Impact of 5-Azacytidine on placental weight, glycoprotein pattern and proliferating cell nuclear antigen expression in rat placenta

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The Impact of 5-azacytidine on Placental Weight, Glycoprotein Pattern and Proliferating Cell Nuclear Antigen Expression in Rat Placenta

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Abstract

In process of placentation, expression of various glycoproteins has important role in embryonal development. Alterations of DNA methylation caused by 5-azacytidine (5azaC) can disturb normal glycoproteins expression as well as proliferative ability of trophoblast cells. In order to assess that, a single dose of 5azaC was injected intraperitoneally to pregnant rats during the day 1 to 19 of gestation. Animals were sacrificed on the day 20 and placental weights as well as glycoprotein composition were analyzed together with immunohistological assessment the of degree of trophoblast cells proliferation. The placental weight was found significantly smaller in animals treated by 5azaC on the days 4 to 14 of gestation ($p<0.01$, Student's *t* test). The treatment on days 4, 5 and 6 resulted in the lack of labyrinth with the strong proliferative activity of the cells in the basal layer. Expression of glycoproteins with molecular mass smaller than 60 kD was reduced on day 6. The 5azaC treatment from day 7 to 10 completely disturbs placental structure and the proliferation of trophoblast cells is poor. During these days GP70 exhibits stronger expression in the treated animals, contrary to GP40 which is stronger in controls. Natural border between labyrinth and the basal layer was established on the days 11 and 12. The basal layer is dominant with lower proliferation of trofoblast cells compared to the controls. With establishment of labyrinth on the day 13, the expression of GP40 was restored. Proliferation of trophoblast cells from day 13 to 15 was higher compared to the controls. The changes of placental mass, proliferative ability of trophoblast cells in rat placenta exposed to 5azaC, represents another proof of the importance of epigenetics in regulation of placental development.

Keywords: Rat; Placenta; Glycoproteins; PCNA; 5-azacytidine

INTRODUCTION

Normal embryonic human development is considered as one among contemporary medicine hottest topics. It is impossible to imagine such development without synchronized cooperation of embryo and placenta [1].

The rat placenta represents one of the most convenient models for study of the molecules and their interactions in processes of human implantation and placentation. It resembles the human placenta in its many characteristics [2]. While the human placenta is villous, the rodent placenta is of labyrinth type, but both of them are haemochorial [3].

Any deviation in gene expression can bring about the significant changes of the placenta, being potentially important for ongoing pregnancy [4]. Considering mammals, gene expression in most cases is epigenetically regulated [5]. The modification of mammalian DNA molecule may consequently change the gene expression at the level of transcription being called DNA methylation [6]. Numerous genes whose methylation appears to be crucial for normal development of mammalian placenta are known. Mash2 is one of them. It is responsible for coding the transcription factor and it is indispensable for the maintenance of trophoblast stem cells [7]. The methylation process is of significant importance for development of the rat placenta. The placental basal layer displays different pattern of methylation from labyrinth layer, suggesting that the normal differentiation of placenta is regulated by precise mechanisms of DNA methylation [8].

In order to investigate the influence of hypomethylation on development of the rat placenta we used demethylating agent 5-azacytidine (5azaC). 5azaC inhibits postreplication methylation by its incorporation into DNA which causes subsequent inhibition of DNA methyltransferase and loss of methylation followed by change in gene expression. In this study we analyzed possible changes in placental weight, proliferative ability of trophoblast cells and glycoprotein expression keeping in mind that glycoprotein expression pattern in human placenta is consistent with a potential role in implantation and placentation [9].

MATERIALS AND METHODS

During the study period (from year 2000 to 2005), we analyzed 1278 rat placentas. Study was approved by Ethical committee, School of Medicine, University of Zagreb. All placentas were analyzed in the laboratory of Department of Biology.

Placental samples

Adult Fischer female rats (three-month-old) were mated overnight with males of the same age. Vaginal plug designated day 0 of pregnancy. Two groups of animals (3 animals in each group) were established for each day of gestation (from day 1 to 19); one was treated with 5azaC (Sigma-Chemie, Deisenhofen, Germany) (study group), and the control group was treated only with phosphate buffer saline (PBS). 5azaC dissolved in PBS was administered to rats in study group by single intraperitoneal injection at concentration of 5mg/kg of body weight. Animals were sacrificed at day 20 of gestation. Immediately after isolation, placentas were weighted and frozen in liquid nitrogen for storage at -80^0 C for further analysis.

Western blotting

Glycoprotein pattern was analyzed by Western blotting with SNA, UEA-I, PHA-E and DBA lectins (Vector, Burlingame, CA, USA), at the days of gestation where placental weight differences between treated and control animals were found; as well as in placentas whose growth was not influenced by 5azaC (i.e. day 3, 15 and 17). Placentas were mechanically homogenized (at least 2 placentas from the same animal in each sample) in homogenization buffer (50 mM Tris HCl, pH 7,5; 100 mM NaCl; 1 mM EDTA) containing 1mM phenylmethylsulphonyl fluoride. The homogenates were centrifuged at 15000g for 10 minutes (4^oC) and supernatants were stored at -80^0 C. Measuring of protein content was performed by Lowry assay method. Gel electrophoresis of glycoproteins was performed in concentration gradient polyacrylamide gels (5-15 %) containing 0,1% sodium dodecylsulfate (SDS, Sigma, St. Louis, MO, USA) according to Laemmli (SDS-PAGE). The protein samples were dissolved in sample buffer (pH 6,8, Tris HCl 50mM, glycerol 10%, β-

mercaptoethanol 5%, SDS 2%, bromphenol-blue 0,1% in distilled H_2O) in ratio 1:1. Samples were denaturized for 5 minutes at 95^0C before further analysis. 40µg of total proteins per slot was used, with constant current of 40 mA. Molecular weight protein markers were loaded simultaneously.

After electrophoresis, proteins were transferred to the PVDF immobilion membrane (Millipore – Bedford, MA, USA) by the semidry blotting system (Pharmacia, Sweden) in the semidry buffer (Tris HCl 48 mM, glycin 39 mM, SDS 1,4 mM, methanol 20%) [10]. Blotting was carried out at 0,8 mA/cm^2 during a period of 60 minutes. After blotting, the part of PVDF membrane with protein markers was separated (SDS-PAGE, SDTS, low range, Bio Rad Lab, Hercules, CA, USA) and the rest of the membrane was blocked overnight with 3% bovine serum albumin (pH 7,5). Membrane was incubated with biotinylated lectins (PHA-E, UEA-I and DBA) in lectin buffer ($MgCl_2$ 1 mM, $CaCl_2$ 1 mM in TBS). Lectin-glycoprotein complexes were detected with streptavidin-alkaline phosphatase conjugate, and visualized with BCIP (5-bromo-chloro-3-indolyl phosphate) and nitro blue tetrazolium (NBT, Sigma, St. Louis, MO, USA) [11].

Histology and immunohistochemistry

Placental samples were immersed in a fixative solution containing 4% paraformaldehide at 4^0C. After fixation and dehydration, the specimens were transferred to paraffin, sectioned in 5 µm slices, deparaffinized and stained with haematoxylin and eosin. Serial sections were put on silanized slides (S 3003; Dako, Glostrup, Denmark) and air-dried for 24 hours at room temperature. Sections were routinely deparaffinized and placed in a jar filled with PBS (pH=7,4). Prior to application to the specimens, primary antibody was diluted with 0,05 mol/dm^3 Tris-HCl buffer, pH=7,6, containing 1% bovine serum albumin and labeled by mixing for 15 minutes in a solution with the biotinylation reagent, a modified biotinylated anti-mouse immunoglobulin. The blocking reagent (normal mouse serum in Tris-HCl buffer containing carrier protein and 15 mmol/dm^3 sodium azide) was added for 5 minutes to the mixture to inhibit the appearance of endogenous signal. Monoclonal Mouse Anti-PCNA, Clone PC10 (M 0879, DAKO) was diluted to 1:100. Negative control was performed by omitting the step of incubation with primary antibodies.

DAKO Animal Research kit (Peroxidase) was used for primary antibody visualization according to the manufacturer's instructions. Haematoxylin was used for counterstaining. The slides were covered with 50% glycerol in PBS.

Quantitative stereological analysis of numerical density (Nv)

Randomly selected paraffin blocks of the placenta were selected for stereological analysis. In order to evaluate immunohistochemistry Proliferating Cell Nuclear Antigen (PCNA) positive cells, five consecutive sections of the placenta were taken in a random fashion from each series. Quantitative stereological analysis of numerical density (Nv) was performed by Nikon Alphaphot binocular light microscop (Nikon, Vienna, Austria) using Weibel's multipurpose test system with 42 points (M 42) at magnification of 400x [12]. The area tested (A_t) was 0,0837 mm^2. For each investigated group the orientation/pilot stereological measurement was carried out in order to define the number of fields to be tested [12]. The numerical density of PCNA-positive cells was determined according to the point counting method [12]. Numerical density (Nv) was calculated by formula: $Nv=N/A_t \times D$, where N is number of PCNA-positive cells on tested area [13,14]. The mean tangential diameter (D) calculated by light microscopy at magnification of 400x and for 300 cells was 0,015mm. The giant trophoblast cells were not counted.

Outcome measure and data analysis

The primary outcome measure was the placental weight difference between control group and group treated by 5azaC at different days of gestation from day 1 to 19. Secondary outcome measures were the comparison of placental proteins glycosylation pattern between treated placenta (study group) and controls, and finally correlation and immunohystochemical analysis of PCNA-positive trophoblast cells. The statistical analysis of placental weight comparing study and control group was performed using Student's *t* test. The stereological data for PCNA-positive cells were evaluated by descriptive statistics. Distribution of the data was assessed by Kolomgorov-Smirnov test, Lilliefors test and Shapiro-Wilks W-test. The homogenicity of the variance was tested by Lavens test. Differences in numerical density of PCNA-positive cells in investigated groups were analyzed with multiple analysis of variance

(MANOVA) with the post hoc LSD test. Statistical significance was set at $p < 0.05$. Statistical analyses were performed using STATISTICA 6,0 software (Stat Soft, Tulsa, USA).

RESULTS

Totally 117 rats were included in the study. Among them 58 were treated by 5azaC (49.6%) and 59 were used as control group (50.4%). Final results were based on 610 placentas treated by 5azaC and 668 controls.

Placental growth after treatment with 5-azacytidine

Significantly smaller placentas were found when 5azaC was administered from the day 4 to 14 (inclusive) of gestation (Fig. 1) ($p<0.01$, Student's *t* test). Before the day 4 and after the day 14 there were no significant weight differences between treated and control placentas (Student's *t* test, $p<0.01$). Placental weights of female rats treated on the day 5 of pregnancy equaled to 59.5% weight of the untreated control placentas; all those treated on the day 8 of pregnancy equaled to 25.4% weight of controls; those treated on the day 9 of pregnancy were only 13.2% weight of control placentas, while placentas from animals treated on day 11 amounted to 15.7% weight of controls. Finally those placentas from animals treated on the day 13 of pregnancy regained the 80.3% of the weight of untreated control placentas.

Comparison of placental proteins glycosylation pattern between treated placentas and controls

No differences in placental glycoprotein pattern/composition were found when animals were treated with 5azaC at days 5 and 14, but significantly smaller placentas were found in this group. On the other hand, in placentas treated with 5azaC on the day 6, absence of glycoproteins with molecular mass under 60 kD was found. The only exception was the presence of GP 34, showing the same intensity in treated and control placentas (Fig. 2 a). Using lectin SNA we detected a novel glycoprotein GP70 in placentas treated with 5azaC on the day 7 of gestation, and glycoprotein GP40 with stronger expression in control samples (Fig. 2 b). Analyses carried out on placentas treated on day 8 of gestation displayed similar GP70 pattern as observed at day 7 (Fig. 2 c). 5azaC treatment on day 9 of gestation resulted in GP40 expression only in control samples, while GP70 was present in those treated by

5azaC (Fig. 2 d). After administration of 5azaC at day 10, GP40 was still restricted only to controls (Fig. 2 e). However, after the administration at day 11, expression of GP40 was stronger in controls and GP70 in treated animals (Fig. 2 f). A similar pattern of GP70 was found at the day 12 of gestation (Fig. 2 g). Finally, administration of 5azaC on the day 13 resulted in nearly complete absence of GP40 in controls. However it was found in treated placentas (Fig. 2 h).

Histological, immunohistochemical and stereological analysis of placentas treated with 5azaC

The polynomial of fifth degree created by using the method of least squares best describes changes of the mean values of Nv by assessed days of gestation (Fig. 3 A). Multivariate analysis of variance (MANOVA) presented that the difference between the mean Nv values is highly significant ($F= 250,76$, $p < 0,0001$).

The significance between the mean Nv values of the analyzed groups was tested by LSD test (post hoc analysis). The differences between Nv of all investigated groups compared to the controls are statistically highly significant (LSD, $p< 0,0001$). The mean values of Nv after the application of 5azaC between day 4 and 6 are statistically significant (LSD, $p = 0,045$), while between day 5 and 14, between day 5 and 15 and between day 8 and 11 those values were not statistically significant. The differences between mean Nv are statistically highly significant between all other groups. (LSD, $p< 0,0001$).

Clearly visible border between the two main parts of rat placenta (the basal layer and the labyrinth) was found in control group. The labyrinth layer was prominent (Fig. 5 D). However, in placentas treated with 5azaC at the days 4,5 and 6, no labyrinth layer was identified (Fig. 4). Although significantly smaller, those placentas showed significantly higher proliferation rate compared to controls (positive PCNA nuclei found in numerous trophoblast cells; LSD, $p<0,0001$). The same demethylating agent applied to animals from day 7 to day 10 of gestation completely disturbs the placental structure, with no recognizable labyrinth layer. Intranuclear PCNA signal was significantly very poor when compared to controls (LSD, $p<0,0001$). The determination of numerical density was performed on the day 8 on treated animals only. Placentas treated on day 7 and 10 had completely disturbed structure and there

was no PCNA signal at all, while on the day 9 there was only one PCNA positive placenta. Therefore the statistical analysis was impossible (Fig. 5 A - C).

Application of 5azaC at day 11 and 12 of gestation demonstrated recurrent establishment of natural border between these two layers although compared to controls labyrinth was significantly reduced with predominant basal layer. Proliferation detected with PCNA was significantly higher compared to previous days but still not comparable to controls (LSD, $p<0,0001$) (Fig. 6 A, B). The characteristic relationship of the basal and the labyrinth layer appears to be present only when 5azaC was applied on the days 13, 14 and 15, with positive intranuclear PCNA signal present in both, trophoblast and in the giant cells (Fig. 6 C). However, numerical density (Nv) of PCNA-positive cells was significantly higher than in the control group (LSD; $p<0,0001$).

DISCUSSION

The role of DNA methylation in growth and differentiation of the rat placenta with special emphasis on glycoprotein expression is opening a new field in reproductive biology. The glycoproteins are crucial molecules during the process of placentation. In this work we wanted to emphasize the necessity in combination of different areas of molecular biology epigenetic and glycoproteomic, both being of particular interest for the scientists in recent years.

The pioneering experiments of Solter and McGrath [15] significantly contributed to the positioning of epigenetic, making it specifically interesting for many of scientists involved in the technology of cloning and methods of assisted reproduction. As well as that, upon the discovery of lectins, the proteins which recognize specific groups of sugars, glycoproteomics flourished as the special branch of glycobiology which is well documented by original research papers in this field [16].

The lack of knowledge of the development of human placenta (17) together with the fact that placentation disturbances representing the origin for many diseases related to pregnancy may potentially explains the relative low fertility in humans compared to some other mammalians. The trophoblast cells, being a crucial for placental development, display a very specific behavioral pattern of invasiveness resembling the tumor cells in some of their life characteristics. The key difference between two of them is defined by spatial and temporal limitation of trophoblast cells [18].

By interruption of normal methylation pattern using demethylating agent 5azaC, we followed several parameters of rat placenta development including: placental weight, expression of glycoproteins, appearance of the two main layers (the basal and the labyrinth), and the degree of cell proliferation. 5azaC was applied to animals in study group at different days of gestation, in order to cover the pregnancy from day 1 to day 19 assessing the importance of methylation in each day of gestation.

The analysis of placental weight after the application of 5azaC to pregnant rats at different stages of gestation showed a significantly reduced weight in study compared to the control group. Weight of placenta from pregnant rats treated on days 4 to 14 of pregnancy were significantly smaller when compared to the untreated control group ($p<0.01$). It has been shown that experimentally induced hypomethylation, has a far more serious impact on embryonic tissue development

compared to extraembryonic one. However, the impact of hypermethylation is directly opposite to that [19]. Also, the development of placenta is less affected by experimentally induced mutations in *Dnmt1* gene, as opposed to the heavy damages in embryonic tissues in those mutants [20]. Hypomethylation of the imprinted genes does not lead necessarily to their reactivation, because they are additionally silenced through the histone modification. So it was postulates that the imprinting mechanism is much more stable, particularly when the extraembryonic tissues are involved [21].

Treatment with 5azaC in study group from day 1 to day 3 day of gestation did not influence the growth of placenta assessed by placental weight. Before day 4 the preimplantation embryo shows the lowest degree of methylation. The process of *de novo* methylation and simultaneous epigenetic reprogramming of the genome starts from day 5 onward [22]. As a consequence of that treatment with 5azaC from day 4 resulted in statistically significant reduction in placental weight; however no difference in glycoprotein composition was noted with any of the lectins.

Application of 5azac on day 6 of pregnancy results in many differences between placentas in study compared to control group. The Western-blot analysis found that the glycoproteins with the molecular mass of less than 60 kD displayed reduced glycosylation in the study group samples. They were barely recognizable with all observed lectins. PHA-E lectin reacts with endothelial cells of the fetal blood vessels and it is particularly strong in labyrinthine layer [23]. The histology of analyzed placentas presented almost totally devoid of labyrinth with dominant basal layer if 5azaC was administered on day 6. Therefore we can conclude that glycoprotein pattern appears to be so poor. The degree of proliferation of these cells in basal layer was exceedingly high, pointing a question about gene affection during our application of 5azaC resulting in the absence of labyrinth development. We believe that inactivation of the gene *Gcm1* is involved in this reaction. Gcm1 codes the transcription factor responsible for initiation of choryoallantoic ramification in healthy individuals and it is a precondition for the establishment of the blood circulation of mother and fetus. It was found that *Gcm1* mutants do not form the labyrinth at all [24]. Also *Gcm1* and its protein gene products are responsible for the differentiation of synciciotrophoblast. The expression of Gcm1 mRNA is visible in mouse chorion cells before the establishment of the contact with allantois [25]. In human, reduced

expression of *GCM1* gene analogue, is observed in pathologic condition called pre-eclampsia [26].

Application of 5-azaC from the day 7 to day 10 of gestation displays different pattern of glycosylation. The expression of the new glycoprotein GP70, being completely absent or slightly expressed in control samples, was clearly detected in placentas treated by 5azaC. This is opposite to glycoprotein GP40 being found only in the controls. By application of 5azaC we probably changed the expression of genes at that very moment when ectoplacental cone, as a primordium for development of final placenta, was developing [27]. This explains the histology findings of hardly detectable labyrinth in 5azaC treated placentas.

Days 11 and 12 of rat pregnancy is the period of particularly pronounced endovascular invasion of giant trophoblast cells into maternal blood vascular system and differentiation of spongioblast into the glycogen-rich trophoblast cells responsible for interstitial infiltration [28]. 5azaC treated placentas showed clear border between labyrinth and basal layer at this particular days of pregnancy. Schreiber and collaborators constructed mutants for the gene *Fra1*, a member of the gene family which codes for synthesis of AP-1 (Activator Protein 1) crucial for embryonic development and carcinogenesis as well. The embryos lacking this gene die in mid gestation with the reduced labyrinth and the major part of placenta is avascular. To the contrary, the basal layer remains without changes [29].

Hemberger and Cross quote a series of genes (i.e. Sos1 and Gab1) whose mutations can result in reduced labyrinth. The expression of *Mash2* gene, epigenetically being regulated by the process of DNA methylation, is responsible for the maintenance of the trophoblast stem cells. Mash2 gene is intensively present in the placenta up to the day 12 of gestation, while in the later stages of pregnancy, its expression diminishes [30]. Although 5azaC probably activates Mash2 when applied after day 12 of gestation, its activity does not disturb the development of labyrinth layer. Western blot analysis using PHA-E lectin shows stronger expression of GP70 in treated placentas. Thus, the stronger expression of this glycoprotein was found in treated rats from day 7 to day 12 of pregnancy, but was not or was slightly visible in control placentas.

The day 13 of rat gestation is the period in placental development when glycogen-rich cells continue the decidual invasion and concentrate around the central maternal artery [31]. In 5azaC treated specimens labyrinth occupies more than 2/3 of the placenta, resembling the normal pregnancy. Moreover, positive intranuclear signal is visible in trophoblast and giant cells. Intensive cellular division is in concordance with endovascular invasion of giant cells and interstitial invasion of glycogen-rich cells. Although the histological picture resembles the picture of the normal, healthy placenta, the expression of glycoproteins in the 5azaC treated animals still does not match the expression pattern in controls. Before day 13 of gestation GP40 showed elevated expression in controls when compared to 5azaC treated placentas. When 5azaC was applied to pregnant females on this very day it caused elevated expression of GP40 in treated placentas. Therefore, it looks like that its expression is important for the establishment of the normal placental structure.

We assumed that preconditions for normal placental structure have been established before day 13 of gestation. Consequently, there was no difference in glycoprotein composition in placentas treated by 5azaC on day 14, 15, and 17, compared to untreated placentas. However, statistically significant difference in placental weight was found in animals treated by 5azaC on day 14 compared to controls. As this is the period when the endovascular invasion of giant cells and interstitial invasion of glycogen-rich trophoblast cells are not completely finished, we can still interfere with the establishment of flow through labyrinth [32].

The proliferative capacity of placental cells was determined by studying expression of PCNA. This is a non-histone nuclear protein, which functions in the system of DNA polymerase δ and serves as an excellent marker for cell proliferation [33]. Many researchers are using it in oncology because it has been proven that its elevated expression speaks in favor of cancer invasiveness, but also about the prognosis of the malignant disease [34].

It is clear that PCNA is included in the process of DNA methylation. The DNA methyltransferase – Dnmt1 is associated with PCNA in the region of replication forks [35]. Dnmt1 demonstrates much stronger affinity for DNA with already bound PCNA, compared to free DNA molecule. Therefore interaction of PCNA and Dnmt1 enhances the process of methylation [36]. The application of 5acac influenced development of the placenta and the degree of trophoblast proliferation in their basal

layer. Statistically significant difference in expression of PCNA positive cells is clearly evident in all analyzed placentas, starting from the day 4 of the 5azac application to the day 15. The most prominent changes occurred in placenta exposed to 5azaC from day 7 to 12. The expression of PCNA positive cells is lower during that period, and the difference is statistically significant compared to the controls. If 5azaC was applied on other days of rat pregnancy the PCNA expression was higher compared to the controls. The PCNA protein, being included in replication of DNA, is also involved in repair of a damaged DNA molecule [37]. However, tumor suppressor protein p53 is also participating in regulation of its activity. The low concentration of protein p53 in cell leads to the activation of PCNA promoter, while the high concentration of p53 results with its inactivation [38]. Based on our results we believe that 5azac administered on days 4, 5, 6 of gestation was so teratogenic that the reaction against its application has mobilized the whole cell machinery for repair of the damaged DNA.

However, there is still open question why there was no differences found in glycoprotein content on the placenta exposed to 5azac on the day 5. As well as thet, in this group of analyzed placentas, there was no clear labyrinth found. Therefore, in our future research projects planned, we intend to expand the number of lectins in order to recognize different glycoproteins. Finally, our results confirm that no research assessing the placenta on molecular level, should avoid the morphological assessment. The results about the outcome of the animals outcome were presented in our previously published paper [39].

These results can have significant impact for further studies pointing the importance for exact identification of detected glycoproteins. According to methylation patterns their expression is prerequisite for normal placentation and fetal development.

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LIST OF FIGURES

Figure 1

The placental weight difference between control group and group treated by 5azaC at different gestation days.

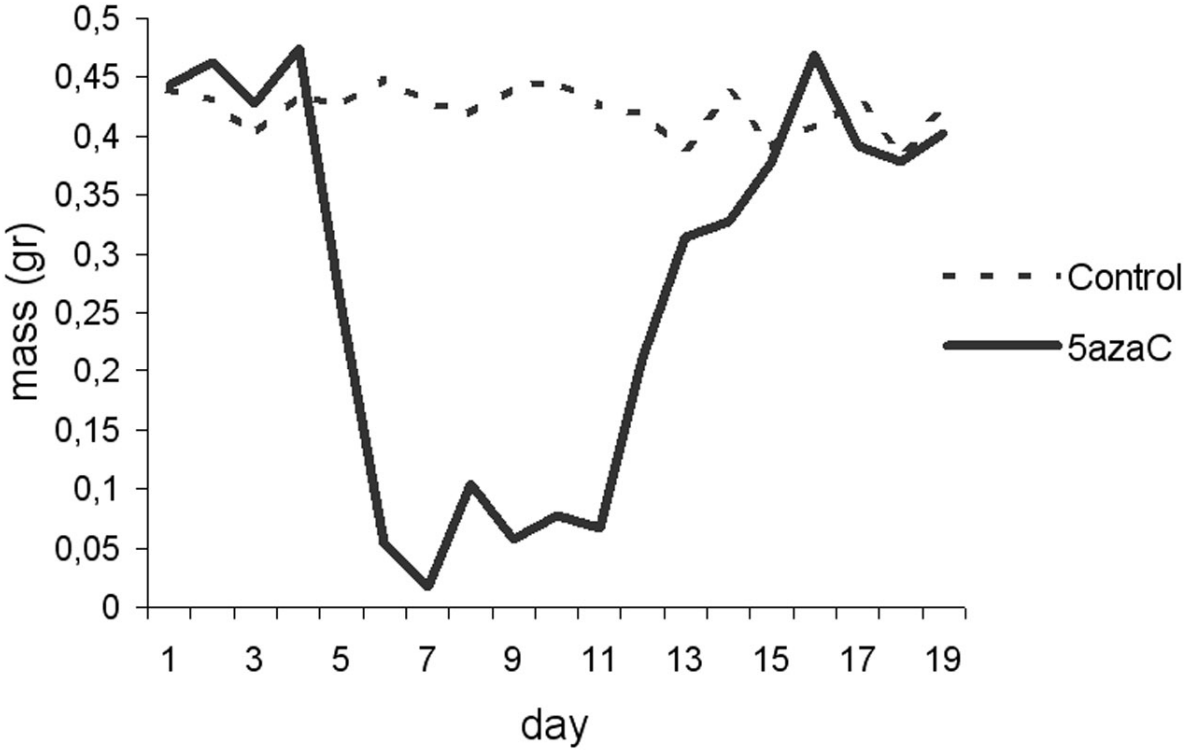

Figure 2

Western blott analysis of placentas treated with 5azaC (2) compared with control (1) detected by lectins SNA, DBA, PHA-E and UEA-I. Placental homogenates (40µg/lane) were loaded onto concentration gradient SDS polyacrylamide gels (5-15 %). Glycoprotein pattern of placenta treated with 5azaC on day 6 (a), day 7 (b), day 8 (c), day 9 (d), day 10 (e), day 11 (f), day 12 (g) and day 13 (h) of gestation, obtained by electrophoresis and detection with lectins (2) in comparison with control placenta (1).

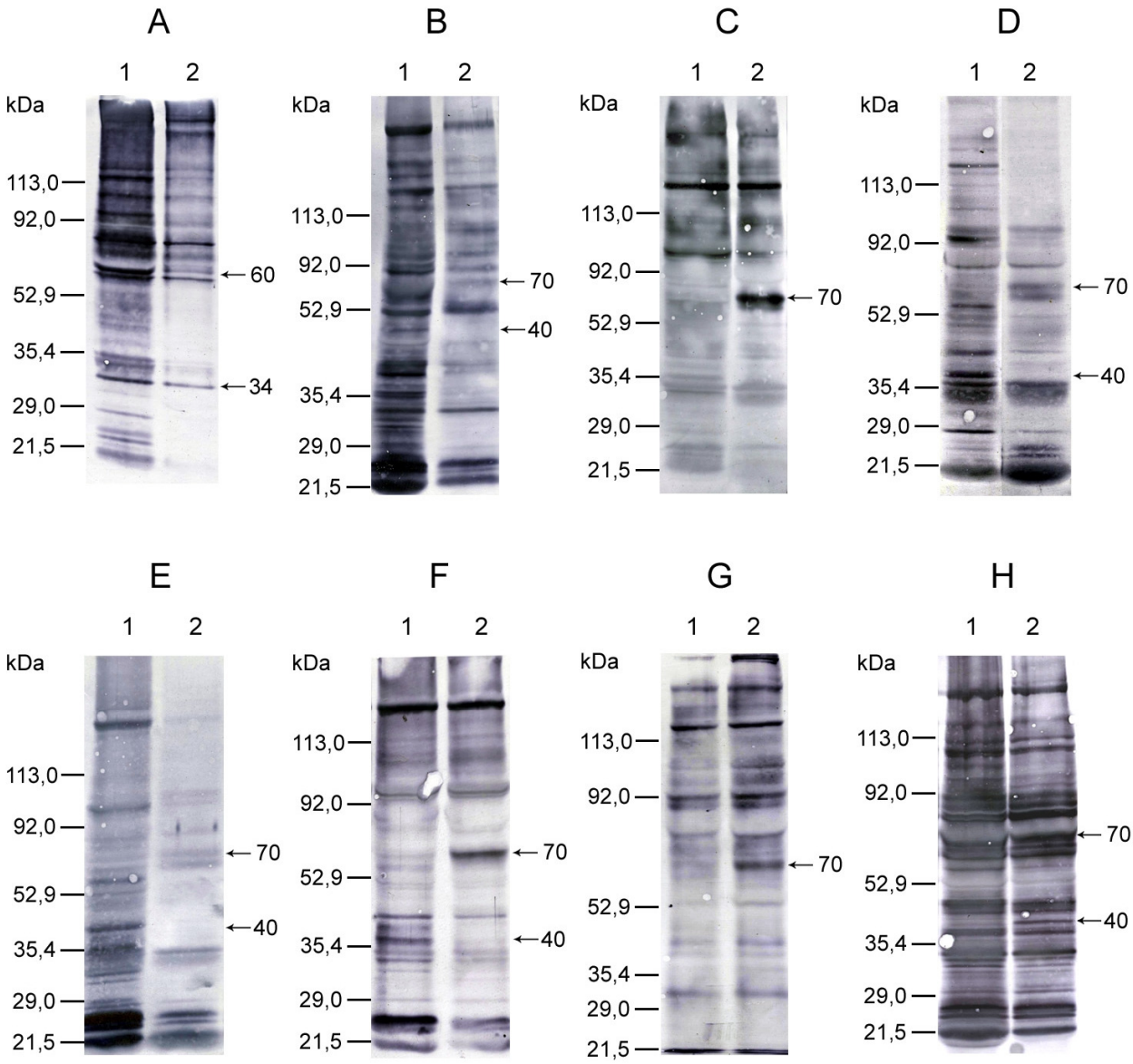

Figure 3 A - Separate values of numerical density (Nv) for PCNA in 20 days old rat placentas presented graphically depending on the day of 5azaC application. The red lines represents the polynomial of fifth degree.

B -The mean values with 95% Confidence intervals (CI) of investigated groups. The red lines represents mean value and 95% CI for control group.

Based on this analysis the relationship can be found regarding the 5azaC application and the ability of trophoblast proliferation of the basal layer cells in rat placenta.

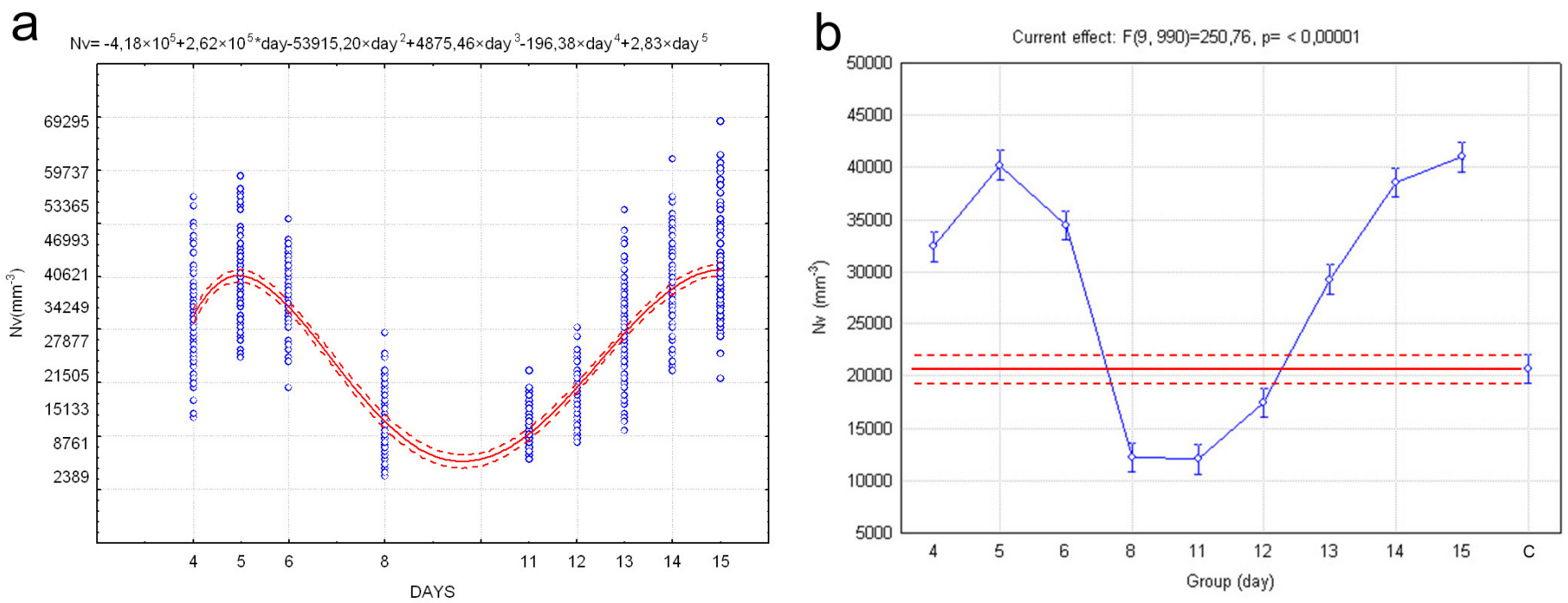

Figure 4

PCNA expression in the placental tissue after the application of 5azaC on day 4 (A), day 5 (B) day 6 (C) and PCNA expression in control group (D). Signal found in trophoblast cells (arrow) b- basal layer, d-decidua, *-giant trophoblast cell, DAB, hemalaun contrast

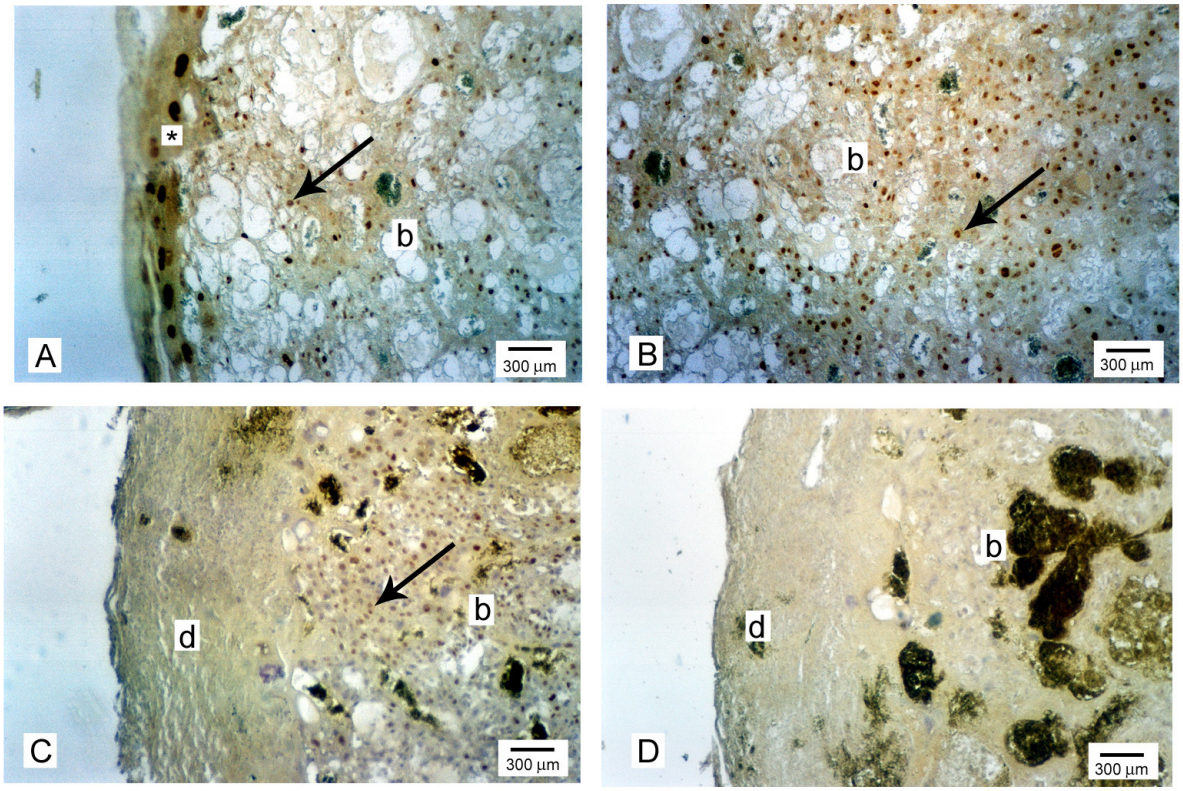

Figure 5

PCNA expression in placental tissue after 5azaC application on the day 8 (A), 9 (B), and negative control (C) as well as normal placenta (D). Signal found in trophoblast cells (arrow) b- basal layer, d-decidua, *-giant trophoblast cell, DAB, hemalaun contrast

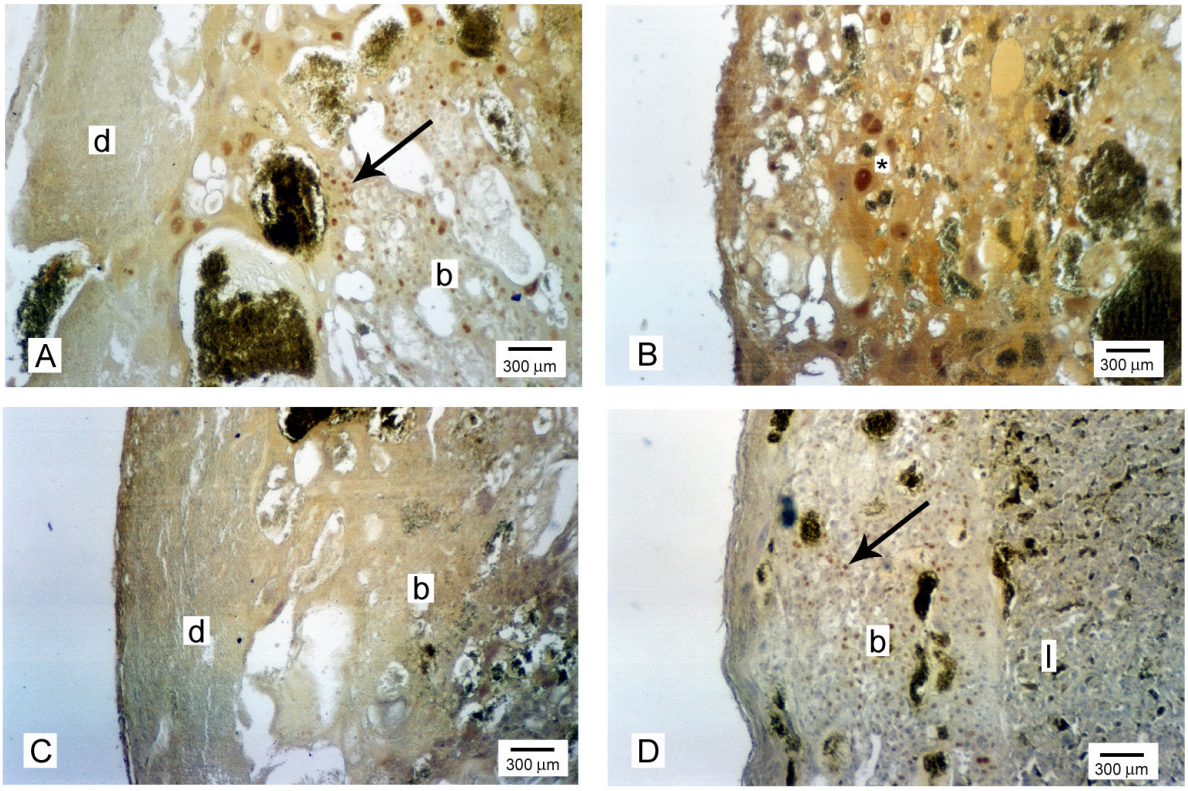

Figure 6

PCNA expression in placental tissue after 5azaC application on day 11 (A), 12 (B), 14 (C) and PCNA expression in negative control (D) Signal found in trophoblast cells (arrow) b- basal layer, d-decidua, *-giant trophoblast cell, DAB, hemalaun contrast