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Serum Lipids Can Convert Bovine Myogenic Satellite Cells to Adipocytes

Jagadish Beloor, Hye Kyeong Kang and Yang Soo Moon*
Department of Animal Science and Biotechnology, Jinju National University,
150 Chilam-dong, Jinju 660-758, Korea

ABSTRACT : Serum lipid (SL) is a commercially available cholesterol-rich, proteinaceous compound extracted from bovine serum. Here we investigated the adipogenic transdifferentiation potential of SL on bovine myogenic satellite cells. Exposure of satellite cells to SL could generate lipid droplets on day 2, and further exposure to SL increased cytoplasmic lipid accumulation giving adipocyte morphology. The expression analysis of PPAR gamma and GPDH adipocyte markers along with Oil-red-O staining results confirmed the transdifferentiation potential of SL. When cells were treated at different concentrations (5, 10, 20, 40 μl/ml) of SL, the results indicated that even levels as low as 5 μl SL /ml could induce transdifferentiation, and maximum induction was obtained at 20 μl SL/ml. After treatment with SL at different concentrations the expression levels of PPAR gamma varied significantly ($p<0.05$), whereas the expression of other adipogenic transcription factors showed no difference, indicating that SL acts through PPAR gamma. The combined effect of SL and troglitazone proved to be the best combination for induction of transdifferentiation compared to the individual effect of SL or troglitazone. Thus, overall results clearly show that SL induces transdifferentiation of bovine myogenic satellite cells to adipocytes. (**Key Words :** Transdifferentiation, Bovine Satellite Cells, Serum Lipids, Myocytes, Adipocytes)

INTRODUCTION

Transdifferentiation is an irreversible switch in postnatal life of one type of already differentiated cell to another type of normal differentiated cell (Tosh and Slack, 2002). Myoblasts and adipocytes arise from same germ layer of the embryo, the mesoderm. Therefore, Li et al. (2005) hypothesized that it is possible to transdifferentiate myoblast cells to adipocytes. Interest in adipocytes located in muscle has developed because of the demand for increased marbling fat and meat quality (Gondret and Lebret, 2002; Van Barneveld, 2003). It has been observed that several methods including rising of intramuscular fat through feed is not economical (Novakofski, 2004). But, recent advances in understanding of transcriptional basis of adipogenesis have allowed meat scientists to directly examine the ability of myogenic cells to enter the adipogenic pathway (Seale et al., 2000). Transcription factors like PPAR gamma and C/EBP alpha (Hu et al., 1995) or the ligands such as thaizolidinediones (TZD) (Singh et al., 2007) or long chain fatty acids (Teboul et al., 1995), which activate these factors, have been shown to induce transdifferentiation in *in vitro* conditions.

Although, the *in vivo* studies in humans and mouse have shown that TZD influences intramuscular fat, several aspects such as the concentration, timing and the type of TZD has to be standardized with respect to different tissue and the species of animal to be treated. Moreover, TZDs are still not approved for their use in meat animals (Hausman et al., 2008). And also, recent study (Ban et al., 2008) has shown that PPAR gamma is insufficient to induce transdifferentiation in mouse under *in vivo* conditions. So these concerns still keep the research for finding the transdifferentiating agent that can be *in vivo* potent and should be feasible for *in vivo* application wide open.

Serum lipid (SL) is a cholesterol rich (9-11 g/L) proteinaceous (15-25 g/L) compound which is obtained from an adult bovine serum. Previously, SL has been shown to have a profound effect on differentiation of bovine subcutaneous and intramuscular preadipocytes (Grant et al., 2008). But, nothing is known about the effect of SL on transdifferentiation of myoblast to adipocyte, so here in our experiments we tested the transdifferentiation potentials of a commercially available compound called serum lipid.

* Corresponding Author: Y. S. Moon. Tel: +82-55-751-3262, Fax: +82-55-751-3267, E-mail: ysmoon@jinju.ac.kr
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MATERIALS AND METHODS

Materials

Dulbecco's Modified Eagle's Medium, fetal bovine serum (FBS) were obtained from HyClone (HyClone Laboratories, Inc., UT, USA); penicillin, streptomycin, amphotericine and trypsin-EDTA were got from GIBCO, NY, USA; pantathenate, caprylic acid, ascorbic acid, biotin, acetic acid, insulin, dexamethasone (DEX), isobutyl methyl xanthine (IBMX) and serum lipids (catalogue # L4646) were obtained from Sigma Aldrich (Saint Louis, MO).

Hind limb skeletal muscle tissues from male Hanwoo cattle were collected from a regional slaughter house. Skeletal muscle samples were collected in air tight plastic bags. The slaughtered cattle were in the age of 24-26 months and with 550-600 kg body weight.

Isolation of bovine satellite cells (BSC) from skeletal muscle

Skeletal muscle was washed in PBS. Then, muscle was minced into fine pieces using sterilized scissors and digested by trypsin-EDTA for 2 h. Digested tissue was centrifuged at 90 g for 3 minutes and upper phase was filtered using 40 μm cell strainer. Filtrate was centrifuged at 2,500 rpm at room temperature for 20 minutes. Digestion media was removed leaving cell pellet in the tube. The collected cell pellet was washed three times by DMEM (Dulbecco's Modified Eagle's Medium) containing 1% penicillin/streptomycin and cultured in 100 mm culture dish using DMEM with 10% FBS, 1% penicillin/streptomycin and 0.1% amphotericine by incubating at 37°C and 5% CO_2. The culture condition was checked every day with change in media every alternate day.

Induction of transdifferentiation of BSC in adipogenic differentiation medium (ADM)

Bovine satellite cells were plated at the density of 2×10^3 cells/cm^2 and grown in proliferation medium containing DMEM, 10% fetal bovine serum and 1% antibiotics. Post confluent cells (d 0) were further cultured in standard ADM containing 17 μM pantathenate, 1 mM caprylic acid, 200 μM ascorbic acid, 33 μM biotin, 10 mM acetic acid, 10 ng insulin, 0.25 μM DEX, 0.5 mM IBMX in DMEM with 10% FBS, antibiotics along with different concentrations of SL for only 2 days. Then the cells were cultured in adipogenic proliferation medium containing 17 μM pantathenate, 1 mM caprylic acid, 200 μM ascorbic acid, 33 μM biotin, 10 mM acetic acid, 10 ng insulin, in DMEM with 10% FBS, antibiotics along with SL for next 8 days with medium changed on alternate days.

Oil-red-O staining of transdifferentiated cells

Cytoplasmic lipid droplets were stained with Oil-red-O. Briefly, Oil-red-O (Sigma) stock solution was prepared by mixing 5 mg/ml of Oil-red-O in 60% tri-ethyl phosphate (TEP, Sigma). Oil-red-O stock solution was diluted into working Oil-red-O solutions (12:8 Oil-red-O/TEP: de-ionized water). The diluted working solution was filtered by Whatman paper (Whatman, Schleicher & Schuell, Whatman International Ltd., Maidstone, England) before use. The cells cultured in 6-well plates were fixed by 10% formalin for 10 minutes after removing the culture media. Then, the formalin fixed cells were washed three times by de-ionized water. The working solutions of Oil-red-O (1 ml/well) were added in the wells. Fifteen minutes later, the staining solution was removed and washed the wells with de-ionized water. After air drying, wells were mounted with de-ionized water and observed under the microscope.

Total RNA extraction

Trizol reagent (Invitrogen, Carlsbad, CA, USA) was used to extract total RNA from cultured cells, according to the standard manufacturer's protocol, and RNA was stored in DEPC-treated water at -80°C until further use. Concentrations of the extracted RNA samples were measured using an ND-1,000 spectrophotometer (NanoDrop Technologies, Wilmington DE, USA).

Semi-quantitative RT-PCR analysis

Semi-quantitative RT-PCR was performed as previously described (Hong et al., 2006) to measure the levels of various adipogenic and myogenic gene expressions. RNA was reverse-transcribed into the first strand cDNA using Superscript-II reverse transcriptase (Invitrogen). Total RNA (1.5 μg in 20 μl total volume) was primed with oligo $(dT)_{20}$ primers (Bioneer Co., Daejeon, Korea), and reverse transcription was carried out to obtain cDNA at 42°C for 50 min and 72°C for 15 min. Subsequently, 1 μl of the cDNA product and 10 pmoles of each gene-specific primers (refer Table 1) were used to check the levels of several adipogenic and myogenic genes. PCR products were resolved on a 1.2% gel. The DNA was visualized by ethidium bromide staining and analyzed using gel viewer software and EasyDoc Gel documentation system (EasyDoc, Korea). The mRNA levels were corrected using the transcription level of the beta-actin gene as an internal standard.

Statistical analysis

The treatment at different time intervals was analyzed by ANOVA using the GLM procedure of SAS (SAS Inst. Inc., Cary, NC). Significant differences were detected ($p<0.05$) by Duncan's multiple range test using SAS (SAS Inst. Inc.).

RESULTS

The transdifferentiation potential of serum lipid was

Table 1. Primers used for semi-quantitative reverse transcription polymerase chain reaction (RT-PCR)

Gene	Primer	Sequence(5'-3')	Size	Tm (°C)
SREBP1c	Forward	ATGATGGTGACACTCAGGCTGCAGTGGTG	1.2	64
	Reverse	TTTCTCTCTCAGAGTAGGGTATCGGTAAGG		
C/EBPβ	Forward	ATGCAACGCCTGGTGGTCTGG	1.2 kb	58
	Reverse	GCAGTGGCCGGAGGAGGCGAG		
PPARγ	Forward	ATGGGTGAAACCCTGGGAGAT	1.4 kb	50
	Reverse	ATACAAGTCCTTGTAGATTTC		
GPDH	Forward	ATGACCGGCAAGAAAGTCTGC	300 bp	58
	Reverse	GATCTTGCCGATGAACTGATG		
Myf5	Forward	ATGGACATGATGGACGGCTGC	750 bp	58
	Reverse	TAGCACATGATAGATGAGCCT		
MyoD	Forward	ATGGAGTTGACTGTCGCCGCCG	250 bp	52
	Reverse	GAGCACCTGGTAAATCGGGTT		
Actin	Forward	GCCACGAGGCCCAGAGCAAG	230 bp	52
	Reverse	GGGGCCACACGCAGCTCATT		

tested on BSCs under the adipogenic conditions. In subsequent experiments to validate our results, the expression of adipogenic genes such as PPAR gamma and GPDH and myogenic genes like Myf5 and MyoD were analyzed, and Oil-red-O staining was performed. As shown in Figure 1, the treatment of SL under adipogenic differentiation medium led to conversion of multinucleated bovine satellite myotubes to small round cells containing numerous lipid oil droplets and subsequently forming the adipoblast. Bovine satellite cells, during differentiation in the presence of the adipogenic mixture plus SL started to swell, and acquired morphology

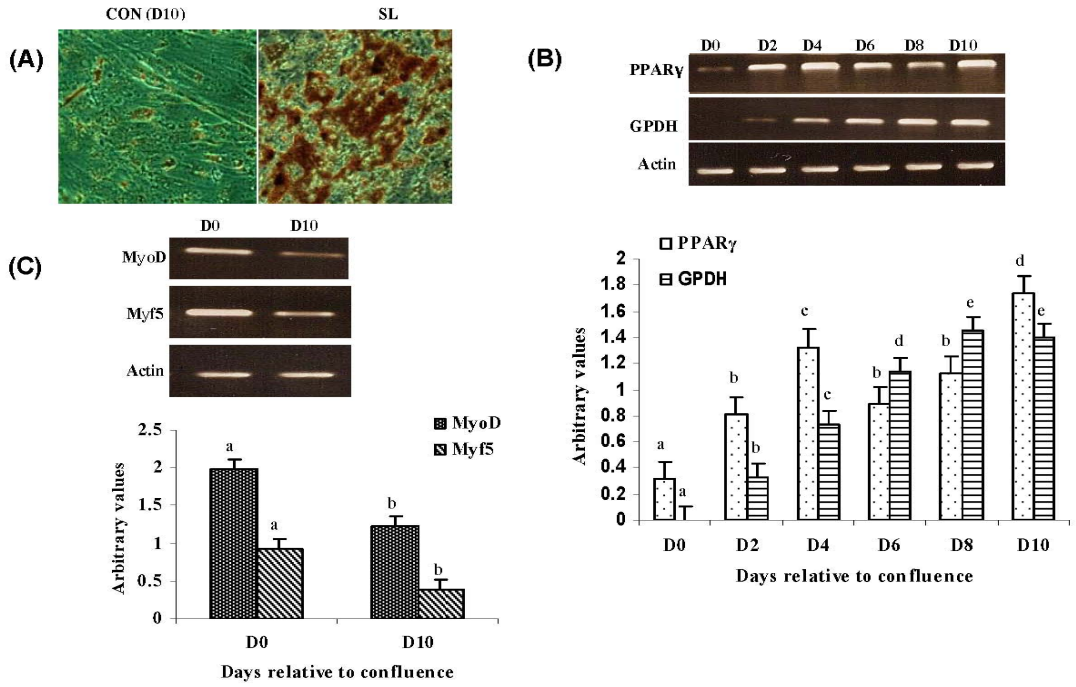


Figure 1. Functional role of serum lipid on transdifferentiation of bovine myogenic satellite cells to adipocytes. (A) The Oil-red-O stained cell pictures on day 10 confirming the transdifferentiation of myocytes to adipocytes after the treatment of serum lipids. (B) Expression of PPAR gamma and GPDH at different intervals of time after the treatment with SL. The ethidium bromide-stained agarose gel shows the amplification products of PPAR gamma, GPDH and actin. Actin was used as the internal control. The gel shows representative RT-PCR results of three separate experiments with the same protocol. The data represent the means±SEM of the three experiments. a-e, Mean values with different superscripts are significantly different * $p<0.05$. (C) Expression of myogenic genes, MyoD and Myf5 after the SL treatment on day 0 and day 10. Actin was used as the internal control. The gel shows representative RT-PCR results of three separate experiments with the same protocol. The data represent the means±SEM of the three experiments. a-b, Mean values with different superscripts are significantly different * $p<0.001$.

similar to adipocytes. The cells, which assumed a large, round shape from day 2 itself (data not shown) of the treatment, stained intensely red with Oil-red-O on day10 confirming these cells as differentiated adipocytes (Figure 1A). Further more, the expression of adipogenic genes (Figure 1B) during the transdifferentiation program was found to have increasing trend ($p<0.05$), while the expression of myogenic genes (Figure 1C) were found to be significantly reduced ($p<0.001$) with the exposure to SL.

There was no data available regarding the minimum and optimum concentration of serum lipid required for the induction of transdifferentiation of bovine satellite cells to adipocytes. So, bovine satellite cells were treated with different concentrations (5, 10, 20 and 40 µl/ml of adipogenic differentiation medium). The results in Figure 2A and B show that, as low as 5 µl/ml of serum lipid can induce the transdifferentiation, and 20 µl/ml being the optimum concentration induces maximum amount of oil droplets and the expression of GPDH gene. When cells were treated with higher concentrations (40 µl/ml) of serum lipids, cell death was observed (data not shown) indicating that serum lipid treatment beyond 20 µl/ml is cytotoxic to bovine myogenic satellite cells.

Similar to thiazolidinediones and long chain fatty acids, serum lipid induces transdifferentiation through PPAR gamma. We analyzed the expression of different adipogenic transcription factors by treating bovine satellite cells with different concentrations of serum lipid. The results in Figure 2C indicates that none other genes except PPAR gamma showed concentration dependent variation in expression, indicating that PPAR gamma was regulated directly by serum lipids.

Adipogenic differentiation medium consists of various components, so optimization of media composition with serum lipid to obtain maximum transdifferentiation was the next attempt. We treated bovine satellite cells with different combinations of adipogenic mixture components along with serum lipids. Our oil droplet accumulation pictures (Figure 3A) or gene expression data (Figure 3B), show that serum lipids with all the components of adipogenic differentiation

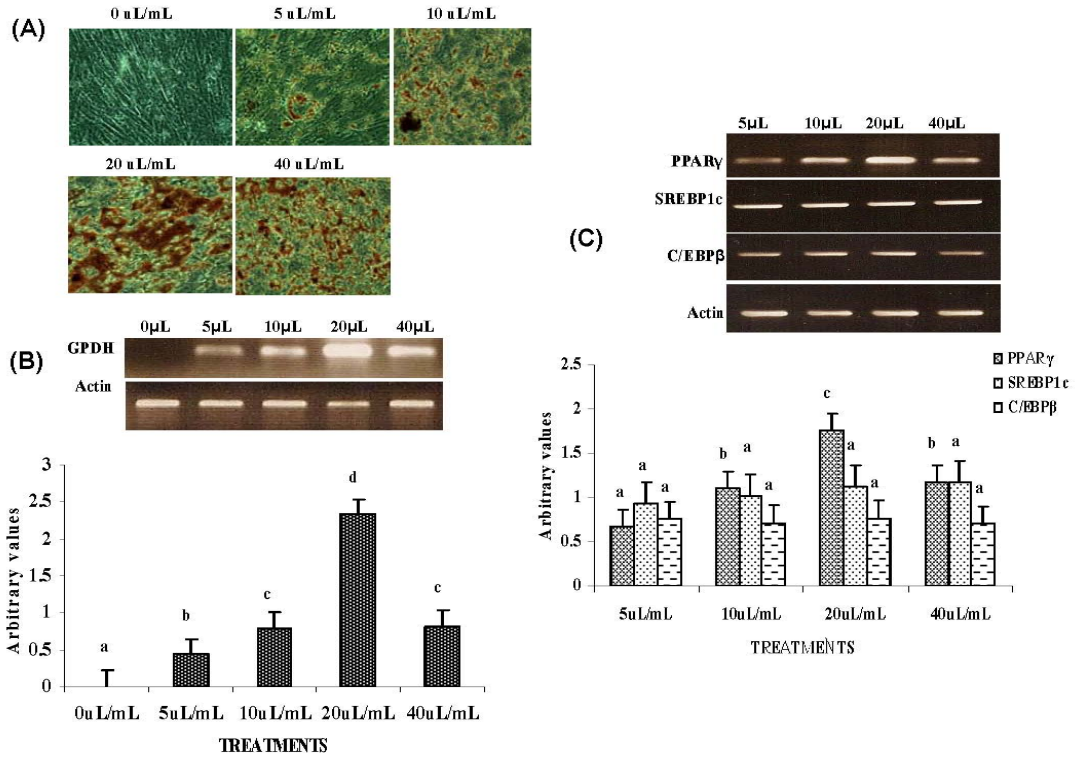
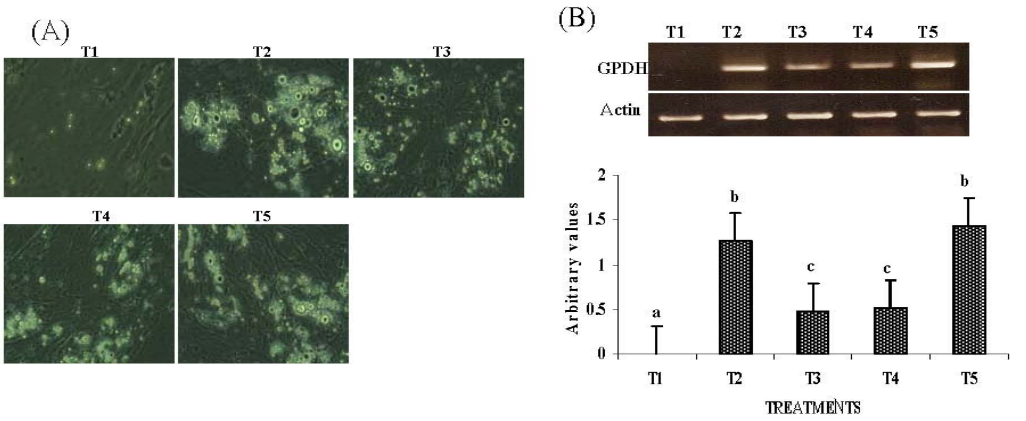


Figure 2. Effects of the different concentration of serum lipid on the transdifferentiation of bovine satellite cells. (A) Oil-red-O stained cells treated with different concentration of the serum lipids on day 10. (B) Effect of different concentration of SL on adipogenic marker gene, GPDH. Upper panel: representative ethidium bromide-stained agarose gel showing amplified GPDH. Lower panel: The RT-PCR results shown are representative of three separate experiments with the same protocol. Data were normalized using beta-actin mRNA and represent the means±SEM of the three experiments. a-d, Mean values with different superscripts are significantly different $p<0.05$. (C) Effect of different concentration of SL on adipogenic transcription factors during transdifferentiation of myogenic BSC to adipocytes. Upper panel: representative ethidium bromide-stained agarose gel showing amplified PPAR gamma, C/EBP beta and SREBP1c. Lower panel: The RT-PCR results shown are representative of three separate experiments with the same protocol. Data were normalized using beta-actin mRNA and represent the means±SEM of the three experiments. a-c, Mean values with different superscripts are significantly different $p<0.05$.



Components	T1	T2	T3	T4	T5
Dexamethasone	+	+	-	+	+
Serum lipids	-	+	+	+	+
Insulin	+	+	+	-	+
Octanoate	+	-	-	-	+
Acetate	+	-	-	-	+

Figure 3. Effect of adipogenic agents on oil droplet accumulation and expression of GPDH gene when treated with SL. Components included (+) in or excluded (-) from the media were dexamethasone (0.25 μ*M*), serum lipids (SL, 20 μl/ml), insulin (280 n*M*), octanoate (1 m*M*), and acetate (10 m*M*). (A) The pictures of cells with accumulated oil droplets after different treatments (T1-T5). (B) The gene expression of GPDH after different treatments. Upper panel: representative ethidium bromide-stained agarose gel showing amplified GPDH. Lower panel: The RT-PCR results shown are representative of three separate experiments with the same protocol. Data were normalized using beta-actin mRNA and represent the means±SEM of the three experiments. a-c, Mean values with different superscripts are significantly different $p<0.05$.

medium or with the removal of octanoate or acetate still induces the maximum transdifferentiation. But, removal of dexamethasone or insulin significantly reduces the effect of serum lipid on transdifferentiation.

We further compared the transdifferentiation potentials of serum lipids (20 μl/ml) and troglitazone (20 μM). The results in Figure 4 clearly indicates that serum lipids can induce a lot better oil droplet accumulation and expression of PPAR gamma ($p<0.01$) after their treatment to bovine satellite cells at the above mentioned concentrations. When cells were treated in combination of serum lipids and troglitazone, the expression of PPAR gamma ($p<0.01$) and oil droplet accumulation was found to be very high compared to the individual treatments.

DISCUSSION

The continuing demand for increased marbling fat and meat quality has, therefore, generated many studies focused on intramuscular adipose tissue, also known as marbling fat, in an attempt to maintain or improve meat quality (Van Barneveld, 2003; Dunshea et al., 2005). Myocyte to adipocyte transdifferentiation is one of the potential way to address the meat quality problem. The current study was based on the observations that myogenic satellite cells transdifferentiate into adipoblasts (Hu et al., 1995; Lee et al., 2000) but the process was applied to adipogenesis by the use of serum lipids. The morphological changes in bovine satellite cells were investigated after culturing postconfluent cells in differentiation medium with the adipogenic mixture plus serum lipids for the period of 10 days (Figure 1A). Postconfluent bovine satellite cells grown only in differentiation medium appeared to form myotubes (control cells), and no restriction to their committed myogenic lineage was observed. Similar observations were reported when C2C12 myoblasts and primary myoblasts (satellite cells) from mice were grown in DMEM with 5% horse serum, which strictly formed myocytes (Asakura et al., 2001). But, the cells cultured along with serum lipids were found to accumulate oil droplets as early as 48 h after treatment, further culturing of cells lead to acquisition of adipocyte morphology. These results are in agreement with the previous findings of Singh et al. (2007) in porcine satellite cells.

To further validate the transdifferentiation effect of serum lipids on bovine satellite cells to adipocytes, we

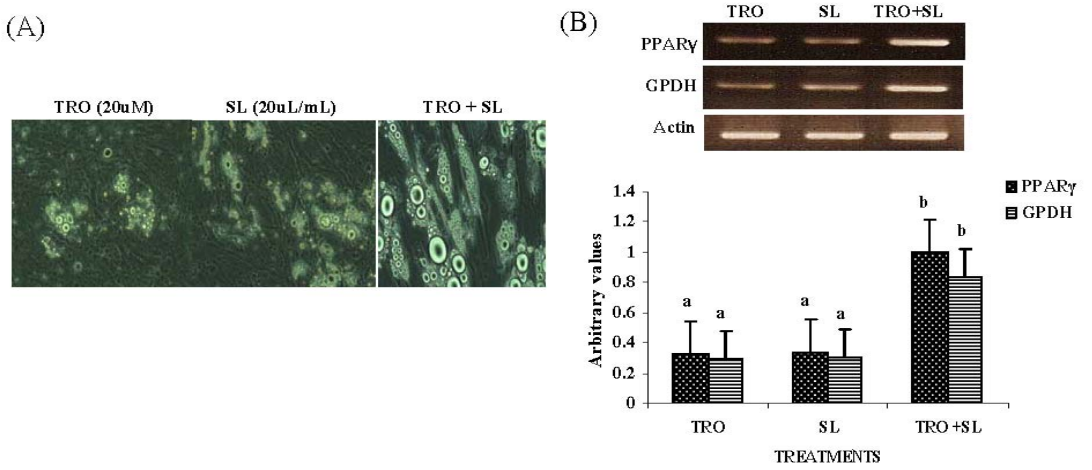


Figure 4. Effects of troglitazone (TRO), serum lipid (SL) and TRO+SL on oil droplet accumulation and adipogenic genes. (A) The pictures of cells with accumulated oil droplets after different treatments (TRO, SL and TRO+SL). (B) The gene expressions of GPDH and PPAR gamma. Upper panel: representative ethidium bromide-stained agarose gel showing amplified GPDH and PPAR gamma. Lower panel: The RT-PCR results shown are representative of three separate experiments with the same protocol. Data were normalized using beta-actin mRNA and represent the means±SEM of the three experiments. a-b, Mean values with different superscripts are significantly different $p<0.05$.

analyzed the expression of adipogenic genes. Important transcription factor such as PPAR gamma and adipogenic marker such as GPDH were (Figure 1B) analyzed. PPAR gamma has a profound effect on the process of adipogenesis, its expression inhibits myogenesis pathway and pushes the cell to follow adipogenic pathways. After the treatment of cells with serum lipids, upregulation of PPAR gamma was observed as early as 48 h. We found that, expression of PPAR gamma was high on day 2 and kept increasing till day 4, however, the expression decreased after day 6. Similar expression pattern was observed after treatment of ciglitazone to porcine satellite cells by Singh et al. (2007). Furthermore, the expression of adipogenic marker gene GPDH was detected on day 2 of the treatment and kept on increasing with the time period. Expression of GPDH supports the idea that few numbers of satellite cells were converted to adipocytes accumulating oil droplets and expressing GPDH gene. These results very well correlate with the fact of observing oil droplets on day 2 of the treatment in our study. In previous studies, Wu et al. (1996) emphasized that signals for adipogenesis begin with the activation of PPAR gamma in the presence of PPAR gamma ligand. McKnight et al. (1989) established that expression of PPAR gamma precedes expression of C/EBP alpha, which is consistent with the results of the current study. Once C/EBP alpha becomes prevalent (Yeh et al., 1995) it regulates terminal adipocyte differentiation and turns on the group of fat-specific genes (such as GPDH) required for the synthesis, uptake, and storage of long-chain fatty acids (Erding et al., 1995).The induction of adipogenic program in bovine satellite cells inhibits the myogenesis. The process of myogenesis is regulated by a family of basic helix-loop-helix transcription factors, including MyoD and Myf5 (Weintraub et al., 1993; Emerson, 1993). So, we analyzed the expression pattern of these myogenic genes (Figure 1C) after the serum lipid treatment. The expression of levels of these myogenic genes were significantly reduced ($p<0.001$), thus inhibiting the process of myogenesis and leading adipogenic pathway. Taken together, these results suggest that serum lipids can convert bovine satellite cells to adipocytes when cultured under adipogenic differentiation medium.

Our next sets of experiments were designed to know the minimum and optimum concentrations of serum lipid to induce transdifferentiation in bovine satellite cells. Results suggest that as low as 5 μl/ml of serum lipids can induce transdifferentiation, and maximum transdifferentiation was obtained using 20 μl/ml of serum lipids. Treatment of cells with higher concentrations (40 μl/ml) of serum lipids had a negative effect on cell morphology and viability, indicating that serum lipids above 20 μl/ml might be cytotoxic. These results are the first for bovine myogenic satellite cells, but similar results have been shown on bovine preadipocytes by Grant et al. (2008).

Adipocyte differentiation is co-ordinatedly regulated by several transcription factors. C/EBP beta, C/EBP delta, and ADD-1/SREBP-1 are active early during the differentiation process and induce the expression and/or activity of the PPAR gamma, the pivotal coordinator of the adipocyte differentiation process (MacDougald and Lane, 1995). So, we wanted to check transcription factor through which serum lipids induces the process of transdifferentiation.

When satellite cells were treated with different concentrations of serum lipids, except PPAR gamma other early expressing transcription factors such as SREBP1c and C/EBP beta did not show concentration dependent change in the gene expression. Similar to previous compounds like long chain fatty acids and thiazolidinediones, serum lipids also target PPAR gamma for its function, and thus inducing transdifferentiation via PPAR gamma.

We then checked the essential ingredients of adipogenic differentiation medium required by serum lipids for induction of maximum transdifferentiation using bovine satellite cells. In Figure 3, the results indicate that the accumulation of oil droplets and the gene expression of GPDH were significantly ($p<0.05$) higher in treatment 5 (with all the components of ADM) and treatment 2 (with the removal of acetate and octanoate components of the ADM). This may be due to the fact that serum lipids mixture of different lipid components which can perform the function of acetate and octanoate. But, the removal of dexamethasone (treatment 3) or insulin (treatment 4) significantly reduces the oil droplet accumulation and gene expression of GPDH. This may be accredited to the roles of dexamethasone, a glucocorticoid hormone required for the induction of early expressing adipogenic genes (Wu et al., 1996). Similarly, insulin increases cellular uptake of long chain fatty acids through stimulating fatty acids transport protein translocation from intracellular pools to the plasma membrane. Therefore, insulin may act as a modulator of cellular differentiation, as well as serve to increase substrate availability for adipogenesis in adipocytes. Thus, the exclusion of insulin may adversely affect the ability of serum lipids to induce transdifferentiation as serum lipid is rich in cholesterol, a long chain fatty acid (Stahl et al., 2002). These results very clearly indicate that serum lipids require insulin and dexamethasone for its maximum activity.

Further, we have tried to compare the effect of troglitazone or serum lipids or the combination of both for the transdifferentiation of bovine satellite cells to adipocytes. Our results (Figure 4) show that the combination of serum lipid and troglitazone had a significantly higher effect when compared to the individual effect. The gene expression levels of GPDH were found to be significantly higher ($p<0.05$) in the cells treated with combination of serum lipids and troglitazone. Previously, similar results have been shown by Soret et al. (1999) in ovine stromal vascular cells and Grant et al. (2008) in bovine preadipocytes.

To conclude, serum lipid is a very potent transdifferentiating agent that can be used for further *in vitro* studies. As serum lipid is a mixture compound which consists of long chain fatty acids, short chain fatty acids and several proteins, it is very important to elucidate which specific component is inducing transdifferentiation in bovine in the near future.

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