# Detrimental Effects of Hypoxia-reoxygenation Injury on Development of Rat Embryos during Organogenesis *In Vitro*

## Ivana Rosenzweig

*Selwyn College, University of Cambridge, Cambridge, United Kingdom*

**Aim.** To analyse the effects of hypoxia-reoxygenation on the development of rat embryos during early stages of gestation *in vitro*.

**Methods.** Whole embryo culture techniques were used to culture Wistar rat embryos under a range of gassing regimes, some of which included periods of mild hypoxia. After the termination of cultures, embryos were morphologically examined and assessed, and their protein content was determined.

**Results.** Cultured embryos exposed to 4 h of mild hypoxia did not show any significant growth or differentiation, as expected during this developmental stage. The ensuing 20 h reoxygenation period appeared to exacerbate the effects of the hypoxia.

**Conclusion.** The effects of hypoxia during the perinatal or late fetal stages are well documented, but less is known of the effects at earlier stages of gestation. Our results indicate that during organogenesis, even short-term exposure to hypoxia may impose detrimental effects on growth and neurodevelopment of embryos.

*Key words:* cell hypoxia; embryo; fetal development; rats, Wistar

During pregnancy, any maternal metabolic disturbance has potentially a detrimental effect on embryonic development. Pathological conditions in the mother, such as diabetes mellitus, pre-eclampsia, smoking, and drug addiction can all lead to placental hypoxia and dysfunction, resulting in the lack of oxygen for the developing offspring (1-4). Recent studies have implicated uterine ischemia-hypoxia injury, followed by reoxygenation, in the incidence of early miscarriage (5,6). However, although the effects of hypoxia during the perinatal or late fetal stages are well documented (7-12), much less is known of the hypoxic effects on embryos at earlier stages of gestation (13-15). Attention has therefore been focused on the consequences of both hypoxia and the subsequent period of reoxygenation during organogenesis on the development of rat embryos.

It has been suggested that hypoxia at preplacental stages can impair reproduction, as shown by retarded embryonic development and increased frequency of embryo death and reabsorption (16). Furthermore, it was shown that long-term exposure to hypoxia during organogenesis can result in growth retardation of the fetus (17). A detailed observation of short-term effects of hypoxia followed by reoxygenation on gross anatomy and differentiation of embryos during organogenesis, to the best of my knowledge, has not been done before.

In the present study, the mammalian postimplantation whole-embryo culture technique was used to highlight this issue. The use of whole-embryo culture techniques to investigate the developmental effects of different teratogens has several advantages. First and foremost, such techniques allow strict manipulation of development, ie, exposure of embryos of known age to a predetermined amount of a single specific teratogen for a set duration of time. Thus, the level and duration of exposure to the teratogen and the developmental stages of the embryo used can all be clearly defined. In addition, the concentration of relevant substances in the culture media surrounding the embryo is known or can easily be measured. Finally, the effects of maternal metabolism are eliminated. Hence, the direct effects of the teratogen on embryonic development can be studied.

### Material and Methods

Female rats were caged with males overnight, and those with sperm in the vagina on the following morning were regarded as being 0.5 days (d) pregnant. Rats were humanely killed at 11.5 d by exposure to either carbon dioxide or ether, followed by exsanguination. The gravid uterine horns were dissected free,

removed, and placed in a dish of Hanks' balanced salt solution (SIGMA, Dorset, UK). Each conceptus was removed from its decidual swelling and the parietal yolk sac opened as previously described (18). The visceral yolk sacs were opened to allow each amniotic sac containing the embryo to be eased out, so that it lay in direct contact with the culture medium and the gas phase. Those littermates that were uniformly developed were further distributed evenly between experimental treatments and some were retained as zero hour (0 h) controls.

The culture medium was composed of 50% immediately centrifuged and heat-inactivated rat serum (19) and 50% Dulbecco's modified Earle's medium (SIGMA) with added L-glutamine. Penicillin (100 U/mL) and streptomycin (60 µg/mL) were added to all culture media. One mL of medium was added for each explant (embryo and yolk sac) cultured. Explants were cultured at 38°C in glass bottles (Wesley Coe / Wingent, Milton, UK) attached to a rotating culture unit (BTC Engineering, Milton, UK) housed within a precision incubator (BTC Engineering). Each bottle contained up to a maximum of 4 embryos and 4 mL of medium. Embryos were either cultured throughout the culture under the gassing conditions that were found to be optimal for their normal development and growth *in vitro* (40% oxygen, 5% carbon dioxide, balance nitrogen; ref. 20), referred to as a "normal oxygenation", or exposed to periods of 4 h of 5% oxygen (90% nitrogen and 5% carbon dioxide) followed by "normal oxygenation" for the remainder of the culture. Cultures were terminated after 4 or 24 h, at which stage the embryos were examined for any morphological abnormalities and assessed for crown-rump (C-R) length, somite number, and protein content. Curvature was regarded as normal in those embryos in which axial rotation had been either completed or nearly accomplished. Somites were counted by light microscopy. Histological examination of the internal anatomy for the staging of embryo development was not undertaken because morphological assessment using scoring system of Brown and Fabro (21) and histological findings closely correlate (22).

Following morphological assessment, the embryos were processed individually for estimation of total protein content, which was determined with a commercial bicinchoninic acid protein assay kit (SIGMA, No. BCA-1) by the colorimetric method (23). Seventy-four embryos were used in total: 10 for 0 h controls, 10 for 4 h hypoxia (4 h, 5% $O_2$), 10 for 4 h normal gassing conditions (4 h, 40% $O_2$), 22 for 24 h hypoxia-reoxygenation (4 h, 5% $O_2$, 20 h, 40% $O_2$) and 22 for 24 h controls (24 h, 40% $O_2$).

Student's *t* test was used for all statistical analyses, with statistical significance defined as $p < 0.05$.

## Results

The 11.5 d explants were examined immediately after explantation or following 4 or 24 h in culture (Table 1). The two groups of embryos cultured for 4 h (normal in 40% $O_2$, and hypoxic in 5% $O_2$) were compared to 0 h controls. The embryos cultured for 24 h under the hypoxia-reoxygenation regime (4 h, 5% $O_2$; 20 h, 40% $O_2$) were compared to 24 h controls (24 h, 40% $O_2$).

The protein content and the development of embryos after 4 h of exposure to 5% $O_2$ was essentially similar to that of embryos in the 0 h controls. They did not show any significant growth or differentiation during those 4 h. By contrast, those cultured under normal conditions (40% $O_2$) for the same 4 h period, had significantly more somites and a higher protein content than the 0 h controls, as ordinarily expected during this developmental stage (20). After a further 20 h of culture under normal conditions (40% oxygen), the control embryos (24 h, 40% $O_2$) had significantly more somites and higher protein content than those subjected to the brief period of hypoxia (4 h, 5% $O_2$; 20 h, 40% $O_2$) (Table 1).

None of the embryos examined immediately after explantation or following 4 h of culture showed any sign of the edema of the neural tubes. Similarly, the controls exposed to 40% $O_2$ throughout the culture had normal or only slightly edematous neural tubes (Fig. 1A). In contrast, in the embryos that were exposed to the hypoxia-reoxygenation regimen, visually considerable edema of the neural tubes could be observed (Fig. 1B). These embryos were also significantly smaller and less well differentiated (as estimated from the somite number) than embryos cultured under normal conditions ($p < 0.001$, Table 1). Visually observed malformations were present in 17 out of 22 (77.3%) embryos cultured under hypoxia-reoxygenation regime, whereas milder malformations were observed in only 3 out of 22 embryos under normal gassing conditions. These differences increased further in the longer cultures (24).
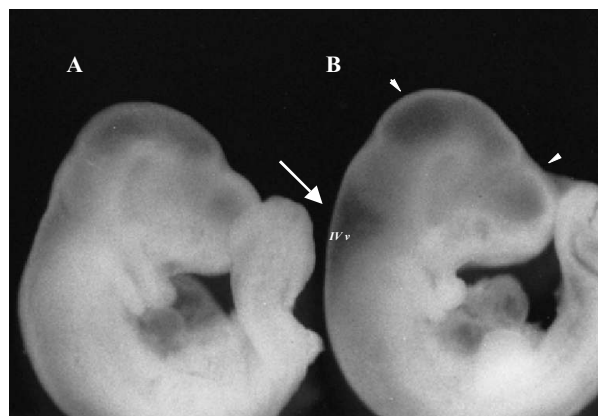


**Figure 1.** Embryos (11.5 d) were exposed to normal (**A**) or hypoxia–reoxygenation gassing regime (**B**). Even short exposure to mild hypoxia, followed by reoxygenation, caused edema of the neural tube (**B**). The effect was particularly prominent in the area of the fourth ventricle (*IV v*, arrow) but was also easily discernible in the regions overlying the future forebrain and midbrain (arrowheads).

**Table 1**. Development of 11.5-day explants in culture exposed to different gassing protocols

| Group of embryos | No. of embryos | Crown-rump length (mm, mean±SEM) | Somite number (mean±SEM) | Protein content (g, mean±SEM) |
|---|---|---|---|---|
| 0 h controls | 10 | 3.7±0.01 | 27.0±0.2 | 240.0±8.0 |
| 4 h 5% $O_2$ | 10 | 3.7±0.1 | 27.0±0.3* | 238.0±8.0* |
| 4 h 40% $O_2$ | 10 | 3.7±0.1 | 29.0±0.4† | 275.0±10.6‡ |
| 24 h 40% $O_2$ | 22 | 4.9±0.2 | 34.4±0.5 | 477.4±8.6 |
| 4 h 5% $O_2$ + 20 h 40% $O_2$ | 22 | 4.6±0.2 | 29.0±0.5§ | 360.2±14.6§ |

*Significantly different from values for appropriate controls (0 h controls)
†Significantly different from values for appropriate controls (0 h controls), $p < 0.005$.
‡Significantly different from values for appropriate controls (0 h controls), $p < 0.05$.
§Significantly different from values for appropriate controls (24 h controls, exposed to 40% $O_2$), $p < 0.001$.

## Discussion

In the present study, the embryos were subjected to short-term hypoxia followed by reoxygenation by use of recently developed *in vitro* model of gassing regimes (25). It was shown that, under these experimental conditions, even relatively short-term exposure to hypoxia during organogenesis was capable of causing significant detriment in the rates of growth and somitogenesis. Johshita et el (26) showed that even a shorter exposure to hypoxia (2 h) was adequate to cause significant decrease in the somite number of embryos, although it did not significantly affect their growth. This finding might reflect a greater detrimental effect of hypoxia on the differentiation of embryos rather than on their growth at this particular stage of embryo development. Furthermore, the results clearly marked the hypoxic period as the period during which detrimental effects on the growth and differentiation of rat embryos occur; the reoxygenation period seemed to exacerbate the effects of the hypoxia.

Remarkably, close observation of embryos exposed to hypoxia-reoxygenation injury also revealed edema of the neural tubes and enlargement of the developing brain ventricles. Severe brain injury is frequently followed by secondary or delayed pathophysiological events, inducing alterations in cerebral metabolism, dysregulation of cerebral blood flow, and the onset of cerebral edema (25). The observed hypoxia–reoxygenation-caused edema in the embryos, however, seems to be the first such record of the similar susceptibility and reaction to injury in embryos undergoing organogenesis. Interestingly, hypoxia alone did not exert this effect; a period of reoxygenation was needed for edema of neural tubes to develop. In adults, post-traumatic cerebral edema has been related to the release or activation of a number of endogenous autodestructive "injury" factors, including radical oxygen species, leukotriens, free fatty acids, and other breakdown products of the arachidonic acid cascade (26). Given that all factors mentioned above have been suggested to induce lipid peroxidation (27) and given that the brain is known to show early and specific evidence of lipid peroxidation during reperfusion (28), it is perhaps not surprising that edema of the neural tubes was only observed after period of reoxygenation, which appeared to allow for some of the subsequent cellular dysregulation to develop.

During the last decade, the causal relationship between smoking and drug intake of mothers-to-be in their early pregnancy, and growth disturbances and malformations in the offspring has gained renewed attention (29-31). Both smoking and drug intake can elicit hypoxic injury in embryos, and hence cause growth retardation and cerebral damage (4). Furthermore, a large body of evidence shows that cerebral damage occurs mainly in the antenatal period and not during the course of labor and delivery as was previously believed (32). Moreover, it has been shown that growth-restricted fetuses are more prone to suffer from intrapartum stress resulting in further damage and potential neurodevelopmental impairment of the child (32).

Surprisingly little is known of either the mechanisms underlying the detrimental effects of hypoxia–reoxygenation injury, or the target for such damage during organogenesis. Faridy et al (17) speculated that growth-retardation observed in fetuses of hypoxic mothers might result directly from inhibition of mitosis and indirectly from a reduction in blood flow. Because of the complexity of the injury that might ensue under hypoxic conditions, and the fact that *in vivo* many parameters, both maternal and fetal, are affected simultaneously, assessment of molecular mechanisms underlying the cell damage in the fetus remains elusive.

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**Correspondence to:**

Ivana Rosenzweig

Selwyn College

University of Cambridge

Cambridge CB39DQ, UK

*ir206@cam.ac.uk*