QUANTITATIVE ASSESSMENT OF BREAST CANCER RESISTANCE PROTEIN DURING PREGNANCY IN RABBITS

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Breast cancer resistance protein (BCRP, ABCG2) is an efflux transporter protein that transports various substrates from the cell to the extracellular space or organ cavities. The aim of this study was a complex assessment of the amount of BCRP during pregnancy in rabbits. The amount of BCRP in samples of the rabbit jejunum, liver, kidney, cerebral cortex, and placenta was determined by enzyme immunoassay, and in human hepatocellular carcinoma (HepG2) cells by the Western blot. To study the mechanisms involved in control of the dynamic BCRP levels during pregnancy, serum concentrations of sex hormones were investigated by radioimmunoassay and relative amounts of constitutive androstane receptor (CAR) and pregnane X receptor (PXR) in these organs were evaluated using the Western blot method. The putative role of CAR and PXR in regulation of the BCRP level by progesterone was evaluated *in vitro* experiments on HepG2 cells. It was found that amount of BCRP in the jejunum of pregnant rabbits was higher than in the placenta, liver, kidneys, and cerebral cortex. An increase in the amount of BCRP in the liver of rabbits was noted on the 21st day of pregnancy and a tendency to the increase was also detected on the 28th day; in the kidney and cerebral cortex increased BCRP levels were detected on the 28th day and 14th day of pregnancy, respectively, as compared with non-pregnant females. *In vitro* experiments with HepG2 cells have shown that the increase in the BCRP level is determined by the activating effect of progesterone on PXR.

Key words: breast cancer resistance protein (BCRP, ABCG2); protein amount; pregnancy; progesterone; constitutive androstane receptor (CAR); pregnane X receptor (PXR)

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INTRODUCTION

Breast cancer resistance protein (BCRP, ABCG2) is an efflux transporter protein that transports a wide range of lipophilic substrates from the cell to the extracellular space or organ cavities. It was originally discovered by Doyle et al. in 1998 in MSF-7/AdrVp resistant breast cancer cells, where it contributed to the active release of antitumor agents from the cytoplasm [1]. Currently, BCRP has been identified in many organs and tissues. It is expressed in placental syncytiotrophoblasts, the apical membrane of the small intestine epithelium, the tubular membrane of the liver, the apical membrane of renal tubular epitheliocytes, on the surface of endothelial cells of brain microvessels, and in the cells of the mammary gland [2-5]. Such localization of the transporter protein determines its involvement in the pharmacokinetics of drugs that are its substrates. BCRP substrates include many antitumor agents, calcium channel blockers, antivirals, fluoroquinolones, antibiotics, statins, proton pump inhibitors, etc. In addition, BCRP transports a number of endogenous substrates: steroid hormones and their metabolites (estrone-3-sulfate, 17β-estradiol, dehydroepiandrosterone), bile acids, urates.

Changes the BCRP functioning can have a significant impact on the pharmacokinetics of drugs that are its substrates. An increase in the amount and activity of the transporter protein will lead to a decrease in the concentration of its substrates in the blood and, consequently, a decrease in the effectiveness of pharmacotherapy. A decrease in the content and activity of BCRP results in an increase of blood concentrations of its substrates and this increases a risk of developing adverse drug reactions.

The ability of sex hormones to regulate the BCRP level has been reported in the scientific literature [6]. Since pregnancy is accompanied by pronounced hormonal changes, it is reasonable to suggest that the BCRP level in various organs changes during pregnancy. Considering that the majority of pregnant women take various pharmacological agents, including substrates of this transporter protein, the study of its functioning during pregnancy is very relevant. Although there are literature data on the content and activity of BCRP in the hematoplacental barrier, a comprehensive quantitative assessment of the transporter protein in various organs during pregnancy has not been carried out so far.

Thus, the aim of this study was to evaluate the amount of BCRP in organs that play a role in the pharmacokinetics of drugs, and to study the mechanisms of its regulation throughout pregnancy.

MATERIALS AND METHODS

In Vivo Studies

Laboratory Animals

The study was performed on 25 female Soviet Chinchilla rabbits weighing 3000-3500 g. The animals were obtained from the "Stolbovaya" nursery (Moscow region), had the appropriate veterinary certificates and were kept under standard vivarium conditions of the Ryazan State Medical University. All animals were divided into 5 series (n=5 for each time point): intact rabbits (control), animals on day 7, day 14, day 21, and day 28 of pregnancy. The first day after intercourse was considered as the first day of pregnancy. Pregnancy was diagnosed by increased serum concentrations of progesterone, visual signs, palpation and autopsy data.

At all-time points serum progesterone, estradiol, testosterone, and prolactin concentrations were determined by radioimmunoassay in all rabbits. The animals were euthanized using an overdose of zoletil ("Virbac", France) and samples of the jejunum, liver, kidney, cerebral cortex, and placenta were taken for research.

Determination of BCRP

The amount of BCRP in tissue samples was determined by enzyme immunoassay using an ELISA kit ("BlueGene", China). Tissue samples homogenized on a DIAX 900 homogenizer ("Heidolph Instruments", Germany) at 26000 rpm in 0.01 M phosphate buffer, pH 7.2, in the cold (1:1, w/v) for 1 min, were then subjected to triple freeze-thaw cycles at -20°C to destroy cytoplasmic membranes, as recommended in the instructions for the kit, then centrifuged at 1500 *g* for 15 min. The supernatant was analyzed. The amount of BCRP was normalized to the total protein content, which was determined by the Bradford method using the Coomassie Plus (Bradford) Assay Kit ("Thermo Fisher Scientific", USA).

Determination of the Relative Amount of the Transcription Factors

In liver samples, the relative amount of the transcription factors, constitutive androstane receptor (CAR) and pregnane X receptor (PXR), was analyzed by Western blot. For this purpose, liver samples were crushed and homogenized in NP40 Cell Lysis Buffer Thermo lysis buffer ("Thermo Fisher Scientific") with addition of a mixture of proteinase inhibitors ("Sigma-Aldrich", Germany) using a Potter homogenizer (16-20 strokes). The ratio of tissue mass (mg):buffer volume (ml) was 1:1 (w/v). The homogenate incubated for 3 h at 4°C under constant stirring was then centrifuged at 22440 *g* for 10 min (AvantiJXN-3, "Beckman Coulter", USA). The supernatant was used for subsequent analysis.

Samples (30 μg of protein) were subjected to electrophoresis using a TGX Stain-Free FastCast Acrylamide Kit ("Bio-Rad", USA) in a Laemmli buffer system ("Bio-Rad"). Prior to loading, samples were processed according to the manufacturer's protocol. They were mixed with the Laemmli sample buffer containing 2.5% 2-mercaptoethanol ("Bio-Rad") (1:1, v/v) and incubated for 5 min at 70°C. Electrophoresis was carried out at 100 V for 90 min. Proteins were transferred to a Trans-Blot Turbo Mini-Size nitrocellulose membrane ("Bio-Rad") using a Mini Trans-Blot module ("Bio-Rad") for 10 min at 20 V and 1.3 A. Proteins on the membrane were blocked with 1% Casein Blocker ("Bio-Rad") containing 0.1% (v/v) Tween 20 ("Sigma-Aldrich") during 1 h incubation at room temperature.

Primary mouse monoclonal antibodies (MB67 CAR Monoclonal Antibody, "Invitrogen" (USA) and MA5-31808 PXR Monoclonal Antibody (1D12G1), "Invitrogen", respectively) at a dilution of 1:200 were used to determine the relative amount of CAR and PXR by Western blot. Visualization of primary antibodies was performed using secondary rabbit antibodies (Rabbit-anti-Mouse IgG (H+L) Secondary Antibody, HRP, "Invitrogen", dilution 1:4000). Proteins were visualized by chemiluminescence using Chemi Doc XRS+ ("Bio-Rad"). The molecular mass of the proteins was confirmed by comparison with molecular mass markers (Precision plus protein standards Dual Color, "Bio-Rad"). The intensity of the resulting bands was analyzed densitometrically using ImageLab software ("Bio-Rad"). The amount of CAR and PXR was normalized to the content of the protein encoded by the GAPDH housekeeping gene (primary GAPDH Loading Control Monoclonal Antibody (GA1R), DyLight 68, "Invitrogen", dilution 1:1000, secondary antibodies (secondary rabbit antibodies to GAPDH primary antibodies) Rabbit-anti-Mouse IgG (H+L) Secondary Antibody, HRP, "Invitrogen", dilution 1:4000).

In Vitro Studies

The mechanisms of the increase in the amount of BCRP during pregnancy were also studied *in vitro* experiments using human hepatocellular carcinoma (HepG2) cells (Central Collective Use of Vertebrate Cell Cultures, St. Petersburg) obtained from the Institute of Cytology of the Russian Academy of Sciences (St. Petersburg).

Culturing Cells

Cells were cultured at 37°C and 5% CO_2 in Dulbecco's Modified Eagle's Medium (DMEM) with a high glucose content (4500 mg/l) ("Sigma-Aldrich") containing 4 mM L-glutamine ("Sigma-Aldrich"), 10% fetal bovine serum ("Sigma-Aldrich"), 100 U/ml penicillin ("Sigma-Aldrich"), and 100 μg/ml streptomycin ("Sigma-Aldrich") in 6-well plates ("Corning", USA). After the formation of a monolayer, the cells were used in experiments.

Experiment Series

The following experimental series were formed: 1) control (n=3): cells incubated in the culture medium without addition of test substances; 2) evaluation of the progesterone effect on the relative amount of BCRP: progesterone ("Sigma-Aldrich") was added to the culture medium at the final concentrations of 10 μM and 100 μM and incubated for 24 h, three repeats were performed for each experiment (n=3); 3) evaluation of the role of CAR in the progesterone-induced increase in the relative amount of BCRP (n=3): cells were incubated with 100 μM progesterone together with a CAR inhibitor, 10 μM 5-[(diethylamino)acetyl]-10,11-dihydro-5H-dibenzo[b,f]azepin-3-yl]carbamic acid ethyl ester (CINPA 1, "Tocris", UK) [7], which was added to the culture medium 30 min before addition of progesterone; 4) evaluation of the role of PXR in the progesterone-induced increase in the relative amount of BCRP (n=3): cells were incubated with 100 μM progesterone together with a PXR inhibitor, 10 μM ketoconazole [8] ("Sigma-Aldrich"), which was added to the culture medium 30 min before progesterone.

Determination of the Relative Amount of BCRP

Determination of the relative amount of BCRP in HepG2 cells was performed by the Western blot method similarly to the determination of CAR and PXR. Supernatant samples (20 μg of protein) were electrophoresed using the TGX Stain-Free FastCast Acrylamide Kit in a Laemmli buffer system. Samples were mixed with the Laemmli buffer containing 50 mM 2-mercaptoethanol (1:3, v/v) and incubated for 10 min at 70°C. Electrophoresis was carried out at 100 V for 90 min. The BCRP protein was detected using primary mouse monoclonal antibodies (CD338 (ABCG2) Monoclonal Antibody (5D3), "Invitrogen") diluted 1:200 in Casein blocker for 2 h at 37°C. Primary antibodies were visualized using secondary rabbit antibodies (Rabbit-anti-Mouse IgG (H+L) Secondary Antibody, HRP, "Invitrogen") diluted 1:4000 and incubated for 1 h at room temperature.

Statistical Analysis

The results obtained were processed using the StatSoft Statistica 7.0 program to determine the type of data distribution according to the Shapiro-Wilk test. In the case of normal data distribution, statistical significance of differences was assessed using the ANOVA test; pairwise comparisons were performed using the Fisher test. When the data did not follow normal distribution, differences between the series were assessed using the Kruskal-Wallis test. At the p-value <0.05, the parameters were subjected to pairwise comparison using the Mann-Whitney test with the Bonferroni correction. The results are given as the arithmetic mean ± standard deviation of the mean (M±SD) in the case of normal data distribution or median, lower, and upper quartiles (Me (Q1; Q3)) in the case of non-normal data distribution.

RESULTS AND DISCUSSION

The amount of BCRP in the jejunum of pregnant rabbits was much greater than the content of this transporter protein in the placenta, liver, kidneys, and cerebral cortex (Table 1). In all the studied periods of pregnancy, the content of the transporter in the jejunum insignificantly differed from control (non-pregnant) animals.

In the liver, the BCRP content increased on the 21st day of pregnancy by 545.5% (p<0.05) and a tendency to the increase was also detected on the 28th day (p=0.07) as compared with non-pregnant females. In the kidney, the content of BCRP increased on the 28th day of pregnancy by 71.0% (p<0.05), and the increase in the cerebral cortex was detected on the 14th day by 145.0% (p<0.05) as compared with the control values. The amount of BCRP in the placenta did not differ significantly at all periods of pregnancy studied.

It is known that the amount of BCRP is controlled by sex hormones. In *in vitro* studies on BeWo cells that mimic the human placental barrier have shown that progesterone, estriol, placental lactogen and prolactin increase, while 17β-estradiol decreases, and testosterone and human chorionic gonadotropin do not influence BCRP expression [6]. At the same time, combinations of 17β-estradiol with progesterone and testosterone increased BCRP expression [6].

Our study of serum concentrations of sex hormones (Table 2) revealed an increase in the progesterone level versus control on day 7 of pregnancy (by 988.1%, p<0.05), on day 14 (by 962.3%, p<0.05), on day 21 (by 1006.3%, p<0.05), on day 28

Table 1. The amount of BCRP protein in the tissues of female rabbits at different stages of pregnancy (Me(Q1;Q3)), ng/g

Tissue	Control	Day 7	Day 14	Day 21	Day 28
Ileum	106.76±46.65	114.23±17.22	104.03±13.45	68.31±34.02	102.25±55.78
Liver	0.66±0.32	1.00±0.40	0.57±0.28	4.32±0.94*	1.4±0.81 (p=0.071)
Kidney	1.14±0.29	1.51±0.45	1.11±0.47	1.17±0.51	1.95±0.85*
Cerebral cortex	0.4±0.12	0.36±0.08	0.98±0.29*	0.32±0.09	0.42±0.12
Placenta	—	4.95±1.14	6.70±1.13	4.31±1.56	5.95±0.91

The asterisk (*) indicates statistically significant differences from the parameters of non-pregnant female rabbits, p<0.05.

(by 378.0%, $p<0.05$) and an increase in prolactin concentration on day 28 (by 103.8%, $p<0.05$). Serum levels of other hormones (estradiol and testosterone) insignificantly differed from pre-pregnancy levels. This suggests that progesterone may be involved in the regulation of BCRP levels during pregnancy.

The most pronounced changes in the BCRP level were detected in the liver, where possible mechanisms of regulation of this transporter protein during pregnancy, including PXR and CAR, were previously studied. PXR and CAR are nuclear factors that regulate functioning of a number of enzymes and transporter proteins involved in the metabolism and transport of xenobiotics, including BCRP [9, 10]. The level of PXR in the liver on day 21 of pregnancy increased by 30.8% ($p<0.05$) as compared with the levels before pregnancy and did not change at all time-points of pregnancy studied. On the contrary, the level of CAR decreased at all terms of gestation. The maximal decrease (by 63.6%, $p<0.05$) (Fig. 1) was found on day 28, when the increase in BCRP was detected. The results obtained are consistent with the literature data. In Caco-2 cells activation of PXR by PCN (5-pregnen-3β-ol-20-one-16α-carbonitrile) promotes an increase in the amount of BCRP [11]. Treatment of mouse TM4 Sertoli cells with PXR agonists 100 μM dexamethasone and 50 μM PCN for 24 h induced BCRP mRNA and protein expression, which was inhibited by the PXR antagonist, 10 μM ketoconazole. Genetic knockdown of PXR in TM4 cells using siRNA also reduced expression of the BCRP protein, thus confirming the direct regulatory role of PXR in the testis [12]. PXR-mediated regulation of BCRP levels has been identified in mouse placentas. An experiment on pregnant C57BL/6 mice revealed that intraperitoneal administration of the PXR PCN agonist at a dose of 50 mg/kg from day 13 to day 17 of pregnancy resulted in an increase in the level of placental BCRP [13].

In order to clarify the mechanisms of the progesterone-induced effect on the BCRP level, we performed *in vitro* studies on HepG2 cells.

Table 2. Serum concentrations of hormones in female rabbits during pregnancy

Hormone	Day 7		Day 14		Day 21		Day 28	
	Control	Pregnancy	Control	Pregnancy	Control	Pregnancy	Control	Pregnancy
Estradiol, pg/ml	323.46±122.50	337.67±139.30	323.70±122.39	304.89±82.93	267.50±41.60	249.35±54.10	269.40±47.92	192.10±118.69
Progesterone, ng/ml	0.46±0.23	4.55±0.77*	0.77±0.38	8.18±1.63*	0.63±0.35	6.97±1.04*	0.73±0.21	3.49±1.63*
Testosterone, nmol/l	1.176±0.52	1.22±0.35	1.13±0.59	1.17±0.48	0.76±0.12	0.94±0.37	0.89±0.31	0.81±0.32
Prolactin, mMU/ml	21.16±4.30	17.26±3.20	22.50±4.70	20.30±2.65	23.00±6.10	23.10±4.90	35.10±14.90	71.53±7.76*

The asterisk (*) indicates statistically significant differences from the parameters of non-pregnant female rabbits, $p<0.05$.

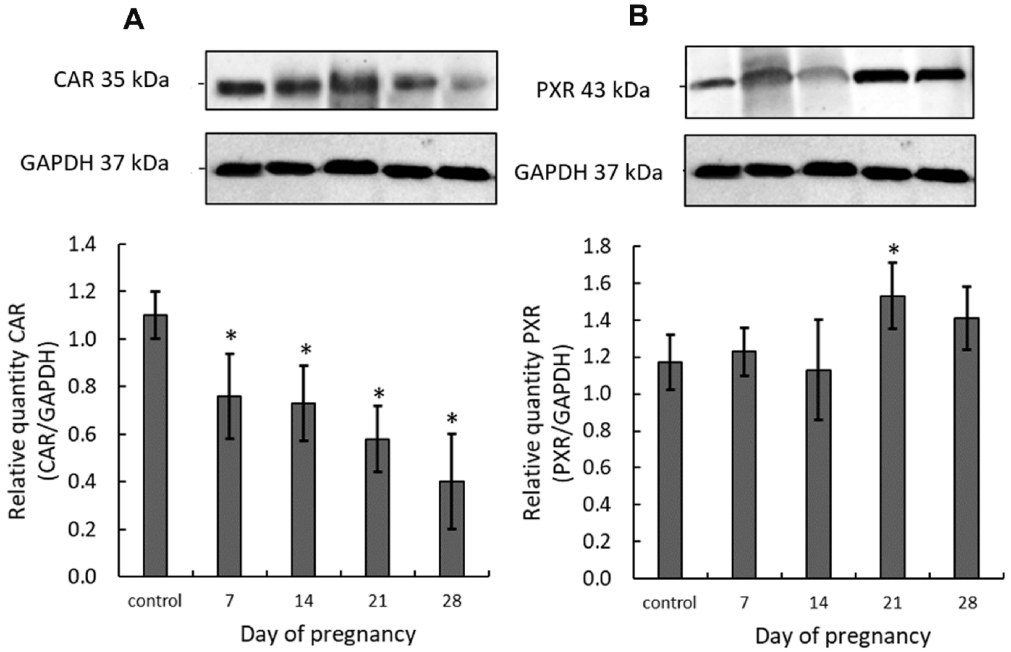

Figure 1. Relative amounts of the constitutive androstane receptor (**A**) and pregnane X receptor (**B**) in the liver of female rabbits at different time points of pregnancy (M±SD). Top – photo of bands obtained with ChemiDocXRS+; bottom: results of densitometric analysis performed using ImageLab software. * – Statistically significant differences from the parameters of non-pregnant female rabbits, $p<0.05$.

Experiments have shown that 10 μM and 100 μM progesterone increased the amount of BCRP by 105.1% and 79.5% (in both cases, $p<0.05$) respectively, as compared with control values (Fig. 2). Inhibition of CAR by CINPA1 did not affect the effect of 100 μM progesterone on the level of BCRP: this parameter increased by 67.5% ($p=0.06$) versus control. The PXR inhibitor ketoconazole prevented an increase in the relative amount of BCRP induced by 100 μM progesterone; in the presence of ketoconazole and progesterone the BCRP level did not differ from the control values (Fig. 2). The data obtained indicate that PXR is involved in the progesterone-induced regulation of BCRP; this is consistent with the literature data on the stimulating effect of progesterone on this nuclear factor [14, 15].

It should be noted that in our study, a significant decrease in the level of CAR in the liver was observed in all the studied periods of pregnancy. In addition, inhibition of HepG2 cell CAR did not eliminate the activating effect of progesterone on the BCRP levels. These results confirm the absence of a role of CAR in the progesterone-induced regulation of the amount of BCRP during pregnancy. In addition, it is known that progesterone is an antagonist of this nuclear factor [16].

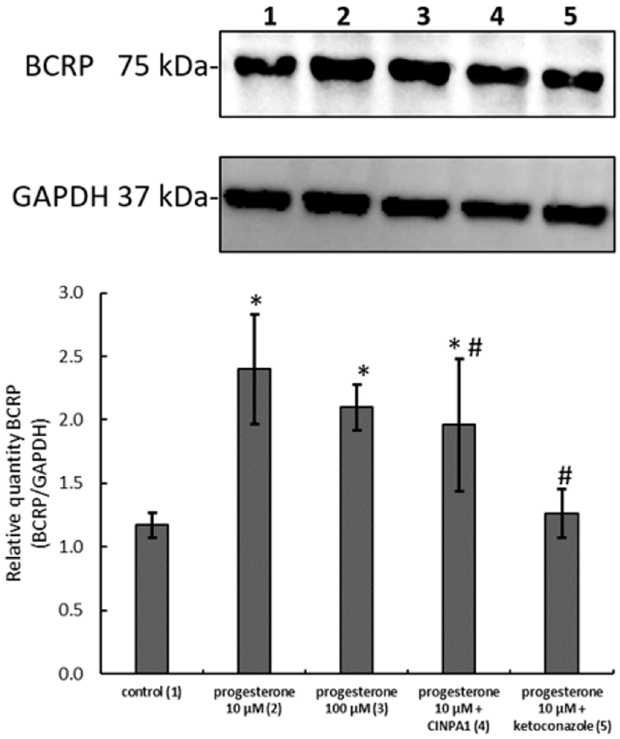

Figure 2. The relative amount of BCRP in intact HepG2 cells in (1), in HepG2 cells incubated with 10 μM progesterone (2), 100 μM progesterone (3), in HepG2 cells incubated with 10 μM progesterone and CAR inhibitor CINPA1 (4), 10 μM progesterone and PXR inhibitor ketoconazole (5) (M±SD). Top – photo of bands obtained with ChemiDocXRS+; bottom: results of densitometric analysis performed using ImageLab software. * – Statistically significant differences from the control group, $p<0.05$; # – statistically significant differences from the 10 μM progesterone group, $p<0.05$.

CONCLUSIONS

Thus, our work has shown that the content of BCRP in the jejunum of rabbits during pregnancy is much higher than in the placenta, liver, kidneys, and cerebral cortex. An increase in the amount of BCRP in the liver of rabbits was noted on the 21st day of pregnancy and a tendency to the increase was also detected on the 28th day; in the kidney and cerebral cortex increased BCRP levels were detected on the 28th day and 14th day of pregnancy, respectively, as compared with non-pregnant females. *In vitro* experiments with HepG2 cells have shown that the increase in the BCRP level is most likely due to an increase in the serum concentration of progesterone and PXR activation.

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COMPLIANCE WITH ETHICAL STANDARDS

Experiments on animals were performed in accordance with the Rules of Laboratory Practice (the Order of the Ministry of Health of the Russian Federation No. 464n of May 18, 2021). The protocol of this study was approved at the meeting of the Commission for the Control of the Keeping and Use of Laboratory Animals no. 46 of June 1, 2022.

CONFLICT OF INTERESTS

The authors declare no conflicts of interests.

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КОЛИЧЕСТВЕННАЯ ОЦЕНКА БЕЛКА РЕЗИСТЕНТНОСТИ РАКА МОЛОЧНОЙ ЖЕЛЕЗЫ ПРИ БЕРЕМЕННОСТИ У КРОЛИКОВ

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Белок резистентности рака молочной железы (breast cancer resistance protein, BCRP, ABCG2) — эффлюксный белок-транспортер, переносящий широкий спектр субстратов из клетки во внеклеточное пространство или полости органов. Целью настоящего исследования была комплексная оценка количества BCRP в различные сроки беременности у кроликов. Содержание BCRP в образцах тощей кишки, печени, почки, коры больших полушарий головного мозга и плаценты кролика определяли методом иммуноферментного анализа, в клетках линии гепатоцеллюлярной карциномы человека (HepG2) — методом вестерн-блот. Для изучения механизмов динамики количества BCRP при беременности были исследованы сывороточные концентрации половых гормонов радиоиммунным методом, а относительное количество конститутивного андростанового рецептора (CAR) и прегнан Х рецептора (PXR) в указанных органах — методом вестерн-блот. Для оценки роли CAR и PXR в регулировании уровня BCRP под влиянием прогестерона были выполнены эксперименты *in vitro* на клетках HepG2. Количество BCRP в тощей кишке беременных кроликов многократно превосходит уровень белка-транспортера в плаценте, печени, почках и коре больших полушарий головного мозга. Увеличение количества BCRP в печени кроликов отмечено на 21 сутки беременности с тенденцией к повышению на 28 сутки, в почке — на 28 сутки беременности, а в коре больших полушарий головного мозга — на 14 сутки беременности по сравнению с небеременными самками. *In vitro* на клетках линии HepG2 установлено, что нарастание уровня изучаемого белка-транспортера обусловлено активирующем влиянием прогестерона на PXR.

Полный текст статьи на русском языке доступен на сайте журнала (http://pbmc.ibmc.msk.ru).

Ключевые слова: белок резистентности рака молочной железы (breast cancer resistance protein, BCRP, ABCG2); количество белка; беременность; прогестерон; конститутивный андростановый рецептор (CAR); прегнан Х рецептор (PXR)

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