The Effect of EDTA on Incorporation and Oxidation of [^{14}C] Glucose and [^{14}C] Pyruvate in the Early Developing Mouse Embryos

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Abstract: *The incorporation and oxidation of glucose and pyruvate were compared when embryos were cultured in M16 medium and M16 plus EDTA. In both culture conditions the incorporation and oxidation of glucose increased a little but then significantly decreased by 40 and 45 h after hCG injection. On the other hand, the incorporation and oxidation of pyruvate increased with the development of embryos, was reduced at the blastocyst stage, but had not significantly decreased by 40 and 45 h after hCG injection. After mouse embryos were cultured for 6 h in M16 medium, the incorporation and oxidation of glucose and pyruvate were significantly decreased compared with culture in M16 plus EDTA at 40, 45 and 50 h after hCG injection. These results suggest that EDTA had the ability to maintain a large degree of viability of mouse embryos for further development.*

Key words: *Glucose, Pyruvate, EDTA, Two-cell block, Mouse embryo.*

In mice, the fertilized one-cell egg requires pyruvate or oxaloacetate; the two-cell embryo can also utilize phosphoenol-pyruvate and lactate, but this is not continued, and glucose utilization usually started at the eight-cell stage and continued [1, 2]. The culture of mouse embryos from the two-cell to the blastocyst stage is now a routine procedure in many laboratories. Nevertheless, *in vitro* development of one cell zygotes to the blastocysts stage remains limited 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 [3–5]. In a previous study [6] we found that the lowest incorporation of [^{3}H] methionine was observed at 45 h after hCG injection at the two-cell stage. Development beyond the two-cell stage is an enhanced rate of total protein synthesis indicating further activation of the transcription process of the embryonic genome, but there is no report about energy substrate in combination with EDTA during the early development of preimplantation mouse embryos. With this idea in view, the aims of this study were to determine the direct or indirect effect of EDTA on incorporation of [^{14}C] glucose and [^{14}C] pyruvate during the early preimplantation stages of development in mouse embryos.

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Materials and Methods

Female ICR mice 5 to 8 weeks old were taken used in this experiment and maintained under controlled lighting conditions (12 h dark and 12 h light). The animals were allowed free access to solid food and water. They were then injected with PMSG (i.p.; 5 iu) and hCG (i.p.; 5 iu) at an interval of 48 h to induce superovulation. They were then mated with male mice of the same strain. One-cell, 2-cell, 4-cell and 8-cell embryos were collected from the oviducts by the scratching method 20 to 30, 40 to 45, 50 and 60 h after hCG injection, respectively. On the other hand, morulae and blastocysts were collected by flushing the uterus 90 and 95 h after hCG injection, respectively.

Incorporation and oxidation experiments with [^{14}C] glucose and [^{14}C] pyruvate were performed according to the method of Brinster [7]. The embryos were pooled with medium and 10 embryos which have normal morphology were transferred to M16 or M16 plus 50 μM EDTA medium in one microtube, and 1 ml Hyamine in another microtube. The contents of the two microtubes were then transferred to a scintillation vial. The scintillation vials were then made airtight and incubated for 3 h at 37°C under 5% CO_2 in air. Then the reaction was stopped by the addition of cold PCA to a final concen-

tration of 5%. The acid insoluble materials were washed several times by millipore filtration (scwp 8 μ) with 5% PCA and ethyl alcohol. The incorporation and oxidation of [^{14}C] glucose and [^{14}C] pyruvate were determined in a scintillation counter and five to eight samples were used for each observation.

Experiment 1. Experiment 1 was design to test the incorporation and oxidation of [^{14}C] glucose and [^{14}C] pyruvate directly after collection of the embryos. In this experiment, embryos were collected 20, 30, 40, 45, 50, 60, 90 and 95 h after hCG injection and incorporated for 3 h with [^{14}C] glucose or [^{14}C] pyruvate as described above.

Experiment 2. The objective of experiment 2 was to determine the incorporation of [^{14}C] glucose and [^{14}C] pyruvate after 6 h culture of the embryos. In this experiment, embryos were collected 40, 45 and 50 h after hCG injection and cultured under paraffin oil for 6 h with a drop of M16 and M16 plus 50 μM EDTA medium in a CO_2 incubator with 5% CO_2 and 95% air at 37°C. After the culture, the embryos were transferred to a microtube for incorporation of [^{14}C] glucose or [^{14}C] pyruvate, and the incorporation experiment also conducted as described before.

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

Results

As shown in Figs. 1 and 2, the lowest ($P<0.05$) incorporation and oxidation of [^{14}C] glucose were observed at 40 and 45 h (2-cell stage) after hCG injection, in both M16 and M16 plus 50 μM EDTA medium. On the other hand the incorporation and oxidation of [^{14}C] glucose were significantly ($P<0.05$) increased at 50–95 h (4-cell to blastocyst stage) after hCG injection in both cases. In the M16 plus 50 μM EDTA medium, the incorporation

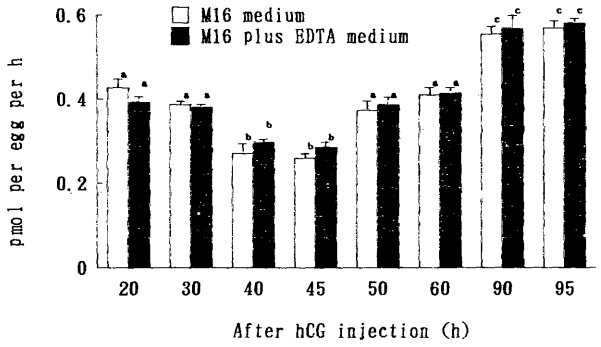

Fig. 1. Effect of EDTA on the incorporation of [^{14}C] glucose in preimplantation mouse embryos. Means ± SE. Values with different superscripts differ ($P<0.05$).

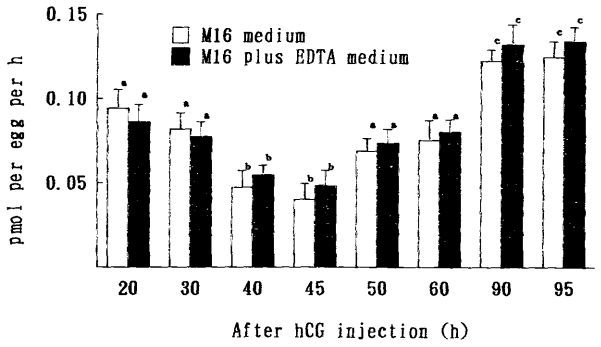

Fig. 2. Effect of EDTA on the oxidation of [^{14}C] glucose in preimplantation mouse embryos. M ± SE. Values with different superscripts differ ($P<0.05$).

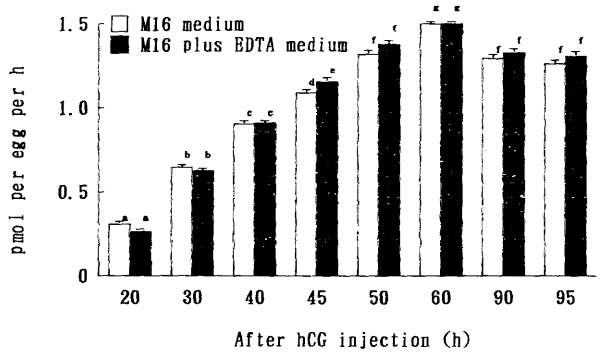

Fig. 3. Effect of EDTA on the incorporation of [^{14}C] pyruvate in preimplantation mouse embryos. Means ± SE. Values with different superscripts differ ($P<0.05$).

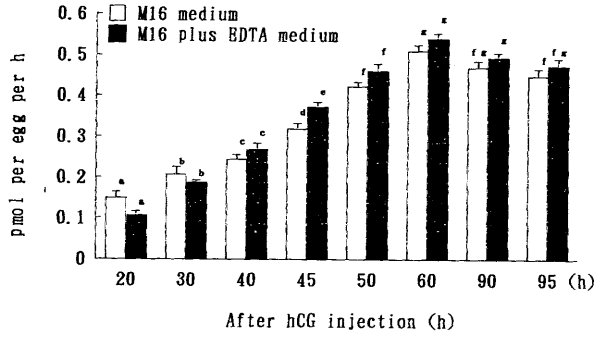

Fig. 4. Effect of EDTA on the oxidation of [^{14}C] pyruvate in preimplantation mouse embryos. Means ± SE. Values with different superscripts differ ($P<0.05$).

and oxidation of [^{14}C] glucose were not significantly different from those of the M16 medium, throughout the experiment, but had slightly increased by 40 h (2-cell) after hCG injection. In both media, the incorporation and oxidation ratios were between 4.31 and 6.08 for each hour.

Incorporation and oxidation of [^{14}C] pyruvate were simultaneously increased with the time spent in both media (shown in Figs. 3 and 4). In M16 plus 50 μM EDTA medium the incorporation and oxidation of [^{14}C] pyruvate were significantly ($P<0.05$) increased at 45 h (2-cell) after hCG injection compared to the M16 medium. In both media the incorporation and oxidation ratios were between 2.05 and 3.38 for each hour. In a comparison of glucose and pyruvate, the incorporation and oxidation of [^{14}C] pyruvate were higher than those of [^{14}C] glucose. The effects of EDTA on the incorporation of [^{14}C] glucose without and with 6 h culture are shown in Table 1. Without culture, the incorporation of [^{14}C] glucose was higher with EDTA than without EDTA (control), but the difference was not significant. On the other hand, in the case of 6 h culture the incorporation of [^{14}C] glucose was significantly higher with EDTA than the control except 50 h (4-cell) after hCG injection.

The effects of EDTA on the incorporation of [^{14}C] pyruvate without or with 6 h culture are shown in Table 2. Without culture, the incorporation of [^{14}C] pyruvate was higher with EDTA than the control, and in all case the difference was significant ($P<0.01$) except at 40 h (2-cell) after hCG injection. On the other hand, in the case of 6 h culture the incorporation of [^{14}C] pyruvate was significantly higher with EDTA than the control ($P<0.05$) in all cases.

Discussion

This study shows that the lowest incorporation of [^{14}C] glucose and carbondioxide output occur at 40 and 45 h (2-cell stage) after hCG injection, but the incorporation and oxidation of pyruvate were not significantly decreased at 40 and 45 h after hCG injection. The incorporation and oxidation were parallel in all the experimental periods for both glucose and pyruvate. This suggested that the energy requirements of the early developing mouse embryos are different.

At the two-cell stage, glucose is not effective as an energy source due to early blockade in glycolysis [8], and the likely site of this blockade should still allow glucose to enter the pentose pathway, in lieu of directly entering the TCA cycle, but mouse zygotes were first successfully cultured in medium containing glucose in addition to lactate and pyruvate [3]. Cross and Brinster [9] obtained 30 to 50% blastocysts in the absence of additional glucose. Initially, Chatot *et al.* [10] found less blastocyst development when glucose was used from the start of the culture but the best development was

Table 1. Effect of EDTA on the incorporation of ^{14}C-glucose in preimplantation mouse embryos (cpm per embryo)

After hCG(h)	without culture		with 6 h culture	
	without EDTA	with EDTA	without EDTA	with EDTA
40	63.16 ± 2.38^{a}	69.35 ± 1.45^{a}	47.53 ± 1.55^{b}	61.24 ± 1.58^{a}
45	60.38 ± 1.38^{a}	66.49 ± 2.36^{a}	21.81 ± 1.86^{c}	68.87 ± 2.26^{a}
50	86.95 ± 4.45^{d}	90.00 ± 3.10^{d}	65.05 ± 2.19^{a}	70.69 ± 2.15^{a}

Means ± SE. Values with different superscripts differ ($P<0.05$).

Table 2. Effect of EDTA on the incorporation of ^{14}C-pyruvate in preimplantation mouse embryos (cpm per embryo, M ± SE)

After hCG(h)	without culture		with 6 h culture	
	without EDTA	with EDTA	without EDTA	with EDTA
40	480.95 ± 3.15^{a}	483.55 ± 2.55^{a}	424.19 ± 5.27^{b}	528.04 ± 6.17^{c}
45	584.40 ± 3.24^{d}	614.25 ± 4.26^{f}	306.82 ± 4.84^{e}	614.74 ± 4.69^{f}
50	701.55 ± 4.45^{g}	724.54 ± 3.25^{g}	598.75 ± 6.21^{f}	660.52 ± 5.58^{h}

$^{a, b, c, d, e, f, g, h}$ Means with different superscripts differ from each other, $P<0.05$.

obtained when glucose was added after day 3. Menezo and Khatchadourian [11] had previously proposed that glucose in the culture medium may lead to a metabolic lock at the level of the glucose-6-phosphate isomerase enzyme. They proposed that glucose may stimulate premature glycogen synthesis in the embryo, whereas fructose, which enters glycolysis downstream of glucose, may not be directed towards glycogen synthesis. Brinster [12] reported that the mouse embryos were able to oxidize only a very small amount of glucose during the early stage of development, but pyruvate and lactate were very actively oxidized to CO_2 in all stages of development of the mouse embryo [7]. Pyruvate oxidation was significantly greater than that of glucose, which corroborates the results obtained by Brinster [7].

In the two-cell block, the transition from maternal to embryonic gene expression occurs at the two-cell stage of development and only the first division exclusively occurs under maternal control. During the first 24 h after fertilization about 30–40% 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 [13–17]. 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 two-cell stage [18]. At the two-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 [19, 20] and further development is blocked by inhibition of transcription with α-amanitin.

The incorporation and oxidation of [^{14}C] glucose and [^{14}C] pyruvate were found to be higher with EDTA than without EDTA both with or without 6 h culture (Tables 1 and 2). These results shows that EDTA had an effect on the incorporation and oxidation of both glucose and pyruvate. Our previous work indicated that the incorporation of [^{3}H]-methionine was lowest at the 2-cell stage [6]. But, the addition of EDTA to M16 medium did not significantly ($P<0.05$) decrease the incorporation of [^{3}H]-methionine at the 2-cell stage. The findings of this experiment are in agreement with our previous work [6]: that EDTA supports further development of 2-cell embryos, and activation of the embryonic genome. The findings of this experiment are consistent with results of other researchers. Goddard and Pratt [21] described the 2-cell block phenomenon and concluded that this phenomenon is due to a culture-induced cytoplasmic defect. Abramczuk *et al.* [22] reported that the effect of EDTA on the development of one-cell embryos *in vitro* is specific and probably due to its chelating action. 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 the medium [23]. These findings together implicate heavy metal contamination of ingredients or apparatus used to prepare culture media, whose detrimental effects can be overcome by using chemical chelators such as EDTA [23]. When EDTA is added to the culture medium, free Fe ions are chelated [24], and ferric ions catalyze the formation of oxygen radicals. The EDTA-Fe complex is known to have SOD activity [25]. Thus the attenuating effects of EDTA on the developmental block may be explained in terms of oxygen toxicity. In mammals, all cells are exposed to the risk of injury by active oxygen, which is formed when molecular oxygen is utilized as an electron acceptor during oxidative reactions in the cells.

In conclusion, we suggest that the two-cell block occurs within 40–45 h after hCG injection in mouse. Culture usually accelerates the cell block but EDTA has the ability to maintain a large degree of viability of mouse embryos for further development.

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