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The Differences of Integrin αvβ3, Leukemia Inhibitory Factors Expression and Superoxide Dismutase Serum Concentration in the Provision of Kebar Extract *(Biophytum petersianum Klotczh)*, Metformin, and Their Combination to Mouse models of Endometriosis

Seindy Glamour[1], Syarief Thaufik Hidayat[1], Adi Setyawan Prianto[1], Widjiati[2]

[1]*Department of Obstetrics and Gynecology Faculty of Medicine, Diponegoro University/ Dr. Kariadi General Hospital- Semarang*
[2]*Faculty of Veterinary Medicine, Airlangga University, Surabaya*.

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Abstract

Background: Papuan use Kebar grass for fertility issues and the use of Kebar grass as cattle feed is also known to be effective to increase the number of livestock because it may improve endometrial receptivity. We use endometriosis model as the disease decrease endometrial receptivity. Kebar grasses and metformin are expected to improve the condition of endometrial receptivity from different pathway, interpreted from the difference of Integrin αvβ3 expression, Leukemia Inhibitory Factors expression and Superoxide dismutase serum concentration.

Objective: To find out the effect of giving Kebar grass extract and metformin on endometrial receptivity

Methods: The study was a simple randomized experimental study. Samples were 60 Balb/c mice grouped into five groups, including control normal mice, one group of endometriosis mouse models given placebo, group of endometriosis mouse models given Kebar grass extract, group of endometriosis mouse models given metformin, and group of endometriosis mouse models given the combination of Kebar grass extract – metformin. The immunohistochemistry of αvβ3 integrin and Leukemia Inhibitory Factors expressions were performed from the endometrium of the uteri and measured by the Rammele Scale Index (Immuno Reactive Score), while the Superoxide Dismutase examination using ELISA was derived from mice serum. These examinations were performed by two veterinarians

Results: The expression of αvβ3 integrin was significantly higher in the group of mouse models given Kebar grass extract as well as the combination of Kebar grass extract and metformin ($p < 0.05$) rather than in the group of endometriosis mouse models with no treatment, whereas LIF expression was significantly higher in the group given Kebar grass extract, metformin and combination of both extracts ($p < 0.05$). Serum SOD levels remained the same.

Conclusion: The expressions of αvβ3 Integrin and LIF are higher due to the provision of Kebar grass extract as well as the combination of Kebar grass extract and metformin

Keywords: Integrin αvβ3; Leukemia Inhibitory Factor; Superoxide Dismutase; Kebar grass; Metformin

INTRODUCTION

Infertility caused by endometriosis can be associated with several mechanisms like anatomical disorders in adnexa that block or interfere the process of capturing ovum during ovulation, impairment of oocyte development or embryogenesis, as well as

decreased endometrial receptivity. Impaired endometrial receptivity is notified by decreased receptor markers such as integrin αvβ3 (integrin) and Leukemia Inhibitory Factor (LIF).[1]

Traditionally, to overcome infertility, people have used various materials from nature. Kebar grass is assumed to be originally from Mali (West Africa). It is widely used in traditional treatments including wound healing, cerebral antimalarial and also antihypertensives proven by joint research as well as its anti-tumors, insulinotropic and hypochestrolemic effects.[2] Papuan use Kebar grass for fertility issues. The use of Kebar grass as cattle feed is also known to be effective to increase the number of livestock[3] most likely because of the content of antioxidants and saponins that help produce steroids.[4] Previous research data showed that providing Kebar grass extract can also increase the level of 17β-estradiol in mice blood. According to previous research, estradiol increases in accordance with the presence of thicker endometrium.[5,6] The increase of estradiol in endometriosis may affect integrin αvβ3, which later influences endometrial receptivity.[7] This condition forces the need to examine whether Kebar grass which is believed to improve reproductive function can also be implemented in the condition of endometriosis, especially in terms of endometrial receptivity.

Metformin is a drug derived from plant that is widely known to be diabetes drug. It also has antioxidant, anti-inflammatory, and anti-proliferative, as well as to control other steroidogenic acute regulatory protein (StAR) in steroidogenesis to produce estradiol. Metformin is known to affect the ovaries, especially to regulate progesterone production and basal 17-estradiol and also to stimulate insulin. This supports the therapeutic potential of endometriosis in which it helps the regression of endometriosis lesions that may progressively increase pregnancy rates in endometriosis-related infertility.[8,9] Although it supports endometriosis regression, considering its effect on estradiol and progesterone which surely influences endometrial condition, the examination of further effect of metformin on endometrial receptivity of endometriosis cases needs to be done.

In this study, endometrial receptivity function will be assessed from endometrial αvβ3 integrin expression and Leukemia Inhibitory Factor (LIF) expression. In addition, we also would like to figure out the result when both are consumed together considering they may be complementary to each other.

As being known, the etiology of endometriosis is also multifactorial, among others most likely are the oxidative stress of estrogen and inflammation.[10] Chronic inflammation of endometriosis can lead to endometrial receptivity disorders as well as oxidative stress which further interferes with reproductive function.[11] The two substances studied namely Kebar grass and metformin are also suspected to have anti-oxidant and anti-inflammatory effects, so we also want to figure out whether the two substances also play a role as a comparable antioxidant which will be examined through Superoxide Dismutase (SOD).[4, 12-15]

In this study, we used endometriosis mice models and given therapy with the extracts of Kebar grass 3 mg / day and metformin 4.0 mg / day.

METHODS

This experiment employs simple experimental randomization in mice and has acquired ethical approval from The Institional Review Board (IRB). The research was conducted at the Obstetrics and Gynecology Fertility Division of Reproductive Endocrinology and Pharmacy Department of Diponegoro University and the examination was conducted at the Faculty of Veterinary Medicine of Airlangga University. The samples were Balb/c female mice aged 3 months weighing 15-20 grams. Sixty female mice were determined to be involved in the research during the stage of sample calculation. Those mice were divided into five groups, each group with 12 mice: control group with normal mice (K), group of endometriosis mouse models given placebo (P1), group of endometriosis mouse models given Kebar grass extract (P2), group of endometriosis mouse models given metformin (P3), and group of endometriosis mouse models given the combination of metformin - Kebar grass extracts (P4). The making of mouse models of endometriosis started from uterine endometriosis tissue which was intraperitoneally injected before cyclosporine injections and estrogen injections were given. The supplementation was gavaged into the endometriosis treatment group for 2 estrous cycles on day 14. On the first day of the third cycle, 6 mice from each group were examined to determine the estrous cycles through microscopic examination of vaginal sweeps. When it came to be estrous phase, 6 mice from each group were mated while the remaining 6 were given ether as anesthesia which has less effect on Superoxide Dismutase (SOD) serum before taking serum samples from their hearts for SOD serum examination with ELISA method.[16] These mice were observed in the next day for the presence of vaginal plug. Mice with vaginal plug were considered as a false pregnancy and followed. Sampling was done on the 4th day of the 3rd estrous cycle. Mice were killed by cervical dislocation, then the uterine organ was taken for immunohistochemical examination. The espression of αvβ3 integrin and Leukemia Inhibitory Factor were measured by using the Rammele Scale Index (Immuno Reactive Score) and examined by two blinded veterinarians.[17]

RESULTS

Integrin αvβ3 Expression

Expression of endometrial tissue of Balb/c endometriosis mouse models in the study group can be seen in Figure 1. It showed that the lowest αvβ3 integrin expression was found in the placebo group (P1) and the highest was in the P4 group. The statistical analysis (Table 1; Figure 2) shown a significant difference in αvβ3 integrin expression among all study groups ($p = 0.001$).

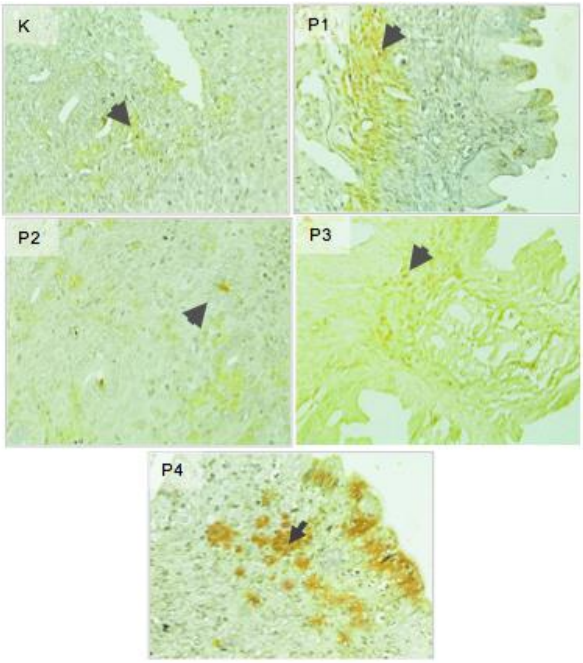

Figure 1. Comparison of Integrin αvβ3 expression with different color intensity. Color intensity from yellowish to chromogenic brown (arrows) on endometrial and uterine gland cells between the treatment groups (histochemical immuno-staining, 400x enlargement, Nikon H600L microscope, DS-Fi2 300 megapixel camera)

The result of comparison between group with Mann-Whitney test showed that the αvβ3 integrin expression of group K was significantly higher than P1 (p = 0.004). Furthermore, the expression of αvβ3 integrin group P1 was significantly lower than P2 (p = 0.004) and P4 (p = 0.002) and was not significantly different than P3 (p = 0.06). Thus, the αvβ3 integrin expression was higher in groups P2 and P4 compared to P1. αvβ3 integrin expression of P2 was significantly lower than P4 (p = 0.002). Similarly, P3 expression was also significantly lower than P4 (p = 0.01).

LIF expression

LIF expression from endometrial tissue of Balb / c mouse models of endometriosis in this study group, as shown in Figure 3, indicated that the lowest average was in the placebo group and the highest average was in the P4 group. The result of statistical test in Table 2 showed a significant difference in the expression of LIF among all the study groups (p = 0.006).

Mann-Whitney test results showed that LIF expression of control group (K) was significantly higher than P1 (p = 0,009). Subsequently, the expression of LIF of P1 group was significantly lower than P2 (p = 0,002), P3 (p = 0,002) and P4 (p = 0,002). Thus, LIF expression of endometrial tissue of Balb / c endometriosis mouse models was higher in the group given Kebar grass extract (P2), metformin (P3), and their combination (P4) compared to placebo / endometriosis without any therapy (P1) as shown in Figure 4.

Table 1. Integrin αvβ3 expression of Balb/c mice endometrial tissue (n=30)

Group	Mean±SD	Median	(Min-Max)	p*
Control	2,8±1,73	2,0	(1,6 - 6,0)	0,001
P1 (Placebo)	1,1±0,30	1,1	(0,8 - 1,6)	
P2 (Kebar)	1,9±,27	1,9	(1,4 - 2,2)	
P3 (Metformin)	2,4±,93	2,7	,(6,0 - 3,0)	
P4 (Kebar+Metformin)	4,9±2,04	4,2	(2,8 - 8,6)	

*Kruskall-Wallis Test

The result of comparison between group with Mann-Whitney test:

K vs P1: p=0,004*	P1 vs P2: p=0,004*	P2 vs P3: p=0,06
K vs P2: p=0,6	P1 vs P3: p=0,06	P2 vs P4: p=0,002*
K vs P3: p=0,8	P1 vs P4: p=0,002*	
K vs P4: p=0,06		P3 vs P4: p=0,01*

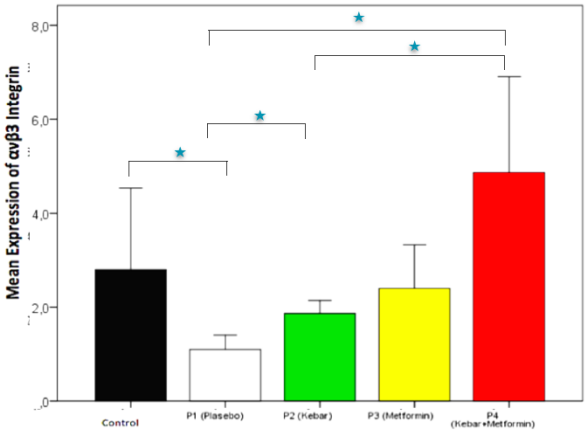

Figure 2. Comparison of αvβ3 Integrin expression of endometrial tissue of Balb/c endometriosis mice model (n = 30). The ★ sign shows p<0.05)

Serum SOD level

Based on SOD level of Balb/c mouse models of endometriosis, the lowest serum level was found in group P4 and the highest was in the control group. The result of statistical test in Table 3 showed a significant difference in SOD levels among overall study groups (p = 0.007). SOD level of group P2 was significantly higher than group P4 (p = 0.01).

The result of comparison between group with Mann-Whitney test showed that SOD level of group K was quite different from P1 (p = 0,03), P2 (p = 0,01), P3 (p = 0,004) and P4 (p = 0,002). SOD level of Balb / c control group mice was higher than Balb / c mouse models of endometriosis, both in placebo group and group with Kebar grass extract, metformin, and their combination as shown in Figure 5.

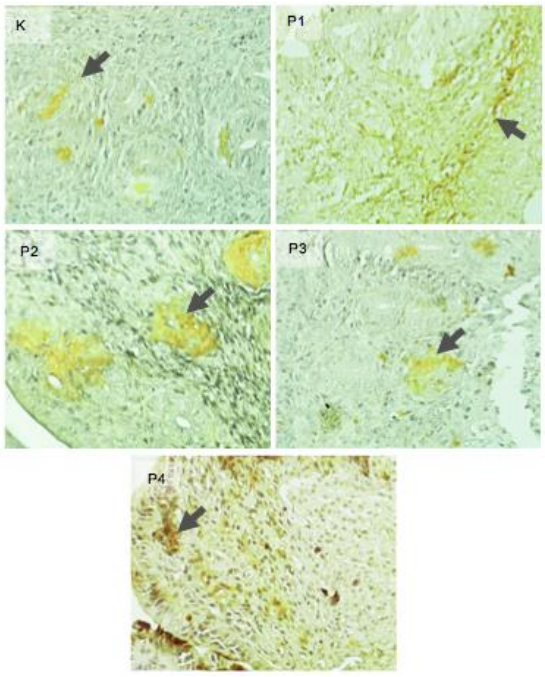

Figure 3. Comparison of LIF expression with different color intensity. Color intensity from yellowish to chromogenic brown (arrows) on endometrial and uterine gland cells between the treatment groups (histochemical immuno-staining, 400x enlargement, Nikon H600L microscope, DS-Fi2 300 megapixel camera).

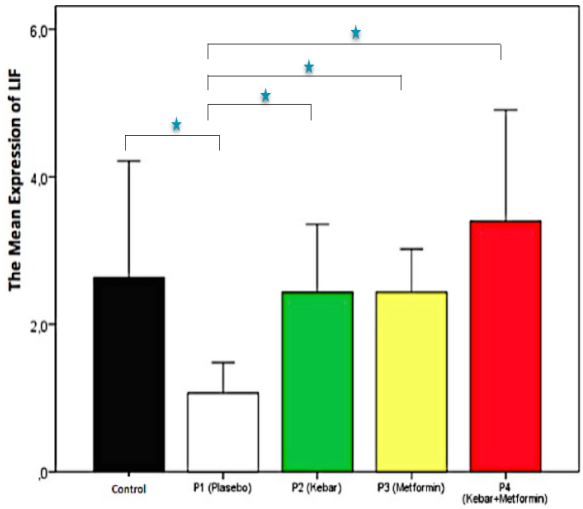

Figure 4. LIF expression comparison of endometrial tissue of Balb / c mice model of endometriosis (n = 30). The ★ sign shows p<0.05).

Table 2. LIF expression of Balb/c mice endometrial tissue (n=30)

Group	Mean±SD	Median	(Min-Max)	p*
Control	2,6±1,58	2,0	(1,4 -5,6)	
P1 (Placebo)	1,1±0,41	1,1	(0,6 -1,6)	
P2 (Kebar)	2,4±0,92	2,0	(1,6 -3,6)	
P3 (Metformin)	2,4±0,59	2,3	(1,8 -3,2)	0,006
P4 (Kebar+ Metformin)	3,4±1,51	3,3	(1,6 -5,8)	

*Kruskall-Wallis Test

The result of comparison between groups with Mann-Whitney test:

K vs P1: p=0,009*	P1 vs P2: p=0,002*	P2 vs P3: p=0,8
K vs P2: p=0,8	P1 vs P3: p=0,002*	P2 vs P4: p=0,2
K vs P3: p=0,7	P1 vs P4: p=0,002*	
K vs P4: p=0,2		P3 vs P4: p=0,3

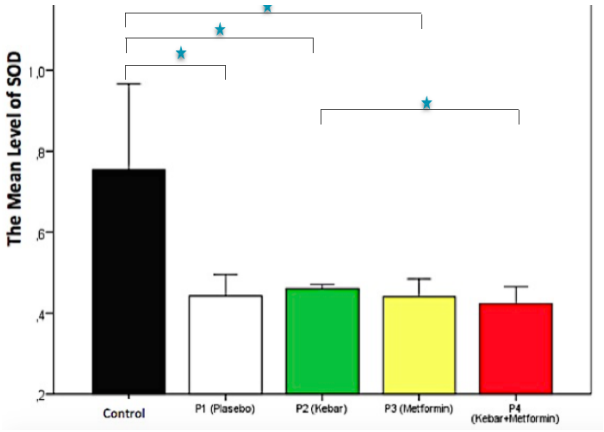

Figure 5. Serum SOD levels of Balb / c mice (n = 30). The ★ sign shows p<0.05).

Group	Mean±SD	Median	(Min-Max)	p*
Control	0,75±0,213	0,88	(0,46 -0,92)	
P1 (Placebo)	0,44±0,053	0,46	(0,36 -0,50)	
P2 (Kebar)	0,46±0,010	0,46	(0,44 -0,47)	
P3 (Metformin)	0,44±0,044	0,45	(0,36 -0,49)	0,007
P4 (Kebar+ Metformin)	0,42±0,042	0,43	(0,34 -0,46)	

*Kruskall-Wallis Test

The result of comparison between group with Mann-Whitney test:

K vs P1: p=0,03*	P1 vs P2: p=0,8	P2 vs P3: p=0,2
K vs P2: p=0,01*	P1 vs P3: p=0,7	P2 vs P4: p=0, 01*
K vs P3: p=0,004*	P1 vs P4: p=0,4	
K vs P4: p=0,002*		P3 vs P4: p=0,3

DISCUSSION

Dysregulation in a large number of genes in endometriosis causes various immune-related disorders, inflammation, and oxidative stress.[18] One of them also causes the failure of estrogen receptor expression whose gene dysfunction results in excessive activity causing various conditions like immune, inflammatory, or hormonal disorders. Furthermore, endometriosis can decrease endometrial receptivity as indicated by a significantly decreased expression of both αvβ3 integrin and LIF in group P1 (placebo) in which endometrial tissue of mouse models of endometriosis were compared with control group of normal mice. It is consistent with other studies on endometrial receptivity in endometriosis and it can be the reference to assess the effects of metformin and Kebar grass provision in enhancing endometrial receptivity. From this study, we found higher endometrial receptivity after treatment by metformin, Kebar grass extract, and the combination

of metformin and Kebar grass extracts in mouse models of endometriosis.

Some studies have shown improvement in endometriosis and even the reduction of endometriosis implants by metformin.[19-21] The broad effects of metformin are assumed to be due to the function of metformin in AMP kinases to prevent prostaglandins and inflammatory reactions.[22] Metformin administration in mouse models of endometriosis in this study resulted in higher endometrial receptivity, whereas the result of the LIF expression examination in this study influenced by immunity and inflammation was found to be higher in LIF expression than in the placebo group.

Research on normal and metformin-treated mice did not indicate any difference in terms of the average of LIF expression. However, in endometriosis-implanted mouse models, there was an implant regression due to metformin administration. Abnormal steroid environment like the high estrogen bioavailability of endometrial stromal endometriosis eventually suppresses the expression of αvβ3 integrin and LIF.[10] It is recognized that metformin decreases the activity of aromatase enzymes and improves the hyperandrogenic environment by increasing SHBG levels including epithelium and stroma, thereby decreasing estradiol levels in circulation and endometrium. [20] Research conducted in endometrial stromal cells by measuring StAR mRNA (Steroidogenic acute regulatory protein) found an increase in StAR mRNA in endometrial stromal cells.[9] Metformin decreases steroid acute regulatory protein (StAR) expression that is PGE2-stimulated by preventing the translocation of *cAMP response element binding protein regulated transcription coactivator 2* (CRTC2) core that is commonly PGE2-induced. This is done by metformin by increasing the phosphorylation of AMP-activated protein kinase (AMPK), so that no complex CREB-CRTC2 is formed. StAR in the process of steroidogenesis plays a very important role of providing a continuous supply of cholesterol for estradiol production. StAR transports cholesterol to mitochondria where cholesterol is converted into pregnenolone, which is then converted to progesterone. Furthermore, androstenedione is converted to estrone and then estradiol. Metformin administration results in the decrease of aromatase activity-dependent stromal cell capacity that converts androstenedione to estrone. Estradiol production decreased significantly, while pregnenolon and progesterone declined slightly.[9] It suggests the need to have more than this mechanism in endometriosis to improve the αvβ3 integrin.

DNA synthesis as a marker of proliferation also decreases the effect of metformin to regulate and activate AMP-activated protein kinase (AMPK) which then inhibits the mammary target of Rapamycin via tuberous sclerosis 2 (TSC2) protein. [9, 23, 22]Metformin also inhibits the production of Reactive Oxygen Species (ROS) thereby limiting pro-inflammatory IL-1β induction and causing macrophages to produce anti-inflammatory cytokines such as IL-10 and TGF-β.29,31,32. This may result in higher LIF expression on metformin administration.

LIF is an example of how hormones act through cytokines.[24] LIF is produced by endometrial epithelial cells, NK cells and Th2-like cells. Factors like interleukin 1α, TGF, PDGF, EGF, induce LIF while inflammatory factors such as RANTES, VEGF, prostaglandins in endometriosis decrease LIF. Besides what has been mentioned previously, metformin functions to suppress the inflammatory response and seem to fix it. Therefore, LIF expression was higher in the group of mouse models of endometriosis given metformin.

The expression of LIF in the group given Kebar grass extract is also higher which means that metformin and Kebar grass extracts are comparable in terms of suppressing inflammation. Kebar is known to contain saponin compounds, namely glycosides with steroid or triterpene nuclei that have extensive pharmacological effects such as anti-inflammatory, immunomodulators, anti-virus, anti-oxidants and anti-tumors.[25] Amentoflavones as well as prosianidin obtained from the genus Biophytum are known not only to suppress COX-2 expression and also prevent NF-kB which is activated by proinflammatory cytokines such as IL-1 and TNF-α.[2, 25] According to several studies, *Biophytum petersianum* Klotzsch is able to induce especially the activation of dendritic cells and also the response to T cells, B cells, and NK cells related to the role of saponin content in inducing the production of cytokines, interleukins, and interferons.[15, 26] The effect of Biophytum on apoptosis as an anti-tumor based on the previous research was gotten by regulating Bcl-2, Caspase-3 and p-53 genes and the production of inflammatory cytokines and tumor activated macrophage.[25, 27] Saponin extract of in vitro studies used human endometrial stromal cells from post-surgical patients with a diagnosis of endometriosis and it was known to able to induce apoptosis in endometrial cells through the modulation of miR-21-5p.[28] This may explain that the higher expression of LIF in the group of mouse models of endometriosis is caused by giving Kebar grass extract due to the way it suppresses inflammation and repairs endometriosis lesions.

Overall, giving Kebar grass extract to the mouse models of endometriosis improves the endometrial receptivity. There was higher αvβ3 integrin expression than the placebo group. The suppression of inflammation and increase of apoptosis may enhance the expression of integrin αvβ3. However, it seems that there is also a hormonal effect that causes higher αvβ3 integrin expression in the administration of Kebar grass extract compared to when metformin is given.

Kebar grass extract is known to cause an increase in 17β-estradiol, which has been abundant in endometriosis. Endometriosis due to aromatase activity increases estron levels. The type-1 IIβ-HSD enzyme in endometriosis lesions transforms estrone into estradiol but there is no 17β-HSD type II expression that inactivates estradiol to convert estradiol to estrone. [29] The estradiol environment suppresses αvβ3 integrin expression so that it appears

more difficult to obtain achievements expression such as LIF. [30] [31]

The regulation of integrin expression is very complex. Integrin enhancement is associated and synchronized with the period in which serum progesterone concentration increases in the met estrus phase of the estrous cycle. It appears that the expression of integrin in the endometrial stromal is affected by progesterone, whereas estrogen decreases the expression of αvβ3 integrin. Mice experienced bilateral oophorectomy and treated with progesterone have been found to have integrin molecule expression. [32]

The study shows the probability of some increase in progesterone. Research on saponin in Korean ginseng found that saponin significantly increases the levels of pre-ovulatory and post-ovulatory progesterone serum.[33] This is thought to be due to increased follicular and oocyte repair thereby resulting in multiple ovulation. This is supported by a PMSG-like substance produced by Kebar grass extract which makes substance similar to FSH-LH which further increases folikulogenesis as well as ovulation in mice.[34, 35] Eventually, progesterone increases. The improvement in the balance of estrogen-progesterone receptors in the endometrium may favor higher expression of αvβ3 integrin as well as LIF even though its expression is not directly related to progesterone level. LIF acts as the mediator for the effects of progesterone on inflammatory condition. High level of progesterone has an anti-inflammatory effect that increases LIF mediated by IL-4 and support implantation.[36]

Steroid receptor balance disturbance is suffered by endometriosis patients. Estrogen receptors are generally decreased during implantation, but in women with endometriosis, estrogen receptors increase.[37] In fact, increasing progesterone relative to estrogen is a must for good endometrial receptivity.[18] This balance disorder is in the form of increased estrogen beta (ERβ) receptor to more than 100 times the normal condition due to methylation deficiency of the promoter ERβ causing excessive expression. Excessive ERβ expression will suppress alpha estrogen receptors (ERα) and lead to reduction of progesterone receptors especially PR-B that results in progesterone resistance in endometriosis and increased levels of cyclo-oxygenase 2 which is closely related to inflammation.[37, 38] The content of isoflavones in Kebar grass needs to be examined further. Isoflavones are known to able to bind estrogen receptors with higher affinity on estrogen-β receptors although its affinity is much lower than estradiol. Based on some studies, flavonoids in Kebar grass can bind estrogen-α receptors and modulate estrogen-α receptor signals. In addition, daidzein content of Kebar grass has more affinity in ERβ that may help achieve balance of estrogen-progesterone receptors besides its anti-inflammatory effect though it can support apoptosis.[39] Furthermore, these things will also lead to higher LIF expression supported by improved endometriosis.

The statements above are strengthened by the achievement of αvβ3 and LIF integrin expressions on the combination of Kebar and metformin grass extracts. The expression of αvβ3 and LIF integrin is similar to the control group, even it tends to be higher than the control group. The same job metformin and Kebar grass do is in terms of suppressing inflammation and inducing apoptosis in favor of hormonal balance uptake and steroid receptors that seem to provide better expression performance.

The superoxide dismutase serum (SOD) level was lower in all groups of mice with endometriosis compared to SOD level of normal mice as controls. This supports an increase in oxidative stress in endometriosis. Increased oxidative stress is known to cause a very high reactive oxygen species (ROS) so that superoxide dismutase (SOD) level is decreased. Increased ROS stimulates PGF2α secretion through activation of nuclear factor kappa β (NFk β) supported by ineffective antioxidant system causing normal cells damage. [40]

SOD level average in the placebo group (mouse models of endometriosis without treatment) was similar to the treatment group. Neither metformin nor Kebar grass extracts provision in this study increased SOD serum.

Different result was obtained in the study using biophytum sensitivum extracts on mouse models for ulcerative colitis by giving 3% acetic acid. This indicates an increase of SOD level in colon tissue. The result of the study demonstrates the role of the biophytum class of plant as antioxidant as well as anti-inflammatory by suppressing TNF-α, IL-1β and IL-6.[41] Similarly, SOD level of renal tissue increased after being administered by biophytum sensitivum extract in SOD test on mice treated as models of renal impairment.[42] The two studies above carried out examination on tissue proteins, both colon and kidney. Similarly, it also happened to metformin administration.[19, 43] Previous studies have shown that levels of SOD increased by administered metformin. Another possible cause of difference outcomes was due to short duration of therapy meaning that both actually worked on the tissue but had not shown any increase in serum yet. In addition, it may be caused by great impact of endometriosis as well as the administration of cyclosporine as immune suppressor and simultaneously as environmental contaminant factors in the induction of endometriosis tissue in mice. It may take longer to increase SOD level in the endometriosis lesion compared to the normal tissues. This possibility is supported by the higher level of SOD in control group as a defensive mechanism after ether was given as anesthesia. [16, 44, 45]

The weakness of this study is the absence of toxicity test against Kebar grass so that it was done to experimental animals. Yet, the whole picture of this study shows that the administrations of metformin, Kebar grass extract, and a combination of metformin and Kebar grass extract supply better effect on endometrial receptivity.

CONCLUSION

The expression of integrin αvβ3 in endometrium is higher when mice were exposed to Kebar grass extract and the combination of both metformin and Kebar grass extract, whereas the expression of LIF is higher through the exposure to Kebar grass extract, metformin and combination of Kebar and metformin grass extract.

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