Injectable ADEP hyaluronic acid-chitosan hydrogels based on Schiff Base enhance angiogenesis, cell proliferation and promote skin regeneration

Junzi Jiang, [1,2] Chang Q[4] ,Gao H[1,2,3], Wen Zhong[3], Feng Lu[4], Malcolm Xing[1,2+]
[1]Mechanical Engineering, University of Manitoba. Winnipeg, MB R3T 2N2, Canada.
[2]Manitoba Institute of Child Health.
[3] Textile Sciences, Faculty of Human Ecology, University of Manitoba,
[4] Plastic Surgery, Nanfang Hospital, Southern Medical University, Guangzhou, China

Introduction Burn injuries and chronic skin ulcer, a major public health issue [1] that can lead to a severe physiological stress and life threatening. Regenerating skin in third-degree burn and chronic wound ulcer are still un- circumvented clinical challenges. Hyaluronic acid (HA) is a widely used in tissue engineering as a biocompatible, non-sulfated, glycosaminogenic and non-inflammatory biomaterial. There are several methods to modify HA for its application in injectable hydrogels. One approach is to oxidize HA to obtain aldehyde groups which can react with amino groups from other polymers to form a hydrogel. This oxidization process, however, involves the uses of HIO_4 and H_2O_2 to break the HA, causing a loss of the integral structure of HA and further degradation problems. Another approach is to endow HA with alkene bonds which can be used in polymerization to obtain various injectable hydrogels including those with thermal sensitivity. Initiators and chemical cross-linkers introduced in this approach, however, cause un-avoidable toxicity to host tissues. Therefore, we developed an injectable bioactive hydrogel using aldehyded aminodiethoxy-propane (ADEP)-HA (AHA) and chitosan. We hypothesize that AHA-CS hydrogel alone, that is, without any additional growth factors, cytokines, or cells, can enhance angiogenic response, cell proliferation, keratinocyte migration, and promote wound healing.

Materials and Methods AHA-CS hydrogel is formed by HA, carbodiimide and ADEP. 24 C57BL/6J mice (6-8 weeks, were used under an approved animal protocol in Southern Medical University**.** A square template with the size of 1cm×1cm was marked on the dorsal skin. For a complete thickness skin defect model, the skin including its panniculus carnosus was excised. The wounds were covered with the Combi DERM dressings (Conva Tec Inc., Skillman, NJ).

RNA was extracted from frozen tissue samples using RNeasy Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's protocol. Real-time reverse-transcriptase polymerase chain reaction was conducted with primers designed for this study in an ABI Prism 7300 system (Applied Biosystems, Foster City, CA) using DyNAmo SYBR Green qPCR Kit (Finnzymes, Espoo, Finland). The amount of each RNA sample was normalized using mouse glyceraldehyde-3-phosphate de-hydrogenase (GAPDH) as an internal control. Primers used were as follows:

MMP3-F: 5′ GACGATGATGAACGATGGA, MMP3-R: 5′ CCATAGAGGGACTGAATACCA
MMP9-F: 5′TCCAGTACCAAGACAAAGC, MMP9-R: 5′GAGCCCTAGTTCAAGGGCAC
SDF1-F: 5′ GCCAGTCCCTCTGTTACAA, SDF1-R: 5′CTGCACTTCCTTGCTAAAGTC
VEGF-F: 5′TGCCGGTTCCAACCAGAA, VEGF-R: 5′GTGGAGGAGCGAGCTGAA
GAPDH-F: 5′ GGCCTCCAAGGAGTAAGAAA, GAPDH-R: 5′ GCCCCTCCTGTTATTATGG

Ct values (cycle threshold) were used to calculate the amount of amplified PCR product in comparison to the housekeeping gene GAPDH. The relative amount of mRNA was calculated as $2^{-\triangle Ct}$.

Statistical significance was tested by using a one way analysis of variance (ANOVA) with 95 % confidence interval. When $P<0.05$, differences were considered to be statistically significant.

Result This hydrogel can enhance wound healing by promoting angiogenesis，cell migration and proliferation. During sol-gel transition, no chemical crosslinking agent was introduced and the structure of polysaccharide HA was not broken as previous reported (Fig. 1). Rheological tests showed the injectability and stability of this AHA-CS hydrogel. The AHA-CA hydrogel significantly accelerated wound closure (Fig. 2), increased cell proliferation (Ki67 marker, Fig. 3) and promoted keratinocyte migration (p63 marker, Fig. 4). Histological examination also demonstrated that the gel significantly advanced granulation tissue and capillary formation in the gel-treated wounds (Fig. 5). mRNA expressions of angiogenesis (VEGF-A), chemotactic factors (SDF-1) and ECM-remodeling MMPs (MMP3 and MMP9) were up-regulated in the hydrogel-treated wounds. Interestingly, neo-formed hair follicles were also found in the gel-treated wound.

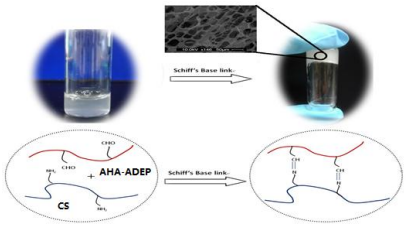

Fig.1 gelation between AHA and chitosan (CS) via Schiff base linkage, insert: photo of the AHA-CS hydrogel.

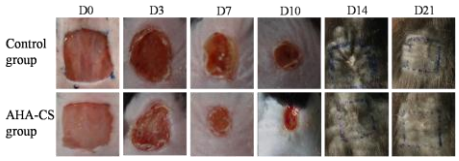

Fig.2 Skin wound contraction Images of representative wound contour of each group on the days 0, 3, 7, 10, 14, and 21.

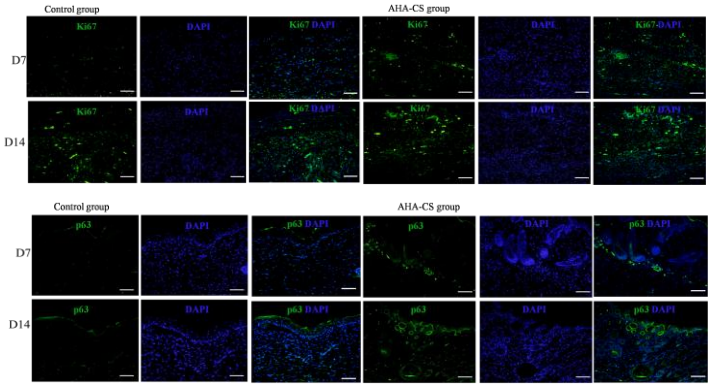

Fig.3 Cell proliferation evaluation using Ki67. Wound healing tissues stained with Ki-67 (green) and nuclear counterstained with DAPI (blue). Scale bar =100 μm

Fig.4 p63 marked keratinocyte migration in wound bed. p63 immunohistochemical staining where p63 positive cells (green) ,and the nuclear was counterstained with DAPI(blue). Scale bar =100 μm

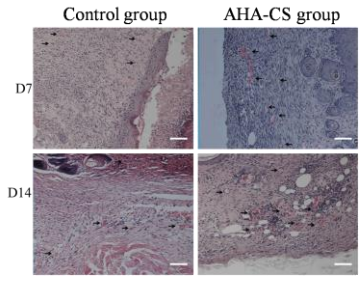

Fig. 5 AHA-CS hydrogel increasing vascularity. (A) Capillary density was observed in H&E stained histological sections of two groups on day 7, 14 Scale bar =100 μm

Reference

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