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SOME BIOCHEMICAL CHANGES IN WOMEN WITH RECURRENT MISCARRIAGE

[1]Ibrahim H. Al-Zuhairi, *[2]Prof. Raid M. H. A-Salih and [3]Asst. Prof. Alaa H. Al-Naser

[1]Alayen University/ College of Health & Medical Techniques.
[2]Thi-QarUniversity/College of Science.
[3]Thi-Qar University/College of Medicine.

***Corresponding Author: Prof. Raid M. H. A-Salih**
Thi-QarUniversity/College of Science.

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ABSTRACT
In this thesis was studied Toxoplasma, Cytomegalovirus, Rubella Antiphospholipid , and Anticardiolipin are tested to distinguish between explained and unexplained of Recurrent Miscarriage Where the study was conducted in Thi-Qar governorate in the south of Iraq. The results revealed that concentration of PC & PS levels showed a significant decrease ($p \leq 0.05$) in the patient group in comparison with the control group, and no significant ($p \leq 0.05$) between explained and unexplained groups.While, MBP level showed significant increase ($p \leq 0.05$) in patient group in comparison with the control group, and significant increase ($p \leq 0.05$) between explained and unexplained groups. The results showed negative correlation between MBP and (PC & PS) in explained and unexplained group.

KEYWORDS: Recurrent Miscarriage (RM), Protein C, Protein S, MBP, MPL.

INTRODUCTION

Recurrent Miscarriage (RM)

Miscarriage is the most common complication of pregnancy, defined as the spontaneous termination of pregnancy itself before the fetus has attained the stage of viability. Although only 15% of miscarriages are clinically recognized, total reproductive losses are closer to 50% of conceptions, and this complication is observed in about 1%– 5% of couples trying to conceive, (Fabro, 2011 ; Christiane Kling et al., 2018).

The term clinical miscarriage is used when ultrasound examination or histological evidence has confirmed that an intrauterine pregnancy has existed. Clinical miscarriages may be subdivided into early clinical pregnancy losses (before gestational week 12) and late clinical pregnancy losses (gestational weeks 12 to 21) (Ayed et al., 2017).

The repetitive miscarriages in some couples suggest that certain women are at particular risk of losing their pregnancy and that there must be an underlying explanation for this. Even though much work has been done to identify these underlying mechanisms, the aetiology of miscarriage is still unknown in many cases. Additionally, on an individual level the exact reason for a particular miscarriage is rarely defined. Despite the extensive medical testing and experimental treatments that many patients undergo, the cause often remains unclear (Plouffe et al., 1992; Clifford et al., 1994; Carrington et al., 2005).

Infection

Infective causes of RM remain speculative, as for any infectious agent to be implicated, it has to be capable of being persistent and undetected in the genital tract and must cause few maternal symptoms. Rubella, toxoplasmosis, cytomegalovirus and herpes infection do not meet these criteria and routine screening for these diseases has now been abandoned (Regan et al., 2001).

TORCH is an acronym which stands for Toxoplasmosis, Rubella virus, Cytomegalovirus infection. These groups of infections are the main threats of serious congenital infection during pregnancy, which may ultimately cause fetal damage or other anomalies. In most cases, the infection can be severe enough to cause serious damage to a fetus than his/her mother. The gestational age of the fetus influenced the degree of severity (Boyer et al., 2004).

Mannose binding lectin (MBL)

The mannose-binding lectin (MBL) is a protein of the innate immune system (Worthley DL, 2005), belongs to the class of collecting in the C-type lectin superfamily, whose function appears to be pattern recognition in the first line of defense in the pre-immune host. MBL recognizes carbohydrate patterns, found on the surface of a large number of pathogenic micro-organisms, including bacteria, viruses, protozoa and fungi. Binding of MBL to a micro-organism results in activation of the lectin pathway of the complement system. Another important

function of MBL is that this molecule binds senescent (Tomaiuolo R, 2012).

MBL could play a critical role in the first line defence during the neonatal period, when the maternal-derived antibodies disappear and the child's own immune system is immature (Scorza M., 2015, Schlapbach M. L. J. , 2010).

Protein C

Protein C is a vitamin K–dependent glycoprotein synthesized in the liver. Protein C is activated by the thrombin-thrombomodulin complex. Protein C circulates as an inactive precursor and exerts its anticoagulant function after activation to the serine protease, activated protein C. Once activated, protein C proteolytically degrades activated coagulation factors Va and VIIIa. More than 160 qualitative or quantitative mutations in protein C have been described. Protein C is inherited as an autosomal dominant trait affecting both males and females equally. Homozygous persons typically have more severe and earlier-onset thrombophilia. (Ardell S, 2018).

The diagnosis of protein C deficiency should be made by means of functional testing based on activation with thrombin-thrombomodulin. Pregnancy and oral contraceptive use can increase plasma protein C levels. Protein C levels are decreased in acute thrombotic events and during therapy with warfarin. In the absence of warfarin therapy and known medical conditions that result in acquired protein C deficiency, patients with a protein C level less than 55% of normal are very likely to have a genetic abnormality, whereas levels from 55% to 65% normal are consistent with either a deficient state or low normal values.(Michael P., 2012).

Protein S

Protein S is a vitamin K–dependent anticoagulant protein that was first discovered in Seattle, Washington in 1979 and arbitrarily named after the city of its discover. The major function of protein S is as a cofactor to facilitate the action of activated protein c (APC) on its substrates, activated factor V (FVa) and activated factor VIII (FVIIIa). Protein S deficiencies are associated with thrombosis. Protein S deficiency may be hereditary or acquired; the latter is usually due to hepatic diseases or a vitamin K deficiency. (Muhammed, 2015).

Protein S deficiency usually manifests clinically as venous thromboembolism (VTE). The association of protein S deficiency with arterial thrombosis appears coincidental or weak at best. Arterial thrombosis is not evident with other hereditary anticoagulant abnormalities (eg, protein C or antithrombin III deficiency, factor V Leiden gene mutation).(Muhammed, 2015).

METHODS

Collection of Blood Sample

About 6mL of blood samples were taken from venous blood of women with recurrent miscarriage and controls,were transferred to gel tube and allowed to clot at room temperature, then the samples were centrifuged at (3600 rpm) for 10 minutes, and the serum was extracted to be used for biochemical assessment. The collected serum transferred to the different tube and was frozen at -20 °C to be analysed later, unless used immediately. Hemolysis samples were discarded.

Estimation of MBP/MBL(Mannose Binding Protein /Mannose Binding Lectin).

Set standard, test sample and control (zero) wells were on the pre-coated plate respectively, and the plate washed 2 times before adding standard, sample and control (zero) wells. Then, standard solutions (0.1ml of 10ng/ml, 5ng/ml, 2.5ng/ml, 1.25ng/ml, 0.625ng/ml, 0.312ng/ml, 0.156ng/ml) were placed into the standard wells., and Add 0.1 ml of Sample/Standard dilution buffer were also placed into the control (zero) well. Later, 0.1 ml of properly diluted sample (Human serum, plasma, tissue homogenates and other biological fluids.) were added into test sample wells. The plate was sealed and incubated at 37°C for 90 min, then the cover was removed and contents were discarded and washed 2times with Wash Buffer. 0.1 ml of Biotin- detection antibody working solution was added into the above wells (standard, test sample & zero wells). Then the plated was Sealed the and incubated at 37°C for 60 min., after that the cover was removed, and wash plate 3 times with Wash buffer. Thereafter, 0.1 ml of the SABC working solution was added into each well, and covered with incubation at 37°C for 30 min then. The plate was washed 5 times with Wash buffer, and each time let the wash buffer stay in the wells for 1-2 min. 90 µl of TMB substrate was added into each well, and incubated at 37°C in dark within 15-30 min., 50 µl of Stop solution was added into each well and mix thoroughly, finally OD. Absorbance at 450nm was used to read solution in the plate.

Estimation of (Vitamin K-dependent protein C)

Set standard, test sample and control (zero) wells were on the pre-coated plate respectively, and the plate washed 2 times before adding standard, sample and control (zero) wells. Then, standard solutions (0.1ml of 4000pg/ml, 2000pg/ml, 1000pg/ml, 500pg/ml, 250pg/ml, 125pg/ml, 62.5pg/ml) were placed into the standard wells., and Add 0.1 ml of Sample/Standard dilution buffer were also placed into the control (zero) well. Later, 0.1 ml of properly diluted sample (Human serum, plasma, tissue homogenates and other biological fluids.) were added into test sample wells. The plate was sealed and incubated at 37°C for 90 min, then the cover was removed and contents were discarded and washed 2times with Wash Buffer. 0.1 ml of Biotin- detection antibody working solution was added into the above wells (standard, test sample & zero wells). Then the plated was

Sealed the and incubated at 37°C for 60 min., after that the cover was removed, and wash plate 3 times with Wash buffer. Thereafter, 0.1 ml of the SABC working solution was added into each well, and covered with incubation at 37°C for 30 min then. The plate was washed 5 times with Wash buffer, and each time let the wash buffer stay in the wells for 1-2 min. 90 µl of TMB substrate was added into each well, and incubated at 37°C in dark within 15-30 min., 50 µl of Stop solution was added into each well and mix thoroughly, finally OD. Absorbance at 450nm was used to read solution in the plate.

Estimation of (Vitamin K-dependent protein S)

Set standard, test sample and control (zero) wells were on the pre-coated plate respectively, and the plate washed 2 times before adding standard, sample and control (zero) wells. Then, standard solutions (0.1ml of 400ng/ml, 200ng/ml, 100ng/ml, 50ng/ml, 25ng/ml, 12.5ng/ml, 6.25ng/ml) were placed into the standard wells., and Add 0.1 ml of Sample/Standard dilution buffer were also placed into the control (zero) well. Later, 0.1 ml of properly diluted sample (Human serum, plasma, tissue homogenates and other biological fluids.) were added into test sample wells. The plate was sealed and incubated at 37°C for 90 min, then the cover was removed and contents were discarded and washed 2times with Wash Buffer. 0.1 ml of Biotin- detection antibody working solution was added into the above wells (standard, test sample & zero wells). Then the plated was Sealed the and incubated at 37°C for 60 min., after that the cover was removed, and wash plate 3 times with Wash buffer. Thereafter, 0.1 ml of the SABC working solution was added into each well, and covered with incubation at 37°C for 30 min then. The plate was washed 5 times with Wash buffer, and each time let the wash buffer stay in the wells for 1-2 min. 90 µl of TMB substrate was added into each well, and incubated at 37°C in dark within 15-30 min., 50 µl of Stop solution was added into each well and mix thoroughly, finally OD. Absorbance at 450nm was used to read solution in the plate.

RESULT AND DISCUSSION

MBP serum level

Table 1 displayed a significant increment in the concentration of serum MBP in patient group than control group ($p \leq 0.05$), the average MBP level in explained group was 2673.60±381.16 while in unexplained group was 2370.18± 323.53, and least average shown in control group 1961.80± 246.63. Also it was found a significant increase in the concentration of serum MBP in explained group in comparison with unexplained groups($p \leq 0.05$).

Table 1. Comparing MBP serum level between the two explained groups.

Groups	No.	MBP (ng/ml) Mean ±SD
Toxoplasma	35	2610.15±381.16[a]
CMV	15	2598.01± 323.53[a]
LSD		125.12

Table 2 showed no significant difference in the serum level of MBP between the patients with CMV infection and patients infected by Toxoplasma. Group with CMV infection recorded an average around 2598.01± 323.53 ng/ml of MBP while the Toxoplasma group recorded 2610.15±381.16 ng/ml of MBP serum level.

Table 2. Comparing MBP serum level between the two explained groups.

Groups	No.	MBP (ng/ml) Mean ±SD
Toxoplasma	35	2610.15±381.16 [a]
CMV	15	2598.01± 323.53 [a]
LSD		125.12

Several studies have shown that Infection with Toxoplasma to the host-cell by involving carbohydrate recognition because *Toxoplasma* Interestingly possess MIC1 and MIC4 which have lectin domains. *Toxoplasma* activated both the lectin (LP) and alternative (AP) pathways, and the deposition of C3b was both strain and lectin-dependent. A flow cytometry-based lectin binding assay identified strain-specific differences in the level and heterogeneity of surface glycans detected. Specifically, increased lectin-binding by Type II strains correlated with higher levels of the LP recognition receptor mannose binding lectin LP (Drickamer Ng, et al., 1996) recognition of carbohydrates on microbial surfaces is mediated by two families of lectin proteins, a subfamily of C-type lectins which include mannose binding lectin (MBL), and ficolins. MBL recognizes terminal monosaccharides such as glucose, mannose, or N-acetylglucosamine (GlcNAc) in a Ca^{2+}-dependent manner(Sikorski et al., 2020).

This study failed to find any association between the MBL level and cytomegalovirus infection but agreed with other study by (Sagedal, 2008)who investigated the association between pre-transplant levels of mannose-binding lectin (MBL) and the occurrence of cytomegalovirus (CMV) infection and symptomatic CMV disease. And find that MBL levels do not influence the incidence of any CMV infection or symptomatic CMV disease . but our results disagreed with (Manuel et al., 2007)who approved that MBL deficiency may be a significant risk factor for the development of CMV infection.

Protein C serum level

Table 3 illustrating a significant difference ($p<0.001$) between control (4.30±0.53) and the both patients groups (the PC level average of explained group equal to

4.03±0.91and the unexplained group PC level equal to (4.05±0.75). while a non-significant deference in the concentration of serum protein C between explained and unexplained groups($p \leq 0.05$).

Table 3. Comparing protein C serum level between the two explained groups.

Groups	No.	Protein C(pg/ml) Mean ±SD
Control	50	4.30±0.53 [a]
Explained	50	4.03±0.91 [b]
Unexplained	50	4.05±0.75 [b]
LSD		0.21

The results in table 4 illustrating a non-significant difference in serum level of protein C between the two explained group (patients with toxoplasma infection and patients with CMV). The group with Toxoplasma showed 4.09±0.78 pg/ml of protein C while CMV group showed 4.01±0.81 pg/ml.

Table 4. Comparing Protein C serum level between the two explained groups.

Groups	No.	Protein C (pg/ml) Mean ±SD
Toxoplasma	35	4.09±0.78 [a]
CMV	15	4.01±0.81 [a]
LSD		0.32

The results of the correlation between MBP and protein C in both of the explained groups are illustrated in figure 1 and have shown a negative correlation with a correlation coefficient (r= - 0.22) and the unexplained group with a correlation coefficient (r= - 0.21).

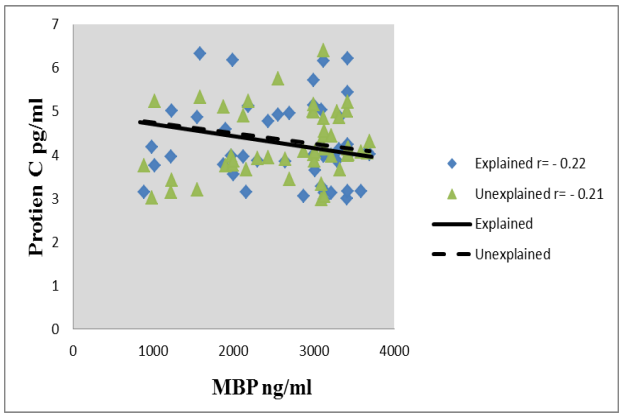

Figure1. Correlation MBP level with the protein C level.

The protein C act as a natural anticoagulant which can serves as a major system for controlling thrombosis, limiting inflammatory responses, and which lead to decreasing n the apoptosis of endothelial cell in response to inflammatory cytokines. (Mölkänen et al., 2010). protein C, an acute-phase marker for underlying systemic inflammation, has long been known to be elevated in patients with acute myocardial infarction. It has even been shown to predict risk for future ischemic events in previously healthy individual(Muhlestein et al., 2000). But this study failed to find any difference between the infection with toxoplasma and CMV. And its considered one of the first studies which invisitgate the correlation between those parameters.

Protein S serum level

The results in table 5 shown a significant decrease in the concentration of serum protein S in patient group in comparison with control group (24.06± 3.90, $p \leq 0.05$). Also it was found no significant deference in the concentration of serum protein S between explained (21.36±5.38) and unexplained groups(22.03±6.12) ($p \leq 0.05$).

Table 5. Comparing protein S serum level between the two explained groups.

Groups	No.	Protein S (ng/ml) Mean ±SD
Control	50	24.06± 3.90 [a]
Explained	50	21.36±5.38 [b]
Unexplained	50	22.03±6.12 [b]
LSD		1.89

The results illustrated in table 6 showed a non-significant differences in the concentration of serum protein S between the two types of infection. And the results of the group with toxoplasma infection showed an average of 21.66±6.13 ng/ml and a group of women with CMV infection showed an average of 20.96±6.01 ng/ml.

Table 6. Comparing Protein S serum level between the two explained groups.

Groups	No.	Protein S (ng/ml) Mean ±SD
Toxoplasmas	35	21.66±6.13 [a]
CMV	15	20.96±6.01 [a]
LSD		2.23

Figure 2 shows the negative correlation between MBP and protein S in explained group with correlation a coefficient (r= - 0.17) and the unexplained group with a correlation coefficient (r= - 0.11).

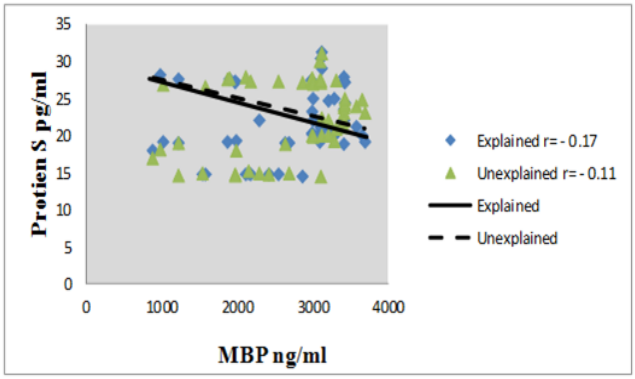

Figure2. Correlation MBP level with the protein S level.

Protein S has an important anticoagulant property. Protein S essential in regulating thrombogenesis and protecting against thrombo-embolic events. When the protein C activated by thrombin formation it will bind to Protein S, to block any new blood activation site, and more especially circulating activated Factors V and VIII (Amiral and Seghatchian, 2019). Protein S not only acts as the activated Protein C cofactor, but also has many other functions such as, cofactor of Tissue Factor Pathway Inhibitor and complement pathway activation. But the disease which can linked to this system is the formation of auto-antibodies against those proteins(Meng et al., 2018). The results of this study similar to previous study done by (Wu et al., 2013).

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