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A novel approach to AVT and IT studies in fish brain and pituitary: *In vitro* perfusion technique

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ABSTRACT

The study was designed to develop a new procedure for perfusion of brain and pituitary explants collected from three-spined stickleback (*Gasterosteus aculeatus*) and round goby (*Neogobius melanostomus*). The procedure was elaborated for studies of arginine vasotocin (AVT) and isotocin (IT) release from explants of both species. AVT and IT, analogs of mammalian vasopressin and oxytocin, are neurohormones produced in hypothalamus and released in neurohypophysis of Teleostei. Both nonapeptides are used as biomarkers of fish well being. Three perfusion sets were applied to test the method of medium transport into gradient container, without or with aeration. Medium supply to the gradient container from the top, without aeration is recommended only for short-term studies. Aeration of the medium with a mixture of 95% O_2 and 5% CO_2 at a pressure of 127.51 mmHg is necessary for a long-term research. Transport of one or two media in the gradient container from the top and the bottom, simultaneously, requires aeration with a mixture of 95% O_2 and 5% CO_2 at a pressure of 315.03 mmHg. Although the presented procedure has been elaborated for studies of AVT and IT in fish explants, after only minor modification, if any, it can serve many other purposes.

1. Introduction

Most of the studies, in which organ perfusion technique is used, are focused on mammals and address pharmacological problems. Recently, there is growing concern about effects of fish farming and environmental pollution on fish well being, thus there is the need for new tests to study the endocrine responses also in fish (Freshney, 2005). So far, organ perfusion methods have not been often used in fish for lack of suitable techniques. Simple organ perfusion systems were applied in pituitary (Habibi et al., 1989; Rotllant et al., 2000; Moriyama et al., 2002; Metz et al., 2004; Weber et al., 2004) and pineal gland (Falcón et al., 1989; Yañez and Meissl, 1996; Okimoto and Stetson, 1999) studies. However, an innovative system for organ perfusion, proposed by Minuth and his co-workers in early 90s, gives more and more options. This gradient perfusion technique meets the requirements for studies of retina, blood–retina and blood–brain barrier, nervous tissues, skin renewal, regeneration of blood vessels, bone and muscular tissue in mammals (Minuth et al., 2010). In this study, for the first time, the system has been adapted for perfusion of brain and pituitary explants in fish to do research on arginine vasotocin (AVT) and isotocin (IT) release.

AVT and IT are teleosts' nonapeptides, closely related to mammalian arginine vasopressin (AVP) and oxytocin (OT) (Acher, 1993). AVT and IT are synthesized in the separate hypothalamic parvo- and magnocellular neurons of the preoptic area (POA) and transported to the neurohypophysis, where they are stored and released into circulation to act as peripheral hormones (Holmqvist and Ekström, 1995; Saito et al., 2004). AVT and IT are also active neurotransmitters and neuromodulators in central nervous system. The physiological role of AVT involves cardiovascular activity, maintenance of water/ions homeostasis, interaction with other endocrine systems and control of reproductive behaviour (Balment et al., 2006; Kulczykowska, 2007; Goodson, 2008). Changes in hypothalamic, pituitary and plasma AVT and IT concentrations were found in many fish species subjected to different type of stress i.e. confinement, disturbance, high density, food deprivation or osmotic. An effect of stress on nonapeptides' synthesis and release depends on the type of stress stimuli (Kulczykowska, 2001; Mancera et al., 2008). AVT and IT can be considered as hormonal markers of the internal state of the individual and thus seems to be good candidates for welfare indicators (Kulczykowska et al., 2010).

Development of sensitive assay for determination of active AVT and IT in brain tissues and plasma samples by Gozdowska and Kulczykowska (2004) has allowed to get new information about the physiological role of both nonapeptides. However, regulation of the release of both peptides within brain and from pituitary to the cardiovascular system is still far from being obvious. Therefore

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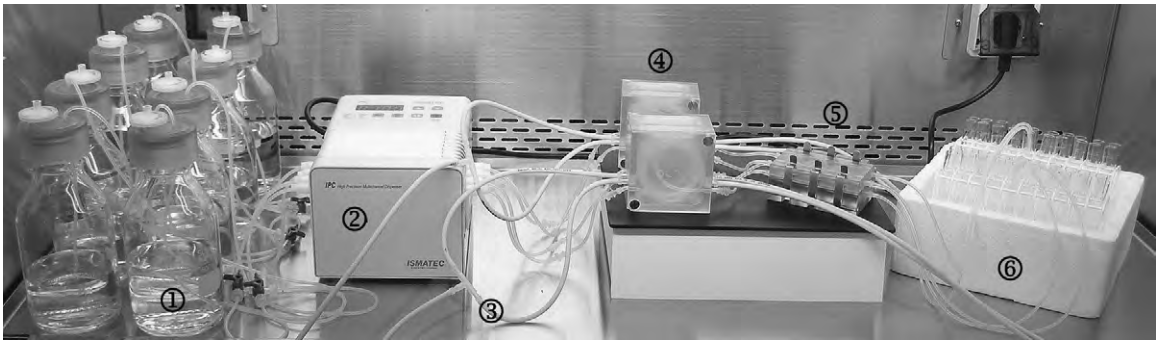

Fig. 1. Components of perfusion culture set. 1 – storage medium bottles; 2 – peristaltic pump; 3 – connecting fittings between air pomp or gas bottle and gas exchange module; 4 – two gas exchange modules; 5 – gradient culture container; 6 – sampling vials. Perfusion culture set 1 used in Experiments 1 and 2 consists of components 1, 2, 5 and 6. Perfusion culture set 2 used in Experiment 3 consists of all components with one gas exchange module. Perfusion culture set 3 used in Experiment 4 consists of all components with double gas exchange module.

we came upon an idea to adapt Minuth's and co-workers' perfusion system to studies of AVT and IT release in fish.

The purpose of this study is to develop the perfusion procedure to monitor the hormones' secretion from brain and pituitary explants of three-spined stickleback (*Gasterosteus aculeatus*) and round goby (*Neogobius melanostomus*) using MINUCELLS and MINUTISSUE Vertriebs GmbH (Germany) technology.

2. Material and methods

2.1. Experimental animals

The experiments reported herein comply with the "Principles of Animal Care", publication No. 86-23, revised 1985 of the National Institute of Health and EC Directive 86/609/EEC for animal experiments as well as with guidelines of The Local Ethics Committee on Animal Experimentation.

Adult three-spined sticklebacks (*G. aculeatus*) of both sexes ($n = 32$) used in this study were caught in the Oliva Stream (Gdańsk, Poland). Adult round gobies (*N. melanostomus*) of both sexes ($n = 52$) were caught in the Gulf of Gdańsk (Gdynia, Poland). Fish were kept in aquariums containing the water from stream (three-spined sticklebacks) or gulf (round gobies) at the Institute of Oceanology PAS (Sopot) at 6 °C under artificial lighting regime (12 h of light: 12 h of dark) for two weeks before the experimentation. At the time of sampling, fish were anesthetized in 0.5% (v/v) 2-phenoxyethanol water solution and after transection of the spinal cord, whole brains were immediately collected. Hypothalamus–pituitary (H–P) complex (three-spined sticklebacks or round gobies) and hypothalamus or pituitary, separately (round gobies) were dissected under stereomicroscope (PZO, Warsaw, Poland). Tissues were washed in appropriate for each species Ringer solution supplemented with 10^{-6} mM Bacitracin before their putting on tissue carriers in gradient culture container.

2.2. Perfusion system

The perfusion culture set can be used in different combinations depending on the sample that is studied. Three perfusion sets were used to test the methods of medium transport into the gradient container: one medium supplied from the top without aeration (set 1), or with aeration (set 2) and one or two media supplied from the top and bottom, simultaneously, with aeration (set 3).

The set 1 used in Experiment 1 and Experiment 2, consisted of 4 storage medium bottles equipped with screw caps, peristaltic pump (ISMATEC, IPC N8, Wertheim, Germany), gradient perfusion container and plastic vials for sampling medium after perfusion (Fig. 1). Gradient perfusion container can hold 6 tissue carriers for the simultaneous culture of several tissues. A specific construction of this container facilitates the uniform supply of medium to the luminal and basal sides. The tissue carrier (MINUSHEET) consists of base and tension rings and a filter with a diameter of 13 mm, placed between them. During culture a peristaltic pump transported the medium (0.1 mL min^{-1}) from the storage bottles to sampling vials. Perfusion medium has been transported to the gradient container from the top and uniformly supplied to the luminal and basal sides of tissue carriers.

The set 2 used in Experiment 3 was enriched with gas exchange module and air pump ELITE 800 (Hagen, Holm, Germany) or bottle of mixture of 95% O_2 and 5% CO_2 (Fig. 1). Medium was transported to the gradient perfusion container from the top and supplied to the luminal and basal side of tissue carrier. Respiratory gases were transported by an air pump or gas bottle and then by gas exchange module. The gas exchange module consists of gas inlet and outlet housing inside and a spiral of long thin walled silicon tube for medium to pass through. The tubes are highly gas-permeable, which guarantees optimal diffusion of gases between culture medium and surrounding atmosphere (0.3% CO_2) or mixture of 95% O_2 and 5% CO_2, during run. The gradient container was supplied with the medium in the same way as in the set 1.

The set 3 applied in Experiment 4 consisted of 8 storage medium bottles, peristaltic pump, 2 gas exchanges modules, gradient perfusion container and plastic vials for sampling buffer after perfusion (Fig. 1). Perfusion medium aerated with mixture of 95% O_2 and 5% CO_2 has been simultaneously transported to the gradient container from the top and bottom and then supplied to the luminal and basal side of tissue carriers. The set 3 makes it possible to perfuse the sample with two different media at the same time during experiment.

In all cases, storage medium bottles and sampling vials were placed on ice and media collected after perfusion, were kept at −70 °C prior to AVT and IT assay. The equipment used in all experiments and procedures was sterilized or autoclaved depending on the material the object was made of. All perfusions were carried out in a laminar air flow (NUAIRE Biological Safety Cabinet Class II, USA). All chemicals were obtained from Sigma–Aldrich (St. Louis, USA) whereas all equipments from MINUCELLS and MINUTISSUE Vertriebs GmbH (Bad Abbach, Germany), except filters and membranes (see text below).

2.3. AVT and IT analysis

Content of AVT and IT in media collected after perfusion were determined by high-performance liquid chromatography (HPLC) with fluorescence and UV detection preceded by solid-phase extraction (SPE) according to modified procedure by Gozdowska

et al. (2006). Media after perfusion were acidified with 1 M HCl to pH 3–4 and loaded on SPE columns. SPE extraction was proceeded on Strata™ – X (30 mg mL^{-1}) columns (Phenomenex, USA). Extraction procedure for perfusion media was as follows: samples were loaded on conditioned columns [1 mL of 100% (v/v) methanol and next 1 mL H_2O], then 2 × 300 μL H_2O and 2 × 300 μL of 0.1% (v/v) trifluoroacetic acid (TFA) in 5% (v/v) acetonitrile were passed through the columns to wash impurities. Hormones were eluted with 2 × 600 μL of 80% (v/v) acetonitrile. Eluates were evaporated to dryness using TurboVap LV™ (Caliper Life Sciences, USA). Then, derivatization of peptides was performed using 4-fluoro-7-nitro-2,1,3-benzoxadiazole (NBD-F). Dried samples were reconstituted with 40 μL of 0.1% (v/v) TFA in H_2O. For derivatization reaction, 20 μL of sample, 20 μL of 0.2 M phosphate buffer (pH 9) and 20 μL of acetonitrile were mixed and next 3 μL NBD-F (30 mg mL^{-1} acetonitrile) was added. The mixture was heated at 60 °C for 3 min, cooled on ice, acidified with 4 μL of 1 M HCl and subjected onto HPLC column. Quantitative analyses were performed on 1200 series Quatemary HPLC system (Agilent Technology, USA) with fluorescence detector and Diode Array Detector. The chromatographic separations of peptides were carried out on ZORBAX Eclipse XDB-C18 (4.6 mm × 150 mm, 5 μm) (Agilent Technology, USA). The following optimized chromatographic conditions were used: mobile phase A – 0.1% TFA (v/v) in H_2O; mobile phase B – 0.1% TFA in acetonitrile:H_2O (3:1); linear gradient system: 45–70% phase B in 12 min. The column temperature was 20 °C and flow rate 1 mL min^{-1}. Fluorescence detection was performed at 470 nm with emission at 530 nm, UV detection at 215 nm.

3. Experimental design

3.1. Experiment 1

The purpose of this experiment was to select a composition of perfusion media (A), type of filter (B) and to determine the time, in which each module of perfusion system is supplied by media (C).

In all experiments, the explants were perfused for 120 min using set 1. During this time, six fractions of 2 mL each were collected every 20 min. After 80 min of perfusion with basal medium, next 40 min were performed with basal medium supplemented with high K^+ concentration (40 mM KCl) to induce an AVT and IT surge after depolarization of cell membranes and verify the reactivity of tissue. A peristaltic pump transported the medium with stable flow of 0.1 mL min^{-1}.

(A) Selection of media composition was carried out in experiments with the whole brains or H–P complex explants collected from three-spined stickleback (n = 8) and hypothalamus and pituitary explants (separately) collected from round goby (n = 6). Two Ringer buffers were tested for perfusion of three-spined sticklebacks explants. The first Ringer buffer (pH 7.8) modified by Takahashi (1992) consisted of: 120 mM NaCl, 2.5 mM KCl, 0.8 mM $CaCl_2$, 1 mM $MgCl_2$, 3 mM 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES) and 10 mM glucose. The second Ringer buffer (pH 7.2) modified by Westerfield (2000) consisted of: 116 mM NaCl, 2.9 mM KCl, 1.8 mM $CaCl_2$, 5 mM HEPES and 0.25% (w/v) glucose. For perfusion of round goby explants, Ringer buffer modified by Rotllant et al. (2000) was tested. This Ringer buffer (pH 7.4) consisted of: 134 mM NaCl, 2 mM KCl, 2 mM $CaCl_2$, 5 mM HEPES, 0.25% (w/v) glucose and additionally 0.03% (w/v) bovine serum albumin (BSA) for pituitary explants.

Ringer buffer modified by Westerfield was selected to the three-spined stickleback's brains and H–P explants perfusion, whereas Ringer buffer modified by Rotllant was chosen for perfusion of round goby hypothalamus and pituitary explants. The AVT and IT efflux in response to the medium supplemented with high K^+ concentration (40 mM KCl) was checked to verify the reactivity of tissue, as was mentioned earlier. Depolarization of synaptic terminals induced by application of medium containing high K^+ concentration is one of the stimuli frequently used to evoke the extracellular release of neurotransmitters. However, to the authors' knowledge, there are no data on the effects of high K^+ concentration on release of AVT or IT in fish. We consider our results as satisfactory, because the medium supplemented with high K^+ concentration (40 mM KCl) caused a 450% increase in AVT release from perfused explants and a 350% increase in that of IT.

We compared our results with AVP and/or OT response to high K^+ concentration observed during *in vitro* studies in rodents (Orłowska-Majdak et al., 2003; Juszczak, 2002). The release of AVP from the rabbit hippocampus increased to 245% of the control value after administration of high K^+ concentration (56 mM), while AVP release from the caudate nucleus, increased to 336% of the control value after administration of the same dose of potassium (Orłowska-Majdak et al., 2003). Moreover, studies of rats' hypothalamo-neurohypophysial explants demonstrated that AVP increased to 636% of the control value, while OT increases to 290% of that after high K^+ concentration (56 mM) treatment (Juszczak, 2002).

(B) Different membranes between rings of tissue carriers in the gradient container were tested during perfusion performed in accordance with the procedure described before for round goby H–P complex (n = 4). Nylon Net Filter 20 μm Milipore (NY2002500), Nylon Membrane 0.2 μm Whatman (7402-001) and MF-Millipore™ Membrane Filters (GSTF01300) were tested. Selection of proper filter is essential to sufficient exchange of medium through the filter inside the perfusion container. It turned out that only Nylon Net Filter 20 μm Milipore (NY2002500) is suitable for perfusion of H–P complex explants of round goby. The same membrane was chosen for studies of sticklebacks' explants.

(C) The experiment was performed to determine the time, in which each module of perfusion system is supplied by media. In this study, the H–P explants of round goby (n = 4) and brain explants of three-spined stickleback (n = 5) were perfused with appropriate for each species Ringer solution in accordance with the procedure described before. Red Hibiscus extract was added as a marker to Ringer buffer supplemented with high K^+ concentration. It was measured that the red marker has been flowing from the extra storage medium bottles to sampling vials for 20 min. This time should be taken into account when buffers are changing during perfusion. This information is crucial for planning various experimental protocols with different treatments and buffers.

3.2. Experiment 2

This experiment was conducted to determine the time required to achieve a stable basal level of AVT and IT release during tissue explants perfusion. The whole brain explants collected from three-spined sticklebacks (n = 7) or hypothalamus and pituitary explants (separately) from round goby (n = 4) were cultured using the set 1. The experimental scheme included 200 min of perfusion, during which 10 fractions of 2 mL each were collected every 20 min. The first 160 min of perfusion was carried out with Ringer buffer modified by Westerfield for three-spined stickleback or Ringer buffer modified by Rotllant for round goby, while next 40 min were performed with appropriate Ringer buffers supplemented with high K^+ concentration (40 mM KCl). The stable basal level of AVT and IT release was achieved between 60 and 80 min of perfusion for both fish species (Fig. 2).

The AVT and IT response to the medium supplemented with high K^+ concentration (40 mM KCl) during last 40 min of perfusion was hardly visible, suggesting reduced tissues' reactivity to

Table 1
The successive stages to establish the acid/base equilibrium of Ringer buffer (RB modified by Metz et al. (2004). The pH was tested 30 min after buffer preparation under air atmosphere and after 30 min of incubation in an atmosphere of 95% O_2 and 5% CO_2 and 24 or 48 h later.

①		
RB without HEPES	30 min – air atmosphere pH 7.05	
	30 min – air atmosphere	30 min – 95% O_2 and 5% CO_2
RB + 15 mM HEPES	pH 5.72	pH 5.35 after 48 h pH 5.4
②	⬇	
RB + 5 mM HEPES	30 min – air atmosphere pH 5.86	
	30 min – air atmosphere	30 min – 95% O_2 and 5% CO_2
RB + 5 mM HEPES + 1 mM $NaHCO_3$	pH 8.36	pH 7.91 after 48 h pH 8.82
RB + 5 mM HEPES + 2 mM $NaHCO_3$	pH 8.44	pH 8.17 after 48 h pH 8.74
③	⬇	
	30 min – air atmosphere	30 min – 95% O_2 and 5% CO_2
RB + 10 mM HEPES + 1 mM $NaHCO_3$	pH 8.14	pH 7.86 after 48 h pH 8.18
RB + 15 mM HEPES + 1 mM $NaHCO_3$	pH 8.04	pH 7.79 after 48 h pH 8.16
RB + 20 mM HEPES + 1 mM $NaHCO_3$	pH 7.97	pH 7.54 after 24 h pH 7.77
RB + 25 mM HEPES + 1 mM $NaHCO_3$	pH 7.85	pH 7.51 after 24 h pH 7.76
RB + 30 mM HEPES + 1 mM $NaHCO_3$	pH 7.73	pH 7.47 after 24 h pH 7.71
④	⬇	
	30 min – air atmosphere	30 min – 95% O_2 and 5% CO_2
RB + 15 mM HEPES + 0.5 mM $NaHCO_3$	pH 7.63	pH 7.57
RB + 20 mM HEPES + 0.5 mM $NaHCO_3$	pH 7.57	pH 7.46
RB + 25 mM HEPES + 0.5 mM $NaHCO_3$	pH 7.47 after 24 h pH 7.48	pH 7.38 after 24 h pH 7.4
RB + 30 mM HEPES + 0.5 mM $NaHCO_3$	pH 7.39 after 24 h pH 7.42	pH 7.23 after 24 h pH 7.49

depolarization stimuli or/and hypoxia at the end of the perfusion. Therefore, during longer perfusion procedures, medium aeration using gas exchange module is necessary.

3.3. Experiment 3

The purpose of this experiment was to modify the procedure used in the previous experiments to allow aeration of the medium during perfusion. Ringer buffer (pH 7.4) modified by Metz et al. (2004) consisted of: 134 mM NaCl, 2 mM KCl, 2 mM $CaCl_2$, 25 mM HEPES, 0.5 mM $NaHCO_3$, 0.25% (w/v) glucose and 0.03% (w/v) BSA was tested. The maintenance of the acid/base equilibrium of tested buffer was established using different configuration of lower $NaHCO_3$ contents (0.5, 1, and 2 mM) in relation to an increasing amount of HEPES (5, 10, 15, 20, 25, and 30 mM). $NaHCO_3$ was applied as a buffering substance and as an essential nutritional component and HEPES was used for the stabilization of pH. The pH was tested 30 min after buffer preparation under air atmosphere and after 30 min of incubation in an atmosphere of 95% O_2 and 5% CO_2 and 24 or 48 h later. The tests established the acid/base equilibrium of perfusion buffer at contents of 25 mM HEPES and 0.5 mM $NaHCO_3$ under 95% O_2 and 5% CO_2 atmosphere and 30 mM HEPES and 0.5 mM $NaHCO_3$ under air atmosphere (see Table 1).

Next, the effect of aeration on AVT and IT release during round goby H–P explants ($n = 8$) perfusion was tested. The experimental scheme included 160 min of perfusion, during which 8 fractions of 2 mL each were collected every 20 min. In last 40 min, perfusion was performed with the same Ringer buffer supplemented with high K^+ concentration (60 mM KCl). The set 2 used in this experiment, complemented with gas exchange module and flowing medium, was aerated by air pump (0.3% CO_2) or mixture of 95% O_2 and 5% CO_2 from the bottle at a pressure of 127.51 mmHg. Our results indicated that only delivery of mixture of 95% O_2 and 5% CO_2 (Fig. 3) offered the proper conditions for perfusion and AVT and IT response to the medium supplemented with high K^+ concentration (60 mM KCl).

3.4. Experiment 4

Experiment was performed to test the method of perfusion, in which gradient container was supplied with aerated medium from the top and bottom, simultaneously. This type of perfusion allows delivery of two different buffers to the perfusion container. H–P explants collected from round goby ($n = 12$) were cultured using the set 3 (Fig. 3). Ringer buffer modified by Metz was aerated with mixture of 95% O_2 and 5% CO_2 from gas bottle. Perfusion was carried out over 160 min and 4 mL fractions were collected every 20 min.

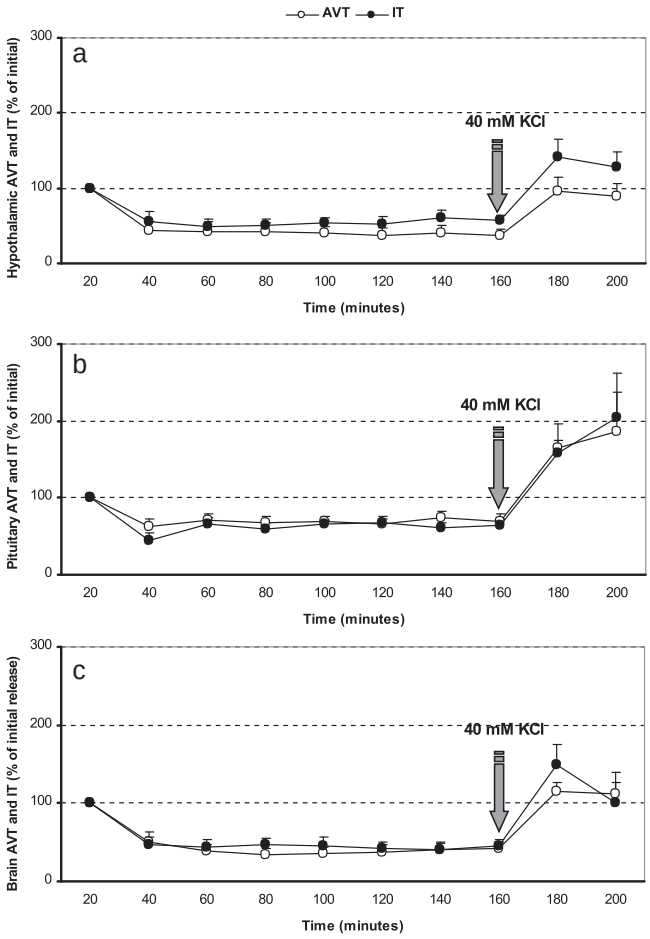

Fig. 2. AVT and IT release from hypothalamus (a) or pituitary (b) of round goby and three-spined stickleback brain (c) during 200 min of perfusion and its response to 40 mM KCl (set 1). AVT and IT content expressed as % of initial concentration. Values are means ± S.E.M.

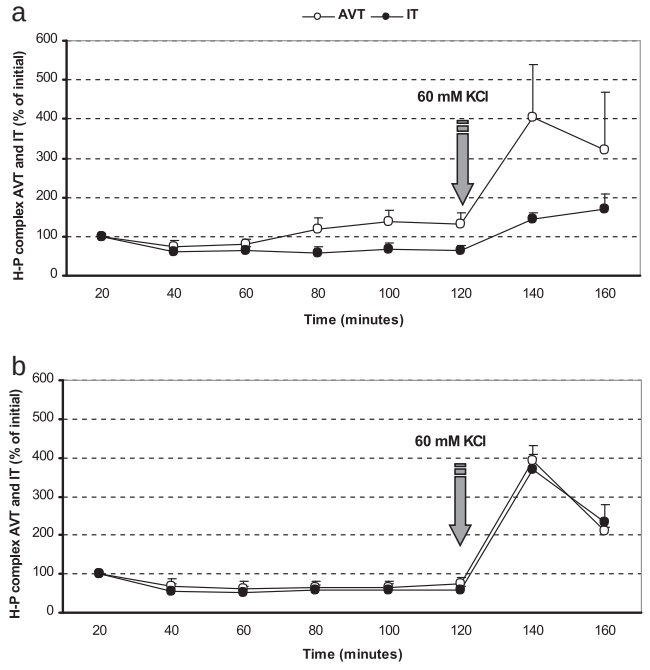

Fig. 3. AVT and IT release from round goby H–P complex in response to 60 mM KCl during perfusion (set 2) with medium aerated by air pomp (a) or mixture of 95% O_2 and 5% CO_2 from the bottle (b). AVT and IT content expressed as % of initial concentration. Values are means ± S.E.M.

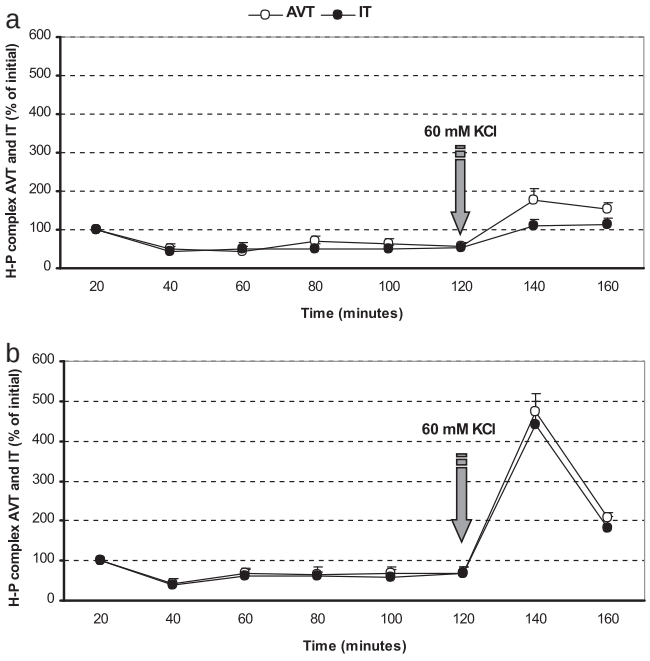

Fig. 4. AVT and IT release from round goby H–P complex in response to 60 mM KCl during perfusion (set 3) with medium aerated mixture of 95% O_2 and 5% CO_2 at the pressure of 127.51 mmHg (a) or 315.03 mmHg (b) at the outlet of the gas bottle. AVT and IT content expressed as % of initial concentration. Values are means ± S.E.M.

In last 40 min, perfusion was performed with basal medium supplemented with high K^+ concentration (60 mM KCl). In order to optimize the conditions of perfusion, various pressure of gas mixture (127.51, 255.02, and 315.03 mmHg at the outlet of the gas bottle) were tested. The gas pressure at the outlet of the air pump is 127.51 mmHg and provides optimal conditions for perfusion in the set 2 with one gas exchange module. To ensure the same pressure conditions in the set 3, with two gas exchange modules with double fittings and one tube connected with the gas bottle, the higher pressure at the outlet of the gas bottle must be applied. The pressure of 315.03 mmHg was sufficient to optimize the conditions of perfusion (Fig. 4).

4. Summary and recommendations

In summary, for the first time, MINUCELLS and MINUTISSUE tissue engineering technique has been applied for perfusion of fish brain and pituitary. In this study, we have developed the procedure and defined the conditions of perfusion. Medium transport from the top of the gradient container (set 1), performed without aeration of perfusion system, turned out to be useful only for a short-term perfusions. However, when aeration is applied (set 2) the system can be used also for long-term studies. Aeration of the medium should be performed with a mixture of 95% O_2 and 5% CO_2 at the pressure of 127.51 mmHg. Sets 1 and 2 allow the transport of only one type of buffer at the same time to the gradient perfusion container. Set 3 allows delivery of two different buffers from the top and bottom to the perfusion container at the same time. In a long-time perfusions model, medium has to be aerated with a mixture of 95% O_2 and 5% CO_2 at the pressure of 315.03 mmHg at the outlet of the gas bottle.

Recently, there is growing concern about effects of anthropogenic impact on fish well being, thus there is a need for new tests to study the endocrine responses in fish. The presented procedure can be successfully used in many research of feedback of fish organism to changing environmental condition.

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