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Análise de padrões homeostáticos em conceptos bovinos clones no Dia 225 da gestação e associação com alterações fenotípicas fetais e placentárias

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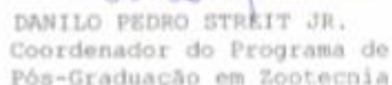

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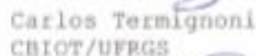

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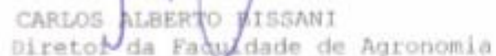

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Análise de padrões homeostáticos em conceptos bovinos clones no Dia 225 da gestação e associação com alterações fenotípicas fetais e placentárias[1]

Autor: Elvis Ticiani

Orientador: Marcelo Bertolini

Resumo: Anormalidades de desenvolvimento após a produção de embriões bovinos por transferência nuclear de célula somática (SCNT) podem interferir no crescimento fetal e placentário em um conjunto de sintomas denominado de Síndrome do Bezerro Anormal (SBA). O objetivo deste estudo foi comparar as características fenotípicas com os perfis bioquímicos, metabólicos e os padrões de expressão gênica de conceptos bovinos no Dia 225 de gestação, estabelecidas *in vivo* (IVD) por superovulação e inseminação artificial, ou *in vitro* tanto por fecundação *in vitro* (IVF) quanto por *Handmade Cloning* (NT-HMC). Avaliações bioquímicas foram realizadas no plasma fetal e materno e nos fluidos fetais, e a expressão de genes chave para o desenvolvimento do concepto foi analisada por PCR quantitativo (RT-qPCR) em tecido hepático fetal e em diferentes tipos de placentônios (A, B, C), classificados de acordo com o aspecto morfológico. Os dados foram analisados pelo procedimento MIXED do sistema SAS. A maioria dos parâmetros fetais para funções hepáticas e renais, metabolismo da hemoglobina e osmolaridade no plasma fetal e nos líquidos amniótico e alantóico, concentrações de glicose, frutose, triglicerídeos e VLDL no plasma fetal, e a quantidade total de glicose e frutose no fluido alantóico foram maiores nos clones do que nos grupos IVD e IVF. No fígado fetal, houve diferença nos níveis de expressão gênica indicando menor atividade nas vias frutolítica, gliconeogênica, via das pentoses fosfato, síntese de colesterol e serina, e aumento de atividades frutogênica e lipogênica em clones do que em controles. Na placenta, não houve diferença entre os grupos para expressão gênica, porém houve padrão de expressão distinto entre os diferentes tipos de placentônios, em uma possível compartimentalização metabólica na placenta de bovinos. Quando comparado a placentônios com morfologia fisiológica (tipos A e B), houve um aumento na expressão de transportadores de glicose e diferença significativa nas vias metabólicas em placentônios tipo C, sugerindo o transporte ativo de glicose e síntese de frutose pelo placentônio tipo C. Como a proporção e massa de placentônios tipo C são significativamente maiores em conceptos clonados, tais funções metabólicas parecem ser intensificadas em clones, levando a alterações bioquímicas que promovem o crescimento fetal e até a processos patológicos, como uma possível lipidose hepática em fetos clonados. Tais mudanças homeorréticas podem estar associadas a mudanças na programação fetal metabólica e oferta excessiva de nutrientes ao fígado em gestações de clones, levando a alterações típicas da SBA em conceptos produzidos pela clonagem por TNCS.

Palavras-chave: Síndrome dos Bezerros Anormais, clonagem por transferência nuclear, placentomas

[1]Tese de Doutorado em Zootecnia – Produção Animal, Faculdade de Agronomia, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brasil. (p.119) Dezembro, 2018.

Analysis of homeostatic patterns in bovine cloned concepti at Day 225 of gestation and association with phenotypical fetal and placental changes[1]

Author: Elvis Ticiani

Advisor: Marcelo Bertolini

Abstract: Developmental abnormalities after production of embryos by somatic cell nuclear transfer (SCNT) may affect fetal and placental growth in a set of symptoms termed Abnormal Offspring Syndrome (AOS). The objective of this study was to compare phenotypic characteristics with biochemical, metabolic and gene expression patterns of bovine conceptus at Day 225 of gestation, established *in vivo* (IVD) by superovulation and artificial insemination, or *in vitro* both by *in vitro* fertilization (IVF) and Handmade Cloning (NT-HMC). Biochemical evaluations were performed in fetal and maternal plasma and in fetal fluids, and expression of key genes for the development of the concept was analyzed by quantitative PCR (RT-qPCR) in fetal liver and in different types of placentomes (A, B, C), classified according to the morphological aspect. The data were analyzed by the MIXED procedure of the SAS system. Most fetal parameters for liver and kidney functions, hemoglobin metabolism, and osmolarity in fetal plasma and in the amniotic and allantoic fluids were significantly higher in clones than in the *in vivo* control group, but no diferences. Glucose, fructose, triglycerides and VLDL concentrations in fetal plasma, and the total amount of glucose and fructose in the allantoic fluid were higher in the clones than in the IVD and IVF groups. In the fetal liver, there was a difference in the levels of gene expression indicating lower fructolysis, gluconeogenesis, phosphate pentoses pathway, cholesterol and serine synthesis, and an increase of fructogenic and lipogenic activities in clones than in controls. In the placenta, there was no difference between the groups for gene expression, but there was a distinct pattern of expression between the different types of placentones, in a possible metabolic compartmentalization of the bovine placenta. When compared to placentomes with physiological morphology (types A and B), there was an increase in the expression of glucose transporters and a significant difference in the metabolic pathways in Type C placentomes, suggesting an active transport of glucose and synthesis of fructose through placentone Type C. The proportion and mass of Type C placentomes are significantly higher in cloned conceptus; so metabolic functions seem to be intensified in clones, leading to biochemical changes that promote fetal growth and even pathological processes such as possible hepatic lipidosis in cloned fetuses. This way homeorrhetic changes may be associated with changes in metabolic fetal programming and excessive nutrients supply to the liver in gestations of clones, leading to typical alterations of AOS in conceptus produced by TNCS.

Keywords: Abnormal Offspring Syndrome, cloning by nuclear transfer, placentome.

[1]Doctoral thesis in Animal Science - Faculdade de Agronomia, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil. (119 p.) December, 2018.

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RELAÇÃO DE ABREVIATURAS E SIGLAS

5mC	Resíduos de citosina 5'
ACACA	Acetil-CoA carboxilase α
ACTB	β-actina
ACTH	Hormônio exógeno adrenocorticotrófico
AKR1B1	Aldose reductase
AKT	Triokinase
ALDOB	Aldolase B
ALP	Fosfatase alcalina
ALT	Alanine aminotransferase
AOS	*Abnormal Ooffspring Syndrome*
AR	Aldose redutase
AST	Aspartato aminotransferase
BAX	*BCL2-associated X protein*
BCL2	*B-cell lymphoma 2*
bPAG1	*Pregnancy-associated glycoprotein 1*
BPL	Lactogênio placentário bovino
chREBP	*Carbohydrate responsive element binding protein*
CIV	Cultivo *in vitro*
CL	Corpo lúteo
CPP	Ciclo das pentoses fosfato
D_2O	Óxido de deutério
DMR	Regiões diferencialmente metiladas
DNMT1	*DNA cytosine-5-methyltransferase 1*
E2	Estradiol
FASN	Ácido graxo sintase
FBP1	Frutose-1,6-bisfosfatase
FIV	Fecundação *in vitro*
G6P	Glicose 6-fosfato
G6PC	Glicose-6-fosfatase
G6PD	Glicose-6-fosfate desidrogenase
GAP	Gliceraldeído-3-fosfato
GGT	γ-glutamil transpeptidase
GLUT	Transportador de glicose
HDL	*High-density lipoproteins*
HMGCR	3-hidroxi-3-metilglutaril-coa redutase
IGF1	*Insulin-like growth factor 1*
IGF1r	Receptor do IGF 1
IGF2	*Insulin-like growth factor 2*
IGF2r	Receptor do IGF 2
IVD	*In vivo-derived*
IVF	*In vitro fertilization*
IVP	*In vitro production*

KHK	Frutoquinase
LDH	Lactato desidrogenase
LDHA	Lactato desidrogenase A
LDHB	Lactato desidrogenase B
LDL	*Low-density lipoprotein*
MBD	Proteínas do domínio de ligação metil-CpG
MIV	Maturação *in vitro*
NT-HMC	*Nuclear transfer-handmade cloning*
P4	Progesterona
P450	Citocromo *P450*
PCK1	Fosfoenolpiruvato carboxiquinase
PEPCK	Fosfoenol piruvato carboxiquinase
PHGDH	Fosfoglicerato desidrogenase
PIV	Produção *in vitro*
PP2A	Proteína fosfatase 2A
PSAT	Fosfoserina aminotransferase
RBC	*Red blood cells*
RPS9	*Ribosomal protein S9*
RT-qPCR	*Real time quantitative PCR*
SBA	Síndrome dos Bezerros Anormais
SCNT	*Somatic cell nuclear transfer*
SLC2A1	*Solute carrier family 2 member 1*
SLC2A2	*Solute carrier family 2 member 2*
SLC2A3	*Solute carrier family 2 member 3*
SLC2A5	*Solute carrier family 2 member 5*
SORD	Sorbitol desidrogenase
TKFC	Dihidroxiacetona quinase 2
TN	Transferência nuclear
TNSC	Transferência Nuclear de Célula Somática
TPP	*Total plasma protein*
TSC2	*AKT-tuberous sclerosis complex 2*
VLDL	*Very-low-density lipoprotein*
Xu-5P	Xilulose-5 fosfato

CAPÍTULO I

1. INTRODUÇÃO

Transcorreram-se mais de três décadas desde o primeiro sucesso na produção de bovinos pela técnica da fecundação *in vitro*, ou FIV (Brackett *et al*., 1982) e desde a primeira clonagem de um mamífero (ovino) por transferência nuclear (TN) utilizando blastômeros de embriões em estádios iniciais de desenvolvimento (Willadsen *et al*., 1986). Com o domínio de todas as etapas de produção de embriões completamente *in vitro*, incluindo a maturação (MIV), fecundação (FIV) e cultivo (CIV) *in vitro*, alcançamos um avanço científico considerável em várias áreas afins de conhecimento (foliculogênese, maturação folicular e ovocitária, fecundação, desenvolvimento embrionário, entre outras). O nascimento de Dolly em julho de 1996 por TN utilizando o núcleo de uma célula somática de um indivíduo adulto (Wilmut *et al*., 1997) representou a queda de um importante dogma biológico, ou seja, que células somáticas diferenciadas não poderiam ser reprogramadas a um estado toti- ou pluripotente que permitisse o desenvolvimento de um novo indivíduo. Subsequentemente, a aplicação da clonagem de adultos por TN com células somáticas (TNCS) foi confirmada em várias espécies mamíferas (Kato *et al*., 1998; Wakayama *et al*., 1998; Baguisi *et al*., 1999; Polejaeva *et al*., 2000; Rahul *et al*., 2011) de animais em risco de extinção (Loi *et al*., 2001; Hammer *et al*., 2001) e mesmo em espécies aquáticas (Ju *et al*., 2004; Sun *et al*., 2005; Jordão *et al.,* 2016). Mesmo que ainda ineficiente, a clonagem por TCNS também tem contribuído muito no entendimento do metabolismo embrionário e fetal, despertando um grande interesse em áreas adicionais, como a fisiologia da gestação e função placentária, a reprogramação epigenética do genoma, engenharia genética, neonatologia, entre outras. Hoje, a produção *in vitro* (PIV) de embriões bovinos por FIV ou TNCS representa uma poderosa ferramenta que tem sido utilizada rotineiramente tanto em nível científico quanto comercial em todas as regiões do mundo. Várias aplicações da tecnologia da TNCS têm sido sugeridas, incluindo a clonagem reprodutiva visando a preservação de indivíduos com genética de importância econômica e a preservação e propagação de espécies em extinção, até a clonagem terapêutica, com implicações diretas à saúde humana. No entanto, os sistemas de PIV de embriões por FIV ou TN em várias espécies

animais, trazem consigo algumas anormalidades de desenvolvimento tanto pré- quanto pós-natais como consequência das manipulações embrionárias (Willadsen *et al*., 1991; Behboodi *et al*., 1995; Farin & Farin, 1995; Bertolini *et al*., 2007, 2012). Tais perturbações que ocorrem durante os primeiros dias de desenvolvimento embrionário podem interferir com o crescimento embrionário fetal e placentário em um conjunto de fenótipos alterados coletivamente denominado em bovinos de Síndrome dos Bezerros Anormais (SBA).

O termo Síndrome dos Bezerros Anormais engloba todos os fenótipos observado em fetos, placentas e neonatos após a transferência de embriões produzidos *in vitro* ou por TNCS (Farin *et al*., 2006). Atualmente, esses fenótipos anormais associados ao SBA são atribuídos a modificações epigenéticas aberrantes que regulam a expressão de genes *imprinting* e não *imprinting*. Como existem diferenças conhecidas na regulação de genes entre espécies, é provável que os fenótipos anormais resultantes da transferência de embriões PIV ou SCNT variem entre as espécies. Por exemplo, o crescimento excessivo fetal e placentário foi observado em bovinos e ovinos (Behboodi *et al*., 1995; Bertolini *et al*., 2002a; Farin & Farin, 1995; Young *et al*., 2001); No entanto, não foi observado em suínos (Archer *et al*., 2003; Park *et al*., 2001). Os suínos clonados exibem outras anormalidades (Archer *et al*., 2003; Park *et al*., 2001) e podem ser específicas para suínos. Essas observações dão apoio à ideia de que os mecanismos subjacentes que geram anormalidades pós-transferência de embriões PIV ou TNCS podem ser semelhantes entre as espécies. No entanto, a variação na manifestação das anormalidades produzidas em fetos, placentas e neonatos são devido as diferenças sutis em como genes específicos são expressos durante o desenvolvimento de cada espécie.

O interesse crescente com respeito ao aparecimento e evolução da SBA pode ser evidenciado pelo número de revisões no assunto (Kruip & Den Daas, 1997; Bertolini & Anderson, 2002, Ogura *et al*., 2013, Verma *et al*., 2015). A SBA foi inicialmente observada em bezerros clonados por TN com blastômeros (Willadsen *et al.,* 1991) para ser subsequentemente também demonstrada a partir da técnica de FIV (Behboodi *et al.,* 1995; Farin & Farin, 1995) e da TNCS (Hill *et al.,* 1999, 2000). A ocorrência da SBA está intrinsicamente associada ao

concepto e não a disfunções maternas. O grau de alterações de desenvolvimento pode variar entre embriões no mesmo cultivo, entre protocolos de FIV e TN, ou mesmo entre laboratórios (Kruip & Den Daas, 1997). Nem todos os produtos, incluindo gêmeos idênticos produzidos por TN, apresentam sintomas da síndrome, o que demonstra o caráter epigenético e/ou ambiental, e não genético, deste fenômeno. Em adição, o crescimento excessivo pré-natal não se mantém na fase pós-natal de desenvolvimento. Sabe-se que as condições de cultivo *in vitro* de embriões em presença de soro e/ou em co-cultivo em monocamadas de células de suporte e a falha de reprogramação epigenética inerente à técnica de TN estão diretamente relacionados ao aparecimento dos sintomas da SBA, dos quais incluem o nascimento de bezerros com peso excessivo e com reduzida sobrevivência pós-natal (Hill, 2014). O peso ao nascer é um dos fatores determinantes na sobrevivência perinatal, sendo o nascimento de bezerros com excesso de peso, fator comum e típico da SBA, a maior causa de mortalidade neonatal em bovinos. Um número relativamente baixo de embriões clonados sobrevive a termo (1 a 5%), os quais frequentemente necessitam atendimento clínico intensivo após o parto (Gavin *et al.*, 2013). O acompanhamento de bezerros clonados até a fase adulta evidenciou taxas de mortalidade de 33% do parto ao desmame, e mais de 8% do desmame aos quatro anos de idade (Wells *et al.*, 2004). Apesar dos problemas pós-parto iniciais, bezerros clonados tendem a normalizar, mas ainda há uma maior morbidade e mortalidade durante o período juvenil (Rudenko *et al.*, 2004).

Outros sintomas menos caracterizados da SBA afetam a fase final da gestação e compreendem o prolongamento da gestação, uma menor preparação fisiológica da fêmea pré-parto, afetando a evolução dos estágios da parição, e um comprometimento da mamogênese pré-parto que parece afetar a lactogênese pós-parto (Ferreira *et al.,* 2013). Porém, muitos dos fenômenos nesta fase são meros reflexos morfológicos, fisiológicos e metabólicos das anormalidades que se iniciam com uma reprogramação inadequada do DNA oriundo da célula somática, e que se traduzem em uma placentação e programação metabólica anormais. A biodisponibilidade de certos substratos e hormônios durante a prenhez é importante para o estabelecimento de padrões

normais de atividade nos sistemas fisiológicos do concepto, um fenômeno comumente chamado de programação metabólica (McMillen & Robinson, 2005). De acordo com esta teoria, mudanças no padrão de suprimento de substratos ao feto podem conduzir a modificações moleculares e celulares permanentes na atividade de órgãos e sistemas que podem persistir por toda a vida do indivíduo, gerando o conceito da origem fetal para as doenças na fase adulta (Bertram & Hanson, 2001), também conhecido como a Hipótese de Barker (Barker, 1999). Por conseguinte, não necessariamente há a continuidade de padrões distintos de expressão gênica em tecidos fetais ou placentários nessa fase, podendo os distintos fenótipos serem manifestações de uma cascata de eventos morfo-fisiológico-metabólicos inicializados por padrões anormais transientes de expressão de genes importantes para as etapas iniciais de desenvolvimento do concepto. Estas são ainda lacunas de conhecimento que necessitam ser melhor esclarecidas, sendo o estudo envolvendo a PIV e a TNCS ferramentas para a compreensão de mecanismos de anormalidades de desenvolvimento, que quando utilizadas comparativamente a indivíduos controle, podem auxiliar na elucidação de eventos de ordem fisiológica ou mesmo patológica que já constituem a natureza das espécies, sendo o bovino um dos modelos de maior possibilidade de avanço de conhecimento científico na área.

2. REVISÃO DA LITERATURA

2.1 Papel do metabolismo no desenvolvimento do concepto na fase embrionária

A variabilidade das alterações decorrentes dos processos de manipulação *in vitro* dos embriões pela clonagem pode interferir no desenvolvimento do concepto, compartilhando desvios de desenvolvimento desencadeados no período embrionário inicial. Tais alterações derivadas das fases iniciais de desenvolvimento embrionário à ativação do genoma sugerem disfunções também no metabolismo energético, quando o embrião utiliza o piruvato como o principal substrato energético. Entretanto, a partir do estádio de 8- a 16-células em bovinos, ou seja, a partir da ativação do genoma embrionário, que ocorre um significativo aumento do metabolismo da glicose em embriões cultivados *in vitro* (Khurana & Niemann, 2000). Este aumento no metabolismo da glicose em embriões de PIV pode e*star* relacionado com um desequilíbrio de rotas metabólicas, concentrações molares não fisiológicas de substratos energéticos, ou mesmo pela expressão anormal de enzimas chave do metabolismo intermediário (Khurana & Niemann, 2000).

Devido ao aumento do consumo de glicose concomitante com a progressão da fase de desenvolvimento embrionário até o estádio de blastocisto, ocorre a elevação na quantidade de transportadores de glicose (GLUT, ou SLC2A). Nas fases iniciais da gestação (fase embrionária), o *SLC2A1* é considerado a principal isoforma para o transporte de glicose por transporte facilitado em conceptos bovinos (Wrenzycki *et al.*, 1998), não exercendo necessariamente um papel dominante na captação de glicose embrionária do ambiente (Augustin *et al.*, 2001), com o *SLC2A3* também exercendo uma função preponderante na captação de glicose materna pelo blastocisto (Pantaleon *et al.*, 1997). Já na fase placentária da gestação em ruminantes, o *SLC2A1* se apresenta como um importante transportador de glicose na interface materno-placentária, enquanto que o *SLC2A3* é considerado o transportador para o fornecimento de glicose da placenta ao feto (Ehrhardt & Bell, 1997). As

expressões dos genes para o SLC2A2 (transportador para vários monossacarídeos) e para o SLC2A5 (transportador específico para a frutose) também são reguladas ao longo do desenvolvimento, sendo que a transcrição de SLC2A5 é iniciada no momento da ativação do genoma embrionário bovino com elevada afinidade para a frutose, o que indica que o embrião é capaz de transportar este substrato energético em estádios iniciais (Augustin *et al.*, 2001).

Diferentes espécies metabolizam substratos energéticos por via do ciclo das pentoses fosfato (CPP) durante as fases iniciais de desenvolvimento embrionário. Esta via está diretamente relacionada à necessidade de nucleotídeos e ribose fosfato durante o início da transcrição gênica na fase embrionária, também sendo regulada de acordo com a maior disponibilidade ou aporte de glicose na célula (O'fallon & Wright, 1986; Loskutoff & Betteridge, 1992). De acordo com Augustin *et al.* (2001), a absorção de frutose, componente comum no fluido uterino e nos fluidos fetais (Casslen & Nilsson, 1984), poderia alterar a via de metabolização de ribose fosfato para a produção de ribose-5-fosfato, um precursor essencial para a síntese de nucleotídeos, e em médio prazo, de lipídeos. Interessantemente, um aporte de glicose mais elevado pode induzir uma ativação do CPP, levando à síntese de ácidos graxos, como sinalizador de excesso de substratos energéticos, uma via proposta para alterações metabólicas em embriões bovinos produzidos *in vitro* e em estádios iniciais de desenvolvimento (Camargo *et al.*, 2008). A ativação da transcrição gênica em resposta a carboidratos foi caracterizada inicialmente em células hepáticas (Yamashita *et al.*, 2001), sendo regulada pela ativação da proteína de ligação ao elemento responsivo a carboidratos (*carbohydrate responsive element binding protein*, ou chREBP). A ativação da chREBP ocorre pela ação da proteína fosfatase 2A (PP2A) dependente de xilulose-5 fosfato (Xu-5P), produto final do CPP (Nishimura & Uyeda, 1995). Uma vez ativado, o chREBP participa da regulação da trascrição de genes do metabolismo energético como para a L-piruvato quinase (LPK) e genes de enzimas lipogênicas como a acetil-CoA carboxilase alfa *(ACACA*) e a ácido graxo sintase (*FASN*), importantes enzimas na lipogênese (Kabashima *et al.*, 2003). Em resumo, um maior aporte de carboidratos ativa o CPP, incrementando a concentração celular de Xu-5P,

que ativa a PP2A, a qual atua sobre o chREBP, que finalmente induz no núcleo a expressão de genes que codificam para enzimas lipogênicas promovendo, portanto, a lipogênese, convertendo carboidratos em lipídeos (Camargo *et al.*, 2008). Não obstante, tal fenômeno ainda está por ser demonstrado no metabolismo embrionário e placentário-fetal.

Independente do sistema de produção de embriões (*in vitro* ou *in vivo*), estudos demonstraram que o metabolismo energético pode culminar com uma maior produção de lactato em blastocistos produzidos *in vitro* em decorrência do estresse oxidativo do sistema de cultivo empregado (Javed & Wright, 1991). O comprometimento no desenvolvimento embrionário pode acarretar distúrbios na formação da placenta que afetam o tamanho e a microarquitetura, causando mudanças na transferência de substrato que podem levar a alterações no padrão de crescimento fetal de conceptos derivados de PIV (Bertolini *et al.*, 2004). Por exemplo, o acúmulo de lactato procedente do metabolismo energético embrionário pode afetar o pH intracelular e gerar uma resposta fisiológica anormal, podendo ser um dos fatores metabólicos que levam à apoptose celular, reduzindo a viabilidade embrionária, expressando um lento crescimento que poderá afetar as fases posteriores de desenvolvimento (Rieger *et al.*, 1995).

Aparentemente, o desenvolvimento de um embrião pós-implantação é afetado pela incidência de apoptose nas fases de pré-implantação (Loureiro *et al.*, 2007). Dentre os diversos fatores que induzem a apoptose destacam-se a alteração nas concentrações dos fatores de crescimento, irradiação ionizante e agentes que danificam o DNA sob estímulo da proteína p53, que atua como fator de transcrição do gene *BAX* (*BCL2 Associated X Protein*), que codifica para uma proteína pró-apoptótica. O *BAX* é um membro da família das proteínas *BCL2* (*B-cell lymphoma 2*), que tem a capacidade de promover (BAD, BAK, *BAX*, BCLxS, BIK, HRK) ou inibir (*BCL2*, BCLW, BCLX, BFL1, GABAR1, MCL1, NR13) a atividade das caspases efetoras de apoptose (Jurisicowa & Acton, 2004). A família de genes *BCL2*, que inclui os genes *BAX* (pró-apoptótica) e *BCL2* (anti-apoptótica), desempenham um papel chave na ocorrência de apoptose em células germinativas femininas (Kim & Tilly, 2004) e são utilizados na análise da apoptose em oócitos e embriões (Pocar *et al.*, 2005; Opiela *et al.*, 2008).

2.2 Papel do metabolismo no desenvolvimento do concepto na fase fetal

A placenta é praticamente a única responsável pela interface de trocas entre os sistemas fetal e materno, tendo um papel importante no crescimento fetal pela regulação da oferta de nutrientes, síntese e transporte de hormônios, substratos e outras substâncias entre os sistemas. Todos os suportes necessários para o crescimento e desenvolvimento feto-placentário de origem direta ou indiretamente do sistema materno, e os valores absolutos e relativos de tais substratos que finalmente atingem o feto são transportados, metabolizados ou modificados pela placenta (Bertolini *et al.*, 2004). A biodisponibilidade de certos substratos ou hormônios durante a gestação é importante para o estabelecimento de padrões normais de atividade dos sistemas fisiológicos no feto em desenvolvimento, um fenômeno habitualmente referido como programação metabólica (McMillen & Robinson, 2005). As alterações no padrão de fornecimento de substrato para o feto, como na modulação ou restrição nutricional em certas fases da gestação, em especial no período de placentação, podem conduzir a alterações moleculares e celulares permanentes ou mesmo novos padrões de atividades em órgãos e sistemas que podem persistir e afetar a vida pós-natal (Figura 1). Consequentemente, as alterações na reprogramação metabólica após manipulações de embriões *in vitro* podem afetar a função da placenta e do padrão de restrição da placenta no crescimento fetal, o que leva a um efeito de promoção do crescimento, alterando os eventos que levam ao início do parto, e comprometer a sobrevivência pós-natal de nascimento à idade adulta (Barker, 1999).

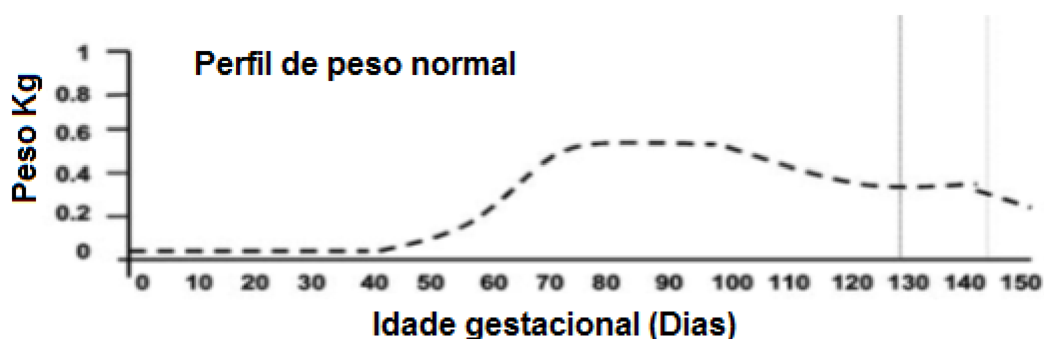

Figura 1. Efeito da restrição da dieta no crescimento pré-natal e peso ao nascer em ovinos (Adaptado de Fowden *et al*., 2006).

Chavatte-Palmer *et al*. (2002) relataram que a termo o sistema endócrino de fetos clones não foi prematuro, como demonstrado por uma resposta normal ao hormônio exógeno adrenocorticotrófico (ACTH). Apesar disso, protocolos de cuidados neonatais de gestações clones reconhecem que muitos recém-nascidos devem ser geridos da mesma forma que para prematuros e, portanto, beneficiar-se-ão de tratamento intensivo para ajudar a transição para a vida extra-uterina (Hill & Chavatte-Palmer, 2002).

O crescimento fetal ocorre lentamente durante as fases iniciais de desenvolvimento gestacional e exponencialmente durante as fases posteriores (Eley *et al*., 1978; Prior & Laster, 1979; Ferrell, 1989; Reynolds *et al*., 1990), com aproximadamente 90% do peso fetal a termo adquirido durante o último trimestre da gestação (Ferrell, 1989). No entanto, a placenta cresce mais rapidamente do que o feto no início da gestação, com o peso placentário sendo maior do que o peso fetal durante o primeiro trimestre da gestação, invertendo-se a taxa de crescimento nos demais períodos de desenvolvimento (Figura 2). Já no terceiro trimestre da gestação, o crescimento fetal é normalmente limitado por fatores maternos e placentários, um mecanismo homeostático fisiológico que garante a sobrevivência materna (Ferrell, 1991a,b; Gluckman *et al*., 1992). O conceito de restrição materna ao crescimento fetal está relacionado com o controle de fornecimento de nutrientes para o feto no final da gestação (Gluckman *et al*.,

1992), e o grau desta restrição pode e*star* associada com o padrão de crescimento da placenta que ocorre durante a fase inicial até meados da gestação (Bell *et al.*, 1999). Alguns pesquisadores têm sugerido a existência de um mecanismo de causa e efeito placentário-fetal, em que o padrão de crescimento da placenta durante a primeira e metade da gestação tem um efeito limitante significativo sobre o crescimento fetal durante a fase final da prenhez (Bell *et al.*, 1999), mesmo quando as demandas fetais de nutrientes são maiores (Prior & Laster, 1979; Reynolds *et al.*, 1990).

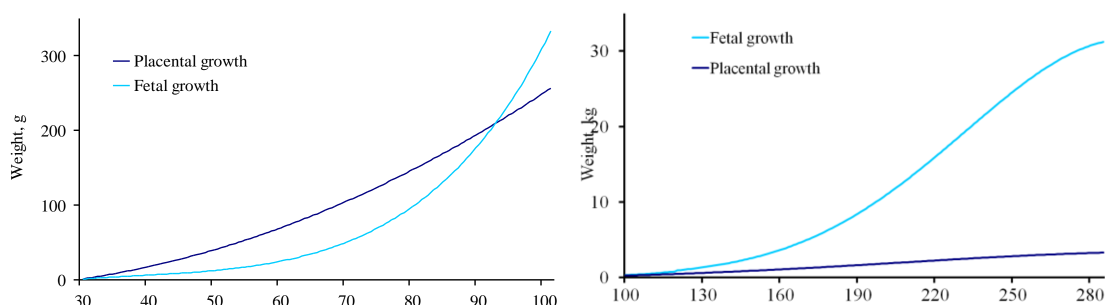

Figura 2. Taxa de crescimento absoluto do concepto bovino durante a gestação (Eley *et al.*, 1978; Prior & Laster, 1979; Ferrell, 1989, 1991ab; Reynolds *et al.*, 1990).

Como a maioria dos nutrientes transferidos através da placenta são metabolizados e/ou produzidos pela mesma (Ferrell *et al.,* 1983; Ferrell, 1989), distúrbios na placentação e na função da placenta podem levar a alterações no padrão de crescimento fetal (Schläfer *et al.*, 2000). Isto não é surpreendente, uma vez que o tecido placentário geralmente demonstra um elevado grau de plasticidade sob condições desfavoráveis, por expressar mecanismos para adaptar-se ao adverso nutricional (Perry *et al.*, 1999) e do ambiente (Ferrell, 1989; Krebs *et al.*, 1997), que podem promover mudanças morfo-histológicas na placenta para modular o crescimento fetal, num padrão típico como o observado após manipulações *in vitro* de embriões produzidos pela FIV e TNCS. Hipóteses recentes têm associado a placentação atípica com anomalias fetais vistas do início da gestação a termo. Na verdade, as associações entre defeitos da placenta e desenvolvimento fetal anormal no início (Stice *et al.*, 1996, Hill *et al*, 2000; Bertolini *et al.*, 2002b) e no final da gestação (Cibelli *et al.*, 1998; Hill *et al.*,

1999, 2001; Bertolini *et al.*, 2000, 2002a; Farin *et al.*, 2010) de conceptos produzidos *in vitro* (PIV) por FIV e TNCS parece desempenhar um papel metabólico chave na ocorrência de excesso de peso ao nascimento e baixa sobrevivência pós-natal (Behboodi *et al.*, 1995; Bertolini *et al.*, 2002a; Batchelder *et al.*, 2005).

Alterações na placenta constituem características preponderantes para o desenvolvimento do concepto e podem interferir nos níveis de glicose dos fluidos fetais em decorrência de desvios de conformação e metabolismo dos placentônios (Chavatte-Palmer *et al.*, 2012), uma vez que o tecido placentário utiliza aproximadamente 60-75% de toda a glicose circulante no útero (Fowden *et al.*, 1994). Na placenta, os transportadores de glicose *SLC2A1* e *SLC2A3* são responsáveis pelo transporte transmembranar de glicose a favor do gradiente de concentração (Ehrhardt; Bell, 1997; Bertolini *et al.*, 2004). Postula-se que, durante o curso da gestação, os *SLC2A1* são importantes para a captação de glicose e utilização placentária, enquanto que os *SLC2A3* podem ser ligados à transferência da glicose fetal (Ehrhardt & Bell, 1997). Para atender ao aumento na taxa de crescimento fetal e requerimentos placentários observados ao final de gestações de FIV e TNCS (Hirayama *et al.*, 2011), a absorção de glicose pode e*star* aumentada com consequente aumento dos seus transportadores e das concentrações de frutose, ao contrário do que acontece em gestações controle (Bertolini *et al.*, 2004; Constant *et al.*, 2006). O glicocorticóide endógeno parece e*star* envolvido na regulação da expressão de transportadores da glicose placentária *SLC2A1* e *SLC2A3* (Hahn *et al.*, 1999), influenciando a quantidade de glicose transportada para o feto e o crescimento fetal. A termo, a expressão do transportador de glicose *SLC2A3* é elevado em placentas de TNCS, havendo uma correlação negativa entre a glicose sanguínea materna pré-parto e o peso fetal (Hirayama *et al.*, 2011). Na ausência de hipoglicemia neonatal, isto implica que a captação e o transporte de glicose na circulação materna são elevados no final de gestações de TNCS, provavelmente para satisfazer as crescentes exigências fetais e placentárias (Hirayama *et al.*, 2011).

Para atender ao aumento da demanda de glicose em bovinos, a gliconeogênese hepática é essencial (Huntington *et al.*, 2006). Como acontece

com qualquer via bioquímica, a regulação da gliconeogênese pode ocorrer através da regulação da oferta de substrato, regulação de atividade de enzimas catalíticas e regulação da utilização do produto final. A fosfoenol piruvato carboxiquinase (PEPCK), a frutose-1,6-bifosfatase (FBP) e a glicose-6-fosfatase (G6Pase) pertencem à via gliconeogênica e se constituem em enzimas importantes para o metabolismo da glicose. A PEPCK foi identificada como uma enzima principal envolvida na produção da glicose a partir do propionato em vacas leiteiras (Greenfield *et al*., 2000). A FBP é a enzima que libera frutose 6-fosfato a partir da via gliconeogênica (Pilkis & Granner, 1992), que, após a conversão para glicose 6-fosfato, pode liberar glicose pela ação da G6Pase. A FBP controla, assim, a produção global da gliconeogênese independentemente dos precursores utilizados.

Em células hepáticas, a frutose é rapidamente fosforilada, permanecendo restrita ao citoplasma celular, sob a forma de frutose-1-fosfato, sendo esta a primeira reação catalisada pela enzima chave do metabolismo da frutose, a frutoquinase (KHK). Esta enzima, quando altamente ativa, tem a capacidade de metabolizar a frutose que chega ao fígado por meio da circulação porta hepática (Pilkis & Granner, 1992). A frutose-1-fosfato é então clivada pela aldolase B (*ALDOB*) hepática em gliceraldeído, que é fosforilado pela enzima triokinase (*AKT*) gerando $NADH+H^+$ na reação, e a di-hidroxiacetona-fosfato, componentes intermediários da via da glicólise, com ambas as moléculas sendo interconvertidas em gliceraldeído-3-fosfato (GAP). A partir deste ponto, o GAP, pode tanto ser degradado pelo metabolismo energético, quanto servir como substrato para a gliconeogênese. A frutose atinge nesta fase o metabolismo sem passar pelos passos de regulação hormonal e alostérica que controlam a via glicolítica. Assim, a frutose proporciona maior aporte de substratos para rotas metabólicas como a síntese de lactato, a glicólise, a gliconeogênese, a lipogênese e a esterificação de ácidos graxos. No entanto, se as concentrações de frutose no plasma forem elevadas (hiperfrutosemia), o metabolismo de GAP é intensificado, uma vez que este percursor não está sob qualquer controle alostérico ou hormonal, gerando um excesso de $NADH + H^+$. Para restaurar o equilíbrio na célula (NADH/NAD), o GAP é metabolizado em piruvato, o que por

sua vez é convertido em lactato para a utilização do excesso de NADH + H^+. Este processo gera lactato em relação direta com a frutosemia, o que em um neonato pode levar a uma acidose láctica e exacerbar qualquer situação de desequilíbrio ácido-básico que já exista, tal como uma acidose respiratória causada por um comprometimento no sistema respiratório (Mayes, 1993).

A maioria dos metabólitos fornecidos à placenta é metabolizada ou modificada pelo tecido placentário, com a glicose sendo o principal substrato necessário para concepto durante a gestação em bovinos (Ferrell, 1989; Ferrell & Reynolds, 1992). Em ruminantes, a frutose é sintetizada pela placenta e se acumula no sangue fetal e nos fluidos fetais (Huggett & Nixon, 1961) em decorrência da conversão de glicose em sorbitol pela aldose redutase (AR), e esta em frutose pela sorbitol desidrogenase (*SORD*) na placenta (Hers, 1960). Desta forma, a capacidade da placenta de clones em sintetizar frutose não é prejudicada, sendo apenas dependente da concentração de glicose (Hay, 1995; Aldoretta & Hay, 1999; Le *et al.*, 2005). Porém, a hiperfrutosemia foi relatada em neonatos de FIV e de TNCS, que podem indicar um aumento na produção placentária ou menor utilização fetal em curto prazo da frutose (Bertolini *et al.*, 2004; Batchelder *et al.*, 2007a). Um maior aporte de glicose em prenhezes de FIV e TNCS, talvez por diferenças na massa, na micro-arquitetura tecidual ou no metabolismo placentário podem promover um aumento na síntese de frutose no plasma e fluidos fetais, também promovendo a aceleração do crescimento fetal no final da gestação.

2.3 Programação Metabólica

Estudos em humanos tem mostrado que perturbações como a redução de aporte de substratos durante a vida fetal levam a uma programação no metabolismo do feto no sentido de garantir sobrevivência a curto prazo, todavia aumentando significativamente as chances de diversos problemas de saúde no decorrer da vida (Barker, 1999). A hipótese de que algumas doenças têm origem durante a fase fetal sugerem que a doença do adulto se origina de adaptações fetais a desafios intrauterinos, que prejudicam o crescimento fetal e resultam em adaptações permanentes nos processos endócrinos e metabólicos (Hales &

Barker 1992, 2001; McMillen *et al.* 2008). O processo pelo qual problemas ou desafios em estágios críticos de desenvolvimento levam a mudanças permanentes na estrutura e função dos tecidos é conhecido como programação intra-uterina (Fowden *et al*., 2006a). A programação intra-uterina da função fisiológica pós-natal tem sido demonstrada experimentalmente em várias espécies, utilizando uma variedade de técnicas para comprometer o ambiente intra-uterino e alterar o desenvolvimento fetal (Fowden *et al*., 2006b; Fowden & Moore, 2012). Basicamente, os estudos demonstraram que o período, a duração e a natureza exata do desafio a ser transposto pelo concepto durante a prenhes são determinantes no padrão de crescimento intrauterino e nos resultados fisiológicos específicos (Bertram & Hanson, 2001).

No caso de bovinos, a capacidade de uma vaca lactante prenhe de manter o concepto é largamente determinada pela forma como ela particiona os nutrientes a fim de sustentar o desenvolvimento embrionário, placentário e fetal, juntamente com a sua própria manutenção e produção de leite (Wathes, 2012). Além disso alterações e adaptações do concepto podem torná-lo mais ou menos eficiente na captação dos nutrientes disponibilizados pela mãe, tornando ainda mais complexa a predição de como fatores estressantes com a produção *in vitro* de embriões podem afetar o desenvolvimento do concepto e a vida pós-natal.

2.3.1 Função da placenta

Uma vez iniciada a placentação, os efeitos de programação dos sinais ambientais podem ser mediados via mudanças no desenvolvimento placentário. No entanto, ainda não se sabe até que ponto os primeiros insultos ou desafios que o concepto passa programam a placenta *per se*. Nos mamíferos placentários, a placenta é o órgão através do qual gases respiratórios, nutrientes e resíduos são trocados entre os sistemas materno e fetal. Portanto, a placenta é essencial na competição entre mãe e feto pela alocação de recursos. O desenvolvimento placentário responde aos sinais fetais da demanda de nutrientes e aos sinais maternos de disponibilidade de nutrientes e, ao adaptar seu fenótipo, regula a distribuição dos recursos disponíveis (Fowden *et al*., 2006b; Fowden & Moore, 2012).

A adaptação placentária pode ocorrer como resultado de alterações na área da superfície placentária para transferência de nutrientes, a espessura da barreira separando as circulações materna e fetal, a abundância de transportadores de nutrientes, taxa metabólica e fluxo sanguíneo, bem como como síntese placentária e metabolismo de hormônios específicos (Fowden & Moore, 2012; Vaughan *et al*., 2012).

O crescimento intra-uterino retardado (IUGR) fetal devido a restrições nutricionais está intimamente ligado ao desenvolvimento e função da placenta (Fowden *et al.,* 2008; Cetin & Alvino, 2009). Basicamente, sob condições de suprimento insuficiente de nutrientes que podem limitar o crescimento fetal normal, a placenta adapta-se de modo a aumentar sua capacidade de transporte (Burton & Fowden, 2012). Estudos anteriores em bovinos de corte e ovelhas demonstraram que as placentas podem adaptar seu fenótipo para acomodar a demanda de nutrientes fetais e que este processo pode incluir mecanismos morfológicos ou funcionais que atuam durante diferentes estágios de desenvolvimento feto-placentário (Long *et al*., 2009; Sullivan *et al.,* 2009; Fowden *et al.,* 2010).

Nos bovinos, a placenta fetal se liga a locais discretos na parede uterina, denominados carúnculas. As carúnculas são locais aglandulares que aparecem como botões ao longo da superfície luminal do útero de animais não prenhes e estão dispostos em duas linhas dorsais e duas linhas ventrais ao longo do comprimento dos cornos uterinos. As membranas da placenta se ligam a esses locais através de vilosidades coriônicas em áreas denominadas cotilédones. A unidade caruncular-cotiledonar é chamada de placentônio e é a principal área funcional de troca fisiológica entre a mãe e o feto. O crescimento cotiledonar aumenta progressivamente ao longo da gestação, em combinação com um aumento na área capilar, tamanho e número (Vonnahme *et al*., 2007; Funston *et al*., 2010).

Recentemente, buscou-se detectar fatores maternos e ambientais associados ao desenvolvimento placentário em bovinos (Van Eetvelde *et al*., 2016). As principais descobertas desse estudo foram que o crescimento materno e a lactação durante a gestação desafiam significativamente o desenvolvimento

placentário. Os resultados sugerem dois potenciais mecanismos compensatórios na placenta. Em primeiro lugar, no início da gestação, a placenta pode lidar com o desenvolvimento de mais cotilédones sobre as carúnculas disponíveis, permitindo que a gestação se mantenha viável. Em segundo lugar, no final da gestaçõ, uma expansão da superfície cotiledonar é sugerida para atender às crescentes demandas nutricionais do feto. Embora a placenta pareça compensar os desafios nutricionais por meio da adaptação das características cotiledonares, sugere-se que a eficiência placentária permaneça mais baixa nessas gestações, com consequências potenciais para o feto em desenvolvimento. Entretanto, a contribuição do tecido materno nesse mecanismo compensatório e as características microscópicas da interface feto-materna devem ser investigadas mais de perto. Além disso, para elucidar ainda mais os mecanismos subjacentes à compensação placentária, mais pesquisas sobre fatores que afetam o desenvolvimento placentário, com especial interesse no nível de produção de leite e no momento da secagem, são necessárias.

2.3.2 Mecanismos epigenéticos da programação metabólica

Fatores ambientais, especialmente nutrientes, disponíveis para fetos moldam o perfil epigenético das células e tecidos. Durante o desenvolvimento inicial do animal, as células-tronco e as células progenitoras mantêm sua pluri- ou multi-potência por meio da inibição reversível de genes específicos, permitindo a expressão de genes de autorrenovação (Meissner *et al.*, 2008ab; Mohn *et al.,* 2008) pelos processos de metilação do DNA e de metilação e acetilação de histonas.

A metilação do DNA em mamíferos é um importante regulador da transcrição gênica, sendo um mecanismo frequentemente utilizado para silenciar e regular genes sem alterar a sequência original do DNA; esta é uma das estratégias mais importantes para modificações epigenéticas do genoma (Jaenisch & Bird, 2003). Bioquimicamente, a metilação do DNA é um processo que envolve a adição de um grupo metil na posição 5 dos resíduos de citosina (5mC) no DNA. Em células somáticas adultas, a metilação do DNA ocorre tipicamente em sequências dinucleotídicas chamadas ilhas CpG, que estão nas

regiões ricas em guanina e citosina. Entre 60 e 90% de todas as ilhas CpGs são metiladas em mamíferos (Tucker, 2001), e essas ilhas geralmente estão presentes nas regiões 5' regulatórias de muitos genes, que também estão associados a regiões diferencialmente metiladas (DMR), diretamente relacionadas ao *imprinting* genômico.

A metilação do DNA é essencial para o desenvolvimento normal e desempenha uma variedade de funções biológicas, sendo associada a processos muito importantes, incluindo a repressão da expressão gênica, *imprinting* genômico, inativação do cromossomo X, supressão de elementos repetitivos e carcinogênese. Assim, a relação entre a metilação do DNA e a estrutura da cromatina é muito importante, pois essa modificação epigenética afeta a função celular, permitindo que células com o mesmo material genético se diferenciem, produzindo múltiplos órgãos ou desempenhem várias funções (Jaenisch & Bird, 2003). A metilação do DNA também pode suprimir a expressão de genes retrovirais endógenos e outras sequências de DNA deletérias que podem ter sido incorporadas ao genoma do hospedeiro ao longo do tempo. A metilação também pode proteger o DNA da clivagem enzimática, porque a maioria das enzimas de restrição é incapaz de reconhecer e se ligar a sequências (epigeneticamente) modificadas externamente.

Como uma das funções mais importantes, a metilação do DNA nas ilhas CpGs têm o efeito específico de reduzir a expressão gênica, sendo encontradas em todos os vertebrados, com ampla evidência de que genes com altos níveis de 5-metilcitosina em sua região promotora são silenciados transcricionalmente (Jaenisch & Bird, 2003). A metilação do DNA pode afetar a transcrição gênica de pelo menos duas maneiras: 1) impedindo fisicamente a ligação de proteínas de transcrição; e/ou 2) via proteínas de ligação conhecidas como MBD (proteínas do domínio de ligação metil-CpG). Essas proteínas podem recrutar proteínas adicionais para o sítio, como as histonas desacetilases e outras proteínas de remodelação da cromatina que podem modificar as histonas, formando assim uma cromatina compacta e inativa, chamada heterocromatina (Nan *et al.,* 1993).

Curiosamente, nenhum padrão significativo de metilação é visto em células-tronco embrionárias (Lister *et al.*, 2009), mas em células somáticas há

altos níveis de metilação do DNA, geralmente transmitidos para células-filhas com alta fidelidade. Portanto, as alterações epigenéticas resultantes são normalmente estáveis, contínuas e unidirecionais, impedindo que um organismo reverta qualquer nível de diferenciação celular ou a conversão de um tecido em outro. Este obstáculo se torna ainda mais desafiador no processo de clonagem, pela necessidade de remoção da metilação do DNA e desdiferenciação de uma célula até o estágio de totipotência.

2.3.3 Padrões anormais de metilação do DNA e consequências no desenvolvimento

O remodelamento da cromatina após a fecundação, associado à reprogramação da metilação do DNA, parece ocorrer de forma semelhante em embriões produzidos *in vivo* e *in vitro* (Han *et al.*, 2003). No entanto, vários estudos em clonagem mostraram que a introdução do núcleo doador somático (altamente metilado) em um citoplasma receptor (oócito) preenchido com componentes de remodelação da cromatina não é suficiente para reprogramar, modificar ou excluir certos marcadores epigenéticos de diferenciação genômica estáveis, levando a padrões de expressão gênica defeituosos (Bird, 2002). Essa falha de reprogramação está associada ao estado de hipermetilação do DNA em embriões de camundongos e bovinos produzidos por TNCS, que podem ter níveis semelhantes de metilação do DNA do núcleo doador de células somáticas (Dean *et al.*, 2001, 2003; Beaujean *et al.*, 2004).

Devido ao seu papel na expressão gênica, a modificação epigenética é considerada essencial para a memória de uma função celular específica durante o desenvolvimento (Bird, 2002) e a regulação da reprogramação nuclear (Han *et al.,* 2003). A eficiência da reprogramação epigenética após a clonagem pela TNCS parece ser fortemente dependente do tipo e estado de diferenciação da células somáticas doadora utilizada (Wells *et al.*, 2003). Em camundongos e bovinos, o tipo de célula em cultivo, linhagem celular ou mesmo subclones celulares podem revelar diferentes capacidades de desenvolvimento antes e após a clonagem (Humpherys *et al.*, 2001; Vichera *et al.*, 2013).

Existe uma aparente relação entre o processo de remodelação da

cromatina, a reprogramação do genoma e o perfil da metilação do DNA. Em camundongos, uma porcentagem significativamente maior de blastocistos é obtida quando embriões clonados são reconstruídos com células com DNA não metilado, como células-tronco embrionárias murinas ou células germinativas primordiais, do que outros tipos de células somáticas (Humpherys *et al.*, 2001). Na ausência de DNMT1 (*DNA cytosine-5-methyltransferase 1*) no núcleo e após a fecundação, a replicação gera apenas cadeias de DNA não metiladas que, com o tempo, levam à desmetilação passiva do DNA, como ocorre com o DNA do zigoto de origem materna. Curiosamente, maiores quantidades de transcritos para DNMT1 foram observados em blastocistos bovinos derivados por clonagem TNCS (Wrenzycki *et al.*, 2001), que coincide com a detecção de um estado mais metilado de embriões clonados. Assim, é possível que o estado hipermetilado de embriões bovinos clonados esteja associado não apenas à desmetilação deficiente do DNA nuclear, mas também pela presença ativa de DNMT1 de origem somática no núcleo durante o desenvolvimento inicial, contribuindo para a hipermetilação.

O *imprinting* genômico parece ser mais suscetível a mudanças epigenéticas, com desregulação de DMRs geralmente resultando em fenótipos anormais (Rancourt *et al.*, 2013). Consequentemente, as manipulações embrionárias, como a FIV e a TNCS, parecem desregular os padrões de *imprinting* (Moore, 2001). Por outro lado, quando a atividade do DNMT1 é reduzida, a metilação do DNA em DMRs associados ao *imprinting* genômico foi geralmente refratária a mudanças epigenéticas, mantendo seus padrões de metilação em células em cultivo (McGraw *et al.*, 2013). No entanto, a perda permanente de DNMT1 por deleção gênica leva a uma redução na expressão mono-alélica de vários genes *imprinting* (McGraw *et al.*, 2013). Curiosamente, embriões de camundongos mutantes para a DNMT1 e homozigóticos, nulos por deleção, morrem entre os dias 9-11 da gestação (Jackson Grusby *et al.,* 2001). A perda de DNMT1, no entanto, não tem efeito sobre o potencial proliferativo de células epiteliais de camundongo em cultivo, mas a indução de diferenciação nessas células também leva à morte celular (Rhee *et al.*, 2002). Por outro lado, a superexpressão de DNMT1 induz uma hipermetilação progressiva de DNA

associada à inativação transcricional de vários genes *imprinting*, levando à letalidade embrionária (Biniszkiewicz *et al.*, 2002). Não é de surpreender que o conjunto de características epigenéticas distintas e mudanças nos níveis de transcritos observados em blastocistos bovinos clonados (Wrenzycki *et al.*, 2001) devam causar um efeito significativo na expressão de uma variedade de genes *imprinting* e não-*imprinting* após a clonagem (Wrenzycki *et al.*, 2001; Bertolini *et al.*, 2002a, 2004). Assim, uma ativação ou inativação inadequada de importantes genes para o desenvolvimento pode predispor os animais a diferentes graus de anormalidades após a clonagem pela TNCS, dependendo da interação entre discrepâncias qualitativas e quantitativas, subsequente ao nível ou grau de reprogramação do genoma obtido após a reconstrução do embrião (Bertolini *et al.*, 2012). Por exemplo, células com alterações em genes *imprinting* podem ser direcionadas para o trofectoderma de embriões clonados, gerando potencialmente membranas fetais defeituosas, como frequentemente observado após a clonagem (Wei *et al.*, 2011). A configuração do genoma modificado de embriões clonados por padrões aberrantes de metilação do DNA pode e*star* intimamente associada a falhas de reprogramação, perfis alterados de expressão gênica e desenvolvimento anormal de embriões.

Em bovinos, padrões anormais de metilação do DNA têm sido associados a anormalidades após a clonagem, que por sua vez podem ser fenotipicamente expressas por de*SORD*ens fetais e placentárias, aumento de perdas pré-natais e menor sobrevida pós-natal (Bertolini & Anderson, 2002; Han *et al.,* 2003; Salilew-Wondim *et al.*, 2013). Entretanto, um certo grau de variação no padrão de expressão gênica após a clonagem, geralmente atribuído a características epigenéticas transmitidas pelo núcleo da célula doadora, é totalmente aceitável e compatível com crescimento, desenvolvimento e sobrevivência normais após o nascimento (Humpherys *et al.*, 2001; Wells *et al.*, 2003).

3. HIPÓTESE E OBJETIVOS

Hipotetizamos que conceptos produzidos pela clonagem por NT-HMC (transferencia nuclear por *Handmade Cloning*) apresentarão um perfil metabólico indicativo de maior aporte de substratos energéticos (maior fluxo total

e/ou partição de nutrientes) em relação ao grupo controle, associado ao desenvolvimento, tanto no perfil bioquímico plasmático quanto na expressão de genes placentários e/ou dos fígados fetais, com este perfil estando associado a diferenças de desenvolvimento fenotípico dos conceptos.

Esta tese visa o entendimento de mecanismos bioquímicos, metabólicos e moleculares associados ao aparecimento da síndrome dos bezerros anormais causada por manipulações embrionárias *in vitro* (FIV e NT-HMC). Para tanto, realizamos a:

a) Análise do perfil bioquímico enzimático, endócrino e protéico dos fluidos fetais (fluido amniótico e alantóico) e do plasma fetal e materno oriundos de prenhezes produzidas *in vivo*, por FIV e por NT-HMC, aos 225 dias de gestação.
b) Análise de expressão de genes relacionados a processos fisiológicos importantes no desenvolvimento de conceptos bovinos, tais como o transporte de glicose (*SLC2A1*, *SLC2A3*) e frutose (SLC2A5), enzimas chave de rotas metabólicas importantes associadas ou não ao metabolismo intermediário, como a glicólise (*LDHA* and *LDHB*), gliconeogênese (G6PC, FBP1 and PCK1), frutólise (KHK, DAK and *ALDOB*), frutogênese (*AKR1B1* and *SORD*), ciclo das pentoses (*G6PD*), síntese de lipídeos (*ACACA* and *FASN*) e de colesterol (*HMGCR*), fatores de crescimento da família IGF e seus receptores (IGF-1, IGF-2, IGF-1r, IGF-2r), apoptose (*BAX*, BCL-2) e função placentária (bLP, b*PAG*-1, *STAR*, $P450_{aro}$) em tecidos placentário, e/ou fígado fetal oriundos de prenhezes produzidas *in vivo*, por FIV e por NT-HMC, aos 225 dias de gestação.
c) Correlação das características fenotípicas fetais e do concepto com as características fisiológicas, bioquímicas, metabólicas e moleculares placentárias e hepáticas mensuradas nos fluidos e tecidos maternos e fetais aos 225 dias de gestação.

CAPÍTULO II

Biochemical and metabolic profiles in *in vivo*- and *in vitro*-derived concepti in late pregnancy in cattle[1]

[1] Artigo elaborado conforme as normas da revista *Reproduction* (Apêndice 1)

Biochemical and metabolic profiles in *in vivo*- and *in vitro*-derived concepti in late pregnancy in cattle

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ABSTRACT

The aim of this study was to compare the metabolic profiles of maternal and foetal blood and the liver gene expression of Day-225 bovine foetuses produced either *in vivo* (IVD) by superovulation and artificial insemination, or *in vitro* by IVF or by Handmade Cloning (NT-HMC). Biochemical evaluations were performed in foetal and maternal plasma and the expression of key genes to foetal development were analysed by RT-qPCR in foetal liver tissue. Data were compared with physical traits between groups by a simple correlation test and by AN(C)OVA. Concentrations of maternal plasma metabolic and gene expression profiles were similar between groups, with a few biochemical and molecular differences. Most foetal parameters for liver and kidney functions, hemoglobin metabolism, and osmolalities in foetal plasma and related fluids were higher in clones than the other groups. Glucose, fructose, tryglycerides and VLDL concentrations in foetal plasma, and fructose levels and total amount of glucose and fructose in the allantoic fluid were higher in clones. Differences in the relative expression levels in the foetal liver were also detected for genes related to fructose and glucose metabolism, sugar transport, and fatty acid and cholesterol metabolism. A significant difference existed in activity in metabolic pathways in cloned concepti, suggesting active fructose accumulation by the foetus, which may be a reflection of an association between changes in metabolic foetal programming and excessive prenatal growth after cloning.

Keywords: Foetal physiology, Abnormal Offspring Syndrome, cloning by nuclear transfer, cattle.

INTRODUCTION

The *in vitro* production (IVP) of bovine embryos by *in vitro* fertilisation (IVF) or cloning by somatic cell nuclear transfer (SCNT) is a powerful tool routinely used for scientific and commercial applications worldwide. However, developmental abnormalities caused by such reproductive technologies can interfere with the pattern of foetal and placental growth that often result in a set of symptoms called Abnormal Offspring Syndrome (AOS), which includes accelerated foetal growth and increased birth weight, anatomical, physiological and metabolic abnormalities, and reduced neonatal survival. The etiology of AOS has been associated with increased concentrations of sugar moieties (glucose, fructose) in the foetal and neonatal plasma and placental fluids, which might be a consequence of changes in placental function (Bertolini *et al.*, 2004). In fact, development of concepti derived from bovine IVP embryos has been shown to be associated with a biphasic growth pattern, with an early growth retardation, which coincides with the period of placentation, followed by foetal and placental accelerated growth in mid- to late pregnancy (Bertolini *et al.*, 2002, 2004; Batchelder *et al.*, 2007; Gerger *et al.*, 2016). Nonetheless, detailed information on placental function and metabolic profile of bovine pregnancies established by IVP embryos is still limited.

In vitro embryo culture conditions, including the presence of serum in media, embryo co-culture with supporting cells, and/or faulty epigenetic reprogramming following cloning by SCNT have been implicated in the occurrence of AOS (Chavatte-Palmer *et al.*, 2012). In fact, the low cloning efficiency is thought to arise from an incomplete reprogramming of the donor cell nucleus, which leads to abnormal expression of developmentally important genes (Sawai, 2009). Therefore, cloned embryos exhibiting abnormal expression of embryonic genes may represent an early indication of incomplete reprogramming that could result in lower subsequent survival outcome. Notwithstanding, the turn of events that allows a viable but epigenetically affected IVP embryo to develop from early to late pregnancy appears to interfere with placental function and conceptus metabolism without lethally compromising homeostasis (Bertolini *et al.*, 2012). Nevertheless, the physiological, metabolic and/or molecular

mechanisms leading to the AOS, defined in early embryonic stages and manifested during and after pregnancy, remain unknown (Gerger *et al*., 2016).

The aim of this study was to compare the gene expression profiles of key metabolic-related enzymes and bioactive molecules in foetal liver tissue and the biochemical profiles in the maternal and foetal plasma and foetal fluids with physical traits from Day-225 bovine concepti produced either *in vivo*- (Control), or *in vitro* by IVF or SCNT cloning. Such moment in pregnancy represents the peak of the relative foetal growth pattern in cattle (Eley *et al*., 1978; Prior & Laster, 1979; Ferrell, 1989; Reynolds *et al*., 1990), also being a period of readily detectable phenotypical changes in bovine IVP concepti (Farin & Farin, 1995).

MATERIAL AND METHODS

This study used biological samples collected in a companion study (Gerger *et al.,* 2016). All procedures performed were approved by the Animal Ethics Committee of the University of São Paulo (São Paulo, SP, Brazil). Figure 1 illustrates some of the intracellular pathways involved in the intermediate metabolism in the liver, for which gene expression patterns of selected key enzymes for pathways involved in the carbohydrate, amino acid, and lipid metabolisms were analysed by RT-qPCR, as well as the interface with some of the maternal and foetal blood and foetal fluid components determined in the biochemical analyses, as below.

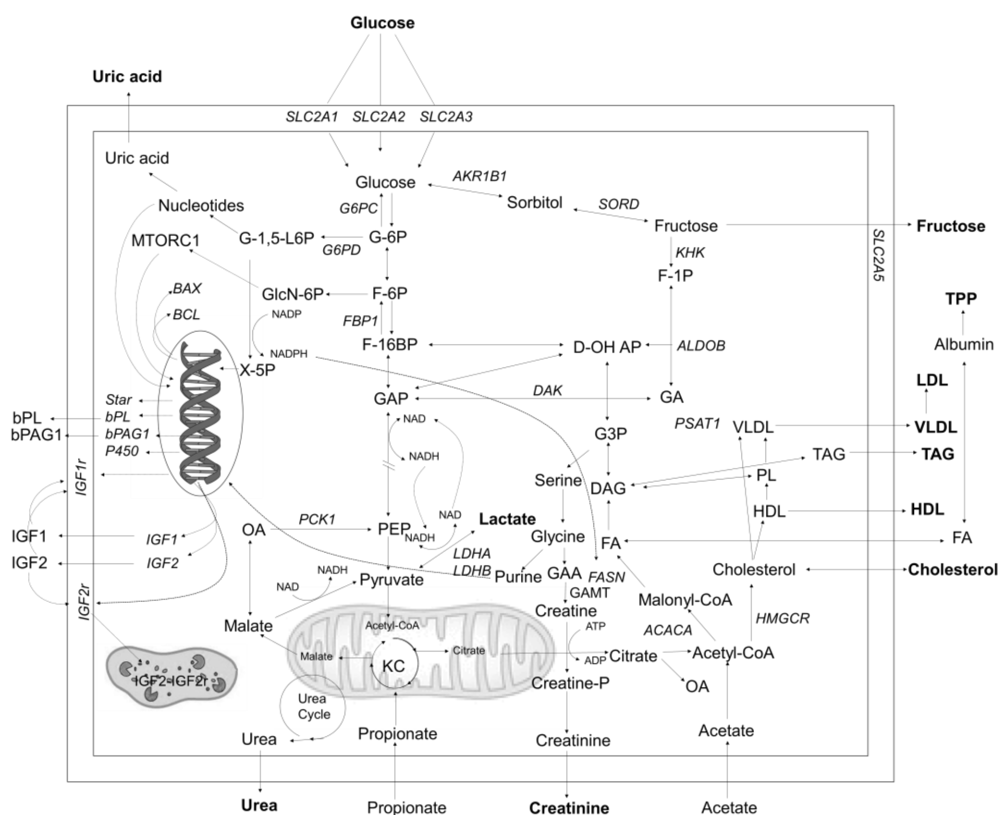

Figure 1. Simplified intracellular pathways involved in the intermediate metabolism in the liver or peripheral tissues, and interface with blood components associated with systemic metabolism. Genes analysed (*italicized*) were related to sugar transport (solute carrier family 2 member 1, *SLC2A1*; member 3, *SLC2A3*; member 5, SLC2A5); glycolysis (lactate dehydrogenase A and B, *LDHA* and *LDHB*); gluconeogenesis (glucose-6-phosphatase, G6PC; fructose-1,6-bisphosphatase, FBP1; phosphoenolpyruvate carboxykinase, PCK1); fructolysis (fructokinase, KHK; dihydroxyacetone kinase 2, *TKFC*; aldolase B, *ALDOB*); fructogenesis (aldose reductase, *AKR1B1*; sorbitol dehydrogenase, *SORD*); pentose phosphate pathway (glucose-6-phosphate dehydrogenase, *G6PD*); fatty acid synthesis (acetyl-CoA carboxylase alpha, *ACACA*; fatty acid synthase, *FASN*); cholesterol synthesis (3-hydroxy-3-methylglutaryl-CoA reductase, *HMGCR*); apoptosis (*BCL2*-associated X protein, *BAX*; *BCL2* B-cell CLL/lymphoma 2, *BCL2*); insulin-like growth factor (IGF) system (*IGF1* and *IGF2*; *IGF1* receptor, *IGF1*r; *IGF2* receptor, *IGF2*r); serine synthesis (phosphoserine aminotransferase, PSAT; phosphoglycerate dehydrogenase, PHGDH); and placental function (steroidogenic acute regulatory protein, *STAR*; cytochrome *P450*, *P450*; bovine placental lactogen, b*PL*; bovine pregnancy-associated glycoprotein 1, b*PAG*1). Fluid biochemical parameters (in **bold**) include total plasma protein (TPP), uric acid, urea, creatinine, lactate, triglycerides (TAG), cholesterol, high-density lipoproteins (HDL), very-low-density lipoprotein (VLDL), low-density lipoprotein (LDL), glucose and fructose.

Bovine embryo production, animals and experimental design

Bovine embryos were produced either *in vivo* (*in vivo*-derived controls, or IVD group), by the superovulation and artificial insemination of donor females or *in vitro*, by *in vitro* fertilisation (IVF group) or nuclear transfer by handmade cloning (NT-HMC group) procedures. The three embryo production systems were previously described in details (Gerger *et al.*, 2016). Fresh Day-7 embryos at the blastocyst or expanded blastocyst stages from the three experimental groups were transferred to synchronous crossbred recipients (*Bos taurus* var. *taurus vs. Bos taurus* var. *indicus*), according to procedures described by Bertolini *et al.* (2002). All recipients were managed together in pasture paddocks with free access to water and mineral premix. Pregnancy diagnosis was performed on Day 30 of gestation by ultrasonography, and pregnant recipients were monitored every two weeks until Day 225 of pregnancy.

A total of 19 bovine pregnancies were established carrying Nellore cattle singleton concepti, produced either *in vivo* (IVD group, n=9, four for *post-mortem* collection, and five for the *in vivo* kinetics study, as below), or *in vitro* by IVF (IVF group, n=4, for *post-mortem* collection) or HMC cloning (NT-HMC group, n=6, for *post-mortem* collection) procedures. Concepti from all embryo production systems were from similar genetic backgrounds, and the same *in vitro* culture (IVC) system was used for NT-HMC- and IVF-derived embryos to minimize developmental deviations in the IVF group, highlighting differences related to cloning procedures *per se* and not to IVC (Gerger *et al.,* 2016). The Nellore breed was chosen due to its rather low birth weights in commercial herds (Ribeiro *et al.*, 1992).

Table 1. Selected physical traits for *in vivo*-derived (IVD), IVF-produced and cloned (NT-HMC) bovine concepti on Day 225 of gestation

Foetal trait	IVD	IVF	NT-HMC	P[1]
Foetal weight (kg)	12.5 ± 2.3[a]	13.7 ± 3.2[a]	27.2 ± 2.0[b]	0.005
Crown-rump length (cm)	58.3 ± 2.2[a]	59.0 ± 3.1[a]	67.5 ± 2.0[b]	0.040
Foetal liver weight (g)	290.3 ± 58.2[a]	345.8 ± 82.4[a]	769.9 ± 52.1[b]	0.001
Total placenta weight (kg)	3.2 ± 0.5[a]	3.2 ± 0.8[a]	7.1 ± 0.5[b]	0.001
Placenta surface area (cm^2)	202 ± 347[ab]	112 ± 347[a]	312 ± 283[b]	0.023
Total placentome number (n)	81.5 ± 8.9[a]	64.8 ± 12.6[a]	79.7 ± 7.9[a]	0.496
Total foetal fluid volume (L)†	7.8 ± 8.4[a]	8.0 ± 11.9[a]	31.0 ± 7.5[a]	0.066

†Foetal fluid volumes measured only from five out of six cloned specimens, as fluids were lost in the largest cloned conceptus (a 42-kg fetus) due to uterine rupture during pregnant tract retrieval

[a,b]:Numbers in each row with distinct superscripts differ, P<0.05

Summarized from Gerger *et al.* (2016)

Post-mortem collection of samples

Fourteen pregnant females carrying IVD (n=4), IVF (n=4) and NT-HMC (n=6) concepti were slaughtered on Day 225 of gestation at a local abattoir, with the harvesting and qualitative and quantitative analyses of maternal and conceptus tissues and fluids, as previously described (Gerger *et al.*, 2016). Briefly, following food restriction for 12 h, maternal jugular blood samples were drawn 30 min prior to slaughter (t=-30 min). Then, pregnant females were stunned and exsanguinated, with the immediate collection of blood samples (t =0 min). The pregnant uterus was removed intact, weighted and carefully dissected at the major curvature. The foetus was exposed with the umbilical cord still intact. Blood samples were collected from the umbilical artery and vein and the foemoral artery (t=0 min), followed by the excision of the cord. The foetus and the empty uterus, containing the foetal membranes and the placenta, were weighed, morphological foetal and placental measurements were performed, and foetal muscles, visceral organs, and endocrine glands were carefully dissected,

weighed, and sampled in 2.5% glutaraldehyde or snap-frozen in liquid nitrogen (LN_2). Blood samples collected from the three groups at t=-30 min (maternal voenous blood) and t=0 min (maternal voenous blood and foetal arterial and voenous blood), and samples of amniotic and allantoic fluids, after careful volume measurement, were centrifuged at 6000 g for 15 min, with plasma and fluids stored at -80ºC.

(i) Biochemical analyses in foetal fluids and maternal and foetal plasma

Samples from maternal (t=-30 min and t=0 min) and foetal (umbilical voenous blood) plasma were used for determination of plasma biochemical parameters for liver (alanine aminotransferase, ALT; aspartate aminotransferase, AST; gamma-glutamyl transpeptidase, GGT; lactate dehydrogenase, LDH; total plasma protein, TPP; alkaline phosphatase, ALP) and renal (uric acid; urea; creatinine) functions; energy status (lactate; triglycerides; cholesterol; high-density lipoproteins, HDL; very-low-density lipoprotein, VLDL; low-density lipoprotein, LDL), hemoglobin metabolism (total bilirubin; conjugated and unconjugated bilirubin), and calcium/phosphorus metabolism, using an automated biochemical analyzer (Labmax 240®, Biotécnica, Brazil). Each sample was run in duplicates. In addition, the osmolalities of maternal and foetal plasma and placental fluids were also measured in duplicates (Osmomat 030®, Gonotec GmbH, Germany).

The determination of glucose and fructose concentrations in maternal (jugular vein at t=-30 min and t=0 min) and foetal (umbilical voenous and foemoral arterial blood) plasma and placental fluids (amniotic and allantoic fluids) was performed by spectrophotometry, in duplicates, according to Bertolini *et al.* (2004). Glucose was determined by an enzymatic assay kit (QuantiChrom™ Glucose Assay Kit, BioAssay Systems, USA), in 96-well plates read at 620 nm (Biotrak II Reader®, Amersham Biosciences, USA), following the manufacturer´s recommendations. Fructose was determined based on Taylor (1995), in microcuvettes (PlastiBrand®, Germany) at 518 nm (Ultrospec 1100 PRO®, Amersham Biosciences, USA), as adapted by Bertolini *et al.* (2004). Due to high fluid fructose concentrations, arterial and voenous foetal plasma and foetal fluids

were diluted in ultrapure water at 1:2 (foetal plasma and amniotic fluid) and 1:4 (allantoic fluid). Samples with an intrassay variation >6% were reanalysed. Glucose and fructose clearance rates (foetal uptake and consumption) were determined in each group using the Fick Principle based on the mean umbilical blood flow (below), by the steady-state diffusion procedure with D_2O for the IVD group, and on the estimation of blood flows in the NT-HMC and IVF groups.

(ii) Analyses of gene expression in foetal liver

Total RNA from foetal liver samples from the three experimental groups was extracted using Trizol® reagent (Invitrogen, USA), and 1800 ng of total RNA from each sample were treated with DNase I (Invitrogen) and used for cDNA synthesis using the GoScript™ Reverse Transcription System (Promega, Madison, WI, USA) according to the manufacturer instructions. Quantification of housekeeping and target transcripts (Table 2) was performed by real time qPCR (RT-qPCR) using the GoTaq® qPCR Master Mix (Promega, USA) and the Fluorescent Quantitative PCR Detection System LineGene 9600 (Bioer Technology, USA). Gene products were evaluated for the housekeeping genes *RPS9* (ribosomal protein S9) and *ACTB* (β-actin), and target genes involved with glycolysis: lactate dehydrogenase A and B (*LDHA* and *LDHB*); gluconeogenesis: phosphoenolpyruvate carboxykinase (*PCK1*), fructose-1,6-bisphosphatase (*FBP1*), glucose-6-phosphatase (*G6PC*); fructolysis: fructokinase (*KHK*), dihydroxyacetone kinase 2 (*TKFC*), aldolase B (*ALDOB*); fructogenesis: aldose reductase (*AKR1B1*), sorbitol dehydrogenase (*SORD*); pentose phosphate pathway: glucose-6-phosphate dehydrogenase (*G6PD*); fatty acid synthesis: acetyl-CoA carboxylase alpha (*ACACA*) and fatty acid synthase (*FASN*); cholesterol synthesis: 3-hydroxy-3-methylglutaryl-CoA reductase (*HMGCR*); glucose transporters: solute carrier family 2 member 1 (*SLC2A1*), member 2 (*SLC2A2*), member 3 (*SLC2A3*), and member 5 (*SLC2A5*); apoptosis: *BCL2*-associated X protein (*BAX*), *BCL2* B-cell CLL/lymphoma 2 (*BCL2*); insulin-like growth factor system: insulin-like growth

Table 2. Gene functions, Gene symbol, GenBank accession codes, primer sequences and amplicon sizes for transcripts analysed by real time qPCR in Day-225 IVD control, IVF-derived and NT-HMC cloned *Bos*

taurus var. *indicus* foetal and maternal liver samples

Function or Pathway	Gene Symbol	Accession Number	Primer sequences (5'-3')	Product (bp)
Glycolysis	*LDHA*	NM_174099.2	F: GATCTCCAACATGGCAGCCT R: TCACGTTACGCTGGACCAAA	154
	LDHB	NM_174100.2	F: AGTGGATTACCCAAGCACCG R: TCCCCCAAAATCCATCCGTG	125
Gluconeogenesis	*G6PC*	NM_001076124.2	F: TTCGAGAAGCTGTGGGCATC R: AAAACCCACCAGTATGGGCG	109
	FBP1	NM_001034447.2	F: AATATCGTCACCGTGACCCG R: TCCCGTCACATTGGTAGTGC	177
	PCK1	NM_174737.2	F: GAGTTCGTCAAGTGCCTCCA R: AGCACTTCTTCCCAAGGAGC	155
Fructolysis	*KHK*	NM_001076351.1	F: ACTCTACGACACGAACCTGC R: TTCACCTGCTCTGATGCGTT	111
	DAK	NM_001024524.3	F: AAAAGGCTTACCCGGACGAG R: GCTAAGAAGGGGGTGTGGAC	136
	ALDOB	NM_001034485.2	F: ACCCATTGTTGAGCCAGAGG R: ACTTCTTGGTGCAGGCATGT	179
Fructogenesis	*AKR1B1*	NM_001012519.1	F: GTGGCAATCGACCTTGGGTA R: TGATACGTGCACCACAGCTT	157
	SORD	NM_001037320.1	F: GTGGAGACCTCCATCCAAGC R: AATCGCCATCGGCCATGTAT	168
Pentose phosphate pathway	*G6PD*	NM_001244135.1	F: GAGCAGCGAAGCACAGAGAG R: AAAGATGTGCGTATCGGCCT	126
Fatty acid synthesis	*ACACA*	NM_174224.2	F: CATGGAGTGGCAGTGGTCTT R: AGG*ACACA*TGAAGGCTGTGG	97
	FASN	NM_001102150.1	F: ATCCTAGCCCAGGAGAGAGC R: CCAGCTAGCACCACCTTCAT	137
Cholesterol synthesis	*HMGCR*	NM_001105613.1	F: CCAAGTTCGCCCTCAGTTCT R: GGTGGGACCTAAAATCGCCA	80
Glucose transporter	*SLC2A1*	NM_174602.2	F: GACTCCATCATGGCAACCA R: GGTTCTCCTCGTTGCGGTTA	149
	SLC2A2	NM_001103222.1	F: TCAGCAACTGGACAGGCAAT R: TTTCGGAACTCTGCTGCGAT	180
	SLC2A3	NM_174603.3	F: GGCGCCTATGTTTTCATCGTC R: ATGATGGGGCCTTTCTCACC	161
	SLC2A5	NM_001101042.1	F: TGTCTTCCAATGTCGTCCCC R: GTCAATCCGAGGAGGATGGG	175
Pro-apoptotic factor	*BAX*	NM_173894.1	F: GCCCTTTTGCTTCAGGGTTT R: ACAGCTGCGATCATCCTCTG	179
Anti-apoptotic factor	*BCL2*	NM_001166486.1	F: GAGTTCGGAGGGGTCATGTG R: GGGCCATACAGCTCCACAAA	158
IGF system	*IGF1*	NM_001077828.1	F: TTCGCTCTGCACGAGTTACC R: TTCATTGGGGGAAATGCCCA	92
	IGF2	NM_174087.3	F: GTGCTTCTTGCCTTCTTGGC R: GCAACACTCTTCCACGATGC	183
	IGF1r	NM_001244612.1	F: CTAGCTCCGGAGGAGGGTC R: GTCCTCGGCCTTGGAAATGA	200
	IGF2r	NM_174352.2	F: ACCTCCGATCCTCAATCCCA R: TGTAGTTGAAGTGCCGGTCC	89
Serine synthesis	*PHGDH*	NM_001035017.1	F: GTGGGAGCGGAAGAAGTTCA R: GACCTCTGGCGAGATGATGG	200
	PSAT1	NM_001102150.1	F: GGTCAGCTAAGGCTGCAGAA R: AGCACTGCTCCCTTGACATC	199
Endogenous control gene	*ACTB*	NM_173979.3	F: CGTGAGAAGATGACCCAGATCA R: GGG*ACACA*GCCTGGA	76
	RPS9	NM_001101152.2	F: CCTCGACCAAGAGCTGAAG R: CCTCCAGACCTCACGTTTGTTC	62

factors 1 (*IGF1*) and 2 (*IGF2*), IGF receptors 1 (*IGF1r*) and 2 (*IGF2r*), and serine synthesis: phosphoserine aminotransferase (*PSAT*) and phosphoglycerate dehydrogenase (*PHGDH*). The amplification cycling conditions used an initial denaturation at 95°C for 2 min, followed by 45 cycles consisting of 95°C for 15 s and 60°C for 60 s. The fluorescence data were recorded at the end of each extension step (60°C). Pilot experiments using six different concentrations of cDNA (spanning a 60-fold range) were run to set up RT-qPCR conditions. The specificity of PCR products was confirmed by analysis of melting curves. All reactions were performed in duplicates.

Determination of umbilical and uterine blood flows – *in vivo* kinetics study

Five pregnant females carrying IVD Nellore concepti were allocated to metabolic cages on Day 205 of pregnancy for animal acclimation to handling and confinement, remaining throughout the experimental period (28 days), receiving water *ad libitum* and balanced food ration according to the NRC (1996). The determination of the uterine and umbilical blood flows was based on the Fick principle, by using the steady-state diffusion procedure with deuterium oxide (D_2O), following procedures by Reynolds & Ferrell (1987), Ferrell (1991b) and Ferrell & Reynolds (1992). Briefly, on Day 220, females were subjected to mid-ventral laparotomy for surgical cannulations with indwelling catheters of uterine artery and vein (pregnant horn), followed by hysterotomy for cannulation of the foetal foemoral artery and vein, and umbilical vein. On Day 225, a priming dose of 2 mL deuterium oxide (D_2O, Icon Isotopes, USA) was infused in the foemoral vein of the foetus in less than 1 min (t=-60 min), followed by a constant infusion (0.2 mL/min) for 7 h (Nutrimat II, B/Braun, Brazil). Blood samples were collected from the foetal (umbilical vein and foemoral artery, 5 mL/sample) and maternal (uterine artery and vein, 10 mL/sample) blood at t=-60, 0, 5, 15, 30, 45, 60, 90, 120, 180, 240, 300 and 360 min. A portion of each blood sample was used for blood chemistry analyses, while the other portion was stored frozen at –80°C for substrate analyses and D_2O quantification to determine the umbilical and uterine blood flows. The measurement of D_2O concentrations was performed by near-infrared spectrophotometry (Isotopic Ecology Lab, Center for Nuclear Energy in

Agriculture, University of São Paulo - CENA/USP, Brazil) according to procedures by Ferrell & Philips (1979) and Ferrell & Reynolds (1992). Then, umbilical blood flows in the NT-HMC and IVF groups were estimated based on the umbilical blood flow:foetal weight ratio from the IVD controls (0.22 L/min per kg), which was similar to Ferrell (1990b) for Brahman concepti carried by Brahman dams on Day 227 of pregnancy (0.21 L/min).

Statistical analyses

Quantitative data comprising physical traits, and biochemical, metabolic, and molecular analyses, were compared between groups (IVD, IVF, NT-HMC) and time periods (t=-30 min and t=0 min for maternal plasma analyses) by using the MIXED procedure of SAS (SAS Institute, 2015), with the model including treatment groups as fixed effect. For gene expression data, the geometric means of the housekeeping genes (*RPS9* and *ACTB*) were used to normalize data of the target genes (Vandesompele *et al.,* 2002). Foetal weight was used as covariate for the analyses of covariance of physical and chemical traits. Data were tested for normality by the Anderson-Darling normality test, with the log transformation of non-normal variables. A simple correlation test was used to evaluate relationships between traits.

RESULTS

Morphological data from Gerger *et al.* (2016) that were most relevant to this study are summarized in Table 1. Maternal physical parameters were similar between groups, with pregnant females (dams) from the three groups having similar body weights, body condition scores, carcass weights and carcass yields (Gerger *et al.*, 2016). The pregnant tract and the empty tract (uterine tract deprived of the foetus and foetal fluids, but including the uterus, foetal membranes and placenta) were significantly heavier in the NT-HMC group than the IVD and IVF groups, which were similar to one another (Table 1; Gerger *et al.*, 2016). Heavier fetuses and/or larger foetal fluid volumes were found in the NT-HMC group (Table 1; Gerger *et al.*, 2016). The uterus weights were similar between groups after further removal of the foetal membranes and placentomes

from the empty tracts (Table 1; Gerger *et al.*, 2016).

Biochemical profiles – postmortem study

(i) Maternal plasma

No differences were observed between collection time periods (t=-30 min *vs.* t=0 min) for any biochemical measurement in the maternal plasma, irrespective of the groups, except for a 1.5-fold increase ($P < 0.05$) in mean glucose plasma concentrations from t=-30 min to t=0 min for all groups (from 4.9 ± 0.6 to 7.3 ± 0.6 mM, respectively), which may be related to pre-slaughter stress.

Maternal plasma glucose concentrations in the IVD, IVF, and NT-HMC groups on t=-30 min (8.1 ± 4.2, 5.4 ± 1.2, and 7.2 ± 4.1 mM) and on t=0 min (7.4 ± 2.5, 7.8 ± 0.7, and 10.5 ± 4.6 mM), and concentration differences between t=-30 min and t=0 min for glucose (-0.7 ± 4.1, 2.4 ± 1.7, and 3.3 ± 5.8 mM) were similar between groups. Even though values for all groups are low, irresepctive of the collection time, as expected (Bertolini *et al.*, 2004), maternal plasma fructose concentrations were higher in the NT-HMC group (0.37 ± 0.21 mM) compared with the IVD (0.07 ± 0.07 mM) and the IVF (0.04 ± 0.03 mM) groups. However, fructose concentration differences in the maternal plasma between t=-30 and t=0 min were similar between groups (0.4 ± 0.1, 0.4 ± 0.1, and 0.3 ± 0.2 mM). Other metabolic maternal plasma components (plasma lactate, cholesterol, HDL, LDL, VLDL, tryglycerides) were similar between groups.

Overall, all parameters for liver and renal functions, hemoglobin metabolism, calcium/phosphorus metabolism, and electrolyte concentration/osmolality measured in the maternal plasma in both collection time periods were within the normal reference values for the species (Smith, 2009). However, plasma of NT-HMC-bearing recipients had higher osmolality (307.5 ± 8.1 *vs.* 295.5 ± 4.0 and 294.8 ± 8.2 mOsm/kg), concentrations of chloride (108.8 ±1.6 *vs.* 102.8 ± 2.0 and 98.0 ± 2.0 mEq/L), conjugated bilirubin (0.18 ± 0.03 *vs.* 0.12 ± 0.03 and 0.13 ± 0.03 mg/dL), and urea (39.0 ± 3.1 *vs.* 13.5 ± 3.8 and 16.0 ± 3.8 mg/dL), and lower ALT concentration (22.8 ± 2.2 *vs.* 34.0 ± 2.7 and 21.0 ±

2.7 mg/dL) compared with IVD- and IVF-bearing recipients, respectively. Total bilirubin concentration was higher in the NT-HMC (0.89 ± 0.15 mg/dL) than the IVD (0.25 ± 0.19 mg/dL) group, with both being similar to the IVF group (0.35 ± 0.19 mg/dL).

(ii) Foetal plasma

Most parameters evaluated for foetal liver function revealed differences between groups, as depicted in Figure 2 (panels a and b). Plasma LDH and GGT concentrations were lower in the IVF and NT-HMC groups than in IVD controls, and alkaline phosphatase (ALP) and total plasma protein (TPP) levels were lower in clones than in the IVD group, with both being similar to the IVF group. On the other hand, plasma ALT was higher in clones and IVF-derived foetuses than IVD controls.

As for products from the hemoglobin metabolism, bilirubin and total bilirubin (conjugated and unconjugated) concentrations were higher in the plasma of clones and IVF foetuses than IVD controls, with conjugated bilirubin being similar between groups (Figure 2, panel c), denoting a higher red blood cell clearance and/or lower liver conjugation activity in cloned and IVF foetuses.

Parameters used for renal function, which are also associated with nitrogen metabolism and muscle activity, were different between groups (Figure 2, panels d and f). Plasma urea and creatinine were respectively higher and lower in cloned concepti than IVD controls and the IVF group. Plasma uric acid concentrations, which also reflect the metabolism of purine nitrogenous bases, were higher in clones when compared with IVD controls, with both being similar to the IVF group.

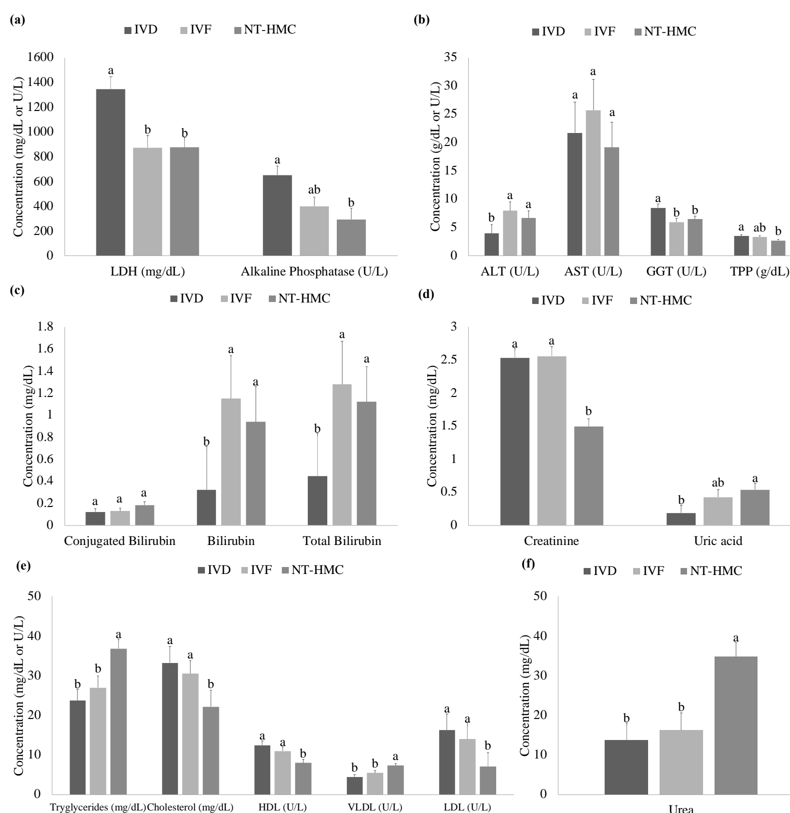

Figure 2. Biochemical parameters for liver and renal functions, hemoglobin metabolism and osmolality in maternal and/or foetal plasma and foetal fluids between Bos taurus var. indicus *in vivo*-derived (IVD) concepti and concepti produced either by *in vitro* fertilisation (IVF) or nuclear transfer by handmade cloning (NT-HMC) procedures, on Day 225 of gestation (LSM ± SEM). (a) Foetal plasma lactate dehydrogenase (LDH) and alkaline phosphatase (ALP) concentrations. (b) Foetal plasma alanine aminotransferase (ALT), aspartate aminotransferase (AST), gamma glutamyl transpeptidase (GGT), and total plasma protein (TPP) concentrations. (c) Foetal plasma conjugated, unconjugated and total bilirubin concentrations. (d) Foetal plasma creatinine and uric acid concentrations. (e) Foetal plasma concentrations of components of the lipid metabolism (tryglycerides, cholesterol, HDL, LDL, VLDL). (f) Foetal plasma urea concentrations. a,b: Columns for each parameter without common superscripts (a,b) differ; P<0.05.

Tryglycerides and VLDL levels were higher, and cholesterol, HDL and LDL were lower in the foetal plasma of clones than in the other groups (Figure 2, panel e). Foetal plasma lactate concentrations tended to be lower (P=0.073) in the IVF group (9.72 ± 0.61 mM) than control foetuses (12.3 ± 0.95 mM), with both being similar to the NT-HMC group (11.43 ± 1.95 mM). However, high plasma lactate

values likely reflect a period of hypoxia in all groups for the time from slaughter to sampling of arterial (8.9 ± 1.0 min) and voenous (10.5 ± 1.0 min) umbilical blood.

Glucose and fructose concentrations in umbilical arterial and voenous plasma (Figure 3, panel a) were higher in clones than in the IVD control and IVF groups. The glucose umbilical voenous-to-arterial concentration differences were similar between groups (Figure 3, panel b), but concentration differences for fructose were lower ($P<0.05$) in the IVF and NT-HMC groups than controls, being similar between the IVF and the NT-HMC groups. Glucose and fructose clearance rates (foetal uptake and consumption) were 0.354 ± 0.113 and 0.581 ± 0.091 g/min in the IVD control group, 0.230 ± 0.057 and 0.212 ± 0.143 g/min in the IVF group, and 0.451 ± 0.134 and 0.696 ± 0.246 g/min in the NT-HMC group, respectively (Figure 3, panel c), with no differences between groups.

Plasma phosphorus (2.52 ± 0.06, 2.33 ± 0.23, and 2.87 ± 0.29 mM), calcium (3.53 ± 0.05, 3.05 ± 0.13, and 3.10 ± 0.30 mM), and chloride concentrations (105.3 ± 1.6, 98.3 ± 2.1, and 100.2 ± 6.6 mEq/L) were similar between IVD controls, IVF, and clones, respectively. To note, osmolalities in foetal plasma from the umbilical vein and artery and in the amniotic fluid were higher in clones than the other groups, with a trend ($P=0.099$) to be also higher in the allantoic fluid (Figure 4).

Several biochemical measurements in foetal plasma and fluids were similar when the foetal weight was used as covariate (Figures 2 to 4), with foetal liver weights in cloned fetuses being significantly larger than the other groups after ANCOVA (Gerger *et al.*, 2016), demonstrating that the liver size in clones was larger than the isometric prediction, which may have a biological significance in liver function, as seen above.

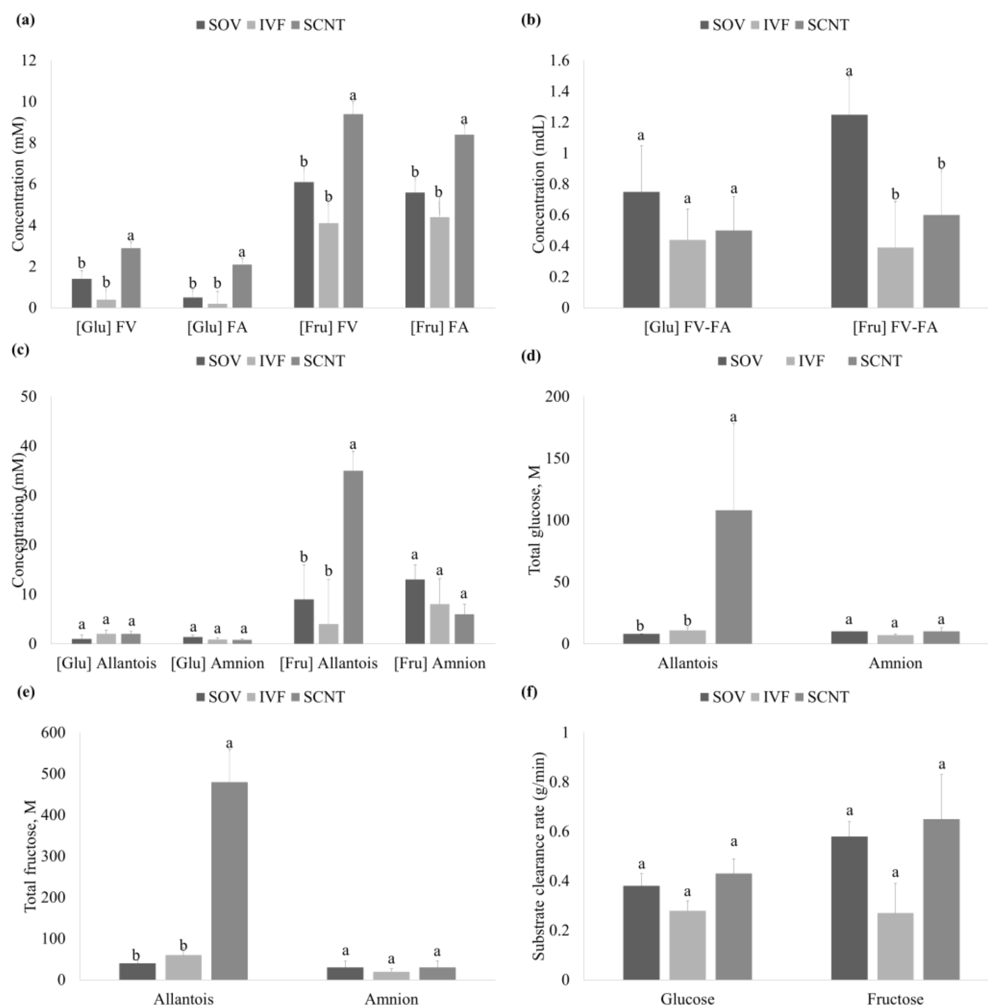

Figure 3. Glucose (Glu) and fructose (Fru) concentrations (mM), concentration differences (mM), and total amounts (mM) in the plasma and foetal fluids between Bos taurus var. indicus *in vivo*-derived (IVD) concepti and concepti produced either by *in vitro* fertilisation (IVF) or nuclear transfer by handmade cloning (NT-HMC) procedures on Day 225 of gestation (LSM ± SEM). (a) Glucose and fructose concentrations in the foetal vein (FV) and artery (FA). (b) Voenous-arterial glucose and fructose concentration differences in the foetal plasma. (c) Glucose and fructose concentrations in the foetal fluids. (d) Total glucose in the foetal fluids. (e) Total fructose in the foetal fluids. (f) Glucose and fructose clearance rates by the foetal system. Columns for each parameter without common superscripts (a,b) differ; P<0.05.

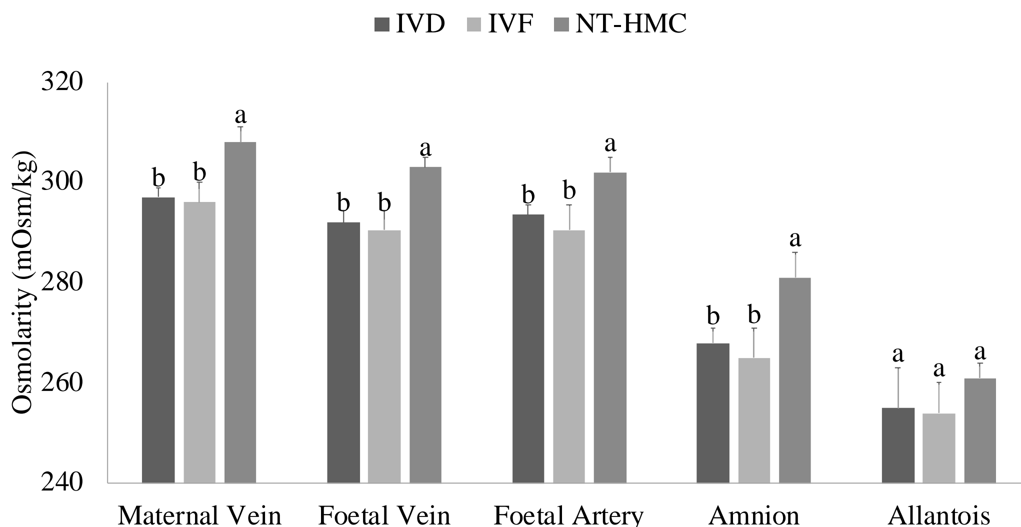

Figure 4. Maternal and foetal plasma and foetal fluids osmolality between *Bos taurus* var. *indicus in vivo*-derived (IVD) concepti and concepti produced either by *in vitro* fertilisation (IVF) or nuclear transfer by handmade cloning (NT-HMC) procedures on Day 225 of gestation (LSM ± SEM). Columns for each parameter without common superscripts ([a,b]) differ; $P<0.05$.

(iii) Foetal fluids

Fructose levels in the allantoic fluid (Figure 3, panel c) were higher in clones than in the IVD control and IVF groups, with no differences in glucose concentrations in both foetal fluids and in fructose in the amniotic fluid between groups (Figure 3, panel c). The total amount of glucose and fructose in the foetal fluids (concentrations by total fluid volume) revealed a 13.3- and 7.1-fold increase in glucose (Figure 3, panel d) and fructose (Figure 3, panel e) in the allantoic fluid of cloned concepti, respectively, than in the IVD control and IVF groups. No significant differences were detected between groups for both sugars in the amniotic fluid (Figure 3, panels d and e).

Gene expression in foetal liver – post-mortem study

The expression patterns of most foetal liver genes related to metabolic pathways were significantly different in the NT-HMC group. On one hand, increased relative transcription patterns for glucose transporters were detected in clones in relation to the IVD group, except for *SLC2A1* that did not differ

between treatments, for no significant SLC2A2 gene expression in any of the groups on Day 225 of gestation (Figure 5, panel a). Also, higher gene expression patterns were seen in the NT-HMC group for G6PC in the glyconeogenic (Figure 5, panel b), and for *LDHA* and *LDHB* in the glycolytic pathways when compared to controls (Figure 5, panel d). On the other hand, lower gene expression patterns were observed in clones, with lower *KHK* and *ALDOB* gene expression (Figure 5, panel e), denoting lower fructolytic activity; a 5.5-fold reduction was also seen in *HMGCR* relative transcription, for cholesterol synthesis; and a 6.2-fold reduction was observed in transcripts for *G6PD*, the key enzyme of the pentose phosphate pathway (Figure 5, panel c).

No significant differences were detected between groups regarding the *BAX*/*BCL2* ratio, as both *BAX* (pro-apoptotic) and *BCL2* (anti-apoptotic) had similar significantly lower expression patterns in the IVF and NT-HMC groups than control counterparts (Figure 5, panel h).

Antagonistic results were observed for expression patterns of genes related to lipogenesis and to the IGF system in the NT-HMC group as compared to the IVF and IVD groups. In the lipogenic pathway, a trend for increased *ACACA* expression (P=0.08) and reduced *FASN* relative transcription were seen in the NT-HMC group (Figure 5, panel c), whereas for the IGF system, *IGF2* and *IGF1r* expression patterns were downregulated by 5.5- and 5.8-fold, respectively, for a 1.7-fold increase in *IGF1* in cloned fetuses (Figure 5, panel g). In addition, the pattern of gene expression in foetal liver revealed significant differences in the relative transcription for the PHGDH and PSAT genes (Figure 5, panel f). As both are important components of the serine synthesis pathway, our data suggests that a downregulation was evident in cloned foetuses on Day 225 of pregnancy, with IVF patterns being intermediate between the NT-HMC and the IVD groups.

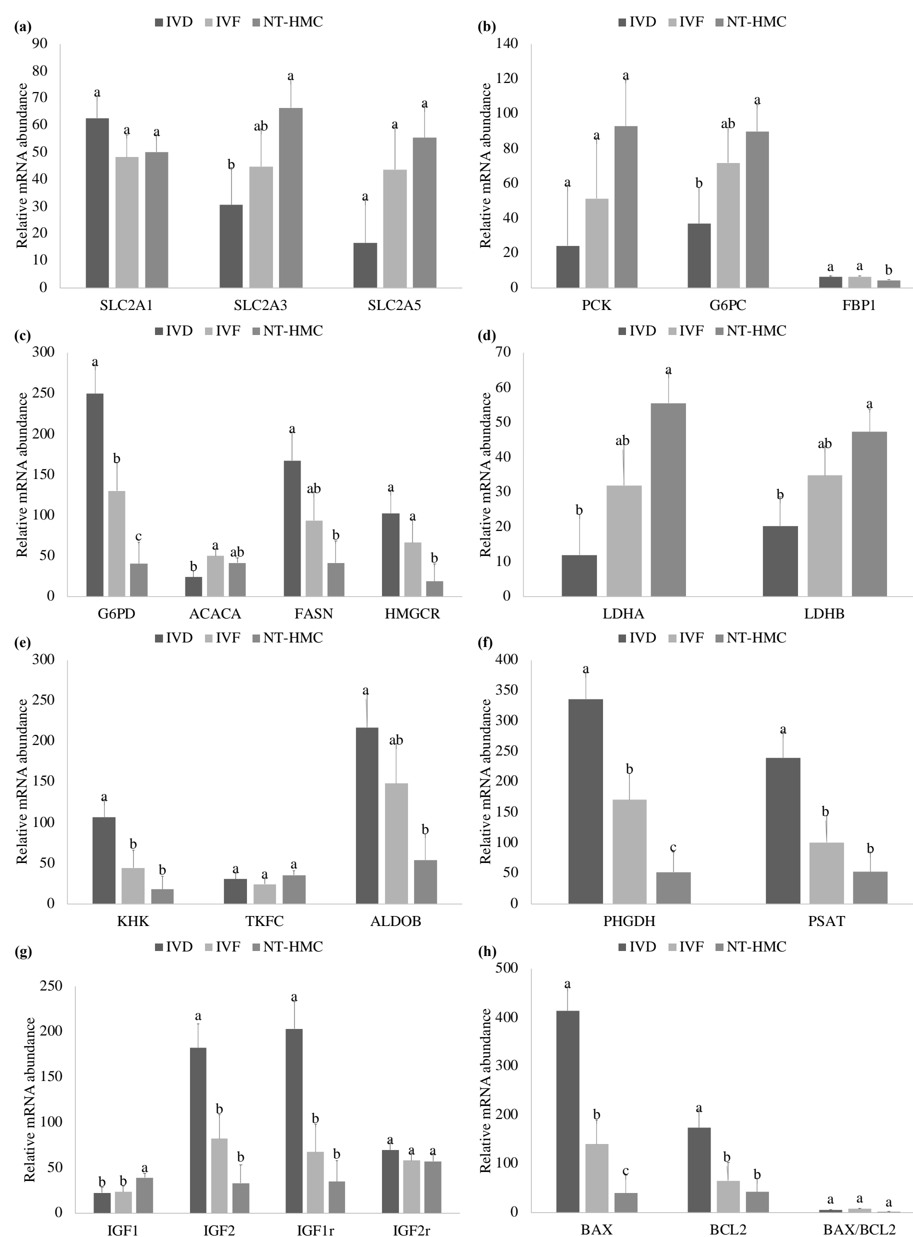

Figure 5. Relative gene transcription in foetal liver (LSM ± SEM) between *Bos taurus* var. *indicus in vivo*-derived (IVD) concepti and concepti produced either by *in vitro* fertilisation (IVF) or nuclear transfer by handmade cloning (NT-HMC) procedures on Day 225 of gestation (LSM ± SEM). Gene products for (a) sodium-independent facilitated hexose transporters (*SCL2A1*, *SCL2A2*, *SCL2A3,* and *SCL2A5*); (b) glyconeogenic enzymes (*PCK*, *G6PC,* and *FBP1*); (c) pentose phosphate pathway (*G6PD*), fatty acid synthesis (*ACACA* and *FASN*) and cholesterol synthesis (*HMGCR*); (d) glycolytic enzymes (*LDHA* and *LDHB*); (e) fructolytic enzymes (*KHK*, *DAK,* and *ALDOB*); (f) serine synthesis (PHGDH and PSAT); (g) IGF system (*IGF1*, *IGF2*, *IGF1*r, and *IGF2*r); (h) apoptosis (*BAX*, *BCL2,* and ratio *BAX/BCL2).* See text and Table 2 for details on genes and abbreviations. Columns for each gene transcript without common superscripts ([a,b]) differ; $P<0.05$.

Estimation of uterine and umbilical blood flows – *in vivo* kinetics study

Two out of five IVD pregnancies subjected to cannulations on Day 220 maintained functional catheters and viable singleton foetuses on Day 225 of pregnancy. Following D_2O maternal and foetal plasma concentration analyses, and after applying the Fick principle of mass conservation, the mean uterine and umbilical blood flow rates for all collection time periods were 5.34 ± 0.89 L/min and 2.80 ± 0.68 L/min, respectively. The flow rate variation during the 7 h flow analysis was slightly greater in the umbilical cord than in the uterus, ranging from 1.72 to 4.71 L/min (39% lower and 68% higher than the mean value) and 4.08 to 8.28 L/min (24% lower and 55% higher than the mean value), respectively. Estimation of umbilical blood flows in the IVF and NT-HMC groups, by using the umbilical blood flow:foetal weight ratio from the IVD controls (0.22 L/min per kg), resulted in the mean umbilical blood flow rates of 3.01 ± 0.63 and 5.98 ± 2.46 L/min for IVF and cloned concepti, varying from 2.64 to 3.52 L/min and 4.03 to 9.24 L/min, respectively. The variation and mean values for the IVF group fell well within the range observed in the IVD group, whereas the mean value for the NT-HMC group was 2.1-fold higher than the mean value from the controls, with a variation ranging from 1.4- to 3.3-fold higher in the umbilical blood flow.

Correlations of physiological significance

Physical traits (foetal body and most organ weights, placenta weight and surface area) positively correlated ($R>0.600$, $P<0.05$) with variables associated with an active and growth-promoting physiological pattern, including key components of glucose (*SLC2A3*), sugar moieties in maternal and/or foetal compartments (foetal plasma glucose, total glucose in the allantoic fluid, and fructose in maternal plasma), lipid metabolism (*ACACA* expression pattern, triglycerides, VLDL), protein and hemoglobin metabolism (urea, conjugated bilirubin). In turn, physical traits negatively correlated ($R<-0.600$, $P<0.05$) with glucose and fructose levels in the amniotic fluid, and with cholesterol metabolism (nonsterified cholesterol, HDL, LDL) and liver function parameters (LDH, AP, GGT, TPP), which may indicate a slower foetal liver activity in larger concepti, despite its allometric larger size.

Not surprisingly, sugar moieties correlated with one another across the distinct foetal fluid compartments. Glucose in maternal and foetal plasma correlated with glucose in the amniotic fluid, fructose in foetal plasma and allantoic fluid, and total fructose in the allantoic fluid. Also, fructose in foetal plasma correlated with fructose concentrations and total amounts in the allantoic fluid.

Parameters for liver function (LDH, GGT, TPP, AP), and calcium and chloride foetal plasma concentrations correlated positively with one another. Urea, tryglycerides, and VLDL also correlated with one another and with *SLC2A3* expression patterns, and with glucose and fructose in the maternal plasma, fructose in the allantoic fluid, and with uric acid (tryglycerides and VLDL). Components of the cholesterol metabolism (nonsterified cholesterol, HDL, and LDL) correlated positively with one another and with calcium, chloride, liver (LDH, AST, AP, GGT, TPP) and kidney (urea, creatinine) function parameters, and negatively with components of the carbohydrate metabolism (foetal plasma glucose, fructose levels in the maternal plasma and allantoic fluid, total fructose in the allantoic fluid and total fluids), as well as the osmolality in the maternal and foetal plasma. Osmolality in maternal and foetal plasma correlated with conceptus weight, foetal plasma glucose, maternal plasma fructose, fructose levels and total fructose in the allantoic fluid and in the total fluids and foetal plasma urea. On the other hand, TPP and AP correlated negatively with foetal plasma glucose and maternal plasma fructose.

DISCUSSION

The IVP of cattle embryos by IVF or cloning by SCNT is a powerful tool routinely used worldwide in science and reproductive programs in commercial herds around the world (Bertolini *et al.*, 2009). However, developmental abnormalities that arise from such embryo manipulations can significantly interfere with the pattern of foetal and placental growth leading to di*SORD*ers in foetal and placental development that may be manifested in animals as symptoms of the AOS (Young *et al.*, 2001). However, in this study, pregnant

females carrying clone foetuses showed no significant physical or physiological distinctions from controls. The wide level of similarities between measured physical, biochemical and molecular measurements in the maternal system between groups, as observed in this study, indicates that robust homeostatic and homeorrhetic mechanisms regulate and keep the maternal system within the boundaries of normalcy during pregnancy, despite the conceptus origin and the overall physiological and/or pathological differences observed into the uterine and foetal compartments. Changes in placental development and function in pregnancies derived either from IVF or NT-HMC procedures have been associated with the delivery of larger and abnormal calves and the appearance of aberrant foetal membranes and placentae (Bertolini *et al.*, 2004), and results from such sort of studies have increasingly contributed to the understanding of the etiology and temporo-spatial evolution of the abnormalities that characterize the AOS.

In previous studies, we observed that growth of foetuses derived from IVF and NT-HMC procedures was characterized by a biphasic growth pattern, with a phase of retardation that coincides with the period of placentation. This phenomenon was followed by a divergence in the development of placental tissue in IVF (Bertolini *et al.*, 2002, 2004) and NT-HMC (Batchelder *et al.*, 2005) pregnancies, restoring the size of the foetus by the end of the first trimester of pregnancy. Such manifestation culminates with the occurrence of larger foetal, placental and uterine weights by the third trimester of pregnancy, with significant morphological changes in placentae of IVF and NT-HMC concepti and the birth of larger calves with reduced postnatal survival (Bertolini & Anderson, 2002; Bertolini *et al.*, 2002, 2004; Batchelder *et al.*, 2005; Gerger *et al.*, 2016). In our study, most foetal physical traits had larger measurements in the cloned group compared to the *in vivo* and IVF groups on Day 225 of pregnancy, with foetal body weights, foetal liver and placentae being heavier in clones than the other groups. Furthermore, the placenta surface area was higher in the cloned than in the IVF group, but similar to the *in vivo* controls.

The physical analyses of linear measurements and weights showed a significant increase in almost all foetal organs and tissues in the NT-HMC group.

However, after statistical adjustment by analysis of covariance, using foetal weight as covariate, such differences mostly disappeared. Such observation was also described by Bertolini *et al.* (2004) for IVF foetuses on Day 180 of gestation, demonstrating that the organ dimensions and weights in the *in vitro* group were larger in absolute values, i.e., as the size of the foetuses increased, organs and tissues generally isometrically grew in proportion as the foetal size, but a few organs followed an allometric growth pattern, such as the liver. The clinical and physiological significance of such findings is still unclear, but analogous results were also found by Hill *et al.* (1999) and Li *et al.* (2005), which is similarly also known to occur after birth (Chavatte-Palmer *et al.*, 2009). Interestingly, foetal liver sizes were allometrically larger in the NT-HMC group, which negatively correlated with several biochemical and molecular parameters used for the analysis of liver function, which may indicate a reduced metabolic liver function capacity.

The excessive foetal growth pattern observed at the middle to late pregnancy in IVP concepti appears to be associated with an increased uptake of energy substrates, especially glucose, by the uterine-placental-foetal tissues (Bertolini *et al.*, 2004). In this study, the concentrations of glucose and fructose in maternal plasma and the difference between the collection times (t=-30 min and t=0 min) were similar between groups. However, although low, fructose concentrations in maternal plasma at t = 0 min were higher in NT-HMC group than in the control group (IVD) and IVF groups. Fructose is synthesized by the placenta from glucose and is the main carbohydrate in plasma and foetal fluids, a characteristic of species with synepithelium- or epithelium-chorial placenta, such as ungulates and cetacea (Goodwin, 1956; Nixon, 1963). Once fructose enters a cell, it is phosphorylated by either *KHK* to produce the metabolite F-1-P or by hexokinase to produce F-6-P by G6PC. In turn, F-1-P is either converted to glyceraldehyde-3-phosphate and dihydroxyacetone phosphate into the glycolytic pathway or it can act as a signaling molecule to induce NFkB activation and subsequent production of cytokines (Wang *et al.*, 2018). In contrast, F-6-P can be metabolised in a different pathway being precursor of hexosamine. The metabolism of fructose via the hexosamine pathway leads to the synthesis of glycolipids, glycosaminoglycans and proteoglycans with functions in cells and

tissues. Also, fructose induces cell proliferation via activation of the AKT-tuberous sclerosis complex 2 (TSC2)-mTOR signaling cascade (Figure 1). Aldoretta & Hay (1999) found in sheep that the fructose production rate seems to depend directly from the glucose supply to the placenta, and once produced at the placental tissue, fructose cannot be transferred significantly into the maternal system, remaining into the uterine and foetal compartments (Bertolini *et al.*, 2004). As maternal fructose was higher in cloned specimens, and as such substrate was also found at higher levels in foetal plasma and allantoic fluid, as more glucose is likely to be converted into fructose by the placenta in the NT-HMC group, more fructose may leak into the maternal system, explaining the differences. However, more studies are necessary to test such concept.

The analyses of expression of genes related to glucose transporters in type C placentomes (Ticiani et al., 2018, unpublished data), which have been shown to be abundant in cloned animals (Gerger et al., 2016), demonstrated a higher capacity to uptake glucose. However, foetal growth is usually limited by maternal and placental factors, which is related to the nutrient supply, which controls foetal growth in late gestation (Ferrell, 1991a,b; Gluckman *et al.*, 1992). Because the placental mass becomes larger in IVF- and NT-HMC-derived pregnancies, an increased use or supply of glucose and fructose to the foetus appears to occur, reducing placental restriction and promoting foetal growth (Bertolini *et al.*, 2004; Gerger *et al.*, 2016). Despite the difference in foetal glucose and fructose plasma concentrations, no differences in minute glucose and fructose clearance (foetal uptake and consumption) were observed between groups. Also, the foetal voenous to arterial fructose concentration difference was lower in the IVF and cloned groups than controls, which may indicate a trend for reducing uptake for excessive substrate supply prior to the period in study (Day 225), as foteal weights were significantly larger in clones than IVF and controls. Alternatively, as umbilical blood flows are dynamic over time, clearance rates are also likely to be dynamic. In addition, subtle differences in substrate clearance may exist as a function of time, which cannot be detected in short term time-point studies, such as this. Thus, long term *in vivo* kinetics studies on the substrate uptake and consumption will better provide clues on the placental transfer

capacity between clones and controls.

In this study, the concentrations of glucose in the allantoic, amniotic fluid and total fluids did not differ between groups, but there was an incread on fructose concentrations to cloned conceptus in the the foetal plasma and allantois. Such results were similar to those reported by Bertolini *et al.* (2004). Nevertheless, a tendency for lower glucose and fructose was seen in the amniotic fluid of clones than in the other groups. However, the total glucose and fructose in foetal fluids showed an increase of 13.4- and 7.1-fold in the allantoic fluid of cloned concepti, respectively, when compared with the control group, but with no differences in the amniotic fluid. These findings, associated with the fact that placentae of cloned concepti had greater weights and surface areas, demonstrate an increase in glucose and fructose usage or supply to the foetus, favoring an increased nutrient supply to foetal liver over time, especially in the last third of pregnancy, which could explain the excessive size observed in the NT-HMC group in this experiment.

The glucose 6-phosphate (G6P) molecule is an important compound present at the intersection of several metabolic pathways, such as glycolysis, gluconeogenesis, pentose phosphate, glucosamine pathway, among others. In glycolysis, G6P is converted by phosphohexose isomerase to fructose 6-phosphate, which involves an aldose-ketose isomerisation (Murray, 2013). However, in our study, a 2.4-fold increase in G6Pase expression was seen in clones when compared to controls, despite other enzymes of gluconeogenic enzymes being similar between groups.

In lipid metabolism, the expression levels of the *ACACA* gene were higher in the liver of cloned foetuses compared to controls. The biosynthesis of long-chain fatty acids occurs in two distinct steps. The first step is the conversion of acetyl-CoA to malonyl-CoA, a reaction catalyzed by a biotin-containing multienzyme system named acetyl-CoA carboxylase, or *ACACA* (Reaction 1). The second step (Reaction 2) is the conversion of acetyl-CoA and malonyl-CoA to palmitate in the presence of NADPH, a reaction catalyzed by the fatty acid synthase (*FASN*) (Wakil *et al.*, 1983). The conversion of acetyl-CoA to malonyl-CoA is the rate-limiting step in fatty acid synthesis and this reaction provides

energy for next step carried out by *FASN*. For this reason, even with the reduction in hepatic *FASN* expression in clones, this could not be representative, since there was an increase in *ACACA* expression in the IVF and NT-HMC groups, which correlated with triglyceride and VLDL foetal plasma concentrations in the NT-HMC group. Another point that contributes to the idea that increased lipogenesis occurred in cloned concepti is that the *IGF1* gene expression in foetal liver was highly correlated with foetal plasma triglyceride concentrations ($R = 0.87$, $p < 0.01$). The hepatic *IGF1* secretion has direct effect on the expression of lipogenic enzymes in the liver, as it may modulate lipogenesis and associated enzymes by indirect mechanisms, also stimulating the production of growth hormone by the adenohypophysis (Samuels & Shipiro, 1973). Growth hormone, in turn, stimulates synthesis and secretion of *IGF1*, accounting for many of the growth-promoting activities of growth hormone (Roberts *et al.*, 1986). The NT-HMC group had an increase in *IGF1* expression, with higher concentrations of triglyceride and VLDL in the foetal plasma, which can possibly lead to lipidosis, because the excess of lipids is stored in the liver, increasing the liver size (Grummer, 1993; Drackley, 1999). Interestingly, the NT-HMC group had enlarged livers in relation to foetal weight, for lower liver function. Liver lipidosis in cloned foetus has already been observed by Hill *et al.* (1999), who analysed five cloned foetuses that died seven days to two months prior to term, with four of them having severe hepatic lipidosis.

Increased concentrations of liver triglycerides are accompanied by decreased concentrations of structural lipids (free cholesterol, cholesteryl ester, and phospholipids), as shown by Bobe *et al.* (2004), and as observed in this study, with reductions in cholesterol levels in foetal plasma and *HMGCR* expression in the foetal liver in the NT-HMC group. Furthermore, lipidosis may lead to microscopic alterations that affect cellular integrity and hepatocyte function and, therefore, may cause necrosis and cellular leakage, which is demonstrated by increased concentrations of liver enzymes and bile constituents in plasma (Bobe *et al.*, 2004). Such observations are interesting because bilirubin and total bilirubin were increased in foetal plasma in the IVF and NT-HMC groups, with bilirubin being correlated with constituents of lipid metabolism, such as

ACACA expression (r=0.57; p<0.05) and triglyceride levels in foetal plasma (r=0.61; p<0.05).

Umbilical blood flow is directly related to foetal weight, determined in a major part by the conceptus genome, whereas the uterine blood flow is less influenced by foetal size, being determined mostly by the maternal genome (Ferrell, 1991b). The umbilical blood flows determined in Nellore IVD concepti carried by *Bos taurus vs. Bos indicus* recipient dams in this study fell well within values reported by Reynolds & Ferrell (1987) for European cattle breeds on Day 224-228 of gestation (2.79 L/min), and by Ferrell (1991b) for Brahman concepti carried by Brahman dams on Day 227 of pregnancy (2.71 L/min), and lower than for Charolais concepti carried by Charolais or Brahman recipient females (3.78L/min; Ferrell, 1991b). Uterine blood flow was similar (5.01 L/min) in Brahman dams carrying Brahman concepti (Ferrell, 1991b), and lower (8.75 L/min) than Charolais recipient females carrying concepti either of European breeds (Reynolds & Ferrell, 1987), Charolais or Brahman (9.24 and 7.18 L/min, respectively; Ferrell, 1991b). Interestingly, mean umbilical blood flows were similar in both studies above, in spite of the difference in foetal weights for the studied cattle breeds, being 1.8-fold higher in European cattle than in Brahman (23.6 *vs.* 13.2 kg, respectively), for the same gestation period. Perhaps the difference was due to the fact that foetal weights in the study by Reynolds & Ferrell (1987) were estimated using an exponential equation for European cattle breeds from studies by Ferrell *et al.* (1976). Nevertheless, estimated foetal weights fell well within values described by Prior & Laster (1979), Reynolds *et al.* (1990) and Ferrell (1990ab) for European breeds for the same gestation period. In addition, a physiologic variation is expected. The umbilical blood flow:foetal weight ratio from Days 137 to 250 (Reynolds & Ferrell,1987) and on Day 227 of gestation (Ferrell, 1991b) ranged from 0.10 to 0.21 L/min per kg of foetus. In this study, the estimation of umbilical blood flow based on exponential relationships of known foetal weights with days of gestation (Ferrell *et al.*, 1976), and on exponential relationships of blood flow rates with the projected days of gestation (Reynolds and Ferrell, 1987), for the foetal weights in the NT-HMC group (from 18.3 to 42.0 kg), resulted in apparently overestimated flow rates ranging from 4.6

to 12.8 L/min, respectively. By using the umbilical blood flow:foetal weight ratio from the IVD controls (0.22 L/min per kg), which was similar to Ferrell (1990b) for Brahman concepti carried by Brahman dams on Day 227 of pregnancy (0.21 L/min), umbilical flow rates for cloned concepti varied from 4.03 to 9.24 L/min, 1.5- to 3.4-fold higher than controls, with values more in agreement for blood flow rates and foetal weights described by Reynolds & Ferrell (1987).

A difference in urea concentration was seen in maternal and foetal plasma between groups, with a higher concentration in the cloned group, unlike findings by Li *et al.* (2005), who observed similar levels in maternal plasma between clones, IVF and artificial insemination (AI) groups. Urea formed by the foetus or by the placenta is constantly removed and eliminated through the maternal circulation. Urea is the final product of nitrogen metabolism secreted in the liver and placenta, and is produced from ammonia by a process requiring energy (Murray, 2013). The foetal parameters for renal function showed differences between groups in urea and uric acid, with higher plasma levels in the cloned group than in the control group, indicating a higher ammonia production possibly from a higher catabolism of amino acids. Moreover, with respect to uric acid in clones, it is possible that higher levels may be due to deficiencies in urate excretion by the kidney, as a product of degradation of purine nitrogenous bases. Plasma creatinine concentrations, one of the main muscle activity indicators (Baptistella, 2009), were lower in cloned concepti than controls and the IVF group. Plasma creatinine is derived, almost in its entirety, from the catabolism of creatine in muscle tissue. In the form of phosphocreatine, creatine metabolite is used to store energy in the muscle, and its degradation to creatinine occurs steadily. The blood concentration of creatinine is proportional to muscle mass. Thus, in case of muscle atrophy and related conditions, there is a decrease in plasma creatinine content (González & Silva, 2006). Chavatte-Palmer *et al.* (2002) examined the biochemical parameters measured sequentially in 21 clones and eight AI controls. The urea, creatinine, AST and ALT levels were within the normal range in all clones.

In relation to the foetal systemic physiology, plasma LDH and TPP concentrations were lower in IVF and in cloned foetuses compared to controls,

indicating that the reversible oxidation of lactate to pyruvate and metabolic rate in those foetuses occured at a smaller rate, in spite of, or perhaps because of, or even as a cause of, the larger liver size in clones. Plasma products from the hemoglobin metabolism (bilirubin, conjugated bilirubin, total bilirubin) were similar between groups with a trend towards a higher level of total bilirubin in the plasma of clones and the IVF group than in controls, possibly indicating higher red blood cells (RBC) degradation. Furthermore, the increased plasma bilirubin levels may be due to increased free bilirubin, which occurs in severe acute hemolysis (González & Silva, 2006), or due to a lower liver conjugation, corroborating with the concept of lower liver metabolic rate. According to Batchelder *et al*. (2007), hemolytic destruction of RBC may cause an increase in total bilirubin concentrations, as observed for both clones and controls in their study.

Phosphorus, calcium and chloride plasma concentrations were similar between groups, but the osmolality of the foetal plasma was higher in the clones than the other groups. Bertolini *et al*. (2004) observed no changes in the composition of fluids in IVF pregnancies after 180 days of gestation. However, an amount two to five times larger in allantoic fluid volume, for higher osmolality values, were observed in IVF pregnancies compared to controls. Since the study also found higher concentrations and total amounts of glucose and fructose in the foetal fluids, and due to the active osmotic nature of those molecules, the authors suggested that the osmotic effect exerted by the carbohydrates may have caused an accumulation of fluids and the increase in total fluid volume, since a positive correlation was found between volume, osmolality, and sugar concentrations in the allantoic fluid. Li *et al*. (2005) found no differences in osmolality in foetal fluids in control, IVF and cloned pregnancies on Day 150, but found a wide variation in biochemical composition in the fluids *per se*. Such studies demonstrate the existing large variation between pregnancies established with embryos produced *in vitro* and *in vivo*, and the difficulty in establishing the actual changes present in each type of pregnancy. The biochemical components of the bovine foetal fluid vary between 115 and 265 days of gestation (Baetz *et al*., 1976). In many species, calcium from foetal blood is maintained at a higher level than in the maternal circulation by the foeto-

placental unit (Kovacs & Kronenberg, 1997). Almost all foetal calcium is associated with the skeleton, with the remaining calcium being involved in important physiological processes, such as intracellular signaling, maintenance of cell membrane stability, and blood coagulation (Wooding *et al*., 1996).

The morphological, biochemical and genetic analyses performed in this study revealed the existence of a large variation between maternal and foetal profiles in clone-bearing pregnancies when compared to the *in vivo* control, with the IVF group resembling the control counterparts. In summary, compared with *in vivo*-derived pregnancies, cloned concepti showed a significant difference in metabolic pathways, suggesting active fructose synthesis by the fetus and possible lipidosis, which may be a reflection of an association between changes in metabolic fetal programming and excessive nutrient supply reaching the liver at the beginning of the second trimester in cloned-bearing pregnancies. Additionally, the results of expression of important genes for foetal and placental development can be used as morphologic markers for applications of advanced reproductive biotechnologies. Studies on the metabolic, biochemical and molecular profiles of cloned concepti may contribute significantly to a better understanding of the effect of IVD embryos in pre- and post-natal development, with an impact in animal welfare, and by finding relationships with naturally occurring problems in herds, which could be of economic and scientific importance. Additional studies involving metabolism, epigenetic reprogramming and control of gene expression should be conducted in *in vitro*-derived concepti, providing better understanding of events involved in the abnormalities in late pregnancy, also allowing the identification of components or causal factors in early embryo development that ultimately determine the phenotypic changes observed during development, including the post-natal period.

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REFERENCES

Aldoretta PW & Hay JR WW 1999 Effect of glucose supply on ovine uteroplacental glucose metabolism. *The American Journal of Physiology – Regulatory Integrative and Comparative Physiology* **277** 947-958.

Baetz AL, Hubbert WT & Graham CK 1976 Changes of biochemical constituents in bovine foetal fluids with gestational age. *American Journal of Veterinary Research* **37** 1047–1052.

Baptistella MF 2009 Atividade sérica das enzimas aspartato aminotrasferase, creatinoquna e lactato desidrogenase em eqüinos submetidos a diferentes intensidades de exercícios. *Anuário da Produção de Iniciação Científica Discente* **3** 33-42.

Batchelder CA, Bertolini M, Mason JB, Moyer AL, Hoffert KA, Petkov SG, Famula TR, Angelos J, George LW & Anderson GB 2007 Perinatal physiology in cloned and normal calves: physical and clinical characteristics. *Cloning and Stem Cells* **9** 63-82.

Batchelder CA, Hoffert KA, Bertolini M, Moyer AL, Mason JB, Petkov SG, Famula TR & Anderson GB 2005 Effect of the nuclear-donor cell lineage, type, and cell donor on development of somatic cell nuclear transfer embryos in cattle. *Cloning and Stem Cells* **7** 238-254.

Bertolini M & Anderson GB 2002 The placenta as a contributor to production of large calves. *Theriogenology* **57** 181-187.

Bertolini M, Beam SW, Shim H, Bertolini LR, Moyer AL, Famula TR & Anderson

GB 2002 Growth, development and gene expression by *in vivo*- and *in vitro*-produced Day-7 and Day-16 bovine embryos. *Molecular Reproduction and Development* **63** 318-328.

Bertolini M, Bertolini LR 2009 Advances in reproductive technologies in cattle: from artificial insemination to cloning. *Revista de Medicina Veeterinaria e Zootecnia* **56** 184–194.

Bertolini M, Moyer AL, Mason JB, Batchelder CA, Hoffert KA, Bertolini LR, Carneiro GF, Cargill SL, Famula TR, Calvert CC *et al*. 2004 Evidence of increased substrate availability to *in vitro*-derived bovine foetuses and association with accelerated conceptus growth. Reproduction 128 341-354.

Bobe G, Young JW & Beitz DC 2004 Pathology, etiology, prevention, and treatment of fatty liver in dairy cows. *Journal of Dairy Science* **87** 3105–3124.

Chavatte-Palmer P, Camousa S, Jammes H, Le Cleac'h N, Guillomot M & Lee RSF 2012 Review: Placental perturbations induce the developmental abnormalities often observed in bovine somatic cell nuclear transfer. *Placenta* **26** 99-104.

Chavatte-Palmer P, Heyman Y, Richard C, Monget P, LeBourhis D, Kann G, Chilliard Y, Vignon X & Renard JP 2002 Clinical, hormonal, and hematologic characteristics of bovine calves derived from nuclei from somatic cells. *Biology of Reproduction* **66** 1596–1603.

Chavatte-Palmer PM, Heyman Y, Richard C, Urien C, Renard JP & Schwartz-Cornil I 2009 The immune status of bovine somatic clones. *Cloning Stem Cells* **11** 309-318.

Drackley JK 1999 Biology of dairy cows during the transition period: the final frontier. *Journal Dairy Science* **82** 2259–2273.

Eley RM, Thatcher WW, Bazer FW, Wilcox CJ, Becker RB, Head HH & Adkinson RW 1978 Development of the conceptus in the bovine. *Journal of Dairy Science* **61** 467-473.

Farin PW & Farin CE 1995 Transfer of bovine embryos produced *in vivo* or *in vitro*: survival and foetal development. *Biology of Reproduction* 52 676-682.

Ferrell CL & Philips FP 1979 Automated procedure for the determination of deuterium oxide in biological fluids. *Journal of Animal Science* SlSuppl. 1

358cAbstr.

Ferrell CL & Reynolds LP 1992 Uterine and umbilical blood flows and net nutrient uptake by fetuses and uteroplacental tissues of cows gravid with either single or twin fetuses. *Journal of Animal Science* **70** 426–433.

Ferrell CL 1989 Placental regulation of foetal growth. In Animal Growth Regulation. Eds Campion DR, Hausman GJ & Martin RJ. *New York: Plenum Press* 1-19.

Ferrell CL 1991a Maternal and foetal influences on uterine and conceptus development in the cow: I. Growth of the tissues of the gravid uterus. *Journal of Animal Science* **69** 1945-1953.

Ferrell CL 1991b Maternal and foetal influences on uterine and conceptus development in the cow: II. Blood flow and nutrient flux. *Journal of Animal Science* **69** 1954-1965.

Ferrell CL, Garrett WN & Hinman N 1976 Growth, development and composition of the udder and gravid uterus of beef heifers during pregnancy. *Journal of Animal Science* **6** 1477-1489.

Gerger RP, Zago FC, Ribeiro ES, Gaudencio Neto S, Martins LT, Aguiar LH, Rodrigues VHV, Furlan FH, Ortigari I, Sainz RD *et al*. 2016 Morphometric developmental pattern of bovine handmade cloned concepti in late pregnancy. *Reproduction, Fertility and Development* **29** 950-967.

Gluckman PD, Morel PCH, Ambler GR, Breier BH, Blair HT & Mccutcheon SN 1992 Elevating maternal insulin-like growth factor-I in mice and rats alters the pattern of foetal growth by removing maternal constraint. *Journal of Endocrinology* **134** R1-R3.

González FHD & Silva SC 2006 Perfil Bioquímico no Exercício. In: Introdução à Bioquímica Clínica Veterinária. Porto Alegre: Universidade Federal do Rio Grande do Sul.

Goodwin RFW 1956 Division of the common mammals into two groups according to the concentration of fructose in the blood of the fetus. *The Journal of Physiology* **132** 146-156.

Grummer RR 1993 Etiology of lipid-related metabolic di*SORD*ers in periparturient dairy cows. *Journal of Dairy Science* **76** 3882–3896.

Hill JR, Roussel AJ, Cibelli JB, Edwards JF, Hooper NL, Miller NW, Thompson JA, Looney CR, Westhusin ME, Robl JM *et al.* 1999 Clinical and pathologic features of cloned transgenic calves and fetuses (13 case studies). *Theriogenology* **51** 1451–1465.

Kovacs CS & Kronenberg HM 1997 Maternal-foetal calcium and bone metabolism during pregnancy, puerperium, and lactation. *Endocrinology Review* **18** 832–872.

Li N, Wells DN, Peterson AJ, Lee RSF 2005 Perturbations in the biochemical composition of foetal fluids are apparent in surviving bovine somatic cell nuclear transfer pregnancies in the first half of gestation. *Biology of Reproduction* **73** 139–148.

Murray RK 2013 Biochemistry of extracellular & intracellular communication. *Harper's Illustrated Biochemistry,* Kindle Edition 29.

Nixon DA 1963 The transplacental passage of fructose, urea and meso-inositol in the direction from foetus to mother, as demonstrated by perfusion studies in the sheep. *Journal of Physiology* **166** 351-362.

NRC Beef 1996 Nutrient Requirements of Beef Cattle (7th Revised Ed.) National Academy of Sciences National Research Council, Washington D. C

Prior RL & Laster DB 1979. Development of the bovine fetus. *Journal of Animal Science* **48** 1546-1553.

Reynolds LP & Ferrell CL 1987 Transplacental clearance and blood flows of bovine gravid uterus at several stages of gestation. *American Journal of Physiology* **253** 735-739.

Reynolds LP, Millaway DS, Kirsch JD, Infeld JE & Redmer 1990 DA Growth and *in vitro* metabolism of placental tissues of cows from day 100 to day 250 of gestation. *Journal of Reproduction and Fertility* **89** 213-222.

Ribeiro MN, Pimenta Filho EC, Santos ES & Costa RG 1992 Genetic and environmental factors affecting pre-weaning weights in Nellore cattle. *Agropecuária Técnica* **13**, 54-61.

Roberts AB, Sporn MB, Assoian RK, Smith JM, Roche NS, Wakefield LM, Heine UI, Liotta LA, Falanga V, Kehrl JH, et al. 1986 Transforming growth factor type beta: rapid induction of fibrosis and angiogenesis *in vivo* and stimulation of

collagen formation *in vitro*. *Proceedings of the National Academy of Sciences of the United States of America* **83** 4167–4171.

Samuels HH & Shapiro LE 1976 Thyroid hormone stimulates de novo growth hormone synthesis in cultured GH1 cells: evidence for the accumulation of a rate limiting RNA species in the induction process. *Proceedings of the National Academy of Sciences of the United States of America* **73**, 3369–3373

Statistical Analysis System SAS 2015. SAS user's guide: statistics. Cary, NC: SAS.

Sawai K 2009 Studies on gene expression in bovine embryos derived from somatic cell nuclear transfer. *Journal of Reproduction and Development* **55** 11-16.

Smith KL, Butler WR & Overton TR 2009 Effects of prepartum 2,4-thiazolidinedione on metabolism and performance in transition dairy cows. *Journal of Dairy Science* **92** 3623–3633.

Vandesompele, J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A & Speleman F 2002 Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biology* **3** RESEARCH0034.

Wakil SJ, Stoops JK & Joshi VC 1983 Fatty acid synthesis and its regulation. *Annual Review of Biochemistry* **52** 537-579.

Wang X, Li D, Wu G & Bazer FW 2018 Functional Roles of Fructose: Crosstalk between O-Linked Glycosylation and Phosphorylation of Akt-TSC2-MTOR Cell Signaling Cascade in Ovine Trophectoderm Cells. *Biology of Reproduction* **95** 1-17.

Wooding FBP, Morgan G, Jones JV & Care AD 1996 Calcium transport and the localization of calbindin-D9K in the ruminant placenta during the second half of pregnancy. *Cell and Tissue Research* **285** 477–489.

Young LE, Fernandes K, McEvoy TG, Butterwith SC, Gutierrez CG, Carolan C, Broadbent PJ, Robinson JJ, Wilmut I & Sinclair KD 2001 Epigenetic change in *IGF2R* is associated with foetal overgrowth after sheep embryo culture. *Nature Genetics* **27** 153-154.

CAPÍTULO III

Evidence of metabolic compartmentation or zonation in the bovine placenta and significance for the regulation of placental function and foetal growth[2]

[2]Artigo elaborado conforme as normas da revista Reproduction (Apêndice 1)

Evidence of metabolic compartmentation in the bovine placenta and significance for the regulation of placental function and foetal growth

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ABSTRACT

The concept of metabolic zonation in the liver proposes that opposing or complementary metabolic pathways are carried out within distinct regions of the liver lobule to maintain optimal metabolic homeostasis. As bovine placentomes differ in mass and shapes within the reproductive tract, which is even more pronounced in cloned-derived concepti, the aims of this study were to investigate the occurrence of metabolic zonation in the bovine placenta by placentome shape and size, and to compare placental functions between control and cloned concepti, which may relate to the abnormal conceptus growth after cloning. Bovine pregnancies established after superovulation/AI (n=4), *in vitro* fertilisation (n=4) or cloning by nuclear transfer (n=6) were slaughtered on Day 225 of gestation. Placentomes were excised, weighed, measured, and classified by shape as types A, B, C, or D; cDNA from each placentome type/group was used for RT-qPCR analyses of 29 target gene products related to carbohydrate and lipid metabolism, placental function, IGF system and apoptosis. No differences in gene expression were detected between clones and controls. However, the gene expression pattern revealed differences in metabolic profile only between placentome types, with an increase in expression in type C in relation to type A placentomes, in special for *IGF2* (5.8-fold) and *IGF2r* (1.6-fold), glucose transporters (1.2-fold), and fructogenesis (2.0- to 2.3-fold), suggesting a potential metabolic zonation in the bovine placenta. Morphological changes in the placenta of clones may influence placental function and metabolic compartmentalization, likely disturbing placental control on foetal growth and programming towards the end of pregnancy.

Keywords: Abnormal Offspring Syndrome, cloning by nuclear transfer, cattle, placentome.

INTRODUCTION

The placenta is the interface between the foetal and maternal systems and plays an important role in foetal growth by regulating nutrient supply, synthesis and transport of hormones, substrates and other substances between systems (Zhang *et al.*, 2015). All substrates necessary for foetal-placental growth and development from the maternal system are directly or indirectly transported, metabolised, or modified by the placenta (Fowden, 1997; Bertolini *et al.*, 2004). The bioavailability of certain substrates or hormones during gestation is important for the establishment of normal patterns of physiological systems in the developing foetus, a phenomenon commonly referred as metabolic programming (McMillen & Robinson, 2005).

Changes in the pattern of substrate delivery to the foetus, such as modulation or nutritional restriction at certain stages of gestation, especially during the placentation period, may lead to permanent molecular and cellular changes or even new patterns of activity in organs and systems that may persist and affect postnatal life (Barker *et al.*, 1999). Consequently, changes in metabolic reprogramming following *in vitro* embryo manipulations may affect placental function and the placental restriction pattern on foetal growth, which may lead to a growth promoting effect, altering events leading to the preparation and onset of parturition, possibly also compromising postnatal survival from birth to adulthood (Barker, 1999).

Initial reports in cattle investigating the effects of *in vitro* embryo production by *in vitro* fertilisation procedures described the occurrence of a set of abnormalities in development, including pregnancy losses, prolonged gestation, excessive foetal growth, and increased post-natal mortality (Walker *et al.*, 1996). Interestingly, such abnormalities are also observed after cloning by somatic cell nuclear transfer (SCNT), which were collectively termed Cloning Syndrome (Wells *et al.*, 2004), Abnormal Offspring Syndrome (AOS; Farin *et al.*, 2006) or even Large Placenta Syndrome (Constant *et al.*, 2006), as various developmental abnormalities seem to be associated with changes in placental function, which has been indicated by some as the cause of AOS.

In cattle cloning, the lower development of the placenta at the early stage

of pregnancy is accompanied by a reduced number of placentomes, nevertheless with a larger size (Constant *et al.*, 2006). Changes in placental formation by *in vitro* embryo manipulations may affect the stereological structure and tissue microarchitecture or even lead to changes in placental function and metabolism, thus affecting the pattern of foetal growth, in a placental-foetal cause-and-effect mechanism. Consequently, morphological, physiological, metabolic and molecular studies that focus on the placenta during the period of major appearance of phenotypical changes (third trimester of gestation) are essential for understanding the relationship between placental pathologies and the physiopathological effects on the foetus, as well as in the newborn.

As in the liver, the placenta may also have a metabolic compartmentalization, perhaps by topographic position in relation to the fetus, or even inside the uterus, since there are generally 4 lines of caruncules along the axis of the uterine horns, which can and should have distinct blood supply and flow. In general, there is an artery and a vein that derive from the umbilical, to each side from the cord, to the smaller curvature of the uterus, which follow its flow to the extremities, sending tributaries to the placentomes, first in those that are more ventral to the most dorsal, and there are no significant anastomoses in the dorsal curvature. This way, the perfusion through the placentomes causes changes in the blood composition, for example, reducing oxygen. In the liver, compartmentalization is continuous in the lobe, from the hepatic artery/portal vein, to the central vein; in the uterus, such pattern is contiguous (Gebhardt, 1992). At the beginning of the lobe in the liver there are more oxidative processes going to non-oxidative processes at the end of the lobe (Gebhardt, 1992), which could also occur at the placenta, through the placentomes. Thus, this study aimed (a) to investigate the occurrence of metabolic zonation in the bovine placenta, and (b) to compare placenta metabolic functions between control and cloned bovine concepti, which may relate to the abnormal conceptus growth pattern seen after cloning.

MATERIALS AND METHODS

This study used samples collected in a companion study (Gerger *et al.*,

2016). Data from Gerger *et al.* (2016) that are relevant to this study are summarized in Table 1 and Figure 1. All procedures performed were approved by the Animal Ethics Committee of the University of São Paulo (São Paulo, SP, Brazil).

Table 1. Selected conceptus physical traits for *Bos taurus* var. *indicus in vivo*-derived (IVD) concepti and concepti produced either by *in vitro* fertilisation (IVF) or nuclear transfer by handmade cloning (NT-HMC) procedures, on Day 225 of gestation

Trait	**Group**			**P value**
	IVD	**IVF**	**NT-HMC**	
Foetal weight (kg)	12.5 ± 2.3[a]	13.7 ± 3.2[a]	27.2 ± 2.0[b]	0.005
Placentome weight (g)	3160.8 ± 242.1[a]	3258.3 ± 279.6[a]	5732.3 ±279.6[b]	<0.001
Placenta surface area (cm^{2})	201.5 ± 29.1[ab]	113.1 ± 33.6[b]	303.3 ± 33.6[a]	0.015
Placentome number (n)	81.5 ± 9.4[a]	65.0 ± 10.8[a]	85.3 ± 10.8[a]	0.409

[a,b:] Numbers with unequal letters on the same row differ, $P < 0.05$

Summarized from Gerger *et al.* (2016)

Animals and experimental groups

A total of 14 bovine pregnancies were established carrying Nellore cattle (*Bos taurus* var. *indicus*) singleton concepti, produced either *in vivo* (*in vivo*-derived controls, or IVD group, n=4), by the superovulation and artificial insemination of donor females, or *in vitro* by either *in vitro* fertilisation (IVF group, n=4) or somatic cell nuclear transfer by Handmade Cloning procedures (NT-HMC group, n=6). Concepti for all embryo production systems were from similar genetic backgrounds, and the same *in vitro* culture (IVC) system was used for NT-HMC- and IVF-derived embryos to minimize developmental deviations in the IVF group, highlighting differences related to cloning procedures *per se* and not to IVC (Gerger *et al.*, 2016). The Nellore breed was chosen due to its rather low birth weights in commercial herds (Ribeiro *et al.*, 1992).

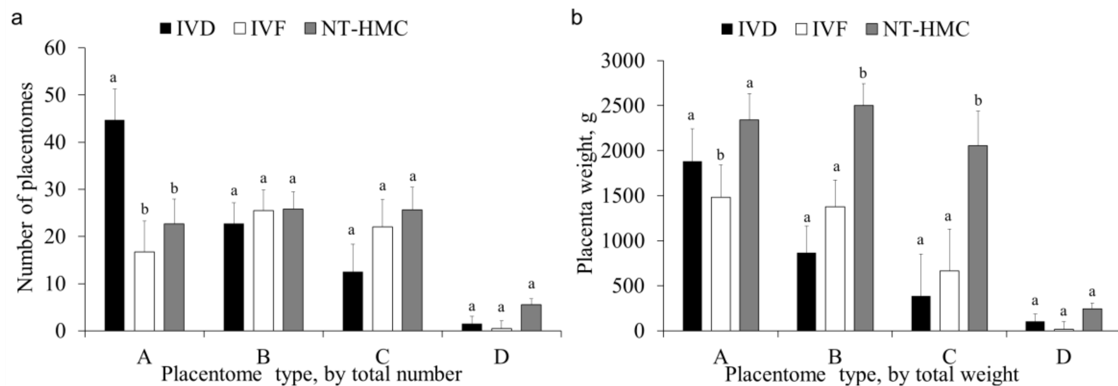

Figure 1. Distribution of placentome numbers and weights (Mean ± SEM) by type in the *in vivo*-derived (IVD), IVF-derived and nuclear transfer–handmade cloning-derived (NT-HMC) groups on Day 225 of gestation. (A) Mean number of placentomes and (B) placentome weights according to placentome type (Types A to D) per animal. [a,b:] Columns with different superscript letters within each placentome type differ significantly ($P < 0.05$). Summarised from Gerger *et al.* (2016).

Post-mortem collection of samples

On Day 225 of pregnancy, pregnancies were terminated by slaughter for harvesting of the uterus, as described by Gerger *et al.* (2016). The foetus and the empty uterus, containing the foetus, the foetal membranes and the placenta, were weighed and morphological foetal measurements were recorded. Placentomes were excised and individually weighed, measured (width and length), and morphologically classified according to Bertolini *et al.* (2006) and Gerger *et al.* (2016) in type A (engulfing, mushroom-like), type B (subengulfing, mushroom-like), type C (flattened, non-engulfing), and type D (semiconvex placentomes), with the placentome surface area calculated based on the equation for the area of an ellipse. Samples of the central longitudinal axis of each placentome type were excised and snap-frozen in liquid nitrogen.

Analyses of gene expression

Total RNA from each placentome type from the three experimental groups was extracted using Trizol® reagent (Invitrogen, USA), and 1800 ng of total RNA from each sample was treated with DNase I (Invitrogen) and used for cDNA synthesis using the GoScript™ Reverse Transcription System (Promega, Madison, WI, USA) according to the manufacturer instructions. Quantification of housekeeping and target transcripts was performed by real time qPCR using the

GoTaq® qPCR Master Mix (Promega) and the Fluorescent Quantitative PCR Detection System LineGene 9600 (Bioer Technology, USA). The genes evaluated included the housekeeping genes ribosomal protein S9 (RPS9) and ß-actin (ACT-B); and key target genes involved with glucose transporters (solute carrier family 2 member 1, *SLC2A1*; member 3, *SLC2A3*; member 5, SLC2A5); glycolysis (lactate dehydrogenase A and B, *LDHA* and *LDHB*); gluconeogenesis (glucose-6-phosphatase, G6PC; fructose-1,6-bisphosphatase, FBP1; phosphoenolpyruvate carboxykinase, PCK1); fructolysis (fructokinase, KHK; dihydroxyacetone kinase 2, *TKFC*; aldolase B, *ALDOB*); fructogenesis (aldose reductase, *AKR1B1*; sorbitol dehydrogenase, *SORD*); pentose phosphate pathway (glucose-6-phosphate dehydrogenase, *G6PD*); fatty acid synthesis (acetyl-CoA carboxylase alpha, *ACACA*; fatty acid synthase, *FASN*); cholesterol synthesis (3-hydroxy-3-methylglutaryl-CoA reductase, *HMGCR*); apoptosis (*BCL2*-associated X protein, *BAX*; *BCL2* B-cell CLL/lymphoma 2, *BCL2*); insulin-like growth factor system (insulin-like growth factor 1 and 2, *IGF1* and *IGF2*; *IGF1* receptor, *IGF1*r; *IGF2* receptor, *IGF2*r); serine synthesis (phosphoserine aminotransferase, PSAT; phosphoglycerate dehydrogenase, PHGDH); and placental function (steroidogenic acute regulatory protein, *STAR*; cytochrome *P450*, *P450*; bovine placental lactogen, *BPL*; bovine pregnancy-associated glycoprotein 1, b*PAG*1). The amplification cycling conditions used an initial denaturation at 95°C for 2 min followed by 45 cycles consisting of 95°C for 15 s and 60°C for 60 s. The fluorescence data were recorded at the end of each extension step (60°C). Pilot experiments using six different concentrations of cDNA (spanning a 60-fold range) were run to set up the RT-qPCR conditions. The specificity of PCR products was confirmed by analysis of melting curves. All reactions were performed in duplicates.

Statistical analyses

Data were analyzed using the MIXED procedure using the SAS statistical software (SAS, Cary, NC, USA), with the model including animal, treatment, and placentome as fixed effects, and interaction between animal and placentome. The geometric means of the housekeeping genes (RPS9 and ACT-B;

Vandesompele *et al.*, 2002) were used as covariates in the model for gene expression data, being neglected when not significant. Data were tested for normality by the Shapiro–Wilk normality test, with the log transformation of non-normal variables. Type D placentomes were removed from the statistical analyses due to their low frequency in the groups.

RESULTS

In general, the patterns of expression of genes for the different metabolic pathways and for physiologically important genes did not differ between the IVD, IVF, and NT-HMC groups (Table 2), inflicting that gene expression was similar on a per unit of tissue basis, as a group, irrespective of the placentome type. However, gene expression in placentomes differed according to the placentome type for most gene products for the investigated metabolic pathways, in special between placentomes types A/B and C, irrespective of the experimental groups, with no significant interactions between placentome types and the experimental groups (P>0.05). Overall, most genes analysed in type C placentomes were overexpressed in relation to types A and B (Table 2).

Table 2. Relative gene transcription levels (LSM ± SEM) in Types A, B and C placentomes between *Bos taurus* var. *indicus in vivo*-derived (IVD) concepti and concepti produced either by *in vitro* fertilisation (IVF) or nuclear transfer by handmade cloning (NT-HMC) procedures, on Day 225 of gestation

Pathway or function	Gene	Placentome type	Group			P_{Group}	$P_{Placentome}$	$P_{Group*Plac}$
			IVD	IVF	NT-HMC			
Sugar transporters	*SLC2A1*	A	2.03±0.43^{aA}	2.33±0.61^{a}	2.99±0.35^{a}	0.59	0.83	0.65
		B	2.22±0.50^{aA}	2.65±0.71^{a}	2.49±0.41^{a}			
		C	2.5±0.34^{aA}	2.76±0.48^{a}	2.48±0.28^{a}			
	SLC2a3	A	2.61±0.28^{aA}	2.03±0.40^{b}	2.85±0.23^{a}	0.71	0.05	0.66
		B	2.55±0.70^{aA}	3.05±0.98^{a}	3.26±0.57^{a}			
		C	3.27±0.55^{aA}	3.87±0.77^{a}	3.27±0.45^{a}			
	SLC2a5	A	2.12±1.65^{aA}	5.1±2.84^{aA}	3.9±1.170^{a}	0.47	0.09	0.72
		B	2.49±0.93^{aA}	4.27±1.32^{a}	2.61±0.83^{a}			
		C	1.93±0.66^{aA}	2.31±0.94^{a}	1.41±0.54^{a}			
Glycolysis	*LDHA*	A	1.85±0.63^{aA}	2.50±0.89^{a}	2.86±0.51^{a}	0.30	0.95	0.74
		B	1.58±0.85^{aA}	3.50±1.20^{a}	2.32±0.69^{a}			
		C	2.08±0.39^{aA}	2.68±0.56^{a}	2.20±0.32^{a}			
	LDHB	A	1.61±0.81^{aA}	1.69±1.14^{a}	3.04±0.65^{a}	0.50	0.61	0.61
		B	2.14±0.66^{aA}	3.48±0.93^{a}	2.23±0.54^{a}			
		C	1.92±0.47^{aA}	2.26±0.67^{a}	2.18±0.38^{a}			
Fructogenesis	*AKR1B1*	A	2.96±0.70^{aA}	1.27±0.99^{b}	1.71±0.57^{b}	0.33	0.02	0.45
		B	2.62±0.64^{aA}	1.86±0.91^{a}	3.57±0.52^{a}			

		C	3.56±0.62^{aA}	3.69±0.88^{a}	3.38±0.51^{a}			
	SORD	A	2.13±0.69^{aA}	1.09±0.98^{a}	0.76±0.56^{b}	0.28	0.03	0.27
		B	0.82±0.88^{aA}	1.09±1.25^{a}	3.57±0.76^{a}			
		C	2.59±0.62^{aA}	2.77±0.88^{a}	2.67±0.51^{a}			
Fructolysis	*ALDOB*	A	0.19±0.57^{aA}	1.36±0.81^{a}	0.50±0.56^{a}	0.10	0.93	0.51
		B	-0.10±0.92^{aA}	0.35±0.95^{a}	1.74±0.55^{a}			
		C	-0.42±0.63^{aB}	0.57±0.89^{a}	1.40±0.52^{a}			
	KHK	A	4.96±0.64^{aA}	3.97±0.91^{a}	3.65±0.57^{a}	0.50	0.58	0.63
		B	4.54±0.47^{aA}	4.24±0.66^{a}	4.3±0.38^{aA}			
		C	4.54±0.32^{aA}	4.90±0.45^{a}	4.41±0.27^{a}			
	TKFC	A	2.36±0.65^{aA}	2.38±0.92^{a}	3.62±0.53^{a}	0.34	0.11	0.45
		B	2.23±0.69^{aA}	3.84±0.98^{a}	2.38±0.57^{a}			
		C	1.98±0.40^{aA}	1.65±0.56^{a}	2.39±0.33^{a}			
Pentose phosphate pathway	*G6PD*	A	3.48±0.67^{aA}	2.57±0.95^{b}	2.74±0.55^{b}	0.98	0.02	0.37
		B	3.32±0.50^{aA}	3.13±0.71^{a}	4.06±0.40^{a}			
		C	4.05±0.47^{aA}	4.83±0.67^{a}	4.06±0.38^{a}			
Fatty acid synthesis	*FASN*	A	2.89±0.14^{aA}	2.81±0.20^{a}	3.03±0.12^{a}	0.19	0.02	0.90
		B	2.82±0.43^{aA}	3.07±0.61^{a}	3.53±0.37^{a}			
		C	3.50±0.34^{aA}	3.50±0.48^{a}	3.40±0.29^{a}			
	ACACA	A	2.99±1.24^{aA}	2.98±1.75^{a}	3.82±1.01^{a}	0.61	0.48	0.48
		B	3.50±0.93^{aA}	5.47±1.31^{a}	2.28±0.86^{a}			
		C	2.38±0.79^{aA}	2.85±1.12^{a}	2.51±0.64^{a}			
Cholesterol synthesis	*HMGCR*	A	3.14±0.88^{aA}	1.38±1.25^{b}	1.07±0.75^{b}	0.47	0.01	0.16
		B	3.13±0.58^{aA}	2.35±0.82^{a}	3.30±0.47^{a}			
		C	3.73±0.60^{aA}	4.37±0.86^{a}	3.71±0.50^{a}			
Placental function	*STAR*	A	1.38±1.55^{aA}	2.47±2.20^{a}	2.80±1.27^{a}	0.42	0.40	0.84
		B	1.22±1.20^{aA}	4.17±1.82^{a}	0.96±1.05^{a}			
		C	0.20±1.10^{aA}	1.02±1.63^{a}	0.63±0.94^{a}			
	bPAG	A	3.14±0.20^{aA}	2.72±0.28^{a}	2.90±0.16^{a}	0.56	0.96	0.96
		B	2.87±0.40^{aA}	2.76±0.53^{a}	2.84±0.33^{a}			
		C	3.14±0.30^{aA}	2.84±0.45^{a}	2.73±0.26^{a}			
	P450	A	1.61±1.20^{aA}	2.52±1.76^{a}	3.75±1.10^{a}	0.55	0.22	0.69
		B	1.22±179^{aA}	4.31±2.53^{a}	0.12±1.57^{a}			
		C	0.28±1.50^{aA}	0.18±1.85^{a}	0.90±1.16^{a}			
	BPL	A	2.89±1.08^{aA}	2.00±1.52^{a}	0.43±0.88^{b}	0.97	0.05	0.22
		B	1.01±1.04^{aA}	0.48±1.47^{a}	3.48±0.85^{a}			
		C	2.83±0.83^{aA}	4.18±1.18^{a}	3.22±0.68^{a}			
IGF system	*IGF1*	A	0.90±1.43^{aA}	1.10±2.02^{a}	1.54±1.17^{a}	0.56	0.69	0.77
		B	1.16±1.17^{aA}	3.60±1.66^{a}	0.50±0.95^{a}			
		C	0.62±1.02^{aA}	1.10±1.44^{a}	0.56±0.83^{a}			
	IGF2	A	1.99±0.63^{aA}	0.81±0.89^{b}	0.49±0.51^{b}	0.47	0.01	0.78
		B	2.70±0.79^{aA}	2.20±1.12^{a}	2.90±0.64^{a}			
		C	3.38±0.78^{aA}	3.34±1.11^{a}	2.85±0.64^{a}			
	IGF1r	A	3.47±0.68^{aA}	2.71±0.97^{a}	2.40±0.56^{b}	0.72	0.01	0.92
		B	3.04±0.84^{aA}	3.59±1.18^{a}	2.95±0.73^{b}			
		C	4.23±0.61^{aA}	4.33±0.86^{a}	3.78±0.50^{a}			
	IGF2r	A	2.35±0.48^{aA}	3.00±0.68^{a}	3.08±0.39^{a}	0.27	0.35	0.45
		B	2.22±0.42^{aA}	3.49±0.59^{a}	2.08±0.34^{a}			
		C	2.22±0.36^{aA}	2.39±0.51^{a}	2.34±0.30^{a}			
Apoptosis	*BAX*	A	3.39±0.73^{aA}	2.51±1.03^{a}	1.92±0.60^{b}	0.70	0.03	0.41
		B	2.94±0.72^{aA}	2.19±1.02^{a}	3.92±0.59^{a}			
		C	3.90±0.42^{aA}	3.95±0.59^{a}	3.62±0.34^{a}			
	BCL2	A	1.85±0.45^{aA}	-	0.84±0.38^{b}	0.70	0.01	0.01
		B	2.12±1.08^{aA}	1.21±1.95^{a}	3.01±0.81^{a}			
		C	2.95±0.73^{aA}	4.90±1.03^{a}	3.34±0.60^{a}			

^{a,b}: Numbers with unequal letters on the same row differ, $P < 0.05$

^{A,B}: Numbers with unequal capital letters on the same column differ, $P < 0.05$

Carbohidrate transport and metabolism. Compared to type A, type C placentomes had increased transcript relative abundance patterns for (i) the glucose transporter gene *SLC2a3*; (ii) the fructogenic pathway, with higher expression of the *AKR1B1* and *SORD* genes; and (iii) the pentose phosphate pathway, with higher *G6PD* gene expression (Table 2 and Figure 2). The type B placentomes had expression patterns that were intermediate and without differences from types A and C (Figure 2). No significant differences were detected between placentome types for genes related to the glycolytic pathway (*LDHA* and *LDHB* genes), or to fructolysis, based on the expression levels of the *ALDOB*, *KHK* and *TKFC* genes (Table 2, P>0.05). No detection of expression was observed for genes related to gluconeogenesis in any of the placentome types analysed, suggesting that such pathway is not active in the bovine placenta, at least for the period under analysis.
Lipid metabolism. Lipid synthesis appeared to be higher in type C placentomes, as the expression levels of the *HMGCR* gene, the key enzyme in the cholesterol synthesis pathway, and the *FASN* gene, part of the fatty acid synthesis, were higher than type A placentomes, with no differences in *ACACA* gene expression between placentome types. No differences in expression were observed between type B placentomes in relation to types A and C.
Placental function, IGF system and apoptosis. Regarding gene expression levels related to placental function, a difference was detected only for bLP expression, with a 2-fold increase in type C than types A and B placentomes (Figure 2, P<0.05). In the IGF system, *IGF2* (P<0.01) and *IGF1r* (P<0.01) expression levels were also higher in type C than types A and B placentomes, with no changes in *IGF2r* (P=0.35) and *IGF1* (P=0.69). As for apoptosis, despite the lower *BAX* (pro-apoptotic) and *BCL2* (anti-apoptotic) expression patterns in type A placentomes (Table 2, P<0.05), both gene products presented the same expression patterns, with no significant differences between placentomes in the *BAX/BCL2* ratio.

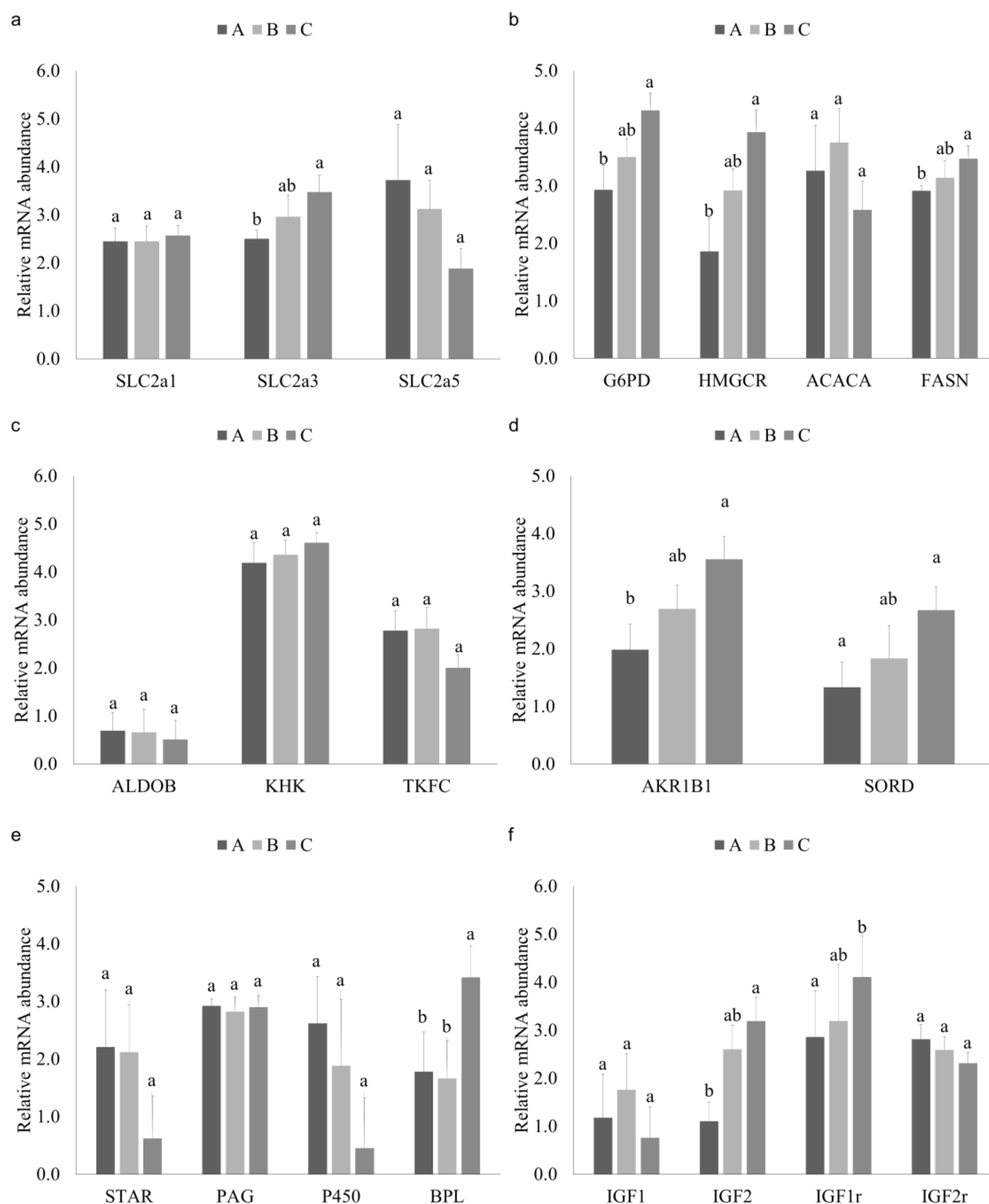

Figure 2. Relative gene expression patterns (LSM ± SEM) in placentomes, by type, between *Bos taurus* var. *indicus in vivo*-derived (IVD) concepti and concepti produced either by *in vitro* fertilisation (IVF) or nuclear transfer by handmade cloning (NT-HMC) procedures on Day 225 of gestation (LSM ± SEM). Gene products for (A) sodium-independent facilitated hexose transporters (*SCL2A1*, *SCL2A3* and *SCL2A5*); (B) lipid metabolism (*G6PD*, *HMGCR*, *ACACA* and *FASN*); (C) fructolysis (*ALDOB*, *KHK* and *TKFC*); (D) fructogenesis (*AKR1B1* and *SORD*); (E) placental function (*STAR*, *PAG*, *P450* and *BPL*); (F) components of the IGF system (*IGF1*, *IGF2*, *IGF1r* and *IGF2*r). [a,b]: Columns for each gene transcript without common superscripts differ; $P<0.05$.

Gene expression patterns for the sugar transporters *SLC2A1* (glucose) and SLC2A5 (fructose) positively correlated with the expression levels for genes related to lipid synthesis (*ACACA* and *FASN*) in types A, B and C placentomes ($r>0.6$; $P<0.05$), suggesting an increase in lipid synthesis as a function of glucose uptake within the placentomes. In type A placentomes, *SLC2A1* and SLC2A5 genes also positively correlated with *IGF1* and *LDHA* and *LDHB* ($r>0.8$; $P<0.05$). In type C placentomes, the *SLC2A1*, *SLC2A3* (glucose) and SLC2A5 sugar transporters positively correlated with the glycolytic (*LDHA*, *LDHB*) and fructolytic (*TKFC*) pathways' expression levels in the foetal liver ($r>0.6$; $P<0.05$).

DISCUSSION

Findings from this study provide evidence regarding the existence of a metabolic compartmentalisation or zonation in the bovine placenta, as significant differences in expression were detected between placentome types, in special between types A/B and type C placentomes. Then, such changes in placentome morphological profile in cloned concepti was correlated with placental metabolic function, which could lead to changes in placental and foetal metabolism and differential foetal growth patterns in the course of development. Our previous studies showed that cloning by SCNT lead to changes in placental morphology followed by an increase in foetal growth (Gerger *et al.*, 2016), with significant impact on the foetal biochemical and metabolic profiles on Day 225 days of gestation (Ticiani *et al.*, 2018, unpublished data). Such pieces of evidence and implications in support of our findings are discussed below.

The differentiation of placentomes may likely be a compensatory approach to provide adequate nutrition for the foetus (Beckett *et al.*, 2014). It is well known that *in vitro*-produced concepti may go through a biphasic growth pattern, with an early growth retardation that coincides with the period of placentation, followed by a foetal and placental excessive growth in mid to late pregnancy, with the birth of larger and less viable calves at term (Bertolini *et al.*, 2002, 2004; Batchelder *et al.*, 2007; Gerger *et al.*, 2016). Thus, placentome sizes and shapes are often different in *in vitro*-derived pregnancies, with a high frequency of enlarged placentomes in clone concepti, with a potential greater role of less frequent and

abnormal, nonetheless heavier, placentome types in placental function (Gerger *et al.*, 2016).

The morphologic and morphometric analyses made by Gerger *et al.* (2016) showed that *in vitro*-derived concepti (IVF and NT-HMC) had more type C (flattened, non-engulfing) and fewer type A (engulfing, mushroom-like) placentomes compared with the IVD group, with similar frequencies of types B (subengulfing, mushroom-like) and D (semiconvex) placentomes. However, despite a lower number of type A placentomes in clones, the absolute total weight for type A placentomes was similar to controls, whereas type B (similar in number as in the IVD group) and type C (increased numbers compared with the IVD group) placentomes were significantly heavier than in the IVD and IVF groups (Figure 1). Altogether, such observations indicated that placentome mass by type increased in clones, with an apparent greater role of types B and C placentomes in placental function and foetal growth in cloned concepti.

The placental pattern in IVF placentae was more similar to the IVD control group, considering the mass of the placentones, even though a lower number and reduced tissue mass was observed in type A placentomes, which may indicate that individually, such placentomes were not different in size when compare to the IVD group, which was not true for clones, as such group had fewer type A placentomes, for a total mass equal to the IVD, indicating a larger size per placentome. In the same way as for types B and C placentomes, the IVF and cloned groups were equal to controls in terms of numbers, but different than for clones, the total weights for types B and C placentomes in the IVF group were similar to controls, which may indicate that no significant metabolic deviations occurred in IVF concepti that could determine phenotypic and pathophysiological changes in development. Such premise can be confirmed by the absolute similarities in physical traits between control and IVF-derived fetuses associated with placentae used for qPCR analysis in this study (Gerger *et al.*, 2016). In clones, in turn, despite the similar numbers as in the IVD and IVF groups, the significant larger total weights for types B and C placentomes indicate larger individual types B and C placentome sizes in clones, which was associated with changes in gene expression, and, therefore, in placental metabolic functions, and

homeostatic and homeorrhetic patterns in cloned concepti.

Interestingly, distinct patterns of differential gene expression between placentome types were observed in this study. An upregulation in the expression of several genes was observed in type C placentomes, including the glucose transporter *SLC2a3*, which is related to glucose transport at the placenta-foetal interface in sheep (Eberhardt & Bell, 1997), being also associated with an imprinted isoform in mice that is stimulated by *IGF2* expressed at the placental tissue (Constancia *et al.*, 2005; Angiolini *et al.*, 2006; Fowden *et al.*, 2006). Even though no expression differences were detected between groups (IVD, IVF, SCNT) for glucose transporters, on a per gram of placenta basis, the cloned group had a greater number of type C placentomes, which were excessively larger. This may represent an increased abundance of glucose transporters for increased total surface area for exchange and, consequently, higher glucose uptake by cloned concepti. Our findings partially corroborate the study by Hirayama *et al.* (2011), in which gene expression patterns for glucose transporters were analysed in late pregnancy in cloned cattle, showing an increase in mRNA abundance for the *SLC2a3* transporter in clones. However, unlike our study, those authors did not classify placentomes according to their morphology, or type (A, B, C, and D). By knowing that cloned concepti change the way placentomes develop and mature, having a higher proportion of flattened (type C) placentomes, it is feasible that more representative samples from type C placentomes were included in the analysis, explaining the expression pattern for *SLC2a3*. The increase in total *SLC2a3* in the placenta and the absence of foetal hypoglycemia implies that glucose uptake and transport from the maternal circulation is increased in clones in mid- to late gestation, probably to meet increasing foetal and placental requirements (Hirayama *et al.*, 2011), as the rate of placental growth in clones is not reduced in late gestation, unlike in normal pregnancies (Constant *et al.*, 2006).

The increased glucose uptake and hyperfructosemia reported by some cloned calves at birth may be expected when considering that another function of the ruminant placenta is to synthesize fructose from glucose via *AKR1B1* and *SORD* enzymes (Batchelder *et al.*, 2007). Additionally, an upregulation was

observed in the expression of genes responsible for fructogenesis in type C placentomes. This is corroborated by the idea that the high concentration of fructose observed in plasma and allantoic fluid in the conceptus from the NT-HMC group (Ticiani *et al.*, 2018, unpublished data) is of placental origin, with type C placentomes potentially being the main responsible for such synthesis, for an increased glucose uptake by the whole collection of type C (and larger) placentomes in clones.

Increased fructose produced by the abnormal cloned placenta may contribute to foetal overgrowth in the NT-HMC group and also for the enlarged umbilical cord. First, fructose metabolism via the hexosamine pathway may also lead to the synthesis of glycosaminoglycans, e.g., hyaluronic acid, uridine diphosphate-N-acetyl glucosamine and uridine diphosphate-N-acetyl galactosamine, which are all involved in the synthesis of glycolipids, glycosaminoglycans and proteoglycans critical to functions of cells and tissues (Kim *et al.*, 2012). Increase in hyaluronic acid and hyaluronidase in the uterine lumen stimulate angiogenesis, morphogenesis and tissue remodeling in the placenta, as reported for the human placenta (Ponting & Kumar, 1995). The accumulation of hyaluronic acidoccurs in the placentae of most mammals and primarily localizes to the umbilical cord, but to a lesser extent to placental blood vessels (Mitchell *et al.*, 2003). Frequently, larger umbilical cords are observed in cloned neonates. Wang *et al.* (2016) showed that fructose has maximum effects on proliferation of ovine trophectoderm cells at molar concentrations well below those that we found in the allantoic fluid (Ticiani *et al.*, 2018, unpublished data). As fructose induces cell proliferation via activation of the AKT-tuberous sclerosis complex 2 (TSC2)-MTOR signaling cascade, and as the activation of such cascade is mediated by O-GlcNAcylation from UDP-N-acetylglucosamine (UDP-GlcNAc), a primary product of the hexosamine biosynthesis pathway (Wang et al., 2018), it is possible that higher fructose synthesis and availability to cloned conceptus may be associated with deviations in umbilical cord development. However, more studies are necessary to address such concept.

The IGF family acts at endocrine, paracrine and autocrine levels to modulate mitogenic and metabolic effects, stimulating the growth and

development of the placenta (Lowe, 1991). In the placenta, at the cell level, IGF signaling can cause a series of biological responses such as growth, proliferation, survival, nutrient uptake, migration and differentiation (Constancia *et al.*, 2002). This family activity is mediated by the interaction of two ligands (*IGF1* and *IGF2*) with three membrane receptors (*IGF1*R, *IGF2R* and insulin receptor), although most of their effects are mediated through the *IGF1*R. The *IGF2* ligand, for instance, is an imprinted gene that has a direct positive pleiotropic effect on development, being expressed in the paternal allele; on the other hand, the *IGF2R* has indirect negative pleiotropic effects, blocking the effect of *IGF2*, being of maternal origin. Although previous studies stated that in cloned animals the parental or imprinting effect could be lost, no changes in gene expression of components of the IGF family were observed between the experimental groups in this study. However, type C placentomes showed higher *IGF2* and *IGF1r* expression, and considering the proportion in numbers and mass of this type of placentomes in the cloned group, it is infered that *IGF2* may have exerted greater pleiotropic effects in cloned concepti in this study.

The bovine placenta is an important source of steroid hormones during pregnancy (Conley & Mason, 1994), and it can produce both progesterone and estrogens. Kohan-Ghadr *et al.* (2011) demonstrated that maternal progesterone profiles were not different between cloned and control pregnancies from about mid-gestation onwards, although transient differences have been observed in the first half of gestation, when progesterone levels were higher in some cloned lines, whereas lower in others. Although no differences in gene expression for a key steroidogenic enzyme were observed in this study, on a per unit of tissue basis, when considering a larger placental mass, the cloned group should have greater capacity to produce steroid hormones. This hypothesis is still under investigation. In addition to steroid hormones, the ruminant placenta secretes several proteins, including the pregnancy-associated glycoproteins (*PAG*s) and placental lactogen. Heyman *et al.* (2002) reported that abnormally high *PAG* concentrations in the plasma of NT-HMC concepti subsequently were associated with placental anomalies. Although no contradicting reports exist about an increase in BNC numbers in cloned placenta (Ravelich *et al.*, 2004; Arnold *et al.*,

2008), Constant *et al.* (2011) showed that elevated *PAG* concentrations do not appear to result from an increased placental expression, nor from a higher proportion of BNC in the placenta, but likely from a higher transport through the placental barrier and/or an increase in *PAG* half-life in the maternal circulation. In agreement with Constant *et al.* (2011), we did not observe changes in *PAG* expression between groups or between placentome types.

The partitioning of nutrients between maternal and fetoplacental compartments is orchestrated by a number of endocrine hormones, including both placental lactogen (PL) and placental leptin, which are also involved in foetal and placental growth (Hoggard *et al.*, 2001). In cattle, bovine PL (*BPL*) is a product of trophoblastic binucleate cells, which are directly involved in the modification of the uterine epithelium (Wooding *et al.*, 1997). In sheep, oPL levels are responsible for about 80% of the variation in total foetal weight (Schoknecht *et al.*, 1991; Kappes *et al.*, 1992) suggesting that PL regulates foetal growth by stimulating repartitioning of maternal nutrients to the foetus, and by stimulating the foetus to use the substrates (Anthony *et al.*, 1995). Thus, even though no differences were detected in PL expression between the *in vitro* and *in vivo* groups, type C placentomes had higher PL expression than other types, supporting our hypothesis that there is a metabolic compartmentalization in the placenta, and the morphological changes in placentomes in the NT-HMC group may direct the overgrowth observed in cloned foetuses.

In summary, the gene expression pattern for relevant genes in the bovine placenta revealed significant differences in the metabolic profile between placentome types, demonstrating a potential metabolic zonation in the bovine placenta. Therefore morpho-physiologic differences observed between clones and controls could be due to changes in size and proportions of placentome types, not due to modifications of tissue function itself. Such changes, as for the increase in glucose transporters in type C placentomes, associated with greater number and mass of such placentomes in cloned concepti, may lead to greater glucose uptake by the placenta. Glucose, in turn, aside from being the basis for energy supply, can also serve as a substrate for both fructose and lipid syntheses. In the case of fructose, such pathway appears to be increased in type

C placentomes, due to the increased expression of genes related to fructogenesis. As a consequence of an increase in fructose synthesis, a significant increase in fructose concentration within the foetal compartments follows, with higher fructose accumulation in the allantoic fluid of cloned concepti. Perhaps, such phenomenon may be associated with hydroallantois in some clone-borne pregnacies, due to higher levels of the osmotically active fructose within the fluid compartment. Regarding lipid synthesis, it is well known that when met the metabolic demands, the excess of nutrients is directed to the synthesis of fatty acids. In this way, we observed an increase in the expression of genes related to fatty acid synthesis in type C placentomes, and a possible hepatic lipidosis in clone concepti, considering the allometric pattern of growth of the fetal liver (Gerger *et al.,* 2016), for a lower liver function capacity (Ticiani *et al.,* 2018). Altogether, such changes corroborate with physical and physiological findings observed in concepti derived from the cloning by SCNT.

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REFERENCES

Anthony RV, Pratt SL, Liang R & Holland MD 1995 Placental–fetal hormonal interactions: impact on fetal growth. *Journal of Animal Science* 73, 1861–1871.

Arnold DR, Fortier AL, Lefebvre R, Miglino MA, Pfarrer C & Smith LC 2008 Placental insufficiencies in cloned animals e a workshop report. Placenta 29 S108-110.

Barker DJ 1999 The fetal origins of Type 2 diabetes mellitus. *Annals of Internal Medicine* 130 322 – 324.

Batchelder CA, Bertolini M, Mason JB, Moyer AL, Hoffert KA, Petkov SG, Famula TR, Angelos J, George LW & Anderson GB 2007 Perinatal physiology in cloned and normal calves: physical and clinical characteristics. *Cloning and Stem Cells* 9 63-82.

Beckett EM, Astapova O, Steckler TL, Veiga-Lopez A, Padmanabhan V 2014 Developmental programing: impact of testosterone on placental differentiation. *Reproduction* 148 199–209.

Bertolini M, Beam SW, Shim H, Bertolini LR, Moyer AL, Famula TR & Anderson GB 2002 Growth, development and gene expression by *in vivo*- and *in vitro*-produced Day-7 and Day-16 bovine embryos. *Molecular Reproduction and Development* 63 318-328.

Bertolini M, Wallace CR & Anderson GB 2006 Expression profile and protein levels of placental products as indirect measures of placental function in *in vitro*-derived bovine pregnancies. *Reproduction* 131 163-173.

Bertolini M, Moyer AL, Mason JB, Batchelder CA, Hoffert KA, Bertolini LR Carneiro GF, Cargill SL, Famula TR, Calvert CC et al. 2004 Evidence of increased substrate availability to *in vitro*-derived bovine foetuses and association with accelerated conceptus growth. *Reproduction* 128 341-354.

Conley AJ & Mason JI 1994 Endocrine function of the placenta. In: Thornburn GD & Harding R, editors. Textbook of fetal physiology. Oxford: *Oxford University Press* 16-29.

Constancia M, Hemberger M, Hughes J, Dean W, Ferguson- Smith A, Fundele R, Stewart F, Kelsey G, Fowden A, Sibley C & Reik W 2002 Placental-specific IGF-II is a major modulator of placental and fetal growth. *Nature* 417 945–948.

Constant F, Camous S, Chavatte-Palmer P, Heyman Y, de Sousa N, Richard C, Beckers JF & Guillomot M 2011 Altered secretion of pregnancy-associated glycoproteins during gestation in bovine somatic clones. *Theriogenology* 76 1006-1021.

Constant F, Guillomot M, Heyman Y, Vignon X, Laigre P, Servely JL, Renard JP

& Chavatte-Palmer P 2006 Large offspring or large placenta syndrome? Morphometric analysis of late gestation bovine placentomes from somatic nuclear transfer pregnancies complicated by hydrallantois. *Biology of Reproduction* 75 122–130.

Ehrhardt RA & Bell AW 1997 Developmental increase in glucose transporter concentration in the sheep placenta. American Journal of Physiology 273 R1132–R1141.

Fowden AL 1997 Comparative aspects of fetal carbohydrate metabolism. *Equine Veterinary Journal* 24 19–25.

Gebhardt R 1992 Metabolic zonation of the liver: regulation and implications for liver function. *Pharmacology Therapeutic* 53 275-354.

Gerger RP, Zago FC, Ribeiro ES, Gaudencio Neto S, Martins LT, Aguiar LH, Rodrigues VHV, Furlan FH, Ortigari I, Sainz RD et al. 2016 Morphometric developmental pattern of bovine handmade cloned concepti in late pregnancy. *Reproduction*, Fertility and Development 29 950-967.

Heyman Y, Chavatte-Palmer P, LeBourhis D, Camous S, Vignon X & Renard JP 2002 Frequency and occurrence of late-gestation losses from cattle cloned embryos. *Biology of Reproduction* 66 6-13.

Hirayama H, Sawai K, Hirayama M, Hirai T, Kageyama S, Onoe S, Minamihashi A & Moriyasu S 2011 Prepartum maternal plasma glucose concentrations and placental glucose transporter mRNA expression in cows carrying somatic cell clone fetuses. *Journal of Reproduction and Development* 57 57-61.

Hoggard N, Haggarty P, Thomas L & Lea RG 2001 Leptin expression in placental and fetal tissues: does leptin have a functional role?. *Biochemistry Society Transactions* 29 57–63.

Kappes SM, Warren WC, Pratt SL, Liang R & Anthony RV 1992. Quantification and cellular localization of ovine placental lactogen messenger ribonucleic acid expression during mid- and late gestation. *Endocrinology* 131 2829–2838.

Kim JY, Song GW, Wu G, Bazer FW 2012 Functional roles of fructose. *Proceedings of the National Academy of Sciences of the United States of*

America 109 E1619–E1628.

Kohan-Ghadr HR, Fecteau G, Smith LC, Murphy BD & Lefebvre RC 2011 Endocrine profiles of somatic nuclear transfer-derived pregnancies in dairy cattle. *Theriogenology* 76 911-920.

Lowe WL Jr 1991 Biological actions of the insulin-like growth factors. In Insulin-Like Growth Factors: Molecular and Cellular Aspects, ed. D. LeRoith, Boca Raton: CRC Press, 49-85.

McMillen IC & Robinson JS 2005 Developmental origins of the metabolic syndrome: prediction, plasticity, and programming. *Physiological Reviews* 85 571-633.

Mitchell KE, Weiss ML, Mitchell BM, Martin P, Davis D, Morales L, Helwig B, Beerenstrauch M, Abou-Easa K, Hildreth T, Troyer D & Medicetty S 2003 Matrix cells from Wharton's jelly form neurons and glia. *Stem Cells* 21 50–60.

Ponting JM & Kumar S 1995 Isolation and characterisation of a hyaluronan binding protein, hyaluronectin, from human placenta and its colocalisation with hyaluronan. *Journal of Anatomy* 186 131–142.

Ravelich SR, Breier BH, Reddy S, Keelan JA, Wells DN, Peterson AJ & Lee RS 2004 Insulin-like growth factor-I and binding proteins 1, 2, and 3 in bovine nuclear transfer pregnancies. *Biology of Reproduction* 70 430-438.

Schoknecht PA, Nobrega SN, Petterson JA, Ehrhardt RA, Slepetis R & Bell AW 1991 Relations between maternal and fetal plasma concentrations of placental lactogen and placental and fetal weights in well-fed ewes. *Journal of Animal Science* 69 1059–1063.

Vandesompele, J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A & Speleman F 2002 Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biology* 3 RESEARCH0034.

Wang X, Li D, Wu G, & Bazer FW 2018 Functional Roles of Fructose: Crosstalk between O-Linked Glycosylation and Phosphorylation of Akt-TSC2-MTOR Cell Signaling Cascade in Ovine Trophectoderm Cells. *Biology of Reproduction* 95 1-17.

Wang Y, Wang Y, Zhang H, Gao Y, Huang C, Zhou A, Zhou Y & Li Y 2016 Sequential posttranslational modifications regulate PKC degradation. *Molecular Biology of the Cell* 27 410–420.

Walker SK, Hartwich KM, Seamark RF 1996 The production of unusually large offspring following embryo manipulation: concepts and challenges. *Theriogenology* 45 111-120.

Wooding FB, Morgan G & Adam CL 1997 Structure and function in the ruminant synepitheliochorial placenta: central role of the trophoblast binucleate cell in deer. *Microscopy Research and Technique* 38 88–99.

Zhang S, Regnault TR, Barker PL, Botting KJ, McMillen IC, McMillan CM, Roberts CT and Morrison JL 2015 Placental adaptations in growth restriction. *Nutrients* 7 360-89.

4. CONCLUSÕES E CONSIDERAÇÕES FINAIS

Com base nos resultados obtidos nos estudos apresentados nos Capítulos II e III acima, podemos concluir que:

a. Foram estabelecidas relações fisiológicas significativas, diretas e indiretas, entre parâmetros físicos, bioquímicos, metabólicos e moleculares com o aparecimento da *Abnormal Offspring Syndrome* (AOS) em conceptos bovinos derivados da clonagem por TNCS, aos 225 dias de gestação.
b. A produção *in vitro* de embriões pela FIV por um sistema de cultivo *in vitro* otimizado gerou conceptos semelhantes aos controles em termos morfológicos e fisiológicos, com algumas alterações ainda detectáveis na morfologia placentária.
c. A morfologia e a distribuição de peso e área dos placentônios nos conceptos clones no Dia 225 de gestação foram significativamente distintas do grupo controle (*in vivo*), com uma redução de placentônios dos tipos normais, em especial do tipo A, e um aumento dos tipos anormais, especialmente do tipo C, com alteração significativa no padrão de expressão de genes importantes no desenvolvimento pré-natal.
d. A origem dos embriões (IVD, IVF, NT-HMC) não causou alterações nas características físicas maternas e na bioquímica plasmática materna aos 225 dias de gestação.
e. Além do crescimento fetal excessivo, vários parâmetros de função hepática, renal e do metabolismo da hemoglobina, dos carboidratos e lipídios foram significativamente distintos em gestações de clones, com um padrão indicativo de maior aporte de substratos (glucose, fructose) ao feto, corroborado por perfis de expressão gênica de enzimas chave de rotas metabólicas que denotam um maior aporte de carboidratos, com um aparente desvio para outras rotas metabólicas, como a via das pentoses e a lipogênese.
f. O padrão de expressão gênica revelou diferenças significativas no perfil metabólico entre os tipos de placentônios, demonstrando uma potencial compartimentalização metabólica na placenta bovina.

g. Os placentônios anormais em forma, mais achatados (tipo C), foram mais abundantes em conceptos clones, e demonstraram ser metabolicamente mais ativos, com aumento na expressão de transportadores de glicose e nas rotas frutogenica e lipogênica.
h. As características físicas de conceptos clones foram correlacionadas positivamente com variáveis associadas a um padrão fisiológico ativo e de promoção do crescimento fetal, incluindo componentes do metabolismo da glicose e da frutose, acúmulo de carboidratos em compartimentos materno e/ou fetal, do metabolismo dos lípidos, de proteínas e da hemoglobina. Por outro lado, as mesmas características físicas foram negativamente correlacionadas com os níveis de glicose e frutose no fluido amniótico, e com o metabolismo do colesterol e parâmetros da função hepática, o que pode indicar uma atividade metabólica menor do fígado fetal de clones, apesar deste órgão ser alometricamente maior em clones. Tal alteração de morfologia e função pode e*star* associado a doenças metabólicas, como a lipidose hepática.
i. A sequência de eventos morfológicos e fisiológicos observados no curso do desenvolvimento de conceptos clones neste estudo corroboram com a hipótese de desregulação do sistema de restrição placentária ao crescimento fetal no final da gestação, e na alteração do padrão de programação metabólica fetal, com consequências potenciais na sobrevida tanto pré- quanto pós-natal.

Com esse estudo foi estabelicedo uma relação entre os padrões fenotípicos útero-placentário e fetal de crescimento e os padrões de uso de substratos pela placenta e aporte ao feto, evidenciando por sua vez a existência de rotas metabolicas alteradas em função de alterações morfofisiológicas da placenta, com efeitos no padrão de drescimento e desenvolvimento de fetos derivados da clonagem por TNCS. Desta forma, forneceu-se uma base que pode ser útil na elucidação dos efeitos da PIV de embriões no desenvolvimento pré- e pós-natal subsequentes, com potencial associação a fenômenos biológicos aberrantes similares de ocorrência natural nos rebanhos.

5. REFERÊNCIAS – Introdução e Revisão de Literatura

ALDORETTA, P. W.; HAY, W. W. Effect of glucose supply on ovine uteroplacental glucose metabolism. **American Journal of Physiology-Regulatory, Integrative and Comparative Physiology**, v. 277, n. 4, p. R947-R958, 1999.

ARCHER, G. S. *et al*. Behavioral variation among cloned pigs. **Applied Animal Behaviour Science**, v. 81, n. 4, p. 321-331, 2003.

AUGUSTIN, L. S. A. *et al.* Dietary glycemic index and glycemic load, and breast cancer risk: a case-control study. **Annals of Oncology**, v. 12, n. 11, p. 1533-1538, 2001.

BAGUISI, A. *et al*. Production of goats by somatic cell nuclear transfer. **Nature biotechnology**, v. 17, n. 5, p. 456-461, 1999.

BARKER, D. J. P. Fetal programming and public health. **Fetal programming: Influences on development and disease in later life**, p. 3-11, 1999.

BATCHELDER, C. A. *et al.* Effect of the nuclear-donor cell lineage, type, and cell donor on development of somatic cell nuclear transfer embryos in cattle. **Cloning and stem cells**, v. 7, n. 4, p. 238-254, 2005.

BATCHELDER, C. A. *et al*. Perinatal physiology in cloned and normal calves: hematologic and biochemical profiles. **Cloning and stem cells**, v. 9, n. 1, p. 83-96, 2007.

BEHBOODI, E. *et al*. Birth of large calves that developed from *in vitro*-derived bovine embryos. **Theriogenology**, v. 44, n. 2, p. 227-232, 1995.

BERTOLINI, M. *et al.* Evidence of increased substrate availability to *in vitro*-derived bovine foetuses and association with accelerated conceptus growth. **Reproduction**, v. 128, n. 3, p. 341-354, 2004.

BERTOLINI, M. *et al*. Growth, development, and gene expression by *in vivo*-and *in vitro*-produced day 7 and 16 bovine embryos. **Molecular reproduction and development**, v. 63, n. 3, p. 318-328, 2002a.

BERTOLINI, M. *et al.* Morphology and morphometry of *in vivo*-and *in vitro*-produced bovine concepti from early pregnancy to term and association with high birth weights. **Theriogenology**, v. 58, n. 5, p. 973-994, 2002b.

BERTOLINI, M.; ANDERSON, G. B. The placenta as a contributor to production of large calves. **Theriogenology**, v. 57, n. 1, p. 181-187, 2002.

BINISZKIEWICZ, D. *et al.* Dnmt1 overexpression causes genomic hypermethylation, loss of imprinting, and embryonic lethality. **Molecular and Cell Biology**, v. 22, p. 2124-2135, 2002.

BIRD, A. DNA methylation patterns and epigenetic memory. **Genes & Development**, v. 16, p. 6-21, 2002.

BRACKETT, B. G. *et al.* Normal development following *in vitro* fertilization in the cow. **Biology of reproduction**, v. 27, n. 1, p. 147-158, 1982.

CAMARGO, F. C. *et al.* Plasma and urinary concentrations of trimetoquinol by LC-MS-MS following intravenous and intra-tracheal administration to horses with heaves. **Journal of veterinary pharmacology and therapeutics**, v. 31, n. 6, p. 501-510, 2008.

CASSLEN, B.; NILSSON, B. Human uterine fluid, examined in undiluted samples for osmolarity and the concentrations of inorganic ions, albumin, glucose, and urea. **American journal of obstetrics and gynecology**, v. 150, n. 7, p. 877-881, 1984.

CHAVATTE-PALMER, P. *et al.* Placental perturbations induce the developmental abnormalities often observed in bovine somatic cell nuclear transfer. **Placenta**, v. 33, p. S99-S104, 2012.

CIBELLI, J. B. *et al.* Cloned transgenic calves produced from nonquiescent fetal fibroblasts. **Science**, v. 280, n. 5367, p. 1256-1258, 1998.

CONSTANT, F. *et al.* Large offspring or large placenta syndrome? Morphometric analysis of late gestation bovine placentomes from somatic nuclear transfer pregnancies complicated by hydrallantois. **Biology of reproduction**, v. 75, n. 1, p. 122-130, 2006.

DEAN, W. *et al.* Conservation of methylation reprogramming in mammalian development: aberrant reprogramming in cloned embryos. **Proceedings of the National Academy of Sciences USA**, v. 98 p. 13734-13738, 2001.

DEAN, W.; SANTOS, F.; REIK, W. Epigenetic reprogramming in early mammalian development and following somatic nuclear transfer. **Seminars in Cell and Developmental Biology**, v. 14, p. 93-100, 2003.

EHRHARDT, R. A.; BELL, A. W. Developmental increases in glucose transporter concentration in the sheep placenta. **American Journal of Physiology-Regulatory, Integrative and Comparative Physiology**, v. 273, n. 3, p. R1132-R1141, 1997.

ELEY, R. M. *et al.* Development of the Conceptus in the Bovine1. **Journal of Dairy Science**, v. 61, n. 4, p. 467-473, 1978.

FARIN, P. W.; FARIN, C. E. Transfer of bovine embryos produced *in vivo* or *in vitro*: survival and fetal development. **Biology of reproduction**, v. 52, n. 3, p. 676-682, 1995.

FARIN, P. W.; PIEDRAHITA, J. A.; FARIN, C. E. Errors in development of fetuses and placentas from *in vitro*-produced bovine embryos. **Theriogenology**, v. 65, n. 1, p. 178-191, 2006.

FERREIRA, A. M. *et al.,* Almeida, The mammary gland in domestic ruminants: a systems biology perspective, **Journal of Proteome**, v. 94, p. 110–123, 2013.

FERRELL, C. L. Maternal and fetal influences on uterine and conceptus development in the cow: I. Growth of tissues of the gravid uterus. **Journal of animal science**, v. 69, n. 5, p. 1945-1953, 1991a.

FERRELL, C. L. Maternal and fetal influences on uterine and conceptus development in the cow: II. Blood flow and nutrient flux. **Journal of animal science**, v. 69, n. 5, p. 1954-1965, 1991b.

FERRELL, C. L. Placental regulation of fetal growth. In: **Animal Growth Regulation**. Springer US, 1989. p. 1-19.

FERRELL, C. L.; REYNOLDS, L. P. Uterine and umbilical blood flows and net nutrient uptake by fetuses and uteroplacental tissues of cows gravid with either single or twin fetuses. **Journal of animal science**, v. 70, n. 2, p. 426-433, 1992.

FOWDEN, A. L. *et al*. Imprinted genes, placental development and fetal growth. **Hormone Research in Paediatrics**, v. 65, n. Suppl. 3, p. 50-58, 2006.

FOWDEN, A. L.; RALPH, M. M.; SILVER, M. Nutritional regulation of uteroplacental prostaglandin production and metabolism in pregnant ewes and mares during late gestation. **Experimental and Clinical Endocrinology & Diabetes**, v. 102, n. 03, p. 212-221, 1994.

GAVIN, W. *et al.* Transgenic cloned goats and the production of recombinant therapeutic proteins. In: Cibelli, J. *et al.* (Ed.). **Principles of Cloning**. London: Academic Press. pp. 329-342, 2013.

GLUCKMAN, P. D. *et al.* Elevating maternal insulin-like growth factor-I in mice and rats alters the pattern of fetal growth by removing maternal constraint. **Journal of endocrinology**, v. 134, n. 1, p. R1-R3, 1992.

GREENFIELD, R. B.; CECAVA, M. J.; DONKIN, S. S. Changes in mRNA Expression for gluconeogenic enzymes in liver of dairy cattle during the transition to lactation1. **Journal of Dairy Science**, v. 83, n. 6, p. 1228-1236, 2000.

HAHN, T. *et al.* Placental glucose transporter expression is regulated by glucocorticoids. **The Journal of Clinical Endocrinology & Metabolism**, v. 84, n. 4, p. 1445-1452, 1999.

HAMMER, C. J. *et al*. Compromised development of calves (Bosgaurus) derived from *in vitro*-generated embryos and transferred interspecifically into domestic cattle (Bostaurus). **Theriogenology**, v. 55, n. 7, p. 1447-1455, 2001.

HAN, Y. M. *et al*. Nuclear reprogramming of cloned embryos produced *in vitro*. **Theriogenology**, v. 59, p. 33-44, 2003.

HAY, W. W. Regulation of placental metabolism by glucose supply. **Reproduction, Fertility and Development**, v. 7, n. 3, p. 365-375, 1995.

HERS, H. G. L. mecanisme de la formation du fructose seminal et du fructose foetal. **Biochimica et biophysica acta**, v. 37, n. 1, p. 127-138, 1960.

HILL, JR. Incidence of abnormal offspring from cloning and other assisted reproductive technologies. **Annual Review Animal Bioscience**, v. 2, p. 307–321, 2014.

HILL J. R.; CHAVATTE-PALMER P. Pregnancy and neonatal care of cloned animals. In Cibelli J, Lanza RP, Campbell KHS, West MD, eds: **Principles of Cloning**, Amsterdam and others, Academic Press, p. 247-266, 2002.

HILL, J. R. *et al*. Clinical and pathologic features of cloned transgenic calves and fetuses (13 case studies). **Theriogenology**, v. 51, n. 8, p. 1451-1465, 1999.

HILL, J. R. *et al*. Evidence for placental abnormality as the major cause of mortality in first-trimester somatic cell cloned bovine fetuses. **Biology of**

reproduction, v. 63, n. 6, p. 1787-1794, 2000.

HILL, J. R. *et al*. Placental anomalies in a viable cloned calf. **Cloning**, v. 3, n. 2, p. 83-88, 2001.

HIRAYAMA, H. *et al.* Prepartum maternal plasma glucose concentrations and placental glucose transporter mRNA expression in cows carrying somatic cell clone fetuses. **Journal of Reproduction and Development**, v. 57, n. 1, p. 57-61, 2011.

HUGGETT, A. St G.; NIXON, D. A. Fructose as a component of the foetal blood in several mammalian species. **Nature**, v. 190, p. 1209, 1961.

HUMPHERYS, D. *et al.* Epigenetic instability in ES cells and cloned mice. **Science**, v. 293, p. 95-97, 2001.

HUNTINGTON, G. B.; HARMON, D. L.; RICHARDS, C. J. Sites, rates, and limits of *star*ch digestion and glucose metabolism in growing cattle. **Journal of animal science**, v. 84, n. 13_suppl, p. E14-E24, 2006.

JACKSON-GRUSBY, L. *et al.* Loss of genomic methylation causes p53-dependent apoptosis and epigenetic deregulation. **Nature Genetics**, v. 27, p. 31-39, 2001.

JAENISCH; R.; BIRD, A. Epigenetic regulation of gene expression: how the genome integrates intrinsic and environmental signals. **Nature Genetics**, 33:245-254, 2003

JAVED, M. H.; WRIGHT, R. W. Determination of pentose phosphate and Embden-Meyerhof pathway activities in bovine embryos. **Theriogenology**, v. 35, n. 5, p. 1029-1037, 1991.

JORDAO, R. *et al.* Induction of multixenobiotic defense mechanisms in resistant *Daphnia magna* clones as a general cellular response to stress. **Aquatic Toxicology**, v. 175, p. 132–143, 2016.

JU, B. *et al*. Cloning zebrafish by nuclear transfer. **Methods in cell biology**, v. 77, p. 403-411, 2004.

JURISICOVA, A.; ACTON, B. M. Deadly decisions: the role of genes regulating programmed cell death in human preimplantation embryo development. **Reproduction**, v. 128, n. 3, p. 281-291, 2004.

KABASHIMA, T. *et al*. Xylulose 5-phosphate mediates glucose-induced

lipogenesis by xylulose 5-phosphate-activated protein phosphatase in rat liver. **Proceedings of the National Academy of Sciences**, v. 100, n. 9, p. 5107-5112, 2003.

KATO, Yoko *et al*. Eight calves cloned from somatic cells of a single adult. **Science**, v. 282, n. 5396, p. 2095-2098, 1998.

KEEFER, C. L. *et al*. *In vitro* culture of bovine IVM-IVF embryos: Cooperative interaction among embryos and the role of growth factors. **Theriogenology**, v. 41, n. 6, p. 1323-1331, 1994.

KHURANA, N. K.; NIEMANN, H. Energy metabolism in preimplantation bovine embryos derived *in vitro* or *in vivo*. **Biology of reproduction**, v. 62, n. 4, p. 847-856, 2000.

KIM, M. R.; TILLY, J. L. Current concepts in Bcl-2 family member regulation of female germ cell development and survival. **Biochimica et Biophysica Acta (BBA)-Molecular Cell Research**, v. 1644, n. 2, p. 205-210, 2004.

KREBS A, C.; LONGOB, L. D.; LEISER, R. Term ovine placental vasculature: comparison of sea level and high altitude conditions by corrosion cast and histomorphometry. **Placenta**, v. 18, n. 1, p. 43-51, 1997.

KRUIP, T. A. M.; DEN DAAS, J. H. G. *In vitro* produced and cloned embryos: effects on pregnancy, parturition and offspring. **Theriogenology**, v. 47, n. 1, p. 43-52, 1997.

Le, N. *et al.* Perturbations in the biochemical composition of fetal fluids are apparent in surviving bovine somatic cell nuclear transfer pregnancies in the first half of gestation. **Biology of reproduction**, v. 73, n. 1, p. 139-148, 2005.

LISTER R, et al. Human DNA methylomes at base resolution show widespread epigenomic difference. **Nature**, v. 462. p. 315- 322, 2009.

LOI, P. *et al*. Genetic rescue of an endangered mammal by cross-species nuclear transfer using post-mortem somatic cells. **Nature biotechnology**, v. 19, n. 10, p. 962-964, 2001.

LOSKUTOFF, N. M.; BETTERIDGE, K. J. Embryo technology in pets and endangered species. **Embryonic development and manipulation in animal production trends in research and applications/editors**, A. Lauria, F. Gondolfi, 1992.

LOUREIRO, B.; BRAD, A. M.; HANSEN, P. J. Heat shock and tumor necrosis factor-α induce apoptosis in bovine preimplantation embryos through a caspase-9-dependent mechanism. **Reproduction**, v. 133, n. 6, p. 1129-1137, 2007.

MAYES, P. A. Intermediary metabolism of fructose. **The American journal of clinical nutrition**, v. 58, n. 5, p. 754S-765S, 1993.

MCGRAW, S. *et al.* Loss of DNMT1o disrupts imprinted X chromosome inactivation and accentuates placental defects in females. PLoS Genet, 9:e1003873, 2013.

MCMILLEN, I. C.; ROBINSON, J. S. Developmental origins of the metabolic syndrome: prediction, plasticity, and programming. **Physiological reviews**, v. 85, n. 2, p. 571-633, 2005.

MEISSNER, A. *et al*. Genome-scale DNA methylation maps of pluripotent and differentiated cells. **Nature**, v. 454, p. 66-770, 2008.

MILES, Jeremy R. *et al*. Angiogenesis and morphometry of bovine placentas in late gestation from embryos produced *in vivo* or *in vitro*. **Biology of reproduction**, v. 71, n. 6, p. 1919-1926, 2004.

MOORE, T. Genetic conflict, genomic imprinting and establishment of the epigenotype in relation to growth. **Reproduction**, v. 122, p. 185-193, 2001.

NAN, X.; MEEHAN, R. R.; BIRD, A. Dissection of the methyl-CpG binding domain from the chromosomal protein MeCP2. **Nucleic Acid Research**, v. 21, p. 4886-4892, 1993.

NISHIMURA, M.; UYEDA, K. Purification and characterization of a novel xylulose 5-phosphate-activated protein phosphatase catalyzing dephosphorylation of fructose-6-phosphate, 2-kinase: fructose-2, 6-bisphosphatase. **Journal of Biological Chemistry**, v. 270, n. 44, p. 26341-26346, 1995.

O'FALLON, J. V.; WRIGHT JR, R. W. Quantitative determination of the pentose phosphate pathway in preimplantation mouse embryos. **Biology of reproduction**, v. 34, n. 1, p. 58-64, 1986.

OGURA, A.; INOUE, K.; WAKAYAMA, T. Recent advancements in cloning by somatic cell nuclear transfer. **Philosophical Transactions of the Royal Society B,** v. 368, n. 1609, p. 20110329, 2013.

OPIELA, J. *et al.* Interactions among activity of glucose-6-phosphate dehydrogenase in immature oocytes, expression of apoptosis-related genes Bcl-2 and *BAX*, and developmental competence following IVP in cattle. **Theriogenology**, v. 69, n. 5, p. 546-555, 2008.

PANTALEON, M. *et al.* Glucose transporter GLUT3: ontogeny, targeting, and role in the mouse blastocyst. **Proceedings of the National Academy of Sciences**, v. 94, n. 8, p. 3795-3800, 1997.

PARK, K. W. *et al*. Production of nuclear transfer-derived swine that express the enhanced green fluorescent protein. **Animal biotechnology**, v. 12, n. 2, p. 173-181, 2001.

PERRY, V. E. A. *et al.* Low dietary protein during early pregnancy alters bovine placental development. **Animal Reproduction Science**, v. 55, n. 1, p. 13-21, 1999.

PILKIS, S. J.; GRANNER, D. K. Molecular physiology of the regulation of hepatic gluconeogenesis and glycolysis. **Annual review of physiology**, v. 54, n. 1, p. 885-909, 1992.

POCAR, P. *et al.* Apoptosis in bovine cumulus–oocyte complexes after exposure to polychlorinated biphenyl mixtures during *in vitro* maturation. **Reproduction**, v. 130, n. 6, p. 857-868, 2005.

POLEJAEVA, I. A. *et al*. Cloned pigs produced by nuclear transfer from adult somatic cells. **Nature**, v. 407, n. 6800, p. 86-90, 2000.

PRIOR, R. L.; LASTER, D. B. Development of the bovine fetus. **Journal of animal science**, v. 48, n. 6, p. 1546-1553, 1979.

RANCOURT, R. C. *et al.* The prevalence of loss of imprinting of H19 and *IGF2* at birth. FASEB J, 27:3335-3343, 2013.

RAHUL, D. *et al.,* Production and characterization of putative ntES cells from handmade cloned goat embryos derived from adult fibroblast donor cell. **Journal of Reproduction and Stem Cell Biotechnology**, v. 2, p. 64-76, 2011.

REYNOLDS, L. P. *et al*. Growth and in-vitro metabolism of placental tissues of cows from day 100 to day 250 of gestation. **Journal of reproduction and fertility**, v. 89, n. 1, p. 213-222, 1990.

RIEGER, D. *et al.* Comparison of the effects of oviductal cell co-culture and oviductal cell-conditioned medium on the development and metabolic activity of cattle embryos. **Journal of reproduction and fertility**, v. 105, n. 1, p. 91-98, 1995.

RUDENKO, L. *et al*. Food consumption risks associated with animal clones: what should be investigated?. **Cloning & Stem Cells**, v. 6, n. 2, p. 79-93, 2004.

SALILEW-WONDIM, D. *et al.* Aberrant placenta gene expression pattern in bovine pregnancies established after transfer of cloned or *in vitro* produced embryos. **Physiology Genomics**, v. 45, p. 28-46, 2013.

SCHLAFER, D. H.; FISHER, P. J.; DAVIES, C. J. The bovine placenta before and after birth: placental development and function in health and disease. **Animal reproduction science**, v. 60, p. 145-160, 2000.

STICE, S. L. et al. Pluripotent bovine embryonic cell lines direct embryonic development following nuclear transfer. **Biology of reproduction**, v. 54, n. 1, p. 100-110, 1996.

SUN, Y. H. *et al*. Cytoplasmic impact on cross-genus cloned fish derived from transgenic common carp (Cyprinus carpio) nuclei and goldfish (Carassius auratus) enucleated eggs. **Biology of reproduction**, v. 72, n. 3, p. 510-515, 2005.

TUCKER, K. L. Methylated cytosine and the brain: a new base for neuroscience. **Neuron**, v. 30, p. 649-652, 2001.

VERMA, G. *et al*. Handmade cloning: recent advances, potential and pitfalls. **Journal of animal science and biotechnology**, v. 6, n. 1, p. 43, 2015.

VICHERA, G.; OLIVERA, R.; SALAMONE, D. Oocyte genome cloning used in biparental bovine embryo reconstruction. **Zygote**, v. 21, p. 21-29, 2013.

WAKAYAMA, T. *et al*. Full-term development of mice from enucleated oocytes injected with cuemulus cell nuclei. **Nature**, v. 394, n. 6691, p. 369-374, 1998.

WEI, Y*. et al.* Unfaithful maintenance of methylation imprints due to loss of maternal nuclear Dnmt1 during somatic cell nuclear transfer**. PLoS One**, v. 6, p. e20154, 2011.

WELLS, D. N. *et al*. The health of somatic cell cloned cattle and their offspring. **Cloning & Stem Cells**, v. 6, n. 2, p. 101-110, 2004.

WELLS, D. N.; OBACK, B.; LAIBLE, G. Cloning livestock: a return to embryonic cells. **Trends Biotechnology,** v. 21, p. 428-432, 2003.

WILLADSEN, S. M. *et al*. The viability of late morulae and blastocysts produced by nuclear transplantation in cattle. **Theriogenology**, v. 35, n. 1, p. 161-170, 1991.

WILLADSEN, S. M. Nuclear transplantation in sheep embryos. **Nature**, v. 320, n. 6057, p. 63-65, 1986.

WILMUT, I. *et al*. Viable offspring derived from fetal and adult mammalian cells. **Nature**, v. 385, p. 810-813, 1997.

WOODS, G. L. *et al*. A mule cloned from fetal cells by nuclear transfer. **Science**, v. 301, n. 5636, p. 1063-1063, 2003.

WRENZYCKI, C. et al. Expression of RNA from developmentally important genes in preimplantation bovine embryos produced in TCM supplemented with BSA. **Journal of reproduction and fertility**, v. 112, n. 2, p. 387-398, 1998.

WRENZYCKI, C. *et al.* Nuclear transfer protocol affects messenger RNA expression patterns in cloned bovine blastocysts. **Biology of Reproduction**, v. 65, p. 309-317, 2001.

YOUNG, L. E. *et al*. Epigenetic change in *IGF2R* is associated with fetal overgrowth after sheep embryo culture. **Nature genetics**, v. 27, n. 2, p. 153, 2001.

6. APÊNDICES

Apêndice 1: Normas para preparação de trabalhos científicos para publicação no periódico *Reproduction*.

6.1 GUIDE FOR AUTHORS - Reproduction

General

Manuscripts should:

Be concise and clear.

Be limited to 5000 words for Research submissions. For information on other manuscript types please see the relevant section below.

Display the word count on the title *PAG*e.

Contain no more than 10 figures and tables and 60 references as recommended by the journal.

Use double line spacing throughout (including reference list and figure legends), and contain continuous line numbering down the left-side of each *PAG*e.

Define all abbreviations when first mentioned.

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The abstract should be a single paragraph of not more than 250 words, clearly stating the objective of the study, the methods used (where applicable), and summarizing results and conclusions. Avoid abbreviations and references in this section.

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Include the source of chemicals, reagents and hormones and give the manufacturer's name and location (town, country) in parentheses.
Give the generic name, dose and route of administration for drugs.
Specify the composition of buffers, solutions and culture media.
Use SI symbols, give concentrations in mol/L and define the term % as w/v or v/v for all solutions. For international units use IU (U should be used for enzyme activity).
Specify the type of equipment (microscopes/objective lenses, cameras, detectors) used to obtain images.
Specify any image acquisition software used, and give a description of specialized techniques requiring large amounts of processing, such as confocal, deconvolution, 3D reconstructions, or surface and volume rendering.

5) Results
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7) Declaration of interest, Funding and Acknowledgements
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Actual or perceived conflicts of interest for all authors must be declared in full.
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Use footnotes to provide any additional explanatory material, cross-referenced to the column entries
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Reproduction publishes reviews on basic mechanisms, recent developments and new hypotheses in reproductive biology. Review articles are made freely available on the journal's website immediately upon publication.
The format of review articles is more fluid but should include the following:
1. Title *PAG*e
2. Abstract
3. Conclusions
4. Declaration of interest, Funding, Author contributions statements (where appropriate)
5. References,
6. Figure legends
7. Figures/ tables.

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(Supplementary Table 1)
(Supplementary Figures 1 and 2)

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State the breed or strain and source of animals, and give details of age, weight, sex and housing
Detail the procedures and anaesthetics used, including doses given

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Gene symbols should be in italics with only the first letter capitalised, e.g. Sox2

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6.2. Publicação do período Sanduíche

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Physiological and cellular requirements for successful elongation of the preimplantation conceptus and the implications for fertility in lactating dairy cows

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Abstract

Elongation of the preimplantation conceptus is a prerequisite for maternal recognition of pregnancy and implantation in ruminants. Failures in this phase of development likely contribute for the subfertility of lactating dairy cows. This review will discuss our current understanding of the physiological and cellular requirements for successful elongation of the preimplantation conceptus and their potential deficiency in subfertile lactating dairy cows. Major requirements include the priming of the endometrium by ovarian steroids, reprogramming of trophectoderm cells at the onset of elongation, and intensification of the crosstalk between elongating conceptus and endometrium. Conceptus elongation and survival in dairy cows does not seem to be affected by lactation per se but seem to be altered in subgroups of cows with endocrine, metabolic and nutritional imbalances or deficiencies. These subgroups of cows include those suffering diseases postpartum, anovular cows enrolled in synchronization programs, and cows with low concentration of circulating steroids and IGF1. Success of conceptus elongation starts long before breeding and entails optimization of health and nutrition programs, especially during the transition period, and might be extended to the supplementation of endocrine and nutritional shortages at the time of breeding. Genetic selection will eventually become more important as researchers unravel the molecular control of reproduction and develop new fertility traits focused on pregnancy survival.

Keywords: conceptus elongation, dairy cow, pregnancy loss.

Introduction

Early pregnancy losses are highly prevalent in lactating cows and lessen production efficiency in dairy herds (Ribeiro *et al*., 2012). Approximately half of the zygotes fail to survive the first 4 weeks of development, contributing meaningfully for the low average conception risk of lactating cows (Santos *et al*., 2004; Wiltbank *et al*., 2016; Ribeiro, 2018; Fig. 1). Ultimately, these losses are caused by impaired developmental competence of the zygote and/or inadequate uterine environment, which in turn are influenced by the genetics of the cow and embryo, and by health, nutritional, endocrine, environmental factors affecting ovarian and uterine biology of the cow. Although embryonic losses in the first week are substantial, failures in the peri-implantation stages of conceptus development seem to account for an important portion of pregnancy losses (Fig. 1A). It has been estimated that 39% of day 6 morulas fail to survive by day 28 of development (Ribeiro, 2018). The moderate efficiency of embryo transfer (ET) as breeding strategy for lactating cows further emphasize the significance of the peri-implantation period for pregnancy success (Table 1).

In cattle, the developing morula enters the uterus around day 4 but implantation starts only around day 20. Therefore, formation of the blastocyst, expansion and hatching from the zona pellucida, formation of ovoid conceptus, elongation and initial differentiation of trophectoderm binucleated cells must all be coordinated by uterine histotroph. Over the years, substantial research efforts have been placed to understand the biology of these events and their connection to pregnancy failures in dairy cows. Large emphasis has been given to the elongation phase of conceptus development because of its complexity and necessity for maternal recognition of pregnancy and implantation. This review summarizes our current understanding of the physiological and cellular requirements for successful elongation of the conceptus and discusses the potential contribution of impaired elongation to subfertility of lactating dairy cows.

Physiological and cellular requirements of elongation

Elongation of the preimplantation conceptus entails remarkable expansion of extraembryonic tissues along the uterine lumen in a short window of development (Betteridge, 1980; Wales and Cuneo, 1989). In cattle, elongation starts around day 14 and, within 3 days, the conceptus grows from <5 mm to approximately 250 mm in length and occupies almost the entire extension of the pregnant uterine horn. The exponential increase in tissue mass is explained mainly by rapid proliferation of trophectoderm cells (Wang *et al*., 2009). The augmented rate of proliferation is induced by driver signals and demands substantial supply of nutrients (e.g. lipids, amino acids, sugar, nucleotides) for energy expenditures and synthesis of biomass. The required signals and nutrients are provided by the uterine histotroph, whose composition is modulated by the activity of ovarian steroids and conceptus-derived molecules (Spencer *et al*., 2004). This section reviews scientific data that provide insights on the physiological and cellular events that coordinate conceptus elongation.

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7. VITA

Elvis Ticiani, filho de José Ticiani e Neide Teresinha Ticiani, nascido em 18 de agosto de 1989, em Chapecó, no Estado de Santa Catarina. Cursou as séries iniciais na Escola Isolada Municipal de Barrado do Camboim, terminando o ensino fundamental na Escola Estadual Dr. Serafin Enoss Bertazo. Cursou o técnico em agropecuária concomitantemente com ensino médio na Escola Agrotécnica Federal de Concordia. No ano de 2008, ingressou no curso de graduação em Zootecnia na Universidade Estadual de Santa Catarina – UDESC, permanecendo na mesma instituição até o final do mestrado em Ciência Animal no ano 2014, sob orientação do Professor Dr. Dimas Estrasula de Oliveira.

Em abril de 2015, ingressou no curso de Doutorado em Produção Animal, do Programa de Pós-Graduação em Zootecnia da Universidade Federal do Rio Grande do Sul – UFRGS, inicialmente sob orientação do Professor Dr. Enio Rosa Prates e posteriormente do Professor Dr. Marcelo Bertolini, com um período de doutarado sanduíche no ano de 2018 na University of Guelph no Canadá, sob orientação do Professor Dr. Eduardo de Souza Ribeiro.