MARCIO DE SOUZA DUARTE

ADIPOGENESIS AND FIBROGENESIS IN MUSCLE AND EFFECTS OF PREGNANCY AND FEEDING LEVEL ON CARCASS MEAT QUALITY AND DEVELOPMENT OF CATTLE FETUS

Thesis submitted to the Animal Science Graduate Program of the Universidade Federal de Viçosa in partial fulfillment of the requirements for the degree of *Doctor Scientiae.*

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Finally, but not least, I dedicate it to all my family and especially to my wife Tahiane Sales de Araújo and my daughter Anita Sales Duarte who makes me stronger every day. I would never get this far without you.

To them all, I leave my heartfelt gratitude.

BIOGRAPHY

Marcio de Souza Duarte, son of José Tarcísio Duarte and Eloisa Helena de Souza Duarte, was born in Viçosa/MG-Brazil on October 21, 1983.

He started the undergrad in Animal Science at *Universidade Federal de Viçosa* in 2002 and became a Bachelor of Science in Animal Science in 2008. At the same year he started the M.S. program with major on meat science and beef cattle production. In February of 2009 he was a visiting researcher at the Meat Animal Research Center of the United States Department of Agriculture (USDA/MARC) in Clay Center/NE-USA for training on meat quality laboratory techniques under supervision of Dr. Tommy Wheeler and Dr. Steven Shackelford.

In February of 2010 he became a M.S. in Animal Science. At the same year he started his D.S. program in Animal Science with major on developmental biology, and meat science. From August of 2011 to June of 2012 he was a visiting scholar at Muscle Biology Laboratory of the Washington State University, Pullman/WA - USA where part of his research was developed under supervision of Dr. Min Du and Dr. Michael Dodson. On June 20th of 2013 Mr. Duarte defended his dissertation to obtain the *Doctor Scientiae* degree in Animal Science.

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ABSTRACT

DUARTE, Marcio de Souza, D.Sc., Universidade Federal de Viçosa, June of 2013. **Adipogenesis and fibrogenesis in muscle and effects of pregnancy and feeding level on carcass meat quality and development of cattle fetus** Adviser: Pedro Veiga Rodrigues Paulino. Co-Advisers: Sebastião de Campos Valadares Filho and Simone Eliza Facioni Guimarães.

The present work was developed based on three experiments. The objective of the first study was to evaluate intramuscular fat (IMF) and collagen deposition in the muscle of Wagyu compared to Angus cattle. Animals were managed under the same condition and slaughtered at an averaging 585 ± 12.1 kg of body weight. Samples of *sternomandibularis* muscle were collected from Wagyu (n = 3) and Angus (n = 3) for molecular and histological investigations of adipogenesis and fibrogenesis. With exception of CCAAT enhancer binding protein β (CEBPβ; $P = 0.2864$), the expression of the adipogenic markers, CCAAT enhancer binding protein α (CEBPα; $P = 0.008$), peroxisome proliferator-activated receptor γ (PPARγ; $P = 0.028$) and zip finger protein 423 (Zfp423; $P = 0.047$) in Wagyu were higher than in Angus muscle, which was consistent with higher IMF deposition in Wagyu ($P = 0.007$). In addition, more adipocytes and pre-adipocytes were detected intramuscularly in Wagyu cattle. Similarly, fibrogenesis was also enhanced in Wagyu, with a higher expression of fibroblast growth factor II (FGF2; $P = 0.028$), FGF receptor 1 ($P = 0.030$), transforming growth factor β (TGFβ; $P = 0.028$), collagen I ($P = 0.012$) and collagen III ($P = 0.025$). Similarly, Wagyu muscle had higher collagen content ($P = 0.002$) and lower collagen solubility ($P = 0.004$). In addition, muscle fiber diameter was larger ($P < 0.0001$) in Wagyu than in Angus cattle, suggesting that the total muscle fiber number was lower in Wagyu cattle. These results clearly show that both IMF and collagen contents are enhanced in Wagyu cattle and more adipogenic cells are detected in Wagyu muscle, indicating intramuscular adipogenesis is enhanced in Wagyu compared to Angus muscle. The second study was developed aiming to evaluate the effects of maternal feed-restriction on development of gastrointestinal tract (GIT) of bovine fetus at different gestational stages. Feed-restricted cows were fed 1.2 times the maintenance level while the control group was fed ad libitum. Pregnant cows were slaughtered at 136, 189, 239, and 269 days of gestation and gastrointestinal tracts of the fetuses were evaluated. No effects of maternal nutrition on body weight ($P = 0.17$) and body length ($P = 0.13$) of the fetuses were observed. No major effects of feed restriction on GIT

mass of the fetuses were observed (P = 0.51). However, the weight of small intestine per unit of body weight was 11.24 % greater (P =0.04) in fetuses from restricted dams. Additionally, the length of small intestine and its villi were 12.93 % and 16.44 % respectively greater ($P < .001$) in fetuses from restricted dams compared to those from non-restricted dams. These data indicates that maternal feed-restriction does not affect the development of most of fetal gastrointestinal parts besides small intestine which in turn increases its surface area as a response of maternal feed restriction. Finally, in the third experiment carcass and meat quality traits of 16 pregnant and 5 non-pregnant cows fed at 1.2 times maintenance and 16 pregnant and 6 non-pregnant fed ad libitum were evaluated. Pregnancy did not affect final body weight (FBW; P = 0.0923), cold carcass yield (CCY; P = 0.0513), longissimus muscle area (LMA; P = 0.8260), rib fat thickness (RFT; P = 0.1873) and shear force (WBSF; P = 0.9707). A lower FBW (P = 0.0028), LMA (P = 0.0048) and RFT (P = 0.0001) were observed in feed restricted cows. However, no differences were found for CCY (P = 0.7243) and WBSF (P = 0.0759) among feeding level groups. These data suggests that carcass and meat quality traits are not affected by pregnancy status in Nellore cows. Moreover, although cows experiencing feed restriction did have reduced deposition of subcutaneous fat and lean tissue, there were no major impacts on meat quality traits.

RESUMO

DUARTE, Marcio de Souza, D.Sc., Universidade Federal de Viçosa, junho de 2013. **Adipogênese e fibrogênese em músculo e efeitos da gestação e nível de alimentação sobre carcaça, qualidade de carne e desenvolvimento de feto bovino.** Orientador: Pedro Veiga Rodrigues Paulino. Coorientadores: Sebastião de Campos Valadares Filho e Simone Eliza Facioni Guimarães

O presente trabalho foi desenvolvido a partir de três experimentos. O Objetivo do primeiro estudo foi de avaliar a deposição de tecido adiposo intramuscular e colágeno em músculo esquelético de animais da raça Wagyu em comparação com animais da raça Angus. Os animais foram manejados sob mesmas condições durante toda fase de desenvolvimento e foram abatidos com peso corporal médio de 585 ± 12.1 kg. Amostras do músculo *Sternomandibularis* foram coletadas de animais Wagyu (n = 3) e animais Angus (n = 3) para avaliações moleculares e histológicas quanto à adipogênese e fibrogênese. Com exceção do marcador CCAATT *enhancer biding protein* β (CEBPβ; P = 0.2864), a expressão dos marcadores para adipogênese CCAAT *enhancer biding protein* α (CEBPα; P = 0.008), *peroxisome proliferator-activated receptor* γ (PPARγ; P = 0.028) e *zip finger protein 423* (Zfp423; P = 0.047) em músclo de animais Wagyu foi maior que em músculo de animais Angus, o que foi consistente com a maior deposição de gordura intramuscular em Wagyu (P = 0.0007). Além disso, maior número de adipócitos e pré-adipócitos intramusculares foram detectados em animais Wagyu. De forma semelhante, maior fibrogênese foi observada em músculo Wagyu tendo-se maior expressão do *fibroblast growth factor II* (FGF2; P = 0.0028), *fibroblast growth factor receptor 1* (FGF 1; P = 0.030), *transforming growth factor β* (TGFβ; P = 0.028), *Collagen I* (COL 1; P = 0.012) e *collagen III* (P = 0.025). Da mesma forma, o músculo de animais Wagyu apresentou maior teor de colágeno total (P = 0.002) e menor solubilidade do mesmo (P = 0.004). Além disso, o diâmetro de fibra muscular em animais Wagyu foi maior (P < 0.0001) em relação a animais Angus, sugerindo que o total de fibras musculares em animais Wagyu fosse menor em comparação a animais da raça Angus. Estes resultados demonstram que além da maior deposição de gordura, há também maior deposição de colágeno intramuscular em animais da raça Wagyu em comparação a animais da raça Angus. O segundo experimento foi realizado com objetivo de avaliar os efeitos da nutrição materna sobre o desenvolvimento do trato gastrintestinal de fetos bovinos em diferentes estágios da

gestação. Dois grupos de vacas gestantes foram aleatoriamente distribuídos em dois tratamentos alimentares em que um grupo foi alimentado *ad libitum* e outro com alimentação restrita em que as vacas foram alimentas a 1,2 x o nível de mantença. As vacas foram abatidas em quatro tempos de gestação (136, 189, 239, 270 dias) sendo que para cada período de gestação avaliada obteve-se o mesmo número de vacas dos grupos alimentares. Não houve efeito da nutrição materna sobre o peso corporal ($P = 0.17$) e comprimento corporal ($P = 0.13$) dos fetos. A restrição alimentar materna durante a gestação não afetou o peso do trato gastrintestinal dos fetos ($P = 0.51$). Entretanto, o peso do intestino delgado dos fetos por unidade de peso corporal foi 11.24% maior ($P = 0.04$) em fetos oriundos de matrizes submetidas a restrição alimentar. Além disso, o comprimento do intestino delgado bem como o tamanho de suas vilosidades foi 12.93% e 16.44% respectivamente maior ($P < 0.001$) em fetos oriundos de vacas sob-restrição alimentar em comparação aos fetos oriundos de vacas alimentadas *ad libitum*. Os dados sugerem que a restrição alimentar materna durante a gestação não afeta o desenvolvimento da maioria dos compartimentos que compõe o trato gastrintestinal fetal, com exceção do intestino delgado o qual por sua vez aumenta a sua superfície de contato como resposta à restrição alimentar materna. No terceiro experimento foram avaliadas características de carcaça e da carne de 16 vacas gestantes e 5 vacas não gestantes alimentadas a 1,2 x nível de mantença e 16 vacas gestantes e 6 vacas não gestantes alimentadas *ad libitum*. A gestação não afetou o peso corporal final (PF; $P = 0.0923$), o rendimento de carcaça fria (RCF; $P = 0.0513$), a área de olho de lombo (AOL; $P = 0.8260$), espessura de gordura subcutânea (EGS; $P = 0.1873$) e força de cisalhamento (FC; $P = 0.9707$). Vacas em restrição alimentar apresentaram menor PF ($P = 0.0028$), AOL ($P = 0.0048$) e EGS ($P = 0.0001$). Entretanto, não foram observadas diferenças quanto a RCF ($P = 0.7243$) e FC ($P = 0.0759$) entre os animais submetidos a diferentes níveis de alimentação. Os dados sugerem que as características de carcaça de vacas Nelore não são afetadas pela gestação. Além disso, embora vacas em restrição alimentar tenham apresentado redução da deposição de gordura subcutânea e tecido muscular na carcaça, não foram observados alterações no aspecto qualitativo da carne destes animais.

INTRODUCTION

Beef cattle production in Brazil is based on pastures which represent the lowest cost food resource for ruminant feeding (Duarte et al., 2011; Sampaio et al., 2010). Although the number of feedlots is increasing in Brazil, the Brazilian beef production system can still be considered grass-fed animals, since animals are kept in the feedlot for a very short time, mainly at the finishing period (Fries and Ferraz, 2006). The production costs of beef in Brazil are estimated to be 60% lower than in Australia and 50% lower than in the United States, which makes the Brazilian beef system highly competitive in the international beef market. In 1961, Brazil exported only 2.6% of the total beef produced, increasing to 17.1% in 2011 (Torres Junior and Neto, 2012). However, even though the great beef production Brazilian beef is considered by the international market as a low quality meat. Consequently, a lot of efforts have been made to provide new technologies and knowledge that may improve the production rates and provide a high quality meat and thus, increase the competitiveness of Brazilian beef in the international market.

For many years most of the production technologies (i.e. supplementation strategies) adopted by a production system was restrict to the post-natal stage of life. Recent studies, however, suggest that nutrient deficiency from early to mid-gestation in ruminant animals may cause challenges in muscle development (Du et al., 2010a) and alters the gastrointestinal tissue growth (Meyer et al., 2010). As a consequence, performance of the progeny is compromised even when no difference is observed on birth weight of the offspring (Wu et al., 2006). Furthermore, during the early phase of fetal development, critical events for normal conceptus development occur, including differentiation, vascularization, fetal organogenesis, and placental development (Funston et al., 2010).

Great efforts have been made in recent years to understand the effects of maternal nutrition on fetus development envisioning a possibility to program the development of the progeny. In livestock production, fetal programming can be simply described as a response to a specific challenge during a critical fetal developmental time window that alters the trajectory of development qualitatively, quantitatively, or both, with permanent effects in the life of the animal. This hypothesis is even more reliable if we think that fetal stage is crucial for skeletal muscle development since there is no

increase in muscle fibers numbers after birth (Du et al., 2010a). Furthermore, the development of vital organs has higher priority in nutrients portioning than skeletal muscle, which makes the muscular tissue more susceptible to variations of maternal nutrition during pregnancy. Therefore, the knowledge of the skeletal muscle development during prenatal stages is crucial for a better understanding of differences in meat quality and animal performance at postnatal phase.

Skeletal muscle development is roughly divided into 3 stages; embryonic, fetal, and adult stages (Du and Dodson, 2012; Du et al., 2010a). Embryonic and fetal skeletal muscle development is collectively referred to as prenatal muscle development, which is critical because it has dramatic impact on postnatal growth (Dauncey and Harrison, 1996). During the prenatal stage, skeletal muscle development mainly involves the formation of muscle fibers (i.e., myogenesis), but also the formation of intramuscular adipocytes (i.e., adipogenesis) and fibroblasts (i.e., fibrogenesis). In livestock, muscle fibers are formed during the prenatal stage and there is no further net increase after birth. Intramuscular adipogenesis during early developmental stages generates sites for fat deposition in offspring muscle, which forms marbling fat in resulting beef; adipogenesis in other depots is undesirable. Intramuscular fibrogenesis creates connective tissue distributed inside muscle. Recent studies show that intramuscular adipocytes and fibroblasts are developed from common progenitor cells (Joe et al., 2010; Uezumi et al., 2010; Uezumi et al., 2011). Consequently, intramuscular adipogenesis and fibrogenesis may be considered as a competitive process, providing that the total density and proliferation of progenitor cells are unaltered; enhancing adipogenic differentiation while reducing fibrogenic differentiation from progenitor cells will increase both the marbling and tenderness of meat (Du et al., 2013; Duarte et al., 2013).

Several myogenic regulatory factors are involved in the differentiation of mesenchymal stem cells into muscle fibers. Prenatal regulation of myogenesis involves expression of regulatory proteins, including Wingless and Int (Wnt), paired box gene (Pax) 3 and Pax 7 (Hyatt et al., 2008). The Wnt signaling regulates the expression of Pax 3 and Pax 7, which are involved in the expression of the myogenic regulatory factors (MRFs) in mesenchymal stem cells (Figure 1). Identified MRFs include myogenin, MRF-4, MyoD and Myf-5 (Stewart and Rittweger, 2006). The expression of MyoD and Myf5 leads undifferentiated stem cells to undergo myogenic differentiation to committed myoblasts. Subsequently, myoblasts express myogenin which is necessary

to form multinucleated myotubes. At the end of skeletal muscle formation, MRF4 is expressed leading to formation of mature muscle fibers and MRF4 becomes the dominant MRF in postnatal muscle (Keren et al., 2006; Kollias and McDermott, 2008).

As previously mentioned, myogenesis, adipogenesis and fibrogenesis appear to be competitive processes, and the commitment of stem cells to one of these events can be altered by *in utero* regulations (Yan et al., 2012). By switching the commitment of multipotent cells from myogenesis to adipogenesis, the number of adipocytes in the body can be increased resulting in a different body composition. The formation of adipocytes from the mesoderm begins during midgestation (Du et al., 2010b). Adipogenesis inside muscle during the fetal stage has a dominant effect on the number of intramuscular adipocytes, an event linked to insulin resistance due to their paracrine effects and close proximity (Aguiari et al., 2008). Adipose tissue growth in later life is due to both hypertrophy and hyperplasia (Fève, 2005). However, new adipocytes generated later in life are mainly located in visceral and retroperitoneal fat depots and also in subcutaneous fat depots, with very few located in intramuscular fat (Faust et al., 1978; Miller et al., 1984; Valet et al., 2002). Mechanisms controlling adipogenesis in fetal muscle *in vivo* are poorly defined, although there are numerous *in vitro* cell culture studies (Rosen et al., 2002). In these studies, pre-adipocytes 3T3 and mesenchymal stem cells 10T1/2 cell lines, are commonly used. These studies have demonstrated that peroxisome proliferator-activated receptor γ (PPARγ) and CCAAT-enhancer-binding proteins (C/EBPs) are crucial intracellular factors controlling adipogenesis. Their expression leads to adipogenesis from pluripotent cells (Cho and Jefcoate, 2004; Giri et al., 2006).

Fibroblasts are the main cell that forms collagen which is the main component of connective tissue and the structural unit of extracellular matrix. In muscle, Type I and Type III are the most abundant collagen in extracellular matrix (Huang et al., 2012). Collagens have a very low turnover rate, and they are degraded by the action of matrix metalloproteinases (MMP). The activity of MMP is inhibited by tissue inhibitor of metalloproteinase. Lysyl oxidase is the key enzyme catalyzing collagen cross-linking. In our studies in sheep and cattle, the expression of collagens, lysyl oxidase and MMP are correlated with each other, showing that all of these components are needed to synthesize extracellular connective tissue (Du et al., 2013).

Recent studies have demonstrated that adipogenesis and fibrogenesis share a common source of ancestor cells known as mesenchymal progenitor cells (Uezumi et

al., 2011). These cells are located in the stromal-vascular fraction of skeletal muscle and are different than satellite cells (Joe et al., 2010; Uezumi et al., 2010). As such, based on this new knowledge it is possible to envision the development of feeding management strategies to manipulate deposition that would allow a greater fat accretion in detrimental of connective tissue deposition in both pre-natal and post-natal stages of life. However, the cellular mechanism that leads to adipogenic and fibrogenic differentiation needs further clarification.

Besides skeletal muscle, the gastrointestinal tract has also been reported as a site of maternal nutrition effects during pre-natal stage of life. During perinatal development of the mammalian the gastrointestinal tract transient absorption of immunoglobulins is one of the main events that affect animal performance (Zabielski et al., 2008). Consequently, as the gastrointestinal tract serves as the main site for nutrient absorption while also being a major energy and nutrient sink due to its increased metabolic activity and rapid turnover, changes in maternal visceral organs during pregnancy may greatly affect the dam and fetus (Meyer et al., 2010). Several studies have suggested that fetuses originated from a feed-restricted dam are born with an immature small intestine may present a decreased immune competence, survival, and growth potential during early neonatal life (Cronj et al., 2003; Greenwood and Cafe, 2007; Trahair et al., 1997; Wu et al., 2006). Studies investigating intrauterine growth restriction in beef cattle have observed differences in postnatal growth with (Martin et al., 2007) and without (Greenwood and Cafe, 2007) altered feed efficiency, which could be due in part to intestinal differences. However, there are only a few studies with this regard in beef cattle and the results reported are far to be conclusive.

In a different context, in regions where grazing beef is the main system used for beef production pregnant cows are often culled in order to relieve pastures or even due to fluctuation of beef prices which in turn are commonly seen in the beef industry (Macedo et al., 2007; Wythes et al., 1990). In a study performed at University of Queensland about it was revealed that in heard not using a pregnancy diagnosis, in herds not using pregnancy diagnosis**,** pregnancy rate in slaughtered cows was 71.6%. On the other hand, prior to disposal of cows resulted in the slaughter of 34% fewer pregnant cows than in undiagnosed herds and most cows, pregnant when culled, were in early pregnancy. The comparatively high (38.1 %) occurrence of pregnancy in cows examined by rectal palpation was the result of these cows being held with bulls for up to 3 months after culling. Of 77 herds selected at random, cows in only 5 (6.4%) were

subsequently found to have been subjected to pregnancy diagnosis at the time of culling (Ladds et al., 1975). However, although harvest of pregnant cows is a reality in grass-based production systems there are a limited number of studies that have evaluated effects of pregnancy on carcass and meat quality traits throughout the gestational period.

Therefore, this study was developed based on three independently experiments with the objective to:

1- Identify the mechanisms underlying the development of connective and intramuscular fat development;
2- Evaluate the influence of maternal nutrition of development of gastrointestinal tissue at prenatal stage of life;
3- Assess the meat quality of pregnant cows under tropical conditions at different stages of gestation.

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Chapter I

Enhancement of adipogenesis and fibrogenesis in skeletal muscle of Wagyu compared to Angus cattle

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Abstract

Intramuscular fat and collagen content are major factors affecting beef quality, but mechanisms regulating intramuscular adipose and connective tissue deposition are far from clear. Japanese Wagyu cattle are well known for their extremely high marbling. The objective of this study was to evaluate intramuscular fat (IMF) and collagen deposition in the muscle of Wagyu compared to Angus cattle. Animals were managed under the same condition and slaughtered at an averaging 585 ± 12.1 kg of body weight. Samples of *sternomandibularis* muscle were collected from Wagyu (n = 3) and Angus (n = 3) for molecular and histological investigations of adipogenesis and fibrogenesis. With exception of CCAAT enhancer binding protein β (CEBPβ; P = 0.2864), the expression of the adipogenic markers, CCAAT enhancer binding protein α (CEBPα; P = 0.008), peroxisome proliferator-activated receptor γ (PPARγ; P = 0.028) and zip finger protein 423 (Zfp423; P = 0.047) in Wagyu were higher than in Angus muscle, which was consistent with higher IMF deposition in Wagyu (P = 0.007). In addition, more adipocytes and pre-adipocytes were detected intramuscularly in Wagyu cattle. Similarly, fibrogenesis was also enhanced in Wagyu, with a higher expression of fibroblast growth factor II (FGF2; P = 0.028), FGF receptor 1 (P = 0.030), transforming growth factor β (TGFβ; P = 0.028), collagen I (P = 0.012) and collagen III (P = 0.025). Similarly, Wagyu muscle had higher collagen content (P = 0.002) and lower collagen solubility (P = 0.004). In addition, muscle fiber diameter was larger ($P < 0.0001$) in Wagyu than in Angus cattle, suggesting that the total muscle fiber number was lower in Wagyu cattle. These results clearly show that both IMF and collagen contents are enhanced in Wagyu cattle and more adipogenic cells are detected in Wagyu muscle, indicating intramuscular adipogenesis is enhanced in Wagyu compared to Angus muscle.

Keywords: Adipogenesis, Angus, beef, collagen, fibrogenesis, intramuscular fat, Wagyu.

Resumo

O Objetivo deste estudo foi de avaliar a deposição de tecido adiposo intramuscular e colágeno em músculo esquelético de animais da raça Wagyu em comparação com animais da raça Angus. Os animais foram manejados sob mesmas condições durante toda fase de desenvolvimento e foram abatidos com peso corporal médio de 585 ± 12.1 kg. Amostras do músculo *Sternomandibularis* foram coletadas de animais Wagyu (n = 3) e animais Angus (n = 3) para avaliações moleculares e histológicas quanto à adipogênese e fibrogênese. Com exceção do marcador CCAATT *enhancer biding protein* β (CEBPβ; $P = 0.2864$), a expressão dos marcadores para adipogênese CCAAT *enhancer biding protein* α (CEBPα; $P = 0.008$), *peroxisome proliferator-activated receptor* γ (PPARγ; $P = 0.028$) e *zip finger protein 423* (Zfp423; $P = 0.047$) em músclo de animais Wagyu foi maior que em músculo de animais Angus, o que foi consistente com a maior deposição de gordura intramuscular em Wagyu ($P = 0.0007$). Além disso, maior número de adipócitos e pré-adipócitos intramusculares foram detectados em animais Wagyu. De forma semelhante, maior fibrogênese foi observada em músculo Wagyu tendo-se maior expressão do *fibroblast growth factor II* (FGF2; $P = 0.0028$), *fibroblast growth factor receptor 1* (FGF 1; $P = 0.030$), *transforming growth factor β* (TGFβ; $P = 0.028$), *Collagen I* (COL 1; $P = 0.012$) e *collagen III* ($P = 0.025$). Da mesma forma, o músculo de animais Wagyu apresentou maior teor de colágeno total ($P = 0.002$) e menor solubilidade do mesmo ($P = 0.004$). Além disso, o diâmetro de fibra muscular em animais Wagyu foi maior ($P < 0.0001$) em relação à animais Angus, sugerindo que o total de fibras musculares em animais Wagyu fosse menor em comparação a animais da raça Angus. Estes resultados demonstram que além da maior deposição de gordura, há também maior deposição de colágeno intramuscular em animais da raça Wagyu em comparação a animais da raça Angus.

Palavras-chave: Adipogênese, Angus, carne, colágeno, fibrogênese, gordura intramuscular, Wagyu.

Introduction

According to recent surveys of beef producers by the National Cattlemen's Beef Association, marbling and tenderness were identified as the top beef quality problems (Garcia et al., 2008; McKenna et al., 2002). Marbling becomes a major quality problem because of the selection for high lean growth, which results in overall reduction of fat accumulation, including intramuscular fat (Albrecht et al., 2011; Du et al., 2010), which is critical for the palatability of meat (Savell and Cross, 1988; Wheeler et al., 1994). The most optimal solution to this problem is to enhance intramuscular adipose deposition without increasing fat deposition in other depots, which necessitates the understanding of molecular and cellular mechanisms regulating intramuscular fat development. The presence of connective tissue, primarily in the form of collagen fibrils and the degree of intermolecular crosslinking, contribute to the background toughness of meat (Du and Carlin, 2012; Duarte et al., 2011). Both marbling adipocytes and connective tissue fibrils such as collagen are located in the extracellular matrix juxtaposed to skeletal muscle fibers.

While muscle cells, adipocytes, and fibroblasts are originated from the same pool of mesenchymal progenitor cells, myogenic progenitor cells and fibrogenic/adipogenic progenitor cells diverge early during development (Du et al., 2012); fibroblasts and adipocytes share immediate progenitor cells, so called fibro/adipogenic cells, located in the evolving extracellular matrix of primordial muscle fibers (Du et al., 2012a; Joe et al., 2010a; Uezumi et al., 2010; Uezumi et al., 2011). Enhancement of adipogenic differentiation of these progenitor cells increases the number of intramuscular adipocytes while fibrogenic differentiation promotes the synthesis of connective tissue in muscle.

Wagyu cattle produce extremely high marbling; in addition to its production value, it is a good animal model to study adipogenesis and lipid metabolism (Lunt et al., 1993; Radunz et al., 2009; Sturdivant et al., 1992). Indeed, the mRNA expression of adipocyte markers, such as peroxisome proliferator-activated receptor γ (PPARγ), is higher in Wagyu compared to other cattle breeds (Albrecht et al., 2011; May et al., 1994; Yamada et al., 2007, 2009). However, intramuscular adipogenic differentiation, fibrogenesis and the structure of Wagyu cattle have not been studied. Wagyu and Angus cattle have been used for comparison by a number of previous studies (Cameron et al., 1993; Cameron et al., 1994; Chung et al., 2007; Lawrence et al., 2007; Lunt et al., 1993; May et al., 1994; May et al., 1993; Radunz et al., 2009; Rhoades et al., 2007; Wertz et al.,

2002; Xie et al., 1996). As such, we investigated the difference in intramuscular adipogenesis and fibrogenesis between Wagyu and Angus cattle.

Material and methods

Animals and Muscle Tissue Sampling

Wagyu (n = 3) and Angus (n = 3) steers with similar age (24 months) and average body weight of 585.0 ± 12.1 kg were obtained from the Beef Center of Washington State University (WSU) and harvested at WSU-Meat Laboratory. Here, we only use 3 steers per breed because of the large marbling difference between Wagyu and Angus, which provides sufficient power to discern breed effects. Steers were managed under the same condition. Animals were slaughtered by desensitization with a nonpenetrating stunner followed by exsanguination in compliance with USDA regulations. Immediately following exsanguination, samples of *sternomandibularis* muscle (50 g approximately) were collected from each animal, minced and snap frozen in liquid nitrogen promptly.

Frozen samples were then powdered in liquid nitrogen, placed in cryovials and stored at -80°C for qRT-PCR and chemical analysis. Another small portion of samples was fixed in 4% fresh paraformaldehyde immediately after slaughter and then processed for paraffin embedding (Huang et al., 2010). We used *sternomandibularis* muscle because: 1) it is a long, uniform muscle clearly separated from the surrounding connective tissue, reducing sampling variation; 2) it only contains one major muscle bundle, without the presence of intermuscular connective tissue and fat, facilitating the separation of pure intramuscular adipose tissue; 3) it is highly accessible in harvested animals; 4) the biological mechanisms regulating adipogenic differentiation is conserved across mammals not to mention in the different muscles of the same animal; thus, data obtained from *sternomandibularis* muscle is applicable to the *Longissimus dorsi* muscle, which is economically important.

Real-Time Quantitative PCR (qRT-PCR) Analysis

Total RNA (1 μg) was extracted from 0.5 g of powdered tissue samples using Trizol® reagent (Invitrogen, Carlsbad, CA), treated with DNase and reverse transcribed into cDNA using an iScript™ cDNA synthesis kit (Bio-Rad, Hercules, CA). The primer sets used are shown in Table 1. RT-PCR was performed on a CFX connected ™ Real-Time PCR detection system (Bio-Rad) using SYBR Green RT-PCR kit from Bio-Rad

and following cycle parameters: 95°C for 3 min and 40 cycles at 95 °C for 10 s and 60 °C for 30 s. The amplification efficiency was 0.90 to 0.99. After amplification, a melting curve (0.01 C/s) was used to confirm product purity, and the PCR products were electrophoresed to confirm the targeted sizes. Results are expressed relative to 18S using the ΔΔCt method. No differences on the expression of control gene were observed between breeds ($P = 0.4611$) as shown in Figure 1F.

Immunohistochemistry

Fixed tissues were sectioned (5 μm) and deparaffinized in xylene followed by graded rehydration in ethanol (100, 95, 80 and 70%) and distilled water. Antigens were unmasked by high-temperature antigen retrieval [10 min boiling in 10 mM sodium citrate buffer (pH 6.0)] and allowed to cool slowly at room temperature. Sections were then washed twice (5 min each) with TBS/T (0.1% Tween-20, 50 mM Tris-HCl, pH 7.6, and 150 mM NaCl) and incubated in blocking solution consisted of TBS/T and 10% BSA for 1 h at room temperature. Sections were incubated overnight at 4 °C with anti-fatty acid binding protein 4 (FABP4) antibody (1:100 dilution) in TBS plus 1% BSA. Then, sections were washed twice with TBS/T and then incubated with the GFP secondary antibody (1:1,000 dilution) in TBS with 1% BSA for 1 h at room temperature. Both primary and secondary antibodies were purchased from Cell signaling technology, Inc. (Danvers, MA). Sections were then washed three times with TBS for 5 min each, drained and mounted in DAPI (4',6-diamidino-2-phenylindole) mounting medium for immunofluorescent observation using an EVOS® fl fluorescence microscope (AMG, Bothell, WA).

Histochemical and Image Analysis

Muscle tissue sections (5 μm) were deparaffinized, rehydrated and used for Masson's trichrome staining (Foidart et al., 1981), which stains muscle fibers red, nuclei black and collagen blue (Huang et al., 2012). Muscle fiber sizes were measured using the ImageJ® software (NIH) and at least 200 muscle fibers per animal were measured (4 images per section and 5 sections per cattle). Similarly, to calculate FABP4:nuclei ratio and the intercellular space in the muscle sections, 4 images per section and 5 sections per cattle were used for analysis using Image J® software. To measure the intercellular area, fluorescent images were initially converted into 8-bit grayscale images and then submitted to a thresholding adjustment for a better

identification of intercellular area. After that, intercellular space was identified as a region of interest (ROI) by using the tracing tool of the Image J® software and then measured. All images were analyzed in 10X magnification.

Collagen Content and Solubility Analysis

Powdered frozen muscle samples (0.1 g) were placed in 2 ml tubes and 400 µl of distilled water was added in each tube. Tubes were capped and placed in a water bath at 80 °C for 120 min. Tubes were then cooled at 4 °C for 15 min and centrifuged for 20 min at 20,000 x *g* at 2 °C using a 5424 R centrifuge (Eppendorf AG, Hamburg, Germany) and a FA-45-24-11 rotor (Eppendorf AG, Hamburg, Germany). After centrifugation the supernatant and pellet of each sample were transferred to two different test tubes with screw caps and 2 ml of hydrochloric acid (HCl 6N) was added to each tube. The tubes were capped and samples were hydrolyzed at 105 °C for 16 h. After HCl digestion, samples were filtered through filter paper (Whatman® #1) and an aliquot was removed and neutralized to pH6.0 with NaOH. Aliquots were then used for hydroxyproline quantification as described by Woessner Jr (1961). To determine collagen content, hydroxyproline amount was multiplied by 7.52 for the supernatant (soluble collagen) and 7.25 for the residual (insoluble collagen) (Cross et al., 1973). Total collagen (mg collagen/g meat) was defined as soluble collagen plus insoluble collagen and percent solubility was calculated as soluble collagen divided by total collagen multiplied by 100.

Intramuscular Fat Content Analysis

The intramuscular fat content was determined on sub-samples of the 50g-samples obtained at the animal's harvest. Intramuscular fat content was quantified by using ether extract according to the method 920 of AOAC (1990).

Statistical Analysis

Statistical analysis was performed using SAS 9.2 (Statistical Analysis System Institute, Inc., Cary, NC, USA). For the gene expression and intramuscular fat content, a unilateral t-test was used with the alternative hypothesis of Wagyu animals showing higher means than Angus animals for these dependent variables. For muscle fiber diameter, and collagen content and solubility, a bilateral T-test was used. Statistical significances were considered at $P < 0.05$.

Results

Expression of Adipogenic Markers and Intramuscular Fat Content in Wagyu and Angus Muscle

Higher expression of late stage adipogenic markers, CEBPα (P = 0.008) and PPARγ (P = 0.028), was observed in Wagyu compared to Angus muscle (Figure 1AC). Similarly, the expression of early adipogenic marker, Zfp423, was also higher in Wagyu muscle (P = 0.047) (Figure 1D). On the other hand, no difference was observed for the expression of CEBPβ (P = 0.2864) (Figure 1B). In agreement with mRNA expression, the IMF content was higher in Wagyu muscle (P = 0.007, Figure 1F).

Expression of Fibrogenesis Markers, Collagen Content and Solubility in Wagyu and Angus Muscle

The mRNA expression of fibrogenic related growth factors, including FGF2 (P = 0.028), FGF-receptor (P = 0.030), and TGFβ (P = 0.028) was higher in Wagyu compared to Angus muscle (Figure 2ABC). Consistently, the mRNA expression of fibrogenic proteins, including fibronectin (P = 0.002), collagen I (P = 0.012) and collagen III (P = 0.025), was also higher in Wagyu muscle (Figure 3AB). Total collagen content (P = 0.002) was greater in Wagyu muscle, while the collagen solubility was higher (P = 0.0046) in Angus muscle (Figure 3CD).

Distribution of Collagen Fibers and Structure of Wagyu and Angus Muscle and mRNA expression of myogenic markers

The higher collagen content in Wagyu muscle was unexpected, which prompted us to examine the location of connective tissue inside muscle. As shown by trichrome staining, Wagyu muscle fibers were loosely packed with significant amount of spaces and collagen between muscle fibers and bundles, while in Angus muscle, collagen was only detected in perimysiun and muscle fibers were densely packed without much space between muscle fibers (Figure 3DE).

Besides overall difference in collagen fiber distribution and muscle fiber packing density, we also noticed the presence of small and large muscle fibers in Wagyu muscle and the overall size of Wagyu muscle fibers appeared larger (Figure 3). Indeed, the average muscle fiber sizes were greater in Wagyu compared to Angus muscle (P < .0001) and the distribution of muscle fiber sizes was also wider in Wagyu muscle (Figure 4AB). Even though differences were observed for muscle fiber size, no

differences were observed for mRNA of MyoD (P = 0.4285; Figure 4D) and MyoG (P = 0.9041, Figure 4E).

Progenitor cell abundance in Wagyu and Angus Muscle

The adipogenic marker, FABP4, is expressed in both pre-adipocytes and adipocytes. To analyze the location of pre-adipocytes and adipocytes, and their relative abundance, we conducted immunohistochemical analysis with FABP4/DAPI antibody. A number of FABP4/DAPI positive cells (green/blue fluorescence stain) were present in intramuscular space in Wagyu compared to Angus (P = 0.0364) indicating a higher number of progenitor cells in Wagyu muscle (Figure 5).

Discussion

Wagyu Cattle Had Larger Muscle Fibers Compared to Angus Cattle

Wagyu cattle are well known for their extremely high marbling, but the mechanisms leading to the high marbling is unclear. We hypothesized that this high marbling is due to the changes in the differentiation of multipotent progenitor cells during skeletal muscle development. Muscle cells, adipocytes, fibroblasts are derived from the same pool of mesenchymal progenitor cells, which are abundant in the skeletal muscle at early developmental stages but still present at significant levels in mature muscle (Du et al. 2010). While the majority of these cells undergo myogenic differentiation, a significant portion differentiate into common progenitor cells committing to both adipocytes and fibroblasts, so called fibro/adipogenic progenitor cells (Du et al., 2012).

Several studies have shown less muscularity in Wagyu cattle when compared to other breeds such as Angus (Gotoh et al., 2009; Lunt et al., 1993). As such, it is possible that a lower number of muscle fibers are formed in Wagyu cattle because of the great number of mesenchymal progenitor cells that shifts to fibrogenic/adipogenic lineage, leading to a greater formation of adipose and connective tissue. Consequently, it would explain the greater muscle fiber size in Wagyu muscle, as they might have a lower number of muscle fibers leading to their greater hypertrophy. The results of muscle fiber size found in the present study corroborates those found by Albretch et al. (2011) who reported that Wagyu present larger muscle fibers compared to Holsteins possibly as consequence of a reduced number of muscle fibers in Wagyu muscle. However, further

studies are needed to clarify the underlying mechanisms of muscle and associate tissues development in Wagyu cattle.

Wagyu Cattle Had Enhanced Adipogenesis and Fibrogenesis Compared to Angus Cattle

Adipogenesis, especially the terminal differentiation of adipocytes requires the concerted action of PPARγ and C/EBPα, which acts synergistically to induce differentiation-linked gene expression (Rosen et al., 2002). Recently, Zfp423 was demonstrated as a very early marker for adipogenesis, which induces adipogenic commitment and the up-regulation of PPARγ expression. (Gupta et al., 2010; Gupta et al., 2012). In this study we observed higher mRNA expression of Zfp423, PPARγ and C/EBPα in Wagyu muscle, consistent with high marbling observed in Wagyu beef. In addition, a greater expression of FABP4 was detected in spaces between muscle fibers of Wagyu compared to Angus muscle, indicating enhanced proliferation and/or adipogenic differentiation of progenitor cells in Wagyu muscle, leading to the higher density of pre-adipocytes and adipocytes. Because the expression of FGF-2 and its receptor was higher in Wagyu compared to Angus muscle, enhanced proliferation is expected for Wagyu progenitor cells compared to Angus muscle.

Both collagen content and its solubility are important factors contributing to meat tenderness. Intermolecular cross-links provide stability to collagen fibrils (Eyre and Wu, 2005), which increases stiffness and reduces tenderness of meat (Duarte et al., 2011). Fibrogenesis, a process leading to collagen synthesis and cross-linking, is mainly mediated by TGF-β signaling pathway (Chen et al., 2005; Salvadori et al., 2005), which promotes fibrosis via activation of the SMAD signaling pathway (Decologne et al., 2007; Gosselin et al., 2004) to induce the expression of fibrogenic genes, including fibronectin and type I collagen (Kennedy et al., 2008). In Wagyu cattle muscle, the expression of fibrogenic markers, fibronectin, type I collagen and TGF-β were higher when compared to Angus cattle, consistent with enhanced fibrogenesis in Wagyu cattle. In addition, we also detected higher collagen content and lower collagen solubility in Wagyu cattle. In our previous study, we observed that collagen content and cross-linking in skeletal muscle are correlated (Huang et al., 2012). The trichrome staining, which stains collagen fibers blue, shows the widespread distribution of collagen in the space between Wagyu muscle fibers. On the other hand, Angus muscle fibers appear to

be tightly attached to each other and collagen is mostly located between the muscle bundles.

Possible Mechanisms Leading to Enhancement of Adipogenesis and Fibrogenesis in Wagyu Cattle

Mesenchymal fibro/adipogenic progenitor cells contribute to ectopic fat formation in skeletal muscle (Uezumi et al., 2010) and also has the ability to differentiate into fibroblasts (Joe et al., 2010b). Due to the dual differentiation capacity of fibro/adipogenic progenitor cells, their differentiation can be considered as a competitive process if the proliferation of progenitor cells is unchanged. We originally hypothesized that the adipogenic differentiation is enhanced while fibrogenic differentiation is reduced in Wagyu muscle. Unexpectedly, our results show that both adipogenesis and fibrogenesis were enhanced in Wagyu muscle, which strongly suggest that the abundance of progenitor cells is enhanced in Wagyu muscle elevating both fibrogenesis and adipogenesis. Indeed, in this study, we observed that there were larger spaces between muscle fibers which were filled with extracellular matrix in Wagyu muscle, and more abundant intramuscular pre-adipocytes and other cells were detected.

To explain possible mechanisms leading to increased abundance of progenitor cells, we also analyzed the expression of FGF-2 and its receptor, which are known to be critical for the proliferation of progenitor cells (Suga et al., 2009; Yun et al., 2010). In this study, we observed that FGF-2 expression was higher in the muscle of Wagyu compared to Angus cattle, which was consistent with the more abundant intramuscular preadipocytes and adipogenic cells in Wagyu cattle muscle. The higher abundance of adipogenic cells in Wagyu muscle also indicates that the overall adipogenic differentiation in Wagyu was higher compared to Angus cattle muscle which warrant further studies.

Conclusions

Both marbling and connective tissue are increased in Wagyu cattle compared to Angus cattle, while myogenesis appears to be reduced in Wagyu cattle. The enhancement of adipogenesis and fibrogenesis is likely due to the higher abundance of fibro/adipogenic progenitor cells in Wagyu cattle. In addition, the richness of adipogenic cells in Wagyu muscle indicates that adipogenic differentiation of progenitor

cells is higher in Wagyu compared to Angus cattle muscle, which warrants further studies.

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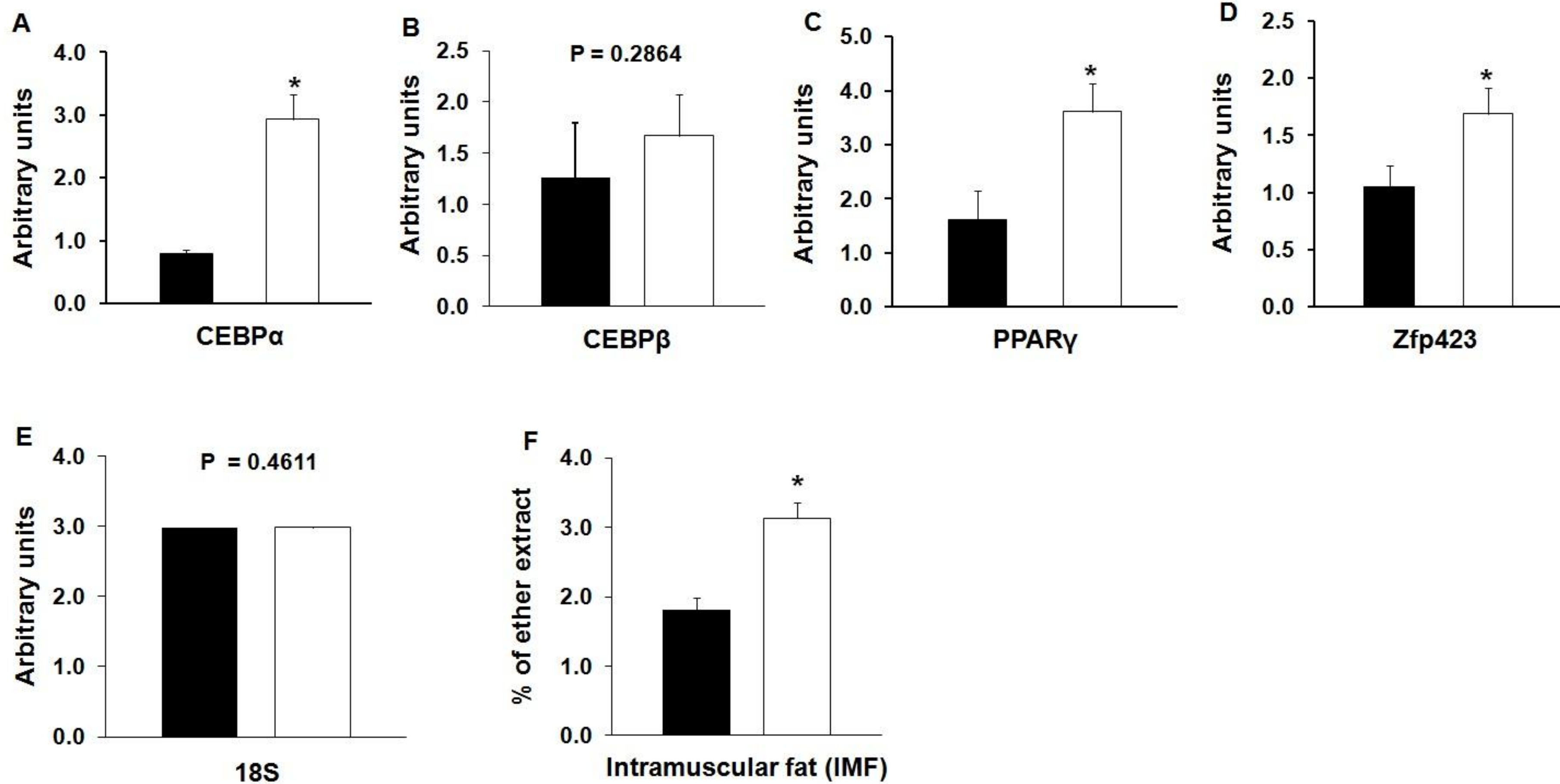

Figure 1. The mRNA expression of C/EBPα (A), C/EBPβ (B), PPARγ (C) and Zfp423 (D), 18S (E), and the intramuscular fat content (F) of Angus (■) and Wagyu (□) *Sternomandibularis* muscle. (* $P< 0.05$; n = 3; Mean ± SE).

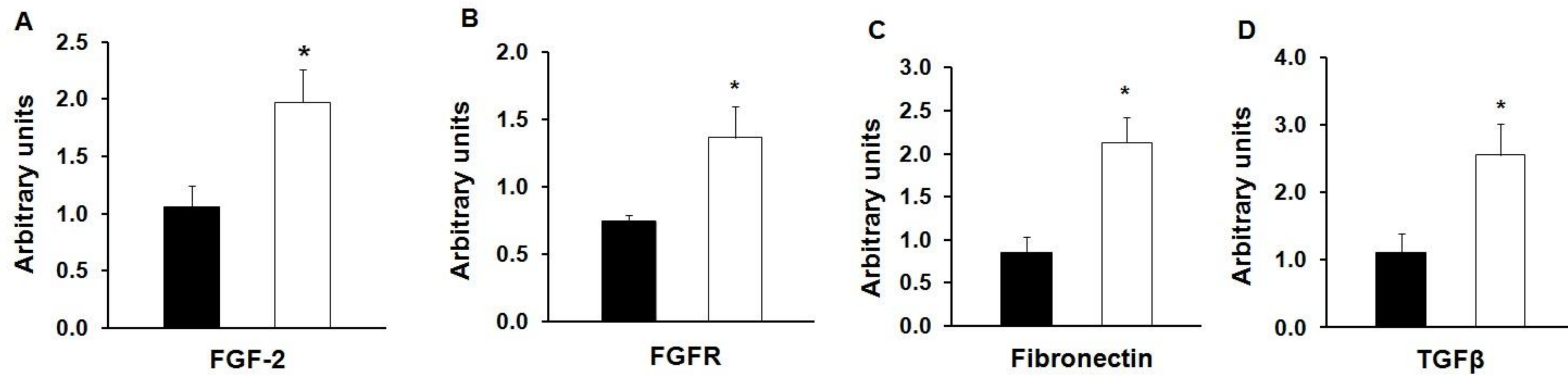

Figure 2. The mRNA expression of FGF-2 (A), FGF receptor (B), fibronectin (C) and TGFβ (D) in *Sternomandibularis* muscle of Angus (■) and Wagyu (□). (*$P < 0.05$; n = 3; Mean ± SE).

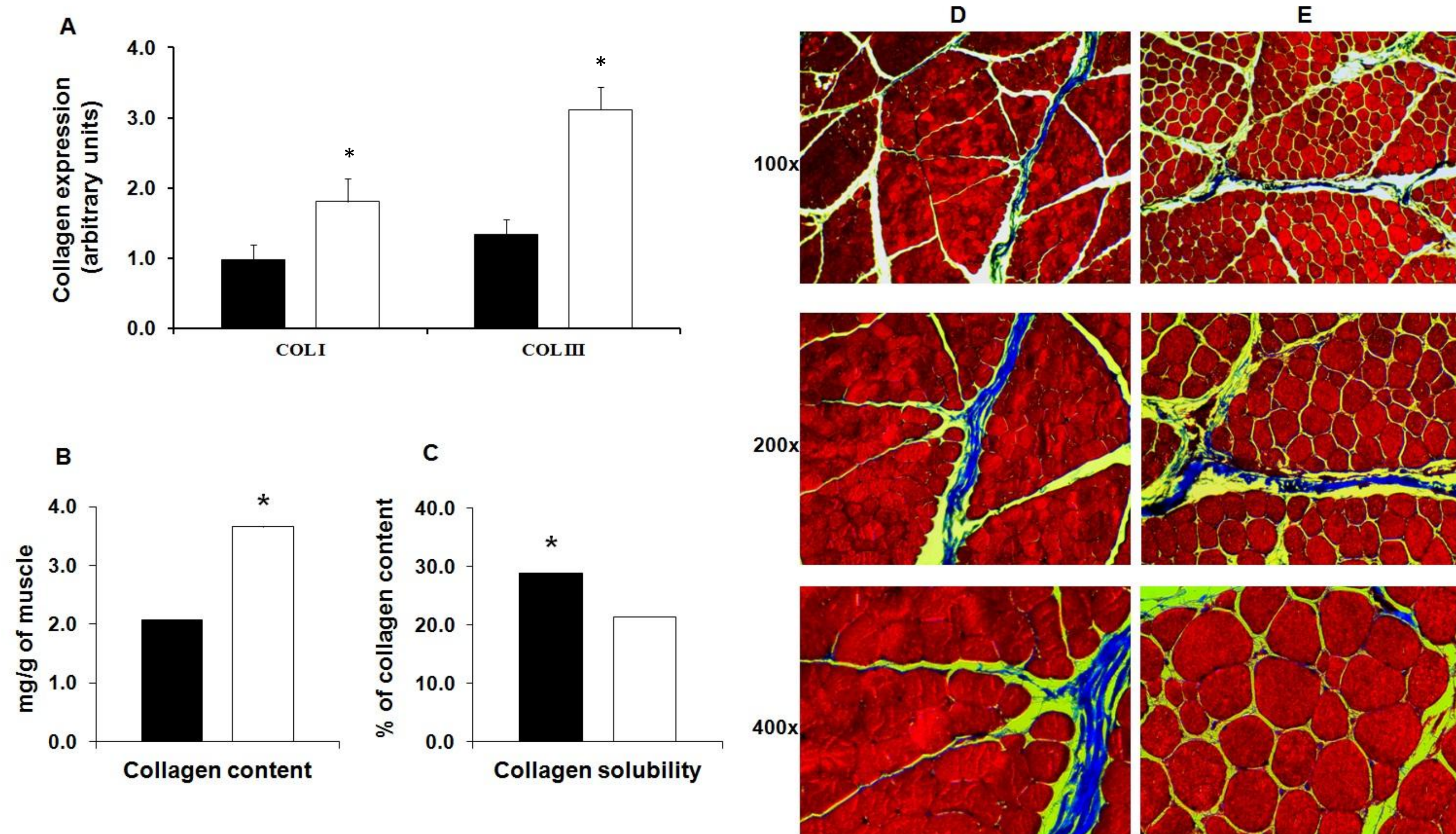

Figure 3. The mRNA expression of Angus (■) and Wagyu (□) *Sternomandibularis* muscle. Collagen I and III (A), collagen content (B), collagen solubility (C), and representative images of muscle stained with Masson's trichrome (D and E). (* $P < 0.05$; n = 3; Mean ± SE).

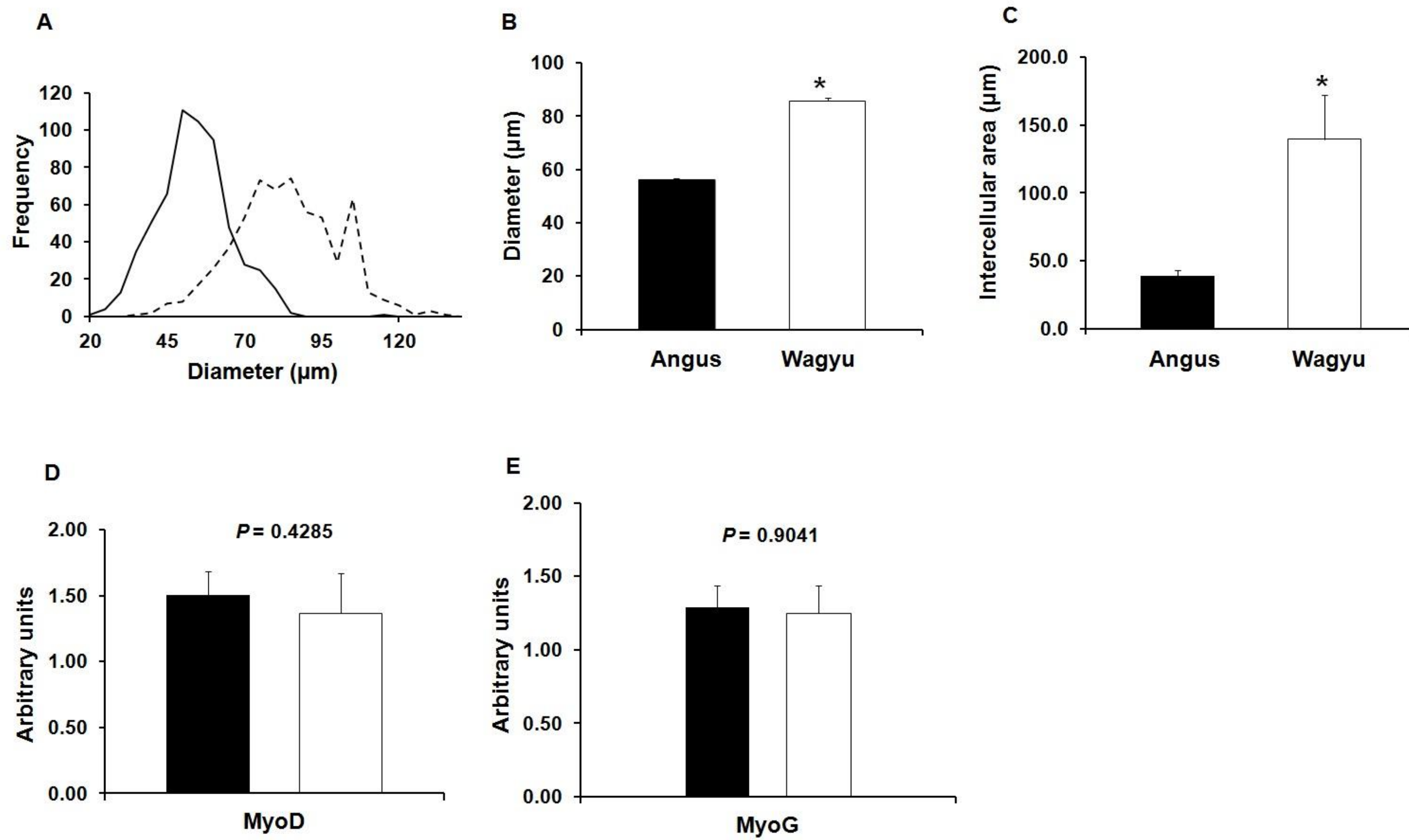

Figure 4. Frequency and size distribution of muscle fibers from Wagyu (dash line) and Angus (solid line) (A); average muscle fiber diameter (B); intercellular space (C); mRNA expression of MyoD (D) and MyoG (E) of Angus (■) and Wagyu (□) *Sternomandibularis* muscle. (*$P < 0.05$; n = 3; Mean ± SE).

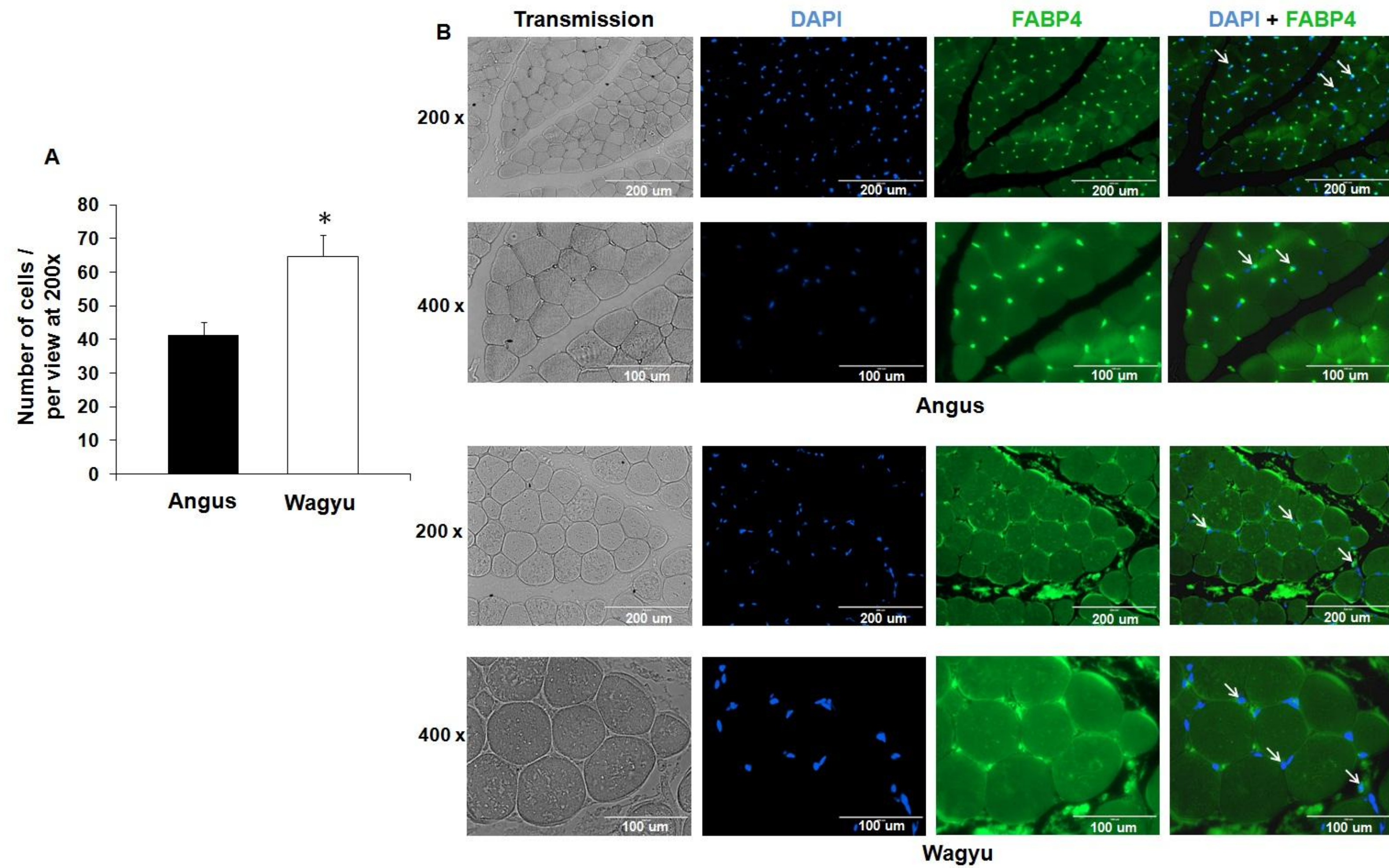

Figure 5. Immunofluorescent staining of Angus and Wagyu *Sternomandibularis* muscle. Fatty acid binding protein (FABP4) stained green and nuclei counterstained with DAPI (4',6-diamidino-2-phenylindole), blue (A). Progenitor cells abundance (B). (*$P < 0.05$; n = 3; Mean ± SE).

Chapter II

Effects of maternal nutrition on development of gastrointestinal tract of bovine fetus at different stages of gestation

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Abstract

This study was developed aiming to evaluate the effects of maternal feed-restriction on development of gastrointestinal tract (GIT) of bovine fetus at different gestational stages. Feed-restricted cows were fed 1.2 times the maintenance level while the control group was fed ad libitum. Pregnant cows were slaughtered at 136, 189, 239, and 269 days of gestation and gastrointestinal tracts of the fetuses were evaluated. No effects of maternal nutrition on body weight (P = 0.17) and body length (P = 0.13) of the fetuses were observed. No major effects of feed restriction on GIT mass of the fetuses were observed (P = 0.51). However, the weight of small intestine per unit of body weight was 11.24 % greater (P =0.04) in fetuses from restricted dams. Additionally, the length of small intestine and its villi were 12.93 % and 16.44 % respectively greater (P < .001) in fetuses from restricted dams compared to those from non-restricted dams. These data indicates that maternal feed-restriction does not affect the development of most of fetal gastrointestinal parts besides small intestine which in turn increases its surface area as a response of maternal feed restriction.

Keywords: cattle, feed-restriction, fetal programming, Nellore, pregnancy, small intestine

Resumo

O presente trabalho foi realizado com objetivo de avaliar os efeitos da nutrição materna sobre o desenvolvimento do trato gastrintestinal de fetos bovinos em diferentes estágios da gestação. Dois grupos de vacas gestantes foram aleatoriamente distribuídos em dois tratamentos alimentares em que um grupo foi alimentado *ad libitum* e outro com alimentação restrita em que as vacas foram alimentas a 1,2 x o nível de mantença. As vacas foram abatidas em quatro tempos de gestação (136, 189, 239, 270 dias) sendo que para cada período de gestação avaliada obteve-se o mesmo número de vacas dos grupos alimentares. Não houve efeito da nutrição materna sobre o peso corporal (P = 0.17) e comprimento corporal (P = 0.13) dos fetos. A restrição alimentar materna durante a gestação não afetou o peso do trato gastrintestinal dos fetos (P = 0.51). Entretanto, o peso do intestino delgado dos fetos por unidade de peso corporal foi 11.24% maior(P = 0.04) em fetos oriundos de matrizes submetidas a restrição alimentar. Além disso, o comprimento do intestino delgado bem como o tamanho de suas vilosidades foi 12.93% e 16.44% respectivamente maior (P < 0.001) em fetos oriundos de vacas sob-restrição alimentar em comparação aos fetos oriundos de vacas alimentadas *ad libitum*. Os dados sugerem que a restrição alimentar materna durante a gestação não afeta o desenvolvimento da maioria dos compartimentos que compõe o trato gastrintestinal fetal, com exceção do intestino delgado o qual por sua vez aumenta a sua superfície de contato como resposta à restrição alimentar materna.

Introduction

During the early phase of fetal stage critical events for normal conceptus development occur including differentiation, vascularization, fetal organogenesis, and placental development (Funston et al., 2010). Several studies have shown that fetuses from dams subjected to nutrient restriction during early to midgestation have decreased growth of the gastrointestinal tract (Harding et al., 1985; Avila et al., 1989; Trahair et al., 1997; Wang et al., 2008), and even with postnatal nutritional intervention, the suboptimal growth causes permanent changes in gastrointestinal functions such as epithelial permeability (Trahair et al., 1997).

Fetal growth restriction due to maternal nutrition has been reported as a problem in livestock production (Wu et al., 2006; Du et al., 2010) since a variety of production conditions may lead to a scenario of fetal growth restriction. As an example, in tropical regions where beef cattle are raised mainly in grazing systems, pregnant cows usually experience feed restriction during the mid gestation period which overlaps with a season of low quantity and quality of forage (Duarte et al., 2012). Therefore, since in cattle the absorption of intact macromolecules such as immunoglobulin across the intestinal epithelium is possible for approximately 24 hours after the calf is born, the well development of gastrointestinal tract during intrauterine stages is crucial to reduce neonatal morbidity and mortality.

Moreover, since the gastrointestinal tract serves as the main site for nutrient absorption, the changes on the development of gastrointestinal tract at the fetal stage may permanently affect the offspring performance and efficiency of nutrient utilization (Wu et al., 2006; Wang et al., 2008) impairing efficiency of animal production. However, there is a very little information regarding this topic using beef cattle as a model (Meyer et al., 2010). The objective of this study was to evaluate maternal nutrition effects on the development of gastrointestinal tract of bovine fetus at different stages of gestation.

Material and methods

Animals and management

All animal care and handling procedures were approved by the Animal Care and Use Committee of the Department of Animal Science of the Universidade Federal de Viçosa, Brazil.

Thirty-two multiparous Nellore cows with average initial body weight of 451 ± 67 (mean ± SE) kg, age of 5.6 ± 1.9 years and body condition score of 4.6 ± 1.1 (1 to 9 scale) were used. Pregnancy was detected by ultrasound 25 days after mating and the day of mating was considered as day 0 of pregnancy. On day 27 of gestation cattle were confined in collective pens (48 m², 6 cows per pen) with individual electronic head gate system (Kloppen Soluções Tecnológicas, Pirassununga, SP, Brazil) for adaptation to individual feeders. At day 47 of gestation, cows were randomly assigned into two groups with different feeding levels where half of the cows (n = 16) were fed at 1.2 times maintenance (NRC, 2000) and the other half were fed ad libitum (n = 16). The restricted feeding level used was estimated to be enough to maintain the pregnancy of the dam throughout the experiment avoiding abortion at any period of gestation. Feed intake of feed-restricted dams was 10.8 ± 1.5 g of dry matter/kg of shrunk body weight (animal's equivalent weight after overnight fast without feed; NRC, 2001) while for dams fed at libitum the feed intake was 16.0 ± 2.0 g of dry matter/kg of shrunk body weight. Cows were fed the same diet with differences only in the feeding level.

Every 28 d cows were weighed in the morning before feeding and after a 16h solid fast to obtain the shrunk body weight and the feed intake was adjusted based on values of shrunk body weight to maintain the feed-restriction throughout the entire gestational period.

Experimental diets consisted of 64.8% of total digestible nutrients and 13.5% of crude protein on dry matter (DM) basis and composed of corn silage (84.3% DM basis), ground corn (8.5% DM basis), soybean meal (5.1% DM basis), urea/ammonium sulphate (1.4% DM basis) and mineral mixture (0.7 % DM basis). The mineral mixture was composed of 15% calcium, 9% phosphorus, 0.53% zinc, 0.13% manganese, 0.2% copper, and 100 mg/kg of cobalt.

To evaluate the effects of maternal feed-restriction on development of gastrointestinal tract at different stages of gestation, pregnant cows were slaughtered at four gestational periods. Each feeding level group (maintenance and ad libitum) was randomly divided into four groups with four cows in each group to be slaughter at 136, 189, 239, and 269 days of gestation. Cows were slaughtered at Universidade Federal de Viçosa abattoir using a captive bolt stunning and exsanguination. Pre-harvest handling was in accordance with good animal welfare practices, and slaughtering procedures followed the Sanitary and Industrial Inspection Regulation for Animal Origin Products (Brasil, 1997).

Tissue sample and data collection

After the exsanguination the gravid uterus was immediately collected and fetus was removed. The dissection of the fetus and isolation of the gastrointestinal tract was performed similarly to that described by Meyer et al., (2010). Briefly, Fetuses were dissected and the whole gastrointestinal tract was collected and gently stripped of fat and digesta. The stomach complex was isolated from the esophagus and the intestine at the pyloric valve and divided into reticulum-rumen, omasum, abomasum and each component was gently emptied and weighed. Small and large intestines were isolated and weigh and length was recorded separately. Then, small intestine was divided into duodenum, jejunum and ileum similarly to that described by Soto-Navarro et al. (2004) as it follows. The duodenum was identified as the segment from the pylorus to a point directly adjacent to the entry of the gastrosplenic vein into the mesenteric vein. The jejunum was the segment from the caudal end of the duodenum to the junction of jejunum and ileum. This junction was determined by measuring 15 cm up the mesenteric vein from the convergence of the mesenteric and ileocecal veins and then up the mesenteric arcade to the point of intestinal intersection. From this point, a 150-cm measurement was made caudally down the small intestine, which was identified as the terminal end of the jejunum and the beginning of the ileum. The ileum measurement was terminated at the ileocecal junction.

Small intestine villi morphology

Tissue samples from jejunum, duodenum and ileum were fixed in fresh 10% (w/v) formalin in phosphate buffer (pH 7.4) and embedded using the HistoResin Mounting Kit (Leica®, Heidelberg, BW, Germany). Fragments of small intestine were carefully embedded to allow the presence of great number of villi longitudinally oriented in each section. Sections were cut at 3μm, stained with toluidine blue, and observed under light microscopy. For each segment of small intestine (duodenum, jejunum and ileum) of each animal only intact villus with evident lamina propria, base and top were selected to measure the villi length. Photomicrographs were taken with a CMOS digital camera (Biocam GmbH®, BAV, Germany) coupled to an Olympus BX50 light microscope (Center Valley, PA, United States). Ten fields and ten villi per field were randomly selected to measure the villus length. Images were analyzed by using the ImageJ® software (National Institutes of Health, USA) and a total of 300 villi per

animal (3 segments x 10 fields x 10 villi per field) were measured. Measurements of curved villus were performed by using the segmented line selection and analyzed by the straighten tool of ImageJ®.

Statistical analysis

Data was analyzed through a model including the fixed effects of gestational period, maternal nutrition, and their interaction as described below:

$$Y_{ijk} = \mu + D_i + G_j + (D * G)_{ij} + e_{ijk}$$

Where:

D_i i^{th} level of the fixed effect of Diet

G_j j^{th} level of the fixed effect of Gestation Period

e_{ijk} random error associated with Y_{ijk}

This model was used to all studied response variables besides villi length. In addition to all the effects in the model, the fixed effect covariate of body length of the fetus was included in order to adjust the values of villi length. For each response variable, outliers were removed in order to achieve normality using Shapiro-Wilks test at $\alpha = 0.05$. Least square means were estimated for all effects and compared using Tukey's method at $\alpha = 0.05$. All statistical procedures were performed using the MIXED procedure from SAS 9.2 (Statistical Analysis System Institute, Inc., Cary, NC, USA).

Results

At the beginning of the experiment (47 days of gestation) cows had similar average shrunk body weight ($P = 0.52$) which was 437 ± 15 kg for the non-restricted cows and 438 ± 14 kg for feed-restricted cows. However, as a result of the feeding levels applied, shrunk body weight was different ($P = 0.0028$) among feeding level groups with average of 563 ± 16 kg for non-restricted cows and 482 ± 16 kg for feed-restricted cows. This was due to the difference of shrunk body weight daily gain ($P < .0001$) among feeding level groups which was 0.86 ± 0.04 kg/day for non-restricted cows and 0.26 ± 0.04 kg/day for feed-restricted cows. Additionally, comparisons within each period of gestation evaluated showed that cows fed ad libitum had greater ($P <$

.0001) shrunk body weight daily gain than feed-restricted cows at all gestational periods evaluated (Table 1), which demonstrates that cows fed 1.2 times maintenance were feed-restricted during the entire gestational period. Together, these data clearly shows the effectiveness of feeding restriction applied in this study.

Significant interaction among feeding level and days of gestation was observed (*P* = 0.02) for body weight of the fetuses. The interaction analysis showed that only at 269 days of gestation fetuses from dams fed ad libitum were heavier (30.47 ± 1.03 kg) than those from feed-restricted dams (23.67 ± 1.03 kg) and no differences were observed in the body weight of fetuses from restricted and non-restricted dams at 136, 189, and 239 days of gestation. Significant interaction (*P* = 0.02) among feeding level and days of gestation was also observed for body weight:body length ratio (Table 2). The difference in body weight of the fetuses observed only at 269 days of gestation is likely because the most fetal growth occurs during the last three months of gestation (Ferrell et al., 1976).

The body length of the fetuses were not affected (*P* = 0.13) by maternal nutrition. However, as expected, the body length of the fetuses increased (*P* <.0001) as the gestation advanced (Table 2).

There was no effect of maternal nutrition (*P* = 0.05) on the absolute weight of gastrointestinal tract, stomach complex (*P* = 0.79), reticulum-rumen (*P* = 0.92), omasum (*P* = 0.18), abomasum (*P* = 0.44), small intestine (*P* = 0.32) and large intestine (*P* = 0.65) (Table 2). When expressed as a function of body weight, no effects of maternal nutrition were found on gastrointestinal tract (*P* = 0.1126), stomach complex (*P* = 0.97), reticulum-rumen (*P* = 0.97), omasum (*P* = 0.52), abomasum (*P* = 0.82) and large intestine (*P* = 0.54). However, the small intestine expressed as function of body weight was greater (*P* = 0.04) in fetuses from restricted dams compared to those from non-restricted dams (Table 2). Similarly, the lengths of small intestine (*P* = 0.0017) and its villi (*P* < .0001) were increased in fetuses from restricted dams (Table 2).

The absolute weight of gastrointestinal tract and abomasum increased (*P* < .0001) with days of gestation but was unaffected (*P* = 0.58) when analyzed as a function of body weight (Table 3). The absolute weight of stomach complex (*P* < .0001), reticulum-rumen (*P* < .0001), and omasum (*P* < .0001) increased with days of gestation. However, the weight per unit of body weight of stomach (*P* = 0.0002), reticulum-rumen (*P* < .0001), and omasum (*P* < .0001) decreased with days of gestation (Table 3). The absolute weight of abomasum (*P* < .0001) and small intestine (*P* < .0001) increased

with days of gestation. However, no differences were observed among days of gestation for weight of abomasum ($P = 0.36$) and small intestine ($P = 0.07$) when analyzed as a function of body weight (Table 3).The lengths of small intestine ($P < .0001$) and its villi ($P < .0001$) increased with days of gestation (Table 3).

Discussion

The phase of a rapid growth of gastrointestinal tract occurs at the third trimester of gestation in species that has a long gestational period (Weaver et al., 1991). However, no effects of maternal feed restriction were observed on the weight of the gastrointestinal tract and its components at any of the gestational stages evaluated. According to Meyer et al., (2010) organogenesis mainly occurs at early to mid-gestation. Thus, in the current study when dams were feed-restricted (mid to late-gestation) the fetal organogenesis may had already been accomplished and the gastrointestinal tract was less vulnerable to changes due to maternal nutrient restriction. Additionally, it has been suggested that even though the dam is undernourished the placental system can compensate to provide the fetus adequate amount of nutrients mainly by increasing the number of caruncles (Bassett, 1991; Clarke et al., 1998) which also possibly have contributed for the lack of effects of maternal feed restriction on fetal gastrointestinal tract mass.

Since the gastrointestinal tract is responsible for nutrient absorption it must have an adequate surface area and therefore, intestinal length is an important characteristic for animal performance (Trahair et al., 1997). Even though no differences was observed among dietary treatments for gastrointestinal tract mass, a greater length of small intestine in fetus of restricted dams was observed, which likely indicates a more efficient growth to compensate the low supply of nutrients. Because the mucosal epithelium is the most labile wall component in the small intestine, intrauterine insults such as feed restriction can alter growth of mucosal components on intestinal the surface area including the size and density of the villi (Trahair et al., 1997). It has been reported previously that intestine from fetuses of restricted dams has an increase in proliferation and vascularization (Meyer et al., 2010) which would explain not only the greater length of small intestine of fetuses from restricted dams but also their greater villi length (Figure 1). Additionally, the lack of correlation ($P = 0.46$) between villi length and body length of fetuses and the use of body length as covariate for villi length

indicates that differences in villi length occurred only as a result maternal feed-restriction (Table 2).

Hammer et al. (2011) have shown that offspring from undernourished dams had increased immunoglobulin transfer compared to non-restricted fed dams. Therefore, from the results observed in the current experiment it can be inferred that fetal gastrointestinal system may be programmed in nutrient restricted animals to be more efficient in extracting nutrients, specifically large molecules like immunoglobulins immediately postnatal (Funston et al., 2010), although this has not been determined in this trial.

Immature ruminants are functionally non-ruminants. To become functional, the stomach compartments, mainly the rumen, must be inoculated with microorganisms, including bacteria, fungi, and protozoa which usually occurs after birth when calves contacts solid feedstuff. Therefore, the lack of effects of maternal feed restriction on the weight of stomach complex and its components may be due to the non-functionality of these compartments at fetal stage since, theoretically, there is no need to expand the surface area of the stomach to increase nutrients absorption as occurs in the small intestine.

With regard to days of gestation, the data of the current study show a disproportionally growth between the stomach complex and the body of the fetus through the pregnancy period (Table 3). When analyzed separately, the weight the stomach parts per unit of body weight of the rumen-reticulum and omasum decreased as the day of gestation increased, suggesting that these compartments have a faster growth at early stages of gestation. Moreover, the decrease of proportional weights (g/kg of body weight) might be due to the non-functionality of the stomach complex at intrauterine stages of life as stated previously. Conversely, the abomasum and small intestine grew proportionally to the fetus as no differences were observed for the weight of the abomasum and small intestine per unit of body weight throughout the gestational period (Table 3). As expected, the length of small intestine and its villi also increased as the days of gestation increased (Table 3).

For a better visualization of the development of different compartments of fetal gastrointestinal tract throughout the gestational period, weights of gastrointestinal tract parts at parturition (285 d) were estimated by extrapolation of a logistic non-linear regression (Koong et al., 1975). The values obtained were 116.1 g, 38.6 g, 126.1 g, 354.5 g and 211.7 g for the reticulum-rumen, omasum, abomasum, small intestine and

large intestine, respectively. Weights of these compartments at different days of gestation were then expressed as percentages of their predicted final weight and presented graphically indicating that reticulum-rumen and omasum development preceded abomasums development (Figures 2 and 3). Similarly, the small intestine development also preceded the large intestine development. Together, these results support the importance of the abomasum in addition to the small intestine as the main source of nutrients absorption for ruminant animals possessing development priory when compared to the other gastrointestinal compartments.

Conclusion

The data suggests that maternal nutrient restriction affects the development of fetal small intestine without major effects on the development of gastrointestinal tract as a whole. Fetal small intestine increase surface area as a response of maternal feed restriction. So far, is not possible to define strategies of supplementation during the pregnancy visioning a better development of the fetal gastrointestinal tract which requires further studies to clarify cellular and molecular mechanisms responsible for changes on fetal gastrointestinal tract due to maternal nutrition.

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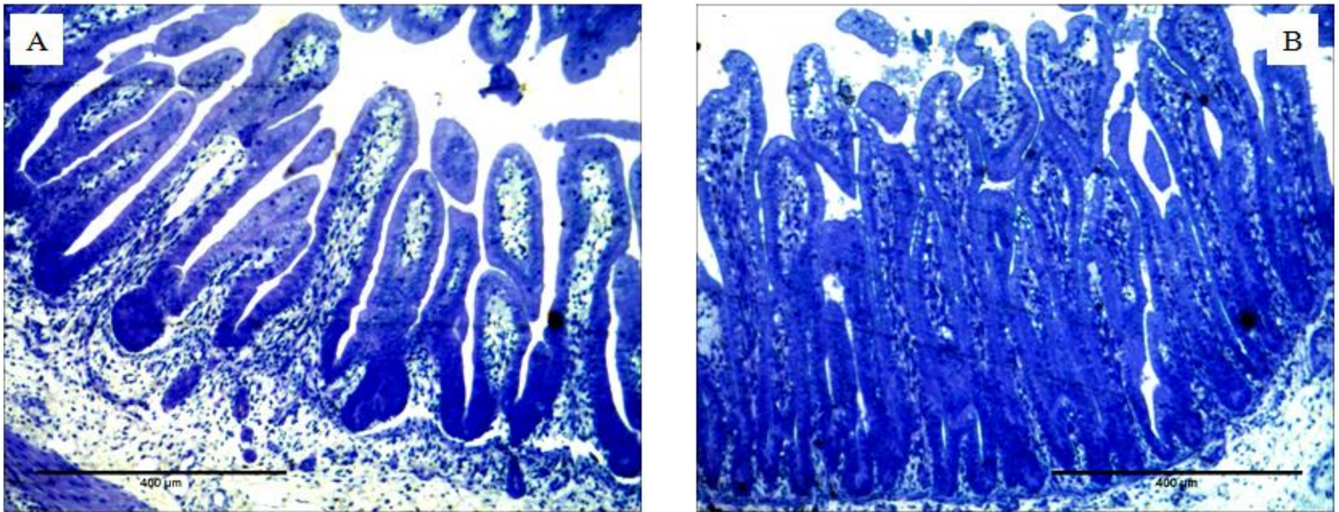
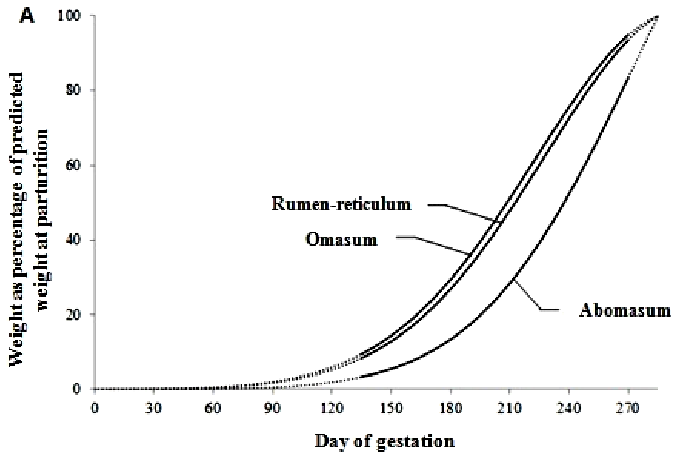


Figure 1: Representative photomicrograph (10x) showing a contrast of small intestine villi of bovine fetus from non-restricted (A) and feed-restricted (B) dams at 190 days of gestation.



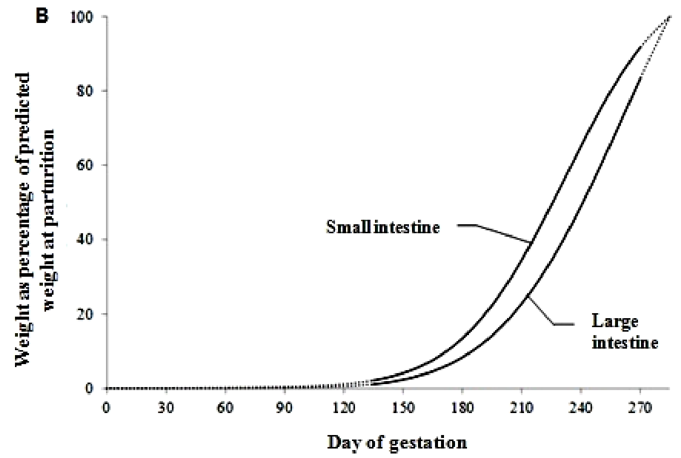


Figure 2: Relationship among weights of the reticulum-rumen, omasum and abomasum, (A) and small and large intestine (B), expressed as percentages of predicted weights at parturition and days of gestation. Solid lines represent actual data range and dash lines are extrapolated from the data.

Table 1. Shrunk body weight daily gain of pregnant Nellore cows fed at different days of gestation and feeding level

Feeding level	Days of gestation			
	136	189	239	269
	Shrunk body weight gain, kg/day			
Ad libitum	0.93 ± 0.09	0.79 ± 0.09	0.85 ± 0.09	0.87 ± 0.09
Maintenance	0.29 ± 0.09	0.18 ± 0.09	0.32 ± 0.09	0.29 ± 0.09
P - value	<0.001	<0.001	<0.001	<0.001

Table 2. Effects of maternal nutrition on development fetal gastrointestinal tract (Mean ± SE).

Item	Feeding level		*P* - value	
	Ad libitum	Maintenance	Diet	Gestation x Diet
Final SBW of the cows, kg	575.00 ± 16.95[a]	479.72 ± 16.95[b]	0.0006	0.4069
Fetus BW, kg	13.75 ± 0.38	13.65 ± 0.38	0.8499	0.0187
Fetus body length, cm	63.58 ± 1.20	60.92 ± 1.20	0.1318	0.2468
Fetus BW/body length ratio	0.19 ± 0.19	0.18 ± 0.19	0.3406	0.0254
Gastrointestinal tract, g	333.31 ± 13.77	346.32 ± 13.77	0.5112	0.5364
g/kg of BW	25.23 ± 0.75	26.98 ± 0.75	0.1126	0.6681
Total stomach, g	119.04 ± 5.16	117.10 ± 5.16	0.7921	0.2834
g/kg of BW	11.41 ± 0.38	11.42 ± 0.38	0.9726	0.6084
Reticulum-rumen, g	55.77 ± 2.48	55.43 ± 2.48	0.9226	0.4525
g/kg of BW	5.18 ± 0.19	5.19 ± 0.19	0.9701	0.2043
Omasum, g	19.32 ± 1.07	17.21 ± 1.11	0.1826	0.2011
g/kg of BW	1.73 ± 0.11	1.83 ± 0.11	0.5197	0.7910
Abomasum, g	42.17 ± 1.07	40.97 ± 10.7	0.4395	0.2925
g/kg of BW	3.09 ± 0.15	3.14 ± 0.15	0.8238	0.4728
Small intestine, g	157.74 ± 7.11	167.73 ± 6.84	0.3217	0.8721
g/kg of BW	11.21 ± 0.46	12.63 ± 0.46	0.0399	0.3712
Large intestine, g	59.40 ± 3.51	61.65 ± 3.38	0.6500	0.3910
g/kg of BW	4.01 ± 0.19	4.18 ± 0.19	0.5394	0.9758
Small intestine length, cm	671.63 ± 20.01	771.43 ± 20.01	0.0017	0.2745
Small intestine villi length, µm	356.37 ± 10.22[b]	430.36 ± 10.22[a]	<.0001	0.6474

Table 3. Development of fetal gastrointestinal tract at different stages of gestation (Mean ± SE)

Item	Days of gestation				*P* - value	
	135	190	240	270	Gestation	Gestation x Diet
Final SBW of the cows, kg	555.63 ± 23.97	551.25 ± 23.97	508.75 ± 23.97	493.81 ± 23.97	0.2046	0.4069
Fetus BW, kg	1.50 ± 0.73	6.32 ± 0.73	21.10 ± 0.79	27.07 ± 0.73	<.0001	0.0187
Fetus body length, cm	32.45 ± 1.70[d]	52.56 ± 1.70	77.37 ± 1.70	86.62 ± 1.70	<.0001	0.2468
Fetus BW/body length ratio	0.05 ± 0.01	0.12 ± 0.01	0.25 ± 0.01	0.31 ± 0.01	<.0001	0.0254
Gastrointestinal tract, g	40.47 ± 20.22[d]	158.69 ± 18.72[c]	510.05 ± 18.72[b]	650.06 ± 20.22[a]	<.0001	0.5364
g/kg of BW	27.35 ± 1.06[a]	25.34 ± 1.06[a]	25.92 ± 1.06[a]	25.81 ± 1.06[a]	0.5817	0.6681
Total stomach, g	19.51 ± 7.30[d]	67.64 ± 7.30[c]	164.24 ± 7.30[b]	220.89 ± 7.30[a]	<.0001	0.2834
g/kg of BW	13.58 ± 0.54[a]	11.89 ± 0.54[ab]	10.06 ± 0.54[b]	10.13 ± 0.54[b]	0.0002	0.6084
Reticulum-rumen, g	11.36 ± 3.51[d]	34.63 ± 3.51[c]	78.79 ± 3.51[b]	97.63 ± 3.51[a]	<.0001	0.4525
g/kg of BW	7.59 ± 0.26[a]	5.51 ± 0.26[b]	4.00 ± 0.26[c]	3.65 ± 0.26[c]	<.0001	0.2043
Omasum, g	3.72 ± 1.51[c]	12.97 ± 1.51[b]	26.70 ± 1.51[a]	29.68 ±1.63[a]	<.0001	0.2011
g/kg of BW	2.49 ± 0.16[a]	2.08 ± 0.16[a]	1.36 ± 0.16[b]	1.20 ± 0.16[b]	<.0001	0.7910
Abomasum, g	4.43 ± 1.46[d]	20.04 ± 1.46[c]	54.66 ± 1.57[b]	87.16 ± 1.57[a]	<.0001	0.2925
g/kg of BW	2.89 ± 0.21[a]	3.18 ± 0.21[a]	2.99 ± 0.21[a]	3.39 ± 0.21[a]	0.3557	0.4728
Small intestine, g	16.18 ± 9.67[d]	69.24 ± 9.67[c]	258.85 ± 9.67[b]	306.65 ± 10.44[a]	<.0001	0.8721
g/kg of BW	10.93 ± 0.65[a]	11.14 ± 0.65[a]	13.18 ± 0.65[a]	12.42 ± 0.65[a]	0.0676	0.3712
Large intestine, g	5.07 ± 4.78[d]	21.81 ± 4.78[c]	86.96 ± 4.78[b]	128.25 ± 5.16[a]	<.0001	0.3910
g/kg of BW	3.45 ± 0.27[b]	3.42 ± 0.27[b]	4.38 ± 0.27[ab]	5.14 ± 0.27[a]	0.0003	0.9758
Small intestine length, cm	361.54 ± 28.30[d]	612.13 ± 28.30[c]	859.25 ± 28.30[b]	1053 ± 28.30[a]	<.0001	0.2745
Small intestine villi length, µm	323.08 ± 54.08	376.99 ± 22.34	414.95 ± 30.18	458.45 ± 45.02	<.0001	0.8323

[a, b, c, d] Means within a row and effect lacking a common superscript letter differ significantly ($P > 0.05$).

Chapter III

Effects of pregnancy and feeding level on carcass and meat quality traits of Nellore cows

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Abstract

Carcass and meat quality traits of 16 pregnant and 5 non-pregnant cows fed at 1.2 times maintenance and 16 pregnant and 6 non-pregnant fed ad libitum were evaluated. Pregnancy did not affect final body weight (FBW; $P = 0.0923$), cold carcass yield (CCY; $P = 0.0513$), longissimus muscle area (LMA; $P = 0.8260$), rib fat thickness (RFT; $P = 0.1873$) and shear force (WBSF; $P = 0.9707$). A lower FBW ($P = 0.0028$), LMA ($P = 0.0048$) and RFT ($P = 0.0001$) were observed in feed restricted cows. However, no differences were found for CCY ($P = 0.7243$) and WBSF ($P = 0.0759$) among feeding level groups. These data suggests that carcass and meat quality traits are not affected by pregnancy status in Nellore cows. Moreover, although cows experiencing feed restriction did have reduced deposition of subcutaneous fat and lean tissue, there were no major impacts on meat quality traits.

Keywords: Ad libitum feeding, beef, gestation, maintenance, R-value, sarcomere length

Resumo

No presente trabalho foram avaliadas características de carcaça e da carne de 16 vacas gestantes e 5 vacas não gestantes alimentadas a 1,2 x nível de mantença e 16 vacas gestantes e 6 vacas não gestantes alimentadas *ad libitum*. A gestação não afetou o peso corporal final (PF; $P = 0.0923$), o rendimento de carcaça fria (RCF; $P = 0.0513$), a área de olho de lombo (AOL; $P = 0.8260$), espessura de gordura subcutânea (EGS; $P = 0.1873$) e força de cisalhamento (FC; $P = 0.9707$). Vacas em restrição alimentar apresentaram menor PF ($P = 0.0028$), AOL ($P = 0.0048$) e EGS ($P = 0.0001$). Entretanto, não foram observadas diferenças quanto a RCF ($P = 0.7243$) e FC ($P = 0.0759$) entre os animais submetidos a diferentes níveis de alimentação. Os dados sugerem que as características de carcaça de vacas Nelore não são afetadas pela gestação. Além disso, embora vacas em restrição alimentar tenham apresentado redução da deposição de gordura subcutânea e tecido muscular na carcaça, não foram observados alterações no aspecto qualitativo da carne destes animais.

Palavras-chave: Alimentação, carne, comprimento de sarcômero, gestação, mantença, valor-R

Introduction

Fluctuation of beef prices is commonly seen in the beef industry and rapid price declines can lead beef producers to harvest dams to reduce feeding costs. Harvest of cull female cattle has as substantial contribution to total beef production world-wide (Graham & Price, 1982; Wooten, Roubicek, Marchello, Dryden, & Swingle, 1979; Wythes, Shorthose, Fordyce, & Underwood, 1990) and in many cases cows are pregnant at slaughter (Macedo, et al., 2007; Wythes, et al., 1990). As pregnancy is marked by substantial homeorhetic controls necessary to support the physiological state such as rates in lipogenesis and lipolysis, carcass and meat quality traits are potentially altered due to pregnancy (Bauman & Bruce Currie, 1980). Even though pregnant cows are often used in beef production, little information is known about the quality of meat from pregnant cows (Wythes, et al., 1990).

In tropical regions such as Brazil, beef cattle production is based on pastures, which represent the lowest cost feed source for ruminant animals. Consequently, the distribution and seasonal variation in quantity and quality of forage is one of the main problems faced by beef producers in these areas (Sampaio, et al., 2010). Considering that the breeding season in most of grazing production systems in Brazil occurs between November and January, pregnant cows usually experience feed restriction at some point during gestation, which overlaps with the dry season in most of the beef cattle production areas.

Both pregnancy and feed restriction causes physiological challenges in the live animal that potentially affect carcass and meat quality traits. A study evaluating effects of feed restriction on calpain and calpastatin activity in skeletal muscle of pregnant cows has shown that calpastatin activity was down-regulated while no differences in calpain activity were observed in muscle of feed-restricted cows (Du, Zhu, Means, Hess, & Ford, 2004). Consequently, meat quality traits such as tenderness and water holding capacity might change since the calpain system plays an important role in proteolysis postmortem. Therefore, this study was developed to evaluate effects of pregnancy and feeding on carcass and meat quality traits of Nellore cows.

Material and methods

Animals and management

All animal care and handling procedures were approved by the Animal Care and Use Committee of the Department of Animal Science of the Universidade Federal de

Viçosa, Brazil (protocol 047/2012). Forty-three multiparous Nellore cows with average initial body weight of 451 ± 67 kg, age of 5.6 ± 1.9 years and body condition score of 4.6 ± 1.1 (1 to 9 scale) were used. Pregnancy of the cows was detected by ultrasound 25 days after mating and the day of mating was considered as day 0 of pregnancy. Thus, on day 27 of gestation cattle were confined in collective pens (48 m², 6 cows per pen) with individual electronic head gate system (Kloppen Soluções Tecnológicas, Pirassununga, SP, Brazil) for adaptation to individual feeders. At day 47 after conception, cows were randomly assigned into two groups with different feeding levels where 16 pregnant and 5 non-pregnant cows were fed at 1.2 times maintenance (NRC, 2000) and 16 pregnant and 6 non-pregnant were fed ad libitum. Cows were fed the same diet with differences only in the feeding level. The experimental diet composition is presented in Table 1. The restricted feeding level used was estimated to be enough to maintain the pregnancy of the dam through the experimental period avoiding abortion at any period of gestation and the *ad libitum* level allowed tissue deposition by the dams.

Harvest, Carcass Traits and Sample Processing

For evaluation of effects of gestational period on carcass and beef quality traits, pregnant cows were slaughtered at four different stages of gestation. Each feeding level group (maintenance and ad libitum) was randomly divided into four groups with four cows in each group to be slaughtered at 136, 189, 239, and 269 days of gestation. Non-pregnant cows were slaughtered at the end of the experimental period. Pre-harvest handling was in accordance with good animal welfare practices, and slaughtering procedures followed the Sanitary and Industrial Inspection Regulation for Animal Origin Products (Brasil, 1997).

After slaughter, all carcasses were refrigerated at 4ºC for approximately 24 h. Carcass temperature chilling rate was monitored by measuring the carcass temperature at 2, 4, 6, 8, 10, 12, 16, 20 and 24 hours post-mortem. After the postmortem chill period the cold carcass weight (CCW), 12th rib fat thickness (RFT) and 12th rib longissimus muscle area (LMA) were measured on the left side of each carcass. Longissimus muscle areas were traced on transparencies and measured later with a planimeter and RFT measurements were taken ¾ the length ventrally over the longissimus muscle (Greiner, Rouse, Wilson, Cundiff, & Wheeler, 2003).

The difference between the chilled carcass weight and hot carcass weight was used to calculate 24 hr shrink loss. Carcass yield percent was calculated using cold carcass weight (CCW) divided by final shrunk body weight (SBW) and then multiplying the result by 100.

A boneless longissimus section 10 cm thick was removed from the 11th to 13th ribs. Longissimus muscle samples were individually vacuum-packaged and held at -20ºC for 2 days. After that, each frozen longissimus muscle sample was standardized from the posterior end into two 2.54 cm thick steak samples (AMSA, 1995) one for Warner-Bratzler shear force measurement and the other for objective color evaluation (*L*, a*, b**) and one 1 cm thick steaks for determination of myofibrillar fragmentation index (MFI). All steaks were vacuum packaged and held at -20ºC for 10 days until the analyses were performed. Another 1 cm thick steak was sampled from the longissimus muscle for water holding capacity analysis right after 24 hours post-mortem.

Estimation of postmortem muscular ATP depletion

Muscular ATP depletion was estimated by determination of R-value throughout the post-mortem period. The determination of R-value is a fast spectrophotometric method for the estimation of the inosine/adenosine ratio in muscle. In the case of postmortem changes in muscle it represents the degree of transformation of ATP to IMP (Honikel, Fischer, Hamid, & Hamm, 1981). For determination of R-value, 5g samples of longissimus muscle collected between the 9th – 11th ribs from the same half of the carcass from which boneless longissimus steak samples were collected. Muscle samples were manually collected by using a metal core at 2, 4, 6, 8, 10, 12, 16, 20 and 24 hours post-mortem in order to determine the time of rigor mortis establishment. After collection, samples were immediately frozen powdered in liquid nitrogen and kept in liquid nitrogen (-196 °C) until the analysis was performed. The determination of R-value was performed as described by Honikel et al. (1981).

Water holding capacity

Water-holding capacity was assessed using a centrifugation method (Honikel & Hamm, 1994) on samples collected right after 24 hours post-mortem. Each 1 cm steak was trimmed of external fat and minced. Duplicate 10-g minced samples were placed into centrifuge tubes and centrifuged for 10 min at 40,000 × *g* at 4°C using a JA-17 (Beckman Coulter, Fullerton, CA) rotor in an Avanti J-25 centrifuge (Beckman

Coulter, Fullerton, CA). After centrifugation, the liquid was removed and the sample meat reweighed. Water lost was also recorded as a percentage of the original weight of the steak.

Cooking Loss

Steak cooking loss was evaluated on the steaks that were also used for WBSF measurement. Cooking loss of each steak was recorded after steaks were oven-broiled. Total cooking loss was calculated as the difference between the weight of the steaks before and after oven-broiling.

Warner-Bratzler Shear Force Measurement

Warner-Bratzler shear force (WBSF) steaks were thawed at 4ºC for 24 h and oven-broiled in an electric oven (Layr, Luxo Inox) preheated to 150ºC. Internal steak temperatures were monitored by 20-gauge copper-constantan thermocouples (Omega Engineering, Stamford, CT) placed in the approximate geometric center of each steak and attached to a digital monitor. When internal steak temperature reached 35ºC, the steak was turned over and allowed to reach an internal temperature of 70ºC before removal from the oven. Cooked WBSF steaks were cooled for 24 h at 4ºC (AMSA, 1995). Eight round cores (1.27 cm diameter) were removed from each steak parallel to the long axis of the muscle fibers (AMSA, 1995). Each core was sheared once through the center, perpendicular to the fiber direction by a Warner-Bratzler shear machine (G-R Manufacturing Company, Manhattan, KS - USA).

Myofibrillar Fragmentation Index

Myofibrillar fragmentation indices (MFI) were determined on fresh muscle according to the procedures of Olson, Parrish and Stromer (1976) and modified by Culler, Parrish, Smith and Cross (1978). The protein concentration of the myofibril suspension was determined by the biuret method (Gornall, Bardawill, & David, 1949). Aliquots of the myofibril suspension were diluted with an isolating medium to reach a protein concentration of 0.5 ± 0.05 mg/ml. The diluted myofibril suspension was stirred and poured into a cuvette; absorbance of this suspension was measured immediately at 540 nm. Absorbance was multiplied by 200 to give a MFI for each sample.

Sarcomere length

Longissimus muscle samples were collected at 24 h post-mortem and small cubes (3.0 x 3.0 x 2.0 cm) were excised in triplicate from each sample. Cubes were then fixed as described by Koolmees et al. (1986). Sarcomere length was measured by laser diffraction using a 05 – LHR – 021 laser, Melles Griot, (Carlsbad, CA) and calculated as described by Cross et al. (1981). From each cube, sarcomere length of six fiber samples was determined and used for sarcomere length average calculation.

Statistical analysis

The response variables were analyzed using PROC MIXED in SAS 9.2. With exception of R-value and carcass temperature, all variables were analyzed as the following model:

$$Y_{ijkl} = \mu + D_i + G_j + (D * G)_{ij} + T_{(j)k} + (D * T)_{i(j)k} + e_{i(j)kl}$$

Where:

D_i = ith level of the fixed effect of Diet

G_j = jth level of the fixed effect of Gestation

$T_{(j)k}$ = kth level of the fixed effect of Time within Gestation

$e_{i(j)kl}$ = random error associated with Y_{ijkl}

The repeated measurements in time (R-value and carcass temperature) were analyzed as the following model:

$$Y_{ijklmn} = \mu + D_i + G_j + (D * G)_{ij} + T_{(j)k} + (D * T)_{i(j)k} + e_{i(j)kl} + Mm + (D * M)_{im} + (G * M)_{jm} + (D * G * M)_{ijm} + (T * M)_{(j)km} + (D * T * M)_{i(j)km} + e_{i(j)klm}$$

Where:

D_i = ith level of the fixed effect of Diet

G_j = jth level of the fixed effect of Gestation

$T_{(j)k}$ = kth level of the fixed effect of Time within Gestation

$e_{i(j)kl}$ = random error associated with the lth replicate of the kth level of T within the jth level of G and ith level of D

M_m = mth level of the fixed effect of Time of Measurement

$e_{i(j)klm}$ = random error associated with Y_{ijklm}

Least square means were estimated for all effects and compared using Tukey's method at $\alpha = 0.05$.

Results

There was no interaction ($P < 0.05$) among treatments. Thus, effects of pregnancy and feeding level were discussed independently.

No effects of pregnancy status were detected on final body weight (FBW; $P = 0.0923$), cold carcass weight (CCW; $P = 0.3125$), cold carcass yield (CCY; $P = 0.0513$), carcass shrink loss (CSL; $P = 0.7875$), longissimus muscle area (LMA; $P = 0.8260$), rib fat thickness (RFT; $P = 0.1873$) and carcass final pH (pHu; $P = 0.0898$). Additionally, no differences were observed for FBW ($P = 0.1274$), CCW ($P = 0.3699$), CCY ($P = 0.4637$), CSL ($P = 0.6208$), LMA ($P = 0.4529$), RFT ($P = 0.2888$), and pHu ($P = 0.1156$) among cows at different days of gestation (Table 2).

Pregnancy status did not affect Warner-Bratzler shear force (WBSF; $P = 0.9707$), myofibrillar fragmentation index (MFI; $P = 0.0917$), cooking loss (CL; $P = 0.5502$), water holding capacity (WHC; $P = 0.3291$), and sarcomere length (SL; $P = 0.2242$). No effects of days of gestation were observed for WBSF ($P = 0.7581$); MFI ($P = 0.4759$), CL ($P = 0.6921$), WHC ($P = 0.5641$), and SL ($P = 0.2195$) (Table 3).

A lower FBW was observed ($P = 0.0028$) in cows fed at maintenance compared to those fed ad libitum which shows the effectiveness of the feed restriction level used in this study. The CCW was also lower ($P = 0.0105$) in cows fed at maintenance compared to those fed ad libitum. No differences were found for CCY ($P = 0.7243$) and CLS ($P = 0.4704$) among the feeding level groups. Cows fed ad libitum had greater LMA ($P = 0.0048$) and RFT ($P = 0.0001$) compared to those fed at maintenance. No differences were observed among feeding level groups ($P = 0.5758$) for pHu (Table 4).

Cows fed ad libitum had similar WBSF values ($P = 0.0759$) to those fed at maintenance. No differences were found among feeding level groups for MFI ($P = 0.5759$), CL ($P = 0.7554$), WHC ($P = 0.5902$), and SL ($P = 0.9628$) (Table 5).

Similar R-value was observed throughout the post-mortem period among cows fed at maintenance and ad libitum ($P = 0.4260$; Figure 1). However, pregnancy status affected the R-value throughout the post-mortem period and rigor onset occurred earlier

in non-pregnant cows ($P = 0.0008$; Figure 2). There was no effect of days of gestation on R-value during the post-mortem period ($P = 0.3551$).

Carcass temperature during post mortem period was affected by feeding level ($P < .0001$) and significant differences were observed after 6 h post mortem where lower temperature was observed on carcass from cows fed at maintenance (Figure 3). No effect of pregnancy status was observed ($P = 0.4476$) for carcass temperature during the postmortem period.

Discussion

In a study evaluating the effects of pregnancy status on beef quality of non-pregnant cows, cows at early to mid gestation (1 to 5 months) and cows at late gestation (more than 6 months) Wythes et al. (1990) reported that females may increase in live and carcass weight during pregnancy but they still have lighter carcasses than their non-pregnant contemporaries. However, in the present study although a greater BW in cows due to pregnancy was expected, differences in BW and CW were not observed among pregnant and non-pregnant cows (Table 2). This might be explained by the mobilization of energy stores in pregnant animals to meet the requirements of the growing fetus. An increase in catabolism in pregnant animals has been demonstrated by Naismith and Morgan (Naismith & Morgan, 1976) who observed a net protein loss due to pregnancy. Thus, the lack of effect of pregnancy on final BW and CW observed in this trial demonstrates the magnitude of the demands of pregnancy on cow's body tissue.

Although there was no difference on CCW and BW throughout the gestational period, both characteristics have increased simultaneously as gestation advanced leading to a similar CCY from cows at different days of gestation. These results are supported by the amount of lean and subcutaneous adipose tissues, which are the main tissues responsible for variations on carcass weight and yield. As presented in Table 2, no differences were observed on LMA and RFT due to pregnancy status and days of gestation, which might explain the lack of differences in CCY.

Pregnancy is characterized by a series of metabolic changes that promote adipose tissue accretion in early gestation, followed by insulin resistance and facilitated lipolysis in late pregnancy. In early pregnancy, insulin secretion increases, while insulin sensitivity is unchanged, decreased, or may even increase (Barbour, et al., 2007). However, in late gestation, maternal adipose tissue depots decline, as the ability of insulin to suppress whole-body lipolysis is reduced during late pregnancy (Valsamakis,

Kumar, Creatsas, & Mastorakos, 2010). Therefore, increase in lipolysis in pregnant cows slaughtered at late gestation might explain the lack of differences for RFT of cows harvested at different days of gestation (Table 2).

Similarly, the lack of effects of days of gestation on LMA may be due to the increase of insulin resistance by skeletal muscle. It has been demonstrated directly in skeletal muscle fibers that pregnancy alone was associated with a marked reduction in insulin-stimulated glucose transport as the gestation advances (Friedman, et al., 1999). As the role of insulin is to increase glucose uptake controlled initially by the transport of glucose across the cell membrane, which takes place by facilitated diffusion through glucose transporters, insulin resistance in skeletal muscle reduces the energy availability for muscle hypertrophy. As a consequence, increase in muscularity may be impaired in pregnant animals leading to similar values of LMA among cows slaughtered at different days of gestation (Table 2).

As expected, cows fed ad libitum had greater FBW and CCW than cows fed at maintenance which shows the effectiveness of the feed restriction level used in this study (Table 4). However, there was a lack of differences in CCY among feeding level groups which is possibly due to greater gastrointestinal contents of cows fed ad libitum (data not shown). Additionally, differences in viscera and internal fat weights have been reported in heifers under different feeding levels (Ferreira, 1998; Lage, et al., 2012). This possibly occurred in this study where cows fed ad libitum likely had greater viscera and internal fat weights than those fed at maintenance, contributing to differences in FBW, and concomitantly, similarity of CCY among feeding level groups (Table 4).

Nutrition management is one of the main factors that affect animal growth. When cattle are fed at maintenance there is a priority for energy utilization to maintain vital functions, as some internal organs of the body such as liver, kidney, heart and gastrointestinal tract are responsible for up to 40 % of energy requirement for maintenance of fasting cattle (Oliveira, et al., 2011). These tissues have relatively higher protein turnover than muscle tissue resulting in high cost of energy for a basal metabolism leading to an impaired muscle growth as observed in this study where cows fed at maintenance had lower values of LMA than those fed ad libitum (Table 4).

It has been reported that transcription factors responsible for adipose tissue deposition reduce their expression in feed restriction situations (Bergen & Burnett, 2012). Moreover, recent studies have shown the ability of the mature adipocyte to

dedifferentiate returning into a proliferative state (Dodson, Jiang, Du, & Hausman, 2012; Fernyhough, Hausman, & Dodson, 2008; Kokta, Dodson, Gertler, & Hill, 2004) resulting in different cell lineages such as myogenesis (Dodson, et al., 2010; Kazama, Fujie, Endo, & Kano, 2008; Matsumoto, et al., 2008; Wei, et al., 2012). Therefore, it is possible that in addition to lipolysis that usually occurs in feed restricted animals as a result of energy store mobilization, reduced RFT in feed restricted animals also occurs due to a dedifferentiation of mature adipocytes for formation of tissues having a higher priority than adipose tissue.

Carcass shrink loss is influenced by carcass fat thickness (Lage, et al., 2012; Savell, Mueller, & Baird, 2005). Increased fatness may decrease shrinkage by serving as a barrier against moisture loss (preventing evaporation from the lean), or it may act to minimize the total moisture content in the carcass (Savell, et al., 2005). Since there are variations in the distribution of back fat on the carcass, a minimum depth of 3 mm of RFT has been recommended to prevent carcass shrinkage and cold shortening during postmortem chill (Lage, et al., 2012; Luchiari Filho, 2000). In this study, regardless of pregnancy status, days of gestation and feeding level, all carcasses had RFT greater than 3 mm. Thus, the absence of differences for CSL among the treatments evaluated can be attributed to adequate RFT depth (Tables 2 and 4).

During the postmortem period hydrolysis of intermediate filaments, titin and nebulin occurs mainly by the activity of calpains (Du, et al., 2004; Koohmaraie, Kent, Shackelford, Veiseth, & Wheeler, 2002) and it can be predicted by measuring the myofibrillar fragmentation index (MFI) which indicates the extent of proteolysis postmortem and has been used as a predictor for postmortem tenderization. Therefore, the lack of effects of pregnancy status and days of pregnancy on WBSF may be partially explained by the lack of differences on MFI among these treatments (Table 3).

According to Whiting et al. (1980), if the ATP level falls below about 1 µMol/g as a result of the postmortem breakdown of ATP, not enough ATP might be available for the operation of the ion pump in the sarcoplasmic reticulum leading to a scenario of Ca^{+2} accumulation in the sarcoplasm and consequently to a shortening of the sarcomere. In bovine longissimus muscle the R-value of 0.967 was reported to be equivalent of 1 µMol/g of ATP (Koh, Bidner, McMillin, & Kim, 1993). In the present study significant interaction between pregnancy status and time of measurement throughout the postmortem period was observed (P = 0.0074) for R-value measured on the longissimus muscle. Differences in r-value among pregnant and non-pregnant cows

were observed (*P* = 0.0008) only after 10 hours postmortem (Figure 1). On the other hand, there was no difference (*P* = 0.4476) in carcass temperature during postmortem period due to pregnancy status (Figure 1). It should be noted that in both pregnant and non-pregnant cows the onset of rigor (R-value = 0.967) (Koh, et al., 1993) occurred before carcass temperature reached the critical range that leads to cold shortening, which explains the similarity in sarcomere length of longissimus muscle from pregnant and non-pregnant cows (Table 3). Together, results of MFI and sarcomere length observed in this study are consistent with the lack of differences in WBSF observed in beef from pregnant and non-pregnant cows.

Carcass temperature was affected by feeding level groups throughout the postmortem period (Figure 2), which is due to the difference in RFT in carcasses of animals fed at maintenance and ad libitum. The subcutaneous fat acts as a thermal insulator of the carcass during chilling, thus reducing the chilling rate of the carcass. As presented in Table 4, cows fed ad libitum had greater values of RFT than those fed at maintenance which explains the higher temperatures of carcasses from cows fed ad libitum throughout the postmortem period. However, although differences in carcass temperature were observed among feeding level groups, sarcomere length did not differ among cows fed ad libitum or at maintenance (Table 5). There was no effect of feeding level on R-value measure on the longissimus muscle during postmortem period (Figure 2). As was previously discussed, the carcass chilling rate was not severe enough to reach temperatures that would cause cold shortening of the muscle and thus, no differences were observed for sarcomere length among feeding level groups. Although cows fed at maintenance had lower RFT than cows fed ad libitum, the amount of subcutaneous fat observed in this study seemed enough to avoid a sudden drop of carcass temperature, preventing toughening of meat by cold shortening.

Conclusions

These data suggests that carcass and meat quality traits are not affected by pregnancy status in Nellore cows. Moreover, although cows experiencing feed restriction did have reduced deposition of subcutaneous fat and lean tissue, there were no major impacts on meat quality traits.

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Table 1. Composition of experimental diet

Item	% Dry matter basis
Ingredients	
Corn silage	84.3
Soybean meal	5.1
Corn meal	8.5
Urea/ammonium sulphate[1]	1.4
Mineral mixture[2]	0.7
Nutritional composition	
Total digestible nutrients	64.8
Crude protein	13.5

[1] Urea/ammonium sulphate proportion = 9:1

[2] Composed of 15 % calcium; 9 % phosphorus; 0.13 % manganese; 0.2 % cooper; and 100mg/kg cobalt

Table 2. Carcass quality traits of non-pregnant and pregnant Nellore cows at different stages of gestation

Item[1]	Pregnancy status		Days of gestation				*P* - value	
	Non-pregnant n = 11	Pregnant n = 32	136 d n = 4	189 d n = 4	239 d n = 4	269 d n = 4	Pregnancy	Days of gestation
FBW, kg	487.38 ± 21.99	531.53 ± 12.84	508.75 ± 25.68	492.56 ± 25.68	555.62 ± 25.68	569.19 ± 25.68	0.0923	0.1274
CCW, kg	287.55 ± 14.32	304.56 ± 8.36	294.94 ± 16.72	284.98 ± 16.72	317.40 ± 16.72	320.93 ± 16.72	0.3125	0.3699
CCY, %	58.94 ± 0.71	57.26 ± 0.42	57.95 ± 0.83	57.83 ± 0.83	57.00 ± 0.83	56.27 ± 0.83	0.0513	0.4637
CSL, %	1.90 ± 0.09	1.87 ± 0.06	1.92 ± 0.11	1.96± 0.11	1.76 ± 0.11	1.84 ± 0.11	0.7875	0.6208
LMA, cm^2	64.31 ± 2.38	64.92 ± 1.42	64.38 ± 2.78	64.09 ± 2.78	68.75 ± 2.78	64.45 ± 2.78	0.8268	0.4529
RFT, mm	6.75 ± 0.89	8.14 ± 0.52	7.24 ± 1.04	8.10 ± 1.04	7.39 ± 1.04	9.83 ± 1.04	0.1873	0.2888
pHu	5.61 ± 0.05	5.50 ± 0.03	5.54 ± 0.06	5.61 ± 0.07	5.43 ± 0.06	5.41 ± 0.06	0.0898	0.1156

[1] FBW = final body weight; CCW = cold carcass weight; CCY = cold carcass yield; CSL = carcass shrink loss; LMA = longissimus muscle area; RFT = rib fat thickness; pHu = carcass final pH.

Table 3. Beef quality traits of non-pregnant and pregnant Nellore cows at different stages of gestation

Item[1]	Pregnancy		Days of gestation				P – value	
	Non-pregnant n = 11	Pregnant n = 32	136 d n = 4	189 d n = 4	239 d n = 4	269 d n = 4	Pregnancy	Days of gestation
WBSF, kgf	4.48 ± 0.22	4.49 ± 0.13	4.69 ± 0.26	4.54 ± 0.28	4.29 ± 0.28	4.44 ± 0.26	0.9707	0.7581
MFI, %	52.35 ± 2.86	50.33 ± 1.67	47.67 ± 3.34	47.94 ± 3.34	51.62 ± 3.34	54.08 ± 3.34	0.0917	0.4759
CL, %	19.91 ± 1.10	20.68 ± 0.64	19.82 ± 1.29	21.67 ± 1.29	21.22 ± 1.29	20.03 ± 1.29	0.5502	0.6921
WHC, %	1.76 ± 0.21	1.53 ± 0.13	1.77 ± 0.26	1.46 ± 0.24	1.58 ± 0.24	1.34 ± 0.26	0.3291	0.5641
SL, µm	2.20 ± 0.08	2.08 ± 0.05	2.01 ± 0.09	2.18 ± 0.09	2.19 ± 0.09	1.96 ± 0.09	0.2242	0.2195

[1] WBSF = Warner-Bratzler shear force; MFI = myofibrillar fragmentation index; TL = thawing loss; CL = cooking loss; WHC = water holding capacity; SL = sarcomere length.

Table 4. Carcass quality traits of Nellore cows fed at two feeding levels

Item[1]	Feeding level		*P* – value[2]		
	Ad libitum n = 21	Maintenance n = 22	FL	FL x PR	FL x DG
FBW, kg	545.0 ± 18.61	473.91 ± 17.39	0.0028	0.5382	0.7179
CCW, kg	315.92 ± 12.12	276.20 ± 11.32	0.0105	0.7544	0.6833
CCY, %	58.03 ± 0.60	58.17 ± 0.56	0.7243	0.4903	0.8064
CSL, %	1.84 ± 0.08	1.93 ± 0.07	0.4704	0.5343	0.4370
LMA, cm^2	67.94 ± 2.01	61.28 ± 1.90	0.0048	0.1269	0.3291
RFT, mm	9.43 ± 0.75	5.46 ± 0.70	0.0001	0.4992	0.3924
pHu	5.53 ± 0.04	5.57 ± 0.04	0.5748	0.5607	0.0702

[1] FBW = final body weight; CCW = cold carcass weight; CCY = cold carcass yield; CSL = carcass shrink loss; LMA = longissimus muscle area; RFT = rib fat thickness; pHu = carcass final pH

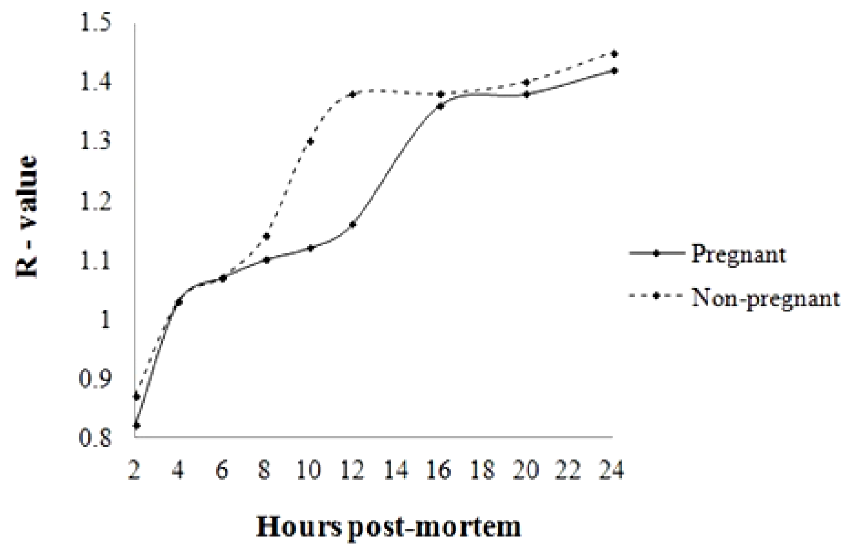
[2] FL = effects of feeding level; FL x PR = effects of interaction of feeding level and pregnancy status; FL x DG = effects of interaction of feeding level and days of gestation

Table 5. Beef quality traits of Nellore cows fed at two feeding levels

Item[1]	Feeding level		*P* – value[2]		
	Ad libitum n = 21	Maintenance n = 22	FL	FL x PR	FL x DG
WBSF	4.26 ± 0.19	4.71 ± 0.18	0.0759	0.5451	0.7785
MFI	53.22 ± 2.42	54.45 ± 2.26	0.5759	0.5394	0.1756
CL	20.35 ± 0.93	20.25 ± 0.87	0.7554	0.1240	0.4796
WHC	1.74 ± 0.18	1.56 ± 0.17	0.5902	0.2225	0.5403
SL, µm	2.13 ± 0.07	2.16 ± 0.06	0.9628	0.2095	0.9181

[1] WBSF = Warner-Bratzler shear force; MFI = myofibrillar fragmentation index; TL = thawing loss; CL = cooking loss; WHC = water holding capacity; SL = sarcomere

[2] FL = effects of feeding level; FL x PR = effects of interaction of feeding level and pregnancy status; FL x DG = effects of interaction of feeding level and days of gestation



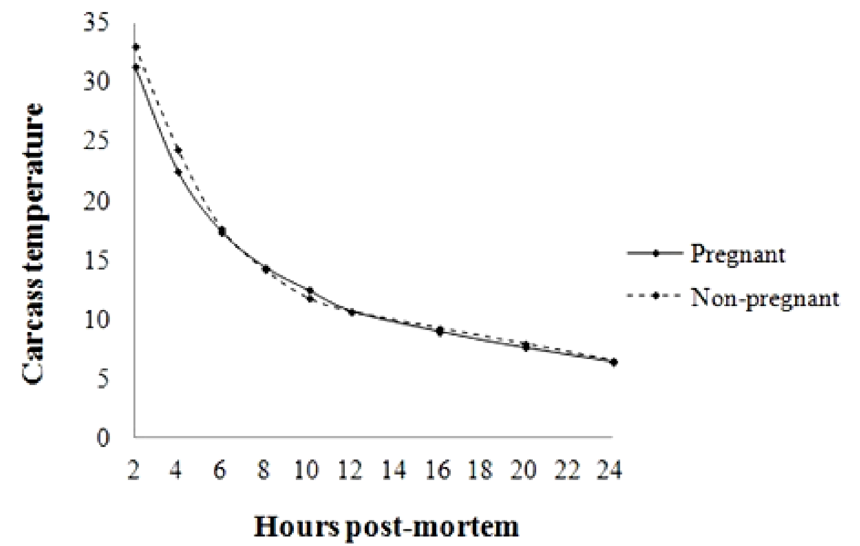
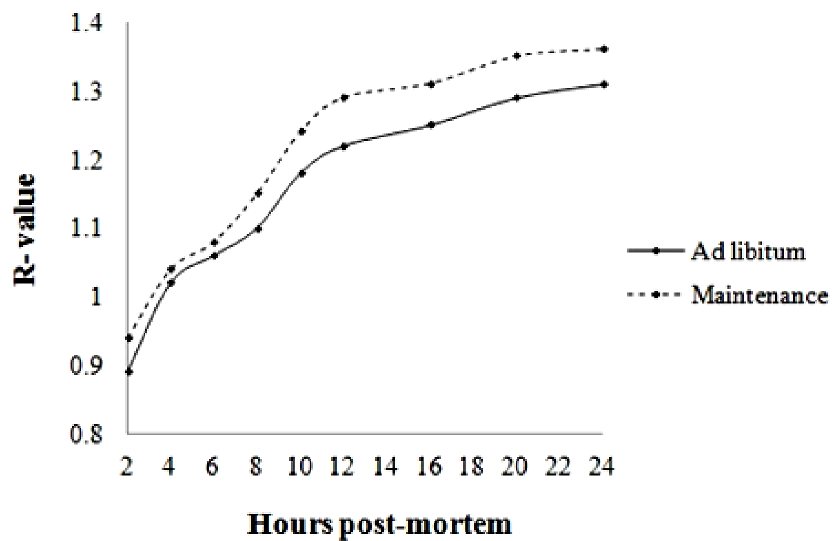


Figure 1: R-value (250/260 nm absorption ratio) and carcass temperature from pregnant and non-pregnant cows throughout the postmortem period.



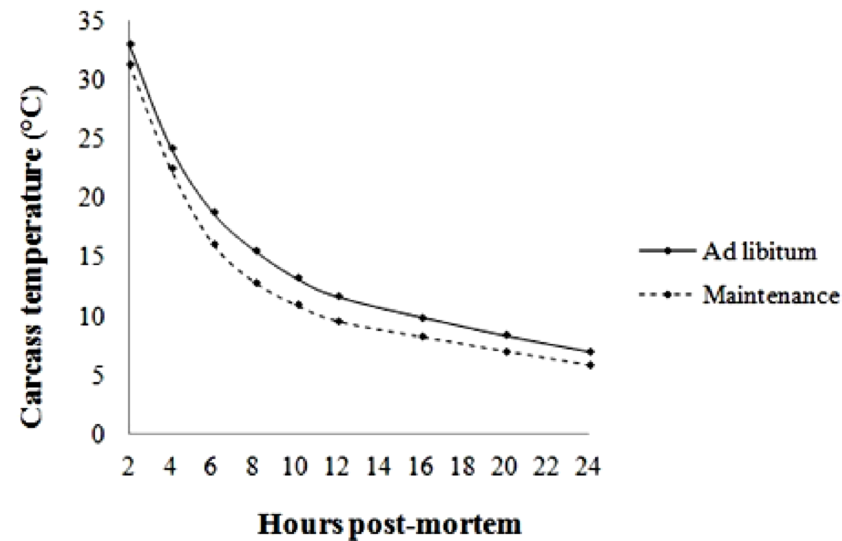


Figure 2: R-value (250/260 nm absorption ratio) and carcass temperature from cows fed different feeding levels throughout the postmortem period.

Appendix

Isolation of mature adipocytes and stromal vascular cells under adverse sampling conditions

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Abstract

Methods are described to obtain adipose tissue for cell isolation, under adverse isolation conditions whereby no scientific controls were in place. Such methods could be used by laboratories of institutions where controlled environments (surgery rooms, abattoirs) are not available. While not ideal, we show that a variety of adipocytes and adipocyte-like cells may be isolated from such methods. These types of procedures may facilitate a greater number of persons entering into the research arena with adipocytes, and are easily adaptable to other animal models.

Key Words: beef; custom pack; adipose tissue; stromal vascular cells; mature adipocytes; isolation; limited facilities.

Resumo

Neste trabalho são descritos métodos para obtenção de tecido adiposo para procedimentos de isolamento de células em condições adversas de coleta. Tais métodos podem ser utilizados em instituições desprovidas de ambientes controlados para obtenção de amostras tais como salas de cirurgia e/ou abatedores. Mesmo não sendo considerada condição ideal, mostrou-se neste trabalho que os métodos aqui propostos foram eficientes para obtenção de adipócitos e células do estroma vascular de tecido adiposo. Tais procedimentos podem facilitar o acesso de pesquisadores ao desenvolvimento de estudos *in vitro* com cultura de células isoladas de tecido adiposo.

Palavras-chave: Adipócitos maduros, carne, células do estroma vascular, isolamento, tecido adiposo.

Introduction

Adipocytes are cells that comprise a portion of any adipose depot. While they are not the only cell present in the adipose depot [10], discernible adipocytes function to assimilate moderate and long-chain fatty acids, store them as triacylglycerol, and release the fatty acids in times of negative energy balance, or in response to a repartitioning agent [1, 2]. Adipocytes of some adipose depots also synthesize and release regulatory cytokines into the blood, which have been postulated to regulate a variety of whole animal and local non-adipose tissue physiology [1,3,4]. As such, in addition to moderating aspects of energy balance, adipocytes also may play a role in altering the body's blood pressure [3, 4], ability to respond to insulin, control the rate of cellular apoptosis, fight inflammation and maintain normal levels of connective tissue surrounding tissues [5, 6]. Adipocytes have been directly linked to obesity, metabolic syndrome, hypertrophy of the heart, and numerous other dynamic pathologies [7-9].

Stromal vascular (SV) cells in adipose depots are present and active in the presence of fully differentiated adipocytes [10], and rodent-derived cells have largely been used as a model for defining aspects of adipogenesis [1]. Are there other animal models that may help in defining aspects of adipocyte physiology, whereby adipocytes from all adipose depots are abundantly available? Research with large animals (such as cattle) provides such an animal model. These types of animals form the foundation of the National Institute of Health/United States Department of Agriculture directive towards use of "dual purpose animals" for both human and animal production-related research, because these large animals are good models/donors for studying molecular/cellular mechanisms of adipocyte physiology. For example, cattle are sufficiently large that all adipose depots may be easily sampled at the same time for comparative adipogenesis studies (depot vs depot), or individually (intramuscular depot) for adipogenesis and lipid metabolism studies [11, 12]. In addition, the expanding marker databases of cattle promote the usage of this model.

Previous research with beef-derived adipocyte isolation utilized existing animal facilities in controlled environments such as either a local surgical suite or a meats laboratory (abattoir). This allows one to be capable of controlling the environment to which tissues are initially prepped/isolated/handled and packaged for transport. Moreover, tissue obtained for cell isolation could be extracted from the donor animal fast, efficiently transferred to a sterile (buffer) environment, and (all) promptly returned to the cell culture laboratory for processing. What about those without such facilities

such as urban medical schools, researchers at (very) small colleges or it other countries whereby facilities are quite limited? Can tissues be isolated from beef cattle if none of the environmentally controlled facilities are available? The focus of this paper was to obtain skeletal muscle from beef animals, which were being terminated and initially processed by a custom-packing/processing company with on-the-farm service. If successful, such tissue procurement results may immediately suggest that research personnel with few facilities might be in a position to conduct research in this area.

Material and methods

Animals and pre-slaughter material preparation

Animals used for tissue sampling were slaughtered in a beef farm located in Viola, Idaho-USA by using a mobile slaughter unit (Fig. 1). No attempt was made to influence their normal procedures. Instead, representatives of the commercial entity physically provided samples to laboratory personnel. Immediately after receipt of the samples, laboratory individuals returned to the cell culture laboratory expediently. However, the locale and personnel responsible for handling of the live animals/slaughter of the same/obtaining the muscle samples, and providing the samples to the laboratory personnel was new and never used for any work like this previously. Thus, we were not able to control any of the steps in tissue isolation--other than to request the specific muscle that should be sampled. Cattle were simultaneously slaughtered by cerebral concussion followed by jugular venesection and the muscle samples were collectively collected immediately after bleeding.

The cell culture laboratory was prepared for cell isolation procedure one day before the tissue sampling. All the supplies needed including plastic and glassware, and buffer solution (PBS) were sterilized prior the tissue collection in order to optimize the cell isolation procedure. The buffer solution was warmed in a water bath to 37 °C and kept in a thermal box during the pre-slaughter until the samples collection in order to keep it as warm as possible.

Tissue samples

At the farm, samples of sternomandibularis muscle from four Angus cattle were collected within 10 min after slaughter and immediately placed in a sterile beaker containing warm phosphate buffered saline (PBS) supplemented with 100 IU/ml penicillin, 100 μg/ml streptomycin , 2.5 ng/ml Fungizone B and 50 μg/ml Gentamicin.

The beakers containing the tissue were placed in a thermal box as soon as the samples were collected in order to keep the samples warm, and then taken to the cell culture laboratory. As soon as the samples arrived at the cell culture laboratory they were immediately processed 50 min after collection and delays were avoided on the way back to the cell culture laboratory in order to short the time between sampling and cell isolation. A timeline of the sample collection and cell isolation procedure is presented in Fig. 2.

Cell isolation procedure

Mature adipocytes (MAs) and stromal vascular (SV) cells from intramuscular fat (IMF) depot were isolated as described by Fernyhough et al. (2004) with minor modifications as it follows (Fig. 3). Preparation of reagents and media used for cell isolation is described in Table 1.

1. Rinse the muscle sample with PBS (37 °C, pH = 7.08) supplemented with antibiotics/antimycotics and trim it prior to muscle dissection in order to reduce chances of contamination.
2. In a laminar flow hood, place the sample in a sterile dish, bath in PBS. Using sterile forceps and scissor dissect the muscle tissue in order to access the intramuscular fat depots. Once beginning the sample dissection, do not turn the tissue upside down since to keep the interior of the sample as clean as possible.
3. Place approximately 5 g of intramuscular fat tissue in a sterile 10 mm dish containing 10 ml of PBS. Cut the isolated fat tissue in small pieces (approximately 1-cm^2) and place the minced tissue in a sterile 50 ml centrifuge tube containing 25 ml of warm (37 °C) sterile collagenase type I solution (0.25% collagenase in PBS). Place the tube in a rocker and incubate for 1 h at 37 °C.
4. After the enzyme digestion, filter the solution through a sterile 1000 μm plastic mesh in a sterile funnel new into a 50 ml tube.
5. Centrifuge the filtrate for 10 min at 186 x *g*. After the centrifugation, an underlying pellet will be seeing at the bottom of the tube which contains the stromal vascular cells and the mature adipocytes will be floating in the top layer of the solution.

6. To isolate the mature adipocytes, gently pipette the top layer of the solution into a new tube. Add the same volume (1:1 v/v) of DMEM/F12 + 10% FBS into a tube and wash the cells twice by centrifugation at 186 x g. After wash the cells, place the isolated mature adipocytes into a 12.5 cm^2 cell culture flask filled with DMEM/F12 + 10% horse serum (HS). Invert the flask so the bottom of the flask is on top and incubate it at 37 °C in a 5 % CO_2 incubator [13].
7. To culture the stromal vascular cells, resuspend the underlying pellet of the digested solution with 10 ml of DMEM/F12 + 10% FBS and filtered once through a 100 μm and subsequently through 40 μm cell strainers into a new 50 ml centrifuge tube. After filtration vortex it vigorously and transfer the solution to a 10 cm dish, rinses with an additional 10 ml of DMEM/F12+10% FBS and transferred into a cell culture dish. Incubate the isolated stromal vascular cells at 37 °C in a 5 % CO_2 incubator.

Photomicrographs

All photomicrographs were taken with a Sony RGB digital camera (3/4–inch chip) coupled to a Nikon Diaphot phase contrast microscope and Image Pro Plus® image analysis software. The major equipment, media and chemicals, glassware and plastic supplies for cell isolation are presented in Table 2, Table 3 and Table 4 respectively.

Results and discussion

It is fairly clear that the environment in which tissue culture is carried out must be clean. This concept leads us to think that for a successful isolation procedure tissue handling must be done with extremely aseptic manner by trained individuals in order to avoid any source of contamination and transported to cell culture laboratory as soon as possible. As such, to isolate cells from a tissue in order to obtained primary cell culture more than an equipped cell culture laboratory is required, being necessary to be as close as possible to the place that the tissues are obtained. Additionally, one of the most important considerations that should be taken prior to cell isolation is the cooperation and collaboration of the clinical staff, which can be easily achieved if a person responsible for tissue isolation is also a member of the cell culture laboratory.

From the moment that a biological sample is removed from its natural environment within the animal, it is susceptible to deterioration from external factors such as moisture loss and temperature fluctuation and excessive passage of time. Another threat to the success of any cell isolation method is contamination from microbial sources including the host's own natural flora. All phases of the isolation process offer opportunities for contamination to occur unless strict adherence to proper aseptic techniques is consistently followed [14]. Additionally, the availability of the cells isolated from a tissue sample depends also depends on how fast the tissue sampling, dissection and digesting can be done, as in most cases contamination problems are related to the time [15].

In this study mature adipocytes (MA; Fig. 4) and stromal vascular (SV; Fig. 5) cells were successfully isolated from bovine muscle collected under non-asepsis conditions. No signs of contamination were detected by microscopy visualization such as cloudiness of media, deterioration of the cells and detachment of the cell monolayer, which would indicate mycoplasm contamination [16, 17]. The photomicrographs results clearly shows that a great number of cells were successful isolated even though the tissue was collected under adverse conditions and was completely exposed to a non-aseptic environment prior to cell isolation procedures, which was never done in this laboratory before. Additionally, due to the distance between the local of tissue sampling and the cell culture laboratory, the samples were not promptly processed as suggested by several previously studies [14, 18-19].

The results obtained in this study encourage new researchers in cell culture field by showing a protocol that allows the isolation of cells from muscle tissue without proper animal facilities. By using the cell isolation procedure developed in our laboratory additionally to the use of basic sterile techniques at the cell culture facility, we were able to obtain non-contaminated, healthy, primary cultures of cells. However, even though we were able to isolate cells in an adverse scenario, it is still recommended the use of sterile techniques during sample collection to avoid contaminations and cells death.

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Figure 1 – Mobile unit used for cattle slaughter and transit to processing facilities. The picture also shows the cold weather conditions of the day that the samples were collected. The snow on the ground contributed for a muddy yard where the cattle were harvested and the muscle samples were collected.

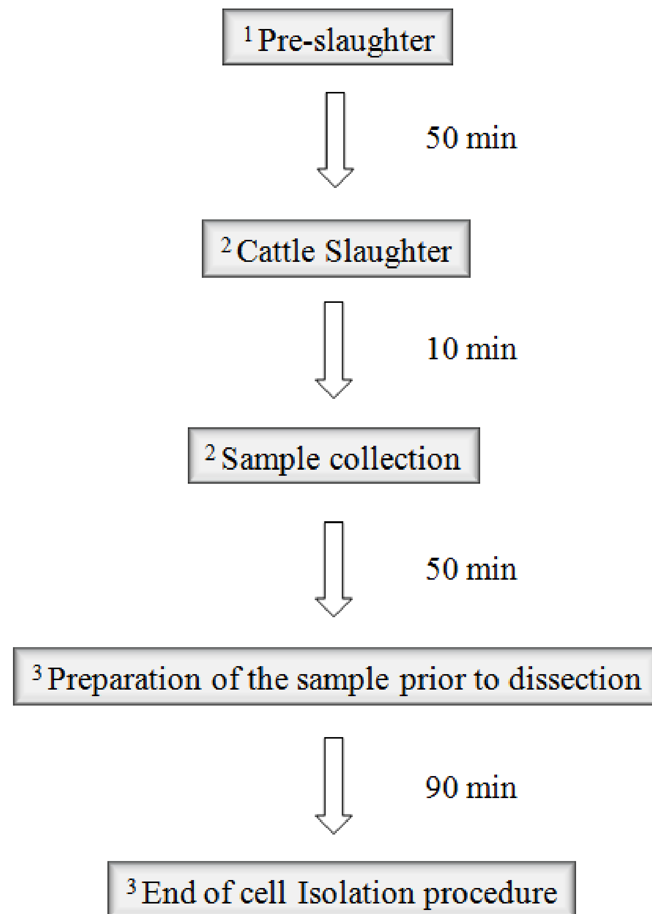


Figure 2 – Flow diagram with the steps of the cell isolation procedure within the cell culture laboratory: 1) Preparation of muscle sample, muscle dissection and mature adipocytes (MA's) and stromal vascular (SV) cells isolation – muscle samples were rinsed several times with PBS containing antibiotics and antimycotics in order to clean the samples as much as possible before take it to the lamina flow hood. Once washed, samples were bath in sterile PBS placed in a dish inside of the lamina flow hood for muscle dissection. Fragments of intramuscular fat (5 g) was isolated from the muscle using sterile forceps and placed in a 10cm dish containing PBS and minced into small pieces in order to increase the surface area to optimize the enzyme digestion. Minced tissue was digested by collagenase type I for 1h at 37 °C. After digestion, the solution was filtered through 1000 μm plastic mesh to separate the digested tissue from the cells. The solution containing isolated cells was centrifuged at 186 x *g* for 10 min and the two types of cells were segregated were MA's were found at the top layer of the solution and SV cells were found at the pellet on the bottom of the tube. ; 2) MA's were transferred into a new tube and washed by centrifugation at 186 x *g* twice with DMEM/F12 + 10% FBS before plating in a 12.5 cm^2 flask completely filled with DMEM/F12 + 10% HS. The SV cells were resuspended with DMEM/F12 +10% FBS and filtered subsequently through a 100 μm and 40 μm mesh filters before plating with the same media in a 10cm dish.

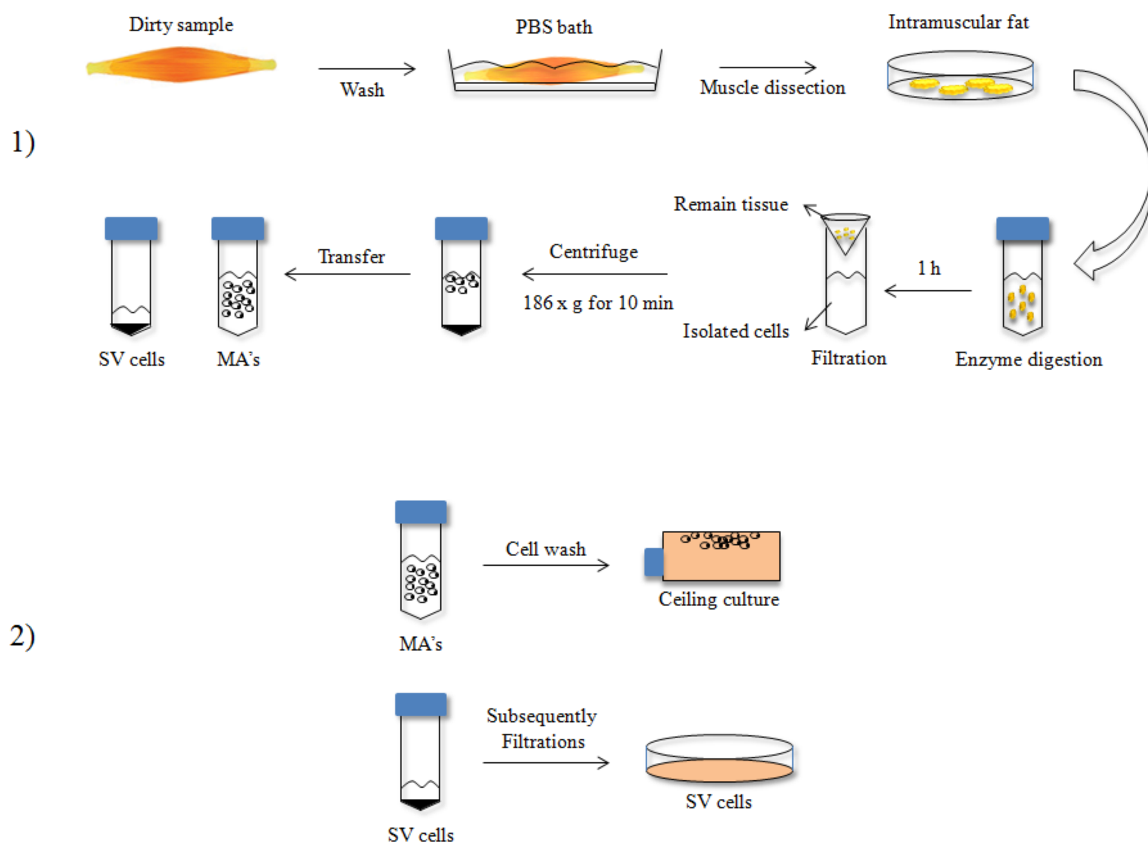


Figure 3 – Approximate time spent at different steps of sampling and cell isolation. [1]Set-up of cell culture laboratory prior the sample collection including media and phosphate buffer saline (PBS) preparation and sterilization of material used for cell isolation procedure. The PBS solution used for sample collection were warmed up to 37 °C and place in a thermal box before leave the cell culture laboratory in order to keep it as warm as possible; [2]At the farm, samples were collected within 10 min after the animals were slaughtered and placed immediately in a beaker containing warm PBS (approximately 37 °C) and kept in a thermal box until arrive at the cell culture laboratory; [3]At the cell culture laboratory samples were rinsed with PBS (37 °C) several times before the sample dissection and cell isolation.

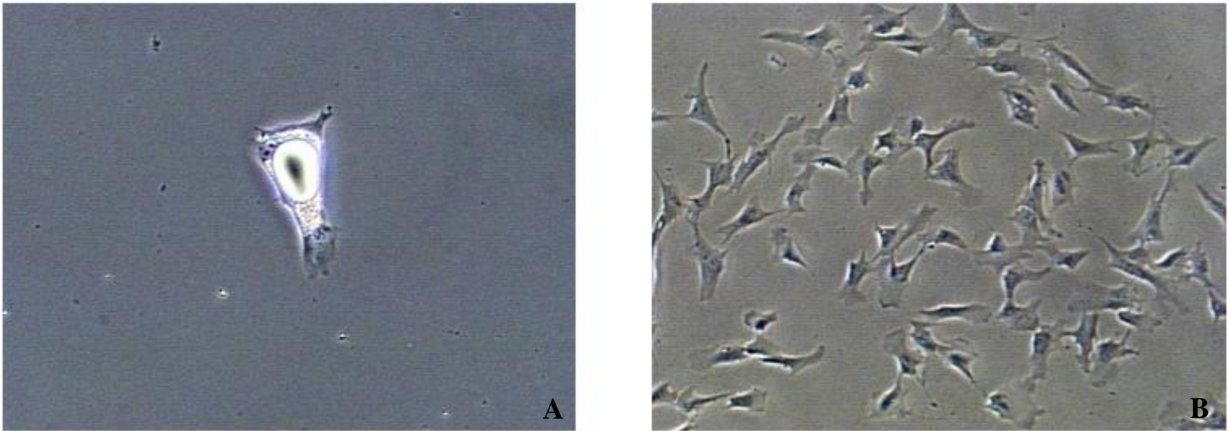
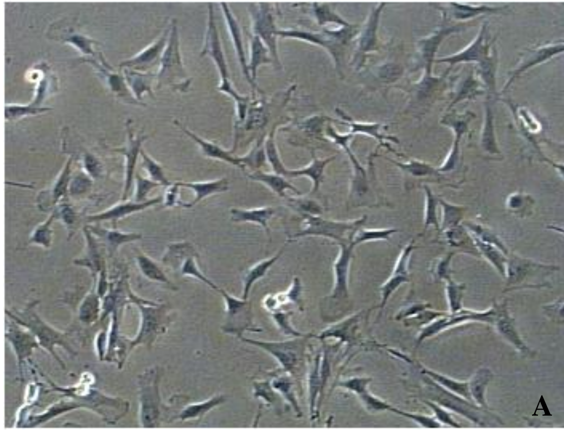


Figure 4 – A) 48 h after cell isolation, mature adipocytes attached the ceiling of flask; B) 7 d after cell isolation, progeny cells derived from mature adipocytes reached confluence. [A, 400 x magnification; B, 100 x magnification]



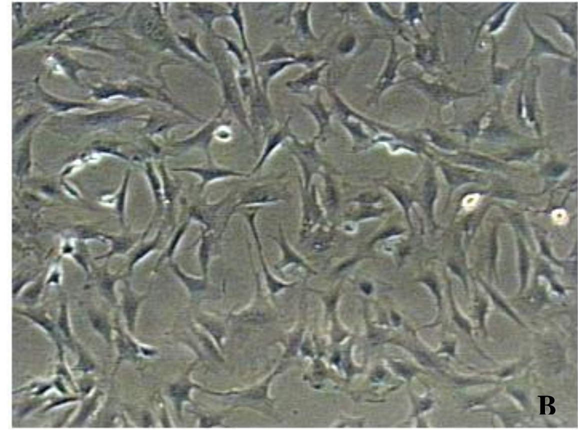


Figure 5 – Photomicrographs (A and B) of stromal vascular cells cultures 72 h after cell isolation [100 x magnification]

Table 1 – Preparation of reagents and media used for cell isolation

Solution	Chemical	Amount	Comments
PBS (pH = 7.08)			
	NaCl	10.0 g	PBS must be autoclaved at 121°C at 15 psi for 30 min in a glass bottle. Before the addition of antibiotics and antimycotics the solution must be cooled. After add the antibiotics and antimycotic the solution must be store at 4 °C.
	KCl	0.25 g	
	Na_2HPO_4	1.44 g	
	Distilled H_2O	1 l (final volume)	
DMEM/F12 (pH = 7.08)			
	DMEM/F12 mixture	1-l envelope	The solution must be filter sterilized in the lamina flow hood passing the media through a 0.22 μm pore filter into a sterile 1-l bottle. Store at 4 °C.
	$NaHCO_3$	2.438 g	
	Distilled H_2O	1 l (final volume)	
Enzyme solution			
	Collagenase type I	0.25 g	Collagenase must be dissolve in 100 ml of PBS (37 °C) and subsequently filter sterilized by passing the solution through a 0.22 μm pore vacuum filter. The solution must be prepared just before use.
	PBS	100 ml	
DMEM/F12 + 10% serum (FBS or HS)			
	DMEM/F12 solution	442.5 ml	Basal medium must be prepared in a lamina flow hood by adding serum and antibiotics to the final volume of 500 ml. Store at 4 °C.
	Serum (FBS or HS)	50 ml	
	Penicillin/streptomycin	5.0 ml	
	Gentamicin	2.5 ml	

Table 2 – Major equipment used for cell isolation procedure

Equipments	Source	Model
Autoclave, gravity air remover type	American Sterilizer Company	P-89501-091
CO_2 water-jacket incubator	Beckman Instruments Inc.	TJ-6
Dry heat gravity oven	The Newell Group	1370 GM
Lamina flow, biological safety cabinet, Labguard Class II	Nu Aire Inc.	NU-425-4000
Peristaltic pump	Milipore Corp.	7015-72
Pipet aid	Drummond Scientific Co.	174
pH meter, digital	Corning Incorporated life Sciences	430
pH electrode	Corning Incorporated life Sciences	47636
Propane torch	Sigma Chemical Company	TS2000
Water bath	Precision Scientific	185
Microscope	Nikon	Diaphot-TMD phase inverted

Table 3 – Media and chemicals used for solutions preparations

Item	Source	Product Number
Dulbecco’s modified eagle’s medium (DMEM)/Ham’s F12 (F12)	Invitrogen	12500-062
Fetal bovine serum	Invitrogen	26140-079
Horse serum	Invitrogen	16050-114
Collagenase Type I	Invitrogen	17100-017
Gentamicin solution, 10mg/ml	Invitrogen	15710-064
Penicilin-streptomycin (pen/strep)	Invitrogen	15140-122
KCl	Sigma Chemical Company	P-5405
KH_2PO_4	Sigma Chemical Company	P-5655
NaCl	Sigma Chemical Company	S-5886
$NaHCO_3$	Sigma Chemical company	S-5761

Table 4 – Glassware and plastic supplies used for cell isolation procedure

Item	Source	Product Number
Media bottles		
125 ml with cap	Wheaton Scientific Products	219715
250 ml with cap	Wheaton Scientific Products	219717
500 ml with cap	Wheaton Scientific Products	219719
Pipettes		
5 ml glass disposable	VWR Scientific Products Corporation	53283-774
10 ml glass disposable	VWR Scientific Products Corporation	53283-776
Flask, dishes and centrifuge tube		
Tissue culture flask, 25 cm^2	Thermo Fisher Scientific, Inc.	163371
Tissue culture dish, 10 cm	Thermo Fisher Scientific, Inc.	172931
Tissue culture dish, 15 cm	Thermo Fisher Scientific, Inc.	168381
50 ml conical tube, plastic	Thermo Fisher Scientific, Inc.	339653