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Research Article

Uterine Cesarean Scar Tissue - An Immunohistochemical Study

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Abstract

The CD31 protein is involved in the wound healing process. Structural proteins (collagens and elastin) have several functions, participation in the formation of tissue scaffolds among others. Type VI collagen after secretion into the extracellular space, creates molecular scaffold and forms interactions between other Basement Membrane (BM) components. As elastin endows tissues with a range of mechanical and cell interactive properties, its inherent properties make it an important inclusion to wound healing. It is possible, that determination of Alpha Smooth Muscle Actin (aSMA) and myosin monomers concentrations may be useful as biomarkers of biomechanical muscle properties. For these reasons, the immunohistochemical analysis of the uterine scar properties was carried out.

The aim of the study was to investigate the immunoexpression and immunoconcentration of myometrial collagen type VI, elastin, aSMA, Smooth Muscle Myosin Heavy Chain (SMMhc) and expression of Platelet and Endothelial Cell Adhesion Molecule 1 (PECAM1) also known as Cluster of Differentiation 31 (CD31) in scarred uteri. Biopsies were obtained from 177 healthy pregnant women during Cesarean Section (CS) operation. Depending on the period that had elapsed since the last CS, women were divided into seven groups: group 1 (primipara, n=52), group 2 (13- 23 months, n=15), group 3 (24-30 months, n=17), group 4 (31-36 months, n=21), group 5 (37-42 months, n=16), group 6 (43-60 months, n=29), group 7 (more than 60 months, n=27). To visualize the tested proteins, mouse anti-human antibodies were used. The slides were analyzed by light microscope, next all slides were scanned for rapid quantitation and comparison of data from multiple samples. A digital computer-assisted analysis technique was based on the use of an image processing program (Cell Sense Dimension 1.5, Olympus) to detect the areas with immunohistochemical staining. Obtained data for slides for each group were presented as the mean of the area [μm^2] and expression index as a percentage of staining. There were no differences in myometrial immunoconcentration of collagen type VI, elastin, SMM, endothelial cell marker CD31 in the analyzed groups. Only the myometrial aSMA concentration was significantly higher in patients, on whom cesarean section was performed in the period shorter than 2 years since the previous CS, than those with a longer interval. In conclusion, we suggest that uterine cesarean myometrial scar contains collagen type VI, elastin, SMMhc, aSMA endothelial cell marker CD31 and their immunoconcentration does not change with the time that has passed since the previous cesarean section. These suggestions still require closely controlled clinical studies.

Keywords: Cesarean section; Immunohistochemistry; Uterine cesarean scar

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Introduction

For a few years Cesarean section has become the most frequent major surgical procedure, and its incidence is observed to have significantly risen in recent times [1]. In many cases, pregnancies are terminated by cesarean sections upon request or in cases after previous cesarean section, patients are cesareaned because of fear of uterine rupture. The myometrial wound healing process is essential for determining the future of uterine muscle morphology, its functional behavior and in consequence risk of uterine rupture in the course of next pregnancies. Although wound healing is a physiological reparation mechanism, many factors may influence this process, leading to impaired wound healing [2]. On the one hand, side factors such as oxygenation, infection, and venous sufficiency may locally alter wound properties, on the other hand, systemic factors such as diabetes, obesity, stress, age, sex hormones, smoking may affect the ability to heal [3]. The normal wound repairing condition is related to fibro-proliferative response involving multiple mediators, as well as blood and extracellular matrix parenchymal cells. This process runs in three phases: inflammation (from the beginning of injury up to 4-6 days), tissue formation (days 4-14), tissue maturation and remodeling (week 1 - year 1) [4]. There is no evidence to confirm, that optimal myometrial structure and functional integrity resulting in low risk of uterine scar rupture in the course of next pregnancies, occurs within 24 months after cesarean section. Therefore, we decided to examine the integrity of the CD31 antigen, which is one of best indicators for assessing tissue density. The CD31 protein is involved in the wound healing process. Due to its properties, it is one of the main antigens assessing the degree of maturity of vessels [5]. Structural proteins (collagens and elastin) are the most widespread proteins and important constituents of the Extracellular Matrix (ECM), which have numerous functions such as cell adhesion and migration, tissue morphogenesis and participation in the formation of tissue scaffolds. They also take part in the repair and healing process. The basement membrane which constitutes an important part of the Extracellular Matrix (ECM) plays a crucial role in mechanical support, homeostasis and repair. Type VI collagen is a unique component of BM,

which is formed from three alpha chains: α1(VI), α2(VI) and α3(VI). After secretion into the extracellular space, it forms networks that facilitate creation of molecular scaffold and form interactions between other BM components. Is possible, that the changes of collagen and morphological characteristics of elastin fibers within ECM may be influenced by abnormal healing and pathological processes leading to tissue fibrosis and atherosclerosis [6,7].

The determination of aSMA concentration in the uterine scar may play a role as a biomechanical muscle properties marker, which under the influence of proinflammatory cytokines (IFN-γ) shows significantly reduced tonic contractility. In normal conditions, during muscle adaptation to longer or shorter length, a variable recruitment of myosin monomers and oligomers into the actin filament lattice is observed. It is possible that in the conditions of scarred lower uterine segment, the concentrations of SMMhc will be changed [8]. We postulate that uterine cesarean scar immunoconcentration of collagen type VI, elastin, SMMhc, aSMA endothelial cell marker CD31 can change in the periods between cesarean sections. The aim of the study was to verify immunohistochemical properties of the postoperative uterine scar in different periods of healing, by analyzing the immunoexpression and immunoconcentration of myometrial elastin, collagen type VI, aSMA, SMMhc, endothelial cell marker CD31 in scarred uteri, depending on interdelivery period, in term gestations.

Materials and Methods

The investigation has been conducted at the Department of Perinatology, Obstetrics and Gynecology, Pomeranian Medical University in Szczecin, Poland, and the study was approved by the Commission of Bioethics at the Pomeranian Medical University in Szczecin. During three years of prospective observation, the total number of deliveries in our department was 4668, and CS was performed in 2395 (51.3%) of cases. 177 healthy pregnant women were included in the study, who voluntarily gave their consent to the collection of biological material. All pregnant women delivered in early term (37 0/7 weeks through 38 6/7 weeks of gestation) and full term (39 0/7 weeks through 40 6/7 weeks of gestation). 52 (29,4%) got pregnant for the first time and 125 (70,6%) previously had cesarean section. The inclusion criteria for the study were as follows:

- uncomplicated term gestations
- absolute and relative indications for cesarean delivery

The mean fetal weight in all groups was 3,271kg and there were no significant statistical differences in maternal age. The size of the trial for the population of 100000 was calculated by estimating the size of the fraction at the level of 4-5%. The confidence level was 95% and the acceptable error margin 10%, which constituted 15 or 18 women in each group.

Depending on the period since the last CS, women were divided into seven groups:

- group 1 (primipara, n=52),
- group 2 (13- 23 months, n=15),
- group 3 (24-30 months, n=17),
- group 4 (31-36 months, n=21),
- group 5 (37-42 months, n=16),
- group 6 (43-60 months, n=29),
- group 7 (more than 60 months, n=27),

All evaluated pregnant women were not in labor and there were no factors that could affect the ability of myometrial healing. None of pregnant women had risk factors for abnormal wound healing such as smoking, gestational diabetes, hypertension or other complicated diseases. The clinical characteristics of women undergoing Cesarean Delivery included in the study are demonstrated in table 1.

Surgical procedures

All patients were operated under epidural anesthesia. CS was performed in sterile conditions. A Pfannenstiel skin incision was made and carried through to the underlying layer of fascia. The fascia was incised in the midline and extended laterally. Once the abdomen cavity was opened, the lower uterine segment was incised in transverse fashion. The infant was delivered atraumatically. After newborns' expulsion, the uterine scarred lower segment was precisely encountered as an irregular scar tissue, and subsequently tissue sample of about 2x2 cm in size was excised and collected. According to current evidence based on randomized trials, which did not support a specific type of uterine closure for optimal maternal outcomes, in our department the uterine incision was closed by using one-layer closure technique, with continuous lock stitches. No hysterectomy was required and there were neither maternal nor neonatal deaths.

Morphological study

Obtained biopsies from three groups of patients were fixed in 4% buffered formalin. Formalin-fixed, paraffin-embedded tissues were sectioned into slices by 4-5 μm thick with a microtome. These sections were then mounted onto poly-L-lysine-coated glass slides. For morphological analysis slides were stained with H-E [9].

Immunohistochemistry

Immunohistochemistry (IHC) has been routinely used as a methodology for the detection of specific protein markers presence: CD31 (PECAM-1) platelet endothelial cell adhesion molecule; α-actin and myosin heavy chain - elements of myofilaments in smooth muscle cells; elastin and collagen type VI - elements of extracellular matrix. To visualize the proteins in the myometrium scar, the following mouse anti-human antibodies (Novocastra distributed by Leica Biosystems, Zalesie Gorne, Poland) were used: anti-CD31 (clone 1A10; dilution of 1:50, primary antibody incubation at 25°C); anti-smooth muscle actin, alpha (clone ASM-1; dilution of 1:50, primary antibody incubation at 25°C); anti-myosin heavy chain (smooth muscle) (clone S131; dilution of 1:25, primary antibody incubation at 25°C); anti-elastin (clone BA-4; dilution of 1:100, primary antibody incubation at 25°C); anti-collagen type VI (clone 64C11; dilution of 1:50, primary antibody incubation at 25°C). The deparaffinized sections of myometrium scar were microwaved in citrate buffer (pH 6.0) for epitope retrieval, induced by heat. Secondly, the slow cooling to room temperature was performed and slides were washed in PBS twice for 5 min and incubated for 60 min with primary antibodies. Next sections were stained by the avidin-biotin-peroxidase system with Diaminobenzidine as the chromogen (EnVision+System-HRP (DAB); Code K4010 DakoCytomation, Glostrup, Denmark) in conformity with staining procedure instruction included in Dako EnVision+System. Sections were washed in distillated water and counterstained with hematoxylin. For negative control, specimens were processed in the absence of

Parameter	Interpregnancy interval (months)	Maternal age (years)	Maternal weight (kg)	Gestational age (weeks)	Birth weight (kg)
Group I	-	28,05±3,71*	75,04±8,09*	38,6 ±1,2	3,256±0,556
Group II	20,07±3,03	32,5±4,46	82,45±18,61	38,67±0,98	3,354±0,279
Group III	26,35±2,45	31,27±4,13	78,63±12,62	38,42±1,52	3,318±0,674
Group IV	34,75±1,29	34,81±5,0	81,21±14,57	37,95±2,85	3,281±0,683
Group V	44,25±3,39	33,71±7,12	81,39±15,92	38,06±2,14	3,215±0,568
Group VI	54,69±5,11	33,36±4,56	80,61±12,36	38,14±1,15	3,334±0,328
Group VII	92,61±33,10	32.82±4,83	81,96±11,63	37,93±2,1	3,231±0,656

Table 1: Clinical characteristics of women delivered by cesarean section, by interpregnancy interval length.

*- the value of $p \leq 0,05$ is the cutoff for statistical significance

primary antibody (the primary antibody was replaced with non-immune mouse serum). Positive staining was defined using a microscope by visual brown pigmentation identification.

The slides were analyzed by a light microscope Olympus BX 46 and an Olympus DP 25 camera. A good and sensitive Platelet Endothelial Cell Adhesion Molecule - CD31 (PECAM-1) not only stain endothelial cells from blood vessels. It can also stain endothelial cells from lymphatic vessels, although not as much as endothelial cells from arteries and veins. In our investigation we did not use a lymphatic-specific marker like LYVE1 or D240, which can also stain many other types of cells, such as mesothelial cells and myoepithelial cells. We distinguished H&E larger-caliber arterioles and venules from lymphatics, because the former two had thicker walls compared to lymphatic vessels, which had thin walls with a thin layer of basement membrane, surrounded by collagen and absent erythrocytes in their lumen. Especially veins and capillaries, due to their very thin walls, mimicked lymphatic in contour.

Digital image analysis (Digital Computer Assisted Analysis Technique)

All stained slides were scanned at 20x objective magnification by high content screening for rapid quantitation and comparison of data from multiple samples. A digital computer-assisted analysis technique was based on the use of an image processing program (Cell Sense Dimension 1.5, Olympus) to detect the areas with immunohistochemical staining for CD31, aSMA, SMMhc, elastin and collagen type VI. Obtained data for slides for each group were presented as the mean of the area [μm^2] and expression index - as the percentage of immunopositive staining concentration.

Statistical analysis

To choose the right statistical analysis, we have checked if the dependent variables were normally distributed using Shapiro-Wilk normality test. As our data have not been normally distributed, a non-parametric Mann-Whitney U test was used to determine the differences between the analyzed groups. We compared mean, median scores of samples and performed one-way analysis of variance with the aid of Statistica10 statistical software. A $p \leq 0.05$ is used as the cutoff for significance.

Results

Semi-quantitative evaluation using immunohistochemical methodology did not bring the expected results. We were hoping to show significant differences in the structure of scar tissue between groups. However, significant differences were demonstrated for actin and CD31 protein in group I with unscarred uterus (primipara) compared to other groups (after caesarean section). The expression of CD31 was observed in endothelial cells of blood vessels as a subtle line in the lumen. Immunoreactivity for actin and myosin was localized in the cytoplasm of myometrial smooth muscle cells, additionally actin was also present in vascular SCM of blood vessels. In relation to myosin and collagen concentrations, the differences between analysed groups remained unclear, whereas immunoexpression / immunoconcentration of elastin did not change in a relevant way between groups (Table 2). Statistically significant differences with calculated probability value P are shown in figures 1-4. Next, in an attempt to explain the existing differences and relations between the groups, the percentage of immunopositive staining cells concentration was calculated (Table 3). The conducted analysis demonstrated the meaning of mutual relationship, clearly indicating that significant differences occurred only between the first group (primipara) and the other groups in all examined parameters except for myosin, as shown in figure 5.

We demonstrate the positive staining patterns in immunohistochemistry with visualization of the presence and abundance of a specific antigen-antibody complex in the tissue section with the aid of a chromogen and microscopy. Evaluating the staining results of IHC and assigning a grade or score has been the most important step in the entire workflow. We did not show any significant differences in the evaluation of the IHC reaction of the preparations, which indicates the invariable content of the analyzed scar promoters, irrespective of the time function.

Discussion

Wound healing is an important biological process, where maintaining organ integrity and proper function is essential. The recognition of molecular mechanisms of this process is still under investigation [10]. Wound healing is achieved through four phases: hemostasis (vascular construction, platelet aggregation, degranulation and fibrin formation), inflammation (neutrophil infiltration, monocyte infiltration with differentiation to macrophage, lymphocyte infiltration), proliferation (re-epithelialization, angiogenesis, collagen synthesis) and remodeling (collagen remodeling, vascular maturation and regression) [11]. The transition of mesenchymal fibroblasts to myofibroblasts plays an important role for connective tissue remodelling [4]. Completely differentiated myofibroblasts are represented by a marker, alpha smooth muscle actin [12]. Histopathological examination of injured myometrium tissues proved that there is evidence of altered healing including myofiber disarray, elastosis, tissue edema and inflammation [13]. Myofiber disarray and elastosis may be markers of aberrancy in wound healing after iatrogenic uterine trauma [13]. In our investigation we noticed no time-dependent differences in percentages in the expression of elastin, collagen type VI, SMMhc, endothelial cell marker

Parameter	Actin area (aSMA)	Myosin area (SMMhc)	Elastin area	Collagen area (collagen t.VI)	CD 31 area (PECAM-1)
Group I	65933,65±33522,77	5870,08±5939,30	5002,57±3823,45	23379,68±14945,39	3553,13±2932,26
Group II	40730,74±27791,32	7198,77±6749,24	5522,69±6390,30	14122,49±11119,03	2490,21±1523,59
Group III	37578,51±33305,28	7554,65±8615,21	4972,82±4437,13	24045,47±21182,52	2013,35±1587,92
Group IV	28598,35±23383,7	8097,26±8845,94	3805,66±4234,11	13136,13±11771,61	2070,71±1417,69
Group V	40118,16±21442,87	10338,99±9192,84	7909,23±9559,51	17991,79±13068,37	2760,05±2155,23
Group VI	37833,75±26360,83	7569,92±6656,56	9693,78±17235,27	26225,81±20777,57	2084,36±1282,20
Group VII	43394,51±30965,22	4837,22±6096,49	5604,20±5449,66	19540,48±15068,75	2380,13±1564,63

Table 2: Myometrial immunoconcentration of elastin, collagen type VI, alpha smooth muscle actin, smooth muscle myosin heavy chain, endothelial cell marker CD31 in analyzed groups (μm²).

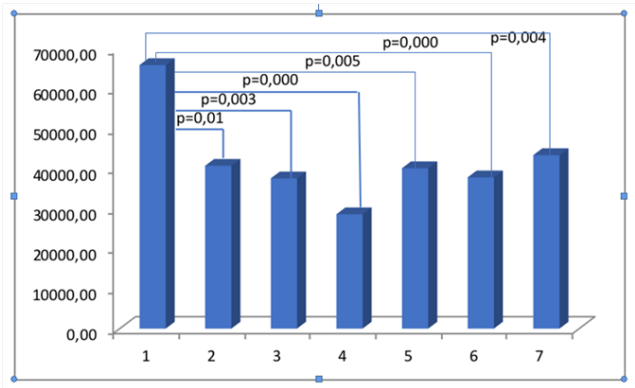

Figure 1: Myometrial immunoconcentration of alpha smooth muscle actin in several groups (μm²).

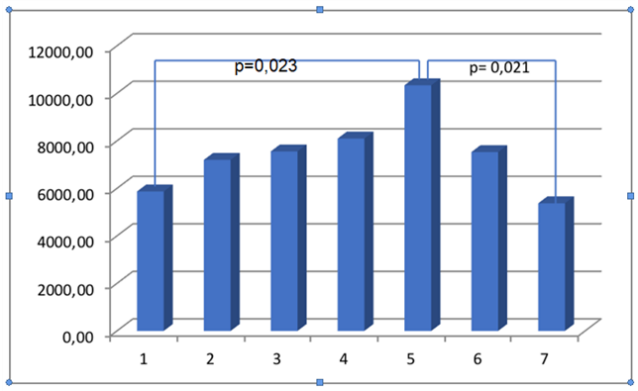

Figure 2: Myometrial immunoconcentration of smooth muscle myosin heavy chain in several groups (μm²).

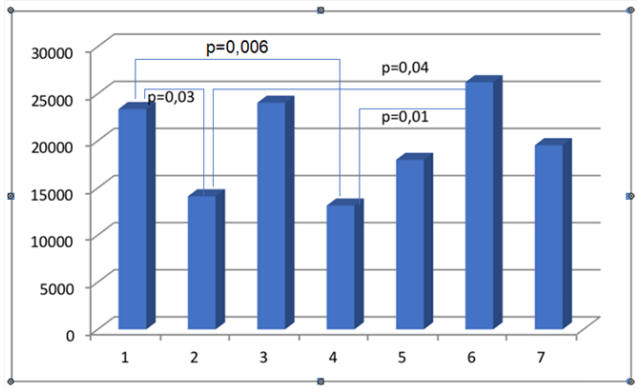

Figure 3: Myometrial immunoconcentration of collagen type VI in several groups (μm²).

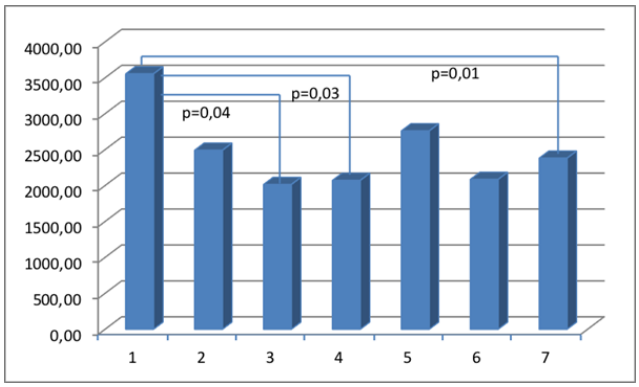

Figure 4: Myometrial immunoconcentration of endothelial cell marker CD31 in several groups (μm²).

CD31 in the scarred lower uterine segments. It is suggested that collagen is an important component of muscular tissue that influences its biomechanical properties. Several collagen types (mainly types I, III, V and VI) are present in the uterine wall. Collagen type VI is abundant in endometrium mainly in a secretory phase and next its level is decreased, in myometrium together with other types of collagen surrounds and associates smooth muscle cells [14]. In pathology, human collagen type VI mutations can result in muscular dystrophy, joint hyperlaxity and contractures. High concentrations of type VI collagen in an in vivo model of post myocardial infarction wound healing alter muscle function and remodeling in the period from days to weeks after injury [15]. Labor and scarring can also alter the collagen network fiber orientation. Scarred lower uterine segment myometrium has a higher collagen content compared with unscarred myometrium specimens that were obtained during labor [16]. In the cases of uterine dehiscence, the scarred lower uterine segments show a higher collagen content, which may be related to altered biochemical behavior of the scarring process [17]. Our investigation demonstrated no significant differences in immunoconcentration of collagen type VI in the scarred uterus in the period between 12-186 months since cesarean section. The mechanical tissue properties are derived from its Extracellular Matrix (ECM) proteins, the most important constituent of which is fibrillary collagen [18,19]. Other important constituents include proteoglycans, hyaluronate, elastin and water. It is well known that during pregnancy the human uterus increases at least 11-fold in wet weight, 7-fold in collagen and 5-fold in elastin content [20]. The

Parameter	Actin % (aSMA)	Myosin % (SMMhc)	Elastin %	Collagen % (collagen t.VI)	CD 31 % (PECAM-1)
Group I	41,33± 14,68	3,07±4,3	2,11±3,19	14,65±10,91	2,06±1,21
Group II	29,92±19,23	4,75±4,93	3,59±4,64	9,94±7,83	1,54±0,83
Group III	21,72±17,8	4,18±5,16	3,19±3,31	12,63±8,14	1,10±0,67
Group IV	19,28±16,38	5,32±5,64	2,27±3,17	9,05±8,43	1,34±1,0
Group V	24,88±15,83	6,33±6,16	2,74±3,64	12,12±9,62	1,45±0,79
Group VI	23,77±17,43	4,32±3,06	4,39±4,78	13,76±9,30	1,24±0,8
Group VII	30,16±22,2	2,69±3,9	3,43±4,07	12,49±10,78	1,43±1,01

Table 3: Myometrial immunoconcentration of elastin, collagen type VI, alpha smooth muscle actin, smooth muscle myosin heavy chain, endothelial cell marker CD31 in analyzed groups (%).

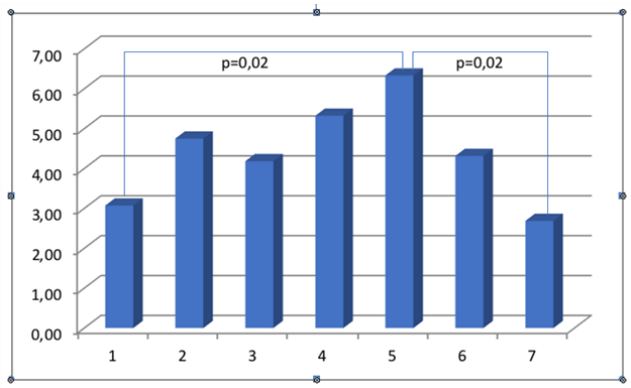

Figure 5: The percentage of concentration of immunopositive myosin staining cells in scar tissue in several groups [%].

studies of reproductive tract in human and animal model have indicated that collagen, elastin and other ECM proteins content of uterus and vaginal wall is drastically altered during gestation [20-22]. During rapid uterus involution after parturition, which is 75% complete within 8-11 days after delivery, elastin and collagen undergo rapid breakdown [23,24].

In women that have undergone six or more pregnancies, the collagen content is double, and elastin is five times higher when compared with their concentrations found in nulliparous uteri [20]. The decreased expression of elastin may correlate with the degree of pelvic organ prolapse in women, which can indicate that elastin plays a certain role in the pathogenesis of this dysfunction [25]. In our investigation we did not find any elastin content differences in the scarred uteri depending on interpregnancy interval length. It means that tensile scar properties remain unchanged after the operation. The potential mechanisms for early scar formation in wounds include activation of fibroblasts, acquisition of aSMA, expression and finally becoming myofibroblasts. These myofibroblasts synthesize and deposit the ECM components and exhibit contractile properties, due to the expression of aSMA, in microfilament bundles or stress fibers, playing a major role in the contraction and maturation of the granulation tissue [26].

Therefore, to identify myofibroblast concentration in scar tissue, the immunohistochemical staining for aSMA, the most reliable marker of the myofibroblastic phenotype was used. The presence of aSMA in the analyzed uterine scars can be proof of an immature wound. Myofibroblasts can also express other contractile proteins, such as SMMhc, or desmin [27]. However, there were no differences in immunoconcentration of aSMA and SMMhc in the tested groups (Figure 1).

In physiology, angiogenesis plays an important role in wound repair and functional vascular network is a critical process [28-31]. Angiogenesis probably facilitates the delivery of oxygen and nutrients to the wound site for the use of rapidly proliferating reparative cells [3,32]. This angiogenic effect can be enhanced by sex steroids [11]. Despite many studies examining angiogenesis and wound repair, the degree to which angiogenesis facilitates healing under normal circumstances is still not known [4,10,29]. The presence of CD31 antigen in the tested caesarian uterine scars, due to its properties, suggest that it is involved in interactive reactions during angiogenesis and wound healing. We did not observe any changes in CD31 scar immunoexpression in relation to interdelivery interval period, while the differences were observed in relation to promipara (Figure 4). Therefore, we pay attention to significant differences in the structure of the uterine myometrium between primipara and women after cesarean section, regardless of the time of the delayed surgery. Although the incidence and consequences of uterine rupture in women with previous cesarean section are more frequently observed, there is no data on the possibility of preventing them [33-38]. There is limited evidence on whether uterine cesarean scar properties are significantly influenced by the number of prior cesarean births, interdelivery length period or type of prior uterine scar [16,39-42]. Based on The Royal College of Obstetricians and Gynaecologists guidelines, vaginal birth after CS less than 2 years since previous cesarean birth can be one of the factors associated with a decreased likelihood of planned vaginal births after cesarean section [43]. There is no sufficient information on whether the risk of uterine rupture is increased in women with previous myomectomy or short interdelivery interval of less than 2 years [44-48].

In conclusion, for the first time we present evidence that longer period between caesarean section deliveries does not seem to affect the immunohistochemical structure of the scar. We suggest that uterine cesarean myometrial scar contains collagen type VI, elastin, SMMhc, aSMA, endothelial cell marker CD31 and their immunoconcentration do not change with the time that has elapsed since the previous cesarean section. These suggestions still require carefully controlled clinical trials on this topic.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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