Caracterização morfológica, morfométrica, de perfis metabólico e molecular de conceptos bovinos produzidos por fecundação *in vitro* e clonagem por transferência nuclear

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EDITORA IN VIVO

CARACTERIZAÇÃO MORFOLÓGICA, MORFOMÉTRICA, DE PERFIS METABÓLICO E MOLECULAR DE CONCEPTOS BOVINOS PRODUZIDOS POR FECUNDAÇÃO IN VITRO E CLONAGEM POR TRANSFERÊNCIA NUCLEAR

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2025

R696c Rodrigues, Victor Hugo Vieira. (Org.).
Caracterização morfológica, morfométrica, de perfis metabólico e molecular de conceptos bovinos produzidos por fecundação in vitro e clonagem por transferência nuclear. [livro eletrônico]. / Organizadores: Victor Hugo Vieira Rodrigues e Natália Holanda e Silva. Fortaleza: Editora In Vivo, 2025.
136 p.

Bibliografia.
ISBN: 978-65-87959-81-8
DOI: 10.47242/978-65-87959-81-8

1. Conceptos Bovinos. 2. Veterinária. I. Título. II. Organizadores.

CDD 599

Denise Marques Rodrigues – Bibliotecária – CRB-3/CE-001564/O

APRESENTAÇÃO

A reprodução bovina por meio de biotecnologias reprodutivas tem se consolidado como um campo estratégico para o avanço da ciência e para o fortalecimento da pecuária moderna. Entre essas técnicas, destacam-se a fecundação in vitro e a clonagem por transferência nuclear, ambas capazes de ampliar o potencial genético dos rebanhos e abrir caminhos para novas aplicações no melhoramento animal, na preservação de espécies e no desenvolvimento de pesquisas biomédicas.

Este livro, intitulado **"Caracterização morfológica, morfométrica, de perfis metabólico e molecular de conceptos bovinos produzidos por fecundação in vitro e clonagem por transferência nuclear"**, apresenta uma análise aprofundada sobre as características dos conceptos oriundos dessas duas metodologias. Trata-se de um esforço científico que vai além da descrição morfológica, abrangendo também aspectos funcionais e moleculares, o que confere ao estudo uma visão integrada dos processos envolvidos no desenvolvimento embrionário.

O mérito da obra está em oferecer aos leitores – sejam estudantes, pesquisadores ou profissionais da área de ciências agrárias e veterinárias – uma compreensão detalhada e atualizada das nuances que diferenciam os conceptos produzidos por técnicas de reprodução assistida. Ao mesmo tempo, o texto estimula a reflexão crítica sobre os desafios, as potencialidades e os impactos dessas tecnologias na produção animal e na ciência contemporânea.

Assim, este livro se insere como uma valiosa contribuição ao conhecimento científico, ampliando horizontes e fortalecendo a ponte entre a pesquisa de ponta e a prática aplicada

Boa Leitura!

Texto: Autores

RESUMO

A produção de embriões bovinos por fecundação *in vitro* (FIV) ou pela clonagem por transferência nuclear (TN) é uma poderosa ferramenta utilizada rotineiramente no mundo tanto em nível científico quanto comercial. No entanto, essa produção está associada ao aparecimento de anormalidades de desenvolvimento, em um conjunto de sintomas denominada Síndrome dos Bezerros Anormais (SBA). O objetivo deste estudo foi comparar as características físicas, e os perfis de expressão gênica e bioquímica de conceptos bovinos derivados de FIV ou TN no Dia 225 de gestação, período considerado o pico de crescimento absoluto do feto na gestação. Prenhezes foram estabelecidas carreando conceptos produzidos por superovulação e inseminação artificial (grupo controle *In vivo*, n = 4), FIV (grupo FIV, n = 4) ou clonagem (grupo Clone, n = 6) utilizando bovinos da raça Nelore. No Dia 225, as fêmeas foram abatidas para análises morfológicas, bioquímicas (parâmetros para funções hepáticas e renais, e do metabolismo energético, do nitrogênio, da hemoglobina e do cálcio e fósforo) e moleculares (perfis de expressão gênica para vias metabólicas, transportadores de carboidratos, componentes do sistema IGF, marcadores de função placentária, e atividade apoptótica por RT-qPCR) de fluidos e tecidos maternos e do concepto. Os dados foram analisados por ANOVA, com comparações pelo teste de Tukey e por correlação simples. A taxa de prenhez foi semelhante entre os grupos, com uma maior perda gestacional no primeiro trimestre no grupo Clone. Um padrão de crescimento bifásico foi observado no grupo Clone, com fetos menores no Dia 51 e duas vezes maiores no Dia 225 da gestação. Houve uma diferença morfológica significativa no formato, tamanho e peso dos placentônios no grupo Clone em relação ao grupo *In vivo*, com o peso total da placenta de clones sendo duas vezes maior do que nos outros grupos. Os conceptos no grupo FIV foram semelhantes ao controle *in vivo*, apenas com ligeiras mudanças nos tipos de placentônios. A maioria dos parâmetros fetais avaliados para as funções hepática e renal, do metabolismo da hemoglobina, e osmolaridade no plasma e nos fluidos amniótico e alantóico foram maiores nos clones do que nos outros grupos. Os triglicerídeos e o VLDL foram maiores nos clones do que nos demais grupos, com o HDL e LDL no plasma sendo mais baixos. Um aumento na atividade da via frutolítica foi observado em fetos clones, com transcritos para a via gliconeogênica correlacionando-se bem com os níveis de glicose e de frutose no fluido alantóico. A expressão de enzimas-chave para a via das pentoses e síntese de ácidos graxos foi associada ao tamanho do feto, e os fígados fetais em clones expressaram mais transportadores de glicose do que o grupo controle. Transcritos para enzimas frutolíticas e glicolíticas se correlacionaram com a maioria das mensurações de glicose e frutose no plasma materno, plasma fetal, e fluido alantóico. Os dados deste estudo indicaram uma diferença significativa na atividade das vias metabólicas em conceptos clones, sugerindo uma síntese ativa de glicose e do catabolismo da frutose pelo feto. As alterações morfológicas influenciaram a função e o metabolismo do concepto, comprometendo o mecanismo de restrição placentária ao crescimento fetal em gestações de clones bovinos.

PALAVRAS-CHAVE: Metabolismo, concepto, TN, FIV, bovino.

ABSTRACT

The production of bovine embryos by *in vitro* fertilization (IVF) or cloning by nuclear transfer (NT) is a powerful tool routinely used in in the world, being, however, associated with the appearance of developmental abnormalities, in a set of symptoms called Abnormal Offspring Syndrome (AOS). The aim of this study was to compare physical traits, and biochemical and metabolic profiles of bovine IVF- and NT-derived pregnancies on Day 225, a period considered the peak in absolute fetal growth in pregnancy. Pregnancies were established carrying concepti produced by superovulation and artificial insemination (*in vivo* control group, n = 4), IVF (IVF group, n = 4) or cloning (Clone group, n = 6) using Nellore cattle. On Day 225 pregnant cows were slaughtered for morphological, biochemical (parameters for liver and kidney functions, and energy, nitrogen, hemoglobin, and calcium and phosphorus metabolisms) and molecular (gene expression profiles of components of metabolic pathways, sugar transporters, IGF system, placental function, and apoptotic activity by RT-qPCR) analyses of the conceptus and maternal tissues and fluids. Data were analyzed by ANOVA, with pairwise comparisons by the Tukey test and a simple correlation test. Pregnancy outcome was similar between groups, with higher pregnancy loss during the first trimester in the Clone group. A biphasic growth pattern was observed in the Clone group, with smaller fetuses on Day 51, for 2-fold larger clone-derived concepti on Day-225 of gestation. A significant morphological difference in placentome shape, size and weight was seen in the Clone group than the *In vivo*, withthe total placenta weight in clones being 2-fold larger than the other groups. Conceptus traits in the IVF group were similar to the *In vivo* controls, with only slight changes in placentome types. Most fetal parameters evaluated for liver and kidney functions, hemoglobin metabolism, and osmolarities in plasma from the umbilical vein and artery and in the amniotic and allantoic fluids were higher in clones than the other groups. Tryglycerides and VLDL levels were higher in clones than the other groups, with plasma HDL and LDL being lower. An increase in the fructolytic pathway activity was seen in cloned fetuses than controls, with transcripts for the gluconeogenic pathway correlating well with glucose and fructose levels in the allantoic fluid. The key enzymes for the pentose phosphate pathway and fatty acid synthesis were associated with fetal size, and cloned fetal livers expressed higher glucose but fructose transportes than the control group. Sugar moieties correlated with one another across the distinct fetal fluid compartments, which were also associated with the pattern of gene expression for important metabolic enzymes. Transcripts for key fructolytic and glycolytic enzymes correlated with most measurements for glucose and fructose in the maternal plasma, fetal plasma, and allantoic fluid. These data indicated that a difference exists in activity in metabolic pathways in cloned concepti, suggesting an active glucose synthesis and in fructose catabolism by the fetus. Morphological changes influenced conceptus function and metabolism, disrupting the placental constraining mechanism on fetal growth in bovine clones during mid- to late pregnancy.

KEYWORDS: Metabolism, conceptus, NT, IVF, cattle.

LISTA DE ABREVIATURAS E SÍMBOLOS

ACACA	Acetil-CoA carboxilase alfa
AI	*Artificial insemination*
ALDOB	Aldolase B
ANCOVA	Análise de covariância
AOS	*Abnormal Offspring Syndrome*
ALP	*Alkaline phosphatase*
ALT	Alanina aminotransferase
AR	Aldose redutase
AST	Aspartato aminotransferase
ATP	Adenosina trifosfato
BAD	*BCL2-associated agonist of cell death*
BAK	*Bcl-2 homologous antagonist killer*
BAX	*Bcl-2 associated protein X*
BCL2	*B cell lymphoma protein 2*
BIK	*BCL2-interacting killer*
BNCs	Células binucleadas (*Binucleate cells*)
bPL	Lactogênio placentário bovino
bPAG-1	Glicoproteína 1 associada à gestação bovina
Ca^{+2}	Cálcio
chREBP	*carbohydrate responsive element binding protein*
CIV	Cultivo *in vitro*
CPP	Ciclo das pentoses fosfato
DAK	*Dihydroxyacetone Kinase*
DMRs	*Differentially Methylated Regions*
FASN	Ácido graxo sintetase (*Fatty acid synthase*)
Fbp	Frutose 1,6-bisfosfatase

FIV	Fecundação *in vitro*
GAP	Gliceraldeído-3-fosfato (*Glyceraldehyde 3-phosphate*)
GAPDH	Gliceraldeído 3-fosfato desidrogenase (*Glyceraldehyde 3-phosphate dehydrogenase*)
G6Pase	Glicose-6-fosfatase
G6PD	Glicose-6-fosfato desidrogenase
GGT	Gama glutamil transferase (*Gamma glutamyl transferase*)
GH	Hormônio do crescimento (*Growth Hormone*)
GLUT	Transportador de glicose (*Glucose transporter*)
HMC	*Handmade cloning*
HMGCR	3-hidroxi-3-metilglutaril-coenzima A redutase
HRK	(*Harakiri, BCL2 interacting protein*)
IA	Inseminação artificial
IGF1	*Insulin-like growth fator 1*
IGF2	*Insulin-like growth fator 2*
IGF2R	*Insulin-like growth factor 2 receptor*
IVF	*In vitro fertilization*
KHK	Frutoquinase
LDH	Lactato desidrogenase (*Lactate dehydrogenase*)
LDH A	Lactato desidrogenase A (*Lactate dehydrogenase A*)
LDH B	Lactato Desidrogenase B (*Lactate dehydrogenase B*)
LDL	Lipoproteína de baixa densidade (*Low-density lipoprotein*)
LN_2	*Liquid nitrogen*
LOS	*Large offspring syndrome*
LPK	L-piruvato kinase
MCL-1	*Myeloid cell leukemia 1*
MIV	Maturação *in vitro*

NADPH	Fosfato de dinucleótido de nicotinamida e adenina *(Nicotinamide adenine dinucleotide phosphate-oxidase)*
NEFA	*Non-esterified fatty acid*
NR13	*Anti-apoptotic protein NR13*
NT	*Nuclear transfer*
PAGs	Glicoproteínas associadas à prenhez (*Pregnancy-associated glycoproteins*)
PCR	*Polymerase Chain Reaction*
PEPCK	Fosfoenolpiruvato Carboxiquinase
PIV	Produção *In Vitro*
PL	Lactogênio placentário (*placental lactogen*)
PPP	*Pentose Phosphate Pathway*
PP2A	Proteína fosfatase 2A (*Protein phosphatase 2A*)
PSPB	Proteína B de soro da prenhez (*Pregnancy-specific protein B*)
RBC	*Red Blood Cells*
RT-qPCR	*Real Time – quantitative Polymerase Chain Reaction*
SBA	Síndrome dos Bezerros Anormais
SBAG	Síndrome do bezerro absolutamente grande
SNA	Síndrome dos Neonatos Anormais
SORD	Sorbitol desidrogenase
TG	Triglicerídeos
TN	Transferência Nuclear
TNCS	Transferência Nuclear de Célula Somática
TK	Trioquinase
TPP	*Total plasma protein*
VLDL	Lipoproteína de muito baixa densidade (*Very-low density lipoprotein*)
XIST	*X-inactive specific transcript*
Xu-5P	Xilulose-5 fosfato (*Xylulose 5-phosphate*)
WT	*Wild Type*

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SUMÁRIO

1 INTRODUÇÃO

Com o domínio das etapas de produção de embriões completamente *in vitro* (PIV) ainda na década de 80 (LU *et al*., 1988), alcançou-se um avanço científico considerável em várias áreas do conhecimento, contemplando a foliculogênese, maturação folicular e oocitária, fecundação e desenvolvimento embrionário. Tais fatos permitiram o desenvolvimento dos primeiros animais de interesse zootécnico produzidos tanto pela fecundação *in vitro* (FIV) no início dos anos 80 (bovino, BRACKETT *et al*., 1982) quanto pela transferência nuclear (TN), utilizando blastômeros de embriões ovinos em estádios iniciais de desenvolvimento há 30 anos (WILLADSEN, 1986), ou células somáticas de uma fêmea ovina adulta (Ovelha Dolly), uma década mais tarde (WILMUT *et al*., 1997).

Assim, vários grupos laboratoriais no país também desenvolveram e dominaram a tecnologia da clonagem bovina por transferência nuclear desde o início do século. Várias aplicações foram sugeridas, incluindo a clonagem reprodutiva visando à reprodução de indivíduos geneticamente superiores e de importância econômica bem como a preservação e propagação de espécies ou raças em extinção, produção de animais transgênicos e até mesmo a clonagem terapêutica, com implicações diretas na saúde humana (BERTOLINI *et al*., 2007).

Apesar dos grandes avanços no desenvolvimento dos sistemas de produção *in vitro* de embriões por FIV ou TN em várias espécies animais, algumas anormalidades de desenvolvimento acarretam consequências inesperadas e imprevisíveis (WILLADSEN *et al*., 1991; BEHBOODI *et al*., 1995; FARIN & FARIN, 1995; BERTOLINI & ANDERSON, 2002; GERGER *et al*., 2016). Denominada coletivamente de *Abnormal Offspring Syndrome*, ou AOS (FARIN *et al*., 2006) essa anormalidade envolve alterações tanto pré- quanto pós-natais. Interessantemente, perturbações que ocorrem durante os primeiros dias de desenvolvimento embrionário de conceptos de PIV podem interferir no crescimento embrionário, fetal e placentário acarretando anormalidades gestacionais, prolongamento da gestação, menor preparação fisiológica da fêmea ao parto, distorcias e o nascimento de bezerros com excesso de peso e reduzida sobrevivência pós-parto (FARIN *et al*., 2006; BERTOLINI *et al*., 2007).

A ocorrência desse conjunto de alterações da AOS parece estar intrinsecamente associada ao concepto (YOUNG *et al*. 1998) e pode variar entre embriões no mesmo cultivo, entre protocolos de FIV e TN, ou mesmo entre laboratórios (KRUIP; DEN DAAS, 1997; WRENZYCKI *et al*., 1998, 1999, 2001; FARIN *et al*., 2001; YOUNG *et al*., 2001).

Além disso, o padrão metabólico anormal identificado nos embriões de PIV interfere no desenvolvimento útero-placentário-fetal (BERTOLINI *et al*., 2004; GERGER *et al*., 2016) e

falhas na expressão gênica em embriões clones em decorrência do processo de reprogramação nuclear ineficiente poderiam ser responsáveis pela alta incidência de perdas gestacionais e anormalidades em neonatos (BOIANI *et al.*, 2003).

O entendimento dos mecanismos fisiológicos das alterações associadas à AOS seria de grande importancia para a detecção de métodos de diagnósticos que permitam prever e prevenir o aparecimento de anormalidades no curso do desenvolvimento. Estudos de caráter morfológico, fisiológico, metabólico e molecular com enfoque na placenta durante o período de maior manifestação de alterações de fenótipo (terceiro trimestre da gestação) são essenciais para a compreensão da etiologia e relação entre patologias placentárias e o efeito fisiológico no feto e no recém-nascido. Além disso, esse conhecimento será de grande importância para o desenvolvimento de protocolos mais eficientes de PIV de embriões bovinos para a redução da ocorrência da AOS, com repercussões econômicas e científicas diretas.

2 REVISÃO DE LITERATURA

2.1 CONJUNTO DE ANORMALIDADES MORFO-FUNCIONAIS E CLÍNICAS EM CLONES BOVINOS: *ABNORMAL OFFSPRING SYNDROME (AOS)*

O uso da fecundação *in vitro* (FIV) na produção *in vitro* de embriões (PIV) em ruminantes foi recebido como mais uma ferramenta para permitir uma rápida multiplicação de animais geneticamente superiores (TERVIT *et al.*, 1972). A clonagem, inicialmente por TN com blastômeros, e posteriormente por TNCS, também surgiu com a mesma promessa. Entretanto, estas biotécnicas geraram uma maior incidência de anormalidades gestacionais com reflexos pós-natal (THOMPSON *et al.*, 1995; BEHBOODI *et al.*, 1995).

Desde os anos 80 os procedimentos de PIV de embriões bovinos por FIV e clonagem por TN têm sido relacionados a alterações pré- e pós-natais, fetais e placentárias, de ordem morfológica, fisiológica, metabólica, bioquímica, molecular e clínica em conceptos e neonatos (WILLADSEN, 1991; BEHBOODI *et al.*, 1995; FARIN e FARIN, 1995; WALKER *et al.*, 1995; WILSON *et al.*, 1995; HILL *et al.*, 1999, 2000, 2001; CHAVATTE-PALMER *et al.*, 2004, 2006; BERTOLINI *et al.*, 2002a,b, 2004, 2006; FARIN *et al.*, 2006; WAKISAKA-SAITO *et al.*, 2006; FLETCHER *et al.*, 2007; BATCHELDER *et al.*, 2007a,b; MIGLINO *et al.*, 2007; EVERTS *et al.*, 2008; ZHOU *et al.*, 2008; SMITH *et al.*, 2012; GERGER *et al.*, 2016). Dentre as principais alterações, destacam-se elevadas taxas de mortalidade embrionária e/ou fetal precoce, hidropsia das membranas fetais, abortos, prolongamento da gestação, associadas ou não a falhas de

sinalização do parto, comprometimento da mamogênese que, por sua vez, parece comprometer a lactogênese, aumento da incidência de partos distócicos, peso anormal de neonatos com elevada morbidade e mortalidade hebdomadal e nas fases juvenil e adulta, malformações, perfis hormonais, bioquímicos e hematológicos alterados, entre outras.

Além das condições *in vitro* a que são submetidos os embriões PIV, sejam provenientes de FIV ou de clonagem, o cultivo *in vitro* parece acentuar as alterações de desenvolvimento (WILLADSEN *et al.*, 1991; BEHBOODI *et al.,* 1995; FARIN & FARIN, 1995; GARRY *et al.*, 1996; WALKER *et al.*, 1996; BATCHELDER *et al.*, 2001; FARIN *et al.*, 2001; BERTOLINI e ANDERSON, 2002). A presença de soro e células suporte no cultivo *in vitro* estão associados ao aparecimento de AOS em embriões de PIV, tanto de FIV quanto em embriões clonados (BERTOLINI *et al.*, 2002b; BATCHELDER *et al.*, 2005). A morfologia de embriões produzidos em cultivo *in vitro* contendo soro demonstrou uma grande quantidade de gotículas lipídicas citoplasmáticas (THOMPSON *et al.*, 1995). Além de soro, outros componentes específicos em meios de cultivo que levaram a anomalias fetais também já foram identificados. A fonte de albumina de soro de bovino também foi identificada como um contribuinte para perdas fetais precoces. A remoção de soro e cessação do co-cultivo com células somáticas resultaram em taxas de prenhezes semelhantes, mas menores taxas de prenhezes anormais (HASLER, 2000; LANE *et al.*, 2003). Estes experimentos mostraram claramente que as perturbações precoces no ambiente embrionário podem causar alterações em longo prazo no desenvolvimento fetal (LANE & GARDNER, 1994), como ilustrado na Figura 1.

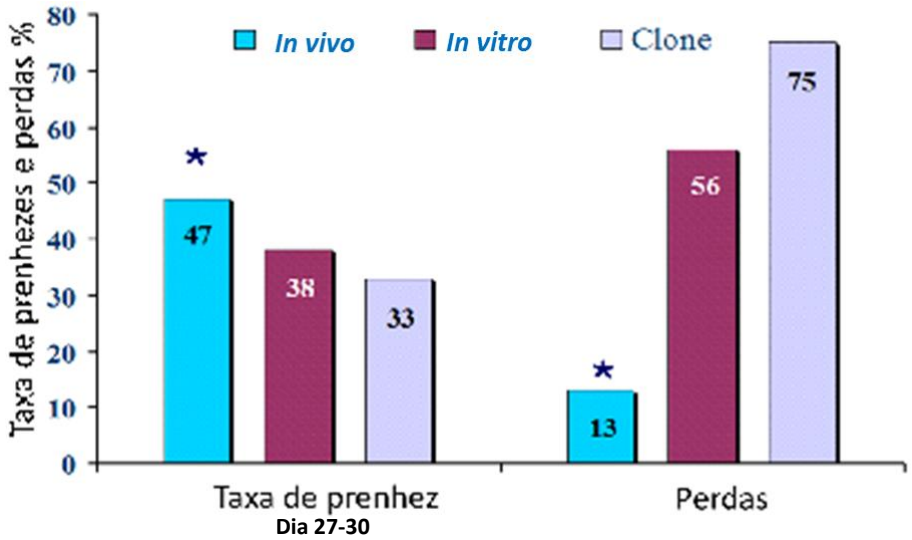

Figura 1. Taxa de prenhezes e perdas em bovinos oriundos de clonagem, *in vitro* e *in vivo* (*P<0,05) (Bertolini *et al.*, 2002b).

Primariamente, uma das anomalias mais evidentes após a PIV em bovinos por procedimentos de FIV e TN se relaciona ao nascimento de animais com peso e tamanho corporal excessivos (Figura 2). O fenômeno foi observado inicialmente em bezerros nascidos de procedimentos de transferência nuclear (WILLADSEN *et al.*, 1991; WILSON *et al.*, 1995), mas posteriormente também sendo relatado em embriões produzidos por procedimentos de FIV (BEHBOODI *et al.*, 1995; FARIN & FARIN, 1995), levando a denominação de *Large Offspring Syndrome* (LOS), ou Síndrome do Bezerro Absolutamente Grande (SBAG). Não obstante, como o peso excessivo ao nascer é apenas um de um conjunto de sintomas de múltiplos distúrbios de desenvolvimento que ocorrem durante e após a gestação, Farin *et al.* (2006) propuseram que a síndrome fosse denominada *Abnormal Offspring Syndrome* (AOS) ou Síndrome do Bezerro Anormal (SBA), por tratar-se de um fenômeno biológico mais amplo. A extensão das alterações para o aparecimento da AOS pode variar individualmente entre embriões no mesmo CIV, entre os protocolos de TN e de FIV, ou entre laboratórios (KRUIP & DEN DAAS, 1997; WRENZYCKI *et al.*, 1998, 1999, 2001; FARIN *et al.*, 2001; YOUNG *et al.*, 2001). Teoriza-se que as alterações de desenvolvimento da AOS sejam desencadeadas pelo desenvolvimento anormal da placenta em gestações de embriões PIV, o que parece estar aliado com uma alta incidência de perdas embrionárias e fetais comumente observadas entre os dias 30 e 60 de gestação (HILL *et al.*, 2000, 2001; DE SOUZA *et al.*, 2001; BERTOLINI *et al.*, 2002b; GERGER *et al.*, 2016). Esse desenvolvimento anormal da placenta e fetos com desenvolvimento retardados foram relatados no primeiro trimestre de gestação de clones bovinos (HILL *et al.*, 2000) e ovinos (DE SOUZA *et al.*, 2001) e de conceptos bovinos oriundos de FIV (BERTOLINI *et al.*, 2002ab, 2004, 2006).

Figura 2. Diferença entre os pesos de um macho bovino produzido por fecundação *in vitro* (esquerda) pesando 53 kg, e uma fêmea controle produzida por IA (direita) pesando 35 kg, ambos da raça Holandesa e nascidos no mesmo dia.

Heyman *et al.* (2002) observaram em 13% de bezerros clonados apresentando sinais compatíveis com AOS no nascimento. Além disso, no mesmo laboratório (CHAVATTE-PALMER *et al.*, 2004), 58 clones alcançaram 74,6% de sobrevivência para a primeira semana após o nascimento, e 64,4% de sobrevivência até os 6 meses de vida. Causas de morte na primeira semana foram anormalidades pulmonares (hipertensão pulmonar) ou músculo-esqueléticas (fraqueza, deformidades dos membros), atrofia do timo, alterações renais, umbigo alargado e placenta edemaciada (HILL *et al.*, 1999; CHAVATTE-PALMER *et al.*, 2004). No Japão, em um estudo de âmbito nacional, relatou-se que 69% de bezerros clones sobreviveram além das 24 h após o nascimento, e 45% sobreviveram até o dia 150, em comparação com 93% e 88% animais de um grupo controle produzido por monta natural, respectivamente (WATANABE & NAGAI, 2009).

As alterações placentárias são consideradas os principais fatores causais determinantes das perdas gestacionais de clones bovinos, estando relacionadas ao comprometimento do desenvolvimento vascular placentário, retardo no processo de implantação, modificação morfo-estrutural dos placentônios, placentomegalia e consequentemente deficiência na funcionalidade da placenta (BORDIGNON *et al.*, 1998; CONSTANT *et al.*, 2006; MIGLINO *et al.*, 2007; KOHAN-GHADR *et al.*, 2008; BAUERSACHS *et al.*, 2009; CHAVATTE-PALMER *et al.*, 2012; GERGER *et al.*, 2016), conforme ilustrado na Figura 3. Dentre as anomalias observadas destacam-se a redução no número de placentônios, com um aumento de tamanho (>15 cm) e massa tecidual (CIBELLI *et al.*, 1998; HILL *et al.*, 1999, 2000; BERTOLINI *et al.*, 2002b; GERGER *et al.*, 2016), como apresentado na Figura 4. Tal achado pode ser um mecanismo compensatório de falhas na placentação, como já descrito em bovinos em casos de interação materno-fetal inadequada, ou mesmo em ovinos após a ablação cirúrgica de carúnculas previamente ao estabelecimento de gestações (BERTOLINI *et al.*, 2002b). No entanto, um número reduzido de placentônios gigantes pode não ser necessariamente prejudicial para viabilidade fetal se a área de superfície total para troca de nutrientes permanecer dentro dos limites normais (BAZER *et al.*, 1979; HILL *et al.*, 1999). No final da gestação e a termo, placentas clones são comumente maiores ou mais pesadas do que o normal em várias espécies, incluindo bovinos e camundongos (KOHAN-GHADR *et al.*, 2008).

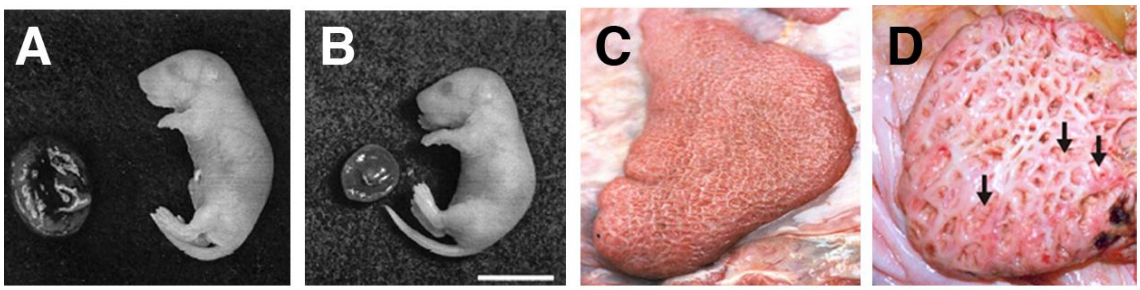

Figura 3. Placentomegalia e anormalidades placentárias após a clonagem por TNCS. Placentas e neonatos de camundongos de clonagem (A) e controle (B), a termo (OGURA *et al.*, 2002). Carúnculas de bovinos controle (C) e clonados (D), a termo (MIGLINO *et al.*, 2007).

Utilizando o modelo bovino, estudos sistemáticos de placentas de gestações bovinas obtidas a partir de embriões produzidos *in vitro* ou *in vivo*, em distintas fases de desenvolvimento, buscam testar a hipótese de que anormalidades do desenvolvimento podem ser detectadas precocemente no curso da gestação, correlacionando-as a alterações pré- e pós-natais e a características das membranas fetais e à fisiologia neonatal (BERTOLINI *et al.*, 2002a,b, 2004, 2006; BATCHELDER *et al.*, 2005, 2007ab; GERGER *et al.*, 2016). Observou-se que embriões produzidos *in vivo* e *in vitro* exibiram características morfológicas, metabólicas, endócrinas e moleculares distintas em vários períodos gestacionais, e tais alterações foram associadas a disfunções e alterações placentárias consistentes manifestadas desde fases iniciais do desenvolvimento. Essas anormalidades da placenta reduzem a viabilidade fetal e aumenta o risco de mortalidade perinatal (CHAVATTE-PALMER *et al.*, 2012).

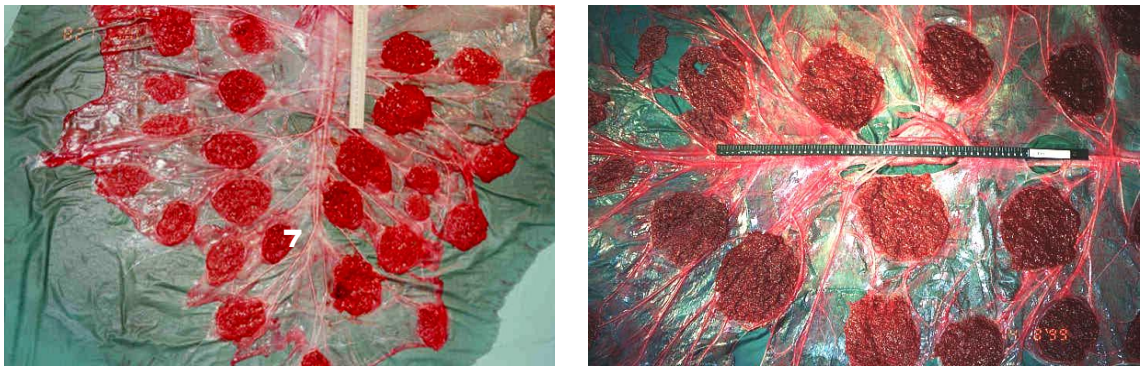

Figura 4. Membranas fetais a termo de neonato bovino produzido *in vivo* (esquerda), e neonato bovino produzido por FIV (direita), do corno fetal, na região do cordão umbilical (Bertolini *et al.*, 2002b).

As perdas gestacionais em bovinos clones ocorrem dentro dos primeiros seis meses, afetando cerca de 30% dos clones que se desenvolvem até o parto, e a baixa viabilidade dos

embriões clones é principalmente expressa pela redução da taxa de implantação, pelo aumento na taxa de mortalidade fetal e perinatal, e pelas diversas anomalias observadas nos animais recém-nascidos (BORDIGNON & SMITH, 1998).

Não obstante aos problemas pré-natais observados após manipulações embrionárias *in vitro*, uma parcela significativa dos bezerros clones que chegam a termo apresentam-se saudáveis (CHAVATTE-PALMER *et al.*, 2002). No entanto, quando comparados a animais controle, uma maior incidência de anomalias congênitas é observada mais comumente em neonatos obtidos pela clonagem por TNCS, e em menor escala em produtos de FIV, com as placentas sendo frequentemente anormais mesmo quando não há alterações morfo-fisiológicas nos fetos e neonatos *per se* (CIBELLI *et al.*, 2002; HILL & CHAVATTE-PALMER, 2002; CHAVATTE-PALMER *et al.*, 2012; GERGER *et al.*, 2016). Estas anomalias morfo-funcionais têm sido associadas a uma inadequada reprogramação epigenética do genoma doador após a produção *in vitro*, tanto após a reconstrução embrionária pela clonagem quanto pelas condições de CIV em embriões FIV e TNCS, levando a padrões de expressão gênica alterados, e incidência de anomalias no desenvolvimento subsequente (BERTOLINI *et al.*, 2007; GERGER *et al.*, 2016).

2.2 REPROGRAMAÇÃO EPIGENÉTICA EM CLONES BOVINOS

A produção de um embrião viável por transferência nuclear a partir de um núcleo de célula somática requer uma mudança extraordinária na expressão gênica na célula doadora, o que requer uma reprogramação epigenética maciça após a reconstrução embrionária. Irregularidades no desenvolvimento durante a vida embrionária, fetal e pós-natal ilustram o processo de reprogramação incompleta (HILL, 2014). Mesmo entre clones com a mesma genética, diferenças fenotípicas podem ser marcantes (WILSON *et al.*, 1995). Estudos apontam a reprogramação nuclear como uma das etapas fundamentais da biotécnica de clonagem, que quando incompleta, leva a falhas de expressão de genes importantes ao desenvolvimento, culminando com anormalidades pré- e pós-natais, contribuindo de forma significativa para a baixa eficiência da clonagem *per se* (LEE *et al.*, 2004). O processo pelo qual um genoma de uma célula diferenciada e/ou especializada readquire o potencial de desenvolvimento envolve o silenciamento de genes somáticos específicos, ativando genes embrionários essenciais, desencadeando a etapa de reprogramação epigenética (LATHAM, 2005).

A fase de pré-implantação, cujo desenvolvimento depende da expressão gênica, é caracterizada por duas fases cinéticas: 1) síntese de proteínas a partir de RNAm de origem

materna após transcrição durante a maturação oocitária, que coordena as etapas iniciais de desenvolvimento após a clivagem; e 2) ativação do genoma embrionário, que orienta a diferenciação celular e o complexo desenvolvimento posterior do concepto (RODRIGUEZ-ALVAREZ & CASTRO, 2010). Durante esta segunda fase, os embriões produzidos pela clonagem por TNCS exibem com frequência padrões anormais de expressão de genes embrionários, sugerindo uma reprogramação incompleta que resulta em uma baixa taxa de sobrevivência (HALL *et al.*, 2005). A consequente falha na reprogramação gênica afeta principalmente o desenvolvimento dos tecidos extra-embrionários e pode provocar anomalias posteriores na implantação e no desenvolvimento da placenta, levando a perdas pré-natais (HILL *et al.*, 2000; FLETCHER *et al.*, 2007).

Dentre os mecanismos de reprogramação epigenética do genoma celular em mamíferos, destaca-se a metilação do DNA como um importante regulador da transcrição gênica. Por ser um mecanismo frequentemente utilizado para silenciar e regular genes sem alterar a sequência original de DNA, este é uma das estratégias mais importantes de modificação epigenética do genoma (JAENISCH & BIRD, 2003). Desta forma, a metilação do DNA é essencial para o desenvolvimento normal, e serve para uma grande variedade de funções biológicas, estando associada a processos chave, incluindo o *imprinting* genômico.

A reprogramação da metilação do DNA parece ser fundamental para o curso de desenvolvimento embrionário normal em mamíferos, já que a remodelação da cromatina após a fecundação está intimamente relacionada a uma rápida desmetilação do genoma parental (REIK *et al.*, 2001), conforme ilustrado na Figura 5. Esta reprogramação genômica fisiológica ocorre rapidamente logo após a fecundação, com o grau de metilação do DNA diminuindo em cerca de 30% do nível médio observado em células somáticas (BIRD, 2002). Seguindo a redução na metilação do DNA durante os primeiros ciclos de divisão embrionária, uma remetilação *de novo* do genoma é iniciada no estádio de 8-células e de blastocisto, em bovinos e camundongos, respectivamente (DEAN *et al.*, 2003).

A reprogramação epigenética em células germinativas é fundamental para repor a marcação parental de *imprintin*g de genes específicos, em decorrência do processo de metilação do DNA durante a gametogênese (DUPONT *et al.*, 2009). Os padrões de expressão uniparental de genes *imprinting* são regulados por regiões diferencialmente metiladas (DMRs). No entanto, durante o processo de TNCS, a metilação aberrante das DMRs de genes *imprinted* aparece frequentemente nesses animais clones (SHEN *et al.*, 2013).

O processo de *imprinting* parece ser mais susceptível aos efeitos ambientais, com a desregulação dos *loci* de *imprinting* ou a perda de *imprinting* geralmente resultando em fenótipos anormais

(MOORE, 2001). Consequentemente, as manipulações embrionárias *in vitro*, como a FIV e a clonagem por TNCS, parecem desregular, mas não causar a perda total de *imprinting* (MOORE, 2001). A reprogramação inadequada de DMRs após a clonagem está correlacionada a anormalidades em clones como, por exemplo, a desregulação do gene *IGF2R,* de *imprinting* materno (YOUNG *et al.*, 2001). Em adição, Shen *et al.* (2013) demonstraram padrões de metilação aberrantes em genes *imprinted* (*IGF2, XIST* e *H19)*, os quais desempenham um papel crucial na regulação das funções do genoma. Tais alterações epigenéticas em fases críticas de desenvolvimento embrionário podem alterar padrões normais de diversas vias metabólicas relacionadas ao desenvolvimento do concepto.

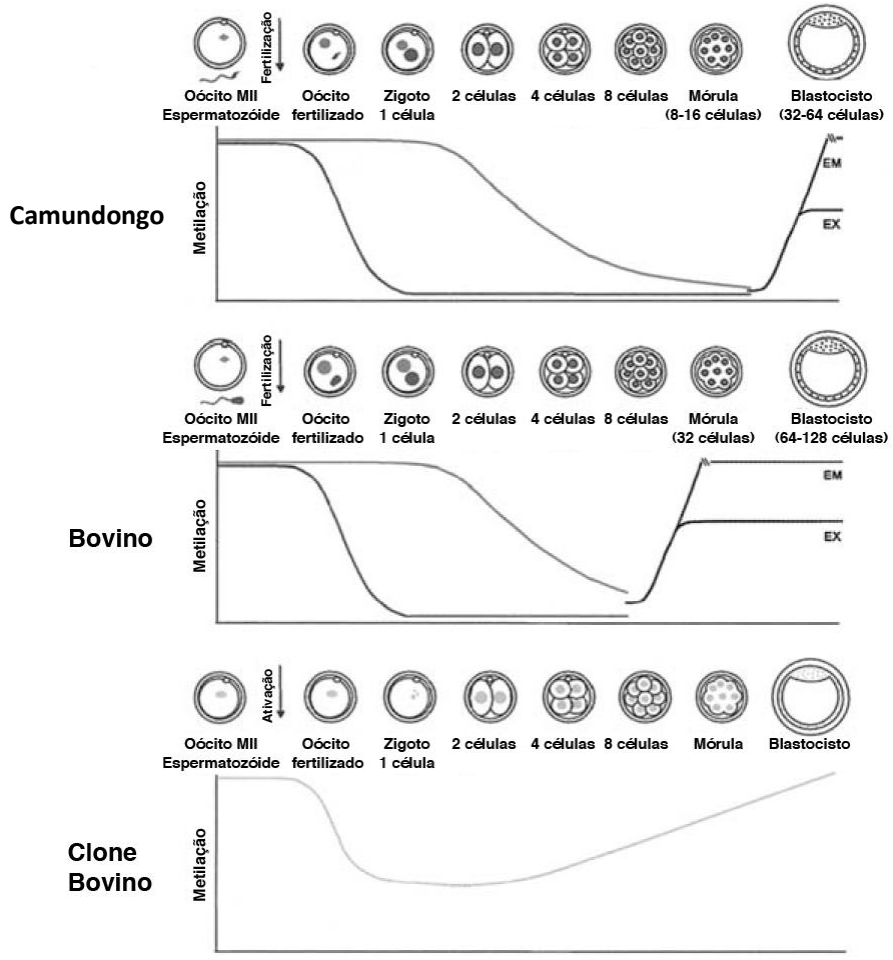

Figura 5. Reprogramação fisiológica da metilação do DNA em embriões mamíferos em estádios de pré-implantação. Desde a fecundação, os genomas paterno e materno passam por um processo de desmetilação ativa e passiva, respectivamente. A remetilação *de novo* inicia no (a) estádio de mórula no camundongo ou (b) no estádio de 8-células nos bovinos. O DNA de embriões bovinos clonados permanece altamente metilado durante o desenvolvimento precoce. Em membranas embrionárias; EX, linhagens extraembrionárias (REIK *et al.*, 2003).

Mudanças epigenéticas podem ser refletidas na expressão gênica e função anormal do tecido placentário, como observado após a clonagem por TNCS (REIK *et al.*, 2003). Sem nenhuma surpresa, as técnicas de manipulação *in vitro* de embriões em ruminantes tem sido associadas a alterações epigenéticas, especialmente em genes de caráter de *imprinting* (TVEDEN-NYBORG *et al.,* 2008), provocando problemas na placentação oriundos de perfis moleculares aberrantes nos tecidos extraembrionários e endometrial, e alterações de diversas funções fisiológicas do concepto no curso do desenvolvimento (ZHOU *et al.*, 2014). Provavelmente, as aberrações no *imprinting* genômico em conceptos originados pelas técnicas de TNCS ou FIV são provocadas durante o processo de reprogramação e/ou manutenção epigenética influenciadas pela reconstrução embrionária na clonagem e condições de cultivo *in vitro* (BERTOLINI *et al.*, 2002ab; BLELLOCH *et al.*, 2006; EVERTS *et al.*, 2008; HERATH *et al.*, 2006; HOCHEDLINGER & JAENISCH, 2006; OISHI *et al.* 2006; GERGER *et al.*, 2016). Everts *et al.* (2008) relataram que 94% dos genes expressos em placentas de clones saudáveis apresentaram perfis de expressão normal quando comparado com placentas de conceptos com hidropsia das membranas fetais. A maioria das falhas nas prenhezes parece ter sido resultado do desenvolvimento anormal da placenta, tais como vascularização reduzida e redução no número de placentônios, porém com tamanho aumentado (DE SOUSA *et al.*, 2001; CHAVATTE-PALMER *et al.*, 2002; HASHIZUME *et al.*, 2002; CHAVATTE-PALMER *et al.*, 2012). Aparentemente, para a sobrevivência fetal, deve-se compensar os defeitos de desenvolvimento causados por erros de reprogramação que levam a aberrações dos genes *imprinted*, e os descendentes clones também devem se adaptar a essas mudanças, o que pode resultar em retardo do crescimento inicial do feto (SMITH *et al.*, 2012), com crescimento compensatório subsequente (BERTOLINI *et al.*, 2004; GERGER *et al.*, 2016). A identificação de genes com funções moleculares importantes e vias associadas com a fisiologia e desenvolvimento da placenta e do feto podem auxiliar no aperfeiçoamento de procedimentos *in vitro* que permitam um roteiro para reduzir perdas gestacionais e anormalidades pré- e pós-natais, permitindo, assim, maiores adaptações fisiológicas do concepto após a clonagem por TNCS.

2.3 ADAPTAÇÕES FISIOLÓGICAS DURANTE A GESTAÇÃO EM CLONES BOVINOS

O desenvolvimento placentário normal é fundamental para a viabilidade e crescimento fetal normal. Condições sub-ótimas no ambiente intrauterino podem resultar em uma variedade

de respostas fetais, como o retardo do crescimento e morte fetal, bem como efeitos mais sutis, como o tamanho alterado dos órgãos (FARIN *et al.*, 2001). A placenta é um órgão endócrino ativo que produz hormônios e bioprodutos ativos, como fatores de crescimento essenciais para o desenvolvimento normal (lactogênio placentário, hormônio do crescimento placentário, entre outros).

Em animais produzidos *in vivo*, há uma incidência baixa mas esperada de perda embrionária e fetal devido a anormalidades do embrião ou placenta, alterações no ambiente uterino materno, ou interações entre vários fatores (WILMUT *et al.*, 1986). No desenvolvimento pré-natal normal, à medida que o concepto cresce, desde zigoto até o feto a termo, há um acentuado aumento de tamanho e peso, concomitantes a várias modificações morfológicas significativas (SLOSS & DUFFY, 1980). O padrão de crescimento relativo, ou seja, o aumento percentual em peso e dimensões por unidade de tempo é muito mais rápido até o segundo trimestre de gestação, diminuindo gradualmente à medida que a gestação progride. Já no terceiro trimestre, o incremento absoluto por unidade de peso aumenta exponencialmente atingindo o máximo no final da gestação (ELLEY *et al.*, 1978; FERRELL, 1989).

A maioria dos problemas descritos em bezerros derivados de PIV de embriões ocorre ao nascimento ou no período hebdomadal imediato. Essas alterações fisiológicas observadas no final da gestação são reflexos de mudanças no período de desenvolvimento, na qual os embriões sobrevivem à fase de implantação, placentação e as primeiras fases da vida fetal (SANGLID *et al.*, 2000).

2.3.1 Alterações no concepto decorrentes de modificações placentárias

A placenta é um órgão multifuncional, diretamente responsável pela mediação e modulação do ambiente materno para o desenvolvimento normal do feto (MÍRYAN *et al.*, 2013). Trata-se de um órgão ativo, com capacidade de sintetizar e secretar proteínas e hormônios esteroides, fatores de crescimento e outras moléculas bioativas de fundamental importância para manutenção da prenhez e viabilidade do concepto (ANTHONY *et al.*, 1995; LUTHER *et al.*, 2007). A placenta de bovinos clones pode apresentar desenvolvimento caruncular insuficiente, além do desenvolvimento atípico de placentônios, tanto em forma quanto em tamanho (BERTOLINI *et al.*, 2002b, 2006; GERGER *et al.*, 2016).

O estímulo para o desenvolvimento fetal via efeitos placentários envolve mecanismos endócrinos e metabólicos, incluindo a síntese e secreção de lactogênio placentário (PL) e glicoproteínas associadas à prenhez (PAGs), os quais são produzidos pelas células binucleadas

e podem influenciar diretamente na morfologia e funcionalidade das estruturas placentárias e do crescimento fetal (BERTOLINI *et al.*, 2006). O atraso no desenvolvimento do trofoblasto durante o estágio inicial da placentação em fêmeas bovinas com gestações com conceptos clones sugere que proteínas específicas da placenta, como o PL e o PAG, sejam indicadores fundamentais para aberrações da gestação e função placentária (HASHIZUME *et al.*, 2002).

Embriões na fase pós-implantacional provenientes de FIV e TNCS apresentam desenvolvimento inicial mais lento que embriões *in vivo*, e por isso podem prejudicar o fenômeno da placentação e explicar a alta perda gestacional associada às biotécnicas (BERTOLINI *et al.*, 2002a; TVEDEN-NYBORG *et al.*, 2005). Dentre as complicações gestacionais comuns no terceiro trimestre e a termo em gestações de FIV e TNCS, constataram-se alterações em bovinos e ovinos na morfologia dos placentônios e da interface materno-fetal, número reduzido de placentônios e aumento na área interplacentomal, presença de placentônios gigantes, achatados e/ou mais delgados, edema e hidropsia das membranas fetais (hidroalantóide, hidroâmnio), espessamento do cordão umbilical, epitélio uterino achatado, densidade celular reduzida, redução da vascularização e dilatação dos vasos sanguíneos, diminuição da vascularização das vilosidades e vasculogênese, hipoplasia e perda da diferenciação do epitélio trofoblástico e hemorragia das vilosidades fetais (BERTOLINI *et al.*, 2002b, 2004, 2006; LEE *et al.*, 2004; BATCHELDER *et al.*, 2005; CONSTANT *et al.*, 2006; FLETCHER *et al.*, 2007; CHAVATTE-PALMER *et al.*, 2012; GERGER *et al.*, 2016). Uma arquitetura microvascular distinta tem sido descrita em placentas de clones bovinos, com a descrição de fusões de placentônios, extensas áreas desprovidas de placentação, e aumento do número de microcotilédones funcionais e acessórios, e presença de criptas carunculares dilatadas acomodando mais de uma vilosidade primária em placentas de clones (MIGLINO *et al.*, 2007). Em resumo, a vascularização anormal, a remodelação inadequada dos tecidos, a diferenciação e a maturação anormal do tecido placentário em gestações oriundas de PIV podem ser umas das principais causas de perdas gestacionais, desenvolvimento anormal de órgãos fetais e do controle homeostático anormal de órgãos e sistemas após o nascimento que podem comprometer a sobrevida neonatal (BERTOLINI *et al.*, 2007).

2.3.2 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-se de 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, 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 estar 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 GLUT1 (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 GLUT3 (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 GLUT1 se apresenta como um importante transportador de glicose na interface materno-placentária, enquanto o GLUT3 é considerado o transportador para o fornecimento de glicose da placenta ao feto (EHRHARDT & BELL, 1997). As expressões dos genes para o GLUT2 (SLC2A2) e o GLUT5 (SLC2A5, transportador específico para a frutose) também são reguladas ao longo do desenvolvimento, sendo que a transcrição de GLUT5 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 sintetase (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 cultivados *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, que codifica para uma proteína pró-

apoptótica. O BAX é um membro da família das proteínas BCL2, 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).

O aparecimento de características morfológicas da apoptose é acelerado pelas condições de cultivo *in vitro* e sofrem interferência positiva ou negativa pela suplementação dos meios com hormônios e fatores de crescimento (BLOCK *et al.*, 2008; JOUSAN *et al.*, 2008a). Diversas vias anti-apoptóticas podem reduzir a morte celular, com destaque ao hormônio do crescimento (GH). Este hormônio atua como fator de sobrevivência durante o cultivo *in vitro* e reduz a taxa de apoptose através da alteração dos níveis da BAX em proporção a BCL2 durante a embriogênese (KÖLLE *et al.*, 2002). Em adição, a suplementação de IGF1 ao meio de cultivo *in vitro*, um fator de crescimento normalmente estimulado pelo GH no âmbito sistêmico, reduziu o número de células apoptóticas nos embriões devido à alteração da transcrição de genes importantes, culminando com a ativação do fosfatidilinositol 3-kinase/Akt (JOUSAN *et al.*, 2008b). Outro fator que exerce atividade anti-apoptótica em embriões bovinos cultivados *in vitro* é a insulina, normalmente utilizada como suplemento nos meios de CIV, que age concomitante com a glicose, importante substrato energético para o desenvolvimento dos blastocistos, especialmente após a ativação do genoma embrionário (JIMENEZ *et al.*, 2003).

Durante o desenvolvimento placentário, a proliferação celular e morte celular por apoptose apresentam processos inversamente proporcionais, sendo relevantes para o desenvolvimento normal do concepto. Rici *et al.* (2008) detectaram uma maior taxa celular apoptótica em placentônios e regiões interplacentomais em clones bovinos, enquanto Barreto Filho e Marques Jr. (1993) explicaram a existência de um processo de eliminação de células desnecessárias, mantendo a homeostase dos tecidos, com papel funcional de maturação e não descolamento da placenta. O aumento no número de células em apoptose no final da gestação contribui para a desconexão materno-fetal da placenta, com a população de células trofoblásticas binucleadas decrescendo significativamente nas últimas semanas da gestação em bovinos (SCHLAFER *et al.*, 2000). Interessantemente, Miles *et al.* (2004) observaram uma redução ainda mais acentuada de vilosidades fetais e número de células binucleadas em placentônios bovinos de conceptos aos 222 dias de gestação oriundos de FIV (FARIN *et al.*, 1995), o que coincidiu com o período de decréscimo nas concentrações séricas maternas de

lactogênio placentário, um bioproduto das células binucleadas, observado por Bertolini *et al.* (2006) também em gestações oriundas de embriões de FIV.

2.3.3 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. 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).

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 6). 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, 1991ab; 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 estar 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).

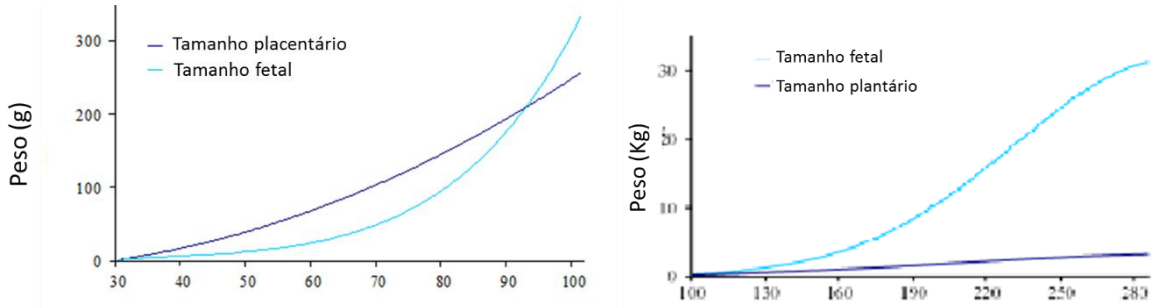

Figura 6. Taxa de crescimento absoluto do concepto bovino durante a gestação (ELEY *et al.*, 1978; PRIOR & LASTER, 1979; FERRELL, 1989, 1991; REYNOLDS *et al.*, 1990).

Como a maioria dos nutrientes transferidos através da placenta são metabolizados e/ou produzido 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 (McEVOY *et al.*, 1997; PERRY *et al.*, 1999) e do ambiente (FERRELL, 1989; KREBS *et al.*, 1997; PENNINGA & LONGO, 1998), 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 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 GLUT1 e GLUT3 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 GLUT1 são importantes para a captação de glicose e utilização placentária, enquanto que os GLUT3 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 estar 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 estar envolvido na regulação da expressão de transportadores da glicose placentária GLUT1 e GLUT3 (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 GLUT3 é elevado em placenta 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 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 frutose 1,6-bisfosfatase 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 hepática em gliceraldeído, que é fosforilado pela enzima triokinase 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 percurso 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 e 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; LI *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 (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.

Existem diferenças essenciais entre o metabolismo da glicose e frutose. As principais enzimas metabólicas reguladoras e ações hormonais, tais como insulina, controlam rigorosamente a homeostase e o metabolismo da glicose ao passo que o metabolismo de frutose, que não está sob controle hormonal, é mais imprevisível (FROESCH, 1976). A frutose ignora o passo regulador que é catalisada pela fosfofrutoquinase no fígado, passando por uma partição mais rápida do que a glicose, aumentando assim o fornecimento de substratos em todas as vias metabólicas (MAYES, 1993). Consequentemente, as concentrações excessivas de frutose no plasma pode afetar profundamente o metabolismo de carboidratos e lipídios, mesmo causando acidose láctica em determinadas condições (MAYES, 1993; VAN DEN BERGHE, 1994). Em estudo realizado em conceptos bovinos derivados *in vitro*, não foram observadas diferenças significativas nas concentrações de glicose e frutose no plasma fetal e líquido amniótico e alantóide no dia 90 de gestação, exceto para o dia 90 em conceptos PIV, que tinha concentrações plasmáticas de glicose inferiores fetais do que nos controles. No dia 180, seguindo um padrão semelhante visto pelas características físicas, foram observadas concentrações mais elevadas de frutose no plasma fetal de conceptos PIV do que com nos controles (BERTOLINI *et al.*, 2004).

2.4 EVIDÊNCIAS PRÉ- E PÓS-NATAIS EM CONCEPTOS CLONES BOVINOS

Prenhezes obtidas após transferências de embriões bovinos produzidos por TN ou FIV comumente resultam em nascimentos de bezerros grandes, um fenômeno associado a numerosas anomalias (BEHBOODI *et al.*, 1995; FARIN e FARIN, 1995). Alterações fenotípicas associadas com a clonagem com células somáticas foram descritas em detalhe por numerosos laboratórios em todo o mundo (HEYMAN *et al.*, 2002; WELLS *et al.,* 2004; BERTOLINI *et al.*, 2007; CHAVATTE-PALMER *et al.*, 2012), incluindo relatos que descrevem vários resultados clínicos observados em neonatos clones de ambos *Bos taurus* (BRISVILLE *et al.*, 2011; BUCZINSKI *et al.*, 2011; KOHAN-GHADR *et al.*, 2011) e bovinos *Bos indicus* (MEIRELLES *et al.*, 2010). Além de anomalias frequentes neo- e pós-natais pela clonagem

bovina, a TNCS é associada a uma alta incidência de perda gestacional (SMITH *et al.*, 2012). Problemas específicos, como o aborto, hidroalantóide, gestação longa, maior incidência de distocia e AOS, tinham sido anteriormente observados e analisados (HEYMAN *et al.*, 2002; MIGLINO *et al.*, 2007; KOHAN-GHADR *et al.*, 2008; MEIRELLES *et al.*, 2010). À medida que a incidência de patologias fetais e placentárias varia consideravelmente de acordo com o genótipo da célula doadora nuclear e os procedimentos TNCS, a baixa eficiência da clonagem em bovinos pode resultar de reprogramação epigenética anormal, que conduz a expressão do gene defeituoso, placenta disfuncional e mais especificamente aos perfis alterados endócrinos (KOHAN-GHADR *et al.*, 2011).

Anomalias durante o período perinatal em 26 bezerros clones foram estudadas por Brisville *et al.* (2011). Apenas três bezerros foram considerados completamente normais e não precisaram de apoio perinatal, enquanto três bezerros foram sacrificados por causa de artrogripose grave. Os demais bezerros sofreram de algum grau de disfunção respiratória, vasos umbilicais alargados, fraco reflexo de sucção e fraqueza generalizada. Duas categorias de disfunção respiratória foram reconhecidas. Uma categoria consistiu de bezerros que sofreram de hipoxemia sem hipercapnia significativa, que foram tratados com suplementação intranasal de oxigênio. A maioria dos bezerros respondeu bem e recebeu alta após suplementação de oxigênio. A outra categoria consistiu de bezerros que sofreram de hipóxia grave associada à hipercapnia, que necessitavam de apoio por ventilação mecânica, sendo que dois destes bezerros morreram, apesar do tratamento.

Em clones, gestações prolongadas são comuns, e a prole viva ocasionalmente apresenta uma síndrome do distresse respiratório, com vários tipos de anomalias que possam comprometer a sobrevivência (CIBELLI *et al.*, 1998; HILL *et al.*, 1999). Há alguma homogeneidade na causa de morte neonatal em animais clones, com a insuficiência cardíaca esquerda e o desconforto respiratório tendo sido frequentemente documentados. Um estudo dá detalhes muito minuciosos sobre o curso clínico de 13 bezerros nascidos vivos de clonagem com células somáticas fetais (HILL *et al.*, 1999). Os 13 bezerros apresentaram sintomas clínicos variando desde normal até sintomas como a síndrome do distresse respiratório grave, hipertensão pulmonar e dilatação do ventrículo esquerdo com cardiopatia. A morte de bezerros neonatos clonados por aspiração de fluido amniótico, conforme descrito, pode ser parte da mesma síndrome de disstresse respiratório (KATO *et al.*, 1998).

Em relação à termoregulação de bovinos clonados neonatos, a temperatura retal média de clones pode ser significativamente mais elevada do que a de animais controle na primeira semana, podendo persistir até 50 dias de idade. Picos característicos da temperatura retal de 41°C ou

mais foram observados comumente em clones sem quaisquer sinais clínicos específicos de enfermidades e sem relação com a temperatura ambiente. Os animais podem não responder a qualquer tratamento anti-térmico e anti-inflamatório não esteróide, com a a temperatura podendo ser manejada por meio de ventilação e resfriamento por banhos de álcool e compressas frias. Apesar de refrigeração agressiva, picos de temperatura elevada podem durar 24 a 36 h (CHAVATTE-PALMER *et al.*, 2002).

Muitas das patologias relatadas em gestações de clones bovinos se assemelham a anormalidades relatadas tanto com mutações ou deleções de genes de *imprinting* ou a desregulação da expressão de genes de *imprinting* (CHAVATTE-PALMER *et al.*, 2012). Há a superexpressão de IGF2 em alguns casos, em camundongos com crescimento placentário excessivo e crescimento excessivo alométrico de alguns órgãos fetais (EGGENSCHWILER *et al.*, 1997). Tais órgãos são muitas vezes os mesmos quese mostram com crescimento excessivo desproporcional em fetos bovinos de TNCS (GERGER *et al.*, 2016). Não obstante, bovinos clonados com sinais clínicos e comportamentais normais podem apresentar algumas particularidades fisiológicas, como alterações na regulação da temperatura, aumento das concentrações de gordura e leptina abdominais, nas primeiras semanas após o nascimento, que não são típicos de síndromes de crescimento excessivo, devido à superexpressão de IGF2.

2.5 PERSPECTIVA DA EVOLUÇÃO TEMPORAL E ESPACIAL DOS MECANISMOS E PROCESSOS BIOLÓGICOS ASSOCIADOS À *ABNORMAL OFFSPRING SYNDROME* EM BOVINOS

Conforme já descrito, em condições fisiológicas, o período inicial da gestação é definido por um maior crescimento placentário, o que é considerado importante para o estabelecimento da placenta, o que dará suporte ao crescimento fetal no segundo e terceiro trimestres da gestação (ELEY *et al.*, 1978; PRIOR & LASTER, 1979; FERRELL, 1989; REYNOLDS *et al.*, 1990). O mecanismo placento-fetal de causa-e-efeito proposto por Bell *et al.* (1999) define que este padrão de crescimento placentário inicial repercutir-se-á em um efeito regulador do crescimento fetal no final da prenhez, quando a demanda fetal por nutrientes atinge o seu máximo (PRIOR & LASTER, 1979; REYNOLDS *et al.*, 1990). Neste conceito, o sistema fetal normalmente sofre uma restrição de crescimento no final da prenhez causada por fatores maternos e placentários, o que representa um mecanismo fisiológico homeostático que garante a sobrevivência materna, resguardando o balanço energético materno e prevenindo o excesso de crescimento fetal (FERRELL, 1991ab; GLUCKMAN *et al.*, 1992). Este conceito está

relacionado ao controle do aporte de nutrientes no terceiro trimestre da gestação (GLUCKMAN *et al.*, 1992), e o grau de restrição naquele período parece estar associado ao padrão de crescimento placentário na primeira metade da prenhez (BELL *et al.*, 1999). O alto grau de plasticidade do tecido placentário frente a condições desfavoráveis, como já descrito, podem promover alterações na placenta que modulam o crescimento placentário e fetal subseqüente. Como a maioria dos nutrientes transferidos através da placenta são metabolizados ou produzidos pela placenta (FERRELL *et al.*, 1983; FERRELL, 1989), alterações na função e metabolismo placentários podem modificar a modulação do efeito regulador após a manipulação embrionária *in vitro* pela FIV e TNCS. De fato, a ocorrência de anormalidades placentárias associadas a problemas de desenvolvimento fetal em estágios iniciais (STICE *et al.*, 1996; HILL *et al.*, 2000; BERTOLINI *et al.*, 2002b) ou tardios da gestação (HILL *et al.*, 1999, 2001; BERTOLINI *et al.*, 2002b, 2004; GERGER *et al.*, 2016) em prenhezes oriundas da PIV de embriões bovinos está correlacionada ao nascimento de bezerros com peso excessivo e menor sobrevivência pós-natal (BERTOLINI *et al.*, 2002b; BATCHELDER *et al.*, 2005, 2007ab), em uma possível desregulação do mecanismo de restrição placentária ao crescimento fetal causado por um crescimento placentário anormal durante a placentação, com comprometimento da função placentária na segunda metade da prenhez.

A placenta é virtualmente a única interface responsável pelas trocas entre os sistemas materno e fetal, exercendo a regulação do crescimento fetal por causa de seu envolvimento na regulação do suprimento de nutrientes e à produção e transporte de hormônios, substratos e outras substâncias para os sistemas fetal e materno durante a prenhez. Levando-se em consideração a teoria evolutiva correlacionando o *imprinting* genômico à placentação (HAIG & GRAHAM, 1991; MOORE & HAIG, 1991; JOHN & SURANI, 2000), e que a expressão de genes com caráter de *imprinting* parece ser mais susceptível a alterações epigenéticas pela PIV de embriões (MOORE, 2001), alterações na expressão destes genes deverão se refletir em desvios de desenvolvimento ou função placentárias (BERTOLINI *et al.*, 2002ab, 2004, 2006). De fato, a disponibilidade e transporte de certos substratos ou hormônios durante a gestação é importante para o estabelecimento de padrões de funcionamento de sistemas fisiológicos no indivíduo em desenvolvimento, um fenômeno denominado de "programação metabólica fetal" (McMILLEN & ROBINSON, 2005). Esta hipótese sugere que alterações no suprimento de substratos ao concepto resultam em modificações moleculares e celulares permanentes ou mesmo novos padrões fisiológicos de funcionamento nos órgãos e sistemas fetais com implicações na sobrevivência pós-natal.

Este fenômeno foi denominado de "Hipótese de Barker" (BARKER, 1999), onde fatores causadores de alterações no ambiente uterino podem resultar em adaptações fisiológicas no feto em desenvolvimento, os quais por sua vez podem predispor o indivíduo a problemas cardiovasculares, metabólicos e endócrinos na vida adulta, conforme evidenciado em várias espécies (GODFREY, 1998), o que parece confirmar o conceito da origem fetal de doenças na fase adulta (BERTRAM & HANSON, 2001). Interessantemente, o desenvolvimento de conceptos de FIV e de TNCS já foi caracterizado por um padrão bifásico de crescimento, com um período de retardo na fase que coincide com o início da placentação, em um processo que parece estar associado a uma insuficiência placentária inicial. Este processo está possivelmente diretamente ligado às elevadas perdas gestacionais que ocorrem no final da fase embrionária e no início da fase fetal. Para os casos de sobrevivência a esta restrição inicial de desenvolvimento, observa-se comumente um desvio subsequente de crescimento do tecido placentário, como que compensatório, e que restaura o tamanho do feto já no final do primeiro trimestre da prenhez. Este crescimento compensatório persiste, culminando com a ocorrência de um maior tamanho e pesos uterinos, placentários e fetais entre o segundo e terceiro trimestres da gestação, com alterações morfológicas óbvias nas placentas e com o nascimento de bezerros absolutamente grandes com menor sobrevivência pós-natal (BERTOLINI *et al.*, 2002b; 2004, 2006, 2007; BATCHELDER *et al.*, 2005, 2007ab; GERGER *et al.*, 2016).

Considerando-se a capacidade placentária em modular seu desenvolvimento e função em face a eventos ambientais, com uma repercussão no crescimento e saúde fetal e pós-natal, uma relação causa-e-efeito morfológica-metabólica pode ser estabelecida na ocorrência da AOS em conceptos derivados de FIV ou TNCS no curso do desenvolvimento pré-natal, com consequências clínicas e metabólicas pós-natais. As alterações morfológicas já bem descritas acima foram correlacionadas a um maior acúmulo de glicose e frutose no plasma e fluidos fetais em prenhezes de FIV e de TNCS, o que demonstra uma maior capacidade de aporte fetal de substratos pela placenta. Uma porção substancial da função placentária está relacionada à regulação e à transferência materno-fetal de nutrientes ao feto, com a maioria dos metabólitos transferidos através da placenta sendo metabolizados e/ou produzidos pelo tecido placentário (FERRELL, 1989), com a glicose sendo o substato de maior relevância do meio para o final da gestação. A frutose, por sua vez, é sintetizada pela placenta a partir da glicose, sendo o principal carboidrato no plasma e fluidos fetais. Um aumento no aporte ou transporte de glicose pela placenta redireciona o uso deste substrato para a síntese de frutose (MEZNARICH *et al.*, 1987; HAY, 1995; ALDORETTA & HAY, 1999).

Alterações nas taxas de transferência, utilização ou partição de nutrientes pela placenta podem comprometer a regulação da restrição placentária ao crescimento fetal no final da gestação (GLUCKMAN *et al.*, 1992). Como a massa placentária torna-se maior em prenhezes de FIV e de TNCS, uma maior utilização ou aporte de glicose parece ocorrer, reduzindo a restrição placentária e favorecendo o crescimento fetal. Em adição, bezerros de FIV e de TNCS são maiores e apresentam concentrações elevadas de frutose imediatamente após o parto, que parece estar associada ao prognóstico favorável ou não no período hebdomadal imediato. Interessantemente, o efeito não somente metabólico, mas também epigenético da frutose foi recentemente demonstrado Meng *et al.* (2016), que demonstraram em murinos que a frutose na dieta pode significativamente alterar o padrão de reprogramação epigenética do cérebro, alterando a expressão de centenas de genes, incluindo genes que podem levar a uma maior predisposição para doenças metabólicas, tais como a diabetes e distúrbios cerebrais. Esta cascata de eventos desencadeada pela frutose parece iniciar com mudanças na metilação do DNA para genes como o BGN e o FMod, um par de genes da matriz extracelular que afeta redes de genes que regulam o metabolismo celular, a comunicação celular, inflamação e sinalização neuronal, com o consumo de fructose levando a uma alteração de metilação em grande escala no hipotálamo e no hipocampo. Logo, as consequências das mudanças metabólicas e no desvio no crescimento pré-natal e sobrevida pós-natal após a PIV de embriões ainda não são completamente elucidadas.

A avaliação do perfil bioquímico e metabólico do concepto em prenhezes de embriões de FIV e de TNCS, especialmente na fase quando a restrição placentária ao crescimento fetal atinge seu pico na gestação fisiológica (Dia 225 de gestação), ainda é necessária para o melhor entendimento dos fenômenos associados aos padrões de anormalidades da AOS em bovinos visando a compreensão da etiologia e relação entre patologias placentárias e o efeito fisiológico no feto e no neonato. Em um estudo sistemático, este estudo comparou o padrão de desenvolvimento, as características fenotípicas, os perfis de expressão gênica do fígado materno e fetal de enzimas-chave de vias metabólicas e de moléculas bioativas, e o perfil bioquímico do plasma/soro materno e fetal entre conceptos bovinos desenvolvidos a partir de embriões produzidos *in vivo* (controles) ou *in vitro* pela FIV ou clonagem por TNCS, aos 225 dias de gestação.

3 OBJETIVOS

3.1. GERAL

Caracterizar de maneira morfológica, morfométrica, molecular e metabolicamente os conceptos bovinos produzidos por fecundação *in vitro* e clonagem por transferência nuclear.

3.2. ESPECÍFICOS

a) Caracterizar o desenvolvimento morfométrico de conceptos bovinos produzidos *in vivo*, por FIV e por TNCS, aos 225 dias de gestação.

b) Determinar o perfil bioquímico dos fluidos fetais e do plasma/soro e soro/plasma fetal e materno de prenhezes bovinas produzidas *in vivo*, por FIV e por TNCS, aos 225 dias de gestação.

c) Determinar o padrão de expressão hepático materno e fetal de genes relacionados a processos fisiológicos importantes na gestação e no desenvolvimento de conceptos bovinos em prenhezes produzidas *in vivo*, por FIV e por TNCS, aos 225 dias de gestação.

d) Correlacionar as características fenotípicas do concepto com as características fisiológicas, bioquímicas e moleculares mensuradas a partir de fluidos e tecidos oriundos de prenhezes bovinas produzidas *in vivo*, por FIV e por TNCS, aos 225 dias de gestação.

e) Contribuir para o melhor conhecimento dos achados relacionados a SBA favorecendo o aperfeiçoamento das biotécnicas utilizadas no estudo.

4 CAPÍTULO I

*MORPHOMETRIC DEVELOPMENTAL PATTERN OF BOVINE HANDMADE CLONED CONCEPTI IN LATE PREGNANCY

Artigo publicado no periódico
Reproduction, Fertility and Development

http://dx.doi.org/10.1071/RD15215
Published online: 9 March 2016
[Epub ahead of print]

*O candidato participou desta publicação dentro do tema do projeto de tese na qualidade de co-autor, de acordo com a Instrução Normativa nº 01 do PPGB-RENORBIO, de 10 de dezembro de 2015.

MORPHOMETRIC DEVELOPMENTAL PATTERN OF BOVINE HANDMADE CLONED CONCEPTI IN LATE PREGNANCY

Short summary: Cloned concepti on Day 225 of gestation were characterized by lower pregnancy rates, high gestational losses, a distinctive biphasic growth pattern, with early growth restriction followed by accelerated growth and foetal weight on Day 225, and significant morphological changes in placental mass and shape when compared with *in vivo*-derived pregnancies. Conversely, Day-225 IVF-derived concepti were widely similar to controls.

Abridged title: Bovine cloned conceptus growth in late pregnancy

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ABSTRACT

Cloning procedures often interfere with conceptus growth and life *ex utero*, in a set of symptoms known as Abnormal Offspring Syndrome (AOS). The aim of this study was to compare the developmental pattern of *in vivo*- (IVD), *in vitro* fertilization- (IVF), and handmade cloning-derived (NT-HMC) Day-225 bovine concepti, using established procedures. Pregnancy diagnosis was performed on Day 30 following blastocyst transfer on Day 7. Conceptus morphometry was assessed by ultrasonography on Day 51, and on Day 225 pregnant cows were slaughtered for morphological examination of concepti. Pregnancy outcome was similar between groups, with greater pregnancy losses in the first trimester (70.6%) and smaller foetuses on Day 51 in the NT-HMC group than the IVD (14.3%) and IVF (20.0%) controls. However, NT-HMC-derived concepti were 2-fold larger on Day-225 of gestation than controls. A higher frequency (63.5%) of placentomes larger than the largest in the IVD group was observed in the NT-HMC group, which may be relevant to placental function. Conceptus traits in the IVF group were similar to IVD controls, with only slight changes in placentome types. Morphological changes in cloned concepti likely influenced placental function and metabolism, disrupting the placental constraining mechanism on foetal growth in mid- to late pregnancy.

Keywords: Conceptus development, handmade cloning, nuclear transfer, *in vitro* fertilization, embryo transfer, placenta, abnormal offspring syndrome, cattle.

INTRODUCTION

The *in vitro* production (IVP) of bovine embryos by *in vitro* fertilization (IVF) or cloning by nuclear transfer (NT) procedures are valuable tools for scientific and commercial purposes. However, such embryo production technologies have been associated with developmental abnormalities that may compromise pre- and postnatal survival (Bertolini *et al.*, 2007). Often, IVF- or NT-derived bovine concepti may present a biphasic growth pattern, initially characterized by a period of growth retardation in early pregnancy, during the period of placentation and embryo-foetal transition, followed by accelerated development detectable in late pregnancy, manifest by enlarged and unusually abnormal placentas and foetal membranes, and heavier concepti and newborn calves with lower postnatal survival (Bertolini *et al.*, 2002a, 2004; Wells *et al.*, 2004; Constant *et al.*, 2006; Batchelder *et al.*, 2007a). Collectively, such symptoms are referred to as Abnormal Offspring Syndrome (AOS, Farin *et al.*, 2006).

Foetal growth occurs slowly during early pregnancy and exponentially during later stages (Eley *et al.*, 1978; Prior and Laster, 1979; Ferrell, 1989; Reynolds *et al.*, 1990). However, the placenta grows faster than the foetus in early pregnancy, with placental weight being greater than foetal weight during the first trimester of gestation. Then, foetal weight surpasses that of the placenta (Eley *et al.*, 1978; Prior and Laster, 1979; Ferrell, 1989; Reynolds *et al.*, 1990). This initial period of faster placental growth is thought to be important for the development of the placenta as an active organ that functions as the interface between the maternal and foetal circulations, allowing the foetus to grow exponentially during the second and third trimesters of gestation (Eley *et al.*, 1978; Prior and Laster, 1979; Ferrell, 1989; Reynolds *et al.*, 1990). However, foetal growth is normally constrained by maternal and placental factors by the end of gestation, a physiological homeorrhetic mechanism that ensures maternal survival (Ferrell, 1991a,b; Gluckman *et al.*, 1992). The concept of maternal/placental constraint to foetal growth is related to the control of nutrient supply to the foetus in late gestation (Gluckman and Liggins, 1984; Gluckman *et al.*, 1992), and the degree of constraint may be associated with the pattern of placental growth occurring during early and mid-pregnancy (Bell *et al.*, 1999). This mechanism implies that the placental growth pattern during early and mid-pregnancy imposes a significant constraining effect on foetal growth during late pregnancy (Bell *et al.*, 1999).

The excessive foetal growth pattern observed in late gestation for some IVP-derived embryos appears to be associated with an increase in the supply of energy substrates to the utero-placental-foetal tissues (Bertolini *et al.*, 2004). Interestingly, changes in conceptus development have also been related to increased glucose and fructose in foetal plasma and fluids

in IVP pregnancies (Bertolini *et al.*, 2004; Batchelder *et al.*, 2007a). As placental mass in IVP-derived concepti is larger than in *in vivo* derived (IVD) concepti, increased glucose supply and fructose synthesis may occur, disrupting the physiological placental restriction to foetal growth. The biological basis for differential growth and developmental patterns observed in some IVF- or NT-derived concepti is still poorly understood. Clones often develop placental and foetal disorders after mid-pregnancy (Constant *et al.,* 2006; Miglino *et al.*, 2007), which result in increased conceptus abnormalities and foetal mortality (Chavatte-Palmer *et al.*, 2012). Placental and foetal growth rates in cattle peak at the beginning of the third trimester of pregnancy, around Days 210 and 225 of gestation, respectively (Eley *et al.*, 1978), determining the level of placental restriction to foetal growth in late pregnancy. Therefore, evaluation of morphologic and physiologic traits at this critical gestational stage may reveal clues for early mechanisms involved in the appearance of abnormalities in IVF- or NT-derived concepti.

Thus, the aim of this study was to compare the developmental pattern and morphological traits of Day-225 bovine concepti produced by (1) NT using Handmade Cloning (NT-HMC); with (2) contempory concepti produced either *in vivo* by superovulation and embryo transfer (IVD); or (3) *in vitro* by IVF procedures. Nellore cattle animals, a breed known to have rather lower birth weights in cattle (under 30 kg; Ribeiro *et al.*, 1992), having the same genetic background were used for all embryo production systems, and the same *in vitro* culture (IVC) system was used for NT- and IVF-derived embryos. The IVC system used was selected aiming to minimize developmental deviations in the IVF group, highlighting differences related more exclusively to cloning procedures *per se* and not with IVC.

MATERIALS AND METHODS

All reagents were from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA), unless stated otherwise. Procedures involving humane treatments of animals in this study were approved by the Animal Ethics Committee of the University of São Paulo, Brazil.

Primary somatic cell culture

Primary fibroblast cell cultures were established from an ear biopsy aseptically collected from an adult Nellore female, based on Gerger *et al.* (2010). Briefly, the biopsy was diced in small pieces and placed in culture dishes containing culture medium (DMEM, Dulbecco´s Modified Eagle´s Medium, Gibco-BRL, NY, USA), supplemented with 0.22 mM

sodium pyruvate, 26.2 mM sodium bicarbonate, 100 UI/mL penicillin G, 100 μg/mL streptomycin sulphate, 0.25 μg/mL amphotericin B, and 10% Foetal Bovine Serum (FBS, Gibco-BRL). Cultures were established, maintained and expanded at 38.5ºC, saturated humidity, and 5% CO_2 in air. Batches of cultured cells up to the third passage were frozen for use as nucleus donors in cloning procedures. In brief, cells at <90% confluence were suspended by trypsinization and diluted (1 x 10^3 cells/μL) in culture medium containing 10% dimethyl sulfoxide (DMSO). Subsequently, 150 μL of the cell suspension was loaded into 0.25 mL straws, which were kept at 2 to 4ºC for 15 min, exposed to nitrogen vapour at -80 to -110ºC for 5 min, and then plunged into liquid nitrogen and stored at -196ºC. Thawing was performed 72 h prior to cloning by exposure to air for 5 s, then in a water bath at 36ºC for 30 s, followed by cell culture in a 4-well dish (Nunclon®144444, Nunc, Roskilde, Denmark), at 38.5ºC and 5% CO_2 in air, aiming a high cell confluence (>95%) about 24 h prior to use for cloning procedures (Gerger *et al.*, 2010).

In vivo-derived (IVD) embryo production by superovulation (SOV)

Eleven multiparous purebred Nellore females, from a similar genetic background, and genetically related to the cell donor female, were selected. Those females were used for *in vivo* embryo production (IVD) by superovulation (SOV) and fixed time artificial insemination using frozen semen from a Nellore bull (father of the cell donor female), based on modified procedures by Bertolini *et al.* (2002a). Briefly, a vaginal progesterone device (CIDR®, Pfizer Animal Health, São Paulo, SP, Brazil) was inserted on Day 0, along with 2 mg oestradiol benzoate IM (Estrogin®, Farmavet, Brazil). Superovulation was induced by the administration of FSH-p (Folltropin-V®, Bioniche Animal Health Inc., Canada), twice a day, 12 h apart, in decreasing doses (27, 27, 20, 20, 13, 13, 6.5, and 6.5 mg), via IM for four days, starting on Day 4. On the sixth FSH dose, 250 μg of a prostaglandin $F_{2\alpha}$ analogue (Dinoprost, Lutalyse®, Pharmacia-Upjohn Co., MI, USA) was given IM, and the progesterone insert was removed on the eighth FSH dose, followed 12 h later by 6.25 mg LH (Lutropin-V®, Bioniche Animal Health Inc, Canada), IM. Fixed-time AI was done 12 and 24 h after the LH dose. Embryo collection was by uterine flushing, on Day 16 of the protocol, or Day 7 of development, according to Bertolini *et al.* (2002a).

In vitro production (IVP) of bovine embryos

The IVP of bovine embryos by IVF and NT procedures was based on Ribeiro *et al.* (2009) and Gerger *et al.* (2010), as described below.

Oocytes from slaughterhouse ovaries as cytoplast source for cloning by NT.

Bovine ovaries were collected at three regional slaughterhouses and transported to the laboratory in saline solution at 30ºC. Follicles <8 mm were aspirated for the retrieval of *cumulus*-oocyte complexes (COCs), which were morphologically selected according to Leibfried and First (1979) and used for NT procedures, as below.

Ovum Pick Up (OPU) for IVF procedures.

Twenty-eight follicular aspirations were performed in five OPU procedures using 10 open, nonstimulated, nonsynchronized, multiparous purebred Nellore females, from a similar genetic background, and genetically related to the cell donor female, for the production of embryos by *in vitro* fertilization (IVF). The OPU procedures were based on Pieterse *et al.* (1988), modified by Seneda *et al.* (2003). Follicular contents were collected in M199H composed by TCM-199 (M2520), supplemented with 2.4 mM $NaHCO_3$, 2.0 mM sodium pyruvate, and 10% heat-inactivated Oestrus Mare Serum (EMS), 0.5 μg/mL FSH (Folltropin-V®, Bioniche Animal Health Inc., Canada), 5 μg/mL LH (Lutropin-V®, Bioniche Animal Health Inc., Canada), and 15 U/mL sodium heparin, and transported to the laboratory at 38ºC. Viable COCs, morphologically selected according to Leibried and First (1979), were used for IVF procedures, as below.

In vitro maturation (IVM).

Selected COCs were matured *in vitro* for either 17 h (COCs from slaughterhouse ovaries, for cloning) or 24 h (OPU-retrieved COCs, for IVF) in 4-well dishes containing 0.4 mL maturation medium composed of TCM-199 (M2520), 26.2 mM $NaHCO_3$, 0.2 mM sodium pyruvate, 0.5 μg/mL FSH, 5 μg/mL LH, and 10% EMS, at 38.5ºC, 5% CO_2 and saturated humidity.

In vitro Fertilization (IVF).

Sperm cells from frozen semen from the same bull used for the *in vivo* production of embryos were segregated by *swim up* by incubation for 60 min at 38.5°C in Sperm-Talp. Then, IVF was performed in 100 μL drops by the co-incubation of 10-15 OPU-derived COCs and 1 x 10^6 sperm cells/mL in IVF-TALP supplemented with 5 IU/mL sodium heparin, 0.72 μg/mL

penicillamine, 0.26 μg/mL hypotaurine and 0.4 μg/mL epinephrine, for 18 to 22 h at 38.5°C, 5% CO_2 in air and saturated humidity.

Nuclear transfer by Handmade Cloning (NT-HMC).

Following IVM, and after cumulus cell removal by pipetting, oocytes presenting the first polar body were subjected to zona pellucida removal by brief exposure to a 0.5% protease solution (P-8811) in M199H + 0.01% polyvinyl alcohol (PVA). Then, zona-free oocytes were incubated for 10 min in 5 μg/mL cytochalasin B (CCB) in M199H + 10% foetal bovine serum (FBS), and then manually bisected into groups of 2-3 in 5 μL microdrops under mineral oil using a bisecting blade (Ultra-sharp Splitting Blade, Bioniche Inc., USA). Hemi-oocytes were incubated in 10 μg/mL *bis*benzimide (Hoechst 33342) in M199H + FBS for 10 min, and then selected by UV light in an inverted epifluorescent microscope (XDY-1, China) for the presence (nucleated) or absence (enucleated) of the metaphase plate. Selected enucleated hemi-oocytes (hemi-cytoplasts) were rinsed in M199H + 10% FBS for use as cytoplasts for embryo reconstruction. Cloned embryos were reconstructed by attaching two hemi-cytoplasts and a somatic donor cell in a linear arrangement, after brief exposure of the cytoplasts to 500 μg/mL phytohaemoagglutinin-p (PHA) in M199H + 0.01% PVA. Only small, round and smooth somatic cells were selected as karyoplasts for embryo reconstruction. Reconstructed structures were subjected to membrane fusion in electrofusion medium composed of 0.3 M mannitol, 0.05 mM $CaCl_2 \cdot 2H_2O$, 0.1 mM $MgSO_4 \cdot 7H_2O$, 0.5 mM Hepes, and 0.01% PVA, in a 3.2-mm fusion chamber (BTX 453, BTX Instruments, Genetronics, CA, USA), coupled to an electrofusion apparatus (BTX Electro Cell Manipulator 200, Biotechnologies & Experimental Research Inc., San Diego, CA, USA), receiving a 15-V AC pulse for 5 s for structure alignment, followed by two 1.25-KV/cm DC fusion pulses of 20 μs. Fused structures were individually cultured in 5 μL microdrops of M199 + 10% FBS under mineral oil, at 38.5°C, 5% CO_2 and saturated humidity for 60 min, when fusion rates were assessed. Fused cloned structures, 2.3 ± 0.6 h after fusion (25 ± 1 h after the onset of IVM) and groups of zona-intact and zona-free oocytes (control groups by parthenogenesis) were chemically activated in 5 μM ionomycin in M199H + 10% FBS for 5 min, and in 2 mM 6-dimethylaminopurine (6-DMAP) for 4 h, in 5 μL microdrops of M199 + 10% FBS, under mineral oil.

In vitro culture of embryos (IVC).

For IVF-derived embryos and zona-intact parthenote control embryos, zygotes were denuded by pipetting and cultured *in vitro* in 400 μL mSOFaaci medium (Holm *et al.*, 1999)

supplemented with 0.34 mM tri-sodium citrate, 2.77 mM myo-inositol, 30 μL/mL essential amino acids (BME), 10 μL/mL nonessential amino acids (MEM), 5 μg/mL insulin, 5 μg/mL transferrin, 5 ng/mL sodium selenite (ITS-X), and 5% EMS, under mineral oil, in 4-well dishes, in the *foil bag* system (Vajta *et al.*, 1997), 5% CO_2, 5% CO_2, 90% N_2, at 38.5ºC, until Day 7 (IVF = Day 0). Zona-free parthenote and cloned embryos were cultured in microwells, in the *Well-of-the-Well* (WOW) system (Vajta *et al.*, 2000), under the same conditions as for zona-intact embryos, with cloned embryos cultured either individually (1 x 100%) or after aggregation of two embryos (2 x 100%) per microwell (Ribeiro *et al.*, 2009), resulting in 100% or 200% of the final embryo volume, respectively. For all groups, cleavage and blastocyst rates were evaluated on Days 2 and 7 of development, respectively.

Embryo transfer, pregnancy diagnosis and pregnancy monitoring by ultrasonography

Fresh Day-7 blastocysts and expanded blastocysts from the three groups (IVD, IVF and NT-HMC embryos) were transferred to crossbred (*Bos taurus* x *Bos indicus*) recipients synchronized following procedures by Bertolini *et al.* (2002a) for fixed-timed ET. For the NT-HMC group, one or two embryos, non-aggregated (1 x 100%) or aggregated (2 x 100%) at the onset of IVC, were transferred per recipient. All recipients were managed together in pasture paddocks, under similar conditions. Pregnancy and foetal gender diagnoses were done by rectal palpation and ultrasonography (Aloka™ SSD-500V, Japan) on Days 30 and 58, respectively (Bertolini *et al.*, 2002a). Pregnant recipients were then re-examined weekly by ultrasonography until Day 93 of pregnancy and by rectal palpation every two weeks until Day 225. For comparisons between groups, and to determine relationships with developmental abnormalities, growth pattern and foetal traits on Day 225 of gestation, sonograms were recorded on Day 51 for morphometric analyses of the concepti from all groups, evaluating heart beat (HB) and linear measurement of the foetal crown-rump (CRL), crown-mouth (CML), amniotic vesicle (AVL) lengths, and the width and length of placentomes surrounding the foetuses, as described by Bertolini *et al.* (2002a). Conceptus traits captured in digital images were analysed using the ImageJ software (http://imagej.nih.gov/ij/).

***Post-mortem* collection and physical analyses of Day 225 conceptus tissues**

Fourteen pregnant females carrying IVD (n=4), IVF (n=4) and NT-HMC (n=6) concepti were slaughtered on Day 225 days of gestation at a local slaughterhouse, according to procedures by Bertolini *et al.* (2004). Briefly, following solid feed restriction for 12 h, with access to water, pregnant females were stunned by captive bolt and exsanguinated. Maternal jugular

blood samples were drawn 30 min prior to and at slaughter time. The pregnant uterus was removed intact, weighed and carefully dissected at the major curvature. Amniotic and allantoic fluids were carefully collected separately, measured, and sampled. The foetus was exposed with the umbilical cord still intact. Blood samples were collected from the umbilical artery and vein by puncture of blood vessels, followed by the excision of the cord. All foetuses had detectable heartbeats at the time of collection. The foetus and the empty uterus, containing the foetal membranes and the placenta, were weighed. Foetal and maternal blood and plasma samples, and foetal fluid samples were stored at -80ºC, pending analyses.

Morphological measurements were performed in the foetuses, including the lengths of the crown-mouth and crown-rump distances, fore- and hindlegs, humerus and humeral diaphysis, femur and femoral diaphysis, heart girth, and eye orbit diameter. Foetal muscles (supraspinatus, longissimus dorsi, semitendinuous, and biceps femoris), visceral organs (heart, lungs, brain, cerebellum, kidneys, liver, spleen, pancreas, thymus, GI tract, trachea, and gonads), and endocrine glands (thyroid, adrenals, and hypophysis) were carefully dissected, weighed, and sampled in 2.5% glutaraldehyde or snap-frozen in liquid nitrogen (LN_2). Foetal white and brown adipose tissues, foetal bone marrow and lymph nodes, and maternal liver samples were also sampled, and fixed in 2.5% glutaraldehyde or snap-frozen in LN_2.

All placentomes were excised and morphologically classified as types A, B, C or D, according to Bertolini *et al.* (2006). Individual placentomes were weighed and measured (width and length) by type. The placentome surface area was calculated based on the equation for the area of an ellipse. Tissue samples from the central (core) area from placentome types were fixed in 2.5% glutaraldehyde or snap-frozen in LN_2. The empty uterus, deprived of placentomes and membranes, was weighed.

Data analyses

Overall pregnancy per embryo transfer and gestational losses were compared by the χ^2 or Fisher tests. Only data for the specimens collected on Day 225 of pregnancy are presented. Conceptus traits were analysed by ANOVA and ANCOVA, using foetal weight as covariate, with pairwise comparisons by the Tukey test, using the GLM procedure (Minitab, State College, PA, USA). Placentome type distributions were compared using the Kruskal-Wallis test, and the placentome length frequency was analysed by the Mann-Whitney test. A simple correlation test was used to evaluate the relationship between traits, and a linear regression analysis was performed between foetal measurements on Day 51 and foetal weight on Day 225 of gestation. The level of statistical significance was 5%.

RESULTS

Embryo production, and pregnancy and foetal gender diagnoses

Pregnancy per embryo transfer was similar between groups. However, pregnancy loss during the first trimester was markedly greater in the NT-HMC (70.6%) than in the other two groups (Table 1).

In vivo-produced embryos (IVD).

A total of 95 structures, with 32 (33.7%) viable embryos, were retrieved from eleven embryo flushing procedures (8.6 ± 3.9 structures and 2.9 ± 3.3 viable embryos per donor), from which, 28 embryos were transferred individually to female recipients, resulting in 13 pregnancies (46.4%) on Day 30 (Table 1). One cow aborted on Day 37 and two on Day 180 of gestation, and another was diagnosed with apparently identical twins and was removed from the experiment. From the nine remaining pregnancies, four were directed to terminal collection after slaughter on Day 225 of gestation (two male and two female foetuses), whereas five were used for a separate study (data not shown).

In vitro-produced embryos by IVF.

A total of 486 viable COCs were retrieved from 28 OPU procedures (17.4 COC per donor), in five OPU sections (97.2 ± 12.0 COC per section), resulting in 29 blastocysts (6.0%) on Day 7 of development after IVM-IVF-IVC. On Day 30, five pregnancies (22.7%) were obtained after the individual transfer of 22 IVF-derived embryos (Table 1). One cow aborted on Day 37 of gestation. The four remaining pregnancies (two males and two females) were slaughtered on Day 225 of gestation, when foetal genders diagnosed on day 58 were confirmed.
Table 1. Pregnancy per embryo transfer (P/ET) and cumulative pregnancy losses up to Day 225 of gestation after the transfer of *in vivo*- (IVD), *in vitro* fertilization- (IVF) and nuclear transfer handmade cloning- (NT-HMC) derived embryos to female recipients.

Group	ET[1]	P/ET on Day 30		Cumulative pregnancy losses[2]							
				Day 37		Day 51		Day 90		Day 225	
		n	(%)	n	(%)	n	(%)	n	(%)	n	(%)
IVD	28	13[3]	46.4[a]	1	14.3[a]	1	14.3[a]	1	14.3[a]	3	28.6[a]
IVF	22	5	22.7[a]	1	20.0[a]	1	20.0[a]	1	20.0[a]	1	20.0[a]

NT-HMC	113	34	30.1[a]	17	50.0[b]	22	64.7[b]	24	70.6[b]	27	79.4[b]
One 1 x 100%[4]	26	7	26.9	4	57.1	4	57.1	4	57.1	4[7]	57.1
Two 1 x 100%[4]	47	12	25.5	5	41.7	6	50.0	8	66.7	10[8]	83.3
One 2 x 100%[5]	23	9	39.1	5	55.5	8	88.8	8	88.8	8[9]	88.8
Two 2 x 100%[5]	17	6[6]	35.3	3	50.0	4	66.7	4	66.7	5[10]	83.3

[a,b:] Numbers with distinct superscripts in the column differ, P<0.05

[1]Total number of transfers (recipients)

[2]Based on pregnancy rates on Day 30 of gestation

[3]One twin pregnancy was removed from the study. From the nine remaining pregnancies, four were directed to terminal collections after slaughter on Day 225 of gestation, as presented in this study, and five were used for a separate study (data not shown)

[4]1 x 100%: transfer of one or two blastocysts derived from 1-cell stage cloned embryos cultured individually into each microwell (non-aggregated embryos)

[5]2 x 100%: transfer of one or two blastocysts derived from two 1-cell stage cloned embryos cultured into the same microwell (embryo aggregation or aggregated embryos)

[6]Two twin pregnancies lost on Days 35 and 196 of gestation

[7]Three remaining pregnancies directed to terminal collections on Day 225 of gestation (Clones 4, 5 and 6, as in Table 6)

[8]Two remaining pregnancies directed to terminal collections on Day 225 of gestation (Clones 1 and 3, as in Table 6)

[9]One remaining pregnancy allowed reaching term on Day 289 of gestation (live born viable female calf, weighing 39 kg at birth, which survived to adulthood)

[10]One remaining pregnancy directed to terminal collection on Day 225 of gestation (Clone 2, as in Table 6)

In vitro-produced embryos by NT-HMC and parthenogenesis.

A total of 10,408 COCs were matured *in vitro* after 20 cloning procedures, resulting in 6,073 matured oocytes (58.3%). Following manual bisection, enucleated hemi-oocytes were used for the reconstruction of 2,107 structures, from which, 1,589 (75.4%) fused and 1,532 were cultured for seven days, with 1010 as non-aggregated (1 x 100%), and 522 as aggregated (2 x 100%, 261 structures) cloned embryos. Cleavage and blastocyst rates on Days 2 and 7 of development were lower for non-aggregated (1 x 100%) NT-HMC-derived embryos (64.6%, 652/1010 and 16.0%, 162/1010, respectively), than for aggregated (2 x 100%) cloned embryos (97.2%, 242/261 and 44.1%, 115/261, respectively), and zona-intact (88.3%, 212/240 and 34.2%, 82/240, respectively), or zona-free (92.0%, 196/213 and 45.1%, 96/213, respectively)

parthenote embryos, respectively. A total of 177 cloned embryos (120 non-aggregated, 1 x 100%, and 57 aggregated, 2 x 100%) were transferred to 113 synchronous recipients (49 as single or 128 as two embryos per recipient), resulting in 34 pregnancies (30.1%) on Day 30 of gestation (Table 1). No differences were observed in pregnancy outcome on Day 30 or in cumulative gestational losses up to Days 37, 51, 90 and 225 of pregnancy after the transfer of either one or two Day-7 cloned blastocysts, derived from non-aggregated (1 x 100%) or aggregated (2 x 100%) 1-cell stage embryos on Day 1, per recipient female (Table 1). Moreover, the transfer of one *vs.* two cloned embryos per recipient, irrespective of the aggregation scheme, did not affect pregnancy rates (16/49, 32.7% *vs.* 18/64, 28.1%, respectively) or cumulative gestational losses up to Day 225 (12/16, 75.0% *vs.* 15/18, 83.3%, respectively), with only two recipients bearing twins after the transfer of two embryos per recipient (both from aggregated embryos), which aborted on Days 35 and 196 of gestation, respectively. Likewise, the transfer of non-aggregated (1 x 100%) or aggregated (2 x 100%) embryos, regardless the number of transferred embryos per recipient, resulted in similar number of pregnancies per ET (19/73, 26.0% *vs.* 15/40, 37.5%, respectively) and cumulative gestational losses up to Day 225 (14/19, 73.7% *vs.* 13/15, 86.7%, respectively). A total of 70.6% (24/34) of the NT-HMC-derived pregnancies established on Day 30 were lost in the first trimester of pregnancy, irrespective of the number of transferred embryos per recipient or aggregation scheme, with 50% (17/34) lost up to Day 37; 14.7% between Days 38 and 51 (5/34); and 5.9% between Days 52 and 73 (2/34). In the second trimester, one recipient aborted on Day 160 (2.9%) and two on Day 196 (5.9%). From the seven remaining pregnancies, six were slaughtered on Day 225 of gestation, with one allowed to go to term, delivering a viable 39-kg female cloned calf by caesarean section on Day 289 of gestation. All cloned concepti were confirmed as females.

Morphometry and morphology of bovine concepti on Days 51 and 225 of gestation

Conceptus traits on Day 51 of gestation.

Foetal traits measured on Day 51 were significantly smaller in NT-HMC than in the IVD and IVF concepti, except for heart beat and amniotic vesicle length that were similar to the IVF group (Table 2; Figure 1). Placentome measurements were similar between groups, with a trend for placentome length to be smaller in the NT-HMC group (P = 0.057) than the other groups. The IVD and IVF groups were similar for all measured traits. Heart beat was highly correlated with the other traits, especially with the crown-rump length (r = 0.809, P = 0.005)

and amniotic vesicle length (r = 0.826, P = 0.003). A positive correlation was also observed between the crown-rump length and the amniotic vesicle length (r = 0.715, P = 0.013).

Table 2. Physical traits (mean and s.d.) of *in vivo*- (IVD), IVF-, and NT-HMC-derived concepti on Day 51 of gestation

Foetal trait[1]	**IVD**	**IVF**	**NT-HMC**	**P**[2]
Heart beat (beats/min)	198.4 ± 1.5[a]	193.7 ± 3.0[a]	184.0 ± 1.5[b]	0.001
Crown-rump length (mm)	34.4 ± 1.6[a]	34.3 ± 3.2[ab]	27.2 ± 1.6[b]	0.034
Crown-mouth length (mm)	15.9 ± 0.5[a]	14.4 ± 0.9[ab]	12.7 ± 0.5[b]	0.020
Amniotic vesicle length (mm)	47.1 ± 1.6[a]	42.1 ± 3.1[ab]	34.3 ± 1.6[b]	0.002
Placentome length (mm)	10.9 ± 0.3[a]	10.5 ± 0.3[a]	8.8 ± 0.3[a]	0.057
Placentome width (mm)	3.7 ± 0.1[a]	3.3 ± 0.1[a]	3.1 ± 0.1[a]	0.156

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

[1]Trait measures based on Day-51 concepti that reached Day 225 of gestation (n=14)

[2]Probability of a Type I error

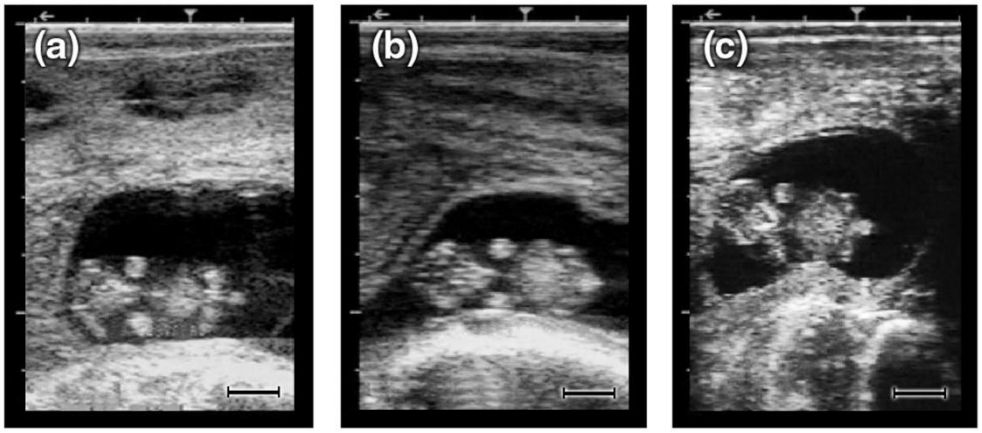

Figure 1. Sonograms of IVD (A), IVF (B), and NT-HMC-derived (C) bovine foetuses on Day-51 of gestation. Day-51 crown-rump lengths (CRL) were 36 mm, 39 mm, and 32 mm for A, B, and C, respectively, resulting in Day-225 foetuses weighing 13.0, 16.0, and 22.5 kg, respectively (scale bars = 10 mm).

Foetal traits on Day 225 of gestation.

The mean foetal weight was up to 2-fold higher in the NT-HMC group than in the IVD and IVF groups, which were similar (Table 3). Nearly half of the foetal variables (21/41) for weights and physical measures of foetal tissues and organs were different or tended to be different between the NT-HMC and the other two groups (Tables 3 and 4). Concepti from IVD and IVF groups were similar for all foetal traits, except the crown-mouth length, which was longer in the IVF group. However, when foetal weight was included as a co-variate (ANCOVA), most traits became similar among all groups (data not shown), demonstrating that the increases in sizes and weights were isometrically related to the increase in foetal growth. Nonetheless, a few differences between the NT-HMC groups and the IVD and IVF groups remained, including the liver, thoracic thymus (smaller for the IVF and NT-HMC group than the IVD controls), and the trachea length and spleen weight, which were 2-fold greater in clones than in the other two groups. The intestine weight tended to be greater in the IVD control group than the NT-HMC group ($P = 0.078$). Positive correlations ($r > 0.650$, $P < 0.05$) were observed between foetal weight and organs and tissues physical measures and weights, except for stomach, CNS, cerebellum, and hypophysis weights, and the lengths of the trachea and diaphysis.

Table 3. Weights and linear measures (mean and s.d.) associated with foetal traits in *in vivo*-(IVD), IVF- or NT-HMC-derived foetuses 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[a]	0.040
Crown-mouth length (cm)	22.0 ± 0.4[a]	24.9 ± 0.6[b]	25.3 ± 0.4[b]	0.000
Optic area (cm^2)	33.8 ± 4.5[a]	41.7 ± 6.3[a]	43.2 ± 4.0[a]	0.195
Heart girth (cm)	48.8 ± 2.4[a]	48.8 ± 3.4[a]	63.1 ± 2.2[b]	0.002
Foreleg length (cm)	58.5 ± 2.6[a]	59.0 ± 3.6[a]	61.5 ± 2.3[a]	0.605
Humerus length (cm)	13.1 ± 0.3[ab]	13.0[a] ± 0.4[a]	14.4 ± 0.3[b]	0.023
Humeral diaphisis length (cm)	7.7 ± 0.3[a]	7.5 ± 0.4[a]	7.1 ± 0.2[a]	0.318
Humerus weight (g)	87.4 ± 10.3[a]	90.5 ± 14.6[a]	149.0 ± 9.2[b]	0.001
Hindleg length (cm)	54.8 ± 1.9[a]	54.5 ± 2.6[a]	60.1 ± 1.7[a]	0.043
Femur length (cm)	15.0 ± 0.3[a]	15.3 ± 0.4[a]	16.4 ± 0.3[a]	0.138
Femoral diaphisis weight (cm)	8.2 ± 0.2[a]	9.0 ± 0.3[a]	8.9 ± 0.2[a]	0.336
Femur weight (g)	120.0 ± 15.2[a]	125.8 ± 21.6[ab]	195.6 ± 13.6[b]	0.004

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

[1]Probability of a Type I error

In general, most parameters measured by ultrasonography on Day 51 of gestation appeared to be good predictors of foetal weight on Day 225 of gestation. There were negative correlations between foetal weight (FW) on Day 225 and heart beat (HB, $r = -0.816$, $P = 0.004$), crown-rump length (CRL, $r = -0.624$, $P = 0.045$), crown-mouth length (CML, $r = -0.716$, $P = 0.013$), and amniotic vesicle length (AVL, $r = -0.617$, $P = 0.043$) on Day 51.

Table 4. Weights and linear measures (mean and s.d.) associated with visceral foetal organs and tissues in *in vivo*- (IVD), IVF- or NT-HMC-derived foetuses on Day 225 of gestation

Tissue/organ		IVD	IVF	NT-HMC	P[1]
Skeletal muscle	Supraspinatus weight (g)	35.4 ± 8.9[a]	35.2 ± 12.5[a]	77.2 ± 7.9[b]	0.003
	Longissimus dorsi	223.4 ±	143.0 ±	462.0 ±	0.027
	Semitendinous weight	40.1 ± 12.1[a]	42.1 ± 17.1[a]	113.9 ±	0.000
	Biceps femoris weight	122.4 ±	127.9 ± 44.7[a]	270.4 ±	0.013
Immune system	Spleen weight (g)	26.1 ± 3.9[a]	29.8 ± 5.5[a]	49.4 ± 3.5[b]	0.001
	Total thymus weight (g)	77.2 ± 24.2[a]	77.9 ± 34.3[a]	155.9 ±	0.019
	Cervical thymus weight	45.7 ± 16.0[a]	32.8 ± 22.6[a]	99.0 ± 14.3[b]	0.007
	Thoracic thymus weight	31.4 ± 11.9[a]	45.1 ± 16.8[a]	56.9 ± 10.6[a]	0.253
Cardio-respiratory systems	Heart weight (g)	99.1 ± 20.6[a]	100.2 ± 29.1[a]	194.5 ±	0.008
	Lung weight (g)	296.5 ±	292.8 ± 32.5[a]	392.5 ±	0.187
	Trachea weight (g)	30.4 ± 3.1[a]	33.0 ± 4.3[a]	44.8 ± 2.7[a]	0.063
	Trachea length (cm)	23.4 ± 1.2[a]	22.3 ± 1.6[a]	22.7 ± 1.0[a]	0.772
GI tract	Pancreas weight (g)	3.0 ± 2.0[a]	6.0 ± 2.4[ab]	8.7 ± 1.5[b]	0.042
	Liver weight (g)	290.3 ±	345.8 ± 82.4[a]	769.9 ±	0.001
	Oesophagus weight (g)	16.6 ± 3.5[a]	12.9 ± 3.5[a]	24.7 ± 2.2[b]	0.008
	Oesophagus length (cm)	26.6 ± 1.9[a]	28.5 ± 2.7[ab]	34.6 ± 1.7[b]	0.021
	Stomach weight[2] (g)	148.7 ±	140.4 ± 12.8[a]	173.2 ± 8.1[b]	0.025
	Total intestine weight	295.9 ±	258.1 ± 55.6[a]	419.8 ±	0.061
	Small intestine weight	207.1 ±	168.3 ± 28.4[a]	238.2 ±	0.088
	Large intestine weight	88.8 ± 28.8[a]	89.8 ± 35.3[a]	181.6 ±	0.071
Urogenital system	Kidney weight (g)	69.3 ± 25.4[a]	79.0 ± 36.0[a]	217.0 ±	0.000
	Gonads weight (g)	0.3 ± 0.1[a]	0.3 ± 0.1[a]	0.6 ± 0.0[a]	0.091
Endocrine glands	Adrenals weight (g)	1.0 ± 0.6[a]	1.4 ± 0.7[a]	3.2 ± 0.4[b]	0.005
	Thyroid weight (g)	4.0 ± 0.8[a]	5.6 ± 1.0[a]	6.1 ± 0.7[a]	0.074
	Hypophysis weight (g)	0.2 ± 0.1[a]	0.3 ± 0.1[a]	0.3 ± 0.1[a]	0.891
Nervous system	CNS weight (g)	136.0 ± 5.2[a]	136.5 ± 6.3[a]	127.9 ± 4.0[a]	0.342
	Brain weight (g)	113.4 ± 6.2[a]	108.0 ± 7.6[a]	111.4 ± 4.8[a]	0.842
	Cerebellum weight (g)	11.0 ± 0.8[a]	10.5 ± 1.6[a]	10.8 ± 0.7[a]	0.957

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

[1]Probability of a Type I error

[2]Includes rumen, reticulum, omasum and abomasum

Pregnant tract and placental traits on Day 225 of gestation.

Pregnant cows from the three groups had similar body weights, body condition scores, carcass weights and carcass yields (Table 5). However, cows in the NT-HMC group had 2- to 6-fold greater pregnant tract and empty tract weights (full and with foetus and foetal fluids

removed, respectively). Nonetheless, the uterus weight (empty uterus), when deprived of all gestational components, and after the removal of all placentomes (which contains the caruncles, a maternal component), was similar between groups, and had no significant correlation with any trait, demonstrating that differences were likely due to the conceptus *per se*.

Table 5. Weights, linear measures and volumes (mean and s.d.) associated with pregnant tracts bearing *in vivo*- (IVD), IVF- or NT-HMC-derived concepti on Day 225 of gestation

	Traits	IVD	IVF	NT-HMC	P[1]
Dam	Body weight (kg)	503.3 ± 19.6[a]	461.5 ± 27.8[a]	535.5 ± 17.6[a]	0.162
	Carcass weight (kg)	238.0 ± 12.6[a]	216.3 ± 17.8[a]	232.5 ± 11.2[a]	0.445
	Carcass yield (%)	47.2 ± 1.5[a]	47.0 ± 2.1[a]	43.5 ± 1.3[a]	0.124
	Body condition score (1	7.4 ± 0.4[a]	6.6 ± 0.6[a]	6.7 ± 0.4[a]	0.527
Pregnant tract	Pregnant tract weight	29.0 ± 8.0[a]	31.7 ± 11.3[a]	76.8 ± 7.1[b]	0.001
	Empty tract weight (kg[4])	7.5 ± 1.7[a]	7.9 ± 2.4[a]	21.8 ± 1.5[b]	0.006
	Uterus weight (kg)	3.0 ± 0.6[a]	3.0 ± 0.8[a]	4.5 ± 0.5[a]	0.073
Foetal membranes and placenta	Foetal membrane weight	0.8 ± 0.8[a]	1.8 ± 1.2[a]	4.8 ± 0.7[b]	0.002
	Total number of	81.5 ± 8.9[a]	64.8 ± 12.6[a]	79.7 ± 7.9[a]	0.496
	Foetal horn (n)	66.0 ± 3.8[a]	56.0 ± 5.4[a]	62.0 ± 3.4[a]	0.538
	Non-foetal horn (n)	31.0 ± 9.0[a]	17.5 ± 12.8[a]	26.5 ± 8.1[a]	0.594
	Total placenta weight (kg)	3.2 ± 0.5[a]	3.2 ± 0.8[a]	7.1 ± 0.5[b]	0.001
	Foetal horn (kg)	2.8 ± 0.6[a]	3.1 ± 0.9[a]	6.4 ± 0.6[b]	0.001
	Non-foetal horn (kg)	0.6 ± 0.3[a]	0.2 ± 0.4[a]	1.0 ± 0.2[a]	0.541
	Foetal weight:placentome	3.9 ± 0.2[a]	4.3 ± 0.2[a]	3.8 ± 0.2[a]	0.258
	Placenta surface area (cm	202 ± 347[ab]	112 ± 347[a]	312 ± 283[b]	0.023
Foetal fluids[3]	Total foetal fluid volume	7.8 ± 8.4[a]	8.0 ± 11.9[a]	31.0 ± 7.5[a]	0.066
	Allantoic fluid volume (L)	3.9 ± 8.0[a]	6.0 ± 11.3[a]	23.6 ± 7.2[a]	0.207
	Amniotic fluid volume	3.9 ± 2.9[a]	3.7 ± 4.2[a]	7.4 ± 2.6[a]	0.634
	Allantoic fluid:Amniotic	1.3 ± 1.3[a]	2.5 ± 1.9[a]	5.1 ± 1.2[a]	0.161

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

[1]Probability of a Type I error

[2]Score from 1 (extremely thin) to 9 (excessively fat)

[3]Values for the NT-HMC group includes data only from five concepti - see text for details

[4]Uterine tract deprived of the foetus and foetal fluids, including the uterus, foetal membranes and placenta

Placenta weight and surface area, and foetal membrane weight were larger in the NT-HMC group than the other groups (Table 5). Foetal fluid volumes and the foetal weight:placentome weight ratio were similar between groups. All parameters were similar between the IVD and the IVF group. The whole reproductive tract weight correlated positively with many foetal tissues and organs, and strong correlations existed with the total foetal fluid volume ($R = 0.908$, $P<0.001$), and the allantoic fluid volume ($R = 0.841$, $P = 0.001$), which in turn was highly correlated with the amniotic fluid volume ($R = 0.957$, $P<0.001$). Total placentome weight from the pregnant horn was highly correlated with foetal fluid volumes, weights and measures of the whole uterine tracts and most foetal measures ($R>0.800$, $P<0.05$), but especially with foetal weight ($R = 0.976$, $P<0.001$) and total placentome weights ($R>0.900$, $P<0.001$).

A systematic analysis was performed in individual placentomes, after morphological classification, as depicted in Figure 2 (types A, engulfing mushroom-like; B, sub-engulfing mushroom-like; C, flattened, nonengulfing; and D, semi-convex placentomes), considering mean numbers and weights, and frequency of occurrence by type (Figure 3). In general, a reduction in size and mass was observed from types A through D, for all groups, with a high correlation between placentome length and width ($R = 0.841$, $P = 0.0001$) or weight ($R = 0.844$, $P = 0.0001$), irrespective of the placentome type and experimental group. In the IVD group, a steady linear decrease occurred in the number and weights of placentomes from types A through D, as visualized in Figure 3. In turn, the NT-HMC group had the largest values for all three measures (weight, length, and width) within each placentome type, especially for placentome weight, with the NT-HMC group presenting the highest values for placentome weight and dimensions (length and width) within the pregnant horn, being different from the IVD and IVF groups, which were similar. Differences between groups were correlated with the pregnant horn, as in general, values for placentome length, width and weight in the non-pregnant horn were lower than in the pregnant horn, with placentome length and mean weight in the non-pregnant horn being similar in all groups. Regardless of the group, the type D had the greatest dispersion in measures between placentome types.

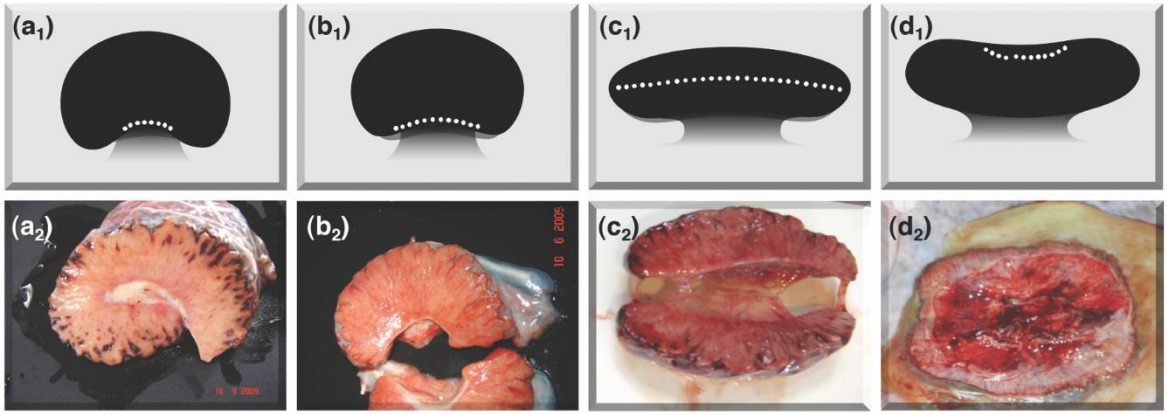

Figure 2. Bovine placentome types based on morphological characteristics on Day 225 gestation. Classification of placentomes by type according to anatomical shapes: (A) engulfing mushroom-like, (B) sub-engulfing mushroom-like, (C) flattened, nonengulfing and (D) semi-convex placentomes (Bertolini *et al.*, 2006).

A rather even distribution in terms of mean numbers and frequency of occurrence between placentomes types A (engulfing mushroom-like), B (sub-engulfing mushroom-like), and C (flattened, nonengulfing) was observed in the NT-HMC group. Even though no differences were observed in the absolute total number of placentomes between groups (Table 5), the total number of type A placentomes was lower in the IVF and NT-HMC groups than in the IVD controls (Figure 3a), with other types being similar in all groups. When the relative proportions of total placentome types were compared, similar frequencies of types B (sub-engulfing mushroom-like) and D (semi-convex) placentomes were observed between groups, but fewer type A (engulfing mushroom-like) and more type C (flattened, nonengulfing) placentomes were observed within both the IVF and NT-HMC groups compared with IVD controls (Figure 3b). These differences represented 47% and 52% decreases in the proportions of type A placentomes, and 123% and 111% increases in the proportion of type C placentomes in the IVF and NT-HMC groups, respectively, relative to the IVD group.

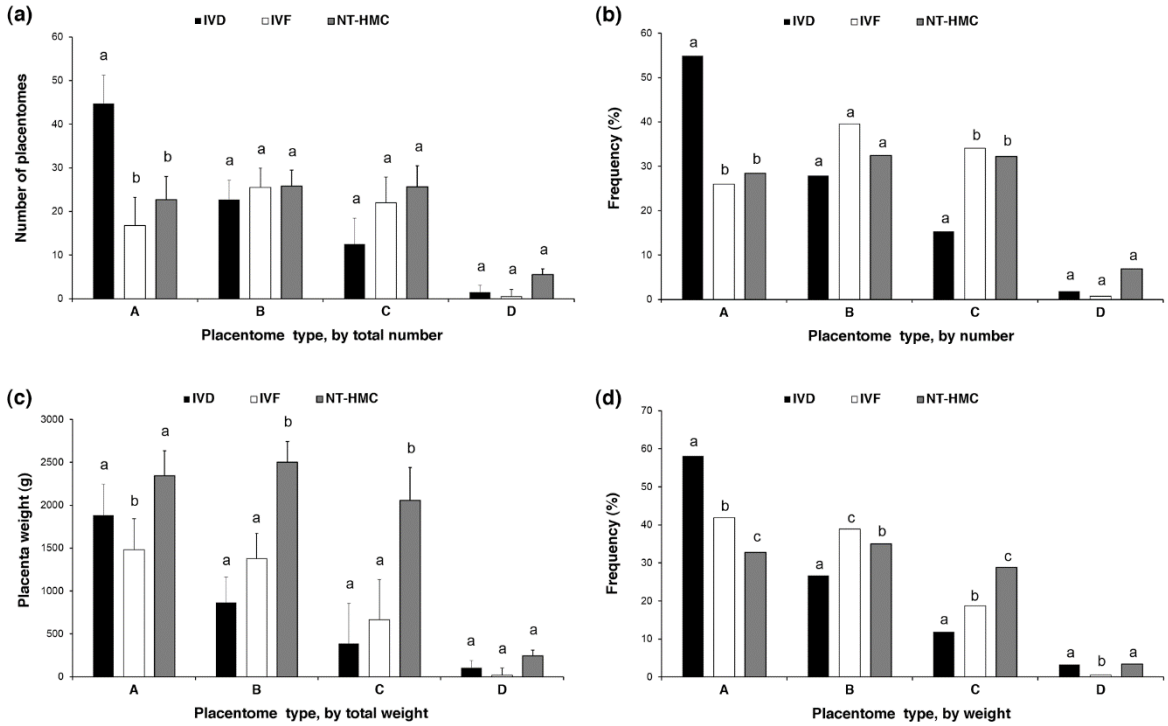

Figure 3. Absolute and relative distribution of placentome numbers and weights by type in the *in vivo*- (IVD), IVF- and NT-HMC-derived groups on Day 225 days of gestation. (a) Mean number of placentomes by type per animal. (b) Placentome relative frequency (proportion) by type per group. (c) Placentome mean weights by type per animal. (d) Placentome relative weight (proportion) by type per group (d). [a,b,c:] Columns with different superscripts within each placentome type differ, $P<0.05$.

Even though the number of type A placentomes differed, the total weight for type A placentomes was similar between the NT-HMC and the IVD control group, demonstrating an increase in size in each individual placentome in the cloned group. Types B and C placentomes were heavier in the NT-HMC group than the IVD and IVF groups (Figure 3c), due to increased weight of individual placentomes in the NT-HMC group. In fact, placentome number and placental mass per placentome type, either by mean placentome number or weight by type per animal (Figure 3a and 3c) or by the proportion of placentome number or weight by type per group (Figure 3b and 3d), demonstrate an indistinctive pattern between types A, B and C placentomes in the NT-HMC group, while in the IVD group, a positive correlation was observed between numbers and weights by type ($r = 0.981$, $P<0.001$).

The NT-HMC group had a higher frequency of giant placentomes (>13 cm length, 12.3%), as depicted in Figure 4. Indeed, the total placenta weight in clones, irrespective of the types, was 122% larger than the IVD and IVF groups. Although the total placenta weight was similar in

IVF and IVD groups, total type A (but not the other types) placentome weight was lower in the IVF group than in the IVD group (Figure 3c).

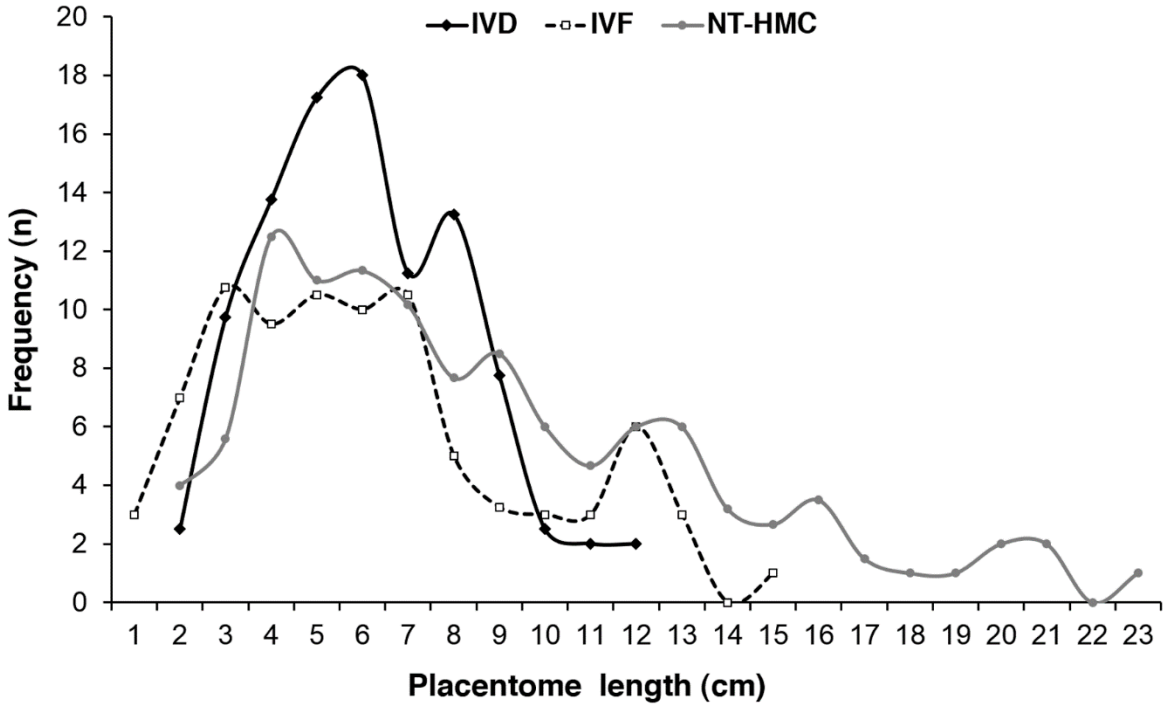

Figure 4. Frequency distributions of placentome lengths (cm) in both uterine horns in the *in vivo*- (IVD), IVF- and NT-HMC-derived groups on Day 225 days of gestation.

Morpho-pathological distinctiveness of bovine concepti on Day 225 of gestation.

Two pregnancies in each group were devoid of placentomes in the non-pregnant horn, having only foetal membranes extending in a variable fashion into the horns. One of the control pregnancies held a foetus in the uterine horn contralateral to the CL-bearing ovary. This specimen was the only one in the control group having type D placentomes (n = 6), also presenting the highest proportion of type C placentomes (23/74, 31.1%), and microplacentomes and caruncular fusions in both horns. The other three control pregnancies also had placentome fusions (at least one per animal), with one presenting more than 100 microplacentomes in the non-pregnant horn, denoting an adventitious placentation. One of the pregnancies had placental oedema, especially at the umbilical cord. Two specimens had caruncular fusions, with one showing microplacentomes in both horns. These findings demonstrate how common these events are in nature, and the wide plasticity of the placenta under physiological conditions. The most significant macroscopic pathologies were observed in NT-HMC-derived concepti, with each foetus displaying distinctive abnormalities. Table 6 summarizes the most pronounced pathological features for all six concepti in the clone group,

in chronological order of harvesting on Day 225 of gestation. Compared with IVD controls, all cloned concepti had enlarged umbilical cords, weighed 1.5- to 3.4-fold more than the mean foetal weight for controls, and displayed 12.3% giant placentomes and mild to intense oedematous foetal membranes. A positive correlation was observed between the number of giant placentomes and foetal weight ($r = 0.772$, $P < 0.05$) within the cloned group.

DISCUSSION

This experiment was part of a comprehensive study on the AOS in cattle following *in vitro* embryo manipulations, such as cloning by NT. Cloning is associated with the occurrence of a larger uterine, placental and foetal size and weight in the third trimester of pregnancy and lower survival after birth (Bertolini *et al.*, 2002a, 2004, 2006; Batchelder *et al.*, 2005, 2007ab). To minimize biological variations in this study, the same genetic background was used for the production of embryos in all experimental groups, in a *Bos taurus* var. *indicus* breed known to result in low birth weights, close to 30 kg (Ribeiro *et al.*, 1994). Most studies reporting abnormalities and AOS symptoms after cloning in cattle have used *Bos taurus* var. *taurus* genetic backgrounds (e.g., Renard *et al.*, 1999; Wells *et al.*, 1999, 2004; Hill *et al.*, 2000, 2001; Kato *et al.*, 2000; Chavatte-Palmer *et al.*, 2002, 2009, 2012; Hashizume *et al.*, 2002; Heyman *et al.*, 2002; Lee *et al.*, 2004; Constant *et al.*, 2006; Batchelder *et al.*, 2005, 2007a), with fewer reports on *Bos taurus* var. *indicus* (Miglino *et al.*, 2007; Barreto *et al.*, 2009; Meirelles *et al.*, 2010; Alberto *et al.*, 2013). Nevertheless, and on the best of our knowledge, even though no systematic comparative cloning studies have been reported between such diverse genetic backgrounds, data from the available literature as well as from our studies have demonstrated cloned *Bos taurus* var. *taurus* embryos, concepti, and calves to be comparable to cloned *Bos taurus* var. *indicus* counterparts in the wide range of manifestations of symptoms of the AOS.

Bovine embryos produced by superovulation and embryo transfer (IVD group) were used as controls for trait comparisons between groups, and as IVF procedures are also usually associated with the appearance of AOS in cattle (Bertolini *et al.*, 2002ab, 2004, 2006), IVF-derived bovine embryos were produced in an IVP system that appears to minimize subsequent phenotypical and physiological developmental abnormalities (Hasler, 2000; Farin *et al.*, 2001; Lazzari *et al.*, 2002; Sakagami *et al.*, 2014). The intent was to establish pregnancies holding IVF-derived concepti with traits that would more closely resemble IVD controls. Cloned (NT-HMC) bovine embryos were produced *in vitro* under the same IVM and IVC conditions as the IVF-derived concepti (Ribeiro *et al.*, 2009). With *in vivo*-derived (IVD) controls as standard for trait

comparisons, abnormalities observed during *in vivo* development up to Day 225 of gestation in cloned concepti reflect negative effects of the NT procedures *per se* on subsequent *in vivo* conceptus development. For most comparative analyses performed in this study, cloned concepti showed distinguished features from IVD control and IVF counterparts, which were largely similar to one another.

Table 6. Morpho-pathological distinctiveness of cloned concepti on Day 225 of gestation

Foetus	Foetal weight (kg)	Pregnant tract (kg)	Allantois (L)	Amnion (L)	Placentome number	Giant placentomes[1]		Caruncular fusions	Placental alterations	Foetal alterations
						n	(%)			
Clone 1	42.0	--[2]	--[2]	--[2]	115	12	10.4	1	Oedematous membranes	Enlarged and oedematous umbilical cord, liver abnormalities[3]
Clone 2	22.0	110.0	62.5	7.9	104	11	10.6	6	Oedematous membranes	Enlarged umbilical cord, liver abnormalities[4], misshaped head[5]
Clone 3	34.7	80.0	17.0	3.5	57	14	24.6	1	Myxoedema of the membranes[6], no placentomes in non-foetal horn	Enlarged and oedematous umbilical cord, limb contractures[7]
Clone 4	18.3	64.5	21.5	2.4	82	7	8.5	6	Myxoedema of the membranes[6], microplacentomes in non-foetal horn	Enlarged umbilical cord, liver[4] and kidney[8] abnormalities, misshaped head[5]
Clone 5	23.5	49.6	6.4	2.0	70	8	11.4	4	Oedematous membranes	Enlarged umbilical cord, limb contractures[7]
Clone 6	22.5	80.0	10.5	21.1	50	7	14.0	0	Myxoedema of the membranes[6], no placentomes in non-foetal horn, microplacentomes in both horns	Enlarged and oedematous umbilical cord

[1]Placentomes >13 cm and >250 g

[2]Not measured due to uterine rupture during tract recovery from the abdominal cavity. Data regarding the weight of the pregnant tract and volume of the foetal fluids was lost and, consequently, not included in the analyses. Such specimen had the heaviest and largest empty tract (39 kg) and foetus (42 kg) in the study. Possible hydrallantois, but as foetal fluids were lost, measurement of osmolarity in the allantoic fluid was not possible to be attained, which could be used to indicate hydrallantois (Wintour *et al.*, 1986)

[3]Superficial blood vessels in the hepatic parenchyma, well visible and pronounced in the *tunica serosa*. Oedematous *tunica serosa* in the gall bladder. A great fat deposit was observed in the mesenterium of the large intestine

[4]Friable parenchyma and presence of blood between the serosa and the parenchyma

[5]Horse-like shape with an elongation of the facial region

[6]Intense oedema of the membranes (mixoedema), with a gelatinous appearance, covering the visualization of some placentomes, especially in the umbilical region

[7]Distal flexor tendons

[8]Adhesion of the renal capsule to the parenchyma, with a rough surface. Enlarged renal pelves and ureters

Early pregnancy outcomes were similar among groups, with two out of five embryo production sessions being necessary to establish four Day-225 pregnancies in the IVD and IVF groups, whereas in the NT-HMC, a total of 20 cloning procedures were required to obtain seven Day-225 pregnancies, with one live term birth. In fact, the lower efficiency in establishing late pregnancies in the NT-HMC group than the IVD and IVF groups was mostly due to the high pregnancy loss (27/34, 79.4%) in the first trimester of gestation (24/34, 70.6%), as also reported elsewhere (Wells *et al.*, 1999; Hill *et al.*, 2000; Heyman *et al.*, 2002; Batchelder *et al.*, 2005; Panarace *et al.*, 2007). Previous studies showed that the increase or decrease in cytoplasmic embryo volume may affect further embryo development (Boiani *et al.*, 2003; Tecirlioglu *et al.*, 2005; Misica-Turner *et al.*, 2007; Ribeiro *et al.*, 2009). As in our previous study, the aggregation of Day-1 HMC-derived bovine embryos significantly improved embryo quality and developmental kinetics up to Day 7 after IVC (Ribeiro *et al.*, 2009), showing an additive effect on the embryo developmental potential for aggregated embryos (2 x 100%) when compared to non-aggregated embryos (1 x 100%), increasing cleavage (96.8% *vs.* 76.9%, respectively) and blastocyst rates (63.7% *vs.* 22.1%, respectively). Such additive effect on development has also been observed by others (Boiani *et al.*, 2003; Tecirlioglu *et al.*, 2005; Misica-Turner *et al.*, 2007), with direct beneficial effects on subsequent *in vivo* development (Boiani *et al.*, 2003; Tecirlioglu *et al.*, 2005; Misica-Turner *et al.*, 2007). Thus, given the diferences in *in vitro* development between aggregated *vs.* non-aggregated cloned embryos, we intended to boost our cloning efficiency by transferring either one or two cloned non-aggregated or aggregated embryos per recipient, seeking an increase in pregnancy rates and/or a reduction in gestational losses. However, despite diferences in *in vitro* development to the blastocyst stage between non-aggregated (1 x 100%) and aggregated (2 x 100%) embryos, neither pregnancies per embryo transfer nor cumulative gestational losses were distinct between sub-groups of cloned embryos in this study. Such findings contrast data in the mouse for SCNT cloned embryos, which seem to benefit from aggregation by compensating epigenetic defects among blastomeres (epigenetic complementation), which can be corrected or adjusted in a paracrine way between adjacent cells (Boiani *et al.*, 2003). However, our data is supported by other studies in cattle cloning , in which no distinct evidence for improvement in overall cloning efficiency has been confirmed after embryo aggegation at early stages in development after cloning (Misica-Turner *et al.*, 2007; Zhou *et al.*, 2008).

The high pregnancy loss rate, especially during the first 51 days of gestation (22/34, 64.7%), suggests a failure in the establishment and/or development of the cloned placenta.

This has been reported previously and has been shown to be associated with retarded conceptus development in early pregnancy (Bertolini *et al.*, 2002a; Chavatte-Palmer *et al.*, 2006). In addition to the higher pregnancy losses in the NT-HMC group, our results indicated significant differences in cloned conceptus development on Days 51 and 225 of gestation, demonstrated by a biphasic growth pattern, with growth retardation in early pregnancy and excessive foetal weight in late pregnancy. In fact, cloned foetuses were 1.5- to 3.4-fold heavier than control foetuses, in a pattern as previously described for bovine IVF-derived (Bertolini *et al.*, 2002a) and cloned (Chavatte-Palmer *et al.*, 2006) pregnancies. The ultrasound morphometric scanning of concepti on Day 51 of pregnancy was effective and was proven instrumental in detecting early foetal growth retardation in the NT-HMC group, as previously suggested (Bertolini *et al.*, 2002a; Chavatte-Palmer *et al.*, 2006). The growth retardation pattern is present even before the implantation of the embryo, as demonstrated with Day-16 bovine IVF embryos (Bertolini *et al.*, 2002b), and with Days-12 and 14 NT-derived porcine concepti (Martin *et al.*, 2007). The negative correlations between foetal trait measurements on Day-51 and foetal weight on Day 225 of pregnancy suggest an association between early growth retardation and accelerated foetal growth in late pregnancy. Alternatively, measurements within the expected range in the IVD and IVF groups, resulted in normal foetal weights (and size) on Day 225 of pregnancy.

In this study, we attempted to isolate the effects of the cloning procedures *per se* on conceptus development up to Day 225 of gestation, a time when the placental metabolic restriction on the foetus should be affecting foetal growth (Eley *et al.*, 1978). Cloned concepti were significantly larger than controls and IVF concepti on Day 225 of pregnancy, demonstrating that the deviation in growth was established prior to the third trimester of pregnancy. According to the placental-foetal cause-and-effect mechanism proposed by Bell *et al.* (1999), such phenomena may be related to changes in placental development in early pregnancy, that in turn appear to be linked to the early growth retardation pattern in clones. The biphasic growth pattern seen in cloned concepti may be a consequence of the loss of the physiological constraint on foetal growth in mid- to late gestation. This mechanism has been proposed to explain the control and variation in birth weights within a given species or breed (Gluckman and Liggins, 1984; Ferrell, 1991b; Gluckman *et al.*, 1992; Bell *et al.*, 1999). When early placental growth is severely retarded, the conceptus may die at the late embryonic or early foetal stages in the first trimester of pregnancy (Bertolini *et al.*, 2002a, 2007; Chavatte-Palmer *et al.*, 2006), as observed in the NT-HMC group in this study. However, if pregnancy is maintained, a compensatory and excessive placental growth pattern may occur up to the

end of the first trimester (Bertolini *et al.*, 2002a), which would impose morphological, metabolic and/or functional changes in the placental tissue, altering placental blood flow, nutrient transfer capacity or partition of substrates. Thus, early changes in cloned concepti may determine the spectrum of deviations in foetal development in the second trimester of pregnancy by the removal or attenuation of the placental constraint on foetal growth, as observed in cloned foetuses in this study. However, in rare cases, smaller-than-normal calves may also occur after cloning (Meirelles *et al.*, 2010), as we have also observed, by the birth of a 2.2-kg term cloned calf with intense growth asymmetry (Aguiar *et al.*, 2013). In humans and sheep, nutrient restriction commonly related to reduced placental development or insufficiency is associated with intrauterine growth restriction (IUGR), which may lead to a pattern of asymmetric foetal growth (Cox and Marton, 2009). For smaller-than-normal clones, the growth-restricted conceptus or placenta may fail to exhibit the compensatory growth described above, leading to placental insufficiency and IUGR.

Most of the measurable foetal traits analysed in this study (96%) were similar between the IVD and IVF groups, with few differences in placenta morphology, for which around 22% of the total measured traits were different between IVD controls and IVF-derived concepti (mostly regarding shape and size of some individual placentomes). The overall similarity between IVD and IVF concepti confirmed that the IVF procedures used in this study were effective in minimizing early effects of the IVP system on subsequent development, as reported previously (Hasler, 2000; Farin *et al.*, 2001; Lazzari *et al.*, 2002; Sakagami *et al.*, 2014). The use of undefined, complex media for the *in vitro* embryo co-culture with supportive somatic cells and 10% serum supplementation has already been intentionally and successfully used for the production of IVF-derived embryos to result in abnormal pregnancies and large calves (Bertolini *et al.*, 2002ab, 2004, 2006). In our IVF model, the use of the modified SOFaaci medium for the IVC, according to Holm *et al.* (1999), even with the supplementation with 5% serum, resulted in no evident changes in the foetal growth pattern. On the other hand, a larger number of foetal and placental traits (including the pregnant tract) in the NT-HMC group were different from the IVD (49% and 28%) and IVF (33% and 28%) groups, respectively. As cloned embryos were produced under the same conditions as IVF embryos, the use of HMC procedures may have also contributed to our findings. As oppose to standard cloning methods that use only one enucleated oocyte to receive the donor nucleus, two hemi-oocytes are fused to a donor cell in the HMC procedure, which may have unpredictable beneficial or detrimental effects on development (Tecirlioglu *et al.*, 2005; Vajta *et al.*, 2005; Schurmann *et al.*, 2006; Ribeiro *et al.*, 2009). Collectively, cell and molecular

mechanisms during cloning seem to be linked to the the subsequent differences in conceptus development and pre-and postnatal survival, as described for AOS, and are likely due to faulty epigenetic reprogramming after cloning by NT (Smith *et al.*, 2012).

On Day 225, foetuses from the NT-HMC group showed a significant increase in many organ weights and dimensions as compared with the IVD and IVF groups. Most differences followed an isometric increase in body size. However, after the ANCOVA, the thoracic thymus (smaller for the IVF and NT-HMC group than the controls), and the liver, trachea length and spleen weight, which were 2-fold heavier in clones than in the other two groups, were allometrically different. The clinical and physiological significance of these findings is uncertain, as conflicting results on the normal immune system after birth have been reported (Chavatte-Palmer *et al.*, 2009). In the neonatal period, cloned calves with thymus and spleen atrophy have been reported (Kubo, 2002; Renard *et al.*, 1999), which appeared to be associated with an increased incidence of infections in young animals (Chavatte-Palmer *et al.,* 2004), and being one possible cause for a reduction in the lower red and white blood cell counts at birth (Batchelder *et al.*, 2007b). In addition, Meirelles *et al.* (2010) reported a case of respiratory acidosis due to low blood oxygen levels associated with stenosis of the distal third part of the trachea in a newborn cloned calf. In the present study, the trachea diameter was not measured, but the reduction in trachea length in one of the NT-HMC foetus was significant when compared to other foetuses. Moreover, a few cloned foetuses displayed morphologic abnormalities, including three foetuses with liver alterations, one of which (Clone 4) also had renal malformations. Chavatte-Palmer *et al.* (2002) and Heyman *et al.* (2002) reported size changes and the presence of hepatic pathological conditions in bovine concepti and newborn clones, as well as alterations in other organs. Similarly, renal abnormalities were also observed in previous studies (Kato *et al.,* 2000; Chavatte-Palmer *et al.*, 2002) and have been suggested to be associated with foetal hydrops due to renal malfunction in *in vitro*-produced animals (Van Wagtendonk-De Leeuw *et al.*, 1998; Li *et al.*, 2005). Lee *et al.* (2004) suggested that organomegaly involving the liver, kidney and heart were related to increased incidence of hydroamnion. In fact, the presence of foetal hydrops in *in vitro*-produced animal pregnancies has been well documented in the literature (Hasler *et al.*, 1995; Cibelli *et al.*, 1998; Li *et al.*, 2005; Constant *et al.*, 2006; Miglino *et al.*, 2007) and is considered a major cause of pregnancy loss in the last third of pregnancy (Edwards *et al.*, 2003). In this study, physiological percentages of foetal fluid volume by uterine weight (20 to 25%; Sloss and Dufty, 1980) were observed in the IVD (26.9%) and IVF (25.2%) groups, but a significant increase in the proportion of foetal fluids was observed in the NT-

HMC group (40.4%). Despite the lack of differences in foetal fluid volumes between groups, the significant increase in the weight of the pregnant uterus in the NT-HMC group, along with its high correlation with the total fluid and allantoic fluid volumes, and the 5:1 allantoic fluid:amniotic fluid volume ratio suggest the occurrence of hydroallantois in some of the cloned pregnancies. Nevertheless, the homeostasis of foetal fluids is not yet fully understood, and the aetiology and pathogenesis of the foetal membrane hydrops still need to be better elucidated (Sloss and Dufty, 1980; Edwards *et al.*, 2003).

In this study, macroscopic placental alterations related to size and mass were also found in NT-HMC and some IVF concepti, following a similar pattern as seen in previous studies (Hill *et al.*, 2000, 2001; Bertolini *et al.*, 2002a, 2004, 2006; Hashizume *et al.*, 2002; Lee *et al.*, 2004; Li *et al.*, 2005; Batchelder *et al.*, 2007a; Miglino *et al.*, 2007; Everts *et al.*, 2008; Chavatte-Palmer *et al.*, 2012). The total placenta weight and surface area in the NT-HMC group was 2.2-fold heavier, and 1.6- and 2.8-fold larger than in the IVD and IVF groups, respectively. Interestingly, measurements related to the maternal traits were similar between groups, with differences in NT-HMC-derived concepti only being detected in the foetus and/or in the placenta, which contains maternal (caruncles) and foetal (cotyledons) components.

The comparison of placentome types by shape in this study was revealing, demonstrating that *in vitro*-derived concepti (IVF and NT-HMC) had more type C (flattened, nonengulfing) and fewer type A (engulfing mushroom-like) placentomes compared to the IVD group, with similar frequencies of types B (sub-engulfing mushroom-like) and D (semi-convex) placentomes. The placentome type frequencies in the IVD and in the NT-HMC groups were very similar to those reported by Batchelder *et al.* (2007a). Also, IVF-derived placentas were demonstrated to have changes in placentome types, with an increase in types C and D placentomes when compared to IVD controls (Bertolini *et al.*, 2006). Differences in placenta size and shape were due to morphological changes within the foetal rather than the nonfoetal horn. However, despite a lower number of type A placentomes in clones, the absolute total weight for type A placentomes was similar to controls, whereas types B (similar in numbers as in the IVD group) and C (increased numbers compared with the IVD group) were heavier than the IVD and IVF groups. These observations indicated that the placentome mass by individual placentome types increased in the NT-HMC group, and an apparent greater role of types B and C placentomes in placental function in cloned concepti. In fact, placentome number and placental mass per placentome type demonstrated a similar pattern between types A, B and C placentomes in the NT-HMC group, whereas in the IVD

group, 85% of the placentomes were from types A and B. Largely, the NT-HMC group had a higher frequency of enlarged placentomes, with the NT-HMC group having the greatest placentome weight (up to 725 g, type A) and dimensions (up to 23 cm in length and 19.5 cm in width, type A) from all groups, with 15.7, 7.3 and 23.0% of the total number of placentomes being longer, wider and heavier than the longest (12.0 cm, type A), widest (10.0 cm, type C) and heaviest (127.7 g, type A) placentome in the IVD group. In effect, the distribution of overall placentome sizes was deviated to the right (Figure 4), so that placentomes that exceeded the largest control placentome represented 63.5% of the total placenta weight in the cloned group, with potentially profound effects on placental function and conceptus growth (Bertolini *et al.*, 2004). On the other hand, the IVF group had only 1.9 and 7.3% longer and heavier placentomes than controls, respectively, representing about 25% of the total placenta weight, with the longest and heaviest placentome measuring 15 cm and weighing 270 g (type C). The IVF group appeared to have an intermediate phenotype, tending to be more similar to controls than clones, with total placenta weight and surface area similar to controls, but also showing slight changes in placentome types that did not seem to have significantly affected conceptus development. Collectively, changes in placental morphology after cloning may affect placental function, metabolic reprogramming and the pattern of placental constraint on foetal growth, generally leading to a growth-promoting effect, which may compromise postnatal survival from birth to adulthood, as predicted by Barker (1999). Investigation on the metabolic profile of placentomes by morphological types may assist in the understanding of mechanisms involved in abnormalities seen after cloning.

Significant morpho-pathological differences were observed in concepti from all groups. Indeed, abnormalities are common findings in bovine concepti, not necessarily being pathological (Alberto *et al.*, 2013). For instance, the presence of caruncular fusions in the bovine placenta has been described as a natural event for *in vivo*-derived pregnancies (Barreto *et al.*, 2009). The three experimental groups had caruncular fusions, but the clone group showed an increase in the frequency and intensity of such fusions, appearing in five out of six animals, with more than two placentomes fusing on occasions, corroborating with previous reports (Miglino *et al.*, 2007; Barreto *et al.*, 2009). Moreover, placental morphological changes may be associated with a reduction in the total number of placentomes in IVF- and NT-derived placentas (Hill *et al.,* 2001; Bertolini *et al.*, 2002a, 2004; Chavatte-Palmer *et al.*, 2002; Batchelder *et al.*, 2007a). An increase in caruncular fusions in cloned placentas was also reported to be commonly associated with a reduction in the number of placentomes and the appearance of regions completely devoid of placentation (Miglino *et al.*, 2007). Such features

were not observed in this study, perhaps due to the presence of microplacentomes (<1 cm) in all NT-HMC-derived pregnancies, and to the prevalence of caruncular fusions, so that placentome numbers were similar among groups. The total number of placentomes in the IVD and in the NT-HMC groups fell well within the expected range for control (Bertolini *et al.*, 2002a) or cloned (Chavatte-Palmer *et al.*, 2002; Batchelder *et al.*, 2007a) pregnancies. However, morphological differences in placentome types and placental surface area and weight were larger in the NT-HMC group than in the IVD and IVF groups, with positive correlations between placental traits and foetal traits, as seen in IVF-derived pregnancies (Bertolini *et al.*, 2002a, 2004, 2006).

The presence of oedema of the foetal membranes in cloned concepti has been reported (Hill *et al.*, 1999; Chavatte-Palmer *et al.*, 2002; Lee *et al.*, 2004; Li *et al.*, 2005; Batchelder *et al.*, 2007a; Miglino *et al.,* 2007), standing out as one of the most frequent occurrences in the placentas of clones. That feature agrees with our results, since all cloned concepti presented this disorder, with three displaying gelatinous oedema (myxoedema) in the foetal membranes, as also described by Miglino *et al.* (2007). In addition, the enlargement of the umbilical cord, with or without oedema, corroborates with other studies (Batchelder *et al.*, 2007a.; Miglino *et al.*, 2007; Meirelles *et al.*, 2010). This outcome is usually linked to persistent urachus after birth, predisposing to ascending infections that may result in morbidity and death of the newborn (Edwards *et al.*, 2003). In this study, the increase in cord diameter was assessed visually, being evident when compared with the umbilical cords from specimens from the other two groups. However, the aetiology underlying the increase in cord size is still elusive, warranting further investigations.

In summary, when compared with *in vivo*-derived pregnancies, Day-225 cloned concepti produced by HMC procedures, using similar Nellore genetic background, were characterized by lower pregnancy rates, high gestational losses during the first trimester of pregnancy, a distinctive biphasic growth pattern, with early growth restriction on Day 51, followed by accelerated growth rate and excessive foetal weight on Day 225, and a variety of morphological abnormalities more pronounced at the placental level. A significant increase in placental mass was accompanied by a striking change in placentome shape in cloned concepti, with 63.5% of the total placenta weight in clones being from placentomes larger and heavier than the largest and heaviest placentomes in the control groups. Such findings likely have profound metabolic implications to foeto-placental physiology and the appearance of the AOS. Alternatively, Day-225 IVF-derived concepti were widely similar to controls, demonstrating the effectiveness of the IVP system used in this study to prevent or

minimize AOS-related abnormalities during pregnancy. However, some differences at the placental level indicate that there is still a need for improved *in vitro* embryo production systems in cattle. Further studies involving morpho-physiology, metabolism, epigenetic reprogramming and gene expression profiles are underway in *in vitro*-derived concepti. This should lead to improved understanding of the AOS, which may also allow the identification of causal factors in early embryonic development that determine the phenotypical changes observed later in development, including the postnatal period.

ACKNOWLEDGMENTS

This study was supported by Procad II Grant no. 192058 funded by CAPES/Brazil. The authors thank Dr. A. Mezzalira, Dr. A.D. Vieira, Dr. A.N. Moraes, Mr. L.U. Ohlweiler, and Ms. J.C. Mezzalira for their technical assistance. Dr. R.P.C. Gerger was supported by a FAPESP/Brazil fellowship.

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5 CAPÍTULO II

BIOCHEMICAL AND METABOLIC PROFILES BETWEEN CLONE, IVF AND IVD CONCEPTI IN LATE PREGNANCY IN CATTLE

Artigo submetido ao periódico

Biology of Reproduction em 28 de maio de 2016

BIOCHEMICAL AND METABOLIC PROFILES BETWEEN CLONE, *IN VITRO* FERTILIZATION AND *IN VITRO* DERIVED CONCEPTI IN LATE PREGNANCY IN CATTLE

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ABSTRACT

The aim of this study was to compare physical traits, biochemical, metabolic and molecular profiles of Day-225 bovine pregnancies established carrying concepti produced *in vivo*, or by *in vitro* fertilization or cloning by somatic cell nuclear transfer. Biochemical evaluations were performed in foetal and maternal plasma and the expression of key genes to foetal development were analyzed by RT-qPCR in maternal and foetal liver tissues. Data were compared by by ANOVA the Tukey test, and a simple correlation. Concentrations of maternal plasma metabolites and gene expression profiles were similar between groups, with few biochemical and molecular differences. Most foetal parameters for liver and kidney functions, hemoglobin metabolism, and osmolarities in fetal plasma/serum and in the amniotic and allantoic fluids were higher in clones than the other groups. Glucose, fructose, tryglycerides and VLDL concentrations in fetal plasma, fructose levels and total amount of glucose and fructose in the allantoic fluid were higher in clones. Foetal 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. An increase in the fructolytic activity was seen in cloned foetuses, and transcripts for fructolytic and glycolytic enzymes correlated with glucose and fructose in the maternal and fetal plasma, and allantoic fluid. A difference exists in activity in metabolic pathways in cloned concepti, suggesting active glucose synthesis and fructose catabolism by the foetus, which may be a reflection of changes in metabolic foetal programming after cloning.

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

INTRODUCTION

The production of bovine embryos by *in vitro* fertilization (IVF) or cloning by nuclear transfer (NT) is a powerful tool routinely used for scientific and commercial applications in the world. However, developmental abnormalities caused by such embryo manipulations can interfere with the pattern of fetal and placental growth in a set of symptoms called Abnormal Offspring Syndrome (AOS), in which accelerated foetal growth has been associated with increased concentration of sugar moieties (glucose, fructose) in the foetal and neonatal plasma/serum and related fluids (Bertolini *et al.*, 2004). In the AOS, IVF and SCNT conceptus development is characterized by a biphasic growth pattern, with a retarded early growth phase coinciding with the period of placentation, followed by a foetal and placental excessive growth in the last third of pregnancy, with the birth of larger and less viable calves at term (Bertolini *et al.*, 2002a, 2004; Batchelder *et al.*, 2007ab; Gerger *et al.*, 2016).

Information on placental function and metabolic capacity in IVF- or NT-derived pregnancies are still lacking. It is known that *in vitro* embryo culture conditions the presence of serum and/or co-culture in supporting cells, and the faulty epigenetic reprogramming following cloning by SCNT are directly related to the occurrence of AOS symptoms (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, SCNT embryos exhibiting abnormal expression of embryonic genes may represent an early indication of incomplete reprogramming that could result in lower survival rates. Notwithstanding, the turn of events that allows a viable but epigenetically affected IVP conceptus to develop from early to late pregnancy appears to interfere with placental function and conceptus metabolism without lethally comprising homeostasis. 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).

As excessive growth in IVP concepti depends primarily on an increased foetal supply of substrates to support the pattern of development, the aim of this study was to compare the gene expression profiles of key enzymes from metabolic pathways and bioactive molecules in the maternal and foetal liver, and the biochemical profile in the maternal and foetal plasma/serum, with phenotypical characteristics obtained bovine concepti developed from *in vivo*- (Control), IVF- and SCNT-derived procedures, on Day 225 of gestation, which represents the peak of the relative fetal growth pattern during pregnancy in cattle (Eley *et al.*,

1978; Prior & Laster, 1979; Ferrell, 1989; Reynolds *et al.*, 1990), also being a period of detectable changes in the phenotype of IVP concepti during pregnancy (Farin & Farin, 1995).

MATERIALS AND METHODS

This set of experiments is part of a systematic study on the Abnormal Offspring Syndrome (AOS) in cloned cattle (Gerger *et al.,* 2016). Procedures involving humane treatments of animals in this study were approved by the Animal Ethics Committee of the University of São Paulo, Brazil.

Animals and experimental groups

A total of 19 bovine pregnancies were established carrying Nellore cattle (*Bos taurus* var. *indicus*) singleton concepti, produced either *in vivo* by superovulation and AI (*in vivo*-derived controls, or IVD group, n=9, four for *post-mortem* collection, and five for the *in vivo* kinetics study, as below), or *in vitro* by *in vitro* fertilization (IVF group, n=4, for *post-mortem* collection) or somatic cell nuclear transfer by Handmade Cloning procedures (NT-HMC group, n=6, for *post-mortem* collection). 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 with IVC (Gerger *et al.,* 2016). The Nellore breed was chosen due to its rather low birth weights in commercial herds (Ribeiro *et al.*, 1992).

Post-mortem collection and physical analyses of Day 225 conceptus tissues

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 fetus was exposed with the umbilical cord still intact. Blood samples were collected from the umbilical artery and vein (t=0 min), followed by the excision of the cord. The fetus and the empty uterus, containing the foetal membranes and

the placenta, were weighed, morphological foetal 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). Placentomes were excised and individually weighed, measured (width and length), morphologically classified by type according to Bertolini *et al.* (2006) and Gerger *et al.* (2016), with the placentome surface area calculated based on the equation for the area of an ellipse. Placentome samples were fixed in 2.5% glutaraldehyde or snap-frozen in LN_2. Blood samples collected from the three groups at t=-30 min (maternal venous blood) and t=0 min (maternal venous blood and foetal arterial and venous blood) were centrifuged at 6000 g for 15 min, with plasma (heparin-based anticlotting agent) and serum stored at -80ºC pending analyses. All volumes of amniotic and allantoic fluids were carefully collected independently, measured, sampled and stored frozen at -80ºC following centrifugation.

Determination of umbilical and uterine blood flows by in vivo tracer study in in vivo-derived pregnancies on Day 225

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 and balanced food ration *ad libitum*. The determination of the uterine and umbilical blood flows by the steady-state diffusion procedure with deuterium oxide (D_2O) was based on the Fick principle, following procedures by Reynolds and Ferrell (1987), Ferrell (1991b) and Ferrell and 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 foetal the foemoral vein infusion catheter (femoral vein) for 10 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 femoral arterial catheters, 5 mL/sample) and maternal (uterine arterial and venous catheters, 10 mL/sample) systems 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 –80ºC for D_2O and substrate analyses 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 and Philips (1979), and Ferrell and Reynolds (1992), which was used for the determination of umbilical and uterine blood flows in the IVD group. Then, umbilical blood flows in the cloned 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).

Biochemical and molecular analyses in maternal and foetal plasma/serum, liver tissues, and foetal fluids

Figure 1 ilustrates some of the intracellular pathways involved in the intermediate metabolism in the liver, for which some of key enzymes for pathways involved in the carbohydrate and lipid metabolisms were analysed by RT-qPCR, as below. The illustration also depicts some of the blood components determined in the blood biochemical analyses.

Biochemical analyses in maternal and foetal plasma/serum, and foetal fluids

Samples from maternal (t=-30 min and t=0 min) and foetal (umbilical venous blood) plasma/sera were used for determination of biochemical parameters of 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 (lactate; triglycerides; cholesterol; high-density lipoproteins, HDL; very-low-density lipoprotein, VLDL; low-density lipoprotein, LDL), hemoglobin (total bilirubin; conjugated and unconjugated bilirubin), and calcium/phosphorus metabolism; and chlorine concentrations; in duplicates, in an automated biochemical analyzer (Labmax 240®, Biotécnica, Brazil). Plasma and foetal fluid osmolarities were measured in duplicates in an osmometer (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 venous and femoral arterial blood) plasma and foetal fluids (amniotic and allantoic fluids) was performed by colorimetry, in duplicates, according to Bertolini *et al.* (2004). Glucose was determined by an enzymatic assay kit (QuantiChromTM Glucose Assay Kit, BioAssay Systems, USA), in 96-well plates 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

venous 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 reanalyzed. 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, 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.

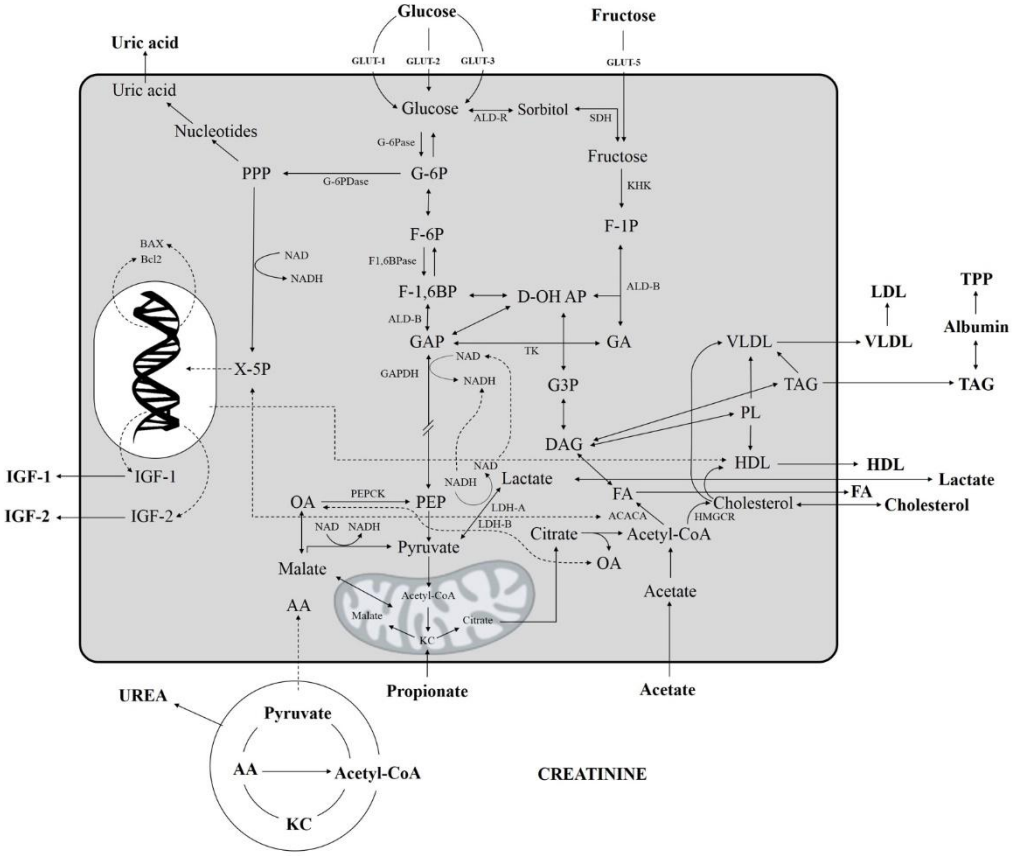

Figure 1. Summary of key molecules and intracellular pathways involved in the intermediate metabolism in the liver cell (gray box), and blood components associated with systemic metabolism (outside the gray box). Carbohydrate, lipid, amino acid and nitrogenous metabolic pathways, related molecules, metabolite fate (intra- or extracellular flow) and other bioactive molecules are depicted. Arrows indicate direction of reaction or metabolite flow. See text, figures and tables for definition of abbreviations.

Analyses of gene expression by RT-qPCR

Total RNA from maternal and foetal liver samples from the three experimental groups was extracted using Trizol® reagent (Invitrogen, USA), and 300 ng of total RNA from each sample were treated with DNase I (Invitrogen) and used for cDNA synthesis with the SuperScript® III kit (Invitrogen), according to the manufacturer instructions. For the qPCR, the Power SYBR Green PCR Mastermix (Applied Biosystems, USA) was used in the StepOne Plus Real Time PCR System (Applied Biosystems), with data normalized using the ribosomal protein S9 (*RPS9*) as the housekeeping endogenous control gene. The target genes of interest for molecular analyses by RT-qPCR were transcripts related to (a) glycolysis: glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), and lactate dehydrogenase A and B (*LDHA* and *LDHB*); (b) gluconeogenesis: phosphoenolpyruvate carboxykinase (*PCK1*), fructose-1,6-bisphosphatase (*FBP1*), glucose-6-phosphatase (*G6PC*); (c) fructolysis: fructokinase (*KHK*), dihydroxyacetone kinase 2 (*TKFC*), aldolase B (*ALDOB*); (d) fructogenesis: aldose reductase (*AKR1B1*), sorbitol dehydrogenase (*SORD*); (e) pentose phosphate pathway (PPP): glucose-6-phosphate dehydrogenase (*G6PD*); (f) fatty acid synthesis: acetyl-CoA carboxylase alpha (*ACACA*); (g) cholesterol synthesis: 3-hydroxy-3-methylglutaryl-CoA reductase (*HMGCR*); (h) glucose transporters: solute carrier family 2 member 1 (*SLC2A1*), member 2 (*SLC2A2*), member 3 (*SLC2A3*), and member 5 (*SLC2A5*); (i) apoptosis: BCL2-associated X protein (*BAX*), BCL2 B-cell CLL/lymphoma 2 (*BCL2*); and (j) components of the IGF system: insulin-like growth factor 1 (*IGF1*) and 2 (*IGF2*), as depicted in Table 1. The amplification cycling conditions used an initial denaturation at 95°C for 10 min followed by 50 cycles consisting of 95°C for 30 s, 60°C for 30 s and 72°C for 30 s. The SYBR Green fluorescence data was recorded at the end of each extension step (60°C). Pilot experiments using five 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 duplicate, with the results normalized using the comparative $2^{-\Delta\Delta CT}$ method (Livak & Schmittgen, 2001).

Table 1. Primer sequences, GenBank accession codes, amplicon sizes, and gene functions for transcripts analyzed by real time qPCR in Day-225 IVD control, IVF-derived and cloned *Bos taurus* var. *indicus* foetal and maternal liver samples

Function or pathway	Gene Symbol	Accession number	Primer sequences (5'-3')	Product (bp)
Glycolysis	*GAPDH*	NM_001034034.2	F: GATTGTCAGCAATGCCTCCT R: AAGCAGGGATGATGTTCTGG	200
	LDHA	NM_174099.2	F: GGAGCATGGTGACTCTAGTGTGCCT R: GGAAATCGGATGCACCCGCC	250
	LDHB	NM_174100.2	F: AGATGGTGGTTGAGAGTGCCTATGA R: ACCTCTTCATCCTTCAGCTTCTGGT	244
Gluconeogenesis	*G6PC*	NM_001076124.2	F: ACAGCAACACCTCGGCACCG R: CAAGCACCGAAATCTGTAGGTTGGC	173
	FBP1	NM_001034447.2	F: CTGGACCCGGCCATTGGAGAGT R: CACGTACCTGGCGCCATAGGG	180
	PCK1	NM_174737.2	F: GCAGAGCACATGCTGATTCTGGGC R: GTTCCCGGAGCGACGCCAAAA	233
Fructolysis	*KHK*	NM_001076351.1	F: GGAGACCCCGTGCTCTTGTTGC R: TCCGGCCCTCAATGTGGATCC	149
	DAK	NM_001024524.3	F: CCTAGTGGGCACCTTCATGT	227

			R: TCTGCTTTGAGGCTTCACCT	
	ALDOB	NM_001034485.2	F: CCCATCTTGCTATCCAGGAA R: TACAGCAGCCAGGACCTTCT	161
Fructogenesis	*AKR1B1*	NM_001012519.1	F: ATCTTAAACAAACCTGGCTT R: TGTAGCTCAGCAAGGTGTTC	350
	SORD	NM_001037320.1	F: CCCGGAGACTTGCGCTTGGAAA R: GGAGCACCAGGCTCGATGGC	248
Pentose phosphate pathway	*G6PD*	NM_001244135.1	F: ATCCGCAAGCAGAGCGAGCC R: GCAGGTCCCTCCCAAACGGC	295
Fatty acid synthesis	*ACACA*	NM_174224.2	F: GCCAGGAGGCCCCAACAACA R: GCTTGGAGGACCCATGAAGGCG	169
Cholesterol synthesis	*HMGCR*	NM_001105613.1	F: CACCAGCAGGGTTGCACGTCT R: ATCCAGTTGATGGCCGCAGGT	225
Glucose transporter	*SLC2A1*	NM_174602.2	F: CATGGAGCCCACCAGCAAGAAGC R: GACCAGAGCGTGGTCAGCGT	198
	SLC2A2	NM_001103222.1	F: AAAGAGGGGGAAGAAGCATC R: GGCACAAACAAACATCCCAC	308

	SLC2A3	NM_174603.3	F: GGGTGGTATGATTGGTTCCTTC R: AAGGCAGCCGCCAGCTAT	105
	SLC2A5	NM_001101042.1	F: GCGCTATTTTCGTGGTGGAA R: CGCAGCAAGCGGTGAGA	80
Pro-apoptotic factor	*BAX*	NM_173894.1	F: GTTGTCGCCCTTTTCTACTTTGC R: CAACTCGGGCACCTTGGT	68
Anti-apoptotic factor	*BCL2*	NM_001166486.1	F: CATGTGTGTGGAGAGCGTCAA R: GCCGGTTCAGGTACTCAGTCA	82
IGF system	*IGF1*	NM_001077828.1	F: ACCCTCTGCGGGGCTGAGTT R: GTACTTCCTTCTGAGCCTTGGGCAT	256
	IGF2	NM_174087.3	F: TCTACTTCAGCCGACCATCC R: TTCGGAAGCAACACTCTTCC	72
Endogenous control gene	*RPS9*	NM_001101152.2	F: CCTCGACCAAGAGCTGAAG R: CCTCCAGACCTCACGTTTGTTC	62

Data 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/serum analyses) by ANOVA and ANCOVA (foetal weight as covariate, adjusted to overall mean foetal weight of 19.3 ± 7.4 kg), using the GLM procedure (Minitab, State College, PA, USA), for $P<0.05$. Pairwise comparisons were scrutinized by the Tukey test. Data were tested for normality by the Anderson-Darling normality test, with the log transformation of non-normal variables (mostly for gene expression data). A simple correlation test was used to evaluate the relationships between traits.

RESULTS

Systemic physiology - Maternal profiles

Physical traits.

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, denoting heavier foetuses and/or larger foetal fluid volumes in the cloned group. In fact, after further removal of the foetal membranes and placentomes from the empty tract, the weight of the uterus *per se* was similar between groups.

Biochemical profiles.

No differences were observed between collection time periods (t=-30 min *vs.* t=0 min) for any biochemical measurement in the maternal plasma/serum, irrespective of the groups, except for a 1.5-fold increase 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 the 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) and fructose (0.4 ± 0.1, 0.4 ± 0.1, and 0.3 ± 0.2 mM) were similar between groups. Maternal plasma fructose concentrations, although very low as expected (Bertolini *et al.*, 2004), were higher in the NT-HMC group (0.37 ± 0.21 mM) than IVD controls (0.07 ± 0.07 mM) and the IVF group (0.04 ± 0.03 mM). Other metabolic maternal plasma components (lactate, cholesterol, HDL, LDL, VLDL, tryglycerides) were similar between groups.

Overall, all parameters for liver and renal function, hemoglobin and calcium/phosphorus metabolism, and electrolyte concentration/osmolarity measured in the maternal plasma in both collection time periods fell well within the normal reference values for the species (Smith, 2009). However, a few biochemical and molecular differences were observed between groups, with the maternal plasma in the NT-HMC group having higher chloride levels (108.8 ±1.6 *vs.* 102.8 ± 2.0 and 98.0 ± 2.0 mEq/L), osmolarity (307.5 ± 8.1 *vs.* 295.5 ± 4.0 and 294.8 ± 8.2 mOsm/kg), conjugated bilirubin (0.22 ± 0.03 *vs.* 0.08 ± 2.0 and 98.0 ± 2.0 mg/dL), and urea (39.0 ± 3.1 *vs.* 13.5 ± 3.8 and 16.0 ± 3.8 mg/dL) concentrations, and lower ALT levels (22.8 ± 2.2 *vs.* 34.0 ± 2.7 and 21.0 ± 2.7 mg/dL) than the IVD and IVF groups, respectively. Total bilirubinemia was higher in clones (0.89 ± 0.15 mg/dL) than in IVD controls (0.25 ± 0.19 mg/dL), with both being similar to the IVF group (0.35 ± 0.19 mg/dL).

Gene expression profiles.

The pattern of expression of maternal liver genes related to fructose, glucose, fatty acid and cholesterol metabolisms and components of the IGF system were widely similar between groups, except for 2- and 9-fold higher relative expression levels for aldolase B (a glycolytic and fructolytic enzyme) and 38- and 14-fold higher expression pattern (P=0.098) for sorbitol dehydrogenase (a fructogenic enzyme) in the liver of clone-bearing dams than in IVD controls and the IVF group, respectively.

Uterine and umbilical blood flows

Only two out of the five IVD pregnancies subjected to cannulations on Day 220 maintained functional catheters and/or had viable foetuses on Day 225, with both rendering valid for the *in vivo* kinetics study. Following D_2O maternal and foetal plasma concentration analysis, the mean uterine and umbilical blood flow rates for all collection time periods for both IVD specimens 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 umbilicus than in the uterus, respectively, ranging from 1.72 to 4.71 L/min (39% lower and 68% higher than the mean value for the foetus), and 4.08 to 8.28 L/min (24% lower and 55% higher than the mean value for the uterus). 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, also respecting the same mean increase in foetal weight, with a variation ranging from 1.4- to 3.3-fold higher in the umbilical blood flow.

Systemic physiology - Foetal profiles

Physical traits.

Significant physical differences between groups emerged at the uterine compartmental level, with pregnant tracts and concepti from clones (NT-HMC group) being significantly larger than the IVD and IVF groups. A summary of some of the most relevant conceptus traits for the IVD, IVF and NT-HMC groups is presented in Table 2, which indicates a greater variation in physical traits and a trend for larger measurements in cloned concepti in comparison to the other groups, as previously reported (Gerger *et al.*, 2016). In fact, the mean values for foetal body, foetal liver and placenta weights, and for placenta surface area and foetal fluid volumes in the NT-HMC group were 1.5- to 4.0-fold larger than the IVD and IVF groups (Table 2), with foetal body, foetal liver, placenta weights, and crown-rump lengths being heavier in clones than the IVD and the IVF groups. The placenta surface area was larger in clones than in the IVF group, with both similar to the IVD group. The total foetal fluid volume in NT-HMC-derived pregnancies tended to be larger than in IVD controls (P=0.066), being both similar to the IVF group, even though the foetal fluid volumes were measured only from five cloned specimens, as the fluids were lost for the largest cloned conceptus (a 42-kg fetus) due to uterine rupture during the pregnant tract retrieval. No differences were observed in the total placentome numbers between groups. After using foetal weight as covariate, most traits were similar between groups, with physical traits regarding mass (e.g., placenta and foetal weights), linear measurements (e.g., placenta

surface area, CRL), and volume (e.g., foetal fluid volumes) strongly correlating positively with one another ($R>0.900$, $P<0.05$), which indicates an isometric tissue growth pattern based on the conceptus size. However, the foetal liver in cloned fetuses continued to be significantly larger than the other groups after ANCOVA, demonstrating that the liver size in clones was larger than the isometric prediction.

Biochemical profiles.

Several biochemical measurements in foetal plasma/serum and fluids were significantly different in the NT-HMC group when compared with the IVD and IVF groups (Figures 2 to 4), as below.

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 fetuses than IVD controls, and AST was higher in the IVF group than in the IVD and NT-HMC groups.

Table 2. 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)

Parameters used for renal function, which are also associated with nitrogen metabolism and muscle activity, were different between groups (Figure 2, panels c and d). 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.

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

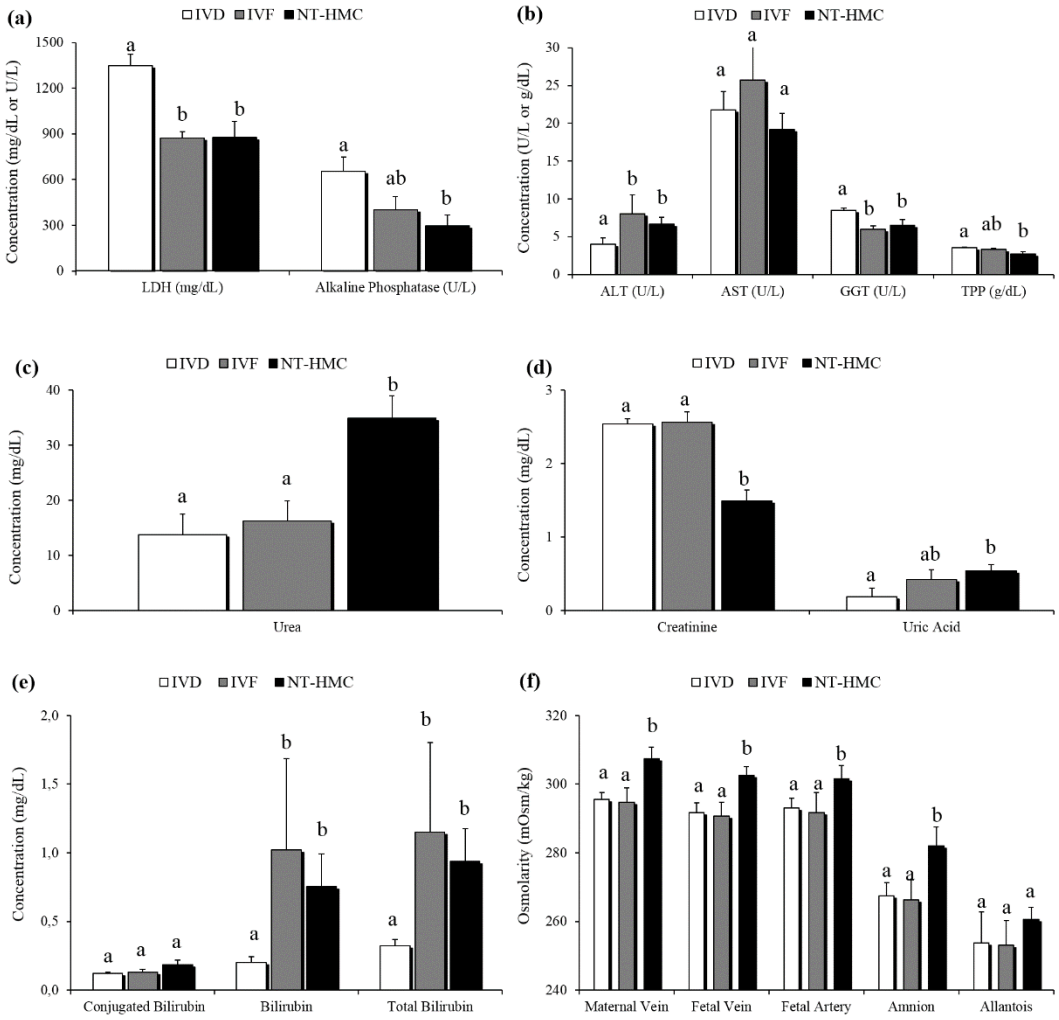

Figure 2. Biochemical parameters for liver and renal functions, hemoglobin metabolism and osmolarity in maternal and/or foetal plasma/serum and foetal fluids between *Bos taurus* var. *indicus in vivo*-derived (IVD) concepti and concepti produced either by *in vitro* fertilization (IVF) or nuclear transfer 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) Fetal plasma urea concentrations. (d) Foetal plasma creatinine and uric acid concentrations. (e) Foetal plasma conjugated, unconjugated and total bilirubin concentrations. (f) Maternal and foetal plasma and foetal fluids osmolarity. [a,b:] Columns for each parameter without common superscripts ([a,b]) differ; $P<0.05$. 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 controls, IVF, and clones, respectively. However, after the ANCOVA, using foetal weight as covariate, foetal calcemia (2.78 ± 0.25, 3.83 ± 0.25, and 3.03 ± 0.25 mM) and plasma chloride concentrations (90.8 ± 5.0, 113.0 ± 5.4, and 104.6 ± 5.1 mEq/L) were lower in clones than in IVD controls, with both being similar to the IVF group. To note, osmolarities 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 2, panel f).

Glucose and fructose concentrations in umbilical arterial and venous plasma (Figure 3, panel a), and 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 the foetal fluids 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, with no significant differences between groups for both sugars in the amniotic fluid (Figure 3, panels d and e). Glucose umbilical venous-to-arterial concentration differences were similar between groups, but concentration differences for fructose were lower ($P<0.05$) in the IVF group, and a trend ($P=0.078$) was observed for the NT-HMC group to be lower than controls, being similar between the IVF and the NT-HMC groups (Figure 3, panel b). 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 f), with no differences between groups.

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 4). Foetal plasma lactate concentrations tended to be lower ($P=0.073$) in the IVF group (9.72 ± 0.61 mM) than control fetuses (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 venous (10.5 ± 1.0 min) umbilical blood.

Gene expression profiles. The pattern of gene expression in foetal liver revealed no significant expression of *IGF1* in all groups, and no differences between groups for *IGF2* and for pro- (*BAX*) and anti-apoptotic (*BCL2*) factors (Figure 5). However, the *BAX*/*BCL2* ratio was 5.5- and 16.5-fold higher in the NT-HMC and IVF groups, respectively, than the IVD control counterpart, indicating a higher pro-apoptotic *BAX* and/or lower anti-apoptotic *BCL2* expression pattern in the former groups.

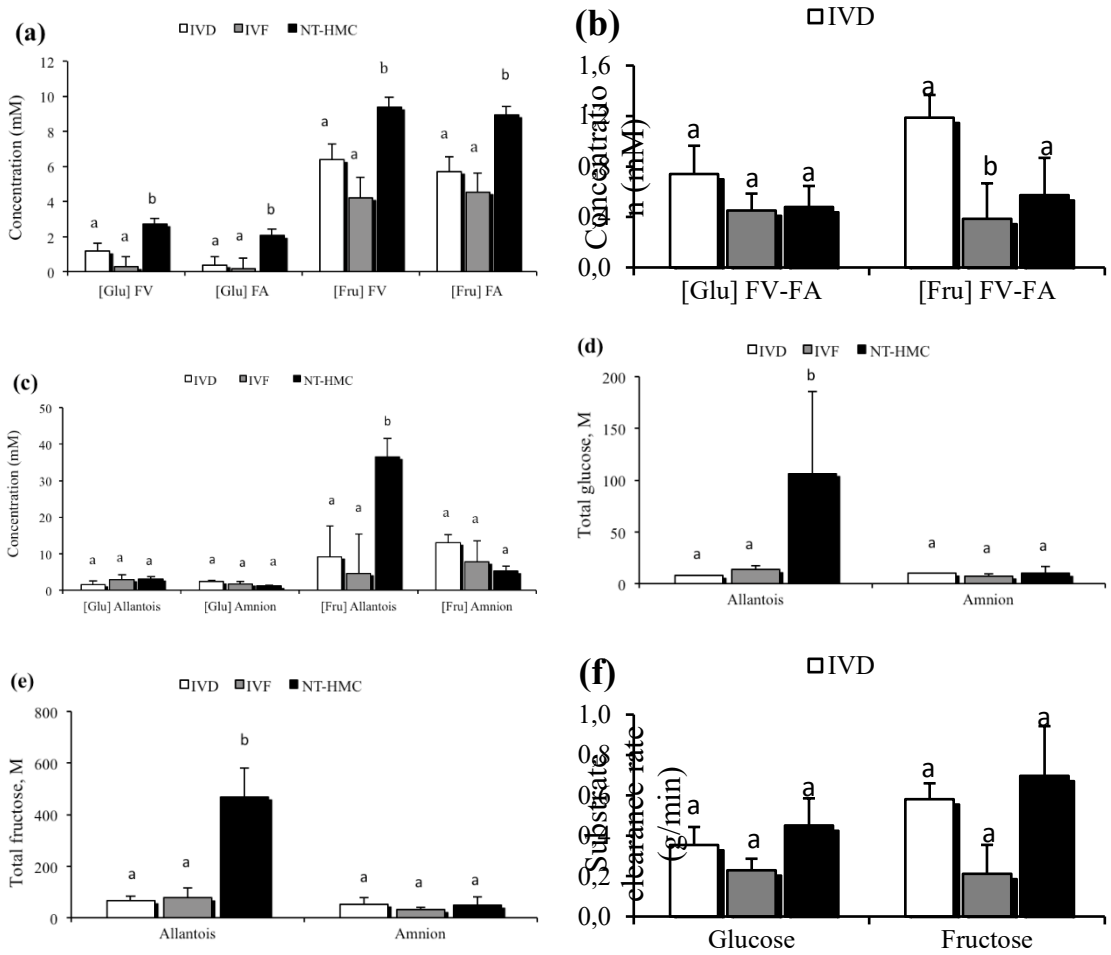

Figure 3. Glucose and fructose concentrations (mM), concentration differences (mM), and total amounts (mM) in the plasma/serum and foetal fluids between *Bos taurus* var. *indicus in vivo*-derived (IVD) concepti and concepti produced either by *in vitro* fertilization (IVF) or nuclear transfer by handmade cloning (NT-HMC) procedures on Day 225 of gestation (LSM ± SEM). (a) Glucose and fructose concentrations in the foetal vein and artery. (b) Venous-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; $P<0.05$.

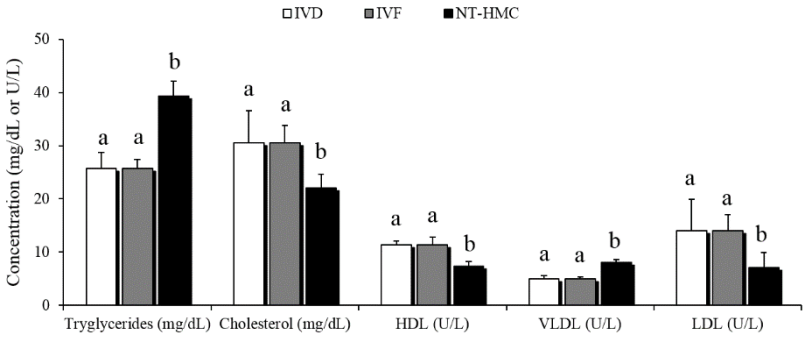

Figure 4. Foetal plasma concentrations of components of the lipid metabolism (tryglycerides, cholesterol, HDL, LDL, VLDL) between *Bos taurus* var. *indicus in vivo*-derived (IVD) concepti and concepti produced either by *in vitro* fertilization (IVF) or nuclear transfer by handmade cloning (NT-HMC) procedures on Day 225 of gestation (LSM ± SEM). Columns for each parameter without common superscripts (^{a,b}) differ; $P<0.05$.

Differences in the relative expression levels in the foetal liver between groups were detected for genes related to fructose and glucose metabolism, sugar transmembranal transport, and fatty acid and cholesterol metabolism. An increase in the fructolytic pathway activity was seen in cloned foetuses, as *KHK, DAK* and *ALDOB* expression levels were 2.6- ($P=0.089$), 7.7- and 30.3-fold higher in clones than in IVD controls. Also, *ALDOB* expression levels in the IVF group were similar to clones, and 156-fold higher than controls (Figure 6, panel a). Interestingly, a 246-fold and 28-fold increase *AR* expression were observed in cloned and IVF foetuses when compared with controls, with no differences in *SRDH* expression between groups (Figure 6, panel b), paradoxically suggesting an increased fructogenic potential in the liver of cloned and IVF-derived concepti.

No expression differences were detected for glycolytic (*LDH* and *GAPDH*) enzymes (Figure 6, panel c), but transcripts for the gluconeogenic enzymes *PEPCK* and *FBP1* were 8.4- and 4.2-fold higher, and *G6Pase* expression levels were 8.3-fold lower in clones in comparison to controls. In addition, *FBP1* expression levels in the IVF group were similar to clones, and 6-fold higher than controls (Figure 6, panel d). The key enzymes for the pentose phosphate pathway (*G6PD*) and fatty acid synthesis (*ACACA*) were associated with foetal size (ANCOVA), with cloned foetuses having a 30- ($P<0.05$) and 15-fold increase in *G6PD* and *ACACA* gene expression levels when compared with the IVD and IVF groups (Figure 6, panel e). Relative transcript abundance for *HMGCR* was similar between groups, despite the lower cholesterol levels in the foetal plasma of cloned foetuses.

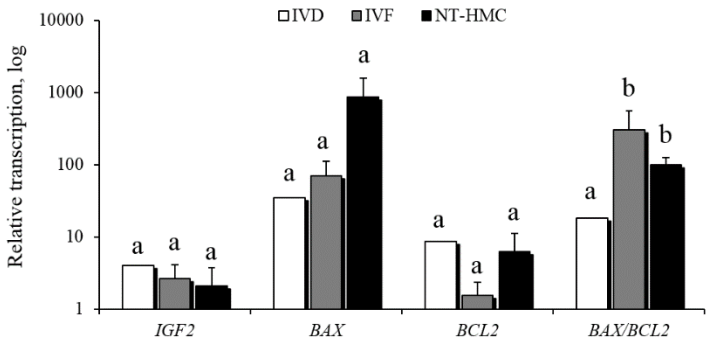

Figure 5. Relative gene transcription (*IGF2*, *BAX*, *BCL2* and ratio *BAX*/*BCL2*) in foetal liver (LSM ± SEM) between *Bos taurus* var. *indicus in vivo*-derived (IVD) concepti and concepti produced either by *in vitro* fertilization (IVF) or nuclear transfer by handmade cloning (NT-HMC) procedures, on Day 225 of gestation (LSM ± SEM). Columns for each gene transcript without common superscripts (^{a,b}) differ; $P<0.05$.

Cloned and IVF foetal livers expressed 4.7- and 10-fold higher *SLC2A1* levels than controls, *SLC2A2* levels were significantly lower and higher in IVF and cloned foetuses, respectively, than IVD control counterparts, and *SLC2A3* levels were 14.6- to 17.5-fold higher in clones than in the IVF or in the IVD groups (Figure 6, panel f). No differences were detected in *SLC2A5* expression levels between groups.

Correlations of physiological significance

Physical traits (foetal body and most organ weights, placenta weight and surface area) positively correlated ($R^2>0.600$, $P<0.05$) with variables associated with an active and growth-promoting physiological pattern, including key components of glucose and fructose metabolism (*TK*, *AR*, *G6PD*), 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 ($R^2<-0.600$, $P<0.05$) 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, which were also associated with the pattern of gene expression for important metabolic enzymes. 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. Transcripts for key fructolytic and glycolytic enzymes (*KHK, TK*, and *GAPDH*) correlated with most measurements for glucose and fructose (concentrations and total amounts) in the maternal plasma, foetal plasma, and allantoic fluid. Transcripts for the gluconeogenic pathway (*PEPCK* and *FBP1*) correlated with glucose and fructose levels in the allantoic fluid. Also, *PEPCK* and *TK* expression levels correlated with one another.

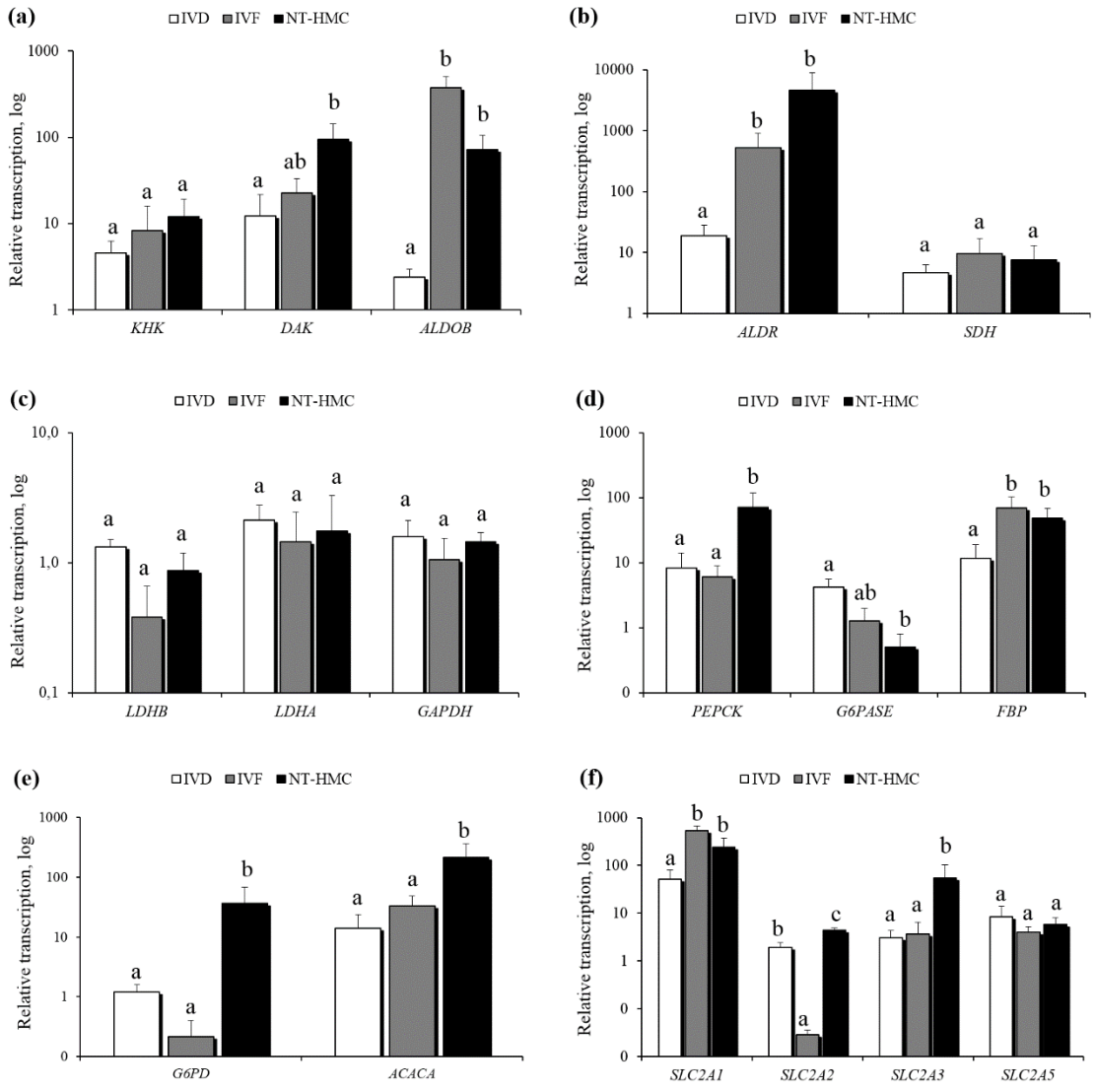

Figure 6. Relative gene transcription in foetal liver (LSM ± SEM) between *Bos taurus* var. *indicus in vivo*-derived (IVD) concepti and concepti produced either by *in vitro* fertilization (IVF) or nuclear transfer by handmade cloning (NT-HMC) procedures on Day 225 of gestation (LSM ± SEM). Gene products for (a) fructolytic enzymes (*KHK*, *DAK* and *ALDOB*); (b) fructogenic enzymes (*ALDR* and *SDH*); (c) glycolytic enzymes (*LDHA*, *LDHB* and *GAPDH*); (d) glyconeogenic enzymes (*PEPCK*, *G6PASE* and *FBP1*); (e) pentose phosphate pathway (*G6PD*) and fatty acid synthesis (*ACACA*); and (f) sodium-independent facilitated hexose

transporters (*SCL2A1*, *SCL2A2*, *SCL2A3* and *SCL2A5*). Columns for each gene transcript without common superscripts (^{a,b}) differ; $P<0.05$.

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 *SLC2A2* and *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 (*TK*, 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 osmolarity in the maternal and foetal plasma. Transcripts for the fructogenic (*AR*) and the pentose phosphate pathway (*G6PD*), and fatty acid synthesis (*ACACA*) correlated with one another, and with glucose in foetal plasma, fructose in the maternal plasma, uric acid, and *IGF2*, *BAX*, and *BCL2*. Osmolarity 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, foetal plasma urea, and *SLC2A1* and *SLC2A2* expression. On the other hand, TPP and AP correlated negatively with foetal plasma glucose and maternal plasma fructose.

DISCUSSION

The production of cattle embryos by IVF or NT is a powerful tool routinely used in both scientific as commercial level in Brazil and worldwide. However, developmental abnormalities from these embryonic manipulations can interfere with foetal and placental growth in a realm of symptoms called Abnormal Offspring Syndrome (AOS). Disorders in foetal and placental development have been presented as one of the types of abnormalities that may be seen in animals with AOS (Young *et al.*, 2001). However, in this study, females who carried clone foetuses showed no significant 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 normality during pregnancy, despite the conceptus type and the overall physiological differences observed in the uterine and foetal compartments. Changes in placental development and function in IVP pregnancies derived either from IVF or SCNT procedures

have been associated with the delivery of larger calves and the appearance of aberrant foetal membrane (Bertolini *et al.*, 2004), and results from these studies have increasingly contributed to the understanding of those abnormalities.

In previous studies, we observed that foetal growth foetuses derived from IVF and SCNT 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.*, 2002a, 2004) and SCNT (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 in the third trimester of pregnancy, with significant morphological changes in placentas of IVF and SCNT concepti and the birth of larger calves with reduced postnatal survival (Bertolini & Anderson, 2002; Bertolini *et al.*, 2002a;. 2004; Batchelder *et al.*, 2005; Gerger *et al.*, 2016). In our study, the physical traits showed a trend for higher measurements in foetuses in the clone group compared to the *in vivo* and IVF groups on Day 225 of pregnancy, with foetal body weights, foetal liver and placentas being heavier in clones than the other groups. Furthermore, the placental surface area was higher in the clone than in the IVF group, but similar to the *in vivo* controls.

The physical analyses of length and weight showed a significant increase in almost all foetal organs and tissues in the SCNT group. However, after statistical adjustment f by analysis of covariance, with foetal weight used as a covariate, such differences mostly disappeared. This observation was also made 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, and as the size of the foetuses increased, organs and tissues generally isometrically shadowed the proportion of 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 it is also known to occur after birth (Chavatte-Palmer *et al.*, 2009).

The excessive foetal growth pattern observed at the end of gestation in IVP embryos appears to be associated with an increased uptake of energy substrates, especially glucose, to 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, the fructose concentrations in maternal plasma at t = 0 min were higher in NT-HMC group than in the control group (IVD) and IVF. Fructose is synthesized by the placenta from glucose and is

the main carbohydrate in plasma and foetal fluids, a characteristic of species with sinepithelium- or chorionic-epithelium placenta, such as ungulates and cetacea (Goodwin, 1956; Nixon, 1963). Aldoretta and Hay (1999) found that in sheep 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 to the maternal system, remaining into the uterine and foetal compartments (Bertolini *et al.*, 2004).

In the analysis of expression of maternal liver genes related to the fructose, glucose, fatty acids and cholesterol metabolism, differences were observed in levels of expression relative to aldolase B and a trend ($P = 0.098$) to an increased expression level of sorbitol dehydrogenase was observed in the liver of females carrying cloned concepti, suggesting that the maternal liver of cloned bovine foetuses have greater of fructolitic and fructogenic activity, for subsequent fructose to glucose conversion, meeting the needs of this metabolite to foetal clones. However, foetal growth is usually limited by maternal and placental factors, which is related to the nutrient supply, which controls foetuses growth in late gestation (Ferrell, 1991a,b; Gluckman *et al.*, 1992). Because the placental mass becomes larger in IVF- and SCNT-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 glucose and fructose clearance (foetal uptake and consumption) were observed between groups. Also, the foetal venous to arterial fructose concentration difference was lower in the IVF and cloned groups than controls, which is rather surprising. However, 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 studies. Thus, long term studies on the substrate uptake and consumption will better provide clues on the placental transfer capacity between clones and controls.

IGF1 and *IGF2* are known to play a role in foetal and postnatal growth. In our study, the pattern of gene expression in foetal liver showed no differences between groups for components of the IGF system. Chavatte-Palmer *et al.* (2002) observed that, in general, no significant differences existed between clones and controls in *IGF1* concentrations, in opposition to *IGF2*, which were higher in clones at birth, but lower on Day 15 after birth compared to controls. This rapid drop in *IGF2* concentrations have also been described in sheep foetuses (Carr *et al.*, 1995).

The *BAX/BCL2* ratio was higher in clones and in IVF foetal livers than in the control group. Previous reports have demonstrated a correlation between the effect of the culture system in the incidence of apoptosis and the level of pro-apoptotic gene *BAX* in quality and clinical normality in embryos produced *in vitro* (Brocco *et al.*, 2003). It is noteworthy that this gene is also abundant in bovine blastocysts cultured *in vitro* in SOF medium supplemented with serum (Yang & Rajamahendran, 1999).

In this study, the concentrations of glucose and fructose in the foetal plasma, and allantoic, amniotic fluid and total fluids did not differ between groups. Such results were similar to those reported by Reynolds *et al.* (1990) and 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. The total glucose and fructose in fetal 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 results, associated with the fact that placenta had a greater weight and surface area in the SCNT group, demonstrate a greater of glucose and fructose usage or supply to the foetus, favoring foetal growth in the last third of pregnancy and explaining the excessive size observed in the SCNT group in this experiment.

Sangild *et al.* (2000) reported that, in the immediate postnatal period, IVP calves have reduced glucose levels in the blood. Changes in the metabolism of glucose were not observed at basal levels or during postprandial conditions in clones. In some other cases, however (those in which insulin tests were performed), hypoglycemia and hypothermia were present in the neonatal period (first 24 h), requiring heating and infusion of glucose solutions, despite the correct supply of colostrum at birth (CHAVATTE-PALMER *et al.*, 2002).

The glucose 6-phosphate (G6P) molecule is an important compound present at the intersection of several metabolic pathways, such as glycolysis, gluconeogenesis, pentose phosphate pathway, among others. In glycolysis, G6P is converted by phosphohexose isomerase to fructose 6-phosphate, which involves an aldose-ketose isomerization (Murray, 2007). In our study, a 8.3-fold decrease in *G6Pase* expression was seen in clones when compared to controls, despite other enzymes of the similarity in other gluconeogenic enzymes between groups.

Interestingly, the expression levels of the *G6PD* and *ACACA* genes were much higher in clones foetuses compared to controls and the IVF group for *G6PD*, or to controls (*ACACA*). Probably, a greater G6P level in the clones, explained by the fact that a greater utilization of NADPH in the fatty acid synthesis could increase the NADP+ levels, could

stimulate *G6PD* to produce more NADPH to supply fatty acid synthesis in the extramitochondrial system. Alternatively, higher glucose intake can induce the activation of the PPP through the G6PD, leading to the synthesis of fatty acids (CAMARGO *et al.*, 2008). The activation of gene transcription in response to carbohydrates was characterized in liver cells (YAMASHITA *et al.*, 2001), being regulated by the activation of the carbohydrate responsive element binding protein (chREBP). The chREBP activation occurs through the action of the phosphatase 2A (PP2A), a protein dependent on the xylulose 5-phosphate (Xu-5P), a final PPP product (Nishimura & Uyeda, 1995). Once activated, chREBP participates in the transcription regulation of energy metabolism genes, such as the L-pyruvate kinase (LPK) and genes for lipogenic enzymes, such as acetyl-CoA carboxylase alpha (ACACA) and fatty acid synthetase (FASN), important enzymes in lipogenesis (Kabashima *et al.*, 2003). In summary, a higher amount of carbohydrates activates the PPP, increasing the cell concentration Xu-5P, activating PP2A, which acts on the chREBP, finally inducing the core expression of genes encoding lipogenic enzymes, promoting, therefore, lipogenesis, converting carbohydrates into lipids (CAMARGO *et al.*, 2008). Acetyl-CoA, the precursor of fatty acids, is formed from glucose by oxidation of pyruvate inside the mitochondria. However, it does not promptly diffuse to the extramitochondrial cytosol, the main site of fatty acid synthesis. Acetyl-CoA carboxylase is an allosteric enzyme activated by citrate, for which concentrations increase in the state of full satiety or substrate surplus, indicating an abundant supply of acetyl-CoA (Murray, 2007).

There are 14 known isoforms of glucose carriers through the membrane (the GLUTs), which are responsible for the diffusion of glucose into lipid bilayers of cell membranes (Simpson *et al.*, 2008). The hepatic expression levels of *GLUT1* were higher in the clone and IVF groups than controls. Moreover, *GLUT2* levels were lower and higher in IVF and clone foetuses, respectively, than controls, and *GLUT3* was higher in clones than in the IVF and *In vivo* groups. In placenta, Bertolini *et al.* (2004) reported no differences in the relative abundance of transcripts of important components of the glucose transport system (*GLUT1, GLUT3, GLUT5*) detected at the placental level (Bertolini *et al.*, 2003). In ruminants, *GLUT2*, located in liver plasma membrane plays a crucial role in the release of glucose in the liver (Zhao *et al.*, 1996).

Fructose is synthesized by the placenta from glucose and is the main carbohydrate in plasma and foetal fluids, a characteristic of species with sinepithelium- or epitheliochorionic placenta, such as in ungulates and cetaceans (Goodwin, 1956; Nixon, 1963). We found an increase in fructolitic activity in the clone group, with a greater expression of the *KHK, DAK*

and *ALDOB* genes when compared with controls. Furthermore, an increase in *AR* expression was detected in IVF and clone foetuses than in the control group, with no differences in expression for *SORD*, suggesting a potential increase in fructogenic activity in liver of IVF and clone concepti.

In our study, umbilical blood flow seems to be 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, 1990ab). The umbilical blood flows determined in Nellore IVD concepti carried by *Bos taurus* x *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 (1990b) 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 (Ferrell, 1990b). Uterine blood flow was similar (5.01 L/min) to Brahman concepti carried by Brahman dams (Ferrell, 1990b), and lower (8.75 L/min) than for European breeds (Reynolds & Ferrell, 1987) or Charolais or Brahman concepti carried by Charolais recipient females (9.24 and 7.18 L/min, respectively; Ferrell, 1990b). Interestingly, mean umbilical blood flows were similar in both studies above, in spite of the difference in foetal weights for the cattle breeds used in those experiments, being 1.8-fold higher in European cattle than 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, 1990b) ranged from 0.10 to 0.21 L/min per kg of fetus. In this study, the estimation of umbilical blood 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 clone group, unlike findings by Li *et al.* (2005), who observed similar levels in maternal plasma between the clone, 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. The foetal parameters of renal function showed differences between groups in urea and uric acid, with higher plasma levels in the clone 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 the 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 clone 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 AI 8 controls. The urea, creatinine, AST and ALT levels were within the normal range in all the clones.

In relation to the foetal systemic physiology, plasma concentrations of LDH, other enzymes and TPP were lower in IVF and clone foetuses compared to controls, indicating that the reversible oxidation of lactate to pyruvate and metabolic rate in these animals occurs 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 tendency towards a higher level of total bilirubin in the plasma of clones and the IVF group than in controls, possibly indicating a higher 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. Moreover, they highlighted that cloned calves tend to experience a greater increase in total bilirubin concentrations, although not significant during the first 24 hours of age, suggesting that anemia was at least partially of hemolytic origin.

Phosphorus plasma concentrations, calcium and chlorides 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 the allantoic fluid volume was observed in IVF pregnancies compared to controls, concomitant to higher osmolality values. Since these study also found a higher concentration 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 fluid 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 fluid 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 of establishing the actual changes present in eah 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 of 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).

Triglyceride levels (TG) in the plasma are increased after the intake of high-fat foods, when there is deficiency of the enzyme lipoprotein lipase activity, and secondarily to other processes such as diabetes mellitus or genetic failure of the activity of this enzyme. TG formed in the liver is transported in the blood in the form of low-density lipoproteins (VLDL). These compounds mainly consist of triglycerides (approximately 60%), also containing cholesterol, phospholipid, and plasma proteins. In our study, the TG and VLDL were higher in the plasma of clones than controls. In addition, cholesterol and LDL were lower in clones foetuses than in controls. Cholesterol circulates in plasma bound to lipoproteins (HDL, LDL and VLDL) and approximately 2/3 of it is esterified with fatty

acids. Plasma cholesterol levels are appropriate indicators of total lipids in the plasma, as it corresponds to approximately 30% of the total (González & Silva, 2006).

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, which resembled the IVF group. Additionally, the results of expression of important genes for feotal 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 IVP embryos in pre- and post-natal development, with an impact in animal welfare, and possible by finding relationships with naturally occurring problems in herds, which could be of great economic and scientific importance. Additional studies involving metabolism, epigenetic reprogramming and control of gene expression should be conducted in *in vitro*-derived concepti, providing greater and better understanding of events involved in the abnormalities in late pregnancy, also allowing the identification of components or causal factors in early embry development that ultimately determine the phenotypic changes observed during development, including the post-natal period.

ACKNOWLEDGEMENTS

This study was supported by Procad II Grant (no. 192058) funded by CAPES/Brazil, and by the RECODISA Grant (no. 0460/08) funded by FINEP/MCT/Brazil. The authors thank Dr. M.Z. Moreira from CENA-USP/SP/Brazil; F.H. Furlan from UFMT/MT/Brazil; and Dr. R.E.G Ricci and P.R. Facciotti from USP/SP/Brazil for their technical assistance. Dr. V.H.V. Rodrigues was supported by a FUNCAP/Brazil doctoral fellowship.

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6 SUMMARY OF THE MOST SIGNIFICANT FINDINGS IN THE STUDY

a. Pregnancy per embryo transfer was similar between groups, but pregnancy loss during the first trimester was greater in the NT-HMC than in the other two groups.
b. Fetal traits measured on Day 51 were smaller in NT-HMC than in the IVD and IVF concepti, placentome measurements were similar between groups, and the IVD and IVF groups were similar for all measured traits.
c. The mean fetal weight was up to 2-fold higher in the NT-HMC group than in the IVD and IVF groups, which were similar.
d. Placenta weight and surface area, and fetal membrane weight were larger in the NT-HMC group than the other groups. Fetal fluid volumes were similar between groups. All parameters were similar between the IVD and the IVF group.
e. Similar frequencies of types B and D placentomes were observed between groups, but fewer type A and more type C placentomes were observed within both the IVF and NT-HMC groups compared with IVD controls. An even distribution in numbers and frequency of placentomes types A, B, and C was observed in the NT-HMC group.
f. The NT-HMC group had a higher frequency of giant placentomes. The total placenta weight in clones was larger than the IVD and IVF groups. Total type A placentome weight was lower in the IVF group than in the IVD group.
g. A few biochemical and molecular differences were observed between groups, with the maternal plasma in the NT-HMC group having higher chloride levels, osmolarity, conjugated bilirubin, and urea concentrations, and lower ALT levels than the IVD and IVF groups. Total bilirubinemia was higher in clones than in IVD controls.
h. Most parameters evaluated for fetal liver and kidney functions, hemoglobin metabolism, and osmolarities in fetal plasma from the umbilical vein and artery and in the amniotic and allantoic fluids were higher in clones than the other groups.

i. Glucose and fructose concentrations in umbilical arterial and venous plasma, and fructose levels in the allantoic fluid were higher in clones than the other groups.
j. The total amount of glucose and fructose in the fetal fluids revealed an increase in glucose and fructose in the allantoic fluid of cloned concepti than in the other groups.
k. Tryglycerides and VLDL levels were higher, and cholesterol, HDL and LDL were lower in the plasma of clones than in the other groups.
l. Differences in the relative expression levels in the fetal liver between groups were detected for genes related to fructose and glucose metabolism, sugar transmembranal transport, and fatty acid and cholesterol metabolism. An increase in the fructolytic pathway activity was seen in cloned fetuses than in IVD controls.
m. Paradoxically, an increase in fructogenic potential in the liver of cloned and IVF-derived concepti was suggested to occur.
n. Sugar moieties correlated with one another across the fetal fluid compartments, which were associated with the pattern of gene expression for important metabolic enzymes.
o. Transcripts for key fructolytic and glycolytic enzymes correlated with most measurements for glucose and fructose in the maternal plasma, foetal plasma, and allantoic fluid. Transcripts for the gluconeogenic pathway correlated with glucose and fructose levels in the allantoic fluid.

7 CONCLUSÕES

Com base nos resultados deste estudo, traçamos as seguintes conclusões:

a. Foram estabelecidas relações fisiológicas da *Abnormal Offspring Syndrome* (AOS) com os parâmetros físicos, bioquímicos, metabólicos e moleculares estudadosem conceptos bovinos derivados da clonagem por TNCS, aos 225 dias de gestação.
b. O crescimento bifásico comum aos clones foi evidenciado, com um retardo de desenvolvimento aos 51 dias de prenhez, associado a elevadas perdas gestacionais no primeiro trimestre de gestação, que se relacionaram com um desvio de crescimento fetal e de alterações placentárias plenamente detectáveis aos 225 dias de gestação.
c. A produção *in vitro* de embriões pela FIV por um sistema de cultivo *in vitro* optimizado gerou conceptos semelhantes aos controles em termos morfológicos e fisiológicos, com algumas alterações ainda detectáveis na morfologia placentária.
d. 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 e um aumento dos tipos anormais.
e. Parâmetros de função hepática, renal e do metabolismo da hemoglobina, dos carboidratos e lipídeos foram distintos em gestações de clones, com um padrão indicativo de maior aporte de substratos ao feto, corroborado por perfis de expressão gênica de enzimas de rotas metabólicas que denotam um maior aporte de carboidratos, com um aparente desvio para outras rotas metabólicas (via das pentoses, lipogênese).
f. As características físicas de conceptos clones foram correlacionadas positivamente com variáveis associadas a um padrão fisiológico ativo e de promoção do crescimento fetal, e negativamente com o metabolismo do colesterol e parâmetros da função hepática, o que indica uma atividade metabólica menor do fígado fetal de clones, apesar deste órgão ser alometricamente maior em clones.

g. A sequência de eventos morfológicos e fisiológicos observados no curso do desenvolvimento de conceptos clones neste estudo corrobora 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.

8 PERSPECTIVAS

Com esse estudo esperamos ter estabelecido uma relação entre os padrões fenotípicos útero-placentário e fetal de crescimento e os padrões de uso de substratos pela placenta ao feto. Possíveis mecanismos fisiológicos de alteração de desenvolvimento em prenhezes de FIV e TNCS forma evidenciados e discutidos, abrindo novas perspectivas de estudos, em especial da função metabólica dos fluidos e tecidos maternos e fetais, os quais poderão contribuir para o melhor entendimento do efeito da PIV de embriões no desenvolvimento pré- e pós-natal subsequentes.

O Brasil ainda necessita de esforços no sentido de congregar idéias e competências e de formular hipóteses relacionadas a problemas correspondentes ao desenvolvimento e/ou ao aprimoramento das tecnologias voltadas à Reprodução Animal. Tais esforços e competências devem estar direcionados à solução de problemas, tais como o incremento dos índices de fertilidade em bovinos, a diminuição das taxas de mortalidade embrionária em bovinos e os fatores relacionados à adaptação das tecnologias, relativas à neonatologia, a prenhez e ao parto. Estas devem ser priorizados com vistas ao aumento da produtividade, ao estabelecimento e multiplicação de competências e a aplicação prática da tecnologia disponível nos diversos laboratórios de pesquisa. Muitos, se não todos os problemas de desenvolvimento, com as consequentes taxas de morbidade e mortalidade que ocorrem após as manipulações *in vitro* de embriões, como a FIV e a TNCS, já ocorrem na natureza e nos rebanhos; o uso destas biotécnicas exacerba a frequência de ocorrência de tais problemas. Desta forma, a identificação de mecanismos causais de cada etapa e processo de anormalidade gera novos conhecimentos sobre os padrões de normalidade *per se*, muitas vezes desconhecidos, e também pode auxiliar na elucidação de problemas de impacto nos rebanhos, como a mortalidade embrionária e pós-natais. As expectativas científica e econômica a serem alcançadas com a conclusão de estudos como os propostos é de obtermos o conhecimento da função fisiológica e metabólica materna e fetal provenientes da FIV e pela clonagem por TNCS e, com isso, por exemplo, diminuir as taxas de mortalidade embrionária, com o nascimento de animais com maior viabilidade. Isto pode se repercutir em uma maior prolificidade e produtividade, reduzindo os custos de produção e aumentando a margem de lucro do produtor. Assim, faz-se importante os estudos sobre a síndrome dos bezerros anormais (SBA), evitando prejuízos aos rebanhos comerciais, o que poderá levar um benefício econômico a médio e longo prazo.

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10 ANEXO – CERTIFICADO DO COMITÊ DE ÉTICA

UNIVERSIDADE DE SÃO PAULO
Faculdade de Medicina Veterinária e Zootecnia

Comissão Bioética

CERTIFICADO

Certificamos que o Projeto intitulado "Fisiologia e metabolismo placentário por canulação cordonal em gestações de bovinos normais, FIV e clonados", protocolado sob o nº902/2006, utilizando 30 (trinta) bovinos, sob a responsabilidade da Profa. Dra. Maria Angélica Miglino, está de acordo com os princípios éticos de experimentação animal da Comissão de Bioética da Faculdade de Medicina Veterinária e Zootecnia da Universidade de São Paulo e foi aprovado em reunião do dia 17/05/2006.

(We certify that the Research "Physiology and placental metabolism by cordonal canulation in pregnancies of normal, IVP and cloned cattle", protocol number 902/2006, utilizing 30 (thirty) bovines, under the responsibility of Profa. Dra. Maria Angélica Miglino, agree with Ethical Principles in Animal Research adopted by Bioethic Commission of the Faculty of Veterinary Medicine and Zootechny of University of São Paulo and was approved in the meeting of the day 05/17/2006).

São Paulo, 18 de maio de 2006

Profa Dra Denise Tabacchi Fantoni
Vice-Presidente da Comissão de Bioética
FMVZ/USP

Av. Prof. Dr. Orlando Marques de Paiva, nº 87 – 05508-270 – Cidade Universitária "Armando de Salles Oliveira". Fax: (11) 3032-2224 – fones: (11) 309107676/7671 – e-mail: fmvz@edu.usp.br

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