

Expression of galectin-3 and apoptosis in placental villi from patients with missed abortion during early pregnancy

QING XIAO^{1,2*}, FANG-LING ZENG^{2*}, GUO-YI TANG², CHUN-YAN LEI³, XIAN-XIANG ZOU²,
XIAO-LE LIU¹, BI-LING PENG¹, SHUANG QIN² and HUI-XIAN LI⁴

¹Department of Obstetrics, The Eighth Affiliated Hospital, Sun Yat-sen University, Shenzhen, Guangdong 518033;

²Department of Obstetrics, Guangzhou Women and Children's Medical Center, Guangzhou Medical University, Guangzhou, Guangdong 510627; ³Department of Research and Development, Guangdong Longsee Biomedical Corporation, Guangzhou, Guangdong 510663; ⁴Department of Pediatrics, Guangzhou Women and Children's Medical Center, Guangzhou Medical University, Guangzhou, Guangdong 510889, P.R. China

Received May 17, 2018; Accepted November 16, 2018

DOI: 10.3892/etm.2019.7227

Abstract. The success of a pregnancy relies on moderate trophoblast apoptosis. If the 'inhibition-induction' balance of apoptosis is broken, a pathological pregnancy may occur. Galectin-3 has an important role in numerous biological processes, including tumor cell apoptosis. However, the association between galectin-3 and missed abortion (MA) has remained elusive. In the present study, the mRNA and protein expression levels of galectin-3 in placental villi, and the apoptotic index of placental cells from patients with MA were assessed and compared with those in a normal spontaneous abortion group. The present study investigated the function of galectin-3 in the process of MA and the possible association between placental apoptosis and galectin-3 expression in MA patients. Furthermore, the role of galectin-3 in patients with MA at different times (<4 and >4 weeks) was explored. The present study provided a potential mechanism of MA from a perspective of apoptosis and also provided potential therapeutic approaches to prevent MA.

Introduction

Missed abortion (MA), also known as abnormal intrauterine pregnancy, refers to the embryo or fetus dying in the uterine cavity but failing to spontaneously excrete in time prior to 20 weeks of gestation (1). MA is a specific type of spontaneous abortion (SA). In the process of MA, with embryonic death, amniotic fluid absorption, embryonic tissue organization, increasingly tight adhesion between the embryonic tissue and the uterine wall occurs, causing difficulty in curettage or induced abortion (2). MA may result in a coagulation dysfunction leading to disseminated intravascular coagulation (DIC), which results in severe bleeding and may threaten the life of affected patients (3). With the accelerated pace of work, increased social pressure and increased gestational age, the proportion of patients with MA has significantly increased among all cases of abortion (4). In the past six years, the proportion of MA among the total abortions encountered at Guangzhou Women and Children's Medical Center (Guangzhou, China) has increased from 24.14 to 30.50% (Fig. 1), and the average increase was 1% per year, indicating that MA is increasingly becoming a vital factor impairing family planning. The recent relaxation of the two-child policy in China has also contributed to this phenomenon. Therefore, investigating the etiology and pathogenesis of MA has become of great interest in the field of pre-natal diagnosis in China and abroad (5,6).

Studies have indicated that the causes of MA are multiple and complex, including abnormalities in chromosome number and structure (7,8), immune dysfunction (9,10), endocrine dysfunction (11), abnormal intrauterine environment (12), hereditary thrombophilia (13), systemic infectious diseases (14,15) and environmental factors (3). However, in ~50% of affected patients, the reason for the occurrence of MA and the associated pathogenesis remain elusive (16). By analyzing the decidua tissue of 11 SA cases and 9 cases of selective termination of pregnancy by immunohistochemistry and the terminal deoxynucleotidyl-transferase mediated dUTP nick end labeling (TUNEL) assay, it was revealed that the rate of positive staining for M30 in decidual tissue cells from the SA was 2.5-fold higher than that in the selective

Correspondence to: Professor Hui-Xian Li, Department of Pediatrics, Guangzhou Women and Children's Medical Center, Guangzhou Medical University, Building 12, 9 Central Jin Hui Road, Guangzhou, Guangdong 510889, P.R. China
E-mail: lhx_hey@126.com

Miss Shuang Qin, Department of Obstetrics, Guangzhou Women and Children's Medical Center, Guangzhou Medical University, Building 5, 123 Central Xiao Yuan Road, Guangzhou, Guangdong 510627, P.R. China
E-mail: qincaicoco@163.com

*Contributed equally

Key words: galectin-3, missed abortion, apoptosis, pregnancy tissue, placenta

termination group, while the apoptotic index was up to 5-fold higher (17). TUNEL-positive cells were occasionally observed in chorionic villi of women with a normal pregnancy-induced abortion but significantly increased in those of MA patients, which mainly concentrated in the nourishment layer cells and extravillous trophoblast cells of villous tissue. However, the factors involved in the apoptosis of trophoblast cells remain unknown and an association with the occurrence of MA remains to be determined.

Apoptosis was also detected in placenta tissue during the early stages of pregnancy (<12 weeks) via the expression of pro-apoptotic genes (18). Numerous other studies have demonstrated this phenomenon (17,19,20). The presence of apoptosis is associated with placental development, including trophoblast invasion, spiral arterial transformation and trophoblast cell differentiation, as well as during birth (20). During pregnancy, the degree of apoptosis of placental tissue gradually increases until delivery (21). However, an imbalance of the 'inhibition-induction' equilibrium leads to a pathological pregnancy (22). Hence, the present study hypothesized that apoptosis of villous trophoblast cells may be associated with the occurrence of MA. Apoptosis-inducing and -inhibiting factors have a potential regulatory role during pregnancy.

Galectin-3, a multifunctional protein, belongs to the family of galectins. Its unique chimeric structure enables it to interact with a plethora of ligands and modulate diverse functions, including cell growth, adhesion, migration, invasion, angiogenesis, immune function, apoptosis and endocytosis, and it has significance in the process of tumor progression (23). Studies have indicated that galectin-3 is involved in embryo implantation, embryogenesis and placental formation, and is closely associated with the success and maintenance of pregnancy (24,25). Numerous studies suggest that high expression of galectin-3 exerts inhibitory effects on apoptotic responses of various cell types (26-29); of note, intracellular galectin-3 has anti-apoptotic effects, while extracellular galectin-3 may induce apoptosis (26,29). Galectin-3 has been reported to inhibit the release of cytochrome C by translocating to the mitochondrial membrane. It was reported to inhibit nitric oxide-induced apoptosis of BT547 human breast cancer cells by activating caspase-9 and caspase-3 (30). Furthermore, Annexin 7, a Ca²⁺-mediated phospholipid binding protein, binds galectin-3 to inhibit the release of cytochrome C to activate the caspase cascade, which prevented mitochondrial damage in cisplatin-treated BT549 cells (29). By contrast, tumor cells have an important role in immune escape mechanisms during tumor progression by secreting soluble galectin-3 to promote T lymphocyte apoptosis (31). Extracellular galectin-3 forms complexes by binding to the polysaccharide CD29/CD7 on the cell surface, which increases cytochrome C release to activate intracellular mitochondrial apoptotic signaling pathways (31,32). Studies have indicated that extracellular galectin-3 secreted by macrophages participates in the apoptosis of neutrophils, and an increase in galectin-3 levels enhances macrophage removal of inflammatory cells (33). Increasing galectin-3 levels also increases macrophage removal of inflammatory cells.

A striking similarity is apparent between the 'pseudo-malignant' blastocyst trophoblastic cells and malignant tumor cells in certain biological aspects, including growth

and development, and the abnormal expression of galectin-3 appears to be involved in the regulation of excessive apoptosis of trophoblast cells in MA patients (34).

Although the functional studies confirm that galectin-3 has an important role in the regulation of apoptosis in endometrial cells (35), the association between abnormal expression of galectin-3 and excessive apoptosis of trophoblast cells in MA patients has not been reported. Thus, in the present study, the apoptosis of placental cells, the expression of galectin-3 at the mRNA and protein level, and the levels of macrophages in the blood were assessed in patients with MA and patients with normal pregnancy induced abortions (spontaneously induced abortions). The possible mechanisms of MA were explored from the perspective of placental apoptosis, and the results may contribute to the clinical treatment as well as the prevention of MA.

Materials and methods

Patient sample collection. After approval by the Institutional Review Board of Guangzhou Women and Children's Medical Center (Guangzhou, China), 32 women with MA and 13 women with normal pregnancy induced abortion (spontaneously induced abortions) were enrolled in the clinical trial (registry no. 20181022) from January 2010 to December 2016. Written informed consent was obtained from each participant. Prior to inclusion in the study, all subjects underwent a standard diagnostic analysis to rule out any abnormal causes for disease, which included the following: i) Hepatitis B or C virus, human immunodeficiency virus, human papilloma virus, ureaplasma urealyticum, *Chlamydia trachomatis* and human cytomegalovirus; ii) medical comorbidities, blood group antibodies, anti-sperm antibodies, anti-cardiolipin antibodies and anti-endometrial antibodies; iii) abnormalities in chromosome number and structure; iv) no unnatural pregnancy, number of gestational weeks of 12, use of sex hormones in the past 6 months, history of miscarriage treatment.

Fresh chorionic villous samples were collected. Briefly, in the lithotomy position a probe was used to detect the direction and depth of the uterine cavity. A thin plastic tube is used to slowly enter the cavity and according to the week of conception and the size of the cavity, a continuous or discontinuous negative pressure (400-500 mmHg) aspiration system was used to obtain samples. The aspiration system is filtered and rinsed with PBS. Each sample was divided into three parts: One part was immediately stored at -80°C for ELISA and reverse transcription-quantitative polymerase chain reaction (RT-qPCR) detection of galectin-3, and the other two parts were fixed in formalin solution and paraffin-embedded to detect galectin-3 by immunohistochemistry (IHC) and apoptosis by using the TUNEL assay. Blood samples were collected from patients in anti-coagulant tubes to determine the macrophage content.

Patients provided consent for the inclusion of their data and samples to the 'Pregnancy tissue sample bank and patient clinical database for MA patients' at the Guangzhou Women and Children Medical Center.

TUNEL assay. Apoptosis of villi was determined using the TUNEL apoptosis detection kit (cat. no. C1098; Beyotime Institute of Biotechnology, Haimen, China). Paraffin sections

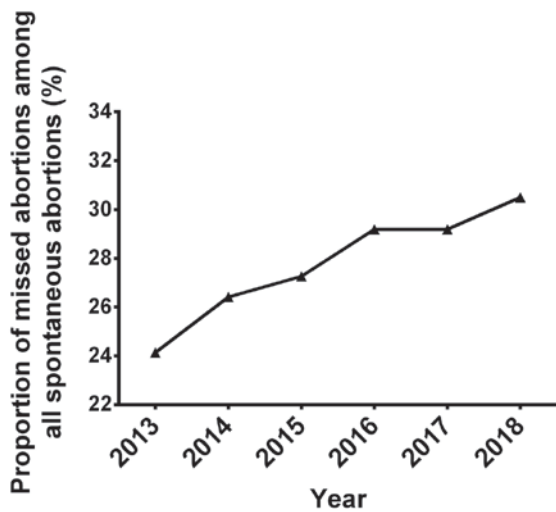


Figure 1. Percentage of missed abortions in SA patients. The percentage of missed abortions in 15,000 SA patients at the Guangzhou Women and Children's Medical Center (Guangzhou, China) during 2013-2018.

of villous tissue were de-waxed with xylene, and the rehydrated with graded ethanol and water. Proteinase K (20 $\mu\text{g}/\text{ml}$; cat. no. A5104530; Sangon Biotech Co., Ltd., Shanghai, China) without DNase was added dropwise to the tissue samples followed by incubation at 37°C for 30 min. Subsequently, the samples were incubated in PBS with 3% hydrogen peroxide for 20 min at room temperature and washed three times with PBS. The tissue was then covered with 50 μl TUNEL assay solution and incubated at 37°C for 60 min in the dark. Following washing with PBS, 0.1 ml labeled reaction stop solution was added and samples were incubated for 10 min at room temperature. Streptavidin-horseradish peroxidase (HRP) working solution (50 μl) was added, followed by incubation for 30 min at room temperature and washing for three times with PBS. Diaminobenzidine (DAB) coloring solution (0.2 ml) was added, samples were incubated for 5 min at room temperature and washed 3 times with PBS. Following staining with hematoxylin for 2 min, slides were washed with pure water, dried and sealed with neutral resin.

Under a microscope, dark brown-yellow granules appeared in the cytoplasm of apoptotic cells. For each slice, 3 non-overlapping higher-power fields (magnification, x250) in the same position were selected. The number of apoptotic cells per 200 cells was counted in each field of view. The apoptotic rate/index was the average of the percentage of positive cells, which represented the degree of apoptosis (apoptotic index=number of TUNEL-positive cells/total count of nuclei x100%).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Tissue samples (10 mg) frozen in liquid nitrogen were ground into powder. Total RNA was extracted from powdered tissue samples using the RNAPrep pure Tissue kit (cat. no. DP431; Tiangen Biotech Co., Ltd., Beijing, China), according to the manufacturer's protocol. Total RNA was reverse transcribed into cDNA using the FastQuant RT kit (cat. no. KR106; Tiangen Biotech Co., Ltd.), according to the manufacturer's protocol. qPCR was performed using 2X Talent qPCR PreMix (cat. no. FP209; Tiangen Biotech Co., Ltd.). The

following primer pairs were synthesized by Sangon Biotech Co., Ltd. (Shanghai, China) and used for qPCR: Galectin-3 forward, 5'-TGCCTTTGCCTGGGGGAGT-3' and reverse, 5'-CTGTTGTTCTCATTGAAGCGTGGG-3'; β -actin forward, 5'-AGCGAGCATCCCCAAAGTT-3' and reverse, 5'-GGG CACGAAGGCTCATCATT-3'. The following thermocycling conditions were used for qPCR: Initial denaturation at 95°C for 3 min; 40 cycles of 95°C for 5 sec, 60°C for 10 sec and 72°C for 15 sec. Galectin-3 mRNA levels were quantified using the $2^{-\Delta\Delta\text{Cq}}$ method and normalized to the internal reference gene β -actin (36).

ELISA. Galectin-3 protein levels in villous tissue were detected using a Galectin-3 Human SimpleStep ELISA[®] Kit (cat. no. ab188394; Abcam, Cambridge, UK). Following grinding of 100 mg tissue in dry ice, 500 μl pre-cooled 1X Cell Extraction Buffer PTR containing 100 mM phenylmethane sulfonyl fluoride was added, followed by mixing and sonication in an ice-water bath. The sample was incubated on ice for 20 min and then centrifuged at 18,000 x g for 20 min at 4°C. The supernatant was transferred to a new tube and the tissue protein concentration was detected using the Pierce BCA Protein Assay Kit (cat. no. 23225; Thermo Fisher Scientific, Inc., Waltham, MA, USA). Determination of galectin-3 protein concentration was then performed according to the manufacturer's protocol for the Galectin-3 Human Simple Step ELISA[®] kit. Each sample was assessed in three replicates. The protein concentration of galectin-3 in the sample was determined by measuring the absorbance at a wavelength of 450 nm using a microplate reader (ELX800; BioTek Instruments, Inc., Winooski, VT, USA).

Immunohistochemistry. Paraffin-embedded slides were baked at 60°C for 1 h. Dewaxing and rehydration were performed using xylene, ethanol and tap water. Following incubation in 3% H_2O_2 at room temperature for 10 min, samples were washed three times with distilled water. The slides were immersed in 0.01 M citrate buffer, heated to boil in a microwave and then allowed to cool to room temperature, followed by blocking with 5% bovine serum albumin (BSA; cat. no. V900933; Sigma-Aldrich, Merck KGaA, Darmstadt, Germany).

The samples were incubated with the primary antibody anti-galectin 3 (1:200; cat. no. PB9081; Boster Biotechnology, Wuhan, China) overnight at 4°C. Following washing with PBS, they were incubated with goat anti-rabbit immunoglobulin G (H+L) secondary antibody, biotin conjugate (1:200; cat. no. BA1003; Boster Biotechnology) at 37°C for 1 h. Subsequent to washing with PBS, samples were incubated with HRP-streptavidin (1:500) for 30 min at 37°C and washed again with PBS, followed by addition of DAB color reagent and incubation for 2 h at room temperature. The slides were counterstained with hematoxylin, rinsed, air-dried and slides were sealed with neutralresinsize and examined under a fluorescent microscope (TE2000-E; Nikon Corporation, Tokyo, Japan). The appearance of brown-yellow granules indicated a positive result. From each slide, 5 high-power fields (magnification, x400) were randomly chosen. The percentage of positive cells was scored as follows: <10%, 0 points; 10-25%, 1 point; 26-50%, 2 points; and >50%, 3 points. The staining intensity was scored as follows: Pale yellow, 1 point; brownish

yellow, 3 points; and an intermediate between the two colours, 2 points. The percentage score and staining intensity score were multiplied to obtain the final score: 0, negative (-); 1-3, weakly positive (+); 4-6, positive (++); and 7-9, strongly positive (+++).

Macrophage assay. A blood analyzer (Sysmex XE-5000; Sysmex Corp., Kobe, Japan) was used to detect the macrophage content of blood samples according to the manufacturer's protocol.

Statistical analysis. All statistical analyses were performed using GraphPad Prism statistical software (version 6.0; GraphPad Inc., La Jolla, CA, USA). Data presented as the mean \pm standard error of the mean. Student's t-test was used to analyze differences between two groups. One-way analysis of variance followed by Student-Newman-Keuls post-hoc test was used to analyze differences among multiple groups. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Clinical information of patients with MA. Since 2013, the proportion of MA among the total abortions encountered at Guangzhou Women and Children's Medical Center (Guangzhou, China) has increased from 24.14 to 30.50%, with an average 1% per year, indicating that MA is an increasing concern.

The age, BMI, number of abortions, number of births, history of miscarriage, history of surgery (gynecological surgery), number of pregnant menopause weeks and the number of weeks gestation at time of miscarriage were recorded for all patients with MA (Table I). Of note, none of the patients with MA had any history of miscarriage treatment, premature rupture of membranes, endometriosis, drug allergies, mental illness, hypertension, thrombosis or trauma during pregnancy. All conceptions were natural without any assistance of reproductive technology. As the embryos stopped developing and remained in the uterus, it was required to perform a negative pressure aspiration.

Apoptotic index of chorionic villi. Apoptosis of villi was determined using TUNEL (Fig. 2A and B). The apoptotic index in the SA group was $2.93 \pm 0.44\%$, while that in the MA group was significantly higher at $4.80 \pm 0.60\%$ ($P < 0.05$; Fig. 2C).

RT-qPCR detection of galectin-3 mRNA in villous tissue. The melting curves for galectin-3 and β -actin had a single peak and the dissolution temperature was $\sim 80^\circ\text{C}$. The results of the RT-qPCR analysis indicated that the relative expression of galectin-3 mRNA in the MA group was significantly higher than that in the SA group ($2^{-\Delta\Delta C_q} = 1.20 \pm 0.02$ vs. 1.00 ± 0.05 ; $P < 0.05$; Fig. 3A). In terms of different time-points of MA occurring, the MA patients were divided into two groups, MA occurring < 4 weeks gestation and MA occurring > 4 weeks gestation. Compared with the SA group, the galectin-3 mRNA levels in the MA at < 4 weeks subgroup were significantly lower ($2^{-\Delta\Delta C_q} = 1.00 \pm 0.00$ vs. 0.47 ± 0.02 ; $P < 0.05$; Fig. 3B). However, in the MA at > 4 weeks subgroup, the galectin-3 mRNA levels were significantly higher than those in the SA group

($2^{-\Delta\Delta C_q} = 1.00 \pm 0.05$; $P < 0.05$; Fig. 3B). Those results suggested that the expression level of galectin-3 was significantly increased in the MA group compared with the SA group. The expression of galectin-3 in early MA group (< 4 weeks) is decreased compared with the SA group. The expression level of galectin-3 in the MA group gradually increases with time.

Protein levels of galectin-3 in chorionic villi detected by ELISA. As presented in Fig. 4A, the mean protein level of galectin-3 detected by ELISA in the SA group was 19.12 ± 3.43 ng/ml and that in the MA group was 21.6 ± 3.15 ng/ml. The protein level of galectin-3 in the MA group was slightly higher than that in the SA group and the difference was statistically significant ($P < 0.05$). Regarding the different time-points of MA occurring, the protein levels of galectin-3 in the subgroup with MA at < 4 weeks (18.26 ± 3.67 ng/ml) were slightly lower than those in the SA group (19.3 ± 3.43 ng/ml), and in the subgroup with MA at > 4 weeks, the protein levels of galectin-3 (22.04 ± 3.68 ng/ml) were higher than those in the SA group; however, the differences were not statistically significant ($P > 0.05$; Fig. 4B).

IHC detection of galectin-3 protein levels in villi. In villous trophoblasts, galectin-3 protein was mainly expressed in the cell membrane, cytoplasm and nuclear membrane. Galectin-3 expression was indicated as brown DAB staining in the cytoplasm (red arrows; Fig. 5A-C). The score of galectin-3 expression in the SA group was 4.84 ± 1.44 and the positive (++) rate was 100%. The score of galectin-3 expression in the MA group was 5.48 ± 1.40 . The rate of weakly positive (++) , positive (++) and strongly positive (+++) staining was 20, 60 and 20%, respectively. As presented in Fig. 6, the difference between the SA and MA in the overall galectin-3 staining score was not statistically significant ($P > 0.05$).

Macrophage levels in the blood. The mean concentration of macrophages detected in the MA group (0.45 ± 0.19 $10^9/l$) was slightly, but not significantly higher than that in the SA group (0.40 ± 0.11 $10^9/l$; $P > 0.05$; Fig. 7A). Regarding the different time-points of MA, the macrophage levels in the subgroup with MA at < 4 weeks (0.35 ± 0.12 $10^9/l$) were lower than those in the SA group (0.40 ± 0.11 $10^9/l$), while those in the subgroup with MA at > 4 weeks (0.57 ± 0.19 $10^9/l$) were higher than those in the SA group ($P < 0.05$; Fig. 7B). These results support the current study hypothesis that galectin-3 accumulation is associated with alternative activation of macrophages following extensive apoptosis.

Discussion

The present study assessed samples from patients with unexplained MA and SA. Neither the patients with MA nor SA included in the present study had any history of pre-mature rupture of membranes, endometriosis, drug allergies, mental illness, hypertension, thrombosis or trauma during pregnancy. RT-qPCR, ELISA and IHC were used to detect the mRNA and protein levels of galectin-3 in villous tissue, and it was indicated that the protein expression of galectin-3 in the MA group was higher than that in the SA group. Furthermore, the apoptotic indices of chorionic villi in the MA group were higher than those in the SA group. Of note, it was observed that galectin-3

Table I. Clinical information of patients with MA.

Age (years)	BMI (kg/m ²)	Number abortion	Gravidity	History of MA	History of surgery	Pregnant menopause week	Gestational week	Missed week
39	23.7	0	2	N	N	11	7	4
31	24.2	1	2	N	N	13	5	8
31	21.9	0	2	N	N	15	6	9
27	22.5	0	1	N	N	17	8	9
31	21.2	0	1	N	N	10	9	1
21	19.8	0	1	N	N	18	11	7
28	18.8	0	1	N	N	11	9	2
26	22.2	0	1	N	N	14	7	7
20	24.1	1	2	N	N	11	6	5
28	26.5	1	1	N	N	12	11	1
40	17.7	1	1	N	Y	14	7	7
29	19.4	1	1	N	N	20	9	11
35	19.3	0	2	N	N	11	11	0
30	27.1	3	4	Y	N	8	7	1
36	23.1	1	3	N	N	18	8	10
46	26.6	1	1	N	N	10	7	3
29	22.2	0	0	N	N	12	9	3
40	20.7	0	3	N	N	11	8	3
28	25	1	2	N	N	13	8	5
26	19.8	1	1	N	N	12	6	6
28	23.3	3	3	Y	N	12	7	5
39	26.9	0	2	N	Y	14	11	3
29	22.1	2	2	Y	N	13	9	4
34	20.5	3	6	N	N	11	6	5
42	19.9	0	2	N	Y	15	7	8
36	18.5	2	2	Y	N	13	8	5
26	18.2	1	2	Y	N	15	8	7
46	40.9	1	1	N	Y	12	6	6
34	34.8	0	1	N	N	13	8	5
30	33.3	0	1	N	N	13	7	6
33	29.2	2	4	N	Y	12	5	7
31	33.0	1	2	N	N	17	8	9

MA, missed abortion; Y, yes; N, no; BMI, body mass index.

mRNA and protein expression levels were decreased in those patients with the time-point of MA of <4 weeks (post-menstruation) compared with those in the SA group. However, in those patients with MA occurring at >4 weeks, the expression of galectin-3 was higher than that in the SA group.

Galectin-3 is a β -galactoside binding protein that has roles in various biological processes, including cell growth, differentiation, cell adhesion and apoptosis (37). It has been reported that galectin-3 is highly expressed in chorionic villi and decidua during the third trimester of pregnancy (38). 17β -estradiol (E2), progesterone and human chorionic gonadotropin (hCG) were indicated to induce the expression and secretion of galectin-3 in BeWo trophoblast cells and 17β -E2 was also demonstrated to participate in the differentiation process of trophoblast cells through regulating galectin-3 (24). Furthermore, hCG was reported to regulate galectin-3 to

prepare for embryo implantation into the endometrium (25). Previous studies demonstrated that galectin-3 may have an important role in embryo implantation through regulation of macrophage levels in the blood, and this was also observed in the current study. Comparison among pregnant women with surgical and medical abortion (gestation age <13 weeks) revealed that galectin-3 expression in the placental villi was significantly reduced. The present study also indicated that galectin-3 expression was decreased in the placental villi of MA patients with a time-point of MA of <4 weeks compared with those in SA patients with a time-point of MA <4 weeks. A reduction of galectin-3 affects the normal development of the embryo, as well as the interaction between the villi and the endometrium, which impairs the invasive ability of trophoblast cells (39). Studies have indicated that overexpression of galectin-3 in tumor cells increases the degree of malignancy

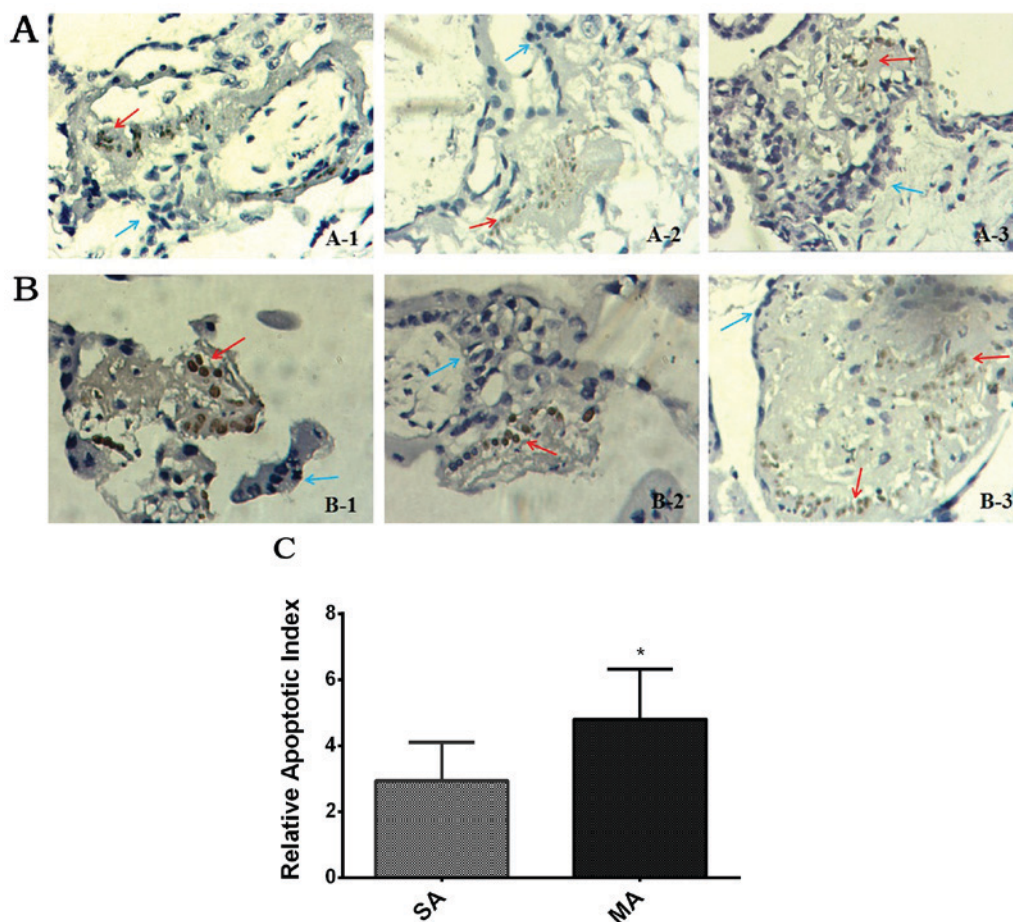


Figure 2. Level of apoptosis in chorionic villi tissue samples from patients in the SA and MA group. The level of apoptosis was determined by TUNEL assay in tissue samples obtained from patients in the (A) SA and (B) MA group (magnification, x250). Red arrows indicate apoptotic cells and the blue arrows indicate cell nuclei counterstained with hematoxylin. SA, spontaneous abortion; MA, missed abortion; TUNEL, terminal deoxynucleotidyl-transferase mediated dUTP nick end labeling. (C) Quantification of apoptosis in chorionic villi tissue samples from patients in the SA and MA group determined by TUNEL assay. * $P < 0.05$ vs. SA group. SA, spontaneous abortion; MA, missed abortion; TUNEL, terminal deoxynucleotidyl-transferase mediated dUTP nick end labeling.

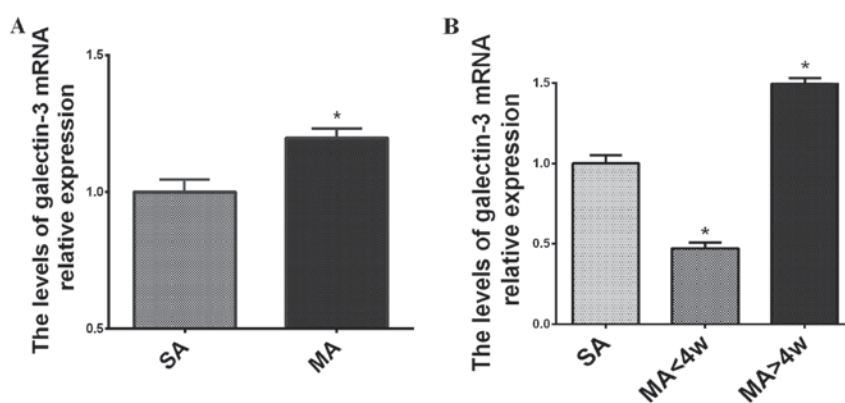


Figure 3. Galectin-3 mRNA expression in chorionic villi of the SA and MA groups. The relative mRNA expression levels of galectin-3 were determined by RT-qPCR in chorionic villi tissue samples from patients in the SA and MA group. (A) The expression level of galectin-3 was significantly increased in the MA group compared with the SA group. (B) The expression of galectin-3 in early MA group (<4 weeks) is decreased compared with the SA group. The expression level of galectin-3 in the MA group gradually increases with time. * $P < 0.05$ vs. SA group. SA, spontaneous abortion; MA, missed abortion; RT-qPCR, reverse-transcription quantitative polymerase chain reaction; 4 w, 4 weeks.

of the tumor, while galectin-3 acts as an anti-apoptotic factor to regulate apoptotic responses (40,41). Based on the striking similarity in the growth and developmental biological processes between the 'pseudo-malignant' trophoblastic cells

of blastocyst and malignant tumor cells, it may be speculated that the low expression of galectin-3 in the villous trophoblast cells led to cell apoptosis. Early MA was associated with downregulation of galectin-3 expression compared with the

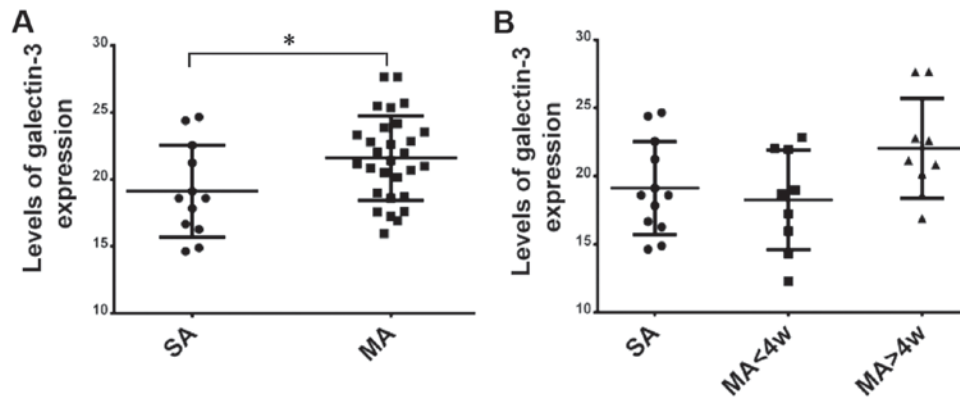


Figure 4. Galectin-3 protein expression in chorionic villi of the SA and MA groups. The relative mRNA expression levels of galectin-3 were determined by ELISA in chorionic villi tissue samples from patients in the SA and MA group. (A) The galectin-3 protein expression level is significantly higher in the MA group compared with the SA group; whilst the galectin-3 protein expression level in the late MA group (>4 weeks) was significantly higher compared with the SA group. * $P < 0.05$ vs. SA group. SA, spontaneous abortion; MA, missed abortion; 4 w, 4 weeks.

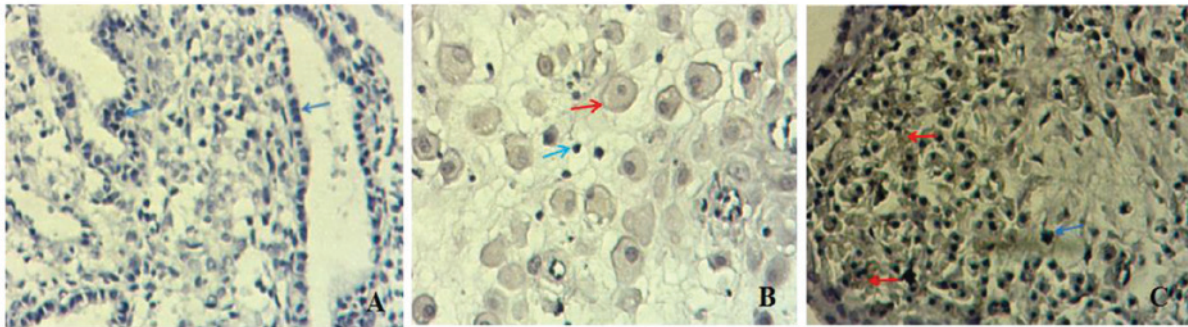


Figure 5. Galectin-3 protein expression in SA and MA groups determined by IHC. Galectin-3 protein expression was examined in chorionic villi tissue samples from patients in the (A) negative control, (B) SA group and (C) MA group (magnification, $\times 400$). Red arrows indicate galectin-3-positive expression in cells stained by DAB and blue arrows indicate cell nuclei counterstained with hematoxylin. SA, spontaneous abortion; MA, missed abortion; IHC, immunohistochemistry; DAB, diaminobenzidine.

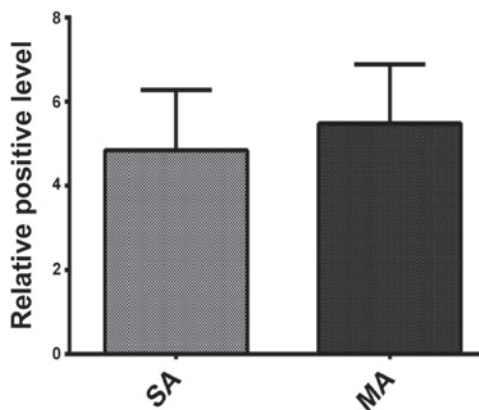


Figure 6. Quantification of galectin-3 protein expression in SA and MA groups determined by IHC. SA, spontaneous abortion; MA, missed abortion; IHC, immunohistochemistry.

SA group, which likely contributes to apoptosis. In addition, galectin-3 expression increased with the advancement of the time-point of MA.

Furthermore, it was reported that BeWo trophoblast cells secrete galectin-3 to inhibit the proliferation of RL95-2 endometrial cells and to mediate endometrial apoptosis by

activating RL95-2 cells to secrete integrin $\beta 1$ (34). It has been indicated that during embryo implantation, endometrial cells undergo proliferation and apoptosis under the regulation of estrogen, progesterone and hCG and the action of galectin-3, which is secreted by trophoblast cells, to facilitate this process. Although galectin-3 secreted by BeWo cells was observed to induce apoptosis in endometrial cells, under induction by 17β -E2, RL95-2 cells exhibit and upregulated galectin-3 expression and reduced apoptosis induced by staurosporine (34). High expression and secretion of galectin-3 may selectively activate macrophages, which have important roles in tissue damage and repair, while it depends on the degree of damage and the effect time whether their role is pathogenic or reparative (42). An effective endometrial environment for embryo implantation is established on the basis of the balance between extracellular galectin-3 with pro-apoptotic and endometrial cell galectin-3 with anti-apoptotic effects. In the present study, it was revealed that galectin-3 was highly expressed in villous trophoblast tissue of MA patients with a time-point of MA of >4 weeks. It was speculated that due to a compensatory mechanism during early MA, trophoblast cells secreted excessive galectin-3 to cause massive apoptosis of endometrial cells. Via this process, the normal development of the endometrium and villi in early pregnancy was affected and

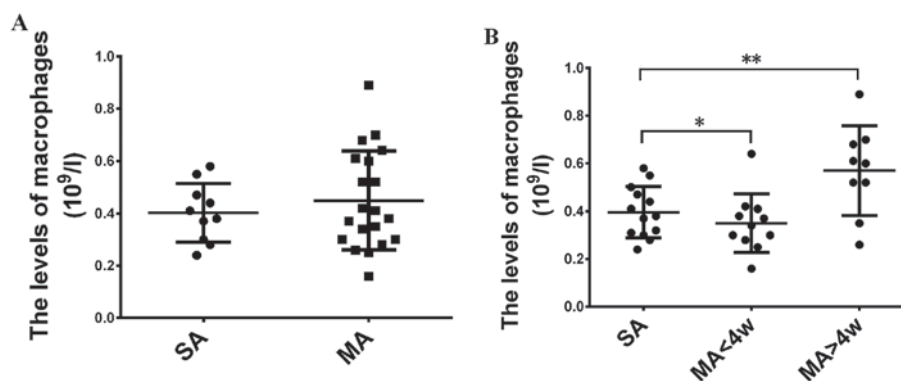


Figure 7. Level of macrophages in blood samples from patients in the SA and MA groups. (A) The level of macrophages in the MA group were significantly higher compared with the SA group. (B) The levels of macrophages in the early MA group (<4 weeks) were decreased compared with the SA group, whilst the level of macrophages in the late MA group (>4 weeks) was significantly higher compared with the SA group. * $P < 0.05$, ** $P < 0.01$ vs. SA group. SA, spontaneous abortion; MA, missed abortion; 4 w, 4 weeks.

the interaction between the villi and the endometrium further promoted MA (34). A limitation of the present study was that galectin-3 was not detected in extracellular fluid or decidual tissues. Detection of the intracellular and extracellular levels of galectin-3 may have had a greater scope of revealing the mechanism of villous trophoblast cells causing apoptosis of endometrial cells by excessive secretion of galectin-3. It may also further prove that extracellular galectin-3 promotes apoptosis, while galectin-3 inside of endometrial cells inhibits apoptosis. An imbalance of intra- and extracellular galectin-3 may trigger embryonic developmental abnormalities, which ultimately result in MA.

In summary, the present study suggested that early MA is associated with apoptosis of placental villi due to down-regulation of galectin-3. With the advancement of the time-point of MA, galectin-3 in placental villi was increased and placental survival may have been extended via compensatory protective mechanisms. Excessive galectin-3 secreted from trophoblast cells likely caused excessive apoptosis of endometrial cells, which affected the normal development of endometrium and villi in early pregnancy. The interaction between villi and endometrium further contributed to the eventual occurrence of MA. As a likely underlying mechanism of MA, it is important to elucidate the exact association between apoptosis and galectin-3 in the corresponding cell types, which will be further assessed in detail in a future study. The present study provided a potential mechanism of MA from a perspective of apoptosis and also provided potential therapeutic approaches to prevent MA.

Acknowledgements

Not applicable.

Funding

This study was supported by the National Natural Science Foundation of Guangdong Province (grant no. 2015A030313689).

Availability of data and materials

Not applicable.

Authors' contributions

QX, SQ, QS and HXL designed the experiments. QX, FLZ, XXZ, XLL and BLP carried out the experiments. QX, FLZ, GYT and CYL collected and analyzed the data. QX and FLZ prepared the manuscript.

Ethics approval and consent to participate

This study was approved by the Ethics Committee of Guangzhou Women and Children's Medical Center (Guangzhou, China) and written informed consent was obtained from each participant (registry no. 20181022).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

- Petersen SG, Perkins AR, Gibbons KS, Bertolone JI and Mahomed K: The medical management of missed miscarriage: Outcomes from a prospective, single-centre, Australian cohort. *Med J Aust* 199: 341-346, 2013.
- Zhang J, Xiao X, Luo F, Shi G, He Y, Yao Y and Xu L: Acute disseminated intravascular coagulation developed after dilation and curettage in an adenomyosis patient: A case report. *Blood Coagul Fibrinolysis* 24: 771-773, 2013.
- Zhang Huijun: Epidemiological analysis on hazards of missed abortion. *Zhongguo Bing An* 8: 71-72, 2012 (In Chinese).
- Zhang X, Li J, Gu Y, Zhao Y, Wang Z and Jia G: A pilot study on environmental and behavioral factors related to missed abortion. *Environ Health Prev Med* 16: 273-278, 2011.
- Li L, Leung PC, Chung TK and Wang CC: Systematic review of Chinese medicine for miscarriage during early pregnancy. *Evid Based Complement Alternat Med* 2014: 753856, 2014.
- Kang MH: The research progress and analysis on pathogenic factor relative to missed abortion. *Medical Recapitulate* 21: 3300-3302, 2011 (In Chinese).
- Chatzimeletiou K, Makrydimas G and Nicolaidis KH: Aneuploidy screening in a coelomic sample from a missed abortion using sequential fluorescence in situ hybridization. *Fertil Steril* 85: 1059.e13-e16, 2006.

8. Halder A and Fauzdar A: Skewed sex ratio and low aneuploidy in recurrent early missed abortion. *Indian J Med Res* 124: 41-50, 2006.
9. Nagima M and Alija A: Immune status parameters significance in pregnancy interruption on type of missed abortion in first trimester. *Med Health Sci J* 12: 25-30, 2012.
10. Cao W, Xu W, Chen T, Wang X, Wang X, Qiu J, Chen N and Mao Y: CD4⁺CD25⁺FoxP3⁺ regulatory T cells and cytokines interact with estradiol in cases of missed abortion. *Exp Ther Med* 7: 417-422, 2014.
11. Fang XX: Predictive value of pregnancy-associated plasma protein A and free β -HCG in missed abortion and ectopic pregnancy. *Chinese Rural Health Service Administration* 9: 980-981, 2012 (In Chinese).
12. Aboujaoude R, Alvarez JR, Alvarez M and Al-Khan A: Management of missed abortion in a patient with congenital uterine anomalies. *Arch Gynecol Obstet* 275: 137-139, 2007.
13. Kosus N, Kosus A, Yildirim M, Duran M and Turhan NO: Mean platelet volume as a marker of thrombosis in patients with missed abortion. *Acta Haematol* 125: 208-209, 2011.
14. Horn LC, Nenoff P, Ziegert M and Höckel M: Missed abortion complicated by Candida infection in a woman with rested IUD. *Arch Gynecol Obstet* 264: 215-217, 2001.
15. Hwang JH, Kim JW, Hwang JY, Lee KM, Shim HM, Bae YK, Paik SS and Park H: Cocksackievirus B infection is highly related with missed abortion in Korea. *Yonsei Med J* 55: 1562-1567, 2014.
16. Guerra Shinohara EM, Pereira PM, Kubota AM, Silva TA, Reis JL, Miyashita GS, D'Almeida V, Allen RH and Stabler SP: Increased MMA concentration and body mass index are associated with spontaneous abortion in Brazilian women: A pilot study. *Clin Chim Acta* 411: 423-427, 2010.
17. Guenther S, Vrekoussis T, Heublein S, Bayer B, Anz D, Knabl J, Navrozoglou I, Dian D, Friese K, Makrigiannakis A and Jeschke U: Decidual macrophages are significantly increased in spontaneous miscarriages and over-express FasL: A potential role for macrophages in trophoblast apoptosis. *Int J Mol Sci* 13: 9069-9080, 2012.
18. Neslon DM: Apoptosis changes occur in syncytiotrophoblast of human placenta villi where fibrin type fibrinoin is deposited at discontinuities in the villous trophoblast. *Placenta* 17: 387-391, 1996.
19. Chen H, Deng X, Yang Y, Shen Y, Chao L, Wen Y and Sun Y: Expression of GRIM-19 in missed abortion and possible pathogenesis. *Fertil Steril* 103: 138-146.e3, 2015.
20. Mayhew TM, Leach L, McGee R, Ismail WW, Myklebust R and Lammiman MJ: Proliferation, differentiation and apoptosis in villous trophoblast at 13-41 weeks of gestation (including observations on annulate lamellae and nuclear pore complexes). *Placenta* 20: 407-422, 1999.
21. Smith SC, Baker PN and Symonds EM: Increased placental apoptosis in intrauterine growth restriction. *Am J Obstet Gynecol* 177: 1395-1401, 1997.
22. Wei D, Wu Q and Shi H: Apoptosis and p53 expression in the placental villi of females with unexplained recurrent spontaneous abortion. *Exp Ther Med* 7: 191-194, 2014.
23. Funasaka T, Raz A and Nangia-Makker P: Galectin-3 in angiogenesis and metastasis. *Glycobiology* 24: 886-891, 2014.
24. Yang H, Taylor HS, Lei C, Cheng C and Zhang W: Hormonal regulation of galectin 3 in trophoblasts and its effects on endometrium. *Reprod Sci* 18: 1118-1127, 2011.
25. Yang H, Lei CX and Zhang W: Human chorionic gonadotropin (hCG) regulation of galectin-3 expression in endometrial epithelial cells and endometrial stromal cells. *Acta Histochem* 115: 3-7, 2013.
26. Yang RY, Hsu DK and Liu FT: Expression of galectin-3 modulates T-cell growth and apoptosis. *Proc Natl Acad Sci USA* 93: 6737-6742, 1996.
27. Akahani S, Nangia-Makker P, Inohara H, Kim HR and Raz A: Galectin-3: A novel antiapoptotic molecule with a functional BH1 (NWGR) domain of Bcl-2 family. *Cancer Res* 57: 5272-5276, 1997.
28. Kim HR, Lin HM, Biliran H and Raz A: Cell cycle arrest and inhibition of anoikis by galectin-3 in human breast epithelial cells. *Cancer Res* 59: 4148-4154, 1999.
29. Yu F, Finley RL Jr, Raz A and Kim HR: Galectin-3 translocates to the perinuclear membranes and inhibits cytochrome c release from the mitochondria. A role for synexin in galectin-3 translocation. *J Biol Chem* 277: 15819-15827, 2002.
30. Moon BK, Lee YJ, Battle P, Jessup JM, Raz A and Kim HR: Galectin-3 protects human breast carcinoma cells against nitric oxide-induced apoptosis: Implication of galectin-3 function during metastasis. *Am J Pathol* 159: 1055-1060, 2001.
31. Fukumori T, Takenaka Y, Yoshii T, Kim HR, Hogan V, Inohara H, Kagawa S and Raz A: CD29 and CD7 mediate galectin-3-induced type II T-cell apoptosis. *Cancer Res* 63: 8302-8311, 2003.
32. Demetriou M, Granovsky M, Quaggin S and Dennis JW: Negative regulation of T-cell activation and autoimmunity by Mgat5 N-glycosylation. *Nature* 409: 733-739, 2001.
33. Karlsson A, Christenson K, Matlak M, Björstad A, Brown KL, Telemo E, Salomonsson E, Leffler H and Bylund J: Galectin-3 functions as an opsonin and enhances the macrophage clearance of apoptotic neutrophils. *Glycobiology* 19: 16-20, 2009.
34. Sengupta J and Ghosh D: Role of progesterone on peri-implantation stage endometrium-embryo interaction in the primate. *Steroids* 65: 753-762, 2000.
35. Yang H, Lei C, Cheng C, Feng Y, Zhang W, Petracco RG and Sak S: The antiapoptotic effect of galectin-3 in human endometrial cells under the regulation of estrogen and progesterone. *Biol Reprod* 87: 39, 2012.
36. Livak KJ and Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2⁻($\Delta\Delta C_T$) method. *Methods* 25: 402-408, 2001.
37. Gao L and Fang AH: Expression and influence of galectin-3 on missed abortion. *J Reprod Contracep* 25: 227-234, 2014.
38. Takenaka Y, Fukumori T, Yoshii T, Oka N, Inohara H, Kim HR, Bresalier RS and Raz A: Nuclear export of phosphorylated galectin-3 regulates its antiapoptotic activity in response to chemotherapeutic drugs. *Mol Cell Biol* 24: 4395-4406, 2004.
39. Califice S, Castronovo V, Bracke M and van den Brûle F: Dual activities of galectin-3 in human prostate cancer: Tumor suppression of nuclear galectin-3 vs. tumor promotion of cytoplasmic galectin-3. *Oncogene* 23: 7527-7536, 2004.
40. Honjo Y, Inohara H, Akahani S, Yoshii T, Takenaka Y, Yoshida J, Hattori K, Tomiyama Y, Raz A and Kubo T: Expression of cytoplasmic galectin-3 as a prognostic marker in tongue carcinoma. *Clin Cancer Res* 6: 4635-4640, 2000.
41. Sokolov DI, Kolobov AV, Lesnichija MV, Kostiouchek IN, Stepanova OI, Kvetnoy IM and Selkov SA: Regulatory mechanisms for apoptosis in placental tissue during normal pregnancy and gestosis-complicated pregnancy. *Bull Exp Biol Med* 148: 766-770, 2009.
42. MacKinnon AC, Farnworth SL, Hodgkinson PS, Henderson NC, Atkinson KM, Leffler H, Nilsson UJ, Haslett C, Forbes SJ and Sethi T: Regulation of alternative macrophage activation by galectin-3. *J Immunol* 180: 2650-2658, 2008.



This work is licensed under a Creative Commons Attribution-NonCommercial-NoDerivatives 4.0 International (CC BY-NC-ND 4.0) License.