Bioprinting of spheroid-based cartilage constructs

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Summary

Bioprinting of spheroid-based cartilage constructs: from engineering to biological optimization aspects

The replacement of damaged, diseased or non-functional organs and tissues through tissue engineering is still a challenge. Within this context, recent advances in additive manufacturing have allowed the development of approaches such as the three-dimensional manufacture of biocompatible constructs associated with cells and bioactive molecules. Using the 3D bioprinting process it is potentially possible to produce tissue in a variety of formats, through reproducible and controllable techniques. Extrusion bioprinting processes with spheroid-loaded bioinks use uniform spherical cell aggregates as building blocks to manufacture tissue constructs. Tissue maturation is more advanced in this case if compared to single cells in suspension, because a much higher cell density is cultured in close contact for an extended period. Evaluating this approach to obtain mature cartilage tissue is of great interest, since chondrogenic differentiation occurs more intensively in aggregates than in single cell cultures. Furthermore, the cartilaginous tissue has low self-repair capacity and the lesions often progress to the development of various pathological conditions in an irreversible way. In this scenario, the present thesis aimed at obtaining cartilage constructs through 3D bioprinting, using spheroid-loaded bioinks. To this end, a reproducible method was developed to obtain uniform spheroids culturing mesenchymal stromal cells, based on the technique of non-adhesive micromolds produced by 3D printing. Subsequently, a natural hydrogel composed of xanthan gum (3.75 % w/v) and alginate (1.12 % w/v) was developed to allow efficient incorporation of the spheroids. The spheroids were mixed with the hydrogel, resulting in a non-cytotoxic and

sufficiently stable bioink. Then, a set of parameters was optimized to implement a high-performance extrusion bioprinting process to obtain a large number of bioprinted constructs. The bioprinting conditions considered optimum were: dry surface; 50 kPa of extrusion pressure; 60 mm/s of extrusion speed rate; a conic nozzle with 250 μm of internal diameter, a concentration of 2,500 spheroids/mL hydrogel (7.6×10^6 cells/mL) and ionic crosslinking with CaCl_2 solution (125 mM, 10 minutes). After bioprinting, the constructs were submitted to a post-printing chondrogenic differentiation process and a biological panel of molecules was analyzed. The biologic constructs showed abundant ECM production with enhanced collagen type II and type IV secretion and maintained collagen type X levels up to 28 days, thus demonstrating a hyaline-like chondrogenic differentiation behavior. Migration movements and fusion process of spheroids dispersed in the hydrogel were identified, and the constructs were viable and stable after a 56-day tissue maturation process. Finally, the cell spheroid production device showed to be feasible and cost-competitive to produce batches of 100, 1000, and 5000 spheroids in a cell culture laboratory, either for academic use or for small-scale companies. Overall, these results show the potential of an effective approach to obtain spheroid-loaded constructs to be applied in the biofabrication of cartilage tissue, as well as in the study of models of cartilage disease, such as osteoarthritis.

Resumo

Bioimpressão de construtos cartilaginosos baseados em esferoides: otimização dos aspectos de engenharia aos biológicos

A substituição de órgãos e tecidos danificados, doentes ou não funcionais por meio da engenharia tecidual é ainda um grande desafio. Dentro desse contexto, avanços recentes na manufatura aditiva têm permitido a fabricação tridimensional de construtos biocompatíveis associadas a células e moléculas bioativas. Com o processo de bioimpressão 3D é potencialmente possível produzir tecidos em uma variedade de formatos, com técnicas reprodutíveis e controláveis. Os processos de bioimpressão de extrusão com biotintas carregados com esferoides celulares usam agregados de células esféricas uniformes como blocos de construção para fabricar tecidos. A maturação do tecido é mais avançada neste caso em comparação com células isoladas em suspensão, porque uma concentração muito maior de células é cultivada em contato próximo por um período prolongado. Avaliar esta abordagem para obter tecido de cartilagem maduro é de grande interesse, uma vez que a diferenciação condrogênica ocorre mais intensamente em agregados do que na cultura de células individuais. Ademais, o tecido cartilaginoso apresenta baixa capacidade de auto reparação e as lesões progridem para o desenvolvimento de diversas condições patológicas de maneira irreversível. Neste cenário, a presente tese teve como objetivo geral a obtenção de construtos de cartilagem por meio da bioimpressão 3D, utilizando biotintas carregadas com esferoides. Para tal, desenvolveu-se um método reprodutível para a obtenção de esferoides uniformes a partir do cultivo de células estromais mesenquimais, baseado na técnica dos micromoldes não adesivos produzidos por impressão 3D. Em seguida, desenvolveu-se um hidrogel natural composto de goma xantana (3.75 % w/v) e alginato (1.12% w/v) para permitir a incorporação eficiente dos

esferoides. Os esferoides foram incorporados ao hidrogel, obtendo-se uma biotinta não citotóxica e suficientemente estável. Na sequência, foi otimizado um conjunto de parâmetros para implementar o processo de bioimpressão por extrusão de alto rendimento para a obtenção de um grande número de construtos bioimpressos. Os parâmetros de bioimpressão considerados ótimos foram: superfície seca; 50 kPa de pressão de extrusão; 60 mm/s de velocidade de extrusão; bico cônico de 250 µm de diâmetro interno; concentração de esferoides de 2,500 por mL de hidrogel (7.6×10^6 células/mL) e reticulação iônica com solução aquosa de CaCl₂ (125 mM, 10 minutos). Após a bioimpressão, os construtos foram submetidos a um processo de diferenciação condrogênica pós-bioimpressão e um painel biológico de moléculas foi analisado. Os construtos biológicos apresentaram produção abundante de ECM, com aumento de expressão de colágeno tipos II e IV, com nível mantido de expressão de colágeno X até 28 dias, demonstrando uma diferenciação condrogênica de caráter hialino. Movimentos de migração e processo de fusão de esferoides dispersos no hidrogel foram identificados, e os construtos se mantiveram viáveis e estáveis após um processo de maturação de 56 dias. Por fim, o dispositivo de produção de esferoides se mostrou viável e de custo competitivo para produzir bateladas de esferoides variando de 100, 1000 e 5000 unidades, em um laboratório de cultura celular para uso de caráter acadêmico ou companhias de pequeno porte. No geral, esses resultados mostram o potencial de uma abordagem eficaz para obter construtos baseados em esferoides para serem aplicados na biofabricação de tecido de cartilagem, bem como no estudo de modelos de doença da cartilagem, como a osteoartrite.

Samenvatting

Bioprinten van sferoïde-gebaseerde kraakbeen constructies: van techniek naar aspecten van biologische optimalisatie

Het vervangen van beschadigde, zieke, of disfunctionele organen en weefsels met behulp van regeneratieve geneeskunde is nog een grote uitdaging. In deze context hebben recente ontwikkelingen in ‘additive manufacturing’ geleid tot de evolutie van drie-dimensionale productie van biocompatible constructies met cellen en bioactieve moleculen. Door gebruik te maken van 3D bioprint processen is het mogelijk om weefsels van verschillende formaten te produceren met behulp van reproduceerbare en controleerbare technieken. Extrusie-bioprinten met sferoïden geladen bio-inkt maakt gebruik van uniforme sferische cel-aggregaten als bouwstenen om weefsel constructies te produceren. De uitrijping van weefsels is in dit geval beter ten opzichte van celsuspensies, omdat een hogere celdichtheid bereikt wordt gedurende een langere tijd. De evaluatie van deze benadering om matuur kraakbeenweefsel te verkrijgen is van groot belang aangezien kraakbeen differentiatie intensiever plaatsvindt in aggregaten dan in normale celweek. Daarnaast heeft kraakbeenweefsel een beperkte mogelijkheid om zichzelf te herstellen waardoor beschadigingen zich vaak tot een onomkeerbare pathologie ontwikkelen. Deze thesis heeft het doel om kraakbeen constructies te creëren door gebruik te maken van 3D bioprinten met behulp van bio-inkt met sferoïden. Hiervoor is een reproduceerbare methode ontwikkeld om uniforme sferoïden te kweken van mesenchymale stromale cellen, gebaseerd op de techniek van 3D geprinte niet-adhesieve micromallen. Daaropvolgend is een natuurlijke hydrogel bestaande uit xanthaan gom (3,75%) en alginaat (1,12%) ontwikkeld om efficiënte inbedding van sferoïden te faciliteren. De sferoïden werden gemengd met de hydrogel, welke

leidde tot een niet-cytotoxische en voldoende stabiele bio-inkt. Daarna werden verschillende parameters geoptimaliseerd om hoogwaardige extrusie bioprinting toe te passen om zo een groot aantal geprinte constructies te verkrijgen. De gevonden optimale conditions waren: droog oppervlak, 50 kPa extrusiedruk, 60 mm/s extrusie snelheid, een conisch mondstuk met 250 μm interne diameter, een concentratie van 2.500 sferoïden/mL hydrogel ($7,6 \times 10^6$ cellen/mL) en ionische crosslinking met CaCl_2 oplossing (125 mM, 10 minuten). Na bioprinten werden de constructies aan het chondrogene differentiatieproces blootgesteld en werd een biologisch paneel van moleculen geanalyseerd. De biologische constructies toonden een overvloed aan extracellulaire matrix met een verhoogde secretie van collageen type II en IV en toonden daarnaast een behoud van collageen type X tot 28 dagen wat een hyaline-achtige chondrogene differentiatie aantoont. Migratie en fusie van sferoïden verspreidt over de hydrogel werd aangetoond en de constructies waren stabiel en levensvatbaar na 56 dagen weefsel maturatie. Als laatste werd aangetoond dat de sferoïden op een kosten-effectieve manier geproduceerd konden worden in oplages van 100, 1000 en 5000 sferoïden in een celkweeklab voor academische maar ook kleine commerciële doeleinden. Over het algemeen tonen de resultaten de mogelijkheid voor een effectieve benadering om constructies met sferoïden toe te passen in biofabricage van kraakbeenweefsel, maar ook onderzoek met kraakbeen ziektemodellen zoals osteoarthritis.