Journal of Pediatric Advance Research

ATHENAEUM SCIENTIFIC PUBLISHERS

Research Article

The Functioning of the Frog's Single Ventricle

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Citation: Corno AF, et al. The Functioning of the Frog's Single Ventricle. J Pediatric Adv Res. 2023;2(3):1-8.

http://dx.doi.org/10.46889/JPAR.2023.2301

Received Date: 07-09-2023

Accepted Date: 25-09-2023

Published Date: 02-10-2023

Abstract

African clawed frog (*Xenopus laevis*) has double-inlet and single-outlet single ventricle supporting both systemic and pulmonary circulations, with normal lifespan of 25-30 years.

The relationship between pulmonary and systemic blood flows was investigated using echocardiography.

Twelve female African clawed frogs, age 18-24 months, mean weight 173±19 grams were studied.

Heart rate, percutaneous oxygen saturation, echocardiography derived 2D and blood flow in all cardiovascular structures were measured in two temperature setting, 17°C (normal environment for frogs) and 22°C. In eight frogs cerebral Near-Infra-Red Spectroscopy (NIRS) was measured.

Mean oxygen saturation was 66±9% with temperature of 22°C and 85±6% with 17°C (P<0.001), while there was no statistical difference between mean heart rates, respectively 48 13 and 42±7 beats/min. Doppler echocardiography showed a mean total pulmonary blood flow (QP) =173.0 ml/min and mean total systemic blood flow (QS) = 99.3 ml/min, with mean QP/QS of 1.74:1.00.

The only statistical correlation between size of the cardiovascular structures and body weight was found for the pulmonary veins, with correlation coefficient = 0.71 (P=0.02).

NIRS measurements showed a reverse correlation (coefficient = -0.21) with oxygen saturation.

The frog's heart has peculiar morphologic and functional characteristics allowing normal quality of life for many years despite the presence of single ventricle.

Baseline QP/QS under general anesthesia was significant for increased total pulmonary blood flow. Exposure to environmental temperature 5°C higher than normal caused peripheral vasodilatation, with subsequent decrease of QP/QS and reduced oxygenation.

Keywords: Amphibians; Congenital Heart Defects; Echocardiography; Frogs; Pathophysiology; Single Ventricle

Introduction

The life expectancy of children born with "functionally" uni-ventricular hearts is severely reduced, as well as the long-term outcomes are negatively affected by heart failure and cyanosis, substantially compromising the quality of life [1-13]. Reports of patients with single ventricle surviving to the adult life either without any surgical treatment or after limited palliative procedures without undergoing Fontan completion are exceptionally rare [12,14-23]. In patients born with this type of complex congenital heart defect at least 2-3 staged operations are needed to achieve Fontan circulation, with the single ventricular chamber pumping the oxygenated blood to the body (systemic circulation), while the desaturated blood returning from the superior and inferior vena cava by pressure difference passively flows directly through the lungs (pulmonary circulation) [1-13]. Abnormally high systemic venous pressure is a consequence of the arrangement of this type of circulation and the chronic elevation of systemic venous pressure may cause liver failure, renal failure, protein-losing enteropathy and plastic bronchitis [4-

11,13,24,25]. The only available approaches to prevent or treat failing Fontan circulation are mechanical assistance or heart and heart and liver transplantation [28,29]. Recent studies explored the potential role of stem cell therapy to support a failing single ventricle [26,27,30,31].

Mathematical and computational fluid dynamic models have investigated the blood flow distribution after a Fontan type of operation for single ventricle with various models of computational simulation with evaluation of alternative surgical approaches to possibly improve the efficiency of blood flows and subsequently the clinical outcomes [32-36]. Unfortunately, due to the limits intrinsic to all theoretical models, the simultaneous introduction of all the biological variables in living individuals and the interferences and interactions resulting from the variation of any biological parameter are not suitable in this type of studies.

Experimental research studies on animals born with single ventricle would be potentially useful, but the only animal models with single ventricle available in nature are amphibians like frogs (*Xenopus Laevis)* and salamanders and reptiles [37-48]. *Xenopus Laevis* (African clawed frog) have a single ventricular chamber providing the systemic and pulmonary circulation, receiving the oxygenated blood returning from the lungs via the left atrium and the less saturated blood returning from the body via the right atrium. The heart ejects the blood back to the lungs and the body through a single ventricular outlet, the conus arteriosus, dividing into right and left truncus arteriosus, each one with the lateral branch for the lung and skin perfusion, the medial one for the body perfusion and the central one for the perfusion of the head [49].

The peculiar anatomy of the frog's heart has been studied many years ago and previous reports of the morphology of the frog's heart tried to speculate on the cardiovascular function but only recently the pathophysiology of the human hearts with single ventricle has been compared with those observations [50-54].

Three different mechanisms are responsible for the gas exchanges in the *Xenopus Laevis*: cutaneous, bucco-pharyngeal and pulmonary respiration [49]. The cutaneous respiration occurs all the time, regardless if the frog is in or out of water; and when the frog is under water or hibernating, this is the only mode of respiration. On land, with the mouth constantly closed and the nostrils open, the bucco-pharyngeal respiration is used. Moist skin and the buccal cavity supplement the amount of oxygen provided through the pulmonary respiration [49]. In addition, amphibians have a substantially lower metabolic rate than mammals, including humans, reflected in the lower requirements for oxygen transport and cardiac output [49].

The heart of the *Xenopus Laevis* remains a heart with single ventricle, as in children with complex congenital heart defects, despite the compensations of oxygen supply provided by skin and mouth; nevertheless, it has been extremely well accounted for, by decades of research in reptiles and amphibians, that the *Xenopus* live a normal life for up to 25-30 years.

While other studies have previously been published on the *Xenopus Laevis* heart anatomy investigated by echocardiography, detailed information on the cardiovascular morphology investigated with microscopic dissecting techniques, as well as the anatomic correlations with echocardiography and cardiac magnetic resonance, were previously reported by us [41,55]. In this study the functioning of the single ventricle of Xenopus Laevis was investigated with echocardiography.

Materials and Methods

Femal *Xenopus laevis* frogs purchased from NASCO (LM00531MX), were housed in the Center for Laboratory Animal Medicine and Care at The UTHealth Science Center at Houston, McGovern Medical School, as in our previous reports [41,56]. All experiments were approved by the UTHealth Institutional Animal Care and Use Committee, Animal Welfare Committee, (protocol # AWC-19-0081). All *Xenopus Laevis* were housed in polycarbonate tanks on a recirculating system at a temperature of 16-19°C, pH of 7.3-7.8, conductivity of 800-1500 μS/cm and on a 12:12 light/dark cycle. Animals were fed frog brittle for *Xenopus Laevis* (SA05961 LM), NASCO) at twice/weekly intervals [41].

General Anesthesia and Recovery

General anesthesia was induced by immersion of the *Xenopus Laevis* in 0.05% Benzocaine solution (neutralized ethyl 3-aminobenzoate methanesulfonate (= MS222, Sigma Aldrich), 1.5 gram in 1 Liter of system water buffered with sodium bicarbonate to a pH of 7.0-7.4 [56].

For induction, *Xenopus Laevis* were placed in a small tank containing MS222 solution, in a position with the head raised and the nostrils above the water line. After checking for areflexia to verify the depth of general anesthesia, the *Xenopus Laevis* were wrapped in a paper towel, soaked in MS222 maintenance solution. Electrocardiography and respiratory rate leads were attached to the skin of the fore limbs for monitoring purposes. The *Xenopus Laevis* was then positioned in supine position for echocardiography [56].

After anesthesia induction and before cardiac investigations each *Xenopus* was weighed (grams) and a photo taken for identification and heart rate and percutaneous oxygen saturation measured, together with the recording of the room temperature, in two rooms with different temperature: 17°C (normal environment for frogs) and 22°C (used for echocardiographic investigations).

Once the echocardiography investigations were completed, the frogs were removed from the wrapping and placed in a solid-sided recovery tank with system water with the head elevated to ensure that the nostrils were above the water line. Frogs were returned to their home tank once they were able to swim around the recovery tank and were checked 24 hours later to verify normal recovery. Feeding was restarted after 24 hours and frogs were re-weighed after 48 hrs and then at monthly intervals. The details of our technique for induction and maintenance of general anesthesia for non-invasive cardiac investigations and the following recovery, were previously reported [56].

Echocardiography

After general anesthesia, twelve female African clawed frogs, age 18-24 months, still wrapped in paper towels soaked in MS222 maintenance solution to maintain proper skin moistening and anesthesic depth, were placed supine on the animal handling platform. Echocardiography investigation was performed using Vevo 3100 imaging system, MX250 transducer with 15-30 MHz range (VisualSonics, Toronto, Canada), with cardiovascular images obtained from the subcostal plane, sagittal plane (long-axis) and axial view (short-axis). After the basic anatomical structures were identified under B-mode, 2D diameters were measured, vascular blood flow recorded by color Doppler, measurements of size and flow taken in the right and left pulmonary veins, right and left atrio-ventricular inflow valves, single ventricular outlet. Conus arteriosus, right and left pulmocutaneous arteries, systemic arteries and carotid arteries respectively. The diameters of all the structures were measured in mm. The following functional measurements were recorded: velocity time integral (VTI in mm), mean velocity (mm/s), mean pressure gradient (mmHg), peak velocity (mm/s) and peak pressure gradient (mmHg). Blood flow of each structure was obtained by multiplying cross sectional area with VTI (ml/min). Size of all structures and flows were statistically analyzed to evaluate if there was a correlation among them and with the body weight. The total pulmonary blood flow (QP) was calculated by adding the flows measured in the right and left pulmocutaneous pulmonary arteries, while the total systemic blood flow (QS) was calculated by adding the flows measured in the right and left systemic and carotid arteries.

Near-Infra-Red Spectroscopy

In eight frogs the cerebral Near-Infra-Red Spectroscopy (NIRS) was measured, simultaneous with the percutaneous oxygen saturation. The NIRS monitor used was a Covidien Invosoximeter (Covidien LLC, Mansfield, MA) and the sensor Covidien Invos Infant Regional Saturation (Covidien LLC, Mansfield, MA).

Statistical Analysis

Mean (± Standard Deviation) and t-test were used for summarizing and testing continuous variables. The Spearman's rank correlation coefficient was used to test the correlation. The Spearman's correlation coefficient ranges from -1 to 1. Its sign indicates the direction of the correlation and its absolute value indicates the magnitude of the correlation. Statistically significant difference was defined as P-value <0.05. All analyses were conducted using R version 4.0.5.

Results

The presence of intact atrial septum and the complete separation of the two atrio-ventricular valves were previously reported with microscopic dissection, echocardiography and cardiac magnetic resonance imaging investigations [41], confirming that in the frog's heart there is complete streaming of the oxygenated blood of the left atrium from the less saturated blood of the right atrium. In all frogs, Doppler investigations were never able to show any evidence of stenosis of regurgitation of either of the two atrio-ventricular valves. The mean (± Standard Deviation) body weight of the frogs was 173±19 grams. The mean oxygen saturation was 66±9 % in the room with temperature of 22°C and 85±6 % in the room with 17°C (P<0.001), while there was no statistical difference between mean heart rates, respectively 48±13 and 42±7 beats/min.

Based on the measurements obtained with Doppler echocardiography, the mean total pulmonary blood flow (QP) was 173.0 ml/min and the mean total systemic blood flow (QS) was 99.3 ml/min. This provided a calculated QP/QS of 1.74:1.00. The only statistical correlation between size of the cardiovascular structures and body weight was found for the pulmonary veins, with a correlation coefficient = 0.71 (P = 0.02). The data of the brain NIRS measurements (Table 1) showed a reverse correlation with the oxygen saturation, with correlation coefficient = -0.21, but P didn't reach statistical significance (0.62), which may be due to the small sample (n=8).

Oxygen Saturation (%)	**Cerebral NIRS**
90	49
90	53
81	51
80	59
75	47
75	52
69	54
67	54
Statistics: Rho = -0.21, P = 0.62	

Table 1: Correlations between NIRS measurements and oxygen saturations.

Discussion

The *Xenopus Laevis*, as the other amphibians, has a functionally uni-ventricular heart, with a single ventricular cavity receiving oxygenated blood from left atrium and less saturated blood from right atrium, with the atrial chambers in situs solitus. The ventricular morphology of the frog is extremely complex, with a structure very spongiform and trabeculated, non-conducive for homogeneous mixing or at least making complete blood mixing extremely unlikely, differently from the mammalian heart with a well-defined cavity. Because of the ventricular morphology, the heart of frogs is not completely separated, but the blood flow is functionally divided into systemic and pulmonary circulations, allowing these animals to change blood volumes, but not pressures, between systemic and pulmonary circulations.

The hemodynamic significance of these anatomical findings suggested us to investigate the streaming of the blood flows and therefore to echocardiographically quantitate the pulmonary and systemic blood flows. To obtain better quantitative measurements with echocardiography, only female frogs were used, as in frogs the female are larger than males and therefore the approach with the echocardiography probe was easier. The results of this study showed that the frogs have a relatively elevated QP/QS (1.74:1.00), which not only provides a sufficient systemic oxygen saturation, but it is well tolerated for many years, without any evident sign of heart failure. This could be explained by a characteristic of the single ventricle in *Xenopus Laevis*, with an evident disproportionate ratio between mass and volume, in favor of the first one. This relationship of relatively large mass for a small volume of blood could explain the efficiency of the peculiar Xenopus myocardium, with a number of myofibrils adequate for a relatively reduced amount of work required to pump a relatively small volume of blood. An adequate cardiac output and therefore oxygen delivery, is maintained with relatively low oxygen consumption, thanks to the good ventricular ejection fraction (49.3±12.5%) and the low heart rate [49,52]. These metabolic and functional characteristics are sufficient to justify the absence of heart failure despite many years with an elevated QP/QS, with a relatively elevated cardiac output maintained by the single ventricle.

Another observation were the changes occurring with the different room temperatures. By moving the frogs from a room with their standard temperature (17°C) to an echocardiography room with higher temperature (22°C), the frogs percutaneous mean oxygen saturation decreased from their baseline 85±6% to 66±9% (P<0.001), with an increase of the mean heart rate from 42±7 to 48±13 beats/min, not statistically significant. These changes are the effects of the peripheral vasodilatation, due to an increase in the room temperature, associated with an increased QS, with relative reduction of the QP/QS and subsequence reduction of the oxygen saturation. Despite these hemodynamic changes lasted for the two-three hours required for the complete echocardiographic investigations, apparently no clinical deterioration was noted in the frogs, as all animals woke up and fully recovered from the general anesthesia. As this study was designed without any invasive approach, we couldn't measure any metabolic parameter requiring invasive manoeuvres to compare the two situations with low and higher temperature, such as lactate value or base excess. Previous experimental studies have investigated the changes of blood and flows distribution induced by acute hemodynamic variations [57-59].

Our observations of a trend toward a negative correlation between cerebral NIRS and percutaneous oxygen saturation is another confirmation of the reverse relationships between the systemic perfusion (cerebral NIRS) and the oxygen saturation, correlated with the QP/QS. However, this finding need be validated in a larger sample. Similarities between the anatomical and physiological findings in frogs and other amphibians and the few reported cases with single ventricle who survived until adult age without surgery have already been proposed [12-17,40,41,54]. The presence of intact interatrial septum, preventing any blood mixing at atrial level, may explain why, in the absence of complete mixing in the single ventricular chamber, the amphibians can live unrestrictive long life. The same is true because of the excessive trabeculated single ventricular chamber, certainly non-conducive for homogeneous mixing.

A large spectrum of ventricular architectural organization exists in nature, suitable for the lives of various animals, with different cardiac working requirements and performance [40]. Some animal's characteristics include to run quite fast while having have large bodies (vertebrates, mammals), while others are mostly sedentary with small bodies (like salamanders) or only intermittently jump (like frogs). Oxygen requirements and capacity at baseline and with exertion are different for different animals living circumstances and habits, even if these have not yet been studied well during exertion [40].

In the evolution in the animal species the more evolved species (larger animals, vertebrates, ectothermic) have taken advantage of split systemic and pulmonary circulatory systems in parallel, which seems to be the most effective and common model [60,61]. In animals living under water, level of activities, diet and access to favorite food, have all selected specific arrangements, especially in the organization of the heart and respiratory systems (lungs or gills or skin) [41]. For example, frogs are usually prone to jumping in place better than running, In the circulatory system of frogs the two circulations, fused at the level of single ventricle, are then split at the pulmonary and systemic arteries, where the amount of blood flow distributed between the outlet arteries is determined by the ratio of the peripheral resistances in the two territories [56]. It is likely that the pulmonary vascular resistance is variable in different functional states, with the resulting QP/QS, depending upon variable circumstances and environments as in human beings with single ventricle [54,56].

Study Limitations

- Amphibians such as the *Xenopus Laevis* have a single ventricle, both in terms of morphology and function, whereas children with functionally single ventricle typically have two ventricles albeit in a setting of one ventricle being severely hypoplastic
- Our observations with flows measurements performed using echocardiography constitute another initial step towards a complete analysis of the function of the frog's single ventricle [62]
- because of logistic reasons the echocardiographic measurements were performed at a room temperature of 22°C, higher than the 17°C, normal environmental temperature for frogs
- Studies of the systemic and pulmonary flows distribution with the more modern methodologies of ECG-gated MRI, validated in small vessels in experimental as well as clinical studies could provide high quality of innovation and creativity for our translational research [63-77]
- We always have to be aware of the species-to-species differences

Conclusion

The frog's heart has peculiar morphologic and functional characteristics allowing unrestrictive quality of life for many years despite the presence of a single ventricle. Our echocardiographic study demonstrated that the frogs have an elevated QP/QS as baseline status, subjected to adaptation to environmental changes. These observations could be confirmed by functional studies performed with cardiac MRI.

Competing Interests

All authors declare no competing interests, either financial or non-financial, professional or persona in relationship to any institution, organization or person.

Acknowledgments

We would like to acknowledge Thomas H Gomez and Noelia E Flores, for the help received at the animal facility of the University of Texas Health Science Center, McGovern Medical School and Callie Lazarine, Memorial Hermann Children's Hospital for the technical assistance. Wen Li was partially supported by UL1TR003167.

Conflict of Interest

The authors have no conflict of interest to declare.

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