FOETAL SURVIVAL DETERMINED BY 5-AZACYTIDINE IMPACT ON THE PLACENTA

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ABSTRACT – DNA methylation as a regulatory mechanism for mammalian gene expression is involved in the process of placentation as well as foetal development. 5azaC is a demethylating agent, which incorporates into DNA instead of cytosine and prevents its methylation, subsequently changes expression of genes involved in normal placental and foetal development. Single dose of 5azaC (5 mg/kg) was administered to pregnant rats at 11th, 12th and 13th day of gestation. Placentas and foetuses were isolated at day 20, weighted and histologicaly analyzed. After 5azaC administration on the 11th day of gestation, treated placentas were significantly smaller and labyrinth was significantly reduced. Consequently complete foetal resorption occurred. When administrated at day 12 of gestation, labyrinth was slightly recovered and 24% foetuses survived, while after its application at 13th day of gestation, almost normal distribution of placental layers was found and survival was 96%. These results confirmed epigenetic influence upon placental development. 5azaC caused changes in its structure, especially the reduction of labyrinth that is crucial for foetal survival. After establishment of normal placental layers distribution, 5azaC has no impact on placental morphology, neither on foetal survival. On the other hand, the influence of 5azaC remains visible in appearance of foetal malformations.

Key words: *placenta, 5-azacytidine, DNA methylation, rat*

Introduction

Methylation process represents one of the mechanisms for modification of DNA molecule, and hence the epigenetic control of gene expression in vertebrates[1]. The very mechanism of methylation refers to the binding of a methyl group to the 5th carbon atom of the cytosine ring, and is carried out with the help of the enzyme DNA methyltransferase (Dnmt) [2]. The result is the formation of a new base, 5-methylcytosine (5mC)[3]. Such modification changes the affinity of access for particular transcription factors towards DNA molecule which prevents the formation of the transcriptional initiation complex, or elongation of those already initiated, that is gene inactivation[4]. The most powerful tool for DNA methylation investigation is 5-azacytidine (5azaC). It incorporates into DNA instead of cytosine, prevents its methylation and subsequently changes the expression of genes involved in normal placental and foetal development[5].

Rat placenta develops in the region of thickened embryonic pole of blastocyst[6]. Until the eighth day of gestation trophoblast cells form pre-placental cone which grows towards the site opposite to the place of implantation and provokes formation of the placental disc[7]. On the ninth day of gestation, maternal lacunas without endothelium are being developed in the ectoplacental cone[8]. Roughly, upon the onset of 11th day of gestation, mesenchyme of allantois comes into contact with ectoplacenta[8]. As it has the capacity for angiogenesis, it differentiates into foetal mesenchyme and blood channels

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surrounded by endothelium[9]. It penetrates deeply into trophoblast of ectoplacenta, among the pre-existing maternal lacunas. In this way, it forms a labyrinth, region in which maternal and foetal blood circulation exist[10]. The role of the labyrinth, basal layer and decidua (including vacuolized subplacental region and maternal glands) in the structure of entire placenta changes during embryonic development. Therefore, on the day 12 of gestation labyrinth occupies only 20%, basal layer 25%, and decidua with associated glands 55%. Before the end of pregnancy, on the day 20, labyrinth occupies almost 60% of total placenta, basal layer 15%, and decidua with glands only 25%. Such growth of labyrinth indicates its crucial role in the exchange processes between mother and foetus[11].

Development of placenta, this exclusive organ for reproduction, is genetically strictly and specifically regulated. Any deviation in expression of genes can bring about changes in embryo development[12].

The purpose of this study was to investigate the influence of DNA methylation on placental development and consequently foetal survival depending on placental development failure caused by demethylating agent 5-azacytidine.

Material and Methods

Experiments were performed on pregnant Fischer female rats on different days of gestation (11-13). Adult (three-month-old) females were mated overnight with males of the same age. Vaginal plug designated day 0 of pregnancy.

5-azacytidine (Sigma) was dissolved in PBS and used at a concentration of 5mg/kg body weight. Administration was done by a single i.p. injection (1ml) of either PBS (controls) or of 5-azacytidine solution. The animals were sacrificed on day 20 of pregnancy.

Placentas were immersed in a fixative solution containing 4% paraformaldehide at 40^{0}C for 72 h. After fixation and dehydration, the specimens were transferred to paraffin, sectioned to 5µm slices, deparaffinized and stained with haematoxylin and eosin. For statistical evaluation Student's t test was used.

Results

Two experimental groups for each day of gestation (from G11 until G13) were established: one treated with 5azaC and the second one treated only with PBS, which served as the control. Histological analysis of placentas treated with 5azaC showed changes in comparison to control placentas (Fig. 1 and 2). After 5azaC administration on the 11th day of gestation, treated placentas were significantly smaller (Table 1). Labyrinth, as a functional main part of the placenta, was reduced. Consequently, complete foetal resorption occurred. The same agent administrated at day 12 of gestation resulted in slightly recovered labyrinth and 24% foetal survival, while after its application only one-day later (13th day of gestation) placentas showed almost normal distribution of placental layers. Foetal survival was significantly improved to 96% (Table 2), but the teratogenic effect of 5azaC was notable in formation of various limb malformations found in survived foetuses.

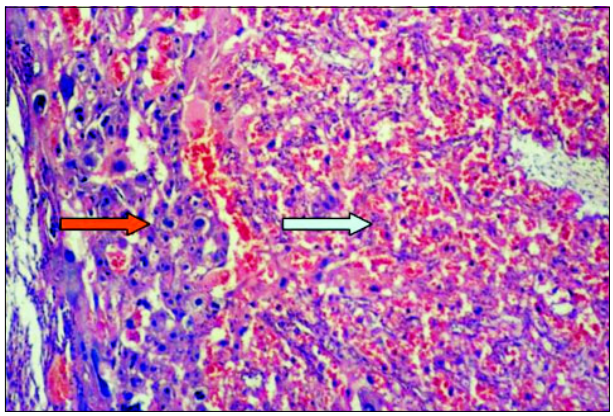

Fig. 1. Basal (⟹) and labyrinth (⇨) layer of control rat placenta (40x).

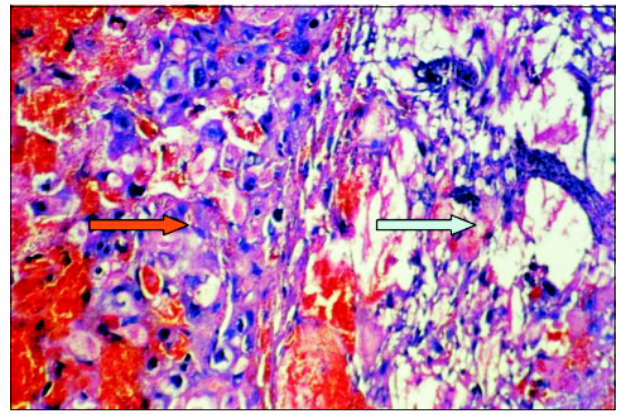

Fig. 2. Cross-section of rat placenta treated with 5azaC on the 12th day of gestation. Note the clear border between basal layer (⟹) and significantly reduced labyrinth (⇨)(40x).

Table 1. Survival of foetuses after treatment with 5-azacytidine on different days of gestation

Treatment on day	11	12	13
No of placentas	20	54	55
No of living foetuses	0	13	53
% of living foetuses	0	24	96

Discussion

Contemporary medicine considers nowadays normal embryonic human development as one among its hottest themes. It is impossible to imagine such development without synchronized cooperation of embryo and placenta[13]. One of the most convenient models for study of molecules and their interactions in processes of implantation and placentation for embryonic human development is the rat placenta, which resembles the human placenta in its many characteristics[14,15]. Although the human placenta is villous and the rodent is of labyrinth type they are both haemochorial[16].

Histological analysis of 11th and 12th day 5azaC treated placentas showed disturbed placental structure, but with notable border between the labyrinth and basal layer. The latter one occupies further more than one-half of the placental tissue. This is the period of particularly pronounced endovascular invasion of giant trophoblast cells into maternal blood vascular system[17]. In addition, it is the time of setting up preconditions for differentiation of spongioblast into the glycogen-rich trophoblast cells responsible for interstitial infiltration into the placental tissue[18]. The reduced labyrinth as well as reduced weight of placentas (Table 2) resulted in embryo lethality (Table 1) similar to results of Schreiber and collaborators[19]. They constructed mutants for the *Fra1* gene, a member of the gene family, which codes for AP-1 (Activator Protein 1). It pertains to the protein family, which plays different roles during embryonic development and carcinogenesis as well. The embryos lacking this gene die in midgestation, with the labyrinth of their placentas reduced and in the major part avascular. To the contrary, the basal layer remains without changes[19]. Consequently, labyrinth reduction resulted in complete foetal resorption. 5azaC induced labyrinth reduction had almost the same effect as *Fra-1* gene mutation on foetal survival.

But when 5azaC was applied at day 13 the characteristic ratio of the two essential placental components appears again and consequently the survival of embryos is almost as in controls (Table 1). At that time in rat gestation, glycogen-rich cells continue further the decidual invasion and concentrate around the central maternal artery[20]. Evidently, all the preconditions for placental development have already been previously established, so the influence of 5azaC application is not any more that intensive[21]. Nevertheless, its teratogenic effect still persists in appearance of various foetal malformations.

Our results indicate a strong epigenetic influence upon placental development. Administration of demethylating agent, 5azaC, caused changes in its structure, specifically the reduction of labyrinth layer, obviously crucial for foetal survival.

Table 2. Weight of 5-azacytidine treated placentas on different days of gestation compared to control placentas by Student's t-test.

Treatment on day		No of placentas	weight (g)	t	p
11 day	5azaC	20	0,062±0,036	34,7	<0,0001
	Control	17	0,420±0,023		
12 day	5azaC	54	0,186±0,049	21,9	<0,0001
	Control	36	0,404±0,041		
13 day	5azaC	55	0,304±0,033	10,7	<0,0001
	Control	54	0,379±0,039		

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Sažetak

PREŽIVLJENJE FETUSA ODREĐENO UTJECAJEM 5-AZACITIDINA NA PLACENTU

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Metilacija DNA kao regulatorni mehanizam ekspresije gena u sisavaca uključena je kako u procese placentacije tako i razvitka fetusa. Demetilacijsko sredstvo 5azaC ugrađuje se u DNA umjesto citozina i sprječava njegovu metilaciju, što ima za posljedicu promjenjenu ekspresiju gena uključenih u normalnu placentaciju i fetalni razvitak. Trudne ženke Fisher soja štakora tretirane su s 5 mg/kg 5azaC jedanaestog, dvanaestog i trinaestog dana gestacije. Dvadesetog dana gestacije izolirani su, izvagani i histološki analizirani kako fetusi tako i placente. Nakon primjene 5azaC jedanaestog dana gestacije, tretirane placente bile su značajno manje, a labirint reduciran, zbog čega je uslijedila potpuna resorpcija fetusa. Dvanaesti dan primjene 5azaC rezultirao je blagim povećanjem labirinta, a preživljenje fetusa bilo je 24%. Tek trinaestog dana aplikacije 5azaC uspostavila se normalna distribucija labirinta i bazalnog sloja s preživljenjem od 96%. Dobiveni rezultati potvrđuju epigenetski utjecaj na razvitak placente. Primjena 5azaC uzrokovala je promjene u strukturi placente, osobito redukciju labirinta čija je uloga očito ključna za preživljenje fetusa. Nakon uspostave normalne građe placente, 5azaC više nije imao utjecaja na njezinu morfologiju niti na preživljenje fetusa. S druge strane, njegov je teratogeni utjecaj ostao i dalje vidljiv na fetusima u obliku različitih malformacija.

Ključne riječi: *placenta*, *5azacitidin*, *metilacija DNA*, *štakor*