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Disruption of Hemoglobin Oxygen Transport Does Not Impact Oxygen-Dependent Physiological Processes in Developing Embryos of Zebra Fish (*Danio rerio*)

Bernd Pelster, Warren W. Burggren

Abstract Embryonic hemoglobin circulated by the developing heart in the early vertebrate embryo is widely assumed (without substantiation) to perform the same vital role of O_2 carriage that it does in fetuses and adults. In order to challenge this assumption, we measured highly O_2-dependent physiological variables like O_2 consumption, cardiac performance, and initial swim bladder filling in the presence and absence of functional hemoglobin in the embryos and early larvae of the zebra fish, *Danio* (=*Brachydanio*) *rerio*. Functional ablation of hemoglobin by carbon monoxide or phenylhydrazine did not reduce whole-animal O_2 consumption, which was ≈85 to 90 $\mu mol \cdot g^{-1} \cdot h^{-1}$. Similarly, no differences in heart variables like ventricular pressure development or heart rate, which increased from 135 to 175 bpm between stages 36h and 96h (indicating developmental stages 36 and 96 hours after fertilization, respectively), were observed in these experiments. Initial opening of the swim bladder was not influenced in the presence of CO-occupied hemoglobin but was significantly impaired when the embryonic hemoglobin was chemically modified by incubation with phenylhydrazine. That aerobic processes continue without hemoglobin O_2 transport indicates the adequacy in the embryo of simple O_2 diffusion alone even in developmental stages with extensive convective blood circulation generated by the heart. (***Circ Res.* 1996;79:358-362.**)

Key Words • hemoglobin • embryonic hemoglobin • O_2 transport • zebra fish

The role of Hb in O_2 and CO_2 transport has been investigated primarily in the context of its function in adult vertebrates, but an extensive literature considers the vital role of blood in general and Hb in particular in O_2 transport and other functions in fetuses.[1-3] Embryos of all vertebrate taxa begin to produce Hb within hours or days of the onset of heart beat. In the absence of direct or even indirect experimental data, it has been universally assumed that the blood of embryos qualitatively functions in vivo as it does in fetuses and adults; ie, embryonic Hb in newly formed blood performs the same vital function of O_2 transport from the gas exchange organ to the developing tissues. However, a combination of the microscopic size and usually difficult access (embryo embedded in a placenta or surrounded by egg shell) has prevented extensive in vivo investigation of blood functions that would parallel the long-standing experiments on later developmental stages and that would confirm the vital role of Hb in early embryonic function suggested by in vitro studies.

The purpose of the present study was expressly to evaluate the importance of Hb function in embryos of the zebra fish, *Danio* (=*Brachydanio*) *rerio*. This species is well suited for our study: it has a relatively high metabolic rate in all developmental stages and thus a considerable demand for effective O_2 transport[4]; it has transparent embryos that are easily manipulated and is increasingly being used in genetic studies of cardiovascular development.[5] Our experimental approach was to focus on three primary functions that depend heavily on Hb function in the adult zebra fish and observe the impact of functional ablation of the Hb in the developing circulation on these functions. The primary functions were swim bladder inflation (used as in indicator of the presence of functional Hb), whole-animal O_2 consumption, and cardiac performance (heart rate, blood pressure, and rate of isometric pressure rise during systole). Functional ablation of the Hb was obtained by raising embryos in an atmosphere containing 2% CO or, chemically, by incubating the embryos in the presence of phenylhydrazine. CO disruption of Hb function has previously been used in studies on both chick embryos[6] and fish embryos.[7] However, CO exposures in both of these studies were highly acute, and animals were not reared in CO-enriched environments as in the present study. Phenylhydrazine causes a chemical ablation of the Hb.[8] A final condition we used to reduce (but not eliminate) functional Hb was hyperoxia, which in fish embryos impairs erythropoiesis and thus reduces the Hb content.[9]

Materials and Methods

Fertilized eggs of the zebra fish (*Danio rerio*) were obtained from a commercial outlet (Scientific Hatcheries, Huntington Beach, Calif). The embryos arrived in Las Vegas, Nev, 24 to 28 hours after fertilization (24h to 28h, according to the Westerfield staging table,[10] which is based on an incubation temperature of 28°C). Because of lower temperatures during initial transportation of the eggs and subsequent raising of the embryos in dechlorinated fresh water at 26±1°C, subsequent development of our embryos was slightly slower than that predicted by the Westerfield staging table. Typically, the developmental stage de-

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Selected Abbreviations and Acronyms

$\Delta P/\Delta T$ = ventricular pressure development
h (in association with preceding number) = hours after fertilization, according to Westerfield staging table
Hb = hemoglobin
TK = Taylor-Kollros

scribed for 84 hours by Westerfield in our embryos was reached after ≈92 to 96 hours. For comparison, developmental stages given in the present study are Westerfield stages, not the actual chronological age.

On arrival, the eggs were transferred to one of several incubation regimes: controls, 2% CO; 1 mg/L phenylhydrazine; 2 mg/L phenylhydrazine; hyperoxia at a P_{O_2} of ≈315 mm Hg; and normoxia without access to the water surface. Larvae were raised in the respective incubation regime until developmental stage 108h. Up to this stage, mortality typically was low (<5%), and no differences between the various incubation regimes were observed.

Swim bladder volume was calculated from dimensional measurements of swim bladders observed through a Wild M3Z dissecting microscope. The swim bladder was assumed to be in the shape of a prolate spheroid. Thus, the volume (V) was calculated from the measured swim bladder width and length using the formula for a prolate spheroid: $V=4/3 \cdot \pi ab^2$.

O_2 consumption was measured at a temperature of 26±1°C using closed glass respirometers (volume, 3 to 4 mL) as described by Burggren et al.[11] Briefly, three or four larvae were incubated in a respirometer, and the decrease in P_{O_2} was measured after the first and again after the second hour. To determine the body weight, the additive weight of seven groups of five larvae each was measured, with the average weight of a single larva determined by regression analysis.

Heart rate of unrestrained embryos and larvae was counted visually under a Wild M3Z microscope. Blood pressure in intact slightly anesthetized larvae was measured according to Pelster and Burggren[12] and Burggren and Fritsche,[13] using a servo-null micropressure system, model 5D (Instrumentation for Physiology and Medicine, Inc). Larvae were anesthetized with 0.05 g/L MS222. With the aid of a Wild M3Z microscope, a glass electrode (5-μm diameter tip) held in a micromanipulator was inserted through the larval body wall into the lumen of either the ventricle or truncus arteriosus to record dynamically central blood pressures. The output from the servo-null system was recorded on a Narco four-channel chart recorder.

Data are presented as mean±SE. Statistical differences between groups were tested with Student's *t* test or by one-way ANOVA using the SigmaStat program provided by Jandel, Inc. Significance of differences was accepted at $P<.05$.

Results

Initial inflation of the swim bladder was observed at about stage 84h. Therefore, swim bladder dimensions were measured, and the swim bladder volume was calculated for stage 84h and stage 96h larvae. No difference in swim bladder development was detectable among larvae reared under control conditions, larvae reared in a submerged closed container without access to air, and larvae reared in an atmosphere containing 2% CO (Fig 1). Ablation of Hb by phenylhydrazine solution, however, significantly reduced swim bladder volume between 84h and 96h. Zebra fish incubated under hyperoxic conditions, which usually delay formation of red blood cells in fish, also reduced swim bladder volume (Fig 1).

O_2 consumption was measured in zebra fish embryos and larvae between 84h and 108h. O_2 consumption of animals reared in the presence of either CO or phenylhydrazine was not significantly different from control values (Fig 2). O_2 consumption was quite constant at ≈16 nmol/h per individual or 85 to 90 $\mu mol \cdot g^{-1} \cdot h^{-1}$, irrespective of the developmental stage and the incubation regime.

Heart rate was counted in unrestrained specimens starting from embryonic stage 36h until larval stage 96h. Within this developmental period, heart rate increased slowly from ≈135 to ≈175 bpm (Fig 3). There was no significant difference between control animals and animals raised in the presence of CO.

Blood pressure traces recorded from within the developing ventricle and truncus of control embryos are shown in Fig 4A. Blood pressure recorded in the ventricle of stage 84h and of stage 96h larvae showed either a brief maximum, rapidly returning to diastolic values (pattern I, Fig 4), or a pattern in which systolic pressure remained high for >100 milliseconds before returning to diastolic pressure (pattern II, Fig 4). In adult vertebrates, high systolic pressure typically is sustained for many milliseconds, so that the second pattern probably represents the more advanced developmental stage. These changes thus may reflect the development of the central cardiac system. Importantly, these same patterns of pressure were evident in CO-incubated larvae (Fig 4B). Both systolic mean pressure and arterial systolic-diastolic blood pressure difference, and also $\Delta P/\Delta T$, were not significantly different ($P>.05$) in controls and animals raised with CO-blocked Hb (Fig 5).

Discussion

Microscopic inspection of the 0.1- to 0.2-mg zebra fish embryos revealed the presence of Hb early during development, and circulating red blood cells could be observed many hours before hatching. Indirect evidence for its functioning in gas transport was obtained from measurements of swim bladder inflation. Many teleost Hbs are characterized by the presence of the Root effect, ie, they show a reduction in O_2-carrying capacity with a reduction in blood pH, as distinct from the Bohr effect.[14,15] The secretion of acidic metabolites from gas gland cells in the swim bladder switches on the Root effect, driving O_2 from Hb passing through the swim bladder blood vessels. By means of countercurrent backdiffusion and concentration, the O_2 partial pressure in swim bladder blood is multiplied many fold, generating gas pressures that can inflate the swim bladder even against transmural pressures as high as several hundred atmospheres.[16,17] The swim bladder of *Danio rerio* inflates several hours after hatching (about stage 84h of total development). In larval teleosts that do not surface and gulp an air bubble for the initial inflation, it is presumed but not known that this initial inflation and subsequent maintenance of inflation involves Hb and the Root effect as in adults. We measured swim bladder volume in populations of zebra fish larvae reared while chronically exposed to either phenylhydrazine or hyperoxia. In both groups, the volume of the swim bladder at stage 84h was significantly reduced compared with controls, indicating that a reduced Hb content impairs swim bladder inflation. Functional ablation of the Hb with CO, however, had no effect on initial swim bladder inflation. Adult fishes can secrete Hb-transported CO into their swim bladders via the Root effect,[18] because acidification induces a transition of Hb to its "deoxygenated" state irrespective of whether it is binding of O_2 or CO.[19,20] This suggests that in an at-

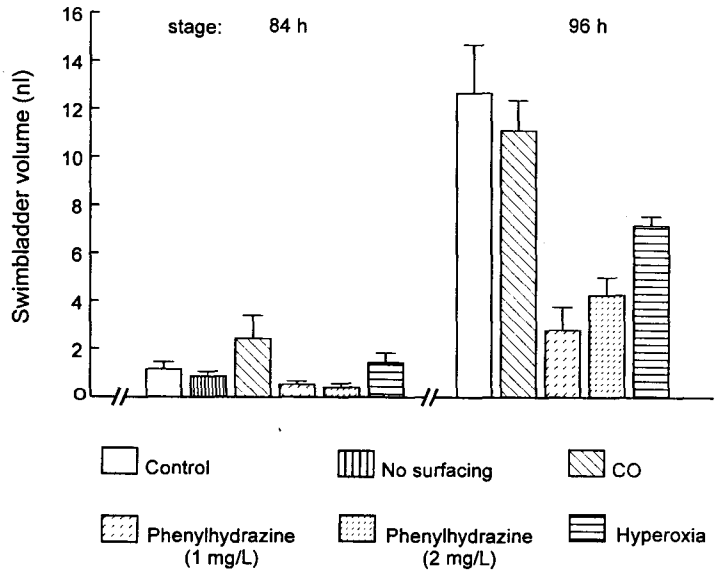

FIG 1. Swim bladder volume measured at stages 84h and 96h. Typically, the swim bladder was first inflated at stage 84h. Swim bladder inflation and volume were impaired by chemical ablation of the Hb with phenylhydrazine or by delaying red blood cell formation in hyperoxia. In the presence of 2% CO, however, swim bladder inflation was not different from control, suggesting that in these animals the swim bladder was filled with CO instead of oxygen. $P<.05$ vs control.

mosphere containing 2% CO, *Danio rerio* were inflating their swim bladders with CO instead of O_2. Collectively, these results indicate the presence of Hb capable of O_2 carriage and with a Root effect for O_2 binding in larval *Danio rerio* at least by stage 84h.

The significance of the convective delivery of O_2 by Hb to O_2-dependent processes in populations of larval zebra fish continuously exposed to either 2% CO or phenylhydrazine from 1 day after fertilization through 3 days after hatching was assessed by measurement of $\dot{M}O_2$. The results demonstrate that for the first 4 to 5 days of development, O_2 delivery to the tissues by simple diffusion is adequate to support normal aerobic metabolism of the highly metabolically active embryos and larvae, even though there is already functional Hb and the convective circulation of blood at this point in development.

Further corroboration of the fully aerobic nature of embryos and larvae deprived of Hb-O_2 transport came from observations of cardiac performance. Cardiac muscle is a highly oxidative tissue, and suboptimal delivery of O_2 to working cardiac muscle would be expected to impact heart contractility and pressure generation. Importantly, there were no qualitative or quantitative differences in the pressure traces between controls and CO-incubated animals. This was confirmed by quantitative analysis of blood pressures recorded in various locations of the central cardiac system and also by the kinetics of pressure development ($\Delta P/\Delta T$), which revealed no difference between controls and animals in which the Hb was functionally blocked for O_2 transport because of the presence of CO. These data show that Hb-O_2 transport is unnecessary and that the diffusional supply of O_2 is adequate to support even the continuous high aerobic metabolic demand for O_2 characteristic of cardiac muscle in early zebra fish larvae.

Interestingly, we observed two different patterns of intraventricular and central arterial blood pressure waves in zebra fish embryos. Both patterns have been observed with the same micropressure system at similar heart frequencies, so that the late pattern cannot be an artifact due to

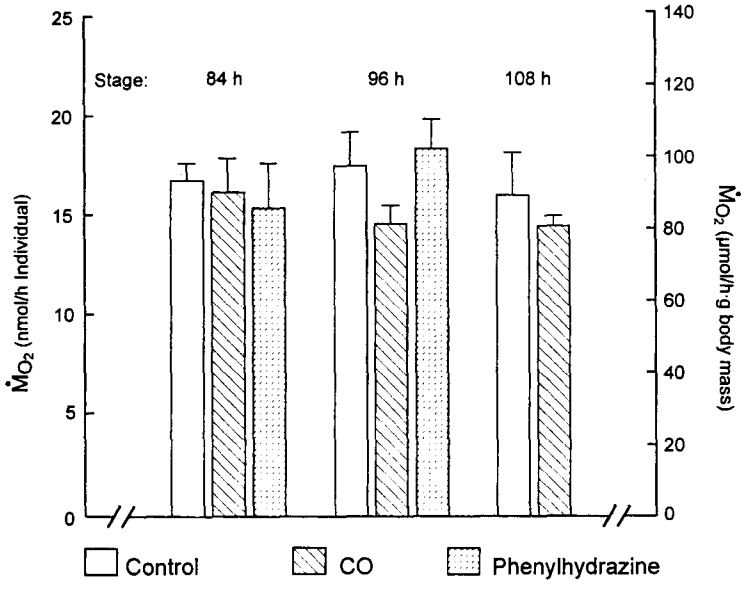

FIG 2. O_2 consumption ($\dot{M}O_2$) as measured in a closed respirometer at stages 84h, 96h, and 108h was not different from control in animals with Hb functionally blocked with an atmosphere containing 2% CO or chemically induced anemia by incubation with phenylhydrazine (1 mg/L).

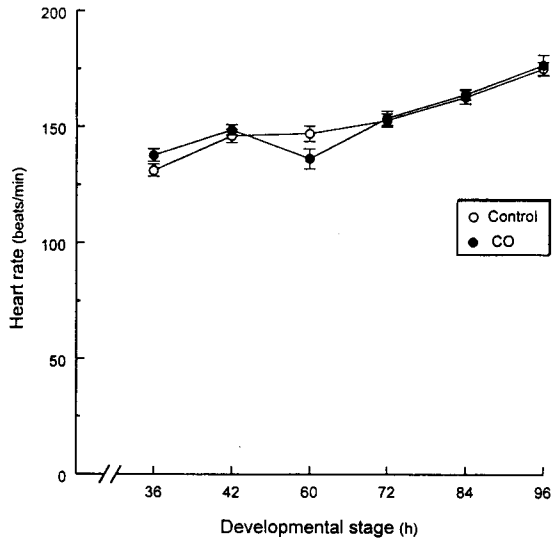

FIG 3. Heart rate slowly increased during development between stages 36h and 96h, but no difference was detectable between animals incubated in 2% CO and control animals.

blunting of the peak. Furthermore, the validity of the micropressure system has been demonstrated by comparison with pressure measurements using fine-needle catheters and conventional pressure transducers in birds[21,22] and in amphibians (authors' unpublished data, 1996). In vitro tests of the frequency response of micropressure systems was flat up to 10 Hz,[23] so that frequencies of up to 2 to 3 Hz, as in the present study, can safely be measured. In the "early" pattern (pattern I) observed in zebra fish larvae, high systolic pressures were observed only for a few milliseconds. In the "adult" or "late" pattern (pattern II), systolic pressure remained high for a short period before decreasing to the diastolic level. Both patterns have been observed in stage 84h larvae as well as in stage 96h larvae. Similar patterns have previously been recorded in larval *Rana catesbeiana*[12] or *Xenopus laevis* (R. Fritsche and W.W. Burggren, unpublished data, 1996) and in embryonic skates *Raja erinacea*.[24] In *Rana catesbeiana* larvae, in which development takes much longer than in the zebra fish and pressure development of the cardiac system could be followed from TK stage II to TK stage XIII larvae, the "early" pattern of pressure development, in which high systolic pressure is only sustained for a few milliseconds, was restricted to early larval stages (up to TK stage VIII-IX). Later stages showed pressure changes qualitatively similar to the adult pressure changes, although maximum systolic pressure was lower than in adults.[12] This suggests that the development of cardiac muscle tissue may in part be responsible for these changes in pressure development.

Collectively, our findings indicate that significant Hb-O_2 transport occurs in early zebra fish larvae (as evidenced by inflation of the swim bladder) but that convective supply of O_2 is not absolutely required to maintain normal levels of $\dot{M}O_2$ by cardiac and other highly aerobic tissues. Presumably, control levels of $\dot{M}O_2$ of cardiac and other tissues are maintained by diffusion rather than convection. Diffusion can supply sufficient O_2 provided that the pathway for O_2 diffusion is short and that a large O_2 diffusion gradient is maintained between the immediate external environment and the O_2-consuming tissues. Additionally, the length of the diffusion pathway for gases can be extended greatly by unstirred boundary layers around the animal.[25,26] Zebra fish larvae begin to show locomotor activity even before hatching, and the intermittent body movements that they develop helps disrupt the boundary layers surrounding their integument.

Why does the zebra fish heart begin to beat and propel blood containing Hb to the growing tissues well in advance of the absolute need for convective O_2 supply (a hypothesis termed prosynchronotropy by Burggren and Territo[27])? Perhaps nutrient and/or electrolyte transport rather than gas transport becomes a limiting factor during early development, because diffusional transport of metabolites and charged ions might be slower than diffusion of small gas molecules. Another reason for "early" beat of the heart could be to assist angiogenesis, because pressurized pulsatile blood flow in itself is crucial for the growth and spreading of the vascular system, which is caused in part by the proliferation of endothelial cells.[28-31] Weinstein et

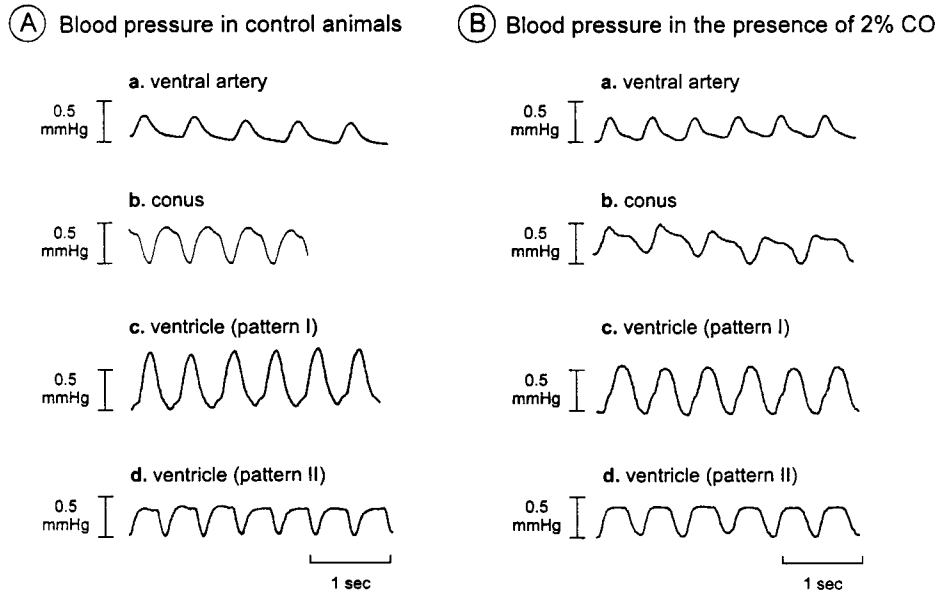

FIG 4. Typical pressure profiles recorded in the ventral artery, the conus arteriosus, or the ventricle of stage 84h and stage 96h larvae under control conditions (A) and in animals raised in the presence of 2% CO (B). Ventricular pressure in some preparations decreased rapidly almost immediately after reaching the maximum (pattern I); in others, a high systolic pressure was generated for >100 milliseconds (pattern II). These changes probably reflect the development of cardiac muscle tissue (see "Results" and "Discussion" for further explanation). Pressure traces recorded from animals incubated in presence of 2% CO were not different from traces recorded in control animals.

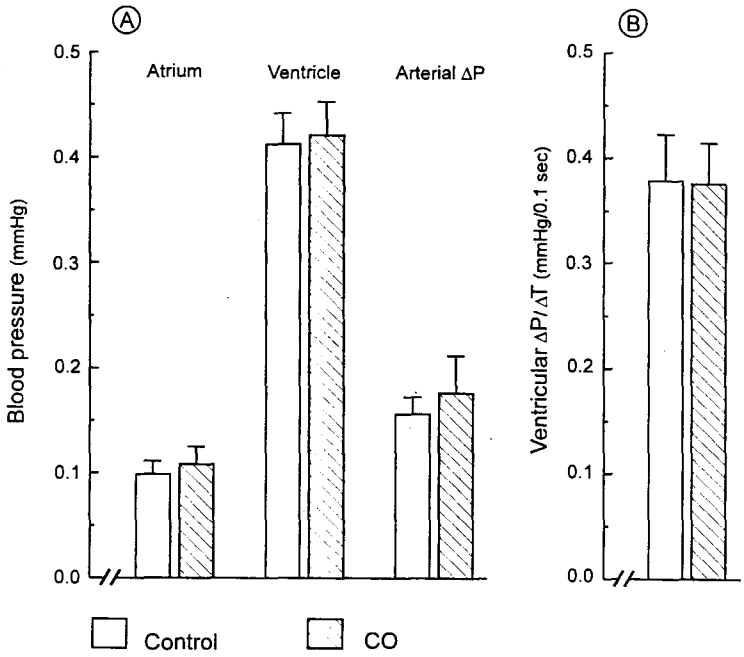

FIG 5. Quantitative analysis of pressure traces from control (A) and CO-incubated (B) animals revealed no difference in absolute pressure generated in the atrium or the ventricle, in the systolic-diastolic pressure difference in the ventral artery, or even in $\Delta P/\Delta T$ ($P > .05$).

al[32] show that in the gridlock mutation of the zebra fish, collateral vessels are generated within the first 60 hours of development as a consequence of the vascular lesion blocking the tail circulation.

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