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RESPIRATORY GASES AND ACID–BASE BALANCE IN SHELL-LESS AVIAN EMBRYOS

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Summary

1. The P_{CO_2} and P_{O_2} levels of oxygenated and deoxygenated embryonic blood were measured in normal and shell-less cultured embryos.

2. Embryos in shell-less culture were both hypoxic and hypocapnic, suggesting that wetting the respiratory membranes and the reduction in their functional area is distorting their normal function.

3. Despite this hypocapnia, there was evidence for an increase in base excess during incubation. This suggests that plasma bicarbonate is derived from renal sources rather than by resorbing the eggshell calcium carbonate, which is, of course, absent from shell-less cultures.

Introduction

The respiration of the avian embryo is of considerable interest for both theoretical and practical reasons. It is, as Wangensteen & Rahn (1970/1971) first pointed out, a model system for studying the diffusion component of vertebrate gas exchange. Thus, the movement of gases between the atmosphere and the animal's vascular system is, in the avian embryo, dictated by the conductance of the eggshell. This has a fixed capacity which appears to be dictated by the need to limit water vapour losses to about 15 % of the original egg weight, a requirement that clearly constrains respiratory gas exchange. The continually increasing metabolic demand by the developing embryo for oxygen is, therefore, met by an increased blood flow through the chorioallantoic respiratory system, increased haematocrit and haemoglobin concentrations and by changes in the oxygen affinity of the haemoglobin. Despite these adaptations, the oxygen pressure in the embryo's blood falls throughout the latter part of incubation and is only reversed when pulmonary respiration is initiated shortly before hatching (Freeman & Misson, 1970; Tazawa *et al.* 1983). It is not surprising, therefore, that the earliest measurements of blood gases in the embryo showed that there was also a progressive respiratory acidosis as carbon dioxide accumulated during development. What was, perhaps, less expected was the discovery that the embryo could compensate for this acidosis by a metabolic alkalosis (Dawes & Simkiss, 1969).

Key words: acid–base, shell-less embryos, acidosis, respiratory gases.

This effect is seen as an increase in blood bicarbonate levels. It is not clear whether the additional base is derived from the secretion of acid into the renal tubules of the embryo (Dawes & Simkiss, 1971), or by the resorption of the eggshell by the chorioallantoic membrane (Crooks & Simkiss, 1974).

The physiology of avian embryos has recently attracted additional interest because of the possibility of genetically manipulating various domesticated birds (Freeman & Messer, 1985). The avian system of reproduction makes it particularly difficult to handle the embryo unless the zygote can be made accessible to the experimenter. For this reason there has been renewed interest in the shell-less *in vitro* techniques developed by Dunn and his coworkers (Dunn & Boone, 1976; Dunn *et al.* 1981) with their subsequent modification to produce a successful 'surrogate egg' system for culturing embryos (Rowlett & Simkiss, 1987).

Shell-less techniques for culturing embryos of the domestic fowl have been successful in producing offspring with a chronological age of 21 days. They have never succeeded, however, in producing viable hatchlings, which clearly suffer from both calcium and protein deficiencies. Another possible reason for their non-viability is the disturbance to their respiratory gas regulation which the absence of a shell induces. This could be due to several causes. Clearly, shell-less cultures have lost the resistance to gas diffusion imposed by the eggshell, but they also have a reduced respiratory area largely dictated by the area of chorioallantoic membrane exposed in the culture vessel. It will also be apparent that if the metabolic alkalosis found in the normal chick embryo is maintained by resorption of base from the eggshell, then this should also be disrupted in shell-less cultures. For these reasons a study was undertaken of the respiratory gases in cultured embryos.

Materials and methods

Fertile eggs of a Rhode-Island Red strain of the domestic fowl were used throughout this work. For normal purposes they were incubated in a commercial forced-air incubator set at 38°C and 60% relative humidity. The shell-less culture system that was used has been described in detail by Rowlett & Simkiss (1987). Eggs were incubated for 72 h and then opened into a 'cling-film' sheet of plastic suspended in a cylindrical plastic holder, and covered with a Petri dish (Fig. 1). These shell-less cultures were incubated in a clean-air cabinet (Bassaire, Southampton) in a constant-temperature room set at 38°C with 80% relative humidity.

Blood samples (100 μl) were taken from the allantoic artery (deoxygenated) and allantoic vein (oxygenated) of embryos with a heparinized needle and syringe. The blood-sampling equipment was kept on ice before and after sampling. With control embryos, the egg was punctured in the air space and suspended with the narrow pole uppermost. A 2 cm^2 hole was made in the shell and the shell membrane dampened with saline so that the underlying allantoic blood vessels could be located and blood samples obtained with the minimum of disturbance.

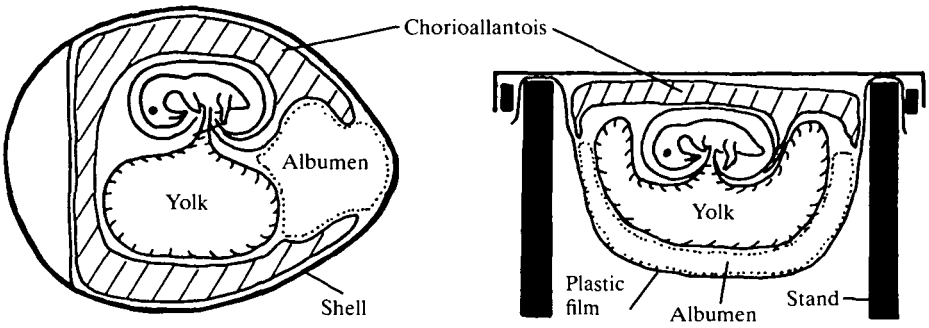

Fig. 1. Orientation of embryo and extraembryonic membranes in a normal egg (A) and shell-less culture (B). Note that the chorioallantois in shell-less culture is only exposed to the air on one restricted surface.

Obtaining samples from shell-less cultures simply involved penetrating the chorioallantois and taking blood from the conspicuous allantoic vessels.

All blood samples were analysed within 10 min by using an automatic blood gas analyser with pH, P_{CO_2} and P_{O_2} electrodes (System 1302, Instrumentation Laboratory, Italy) which were calibrated with precision buffers and analysed gas mixtures.

All the embryos were killed and weighed immediately after taking blood samples.

Results

Results of analyses for pH, P_{CO_2} and P_{O_2} for oxygenated and deoxygenated blood samples are shown for control embryos and for shell-less cultures (Fig. 2). 46 control and 37 shell-less embryos were studied at various stages of development, but it was not always possible to obtain both arterial and venous blood samples from each specimen. The numbers of samples are shown in Fig. 2, with means and standard error values for each stage. Student's *t*-test was used to test for statistical significance between the control and shell-less values for each parameter on each day of incubation. Apart from those on day 12, all P_{CO_2} values were significantly different for both oxygenated ($P<0{\cdot}05$) and deoxygenated ($P<0{\cdot}001$) blood. The P_{O_2} values for oxygenated blood were also significantly different ($P<0{\cdot}05$), but there were no significant differences for P_{O_2} in deoxygenated blood or for blood pH.

Plasma bicarbonate concentrations were calculated from the mean values shown in Fig. 2 by using the Henderson–Hasselbalch equation:

$$pH = pK_1 + \log\frac{[HCO_3^-]}{\alpha P_{CO_2}},$$

where the dissociation constant, pK_1, is 6·10 and the absorption coefficient, α, is $0{\cdot}2213\,\mu mol\,dm^{-3}\,Pa^{-1}$ for embryonic chick blood (Tazawa *et al.* 1983). The bicarbonate values are given in Table 1.

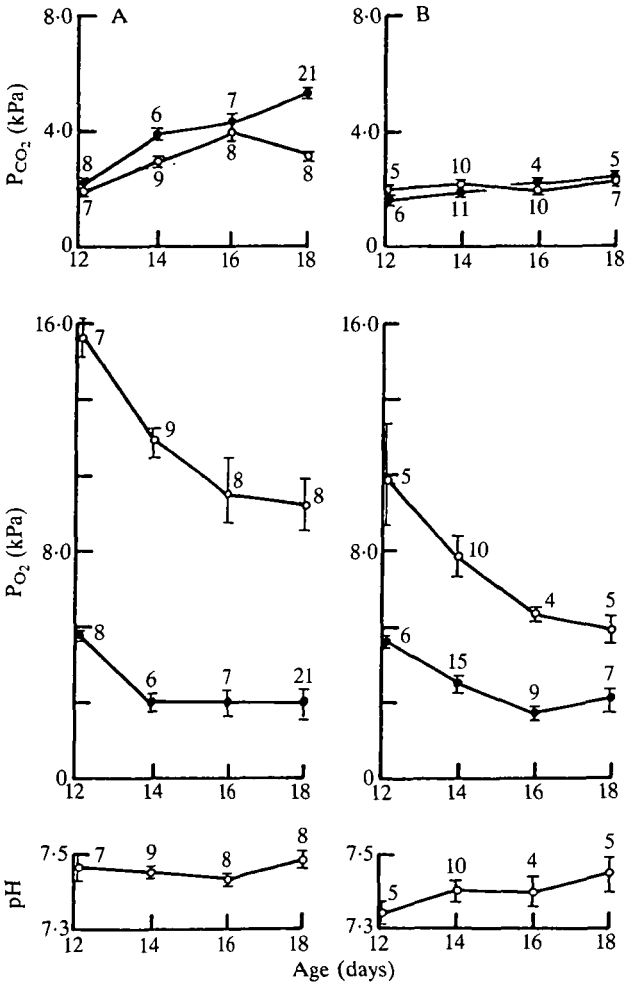

Fig. 2. Carbon dioxide pressure (P_{CO_2}), oxygen pressure (P_{O_2}) and pH in the blood of the control (A) and shell-less (B) embryos. Samples were taken from oxygenated (open symbols) and deoxygenated blood (solid symbols). Values are means ± S.E.M. Numbers refer to number of samples.

Table 1. *Plasma bicarbonate levels (mmol dm^{-3}) for the oxygenated (allantoic vein) and deoxygenated (allantoic artery) blood values shown in Fig. 2*

Age (days)	Normal		Shell-less cultures	
	Oxygenated	Deoxygenated	Oxygenated	Deoxygenated
12	11·0	11·8	7·8	7·2
14	14·9	17·9	9·2	6·9
16	19·3	21·4	8·4	9·9
18	17·4	20·5	11·3	10·4

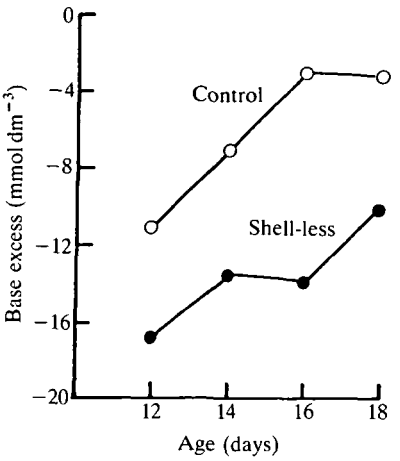

Fig. 3. Changes in base excess in oxygenated blood of control (open symbols) and shell-less embryos (solid symbols).

Table 2. *P_{CO_2} and P_{O_2} values for oxygenated blood from shell-less embryo cultures and for embryos of equivalent mass calculated according to the wet mass relationships found by Tazawa* et al. *(1971)*

Age of embryo (days)	*N*	Mean wet mass ±S.E.M. (g)	Blood P_{O_2} levels		Blood P_{CO_2} levels	
			Actual (kPa)	Calculated (kPa)	Actual (kPa)	Calculated (kPa)
12	15	3·8 ± 0·2	9·4	10·8	2·0	1·9
14	23	7·1 ± 0·3	7·7	10·1	2·1	2·5
16	15	9·3 ± 0·4	5·7	9·7	2·0	2·9
18	7	11·3 ± 1·1	5·2	9·3	2·3	3·2

An estimate of the non-respiratory component of the blood bicarbonate, i.e. base excess, was determined from nomograms published for human blood (Siggaard-Andersen, 1965). Haemoglobin values of 7, 8, 9 and 10 g 100 cm^{-3} for 12-, 14-, 16- and 18-day embryos were used for these estimates, based on the results of Tazawa *et al.* (1971). The results are shown in Fig. 3 for both normal and shell-less embryos.

Discussion

During the period from 10 to 18 days of incubation, the blood of the normal avian embryo shows a decrease in P_{O_2} from about 10·6 to 7·6 kPa, an increase in P_{CO_2} from 1·6 to 4·3 kPa, a rise in blood bicarbonate from 12 to 22 mmol dm^{-3} and an increase in base excess from −8 to 1 mmol dm^{-3} (Tazawa *et al.* 1971). The results shown in Table 1 and Figs 2 and 3 for the control embryos agree with these

data, and may be interpreted as the product of a resistance to gas diffusion across the avian eggshell and increasing metabolic activity from the growing embryo. Accordingly, there is increased respiratory acidosis, which is compensated by metabolic alkalosis (Dawes & Simkiss, 1969).

This interpretation of the respiratory adaptations of the avian embryo has been confirmed elsewhere (Tazawa & Piiper, 1984). It has also been tested experimentally by effectively reducing the area of the eggshell available for respiratory exchange by coating roughly one-quarter of the surface with epoxy resin. Under these conditions blood P_{O_2} falls and P_{CO_2} rises, inducing an increase in blood base excess which tends to regulate the blood pH at about 7·4 (Tazawa *et al.* 1971).

The area of the chorioallantoic membrane available for respiratory exchange in shell-less culture systems is constrained by the morphology of the system (Fig. 1). The average surface area unobstructed by plastic film was only 38 cm^2, roughly half of what is normally available (Dunn & Boone, 1976). It is possible that further gas exchange occurs across the plastic film, but this has an oxygen permeability of $3{\cdot}1\times10^{-4}$ $cm\,min^{-1}\,Pa^{-1}$, i.e. roughly half that of the normal eggshell (Romijn, 1950; Kutchai & Steen, 1971) and the chorioallantois is separated from this by varying thicknesses of albumen. As a consequence, it seemed likely that shell-less cultures would be subjected to hypoxia and hypercapnia in a similar way to embryos in coated eggshells. The results shown in Fig. 2 demonstrate that this is not the case. The blood oxygen levels in the allantoic vein, which drains the respiratory organ, decrease whereas the levels in the allantoic artery do not. The cultured embryos are, therefore, subjected to a considerable decrease in the arteriovenous oxygen gradient (Fig. 2.). However, instead of being hypercapnic, they show reduced levels of blood carbon dioxide. The decreased carbon dioxide levels could be due to the embryos in shell-less culture being retarded in size (Table 2). It is, however, possible to compensate for this, as Tazawa *et al.* (1971) and Tazawa & Mochizuki (1977) have produced equations relating blood gas levels to chick embryo mass. The observed and calculated blood gas levels (Table 2) indicate that the abnormal levels in shell-less embryos are not simply due to their small size. In fact, the low oxygen and carbon dioxide levels of the blood of shell-less cultured embryos are more typical of amphibious vertebrates in which respiratory exchange is strongly influenced by the solubility of the respiratory gases in water. Thus, in the Amphibia, aerial respiration accounts for much of the oxygen uptake whereas diffusion across the skin into the water is responsible for the removal of much of the carbon dioxide. The efficiency of these systems decreases with an increase in temperature (Rahn & Howell, 1976), but it appears likely that the reduced aerial part of the chorioallantois is the main region for oxygen uptake in the cultured embryo and the submerged lower part of the chorioallantois may contribute to the removal of carbon dioxide.

A second feature of the respiratory physiology of cultured embryos is also of interest. As was originally noted by Dawes & Simkiss (1969, 1971), the avian embryo is able, from a very early age, to exert considerable control over its acid–base balance. An increase in P_{CO_2} is counterbalanced by a metabolic influx of

bicarbonate base so as to keep the blood pH virtually constant. The embryo exerts this control from about day 12 of incubation onwards. The bicarbonate could be derived either from the eggshell, which the embryo is resorbing at about this time:

$$CaCO_3 + CO_2 + H_2O \longrightarrow Ca(HCO_3)_2 ,$$

or from the excretion of protons into the urine, which is also becoming acidified at this time (Simkiss, 1980):

$$CO_2 + H_2O \longrightarrow H^+ + HCO_3 .$$

Clearly, embryos developing in shell-less culture do not have access to the base reserve in the eggshell. It is interesting to note, therefore, that such embryos still show a small rise in base excess (Fig. 3). This strongly suggests that it is a metabolic compensation (Atkinson & Bourke, 1987), probably acting *via* the embryonic kidney, that is responsible for acid–base regulation during the latter half of incubation. Presumably it is the P_{CO_2} of the blood which drives the secretion of protons into the urine with a consequent release of bicarbonate ions into the blood. The decreased level of P_{CO_2} in cultured embryos would therefore be responsible for an equivalent decrease in the formation of base excess by the kidney.

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