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Immunohistochemical study of matrix metalloproteinases 2 and 9 in the placenta of spontaneous miscarriage

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ABSTRACT: Miscarriage is the termination of pregnancy by spontaneous expulsion of embryo/fetus before 25 weeks of gestation or when the fetus weighs less than 500 grams. It is the most common pregnancy problem, impacting around 10% to 15% of all pregnancies. Matrix metalloproteinase 2 and 9 (MMP-2 and MMP-9) play a crucial role in embryo implantation and placentation; spontaneous abortion has been observed when there is abnormal expression of MMP-2 and MMP-9 levels. This research aims to evaluate the expression of MMP-2 and MMP-9 in the placenta of women with spontaneous miscarriage. The placental specimens were collected from 30 women who had delivered normally as a control group, 30 women who had miscarried in the first trimester, and 30 women who had miscarried in the second trimester. The specimens included the decidua basalis and trophoblast. The expression patterns of MMP-2 and MMP-9 were examined using the immunohistochemical study. Antibodies were used against MMP2 and MMP9. The results revealed a strong expression of MMP-2 and MMP-9 in the placenta of normally delivered women; 18 and 20 cases of first and second-trimester miscarriage showed weak expression of MMP-2, while 12 and 10 cases of both trimesters revealed moderate expression. MMP-9 showed weak expression in decidua and trophoblastic tissue of 26 and 24 cases of the first and second trimester, 4 and 6 specimens reported moderate expression in the first and second-trimester miscarriage, and significant differences were detected between the study groups (p-value <0.01). We conclude that MMP-2 and MMP-9 immunopositivity could indicate uncontrolled invasion of trophoblasts, leading to their destructive invasion and contributing to spontaneous miscarriage pathogenesis.

KEYWORDS: Immunohistochemistry; Miscarriage; MMP2; MMP9; Placenta.

1. INTRODUCTION

Miscarriage is the termination of pregnancy by spontaneous expulsion of embryo/fetus before 25 weeks of gestation or when the fetus weighs less than 500 grams. It is the most common pregnancy problem, impacting around 10% to 15% of all pregnancies that are clinically established. Approximately 1 in 5-6 pregnancies terminate before reaching 12 weeks of gestation. An estimated 50% of miscarriages are believed to be caused by fetal chromosomal disorders [1,2]. Spontaneous miscarriage can be classified as either the first or second trimester, depending on whether it occurs before or after the 12 weeks of gestation. Most cases of spontaneous miscarriage occur during the first trimester of pregnancy, which accounts for approximately 80% of clinically recognized pregnancy loss. Risk factors for spontaneous miscarriage include genetic predisposition, advanced maternal age, previous early pregnancy loss, maternal diseases such as endocrine disorders, and autoimmune disturbance [3]. The ability of the embryo to enter the uterine stroma and cause breakdown of the basement membrane of the uterine epithelium is fundamental to the embryo's ability to implant itself in the uterus successfully. The blastocyst is the source of a specific type of cells known as cytotrophoblastic cells, originating from trophoectodermal cells. Cytotrophoblastic cells go through two unique processes: either they grow into villous cytotrophoblastic cells, which subsequently proliferate and differentiate by fusion to generate the syncytiotrophoblast, or they evolve into mononuclear multilayered invasive extravillous cytotrophoblastic cells. The temporal and spatial regulation of trophoblast invasion is promoted by trophoblastic and uterine factors. A healthy pregnancy is characterized by the presence of cytokines, growth factors, hormones, chemical mediators, and matrix metalloproteinases (MMP), which are

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responsible for controlling the biological process of embryo implantation. This process enables the migration of extravillous cytotrophoblastic cells and penetrates the endometrium [4,5].

MMPs are crucial in embryogenesis and fundamental physiological processes, including cell proliferation, remodeling, wound healing, angiogenesis, and other critical reproductive events [6]. They can effectively engage in remodelling the extracellular matrix (ECM). In conjunction with their tissue endogenous inhibitors (TIMP), they can establish a state of equilibrium that allows for the maintenance of pregnancy and the appropriate development of the placenta. Several disorders are associated with dysregulation of MMP and TIMP expression. The invasion of extravillous trophoblasts (EVTs) into the maternal endometrium begins around the sixth week of gestation. Furthermore, it has been proven that MMPs are associated with the lysis of the foetal membrane throughout the entirety of pregnancy and birth process [7,8]. Gelatinase A, also known as MMP-2, is one of the two human gelatinases identified for their proteolytic enzyme activity in breaking down gelatine. MMP-2 generally takes the form of a proenzyme with a molecular weight of 72 kilodaltons and is highly glycosylated. MMP-2 has the ability to hydrolyze gelatine as well as collagens of types I, IV, and V, as well as vitronectin and elastin. The basal membranes of blood vessels are the sites where gelatinases are responsible for collagen degradation. In addition, MMP-2 has the ability to facilitate cell migration by directly destroying the basement membrane. This makes it possible for neutrophils and lymphocytes to permeate the membrane and release chemoattractants. MMP-2 is involved in both the stimulation and suppression of inflammation. MMP-9, known as gelatinase B, when activated, MMP-9, which is initially expressed as a 92-kilodalton proenzyme, can produce an 83-kilodalton mature enzyme. It has been demonstrated that MMP-9 is capable of degrading collagen types IV, V, VII, X, and XIV, in addition to fibronectin and laminin. It is essential for the process of angiogenesis since it can release the physiologically active form of vascular endothelial growth factor (VEGF). The direct proteolytic breakdown of vascular basement membrane proteins reveals that MMP-9, even more so than MMP-2, has the potential to contribute to the development of new blood vessels considerably [9,10].

MMP-2 and MMP-9 promote the invasion of trophoblast cells into the uterine lining, a critical process for complete placental implantation and the formation of maternal-fetal circulation [11]. These enzymes degrade extracellular matrix components, facilitating trophoblast invasion and the restructuring of uterine tissue. They are recognized as the primary mediators of human placentation and parturition [12]. In addition, MMP-2 and MMP-9 facilitate the remodeling of spiral arteries, adapting maternal blood vessels to ensure enough blood flow to the growing fetus. Optimal control of MMPs is crucial to avoid excessive and insufficient invasion of trophoblasts, which can result in pregnancy complications such as preeclampsia [13]. Recent research has shown that MMP-2 and MMP-9 play a crucial role in embryo implantation, and spontaneous abortion has been observed when there is an overwhelming elevation of MMP-2 and MMP-9 levels [14,15]. This research aims to evaluate the expression of MMP-2 and MMP-9 in the placenta of women with spontaneous miscarriage.

2. RESULTS AND DISCUSSION

It was found that MMP-2 was diffusely expressed throughout the decidual, trophoblastic, and mesenchymal cells of the chorionic villi in placentas of patients who had miscarriage as well as placentas of patients who had normal delivery who served as controls. The intensity of expression was high in the trophoblastic and mesenchymal cells of the chorionic villi in the placenta that was delivered normally Figure 1. In contrast, the expression of MMP-2 was low in the decidual cells, as seen in Figure 2, and weak to moderate in the trophoblast and mesenchymal cells of villi from the placenta that was aborted in Figures 3 and 4. As shown in Table 1, the first-trimester group had a significant proportion of cases, with sixty percent falling into the weak grading category (five to twenty-five percent) and forty percent falling into the moderate grading area (twenty-six to fifty percent). Not even one of them fell into the negative or strong category. Similarly, the group of second-trimester miscarriages demonstrated that 66.67 percent of cases were classified as weak (5-25%), as seen in Figure 5, and 33.33 percent of cases were classified as moderate (twenty-six to fifty percent), As illustrated in Figure 6. The statistical analysis found that there were very significant differences between the groups that experienced a normal delivery and those that experienced a miscarriage in either the first or second trimester.

MMP-9 protein expression was seen in decidual cells and chorionic villi (trophoblast and mesenchymal cells) of normal deliveries and aborted placentas. The expression was strong in normal delivered cases, as illustrated in Figure 7, and weak to moderate in aborted cases. Table 2 revealed that the first-trimester group had 26 cases in the weak (5-25%) category in decidua and chorionic villi, as illustrated in Figures 8 and 9, while

four cases were in the moderate (26-50%) category, as illustrated in Figures 10 and 11. None were in the negative or strong categories. Similarly, the second-trimester group showed that 24 cases were graded in the weak (5-25%) category, as seen in Figure 12, and 6 cases (26-50%) in the moderate category, as illustrated in Figure 13. Statistical analysis revealed highly significant differences between the normal delivery group and both the first and second-trimester miscarriage groups.

Table 1. Expression of MMP-2 in placental tissue of each group

Groups No=30	Grading			
	- <5%	+ 5-25%	++ 26-50%	+++ >50%
Normal delivered placenta (Control)	0 (0.00%)	0 (0.00%)	0 (0.00%)	30 (100%)
First -trimester Miscarriage	0 (0.00%)	18 (60.00%)	12 (40.00%)	0 (0.00%)
Second-trimester Miscarriage	0 (0.00%)	20 (66.67%)	10 (33.33%)	0 (0.00%)
P-value	0.00 NS	0.0001 **	0.0084 **	0.0001 **

**** (P≤0.01)**

Table 2. Expression of MMP-9 in placental tissue of each group

Groups No=30	Grading			
	- <5%	+ 5-25%	++ 26-50%	+++ >50%
Normal delivered placenta (Control)	0 (0.00%)	0 (0.00%)	0 (0.00%)	30(100%)
First -trimester Miscarriage	0 (0.00%)	26 (86.67%)	4 (13.33%)	0 (0.00%)
Second-trimester Miscarriage	0 (0.00%)	24 (80.00%)	6 (20.00%)	0 (0.00%)
P-value	0.00 NS	0.0001 **	0.074 NS	0.0001 **

**** (P≤0.01).**

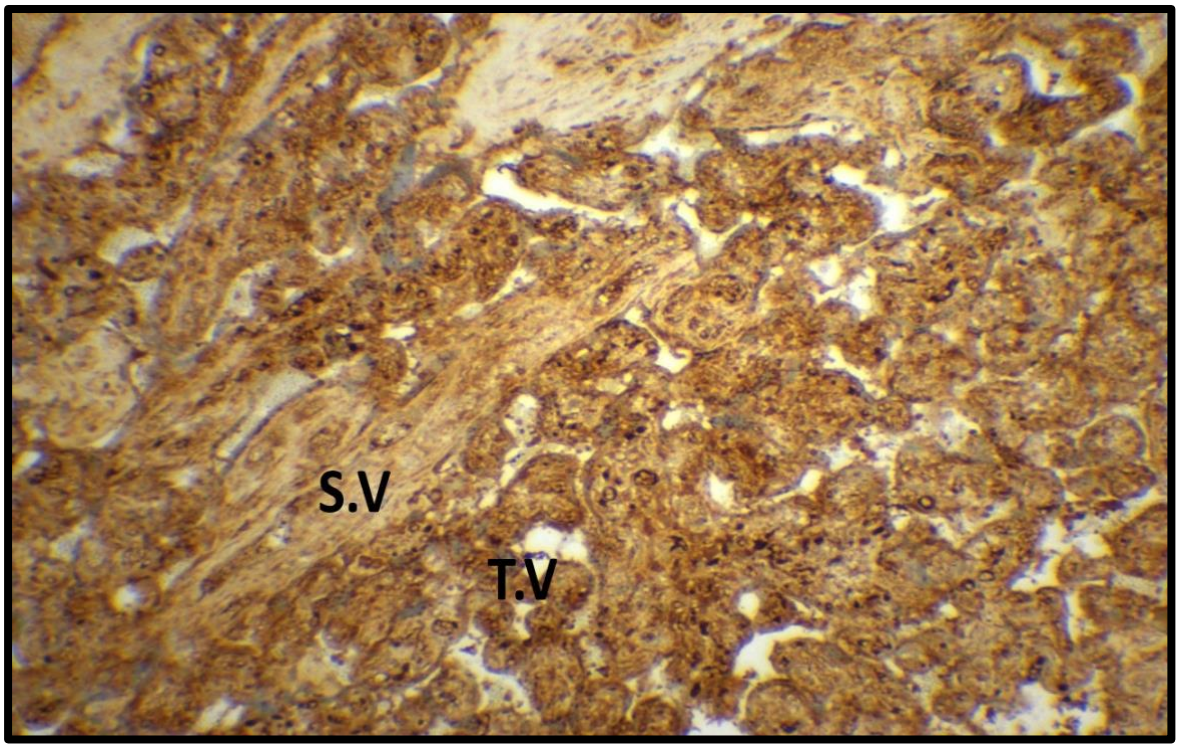

Figure 1. Photomicrographs demonstrating Immunohistochemical staining of MMP-2 in the placenta from women of normal delivery showed strong expression in the chorionic villi, Stem villi (S.V), Terminal villi (T.V). 100X.

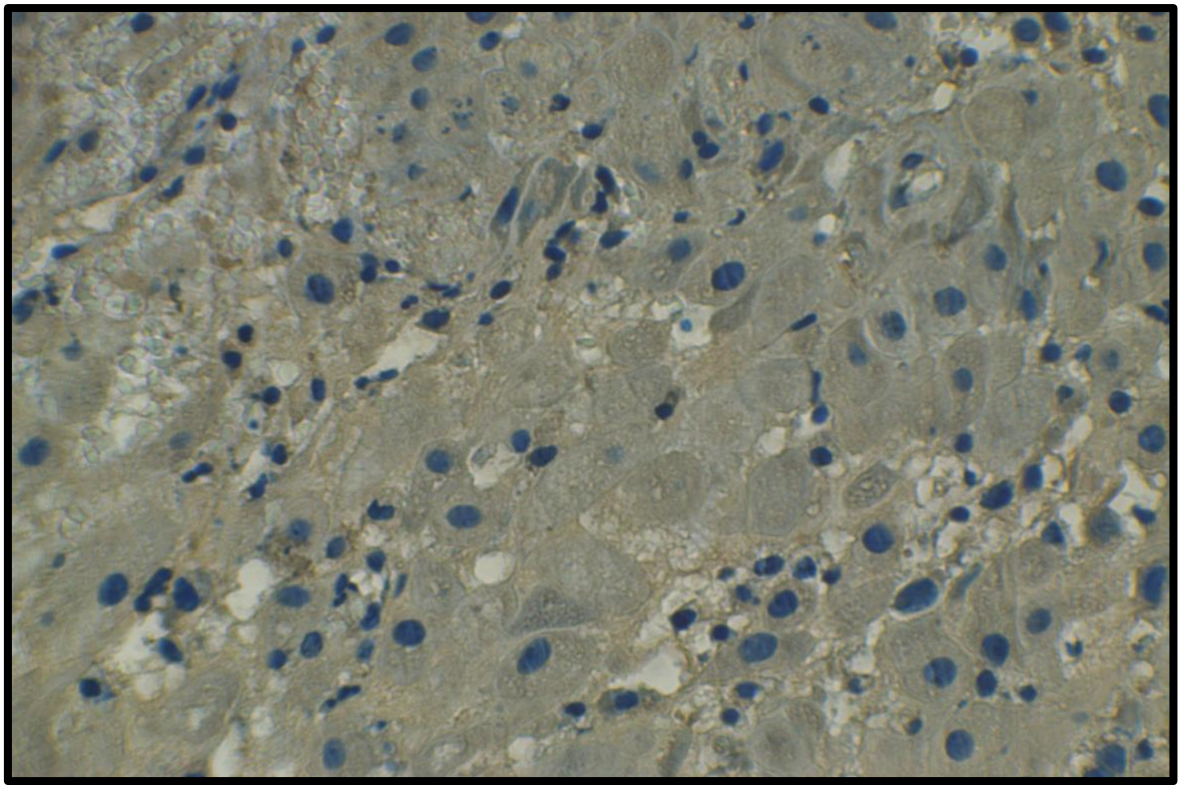

Figure 2. Photomicrographs demonstrating Immunohistochemical staining of MMP-2 in the placenta from first-trimester miscarriage showed weak expression in the decidual cells. 400X.

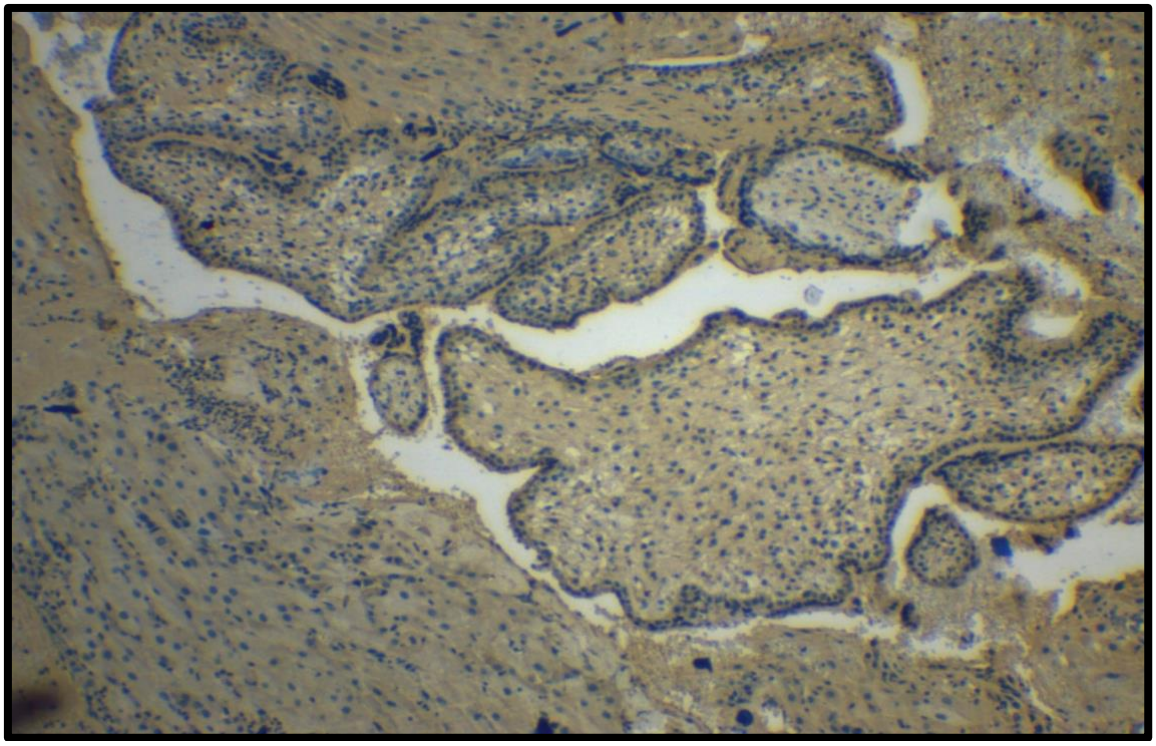

Figure 3. Photomicrographs demonstrating Immunohistochemical staining of MMP-2 in the placenta from first-trimester miscarriage showed weak expression in the chorionic villi. 100X.

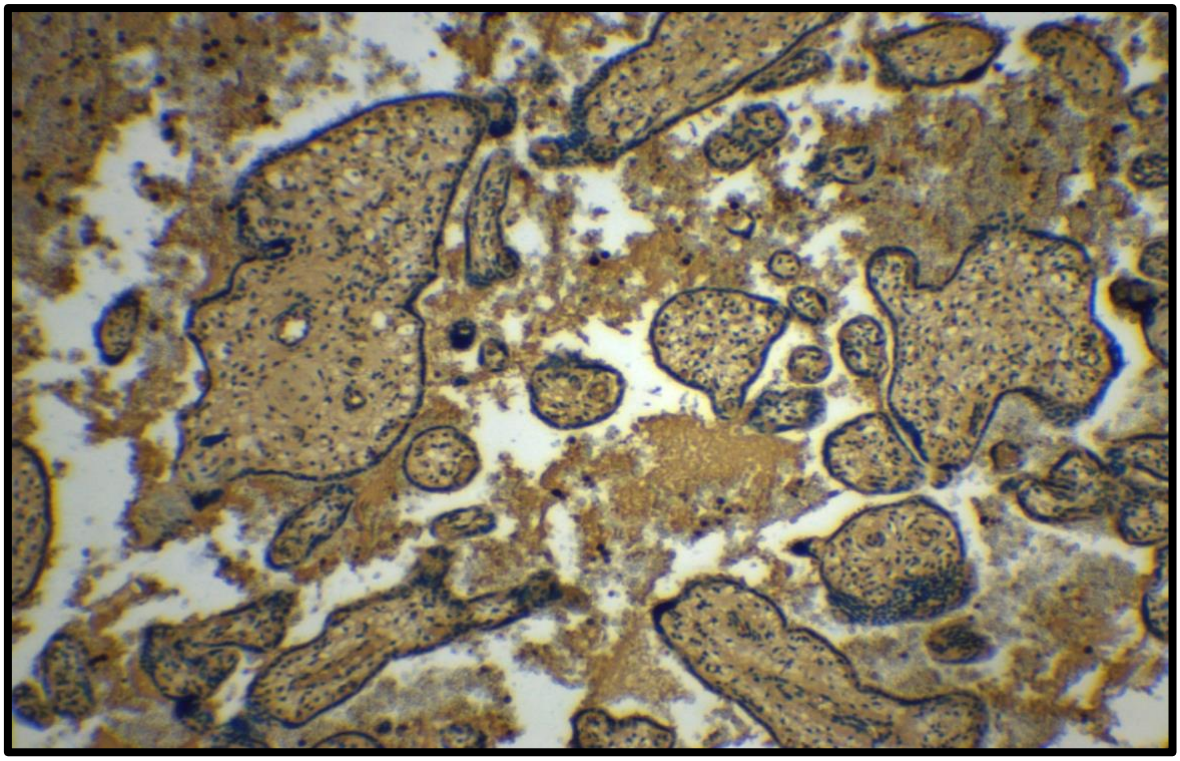

Figure 4. Photomicrographs demonstrating Immunohistochemical staining of MMP-2 in the placenta from first-trimester miscarriage showed moderate expression in the chorionic villi. 100X.

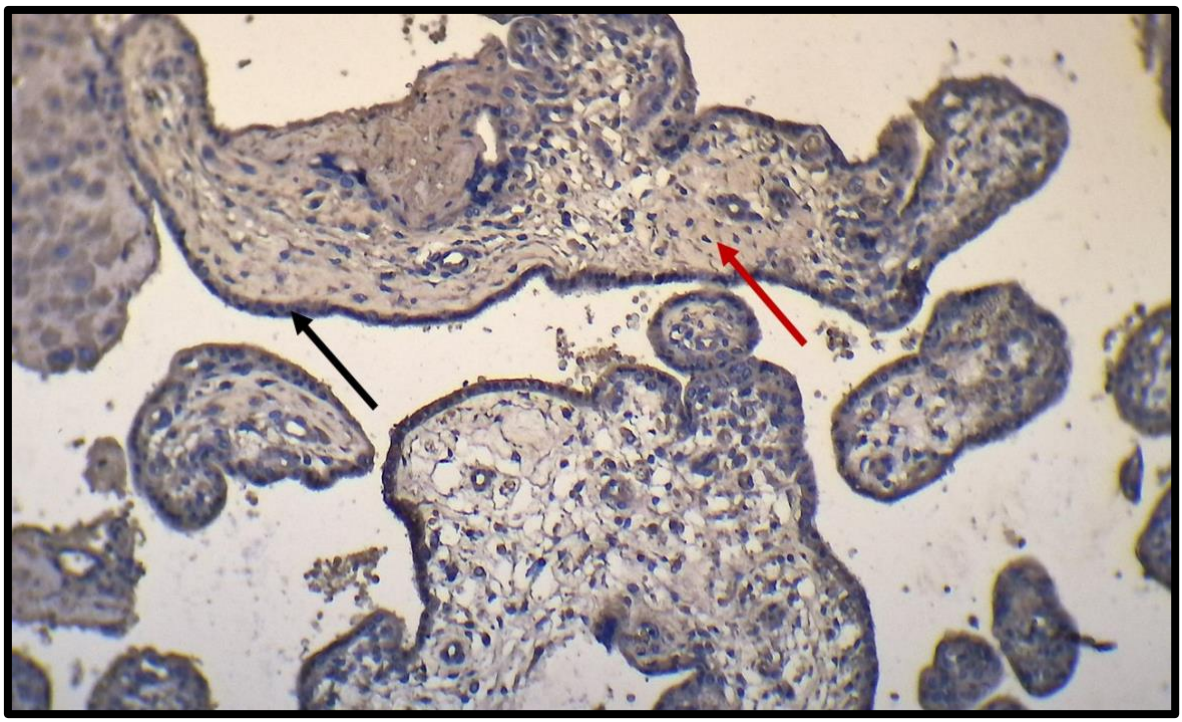

Figure 5. Photomicrographs demonstrating Immunohistochemical staining of MMP-2 in the placenta from second-trimester miscarriage showed weak expression in the chorionic villi, trophoblastic cells (black arrow), stromal cells (red arrow). 100X.

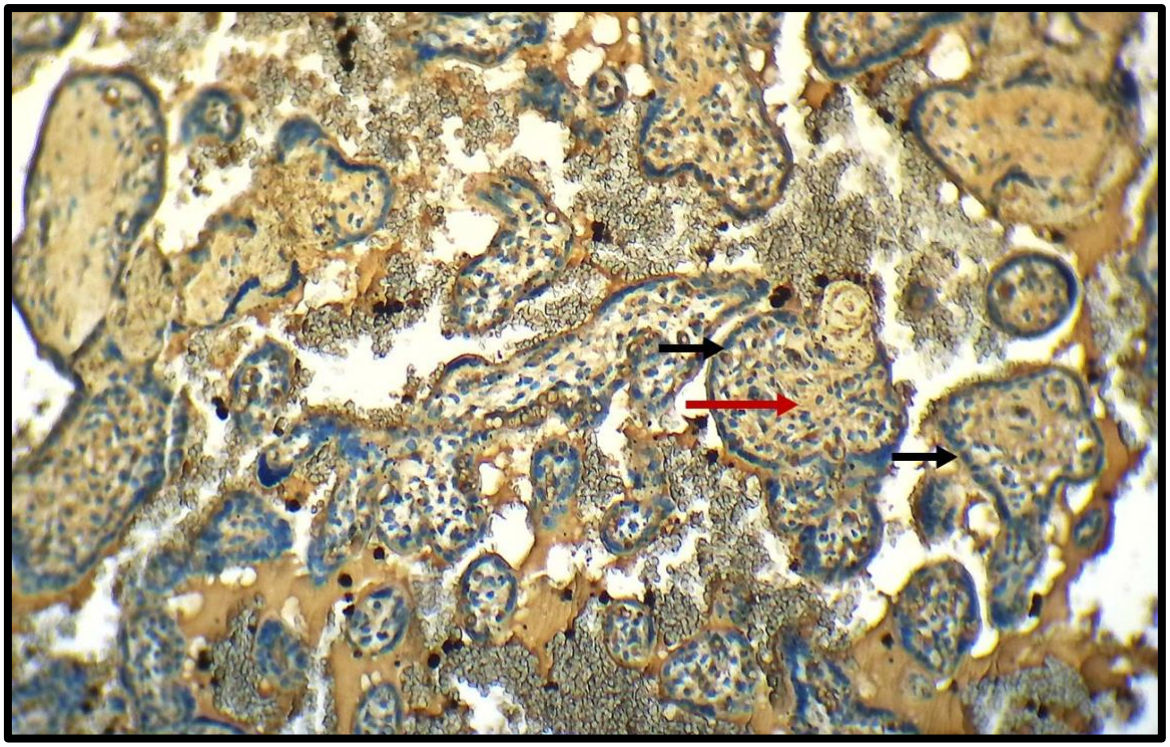

Figure 6. Photomicrographs demonstrating Immunohistochemical staining of MMP-2 in the placenta from second-trimester miscarriage showed moderate expression in the chorionic villi, trophoblastic cells (black arrow), stromal cells (red arrow). 100X.

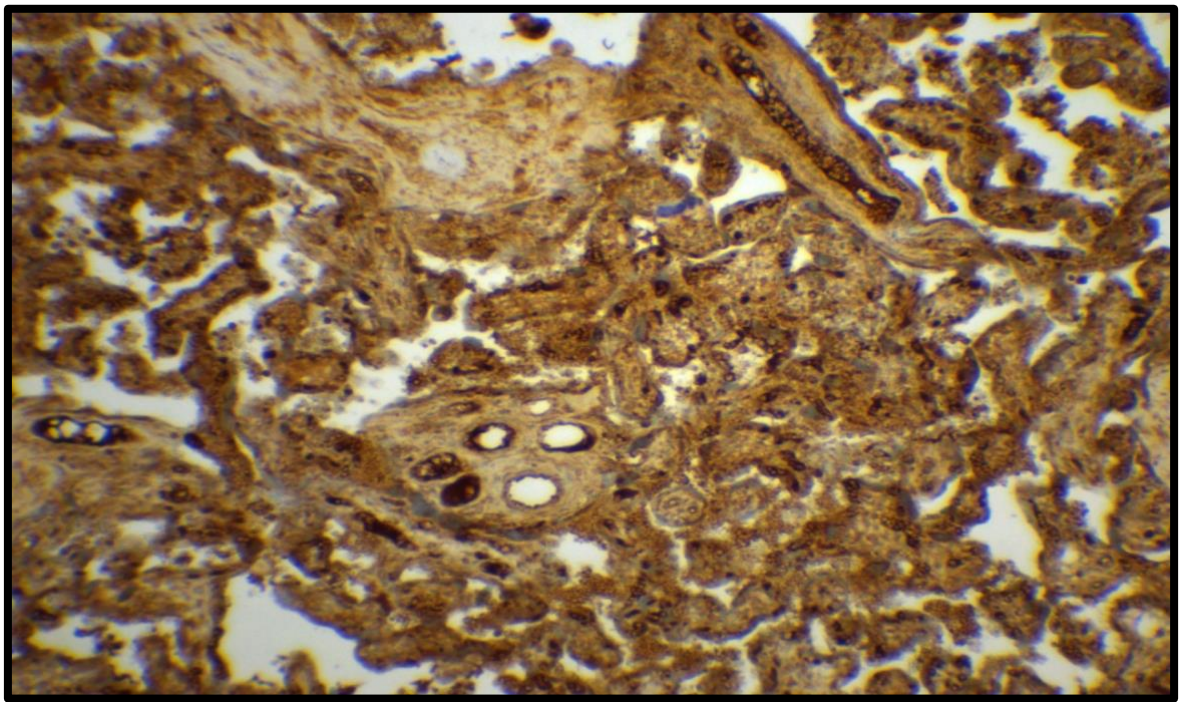

Figure 7. Photomicrographs demonstrating Immunohistochemical staining of MMP-9 in the placenta from women of normal delivery showed strong expression in the chorionic villi, Stem villi, and Terminal villi. 100X.

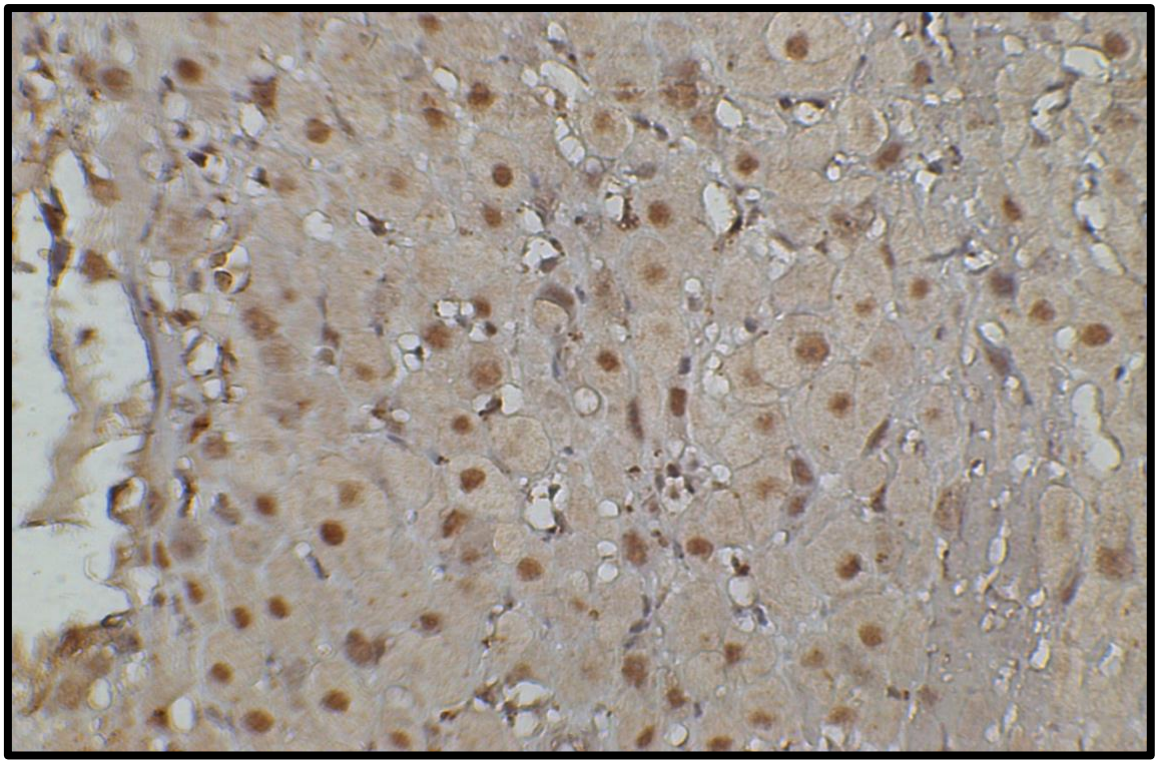

Figure 8. Photomicrographs demonstrating Immunohistochemical staining of MMP-9 in the placenta from first-trimester miscarriage showed weak expression in the decidual cells. 400X.

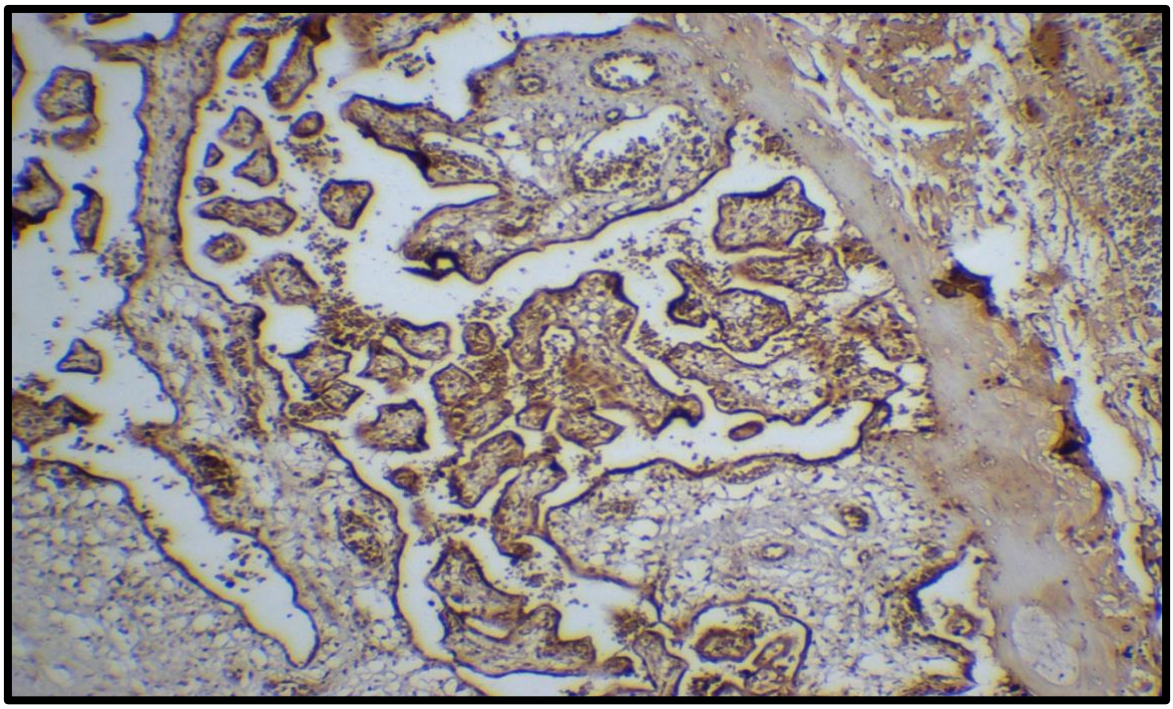

Figure 9. Photomicrographs demonstrating Immunohistochemical staining of MMP-9 in the placenta from first-trimester miscarriage showed weak expression in the chorionic villi. 400X.

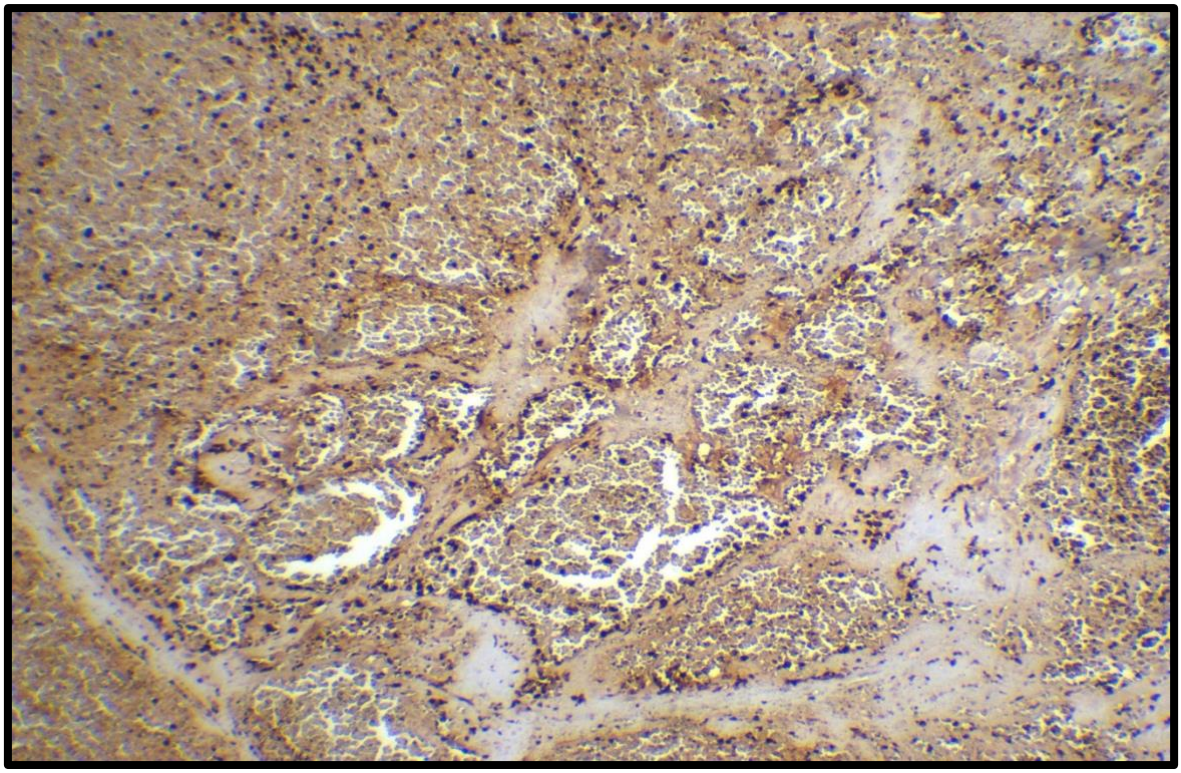

Figure 10. Photomicrographs demonstrating Immunohistochemical staining of MMP-9 in the placenta from first-trimester miscarriage showed moderate expression in the chorionic villi. 100X.

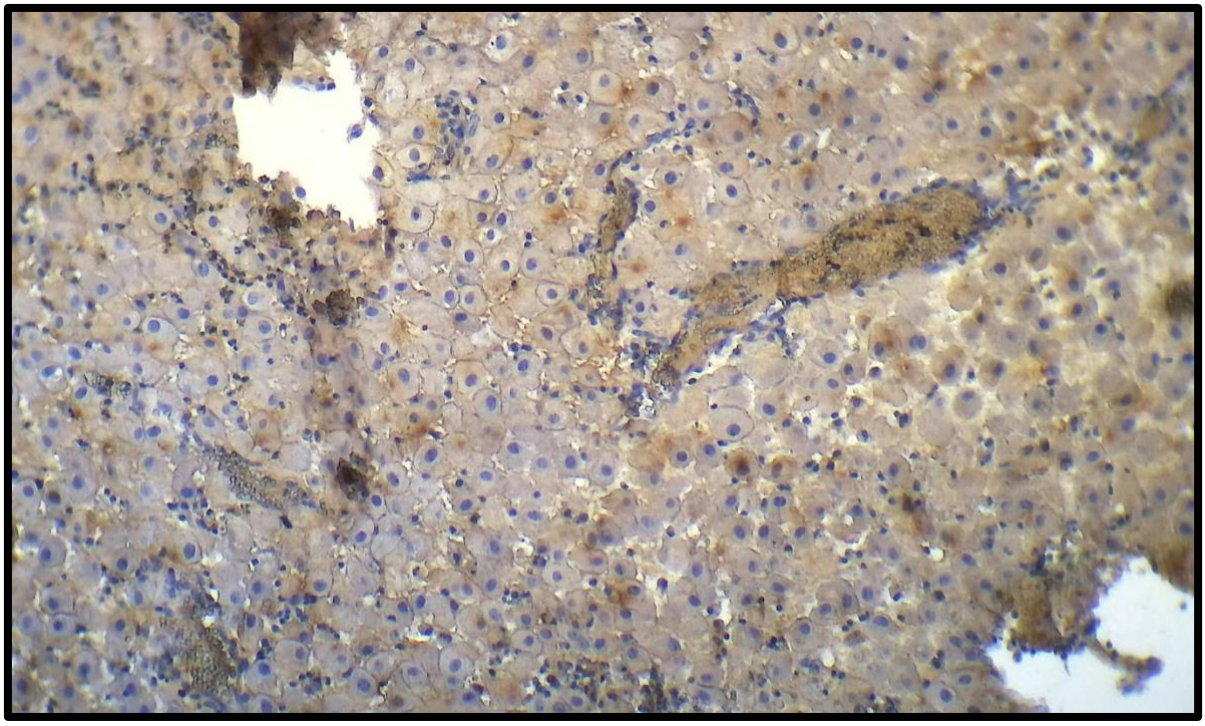

Figure 11. Photomicrographs demonstrating Immunohistochemical staining of MMP-9 in the placenta from second-trimester miscarriage showed moderate expression in the decidual cells. 100X.

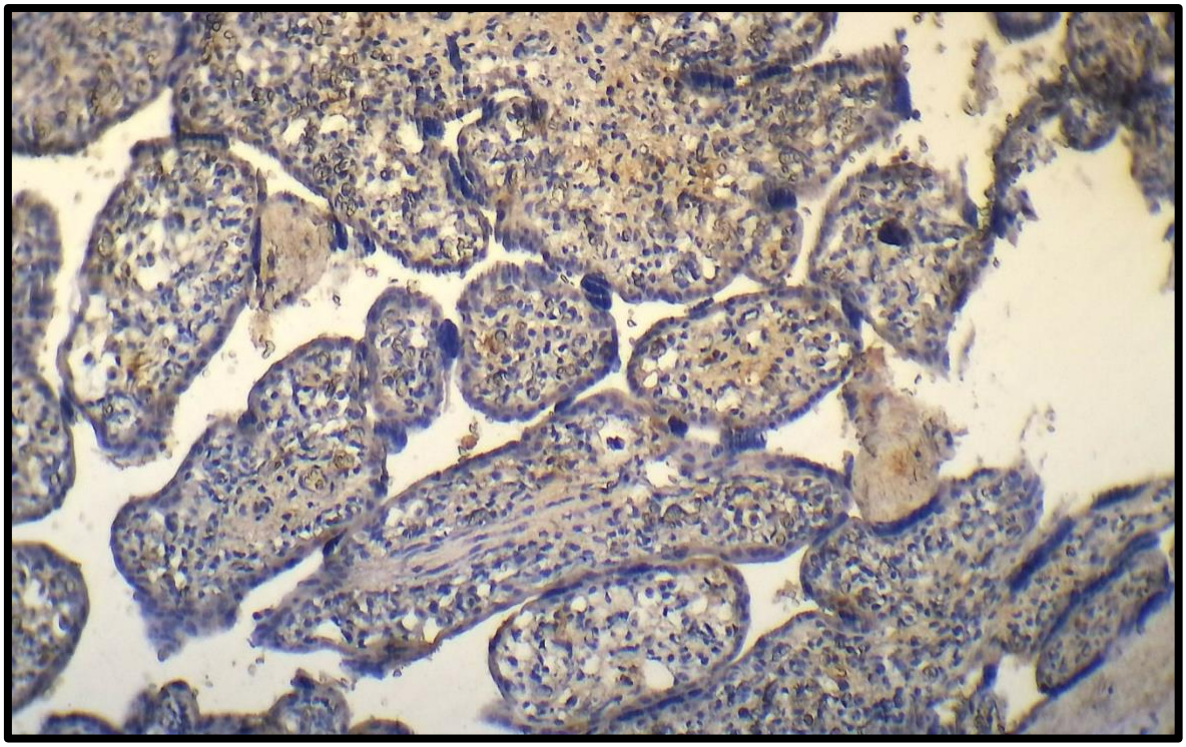

Figure 12. Photomicrographs demonstrating Immunohistochemical staining of MMP-9 in the placenta from second-trimester miscarriage showed weak expression in the chorionic villi. 100X.

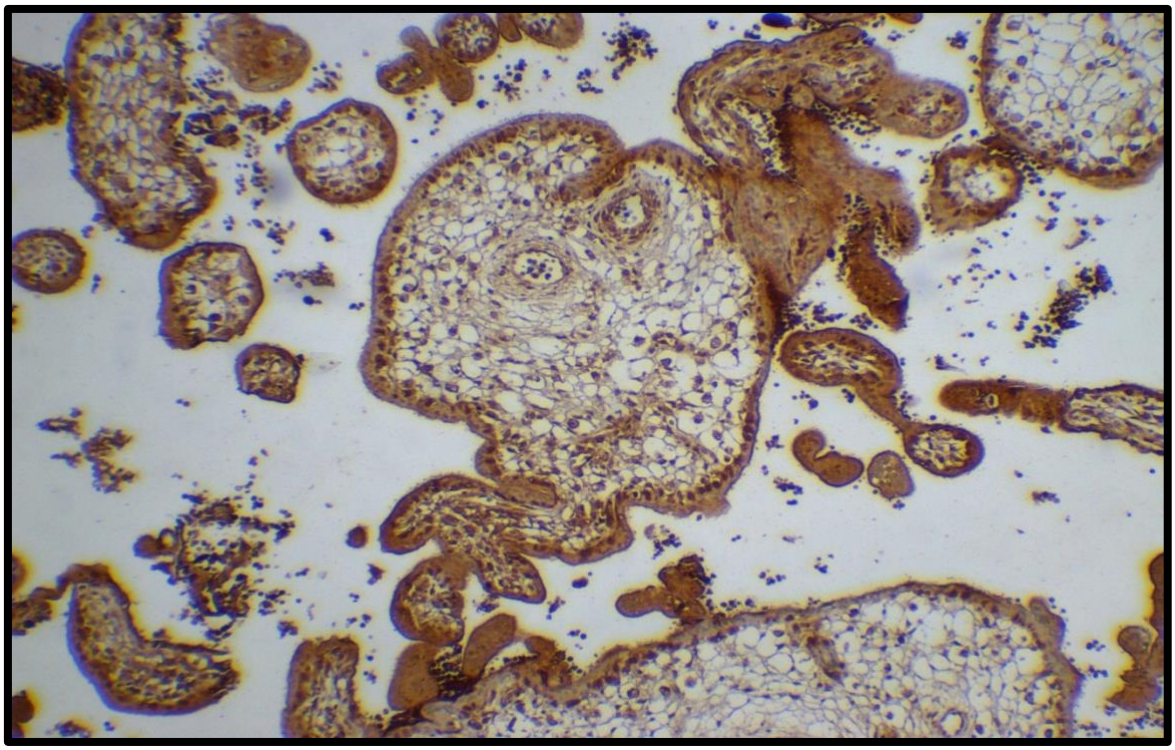

Figure 13. Photomicrographs demonstrating Immunohistochemical staining of MMP-9 in the placenta from second-trimester miscarriage showed moderate expression in the chorionic villi. 100X.

Proteolytic enzymes are classified into a group known as MMPs. These enzymes are recognized by their consistent structures and complex functions. During the matrix degradation process, MMPs are important enzymes. There is a relatively low level of MMP-9 production in the placental bed during the first few weeks of pregnancy (weeks 6 and 7 especially). A large amount of MMP-9 is produced by the cells by the eleventh week of the experiment, despite the fact that the secretion gradually increases after the eighth week. On the

other hand, during the early stages of pregnancy, there is a very noticeable increase in the amount of MMP-2 that is being produced. It is generally agreed that MMP-2 is the most important regulator of trophoblast invasion throughout the early stages of pregnancy. MMP-2 is found in the placental bed during the early stages of pregnancy. It is shown to be more prevalent than MMP-9 in trophoblasts, and it begins to occur between 6 and 8 weeks of gestation. During labour, the primary function of MMP-9 is to increase gelatinolytic activity in the membranes of the uterus. Trophoblasts that originate from the human placenta have the potential to develop into extravillous trophoblasts (EVT), which are characterized by their ability to invade healthy tissue. It is very necessary for EVT cells to possess proteolytic enzymes, more especially MMP-2 and MMP-9, in order for them to invade the endometrial stroma [15,16].

MMPs are vital in controlling vascular and uterine remodeling, the gestational transformation of the spiral arteries, and the formation of the placenta [17]. Elevations in MMP-2 and MMP-9 have been associated with the dilatation of blood vessels, implanting of the placenta, and enlargement of the uterus in a normal pregnancy [18]. Evidence on MMP-2 and MMP-9 displays inconsistencies about their involvement with miscarriage, such as recurrent pregnancy loss. Although several studies found elevated amounts of MMPs and their tissue inhibitors in pregnancy loss, others observed reduced levels of these components. Our result revealed that the expression of MMP-2 was weak to moderate in both abortion groups. Similarly, MMP-9 was detected as weak to a moderate extent in the abortion groups; these results were consistent with a study by Karachrysafi et al. that reported mild to moderate expression of MMP-2 in decidua and trophoblast cells of recurrent pregnancy loss; they also reported moderate expression of MMP-9 in decidua and trophoblastic tissue in the placenta of pregnancy loss. There is so much evidence of MMP-9 participation in recurrent pregnancy loss [19].

Some recent investigations have shown that MMP-9 plays a crucial role in embryo implantation into the endometrium. Spontaneous abortion is observed when there is an excessive elevation of MMP-9 levels; this occurs when decidual membrane destruction and subchorionic bleeding occur, followed by MMP-9 activation; this activation causes damage to the basement membrane and thus leads to spontaneous miscarriage [14]. Similarly, a study reported that the immunopositivity of MMP-9 may suggest highly aggressive invasion that contributes to the development of spontaneous miscarriage pathogenesis [20]. Another research revealed that spontaneous termination of pregnancy due to unknown causes was found to be correlated with increased serum levels of MMP-9 [5]. In addition, A higher level of MMP-9 mRNA expression was observed in the spontaneous abortion group compared to the normal pregnancy group [21].

Some studies have demonstrated that the synthesis and activation of MMP-2 and MMP-9 are essential for the invasion of trophoblasts. They have identified either MMP-9 or MMP-2 as more prominent during the initial phase of trophoblast invasion and also detected distinct expression of MMP-2 and -9 in trophoblast cells during the first trimester, with MMP-2 being the primary gelatinase released until 9 weeks gestation and after that MMP-9 [22]. A study by Woolner et al. reported that an obstetric condition, such as preterm birth and preeclampsia, could have a similar pathophysiology to spontaneous miscarriage [3]. Uterine trophoblasts and vascular cells were shown to be the primary sources of (MMPs), whereas collagen-IV was found to be a significant target for MMP-2 and MMP-9. MMP-2 and MMP-9 play a role in the endometrial tissue remodelling that occurs during pregnancy. There is a minimal infiltration of trophoblasts into the superficial decidual vessels that occurs in preeclampsia. This helps to preserve the integrity of the endothelial lining and the musculoelastic tissue that is present in the deeper spiral arteries. As a consequence, this leads to a partial dilation of the spiral arteries, which reduces their size to half during a normal pregnancy and causes placental ischaemia and hypoxia. Progressive placental ischemia/hypoxia, in addition to its role in the inflammatory response, can result in the loss of trophoblasts and their subsequent entry into circulation, as well as uteroplacental rarefaction and attrition [13]. A hypoxia response element is present in the proteolytic enzymes of MMPs, which has the potential to influence the generation and activity of MMPs as well as the proteolysis of the extracellular matrix (ECM) that is mediated by MMPs [23]. The findings of this investigation corroborated the findings of a prior study that suggested considerable protein expression of MMP-2 and MMP-9 in the trophoblast of chorionic villi and decidua. This study demonstrated robust expression of both MMP-2 and MMP-9 in the placenta of normal births [24].

3. CONCLUSION

In the course of our work, we came to the conclusion that MMP-9 and MMP-2 have a role in the process of uterine tissue remodelling that occurs during the beginning of pregnancy and during a miscarriage. A component of the pathophysiology of pregnancy loss is unregulated trophoblastic invasion, which was found

to be responsible for the shift in MMP-2 and MMP-9 expression that occurs in spontaneous miscarriage, according to the findings. To assess the risk of spontaneous miscarriage or premature birth, a predictive tool could potentially be created through the analysis of normal and abnormal expression patterns of MMP-2 and MMP-9.

4. MATERIALS AND METHODS

4.1. Materials

- Hydrogen peroxide blocking reagent (15ml); Abcam: UK
- Protein block (15 ml); Abcam: UK
- Mouse specifying reagent (monoclonal 6E3F8 to MMP 2) (15 ml); Abcam: UK
- Rabbit specifying reagent (monoclonal EP1254 to MMP 9) (15 ml); Abcam: UK
- HRP polymer (15 ml); Abcam: UK
- DAB chromogen (0.5 ml); Abcam: UK
- DAB substrate (15 ml); Abcam: UK

4.2. Study Design

In a case-control study, we collected 30 placentas from women of normal delivery who were chosen as a control group, and 60 reproductive-aged women with different clinical types of spontaneous miscarriage before 12 weeks of gestation were evaluated as the first-trimester miscarriage group, and after 12 weeks of gestation were considered a second-trimester miscarriage group. Those women were hospitalized due to vaginal bleeding and abdominal pain during the first and second trimester of their pregnancies. An obstetric history of preeclampsia, diabetes, and endocrine disorders were excluded. Products of conception were collected from aborted women as they were admitted for uterine evacuation at the women's emergency department in Fatema AL-Zahraa Hospital in Baghdad province.

4.3. Immunohistochemistry staining

The specimens of the placenta were recovered through the process of curettage. The tissues of the placenta were preserved in formalin at a concentration of 10%, embedded in paraffin blocks, and sectioned at a thickness of 5µ. Subsequently, the sections were put on adhesive microscope slides. Deparaffinization of placental tissue sections was performed using xylene, and then the sections were rehydrated by a series of alcohol washes in sequential order. Following the retrieval of antigen, the endogenous peroxidase activity was suppressed by incubation with 3% hydrogen peroxide at 37 degrees Celsius for ten minutes. Two washes in phosphate-buffered saline (PBS) were performed on the slides after they had been thoroughly rinsed with distilled water for a period of five minutes. In order to prevent nonspecific background staining, a protein block was given to the participant and then incubated for ten minutes at 37 degrees Celsius. At 37 degrees Celsius and in a humidified environment, slides were treated with primary antibodies targeting MMP-2 (6E3F8, diluted 1:1000) and MMP-9 (EP1254[ab76003], diluted 1:1000) for a period of one hour. Every one of the three washes in PBS that were performed on the slides lasted for five minutes. After applying the mouse/rabbit specific reagent and incubating the slides at 37 degrees Celsius for ten minutes, the slides were washed with distilled water for five minutes, and then they were rinsed twice in phosphate-buffered saline (PBS). There was an administration of mouse anti-MMP2 and rabbit anti-MMP9 conjugates, which were then incubated at 37 degrees Celsius for fifteen minutes. A total of four washes in PBS were performed on the slides. After adding chromogen and diaminobenzidine (DAB) to 1.5 millilitres or fifty drops of DAB substrate, the mixture was swirled and then applied to the tissue. After incubating the tissue for seven to ten minutes, the tissue was rinsed with distilled water for one minute. The slides were cleaned in PBS many times. Harris Haematoxylin was used to perform the counterstaining procedure. Increasing concentrations of ethyl alcohol (50 percent, 70 percent, 80 percent, 90 percent, and 100 percent) are used to dehydrate slides for one minute each. This is then followed by immersion in xylene, which has been cleaned for two minutes. After that, the slides are mounted with mounting media (DPX – SYRBIO), and a coverslip is placed over them before they are examined with a light microscope. In 2022, the Baghdad Health Department provided permission number 9559 to the ethics committee. This number was issued by the committee.

4.4. Assessment of Immunohistochemical staining

The immunohistochemistry staining was evaluated independently by a pathologist who had prior expertise using a semi-quantitative grading system. Based on the cytoplasmic staining of syncytiotrophoblast, cytotrophoblast, and decidual stromal cells, the cells were categorized as either negative (zero), faintly(weak) positive (1+), moderately positive (2+), or strongly positive (3+). A distinct brown stain is scored as positive. A strong positive outcome was considered when over 50% of the cells displayed strong staining. Furthermore, a weakly positive outcome was reported when fewer than 25% of the cells exhibited strong staining. An intermediately positive staining outcome (2+) was seen when 25% to 50% of the cells of interest exhibited strong staining.

4.5. Statistical Analysis

The SPSS program, version 25.0 (SPSS, Chicago), was utilized for each and every statistical analysis that was processed. In order to conduct a comparative examination of the levels of immunohistochemistry expression between the different study groups, the Chi-square test was utilized. This test performed a statistically significant comparison of the percentages of likelihood (0.05 and 0.01). A difference that was judged to be statistically highly significant was considered to have a p-value that was less than 0.01 [25,26].

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Author contributions: Concept – M.H.; Design – R.H., M.H.; Supervision – M.H.; Resources – R.H.; Materials – R.H.; Data Collection and/or Processing – R.H.; Analysis and/or Interpretation – R.H.; Literature Search – R.H., M.H.; Writing – R.H.; Critical Reviews –M.H.

Conflict of interest statement: The authors declared no conflict of interest" in the manuscript.

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