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World J Gastroentero, 2001; 7(1):140 - 142
World Journal of Gastroenterology
 ISSN 1007 - 9327

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

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Subject headings intestine, small; intestinal epithelial cell; radiation; cells, cultured; radiation dosage; immunohistochemistry; microscopy, electron

Ran XZ, Su YP, Wei YJ, Ai GP, Cheng TM, Lin Y. Influencing factors of rat small intestinal epithelial cell cultivation and effects of radiation on cell proliferation. ***World J Gastroenterol***, 2001;7(1):140-142

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 electron microscopy, immunihistochemistry and enzymatic-histoch-emistry. 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, ^{3}H-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.

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Supported by the National Natural Science Foundation of China, No. 39100119
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Received 2000-08-08 **Accepted** 2000-09-29

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 10^5U, 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℃ 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% CO_2, 18.6% O_2 100% relative humidity, 37℃). 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℃. 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. ^{3}H-TdR, 1.5uCi per well, was added at the 12th hour before the culture was stopped. At the end of the culture, the cells were digested with 0.02% EDTA, harvested on the glass fiber filter membrane, and heated at 80℃. When the membrane cooled down to the room temperature, 8 mL scintillation liquid was added, Cpm was measured with automatic liquid scintillation counter.

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

RESULTS AND DISCUSSION

Effect of IEC-6 density

IEC-6 cells at various densities in microplate wells were labeled with ^{3}H-TdR 18.5kBq and cultured for 72 hours to investigate its effect on proliferation. Table 1 shows that at a certain range of densities, ^{3}H-TdR incorporation increased with the IEC-6 amount, the peak was at 10×10^4/well. Positive

correlation was found between cell density (X) and ^{3}H-TdR incorporation (y) at the range of 1.25×10^4-10×10^4/well with the correlation coefficient $r = 0.956$ and regression equation $y = 2177X+3575$(min^{-1}). When the cell density was more than 11.25×10^4/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 ($\bar{x} \pm s$)

Density ($\times10^4$/well)	n	Min^{-1}	Density ($\times10^4$/well)	n	Min^{-1}
1.25	18	3547 ± 681	11.25	20	23648 ± 1398
2.50	20	9941 ± 413	12.50	18	20593 ± 2245
5.00	18	17931 ± 2051	13.75	20	19812 ± 2310
7.50	18	19825 ± 2135	15.00	18	17638 ± 959
10.00	24	23789 ± 2536	17.50	22	14874 ± 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 ± 146 to 25727 ± 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 ($\bar{x} \pm s$)

Culture time (h)	n	Min^{-1}	Culture time (h)	n	Min^{-1}
6	24	1846 ± 146	48	24	21258 ± 1240
12	26	4038 ± 363	72	24	25727 ± 2006
24	24	6367 ± 588	96	24	24355 ± 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 55.5KBq/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 10×10^4 cell yielded a satisfactory result of incorporation 2.5×10^4.

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

^{3}H-TdR dosage (kBq/well)	n	Min^{-1}	^{3}H-TdR dosage (kBq/well)	n	Min^{-1}
0.00	20	139 ± 29	27.75	21	27555 ± 1637
2.31	22	1333 ± 118	37.00	21	37235 ± 1485
4.62		10136 ± 1083	46.25	22	41874 ± 1213
9.25	24	16880 ± 1447	55.05	24	48072 ± 1676
18.50	24	24890 ± 1623	74.00	20	42430 ± 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 ($\bar{x} \pm s$)

pH	n	Min^{-1}	pH	n	Min^{-1}
6.0	20	4528 ± 660	7.6	28	12897 ± 1301
6.6	24	18771 ± 920	8.0	20	1305 ± 146
7.26	24	22510 ± 1448	8.8	20	636 ± 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 ($\bar{x} \pm s$)

Fetal bovine serum(%)	n	Min^{-1}	
		Insulin group	Control group
0.0	18	476 ± 22	510 ± 101
2.5	20	13111 ± 978	1901 ± 580
5.0	22	14756 ± 1094	9097 ± 1069
7.5	22	20262 ± 2012	14569 ± 1136
10.0	24	23666 ± 1114	18775 ± 1361
15.0	20	22743 ± 1728	17645 ± 1289
20.0	18	22590 ± 1603	16965 ± 1147

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 ± 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 ± 808. Incorporation after 4Gy, 8Gy, 16Gy, 2Gy and 26y 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 ($\bar{x} \pm s$)

Dosage(Gy)	Min^{-1}	(%)	Dosage(Gy)	Min^{-1}	(%)
0	13427 ± 803	100.0	16	3698 ± 371	15.2
4	7736 ± 765	31.8	18	3381 ± 235	13.9
6	7249 ± 472	29.8	20	3041 ± 327	12.5
8	5863 ± 594	24.1	22	2725 ± 348	11.2
10	4865 ± 586	20.0	24	2481 ± 263	10.2
14	4136 ± 424	17.0	26	2019 ± 154	8.3
			r = -0.970		

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 radioactivity. These will provide an ideal method for the research[23-35] on intestinal epithelimm[36-42].

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Edited by Ma JY