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ADMINISTRATION OF AUTOLOGOUS PLATELET RICH PLASMA ON THE IN VITRO FERTILIZATION ANTAGONIST PROTOCOL CAUSED HIGHER EXPRESSION OF INTEGRIN B3 IN THE ENDOMETRIUM OF WISTAR STRAIN RATS THAN WITHOUT PRP AUTOLOGOUS ADMINISTRATION

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Autologus PRP, Antagonis FIV, Integrin β3, Endometrium

ABSTRACT
The purpose of determining the administration of Autologous Platelet-rich plasma (PRP) in the In Vitro Fertilization anthogonist protocol caused the expression of β3 integrin in the endometrium of wistar strain rats was higher than without Autologous PRP administration. This study used the experimental design of the randomized posttest only controlled group design using 40 female rats of the Wistar strain type which were divided into 2 treatments, namely the treatment group with PRP and the group without PRP with simple random sampling. The research procedure consisted of ovarian stimulation with Cetrotide (GnRH anatagonis), immunohistochemical examination of integrin ß3, and analysis of integrin ß3 expression with a microscope. The data were then tested for normality and homogeneity with Shairo Wilk and Levene's test, and continued with comparison tests using the Mann whittney test and Chi Square. The average H-score of integrin ß3 was obtained in the treatment group of 2.90 and the control group of 1.54 with P<0.001. In the treatment group, the expression of integrin ß3 was strong – very strong in all samples, compared to the control group obtained 18 weak – medium and 2 strong – very strong (P<0.001). In conclusion, administration of Autologous Platelet Rich Plasma (PRP) on the In Vitro Fertilization antagonist protocol led to higher expression of β3 integrin in the endometrium of wistar strain rats.

INTRODUCTION

Infertility is one of the many problems that occur throughout the world, including Indonesia. Infertility is the inability to get pregnant within 1 year and have had sex without contraception. Infertility can be caused by various aspects. The psychic, physical, sexuality and social aspects of the infertile couple are important aspects that are influential, so they require appropriate intervention.

Assisted reproductive technology (ART) has advanced rapidly and researchers have tried to develop therapeutic options for each infertile couple. In recent years, developments in ART have aimed to increase success rates in In-Vitro Fertilization (FIV). But pregnancy rates are still not satisfactory enough. In-Vitro fertilization with the involvement of various factors such as endometrial receptivity can affect its success rate (Fathi Kazerooni et al., 2018)

In the past decade, the success of In-Vitro Fertilization has been low. Especially in terms of implantation rate (IR). The average IR FIV-ISIS data from 28 IVF service centers in Indonesia is 13.30%. In America IR has also been reported at 29%. In the last five years, this percentage has not increased comparatively. (Anantasika, Suwiyoga, Bakta, & Astawa, 2018) . 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-placentation process.

Endometrial receptivity is one of the most important factors in predicting pregnancy after In-Vitro Fertilization and embryo transfer. Endometrial thickness is utilized as an individual indicator for endometrial receptivity and is measured on the midsagittal plane through transvaginal ultrasound, which is considered a non-traumatic and simple method.

Adhesion molecules such as the integrin ß3 are thought to play a very important role in eliciting endometrial receptivity. Integrin ß3 is a heterodimer transmembrane glycoprotein composed of α and β subunits (Takada, Ye, & Simon, 2007). A number of integrins are found in the lumen of the epithelium and glands of endometrial tissue (Malaiyandi et al., 2018). Integrin β3 beserta ligannya (osteopontin) terdeteksi dengan pemeriksaan imunohistokimia pada permukaan epitel endometrium saat pertama kali berinteraksi dengan trofoblas. Based on its location and expression pattern, the β3 integrin is thought to be a receptor for embryo implantation (Capranica & Millard-Stafford, 2011) and also a good marker of endometrial receptivity (Franasiak et al., 2014). Integrins and their ligands are expressed in the endometrial lumen of the menstrual cycle at the moment that coincides with the implantation window. This protein plays a role in the adhesion of endometrial cells that initiate the implantation process. Integrin ß3 is present in luminal epithelium and glands expressed after day 19 of the menstrual cycle. Proteins expressed on the surface of the endometrial luminal epithelium are thought to play a role in the first interaction with the embryonic trophoblast. Integrins are markers for measuring endometrial receptivity whose expression is regulated by estrogen and progesterone via HOXA10 (Michelson et al., 2000).

In the In Vitro Fertilization program, ovarian stimulation is carried out with two types of protocols, namely antagonists and agonists. The antagonist protocol is preferred by clinicians because it is cheaper, easier to perform, and requires a shorter time so that it is more comfortable for the patient than the agonist protocol. Meanwhile, ovarian stimulation with both types of protocols will disrupt the physiological endocrine environment which will result in impaired regulation of estrogen and progesterone receptors in stroma and endometrial epithelial cells. Supraphysiological levels of estrogen and progesterone hormones in the early luteal phase are thought to indirectly affect the expression of adhesion molecule cells, especially β3 integrins in endometrial epithelial cells where this can reduce endometrial receptivity, so that the implantation rate is lower.

Autologous Platelet-rich plasma (PRP) is an autologous blood product that carries platelet-rich products three to five times the normal amount. In Autologous PRP contains growth factors, chemokines, cytokines, nutritional hormones, stabilizing proteins, such as albumin and other products that can function for cell growth and homeostasis (Zadehmodarres, Salehpour, Saharkhiz, & Nazari, 2017). (Dhurat & Sukesh, 2014) and (Norrving et al., 2018) states that in cell membranes, PRP contains many α 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). The study of Chang, et al., (2019) with the administration of Autologous PRP in women undergoing FIV with thin endometrium was very satisfying.

It is supported by (Jang et al., 2017) which states the proliferative and anti-fibrotic effects on damaged endometrium where administration of Autologous PRP can also trigger angiogenesis and cell migration associated with increased endometrial thickness. Thus, administration of Autologous PRP can improve endometrial receptivity associated with increased implantation rates. Thus, the increased implantation rate in FIV in pathological endometrial conditions or endometrial damage given by Autologous PRP is thought to be related to the role of β3 integrin.

This study aims to determine the effect of Autologous PRP administration on endometrial receptivity through the role of β3 integrins in the FIV antagonist protocol. Based on theoretical studies, administration of Autologous PRP is thought to increase the expression of mouse β3 integrins which is directly related to endometrial resptivity.

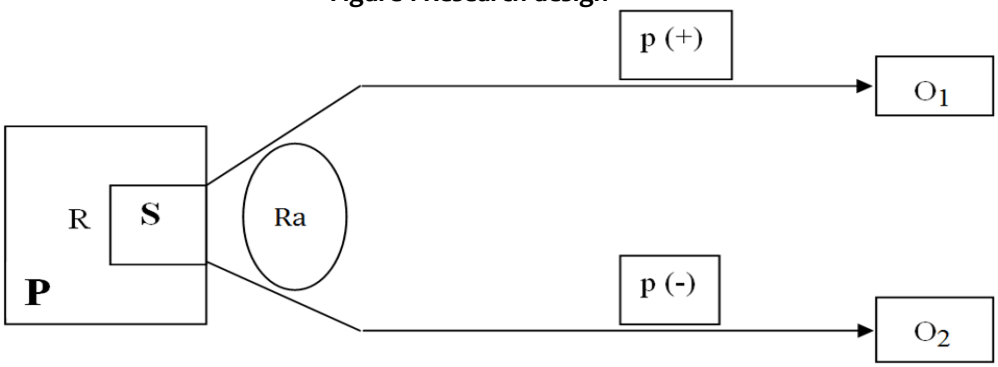
Based on the above background, the formulation of this study Does the administration of Autologous Platelet-rich plasma (PRP) in the In Vitro Fertilization anthogonist protocol cause the expression of β3 integrin in the endometrium of wistar strain rats is higher than without Autologous PRP administration?

This study aims to determine the administration of Autologous Platelet-rich plasma (PRP) in the In Vitro Fertilization anthogonist protocol causes the expression of β3 integrin in the endometrium of wistar strain rats is higher than without Autologous PRP administration. Benefits of this study If it can be proven that the administration of Autologous Platelet Rich Plasma (PRP) in ovarian stimulation with antagonistic protocol resulted in higher integrin expression in rat endometrium compared to ovarian stimulation antagonist protocol without Autologous PRP administration, then it can be used as an alternative therapy that needs to be considered, especially related to implantation rate (IR) in the endometrium.

RESEARCH METHODS

The design of this study is experimental the randomized posttest only controlled group design using experimental female rats type Wistar strains. The research design is described as follows

Figure 1 Research design



Information:
P = Population
R = Randomization
S = Sample.
Ra = Random allocation.
p(+) = Ovarian stimulation group with antagonist + PRP protocol. p(-) = Ovarian stimulation group with antagonist protocol.
O1 = Observation of β3 integrin expression
endometrium in the PRP therapy group
O2 = Observation of β3 integrin expression
endometrium in the group without PRP administration

The observation of this study was carried out at the Biomedical Lab. Animal Lab unit, Udayana University, Denpasar in April - June 2022. The target population is female Wistar strain rats. The size of the study sample was calculated based on the Federer formula.

$(n - 1) (t - 1) > 15$

n = sample size,

t= number of treatment groups = 2

So the minimum sample size obtained is 18 for each treatment group. Add to that the 10% (1.8) drop-out probability during the study, the minimum number of groups was 20.

Samples that have been selected by veterinarians at the Biomedical Lab of Faculty of Medicine UNUD are taken from the target population with a simple random sampling technique using a random number table in the Microsoft Excel for Windows program. Next, rats number 1-20 were put into the treatment group with PRP, and mice number 21-40 were put into the group without PRP.

Research Instruments, namely; (a) Cages with a capacity of 4-6 mice, complete with a light source for adaptation of 12 hours of light – 12 hours of darkness and an air humidity meter. (b) Rats food (standard pellet diet). (c) Veterinary surgical table + surgical kit, diehil ether, cotton, sealed cup. (d) Syringes, syringes, sterile gauze, 70% alcohol, Tissue preparation bottles. (d) Glas object for examination of vaginal secretions of rats. (e) Stationery and paper. The examination kit and collection of tissue materials used are those in the Biomedical Lab. Animal Lab unit, Udayana University, Denpasar and immunohistochemical examination at the Biomedical Lab. FK UNUD, Denpasar.

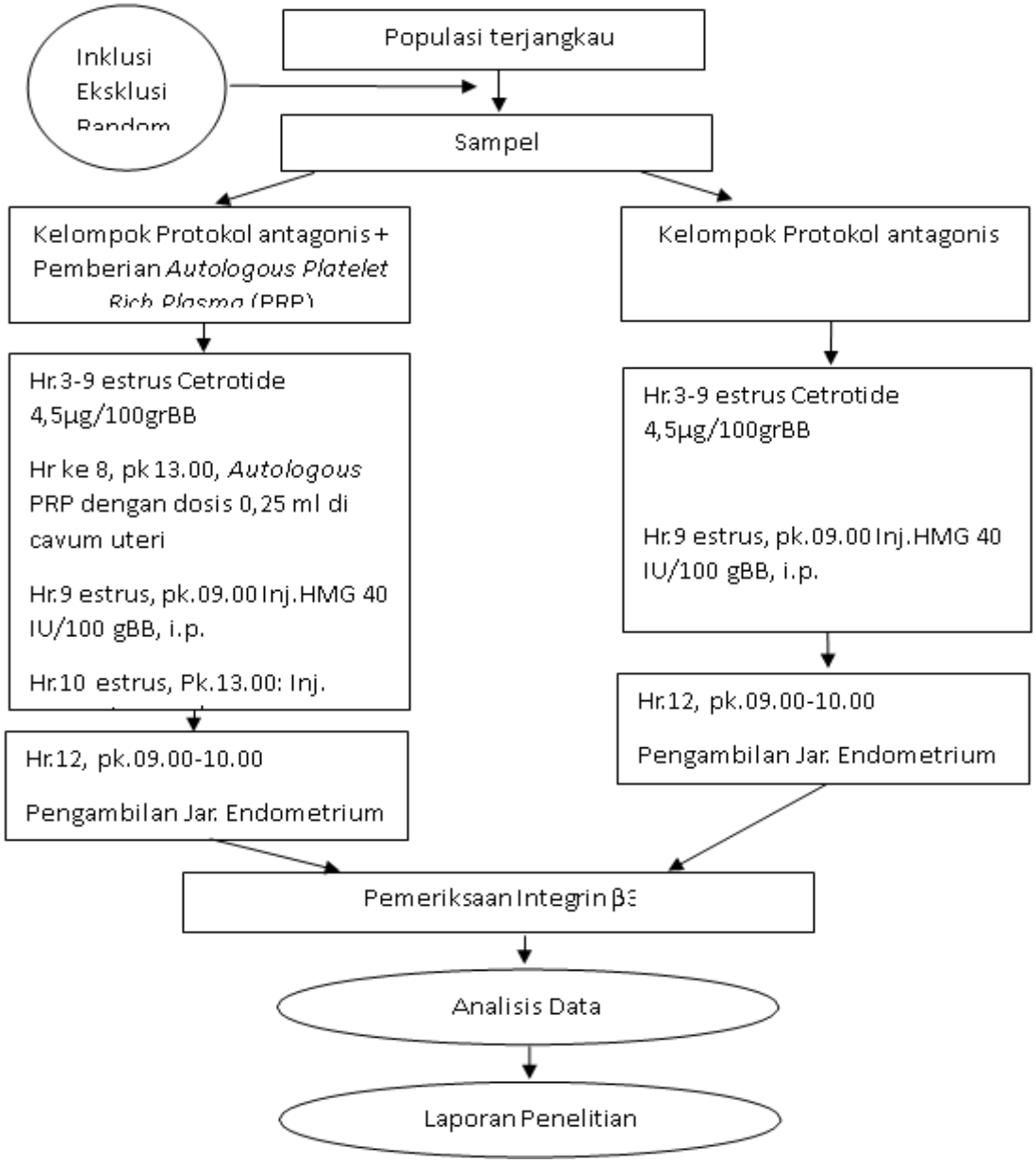


Figure 2 Research Flow

Descriptive statistical tests were carried out to describe the basic characteristics of each group and the frequency distribution of various variables in the form of weight data and Autologous PRP administration, data on immunohistochemical examination of rat endometrial preparations Wistar / c strain in the form of: H-score results Integrin subunit β3 and its categories. And displayed the proportion of H-score Integrin Subunit β3 in the group administering Autologous PRP therapy and without Autologous PRP.

RESULTS AND DISCUSSION

This research is an experimental study with the randomized posttest only controlled group design. The study was conducted on 40 rats divided into 2 groups, namely 20 as a treatment group and 20 as a control. This research was carried out at the integrated Biomedical Laboratory, Faculty of Medicine, Udayana University, Denpasar.

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. The affordable population contained in the Pharmacology Laboratory during the study period amounted to 40 mice. Sampling is done through a simple random sampling technique using a random number table in the Microsoft Excel for Windows program. Furthermore, mice in column A numbered 1-20 were put into the treatment group with Autologous PRP administration, and mice in column B were given numbers 21-40 were put into the control group without Autologous PRP administration.

Distribution of Research Subjects

In this study, the average age of mice when the study began in the treatment group was 62.95 days, while in the control group was 62.45 days. There was no significant difference in mean age between the treatment and control mice at the time the study began. The t-test gets p = 0.288.

Table 1 Demographic characteristics of treatment and control groups

Characteristic	Treatment Group (N=20)		Control Group (N=20)		
	Average	SD	Average	SD	p*
Age (days)	62.95	1.31	62.45	1.60	0,288
Initial BB (grams)	211,24	1.73	211,85	1.61	0,257
Final BB (grams)	227,69	1.36	228.30	1.36	0,152

The average body weight of mice from the treatment group when the study began was 211.24 grams, while the average body weight of the control group was 211.85 grams. There was no significant difference between the body weight of the mice from the treatment and control groups at the time the study began (p = 0.257). At the end of the study, the average body weight of the treatment group was 227.69 grams, while the average body weight of the control group was 228.30 grams. Similar to the beginning of the study, at the end of the study there was also no significant difference between the average body weight of mice from the treatment group and the control group. The t-test gets p = 0.152.

Effect of Autologous PRP Addition to In Vitro Fertilization Antagonist Protocol on Mouse Endometrial β3 Integrin Expression

In this study, examination of β3 integrin expression in the endometrium of rats from both research groups with immunohistochemical techniques. Based on the data normality test (Kolmogorov-Smirnov test), it was found that the H-score data integrin β3 of the treatment and control groups was abnormally distributed (p = 0.000), then the test used for data comparison was the Mann-whitney test.

Based on immunohistochemical examination of rat endometrial tissue, the average expression (H-score) of β3 integrin in the endometrial epithelium in the treatment group was 2.90 + 0.15, while in the control group the average H-score of β3 integrin was 1.54 + 0.26. The mean H-score in the treatment group was significantly higher than in the control group. The Mann-Whitney test gets p<0.001.

Table 2 Mean H-score Distribution of Integrin β3 Treatment and Control Groups

Rerata	Kelompok (n=20)	Perlakuan	Kelompok (n=20)	Kontrol	*P*
	Rerata	SD	Rerata	SD	
H-score integrin β3	2.90	0,15	1,54	0,26	<0,001*

Selanjutnya, dilakukan pengelompokkan H-score integrin β3 sebagai berikut: (a) ekspresi lemah (Hscore < 1,1), (b) ekspresi sedang (Hscore = 1,1 – 2), (c) ekspresi kuat (Hscore = 2,1- 3), dan (d) ekspresi sangat kuat (Hscore = 3.1 – 4). Uji Chi Square kemudian digunakan untuk membandingkan ekspresi integrin antara kelompok perlakuan dengan kelompok kontrol. Pada kelompok perlakuan, keseluruhan 20 tikus (100%) memiliki ekspresi integrin β3 kuat-sangat kuat dan tidak ada tikus yang memiliki ekspresi integrin β3 lemah dan sedang.

Sebaliknya, pada kelompok kontrol, 18 tikus (90%) memiliki ekspresi integrin β3 lemah dan sedang dan hanya 2 tikus (10%) yang memiliki ekspresi kuat-sangat kuat. Dengan Uji Chi Square didapatkan bahwa nilai χ^2=32.72 dan nilai p < 0,001. Hal ini menunjukkan bahwa ekspresi integrin β3 lebih tinggi secara bermakna pada kelompok perlakuan (p<0,05). Dimana penambahan autologous PRP pada protokol antagonis FIV dapat secara bermakna meningkatkan ekspresi kuat-sangat kuat integrin β3 pada epitel endometrium tikus.

Table 3 Differences in β3 Integrin Expression between Treatment Groups with Control Group

Kelompok	Ekspresi Integrin β3		χ^2	*p*
	Lemah Sedang	Kuat Sangat Kuat		
Perlakuan	0	20		
			32.72	<0,001

Table 3 shows the results of immunohistochemical examination of integrin β3 in rat endometrial epithelium. The intensity of β3 integrin staining is indicated by brown color in the endometrial epithelium. The gradation of coloring intensity is expressed as 0=uncolored/negative, 1=weak intensity, 2=moderate intensity and 3=strong intensity. The H-score is calculated by the following formula:

$$\text{H-score} = \Sigma Pi\ (i + 1)$$

where Pi is the proportion of colored cells in each intensity category, represents the intensity of staining (Budwit-Novotny, et al., 1986; Lessey, 2000; Cassals, 2012).

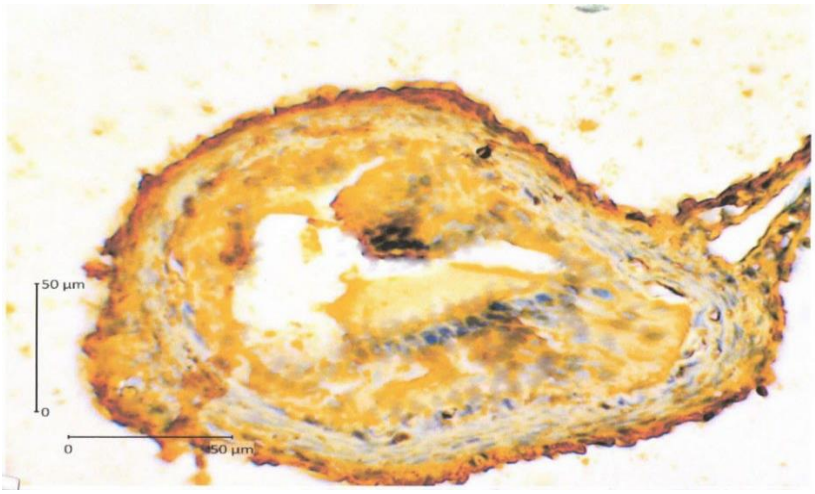


Figure 1 Integrin expression β3 treatment group (sample no 8).

Description: H-score = 3.24. The expression is very strong. Of the 279 epithelial cells, 112 epithelial cells were stained with strong intensity, 123 epithelial cells were stained with moderate intensity. 44 colored epithelium with weak intensity, 11 uncolored epithelium 400 X magnification. Description: intensity 0 = absent, 1 = weak, 2 = medium, 3 = strong.

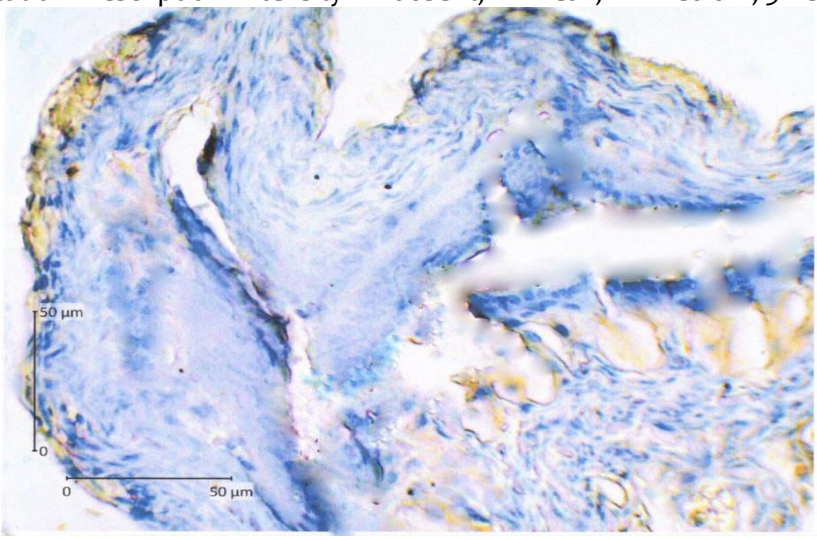


Figure 2 Integrin expression β3 control group (sample no 19).

Description: H-score = 1.21. Weak expression. Of the 137 epithelial cells, 2 epithelial cells were stained with strong intensity, 0 epithelial cells were stained with moderate intensity, 23 epithelium were stained with weak intensity, 112 epithelium were not stained. Magnification 400 X. Description: intensity 0 = absence, 1 = weak, 2 = medium, 3 = strong.

Characteristics of Demographic Data

The average age of female rats in the treatment group was 61.9 days and in the control group 62.5 days belonging to the adult stage. Rodents older than 60 days are classified into the adult stage, where growth and development have been completed (Barbieri et al., 2021). The body weight of the mice was affected by age, sex, and diet. (Sulistiani, Putra, Rahmanto, Fahrizqi, & Setiawansyah, 2021) In this study, the increase in average body weight in mice in both groups from the start of the study to the end of the study reflected the good health

condition of the study subjects. It is known that weight, age and stage perkembangan hewan percobaan dapat mempengaruhi hasil penelitian. (Jackson et al., 2017; Kilkenny et al., 2009).

Effect of Platelet-Rich Plasma on β3 Integrin Expression

The process of embryo implantation for successful pregnancy involves the adhesion of the trophoblast to the receptive endometrium. Significant changes in the expression of adhesion molecules in the endometrium have an effect on endometrial reception. Increased expression of integrin Vβ3, as an important marker of endometrial reception, has been reported in the luminal epithelium during the implantation window.(Cronin et al., 2019).

In detecting extracellular matrix proteins, cell adhesion molecules in the form of integrins are important. Integrins can trigger the production of angiogenesis factors, mediate cell adhesion with the extracellular matrix, and increase the receptivity of the endometrium (Abbott et al., 2009). Defects in endometrial receptivity can develop for reasons other than inadequate progesterone production. It has been shown that the expression of vβ may be influenced by other factors, such as cytokines or growth factors that may also cause cases of subfertility. (Thomas, 2003).

Integrins are one of the best biomarkers of uterine acceptance, and their role in the implantation process has been widely studied. The β3 integrin is expressed by epithelial cells in the area of the proliferating endometrium. Increased regulation of β3 integrins by blastocysts has been demonstrated in human endometrial epithelial cell cultures, possibly mediated by the embryonic IL-1 system.(Ma, Chen, Li, & Huang, 2016) Elnaggar et al. in their study reported that the expression level of the integrin Alpha-v Beta-3 was significantly lower in the endometrium of sampled patients with unexplained infertility.

This suggests that deficient expression of Alpha-v Beta-3 integrins in the endometrium may be associated with poor uterine receptivity and that these integrins play an unknown cause of infertility. (Elnaggar et al., 2017). The use of PRP in IVF is new, first published in 2015. (Chang et al., 2015) Until now, the molecular mechanism underlying the influence of platelet-rich plasma (PRP) in increasing endometrial receptivity has not been fully elucidated. In this study, PRP administered intrauterine had a significant effect on increasing the expression of β3 integrins in the endometrium of Wistar rats so that it can be concluded that increasing the expression of β3 integrins can be one of the mechanisms of increasing endometrial receptivity.

PRP contains high platelet concentrations that supply growth factors that are essential to provide regenerative stimulus to tissues with low potential for healing. Several studies and randomized controlled trials report that PRP administration is effective in promoting endometrial growth and pregnancy in patients with thin endometrium. (Agarwal et al., 2020; Coksuer et al., 2019; Du et al., 2020; Wang et al., 2019)

Research in Korea by Hang-Yong Jang et al. has been conducted to determine the therapy of autologous platelet-rich plasma (PRP) can increase regeneration from endometrial damage in experimental models induced by ethanol. Sixty Sprague-Dawley rats divided into control group, ethanol group and PRP-treated group, hematoxylin-eosin (H&E) and Masson trichrome (MT) staining analyses confirmed a significant reduction in fibrosis and increased cell proliferation in the PRP treated group, compared to other groups.

Penelitian ini menunjukan administrasi dari PRP menggunakan efek proliferasi dan anti-fibrotik pada endometrium yang rusak. PRP diketahui mengandung beberapa growth factor dan sitokin yang dapat membantu percepatan dari proliferasi sel, angiogenesis, dan migrasi sel, menghasilkan penyembuhan dan regenerasi jaringan yang cepat. Mayor Growth factor

In large numbers, platelet-derived growth factor has demonstrated an important role in cell proliferation in the endometrium. (Jang et al., 2017)

A systematic review and meta-analysis by Maleki-hajiagha et al. showed that administration of Autologous PRP in patients undergoing frozen-thawed embryo transfer improved pregnancy rates. Of the seven studies, there were two studies that assessed endometrial thickness after administration of Autologous PRP with the results of a significant increase in endometrial thickness with an average thickness difference of 0.94 mm ($p < 0.001$). Similar results were obtained at the implantation rate, where there was a significant increase in implantation rates in patients receiving PRP compared to controls.(Sepidarkish et al., 2020)

Wang et al.'s research found that Autologous PRP significantly stimulated the growth, migration, and adhesion of endometrial mesenchymal stem cells (EnMSCs) when compared to the control group. Data showed that endometrial expansion and pregnancy occurred in 12 out of 20 patients after Autologous PRP infusion, resulting in a pregnancy rate of 60%. Despite this, the molecular mechanism of EnMSC proliferation is also still unknown. (Wang et al., 2019)

In addition to the stimulation of migration of human primary endometrial epithelial cells, endometrial stromal fibroblasts, and EnMSCs, several other mechanisms have been proposed that are thought to explain the relationship of PRP with increased endometrial receptivity, including: (1) Regulation of proliferation, apoptosis, inflammation, cell adhesion, chemottaxis, and immune response during blastocyst implantation, (2).

Stimulation of cell regeneration, proliferation and vascularization by several growth factors, such as VEGF, TGF-b, PDGF, IGF1, EGF, HGF, (3) Cell migration through the process of chemoattraction, differentiation of mesenchyma into epithelium and inflammation, and (4) Stimulation of expression of pro-inflammatory cytokines (IL1A, IL1B, IL1R2), chemokines (CCL5, CCL7, CXCL13), and matrix metalloproteins (MMP3, MMP7, MMP26). (Maleki-Hajiagha et al., 2020).

CONCLUSION

This study proved that administration of Autologous Platelet Rich Plasma (PRP) on the In Vitro Fertilization antagonist protocol caused higher expression of β3 integrin in the endometrium of wistar strain rats compared to without Autologous PRP administration.

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