The Effect of EDTA on Incorporation of [^{3}H] Methionine into Two-Cell Mouse Embryos

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Summary

To investigate the effect of EDTA on the in vitro development of mouse embryos from 1-cell to blastocyst, the embryos were cultured in M 16 and M 16 plus EDTA. The incorporation of [^{3}H] methionine was also compared. A significant increase in the number of four-cell embryos (87.5 %) and blastocysts (71.9 %) was observed when embryos were cultured in M16 with EDTA compared to basic M16 medium (23.5 % and 2.9 %, respectively). When considering the results of first two incorporation experiments together, the lowest incorporation of [^{3}H] methionine was observed at 45 h after hCG injection in both medium. So, we may explain that cell block in the in vitro embryo development occurred within a relatively narrow range of time, during 40-45 h after hCG injection. On the other hand the incorporation of [^{3}H] methionine differed between ICR and C57B/6 strains and indicated that in blocking strain mice care must be taken to ensure that embryos at the 2-cell stage are recovered from the oviducts late enough in the second cell cycle for their normal development.

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Key words : two-cell block, incorporation of methionine, EDTA, mouse

Introduction

In the mouse, 2-cell embryos developed into blastocysts when simple medium was supplemented with lactate or pyruvate (Whitten, 1957; Brinster, 1963). The culture of mouse embryos from two-cell to blastocyst is now a routine procedure in many laboratories. However, in vitro development of one-cell zygotes to the blastocyst stage remains to limit in certain inbred strains and several F1 hybrids of mice; embryos from randomly bred strains undergo the first cleavage to the two-cell stage but further development is generally blocked (Whitten and Biggers, 1968; Biggers, 1971; Shire and Whitten, 1980).

In vitro blocks during embryonic development in mammals are not unique. For

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example, rat embryos cease development at the four-cell stage (Whittingham, 1975), cattle embryos at 8- to 16-cell stage (Thibault, 1966), and hamster embryos at two-cell stage (Yanagimachi and Chang, 1964; Bavister et al., 1983). Various methods have been employed to circumvent the obvious limitations imposed by these blocks of preimplantion embryo development. There is good evidence that chelating agents such as EDTA stimulate development of mouse embryos(Abramczuk et al., 1977 and Charot et al., 1989). EDTA may overcome two-cell block in mouse. The present study therefore, was conducted to examine the effect of EDTA on incorporation of [^{3}H] methionine to overcome 2-cell block in the mouse.

Materials and Methods

Five to eight week old female ICR and C57B/6 mice were used for this experiment and maintained under controlled lighting conditions (12 h dark and 12 h light). Animals were allowed free access to solid food and water. The animals were then injected with PMSG(i.p.;5IU) at an interval of 48 h to induce superovulation. They were then mated with male mice of the same strain. Vaginal plug formation on the next morning was confirmed on d 1 of pregnancy. Female mice were then killed by cervical dislocation and embryos were collected from the oviducts by the scratching method 20, 30, 40, 45, 50, and 60 h after hCG injection.

Embryo Culture : The basic medium for embryo culture was M16 (Quinn et al., 1982) and M16 plus 50 μM EDTA (Loutradis et al., 1987). The medium used for collection and washing of embryos was M16 or M16 plus 50 μM EDTA. Embryos were then selected and incubated with a drop (50μl) of medium (M16 or M16 plus 50μM EDTA) in a plastic petridish covered with paraffin oil under an atmosphere of 5 % CO_2 in air at 37 °C and observed every 24 h under a microscope. The efficacy of the experimental culture was evaluated by determining the proportion of embryos reaching the four-cell and blastocyst stages.

Incorporation of [^{3}H] Methionine : The embryos were pooled in the medium and 10 embryos that had normal morphology were transferred to M16 and M16 plus 50μM EDTA medium in a microtube. After the preincubation at 37.5°C for 5 minute, 18.5 KBq/ml [^{3}H] methionine (Spec.act.1.67 GBq/mmol) were added and then incubated for 1 h at 37°C under 5 % CO_2 in air. Then the reaction was stopped by addition of cold TCA to a final concentration of 5 %. The acid-insoluble materials were washed by Millipore filtration (scwp 8μ) with 5 % TCA and ethyl alcohol. The incorporation of [^{3}H] methionine into proteins were determined in a scintillation counter (Tsujii et al., 1970) and five to eight samples were used for each experiment.

Experiment 1 : To determine the developmental response of mouse embryos from ICR strain, one-cell embryos were collected 20 h after hCG injection. After collection and

washing, the embryos were cultured in M16 and M16 plus 50μM EDTA.

Experiment 2: To test the incorporation of [^{3}H] methionine directly, after collection and washed, embryos incorporated for 1 h with [^{3}H] methionine as described above.

Experiment 3: To determine the incorporation of [^{3}H] methionine after a 6 h culture, embryos were cultured under paraffin oil for 6 h with a drop of M16 and M16 plus 50 μM EDTA medium as described above. After culture, the embryos were transferred into a microtube for incorporation of [^{3}H] methionine. The incorporation experiment was also conducted as described above.

Experiment 4: For comparing the incorporation of [^{3}H] methionine by ICR and C57B/6 mouse embryos, the embryos were collected from both strains and incorporation of [^{3}H] methionine was conducted as experiment 2.

Data for each experiment were analyzed for each treatment differences by chi-square using a two-way contigency table.

Results

Experiment 1: As shown in Table 1, the developmental responses following collection of embryos in either of M16 or M16 plus 50μM EDTA medium were varied. Development to the four cell and subsequently blastocyst stage of one-cell embryos cultured in M16 plus 50μM EDTA medium was significantly ($p<0.05$) greater in the number and percentage than that in M 16 medium.

Experiment 2: As shown in Figure 1, the lowest incorporation of [^{3}H] methionine was observed at 45 h after hCG injection in embryos cultured in the M16 medium and M16 plus 50μM EDTA medium, during 30-60 h after hCG injection. However, the incorporation of [^{3}H] methionine increased 50 and 60 h after hCG injection in both media. The presence of EDTA in medium at 30 h and 40 h after hCG injection significantly ($P<0.05$) reduced the amount of [^{3}H] methionine incorporated into the protein but the incorporation increased significantly ($P<0.05$) at 50 h and 60 h after hCG injection.

Table 1. Development of 1-cell ICR mouse embryos cultured in M16 medium supplemented with or without EDTA.

Medium	n	Development (%)		
		2-cell (43h)	4-cell (88h)	blastocyst (136h)
M16	34	31 (91.2)	8 (23.5)	1 (2.9)
M16+EDTA50μM	32	29 (90.6)	28 (87.5)*	23 (71.9)*

*$P<0.05$

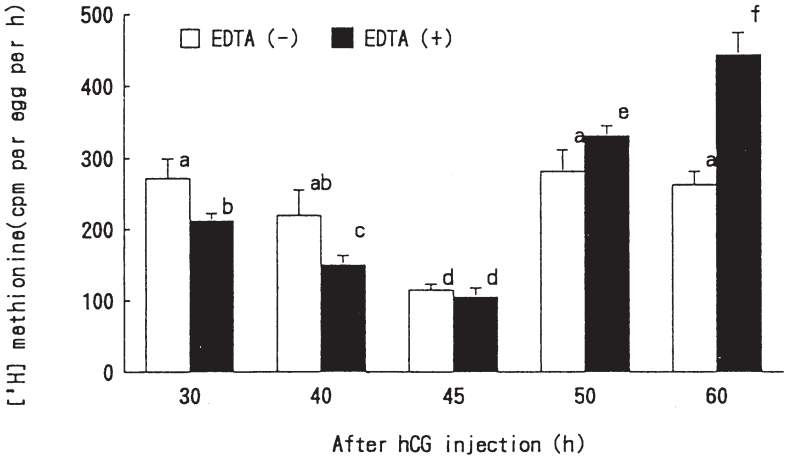

Fig. 1. Incorporation of [³H] methionine in M16 medium with or without EDTA. Means ± SE. Values with different superscripts differ significantly ($P < 0.05$).

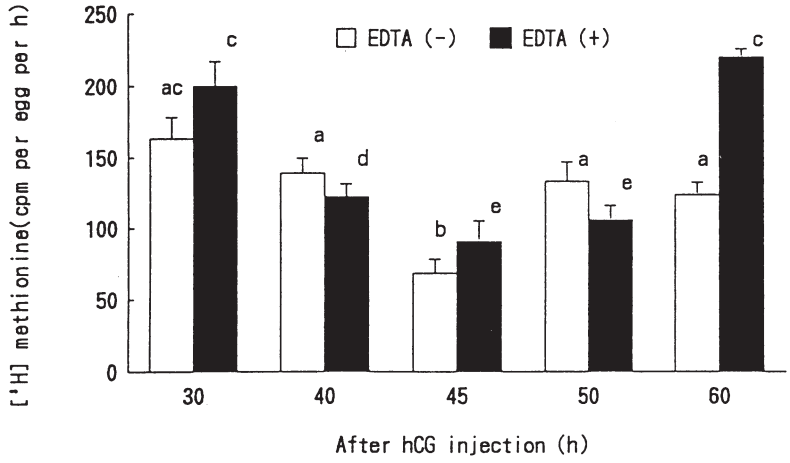

Fig. 2. Incorporation of [³H] methionine in medium M16 with or without EDTA after 6 h in vitro culture. Means ± SE. Values with different superscripts differ significantly ($P < 0.05$).

Experiment 3: As shown in Figure 2, the lowest incorporation of [³H] methionine observed at 45 h after hCG injection in both media during 30 to 60 h. If embryos were exposed continuously to M16 medium in culture the values of incorporation of [³H] methionine was not significant at 30, 40, 50, and 60 h after hCG injection. In contrast, when embryos were cultured in M16 medium plus EDTA, the incorporation was differ significantly ($P < 0.05$) at 30, 45 and 60 h after hCG injection. Also, in culture for 6 h (Exp. 3), significant ($P < 0.05$) decrease in the incorporation of [³H] methionine during 30 to 60

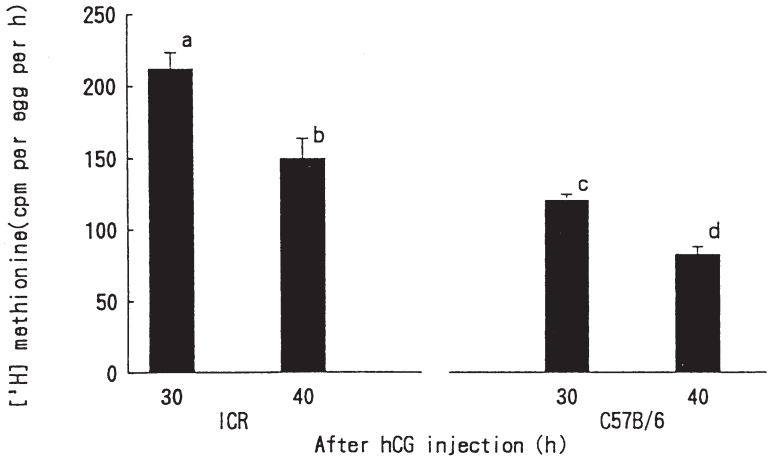

Fig. 3. Comparison of [^{3}H] Methionine incorporation in condition with EDTA between in vivo embryos derived from ICR and C57B/6 strain mouse. Means ± SE. Values with different superscripts differ significantly ($P < 0.05$).

h after hCG injection was observed, compared to without culture (Exp.2). On the other hand, the addition of 50μM EDTA to M16 medium did not significantly ($P<0.05$) decrease the incorporation of [^{3}H] methionine at 30, 40, and 45 h after hCG injection, but it was significantly ($P<0.05$) decreased the incorporation of [^{3}H] methionine at 50 h after hCG injection, compared without (Exp.2) and with 6 h culture (Exp.3).

Experiment 4: The results of incorporation of [^{3}H] methionine of C57B/6 mouse embryos in M16 medium plus EDTA at 30 and 40 h after hCG injection are shown in Figure 3. The results indicate that the incorporation of [^{3}H] methionine at 40 h was lower ($P<0.05$) than at 30 h after hCG injection and also reveal a significantly ($P<0.05$) lower incorporation of ^{3}H-Methionine in C57B/6 mouse embryos at 30 h and 40 h after hCG injection than that of ICR mouse embryos.

Discussion

These experiments demonstrated three clear-cut effects of EDTA on incorporation of [^{3}H] methionine and the development of one-cell mouse embryos into blastocysts. The first effect is the promotion of growth from the one-cell stage to blastocysts in medium M16 with EDTA. Several investigatiors have reported that inclusion of EDTA in the chemically defined culture medium improves the development of mouse embryos or is a requirement (Abramczuk et al., 1977; Chatot et al., 1989; Nasr-Esfahani et al., 1992). In one study, a block to development of mouse embryos at the two-cell stage was overcome by using either EDTA or apotransferrin, an iron-carrier protein (Nasr-Esfa-

hani et al., 1990). Most recently, it was found that zinc contamination of oil used to overlay culture media was responsible for blocking mouse embryo development, an effect that was alleviated by EDTA in medium (Bavister, 1995). These findings together implicate heavy metal contamination of ingredients or the apparatus used to prepare culture media, the detrimental effects of which can be overcome by adding chemical chelator such as EDTA. When added to the culture medium, free Fe ions are chelated (Gutteridge, 1982) and ferric ions catalyze the formation of oxygen radicals. The EDTA-iron complex is known to have SOD activity (Halliwell, 1975). Thus the attenuating effects of EDTA on the developmental block may be explained in terms of oxygen toxicity.

Secondly, the lowest incorporation of [^{3}H] methionine occurred in embryos at 45 h after hCG injection. If mouse embryos before the mid two-cell stage are taken for culture the development in G2 phase of the second cell cycle will arrest and result in the phenomenon referred to as two-cell block (Biggers, 1971). Most strains of mice show this phenomenon. In the 2-cell block, the transition from maternal to embryonic gene expression occurs at the 2-cell stage of development and only the first division exclusively occurs under maternal control. During the first 24 h after fertilization about 30 to 40 % of the total maternal RNA, about 70 % of the polyadenylated RNA fraction and around 90 % of the specific messages for histone H3 and actin are degraded (Levey et al., 1978; Bachvarova and Deleon, 1980; Piko and Clegg, 1982; Giebelhaus et al., 1985 and Graves et al., 1985). There seems to be only very little, if any transcription from the embryonic genome during this time. In accordance with this, transcriptional inhibitors such as α-amanitin do not block development of fertilized eggs to the 2-cell stage (Braude et al., 1979). At the 2-cell stage, transcription from the embryonic genome occurs and it is required for normal development to proceed. All classes of new RNA are usually synthesized (Clegg and Piko, 1977 and 1983) and further development is blocked by inhibition of transcription with α-amanitin. So, if recovery from the oviduct is delayed until embryos have reached the late two-cell stage (48 h after hCG), then in vitro development in simple defined media is unimpaired.

Thirdly, we found that the incorporation of [^{3}H] methionine differed between ICR and C57B/6 strains of mice. It indicated that the difference in the strain of mice used may be a cause of the inconsistency. This finding indicated that in the case of blocking-strain mice (if the mice are outbred this is most likely to be the case) care must be taken to ensure that embryos at the two-cell stage are recovered from the oviducts late enough in the second cell cycle for their normal development to blastocysts in vitro (Pratt, 1987). This is likely to be approximately 36 to 40 h after hCG injection, but it is best to determined for each strain of mouse available (Goddard and Pratt, 1983).

In conclusion, we may suggest that culture usually accelerates the cell block but EDTA has the ability to maintain a large degree of viability of mouse embryos for

further development. Two-cell block occurs within 40-45 h after hCG injection in mouse. So, in the case of blocking-strain mice care must be taken to ensure that embryos at the two-cell stage are recovered from the oviducts late enough in the second cell cycle.

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マウス2細胞卵子における^{3}H-メチオニンの取込みに及ぼすEDTAの影響

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要　　約

マウス初期胚の培養及び^{3}H-メチオニンの取り込みに及ぼすEDTAの効果について調べた。マウス1細胞をM16に50μM EDTA添加した培養液での発育を観察した。その結果4細胞への発生率は，無添加区23.5%に対して87.5%,胚盤胞への発生率は無添加区の2.9%に対して71.9%と有意に増加した。^{3}H-メチオニンの取り込みを調べたところ，hCG投与後45時間の2細胞期に取り込みが低下するのがM16およびM16+EDTAで観察された。このことからin vitroで起こるマウス初期胚のcell blockはhCG投与後40～45時間に起こることが判明した。一方，ICR系マウスとC57B/6系マウスの卵子の^{3}H-メチオニンの取り込みを比較したところ違いがみられた。これらのことから，M16にEDTAを添加した培養液は2細胞以降のタンパク合成を高め，卵子のゲノムを活性化すると思われた。

キーワード：2-cell block，メチオニンの取込み，EDTA，マウス