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CÉLULAS-TRONCO DE CORDÃO UMBILICAL EM MODELO EXPERIMENTAL DE ASFIXIA NEONATAL EM SUÍNOS

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Lista de abreviaturas

BDNF	Brain-derived neurotrophic factor
BE	Basic excess
BLAST	Basic Local Alignment Search Tool
bp	Base pairs
bpm	Breaths per minute
CNS	Central nervous system
CO_2T	Total CO_2
CVA	Cerebrovascular Accident
DBPS	Disinfection by products
DNA	Deoxyribonucleic acid
FSC	Fluxo sangüíneo cerebral
HCO_3	Bicarbonate
H-I	Hypoxic-ischemic insult
HLA	Human leukocyte antigen system
HR	Heart rate
HUCSC	Human umbilical cord stem cells
IV	Intravenous
MAP	Mean arterial pressure
mM	Millimolar
mm^3	Cubic millimeter

mm Hg	Millimeter of mercury
NGF	Nerve growth factor
NT3	Neurotrophin-3
PBS	Public broadcasting service
PCR	Polymerase Chain Reaction
pCO_2	Partial CO_2 pressure
pO_2	Partial O_2 pressure
PUC-RS	Pontifícia Universidade Católica do Rio Grande do Sul
SNC	Sistema nervoso central
RPM	Rotations per minute
µm	Micrometer
SA	Sum of the areas
Sat O2	Saturation
SD	Standard deviation
T	Distance between the analyzed sections
V(est)	Volume estimation

Resumo

EFEITOS DO TRANSPLANTE DE CÉLULAS-TRONCO DE CORDÃO UMBILICAL EM MODELO EXPERIMENTAL DE ASFIXIA NEONATAL EM SUÍNOS

Introdução: A asfixia neonatal é a principal causa de lesão cerebral no período perinatal, tendo como conseqüências alta mortalidade e grande número de seqüelas neurológicas. Atualmente, várias estratégias neuroprotetoras estão sendo avaliadas em modelos animais na tentativa de reduzir a morte celular e melhorar os desfechos neuro-comportamentais dos recém nascidos, mas os resultados são pouco expressivos. Estudos sugerem que o transplante de células-tronco limitaria a expansão de lesões e facilitaria o reparo de tecidos lesados, podendo se constituir numa opção terapêutica em casos de asfixia. Os pesquisadores optaram pelo uso de um modelo em suínos recém-nascidos devido ao fácil manejo, baixo custo e similaridade de peso e tamanho em relação aos bebês.

Objetivo: O objetivo deste estudo é analisar de que forma células-tronco de cordão umbilical humano, infundidas via intra-arterial entram no cérebro, sobrevivem neste micro ambiente, e promovem a recuperação da função neurológica após insulto hipóxico-isquêmico, usando dois tipos diferentes de acessos arteriais.

Materiais e métodos: Foram utilizados 36 suínos com até dois dias de vida divididos em 4 grupos: Grupo I (Sham), Grupo II de controle, Grupo III tratado com células-tronco via artéria umbilical, e Grupo IV com células-tronco injetadas pela artéria carótida comum.

Para a indução da asfixia utilizou-se a associação simultânea de procedimentos que causavam hipóxia e isquemia. As células-tronco foram obtidas a partir de sangue umbilical humano.

Com 2, 7, 14 e 21 dias de vida os animais eram examinados e era aplicado um escore neurológico. O tecido cerebral de animais tratados com células tronco que morreram antes de completar 21 dias foi utilizado para pesquisa de PCR para DNA humano. Aos 21 dias os animais sobreviventes eram novamente levados a sala cirúrgica, anestesiados profundamente a fim de serem sacrificados e realizar-se uma perfusão trans-cardíaca com paraformaldeído para a extração dos encéfalos. Logo após, era aplicada a técnica histológica de Nissl e realizada a estimativa de volume encefálico para avaliação do grau de lesão cerebral.

Resultados:. Aos 21 dias houve diferença entre a média dos escores do grupo que recebeu células pela carótida quando comparada as dos grupos controle e o que recebeu células pela artéria umbilical. Na pesquisa através de PCR em animais do grupo das células-tronco pela artéria carótida comum foi possível a visualização da banda correspondente ao gene β-globina humano em dois dos quatro animais em diversos pontos de tecido cerebral em amostras obtidas 15 e 24 horas após o procedimento de asfixia. Não se identificou PCR positivo nas coletas realizadas 7 dias e 15 dias deste mesmo grupo bem como em nenhuma das amostras dos animais do outro grupo pesquisado . Não houve diferença entre as médias dos volumes encefálicos nos quatro grupos. O volume cerebral e o peso final dos animais apresentaram uma correlação positiva moderada.

Conclusão: Os resultados deste estudo sugerem que a administração de células-tronco de cordão umbilical humano via artéria carótida comum em modelo de hipóxia-isquemia em suínos está associada a presença de PCR positivo para o gene da β-grobina humana e a uma melhora na função neurológica com 3 semanas embora sem evidência de diminuição da área de lesão.

Descritores: 1. Células-tronco, 2. Asfixia Neonatal, 3. Cordão Umbilical, 4. Hipóxia-isquemia encefálica, 5. Modelos Animais, 6. Animais recém-nascido,. 7. Suínos, 8.Humanos .

Abstract

EFFECTS OF THE UMBILICAL CORD STEM CELL TRANSPLANT IN A PIGLET MODEL OF NEONATAL ASPHYXIA

Introduction – Neonatal asphyxia is the main cause of brain damage in the perinatal period. Studies suggest that the stem cell transplant would curb the expansion of damages and facilitate the repair of damaged tissues, and could thus become a therapeutic option in cases of asphyxia.

Objective: In the present study we tested whether intra-arterialy infused human umbilical cord stem cells enter brain and survive in the brain microenvironment, and improve neurological functional recovery after hypoxic-ischemic insult using two two different arterial access.

Materials and methods: Thirty-six healthy piglets not older than two days were divided into four groups: Group I (Sham), Group II, which was the control group, Group III, treated with stem cells infused through the umbilical artery, and Group IV, treated with stem cells injected via the common carotid artery. Stem cells were obtained from human umbilical cord blood.

For induced asphyxia, a simultaneous association of procedures that caused hypoxia and ischemia was used. The brain tissue of treated animals that died before completing the twenty-one days was used for PCR research for human DNA. At two, seven, fourteen, and twenty-one days after the procedures, the animals a neurologic score was applied. After twenty-one days, the survivors were taken to the surgery room again, deeply anesthetized and a transcardiac perfusion was performed in order to be sacrificed. After this, the animal brains were slowly extracted and the Nissl histological staining technique was used to assess the degree of brain damage.

Results : At 21 days there were differences among the average scores of group treated via carotid, when compared to those of control group and treated via umbilical artery. At other assessment moments no differences were found. In the PCR research of animals that received stem cells via the common carotid artery catheter it was possible to visualize the band corresponding to the human β-globin in several points of the researched brain tissue samples of two of the four animals. The samples with positive PCR were obtained fifteen and twenty-four hours after the asphyxia procedure. Likewise, no positive PCR was found in any of the samples of the animals in group III. The averages and SD of encephalic volume in four groups didn't show differences and brain volume and final body weight of the animals had a moderate positive correlation

Conclusion : The results of this study suggest that the administration of human umbilical cord stem cells via the carotid artery in a hypoxia-ischemia model in piglets is associated with the presence of positive PCR for the human β-globin gene, and led to a significant improvement in neurological function with 3 weeks, although there was no evidence of decreased lesion area.

Key-words: 1.Stem cells, 2.Neonatal asphyxia, 3.Umbilical cord blood, 4. Hypoxic-ischemic brain injury, 5.Animal models, 6.Animal newborns, 7.Piglets, 8. Humans.

CAPÍTULO I

1. INTRODUÇÃO E JUSTIFICATIVA

Esta tese foi redigida sob forma de dois artigos originais conforme as normas do programa de Pós-Graduação em Pediatria. O primeiro é apresentado no capítulo II sob o título "*DEVELOPMENT OF AN EXPERIMENTAL PIGLET MODEL OF NEONATAL ASPHYXIA WITH 21-DAY SURVIVAL*", e descreve o desenvolvimento de um modelo experimental de asfixia neonatal em suínos em que os animais apresentam sobrevida por um período longo. Já no capítulo III, descrevemos o segundo artigo, intitulado **"***EFFECTS OF THE UMBILICAL CORD STEM CELL TRANSPLANT IN A PIGLET MODEL OF NEONATAL ASPHYXIA*" que tem como objetivo principal analisar de que forma células-tronco de cordão umbilical humano, infundidas pela corrente sanguínea se instalam no cérebro, sobrevivem neste micro ambiente, e promovem a recuperação da função neurológica após insulto hipóxico-isquêmico, utilizando-se dois tipos diferentes de acessos intra-arteriais no modelo experimental desenvolvido no estudo anterior. Os artigos foram redigidos no formato da revista *Pediatric Research*.

1.1. Anóxia neonatal

De acordo com a *American Academy of Pediatrics* e o *American College of Obstetrician and Gynecologists*, anóxia ou asfixia neonatal é definida como uma agressão hipóxico-isquêmica grave ao feto ou ao recém-nascido que tem como resultado um percentual elevado de danos neurofisiológicos permanentes e alta mortalidade(1). Asfixia é conseqüência do bloqueio da troca gasosa que leva a três efeitos bioquímicos: hipoxemia, hipercapnia, e acidose metabólica. As condições a seguir caracterizam a asfixia neonatal:

- Evidência de acidose no sangue de cordão umbilical obtido no parto;
- Escore de Apgar de 0-3 por 5 minutos ou mais;
- Evidência de seqüela neurológica e em um ou mais dos sistemas orgânicos a seguir: cardiovascular, gastrintestinal, hematológico, pulmonar, hepático ou renal.

Os recém-nascidos são particularmente vulneráveis a asfixia durante o parto ou imediatamente após este. Quando ela começa no útero, pouco antes ou durante o trabalho de parto, decorre geralmente de comprometimento do fluxo sanguíneo da placenta ou do cordão umbilical e após este período muito provavelmente se origine de problemas na passagem do ar pelas vias aéreas. (2, 3).

A suscetibilidade do cérebro imaturo à asfixia perinatal depende do estado temporal e regional do processo de desenvolvimento, bem como, da proliferação, migração, diferenciação, mielinização, morte programada de células e da regulação no fluxo sangüíneo cerebral e metabolismo(3).

Atualmente, várias estratégias neuroprotetoras estão sendo avaliadas em modelos animais na tentativa de reduzir a morte celular apoptótica e, assim, melhorar os desfechos comportamentais. Dentre os novos tratamentos propostos para recuperar o tecido cerebral lesado pelos efeitos da anóxia neonatal inclui-se: inibidores de aminoácidos excitatórios e radicais livres, óxido nítrico, caspases, topiramato e hipotermia(4, 5). Mais recentemente o transplante com células-tronco também tem sido considerado como alternativa terapêutica(6).

1.2. Modelos animais de anóxia neonatal

Nenhum modelo de anóxia perinatal é considerado ideal, apesar das pesquisas em animais têm sido de grande importância neste campo. Modelos com porcos e ovelhas parecem ser os mais apropriados para os estudos de curto prazo (até aproximadamente uma semana) e com ratos mais apropriados para estudos mais longos (com duração de várias semanas). O modelo suíno tem se mostrado bom para estudos de fluxo sangüíneo cerebral (FSC) e metabolismo, sendo que atualmente está bem padronizado. Os estudos em curto prazo têm nos ajudado a entender a fisiopatologia da asfixia, mas estudos de maior prazo têm maior possibilidade de oferecer evidências histopatológicas de lesão cerebral. Nestes últimos é possível também avaliar achados clínicos e neurológicos(7-10).

Lê blanc et al.(11, 12) desenvolveram um modelo suíno com sobrevivência de prazo relativamente longo utilizando a combinação de oclusão de vasos cerebrais, seguidos por um período de hipotensão hemorrágica e hipóxia. Neste modelo a mortalidade foi de 30% e aproximadamente 70 a 80% dos animais sobreviventes apresentaram déficits neurológicos. Munnkeby et al(13, 14) desenvolveram dois modelos agudos de asfixia utilizando mistura gasosa de oxigênio a 8%. Em um deles a manutenção da hipóxia durou até a pressão sanguínea média alcançar 15 mm Hg ou o excesso de base alcançar -20 mM. No outro modelo deste autor os animais foram submetidos a 30 minutos de hipóxia simultâneo a um clampeamento bilateral das carótidas comuns. Nossa idéia foi fazer uma associação entre os estudos destes dois autores e desenvolver um modelo suíno de prazo maior, em que pudéssemos avaliar os efeitos da infusão de células tronco. Fatores como o peso e tamanho dos suínos recém-nascidos que são parecidos com o de bebês, o fácil manejo e o baixo custo, fazem com que este modelo seja muito atrativo e factível. Além disso, existe uma quantidade considerável de literatura relacionada a metabolismo e FSC em suínos(7, 15, 16).

1.3. Terapia com células-tronco

As células-tronco são definidas funcionalmente como células que tem a capacidade de auto-renovação associada a habilidade de gerar diferentes células, ou seja, elas podem gerar células filhas idênticas à mãe (auto-renovação), além de produzir linhagem com potencial mais restrito (células diferenciadas) (17, 18).

Pode-se classificá-las em dois tipos principais: as do tipo embrionário existente nas primeiras semanas do período fetal, e as do tipo adulto que predominam após esta fase. Funcionalmente, as do tipo adulto são responsáveis pelo reabastecimento tecidual ao longo da vida e estão presentes na maioria dos tecidos humanos, tais como, o sangue, a pele, o fígado, o coração e o cérebro. Há muitos anos doenças hematológicas malignas têm sido tratadas através do transplante de células-tronco tipo adulto de medula óssea ou de sangue de cordão umbilical e atualmente estudos em diversas doenças de variados órgãos e sistemas vêm testando esta terapia(18-25).

1.4. Células-tronco e doenças neurológicas

Nos últimos 30 anos ocorreram grandes avanços no campo do transplante neural, e muitos estudos clínicos vem sendo propostos. Em boa parte dos trabalhos células embrionárias são transplantadas no cérebro de pacientes com doenças neurológicas, incluindo doença de Parkinson e Huntington e, apesar de alguns resultados controversos, existe uma concordância geral de que esta terapia trouxe benefícios aos pacientes (26-29). Todavia, a aplicação do transplante de células embrionárias em terapia de larga escala encontra séria resistência relacionada a aspectos éticos e metodológicos, uma vez que se utiliza material abortivo. Conseqüentemente, um grande esforço tem sido devotado para encontrar fontes doadoras alternativas, dentre as quais as células-tronco do tipo adulto provenientes da placenta e cordão umbilical (4, 20, 28, 30-32).

1.5. Aspectos Éticos

Na coleta de sangue de cordão umbilical, embora se trate de material de descarte, optamos por solicitar o consentimento informado de todas as gestantes cujo material da placenta tenha sido utilizado.

O presente trabalho baseia-se no princípio de valorizar a vida animal, considerando sua sensibilidade e procurando sempre reduzir ou evitar sofrimentos desnecessários. Russell et al l(33) conseguiram sintetizar com 3 palavras o Princípio Humanitário da experimentação animal, o que ficou definido como o princípio dos 3 Rs devido a sua grafia em inglês.

Replasements, ou seja, Alternativas, indicando que sempre que possível devemos usar, no lugar de animais vivos, materiais sem sensibilidade. No caso do presente trabalho, que busca uma avaliação de aspectos clínicos e terapêuticos embora haja uma fundamentação *in vitro* bem estabelecida, a complexidade dos processos envolvidos e a impossibilidade de avaliação dos resultados em seres humanos, não deixam alternativas senão a experimentação em animais. Trata-se, portanto, de um indispensável estudo pré-clínico, a fim de que se obtenha indicações prévias sobre a possibilidade das células-tronco apresentarem efeito terapêutico e proporcionarem segurança.

Reduction, quer dizer, o número utilizado deverá ser o menor possível, baseado em um cálculo amostral e o minimamente suficiente para que se alcance resultados confiáveis pelos métodos estatísticos disponíveis.

Refinement, aprimoramento, refere-se a técnicas menos invasivas ou ao manejo de animais somente por pessoas treinadas. No presente estudo, os protocolos experimentais utilizados seguirão as normas internacionais de experimentação animal. Os procedimentos anestésicos, sedativos, e as técnicas utilizadas estarão de acordo com a prática veterinária correntemente aceita, evitando-se ao máximo a dor e o sofrimento. Durante a fase de recuperação os animais ficarão em local apropriado onde pessoas treinadas serão encarregadas da alimentação, recuperação dos ferimentos e cuidados com a temperatura e a higiene.

1.6. Justificativa

O sistema nervoso central (SNC) é um dos principais sistemas acometidos por lesão tecidual no período perinatal, e a anóxia neonatal é a causa mais importante de dano neurológico ocorrendo em aproximadamente 2-4:1000 nascidos vivos a termo. (6, 34). Recentemente, muitas pesquisas estão avaliando a aplicação de células-tronco nas mais diversas doenças, em especial no campo da neurologia.

A terapia celular poderia facilitar o reparo de tecidos lesados e exercer efeito protetor, limitando a expansão de lesões. A capacidade potencial destas células em responder a sinais sistêmicos de tecidos lesados, de migrar para estas regiões, de substituir tecidos mortos ou de proporcionar proteção por secreção de hormônios de crescimento e fatores de proteção específicos, são consideradas características desejáveis às necessidades terapêuticas da medicina perinatal. Há também algumas particularidades dos recém nascidos poderiam oferecer vantagens. As dimensões relativamente pequenas, a perspectiva de futuro desenvolvimento do bebê e a disponibilidade de material da placenta, que é um grande reservatório de sangue fetal, favorecem a obtenção, a aplicação e os efeitos do tratamento(6, 28, 35).

Existe, porém, um número menor de estudos nesta faixa etária. Além disso, várias questões carecem de respostas mesmo considerando os bons resultados em estudos com modelos adultos. Exatamente que mecanismos moleculares, celulares,

fisiológicos estariam implicados? Quais os tecidos seriam mais suscetíveis à sua utilização? Qual a duração dos possíveis efeitos? Que via de administração e momento de aplicação seriam mais adequados (2, 6, 17, 20, 22, 23)?

Um estudo pré-clínico visando contribuir na elucidação de algumas destas questões é de fundamental importância, especialmente quando levamos em consideração o uso desta terapia durante o período perinatal.

1.7.Objetivos

Objetivo geral:

Avaliar os efeitos da injeção de células-tronco de cordão umbilical humano em um modelo experimental em suínos de anóxia neonatal.

Objetivos específicos:

1. Testar um modelo experimental de asfixia neonatal em suínos recém-nascidos;

2. Comparar as alterações histológicas, e neurocomportamentais entre animais não tratados e tratados com células-tronco administradas por duas vias arteriais diferentes, após serem submetidos à asfixia neonatal;

3. Detectar a migração das células-tronco transplantadas nas regiões encefálicas acometidas pelo insulto hipóxico-isquêmico através de PCR humano.

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CAPÍTULO II

2. DEVELOPMENT OF AN EXPERIMENTAL PIGLET MODEL OF NEONATAL ASPHYXIA WITH 21-DAY SURVIVAL

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ABSTRACT: Perinatal Asphyxia is the most frequent cause of neurological damage in newborn. The purpose of this study was to develop a swine model of Neonatal Asphyxia in which the animals remain alive for a period up to twenty-one days, keeping evidence of damage throughout the study. We used combined hypoxia and ischemia induction techniques in two different time regimes. Twenty animals were divided into three groups: Group I (n-5) or Sham , Group II(n-5), submitted to hypoxia and ischemia for forty-five minutes, and Group III(n-10), submitted to hypoxia and ischemia for variable periods of time, based on a combination of hypotension and acidosis. At 2, 7, 14, and 21 days after the procedures, the animals were examined ,any abnormalities found were recorded, and a neurological score was performed. After twenty-one days, the piglets were deeply anesthetized and a transcardiac perfusion was performed to the brain extraction . To estimate the degree of brain damage, we applied a brain volume method. Most part of animals in Group II presented changes forty-eight hours after the procedure but seven days later, such changes were still found in just one of the animals. In Group III, just one of survivors did not present any kind of abnormality after forty-eight hours, and after the twenty-one days, four of the five survivors did not demonstrate effective sucking. There was a significant difference in the neurological score mean of Group III, if compared with the other groups, in the four evaluations. Brain volume and final body weight of Group III were significantly higher, compared with the other two groups. In conclusion, this model of neonatal asphyxia with association of hypoxia and ischemia was useful in a more prolonged follow-up, thus enabling the identification of neurological changes throughout the twenty-one days of the study, mainly when the time of the insult was based on parameters of hypotension and acidosis. The brain damage estimation based on brain volume was not effective to determine damage.

The central nervous system (CNS) is one of the main systems affected by tissue injury in the perinatal period, and Perinatal Asphyxia is the most frequent cause of neurological damage. It occurs

in approximately 2-4 out of every 1,000 living infants born at full term. The damages caused by Asphyxia to the newborn infant are the result of the association between hypoxia and ischemia, affecting the organism in a generalized manner, and oftentimes leading to a severe neurological damage, difficult to recover from, and in many instances related to long-term repercussions (1-3). According to the American Academy of Pediatrics and the American College of Obstetricians and Gynecologists, neonatal anoxia or asphyxia is defined as a severe hypoxic-ischemic aggression to the fetus or newborn, which results in a high percentage of permanent neurophysiological damage and high mortality rate (4) (5) .

Although research conducted with animals has been of great importance in this field, no model of perinatal asphyxia is considered ideal. Models with pigs and sheep have been more widely used in short-term studies (of approximately up to one week), whereas rats are used in longer studies (lasting several weeks). The short-term studies are used to study the physiopathology of asphyxia, and the more prolonged ones are used to assess clinical aspects, and these are more favorable to the detection of histopathologic evidence of brain damage (6-10). In this study, we chose a model in swine. Several characteristics are interesting in this model. The gyroenchephalic anatomy and the brain vascularization of the swine are similar to those of humans, and there is a considerable amount of literature concerning metabolism and brain blood flow (6, 8, 11-13) (14). Due to the size and body weight of the animals, the swine models enable easy obtaining of vascular accesses, as well as cardiovascular and respiratory monitoring, thus also allowing the use of neonatal intensive treatment equipment. Nevertheless, the number of longer-term studies is relatively small.

The objective of this study is to use a swine model of Neonatal Asphyxia in which the animals remain alive for a period up to twenty-one days, keeping evidence of damage throughout the study. For this purpose, we planned to use combined hypoxia and ischemia induction techniques in two different time regimes.

METHODS

Twenty-nine healthy piglets of the *Sus scrofa* race, obtained from a local farm and not older than two days old, were used in this study. Initially, a pilot study was conducted, in which nine animals were subjected to the hypoxia and ischemia association, with different durations. Based on the results of the pilot, the duration of the hypoxia and ischemia was established for one of the groups in the subsequent phase of the study. In the next stage, twenty animals were divided into three groups: Group I (Sham), Group II, submitted to hypoxia and ischemia for forty-five minutes, and Group III, submitted to hypoxia and ischemia for variable periods of time, based on a combination of hypotension and acidosis. The first two groups were composed of five animals, whereas the third group had ten animals. The protocol of this study was approved by the Research Ethics Committee of the Medical School of PUC-RS, under the following registration number: CEP-PUC 06/03425.

Anaesthesia, ventilation and monitorin of physiological variables .After the stabilization period, the animals were subjected to anesthetic induction with inhalational halothane (3%),

endotracheal intubation, and umbilical cord vessel catheterization. For anesthetic maintenance, ketamine(15-20 mg/kg IV or IM) and xilazine (2mg/Kg IV or IM) was used every two hours. After intubation the animals were kept on a mechanical ventilator (BP 400, Pró Médico,São Paulo,Brasil) with respiratory rate of 20 breaths per minute(bpm), PIP of 15 mmHg and PEEP of 3 mmHg and oxygen concentration of 21%.

The heart rate, rectal temperature, and saturation of all animals were monitored by transcutaneous monitoring(Ohmeda 3800,GE,Helsinki,Finland). In the group III the mean arterial pressure was obtained via catheter installed through an umbilical artery and connected to a pressure monitoring device(Kananda 2, Belo Horizonte, Brasil) . For the same catheter samples were collected for determination of arterial blood gases.

Protocol for hypoxic-ischemic insult and experimental groups. For induced asphyxia, a simultaneous association of procedures that caused hypoxia and ischemia was used. Hypoxia was obtained by administering an inhalational mixture of 8% O2 and 92% nitrogen through an endotracheal tube connected to a mechanical ventilator, and ischemia was induced by clamping both common carotid arteries. The procedure was completed with the reversion of the arterial occlusion and the replacement of the hypoxic mixture with ambient air.

In Group I, the common carotid arteries of the animals were dissected, but not occluded. The animals in Group II were subjected to the inhalation of a hypoxic mixture associated with the bilateral occlusion of the common carotid artery for a fixed time of forty-five minutes. And the animals in Group III were submitted to hypoxia and occlusion of the carotid arteries until their mean arterial pressure (MAP) reached less than 30 mm Hg, associated with an arterial pH of 7.28 or below. All animals in Group III had one of their umbilical arteries dissected and catheters were introduced into them for the monitoring of the arterial pressure. Arterial blood gas samples were taken immediately before asphyxia was induced and at the moment the animals' mean arterial pressure reached 30 or below.

After the surgical procedures, the animals remained under mechanical ventilation until they regained spontaneous breathing and were fit to be transported to a shelter. In the shelter, the animals stayed in incubators, warmed by radiant heat and gavage-fed bovine milk until they were able to suck effectively to drink the milk directly from the bottle.

After twenty-one days, the survivors were taken to the surgery room again, deeply anesthetized with thiopental 50 mg/kg administered intraperitoneally for them to be sacrificed, and so that a transcardiac perfusion with physiological serum and 4% paraformaldehyde could be performed.

Neurological Evaluation. At 2, 7, 14, and 21 days after the procedures , the animals

were examined and any abnormalities found were recorded, such as gait changes, palsies, difficulty sucking, hypoactivity, and abnormal movements. A neurological score previously used for swine models was adapted and included the assessment of consciousness level (0 to 15 points), brainstem function (0 to 22 points), sensory response (0 to 20 points), muscle tone (0 to 8 points),postural reflexes (0 to 8 points), mobility (0 to 30), spatial orientation (0 to 20 points), activity (0 to 16 points) and seizures (0 to 10 points) (Table 1) (12).The results were recorded and scored from 0 to 149

Table 1- *Neurobehavioral Scoring Tool for piglets*

Item	Scoring code
Consciousness	0=Normal 5=clouded 10=stupor 15=coma
Brainstem function	
Respiration	0=Normal 5=Present but abnormal 10=Absent
Pupilar light reflex	0=Normal 2=Present but abnormal 4=Absent
Corneal reflex	0=Normal 2=Present but abnormal 4=Absent
Gag reflex	0=Normal 2=Present but abnormal 4=Absent
Sensory responses	
Olfaction	0=Normal 2=Present but abnormal 4=Absent
Visual threat/orienting	0=Normal 2=Present but abnormal 4=Absent
Auditory startle/arousal	0=Normal 2=Present but abnormal 4=Absent
Pain withdrawal	0=Normal 2=Present but abnormal 4=Absent
Tactile localization	0=Normal 2=Present but abnormal 4=Absent
Muscle tone	
Muscle tone, trunk	0=Normal 2=Present but abnormal 4=Flaccid
Muscle tone, limbs	0=Normal 2=Present but abnormal 4=Flaccid
Postural reflexes	
Extensor thrust	0=Normal 2=Present but abnormal 4=Absent
Wheelbarrow	0=Normal 2=Present but abnormal 4=Absent
Mobility	0=Normal postural righting and gait 5=Ataxic, walk, but walks without falling 10=Ataxic, walks but falls frequently 15=Can't walk, but stands without assistance 20=Stands only with assistance 25=Cannot stand, but attempts to right head &trunk 30=Unable to right head, no purposeful movement
Spatial orientation	
During locomotion	0=Normal 4=Abnormal 8=Absent
With sniffing	0=Normal 2=Abnormal 8=Absent
Toward depth	0=Present 4=Absent
Activity	
Appetite	0=Present 2=Abnormal 4=Absent
Vocalization	0=Present 2=Abnormal 4=Absent
Psychomotor activity	0=Present 2=Abnormal 4=Absent
Social Interativiness	0=Normal(seeks contact) 2=Abnormal(aggressive) 4=Absent (no social responsiveness)
Seizures	
Stimulus-induced myoclonus	0=Absent 5=Present
Clonic or tonic or seizures	0=Absent 10=Present
Total	Range 0 (no deficit) – 149(maximum deficit)

Brain Volume Estimation and histology. Brain volume estimation was chosen to assess the degree of brain damage. Brain damage is associated with the loss of brain tissue and the consequent decrease of brain volume.

After being extracted, the brains were photographed and stored for twenty-four hours in 4% paraformaldehyde, and then embedded in paraffin. 20- µm thick coronal sections at 1,200-µm intervals were obtained by microtome. The Nissl histological staining technique (cresyl violet method) was used. Images of the histological sections were obtained through a video camera installed on an Olympos (BX40) microscope, and later analyzed with the aid of the software Image Pro-Plus 6. 1. The brain volume estimation was determined using the Cavalieri principle, according to the following equation: V(est) = T. AS, where V(est) = volume estimation; T = distance between the analyzed sections; SA = sum of the areas (15).

Data analysis. Normal distribution variables were presented as mean and standard deviation. Comparisons between the groups were analyzed using one-way analysis of variance (ANOVA), followed by the Tukey test. In the neurologic evaluation results, when appropriate, the Kruskal-Wallis non-parametric analysis and post-hoc Mann-Whitney tests, followed by the Finner-Bonferroni correction for multiple testing were performed. Differences were considered significant at $P\_< 0.05$.

RESULTS

A total of twenty-nine animals were used. Nine of them were used in the pilot study, Group I had five animals, Group II had also five animals, and the remaining ten piglets were in Group III. In the pilot study, none of the animals submitted to hypoxia and ischemia (H-I) for less than forty-five minutes presented any neurological changes forty-eight hours after the procedure, whereas no animal submitted to the procedure for one hour or more survived for over three days.

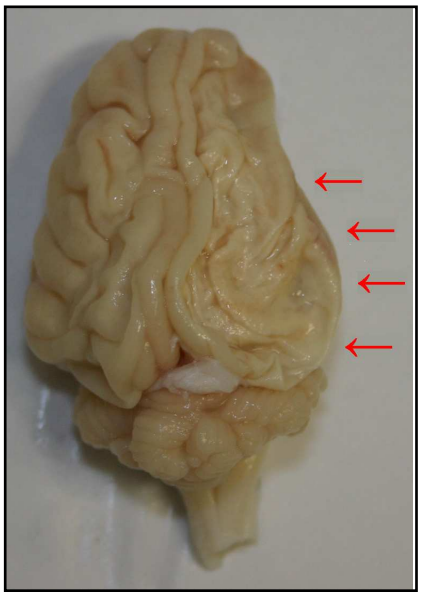

Figure 1. *Photograph illustrating the brain taken from a piglet of Group III with severe brain damage.*

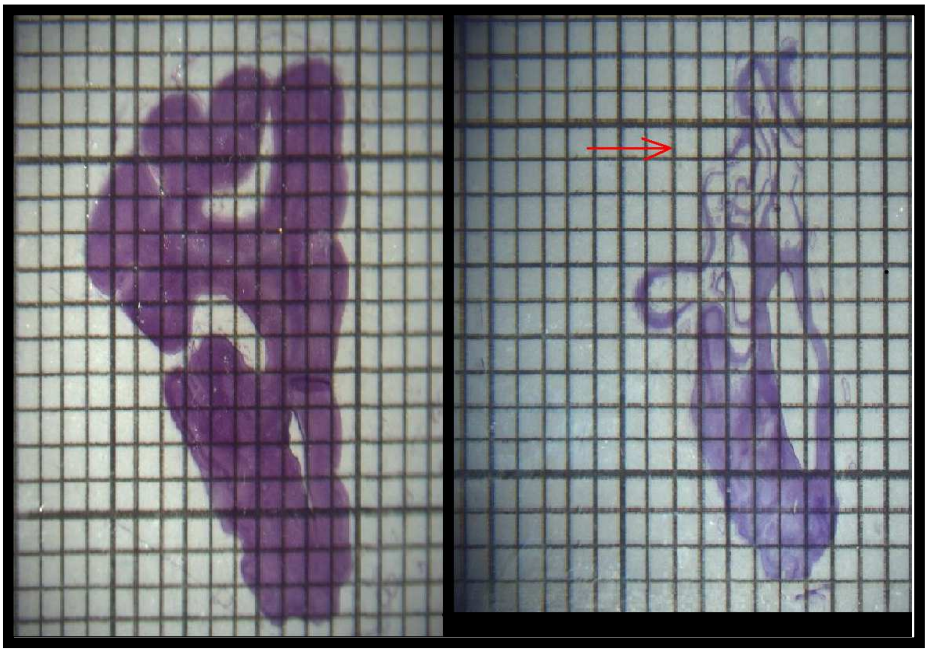

Figure 2 - *Photomicrographs of the ischemic core to marginal zone with Nissl staining. The right picture shows a hemisphesric brain damage in place indicated by red arrow, and the left one the same region in a normal brain.*

Physiologic variables before and during the hypoxic-ischemic insult. The groups did not demonstrate any differences concerning body weight, age, temperature, heart rate, and arterial saturation obtained in the pre-operative period (table 2)

Table 2 – *Physiological variables in the pre-operative period*

GROUPS	***Age (h)***	***Initial body weight (g)***	***HR (bpm)***	***Temp. (ºC)***	***Sat***
I (n-5)	10,6 ± 4,1	1430 ± 327,1	132,6 ± 29,72	36,54 ± 0,68	93,6 ± 9,86
II (n-5)	13,67 ± 7	1560 ± 243,41	156 ± 12,94	36,8 ± 0,8	98,2 ± 1,8
III (n-10)	13,6 ± 8,5	1635 ± 270,85	125,8 ± 26,55	35,9 ± 1,19	97,6 ± 1,57

Values are presented as means ± S.D.

HR = Heart rate MAP = Mean Arterial Pressure PaO2 = Partial oxygen pressure PaO2 = Partial carbon dioxide pressure Sat –O2. saturation SD = Standard deviation

In the comparison between the mean arterial pressures and the variables of the arterial blood gas samples calculated immediately before the induction and with those conducted at the end of the hypoxia and ischemia mean values for the Group III was significantly lower ($p < 0.001$), as shown in table 3.

Table 3 - *Variables of Arterial Pressure and Arterial Blood Gas of piglets from Group III*

	MAP	pH	pO2	pCO2	HCO3	CO2T	Sat	BE
Pre-Induction	57,8 ± 8,5	7,53 ± 0,1	81,8 ± 14,1	34 ± 3,1	27,7 ± 2,8	28,4 ± 3,1	96,2 ± 2	5,1 ± 2,6
Final	27,8 ± 8,5	7,15 ± 0	17,5 ± 6,8	61,7 ± 3,3	20,5 ± 3,3	22,4 ± 3,5	16,5 ± 11,8	-8,8 ± 3,4

Values are presented as means ± S.D. MAP = Mean Arterial Pressure pO2=partial O2 pressure pCO2=partial CO2 pressure HCO3=Bicarbonate CO2T= Total CO2 Sat – saturation BE= Basic excess

Survival and posthypoxic neurological examination. Signs of neurological impairment and longer survival were only identified in those animals whose pre-established procedure time was forty-five minutes. For this reason, a fixed period of forty-five minutes of H-I was established for Group II in the subsequent stage. All the five animals in Group II survived, and four of them presented changes forty-eight hours after the procedure. However, seven days later, such changes were still found in just one of the animals.

In Group III, all of them presented some sort of change forty-eight hours after the procedure, and out of the animals that survived until the seventh day, just one did not show any changes. Out of the ten animals, five survived. Nonetheless, just one of them did not present any kind of abnormality after forty-eight hours, and after the twenty-one days, four of the

five survivors did not demonstrate effective sucking, thus requiring the introduction of a gastric feeding tube (Table 4).

Table 4- *Duration of the hypoxic-ischemic insult (HI), Neurological findings at four different periods (2^{nd}, 7^{th}, 14^{th} and 21^{st} days, Brain Volume, and Survival period.*

Group	Animal number	Duration of HI insult(min)	2 days	7 days	14 days	21 days	Brain Vol. (mm^3)	Survival period after HI insult (days)
I	10	0	NA	NA	NA	NA		21
	4	0	NA	NA	NA	NA		21
	1	0	NA	NA	NA	NA	2700	21
	6	0	NA	NA	NA	NA	2956	21
	7	0	NA	NA	NA	NA	2481,6	21
II	2	45	Normal	Normal	Normal	Normal	2560,8	21
	9	45	Unilateral facial palsy Lethargy ataxia	Ataxia	Normal	Normal	2286,38	21
	11	45	ataxia	Normal	Normal	Normal	2459,18	21
	12	45	bilateral facial palsy ataxia	Normal	Normal	Normal		21
	13	45	bilateral facial palsy ataxia	Normal	Normal	Normal		21
III	19	107	Unilateral facial palsy; Lethargy Ataxia Tremulousness poor sucking	Unilateral facial palsy; Lethargy				12
	25	60	coma					1
	30	47	coma Seizure	Bilateral facial palsy Ataxia Lethargy poor sucking	Ataxia Lethargy poor sucking	poor sucking Ataxia Lethargy	3190,36	21
	41	35	Lethargy Not stand poor sucking					2
	42	50	Lethargy Ataxia poor sucking	Normal	Normal	Normal	3912	21
	43	45	Unilateral facial palsy Lethargy	Lethargy poor sucking	poor sucking	poor sucking	4128	21
	45	49	Ataxia poor sucking Lethargy Ataxia poor sucking	poor sucking	poor sucking	poor sucking	3639,98	21
	46	65	Coma					2
	49	40	Lethargy Ataxia poor sucking	Lethargy poor sucking	poor sucking	poor sucking	4001,56	21
	57	100	Coma					2

When the neurological score was applied, there was a significant difference in the mean score of Group III, if compared with the other groups, in the four evaluations (figure 3).

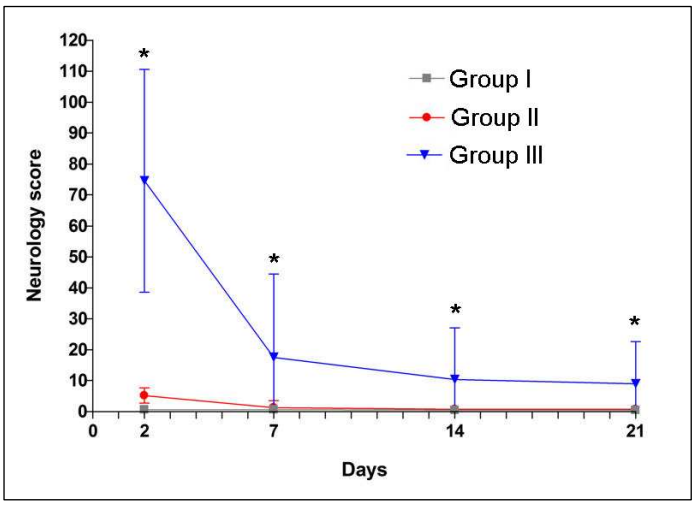

Figure 3 – *Graph shows neurological score o on the second, seventh, fourteenth, and twenty-first days.*

Assessment of Brain Volume. The means of brain volume and final body weight of the three groups are shown in figure 4.

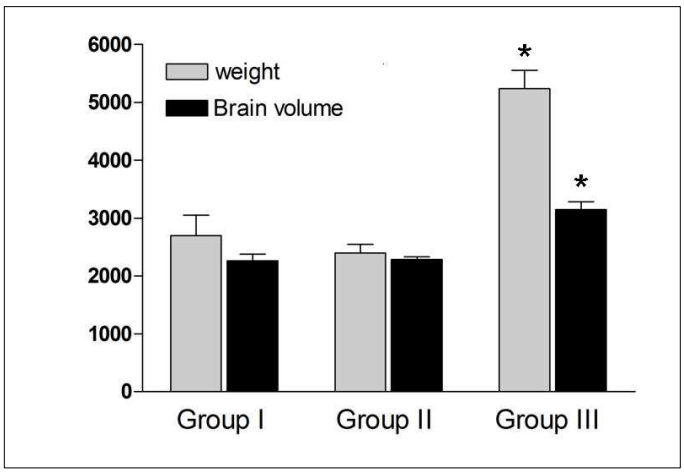

Figure 4 – *Brain volume and final body weight of Group III, compared with the other two groups. Values are presented as means + S.D.* P< 0,0001 Group III vs. I and II. Error bars are mean ± SEM.*

By using the correlation test between the body weight verified at the end of the follow-up period and the brain volume of all the animals, a positive correlation of 0.92 ($p < 0.001$) was found (figure 5).

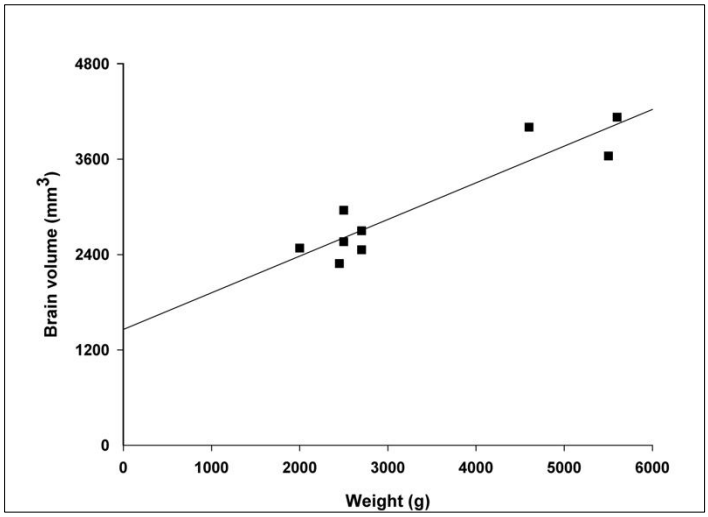

Figure 5 – *Scatter plot of brain volume (ordinate) versus body weight (abscissa) at the end of the study. There was a significant correlation of 0.92 ($p < 0.001$).*

DISCUSSION

We have developed a model of hypoxic-ischemic brain damage in piglets , in which the animals survived for a prolonged period of time. This model is suitable for examining mechanisms of damage and evaluation of potential protective therapies after birth asphyxia. The animals whose duration of the asphyxia was based on the presence of acidosis and hypotension presented longer-lasting neurological manifestations. Moreover, mortality rate, final mean body weight, and brain volume were significantly higher in the group where the number of neurological changes was larger.

Finding an ideal model to study Neonatal Asphyxia remains a great challenge for researchers.

Models with rats are well standardized. Such animals are available in large numbers and are widely used in testing new therapeutic strategies. However, they become limited when physiological monitoring is important (3, 6, 7, 16, 17). These models also present difficulty reproducing neurological changes in the long run. Pigs are relatively available and allow the access to physiological monitoring. Throughout this work it was possible to utilize equipment used in neonatal Intensive Care Units for various functions, such as heating, mechanical ventilation, and umbilical catheterization. This fact contributed to the performance of the experiment and to the maintenance care.

For the purpose of sham neonatal asphyxia, our choice was for newborn animals. In this experiment, notwithstanding the severe insult, several animals recovered completely in a short time. The damage and repair mechanisms vary according to the stage of neuronal maturation (18,19). Differently from the adult brain, in the immature brain we find neuronal

proliferation, myelination process and apoptosis, low ratio of glial cells, low metabolic need, dependence on different substrate, and different enzymatic activity (8). Therefore, the choice was for animals not older than two days old. Research suggests that although in immature animals the groups of neurons are more vulnerable to damage, they are in general more resistant to hypoxia and ischemia, and present low rates of energy use, which makes it difficult to study sequelae in the long run. All these aspects explain why it is difficult to develop a neonatal asphyxia model with a longer observation period.

An association between hypoxia and ischemia techniques has been used in models of different species (14, 20-24). . Munkeby et al(21, 22) developed a swine model of short-term asphyxia by using a gaseous mixture of 8% oxygen. In one of them, the maintenance of hypoxia lasted until the mean blood pressure reached 15 mm Hg, or until the basic excess reached -20mM. In another one, the animals were submitted to thirty minutes of hypoxia simultaneously with the bilateral clamping of the common carotid arteries. In these studies, the animals were maintained during a maximum period of forty-eight hours. Lê blanc et al(23-25) developed a model in piglets with a relatively long-term survival by using a combination of occluded brain vessels, followed by a period of hemorrhagic hypotension and hypoxia with a concentration of 6% O2. In this model, the mortality rate was approximately 30%, and 70% to 80% of the surviving animals demonstrated neurological deficits. He kept the animals alive for three days. However, we do not know whether these animals would have sustained damage if they had been kept alive for a longer period of time.

Nevertheless, some authors claim that the use of vascular occlusion with regional ischemia would represent an important limitation, due to the fact that it does not occur in human neonatal asphyxia (8). The animal diffusely exposed to aggression, with subsequent impairment of organs such as kidneys, heart, and intestines would reproduce more faithfully the actual conditions. Nevertheless, for the objective of this study, this proposal would pose additional difficulties, since a larger number of severely affected systems would represent a higher level of complication to keep the survival of the animals, and consequently the continuation of the neurological manifestations.

In this model, the challenge lies in provoking a sufficiently severe damage that may lead to prolonged sequelae, but not so strong as to impede the survival of the animal. When it comes to balance, there is no doubt that the duration of the hypoxic-ischemic insult is an important variable. At the other end of the scale, factors like the use of mechanical ventilation equipment and the anesthetic regimen employed may influence the survival time (14). Based on this work, a suggestion is that, in addition to the above-mentioned items, two other highly

useful items be also included. The use of an umbilical catheter for hydration and anesthetic maintenance, especially during the immediate post-operative period, as well as the use of an orogastric tube until the animals are able to suck, which may take several days. It was observed that animals initially in a more severe condition needed the gastric tube for a longer period of time, which is a fact that also occurs with human newborns.

Through the analysis of neurological manifestations, it was observed that the fixed time model was capable of provoking briefer changes, but the model in which MAP and acidosis as a reference were used led to more prolonged changes, despite its higher mortality rate. The scale used was effective in detecting conspicuous changes, although it may not have been sensitive to detect subtle signs. New studies are necessary to develop a more sensitive and specific scale for this model.

Brain volume determination is a widely used method in brain damage models in rats (17, 26-29). Contrary to what we thought, the results of this study showed that the brain volume in the group with more neurological changes was significantly larger than that of the other ones. Furthermore, the mean body weight of these animals was also greater at the end of the experiment. The animals with a higher level of impairment stayed inactive or hypoactive for longer periods of time, and were often tube-fed, which would explain a greater weight gain in this group. The comparison between the brain volume and the final body weight of all the animals included in the study showed that there was a positive correlation, which suggests that the brain volume in newborns of this species has a direct relation to the animal's nutritional state. Our hypothesis is that the volume gain brought about by better nutrition may have compensated for the possible decrease in volume caused by the loss of brain mass.

In conclusion, this model of neonatal asphyxia obtained through the association of hypoxia and ischemia was useful in a more prolonged follow-up, thus enabling the identification of neurological changes throughout the twenty-one days of the study, mainly when the time of the insult was based on parameters of hypotension and acidosis.

The brain volume of newborn piglets is related to the animals' nutritional state throughout the experiment. In this study, brain damage estimation based on brain volume was not effective to determine damage. We suggest that further research be conducted to evaluate the use of this brain damage estimation method in experimental models with piglets.

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CAPÍTULO III

3. EFFECTS OF THE UMBILICAL CORD STEM CELL TRANSPLANT IN A PIGLET MODEL OF NEONATAL ASPHYXIA

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ABSTRACT: Neonatal asphyxia is the main cause of brain damage in the perinatal period. Studies suggest that the stem cell transplant would curb the expansion of damages and facilitate the repair of damaged tissues, and could thus become a therapeutic option in cases of asphyxia. In the present study we tested whether intra-arterialy infused human umbilical cord stem cells enter brain and survive in the brain microenvironment, and improve neurological functional recovery after hypoxic-ischemic insult using two two different arterial access. Thirty-six healthy piglets not older than two days old were used in this study. The animals were divided into four groups: Group I (Sham), Group II, which was the control group, Group III, treated with stem cells infused through the umbilical artery, and Group IV, treated with stem cells injected via the common carotid artery. Stem cells were obtained from human umbilical cord blood. For induced asphyxia, a simultaneous association of procedures that caused hypoxia and ischemia was used. The brain tissue of treated animals that died before completing the twenty-one days was used for PCR research for human DNA. At two, seven, fourteen, and twenty-one days after the procedures, the animals a neurological score was applied. After twenty-one days, the survivors were taken to the surgery room again, deeply anesthetized and a transcardiac perfusion was performed in order to be sacrificed. After this, the animal brains were slowly extracted and the Nissl histological staining technique was used to assess the degree of brain damage. At 21 days there were differences among the average scores of group treated via carotid, when compared to those of control group and treated via umbilical artery. At other assessment moments no differences were found. In the PCR research of animals that received

stem cells via the common carotid artery catheter it was possible to visualize the band corresponding to the human β-globin in several points of the researched brain tissue samples of two of the four animals. The samples with positive PCR were obtained fifteen and twenty-four hours after the asphyxia procedure. Likewise, no positive PCR was found in any of the samples of the animals in group III. The averages and SD of encephalic volume in four groups didn't show differences and brain volume and final body weight of the animals had a moderate positive correlation. The results of this study suggest that the administration of human umbilical cord stem cells via the carotid artery in a hypoxia-ischemia model in piglets is associated with the presence of positive PCR for the human β-globin gene, and led to a significant improvement in neurological function within 3 weeks, although there was no evidence of decreased lesion area.

Neonatal asphyxia is the main cause of brain damage in the perinatal period. Epidemiological studies report on a high mortality rate and a considerable proportion of permanent neurological sequels (6, 34, 36). Currently, various neuroprotective strategies have been evaluated in animal models in an attempt to reduce apoptotic cell death, and thus improve the neurobehavioral outcomes of newborns. However, results have not been very expressive (4, 5). Studies suggest that the stem cell transplant would curb the expansion of damages and facilitate the repair of damaged tissues, and could thus become a therapeutic option in cases of asphyxia. These cells would respond to systemic signs of damaged tissues and would then migrate to the damaged sites to enable the replacement of dead tissues and the protection of the healthy areas by secreting growth hormones and specific protective factors (6, 35).

Stem cells are functionally defined as cells that have the ability of self-renewal and differentiation, that is, on the one hand, they can generate daughter cells identical to those of the mother, and on the other hand, they can produce a lineage that will develop cellular characteristics of some particular tissue. (17, 18) They can be classified into two major types: those of the embryonic type, found in the first weeks of the fetal period, and those of the adult type, which predominate after that phase (18). Functionally, the ones of the adult type are responsible for the tissue replenishment throughout life, and are present in most human tissues. Human umbilical cord blood cells are rich in mesenchymal progenitor cells and contain a large number of

endothelial cell precursors(37). Cord blood cells contain many immature stem/progenitor cells and have been used as a source of marrow-repopulating cells for the treatment of malignant

hematologic diseases. More recently, studies of several diseases affecting various organs and systems have been testing this therapy(19-25, 38, 39). Parkinson's disease, Huntington's disease, Multiple Sclerosis, and Cerebrovascular Accident (CVA), are some of the pathologies studied, also in clinical trials. Based on these studies, there is a certain agreement that such techniques are beneficial to patients, in spite of some controversial results(26-29, 40).

Some particular characteristics of newborns could offer some advantages. The relatively small dimensions, the perspective of future development (6, 35), and the availability of the material in the placenta, which is a large reservoir of fetal blood (37), favor the obtaining, the application, and the effects of the treatment.(37). However, the number of studies covering this age range is smaller. Regarding to the potential therapeutic use of infused Human umbilical cord stem cells (HUCSC) in newborns with hypoxic-ischemic insult several questions needed to be answered What exactly would be the molecular, cellular, and physiologic mechanisms involved? Which tissues would be more susceptible to its use? What would be the duration of the possible effects? What would be the most adequate mode of administration and optimum moment for the application of the therapy(6, 28, 35, 37, 41, 42)? These are questions that need to be answered, especially when we take into consideration the use of this treatment during the perinatal period.

We opted for the use of newborn piglets as an experimental model for hypoxic-ischemic insult due to their easy handling, low cost, and to the fact that their weight and size are similar to those of human babies (7-10).

In the present study we tested whether intra-arterialy infused Human umbilical cord stem cells enter brain and survive in the brain microenvironment, and improve neurological functional recovery after hypoxic-ischemic insult using two two different arterial access.

METHODS

Thirty-six healthy piglets of the *Sus scrofa* race, obtained from a local farm and not older than two days old were used in this study. The animals were divided into four groups: Group I (Sham), Group II, which was the control group, Group III, treated with stem cells infused through the umbilical artery, and Group IV, treated with stem cells injected via the common carotid artery.

Anesthesia, ventilation and monitoring of physiological variables. After the stabilization period, the animals were subjected to anesthetic induction with inhalational halothane (3%), endotracheal intubation, and umbilical cord vessel catheterization. For

anesthetic maintenance, ketamine (15-20 mg/kg IV or IM) and xilazine (2mg/Kg IV or IM) was used every two hours. The heart rate, rectal temperature, and saturation of all animals were monitored.

Protocol for hypoxic-ischemic insult and experimental groups. For induced asphyxia, a simultaneous association of procedures that caused hypoxia and ischemia was used. In Group I, the common carotid arteries of the animals were dissected, but not occluded. In the other groups, hypoxia was obtained by administering an inhalational mixture of 8% O2 and 92% nitrogen through an endotracheal tube connected to a mechanical ventilator, and ischemia was induced by reversible clamping of both common carotid arteries. The procedure was completed when the mean arterial pressure (MAP) reached less than 30 mm Hg, associated with an arterial pH of 7.28 or below. All animals in Groups II, III and IV had one of their umbilical arteries dissected and catheters were introduced into them for the monitoring of the arterial pressure. Arterial blood gas samples were taken immediately before asphyxia was induced and at the moment the animals' mean arterial pressure reached 30 or below.

Sources and Preparation of HUCSC. Stem cells were obtained from human umbilical cord blood of placentas of newborn females. Although this material disposal, we decided to seek the informed consent of all pregnant women whose placental material has been used. The collection of umbilical cord blood was done not later than twenty hours prior to the procedure whereby the stem cells were administered to the animals in the experiment. The material was kept under a temperature of 4°C during the transport and storage, until the moment the cells were separated. For the collection, a closed system was used, which was constituted of a collection bag connected to a puncture needle by a catheter(43). After the baby's birth, immediately prior to or following the delivery of the placenta, local asepsis was performed and the needle was inserted into the vessel of larger caliber of the distal portion of the umbilical cord. The bag was placed at a lower position in relation to the site of the puncture, so the blood could run down by gravity until its flow stopped completely. Finally, the bag was homogenized and stored in a thermal container with recyclable ice and a properly adjusted thermo recorder. Immediately after the collection of approximately 40 ml of blood, the material was sent for preparation. For the separation of the stem cells, the blood obtained from the human umbilical cord was diluted in RPMI 1640 medium (1:1) (Gibco®, USA): This suspension was fractionated in a density gradient generated by centrifugation over

Histopaque® with a density of 1.077 g/L (Sigma-Aldrich®, USA), at 400 g for 30 minutes at 25°C. The mononuclear fraction located on the interface with Histopaque® was collected and washed twice with 0.9% sterile saline solution. Cellular viability was assessed by the exclusion method with Trypan Blue Stain 0.4%. Cells were filtered through a 100-µm 3M© Steri-Dual filter.

Flow cytometric analysis. We performed flow cytometry on one sample of umbilical cord blood that was used in the transplantation of some animals. Immunophenotyping surface of the mononuclear fraction of umbilical cord blood was performed using the technique of flow cytometry using FACS Calibur cytometer (Becton Dickinson). We used the following markers for analysis: anti-CD45, anti-CD135, anti-CD34, anti-CD117 and corresponding isotype control. Samples containing 104 cells were incubated with antibodies to 5 ° C for 20 minutes in the dark. Was added 3 ml of DPBS and centrifuged at 500g for 5 minutes. The supernatant was discarded and the precipitate was dissolved in 1 ml of paraformaldehyde 3.6%. The analysis was performed two days after incubation in buffer cytometry (DBPS + sodium azide 0.2%).

HUCSC administration. The animals in group I were not treated. Approximately twenty-four hours following the procedure, the animals in group II were submitted to the infusion of 5 ml of physiological serum via the arterial umbilical catheter. Groups III and IV received an injection of 1 x 10^8 mononuclear cells from the human umbilical cord diluted in saline. In group III, the injection was through an arterial umbilical catheter approximately twenty-four hours following the asphyxia, whereas in group IV it was via a puncture into the common carotid artery between three and four hours after the asphyxia.

Maintenance Care. After the surgical procedures, the animals remained under mechanical ventilation until they regained spontaneous breathing and were fit to be transported to a shelter. In the shelter, the animals stayed in incubators, warmed by radiant heat and gavage-fed bovine milk until they were able to suck effectively to drink the milk directly from the bottle. After twenty-one days, the survivors were taken to the surgery room again, deeply anesthetized with thiopental 50 mg/kg administered intraperitoneally, and a transcardiac perfusion with physiological serum and 4% paraphormaldehyde was performed in order to be sacrificed. Immediately after this, the animal brains were slowly extracted.

Neurological Evaluation. At two, seven, fourteen, and twenty-one days after the procedures, the animals were examined and a neurologic score previously used for swine models was applied, which included the assessment of consciousness level (0 to 15 points), brainstem function (0 to 22 points), sensory response (0 to 20 points), muscle tone (0 to 8

points),postural reflexes (0 to 8 points), mobility (0 to 30), spatial orientation (0 to 20 points), activity (0 to 16 points) and seizures (0 to 10 points) (Table 1) (12).The results were recorded and scored from 0 to 149. The completing form of score was based, in part, on observations of animal caregivers which had been instructed to observe and record every abnormalities found and completed by assessment of examiners. As higher score number, as worse neurologic function (Table 1).

Table 1- *Neurobehavioral Scoring Tool for piglets*

Item	Scoring code
Consciousness	0=Normal 5=clouded 10=stupor 15=coma
Brainstem function	
Respiration	0=Normal 5=Present but abnormal 10=Absent
Pupillary light reflex	0=Normal 2=Present but abnormal 4=Absent
Corneal reflex	0=Normal 2=Present but abnormal 4=Absent
Gag reflex	0=Normal 2=Present but abnormal 4=Absent
Sensory responses	
Olfaction	0=Normal 2=Present but abnormal 4=Absent
Visual threat/orienting	0=Normal 2=Present but abnormal 4=Absent
Auditory startle/arousal	0=Normal 2=Present but abnormal 4=Absent
Pain withdrawal	0=Normal 2=Present but abnormal 4=Absent
Tactile localization	0=Normal 2=Present but abnormal 4=Absent
Motor function	
Muscle tone, trunk	0=Normal 2=Present but abnormal 4=Flaccid
Muscle tone, limbs	0=Normal 2=Present but abnormal 4=Flaccid
Postural reflexes	
Extensor thrust	0=Normal 2=Present but abnormal 4=Absent
Wheelbarrow	0=Normal 2=Present but abnormal 4=Absent
Mobility	0=Normal postural righting and gait 5=Ataxic, walk, but walks without falling 10=Ataxic, walks but falls frequently 15=Can't walk, but stands without assistance 20=Stands only with assistance 25=Cannot stand, but attempts to right head &trunk 30=Unable to right head, no purposeful movement
Spatial orientation	
During locomotion	0=Normal 4=Abnormal 8=Absent
With sniffing	0=Normal 4=Abnormal 8=Absent
Toward depth	0=Present 4=Absent
Activity	
Appetite	0=Present 2=Abnormal 4=Absent
Vocalization	0=Present 2=Abnormal 4=Absent
Psychomotor activity	0=Present 2=Abnormal 4=Absent
Social Interativiness	0=Normal(seeks contact) 2=Abnormal(aggressive, Withdrawn) 4=Absent (no social responsiveness)
Seizures	
Stimulus-induced myoclonus	0=Absent 5=Present
Clonic or tonic or seizures	0=Absent 5=Present
Total	Range 0 (no deficit) – 149(maximum deficit)

PCR research of human cord blood stem cells. The brain tissue of treated animals that died before completing the twenty-one days was used for PCR research for human DNA. The samples were taken from many places of brain according showed in figure 1. The group III ones were collected twelve and twenty-four hours, four and ten days after the procedure; and the group IV ones fifteen and twenty-four hours, seven and fifteen days after the cellular infusion.

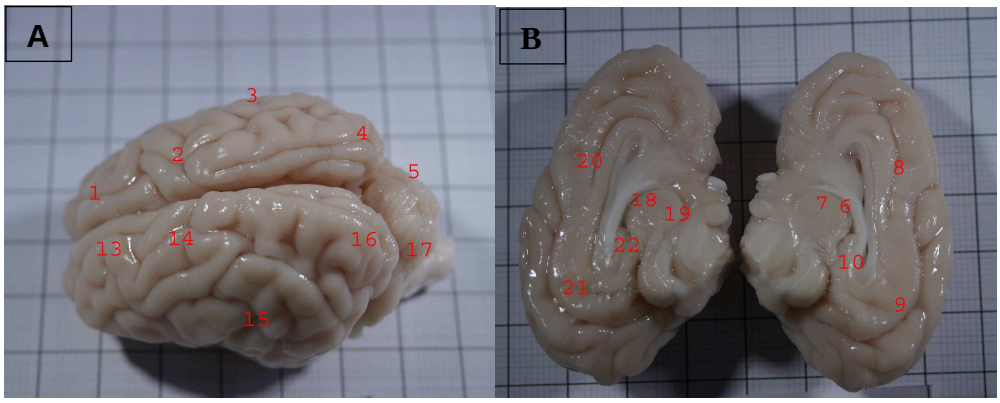

Figure 1 – Piglet brain**.** A) Top view. B) Left and right hemispheres. Numbers identify brain regions used as sample. The points 11, 12, 23 and 24 were used as polled tissue macerated from all brain areas.

DNA extraction was performed with phenol / chlorophorm based on the method described by Isola *et al*. (44). The material collected was macerated in the microtubes containing the 300 µl of PBS buffer. 600 µl of Brazol (LGC Biotecnologia®) and 120µl of chlorophorm were added. The contents were homogenized with the use of vortex (Genie™) and centrifuged at 4000g during ten minutes (Eppendorf 5417C™). The supernatant was preserved and isopropyl alcohol was added to it at a ratio of 70% of its volume. The contents were resuspended and stored at -20°C *overnight*. The material was centrifuged again at 4000g during ten minutes and the supernatant was discarded. The *pellet* formed was resuspendend in 40 µl of ultrapure water. The DNA of the samples was quantified spectrophotometrically, and presented results between 3.05 ng/ µl and 13.53ng/µl (FEMTO 700®).

Human DNA detection. For the identification of the human DNA (derived from the stem cell administration) the Polymerase Chain Reaction (PCR) was used. This technique was devised by Kay Mullis and enables the amplification of a specific DNA region, which can be viewed by agarose gel electrophoresis (45). The amplification was performed with the use of

a thermocycler (PTC-200/MJ Research™), by employing complementary *primers* in the DNA sequence of the human ß-globin gene.

As a positive control of each reaction, DNA samples obtained from human peripheral blood were tested together with each battery of DNA of samples of transplanted animals. The specific *primers* for the human ß-globin gene were designed so as to amplify specifically the desired gene. Proper care was taken to prevent the amplification of homologous swine regions, which was confirmed by the alignment using the data bank of the Basic Local Alignment Search Tool (BLAST), on the NCBI website (http:// www.ncbi.nlm.nih.gov/blast).

The conditions for the polymerase chain reaction, the *primers*, and the concentration of the reagents are described in the Table 2.

Table 2 - *Primers used, reagents and amplification conditions*

Primers	Reagents	PCR Conditions
F - 5'-caacttcatccacgttcacc-3"	36µl - H2O	95°C - 5 min.
R - 5'-gaagagccaaggacaggtac-3"	5µl – Buffer (10x)	95°C - 1 min.
	1,5µl - MgCl2 (1,5mM)	52°C - 40 sec.
	2µl - dNTP (200µM)	72°C - 30 sec
	1µl - Pf (10pmol)	72°C - 5 min
	1µl Pr - (10pmol)	4°C - Maintenance.
	0,5µl - Taq (2,5U)}	
	5µl - DNA	

The products generated by the PCR were subjected to 2% agarose gel electrophoresis containing ethidium bromide in 1x TBE buffer during thirty minutes, at a voltage 100 V and amperage of 400 mA. Gels were visualized with an ultraviolet transilluminator (3UV™) and the images were captured from the agarose gels resulting from the work using photodocumentation equipment through the software Quantity One™. The product of amplification of the polymerase chain reaction with the use of complementary *primers* in the human ß-globin gene sequence generates a fragment of 276 base pairs (bp).

Brain Volume Estimation. Brain volume estimation was chosen to assess the degree of brain damage. The presence of damage is associated with the loss of brain tissue and the consequent decrease of brain volume. After being extracted, the brains were photographed and stored for twenty-four hours in 4% paraformaldehyde, and then embedded in paraffin. 20 µm thick coronal sections at 1,200 µm intervals were obtained by microtome. The Nissl

histological staining technique (cresyl violet method) was used. Images of the histological sections were obtained through a video camera installed on an Olympus microscope (BX40), and later analyzed with the aid of the software Image Pro-Plus 6. The brain volume estimation was determined using the Cavalieri principle, according to the following equation: V(est) = T. AS, where V(est) = volume estimation; T = distance between the analyzed sections; SA = sum of the areas (30).

Data analysis. Normal distribution variables were presented as mean and standard deviation. Comparisons between the groups were analyzed using one-way analysis of variance (ANOVA), followed by the Tukey test. In the neurologic evaluation results, when appropriate, the Kruskal-Wallis non-parametric analysis and post-hoc Mann-Whitney tests, followed by the Finner-Bonferroni correction for multiple testing were performed. . Differences were considered significant at $P\_ < 0.05$.

RESULTS

A total of thirty-six animals were used. Five of them were in Group I (SHAM); ten were in Group II, eleven in Group III, and ten in Group IV. The groups were not different concerning weight, temperature, heart rate, and arterial saturation obtained in the pre-operative period (Table 3).

Table 3 – *Body weight and Physiological variables before H-I induction*

Values are presented as means ± S.D.

GROUPS	***Initial body weight (g)***	***HR (bpm)***	***Temp. (ºC)***	***sat***	***MAP***
I (n-5)	1430 ± 327,1	132,6 ± 29,72	36,54 ± 0,68	93,6 ± 9,86	-
II (n-10)	1635 ± 270,8	125,8 ± 26,55	35,9 ± 0,8	97,6 ± 1,5	57,8 ± 8,56
III (n-11)	1615 ± 252,9	123,18± 18,24	35,44 ± 1,2	95,8 ± 5,8	52,09 ± 9,7
IV (n-10)	1564 ± 296,2	123 ± 18,4	35,87 ± 1	97,5 ± 1,7	58,9 ± 8,8

HR = Heart rate Temp. = Temperature Sat –O2. Saturation MAP = Mean Arterial Pressure

There was no difference regarding the time of asphyxia, mean arterial pressure (MAP), or the arterial blood gas data during the asphyxia among groups II, III, and IV. The averages and SD of pH and MAP at the end of procedure in groups II, III and IV were respectively 7,15 ± 0,05, 7,11 ± 0,11 and 7,11± 0,14, and 27,8 ± 5,6, 29,9 ± 4,3 and 29,6 ± 5,4.

At the end of study the body weight in Groups I, II, III and IV had average and SD of 2700 g ± 771,3, 5233 g ± 550,7, 3270 ±1803g and 3191g ± 770. There was statistically significant differences among the final weight averages of groups III and IV (p=0.009).

Flow cytometric analysis. The major cell population of the mononuclear fraction of umbilical cord blood cells sample was CD 45 +, reaching 34.35% of the population. CD34 + had 2,43%, CD 105+ 0,38% and CD 117 + 0,06% (Figure 2).

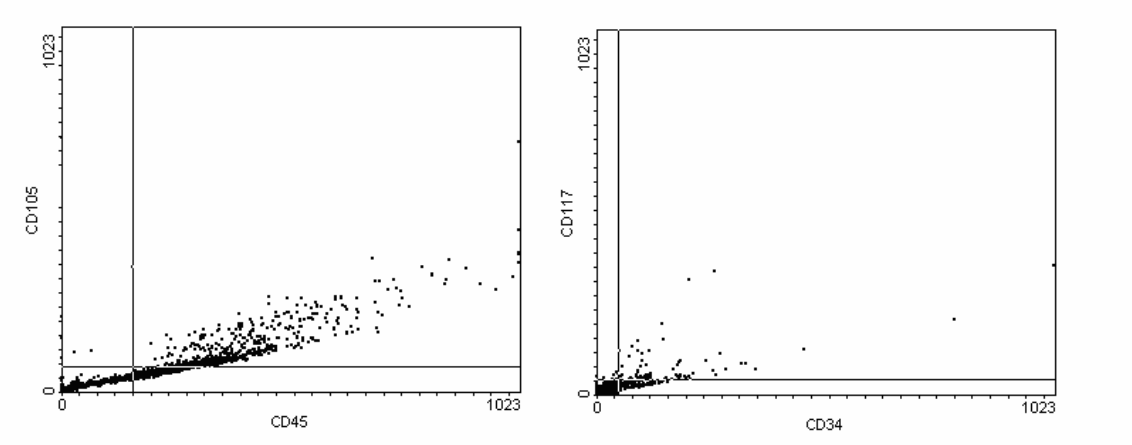

Figure 2 - *Immunophenotyping of the mononuclear fraction of umbilical cord blood. Monoclonal antibodies to CD34/45 FITC and PE CD117/105. The reading was done with 100,000 events. Fraction indicates negative cells not marked.*

Neurological Evaluation. The comparison among the neurologic scores in the four proposed moments for the groups subjected to asphyxia showed that, the three hypoxic-ischemic (H-I) groups were different from the SHAM at first assessment. At 21 days there were differences among the average scores of group IV, when compared to those of groups II and III (p=0.02). At other assessment moments no differences were found among H-I groups (Table 4).

Table 4 - *Neurological scores in four different periods*

	2 days	7 days	14 days	21 days
Group II	77 ± 37,4	17,5 ± 27	10,4 ± 16,7	9 + 13,6
Group III	55,33 ± 36	28 ± 38	21 ± 20	16,8 ± 18,1
Group IV	44,9 ± 35	4,3 ± 7	2,6 ± 4,4	0 *
P value	0,23	0,29	0,13	0,02

Values are presented as means ± S.D. * p < 0, 05 vs. Group II and III

PCR Research of human cord blood stem cells. In the PCR research of animals that received stem cells via the common carotid artery catheter it was possible to visualize the band corresponding to the human β-globin in several points of the researched brain tissue samples of two of the four animals. (Figure 3)

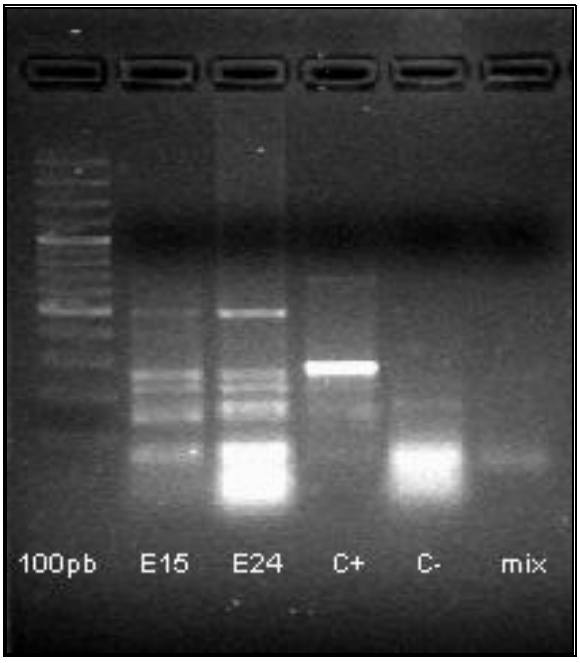

Figure 3 *– Agarose gel with the result of the amplification of different brain regions in two piglets. Representation of the presence of the band corresponding to the gene human β-globin gene (276bp) in the swine brain tissue sample treated with stem cells injected via carotid artery at 15(E 15) and 24(E 24) hours . Molecular weight marker (100bp) positive control (C +) negative control (C-)*

The samples with positive PCR were obtained fifteen and twenty-four hours after the asphyxia procedure (Table 5). No positive PCR was identified in the samples collected on days seven and fifteen in group IV. Likewise, no positive PCR was found in any of the samples of the animals in group III.

Table 5 – *Result of the PCR for presence of the human β-globin in two animals of group IV*

	R01	R03	R04	R06	R09	R10	R11	R12	R13	R14	R15	R16	R17	R21	R23
A1	X	X		X			X		X	X		X		X	X
A2			X		X	X	X	X	X		X		X		

A1 = 15 hours after infusion; A2= 24 hours after infusion. X: positive results. Regions 2, 5, 7, 8, 18, 19, 20, 22, and 24 were negative.

Estimation of brain volume. The averages and SD of encephalic volume in four groups were 2826 $\pm$ 194 mm^3 in Group I, 3145 $\pm$ 310 mm^3 in Group II, 2859 $\pm$ 896 mm^3 in Group III and 2936 $\pm$ 378 mm^3 in Group IV (p=0,19). The brain volume and final body weight of the animals showed a moderate positive correlation (Pearson= 0,629 p=0.021).

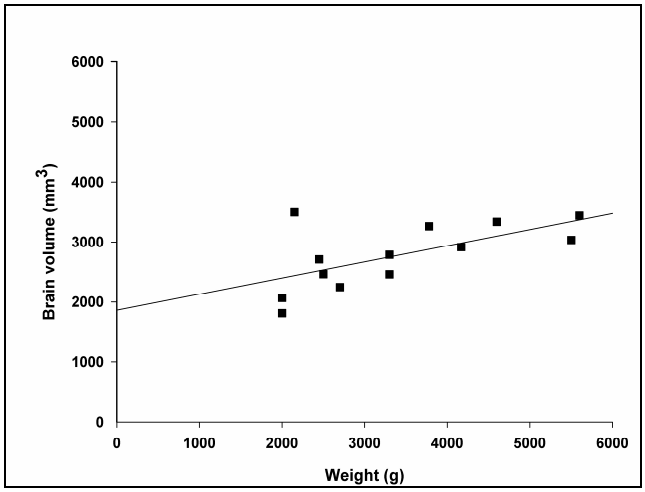

Figure 4 – *Scatter plot of brain volume (ordinate) versus body weight (abscissa) at the end of the study. There was a significant correlation of 0.629 (p< 0.021).*

DISCUSSION

According to the results of this study in the comparison of the average scores of the neurologic evaluation at the end of the twenty-one days of observation, the group that received stem cells administered via common carotid artery showed a better result than the control group or the group that received stem cells via the umbilical artery. Additionally, it was also possible to identify through the PCR the presence of human β-globin gene in two of the four animals tested in the group that was treated via the carotid artery, which was not evidenced in any sample of the group treated through the umbilical cord artery.

The positive PCR suggests that there was cell migration from the carotid artery to the damaged site. Probably, the pattern diffuse of cells distribution was because there was a generalized hypoxic- ischemic damage of this experiment. These findings is compatible with many studies showing that adult stem cells could find their way to the damaged area in appropriate circumstances, and then differentiate themselves into neurons, astrocytes, and oligodendrocytes (46-51). However, we observed that the identification of the human β-globin gene was positive just until twenty-four hours after the administration, and negative in the samples collected later. Other authors described this evolution, for example, Keimpema et al.(52). They administered stem cells in a model of ischemia in adult rats through the common carotid artery, and observed that six hours after the injection the cells started being detected around the lesion, increasing in the first twelve hours, but suddenly decreasing after twenty-four hours. Two weeks after the lesion, no transplanted cell was detected. Also, studies reporting improvement in animal models show a relative lack of correlation between these results and the migration and differentiation of the donated cells. These dates suggest the

implication of additional mechanisms. Perhaps the action of this therapy is based more on protection than regeneration. The transplanted cells could secrete neurotransmitters such as dopamine, acetylcholine, or GABA, or even produce or stimulate the production of neurotrophic factors or neuroprotectors, which would stop degeneration or promote endogenous regeneration. Studies have demonstrated that umbilical cord blood contains growth factors similar to the ones that exert neurotrophic effects. Neurotrophins such as brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT3), and nerve growth factor (NGF) have been found in the umbilical cord. These neurotrophins are widely known for their great effects on brain plasticity. Particularly, studies with BDNF suggest that this neurotrophin plays a critical role in the development and maturation in the axonal and dendritic connections. Therefore, the effects that we observed in this study might have been partially mediated by the secretion of neurotrophins(6, 21, 28, 39). Alternatively, recent studies have attributed beneficial effects of stem cells by their modulation of inflammatory and immune responses, apparently by alternative activation of microglia and/or macrophages. The fate of the noncommitted neural stem cells and its differentiation potency are often under strict regulation, and these proinflammatory mediators seem to disrupt this critical balance and finely tune the neurogenesis pattern in the two niches of neurogenesis, the subventricular zone and the subgranular zone of the hippocampus. Moreover, the migration ability of these stem cells, which is important for localization to the proper site, is also affected in a major way by the chemokines released following inflammation(53-56).

We compare 2 different kinds of arterial access to stem cell administration, one of them central (carotid) and another peripheral (umbilical). The animals treated with stem cells through carotid had the better performance in the end of the observation. There is little literature comparing the infusion through the different kinds of administration in newborn animals(57). Procedures using the injection of intraparenchymal stem cells are associated with tissue lesion and high surgical risk. Most studies use intravenous stem cell infusion(40). The umbilical vases are often used in newborns to infusion of drugs, access of laboratorial samples and get invasive monitorization. In this study, umbilical artery didn’t seem an effective way. It’s probable that stem cells injected by peripheral administration couldn’t get through several barriers in the body, due to having to do a longer path. Lundberg *et al(58)* analyzed the amount of mesenchymal cells transplanted after the administration via internal carotid artery, compared with intravenous administration in an experimental model of brain trauma in rats, and showed that the administration via carotid artery is significantly more efficient. We speculate that the administration of cells via the common carotid artery enabled the migration

of a larger number of cells toward the damaged site, and, consequently, a better performance on the neurologic score. According to our observation, no complication seems to be related clearly to the infusion through the carotid artery.

In the first 14 days of follow-up found no difference in neurological performance of the groups that received stem cells compared to control. The use of cells from cord blood could explain a apparently slower response to treatment as happened in this experiment. In umbilical cord stem cells transplant, the engraftment of some cells, such as neutrophils and platelets, is delayed. Furthermore, the limiting number of stem-progenitor cells in one single cord blood collection, the immature nature of these cells, the difficulty in programming themselves toward differentiation, or all of these factors combined may be responsible for the relatively delayed blood cell engraftment(37, 39). On the other hand, some of umbilical cells features may have contributed to the favorable outcome at 21 days. These are young cells, which, therefore, have not been exposed to the main harmful environmental factors yet and demonstrate a low incidence of graft-host disease, thus allowing the use of cord blood with a greater HLA-disparity than is usually acceptable for bone marrow transplantation(38, 39).

One of our biggest challenges was to develop an experimental model in large animal that can be monitored for a longer period. This model is based in an association of hypoxia and ischemia. Although in some animals the pH hadn't achieved a significant reduction of pH (maximum 7.28) on average in all groups the pH was approximately 7.15, and has always been associated with hypotension (MAP less than 30) and reversible ischemia of the common carotid by an average of 45 min. Most of the experimental hypoxia and ischemia studies followed by stem cell infusion in newborns use rats and mice. Compared with swine, models with rats are better standardized and the animals are available in large numbers, which might facilitate the detection of clinical signs. Nonetheless, small animal models also present difficulty in reproducing neurological changes in the long run(5, 7, 8, 36, 59). Piglets are relatively available and allow the access to physiological monitoring, reproducing more accurately the environment of a neonatal Intensive Care Unit(60-63).

An additional difficulty was to find a method that would allow a proper assessment of the neurological findings. The test used was able to detect gross abnormalities, such as consciousness level, motor activity and appetite, but many times we observed that animals had different degree difficulty, and the score provided only the framework in presence or absence of the clinical signal. Considering the peculiar difficulties of the assessment of newborns, the availability of a more sensitive technique would be useful to the comparison of neurological outcome in this study(64, 65). Several authors suggest that although in immature

animals some groups of neurons are more vulnerable to damage, they are in general relatively more resistant to hypoxia and ischemia, and present low rates of energy use, which makes it difficult to study sequelae in the long run (9). Unfortunately, well-standardized assessment tools for this model are practically nonexistent, since piglets are mainly used for shorter studies.

A further limitation of our study is the fact of the neurologic score evaluators weren't blinded to the groups. However, it should be considered, that the data were strongly based on observation of the animal caregivers who were not aware of the treatment previously applied and the most part of score items were objectives and easily recognized.

Regarding the estimation of damaged brain area, no differences were found in the average brain volume among the groups. Therefore, it was not possible to determine whether there was a greater neuronal replacement in the treated groups. A positive correlation between the brain volume and the weight at the end of the study suggests that the nutritional state could influence this variable. Estimation of brain damage, therefore, showed not to be the most appropriate method for this model in swine. Possibly, a neuronal counting method would be more appropriate.

In conclusion, the results of this study suggest that the administration of human umbilical cord stem cells via the carotid artery in a hypoxia-ischemia model in piglets is associated with the presence of positive PCR for the human β-globin gene, and led to a significant improvement in neurological function within 3 weeks, although there was no evidence of decreased lesion area.

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ANEXOS

ANEXO I

TERMO DE CONSENTIMENTO LIVRE E ESCLARECIDO

CONSENTIMENTO PARA DOAÇÃO DE SANGUE DE CORDÃO UMBILICAL PARA REALIZAÇÃO DO PROJETO DE PESQUISA:

"AVALIAÇÃO DO POTENCIAL TERAPÊUTICO DAS CÉLULAS-TRONCO EM LESÃO CEREBRAL POR ANÓXIA NEONATAL EXPERIMENTAL"

A – PROPOSTA DO PROGRAMA:

Você está sendo convidado a participar do projeto de pesquisa: "Efeitos da injeção de células-tronco de cordão umbilical humano em modelo experimental de anóxia neonatal"

B – OBJETIVOS DO PROGRAMA:

Durante a gravidez, o oxigênio e nutrientes essenciais passam do sangue materno para o bebê através da placenta e do cordão umbilical. Após o parto, o sangue que permanece no cordão umbilical e na placenta é geralmente descartado. Este sangue contém um grande número de células-tronco, que são células jovens, que conseguem se reproduzir em células de seus respectivos tecidos. Pesquisas em andamento buscam utilizar essas células na regeneração de órgãos, como o coração e o cérebro, pois estas células podem se transformar em diversas outras células, tais como, células sangüíneas, musculares e nervosas (neurônios).

Você está sendo convidada a participar deste projeto de pesquisa porque acaba de dar a luz a um bebê. A coleta ocorrerá após o nascimento do seu bebê e não afetará de nenhuma maneira o parto ou os cuidados dispensados ao seu filho.

O objetivo deste trabalho é avaliar se as células-tronco de cordão umbilical humano podem tratar a lesão cerebral de animais que sofreram asfixia no período do nascimento.

Com a sua permissão, o sangue do cordão umbilical de seu filho, poderá ser usado para os objetivos citados anteriormente. Porém, o sangue do cordão umbilical de seu filho não será usado para doação para pacientes que precisam de transplante, mas apenas para pesquisa.

C – RISCOS E DESCONFORTOS POTENCIAIS:

Não existe qualquer tipo de risco na participação do programa. O sangue coletado da placenta não é necessário para o seu bebê após o cordão umbilical ter sido cortado, uma vez que este material seria descartado.

Todas as informações coletadas serão mantidas confidenciais. Nem você e nem seu bebê serão identificados em qualquer publicação dos dados que seja realizada.

D – BENEFÍCIOS:

O único benefício em participar deste programa é que, doando o sangue da placenta de seu bebê para pesquisa, você poderá ajudar-nos a conhecer o tratamento de lesões no cérebro de crianças com o uso de células-tronco de cordão umbilical.

E – CUSTOS:

Não haverá, para o participante, nenhum custo com a realização da pesquisa proposta. Caso você decida não participar do programa, não sofrerá nenhum prejuízo no atendimento dispensado à você e a seu filho. A decisão de não participar do programa não irá afetar o seu atendimento no hospital.

F – TERMO DE PARTICIPAÇÃO:

Assuntos de importância para você serão esclarecidos antes de o consentimento ser assinado.

G – ALTERNATIVAS PARA PARTICIPAÇÃO:

Você pode decidir não doar o sangue da placenta de seu filho para o projeto. Caso decida não participar do programa, o sangue da placenta não será coletado, ou se já coletado, será desprezado, não sendo utilizado na pesquisa.

CONSENTIMENTO PARA DOAÇÃO DE SANGUE DE PLACENTA

Declaro que li as informações anteriormente descritas a respeito do projeto de pesquisa "Efeitos da injeção de células-tronco de cordão umbilical humano em modelo experimental de anóxia neonatal" e tive a oportunidade de esclarecer todas as minhas dúvidas.

Aceito participar da doação de sangue de placenta para o projeto proposto, com o objetivo de pesquisa, não sendo usado para doação.

_____ _____ ___/___/___

Assinatura da mãe Nome da mãe

_____ _____ ___/___/___

Assinatura do Pesquisador Nome do Pesquisador

Este formulário foi lido para _____ (paciente) em ____/____/____ pelo _____ (pesquisador) enquanto eu estava presente.

_____ _____ ___/___/___

Assinatura do testemunha Nome da testemunha

AFIRMAÇÃO DO INVESTIGADOR: foi oferecido ao participante a oportunidade de tirar dúvidas a respeito dos riscos e desconfortos que podem se associar à participação no programa. Todas as respostas foram devidamente respondidas.

_____ ___/___/___

Assinatura do Pesquisador

Telefones de contato do Pesquisador: (51) 3320-3000 ramal 2173 ou (51) 99883365

Aprovado pelo Comitê de Ética em Pesquisa do Hospital em ___/___/___

Número: _____