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Plasma FITC-dextran exchange between the primary and secondary circulatory systems in the Atlantic cod, *Gadus Morhua*

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Abstract Fluorescein isothiocyanate dextran (FITC-dextran) exchange between the primary (PCS) and secondary (SCS) circulatory systems in the Atlantic cod, *Gadus morhua* (Linnaeus, 1752), were studied using 20-kDa ($n = 4$) and 500-kDa ($n = 4$) FITC-dextran. In order to give a qualitative perspective of the general connection between the PCS and SCS, distribution of plasma-borne tracers (FITC-dextran) in the PCS and SCS were examined. In this study, a total of eight cod were cannulated in the ventral aorta (PCS) and dorsal cutaneous vessel (SCS), for investigation of FITC-dextran disappearance in the PCS and its subsequent appearance in the SCS. FITC-dextran of both sizes was found to be in equilibrium between the PCS and SCS in less than 20 min. This indicates a profound connection between the PCS and SCS in the Atlantic cod, and rapid mixing of tracers between the PCS and SCS. The destination of the injected 500-kDa FITC-dextran was also examined, and it was observed that of the 500-kDa FITC-dextran lost from the primary and secondary vascular systems, 63.0 ± 9.2% could be recovered from the liver

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Introduction

The morphology and physiology of the circulatory system of teleosts have been thoroughly investigated, especially since Vogel and Claviez (1981) reported a connection between the so-called lymphatic circulation and the vascular system, through *inter-arterial-anastomoses*. These findings led to the acknowledgement that fish do not possess a lymphatic system but have two circulatory systems, i.e. the primary (PCS) and secondary (SCS) circulatory systems (Vogel and Claviez 1981). The secondary circulatory system originates directly from the PCS through the anastomoses, and the vessels show a construction similar to that of the PCS, as the SCS contains arteries, capillaries, and veins. Most studies of the secondary circulatory system have been of mere morphological and anatomical origin, and the SCS was found to form capillary beds situated in the outer surfaces of fish, such as the skin, fins, gills, mouth and pharynx, but were absent in mesenteric and renal tissues (Vogel 1985a, b; Steffensen and Lomholt 1992; Skov and Bennett 2003). The rate of plasma exchange between the PCS and SCS in fish circulation has been studied intensively by monitoring the disappearance of dye from the primary circulatory system as a

function of time (Duff et al. 1987; Nichols 1987; Brill et al. 1998; Bushnell et al. 1998; Skov and Steffensen 2003). These studies have all focussed on the disappearance of tracer from the PCS, whereas the appearance of tracer in the SCS has never been studied, probably due to difficulties in gaining chronic access to the secondary circulatory system. In the present study, the PCS and the SCS were chronically cannulated, with the aim of describing the magnitude of plasma-borne fluorescein isothiocyanate-labeled dextran (FITC-dextran) exchange from the PCS to the SCS in the Atlantic cod.

Commercially produced FITC-dextran of different molecular mass has been used for decades to investigate plasma distribution kinetics (Arfors et al. 1979; Brill et al. 1998; Mehvar 2000). Regardless of the reported stability of dextran, the use of labeled dextrans should be approached with concern, as dextrans are eventually eliminated by dextranase in the liver (Larsen 1989; Mehvar 2000). Furthermore some authors have found photoactivated FITC-labeled conjugates to induce damage to microvessels, mediated by reactive oxygen species, mostly singlet oxygen ($^1O_2$) (Zhang et al. 1997; Rumbaut and Sial 1999). Reactive oxygen species have been reported to play a part in the induction of membrane damage, cardiotoxicity, neurotoxicity, and oxidative stress in cells, and the action of $^1O_2$ is thought to be one of the most damaging among reactive oxygen species (Niesink et al. 1996; Rumbaut and Sial 1999). Furthermore, singlet oxygen may cause an increase in cytosolic Ca^{2+} concentration, and disturbance of Ca^{2+} homeostasis ultimately leads to changes in cellular membranes' tight junctions, by increased phosphorylation of the myosin light chain, which in turn mediates formation of intercellular gaps through activation of the tight junction actin–myosin contractile mechanisms (Lum and Malik 1996; Niesink et al. 1996). In this study, FITC-dextran was used with caution and kept away from light sources prior to injection. The possible phototoxicity of the FITC-dextran used in this study was therefore considered negligible. Commercially produced macromolecules used in measuring capillary permeability can be regarded as non-native xenobiotic substances (i.e. chemical substances that are foreign to the biological system, including naturally occurring compounds and drugs). These xenobiotic substances may therefore hypothetically be eliminated from the vasculature based on their foreign composition. However several of these commercially produced macromolecules have been designed to act as pharmaceutical drug-carriers, specifically ensuring a long plasma half-life, stabilization, and target-specific action of the pharmaceutical agent (Larsen 1989; Mehvar 2000). This implies that xenobiotic molecules may be found suitable for investigation of capillary permeability. Commercially produced dextrans have proven to be good candidates as they can be designed to be an inert and extremely stable tracer, and to a great extent remain within the vasculature during in-vivo experiments. Dextrans are used as plasma expanders to restore and maintain blood volume in the treatment of shock, haemorrhage and extensive burns, and dextrans are believed to be the most promising drug-carrier candidates due to excellent physicochemical properties and physiological acceptance (Larsen 1989).

Materials and methods

General

Atlantic cod, *Gadus morhua*, ($N = 8$) were caught by trawl, at 17–20 m depth, in the northern part of Øresund, Denmark. In the laboratory, fish were kept in 500-L tanks and provided with continuously recirculating (8 L min^{-1}) aerated filtered seawater (10°C, 30‰ salinity). Experimental animals had a body mass of 483.8 ± 84.7 g (mean ± S.D.), length (l) of 35.6 ± 1.5 cm (mean ± S.D.), and a condition factor (condition factor = body mass × l^{-3} × 100) of 1.1 ± 0.2 (mean ± S.D.). The experimental animals were acclimated to laboratory conditions for 1–3 weeks, with a 12:12 h light:dark photoperiod. During acclimation the cod was fed a diet of chopped herring (*Clupea hargenus*, L.) ad libitum twice weekly.

Experimental procedure

Surgical procedure

Fish were anaesthetized in a 0.025 g L^{-1} benzocaine (ethyl *p*-amino benzoate) solution, weighed, measured for body length, and subsequently placed with

the ventral side up on a wet foam rubber mat. Aerated seawater (10°C, 30‰ salinity) containing 0.025 g L^{-1} benzocaine was pumped across the gills to maintain anaesthesia and ensure adequate oxygen supply. The PCS was cannulated in the ventral aorta in the second gill arch using PE-50 tubing (Portex, UK), and the cannula was fastened using a silk suture (Kruuse, Denmark). The SCS was cannulated in the dorsal caudal cutaneous vein approximately 2–4 cm anterior to the peduncle. A continuous flow trough the dorsal caudal cutaneous vein was ensured by a PE-50 loop fitted to a homemade miniature three-way connector, fastened to the tail by a silk suture. The cannulas from the PCS and SCS were filled with heparinised saline (HS) (Sigma Chemicals, St Louis, MO, USA), at a concentration of 25 i.u mL^{-1} in 1.0% NaCl solution, and connected to a large three-way stopcock enabling administration of HS and withdrawal of blood samples. The entire surgical procedure was completed in 5–10 min.

After surgery the cod were placed in non-transparent plastic tubes, with a slit along the top, allowing access to the cannula without disturbing the fish. Plastic grids, ensuring adequate circulation of water, closed the ends of the tubes. Experimental animals were allowed acclimatization for approximately 24 h before the experiments.

At the end of the experiment tissue samples were collected from the liver ($n = 4$) of the fish injected with 500-kDa FITC-dextran.

Injection and measurement of plasma FITC-dextran

Prior to the FITC-dextran injections in the PCS, 2 mL blood was removed from the PCS to balance and minimize the increase in circulatory system volume, caused by the injection of 2 mL FITC-dextran. From this blood sample the initial haematocrit was measured visually using a haematocrit kit (Mikro-Hämatokrit, Heraeos Sepatech, Germany), and red blood cells were harvested by centrifugation and re-injected after FITC-dextran to replace the amount of red blood cells lost. FITC-dextrans of 20 and 500 kDa (Sigma Diagnostics, St Louis, MO, USA) were used to measure plasma exchange between the PCS and SCS. Preliminary experiments showed that FITC-dextrans of 20, 70, and 150 kDa showed no clear difference in permeability (data not shown), indicating the permeability of these FITC-dextran sizes was of similar magnitude. The 20-kDa and 500-kDa sizes produced dye-dilution curves which were significantly different ($P < 0.05$) and these sizes were therefore used. For both FITC-dextran sizes approximately 50 mg kg^{-1} body weight (BW) was suspended in 2 mL HS and injected via the cannula. After injection of the dye the cannula was flushed with red blood cells suspended in HS, and HS was used to fill the cannula. Samples were taken as follows: an initial 100–200 μL (the volume of the cannula) was removed to empty the cannula. Hereafter approximately 100–200 μL sample was collected, the initial HS sample was re-injected and the cannula was filled with HS. Blood samples were taken from the PCS and SCS at 5, 10, 20, 40, 60, 90, 120, 180, 240, 300, 360, 420, and 480 min post injection. The entire blood-sampling procedure was completed in less than 60 s. The total amount of plasma removed using this procedure was negligible, as it was approximately 4% of the total plasma volume. The samples were centrifuged in Eppendorff tubes and supernatant was harvested and stored at –80°C for later analysis. Plasma samples were analyzed for FITC-dextran content spectrophotometrically, using a Hitachi (Tokyo, Japan) F2500 fluorescence spectrophotometer at 492 nm excitation and 520 nm emission wavelengths.

Statistical procedure

All values are given as mean ± 95% confidence interval (CI) unless otherwise specified. Significant difference between means was determined using *t*-tests and ANOVA, and, when normality failed a non-parametric variant, Mann–Whitney rank sum test and Tukey test, using SigmaStat 2.0 (SPSS. Chicago, IL, USA).

Results

FITC-dextran exchange between the PCS and SCS gave a textbook example of rapid exchange between two compartments. The time for complete mixing of both 20-kDa and 500-kDa FITC-dextran between the compartments is remarkably fast (Fig. 1A and B), and after 20 min no significant difference between

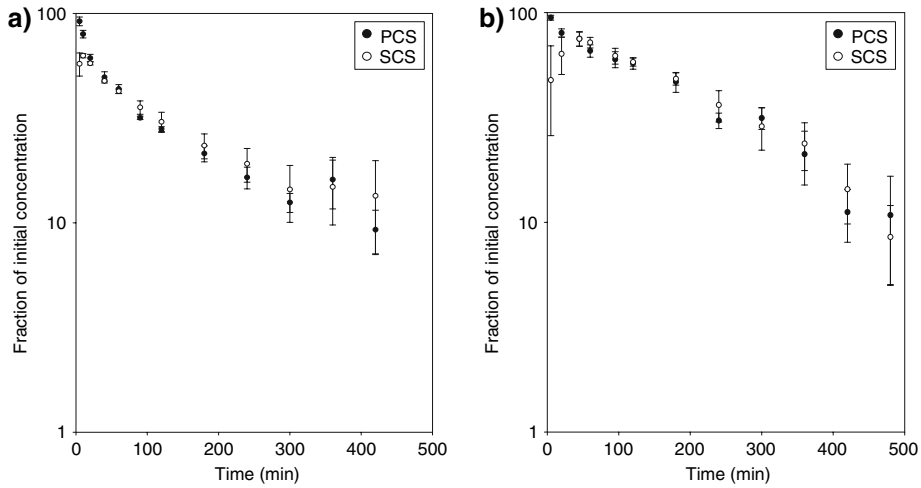

Fig. 1 20-kDa (**A**) and 500-kDa (**B**) FITC-dextran exchange between the PCS (*solid circles*) and SCS (*open circles*). All values are given in mean ± 95% CI

FITC-dextran concentrations in the PCS and SCS ($P > 0.05$) were detected.

The haematocrit values for the cod used in the experiments were 22.5 ± 1.9% (mean ± 95%CI) in the PCS and 2.1 ± 0.4% in the SCS, with white blood cells constituting a large fraction of the total amount of red and white blood cells (data not shown).

Of the 500-kDa FITC-dextran lost from the primary and secondary vascular systems, 63.0 ± 9.2% can be recovered from the liver, based on a hepatosomatic index (HIS = (liver weight/body weight) × 100) of approximately 9% in the cod (Jobling 1988).

Discussion

Chronic cannulation of both the PCS and SCS was successful in this study. This procedure of double chronic cannulation made it possible to investigate the disappearance of FITC-dextran from the PCS and its subsequent appearance in the SCS. The complete mixing of FITC-dextran between PSC and SCS within 20 min indicates a profound connection between these compartments, and this is in agreement with the observations of flow between the primary and secondary vessels in the glass catfish, *Kryptopterus bicirrhis* (Valenciennes, 1839) (Steffensen et al. 1986). The fast mixing of FITC-dextran between the PCS and SCS suggests the SCS receives a substantial amount of plasma from the PCS. This is in contrast with the assumptions made by Olson et al. (2003), as these authors assumed that fluid lost from the PCS entered the interstitial space and not the SCS. Actually Olson et al. (2003) went so far as to assume the flow of plasma from the PCS to the SCS was absent, however this assumption seems rather doubtful as flow between the PCS and SCS, following in-vivo observations of the primary and secondary vessels in the glass catfish, *Kryptopterus bicirrhis* (Valenciennes, 1839), was reported in 1986 (Steffensen et al. 1986).

Authors investigating plasma distribution kinetics, using tracers that are not fully mixed between the compartments after approximately 20 min (Brill et al. 1998; Bushnell et al. 1998; Skov and Steffensen 2003), might find the tracer is not retained in the vascular compartments but is extravagated into other tissues, i.e. muscle interstitial fluid.

In the current study the destination of the injected FITC-dextran was investigated. It was hypothesized that 20-kDa FITC-dextran, due to its small size, was eliminated from the blood plasma primarily via the hepatic circulation and renal excretion, but also by the spleen, and to some extent accumulation in tissues. However, the 500-kDa FITC-dextran was believed to be retained within the vascular compartment, until eliminated in the liver. These assumptions were based on the fact that 500-kDa FITC-dextran was proposed to be eliminated by the liver (Larsen 1989; Randall et al. 1997; Mehvar 2000). It was

therefore hypothesized that 500-kDa FITC-dextran could be found in high doses in liver tissue, and the current results show that of the 500-kDa FITC-dextran injected into the PCS 63.0 ± 9.2% could be recovered from the liver at the end of the experiment. This indicates that the liver is the general elimination site for FITC-dextran in cod, which is supported by the sinusoidal capillary supply and the large amount of dextranase found in liver tissue (Larsen 1989; Randall et al. 1997; Mehvar 2000).

In the light of these results, it seems reasonable to conclude that plasma exchange between the primary and secondary circulatory systems in the Atlantic cod is remarkably fast. Furthermore we conclude that 500-kDa FITC-dextran is retained within the vascular compartment until eliminated by the liver, making it a useful compound for investigating plasma exchange between the primary and secondary circulatory systems.

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