Neural Crest Ablation *Versus* Sham Surgical Effects in a Chick Embryo Model of Defective Cardiovascular Development

LINDA LEATHERBURY, DAVID M. CONNUCK, AND MARGARET L. KIRBY

Departments of Pediatric Cardiology [L.L., D.M.C., M.L.K.], and Cellular Biology and Anatomy [D.M.C., M.L.K.], Medical College of Georgia, Augusta, Georgia, 30912-3710

ABSTRACT. In chick embryos, ablation of premigratory neural crest destined for the third, fourth, and sixth pharyngeal arches results in persistent truncus arteriosus and interrupted aortic arch. Studies of cardiogenesis in these embryos have shown decreased ejection fraction and ventricular dilation with normal cardiac output. The experimental embryos that survive to d 11 of incubation have a higher cardiac output than nonsurvivors at the same earlier stage of development. We hypothesize that this survival is due to a surgically induced decrease in vascular resistance of the embryonic and vitelline vessels. Embryos from 15 opened eggs, eight sham-operated embryos, and 13 neural crest-ablated embryos were examined at stage 18. The sham-operated embryos were treated identically with the experimental embryos except that neural crest was not ablated. Ejection fraction and cardiac output were determined by cinephotography. Mean dorsal aortic and diastolic ventricular (approximates mean atrial) pressures were determined by a servo-null pressure technique and used to calculate vascular resistance. The experimental embryos had a significantly decreased ejection fraction in comparison with either sham-operated embryos or embryos from eggs that remained unopened until just before cinephotography. The experimental and sham-operated embryos had a higher cardiac output than embryos from unopened eggs. The calculated vascular resistance was also significantly lower in both the experimental and sham-operated embryos than in the embryos from unopened eggs. Neural crest ablation appears to cause a decreased ejection fraction. Sham surgery is associated with a higher cardiac output and lower vascular resistance. These hemodynamic changes may be related to opening and resealing the egg-shell, which would interfere with oxygen delivery and water vapor exchange. (*Pediatr Res* 33: 628–631, 1993)

The neural crest has been shown to be very important in normal cardiovascular development (1). The region of neural crest contributing to cardiovascular development extends from the midotic placode to the caudal limit of somite three. Cells from this region migrate from the neural fold into pharyngeal arches three, four, and six (2–4). A population of the neural crest-derived cells continue their migration through the pharyngeal region into the outflow tract of the heart, where they participate in outflow septation. Ablation of the premigratory neural crest destined for the third, fourth, and sixth pharyngeal

arches and outflow septa produces a high incidence of persistent truncus arteriosus and anomalous development of the derivatives of the aortic arch arteries.

Nishibatake *et al.* (5) demonstrated that ablation of premigratory neural crest destined for pharyngeal arches four and six resulted in a 73% incidence of persistent truncus arteriosus. An additional 10% had other outflow anomalies, most commonly dextroposed aorta associated with double-outlet right ventricle. There was also a 50% incidence of severe aortic arch anomalies, such as interruption of the aortic arch, and a 20% incidence of inflow tract anomalies associated with maldevelopment of the tricuspid valve. A study by Tomita *et al.* (6) demonstrated that ablation of premigratory neural crest destined for the third and fourth pharyngeal arches produced an 83% incidence of persistent truncus arteriosus, and 100% incidence of anomalies of the derivatives of the aortic arch arteries.

Microcinephotography studies in early cardiogenesis, while the heart is a looped tube (Hamburger-Hamilton stage 18), have shown that morphologic and functional changes occur long before the time when the developing heart would be recognized as phenotypically abnormal. Depressed contractility and dilation of the primitive ventricle have been reported from visual assessment by microcinephotography (7). This visual impression was later confirmed by quantitative data showing the primitive ventricle to be dilated in both end-diastole and end-systole with decreased shortening and ejection fractions in embryos with neural crest ablations (8). In these studies, it appeared that the experimental embryos were able to maintain an adequate cardiac output for survival due to ventricular dilation, which was thus considered to be a compensatory mechanism.

In a subsequent study by Tomita *et al.* (6) microcinephotography was used to determine cardiac output in stage 18 embryos with subsequent reincubation of the embryos until an age when the cardiovascular anatomy could be evaluated (d 11 of incubation). This study showed that experimental embryos surviving to d 11 had a significantly higher cardiac output, as compared with nonsurvivors. However, the cardiac output of the whole experimental group had a similar mean in comparison with a control group of embryos from eggs that were opened at 24 h of incubation but had no other treatment. Other studies in the stage 18 embryos showed that the dorsal aortic blood pressure showed a tendency toward lower diastolic pressure (9, 10), whereas the vitelline arteries of neural crest-ablated embryos had lower systolic, diastolic, and mean pressures (10).

The observation, from microcinephotography, that 50% of embryos with neural crest ablations did not have apparent blood flow through the right fourth aortic arch artery at stage 18 correlates well with the finding that 50% of the embryos at d 11 have an interrupted aortic arch (5, 7). It was hypothesized that the lower pressures distal to the aortic arch arteries might represent a pressure gradient due to obstruction across the aortic arch arteries. Subsequent experiments measuring ventricular and dor-

Received August 11, 1992; accepted January 22, 1993.

Correspondence and reprint requests: Linda Leatherbury, M.D., Pediatric Cardiology, BAA8302, Medical College of Georgia, Augusta, GA 30912-3710.

Supported by PHS Grant HL-36059.

sal aortic pressures showed that there was a similar systolic gradient across the conotruncus and across the total sum of the aortic arch arteries (9) in embryos from unopened eggs and sham-operated and experimental embryos. Considering that embryos with cardiac neural crest ablations have an increased cardiac output and a tendency toward lower diastolic dorsal aortic pressure, we proposed the hypothesis that there may be a decrease in the embryonic and vitelline vascular resistance in the experimental embryos.

Most previous studies of hemodynamic parameters in the neural crest model of defective cardiovascular development have used an unopened egg control group and a neural crest-ablated experimental group. Unpublished microcinematography observations of a sham-operated control group suggest that there may be surgical effects on the developing cardiovascular system that are independent of the neural crest ablation, as well as specific effects due to the neural crest ablation. The second hypothesis of this study was that opening the eggshell and/or preparation of the embryo for neural crest ablation may be causing changes in the ejection fraction, cardiac output, dorsal aortic pressures, and vascular resistance in early cardiovascular development in addition to those caused by neural crest ablation.

MATERIALS AND METHODS

Animal preparation. Fertilized Arbor Acre chicken eggs (Seaboard Hatchery, Athens, GA) were incubated in a forced-draft incubator at 37.5°C. Three groups of embryos (unopened controls, sham-operated, and neural crest-ablated) were used in this experiment. The sham-operated and neural crest-ablated embryos were incubated for 25 to 30 h. They were "windowed" by sanding a 1-cm² hole in the eggshell. The airspace was punctured, and the egg shell membrane was removed. The embryo was stained using neutral red-impregnated agar, and the stage of development of the embryo was determined according to the procedure of Hamburger and Hamilton (11). The vitelline membrane overlying the cardiac neural crest was torn. In the experimental group, the neural fold was ablated by bilateral microcauterization between the midotic placode and somite three. Embryos in the sham-operated group were treated the same as those in the experimental group with the exception that the premigratory neural crest was not ablated. The unopened control group of eggs remained unopened but were incubated and transferred in parallel with the sham-operated and experimental eggs. In the sham-operated and experimental groups, the hole in the eggshell was sealed with cellophane tape and the eggs reincubated in a high-humidity incubator. Twenty-four h after surgery, the eggs were transferred to a second incubator at 37.5°C and 70% relative humidity. The eggs in the sham-operated and experimental groups were reopened by removing the cellophane tape at d 3 for staging of the embryo in preparation for microcinematography at stage 18. The unopened control eggs were "windowed" on d 3, at least 30 min before microcinematography at stage 18.

Microcinematography and film analysis. Analysis of microcinematography films as described by Leatherbury *et al.* (8) was used to determine the shortening fraction, ejection fraction, ventricular diastolic and systolic areas, and cardiac output. In preparation for filming, each egg was transferred to a heated sand bath. Using a thermistor probe placed on the surface of the yolk sac adjacent to the embryo, the temperature of the sand bath was adjusted so that the embryo was maintained at a constant temperature of 37.5°C. At stage 18, embryos were selected for filming. Embryos with axial torsion that altered or obscured the visual characteristics of the cardiovascular system were not filmed. Survival rate, incidence of persistent truncus arteriosus, and aortic arch anomalies have been shown to be identical in embryos selected for microcinematography by these criteria and embryos eliminated from study (6). Filming was done with a high-speed camera mounted on a stereomicroscope, with lighting

provided by two fiberoptic light sources. The primary light source was focused through a lens attached to a strobe that was triggered by the camera's shutter opening. Each embryo was filmed for 5 s at a film rate of 100 frames/s, which resulted in 40 frames/cardiac cycle for a typical embryo. Each embryo was positioned in the sand bath at the same distance from the microscope lens, yielding a magnification factor of approximately 30× on the film. A stage micrometer was filmed at the same focus plane as the embryo to allow exact calibration of the magnification factor.

At stage 18, the embryo lies with its right side up. The internal boundaries of the developing cardiovascular structures can be seen through the transparent embryonic tissue, delineated by the presence of red blood cells. At this stage of development, the heart is a looped tube with a well-defined sinus venosus and atrium, primitive left and right ventricle, and outflow tract. Aortic arch arteries two, three, and four are present bilaterally.

The frame selected for end-diastolic measurements showed the largest amount of blood in the ventricle, whereas that selected for end-systolic measurements showed the least amount. Using a digitizing pad, the endocardial boundaries of the primitive right ventricle and outflow tract were measured using a planimeter, yielding areas that were summed for the bulbus cordis area. Midventricular linear measurements were taken to compute the shortening fraction. Using a computer software program specifically designed for electronic planimetry (12), the areas were rotated on their long axis to complete 360° for volume determinations (8). The exact number of film frames counted between an end-diastolic frame and the subsequent end-systolic frame and the film speed of 100 frames/s were used to calculate the heart rate of each embryo.

Pressure measurements and resistance calculations. Intraventricular and dorsal aortic pressures were measured using a sharpened glass microelectrode (3- to 5-μm tip) filled with 2 μM NaCl attached to a WPI (Sarasota, FL) 900 servo-null pressure system (10). The signal from the servo-null transducer was digitized (12-bit 100 Hz) by an IBM AT-based data acquisition system and stored on a disk for later analysis. In half of the embryos, the intraventricular pressures were measured first, followed by dorsal aortic pressures. This process was reversed in the remainder of the embryos. Peak systolic, mean diastolic, and end-diastolic ventricular pressures were determined from these digitized signals, although only the mean diastolic ventricular pressure was used in this study. As there is no significant gradient across the atrioventricular canal in stage 18 chick embryos, the mean diastolic ventricular pressure was used as an equivalent to the mean systemic venous pressure. Peak systolic, mean, and diastolic dorsal aortic pressures were measured distal to the entrance of the aortic arch arteries into the dorsal aorta. Pressure measurements were made immediately after completion of the microcinematography. Any embryo that bled on insertion of an electrode was discarded from the study. Heart rates were also determined from the digitized pulsatile pressure records and were compared with heart rate determined from cinematography. If the heart rates differed more than 10%, the embryo was not included in the analysis.

To interrelate the cardiac output and pressure determinations, resistance values were calculated for blood flow across the embryonic and vitelline vasculature. The pressure difference across this vascular bed was calculated by subtracting the ventricular mean diastolic pressure, as an approximation of the systemic venous mean pressure, from the dorsal aortic mean pressure. This pressure difference was divided by the cardiac output to yield resistance units in mm Hg × min/mm³.

Statistical analysis. Two independent observers, who were not aware of the treatment groups of the embryos being analyzed, digitized the cinematographic films. The study described above used the three independent variables of the unopened control, sham-operated, and neural crest-ablated embryos. The numbers of embryos in which microcinematography and pressure variables, respectively, were obtained were as follows: 15 and 21 in

the unopened egg control embryos, 15 and 14 in the sham-operated embryos, and 13 and 15 in the experimental embryos. Microcinéphotography and pressure variables from the same embryo for 15 unopened control embryos, eight sham-operated embryos, and 13 neural crest-ablated embryos were used to calculate the vascular resistance. Analysis of variance was performed, and significant interactions were subjected to a followup analysis by Tukey's honest significant difference procedure. Significant interactions were taken as $p < 0.5$.

RESULTS

The shortening fraction as well as the ejection fraction of stage 18 embryos were significantly lower in embryos with neural crest ablations than in either the sham-operated or unopened control embryos (Table 1). Similarly, the systolic area of the primitive ventricle was larger in experimental embryos than in either sham-operated or unopened control embryos (Table 2). These results suggest that the decreased indices of contractility, reported previously (8), and confirmed here, are a direct result of neural crest ablation.

However, the diastolic area of the primitive ventricle was larger in both the experimental and sham-operated embryos than in the unopened control embryos (Table 2). These results suggest that diastolic dilation of the primitive ventricle was, in part, due to the manipulation of the egg and embryo in preparation for surgery rather than the neural crest ablation *per se*. Table 3 shows that the stroke volume was higher in both the experimental and sham-operated embryos than in the unopened control embryos. The heart rate was similar for all three groups. Therefore, both the experimental and sham-operated embryos had a higher cardiac output than the unopened control embryos.

The dorsal aortic pressures measured distal to the aortic arch arteries can be seen in Table 4. There was a tendency for the diastolic pressures to be lower in the experimental and sham-operated embryos than in the unopened control embryos. The estimated resistances of the embryonic and vitelline vasculature

Table 1. *Indices of contractility at stage 18**

	Shortening fraction (%)	Ejection fraction (%)
Unopened controls	78 ± 3.8†	96 ± 0.9†
Sham-operated	69 ± 3.8†	94 ± 2.5†
Experimental embryos	53 ± 3.4	87 ± 2.7

* Values are mean ± SEM.

† $p < 0.001$ vs experimental embryos.

Table 2. *Dilation of ventricle at stage 18**

	Area diastolic (mm ²)	Area systolic (mm ²)
Unopened controls	0.31 ± 0.025	0.003 ± 0.008†
Sham-operated	0.42 ± 0.030‡	0.015 ± 0.008†
Experimental embryos	0.45 ± 0.030‡	0.028 ± 0.007

* Values are mean ± SEM.

† $p < 0.005$ vs experimental embryos.

‡ $p < 0.005$ vs unopened controls.

Table 3. *Cardiac output at stage 18**

	Stroke vol. (mm ³)	Heart rate (bpm)	Cardiac output (mm ³ /min)
Unopened controls	0.15 ± 0.017	151 ± 3	23.4 ± 3.7
Sham-operated	0.22 ± 0.018†	151 ± 3	32.9 ± 4.2‡
Experimental embryos	0.21 ± 0.019†	150 ± 4	32.3 ± 4.4‡

* Values are mean ± SEM. Stroke volume × heart rate = cardiac output.

† $p < 0.001$ vs unopened controls.

‡ $p < 0.05$ vs unopened controls.

Table 4. *Dorsal aortic pressures at stage 18**

	Systolic (mm Hg)	Diastolic (mm Hg)	Mean (mm Hg)
Unopened controls	0.91 ± 0.05	0.51 ± 0.04	0.72 ± 0.05
Sham-operated	0.91 ± 0.05	0.45 ± 0.04	0.69 ± 0.04
Experimental embryos	0.91 ± 0.05	0.43 ± 0.04	0.66 ± 0.04

* Values are mean ± SEM. There were no significant differences between groups.

are listed in Table 5. The dorsal aortic mean pressure was used as the pressure seen by the largest component of the embryonic and vitelline vasculature beds.

The cardiac output determined supports the entire embryo and the vitelline vasculature. The vascular resistance was significantly lower in both the experimental and sham-operated embryos than in the unopened control embryos, suggesting that there was a strong "sham" effect not attributable to neural crest ablation.

DISCUSSION

Previous studies (7, 9, 10) have shown early hemodynamic changes in neural crest-ablated embryos in comparison with embryos from unopened eggs. The results of this study show that sham-operated embryos are an important control group because there are hemodynamic changes in sham-operated embryos that are distinct from those found after neural crest ablation.

The etiology of the hemodynamic changes seen in the sham-operated embryos can be inferred from a review of several experiments using different types of control groups. The "unopened egg" control group used in this study actually had a 1-cm² portion of eggshell removed 30 min before microcinéphotography. The hemodynamic measurements did not show an increase in the diastolic volume or stroke volume in these embryos that could be measured in the sham-operated embryos. However, another control group in which the eggs were windowed at stages 9 to 10 did show an increased stroke volume at stage 18 similar to that seen in sham-operated embryos (6). The vitelline membrane was not torn, and the embryos were not stained with neutral red for the "opened egg" control group. Thus, the process, of puncturing the air sac or cell, removing a portion of eggshell and shell membrane, and resealing the eggshell with cellophane tape leads to the hemodynamic changes seen in our sham-operated embryos.

Both respiration and intravascular volume of the early developing avian embryo are dependent on the exchange of water vapor, oxygen, and carbon dioxide through conductance across the area vasculosa and chorioallantoic vessel walls, the air cell (air-filled space at the blunt end of the egg), and the gas-filled pores in the eggshell (13). Chick embryos in early development (d 5) actually have a high air-cell oxygen tension or PO₂ of 140 torr (18.7 kPa) with an oxygen tension differential across the porous shell of 40 torr (5.3 kPa) (14). The oxygen supply available to the embryo is progressively decreased during development to a PO₂ in the air cell of 98 to 105 torr (13.1 to 14.0 kPa) just before hatching (15, 16). Puncturing the air cell is a part of the procedure of opening the egg. This would decrease the air-cell oxygen tension, which would presumably result in acute hypoxemia. Hypoxemia is a known acute vessel vasodilator that is probably the stimulus for the decreased vascular resistance seen in our sham-operated or "opened egg" control embryos. Decreased vascular resistance would be compensated acutely with an increase in stroke volume to maintain blood pressure. This appears to be exactly what happened in the sham-operated embryos in this study.

Disruption of a portion of the normal shell surface and its replacement with the impermeant cellophane tape would also cause chronic hypoxemia due to decreasing the surface area of the egg available for gas exchange. Eggs incubated with increasing

Table 5. Estimated resistance of embryonic and vitelline vasculature at stage 18*

	Dorsal aorta mean pressure (mm Hg)	Ventricular mean diastolic pressure (mm Hg)	Cardiac output (mm ³ /min)	Resistance of vasculature (mm Hg × min/mm ³)
Unopened controls	0.72 ± 0.05	0.20 ± 0.02	23.4 ± 3.7	0.022 ± 0.004
Sham-operated	0.69 ± 0.04	0.19 ± 0.02	32.9 ± 4.2†	0.015 ± 0.005†
Experimental embryos	0.66 ± 0.04	0.21 ± 0.02	32.3 ± 4.4†	0.014 ± 0.006†

* Values are mean ± SEM. (Dorsal aorta mean pressure – ventricular mean diastolic pressure)/cardiac output = resistance of vasculature.

† $p < 0.05$ vs unopened controls.

paraffin strips in room air, at sea level, 37°C, and elevated relative humidity show progressively decreasing growth rate (weight) and decreased survival (17). Oxygen availability is the most important factor in both survival and growth in chick embryos (18). If the oxygen-diffusing capacity is reduced by 20% due to a neoprene coating on the egg, there is a decrease in growth and survival. The effect of the neoprene coating can be counteracted by incubating the egg in increased environmental oxygen. Cessation of growth is a mechanism for conserving oxygen that is not available to an adult animal (18). Decreased embryonic growth (Creazzo TL, unpublished data) as well as survival (4) have been seen routinely in experimental and sham-operated chick embryos.

Before death or growth retardation, the embryo compensates for hypoxemia or hypoxia caused by decreased oxygen availability. If one quarter of the shell is coated to prevent gas exchange, blood oxygen tension decreases, causing hypoxemia that can be correlated with an increase in Hb (19). Hypoxemia can also result from lack of egg turning during incubation, which further results in an increased hematocrit (20). Experimental and sham-operated embryos in our lab are not turned during incubation, which would tend to exaggerate any hypoxic effect caused by puncture of the air cell and replacing a portion of the porous eggshell with impermeant cellophane tape. Thus, we propose that the chronic "sham" effect seen in our embryos decreases oxygen availability, resulting in chronic hypoxemia or hypoxia. Hypoxemia then causes an increased blood volume, which is a chronic cause for the increased diastolic volume. As mentioned, the decreased vascular resistance is the acute cause for the increased stroke volume and cardiac output in response to hypoxemia.

This study confirms and clarifies results from previous studies (6, 7, 9, 10) showing abnormal hemodynamic effects due to neural crest ablation early in cardiovascular development before the time when structural heart defects are present. Complete cardiac neural crest ablation is associated with decreased shortening fraction or ejection fraction. Tomita *et al.* (6) did not find a significant difference in ejection fraction between embryos with neural crest ablations and "opened egg" control embryos. The difference in results is most likely due to the difference in ablations used by the two studies. In the current study, the entire cardiac neural crest was ablated (arches 3 to 6), whereas Tomita's study used embryos with incomplete ablations of the cardiac neural crest (3rd and 4th arches).

Other studies have also demonstrated myocardial dysfunction after ablation of the complete cardiac neural crest. Creazzo (21, 22) observed reduced L-type calcium current in d-11 embryonic chick hearts with truncus arteriosus induced by neural crest ablation. Recent studies by Fogaca *et al.* (23) have demonstrated, at d 7, a decreased maximal twitch force in intact myocardial trabeculi at 0.48 to 20 mM in high extracellular calcium from embryonic chick hearts after ablation of the cardiac neural crest. When the fibers were chemically skinned, they showed that the maximal calcium-activated force was decreased in comparison with sham-operated embryos. Thus, the effect of complete cardiac neural crest ablation appears to be decreased myocardial contractility observed before structural defects are present.

We have demonstrated that an intact shell may be important

for normal cardiovascular development. The knowledge gained from studying the factors that decrease viability in chick embryos helps in elucidating the contribution of various factors, including the neural crest, to normal heart development.

Acknowledgments. The authors thank Hal Gauldin, Rennie Wolfe, Harriett Stadt, and Donna Kumiski for technical assistance, Harry Davis for statistical consultation, and Carol Pennington for manuscript preparation.

REFERENCES

1. Kirby ML, Waldo KL 1990 Role of neural crest in congenital heart disease. *Circulation* 82:332–340
2. Miyagawa S, Waldo K, Tomita H, Kirby ML 1990 Migration of cardiac neural crest cells: a temporospatial study in early quail-chick chimera. In: Bockman DE, Kirby ML (eds) *Embryonic Origins of Defective Heart Development*. Ann NY Acad Sci 588:427–429
3. Kirby ML 1987 Cardiac morphogenesis: recent research advances. *Pediatr Res* 21:219–224
4. Kirby ML, Turnage III KL, Hays BM 1985 Characterization of conotruncal malformations following ablation of "cardiac" neural crest. *Anat Rec* 213:98–93
5. Nishibatake M, Kirby ML, van Mierop LH 1987 Pathogenesis of persistent truncus arteriosus and dextroposed aorta in the chick embryo after neural crest ablation. *Circulation* 75:255–264
6. Tomita H, Connuck DM, Leatherbury L, Kirby ML 1991 Relation of early hemodynamic changes to final cardiac phenotype and survival after neural crest ablation in chick embryos. *Circulation* 84:1289–1295
7. Leatherbury L, Gauldin HE, Waldo K, Kirby ML 1990 Microcinematography of the developing heart in neural crest-ablated chick embryos. *Circulation* 81:1047–1057
8. Leatherbury L, Connuck DM, Gauldin HE, Kirby ML 1991 Hemodynamic changes and compensatory mechanisms during early cardiogenesis after neural crest ablation in chick embryos. *Pediatr Res* 30:509–512
9. Leatherbury L, Braden DS, Tomita H, Gauldin HE, Jackson WF 1990 Hemodynamic changes: wall stress and pressure gradients in neural crest-ablated chick embryos. In: Bockman DE, Kirby ML (eds) *Embryonic Origins of Defective Heart Development*. Ann NY Acad Sci 588:305–313
10. Stewart DE, Kirby ML, Sulik KK 1986 Hemodynamic changes in chick embryos precede heart defects after cardiac neural crest ablation. *Circ Res* 59:545–550
11. Hamburger V, Hamilton HL 1951 A series of normal stages in the development of the chick embryo. *J Morphol* 88:49–92
12. Santi PA, Fryhofer J, Hansen G 1980 Electronic planimetry. *Byte* (March):114–120
13. Ackerman RA, Rahn H 1980 *In vivo* O₂ and water vapor permeability of the hen's eggshell during early development. *Respir Physiol* 45:1–8
14. Hoyt DF, Rahn H 1980 Respiration of avian embryos: a comparative analysis. *Respir Physiol* 39:255–264
15. Wangenstein OD, Rahn H 1970/71 Respiratory gas exchange by the avian embryo. *Respir Physiol* 11:31–45
16. Wangenstein OD, Wilson D, Rahn H 1970/71 Diffusion of gases across the shell of the hen's egg. *Respir Physiol* 11:16–30
17. Weiss HS 1978 Role of shell diffusion area in incubating eggs at simulated high altitude. *J Appl Physiol Respir Environ Exercise Physiol* 45:551–556
18. Metcalfe J, McCutcheon IE, Francisco DL, Metzner AB, Welch JE 1981 Oxygen availability and growth of the chick embryo. *Respir Physiol* 46:81–88
19. Metcalfe J, Bissonnette JM, Bowles RE, Matsumoto JA, Dunham SJ 1979 Hen's eggs with retarded gas exchange. *Respir Physiol* 36:97–101
20. Tazawa H 1980 Adverse effect of failure to turn the avian egg on the embryo oxygen exchange. *Respir Physiol* 41:137–142
21. Creazzo TL 1990 Reduced L-type calcium current in the embryonic chick heart with persistent truncus arteriosus. *Circ Res* 66:1491–1492
22. Aiba S, Creazzo TL 1992 Calcium currents in hearts with persistent truncus arteriosus. *Am J Physiol* 262:H1182–H1190
23. Fogaca RTH, Warren KS, Lin JJC, Nosek TM, Godt RE 1993 Contractile alterations during myocardial development in the chick subsequent to ablation of cardiac neural crest. *J Cell Biochem Suppl* 17D:211