Elevated expression levels of matrix metalloproteinase-9 in placental villi and tissue inhibitor of metalloproteinase-2 in decidua are associated with prolonged bleeding after mifepristone-misoprostol medical abortion

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Objective: To determine whether the expression levels of matrix metalloproteinases 2 and 9 (MMP-2 and -9) and tissue inhibitors of metalloproteinases 1 and 2 (TIMP-1 and -2) in the villi and the decidua are associated with prolonged bleeding after medical abortion.

Design: Case-controlled study.

Setting: University hospital.

Patient(s): Mifepristone-misoprostol medical abortion patients were divided into two groups (20 women each) based on the length of time (>14 or ≤14 days) of bleeding after the abortion.

Intervention(s): Discharged villi and deciduas were collected.

Main Outcome Measure(s): The expression levels of MMP-2 and -9 and TIMP-1 and -2 in the villi and deciduas were assessed with semiquantitative immunohistochemistry.

Result(s): The median semiquantitative immunohistochemistry staining index (SI) scores for MMP-9 expression in the villi were elevated in the bleeding group compared with the control group (median SI scores 0.31 and 0.03, respectively). TIMP-2 expression was elevated in the decidua in the bleeding group compared with the control group (median SI scores 1.00 and 0.20, respectively). No significant differences were observed between the two groups in the expression levels of MMP-2 in the villi or of MMP-2, MMP-9, or TIMP-1 or of the ratios of MMP-9/TIMP-1 or MMP-2/TIMP-2 in the decidua.

Conclusion(s): Elevated expression levels of MMP-9 in the villi and of TIMP-2 in the decidua were associated with prolonged bleeding after medical abortion. (Fertil Steril® 2014;101: 166–71. ©2014 by American Society for Reproductive Medicine.)

Key Words: Matrix metalloproteinase, tissue inhibitor of matrix metalloproteinase, abortion, immunohistochemistry

Discuss: You can discuss this article with its authors and with other ASRM members at http://fertstertforum.com/huangl-mmp9-timp2-mifepristone-misoprostol-abortion/

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Mifepristone-misoprostol medical abortion is well established as a safe, effective, feasible, and acceptable option for early pregnancy termination (1–4). In China, most hospitals currently use repeated small doses of 25 mg (total 150 mg) of mifepristone in combination with misoprostol (total 600 μg) as the
Materials and Methods

This study was conducted with the approval of the Institutional Review Board, and informed consent was obtained from each sample provider. Forty women were recruited after they had undergone medical abortions. Entry criteria included: age 20–40 years; normal menstrual cycles; singleton intrauterine pregnancy of ≤49 days’ gestation (based on the onset of the last menstrual period, bimanual examination, and ultrasound); absence of genital inflammation and neoplasms; and lack of treatment with exogenous hormones within the previous 6 months.

Medical abortion patients were treated with 50 mg mifepristone administered orally on their first visit, followed by 25 mg mifepristone administered orally every 12 hours (total 150 mg) at home. During a second visit 48 hours later, women received 600 μg oral misoprostol and remained in the hospital under observation for 4 hours. During this observation period, tissues discharged from the vagina (including placental villi and decidua) were collected, fixed overnight in 10% buffered formalin, dehydrated, and embedded in paraffin. The women returned to the hospital for a follow-up visit 15 or 16 days after the day that misoprostol was administered. In China, large-scale studies of medical abortions have shown that vaginal bleeding after a complete medical abortion lasts for ~14 days on average (18). Therefore, we selected a time period of >14 days to define prolonged uterine bleeding. Women were assigned to the bleeding or the control group (n = 20 women each) according to whether their duration of bleeding after the medical abortion was >14 days or ≤14 days, respectively.

Ultrasounds were performed for all 40 women. The ultrasound evaluation revealed the presence of tissue remaining in the uterine cavity of those women with a duration of bleeding >14 days. No abnormal ultrasound images were obtained from women who had a duration of bleeding ≤14 days.

Formalin-fixed paraffin-embedded decidua tissue blocks from each case were cut into 4-μm-thick sections. Four serial sections were examined for the expression of MMP-2, MMP-9, TIMP-1, and TIMP-2. Formalin-fixed paraffin-embedded villi tissue blocks from each case were cut into 4-μm-thick sections, and two serial sections were examined for the expression of MMP-2 and MMP-9.

Immunohistochemical staining for MMP-2, MMP-9, TIMP-1, and TIMP-2 was performed with the Envision System according to the manufacturer’s instructions. Briefly, antigen retrieval was performed before immunostaining for each section. After endogenous peroxidase was quenched with 3% hydrogen peroxide in phosphate-buffered saline solution (PBS) for 15 minutes at room temperature, the slides of villi were incubated with mouse antihuman monoclonal antibodies for MMP-2 (1:50 dilution; Maixin) or MMP-9 (1:50; Maixin), and the slides of decidua were incubated with mouse antihuman monoclonal antibodies for MMP-2 (1:50; Maixin), MMP-9 (1:30; Maixin), TIMP-1 (1:30; Maixin), or TIMP-2 (1:100; Zhongshan) for 60 minutes at room temperature. Antibody binding was visualized and photographed under a light microscope.

A color reaction was developed by staining with diaminobenzidine (Maixin) and counterstaining with hematoxylin. Negative and positive control slides were incorporated into each slide run. Negative control samples were incubated similarly, but the primary antibody was replaced with PBS.

Immunostaining was scored by two independent experienced pathologists who were blinded to the...
clinical-pathologic data and the clinical outcomes of the patients. First, the percentages of endometrial cells staining positive were categorized and awarded a score of 0–4 as follows: 0, <5%; 1, 5%–25%; 2, 26%–50%; 3, 51%–75%; and 4, >75%. The intensity of immunostaining was scored on a 3-point scale, as follows: 1, weak; 2, moderate; and 3, intense (19). A weighted score was produced for each section by multiplying the percentage by the intensity score. The mean staining index (SI) scores were calculated from the average scores from the two independent observers.

Statistical Analysis
We used a two-sided Wilcoxon–Mann–Whitney test to determine whether the level of TIMP-2 varied between the two groups. The test was performed at the 5% significance level (α = 0.05). The test parameters were set so that an 80% chance existed for rejecting the null hypothesis of equality if the difference in the TIMP-2 was 1.0 in either direction (80% power; β = 0.2). Previous measurements suggested that the common standard deviation for the level of TIMP-2 in both groups is 0.8. Using the sample size formula from Divine et al. (20), we estimated the appropriate sample size to be 34 (17 per group). For statistical robustness, we enrolled 20 subjects per group. Statistical analysis was performed with the SPSS 11 software package. The Mann–Whitney U test was used to compare the expression levels of MMP-2/9 and TIMP-1/2 (weighted scores) between the two groups. The statistical analysis of demographic characteristics was performed with Student t test or χ² analysis as appropriate.

RESULTS
Supplemental Table 1 (available online at www.fertstert.org) shows the demographic characteristics of the participants. Overall, no significant differences were found between the two groups regarding age, gestational age, embryo sac size, gravidity, or parity. Results of the semiquantitative evaluation of MMP-2 and -9 immunoreactivity in the villi are presented in Table 1. Results of the immunoreactivity toward MMP-2/9 and TIMP-1/2 in the decidua are presented in Table 2. The MMP-2/TIMP-2 and MMP-9/TIMP-1 ratios are reported in Supplemental Table 2 (available online at www.fertstert.org).

MMP-2 staining was observed in the membrane and cytoplasm of cytotrophoblast cells in villus samples of both groups (Figs. 1A and 1B), and MMP-9 staining was observed in the membrane and cytoplasm of cytotrophoblast and syncytiotrophoblast cells in villus samples from both groups (Figs. 1C and 1D). The median SI scores for MMP-2 in the villi (0.44 for the bleeding group and 0.20 for the control group) were not significantly different (Table 1). The median SI score of MMP-9 in the villi of the bleeding group was 0.31, compared with 0.03 in the control group; thus, MMP-9 expression differed between the two groups (P<.05; Table 1).

Immunostaining for MMP-2/9 and TIMP-1/2 was detected in all major tissue compartments of the decidua, including the epithelium and stromal cells, but it was predominantly observed in the glandular epithelial cells. Immunoreactivity for MMP-2 and -9 was localized to the cytoplasm and membrane (Figs. 2A–2D). The median SI scores for MMP-2 in the decidua were not significantly different between the bleeding and control groups (1.44 and 0.82, respectively), nor were the median SI scores significantly different for MMP-9 in the decidua (0.64 and 0.50 in the bleeding and control groups, respectively), as presented in Table 2.

The pattern of TIMP-1 and TIMP-2 immunoreactivity observed in the decidua was also cytoplasmic and membranous (Figs. 2E–2H). The median SI score for TIMP-1 in the decidua was 1.00 for both groups (P>0.05), whereas the median SI scores for TIMP-2 in the decidua were 1.00 and 0.20 in the bleeding and control groups, respectively (P<.05; Table 2). A negative control sample is shown in Figure 2L.

To evaluate the relative inhibition of MMP activity, we calculated the ratios of MMP-9/TIMP-1 and MMP-2/TIMP-2 in the decidua. In the bleeding group, no significant difference was found for the median MMP-9/TIMP-1 ratio between the bleeding group (0.76) and the control group (0.58; P>.05), nor was a significant difference observed for the median MMP-2/TIMP-2 ratio between the bleeding group (1.97) and the control group (1.00; P>.05; Supplemental Table 2).

DISCUSSION
In this study, we collected endometrial tissue samples that were discharged from medical abortion patients on the day that misoprostol was administered. Abnormal expression levels of MMP-9 in the villi and of TIMP-2 in the decidua were found to be associated with prolonged bleeding. These findings may help to explain the mechanisms contributing

| TABLE 2 |

| Immunoreactivity in decidua (SI). | Control group (n = 20) | Bleeding group (n = 20) | \( P \) value

<table>
<thead>
<tr>
<th>Percentile</th>
<th>25th</th>
<th>50th</th>
<th>75th</th>
<th>25th</th>
<th>50th</th>
<th>75th</th>
</tr>
</thead>
<tbody>
<tr>
<td>MMP-2</td>
<td>0.36</td>
<td>0.82</td>
<td>2.48</td>
<td>0.80</td>
<td>1.44</td>
<td>3.81</td>
</tr>
<tr>
<td>MMP-9</td>
<td>0.07</td>
<td>0.50</td>
<td>1.44</td>
<td>0.36</td>
<td>0.64</td>
<td>1.44</td>
</tr>
<tr>
<td>TIMP-1</td>
<td>0.07</td>
<td>1.00</td>
<td>2.24</td>
<td>0.30</td>
<td>1.00</td>
<td>2.04</td>
</tr>
<tr>
<td>TIMP-2</td>
<td>0.04</td>
<td>0.20</td>
<td>0.64</td>
<td>0.64</td>
<td>1.00</td>
<td>2.41</td>
</tr>
</tbody>
</table>

Note: TIMP = tissue inhibitor of metalloproteinases; other abbreviations as in Table 1.

a Calculated with the Mann–Whitney U test.

to the prolonged bleeding that can occur after medical abortions.

A previous histologic analysis showed that prolonged bleeding after medical abortions is caused by retained tissues and the impaired repair and regeneration of the endometrium (21). Another study demonstrated an overall increase in the endometrial leukocyte population in women with prolonged bleeding, suggesting that leukocytes or their products may contribute to persistent bleeding (22). Prolonged bleeding is primarily thought to reflect the uterine retention of small pieces of trophoblast and decidua that remain viable after embryonic expulsion (6, 7). Interestingly, we documented a significant increase in MMP-9 expression in villi but no increased MMP-9 expression in decidua from women with a bleeding duration >14 days, compared with the control group.

The invasiveness displayed by human cytotrophoblast cells reportedly depends on the production of MMP-9 (23). The MMP-9 expression also coincides with the maximal invasive potential of the cytotrophoblast cells during the first trimester (14). Trophoblast cells exhibit up-regulated secretion and expression of MMP-9 from week 6 to week 11 of gestation, which may be a prerequisite for trophoblast invasiveness during placentation. Cytotrophoblast cells isolated from preeclamptic placentas reportedly express almost no MMP-9 (16). In this study, MMP-9 expression in the bleeding group was elevated compared with the control group. This result suggests that trophoblast cells in the bleeding group were more invasive than in the control group. As a result, these cells were prevented from shedding from the uterine wall and remained in the uterine cavity.

TIMP-1 inhibits all MMPs in their activated form and preferentially binds MMP-9 in both its latent and its active forms (24). TIMP-2 binds both active and latent forms of MMP-2 but with less inhibitory activity than it has toward other MMPs (25–27). Therefore, we evaluated the ratios of MMP-9/TIMP-1 and MMP-2/TIMP-2 expressions in the decidua. Although the ratios did not differ significantly between the two groups, the TIMP-2 expression level was higher in the bleeding group than in the control group. Therefore, in some cases, TIMP-2 inhibited the activity of MMP-2 in the bleeding group. We propose that TIMP-2 inhibits the activity of MMP-2 and that this inhibition may have resulted in the incomplete breakdown of the extracellular matrix, making the complete shedding of the products of conception difficult. This phenomenon may help to explain why women who experience prolonged bleeding after a medical abortion still retain tissue.

A limitation of the present study is that because the sample size is small owing to difficulties in sample collection, the possibility of a type II error was increased. Therefore, the power of this study may be too low to reject the null hypothesis that the expression levels of MMP-9, MMP-2, and TIMP-1 are equal between the two groups.

In summary, elevated expression levels of MMP-9 in the placental villi and of TIMP-2 in the decidua were associated with prolonged bleeding after medical abortions. Additional research and an increased number of samples are needed to
Expression levels of matrix metalloproteinase (MMP) 2, MMP-9, tissue inhibitor of metalloproteinases (TIMP) 1, and TIMP-2 in the decidua after medical abortion. Formalin-fixed paraffin-embedded tissues were stained as indicated. Samples are shown at ×200 magnification. MMP-2 expression in (A) bleeding group and (B) control group; MMP-9 expression in (C) bleeding group and (D) control group; TIMP-1 expression in (E) bleeding group and (F) control group; TIMP-2 expression in (G) bleeding group and (H) control group; (I) negative control.

determine whether additional MMPs and TIMP-3 or -4 are also involved in this process.

REFERENCES

## SUPPLEMENTAL TABLE 1

Demographic characteristics of participants.

<table>
<thead>
<tr>
<th></th>
<th>Control group (n = 20)</th>
<th>Bleeding group (n = 20)</th>
<th>P value (^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (y) (^a)</td>
<td>26.90 ± 5.37</td>
<td>28.15 ± 4.20</td>
<td>&gt;.05</td>
</tr>
<tr>
<td>Gestational age (d) (^a)</td>
<td>41.95 ± 4.29</td>
<td>43.00 ± 3.49</td>
<td>&gt;.05</td>
</tr>
<tr>
<td>Size of embryo sac (cm) (^a)</td>
<td>1.25 ± 0.43</td>
<td>1.37 ± 0.41</td>
<td>&gt;.05</td>
</tr>
<tr>
<td>Parity (no. of times)</td>
<td></td>
<td></td>
<td>&gt;.05</td>
</tr>
<tr>
<td>0</td>
<td>15</td>
<td>14</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>5</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>Gravidity (no. of times)</td>
<td></td>
<td></td>
<td>&gt;.05</td>
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<tr>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>11</td>
<td>12</td>
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</tr>
<tr>
<td>2</td>
<td>4</td>
<td>6</td>
<td></td>
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<tr>
<td>≥ 3</td>
<td>1</td>
<td>1</td>
<td></td>
</tr>
</tbody>
</table>

\(^a\) Mean ± SD.

\(^b\) Student t-test and \(\chi^2\) analysis.

### SUPPLEMENTAL TABLE 2

**MMP/TIMP ratios in decidua.**

<table>
<thead>
<tr>
<th>Percentile</th>
<th>Control group (n = 20)</th>
<th>Bleeding group (n = 20)</th>
<th>P value$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>25th</td>
<td>50th</td>
<td>75th</td>
</tr>
<tr>
<td>MMP-2/TIMP-2</td>
<td>0.13</td>
<td>1.00</td>
<td>5.17</td>
</tr>
<tr>
<td>MMP-9/TIMP-1</td>
<td>0.11</td>
<td>0.58</td>
<td>1.73</td>
</tr>
</tbody>
</table>

Note: MMP = matrix metalloproteinase; TIMP = tissue inhibitor of metalloproteinases.

$^a$ Calculated with the Mann-Whitney U test.