

Analysis of serum protein fractions from women with recurrent miscarriage

Analiza frakcji białkowych surowicy w grupie kobiet z poronieniami nawracającymi

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Abstract

Recurrent miscarriage occurs in 1 - 5 % of women at reproductive age. The most common cause of recurrent miscarriage is chromosomal abnormalities of the embryo (41%), chromosomal aberrations parents (10%), anatomical abnormalities of the uterus (5%), infectious and hormonal factors. In about 25% of women, no cause of recurrent miscarriage is usually found. Therefore it seems important to study all factors possibly inducing pregnancy disorders.

Objective: The aim of this study was to find a difference in serum protein fractions between women with primary and secondary recurrent miscarriage.

Methods: The study group consisted of 52 women (aged 36.0±4.9) with recurrent miscarriage. Nine of them (17%) reported one earlier regular pregnancy ending with childbirth without complications. Control group comprised 30 non-pregnant women (aged 36.1±3.6), who had given vaginal birth to healthy children at least twice. Serum protein fractions were separated by electrophoresis in the SDS PAGE buffer system using a Mini PROTEAN 3 cell device. BioRad SDS PAGE Molecular Weight Standards covering mass range of 6.5-200 kDa were used as a reference. Gels were stained with Coomassie Blue R 250 solution. BioRad QuantityOne software was used for the assessment of molecular weight of each protein fraction.

Results: Electrophoretic separation revealed 39 protein fractions of 10 243 kDa. Particularly interesting was a 38 kDa fraction present exclusively in serum of women with recurrent pregnancy, who had never given birth. Another fraction (74 kDa), not detected in the control group, was found in all women with recurrent pregnancy loss. Protein fractions of 76 and 151 kDa were present only in the control group.

Conclusions: The presence of the protein fractions of low- or mid-weight in serum from women with recurrent miscarriage may potentially play a role in the pathomechanism of this disorder.

Key words: **recurrent miscarriage / protein fractions / electrophoresis /**

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Streszczenie

Wstęp: Uważa się, że poronienia nawracające dotyczą od 1 do 5% par w populacji w wieku rozrodczym. Najczęstszą przyczyną nawracających poronień są aberracje chromosomowe zarodka (41%), aberracje chromosomowe rodziców (10%), nieprawidłowości anatomiczne macicy (5%), czynniki infekcyjne i hormonalne. U około 25% kobiet nie udaje się znaleźć przyczyny poronień nawracających. Dlatego tak ważne wydaje się zbadanie wszystkich możliwych czynników wpływających na zaburzenia przebiegu ciąży.

Cel pracy: Celem pracy było znalezienie różnicy we frakcjach białkowych w surowicy między kobietami z pierwotnymi i wtórnymi nawracającymi poronieniami.

Materiał i metody: Grupę badaną stanowiły 52 kobiety ($36,0 \pm 4,9$ lat), u których występowały poronienia nawracające. U 9 (17%) z tych kobiet odnotowano wcześniejsze wystąpienie jednej ciąży o przebiegu prawidłowym i porodu bez powikłań. Do grupy odniesienia zakwalifikowano 30 nieciążarnych kobiet ($36,1 \pm 3,6$ lat), które co najmniej dwa razy były w ciąży i urodziły zdrowe dzieci siłami natury. Materiałem badanym była surowica krwi, którą uzyskano z naktucia żyły łokciowej. Frakcje białkowe zostały rozdzielone przy pomocy elektroforezy w systemie buforów SDS-PAGE (Laemmli) przy użyciu aparatu Mini-PROTEAN 3-cell. Jako wzorzec masowy zastosowano SDS-PAGE Molecular Weight Standards firmy BioRad z zakresem mas od 6,5 do 200 kDa. Żele wybarwiono w roztworze Coomassie Blue R-250. Do oceny mas cząsteczkowych poszczególnych frakcji białkowych zastosowano program QuantityOne firmy BioRad.

Wyniki: W rozdziale elektroforetycznym ujawniono 39 frakcji białkowych w zakresie od 10 do 243 kDa. Na szczególną uwagę zasługuje frakcja białkowa o masie cząsteczkowej 38 kDa, która była obecna tylko w surowicy kobiet z poronieniami, które przed wystąpieniem co najmniej 3 poronień jeden raz rodziły siłami natury.

Wnioski: Zaobserwowano znaczące różnice w częstości występowania frakcji białkowych między grupą odniesienia, a kobietami z grupy badanej.

Słowa kluczowe: **poronienia nawracające / frakcje białkowe / elektroforeza /**

Introduction

Miscarriage, the commonest complication of pregnancy, is the spontaneous loss of a pregnancy before the fetus has reached viability. The highest incidence of pregnancy loss occurs before implantation and reaches approx. 50%. In the post-implantation period miscarriage incidence decreases to 12-24% [1].

Miscarriage incidence correlates with, among other factors, gestational age, women's age and the number of previous abortions [2, 3]. The incidence of miscarriages increases significantly in women over the age of 35 and in women aged over 45 approx. 75 % of pregnancies are lost [4]. Reproductive history is an independent predictor of the pregnancy outcome [1]. In women who have had two miscarriages, the probability of experiencing a third pregnancy loss increases to 17-45% and the probability of further miscarriages is 26-46% [4-7]. A succession of at least three consecutive cases of early pregnancy loss is classified as recurrent miscarriage. It is believed, that recurrent pregnancy loss occurs in 1-5% of women at reproductive age [8-10]. Etiological factors, tests and treatments for recurrent miscarriage are still controversial [11].

The most common causes of recurrent pregnancy loss are: chromosomal abnormalities of the embryo, chromosomal aberrations parents, anatomical abnormalities of the uterus, infectious and hormonal factors [12]. In about 25% of women, no cause of recurrent miscarriages is usually found. Therefore it seems vital to study all factors possibly inducing pregnancy disorders [8, 9, 13].

Aim of the study

The aim of this study was to find a difference in serum protein fractions between women with primary and secondary recurrent miscarriages.

Materials and methods

The examined cohort consisted of 52 women aged 24 to 44 (36.0 ± 4.9) with early recurrent miscarriage, who reported to the Genetics Clinic of the A. Jurasz University Hospital, at Collegium Medicum in Bydgoszcz, Poland.

Based on examination results, the existence of comorbid chronic diseases, anatomical abnormalities of the uterus, parental chromosomal aberrations, acquired and innate thrombophilias, as well as autoimmune and alloimmune disorders was excluded in all women. The group of women with recurrent miscarriage was divided according to earlier pregnancy occurrences and for 9 patients (17%) one earlier pregnancy with normal course and regular delivery was reported.

The control group consisted of 30 non-pregnant women aged 30 to 41 (36.1 ± 3.6), who had given vaginal birth to healthy children at least twice. Comorbid chronic diseases were excluded in those patients based on the anamnesis conducted prior to their inclusion in the study.

Blood (5 ml) was collected by vein puncture and serum was obtained after centrifugation in standard conditions. Collection of blood samples from the women with recurrent miscarriage was performed at least 6 weeks after a miscarriage, i.e. after the reversal of changes occurring in woman's organism due to pregnancy. Sera were divided into 200 ml portions and stored at -70°C until the assays were performed.

1. Isolation of circulating immune complexes from serum from women with recurrent pregnancy loss

Serum samples (0.5 ml) were added to 0.5 ml of 7% polyethylene glycol (PEG-6000) in 0.1 M borate buffer at pH 8.4. The obtained mixtures were incubated at 4°C for 18 h and centrifuged afterwards. The sediments containing precipitated complexes were washed twice with 3.5% PEG in borate buffer. Subsequently, the samples were centrifuged for 10 min. at 2500 RPM and 4°C. The sedimented complexes were then dissolved in 0.5 ml PBS. Finally, thus prepared samples served as input material for electrophoretic separation.

2. Electrophoretic separation of protein fractions of circulating immune complexes

Protein fractions of circulating immune complexes were separated electrophoretically according to their molecular weight in the SDS-PAGE buffer system (by Laemmli, a discontinuous system) using a Mini-PROTEAN 3-cell device. Electrophoreses were conducted at the constant voltage of 200 V for approx. 45 min. BioRad SDS-PAGE Molecular Weight Standards covering mass range of 6.5-200 kDa were used as a reference. After fraction separation, the gels were placed in fixing solution composed of acetic acid, methyl alcohol and double-distilled water at the ratio of 1:3:6. To visualize protein fractions, the gels were subjected to staining with Coomassie Blue R-250 solution for 1 h at room temperature. The staining enables to visualize bands containing approx. 0.1 µg of proteins.

3. Molecular weight assessment of protein fractions obtained in electrophoretic separation

BioRad QuantityOne software was used for the assessment of molecular weight of each protein fraction. Images and archived results were generated using BioRad GelDoc EZ system.

Statistical analysis of the results of the study was performed using Excel 2007 of the Microsoft® Office® 2007 package and Statistica 9.1 by StatSoft, Inc. (2010).

Significance assessment of the differences in fraction occurrence between each group was carried out using the χ^2 tests (χ^2 , χ^2 NW). In the case of expected low numbers, test results were corrected using Yates's correction (χ^2 Y).

Results

Table I presents the results of a comparative analysis of the CIC (circulating immune complexes) protein fractions isolated from serum of women with recurrent miscarriage, who had never given birth and those who had given birth once, as well as the control group. Most of the protein fractions were present in the serum of both, the women with recurrent miscarriage and the women from the reference group. Some fractions present in the women with recurrent miscarriages were absent among those from the control group.

Special attention was paid to several protein fractions. A fraction with molecular weight of 38 kDa was present only in the women with recurrent miscarriage who had never given birth and absent in both the women who had given birth once and those from the control group. Another fraction (molecular weight of 74 kDa) was not detected in the control group but was found in all the women with recurrent miscarriage, whether they had given birth or not. Protein fractions of 133, 145 and 154 kDa

were also absent in serum of women from the control group and present exclusively in women with recurrent miscarriages with high frequency. Protein fractions of 76 and 151 kDa were only found in the control group with frequency of 60% and 46.7%, respectively. Other fractions occurred with a similar frequency in all analyzed groups of patients.

Discussion

Currently, sensitive and specific tests useful in routine laboratory diagnostics in women with recurrent miscarriage are being sought. It is vital to diagnose all factors possibly inducing pregnancy disorders, prevent the occurrence of such disorders and introduce efficient therapeutic methods.

In this study, we performed electrophoretic separation of protein fractions from immune complexes, isolated from serum of women who had never given birth and women with recurrent pregnancy loss who had given birth once, as well as the control group. Electrophoretic separation revealed 39 protein fractions ranging from 10 to 243 kDa.

Special attention was paid to several protein fractions. For example, a fraction with molecular weight of 38 kDa, which was present only in women with recurrent miscarriage who had never given birth. Although the fraction was detected in less than 1/3 of the women, it may play a role in reproduction disorders. In their studies, Myung-Sun et al. assessed the presence of serum factors involved in recurrent pregnancy loss using proteomic techniques. They employed 2D electrophoresis and identified protein fractions with mass spectrometry and Western Blot [14]. The results demonstrated, that in serum from women with recurrent miscarriages a higher concentration of the ITI-H4 (inter- α -trypsin inhibitor heavy chain) protein fragments was detected, especially of the 36 kDa fraction. The authors suggested, that ITI-H4 fragments may play the role of a biomarker of recurrent miscarriages. In our study, the most characteristic fraction in women with recurrent miscarriage was that of 38 kDa, which seems to be similar to that described by Kim Myung-Sun et al. The difference in fraction size may be due to the use of different experimental methods.

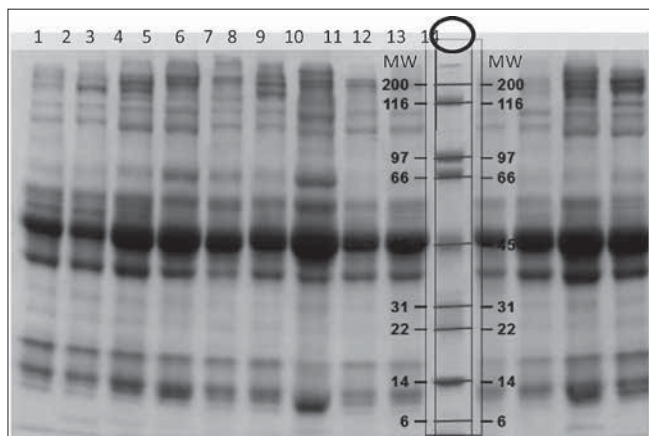
In this study, we observed that protein fractions with molecular weights of 74, 133, 145 and 154 kDa occurred at a similar frequency exclusively in serum from the women with recurrent miscarriage. Analyses of other protein fractions revealed, that in all the women with recurrent miscarriage who had given birth once, fractions of 10, 15, 25, 53, 70, 74 and 82 kDa were present. These fractions were also present in women with recurrent miscarriage who had never given birth, but with slightly lower percentage. There is little information in the literature on the serum protein profile in women with recurrent miscarriage. Our results are similar to those obtained by Pasińska et al., who also detected protein fractions of 59, 95 and 145 kDa mainly in women with recurrent miscarriage [15]. In our study, fractions of 59 and 95 kDa were revealed in a minor percentage of the control group (6.7 and 10%) and occurred chiefly in the patients with recurrent miscarriages (53.5-83.7%). The lack of further information on the protein fractions present in serum from women with recurrent miscarriage poses a difficulty in subsequent assessment of the results obtained in this study. The only available results are those of isolated serum protein analyses in healthy people and cancer patients [16-19].

Table I. Evaluation the occurrence of serum protein fractions from women with recurrent pregnancy loss, compared to the reference group.

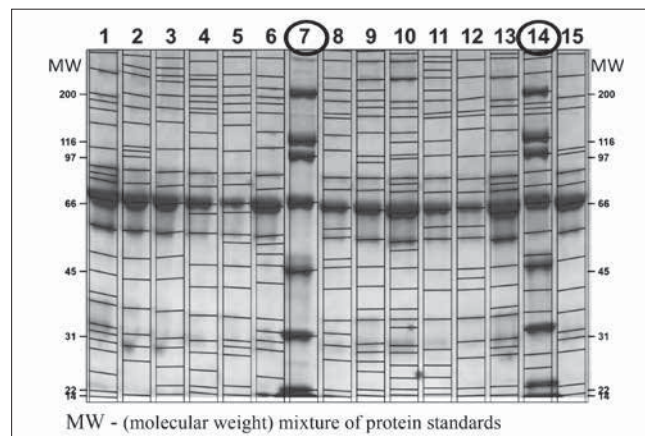
No	The molecular weight [kDa]	Reference group		Studied group II N=52				p-value I vs II
		I N=30		RPL 0 N=43		RPL 1 N=9		
		N	[%]	N	[%]	N	[%]	
1	10	30	100,00	37	86,05	9	100,00	0,13560
2	11	21	70,00	14	32,56	3	33,33	0,00110
3	12	6	20,00	27	62,79	7	77,78	0,00007
4	15	30	100,00	38	88,37	9	100,00	0,20280
5	17	3	10,00	22	51,16	5	55,56	0,00015
6	19	11	36,67	14	32,56	3	33,33	0,71469
7	22	28	93,33	37	86,05	8	88,89	0,56097
8	25	26	86,67	42	97,67	9	100,00	0,10943
9	28	29	96,67	35	81,40	8	88,89	0,13045
10	32	13	43,33	8	18,60	2	22,22	0,01928
11	35	24	80,00	28	65,12	7	77,78	0,21784
12	38	0	0,00	13	30,23	0	0,00	0,00755
13	45	29	96,67	40	93,02	8	88,89	0,75239
14	53	24	80,00	39	90,70	9	100,00	0,19699
15	59	2	6,67	23	53,49	6	66,67	0,00001
16	66	27	90,00	31	72,09	8	88,89	0,09876
17	70	15	50,00	41	95,35	9	100,00	0,00000*
18	74	0	0,00	43	100,00	9	100,00	0,00000*
19	76	18	60,00	0	0,00	0	0,00	0,00000*
20	82	27	90,00	41	95,35	9	100,00	0,52045
21	88	19	63,33	40	93,02	7	77,78	0,00291
22	95	3	10,00	36	83,72	7	77,78	0,00000*
23	106	19	63,33	21	48,84	5	55,56	0,24253
24	116	10	33,33	27	62,79	3	33,33	0,03354
25	121	6	20,00	8	18,60	2	22,22	0,93253
26	133	0	0,00	34	79,07	7	77,78	0,00000*
27	145	0	0,00	26	60,47	5	55,56	0,00000*
28	151	14	46,67	0	0,00	0	0,00	0,00000*
29	154	0	0,00	34	79,07	8	88,89	0,00000*
30	163	2	6,67	15	34,88	5	55,56	0,00175
31	168	1	3,33	14	32,56	1	11,11	0,00499
32	173	23	76,67	24	55,81	5	55,56	0,05845
33	188	7	23,33	36	83,72	8	88,89	0,00000*
34	198	15	50,00	37	86,05	7	77,78	0,00078*
35	203	2	6,67	33	76,74	4	44,44	0,00000*
36	214	5	16,67	25	58,14	3	33,33	0,00094
37	221	6	20,00	20	46,51	5	55,56	0,01155
38	232	8	26,67	34	79,07	7	77,78	0,00000*
39	243	5	16,67	28	65,12	5	55,56	0,00004*

RPL 0 – Women with recurrent miscarriages, who had never given birth;

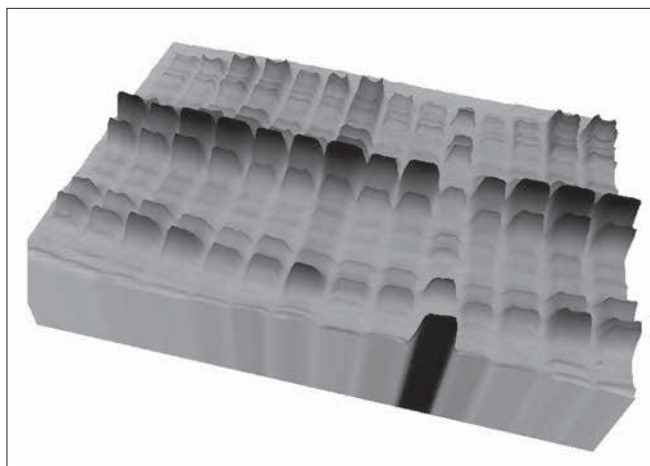
RPL 1 – Women with recurrent miscarriages, who gave vaginal birth once prior to having at least 3 miscarriages; N – number of patients

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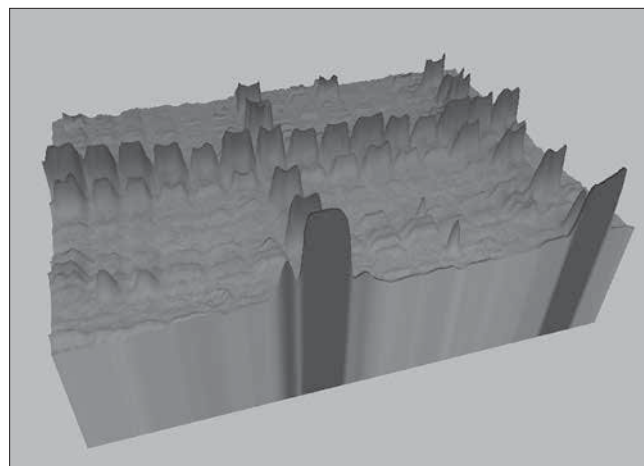
Picture 1. CCI dissociated protein fractions isolated from the sera of women with recurrent miscarriages (lane 1-9, 11-14). Lane 10 – a mixture of protein patterns. MW – (molecular weight) mixture of protein standards SDS-PAGE.



Picture 3. Circulating immune complexes dissociated protein fractions isolated from the sera of women with recurrent miscarriages (lane 1-6, 8-13, 15). Lane 7 and 14 – a mixture of protein patterns.



Picture 2. 3D image of protein fraction dissociated immune complexes isolated from the sera of women with recurrent miscarriages (lane 1-9, 11-14). Lane 10 – a mixture of protein patterns. MW – (molecular weight) mixture of protein standards SDS-PAGE.



Picture 4. 3D image of protein fraction dissociated Circulating immune complexes isolated from sera of women with recurrent miscarriage together with a mixture of protein patterns.

The obtained results brought some new information, however, it should only be considered as an introduction to further studies. It seems reasonable to conduct similar experiments with a larger group of women with recurrent miscarriage. The aforementioned protein fraction studies should be expanded with 2D electrophoresis, in which the fractions would be separated according to their isoelectric point and molecular weight, leading to higher resolution. Western blotting may also be applied to characterize a particular protein. It also seems reasonable to perform analyses of the ITI-H4 protein fragments in women with recurrent miscarriage. Confirmation of the above mentioned discovery may putatively enable the development of new diagnostic and therapeutic tools in the management of recurrent miscarriage.

Conclusions

The presence of the protein fractions of low- or mid-weight in serum from women with recurrent miscarriage may potentially play a role in the pathomechanism of this disorder.

Performed studies constitute an introduction to wider diagnostics anticipated in the future, which may support the development of knowledge on the reasons underlying recurring miscarriages, as well as help create further diagnostic.

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