Investigation of blood flow and the effect of vasoactive substances in cutaneous blood vessels of *Xenopus laevis*

Aleš Škorjanc and Gregor Belušič

Department of Biology, Biotechnical Faculty, University of Ljubljana, Ljubljana, Slovenia

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REGULATION OF BLOOD FLOW is a core concept in animal physiology. In physiology courses, peripheral blood flow can be conveniently observed in a number of preparations such as the frog web (swimming skin in the frog foot) or fish tail (2, 5). It is, however, important during classroom experiments and demonstrations to step further from qualitative observations and perform quantitative experiments. Blood flow can be experimentally manipulated by controlling a number of factors, such as temperature, hydration, heart rate, stroke volume, and by applying vasoactive substances. The choice of experimental objects should conform with Krogh’s rule, preferring easily accessible, durable, and illustrative preparations. The frog cutaneous circulation fulfills all these criteria. Frog web has been traditionally used as a preparation for observations in immobilized frogs (2, 5, 7, 8). In a euthanized frog, however, the circulation can be observed in an exposed patch of skin from its internal side, where the vasculature is even better visible and accessible for pharmacological assays.

Frog skin is an important respiratory surface, where CO₂ and O₂ are exchanged between the blood and external medium (6). The vasculature of the skin thus represents a part of the respiratory or pulmocutaneous circulation. Cutaneous arteries deliver to the skin less oxygenated blood with higher levels of CO₂. Respiratory gases are then exchanged with the external medium via an abundantly developed network of capillaries and the superficial mucus. Hence, cutaneous veins carry blood with a higher level of O₂ and lower level of CO₂. Blood flow through the skin is regulated via the diameter of arteries and arterioles. The diameter depends on the tension of smooth muscles in arterial walls. Smooth muscles are virtually absent in venous walls; hence, the veins do not contract actively and do not contribute to regulation. The tension of arterial smooth muscles depends on the levels of catecholamines. Norepinephrine (NE) is released from the terminals of the sympathetic autonomous neurons, whereas epinephrine is released from the suprarenal gland and acts as a hormone. These catecholamines bind to α- and β-adrenergic receptors in the membranes of vascular smooth muscle. These receptors can also be activated by exogenous addition of catecholamines and blocked by certain pharmacological agents, intended for decreasing blood pressure. Additionally, a number of other mechanisms contribute to the local autoregulation of cutaneous blood flow (1).

Here, we present a simple experiment in frog skin suitable for university as well as high school practical courses. It is very simple to perform, yet graphical and serves well to demonstrate the physiological difference between arteries and veins, the fundamental concepts of peripheral blood flow and its regulation, and the special features of the cutaneous respiratory circulation.

MATERIALS AND METHODS

Female African clawed frogs (*Xenopus laevis*) were used in the experiments. The approximate age of the frogs was 20 mo, their body mass was ~80 g, and their size was ~8 cm (snout-vent length). Animals were obtained from a raniculture (*Xenopus Express*, Vernasal, France). They were kept in an animal facility room in a water tank at room temperature and an artificial 12:12-h light-dark cycle. Animals were fed with flaked fish food once a day. The water tank was filled with municipal water (hardness: 12–19 degrees of general hardness, equivalent to 120–190 mg/l CaO) that was continuously aerated. The tank had no filtering system; therefore, the water was exchanged with clean water every second day.

Identical experiments were repeated in two animals. Before the experiments, the frogs were euthanized by brain concussion. Immediately after the concussion, the brain and spinal cord were destroyed with a steel probe inserted through the palatum. This procedure provoked minimal bleeding, which ceased after ~2 min. Experiments were performed in cadavers with a preserved functioning circulatory system. Permission was obtained from the Administration of the Republic of Slovenia for Food Safety, Veterinary Sector and Plant Protection (Permission no. U34401-32/2014/4).

The frog cadaver was pinned to a cork board with its ventral side up (Fig. 1A). The skin was cut with scissors adjacent to the body axis. Perpendicular cuts were made from the midline cut to the side of the body to create a large rectangular patch of skin. To reduce blood loss, care was taken not to cut large blood vessels during the preparation. The vessels can be easily avoided by cutting from the midline since they permeate the skin from the dorsal lateral side of the body wall.
The lateral edge of the patch remained intact, which ensured sufficient blood flow through the blood vessels of the patch during the time of the experiment. To expose the inner side of the skin, the patch was turned over and pinned to the cork board. On the inner side of the skin, the blood vessels were clearly visible already by bare eye.

Blood vessels were imaged from the top with a USB digital microscope Dino-Lite Edge AM4515ZT (AnMo Electronics). Images and movies were viewed on a PC and saved to the hard drive with DinoCapture 2.0 software (AnMo Electronics). The diameter of blood vessels was measured in micrometers with DinoCapture 2.0 measuring tool. Before the experiment, the system was calibrated according to the manufacturer’s instructions with a calibration target that was included with the microscope. The precision of the measurement was limited by the size of the sensor pixel (i.e., 2 μm at ×240 magnification).

The skin was illuminated with a white LED ring illuminator of the microscope. The glare from the illuminated skin was reduced with crossed polarizing filters that were commercially fitted to the objective of the microscope and illuminator. The use of the filters was essential for obtaining clear, contrasted images of blood vessels (Fig. 1, B and C).

The skin patch and abdominal wall of the animal were kept moist with frog Ringer solution (110 mM NaCl, 2.5 mM KCl, 2.4 mM CaCl₂, 0.07 mM NaH₂PO₄, and 11 mM glucose, pH 7.2). In contrast to mammalian and reptilian preparations, in amphibian preparations the external surface of the skin must also be kept moist throughout the experiment. To provoke changes in the diameter of blood vessels, three vasoactive substances were used: NE (N-α-norepinephrine hydrochloride, Aldrich), epinephrine (L-epinephrine hydrochloride, Sanofi), and the α-adrenergic receptor blocking agent doxazosin mesylate (obtained as Ebrantil, Nycomed). The substances were superfused over the exposed blood vessels with a transfer pipette. The skin patch was slightly tilted to allow solutions superfused over the patch to drain away. Otherwise, the accumulated liquid severely defocused the image. Concentrations were prepared by diluting the substances in Ringer solution. Dose-response experiments were performed by the application of a range of concentrations to the skin patch. At each concentration, the diameter of blood vessels was measured before the application and in 30-s intervals during the first 3 min after the application. The effect of all applied substances developed maximally at 30 s after the application. To quantify the dose response, EC₅₀ was determined by fitting the blood vessel diameter versus substance concentration relation with the following four-parameter logistic equation using Prism 6.0 (Graphpad, La Jolla, CA):

\[
y = \text{bottom} + \frac{(\text{top} - \text{bottom})}{1 + 10^{(\text{LogEC}_{50}-x)/\text{Hill slope}}}
\]

To remove the substance from the skin, the patch was thoroughly rinsed with Ringer solution. All experiments were performed at a room temperature (22°C). To eliminate any influence of temperature on the results, all superfusion solutions were also kept at room temperature except when testing the effect of temperature on vessel diameter.

During the entire experiment, heart rate was monitored via ECG. Two chlorinated silver wire electrodes were inserted inbetween the skin and muscle on the two opposite sides of the patch where the skin was removed. An additional silver wire was used for grounding the animal. Electrodes were connected to a DAM 50 amplifier (World Precision Instruments, Sarasota, FL). The signal was amplified in alternating current mode with the high-pass filter set to 0.1 Hz and the low-pass filter to 100 Hz. The conditioned signal was sampled at 1,000 Hz, saved to the PC, and analyzed using Power Lab 4/25T laboratory interface and Chart software (AD Instruments).

**RESULTS**

The internal side of the exposed patch of *Xenopus* skin is extensively vascularized, with arteries and veins branching in parallel (Fig. 1C). In contrast to the systemic circulatory system, the arteries are darker and the veins are brighter. Frog skin is an area of gas exchange; therefore, the blood carried by the veins from the skin to the heart is more oxygenated and brighter than the deoxygenated blood in the arteries (3).

Upon closer inspection of live image or by viewing video footage, blood flow can be observed in the blood vessels, which are lined with melanophores. Arteries and veins can be distinguished by the direction of the blood flow from the arteries to arterioles and capillaries and from the capillaries to the veins. Blood flow could be observed properly only with a sufficiently high video refresh rate at low resolution (640 × 480)
480, 30 frames/s). At high resolution (1,280 × 1,024, 10 frames/s), the low refresh rate of the image on the display resulted in significant aliasing artifacts, which even caused the blood to appear to be flowing in the opposite direction. In small blood vessels, individual red blood cells were clearly distinguishable, making the blood flow easy to visualize. In larger vessels, this was more difficult due to the higher velocity of the flow and thickness of the observed part.

Borders of individual blood vessels could be easily identified in the microscope image. Due to the dense vascularization, it was possible to observe adjacent arteries and veins at the same time even at a high magnification (×240), allowing direct comparison between them and hence simultaneous measurements of vessel diameter. For quantitative observations of blood vessel diameter, we chose an area of the skin with a parallel branch of arterioles and veins. The part of the vessels where the diameter was measured was selected so that the initial diameter was sufficiently large to allow reasonably accurate measurements (Fig. 2, “reference” image; diameter of vein: 190 μm, diameter of arteriole: 128 μm, pixel size: 2 μm).

Before the application of vasoactive substances, the diameter of the blood vessels and blood flow were constant. Heart rate changed very little throughout the whole experiment, declining by only 3 beats/min in 2 h (Fig. 3A; 37 beats/min at 0 min, 36 beats/min at 60 min, and 34 beats/min at 120 min). This was probably due to modest deterioration of the preparation; nevertheless, the blood vessels remained responsive to the vasoactive agents tested.

At the beginning of the experiment, we superfused the skin patch with cold and warm Ringer solution (10 and 30°C, respectively) to see if vasoconstriction could be triggered by changes in temperature. However, we observed no significant change in either the diameter of the blood vessels or heart rate (Fig. 3, A and B, 0–14 min). Subtle changes occurred only in the smallest arterioles, which constricted slightly at the lower temperature.

To provoke the response of arteries and veins to vasoactive substances, we first superfused the skin patch with a series of NE solutions (0.01, 0.1, 1, 2, 4, 6, 8, and 10 μM). The application of NE caused arteries to constrict in a graded, dose-dependent manner (vessel images, Fig. 2; vessel diameter, Fig. 3B; dose-response curve, Fig. 4A). The EC50 in both...
animals was 1.34 and 1.98 μM, respectively. At high NE concentrations, the constriction was so severe that blood flow was completely obstructed. We also observed an interesting phenomenon during intermediate constrictions. In some of the smaller blood vessels, blood flow not only stopped but even switched its direction. In contrary to the arteries, the reduction of the diameter of the veins was much smaller. The veins, however, did not react directly to NE but rather constricted passively due to the reduced inflow of the blood from the constricted arteries. NE had no effect on heart rate (Fig. 3A).

After the application of the highest concentration of NE (Fig. 3, 30') the skin patch was rinsed with Ringer solution. Rinsing the skin had little immediate effect on the blood vessels. We then applied 1 μM doxazosin, an α-adrenergic receptor blocker. The application was immediately followed by a dilation of the arteries (Fig. 3, 33–36 min), which was very reduced in inflow of blood from the arteries. The subsequent application of 4 μM NE to the skin elicited only slight vasoconstriction of the arteries (Fig. 3, 33–36 min), which was very reduced compared with the first 4 μM NE application before doxazosin treatment (Fig. 4A).

To test the effect of epinephrine, we first rinsed the skin for ~2 min with Ringer solution. Vessels returned to ~80% of their original diameter (100 μM at 37 min and 125 μM at 0 min). Afterward, we superfused the skin with epinephrine solutions, progressively increasing epinephrine concentrations (0.01, 0.1, 1, 10, and 100 μM). Epinephrine exerted a similar effect on artery diameter as NE. The application of epinephrine caused a graded, dose-dependent constriction of arteries (vesSEL diameter, Fig. 3B; dose-response curve, Fig. 4B) followed by a passive constriction of veins. The EC50 in both animals was 0.64 and 1.72 μM, respectively. Subsequent application of doxazosin had a similar effect as in the case of NE, facilitating the dilation of the arteries (Fig. 3, 45–52 min).

Finally, we tested the influence of the α-adrenergic receptor blocker doxazosin mesylate on the dose-response dependence of blood vessel diameter to NE. Without any rinse after the application of doxazosin, we superfused the skin with NE solutions (6, 10, and 100 μM). As expected, blood vessels constricted at higher NE concentrations, shifting the dose-response curve of NE to higher concentrations by one order of magnitude (Fig. 3, 52–68 min; Fig. 4B). The EC50 was 11.27 μM.

DISCUSSION

This experiment is a very simple and graphical demonstration of peripheral blood flow and its regulation. Xenopus is a commonly used experimental animal in high school and university practical courses. The animals can often be easily obtained, frequently reared at university facilities or purchased as obsolete animals from companies that harvest their oocytes. The preparation of frog skin is quick, simple, and robust. Although the animal has to be euthanized by a professional, the preparation procedure itself can be accomplished by an unskilled person in <10 min. Selective vasoconstriction of the arteries and not veins by NE or epinephrine can be conveniently demonstrated even in a completely excised skin patch, albeit only once, until all blood is squeezed out. The setup of the whole experiment can be done in <30 min. The length of the whole experiment depends on the exact experimental protocol, but it is probably most suitable for a 2-h course, although the preparation remains functional for at least a couple of hours longer. The required instrumentation is relatively cheap, yet the magnification, working distance, field of view, and image quality are very good. One must take care, though, to avoid image defocusing due to the accumulation of solutions over the skin patch. This can be easily avoided by tilting the patch and allowing the solution to drain away. We also recommend using a sturdy heavyweight stand for the microscope; otherwise, it is difficult to change the magnification without losing the image from the microscope visual field. The image acquired with a USB microscope can be observed by a group of students on a computer display. The regular branching of arteries and veins allows for simultaneous observation of both types of blood vessels. Arteries and veins can be easily distinguished by the color of the blood, but the fact that their colors are swapped, compared with the systemic circulation, makes the observation even more appealing. The experiment represents a great opportunity to explain the particularities of the amphibian circulatory system. The difference in color of arterial blood compared with that of venous blood in the frog cadaver is perhaps exaggerated, though, with respect...
to the state in a living animal performing ventilatory movements and breathing with lungs, which maintain a high level of blood oxygenation. The live image also enables the students to observe the complexity of hemodynamics. In small arteries and capillaries, individual erythrocytes can be distinguished, which allows for a direct observation of blood flow. The velocity of the flow varies between different blood vessels and during the experiment. Blood flow can even reverse its direction in some blood vessels during intense vasoconstriction.

Students are usually familiar with the concept of temperature-dependent autoregulation of cutaneous blood flow in mammals. Thus, they intuitively expect to observe similar changes in cutaneous blood flow in ectothermic animals such as frogs. The skin vessels of frog cadaver, however, respond poorly to temperature changes.

The experiment was designed to allow the observation of physiological effects of two closely related signaling molecules, epinephrine and NE. Both provoked vasoconstriction only in the arteries, which beautifully demonstrated the structural and physiological differences between the arteries and veins (presence of smooth muscle and α-adrenergic receptors in the arteries and not in the veins). Epinephrine is a powerful vasoconstrictor in frogs (5). Mammalian arteries respond to both substances with somewhat opposing effects in a complex, concentration-dependent manner, i.e., low concentrations of epinephrine elicit vasodilation in skeletal muscle [“β-adrenergic effect” (1)] and high concentrations elicit vasoconstriction, whereas NE elicits vasoconstriction [“α-adrenergic effect” (1)]. We could not distinguish between the effects of the two catecholamines. Frog arteries never dilated but constricted upon the addition of either agent at similar concentrations. We assume that the epinephrine-provoked dilation could not be observed due to the absence of sympathetic vascular tone in the cadaver with a destroyed central nervous system. Besides that, we had no control over the endogenous level of catecholamines in either the blood or released from sympathetic terminals. The substances that we applied had to cross several layers of tissue, and their concentration at the site of action was unknown. Anyhow, our simple pharmacological assay yielded surprisingly repeatable results. In the four subsequent experiments in the student practicum, vasoconstriction always occurred in a similar range of agonist concentrations (1–10 μM NE; we abandoned the use of epinephrine for the sake of simplicity). The same holds for the α-adrenergic antagonist doxazosin mesylate, originally intended for human medical use, effective in frog cutaneous arteries at a concentration of 1 μM.

Students embraced the experiment due to its robustness and elegance, great visualization, and possibility of pharmacological intervention. The experiment could be easily expanded and modified for educational or even research purposes. Various receptor antagonists and agonists as well as pharmacological agents could be tested. Tissues could be collected for protein or gene expression analysis. In any experiment, the skin patch on the contralateral side could serve as the control. The use of frogs could also be substituted by the graphic material in this article and in the Supplement Material or provided from the authors upon request.1 However, frog experimentation in the classroom is far from obsolete. Students of experimental life sciences should always have the opportunity to perform hands-on experiments. Furthermore, the frog physiology course constitutes an important part of the curriculum at the International Biology Olympiad (4). Secondary school pupils compete in the practical part by isolating functional frog organs and demonstrating their ability to perform real experiments. This certainly sets high criteria for the knowledge and skills taught to university students.

DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS
Author contributions: A.Š. and G.B. conception and design of research; A.Š. and G.B. performed experiments; A.Š. and G.B. analyzed data; A.Š. and G.B. interpreted results of experiments; A.Š. and G.B. prepared figures; A.Š. and G.B. drafted manuscript; A.Š. and G.B. edited and revised manuscript; A.Š. and G.B. approved final version of manuscript.

REFERENCES

1 Supplemental Material for this article is available at the Advances in Physiology Education website.