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THE EFFECT OF MUCİN-1, GLYCODELİN-A, LEUKEMİA INHİBİTORY FACTOR, INTERLEUKİN-15 AND GRANULOCYTE COLONY STİMULATİON FACTOR IN THE UNEXPLAINED RECURRENT PREGNANCY LOSS - A CASE CONTROL STUDY

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ABSTRACT

Purpose: To examine the effect on recurrent pregnancy loss of Mucın-1, Glycodelin-a, Leukemia Inhibitory Factor, Interleukin-15 and Granulocyte Colony Stimulation Factor blood serum levels and for those with the greatest effect to be able to contribute to alternative treatment approaches.

Materials and Methods: The study was conducted between November 2016 and December 2016 in the Obstetrics and Gynaecology Clinic of Evliya Celebi Training and Research Hospital, Dumlupinar University Medical Faculty. A total of 87 patients were included, comprising 42 patients who had experienced at least 2 consecutive pregnancy losses before the 12th week, and a control group of 45 patients with at least 1 live birth and no pregnancy loss. Examination was made of the blood plasma levels of Mucın-1, Glycodelin-a, Leukemia Inhibitory Factor, Interleukin-15 and Granulocyte Colony Stimulation Factor.

Results: In the patient and control groups the Mucin-1 plasma levels were determined as 0.88±0.45 ng/ml and 1.17±0.57 ng/ml (p=0.008) respectively, Glycodelin-a as 23.73 ng/ml and 25,92 ng/ml (p=0.006). Leukemia Inhibitory Factor as 64.59 pg/mL, and 72.14 pg/mL (p=0,011), Interleukin-15 as 38.57 ng/ml and 31.44 ng/ml (p=0.013), and Granulocyte Colony Stimulation Factor as 13.07 pg/mL and 14.11 pg/mL (p=0.056).

Conclusion: According to the results of this study, Mucın-1, Glycodelin-a, Leukemia Inhibitory Factor, and Interleukin-15 play an imprtant role in the pathology of RPL. Further studies conducted on these factors could be successful in the treatment of RPL.

KEYWORDS : Recurrent pregnancy loss, Mucin-1, Glycodelin-a, Leukemia Inhibitory Factor, Interleukin-15, Granulocyte Colony Stimulation Factor

INTRODUCTİON

Recurrent pregnancy loss (RPL) is defined as at least 2 consecutive spontaneous abortions before the 20th week of pregnancy with the same partner [1, 2]. The American Society of Reproductive Medicine (ASRM) has defined RPL as 2 or more consecutive pregnancy losses [3], whereas in the most recent guidelines of the Reproductive Health and Infertility Association, RPL is defined as 3 or more consecutive pregnancy losses [4]. In a study by Wilcox et al, a strong relationship was shown between early pregnancy loss in particular and endometrial receptivity [5]. These receptors play a role in the control mechanism during embryo implantation and this receptivity is thought to be imapired in patients with RPL [6].

Implantation of the blastocyst to the endometrium may only be possible for a very short period and this is known as the implantation window. As this is a very complicated event, both the embryo and the endometrial receptors must be well-synchronised [7].

Of these receptors examined in the current study, Mucin-1 (MUC-1) has been previously investigated in patients with recurrent implantation disorder applied with invitro fertilisation and both the tissue and blood levels were found to be lower compared to the control group [8]. In the same study, Glycodelin-A (GdA) levels were also found to be significantly lower both in tissues and blood in comparison with the control group.

In a study of endometrial biopsies of RPL patients, Leukemia Inhibitory Factor (LIF) was shown to be lower in the endometrium compared to a control group, but the difference was not reported to be statistically significant [9]. In another study that examined LIF and Interleukin-15 (IL-15) in the endometrial biopsies of patients with recurrent implantation disorder, lower levels were observed in the patients than in the control group [10].

Granulocyte Colony Stimulating Factor (G-CSF) is a hematopoietic cytokine which stimulates granulocyte proliferation and differentiation, produces the maternofetal interface during embryo implantation and is thought to have a role in decidual and placental functions [11, 12]. Some studies have shown that the application of G-CSF to patients with recurrent implantation disorder and RPL patients could be useful in respect of pregnancy outcomes [13-15].

From the starting point of the above-mentioned studies, the aim of this study was to compare the serum levels of MUC-1, GdA, LIF, IL-15 and G-CSF in RPL patients and in patients who had not experienced any pregnancy loss and had at least one live birth, and to be able to establish an alternative treatment approach based on the factors with the greatest effect.

MATERİALS AND METHODS

Patient Selection

The study was conducted in the Obstetrics and Gynaecology Clinic of Evliya Çelebi Training and Research Hospital of Dumlupinar University, Kutahya, Turkey. A total of 87 patients were included in the study as 42 RPL patients and 45 healthy patients. Approval for the study was granted by the Ethics Committee of Aydin Adnan Menderes University Medical Faculty Non-Interventional Ethics Committee (Decision no: 11/11/2016-11).

Patients presenting at the Obstetrics and Gynaecology Clinic of Dumlupinar University Medical Faculty with a history of recurrent pregnancy loss were informed about the study, and those who wished to participate and met the inclusion criteria were included as the study group. The inclusion and exclusion criteria are presented below in Section 2-2.

The control group was formed of patients selected from those who presented at the polyclinic, met the study inclusion criteria and wished to participate. Demographic data of age, abortus, parity and gravida were recorded. Medical histories were taken including medication use and chronic diseases.

Inclusion and Exclusion Criteria

Inclusion criteria for the RPL group:

1. At least 2 consecutive abortus,

2. Aged at least 18 years,
3. Maximum age of 35 years to discount reduced ovarian reserve,
4. No previous live birth.

Inclusion criteria for the control group

1. No history of pregnancy loss,
2. Age 18 years – 35 years,
3. At least one live birth.

Exclusion criteria for both the study group and the control group:

1. Uterine anomaly (septate, bicornate, unicorn uterus etc.),
2. Myoma uteris
3. Endometrial polyps
4. Endocrinological disorders (diabetes, polycystic ovary syndrome, thyroid function disorder)
5. Known chromosome anomaly that could cause RPL
6. Cigarette smoking and alcohol consumption
7. Active infection (as it could affect the molecule levels in the study)
8. Oligomenorrhea -amenorrhea with a history of abnormal uterine bleeding which could affect endometrial receptivity, or use of intra-uterine device or oral contraceptives
9. At least one diagnosis of thrombophilia

Blood Sample Collection

As the molecules to be studied are at the highest amount in the implantation window, blood samples were taken from all the participants in this period. The blood samples were taken one week before the expected menstruation as LH is at a peak 7-10 days later. After collection, the samples were centrifuged at 10,000 rpm/min for 10 mins and serums were obtained and placed in 2ml, sterile, clear Eppendorf cryo tubes. The samples were stored at -20˚C out of direct light until assay. Patients with hemorrhagic, icteric or lipemic serums were excluded from the study.

Biochemical Analysis

The samples were examined using the Enzyme-linked Immunosorbent Assay (ELISA) method in a private biochemistry laboratory by a medical biochemistry specialist.

MUC-1 was examined with the ELISA kit of Elabscience Biotechnology Co. Ltd., which uses the Sandwich-ELISA method. In this kit, the micro ELISA plate is first covered with antibody specific to human MUC-1. Standards or samples are added to the appropriate micro ELISA plate wells and combined with specific antibody. Then a determinant antibody with biotin, specific to human MUC-1 and Avidin-Horseradish Peroxidase (HRP) conjugate is added to each micro-plate and incubated. Free elements are washed and substrate solution is added to each well. Wells containing only human MUC-1, determinant antibody with biotin and Avidin-HRP conjugate are seen with a blue colour. The enzyme-substrate reaction is terminated by adding sulphuric acid solution and the colour becomes yellow. Optic density (OD) is measured spectrophotometrically at a wavelength of 450 nm± 2nm. The OD value is proportional to the human MUC-1 concentration. The colour formed is measured spectrophotometrically. Then the concentrations of the samples examined are calculated from the drawn calibration curve.

GdA was examined with the ELISA kit of Elabscience Biotechnology Co.Ltd, which uses the Sandwich-ELISA method. In this kit, the micro ELISA plate is first covered with antibody specific to GdA. Standards or samples are added to the appropriate micro ELISA plate wells and combined with specific antibody. Then a determinant antibody with biotin, specific to GdA and Avidin-Horseradish Peroxidase (HRP) conjugate is added to each micro-plate and incubated. Free elements are washed and substrate solution is added to each well. Wells containing only GdA, determinant antibody with biotin and Avidin-HRP conjugate are seen with a blue colour. The enzyme-substrate reaction is terminated by adding sulphuric acid solution and the colour becomes yellow. Optic density (OD) is measured spectrophotometrically at a wavelength of 450 nm± 2nm. The OD value is proportional to GdA concentration. The colour formed is measured spectrophotometrically. Then the concentrations of the samples examined are calculated from the drawn calibration curve.

LIF was examined with the ELISA kit of Elabscience Biotechnology Co.Ltd, which uses the Sandwich-ELISA method. In this kit, the micro ELISA plate is first covered with antibody specific to LIF. Standards or samples are added to the appropriate micro ELISA plate wells and combined with specific antibody. Then a determinant antibody with biotin, specific to GdA and Avidin-Horseradish Peroxidase (HRP) conjugate is added to each micro-plate and incubated. Free elements are washed and substrate solution is added to each well. Wells containing only LIF, determinant antibody with biotin and Avidin-HRP conjugate are seen with a blue colour. The enzyme-substrate reaction is terminated by adding sulphuric acid solution and the colour becomes yellow. Optic density (OD) is measured spectrophotometrically at a wavelength of 450 nm± 2nm. The OD value is proportional to LIF concentration. The colour formed is measured spectrophotometrically. Then the concentrations of the samples examined are calculated from the drawn calibration curve.

IL-15 was examined with the ELISA kit of Elabscience Biotechnology Co.Ltd, which uses the Sandwich-ELISA method. In this kit, the micro ELISA plate is first covered with antibody specific to IL-15. Standards or samples are added to the appropriate micro ELISA plate wells and combined with specific antibody. Then a determinant antibody with biotin, specific to IL-15 and Avidin-Horseradish Peroxidase (HRP) conjugate is added to each micro-plate and incubated. Free elements are washed and substrate solution is added to each well. Wells containing only IL-15, determinant antibody with biotin and Avidin-HRP conjugate are seen with a blue colour. The enzyme-substrate reaction is terminated by adding sulphuric acid solution and the colour becomes yellow. Optic density (OD) is measured spectrophotometrically at a wavelength of 450 nm± 2nm. The OD value is proportional to IL-15 concentration. The colour formed is measured spectrophotometrically. Then the concentrations of the samples examined are calculated from the drawn calibration curve.

G-CSF was examined with the ELISA kit of Elabscience Biotechnology Co.Ltd, which uses the Sandwich-ELISA method. In this kit, the micro ELISA plate is first covered with antibody specific to human G-CSF. Standards or samples are added to the appropriate micro ELISA plate wells and combined with specific antibody. Then a determinant antibody with biotin, specific to human G-CSF and Avidin-Horseradish Peroxidase (HRP) conjugate is added to each micro-plate and incubated. Free elements are washed and substrate solution is added to each well. Wells containing only G-CSF, determinant antibody with biotin and Avidin-HRP conjugate are seen with a blue colour. The enzyme-substrate reaction is terminated by adding sulphuric acid solution and the colour becomes yellow. Optic density (OD) is measured spectrophotometrically at a wavelength of 450 nm± 2nm. The OD value is proportional to G-CSF concentration. The colour formed is measured spectrophotometrically. Then the concentrations of the samples examined are calculated from the drawn calibration curve.

Statistical Analysis

All analyses of the study data were performed using SPSS vn 21 software. Continuous quantitative variables were stated as number, mean and standard deviation and qualitative variables as number, median, 25th and 75th percentiles. For continuous variables from independent measurements that did not show normal distribution, the Mann Whitney Rank Sum Test was applied. A value of $p<0.05$ was accepted as statistically significant.

RESULTS

As a result of the analysis of the demographic data of the study patients, the mean age was determined as 30.41±3.76 years in the whole group and 30.44±3.91 in the RPL patient group and 30.40±3.68 years in the control group (p=0,972).

Mean gravida was determined as 3.83±1.61 in the RPL group and 1.80±0.73 in the control group. Mean parity was 0 in the RPL group and 1.80±0.73 in the control group. The number of abortus was 3.86±1.20 in the RPL group and 0 in the control group. These results were summarized in Table 1.

Table 1: comparison of the pregnancy results between the groups.

Variable	Patient group (n=42) mean±SD	Control group (n=45) mean±SD	P
Gravida	3,83±1,61	1,80±0,73	<0,001
Parita	0±0	1,80±0,73	<0,001
Abortus	2,86±1,20	0±0	<0,001

The MUC-1, GdA, LIF, IL-15 and G-CSF plasma levels of the patients were determined and compared between the groups. The MUC-1 level of the RPL group was found to be statistically significantly lower than that of the control group (0.88±0.45 ng/ml vs. 1.17±0.57 ng/ml, p=0.008). The GdA level of the RPL group was found to be statistically significantly lower than that of the control group (23.73±3.72ng/ml vs. 25.92±4.20 ng/ml, p=0.006). The LIF level of the RPL group was found to be statistically significantly lower than that of the control group (64.59±21.13 pg/ml vs. 72.14±20.89 pg/ml, p=0.011). The IL-15 level of the RPL group was found to be statistically significantly higher than that of the control group (38.57±55.70 ng/ml vs. 31.44±43.65 ng/ml, p=0.013). No statistically significant difference was determined between the RPL group and the control group in respect of the G-CSF levels (13.07±3.84 pg/mL vs 14.11±3.62 pg/mL, p=0.056). All these findings were summarized in Table 2.

Table 2: Comparison between the groups according to MUC-1, GdA, LIF, IL-15 and G-CSF values.

Parameter	Total (n=87) mean±SD	Patient group (n=42) mean±SD	Control group (n=45) mean±SD	P
MUC-1 (ng/l)		0,88±0,45	1,17±0,57	0,008
GdA (µg/l)	24,86±4,09	23,73±3,72	25,92±4,20	0,006
LIF(pg/l)	68,49±21,22	64,59±21,13	72,14±20,89	0,011
IL-15 (ng/l)	34,88±49,67	38,57±55,70	31,44±43,65	0,013
G-CSF (pg/l)	13,60±3,73	13,07±3,84	14,11±3,62	0,056

DİSCUSSİON

Recurrent pregnancy loss (RPL) is 2 or more consecutive losses before the 20th week of pregnancy. However, there are different definitions of this disease [16]. For example, in the 2016 guidelines of the Reproductive Health and Infertility Association, RPL is defined as 3 or more consecutive pregnancy losses before the 20th week of pregnancy [4]. The American Society of Reproductive Medicine (ASRM) has defined RPL as 2 or more pregnancy losses, but these do not need to be consecutive [3].

Although no clear consensus has been reached on the definition of RPL, several causes have been held responsible in the etiology. However, in approximately half of patients, the etiology cannot be fully clarified. Despite great efforts made by physicians in the diagnosis and treatment of patients where the etiology cannot be fully established, no corresponding reasons can be found in most cases. Thus, there is no chance of treating approximately half of patients when the etiology cannot be determined. The only current method for RPL of unknown causes is close follow-up and monitoring. When it is considered that 0.5%-2% of women are affected by this disease [16], there can be seen to be a great burden in respect of the healthcare costs of diagnosis and treatment. Moreover, these patients may develop psychological problems and the marriage may even be at risk [17].

Pregnancies which continue in women with a history of RPL are generally seen to be delivered by caesarean section. When the indications for caesarean delivery are examined, the most common reason has been found to be that it is a valuable pregnancy [18, 19].

As there are different definitions of RPL made by prestigious societies in the field of obstetrics and gynaecology and etiological causes have not been clarified in approximately half of cases, this suggests that there are several scientific points that are unknown and require clarification. In this context, the aim of this study was to investigate possible endometrial implantation disorders among the as yet unexplained etiological causes of RPL and to evaluate the plasma levels of a series of cytokines which are effective in implantation.

Successful embryo implantation requires both a healthy embryo and a healthy endometrium. There has to be correct and healthy communication between the embryo and the endometrium for the implantation to be successful. In IVF patients with recurrent failure of implantation, one of these two elements is usually seen to be impaired. Several studies have shown impaired endometrial receptivity in IVF patients with recurrent implantation failure [8, 10, 20]. Consequently, there may be a significant difference between patient and control groups in plasma concentrations that reflect the endometrial expression levels of a series of markers that are required for implantation. With this hypothesis, the aim of the current study was to compare RPL paitents and a control group in respect of blood plasma levels of MUC-1, GdA, LIF, IL-15 and G-CSF, which play a role in endometrial receptivity and implantation and have been previously studied in IVF patients with recurrent implantation failure.

The results of the study demonstrated that the MUC-1, GdA and LIF mean plasma values were statistically significantly lower in the patient group than in the control group. The blood plasma IL-15 level was determined to be statistically significantly higher in the patient group than in the control group. Although the G-CSF blood plasma values were lower in the patient group than in the control group, this difference was not statistically significant (p=0.56).

As previously mentioned, MUC-1 is both an anti-adhesion molecule and a molecule that has immunomodulator effects on T-lymphocytes [21, 22]. The implanted embryo is protected against foreign agents with the immunomodulator effect. Although it has been suggested in previous studies that a relatively low level of MUC-1 in the implantation window, is important for implantation, patients with a history of RPL have been shown to have a significantly lower MUC-1 level than the normal population [8, 23-24]. Consistent with the findings of previous studies, the MUC-1 level of the RPL patients in the current study was found to be lower than that of the control group. This paradoxical situation can be attributed to the fact that although a reduction in MUC-1 levels during the implantation window is important in respect of implantation of the blastocyst to the endometrium, the excessive reduction in MUC-1 levels in RPL patients cannot function to protect the embryo and therefore leaves the embryo defenceless against external factors [(22, 25].

It has been proposed that by stimulating apoptosis in pro-inflammatory monocytes, GdA could have a role in maintaining the anti-inflammatory environment that is necessary for pregnancy [26]. In a study by Tulppala et al, the GdA serum levels in the implantation window were compared between patients with a history of RPL and a healthy control group, and the GdA level was found to be lower in the RPL group [27]. Dalton et al also compared patients with a history of RPL and a control group, through the examination of the GdA level in fluid adminstered to the uterine cavity as 10 ml sterile saline and then re-aspirated during the implantation window. The GdA level was determined to be lower in the RPL group than in the control group [28].

In another study conducted on patients with implantation failure, GdA levels both in the serum and in endometrial tissue samples were found to be lower when compared to a healthy control group [8]. In the current study, the blood plasma GdA levels were found to be lower in the RPL patients than in the control group, which was consistent with findings in literature.

Several previous studies have examined the relationship between LIF and embryo implantation. It is known that immediately before implantation, LIF synthesis in the glandular epithelium of the endometrium reaches a maximum level [29-30]. In the peri-implantation stage, the effects of LIF reaching peak levels has been shown with trophoblast function and placental vascular formation [30, 31].

In a study by Laird SM et al, the LIF concentration in the uterine irrigation fluid of patients with implantation failure was found to be lower than that of the normal population [32]. In contrast, Xu B et al found no difference between patients with a history of RPL and a control group in respect of endometrial expression of LIF [9]. However, in another study, LIF levels were found to be significantly lower in both endometrial tissue and blood plasma in patients with a history of RPL compared to a control group [33]. In the current study, the LIF levels of the RPL patients were determined to be statistically significantly lower than those of the control group. These results were consistent with the findings of several studies in literature that LIF levels, which have a very important role in the implantation process, can be expected to be lower in patients with a history of RPL.

In a study by Chegini et al, endometrial IL-15 synthesis was shown to be increased in patients with a history of RPL [143]. Mariee et al also showed increased levels of endometrial IL-15 synthesis in patients with recurrent implantation failure compared to a control group [10]. In the current study, the blood plasma levels of the RPL patient group were determined to be higher than those of the control group, which was consistent with the findings of previous studies that have measured endometrial levels.

IL-15 is known to be effective on the proliferation of uNK cells and increases the cytotoxicity of these cells [34]. It has been suggested that IL-15 plays an important role in the transformation of NK cells to uNK cells [35, 36] and the number of uNK is known to be increased in patients with a history of RPL [37, 38, 39]. From this point, it can be thought that the greater transformation of NK cells to uNK cells could be a reason for impaired implantation. This could even be a reason for the loss of implanted healthy embryos.

In a recent, randomised, placebo-controlled study, patients with a history of RPL for whom IVF was planned were separated into 3 groups. One group was administered with intrauterine G-CSF, one group with low-molecular weight heparin, acetylsalıcylic acid and prednisolone and one group with a placebo. The results showed that the rates of live births were higher and pregnancy losses were lower in the G-CSF group than in the other groups [13]. In the current study, it was aimed to evaluate how the G-CSF serum values differed in the RPL patients from the control group. The results of the study showed that although the serum G-CSF values were lower in the RPL group than in the control group, the difference was not statistically significant.

Strong aspects of the current study can be said to be that in literature the studies made on the concept of endometrial receptivity and the molecules that have a function in that, have generally been conducted on patients who have undergone IVF with implantation failure. There is a limited number of studies that have examined these molecules in patients with a history of RPL.

In literature, although MUC-1 has been previously examined in RPL, it has been examined in uterine irrigation fluid and endometrial biopsies. To the best of our knowledge, there has been no previous study that has examined blood plasma MUC-1 concentrations in RPL compared with a healthy population. Therefore, the current study is the first in this respect. However, there is a need for further studies to be made of the MUC-1 blood plasma levels in RPL patients to support the results of this study.

The only study in literature to have studied GdA serum levels is that by Tulppala et al [27]. The similar results obtained in the current study suggest that this molecule has an important role in RPL. Nevertheless, further studies are required on this subject to support the results.

Only 1 study could be found that has examined LIF blood plasma levels in RPL, the results of which were similar to those of the current study. Further studies are required on this subject.

Another strong aspect of the current study is that it is the first to have examined IL-15 blood plasma levels in RPL patients. In the 2 previous studies that evaluated the relationship between IL-15 and RPL, expression levels were examined in endometrial tissues. Although the results of the current study are consistent with the findings of those 2 studies, there can still be considered a need for further research to shed light on this subject.

As this study examined the molecules in the plasma levels obtained from venous blood samples rather than using an invasive method such as endometrial biopsy, this can be considered a useful method for the examination of endometrial receptivity in RPL.

Although there are dozens of molecules that function in embryo implantation, the plasma levels of only 5 were examined in this study because of time and financial restraints. If a study could be conducted which could evaluate all these molecules, it could be determined which molecules were related to RPL and could be used as predictive markers.

CONCLUSİON

According to the results obtained in this study, statistically significantly low levels of MUC-1, GdA and LIF were determined in RPL patients, which was consistent with previous findings in literature, and these could be considered for use at both the diagnosis and treatment stage in future clinical practice. Similarly, that the IL-15 values were found to be higher in the RPL patients than in the control group is of clinical value. Although the G-CSF levels of the RPL patients were lower than those of the control group, the difference was not of a statistically significant level, and as it has been shown in literature that the use of this molecule improved results in cases of recurrent IVF failure, there can be considered to be a need for further more extensive studies of the effects of this molecule on patients with a history of RPL.

The etiology of RPL has not been fully clarified and therefore a patient group of approximately 50% who cannot be diagnosed with known causes do not have full access to treatment options. In this context, it can be considered that further descriptive and randomised, controlled studies will significantly contribute to implementations in clinical practice.

Endometrial receptivity is an important part of embryo implantation and can be thought to play an important role in the clarification of the etiology of this disease. Generally, samples are taken with endometrial biopsy in the implantation window to evaluate endometrial receptivity. The samples taken are usually examined under electron microscope or with immunohistochemical methods. These examinations are both expensive and impractical and include the invasive method of biopsy. The use of venous blood samples to evaluate endometrial receptivity, as in the current study, can be considered a more practical, cheaper and less invasive method.

Further similar studies to this investigating the etiology of RPL, conducted on more extensive patient series, are necessary and important in respect of contributing to literature and the development of new diagnostic and treatment methods for this disease.

Compliance with Ethical Standards:

Conflict of interest: All authors (Nadi Keskin, Murat Polat, and Ghanim Khatib) declare that there is no conflict of interest.

Ethical approval: All procedures performed in this study involving human participants were in accordance with the ethical standards of the institutional research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed consent: Informed consent was obtained from all individual participants included in the study.

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