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Effect of β-Carotene on Differential Gene Expression of *BCO2* and Related Genes During Bovine Adipocytes Differentiation

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Abstract: The effects of β-carotene on differentiation of Bovine Intramuscular Preadipocytes (BIP) and Subcutaneous Preadipocytes (BSP) via *BCO2* and related genes were studied. After treatment of cells with 0, 10, 20, 30, 50 and 80 μM β-carotene, cellular lipid content was assessed by Oil Red O staining and a Triglyceride (TG) assay. The relative expression of β-Carotene-15, 15'-Momoxygenase 1 (BCMO1), β-Carotene-9', 10'-momoxygenase2 (BCO2), Peroxisome Proliferator-Activated Receptor-γ (PPARγ) and Retinoic acid X Receptor α (RXRα) were also investigated. Results showed that β-carotene can suppress adipocytes differentiation as a whole. And different concentration β-carotene addition to BSP had not obvious variation of BCO2, PPARγ and RXRα while BIP of same treatment had a highest expression of 30 μM β-carotene at 5 h which can be showed through the results of both Oil Red O staining of cells and fluorescence RT-qPCR. Besides, researchers found that BCMO1 did not nearly express in adipocytes of bovine but BCO2 had a good expression. The present study provides further evidence of the effects of β-carotene on lipid metabolism and suggests that BCO2 may play an important role in the development of bovine adipose tissue *in vivo*.

Key words: β-carotene, β-carotene-15, 15'-momoxygenase1, β-carotene-9', 10'-momoxygenase2, peroxisome proliferator-activated receptor-γ, retinoic acid X receptor α, bovine adipocytes

INTRODUCTION

Intramuscular fat and backfat thickness are very important production traits of beef that related with fat deposition. Fat deposition is the result of proliferation, differentiation and apoptosis of preadipocytes also the result of selective expression of adipogenic genes in different time and space (Liu *et al*., 2009). Therefore, an understanding of mechanisms regulating fat deposition and metabolism in bovine is essential as the adipocyte is of great importance in overall metabolic regulation (Morrison and Farmer, 2000).

In mammals, β-carotene is the natural precursor for vitamin A and its derivatives. Two different types of β-carotene metabolizing enzymes have been identified to express in various tissues of many species (Kiefer *et al*., 2001; Berry and Noy, 2009; Redmond *et al*., 2001). BCMO1 converts β-carotene to all-trans-retinal, development of a knock out model and identification of a loss-of function mutation have pointed out BCMO1 as being probably the sole enzyme responsible for provitamin A conversion into retinal (Redmond *et al*., 2001). Besides BCMO1, mammalian genomes encode a second β-carotene metabolizing enzyme known as BCO2 which cleaves carotenoids at position 9, 10 resulting in the formation of one molecule β-ionone and one molecule β-10'-apocarotena (Kiefer *et al*., 2001).

Some observations suggest a relationship between β-carotene/retinoid and lipid metabolism through studies investigating the effect of retinoids on body fat content. Increasing evidence has been provided that β-carotene-derived apocarotenoid signaling molecule can influence adipocyte physiology (Amengual *et al*., 2011). Retinoids such as retinoic acid and retinaldehyde are ligands for nuclear receptors including RXRγ and PPARγ that help to control adipogenesis and adipocyte metabolism. Treatment with retinoic acid has been shown to inhibit differentiation of preadipocytes in culture by blocking PPARγ induction in an RXR-mediated manner (Schwarz *et al*., 1997; Kamei *et al*., 1994) and to result *in vivo* in mice in reduced PPARγ expression levels in WAT and reduced whole body adiposity (Berry and Noy, 2009; Paik *et al*., 2001). PPARγ controls the expression of genes for lipid and glucose metabolism (Feige *et al*., 2006; Grimaldi, 2007) and PPARγ is pivotal for adipocyte differentiation and lipogenesis in mature

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adipocytes (Lefterova and Lazar, 2009). Part of these effects are mediated via RXRα (Kuri-Harcuch, 1982). These findings implicate that a tissue-specific conversion of β-carotene via β-carotene-oxygenases can influence the activities of key transcription factor that control adipocyte physiology (Amengual *et al*., 2011). In brief, β-carotene-derived retinoids influence adipocyte physiology.

Though one study analyzed the effects of β-carotene on cell proliferation and the expression of BCMO1 during mice preadipocytes differentiation (Hessel *et al*., 2007), the underlying mechanism on lipid metabolism in bovine preadipocytes is not yet fully understood. Therefore, Luxi adult yellow steers were used to isolate and culture preadipocyte *in vitro* and investigate differentiation in bovine adipocytes in the presence of β-carotene for improving the beef quality based on local breed beef cattle.

MATERIALS AND METHODS

Cell culture and induction of differentiation of preadipocytes: To investigate the induction of preadipocyte differentiation, cells from subcutaneous and intramuscular adipose tissues of Luxi yellow steers were isolated and cultured. In brief, the cells were propagated and maintained in DMEM/F12 (Hyclone, Logan, UT, USA) containing 10% FBS (Gibco, Grand Island, NY, USA) with an antibiotic-antimycotic agent (containing 100 IU mL^{-1} penicillin and 100 μg mL^{-1} streptomycin; Gibco). The cells were incubated at 37°C under a humidified 5% CO_2 atmosphere. To induce adipogenesis of bovine pre-adipocytes, 2 days post-confluent preadipocytes (designated day 0) were incubated in differentiation-induction medium with DMEM/F12 containing 10 μg mL^{-1} insulin (Sigma, St. Louis, MO, USA), 0.25 μM DEX (Sigma) and 0.5 mM IBMX (Sigma) for 48 h (Liu *et al*., 2009). Then, change the basal culture medium added 0, 10, 20, 30, 50 and 80 μM β-carotene. Cells were cultured for 0, 5, 10 and 15 h prior to mRNA extraction.

Addition of β-carotene to medium of cell culture: The 0.8 pmol of β-carotene in hexane and 8 pmol of α-tocopherol in ethanol were introduced in a glass tube, solvents were evaporated and the residue dissolved in 0.5 mL of THF. The THF solution was then rapidly injected into 50 mL of DMEM/F12. For controls the same procedures described above were followed except β-carotene was not added.

Oil red O staining and extraction: Cells were seeded into 6 well culture plates at a density of $5\times10^4/cm^2$ to examine lipid accumulation. After 48 h, the medium was removed and the cells were washed 3 times with Phosphate-Buffered Saline (PBS) and fixed with 10% formaldehyde for 30 min at room temperature. After 3 washes with PBS, the cells were stained for at least 1 h with 1% filtered Oil Red O (6:4 Oil Red O stock solution-H_2O where Oil Red O stock solution comprises 0.5% Oil Red O in isopropyl alcohol). Finally, the cell morphology was examined and photographed under a light microscope.

At 48 h, the intracytoplasmic lipid contents were determined. The steps for Oil Red O extraction were similar to those for Oil Red O staining. The cells were washed 3 times with PBS and fixed with 10% formaldehyde for 30 min at room temperature. After 3 washes with PBS, the cells were stained with 1% filtered Oil Red O for 40 min at room temperature. Subsequently, the Oil Red O solution was removed no additional washes were required. Intracellular triglycerides were extracted by agitation with 100% isopropanol solution for 15 min on a shaker. Absorbance at 500 nm was measured on a biochemical analyzer.

RNA extraction and fluorescence RT- qPCR: Total RNA was isolated from fat tissue and cells using Trizol reagent (Invitrogen, Carlsbad, CA, USA). For each sample, 1 μg of total RNA was used for first-strand cDNA synthesis according to the previously published procedures (Liu *et al*., 2009).

Fluorescence RT-qPCR primers were designed and synthesized (BIOSUNE, Shanghai, China) to assay differentially expressed genes (Table 1). The *β-actin* gene was used as the house keeping gene. Fluorescence qPCR was performed using the LightCycler 480 II System (Switzerland) in a final volume of 20 μL containing SYBR Green II (TAKARA). The cycling conditions for qPCR were as follows: stage 1 of 95°C for 30 sec, stage 2 of 35 cycles of 95°C for 5 sec and 60°C for 20 sec, stage 3 of 95°C for 0 sec, 65°C for 15 sec and 95°C for 0 sec. The data were analyzed and the ΔΔCt values were obtained using the LC 480 System SDS Software. The relative gene expression levels were determined using the $2^{-\Delta\Delta C}{}_T$ Method. The gene expression levels of the BIPs on day 0 were set to 1 to compare the relative levels of gene expression in the experimental groups. The primer sequences are shown in Table 1.

Table 1: Primer sequences of genes selected for analysis by real-time qRT-PCR

Genes	Primers	bp	Temp. (°C)
BCO2	F: 5' TCTTCTATGGCTGTGGCTTCC 3'	153	57.0
	R:5' CATCATCTTCTTTACTGGCTCCTG 3'		
RXRα	F: 5' CCTCAATGGTGTCCTCAAAGTG 3'	200	57.0
	R: 5' TCAGGCAGTCCTTGTTGTCC 3'		
PPARγ	F: 5' CACCACCGTTGACTTCTCCAG 3'	158	57.5
	R: 5' GATACAGGCTCCACTTTGATTGC 3'		
β-actin	F: 5' TGACCCAGATCATGTTTGAGA 3'	256	57.0
	R: 5' CAAGGTCCAGACGCAGGAT 3'		

Statistical analysis: All data were analyzed by one-way Analysis of Variance (ANOVA) using the SPSS 19.0 Software. If the one-way ANOVA results were significant, the Duncan's multiple-range test was performed for multiple comparisons with $p<0.05$ and $p<0.01$ accepted as statistically significant and highly significant respectively.

RESULTS AND DISCUSSION

Effect of β-carotene on differentiation of bovine preadipocytes: Bovine preadipocytes were treated with 0, 30 or 50 μM β-carotene. After 48 h, the cells were harvested and differentiation was confirmed by Oil Red O staining of cells and measurement of TG accumulation. Researchers can see clearly that lipid reduced obviously after β-carotene was added. And 30 μM of BIP produced >50 μM which can not been seen in BSP (Fig. 1 and Table 2).

β-carotene results in a general regulation of gene expression in adipose tissue

gene expression with increasing of incubation time after adding β-carotene: After adding β-carotene into the medium, the expression of BCO2, PPARγ and RXRα reduced immediately. Then the amplitude of variation was not obvious along with increasing of incubation time (Fig. 2).

The result of BCO2 expression in BIP and BSP: BCO2 expressed in both BSP and BIP and more obviously in BIP ($p<0.05$). At 0 h, different concentration has not obvious variation (Fig. 3a). Till 5 h this gene expression reduced as a whole but had not obvious variation among the concentration of BSP while BIP with 30 μM β-carotene was much higher than others (Fig. 3b). At 10 h, BCO2 had a slightly higher and higher expression with increasing concentration of β-carotene until 20 μM following expression gradual lower. And BIP had a slightly higher expression at the concentration of 30 μM (Fig. 3c). At 15 h, BIP had a much lower expression at 20 and 50 μM while still having not obvious variation among the concentration of BSP (Fig. 3d).

The result of PPARγ expression in BIP and BSP: PPARγ expressed in both BSP and BIP and more obviously in BSP ($p<0.05$). At 0 h, different concentration has not obvious variation (Fig. 4a). Till 5 h this gene expression reduced as a whole but had not obvious variation among the concentration of BSP while BIP with 30 μM β-carotene was much higher than others (Fig. 4b). At 10 h there was not obvious variation among

Table 2: Bovine preadipocytes by Oil Red O extraction

Cells	Concentrate	OD values
BSP	0 μM	0.4051 ± 0.0035^{a}
	10 μM	0.2775 ± 0.0054^{b}
	30 μM	0.2713 ± 0.0017^{b}
	50 μM	0.2824 ± 0.0034^{b}
BIP	0 μM	0.3990 ± 0.0051^{a}
	10 μM	0.2153 ± 0.0007^{c}
	30 μM	0.2936 ± 0.0042^{b}
	50 μM	0.2190 ± 0.0017^{c}

OD value = Mean±SE; experiments were repeated 3 times; $^{a-c}$ Values in the same row with different superscripts differ significantly ($p<0.05$)

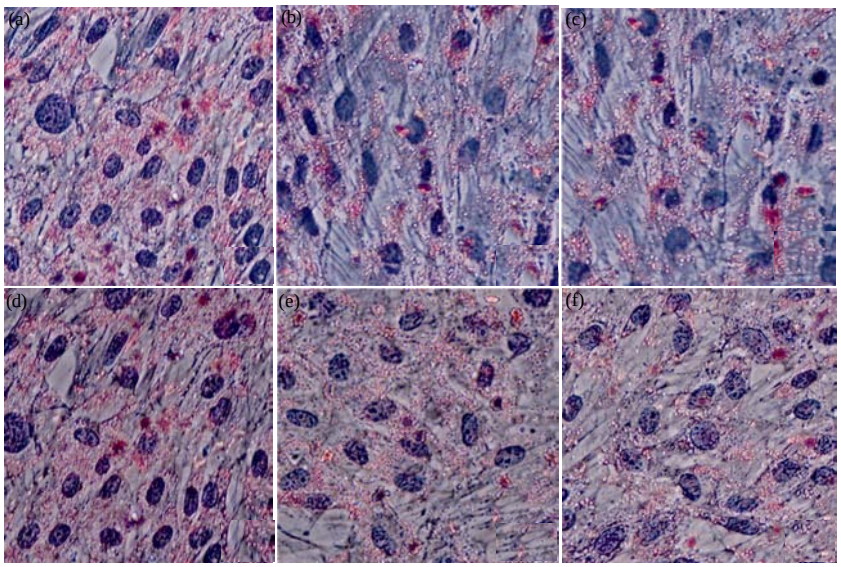

Fig. 1: The result of Oil Red O staining of cells after 0, 30 and 50 μM β-carotene concentration treatment to BSP and BIP (x200), a-c) shows the result of BIP after treatment of 0, 30, 50 μM β-carotene addition, respectively and d-f) shows the result of BSP after treatment of 0, 30 and 50 RM β-carotene addition, respectively

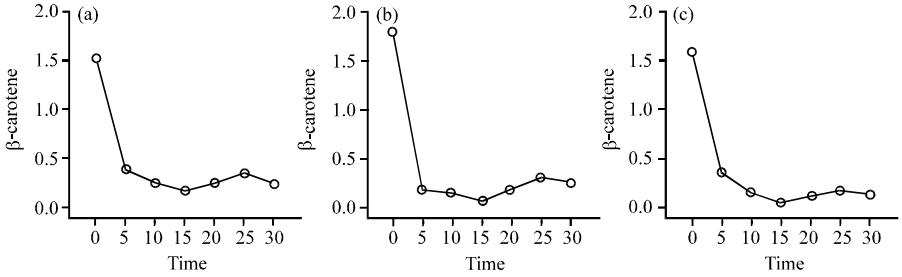

Fig. 2: a-c) Show BCO2, PPAR, RXR expression with increasing of incubation time after adding β-carotene, respectively

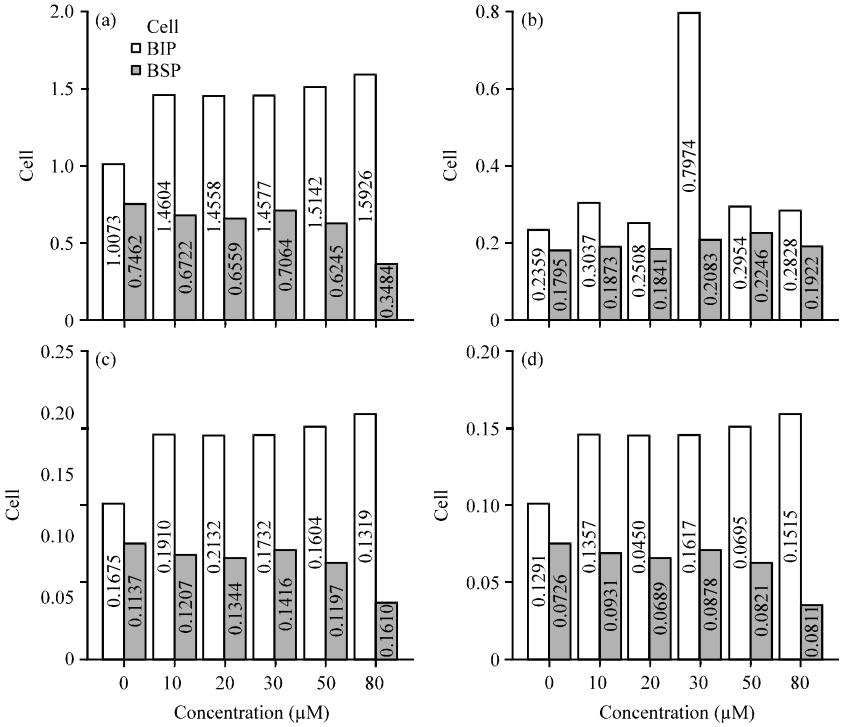

Fig. 3: Effect of β-carotene on the expression of BCO2; a-d) shows the results of 0, 5, 10 and 15 h, respectively

the concentration of BSP and BIP (Fig. 4c). At 15 h, BIP had a much lower expression at 20 and 50 μM while having a slightly higher and higher expression with increasing concentration of β-carotene except 20 μM in BSP (Fig. 4d).

The result of RXRα expression in BIP and BSP: RXRα expressed in both BSP and BIP and more obviously in BIP ($p<0.05$). At 0 h, different concentration has not obvious variation (Fig. 5a). Till 5 h this gene expression reduced as a whole but had not obvious variation among the concentration of BSP while BIP with 30 μM β-carotene was much higher than others (Fig. 5b). At 10 h there was a highest expression at 20 μM following gradual lower (Fig. 5c). At 15 h, BIP had a much lower expression at 20 and 50 μM while having a slightly higher and higher expression with increasing concentration of β-carotene, while expression of 30, 50, 80 μM was obvious higher than that of 10 and 20 μM in BSP (Fig. 5d).

From the above there was not obvious variation among different concentration β-carotene addition to BSP while BIP had a better differentiation of 30 μM β-carotene addition than the others. Besides, the best time was 5 h.

In mammals, β-carotene is the natural precursor for vitamin A and its derivatives. Two different types of β-carotene metabolizing enzymes of BCMO1 and BCO2 have been identified to express in various tissues of some animals. And BCMO1 have been pointed out as being probably the sole enzyme responsible for provitamin A conversion into retinal (Redmond *et al*., 2001). But the

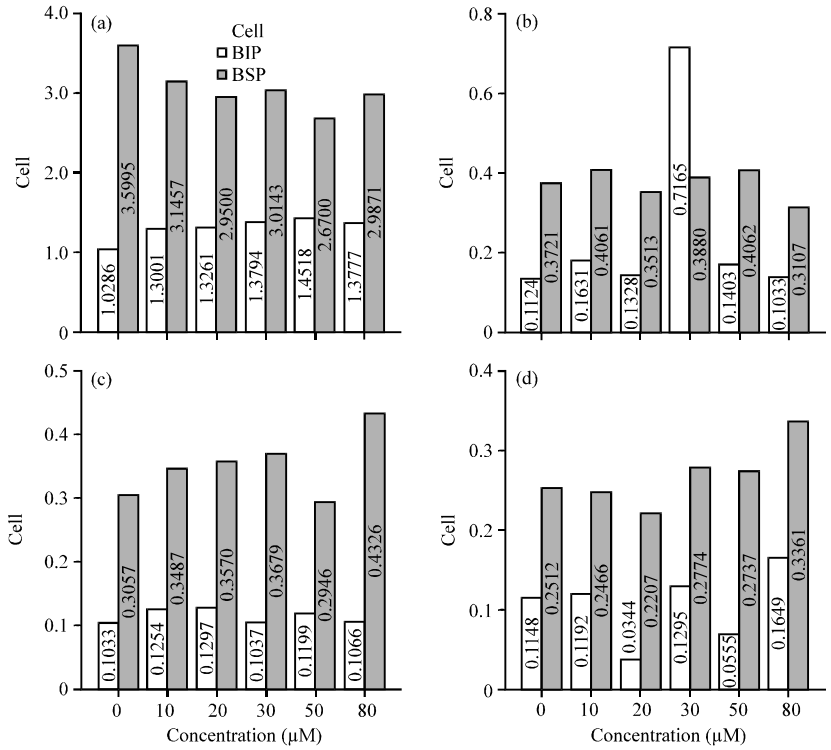

Fig. 4: Effect of β-carotene on the expression of PPAR; a-d) shows the results of 0, 5, 10 and 15 h, respectively

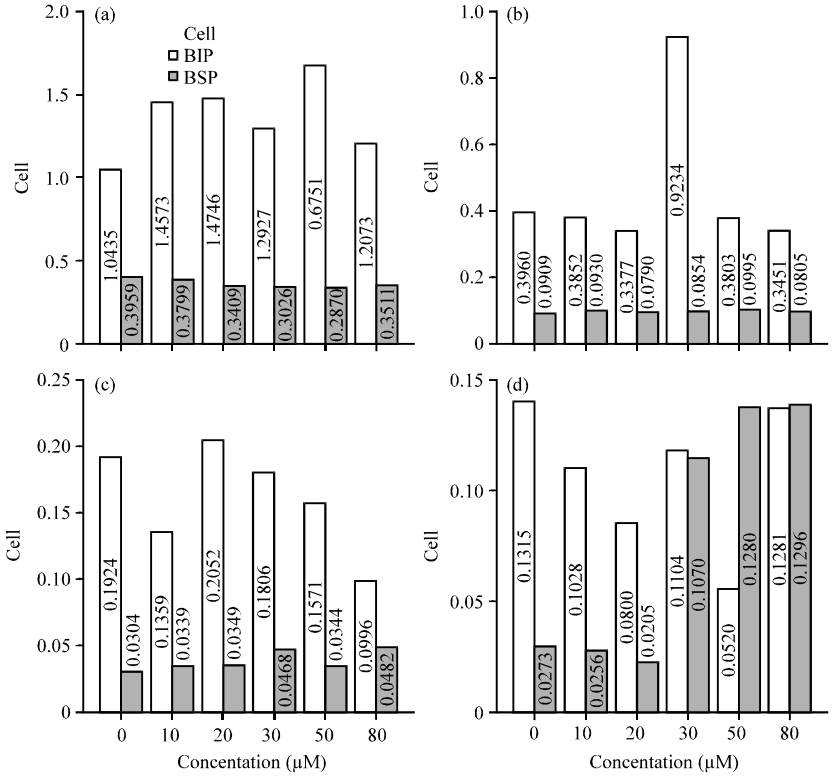

Fig. 5: Effect of β-carotene on the expression of RXR; a-d) shows the results of 0, 5, 10 and 15 h, respectively

result had a very difference with these studies. Researchers found that BCMO1 did not nearly express in adipocytes of boving but BCO2 had a good expression. So, researchers forecasted preliminarily BCO2 being probably the sole enzyme responsible for provitamin A conversion into retinal in adipocytes of bovine. At least, researchers can see that BCO2 plays an important role in the differentiation of adipocytes *in vivo*. However, this result requires researchers to do more research to confirm.

Preadipocytes can differentiate to adipocytes. This differentiation is triggered by nutritional and hormonal signals that activate a cascade of transcription factors including the CCAAT/Enhancer Binding Proteins (C/EBPs) and PPARγ, the latter being considered as the master regulator of adipogenesis. PPARγ control the expression of genes for lipid and glucose metabolism (Feige *et al*., 2006; Grimaldi, 2007) and PPARγ is pivotal for adipocyte differentiation and lipogenesis in mature adipocytes (Lefterova and Lazar, 2009). The primary β-carotene cleavage product retinaldehyde has been shown to inhibit PPARγ activity both in adipocyte cell cultures and mouse models (Ziouzenkova *et al*., 2007a, b). Part of these effects are mediated via RXRα which upon retinoic acid binding regulate the expression of direct target genes and interfere with the activity of other transcription factors including early adipogenic transcription factors such as PPARγ (Kuri-Harcuch, 1982). In addition, retinoic acid may influence PPAR-mediated responses by activating RXR moiety of permissive PPAR:RXR heterodimers and possibly by serving as an agonist of PPAR (Berry and Noy, 2009). Finally, β-derived long chain apocarotenoids such asb-apo-14-carotenal can inhibit PPARγ activity and adipogenesis in cell culture (Ziouzenkova *et al*., 2007a, b). These findings implicate that a tissue-specific conversion of β-carotene via β-carotene-oxygenases can influence the activities of key transcription factor that control adipocyte physiology (Amengual *et al*., 2011). Researchers found that the expression of PPARγ and RXRα reduced obviously as soon as β-carotene was added though Oil Red O staining of cells, measurement of TG accumulation and Fluorescence RT-qPCR. And this two results were very consistent. In this guard, researchers can certify that β-carotene can inhibit PPARγ activity.

Furthermore, researchers can see from the earlier results, the addition of β-carotene had obvious inhibition to both BSP and BIP. However, BSP and BIP had a greatly variance. There were not obvious variation among the concentration of BSP at any timing but BIP had a better differentiation of 30 μM β-carotene addition than the others. Besides, the best time was 5 h. This result can been seen from Oil Red O staining of cells, measurement of TG accumulation and Fluorescence RT-qPCR.

Intramuscular fat and backfat thickness are very important production traits of beef that related with fat deposition. Fat deposition is the result of proliferation, differentiation and apoptosis of pre-adipocytes also the result of selective expression of adipogenic genes in different time and spacel. The proliferation and differentiation of preadipocytes is a dynamic and plastic process under the influence of various hormones, genes and signaling pathway (Liu *et al*., 2009). Adipose tissue is highly plastic. It has an enormous capacity to expand through hypertrophy and hyperplasia of adipocytes (Farmer, 2006).

CONCLUSION

In the present study, BCMO1 did not nearly express in bovine adipocytes but BCO2 had a good expression. The present study provides further evidence of the effects of β-carotene on lipid metabolism and suggests that BCO2 may play an important role in the development of bovine adipose tissue *in vivo*. Researchers judged preliminarily through this study that researchers can use earlier result of 30 μM β-carotene of 5 h till 10 h to improve the beef quality. But this conclusion requires us to do more research to confirm.

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REFERENCES

Amengual, J., E. Gouranton, Y.G.J. van Helden, S. Hessel, J. Ribot *et al*., 2011. β-carotene reduces body adiposity of mice via BCMO1. PLoS ONE, Vol. 6. 10.1371/journal.pone.0020644

Berry, D.C. and N. Noy, 2009. All-*trans*-retinoic acid represses obesity and insulin resistance by activating both peroxisome proliferation-activated receptor β/σ and retinoic acid receptor. Mol. Cell Biol., 29: 3286-3296.

Farmer, S.R., 2006. Transcriptional control of adipocyte formation. Cell Metab., 4: 263-273.

Feige, J.N., L. Gelman, L. Michalik, B. Desvergne and W. Wahli, 2006. From molecular action to physiological outputs: Peroxisome proliferator-activated receptors are nuclear receptors at the crossroads of key cellular functions. Prog. Lipid Res., 45: 120-159.

Grimaldi, P.A., 2007. Peroxisome proliferator-activated receptors as sensors of fatty acids and derivatives. Cell. Mol. Life Sci., 64: 2459-2464.

Hessel, S., A. Eichinger, A. Isken, J. Amengual and S. Hunzelmann *et al*., 2007. CMO1 deficiency abolishes vitamin A production from β-carotene and alters lipid metabolism in mice. J. Biol. Chem., 282: 33553-33561.

Kamei, Y., T. Kawada, J. Mizukami and E. Sugimoto, 1994. The prevention of adipose differentiation of 3T3-L1 cells caused by retinoic acid is elicited through retinoic acid receptor α. Life Sci., 55: PL307-PL312.

Kiefer, C., S. Hessel, J.M. Lampert, K. Vogt and M.O. Lederer *et al*., 2001. Identification and characterization of a mammalian enzyme catalyzing the asymmetric oxidative cleavage of provitamin A. J. Biol. Chem., 276: 14110-14116.

Kuri-Harcuch, W., 1982. Differentiation of 3T3-F442A cells into adipocytes is inhibited by retinoic acid. Differentiation, 23: 164-169.

Lefterova, M.I. and M.A. Lazar, 2009. New developments in adipogenesis. Trends Endocrinol. Metab., 20: 107-114.

Liu, X.M., J.L. Fu, E.L. Song, K. Zang, F.C. Wan, N.K. Wu and A.G. Wang, 2009. Effect of nicotinamide on proliferation, differentiation and energy metabolism in bovine preadipocytes. Asian-Aust. J. Anim. Sci., 22: 1320-1327.

Morrison, R.F. and S.R. Farmer, 2000. Hormonal signaling and transcriptional control of adipocyte differentiation. J. Nutr., 130: 3116S-3121S.

Paik, J., A. During, E.H. Harrison, C.L. Mendelsohn, K. Lai and W.S. Blaner, 2001. Expression and characterization of a murine enzyme able to cleave β-carotene: the formation of retinoids. J. Biol. Chem., 276: 32160-32168.

Redmond, T.M., S. Gentleman, T. Duncan, S. Yu and B. Wiggert *et al*., 2001. Identification, expression and substrate specificity of a mammalian β-carotene 15,15'-dioxygenase. J. Biol. Chem., 276: 6560-6565.

Schwarz, E.J., M.J. Reginato, D. Shao, S.L. Krakow and M.A. Lazar, 1997. Retinoic acid blocks adipogenesis by inhibiting C/EBPβ-mediated transcription. Mol. Cell. Biol., 17: 1552-1561.

Ziouzenkova, O., G. Orasanu, G. Sukhova, E. Lau and J.P. Berger *et al*., 2007a. Asymmetric cleavage of β-carotene yields a transcriptional repressor of retinoid X receptor and peroxisome proliferator-activated receptor responses. Mol. Endocrinol., 21: 77-88.

Ziouzenkova, O., G. Orasanu, M. Sharlach, T.E. Akiyama and J.P. Berger *et al*., 2007b. Retinaldehyde represses adipogenesis and diet-induced obesity. Nat. Med., 13: 695-702.