THAÍS CORREIA COSTA

EFFECT OF MATERNAL FEED RESTRICTION AT DIFFERENT STAGES OF GESTATION ON SKELETAL MUSCLE DEVELOPMENT AND ENERGY METABOLISM OF NEWBORN GOATS

Dissertação apresentada à Universidade Federal de Viçosa, como parte das exigências do Programa de Pós-Graduação em Zootecnia, para obtenção do título de *Magister Scientiae.*

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BIOGRAFIA

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RESUMO

COSTA, Thaís Correia, M.Sc., Universidade Federal de Viçosa, julho de 2018. **Efeito da restrição alimentar materna em diferentes estágios de gestação sob o desenvolvimento do musculo esquelético e metabolismo energético de cabritos recém-nascidos.** Orientador: Márcio de Souza Duarte.

O desenvolvimento do tecido muscular esquelético inicia-se no período pré-natal e sua formação pode ser influenciada por diversos fatores, dentre estes, os efeitos da nutrição materna vem sendo amplamente estudado. Matrizes ruminantes, frequentemente, passam por um período de restrição alimentar durante a gestação, devido à baixa disponibilidade de alimentos em determinada época do ano. Como consequência disso, o desenvolvimento fetal pode ser afetado em diversos aspectos, dentre os principais efeitos, encontram-se alterações no comprometimento de células mesenquimais indiferenciadas, que dependendo do estímulo sofrido, irão se diferenciar em linhagens celulares especificas, definindo a composição corporal do concepto, e consequentemente seu desempenho futuro. Além disso, a restrição alimentar materna influencia o metabolismo energético do concepto, visto que, há indícios que um menor aporte de nutrientes recebido pela matriz ocasiona meios adaptativos no metabolismo fetal que favorecem a captação de glicose pelos tecidos periféricos. Apesar do conhecimento atual sobre o efeito da nutrição materna no desenvolvimento do músculo esquelético da progênie, algumas divergências ainda são encontradas na literatura, possivelmente devido aos diferentes tipos e duração dos insultos testados. Contudo, cabe elucidar em que momentos da mesma a restrição nutricional da matriz pode afetar de forma negativa a formação do tecido muscular esquelético e o metabolismo energético da progênie. Assim, o primeiro capítulo deste trabalho, corresponde a uma revisão de literatura que aborda aspectos e conceitos relacionados com o desenvolvimento do tecido muscular esquelético e as vias e mecanismos que atuam sobre o mesmo, tais como, via de sinalização da insulina e mecanismos epigenéticos. No segundo capítulo, objetivou- se avaliar os efeitos da restrição nutricional da matriz durante diferentes estágios (primeira ou segunda metade) da gestação sobre o desenvolvimento e metabolismo energético do tecido muscular esquelético da progênie. A partir dos resultados obtidos, concluímos que restrição alimentar materna na segunda metade da gestação não afeta a expressão de mRNA dos marcadores de miogênese, adipogênese e fibrogênese e também não altera a população de células mesenquimais no tecido muscular esquelético de

cabritos recém-nascidos. No entanto, pode prejudicar o metabolismo energético pela redução da expressão de Hexoquinase II (*HKII*) no musculo esquelético do recém-nascido. Coletivamente, nossos dados sugerem que a restrição alimentar materna na primeira metade da gestação seguida de realimentação pode ter sustentado um crescimento compensatório no músculo esquelético do recém-nascido.

ABSTRACT

COSTA, Thaís Correia, M.Sc., Universidade Federal de Viçosa, July, 2018. **Effect of maternal feed restriction at different stages of gestation on skeletal muscle development and energy metabolism of newborn goats.** Advisor: Márcio de Souza Duarte.

The skeletal muscle development begins in the prenatal period and its formation can be influenced by several factors, among them, the effect of maternal nutrition has been widely studied. Ruminant dams, frequently, experience feed restriction in certain period of gestation, due to a season of low availability of food. As a consequence, fetal development can be affected in several aspects. The main effects, include alterations in the commitment of mesenchymal stem cells, which depending on the stimuli, will differentiate into specific cell lineage, determine the body composition of the offspring, and consequently define their future performance. In addition, maternal feed restriction impairs the energy metabolism of the offspring, since there are evidences that low intake of nutrients by the dam, cause adaptative response in the fetal metabolism that favor the uptake of glucose by peripheral tissues. Despite the current knowledge about the effect of maternal nutrition on skeletal muscle development of the offspring, some divergences are still found, possibly due to the different types and duration of the insults tested. Thus, the first chapter of this study corresponds to a literature review, which addresses aspects and concepts related with the skeletal muscle development, its pathways and mechanisms, such as, insulin signaling pathway and epigenetic mechanisms. In the second chapter we aimed to investigate the effect of maternal feed restriction at different stages of gestation (first or second half) on skeletal muscle development and energy metabolism in the progeny. With the results obtained, we conclude that maternal feed restriction at early or late gestation does not affect mRNA expression of myogenesis, adipogenesis and fibrogenesis markers neither change the mesenchymal stem cell population in skeletal muscle of newborn goats in a different manner. However, energy metabolism may be impaired by reducing Hexokinase II (*HKII)* expression in skeletal muscle of newborns. Collectively, our data suggest that maternal feed restriction in first half of gestation followed by realimentation may have sustained a compensatory growth in newborns skeletal muscle.

CAPÍTULO 1

Introdução Geral

Nutrição gestacional impacta o desenvolvimento da progênie. Os constituintes do tecido muscular (miócitos, adipócitos e fibroblastos) são originados do mesmo *pool* de células mesenquimais. Estímulos externos sofridos durante a fase gestacional podem comprometer a formação desse tecido, determinando a constituição corporal e consequentemente o desempenho pós-natal (Du et al., 2013). Coletivamente, durante esta fase, o tecido muscular esquelético é extremamente suscetível à oscilações de suprimento de nutrientes, uma vez que tem menor prioridade de deposição durante a organogênese frente à outros tecidos vitais, como órgãos e vísceras (Du et al., 2010; Zhu et al., 2006). Estudos anteriores têm associado à restrição materna nos estágios iniciais da gestação com redução do numero de fibras musculares (Zhu et al., 2006, 2004), enquanto que a restrição nutricional nos estágios finais na gestação leva a uma diminuição na hipertrofia muscular e redução da adipogênese e fibrogênese (Du et al., 2010; Underwood et al., 2010).

Além disso, há indícios que alterações no metabolismo energético do tecido muscular esquelético durante a fase pós-natal pode ser consequência de insultos sofridos durante a fase gestacional, fazendo com que o mesmo possa vir a apresentar maior afinidade por ácidos graxos do que carboidratos para síntese de ATP(Aragão et al., 2014). O tecido muscular esquelético apresenta alta sensibilidade à insulina, ou seja, são capazes de captar e utilizar a glicose circundante por meio de ação hormonal (Beauchamp and Harper, 2016). Sendo assim, o comprometimento da utilização de glicose durante a fase intrauterina pode ocasionar uma disfunção pós-natal e impactar no desempenho na progênie.

Nutrigenômica consiste no estudo do efeito da nutrição sobre genoma, ou seja, de que forma os nutrientes da dieta podem afetar a expressão de genes, que posteriormente afetará o metabolismo das proteínas e de todo o organismo (Osorio et al., 2017). Essa regulação pode ser mediada por modificações epigenéticas, que consiste em alterações hereditárias na expressão de genes. Sendo assim, esta revisão tem como objetivo abordar e associar a nutrição materna com o desenvolvimento do tecido muscular esquelético e metabolismo energético da progênie.

Desenvolvimento do tecido muscular esquelético

O tecido muscular esquelético é composto por fibras musculares, tecido adiposo e tecido conjuntivo. Estes são derivados de um mesmo *pool* de células mesenquimais multipotentes (Du et al., 2011), sendo assim, a partir de estímulos externos estas células são determinadas em linhagens especificas a partir de processos denominados miogênese, adipogênese e fibrogênese.

Miogênese

A formação de fibras musculares ocorre através do processo denominado miogênese. A miogênese se inicia no período embrionário, mediante a ação sinalizadora de tecidos vizinhos, tais como notocorda e tubo neural (Chargé et al., 2008). Estes sinais agem na ativação de genes responsáveis pela determinação de células não musculares em células com fenótipo muscular.

O processo de formação das fibras musculares ocorre em etapas, sendo regulado pela ação de diversos fatores regulatórios. A primeira etapa ocorre no período embrionário e envolve a formação de fibras primárias por meio da fusão dos mioblastos. Estas são utilizadas como molde em uma segunda onda de diferenciação

para a formação das fibras secundárias (Rehfeldt et al., 2000; Zhu et al., 2004), as quais ocorrem durante o estágio fetal e também representam a maioria das fibras musculares presentes em adultos.

A ação sinalizadora para a formação das fibras musculares inicia-se com o complexo Wnt/β-catenina e SHH, estes são responsáveis pela regulação dos fatores de transcrição Pax3 e Pax7. A expressão de Pax3 e Pax7 nas células progenitoras induz a expressão dos fatores de regulação da miogênese (MRFs). Dentre eles, Myf-5 e MyoD ocasionam a diferenciação das células precursoras em mioblastos. Os mioblastos então formados começam a expressar outros fatores de transcrição (MRFs), dentre eles, Miogenina e MRF-4. A Miogenina proporcionar a fusão dos mioblastos em miotubos, enquanto MRF-4 atua com o intuito de manter a identidade das células musculares (Du et al., 2010). Esses fatores de transcrição miogênicos então iniciam a expressão de genes específicos, incluindo as isoformas da cadeia pesada da miosina (MHC) (Du et al., 2015).

Parte dos mioblastos não se diferenciam e permanecem justapostos entre a membrana basal e o sarcolema, formando uma população de células denominadas células satélites (Chargé et al., 2008). Em resposta a estímulos, como crescimento ou traumas, estas células se tornam ativas, se proliferam e se fundem com fibras musculares já existentes ou com outras células satélites para gerar novas fibras musculares. Além disso, uma pequena porcentagem dessas células apresenta caráter multipotente o que as tornam capazes de se diferenciarem também em adipócitos ou fibroblastos (Aguiari et al., 2008; Kuang et al., 2008; Yablonka-Reuveni et al., 2008).

A massa muscular é determinada pelo número (hiperplasia) e pelo tamanho (hipertrofia) de fibras musculares. A hiperplasia ocorre exclusivamente no período

pré-natal, sendo que o número de fibras é fixado na ocasião do parto. Além disso, a hipertrofia muscular também se inicia no período pré-natal, no entanto é responsável pelo aumento em tamanho das fibras no pós-natal. Diante disso, garantir um maior número de fibras musculares ao nascer é um fator preponderante na determinação do desempenho animal pós-natal. Dessa forma, garantir adequado aporte nutricional e ambiental no período intrauterino favorece a hiperplasia muscular e consequentemente determina o desempenho animal.

Adipogênese

Os adipócitos são provenientes de células mesenquimais multipotentes e se estabelecem através de estímulos que comprometem essas células com a linhagem adipogênica. Primeiramente na fase de determinação as células mesenquimais tornam-se pré-adipócitos e perdem a habilidade de se diferenciar em outras linhagens celulares. Após isso, na segunda fase denominada diferenciação terminal, os pré-adipócitos adquirem as caraterísticas de adipócitos maduros e tornam-se capazes de responder a estímulos hormonais, estocar lipídios, e secretar hormônios que irão agir em outros tecidos, com o intuito de manter a homeostase energética (Stephens, 2012).

Em ruminantes a adipogênese inicia-se no terço médio da gestação, concomitantemente com a miogênese secundária (Feve, 2005; Gnanalingham, 2005; Muhlhausler et al., 2007), e sua regulação é mediada pela ação de fatores de transcrição. Existem inúmeras famílias de fatores de transcrição que promovem a diferenciação dos adipócitos (Stephens, 2012), no entanto dois deles são apontados como principais reguladores centrais do processo adipogênico. São estes os membros da família *CCAAT (CCAAT/enhancer binding proteins, C/EBPs)* e *PPARγ* (receptor

gama ativado por proliferadores de peroxissomas) (Moisá et al., 2014). Eles funcionam como parte de uma cascata de eventos, com a indução precoce do *C/EBPβ* e do *C/EBPδ* levando à indução do *C/EBPα*. O *C/EBPα* é um transativador do *PPARγ*, e ambos os reguladores da transcrição atuam juntos para promover a adipogênese (Wu et al., 1999). Adicionalmente, estudos identificaram o fator Zfp42 como marcador para estágios iniciais do processo de adipogênese, promovendo a adipogênese através do aumento da expressão de PPARγ (Gupta et al., 2012, 2010). Além disso, o Zfp423 pode regular a expressão do PPARγ em fibroblastos e induzi-los a se diferenciarem em adipócitos (Gupta et al., 2010). Além dos fatores que regulam positivamente a adipogênese, outros atuam como agentes inibitórios, incluindo a ação da proteína de membrana Pref-1(fator pré-adipocito 1) (Campos et al., 2016). A inibição da adipogênese pela Pref-1 ocorre via Sox9, a qual se liga as regiões promotoras de *C/EBPβ* e *C/EBPδ* e suprime sua transcrição (Sul, 2009).

Fibrogênese

A formação do tecido conjuntivo a partir dos fibroblastos ocorre através do processo denominado fibrogênese. A formação deste tecido tem inicio durante o final da gestação, sendo necessário para a geração das estruturas que revestem o musculo esquelético, como o perimísio e epimísio (Du et al., 2012). Além disso, a proporção do tecido conjuntivo correlaciona-se negativamente com aspectos de maciez da carne (Duarte et al., 2011).

A regulação da fibrogenese é mediada por citocitas e fatores de crescimentos. A citocina profibrogenica mais importante é o fator de crescimento transformador beta (TGF-β) e suas isoformas (Liu and Gaston Pravia, 2010). Todas as isoformas de TGF-β via fosforilação ativam as proteínas do complexo SMAD (Letterio and

Roberts, 1998). Este complexo ativo é translocado para o núcleo e inicia a transcrição de genes fibrogênicos (Decologne et al., 2007; Gosselin et al., 2004), incluindo fibronectina e colágeno tipo I (Kennedy et al., 2008).

O colágeno tipo I e III são os mais abundantes no musculo esquelético (Light et al., 1985). Eles se contrastam pela espessura de seus filamentos e pela proporção que se encontram nas fibras musculares, dependendo de sua capacidade oxidativa (Huang et al., 2012). O colágeno tipo I apresenta filamentos mais espessos e são encontrados em maior proporção em fibras musculares de caráter oxidativo, enquanto que o colágeno tipo III contem filamentos menos espessos e encontra-se em maior proporção em fibras musculares de caráter glicolítico.

Adicionalmente, estudos anteriores demostraram a capacidade de algumas células progenitoras mesenquimais em se comprometerem tanto em linhagens adipogênicas quanto fibrogênicas (Joe et al., 2010; Uezumi et al., 2010), o que contribui para o desenvolvimento do tecido adiposo e conjuntivo respectivamente (Uezumi et al., 2011, 2010). Essas células expressam especificamente o marcador PDGFRα no músculo esquelético e representam uma população distinta das células satélites (Uezumi et al., 2014).

Via de sinalização da insulina

A insulina é um hormônio anabólico secretado pelas células β do pâncreas em resposta aos níveis circundantes de glicose e aminoácidos após a refeição, além de ser essencial para a manutenção da homeostase de glicose e para o crescimento e diferenciação celular. A insulina regula a homeostase da glicose em vários níveis, diminuindo a gliconêogenese e glicogenólise a fim de reduzir a produção hepática de glicose, e aumentando a captação periférica de glicose, principalmente no tecido

muscular e adiposo. No fígado e adipócitos, a insulina estimula a lipogênese e reduz a lipólise, bem como aumenta a síntese e inibe a degradação proteica (Carvalheira et al., 2002).

A via de sinalização da insulina inicia-se com a ligação de insulina ao seu receptor de membrana (IR) que apresenta duas subunidades, α e β. A ligação da insulina com a subunidade α de seu receptor promove a auto-fosforilaçao da subunidade β que provoca um aumento na atividade tirosina-quinase do receptor que fosforila substratos proteicos, como (IRS)-1/2. Estes substratos (IRS)-1/2 atuam na ativação da proteína fosfatidilnositol 3- quinase (PI3K), essencial na regulação mitogênica, diferenciação celular e transporte de glicose (Saad et al., 1992). Em paralelo à ativação da via PI3K, a via CAP/Cbl é ativada e sinaliza para a translocação da proteína transportadora de glicose GLUT4 (Chiang et al., 2001). Em conjunto, as proteínas PI3K e PDK1 ativam a proteína quinase B (Akt) (Hemmings and Restuccia, 2012). A completa ativação da via IR/PI3K/Akt medeia inúmeras funções celulares, incluindo angiogênese, metabolismo, crescimento, proliferação, sobrevivência, síntese de proteínas, transcrição e apoptose (Hemmings and Restuccia, 2012).

No tecido muscular, a ativação da proteína alvo da rapamicina em mamíferos (mTOR) *dowstream* a Akt promove o aumento da síntese proteica e consequentemente hipertrofia muscular (Bentzinger et al., 2008; Bodine et al., 2001). A ativação de mTOR via fosforilação promove a ativação de seus substratos envolvidos na tradução do mRNA, incluindo a proteína quinase ribossomal S6K (S6K1 e 2) e o fator de iniciação de tradução eucarioto (eIF4E) ligante à proteína 1 (4E-BP1) (Zhu et al., 2008; Zoncu et al., 2011). Uma vez que a ativação de mTOR é regulada pela disponibilidade energética, em resposta ao déficit energético ocorre a

inibição dessa via atuante nos processos anabólicos, mediante atuação da proteína quinase dependente de AMP (AMPK) (Howell et al., 2017; Laplate, 2012).

Em relação à regulação do metabolismo de glicose, a insulina via IR/PI3K/Akt bloqueia a gliconeogênese e glicogenólise, e estimula a glicólise e glicogênese (formação de glicogênio). Um aumento fisiológico da insulina suprime a produção hepática de glicose, inibindo a glicogenólise e promovendo a síntese de glicogênio, estimulando o fluxo glicolítico e redirecionando o carbono derivado da gliconeogênese para o glicogênio (Ramnanan et al., 2009). Na glicogênese a ativação de Akt inativa por meio da fosforilação a enzima glicogênio sintase quinase 3 (GSK3), essa desativação reduz a fosforilação da enzima glicogênio-sintetase, aumentando sua atividade e consequentemente o acumulo de glicogênio (Cross et al., 1995). Além disso, em um processo dependente PI3K, a insulina atua na ativação da proteína fosfatase-1 (PP1), que desfosforila a enzima glicogênio-sintetase diretamente aumentando sua atividade (Brady et al., 1997). A regulação da gliconeogênese pela hiperinsulinemia ainda não é bem compreendida, no entanto estudos recentes apontaram que a modulação da gliconeogênese envolve a regulação transcricional que diminui a expressão dos genes gliconeogênicos fosfoenolpiruvato-quinase 1 (PCK1) e glicose-6-fosfatase (G6PC), através do fator de transcrição FOXO1 e do receptor nuclear HNF4 e seu coativador transcricional (PGC-1) (Hatting et al., 2018). Por outro lado a insulina atua positivamente na glicólise estimulando a transcrição de genes relacionados às enzimas glicolíticas, como a glicoquinase (GK) e a piruvato-quinase (PK) (Pilkis and Granner, 1992; Sutherland, 1996).

A regulação das atividades metabólicas celulares é dependente da disponibilidade de energia, advindos dos nutrientes que permitem respostas

biológicas em um determinado organismo. Em resposta a falta de energia alguns mecanismos são ativados a fim de manter a homeostase intracelular e garantir aporte energético para o metabolismo celular. Um importante sensor e regulador de energia celular é a proteína AMPK. A AMPK é ativada por fosforilação e pelo aumento da relação AMP: ATP intracelular. No músculo esquelético, sua ativação estimula a captação de glicose aumentando a translocação do transportador de glicose GLUT4, a oxidação de ácidos graxos, a biogênese mitocondrial e inibe a síntese proteica via inibição via mTOR e inibe a glicogênese (Coughlan et al., 2014; Wang et al., 2012). Também quando ativada, a AMPK estimula a via glicolítica, a fim de promover a quebra da glicose para extrair energia para o metabolismo celular. Os metabolismos regulados pela AMPK podem ser vistos na Figura 1.

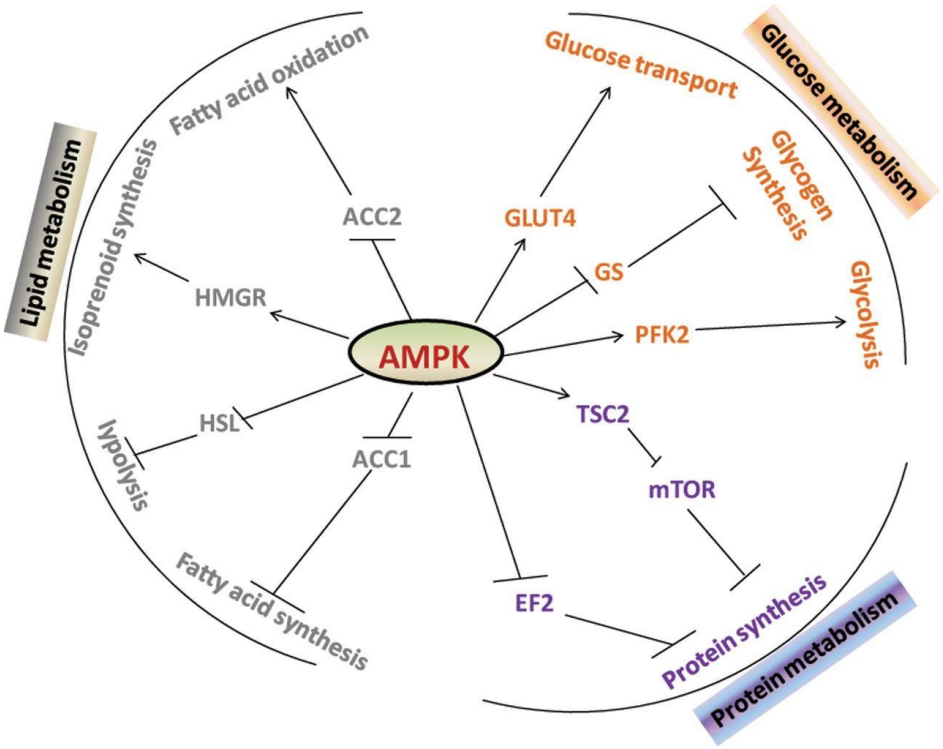

Figura1. Metabolismos regulados via ativação de AMPK (Wang et al., 2012).

Programação Fetal e Nutrição Materna

O termo "programação fetal" se baseia no conceito de plasticidade do desenvolvimento. Ou seja, a exposição a diferentes condições ambientais durante um período crítico de desenvolvimento modula e resulta em diferentes respostas fisiológicas, morfológicas e patológicas (Barker et al., 2002; Lopes et al., 2017). Durante a gestação, o feto é totalmente dependente do status nutricional materno para o atendimento de suas exigências e para um adequado desenvolvimento no ambiente intrauterino. Diante disso, insultos ou estímulos sofridos durante a fase gestacional influenciam o desenvolvimento e desempenho da progênie, com resultados que persistem por toda a vida do indivíduo (Barker et al., 2002).

Nas áreas médicas esse conceito tem sido aplicado, associando a nutrição maternal durante a gestação com o desenvolvimento e incidências de doenças crônicas no pós-natal (Barker et al., 2002). Enquanto que estudos com animais de produção têm sido realizados com o intuito de elucidar os efeitos da nutrição gestacional sob o desempenho pós-natal e como isso afeta a eficiência de produção (Du et al., 2010; Duarte et al., 2014; Marquez et al., 2017; Ojuka et al., 2000).

Aliado a esse fato, o entendimento do desenvolvimento do tecido muscular esquelético tem sido o alvo de maior interesse das pesquisas. Contudo, a formação do desse tecido é extremamente suscetível a oscilações no suprimento de nutrientes, uma vez que tem menor prioridade na partição de nutrientes durante a organogênese em relação a outros tecidos vitais, como órgãos e vísceras (Du et al., 2010; Zhu et al., 2006). Cabe ressaltar ainda que constituintes do tecido muscular esquelético (miócitos, adipócitos e fibroblastos) são originados do mesmo *pool* de células mesenquimais, e que estímulos externos sofridos durante a fase gestacional (ex.

nutrição, estresse térmico), influenciará a diferenciação e determinação de células para uma linhagem específica (Du et al., 2011).

Coletivamente, estudos indicam que restrição nutricional materna impacta negativamente na miogênese fetal, quando aplicada nos primeiros dois terços da gestação (aproximadamente no dia 105 de gestação de ovelhas), enquanto que a restrição nutricional da matriz durante o terço final impacta negativamente na hipertrofia muscular e na formação do tecido adiposo e conjuntivo (Du et al., 2010). Além disso, o metabolismo energético este intimamente relacionado com as alterações observadas em indivíduos que passaram por um período de restrição nutricional no período pré-natal. Alguns dos mecanismos de adaptação adotados pelo feto incluem o aumento da sensibilidade à insulina para favorecer a captação de glicose pelos tecidos periféricos (ex. tecido muscular e adiposo) e aumento da gliconeogênese hepática com o intuito de suprir as necessidades de glicose para os órgãos vitais (Nijland et al., 2010; Thorn et al., 2011, 2009a). Se persistirem após o nascimento, tais adaptações podem contribuir para a ocorrência de hiperglicemia, desenvolvimento de diabetes e obesidade (Thorn et al., 2013, 2011). Adicionalmente, fetos que passaram por déficit energético no ambiente intrauterino, podem apresentar alterações na composição das fibras muscular. A composição da fibra muscular esta relacionada com a capacidade oxidativa e a sensibilidade à insulina (Coen et al., 2010; He et al., 2001; Oberbach et al., 2006; Stuart et al., 2013). Fibras do tipo I ou de contração lenta possui metabolismo predominantemente aeróbio, utilizam principalmente gordura para geração de energia e são resistentes a fadiga. Enquanto que as fibras do tipo II ou de contração rápida geram energia através de vias glicolíticas e não apresentam alta resistência a fadiga.

Modificações epigenéticas

Atualmente, sabe-se que fatores como dieta e condições ambientais não afetam apenas a expressão de genes em curto e médio prazo, mas também há uma regulação de genes de médio a longo prazo. As alterações na expressão de genes de médio a longo prazo são realizadas principalmente através de alterações na disponibilidade de sequências genéticas a serem transcritas em mRNA(Osorio et al., 2017). Este conceito refere-se a alterações epigenéticas, que consiste no estudo de potenciais alterações hereditárias na expressão genica, sem que haja alteração na sequencia do DNA. Os mecanismos epigenéticos estão intimamente relacionados ao DNA e as proteínas associadas (cromatina) e incluem metilação do DNA, modificações nas proteínas histonas (pertencentes a cromatina), posicionamento do nucleossomo, e alterações pós-trascricionais mediada por microRNAs (Lopes et al., 2017).

A metilação do DNA é uma das marcas epigenéticas, que vem sendo amplamente estudada. Esta alteração consiste na adição do grupo metil ao carbono 5 de citosinas localizadas nas ilhas CpG na região promotora de um gene. O elevado grau de metilação acarretara em diminuição da expressão genica (Lopes et al., 2017). As proteínas doadoras de metil que contribui na metilação das citosinas são denominadas metiltransferases (DNMT). A metionina é um aminoácido que desempenha um importante papel para a formação das metiltranferases, uma vez que seu metabolismo produz S-adenosilmetionina (SAM) que atua com um doador de metil as DNMT (Osorio et al., 2017). Ao contrario das DNMTs, as proteínas TET1 catalisam as etapas iniciais de demetilação das citosinas do DNA (Maiti and Drohat, 2011). Yang et al. (2013) observou um aumento na expressão de Zfp423 e na

diferenciação adipogenica, decorrente da redução da metilação da região promota desse gene, ocasionado pela obesidade materna.

Além disso, as proteínas histonas estão sujeitas a inúmeras modificações pós-traducionais que controlam a estrutura e função da cromatina (Lopes et al., 2017). Algumas marcas epigenéticas estão relacionadas à indução ou repressão da expressão genica. O complexo repressor Polycomb 2 (PCR2) possui metiltransferases que catalisam a trimetilação da lisina 27 da histona H3 (H3K27me3), responsável pelo silenciamento gênico (Bernstein et al., 2006). Enquanto que o grupo de proteínas trithorax (trxG), catalisam a trimetilação da lisisa 4 da histona H3 (H3K4me3), que ativa a transcrição gênica (Schuettengruber et al., 2011). A regulação da diferenciação de células satélites é associada ao recrutamento do complexo trithorax (trxG), que promove o remodelamento da cromatina, favorecendo a transcrição dos fatores de regulação da miogênese (MRFs) (Sincennes et al., 2016). Adicionalmente, estudos anteriores com animais de produção demonstraram que inúmeros microRNAs são expressos no estagio pré-natal e estes estão ligado diretamente com a degradação ou repressão a tradução de mRNAs alvos presentes no musculo esquelético (McDaneld et al., 2009; Mobuchon et al., 2015; Qin et al., 2013; Wang et al., 2014).

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CAPÍTULO 2. Effect of maternal feed restriction at different stages of gestation on skeletal muscle development and energy metabolism of newborn goats

OBS.: Capítulo escrito de acordo com as normas da revista Animal Reproduction Science.

ABSTRACT

We aimed to investigate the effect of maternal feed restriction at different stages of gestation (first and second half) on skeletal muscle development and energy metabolism in newborn kids. Six pregnant goats were fed 50% of total digestible nutrients (TDN) and crude protein (CP) following the NRC (2007) recommendations in the first half of gestation and then fed to 100% of recommendation in the second half of gestation (treatment R-M). In the other treatment, eight pregnant goats were fed 100% of TDN and CP of NRC recommendation in the first half of gestation and then received a restriction of 50% of recommendation in the second half of gestation (treatment M-R). Birth weight, blood glucose concentration, muscle fiber number, and size were not influenced by maternal feed restriction. With the exception of the fibrogenic marker *COL III*, which tended ($P = 0.08$) to be less expressed in newborns from treatment M-R, the mRNA expression and protein abundance of myogenic, adipogenic and fibrogenic markers evaluated were not impaired ($P > 0.05$) by maternal feed treatment. With regards to newborns' energy metabolism, the mRNA expression of the glycolic enzyme *HKII* was less expressed ($P = 0.03$) in the M-R treatment. In conclusion, maternal feed restriction in the first or second half of gestation does not affect mRNA expression of myogenic, adipogenic, and fibrogenic markers neither change the mesenchymal stem cell population in skeletal muscle of newborn goats. However, it may impair the energy metabolism by reducing *HKII* expression in skeletal muscle of newborns.

Keywords: Caprine; Skeletal muscle development; Energy metabolism; Fetal programming

INTRODUCTION

The constituents of skeletal muscle, such as myocytes, adipocytes, and fibroblasts are originated from the same pool of mesenchymal cells. As a consequence, external stimuli during gestation may influence the activation, differentiation, and determination of these cells for a specific lineage (Du et al., 2012). Studies have shown that maternal undernutrition in early to mid-gestation reduces the number and composition of muscle fibers (Zhu et al., 2006, 2004), while a lack of nutrients during mid-to-late gestation affects adipogenesis and muscle fiber size (Du et al., 2010; Underwood et al., 2010).

Besides the effect of maternal nutrition during gestation on the cell fate in the skeletal muscle, changes in skeletal muscle energy metabolism may also occur in the offspring (Aragão et al., 2014; George et al., 2012; Yang et al., 2016). A higher affinity for fatty acids utilization than carbohydrates for ATP synthesis (Aragão et al., 2014) in the offspring's skeletal muscle tissue has been observed when dams are feed-restricted during gestation. Because fetal skeletal muscle is also the largest insulin-sensitive tissue in the body and the primary site for insulin-stimulated glucose utilization (Beauchamp and Harper, 2016), impairment of glucose uptake and insulin signaling may lead to changes in the animal performance after birth and, thus, it is an important trait that must be investigated as a consequence of in-utero changes.

Despite the current knowledge about the effects of maternal nutrition on the skeletal muscle development of the offspring, some divergences are still found, possibly due to the different types and duration of feeding strategies. It has been suggested that maternal feed restriction during early gestation accompanied by an adequate nutrition in the remaining gestational period may allow for fetal compensatory growth (Gonzalez et al., 2013). Thus, the objective of this study was to

evaluate the effects of feed restriction of the dams during different stages (first or second half) of gestation on the skeletal muscle development and energy metabolism of the newborn. Under this circumstance, we hypothesized that feed restriction in different stages of gestation alters the commitment of mesenchymal stem cell and energy metabolism in skeletal muscle of newborn kids.

MATERIAL AND METHODS

Animal husbandry

All animal care and handling procedures were approved by Animal Care and Use Committee of the Department of Animal Science at *Universidade Federal de Viçosa, Viçosa, Minas Gerais,* Brazil (protocol 09/2017). Initially, 60 nulliparous dairy goats were submitted to estrus synchronization using prostaglandin with a 7-day interval during the first and last application and then artificially inseminated. All dams were bred using semen from a single male, to avoid paternal effects. The day of insemination was considered day 0 of pregnancy and the beginning of the experiment. On the same day, dams were confined in individual pens ($3m^2$), and submitted to an adaptation period of 7 days, receiving the experimental diet and water *ad libitum*. Pregnancy was confirmed 30 days after insemination, and a total of 14 nulliparous dairy goats, with 50 ± 13 kg and 19 ± 7 months had pregnancy confirmed and were maintained in this study.

At the 8th day of gestation, dams were randomly assigned into two treatments with different feeding levels. Six pregnant goats were fed 50% of total digestible nutrients (TDN) and crude protein (CP) following the National Research Council (NRC, 2007) recommendations from day 8 to day 84 of gestation and then fed to 100% of TDN and CP of NRC recommendations from day 85 of gestation until the

parturition (term ~ 150 days) (treatment restriction-maintenance, R-M). In the other treatment, eight pregnant goats were fed 100% of TDN and CP of NRC recommendations from day 8 to day 84 of gestation and then received a restriction of 50% of TDN and CP of NRC recommendations from day 85 of gestation until the parturition (treatment maintenance-restriction, M-R). Those treatments consisted of the same diet for both groups with differences only in the feeding levels and gestational stage.

Both groups were fed once daily (at 7 a.m.) and every 7 days the dams were weighed in the morning before feeding. Dry matter intake and daily supply of feed were adjusted weekly based on the body weight and week of gestation of the dams.

Experimental diets consisted of 111.6 g/kg of crude protein and 676 g/kg of total digestible nutrients on dry matter (DM) basis composed of corn silage (723 g/kg DM basis), soybean meal (96 g/kg DM basis), ground corn (165 g/kg DM basis), and mineral mixture (16 g/kg DM basis), considering dairy goats' nutritional requirements (NRC, 2007) (Table 1).

Feed sampling and chemical analysis

To evaluate the nutritional characteristics of the diet, samples of roughage and concentrate were collected weekly, while leftovers were measured daily, sampled, grouped into composite samples and stored under -20° C for further chemical analysis.

To evaluate the intake of dry matter (DMI), crude protein (CPI) and total digestible nutrient (TDNI), a five-day assay was performed, in the middle of each experimental period (maintenance or restriction), in the day 46 and 117 of gestation respectively. The diet provided, the leftovers and total feces were collected and

weighed every day during the assay. After the collection period, daily samples of each animal were grouped into composite samples, one for each animal.

The roughage, concentrate, feces, and leftovers were oven-dried (55°C/ 72 hours), grounded with 1 mm knife in a Wiley mill (Willye® TE-680), and analyzed according to the standard analytical procedures of the Brazilian National Institute of Science and Technology in Animal Science (INCT- CA) for dry matter (DM; INCT-CA method G-003/1), ash (INCT-CA method M-001/1), crude protein (CP; INCT-CA method N-001/1), ether extract (EE; INCT-CA method G-004/1) and neutral detergent fiber (NDFap; INCT-CA method F-002/1), corrected for ash (NDIA; INCT-CA method N-002/1) and protein (NDIP; INCT-CA method N-004/1) (Detmann et al., 2012).

Maternal performance

Goats were weighed in the morning at day 8, 84, 85 of gestation, and right before parturition to estimate the maternal total ADG. The pregnant compound (PREG) approach (Gionbelli et al., 2015) was used to separate the maternal and gestational ADG. However, the bovine data from Gionbelli et al. (Gionbelli et al., 2015) was replaced by goat data considering the following equation as described by Castagnino et al.,(2015):

$$npEBW = -7.77 + 1.03 * BW$$

where, *npEW* is the non-pregnant body weight and BW is the body weight.

Newborn goat tissue sampling

After birth, newborns were immediately separated from the dam to avoid milk sucking. This procedure was done to avoid changes in blood glucose level as

well as changes in transcriptome profiling of skeletal muscle tissue that might occur due to increase in glucose/insulin blood levels. At birth, newborns were weighed, stunned by a nonpenetrating captive bolt, and exsanguinated. In case of twins (treatment R-M, n = 4; treatment M-R, n = 5), the heaviest offspring was taken. Blood samples were collected at exsanguination and submitted to glucose level analysis. After slaughter two skeletal muscle samples were taken from *Longissimus dorsi* muscle. One of the samples was immediately snap frozen and stored in liquid nitrogen for further RNA and protein extractions. The second sample was immediately fixed in fresh formalin 10% (wt/vol) buffered to pH = 7.4. Liver samples were taken from each offspring and immediately snap-frozen and stored in liquid nitrogen until RNA extraction was performed.

Skeletal muscle morphometric evaluation by histochemical and image analysis

Skeletal muscle samples, previously fixed in fresh 10%(wt/vol) formalin in phosphate buffer, were dehydrated in crescent ethanol series and embedded in resin using the HistoResin Mounting Kit (Leica, Solmos, Hessen, Germany). Sections of 3µm were obtained using a rotary microtome (RM 2265, Leica Biosystems, Nussloch, Germany) and stained with toluidine blue. For observation of number and diameter of muscle cells ten digital images of muscle sections per animal were taken by photomicroscope Olympus AX70 coupled with an AxioCam HRc- Zeiss camera at a magnification of 40x and analyzed with ImageJ software (National Institute of Health, Baltimore, MD, USA).

To quantify the collagen content, samples were embedded in resin using HistoResin Mounting Kit (Leica, Solmos, Hessen, Germany), sections of 3µm cut with microtome, and stained with Sirius-red solution (0.1% (wt/vol) of Direct Red 80

(Sigma-Aldrich) in 1.3% (wt/vol) aqueous picric acid solution) over-night at 60°C, washed with running water and mounted with DPX (Sigma-Aldrich). Ten digital images per animal were taken under polarized light by using a photomicroscope Olympus AX70 coupled with an AxioCam HRc- Zeiss camera at a magnification of 4x and analyzed with ImageJ software (National Institute of Health, Baltimore, MD). For quantification of the collagen content, the images were converted into grayscale, threshold to the same level to highlight and quantified as percentage of the total image area as previously described (Duarte et al., 2014).

Total RNA extraction and mRNA expression analysis

The frozen samples were powdered in liquid nitrogen and total RNA was extracted from 0.1 g of tissue using Trizol® (InvitrogenTM, Thermo Fisher Scientific®, Oregon, USA) following the manufacturer's recommendations. Total RNA was quantified by NanoVue spectrophotometer (GE Healthcare Life Sciences Inc.) and integrity assessed in 1% agarose gel.

The RNA samples were then reverse transcribed into cDNA using the GoScriptTM Reverse Transcription System Kit (Promega Corporation, Madison, WI, USA), and quantified by NanoVue spectrophotometer (GE Healthcare Life Sciences Inc.).

The primers (Table 2) for amplification of target gene and endogenous gene were designed using PrimerQuest software (www.idtdna.com/Scitools/Applications/PrimerQuest) with sequences obtained from GenBank (www.ncbi.nlm.nih.gov). Real-time quantitative PCR was performed in thermal cycler ABI Prism 7300 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) using the detection method SYBR Green (Applied

Biosystems - Foster City, CA, USA) and GoTaq® qPCR Master Mix kit (Promega Corporation, Madison, WI, USA) following cycle parameters: 95°C for 2 min and 40 cycles at 95°C for 15 s and 60°C for 60 s. Results were expressed relative to 18S using the ΔΔCt method (Bustin, 2002).

Protein abundance quantification by western-blot analysis

Total protein of *Longissimus dorsi* muscle was extracted from 0.1 g of powdered tissue in 1mL of lysis buffer (10 mM Tris HCl, 100 mM of NaCl, 0.5 mM of DDT (dithiothreitol), 2.5 mM of MgCl2, 0.5% triton X-100, and 1% protease inhibitor cocktail (Sigma-Aldrich®). Total protein content was estimated by Bradford protein assay, (Bio-Rad, Hercules, CA, USA) and stored at -80°C.

Proteins were separated by SDS-PAGE 10% gels loaded with 80 µg of protein per sample, transferred to PVDF (Polyvinylidene Difluoride) membrane and blocked for 1h at room temperature with 3% BSA (Bovine Serum Albumin, Sigma-Aldrich®) in TBS1x (Tris-Buffered Saline) for phosphorylated proteins or 3% nonfat dry milk in TBS1x for non-phosphorylated proteins. Subsequently, the membranes were incubated for 12 h at 4°C with the primary antibodies anti-INSR (0161212c032031, rabbit polyclonal IgG, Boster Bio-engineering®, Wuhan, China), anti-IRS-1 (0921412Da7123120, rabbit polyclonal IgG, Boster Bio-engineering®, Wuhan, China), GLUT4 (0911412Da270995, rabbit polyclonal IgG, Boster Bio-engineering®, Wuhan, China), anti-AMPK (2603, rabbit monoclonal IgG, Cell signaling Technology®, Massachusetts, USA), anti-p-AMPK (2535, rabbit monoclonal IgG, Cell signaling Technology®, Massachusetts, USA), anti-p-mTOR (293133, mouse monoclonal IgG, Santa Cruz Biotechnology®, Dallas, TX, USA), anti-PDGFRα (0001712M66126, rabbit monoclonal IgG, Cell signaling

Technology®, Massachusetts, USA), anti-PPARγ (0131212042027, rabbit polyclonal IgG, Cell signaling Technology®, Massachusetts, USA), anti-MYO1B (PA522347, rabbit polyclonal IgG, Thermo Fisher Scientific®, Waltham, USA), and anti-MYO2B (RE2207281C, rabbit monoclonal IgG, Cell signaling Technology®, Massachusetts, USA), diluted 1:500 in blocking solution.

After 12 hours of incubation, membranes were washed three times for 5 min with Tris-Buffered Saline and 0.1% Tween® (TBSt) and incubated with the secondary antibody (anti-rabbit IgG- Cell signaling® and anti-mouse IgG - Sigma-Aldrich®) diluted 1:5000 in blocking solution for 1h at room temperature. Afterward, membranes were washed with TBSt, revealed by ECL ClarityTM substrate (Bio-Rad, Hercules, CA), the images were generated by c-Digit® Blot scanner (Licor Biosciences, Nebraska, USA), and analyzed with the software Image Studio Digits Lite Version 5.2 (Licor Biosciences). Each SDS-PAGE gel contained protein extracted from all the treatments, as well as a loading control sample used for signal normalization.

Statistical analysis

Data collected from dams and newborn kids were analyzed in a similar fashion. For both cases, a full fixed-effect model was used and specific model terms were removed from the model when *P*-value > 0.10. The following full model was tested:

$$Y_{ijk} = \mu + D_i + T_j + (DT)_{ij} + BW_{ijk} + e_{ijk}$$

where, Y_{ijk} is the observed measurement; μ is the overall mean; D_i is the fixed-effect of the i^{th} level of maternal dietary treatment (2 levels); T_j is the fixed effect of the j^{th} level of twins (2 levels; “yes” or “no”); DT_{ij} is the interaction between

D and *T*; BW_{ijk} is the covariate of initial body weight of the kth dam or birth weight of the kth kid; and e_{ijk} is the random error associated with Y_{ijk}, with $e_{ijk} \sim N(0, \sigma_e^2)$.

For each of the characteristics analyzed, effects in the model, with the exception of *D* and *T*, were removed when *P*-value > 0.10. Prior to the final analyses, extreme data were removed when Studentized residuals were not within ± 3 standard deviations, and normality (*P*-value > 0.05) was assessed using Shapiro-Wilk's test. As expected, the gene expression data was not normal and it was transformed using the ΔΔCt method (Bustin, 2002). Least-squares means were separated using Fisher's least significant difference test. Results were deemed significant when *P*-value ≤ 0.05 and trending when 0.05 < *P*-value ≤ 0.10. All analyses were performed using SAS 9.4 (Statistical Analysis System Institute, Inc., Cary, NC, USA).

RESULTS

Maternal intake according to feed restriction

The maternal dry matter intake (DMI), crude protein intake CPI and total digestible nutrients intake (TDNI) during the first experimental period (8-84d of gestation) differed between treatments. The dams who were feed restricted in this period (treatment R-M) presented less intake ($P < 0.001$) than dams from treatment M-R. During the last experimental period (85d-parturition), with exception of TDNI ($P = 0.249$), the dams who were feed restricted during this period (treatment M-R) presented less DMI and CPI ($P < 0.001$) than the dams from treatment R-M (Table 3).

Maternal performance according to feed restriction

The total average daily gain (ADG) of the dams, during the first (8-84d of gestation) and last (85d-parturition) experimental periods differed ($P < 0.001$) between treatments, however, when observed the total ADG from the entire gestation, no differences ($P = 0.41$) were observed between treatments (Table 4).

Similarly, the ADG of maternal tissues was different ($P < 0.001$) between treatments and experimental periods (Table 4). The dams from treatment R-M, while restricted in the first experimental period, gained less weight than dams from treatment M-R. Conversely, the dams from treatment M-R presented less weight gain than the dams from treatment R-M in the last experimental period. The ADG of maternal tissues during the whole gestation were similar between treatments ($P = 0.57$).

Collectively, this data shows the effectiveness of maternal dietary treatments as it demonstrates that the total ADG and maternal tissue ADG at the end of experimental period was the same between treatments, and only differed during different periods (feed-restriction x maintenance at 8-84 days of gestation or 85 days of gestation to parturition). Moreover, the increase in body weight gain of the dams in both treatments observed during the last period can be attributed due to the greater fetal growth that occurs at this stage compared to the first half of the gestation

No differences were observed in the gestation ADG. That means that the weight of the fetal attachments remained similar between treatments during the first ($P = 0.99$), the last ($P = 0.93$) experimental periods, and also when evaluated during the entire gestation ($P = 0.89$) (Table 4).

Birth weight and blood glucose levels of newborn goats

No differences were observed for birth weight (P = 0.46), or blood glucose levels (P = 0.65) of offspring born from dams that were feed-restricted from 8 to 84 days of gestation (R-M) compared to those born from dams that were feed-restricted from 85 days of gestation to parturition (M-R) (Fig. 1).

Skeletal muscle cell number and size of newborn goats

No differences were observed in cell number (P = 0.87) or in cell size (P = 0.12) of offspring born from dams that were feed-restricted from 8 to 84 days of gestation (R-M) compared to those born from dams that were feed restricted from 85 days of gestation to parturition (M-R) (Fig. 2).

Molecular markers of cell fate in skeletal muscle of newborn kids

No difference was observed for mRNA expression of *PAX3* (P = 0.94), a marker of myogenic events, in skeletal muscle of newborn goats between the treatments R-M and M-R (Table 5). However, mRNA expression of *PAX7,* a marker of satellite cells, showed effects of interaction between number of fetuses (single or twin) and maternal nutritional treatment during gestation (P < 0.001; Fig. S1).

Expression of adipogenic markers such as *Zfp423* (P = 0.79), *PPARγ* (P = 0.19) and the inhibitor of adipogenesis *Pref-1* (P = 0.84) were not different between treatments (Table 5). No difference was observed for mRNA expression of *COL I* (P = 0.28), a fibrogenic marker, neither in total collagen content (P = 0.14; Fig. 3) between the treatments R-M and M-R (Table 5). On the other hand, mRNA expression of *COL III* (P = 0.08) tended to be greater in the treatment R-M than treatment M-R. Moreover, the mRNA expression of *PDGFRα*, a growth factor

receptor and mesenchymal stem cell marker, did not differ between treatments (P = 0.87) (Table 5). The abundance assessed by western-blot analysis of PDGFRα (P = 0.82) and the adipogenic marker PPARγ (P = 0.86) revealed no differences between treatments (Fig. 4).

Molecular markers of energy metabolism in skeletal muscle and liver of newborn goats

No differences were observed for mRNA expression of the markers related with energy status in the skeletal muscle, including *INSR* (P = 0.75), *IRS-1* (P = 0.91), and *GLUT4* (P = 0.19) between the treatments R-M and M-R (Table 6). The mRNA expression of *HKII* (P = 0.03) was greater in skeletal muscle of newborn kids from treatment R-M than treatment M-R, while mRNA expression of *PFKM* (P = 0.15) and *PKM* (P = 0.63) were not different between treatments (Table 6).

No differences were observed in the abundance of proteins related with energy metabolism, such as IRS-1 (P =0.94), GLUT4 (P = 0.54), AMPK (P = 0.89), the active form p-AMPK (P = 0.99), and p-mTOR (P = 0.57) (Fig. 5). However, the abundance of INSR showed effects of interaction between number of fetuses (single or twin) and maternal nutritional treatment during gestation (P = 0.05; Fig. S2). The type of skeletal muscle fiber was evaluated by protein abundance of MYO1B (P =0.93) and MYO2B (P = 0.70) which did not differ between treatments (Fig. 6).

With regard to energy metabolism markers in the liver, no differences were observed for mRNA expression of *INSR* (P = 0.22), *IRS-1* (P = 0.80), *HK4* (P = 0.32) and *PKLR* (P = 0.28) between the treatments R-M and M-R (Table 6).

DISCUSSION

Maternal feed restriction at different stages of gestation and skeletal muscle development

The difference observed in maternal total ADG, and especially the maternal tissues ADG between periods highlights the efficacy of maternal nutritional treatment applied. Moreover, the difference in maternal weight gain did not appear to impact fetal growth, as well as the gestation ADG and newborns weight were similar, which corroborates with other studies (Du et al., 2010; Underwood et al., 2010; Wu et al., 2006) and indicates that birthweight might not be affected by maternal nutrition status.

Maternal feed restriction was demonstrated to decrease muscle fiber number in the offspring when applied before the second wave of myogenesis (approximately 105d of gestation in ewe and 210d in cattle) (Du et al., 2010). After that, there is no evidence of increase in number of muscle fibers, thus, postnatal muscle growth is mainly due to increase of muscle fiber size. Therefore, we hypothesized that newborns from the R-M treatment would present less and larger muscle fibers than newborns from the M-R treatment. However, there were no differences in cell number and size between treatments. At the day in which the treatment was changed (day 85), secondary myogenesis was still occurring and despite the application of restriction since day 8 of gestation, it is suggested that there was a compensation when the treatment was changed, resulting in the equivalence between the newborns of both treatments, in terms of number and size of muscle cells.

Results involving skeletal muscle formation related to maternal nutritional status lead to investigate molecular markers and events related to cellular commitment. A genome-wide analysis showed that overexpression of *PAX3* and

PAX7 upregulates genes associated with growth and proliferation and downregulates genes related to myogenic differentiation (Buckingham and Relaix, 2015). The *PAX3* and *PAX7* positive-cell population also assume the characteristic of satellite cell, a quiescent cell that when activated contributes to postnatal muscle growth (Lagha et al., 2008). Given the critical need of the transcriptional factors *PAX3* and *PAX7,* for muscle development and growth, it is essential that these cells remain available to the fetus throughout gestation and adult life (Gonzalez et al., 2013). Based on this, the results of *PAX7* expression presented significant interaction among number of fetuses (single or twin) and maternal nutritional treatment. Where greater expression of *PAX7* in singletons from treatment R-M and twins from treatment M-R may be related with greater number of satellite cells postnatal and further increase the number of muscle fibers and form new fibers when required. PAX7 recruits histone methyltransferase complex (Trithorax complex), which leads to chromatin modification by the methylation of histone H3 lysine 4 (H3K4), an epigenetic marker that stimulates transcriptional activation of myogenic regulatory factors (MRFs) (Buckingham and Relaix, 2015; Sincennes et al., 2016). Additionally, it was demonstrated changes in temporal effects of MRFs and decreased fusion index in satellite cells cultured from offspring of restricted-fed ewes (Raja et al., 2016), which may lead to impaired muscle growth postnatal.

Besides myogenesis, events related with adipogenesis and fibrogenesis can be influenced by maternal nutrition (Duarte et al., 2014; Huang et al., 2012; Marquez et al., 2017; Paradis et al., 2017). It is known that the formation of muscle cells is diminished as gestation progress concomitantly with the increase of adipogenesis and fibrogenesis (Du et al., 2010). The intrauterine growth restriction environment trigger enhancement of adipogenesis postnatal and consequently to obesity (Ross and Desai,

2013). It was demonstrated that the mechanisms involved on enhanced adipogenesis resulted in programmed appetite/satiety and function (lipogenesis) (Fukami et al., 2012; García et al., 2010; Ross and Desai, 2013). Moreover, previous studies have shown the ability of some mesenchymal progenitor cells in commit in both adipogenic and fibrogenic lineages (Joe et al., 2010; Uezumi et al., 2010), which directly contribute to intramuscular fat and connective tissue development (Uezumi et al., 2011, 2010). These cells specifically express the marker PDGFRα in skeletal muscle and represent a cell population distinct from satellite cells (Uezumi et al., 2014). Therefore, greater expression of PDGFRα would contribute for the increase of fat or connective tissue postnatal. As such, we hypothesized that feed restriction mainly during the second half of gestation would impair the formation of adipocytes and fibroblasts.

In this study, we used PDGFRα content as a marker of mesenchymal progenitor cell, and the results suggested that maternal feed restriction in both stages evaluated did not impact the number of these cells in the newborn. Concomitantly the mRNA expressions of the markers related with adipogenesis were also not altered. Similarly, collagen content and the mRNA expression of *COL I* did not present differences between treatments, indicating that the maternal restriction in both periods applied did not differently impact in fibrogenesis. However, the expression of the fibrogenic marker *COL III* was tended to be lower in newborns from the M-R treatment. These data suggest that maternal feed restriction in the second half of gestation does not impact the adipose tissue formation/ fat deposition and may also acts decreasing the formation of connective tissue by decreasing *COL III* expression.

Maternal feed restriction at different stages of gestation and skeletal muscle energy metabolism

Several studies have proposed a strong connection between intrauterine growth restrictions with alterations in energy metabolism, including insulin pathways (Aragão et al., 2014; George et al., 2012; He et al., 2013). Compared with vital organs, skeletal muscle has lower priority for nutrient portioning in the developing fetus. Therefore, maternal feed restriction may lead to adaptive response in the newborn energy metabolism, in order to maintain glucose supply to vital organs (Thorn et al., 2013). In response to glucose, insulin is secreted and its pathways are highly regulated and sensitive to changes in oxygen, nutrient status, and cell stress via input from several major energy and stress sensors including AMP-activated protein kinase (AMPK) (Thorn et al., 2009b). The AMPK acts as an important cellular energy sensor and is activated by phosphorylation and increase in the intracellular AMP:ATP ratio. In skeletal muscle, its activation leads to stimulate glucose uptake by enhancing the glucose transporter GLUT4 translocation, fatty acids oxidation, mitochondrial biogenesis, inhibition of glycogen synthesis, and protein synthesis via inhibition of mTOR (Coughlan et al., 2014; Wang et al., 2012). Also, when activated, AMPK leads to increase glycolysis pathways in order to promote breakdown of glucose to extract energy for cellular metabolism. Hence, maternal feed restriction would promote an increase in the activated AMPK. However, no differences were observed in protein abundance of p-AMPK neither p-mTOR. Our findings suggest that even on restriction the dams were able to provide sources of ATP, such as glucose to supply fetal growth.

It has been shown that decreased muscle glucose uptake is related with decreased expression of *HKII* (Vestergaard et al., 1995; Wang et al., 2016). Despite

the absence of difference in GLUT4 abundance, newborns from treatment M-R displayed lower mRNA levels of *HKII*. The principal role of HKII is to convert glucose to glucose-6-phosphate (G6-P) (Roberts and Miyamoto, 2015), ensuring the permanence of this metabolite inside the cell. The G6-P can be further metabolized through glycolysis or glycogenesis (Long et al., 2005). Interestingly, mRNA levels of the enzymes that catalyze glycolysis downstream G6-P were not influenced by maternal feed treatment during gestation. Although enzymes associated with glycogen synthesis were not evaluated in this study, our findings suggest that maternal feed status during the second half of gestation impairs the storage of glucose as glycogen in skeletal muscle of the offspring. In pigs, impaired glucose tolerance was associated with decreased muscle glycogen synthesis, caused by downregulation of *HKII,* glycogen synthase (*GS*) expression and glycogen content (Wang et al., 2016).

In response to lack of energy, fetuses that experienced intrauterine growth restriction may present changes in fiber composition which is closely related with oxidative capacity of muscle and insulin sensitivity (Coen et al., 2010; He et al., 2001; Oberbach et al., 2006; Stuart et al., 2013). Previous studies showed a positive correlation between slow (type I) muscle fibers and insulin sensitivity. Results involving changes in fiber composition have been variable between studies. In response to maternal undernutrition during early to mid-gestation, the offspring increased (Daniel et al., 2007; Zhu et al., 2006) or decreased the number of fast (type II) muscle fiber in the post-natal. While the undernutrition during late gestation triggered low slow (type I) muscle fiber in the fetuses (Costello et al., 2008). Moreover, lambs that were restricted in the intrauterine period had greater abundance of type IIa fibers at birth, but at 3 months of age, no difference was observed

between the treatments, suggesting the adequate diet postnatal allowed fiber type switching (Reed et al., 2014). Thus, the absence of difference between the newborns from both treatments observed in our results suggests that maternal feed restriction during the first half of gestation did not contribute for changes in muscle fiber composition or there was a compensation which allowed restore the fiber composition when the dams received appropriate conditions that favored fetal growth after a period of feed restriction.

CONCLUSION

Our results indicate that maternal feed restriction at early or late gestation does not affect mRNA expression of myogenic, adipogenic and fibrogenic markers neither changes the mesenchymal stem cell population in skeletal muscle of newborn goats in a different manner. However, energy metabolism may be impaired by reducing *HKII* expression in skeletal muscle of newborns. Collectively, our study suggests that maternal realimentation during the second half gestation of dams that were feed-restricted during the first half of gestation is able to overcome the impairment of skeletal muscle development in goats.

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Table 1

Feeds and chemical composition of the experimental diet

Item[a] (g/kg)	Ingredients	
	Corn Silage	**Concentrate**
DM[b]	257±6.93	845±0.04
OM[c]	949±2.00	951±0.14
CP[c]	71.2±1.55	145±3.07
EE[c]	23.4±1.44	7.00±0.50
NDFap[c]	542±9.21	147±26.2
NFC[c]	313±9.94	652±29.7

[a]Least square means ± standard errors of: DM: dry matter; OM: organic matter; CP: crude protein; EE: ether extract; NDFap: neutral detergent fiber corrected for ash and protein; NFC: non-fiber carbohydrate (NCF= [100-(%NDF + %CP + %EE + %Ash)].

Concentrate composition: 96 g/kg of soybean meal, 165 g/kg of ground corn, 3 g/kg of CaCO3 and 3 g/kg of CaHPO4 (DM basis).

[b]g/kg as feed

[c]g/kg DM

Table 2

List of primers for gene expression analysis by RT-qPCR

Gene	Gene abbreviation	NCBI access code	Primer
Paired box 3	PAX3	XM_005676604.3	F: GGACTAAGAGCGAGCAAAC R: CAGGTGAAGGCGAAATAGAC
Paired box 7	PAX7	XM_018054740.1	F: CATGCTTCCTCCAACTTCTC R: AGCACATCACCACGTTTC
Platelet-derived growth factor receptor A,	PDGFRα	XM_018049462.1	F: GGTGATGCTTTGGGAGATG R:GCTCAGTCTTCACGCTTAC
Preadipocyte factor 1	PREF-1	NM_001314212.1	F: CATGACCACCTTCACCAAG R: AACAGACCGCACAGAGA
Zinc finger protein 423	Zfp423	XM_018061954.1	F: GAAGAAGATGCGGGATGAC R: TGGTCTTCAGGTGGATCTC
Peroxissome proliferator actiated-receptor gamma	PPARγ	NM_001285658.1	F: GACATCGACCAACTGAACC R: TCAGCGGGAAGGACTTTA
Collagen type I, alpha 1	COL1A1	XM_018064893.1	F: GCTTCCTGTAAACTCCTTCC R: GGCTTCAGTTTGGGTTGT
Collagen type III, alpha 1	COL3A1	XM_005675869.3	F: AGGTGAACCCGGTAAGAA R: CACCCTTAGGTCCTGGAATA
Hexokinase II	HKII	XM_018054976.1	F: CATGATGACCTGTGGCTATG R: GCGCATCTCTTCCATGTAG
Phosphofructokinase muscle	PFKM	XM_018047861.1	F: CTGAGTGGAGTGACTTGTTG R: AGGTAGCTGGACTTGGTAG
Pyruvate kinase muscle	PKM	XM_005685176.3	F: GGGATGAAGGAGGGATACA

			R: CTGAATCGGGTACACAAAGG
Insulin receptor	INSR	XM_018051135.1	F: CGGACGGATTCTGACTTTG
			R: GCCTTTGAACCAGAGAGAAG
Insulin receptor substrate 1	IRS-1	XM_018058864.1	F: GTCCCTCCACAGCTCTATAA
			R: CACCTCCTCTCAGCAACTA
Solute carrier family 2 member 4	GLUT4	NM_001314227.1	F: CCCGCTACCTCTACATCAT
			R: AGCCAACACCTCAGACA
Glucokinase (Hexokinase 4)	HK4	XM_013963394.2	F: GAAGGTGATGAGGCGAATG
			R: TAGGTGGGCAGCATCTT
Pyruvate kinase liver and red blood cell	PKLR	XM_018046254.1	F: CTCTCAACTGGTCCCTAAGA
			R: GAGACTGTGGCCATGATTAC
18 S ribosomal	18S	NM_001033614	F:CCTGCGGCTTAATTTGACTC
			R: AACTAAGAACGGCCATGCAC

Table 3

Intake according to feed restriction of the dams during different stages (first or second half) of gestation.

Item[a] (g/day)	Treatment		*P*-value
	M-R[b]	R-M[c]	
	8 – 84d of gestation		
DMI	851±29.7	432±34.3	<0.001
CPI	79.0±4.03	41.2±4.67	<0.001
TDNI	581±22.2	393 ± 25.7	<0.001
	85d - parturition		
DMI	719±32.3	982±37.3	<0.001
CPI	64.0±2.48	87.9±2.86	<0.001
TDNI	533±16.4	503±18.9	0.249

[a]Least square means ± standard errors of dry matter intake (DMI), crude protein (CPI) and total digestible nutrients intake (TDNI);

[b]M-R: maintenance-restriction treatment;

[c]R-M: restriction-maintenance treatment

Table 4

Performance according to feed restriction of the dams during different stages (first or second half) of gestation.

Item[a] (g/day)	Treatment		*P*-value
	M-R[b]	R-M[c]	
	8 – 84d of gestation		
Total ADG	95.5±7.99	-1.24±9.23	<0.001
Maternal tissues ADG	75.0±8.24	-24.6±9.25	<0.001
Gestation ADG	22.2±2.44	22.2±2.83	0.999
	85d - parturition		
Total ADG	27.3±15.4	191±17.8	<0.001
Maternal tissues ADG	-80.5±15.3	82.1±17.8	<0.001
Gestation ADG	107±11.4	109±13.2	0.931
	8d - parturition		
Total ADG	77.0±8.61	88.2±9.94	0.411
Maternal tissues ADG	17.4±8.48	24.9±9.79	0.569
Gestation ADG	60.9±6.37	62.2±7.36	0.894

[a]Least square means ± standard errors of total average daily gain (ADG), maternal tissues ADG and gestation ADG of dams from the first (8 to 84d of gestation), the last (85d to parturition) experimental periods, and during the entire experimental period (8d to parturition)

[b]M-R: maintenance-restriction treatment;

[c]R-M: restriction-maintenance treatment

Table 5

Least square means ± standard errors for mRNA expression of myogenic, adipogenic, fibrogenic and mesenchymal stem cell markers evaluated on newborns skeletal muscle according to maternal nutritional treatment

Item[a] (Arbitrary units)	Treatment		*P*-value
	M-R[b]	R-M[c]	
Myogenic marker			
PAX3	2.56±0.48	2.50±0.55	0.938
Adipogenic markers			
Zfp423	1.66±0.25	1.76±0.28	0.791
PPARγ	1.40±0.18	1.79±0.21	0.191
PREF-1	4.28±0.80	4.03±0.92	0.842
Fibrogenic markers			
COL1	4.19±0.37	3.55±0.43	0.283
COL3	1.39±0.11	1.72±0.13	0.081
Mesenchymal stem cell marker			
PDGFRα	2.04±0.42	1.94±0.48	0.873

[a]PAX3: Paired box 3; Zfp423: Zinc finger protein 423; PPARγ: Peroxissome proliferator activated-receptor gamma; PREF-1: Preadipocyte factor 1; COL1: Collagen type I, alpha 1; Collagen type III, alpha 1; PDGFRα: Platelet-derived growth factor receptor A.

[b]M-R: maintenance-restriction treatment

[c]R-M: restriction-maintenance treatment

Table 6

Least square means ± standard errors for mRNA expression of makers related with energy metabolism evaluated on newborns skeletal muscle and liver according to maternal nutritional treatment

Item[a] **(Arbitrary units)**	**Treatment**		***P*-value**
	M-R[b]	**R-M**[c]	
Skeletal muscle			
INSR	2.27±0.32	2.43±0.37	0.748
IRS-1	1.63±0.29	1.58±0.34	0.909
GLUT4	0.89±0.06	1.02±0.07	0.189
HKII	1.00±0.09	1.31±0.08	0.034
PFKM	0.91±0.08	1.10±0.09	0.146
PKM	5.41±0.90	4.73±1.04	0.629
Liver			
INSR	1.25±0.17	0.92±0.18	0.223
IRS-1	2.28±0.47	2.10±0.51	0.805
HK4	3.74±0.99	5.30±1.15	0.325
PKLR	3.06±0.67	1.91±0.77	0.282

[a]INRS: Insulin receptor; IRS-1: Insulin receptor substrate 1; GLUT4: Solute carrier family 2 member 4; HKII: Hexokinase II; PFKM: Phosphofructokinase muscle; PKM: Pyruvate kinase muscle; HK4: Glucokinase (Hexokinase 4); PKLR: Pyruvate kinase liver and red blood cell.

[b]M-R: maintenance-restriction treatment

[c]R-M: restriction-maintenance treatmen

t

Figure captions

Fig. 1. Average birth weight (A) and blood glucose level (B) of newborn kids from dams that were fed at maintenance from 8-84 days of gestation followed by feed-restriction from 85 days of gestation to parturition (M-R), and newborn goats from dams that were feed restricted from 8-84 days of gestation followed by fed at maintenance from 85 days of gestation to parturition (R-M).

Fig. 2. Cell number (A) and sizer (B) of *Longissimus dorsi* muscle of newborn goats from dams that were fed at maintenance from 8-84 days of gestation followed by feed- restriction from 85 days of gestation to parturition (M-R) (C), and newborn goats from dams that were feed restricted from 8-84 days of gestation followed by fed at maintenance from 85 days of gestation to parturition (R-M) (D). Skeletal muscle samples were embedded in resin, 3µm sectioned, stained with toluidine blue and visualized in 40-fold magnification.

Fig. 3. Collagen content in skeletal muscle of newborn goats from dams that were fed at maintenance from 8-84 days of gestation followed by feed- restriction from 85 days of gestation to parturition (M-R), and newborn goats from dams that were feed restricted from 8-84 days of gestation followed by fed at maintenance from 85 days of gestation to parturition (R-M) (A). Representative images of skeletal muscle stained with Sirius-red and observed under brightfield light (B and C) and polarized light (D and E) at 4-fold magnification.

Fig. 4. Representative images of western-blot analysis and protein abundance for PDGFRα (A), PPARGγ (B). The protein abundance was measured in skeletal

muscle of newborn goats from dams that were fed at maintenance from 8-84 days of gestation followed by feed- restriction from 85 days of gestation to parturition (M-R), and newborn goats from dams that were feed restricted from 8-84 days of gestation followed by fed at maintenance from 85 days of gestation to parturition (R-M).

Fig. 5. Representative images of western blotting analysis and protein abundance for IRS-1 (A), GLUT4 (B), AMPK (C), P-AMPK (D) and P-mTOR (E). The protein abundance was measured in skeletal muscle of newborn goats from dams that were fed at maintenance from 8-84 days of gestation followed by feed- restriction from 85 days of gestation to parturition (M-R), and newborn goats from dams that were feed restricted from 8-84 days of gestation followed by fed at maintenance from 85 days of gestation to parturition (R-M).

Fig. 6. Representative images of western blotting analysis and protein abundance for MYO1B (A) and MYO2B (B). The protein abundance was measured in skeletal muscle of newborn goats from dams that were fed at maintenance from 8-84 days of gestation followed by feed- restriction from 85 days of gestation to parturition (M-R), and newborn goats from dams that were feed restricted from 8-84 days of gestation followed by fed at maintenance from 85 days of gestation to parturition (R-M).

Supplementary Figures

Fig. S1. Effects of the interaction between the number of fetuses (single or twin) and maternal nutritional treatment during gestation on PAX 7 mRNA expression measured by RT-qPCR analysis. The mRNA abundance was measured in skeletal muscle of newborn goats from dams that were fed at maintenance from 8-84 days of gestation followed by feed- restriction from 85 days of gestation to parturition (M-R), and newborn goats from dams that were feed restricted from 8-84 days of gestation followed by fed at maintenance from 85 days of gestation to parturition (R-M). Different letters indicate significant differences among the groups ($P < 0.05$).

Fig. S2. Effects of the interaction between the number of fetuses (single or twin) and maternal nutritional treatment during gestation on Insulin Receptor (INSR) protein abundance measured by western blotting analysis. The protein abundance was measured in skeletal muscle of newborn goats from dams that were fed at maintenance from 8-84 days of gestation followed by feed- restriction from 85 days of gestation to parturition (M-R), and newborn goats from dams that were feed restricted from 8-84 days of gestation followed by fed at maintenance from 85 days of gestation to parturition (R-M).

Figure 1.

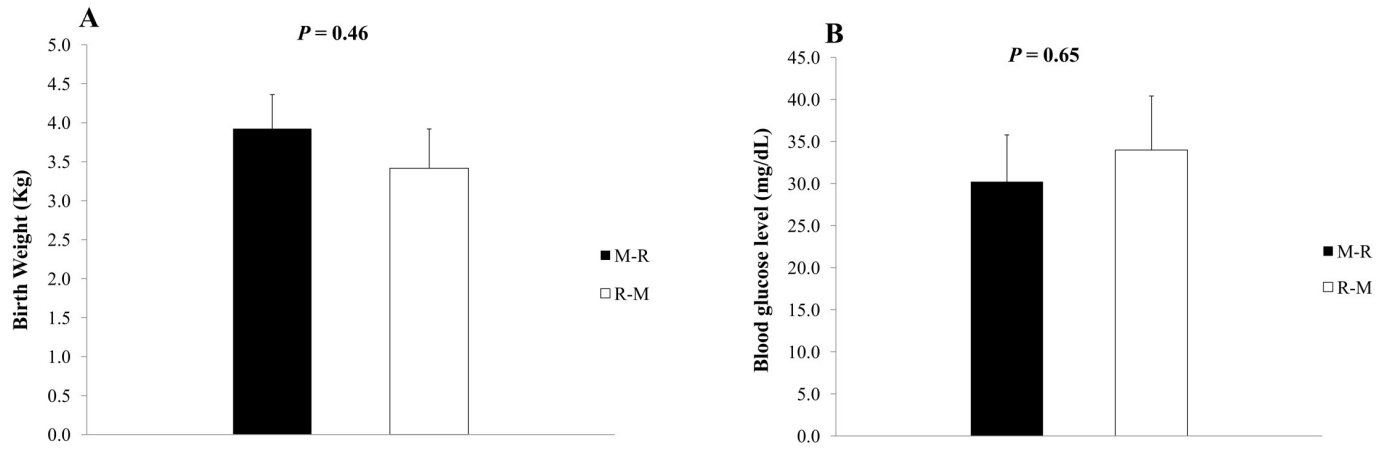

A
P = 0.46
Birth Weight (Kg)
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
M-R
R-M
B
P = 0.65
Blood glucose level (mg/dL)
45.0
40.0
35.0
30.0
25.0
20.0
15.0
10.0
5.0
0.0
M-R
R-M

Figure 2.

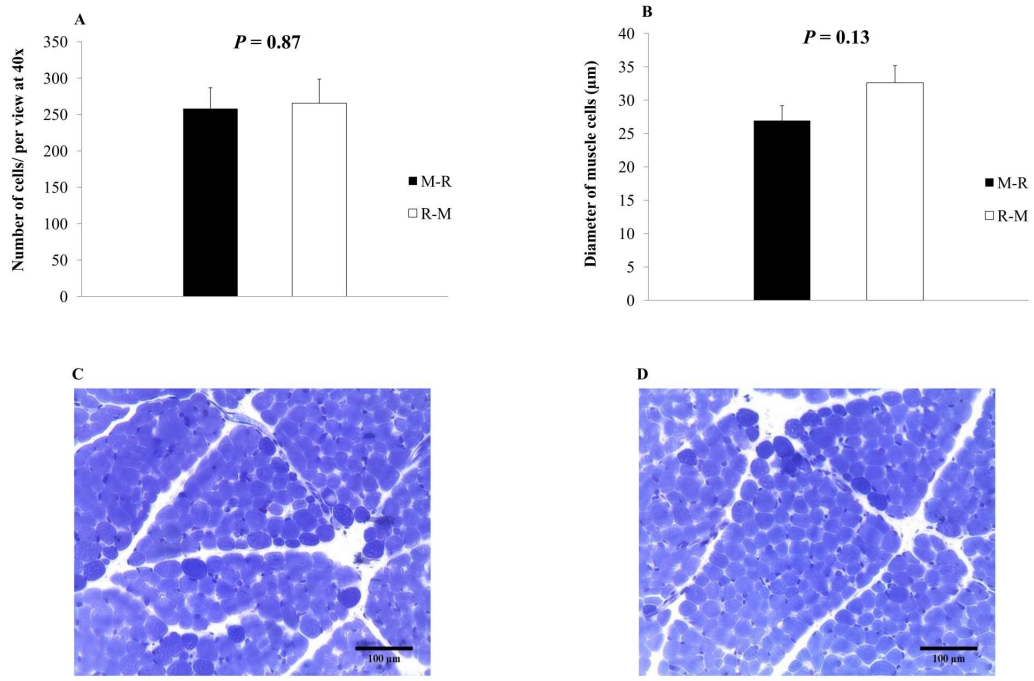

A
P = 0.87
Number of cells/ per view at 40x
350
300
250
200
150
100
50
0
M-R
R-M
B
P = 0.13
Diameter of muscle cells (µm)
40
35
30
25
20
15
10
5
0
M-R
R-M
C
100 µm
D
100 µm

Figure 3.

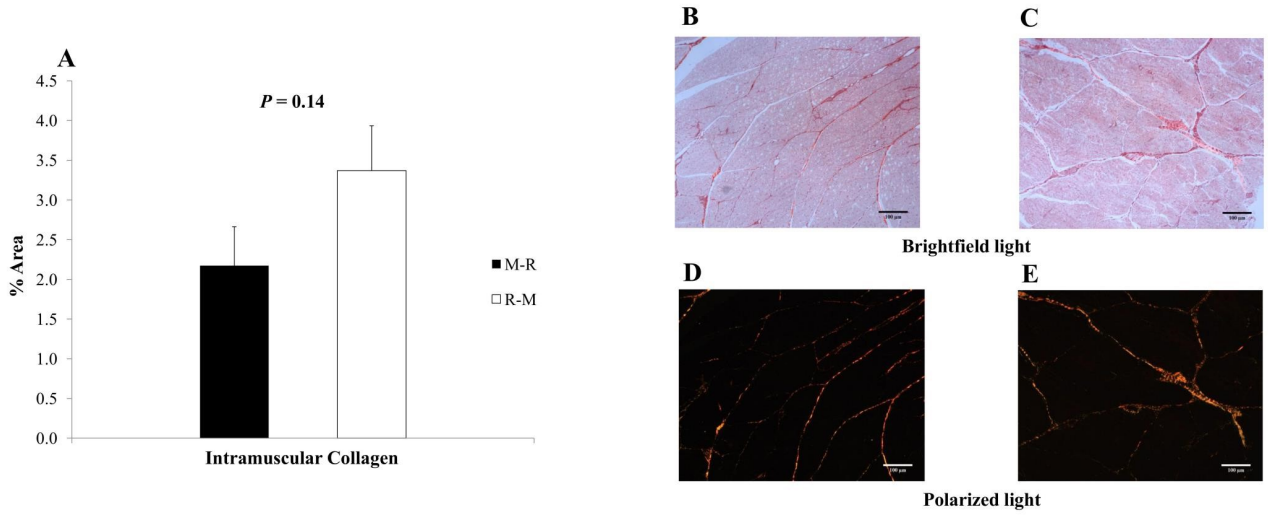

A
P = 0.14
% Area
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
M-R
R-M
Intramuscular Collagen
B
C
Brightfield light
D
E
Polarized light

Figure 4.

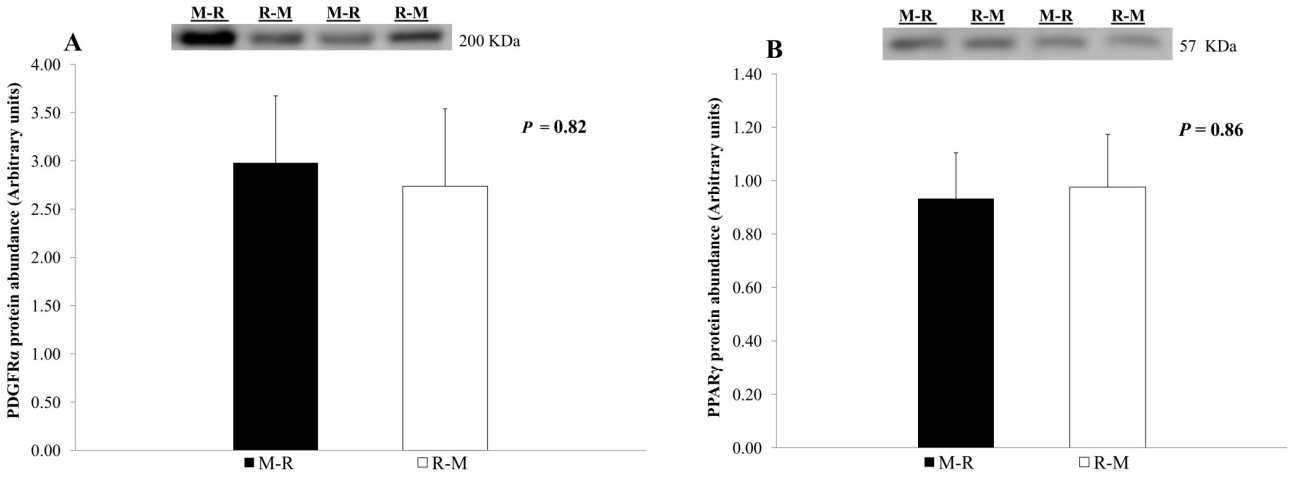

A
M-R
R-M
M-R
R-M
200 KDa
PDGFRα protein abundance (Arbitrary units)
4.00
3.50
3.00
2.50
2.00
1.50
1.00
0.50
0.00
P = 0.82
■ M-R
□ R-M
B
M-R
R-M
M-R
R-M
57 KDa
PPARγ protein abundance (Arbitrary units)
1.40
1.20
1.00
0.80
0.60
0.40
0.20
0.00
P = 0.86
■ M-R
□ R-M

Figure 5.

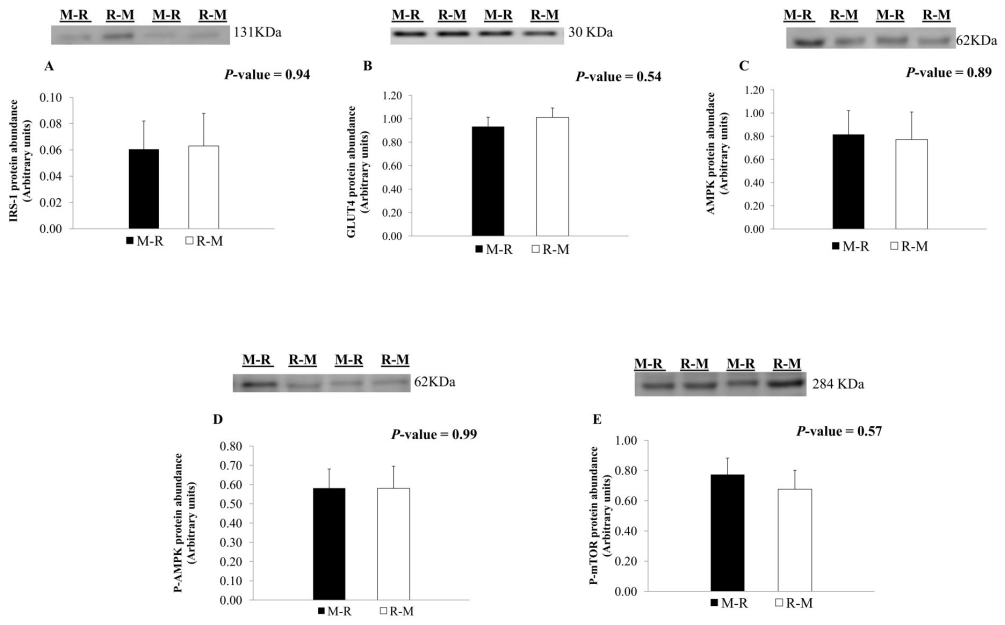

M-R R-M M-R R-M
131KDa
A
P-value = 0.94
IRS-1 protein abundance (Arbitrary units)
0.10
0.08
0.06
0.04
0.02
0.00
M-R R-M
M-R R-M M-R R-M
30 KDa
B
P-value = 0.54
GLUT4 protein abundance (Arbitrary units)
1.20
1.00
0.80
0.60
0.40
0.20
0.00
M-R R-M
M-R R-M M-R R-M
62KDa
C
P-value = 0.89
AMPK protein abundance (Arbitrary units)
1.20
1.00
0.80
0.60
0.40
0.20
0.00
M-R R-M
M-R R-M M-R R-M
62KDa
D
P-value = 0.99
P-AMPK protein abundance (Arbitrary units)
0.80
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0.00
M-R R-M
M-R R-M M-R R-M
284 KDa
E
P-value = 0.57
P-mTOR protein abundance (Arbitrary units)
1.00
0.80
0.60
0.40
0.20
0.00
M-R R-M

Figure 6.

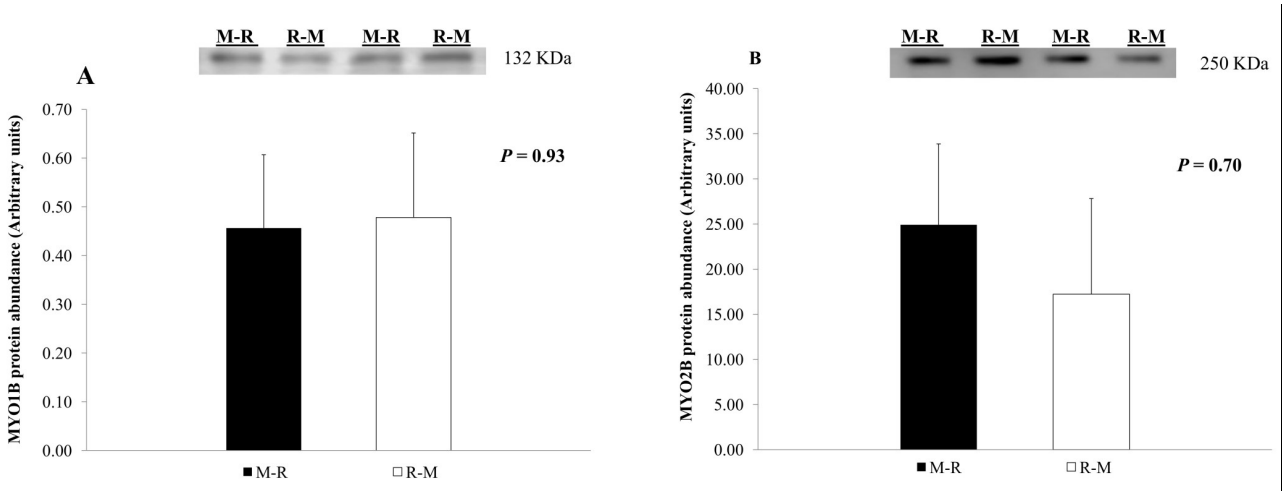

A
M-R
R-M
M-R
R-M
132 KDa
MYO1B protein abundance (Arbitrary units)
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0.00
P = 0.93
M-R
R-M
B
M-R
R-M
M-R
R-M
250 KDa
MYO2B protein abundance (Arbitrary units)
40.00
35.00
30.00
25.00
20.00
15.00
10.00
5.00
0.00
P = 0.70
M-R
R-M

Figure S1.

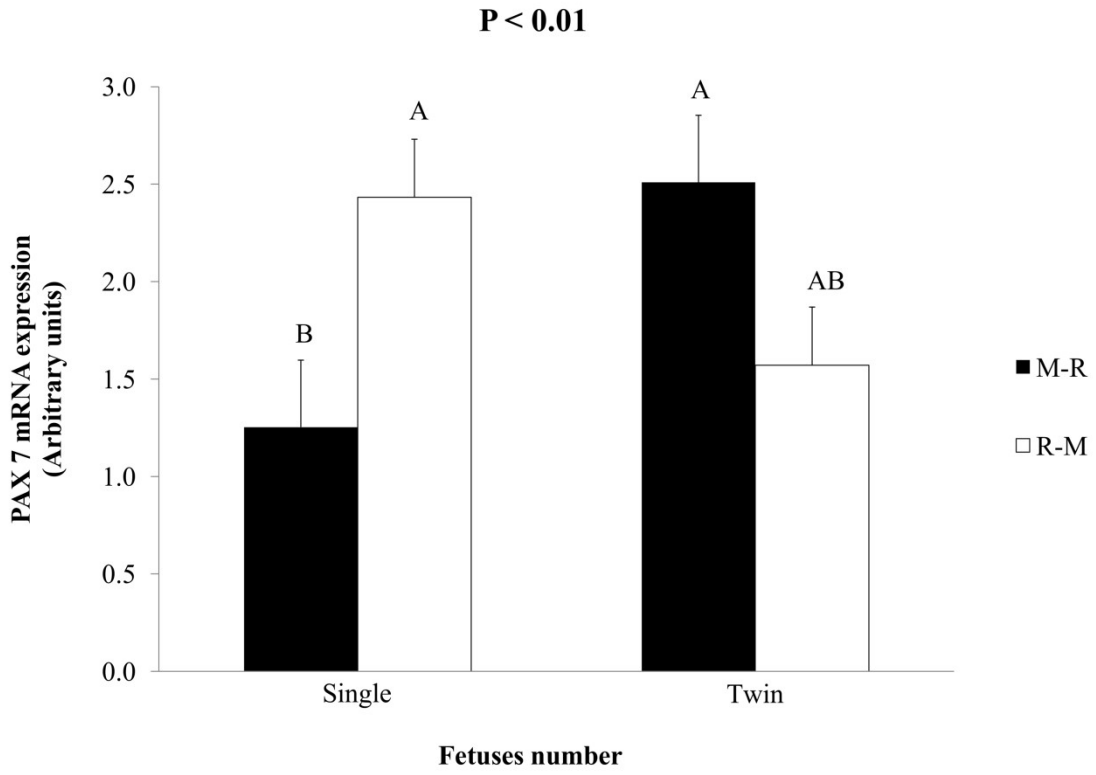
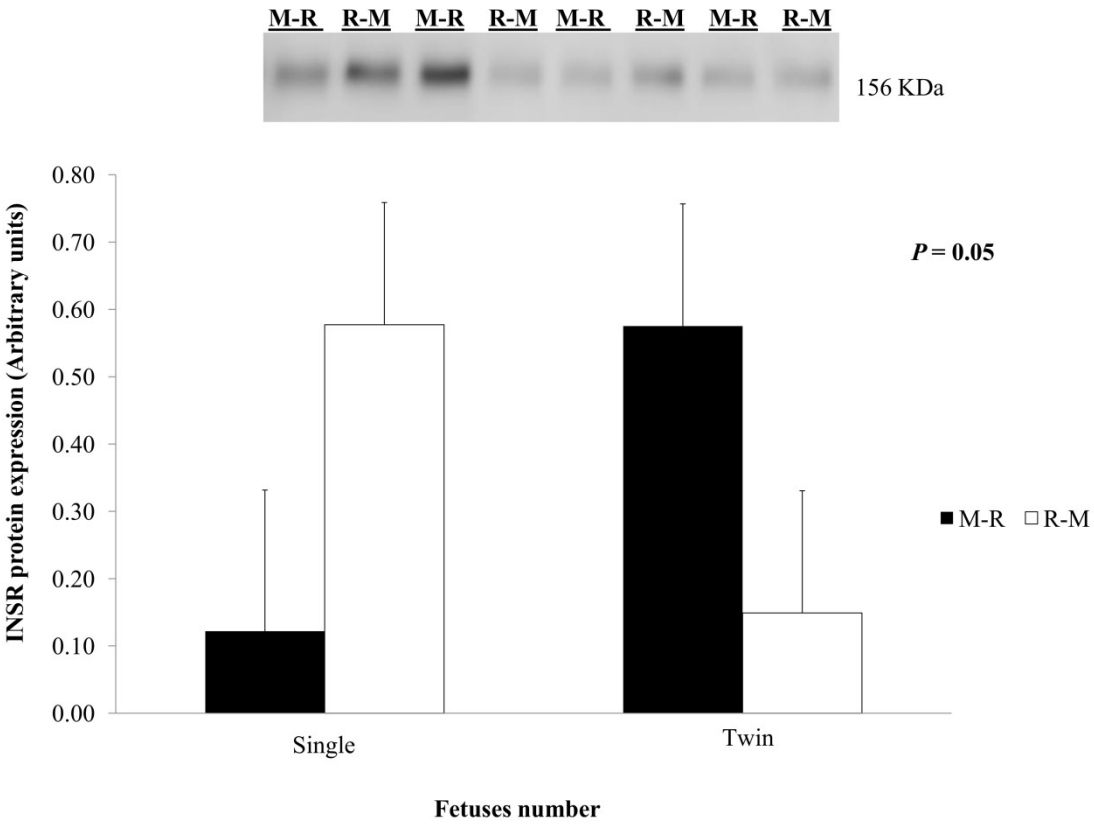

P < 0.01
PAX 7 mRNA expression (Arbitrary units)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
A
A
B
AB
Single
Twin
Fetuses number
M-R
R-M

Figure S2.


M-R
R-M
M-R
R-M
M-R
R-M
M-R
R-M
156 KDa
INSR protein expression (Arbitrary units)
0.80
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0.00
P = 0.05
M-R
R-M
Single
Twin
Fetuses number