagonist protocol.

Based on the above background, research was conducted on the effect of autologous platelet rich plasma (PRP) administration on ovarian stimulation in vitro fertilization antagonist protocol on homeobox A 10 (HOXA10) endometrial expression of wistar strain rats.

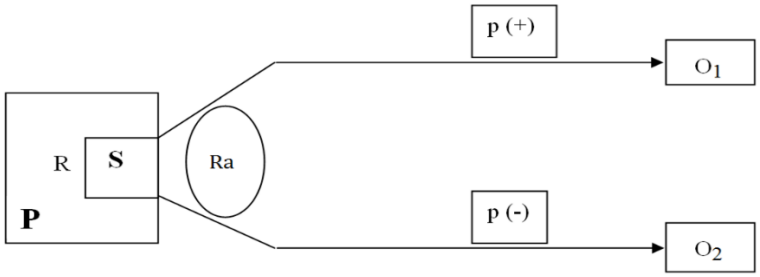
This study aims to prove the effect of autologous PRP administration on the IVF antagonist protocol on endometrial receptivity and specifically to prove that administration of autologous PRP on antagonist protocol causes homeobox A 10 (HOXA10) expression in the endometrium of wistar strain rats is higher than without autologous PRP administration.

Through this study, it is hoped that benefits can be obtained regarding the latest understanding related to the administration of autologous PRP on ovarian stimulation protocols with GnRH antagonists to the expression of homeobox A 10 (HOXA10) endometrium of wistar strain rats. High HOXA10 expression will be associated with increased endometrial receptivity and implantation rate in pregnancy success.

METHODS

The design of this study was experimental the randomized posttest only controlled group design using experimental animals of female rats of the wistar strain. The research design is described as follows:

Figure 1. Research Design



Information:

P : Population
R : Randomization
S : Sample
Ra : Random allocation
p (+) : PRP therapy group on ovarian stimulation with GnRH antagonist protocol
p (-) : Group without PRP administration on ovarian stimulation with GnRH antagonist protocol
O1 : Observation of endometrial HOXA10 expression in the PRP therapy group
O2 : Observation of endometrial HOXA10 expression in the group without PRP administration

Observation, maintenance, physical examination, vaginal swab examination, randomization of samples, and collection of rat endometrial tissue were carried out at the Biomedical Laboratory of the Animal Lab Unit, Faculty of Medicine, Udayana University,

Denpasar. Immunohistochemical examination is carried out at the Biomedical Laboratory of the Faculty of Medicine, Udayana University, Denpasar. The study was conducted in April – June 2022.

RESULTS AND DISCUSSION

Characteristics of the Research Subject

This research is an experimental study with the randomized posttest only controlled group design. The study was conducted on 40 wistar strain rats which were divided into 2 groups, namely 20 as the treatment group and 20 as the control group. Wistar rats with a regular estrus cycle of 4 days, aged 8-12 weeks, with a body weight of 200-230 grams were used as the target population of the study. This research was conducted at the Integrated Biomedical Laboratory, Faculty of Medicine, Udayana University, Denpasar.

This research has received approval from the Integrated Biomedical Laboratory Unit, Faculty of Medicine, Udayana University with a research implementation approval letter No: 1112/UN14.2.2.VII.6/LT/2022 and ethical feasibility approval from the Research Ethics Commission Unit, Faculty of Medicine, Udayana University dated December 31, 2021, in the form of Ethical Clearance Number: 2898/UN14.2.2.VII.14/LT/2021.

All data from treatment and control groups that had met the inclusion and exclusion criteria in this study were then tested for normality using the Kolmogorov-Smirnov test. Descriptively, the characteristics of research subjects in the treatment and control groups are presented in the following table.

Table 1. Characteristic Distribution of Treatment and Control Groups

Average	Treatment Group (N=20)		Control Group (N=20)		*p-value**
	Rerata	SD	Rerata	SD	
Age	62.95	1.31	62.45	1.60	0.288
Initial Weight Loss	211.24	1.73	211.85	1.61	0.257
Final Weight Loss	227.69	1.36	228.30	1.26	0.152

T-independent test

Based on table 1. It can be seen that the average age of wistar strain rats during the study in the treatment group was 62.95 ± 1.31 days, while in the control group was 62.45 ± 1.60 days. Body weight of wistar strain rats was measured before and after the study. The average initial body weight of wistar strain rats in the treatment group was 211.24 ± 1.73 grams, while in the control group was 211.85 ± 1.61 grams. At the end of the study, there was an increase in body weight with the average body weight of the treatment group wistar strain rats was 227.69 ± 1.36 grams, while in the control group was 228.30 ± 1.26 grams. The Kolmogorov-Smirnov test obtained a significance value of more than 0.05 which means that the data are normally distributed on age, intial and final body weight of wistar

strain rats. Furthermore, in the T-independent test, a significance value of more than 0.05 was obtained which stated that there was no significant difference in average age, initial and final body weight between the treatment and control group wistar strain rats. Other control variables have been adjusted by conditioning wistar strain rats to the same conditions, including cage size, temperature, humidity, type of food and how to give it, adaptation to light and dark environments and the process of health checks to rule out infections or abnormalities.

HOXA10 Expression in the Endometrium of Wistar Strain Rats

To assess HOXA10 expression in the endometrium of wistar strain rats was carried out by immunohistochemical examination and H-score calculation. H-score is calculated by the following equation: H-Score = ΣPi (i + 1). Intensity (i) represents the core staining of HOXA10 indicated by a value of 0, 1, 2, or 3 (negative, weak, medium or strong) and Pi is the percentage of core staining for each intensity, ranging from 0-100% (Rackow et al., 2008). The following are the results of HOXA10 H-Score in the treatment and control groups (Figure 2).

Figure 2. HOXA10 H-Score Graph

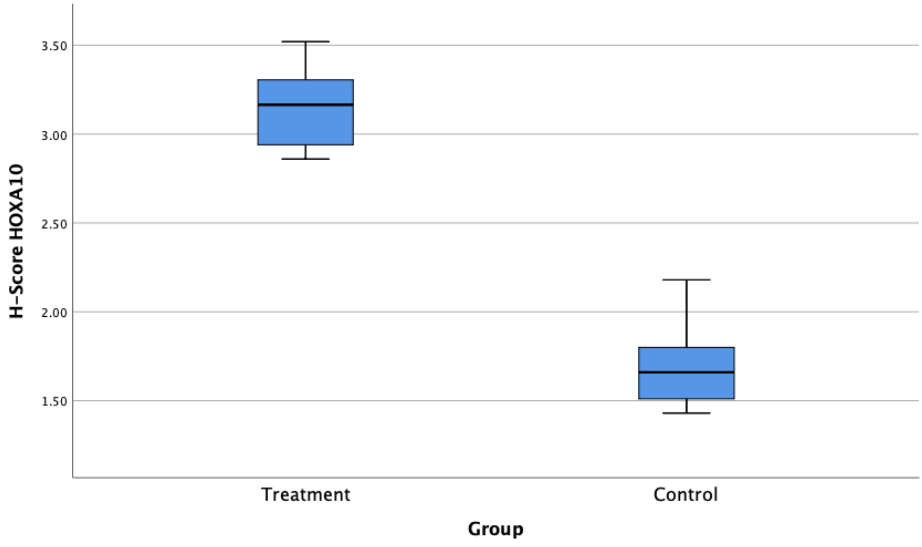


Figure 3 shows the results of immunohistochemical examination in sample treatment group no. 14 with a total of 343 epithelial cells with negative staining of 11 epithelial cells, weak intensity 72 epithelial cells, moderate intensity 103 epithelial cells and strong intensity 157 epithelial cells. The H-score obtained is 3.18 which means the expression is very strong.

Figure 3. HOXA10 Expression in The Treatment Group

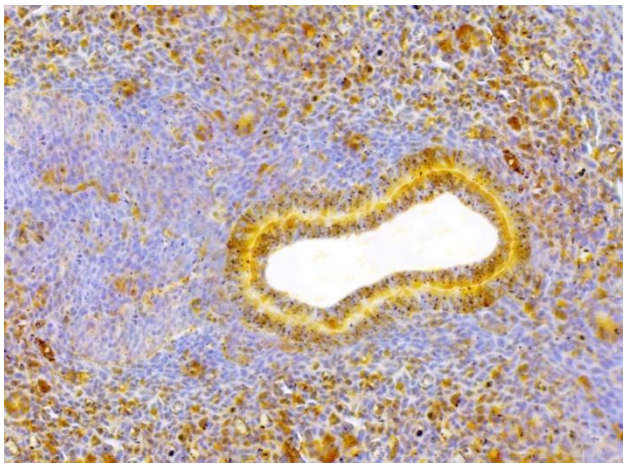
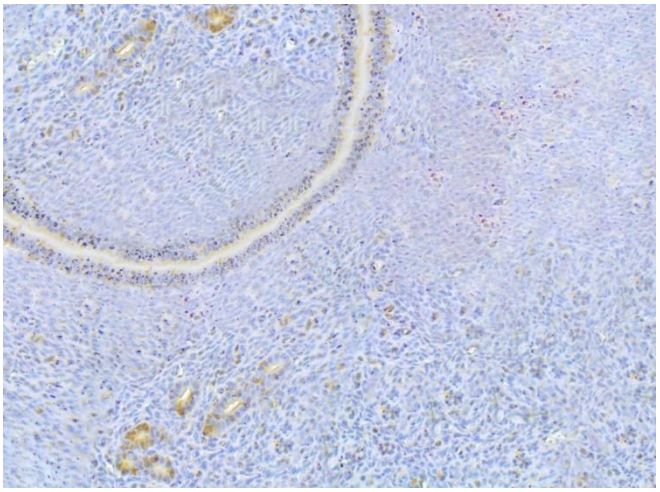


Figure 4 shows the results of immunohistochemical examination in sample control group no. 21 with a total of 328 epithelial cells with negative staining of 193 epithelial cells, weak intensity 87 epithelial cells, moderate intensity 32 epithelial cells and strong intensity 4 epithelial cells. The H-score obtained is 1.52 which means medium expression.

Figure 4. HOXA10 Expression in The Control Group



Effect of Autologous PRP Administration on HOXA10 Expression in the Endometrium of Wistar Strain Rats

In this study, the results of HOXA10 H-score were tested for data normality using the Kolmogorov-Smirnov test, it was found that the HOXA10 H-score data of the treatment

and control groups were normally distributed, so that to conduct a data comparison test used was a T-independent test.

Table 2. HOXA10 H-score Normality Test

Parameters	*p-value**
HOXA10 H-score Treatment Group	0.200
HOXA10 H-score Control Group	0.200

Kolmogorov-Smirnov test

Based on immunohistochemical examination of endometrial tissue of wistar strain rats, HOXA10 H-score results were obtained in the treatment group, namely 2.86 to 3.52 with an average of 3.15 ± 0.20. A lower HOXA10 H-score result was obtained in the control group, which was 1.43 to 2.18 with an average of 1.68 ± 0.20. In table 3 it has been described that the T-independent test with p-value results of 0.000 < 0.05. These results showed that the average H-score in the treatment group was significantly higher than in the control group.

Table 3. HOXA10 H-score Expression in Endometrial of Wistar Strain Rats

Average	Treatment Group (N=20)		Control Group (N=20)		*p-value**
	Average	SD	Average	SD	
HOXA10 H-score	3.15	0.20	1.68	0.20	0.000

T-independent test

Furthermore, the HOXA10 H-score was grouped into HOXA10 expression levels as follows: (a) weak expression (H-score < 1.1), (b) medium expression (H-score = 1.1 – 2), (c) strong expression (H-score = 2.1-3), and (d) very strong expression (H-score = 3.1 – 4). The Chi-square test was then used to compare HOXA10 expression between the treatment group and the control group (Table 4).

In the treatment group, 20 rats (100%) had strong and very strong HOXA10 expression and no mice had weak and moderate HOXA10 expression. In contrast to the control group, 18 mice (90%) had weak and moderate HOXA10 expression and only 2 mice (10%) had strong and very strong expression. With the Chi-Square test it was found that the value of χ^2 = 32.7 and the p-value 0.000 < 0.05. This indicates that there is a significant difference in HOXA10 expression between the treatment and control groups. Thus the initial hypothesis was proven, namely the administration of PRP in the in vitro antagonist protocol showed significantly higher endometrial HOXA10 expression of wistar strain rats compared to without PRP administration.

Table 4. Differences in HOXA10 Expression between the Treatment Group and the Control Group

		HOXA10 Expression		$\chi 2$	*p-value**
		Weak and Moderate	**Strong and Very Strong**		
Group	Treatment	0 (0%)	20 (100%)	32.7	0.000
	Control	18 (90%)	2 (10%)		

Chi-square test

DISCUSSION

Characteristics of the Research Subject

In this study, the average age of wistar strain rats when the study began in the treatment group was 62.95 days and the control group was 62.45 days. Research subjects from both research groups have entered the period of reproductive maturity, where the age of wistar strain rats more than 60 days is classified into the adult stage (Jackson et al., 2017).

The body weight of the mice was affected by age, sex and diet. In this study, the average body weight increase of wistar strain rats in the treatment group was 211.24 grams to 227.69 grams and the control group by 211.85 grams to 228.30 grams. The increase in body weight of study subjects from the beginning to the end of the study reflected a good state of health. It is known that the body weight, age and stage of development of experimental animals can affect the results of studies (Kilkenny et al., 2009); (Ghasemi et al., 2021).

Effect of Platelet Rich Plasma Administration on HOXA10 Endometrial Expression of Wistar Strain Rats on Ovarian Stimulation in Vitro Fertilization Antagonist Protocol

In this study, HOXA10 H-scores were obtained from 1.43 to 2.18 with a mean of 1.68 ± 0.20 in the endometrium of the control group of wistar strain rats that had received ovarian stimulation with GnRH antagonists. In the treatment group with ovarian stimulation with GnRH antagonists followed by PRP administration, the HOXA10 H-score was higher, from 2.86 to 3.52 with a mean of 3.15 ± 0.20 compared to the control group.

HOXA10 plays an important role in both endometrial implantation and decidualization. HOXA10 expression has been widely observed in endometrial stromal cells, endometrial glands and endometrial epithelial cells. Loss of HOXA10 has no adverse impact on embryo survival during the embryo transfer period, but profoundly affects function and implantation in the endometrium. In humans, it is known that defects in the implantation process occur along with lower HOXA10 expression. The important role of

HOXA10 during implantation is proven by transgenic mouse model experiments and decreased implantation rates through changes in HOXA10 expression (Jang et al., 2017).

In women, HOXA10 expression increases in the midluteal phase at implantation which plays a role in endometrial receptivity (Du & Taylor, 2016). Strong immunoreactivity in HOXA10 was found in the endometrial stroma of fertile women with an H-Score of 2.1 (Fischer et al., 2011). HOXA10 is a good biomarker for endometrial receptivity and changes in its methylation have been shown to be associated with impaired endometrial receptivity in several pathological endometrial conditions, including diseases of the reproductive system and external factors that disrupt the endocrine system (Li et al., 2015).

Administration of GnRH antagonists in ovarian stimulation, can interfere with the expression of HOXA10 in endometrial stromal cells, thereby affecting endometrial receptivity. There was an increase in oocyte production, but a relatively low implantation rate was found so that most embryo implantation failures occurred. However, GnRH antagonists have been shown to be effective, safe and the therapeutic benefits outweigh the negatives, so they should continue to play a role in controlled ovarian stimulation. The results of the study support the molecular basis for the lower pregnancy rates seen clinically with the use of GnRH antagonists. Based on the results of that study, it was found that in female endometrial stromal cells, the HOXA10 H-score was significantly lower in the administration of GnRH antagonists of 1.50 ± 0.18 compared to the administration of GnRH agonists of 2.51 ± 0.12 and the control group of normal cycles of 2.31 ± 0.07 (Rackow et al., 2008). Other studies also obtained the same results, namely HOXA10 protein expression was found to be lowest in the rat endometrium of the treatment group with GnRH antagonists and highest in the natural cycle control group followed by GnRH agonists (Li et al., 2015).

Decreased expression of HOXA10 during the midluteal phase of the menstrual cycle is seen in other conditions associated with implantation disorders, such as PCOS, submucosal uterine myoma, hydrosalfing and endometriosis (Fischer et al., 2011). The expression of HOXA10, LIF and integrin-β3 proteins is also decreased in Ovarian Hyperstimulation Syndrome (OHSS). Studies have shown that during the IVF cycle, the incidence of moderate OHSS is 3-6% and the incidence of severe OHSS is 0.2-1%. Pregnancies associated with OHSS have been found to have a higher incidence of complications, including gestational diabetes mellitus, placental abruption, prematurity, stillbirth, low birth weight and preterm birth (Xu & Tang, 2014).

Understanding the mechanism of HOXA10 expression is critical in finding potential therapies to improve the condition of implantation disorders with decreased HOXA10 expression. The result of research by Jang et al., (2017) demonstrated that intrauterine administration of autologous PRP exerts proliferative and antifibrotic effects on damaged endometrium. PRP is known to contain several growth factors and cytokines that can help accelerate cell proliferation, angiogenesis, cell migration, accelerate healing and tissue regeneration. Analysis on the expression of Ki-67, CK, VEGF, and HOXA10 showed significant semi-quantitative differences with PRP administration. HOXA10 was

found to be an important transcription factor for many target genes involved in regulating endometrial function and development during the menstrual cycle, together with endometrial receptivity to establish the necessary conditions for implantation in humans and mice. In that study, the expression of HOXA10 upon PRP administration increased significantly on IHC staining. Similar results were also observed for HOXA10 mRNA expression by RT-PCR. These results provide correlative evidence for the possible use of PRP in achieving implantation with increased uterine vascularity and endometrial receptivity (Jang et al., 2017).

PRP contains several growth factors and cytokines, including platelet-derived growth factor (PDGF), fibroblast growth factor (FGF), vascular endothelial growth factor (VEGF), epidermal growth factor (EGF), insulin-like growth factors I, II (IGF I, II), fibroblast growth factor (FGF), transforming growth factor (TGF), connective tissue growth factor (CTGF) and interleukin 8 (IL-8) that promote stimulation, proliferation and regeneration. It has been shown that intrauterine infusion of PRP can increase endometrial thickness and pregnancy rates in the IVF cycle (Chang et al., 2019); (Zadehmodarres et al., 2017).

PRP significantly stimulated growth, migration and adhesion of endometrial mesenchymal stem cells when compared to the control group. Data showed that there was endometrial expansion and pregnancy with the use of PRP infusion so that the pregnancy rate reached 60% (Wang et al., 2019). Other studies have shown that PRP is effective in increasing pregnancy rates in recurrent implantation failures. In the PRP group compared to the control group, the results of a higher chemical pregnancy rate of 53.06% vs 27.08% and a higher clinical pregnancy rate of 44.89% vs 16.66% (Nazari et al., 2019).

CONCLUSION

Based on the results of this study, it can be concluded that PRP administration in ovarian stimulation antagonist protocol showed significantly higher endometrial HOXA10 expression of wistar strain rats compared to without PRP administration. In the administration of PRP, the results of HOXA10 expression were significantly strong and very strong.

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