Influencing factors of rat small intestinal epithelial cell cultivation and effects of radiation on cell proliferation

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**INTRODUCTION**

Crypt epithelial cells in normal small intestine proliferate at a high speed. But they are very difficult to culture in vitro and passage stably. A lot of studies have been done [1-16]. Some domestic labs isolated and cultured crypt cells from embryonal intestines and aseptic animal intestine, but failed. We introduced normal rat epithelial cell line IEC-6 from the USA and its living condition for stable passage was successfully established after trials. The cell line was testified to be the small intestinal epithelial cell by electronmicroscopy, immunohistochemistry andenzymatic-histoch-emiistry. It has been applied to some related research work[17-21]. It was found that many factors were involved in the culture system. Our present study focuses on the culture method and the influencing factors on IEC-6.

**MATERIALS AND METHODS**

**Reagents**

Dulbecco’s modified Eagle Medium (DMEM), HEPES from Sigma Cooperation, 3H-TdR with the radioactive concentration of 37 MBq/mL and activity ratio of 740GBp/mL is the product of the Chinese Nuclear Science Institute.

**Apparatus**

Carbon dioxide culture case, Model Queue 2721, USA; automatic liquid scintillation counter, Model 1217, Sweden; cell harvester, Model 2T-II, Zhejiang Province; and microplate, Japan.

**RESULTS AND DISCUSSION**

**Effect of IEC-6 density**

IEC-6 cell line was immediately put into 40°C water bath to thaw after being taken from liquid nitrogen, centrifuged for 10 minutes at 1000r/min. Liquid medium was added per bottle after the supernatant was deserted. Then the bottle was put into the carbon dioxide case (10% CO₂, 18.6% O₂ 100% relative humidity, 37°C). After the cells adhered to the wall, change the liquid once, then passage on the 5th day. When the cell was passaged or the activity was detected, the liquid medium was deserted and 0.02% EDTA 8 mL was added for digestion of 30 minutes at 37°C. The incompletely digested cells were scraped softly with curved tube, passed into centrifuge tube and centrifuged for 8 minutes at 1000r/min. Supernatant was deserted and the liquid medium was added to a certain concentration. The cells were then seeded onto 96-well plates and cultured for 72 hours. Then the cells were digested with 0.02% EDTA, harvested on the glass fiber filter membrane, and heated at 80°C. When the membrane cooled down to the room temperature, 8 mL scintillation liquid was added, Cpm was measured with automatic liquidscintillation counter.

**Scintillation liquid contained** POPOP 0.4 g, PPo 4 g, xylene 1000 mL.

**Cell line**

Intestinal epithelial cell line, No. 6, IEC-6 was provided by the General Hospital of Massachusetts, Boston, USA.

**Culture liquid medium**

Liquid DMEM/L was made up according to the protocol supplemented with HEPES 10 mmol, penicillin 10U, streptomycin 100 mg, sodium carbonate 3.7 g and fetal bovine serum 100 mmol. L-glutamine 200 mmol/L was added before use.

**Establishment of cell passage and detection of activity**

IEC-6 cell line was immediately put into 40°C water bath to thaw after being taken from liquid nitrogen, centrifuged for 10 minutes at 1000r/min. Liquid medium was added per bottle after the supernatant was deserted. Then the bottle was put into the carbon dioxide case (10% CO₂, 18.6% O₂ 100% relative humidity, 37°C). After the cells adhered to the wall, change the liquid once, then passage on the 5th day. When the cell was passaged or the activity was detected, the liquid medium was deserted and 0.02% EDTA 8 mL was added for digestion of 30 minutes at 37°C. The incompletely digested cells were scraped softly with curved tube, passed into centrifuge tube and centrifuged for 8 minutes at 1000r/min. Supernatant was deserted and the liquid medium was added to a certain concentration. The cells were then seeded onto 96-well plates and cultured for 72 hours. Then the cells were digested with 0.02% EDTA, harvested on the glass fiber filter membrane, and heated at 80°C. When the membrane cooled down to the room temperature, 8 mL scintillation liquid was added, Cpm was measured with automatic liquid scintillation counter.

IEC-6 cells at various densities in microplate wells were labeled with ³H-TdR 18.5kBq and cultured for 72 hours to investigate its effect on proliferation. Table 1 shows that at a certain range of densities, ³H-TdR incorporation increased with the IEC-6 amount, the peak was at 10 × 10⁴/well. Positive
correlation was found between cell density ($X$) and $^3$H-TdR incorporation ($y$) at the range of $1.25 \times 10^4$ to $10 \times 10^4$/well with the correlation coefficient $r = 0.956$ and regression equation $y = 2177X + 3575$ (min$^{-1}$). When the cell density was more than $11.25 \times 10^4$/well, there was negative correlation ($r = 0.986$, $y = 36782 - 1253 X$), possibly due to the limit amount of nutrition, liquid evaporation and subject to changes of culture condition[22].

### Table 1 Effect of density on $^3$H-TdR incorporation in culture cell ($\overline{x} \pm s$)

| Density ($\times 10^4$/well) | $n$ | $\overline{y} \pm s$ | $\overline{x}$ | $n$ | $\overline{y} \pm s$ |
|-----------------------------|-----|----------------------|---------------|-----|----------------------|
| $1.25$                      | 18  | $3547 \pm 681$       | $11.25$       | 20  | $23648 \pm 1398$    |
| $2.50$                      | 20  | $9941 \pm 413$       | $12.50$       | 18  | $20593 \pm 2245$    |
| $5.00$                      | 18  | $17931 \pm 2051$     | $13.75$       | 20  | $19812 \pm 2310$    |
| $7.50$                      | 18  | $19825 \pm 2135$     | $15.00$       | 18  | $17638 \pm 959$     |
| $10.00$                     | 24  | $23789 \pm 2536$     | $17.50$       | 22  | $14874 \pm 881$     |

**Effect of culture time**

Incorporation of $^3$H-TdR was different after IEC-6 was cultured for 6, 12, 24, 48, 72 and 96 hours (Table 2). Within 72 hours, incorporation increased from $1846 \pm 146$ to $25727 \pm 4006$ (min$^{-1}$) along with the time prolongation and it reached its peak at 72 hour. But when the culture time extended to 96 hours, the incorporation decreased. This may be caused by the activity inhibition of some IEC-6 under non-physical conditions.

### Table 2 Effect of culture time on IEC-6 cell proliferation ($\overline{x} \pm s$)

| Culture time (h) | $n$ | $\overline{y} \pm s$ | $\overline{x}$ | $n$ | $\overline{y} \pm s$ |
|-----------------|-----|----------------------|---------------|-----|----------------------|
| $6$             | 24  | $1846 \pm 146$       | $48$          | 24  | $21258 \pm 1240$    |
| $12$            | 26  | $4038 \pm 363$       | $72$          | 24  | $25727 \pm 2006$    |
| $24$            | 24  | $6367 \pm 588$       | $96$          | 24  | $24355 \pm 2079$    |

**Effect of different $^3$H-TdR dosage**

In this study, different dosage of $^3$H-TdR was administered in the IEC-6 culture system. A linear correlation was found between the $^3$H-TdR incorporation and dosage when the dose was below $55.5$KBq/well. When larger dosage was used, the incorporations slightly increased or decreased (Table 3). The radioactive damage to cells and consequent inhibition of DNA synthesis by high concentration of $^3$H-TdR contributed to the incorporation decrease. Generally, the dosage of 18.5 KBq/well $^3$H-TdR to $10 \times 10^4$ cell yielded a satisfactory result of incorporation $2.5 \times 10^4$.

### Table 3 Effect of $^3$H-TdR dosage on IEC-6 cell proliferation ($\overline{x} \pm s$)

| $^3$H-TdR dosage (KBq/well) | $n$ | $\overline{y} \pm s$ | $\overline{x}$ | $n$ | $\overline{y} \pm s$ |
|-----------------------------|-----|----------------------|---------------|-----|----------------------|
| $0.00$                      | 20  | $139 \pm 29$         | $27.75$       | 21  | $27555 \pm 1637$    |
| $2.31$                      | 22  | $1333 \pm 118$       | $37.00$       | 21  | $37235 \pm 1485$    |
| $4.62$                      | 22  | $10136 \pm 1083$     | $46.25$       | 22  | $41874 \pm 1213$    |
| $9.25$                      | 24  | $16880 \pm 1447$     | $55.05$       | 24  | $48072 \pm 1676$    |
| $18.50$                     | 24  | $24890 \pm 1623$     | $74.00$       | 20  | $42430 \pm 1735$    |

**Effect of pH in lipid medium**

pH of culture medium is one of the most important factors in cell culture. To optimize the culture condition, the pH value was set at 6.0, 6.6, 7.26, 7.6, 8.0 and 8.8, and $^3$H-TdR incorporation was measured respectively (Table 4). The incorporation was the highest at pH 7.26, lower pH at 6.6 and 7.6, and the lowest at pH 6.0 and 8.0. In the common sense, cells can survive when pH ranged from 6 to 8. Variant cells and animal species do not have the same optimal pH. It is believed that optimal pH has an effect on the survival of cells in vitro by adjusting the intracellular enzymes and proliferation factors. We therefore set the optimal pH 7.26 in IEC-6 culture medium.

### Table 4 Effect of pH of culture medium on $^3$H-TdR incorporation ($\overline{x} \pm s$)

| pH   | $n$ | $\overline{y} \pm s$ | $\overline{x}$ | $n$ | $\overline{y} \pm s$ |
|------|-----|----------------------|---------------|-----|----------------------|
| 6.0  | 20  | $4528 \pm 660$       | $28$          | 28  | $12897 \pm 1301$    |
| 6.6  | 24  | $18771 \pm 920$      | $20$          | 20  | $1305 \pm 146$      |
| 7.26 | 24  | $22510 \pm 1448$     | $20$          | 20  | $636 \pm 102$       |

**Effect of insulin and concentration of fetal bovine serum**

Fetal bovine serum is one of the essential factors in cell culture in vitro. If the concentration of fetal bovine serum is too low, cells will die or have proliferation prohibited. When the concentration is too high, the osmotic pressure in culture medium will change and it will influence the survival of cells. In this study, we found that 10% of fetal bovine serum was optimal in culture medium. Content of glucose in DMEM was high (4500 mg/L) and insulin can speed up glucose oxygenolysis and transportation through cell membrane, so the use of glucose was accelerated in the cells. The results showed that incorporation of $^3$H-TdR was higher in cells treated with insulin than in the cells (Table 5) without insulin treatment.

### Table 5 Effect of insulin and concentration of fetal bovine serum on IEC-6 cell proliferation ($\overline{x} \pm s$)

| Fetal bovine serum concentration(%) | $n$ | $\overline{y} \pm s$ | $\overline{x}$ | $n$ | $\overline{y} \pm s$ |
|------------------------------------|-----|----------------------|---------------|-----|----------------------|
| Insulin group                       | 18  | $476 \pm 22$         | $27$          | 21  | $27555 \pm 1637$    |
| Control group                       | 20  | $1311 \pm 978$       | $40$          | 21  | $37235 \pm 1485$    |
| Insulin group                       | 20  | $1311 \pm 978$       | $40$          | 21  | $37235 \pm 1485$    |
| Control group                       | 22  | $14756 \pm 1094$     | $50$          | 22  | $41874 \pm 1213$    |
| Insulin group                       | 22  | $20262 \pm 2012$     | $50$          | 22  | $41874 \pm 1213$    |
| Control group                       | 24  | $23666 \pm 1114$     | $55$          | 24  | $48072 \pm 1676$    |
| Insulin group                       | 24  | $22743 \pm 1728$     | $60$          | 24  | $48072 \pm 1676$    |
| Control group                       | 20  | $22590 \pm 1603$     | $65$          | 20  | $48072 \pm 1676$    |

**Repeatability measurement**

To investigate the experimental method, stability and the researcher’s error, repeatability was measured by dividing the same culture system of IEC-6 into 30 parts. The incorporation of $^3$H-TdR was $24327 \pm 808$ (min$^{-1}$). The value ranged from 23921 to 24733 when $P<0.01$ and coefficient of variation was 3.32%.
Effect of ionizing radiation on IEC-6

Intestinal epithelial cells are sensitive to ionizing radiation. The changes of incorporation of $^3$H-TdR showed the damage of ionizing radiation on cells which reflected the cell biological characteristics. When IEC-6 was not exposed to radiation, the incorporation was $24327 \pm 808$. Incorporation after 4 Gy, 8 Gy, 16 Gy, 2 Gy and 26 Gy irradiation were 31.8%, 24.1%, 15.2%, 11.2% and 8.3% of control. Significantly negative dose-effect relation was found with the relative coefficient $r = -0.970$ (Table 6).

### Table 6 Effect of ionizing radiation dosage on $^3$H-TdR incorporation in IEC-6 cell ($\pm s$)

| Dosage(Gy) | Min $^1$ | (%) | Dosage(Gy) | Min $^1$ | (%) |
|------------|----------|-----|------------|----------|-----|
| 0          | 13427 $\pm$ 803 | 100.0 | 6          | 3041 $\pm$ 327 | 12.5 |
| 4          | 7736 $\pm$ 765 | 31.8 | 12         | 2019 $\pm$ 154 | 8.3 |
| 6          | 6249 $\pm$ 424 | 20.0 | 14         | 2461 $\pm$ 263 | 10.2 |
| 8          | 5863 $\pm$ 594 | 24.1 | 14         | 2019 $\pm$ 154 | 8.3 |
| 12         | 4136 $\pm$ 424 | 17.0 | 14         | 2461 $\pm$ 263 | 10.2 |
| 16         | 3773 $\pm$ 770 | 12.5 | 14         | 2461 $\pm$ 263 | 10.2 |

In summary, methods of IEC-6 culture, passage and activity detection established in this study have the advantage of easy handling, being reliable in results, using less amounts of cells and a good repeatability. Subjective error can be avoided in measurement of epithelial proliferation with radioactive activity. These will provide an ideal method for the research on intestinal epithelium.