Research on apoptotic signaling pathways of recurrent spontaneous abortion caused by dysfunction of trophoblast infiltration

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Abstract. – **OBJECTIVE:** To study the apoptotic signaling pathways of recurrent spontaneous abortion caused by dysfunction of trophoblast infiltration.

PATIENTS AND METHODS: 60 patients with recurrent spontaneous abortion and normal abortion were selected consecutively as recurrent spontaneous abortion group and abortion group, respectively. Villous tissues were obtained and cell apoptosis was observed under a microscope; terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling (Tunel) method was used to test the apoptosis rate. In situ hybridization was adopted to detect expressions of Fas messenger RNA (Fas mRNA) and Fas ligand messenger RNA (FasL mRNA); expression of Fas, FasL and protein kinase C (PKC) were examined by immunohistochemistry at protein level; fluorescence spectrophotometer was used to test Ca2+ level.

RESULTS: The apoptosis rate, expressions of Fas mRNA, and FasL mRNA, expressions of Fas and FasL proteins, as well as Ca2+ level, were significantly higher in the recurrent spontaneous abortion group than in abortion group. The level of PKC protein was significantly lower in recurrent spontaneous abortion group than in abortion group ($p<0.05$).

CONCLUSIONS: Fas-FasL and PKC signaling pathways, as well as Ca2+, may mediate the dysfunction of trophoblast infiltration, which leads to recurrent spontaneous abortion.

Key Words:
Trophoblast cells, Recurrent spontaneous abortion, Apoptosis, Fas, PKC proteins, Ca^{2+}.

Introduction

Recurrent spontaneous abortion can be caused by a variety of factors[1]. Studies on animal model and clinical studies have shown the presence of trophoblast infiltration dysfunction[2,3] and apoptosis[4], as well as multiple signal transduction pathways, which are involved in the progression of recurrent spontaneous abortion[5,6]. In our work, the villous tissues of 60 patients with recurrent spontaneous abortion were subject to different experimental observation and detection. The results indicated that Fas-FasL and PKC signaling pathways were involved in the development of disease and that Ca^{2+}, the second messenger, also plays an important role in this process. Our study provided new targets for recurrent spontaneous abortion treatment and medicine-induced abortion.

Patients and Methods

Patients

A total of 60 patients who were diagnosed with recurrent spontaneous abortion were selected consecutively in our hospital from June 2015 to January 2016. All patients were not treated with surgery or medicine before the selection. Patients with genetic defects, anatomical abnormalities of the reproductive system, endocrine disorders, immune deficiency, infections and thrombus were excluded; reproductive system and semen functions of their male partners were normal and there were no ABO-incompatible phenomena. The age of patients ranges from 20 to 35 years with an average age of (23.4±5.5) years; the gestational age ranged from 4 to 7 weeks with and averaged age of (8.5±3.4) weeks. At the same time, 60 healthy pregnant people who wanted to receive artificial abortion were selected as abortion group. The age of abortion group ranged from 20 to 35 years with an average age of (24.2±5.7) years; the gestational age ranged from 4 to 7 weeks with an average age of (8.5±3.4) weeks. The differences in age and gestational age betwe-

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en the two groups were not statistically significant (p>0.05). The study was approved by the Ethics Committee of Zaozhuang Municipal Hospital. All the patients signed informed consents.

Research Methods

Villous tissues were obtained and cell apoptosis was observed under microscope; Tunel method was used to calculate the apoptosis rate; *in situ* hybridization method was adopted to detect expressions of Fas mRNA and FasL mRNA; expression of Fas, FasL and PKC were examined by immunohistochemistry at protein level; fluorescence spectrophotometer was used to quantify Ca^{2+} level. Villous tissues were fixed in 3% glutaraldehyde. After washing 3 times with 0.1%mol/L phosphate-buffered saline (PBS), the tissue was fixed in 1% osmium tetroxide for 1 h. After that, the tissue was washed 3 times with 0.1 mol/L PBS, following the dehydration step by passing a series graded concentration of ethanol. After that, tissue was embedded in Epon 812 and cut into 60-70 nm sections. Uranyl acetate and lead citrate were used for double staining. The tissue sections were observed under JEM-1200EX transmission electron microscope.

Detection of Apoptosis Rte by Tunel Method

After deparaffinization, tissue sections were incubated with proteinase K at room temperature for 7-20 min. After that, 50 µL Tunel reaction mixture was added and incubated with tissue sections in a wet box at 37˚C for 60 min. Tissue sections were then incubated in a wet box at 37˚C for 30 min with 50 µL converter-peroxidase (POD). 3,3'-diaminobenzidine tetrahydrochloride (DAB) was used for color development. After counterstaining with hematoxylin, tissue sections were dehydrated, transparentized and mounted. Negative control sections were incubated in the solution without terminal deoxynucleotidyl transferase. Positive signals, which were yellowish-brown color, can be found in nuclei. Determination of apoptotic cells was: discrete distribution, apoptotic nuclear morphology (including karyopyknosis, condensation and margination of chromatin or nuclear fragmentation) and no inflammatory responses around. Five non-overlapping visual fields (100×) were selected randomly and the percentage of positive cells was taken as the apoptosis rate (AR).

In Situ Hybridization Method

Specimens were fixed in 10% neutral formalin overnight, followed by dehydration by passing a series of a graded concentration of ethanol. Tissue was then transparentized in xylene. After embedding in wax, tissue was cut into 4 µm sections; after routine dewaxing and rehydration, the specimens were incubated with 3% H_2O_2 at room temperature for 10 min, followed by washing 3 times with distilled water. RNA was exposed after digestion with fresh 3% citric acid solution containing pepsin at 37°C for 120 s. r After washing 3 times with PBS (5 min for each time); prehybridization was carried out (the sections were incubated 20 µL prehybridization solution in a thermostat at 40°C for 2-4 h; excessive solution was absorbed but not washed); followed by hybridization (the sections were incubated with 20 µL hybridization solution overnight at 40°C). Tissue sections were washed after hybridization; after removing the cover glass, the sections were washed twice (5 min for each time) with 2× saline sodium citrate (SSC) at 37°C, followed by washing with 0.5× SSC once at 37°C for 15 min and washing with 0.2× SSC at 37°C for 15 min. After that, tissue sections were incubated with blocking buffer at 37°C for 30 min and the excessive solution was removed but not washed. The tissue sections were incubated with biotinylated mouse anti-digoxin at 37˚C for 60 min, followed by washing 4 times with PBS (5 min for each time). Tissue sections were then incubated with strept avidin-biotin complex (SABC) at 37°C for 20 min, followed by washing 3 times with PBS (5 min for each). After that tissue sections was incubated with biotinylated peroxidase at 37°C for 20 min, followed by washing 3 times with PBS 3 times (5 min for each). Color development was performed with diaminobenzidine (DAB) and counterstaining was performed with hematoxylin. Tissue sections were dehydrated in ethanol and transparentized in xylene. After mounting, the sections were observed under microscope.

Immunohistochemistry

Rabbit anti-Fas, FasL, PKC and human monoclonal antibodies (Sigma Company, St. Louis, MO, USA) and immunohistochemical streptavidin-peroxidase (SP) method were utilized. The paraffin sections were dewaxed hydrated. Tissue sections were then incubated with 3% H_2O_2 at room temperature for 10 min, followed by washing three times with distilled water (2 min for each time). Tissue sections were then soaked in PBS for 5 min; antigen retrieval was performed in a microwave oven; the sections were blocked in 10% normal goat serum at room temperatu-

re for 10 min; the serum was discarded but the sections were not washed. After that, antibodies with appropriate concentration were incubated with tissue sections overnight at 4°C, followed by washing 3 times with PBS (5 min for each); horseradish peroxidase-labeled secondary antibodies with appropriate concentration were then incubated with the sections at 37°C for 30 min, followed by washing 3 times with PBS 3 times (5 min for each time). Finally, color development was performed with DAB. After washing with tap water, the sections were counterstained and mounted. Leica QWin_V3 image analysis software was used for result analysis; the lower gray scale value indicated higher expression levels.

Fluorescence Spectrophotometer

The specimen was frozen in liquid nitrogen immediately after collection, the villous cell suspension was prepared by mechanical methods after thawing and the concentration was adjusted to $2*10^6$/mL; 0.4% trypan blue was mixed with cells suspension at a proportion of 1:1; the cells were counted under a light microscope to make sure that ratio of living cells was higher than 80%. Fura-2/acetoxymethyl (AM) loading and fluorometric determination were performed. The excitation grating of Hitachi F-4500 type fluorescence spectrophotometer was set as 5 nm and 10 nm for emission grating. The temperature was 37°C; the excitation spectrum of Fura-2/AM standard solution and loaded solution was measured in a spectral mode; the decrease of the maximum wavelength from 380 nm to 340 nm, indicated the entry of Fura-2/AM into the cells. The fluorescence intensity (F value) of Fura-2 loaded solution was measured in a manner of time; 10% Triton X-100 was added to a final concentration of 0.1% and the maximum fluorescence intensity (Fmax) was tested 30 min later; ethylenediamine tetraacetic acid (EDTA) was added to a final concentration of 5 mmol/L and the minimum fluorescence intensity (Fmin) was detected 30 min later. The concentration of Ca^{2+} was calculated according to the following formula: Ca^{2+} concentration (nmol/L) = Kd (Fmin)/(Fmax-F); as the dissociation constant of Fura-2 and Ca^{2+}, Kd is equal to 224 nmol/L

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 19.0 statistical software (SPSS Inc. Chicago, IL, USA) was used for data analysis; quantitative data were presented as means ± standard deviations and *t*-test was used for comparisons between groups; qualitative data were presented as cases or percentage (%) and χ^2 test was used for comparison between groups; $p<0.05$ meant the differences were statistically significant.

Results

Comparisons of Apoptosis and AR Observed by Electron Microscope

In the recurrent spontaneous abortion group, the trophoblastic nuclear morphology was irregular, the nucleoli disappeared, the euchromatin was in massive shape and margination of heterochromatin, karyopyknosis and nuclear fragmentation were visible; the number of organelles was declined, the cytoplasmic mitochondria were swollen and the sheath in mitochondria was decreased and arranged irregularly; rough endoplasmatic reticulum was expanded and surface degranulation was visible; the quantities of Golgi apparatus and glycogen granule were decreased; cytoplasmic vacuoles were increased. The gap junctions between cells were broadened and vague; the basement membrane was thickened; typical apoptotic bodies containing mitochondria and other organelles were observed. In the abortion group, the cells were in regular morphology and nuclei, cytoplasm and organelles were generally in normal forms; the cells were strongly attached to each other and no apoptotic bodies were observed (Figure 1). The AR of the recurrent spontaneous abortion group was significantly higher than that of the abortion group [(2.2±0.5) *vs.* (0.6±0.2) %, t=12.325, $p<0.001$] (Figure 2)].

Comparisons of Expressions of Fas mRNA and FasL mRNA

The expressions of Fas mRNA and FasL mRNA in recurrent spontaneous abortion group were significantly higher than those in abortion group [(123.5±23.2) *vs.* (178.6±30.4), t=8.625, $p<0.001$; (144.2±36.7) *vs.* (196.8±40.5), t=7.649, $p<0.001$] (Figure 3)].

Comparisons of Expressions of Fas, FasL and PKC Proteins

The expressions of Fas and FasL proteins in recurrent spontaneous abortion group were significantly higher than those in abortion group, but the level of PKC protein of recurrent spontaneous abortion group was significantly lower than that of abortion group [(96.4±10.3) *vs.* (132.6±15.8), t=7.648, $p<0.001$; (85.3±12.4) *vs.* (112.7±13.2),

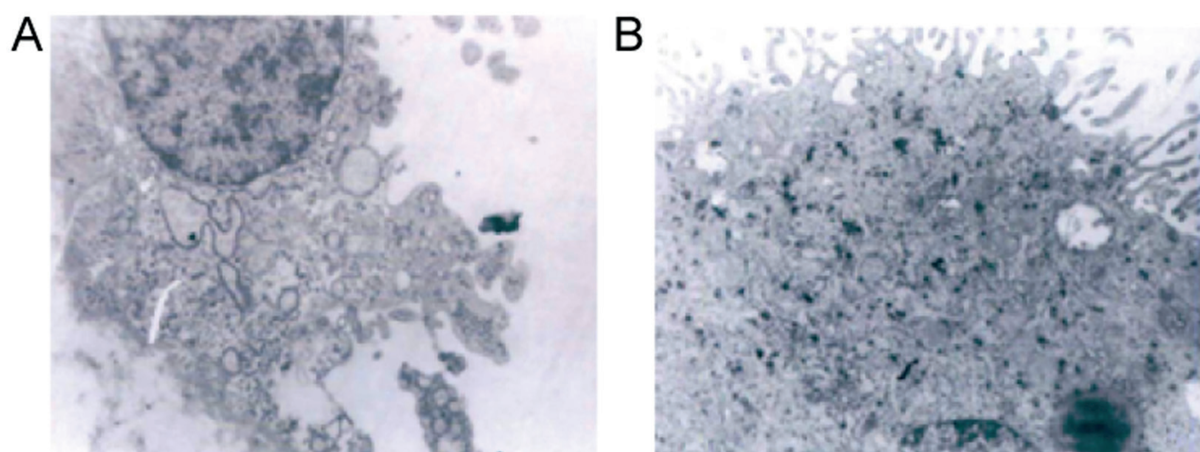

Figure 1. Apoptosis of trophoblast cells observed under electron microscope. (8000×, ***(A)*** represents for the recurrent spontaneous abortion group and ***(B)*** for the abortion group).

t=9.230, p<0.001; (186.9±18.2) *vs.* (125.3±14.4), t=10.325, p<0.001] (Figure 4)].

Comparisons of Ca^{2+} Concentration

The Ca^{2+} level in recurrent spontaneous abortion group was significantly higher than that in abortion group [(106.3±11.5) *vs.* (72.5±12.4) nmol/L, t=15.628, p<0.001] (Figure 5)].

Discussion

Our study showed that AR of the recurrent spontaneous abortion group was increased significantly compared with abortion group. Previous reports also showed[7] that a certain degree of cell apoptosis can also be observed in medicine-induced abortion, but not in mechanical abortion, indicating that apoptosis may be an important cause of the occurrence of spontaneous abortion or recurrent spontaneous abortion. Several scholars[8,9] have confirmed the presence of trophoblast cells apoptosis in early pregnancy, and implantation and growth of blastocyst, regression and reconstruction of decidual tissues, remodeling of placental structure and other processes are closely related to apoptosis. There is a balance between apoptosis and proliferation of villous and decidual cells during pregnancy and pathological pregnancy, such as spontaneous abortion, which occurs with the presence of excessive apoptotic cells. Kokawa et al[10] analyzed the apoptotic DNA fragments by autoradiography and the DNA fragments of histological sections using *in situ* hybri-

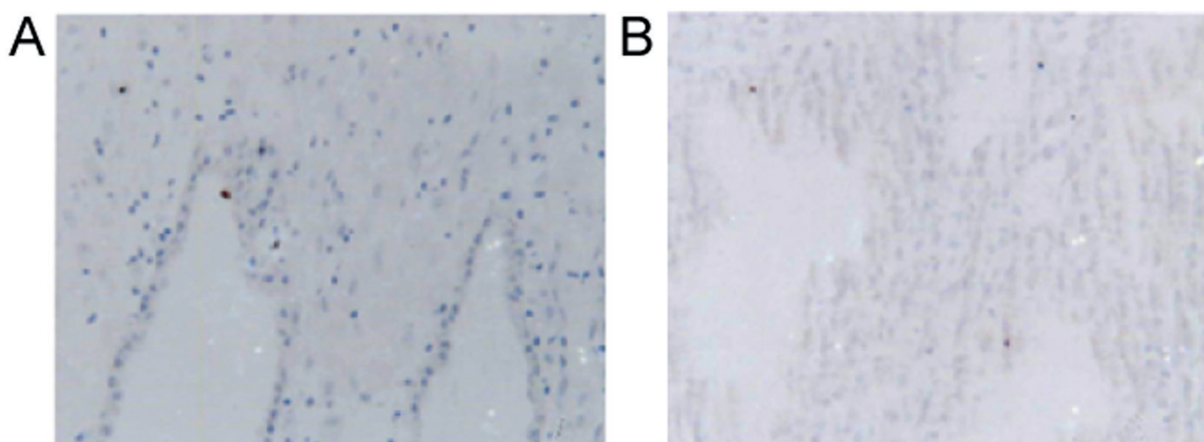

Figure 2. AR tested by Tunel method (200×, ***(A)*** represents for the recurrent spontaneous abortion group and ***(B)*** for the abortion group).

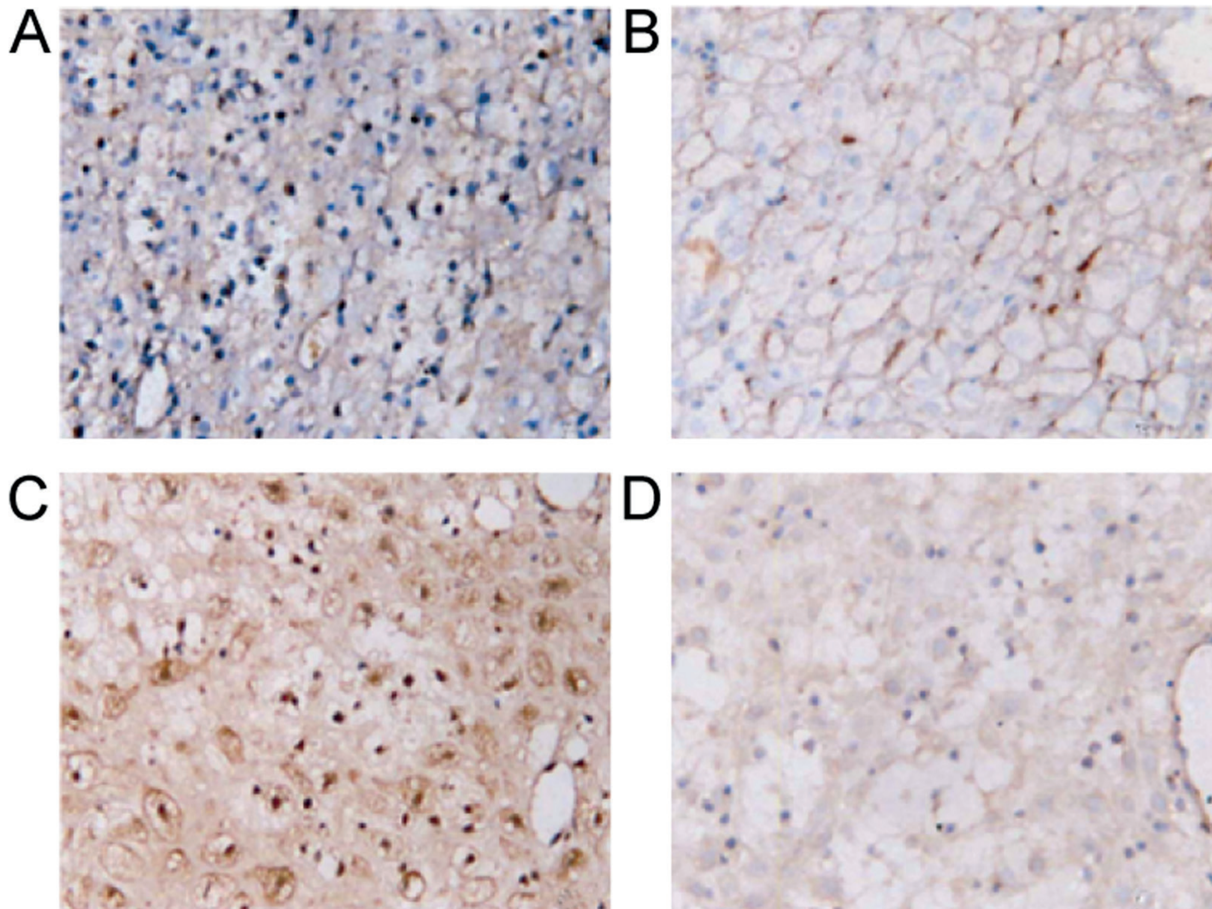

Figure 3. Expressions of expressions of Fas mRNA and FasL mRNA detected by *in situ* hybridization method (200×, ***(A-B)*** represent for the expressions of Fas mRNA in the recurrent spontaneous abortion group and the abortion group, respectively; ***(C-D)*** represent for the expressions of FasL mRNA in the abortion group and the abortion group, respectively).

dization. The study found that the apoptotic cells of normal pregnancy are mainly located in cytotrophoblast while those of spontaneous abortion are mainly in syncytiotrophoblast; syncytium is a kind of cell which directly connects fetus with the mother and the apoptosis of syncytium may cause pregnancy loss. Meanwhile, syncytiotrophoblast is the major bearer of the secretory functions of placental villi, and excessive apoptosis of syncytiotrophoblast can affect the normal functions of the placenta, impair the maternal and fetal barrier functions, enhance self-rejection and cause spontaneous abortion[11]. The occurrence of apoptosis is related to multiple cellular signaling pathways. In this study we found that expressions of Fas mRNA and FasL mRNA, expressions of Fas and FasL proteins, as well as Ca^{2+} level in recurrent spontaneous abortion group, were significantly higher than those in abortion group, but the level of PKC protein of recurrent spontaneous abortion group was significantly lower than that of the abortion group. Fas, also known as Apolipoprotein A1 (Apo-1) is a kind of cell surface receptor, which is also called apoptosis-related molecule. Heterodimer can be formed when Fas is combined with its ligand FasL, and the heterodimer can recruit caspase-8 to activate downstream caspases and cause apoptosis[12]. PKC is one of the important components of cell growth signaling pathways. Activated PKC can mediate a variety of cellular biological effects (including acceleration of proliferation, differentiation of cells and inhibition of apoptosis) via phosphorylation of serine/threonine residues in target proteins. Nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) can form a complex with its inhibitor I-kappa-B (IκB), which exists in the cytoplasm; PKC activation can lead to the phosphorylation of IKB to release IKB from IKB-NF-κB complex. Detached NF-κB can enter into

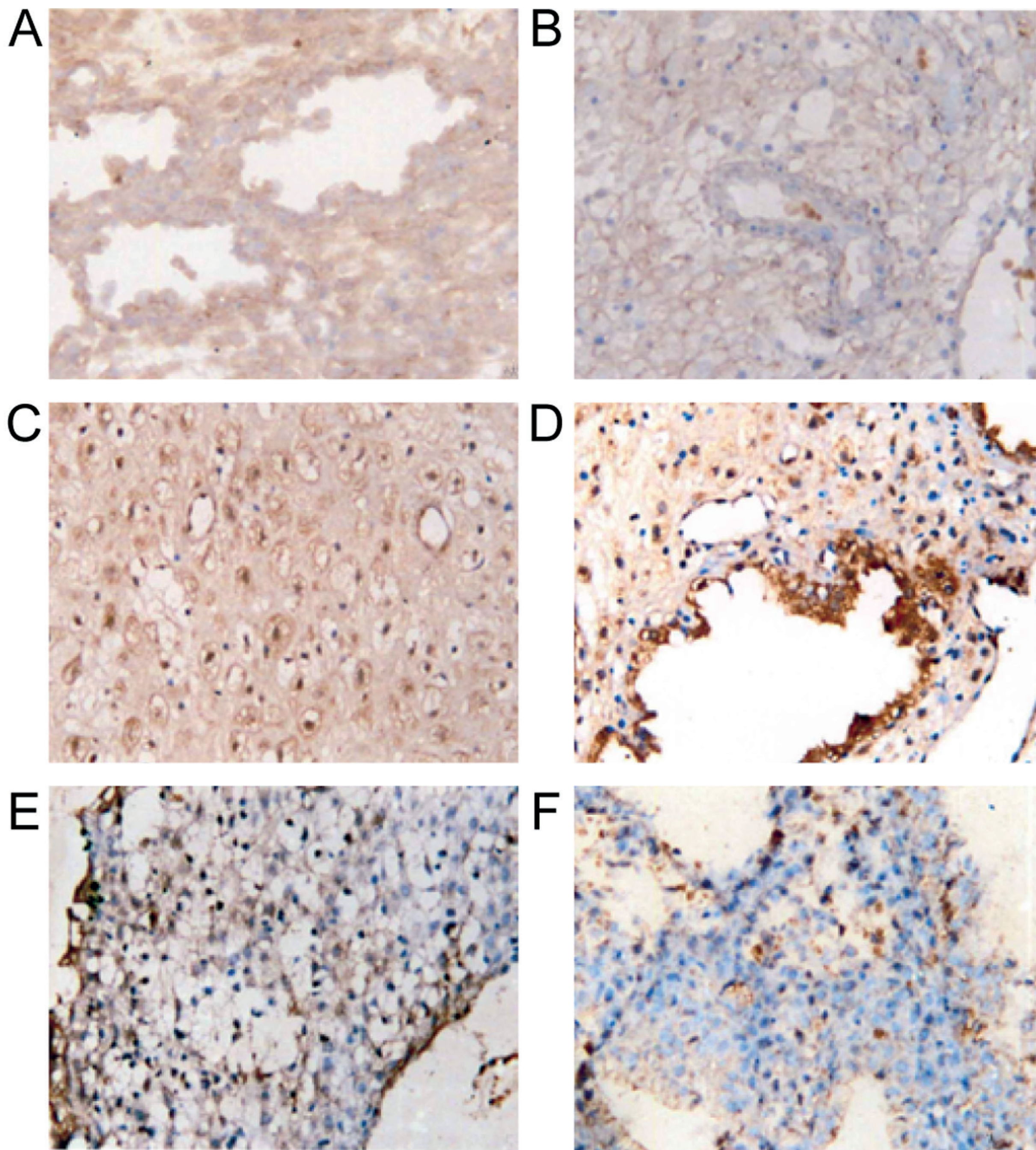

Figure 4. Expressions of Fas, FasL and PKC proteins examined by immunohistochemistry (200×, ***(A-B)*** represent for the expressions of Fas proteins in the recurrent spontaneous abortion group and the abortion group, respectively; ***(C-D)*** represent for the expressions of FasL proteins in the abortion group and the abortion group, respectively; ***(E-F)*** represent for the expressions of PKC proteins in the abortion group and the abortion group, respectively).

nuclei to accelerate cell proliferation by regulating relevant genes[13]. Also, PKC can also promote cell proliferation by activating Myc (c-Myc) and other factors[14]. Ca^{2+} is the hub of multiple signal transduction pathways. Ca^{2+} can participate in apoptosis by regulating the permeability transition (PT) of mitochondria[15,16]. Also, it can act on Ca^{2+}/Mg^{2+} dependent endonuclease to cut DNA into fragments[17]; moreover, Ca^{2+} can activate PKC or induce the expression of genes involving in apoptosis to transmit apoptosis signals and mediate apoptosis [18].

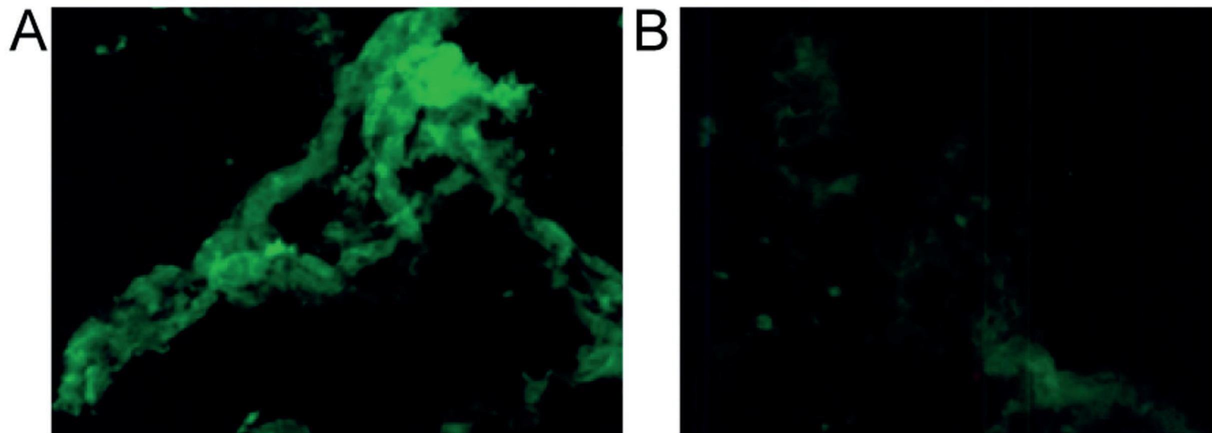

Figure 5. Ca^{2+} concentration tested by fluorescence spectrophotometer (200×, ***(A)*** represents for the recurrent spontaneous abortion group and ***(B)*** for the abortion group).

Conclusions

The signaling pathways of Fas-FasL and PKC, as well as Ca^{2+}, may mediate dysfunction of trophoblast infiltration, which leads to recurrent spontaneous abortion.

Conflict of interest

The authors declare no conflicts of interest.

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