ROLES OF CHOLESTEROL IN PROLIFERATION AND DIFFERENTIATION OF BOVINE MYOBLASTS

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Keywords: cattle, myoblasts, cholesterol, proliferation, differentiation

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ABSTRACT (Academic)

The objective of this study was to assess the potential role of extracellular, cytosolic, and membrane cholesterol in the proliferation and differentiation of bovine myoblasts. In the first experiment, myoblasts isolated from Angus or Angus crossbred steers were cultured with 2% lipoprotein deficient fetal calf serum (LPDS) or normal fetal calf serum. Culturing with LPDS did not alter the cytosolic or membrane cholesterol content, or myoblast differentiation, but inhibited myoblast proliferation, compared to culturing with normal fetal calf serum. In the second experiment, myoblasts were cultured with or without lovastatin, a selective inhibitor of cholesterol synthesis. Culturing with 5 μM lovastatin did not affect medium concentration of cholesterol, but reduced cytosolic and membrane cholesterol contents, compared to culturing with vehicle control. Culturing with 5 μM lovastatin inhibited both myoblast proliferation and differentiation. In the third experiment, myoblasts were cultured with or without methyl-β-cyclodextrin (MβCD), a chemical that depletes cholesterol from cell membranes. Treating myoblasts with 10 mM MβCD for 30 minutes reduced membrane and cytosolic cholesterol contents while increasing medium cholesterol concentration. Treating with MβCD inhibited both myoblast proliferation and differentiation compared to treating with vehicle control. Overall, this study showed that lovastatin- or MβCD-induced reductions in cytosolic and membrane cholesterol contents were associated with reduced proliferation and differentiation in bovine

myoblasts. These associations suggest that cytosolic cholesterol, membrane cholesterol, or both may play a role in bovine myoblast proliferation and differentiation.

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ABSTRACT (Public)

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Chapter I

Literature Review

Introduction

Skeletal muscle contraction is critical for breathing, thermal homeostasis, maintaining body posture or position, and exercise capacity (Hoppeler and Fluck, 2003; Yin et al. 2013). A skeletal muscle is composed of multiple bundles of cells shaped into cylinders called muscle fibers or myofibers. Muscle fibers are formed from the fusion of myoblasts in a process known as myogenesis (Zammit et al., 2006). Satellite cells are the muscle progenitor cells in postnatal animals (Charge and Rudnicki, 2004). In the postnatal stage, the number of myofibers is believed to stay consistent and the hypertrophy of myofibers depends on satellite cells fusing into existing myofibers (Yablonka-Reuveni, 2011). Skeletal muscle development and hypertrophy is a biological process of particular interest to beef cattle researchers and producers because it determines beef quantity and quality.

Cholesterol is the precursor of bile acid, vitamin D, and steroid hormones. Cellular cholesterol is essential in maintaining membrane fluidity and permeability, and also acts as an important regulator of the function of a broad range of membrane proteins, including receptors, transporters, and ion channels. Skeletal muscle is rich in cholesterol (Nikkilä et al., 1978). Emerging evidence suggests that cholesterol may play a role in myogenesis.

This review summarizes the major events in the myogenic process and the roles of cholesterol in the body, with a focus on the potential function of cholesterol in skeletal muscle

development and growth.

Myogenic progression of satellite cells

Satellite cells

Satellite cells are muscle stem cells that were identified by using electron microscopy and named based on their unique morphology and anatomical localization (Asakura, 2003; Mauro, 1961). They are located between the plasma membrane and the basal membrane of a muscle fiber and are mononuclear cells that spread out along the whole muscle fiber (Mauro, 1961). Early studies of satellite cells showed satellite cells contribute to myogenic differentiation, postnatal muscle maturation, adult skeletal muscle regeneration after injury, myofiber hypertrophy, and myofiber maintenance (Kuang et al., 2008). Subsequent studies demonstrated that satellite cells are multipotential stem cells that exhibit not only myogenic ability, but also adipogenic and osteogenic differentiation adaptability under different culture environments or stimuli (Asakura et al., 2001; Harding et al., 2015).

Adult skeletal muscle contains a large amount of satellite cells (Charge and Rudnicki, 2004). Satellite cells contribute to the muscle mass through two unique approaches. In response to muscle damage, satellite cells become activated from the quiescent stage and proliferate to establish the self-renewal ability in the damaged area, and then replace the damaged muscle fibers with new muscle fibers. Satellite cells could add the number of nuclei to pre-existing muscle fibers through fusion. Thus, satellite cells can repair damaged muscle and also contribute to muscle hypertrophy (Schiaffino et al.,1976).

The activation, specific differentiation and fusion of satellite cells are necessary for forming new myotubes. This process maintains the original myofiber number in the muscle and

normally will not cause hyperplasia (Rosenblatt et al., 1994). These paradigms suggest that satellite cells have clinical application potential in the treatment of myopathies such as muscular dystrophy (Anderson, 2006; Asakura et al., 2001). However, the role of satellite cells in muscle hypertrophy has long been a debated issue. Satellite cells-deficient muscle exhibits muscular hypertrophic traits with enhanced muscle mass and size under gradually overloading stimuli; meanwhile, satellite cell depletion is also associated with the inhibition of regeneration (McCarthy et al., 2011). Even though satellite cells might not be necessary for muscle fiber hypertrophy, activation of satellite cells occurs in virtually all hypertrophy models (Blaauw et al., 2009; Relaix et al., 2012). These facts underline the need of further study on the role of satellite cells during muscle hypertrophy.

Satellite cell proliferation, differentiation, and fusion

From satellite cells to myotubes, skeletal muscle myogenesis is tightly regulated by various gene subsets (Rudnicki and Jaenisch, 1995; Weintraub et al., 1991). Stimuli in the micro-environment can activate and induce quiescent satellite cells to enter myogenic differentiation. Activation or repression of paired-box transcriptions factors (PAX) and myogenic regulatory factors is required for the progression of satellite cells through myogenesis. The PAX genes include PAX3 and PAX7, and the myogenic regulatory factors include MYOD, myogenic factor 5 (MYF5), myogenin (MYOG), and MYF6.

PAX7 has widely been recognized as a satellite cell survival and anti-apoptotic factor. It is essential for postnatal maintenance and matured muscle self-renewal (McCarthy et al., 2011; Seale et al., 2000). In gene-knockout animal models, severe cell death was detected and the

quantity of satellite cells kept declining due to the absence of PAX7 (Seale et al., 2000). Besides, over-expression of PAX7 delayed MYOG expression, suggesting a dual role of PAX7 in maintaining myoblast proliferation and preventing differentiation (Relaix et al., 2006; Zammit et al., 2006). PAX3 and PAX7 have overlapping and distinct functions (Kassar-Duchossoy et al., 2005; Relaix et al., 2005). The roles of PAX3 in muscle regeneration were defined by using a stable green fluorescent protein reporter instead of gene-knockout animal models, because PAX3-null mice die before birth (Kassar-Duchossoy et al., 2005; Relaix et al., 2005). Like PAX7, PAX3 has the ability to mediate satellite cell myogenic proliferation, but PAX3 cannot replace the anti-apoptotic function of PAX7 (Kassar-Duchossoy et al., 2005; Yang et al., 2016). Furthermore, satellite cells from $PAX3^{+}/PAX7^{-}$ and $PAX3^{-}/PAX7^{+}$ mice demonstrated various myogenic differentiation rates (Yang et al., 2016).

When satellite cells-derived myoblasts commit to the early stage of differentiation, they stop cycling and gradually lose the expression of PAX3 and PAX7. Experiments in rats clearly demonstrated that myoblasts expressed myogenic markers such as MYOD, MYF5 and desmin in this myogenic stage. However, desmin is not present in bovine muscle satellite cells (Allen et al., 1991). Quiescent satellite cells enter the cell cycle and proliferate as myoblasts with increasing expression of MYOD and MYF5 (Cooper et al., 1999; Cornelison and Wold, 1997). MYF5 regulates the rate of proliferation and homeostasis of myoblasts. MYF5 deficiency in mice by gene knockout resulted in a lack of myoblast amplification, supporting a role of MYF5 in formation of myoblasts during embryogenesis (Beauchamp et al., 2000; Kassar-Duchossoy et al., 2004). MYOD is required for the differentiation potential of skeletal myoblasts, and it also participates in the early stage of differentiation (Davis et al, 1987). As such, MYOD gene

knockout mice ($MYOD^{-/-}$) showed a withdrawal tendency in myogenic differentiation (Beauchamp et al., 2000).

In the terminal stage of differentiation, myoblasts cease division and differentiate into myotubes. There are two main stages in the formation of myotubes. In the first stage, most proliferating myoblasts exit cell cycle and fuse with each other and generate myotubes containing a few nuclei with the up-regulated expression of MYOG and MYF6, the latter also known as MRF4 (Cornelison and Wold, 1997; Cornelison et al., 2000). MYOG as a muscle specific basic-helix-loop-helix transcription factor can effect skeletal muscle development by activating and modulating expression of important myogenesis genes, then altering the content of muscle contractile proteins such as actin, myosin, and troponin (Funk and Wright, 1992). The expression of MYOG is strictly regulated by protein kinases A and C (Biesiada et al., 1999). In the second stage of myotube formation, myoblasts fuse into existing myotubes and form larger myotubes with increased size and number of nuclei (Knudsen and Horwitz, 1977; Przybylski and Blumberg, 1966). MRF4 is highly expressed in terminally differentiated myotubes (Rudnicki and Jaenisch, 1995; Weintraub et al., 1991). MRF4 does not contribute to satellite cell development or maintenance but is important for the formation and hypertrophy of myofibers (Lowe and Alway, 1999).

Fusion of myoblasts is essential for the formation of multi-nucleated muscle fibers. Myomaker (also called TMEM8C) is a specific myoblast cell surface activator protein that mediates the myoblast fusion process and also participates in cell fusion for skeletal muscle hypertrophy (Goh and Millay, 2017; Landemaine et al., 2014; Millay et al., 2013). MYOD and MYOG regulate Myomaker expression and upregulated Myomaker promotes myoblast fusion

(Luo et al., 2015). In mice, gene knockout of Myomaker leads to perinatal death due to the suppression of myotube formation and absence of multi-nucleated muscle fibers (Millay et al., 2013). Overexpression of Myomaker in myoblasts dramatically enhances fusion and also promotes myoblasts fusion with existing myofibers (Millay et al., 2014).

Role of cholesterol in myogenesis

The function of cholesterol

Cholesterol is the precursor of steroid hormones such as sex steroid hormones testosterone, progesterone, and estradiol, as well as corticosteroid hormones such as cortisol, corticosterone, and aldosterone synthesized in the gonads and adrenal glands (Lynch et al., 2012). Cholesterol is also the precursor for the synthesis of bile acid in liver cells. Bile acid is critical for dietary fat digestion and vitamin absorption (Chiang, 2009). Bile acid synthesis is a major extracellular catabolic route for cholesterol disposal, which is tightly regulated by the activity of cholesterol 7α-hydroxylase (Parks, 1999). In addition, cholesterol is an important biological molecule involved in both the creation and maintenance of cell membranes. In cells, the majority of cholesterol is located in the plasma membrane and only a small amount is in the intracellular membranes (Finegold, 1992). The change of cholesterol content in plasma membrane alters the membrane fluidity and permeability (Yeagle, 1985). Besides, the cholesterol content in plasma membrane is also associated with the functions of membrane proteins that participate in transmembrane signaling processes (Cherezov et al., 2007; Ikonen, 2008). Membrane cholesterol depletion immediately changed the G protein-coupled receptor signaling (Simons and Toomre, 2000). Glucose transport mediated by insulin-sensitive glucose transporter GLUT4 was affected by membrane cholesterol levels (Barrientos et al., 2017). Abnormal levels of membrane cholesterol led to serious cellular consequences and disease (Ikonen, 2006; Maxfield and Tabas, 2005).

Skeletal muscle has a high cholesterol content. Formation of new plasma membrane in skeletal muscle requires a large amount of cholesterol, which is primarily from the endogenous

cholesterol synthesis (Bloor, 1936; Chang et al., 2006). Adipocytes also have a high cholesterol content due to the size of adipocytes and the content of lipid raft (Le Lay et al., 2001). Raised under similar condition, steer carcasses contain more cholesterol than bull carcasses mainly because the former have a higher percentage of fat (Eichhorn et al., 1986).

Cellular regulation of cholesterol homeostasis

Cholesterol is mainly synthesized in the liver and intestines, although every tissue has the capability to synthesize cholesterol (Lopez et al., 2017). The resource of cholesterol from *de novo* synthesis and dietary intake has been estimated as a ratio of 7:3 in non-ruminant mammals (Grundy, 1983). Both dietary and synthesized cholesterol can be used by the body to produce bile acid, vitamin D, and steroid hormones (Hu et al., 2010; Payne and Hales, 2004). Dietary cholesterol is absorbed by small intestines and transported to the liver for systemic delivery. The biosynthesis of cholesterol could be inhibited by cholesterol intake. In rats and chickens, the dietary cholesterol intake suppresses cholesterol synthesis in the liver and most of the biosynthetic cholesterol is from other tissues. Diets for ruminant species normally contain low cholesterol contents. In a beef study, the cholesterol content in skeletal muscle was not significantly altered by the dietary cholesterol supplement (Bohac and Rhee, 1988), perhaps because the *de novo* cholesterol synthesis is sufficient for daily total cholesterol requirements in cattle.

Considerable evidence suggests a great difference in the rate of cholesterol synthesis among species, as well as within the species depending on the type and quantity of muscles (Dayton and White, 2008; Dinh et al., 2011; Du and McCormick, 2009). Cholesterol biosynthesis is commonly quantitated by incorporating radiolabeled cholesterol precursors. The mevalonate

pathway is the main pathway to mediate cholesterol biosynthesis in muscle, and 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase (HMG-CoA reductase or HMGCR) is the rate-controlling enzyme in this pathway. Sequences of HMGCR cDNAs derived from different animal species suggest that the structure of the membrane-associated and catalytic domains of HMGCR is highly conserved (Luskey and Stevens, 1985). Sterol regulatory element–binding proteins (SREBPs) are transcription factors that facilitate and regulate various enzymes including HMGCR (Horton, 2002). SREBPs are synthetized as inactive forms located on endoplasmic reticulum membranes. SREBP activation is mainly controlled by cellular sterol content. When cellular sterol content drops, low cholesterol content acts on a cleavage protein called sterol regulatory element-binding protein cleavage-activating protein (SCAP) and induces it to interact with the inactive precursor of SREBPs. SCAP-causes SREBP to undergo a sequential two-step cleavage. The cleavage of SREBPs exposes the active domain, which alters the expression of HMGCR, and ultimately, results in the upregulated synthesis of cholesterol (Brown and Goldstein, 1980). Conversely, when the cholesterol content is high within cells, the expression of HMGCR is suppressed and hence *de novo* synthesis of cholesterol is decreased.

Cellular cholesterol homeostasis is also regulated through the low-density lipoprotein (LDL) receptors. When the content of extracellular cholesterol or its derivative is high, the LDL receptor gene transcription is reduced in the nucleus, thereby decreasing the synthesis of new LDL receptors and turning off cellular cholesterol uptake (Brown and Goldstein, 1980). Conversely, LDL receptor synthesis will increase when a cell is starved for cholesterol or cholesterol synthesis is inhibited.

Role of cholesterol in myogenesis

Skeletal muscle is rich in cholesterol (Bloor, 1936). In rat skeletal muscle, cholesterol is predominantly localized in the sarcoplasmic reticulum membrane (Clarke et al., 2000). The potential function of cholesterol in skeletal muscle has been investigated in cellular studies. These studies involved several common ways to deplete cellular cholesterol content: treating cells with metabolic inhibitors such as statins, growing cells in lipoprotein-deficient serum, or using chemicals such as MβCD to remove cholesterol from cell membranes (Mahammad and Parmryd, 2015).

A class of cholesterol-lowering drugs called statins has been widely used as HMGCR inhibitors to prevent hypercholesterolemia-related diseases. Examples of statins are atorvastatin, fluvastatin, lovastatin, pravastatin, and simvastatin. However, long-term use of statins can be accompanied by side effects such as myopathy, which is more severe in patients with type II diabetes (Sinzinger and Wolfram, 2002). The transverse tubule (T-tubule) system in skeletal muscle has a high cholesterol content (Draeger et al., 2006). A decrease in membrane cholesterol in this area alters both muscle contraction and the location of glucose transporter GLUT4, indicating the possible function of membrane cholesterol in insulin resistance (Barrientos et al., 2017). Consistently, use of statins resulted in reduced intracellular cholesterol content and also depleted the secondary metabolic intermediates formed in the cholesterol synthesis pathway. This effect diminishes plasma cholesterol levels and subsequently increases LDL receptor expression on the cell surface (Alberts et al., 1980). In human bone marrow stromal cells (BMSCs), simvastatin has positive effects on osteoblastic differentiation, but it has negative effects on their proliferative potential (Baek et al., 2005). Lovastatin reduces the intracellular cholesterol content in striated muscle cells and the viability of mouse C2C12 cells (Gadbut et al., 1995; Mullen et al., 2010). Statins inhibit HMGCR and decrease its downstream signals such as

mevalonate, which is also an intermediate precursor of ubiquinone. Reduced ubiquinone biosynthesis by statins is related to mitochondrial myopathies (Kaufmann et al., 2006). Statin treatment reduced cholesterol synthesis in both C2C12 and HepG2 cells. However, unlike in HepG2 cells, it did not affect total ubiquinone levels and LDL receptor mRNA expression in C2C12 myotubes (Mullen et al., 2010). Statins inhibit the AKT signaling pathway, which is associated with accelerated myofibrillar degradation and myotube atrophy. Statins activate Caspases and poly ADP ribose polymerase (PARP), thereby inducing apoptosis (Bonifacio et al., 2015). Interestingly, IGF-I can prevent simvastatin-induced myotoxicity in C2C12 myotubes (Bonifacio et al., 2016).

Statin-induced myopathy is associated with decreased mitochondrial NADH content, oxidative stress, mitochondrial membrane depolarization, and alteration of Ca^{2+} homeostasis (Sirvent et al., 2005; Sirvent et al., 2005). Stimulating the synthesis of cholesterol or supplying exogenous cholesterol can efficiently restore mitochondrial activity (Cornell, 1980). The risk of statin-associated myopathy can be minimized by specific exercise management or supplement of CoQ10 (Banach et al., 2015; Kobayashi et al., 2008). CoQ10 shares parts of the biosynthesis pathway with cholesterol. However, evidence from randomized controlled trials does not support the idea that CoQ10 is an effective treatment for statin-associated myopathy (Banach et al., 2015). This is perhaps because myotubes mainly contain CoQ9, not CoQ10.

Cholesterol plays a pleiotropic role in cell membranes. It is involved in crucial membrane functions such as maintaining the intactness, fluidity, permeability, and lipid-protein interaction (Wüstner et al., 2012). An alternation of cholesterol content in membrane is required for plasma membrane fusion (Nakanishi et al., 2001). MβCD is widely used to exclude

cholesterol from cell membranes (Simons et al., 1998; Song et al., 2013). Cholesterol depletion by MβCD increases cell membrane stiffness in various cell types (Brown et al., 2010; Byfield et al., 2004), affects the transmembrane activity, and changes the proliferation, differentiation, or fusion of cells.

Although cholesterol synthesis and transportation is a conserved process in mammals, research in different animal models or different cell types has achieved different results. When smooth muscle cells were exposed to MβCD, vasoconstriction was significantly impaired, whereas this MβCD effect was attenuated by excess exogenous cholesterol (Potocnik et al., 2007). When C2C12 cells (a mouse skeletal muscle cell line) were exposed to MβCD, their fusion was inhibited, and the inhibited fusion was restored by subsequent cholesterol supplementation (Taulet et al., 2009). Controversially, membrane cholesterol depletion by MβCD speeded up myoblast fusion and formation of large multinucleated myotubes in chicken myoblasts (Mermelstein et al., 2005). MβCD increased the proliferation of primary chick myogenic cells too (Portilho et al., 2012). It was suggested that membrane cholesterol participated in the early myogenic differentiation stage by activating the Wnt / β-catenin signaling pathway in chicken myoblasts (Mermelstein et al., 2007). In chicken cardiomyocytes, depletion of cholesterol by MβCD suppressed their proliferation but induced their differentiation (Soares et al., 2010), indicating dual function of membrane cholesterol in chicken cardiomyocyte development.

Conclusion and perspectives

Studies using primary human skeletal muscle cells, mouse myoblast lines and primary chicken myoblasts point to cholesterol as a potential functional element in myogenesis, although the results are somewhat conflicting. Little is known about the role of cholesterol in ruminant skeletal muscle development. As meat, skeletal muscle is a major product of cattle production. A better understanding of the function of cholesterol in bovine myoblast proliferation and differentiation could lead to the development of novel strategies to improve meat production or quality in cattle.

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Chapter II

Roles of Cholesterol in Proliferation and Differentiation of Bovine Myoblasts

ABSTRACT

The objective of this study was to assess the potential role of extracellular, cytosolic, and membrane cholesterol in the proliferation and differentiation of bovine myoblasts. Satellite cells, myogenic cells in adult skeletal muscle, were isolated from Angus or Angus crossbred steers, expanded as myoblasts in growth medium (DMEM and 10% fetal bovine serum), and used in three experiments. In the first experiment, myoblasts were differentiated and fused into myotubes in 2% lipoprotein deficient fetal calf serum (LPDS) or normal fetal calf serum. The use of LPDS did not alter cytosolic or membrane cholesterol content in myoblasts, or the differentiation status of myoblasts. But myoblasts cultured in 2% LPDS had 20% less ($P < 0.01$) 5–ethynyl–2′–deoxyuridine (EdU)-positive cells (i.e., proliferating cells) at 24 h, and 50% less ($P < 0.01$) at 48 h of culture, than those cultured in normal fetal calf serum. In the second experiment, myoblasts were cultured with or without lovastatin, a selective inhibitor of 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMGCR), which is a key enzyme involved in cholesterol synthesis. Treating myoblasts with 0.2 μM, 1 μM or 5 μM lovastatin for 48 or 72 h did not affect medium concentration of cholesterol, but reduced cytosolic content of cholesterol by 30% ($P < 0.05$) and 40% ($P < 0.05$), respectively. Treating myoblasts with 5 μM lovastatin for 48 h did not change membrane cholesterol content, but treating myoblasts with 5 μM lovastatin for 72 h reduced membrane cholesterol content by 20% ($P < 0.05$). Myoblasts treated with lovastatin in growth medium had 45% less ($P < 0.05$) EdU-positive cells than did control myoblasts. Myoblasts treated with lovastatin in differentiation medium for 72 h had less ($P < 0.05$) mRNA expression

for 4 of 6 selected myoblast differentiation and fusion markers (MYOG, MYH3, CKM, TMEM8C, MYL2, and ACTA1) compared to control myoblasts. In the third experiment, myoblasts were cultured with or without methyl-β-cyclodextrin (MβCD), a chemical widely used to remove cholesterol from cell membranes. Treating myoblasts with 10 mM MβCD for 30 minutes reduced membrane cholesterol content by 50% ($P < 0.05$) and cytosolic cholesterol content by 30% ($P < 0.05$) while increasing medium cholesterol concentration by nearly 50% ($P < 0.05$). Myoblasts treated with MβCD in growth medium had approximately 20% less EdU-positive cells than did control myoblasts ($P < 0.05$). Myoblasts treated with MβCD in differentiation medium had less ($P < 0.05$) mRNA expression for 5, 5, and 4 of 6 selected differentiation and fusion markers than control myoblasts at 24, 48, and 72 h of differentiation, respectively. Overall, this study showed that lovastatin- or MβCD-induced reductions in cytosolic and membrane cholesterol contents were associated with reduced proliferation and differentiation of bovine myoblasts. These associations suggest that cytosolic cholesterol, membrane cholesterol, or both are needed for the proliferation and differentiation of bovine myoblasts.

Keywords: cattle, myoblasts, cholesterol, proliferation, differentiation

INTRODUCTION

Satellite cells are a kind of mononuclear progenitor cells found in the basal lamina that surrounds skeletal muscle fibers (Collins et al., 2005). Satellite cells are usually quiescent. When muscle is damaged, satellite cells are activated to fulfill their roles in muscle repair and regeneration. Satellite cells may also contribute to muscle maintenance and hypertrophy (Pallafacchina et al., 2013). Skeletal muscle development or regeneration is marked by the expression of various gene subsets that control the differentiation of the myogenic progenitors into myotubes. These genes include myogenin (MYOG), a muscle-specific transcription factor, which belongs to myogenic regulatory factors (MRF) (Funk and Wright, 1992); the creatine kinase, M-type (CKM), which functions as a muscle-specific enzyme; myomaker (TMEM8C), which is essential for the membrane fusion reaction to form the multi-nucleated muscle fibers (Millay et al., 2013); and various myosin heavy chain (MYH) and myosin light chain (MYL) proteins, and skeletal α-actin (ACTA1), which are muscle structural proteins (Levine et al., 1996; Mahdavi et al., 1987). The expression levels of these genes are commonly used to indicate the differentiation level of muscle cells (Rudnicki and Jaenisch, 1995; Weintraub et al., 1991).

Cell membranes are rich in cholesterol. Membrane cholesterol is involved in crucial membrane functions such as membrane fluidity, permeability, and lipid-protein interaction (Wüstner et al., 2012). Cholesterol is also used by the body to synthesize bile acid, vitamin D, and steroid hormones (Hu et al., 2010; Payne and Hales, 2004). The body obtains cholesterol through diet intake and *de novo* synthesis. Cholesterol is mainly synthesized in the liver and intestines, but every tissue has the capability to synthesize cholesterol with varied rates (Lopez et al., 2017; Siperstein, 1970). Intracellular cholesterol uptake and synthesis are regulated by

multiple feedback mechanisms (J. E. Vance and Vance, 2008). In the cholesterol biosynthesis pathway, the rate-limiting step is the conversion of HMG-CoA to mevalonate catalyzed by 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase (HMGCR). Sterol regulatory element-binding proteins (SREBPs) are transcription factors that facilitate and regulate various enzymes including HMGCR in the cholesterol biosynthesis pathway (Horton, 2002). In non-ruminant animals, excess cholesterol intake inhibits *de novo* synthesis of cholesterol (Siperstein, 1970). However, dietary cholesterol has little effect on cholesterol synthesis in the ruminant (D'Mello, 2000; Nestel et al., 1978).

Skeletal muscle is rich in cholesterol (Bloor, 1936). The concentration of membrane cholesterol in myoblasts changes prior to membrane fusion (D. Vance and Vance, 1996). Cholesterol depletion by methyl-β-cyclodextrin (MβCD), a common treatment that is used to selectively exclude cholesterol from cell membrane (Simons et al., 1998; Song et al., 2013), enhanced both proliferation and differentiation in primary chicken myoblasts (Portilho et al., 2012; Possidonio et al., 2014). Membrane cholesterol depletion also enhanced chicken myoblast fusion and induced the formation of large multinucleated myotubes (Mermelstein et al., 2005). It was further demonstrated that membrane cholesterol is involved in the early steps of myogenic differentiation by activating the Wnt/β-catenin signaling (Mermelstein et al., 2007). However, in the mouse myoblast cell line C2C12, depletion of membrane cholesterol impaired myoblast fusion, which was restored by cholesterol supplement (Nakanishi et al., 2001).

The function of cholesterol in bovine skeletal muscle development and growth is unknown. Thus, we investigated the potential function of extracellular cholesterol, intracellular

cholesterol, and membrane cholesterol in proliferation, differentiation, and fusion of bovine myoblasts in this study.

MATERIALS AND METHODS

Isolation of bovine satellite cells

Extensor Carpi Radialis muscle was collected from Angus or Angus crossbred steers at the Virginia Tech Meat Science Center or a local slaughter house and transported to the lab in ice-cold sterile phosphate buffered saline (PBS) containing 1% antibiotics and antimycotics (ABAM; Mediatech, Manassas, VA) within 30 minutes of collection. Satellite cells were isolated as described previously (Dayton and White, 2008; Kamanga-Sollo et al., 2004). Briefly, muscle was washed with 70% ethanol for 1 minute and rinsed with cold PBS twice to remove potential surface contaminants and blood. Fat and connective tissue were removed from muscle with scissors. Muscle was cut into small pieces and then ground using a sterilized meat grinder. Ground tissues were transferred into a flask that contained PBS, 1 mg/ml Pronase (Calbiochem, San Diego, CA) and 1% ABAM. The flask was incubated in a 37°C water bath for 10 minutes and then in a 37°C dry incubation shaker for 50 minutes. The muscle samples were then transferred into the centrifuge bottles and centrifuged at 1,500 ×g for 10 minutes at room temperature. The supernatant was discarded, and the pellet was resuspended in fresh PBS. The resuspension was centrifuged at 400 ×g for 5 minutes at room temperature. The supernatant from this centrifugation was transferred into a new centrifuge bottle while the pellet was discarded. This washing and differential centrifugation procedure was repeated three more times. The pellet from the last centrifugation at 1,500 ×g was re-suspended in fetal bovine serum (FBS; Atlanta Biologicals, Lawrenceville, GA) supplemented with 10% DMSO and stored in a -80°C freezer.

Cell culture

Satellite cells from 6 steers were pooled at equal proportions and the pooled cells were used for all experiments. Satellite cells were proliferated as myoblasts in growth medium composed of Dulbecco's Modified Eagle Medium (DMEM; Mediatech, Manassas, VA), 10% FBS and 1% ABAM at 37°C and 5% CO_2 for no more than 12 days, during which the medium was refreshed every two days. In the myoblast proliferation experiments, satellite cells were cultured in DMEM supplemented with 10% lipoprotein-deficient fetal calf serum (LPDS; Sigma-Aldrich, St. Louis, MO) or 10% fetal calf serum (FCS). In the differentiation experiments, satellite cells were grown to 80% confluence in growth medium and then cultured in differentiation medium composed of DMEM, 1% ABAM, and 2% FCS or 2% LPDS.

Chemicals and treatment preparation

Lovastatin (Cayman Chemical, Ann Arbor, MI) stock solution (10 mM) was made by dissolving it in filtered ethanol and stored at -20 ^{o}C in darkness for no more than 12 months. Lovastatin was further diluted in ethanol and added to cell culture medium at 0.2 μM, 1 μM, or 5 μM. Control cells were added with ethanol only. All treatments and control contained the same amount (0.5%) of ethanol. Lovastatin treatment and culture medium were refreshed every day.

The stock solution of methyl-β-cyclodextrin (MβCD; C4555, Sigma-Aldrich, St. Louis, MO) was made in DMEM and stored at -20^{o}C in darkness for no more than 6 months. MβCD was added to cell culture at 0.4 mM, 2 mM, or 10 mM. Control cells were added with an equal amount of DMEM. Cells were treated with MβCD for 30 minutes every 24 hours. Longer MβCD treatment caused apoptosis in bovine satellite cells.

Giemsa staining of cells

The Giemsa staining was applied to visualize myotubes and cell nuclei (Velica and Bunce, 2011). Cells used for Giemsa staining were cultured in 24-well culture plates. Before staining, myoblasts were washed twice with 37^{o}C PBS, and then fixed with methanol and PBS mix (1:1) at room temperature for 2 minutes. Cell membranes were permeated with fresh methanol for 10 minutes. To stain the cells, the Giemsa buffer (Ricca Chemical Company, Arlington, TX) was added to the wells as drops. The plates were then incubated at room temperature for 2 minutes with gentle shaking. An equal volume of water was added to each well. The plates were agitated gently for another 2 minutes before being rinsed with ddH_2O and air dried. Images were taken at 10× magnification of at least 15 randomly selected areas from each culture well using a microscope with an Olympus digital camera. The number of nuclei in all cells and number of nuclei in myotubes were counted using ImageJ software (NIH, Bethesda, USA).

Total RNA isolation and real-time RT-PCR

Total RNA from cells was extracted using TRIzol Reagent (Molecular Research Center, Cincinnati, OH), according to the manufacturer's instructions. Nano-Drop 2000 Spectrophotometer (ThermoFisher Scientific, Pittsburgh, PA) was used to determine the quantity of extracted RNA. Real-time reverse transcription polymerase chain reaction (Real-time RT-PCR) was used to measure mRNA expression. Reverse transcription was performed using the Reverse Transcription System from Promega (Madison, WI). Real-time PCR was performed with 10 ng cDNA in a total volume of 10 µl containing 5 µl of iTaq Universal SYBR Green Supermix (Bio-Rad Laboratories, Hercules, CA) and 0.5 µM gene-specific forward and reverse

primers (Table 2.1), under the conditions suggested by the manufacturer. Primers were designed using the Primer-BLAST program (https://www.ncbi.nlm.nih.gov/tools/primer-blast/). Specificity of primers was validated by melting curve analysis and PCR product sequencing. PCR data were analyzed by the $2^{-\Delta\Delta Ct}$ method. Geometric mean of 18S, HMBS and SF3A1 was used for normalization. These genes were chosen as reference genes because their expression was stable in bovine skeletal muscle (Perez et al., 2008; Vandesompele et al., 2002; Zhang et al., 2010). The stable expression of these genes in this study was also confirmed by their consistent Ct values among treatments ($P > 0.1$).

Total cellular protein isolation and sub-cellular fractionation

Cells were lysed on ice for 30 minutes with radio immunoprecipitation assay (RIPA) buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Triton-X 100, 0.25% Na-deoxycholate, 1 mM EDTA, 0.1% SDS) added with protease inhibitors (ThermoFisher Scientific). The lysates were centrifuged at 12,000 ×g for 15 minutes at 4 °C and the supernatants were collected. Protein concentration was measured using a BCA Protein Assay Kit (ThermoFisher Scientific).

Cytosol and membrane fractions were prepared as described previously (McDaniel, et al., 1983; Nicholson and Ferreira, 2009). Cells were scraped off a 6-cm plate in 100 µl fractionation buffer (0.25 M sucrose, 2 mM $MgCl_2$, 10 mM KCl, 1 mM EGTA, 1 mM EDTA, and 20 mM HEPES, pH 7.4), transferred to a microcentrifuge tube, mixed by brief vortexing, and incubated on ice for 10 minutes. Cells were then sonicated on ice using an ultra-sonicate probe (Sonic Dismembrator Model 100, ThermoFisher Scientific) for 10 seconds at power level-2. Cells were incubated on ice for another 10 minutes and sonicated again as before. Samples were centrifuged at 10,000 ×g for 10 minutes. The pellet contained mainly nuclei and

mitochondria while the supernatant contained mainly cytosol and membranes. The supernatant was transferred into a new tube and kept on ice. The pellet was washed once with 200 µl of fractionation buffer and resuspended in 200 µl fractionation buffer added with 5% Triton X-100 by brief sonication. The supernatant was centrifuged in an ultracentrifuge (Beckman Coulter Optimal TL ultracentrifuge, Miami, FL) at 100,000 ×g and 4^{o}C for 1 hour. The supernatant from this centrifugation contained mainly the cytosol and the pellet contained mainly the membrane fraction. The pellet was resuspended in 500 µl of fractionation buffer, and then centrifuged again at 100,000 ×g for 45 minutes at 4^{o}C. The supernatant was discarded and the membrane pellet was resuspended in 50 µl of membrane protein isolation buffer (1.25 M sucrose, 1.5 M NaCl, 0.5M EGTA, 1M Tris,pH 7.6) by brief sonication.

Amplex Red cholesterol quantification assay

Cholesterol contents in culture medium, whole-cell lysates, membrane fraction, nuclear fraction, and cytosol fraction were quantified by the Amplex Red Cholesterol Assay (ThermoFisher Scientific). The cholesterol reference standard was diluted to 25 ng/µl by adding 20 µl standard solution into 1,540 µl 1× reaction buffer. The cholesterol standard curve was based on 0, 4, 8, 12, 16, and 20 µl of 25 ng/µl cholesterol. The total volume of each standard was adjusted to 50 µl with reaction buffer. The total volume of each sample was adjusted to 50 µl with fractionation buffer (Watterson et al., 2013). To each 50 µl standard or sample in a 96-well plate, 50 µl of Amplex Red working solution containing 2 U/ml cholesterol oxidase, 2 U/ml cholesterol esterase, and 2 U/ml horseradish peroxidase were added. Each standard or sample was assayed in duplicate. The plate was incubated at 37°C for 30 minutes while protected from light. Fluorescence was measured with a Tecan Infinite M200 PRO microplate reader (Tecan,

Mannedorf, Switzerland) using excitation at 540 nm and emission detection at 590 nm after the plate had cooled down to room temperature. The background was adjusted by subtracting the value derived from the no-cholesterol negative control. The positive control was prepared by diluting 20 mM H_2O_2 to 10 μM in 1× reaction buffer. Protein content in each sample was measured by a BCA assay kit. Cholesterol concentration in each sample was normalized to its protein concentration.

EdU assay

The effect of treatments on cell proliferation was measured using an EdU assay kit (ThermoFisher Scientific). To determine the effect of MβCD on myoblast proliferation, 1×10^4 bovine myoblasts in 1 ml DMEM containing 10% LPDS were seeded to each well of a 24-well plate. Following incubation overnight, cells were treated with different concentrations of MβCD for 30 minutes. Cells were then cultured in fresh growth medium supplemented with 10 μM EdU for 24 hours. To determine the effects of LPDS on satellite cell proliferation, bovine myoblasts were cultured in DMEM supplemented with 10% FCS or 10% LPDS and 10 μM EdU for 24 hours. To determine the effect of lovastatin on myoblast proliferation, bovine myoblasts were cultured in DMEM supplemented with 10% FCS and different concentrations of lovastatin and 10 μM EdU for 24 hours. To visualize EdU labeling, cells were fixed with 4% paraformaldehyde for 15 minutes and permeated with 0.5% Triton X-100 in PBS for 20 minutes at room temperature. After being washed with PBS three times, the cells were incubated in 1× Click-iT reaction cocktail from the kit for 30 minutes at room temperature. Subsequently, the cells were incubated with Hoechst 33342 (5 μg/ml, 100 μl/well) for 30 minutes to stain all nuclei. EdU labeling and Hoechst 33342 staining were detected with a fluorescence microscope. The EdU

positive cells (red) and Hoechst 33342 positive cells (blue) were counted using NIS software (Nikon, Tokyo, Japan). The EdU incorporation rate was expressed as the ratio of EdU positive cells to total Hoechst 33342 positive cells.

Statistical analyses

Statistical analyses were performed using JMP Pro 13.0 (SAS Institute Inc, Cary, North Carolina). All data are expressed as mean ± SEM (standard error of the mean). The experimental unit was cell culture. The effects of LPDS and FCS on cell proliferation and gene expression were compared using 2-tailed Student's t-test. For data from the lovastatin and MβCD experiments, statistical analysis was performed using one-way ANOVA, followed by the Tukey-Kramer post hoc test. Alpha level was set at 0.05.

RESULTS

Effect of extracellular cholesterol deficiency on cholesterol content in bovine myoblasts

To determine the effect of extracellular cholesterol deficiency on cholesterol content in bovine myoblasts, bovine myoblasts were cultured in medium containing 2% FCS or 2% LPDS for 48 and 72 hours. Cholesterol concentration in 48- or 72-hour culture medium containing 2% FCS was 10 times greater than in medium containing 2% LPDS ($P < 0.05$, Fig. 2.1A). However, total, cytosol, membrane, or nuclear cholesterol content was not different between myoblasts cultured with 2% LPDS and those with 2% FCS for 48 hours (Fig. 2.1B) or 72 hours (Fig. 2.1C). Based on the ratio of the amount of cholesterol to that of protein, the membrane fraction of bovine myoblasts had the highest level of cholesterol, followed by the nuclei and then cytosol (Fig. 2.1).

Effect of extracellular cholesterol deficiency on bovine myoblast proliferation

To investigate the effect of extracellular cholesterol on myoblast proliferation, bovine myoblasts were cultured in the presence of 10% FCS or 10% LPDS for 24 and 48 hours, and proliferating myoblasts were labeled with EdU. Myoblasts cultured with 10% LPDS for 24 hours had 20% less EdU-positive cells than myoblasts cultured with 10% FCS ($P < 0.01$, n = 4, Fig. 2.2). Myoblasts cultured with 10% LPDS for 48 hours had 50% less EdU-positive cells than those cultured with 10% FCS ($P < 0.01$, n = 4, Fig. 2.2). These differences indicated that extracellular cholesterol deficiency inhibited the proliferation of bovine myoblasts.

Effect of extracellular cholesterol deficiency on bovine myoblast differentiation

To evaluate the effect of extracellular cholesterol on myoblast differentiation and fusion, bovine myoblasts were cultured with 2% FCS or 2% LPDS for 24, 48 or 72 hours, and the degree of myogenic differentiation was determined by measuring the mRNA levels of myogenic marker genes. Among 6 myogenic marker genes measured, 5 (MYOG, CKM, TMEM8C, MYL2 and ACTA1) were expressed at higher levels in myoblasts cultured with 2% FCS than with 2% LPDS at 24 hours of differentiation (Fig. 2.3, $P < 0.05$, n = 6). However, none of the marker genes were differentially expressed between 2% FCS and 2% LPDS at 48 hours or 72 hours of differentiation, except for MYH3 and MYL2, which were expressed at higher levels in myoblasts cultured with 2% LPDS than in myoblasts with 2% FCS at 72 hours of differentiation ($P < 0.05$). These data indicated that myoblasts cultured with 2% LPDS were more differentiated than myoblasts with 2% FCS at 24 hours of differentiation, but that they had similar differentiation levels at 48 hours or 72 hours of differentiation.

Effect of lovastatin on cholesterol content in bovine myoblasts

Bovine myoblasts were cultured in medium containing 2% LPDS and 0 (control), 0.2, 1, or 5 μM lovastatin for 48 hours and 72 hours. Treating myoblasts with lovastatin at 0.2 and 1 μM for 48 hours had no effect on total cellular, nuclear, or membrane cholesterol content or medium cholesterol concentration compared to control (Fig. 2.4A). Treating myoblasts with 5 μM lovastatin for 48 hours reduced cytosolic cholesterol content by 30% compared to control (Fig. 2.4A, $P < 0.05$, n = 4) but did not change other subcellular cholesterol contents or medium cholesterol concentration (Fig. 2.4A). Treating myoblasts with 1 μM lovastatin for 72 hours reduced total and cytosolic cholesterol contents ($P < 0.05$, n = 4), but did not alter membrane or nuclear cholesterol content or medium cholesterol concentration (Fig. 2.4B). Compared to

control, treating myoblasts with 5 μM lovastatin for 72 hours reduced total cellular cholesterol content by 30%, cytosolic cholesterol content by 40%, membrane cholesterol content by 20%, (Fig. 2.4B, $P < 0.05$, n = 4), but did not alter nuclear cholesterol content or medium cholesterol concentration (Fig. 2.4B).

Effect of lovastatin on bovine myoblast proliferation

To determine the potential role of intracellular cholesterol in the proliferation of bovine myoblasts, bovine myoblasts were cultured in medium containing 10% LPDS and 0, 0.2, 1, or 5 μM lovastatin for 48 hours and 72 hours. Proliferating cells were identified by EdU labeling. The percentage of EdU-positive myoblasts was not different between myoblasts treated with lovastatin for 24 hours and control myoblasts (Fig. 2.5B). The percentage of EdU-positive myoblasts was lower in myoblasts treated with 5 μM lovastatin for 48 hours than in control myoblasts (Fig. 2.5B, $P < 0.01$, n = 4).

Effect of lovastatin on bovine myoblast differentiation

To determine the potential role of intracellular cholesterol in myoblast differentiation, bovine myoblasts were cultured in medium containing 2% LPDS and 0, 0.2, 1, or 5 μM lovastatin for 24, 48, and 72 hours. Myoblasts differentiated in the presence of 5 μM lovastatin for 72 hours had less mRNA expression for 4 (MYOG, TMEM8C, MYL2 and ACTA1) of 6 differentiation marker genes than did control myoblasts (Fig. 2.6B, $P < 0.05$, n = 6). Myoblasts differentiated in the presence of 0.2 or 1 μM lovastatin for 24 hours or 48 hours had similar mRNA expression for all or the majority of 6 differentiation marker genes as compared with control myoblasts (Fig. 2.6B). Overall, these data indicated that bovine myoblast differentiation

was inhibited by 5 μM lovastatin treatment for 72 hours. Myoblast differentiation was not affected by lovastatin treatment at lower concentrations or for shorter times.

Effect of MβCD on cholesterol content in bovine myoblasts

To determine if MβCD is effective in removing cholesterol from bovine myoblasts, we treated bovine myoblasts in serum-free medium with 0 (control), 0.4, 2, or 10 mM of MβCD for 30 minutes. Myoblasts treated with 10 mM MβCD had 40%, 20%, 50% and 40% less total cellular, cytosolic, membrane, and nuclear cholesterol, respectively, than control myoblasts (Fig.2.7A, $P < 0.01$, n = 4). Cholesterol concentration in culture medium of myoblasts treated with 10 mM MβCD was approximately 30% greater than in control medium (Fig.2.7B, $P < 0.05$, n = 4). Treating myoblasts with 0.4 or 2 mM MβCD had no significant effect on total cellular, cytosolic, membrane or nuclear cholesterol content, although both increased medium concentration of cholesterol (Fig.2.7, $P < 0.05$, n = 4).

Effect of MβCD on bovine myoblast proliferation

To determine the role of membrane cholesterol in myoblast proliferation, bovine myoblasts were treated with 0 (control), 0.4, 2, or 10 mM MβCD for 30 minutes and then cultured in medium containing 10% LPDS for 24 and 48 hours. Proliferating myoblasts were identified by EdU incorporation. Treating myoblasts with 10 mM MβCD for 30 minutes reduced ($P < 0.05$, n = 4) the percentage of EdU-positive cells at 24 hours of culture but not at 48 hours of culture compared to control (Fig. 2.8B). Treating myoblasts with 2 mM MβCD for 30 minutes reduced ($P < 0.05$, n = 4) the percentage of EdU-positive cells at 48 hours of culture but not at 24

hours of culture (Fig. 2.8B). Treating myoblasts with 0.4 mM MβCD had no effect on the percentage of EdU-positive cells at either 24 or 48 hours of culture (Fig. 2.8B).

Effect of MβCD on bovine myoblast differentiation

To investigate the potential role of membrane cholesterol in myoblast differentiation, we differentiated bovine myoblasts in medium containing 2% LPDS for 24, 48, and 72 hours and treated the cells with 0 (control), 0.4, 2, or 10 mM MβCD in the first 30 minutes of every 24 hours. Myoblasts treated with 10 mM MβCD appeared to form fewer and smaller myotubes than control myoblasts by 24, 48, or 72 hours of differentiation (Fig. 2.9A). At least 4 (MYOG, CKM, TMEM8C and ACTA1) of 6 myogenic differentiation marker genes were expressed at lower levels ($P < 0.05$, n = 4) in myoblasts treated with 10 mM MβCD than in control myoblasts at 24, 48, or 72 hours of differentiation (Fig. 2.9B). The expression levels for the majority of these marker genes were not different between myoblasts treated with 0.4 or 2 mM MβCD and control myoblasts at 24, 48, or 72 hours of differentiation (Fig. 2.9B). Overall, the data showed that treating myoblasts with 10 mM MβCD inhibited bovine myoblast differentiation and myotube formation.

DISCUSSION

In this study, we assessed the importance of extracellular cholesterol, intracellular cholesterol, and plasma membrane cholesterol to bovine myoblast proliferation and differentiation. Culturing bovine myoblasts with cholesterol-deficient fetal calf serum (LPDS) did not change their intracellular or membrane cholesterol content. This result together with the in vivo observation that skeletal muscle cholesterol content in cattle is not affected by dietary cholesterol intake (Bohac and Rhee, 1988), suggest that extracellular cholesterol availability has little effect on cholesterol content and distribution in bovine muscle cells. However, bovine myoblast proliferation was inhibited in LPDS compared to that in normal fetal calf serum. This data suggests that extracellular cholesterol may be beneficial to the proliferation of bovine myoblasts. Because LPDS did not change the intracellular or membrane cholesterol content in myoblasts, the inhibitory effect of LPDS on myoblast proliferation is unlikely mediated through intracellular or membrane cholesterol. Cholesterol has been recently discovered to be the long-sought activator of the G-protein coupled receptor Smoothened (Luchetti et al., 2016). Signals from Smoothened can affect cell proliferation (Gritli-Linde et al., 2002). Thus, it is possible that extracellular cholesterol affects bovine myoblast proliferation through Smoothened. Lipoprotein-deficient serum is deficient in phospholipids and triglyceride in addition to cholesterol compared to normal serum. Therefore, we cannot rule out the possibility that myoblast proliferation is inhibited in LPDS due to changed availability of phospholipids or triglyceride. Compared to differentiation in normal serum, differentiation pf bovine myoblasts in LPDS was enhanced in the first 24 hours of culture but was not different in the second or third 24 hours. Chicken cardiac cells cultured in LPDS did not display any detectable morphological change or protein content difference as compared with cells cultured in normal fetal calf serum (Renaud et al., 1982). Thus,

extracellular cholesterol is probably not important to myoblast differentiation. The initial enhancement in myoblast differentiation was probably secondary to the effect of LPDS on myoblast proliferation rather than a direct effect of cholesterol deficiency.

Statins have been widely used as HMGCR inhibitors to reduce cholesterol in humans with hypercholesterolemia (Istvan and Deisenhofer, 2001). In this study, we found that treating bovine myoblasts with 5 μM lovastatin for 48 hours caused a significant reduction in cytosolic cholesterol content and that treating bovine myoblasts with 5 μM lovastatin for 72 hours caused a significant reduction not only in cytosolic but also membrane cholesterol content. These results suggest that lovastatin is effective in inhibiting the bovine HMGCR. These results also suggest that membrane cholesterol homeostasis in bovine myoblasts, like in other cells (Yokoyama, 2000), is maintained by cytosolic synthesis of cholesterol. Our study demonstrated that lovastatin-inhibited intracellular synthesis of cholesterol was associated with reduced differentiation in bovine myoblasts, suggesting that intracellular cholesterol synthesis is necessary for differentiation of bovine myoblasts. This finding agrees with previous results from inhibiting cholesterol synthesis using simvastatin in rat L6 myoblasts (Matzno et al., 1997), and zebrafish myoblasts (Campos et al., 2015). In this study, we also found that lovastatin-inhibited cholesterol synthesis in bovine myoblasts was associated with inhibited proliferation. Thus, not only differentiation but also proliferation in bovine myoblasts requires intracellular cholesterol synthesis.

Methyl-β-cyclodextrin (MβCD) is the most widely used agent to deplete cholesterol from plasma membrane of cells (Mahammad and Parmryd, 2015). In this study, a short incubation with MβCD caused a significant reduction in membrane cholesterol content in bovine myoblasts

and a significant increase in medium cholesterol concentration. These changes demonstrate that MβCD is highly effective in removing cholesterol from bovine myoblast membrane. In this study, MβCD reduced not only cholesterol content in membrane but also cholesterol content in cytosol of bovine myoblasts. These changes suggest the existence of an equilibrium between plasma membrane cholesterol and cytosolic cholesterol in bovine myoblasts. A short MβCD treatment inhibited proliferation, differentiation, and fusion in bovine myoblasts. These effects of MβCD are consistent with the effects of inhibiting cholesterol synthesis and cholesterol deposition in membrane with lovastatin, suggesting that membrane cholesterol, cytosolic cholesterol, or both are important to bovine myoblast proliferation, differentiation, and fusion. Intriguingly, MβCD depletion of membrane cholesterol stimulated proliferation, differentiation, and fusion in chicken myoblasts (Mermelstein et al., 2007; Possidonio et al., 2014). Thus, the role of membrane or intracellular cholesterol in myoblast proliferation, differentiation, and fusion may be species dependent. However, at least one study demonstrated a similar beneficial role of membrane cholesterol to differentiation and fusion in chicken myoblasts (Cornell et al., 1980). One limitation of using MβCD to remove cholesterol from cell membrane is that it is not specific for cholesterol, and its effects depend on its dose and length of action (Mahammad and Parmryd, 2015). Thus, the conflicting effects of MβCD on myoblast proliferation and fusion between studies could reflect the differences in the dose and/or length of MβCD treatment.

In this study, treating the bovine myoblasts with MβCD and lovastatin both led to inhibition of proliferation and differentiation in bovine myoblasts. These overlapping results from using different approaches further support the function of cholesterol in bovine myoblast proliferation and differentiation. Because both membrane and cytosolic cholesterol contents are altered following lovastatin and MβCD treatments, we cannot attribute the changes in myoblast

proliferation or differentiation to membrane or cytosolic cholesterol. As a primary structural component of animal cell membranes, depletion of membrane cholesterol interferes with the interactions between fatty acid chains, thereby increasing the stiffness of cell membranes (Cooper, 1978). Moreover, depletion of membrane cholesterol may also affect the function of membrane receptors, transporters, and channels (Ridgway and McLeod, 2015; Guerra et al., 2016; Sekine et al., 2010). How intracellular cholesterol affects proliferation, differentiation and fusion in bovine myoblasts independent of membrane cholesterol is not obvious, but a recent study indicates that intracellular cholesterol mediates differentiation in C2C12 myoblasts through the nuclear receptor estrogen related receptor α (ERRα)(Wei et al., 2016).

In summary, this study indicates that extracellular cholesterol deficiency inhibits proliferation in bovine myoblasts without affecting the intracellular or membrane cholesterol content, and that reducing membrane and intracellular cholesterol contents through lovastatin or MβCD inhibits the proliferation and differentiation in bovine myoblasts. These results imply the importance of de novo cholesterol synthesis or proper cholesterol supply to skeletal muscle development and growth in cattle.

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Table 2.1. Nucleotide sequences of the primers used for real-time RT-PCR

Gene	Direction	Primer sequence	GenBank Accession #
SF3A1	Forward	5’-GCGGGAGGAAGAAGTAGGAG-3’	NM_001081510.1
	Reverse	5’-TCAGCAAGAGGGACACAAA-3’	
18S	Forward	5’-GTAACCCGTTGAACCCATT-3’	NR_036642
	Reverse	5’- CCATCCAATCGGTAGAGCG-3’	
HMBS	Forward	5’-CTTTGGAGAGGAATGAAGTGG-3’	NM_001046207.1
	Reverse	5’-AATGGTGAAGCCAGGAGGAA-3’	
MYOG	Forward	5’-TGGGCGTGTAAGGTGTGTAA-3’	NM_001111325
	Reverse	5’-TATGGGAGCTGCATTCACTG-3’	
MYH3	Forward	5’-CTGGAGGAAATGAGGGATGA-3’	NM_001101835.1
	Reverse	5’-CACTCTTGAGAAGGGGCTTG-3’	
CKM	Forward	5’-TGGAGATGATCTGGACCCCA-3’	NM_174773.4
	Reverse	5’-TTTCCCCTTGAACTCACCCG-3’	
TMEM8C	Forward	5’-GCTCGGCCATCCTCATCATT-3’	XM_015473661.1
	Reverse	5’-GTCCCAGTCCTCGAAGAAGAA-3’	
ACTA1	Forward	5’-AATCGGCGGGAATCCATGAG-3’	XM_005226192.2
	Reverse	5’-CAGGTGGGGCGATGATCTTGA-3’	
MYL2	Forward	5’-TCGGGGAGAAACTTAAAGGAGC-3’	XM_005217753.3
	Reverse	5’-AGTTGCCAGTCACATCAGGG-3’	

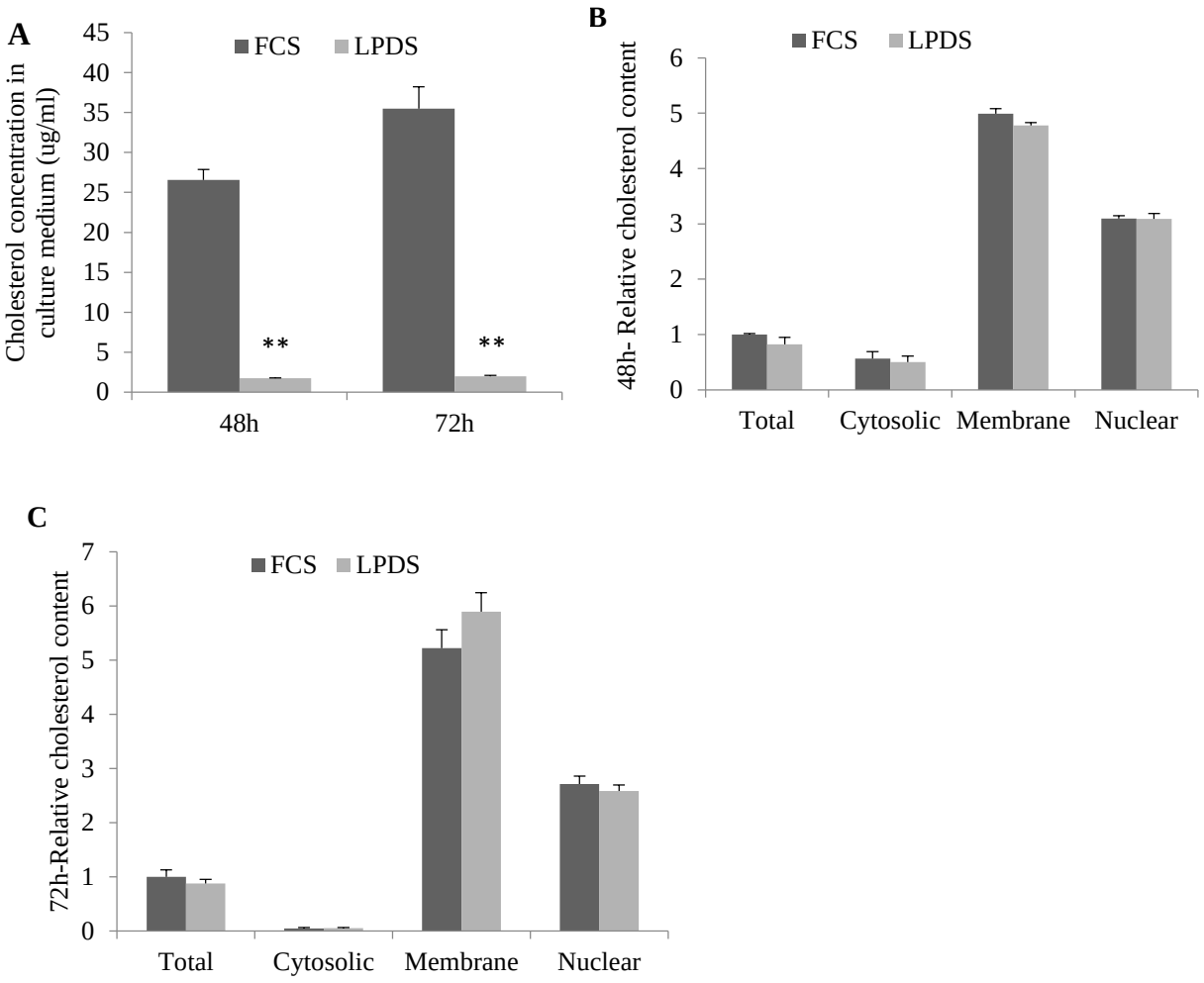


Figure 2.1. Extracellular cholesterol deficiency had no effect on cellular cholesterol content in bovine myoblasts. Myoblasts were cultured in medium supplemented with 2% fetal calf serum (FCS) or lipoprotein-deficient fetal calf serum (LPDS) for 48 and 72 hours. (A) Cholesterol concentration in culture medium. ** $P < 0.01$ within time, n = 4. (B) Relative cholesterol content in total lysates, cytosolic, membrane, and nuclear fractions at 48 hours of culture. The concentration of cholesterol in each subcellular fraction was normalized to that of protein. No difference was detected within each category ($P > 0.1$, n = 4). (C) Relative cholesterol content in

total cell lysates, cytosolic, membrane, and nuclear fractions at 72 hours of culture. No difference was detected within each category ($P > 0.1$, n = 4).

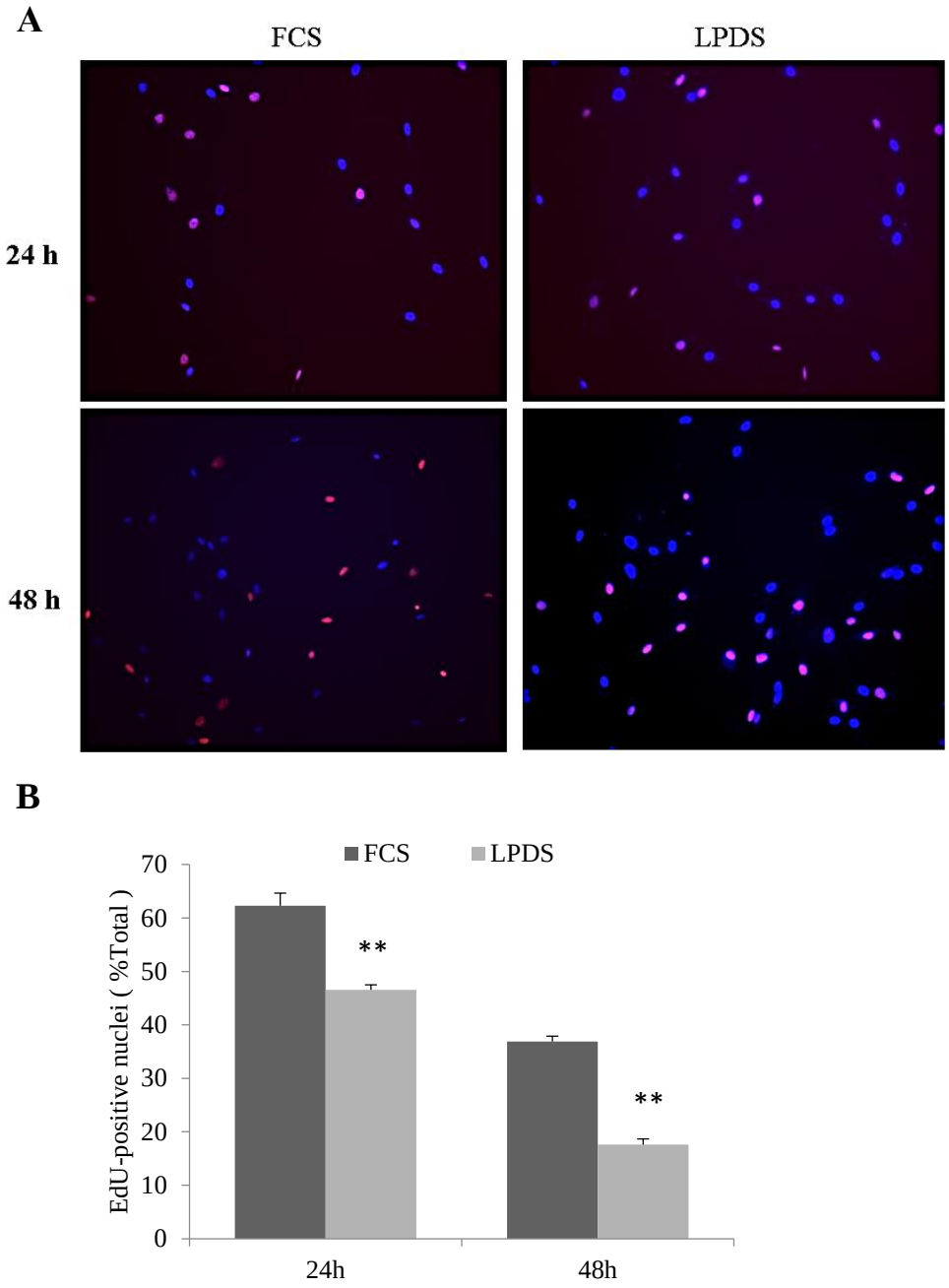
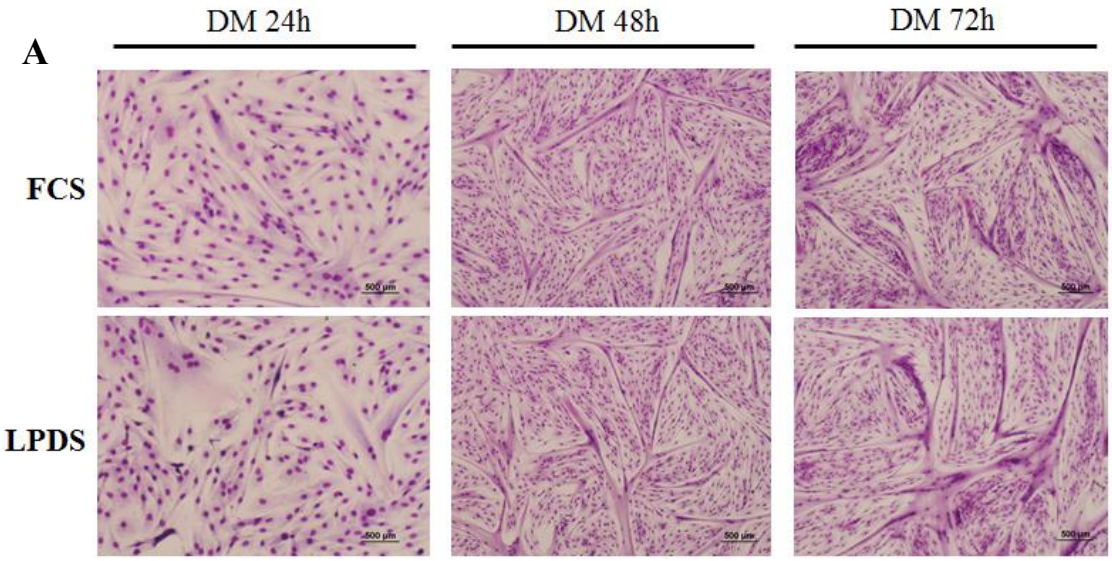
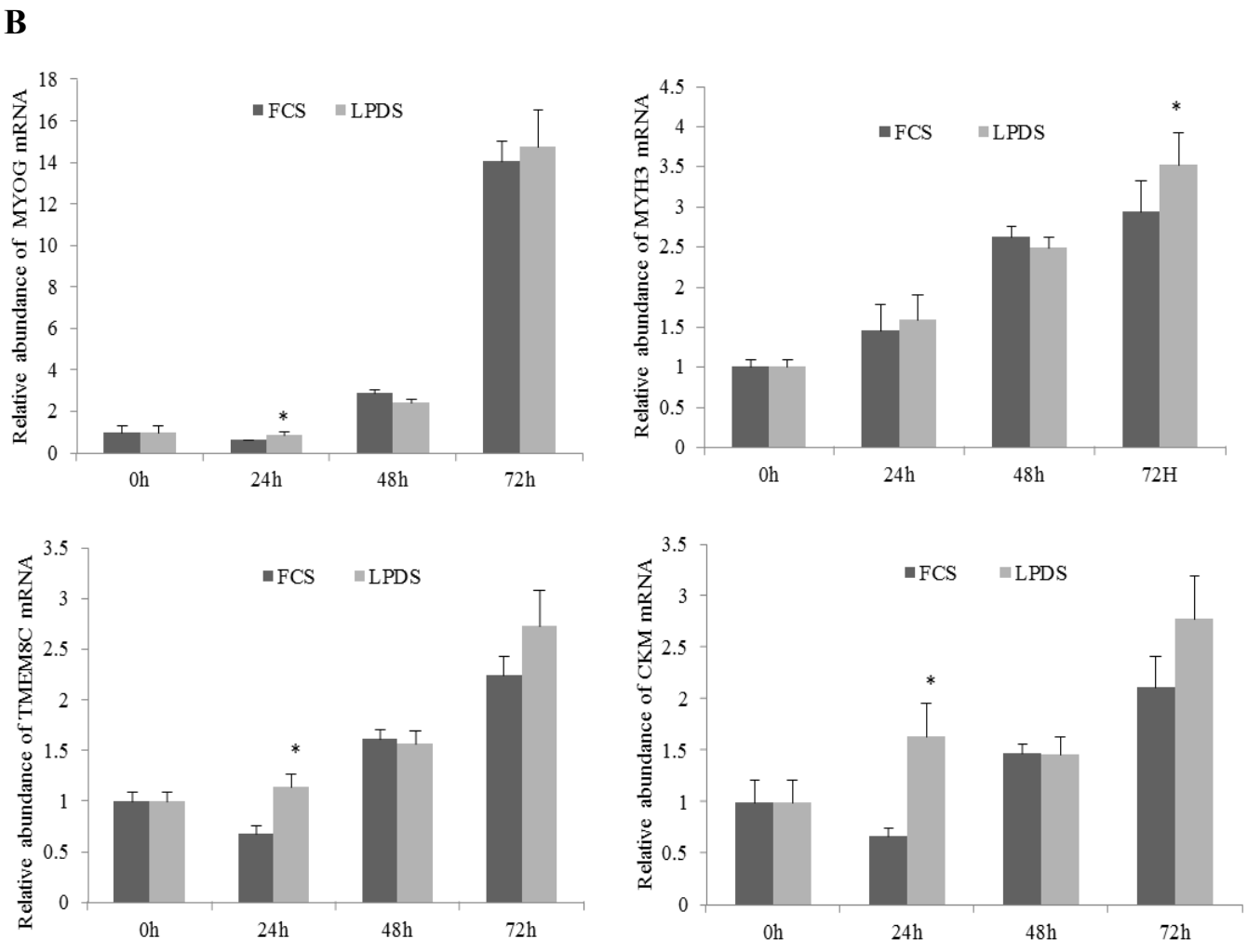


Figure 2.2. Extracellular cholesterol deficiency inhibited the proliferation of bovine myoblasts. Cells were cultured in medium containing 10% FCS or 10% LPDS for 24 and 48 hours. (A) Representative images of stained cells. Nuclei of proliferating cells were labeled with EdU (red). All nuclei were labeled with Hoechst 33342 (blue). (B) Percentage of EdU-positive cells. ** $P <$ 0.01 within time, n = 4.


A
DM 24h
DM 48h
DM 72h
FCS
LPDS


B
Relative abundance of MYOG mRNA
FCS
LPDS
0h
24h
48h
72h
Relative abundance of MYH3 mRNA
72H
Relative abundance of TMEM8C mRNA
Relative abundance of CKM mRNA

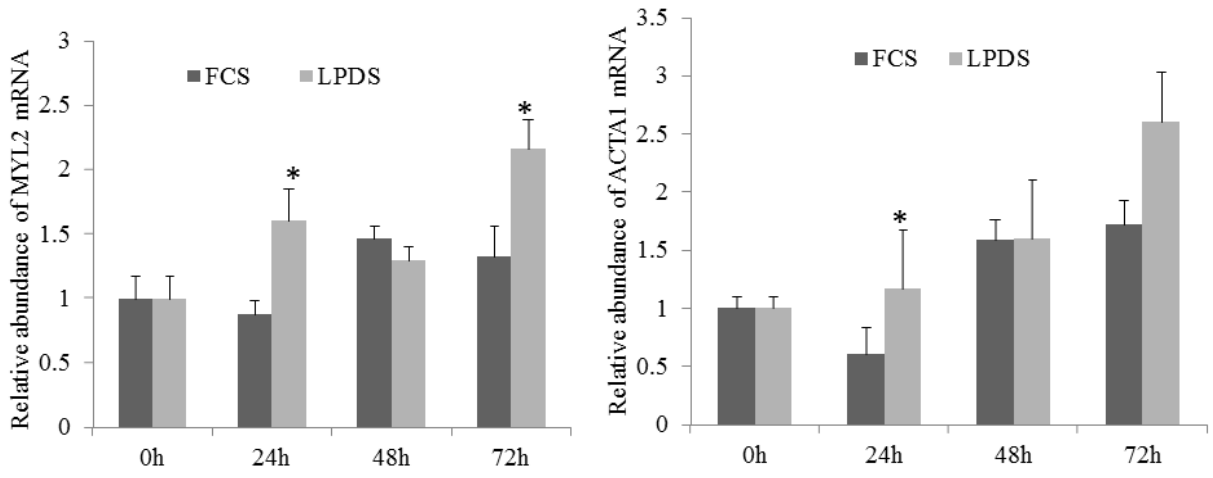


Figure 2.3. Effects of extracellular cholesterol deficiency on bovine myoblast differentiation. Myoblasts were differentiated in medium containing 2% FCS or 2% LPDS for 0, 24, 48 and 72 hours. (A) Representative pictures of Giemsa-stained myoblasts at 24, 48, and 72 h of differentiation. (B) The relative expression levels of myoblast differentiation marker genes MYOG, CKM, TMEM8C, MYH3, MYL2 and ACTA1 at 0, 24, 48, and 72 h of differentiation (* $P < 0.05$ within time, n = 6).

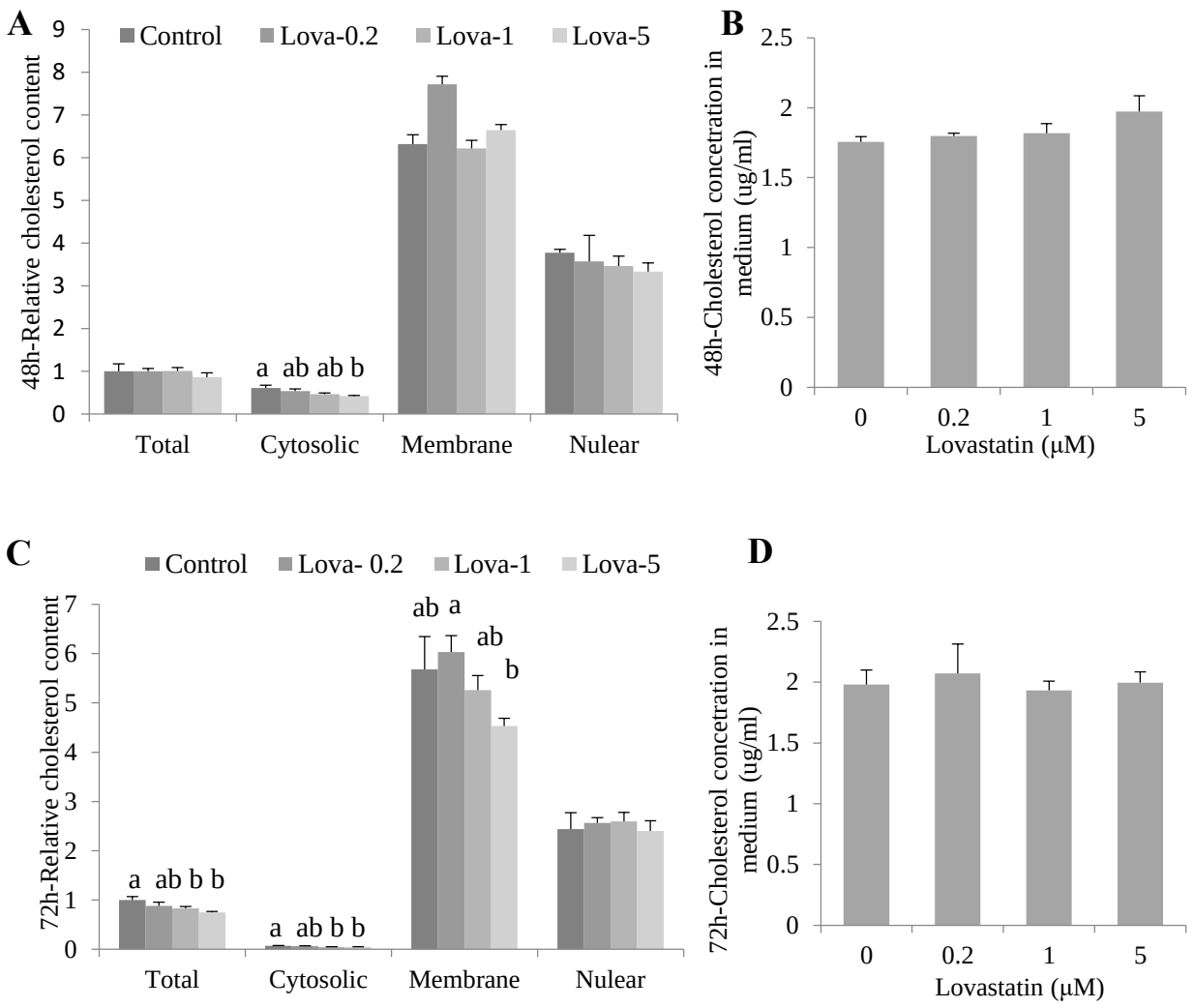


Figure 2.4. Effect of lovastatin on cholesterol content in myoblasts. Bovine myoblasts were cultured in medium containing 2% LPDS and 0, 0.2, 1, or 5 μM lovastatin for 48 and 72 hours. Cholesterol content in total lysates, cytosolic, membrane, and nuclear fractions and cholesterol concentration in culture medium were analyzed. (A) Cholesterol content and concentration at 48 hours of treatment. Means labeled with different letters are statistically different within category ($P < 0.05$, n = 4). (B) Medium cholesterol concentration at 48 hours of treatment ($P > 0.1$, n = 4). (C) Cholesterol content and concentration at 72 hours of treatment. Means labeled with different

letters are statistically different within category ($P < 0.05$, n = 4). (D) Medium cholesterol concentration at 72 hours of treatment ($P > 0.1$, n = 4).

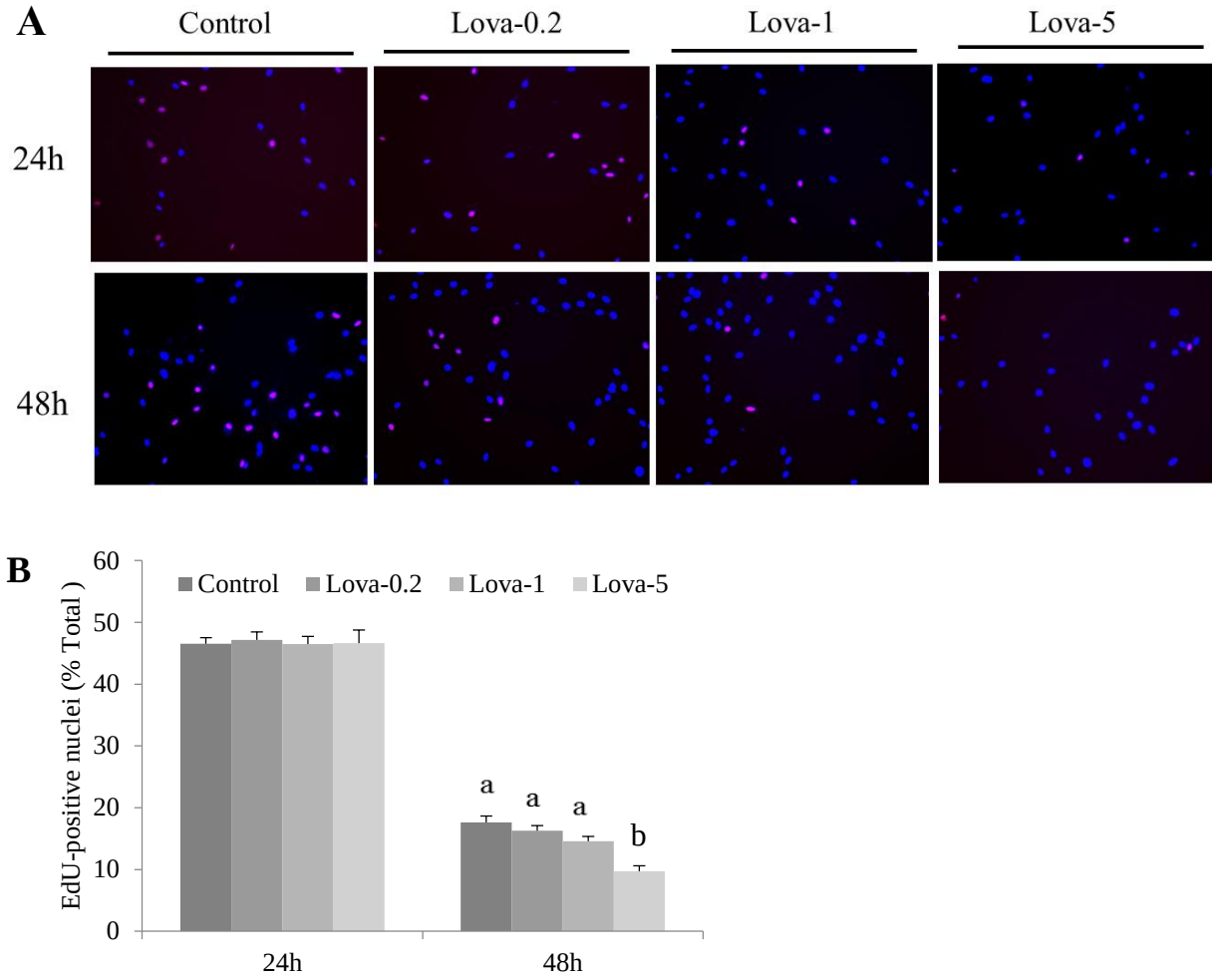
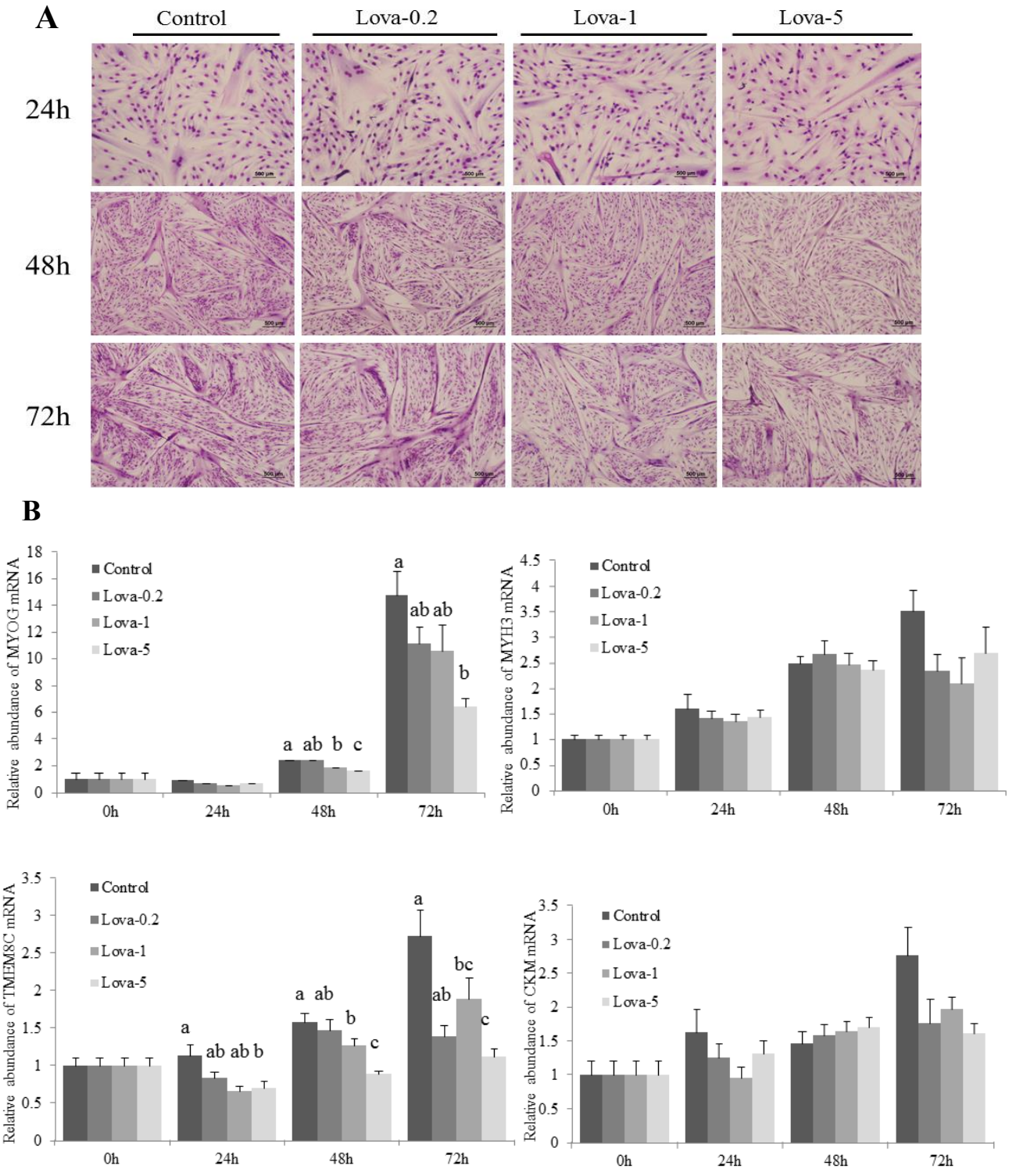


Figure 2.5. Effect of lovastatin on bovine myoblast proliferation. Cells were cultured with 0 (control), 0.2, 1, or 5 μM lovastatin in medium containing 10% LPDS for 24 and 48 hours. Proliferating cells were stained with EdU (red). All nuclei were stained with Hoechst 33342 (blue). (A) Representative images of stained cells. (B) Percentage of EdU-positive myoblasts. Bars labeled with different letters are different within time ($P < 0.05$, n = 4).


A
Control
Lova-0.2
Lova-1
Lova-5
24h
48h
72h
B
Relative abundance of MYOG mRNA
Relative abundance of MYH3 mRNA
Relative abundance of TMEM8C mRNA
Relative abundance of CKM mRNA
Control
Lova-0.2
Lova-1
Lova-5
0h
24h
48h
72h

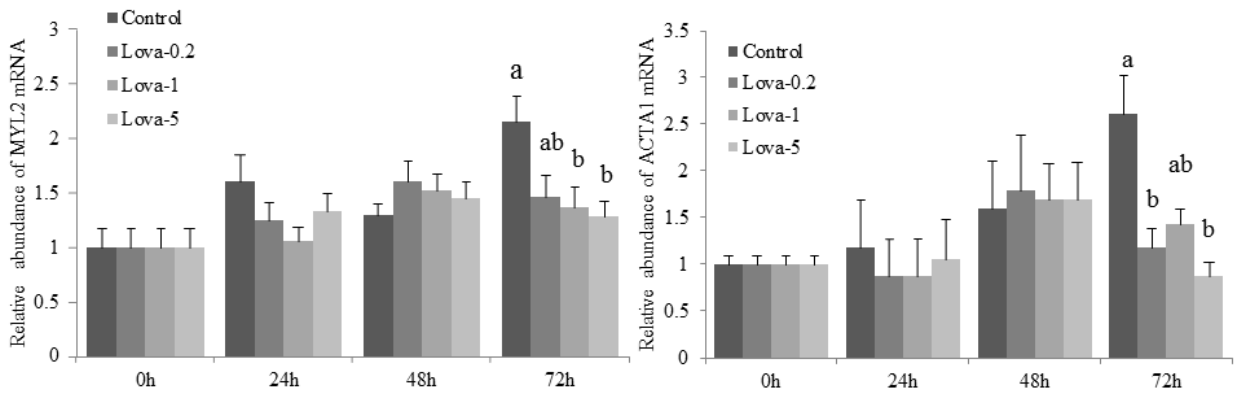


Figure 2.6. Effect of lovastatin on differentiation of bovine myoblasts. Myoblasts were induced to differentiate in medium containing 2% LPDS in the presence of 0 (control), 0.2, 1, or 5 μM lovastatin for 0, 24, 48 and 72 hours. (A) Representative images of Giemsa-stained cells. (B) Relative mRNA abundance of myoblast differentiation marker genes. Bars labeled with different letters are different within time ($P < 0.05$, n = 6).

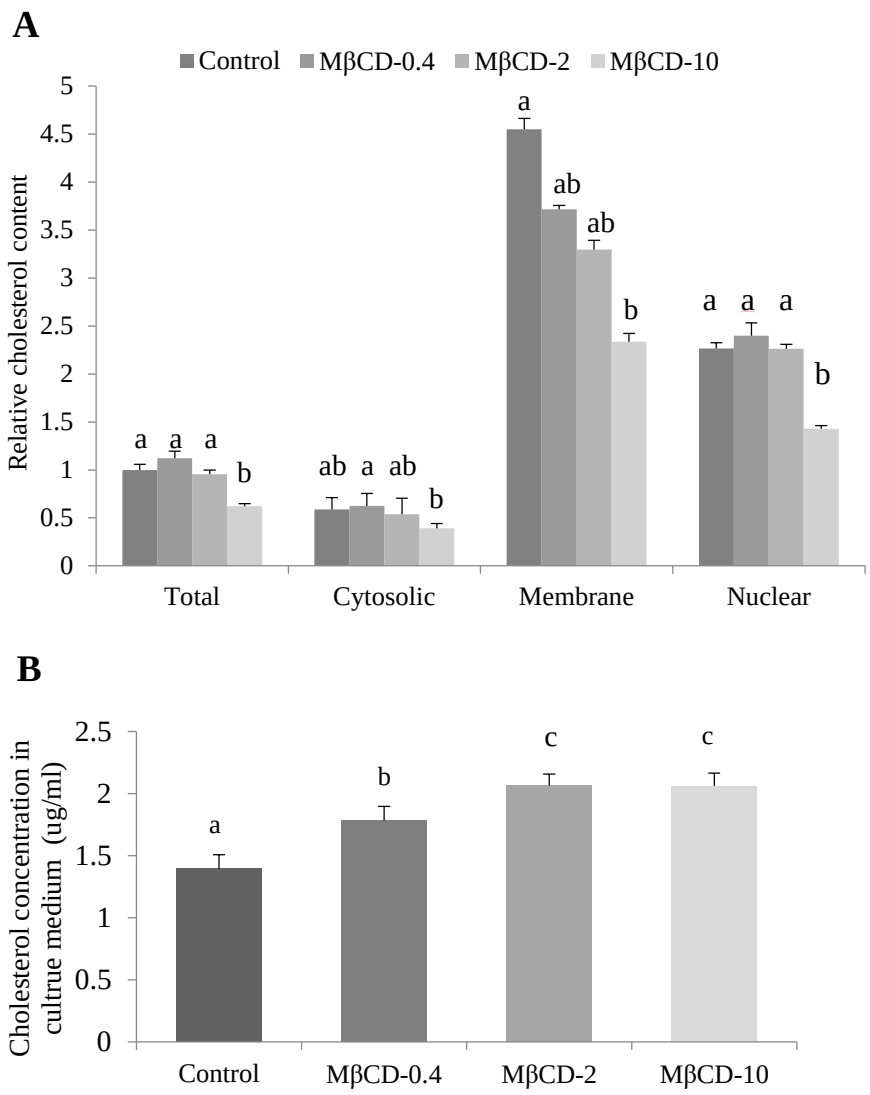


Figure 2.7. Effect of MβCD on cholesterol content in bovine myoblasts. Myoblasts were treated with 0 (control), 0.4, 2, or 10 mM MβCD for 30 minutes. (A) Cholesterol content and concentration after MβCD treatment. Bars labeled with different letters are different within category ($P < 0.05$, n = 4). (B) Medium cholesterol concentration. Bars labeled with different letters are different ($P < 0.05$, n = 4).

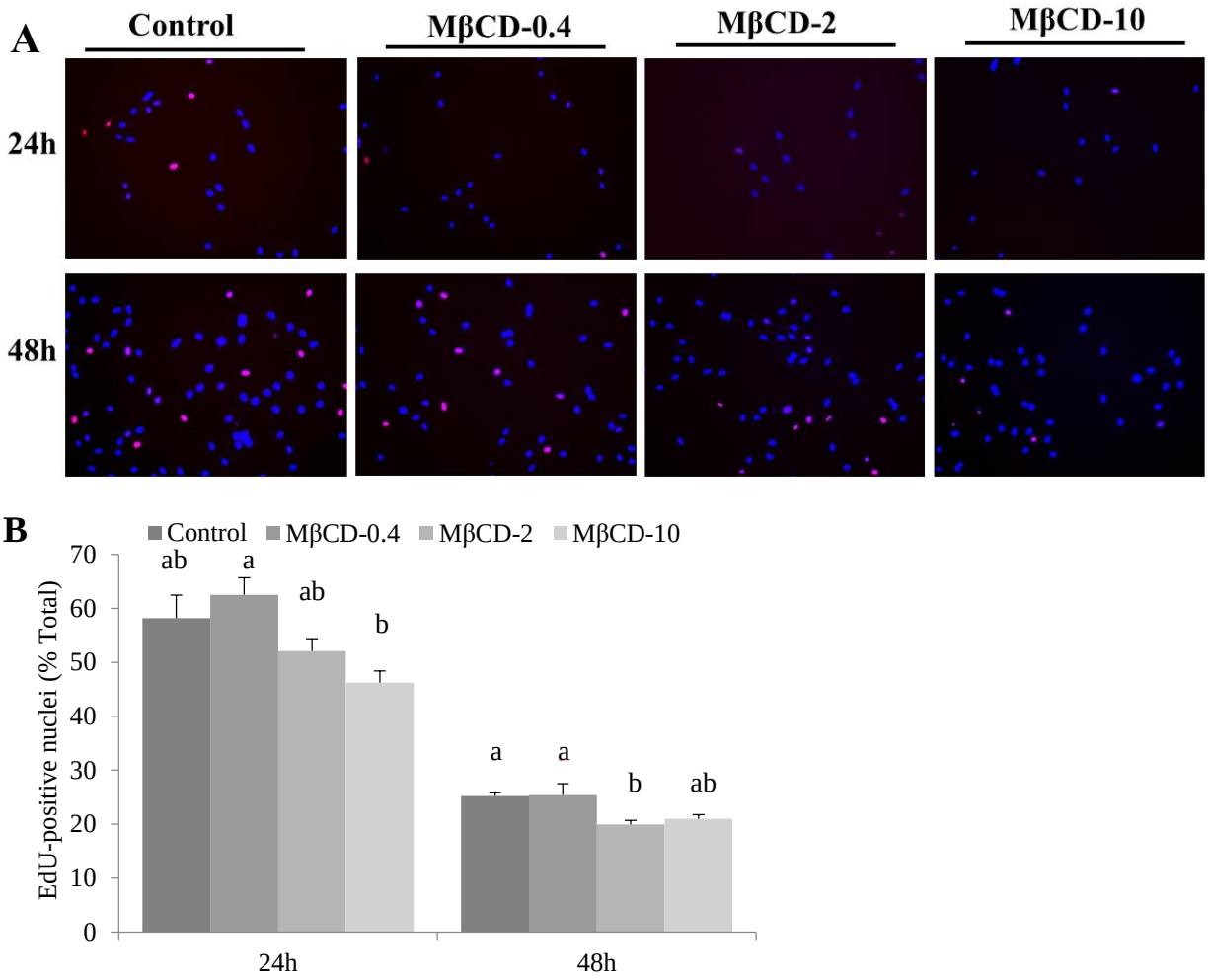
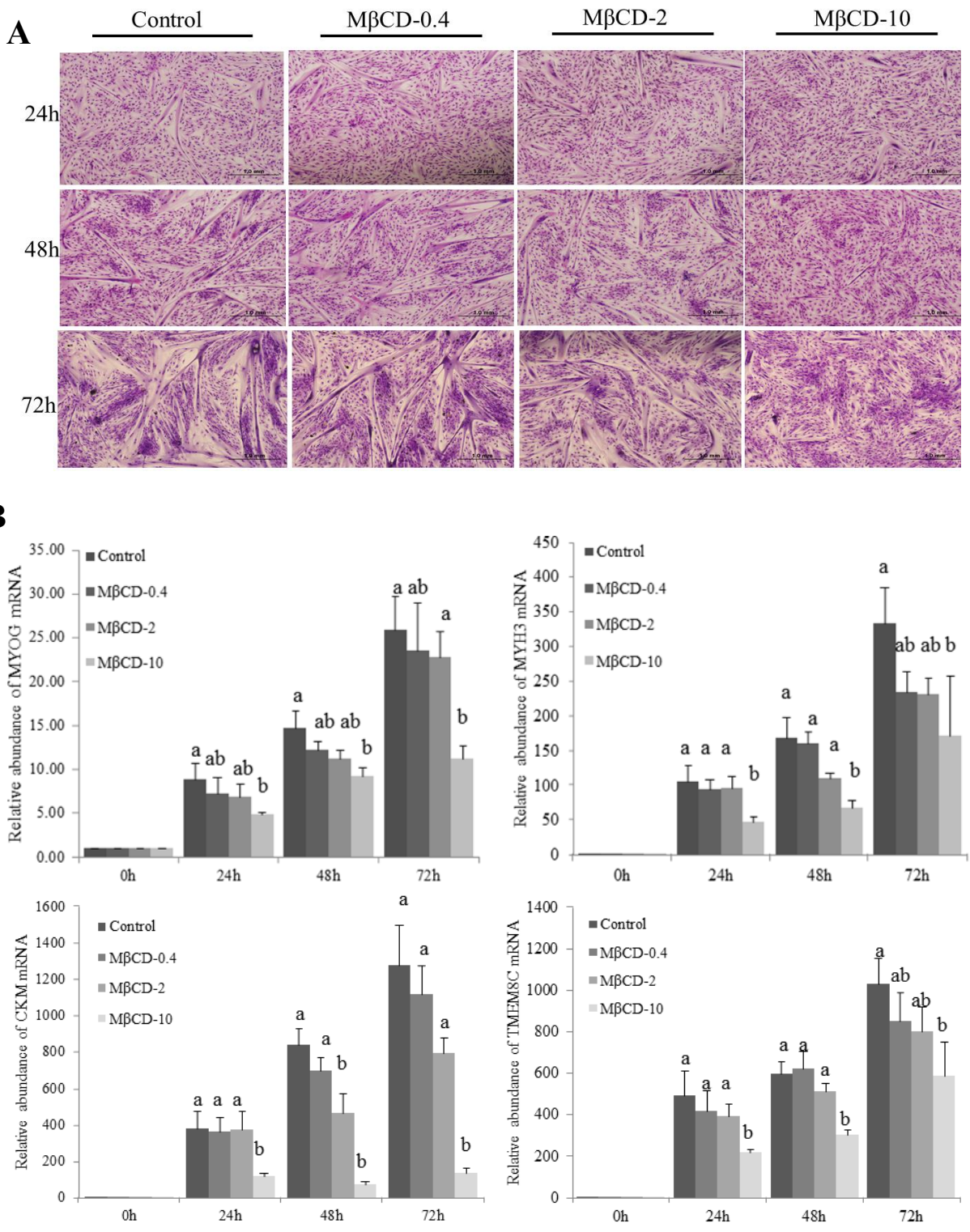


Figure 2.8. Effect of MβCD on bovine myoblast proliferation. Myoblasts were treated with indicated concentrations of MβCD for 30 minutes and then cultured in medium containing 10% LPDS for 24 and 48 hours. (A) Representative images of cells. All nuclei stained for Hoechst 33342 (blue). Proliferating cells stained for EdU (red). (B) Percentage of EdU-positive cells. Bars labeled with different letters are different within time ($P < 0.05$, n = 6).


A
Control
MβCD-0.4
MβCD-2
MβCD-10
24h
48h
72h
B
Relative abundance of MYOG mRNA
Relative abundance of MYH3 mRNA
Relative abundance of CKM mRNA
Relative abundance of TMEM8C mRNA
Control
MβCD-0.4
MβCD-2
MβCD-10
0h
24h
48h
72h

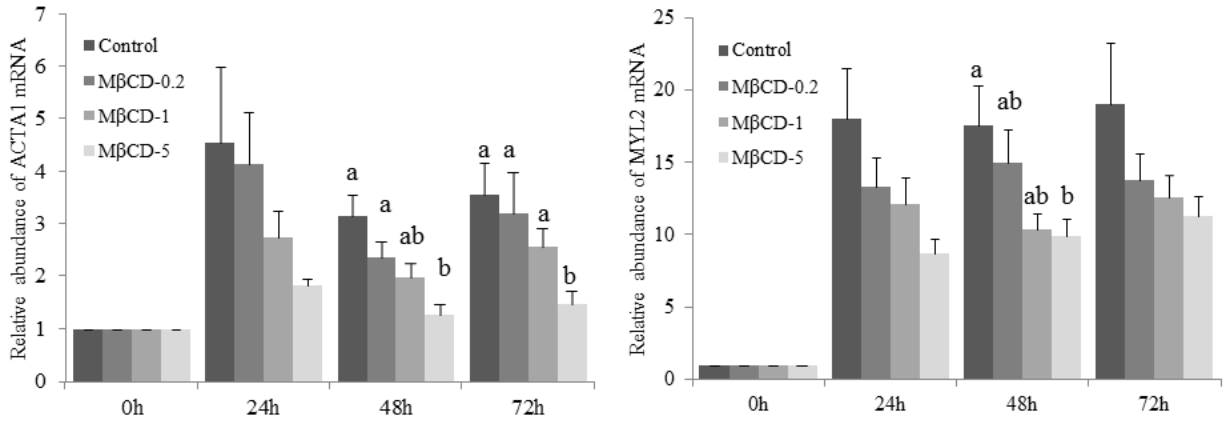


Figure 2.9. Effect of MβCD on differentiation of bovine myoblasts. Myoblasts were induced to differentiate in medium containing 2% horse serum for 0, 24, 48 and 72 hours. Myoblasts were treated with indicated concentrations of MβCD in serum-free medium in the first 30 minutes of every 24 hours. (A) Representative images of Giemsa-stained cells. (B) Relative expression levels of 6 differentiation marker mRNAs (MYOG, CKM, TMEM8C, MYH3, MYL2 and ACTA1) at 0, 24, 48, and 72 hours of differentiation. Bars not sharing the same letter labels are different within time ($P < 0.05$, n = 6).