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Research Article

Recovery of the organism of poikilothermic hydrobionts using mammalian stem cells

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Abstract. In this paper, we present the results of four experiment variants on the effect of stem cells from mice donor on different species of hydrobionts: *Pontastacus leptodactylus* long-sided river crayfish, carp *Cyprinus carpio* L., Axolotl - neotenic larva Mexican *Ambystoma mexicanum Ambystoma*. There were investigated their reproductive activity and the restoration of the organism after various kinds of damage. In the first experiment variant, the lipid and protein dystrophy of hepatopancreas was modeled in the fish of the control and experimental groups by administering per os of paracetamol at a dose of 15 g / kg seven times for 14 days. The investigated fish were then intravenously injected with 10 million bone marrow cells (BMC). In contrast to the control group, 14 days after the administration of BMC in the experimental group, restoration of the histological structure of hepatopancreas was observed. The second variant of the experiment included the modeling of the pathology of hepatopancreas of crayfish with a single injection of alloxane in the ventral sinus at a dose of 50 mg / kg. 14 days later, in an experimental group of crayfish, the stem cells of from donor mice were injected once into the ventral sinus at a dose of 10 million BMC. In the control group there was a disruption in the structure of hepatopancreas: vacuolation of the cytoplasm of resorptive (R) cells. In the experimental group, restoration of the organ structure was noticed, which is confirmed by histological studies investigation. In the third variant of the experiment, the anterior limb was amputated from the axolotls to the shoulder joint. The specimens of the experimental group were simultaneously injected with mouse stem cells at a dose of 10 million BMC. The axolotls of the experimental group regenerated the lost limb more quickly. The fourth variant of the experiment was conducted on mature males and females *Pontastacus leptodactylus*. In crayfish of the experimental group were introduced stem cells from donor mice. There was no temperature stimulation of reproduction. As a result, in the experimental group of crayfish, in contrast to the control, there was observed the laying and fertilization of caviar.

Key Words: stem cells, hydrobionts, fish, crayfish, axolotl, regeneration, reproductive activity.

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Introduction

Recently, much attention is being paid to the study of the effect of stem cells on the recovery of the organism. Entered into the body, stem cells release active substances (growth factors, cytokines and others), have a contact effect on surrounding cells merging with damaged cells. Due to its ability to proliferate and differentiate, stem cells are the ideal cellular material for therapy. The use of embryonic stem cells (ESC) is limited due to the fact that the processes of their division and development after transplantation into the recipient organism cannot be controlled. Transplanted into an adult organism, they follow process of multidirectional differentiation form (teratomas and teratocarcinomas), consisting in the derivatives of three embryonic leaves (ectoderm, endoderm and mesoderm). An inflammatory response to graft rejection is also possible (Keirstead et al 2005; Stadtfeld et al 2014).

Fetal stem cells (CSFs) are precursor cells of a higher degree of differentiation and do not pose a risk of carcinogenesis. However, with their transplantation, there is a risk of their immune rejection (Deans & Moseley 2000; Sottile 2007).

For these reasons, researchers are attracted to adult stem cells, which are known to be found in a number of tissues, in particular adipose tissue, skin, kidneys, peripheral blood, brain, and bone marrow. Most of these cells are capable of giving rise only to the elements of the tissue from which they originate, but the number of such regional stem cells (SCs) is very small, and they normally serve rather for physiological tissue renewal than for its posttraumatic regeneration (Lu et al 2003; Tomchuck et al 2007; Michalopoulos 2010).

From all adult stem cells of mammals, including humans, the most accessible are the so-called mesenchymal stem cells (MSCs) present in the bone marrow stroma, cultured and subsequent used. It is possible to transplant these cells to recipients by intravenous infusion.

Studies of the biology of adult stem cells are an urgent task of modern experimental medicine and cell biology (Muraglia et al 2000; Toma et al 2002; Pettinger & Martin 2004).

Mesenchymal stem cells (MSCs) are used in cardiology, neurology, traumatology. A small number of MSCs are represented (1: 104-105 mononuclear cells) in the human bone marrow (Zhao et al 2004), where they participate in the formation of

the stroma necessary to maintain homeostasis and the functioning of intrinsic and transplanted hemopoietic cells (Koc et al 2000; Noort et al 2002).
MSCs promotes the growth of hematopoietic progenitors by secretion of a number of cytokines such as IL-6, IL-7, 8, 11, 12, 14, 15, LIF factor (leukemia-inhibitory factor), macrophage colony-stimulating factor (M-CSF), granulocyte- macrophage colony stimulating factor (GM-CSF), stem cell growth factor (Jorgensen et al 2003; Zhao et al 2004).
Pluripotency of MSCs, specific migration ability in the area of damage and adhesive properties cause their restoration function. MSCs are able to migrate to the area of damage, fix, differentiate and carry out the function of substituted cells. These properties make it possible to use MSCs for tissue repair and regeneration (Azizi et al 1998; Jin et al 2002; Horwitz et al 2002).
Several studies showed that MSCs are not rejected for allo- and xenotransplantation (Liechty et al 2000; Di Nicola et al 2002; Chiu 2004).
A variant of xenotransplantation is the method of using animal cells extracted from an embryo or fetus of a sheep which is further injected into the patient's body in order to achieve a revitalization effect (Tomchuck et al 2007; Sottile 2007; Ryu 2009, Williams et al 2013). Of course, animal cells are not able to integrate into the patient's body, but they supply it with humoral factors that promote healing and activate its immune system.
Organoids from pluripotent stem cells are generated in vitro using two-dimensional and 3D cultures that repeat the properties of various specific sub-regions of a variety of human organs (Hisha et al 2013; Ader & Tanaka 2014; Aihara et al 2015).
The use of stem cells in medicine and even more, in veterinary medicine, is limited due to economic reasons, namely due to poor availability of stem cell sources. Therefore, the search for alternative methods of obtaining stem cells is a task of the utmost importance. For humane reasons and due to high availability, use of hydrobionts as donors of these cells may be considered as a possible opportunity. First step is the use of mammalian stem cells in hydrobionts. In an experiment, we observed restoration of affected organs and tissues of crayfish and fish following administration of mouse stem cells and progenitor cells. This work is a continuation of the results obtained earlier. The goal of the study was to analyze the effects of mouse stem cells on poikilothermic hydrobionts.

Materials and methods

This article does not contain any studies with human participants performed by any of the authors.
The experiments were carried out in accordance with the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes, ETS №123, Strasbourg, 1986. The experimental protocol was approved by the Ethics Committee of the Federal State-Funded Scientific Institution All-Russian Research Institute of Irrigation Fish-Breeding.
The investigations were performed on two-years old mirror and scaly carps (*Cyprinus carpio* L.), weighing 30-32 g, two-years old axolotls (*Ambystoma mexicanum*), weighing 15-16 g, and mature crayfish (*Pontastacus leptodactylus*), weighing 31-33 g. Hydrobionts were kept in 160 L aquariums equipped with water purification system and pump aeration. The animals were fed with the larvae of *Chironomidae* ad libitum. Water temperature was 15°C.
No cases of death of tested hydrobionts were observed.
To study the effect of mouse stem cells on the organism of hydrobionts, four variants of the experiment were conducted:
1. In fish control (n = 16) and experimental group (n = 16), lipid and protein dystrophy of hepatopancreas was modeled by administration of per os of paracetamol at a dose of 15 g / kg seven times for 14 days. On the 15th day, 10 million BMC were administered intravenously to experimental fish.
2. The simulation of hepatopancreas pathology of crayfish (n = 24) with a single injection of alloxane in the ventral sinus at a dose of 50 mg / kg was performed. For the experimental group of cancers (n = 12) on day 15, the stem cells of donor mice were injected once into the ventral sinus at a dose of 10 million BMC.
3. The experimental group of axolotls (n = 14) the anterior limb to the shoulder joint was amputated. Control axolotls stem cells were not introduced. The animals of the experimental group (n = 7) at the same time were injected with stem cells of mice at a dose of 10 million BMC.
4. The crayfish *Pontastacus leptodactylus* without pathology were divided into 2 groups: the control (4 males and 4 females) and the group for experiment (4 males and 4 females). In cancers of the experimental group were introduced stem cells of a donor mice at a dose of 10 million BMC. A temperature stimulation of reproduction (temporary decrease in water temperature) was not performed.
The procedures of bone marrow cell (BMC) isolation and culture were conducted according to general rules of culture-based studies of cells obtained from live and dead donors (within 30-40 minutes from death of test animals). To evaluate the viability of hematopoietic and stromal fractions of BMC, the cells were analyzed using trypan blue staining.
Bone marrow cell sampling was performed in murine donors (with GFP gene expression) under ether anesthesia. Under sterile conditions, the forearm, shoulder, shank and thigh bones were dissected together with the respective joints and separated from the muscles. Then the bones were placed in 70% alcohol; under sterile conditions, the joints were dissected with scissors and BMC were washed out from the medullary cavity with Ca+2 and Mg+2 free Hank's solution using a syringe (1 mL or 2 mL). The obtained mixed cell suspension was centrifuged with lysis solution (114 mM NH4Cl; 7.5 mM KHCO3; 100 µm EDTA) in the ratio 1:4 for 5 min at 1,500 rpm at room temperature (t = 22^0C).
Then the supernatant was removed by aspiration. The obtained cell mixture was washed to removed red blood cells and resuspended in DMEM culture medium (PanEco) containing 25 mM HEPES, 0.58 g/L glutamine, 100 µg/L gentamycin, 10% fetal bovine serum (HyClone, USA), 5 µg/L insulin. The cells were cultured in vials at +37^0C in a CO2 incubator with 5% CO_2 content and 95% humidity for 3 days.
In 3 days, murine BMC culture contained up to 50% free floating in the suspension with culture medium unattached hematopoietic cells at different stages of differentiation (hematopoietic cells, lymphocytes, monocytes) and up to 50% fibroblast-like cells attached to the surface of the plastic (multipotent mesenchymal stromal cells [MMSC]).

Transplantation of cultured BMC was performed via intravenous injection to fish recipients, via injection into the ventral sinus to crayfish, and via intraperitoneal injection to axolotls. Necropsies of tested animals were performed on Days 7, 14 and 21 following BMC administration.

Organs and tissues obtained from test hydrobionts were taken for histological examination only. Impression smears were made from dissected stump of axolotls.

Studies of structural impairment in the parenchyma of the internal organs were conducted using morphological methods.

The internal organs were fixed in 10% formalin solution. Following dehydration and embedding in paraffin, the specimens were cut using a microtome to obtain histological sections. The sections were further stained with hematoxylin-eosin and examined for severity of structural impairment in the organs. Histological examinations were performed using digital microscopes.

Statistical data analysis was conducted by means of variation statistics method using Microsoft Excel software; the results were interpreted using Student's t test. The differences were considered statistically significant if $p<0.05$.

Polymerase Chain Reaction (PCR). For construction of specific primers and a GFP-specific probe, bioinformation analysis using Vector NTI Advance 9.0 (PC), DNASTAR, BLAST software package was applied.

Extraction of DNA from the examined organs and tissues was conducted using an AmpliPrime RIBO-sorb reagent set for RNA/DNA extraction from clinical material (AmpliSense, Moscow).

For detection of GFP gene expression, specific oligonucleotide primers and probes were used. Synthesis of primers and the probe was performed using an automated DNA synthesizer ASM-800 by amidophosphite method at "DNA-Synthesis" CJSC (Moscow).

Amplification stage was conducted in 25 μL of the mixture composed of: PCR buffer (x10): 700 mM Tris-HCl, pH 8.6 / 25^0C, 166 mM (NH4)2SO4, 25 mM MgCl2, 0.2 mM dNTPs, Taq - polymerase, using a CFX-96 amplifier (Bio-Rad, USA). Primers: GFPF, GFPR, FAM-containing probe and a BHQ1:GFPZ quencher.

DNA-probe is a DNA fragment labeled by any method and used for hybridization with a specific DNA site. It allows identifying complementary nucleotide sequences.

The following reaction conditions were used for GFP fragment amplification: 95°C - 15 min, then 40 cycles as follows: 95°C – 20 sec, 54°C – 30 sec, 72°C – 20 sec and cooling down to 4°C.

Analysis of the results was conducted using conventional PCR technique with subsequent visualization, and real-time PCR.

After the end of amplification, 10 μL of amplicons were mixed with 6X buffer for loading and introduced into 2% agarose gel (0.5 μg/mL ethidium bromide). The size of an amplified product was determined using a restriction fragment length marker of about 1000-50 bp at concentration of 0.5 μg/mL in a buffer for application on the gel ("Sileks" CJSC, Moscow).

PCR specificity was evaluated based on comparison of amplification product size with the size estimated for each couple of GFP gene primers for a specific specimen examined.

Results

Fish with paracetamol-induced liver pathology demonstrated dystrophic changes in hepatocytes (fatty liver and protein-related liver dystrophy). These changes were of different severity (from focal to subtotal). Some areas demonstrated death of hepatocytes, foci with heterogeneous cytoplasm staining, mild or moderate round cell infiltration of portal tracts, moderate serous inflammation in the wall of adjacent large bile duct showing vacuolar dystrophy of the lining epithelium (Fig 1A).

The hepatopancreas tissue of crayfish with alloxan-induced pathology showed increased vacuolization of R-cells, areas of necrosis of groups of these cells and isolated tubules. Other findings included moderate to severe stromal edema, mild to moderate hemocyte infiltration of the intertubular stroma. Fourteen days after alloxan administration, the hepatopancreas consisted of isolated tubules lined with flattened epithelial cells. The tubules had significantly dilated lumens and were filled with thick secretion (Fig. 2A).

Following stem cell administration to crayfish (in 14 days), the hepatopancreas with alloxane-induced pathological changes demonstrated signs of restoration (Fig 2B): reduced vacuolization of the cytoplasm of R-cells (most vacuoles were small). The shape and position of the nucleus were intact. Some areas showed mild to moderate edema of the intertubular stroma with heterogeneous, mainly insignificant infiltration, and loosely distributed hemocytes. The lumens of the tubules in most fields of view are patent.

The study of the effects of stem cells on limb regeneration in *Ambystoma larvae* - axolotls of the control group showed the second stage of regeneration in 3 weeks: about 2-mm apical epithelial cap (table 1).

Axolotls with amputated limbs which were injected with stem cells, demonstrated significantly larger apical epithelial cap (by 50%). The growing part of the stump showed a bump of a millet seed size - third stage of regeneration; 6 weeks following stem cell administration, finger formation (4th stage of regeneration) was observed.

Interestingly, after repeated limb amputation axolotls demonstrated similar tendency - study group showed more rapid regeneration as compared to control group.

The studies of reproductive activity demonstrated that three females of narrow-clawed crayfish showed spawning, fertilization and egg attachment two weeks after stem cell administration. The attached spermatophores were clearly visible on their ventral side between the cephalothorax and the abdomen (Fig 3). The control group of animals did not demonstrate female fertilization throughout the whole experiment.

The obtained results indicate initiation of sexual activity.

None of the tissue samples obtained from hydrobionts injected with stem cells showed the presence of green fluorescent protein (GFP) gene by PCR.

Discussion

Restoration of damaged structures of the parenchymal organs of fish and crayfish, which were injected with stem cells from donor mice, is probably due to the release of biologically active substances by these cells that trigger a cascade of enzymes that stimulate reparative regeneration. The absence of the green protein gene (GFP) in the experimental samples indicates that replacement of the hepatopancreas defect of hydrobionts does not occur due to the embedding and differentiation of mouse stem cells. Which is natural: too great differences in the genome, as

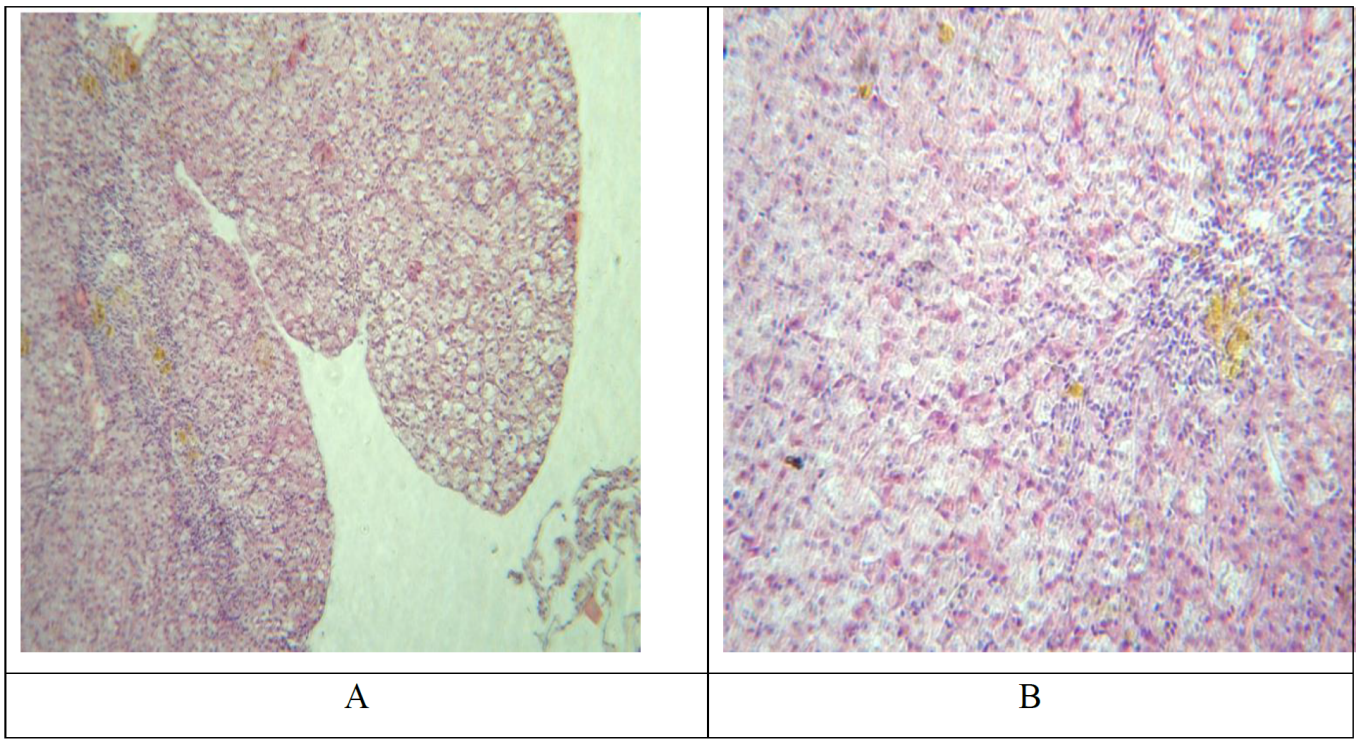


Figure 1 - Restoration of the structure of the carp liver following administration of stem cells.
A. Pathological changes in the carp liver following paracetamol administration: foci of hepatic steatosis, foci with significant dystrophic changes in hepatocytes (small number of them are anucleate). Magnification 200.
B. The carp liver after administration of stem cells. Magnification 400.

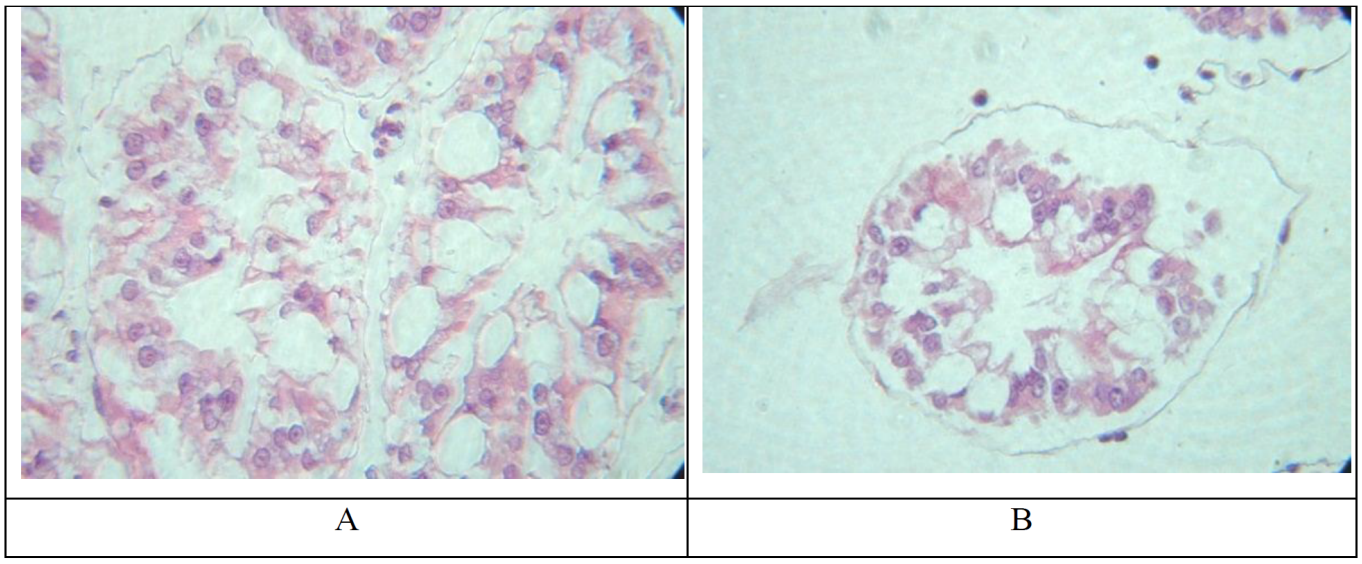


Figure 2 - Changes in the structure of the crayfish hepatopancreas with pathology compared to that after mouse stem cell administration. Magnification 400.
A. Alloxan-induced pathological changes in the hepatopancreas.
B. Signs of regeneration of pathologically altered hepatopancreas in 14 days after stem cell administration.

donors and recipients occupy a different, far-behind from each other systematic position.

Acceleration of regeneration of the lost limb of axolotls after the introduction of mouse stem cells is apparently due to the fact that stem cells of mice have an indirect effect on the body of hydrobionts by secretion of cytokines that activate the growth of their own hematopoietic progenitors. Correspondingly, the proliferation of blast cell forms, their maturation, migration to the defect zone and replacement of tissue is intensified.

Stimulation of the reproductive activity of crayfish is also probably associated with the action of secreted stem cells of biologically active substances. The circulatory system of crayfish is not closed, therefore, stem cells of the mouse inserted into the ventral sinus, go directly to the hemolymph. All enzymes

Table 1. Regeneration of amputated limbs in axolotls

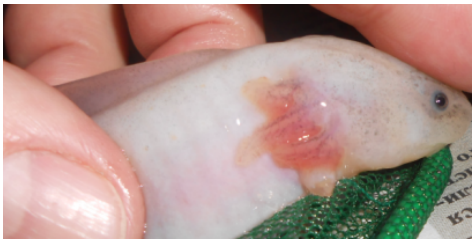
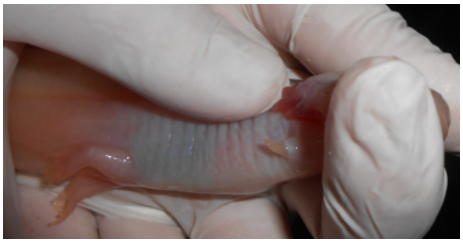
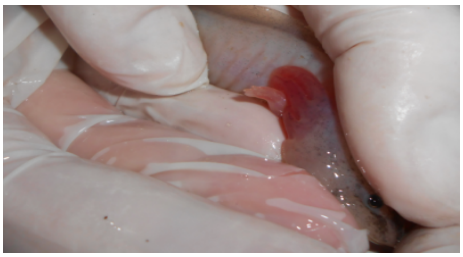
	Time after stem cell administration to the experimental group of axolotls with amputated limbs	
	3 weeks	6 weeks
Control group (no stem cell administration)		
Study group (stem cell administration)		

Figure 3 - A female of narrow-clawed crayfish *Pontastacus leptodactylus* with eggs after stem cell administration

involved in the regulation of reproduction of crayfish are currently unknown. But we can assume that the cytokines of stem cells activate cytochrome P450 enzymes (CYP enzymes), which in turn trigger a cascade of reactions that stimulates the reproduction function.

We made such an assumption, since it was revealed that these enzymes catalyse important metabolic reactions of exogenous and endogenous substrates, including steroid hormones of river cancer. Two complete cDNAs isolated from crab hepatopancreas encode CYP enzymes named CYP330A1 and CYP4C39. It is interesting that CYP4C39 on 59.5% is identical to crayfish CYP4C15, and CYP330A1 on 37.3% is identical to mouse CYP2J6 (Rewitz et al., 2003).

Conclusions

The administration of murine donor-derived stem cells to hydrobionts results in restoration of pathologically altered parenchymal organs of fish and crayfish (the liver and the hepatopancreas), accelerates regeneration of amputated axolotl limbs and stimulates reproductive activity of crayfish. The obtained results give an opportunity to extend use of stem cells in hydrobionts in order to accelerate regeneration of pathologically altered organs and tissues, as well as maturation of crayfish. These data also allow considering further research regarding use of hydrobionts as an alternative source of stem cells.

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