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REVIEWS

Organotypic Culturing as a Way to Study Recovery Opportunities of the Eye Retina in Vertebrates and Humans

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Abstract—Using the literature and the authors' own data, the review provides information on experiments carried out at different times with organotypic culturing of the retina in vertebrates and humans. The method allows one to maintain the structure and viability of the retina, model a number of its pathological conditions, and observe the processes of retinal development, regeneration, reconstruction, death, and growth of neuron outgrowths. In addition, organotypic culturing makes it possible to affect all indicated processes by different regulating factors as well as protective/damaging agents at strictly specified concentrations and under controlled conditions. Particular attention is paid to the behavior of retinal pigment epithelium cells, photoreceptor cells, Müller glia cells, and ganglion cells and their axons (that is, to those cell populations that are most often affected in cases of different pathological conditions and retinal diseases). A separate section is devoted to the production and culturing of so-called retinal organoids, which is being actively developed at present. The direction promises opportunities for the transplantation of retinal cells, conducting experiments on gene therapy, and testing ophthalmic pharmacological drugs.

Keywords: vertebrates, retina, culturing, regeneration, reconstruction, organoids

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INTRODUCTION

In vitro cell cultures are an important tool in studying individual cell populations in conditions of their isolation and a significant addition to in vivo experiments. The cell cultures allow one to study viability, morphology, behavior, and function of the cells and their populations as well as the effect of different substances with toxic, protective, or regulatory effect on these processes. In addition, cell culturing is an indispensable tool in studying molecular factors of regulation of cell proliferation and differentiation and changes or conversions of the cell phenotype. And finally, in vitro cell culturing is a tool for the production and accumulation of stem or poorly differentiated cell populations for the most diverse needs of modern biomedicine.

Despite all the advantages of the cell culturing, isolated planted in vitro cells are deprived of familiar surroundings and effects from the adjacent tissues. As is known, this is a definite obstacle for extrapolating of the information received on the situation in situ. In this regard, much attention has been paid in the last two decades to organotypic culturing, which is more adequate to simulate the conditions of the whole organism; therefore, this technology is regarded in the literature as "in vivo-like in vitro technology" (Schutgens et al., 2016), while the cultured tissue as "in vivo-like in vitro model" (Cho et al., 2018). To a large extent, this concerns the neural tissue, in the study of which it is very important to preserve the existing initially complex structural and synaptic organization (Humpel, 2015).

The eye retina in vertebrates is a complex sensor organ, in the functioning of which different types of neurons and glial cells, as well as retinal pigment epithelium, are involved (Hoon et al., 2014). The main types of retinal cells have a wide range of subtypes organized in a complex, formed in the development cell ensemble, where the interaction is performed due to a large number of neural and intercellular connections (Amini et al., 2018). This circumstance requires researchers to use the entire retinal tissue ("whole-mount") in the work without the cell separation and destruction of its native structure. Organotypic culturing of the retina, currently attracting a lot of attention, corresponds to this condition.

METHOD FOR ORGANOTYPIC CULTURING OF EYE RETINA

The term "organotypic" was for the first time used in 1954 when describing the differentiation of developing a chicken eye retina in vitro (Reinbold, 1954). During the development of the method, a technique of

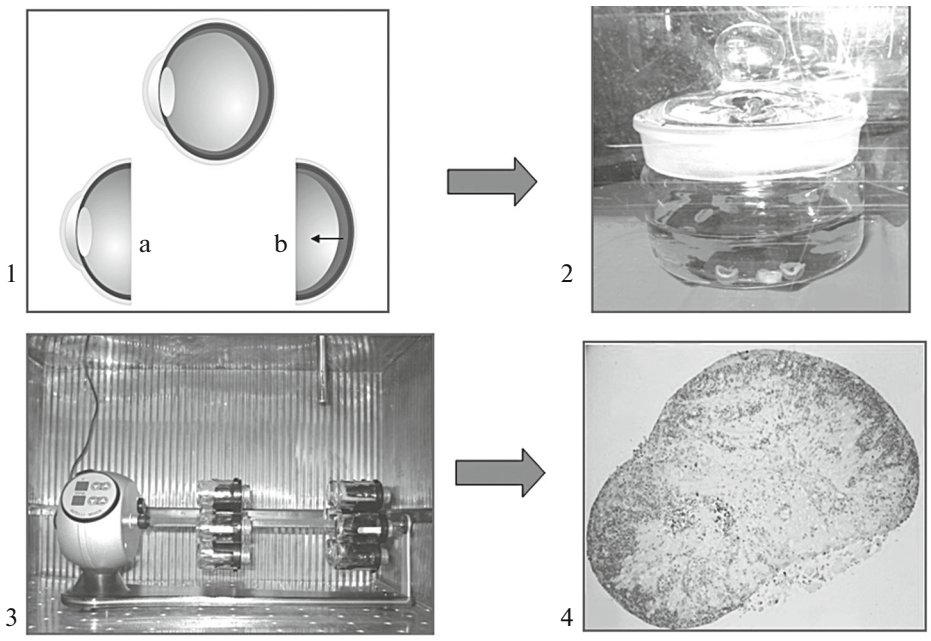

Fig. 1. Stages of organotypic rotational culturing of the eye retina. 1, microsurgical isolation of retina: (a) isolation of anterior sector of the eye, (b) retina in the posterior eye wall and its isolation (arrow); 2, placing samples in a cold culture medium; 3, exposure of isolated retinas in a medium with constant rotation; 4, closed sphere of the rat retina by the end of exposure.

rotational organotypic culturing in rotating tubes or culture bottles began to be used in addition to the stationary culture of the whole retina or its sections (Gähwiler, 1988; Braschler et al., 1989; Gähwiler et al., 1997, 2001; Victorov et al., 2001). Due to the permanent entry of a fresh portion of the medium into the tissue, such method provided its great safety and increased culturing time (Fig. 1).

Organotypic culturing is an indispensable tool in the studies on the mechanisms of development and regeneration of the retina and their constituent processes (cell proliferation, migration, differentiation, and morphogenesis) (Amini et al., 2018). During the study, it was possible to fully cultivate the optic cups of 2–5-day-old chicken and quail embryos for 2 days (Halfter and Deiss, 1986). The retinas of 5–6-day-old chicken embryos were cultivated for 6 days together with RPE and without it (Liu et al., 1988). The retina of neonatal rabbits was cultivated for 2 weeks (Germer et al., 1997; Pinzon-Duarte et al., 2000), while retinal explants of neonatal mice were placed in serum-free medium for 4 weeks (Caffe et al., 1993; Ogilvie et al., 1999). Culturing of retinal radial sections of various thicknesses obtained both from higher and lower vertebrates was frequently used (Mack and Fernald, 1991; Feigenspan et al., 1993; Sassoe-Pognetto et al., 1996).

Today, wholemount organotypic retinal culturing is widely used for a variety of purposes, including for morphological, biochemical, and pharmacological studies of this tissue (see review Li et al., 2018). Organotypic culturing conditions are used for genetic engineering manipulations (Moritoh et al., 2010), particularly, transfer of the genes that are capable of being transiently expressed in the cells of cultivated retina (Moritoh et al., 2013). In addition, organotypic culturing allows one to conduct numerous pharmacological tests, during which it is possible to strictly control the conditions of testing the drugs with a protector or toxic effect.

We should recall here that ophthalmic drugs are most often tested in vivo on rabbits, cats, dogs, pigs, and monkeys (Shafaie et al., 2016). Organotypic cultures are an irreplaceable alternative to these experiments, are economical in relation to the volumes of the test substances, and allow one to significantly decrease the number of animals used in the experiment. In experiments on the vertebrate retina, it was possible to identify the efficiency of different factors in increasing the viability and reducing the risk of neuron damage. Among such substances, growth factors, neurotrophins, cytokines (LaVail et al., 1998), cell adhesion proteins (Rattner et al., 2001), and antioxidants (Grigoryan et al., 2012) were noted.

As mentioned above, the material obtained from developing animals in which the formation of the eye retina was not yet completed was used in many works on the retina of vertebrates. In the experiments carried out using organotypic rotation culturing, we used the wholemount isolated retina of adult animals in order to study its capability for regeneration and/or reconstruction (Novikova et al., 2010; Grigoryan et al., 2016) as well as the factors potentially capable of increasing the cell viability (Novikova, 2010; Grigoryan et al., 2012, 2013).

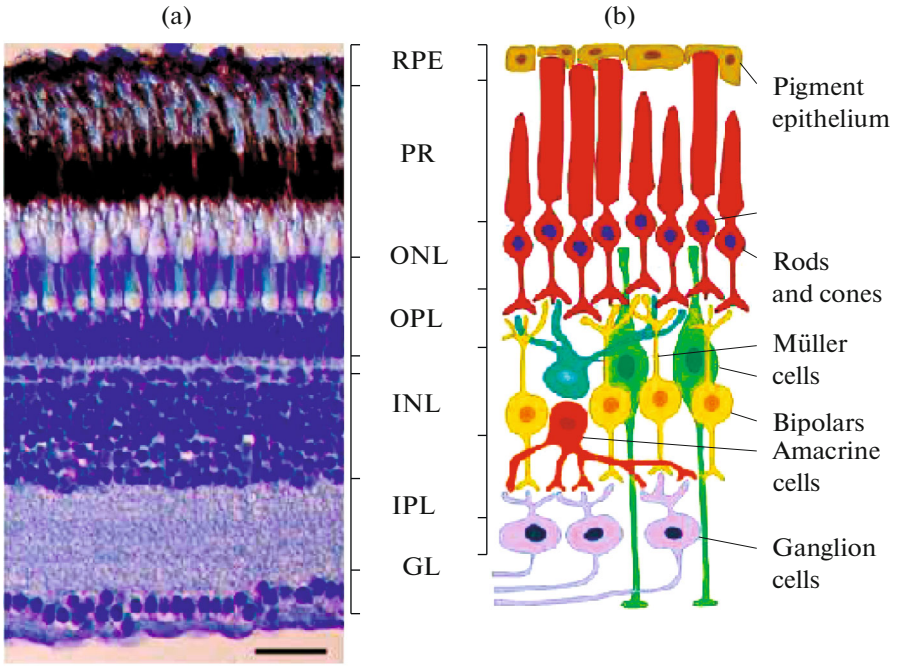

Fig. 2. Retinal structure (a) on histological section and (b) in a schematic representation of the main cell types. From Gramage and Hitchcock, 2014 (with modifications).

RETINAL STRUCTURE AND SOURCE CELLS OF ITS RECOVERY

In the context of this article, we need to stop briefly at the eye retina structure. The retina is a part of CNS and provides receiving, processing visual information and its transfer to the appropriate region of the brain (Dowling, 2012). The retina is highly organized to perform such functions. In vertebrates, it is inverted: the light must pass through all layers of the retina in order to reach the photoreceptors (the main light acceptors). The retina has a common and universal structure plan in the evolutionary series of vertebrates. Schematically, the retina layered organization looks as follows (Fig. 2). It is lined outside with a layer of pigmented epithelial cells (RPE) interacting with the retina photoreceptors (PR) and providing, together with them, the light perception and its processing for the transmission in the visual cascade. In turn, the cascade is provided by the cells of nuclear (outer and inner, ONL and INL) and plexiform (outer and inner, ONL and IPL) layers, as well as by the ganglion cells sending their outgrowths (axons) to the optic nerve. ONL is presented by the bodies of photoreceptor cells, while INL by the bodies of interneurons (bipolars and amacrine cells) as well as a number of horizontal cells. OPL contains the outgrowths of photoreceptors interacting with the outgrowths of interneurons, while IPL consists of the outgrowths of interneurons in the interaction with the outgrowths of the ganglion cells.

Müller glia is the only population of macroglial cells in the retina. Müller cells penetrate the thickness of the retina, provide its mechanical support, sending its long outgrowths outward and inward, and are involved in the formation of external and internal limiting retinal membranes (MacDonald et al., 2015). In addition, Müller cells provide a trophic support of all retinal neurons without exception (Reichenbach and Bringmann, 2013) and serve for light perception (Franze et al., 2007).

It is necessary to separately stop at the cells that are sources of retinal recovery. The question is significant in the aspect of our studies using organotypic culturing; however, it is too broad for a detailed presentation in this article. The spectrum of endogenous retina restoration source cells has been described in recent literature (Jeon and Oh, 2015; Chohan et al., 2017; Grigoryan, 2018, 2019). In the eye retina, the cells of the ciliary eye area belong to the cells that potentially can or are really involved in the retina recovery depending on the species and age of animals. In lower vertebrates, this is the ciliary marginal zone (CMZ); in higher vertebrates, this is the region of the retina edge and ciliary body. In addition, regenerative resource is presented by RPE and Müller cells. The latter are regarded as the most promising for the retina regeneration in higher vertebrates and human (see the review Grigoryan, 2019). The growth of neural outgrowths and the recovery of synaptic connections between different types of neurons in the damaged retina is one more important mechanism of the retina recovery. The method of retinal organotypic culturing also contributes significantly to the study of this mechanism (Al-Ali et al., 2017).

RETINAL ORGANOTYPIC CULTURING IN LOWER VERTEBRATES

In the studies using organotypic culturing of retinas in lower vertebrates, fishes and caudate and tailless amphibians were used as objects. There are few such works, which is apparently due to the easy accessibility of these animals for in vivo studies. However, as we will see below, they also contribute significantly to the study of the retina recovery opportunities.

Culturing of retinal sections of adult bony fish for 5 days allowed for the detection that not only the main morphological properties of the tissue (particularly, stratification) but also the main (detectable immunochemically) cell types inherent to the normal retina are preserved in vitro. Moreover, the signs of retinal regeneration (the growth of CMZ cells from the periphery, as well as the replenishment of photoreceptor layer due to the precursor cells localized in INL), were found. The dynamics of CMZ cell proliferation according to the results of thymidine radioautography was similar to that observed in vivo (as well as the emergence of new cells with a photoreceptor immunophenotype in ONL). They were descendants of the cell source localized in INL (Mack and Fernald, 1991, 1992). Later, organotypic culturing of isolated retina of *Danio rerio* fish allowed the study into some details of retinal recovery due to glial Müller cells (Lahne et al., 2017). Regeneration of the retina damaged by a bright light occurred due to the entry of Müller cells into the cell cycle, the production of progeny cells by them, and their subsequent differentiation in photoreceptors lost when irradiated with light. At the same time, there was interkinetic migration of Müller cell progeny cells from INL to ONL. It was possible to follow this interesting process, which is an important but, until recently, obscure stage of regeneration, due to a preliminary labeling of the cells with fluorescent label (Tg[gfap:nGFP]) as well as using multiphoton intravital microscopy. Based on obtained data on intravital registration of Müller cell migration, the dynamics of their movement along the outer and inner limiting membranes of the retina was estimated in the same experiment (Lahne et al., 2017). The effect of taurine and serotonin on the growth of ganglion cells' axons was tested on retinal explants of a goldfish (*Carassius auratus*) in vitro for 5 days. We note that the growth of axons is one of the important, constantly studied mechanisms of retinal recovery. The results demonstrated an increase in the process rate when adding taurine and a decrease in the presence of serotonin in culturing medium (Matus et al., 1997).

There are few examples of organotypic culturing of the retina in tailless amphibians. When using the retina of the clawed frog (*Xenopus laevis*) in culture, it was possible to establish that the return of the interactions of photoreceptor and RPE cells (lost during tissue isolation) occurs after the separation of neural retina and RPE for 3 h. This occurred due to a rapid recovery, native properties of RPE in situ, particularly, adhesive, key during the interaction of apical RPE outgrowths with outer segments of photoreceptors (Defoe and Easterling, 1994). Organotypic culturing of the *Xenopus laevis* retina in the posterior wall of the eye (that is, together with RPE, choroid and scleral coats underlying the retina) allowed for the confirmation of this model's suitability for analyzing the process of recovery of the interaction between RPE cells and photoreceptors. As a result, not only viability of the outer retina cells but also the behavior of light transduction proteins arrestine and transducine were estimated (Reidel et al., 2006).

We carried out organotypic roller culturing of the newt (*Pleurodeles waltl* (Urodela)) retina (Novikova et al., 2010). The study was aimed at clarification of regeneration methods and involvement of internal retinal sources in it in these animals in the absence of RPE. At the same time, the latter is well known as the main source of retinal cells for the recovery in the newt after its removal, optic nerve transaction, and retinal detachment in vivo (Keefe, 1973; Mitashov, 1996, 1997; Grigoryan, 2012). It was found that selected conditions for long-term culturing (within 1 month) induce the activation, proliferation, and migration of the cells that are internal sources for neural retina regeneration as well as the growth of neural processes of viable neurons. Since the retina was isolated without RPE, the recovery could only occur due to its own cellular resource of neural retina (CMZ cells, bipolar-like cells, and, probably, Müller cells) (Grigoryan, 2019). Details of the isolation of neural retina and long-term culturing process as such have been described (Novikova et al., 2010; Grigoryan et al., 2016).

With rotational organotypic in vitro culturing, closed structures (spheroids with outwardly facing photoreceptor layer) were initially formed as a result of closing the edges (periphery) of the newt retina (Fig. 3). Despite the death of individual photoreceptors and internal retinal cells, a layered organization persisted in the formed spheroids in 2 weeks. Multiple mitoses were found near the spheroid closing zone in ONL and INL. In ONL, mitotic cells were located among the bodies of photoreceptors; those in INL were often close to the long outgrowth of Müller cells (Novikova et al., 2010; Grigoryan et al., 2016).

The hypertrophy of Müller cells, which is expressed in a significant increase in the sizes of nuclei, number of nucleoli, and thickness of long outgrowths of these cells, was obvious on semithin sections. In this population, individual cells were also in the mitotic phase. The flows of cells migrating from INL to ONL, as well as multiple mitoses, were seen in 2 weeks of culturing in the central area of the retina far from the periphery. All this was evidence of the active involvement of internal reserve of the newt retina regeneration (CMZ cells, as well as probably descendants of Müller cells

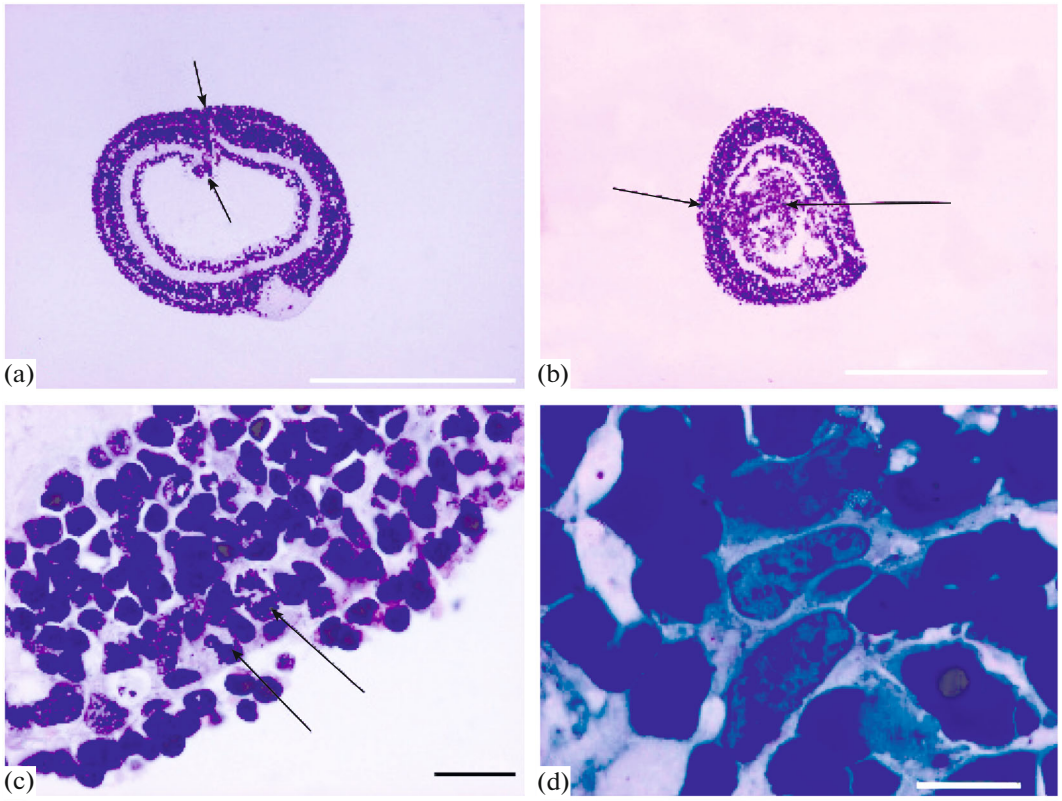

Fig. 3. Newt retina exposed in conditions of rotational organotypic culturing. Total view in (a) two and (b) four weeks of culturing. (a) Arrows indicate the spheroid closure area, scale bar: 500 μm; (c) mitoses in internal nuclear layer of the retina (arrows), scale bar: 20 μm; (d) cells having a neuroblast phenotype, among initial neurons of internal part of the retina (arrows), scale bar: 10 μm.

and bipolar-like cells localized in INL (Novikova et al., 2010).

In 4 weeks of the newt retina's culturing, mixing of ONL and INL and sprouting of neural outgrowths of the cells into the spheroid cavity occurred. The cells in the mitotic phase were more common than those in 2 weeks. As a result, a significant portion of the cells in such spheroids analyzed on a series of semithin sections had typical signs of neuroblasts (Fig. 3d).

Thus, the recovery processes and reconstruction of adult neural retina (outside RPE), which uses internal cell sources and mechanisms for recovery in the newt, take place in the process of continuous organotypic in vitro culturing inevitably accompanied by a limited cell death, particularly in the population of photoreceptor cells.

The study of the newt retina during organotypic culturing using molecular methods confirmed our morphological evidence. The presence of a significant number of poorly differentiated cells in the cultivated newt retina for 4 weeks is indicated by data from a study performed using PCR. A high expression of the genes encoding ßII-tubulin (ßII-tub) and nucleostemin (Ns) (marker proteins of low differentiation level) was found. Simultaneously, the transcripts of regulatory Fgf2 factor responsible for the cell differentiation and proliferation were detected (Novikova, 2010; Markitantova et al., 2014).

On this model, the efficiency of mitochondrial SkQ1 antioxidant in the maintenance of viability, proliferative activity, and ability to change the cell phenotype was also determined. Differences from the control (without introducing a factor into the medium) were expressed not only in an increase in the number of viable cells but also in total level of proliferative activity and cell dedifferentiation in cultivated retinas. In 30 days of culturing, the number of dedifferentiated cells (in the presence of SkQ1 in the medium) in some cases reached 80% of the total cell number (Novikova, 2010).

Using organotypic culturing, one more aspect of retinal regeneration in the newts (*Cynops pyrrhogaster*) was studied; namely, the role of the interaction between RPE and underlying tissues (choroid and scleral coat included in the posterior eye wall) in the regeneration of neural retina not from their own internal reserve but from RPE cells (Mitsuda et al., 2005). It was found that the choroidal (vascular) coat is required to perform the process of regeneration. RPE isolated from the choroid did not proliferate but it

could show its regenerative capabilities (proliferation and transdifferentiation in retinal cells) in the presence of vascular membrane (even when separated by a membrane filter). The study on the role of the growth factors, for which the choroid could be a source (Fgf2 and Igf1) indicating the leading role of Fgf2, while Igf1 could affect the initiation and progress of retinal regeneration from RPE only together with Fgf2. According to real-time PCR, an increase in the expression of the genes encoding both these factors in the tissues of the posterior eye wall shortly after retinal removal. This confirmed the assumption about the role of choroid as a source of factors in retinal regeneration in the newt due to RPE cells (Mitsuda et al., 2005).

RETINAL CULTURING IN BIRDS

The models of cultivated retina in birds were mainly used to address the issues related to the development of this tissue. At the same time, the sections and reaggregation (formed from the cell suspension) cultures of the retina of chicken embryos were frequently used. Thus, the work on the sections of embryonic chick retina under rotational culturing determined that the model fully satisfies the study objectives (the study of differentiation of neurons and retinal glial cells). Moreover, a dose-dependent effect of mexiletine (Na+ channel blocker) on retinogenesis was successfully studied on the model, which confirms that the model is also suitable for toxicological studies (Hoff et al., 1999). The isolated chicken retina (E6) was organotypically cultivated at a constant rotation, which provided its good viability and allowed for performing a fractional fixation of the samples for 3 weeks for immunochemical study of the course of development. The development of photoreceptors in the absence of RPE was an interesting observation. Using electron microscopy, the growth of external outgrowths of photoreceptor cells out of contact with retinal pigment epithelium was found (Thangaraj et al., 2011).

A targeted study on the role of growth factors in the development of chicken retina was performed using suramin (a blocker of their effect) (Cirillo et al., 2001). Culturing of embryonic chick retina for 3 days in the presence of suramin (50–200 μM) led to the suppression of proliferation, cell disintegration, and violation of the formation of the outer limiting membrane. The manifestation of the effect of suramin was dose-dependent and could be leveled by the presence of FGF-2 in the medium, which confirmed a leading role of this growth factor in the development of the retina in vertebrates.

A wide series of works was carried out on reaggregation cultures of the chick retina in order to identify patterns of the development and recovery of the retina as well as the effect of different factors on this process (Layer and Willbold, 1989; Wolburg et al., 1991; Willbold and Layer, 1992). With such an approach, reaggregation cultures could be obtained by eliminating (or, on the contrary, saving) certain areas of the retina with its isolation (only central region of the retina, retina with the edge (CMZ) zone, retina outside, and with RPE were taken). Thus, while maintaining CMZ together with adjacent pigmented cells during the isolation of the retina, hidden regenerative capabilities of this growth area of the eye were found in forming retinotypic stratified spheroids in the case of reaggregation of chicken retinal cells (stage E9). The proliferation and differentiation of CMZ cells was observed for a long time, while the process of retinal replenishment due to this source in vivo is already suppressed at the stage E4 (Willbold and Layer, 1992). At the same time, CMZ cells were in a proliferative phase for a long time with the formation of retinal neurons *de novo*. This period was accompanied by the presence of butyrylcholinesterase (the enzyme whose expression is correlated with the phase of cell transition from proliferation to differentiation). As a result, an important conclusion was made that the potencies of CMZ cells to proliferation and replenishment of the cell composition of the chicken retina persist at least until the stage E9 (Willbold and Layer, 1992). The role of the peripheral part of RPE in histogenesis of retinotypic stratified spheroids was studied in the work (Wolburg et al., 1991). The authors managed to find out that the correct, comparable to normal in vivo layered organization and the growth of long processes of Müller glial cells and the formation of limiting outer and inner membranes with their involvement depend on the presence of RPE cells in reaggregation cultures.

As was demonstrated relative to the role of choroidal membrane in regeneration of the retina from RPE cells in the newt, a significant role of this structure was established in stimulation of the growth of neural cell outgrowths with culturing dissociated cells of chicken retinal explants. The effect of chicken eye choroid extract (E18) was compared with the effect of the well-known CNTF neurotrophic factor. For both factors, both stimulating effect and dose dependence of the axon growth on the concentrations of factors in the medium was found (Carri et al., 1994). This suggested that the vascular membrane has a wide range of factors having signal and trophic significance during the regeneration and development of the eye retina in amphibians and birds.

RETINAL ORGANOTYPIC CULTURING IN MAMMALS

For mammals, an organotypic culturing tool was no less efficient to address many issues associated with the development and regeneration of the retina. It was demonstrated (Pinzón-Duarte, 2000) that the retina of neonatal mice that were born blind is in a state of histotypic development and it not only successfully survives the conditions of explantation and culturing

but also demonstrates the completion of the formation of layers and cell types similar to that formed in vivo. It is interesting that RPE was required for the development of photoreceptors and the outer limiting membrane; however, it was not required for the correct development of internal retina, differentiation of its cells, growth of neuron outgrowths, and synaptogenesis (Pinzón-Duarte, 2000). The observations indicating that the development of the retina in neonatal mice can also successfully occur in serum-free medium are also known (Caffe et al., 2001).

As for culturing of the postnatal mouse retina, its already largely formed structure not only persists in the medium without serum in this case but the continued growth of neuronal outgrowths is also observed, although a loss in the population of ONL cells is found (Caffe et al., 2001). In the work of Bandyopadhyay and Rohrer (2010), organotypic culturing of postnatal mouse retinas also did not interfere with normal tissue development, particularly, the formation of photoreceptors. The retina tissue was placed in the conditions of organ culturing at the stage P7, when INL was formed, but the precursors of rods have not yet completed the active migration towards ONL (the layer, the complete formation of which is completed at the stages P10–P12). Subsequently, the growth of external outgrowths of photoreceptors and the formation of reticular layers occurs in vivo. After 11 days of culturing in vitro, the comparison was made with retinal tissue under normal development in vivo at the stage P18, when mouse retinal histogenesis generally ends. The comparison demonstrated not only the preservation of the structure of the retina formed before placing the tissue in the culture but also the subsequent correct formation of ONL. In addition to these results, the production of 11-cis retinal by RPE cells, which provides the support for the development of cones and signaling phototransmission, was detected (Bandyopadhyay and Rohrer, 2010).

Retinal explants of developing (stage P3 and P10) and adult (P60) mice were cultivated for 10 days. Better viability of neurons of the retina obtained from the developing mouse was detected as compared with the retina of adult animals. The methods of cytofluorimetry and immunocytochemistry helped to establish the level of cell death as well as its association with the volumes and distribution of microglia/macrophages cells (population representing a system of phagocytes, the volumes of which can indicate the level of retinal degeneration) in the retina (Ferrer-Martin et al., 2014).

Organotypic long-term (up to 2–3 weeks) culturing of the developing retina in rodents seems now to be a convenient model for retroviral transfection. The attempts to transfer the genes into retinal progenitors for understanding the role of certain regulatory transcriptional factors in differentiation and sequence of maturation of certain specific cell types of the retina are the main direction of these studies (Hatakeyama and Kageyama, 2002; Zhang et al., 2002).

There are examples of successful organotypic culturing of the retinas of not only embryos or postnatal mice but also those obtained from adult individuals. Thus, it was possible to demonstrate in a work conducted using immunochemistry and PCR analysis (Müller et al., 2017) that in vitro conditions are capable of maintaining the tissue at least 10 days. It was also found that typical changes, particularly the activation of Müller cells and changes in photoreceptor outgrowths and the bipolar cell neuritis network, take place in the first 4 days.

We attempted rotational organotypic culturing of the isolated whole (without RPE) retina of adult (2-month-old) rat for 10 days. The procedures of isolation, culturing, and subsequent analysis are described in detail in Novikova et al. (2010). By the end of culturing, the rat retina maintained viability but underwent significant morphological changes. The rat retinas in vitro formed spheroids of varying degrees of closure. The latter determined the cell viability. In open spheroids, while maintaining layered organization, the cell death was high and there were no signs of proliferation of nonneuronal cells, while resident macrophages actively populated the ganglion layer desolating as a result of the cell death. Resident macrophages in the rat retina are included in a relatively large heterogeneous population of microglial cells and macrophages differing depending on the state of the retina in distribution by its thickness and by the expression of some antigens as well as morphologically (Chang et al., 2005). We relied on morphological characteristics that significantly differ these cells from other retinal cells (large sizes, round, or amoeboid cell shape).

In the case of a complete closure of the rat retina spheroid, reorganization in the tissue and cell responses (indicating a reconstruction) were initiated (Fig. 4). The bodies of outward-facing photoreceptor cells lost the outgrowths, partially died, and shifted to the inside of the spheroid, as a result of which INL cells were in its external part (close to the outer limiting membrane). Such cell behavior is observed in rats and when illuminated by a bright light (Grigoryan et al., 2016). At the same time, the cells of a ganglion layer retained the previous localization. The cell bodies in the spheroid thickness had a high viability, while some (not neuronal) cells also had mitotic activity: we found 1–3 pictures of mitotic divisions on each of the serial sections of such spheroids. The study of the cells at stage M and their localization indicated that two cell populations have proliferative activity: large macrophage cells and individual small cells belonging to INL (Figs. 4c, 4d). The latter represented with a high probability activated Müller cells; populations, a potential source of retinal cell regeneration, particularly, photoreceptors in mice (Jorstad et al., 2017).

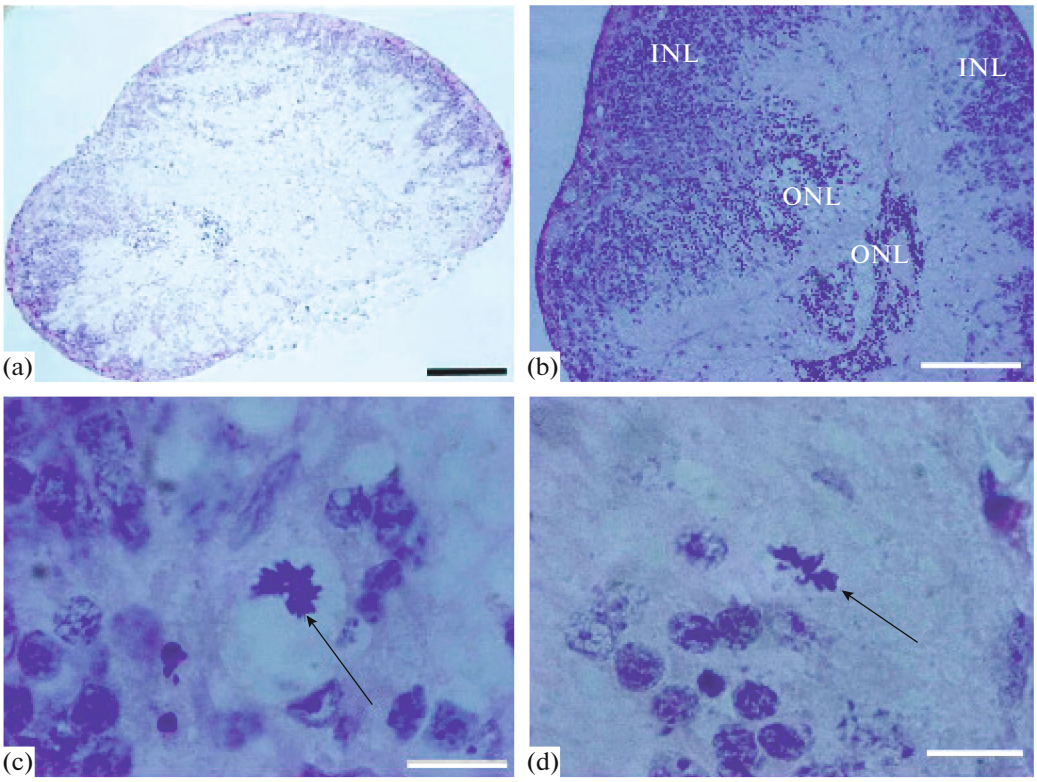

Fig. 4. Rat retina exposed 10 days in conditions of rotational organotypic culturing. (a, b) Total view upon completion of culturing; ONL, outer nuclear layer; INL, inner nuclear layer; scale bar: (a) 500 μm, (b) 100 μm; (c, d) mitoses of nonneural cells in inner part of the retina (arrows), scale bar: 10 μm.

Thus, data obtained using organotypic culturing of the retina in adult rats indicate the possibility of structural changes and activation of regenerative responses in surviving in vitro retinal tissue. The translocation of INL cells outside against the background of a partial death in ONL, displacement of photoreceptor bodies inward, reaction from macroglia, and relative stability of layered organization and limited proliferation of nonneuronal cells are the mechanisms used (Novikova et al., 2010; Grigoryan et al., 2016).

Using organotypic culturing, the effect of a toxin and one of the factors with neuroprotective effect on retinal cells in rats were studied. Particular attention was paid to retinal cells of the ganglion layer and their outgrowths vulnerable to various pathologies. For this purpose, a fluorescent label (FluoroGold) was preliminary delivered to the end zone of the optic nerve in the area of the visual analyzer in the brain by intracranial administration (before retinal explantation, in vivo); distributing throughout the nerve fibers, it allowed the coloring of both bodies and outgrowths of the ganglion cells. After this, such retinas were isolated and cultivated in the presence of gentamicin added in the medium in toxic concentrations (Smedowski et al., 2018). Oxidative stress induced by gentamicin led to a significant damage of the cells, destruction of their outgrowths, accompanied by a rapid release of lactate dehydrogenase enzyme (a marker of cellular destruction). The addition of ciliary neurotrophic factor (CNTF) led to a significant (although incomplete) removal of these negative indicators in the population of ganglion cells and their outgrowths (forming optic nerve) in response to the effect of toxin (Smedowski et al., 2018).

We used SkQ1 as an efficient antioxidant and neuroprotector to prevent the cell death of the rat retina cultivated in vitro in the posterior wall of the eye. The addition of 20 nM of this efficient (protecting the cell mitochondria) drug led to a significant reduction of the cell loss in the ganglion layer on the seventh day and prevention of the death and disintegration of INL cells on the 14th day of rotational organotypic culturing (Grigoryan et al., 2013).

The work on the study of the effect of oxidative stress on retinal cells was also carried out on the pig retina (which is very close in structure to the human retina) (Hurst et al., 2017). Hydrogen peroxide (H_2O_2) at concentrations 100, 300, and 500 μM was used as a stress-inducing agent. The estimation of a decrease in the number of viable cells of the ganglion layer, expression of genetic markers of oxidative stress, heat shock protein Hsp70, inflammation markers, an increase in the population of microglia, and activation of macroglial cells was conducted. This work allowed suggesting

the model for testing of not only factors of retinal protective from the oxidative stress but also other retinal protectors as well as to study molecular mechanisms of retinal cell death (Hurst et al., 2017).

When cultivating the pig retina (Kaempf et al., 2008), it was found that RPE is responsible for the degree of manifestation of reactive gliosis (the response of Müller glial cells to structural and metabolic violations in the retina) (deHoz et al., 2016). The activation of Müller cells, which is expressed in a hypertrophy, increase of the proteins of intermediate filaments (glial fibrillar protein (Gfap) and vimentin), was significantly decreased while maintaining the relationship of neural retina and RPE. Along with this, the cell death in outer, INL, and the retinal ganglion layer was decreased (Kaempf et al., 2008). In another study on the pig retina model (complex: choroid–RPE–neural retina), the conditions were created for a constant perfusion of the fresh portion of the medium and, thus, maintaining a complex wholemount and carrying out fractional fixation of the samples for 10 days (Kobuch et al., 2008). To estimate the state of the retina, a wide range of parameters was analyzed immunohistochemically (cell proliferation, viability, growth of outgrowths, and immunophenotypes of photoreceptor and glial cells). This allowed one to demonstrate convincingly that the model can be used to test ophthalmic drugs, which allows the reduction in the number of studies on animals in vivo.

ORGANOTYPIC CULTURING OF RETINA IN HUMANS

Organotypic culturing of the human explanted retina appears to be a good tool to conduct a wide variety of biomedical studies (modeling of diseases and pathologies of the retina, for preclinical trials of the drugs and toxicological tests). However, there are only a small number of works in the literature that were conducted using human retina explants. In early works, the growth of outgrowths of human retinal neural cells in vitro was described, and differences in this regeneration parameter were described in different donors depending on the substrate and the presence of stimulators (particularly, the presence of Schwann cells in the medium contributing to the growth of neural outgrowths (Thanos and Thiel, 1990; Hopkins and Bunge, 1991). An attempt was made later in the work of Osborne et al. (2016) to model chronic, sequential degeneration of ganglion cells that were successfully marked using antibodies to neuronal nuclear antigen (NeuN), βIII tubulin (βIII-tub), and C90 surface antigen (Thy-1) regardless of donor and material sampling time (within 24 h after death). Simultaneous study on the expression of these marker molecules, as well as apoptosis, allowed them to estimate the dynamics of ganglion cell death by the retinal space (particularly, macula area, its highly sensitive fovea area and near it) (Osborne et al., 2016). Recently, testing of some promising human retinal neuroprotectors was conducted on the same model. Among them, the efficiency of human mesenchymal stem cells and neuroprotector factors produced by them, as well as PDGF (isolated from growth factor platelets) was detected relative to the protection of retinal ganglion cells (Osborne et al., 2018).

Human retinal explants in vitro were also used in the studies on its regenerative potencies. It was found (Mayer et al., 2005) that the cells that are potential sources of recovery (called neural precursors by the authors) (the presence of which in the retina is suggested and widely discussed; Grigoryan, 2019) are able to change their phenotype in the human retina in vitro and then to differentiate in different types of neurons. In the work by Mayer et al., the explants of retinal fragments taken from its different areas, as well as separately from the *pars plana* edge area, were used. The explants were obtained from adult donors (15–87 years) immediately after death and were cultivated in the presence of a wide set of growth factors, including FGF-2. In the cells evicted and migrating in vitro from such explants, individual signs of neural progenitors were found: proliferation, nestin expression, and the ability of such cells to form neurospheres. Unfortunately, the authors do not answer the question about the origin of such cells in the human adult retina but suggest a model for further study on regenerative capabilities of mature human retina under organotypic culturing for therapeutic purposes (Mayer et al., 2005).

It was also found that the precursors of photoreceptor cells in human fetal retinal explants (as well as mouse) differentiate faster in the presence of both human retina and RPE obtained from human embryonic stem cells (ESC) (Yanai et al., 2015). Several attempts to transfer the genes using a viral transfection were made on human retinal cultures with the prospect of further gene therapy. Using this technology, there was a successful attempt to reactivate photoreceptor cells in the absence of an external segment in them (and therefore of sensitivity) as well as to restore the activity of ganglion cells of human explanted retina using lentiviral and adenoviral vectors (Sengupta et al., 2011).

The accumulated experience of human retinal culturing *ex vivo* was recently presented in the review by Murali et al. (2018). It is particularly indicated there that, despite the attractiveness of the model of explanted human retina and its culturing for the studies on the development and regeneration, modeling of pathologies, and toxicological tests and preclinical studies, there are a lot of limitations for widespread use of the model. First of all, this is a complexity in obtaining postmortal material of the retina from donors, the absence of information about the state of the retina (the presence of certain diseases that can affect the eye retina in a donor). It should also be noted that the above-mentioned axotomy, which is accompanied by

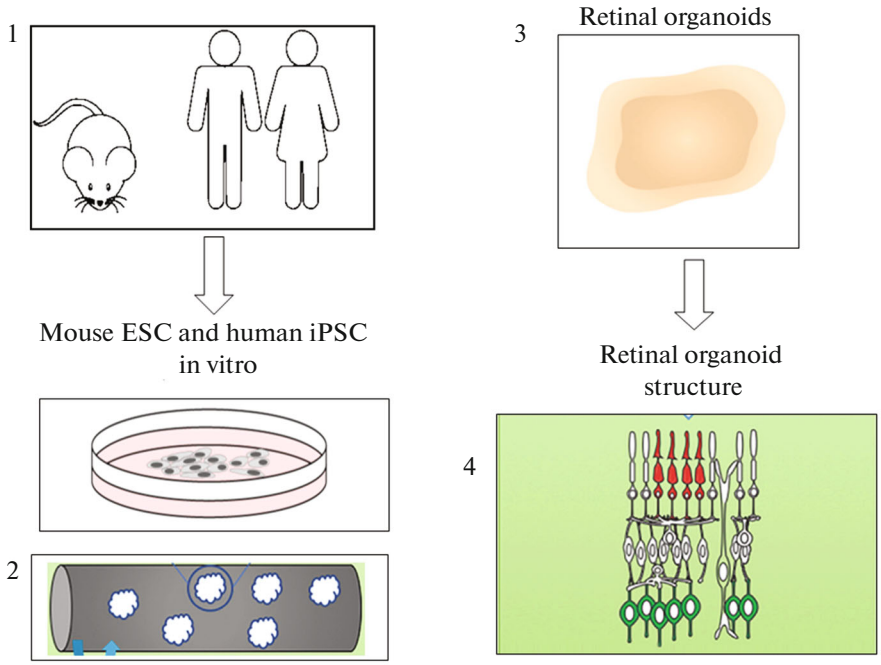

Fig. 5. Stages of obtaining retinal organoids. 1, stem cell isolation (ESC and iPSC); 2, stem cell culturing and transfer to rotating bioreactors; 3, forming organoids; 4, internal structure of an organoid.

a retrograde death of outgrowths (and then the ganglion cell bodies), is inevitable when isolating the retina. The cell stress, as well as metabolic changes (that are only strengthened during culturing in conditions of isolation of the retina from the surrounding eye tissues), also takes place during the isolation. At the same time, getting the controls (normal functioning tissue of healthy human retina) is also impossible for obvious reason (Murali et al., 2018).

The above works performed using organotypic culturing of the retina of embryos and adult animals from different vertebrate classes and species, as well as on human retina, against the background of constantly improving culturing techniques and advances in the stem cell studies laid the foundation for using the method of organotypic culturing to obtain so-called retinal organoids.

RETINAL ORGANOIDS ARE A PROSPECT OF APPLYING THE ORGANOTYPIC CULTURING METHOD

As we repeatedly mentioned above, the viability of retinal tissue significantly increases in renewing culturing medium allowing better substance delivery as well as elimination of metabolic products. In addition to organotypic rotational and stationary culturing, so-called bioreactors significantly increasing culturing time and allowing analysis of biological and biochemical processes in the retina under a strict monitoring of the conditions in vitro (such as temperature, pH, oxygen level, etc.) are used at present for this purpose (Martin et al., 2004; Antoni et al., 2015).

These new technological opportunities are of particular importance to develop the technique of obtaining so-called retinal organoids for the purpose of their further use for transplantation in cases of cell loss in diseases or retinal damages in mammals and humans (Ader and Tanaka, 2014; Llonch et al., 2018; DiStefano et al., 2018). The use of bioreactors (Ovando-Roche et al., 2018) and the technology of obtaining retinal organoids for 3D culturing are constantly improving (Reichman et al., 2017; DiStefano et al., 2018; Capowski et al., 2019). It is noted that these achievements have good prospects for obtaining differentiated (to varying degrees from precursors to mature neurons) retinal cells for transplantation, particularly, most commonly dying photoreceptors, RPE cells, as well as for testing different drugs and gene modification procedures. Very slowly developing retinal organoids also are of a particular importance for studying the differentiation of retinal cell types and molecular regulators of this process.

Mouse and human embryonic stem cells (ESC) and induced pluripotent stem cells (iPSC) are material for obtaining and forming retinal organoids in vitro (Fig. 5). Previously, it was demonstrated that a spontaneous reproduction of the processes comparable with those during retinogenesis in vivo takes place during the formation and development of retinal organoids (Eiraku et al., 2011; Nakano et al., 2012). Under adequate conditions in vitro, the cultivated

stem cells begin to develop in a neuroepithelial direction, after which the resulting neuroepithelium invaginates in the same way as happens with the development of the optic cup in vivo. Subsequently, this structure undergoes differentiation of the cell types and morphogenesis as a result of self-organization, forming a stratified tissue containing differentiated interneurons and photoreceptor cells (Eiraku et al., 2011; Nakano et al., 2012). It is interesting that the process of retinal organoid maturation is faster under conditions of hypoxia simulating the conditions of retinal development in embryogenesis in vivo (Chen et al., 2016).

Moreover, in addition to structural similarity to the retina, recapitulation of existing in vivo biological and metabolic its parameters can be also achieved in cultivated organoids (Yin et al., 2016; Browne et al., 2017). However, there are shortcomings of retinal organoids obtained in vitro that have not yet been overcome. It is noted that they are poorly accessible for the penetration of various factors, and they have no blood supply required for this purpose (McMurtrey, 2016). Besides this, the absence of the formation of retinal synaptic layers (ERL and IRL), as well as the ganglion cell layer, was noted. Details about retinal organoid culturing methods obtained from human iPSC in order to obtain the cell material for transplantations were recently presented in the review by Llonch et al. (2018).

The use of retinal organoids obtained from iPSC of patients (patient-specific organoids) suffering from a particular retinal genetic disease is a special direction developed recently (Foltz and Clegg, 2019). For now, retinal pathology models, for which genetic mutations and their cellular and functional consequences are already well-known, are used. One of the few of such works is devoted to the correction of consequences of retinitis pigmentosa (RP) (common retinal genetic disease leading to blindness). When using CRISPR-Cas9 technique on the cells of organoids obtained from iPSC of patients suffering from this disease, Deng et al. (2018) carried out a correction of the mutation-carrying *RPGR* gene (GTPase regulator, one of the main genes responsible for the development of RP). This allowed them to prevent a violation of the structure of photoreceptors and RPE, return the electrophysiological properties to the cells, and to restore the genetic expression of *RPGR* in photoreceptor cells to the control level. It can be foreseen that this direction will receive a special development in the coming years, opening up the prospects for the gene therapy of hereditary, yet incurable retinal diseases, with the development of methods for producing organoids (including personified), with further improvement of organotypic culturing methods, and the methods of corrective gene modification.

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COMPLIANCE WITH ETHICAL STANDARDS

The authors declare that they have no conflict of interest. This article does not contain any studies involving animals or human participants performed by any of the authors.

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