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ADRENOMEDULLIN (ADM) AND SOLUBLE FMS-LIKE TYROSINE KINASE-1 (sFlt-1) EXPRESSION IN PLACENTAS WITH GESTATIONAL DIABETES MELLITUS

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Abstract

Aim: In this study, we aimed to examine the expression levels of Adrenomedullin (ADM) and soluble fms-like tyrosine kinase-1 (sFlt-1), two novel proteins found to be involved in vascular regulation in gestational diabetes mellitus (GDM), to compare the expression levels of these proteins in the histopathology of the disease and to observe the correlation of the expression intensity of these proteins with the disease.

Methods: In our study, 20 healthy and 20 GDM placenta samples were obtained. Histologic follow-up was performed. 5µm thick sections were taken from these tissues and stained with Hematoxylin-Eosin and Periodic Acid Schiff (PAS). Immunohistochemically, ADM and sFlt-1 antibodies were studied.

Results: In the GDM group, vascular dilatation and congestion in stem villus, hyperplasic endothelial cells, and increased syncytial bridges in the external part of the villi, mononuclear cell infiltration, pyknotic nuclei and cytoplasm loss in some of the decidual cells in the maternal region were observed. In the immunohistochemical examination, cytotrophoblast and syncytiotrophoblast cells of villous and syncytial nodes showed negative ADM expression. ADM was positively expressed in some cytotrophoblast cells of small villi, vascular endothelial cells and decidual cells. In the GDM group, sFlt-1 expression was positive in endothelial cells, some Hofbauer cells of mesenchymal connective tissue, decidual cell nuclei and membranes.

Conclusions: ADM may be an important receptor in insulin metabolism to determine the glucose level because we found positive ADM expression in cytotrophoblasts and membranes of decidual cells. In addition, changes in endothelial cells of maternal and fetal regions and sFlt-1 expression in Hofbauer cells suggest that this molecule possibly plays a key role in the angiogenic effect.

Keywords: *Placenta, GDM, PAS, hematoxylin-eosin, sFlt-1, adrenomedullin*

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Introduction

Gestational diabetes mellitus is a clinical condition in which a woman without diabetes has high blood glucose levels during pregnancy. GDM is caused by insufficient insulin to regulate insulin resistance and its etiology includes being overweight, previous gestational diabetes, a family history of type 2 diabetes and polycystic ovary syndrome. GDM is diagnosed with a blood test. The screening test used as one of the universal diagnostic criteria for GDM is the 75 g oral glucose tolerance test performed at 24-28 weeks of gestation[1]. GDM affects at least one in 10 pregnant women worldwide and accounts for a range of adverse perinatal outcomes, including excessive fetal fat addition (macrosomia), fetal hypoglycemia, and the need for neonatal intensive care and mortality. Furthermore, GDM not only causes short-term complications in the newborn, but is also associated with an increased risk for chronic conditions such as cardiovascular disease, obesity and diabetes [2,3].

The placenta is a temporary organ that provides the developing fetus with nutrient intake through maternal blood, thermoregulation, waste removal, gas exchange and connects the fetus to the uterine wall via the umbilical cord. It also has functions such as providing fetal immunity and secreting hormones for the continuation of pregnancy[2]. Increased vascularization is seen in GDM placentas compared to normal pregnancies. This may be a manifestation of maternal nutritional overload but reflects the increased oxygen demand of the fetus due to increased fetal aerobic metabolism stimulated by insulin. The mechanisms underlying increased vascularization have not been fully elucidated [4,5].

Adrenomedullin is a vasodilator peptide encoded by the ADM gene in humans and acts as a hormone. It is expressed by all tissues and found in the circulation. It is thought to be involved in hypertension, myocardial infarction, chronic obstructive pulmonary disease (COPD) and other cardiovascular diseases [6,7].

Soluble fms-like tyrosine kinase-1 is a tyrosine kinase protein with anti-angiogenic properties. sFlt-1, a splice variant of sFlt-1 with no membrane binding, binds to the angiogenic factors vascular endothelial growth factor (VEGF) and placental growth factor (PlGF) and prevents blood vessel growth by reducing their free circulation. In humans, sFlt-1 is synthesized from various tissues including kidney, cornea and uterus and is important in the regulation of blood vessel formation [8,9].

The aim of this study was to examine the expression levels of ADM and sFlt-1, which are two proteins found to be involved in vascular regulation in gestational diabetes mellitus, to compare the expression levels of these proteins in the histopathology of the disease and to examine the correlation of the expression intensity of these proteins with the disease.

Materials and Methods

The study was initiated after obtaining ethics committee approval from Dicle University Faculty of Medicine Ethics Committee. In our study, placentas were obtained from a total of 40 pregnant patients (regardless of age), including 20 GDM and 20 normotensive pregnant patients, by obtaining informed consent form approval. In the study, a consent form was obtained to use the postpartum placentas of the mothers who applied to the Gynecology and Obstetrics Clinic of Dicle University Faculty of Medicine Hospital. The placentas taken from the operating room were washed with saline and then placed in 10% buffered neutral formalin under appropriate conditions for tissue tracking and brought to the laboratory of the Department of Histology and Embryology, Faculty of Medicine, Dicle University.

1x1 cm^3 pieces were taken from the placentas. Tissue pieces were transferred to 10% formalin. Fixation was completed in 16 hours. The fixed tissues were washed in tap water for 1 night. For dehydration, tissue pieces were kept in a series of alcohols (50%, 70%, 80%, 90%, 90%, 96%, 99.9%, 99.9%)

and kept in xylol for 2x15 minutes for transparency. For infiltration, they were kept in liquid paraffin for 2 hours in an oven set at 58°C and then blocked. 5µm thick sections were taken from the paraffin blocks using a rotary microtome (Leica RM2265, Germany). The sections were subjected to routine Hematoxylin-Eosin (HE) and PAS staining and immunohistochemistry with ADM and sFlt-1 antibodies.

Periodic Acid Schiff (PAS) Staining

Tissues obtained from paraffin block were deparaffinized by soaking in xylol for 2x10 minutes. Tissues were passed through decreasing alcohol series (100%, 96%, 90%, 70% ethyl alcohol) for 10 minutes and brought to distilled water for 5 minutes. Tissue sections were prepared by following the procedure of Periodic Acid Schiff (cat#04-130801, Bio Optica, Milano, 20134, Italy) ready kit and the following solutions were used.10 drops of solution A were poured onto the sections and waited for 30 minutes. Tissue sections were washed and 15 drops of solution B were poured onto the sections and waited for 10 minutes. The sections were washed in tap water in a chalice for 5 minutes and then washed in distilled water for 2 minutes.10 drops of solution C were added on the sections and kept for 10 minutes and then washed in DS. 10 drops of solution D were added on the sections and kept for 20 minutes and then washed in DS.10 drops of solution E were added on the sections and kept for 2 minutes. The solution on the tissue sections was poured without washing, 10 drops of solution F was added on them and waited for 3 minutes and then washed in DS. The sections were washed with 10 drops of solution G for 2 minutes and then washed in tap water for about 5 minutes until the blue dye was removed. Tissue sections were passed through ascending alcohol series. They were kept in absolute alcohol (100%) for 1 minute. Tissue sections were passed through Xylol series for 2x10 minutes for polishing and cleaning, then Entellan was dripped on the stained tissue, covered with a coverslip and after drying, it was made ready for microscope examination.

Immunohistochemical Staining

Immunohistochemical protocol was performed according to Aktas et al[10]. 5 µm thick sections were taken from paraffin blocks and passed through xylol and decreasing alcohol series and brought to distilled water. The sections were then washed in PBS (3x5 min). For antigen retrieval, the sections were heat treated in Ethylenediamine tetraacetic acid (EDTA) solution. The sections were washed in PBS and outlined with a delimiter pen. Sections were incubated with 3% H2O2 (w/Methanol) for 30 minutes at room temperature for endogenous peroxidase blockade. Goat Serum Blocking solution was added to the sections washed in PBS and incubated for 9 minutes at room temperature. After protein blocking, Soluble Fms-like tyrosine kinase-1 (Abcam, cat#ab9540, Cambridge, MA 02139- 1517, USA) and Adrenomedullin (Abcam, cat#ab69117, Cambridge, MA 02139- 1517, USA) primary antibodies diluted in PBS were added. Dilution of both antibodies was performed 1/100 in PBS. The primary antibody drop sections were incubated at +4°C overnight. At the end of incubation, the sections were washed in PBS for 3x5 min. Biotinylated secondary antibody drops were kept at room temperature for 14 minutes. After the sections were washed in PBS for 3x5 min, Streptavidin-peroxidase was dripped on them and kept for 15 minutes. Then the samples were washed with PBS for 3x5 minutes. DAB was added to the sections and kept for 10-15 minutes. The samples were washed free of DAB and counterstained with Mayer hematoxylin for 45 seconds and washed in tap water for 5 minutes. Finally, the sections were rapidly passed through the increasing alcohol series, kept in xylol for 2x15 minutes and covered with entellan. The preparations were examined under a light microscope using Zeiss Imager A2 and Zen 3.00 software program.

Results

Histopathologic Findings

In the control group hematoxylin-eosin staining, it was observed that the decidual cells in the maternal placenta had a polyhedric appearance and the nuclei were rich in chromatin. In the mesenchymal tissue, collagen fibers were parallel to each other, irregularly distributed and connective tissue cells were solitary (Figure 1a). In the fetal area of the control group, villi, small capillary vessels had normal morphology and connective tissue cells and Hofbauer cells were localized regularly and solitary. Pyknotic nuclei, loss of cytoplasm and thickening of the membrane were observed in the GDM group decidual cells. Dilatation and congestion of blood vessels in the root villi of the GDM group were prominent, hyperplasia of endothelial cells, increase in syncytial bridges and nodes in the outer part of the villi, and mononuclear cell infiltration were observed (Figure 1b). Occasional vacuolar areas were detected in the mesenchymal connective tissue. In the control group, PAS staining showed PAS positive reaction in the cytotrophoblastic basement membrane layer and connective tissue collagens and blood vessels (Figure 1c). In the GDM group, thickening of the membrane in the syncytial regions of the villi and PAS positive reaction was observed in the capillary vessel membrane inside the small villi. PAS positive fibrinoid areas were observed in the outer part of decidual plaques. In addition, PAS positive reaction was detected in some collagen fibers within the mesenchymal connective tissue area and numerous vacuolar structures were observed within the villi (Figure 1d).

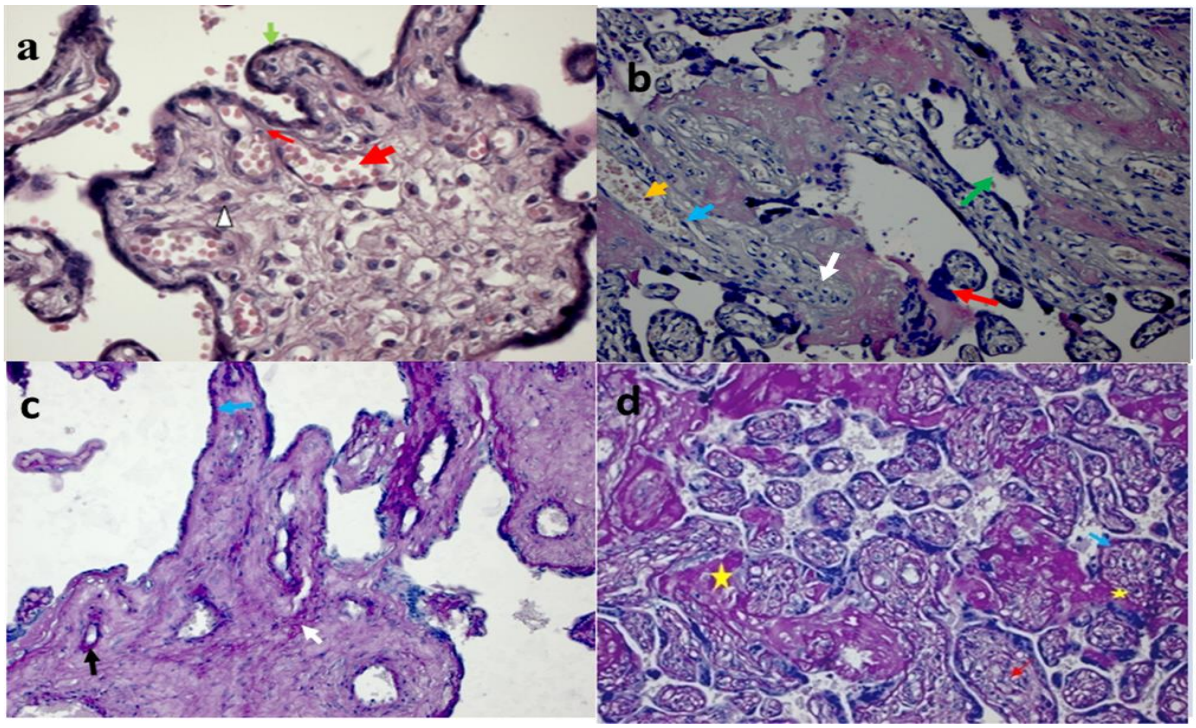

Figure 1. Histopathologic appearance of placenta samples.
a) Syncytiotrophoblasts (green arrow), normal looking villous capillaries (red arrow) and embryonal connective tissue elements were observed in the fetal placenta of the control group (Staining: H&E, x40), b) Root villi of the GDM group showed vascular dilatation (yellow arrow), endothelial hyperplasia (blue arrow), increased syncytial bridges (red arrow), mononuclear cell infiltration (white arrow) and increased syncytial nodes (green arrow) (Staining: H&E, x40), c) PAS positivity was observed in cytotrophoblast basement membrane (blue arrow), capillary basement membrane (black arrow) and occasional connective tissue collagen (white arrow) in control group PAS samples (Staining: PAS, x20), d) GDM group fetal placenta shows thickening of villus basement membrane (blue arrow), PAS positive fibrinoid structures in mesenchymal connective tissue (yellow star), vacuolized structures in villi (red arrow) Staining: PAS, x20.

Immunohistochemical Findings

Control group in immunohistochemical staining, sFlt-1 expression was occasionally positive in the endothelial cells in the blood vessels of the sections, while expression was generally negative in the endothelial cells (Figure 2a). Some small diameter capillaries in the mesenchymal connective tissue showed mild positive sFlt-1 expression. In GDM group samples, positive sFlt-1 expression was observed in the endothelial cells of the arteiole, a branch of the umbilical cord artery in the maternal region, villous Hofbauer cells, decidual cell nuclei and membranes, endothelial cells in the capillary vessels expanding in the villus and cells in the mesenchymal connective tissue. Negative expression was observed in muscle cells (Figure 2b). In control group ADM samples, ADM positive signals were observed in arteriole endothelial cells in the maternal region, fibroblasts in connective tissue, some collagen fibers, syncytial cells of root villi and a few decidual cell nuclei. In contrast, ADM expression was negative in placental muscle cells, membranes of decidual cells and most of the nuclei of decidual cells (Figure 2c). In the mesenchymal connective tissue of the root villi of the GDM group, ADM expression was positive in the cells around the vessels, endothelial cells in the membranes of capillary vessels, some cytotrophoblast cells of small villi, and some mesenchymal cells in the vascular endothelium and outside the vessels. In addition, intense ADM expression was detected in the membranes of decidual cells. On the other hand, ADM expression was negative in the syncytio and cytotrophoblast cells of the villi, syncytial bridge and syncytial nodes at the junction of small villi and in the nuclei of the decidua (Figure 2d).

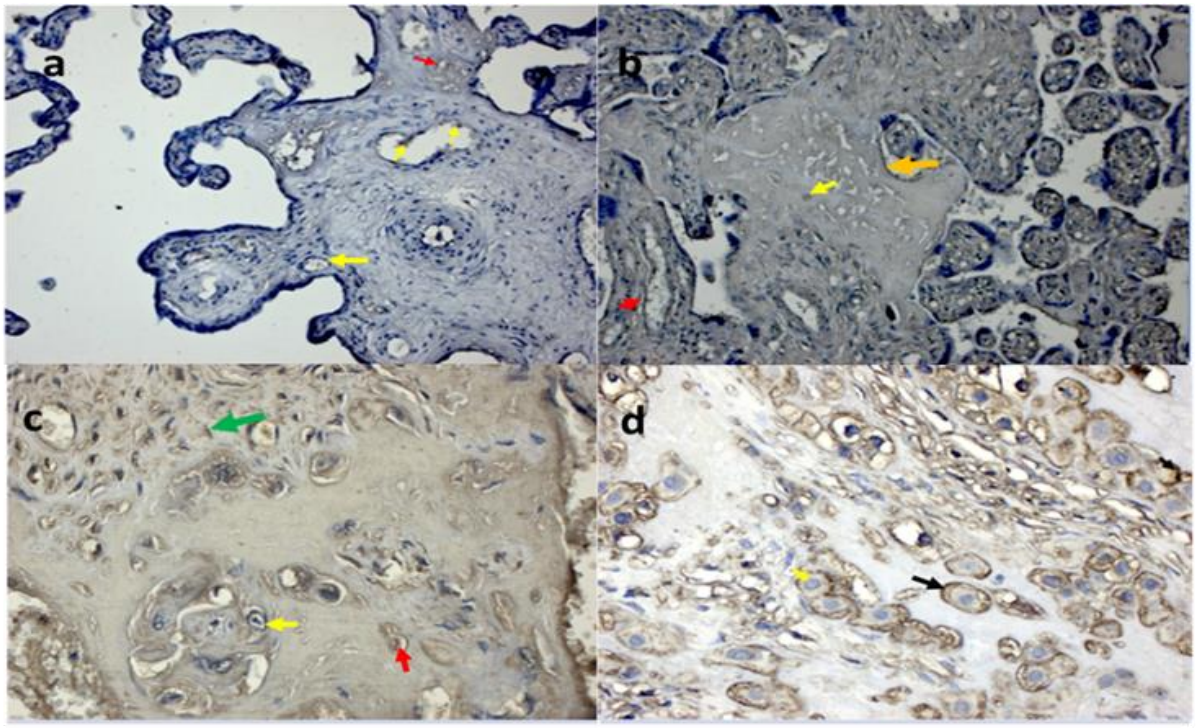

Figure 2. sFlt-1 and ADM immunohistochemistry.
a) Control group sFlt-1 positive capillary endothelium (yellow arrow) and connective tissue cells (red arrow) (Staining: sFlt-1 immunohistochemistry x20),
b) GDM group placenta section shows positive staining in decidua cells (yellow arrow), capillary membranes (red arrow) and syncytial areas (orange arrow) (Staining: sFlt-1 immunohistochemistry x20),
c) In control group placentas, the nuclei of the decidua were ADM negative (yellow arrow), while some decidua cells (red arrow) and fibroblasts (green arrow) showed positive reaction (Staining: ADM immunohistochemistry x40),
d) GDM group placentas showed negative signals in the nuclei of decidua cells (yellow arrow) but positive signals in the cell membrane (black arrow) Staining: ADM immunohistochemistry x40.

Discussion

Gestational diabetes mellitus is a common metabolic disease characterized by chronic hyperglycemia and is a risk factor for both mother and fetus during pregnancy[11]. Gestational diabetes mellitus is a tolerance disorder that develops due to glucose disorder during pregnancy. In gestational diabetes pregnancies, blood glucose levels should be around 72-126 mg/dl to avoid hypoglycemia during delivery. Patients diagnosed with gestational diabetes before the 24th week of gestation have a higher risk of developing diabetes in the future[12]. It has been shown that maternal obesity and adipose tissue increase in GDM are related with inflammation and it has been reported that there is a relationship between insulin resistance and diabetic pregnancies caused by secreted cytokines[13,14].

In histopathological examination of gestational diabetes, it has been described that there are immature villi, proliferation of stromal fibroblast cells and hafbouer cells, degeneration of cytotrophoblast cells and thickening of the basement membrane with changes such as syncytial nodes and villus edema[15,16]. In a study by Desoye G et al.[17], it was reported that histopathologic changes occurring in early periods in diabetic mothers caused high rates of perinatal mortality, and fetal hypoxia and fetal pulmonary failure developed due to insulin index. In our study, degeneration in decidual cells, increased intercellular collagen fibers and fibrinoid accumulation were observed in the maternal region of the placenta. In the root villi, dilatation of vessels and significant increases in collagen fibers, syncytial bridges and nodes were observed.

In a study by Sela et al.[16] a high rate of glycogen accumulation was observed in stromal cells of diabetic placentas. Asmusen[18] and Gheorman[19] reported that basement membrane thickness showed PAS(+) reaction in terminal villi and in areas where syncytial cells were localized and PAS (+) reaction was higher in diabetic placentas compared to normal placentas. In our study, PAS (+) reaction was observed in the outer part of the decidual plaques in the maternal region with thickening of the membrane containing syncytial cells in fibrinoid areas. A positive PAS reaction was observed in the basement membranes, syncytial bridges and nodes in the inner part of the small villi. It was concluded that PAS reaction was higher in diabetic placentas compared to normotensive placentas, glycogen accumulation in membranes and cell cytoplasms was higher and may induce complications that may develop.

Soluble fms-like tyrosine kinase-1 acts as an antiangiogenic protein in the extracellular space as a vascular endothelial receptor and in the circulation as an endothelial growth factor and by antagonizing the effects of placental growth factor. sFlt-1 has also been named as Vascular Endothelial Growth Factor (VEGF) receptor or VEGFR1[20]. In a study by Jonata et al.[21], when patients with high hyperglycemia index were compared with normotensive patients, it was observed that VEGF and VEGF2 receptor levels were high, but VEGF receptor-1 (sFlt-1) levels were decreased. However, the determination of VEGF1 levels in placentas with GDM has not been clearly stated. In a study by Dong et al.[6], decreased levels of sFlt-1 in placentas with preeclampsia were thought to cause endothelial dysfunction and trigger preeclampsia. In our study, sFlt-1 expression in blood vessel endothelial cells within the root villi in the maternal region in normotensive placenta was positive in some regions and negative in others. Low levels of sFlt-1 expression were detected in mesenchymal connective tissue. While negative sFlt-1 expression was observed in the muscle layer of the vessels, positive sFlt-1 expression was observed in mesenchymal connective tissue cells. One of the remarkable features of our study is that Hofbauer cells in the mesenchymal connective tissue within the chorionic villi showed positive sFlt-1 expression, suggesting that it may be a sign that induces angiogenesis.

The role of adrenomedullin in gestational diabetes and its role in impaired insulin production leads to elevation of beta cells in the pancreas, and adverse pancreatic beta cell adaptation during pregnancy has been reported to

play an important role in the pathology of gestational diabetes[22]. Di Iorio et al.[7] investigated the effects of adrenomedullin, a hypotensive peptide in the insulin regulatory system, in maternal and fetal placenta and reported that ADM production in diabetic pregnancy was important in preventing vasoconstriction of placental vessels. Maynard et al.[23] reported that elevated glucose levels in the early stages of pregnancy may have a stimulatory effect for upregulated adrenomedullin receptors in adipose tissue and that ADM regulation in GDM patients, especially in adipose tissue, is sensitive to glucose. In our study, although some capillary vessels in the maternal region in the GDM group appeared to be vasoconstricted in the maternal region, it was observed that vasoconstriction continued in the medium type and small type vessels in the maternal and fetal region in general. Kanensihi et al.[24] found that ADM expression was lower in preeclampsia patients compared to normal placentas, especially in villi, amnion membrane and syncytiotrophoblasts.

Conclusion

Gestational diabetes mellitus is an important disease with increasing incidence during pregnancy and is associated with both perinatal and long-term morbidity for the mother and fetus. During the comparison of placental tissues with a high hyperglycemia index with normal placentas, degenerative changes in decidua cells, which are considered glucose precursors in the maternal region, thickening of basement membranes, fibrin deposition, and an increase in syncytial nodes and bridges in chorionic villi induced circulatory disorders between mother and fetus. Decreased and increased levels of VEGFR1/sFlt-1 in maternal and fetal areas of the placenta, as well as increased expression of Hofbauer cells, which have macrophage characteristics, suggest that this molecule may play a key role in the angiogenetic effect. When the effects of ADM, an insulin-regulating hypotensive peptide, on maternal and fetal placenta were examined, it was found that ADM was positively expressed especially in the membranes of decidual cells in GDM placentas, suggesting that these receptors are effective in determining glucose levels. In addition, ADM expression in cytotrophoblasts suggests that cytotrophoblasts may be involved in the insulin regulatory system.

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

Ethical approval
Ethical approval was obtained from Dicle University Faculty of Medicine Ethics Committee with the protocol number (2018/266).

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