Effect of Rare Earth Elements on Proliferation and Fatty Acids Accumulation of 3T3-L1 Cells

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ABSTRACT : The present study including two experiments was designed to determine the effect of media containing different rare earth elements (REE) on proliferation and fatty acids accumulation in 3T3-L1 cell cultures. In Experiment 1, 3T3-L1 preadipocytes in 96-well plates (1.5×10^4 cells/ml) were cultured with Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) for 24 h. Then the media were changed to the following 10 different media for 48 h: DMEM containing 10% FBS for the control; the above media containing 5 μM, 10 μM or 15 μM of $LaCl_3$, $CeCl_3$ or the mixture of these REE chlorides. The proliferation rate of the cells was measured and compared by a non-isotope method-XTT method. In Experiment 2 the cells in 24-well plates (1.5×10^4 cells/ml) were cultured in DMEM containing 10% FBS for 7 days until confluent and then were changed to above DMEM containing dexamethasone, methyl-isobutylxanthine and insulin (DMI) for two days. Afterwards the media were changed to the 10 different media with REE supplements as in Experiment 1 and cultured for 6 days. The cells were then harvested for fatty acids analysis by gas chromatography. It was found that supplementation of La (5, 10 and 15 μM), Ce (5 μM and 15 μM) and the mixture REE (5, 10 and 15 μM) stimulated ($p<0.05$) the proliferation of 3T3-L1 preadipocytes (Experiment 1). In the differentiating 3T3-L1 cells supplementation of La (5 μM and 10 μM), Ce (5 μM) and the mixture REE (5 μM and 15 μM) decreased ($p<0.05$) the concentration of monounsaturated fatty acids (MUFA) per 10^5 cells, while the supplementation of La (5 μM), Ce (5 μM) and the mixture REE (15 μM) increased ($p<0.05$) the ratio of saturated fatty acids (SFA) to MUFA. These results indicate that the supplementation of REE to the media may affect proliferation, differentiation and lipogenesis rates of 3T3-L1 cells. However, the effect may depend upon the level or type of REE applied. ***(Asian-Aust. J. Anim. Sci. 2006. Vol 19, No. 1 : 119-125)***

Key Words : Rare Earth Elements, 3T3-L1 Cells, Adipocytes, Proliferation, Fatty Acids

INTRODUCTION

Rare earth elements (REE) including Lanthanum (La), Cerium (Ce) and other lanthanides are a group of elements with a lot of similarities in chemical and biochemical characters. The chlorides of REE such as $LaCl_3$, $CeCl_3$ and a mixture of lanthanum chlorides have been reported as possible growth promoters to improve the performance of growing pigs, chicken and Wistar rats when added to diet with concentration of 100-300 mg/kg (He et al., 2001, 2003; Halle et al., 2003). The REE have been used as a kind of feed additive in animal production in China for years. Recently, the REE obtained a temporary registration as feed additive also in Switzerland (Rambeck et al., unpublished). The supplementation of REE to the diet improved the daily body weight gain or feed conversion ratio in the growing animals. Only a very small amount of REE could be absorbed into body when they were supplemented orally (He and Rambeck, 2000). However, such small amount (micromoles) of REE in animal body may have effect on the metabolism through influence the hormones such as triiodothyronine (He et al., 2003) or growth hormone (Wang and Xu, 2003). Although there are some early studies on effect of REE in animal tissues and cells (Evans, 1990), little information was available on their effect on animal adipose tissues or development of adipocytes.

3T3-L1 cell lines have been used as a model to study the effect of various supplements in the development of adipocytes (Green and Kehinde, 1974). The early study found that supplementation of $LaCl_3$ to the media could stimulate the initiation of DNA synthesis in quiescent Swiss 3T3 and 3T6 cells (Smith and Smith, 1980). However, little information was available concerning the effect of various REE on 3T3-L1 preadipocytes during the proliferation period, neither on accumulation of fatty acids in differentiating 3T3-L1 cells.

Therefore, the present study including two experiments was designed to determine the effect of different levels of $LaCl_3$, $CeCl_3$ and a lanthanum chlorides mixture on the proliferation and fatty acids accumulation in 3T3-L1 cell cultures. Experiment 1 was conducted to compare the effect of REE on the proliferation of preadipocytes while Experiment 2 was to compare the effect of REE on the fatty acids concentration and composition in differentiating 3T3-L1 cells.

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MATERIALS AND METHODS

Experimental groups and cell culture

3T3-L1 cell lines were from ATCC™ (VA, USA). In Experiment 1, 3T3-L1 cell suspensions in a density of 1.5×10^4 cells per ml (200 μl each well) were seeded to 96-well plates. The cells were incubated overnight for an attachment at 37°C with Dulbecco's modified Eagle's medium (DMEM, without phenol, Sigma, MO, USA) containing 10% fetal bovine serum (FBS, Sigma, MO, USA). In the next 48 h the cells were incubated with the following 10 different experimental media: the DMEM medium supplemented with 10% FBS for the control; the DMEM media supplemented with 10% FBS and 5 μM, 10 μM or 15 μM of $LaCl_3$, or the same amount of $CeCl_3$ and the mixture of lanthanum chlorides for all the other 9 treatments. In Experiment 2, 3T3-L1 cells were seeded to 24-well plates in a density of 1.5×10^4 cells/ml and cultured in a proliferation medium of DMEM (Sigma, MO, USA) containing 10% FBS for 7 days until confluent. Then all the media were changed to a differentiation medium containing 0.1 μM dexamethasone (D, Sigma, MO, USA), 1 mM methyl-isobutylxanthine (M, Sigma, MO, USA) and 0.1 μM insulin (I, Bovine insulin, Sigma, MO, USA) for 2 days. Afterwards the media were changed to the following 10 different media and cultured for 6 days: the control medium was the DMEM (Sigma, MO, USA) supplemented with 10% FBS; the treatment media were the REE-supplemented control media containing 5 μM, 10 μM or 15 μM $LaCl_3$, $CeCl_3$ or a mixture of lanthanum chlorides. Pure $LaCl_3{\cdot}7H_2O$, and $CeCl_3{\cdot}7H_2O$ were the products of Sigma, MO, USA. The mixture of lanthanum chlorides that derived from Baotou area, Innermongolia, China, contains 38.0% of $LaCl_3{\cdot}6H_2O$, 52.1% of $CeCl_3{\cdot}6H_2O$, 3% of $PrCl_3{\cdot}6H_2O$ and 6.9% chlorides of other REE.

Measurement of cell proliferation

In Experiment 1, 3T3-L1 preadipocytes in the plates were incubated in different media for 48 h. Afterwards, all the media were changed to a FBS-free DMEM media (200 μl) and 40 μl solution containing 1 mg/ml mixture of sodium-2, 3-bis[2-Methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium -5-carboxyanilide (XTT, Sigma, MO) and 1% phenazine methosulfate (PMS, Sigma, MO, USA) were added (Scudiero et al., 1988). The incubation time was 3 h. Then the absorbance at a wavelength of 420 nm was measured in a plate reader (MRX, Dynatech, VA, USA). In both experiments cell number in different cultures were also determined after dissociated by incubation with phosphate buffered saline (PBS, pH 7.08) containing 0.25% trypsin and 100 mg/L EDTA. The cells harvested from each well were counted on a haemocytometer. In Experiment 2 the harvested cell suspensions were stored under -30°C for the further extraction of lipids and fatty acids analysis.

Staining and photographing of the cells

In Experiment 2 the cell cultures were photographed through using an inverted microscope equipped with a microphotograph system. One set of the treatment cultures were stained with Oil Red-O (Sigma, MO, USA) and Mayer's Hematoxylin (Sigma, MO, USA). Buffered formalin (10%, 1 ml) at 4°C was added to the cell cultures and stood for 30 min at room temperature for a preliminary fixation. Then fixation was carried on again by 2 ml buffered formalin for 1 h at room temperature. Finally they were washed with deionized water and stained by 1 ml mixture of 0.5% Oil Red-O in isopropanol:deionized water (3:2) for 1 h. After washing with deionized water, the cells were counterstained with Mayer's Hematoxylin (1 g/L, Sigma, MO, USA) for 3 min.

Determination of fatty acids in lipids of the cells

In Experiment 2 total lipids of the cells were extracted by a method using hexane and isopropanol for determination of fatty acids (Jiang et al., 1996). The extracted lipids were methylated by a combined base/acid methylation method (Kramer et al., 1997) with modifications. Henicosanoic acid (C21:0) methyl ester (Nu-Chek Prep, Inc., MN, USA) was used as internal standard. The fatty acids profiles were analyzed by Shimazu gas chromatography (GC-14B, Japan) with a capillary column Rtx®-2330, 30 m×0.32 mm ID×0.2 μm df (Restek, USA). Initial GC temperature was 100°C and then increased to 175°C at a rate of 25°C per min. After held for 5 min the temperature was continually increased to 225°C at a rate of 5°C per min and held for 10 min. Helium was used as the carrier gas. The fatty acids were identified by comparison to retention times of known standards. The concentration of identified fatty acid was then calculated based on the ratio of the fatty acid to the internal standard.

Statistic analysis

Analysis of variance (ANOVA) was used for testing the differences on the absorbance data in XTT assay (Experiment 1) and ratio or concentration of the fatty acids (Experiment 2) among the experimental groups. Tukey test was used to compare the differences among the treatments after a significant difference ($p<0.05$) was found (SPSS, 1999).

RESULTS

Proliferation of the 3T3-L1 cells

The effects of media containing different REE on cell proliferation is shown in Figure 1 (Experiment 1). The

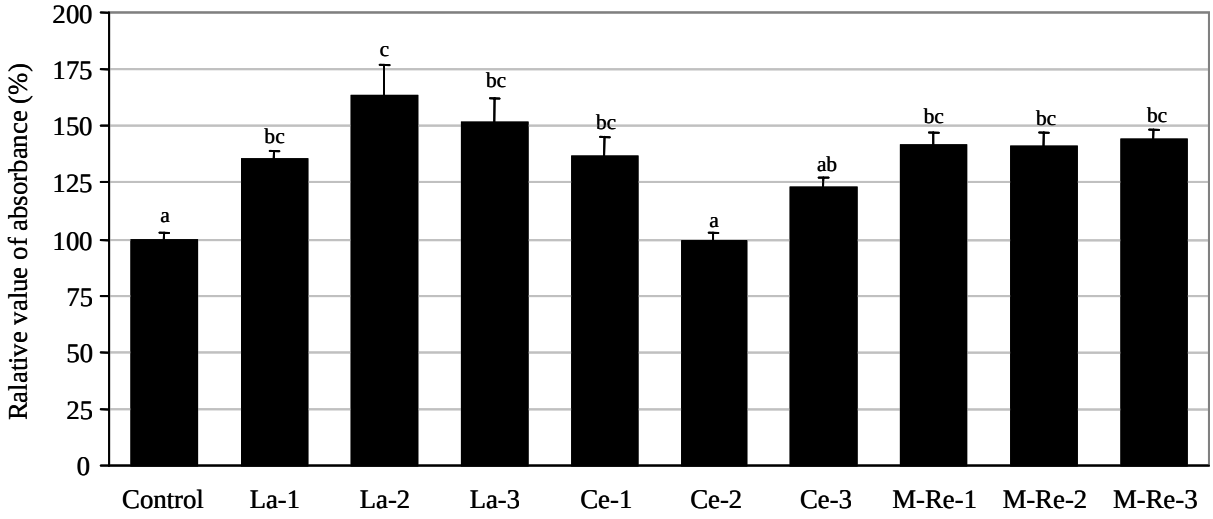

Figure 1. Comparison on the relative value of absorbance in XTT assay among 3T3-L1 preadipocyte cultures treated in the media containing various rare earth chlorides (Experiment 1). Concentration of rare earth chlorides supplemented to the media was 0 for the control; 5 μM for La-1, Ce-1 and M-Re-1; 10 μM for La-2, Ce-2 and M-Re-2; 15 μM for La-3, Ce-3 and M-Re-3. [a-c] Means without the same letter differ significantly ($p<0.05$), n=8.

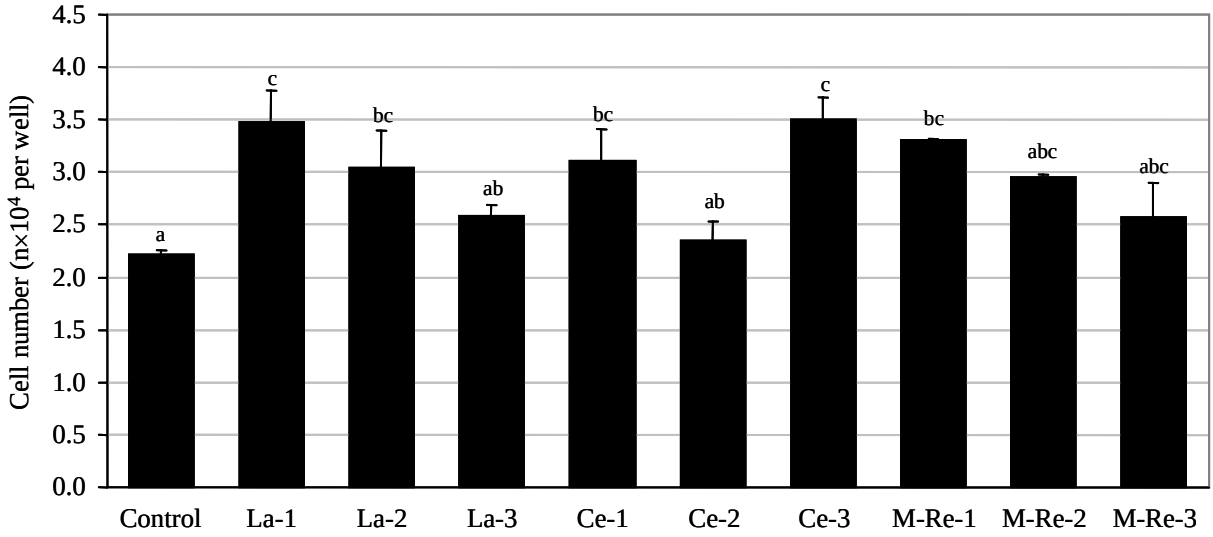

Figure 2. Comparison on the cell number among 3T3-L1 preadipocyte cultures treated in the media containing various rare earth chlorides (Experiment 1). Concentration of rare earth chlorides supplemented to the media was 0 for the control; 5 μM for La-1, Ce-1 and M-Re-1; 10 μM for La-2, Ce-2 and M-Re-2; 15 μM for La-3, Ce-3 and M-Re-3. [a-c] Means without the same letter differ significantly ($p<0.05$), n=4.

media containing $LaCl_3$, $CeCl_3$ or a mixture of lanthanum chlorides at certain low concentrations (5-15 μM) stimulated ($p<0.05$) the proliferation of 3T3-L1 preadipocytes. The supplementation of 5, 10 and 15 μM of $LaCl_3$ to the media significantly increased ($p<0.05$, Figure 1) the relative ratio of absorbance via the XTT assay, which indicated that the proliferation of the cells was improved with supplementation of $LaCl_3$ compared to that of the control. Similar promoting effects ($p<0.05$) on the cell proliferation were found in those treated with media containing 5 μM and 15 μM of $CeCl_3$, 5 μM, 10 μM and 15 μM REE mixture (Figure 2).

The comparison on cell number in the culture treated with or without various REE is shown in Figure 2 (Experiment 1). The cell number was significantly higher ($p<0.05$) for media containing 5 μM or 10 μM $LaCl_3$, 5 μM or 15 μM $CeCl_3$, or 5 μM REE mixture than for that in the control group.

Fatty acid accumulation in the differentiating 3T3-L1 cells

At the end of culture experiment (Experiment 2), it was

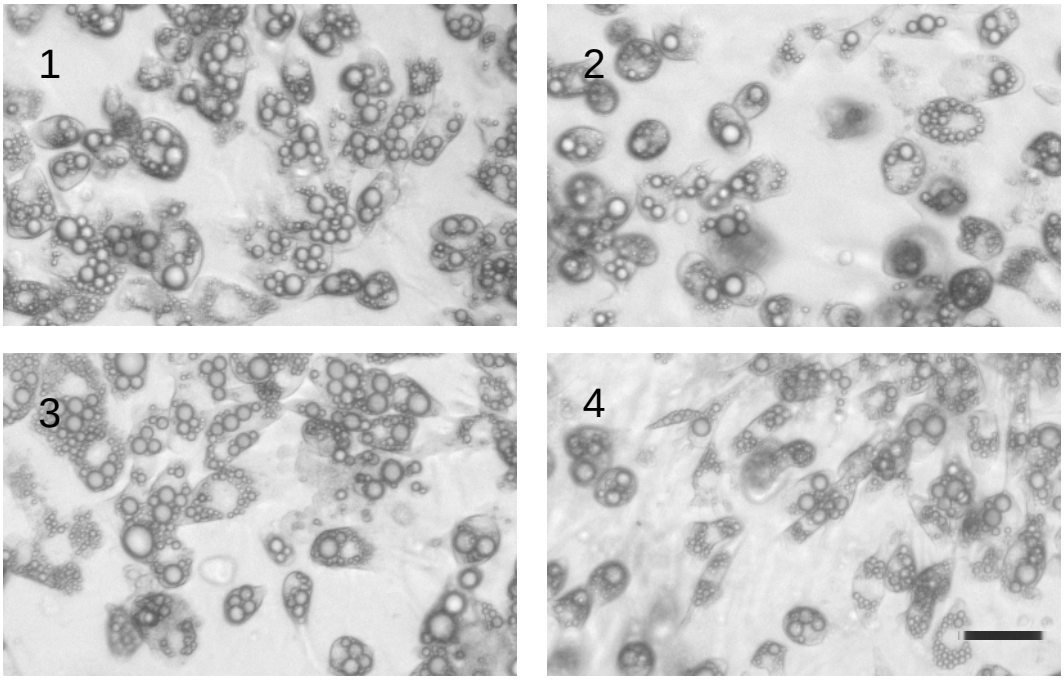

Figure 3. Photos of the 3T3-L1 adipocytes after treated with or without REE for six days (Experiment 2). Photos 1-4: the control, La-1, Ce-1 and M-Re-1, respectively. Bar: 50 μm.

Table 1. Effect of various rare earth chlorides on the fatty acids accumulation of differentiated 3T3-L1 adipocytes (Experiment 2)

Treatment	Control	La-1	La-2	La-3	Ce-1	Ce-2	Ce-3	M-Re-1	M-Re-2	M-Re-3	SEM	p-value
FA (μg/10^5 cells)												
C12:0	0.36	0.33	0.23	0.31	0.24	0.25	0.34	0.22	0.28	0.27	0.03	0.15
C14:0	3.95	3.37	3.08	3.71	2.95	3.36	3.87	3.03	3.32	3.04	0.29	0.30
C14:1	0.86	0.63	0.67	0.76	0.56	0.69	0.82	0.66	0.69	0.59	0.07	0.13
C15:0	4.81 ^{c}	3.24 ^{a}	3.56 ^{ab}	4.19 ^{abc}	3.10 ^{a}	3.38 ^{a}	4.64 ^{bc}	3.33 ^{a}	3.76 ^{abc}	3.26 ^{a}	0.34	0.02
C16:0	39.90	32.30	30.60	35.80	29.30	34.10	37.50	30.50	32.80	29.70	2.40	0.12
C16:1	28.60 ^{c}	20.20 ^{a}	21.80 ^{ab}	23.90 ^{abc}	18.20 ^{a}	23.10 ^{abc}	26.40 ^{bc}	21.70 ^{ab}	22.10 ^{ab}	19.00 ^{a}	1.81	0.03
C18:0	3.07	3.28	2.38	3.67	2.52	2.81	3.19	2.45	3.21	2.67	0.30	0.24
C18:1$_{c9}$	8.68	7.23	6.58	8.09	6.11	7.42	8.01	6.36	7.79	6.53	0.56	0.08
C18:1$_{c11}$	2.18 ^{c}	1.82 ^{abc}	1.72 ^{ab}	2.07 ^{bc}	1.56 ^{a}	1.94 ^{abc}	2.17 ^{c}	1.77 ^{ab}	1.95 ^{abc}	1.61 ^{a}	0.12	0.02
C18:2	1.10 ^{c}	0.87 ^{ab}	0.85 ^{ab}	1.05 ^{bc}	0.80 ^{a}	0.97 ^{abc}	1.09 ^{c}	0.98 ^{abc}	0.98 ^{abc}	0.80 ^{a}	0.07	0.02
C20:4	0.81 ^{c}	0.68 ^{abc}	0.66 ^{ab}	0.66 ^{ab}	0.56 ^{a}	0.75 ^{bc}	0.76 ^{bc}	0.57 ^{a}	0.72 ^{bc}	0.65 ^{ab}	0.05	0.02
SFA	52.10	42.50	39.90	47.60	38.10	43.90	49.50	39.50	43.30	39.00	3.26	0.12
MUFA	40.30 ^{d}	29.90 ^{abc}	30.70 ^{abc}	34.80 ^{bcd}	26.40 ^{a}	33.20 ^{abcd}	37.40 ^{cd}	30.50 ^{abc}	32.50 ^{abcd}	27.80 ^{ab}	2.50	0.04
Total FA	94.30	73.90	72.10	84.20	65.90	78.80	88.80	71.50	77.60	68.20	5.81	0.09

Concentration of rare earth chlorides supplemented to the media was 0 for the control; 5 μM for La-1, Ce-1 and M-Re-1; 10 μM for La-2, Ce-2 and M-Re-2; 15 μM for La-3, Ce-3 and M-Re-3. FA: the detected fatty acids. SFA: the detected saturated fatty acids. MUFA: the detected monounsaturated fatty acids. $^{a-d}$ Means within the same raw without the same letter differ significantly ($p<0.05$), n = 7.

shown that neutral lipids accumulated in the cells during the experiment period in Experiment 2 (Figure 3), which was confirmed at the end of culture experiment through using Oil Red-O and Mayer's Hematoxylin staining. The most of preadipocytes were differentiated to the adipocytes after treated with DMEM media containing DMI differentiation factors for 2 days.

The concentrations of cellular fatty acids in differentiating adipocytes incubated in media containing various REE are shown in Table 1. Supplementation of La (5 μM and 10 μM), Ce (5 μM) and the mixture REE (5 μM and 15 μM) decreased ($p<0.05$) the concentration of monounsaturated fatty acids (MUFA). Supplementation of REE also tended ($p = 0.09$) to decrease the concentration of total fatty acids (FA) in the cells, which was mainly due to the decrease in MUFA and SFA. Among the detected fatty acids, palmitic acid (C16:0) was the main saturated fatty acid which ranged from 30-40 μg/10^5 cells, while

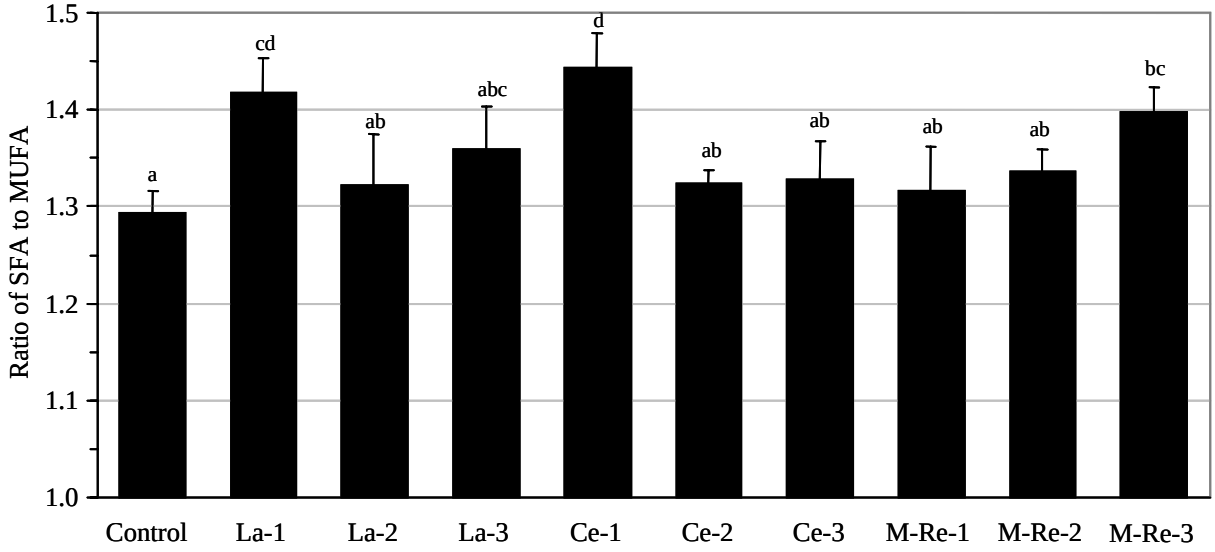

Figure 4. Comparison on the ratio of saturated fatty acids (SFA) to monounsaturated fatty acids (MUFA) in lipids extracted from differentiated 3T3-L1 adipocytes treated in the media containing various rare earth chlorides (Experiment 2). Concentration of rare earth chlorides supplemented to the media was 0 for the control; 5 μM for La-1, Ce-1 and M-Re-1; 10 μM for La-2, Ce-2 and M-Re-2; 15 μM for La-3, Ce-3 and M-Re-3. [a-d] Means without same letter differ significantly ($p<0.05$), n = 7.

palmitoleic acid (C16:1) was the major monounsaturated fatty acid that ranged from 18 to 29 $\mu g/10^5$cells. Supplementation of La (5 and 10 μM), Ce (5 μM) and the mixture REE (5, 10 and 15 μM) decreased ($p<0.05$) the C16:1 fatty acids. A similar effect (or trend effect) of REE was also found in concentration of oleic acid (C18:1) and C16:0.

The ratio of saturated fatty acids (SFA) to monounsaturated fatty acids (MUFA) is shown in Figure 4. Significant higher ($p<0.05$) ratio of SFA to MUFA was observed in the lipids extracted from differentiating 3T3-L1 cells incubated in media containing those treatments of 5 μM of $LaCl_3$, 5 μM of $CeCl_3$, and 15 μM of the REE mixture compared to that of the control.

DISCUSSION

The supplementation with micromoles of REE ions to the media significantly increased the proliferation of 3T3-L1 preadipocytes (Experiment 1), which was consistent with those reported by Smith and Smith (1984) that La ions stimulated the arrested Swiss 3T3 and 3T6 cells in G1/G0 phase to the S phase in the cell cycle. The promoting effects of REE found in the present study were varied, which depend upon the treatment doses and types. The treatment of La with a dose of 10 μM had significant effective while the same amount of Ce had no effect on it. However, the treatment of Ce with both 5 and 15 μM could significantly improve the proliferation of the cells. The effect of REE ions on the cells found in the present study might be achieved through affecting transmembrane fluxes of Ca ions. It was found that calcium formed in the membrane surface during 3T3 cells approach quiescence (Tupper and Zorgniotti, 1977; Smith and Smith, 1984). REE ions had a high affinity for the external surface of cells (Flatt et al., 1980). Ions of La and Ce could take over calcium binding sites or inhibit the uptake of Ca ions in the membrane because of the similar ionic radium among them. With only certain concentration in the media REE ions could effectively displace the calcium in the membrane surface of the quiescent or in proliferating cells and affected the cell cycle. Further study is needed to compare the affinity and accumulation of various REE in the cells when different doses are applied.

Proliferation rate of cells during their growing period can be indicated by the cell number counted on a haemocytometer after dissociated by a trypsin-EDTA solution, or by measuring the activity of DNA synthesis indicated by the incorporation rate of radioactive thymidine to the cells. As a non-isotope method, XTT assay (Scudiero et al., 1988), as well as MTT assay or WST-1 assay (Ishiyama et al., 1993; Brodie et al., 1999) based on tetrazolium salt/formazan system. It has been used for measuring cell proliferation and viability. The formazan formed was used as an indicator for the activity of living cells via mitochondrial dehydrogenases and therefore reflected the number of living cells. Previous studies (He et al., unpublished) also found that there was a strong correlation between the absorbance of cultures measured by using XTT assay and the number of attached 3T3-L1 preadipocytes.

Most of the preadipocytes could differentiate to

adipocytes after treated with differentiation media containing dexamethasone, methyl-isobutylxanthine and insulin for 2-4 days (Weiss et al., 1980). The further incubation with DMEM media containing 10% FBS caused the cell enlargement together with the lipids accumulation. The culture system could be used for testing effect of trance minerals on the differentiation and lipogenesis of adipocytes through supplement to the media (Oh and Choi, 2004). The concentration of cellular fatty acids, especially those de novo synthesized fatty acids, could reflect the degree of differentiation and lipogenesis in differentiated 3T3-L1 adipocytes. The concentration and composition of fatty acids accompanying the lipids accumulation in the cells could be determined by gas chromatography after extraction of lipids by hexane (Satory and Smith, 1999; Mir et al., 2000). The present study found that supplementation of REE to the media resulted in a less accumulation of the main synthesized fatty acids, especially the main monounsaturated fatty acids such as C16:1 and C18:1. It was reported that the concentration of cellular monounsaturated fatty acids were generally increased during adipocyte differentiation, which was due to the increased expression of stearoyl coenzyme A desaturase (SCD) mRNA (Wang et al., 2004). The decrease in the monounsaturated fatty acids reflected an inhibition effect of REE on the differentiation of the cells. Furthermore, the ratio of SFA/MUFA was also significantly increased. The change in the ratio may have effect on the metabolic rate and physiological functions through affecting membrane fluidity of cells (Field et al., 1990).

The REE ions may directly or indirectly affect the lipid metabolism in differentiating adipocytes. Segal (1986) reported that REE can increase cytoplasmic free calcium concentration by enhancing calcium influx and this eventually affected the metabolism of the lipids. The direct and indirect evidences had shown that REE ions were difficult to penetrate the cellular membrane (Langer and Frank, 1972; Szasz et al., 1978; Flatt et al., 1980). However, Pawis et al. (1994) found that REE could be transported by the sodium-calcium exchange pathway and directly triggered hormone release. The lipid metabolism in adipose tissue is mediated by the change of intracellular concentration of adenosine 3', 5' cyclic monophosphate (cAMP) which is known as a second messenger produced from adenosine triphosphate (ATP) by adenylate cyclase after the cells were activated (Waterman et al., 1985). During the differentiation of 3T3-L1 preadipocytes to adipocytes the synthesis of lipogenic enzyme could be affected by cAMP-mediated control (Spiegelman and Green, 1981). In adipose tissue acetyl-CoA carboxylase was phosphorylated and deactivated when exposed to epinephrine or cAMP (Lee and Kim, 1979). Yajima et al. (1994) found that REE ions could catalyze the formation of cAMP from ATP under physiological conditions. It is suggested that the effect of REE on the fatty acid synthesis in differentiating 3T3-L1 cells may be achieved through affecting the concentration of intracellular cAMP. On the other hand, the variation in cell number among the treatments (Experiment 2) may also affect the value of fatty acids concentration, because the data on fatty acids were based on per 10^5 cells. After being treated with differentiation media containing DMI for 2 days most of the preadipocytes were differentiated to adipocytes but there were still some undifferentiated cells existed in the culture. The supplementation of REE in the media may stimulate the proliferation of those quiescent undifferentiated cells (Smith and Smith, 1984). A study through using human preadipocytes found that the proliferation and differentiation could occur simultaneously on the same plate (McNeel et al., 2003).

In conclusion, the supplementation of $LaCl_3$, $CeCl_3$ or a mixture of lanthanum chlorides to the media significantly stimulated proliferation of 3T3-L1 cells. The supplementation of REE also decreased the concentration and composition of monounsaturated fatty acids in the differentiating cells. These results indicate that the supplementation of REE to the media may affect adipogenesis and lipogenesis rates of 3T3-L1 cells. However, the effect may depend upon the dose or type of REE applied.

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