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ORIGINAL RESEARCH ARTICLE

Placental Expression and Relative Role of Anti-inflammatory Annexin A1 and Animal Lectin Galectin-3 in the Pathogenesis of Preeclampsia

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Abstract Preeclampsia (PE) remains the major cause for maternal and foetal mortality and morbidity all over the world. Preeclampsia is associated with maternal, placental aggravated inflammatory response and generalized endothelial damage. AnnexinA1 (AnxA1) is glucocorticoid regulated protein regulates a wide range of cellular and molecular steps of the inflammatory response and is implicated in resolution of inflammation. Galectin-3 (Gal-3), β-galcotoside-binding lectin participates in many functions, both intra- and extracellularly. Recently it has been shown that galectin-3 modulates the inflammation. Role of AnxA1 and Galectin-3 is poorly studied in context with human reproductive disease like Preeclampsia. Therefore, the present study examined the expression of AnxA1 and Gal-3 which are involved in modulation of inflammation and their association in the placental bed of pregnancy with and without PE. The study group consisted of placental bed biopsy tissues obtained from pregnancies with PE (n = 30) and without (n = 30) PE. The expression of AnxA1 and Gal-3 in the placental bed tissues was evaluated quantitatively using Immunohisto-chemistry (IHC), western blot and mRNA expression analysis by quantitative RT-PCR. Our IHC, western blot and RT PCR analyses showed the increase in the expression of AnxA1 and Gal-3 in PE group compared with the normotensive control group ($P < 0.001$). The increased expression of AnxA1 and Gal-3 in placental bed may be associated with a systemic inflammatory response in PE, suggesting role of AnxA1 and Gal-3 in PE pathogenesis.

Keywords Preeclampsia · Annexin A1 · Galectin-3 · Placenta · Inflammation

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Introduction

Preeclampsia (PE) is a disorder of pregnancy characterized by onset of high blood pressure and proteinuria developing after the 20th week of gestation in a previously normotensive woman. It is a severe complication of human pregnancy with a worldwide incidence of 2–10%. It is one of the leading causes of maternal, as well as perinatal morbidity and mortality all over the world even in developed countries. In spite of in depth research, the pathophysiology of PE is not completely understood. An exaggerated maternal systemic inflammatory response to pregnancy with activation of both the innate and the adaptive arms of the immune system play a pivotal role in the development of the disease [1]. It has been proposed

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that the ischemic placenta can release soluble factors into the maternal circulation that cause endothelial cell activation and/or dysfunction and a systemic inflammatory response [2]. Redman and Sargent [3] earlier proposed that the features of the systemic inflammatory response seen in normotensive pregnant women are also seen in PE women, but in a greater severity. Annexin A1 (AnxA1), previously known as lipocortin-1 is a member of the calcium-dependent phospholipid-binding protein superfamily of Annexins, which regulate diverse cellular functions in various cellular types [4]. AnxA1 was predominantly delineated as a glucocorticoid-regulated protein having anti-phospholipase activity, but the protein also exhibits many other anti-inflammatory and pro-resolving properties, which primarily include profound inhibitory action on leucocyte transmigration and activation, leading to resolution of inflammation [5]. Gal-3 is involved in numerous biological processes associated with cell growth and differentiation [6]. This protein has also been implicated in numerous clinical states, such as inflammation [7]. Gal-3 has controversial pro- or anti-inflammatory activities depending on various factors including its intracellular or extracellular localization and the target cell implicated in these processes [8]. Although it may contribute to resolution of inflammation by clearing apoptotic neutrophils [9]. Gal-3 has also been identified in the human placenta and its abundance was found to be inversely correlated with trophoblast invasiveness during the course of gestation [10]. Considerable body of evidence illustrates that AnxA1 and Gal-3 participates in anti-inflammatory and proresolving function. PE is associated with an exacerbated inflammatory state, therefore it is rational to hypothesize that AnxA1 and Gal-3 may be altered in PE women. Therefore, the present study examined the expression of above proteins which are involved in modulation of inflammation and their association in the placental bed of pregnancy with and without PE.

Material and Methods

This study was approved by Institutional ethics committee at SDM College of Medical Sciences and Hospital Dharwad, Karnataka. PE was diagnosed based on increased blood pressure (140/90 mmHg) in a pregnant woman after 20 weeks of amenorrhea, accompanied by proteinuria (0.3 g/24 h or 1+ dipstick), as defined by the report of National High Blood Pressure Education Program Working Group on High Blood Pressure in Pregnancy [13]. Cases of chronic hypertension or superimposed PE were excluded from the study. Body mass index (BMI) was evaluated pre pregnancy and prior to caesarean delivery.

Placental Bed Biopsies

Fresh placental bed biopsy tissues were obtained from 60 term pregnancies at the time of caesarean delivery after the patient's informed consent between January 2017 and December 2018. The study groups consisted of pregnant women with PE (n = 30, PE group) and without PE (n = 30, normotensive control group). All pregnancies were also free of other complications, such as gestational diabetes, chronic hypertension, and autoimmune disease. For expression study, chorionic villous tissue from maternal side of the placenta is collected. The expression of AnxA1 and Gal-3 were analysed by using immunohistochemistry, western blot and real time PCR.

Immunohistochemistry

By using scalpel, 4–5 biopsies of villous parenchyma (1 cm^3 each) from the central and marginal regions of part of the placental disc are collected. Tissue fragments from the placenta consisting of homogeneous villous tissues were cut longitudinally from the maternal side to the foetal side and infarct areas were excluded from the study. Expression of Anx A1 and Gal-3 was analysed in 60 placental villous tissues. 3 µm thick sections were obtained from formalin fixed and paraffin embedded placental tissues. The sections were treated according to standard Immunohistochemical staining procedure for the detection of protein.

The endogenous peroxidase activity was blocked by incubating the tissue with 0.3% hydrogen peroxide. Non-specific binding sites were blocked by incubating the sections with normal horse serum (vector laboratories) and then incubated with primary antibody against AnxA1 (DIL 1:100, Santa Cruz Biotechnology Inc; Santa Cruz, CA catalogue No. SC-12740) and Gal-3 (DIL1:100 Santa Cruz Biotechnology Inc; Santa Cruz, CA catalogue No. SC-23938). This was followed by sequentially incubating the sections with biotynylated secondary antibody (Vector Laboratories, Burlingame, CA) and avidin biotin horse raddish peroxidase complex (ABC; VECTOR). The antigen of interest was detected by use of a 3, 31-diamino benzidine (DAB) chromogen and by counterstaining with haematoxylin.The primary antibody was replaced by anti-rabbit immunoglobulin G (IgG) whole molecule (Sigma-Aldrich, USA, Catalogue No. A0545), at 1:1000 dilution, as negative control, while triple negative breast cancer cell sections were used as positive controls. The tissues were evaluated under light microscope with Lieca image Centre. The intensity and localization of the staining reaction in syncytiotrophoblasts membrane chorionic villous stromal cells, and villous vascular endothelial cells and was assessed by using semiquantitative immunoreactive score

(IRS) and all the samples were blinded. The IRS was derived by multiplication of staining intensity graded (as 0 negative, 1 weak, 2 moderate, and 3 strong staining) and percentage of positively stained cells (0 = no staining, 1–10% as 1, 11–50% as 2, 51–70% as 3, 71–100% as 4). The localization of AnxA1 and Gal-3 protein was counted in 10 random fields in placental villi.

Western Blot Analysis

Human placental bed samples were homogenized at 4°C in 500 µL RIPA lysis buffer. The lysates were centrifuged at 14,000 rpm at 4°C for 45 min to remove the cell debris. Bicinchonic acid assay (BCA assay) was used to determine the protein concentrations. Whole cell lysates (40 µg) were subjected to SDS-PAGE using Tris–Hcl buffer and the proteins were transferred to nitrocellulose membranes (Himedia) using a transfer apparatus at 65 V for 90 min. The antibodies were used against AnxA1 (Mouse monoclonal, BD Biosciences, CA-12740), Gal-3 (Santa Cruz Biotechnology, SC-23938) and GAPDH (Santa Cruz Biotechnology, SC-166574). Appropriate secondary antibodies conjugated to horseradish peroxidase (BioRad) were incubated with respective membranes for 2 h at room temperature. The membranes were developed using ECL plus (BioRad) and the image was captured using enhanced Chemiluminescence system, G: BOX Chemi XX6/XX9. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) housekeeping gene was used as an internal control for loading. The densities of protein bands were determined with Image J, version 1.35d.

RNA Preparation, RT-PCR and Real-Time PCR

Total RNA was extracted using Trizol reagent (Thermofisher scientific invitrogen). Complementary DNA (cDNA) was synthesized from 2 µg of total RNA by using Takara cDNA synthesis kit using a random hexamer at 42°C for 1 h. Template cDNA was subjected to PCR amplification using gene-specific sense and antisense primers Annexin A1 (forward primer: 5′-ATCAGCGGTGA GCCCCTATC-3′ reverse primer 5′-TTCATCCAGGG GCTTTCCTG-3′), Galectin-3 (forward primer 5′-5CAA TACAAAGCTGGATAATAACTGG-3' reverse primer 5′-GATTGTACTGCAACAAGTGAG-3) and reference gene β actin (forward primer 5′-GGGAAATCGTGCGT GACATTAAG-3′, reverse primer 5′-TGTGTTGGCGTA CAGGTCTTTG-3′) were generated (Juniper life sciences) RT-PCR conditions were at 95 °C for 5 min, followed by 40 cycles of 95 °C for 30 s, annealing at 60 °C for 30 s, and extension at 72 °C for 30 s in a thermal cycle (Quant Studio 5 by Applied Bio systems). The quantitative amount of each gene was standardized against the house-keeping gene β-actin. The RNA levels were expressed as a ratio, using the 'delta-delta' method for comparing the relative expression results between normotensive control and patients with PE.

Statistical Analysis

All statistical analysis was carried out by using Graph Pad Prism version 7.04. Results for normally distributed data were shown as ± SD. Statistical analysis of expression of AnxA1 and Gal-3 for IHC was carried out with the Mann–Whitney U-test. Spearman correlation coefficients were used to detect correlation between AnxA1 and Gal-3 expression. "*P* value" less than 0.05 were considered to be statistically significant. Results for normally distributed data were analysed using student t test.

Results

The Demographic characteristics of the normotensive women and preeclamptic patients are shown in Table 1. There were no statistical differences between the PE and normotensive control groups with respect to their age, BMI, neonatal gender. Almost all the deliveries in the control group were at full term. Compared to normal control group the mean gestational age is shorter in PE group. In patients with PE, when compared with the normotensive control group, birth weight of the baby is reduced and the systolic and diastolic blood pressures were significantly higher ($P < 0.05$).

Histopathological Changes in Placenta of Preeclampsia

Microscopic examination revealed the increase in the number, density and volume of villi in PE compared to controls. But villous surface area and diameter of villi reduced in PE compared to controls. Intervillous space volume is reduced in PE. The endothelial cells of the tunica intima were activated with a swollen morphology in the PE group, whereas the endothelial cells of the tunica intima in the control group appeared normal with a flattened morphology. This activation of endothelial cells means that the cells were damaged. Other villous abnormality observed in PE placenta was increased stromal fibrosis, fibrinoid necrosis, thickening of syncytiotrophoblast basement membrane and increased syncytial knot formation (Fig. 1). All the change observed in PE placenta may be response of the placenta due to inflammation and disturbance in the blood flow.

Table 1 Demographic characters in preeclampsia (PE) and normotensives

Clinical data	Normotensives (n = 30)	Preeclampsia (n = 30)	P value
Age (years)	25.9 ± 3.9	24.93 ± 2.7	0.275
Gestational age	38.73 ± 0.94	35.07 ± 1.87	0.000*
BMI (kg/m2)	25.94 ± 2.01	25.83 ± 2.1	0.851
Gravid	2.0 ± 0.78	1 ± 0.0	0.000*
Parity	1.27 ± 0.45	1 ± 0.0	0.002*
Birth weight	2.94 ± 0.29	2.47 ± 0.35	0.000*
SBP (mm Hg)	105.9 ± 8.66	153.9 ± 10.71	0.000*
DBP (mm Hg)	71.67 ± 6.08	96.13 ± 7.4	0.000*
Platelet s (10^3/μL)	2.67 ± 0.21	2.66 ± 0.41	0.887

Statistical analysis is carried out by Student's t test (*P < 0.05). Above data is expressed as Mean ± SD

BMI Body mass index, *SBP* systolic blood pressure, *DBP* diastolic blood pressure

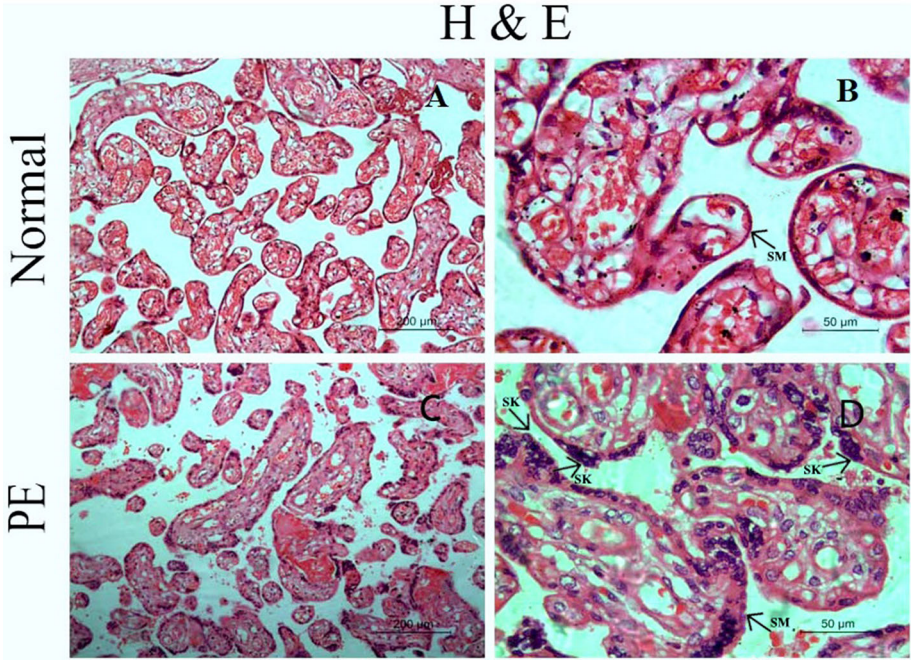

Fig. 1 **a**, **b**: H and E staining showing the morphology of normal placenta, **b**: black arrow shows the normal thickness of syncytiotrophoblast membrane (SM), **c**, **d**: H and E staining showing the morphology of PE placenta, **d**: increased thickness of syncytiotrophoblast membrane (SM) and increased syncytial knot (SK) formation. Magnification 10× = 200 μm and 40× = 50 μm

Expression of Annexin A1 and Galectin 3 in Placental Bed

Immunostaining of placental bed sections confirmed the increased expression of AnxA1 and Gal-3 in PE group compared to the normotensive control group. Several different cell types in both placental bed biopsies of the PE and normotensive control groups were positive for AnxA1 and Gal-3 including of syncyotiotrophoblastic cells, chorionic villous stromal cells, and villous vascular endothelial cells. Expression in placental villous tissues was semi-quantified (Table 2). In preeclamptic placentas, immunostaining was strong and located predominantly in the syncytiotrophoblasts and mild staining was observed in villous stromal cells and villous vascular endothelial cells. In normal placenta moderate staining was obtained with AnxA1 and Gal-3 primary antibodies (Fig. 2).

The technique of Western blot and RT-PCR was performed because the technology of immunohistochemistry does not impart itself to quantification. In western blot AnxA1 expression is increased in PE placenta by 3.2-fold ($P = 0.011$) while Gal-3 is increased by 3.14 fold ($P = 0.031$) compared to normal placenta.

Relative mRNA expression of AnxA1 and Gal-3 was increased in placenta in PE placenta compared to controls ($P = 0.0001$ and 0.035 respectively). Levels of mRNAs are expressed as arbitrary units. Unpaired t test is used to evaluate the potential difference (Figs. 3, 4). We also studied the statistical spearman correlation of expression level of AnxA1 and Gal-3 as both proteins are involved in modulation of inflammation. A statistically significant

Table 2 Localisation and Immunostaining intensity of AnxA1 and Gal-3 expression in placental villous tissues

	AnxA1				Gal-3		
	SCORE	STM	CVSC	VVEC	STM	CVSC	VVEC
Normal (n = 30)	0	0	4 (13)	4 (13)	0	11 (36)	9 (30)
	1+	7 (23)	20 (66)	21(70)	17 (56)	14 (46)	21 (70)
	2+	21 (70)	6 (20)	5 (16)	12 (40)	5 (16)	0
	3+	2 (6)	0	0	1 (3)	0	0
PE (n = 30)	0	0	1 (3)	1 (3)	0	7 (23)	7 (23)
	1+	0	7 (23)	17 (56)	7 (23)	15 (50)	20 (66)
	2+	15 (50)	22 (73)	12 (40)	14 (46)	8 (26)	3 (10)
	3+	15 (50)	0	0	9 (30)	0	0
P value		0.000*	0.000*	0.000*	0.000*	0.140	0.000*

Statistical analysis of expression of AnxA1 and Gal-3 for IHC was carried out with the Mann–Whitney U-test (*$P < 0.05$)

Immunohistochemical staining for AnxA1, 0 = no staining, 1–10% as 1+ , 11–50% as 2+ , 51–70% as 3+ , 71–100% as 4 + . (Numbers in bracket are in the percentage)

STM Syncytiotrophoblast membrane, *CVSC* chorionic villous stromal cells, *VSMC* vascular smooth muscle cell, *VVEC* villous vascular endothelial cells

correlation in the expression of AnxA1 and Gal-3 was observed in syncytiotrophoblast membrane ($P < 0.0048$).

Discussion

This study showed increased expressions of AnxA1 and Gal-3 in the third trimester placental bed from pregnancies with PE compared with the normotensive control group. The result of western blotting and Real time PCR in the study revealed an increased expression of AnxA1 and Gal-3 in the preeclamptic placental samples compared to normotensive placenta. The increased expression of protein in different placental compartment was confirmed by immunohistochemistry analysis. Immunohistochemistry findings revealed protein is expressed strongly in syncytiotrophoblast layer of the preeclamptic placental villous in comparison to controls. Expression of AnxA1 has been thoroughly studied in models of sterile inflammation, recognising its central role as key modulator of both of the innate and adaptive immune systems [11]. However, in the context of Preeclampsia only one study have been reported on the altered expression of AnxA1 in the plasma [12]. Very little is known about the expression status of Annexin A1 in PE. Our study shows the differential expression of AnxA1 in the placenta of normotensive and preeclamptic women. This result was consistent with the study by Perucci et al. [12] who measured the protein in the plasma [12]. This result implies that the increase AnxA1 expression in PE placental bed could be an important factor in the aetiology of PE. In normal pregnancy it is established that there is a homeostatic balance between inflammatory and regulatory response [13], which suggests that regulatory molecular mechanisms are sufficient to reduce the mild inflammatory response. Preeclampsia is associated with chronic activation of immune system which leads to an increased production of inflammatory cytokines by pro-inflammatory T cells, and a decrease in regulatory and anti-inflammatory cytokines, which further promotes an inflammatory state during PE [14, 15]. In preeclamptic pregnancy, the imbalance between pro-inflammatory and regulatory cytokines is correlated with placental ischemia. This imbalance exacerbates as the pregnancy progresses [16]. AnxA1 has been shown to be capable of regulating a large number of biological events such as chronic inflammation, growth of the tissue, and programmed cell death. It has been shown that the decreased expression of AnxA1 is associated with the development of more severe inflammation in inflammatory diseases [17]. Additionally, AnxA1 helps in monocyte augmentation and elimination of apoptotic leukocytes by macrophages, resulting in reduced production of pro-inflammatory cytokines and increased release of immunosuppressive and pro-resolving molecules [18].

Our data may imply that AnxA1 is increased in patients with preeclampsia in an attempt to attenuate the exacerbated inflammatory response in these patients. Chronic inflammation in PE suggests that the resolution of inflammation pathway is dysfunctional. Consequently increased AnxA1 expression seems to be inadequate to resolve inflammation. In other chronic inflammatory disease such as inflammatory bowel disease and Alzheimer's disease systemic levels of proresolving mediators are increased [19]. In acute inflammatory response during the initiation phase, mediators derived from arachidonic acid become up-regulated and contribute to changes in vascular

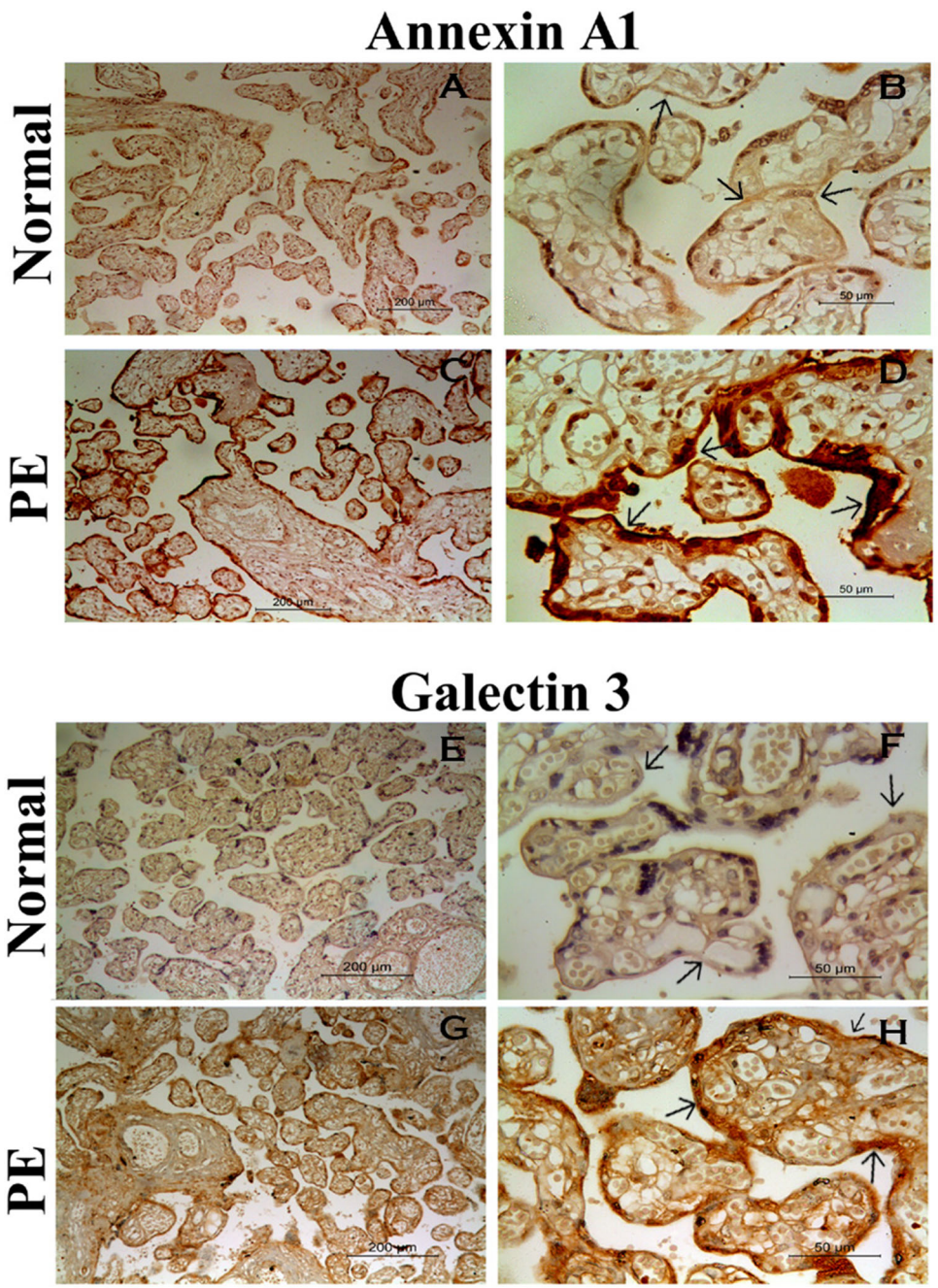

Fig. 2 Representative photomicrograph showing the expression of AnxA1 and Gal-3 in placental villi of normal and PE placenta under 10× and 40× respectively. **A**, **B**: note moderate AnxA1 immunostaining predominantly in the syncytiotrophoblastic membrane of normal placenta as indicated by black arrow, **C**, **D**: strong AnxA1 immunostaining in PE placenta, **E**, **F**: moderate membranous Gal-3 immunostaining in syncytiotrophoblast of normal placenta, **G**, **H**: intense Gal-3 staining in PE placenta. Bright-field microscopy images, representative of n = 30 per group. Mann–Whitney U-test was used to evaluate potential difference ($P < 0.05$)

permeability and Polymorphonuclear leukocytes recruitment. However, the generation of these pro-inflammatory mediators in due course terminated by successive dynamic changes in prostaglandins E2 and D2 [20]. This can be seen as switch where elevated levels of pro resolving mediators, including pro-resolving lipoxins A4 decreases inflammatory molecules such as prostaglandins, leukotrienes, and cytokines [21]. Pro-resolving and anti-inflammatory actions of AnxA1 are mediated by a G-protein-coupled receptor named formyl peptide receptor like-2 (FPR2)/lipoxin A4 receptor (ALXR) [22]. Decreased ALX expression has been observed in patients with asthma, a chronic inflammatory disease [23]. These mechanisms might explain the noticeable ineffectiveness of AnxA1 up-regulation in some human inflammatory diseases. More studies are required to solve the mystery whether these dysfunctional mechanisms in AnxA1 resolution pathway are present in PE. Proteins from galectin family have emerged as master regulators of immune system homeostasis, playing central role in the amplification and/or resolution of inflammatory processes. Gal-3 functions as pro- or anti-inflammatory activities depending on various

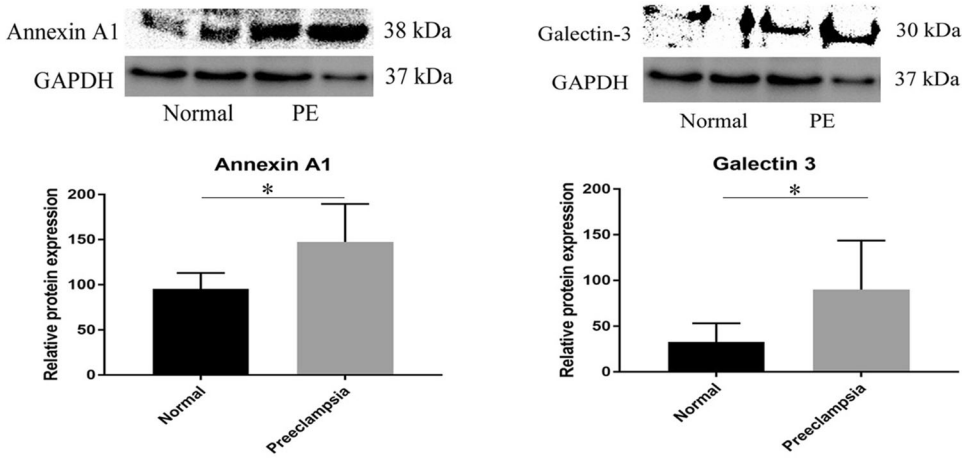

Fig. 3 Western blot analysis of AnxA1 and Gal-3 protein in Normal and Preeclamptic placenta. Expression levels were confirmed by densitometry. AnxA1 and Gal3 expression was significantly increased in PE placentas compared to normal placenta. Unpaired t test is used to evaluate the potential difference ($P < 0.05$)

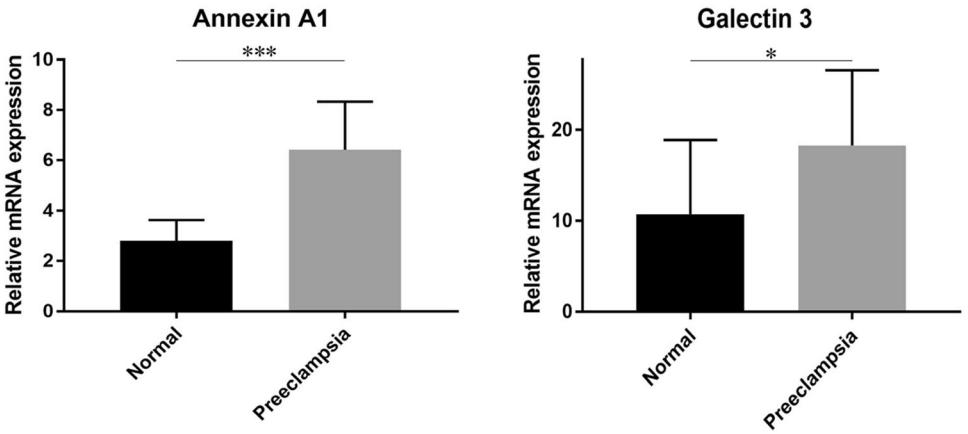

Fig. 4 Expression of Annexin A1 and Gal-3 mRNA in placental tissues as determined by RT-PCR. Levels of mRNAs are expressed as arbitrary units. Unpaired t test is used to evaluate the potential difference (*$P < 0.05$, ***$P < 0.001$)

factors including its intracellular or extracellular localization and the target cell involved in these processes [8]. Even though it may contribute to resolution of inflammation by clearing apoptotic neutrophils [9] this lectin exhibits mostly pro-inflammatory effects by increasing activation of macrophages, mast cells, and natural killer cells, as well as T and B lymphocytes [24]. Studies have shown that Gal-3 has a role in implantation of embryo, embryogenesis and placental formation, and is closely connected with the success and maintenance of pregnancy [25]. Proliferation and programmed cell death are crucial components of the trophoblast life cycle. There are aberrant cell turnover including an increased apoptosis in placental villous trophoblast of preeclamptic pregnancies [26]. Numerous studies suggest that high expression of Gal-3 exerts regulatory effects on apoptotic responses of various cell types [27]. Our results may suggest that the marked increase in expression of Gal-3 in the syncytiotrophoblast cells in preeclamptic placenta could be important to turn on the intracellular machinery of these cells required for defence against a rapid process of apoptosis. In our earlier study, we have reported the interaction of Gal-3 and Annexin A2 resulting in the cancer progression in Triple negative breast cancer cells [28]. Annexin A2 is the pro-inflammatory molecule and AnxA1 being the anti-inflammatory, which are reciprocally regulated [29]. As far as PE is concerned, Annexin A2, a (proinflammatory fibrinolytic) protein level decreases and which should result in simultaneous increase in AnxA1 and which we are seeing in our current data. With respect to Annexin A2 and AnxA1 reciprocal regulation, but increase in Gal-3 in preeclampsia reveals that it could be the apoptotic activity of the protein not the proliferative function which associates with Annexin A2 is the causative factor in preeclampsia. Present study data illustrates that, there is a significant positive correlation in the expression of AnxA1 and Gal-3.These facts presumably indicate that expression of these two factors may be dependent and regulated in the placental bed in response to the same pathogenic stimulator such as inflammation. ANXA1 and Gal-3 undergo changes in their content and localization when neutrophil adheres to the endothelium, and this could be indicative of a process of favouring and counter-balancing between two endogenous anti- and pro-inflammatory mediators [30].

Conclusion

The increased expression of AnxA1 and Gal-3 in placental bed may be associated with an altered systemic inflammatory response in PE, suggesting role of AnxA1 and Gal-3 in PE pathogenesis. Although more studies are needed to clarify the role of these inflammation modulatory proteins and other pro-resolving molecules in the context of the systemic inflammatory response in preeclampsia Additionally, further studies performed over the course of gestation are needed to confirm the role of these proteins in the development of preeclampsia and to determine whether assessment of these proteins may be a good predictive marker for the management of preeclampsia.

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Author contributions Conceived the idea and designed the experiments: PKS and KR; Helped in clinical sample collection and correlated the clinical relevance to the study: KR, PKS, MA and VK; Performed the experiments: KR, SE, AB, and RS; Analysed the data: USD, KR, PKS, PP, AB and VK; Manuscript preparation: KR, PKS and PP; Supervised the overall study: PKS and MA.

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Compliance with Ethical Standards

Conflict of Interest The author declare that they have no conflict of interest.

Ethical Approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. The study was approved by the Ethical Committee of SDM College of Medical Sciences and Hospital, Dharwad, Karnataka, India (SDM IEC: 0748: 2016).

Informed Consent Informed consent was obtained from all individual participants included in the study.

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