The influence of carbon dioxide concentration on the neurite outgrowth of mouse embryonic cortical neurons in vitro

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SUMMARY

The carbon dioxide (CO_2) concentration of the incubation medium in nerve cell culturing has always been a matter of debate and is usually adjusted in a somewhat empirical manner. In the many laboratory protocols and manuals published to date, the data concerning this aspect differ. The goal of this study was thus to follow up the neurite outgrowth of cortical neurons in dissociated primary cultures obtained from seventeen-day-old embryonic mouse brains incubated in 90% air and 10% CO_2, and in 95% air and 5% CO_2 respectively. We recorded the density and confluence of the neuronal distribution *in vitro* on the second, third and fifth day after establishing the cultures. Morphological evaluation to demonstrate viable cells was carried out by applying immunocytochemistry for microtubule-associated protein-2 due to its ubiquity in neurons. The results showed that neuronal survival, the rate of neurite outgrowth and the establishment of intercellular networks and synaptic contacts were more pronounced in cortical cultures raised in 10% CO_2-containing incubation medium than in 5% CO_2-containing incubation medium. From these results, it can be inferred that CO_2 at a higher concentration plays a significant role in influencing the metabolic activities of embryonic cortical neurons *in vitro*, thus probably determining their synaptogenesis and viability.

Key Words: Carbon dioxide – Cortical neurons – Dissociated cultures – MAP-2 – Mouse

INTRODUCTION

As the epitome of a heterogeneous tissue, nerve tissue was the first type to be introduced in cell culturing methods. Despite the time span, over a century, during which nerve cell cultures have been elaborated, there still exist many pitfalls and hard-to-explain phenomena in the approach (Fedoroff and Richardson, 1992; Martin, 1994). Besides the complexity of the chemical factors, both cell-produced and media-contained, it should also be borne in mind that alterations to the environmental conditions in which the cells are grown and maintained may be of crucial significance for their fate. This can also doubtless be used as an experimental tool, especially by changing the incubator conditions; that is, temperature and the mixture of gases. In the case of the latter, standard conditions for incubating most cellular types involve a mixture of 95% air and 5% carbon dioxide (CO_2) (Martin, 1994). In the case of nerve cells, however, the views of various authors and laboratory protocols differ considerably with regard to the appropriate CO_2 concentration, and many authors have advised using either higher or lower concentrations than the standard (Schilling et al., 1991; Copray and Liem, 1993; Brewer and Price, 1996; Juurlink and Walz, 1998; Sim and Allen, 1998; Diaz et al., 1999). Therefore, in order to study the influence of varying CO_2 concentrations on neuronal development, we used mouse embryonic cortical neurons as a paradigm to find out how their developmental features, neurite outgrowth and viability are affected by this environmental factor.

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Materials and Methods

For the present study, we used 8 seventeen-day-old embryonic (E17) mouse brains. All the procedures described were performed in a laminar flow hood. After routine dissection of the cortexes, they were thoroughly minced and trypsinized in a 10 ml solution of 2x trypsin-EDTA (Gibco BRL) for exactly 15 min at 37°C in a water bath. The trypsinization process was stopped by adding 3 ml fetal calf serum (FCS) (Gibco BRL) in 17 ml PBS. The cell preparation was then passed through a fine meshwork (50 µm large pores) to obtain mostly separate cells and further centrifuged at 1,500 rt. for 9 min. The pellets were then gathered in Fisher's medium (for reference cf. Schilling et al., 1991) containing 5% FCS, re-stirred, and cell numbers and viability prior to establishing the cultures were assessed using 0.4% Trypan Blue stain (Gibco BRL) and standard hemocytometer counting (Romijn et al., 1984; Schilling et al., 1991). The cultures were seeded in ten four-well manufacturer's dishes, preliminarily coated with poly-L-lysin (Sigma Chemicals Co, St. Louis, Mo., USA) and, after a starvation period of 24 hours, the whole medium was changed and the cultures were then fed with Fisher's modified medium containing 5% FCS on an "every other day" routine. Five of the dishes were placed in an incubator supplied with 90% air and 10% CO_2 gas inflow and the remaining dishes were maintained in an incubator supplied with 95% air and 5% CO_2. On the sixth day, the neuronal cultures were fixed in 4% paraformaldehyde for 30 min., processed for standard immunocytochemistry against microtubule-associated protein-2 (MAP-2) (Sigma Chemicals Co, St. Louis, Mo., USA) and incubated overnight in the primary antibody diluted 1:1500 at 4°C. The reaction was further developed applying the avidin-biotin method (ABC) (Vector) and diaminobenzidine (DAB) was used as a chromogen.

Results

Cortical cultures grown in 95% air and 5% CO_2 inflow

The results of the study in this series demonstrated that cortical cultures maintained at a lower concentration of CO_2 tended to attach to a lower extent to the well bottoms; their viability was compromised and the rate of cell death was relatively extensive 24 hours following their establishment. On the sixth day, a sparse neuronal population was observed, whose neurites were finer as well as relatively lacking dendritic spines, while their axons did not show the typical bead-like morphology (Figures 1 and 2).

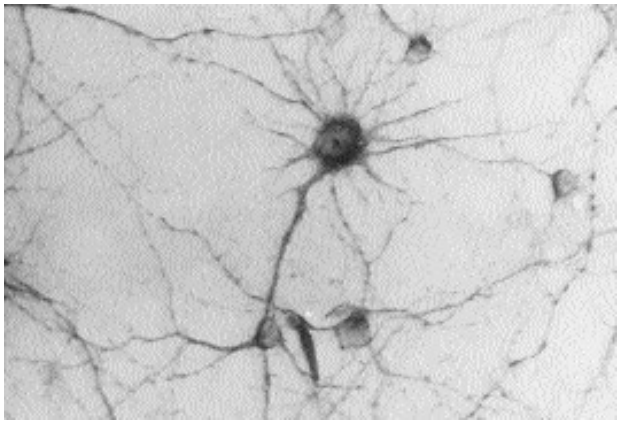

Figure 1. Six-day old cortical E17 mouse neurons grown in 95% air and 5% CO_2 inflow. MAP-2 immunocytochemistry. Note the relatively sparse presence of neuronal preikarya and the reduced appearance of the neurites. x 250.

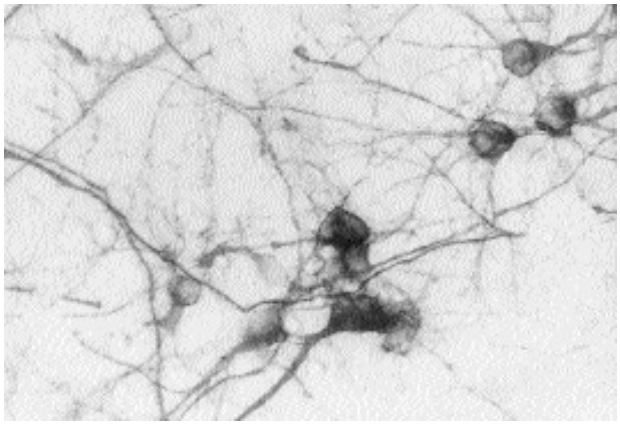

Figure 2. Six-day old cortical E17 mouse neurons grown in 95% air and 5% CO_2 inflow. MAP-2 immunocytochemistry. Although the neuronal network appears to be denser than in Fig. 1, it can still be noted that the neurites are rather feeble in appearance and do not thoroughly traverse through the field to make synaptic contacts the neighboring neurons. x 250.

Cortical cultures grown in 90% air and 10% CO_2 inflow

The neuronal cultures in this series were characterized by a greater tendency to attach to the well bottoms; their viability was well expressed and the cell death rate was minimal by 24 hours after seeding. The morphological results concerning the immunocytochemical reaction revealed a very well developed and dense intercellular network. The neurites of the nerve cells were numerous, with an abundance of dendritic spines, and their axons were long and had a typical bead-like appearance (Figures 3 and 4).

Discussion

The doubtless role of CO_2 in maintaining a normal homeostasis in living cells both *in vivo* and *in vitro* has been well documented in thou-

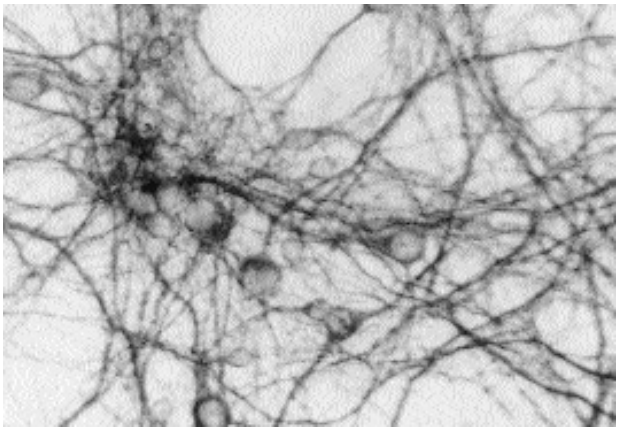

Figure 3. Six-day old cortical E17 mouse neurons grown in 90% air and 10% CO_2 inflow. Note that the neuronal network is prominent in appearance and the number of perikarya is considerably larger. The neurites are intertwined and it is apparent that synaptic contacts are well established. x 250.

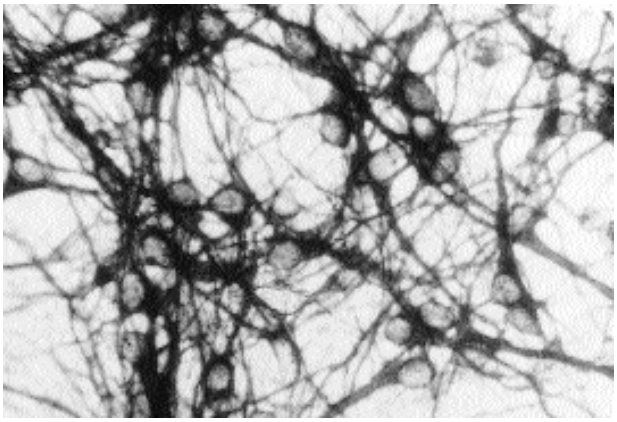

Figure 4. Six-day old cortical E17 mouse neurons grown in 90% air and 10% CO_2 inflow. Again, it can be observed that the neuronal number is greater and the intercellular network is prominent. x 250.

sands of studies (Hertz et al., 1998; Yoshida and Oka, 1998; Winkler et al., 2000). Nevertheless, in the living organism both pre- and postnatally a higher partial CO_2 pressure has always been associated with cell damage and it is well known that neurons in the central nervous system under hypoxic conditions are particularly susceptible to injury and death (Fedoroff and Richardson, 1992; Juurlink and Walz, 1998). Thus, contrary to what can be expected, it is interesting to note that in the presence of a higher concentration of CO_2 in the incubation medium neuronal survival *in vitro* and the outgrowth of neurites, as well as the establishment of denser intercellular networks, are much more enhanced and better expressed in comparison with the same phenomena observed in neuronal cultures maintained at a lower CO_2 concentration. It can be inferred from the results obtained that carbon dioxide as an environmental factor for embryonic nerve tissue can be of certain importance not only as a participant in cellular metabolic processes but also as a promoter of neurite outgrowth and synaptogenesis, possibly through interfering with perikaryal behavior at the stages of cell attachment, cell type recognition and compatibility (Sim and Allen, 1998). Further studies should attempt to clarify what precisely makes neurons require such environmental conditions and whether a higher CO_2 concentration in the incubation medium is indeed optimal for their survival and population stability.

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