Simulated biological effects of microgravity on phospholipid and energy metabolism of chicken embryonic brain cells studied by ^{31}P-NMR spectroscopy

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Abstract Levels of phosphomonoester (PME), phosphodiester (PDE), ATP and pH in brain cells of chicken embryos rotated for 24 h in a clinostat during the period of hatching the 13th day (E13) and 15th day (E15) embryos were investigated by using ^{31}P-NMR spectroscopy. Significant increases in the values of PME, ATP and pH were identified after E13 rotating for 24 h. With the same treatment, differences were obtained in the phospholipid and energy metabolism of E15, but no significant levels have been reached . The calorimetric assay (malachite green method) was used for measuring the activity of total ATPase. A dramatic decrease was evident in the activity of ATPase in brain cells of rotated E13 and E15. The former is more sensitive than the latter. All the levels mentioned above could restore in 24 h after the rotation stopped, except that the level of ATP was still higher than the control.

Keywords: microgravity, biological effects, phospholipid metabolism, ATP, ATPase, brain cells.

Manned space flight and establishment of space lab and space station have raised a serious question for space biology: if and how the microgravity affects living organisms, including human beings. Physical examination of astronauts indicates that microgravity (weightlessness) could induce many physiological and pathological changes, which may be related with nerve system to some degree although they only happened in some other parts. Therefore, it is very important to study the biological effects of changed gravity on nerve cells, especially on brain cells for answering the above question. Due to the costs and time limitation of space flight, various methods have been used to simulate the biological effects of microgravity on the earth. The clinorotation is a commonly accepted method[1, 2]. We have studied previously the effects of clinorotation on movement of embryonic chick neuron *in vitro*[3], on cell cycle phases distribution of brain cells from developmental embryo[4] and on intracellular signal molecule, cytosolic free calcium[5,6]. ^{31}P-NMR spectroscopy can deduce small phosphor-containing molecules according to the chemical shifts on the spectra[7]. The values of pH, PMT, PDT, ATP and Pi in living cells can be measured at the same time. Because neurons contain rich phospholipids, ^{31}P-NMR spectroscopy is an important method in studying the metabolism of phospholipid and energy. Reports[8—11] have appeared recently in this area. We report here the experimental results concerning the clinorotation

effects on metabolism of phospholipid and energy in chicken embryonic brain cells using ^{31}P-NMR spectroscopy.

1 Materials and methods

1.1 Materials and apparatus

Laiheng eggs were purchased from the farm of Chinese Agriculture University. Dulbecco modified medium (DMEM) was from USA GIBCO Company. Fetal calf serum was the product from Chinese Academy of Medical Sciences. $PCrNa_2$ was from Serva. Other chemicals were from China (A.R.). The clinotat was designed and constructed in our institute. The gravity center of eggs was set in coincidence with the rotating axis that rotated anti-clock at 60 r/min. The clinostat with the eggs was placed in the incubator where the temperature was kept at 37°C.

1.2 Sample preparation

Fertilized eggs were hatched in an incubator at 37°C. Each measurement was repeated at least three times. Depending on the ages, 2—18 embryos were used for making samples for each measurement. The concentration of cells was adjusted at about 2×10^8—3×10^8/mL. The whole brain was laminated slowly, washed with Hank's (pH 6.8) twice, then cut with scissors blown with dropper gently, and finally filtered with 200 net. The cells were washed again with Hank's and resuspended in DMEM containing 10% FCS and 0.2 g/mL sucrose. Cells were put into a 10-mm NMR tube. DMEM (containing 10% FCS and 0.2 g/mL sucrose) and 0.5 mL D_2O were added to a final volume of 2.5—3.0 mL.

1.3 ^{31}P-NMR spectroscopy

^{31}P-NMR measurement was carried out in a Varian AM 400 spectrometer operating in the Fourier transform mode. NMR spectra were obtained at a frequency of 161.94 mHz, with pulse of 15 μs, 80° flip angle, 2 s interscan delay, data size 8 K, accumulation of 800 times.

1.4 Measurement of pH

The value of pH was determined using the following equation:

$$d\ (\mathrm{Pi}) = 3.22 + 2.51/(1+10^{6.8-\mathrm{pH}}).$$

Here d (Pi) represents the chemical shift of Pi. Since on the ^{31}P-NMR spectrum, the chemical shift of PCr in the physiological range remained stable and unchangeable with pH, the chemical shift of Pi was very sensitive to the pH value. Therefore, pH in cell could be calculated. After the sample was accumulated to the first 800 times in every measurement, 50 μL $PCrNa_2$ (50 mg/mL) was added. Another 50 times were accumulated again. The shift of Pcr was set 0 and used as the inter-reference .

1.5 ATPase assay

The activity of ATPase was determined by the method of Chan et al.[12]. The following solution (in mmol/L) was used for enzyme reaction: Hepes (pH 7.2) 20, KCl 100, NaCl 20, $MgCl_2$ 5,

EGTA 2, $CaCl_2$ 0.6, 12% TCA was added to terminate the reaction of enzyme. Protein concentration was measured by the method described by Lowery[13].

2 Results

2.1 ^{31}P-NMR spectra of brain cells in developmental embryos

Fig. 1 shows the ^{31}P-NMR spectra of suspended brain cells from embryos in different developmental stages but obtained under the same conditions. The peaks of PME, Pi, PDE, ATP-α, β, γ could be seen clearly in the figure. With the increase of embryonic age, the area of peaks for PME reduces, whereas that for PDE increases gradually. The statistical results are given in table 1, from which we can see that the level of ATP reaches its extreme value in E14 embryos.

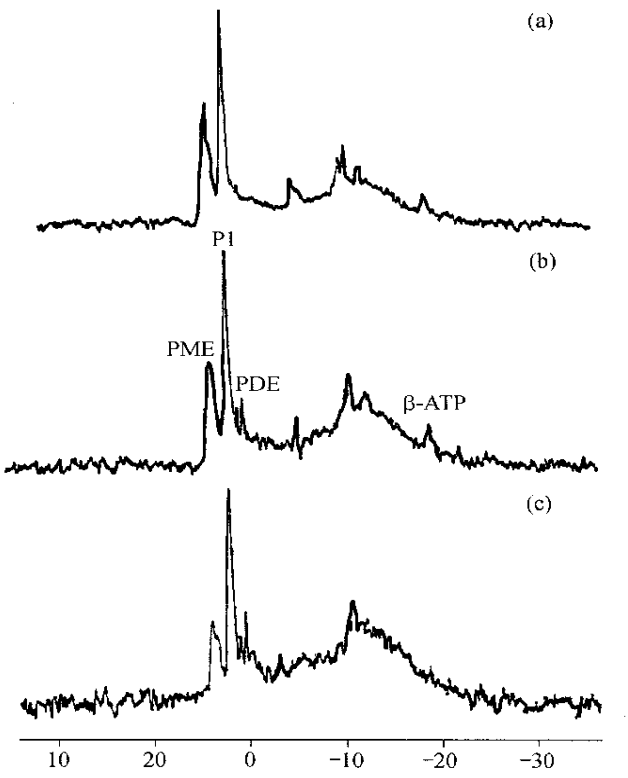

Fig. 1. ^{31}P-NMR spectra of brain cells in different embryonic stages. (a) E10. The sample was made by mixture of 18 embryonic brains. (b) E14. Mixture of 8 brains. (c) E19. Mixture of 2 brains.

In order to limit the variation from the sample, the ratio of content of different products to Pi was used to express the relative values of the product. The peak of ATP-β was used to represent the component of ATP since in the peaks of ATP-α and ATP-γ were involved the contributions from AMP and ADP as well.

Table 1 Different intermediate products during the development from E14 to E19

Embryo age/d	*n*	ATP/Pi	PME/Pi	PDE/Pi	pH
E10	3	0.251 ± 0.02	0.771 ± 0.07	0.451 ± 0.03	6.71 ± 0.04
E14	4	0.304 ± 0.09[a)]	0.680 ± 0.07	0.510 ± 0.06	6.61 ± 0.13
E16	5	0.272 ± 0.07	0.636 ± 0.08	0.519 ± 0.08	6.51 ± 0.12
E19	5	0.261 ± 0.01	0.455 ± 0.06[a)]	0.589 ± 0.10[a)]	6.58 ± 0.30

a) $p<0.01$ compared with E10. The data are mean ± SD.

2.2 Influence of clinorotation on metabolism in brain cells and recovering

In this study the experiments were only performed with E13 and E15 since making one NMR measuring sample needs a large number of cells. The samples made from E13 clinorotated for 24 h showed an increase in ATP, PME and pH compared with controls (see table 2); with the same treatment a similar change in ATP, PME and pH for E15 was also observed while no significant levels were identified (table 3). The eggs were continuously incubated for 24 h after stopping ro-

tation, then levels of PME were restored to normal but ATP level still increased (see table 4). Therefore, we concluded that after clinostating for 24 h, the energy metabolism in brain cells of E13 may keep unchanged.

Table 2 Levels of different intermediate products of brain cells in E13 rotated for 24 h

Groups	*n*	ATP/Pi	PME/Pi	PDE/Pi	pH
Control (E14)	4	0.304±0.09	0.678 ± 0.07	0.510 ± 0.06	6.61 ± 0.13
Revolved (E13→E14)	3	0.453 ± 0.07[a)]	0.978 ± 0.166[b)]	0.498 ± 0.07	7.09 ± 0.07[a)]

a) $p<0.01$; b) $p<0.05$, compared with control. E13→ E14 represents the E13 rotated for 24 h.

Table 3 Levels of different intermediate products of brain cells in E15 rotated for 24 h

Groups	*n*	ATP/Pi	PME/Pi	PDE/Pi	pH
Control (E16)	5	0.272 ± 0.07	0.636 ± 0.08	0.519 ± 0.08	6.51 ± 0.12
Resolved (E15→E16)	5	0.291 ± 0.11	0.690 ± 0.14	0.510 ± 0.07	6.62 ± 0.01

E15→ E16 represents the E15 rotated for 24 h.

Table 4 Levels of different intermediate products of brain cells in E13 rotated for 24 h and then recovering for 24 h

Groups	*n*	ATP/Pi	PME/Pi	PDE/Pi	pH
Control (E15)	5	0.248 ± 0.02	0.613 ± 0.05	0.499 ± 0.09	6.59 ± 0.17
Recovered (E13 →E14 →E15)	4	0.317 ± 0.04[a)]	0.627 ± 0.06	0.546 ± 0.08	6.81 ± 0.22

a) $p<0.01$, compared with control. E13→ E14→ E15 represents the E15 rotated for 24 h and then recovering for 24 h.

2.3 ATPase activity of brain cells in developmental embryos

Comparison of activities of ATPase in brain cells from E14 to E19 is given in fig. 2, from which it can be seen that as the embryonic chicken developed the levels of ATPase increased significantly. The activity of ATPase was presented in Pi μmol · L^{-1}/30 μg protein, 10 min.

2.4 Effect of clinorotation on activity of ATPase and recovery

The activities of ATPase in brain cells of the controls (E13, E15) and of those rotated for 24 h are shown in fig. 3.

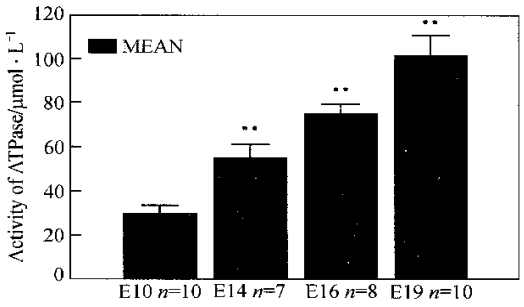

Fig. 2. Comparison of activities of ATPase in brain cells from E14 to E19. ** $p<0.01$.

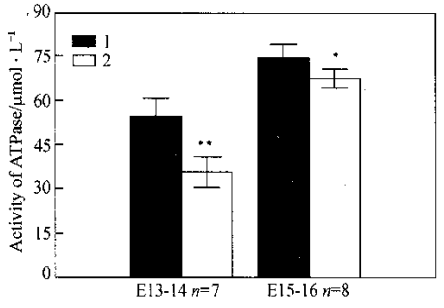

Fig. 3. Effects of clinorotation on ATPase in brain cells of E13 and E15. ** $p<0.01$; * $p<0.05$ compared with the controls. 1, Control; 2, rotate.

Compared with the controls, the activities of ATPase in brain cells of rotated E13 and E15 embryos showed dramatic decrease. The former decreased about 34.8%, while the latter decreased about 9.3%. After the rotation stopped, the activity of ATPase could be restored (table 5).

Table 5 ATPase in brain cells of E13 embryos rotated for 24 h and then recovering for 24 h

Group	*n*	Activity of ATPase (Pi μmol · L^{-1}/30 μg protein 10 min)
E14	7	54.62 ±6.08
E13 →E14	7	35.62 ±5.15[a)]
E13 →E14 →E15	10	54.29 ± 10.04
E15	10	60.03 ±11.72

a) $p<0.01$ compared with the controls. E13 →E14:E13 rotated for 24 h. E13 →E14 →E15: E13 rotated for 24 h and then recovering for 24 h.

3 Discussion

Phospholipid is a very important component of cell membrane. Using ^{31}P-NMR spectroscopy, we could access more information about biosynthesis of cell membrane by analyzing intermediate products in metabolism of phospholipid. Using multiple NMR spectroscopy Serkova et al.[9] investigated the changes in cellular metabolism of glial cells, neuronal cells. The authors found that there was a significant increase in the intermediate products of phospholipid after incubation with immunosuppressants. From tables 2 and 3 we can conclude that the clinorotation of embryonic chicken induced some changes in anabolism and catabolism of phospholipid in their brain cells. The experimental results also showed that with the development of chicken embryos from E10 to E19, PME (the intermediate products of anabolism of phospholipid) gradually decreased, but PDE (the intermediate products of catabolism of phospholipid) increased, suggesting that the anabolism in E10 is greater than in E19, whereas catabolism in the latter is stronger than in the former.

About 25% of ATP in nerve cells is used to maintain the ionic permeability. Na^+-K^+ exchange and Ca^{2+} gradient mainly depends on Na^+, K^+-ATPase and Ca^{2+}-ATPase. Actually the level of ATP measured in this study represents a complex result of its anabolism and catabolism. The activity of ATPase increased during the development of the embryos. The level of ATP decreased after E14, which may be partly caused by more ATP consumed with the increase activity of ATPase. There may be some relationship between the highest level of ATP and biophoton emission of chicken embryo of E14[14].

All objects on the earth would be affected by gravity field from which none could escape. Therefore from the viewpoint of physics it is impossible to simulate the microgravity on the earth. However its biological effects could be simulated by some methods. The simulation using clinostat is based on the fact that living organisms have some delay time for recognizing changes in the direction of the gravity. According to the capability of the organisms in resolving the delay time, they need different rotation speeds to simulate biological effects. Up to now, though it is impossible to avoid other influences on biological systems, like centrifugation force, the clinorotation is one of the most popular methods to simulate biological effects of microgravity on the earth. Embryonic development is a kind of process which is sensitive to the environmental factors.

Some researches about the effect of microgravity on embryonic development of Drosophila, fishes and birds have been done in space. As mentioned above, experiments on the earth are much more practical than in space because of many reasons such as high cost and the limitation of time in space. The previous research on changes of calcium ions in brain cells of chicken embryos by rotating in a clinostat made it clear that the younger the embryo is, the more sensitive it is. We have obtained the similar results from the measurement of ^{31}P-NMR and activity of ATPase. Additionally, the influence of clinorotation on metabolism and activity of ATPase is reversible. The results reported in this paper conform with the previous studies dealing with intracellular Ca^{2+}.

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