**USO DE BIOCORRETORES NA ESTIMULAÇÃO DA REGENERAÇÃO
HEPÁTICA FETAL DE RATOS LESIONADOS MECANICAMENTE****USING OF BIOCORRECTORS IN REGENERATION PACING
OF MECHANICALLY INJURED RATS' FETAL HEPATIC**

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RESUMO

O artigo é dedicado ao estudo da influência de substâncias biologicamente ativas na regeneração de órgãos internos de mamíferos em estágios iniciais de desenvolvimento. A recuperação completa do órgão após o dano não ocorre. É necessário procurar novas maneiras que revelem os processos que contribuem para a regeneração. O estudo dos processos que ocorrem no corpo antes do nascimento dá a oportunidade de entender os mecanismos do que acontece no período pós-natal nos processos patológicos gerais. Foi estudado o dano mecânico ao fígado em fetos de ratos sob a influência dos biocorretores "Trepel" e "Suvar". Métodos morfológicos, morfométricos, histoquímicos e imuno-histoquímicos foram utilizados para o processamento do material. Os resultados do trabalho indicam que o uso combinado desses biocorretores após o desenvolvimento de um foco de necrose no fígado reduz as manifestações alteradas de hepatócitos, inibe a inflamação reativa ao redor do local da lesão, retarda o recrutamento de fibroblastos para a zona de lesão e inibe a colagenogênese. Ao mesmo tempo, os biocorretores têm um efeito estimulante pronunciado na proliferação de hepatócitos, que, no contexto de atividade enzimática aumentada, se manifesta na forma de ativação da divisão mitótica e poliploidização de hepatócitos. Apesar dos sinais de estimulação regenerativa, como resultado a recuperação completa do fígado no local do tecido morto em ratos experimentais não ocorre; há apenas uma redução de 34,7% no foco da fibrose formada no local do tecido hepático morto, em comparação aos animais de controle.

Palavras-chave: *Feto, Hepático, Lesão mecânica, Biocorretores, Hepatócitos.*

ABSTRACT

The article is devoted to studying the influence of biologically active substances on the regeneration of internal organs in mammals at the early stages of development. Complete recovery of the organ after damage does not occur. It is necessary to look for new ways that reveal the processes that contribute to regeneration. The study of processes that occur in the body before birth allows understanding the mechanisms of what happens in the postnatal period in general pathological processes. It was studied the mechanical damage to the liver in rat fetuses under the influence of biocorrectors "Trepel" and "Suvar." Morphological, morphometric, histochemical, and immunohistochemical methods were used for processing the material. The results of the work indicate that the combined use of these biocorrectors after the development of a necrosis focus in the liver reduces alternative manifestations of hepatocytes, inhibits reactive inflammation around the injury site, slows down the recruitment of fibroblasts to the injury zone, and inhibits collagen genesis. At the same time, biocorrectors have a pronounced stimulating effect on proliferation of hepatocytes, which, against the background of increased enzymatic activity, manifests itself in the form of mitotic division activation and polyploidization of hepatocytes. Despite the signs of regenerative stimulation, as a result, complete recovery of the liver in the site of the dead tissue in experimental rats does not occur; there is only a 34.7% decrease in the focus of fibrosis formed in the place of dead liver tissue, compared to control animals.

Keywords: *Fetus, Hepatic, Mechanical injury, Biocorrectors, Hepatocytes.*

1. INTRODUCTION:

Mechanical injury hepatic does not show any regenerative progress neither in late perinatal life (Eltschaninov, 2011), nor after birth (Tournier-Thurneyssen, Feldmann, & Bernuau, 1992). The reason for this is those hepatic hepatocytes belong to highly specialized tissue, unable to regenerate through epimorphosis. Although, nowadays, searching for highly efficient means for the pacing of hepatic regeneration is still in progress, apart from the parallel investigation of stem cells (Wang, Zhao, Fish, Logan, & Nüsse, 2015).

From (Sigareva, Gerashev, Svyatash, & Lucanina, 2001; Solopaeva & Solopaev, 1991; Sosina, Molotkov, & Reshedko, 1993) it is known that during long decades many attempts have been made to pace hepatic regeneration after various injuries until complete organ recovery was achieved. Forth is the purpose, to activate hepatocyte division, operating (partial hepatectomy) (Andreev, Ostroushko, Laptiyova, & Glukhov, 2018; Berg et al., 2018; Soltys & Zuevkiy, 2000) and pharmacological methods Grevtsov & Belkina, 2002; Sigareva et al., 2001; Sosina et al., 1993) were used in experimental and clinical conditions.

In present days, for veterinary medicine performance improvement (G. I. Ivanov & Grigoryev, 1998), as well as for treatment of different human diseases (Grevtsov & Belkina, 2002; L. N. Ivanov & Kolotilova, 2005; Kolotilova & Ivanov, 2003; Malov, Ivanov, & Kolotilov, 2018; Malov, Ivanov, Kolotilova, & Karpunina, 2018; Vasiliev, Skrebkov, & Pavlova, 1988), zeolites of Alatyrskoe field, Chuvash Republic of Russian Federation are used. "Trepel" is a natural loses sedimentary rock with a silicon base and rich microelement composition (Malov, 2017).

"Suvar" biocorrector is obtained in the laboratory of I.N. Yulianov Chuvash State University and represents natural terpenoids compound with significant microelement content. Technically it is conifer crude turpentine derivative product (Zazhivikhina, 1998). "Suvar" possesses a stimulating effect on human and animal organisms (Frolov, 2003; Petryankin, & Sherne, 1999; Zazhivikhina, 1998). It is proved that the joint application of "Trepel" and "Suvar" favors the development of various mammal organism functional systems, and has immunostimulating and growth promotion effect (Arkhipova, 2008; Sigareva et al., 2001; Tarasov, 2001).

This study aimed to determine the positive influence of the combined application of "Trepel"

and "Suvar" biocorrectors on rats' fetus hepatic regeneration after mechanical injury.

2. MATERIALS AND METHODS:

The experiment included 23 pregnant females of outbred rats 257-310 grams of weight. Rat's fetus at the prenatal stage received a mechanical injury of hepatic. Using the innovator method (Chuvash State University named after I.N. Ulyanov Patent RU 2262135, 2005), pregnant female shave undergone an autopsy of the abdominal cavity, in sterile conditions under deep ether-mask anesthesia on the 17th – 19th day of pregnancy.

Uterus, together with a fetus inside, was taken through the operating wound; afterward, along the upper pole, an incision was made. Then a steel stop needle was used (Chuvash State University named after I.N. Ulyanov Patent RU 2262135, 2005), and firstly, membrane rupture was implemented, and later on, the same procedure was made for a well visible fetus (under visual control), in the right hypochondriac region, in hepatic projection point at the depth 0.4-0.5 cm. As are sultofrupturein the hepatic, there appeared stereotypic damage is with dimensions $3085.7 \pm 368.3 \mu\text{m}^2$.

At the end of the rapture procedure, the needle was extracted, and the uterus incision was suture closed. After all, fetuses have systematically undergone the same process; the skin was closed with knot silk suture. At the early stages of observation (1st and 3rd day after the operation), live fetuses were extracted from the uterus employing cesarian operation; later (5 and more days after the operation), female rats independently delivered viable infant rats, which grew and developed according to age. Infant rats got ether-killed in 1, 2, 3, 4, 5, 7, 9, 11, 15, 20, and 30 days after hepatic mechanical injury operation, in the morning from 7.10 to 9 o'clock.

In total, 112 operated fetuses were taken for experiment. The investigation was conducted in accordance with the order of Ministry of Higher and Secondary Education of the USSR dated 13.11.1984 "On the rules of work using experimental animals" (Interstate Council for Standardization, Metrology, and Certification, 2016), and according to Declaration of Helsinki 1975, revision 2000 "On the rules of work using experimental animals." Stillbirth part among infant rats comprised 3% and was not included in analyzed material. Female rats bore an operation satisfactorily and soon afterward were able to pair and get pregnant again.

Rat females from the moment of operation to together with regular feed started receiving biocorrectors "Trepel" at a dose of 1.25 g/kg and "Suvar" at a dose of 0.05 g/kg of weight. After birth and along with maturity, infant rats, apart from milk feeding, started having common feed containing biologics.

Control was performed on fetuses of the same age (p-78) similarly operated, but the dietary intake did not include biocorrectors.

Obtained material processing included histological staining with hematoxylin, eosin, and cerasine red, as well as histochemical methods. Tetrazolium method (according to Z. Lloyd) was used to determine SDH (succinate dehydrogenase), NADH (nicotinamide adenine dinucleotide, reduced form) and NADPH (nicotinamide adenine dinucleotide, reduced form). Acid phosphatase was defined through method of simultaneous combination with naphthol phosphates AS and permanent salts of diazonium (according to Z. Lloyd). Alkaline phosphatase was determined by Barston method (Lloyd, Gossrau, & Shibler, 1982).

Quantitative assessment of the enzymatic activity of hepatocytes in dynamics was carried out using photometric measurements, conducted in transmitted light on a "Micromed" microscope with a photomodule FMEL-1 and FEU-79 and an output voltage of the amplifier of 1200 V. In order to obtain a monochromatic light beam in the red region of the spectrum passing through the preparation, an interference filter was used with a maximum light transmission at a wavelength of 620 nm. The light transmission was recorded using a Sch-4300 digital voltmeter, after which, by negative decimal logarithm, the light transmission level was transformed into light absorption, which was expressed in units of optical density. According to the law of Lambert-Bouguer-Baer, the optical density of the preparation is proportional to the amount of stain. In turn, the described method meets the requirements of proportionality of stain concentration and enzyme activity. Optical density is calculated as follows:

$$O.D. = LgUi/100.$$

To detect the DNA content in the nuclei of hepatocytes, the Fölgen reaction was performed (hydrolysis in 5N HCL at 20 degrees for 20 minutes, followed by staining with Schiff's reagent for 1 hour at room temperature). The amount of DNA in the nuclei was determined using a Biolam-70 light microscope with photometry using an FMEL-1 component and an FEU-79A photometer in transmitted light with a blocking filter of maximum light transmission at a wavelength of

570 nm and a supplied voltage of 900 V. The results were subjected to the inverse decimal logarithm according to the formula:

$$A=LgUi/U_0,$$

where A – optical density;

Ui – voltage meter reading on the nucleus;

U₀ - voltmeter readings on unstained areas of the preparation.

Peripheral blood lymphocytes and small lymph nodes lymphocytes served as a diploid standard.

The immunohistochemical method was used to further evaluate the proliferative activity of hepatocytes after a mechanical injury to the hepatic that takes place under the use of biologically-active agents (Kumar & Rudbeck, 2011). For immunohistochemical studies, two commercial monoclonal antibodies by Santa Cruze were used: 1) Ki-67 proliferative activity marker; 2) a marker of apoptosis Bcl-2. Immunohistochemical studies were performed according to a standard protocol. Sections 3 μm thick were applied to high-adhesive glass treated with L-polisine and dried at room temperature for 24 hours. Staining was carried out manually and by hardware using immunohistochemical containers AUTOSTAINER-360 (THERMO, UK) and Leica BOND-MAX (Germany), as well as through Envision imaging systems (DACO Denmark) and NovoLincpolimer (NovoCastr, UK). Nonimmune rabbit and mouse sera, as well as sections of control hepatic tissues, served as a control of the sensitivity and specificity of the reaction. Results of the responses were evaluated by counting the number of stained nuclei per 200 hepatocyte nuclei in six fields of view. Then, the ratio of stained hepatocytes to the total number of hepatocytes was calculated, and the results were expressed as a percentage.

Statistical processing of digital data was carried out according to the particular program "Statistics" with the assistance of the Microsoft office software package (MS Word and MS Excel) on a Pentium 166 MMX computer. Statistical significance was determined by Student's t-test.

3. RESULTS AND DISCUSSION:

Histological changes one day after the operation were characterized by a predominance of dystrophic and necrobiotic changes in hepatocytes. Microscopic preparations of the hepatic taken from the site of injury revealed slit damage to the hepatic tissue, in which dead

hepatocytes and erythrocytes were noted. These changes have occurred in both experimental and control animals.

On the 3rd and, especially, the 5th day, a cluster of cells appeared on the periphery of the necrotic focus, represented by macrophages and lymphocytes. In control of infant rats, the intensity of the cellular inflammatory response was more pronounced in comparison with experimental animals, which visually showed a weaker presence of inflammatory cellular elements, macrophages, and lymphocytes around the injury site. On the 7th-9th day, in experimental animals, fibroblasts are fixed among inflammatory cells; fibrous connective tissue is formed 11 days after the operation. In control of infant rats, the appearance of fibroblasts is noted earlier (on the 5th-7th day), and, respectively, the formation of fibrous connective tissue occurs in them earlier - on the 9th day. Thus, among experimental rats, as well as in control, the fibrous connective tissue is found in place of the dead hepatic tissue (Figure 1). However, in experimental animals, it formed later, and the area of the connective tissue scar was 34.7% less than among control rats.

Starting from the first day after operation, hepatocyte proliferation was activated in hepatic of both experimental and control rats (Michalopoulos, 2010). Microscopic preparations revealed all phases of the mitotic cycle, from metaphases to late telophases. The number of mitoses in experimental animals was statistically significantly higher than in control animals ($p < 0.001$). The peak of hepatocyte proliferation in experimental rats was observed in the period from 3 to 11 days; in control animals, the highest number of mitoses was recorded on days 3–7. Mitotic division in experimental pups was observed up to 20 days after the operation. In control, they were absent in the hepatic so far after 11 days, and until the end of the experiment.

Along with mitoses, starting from the 7th day after the operation, the number of binuclear hepatocytes in the hepatic increased; this was the case in animals of both groups, however, compared with the control, in experimental animals, there was a statistically significantly greater number ($p < 0.001$). This trend was observed until the end of the experiment.

An immunohistochemical study confirmed the activation of hepatocyte proliferation: a statistically significant increase in Ki-67 positive nuclei was observed in experimental animals compared to control ones (Figure 2).

Most often, Ki-67 positive nuclei are determined on the 3.5 and 9 days after operation ($p < 0.001$). Almost simultaneously with the activation of hepatocyte proliferation, their hypertrophy and polyploidy increased. From 3 days to 30 days, tetraploid nuclei began to predominate in animals. Moreover, in experimental rats, polyploidy was more pronounced, which manifested itself in the fact that tetraploid cells prevailed over diploid cells, in contrast to the hepatic of control rats. In experimental fetuses, hepatocytes of higher ploidy (8p, 16p, and even 32p) were often fixed, which was absent in control (Table 1).

Hypertrophy of hepatocytes and their nuclei began to increase from the 5th and, especially, from the 7th day, reaching the greatest value on the 20th and 30th days after the operation; in experimental fetuses, it was more pronounced. Restorative processes in the hepatic, especially in experimental rats, took place against the background of an increase in the vital activity of hepatic cells: an increase in the activity of SDH, NADH, NADPH was noted, the number of binucleate hepatocytes went up significantly, which indicates an increase in the protein-synthetic function of hepatic cells (Brodskaya et al., 2010) (Figure 3).

Electron microscopy revealed changes of two types. Hepatocytes with signs of degeneration, manifested by swelling of the mitochondria, focal enlightenment of the matrix, and also a change in the orientation of the cristae up to a decrease in their number in the cells, were observed. There was disorganization of the membranes of the organelles. At the same time, there were cells with changes that indicated the intensity of intracellular hyperplastic processes. This was manifested by hypertrophy and swelling of mitochondria, the convergence of the membranes of the endoplasmic reticulum, hypertrophy, and hyperplasia of ribosomes. These changes could be interpreted as a sign of intracellular regeneration.

Data obtained in the course of this work are generally consistent with the literature references on the morphogenesis of the healing of hepatic injury of various origins and development of regenerative manifestations (Baidyuk, Korshak, Karpov, Kudryavtsev, & Sakuta, 2012). In particular, present research revealed confirmation that healing of hepatic damage always occurs due to regenerative hypertrophy (Eltschaninov, 2011; Tournier-Thurneysen et al., 1992). Increase in the proliferation of hepatocytes was also expected, as it was repeatedly indicated in the

works of various authors (Celton-Morizur & Desdouets, 2010; Eltschaninov & Bolshakova, 2012; Malato et al., 2011; Sakuta, Baidyuk, Zhumagalieva, & Kudryavtsev, 2011; Solopaeva & Solopaev, 1991; Soltys & Zuevkiy, 2000; Yanger et al., 2014).

Also, in this study, the fact of an increasing number of binuclear hepatocytes (Berezovskiy, Yanko, Litovka, & Volovich, 2012; Stupin et al., 1995), and development of hypertrophy and polyploidy of hepatocytes (Soltys and Zuevkiy, 2000) was noted. Proliferation was activated against the background of increased protein-synthetic and enzymatic activity of hepatocytes, also earlier reported (Soltys and Zuevkiy, 2000). Even though all manifestations of a regenerative nature in experimental rats were more intense, complete organ restoration did not take place. Nevertheless, the focus of fibrosis that occurs at the site of dead hepatic tissue in experimental rats was visually much smaller than in control animals, which can be regarded as an encouraging fact, indicating that stimulation of hepatic regeneration is fundamentally possible (Koroy, Yagoda, Gilyazova, & Mukhoramova, 2014).

In previously published works (Juan, Benito, Alvarez, & Fabregat, 1992; Romanova, 2012; Romanova & Malyshev, 2012), an effect on the regeneration of fetal hepatic after a mechanical injury was studied separately for "Trepel" and "Suvar." "Trepel" when introduced into the diet of animals after the operation, is a powerful detoxification agent, which lowered the alternative processes in the hepatic of rat fetuses, suppressed the inflammatory reaction along the periphery of the injury site, and slowed the recruitment of fibroblasts into inflammatory infiltrate.

When only "Suvar" was used in the animal diet, in response to mechanical damage to the hepatic in rat fetuses, hepatocyte proliferation was activated (Romanova, 2012). Thus, "Trepel" and "Suvar", introduced into rat food in isolation, peculiarly influenced the healing morphogenesis but did not lead to a decrease in the size of the connective tissue scar that occurs in the hepatic at the area of dead tissue.

Significant in the study were the results of the combined effect of "Trepel" and "Suvar" biocorrectors on healing of mechanical injury of rat fetuses, which was realized in reducing the area of post-traumatic fibrous connective tissue by 34.7% compared with the control. This follows from the fact that combined action of "Trepel" and "Suvar" biocorrectors led to decrease in the intensity of

alternative manifestations in hepatocytes, reduced inflammatory changes around the injury site, and slowed the recruitment of fibroblasts to the area of injury, which, in turn, reduced the intensity of collagen formation. The described processes proceeded against the background of a revitalization of hepatocyte proliferation, which manifests itself in the form of activation of mitotic division, an increase in the number of binuclear hepatocytes, polyploidy and hypertrophy.

The theoretical significance of the results is that for the first time, data was obtained on the stimulating effect on rat hepatic regeneration using "Trepel" and "Suvar" biocorrectors. This may be a prerequisite for their further study in various pathological processes in mammals, both in the prenatal and postnatal periods. Successful surgical interventions in the organs of the mammals' fetuses, including hepatic (Chuvash State University named after I.N. Ulyanov Patent RU 2262135, 2005), were first performed in Russia; biocorrectors "Trepel" and "Suvar" is a purely Russian development. Literature references search established that abroad there are no works even closely related to this subject. Therefore, this study can attract attention abroad and stimulate further development of the problem.

4. CONCLUSION:

Thus, in experimental animals, the size of the focus of fibrous connective tissue developing at the area of dead hepatic tissue was 34.7% smaller than in infant rats of the control group. The stimulating effect of biocorrectors on organ restoration is explained by their mechanism of action. It has been established that combined use of the "Trepel" and "Suvar" biocorrectors in veterinary medicine is accompanied by hypertrophy of the endocrine glands: adrenal glands (mainly their cortex), thyroid gland (follicular epithelium and follicles themselves), testes. This leads to an increase in production of the corresponding hormones. Female rats receiving biocorrectors as a food supplement feed their newborn rats with milk with a significantly higher hormone content.

The excess content of glucocorticoids in the body of experimental infant rats explains the absence of a pronounced inflammatory reaction around the injury and inhibition of collagen genesis. Iodine-containing thyroid hormones, activating all types of metabolism, sharply increase cellular metabolism, and stimulate the proliferation of hepatocytes. Thus, based on a change in the hormonal background of the

organism of experimental rats, it can be concluded with a high degree of probability that hypertrophied hepatic cells, while slowing down the development of connective tissue, mechanically compress the site of organ damage. Proliferating hepatocytes gradually fill the post-traumatic hepatic defect, as a result of which, along with the connective tissue, it is replaced for a considerable extent by newly formed and hypertrophic hepatocytes. This led to the fact that the size of the focus of fibrosis in experimental infant rats was 34.7% smaller than in control animals.

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Table 1. Karyometric data on the ploidy of hepatocyte nuclei in infant rats depending on time after operation

Time after operation, day	Diploid nuclei, %	Tetraploid nuclei, %	Octaploid nuclei, %	16-ploid nuclei, %	32-ploid nuclei, %
3	40.6±3.7	56.2±6.2	1.0±0.6	8.4±2.7	1.6±0.5
5	36.4±4.3	60.6±5.3	2.7±0.9	9.1±3.6	3.0±1.4
7	29.1±4.6	62.8±7.2	6.1±1.4	8.5±2.8	2.8±0.8
9	37.2±3.8	59.1±6.3	2.4±1.2	7.0±3.4	2.1±0.8
11	35.4±4.5	61.5±7.4	2.1±0.8	3.3±1.6	0.9±0.3

Figure 1. Connective tissue portion which appeared in place of injury: a - experiment, b – control. Staining with hematoxylin and eosin x240

Figure 2. Stained with hepatocyte proliferation marker Ki-67 in the experiment (a) and in control (b)

Figure 3. *NADH activity in 5 days after operation (a – experiment, b – control), Biolamx 900*