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A DISSERTATION

FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

Effects of castration, cold temperature, and dietary glycerol on gluconeogenesis and lipid metabolism, growth performance, and meat quality in Korean cattle

거세, 저온 환경 및 사료 내 글리세롤 첨가가 한우의
gluconeogenesis와 지방대사, 성장능력 및 육질에 미치는 영향 연구

by

Dilla Mareistia Fassah

DEPARTMENT OF AGRICULTURAL BIOTECHNOLOGY

GRADUATE SCHOOL

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Effects of castration, cold temperature, and dietary glycerol on gluconeogenesis and lipid metabolism, growth performance, and meat quality in Korean cattle

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Overall Summary

Castration of bulls results in better beef and carcass quality through increased fat deposition. The deposition of intramuscular fat required incorporation of triglyceride in muscle, after the absorption of dietary fatty acids or *de novo* synthesis of fatty acid. Intramuscular adipose tissue uses a high proportion of glucose for fatty acid synthesis, either to supply a carbon source or to produce NADPH. Testosterone deficiency is associated with an increased in fat mass, reduced insulin sensitivity, and impaired glucose metabolism. Therefore, alteration of glucose and lipid metabolism following castration may affect beef quality.

Performance of the cattle is influenced by the climatological factors, such as ambient temperature. Temperature stress occurs when the ambient temperature is higher or lower than that of the cattle's thermo-neutral zone. Cold stress increases the metabolic rate and maintenance requirement of cattle, which may lead to reduced performance and productivity. Increasing the energy density in the diet is an effective strategy to maintain the productivity of stressed cattle. Glycerol, a by-product of biodiesel production can be used as a glucogenic precursor to improve energy status of cattle. It is preferable since fat source from animal or plant origin has some limitation to be used in the ruminant diet. The fat content in ruminant diet is not recommended to exceed 5% since it may inhibit rumen fermentation.

Limited information is available about the effects of castration, cold temperature, and dietary glycerol on gluconeogenesis and lipid metabolism, growth performance, and meat quality in Korean cattle. Thus, this study was conducted to 1) examine effects of castration of bulls on transcriptional changes in the genes involved in gluconeogenesis and glycolysis pathways and 2) to investigate effects of dietary glycerol and cold temperature on growth performance, rumen characteristics, blood metabolites, carcass characteristics, meat quality and expression gluconeogenesis and lipid metabolism of in finishing Korean cattle steers.

In study 1, castration increased the mRNA (3.6 fold; $P < 0.01$) and protein levels (1.4 fold; $P < 0.05$) of *pyruvate carboxylase* (*PC*) and *mitochondrial phosphoenolpyruvate carboxykinase* genes (*PCK2*; 1.7 fold; $P < 0.05$). Hepatic mRNA levels of genes encoding the glycolysis enzymes were not changed by castration. Castration increased mRNA levels of both *lactate dehydrogenase A* (*LDHA*; 1.5 fold; $P < 0.05$) and *lactate dehydrogenase B* (*LDHB*; 2.2 fold; $P < 0.01$) genes for lactate utilization. Castration increased mRNA levels of *glycerol kinase* (*GK*; 2.7 fold; $P < 0.05$) and *glycerol-3-phosphate dehydrogenase 1* (*GPD1*; 1.5 fold; $P < 0.05$) genes for glycerol utilization. Castration also increased mRNA levels of *propionyl-CoA carboxylase beta* (*PCCB*; 3.5 fold; $P < 0.01$) and *acyl-CoA synthetase short chain family member 3* (*ACSS3*; 1.3 fold; $P = 0.06$) genes for propionate incorporation.). Positive correlations were found between hepatic *fructose 1,6-biphosphatase* (*FBP1*), *LDHA, propionyl-CoA carboxylase alpha, mitochondrial* (*PCCA*), *GK, and*

GPD1 gene expressions with backfat thickness. Hepatic gluconeogenic gene (*PC, PCK2, LDHA, LDHB,* and *PCCB*) expression levels were associated with marbling score (MS) and/or quality grade (QG).

In study 2, the ambient temperature in December was lower ($P < 0.001$) than those in other months. Neither month nor glycerol supplementation (GS) affected ($P > 0.05$) ADG and feed efficiency. Cold weather affected ($P < 0.001$) the amount of concentrate intake and total feed intake, whereas GS decreased ($P < 0.05$) the concentrate intake. Cold weather increased ($P < 0.05$) ruminal pH and ammonia nitrogen (NH_3-N) concentration. Cold weather decreased ($P < 0.05$) total volatile fatty acid (VFA) molar concentration, and the molar proportions of propionate and valerate, while it increased ($P < 0.05$) molar proportion of butyrate. Glycerol supplementation increased ($P<0.05$) the valerate molar concentration. Cold weather affected ($P < 0.05$) serum concentrations of glucose, cholesterol, and triglyceride. Glycerol supplementation increased ($P < 0.05$) MS and color lightness (CIE L^*) of *longissimus thoracis* (LT), while GS decreased ($P < 0.05$) ultimate pH of beef. Glycerol supplementation increased flavor and overall acceptance ($P < 0.05$) of LT. Glycerol supplementation did not change mRNA levels of hepatic gluconeogenesis genes. However, it tended to upregulate ($0.05 < P \leq 0.10$) *fatty acid translocase* (*CD36*), *lipoprotein lipase* (*LPL*), and *fatty acid binding protein 4* (*FABP4*) genes in LT. These results indicate that cold condition did not affect growth performance of Korean cattle, although some changes in blood metabolites and ruminal parameters were observed. Diet with 3.15% GS

of concentrate DM did not affect growth performance, ruminal VFA concentrations, and blood metabolites. However, GS increased MS and sensory traits. Glycerol supplementation could be used to improve beef quality during cold conditions without affecting performance.

From this study, it can be concluded that beef quality can be improved by castration and dietary glycerol supplementation, which influence hepatic gluconeogenesis and lipid metabolism of Korean cattle.

Key words: Glycerol, glucose metabolism, lipid metabolism, performance, carcass characteristics, meat quality, sensory quality, cold temperature, Korean cattle steers

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ACC: acetyl CoA carboxylase

ACS: acyl CoA synthetase

ACSS3: acyl-CoA synthetase short-chain family member 3

ADF: acid detergent fiber

ADG: average daily gain

AGPAT1: 1-acylglycerol-3-phosphate O-acyltransferase 1

ATGL: adipose triglyceride lipase

ATP: adenosine tri phosphate

BMS: beef marbling score standard

BSE: bovine spongiform encephalopathies

BW: body weight

CD36: fatty acid translocase CD36

CP: crude protein

DDGS: distiller's dried grains with solubles

DGAT1: diacylglycerol O-acyltransferase 1

DHAP: dihydroxy acetone phosphate

DLAT: dihydrolipoamide S-acetyltransferase

DLD: dihydrolipoamide dehydrogenase

DM: dry matter

DNA: deoxyribonucleic acid

EE: ether extract

FABP: fatty acid binding protein

FABP4: fatty acid binding protein 4

FASN: fatty acid synthase

FATP: fatty acid transport protein

FBP1: fructose-1,6-bisphosphatase 1

G6PC: glucose 6-phosphatase

GK: glycerol kinase

GPAM: glycerol-3-phosphate acyltransferase, mitochondrial

GPD: glycerol-3-phosphate dehydrogenase

GPD1: glycerol-3-phosphate dehydrogenase 1

GPD2: glycerol-3-phosphate dehydrogenase 2

GS: glycerol supplementation

HK2: hexokinase 2

HMGCR: 3-hydroxy-3-methylglutaryl-Coenzyme A reductase

HSL: hormone sensitive lipase

IMF: intramuscular fat

LDH: lactate dehydrogenase

LDHA: lactate dehydrogenase A

LDHB: lactate dehydrogenase B

LPL: lipoprotein lipase

LT: longissimus thoracis

MCEE: methylmalonyl-CoA epimerase

ME: metabolizable energy

NDF: neutral detergent fiber

MS: marbling score

MUFA: mono unsaturated fatty acid

MUT: methymalonyl-CoA mutase

NADH: nicotinamide adenine dinucleotide

NADPH: nicotinamide adenine dinucleotide phosphate

NEg: net energy for gain

NEl: net energy for lactation

NEm: net energy for maintenance

OAA: oxaloacetate

PC: pyruvate carboxylase

PCCA: propionyl-CoA carboxylase, alpha

PCCB: propionyl-CoA carboxylase, beta

PCK1: phosphoenolpyruvate carboxykinase, cytosol

PCK2: phosphoenolpyruvate carboxykinase, mitochondrial

PCR: polymerase chain reaction

PDHA1: Pyruvate dehydrogenase (lipoamide) alpha 1

PEP: phosphoenolpyruvate

PFKL: Phosphofructokinase-1

PKLR: pyruvate kinase

PPARG: peroxisome proliferator-activated receptor gamma

PUFA: poly unsaturated fatty acid

RNA: ribonucleic acid

QG: quality grade

RPS9: Ribosomal protein S9

SLC2A2: Solute carrier family 2 member 2

SLC2A4: Solute carrier family 2 member 4

SREBP1: Sterol regulatory element binding transcription factor 1

TCA: tricarboxylic acid

TDN: total digestible nutrient

THI: temperature humidity index

UDP: uridine 5’-diphosphate

VFA: volatile fatty acid

WHC: water holding capacity

YG: yield grade

UNIT AND MARKS

%: percent

cm: centimeter

dL: deciliter

g: gram

kDa: kiloDalton

kg: kilogram

m: meter

Mcal: megacalori

mg: miligram

mL: mililiter

mM: miliMolar

mm: millimeter

mmol: millimol

nm: nanometer

^{o}C : celcius degree

μL : microliter

μm: micrometer

μM: microMolar

CHAPTER ONE

INTRODUCTION

Glucose metabolism in ruminant is different from monogastric animals. In ruminants, glucose is mainly supplied by hepatic gluconeogenesis to maintain the blood glucose level. The major precursors of hepatic gluconeogenesis are including propionate, amino acids and lactate are major precursors. Most of the carbohydrate on ruminant's diet is fermented into a short-chain fatty acid in the rumen, and prior to liver entry, all of the remaining glucose is removed by cells of the gastrointestinal tract.

In South Korea, the carcass grading system is judged by a combination of a QG and a yield grade (YG) (KAPE, 2017). Although the QG is estimated by several factors exposed in LT at the 13^{th} rib interface, the MS is the major determinant (NLCF, 1998). Since Korean consumers prefer the high marbled beef, the farmers generally apply castration to bulls to increase the intramuscular fat (IMF) and MS combined with intensive grain-based feeding system.

Beef cattle industry uses castration to provide advantages on beef cattle production, including to modify behavior, to improve safety management, and to produce better carcass and meat quality. However, the castrated bull exhibited slower growth and lower feed efficiency (Seideman et al., 1982). Castration of bulls either with physical method or immune castration, decrease

testosterone level (Amatayakul-Chantler et al., 2013). In Korean cattle, castration is an efficient way to enhance marbling and improved beef quality (Bong et al., 2012). Carcasses of steers slaughtered at later age and higher body weights had a lower percentage of valuable cuts and higher fat content (Nogalski et al., 2017). Study in rats reported that castration decreases the circulation of testosterone, and enhances gluconeogenesis in the liver (Xia et al., 2013). Smith and Crouse (1984) demonstrated that a high concentrate diet increased glucose incorporation into fatty acids in intramuscular adipose tissue than subcutaneous adipose tissue. Furthermore, glucose was the primary lipid precursors in intramuscular adipose tissue, whereas subcutaneous adipose tissue uses acetate (Smith and Crouse, 1984). Currently, little information is available about the effect of castration on gluconeogenesis in the liver at the molecular level and its correlation with MS.

The cold environment can alter nutrient utilization and metabolism, which has noticeable effects on cattle's growth performance (Young, 1981) and may also change meat quality (Hocquette et al., 1998). Cold environment increases the maintenance energy of about 30% to 40% due to the higher energy requirement of animal for maintaining the body temperature (Webster et al., 1970). Shivering increasing in cold adaptation involves biochemical mechanisms similar to those underlying muscle contraction and subsequent relaxation. Shivering also increases the adenosine tri phosphate (ATP) utilization enhancing in the oxidation rate of energy substrates. Gluconeogenesis partly supplies energy to muscles shivering to maintain a

body temperature of animal in response to cold temperature. Cold exposure leads animals to change carbohydrate metabolism, which is modulated by hormones such as insulin and glucagon. Increased cortisol during cold stress enhances gluconeogenesis, promotes the breakdown of lipids and proteins, and mobilization of extrahepatic amino acids and ketone bodies, thus counteracting with insulin (Bhimte et al., 2018).

The cold adaptation changes the muscle structure and metabolism. In the non-ruminant studies, cold exposure induced an increase in muscle oxidative metabolism which is associated with a rise in lipid content and glycolytic potential (Lefaucheur et al., 1991; Duchamp et al., 1992). Though there are no clear-cut effects of cold exposure on meat quality through muscle's biochemical changes, Lefaucheur et al. (1991) reported that cold exposure decreased the water holding capacity (WHC) of red muscles and increased the proportion of unsaturated lipids leading to a softer fat.

To increase the energy supply and to improve the metabolic status of the animal, increasing the energy density in concentrate by fat supplementation was generally used in ruminant's diet. However, fat sources may have negative effects on dry matter (DM) intake (Nawaz and Ali, 2016) and fibrous carbohydrate digestibility (Doreau and Chilliard, 1997). Utilization of fat from animal origins, such as tallow and lard, is limited due to the concern of Bovine spongiform encephalopathies (BSE) in cattle. Glycerol, a by-product of the biodiesel industry, has been reported to have a beneficial effect on improving energy status, though it is not always matched with the increased of

performance. According to FDA (2007), glycerin or crude glycerol is categorized as a safe feed ingredient when used in accordance with good manufacturing or feeding practice. Previously reported, glycerol supplementation in the diet showed the better result in performance, rumen parameters, and blood metabolites than rumen-protected fat supplementation (Kang, 2019).

Several studies used either pure or crude glycerol on a diet did not observe any adverse effects of glycerol on cattle performance (Mach et al., 2009; Chanjula et al., 2014; Del Bianco Benedetti et al., 2016; Kang et al., 2017). Cows fed glycerol tended to increase body weight at a higher rate relative to those supplied in the control diet. Some studies conducted in beef cattle showed that glycerin utilization in the diet does not affect carcass characteristics and meat quality (Gunn et al., 2010; Barton et al., 2013). A potential effect of dietary glycerol for improving ruminant meat quality is due to the increased availability of gluconeogenic compounds, which allow a greater deposition of intramuscular fat (Mach et al., 2009). Glycerol supplementation in the beef cattle's feed reared under cold condition also might reduce lipolysis, which is occurred to maintain body temperature. The effects of glycerol supplementation on beef cattle's diet have been investigated, but limited studies were conducted to evaluate its effect on beef cattle in cold condition. The studies on utilization of glycerol as a feed supplement to cope cold condition on of beef cattle would provide comprehensive knowledge to the performance, meat quality, gluconeogenesis, and lipid metabolism in cattle.

This study was conducted to 1) examine effects of castration of bulls on transcriptional changes in the genes involved in gluconeogenesis and glycolysis pathways and to 2) investigate effects of dietary glycerol and ambient temperature on growth performance, rumen characteristics, blood metabolites, carcass characteristics, meat quality and expression gluconeogenesis and lipid metabolism of in finishing Korean cattle.

CHAPTER TWO

LITERATURE REVIEW

1. Glucose metabolism in ruminant

Glucose metabolism is tightly regulated to guarantee a sufficient energy requirement to glucose-dependent organs (Han et al., 2016). Input of glucose is essential to meet certain obligatory function, such as lactose synthesis in milk production, supply energy for fetus, nervous system, brain cells and working muscle, synthesis of structural polysaccharides, glycoproteins, and glycolipids of cell membranes, and synthesis of triglycerides in adipose tissue (Hamada, 1984; Ortega Cerilla and Martínez, 2003). In contrast to non-ruminants, most of the ingested carbohydrates in ruminant are fermented into short chain fatty acid by microbes in the rumen, so that little glucose is absorbed from small intestine (Reynolds et al., 1995). As a consequences, gluconeogenesis in the liver and kidney in ruminants is quantitatively more important than in non-ruminant. Ruminants have adapted to limited availability of glucose by high rates of hepatic gluconeogenesis, limited use of glucose for oxidation, and use acetate instead of glucose for fatty acid synthesis (Brockman and Laarveld, 1986).

Carbohydrate digestion

Several carbohydrates are found in feedstuffs, such as cell wall insoluble or soluble carbohydrates, starch, and water-soluble carbohydrates. According to Nozière et al. (2010) cell wall insoluble carbohydrates characterized as fraction insoluble in neutral detergent solution, such as cellulose, hemicellulose and lignin, whereas cell wall soluble carbohydrate include soluble material such as pectin. Starch and water-soluble carbohydrate are consisted of xylans, glucans, and organic acids (Nozière et al., 2010).

Nearly all carbohydrate digestion occurs within the rumen, but under certain circumstance (high rate passage), a significant amount of carbohydrate is digested in the small and large intestines (Nocek and Tamminga, 1991). Young et al. (1977) reported that less than 10% of body glucose requirements are absorbed as pre-formed glucose from the ruminant digestive tract. In addition, Aschenbach et al. (2010) reported that feeding a diet containing high bypass rumen starch only results in the absorbed glucose less than 5% of the total glucose supply because of limitation in intestinal glucose absorption.

The rumen microbes are able to hydrolyze variety of carbohydrates found in feedstuffs, and convert them to microbial protein and VFAs. According to Van Soest (1994), the major VFAs produced in the rumen are acetate, butyrate, and propionate. A significant portion of protein which is also digested in the rumen contribute to the small concentrations of branched chain VFAs (isovalerate, isobutyrate, 2-methylbutyrate) production (Nozière et al., 2010). The VFA composition is mainly determined by the composition of microbial

population in the rumen, which is largely determined by dietary composition and dietary regimen (Warner, 1962). Djikstra and Tamminga (1995) reported that approximately 80% of carbohydrate degradation being carried out by bacteria, along with fungi and protozoa. The development of cellulolytic bacteria induce high levels of acetate, whereas amylolytic bacteria induces an increase in propionate proportion (Castillo-Gonźales et al., 2014). Diets rich in water soluble carbohydrate may promote protozoa population that induces an increase in butyrate (Hristov et al., 2001; Denton et al., 2015).

Volatile fatty acids produced in the rumen are absorbed directly into the portal blood circulation primarily via ruminal epithelium (Djikstra, 1994). Absorption of VFA through the ruminal epithelium accounts for 65% to 85% of the ruminal production, depending on the balance between the absorption and turnover rates of the liquid phase in the rumen (Gäbel and Aschenbach, 2006). Moreover, Merchen and Borquin (1994) reported that these VFAs absorbed in the rumen contribute to approximately 80% of the total energy requirements of the host animal.

Gluconeogenesis

It is generally established that most of carbohydrates in diets is fermented to VFA in the rumen, glucose requirements in ruminants arise mainly from gluconeogenesis in the liver (Reynolds, 1995) with a little contribution by kidneys (Stumvoll et al., 1999). Approximately 90 to 100% of glucose supply in ruminants depends on a state of continuous gluconeogenesis.

Gluconeogenesis refers to the pathway for *de novo* synthesis of glucose from non-carbohydrate precursors, such as lactate, amino acids, glycerol, and propionate (Mayes and Bender, 2003).

Large quantities of VFAs produced by rumen fermentation are converted into glucose by hepatic gluconeogenesis. According to Aschenbach et al. (2010), propionate, glycerol, amino acids, lactate and pyruvate play an important role as a significant glucogenic subtrates in ruminants. Reynolds et al. (1988) reported that carbon in glucose synthesized is mostly derived from propionate (55.1%), L-lactate (17.4%) and amino acids (16.5%). Glycerol is contributed to about 0.5-3.0 % of carbon glucose synthesized in cattle (Aschenbach et al., 2010). In the fasted state, the liver secretes glucose not only from gluconeogenesis but also through breakdown of glycogen (glycogenolysis) (Rui, 2014).

In animals fed with grain, about 90% of the propionate in portal blood is removed in every circulation through the liver and metabolized (Cook and Miller, 1965). According to Aschenbach et al. (2010), 80% of the total glucose production in cattle is originated from propionate. Glycerol released by lipolysis in adipose tissue during fasting, may be used for a substrate for gluconeogenesis (DiMarco et al., 1981). Almost all amino acids are glucogenic, with lysine, leucine and taurine being exceptions (Berg et al., 2002). In the fasted state, amino acids derived from muscle protein are important substrates for gluconeogenesis (Cahill, 1970). Pyruvate is the end-product of glycolysis, and it is reduced into lactate when actively working muscle become anaerobic.

In ruminants fed with high concentrate diet, ruminal lactate may become a substrate in gluconeogenesis, though the conversion rate is low (Aschenbach et al., 2010).

Hepatic gluconeogenesis involves utilization of several precursors to be incorporated into glucose by several reactions. Gluconeogenesis shares many common reactions with glycolysis, except for three futile cycles, namely the glucose / glucose-6-phosphate cycle, the fructose-6-phosphate / fructose-1,6-biphosphate cycle, and the pyruvate / phosphoenolpyruvate cycle.

Hepatic gluconeogenesis is tightly regulated by various mechanisms, such as substrate availability, rate-limiting enzyme activities, or both (Exton, 1972). Oxaloacetate is the common entry point for most of the gluconeogenesis precursor, except for glycerol which enters the gluconeogenesis pathway by glycerol-3-phosphate. Propionate incorporation is catalyzed by several enzymes, such as propionyl-CoA carboxylase, methylmalonyl-CoA mutase, and methylmalonyl-CoA epimerase to form succinyl-CoA which is next converted to oxaloacetate through part of the tricarboxylic acid (TCA) cycle (Aschenbach et al., 2010). Oxaloacetate can be metabolized to phosphoenolpyruvate (PEP) by phosphoenolpyruvate carboxykinase (PCK) or metabolized in TCA cycle. Phosphoenolpyruvate also can be metabolized to glucose or recycled to pyruvate by pyruvate kinase. First of all, lactate and gluconeogenic amino acids are converted to pyruvate in the cytosol, which later transported to mitochondria and converted to oxaloacetate by PC. Glycerol is phosphorylated to glycerol-3-phosphate by glycerol kinase to enter

gluconeogenesis. Therefore, glycerol is an efficient energy substrate because it is not affected by two of the rate-limiting gluconeogenesis enzymes, i.e., PC and PCK (Krehbiel et al., 2008).

There are two forms of PCK in mammalian tissues, the mitochondrial (PCK2) and the cytosolic form (PCK1). It is reported that PCK1 in liver and kidney is essential for gluconeogenesis, while the role of PCK2 is uncertain (Wiese et al., 1991). According to Aschenbach et al. (2010), the PC and PCK2 activities regulate entry of lactate to gluconeogenesis; the entry of gluconeogenic amino acids is regulated by PC and PCK1 activities, whereas entry of propionate is regulated by PCK1 activity. Pyruvate carboxylase catalyzes the carboxylation of pyruvate to oxaloacetate for gluconeogenesis. Pyruvate carboxylase expression plays a pivotal role in promoting entry of endogenous precursors in gluconeogenesis when intake is compromised such as during a transition to calving, whereas PCK expression is linked to controlling gluconeogenesis when feed intake is not constrained (Aschenbach et al., 2010). Fructose-1,6 biphosphatase and glucose-6 phosphatase (G6PC) catalyzed the next steps of gluconeogenesis. Fructose-1,6 biphosphatase catalyzed the conversion of fructose-1,6 biphosphate to fructose-6 phosphate. Glucose 6 phosphatase catalyzes the hydrolysis of glucose-6 phosphate to glucose and inorganic Pi (Nutall et al., 2008).

The rate of hepatic gluconeogenesis is under the control of various hormones. Among them, glucagon and insulin play vital roles in maintaining normal blood glucose concentration and gluconeogenesis. Insulin and glucagon

are secreted by the islets of Langerhans of pancreas into the bloodstream (Da Silva Xavier, 2018). Insulin has a hypoglycemic effect that promotes the storage of metabolites in peripheral tissues, whereas glucagon is a hyperglycemic hormone that activates gluconeogenesis and lipolysis (Brockman, 1978). Secretion of both hormones can be influenced by glucose, certain amino acids, VFA, nervous stimulation, and hormones.

Insulin is secreted into the bloodstream to promote glycolysis to lower glucose levels by increasing the removal of glucose from the bloodstream. Also, it participates in directing absorbed acetate and amino acids into peripheral tissues (Qaid and Abdelrahman, 2016). In the absence of insulin, glucose uptake decreases in the tissues and increases the mobilization of lipids in adipocytes (Frayn, 2010). Insulin directly controls the enzyme activities by phosphorylation or de-phosphorylation and may suppress gluconeogenesis through both direct effects on the liver and indirect extrahepatic mechanisms. Insulin increases the uptake rate of glucose by stimulating glucose transporter in skeletal muscle and adipose tissue (Kahn, 1996). Insulin is also known to inhibit the transcription of hepatic PCK (Sutherland et al., 1996), and it causes a greater decrease in the activity of G6PC in the liver of rat (Ashmore et al., 1956).

When the glucose concentration drops, glucagon is released to promote hepatic glucose output to raise the blood glucose levels by increasing glycogen breakdown and gluconeogenesis, and by decreasing glycogenesis and glycolysis (Jiang and Zhang, 2003). According to Brockman and Manns (1974),

glucagon enhanced gluconeogenesis is associated with increased activity of hepatic PC. Williams et al. (2006) reported that the administration of glucagon in subcutaneous adipose tissue resulted in a 1.5 fold increase in hepatic mRNA concentration of *PC*, *PCK1*, and *PCK2*. However, the authors concluded that glucagon does not make immediate alterations in total PCK enzyme activity and gluconeogenic capacity. Other study reported that the mRNA concentration of *PC*, *PCK2*, and *PCK1* in liver increased with subcutaneous glucagon injection in dairy cows (Bobe et al., 2009).

Different dietary ingredients could change the gene expression of hepatic gluconeogenesis pathway, especially *PCK* and *G6PC* (Li et al., 2013). The activity of G6PC increases with the presences of free fatty acid, amino acids, and possibly fructose, while it suppresses following carbohydrate feeding (Radziuk and Pye, 2001). It is possible that nutrients availability participates in the regulation of gluconeogenesis. Increasing glucose supply generally does not negatively affect the activity of important gluconeogenesis enzymes in mid lactating cows (Al-Trad et al., 2010). Ruminal infusions of propionate and casein increased rates of glucose synthesis in the sheep, and the rate of gluconeogenesis is related directly to the availability of gluconeogenic precursors absorbed from the digestive tract (Judson and Leng, 1973). Nevertheless, increased glucogenic precursor supply, either from food or ruminal infusions, does not always produce predicted increments in glucose production (Vanhatalo et al., 2003; Lemosquet et al., 2009).

In addition to glucose, other nutrients derived from protein or fat also could regulate the hepatic gluconeogenesis, either by serving as a substrate or by modulating the secretion of pancreatic hormones (Roden and Bernroider, 2003). Cummins and Sartin (1987) reported that feeding a high-fat diet increases basal insulin concentration and insulin to glucagon ratio, which may decrease glucose synthesis in lactating cows. Rumen-protected fat feeding in dairy cows during mid-lactation lowers hepatic glucose output by decreasing the plasma insulin concentration, and increases the glucagon to insulin ratio, thus down-regulating the mRNA abundance of *G6PC* (Lohrenz et al., 2010). Different fatty acids might have caused different effects to hepatic gluconeogenesis. Mashek and Grummer (2003) reported that the addition of C22:6 to the monolayer cultures of bovine hepatocytes resulted in increased metabolism of palmitic acid and decreased gluconeogenesis. According to Santos et al. (2011), oleic acids increase insulin secretion. Furthermore, 2 mM oleic acid were reduced rates of gluconeogenesis from propionate in bovine hepatocytes (Cadórniga-Valiño et al., 1997). In contrast, Strang et al. (1998) reported that triglyceride infiltration increased propionic metabolism and gluconeogenesis rates.

In a human study, the insulin concentration to feeding with protein solution was closely related to specific plasma fatty acid, especially leucine, isoleucine, valine, phenylalanine, and arginine, while glucagon level was linearly related with the increase in plasma fatty acid concentrations (Calbet and MacLean, 2002). An increase of DL-methionine level in the diet did not upregulate key glucogenic genes but decreased the expression of *PCK2* of Holstein calves

(Zhang et al., 2016). Chandler and White (2019) reported that choline and DL-methionine interact differently with hepatic gluconeogenesis. Choline increased the mRNA expression of *PCK1* and *G6PC*, whereas DL-methionine decreased mRNA expression of *PCK1* without any change on *G6PC*. Nonprotein nitrogen supplementation in sheep did not change insulin concentration; however, it increases the liver PCK activity associated with an increase of gluconeogenic capacity from propionate (Noro et al., 2012).

Glycerol supplementation during the prepartum and post-partum periods did not change the plasma insulin and glucose concentration (DeFrain et al., 2004). Study in dairy cattle reported that substitution of corn with glycerol increased the expression of *PCK1* mRNA during the transition to lactation suggesting that dietary energy source alters hepatic gene expression due to changes in rumen propionate production (White et al., 2016). When the energy supply is relatively enough to body demand, glycerol can enter the glycolysis and can be converted to Acetyl CoA, and further metabolize via TCA cycle (Allen et al., 2009). Nye et al. (2008) also reported that glycerol as the predominant source of triglyceride glycerol in adipose tissue, skeletal muscle, and liver of rat during fasting and high sucrose feeding. Therefore, glycerol can be an important source of glucose production since the concentrations increase because of lipid mobilization during negative energy balance.

Glycogen metabolism

Glycogen is defined as the store of body sugar. The chemical structure of glycogen is a relatively simple branched-chain polymer of glucose with a small protein core. Liver and muscle account for most of the body's glycogen stores. Glycogen plays a vital role in controlling the blood glucose homeostatic and energy balance of the animal. Jensen (2011) reported that hepatic glycogen acts as a source of blood glucose when the energy is deficit, whereas glycogen in skeletal muscle is thought to be contributed to local energy demand.

Synthesis of new glycogen is initiated by glucose which comes from either the ingested food (direct pathway) or gluconeogenesis (indirect pathway), being phosphorylated into glucose-6-phosphate by hexokinase (Adeva-Andany et al., 2016). Then, glucose-6-phosphate is going through isomerization into glucose-1-phosphate by phosphoglucomutase-1. After that, UDP-glucose is formed from glucose-1-phosphate, which is catalyzed by uridine 5'-diphosphate (UDP)-glucose phosphorylase. Glycogenin initiates the synthesis of glycogen by auto-glycosylation transporting glucose from UDP-glucose to itself and forming a short glycogen chain containing 10-20 glucose moieties. Then, it goes to elongation process catalyzed by glycogen synthase that transfers a glycosyl moiety from UDP-glucose to the growing glycogen strand with α-1,4-glycosidic between glucose residues. The branching enzyme introduces branch points in the glycogen particle, by creating α-1,6-glycosidic bonds.

Glycolysis is the first step in the breakdown of glucose to extract energy for cellular metabolism. Enzymatic control of glycolysis is applied by 3 non-

reversible steps mediated by the enzymes hexokinase, phosphofructokinase 1, and pyruvate kinase (Murray et al., 1996). Glucose-6-phosphatase mediates the first step of glycolysis secures glucose within the cell. Glucose ring is phosphorylated by hexokinase resulting in a molecule called glucose-6-phosphate. Glucose-6-phosphate is converted into its isomer, fructose-6-phosphate, then transferred to fructose-1,6-biphosphate by phosphofructokinase. Fructose-1,6-biphosphate splits to form dihydroxyacetone phosphate (DHAP) and glyceraldehyde-3-phosphate (Guo et al., 2012). Only glyceraldehyde-3-phosphate can enter to the next step of glycolysis. By several reactions, glyceraldehyde-3-phosphate is converted to PEP. Pyruvate kinase catalyzed the transfer of a phosphate group from PEP donates to ADP, producing a molecule of pyruvate, the end product of glycolysis (Israelsen and Vander Heiden, 2015).

Glycogen deposition is related to the nutritional state. High concentrations of glucose (15-25 mM), lactate (10 mM), and pyruvate (1 mM) had a synergistic effect on glycogen deposition in both periportal and perivenous rat hepatocytes. In the presence of insulin, the initial rates of glycogen deposition were increased about 20-40%, and the length time for glycogen deposition continued for more than 4 hours than when it was absent (Agius et al., 1990). The physiological state of cattle can alter liver glycogen metabolism. Liver glycogen concentration is diminished by 85% in the 3 days either side of calving in ad-libitum dairy cows (Van Den Top et al., 1996). Cold stress also plays a role in increasing glycogen turnover and/or depletion. Cattle with poor nutritional

status and exposed to acute cold temperature may have lower glycogen reserves and survival rates (Bell et al., 1975).

Glycogen metabolism in muscle is influenced by hormones, such as insulin and adrenaline, and by nutritional factors such as amino acids and glucose (Yeaman et al., 2001). Halse et al. (1999) reported that insulin activates glycogen synthesis in muscle, acting principally via the PKB/GSK3 pathway but with contribution from a rapamycin-sensitive component that lies downstream of PI-3 kinase. Long-term feeding of whey protein stimulated glycogen synthase protein levels increasing muscle glycogen storage in mice (Kanda et al., 2012). Muscle glycogen increases slightly by a large amount of intake of carbohydrates (Hawley et al., 1997). The prolonged consumption of a high carbohydrate increases the conversion of carbohydrate to lipid in muscle, but not glycogen (Acheson et al., 1984).

Hormone and allosteric control are important regulators of glycolytic enzymes. Among the three rate-limiting enzymes, phosphofructokinase plays a central role and is considered as the pacemaker in glycolysis (Sola-Penna et al., 2010). Fructose-2,6-biphosphate exerts its allosteric effects on phosphofructokinase 1, promoting glycolytic than gluconeogenic flux. Glucagon promotes a reduction in fructose-2,6-biphosphate that causes a rapid decrease in the rate of glycolysis (Miller and Birnbaum, 2016). The lack of insulin significantly impacts the expression of phosphofructokinase genes in liver and muscle, which influences the regulation of glycolysis in the cell (Ausina et al., 2018). Glycolysis shares metabolites with other pathways,

including glycogen synthesis, TCA cycle, fatty acid synthesis, and gluconeogenesis. Therefore, products from a reaction in one pathway can become allosteric control of glycolytic enzymes (Murray et al., 1996).

2. Lipid metabolism in ruminant

The balance between fat synthesis (lipogenesis) and fat breakdown (lipolysis) determine accumulation of triglyceride in adipocytes. Triglyceride stored in adipocytes might be originated either from the uptake of fatty acid from blood or *de novo* synthesis (lipogenesis). Lipogenesis in ruminants mainly occurred in adipose tissue, rather than in the liver (Pethick et al., 2004). In opposite, lipolysis leads to the breakdown of triglyceride stored in adipocytes and release them into free fatty acids and glycerol (Luo and Liu, 2016).

Nutritional status, such as VFAs produced by ruminal fermentation, is the main factor of fat deposition rate in ruminants. In addition, glucose is also used as the primary precursors for biosynthesis of fatty acids in ruminants. Intramuscular adipocytes prefer glucose, while subcutaneous adipose tissue prefers acetate as lipogenic substrates (Smith and Crouse, 1984). The ratio of acetate: glucose use for lipogenesis in subcutaneous adipose tissue and intramuscular adipose tissue of Korean cattle are 1.61 and 1.23, respectively (Lee et al., 2000). Increasing lipogenesis, fatty acid uptake, and decreasing lipolysis are associated with higher IMF deposition (**Figure 1**).

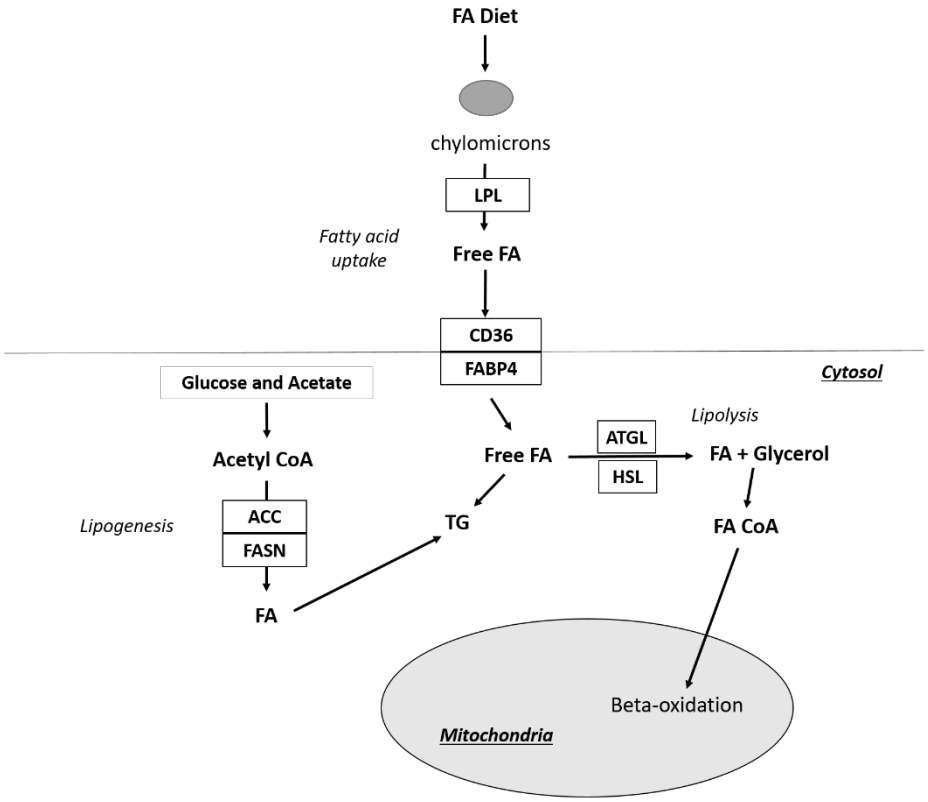

Figure 1. Lipid metabolism in ruminant adipose tissue. ACC = Acetyl CoA carboxylase, ATGL = adipose triglyceride lipase, CD36 = fatty acid translocase, FABP4 = fatty acid binding protein 4, FASN = fatty acid synthase, HSL = hormone sensitive lipase. FA = fatty acid, TG = triglyceride. (Modified from Ladeira et al., 2018).

After absorption across the ruminal epithelium and distribution to peripheral tissue, acetate is converted in adipose cells into acetyl-CoA through the action of acetyl-CoA synthetase enzyme. According to Ladeira et al. (2016), acetyl CoA carboxylase and fatty acid synthase are involved in the *de novo*

synthesis of fatty acid. Acetyl CoA will go through carboxylation process to malonyl-CoA by the action of enzyme acetyl-CoA carboxylase, encoded by *ACC* gene. Then, the fatty acid synthase, a multi-enzyme complex encoded by *FASN* gene catalyzes seven different reactions, whereby another acetyl-CoA molecule is united to malonyl CoA molecules through multiple enzymatic reactions, producing a long-chain fatty acid (palmitic acid).

Glucose enters fatty acid synthesis via glycolysis, which produces pyruvate that is further transformed into acetyl-CoA within the mitochondria. Acetyl CoA should be converted into citrate to be transported out of mitochondria into the cytosol via citrate shuttle pathway. In cytosol, citrate is condensed into oxaloacetate and acetyl CoA by ATP citrate lyase (Pietrocola et al., 2015). Glucose also required for producing reduced nicotinamide adenine dinucleotide phosphate (NADPH). The presences of acetyl CoA and NADPH are essential for *de novo* fatty acid synthesis.

In most tissue, triglyceride can also be produced through the glycerol phosphate pathway (Yu et al., 2018). Glucose can be converted into glycerol-3-phosphate catalyzed by glycerol-3-phosphate dehydrogenase, which reduces dihydroxyacetone phosphate from glycolysis pathway. According to Yu et al. (2018), there are a series of enzymes participating in the catalytic reaction of glycerol-3-phosphate. Glycerol-3-phosphate acyltransferase catalyzes the acylation of glycerol-3-phosphate and fatty acyl CoA to produce lysophosphatidic acid. Subsequently, lysophosphatidic acid will be converted into phosphatidic acid, which is catalyzed by 1-acyl glycerol-3-phosphate

acyltransferase. Next, phosphatidic acid phosphatase catalyzes the dephosphorylation of phosphatidic acid to form diacylglycerol. Diacylglycerol acyltransferase catalyzes the conversion of di-acyl glyceride to triglyceride.

Nutritional status and hormones regulate lipogenesis. Baldwin et al. (2007) reported that post-ruminal carbohydrate infusion stimulates lipogenesis from acetate and glucose in growing beef steers. High carbohydrate diet increased propionate production in the rumen, which is subsequently converted into glucose in the liver. Thus, glucose might enter the glycolytic pathway, enter the TCA cycle, and become a carbon donor in *de novo* fatty acid synthesis. Increase of plasma glucose levels stimulates lipogenesis by stimulating the release of insulin and inhibiting the release of glucagon (Kersten, 2001). Insulin potentially stimulates lipogenesis by increasing the glucose uptake in the adipose cell via recruitment of glucose transport to the plasma membrane, as well as activating lipogenic and glycolytic enzymes (Kersten, 2001). Furthermore, decreases the rate of lipolysis and utilization of fat in adipose tissue by insulin contribute to increase in fat deposition (Qaid and Abdelrahman, 2016).

Insulin and glucose stimulate the expression of lipogenic gene *sterol regulatory element binding protein 1* (*SREBP1*) which regulates the expression of genes associated with cholesterol, fatty acids, triglyceride, and phospholipid synthesis (Ferré and Foufelle, 2007). Another crucial transcription factor, *peroxisome proliferator-activated receptor gamma (PPARG)* is stimulated by insulin and *SREBP1* (Vidal-Pluig et al., 1997). The action of enzymes

lipoprotein lipase, fatty acid translocase, and fatty acid binding protein 4 facilitates uptake of fatty acid from blood into adipose tissue. Lipoprotein lipase is the key enzyme for hydrolyzing one fatty acid from circulating triglyceride, and FABP4 responsible for transporting fatty acids into cells. Fatty acid translocase contributes to uptake long chain fatty acids and contributes to excessive fat supply to lipid accumulation and metabolic dysfunction (Pepino et al., 2014).

Opposite of lipogenesis, lipolysis leads to the breakdown of triglyceride stored in adipocytes and subsequently releases fatty acids and glycerol. In response to nutritional state, lipolysis is regulated by hormonal and biochemical signals to maintain the supply for energy substrates. Hydrolysis of triglyceride into fatty acids and glycerol requires several steps involving at least three different enzymes adipose triglyceride lipase, hormone-sensitive lipase, and monoglyceride lipase (Zechner et al., 2012). Adipose triglyceride lipase catalyzes the initial step of lipolysis, converting triglyceride to diacylglycerol. Hormone-sensitive lipase is the primary diglyceride lipase found in adipose tissue. Together with adipose triglyceride lipase, hormone-sensitive lipase hydrolyzes triglyceride in a coordinated manner (Schweiger et al., 2006). Both enzymes were inhibited by insulin. Monoglyceride lipase is the rate-limiting enzyme for the breakdown of monoglycerides derived from extracellular triglyceride hydrolysis, intracellular triglyceride hydrolysis, and intracellular phospholipid hydrolysis (Zechner et al., 2012).

Fasting or energy restriction stimulated lipolysis, which is activated by catecholamine. During fasting, catecholamine enhances the sympathetic nervous activity, thereby stimulating lipolysis (Vernon, 1992). Refeeding suppressed lipolysis, which is primarily through the anti-lipolytic action of insulin (Duncan et al., 2007). Lipolysis is not only sensitive to nutrient availability, but also the composition of the diet. Carbohydrate ingestion may elevate plasma insulin concentration, thus suppress lipolysis during exercise in human (Horowitz et al., 1997). High energy diet increased the adipose tissue lipogenic gene expressions (*PPARG, LPL, FASN, SREBP1, ACC, stearoyl CoA desaturase*, and *FABP*), while decreased lipolytic gene expressions (*hormone sensitive lipase* and *carnitine palmitoyltransferase-1*) in Yellow breed x Simmental cattle (Zhang et al., 2016).

3. Korean cattle

Characteristics of Korean cattle

Korean cattle or Hanwoo has been raised in Korean peninsula since at least 2000 years ago. Korean cattle is originated from crossbred of *Bos indicus* and *Bos primigenius*. It was used extensively for drafting purposes, such as farming and transportation. Over time, the meat consumption is increased, and Korean cattle is developing into a meat type cattle. Since early of the 1960s, there has been a program for improvement through pure-breeding and crossbreeding system to select the dominant sires to produce the greatest meat productivity

and quality (Na, 1994). Korean cattle have been maintaining stable traits through pure breeding; the present blood lineage is very valuable and is mainly widespread in the Korean peninsula (Kim and Lee, 2000). After the implementation of the breeding system, the performance and carcass characteristics of Korean cattle increased dramatically. The average carcass weight of Hanwoo steers increased from 343 kg in 2000 to 437 kg in 2016. In addition, the MS also improved from 3.6 in 2000 to 5.6 in 2016 (Chung et al., 2018).

In Korea, approximately 3.1 million head of beef cattle were raised in 2016. The total number of slaughter cattle was 859,472, including 737,476 Hanwoo cattle, 57,642 Holstein cows, and 64,354 Holstein heifers and bulls (KAPE, 2016). The number of cattle farming households was 89,879, including 85,040 Hanwoo farmers (KAPE, 2016). However, the number of households raising Hanwoo cattle decreased from 186,000 households in 2006 to 85,040 in 2016 (KAPE, 2017).

In order to produce high quality beef, Korean cattle are fed a high-energy diet for long fattening period to increase the fat percentage in the loin muscle (Chung et al., 2018). The fattening periods are divided into three stages, namely: the growing stage (6 to 11 months), the early fattening stage (12 to 20 months), and the final fattening stage (21 to 29 months). During the early fattening stage, Korean cattle are fed with a concentrate (TDN 71%, CP 13%) and *ad libitum* rice straw (Chung et al., 2018). During the finishing stage, 90%

concentrate and 10% rice straw is provided to maximize IMF development (Jo et al., 2012).

Beef quality and yield grade in South Korea

Hanwoo beef is characterized by high IMF content and unique palatability. Also, it has relatively thin muscle fiber and a lower content of connective tissues compared to other breeds (Joo et al., 2017). It has been reported that the IMF was two-fold higher in Hanwoo compared to Angus cattle at 24 months old (Cho et al., 2005). The LT of Hanwoo steers had the highest fat content resulting in higher sensory traits (flavor, tenderness, juiciness, and overall acceptance) than Holstein and Angus steers (Piao et al., 2019). However, IMF in Hanwoo beef is less than high marbled Wagyu (28% vs. 40%) (Gotoh and Joo, 2016).

The producer usually extends the slaughter age of Hanwoo beyond 24 months to increase the IMF content, even the IMF deposition is plateaued at that age. Commonly, Hanwoo has been extended to an average of 31 months with weight around 719 kg to fatten the cattle (KAPE, 2017). The high content of IMF in Hanwoo beef can improve the texture and juiciness of beef, improving its overall acceptability (Jung et al., 2015). The IMF content also increases the water-holding capacity and chemical composition of meat. According to Jo et al. (2012), the increase of IMF from 6.6% into 21.5% decreases by about 17% of the moisture content (73.7% to 60.9%). This change makes lesser drip loss and water loss during cooking in meat with high IMF.

The higher IMF content in Hanwoo beef results in the superior sensory properties compared to other beef from other breeds. Hanwoo beef with QG1++ and 1+ had higher scores in tenderness, juiciness, flavor, and overall likeness of *longissimus dorsi* muscle than imported beef (Jo et al., 2012).

Korea's carcass grading system is developed due to the preference of Korean consumers for highly marbled beef. In addition, since the beef market was opened for imported beef since 2001, the quality of domestic beef needs to be improved. The carcass grading system in Korea was established and introduced with the Livestock Industry Act in 1993 (KAPE, 2016). The grading system consists of two kinds of categories: 1) YG, including YG A, B, and C for evaluating the amount of meat; 2) QG, including QG 1++, 1+, 1, 2, and 3 for assessing the meat quality (KAPE, 2017). The professional grader assesses beef grades at 24-hour post-mortem. The beef grading is performed at loin muscle between the 13^{th} rib and the 1^{st} lumbar vertebra. The grading parameters include carcass weight, longissimus muscle area, backfat thickness, MS, meat color, fat color texture, and maturity. Marbling score is the most important criterion for determining QG. The beef marbling score standard (BMS) are from 1 (devoid) to 9 (abundant) which have a relation with QG. The MS 8-9 is for QG 1++, 6-7 is for QG1+, 4-5 is for QG 1, 2-3 is for QG 2, and 1 is for QG3, respectively.

4. Effects of castration of bulls on growth and beef quality

Castration is an ancient husbandry procedure used to reduce aggressive behavior and sexual activity by lowering the testosterone levels as well as to modify carcass quality. Methods for castration are typically associated with physical, chemical, or hormonal impairment to the testicles. Among these, physical castration method is commonly applied to bulls. Castration reduced the natural anabolic steroid hormones, testosterone, and estrogen, which is served to accelerate linear growth and weight gain of male animals (Spencer, 1985).

Androgens, including testosterone are a subclass of steroid hormones that are primarily synthesized in the testes and adrenal glands through a series of enzymatic bioconversions from cholesterol (Locke et al., 2009). The androgen synthesis pathway is initiated in mitochondria of steroidogenic cells, therefore cholesterol must be transported across the mitochondrial membrane where the steroidogenic enzymes are present. Once cholesterol present in mitochondria, the androgen synthesis process continues through a number of enzymatic enzymes mainly producing testosterone (Twiddy et al., 2011).

Cholesterol can be obtained exogenously from the diet and endogenously via *de novo* synthesis within the liver (Ikonen, 2008). Exogenous cholesterol supply involves uptake of cholesterol from circulating lipoproteins, while endogenously cholesterol is synthesized from acetyl-CoA through mevalonate pathway in endoplasmic reticulum (Kim et al., 2014). A 3-hydroxy-3-

methylglutaryl-Coenzyme A reductase (HMGCR) is a crucial enzyme, which converts 3-hydroxy-3 methyl-glutary-Coenzyme A into mevalonate in *de novo* cholesterol synthesis (Swyer, 1942). Mevalonate further undergoes multiple reactions to be converted into cholesterol downstream.

A study in castrated rats showed that castration decreased the serum testosterone concentrations and did not change the estrogen concentrations compare to those in non-castrated one. On the other hand, it increased insulin concentrations and hepatic glucose production. Therefore, castration –induced testosterone deficiency decreased hepatic insulin sensitivity results in hepatic gluconeogenesis increase (Xia et al., 2013). Insulin resistance can be central (liver) or peripheral (muscle and adipose tissue). Peripheral insulin resistance impairs glucose uptake from the blood into muscles, while central insulin resistance unrestrained the hepatic glucose production (Xia et al., 2013).

Christoffersen et al. (2010) reported that castration induced testosterone and estradiol deficiency in male-Göttingen mini pigs results in insulin resistance, glucose intolerance, and hyperglucagonemia, which disturbed glucose metabolism. In addition, lowering testosterone concentrations are associated with a reduction in muscle protein synthesis and an increase in protein degradation. Testosterone deficiency also leads to an increase in fat mass (Bhasin et al., 2003). Previously reported, the strong relationship between free testosterone and growth hormone is a function of excess adiposity rather than a direct relationship (Makimura et al., 2010). Steers exhibit lower growth performance, dressing percentage, and meat yields, but greater fatness and

sensory characteristics in comparison with intact males (Wierbicki et al., 1955). Another study showed the growth performance was unaffected by castration; however, steers had lower carcass yield and greater fat thickness (Aricetti et al., 2008). Therefore, insufficient hormones for supporting maximal growth may cause reduced growth efficiency in steers in comparison to bulls (Lee et al., 1990).

The reduction of testosterone levels following castration results in higher carcass fat deposition (Lee et al., 1990; Knight et al., 2000). Acetyl CoA is used as a precursor for various metabolites, such as lipids, triglycerides, steroid hormones, cholesterol, and bile salt. Steroid hormones, such as testosterone is important in modulating adipose mass and insulin sensitivity. In a mice study, adiponectin levels in adipose tissue of male mice increase after castration, which does correlate with increased insulin sensitivity (Macotela et al., 2009). Floryk et al. (2011) reported that castration reduces tissue mass and the expression of androgen receptor, while it increased FASN expression in white adipose tissue of mice. Previously reported, castration of bulls increased circulating blood cholesterol concentrations, but decreased the hepatic HMGCR expression (Baik et al., 2015). These may explain effect of castration which may, at least in part, shift the utilization of unused acetyl CoA toward lipogenesis.

Castration changes the expression of genes related to lipid metabolism in several fat depots of cattle. Increases body fat cell sizes at various fat depots following castration are associated with upregulations of several genes

involved in adipogenesis, lipogenesis, and cellular fatty acid transport (Baik et al., 2014). Castration contributes to increases in expression of genes involved in lipid uptake and lipogenesis, and to decrease in lipolysis in the muscle of Korean cattle (Bong et al., 2012). Transcriptions of several genes involved in adipogenesis, fatty acid synthesis, and fatty acid oxidation in the muscle are upregulated by castration (Jeong et al., 2012). Following the decrease of testosterone levels in steers, the genes associated with lipid metabolism, lipogenesis, and fatty acid transportation are increased in intramuscular fat tissue (Zhang et al., 2017). In addition to lipid metabolism, castration increases transcription levels of several critical genes encoding enzymes for the incorporation of glucogenic substrates including lactate, glycerol, and propionate into gluconeogenesis in the liver. Furthermore, the increase of hepatic gluconeogenic gene expression following castration is associated with increased backfat thickness, fat deposition, and quality grade of Korean cattle (Fassah et al., 2018).

The increase of transcription of genes contributed to lipid metabolism in muscle causes steers to produce significantly more marbling and more tender meat than bulls. A castration study in Chinese Simmental calves reported that steer meat had lower shear force values and was fatter than bull (Zhou et al., 2011). Castration age seems to affect performances and quality traits of beef cattle. Early castration (10 months old vs. 17 months old) on Simmental bulls leads to an increase in meat fatness, color, texture, and flavor (Segato et al., 2005). Castration improved meat tenderness, meat pH, and had more lightness,

redness, and yellowness than meat from bulls (Marti et al., 2013). Destefanis et al. (2003) reported that castration of Piemontese bulls at 5 or 13 months of age lower the water content of beef, while increase contents of protein and ether extract; however, physicochemical and sensory analyses of beef from castrated cattle are not different in comparison with intact cattle. Castration in Korean cattle bulls negatively affects meat yield but increases the meat quality (Park et al., 2002).

5. Effects of temperature stress on cattle performance and beef quality

Growth and production performances of farm animals have generally depended on the environment temperature. The thermoneutral zone is the range of ambient temperatures in which the heat production of an individual is independent of the environmental temperature (Webster, 1974). The thermoneutral zone is surrounded by lower and upper critical temperatures. The lower or upper critical temperature is defined as the effective ambient temperature at which energy intake increases to minimize the reduction of weight gain in growing cattle or preventing weight loss in mature cattle. Climate change has adverse effects on animal production when the animal is subjected to heat and cold stress condition. According to Baumgard and Rhoads (2012), when the temperature is either above or below the thermoneutral zone for optimum animal production, the efficiency is compromised because

nutrients were diverted to maintain the body temperature which more priority than product syntheses, such as milk, meat or fetus.

It has been reported by Young (1981) that the lower critical temperature values were approximately 8 ^{o}C for newborn calves, -5^{o}C for 50-200 kg growing calves, -22 ^{o}C for growing cattle, -28 ^{o}C for dairy cows at peak lactation, and -35 ^{o}C for finishing feedlot cattle, respectively (Young, 1981). However, the wind chill index, the combined effects of temperature and wind, are often used to estimate effective temperature for considering cold stress. Piao and Baik (2015) reported that winter in South Korea might be within mild or moderate cold stress categories for beef cattle based on the wind chill index. Moreover, in the northern part of South Korea (Gangwon-do province), the wind chill index was approximately 4 ^{o}C to 5 ^{o}C colder than the southern parts of South Korea (Piao and Baik, 2015).

The upper critical temperature has been defined in dairy cows as 25 to 26 °C (Berman et al., 1985). The temperature humidity index (THI), which combines the effects of temperature and humidity has been used to indicate heat stress level (Brown Brandl, 2018). LCI (1970) developed livestock weather safety index based on four THI categories, namely: normal (THI < 74), alert (74 < THI < 79), danger (79 < THI < 84), and emergency (THI > 84). The THI on summer until fall in South Korea were classified as mild to alert heat stress range (Kang et al., 2017, 2019).

Effects of temperature stress on growth performance

Animals exposed to cold or heat stress must spend more energy to maintain body temperature, diverting it from growth and production; therefore, growth performance and feed efficiency are reduced during winter (Ames et al., 1980; Young et al., 1981; Ronchi et al., 1997). The net energy for maintenance might be increased to avoid shivering and maintain the body temperature of cattle in a colder environment (Young, 1983). Heat-stressed cattle would compensate for increased body temperature by homeostatic mechanisms (panting, sweating, and urination), reduced activity, increased water intake, and reduced feed intake (Summer et al., 2019). Temperature stress may affect rumen fermentation and digestibility. The cold condition has been reported to increase rumination activity, reticulorumen motility, and digesta passage rate (Kennedy et al., 1976). The cold ambient temperature increased the rate of feed passage through the gastrointestinal tract and reduced digestion efficiency (NRC, 2001). Concentrations of volatile fatty acid in the rumen were also changed by cold ambient temperature (Christopherson and Kennedy, 1983). Several studies have reported that the cattle digestibility was reduced even at a temperature above the lower critical temperature (Webster et al., 1970; Christopherson, 1976; NRC, 1981). According to Beede and Collier (1986), heat stress reduced dry matter intake, because it increased the retention time of digesta in the digestive tract, thus enhanced the feed digestibility. The blood flow to the intestines decreased during heat stress reducing the nutrient absorption and intake (Beede and Collier, 1986).

Temperature stress induces several neuroendocrine responses in an animal by releasing hormones which mediate the adaptive and behavioral responses in the animal. Cortisol mainly plays a role during heat stress by mobilization of energy for maintenance of muscular and neural functions. Cortisol helps to restore the energy homeostasis by stimulating glycogenolysis, lipolysis, and proteolysis in the stressed animal (Bhimte et al., 2018). Cold exposure induces adrenergic signaling, which also induces lipolysis via the activation of the major triglyceride hydrolases, adipose triglyceride lipase, and hormone-sensitive lipase (Zechner et al., 2012) to maintain body temperature. Mader (2003) reported that during the cold condition, the circulating glucose and non-esterified fatty acid are increased probably due to the mobilization of substrates for energy metabolism in the adipose tissue and liver. On the other hand, heat stress has been reported to increase the circulating glucose, but reduce non-esterified fatty acid in cattle (Ronchi et al., 1999).

Heat stress and cold stress increase blood concentrations of glucocorticoids and glucose circulation in ruminants. Elevated levels of glucocorticoids promote glycogenolysis and gluconeogenesis in the liver, while decrease the glucose uptake and utilization by antagonizing insulin response in skeletal muscle and white adipose tissue (Collin et al., 2001; Febbraio, 2001; Kuo et al., 2015). Published report in the rat (Nakagawa and Nagai, 1971) showed that activities of major gluconeogenic enzymes, such as G6PC, fructose diphosphatase, PC, and PCK were elevated when rats exposed to the cold condition. Pyruvate carboxylase, a critical enzyme in gluconeogenesis is

increased during heat stress, indicating higher alanine and lactate utilization in gluconeogenesis (Wheelock et al., 2008; O'Brien et al., 2008; Rhoads et al., 2011). Cold exposure increases the hepatic uptake of lactate and alanine, resulting in double hepatic glucose uptake and blood glucose concentration in sheep (Thompson et al., 1978). Shore et al. (2013) reported that cold exposure down-regulated genes involved in oxidoreductase activity, lipid metabolic processes, and protease inhibitor activity in both brown adipose tissue and liver. Moreover, the authors suggest that the response of cold stress involves decreased gene expression in a range of cellular processes to maximize pathways which are involved in heat production. Faylon et al. (2015) reported that acute heat stress increases the response of bovine primary adipocytes to lipolytic signals which is partly mediated through increased PKA phosphorylation of hormone sensitive lipase and periplin; however, acute heat stress diminished the insulin-stimulated activation of acetyl CoA carboxylase, a critical enzyme in lipogenesis.

Effects of temperature stress on beef quality

The sufficient energy is required to support hyperplasia and hypertrophy for deposition of intramuscular fat. Therefore, the beef quality may be decreased during cold or heat stress because cattle spend more energy to maintain body temperature and energy homeostasis. The occurrence of dark-cutting beef is high during very warm or very cold weather, especially if there are wide fluctuations in ambient temperature for very short periods (Arias et al.,

2018). Carcass weight and dressing percentage of beef cattle raised in the hot or cold season were lower than that of a warm season (Demircan et al., 2007). Between autumn to winter, the incidence of dark cutting beef may be caused by higher ultimate pH values of beef (pH $\geq$ 6.0) (Tarrant and Sherington, 1980). The occurrence of dark firm dry was also high in the hot season, between May to August (Fabiansson et al., 1984). A marked seasonal difference in dark firm dry incidence was obviously seen in steers than bulls (Tarrant, 1981).

6. Effects of glycerol on growth performance and beef quality

Properties and energy contents of glycerol

The term of glycerol is derived from the Greek word "*glykys*", which means "sweet". Glycerol (1,2,3-propanetriol) is derived from both natural and petrochemical feedstocks. It is characterized as a colorless, odorless, and viscous liquid with a sweet taste that is generated approximately 10 to 13% as the main byproduct from the total volume of the biodiesel industry (Dasari et al., 2005). Fatty acids are hydrolyzed from the glycerol backbone of the triglyceride molecule by a trans-esterification process using methanol to yield biodiesel and crude glycerol. The biodiesel is separated from the glycerol by gravity separation or by centrifugation. Excess alcohol and salt are removed from glycerol by flash evaporation or distillation. This process resulting in glycerol contains unused catalyst and soaps, which are then neutralized by the addition of acid to produce crude glycerin (Donkin, 2008). Glycerin or crude

glycerol contains 70 to 80% of pure glycerol, and it is often concentrated and purified into 95.5 to 99% purity (Pagliaro and Rossi, 2010).

Glycerol has a potential energy source for ruminants. The energy value of glycerol can be assumed to be 4.03 Mcal/kg (Mach et al., 2009), which is higher than corn starch. Therefore, it has great potential for replacing cereals in the diets. Recently, glycerol has been examined in order to avoid metabolic problems associated with negative energy balance in transition dairy cows. Also, crude glycerol has widely utilized to prevent ketosis in dairy cows (Johnson et al., 1954; Fisher et al., 1973; DeFrain et al., 2004).

Glycerol ca be used as an alternative feedstuff for ruminant to improve their productivity (Gunn et al., 2010). Donkin (2008) showed that corn can be replaced by crude glycerol, which is the main ingredient of finishing cattle diets (Donkin, 2008). Previous study showed that the replacement of corn with crude glycerin did not change the performance and carcass characteristics of Nellore bulls (Del Bianco Benedetti et al., 2016). Glycerol supplementation on finishing period diets may contribute to the prevention of dark firm dry meat by alleviating some stressful conditions. Administration of 2 g/kg glycerol at 24h before slaughtering did not affect the plasma stress markers, but it seems to improve WHC of beef (Egea et al., 2015). Krueger et al. (2010) reported that dietary GS enhanced passage rate of unsaturated fatty acids from the rumen and improved nutrient absorption, which makes mono unsaturated fatty acids and poly-unsaturated fatty acid more available to be incorporated in meat. Lage et al. (2004) also demonstrated that Nellore bulls fed diets with the replacement

of corn or soybean meal with crude glycerin had higher deposition of mono unsaturated fatty acid and conjugated linoleic acid contents than those fed diets without crude glycerin.

The advantage of glycerol utilization compared to fat source in ruminant diet

To increase the energy supply and to improve the metabolic status of the animal, increasing the energy density in concentrate by fat supplementation was generally used in ruminant's diet compare to glucogenic feed ingredient. Fat supplementation has a positive effect of reducing heat increment instead of the grains (NRC, 2001). However, several factors may limit fat utilization in ruminant's diet. Fat sources can have negative effects on dry matter intake (Chilliard et al., 1993; Nawaz and Ali, 2016) and fibrous carbohydrate digestibility (Doreau and Chilliard, 1997).

Fat sources in the ruminant diet may be originated from different type and origin (animal, plant, processed or whole oilseeds, and protected fat). Supplemental fat sources, depending on the degree of unsaturation, have variable effects on ruminal fermentation. Oils containing poly-unsaturated fatty acid have a more toxic effect on rumen bacteria and ruminal fermentation than saturated fatty acids (NRC, 2016). Therefore, the utilization of fat sources from plants and oilseeds origin are limited in ruminant's diet. According to NRC (2016), ruminants fed a high concentrate diet can receive a maximum 6% of supplemental fat in the diet.

Utilization of fat from animal origins, such as tallow and lard, is limited due to the concern of BSE in cattle and variant Creutzfeldt-Jakob disease in human (Brown, 2004). Tallow is prohibited in all animal feed if it contains more than 0.15% insoluble impurities (protein free) and is derived from rendering cattle materials prohibited in animal food (FDA, 2017). Compared to the fat source, glycerin (crude glycerol) is generally recognized as safe when used in accordance with good manufacture and feeding practices (FDA, 2007). As the demand for renewable energy source continues to rise, glycerol will likely become more affordable feed ingredient for ruminant diet.

Glycerol fates in ruminant digestive tract

Most of the dietary glycerol (44%) was fermented to VFAs in the rumen, and the glycerol which escapes rumen fermentation may be absorbed by rumen epithelium (43%) or the small intestine (13%), reaching the liver by bloodstream Krehbiel (2008). Bergner et al. (1995) reported that the disappearance rate of glycerol is depended from the amount glycerol addition and incubation time. Glycerol absorption appeared to occur mainly by passive diffusion rather than facilitated diffusion and it increases linearly with the inclussion levels (Werner Omazic et al., 2015). The inclusion of dietary glycerol in the ruminant diet may shift in VFA profiles, favoring to propionate production than acetate (Boyd et al., 2013). Rémond et al. (1993) pointed out that 35 - 69% of the dietary glycerol are transformed into propionate. Increasing glycerol inclusion level in dairy cattle diets increased propionate and linearly

decreased acetate concentrations resulting in lower acetate to propionate ratio (Kijora et al., 1998; Paiva et al., 2015).

Effect of glycerol on cattle performance and beef quality

Glycerol can be converted to glucose in the liver, providing energy for cell metabolism, which is an important carbon source used for fatty acid synthesis (Schoonmaker et al., 2004). In the liver and adipocytes, glycerol is a precursor for triglycerides and phospholipids syntheses. Therefore, dietary glycerol inclusion may increase fatty acid synthesis leading to an increase in beef marbling score. Additionally, the supplementation of dietary crude glycerol did not show negative effects on growth performance, and carcass characteristics of beef cattle (Ramos and Kerley, 2012). Previous report also found that increasing glycerin levels up to 178 g/kg (DM) diet increased the apparent digestibility of nutrients, and feed efficiency of crossbred bulls (Eiras et al., 2014). Glucose and insulin levels were not changed by dietary glycerol in dairy cows (DeFrain et al., 2004; Mach et al., 2009). Glycerol inclusion into concentrate diets showed in different results in beef quality. Some studies showed glycerol supplementation had no significant effects on carcass percentage and meat quality of beef cattle (Leao et al., 2013; Ribeiro et al., 2016; Almeida et al., 2018). On the other hand, several studies reported that glycerol inclusion improved carcass characteristics, chemical composition and sensory traits of beef (Carvalho et al., 2014; Egea et al., 2015; Chanjula et al., 2016).

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CHAPTER THREE

Hepatic transcriptional changes in critical genes for gluconeogenesis following castration of bulls

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1. Abstract

This study was performed to understand transcriptional changes in the genes involved in gluconeogenesis and glycolysis pathways following castration of bulls. Twenty Korean bulls were weaned at average 3 months of age, and castrated at 6 months. Liver tissues were collected from bulls (n=10) and steers (n=10) of Korean cattle, and hepatic gene expression levels were measured using quantitative real-time polymerase chain reaction. We examined hepatic transcription levels of genes encoding enzymes for irreversible reactions in both gluconeogenesis and glycolysis as well as genes encoding enzymes for the utilization of several glucogenic substrates. Correlations between hepatic gene expression and carcass characteristics were performed to understand their associations. Castration increased the mRNA (3.6fold; *P*

<0.01) and protein levels (1.4fold; *P* <0.05) of *PC* and *PCK2* genes (1.7fold; *P* <0.05). Hepatic mRNA levels of genes encoding the glycolysis enzymes were not changed by castration. Castration increased mRNA levels of both *LDHA* (1.5fold; *P* <0.05) *LDHB* (2.2fold; *P* <0.01) genes for lactate utilization. Castration increased mRNA levels of *GK* (2.7fold; *P* < 0.05) and *GPD*1 (1.5fold; *P* < 0.05) genes for glycerol utilization. Castration also increased mRNA levels of *PCCB* (3.5fold; *P* < 0.01) and *ACSS3* (1.3fold; *P* = 0.06) genes for propionate incorporation. Castration increases transcription levels of critical genes coding for enzymes involved in irreversible gluconeogenesis reactions from pyruvate to glucose and enzymes responsible for incorporation of glucogenic substrates including lactate, glycerol, and propionate. Hepatic gluconeogenic gene expression levels were associated with intramuscular fat deposition.

2. Introduction

Glucose metabolism is tightly regulated to meet the energy demands of animals, and significantly contributes to animal growth, as well as the quantity and quality of beef production (Nafikov et al., 2007). Glucose metabolism in adult ruminants is different from that of monogastric animals. In ruminants, large portions of ingested carbohydrates are fermented by anaerobic rumen microbes into short-chain fatty acids (mainly acetate, propionate, and butyrate), resulting in much less glucose absorption from the gastrointestinal tract

compared to monogastric animals (Huntington et al., 2006). Therefore, the glucose requirement of ruminants is largely met through *de novo* glucose synthesis via gluconeogenesis, which occurs predominantly in the liver (Nafikov et al., 2007).

Gluconeogenesis and glycolysis pathways are important for hepatic glucose homeostasis. These are not identical pathways operating in opposite directions, although they share several steps. There are three bypass (irreversible) reactions within the gluconeogenesis and glycolysis pathways. The first bypass reaction in gluconeogenesis is the conversion of pyruvate to PEP, involving the enzymes PC and PCK. The next irreversible reaction is the conversion of fructose 1,6-bisphosphate to glucose 6-phosphate by the enzyme FBP1. The third bypass reaction is the conversion of glucose 6-phosphate to glucose by the enzyme G6PC. In the hepatic glycolysis pathway, the first irreversible reaction is the conversion of glucose to glucose 6-phosphate by glucokinase. In the second irreversible reaction, phosphofructokinase-1 is involved in the conversion of fructose 6-phosphate to fructose 1,6-bisphosphate. In the third irreversible reaction, PEP is converted to pyruvate by pyruvate kinase (PKLR).

In ruminants, the important glucogenic substrates of gluconeogenesis are lactate, glycerol, and propionate as well as certain amino acids (Aschenbach et al., 2010). Several enzymes are involved in the incorporation of these substrates in the gluconeogenesis pathway (Aschenbach et al., 2010): lactate dehydrogenase (LDH) for lactate; GK and GPD for glycerol; and acyl-CoA

synthetase short-chain family member 3, propionyl-CoA carboxylase (PCC), methylmalonyl-CoA epimerase, and methylmalonyl-CoA mutase for propionate. Our previous study showed higher circulating glucose levels in steers than in bulls (Bong et al., 2012). This increase may be due in part to changes in the activities of the gluconeogenesis and/or glycolysis pathways following bull castration. However, the molecular events associated with gluconeogenesis and glycolysis pathways in the liver following bull castration remain poorly understood.

Castration is an important method used in the beef cattle industry to improve meat quality, reduce aggressive behavior, and improve herd management. In Korean cattle, beef QG is determined mainly by the amount of marbling (NLCF, 1998). Castration is an efficient way to enhance marbling, resulting in improved beef quality (Bong et al., 2012), although it decreases growth rate, feed efficiency, dressing percentage, and meat yield (Ribeiro et al., 2004). Castration of male rats reduces circulating testosterone levels and enhances hepatic gluconeogenesis (Xia et al., 2013). Our previous study revealed a significant reduction in circulating testosterone levels in bulls following castration (Bong et al., 2012). Little is currently known about the effects of bull castration on gluconeogenesis processes.

The objective of this study was to elucidate transcriptional changes in the genes involved in the gluconeogenesis and glycolysis pathways following bull castration. We examined whether castration affects the expression of genes encoding enzymes for three bypass (irreversible) reactions in gluconeogenesis

and glycolysis from pyruvate to glucose and vice versa. We also determined whether castration affects the expression of genes encoding enzymes responsible for the incorporation of glucogenic substrates (lactate, glycerol, and propionate) into the gluconeogenesis pathway. The associations of gene expression levels with backfat thickness, MS, and QG were also examined by correlation analyses.

3. Materials and methods

Animals and sampling

We used liver tissue from 10 bulls and 10 steers from a previous study (Bong et al., 2012). Briefly, Korean bulls were weaned at a mean age of 3 months, and fed with 30% concentrates/70% roughage until they reached 6 months of age. Bulls were castrated at 6 months. After 6 months of age, bulls and steers were fed with concentrates consisting of 15% crude protein (CP)/71% total digestible nutrients (TDN) until 14 months of age, and 11% CP/73% TDN after 21 months of age. Roughage was offered *ad libitum*, and the animals had free access to fresh water during the entire experimental period. To study the effects of castration on carcass traits and the associations with gene expression levels, we used both bulls and steers of conventional slaughter age in Korea beef industry. Slaughter ages were 26 months and 32 months for bulls and steers, respectively. Carcass weight was 445 kg and 437 kg for bulls and steers, respectively. We used 10 steers (Bong et al., 2012) with high MS values to

detect differences in liver metabolism between bulls and steers clearly. In our study, steers (6.6 ± 0.3) had a 6-fold higher ($P < 0.001$) MS than bulls (1.1 ± 0.1).

Animals were transported for 4 hours and slaughtered the following day. After undergoing captivate-bolt stunning, the animals were slaughtered in a conventional manner. After slaughter, liver tissue samples were collected immediately, snap-frozen in liquid nitrogen, and stored at –80°C. The carcasses were moved to a cold room (5°C). After 24 hours, the carcasses were graded using the carcass grading system designed by the Korea Institute for Animal Products Quality Evaluation (KAPE, 2013).

We used previously reported carcass characteristics (Bong et al., 2012) to calculate correlation coefficients. Briefly, backfat thickness, MS, meat color, fat color, texture, and maturity were determined by an official meat grader. Among these parameters, MS was the main determinant of QG. Five QGs (QG 1++, 1+, 1, 2, and 3) were assigned by meat graders. The Beef Marbling Standard (BMS) marbling score ranges from 1 (devoid) to 9 (abundant); 8 or 9 was the MS for QG1++, 6 or 7 was the MS for QG1+, 4 or 5 was the MS for QG1, 2 or 3 was the MS for QG2, and 1 was the MS for QG3. Meat color, fat color, texture, and maturity of the exposed longissimus muscle (LM) at the 13th rib interface were used for QG determination (NLCF, 1998). Back fat thickness was evaluated in terms of the thickness of the fat over the LM measured perpendicular to the outside surface at a point two-thirds the length of the rib eye from its chine bone end.

RNA extraction and real-time polymerase chain (PCR) reaction

Total RNA was isolated from liver tissues using Trizol reagent (Molecular Research Center, Cincinnati, OH, USA) according to the supplier's protocol. Total RNA concentration and integrity were verified by checking the absorbance at 260 nm and analyzing 2 μL of each sample in 1% agarose gel electrophoresis. RNA quality was also checked by an RNA 6000 Nano LabChip kit and Agilent 2100 BioAnalyzer (Agilent Technologies, Palo Alto, CA, USA).

Total RNA was transcribed into cDNA using the iScript cDNA synthesis kit (Bio-Rad, Hercules, CA, USA) according to the supplier's protocol. All primers were designed using Pick Primer program from the National Center for Biotechnology Information (**Table 1**). We used two different exons for forward and reverse primers to prevent amplification of the DNA template. We have tried to set melting temperature around 60°C and indicated the melting temperatures (Tm) of all primers in Table 1. Gene expression was evaluated using real-time PCR analysis in a 25-μL total reaction volume containing 200 ng cDNA, 12.5 μL of SYBR Green RT-PCR Master Mix and 1.25 μL of 10 μM primers. Reactions were performed under thermal cycling condition as follows: 95°C for 15 minutes (initial step) followed by 40 cycles at 94°C for 15 s (denaturation), 55°C for 30 s (annealing), and 72°C for 30 s (extension). Real-time PCR data were normalized using the ΔΔCT method to determine relative fold changes (Livak and Schmittgen, 2001), and all data were normalized with *ribosomal protein s9* (*RPS9*) as the housekeeping gene.

Table 1. Primer sequences for real-time PCR analysis

Gene name (Symbol)	Gene bank accession no.	Primer	Sequence (5'-3')	Length (bp)	Tm[1] (°C)
Ribosomal protein S9 (RPS9)[2]	NM_001101152	Forward	CCTCGACCAAGAGCTGAAGCCTC	64	62.1
		Reverse	CAGACCTCACGTTTGTTC		53.3
Gluconeogenesis					
Pyruvate carboxylase (*PC*)	NM_177946	Forward	TCCCCAACATCCCATTCCAG	116	59.4
		Reverse	TGTCCATGCCATTCTCCTTG		57.9
Phosphoenolpyruvatecarboxykinase, mitochondrial (*PCK2*)	NM_001205594	Forward	TGGATGAGGTTTGACAGTGATG	127	58.3
		Reverse	TGGTGTTACTCTGGATTGTGG		57.6
Phosphoenolpyruvatecarboxykinase, cytosolic (*PCK1*)	AY145503	Forward	GGTGTGATCAAGAGGCTGAAG	125	58.6
		Reverse	ATGGGCACCGTATCTCTTTG		57.7
Fructose-1,6-bisphosphatase 1 (*FBP1*)	NM_001034447.2	Forward	TCACCGAGTATGTCCAGAGGA	146	60.0
		Reverse	GGGGCTTTTCTTGTTAGCTGG		59.4
Glucose 6-phosphatase (*G6PC*)	NM_001076124	Forward	AGCCAACCTACAGATTTCG	95	54.9
		Reverse	AGCAAGGTAGATTCGTGACAG		57.8
Glucogenic substrate-lactate/pyruvate					
Lactate dehydrogenase A (*LDHA*)	NM_174099	Forward	CGTGTTATTGGAAGTGGTTGC	139	58.1
		Reverse	CACTCCATACAGGCACACTAG		57.8

Lactate dehydrogenase B (*LDHB*)	BT021009	Forward	CTTGCTCTTGTGGATGTTTTGG	127	58.3
		Reverse	TGGAATTGGCAGTGACAGAG		57.8
Pyruvate dehydrogenase (lipoamide) alpha 1 (*PDHA1*)	NM_001101046.2	Forward	GTGGCATCTCGTCATTTTGC	98	58.1
		Reverse	GTAAGCACTGTAGTGACAGG		55.5
Dihydrolipoamide S-acetyltransferase (*DLAT*)	NM_001205730.2	Forward	GACCATGGGCACAGTTCAGA	130	60.0
		Reverse	TGCCAGGTAACCTTCTTCCTG		59.6
Dihydrolipoamide dehydrogenase (*DLD*)	NM_001206170.1	Forward	GACACAGATGGCATGGTGAA	143	58.2
		Reverse	GCTATGTCTTCACAGGATGC		56.3
Glucogenic substrate-propionate					
Acyl-CoA synthetase short-chain family member 3 (*ACSS3*)	NM_001102137	Forward	GAGGCACTGAGAATAGGACAAC	133	58.5
		Reverse	CATGTGACTGTGCTTTTGAGAG		57.9
Propionyl-CoA carboxylase alpha, mitochondrial (*PCCA*)	NM_001083509	Forward	AAATGAACACGAGACTCCAG	135	55.4
		Reverse	AGCCGTTGATGGGAATATCG		57.8
Propionyl-CoA carboxylase beta, mitochondrial (*PCCB*)	NM_001038548	Forward	CACATGCCCAAAAGATCTGC	115	57.7
		Reverse	GCCAAAGATTCCACTCCCTC		58.2
Methymalonyl-CoA mutase (*MUT*)	NM_173939	Forward	GCTGATCTTGGTTTTGATGTGG	138	58.2
		Reverse	CTCAGGAACTAGGGTCTTGTG		57.4
Methylmalonyl-CoA epimerase (*MCEE*)	NM_001045995.2	Forward	TGGAGGGATGCACCATGTCT	114	60.9
		Reverse	CCATGTGCTCCTATTTTGGC		57.1

Glucogenic substrate-glycerol					
Glycerol kinase (*GK*)	NM_001075236.1	Forward	GCTTCGTTGGCTCCTTGACA	131	60.9
		Reverse	TACAATGGACCCCTCCACTG		58.7
Glycerol-3-phosphate dehydrogenase 1 (*GPD1*)	NM_001035354.1	Forward	CACCCAATTTCCGCATCACG	126	60.2
		Reverse	CCTTGGTGTTGTCGCCAAAG		60.0
Glycerol-3-phosphate dehydrogenase 2 (*GPD2*)	NM_001100296.1	Forward	GACAGGGAAAGAGCGTGTGA	104	60.0
		Reverse	TGAAAGGTCCCGTGGCATTG		60.9
Solute carrier family 2 member 2 (*SLC2A2*)	NM_001103222	Forward	TGCTGTCTCTGTGTTCCTTG	127	57.7
		Reverse	CCAAGGGAATTTACTCAGGAGC		58.7
Hexokinase 2 (*HK2*)	XM_002691189	Forward	TTTCACTTTCTCCTTCCCCTG	82	57.5
		Reverse	AGACGCCTTGAAGCCTTTAG		57.9
Phosphofructokinase-1 (*PFKL*)	NM_001080244	Forward	TCAGAGCCGTGACTCGTATG	131	59.3
		Reverse	GATGTTGGAGACGCTCAG		55.2
Pyruvate kinase (*PKLR*)	NM_001076176.1	Forward	ATCGACTCTGAGCCTGTGGT	97	60.6
		Reverse	CCTCGATCATCTCCTTGAGAC		57.4

[1]Tm = melting temperature; [2]Housekeeping gene

Western blot

Samples were homogenized in a Polytron homogenizer for 30 s with cold Pro-PREP protein extraction solution (Intron Biotechnology, Seongnam, Korea), and the homogenized samples were incubated at 4°C for 30 min. Samples were centrifuged (13,000× *g*, 30 min, 4°C) and the protein content of each supernatant was determined using a BCA protein assay kit (Pierce Rockfold, IL, USA). Total proteins were prepared for Western blot analysis by boiling in 5X sample buffer (50 mM Tris, 2% SDS, 5% glycerol, and 10% 2-mercaproethanol, pH 6.8). We separated 20 μg of proteins by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using 6% polyacrylamide separating gels for PC (130 kDa), with 5% stacking gels. Proteins were transferred into polyvinylidene flouride membranes (BioRad Laboratories, Inc., Hercules, CA, USA) blocked with 1 × TBS/0.1% Tween 20 and 5% nonfat dried milk,and incubated using commercial primary antibodies (1:200 dilution for antibodies against PC), including goat polyclonal antibody (sc-46228; Santa Cruz Biotechnology, Santa Cruz, CA, USA). The blots were treated with secondary horseradish peroxidase–conjugated anti-goat antibody, anddeveloped using an enhanced chemiluminescence detection kit (Amersham Biosciences, Buckinghamshire, UK). The signal intensity was quantified using image processing scan analysis (ChemiDoc XRS, BioRad) and measured using ImageJ software (NIH, Bethesda, MD, USA). Band densities were normalized with actin content.

Statistical analysis

All data are presented as the mean ± standard error of the mean. Statistical differences between bulls and steers were examined using the general linear model procedure in the SAS software 9.1 (SAS Institute). Correlations of gene expression levels with back fat thickness, MS, and QG were calculated using the CORR procedure in SAS.

4. Result and discussion

Changes in expression of genes for gluconeogenesis pathway from pyruvate to glucose following bull castration

Study about effects of castration on gluconeogenesis is limited in ruminant. In male-Göttingen mini pigs, castration induced testosterone and estradiol deficiency, results in insulin resistance, glucose intolerance, and hyperglucagonemia, which disturbed glucose metabolism (Christoffersen et al., 2010). In this study, we examined changes in the expression of genes encoding enzymes responsible for these bypass reactions in the gluconeogenesis pathway following castration. Pyruvate carboxylase is the regulatory enzyme of gluconeogenesis, and it catalyzes the conversion of pyruvate to oxaloacetate (OAA) (Pershing et al., 2002). We found higher (3.6 fold; P <0.01) *PC* mRNA levels in steers than in bulls (**Figure 2**). We examined the existence of PC protein expressions by Western blot. Steers exhibited higher existence (P <0.05) PC protein in the liver than did bulls, reflecting the transcriptional activity trend.

Upregulation of *PC* gene expression may contribute to increased OAA production and subsequent activation of the gluconeogenesis pathway following castration.

Phosphoenolpyruvate carboxykinase catalyzes the decarboxylation of OAA to produce PEP (Yang et al., 2009). In gluconeogenesis, the conversion of OAA into PEP by PCK can occur in both the cytosol and mitochondria. One of two isoforms of PCK, the cytosolic form (PCK1) and the mitochondrial form (PCK2), works in each compartment. In the mitochondria, OAA can be converted into PEP by PCK2 and transported to the cytoplasm, where it is converted into glucose via the remaining gluconeogenic enzymes. In this study, we found higher (1.7 fold; $P < 0.05$) *PCK2* mRNA levels in steers than in bulls (Figure 5). The *PCK2* reaction predominates when lactate is the glucogenic precursor (Aschenbach et al., 2010). Upregulation of *PCK2* gene expression may indicate that castration increases utilization of lactate as a glucogenic precursor in the gluconeogenesis pathway. In this study, castration did not affect ($P > 0.05$) *PCK1* mRNA levels.

In this study, the mRNA levels of *FBP1* and *G6PC* remained unchanged ($P > 0.05$) following castration (**Figure 2**). Al-Trad et al. (2010) also reported no significant correlation between glucose release from the gluconeogenesis pathway and the change in *FBP1* and *G6PC* mRNA abundance, which was mainly regulated post-transcriptionally.

Glucose is the major fuel for metabolism in most body tissues. Hepatic glucose homeostasis is determined by the rates of glucose uptake and glucose

production. In ruminants, glucose requirements are largely met through gluconeogenesis, which occurs predominantly in the liver. There are three bypass reactions of gluconeogenesis pathway: conversion of pyruvate to PEP by enzymes PC and PCK, conversion of fructose 1,6-bisphosphate to glucose 6-phosphate by FBP1, and conversion of glucose 6-phosphate to glucose by G6PC. In this study, we found that castration increased the expression of PC mRNA and protein levels. Upregulation of *PC* gene expression may contribute to increased OAA production and subsequent activation of the gluconeogenesis pathway following castration.

In this study, we found that castration upregulated *PCK2* gene expression. The *PCK2* reaction predominates when lactate is the glucogenic precursor (Aschenbach et al., 2010). Upregulation of *PCK2* gene expression may indicate that castration increases utilization of lactate as a glucogenic precursor in the gluconeogenesis pathway. Castration of male animals reduces the concentration of circulating androgen. In a rat study, castration was found to reduce circulating testosterone levels and increase body fat mass (Xia et al., 2013). In our previous study, castration also decreased testosterone levels and increased intramuscular fat deposition in beef cattle (Bong et al., 2012). Insulin is known to suppress lipolysis and gluconeogenesis (Rui, 2014).

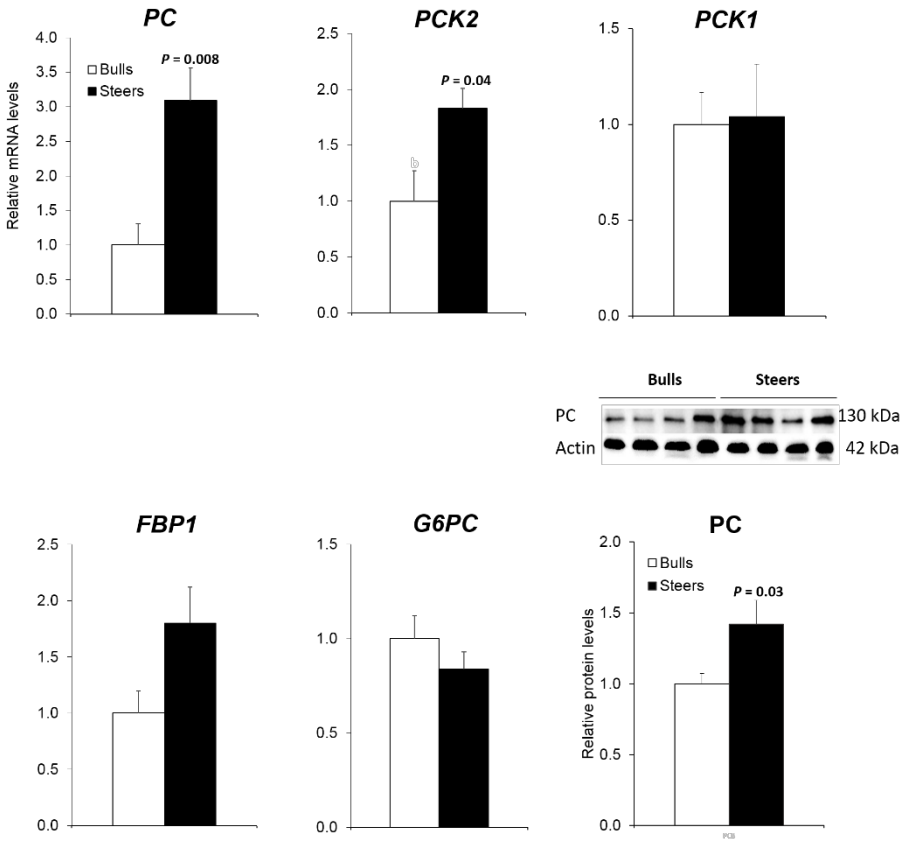

Figure 2. Hepatic expression levels of genes for gluconeogenesis from glucose to pyruvate in Korean cattle. mRNA levels (n = 10) were determined using real-time polymerase chain reaction and normalized with a control ribosomal protein s9 gene. mRNA levels in bulls were normalized to 1.0. Protein levels (n = 4) were determined by Western blot analysis and normalized with actin levels. Values are expressed as the mean + SE. *PC = pyruvate carboxylase, PCK2 =mitochondrial phosphoenolpyruvate carboxykinase, PCK1 =cytosolic phosphoenolpyruvate carboxykinase, FBP1 = fructose 1,6-bisphosphatase,G6PC = glucose 6-phosphatase.*

Castration alone (Xia et al., 2013) or a combination of castration and a high-fat diet (Dubois et al., 2016) induced insulin resistance in the liver and other peripheral tissues, such as adipose and muscle tissue, in rodent studies. The authors suggested that increased gluconeogenic *Pck* gene expression is associated with hepatic insulin resistance, which was induced by androgen deficiency under a combination of castration and feeding a high-fat diet. Thus, the increased hepatic *PCK2* transcription levels observed in this study may be also associated with insulin resistance caused by testosterone deficiency following castration. In the cytosol, OAA can also be converted into PEP by PCK1. This pathway is predominant when an amino acid such as alanine is the glucogenic precursor. The mitochondrial membrane possesses no OAA transporter. Thus, OAA is reduced to malate at the expense of NADH, and malate is then transported to the cytosol where it is reoxidized to OAA with the generation of cytosolic NADH. The cytosolic OAA is converted into PEP to enter the gluconeogenesis pathway. In this study, mRNA levels of *PCK1* gene did not differ between bulls and steers. It has been proposed that PCK1 is required for gluconeogenesis from amino acids, whereas PCK2 is better suited for gluconeogenesis from lactate (Hanson and Reshef, 1997). Thus, our study implies that castration may increase gluconeogenesis by using lactate as glucogenic substrate with no change in amino acid availability for gluconeogenesis.

Expression of genes for the incorporation of glucogenic substrates in the gluconeogenesis pathway

In this study, we examined changes in the hepatic expression levels of genes encoding enzymes for the incorporation of glucogenic substrates (lactate, glycerol, and propionate) following castration (**Figure 3**). Lactate is first converted to pyruvate by LDH in the cytosol. In this study, castration increased hepatic expression levels of both *LDHA* (muscle type; 1.5 fold; $P < 0.05$) and *LDHB* (liver type; 2.2 fold; $P < 0.01$) genes.

Pyruvate can either be converted to OAA by PC or reduced to acetyl-CoA by the pyruvate dehydrogenase complex. The complex consists of three enzymes: pyruvate dehydrogenase (lipoamide) alpha 1, dihydrolipoamide S-acetyltransferase, and dihydrolipoamide dihydrogenase. In this study, hepatic mRNA levels of *pyruvate dehydrogenase (lipoamide) alpha 1*, *dihydrolipoamide S-acetyltransferase*, and *dihydrolipoamide dihydrogenase* genes did not differ ($P > 0.05$) between bulls and steers. Our study demonstrated that castration did not change acetyl-CoA generation from pyruvate.

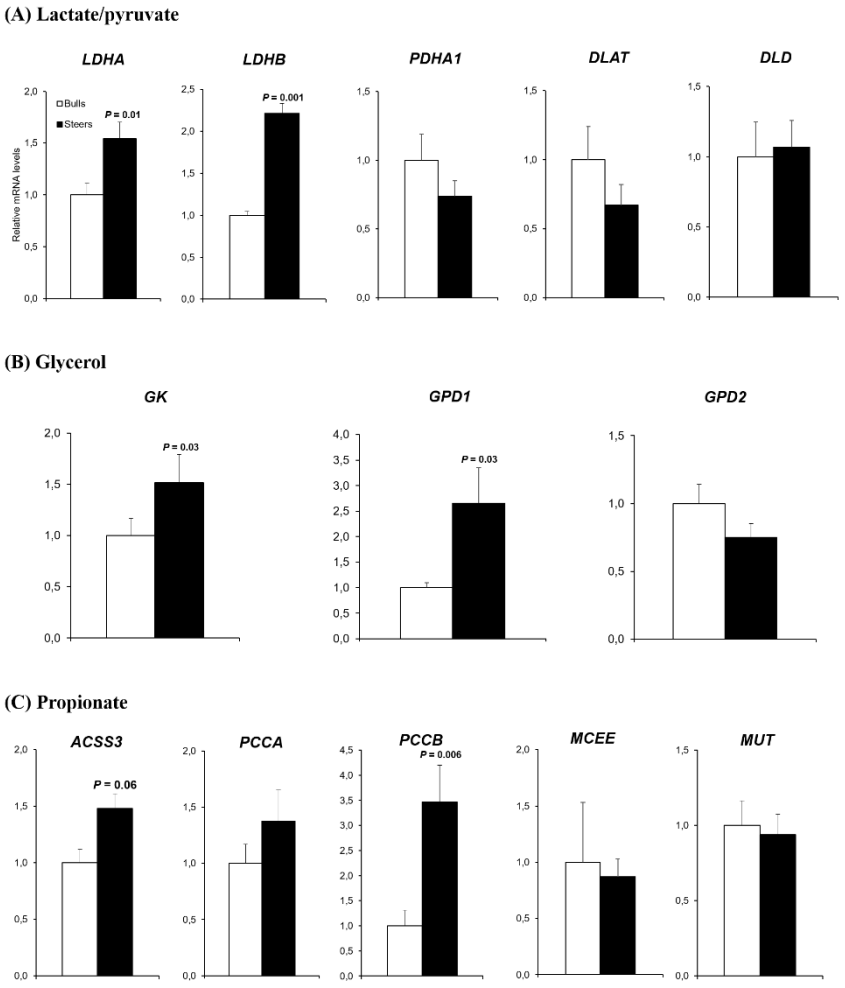

Figure 3. Hepatic mRNA levels of genes for glucogenic substrate incorporation in gluconeogenesis pathway in Korean cattle. mRNA levels were determined by real-time PCR and normalized with a control ribosomal protein s9 gene. mRNA levels in bulls were normalized to 1.0. Values are expressed as the mean + SE (n = 10). *LDHA = lactate dehydrogenase A, LDHB = lactate dehydrogenase B, PDHA1 = pyruvate dehydrogenase (lipoamide) alpha 1, DLAT = dihydrolipoamide S-acetyltransferase, DLD = dihydrolipoamide*

dehydrogenase, GK = glycerol kinase, GPD1 = glycerol-3-phosphate dehydrogenase 1, GPD2 = glycerol-3-phosphate dehydrogenase 2, ACSS3 = acyl CoA synthetase short chain family member 3, PCCA = propionyl CoA carboxylase alpha, PCCB = propionyl CoA carboxylase beta, MCEE = methylmalonyl CoA epimerase, MUT = methylmalonyl CoA mutase.

Glycerol can be utilized as a glucogenic precursor. Glycerol is converted to glycerol-3-phosphate by GK in the liver. Glycerol-3-phosphate is then oxidized to dihydroxyacetone phosphate by GPD and utilized for gluconeogenesis. Two isoforms (GPD1 in the cytosol and GPD2 in the mitochondria) are present; both enzymes catalyze the reversible conversion of glycerol-3-phosphate to dihydroxyacetone phosphate. We examined hepatic transcript levels of genes for the incorporation of glycerol into the gluconeogenesis pathway in bulls and steers. Castration increased mRNA levels of the *GK* (2.7 fold; $P < 0.05$) and *GPD1* (1.5 fold; $P < 0.05$) genes (Figure 6), but did not affect hepatic expression of the *GPD2* gene.

Propionate is the major substrate for gluconeogenesis in ruminants (Aschenbach et al., 2010). Castration affected the expression of several genes for the incorporation of propionate into the gluconeogenesis pathway (Figure 6). Acyl-CoA synthetase short-chain family member 3 plays a role in the activation of acetate to specific metabolic fate, and has also a high activity to activate propionate (Orchard and Anderson, 1996). In mitochondria, acyl-CoA synthetase short-chain family member 3 catalyzes the conversion of propionate

to propionyl-CoA at the first step of the propionate-originated gluconeogenesis pathway (Wang et al., 2015). In this study, steers exhibited higher (P = 0.06) *acyl-CoA synthetase short-chain family member 3* mRNA levels than did bulls (Figure 6). Propionyl-CoA is then converted by mitochondrial PCC to methylmalonyl-CoA, which is converted to succinyl-CoA by methylmalonyl-CoA epimerase and methylmalonyl-CoA mutase. Succinyl CoA is subsequently incorporated into part of the tricarboxylic acid cycle to generate OAA (Aschenbach et al., 2010). Mitochondrial PCC is composed of two subunits, alpha (PCCA) and beta (PCCB), which are encoded by two separate genes (NCBI, 2017).In this study, castration increased (P <0.01) mRNA levels of *PCCB*, although it did not affect (P >0.05) *PCCA* mRNA levels (**Figure 3**). Castration did not affect (P >0.05) mRNA levels of the *methylmalonyl-CoA epimerase*and *methylmalonyl-CoA mutase* genes (**Figure 3**).

In ruminants, the major glucogenic precursors of gluconeogenesis in the liver are lactate, propionate, glycerol, and amino acids. We found that castration increased hepatic gene expression levels of both *LDHA* and *LDHB*, which are involved in conversion of lactate to pyruvate. Lactate can be produced endogenously from the anaerobic glycolysis pathway, mainly in skeletal muscle, but also in other tissues including the brain, gastrointestinal tract, renal medulla, adipose tissue, skin, and erythrocytes (Guo et al., 2012). Kuhla et al. (2011) suggested that increased lactate production in skeletal muscle might serve as a substrate for hepatic gluconeogenesis during the adaptation period in the early lactation of dairy cows. Several studies have reported that lactate metabolism

and hepatic lactate uptake are influenced by insulin action. Insulin decreases hepatic lactate uptake in rats (Edgerton et al., 2009). Insulin resistance in obese patients is associated with elevated circulating basal lactate levels in humans (Lovejoy et al., 1992). Insulin resistance in obese Zucker rats was also associated with higher circulating lactating concentrations and impaired lactate metabolism in skeletal muscle compared with normal Wistar rats (Py et al., 2001). As described above, several studies have shown that castration induces insulin resistance in the skeletal muscle of male rats (Xia et al., 2013; Dubois et al., 2016). Therefore, castration may induce insulin resistance, which limits glucose utilization in skeletal muscle and subsequently increases its lactate output, contributing to increased hepatic lactate uptake. Increased hepatic lactate uptake following castration may in part contribute to increased hepatic gluconeogenic activity through the activation of LDH expression. After pyruvate is transported into the mitochondria, it is converted to OAA by PC, as described above. Our findings on the upregulation of *PC* and *PCK2* gene expression following castration maybe due to the increase in lactate availability for the glucogenic substrate. In a rat study, Stark and Kibbey (2014) found that *Pck2* has a direct role in gluconeogenesis from a lactate substrate.

In addition to endogenous lactate production from peripheral tissues, lactate can also be produced in the rumen of ruminant animals by bacterial fermentation if they are fed high amounts of concentrate diet, including starch, and if ruminal pH is relatively low (Aschenbach et al., 2010). This lactate can also be used as a gluconeogenesis substrate. In the conventional Korean beef

cattle feeding system, steers consume a higher concentrate diet than do bulls during the finishing period to increase MS (Baik et al., 2017). In steers, this may induce higher lactate production, which may be used as a glucogenic substrate. Further study is warranted to understand the link between hepatic gluconeogenesis metabolism and lactate production in the skeletal muscle and rumen of cattle.

We found that castration increased gene expression for *GK* and *GPD1*, which are involved in the incorporation of glycerol into the gluconeogenesis pathway. This result indicates that castration may increase the utilization of glycerol as a glucogenic source. In a mouse study, a deficiency of *Gpd1* activity inhibited the use of glycerol for gluconeogenesis (Sato et al., 2016). In mouse and rat studies, insulin resistance has induced lipolysis, resulting in increased generation of fatty acid and glycerol production, which caused hepatic increases in acetyl-CoA production and glycerol input (Perry et al., 2015). Adeva-Andany et al. (2016) reported positive correlation of the rates of gluconeogenesis and fatty acid oxidation. Increased hepatic fatty acid oxidation activates gluconeogenesis, in part, due to increased production of acetyl-CoA, which is an activator of *PC* (Adina-Zada et al., 2012). Our previous study showed that castration increases fatty acid oxidation in skeletal muscle of Korean cattle (Jeong et al., 2014). Perry et al. (2015) suggested that allosteric regulation of *PC* activity by hepatic acetyl-CoA is an important factor regulating hepatic glucose production. In their study, insulin resistance increased gluconeogenesis through increased *PC* activation by acetyl-CoA and

increased utilization of glycerol as a glucogenic source. Xia et al. (2013) also reported that castration-induced testosterone deficiency decreased insulin sensitivity and that it increased hepatic gluconeogenesis, especially from a glycerol substrate. Thus, our observation that increased transcription levels of genes encoding PC, GK, and GPD1 for the utilization of glycerol as a glucogenic substrate may be also associated with insulin resistance caused by testosterone deficiency following castration. Further study is needed to verify whether castration increases hepatic fatty acid oxidation, thus increasing production of acetyl-CoA and glycerol, contributing to activation of the gluconeogenesis pathway.

Overall, the current study showed that the combined effects of bull castration and a high-concentration diet during the fattening period might cause insulin resistance, resulting in increased lipolysis and subsequent increased production of lactate, glycerol, and acetyl-CoA. These increases then may activate several enzymes (LDH, PC, PCK2, GK, and GPD1) responsible for gluconeogenesis.

Collectively, our results indicate that castration increases transcriptional activities of the hepatic gluconeogenesis pathway from propionate through the activation of *ACCS3* and *PCCB* gene expression. In ruminants, feeding a higher-concentrate produces relatively high propionate levels in the rumen (Chen et al., 2015). In Korea, steers are typically fed a higher-concentrate during the late fattening period than bulls are, probably causing higher propionate production in steers than in bulls (Jeong et al., 2016). Thus,

increased transcriptional activities of propionate utilization for gluconeogenesis precursors following castration may in part be due to the consumption of a high-concentrate during the late fattening period in steers.

Gluconeogenesis from propionate is less responsive than other substrates to changes in insulin concentration (Brockman, 1990). When lactate and glucogenic amino acids are more available for other synthetic processes, propionate could preferentially be used for gluconeogenesis (Brockman, 1990; Huntington et al., 2006), increasing the proportion of glucose derived from propionate. Our results indicate that expression levels of genes encoding enzymes for the gluconeogenesis pathway using several substrates including lactate, glycerol, and propionate are upregulated following castration.

Expression of genes encoding enzymes regulated glucose transport and glycolysis following castration.

We have examined the expression of genes for hepatic glucose transporter *(SLC2A2*) and glycolysis. The *SLC2A2* gene is a major glucose transporter in the cattle liver (Li et al., 2013). We found no difference ($P > 0.05$) in *SLC2A2* mRNA levels between bulls and steers (**Figure 4**). The mRNA levels of glycolysis genes encoding enzymes *HK2* and *PFKL* did not differ ($P > 0.05$) between bulls and steers. Our study indicates that castration does not affect the transcriptional activities of glucose uptake and upstream of the glycolysis pathway in the liver.

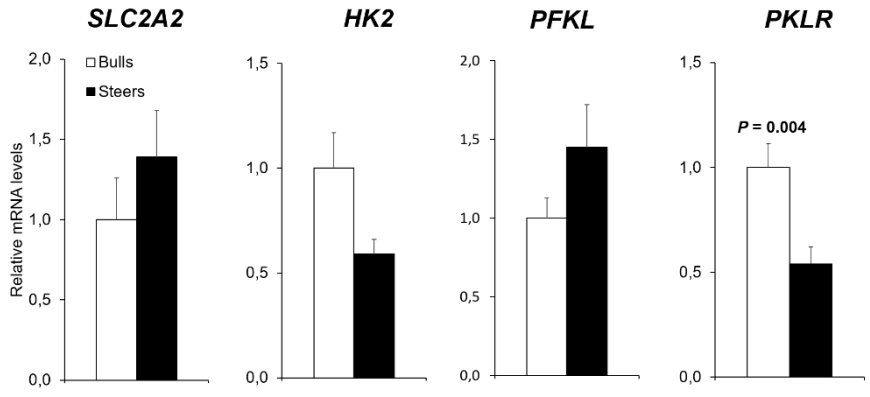

Figure 4. Hepatic mRNA levels of glucose transporter and glycolysis genes in Korean cattle bulls and steers. mRNA levels were determined by real-time PCR and normalized with a control ribosomal protein s9 gene. mRNA levels in bulls were normalized to 1.0. Values are expressed as the mean + SE (n = 10). *SLC2A2 = solute carrier family 2 member 2*, *HK2 = hexokinase 2*, *PFKL = phosphofructokinase-1*, *PKLR = pyruvate kinase.*

Pyruvate kinase catalyzes the transfer of phosphate from PEP to adenosine diphosphate to produce ATP and pyruvate. In this study, hepatic mRNA levels of the *PKLR* gene were lower ($P < 0.01$) in steers than in bulls. This may indicate lower PEP conversion for ATP production following castration.

In this study, steers showed the higher hepatic mRNA levels of the *PKLR* gene than bulls indicating lower PEP conversion for ATP production following castration. In a mouse study, insulin resistance in the liver led to a decrease in the expression of *Pklr* (Michael et al., 2000). As described above, we found that

castration increased the mRNA levels of the *LDH* gene, which is responsible for gluconeogenesis from lactate. Thus, our study demonstrates the shift of PEP utilization from energy (ATP) production to lactate utilization for hepatic gluconeogenesis.

Correlation between mRNA level of glucose metabolism genes and backfat thickness, marbling score, and quality grade

We analyzed the relationships of hepatic gene expression levels with backfat thickness, MS, and QG using pooled steers and bulls. Several significant correlations were observed (**Table 2**). Backfat thickness showed positive correlations with the hepatic mRNA levels of genes for gluconeogenesis, including *FBP1* (r=0.45, $P<0.05$), *LDHB*(r=0.66,$P<0.01$), *PCCA* (r=0.45,$P<0.05$), *GK* (r=0.53,$P<0.05$), and *GPD1* (r=0.56,$P<0.05$). MS and/or QG were positively correlated with the hepatic mRNA levels of *PC* (r = 0.55, $P< 0.05$; r = 0.55, $P< 0.05$), *PCK2* (MS r = 0.47, $P< 0.05$), *LDHA* (r = 0.51, $P< 0.05$; r = 0.52; $P< 0.05$), *LDHB* (r = 0.79, $P< 0.01$; r = 0.80, $P< 0.01$), and *PCCB* (r = 0.58, $P < 0.01$; r = 0.57, $P< 0.01$)genes. Negative correlations of MS and QG with hepatic mRNA levels of the *G6PC* (r= –0.51, $P< 0.05$; r= –0.45, $P< 0.05$) and *PKLR* (r = –0.58, $P<0.01$; r = –0.64, $P<0.01$) genes were observed.

By correlation analysis, we found significant correlations of expression levels of several genes including *fructose 1,6-bisphosphatase*, *LDHB*, *PCCA*, *GK*, and *GPD1* with backfat thickness. The quality grade of Korean cattle beef

is determined mainly by MS. We also found that hepatic mRNA levels of *PC*, *PCK2*, *LDHA*, *LDHB*, and *PCCB* genes were positively correlated with the MS and/or QG. Insulin resistance increased hepatic glucose production; however, it was associated with diminished glucose utilization in muscle and enhanced utilization in white adipose tissue (Cettour-Rose et al., 2005). Prior and Scott (1980) suggested that increased availability of glucogenic precursors (lactate and propionate) or glucose may be responsible for the induction of lipogenesis in steer fat tissues. Therefore, significant correlations of glucogenic gene expression with carcass traits may support that increased incorporation of glucogenic substrates from lactate and glycerol or propionate enhance hepatic gluconeogenesis, which contributes to glucose utilization in adipose tissue, resulting in increased backfat thickness and intramuscular fat deposition, and thus MS and QG following castration. Some genes for gluconeogenesis may therefore be used as genetic markers to predict backfat thickness, MS, and QG.

Table 2. Correlation between hepatic gene expression levels and backfat thickness, marbling score, and quality grade in Korean cattle

Genes	Backfat thickness	Marbling score	Quality grade
Pyruvate carboxylase (PC)	0.27	0.55*	0.55*
Phosphoenolpyruvate carboxykinase, mitochondrial (PCK2)	0.29	0.47*	0.44
Phosphoenolpyruvate carboxykinase, cytosolic (PCK1)	-0.06	-0.06	-0.02
Fructose 1,6-bisphosphatase(FBP1)	0.45*	0.30	0.35
Glucose 6-phosphatase (G6PC)	-0.22	-0.51*	-0.45*
Lactate dehydrogenase A (LDHA)	0.33	0.51*	0.52*
Lactate dehydrogenase B (LDHB)	0.66**	0.79**	0.80**
Pyruvate dehydrogenase (lipoamide) alpha 1 (PDHA1)	-0.33	-0.30	-0.26
Dihydrolipoamide S-acetyltransferase (DLAT)	-0.29	-0.31	-0.27

Dihydrolipoamide dehydrogenase (DLD)	0.03	0.04	0.06
Acyl-CoA synthetase short-chain family member 3 (ACSS3)	0.23	0.31	0.33
Propionyl-CoA carboxylase alpha, mitochondrial (PCCA)	0.45*	0.24	0.25
Propionyl-CoA carboxylase beta, mitochondrial (PCCB)	0.27	0.58**	0.57**
Methymalonyl-CoA mutase (MUT)	-0.04	-0.12	-0.10
Methylmalonyl-CoA epimerase (MCEE)	-0.09	-0.06	-0.04
Glycerol kinase (GK)	0.53*	0.43	0.44
Glycerol-3-phosphate dehydrogenase 1 (GPD1)	0.56*	0.39	0.44
Glycerol-3-phosphate dehydrogenase 2 (GPD2)	-0.10	-0.31	-0.34
Hexokinase 2 (HK2)	-0.39	-0.44	-0.44
Phosphofructokinase-1 (PFKL)	0.36	0.27	0.31
Pyruvate kinase (PKLR)	-0.40	-0.58**	-0.64**

N = 20; Significant correlations: *P<0.05; ** P<0.01.

In addition, castration may change the myofiber composition and size in the longissimus muscle. Castration decreased the type IIA myofiber (fast-twitch oxidative) and increased the type IIB myofiber (fast-twitch glycolytic) in longissimus muscle (Young and Bass, 1984). Increased proportion of type IIB muscle fibers has been found to correlate with insulin resistance, which may alter the metabolism and/or storage of lipids in the muscle, partly contributing to increased intramuscular fat deposition following castration.

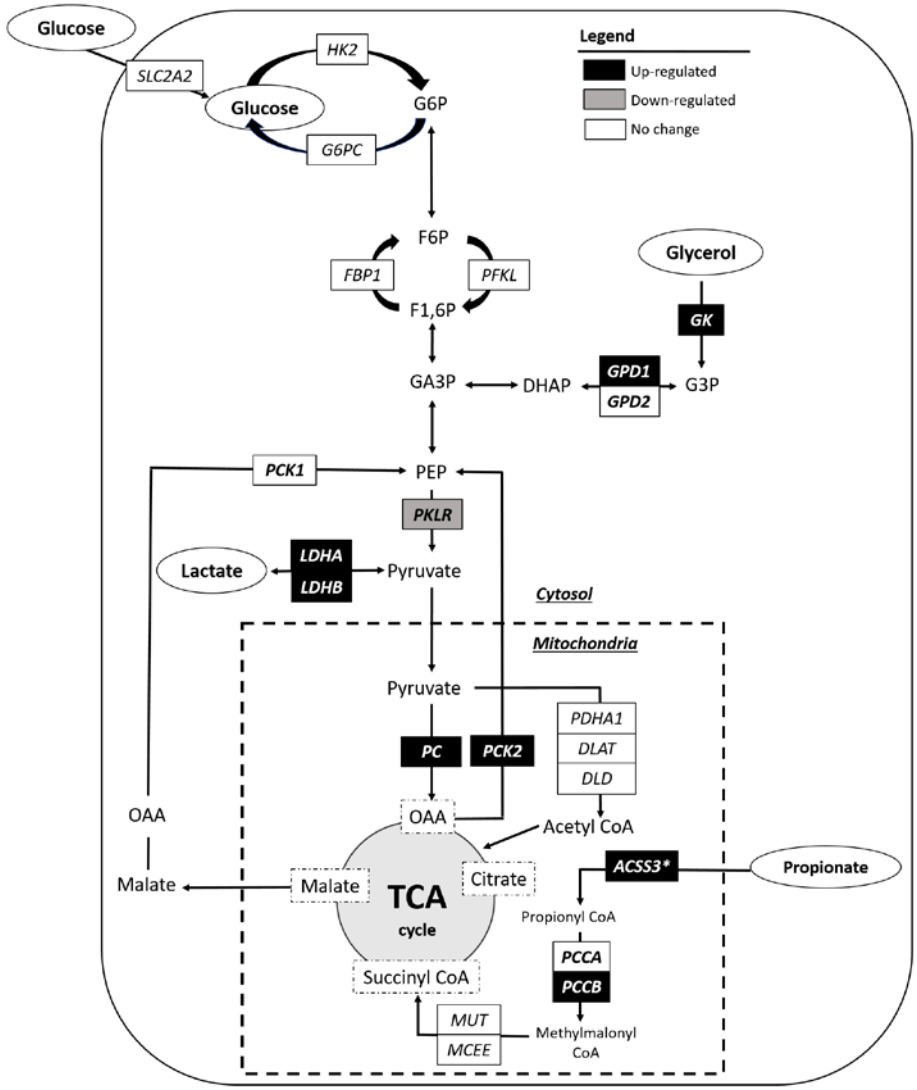

Figure 5. Changes in the hepatic expression levels of genes for gluconeogenesis pathway following castration of bulls. Castration upregulated the mRNA levels of several gluconeogenesis genes, including *PCCB*, *ACSS3** (*P* = 0.06), *PC*, *PCK2*, *LDHA* and *LDHB*, *GK*, and *GPD1*, demonstrating that castration increases the transcriptional activities of hepatic gluconeogenesis

from several glucogenic substrates (propionate, lactate, and glycerol). *ACSS3 = acyl-CoA synthetase short-chain family member 3, DLAT =dihyrolipoamideS-acetyltransferase, DLD = dihydrolipoamide dehydrogenase, FBP1 = fructose 1,6-bisphosphate, G6PC = glucose 6-phosphatase, GK = glycerol kinase, GPD1 = glycerol-3-phosphate dehydrogenase-1, GPD2 = glycerol-3-phosphate dehydrogenase-2, HK2 = hexokinase 2, LDHA = lactate dehydrogenase A, LDHB = lactate dehydrogenase B, MCEE = methymalonyl CoA epimerase, MUT = methylmalonyl CoA mutase, PC = pyruvate carboxylase, PCCB = propionyl CoA carboxylase beta, PCCA = propionyl CoA carboxylase alpha, PCK1 = cytosolic phosphoenolpyruvate carboxykinase, PCK2 = mitochondrial phosphoenolpyruvate carboxykinase, PDHA1 = pyruvate dehydrogenase (lipoamide) alpha 1, cytosolic, PFKL = phosphofructokinase-1, PKLR = pyruvate kinase, SLC2A2 = solute carrier family 2 member 2*.G6P = glucose 6-phosphate, F6P = fructose 6-phosphate, F1,6P = fructose 1,6-bisphosphate, GA3P = glyceraldehyde-3-phosphate, DHAP = dihydroxyacetone phosphate, G3P = glycerol-3-phosphate, PEP = phosphoenolpyruvate, OAA = oxaloacetate, TCA = tricarboxylic acid cycle.

5. Conclusion

Our results reveal that castration of bulls upregulates the transcriptional levels of several genes involved in gluconeogenesis, including *PCCB*, *PC*, *PCK2*, *LDHA* and *LDHB*, *GK*, and *GPD1* (**Figure 5**). These results demonstrate that castration may increase gluconeogenesis pathways from several substrates, including lactate, propionate, and glycerol. The castration of male animals significantly reduces circulating androgen concentrations, and androgen deficiency often induces insulin resistance. Insulin resistance is known to increase gluconeogenesis, especially from lactate and glycerol. In contrast, gluconeogenesis from propionate is known to be less responsive to insulin. Overall, castration may increase gluconeogenesis through both insulin-dependent (from lactate and glycerol) and insulin-independent (from pyruvate) pathways in beef cattle. We found positive correlations of gluconeogenic gene expression in the liver with backfat thickness, MS, and QG. These results imply that increased hepatic gluconeogenic gene expression following castration is associated with increased backfat thickness and intramuscular fat deposition, and thus QG.

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CHAPTER FOUR

Effect of glycerol supplementation and ambient temperature on growth performance, carcass and meat quality traits, and lipid metabolism gene expression in Korean cattle steers

*This study will be published in elsewhere as a fulfillment of Dilla Mareistia Fassah's Ph.D program

1. Abstract

This study investigated the effects of cold temperatures and the glycerol supplementation (GS) on the growth performance, rumen fermentation, blood metabolites, carcass characteristics, beef sensory traits, and gene expressions related to glucose and lipid metabolism during the fattening stage of steers. Fourteen Korean cattle steers (733 ± 15.6 kg BW, 28.4 ± 0.23 months of age) were divided into a conventional control concentrate group (n = 7) and a glycerol-supplemented (3.15% of concentrate DM) group (n = 7). A concentrate (1.2% of BW) and 1.0 kg of ryegrass were individually fed twice daily. Body weight was measured, and blood and rumen fluid were collected at

the start of the experiment and a 4-week interval thereafter during 16 weeks of feeding trial. At the end of feeding trial, steers were slaughtered, and LT and liver samples were collected for beef quality and gene expression analysis. The ambient temperature in December was lower (P<0.001) than those in other months. Neither month nor GS affected (P>0.05) ADG and feed efficiency. Cold weather affected (P < 0.001) the amount of concentrate intake and total feed intake, whereas GS decreased (P < 0.05) the concentrate intake. Cold weather increased (P < 0.05) ruminal pH and NH_3-N concentration. Cold weather decreased (P < 0.05) total VFA molar concentration, and the molar proportions of propionate and valerate, while it increased (P < 0.05) molar proportion of butyrate. Glycerol supplementation increased (P<0.05) the valerate molar concentration. Cold weather affected (P < 0.05) serum concentrations of glucose, cholesterol, and triglyceride. Glycerol supplementation increased (P < 0.05) MS and color lightness (CIE L*) of LT, while GS decreased (P < 0.05) ultimate pH of beef. Glycerol supplementation increased flavor and overall acceptance (P < 0.05) of LT. Glycerol supplementation did not change mRNA levels of hepatic gluconeogenesis genes. However, it tended to upregulate ($0.05 < P \leq 0.10$) *CD36*, *LPL*, and *FABP4* genes in muscle. These results indicate that cold condition did not affect growth performance of Korean cattle, although some changes in blood metabolites and ruminal parameters were observed. The diet with 3.15% GS of concentrate DM did not affect growth performance, ruminal VFA

concentrations, and blood metabolites. However, GS increased MS and sensory traits. Glycerol supplementation could be used to improve beef quality during cold conditions without affecting performance.

2. Introduction

Cold exposure induces metabolic acclimatization, resulting in decreased animal performance and production efficiency (Ames et al., 1980; Young, 1981). In animal acclimated to cold temperatures, heat production at maintenance may be increased 30-40% (Webster et al., 1970; Delfino and Mathison, 1991). Severe cold stress can negatively affect the backfat thickness and meat quality (Mader et al., 2003). Winter cold weather in South Korea may cause cold stress, resulting in decreased YG (Piao and Baik, 2015) and increased prevalence in dark cutting beef (Kim et al., 2003). Therefore, providing additional energy to cattle may help to overcome the decline of beef quality in cold condition. Supplementing the diet of beef cattle with glycerol, which could increase energy density should be applied to alleviate cold stress on finishing stage of Korean cattle steers.

The energy value of glycerol is similar to that of corn grain (Mach et al., 2009). Feeding crude glycerol to the ruminant animals increased propionate and butyrate molar concentrations, and reduce acetate to propionate ratio (Wang et al., 2009), then those can be used as precursors for gluconeogenesis in the liver

(Rèmond et al., 1993; Krehbiel, 2008). Substitution of corn with glycerol resulted in similar plasma glucose concentrations in dairy cattle, suggesting that glycerol has the potential to act as an energy substitution for ruminant animals (DeFrain et al., 2004). Moreover, the availability of gluconeogenic compounds increase would be used as precursors for fatty acid synthesis leading to an increase in marbling (Ladeira et al., 2016a).

The use of crude glycerol in feedlot diets has been evaluated in several studies in beef cattle (Mach et al., 2009; Parsons et al., 2009; Françozo et al., 2013; Eiras et al., 2014a). Some studies have been demonstrated that the inclusion of this by-product did not affect carcass characteristics (Almeida et al., 2018), but it increased IMF (Carvalho et al., 2014), and improved fatty acid composition (Lage et al., 2014). However, most of the previous studies of the effects of dietary glycerol inclusion on growth performance and beef quality have been performed in the normal ambient temperature or no indication of weather conditions studied.

Substituting corn with glycerol increased the hepatic gene expression of *PCK1* during the transition period of dairy cattle (White et al., 2016). Other study reported that glycerol inclusion in the diet of young bull down-regulated *PCK1* and up-regulated *GK* gene expressions (Ladeira et al., 2016a). In addition, the relative mRNA expression of lipogenic genes (*ACC*, *FABP4*, *FASN*, and *glycerol-3-phosphate acyl transferase (GPAM)*) and transcription factors, *PPARG* were not changed by GS via drinking water in the muscle of

finishing cattle (Volpi-Lagreca and Duckett, 2016). Currently, no comprehensive study was performed to evaluate expressions of genes contributed to gluconeogenesis and lipid metabolism in finishing cattle fed with GS diet.

Previously, we have evaluated the effects of GS fed to cattle during the hot season. The 2% glycerol inclusion on a diet did not affect growth performance in Korean steers reared under hot condition, although some blood metabolites changed (Kang et al., 2017). On the other study, 3% GS improved growth performance, decreased ruminal acetate:propionate ratio, and increased serum cholesterol of Korean steers (Kang et al., submitted). Furthermore, the inclusion of 3.17% glycerol as replacement of distiller's dried grain with solubles (DDGS) did not affect performance and carcass traits but resulted in minor changes in blood glucose concentrations and ruminal acetate:propionate ratio (Piao et al., submitted). To the best of our knowledge, limited study was conducted to evaluate glycerol inclusion on a diet to performance, rumen fermentation, blood metabolites, meat quality, and expression of genes related to gluconeogenesis and lipid metabolism in finishing cattle reared under cold environment. We investigated the effects of cold temperatures and 3.15% GS of concentrate DM on the growth performance, rumen fermentation, blood metabolites, carcass characteristics, meat quality, and expression of gluconeogenesis and lipid metabolism genes of Korean cattle steers.

3. Materials and methods

The experiment was approved by The Seoul National University Institutional Animal Care and Use Committee (SNUIACUC), Republic of Korea, and conducted in accordance with the Animal experimental guidelines provided by SNUIACUC.

Animals and dietary treatments

Fourteen Korean cattle steers were reared at a farm located on Uijeongbu, Gyeonggi province, South Korea. To assign the cattle uniformly into two groups, the estimated MS and backfat thickness were obtained by ultrasound scanning. Based on BW, age, and estimated MS and backfat thickness, the steers were assigned to one of two feeding treatments: a control group and a glycerol-supplemented group. During a 4-week adaptation period prior to the experiment, a concentrate (approximately 1.2 % of BW) and 1.0 kg ryegrass were individually fed twice daily. The glycerol supplement was prepared by adding 99.7% purified glycerol (Palm Oleo SDN. BHD., Selangor, Malaysia) to the ground wheat bran (63% of glycerol adsorbed to 37% of wheat bran on DM basis). Steers in the glycerol-supplemented group received glycerol supplement at 3.15% of concentrate DM daily, whereas those in the control group received wheat bran at 1.85% of concentrate DM, which is the same amount as the glycerol-supplemented group.

Table 3. Ingredients of concentrate and chemical composition of experimental diets for Korean cattle steers

	Diet or supplement		
Item	Concentrate	Ryegrass	Wheat bran
Ingredient	Dry matter basis %		
Tapioca	5.00	-	-
Cottonseed	0.50	-	-
Rice grain, ground	5.00	-	-
Wheat bran	8.00	-	-
Wheat flour	5.00	-	-
Corn gluten feed	12.5	-	-
Corn gluten meal	2.00	-	-
Rapeseed meal	1.59	-	-
Distiller's dried grains with solubles	2.00	-	-
Palm kernel meal	5.50	-	-
Coconut meal	8.00	-	-
Molasses	5.00	-	-
Limestone	1.10	-	-
Di calcium phosphate	0.35	-	-
Yeast culture	0.12	-	-
Sodium bicarbonate	1.00	-	-
Salt	0.60	-	-
Vitamin mineral premix[1]	0.22	-	-
Sweetener	0.04	-	-
Corn, flaked	25.0	-	-
Corn, ground	2.49	-	-
Wheat	6.00	-	-

Lupin seed	3.00	-	-
Total	100	-	-
Chemical composition			
DM, %	91.4	95.4	88.3
DM basis, %			
CP	13.7	3.56	14.3
EE	3.08	0.88	3.59
Ash	6.97	5.59	4.30
NDF	21.1	72.5	35.1
ADF	8.85	47.5	10.8
Ca	0.80	0.26	0.08
P	0.50	0.06	0.80
TDN[2], %	88.2	50.4	71.9[5]
DE[3], Mcal/kg	3.89	2.22	3.17[5]
ME[4], Mcal/kg	3.48	1.78	2.60[5]

[1] Mineral and vitamin premix contained Vit. A, 2,650,000 IU; Vit. D3, 530,000 IU; Vit. E, 1,050 IU; Niacin, 10,000 mg; Mn, 4,400 mg; Zn, 4,400 mg; Fe, 13,200 mg; Cu, 2,200 mg; I, 440 mg; Co, 440 mg (Grobic-DC, Bayer Health Care, Leverkusen, Germany).

[2] TDN (%) = NFC + CP + ((EE - 1) x 2.25) + NDF -7 (NRC, 2016)

[3] DE (Mcal/kg) = 0.04409 x TDN (%) (NRC, 2016)

[4] ME (Mcal/kg) = (1.01 x (DE) – 0.45) + 0.0046 x (EE-3) (NRC, 2016)

[5]NRC (2016)

The steers were weighed, and the experiment was started with an initial BW of 733 ± 15.6 kg, and at 28.4 ± 0.23 months of age. During the experimental

period steers were fed the concentrate (1.2% BW) and a ryegrass (1.0 kg/d) at 0900 h and 1800 h in a stanchion for 16 week [September 8 to October 6(P1), October 7 to November 4 (P2), November 5 to December 2 (P3), and December 3 to December 30 (P4)] following the Purina finishing steer feeding program (Cargill Purina, 2017). The supplements were individually provided by top-dressing during roughage feeding. The ingredients and chemical compositions of the diets are presented in **Table 3**. The pellet form of basal concentrate was made in Yangjoo Livestock Cooperatives (Yanjhoo-si, Republic of Korea). The supplement amount of the control and the glycerol group was presented in **Table 4**. Concentrate amounts were adjusted based on the BWs of individual animals at 4-week intervals. Residual diets were weighed before the morning feeding. The steers had free access to water. Body weight was measured at 0900 h before the morning feeding on the start day and at 4-week intervals thereafter. Samples of concentrate and ryegrass were collected monthly and stored at -20 °C until analysis.

The chemical composition (DM, CP, ether extract, ash, Ca, and P of the concentrate diet and ryegrass was determined using AOAC (1996), and neutral detergent fiber and acid detergent fiber contents were analyzed using the sequential method with ANKOM200 Fiber Analyzer (Ankom Technology Corp., Macedon, NY, USA) and reagents. Details of the method and reagents used are described in Van Soest et al. (1991). Total digestible nutrient and metabolizable energy were calculated based on NRC (2016).

Table 4. Supplementation amount of control and glycerol groups

Item	Control	Glycerol
Supplements, % of concentrate DM		
Wheat ban	1.85	1.85
Glycerol	—	3.15
Energy value of supplements		
ME[1], Mcal/kg of DM	3.82	4.13

[1] ME (Mcal/kg) = (1.01 x (DE) – 0.45) + 0.0046 x (EE-3) (NRC, 2016)

Measurements of climate temperatures

Temperatures and relative humidities of indoor and outdoor were recorded at 1 h intervals using two HOBO data loggers (Onset Computer Corp., Cape Cod, MA, USA). Minimum, maximum, and mean temperatures and relative humidity were recorded daily. The steers were raised indoors in an experimental farm covered with a roof; therefore, steers were protected from rainfall and direct sunlight. Doors on both sides of the barn were kept open, allowing the steers to be exposed to cold temperatures.

Blood and rumen fluid collections and measurements

Blood was collected at 3 h post feeding at 4-week intervals by jugular venipuncture and transferred into both a non-heparinized vacutainer (20 mL; Becton-Dickinson, Franklin Lakes, NJ, USA) for serum and ethylenediaminetetraacetic acid-treated vacutainer (20 mL) for plasma. Both

serum and plasma were separated by centrifugation at 1,500 x g at 4 ˚C for 15 min, and stored at -80 ˚C until analysis. The blood serum was used for glucose, triglyceride, and cholesterol analysis. Reagents to analyze glucose, triglyceride, and cholesterol were purchased from JW Medical (Seoul, Republic of Korea). All of the parameters were analyzed using an automated chemistry analyzer (Hitachi 7180; Hitachi, Tokyo, Japan). All of the analysis methods were verified in our laboratory, as previously reported by Kang et al. (2016). Plasma glycerol was analyzed using a glycerol assay kit (Sigma-Aldrich, St. Louis, USA, Cat. No: MAK117).

Rumen fluid was collected at 3.5 h of post-feeding using oral-stomach tube method as described by Shen et al. (2012) after blood collection. The rumen fluid pH was measured immediately with pH meter (Ohaus Corp., 7 Campus Drive, Suite 310, Parsippany, NJ07054, USA). For VFA analysis, 1 mL of rumen fluid was mixed with 0.2 mL of 25% meta-phosphoric acid, and stored at -20 ˚C until analysis, while a portion of rumen fluid for ruminal NH_3-N was also stored at -20 ˚C until analysis. The VFA and NH_3-N concentrations were determined as described previously by Kang et al. (2019a). Briefly, the NH_3-N concentration was determined using a modified colorimetric method (Chaney and Marbach, 1962), and the VFA concentrations were determined by gas chromatograph using an Agilent Tech 7890A (Hewlett Packard, Waldbronn, Germany) with a SUPELCOWAX® 10 Capillary GC Column (30 m x 0.25 mm x 0.25 μm).

Slaughter procedure and tissue sample collection

Steers were transported to the commercial abattoir (Bucheon, Korea), and slaughtered by captivate-bolt stunning, as reported in our previous study (Bong et al., 2012). Immediately after slaughter, liver samples were collected, snap-frozen in liquid nitrogen, and stored at -80 ^{o}C. Also immediately after slaughter, a piece of longissimus thoracis (LT) samples were taken from between the 12th and the 13th rib of the hot carcass, frozen in liquid nitrogen, and stored at -80 ^{o}C until glycogen and mRNA analyses.

At 24 h post-mortem, carcasses of Korean cattle steers were evaluated by professional meat grader using the Korean carcass grading system of Korea Institute of Animal Products Quality Evaluation (KAPE, 2013). Carcass characteristics for meat quality and yield grades were examined by an official meat grader as described (Piao et al., 2017). The LT (cold carcass) was obtained from the 12th vertebrae, vacuum-packaged, and transported on ice (4 ℃) to a laboratory.

After transportation to the laboratory, the LT samples were subsequently stored at 4 ℃ for 4 d. After a total 5 d of storage post-mortem, the packages containing the LT samples were opened, and the external fat was trimmed away. The LT samples were minced using a mini chopper (CH180, Kenwood, Shanghai, China) for 30 s. Minced LT samples from various locations were pooled, and some samples were used immediately for the analysis of pH, WHC,

and chemical composition, while others were stored at -70 ºC for the analysis of reducing sugar content. Samples for shear force, meat color, and sensory trait analyses were collected but not minced, and the shear force and meat color were immediately determined, whereas samples for sensory evaluation were stored at -70 ˚C for 6 days.

Chemical and physiochemical composition of LT

The proximate composition (moisture, crude protein, and crude fat) were analyzed according to AOAC methods (AOAC, 1996). Surface-color values (International Commission on Illumination [CIE]; *L**, *a**, and *b** values represent lightness, redness, and yellowness, respectively) were measured using a colorimeter (CM-5, Minolta Co., Ltd., Osaka, Japan) as described in Piao et al. (2019). Samples were bloomed for 20 min before color measurement to ensure stable data (Brewer et al., 2001). Chroma and hue-angle were also calculated from *a** and *b** values. The pH of beef samples was measured using a pH meter (SevenGo, Mettler-Toledo Inti, Inc, Schwerzenbach, Switzerland) as described (Piao et al., 2015). The maximum shear force value (kg) was measured using a Warner-Bratzler shear attached to a texture analyzer (CT3 10K, Brookfield Engineering Laboratories., Middleboro, MA, USA), as described previously (Piao et al., 2015). Meat sample (5 g) was placed into a centrifugation tube with a filter paper (Whatman No. 4, Whatman PLC., Kent, UK), and centrifuged at 2,265 × g for 10 min. After centrifugation, WHC was

calculated as the remaining moisture in the meat sample based on the moisture content of the original meat sample.

Glycogen contents

Glycogen of LT was analyzed as indicated by Dreiling et al. (1987). Two g of grounded meat was suspended with 8.5% perchloric acid (10 mL) and homogenized at Lv. 6 for 30 s (T10, Ika Works, Staufen, Germany). The homogenate was centrifuged (Combi 514R, Hanil Co., Ltd., Incheon, Korea) and filtered through glass wool. The pellet was re-extracted with 8.5% perchloric acid (5 mL), and supernatants were obtained in the same manner. The 92.6 mL of iodine color reagent [1.3 mL of a solution containing 0.26 g of iodine and 2.6 g of potassium iodide (in 10 mL distilled water) with 100 mL calcium chloride] was added to glycogen extracts (0.4 mL) and measured the absorbance at 460 nm (X-ma 3100, Human Co., Ltd., Seoul, Korea) after 30 min. The amount of glycogen was calculated using a standard curve developed with glycogen (Sigma Aldrich, St. Louis, MO, USA). The method was verified in our previous study (Piao et al., 2019).

Reducing sugars

Reducing sugar contents of LT were analyzed following Jayasena et al. (2015). The sugars were extracted from LT sample (1 g) twice using 5 mL of hot 80% ethanol (50 ℃). The extracts were then centrifuged (Continent 512R, Hanil Co., Ltd., Incheon, Korea) at 2,265 × g for 10 min. The resulting supernatants were filtered (filter paper No. 1, Whatman International Ltd., Springfield Mill, Kent, England) separately into 15 mL tubes and evaporated using N_2 gas (99.999%). Next, distilled water (2 mL) was added to each tube and vortexed to dissolve the sugars. This mixture was transferred to a 2 mL microtube and centrifuged at 18,500 × g for 10 min (HM-150IV, Hanil Science Industrial Co. Ltd). A total of 1mL of each extract was mixed with 2 mL of 3,5-dinitrosalicylic acid solution (0.5 g of 3,5-dinitrosalicylic acid, 8.0 g of NaOH, and 150 g of Rochelle salt in 500 mL of distilled water) in a 15 mL test tube and heated in a water bath (90 ^{o}C) for 10 min. The mixture was then cooled under running water for 5 min, and the absorbance was measured at 550 nm using a spectrophotometer (X-ma 3100, Human Co., Ltd., Seoul, Korea). The reducing sugar content was calculated using a standard curve developed with glucose (Sigma Corp., St. Louis, MO, USA). The method was verified in our previous study (Piao et al., 2019).

Sensory evaluation

Human ethics approval for the sensory evaluation procedures were granted by Seoul National University Institutional Review Board (IRB No.: 1905/003-002), Republic of Korea. Sensory traits of LT samples were evaluated as reported previously (Piao et al., 2019). Briefly, samples were cut into sections (15 × 40 × 15 mm) and cooked. The internal temperature was monitored using a digital thermometer (YF-160A Type-K; YFE, Hsinchu City, Taiwan) that was placed in the center of the LT samples; the samples were removed from the grill after they reached an internal temperature of 72 °C. Twelve semi-trained panelists evaluated the cooked samples for appearance, odor, taste, flavor, tenderness, juiciness, and overall acceptability. A 9-point hedonic scale, where 9 indicates “extremely like” and 1 indicates “extremely dislike”, was employed to evaluate all of the parameters. For evaluation, samples were placed into randomly coded white dishes and served together with drinking water to rinse the panelist’s oral cavity, following a test of each sample. The sensory characteristics were evaluated in three independent experiments.

Quantitative Real-time PCR (qPCR)

Total RNA extraction and qPCR were performed as described (Park et al., 2018). Briefly, total RNA from liver and LT were transcribed into cDNA using the iScript cDNA synthesis kit (ENZO, Farmingdale, NY, USA) according to the supplier’s protocol. Gene expression was evaluated using quantitative

polymerase chain reaction (qPCR) analysis performed using AMPIGENE® qPCR Green Mix Lo-Rox (Dong In Biotech Co., Ltd., Seoul, Republic of Korea). qPCR analyses were performed using a Rotor-Gene Q instrument (Qiagen, Germany). Information on the primers used is presented in **Table 5**. All primers were designed using Pick Primer program from the National Center for Biotechnology Information. We used two different exons for forward and reverse primers to prevent amplification of the DNA template. We indicated the melting temperatures of all primers in Table 5. The ΔΔCT method was used to determine relative fold changes (Livak and Schmittgen, 2001). Liver and LT data were normalized with *RPS9* and *β-actin* as the housekeeping genes, respectively.

Table 5. Primer sequences for real-time PCR analysis

Gene name (Symbol)	Gene bank accession no.	Primer	Sequence (5'-3')	Length (bp)	Tm[1] (°C)
Gluconeogenesis					
Glycerol kinase (*GK*)	NM_001075236.1	Forward	GCTTCGTTGGCTCCTTGACA	131	60.9
		Reverse	TACAATGGACCCCTCCACTG		58.7
Acyl-CoA synthetase short-chain family member 3 (*ACSS3*)	NM_001102137	Forward	GAGGCACTGAGAATAGGACAAC	133	58.5
		Reverse	CATGTGACTGTGCTTTTGAGAG		57.9
Propionyl-CoA carboxylase alpha, mitochondrial (*PCCA*)	NM_001083509	Forward	AAATGAACACGAGACTCCAG	135	55.4
		Reverse	AGCCGTTGATGGGAATATCG		57.8
Propionyl-CoA carboxylase beta, mitochondrial (*PCCB*)	NM_001038548	Forward	CACATGCCCAAAAGATCTGC	115	57.7
		Reverse	GCCAAAGATTCCACTCCCTC		58.2
Fructose-1,6-bisphosphatase 1 (*FBP1*)	NM_001034447.2	Forward	TCACCGAGTATGTCCAGAGGA	146	60.0
		Reverse	GGGGCTTTTCTTGTTAGCTGG		59.4
Glucose 6-phosphatase (*G6PC*)	NM_001076124.2	Forward	AGCTGTGGGCATCAAACTCC	116	60.6
		Reverse	AGTAGTCGGTATCCAAAACC		54.5
Lipid uptake and glucose transporter					
Solute carrier family 2 member 2 (*SLC2A2*)	NM_001103222	Forward	TGCTGTCTCTGTGTTCCTTG	127	57.7
		Reverse	CCAAGGGAATTTACTCAGGAGC		58.7

Solute carrier family 2 member 4 (*SLC2A4*)	NM_174604.1	Forward	CCACCAGGCACACTTACCAC	113	60.9
		Reverse	CTCTTCCTTCCCAGCCACTG		60.0
Lipoprotein lipase (*LPL*)	NM_001075120.1	Forward	CTTGCCACCTCATTCCTG	118	55.3
		Reverse	ACCCAACTCTCATACATTCC		54.4
Fatty acid translocase (*CD36*)	NM_001278621.1	Forward	ACTGTCCTCTGTGTCTAAGC	124	56.6
		Reverse	GTTGTGTCTGCCTCAAGTGC		59.7
Triglyceride synthesis					
1-acylglycerol-3-phosphate O-acyltransferase 1 (*AGPAT1*)	NM_177518.1	Forward	GCCATCAGTGTCATGTCTG	85	56.0
		Reverse	GGTTTCTCGTGCCCTCAG		57.7
Diacylglycerol O-acyltransferase 1 (*DGAT1*)	NM_174693.2	Forward	TCTTCCACTCCTGCCTGAAC	96	59.3
		Reverse	AGTAGGTGATGGACTCGGAG		57.9
Glycerol-3-phosphate acyltransferase, mitochondrial (*GPAM*)	NM_001012282	Forward	GTGCTATCTGCTCTCCAATG	115	56.0
		Reverse	CTCCGCCACTATAAGAATG		52.6
Lipogenesis					
Peroxisome proliferator activated receptor gamma (*PPARG*)	NM_181024.2	Forward	TTCCGTTCCCAAGAGCTGAC	98	60.0
		Reverse	TGGGGATACAGGCTCCACTT		60.2

Sterol regulatory element binding transcription factor 1 (*SREBP1*)	NM_001113302	Forward	ACCGCTCTTCCATCAATGAC	120	58.0
		Reverse	GCTGAAGGAAGCGGATGTAG		58.4
Fatty acid binding protein 4 (*FABP4*)	NM_174314.2	Forward	GCTGCACTTCTTTCTCACCT	140	58.1
		Reverse	TTCCTGGTAGCAAAGCCCAC		60.2
Acetyl-CoA carboxylase alpha (*ACC*)	NM_174224.2	Forward	AGGAGGGAAGGGAATCAGAA	69	57.3
		Reverse	GCTTGAACCTGTCGGAAGAG		58.6
Fatty acid synthase (*FASN*)	NM_001012669	Forward	ATCGAGTGCATCAGGCAAGT	92	59.7
		Reverse	TGTGAGCACATCTCGAAAGCCA		62.5
Ribosomal protein S9 (*RPS9*)[2]	NM_001101152	Forward	CCTCGACCAAGAGCTGAAGCCTC	64	62.1
		Reverse	CAGACCTCACGTTTGTTC		53.3
β-actin[2]	NM_173979	Forward	AGCAAGCAGGAGTACGATGAGT	120	61.5
		Reverse	ATCCAACCGACTGCTGTCA		58.9

[1]Tm = melting temperature. [2]Housekeeping genes: *RPS9* for liver; *β-actin* for muscle.

Statistical analysis

All statistical analyses were carried out using SAS software 9.1 (SAS Institute, Cary, NC, USA). Climate data, carcass characteristics, meat quality, glycogen and reducing sugar contents, sensory traits, and gene expression were analyzed by General Linear Model. The performance, rumen fermentation parameters, and blood metabolites were analyzed by a repeated measure two-way ANOVA, with the main effects being the month (September, October, November, or December) and diet (control or GS). The statistical method included month, diet and their interaction. Differences were considered significant at the $P \leq 0.05$ level, and a tendency was indicated by $0.05 < P \leq 0.10$. Pearson or Spearman correlation analyses were performed respectively, to analyze the relationship between IMF content (continuous trait) and MS (discrete trait) and mRNA levels of gluconeogenesis and lipid metabolism genes.

4. Results and Discussion

Climate conditions

The mean (-.3.50 °C) and minimum (8.15 °C) indoor temperatures in December (P4) were lower ($P < 0.001$) during P4 than those in other months [September (P1), October (P2), November (P3)] (**Table 6**). Mean indoor temperatures were also recorded on days that blood and rumen fluid were collected. These temperatures were 23.5 °C on September 8, 17.4 °C on October 6, 7.39 °C on November 4, 2.84 °C on December 2, and -0.39 °C on December 30. The minimum relative humidity (39.1) in P3 was lower than those in other periods.

Cold stress has been categorized as "mild" (0 °C to -6.7 °C), "moderate" (-7.2 °C to -13.9 °C), and "severe" (≤ -13.9 °C) under dry winter cattle coat condition (Grzych, 2010). Therefore, the mean indoor temperatures during P4 is associated as mild cold stress, whereas P1, P2, and P3 are considered thermoneutral. The minimum indoor temperature on P3 (-2.46 °C) and P4 (-8.15 °C) were categorized as "mild" and "moderate" cold stress condition; however, P1 and P2 were under "threshold" condition. The mean and maximum indoor temperatures, recorded in P1 (19.8 °C and 27.1 °C, respectively) 2017 were similar than those recorded during our previous study in September (18.4 °C and 28.8 °C, respectively) 2015 (Kang et al., 2019b). In addition, the mean indoor temperatures on the last day that blood and rumen fluid were collected

were associated with mild cold stress. However, the temperature ranges associated with cold stress have not been defined clearly in Korean cattle breed.

Table 6. Mean, maximum and minimum values of indoor and outdoor temperatures, and relative humidity from September to December 2017

Item	September (P1)[1]	October (P2)[2]	November (P3)[3]	December (P4)[4]	SEM	*P*- value
Indoor temperature, ˚C						
Mean	19.8[d]	14.1[c]	2.88[b]	-3.50[a]	0.93	<0.001
Maximum	27.1[d]	21.0[c]	10.5[b]	1.85[a]	0.97	<0.001
Minimum	14.1[d]	8.96[c]	-2.46[b]	-8.15[a]	0.91	<0.001
Outdoor temperature, ˚C						
Mean	19.2[d]	13.6[c]	2.97[b]	-3.29[a]	0.89	<0.001
Maximum	26.4[d]	21.1[c]	9.90[b]	2.17[a]	0.96	<0.001
Minimum	13.5[d]	7.91[c]	-2.65[b]	-8.41[a]	0.90	<0.001
Relative humidity						
Mean	73.9	72.9	68.2	71.5	0.94	0.16
Maximum	91.4	89.7	88.0	86.9	0.71	0.13
Minimum	48.3[a]	46.4[a]	39.1[b]	48.3[a]	1.31	0.04

[a-d] means with different letter within the same row differ (P<0.05).

[1]Experiment period was 4 weeks from September 8 to October 6

[2]Experiment period was 4 weeks from October 7 to November 4

[3]Experiment period was 4 weeks from November 5 to December 2

[4]Experiment period was 4 weeks from December 3 to December 30

Growth performance

No interactions between diet and month were observed on all growth performance parameters. There were no differences ($P > 0.05$) in ADG, and feed efficiency of Korean cattle steers among months (**Table 7**). However, both amount and % BW of the daily concentrate intake and total feed intake were higher ($P < 0.001$) in P4 than in other periods. These results showed that colder temperature increased feed intake. Kang et al. (2017) reported concentrate intake increased when the ambient temperature was colder. Heat or cold stress leads to change carbohydrate metabolism, which is modulated by hormones, such as insulin and glucagon to maintain energy availability (Bhimte et al., 2018). Energy requirements increase during winter or when animals are under cold stress due to the higher needs for increasing resting heat production to maintain body temperature by shivering or other thermogenic processes (Young, 1983). In this study, the higher total feed intake on December may reflect the increase of concentrate intake due to colder ambient temperature. It can be assumed that cattle performance was not affected by cold temperature since the energy requirements for maintaining body temperature and growth were sufficient.

Glycerol supplementation did not affect ($P > 0.05$) final BW, ADG, total feed intake, and feed efficiency. Glycerol supplementation decreased ($P = 0.03$) the concentrate intake of Korean cattle steers during all experimental period. In this study, glycerol was administered with ryegrass first before feeding

concentrate at each feeding time. According to National Institute of Animal Science (2017), the TDN and ME requirements for steer (BW 700 kg and ADG 0.8 kg) are 81% and 26.87 Mcal, respectively. The average ME intake for control and glycerol groups were 27.2 Mcal/d and 27.6 Mcal/d, respectively. Therefore, the amount of our basal diet feeding may provide enough energy to meet growth since cattle in glycerol group ate less amount of basal concentrate with 3.15% GS. In agreement with our study, no negative effect occurred on total feed intake (Chung et al., 2007; San Vito et al., 2016), ADG, and feed efficiency (Mach et al., 2009; Del Bianco Benedeti et al., 2016) when diet was supplemented with crude glycerin.

Table 7. Growth performance of Korean cattle steers fed either the control or the glycerol-supplemented diet from September to December 2017

Item	Diet	Month				SEM	*P*-value		
		September (P1)[1]	October (P2)[2]	November (P3)[3]	December (P4)[4]		Month	Diet	Interaction
Initial BW, kg	Control	731	747	763	778	7.79	<0.001	0.85	0.37
	Glycerol	734	741	757	771				
Final BW, kg	Control	747	763	778	796	7.96	<0.001	0.71	0.73
	Glycerol	741	757	771	785				
ADG, kg	Control	0.51	0.56	0.53	0.64	0.06	0.90	0.54	0.95
	Glycerol	0.43	0.59	0.47	0.48				
Daily feed intake, kg DM/d									
Total feed intake	Control	8.06	8.54	8.50	10.1	0.11	<0.001	0.51	0.93
	Glycerol	8.08	8.66	8.58	10.2				
Concentrate intake	Control	6.90	7.38	7.35	8.99	0.12	<0.001	0.03	0.93
	Glycerol	6.59	7.18	7.10	8.69				
Ryegrass intake	Control	0.95	0.95	0.95	0.95	-	-	-	-
	Glycerol	0.95	0.95	0.95	0.95				

Glycerol intake	Control	0.00	0.00	0.00	0.00	-	-	-	-
	Glycerol	0.33	0.33	0.33	0.33				
Wheat bran intake	Control	0.20	0.20	0.20	0.20	-	-	-	-
	Glycerol	0.20	0.20	0.20	0.20				
Daily feed intake, % BW									
Total feed intake	Control	1.08	1.12	1.09	1.28	0.01	<0.001	0.43	0.98
	Glycerol	1.09	1.15	1.12	1.30				
Concentrate intake	Control	0.93	0.97	0.95	1.13	0.01	<0.001	0.26	0.96
	Glycerol	0.89	0.95	0.92	1.11				
Feed efficiency	Control	0.084	0.067	0.061	0.061	0.01	0.78	0.17	0.87
(G:F)	Glycerol	0.053	0.070	0.054	0.047				

n = 7 per group.

Ruminal fermentation parameters

Ruminal pH and NH_3-N were higher ($P < 0.001$) during colder months (**Table 8**). Cold temperatures decreased ($P < 0.05$) the molar concentrations of total VFA. In addition, cold temperatures decreased ($P < 0.01$) the molar proportions of propionate and valerate, whereas it increased ($P < 0.05$) the molar proportion of butyrate and the ratio of acetate to propionate. The significant ($P < 0.05$) month and treatment interaction were observed for molar concentrations of total VFA. The ruminal pH and NH_3-N concentration in late December were higher ($P < 0.001$) than the other months The increase in rumen pH observed in the present study may be associated with the increased of NH_3-N concentration and decreased of VFA production during cold exposure (Kennedy, 1985). Lower rumen pH was mainly as the consequence of VFA production decline and increase VFA absorption in the rumen (Plaizier et al., 2009). Penner et al. (2009) reported that increased VFA absorption across the rumen wall resulted in additional proton removal from the rumen and subsequently increasing rumen pH. In this study, the higher NH_3-N concentration may reflect the increased of total protein intake due to increase total feed intake by colder temperature. The concentration of NH_3-N in ruminal fluid is a function of NH_3-N production, NH_3-N uptake by the ruminal microbes, and diffusion through the rumen wall (Agle et al., 2010). According to Tamminga (1979), dietary proteins metabolized within rumen produces amino acids, which may be incorporated into microbial proteins or fermented as an

energy source. During amino acid fermentation NH_3-N is produced. The total CP intake on P1, P2, P3 and P4 were 1.01 kg/d, 1.07 kg/d, 1.07 kg/d, and 1.29 kg/d for control group, and 0.96 kg/d, 1.04 kg/d, 1,03 kg/d, and 1.25 kg/d for GS group, respectively. Another possible reason for higher NH_3-N concentrations in this study is the irreversible loss of both plasma urea and rumen NH_3-N, and the conversion of plasma urea-nitrogen into rumen NH_3-N which are greater by cold exposure (Kennedy et al., 1982). In conclusion, decreased VFA production and increase NH_3-N concentration may contribute to higher rumen pH.

Table 8. Ruminal parameters in Korean cattle steers after 3 h of feeding on the control or the glycerol-supplemented diet

		Month						*P*-value		
Item	Diet	September 8	October 6	November 4	December 2	December 30	SEM	Month	Diet	Interaction
Temperature[1], ˚C		23.5	17.4	7.39	2.84	-0.39				
pH	Control	5.70	6.48	6.47	6.60	6.67	0.10	<0.001	0.28	0.19
	Glycerol	5.96	6.33	6.19	6.56	6.48				
NH_3-N, mg/100 mL	Control	4.36	7.36	7.34	10.1	9.57	0.62	<0.001	0.89	0.82
	Glycerol	4.23	6.63	8.01	9.17	9.17				
Total VFA, mmol/L	Control	86.0	80.6	78.4	73.9	64.8	2.45	0.04	0.47	0.03
	Glycerol	87.2	76.9	90.0	59.9	84.0				
VFA proportions, mol/100mol										
Acetate	Control	54.4	56.0	51.6	51.3	60.3	0.93	0.29	0.26	0.22
	Glycerol	51.0	51.0	51.5	53.1	51.4				
Propionate	Control	30.2	24.5	27.6	25.4	19.4	0.89	<0.01	0.60	0.48
	Glycerol	28.4	26.8	28.3	25.0	25.3				
Iso-butyrate	Control	0.43	0.60	0.51	0.69	0.73	0.03	0.76	0.46	0.16

	Glycerol	0.62	0.48	0.54	0.56	0.49				
Butyrate	Control	12.4	15.8	17.6	15.6	17.0	0.47	0.03	0.21	0.45
	Glycerol	15.9	16.4	16.3	17.5	19.6				
Iso-valerate	Control	1.12	1.59	1.34	1.86	1.59	0.08	0.10	0.18	0.30
	Glycerol	1.73	1.40	1.60	2.28	1.31				
Valerate	Control	1.51	1.46	1.33	1.12	0.90	0.07	<0.01	0.02	0.67
	Glycerol	2.30	2.04	1.85	1.55	1.72				
Acetate/propionate	Control	1.85	2.45	2.10	2.41	3.32	0.11	0.003	0.34	0.06
	Glycerol	1.98	2.18	2.13	2.25	2.22				

n = 7 per group.

[1]Average ambient temperature of sampling times of each day.

The reduction in total VFA concentrations seen in this study with cold exposure may have resulted from the shorter total retention time of the diets due to an increased rate of passage through the reticulorumen (Westra and Christopherson, 1976) which reduces the fermentation time in the rumen. In addition, Christopherson and Kennedy (1983) reported that cold exposure might alter chemoreceptor reflexes by influencing rumen blood flow and hence absorption. Increased blood flow during cold exposure might enhance VFA uptake into the portal circulation and subsequently reduce VFA concentrations in the rumen. In line with our study, Kennedy (1985) reported a decrease in ruminal VFA production rate during cold exposure in sheep.

Some changes in molar proportions of propionate, butyrate, and valerate in this study may indicate a shift in the microbial population. Romero-Perez et al. (2011) reported that change in rumen bacteria population might be caused by the poor access of bacteria to nutrients as a result of higher motility and rate of digesta passage throughout the gut on cold exposure. The increase in molar proportion of butyrate in this study may be caused by the increase of lactate-producing bacteria population, such as *Megasphera elsdenii* and *Butyvibrio fibrisolvens*. Lactate-producing bacteria proliferate rapidly in response to concentrate feeding, thus convert lactic acid into acetate, propionate, and butyrate (Thieszen et al., 2015). Genus *Butyrivibrio* and *Megasphera elsdenii* are known as producers of butyrate (Mohammed et al., 2014). According to Counotte et al. (1981), as a rumen pH declined, lactic acid fermentation by

Megasphera elsdenii is shifted from propionate to butyrate. In this study, high rumen pH was occurred during colder condition. It may be explained by butyrate accumulation alters the metabolic flux of the rumen, favoring the neutralization of H+ through the efflux of bicarbonate (Dionissopoulos et al., 2013). Similar to our study, high ruminal butyrate concentration increased rumen pH in lactating dairy cows (Herrick et al., 2017).

In this study, changes in the molar proportions of propionate and butyrate by cold exposure may also contribute to the alterations of the ratios of acetate to propionate. In contrast with our present study, Kang et al. (2019a) reported that cold condition from January (mean ambient temperature = -7.37 ˚C) to April (mean ambient temperature = 6.22 ˚C) in 2016 increased concentrations of ruminal acetate, propionate, and total VFA. In this study, VFA concentrations were measured starting from early September 2017, which had a mean ambient temperature of 23.5 ˚C, until late December, which had a mean ambient temperature of -0.39 ˚C. Thus, the ambient temperature in this study was higher than in 2016 study (Kang et al., 2019a). Therefore, a different pattern of VFA concentrations under cold condition among studies may be due to differences in cold intensity.

Glycerol supplementation did not change the ruminal pH and NH_3-N ($P > 0.05$). The GS increased ($P < 0.05$) the molar proportions of valerate. No differences were observed in other VFA molar proportions with GS during the entire experimental period. The results of the present study were in line with

Paschoaloto et al. (2016) who reported that ruminal pH and NH_3-N were not affected by glycerin addition in the diet of beef cattle. The ruminal pH observed in the current study was ranged from 5.70 to 6.68 which was the normal levels in cattle (Nocek, 1997). In the present study, NH_3-N concentration was ranged from 4.23 to 10.1 mg/100mL which were adequate to support the microbial growth, as demonstrated in Holstein cows by Kang-Meznarich and Broderick (1980). Most of the previous studies reported that propionate molar proportion was increased with the inclusion of crude glycerin in the diet (Boyd et al., 2013; Paiva et al., 2016). Moreover, DeFrain et al. (2004) reported that crude glycerol supplementation had a greater rumen VFA with greater propionate molar proportions and lower acetate to propionate molar ratio. Shin et al. (2012) reported that feeding crude glycerin may increase the numbers of ruminal microbes that shift VFA production toward propionate and butyrate, and that may decrease the acetate and the acetate/propionate ratio. However, in the present study glycerol supplementation did not alter most of the molar proportions of VFA.

The increase in valerate molar proportion by glycerol supplementation may have resulted from the increased production of lactate by fermentation of glycerol (Trabue et al., 2007) providing a substrate for *Megasphaera elsdenii* (Klieve et al., 2003) which mainly involve on valerate production. Trabue et al. (2007) reported that fermentation of a portion of glycerol produces some valerates. In line with our results, San Vito et al. (2016) reported that the

concentrations of propionate and valerate increased when crude glycerol was supplemented on the feed of Nellore steers. On this study, the valerate concentrations were increased in late December. Propionate is more hypophagic than acetate because it has a better ability to stimulate oxidation in the liver (Allen, 2000) for providing energy for the cattle. Valerate can provide carbon sources for glucose biosynthesis and are considered to be glucogenic, although the contributions to gluconeogenesis are smaller than propionate (Aschenbach et al., 2010). Overall, dietary GS increased ruminal valerate levels with no changes in other VFA proportions.

We found significant interaction between month and diet in total VFA concentration: lower total VFA concentrations were observed during the colder months in control group, but this was not true in glycerol group at some months. This means that total VFA concentrations were not decreased at some of cold months in glycerol group. Cold exposure decreased ruminal VFA production rate in sheep (Kennedy, 1985). Feeding crude glycerol to transition dairy cows increased the total VFAs (DeFrain et al., 2004). Our findings implicate that glycerol supplement may restore the decreased VFA production at some of cold months to the normal levels.

Blood metabolites

The concentrations of serum glucose, triglyceride, and cholesterol were affected ($P < 0.05$) by month (**Table 9**), but these variations do not seem to be

associated with cold conditions since glucose and cholesterol concentrations were lowest at October and triglyceride was highest at November, respectively during entire experimental period (September through December). Plasma glycerol concentrations were not affected ($P > 0.05$) by month. Blood metabolites reflect the health status of cattle. In contrast with our previous studies (Kang et al., 2019a; Kang et al. submitted), serum glucose concentrations were lowest in October. Higher glucose concentrations during colder months may be associated with increased metabolic rates (Young, 1975). Therefore, it can be assumed that the rate of gluconeogenesis was remained stable to maintain the blood glucose circulation during colder condition. The present results indicated that cold exposure in November increased the concentration of serum triglyceride concentration. Kang et al. (2016) reported that lipolysis might occur at colder temperatures to generate heat and maintain body temperature. It is likely that the fat mobilization and lipolysis from body fat depots could contribute to these results. In mature animals, fat mobilization from stored lipids is an important mechanism to make up the energy deficit. The fatty acid is a preferred substrate as an energy source for skeletal muscle, heart muscles, white and brown adipose tissues, and liver (Berg et al., 2002). Cold exposure increases the plasma free fatty acids, indicating an increase in lipolysis (Bell and Thompson, 1979). Cortright et al. (1997) reported that free fatty acids may be derived from plasma non-esterified fatty acid, circulating triglyceride, and endogenously stored triglycerides. Thus, the higher serum

triglyceride and cholesterol in several months may indicate mobilization of lipid stores to provide alternative fuels during the cold condition.

Glycerol supplementation did not affect ($P > 0.05$) all the blood metabolites. The absence of differences in plasma glycerol concentrations between two diet groups suggests that all dietary glycerol may be completely metabolized in the body. In this study, the plasma glycerol concentration was not changed by GS, which also may contribute to no change in serum glucose concentration. In this study, the actual glycerol supply was 528 g/d by top-dressing in glycerol group, which is less than 860 g/d that was provided in the study of DeFrain et al. (2004), which showed no difference in plasma glucose concentration. In our previous study, 2% glycerol supplementation did not affect blood triglyceride, glucose, and cholesterol concentrations of Korean cattle (Kang et al., 2017). Our results show that glycerol supplementation may maintain the blood metabolites homeostasis in finishing cattle.

Table 9. Blood metabolites concentrations at 3 h post-feeding of Korean cattle steers fed either the control or the glycerol-supplemented diet

		Month						*P*-value		
Item	Diet	September 8	October 6	November 4	December 2	December 30	SEM	Month	Diet	Interaction
Temperature[1], ˚C		23.5	17.4	7.39	2.84	-0.39				
Glucose, mg/dL	Control	81.0	75.7	79.4	84.1	79.1	0.75	0.04	0.38	0.21
	Glycerol	77.8	76.3	78.5	78.7	77.2				
Triglyceride, mg/dL	Control	20.7	24.1	29.9	21.3	22.1	0.74	<0.001	0.20	1.00
	Glycerol	18.1	21.8	27.1	18.4	19.1				
Cholesterol, mg/dL	Control	146	121	140	156	154	3.28	0.007	0.99	0.64
	Glycerol	150	131	134	149	150				
Glycerol, mg/dL	Control	1.31	0.79	1.48	1.00	1.51	0.09	0.06	0.27	0.39
	Glycerol	0.90	1.39	2.05	1.23	1.48				

n = 7 per each group.

[1]Average ambient temperature of sampling times of each day.

Carcass Traits and Chemical Composition of the LT

The carcass traits and beef quality may be decreased during cold or heat stress because more energy is spend to maintain body temperature which is more priority than meat production. Glycerol supplementation increased ($P < 0.05$) MS (**Table 10**). In line with our result, glycerin inclusion in the feedlot diet increased beef marbling in young bulls (Ladeira et al., 2016a). Other carcass traits, including the carcass weight, LM area, backfat thickness, yield index, YG, QG, meat color, texture, and maturity were not affected ($P > 0.05$) by GS. Glycerol inclusion on the finishing cattle diet did not affect carcass weight and backfat thickness (Leão et al., 2013; de Barros et al., 2018). Crude glycerin inclusion in the diets of crossbred bulls did not affect backfat thickness and LM area (Eiras et al., 2014b). Similar with our study, most of carcass parameters were not affected by GS of finishing steers (Chanjula et al., 2016). In conclusion, 3.15% GS of concentrate DM on finishing feed did not affect most of the carcass traits of Korean cattle steers reared under cold condition.

The chemical composition of the LT, including crude fat, crude protein, and moisture, were not affected ($P > 0.05$) by GS (**Table 10**). Our results were in agreement with previous studies (Volpi-Lagreca and Duckett, 2016; Van Cleff et al., 2017), which did not find any differences in moisture, protein and fat contents of LM by inclusion of crude glycerin in cattle diets. The lack significant differences on the chemical composition of beef with GS may confirm that the nutritional quality of beef was maintained without any loss in

the composition of final product. In this study, we found the increased MS by GS. It seems that GS was insufficient to significantly affect fat content in the LT.

Table 10. Carcass characteristics of Korean cattle steers fed the control or glycerol-supplemented diet

Item	Control	Glycerol	SEM	*P*-value
Slaughter age, month	33.3	33.6	0.22	0.55
Carcass weight, kg	464	470	12.3	0.68
LMA[1] area,cm[2]	91.1	94.6	1.30	0.20
BF[1] thickness, mm	17.3	15.9	1.85	0.72
Marbling score[2]	6.14	7.28	0.31	0.01
Yield index[3]	61.3	62.5	1.40	0.69
Yield grade[4]	15.7	17.1	1.69	0.69
Quality grade[5]	37.1	41.4	1.65	0.21
Meat color[6]	5.14	4.71	0.16	0.21
Fat color[7]	3.00	3.00	0.00	-
Texture[8]	14.6	13.7	1.04	0.70
Maturity[9]	2.14	2.14	0.10	1.00

n = 7 per group.

[1]LMA = loin muscle area; BF = back fat

[2]Marbling score = 1 = trace marbling, 9 = highly abundant marbling.

[3]Yield index = 68.184-[0.625 x back fat thickness (mm)] + [0.130x rib eye area (cm2)] - [0.024 x carcass weight (kg)] + 3.23.

[4]Yield grade: C (yield index < 63.3) = 10, B (63.3 ≤ yield index < 67.2) = 20, A ((yield index ≥ 67.2) = 30.

[5]Quality grade: 3 (lowest) = 10; 2 = 20, 1 = 30, 1+ = 40, 1++ (highest) = 50.

[6]Meat color: 1 = bright red, 7 = dark red.

[7]Fat color: 1= white, 7= yellowish.

[8]Texture: 1= very fine, 3=very course.

[9]Maturity: 1 =youthful, 9= mature.

The glycogen contents in LT and liver and the LT reducing sugar contents were not affected ($P > 0.05$) by GS (**Table 10**). Pre-slaughter administration (2 g/kg BW) of crude glycerin via nasogastric tube or in drinking water in young bulls did not affect liver glycogen contents (Egea et al., 2015). However, high-energy supplementation increased glycogen levels of biopsied muscle in pasture-fed beef steers (Knee et al., 2007). It has been suggested that increased ingestion of carbohydrate leads to increased glycogen storage in liver and muscle (Qaid and Abdelrahman, 2016). The concentrate-based high- energy diet has had protective effects against glycogen depletion in glycogen-depleting conditions such as high temperatures and transportation (Immonen et al., 2000a). Gardner et al. (2001) suggest that glycerol and propylene glycol supplements may not be applicable for enhancing muscle glycogen repletion in normal diet condition, although these supplementations may enhance the

repletion of muscle glycogen following a marked level of depletion. In our study, cattle received about 89% of finishing concentrate with high-energy level and 11% of roughage in order to produce high-marbled beef following Korean cattle feeding system (Park et al., 2018). Thus, this kind of high energy diet feeding may saturate glycogen storage levels in the muscle, resulting no effect on glycogen levels by GS in this study.

Physicochemical and sensory traits of the LT

Glycerol supplementation decreased (P = 0.05) the ultimate pH of LT (**Table 11**). Higher glycogen contents at early post mortem was significantly associated with lower ultimate pH of beef (Lahucky et al., 1998). However, our study showed no difference in muscle glycogen contents between two groups. The glycolytic potential is commonly used for measuring the ability of a muscle to generate lactic acid, which is calculated based on the contents of glycogen, glucose-6-phosphate, glucose and lactic acid (Monin and Sellier, 1985). The post-mortem glycolytic potential was negatively related with ultimate pH in pork (Hamilton et al., 2003). It is possible that GS changes the glycolytic potential of beef in this study. However, the reason for decreased meat pH by GS remains unclear. In unstressed animals with large reserves of muscle glycogen, pH decreases typically from an initial 7.0-7.2 to final values 5.4-5.8 postmortem (Young et al., 2004). A sufficient pH decrease is related to the WHC, color, and tenderness of muscle. The dark firm dry meat will have a

much higher pH than 6.0 (Mounier et al., 2006; Adzitey and Nurul, 2011). Therefore, the ultimate pH of LT on the present study showed a sufficient pH decrease for beef.

Table 11. Chemical, physico-chemical composition and sensory traits of *longissimus thoracis* from Korean cattle steers fed the control or glycerol-supplemented diet

Item	Control	Glycerol	SEM	*P*-value
Chemical composition				
Moisture, %	61.9	59.4	0.82	0.14
Crude protein, %	19.6	18.6	0.36	0.15
Crude fat, %	18.5	21.1	1.01	0.20
Reducing sugar content, mM	10.9	10.8	0.37	0.87
Glycogen				
Liver glycogen content, mg/g	15.7	16.4	1.04	0.76
Muscle glycogen content, mg/g	2.43	3.03	0.32	0.36
Physico-chemical composition				
pH	5.53	5.45	0.02	0.03
Water holding capacity	71.3	74.0	0.76	0.08
CIE *L**, lightness	34.2	37.7	0.63	<0.001

CIE *a*, redness*	12.4	12.7	0.43	0.72
CIE *b**, yellowness	10.0	10.4	0.26	0.49
Shear force, kg	15.9	16.4	0.45	0.60
Sensory traits[1]				
Appearance	6.00	6.09	0.18	0.81
Aroma	6.08	6.13	0.13	0.84
Taste	6.20	6.30	0.16	0.75
Flavor	6.04	6.71	0.14	0.01
Tenderness	5.18	6.14	0.24	0.10
Juiciness	5.98	6.49	0.17	0.15
Overall acceptance	5.94	6.82	0.17	<0.01

n = 7 per group.

[1]The score was evaluated with 12 semi-trained panelists (1, extremely dislike; 5, neither dislike nor like; 9, extremely like).

Glycerol supplementation tended to increase ($P = 0.08$) WHC. The ultimate pH of beef was decreased by GS. According to Cheng and Sun (2008), increasing or decreasing the pH on either side of isoelectric point (pH 5.2 to 5.3) will result in an increased WHC by improving ionic strength steadily. According to Van Laack et al. (1994), ultimate pH accounted only 24% of the variation in WHC. Water holding capacity increased with the increasing fat contents (den Hertog-Meischke et al., 1997; Carvalho et al., 2014) Intramuscular fat loosens up the microstructure of meat, causing more water to

be incorporated (Lawrie, 1964). The crude fat content was 14% higher by glycerol supplementation with no statistical significance. Collectively, changes of ultimate pH and crude fat contents in this study may contribute partially to the higher trend of WHC of LT by GS.

The glycerol supplementation increased ($P < 0.001$) the color lightness (CIE L^*) of the LT, but it did not affect ($P > 0.05$) the color redness (CIE a^*) and yellowness (CIE b^*). Beef with higher MS exhibited higher lightness values compared to the lower one (Pflanzer and de Felicio, 2011). Thus, the increased MS may in part contribute to the increased color lightness by GS in this study. Another possible reason is the contribution of pH to keep the amount of water in the meat surface (Pearce et al., 2011). According to Swatland (2008), muscles with lower pH undergo more myofibrillar and fiber shrinkage and consequently scatter more light. At a lower muscle pH, proteins do not bind water very tightly, allowing more free water to reflect light (Ledward et al., 1992). In addition, low pH will cause myoglobin fraction to be more readily oxidized to metmyoglobin which has low color intensity (Walters, 1975). Previously reported, beef carcass with a low ultimate pH (5.4) have a larger percentage of lighter color scores (Hughes et al., 2014). The ultimate pH was negatively correlated with muscle *CIE L** values in pork (Hamilton et al., 2003). Similarly, *CIE L**, a* and b*values were negatively associated with beef muscle pH (Page et al., 2001). Thus, the decreased pH may also be in part responsible for the increased color lightness by GS in this study.

The glycerol supplementation did not affect ($P > 0.05$) shear force values. Similarly, crude glycerin inclusion in the diets of bulls did not affect shear force (Mach et al., 2009; Carvalho et al., 2014). In this study, GS increased flavor ($P = 0.01$) and overall acceptance ($P < 0.01$) of LT and it tended to increase ($P = 0.10$) tenderness, although other sensory parameters (appearance, aroma, taste, juiciness) were unaltered ($P > 0.05$) (**Table 11**). Similarly, partial replacing of corn with crude glycerin in the diet of crossbred bulls increased flavor, tenderness, and overall acceptance of meat (Prado et al., 2016). Marbling score plays an important role in beef palatability (O'Quinn et al., 2018). Overall beef palatability is dependent upon the acceptance of three primary traits, tenderness, juiciness, and flavor (O'Quinn et al., 2018). Thus, increased overall acceptance is likely attributed to the increased MS and flavor and increased trend of tenderness by GS in this study. Thus, the purified glycerol supplementation may be used to improve the quality and acceptability of beef.

Expression of genes related to gluconeogenesis and lipid metabolism in the muscle

Several genes related to gluconeogenesis pathway from glycerol and propionate were analyzed in this study. Glycerol supplementation did not affect ($P > 0.05$) hepatic mRNA expression of genes for glycerol (*GK*) and propionate substrate incorporation (*PCCA*, *PCCB*, and *ACSS3*) in hepatic gluconeogenesis

pathway in Korean cattle steers (**Figure 6).** Our results demonstrate that the 3.15% GS of concentrate DM does not significantly affect the transcript levels of genes related to the incorporation of glycerol and propionate substrates in the gluconeogenesis pathway. These may reflect no changes in ruminal propionate and serum glycerol concentrations by GS. In gluconeogenesis pathway, fructose 1,6-biphosphatase is involved in conversion of fructose 1,6-bisphosphate to fructose 6-phosphate, and glucose 6-phosphatase is involved in conversion of glucose 6-phosphate go glucose (Pilkis and Granner, 1992). In the liver, glucose transporter 2 is involved in glucose uptake and release processes (Zhao and Keating, 2007). In the present study, GS did not affect ($P > 0.05$) mRNA levels of *FBP1, G6PC,* and *solute carrier family 2 member 2 (SLC2A2),* demonstrating that GS does not alter the gluconeogenesis and glucose transport in the liver of Korean cattle steers.

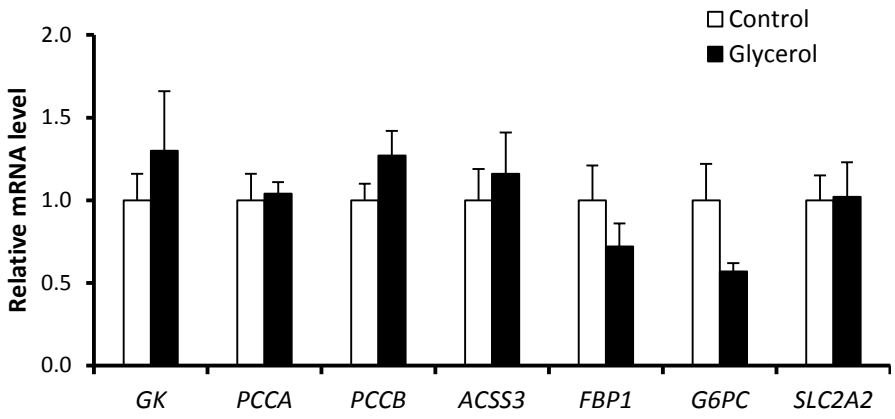

Figure 6. Comparison of the hepatic mRNA levels of genes related to gluconeogenesis and glycerol metabolism between the control and glycerol supplementation groups in Korean cattle steers. mRNA levels were determined by real-time polymerase chain reaction, and the results were normalized with *ribosomal protein S9* gene. The mRNA levels of the control group were normalized to 1.0. Values are the means ± SEM (n=7). *GK*, glycerol kinase; *PCCA*, propionyl CoA carboxylase alpha; *PCCB*, propionyl CoA carboxylase beta; *ACSS3*, acyl-CoA synthethase short chain family member 3; *FBP1*, fructose 1,6 biphosphatase; *G6PC*, glucose-6-phosphatase; *SLC2A2*, solute carrier family 2 member 2.

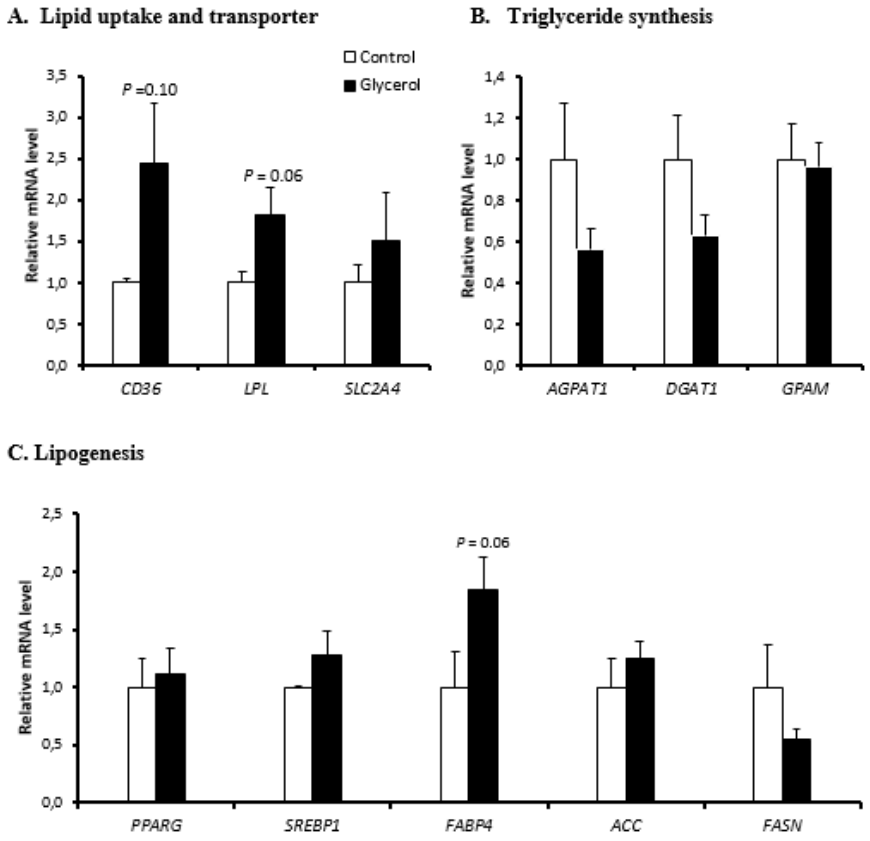

Figure 7. Comparison of the mRNA levels of genes related to lipid uptake and transporters (A), triglyceride synthesis (B), and lipogenesis (C) in muscle between the control and glycerol supplementation groups in Korean cattle steers. mRNA levels were determined by real-time polymerase chain reaction, and the results were normalized with *β-actin* gene. The mRNA levels of the control group were normalized to 1.0. Values are the means ± standard error (n=7). **P*<0.05. *CD36*, fatty acid translocase; *LPL*, lipoprotein lipase; *SLC2A4*, solute carrier family 2 member 4; *AGPAT1*, 1-acylglycerol phosphate acyltransferase 1; *DGAT1*, diacylglycerol transferase 1; *GPAM*, glycerol 3-phosphate

acyltransferase, mitochondrial; *PPARG*, peroxisome proliferator-activated receptor gamma; *SREBP1*, sterol regulatory element binding protein 1; *FABP4*, fatty acid binding protein 4; *ACC*, acetyl CoA carboxylase; *FASN*, fatty acid synthase.

In this study, we found that GS increased MS. Marbling is determined by the balance between fat deposition and fat removal in the longissimus muscle (Bong et al., 2012). In this study, we examined mRNA levels of lipid uptake and transport, triglyceride synthesis, and lipogenic genes in the LT. Glycerol supplementation tended to upregulate *LPL* ($P = 0.06$) and lipid uptake *CD36* ($P = 0.10$) mRNA levels in the LT (**Figure 7**). Lipoprotein lipase hydrolyzes circulating lipoprotein triglycerides, releasing free fatty acids that are taken up by adipose tissue and muscle (Wang and Eckel, 2009). Fatty acid translocase CD36 is an important protein for fatty acid uptake by catalyzing translocation of fatty acid across the plasma membrane (Xu et al., 2013). Glycerol supplementation tended to upregulate ($P = 0.06$) *FABP4*. FABP4 is consisted of a group of small cytosolic proteins that bind and transport fatty acids intracellularly (Storch and Thumser, 2000). Collectively, our results demonstrate that up-regulated tendency of release of FA from circulating lipoprotein and lipid uptake and subsequent cytosolic transport may be in part responsible for the increased MS by GS. *Solute carrier family 2 member 4* (*SLC2A4*) was not altered with GS ($P > 0.05$) in the LT. *Solute carrier family 2 member 4* encodes glucose transporter 4 protein, which is an insulin-

responsive glucose transporter (Ostrowska et al., 2015). Glucose transporter 4 is a major regulator of glucose uptake in skeletal muscle adipocytes (regulated by insulin), which participates in adipogenic stimulation process (Cesar et al., 2015). mRNA levels of genes for triglyceride synthesis including 1-*acylglycerol phosphate acyltransferase 1 (AGPAT1), diacylglycerol transferase (DGAT1),* and *glycerol-3-phosphate acyltransferase, mitochondrial* (*GPAM*) were not altered ($P > 0.05$) by GS in the LT. mRNA levels of adipogenic *PPARG* and *SREBP1 genes* and of lipogenic *ACC* and *FASN* genes were also not altered ($P > 0.05$) by GS in the LT. In our recent study, mRNA levels of genes related to triglyceride synthesis and lipogenesis-related genes (*ACC* and *FASN*) in muscle were not altered by GS. Similar with our result, supplementation of glycerol or fructose via drinking water did not affect mRNA levels of genes related to lipogenesis and triglyceride synthesis (*ACC, FASN, FABP4, PPARG,* and *GPAT*) in Angus-cross steers (Volpi-Lagreca and Duckett, 2016). Thus, up-regulation of lipid uptake and cytosolic transport by GS may contribute partially to increase MS.

Correlation between gene expression levels and marbling score and intramuscular fat content

Several significant correlations were observed between the expression of glucose and lipid metabolism genes with MS and IMF content (**Table 12**). The hepatic *PCCB* mRNA levels were positively correlated ($r = 0.56$, $P < 0.05$) with

MS. The hepatic mRNA levels of *GK, PCCB, and solute carrier family 2 member 2 showed positive correlations* ($r = 0.72$, $P < 0.01$; $r = 0.60$, $P < 0.05$; $r = 0.60$, $P < 0.05$) with IMF. Triglyceride synthesis is a key factor in IMF deposition and MS. Both non-esterified fatty acids and glycerol are required for triglyceride synthesis. Glycerol kinase is an important enzyme at the interface of carbohydrate and lipid metabolism, catalyzing the interconversion of glycerol and glycerol-3-phosphate (Dipple et al., 2001). Propionyl CoA carboxylase beta catalyzes the carboxylation of propionyl-CoA with bicarbonate producing methylmalonyl-CoA which is then converted to succinyl-CoA, an intermediate in TCA cycle (Wongkittichote et al., 2017). Our previous study reported that hepatic *PCCB* was positively associated with MS (Fassah et al., 2018). Although *GK, PCCB,* and *solute carrier family 2 member 2* mRNA levels were positively correlated with IMF and/or MS, the expressions of those genes were not changed with GS. In addition, GS did not change the gene expressions of lipogenesis and triglyceride synthesis in muscle. Thus hepatic gluconeogenesis may contribute partially to glucose utilization in fatty acid synthesis in muscle, resulting in higher IMF deposition and MS following GS in Korean cattle steers.

Table 12. Correlation of mRNA levels of gluconeogenesis and lipid metabolism genes with marbling score and intramuscular fat content in liver and muscle

Genes	Marbling score	Intramuscular fat content
Liver		
Gluconeogenesis		
Glycerol kinase (*GK*)	0.14	0.74**
Propionyl CoA carboxylase alpha (*PCCA*)	0.24	0.19
Propionyl CoA carboxylase beta (*PCCB*)	0.58*	0.65*
Acetyl CoA synthetase short-chain family member 3 (*ACSS3*)	0.41	0.41
Fructose 1,6-biphosphatase (*FBP1*)	0.10	0.39
Glucose 6-phosphatase (*G6PC*)	0.01	0.15
Solute carrier family 2 member 2 (*SLC2A2*)	0.09	0.64*
Muscle		
Lipid uptake and transporter		
Lipoprotein lipase (*LPL*)	0.63*	0.03
Fatty acid translocase (*CD36*)	0.32	-0.19
Solute carrier family 2 member 4 (*SLC2A4*)	0.09	0.38
Triglyceride synthesis		
1-acylglycerol-3-phosphate O-acyltransferase 1 (*AGPAT1*)	0.13	0.15
Diacylglycerol O-acyltransferase 1 (*DGAT1*)	-0.01	-0.03

Glycerol-3-phosphate acyltransferase, mitochondrial (*GPAM*)	-0.20	-0.48
Lipogenesis		
Peroxisome proliferator activated receptor gamma (*PPARG*)	0.29	0.30
Sterol regulatory element binding transcription factor 1 (*SREBP1*)	0.11	-0.25
Fatty acid binding protein 4 (*FABP4*)	0.34	0.38
Acetyl CoA carboxylase (*ACC*)	0.47	0.33
Fatty acid synthase (*FASN*)	-0.08	0.16

n = 14

Significant correlations: *P<0.05; **P<0.01

In this study, though 3.15% GS of concentrate DM was insufficient to show a significant difference in IMF content in this study, we found that the expression of lipid uptake gene (*CD36* and *LPL*) and *FABP4* in muscle tended to increase by GS (**Figure 7**). Therefore, lipid uptake may contribute partially to IMF deposition following GS in Korean cattle steers. The mRNA levels of *LPL* in muscle were positively correlated (r = 0.63, P < 0.05) with MS. A variety of genes may be used as markers of adipocyte development and adipogenesis, including *LPL* and *PPARG,* and they have a great potential for predicting subsequent IMF development (Hocquette et al., 2010). Along with MS increased, GS tended to upregulate mRNA levels of *LPL* in muscle. Marbling score represents the distribution of white flecks or streaks of fatty tissue between muscle fiber within a muscle. Kruk et al. (2002) reported a possible relationship between MS and fat distribution, which is not precisely evaluated. Since MS is determined by the visual appraisal by professional evaluators using the MS standard, the fat distribution on the muscle surface may influence the MS. Our study suggests that GS increased fatty acid uptake in the muscle contributes to increased marbling. Overall, our correlation results suggest that hepatic *GK, PCCB, solute carrier family 2 member 2*, and *LPL* in muscle could be used as a genetic markers to predict beef quality

5. Conclusion

Our results reveal that mild cold stress does not affect the growth performance. However cold temperatures affect rumen fermentation and blood metabolites levels. Glycerol supplementation at 3.15% of concentrate DM could be fed to finishing Korean steers with little or no effect on growth performance, rumen fermentation parameters, and blood metabolites. Glycerol supplementation increased MS. Glycerol supplementation in the diet improved pH value, color lightness, and flavor followed by the increase in overall acceptance rate. In the muscle, GS tended to upregulate mRNA expression of lipid uptake genes. Positive correlations were observed between mRNA levels of *PCCB* in liver and *LPL* in muscle with MS. Positive correlations were also observed between hepatic mRNA levels of *GK*, *PCCB*, and *SLC2A2* with IMF. These results imply that increased lipid uptake in the muscle of Korean cattle steers reared under cold condition following GS is associated with increased MS. Glycerol could be used as an energy supplement in beef cattle to improve beef quality.

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CHAPTER FIVE

General Conclusion

Effect of castration on gluconeogenesis

In study 1, the expression of critical genes in hepatic gluconeogenesis pathway between bulls and steers of Korean cattle were compared. The associations between those gene expressions level with backfat thickness, marbling score, and quality grade of Korean cattle were examined. The castration of bulls upregulates the transcriptional levels of several genes involved in gluconeogenesis, including *PCCB, PC, PCK2, LDHA, LDHB, GK* and *GPD1*. Positive correlations were found between hepatic *FBP1, LDHA, PCCA, GK,* and *GPD1* gene expressions with backfat thickness. Also, hepatic *PC, PCK2, LDHA, LDHB,* and *PCCB* gene expressions were positively correlated with MS and/or QG. These results suggest that castration may increase gluconeogenesis process by several pathways including lactate, propionate, and glycerol. It can be imply that increased hepatic gluconeogenic gene expressions following castration is associated with backfat thickness, IMF deposition and QG.

Effect of glycerol supplementation on gluconeogenesis and lipid metabolism, growth performance, and meat quality

In study 2, a feedlot trial (using 3.15% GS of concentrate DM) was conducted during fall through winter. The effect of ambient temperature, glycerol supplementation, and their interactions on feed intake, rumen parameters, blood metabolites, carcass characteristics, meat quality and expression of genes related to gluconeogenesis and lipid metabolism were examined. Neither cold temperature nor GS significantly affected the performance of Korean cattle steers. Cold exposure between months increased ruminal pH, NH_3-N concentration, and molar proportion of butyrate, while total VFA molar concentration and molar proportions of propionate and valerate were decreased. Glycerol supplementation increase the molar proportions of ruminal valerate; however, the other ruminal VFA proportions were not changed. Interaction between the cold temperature and GS affected the molar concentration of total VFA. Cold exposure between months affected the concentrations of blood glucose, cholesterol, and triglyceride, while GS did not affect the blood metabolites. Glycerol supplementation in the diet increased MS and color lightness (CIE *L**) of LT, while it decreased the ultimate pH. Though most of the sensory traits were not different after glycerol was supplemented, the flavor and overall acceptance of LT were increased. Glycerol supplementation did not change the mRNA levels of hepatic gluconeogenesis

genes from glycerol oxidation. The mRNA expression levels of *CD36*, *LPL*, and *FABP4* genes in muscle were upregulated by GS. The mRNA levels of hepatic *PCCB* and *LPL* in muscle have positive correlations with MS. The hepatic mRNA levels of *GK*, *PCCB*, and *SLC2A2 were* positively correlated with IMF. Considering the increased of several gene expression contributed to lipid uptake, and positive correlations between genes in gluconeogenesis and lipid with MS and IMF, it can be assumed that GS can be used as a supplement to improve MS and beef quality.

SUMMARY IN KOREAN

수소의 거세는 지방침착을 증가시켜 좋은 육질과 도체성적을 나타낸다. 근내지방의 침착은 지방산섭취 및 흡수나 지방산 신생합성 이후 근육내 트리글리세라이드의 결합을 필요로 한다. 탄소원의 공급, NADPH 생산 또는 지방산 합성을 위해 근내지방조직은 높은 비율로 포도당을 이용한다. 테스토스테론 결핍은 지방질량의 증가, 인슐린 감수성 저하, 포도당 대사 약화와 연관이 있다. 따라서 거세 이후 나타나는 포도당과 지질대사의 변화는 소고기 품질에 영향을 미칠 것이다.

소의 성장능력은 주위 온도 같은 기후적인 요소에 영향을 받는다. 온도스트레스는 소의 열중립대보다 주위 온도가 높거나 낮을 때 발생한다. 저온스트레스는 소의 대사율과 유지요구량을 증가시키는데 이것은 성장능력과 생산성을 감소시킨다. 사료내 에너지밀도를 높여주는 것은 온도스트레스를 받은 소에게 있어 생산성을 유지시킬 수 있는 효과적인 방법이다. 바이오디젤 생산과정의 부산물인 글리세롤은 소의 에너지 상태를 향상시키기 위한 당 생성의 전구체로 이용될 수 있다. 동물성, 식물성 지방공급원은 반추동물의 사료로 이용되기에 한계가 있기 때문에 글리세롤이 선호된다. 사료내 조지방 함량은 5%를

넘지 않는 것이 권장되는데 이는 반추동물의 반추위내 발효를 저해할 수 있기 때문이다.

한우에서 당신생과 지질대사, 성장능력, 육질등에 대한 거세, 저온, 사료내 글리세롤 첨가 효과의 정보는 제한적이다. 따라서, 본 연구는 1) 한우 수소의 거세가 당 신생합성 및 해당 과정과 관련 있는 유전자들의 전사에 미치는 영향, 2) 글리세롤의 섭취와 주위 온도가 비육 후기 한우 거세우의 성장률, 반추위 성상, 혈 중 대사물질, 도체 특성, 육질과 당 신생합성 및 지질 대사에 미치는 영향을 알아보고자 수행되었다.

첫 번째 연구에서, 거세는 *pyruvate carboxylase* (*PC*) 의 mRNA 발현량 (3.6 배; $P < 0.01$) 단백질 수준 (1.4 fold; $P < 0.05$)을 증가시켰으며 *mitochondrial phosphoenolpyruvate carboxykinase* genes (*PCK2)*의 단백질 수준을 (1.7 fold; $P < 0.05$) 증가시켰다. 해당과정의 효소들을 지정하는 유전자들의 간 내 mRNA 발현량은 거세에 의해 변하지 않았지만 젖당 이용에 필요한 *lactate dehydrogenase A* (*LDHA*; 1.5 배; $P < 0.05$) 와 *lactate dehydrogenase B* (*LDHB*; 2.2 배; $P < 0.01$) 유잔자의 발현량은 증가하였다. 더하여, 거세는 글리세롤 이용과 관련 있는 *glycerol kinase* (*GK*; 2.7 배; $P < 0.05$) 와 *glycerol-3-phosphate dehydrogenase 1* (*GPD1*; 1.5 배; $P < 0.05$) 유전자의 mRNA 수준을 증가시켰으며, 또한 프로피온산 결합에 이용되는 *propionyl-CoA*

carboxylase beta (*PCCB*; 3.5 fold; $P < 0.01$) 와 *acyl-CoA synthetase short chain family member 3* (*ACSS3*; 1.3 fold; $P = 0.06$) 유전자의 발현도 증가시켰다. 두 번째 연구에서 12 월의 외기 온도가 가장 낮았다 ($P < 0.001$). 날짜 (월 단위)와 글리세롤 첨가는 평균 일당 증체와 사료 효율에 영향을 미치지는 않았다 ($P > 0.05$). 추운 날씨는 농후 사료 섭취량과 총 사료 섭취량에 영향을 미쳤지만 ($P < 0.001$), 글리세롤 첨가는 농후 사료 섭취량을 감소시켰다 ($P < 0.05$). 추운 기간 동안 반추위 pH 와 암모니아 질소 (NH_3-N) 농도, 뷰티르산의 몰 농도 비율은 유의적으로 높았지만 ($P < 0.05$), 총 휘발성 지방산 (volatile fatty acid, VFA)의 농도와 프로피온산, 발레르산의 몰 농도 비율은 낮았다 ($P < 0.05$). 글리세롤 첨가는 유의적으로 발레르산의 몰 농도를 증가시켰다($P<0.05$). 추운 날씨는 혈 중 글루코스, 콜레스테롤, 트리글리세리드, 비에스테르형 (non-esterified fatty acid, NEFA) 지방산의 농도에 영향을 미쳤다 ($P < 0.05$). 글리세롤 첨가는 등심의 마블링 스코어와 육색 밝기를 증가시켰지만 ($P < 0.05$), 최종 도달 pH 는 감소시켰다 ($P < 0.05$). 더하여, 글리세롤 첨가는 관능적 특성인 풍미와 전반적인 기호 또한 증가시켰다 ($P < 0.05$). 글리세롤의 첨가는 간 내 당 신생 합성 관련 유전자들의 발현량에 영향을 미치지는 않았지만, 등심 조직 내 *fatty acid translocase* (*CD36*), *lipoprotein lipase* (*LPL*), and *fatty acid*

binding protein 4 (*FABP4*) 유전자들의 mRNA 는 증가하는 경향을 보였다 ($0.05 < P \leq 0.10$). 위 결과들로 미루어보아, 추운 환경으로 인해 한우 거세우의 몇가지 혈 중 대사 물질과 반추위 성상에 변화가 일어나지만 성장에는 영향을 미치지 않는다는 것을 알 수 있다. 더하여, 사료 내 3.15%의 글리세롤의 첨가는 성장률과 반추위 VFA 농도, 혈 중 대사 물질에 영향을 미치지 않으면서 마블링 스코어와 관능적 기호도는 증가시키므로 저온 기간 동안 성장률에 대한 악영향없이 소고기의 품질을 향상시킬 수 있는 방법으로 판단된다.

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