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CENTRO DE BIOCIÊNCIAS
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GISELLE MACHADO MAGALHÃES MORENO

ANÁLISE DA BIOCOMPATIBILIDADE DO BIOPOLIMERO DA CANA-DE-AÇÚCAR EM MODELO DE TRAUMATISMO CRANIANO E EM CULTURAS DE CÉLULAS NEURAIS

Recife
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Aprovada em 28 de junho de 2018:

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E ao meu pai: Pai, você moldou sua vida em um exemplo impossível de não seguir.

(Filha, a você dedico minha vida.)

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RESUMO

Polissacarídeos sintetizados por bactérias têm atraído interesse para engenharia de tecidos como um material promissor para ser usado em suportes de células e implantes. O presente estudo testou a hipótese de que o exopolissacarídeo celulósico produzida a partir do melaço da cana-de açúcar (CEC), pode ser viável para neuroengenharia de tecidos. Sua biocompatibilidade como substrato bi e tridimensional para culturas primárias de neurônios e/ou astrócitos foi avaliada em relação à adesão, crescimento e viabilidade celular, além disso foi realizada análise funcional baseada em imageamento de cálcio onde foi observado o influxo de cálcio, em células de culturas mistas, frente a estimulação por agonista glutamatérgico, KCl e ATP. Foi também adotado um modelo de traumatismo craniano para avaliar, *in vivo*, a biocompatibilidade desse CEC na forma de hidrogel coloidal, para incorporação de células neurais durante o processo de recuperação tecidual. As propriedades da CEC nas configurações 2D (membranas) e 3D (hidrogel) foram adequadas para a diferenciação morfológica de neurônios e astrócitos corticais em culturas puras ou mistas, mesmo sem estar conjugada a proteínas da matriz extracelular. Não foi observada toxicidade induzida pelo CEC em culturas puras de neurônios embrionários. Em culturas mistas, os neurônios também apresentaram resposta funcional de influxo de cálcio similar ao controle, diante dos estímulos despolarizantes induzidos por N-Metil-D-aspartato (NMDA) e KCl. Em culturas puras de astrócitos, tanto o tamanho como a diferenciação fenotípica foi similar ao observado no controle. No entanto, a resposta dos astrócitos crescidos em culturas mistas diante do estímulo induzido pelo ATP foi significativamente reduzida em relação ao controle. O implante do CE no local da lesão mecânica mostrou a capacidade de permitir a incorporação de células. Os resultados abrem perspectiva para o desenvolvimento de um novo suporte funcional para células neurais, no entanto, futuros estudos precisam ainda ser feitos para investigar interações recíprocas entre neurônios e células gliais nas estruturas bi e tridimensionais deste CE. Torna-se necessário esclarecer que mecanismos estão envolvidos na menor resposta astrocitária diante do estímulo induzido pelo ATP em condições de cultura mista.

Palavras-chaves: Biopolímero. Exopolissacarídeo. Celulose bacteriana. Engenharia de tecido neural. Culturas primárias de neurônios e astrócitos. Imageamento de cálcio.

ABSTRACT

Polysaccharides synthesized by bacteria have attracted interest in tissue engineering as a promising material to be used as cell scaffold and implants. The present study tested the hypothesis that bacterial cellulose (BC), produced from sugar cane molasses may be feasible for tissue neuroengineering. Its biocompatibility as a bi-or three-dimensional substrate for primary cultures of neurons and / or astrocytes was evaluated regarding to cell adhesion, growth and viability. In addition, a functional analysis based on calcium imaging was performed, where calcium influx was observed in neuron and astrocytes, after stimulation by a glutamatergic agonist, KCl and ATP. A traumatic brain injury model was also used to evaluate *in vivo* the biocompatibility of this BC in the form of a colloidal hydrogel for the incorporation of neural cells during the tissue recovery process. BC properties in 2D (membrane) and 3D (colloidal hydrogel) configurations were suitable for the morphological differentiation of neurons and cortical astrocytes in pure or mixed cultures, even without being conjugated to extracellular matrix proteins. No toxicity was induced by BC in pure cultures of embryonic neurons. In mixed cultures, the neurons also presented functional response of calcium influx similar to the control, after depolarizing stimuli induced by N-Methyl-D-aspartate (NMDA) and KCl. In pure astrocyte cultures, both size and phenotypic differentiation were similar to those observed in the control. However, the response of astrocytes grown in mixed cultures to the ATP-induced stimulus was significantly reduced relative to the control. The BC implant at the site of the mechanical lesion was able to allow the incorporation of cells. The results open up the perspective for the development of a new functional support for neural cells, however, future studies still need to be done to investigate reciprocal interactions between neurons and glial cells in the bi and tridimensional structures of this CEC. It is also necessary to clarify which mechanisms are involved in the lower astrocytic response to the stimulation induced by ATP under mixed culture conditions.

Keywords: Biopolymer. Exopolysaccharide. Bacterial cellulose. Neural tissue engineering. Neuron and astrocyte primary cultures. Calcium imaging.

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AMPA	A-AMINO-3-HIDROXI-METIL-5-4-ISOXAZOLPROPIÓNICO
AVE	ACIDENTE VASCULAR ENCEFÁLICO
ATP	ADENOSINE TRIPHOSPHATE
BC	BACTERIAL CELLULOSE
CEC	EXOPOLISSACARÍDEO CELULÓSICO OBTIDO A PARTIR DO MELAÇO DA CANA-DE-AÇÚCAR
DAB	DIAMINOBENZIDINE
DMEM	DULBECCO'S MODIFIED EAGLE'S MEDIUM
EA	ETIL-ACRILATO
EDTA	2,2',2'',2'''-(ETHANE-1,2-DIYLDINITRILO) TETRAACETIC ACID
FBS	FETAL BOVINE SERUM
FGF-2	FATOR DE CRESCIMENTO DE FIBROBLASTO-2
FITC	FLUORESCEIN ISOTHIOCYANATE GFAP - GLIAL FIBRILLARY ACIDIC PROTEIN
GFAP	GLIAL FIBRILLARY ACIDIC PROTEIN
HE	HEMATOXYLIN-EOSIN
HEA	HIDROXIL-ETIL-ACRILATO
HEPES	4-(2-HYDROXYETHYL)-1-PIPERAZINEETHANESULFONIC ACID
HUCMSCS	CÉLULAS TRONCO MESENQUIMAIS DO CORDÃO UMBILICAL
IBA-1	IONIZED CALCIUM BINDING ADAPTOR MOLECULE 1
MSCS	CÉLULAS TRONCO DA MEDULLA ÓSSEA
NGN2	NEUROGENINA-2
NMDA	N-METIL-D-ASPARTATO
NMDARS	NMDA RECEPTORS PB - PHOSPHATE BUFFER
PBS	PHOSPHATE BUFFERED SALINE
PCL	POLI- E –CAPROLACTONA
PDMS	POLYDIMETHYLSILOXANE
PFA	PARAFORMALDEHYDE
PLGA	POLI-L-LACTICO-CO-GLYCOLIC

PND	POSTNATAL DAY
PSD-95	POSTSYNAPTIC DENSITY PROTEIN 95
SNGR	MOLÉCULA SOLÚVEL RECEPTOR NOGO
TBI	TRAUMATIC BRAIN INJURY
TE	TISSUE ENGINEERING
TCE	TRAUMATISMO CRANIOENCEFÁLICO

SUMÁRIO

1 INTRODUÇÃO

A Engenharia de tecidos é uma importante área interdisciplinar de inovação tecnológica que possui foco no uso de biomateriais para facilitar ou promover regeneração ou substituição do tecido lesionado (Wang et al. 2012). Seus avanços, diretamente relacionados ao desenvolvimento biotecnológico, ocorrem nos mais variados sistemas, como exemplo: reconstrução completa de tecido cartilaginoso (orelha) (Reighard, Hollister, and Zopf 2018); reparo ósseo (Pearlin et al. 2018), substituição de válvulas cardíacas (Stassen et al. 2017) e auxílio à regeneração no tecido nervoso (Orive et al. 2009; Wieringa et al. 2018) constituindo uma recente e promissora área dentro das neurociências que é a neuroengenharia de tecidos.

As desordens que acometem o sistema nervoso costumam cursar com déficits funcionais que têm grande impacto nos pacientes por elas acometidos além de grande impacto socioeconômico. O arsenal terapêutico atualmente disponível é ineficiente em promover recuperação de danos sofrido pelo tecido neural sendo capaz apenas de limitar o dano, como ocorre no acidente vascular encefálico (AVE) ou traumatismo cranioencefálico (TCE), ou retardar processos degenerativos, como na doença de Alzheimer. A busca por estratégicas terapêuticas capazes de promover reparo tecidual funcional de lesões neurológicas é válida e urgente.

Os biomateriais podem ser de origem natural ou sintética, e sua adequação às particularidades de cada tecido ocorre por meio de extensa investigação *in vitro* e *in vivo* para avaliar seu comportamento físico, químico e biofuncional (Koss and Unsworth 2016). O desempenho de diversos materiais tem sido avaliado pela neuroengenharia de tecido desde seu uso para cultivo de células neurais (Murphy et al, 2017) até em suturas diretas e construção de pontes no reparo de nervos periféricos em humanos (Safa and Buncke 2016), passando por uma variedade de modelos experimentais que utilizam implantes diretamente em contato com o parênquima cerebral lesionado (Tate et al. 2009), biomateriais como arcabouço para implante de células tronco no sistema nervoso central (Aligholi et al. 2016) e entrega de fármacos ou fatores tróficos com especificidade no alvo.

Uma variedade de materiais tem encontrado êxito nos estudos experimentais com tecido neural, no entanto existem limitações translacionais importantes na neuroengenharia (Cousin et al. 2016). Tendo em vista as maiores dificuldades para obter êxito nas intervenções em humanos e o alto custo de processamento de alguns materiais, torna-se necessária uma continuidade na busca por novos biomateriais. Nesse sentido

celulose bacteriana obtida a partir do melaço da cana-de-açúcar foi sintetizado inicialmente na Estação Experimental de Cana de Açúcar de Carpina, da Universidade Federal Rural de Pernambuco - UFRPE, a partir de 1990, e mostra resultados promissores em diversas áreas da engenharia de tecidos, apresentando biocompatibilidade, atoxicidade e capacidade de ser processada e apresentada sob diferentes formas: membranas, hidrogel, esponjas entre outras.

A disposição de um promissor produto local, a crescente necessidade de novas estratégias terapêuticas dentro das Neurociências e os avanços obtidos pela neuroengenharia de tecidos configuraram o cenário ideal para desenvolvimento deste trabalho que se propôs a investigar o comportamento da celulose bacteriana obtida a partir da cana-de-açúcar frente ao tecido nervoso em culturas bi e tridimensionais e em modelo experimental de traumatismo cranioencefálico em ratos. A relevância do estudo estende-se ainda ao fato do exopolissacarídeo da cana-de-açucar resultar da produção intelectual nacional mais especificamente de uma colaboração entre a UFPE e a UFRPE, que vem agregar valor a um produto regional, o melaço da cana de açúcar. A consolidação da produção do biopolímero na unidade de produção da EECAC - UFRPE, já levou à implantação de uma empresa, a POLISA Biopolímeros para a Saúde Ltda, incubada pela INCUBATEC Rural, UFRPE com a finalidade de pesquisar e desenvolver a produção em escala e a comercialização de produtos do biopolímero. A expansão do uso desse exopolissacarídeo contribui para o desenvolvimento econômico e social da região.

1.1 HIPÓTESE

O exopolissacarídeo celulósico produzida a partir do melaço da cana-de açúcar pode ser viável para neuroengenharia de tecidos.

1.2 OBJETIVOS

1.2.1 Geral

Investigar a biocompatibilidade do CEC em modelo de traumatismo craniano e em culturas de células neurais.

1.2.2 Específicos

1) Investigar a potencialidade do CEC como suporte adequado para obtenção de culturas bi e tridimensionais de neurônios e astrócitos do córtex cerebral;

2) Avaliar sua biocompatibilidade, na forma de hidrogel, para recuperação do tecido cerebral em um modelo experimental de traumatismo cerebral.

2 FUNDAMENTAÇÃO TEÓRICA

2.1 ENGENHARIA DE TECIDOS

O uso de biomateriais no reparo de diversas lesões teciduais vem de longa data (Figura 1). Há registros do uso de ouro para reposição de dentes perdidos ou fixação dentária em 50dC (Minozzi et al. 2007), e uso de vidro, na Grécia antiga, nos casos de perda do globo ocular e, no início do século XX, para implante articular (Coburn and Pandit 2007). Uma característica que os materiais apresentavam, à época, era ser inerte no contato com os tecidos no local do implante (Coburn and Pandit 2007). A utilização de enxertos naturais heterólogos (espécies diferentes), homólogos (tecido para enxerto retirado de indivíduo da mesma espécie) e autólogos (tecido para enxerto retirado do próprio paciente) (Lopes et al. 2011) foi um importante passo na direção da interação entre os tecidos lesionados e o material usado para favorecer o reparo (Coburn and Pandit 2007). Esta técnica oferece, nos enxertos autólogos, a desvantagem de um segundo procedimento invasivo e do processo de cicatrização que o local doador do enxerto terá que passar.

Figura 1: Vistas superior e fronto-lateral de arcada dentária datada do primeiro ou segundo século a.C., mostrando que o uso de fios de ouro para manutenção dos dentes é ainda anterior aos primeiros registros encontrados (adaptada de Minozzi et al, 2007).

Da interação multidisciplinar entre a biologia, química e engenharias surgiu a área de pesquisa em engenharia de tecido, que atualmente utiliza o avanço de processos biotecnológicos para o uso de biomateriais investigando a capacidade dos mesmos de interagir com o tecido hospedeiro, com alta especificidade, auxiliando sua regeneração e sendo por ele reabsorvido. A busca por uma interação ótima entre os implantes e os diferentes tecidos conduziu ao aprimoramento de biomateriais capazes de mimetizar a matriz extracelular do tecido de interesse, permitindo reconhecimento biomolecular pelo tecido hospedeiro (Tam et al. 2014). Ainda dentro da engenharia de tecidos é comum vermos o uso de *scaffolds* para o crescimento de células. São colocados nesses *scaffolds* células e fatores de crescimento para que permitam

o crescimento do tecido celular até que substitua completamente a superfície do suporte. Os *scaffolds* têm a função de guiar o crescimento celular, sintetizar uma matriz celular e outras moléculas biológicas e facilitar a formação de tecidos e órgãos funcionais, para permitir o crescimento de tecidos precisam apresentar alta porosidade e tamanho e formato de poros adequados, e para que haja boa vascularização é importante grande área superficial (Machado et al. 1996).

De acordo com a origem os materiais são classificados em sintéticos ou naturais, e sua adequação à engenharia de tecidos se dá através de avaliações minuciosas de sua biocompatibilidade *in vitro,* especialmente avaliando toxicidade, capacidade de permitir adesão, diferenciação e funcionalidade celular, e *in vivo*, com extensa avaliação da interação do implante com o tecido hospedeiro. Embora muitos materiais sejam biocompatíveis nem todos são bons candidatos à engenharia de tecidos. Dentre as características desejáveis estão: (1) adaptabilidade e processabilidade (biomaterial deve permitir acoplamento de diferentes moléculas, como fatores tróficos, além de permitir apresentação sob formas diversas, como hidrogel, membrana, *scaffold*); (2) baixa imunogenicidade; (3) biodegradabilidade; (4) estabilidade química e mecânica; (5) pemitir adesão, migração e diferenciação celular e (6) atoxicidade (Orive et al. 2009).

A biocompatibilidade é uma propriedade muito relevante e por isso tornam-se necessários estudos *in vitro* e *in vivo* para que seja evidenciado o comportamento celular frente a esses materiais. Tratando-se de relações biológicas de um implante, o modelo animal torna-se essencial para o estudo, porém o mesmo pode nos fornecer informações que dificultam a interpretação dos resultados ao nível celular. Em vista disso, abordagens *in vitro* tornam-se fundamentais por superar essas limitações e por oferecer vantagens como: rapidez, simulação do desempenho do material antes da sua implantação e fornece um maior número de resultados em relação ao comportamento do material (Cruz, 1987).

Nos últimos anos, cresce o número da confecção de biomateriais que sirvam como suporte físico e permitam o crescimento das células sobre o substrato *in vitro* para posterior implantação (Zhao et al. 2017). Para que haja uma boa interação polímero-célula também se faz necessário uma boa adesão celular ao substrato. O substrato não precisa obrigatoriamente ter características semelhantes às da matriz extracelular para que as células sofram adesão, porém a similaridade físico-química é importante quando a meta é promover uma diferenciação celular ou para que ocorra uma interação mais efetiva. A figura 2 esquematiza as etapas necessárias para a caracterização e avaliação da biocompatibilidade de um material com potencial aplicação em engenharia de tecido.

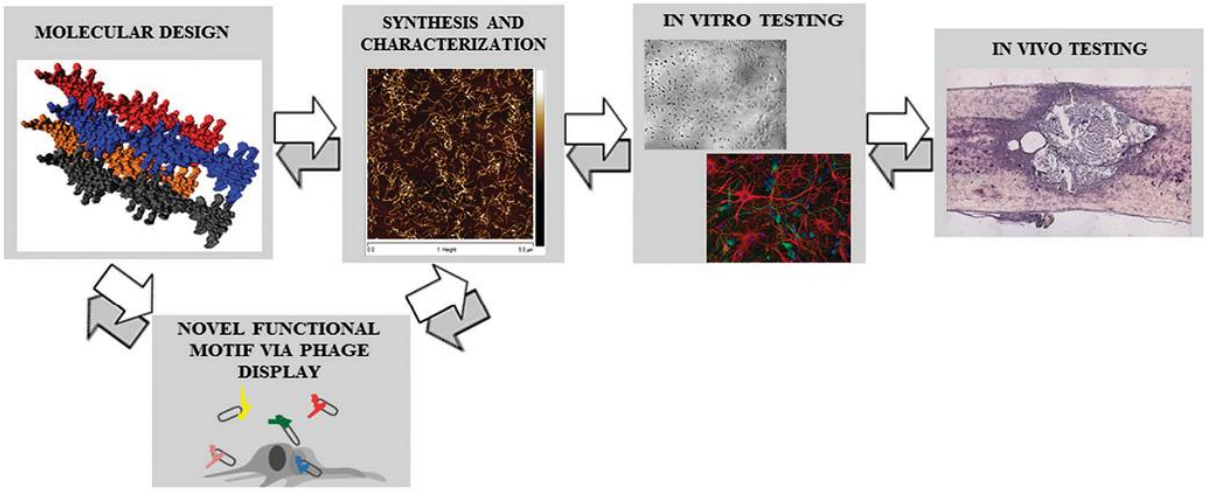

Figura 2: Esquema evidenciando métodos adotados durante o desenvolvimento e teste de um material como potencialidade para uso em Engenharia de tecido (Retirado de Saracino et al. 2013)

2.1.1 Neuroengenharia de tecidos

A Neuroengenharia é uma área de pesquisa que integra métodos de Neurociência e de Engenharia para desenvolver soluções voltadas às limitações e disfunções associadas ao sistema nervoso. Essa área engloba aspectos de pesquisa experimental, computacional, teórica, clínica e aplicada em níveis moleculares, celulares e sistemas, em diversas áreas, dentre as quais estão interface cérebro-máquina; neurotecnologia, neuroreabilitação, regeneração de tecido neural e neurociência translacional.

A Neuroengenharia de tecidos chama atenção da comunidade científica tanto por ser uma promissora via terapêutica, considerando a baixa taxa de regeneração do tecido neural adulto, quanto por proporcionar a oportunidade de investigar aspectos funcionais do sistema nervoso em um "tecido" cultivado em ambiente controlado e passível de interferências específicas e direcionadas (Shi et al. 2012). O sistema nervoso possui funcionamento complexo: de um lado neurônios, células especializadas com morfologia e função variáveis e intrinsecamente relacionadas, são estruturas frágeis e dependentes do suporte oferecido por diferentes tipos de células da glia bem como da microglia. A glia, por outro lado, compreende diferentes tipos celulares, com funções específicas e essenciais que participam ativamente dos processos fisiológicos que ocorrem no SNC e SNP. Além disso, é a maneira como as células se dispõem e se conectam que confere funcionalidade ao tecido neural e esse talvez seja o grande desafio ao reparo de lesões. A neuroengenharia de tecidos engloba três grandes áreas

experimentais que visam analisar interações em cultura de células, reparo de lesões no SNP e reparo de lesões no SNC, e tem feito importantes avanços em todas elas

Uma variedade de biomateriais tem sido usada para o cultivo de células neurais. Dentre eles, podemos citar géis e superfícies semi-sintéticas ou totalmente sintéticas ou ainda constituídas por proteínas da matriz extracelular (Little, Healy, and Schaffer 2008), *scafolds* de nanofibras (Vasita and Katti 2006) polímeros híbridos (natural-sintético) de quitosana (García Cruz et al. 2008; Guerra et al. 2011), fibras produzidas pelo bicho da seda *Bombix mori* (Flanagan et al. 2015) *scafolds* tubulares de amido-policaprolactona vem sendo testados como suporte para células de Schwann, para recuperação de injúrias na medula espinhal (Tukmachev et al. 2016; Fan et al. 2017,).

O sistema nervoso periférico possui capacidade de regeneração superior ao sistema nervoso central. No entanto, injúrias que promovam destruição da matriz extracelular de nervos periféricos reduzem significativamente suas chances de recuperação funcional (Ide 1996). Nesse sentindo a neuroengenharia de tecidos tem obtido resultados animadores no tratamento de lesões de nervos periféricos. Inicialmente foram usados tubos ocos, como conduites, que uniam as duas extremidades de um nervo seccionado e serviram como isolamento para regeneração do nervo (Wieringa et al. 2018). Alguns autores, em revisões de literatura, apontam para os bons resultados deste tipo de implante, mas ressaltam o fato de se tratar de uma estrutura que não possui arquitetura interna para guiar o crescimento do nervo (Daly et al. 2013). Uma outra alternativa terapêutica para transecção de nervos pertiféricos são os enxertos autológos e heterólogos (xenograf), estes no entanto encontram limitações importantes devido à resposta imune que provocam (Lee and Wolfe 1999). Mais recentemente começaram a ser desenvolvidos *scaffolds* para reparo de nervos, com estrutura interna para servir de suporte aos neuritos e ao cone axonal. Panseri e colaboradores (2008) utilizaram um implante 3D a base de PGLA/PGL e observaram regeneração e recuperação funcional na maioria dos animais submetidos à lesão de 10mm no nervo ciático.

No SNC, biomateriais vem sendo utilizados para: (1) servir como arcabouço para manutenção de células tronco ou neurais transplantadas (Jahanbazi Jahan-Abad et al. 2018), (2) servir como ponte estrutural, facilitando a regeneração do tecido após injúria (Zhou et al. 2016), ou ainda como ponto de liberação para fatores tróficos e medicamentos para a manutenção de células transplantada e/ou células tecido hospedeiro (Xu et al. 2018). É crescente o número de estudos que avalia o comportamento, *in vivo,* de diferentes materiais como ferramentas para o tratamento de injúrias cerebrais traumáticas. Os modelos de TCE por

produzirem lesão com características favoráveis ao implante de biomateriais (Skop et al. 2016) e grande relevância epidemiológica, como será abordado adiante.

2.2 TRAUMATISMO CRANIOENCEFÁLICO (TCE)

O traumatismo cranioencefálico pode ser definido como uma interrupção na função normal do cérebro provocada por uma colisão, golpe, choque ou lesão penetrante na cabeça, além disso movimentos bruscos de aceleração ou desaceleração da cabeça também são capazes de gerar lesão anatômica e comprometimento da função cerebral, configurando também um TCE (Ling, Hardy, and Zetterberg 2015). A ação das forças mecânicas sobre o tecido encefálico promove dano e morte celular, ruptura de vasos e descontinuidade da barreira hematoencefálica, configurando a lesão cerebral primária que ocorre no momento do trauma (Lee et al. 2015). Nas horas e semanas posteriores ao trauma se seguem processos inflamatórios, neuroquímicos e metabólicos que afetam as células neurais sobreviventes e podem, progressivamente aumentar o comprometimento neurológico, essas cascatas de eventos configuram a lesão cerebral secundária ao trauma (Okano 2002) e será abordada em mais detalhes posteriormente neste tópico.

Considerado um problema de saúde pública em todo o mundo, o TCE atinge um amplo espectro da população e apresenta elevada incidência em crianças e adultos jovens (Tian, Prabhakaran, and Ramakrishna 2015) acarretando um grande impacto socioeconômico. Nos Estados Unidos aproximadamente 1,7 milhões de pessoas sofrem TCE por ano, representando um custo anual de cerca de 60 bilhões de dólares (Roozenbeek, Maas, and Menon 2013). No Brasil os dados epidemiológicos a respeito de vítimas de TCE são escassos, mas estudos baseados em dados do DATASUS de 2008 a 2012 revelam 125.500 internações hospitalares por ano associadas a TCE estima-se que mais de um milhão de pessoas vivam, no Brasil, com sequelas neurológicas irreversíveis decorrentes de TCE (Magalhães et al. 2017).

O TCE está entre as principais causas de morte e perda funcional que acomete a população e ocupa primeira posição quando se considera a faixa de idade que compreende os adultos jovens (Buckley et al. 2017). A variedade de mecanismos causadores e a extensão das lesões torna difícil um prognóstico linear. Uma classificação amplamente aceita e usada na prática clínica inclusive para fins prognósticos é a divisão do quadro clínico em: TCE leve, moderado e severo. Essa classificação utiliza como principais critérios presença de alterações nos exames imagem, perda da consciência, alteração da consciência, amnésia pós-traumática e score na escala de coma de Glasgow (de Almeida-Pititto, Almada Filho, and Cendoroglo 2008).

Apesar do crescente esforço científico, sua terapêutica ainda é desafiadora devido à heterogeneidade dos mecanismos de lesão, imprecisão das áreas afetadas e variedade de processos patológicos potencialmente desencadeados pelo trauma (Jassam et al. 2017). Conforme abordado anteriormente a lesão cerebral decorrente do evento traumático é classificada em primária, consiste no dano tecidual mecânico direto, e secundária, que ocorre no nível celular e é mediada pelas seguintes vias principais: (1) excitotoxicidade, causada pelo excesso local de neurotransmissores excitatórios tais como o glutamato (LaPlaca et al. 2007); (2) aumento na produção de radicais livres, que irão promover danos à biomoléculas (Anthonymuthu, Kenny, and Bayır 2016); e (3) resposta imune, por ativação do sistema imune local ou sistêmico (Simon et al., 2017).

2.2.1 Fisiopatologia do TCE

A excitotoxicidade consiste uma cascata de eventos celulares desencadeados pelo aumento na presença de neurotransmissores no espaço extracelular e na estimulação excessiva dos neurônios que pode levar à lesão e/ou morte celular. A via de ativação glutamatérgica é a mais investigada na fisiopatologia do TCE. A hipótese da excitotoxicidade glutamatérgica coloca o glutamato, como peça central para progressão e propagação da lesão secundária no cérebro após o TCE (McAllister et al. 2011), no entanto é crescente o número de estudos que investiga a ação excitotóxica de outros neurotransmissores em populações específicas de neurônios, como por exemplo a excitotoxicidade GABAérgica em neurônios imaturos do giro denteado do hipocampo em modelo de TCE (Deng, Aimone, and Gage 2010).

Além da presença local de fatores nocivos resultados da lesão cerebral primária, dois mecanismos podem explicar o aumento não fisiológico na liberação de neurotransmissores após a injúria cerebral traumática. O estresse mecânico a que as células são submetidas no momento do trauma levaria ao aparecimento de poros na membrana e consequente influxo de ións por difusão simples, ou a presença de receptores mecânicos na membrana que seriam ativados no momento da injúria e permitiriam também influxo de cátions (Krishnamurthy and Laskowitz 2016). Ambos mecanismos culminariam com alteração do potencial de membrana e ativação de canais de cálcio voltagem-dependentes, produzindo um aumento na concentração de Ca^{2+} intracelular, que por sua vez sinaliza para a liberação de neurotransmissores pelas vesículas sinápticas. Na excitotoxicidade glutamatégica há estimulação de receptores α-amino-3-hidroxi-metil-5-4-isoxazolpropiónico (AMPA), cainato e N-metil-D-aspartato (NMDA) que promoverão despolarização do neurônio pós-sináptico de forma não fisiológica (Werner and Engelhard 2007). A ativação do receptor NMDA induz aumento na concentração de Ca^{+2}

citosólico o que leva a ativação de proteases, fosfolipases, geração de radicais livres e disfunção mitocondrial (Quillinan et al. 2017).

Secundariamente ao insulto cerebral ocorre aumento na presença de espécies reativas de oxigênio e nitrogênio, moléculas que possuem elétrons livres e alta capacidade de reagir com elementos celulares, produzindo, entre outros danos, prejuízos à função mitocondrial. A falência mitocondrial leva a maior depleção energética e redução do pH, fatores que tendem a aumentar o dano tecidual (Giza and Hovda 2001).

Por fim a resposta neuroimune é considerada um importante aspecto na evolução da lesão secundária. A resposta imune inicial se desenvolve minutos após a injúria cerebral por sinalização das células da glia, neurônios e meninges danificadas, através da liberação de moléculas associadas ao dano que são inicialmente reconhecidas pela micróglia, segue-se um infiltrado de outras células imunes (como neutrófilos e macrófagos), produção e liberação de diversos fatores inflamatórios (Jassam et al. 2017). A magnitude da resposta imune está relacionada com os desfechos possíveis para o TCE, uma vez que sua ativação é necessária inclusive aos eventos plásticos que se seguirão ao trauma e, por outro lado, essa mesma ativação em maior magnitude pode ser responsável por aumento da lesão (Bergold 2016). A figura 3 resume a sequência temporal de alguns dos eventos que ocorrem após o TCE os quais envolvem respostas imunomodulatórias.

Vários modelos experimentais in vitro e in vivo de TCE vêm sendo adotados na tentativa de entender melhor mecanismos celulares e moleculares envolvidos na sua complexa fisiopatologia, embora discuta-se que nenhum modelo isolado será capaz de reconstituir todos os tipos de danos primários e secundários observados no TCE em humanos bem como a complexa diversidade de mecanismos de injúria que contribuem para os resultados observados em cada indivíduo (Saatman et al. 2008).

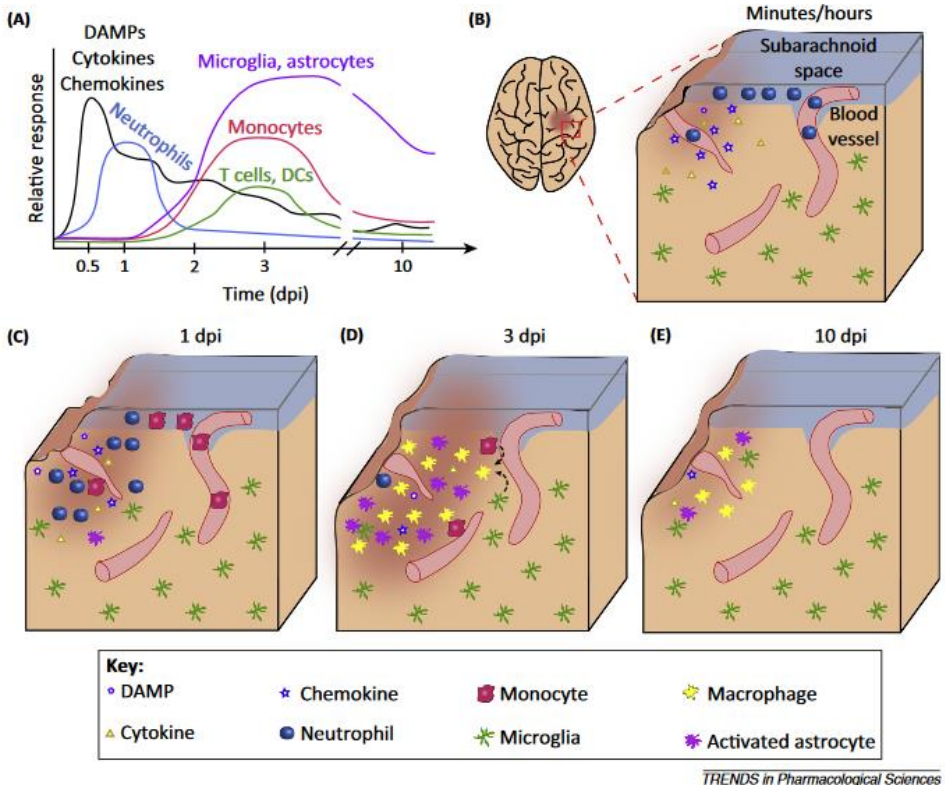

Figura 3: Resposta inflamatória após um TCE. A: Evolução temporal das respostas de potenciais mediadores celulares e moleculares ativados após um TCE. B-E: Representação histológica da reação inflamatória após minutos ou horas (B) e após dias (D-E) (dpi – dias pós-lesão). Retirado de Gyoneva e Ransohoff (2015).

2.2.2 Neuroengenharia de tecidos no TCE

Os tratamentos atualmente disponíveis para quadros agudos de lesão cerebral como no TCE são farmacológicos e possuem como principais alvos a limitação do dano e retardo de processos degenerativos (Brasure et al. 2013). Além disso os fármacos encontram algumas dificuldades para atingir seus alvos no sistema nervoso central, como a alta seletividade da barreira hematoencefálica e a ação inespecífica resultante da administração sistêmica (Orive et al. 2009). A busca por estratégias terapêuticas mais eficientes encontrou na Engenharia de tecidos um campo promissor.

Nesse sentido é crescente o número de estudos que avalia o comportamento, *in vivo*, de biomateriais como ferramentas para o tratamento de injúrias cerebrais traumáticas. Os modelos experimentais de TCE produzem lesão focal (única ou associada à outras lesões difusas) com extensa perda celular e de matriz extracelular (Skop et al. 2016) que configuram cavidades que serão sítio de aplicação do biomaterial. Além disso modelos experimentais de AVE e TCE possuem a característica de cursar com elevada taxa de morte celular e degradação da matriz

extracelular que levam ao surgimento ou aumento no volume dessas cavidades dificultando ainda mais os processos fisiológicos de reparo neural (Aertker, Bedi, and Cox 2016).

Em levantamento bibliográfico recente na base de dados *pubmed* usando os descritores na língua inglesa, indexados no DeSC/MeSH: *Neural tissue engineering AND traumatic brain injury*, foram encontrados 106 artigos dos quais (21) utilizaram implante de diferentes biomateriais em modelos experimentais de traumatismo craniano. A tabela 1 resume o tipo de biomaterial utilizado por esses estudos, se o material foi usado para implante de células (e qual tipo de célula), e se os biomateriais foram funcionalizados com alguma biomolécula; além de indicar o modelo de TCE adotado pelos experimentadores. Os aspectos gerais dos artigos selecionados serão abordados a seguir.

Quadro 1: Quadro esquemático apresentando trabalhos que utilizaram modelos experimentais de traumatismo cranioencefálico para implante de biomateriais.

ARTIGO	MATERIAL	CÉLULAS	FATOR TRÓFICO	MODELO
Tian et al. 2005	Hidrogel de um copolímero de ácido hialurônico – poli-d-lisina	_____	_____	Lesão por incisão direta
Wei et al. 2007	Hidrogel de /ácido hialurônico(HA)	_____	Sequência peptídica IKVAV - promotora de crescimento neurítico	Lesão por incisão direta
Wong et al. 2007	Scaffolds de ácido poli-l-lactico-co-glycolic (PLGA) e poli- ε -caprolactona (PCL)	_____	_____	Lesão por incisão direta
Wong et al. 2008	Scaffold de poli-ε-caprolactona (PCL)	_____	_____	Lesão por incisão direta
Tate et al. 2009	Scaffolds baseado em laminina ou fibronectina	Células tronco neurais	_____	Lesão por impacto cortical controlado
Wang et al. 2012	Poli-ε-caprolactona (PCL)	Células neurais corticais	iGDNF	Lesão por incisão direta
Sarnowska et al. 2013	Scaffolds de laminina ligada a gelatina	Células tronco mesenquimais do cordão	_____	Modelo de injúria induzida por oubaína

		umbilical (HUCMSCS)		
Mahmood et al. 2014	Scaffolds de colágeno	Células tronco da medulla óssea (MSCS)	_____	Lesão por impacto cortical controlado
Álvarez et al. 2014	Scaffold a base de poli-l/d ácido lactico com nanofibras de electrospun.	_____	L-lactato (indutor de angiogênese)	Lesão por sucção através de seringa
Martinéz-Ramos et al. 2015	Scaffold baseados em etil-acrilato (EA) e hidroxil-etil-acrilato (HEA)	_____	_____	Criolesão
Shin et al. 2015	Matriz semissintética de colágeno	_____	_____	Lesão por impacto cortical controlado.
Elias and Spector 2015	Scaffold a base de colágeno	_____	Molécula solúvel receptor NOGO (SNGR) (evita colapso do cone de crescimento axonal)	Modeno de lesão por expansão rápida de projétil.
Rivet et al. 2015	Hidrogel de agarose/metilcelulosec om fibras de electrospun dispersas	_____	Fibronectina	Colocação direta do scaffold na região do estriado.
Vaysse et al. 2015	Implante de polydimethylsiloxane (PDMS) micromodelado	Células neurais Hnt2	_____	Lesão obtida por injeção da toxina de malonato.
Li et al. 2016	Hidrogel de ácido hialurônico	Células tronco neurais	Neurogenina-2 (NGN2) – fator de transcrição envolvido na neurogênese.	Lesão por impacto cortical controlado.
Aligholi et al. 2016	Hidrogel de puramatrix	Células tronco neurais	_____	Lesão por incisão direta.
Skop et al. 2016	Scaffolds baseados em quitosana	Linhagem de células Rg3.6 – precursors neurais	FGF-2 – Fator de crescimento de fibroblasto - 2	Lesão por impacto cortical controlado.

Duan et al. 2016	Scaffold de colágeno e hialuronato de sáodio	Células tronco neurais.	BFGF (induz diferenciação de células tronco neurais	Lesão por sucção através de seringa.
Zhou et al. 2016	scaffolds de microfibras de electrospun, grafeno e poli-ε-caprolactona (PCL)	_____	_____	Colocação direta do scaffold nas regiões do estriado e svz
Xu et al. 2018	Matrigel	_____	semaphorin 3A (Sema3A)	Lesão por incisão direta
Jahanbazi Jahan-Abad et al. 2018	Hidrogel puramatrix	Células tronco neurais humanas e células tronco derivadas de tecido adiposo	_____	Lesão por incisão direta

Conforme abordado anteriormente a neuroengenharia de tecidos envolve o implante de biomateriais seja para agir como suporte na recuperação tecido lesionado, seja para servir na entrega de fatores neurotróficos, fármacos, implante ou transplante de células. Wong e colaboradores (2008) ressaltam a importância da realização de experimento iniciais usando o biomaterial puro como implante, para que se conheçam seus potenciais e problemas na interação com o tecido hospedeiro. Estudos que utilizaram diferentes biomateriais, *scaffold* de ácido poli-L-láctico-co-glicolítico e uma matriz de colágeno, observaram redução no volume final da lesão e redução da perda celular (Wong et al. 2007; Shin et al. 2015), indicando que a simples presença de um biomaterial na cavidade produzida pelo trauma pode ter efeitos positivos na evolução da lesão. O processo inflamatório que se segue ao trauma e a formação da cicatriz astrocitária estão intimamente relacionados à gravidade e progressão da lesão, o uso de um scaffold baseado em um copolímero de etil-acrilato (EA) e hidroxi-etil-acrilato (HEA) reduziu a cicatriz glial, além de permitir infiltrado de neurônios, células da glia e presença de neovasos (Martínez-Ramos et al. 2015). Redução na formação da cicatriz glial também foi observada por Zhou e colaboradores (2016) ao utilizar *scaffold* de *eletrospun* associado a grafeno.

A forma de apresentação do biomaterial também influencia sua interação com o tecido circundante. O uso de *scaffolds* de poli-(ε-caprolactona), sob duas diferentes conformações 3D, em lesões corticais, resultou em maior crescimento tecidual total e maior infiltração astrocitária nos *sacaffolds* com estrutura ortogonal, quando comparados àqueles com canais unidirecionais

orientados longitudinalmente (Wong, Krebsbach, and Hollister 2008). Evidenciando que a arquitetura do andaime pode beneficiar e direcionar a regeneração do tecido cerebral. Outra importante característica em relação às características físicas dos implantes está relacionada à sua adaptação ao sítio de lesão, que em sua maioria é irregular, nesse sentido o uso do material na forma de hidrogel permite maior contato e interação com o tecido hospedeiro (Rivet et al. 2015). Exemplificando essa abordagem, hidrogel de copolímero de ácido hialurônico-poli-D-lisina utilizado em cavidade pós-TCE permitiu contiguidade com o parênquima cerebral nas semanas que se seguiram ao trauma (Tian et al. 2005).

Alguns biomateriais permitem acoplamento de biomoléculas, que podem ser usadas no intuito de melhorar a interação com o tecido hospedeiro, estimular neuroregeneração, crescimento e migração celular, entre outros. O uso de biomoléculas em implantes para reparo tecidual em modelos experimentais de TCE tem mostrado bons resultados. O implante de scaffold biomimético com liberação de L-lactato, uma via celular de indução de angiogênese, em ratos neonatos resultou em completa vascularização, manutenção da neurogênese, sobrevivência e integração dos neurônios neoformados. As observações foram feitas em até 15 meses após a cirurgia (Álvarez et al. 2014). Em animais adultos o uso de *scaffolds* associados a substancias que induzam crescimento neurítico como a sequência de pepitídeos IKVAV, que promove ainda adesão celular e angiogênese (Wei et al. 2007), ou que reduza bloqueios fisiológicos à regeneração, como exemplo a molécula solúvel receptor NOGO (SNGR), um neutralizador de proteínas da mielina que impedem regeneração axonal induzindo colapso do cone de crescimento (Elias e Spector, 2015), embora não tenha atingido os resultados específicos das biomoléculas acopladas, tiveram efeitos positivos na interação com o tecido hospedeiro.

Outro aspecto importante da neuroengenharia de tecidos é a terapia celular. O uso de células tronco e o transplante de células neurais para reparo de lesões centrais é uma esperança dentro da neurociência, no entanto uma limitação dessa técnica se relaciona com a manutenção e diferenciação das células no tecido hospedeiro. A inserção de células livres no ambiente hostil pós-lesão não obteve sucesso até então, acredita-se que a falta de fatores de crescimento e de adesão celular, além da presença de células imunes levam essas células à morte ou migração (Harting et al. 2009). Os biomateriais podem servir como barreira física ao ambiente desfavorável e permitir contenção dessas células no sítio de interesse e ainda como fonte de liberação de fatores tróficos e de crescimento. O implante de células tronco neurais (Aligholi et al. 2016; Duan et al. 2016) (Tate et al, 2009; Li et al, 2016; Skop et al, 2016;), células tronco mesenquimais do cordão umbilical (Sarnowska et al. 2013), células tronco do estroma ósseo

(Mahamood et al, 2014), células neurais corticais (Wang et al. 2012) e células neurais HNT2 (Vaysse et al. 2015) em *scaffolds* e hidrogéis de diferentes biomateriais, associados ou não à fatores tróficos (Tabela 1) resultou em sobrevivência e contenção dessas células na área de lesão. Evidenciando que o uso de biomateriais é peça fundamental para a terapia celular.

2.3 EXOPOLISSACARÍDEO CELULÓSICO OBTIDO A PARTIR DO MELAÇO DA CANA-DE-AÇÚCAR (CEC)

A celulose bacteriana originária da cana-de-açúcar é um exopolissacarídeo obtido por via microbiológica a partir do melaço de cana-de-açúcar e sua constituição química está detalhada na tabela 1 (Paterson-Beedle et al. 2000). Esta celulose foi sintetizada inicialmente na Estação Experimental de Cana de Açúcar de Carpina, da Universidade Federal Rural de Pernambuco – UFRPE. Desde 2001, o Núcleo de Cirurgia Experimental da Universidade Federal de Pernambuco vem desenvolvendo uma série de ensaios químicos com o objetivo de adequar a utilização da CEC para diversas aplicações farmacêuticas e cirúrgicas (Figura 4).

Tabela 1: Monossacarídeos que compõem a CEC (percentual)

Monossacarídeos	Composição (%)
Glicose	87,57
Xilose	8,58
Ribose	1,68
Acido glicurônico	0,83
Manose	0,82
Arabinose	0,37
Galactose	0,13
Ramnose	0,01
Fucose	0,01

A CEC atende a uma importante prerrogativa que os biomateriais devem apresentar para serem viáveis à engenharia de tecidos: ela pode ser processada em uma variedade de formas de apresentação, tais como: hidrogel, membranas, esponjas e fios de sutura. Preparações de hidrogel da CEC adquirem propriedades viscoelásticas e permanecem estáveis na temperatura de fluidos biológicos e temperatura ambiente, em concentrações de 0,6 a 0,8% (Paterson-Beedle

et al. 2000). Pita e colaboradores (2015) testaram, através de implante subcutâneo em coelhos, a biocompatibilidade e reação de sensibilidade local do hidrogel da CEC, observando ausência de toxicidade e boa interação com o tecido adjacente permitindo remodelagem e vascularização. Ainda na forma de hidrogel a celulose bacteriana obteve boa performance no reparo de olhos eviscerados de coelhos (Cordeiro-Barbosa et al. 2012) e, em ratos *Wistar*, na cicatrização óssea (Albuquerque et al. 2011), na cicatrização óssea associado à proteínas morfogênica ósseas (Medeiros Junior et al. 2013) e como barreira isolante para evitar aderência peritoneal após cirurgia abdominal (Coelho Junior et al. 2015).

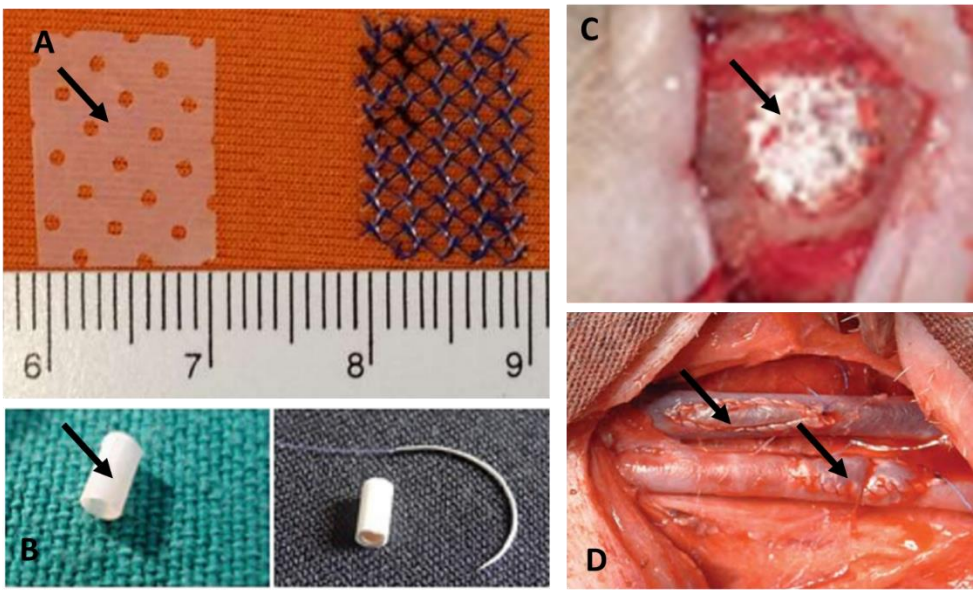

Figura 4: Deferentes apresentações e aplicações do CEC. A) Tela de CEC (seta preta), ao lado de tela de polipropileno, usada em cirurgia para tratamento de incontinência urinária (Silveira et al, 2014); B) tubo de CEC (seta preta), ao lado de seu equivalente de poliuretano, usado como cateter implantado na bexiga (Lima et al, 2018); C) CEC granulado usado para reparo ósseo em calota craniana (Abreu et al, 2016) e D) membrana de CEC usada na reconstrução de veia e arteia femorais (Andrade-Aguiar et al, 2007).

A partir do hidrogel do CEC podem ser obtidas membranas e esponjas em diferentes configurações. Tavares e colaboradores (2014) utilizaram esponjas de CEC para hemostase e para favorecer o reparo do parênquima renal em modelo de trauma, o resultado foi semelhante ao grupo controle que usou uma esponja hemostática de comum uso clínico. Membranas de CEC produzidas a partir do gel 0,8%, de tamanho e espessura variáveis de acordo com o uso a que se destina, foram testadas em diversos modelos cirúrgicos e apresentação boas adaptação mecânica e aceitação pelos tecidos hospedeiros. Usada em arterioplastia de artéria femoral em cachorros apresentou similar eficiência em relação ao ePTFE, material comumente usado na clínica (Aguiar et al. 2007; Romero et al. 2007). Foi observada boa adaptação mecânica evidenciada pela ausência de dilatações, falso aneurisma ou rupturas do implante (Romero et

al., 2007). Resultados igualmente positivos ocorreram quando membranas de CEC foram usadas em angioplastia de veia femoral em cães (Barros-marques et al. 2012).

Atuando como enxerto na cirurgia de Sling em ratos *Wistar*, a membrana do CEC foi mais eficaz em promover deposição de fibras colágenas que a tela de polipropileno, usual neste tipo de cirurgia (Silveira et al. 2014). Ensaios experimentais realizados em ratos também evidenciaram que esse biomaterial pode ser utilizado com sucesso como substituto da dura-máter em cirurgias neurológicas que envolvem, por exemplo, craniotomia fronto-parietal (Lima et al. 2017), onde o CEC apresentou propriedades mecânicas adequadas e não foram observados sinais de neurotoxicidade. O processamento dessas membranas permitiu ainda a apresentação da celulose bacteriana na forma de fios de sutura (Carvalho-Júnior et al. 2012) e tubos para implantação na bexiga para coleta de urina (Lima et al. 2015), obtendo bons resultados nos dois casos.

Em estudos *in vitro* o CEC na forma de membrana foi testada inicialmente para análise de sua citotoxicidade em culturas de macrófagos alveolares, demonstrando que o mesmo é um produto atóxico, não comprometendo a viabilidade celular (Castro *et al*., 2004). Foi também demonstrado que o CEC é um suporte adequado para adesão e crescimento de células tronco da geléia do cordão umbilical humano (Fragoso et al. 2014). Atualmente esta celulose bacteriana é produzida também na forma de *hidrogéis coloidais* permitindo que sejam realizadas culturas tridimensionais, além das bidimensionais obtidas nas membranas (Gonçalves-Pimentel et al. 2018).

Alguns biomateriais apresentam importante limitação translacional e esse aspecto pode culminar na inviabilidade de seu uso na engenharia de tecidos. O uso do CEC em humanos tornou-se realidade nos últimos anos e a pesquisa clínica avança em diferentes tecidos. Finas membranas de CEC foram usadas como curativo no tratamento de pacientes com úlceras venosas de membros inferiores (Cavalcanti et al. 2017) e como curativo após cirurgias na região genital masculina (Vilar et al. 2016; Martins et al. 2013) apresentando resultados satisfatórios semelhantes àqueles obtidos com os materiais padrões normalmente utilizados. Nos dois estudos chama atenção o fato que os pacientes tratados com CEC relataram redução significativa da dor e desuso precoce de analgésicos. (Silveira et al. 2016) compararam, na reconstrução de membrana timpânica perfurada, o desempenho de membrana CEC com enxerto autólogo oriundo da fáscia temporal. Neste estudo os achados fisiopatológicos foram semelhantes para os dois grupos, no entanto outros aspectos chamaram atenção: o tempo de duração da cirurgia foi 5 vezes menor e o custo da cirurgia, cerca de 13 vezes menor para os pacientes que receberam o biomaterial proveniente da cana-de-açúcar. Estudo experimental

para avaliar o comportamento deste material em lesão de ouvido médio havia mostrado uma reação inflamatória inicial mais intensa no grupo que recebeu a membrana de CEC quando comparado ao enxerto autólogo e diminuía ao longo das semanas (Lopes et al. 2011). O quadro 2 resume dados obtidos até então nos estudos que utilizaram a CEC.

Quadro 2: Quadro esquemático apresentando trabalhos que utilizaram CEC e testaram seus resultados em variadas condições patológicas, em humanos e animais.

ARTIGO	FORMA DO CEC	SUJEITOS	APLICAÇÃO
Lima et al. 2018	Tubos	Ratos *Wistar*	Cateter para a bexiga
Tavares de Lima et al. 2017	Membranas	Ratos *Wistar*	Duraplastia
Cavalcanti et al. 2017	Membranas	Humanos	Curativo para úlceras venosas de membros inferiores
Vilar et al. 2016	Membranas	Humanos	Curativo pós-cirúrgicos em região genital masculino.
Abreu et al. 2016	Particulado	Ratos *Wistar*	Reparo ósseo
Silveira et al. 2016	Membrana	Humanos	Tratamento de perfuração da membrana timpânica.
Silveira et al. 2016	Membrana (perfurada e compacta)	Ratos *Wistar*	Reparo de lesão aguda induzida na aponeuroses de músculo abdominal.
Pita et al. 2015	Hidrogel	Coelhos	Reparo cartilaginoso
Albuquerque et al. 2015	Hidrogel	Coelhos	Reparo ósseo
Albuquerque et al. 2015	Hidrogel	Coelhos	Reparo ósseo
De Lucena et al. 2015	Membrana	Ratos *Wistar*	Cicatrização de lesão cutânea.
Lima et al. 2015	Hidrogel	Coelhos	Testar biocompatibilidade.
Coelho-Júnior et al. 2015	Hidrogel	Ratos *Wistar*	Barreira para prevenção de adesão peritoneal pós-operatória.
Teixeira et al. 2014	Membrana esponjosa	Ratos *Wistar*	Cicatrização de lesões aftosas na mucosa oral.
Tavares et al. 2014	Esponja	Coelhos	Restauração do parênquima renal em modelo de trauma.
Silveira et al. 2014	Tela (membrana perfurada)	Ratos *Wistar*	Cirurgia de Sling (padrão ouro no tratamento da incontinência urinária de esforço)

Martins et al. 2013	Membrana	Humanos	Curativo pós-cirúrgicos em região genital masculina.
Fragoso et al. 2013	Membrana	*In vitro*	Cultura de células tronco do cordão umbilical humano
Medeiros Júnior et al. 2013	Hidrogel (+BMPs)	Ratos *Wistar*	Reparo ósseo
Barros-Marques et al. 2012	Membrana	Cães	Angioplastia de veia femoral.
Cordeiro-Barbosa et al. 2012	Hidrogel	Coelhos	Olhos eviscerados de coelhos (bolsa escleral suturada e preenchida com biopolimero).
Carvalho-Júnior et al. 2012	Fios de sutura	Ratos *Wistar*	Suturas na bexiga.
Albuquerque et al. 2011	Hidrogel	Coelhos	Preenchimento e reparo de defeitos osteocondrais.
Lopes et al. 2011	Membrana	Ratos *Wistar*	Reparo de perfuração timpânica
Romero et al. 2007	Membrana	Cães	Arterioplastia femoral (enfoque histológico e na hemodinâmica)
Andrade Aguiar et al. 2007	Membrana	Cães	Arterioplastia femoral.
Falcão, Coelho, and Evêncio Neto 2008	Membrana	Ratos *Wistar*	Avaliação biomecânica em cirugias abdominais

O uso do CEC tem encontrado êxito em diferentes tecidos, condições patológicas e espécimes. A mesma constitui um material adaptável, permitindo não apenas diferentes formas de apresentação como também a associação com moléculas, característica essencial à Engenharia de tecidos. Possui boa biocompatibilidade, baixa imunogenicidade e interação com o tecido hospedeiro, além de boa estabilidade química e mecânica. Permite ainda adesão e diferenciação celular nos experimentos *in vitro*. As características agora listadas são as principais prerrogativas para eleição de um biomaterial promissor à neuroengenharia de tecidos (Orive et al. 2009). Os trabalhos nesse campo foram iniciados pelo Laboratório de Neurofisiologia da UFPE, e visam testar a hipótese de que o CEC pode ser um biomaterial adequado como suporte para células do sistema nervoso, com potencial aplicação na recuperação do tecido neural submetido a traumatismo craniano.

3 BIOCOMPATIBILITY OF A CELLULOSIC EXOPOLYSACCHARIDE IMPLANT FOR THE REPAIR OF DEFECTS IN EXPERIMENTAL MODEL OF TRAUMATIC BRAIN INJURY

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Running title: Bacterial cellulose as a cell scaffold for brain tissue defects

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Introduction

Traumatic brain injury (TBI) can be induced by different types of insults with deleterious effects on the skull, brain parenchyma, and meninge integrity. TBI is considered the most prevalent cause of death and morbidity in the World, especially in the young population (Leon-Carrion et al., 2005). Pharmacological intervention has been used for reducing the sequelae in the acute and chronic stage of brain injury (Faden et al., 1996; Gultekin et al., 2016). Nevertheless, early and recent studies have adopted techniques of tissue engineering (TE) for intracerebral implantation of synthetic or natural polymers as a new perspective and a promising option for cellular replacement and rescue of resident cells in the brain defects (review in LaPlaca et al., 2011; Forraz et al., 2012; Khaing et al., 2014). Scaffolds of poly ε-caprolactone (PCL; Wong et al., 2007), hydrogels of components of extracellular matrix such as hyaluronic acid (Wei et al., 2007), fibronectin (Tate et al., 2009), collagen, copolimers of acrilate (etilacrilate and hidroxietilacrilate; Ramos et al., 2015), among others materials have been tested in different experimental models of TBI with variable results. The efficacy of these materials have been evaluated regarding their adaptability to the brain tissue, possibility to be conjugated to diverse molecules including trophic factors, low immune response, mechanical and chemical stability, low toxicity providing architectural support for the adjacent brain parenchyma, while allowing cell adhesion, migration, and differentiation (Pettikiriarachchi et al., 2010*).*

Cellulosic exopolysaccharide obtained by biotechnological synthesis via bacteria has attracted interest for biomedical applications especially in bone, cartilage and skin tissue engineering (Czaja et al., 2006; Khan and Ahmad, 2013). Bacterial cellulose produced by *Acinetobacter* or *Acetobacter* bacteria fermentation was used for the formation of a neoduramater following prenatal correction of meningomyelocele in fetal sheep (Sanchez e Oliveira et al., 2007) or as dural substitute in humans (Mello et al., 2012) or rabbits (Xu et al., 2014). A modified bacterial cellulose subjected to a mercerization process has been also tested as an alternative neuro tube for regeneration of peripheral nerve (Kowalska-Ludwicka et al., 2013). Biocompatibility of cellulosic exopolysaccharide, produced via bacterial action on sugarcane molasses (CEC) has been investigated in diverse biomedical assays considering its low toxicity and ability to be incorpored, permitting vascularization of the new tissue (Barros Marques et al., 2012; Pinto et al., 2016). CEC is also a competitive biomaterial considering its low production cost and capability of integration with different living tissues (Carvalho-Junior et al., 2012; Medeiros-Junior et al., 2013; Lima et al., 2015; de Lucena et al., 2015; Silveira et al., 2016). Colloidal hydrogels of CEC in concentrations of 0.6 to 0.8% exhibit viscoelastic properties and stability at temperatures adequate to biological fluids (Paterson-Beedle et al,

2000). In a recent study, it was also reported the biocompatibility of CEC films as *dura mater* substitute for up 120 days, without cause inflammation or other adverse reaction in the rat cerebral cortex (Lima et al., 2017). *In vitro* study using electrical impedance spectroscopy (EIS) have demonstrated that biophysical properties of CEC are adequate to adhesion and growth of human umbilical cord Wharton's jelly mesenchymal stem cells (Fragoso et al. 2014).

Using 2D (films) and 3D (colloidal hydrogel) configurations of CEC we have shown its biocompatibility as a substrate for neuron and/or astrocyte primary cultures analyzing cell viability, adhesion, growth, phenotypic differentiation and the NMDA-induced Ca^{2+} influx in neurons (Pimentel et al., 2018, submitted paper). The purpose of the current study is to test the hypothesis that CEC colloidal hydrogel can be a promising scaffold material for the repair of brain defects. Adopting an experimental model of mechanic TBI, it was evaluated the ability of CEC implantation into the cerebral cortex to serve as a bridge for endogenous cell migration reducing the impact of primary and secondary cellular effects of the lesion. It is recognized that mechanical TBI is able to induce disruption of intracellular structures and activation of purinergic receptors that can lead to transient calcium cascades as one of the steps involved in the insult-induced astrocyte reactivity (Neary et al., 2003; Floyd et al., 2004; Neary et al., 2005). On the other hand, the ionic disequilibrium caused by the trauma can provoke excitotoxicity and neuronal depolarization (Lea et al., 2002). Therefore, in order to test the ability of CEC to keep these cellular reactive responses, we also investigated in mixed cultures grown over CEC films, intracellular Ca^{2+} mobilization in neurons or astrocytes, induced respectively by KCl or ATP in the extracellular medium.

Materials and Methods

Animals and Cellulosic exopolysaccharide

All procedures adopted were approved by the Ethics Committee for Animal Research of the Federal University of Pernambuco (# 23076.000254/2013-72), in accordance with the Brazilian College for Animal Care guidelines and following the "Principles of Laboratory Animal Care" (NIH, Bethesda, USA).

A total of thirty six adult *Wistar* rats weighing 280-350 g were used in the present study. For the *in vivo* experiments, only male animals were subjected to an experimental model of TBI and divided into 2 groups (n= 18 animals per group) according to the surgical procedure. A control group (C group) where no material was put in the injury site and an experimental group (CEC group) where the colloidal hydrogel was injected into the injury site. Each one of these

groups was subdivided into 2 subgroups: C21 and CEC21 or C130 and CEC130 sacrificed 21 or 130 days, respectively after the surgery.

For *in vitro* studies, 14 progenitors (10 females and 4 males) and 20 pups from different litters (2-3 per litter) were grouped to obtain cerebral cortex primary cultures. All the animals were maintained in a room at 22 ± 2 ◦C with 67% relative humidity and 12 h light/dark cycle (lights on at 06:00 am) and were fed a commercial balanced diet (*Purina*® rodent *chow, Brazil*) ad libitum.

The CEC obtained from sugarcane molasses was produced by Polisa® Biopolymers for Health Ltd, linked to the Federal Rural University of Pernambuco (UFRPE). It was supplied as thin films or in the form of a colloidal hydrogel. CEC films were fixed on the surface of 15 mm glass coverslips. Before used as templates for cells or brain implantation, all of the CEC presentations were sterilized using gamma radiation.

Surgery procedure

Under the aseptic condition, the rats were anesthetized with a mix of xylazine (10 mg/Kg) and Ketamine (90 mg/Kg) intraperitoneally and were fixed on a stereotaxic apparatus (Insight, São Paulo, Brazil). After this step, a small incision was done in the skin to expose the skull and a circular piece (~3 mm diameter) of the bone was removed to expose the cerebral cortex. A small lesion about 3 mm in depth from the cortex was produced unilaterally in the left hemisphere by scalpel at a site 2 mm to the left and 2 mm posterior to the frontal suture. Bleeding was stopped before the bone incision with 10 % H_2O_2 and after the cerebral cortex exposition, with a hemostatic gelatin (Gelfoam). Ten microliters of sterile CEC hydrogel or saline (NaCl 0.9%) were placed into the cavity in the BC or control groups respectively. After this step, a thin membrane of BC was placed as a substitute of the dura mater removed during the surgical procedure to avoid cerebrospinal fluid leaking. The overlying skin was sutured and the rats were kept in individual cages during the recovery period. The animals were analyzed every day to check potential modifications in the motor behavior. Twent one or 130 days after implantation surgery the rats were sacrificed with a lethal dose of ketamine/xylazine mix (100 mg/kg, intraperitoneal) and transcardially perfused with 300 mL of saline followed by 400 mL of 4% paraformaldehyde in 0.01 M PBS, pH 7.4 (4% PFA) using a perfusion pump (Harvard Equipments) at a flux of 5 ml/min. After perfusion, the brain was additionally immersed in a solution of 4% PFA for 24 h and then rinsed in PBS, pH 7.4 followed by increasing concentrations of sucrose in phosphate buffer (10, 20 and 30%) for posterior sectioning in a

cryostat (Leica). Coronal sections of the brain (40 um) were obtained and divided in 6 series for posterior morphological and immunohistochemistry analysis.

Morphological and Immunohistochemical staining

Immunostaining of one series of sections was performed to visualize the glial reactivity and glial migration to the CEC hydrogel implanted. Adjacent series were stained with antibodies for the astrocyte marker glial fibrillary acidic protein (GFAP, diluted 1:1000; Sigma), microglial marker, IBA-1 and macrophages (ED-1). The incubation in the primary antibodies overnight was followed by biotinylated secondary antibodies (1:1000, Jackson Labs) and then for streptavidin-HRP (Vector Labs). Antibody binding was revealed with diaminobenzidine tetrahydrochloride 0.05% (DAB, Sigma). Subsequently, the free-floating sections were rinsed in PB and mounted on gelatin-coated glass slides. These procedures were carried out simultaneously in brain sections from both control and experimental animals. As for the control of the staining specificity, some sections were subjected to the immunohistochemical procedure omitting the primary antiserum.

To quantify the tissue reactions adjacent to the implant, the number of ED1- or GFAP-positive cells in the interfacial area was counted in 10 regions randomly selected along the implant surface. The cell numbers are given as numbers per square millimeter, based on the actually counted area.

Morphological analysis of one series of brain sections was done using Hematoxylin-eosin staining. After mounted and dried in slides the sections were immersed 3 times in absolute alcohol, followed by water and then stained by hematoxylin for 5 min. After this step, they were rinsed using tap water and stained by Eosin for 1 min, dehydrated by 3 changes in absolute alcohol, alcohol/xylene (1:1) mix, and absolute xylene (2 changes)

Single cell calcium imaging

Mixed neuron and astrocyte primary cultures obtained from the cerebral cortex of neonatal rats (PND2) were plated in 15mm glass coverslips, with or without CEC films, in the concentration of $1x10^5$ cells and maintained for ten days in vitro. Changes in intracellular calcium levels ([Ca2 +] i) were obtained according to De Melo Reis et al (2011). Cells were washed with Krebs solution (132mM NaCl, 4mM KCl, 1.4mM MgCl2, 2.5mM CaCl2, 6mM glucose, 10mM HEPES, pH 7.4) and then incubated for 40 minutes with 5μM Fura-2 / AM (Molecular Probes) , and 0.02% pluronic acid F-127 (Molecular Probes) in Krebs solution, and incubated with 5% CO2 and 95% atmospheric air at 37 ° C. After incubation the cells were

washed again in Krebs solution and mounted in a coupled chamber under an inverted fluorescence microscope (Axiovert 200; Carl Zeiss). Cells were maintained under continuous perfusion of Krebs solution and stimulated with 50mM KCl and 100μM ATP for about 30 and 45 seconds respectively. Between these stimuli, the cells were washed in Krebs solution. The variations in [Ca 2+] I were evaluated by quantifying the fluorescence ratio emitted at 510nm followed by excitation (750 ms) at 340 and 380nm using a Lambda DG4 device (Sutter Instrument, Novato, CA) prior to fluorescence acquisition with a 40x objective lens coupled to CoolSNAP digital camera (Roper Scientific, Trenton, NJ). The values obtained were processed using MetaFluor Software (Universal Imaging Corp., West Chester, PA). Only increases of at least 15% in the fluorescence ratio of FURA-2 were considered an effective response.

Immunocytochemical analysis of the mixed cultures was done using antibodies against the glial fibrillary acidic protein (GFAP) and beta-tubulin III as specific markers for astrocytes and neurons respectively.

Analysis of post-synaptic marker in mixed cultures

In order to investigate the presence of a post-synaptic marker related to glutamate signaling, immunostaining for the postsynaptic density protein 95 (PSD-95) (monoclonal antibody, Cell Signaling, 1:300 dilution) was carried out in neuron/astrocyte mixed cultures grown over glass coverslips covered or not with CEC. The puncta analysis was carried out in more than 50% of the coverslip area in 8 random sampling windows according to the protocol reported by Ippolito and Eroglu, 2010. Briefly, it was used the plugin Puncta analyzer written by Bary Wark available (c.eroglu@cellbio.duke.edu) under Image J 1.26 analysis Software platform adopting the threshold tool. We set the minimum puncta size as 4 pixels. DAPI staining was used to label the nuclei.

Statistical analysis

Results are representative of at least three experiments. The statistical analysis was done using ANOVA followed by Tukey as the post-hoc test or Student's t-test. The non-parametric Kruskal–Wallis ANOVA Ranks test was used when data did not follow a normal distribution, previously analyzed using Shapiro-Wilk test. In this cases, the Dunn's test or Mann Whitney U test was used as post hoc tests. $p \leq 0.05$ was considered statistically significant. Unless stated otherwise, all values are expressed as the mean ± standard deviation (SD).

Results

In vivo assay

Clinical aspects of the rats after surgery

Most of the animals survived the surgical procedure and until 130 days following the CEC implant. No signs of tremors and convulsions or deficiency in the motor or feeding behavior were observed during this period. The healing of the post-operative wound was similar between the two groups. The individual and average body weight of the animals is described in Table 1.

Table 2 : Individual body weight (g) at initial time.

Body weight (g)		
Group	**Control**	**CEC**
21 days	282	311
	334	283
	324	296
	302	349
	297	353
Mean ±SD	307,8 ± 21	318,4 ± 31,4
130 days	295	296
	293	393
	343	337
	325	335
	357	335
	302	322
	339	308
Mean ±SD	322 ± 25,6	332,28 ± 30,9

Morphological analysis of the brain defect using Hematoxylin-Eosin staining

The Figure 1 illustrates brain coronal sections (40 um thickness) stained by HE 21 days after CEC implant. The presence of the CEC in the colloidal hydrogel configuration can be visualized into the brain parenchyma with cells inserted in this space.

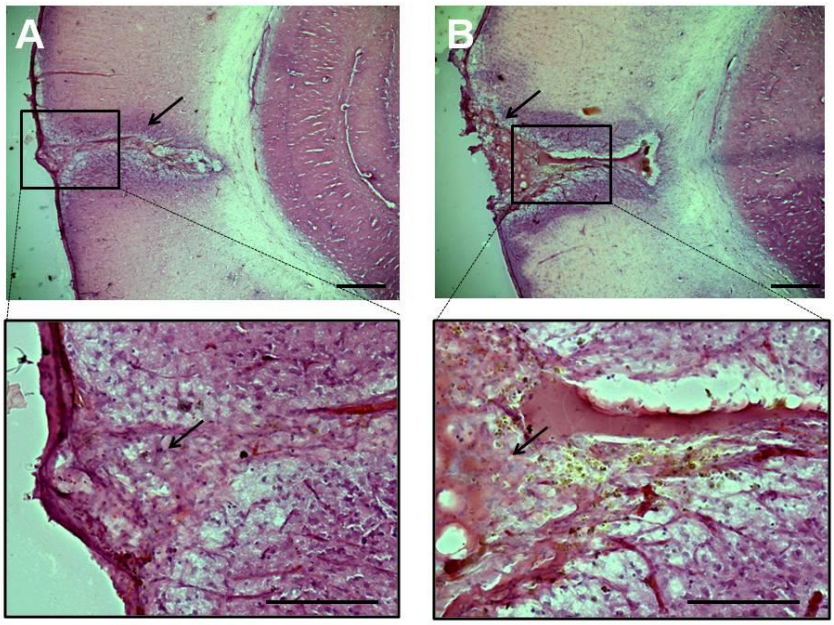

Figure 5: Brain coronal sections of a representative CEC 21animal stained for Hematoxylin-Eosin, showing the site of hydrogel implant and the presence of cells in this region. Calibration bar in A and B= 1 mm; in the insets = 0.5 mm.

In animals whose hydrogel implant was kept into the brain parenchyma for 130 days, it was observed a better integration of the biomaterial into the brain tissue and a better preservation of this tissue when compared to animals from the control group (*sham*). Figure 2 illustrates in different magnifications coronal sections of a representative animal of subgroups CEC130 and C130.

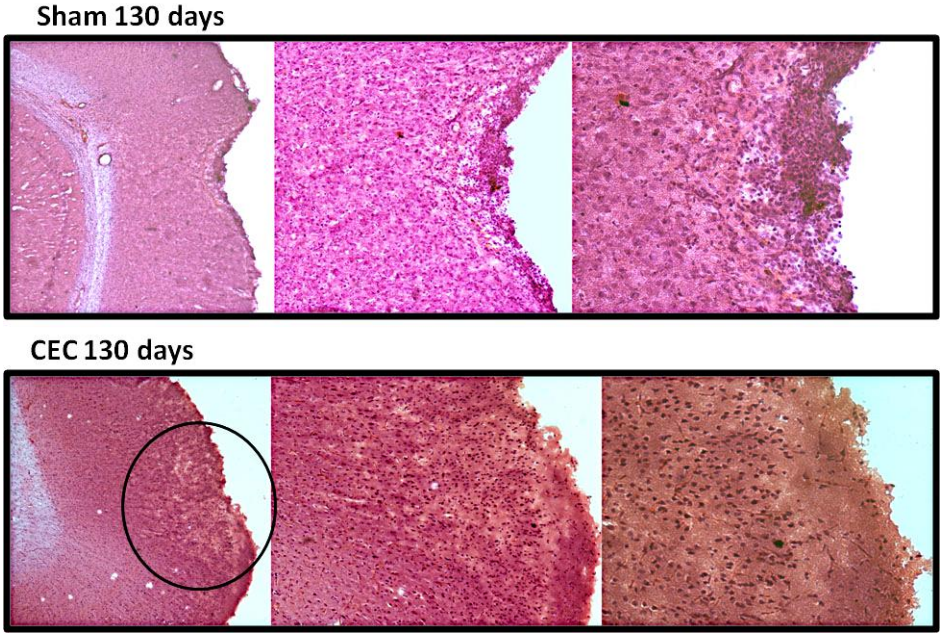

Figure 6: Brain coronal sections of representative C130 and CEC130 animals, stained for Hematoxylin-Eosin, showing the site of the brain traumatic injury were. Note the apparent incorporation of the hydrogel in the brain parenchyma and better preservation of the cortical tissue in the CEC 130 animal.

Immunohistochemical analysis of cellular infiltration

Macrophages immunoreactive to ED-1 antibody were seen inside the implant region in animals of CEC 21 group (Figures 3). In the borders of the lesion, these cells were also detected in animals whose implant was removed during brain dissection (Figure 4).

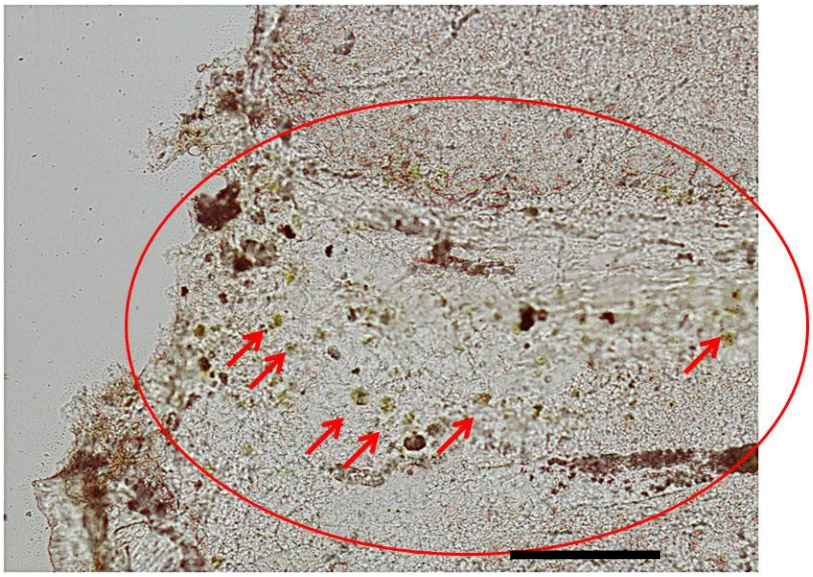

Figure 7: Brain coronal section of a representative animal of CEC 21 group, immunoreacted for the ED-1 antibody. Red arrows indicate the presence of macrophages inside the hydrogel implant. Calibration bar: 0.5 mm.

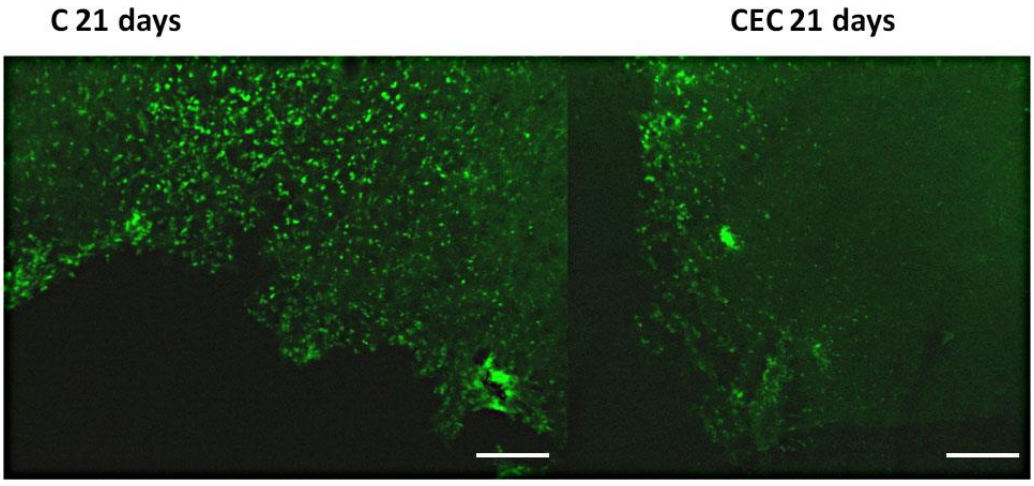

Figure 8: Brain coronal section of a representative animal of CEC 21 group, immunoreacted for ED-1 antibody indicating the presence of macrophages in the borders of the injury site. Calibration bar: 0.5 mm.

The presence of astrocytes inside and within the borders of the hydrogel was also detected using specific markers such as GFAP, vimentin, and S100. Figures 5 and 6 illustrate representative brain sections of CEC 21 groups immunoreacted for these proteins. In Fig. 5 A and 5B labeling for DAPI shows also the presence of cells inside the hydrogel. While GFAP was detected in cells inside the implant, s100 was visualized in astrocytes in the borders of the injury.

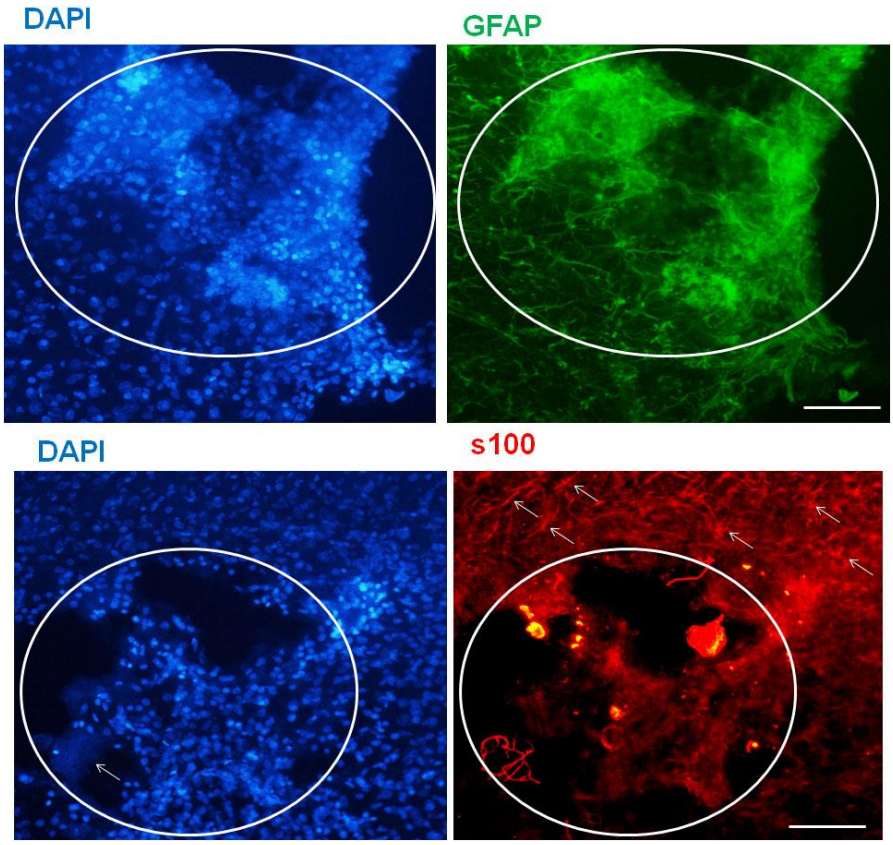

Figure 9: Brain coronal sections of a representative animal of CEC 21 group, immunoreacted for GFAP and S100 antibodies indicating the presence of astrocytes inside (GFAP) or in the borders of the injury site. Note the presence of several nuclei of non-identified cells into the implant labeled for DAPI. Calibration bar: 0.5 mm.

Vimentin-positive astrocytes were also seen inside the implant in the CEC 21 group or in the border of the lesion in the C21 group (Figure 6).

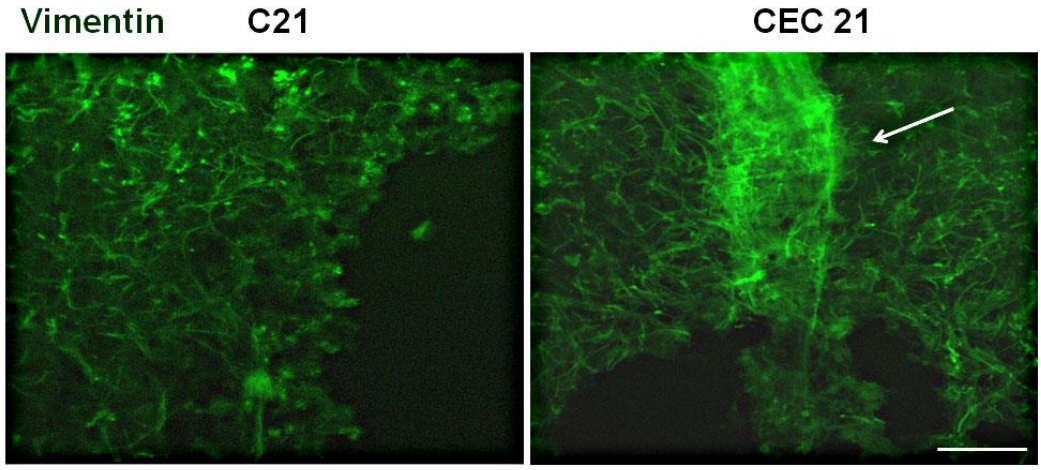

Figure 10: Vimentin-immunoreactivity in the site of injury in representative C21 and CEC21 animals. Calibration bar: 0.5 mm.

In vitro assays

KCl and ATP induced Ca^{2+} influx in 2D mixed cultures grown on CEC

Neuronal and astrocyte responses to depolarizing stimulus induced by KCl and ATP, respectively, were investigated using Ca^{2+} imaging in four independent 2D mixed cultures kept for 10 div. Treatment with 50 µM KCl was able to induce similar number of responsive cells as well as an increase in the Ca^{2+} influx (assessed by Fura-2 fluorescence) in cells grown over CEC, compared to the control condition. However, the average response of astrocytes to ATP stimulus was significantly lower to that observed in the control (Table 2) despite the similar morphological differentiation of astrocytes in these cultures.

Figure 7 shows digital images of representative mixed cultures grown on CEC or control condition, immunoreacted for GFAP and beta-tubulin III.

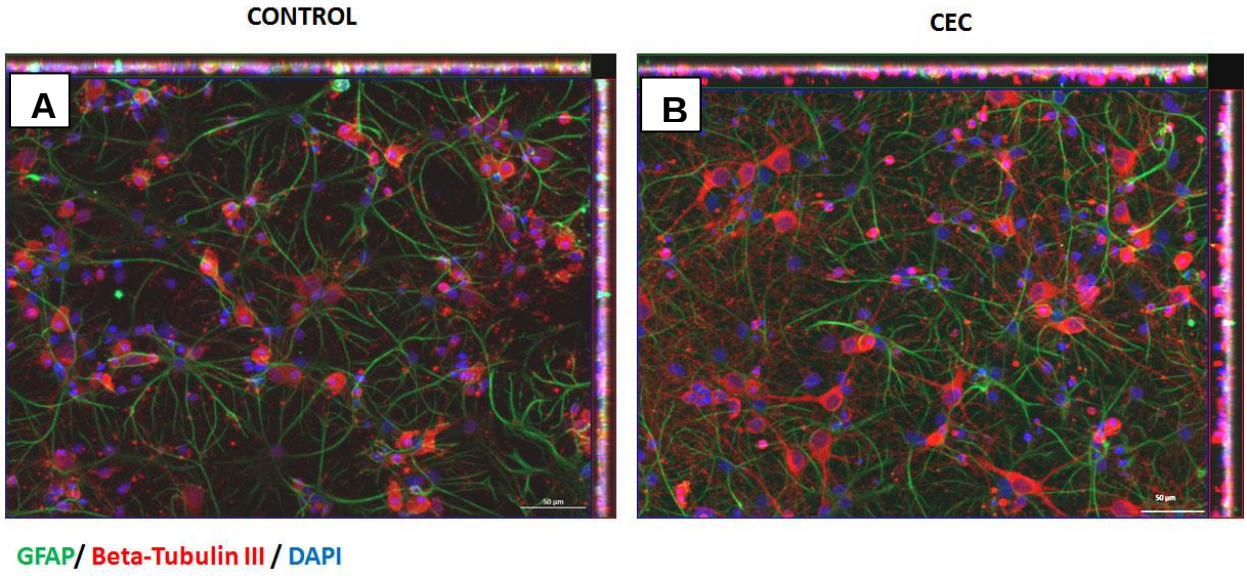

Figure 11: Two-dimensional mixed neuron-astrocyte cultures grown on CEC or control condition: Z-stack images of immunolabeled cortical neurons and astrocytes (green: GFAP; red: β-tubulin-III; blue: DAPI) cultured for 10 div glass coverslips covered (a) or not with CEC (b). Note the similar topographic distribution of these cells, with most of astrocyte cell bodies involving neuronal cell bodies. Scale bar = 20 µm

Figure 8A and B shows phase contrast and fluorescent images of cells labeled for Fura 2 before stimulus for intracellular Ca^{2+} responses in cultures grown over the two substrates. Figure 8 C, D, E, F compares the curves of all the cells responsive to KCl or ATP stimulus during these 4 independent experiments.

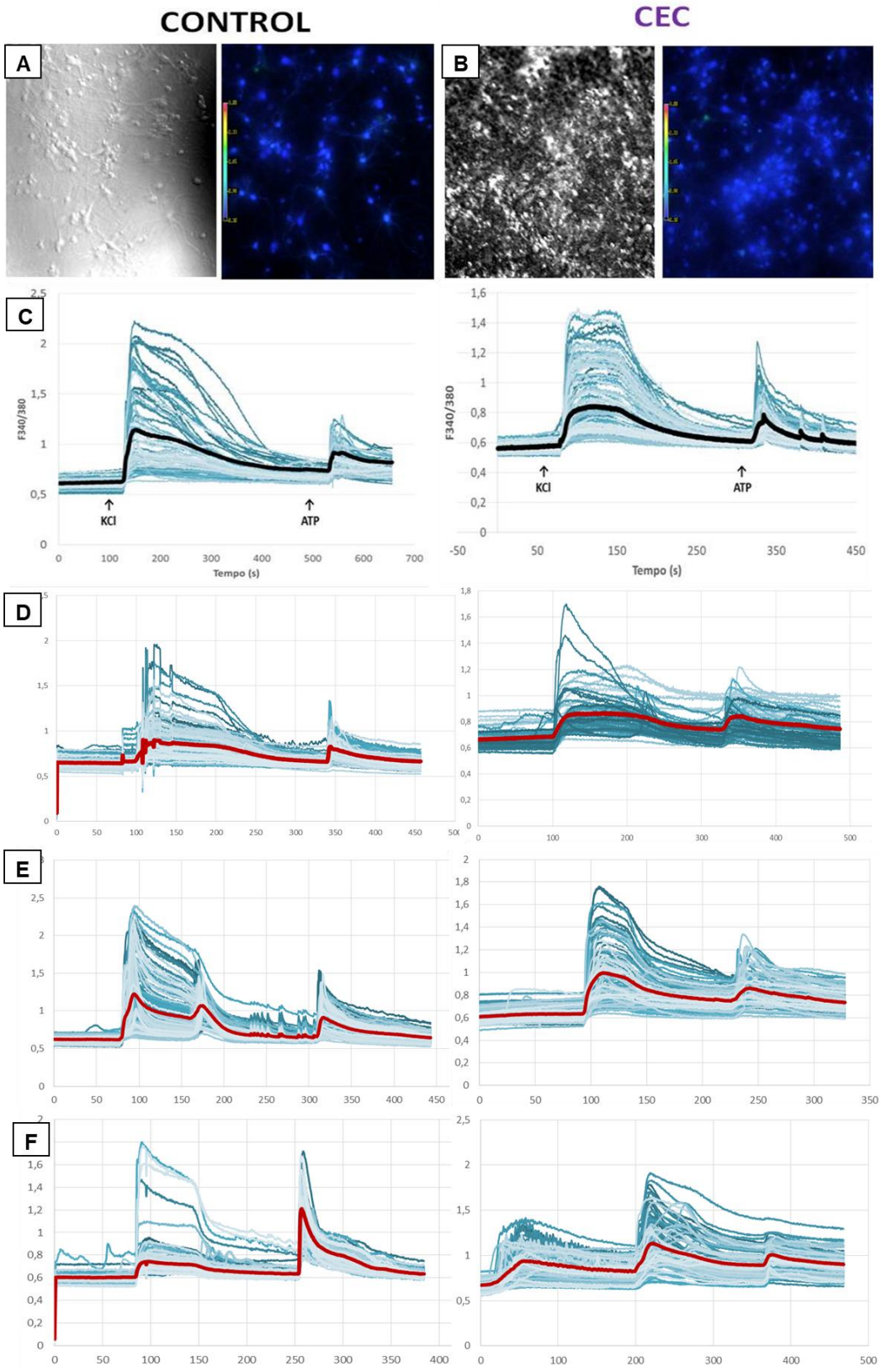

Figure 12: Photomicrographs in phase contrast (A) and fluorescent labeling for Fura 2 (B; blue cells) of 2D neuron and astrocyte mixed cultures before the depolarizing stimulus induced by 50 mM KCl or 100 uM ATP. The response of intracellular calcium mobilization in the 4 independent cultures (C, D, E, and F) whose results are expressed in Table 2.

Distribution of the post-synaptic marker PSD-95 in mixed cultures

Preliminary analysis of synaptic markers in mixed cultures revealed a similar number of puncta characterized by synaptic development in cells grown on the CEC when compared to control condition. Figure 9A and B illustrates digital images of PSD-95 immunoreactive puncta obtained in one coverslip of a culture grown for 10 div in control or CEC substrates, respectively. Quantitative analysis of these cultures carried out in 8 random sampling windows is expressed in Figure 9C.

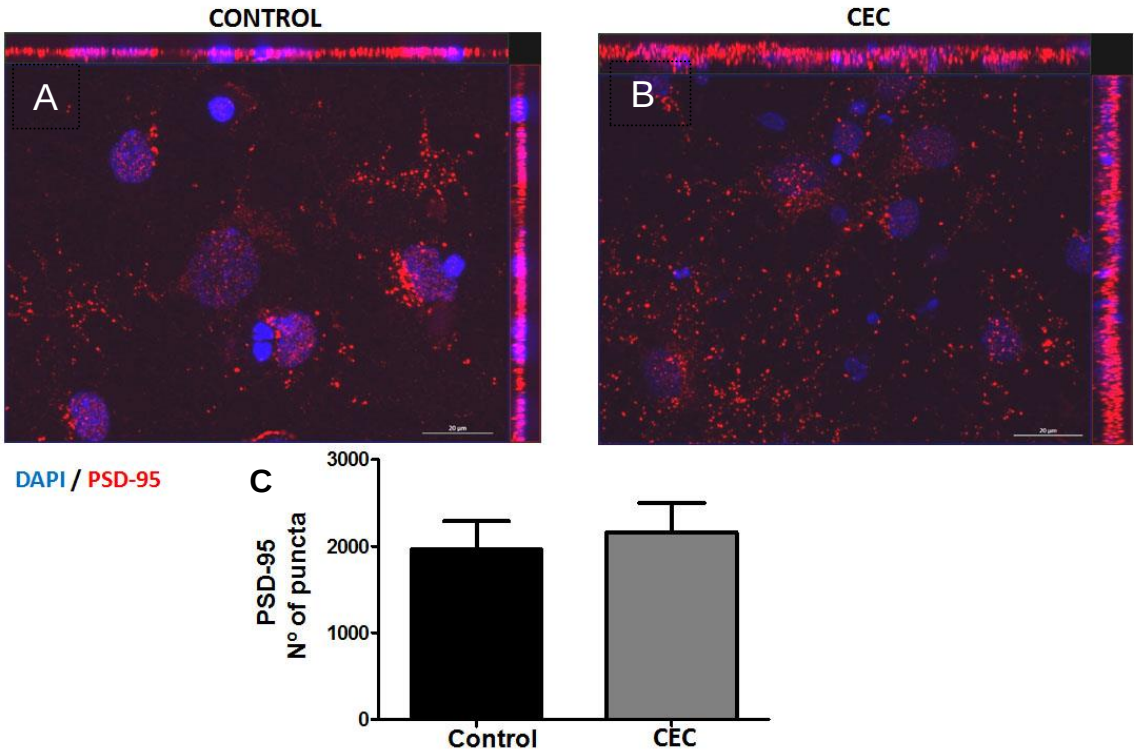

Figure 13: Digital images of neuron/astrocyte mixed cultures grown for 10 days over the control (A) or CEC (B) substrates, immunoreacted for PSD-95 protein, illustrating the puncta (red) of post-synaptic markers related to glutamatergic signaling. DAPI staining in blue indicate the nuclei of the cells. Quantitative analysis in this culture showed similar distribution between the groups. Calibration bar: 20 um.

Discussion

The main purpose of the present study was to test the hypothesis that colloidal hydrogel of CEC can be suitable as a potential tissue engineering scaffold for bridging injured brain tissue. This bacterial cellulose was clearly visualized at 21 days post-injection into the cavity and

infiltrated by macrophages and glial cells. The qualitative analysis carried out until this moment indicated that this polymer did not exacerbate the injury response at the time periods examined. 130 days after the implantation, the biomaterial was incorporated into the brain parenchyma and the cell distribution in this tissue was more organized than in the sham condition. Such results are similar to that reported by Lima et al., (2017) using CEC films as dura mater substitute for 120 days reinforcing the ability of this biopolymer to be absorbed and incorporated to the brain tissue.

One of the most important issues when biopolymers are implanted in the brain is the possible toxicity of the material. Usually, the presence of brain macrophages in the cavity area after injury is a normal response to cortical injury in the mammalian brain (Gyoneva and Ransohoff, 2015). The immunodetection of ED1 indicates macrophage and/or phagocytic microglia activity (Turtzo et al., 2014). This type of reaction was also detected in injured rats with no CEC. Nevertheless, intracerebral grafting of CEC did not cause an enhanced brain tissue response relative to sham condition at the two-time points analyzed. The presence of vimentin-positive astrocytes in the implant and in the borders of the injury site also indicated an astroglial proliferative and reactive state. S100 –positive astrocytes in the neighbor region also reinforce this condition, especially considering that the presence of this calcium modulating protein in the liquor and serum has been considered a biomarker of traumatic brain injury (Gonçalves et al., 2008; Thelin et al. 2017).

Similar tissue engineering strategies using other types of hydrogels have been investigated to alleviate neural damage (Tate et al., 2001; Tian et al 2005). The initial lesion subsequent to the traumatic impact to the brain usually leads to several types of damage and provokes the formation of an irregular cavity as was here observed in the sham condition. It is expected that an injectable scaffolding material reduces the gap between brain tissue and implant enhancing the cellular integration. The absence of toxicity and the mechanical properties of CEC recently reported (Silveira et al., 2016, Gonçalves-Pimentel, 2018) has been favorable to its incorporation in several types of tissue, where vascularization was also induced (Carvalho-Junior et al., 2012; Medeiros-Junior et al., 2013; Lima et al., 2015; de Lucena et al., 2015; Silveira et al., 2016). The reduction in the brain cavity size observed after 130 days of CEC implant and the apparent reorganization of the cortical tissue reinforces this previous evidence and suggest that the CEC properties enable the hydrogel to integrate into the neural tissue without cause undesirable mechanical pressure. The histopathological analysis in semithin brain sections is the next step necessary to analyze the time course of this process of integration into the brain parenchyma.

It is well established that mechanical TBI induces ionic disequilibrium which can provoke neuronal depolarization (Lea et al., 2002) and leads to transient calcium cascades as one of the steps related to neuron and astrocyte reactivity (Neary et al., 2003; Floyd et al., 2004; Neary et al., 2005). Therefore, with the purpose of investigating the ability of CEC to keep these cellular responses, we also analyzed the intracellular calcium mobilization induced by KCl or ATP respectively in cultured neurons or astrocytes. The results indicated an adequate neuronal response to KCl which is consistent with the ability of these cells to be depolarized, indicating the functional activity of Ca^{2+} voltage-dependent channels in their membranes (Scemes and Giaume, 2006). We previously demonstrated a similar response to NMDA receptor activation in mixed cultures kept in vitro for 7 days (Gonçalves-Pimentel et al., 2018, submitted). The evidence obtained in the present study demonstrating a similar distribution of the PSD-95 protein in mixed neuron/astrocyte cultures grown on the CEC or control condition for 10 div reinforce the idea of a suitable synaptic development. NMDA receptors (NMDARs) are principal regulators of synaptic signaling in the brain, and especially in the cerebral cortex. Modulation of NMDARs' function and trafficking is important for the regulation of glutamatergic synaptic transmission and several forms of synaptic plasticity. PSD-95 acts as a scaffolding protein and stabilizes the surface and synaptic expression of NMDARs (Collingridge et al., 2004). The interaction between the functional activity of this postsynaptic protein and NMDARs has been also investigated for a potential strategic therapeutic approach in the treatment of brain ischemia (Aarts et al., 2002). Our present analysis of PSD-95 puncta distribution has been done after 10 div when synaptic communication begins to be established in mixed neuron/astrocyte cultures. Therefore, these preliminary results also indicate the ability of CEC in keeping functional properties of neurons during maturation of this glutamatergic system. Investigating the presence of pre-synaptic markers in these cells is necessary to reinforce this hypothesis and these experiments are in course at this moment.

Purinergic signaling is also an important step involved in the brain reactivity under conditions of traumatic injury. ATP–induced calcium waves in astrocytes are able to mediate synchronization of neuronal spiking (Kurnaria et al., 2008). Two types of purinergic receptors present in glial cells are involved in the transient calcium cascades as one of the steps involved in the injury- provoked astrocyte reactivity (Neary et al., 2003; Floyd et al., 2004; Neary et al., 2005) and has been analyzed in in vitro models of TBI (Morrinson et al., 2011). For this reason, we investigated the intracellular calcium mobilization as a functional glial response induced by ATP in mixed neuron/astrocyte cultures. Despite the apparent morphological differentiation of astrocyte in our mixed cultures grown on the CEC, the confluence of these cells and their normal

widespread distribution in the mixed cultures were not similar among the 4 independent experiments. The results were variable and demonstrated a significant average reduction in the intracellular calcium induced by 100 uM ATP. The mechanisms involved in these unfavorable responses are ben investigated at this moment, especially considering the influence of Neurobasal medium on the functional activity of these astrocytes. Experiments using pure astrocyte cultures and a more adequate medium for astrocytes in mixed cultures will clarify methodological issues involved in these results. Considering that co-cultures of embryonic neurons over astrocytes grown on CEC were able to provide an adequate neuronal growth and differentiation, we conclude that several aspects of the functional activity of astrocytes and their interaction with neurons are preserved when they are plated on the CEC. The incorporation of vimentin or GFAP-positive astrocytes into the CEC hydrogel implant also reinforce the ability of this biomaterial in keeping the functional activity of this type of glial cell.

Although additional histopathological analysis needs to be done to analyze the time course of interaction of CEC with the brain tissue, taken together, the present *in vivo* and *in vitro* findings suggest that this bacterial cellulose can be a promising biomaterial to be used as an injectable scaffold for the repair of defects in the brain.

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4 CONCLUSÃO

Os resultados aqui expostos nos permitem concluir que o exopolissacarídeo celulósico obtido a partir do melaço da cana-de-açúcar fornece um substrato promissor para importantes áreas da neuroengenharia de tecido, tendo apresentado boa biocompatibilidade *in vitro* e *in vivo.* A boa capacidade de permitir adesão e crescimento de células neurais e de manutenção da sua funcionalidade sugerem que o uso da CEC como scaffolds para o desenvolvimento de estruturas 3D neurofuncionais possui um bom potencial além de um baixo custo. Por outro lado a boa interação que teve com o tecido hospedeiro, quando implantado em modelo de traumatismo crânio-encefálico, permitindo infiltrado celular e não provocando reação local indesejada ou sistêmica nos faz acreditar na sua viabilidade para a medicina regenerativa.

REFERÊNCIAS

AERTKER, Benjamin M., Supinder BEDI, Charles S. COX. Strategies for CNS Repair Following TBI. **Experimental Neurology** 275. Elsevier Inc.: 411–26. https://doi.org/10.1016/j.expneurol.2015.01.008.2016.

AGUIAR, J. L. A. *et al.* Surgarcane Biopolymer Patch in Femoral Artery Angioplasty on Dogs." **Acta Cirúrgica Brasileira** 22 (1): 77–81. 2007.

ALBUQUERQUE, P.C.V.C *et al.* Comparative Study of the Areas of Osteochondral Defects Produced in the Femoral Condyles of Rabbits Treated with Gel of Sugarcane Biopolymer. **Acta Cirurgica Brasileira** 26 (5): 383–86. https://doi.org/10.1590/S0102-86502011000500010. 2011.

ALIGHOLI, H. *et al.* Preparing Neural Stem/progenitor Cells in PuraMatrix Hydrogel for Transplantation after Brain Injury in Rats: A Comparative Methodological Study. **Brain Research** 1642. Elsevier: 197–208. https://doi.org/10.1016/j.brainres.2016.03.043. 2016.

ALMEIDA-PITITTO, Bianca de, CLINEU de M ALMADA FILHO, Maysa S CENDOROGLO. D{é}ficit Cognitivo: Mais Uma Complica{ç}{ã}o Do Diabetes Melito?" **Arquivos Brasileiros de Endocrinologia {&} Metabologia**, no. Dm: 1076–83. https://doi.org/10.1590/S0004-27302008000700003. 2008.

ÁLVAREZ, Z. *et al.* "Neurogenesis and Vascularization of the Damaged Brain Using a Lactate-Releasing Biomimetic Scaffold." **Biomaterials** 35 (17). Elsevier Ltd: 4769–81. https://doi.org/10.1016/j.biomaterials.2014.02.051. 2014.

ANTHONYMUTHU, Tamil Selvan, Elizabeth Megan KENNY, Hülya BAYIR. Therapies Targeting Lipid Peroxidation in Traumatic Brain Injury HHS Public Access. **Brain Res** 1640: 57–76. https://doi.org/10.1016/j.brainres.2016.02.006. 2016.

BARROS-MARQUES, S. R.E. Sugarcane Biopolymer Patch in Femoral Vein Angioplasty on Dogs.**YMVA** 55 (2). Elsevier Inc.: 517–21. https://doi.org/10.1016/j.jvs.2011.05.047. 2012.

BERGOLD, Peter J. Treatment of Traumatic Brain Injury with Anti-Inflammatory Drugs. **Experimental Neurology** 275. Elsevier Inc.: 367–80. https://doi.org/10.1016/j.expneurol.2015.05.024. 2016.

BRASURE, M. *et al.* Participation after Multidisciplinary Rehabilitation for Moderate to Severe Traumatic Brain Injury in Adults: A Systematic Review. **Archives of Physical Medicine and Rehabilitation** 94 (7): 1398–1420. https://doi.org/10.1016/j.apmr.2012.12.019. 2013.

BUCKLEY, L. *et al.* Traumatic Brain Injury and Aggression: A Systematic Review and Future Directions Using Community Samples. **Aggression and Violent Behavior** 37 (September). Elsevier: 26–34. https://doi.org/10.1016/j.avb.2017.08.004. 2017.

CAVALCANTI, L.M. *et al.* Efficacy of Bacterial Cellulos/e Membrane for the Treatment of Lower Limbs Chronic Varicose Ulcers: A Randomized and Controlled Trial. **Revista Do Colégio Brasileiro de Cirurgiões** 44 (1): 72–80. https://doi.org/10.1590/0100-

69912017001011. 2017.

COBURN, J.C., A. PANDIT. Development of Naturally-Derived Biomaterials and Optimization of Their Biomechanical Properties." **Topics in Tissue Engineering** 3: 1–33. http://www.oulu.fi/spareparts/ebook_topics_in_t_e_vol3/abstracts/coburn_01.pdf. 2017.

COELHO JUNIOR, E.R. *et al.* Prevention of Peritoneal Adhesion Using a Bacterial Cellulose Hydrogel, in Experimental Study. **Acta Cirurgica Brasileira** 30 (3): 194–98. https://doi.org/10.1590/S0102-865020150030000005. 2015.

CORDEIRO-BARBOSA, F. A. *et al.*Use of a Gel Biopolymer for the Treatment of Eviscerated Eyes: Experimental Model in Rabbits. **Arquivos Brasileiros de Oftalmologia** 75 (4): 267–72. https://doi.org/10.1590/S0004-27492012000400001. 2012.

COUSIN, M.A. The Value of Systematic Reviews in Estimating the Cost and Barriers to Translation in Tissue Engineering. **Tissue Engineering Part B: Reviews** 22 (6): 430–37. https://doi.org/10.1089/ten.teb.2016.0060. 2016.

DALY, W.T. Comparison and Characterization of Multiple Biomaterial Conduits Forperipheral Nerve Repair. **Biomaterials** 34 (34). Elsevier Ltd: 8630–39. https://doi.org/10.1016/j.biomaterials.2013.07.086. 2013.

DENG, Wei, James B. AIMONE, Fred H. GAGE. New Neurons and New Memories: How Does Adult Hippocampal Neurogenesis Affect Learning and Memory?" **Nature Reviews Neuroscience** 11 (5). Nature Publishing Group: 339–50. https://doi.org/10.1038/nrn2822. 2010.

DUAN, H.M. *et al.* Functional Hyaluronate Collagen Scaffolds Induce NSCs Differentiation into Functional Neurons in Repairing the Traumatic Brain Injury." **Acta Biomaterialia** 45. Acta Materialia Inc.: 182–95. https://doi.org/10.1016/j.actbio.2016.08.043. 2016.

FALCÃO, S. C., A. R. de BARROS COELHO, J. E. NETO. Biomechanical Evaluation of Microbial Cellulose (Zoogloea Sp.) and Expanded Polytetrafluoroethylene Membranes as Implants in Repair of Produced Abdominal Wall Defects in Rats." **Acta Cirurgica Brasileira** */ Sociedade Brasileira Para Desenvolvimento Pesquisa Em Cirurgia* 23 (2): 184–91. https://doi.org/10.1590/S0102-86502008000200012. 2008.

FLANAGAN, K. E. *et al.* Development of a Sutureless Dural Substitute from Bombyx Mori Silk Fibroin." **Journal of Biomedical Materials Research Part B: Applied Biomaterials** 103 (3): 485–94. https://doi.org/10.1002/jbm.b.33217. 2015.

FRAGOSO, A. S. *et al.* Dielectric Study of the Adhesion of Mesenchymal Stem Cells from Human Umbilical Cord on a Sugarcane Biopolymer." **Journal of Materials Science: Materials in Medicine** 25 (1): 229–37. https://doi.org/10.1007/s10856-013-5056-5. 2014.

GARCÍA CRUZ, D. M. Chitosan Microparticles as Injectable Scaffolds for Tissue Engineering." **Journal of Tissue Engineering and Regenerative Medicine** 2 (6): 378–80. https://doi.org/10.1002/term.106. 2008.

GIZA, C. C., D. A. HOVDA. The Neurometabolic Cascade of Concussion." **Journal of**

Athletic Training 36 (3): 228–35. https://doi.org/10.1227/NEU.0000000000000505. 2001.

GONÇALVES-PIMENTEL, C. *et al.* Cellulose Exopolysaccharide from Sugarcane Molasses as a Suitable Substrate for 2D and 3D Neuron and Astrocyte Primary Cultures. **Journal of Materials Science: Materials in Medicine** 29 (9). Springer US: 139. https://doi.org/10.1007/s10856-018-6147-0. 2018.

GUERRA, G. D., N. BARBANI, M. C. GAGLIARDI, Elisabetta ROSELLINI, AND Caterina CRISTALLINI. Chitosan-Based Macromolecular Biomaterials for the Regeneration of Chondroskeletal and Nerve Tissue. **International Journal of Carbohydrate Chemistry** 2011: 1–9. https://doi.org/10.1155/2011/303708. 2011.

GYONEVA, Stefka, Richard M RANSOHOFF. Inflammatory Reaction after Traumatic Brain Injury: Therapeutic Potential of Targeting Cell-Cell Communication by Chemokines. **Trends in Pharmacological Sciences** 36 (7): 471–80. https://doi.org/10.1016/j.tips.2015.04.003. 2015.

HARTING, M T. *et al.* Subacute Neural Stem Cell Therapy for Traumatic Brain Injury. **Journal of Surgical Research** 153 (2). Elsevier Inc.: 188–94. https://doi.org/10.1016/j.jss.2008.03.037. 2009.

IDE, C. Peripheral Nerve Regeneration. **Neuroscience Research** 25 (2): 101–21. https://doi.org/10.1016/S0168-0102(96)01042-5. 1996.

JAHANBAZI JAHAN-ABAD, A. *et al.* Human Neural Stem/Progenitor Cells Derived From Epileptic Human Brain in a Self-Assembling Peptide Nanoscaffold Improve Traumatic Brain Injury in Rats. **Molecular Neurobiology**. Molecular Neurobiology, 1–17. https://doi.org/10.1007/s12035-018-1050-8. 2018.

JASSAM, Y.N. *et al.* Neuroimmunology of Traumatic Brain Injury: Time for a Paradigm Shift. **Neuron** 95 (6). Elsevier Inc.: 1246–65. https://doi.org/10.1016/j.neuron.2017.07.010. 2017.

KOSS, K. M., L. D. UNSWORTH. Neural Tissue Engineering: Bioresponsive Nanoscaffolds Using Engineered Self-Assembling Peptides. **Acta Biomaterialia** 44: 2–15. https://doi.org/10.1016/j.actbio.2016.08.026. 2016.

KRISHNAMURTHY, K., D. T. LASKOWITZ. Cellular and Molecular Mechanisms of Secondary Neuronal Injury Following Traumatic Brain Injury. **Translational Research in Traumatic Brain Injury.** http://www.ncbi.nlm.nih.gov/pubmed/26583177. 2016.

LAPLACA, M. C. *et al.* CNS Injury Biomechanics and Experimental Models. **Progress in Brain Research** 161: 13–26. https://doi.org/10.1016/S0079-6123(06)61002-9. 2007

LEE, S. K., S. W. WOLFE. [REVIEWED] Peripheral Nerve Injury and Repair. *The* **Journal of the American Academy of Orthopaedic Surgeons** 8 (4): 243–52. https://doi.org/10.1097/00006534-198804000-00086. 1999.

LEE, S. *et al.* The Incremental Induction of Neuroprotective Properties by Multiple Therapeutic Strategies for Primary and Secondary Neural Injury. **International Journal of Molecular Sciences** 16 (8): 19657–70. https://doi.org/10.3390/ijms160819657. 2015.

LIMA, F.M.T. *et al.* Biocompatible Bacterial Cellulose Membrane in Dural Defect Repair of Rat. **Journal of Materials Science: Materials in Medicine** 28 (3). Springer US. https://doi.org/10.1007/s10856-016-5828-9. 2017.

LIMA, S.V.C. *et al.* The Biocompatibility of a Cellulose Exopolysaccharide Implant in the Rabbit Bladder When Compared with Dextranomer Microspheres plus Hyaluronic Acid." **Urology** 85 (6): 1520.e1-1520.e6. https://doi.org/10.1016/j.urology.2015.02.028. 2015.

LING, H., J. HARDY, H. ZETTERBERG. Neurological Consequences of Traumatic Brain Injuries in Sports. **Molecular and Cellular Neuroscience** 66 (PB). Elsevier B.V.: 114–22. https://doi.org/10.1016/j.mcn.2015.03.012. 2015.

LITTLE, L., K. E. HEALY, D. SCHAFFER. Engineering Biomaterials for Synthetic Neural Stem Cell Microenvironments. **Chemical Reviews** 108 (5): 1787–96. https://doi.org/10.1021/cr078228t. 2008.

LOPES, D. *et al.* Engineering Biomaterials for Synthetic Neural Stem Cell **Middle Ear** 77 (1): 44–50. 2011.

LUCENA, M. T. Biocompatibility and Cutaneous Reactivity of Cellulosic Polysaccharide Film in Induced Skin Wounds in Rats. **Journal of Materials Science: Materials in Medicine** 26 (2). https://doi.org/10.1007/s10856-015-5410-x. 2015.

MAGALHÃES, A. L. G. Epidemiologia Do Traumatismo Cranioencefálico No Brasil TT - Epidemiology of Traumatic Brain Injury in Brazil. **Rev. Bras. Neurol** 53 (2): 15–22. http://fi-admin.bvsalud.org/document/view/b8k64. 2017.

MARTÍNEZ-RAMOS, C. Neural Tissue Regeneration in Experimental Brain Injury Model with Channeled Scaffolds of Acrylate Copolymers. **Neuroscience Letters** 598. Elsevier Ireland Ltd: 96–101. https://doi.org/10.1016/j.neulet.2015.05.021. 2015

MARTINS, A.G.S. A Wet Dressing for Hypospadias Surgery. **International Braz J Urol** 39 (3): 408–13. https://doi.org/10.1590/S1677-5538.IBJU.2013.03.15. 2013.

MEDEIROS JUNIOR, M. D. Hydrogel of Polysaccharide of Sugarcane Molasses as Carrier of Bone Morphogenetic Protein in the Reconstruction of Critical Bone Defects in Rats. **Acta Cirurgica Brasileira** / *Sociedade Brasileira Para Desenvolvimento Pesquisa Em Cirurgia* 28 (4): 233–38. https://doi.org/10.1590/S0102-86502013000400001.2013.

MINOZZI, S. *et al.* A Gold Dental Prosthesis of Roman Imperial Age. **American Journal of Medicine** 120 (5): 4–5. https://doi.org/10.1016/j.amjmed.2006.05.062. 2007.

OKANO, Hideyuki. Stem Cell Biology of the Central Nervous System. **Journal of Neuroscience Research** 69 (6): 698–707. https://doi.org/10.1002/jnr.10343. 2002.

ORIVE, G. *et al.* Biomaterials for Promoting Brain Protection, Repair and Regeneration. **Nature Reviews Neuroscience** 10 (9): 682–92. https://doi.org/10.1038/nrn2685. 2010.

PANSERI, S. *et al.* Electrospun Micro- and Nanofiber Tubes for Functional Nervous Regeneration in Sciatic Nerve Transections." **BMC Biotechnology** 8 (1): 39.

https://doi.org/10.1186/1472-6750-8-39. 2008.

PATERSON-BEEDLE, M. *et al.* A Cellulosic Exopolysaccharide Produced from Sugarcane Molasses by a Zoogloea Sp. **Carbohydrate Polymers** 42 (4): 375–83. https://doi.org/10.1016/S0144-8617(99)00179-4. 2000.

PEARLIN, S.N., G. MANIVASAGAM, D. SEN. Progress of Regenerative Therapy in Orthopedics. **Current Osteoporosis Reports** 16 (2): 169–81. https://doi.org/10.1007/s11914-018-0428-x. 2018.

PITA, P. C. C. *et al.* Biocompatibility of the Bacterial Cellulose Hydrogel in Subcutaneous Tissue of Rabbits." **Acta Cirurgica Brasileira** 30 (4): 296–300. https://doi.org/10.1590/S0102-865020150040000009. 2015.

QUILLINAN, N. *et al.* A Cellulosic Exopolysaccharide . **HHS Public Access** 34 (3): 453–64. https://doi.org/10.1016/j.anclin.2016.04.011.Neuropathophysiology. 2017

REIGHARD, C. L., S. J. HOLLISTER, D. A. ZOPF. Auricular Reconstruction from Rib to 3D Printing. **Journal of 3D Printing in Medicine** 2 (1): 35–41. https://doi.org/10.2217/3dp-2017-0017. 2018.

RIVET, C. *et al.* Classification of Traumatic Brain Injury. **Implanted in the Brain** no. May: 1–7. 2015.

ROOZENBEEK, B., A. I. R. MAAS, D. K. MENON. Changing Patterns in the Epidemiology of Traumatic Brain Injury. **Nature Reviews Neurology** 9 (4): 231–36. https://doi.org/10.1038/nrneurol.2013.22. 2013.

SAATMAN, K. *et al.* Classification of Traumatic Brain Injury for Targeted Therapies. **Journal of Neurotrauma** 25 (7): 719–38. https://doi.org/10.1089/neu.2008.0586. 2008.

SAFA, B., G. BUNCKE. Autograft Substitutes." **Hand Clinics** 32 (2): 127–40. https://doi.org/10.1016/j.hcl.2015.12.012. 2016.

SARACINO, G.A.A. Nanomaterials Design and Tests for Neural Tissue Engineering. **Chemical Society Reviews** 42 (1): 225–62. https://doi.org/10.1039/c2cs35065c. 2013.

SARNOWSKA, A. *et al.*. Encapsulation of Mesenchymal Stem Cells by Bioscaffolds Protects Cell Survival and Attenuates Neuroinflammatory Reaction in Injured Brain Tissue after Transplantation. **Cell Transplantation** 22 (SUPPL.1): 67–82. https://doi.org/10.3727/096368913X672172. 2013.

SHI, W. BDNF Blended Chitosan Scaffolds for Human Umbilical Cord MSC Transplants in Traumatic Brain Injury Therapy. **Biomaterials** 33 (11). Elsevier Ltd: 3119–26. https://doi.org/10.1016/j.biomaterials.2012.01.009. 2012.

SHIN, S. S. Neuroprotective Effects of Collagen Matrix in Rats after Traumatic Brain Injury. **Restorative Neurology and Neuroscience** 33 (2): 95–104. https://doi.org/10.3233/RNN-140430. 2015.

SILVEIRA, F.C.A. *et al.* Treatment of Tympanic Membrane Perforation Using Bacterial Cellulose: A Randomized Controlled Trial. **Brazilian Journal of Otorhinolaryngology** 82 (2). Associação Brasileira de Otorrinolaringologia e Cirurgia Cérvico-Facial: 203–8. https://doi.org/10.1016/j.bjorl.2015.03.015. 2016.

SILVEIRA, R. K. *et al.* Bioprosthetic Mesh of Bacterial Cellulose for Treatment of Abdominal Muscle Aponeurotic Defect in Rat Model. **Journal of Materials Science***: Materials in Medicine* 27 (8). https://doi.org/10.1007/s10856-016-5744-z. 2016.

SKOP, N. B. *et al.* Optimizing a Multifunctional Microsphere Scaffold to Improve Neural Precursor Cell Transplantation for Traumatic Brain Injury Repair. **Journal of Tissue Engineering and Regenerative Medicine** 10 (10): E419–32. https://doi.org/10.1002/term.1832. 2016.

STASSEN, O. M. J. A., *et al.* Current Challenges in Translating Tissue-Engineered Heart Valves. **Current Treatment Options in Cardiovascular Medicine** 19 (9): 71. https://doi.org/10.1007/s11936-017-0566-y. 2013.

TAM, R. Y. *et al.* Regenerative Therapies for Central Nervous System Diseases: A Biomaterials Approach. **Neuropsychopharmacology** 39 (1). Nature Publishing Group: 169–88. https://doi.org/10.1038/npp.2013.237. 2014.

TAVARES, L. H. M. *et al.* Biopolymer Sponge for High Grade Renal Trauma: An Experimental Study in Rabbits. **Open Journal of Urology** 4 (1): 1–6. https://doi.org/10.4236/oju.2014.41001. 2014.

TEIXEIRA, F. M. *et al.* Spongy Film of Cellulosic Polysaccharide as a Dressing for Aphthous Stomatitis Treatment in Rabbits. **Acta Cirurgica Brasileira** 29 (4): 231–36. https://doi.org/10.1590/S0102-86502014000400003. 2014.

TIAN, L., M. P. PRABHAKARAN, S. RAMAKRISHNA. Strategies for Regeneration of Components of Nervous System: Scaffolds, Cells and Biomolecules." **Regenerative Biomaterial**s 2 (1): 31–45. https://doi.org/10.1093/rb/rbu017. 2015.

TIAN, W.M. *et al.* Hyaluronic Acid–Poly-D-Lysine-Based Three-Dimensional Hydrogel for Traumatic Brain Injury. **Tissue Engineering** 11 (3–4): 513–25. https://doi.org/10.1089/ten.2005.11.513. 2005.

TUKMACHEV, D. *et al.* Injectable Extracellular Matrix Hydrogels as Scaffolds for Spinal Cord Injury Repair. *Tissue* **Engineering Part A** 22 (3–4): 306–17. https://doi.org/10.1089/ten.tea.2015.0422. 2016.

VASITA, R., DHIRENDRA, S. K. Nanofibers and Their Applications in Tissue Engineering. **International Journal of Nanomedicine** 1 (1): 15–30. http://www.ncbi.nlm.nih.gov/pubmed/17722259. 2006.

VAYSSE, L. *et al.* Micropatterned Bioimplant with Guided Neuronal Cells to Promote Tissue Reconstruction and Improve Functional Recovery after Primary Motor Cortex Insult. **Biomaterials** 58. Elsevier Ltd: 46–53. https://doi.org/10.1016/j.biomaterials.2015.04.019. 2015.

VILAR, F. O. *et al.* A Wet Dressing for Male Genital Surgery: A Phase II Clinical Trial.”**International Braz J Urol** 42 (6): 1220–27. https://doi.org/10.1590/S1677-5538.IBJU.2016.0109. 2016.

WANG, T. Y. *et al.* Promoting Engraftment of Transplanted Neural Stem Cells/progenitors Using Biofunctionalised Electrospun Scaffolds. **Biomaterials** 33 (36). Elsevier Ltd: 9188–97. https://doi.org/10.1016/j.biomaterials.2012.09.013. 2012.

WEI, Y. *et al.* Hyaluronic Acid Hydrogels with IKVAV Peptides for Tissue Repair and Axonal Regeneration in an Injured Rat Brain. **Biomedical Materials** 2 (3). https://doi.org/10.1088/1748-6041/2/3/S11. 2007.

WERNER, C., K. ENGELHARD. Pathophysiology of Traumatic Brain Injury. **British Journal of Anaesthesia** 99 (1). British Journal of Anaesthesia: 4–9. https://doi.org/10.1093/bja/aem131. 2017.

WIERINGA, P. A. *et al.* Biomimetic Architectures for Peripheral Nerve Repair: A Review of Biofabrication Strategies. **Advanced Healthcare Materials** 1701164: 1–19. https://doi.org/10.1002/adhm.201701164. 2018.

WONG, D. Poly(ε-Caprolactone) and Poly (L-Lactic-Co-Glycolic Acid) Degradable Polymer Sponges Attenuate Astrocyte Response and Lesion Growth in Acute Traumatic Brain Injury. **Tissue Engineering** 13 (10): 2515–23. https://doi.org/10.1089/ten.2006.0440. 2007.

WONG, D. Y., P. H. KREBSBACH, S. J. HOLLISTER.. “Brain Cortex Regeneration Affected by Scaffold Architectures.” **Journal of Neurosurgery** 109 (4): 715–22. https://doi.org/10.3171/JNS/2008/109/10/0715. 2008.

XU, Z. *et al.* Regeneration of Cortical Tissue from Brain Injury by Implantation of Defined Molecular Gradient of Semaphorin 3A. **Biomaterials** 157. Elsevier Ltd: 125–35. https://doi.org/10.1016/j.biomaterials.2017.12.010. 2018.

ZHAO, Z. *et al.* Cellular Microparticles and Pathophysiology of Traumatic Brain Injury. **Protein and Cel***l* 8 (11). Higher Education Press: 801–10. https://doi.org/10.1007/s13238-017-0414-6. 2017.

ZHOU, K. *et al.* Graphene Functionalized Scaffolds Reduce the Inflammatory Response and Supports Endogenous Neuroblast Migration When Implanted in the Adult Brain. **PLoS ONE** 11 (3): 1–15. https://doi.org/10.1371/journal.pone.0151589. 2016.

APÊNDICE A – ARTIGO PUBLICAO NA REVISTA *JOURNAL OF MATERIALS SCIENCE*

“Bacterial cellulose from sugarcane molasses as a suitable substrate for 2D and 3D neuron and astrocyte primary cultures”

Dra. Catarina Gonçalves Pimentel e Giselle Machado Magalhães Moreno dividem a primeira autoria deste artigo.

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TISSUE ENGINEERING CONSTRUCTS AND CELL SUBSTRATES

Original Research

Cellulose exopolysaccharide from sugarcane molasses as a suitable substrate for 2D and 3D neuron and astrocyte primary cultures

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Abstract

Bacteria-synthesized polysaccharides have attracted interest for biomedical applications as promising biomaterials to be used as implants and scaffolds. The present study tested the hypothesis that cellulose exopolysaccharide (CEC) produced from sugarcane molasses of low cost and adequate purity would be suitable as a template for 2D and 3D neuron and/or astrocyte primary cultures, considering its low toxicity. CEC biocompatibility in these primary cultures was evaluated with respect to cell viability, adhesion, growth and cell function (calcium imaging). Polystyrene or Matrigel® matrix were used as comparative controls. We demonstrated that the properties of this CEC in the 2D or 3D configurations are suitable for differentiation of cortical astrocytes and neurons in single or mixed cultures. No toxicity was detected in neurons that showed NMDA-induced Ca^{2+} influx. Unlike other polysaccharides of bacterial synthesis, the CEC was efficient as a support even in the absence of surface conjugation with extracellular matrix proteins, maintaining physiological characteristics of cultured neural cells. These observations open up the perspective for development of a novel 3D biofunctional scaffold produced from bacterial cellulose and obtained from renewable sources whose residues are not pollutants. Its low cost and possibility to be manufactured in scale are also suitable for potential applications in regenerative medicine.

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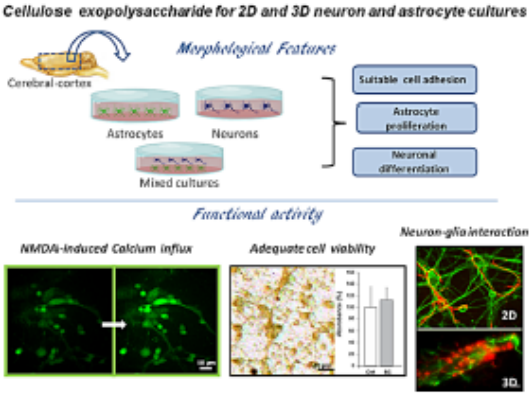
Graphical Abstract



1 Introduction

The use of biomaterials as substrates for tissue engineering and cell cultures has been largely investigated as a suitable alternative to grafts in regenerative medicine [1, 2]. The main advantage of biomaterials over synthetic products is their ability to be incorporated in the host tissue without inducing undesirable inflammatory responses [2].

A number of materials have been tested as support to neural cells [3]. Among them are collagen gels, fully- or semi-synthetic surfaces conjugated or not with extracellular matrix proteins [4], scaffolds of nanofibers [5] - and hybrid or natural polymers of chitosan [6, 7]. Special attention has been given to tubular scaffolds as support to Schwann cells to improve nerve regeneration [8]. It is also noteworthy that tissue engineering in traumatic brain injury represents a promising alternative for cellular replacement and rescue, providing a scaffold for resident cells [9].

Important requirements for using biopolymers in the central nervous system are the absence of toxicity, suitable hydrophilic properties, ability to be absorbed by the host tissue and the induction of vascularization, especially due to the poor regenerative capability of the brain [10]. Bacteria-synthesized polysaccharides have attracted interest for biomedical applications as unique and promising materials to be used as implants and scaffolds, especially in bone, cartilage and skin tissue engineering [11]. A recent study using modified bacterial cellulose (BC) synthesized by *Acetobacter xilinus* has indicated the compatibility of this biomaterial when subjected to a mercerization process, to form cartilage-like constructs used as neurotubes for regeneration of damaged peripheral nerve [12]. In combination with recombinant proteins or other molecules, modified BC has also been tested as a substrate for rat mesenchymal cells and neuron-like cells such as PC12 cell lineage and the human neuroblastoma cell line SH-SY5Y [13–15]. In these previous studies, purified BC was used as a scaffold in the shape of a pellicle with a textured surface for the cells to attach to. However, it has not yet been established if this type of biomaterial *per se* is sufficient to keep primary cultures of neural cells in favorable conditions for cell interactions.

The potential biotechnological use of *Zoogl*ea-synthesized cellulose exopolysaccharide (CEC) produced from sugarcane molasses [16] has been tested in different types of biomedical assays taking into account its high purity, low cytotoxicity, and genotoxic effects as well as the ability to be absorbed, permitting neovascularization of the new tissue [17–21]. Other important features of this CEC are the low-cost large-scale production from abundant renewable raw materials and the fact that their residues are not pollutants. Translational applications of this CEC in phase II clinical trials in humans has been recently demonstrated, as a wound dressing in the treatment of venous ulcers of the lower limbs, [22] or as a wet dressing for male genital surgery: [23]. It is noteworthy that in these two studies, the patients treated with CEC reported a significant reduction of pain. Moreover, the CEC was effective in removing exudate material, offered protection to the region and promoted tissue regeneration [23]. In this latter study, the estimated cost of CEC was low (US$4 per patient) when compared to other similar materials used as wound dressing (US$17-US $25) or even when compared to the clinically used polyurethane (US$5 to US$7). The use of CEC was also adequate for treatment of tympanic membrane perforation in humans in a randomized controlled trial [24]. Compared to the conventional approach with autologous fascia, the average surgery time using CEC was 5 times lower and its use provided a reduction of 13 times in hospital costs in the public health system [24].

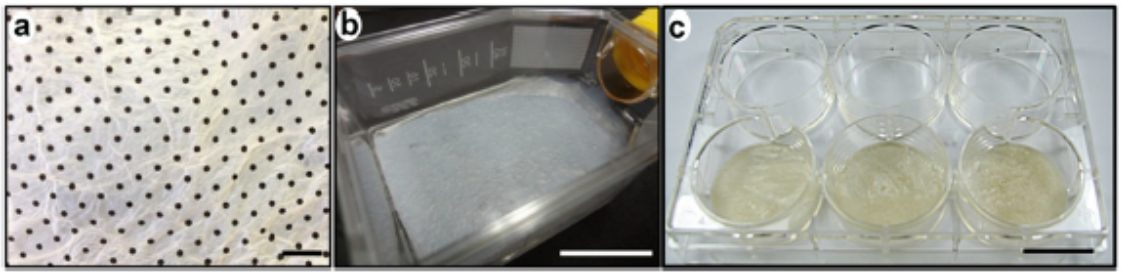


Fig. 1 Low magnification images of representative samples of CEC films, perforated **a** or homogeneous **b**, fixed on the inner surface of a polystyrene culture flask and CEC hydrogel colloidal fixed on 6-well plate Scale bars: **a** = 6 mm; **b** = 1 cm and **c** = 2 cm

Recent evidence has also shown CEC biocompatibility as a *dura mater* substitute [25] and as a substrate for human umbilical cord Wharton's jelly mesenchymal stem cells [26]. The present study aims to test whether this bacterial cellulose is a suitable biopolymer for neural cells. In order to address this, we investigated the adequacy of this biomaterial as a substrate for neuron and astrocyte primary cultures using 2D (films) and 3D (colloidal hydrogel) configurations. It was hypothesized that the CEC would be able to keep functional properties of these neural cells, and in the 3D arrangement, it would be appropriate as an interconnective network where neurons and astrocytes can penetrate, grow and interact in all directions as occurs in vivo conditions. Therefore, the main purpose of this study is to open up a perspective for the development of a low-cost material that could be used as a novel 2D and 3D biofunctional scaffold with potential application in future studies on cell neurobiology and regenerative medicine.

2 Materials and Methods

2.1 Ethical procedures, animals and bacterial cellulose from sugarcane molasses

All procedures adopted were approved by the Ethics Committee for Animal Research of the Federal University of Pernambuco (# 23076.000254/2013-72), in accordance with the Brazilian College for Animal Care guidelines, following the "Principles of Laboratory Animal Care" (NIH, Bethesda, USA). Twenty-six adult *Wistar* rats weighing 250–300 g were used as progenitors. These animals mated and were maintained in a room at 22 ± 2 °C with 67% relative humidity and 12 h light/dark cycle (lights on at 06:00); and they were fed a commercial balanced diet. On the 16th or 18th embryonic day (E16 or E18) and on the 1st or 3rd postnatal day (PND1 or PND3) pups from different litters (2–3 per litter) were grouped to obtain cerebral cortex primary cultures, as described below.

The CEC obtained from sugarcane molasses was produced by Polisa® of the Research Group of Sugarcane Polymer Laboratory, from the Rural Federal University of Pernambuco (UFRPE). As described previously [16], this exopolysaccharide is mainly composed by glucose (87.57%) followed by xylose (8.58 %) ribose (1.68%), mannose (0.82 %), glucuronic acid (0.68%), arabinose (0.37%), galactose (0.13%), rhamnose (0.01%) and fucose (0.01%). Colloidal hydrogels of CEC in concentrations of 0.6 to 0.8% exhibit viscoelastic properties and stability at temperatures adequate to biological fluids [16]. For the present study, it was supplied as thin 2D films (75–95 µm thickness) or in the form of a 3D colloidal hydrogel (500 µm or 2 mm thickness). Continuous or perforated CECfilms (pore diameter = 1 mm) were fixed on the inner surface of polystyrene culture flasks or in 6-, 12-, 24- or 96-well plates or on glass coverslips. CEC as colloidal hydrogel was fixed on 6-well plates (Fig. 1). Before used as templates for cells, all of the CEC presentations were sterilized using gamma radiation. Scanning electron micrographs of homogeneous or perforated CEC films are illustrated in Fig. 2, showing the structural organization of their surface and lateral plans. Note the random ultrafine nanofibril network structure of homogeneous bacterial cellulose (a) and the grid-like arrangement of the perforated BC film (c).

2.2 Mechanical properties of CEC films

Mechanical properties including maximal loading (N), tensile strength (stress, MPa) and elongation (strain, %) of CEC films (previously moistened with isopropyl alcohol) were measured by conducting a standardized tensile test in a United Universal Mechanical Testing Machine (EMIC, model DL 500 MF) with a gap distance of 2.5 cm and a crosshead speed of 250 mm/min. Stress was calculated as F/A, where F is the loading force and A is the area of the cross-section of materials. The strain was calculated considering DL/L, where L is the initial length and DL is the difference between the lengths at the break and the initial length. The samples ($n = 10$) were cut into test pieces each

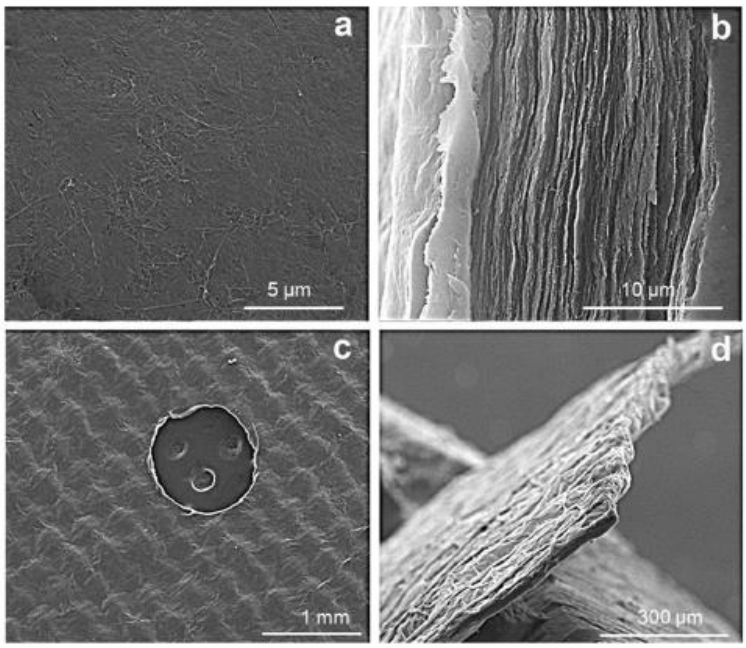


Fig. 2 Scanning electronic micrographs in low and high magnification of homogeneous **a**, **b** and perforated **c**, **d** CEC films, showing their structural organization in the surface and lateral plans. Note the random ultrafine nanofibril network structure of homogeneous **a** and the grid-like arrangement of the perforated film Magnifications: **a** = ×14,000; **b** = ×10,000; **c** = ×70; **d** = ×300

2 × 7 cm. A Digimatic point micrometer (Mitutoyo Series 342, Japan; Resolution: 0.001 mm; Graduation: 0.01 mm) was used to measure the thickness prior to loading the samples into the testing machine.

2.3 Two-dimensional astrocyte primary cultures

Primary cultures of cortical astrocytes from PND1-3 neonates were prepared according to the protocol previously described [27]. Pups from different litters per group were decapitated; cerebral cortices were dissected in a phosphate buffer solution containing 0.6% glucose (PBS-glucose) and then mechanically dissociated. Cell suspension was diluted in Dulbecco's Modified Eagle medium and nutrient mixture F-12 (DMEM-F-12; Gibco) supplemented with 10% fetal bovine serum (FBS; Gibco), 33 mM glucose (Merck), 2 mM glutamine (Calbiochem), 3 mM sodium bicarbonate (Merck), 0.5 mg/mL penicillin/streptomycin (Invitrogen), 2.5 µg/mL amphotericin (Sigma-Aldrich) and then centrifuged for 5 min (1500 r.p.m.). The cells re-suspended in DMEM-F-12 were plated in polystyrene flasks (25 cm^2) and incubated at 37 °C in a humidified 5% CO_2-95% O_2 air atmosphere. The medium was replaced every 2 days. After 10 days in vitro (div), the cells were trypsinized (0.25% trypsin + 0.2% EDTA; Sigma-Aldrich) for 5 min to obtain a more purified astrocyte culture and then re-plated in DMEM-F-12 into polystyrene flasks or on 24-well plates coated or not with CEC films for 5 div.

2.4 Two-dimensional embryonic neuron primary cultures and cell viability assay

The presence of spermatozoids in the vaginal smear was used to determine the first day of pregnancy. On the 16th day, pregnant females were anesthetized with isofluorane and submitted to surgery to remove the embryos and then were sacrificed by an overdose of isoflurane. Embryonic cerebral cortices were dissected, dissociated in PBS-glucose and centrifuged for 5 min at 1500 r.p.m. The cell suspension was diluted in Neurobasal medium (Gibco) supplemented with 2 mM glutamine (Calbiochem), 10 µl/mL penicillin/streptomycin (Sigma-Aldrich) and 1% B-27 (Gibco). Cells were seeded onto 24-well plates (50,000 cells/well) or 96-well plates (10,000 cells/well) coated or not with CEC films and pre-treated with 1 µg/mL poly-L-lysine (Sigma-Aldrich) as an attachment factor necessary for increasing the number of positively-charged sites available for neuronal cell binding. Cells were incubated in a humidified 5% CO_2-95% O_2 air atmosphere for 48 h. Cell viability of neuron cultures was assessed by the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, Invitrogen) reduction assay [28].

2.5 Two and three-dimensional embryonic neuron and astrocyte mixed cultures

As described above, anesthetized pregnant females were submitted to surgery to remove E18 embryos and then

sacrificed by an overdose of anesthetic. Cerebral cortices of three embryos per experiment were dissected in Hanks' Balanced Salt Solution (Invitrogen) plus 20 mM Hepes (HBSS-Hepes, pH 7.4) and then incubated in 0.25% trypsin diluted in the same dissection solution for 15 min (37 °C). After rinsed in HBSS-Hepes, the cells were mechanically dissociated in attachment medium composed of Minimum Essential Medium (Gibco) with 2 mM glutamine, 10% horse serum, 1 mM Na^+ pyruvate; and then seeded on 12-(16×10^4 cells/mL) or 6-well (20×10^4 cells/mL) plates, pre-treated with 50 µg/ml poly-L-lysine. In 12-well plates, 2D cultures were obtained by seeding the cells over 18 mm diameter glass coverslips (Marienfeld Gmbh & Co. KG) coated or not with CEC as thin films (~85 µm thickness). In 6-well plates, the cells adhered directly onto the polystyrene or to CEC films fixed on the polystyrene. 3D cultures were obtained by seeding the cells over CEC colloidal hydrogel (diluted 0.8%, 500 µm or 2 mm thickness) or using Matrigel® Basement membrane matrix phenol red free (Corning) as a positive control. In this case, 3D cultures were obtained by dispersing the cells in a liquid Matrigel® diluted 2–4% in the attachment medium followed by solidification at 37 °C for 1 h, before adding Neurobasal medium (Gibco™). After 4 h of incubation in the attachment medium, the mixed cultures were maintained in Neurobasal medium containing 2 mM glutamine, 10 µg/ml penicillin/streptomycin and 2% B-27 50X supplement (Gibco). They were kept at 37 °C in a humidified 5% CO_2-95% O_2 air atmosphere for 10 days (30% of the medium was replaced every 5 days).

2.6 Ca^{2+} Imaging

Two-dimensional mixed cultures containing a higher density of neurons than astrocytes were kept on 12-well plates (over coverslips covered or not with CEC) for 7–8 days and then treated with the glutamate receptor agonist NMDA (Sigma-Aldrich) for analysis of intracellular calcium mobilization. On the day of the experiment, the cells were loaded with 0.5 µM Fluo-4 AM (Invitrogen) for 5 min at 37 °C in MEM phenol red-free containing 33 mM glucose, 20 mM HEPES (Sigma), 2 mM glutamine (Sigma), 1 mM Na^+ pyruvate (Sigma-Aldrich). Cells were then rinsed for 5 min in the same medium to allow complete de-esterification of the intracellular AM esters. Fluo-4 AM fluorescence was assessed before the treatment (time 0) and every minute for 10 min after adding 50 µM NMDA plus 2 µM glycine (Sigma-Aldrich). The imaging was carried out at 37 °C under an inverted Leica DMI 4000B microscope equipped with spinning disc Yokogawa CSU-x1, an HCX Plan Apo 40×/0.85 CORR CS dry objective and motorized piezo stage. A cooled CCD camera (Quant EM 5125C; Photometrics, USA) with 512 × 512 imaging array, 16 × 16 pixels and acquisition at 10 MHz using a filter for Fluo4 AM (excitation: 491 nm; emission: 525 nm) was used. At each time point, the image acquisition consisted of a 33-frame stream recording, which was averaged for quantification. The cell body fluorescence intensity was measured. Images of extracellular regions were taken and averaged to obtain the background fluorescence. The fluorescence intensity compared to the initial values (F10/F0) was calculated after subtraction of the background fluorescence. If the fluorescence increased more than 10%, the cell was considered as a responding cell.

2.7 Immunocytochemical assay

Immunostaining for neuron and astrocyte markers was carried out for all types of cultures. In all cases, the cells were fixed with 4% paraformaldehyde in PB (pH 7.4) for 20 min followed by 3 washes in PB. In pure astrocyte or neuron cultures, the cells were first treated with 3% bovine serum albumin in PBS + 0.3% Triton X-100 (PBS-T) for 1 h and then incubated (for 18 h at 4 °C) with rabbit anti-GFAP (Invitrogen; 1:500) or mouse anti-β tubulin III (Santa Cruz Biotechnology; 1:500) antibodies diluted in PBS-T. The secondary biotinylated antibodies goat-anti-mouse or goat-anti-rabbit (Jackson Immuno Research, 1:1000) were used followed by ABC kit (Vectastain; Vector Labs) and diaminobenzidine (DAB). In the 2D or 3D embryonic mixed cultures, the cells were incubated simultaneously with rabbit anti-GFAP (Dako;1:1000) and mouse anti-β tubulin III (Santa Cruz Biotechnology; 1:1000) in PBS-T for 18 h at 4 °C and then with FITC or Cy-3-labeled anti-rabbit or anti-mouse IgGs (1:500, Jackson Immuno Research) diluted in PB for 3 h. Digital images of the pure cultures were obtained using an inverted microscope (Leica DMIL) coupled to a Moticam 2300 camera and analyzed with Motic Image Plus 2.0 (2007) software. Images of 2D or 3D mixed cultures were obtained using an inverted microscope (ZEISS Axio Imager Z1, the motorized version with high-precision Z drive) equipped with Digital CCD camera AxioCam MRm, ApoTome slider, and the imaging software AxioVision 4.8.

2.8 Statistical analysis

Unless stated otherwise, all experiments were done in triplicate. Statistical analyses were carried out using the Shapiro–Wilk normality test and then Ranks ANOVA (Kruskal Wallis test) followed by the Mann Whitney test or the unpaired Student´s *T*-test. Image analysis was performed using ImageJ software (version 1.45r, NIH). GraphPad Prism 4.0 software was used with $p \leq 0.05$ considered statistically significant.

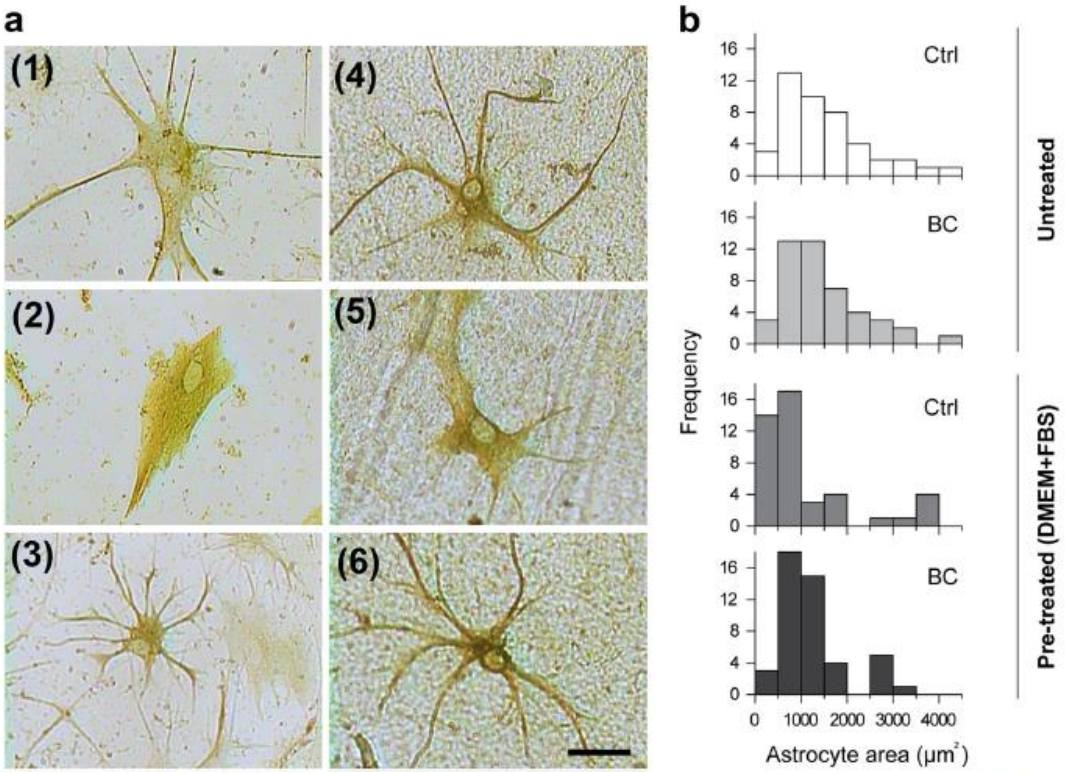


Fig. 3 Characterization of 2D astrocyte primary cultures grown on treated or non-treated perforated CEC films. **a:** High magnification images of GFAP-positive astrocytes grown for 10 days on perforated CEC films Similar types of astroglia morphological phenotypes are seen over the polystyrene in the holes (1), (2), (3) or over the CEC film (4), (5), (6) in the same flask. Scale bar = 30 μm. **b**: Histograms of the astrocyte's size grown on the perforated CEC untreated or pre-treated with the DMEM-F12 + FBS 10% in a representative culture. No significant difference was found in the astrocyte size profile grown over CEC or polystyrene nor between untreated or DMEM-F12 + 10% FBS pre-treated CEC (Kruskal–Wallis test)

3 Results

3.1 Mechanical properties of the CEC films

The average thickness of 10 CECfilms analyzed regarding their physical properties was 85 ± 9.7 μm. Their mechanical properties were a maximal loading of 70.71 ± 23.59N, a tensile strength of 31.61 ± 9.77 MPa, an elongation of 8.29 ± 1.58% and an elastic modulus of 497.03 ± 113.33 MPa.

3.2 CEC as a substrate for 2D astrocyte cultures

Using perforated CEC films, we analyzed in a single flask the efficacy of the CEC regarding astrocyte's adhesion and spreading when compared to the polystyrene. As can be seen in the high magnification images in Fig. 2, no apparent qualitative difference was detected in the morphological phenotypes of GFAP-immunoreactive astrocytes grown over the polystyrene (Figs. 3a (1), (2), (3)) or the BC (Figs. 3a (4), (5), (6)) in the same flask. Morphometric analysis of the astrocyte size carried out in two independent cultures did not show any significant difference between the cells adhering to the two substrates present in the same flask either when they were untreated or pre-treated with DMEM-F12 + 10% FBS (Kruskal Wallis followed Mann Whitney test; $p < 0.05$). Figure 3b compares the histograms of the astrocyte's size grown on the perforated CEC, untreated or previously treated with DMEM-F12 + 10% FBS, in a representative culture.

In flasks fully covered with homogeneous CEC untreated with DMEM-F12 + 10% FBS or other compounds, the astrocytes were evenly distributed with similar levels of confluence and differentiation when compared to those seeded on non-covered polystyrene flasks. Figure 4 illustrates low and medium magnification images of two independent astrocyte primary cultures that were kept over polystyrene (Fig. 4a, b) or CEC (Fig. 4c, d) for 10 div.

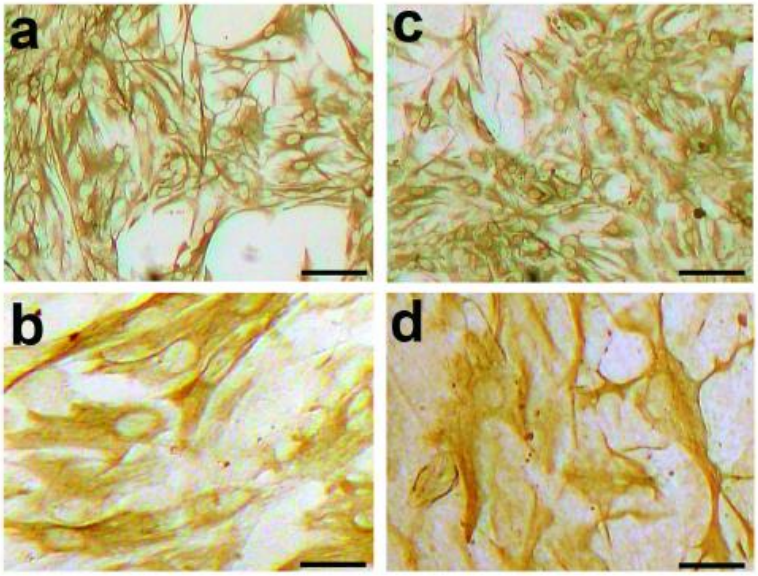


Fig. 4 Morphological differentiation of 2D astrocyte primary cultures on non-treated and homogeneous CEC **a**: Low and medium magnification images of two representative 2D cortical astrocyte cultures immunolabelled for GFAP, grown for 10 days in vitro over polystyrene **a**, **b** or homogeneous CEC membranes **c**, **d**. Scale bar = 70 µm for **a** and **c**; 20 µm for **b** and **d**

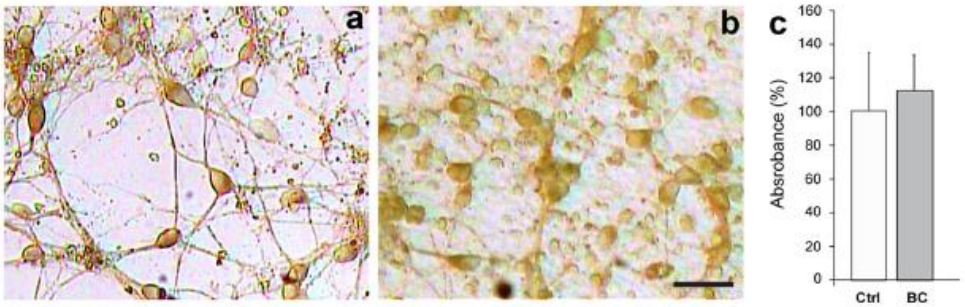


Fig. 5 Differentiation and cell viability of 2D embryonic neuron cultures grown on CEC films Medium magnification images of representative 2D pure embryonic cortical neuron cultures labeled for β-tubulin III, that were kept for 2 days over glass coverslips **a** or homogeneous CEC membrane **b**. Scale bar = 20 µm. **c** Cell viability quantified by MTT assay in pure neuron cultures grown for 2 days over the control condition or CEC. Data are normalized for the control condition and expressed as the mean ± SD of three independent cultures. No intergroup difference was found (Student *T*-test; $p = 0.612$)

3.3 Biocompatibility of CEC as a substrate for 2D pure embryonic neuron cultures

Pure embryonic neuron cultures obtained from E16 and kept for two days over the CEC displayed similar adhesion and neurite outgrowth when compared to cells plated onto the control substrate. Figure 5 shows mild magnification images of representative primary neuron cultures immunoreacted for β-tubulin III that were kept for 48 h in a Neurobasal medium + 1% B27 supplement over glass coverslips uncovered (5a) or covered with CEC (5b). No intergroup difference was found in the MTT cell viability assay in pure neuron cultures grown over the CEC or control substrate (unpaired Student´s *T*-test). The results (mean ± standard deviation) expressed in percentage of control condition were: 100 ± 48.22, 100 ± 35.42 and 100 ± 20.65 in uncovered glass coverslips, and 121.23 ± 35.58, 110.32 ± 9.45, 105 ± 20.30 in the CEC. Figure 5c depicts the averaged values obtained for these three independent cultures.

3.4 CEC as a substrate for 2D embryonic neuron and astrocyte mixed cultures

Embryonic neuron and astrocyte mixed cultures obtained at E18 and kept for 10 div over the CEC also exhibited adequate adhesion, differentiation and neurite outgrowth when compared to cells plated onto the polystyrene. Figure 6 shows digital images obtained using a Z stack of representative cultures grown over the polystyrene (Fig. 6a) or CEC (Fig. 6b) showing neurons stained by β-tubulin III and astrocytes stained by GFAP and the merge.

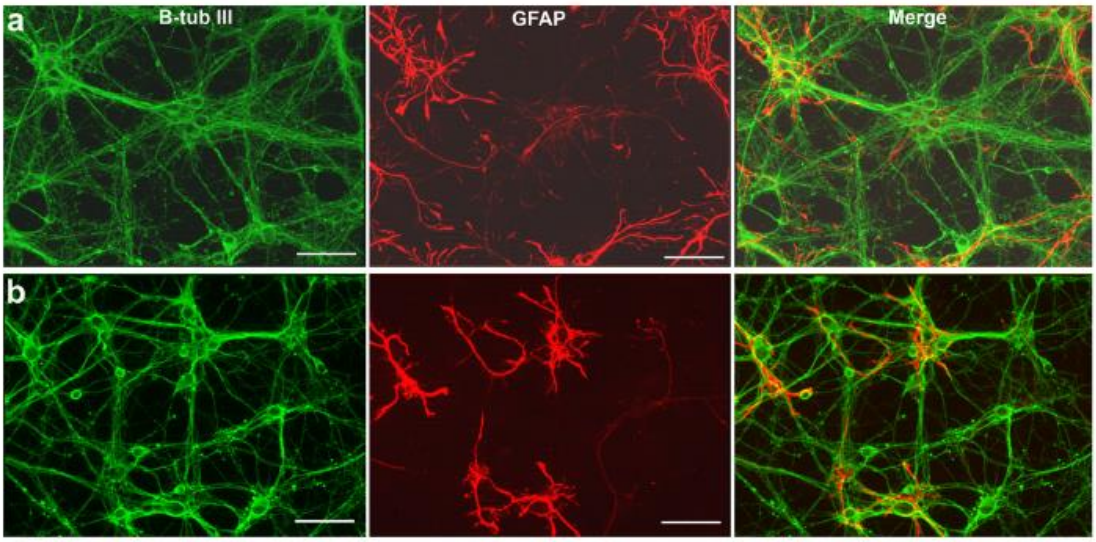


Fig. 6 Two-dimensional mixed neuron-astrocyte cultures. Z-stack images of immunolabeled cortical neurons (green: β-tubulin III) and astrocytes (red: GFAP) kept for 10 days in vitro on 6-well plates, directly onto the polystyrene **a** or CEC films **b**. Note in the merged images the similar topographic distribution of these cells, with most of astrocyte cell bodies involving neuronal cell bodies. Scale bar = 20 µm

3.5 NMDA induced Ca^{2+} influx in 2D mixed cultures grown on CEC

Neuronal response to a glutamatergic stimulus was investigated using Ca^{2+} imaging in 2D mixed cultures kept for 7–8 div. Treatment with 50 µM NMDA in the presence of 2 µM glycine was able to induce a similar increase in the Ca^{2+} influx (assessed by Fluo-4-AM fluorescence) in cells grown over glass coverslips covered with CEC, compared to the control condition. Figure 6 shows digital images of intracellular Ca^{2+} responses in 2 independent cultures grown over these two substrates, before (Fig. 7a–d) or 10 min after NMDA + glycine application (Fig. 7a*–d*). Quantitative analysis using Mann Whitney Test showed that compared to the basal state, the increase in the Fluo-4-AM fluorescence in 28 responsive cells (56–70% of total cells analyzed) plated on control glass coverslips reached a median of 61.59% (minimum = 10.40% and maximum = 193.19%) and in cultures grown over the CEC the values obtained were 80.57% (min = 10.88% and maximum = 377.80%). In these independent cultures, the range of response did not differ between the groups ($p = 0.176$; Fig. 7e).

3.6 CEC as colloidal hydrogel is a suitable biomaterial for 3D mixed cultures

Embryonic astrocyte and neurons were able to penetrate and grow at several depths of the 3D configuration of CEC and displayed morphological phenotype characterized by round or elongated neuronal cell bodies dispersedly distributed or grouped. Most of them are involved by astrocytes with invasive processes in all directions. Figure 8 compares two representative 3D cultures kept on Matrigel® (Fig. 8a, b) or CEC (Fig. 8c, d). Figures 8b*, 8d* show neurons and astrocytes in deeper regions of Matrigel® and CEC respectively, which are not in focus in 8b or 8d.

4 Discussion

Present findings show that bacterial cellulose obtained from sugarcane molasses is a versatile biomaterial with potential biomedical applications in neural tissue engineering. As previously demonstrated for mesenchymal stem cells from human umbilical cord Wharton's jelly [26], this type of biopolymer was a suitable template for the seeding of astrocyte and neuronal cells, allowing adhesion and growth in vitro. This biocompatibility with neural cells is comparable to what has been reported for other materials such as methylcellulose [9], nanofibers of polymers conjugated or not with proteins of extracellular matrix [4], a polymer made from poly-l-lactic-acid [29] and *Bombyx mori* silk fibroin [30]. Evidence has indicated that a higher efficiency of bacterial nanocellulose scaffolding produced by *Gluconacetobacter xylinus* for SH-SY5Y neuroblastoma [15] or PC12 [14] cell lines was obtained when it was conjugated with polypyrroles or collagen I, respectively. Similar results were obtained for PC12 cell line and mesenchymal stem cell adhesion and growth when cellulose membranes obtained by *Gluconacetobacter xylinus* were conjugated

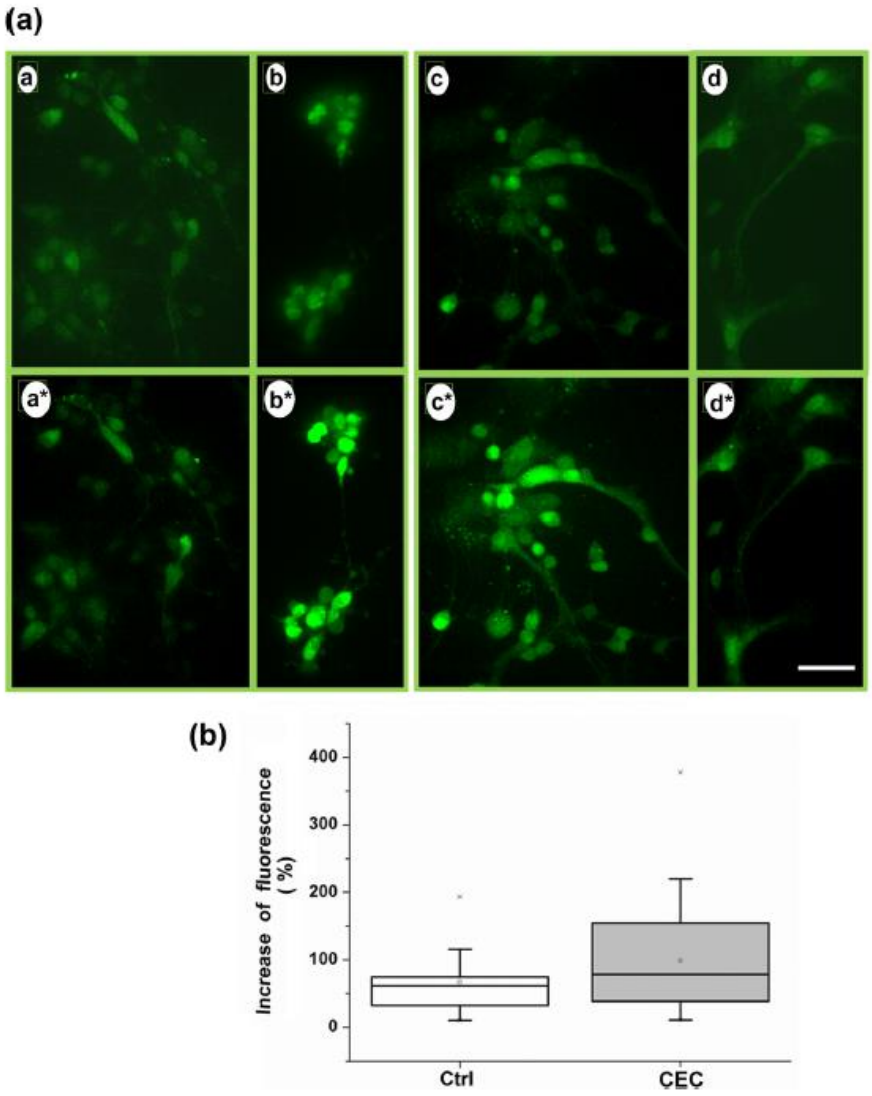


Fig. 7 Calcium imaging in 2D mixed neuron-astrocyte cultures. **a**; Digital images of intracellular Ca^{2+} responses in 2 independent cultures grown over the glass coverslip (**a**, **a***, **b**, **b***) or CEC (**c**, **c***, **d**, **d***) before 50 µM NMDA + 2 µM glycine application (F0; **a**–**d**) and 10 min after this treatment, (F10; **a***, **b***, **c***, **d***). **b**: Quantitative analysis using Mann Whitney Test showed a similar increase in the Fluo-4-AM fluorescence between responsive cells of the two groups ($p = 0.176$). Data are expressed as a percentage of increase compared to basal condition (F10/F0 ratio) in 28 responsive cells in a total of 40–50/group. Scale bar = 20 µm

with recombinant proteins [13]. It is known that primary proliferating astrocytes can adhere and divide when plated on chemically un-functionalized surfaces because they have effective extracellular matrix secretion. In our pure astrocyte cultures, a similar growth profile was achieved over polystyrene and CEC membranes untreated or pre-treated with DMEM-F12 plus 10% FBS. Therefore, the CEC films *per se* were able to maintain the ability of astrocytes to release proteins necessary for their adhesion, even in the absence of surface conjugation with other compounds. In pure neuronal-cultures, the MTT assay evaluating the activity of NAD(P)H-dependent oxidoreductase enzymes also indicated a similar and adequate metabolic activity and viability of these embryonic neurons over the CEC, compared to the polystyrene substrate.

Neurons and astrocytes respond differently to substrates of varied stiffness [31, 32]. Usually, neurons show enhanced attachment and growth on softer surfaces when compared to astrocytes [31]. The physical properties here described for CEC films reinforce previous evidence that the elasticity and tensile strength of this cellulose are adequate for surgical repair of acute muscle aponeurotic defect-induced in rats [33] and for suturing femoral vein without tearing [20]. Since mechanical properties of cellulosic films strongly depend on the properties of the network, Silveira et al. [33] have reported the mechanical properties of CEC after associated with the host specimen. They have found satisfactory biomechanical parameters of this biomaterial for use as a coating of biological tissue. Recent evidence using neural stem cells from subventricular zone of

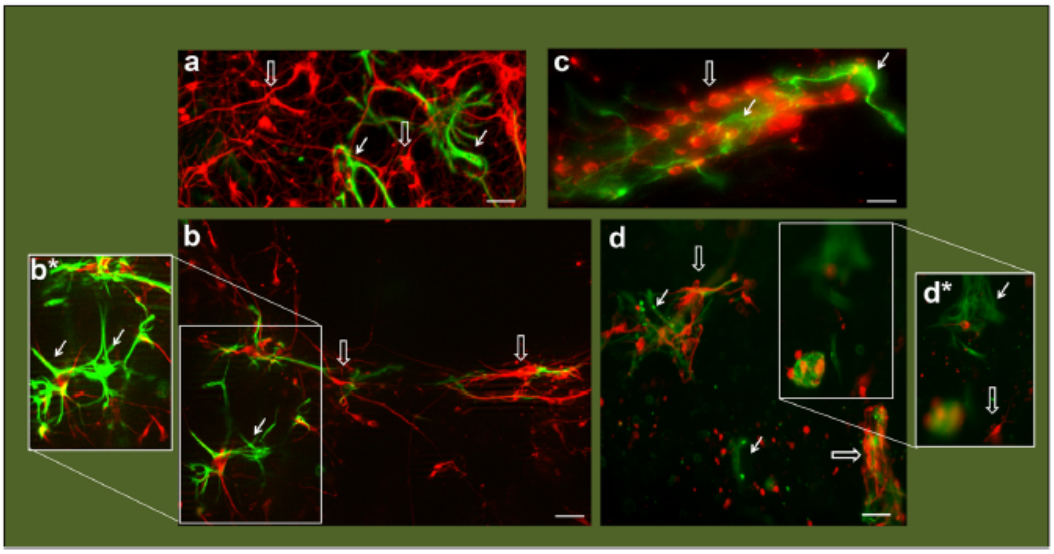


Fig. 8 Growth and differentiation of neurons and astrocytes in three-dimensional mixed neuron-astrocyte cultures grown on colloidal hydrogel CEC: Z-stack images of immunolabeled cortical neurons and astrocytes (red: β-tubulin III, green: GFAP) of two independent cultures grown for 10 days in vitro on the Matrigel® (**a**, **b**, **b***) or BC colloidal hydrogel (**c**, **d**, **d***). Note the presence of neurons with elongated or round cell bodies and neurite outgrowth at different depths (**b*** and **d***) and astrocytes with invasive processes in all directions and involving groups of neurons, in both substrates. Scale bar = 20 µm

postnatal balb/C mice indicated that random or line structured bacterial cellulose produced by *Acetobacter xylinum* favor neuron and astrocyte differentiation compared to grid structured that is more adequate for astrocyte differentiation [34].

In this respect, it is noteworthy that the levels of stiffness, porosity and the random fiber arrangement of the homogeneous CEC films here used were sufficient to allow not only cellular adhesion but a signaling between astrocyte and neurons necessary for their outgrowth. This type of bilateral communication usually involves the release of trophic factors and other molecules [35]. Preliminary experiments using co-cultures showed that the conditioned medium of a confluent layer of astrocytes grown over the BC was also able to support neurite outgrowth of the E16 cortical neurons (data not shown).

Functional activity of neurons in 2D mixed cultures was investigated using Ca^{2+} imaging. It is well established that Ca^{2+} plays an integral role in neuron function. Modifications in its intracellular levels are involved in a number of mechanisms related to activity-induced adaptations [36]. These cellular responses can be activated by NMDA receptors located in the synaptic or extrasynaptic regions [37]. Their expression can be detected at early stages of rat cerebral cortex development and continuously increases, reaching a peak in the third postnatal week [38]. Ca^{2+} responses induced by 50 µM NMDA in neurons grown for 7–8 days over the CEC indicate that this biomaterial is a permissive substrate for maintaining glutamatergic signaling during the maturation of these cells. This invites further research to analyze the potential use of BC in experimental models of synaptic plasticity in mature neurons.

The restricted capacity of the mammalian cerebral cortex to restore itself after insults such as stroke, trauma or neurodegeneration has stimulated the use of engineered biomaterials that resemble the 3D innate tissue and that can modulate properties involved in neural cell survival [39]. Recent studies using biodegradable matrices, as for example those composed of collagen plus hyaluronic acid plus Matrigel®, at the site of injury have demonstrated the efficacy of these optimized hydrogels in reducing reactive astrogliosis and glial scarring [40]. Moreover, tissue-engineered 3D culture models can offer a useful instrument to gain insight into the biological processes that occur as a consequence of central nervous system injury [41]. Despite the difference in the chemical composition of the CEC colloidal hydrogel when compared to Matrigel®, the morphological differentiation in round or elongated cell bodies and widespread distribution of neurons and astrocytes occurred in all directions and depths. The presence of glial cells involving groups of neurons inside the CEC colloidal hydrogel is also a feature similar to the in vivo architecture complexity and expected for an adequate 3D culture, where cell interactions and networks can be established [42]. Although future studies are necessary to analyze neuron-astrocyte metabolic coupling in this 3D

configuration of CEC, the present findings obtained in this first step necessary to test the suitability of this biomaterial in neural tissue engineering are promising. The purity, absence of neurotoxicity and the low cost of this renewable biomaterial encourage its use in functional assays where the integration of the colloidal hydrogel into the brain parenchyma could be assessed. The biocompatibility of CEC-films as a *dura mater* substitute has been previously demonstrated for periods of up to 120 days, without causing adverse effects to brain parenchyma [25]. In this latter study, the ability of CEC to be absorbed and induce vascularization and tissue regeneration were superior to that observed using e-PTFE as an inert standard material. Therefore, the present results suggest that the use of CEC in the form of colloidal hydrogel could provide an alternative scaffold for migration and rescue of neural resident cells in experimental models of traumatic brain injury.

In conclusion, we demonstrated that a cellulose exopolysaccharide produced from sugarcane molasses provides a suitable substrate for adhesion and growth of cultured primary neurons and astrocytes, maintaining the basic functional properties of these cells. These results suggest that it can be a promising construct for development of a novel and low cost 3D biofunctional scaffold, with potential application in neural tissue engineering, either for studies on cell neurobiology or regenerative medicine.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

References

1. Blunk T, Göpferich A, Tessmar T. Special issue: biomimetic polymers. Biomaterials. 2003;24:4335.
2. O'Brien FJ. Biomaterials and scaffolds for tissue engineering. Mater Today. 2011;14:88–95.
3. Ai J, Kiasat-Dolatabadi A, Ebrahimi-Barough S, Ai A, Lotfi-bakhshaiesh N, Norouzi-Javidan A, et al. Polymeric scaffolds in neural. Tissue Eng. 2013;1:15–20.
4. Little L, Healy KE, Schaffer D. Engineering biomaterials for synthetic neural stem cell microenvironments. Chem Rev. 2008;108:1787–96.
5. Vasita R, Katti DS. Nanofibers and their applications in tissue engineering. Int J Nanomed. 2006;1:15–30.
6. Fabela-Sánchez O, Zarate-Trivino D, Parra-Cid C, Gómez-García R, Munoz-Sánchez C, Solis L, et al. A hybrid natural -synthetic chitosan-based hydrogel as scaffold for mammalian cell culture for tissue engineering applications. Tissue Eng Cell Ther Regen Med Lab. 2008;22:6117575.
7. Guerra GD, Barbani N, Gagliardi M, Rosellini E, Cristallini C. Chitosan-based macromolecular biomaterials for the regeneration of chondroskeletal and nerve tissue. Int J Carbohydr Chem. 2011;14:1–9.
8. Zamani F, Amani-Tehran F, Latifi M, Shokrgozar MA, Zaminy A. Promotion of spinal cord axon regeneration by 3D nanofibrous core-sheath scaffolds. J Biomed Mater Res Part A. 2014;102:506–13.
9. Tate MC, Shear DA, Hoffman SW, Stein DG, LaPlaca MC. Biocompatibility of methylcellulose-based constructs designed for intracerebral gelation following experimental traumatic brain injury. Biomaterials. 2001;22:1113–23.
10. Clausen F, Lindh T, Salimi S, Erlandsson A. Combination of growth factor treatment and scaffold deposition following experimental traumatic brain injury show a temporary effect on cellular regeneration. Brain Res.2014;1588:37–46.
11. Khan F, Ahmad SR. Polysaccharides and their derivatives for versatile tissue engineering application. Macromol Biosci. 2013;13:395–421.
12. Kowalska-Ludwicka K, Cala J, Grobelski B, Sygut D, Jesionek-Kupnicka D, Kolodziejczyk M, Bielecki S, Pasieka Z. Modified bacterial cellulose tubes for regeneration of damaged peripheral nerves. Arch Med Sci. 2013;9:527–34.
13. Pértile RAN, Moreira S, Andrade FK, Domingues L, Gama M. Bacterial cellulose modified through recombinant proteins as a scaffold for neuronal cell culture. Sem Eng. 2010;1:11–2.
14. Muller D, Silva JP, Rambo CR, Barra GMO, Dourado F, Gama FM. Neuronal cells' behavior on polypyrrole coated bacterial nanocellulose three-dimensional (3D) scaffolds. J Biomater Sci Polym. 2013;24:1368–77.
15. Innala M, Riebe I, Kuzmenko V, Sundberg J, Gatenholm P, Hanse E, Johannesson S. 3D Culturing and differentiation of SH-SY5Y neuroblastoma cells on bacterial nanocellulose scaffolds, artif cells, nanomedicine. Biotechnol. 2014;42:302–8.
16. Paterson-Beedle M, Kennedy JF, Melo FAD, Lloyd LL, Medeiros V. Cellulosic exopolysaccharide produced from sugarcane molasses by a Zoogloea sp. Carbohydr Polym. 2000;42:375–83.
17. Cavalcante AHM, Carvalho LB, Carneiro-da-Cunha MG. Cellulosic exopolysaccharide produced by Zoogloea sp. as a film support for trypsin immobilization. Biochem Eng J. 2006;29:258–61.
18. Carvalho AM Jr, Santos MM, Barkokébas BB, Aguiar JLA, Lima SVC, Dambros M. Characterization of the deposition of collagen fibers and lithogenic potential in bladder of rats submitted to a sugar cane biopolymer graft. Int Braz J Urol. 2012;38:544–51.
19. Medeiros MD Jr, Carvalho EJDA, Catunda IS, Bernardino-Araujo S, Aguiar JLDA. Hydrogel of polysaccharide of sugarcane molasses as carrier of bone morphogenetic protein in the reconstruction of critical bone defects in rats. Acta Cir Bras. 2013;28:233–38.
20. de Barros-Marques SR, Marques-Lins E, de Albuquerque MC, de Andrade-Aguiar JL. Sugarcane biopolymer patch in femoral vein angioplasty on dogs. J Vasc Surg. 2012;55:517–21.
21. Pinto FC, De-Oliveira AC, De-Carvalho RR, Gomes-Carneiro MR, Coelho DR, Lima SV, Paumgartten FJ, Aguiar JL. Acute toxicity, cytotoxicity, genotoxicity and antigenotoxic effects of a

cellulosic exopolysaccharide obtained from sugarcane molasses. Carbohydr Polym. 2016;137:556–60.
22. Cavalcanti LMA, Pinto FCM, Oliveira GMA, Lima SVC, Andrade Aguiar JL, Lins, Lins EM. Efficacy of bacterial cellulose membrane for the treatment of lower limbs chronic varicose ulcers: a randomized and controlled trial. Rev do Colégio Bras De Cir. 2017;44:72–80. https://doi.org/10.1590/0100-69912017001011
23. Vilar FL, Pinto FCM, Albuquerque AV, Martins AGS, Araújo LAP, Andrade Aguiar JL, Lima SVC. A wet dressing for male genital surgery: a phase II clinical trial. Int Braz J Urol. 2016;42:1220–7.
24. Silveira FCA, Pinto FCM, Caldas Neto SS, Leal MC, Cesário J, Aguiar JLA. Treatment of tympanic membrane perforation using bacterial cellulose: a randomized controlled trial. Braz J Otorhinolaryngol. 2016;82:203–8.
25. Lima FM, Pinto FC, Andrade-da-Costa BL, Silva JG, Campos Júnior O, Aguiar JL. Biocompatible bacterial cellulose membrane in dural defect repair of rat. J Mater Sci Mater Med. 2017;28:37.
26. Fragoso AS, Silva MB, de Melo CP, Aguiar JL, Rodrigues CG, de Medeiros PL, et al. Oliveira MD. Dielectric study of the adhesion of mesenchymal stem cells from human umbilical cord on a sugarcane biopolymer. J Mater Sci Mater Med. 2014;25:229–37.
27. Gomes FCA, Garcia-Abreu J, Galou M, Paulin D, Moura Neto V. Neurons induce GFAP gene promoter of cultured astrocytes from transgenic mice. Glia. 1999;26:97–108.
28. Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods. 1983;65:55–63.
29. Lai Y, Cheng K, Kisaalita W. Three dimensional neuronal cell cultures more accurately model voltage-gated calcium channel functionality in freshly dissected nerve tissue. PLoS One. 2012;7:e45074.
30. Flanagan KE, Tien LW, Elia R, Wu J, Kaplan D. Development of a sutureless dural substitute from Bombyx mori silk fibroin. J Biomed Mater Res Part B. 2015;103:485–94.
31. Georges PC, Miller WJ, Meaney DF, Sawyer ES, Janmey PA. Matrices with compliance comparable to that of brain tissue select neuronal over glial growth in mixed cortical cultures. Biophys J. 2006;90:3012–8.
32. Ali MY, Chuang CY, Saif MTA. Reprogramming cellular phenotype by soft collagen gels. Soft Matter. 2014;10:8829–37.
33. Silveira RK, Coelho AR, Pinto FC, de Albuquerque AV, de Melo Filho DA, de Andrade Aguiar JL. Bioprosthetic mesh of bacterial cellulose for treatment of abdominal muscle aponeurotic defect in rat model. J Mater Sci Mater Med. 2016;129:1–9.
34. Geisel N, Clasohm J, Shi X, Lamboni L, Yang J, Mattern K, Yang G, Schäfer KH, Saumer M. Microstructured multilevel bacterial cellulose allows the guided growth of neural stem cells. Small. 2016;12:5407–5413.
35. Li D, Agulhon C, Schmidt E, Oheim M, Robert N. New tools for investigating astrocyte-to-neuron communication. Front Cell Neurosci. 2013;7:193.
36. Brini M, Calì T, Ottolini D, Carafoli E. Neuronal calcium signaling: function and dysfunction. Cell Mol Life Sci. 2014;71:2787–814.
37. Hardingham GE, Hilmar B. Synaptic versus extrasynaptic NMDA receptor signaling: implications for neurodegenerative disorders. Nat Rev Neurosci. 2010;11:682–696.
38. Sheng M, Cummings J, Roldan LA, Jan YN, Jan LY. Changing subunit composition of heteromeric NMDA receptors during development of rat cortex. Nature. 1994;368:144–7.
39. Katz JS, Burdick JA. Hydrogel mediated delivery of trophic factors for neural repair. Wiley Interdiscip Rev Nanomed Nanobiotechnol. 2009;1:128–39.
40. Placone AL, McGuigan PM, Bergles DE, Guerrero-Cazares H, Quiñones-Hinojosa A, Searson PC. Human astrocytes develop physiological morphology and remain quiescent in a novel 3D matrix. Biomaterials. 2015;42:134–43.
41. Hopkins AM, De Simone E, Chwalek K, Kaplan DL. 3D in vitro modeling of the central nervous system. Prog Neurobiol. 2015;125:1–25.
42. Phillips JB. Monitoring neuron and astrocyte interactions with a 3D cell culture system. Methods Mol Biol. 2014;1162:113–24.

ANEXO A – CARTA DE APROVAÇÃO DA COMISSÃO DE ÉTICA

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Recife, 02 de maio de 2013.

Ofício nº 562/13

Da Comissão de Ética no Uso de Animais (CEUA) da UFPE
Para: **Profª Belmira Lara da Silveira Andrade da Costa**
Universidade Federal de Pernambuco
Departamento de Fisiologia e Farmacologia
Processo nº 23076.000254/2013-72

Os membros da Comissão de Ética no Uso de Animais do Centro de Ciências Biológicas da Universidade Federal de Pernambuco (CEUA-UFPE) avaliaram seu projeto de pesquisa intitulado, **"Análise da Funcionalidade de Astrócitos e Neurônios Corticais Cultivados Sobre Membrana Bi e Tridimensionais de Biopolímero da Cana-de-açúcar: Uma Abordagem Neuroquímica e Eletrofisiológica".**

Concluímos que os procedimentos descritos para a utilização experimental dos animais encontram-se de acordo com as normas sugeridas pelo Colégio Brasileiro para Experimentação Animal e com as normas internacionais estabelecidas pelo National Institute of Health Guide for Care and Use of Laboratory Animals as quais são adotadas como critérios de avaliação e julgamento pela CEUA-UFPE.

Encontra-se de acordo com as normas vigentes no Brasil, especialmente a Lei 11.794 de 08 de outubro de 2008, que trata da questão do uso de animais para fins científicos e didáticos.

Diante do exposto, emitimos **parecer favorável** aos protocolos experimentais a serem realizados.

Origem dos animais: Biotério do Departamento de Nutrição da UFPE. Animais: ratos heterogênicos; Linhagem: Wistar; Idade: progenitores adultos e prole adulta; Peso: adultos:250-300g; sexo: machos e fêmeas; nº total de animais: 130 ratos.

Atenciosamente,

Profa. Maria Teresa Jansem
Presidente do CEEA
UFPE

CCB: Integrar para desenvolver