Differential *pro*MMP-2 and *pro*MMP-9 Secretion in Human Pre-implantation Embryos at Day 5 of Development.

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Research

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Abstract

Background: The most commonly used non-invasive criterion for evaluating the probable success of transferring *in vitro* human embryos for implantation is their morphological development. With this criterion, however, embryos in cellular arrests go unnoticed. Extracellular matrix metalloproteases type 2 (MMP-2) and MMP-9 are key markers of embryonic development and the implantation process, according to various animal studies. The current study investigated the *pro*MMP-2 and *pro*MMP-9 expression in the culture media developing human embryos that were transferred for implantation.

Methods: Forty-two patients were accepted in the Department of Reproductive Biology of a Hospital in México City, based on the Institutional inclusion criteria for *in vitro* fertilization. On day 5 of development, embryos were transferred to women, and the culture medium was stored at -70 to await assessment of the activity of *pro*MMP-2 and *pro*MMP-9 in substrate gel zymography.

Results: The patients showing embryo sac development were assigned to the pregnant group (n =17) or non-pregnant (n =25). In both groups, the activity of *pro*MMP-2 and *pro*MMP-9 was evaluated in substrate gel zymography. Our results indicate for all 17 women able to achieve a full-term pregnancy, the activity band of *pro*MMP-2 was found in the corresponding culture medium. For 11 of them, the band of *pro*MMP-9. Regarding the other 25 patients, the expression band for *pro*MMP-2 detected in 3 and that *pro*MMP-9 in 11 individuals.

Conclusions: On day 5 of embryo development, the evaluation of *pro*MMP-2 and *pro*MMP-9 in the embryo culture medium is a reliable indicator of embryo quality and capacity to establish pregnancy.

Background

The success growth and implantation of blastocysts is a complex event involving maternal and embryonic signals [1, 2]. Cytokines [3, 4], growth factors [5, 6], and matrix metalloproteinases [7, 8] are associated with an adequate interaction between the blastocyst and uterine endometrium after implantation [9, 10].

Taskin *et al*. (2012) detected the secretion of interleukin (IL)-1β in the culture media of human embryos at distinct stages of development [11]. Among the signaling pathways regulated by inflammatory cytokines is the activation of matrix metalloproteinases (MMPs) [12], a family of zinc-dependent endoproteases MMPs participate in tissue remodeling and the degradation of various proteins in the extracellular matrix including collagen [13, 14], elastin [15, 16], gelatin [17, 18], matrix glycoproteins and proteoglycans [19, 20]. The substrates degraded by MMPs are the basis of classifying the latter commonly known MMPs are stromelysin-1 (MMP-3), -2 (MMP-10), -3 (MMP-11), collagenase-1 (MMP-1), -2 (MMP-8), -3 (MMP-13), gelatin-A (MMP-2), and gelatin-B (MMP-9), matrilysin type I (MMP-7), and type II (MMP-26). Membranal type I (MMP-14, -15, -16, and − 24), and Type II (MMP-23) [21–23]. In addition to their role in pregnancy [24, 25]. MMPs promote cell proliferation [26, 27], migration [28, 29], differentiation [30, 31].

Regarding the evaluation of embryo quality prior to implantation, there is as yet no quantitative method. Embryonic morphology is the criterion employed in clinical practice as a qualitative marker of the viability of embryos to be transferred to patients [32, 33]. However, this method has not shown an acceptable clinical correlation, since even aneuploid embryos can have normal morphology, and some euploid embryos can have aberrant morphology [34]. Thus, the aim of present study, was to analyze the culture medium of embryos with good morphological development in order to determine whether significant differences exist in the expression of *pro*MMP-2 and *pro*MMP-9 between the groups of embryos with successful and implantation.

Methods

Ethics statements

The current protocol was reviewed and approved by the Ethics and Research Committees of the Instituto Nacional de Perinatología in México City (212250 – 22661). The purpose of the study was explained to all patients, and informed consent was signed by those who decided to participate.

Patients

Forty-two patients diagnosed with infertility were admitted to the Department of Reproductive Biology (Instituto Nacional de Perinatología) for *in vitro* fertilization. The following constituted the inclusion criteria: ≤37 years of age, a regular menstrual cycle, a normal uterine cavity confirmed by hysteroscopy, an absence of intrauterine adhesion or inflammation, an endometrial thickness in the late follicular phase of ≥ 7 mm measured by ultrasonography, a normal ovarian reserve (follicle-stimulating hormone < 9.0mU/mL), a normal ovarian response to the stimulation protocols (> 8 oocytes retrieved in a controlled ovary hyperstimulation cycle), and the lack of hormone (estradiol/progesterone) treatment during the endometrial cycle.

Hormonal stimulation of patients

The patients received controlled ovarian stimulation with FSH/LH. When the follicular diameter measured 18 mm, oocyte maturation was stimulated with human chorionic gonadotropin. Thirty-six hours later, follicular oocytes were obtained using ultrasound guidance.

In vitro fertilization and developing embryos

Oocytes were fertilized *in vitro* by exposing them to $1x10^6$ capacitated spermatozoa/mL for 18 hours. This process was carried out in HTF/HEPES medium supplemented with 5% albumin under controlled conditions (37°C, 54% CO_2 and 95% air). Fertilization was assessed by the presence of a second polar corpuscle body. Oocytes were each maintained in 50 µL of G-1 PLUS culture medium (Sweden). Morphological development was observed daily up to the 36-cell stage (5 day), at which time the culture medium was retained for matrix metalloproteinase analysis.

On day 5 of embryo development two embryos (with quality type I, II, or III quality) were transferred to the uterine cavity with Flexible Pass intrauterine transfer cannula based on the Soft Cook technique. The process was assisted by abdominal ultrasound guidance and a real-time, 5-MHz sector electronic array endovaginal test (Philips Epiq CVx; MO, USA).

Blood samples

Five milliliters of peripheral blood were obtained from the patients by puncture of the cephalic vein, placed in EDTA-K2 tubes (BD Vacutainer), and centrifuged at 14,000 rpm for 10 minutes. Serum was collected in Eppendorf tubes and stored at -70°C until to quantification hormones assay.

Assay to determine the levels of sex hormones

All evaluations of hormones were performed in the central laboratory of the Instituto Nacional de Perinatología on the Modular Analytical apparatus cobas e 411 (Roche, USA). A commercially available assay kit was used to measure the serum levels of P4, E2, T4, FSH, LH, AMH, and hCG (Roche system, USA), with according to the manufacturer´s recommendations and as previously described [35–37]. The lower limit of detection for these hormones was 0.4 ng/mL, 5.0 pg/mL, 0.025 pg/mL, 0.100mIU/mL, 0.100 mIU/mL, and 0.2 ng/mL, 0.1 mIU/mL, respectively. The intra-assay coefficient of variation was 3%, 5%, 5%, 3%, 2%, 3%, and 5% respectively.

Determination of pregnancy outcome

The serum concentration of P4, E2, T4, FSH, LH, AMH, and hCG was assessed 14 days after embryo transfer. The apparent successful implantation of the embryo and endometrial receptivity were confirmed by ultrasound, embryo sac development. In accordance with the finding of this analysis, the patients were assigned to one of two groups: with and without implanted embryos (n = 17, pregnant and n = 25, non-pregnant patients, respectively).

Protein quantification and matrix metalloproteinase activity

The concentration of *pro*MMP-2 and *pro*MMP-9 proteins in the culture media, indicating their secretion into the media by the embryos, was measured in the two groups by the colorimetric Bradford method [38] and activity using SDS-polyacrylamide gels with porcine gelatin (1 mg/mL), as described previously [39]. The internal control of electrophoretic mobility was a used a culture medium from U937 promyelocyte cells (ATCC, Manassas, VA, USA). Each sample was loaded with 0.75 μg of protein and the activity band was captured with the EpiChemi Darkroom gel documentation system (UVP, CA, USA). Optical densitometry was quantified on the NIH ImageJ program.

Statistical analysis

Difference between the two groups of embryos (implanted vs. non-implanted) base on the mean optical density values of the corresponding culture media, were examined with the Student´s t- test. All values are expressed as the mean ± SD, and statistical significance was considered at $p \leq 0.05$. Statistical analysis was performed on GraphPad Prism version 8.0 (GraphPad Software, San Diego, CA, USA). STATA

software was used to plot the receiver operating characteristic (ROC) curves and calculate the area under the curve (AUC-ROC) (StataCorp LLC; v16, TX, USA). The cutoff value selected (based on the ROC curve) was that at which the sensitivity and specificity were best and the distance to the top-left corner of the ROC curve was the least. Sensitivity, specificity, and positive and negative predictive values were determined on the STATA software.

Results

Patient characteristics

Table 1 shows the characteristics of the 42 patients were compared between the two groups: pregnant (n = 17); and 2) non-pregnant (n = 25) patients. No significant difference existed in regard to any of these parameters: age (p = 0.23), body mass index (p = 0.43), and years of infertility (p = 0.39).

Table 1
Clinical data on the patients participating in the study.

Characteristics	**Pregnancy (n = 17)**	**Non-pregnancy (n = 25)**	***p*-value**
Age (years)	35.7 ± 2.4	36.8 ± 3.1	0.23
BMI (Kg/mL)	27.2 ± 3.7	26.3 ± 3.2	0.43
Year of infertility	4.8 ± 3.0	5.4 ± 3.2	0.39
Number of transferred embryos			
1, *n* (%)	0 (0.0)	4 (10.0)	0.085
2, *n* (%)	17 (100)	19 (45)	
Quality of the transferred embryos			
I, *n* (%)	7 (22.0)	4 (10)	0.137
II, *n* (%)	25 (71.0)	25 (60.0)	0.04
III, *n* (%)	2 (7.0)	13 (30.0)	0.001
Hormone concentration in serum			
hCG	2088.2 ± 2300.4	1254.0 ± 1149.2	0.70
E_2	1496.7 ± 793.9	1674 ± 513.3	0.71
P_4	0.65 ± 0.4	0.55 ± 0.3	0.90

BMI, body mass index. hCG, human chorionic gonadotropin; E_2, estradiol; P_4, progesterone. The criterion for assigning patients to the groups was embryo sac development (or lack thereof). Data are reported as the mean ± standard deviation.

Hormone profiling

The concentration of hormone was compared between pregnant and non-pregnant patients (Table 2). No significant differences existed with respect to P4 on the day of final oocyte maturation (p = 0.664), E2 no-follicular phase (p = 0.684), and follicular phase (p = 0.326), T4 (0.326), LH (p = 0.095), and the Anti-Müllerian Hormone (p = 0.263); however, significant difference was indeed found for FSH (p = 0.011), and hCG (p < 0.0001; Table 2).

Table 2
Comparison of the hormonal concentration between women with implanted and non-implanted embryos.

Variable	**Pregnancy (n = 17)**	**Non-pregnancy (n = 25)**	***p*-value**
P4 on day of final oocytes maturation, (ng/mL)	0.49 ± 0.20	0.45 ± 0.28	0.664
E2 no-follicular phase, (pg/mL)	60.7 ± 16.4	63.7 ± 2710	0.684
E2 follicular phase, (pg/mL)	1368.0 ± 582.1	1889.0 ± 849.3	0.326
T4 follicular phase, (pg/mL)	50.4 ± 20.5	46.2 ± 7.8	0.336
FSH follicular phase, (mIU/mL)	4.6 ± 1.3	6.3 ± 2.5	0.011
LH follicular phase, (mIU/mL)	4.9 ± 1.2	5.8 ± 1.6	0.095
AMH, (ng/mL)	1.56 ± 0.61	1.39 ± 0.37	0.263
hCG, (mIU/mL)	61.8 ± 32.7	2.7 ± 1.4	< 0.0001
Data are presented as the mean ± standard deviation.			

Expression of proMMP-2 and proMMP-9 in the culture medium of the embryos.

On day 5 of development, the embryo was transferred to the patients and the secretion profile of *pro*MMP-2 and *pro*MMP-9 was determined in the culture medium (Fig. 1).

The presence of *pro*MMP-2 was detected in the culture media corresponding to 100% of the pregnant patients (17 of 17; lane 1 to 14) and in 11% (3 of 25; lane 15 to 57) of the non-pregnant patients (Fig. 1). The latter group included three women who became pregnant and underwent a spontaneous abortion (P52, P57, and P58; Fig. 1). The optical density of *pro*MMP-2 quantified for each of the activity bands (Fig. 2). Was significantly (1.4-fold) lower in the three non-pregnant patients with *pro*MMP-2 expression than in the pregnant women (Fig. 2; p = 0.045).

On the other hand, *pro*MMP-9 expression was found in the culture media corresponding to 11 of 14 pregnant and 39% of non-pregnant patients (11 of 25). The optical density of the bands of *pro*MMP-9 showed a significantly (1.2-fold; Fig. 2) lower value in the culture media corresponding to the 11 non-pregnant patients with *pro*MMP-9 expression versus the culture media corresponding to the 11 pregnant women with *pro*MMP-9 expression (Fig. 2; p = 0.002).

Predictive values of proMMP-2 and proMMP-9 in the culture medium of developing embryos from pregnant patients.

The ROC curve was used to evaluate the sensitivity and specificity of *pro*MMP-2 and *pro*MMP-9 for determining the capacity of transferred embryos to result in pregnancy (Fig. 3). For *pro*MMP-2, the optical density of 423 was taken as the cutoff value and showed statistical significance ($p = 0.02$) which exhibited a sensitivity of 100% and a specificity of 100%. For *pro*MMP-9, the optical density of 550 was adopted as the cutoff value and showed statistical significance ($p = 0.0035$) having a sensitivity of 81.8% and a specificity of 72.7%.

Discussion

MMPs play an important role in the remodeling of different structural and support components during ovulation [40, 41] decidualization [42, 43], and implantation [44–46]. The main findings of the present study can be summarized in five points in relation to the activity of *pro*MMP-2 and *pro*MMP-9 in the culture media of the embryos on day 5 of their development 1) the activity of *pro*MMP-2 was found in 14 of 14 culture media corresponding to the patients who achieved a full-term pregnancy and in 3 of 25 culture media associated with women who did not have this outcome. It was 1.4-fold greater in the former group; 2) The activity of *pro*MMP-2 was observed in 11 of 14 culture media corresponding to the patients who carried their pregnancy to term and in 11 of 25 culture media associated with women who did not have this outcome. It was 1.2-fold greater in the former group (Fig. 1); 3) In the three patients who did not carry their pregnancy to term, only proMMP-9 activity was detected; 4) the activity of both *pro*MMP-2 and *pro*MMP-9 was identified in three non-pregnant patients who had some pre-pregnancy complications (Fig. 1); and 5) There were no significant differences in the concentration of the hormones HCG, E2, or P4 between pregnant and non-pregnant patients (Table 1).

Gu et al. (2015) reported the concentration of the active form of MMP-9 at 0.698 ± 0.022 ng/mL in the culture medium of developing human embryos, which resulted in pregnancy for 77.0% of the participating patients [47–49]. According to the present study, the activity of *pro*MMP-2 and *pro*MMP-9 (Fig. 2) was significantly higher in the culture media of the embryos yielding a full-term pregnancy than in the culture media of the other cases (Table 1). The culture media exhibited a 1.4-fold greater *pro*MMP-2 activity (p = 0.045) for the embryos generating a full term pregnancy compared the other cases (3/25) showing *pro*MMP-2 activity. Similarly, the culture medium displayed a 1.2-fold greater *pro*MMP-9 activity (p = 0.002) for the embryos engendering a full term pregnancy (11/14) compared to the other cases (11/28), considering the media with *pro*MMP-9 activity.

A conceptual model is herein provided (Fig. 4) how MMPs are activated by epidermal growth factors [47], interleukin (IL) -1β, and tumor necrosis factor (TNF)-α [50, 51]. Sequeira et al. (2015) reported a significantly (15.4-fold) greater value for the level of IL-1β in the culture media of developing human embryos that became implanted in patients versus the culture media of non-implanted embryos (0.55 ± 0.25 pg/mL), finding implantation in 42.0% of the total participants [52].

In the first and second trimesters of pregnancy, syncytiotrophoblast cells have been described to secrete a 2.4- and 3.8-fold higher amount of IL-1β, respectively, than the pre-pregnancy level. The secretion of IL-1β is associated with an increase in MMP-9 expression [53]. After interacting with its receptor, IL-1β regulates the signaling pathway that involves the activation of the mitogen-activated protein kinase (MAPK), p38 MAPK, c-Jun N-terminal kinase (JNK), and the extracellular regulatory kinase (ERK) [54, 55]. As a consequence, IL-1β promotes the activation of nuclear factor kappa-beta (NFkβ) and the expression of MMPs [56].

The collagenolytic activity of MMPs is regulated by the specific tissue inhibitors of these proteinases [49, 57]. Cytotrophoblast cells, treated with 50 nM of their tissue inhibitor, known as tissue inhibitor of metalloproteinase-2 (TIMP-2) exhibit an up to 40% reduction in invasiveness according to Librach (1991) [51, 58]. The present results showed a 1.4-fold and 1.2-fold decrease in the activity of *pro*MMP-2 and *pro*MMP-9 respectively (Fig. 2), in the culture media corresponding to the cases of patients who were pregnant but did not carry to term. However, the expression of TIMPs was not herein evaluated in the culture medium of developing embryos. It would be interesting to determine the MMP/TIMP relationship is involved in the mechanism responsible for regulating the progress of implantation and pregnancy.

Recently, localized polymorphisms identified in the promoter region of MMP-2 (-1306 C/T; rs 243865) and MMP-9 (-1562 C/T; rs 3918242) were found to induce changes in the levels of transcription and or expression of the protein. These mutations have been proposed as a risk factor for spontaneous abortion [50, 59]. Regarding the three patients of the present study with implanted embryos that spontaneously aborted (P52, P57, and P58), the corresponding culture media displayed *pro*MMP-2 and *pro*MMP-9 (Fig. 2) activity. Future research should explore the possible relation of such pregnancy complications to mutations.

MMP-2 has the ability to degrade fibronectin, elastin, and collagen type IV, V, VII, and. In contrast MMP-9 degrades proteoglycans, elastin, and collagen I, IV, V, and XI [51, 60–62] which allows the cytotrophoblast cells to invade the endometrium to prepare the way for implantation) [63] (Fig. 5). During the implantation window, according to *in vivo* (in animal models [64] and *in vitro* studies [65], the epithelial cadherin-like binding protein (E-cadherin) enables the embryo to adhere to the endometrial epithelium, which is degraded by MMP- 9 [66]. Hence, previous reports evidence a key role by MMP-2 and MMP-9 in embryonic development. The present analysis based on the cutoff points of the ROC curve for both proteinases suggests that their evaluation in the culture medium of developing embryos has plausible predictive power in relation to the success of implantation.

Conclusions

These results of the current contribution demonstrate the feasibility of detecting *pro*MMP-2 and *pro*MMP-9 activity in the culture medium of embryos on day 5 of development by using *in gel* zymography. Additionally, such activity is associated with the capacity of embryos for implantation. Thus this evaluation could serve as a non-invasive method for determining the viability of human embryos developed *in vitro* conditions.

Abbreviations

BMI: Body mass index; IVF: in vitro fertilization; MMP: matrix metalloproteases.

Declarations

Ethics approval and informed consent

Each patient was informed that, by agreeing to participate in the study, the culture medium of the embryo would be used to perform MMPs assays after embryo transfer, and that this procedure would not affect the development of the embryos. All candidates signed the consent form after the explication. The present protocol was reviewed and approved by the Ethics and Research Committees of the Instituto Nacional de Perinatología (212250-22661).

Consent for publication

All authors carefully read the final version of the manuscript and gave their permission to submit to the journal of Reproductive Biology and Endocrinology.

Availability of data and materials

All of the relevant information from the study is described in the manuscript.

Competing Interest

All authors declare that they have no competing interests with respect to the research, or the authorship publication of this article.

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Authors´ contributions

MOV performed the quantification of hormones in the serum of the patients. MOV and RJAG carry out the evaluating of MMP activity. RJAG and EIC performed *in vitro* fertilization, obtained the culture medium for the development embryos of the embryos. RJAG monitoring pregnancy and gestational sac formation. PVC performed the statistical analysis of the ROC curve, and participated in the discussion of results and writing of manuscript. MOV, RJAG, JSLC and JLC participated in the analysis and discussion of the results. MOC performed the analysis of MMP activity and participated in the writing of the manuscript. HFH participated in the design of the study, analysis of results, and writing the manuscript, as well as obtaining financial and material support for the study.

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Figure

Figure 5 is not available with this version.

Figures

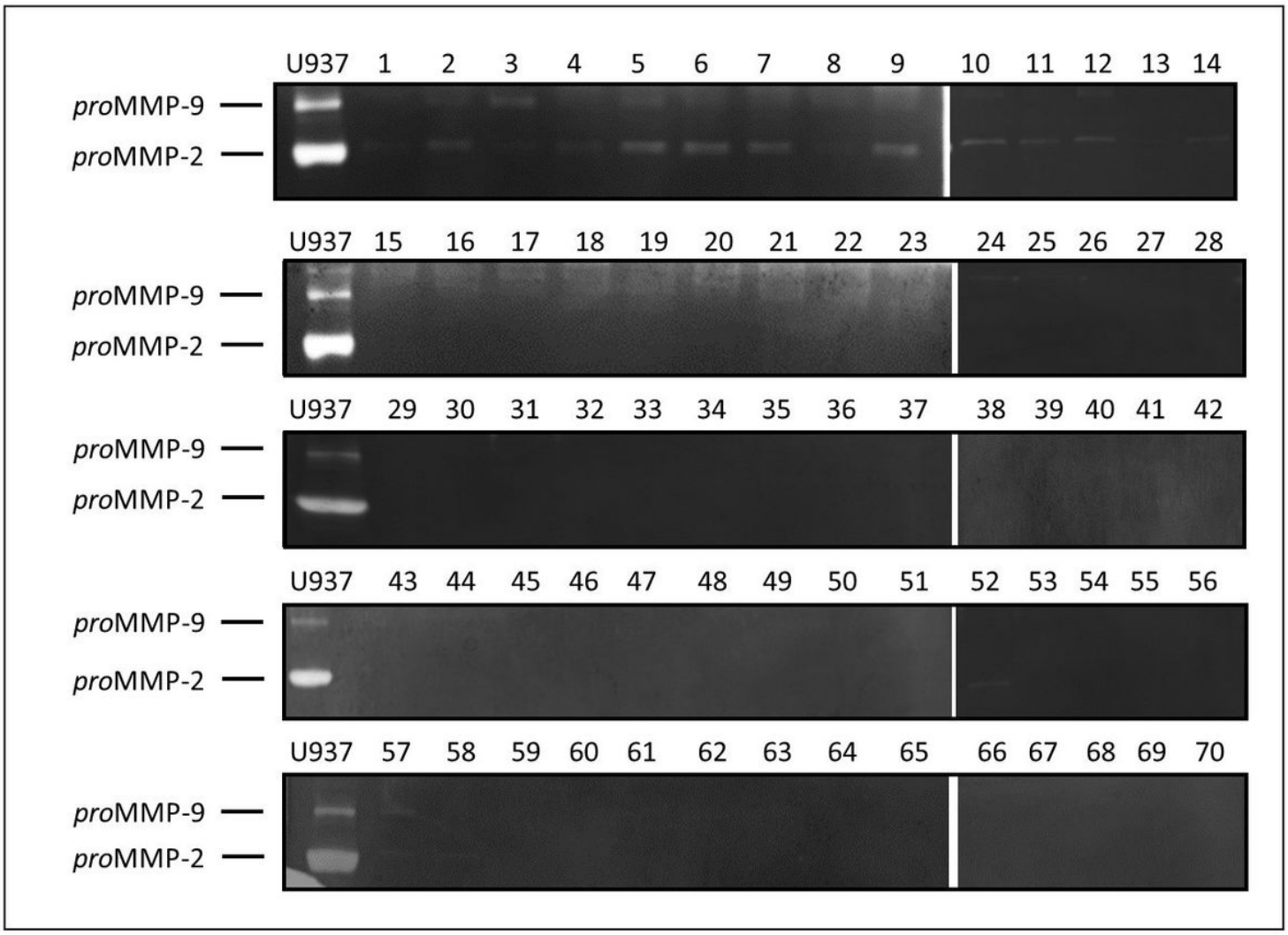

Figure 1

Activity of proMMP-2 and proMMP-9 in the culture medium of human embryos on day 5 of development. The culture medium from both implanted (pregnant patients; lanes 1-14) and non-implanted embryos (non-pregnant patients; lanes 15-70) was analyzed using gel. The activity of proMMP-2 (62 KDa) and proMMP-9 (92 KDa) was identified with respect to the electrophoretic mobility of the culture medium of the promyelocyte cell line (ATCC, U937), as previously reported by Flores-Herrera [36].

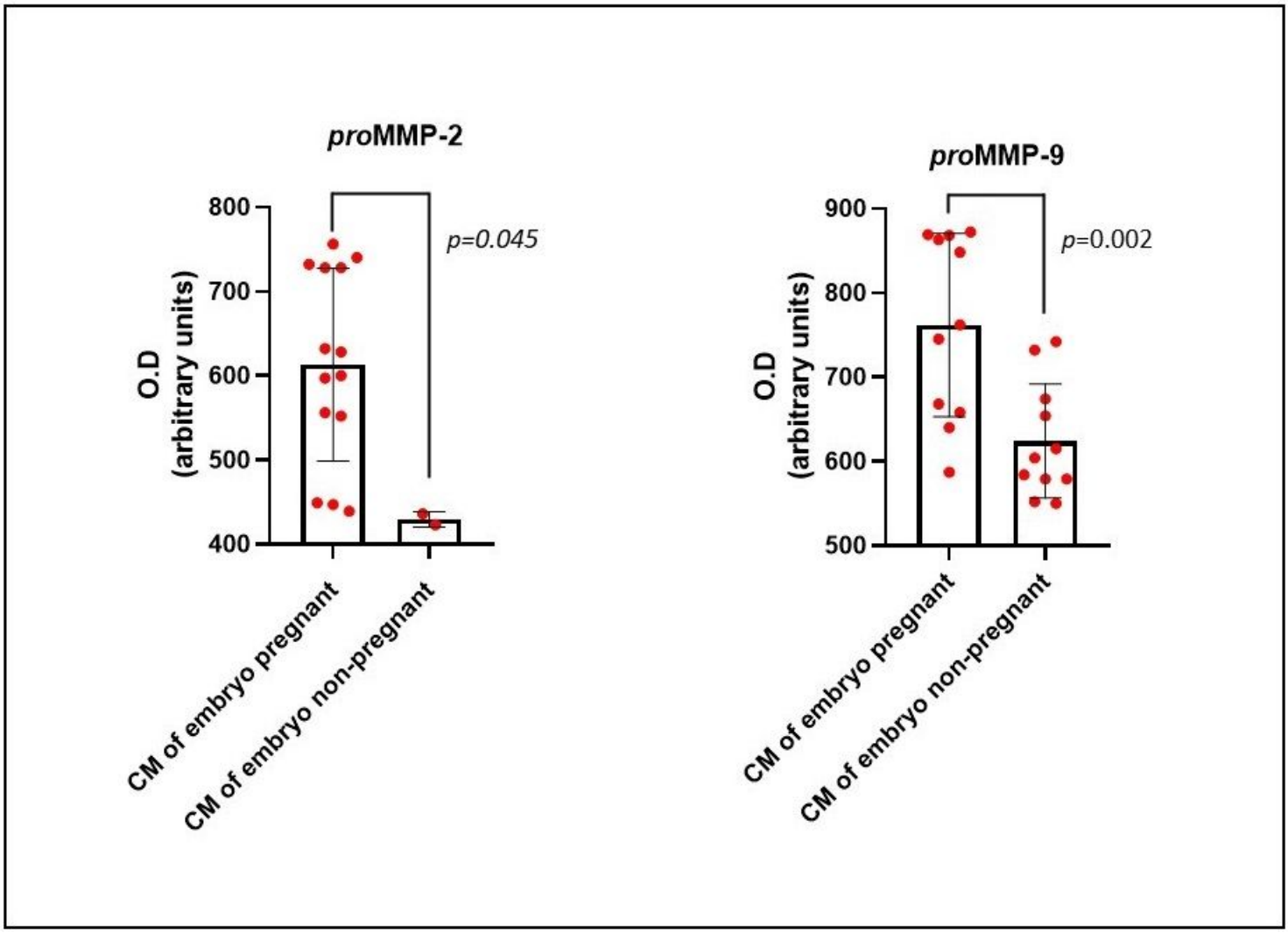

Figure 2

Optical density of proMMP-2 and proMMP-9. The points indicate the values of each activity band for implanted (pregnant patients, n=14) and non-implanted embryos (non-pregnant patients, n=28). Optical density (O.D) was determined using the JImage program (USA).

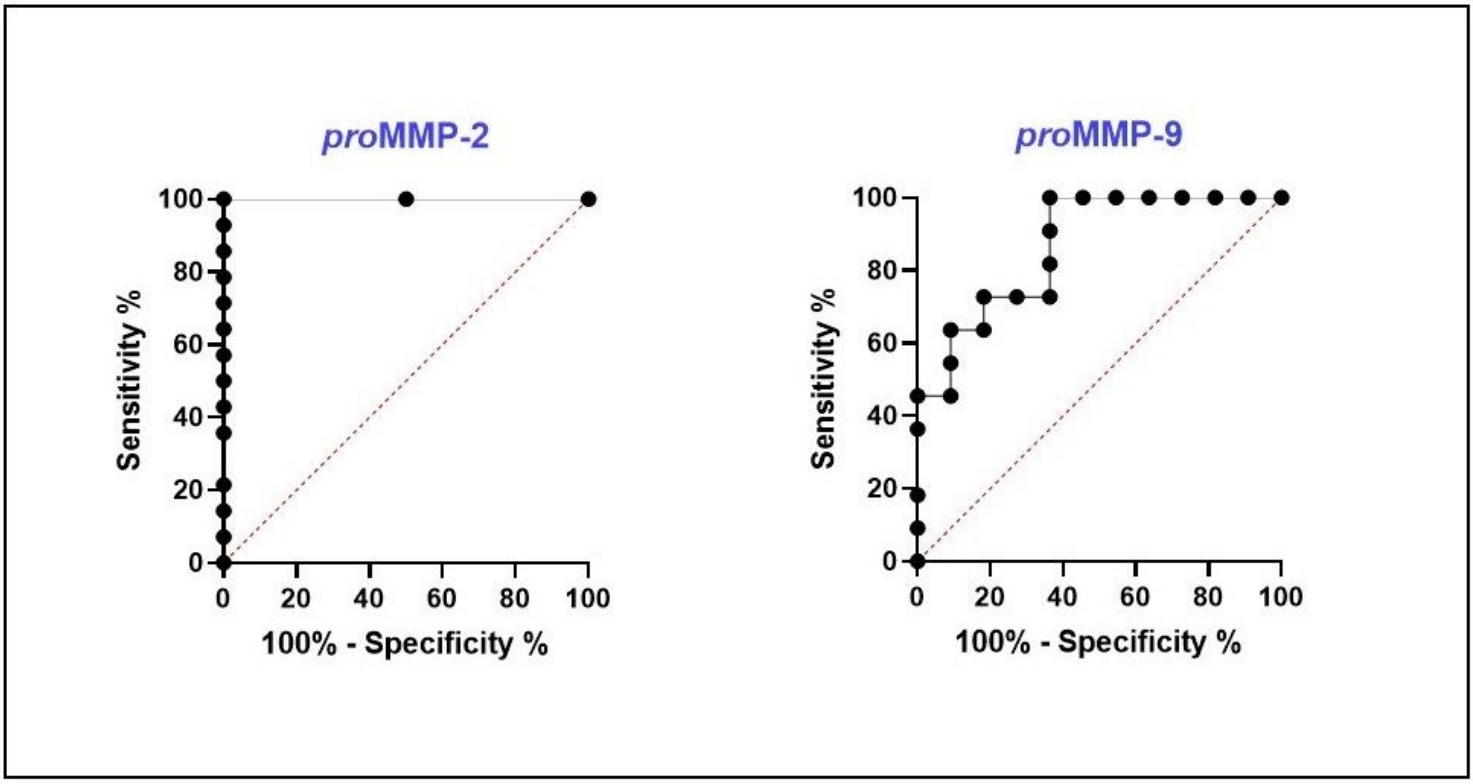

Figure 3

Predictive values of the ROC curves. The cutoff value selected (based on the ROC curve) was that at which the sensitivity and specificity were best for proMMP-2 and proMMP-9 as biomarkers of the success of implantation.

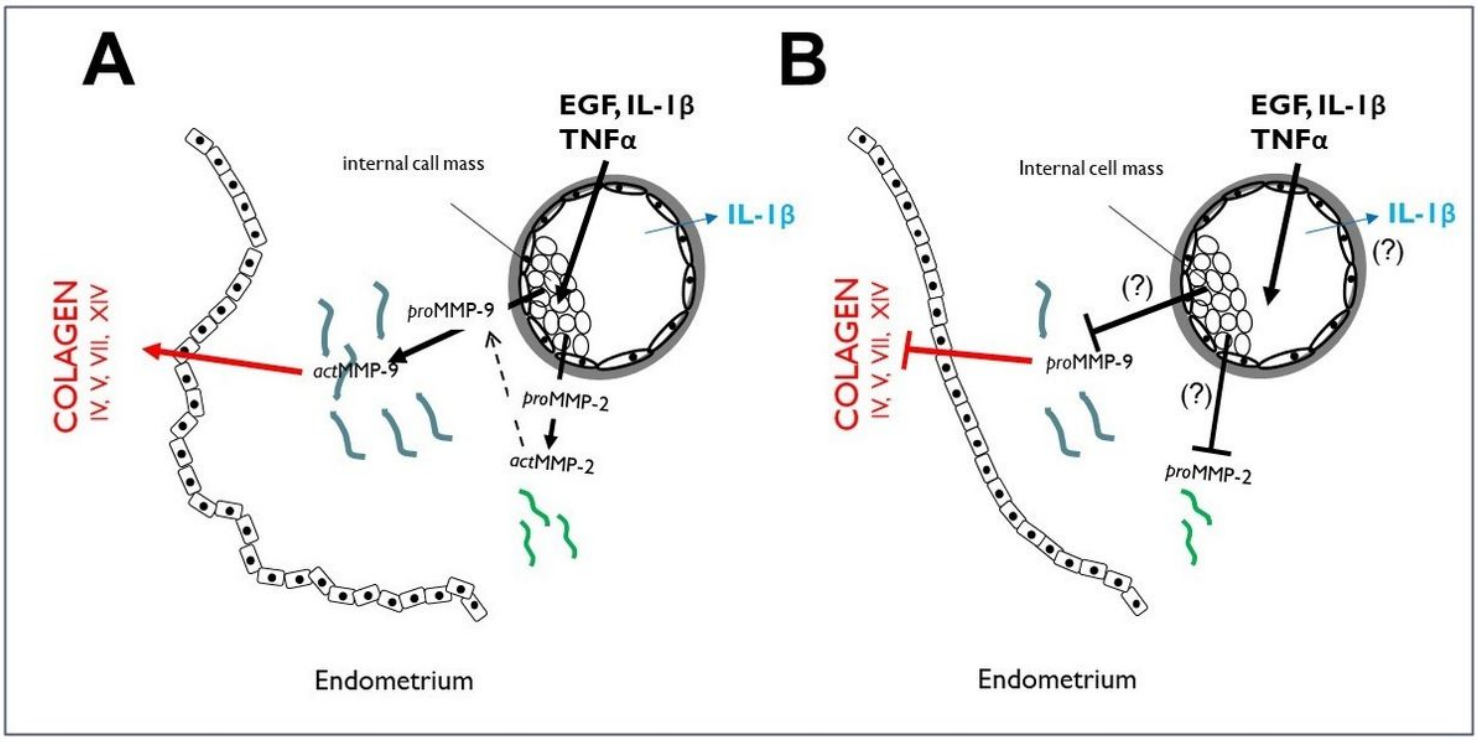

Figure 4

Model of active proMMP-2 and proMMP-9 secreted from embryos into the culture medium. The developing embryos secrete interleukin type 1-beta (IL-1β) [49], tumor necrosis factor-alpha (TNFα) [53, 64] and epidermal growth factor (EGF) [65], promoting the expression of the collagenolytic protein extracellular matrix [45, 66]. The actMMP-2 protein form has been shown to remove propeptides associated with the catalytic site of proMMP-9, leading to activation of its degradative function [67, 68].