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Cross-Linked Hyaluronic acid can Prevent the Capsular Contracture Formation

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Abstract

Introduction: Breast reconstruction using silicone implants is a widespread choice, however, breast capsular contracture (BCC), a response of the immune system to foreign materials, is the most affliction and frustration fibrosis complications after silicone implantation. Chronic inflammation-associated fibrosis is regarded as a main cause of BCC. Hyaluronic acid (HA) is an effective anti-adhesive and anti-fibrotic agent. Furthermore, 1,4-butanediol diglycidyl ether-cross-linked hyaluronan hydrogel (cHA) is a non-resorbable cross-linked hyaluronan-derived polymer with no cytotoxicity and high stability. This study aims to evaluate the potential of cHA on preventing BCC in breast surgery using silicone implants.

Methods: The effect of cHA on BCC will be examined in breast BCC rat model.

Results: Our study showed that cHA decreased fibrosis-regulated factors transforming growth factor beta 1 (TGF-β1) and tissue inhibitor of metalloproteinase (TIMP) expressions and increased metalloproteinase (MMP) MMP-9 expression in capsule tissue. cHA significantly decreased inflammatory mediators tumor necrosis factor-alpha (TNF-α), interleukin IL-6, nitric oxide (NO), as well as inducible nitric oxide synthase (iNOS) expressions. In addition, cHA significantly decreased transcription factor nuclear factor kappa B (NF-κB) activation in silicone surrounded tissue.

Conclusions: cHA showed prevention in BCC formation through inhibiting NF-κB -associated inflammation in rats with silicone implantation.

Keywords: Cross-linked hyaluronan hydrogel; Breast augmentation; Capsular contracture; Fibrosis; Nuclear factor kappa B

Introduction

Breast augmentation Silicone implants have been used extensively for breast reconstruction following mastectomy since 1964. However, silicone implants combined with autologous proteins trigger a specific local immune response which leads to fibrosis promoted by the production of profibrotic cytokines. The chronic inflammation and fibrosis leads to development of breast capsular contracture (BCC) [1]. BBC causes breast firmness, uncomfortableness, pain, a change in the appearance of the breast, and palpability of the silicone implants. In clinical settings, the incidence of symptomatic BCC ranges from 5 to 20% [2,3] and additional surgeries may be required to clean the scar tissue which may involve removal or replacement of the implants [4]. Currently, there is no effective treatment to avoid BCC.

Chronic inflammation and fibrosis participate in the development of capsular contracture. BCC is result Hyaluronic acid (HA) is an effective anti-adhesive and anti-fibrotic agent used to inhibit peripheral nerve adhesion, scar formation, and post-laminectomy fibrosis formation [5-9]. To improve the mechanical properties and lengthen the residence time, the polymer chains of HA are cross-linked with linker molecules, such as divinyl sulfone, homobifunctional glycidyl ethers, glutaraldehyde, and formaldehyde 1,4-butanediol diglycidyl ether-cross-linked hyaluronan hydrogel (cHA) is a non-resorbable cross-linked hyaluronan-derived polymer with no cytotoxicity and adverse changes in electrophysiology and neuro behavior [10]. Therefore, topical treatment of cHA on implant material may be effective in preventing BCC; however, the effect of cHA on BCC-

associated fibrosis and inflammation has never been investigated. The aim of the present study is to evaluate the potential of cHA on BCC prevention after silicone implantation in rats.

Materials and Methods

Animals

Female SPF Wistar rats weighing 200-300 g were obtained from our institution's Laboratory Animal Center. They were individually housed in a room with a 12-hour dark/light cycle and central air conditioning (25°C, 70% humidity), allowed free access to tap water, and fed a rodent diet from Richmond Standard, PMI Feeds, Inc (St Louis, MO), with or without a sesame oil supplement. The animal care and experimental protocols were in accordance with nationally approved guidelines. (IRB No: B-ER-102-262)

Inducing capsule contracture in rats

The rats were anesthesia with Zoletil (1 ml. Zoletil with concentration of 10 mg/ml combined with Rompun 0.1 ml. for intraperitoneal injection, the dosage: 0.05-0.06 ml/10 gm) to sedation for the surgery. Before surgery, the dorsum of 20 anesthetized rats (n=10 for each group) were shaved and cleansed with povidone iodine, and all surgical procedures were carried out under sterile conditions. Two 1-cm skin incisions were made on the back of each animal at a distance sufficient to avoid merging of the pockets. Pockets were created by blunt dissection beneath the panniculus carouses plane with dimensions barely sufficient to accommodate the implant. One silicone implant (The MENTOR® ARTOURA™ Breast Tissue Expander, cut the expander into size: 1 × 2 cm^2) covered with HA with 100 μl (HA group, 0.5% BDDE-cross-linked HA Maxigen Biotech Inc.) was placed in left pocket while another one without HA was placed in right pocket (C group). Incisions were then closed with stitches making sure the suture line was not directly over the implant. The rats were kept and observed the behavior to evaluate the wound condition; if the animals showed the weight loss over than 10%, reduced mobility, poor appetite, weakness and self-mutilation, the animal were intraperitoneal injected with the Butorphanol 0.1-0.5 mg/100 g. At the sacrificed time point, the rats were anesthetized with Barbiturate (100 mg/kg) intraperitoneal injection to over dose sedation. The silicone implants were removed together with their capsules 4 months after implantation.

Immunohistochemistry staining (IHC)

The collected samples were fixed with 4% formaldehyde. It should be the samples were dehydrated and embedded in the paraffin for the slice into the slide to examine the protein expression with the immunohistochemistry staining (Figure 1). The slides sections were immersed within xylene, and rehydrated in alcohol gradient and then rinsed in phosphate buffer for primary antibody detection. The samples were incubated with Anti-TGF-β1 primary antibody for 12 hours at 4°C after being blocked with 3% hydrogen peroxide, avidin and biotin for 15 minutes each. The slides were then incubated with a biotinylated secondary antibody for 1 hour, following by a 15-minutes-incubation with streptavidin peroxidase, 1-minute-hematoxylin-stain. And then, the slides were dehydrated by graded ethanol, dehydrated by xylene, and mounted with rhamsan gum. The results were observed under optical microscope and photographed.

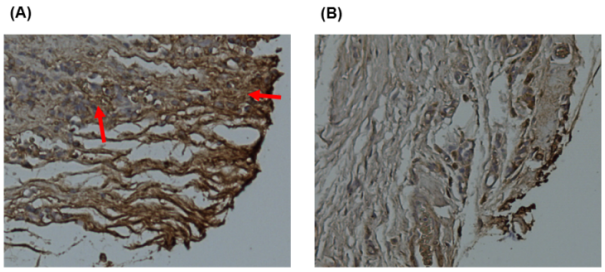

Figure 1: Immunohistochemistry study of TGF-β1 expression in capsule tissue. Immunohistochemistry staining for TGF-β1 protein expression (brown color; arrows) in capsule tissue from non-HA group (C group) (A) and cHA-treated groups (HA group) (B) (X100).

Western blotting

Nuclear extraction kit (Sigma, Inc., St. Louis, MO) was used to separate nuclear and cytosolic protein. Fifty micrograms of protein was loaded on SDS–PAGE, and then transferred to nitrocellulose sheets (NEN Life Science Products, Inc., Boston, MA). After blocking, the blots were incubated with TGF-β1, matrix metalloproteinases (MMP)-2, MMP-9, inducible nitric oxide synthase (iNOS), nuclear factor-kappa B NF-κB, or β-actin antibody (dilution 1:1000) in 5% non-fat skim milk (using β-actin as a loading control). After washing, the blots were incubated with secondary antibodies conjugated with alkaline phosphatase (dilution 1:3000) (Jackson ImmunoResearch Laboratories, Inc., Philadelphia, PA). Immunoblots were developed using bromochloroindolyl phosphate/nitroblue tetrazolium solution (Kirkegaard and Perry Laboratories, Inc., Baltimore, MD).

Measuring tumor necrosis factor TNF-α and interleukin (IL)-6 in tissue

Cytokines including TNF-α and IL-6 levels were quantitatively measured by using ELISA kits (Duo-Set; R&D Systems Inc., Minneapolis, MN) followed the manufactory protocol. The protein concentration in tissue homogenate was determined by using protein assay dye (Bio-Rad Laboratories, Hercules, CA, USA).

Assessing Nitric oxide (NO) concentration in tissue

Briefly, the amounts of nitrite in serum were measured following the Griess reaction by incubating 100 ml of sample with 100 ml of Griess solution at room temperature for 20 min. The absorbance was measured at 550 nm by a spectrophotometer. Nitrite concentration was calculated by comparison with a standard solution of known sodium nitrite concentrations.

Statistical analysis

Data were expressed as the means ± standard deviation (SD). The data were used Wilcoxon signed rank test and paired student t test to make pairwise comparisons between 2 groups. Statistical significance was set at $p<0.05$. The number for each group was 10 animals.

Results

The effect of cHA on fibrosis

To investigate the effect of HA on fibrosis regulation of capsule formation during silicone implantation, TGF-β1, MMP-9, and TIMP expressions were detected. In immunohistological results, TGF-β1 protein (brown color; arrows) expressed more in non-HA group (C group) than HA-treated groups (HA group). Western blotting results show that capsular active TGF-β1 expressions were 5.3 ± 0.03 relative units in C group and 1.10 ± 0.04 relative units in HA group (p value<0.0001). Non-activated TGF-β1 expressions were 4.29 ± 0.54 relative units in C group and 0.98 ± 0.13 relative units in HA group (p value<0.0001). Capsular expressions of MMP-9 were 0.47 ± 0.19 relative units in C group and 1.43 ± 0.36 relative units in HA group (p value<0.0001). Capsular expressions of TIMP were 6.09 ± 2.81 relative units in C group and 0.82 ± 0.13 relative units in HA group (p value=0.0023). These differences also reached statistical significances (Figure 2).

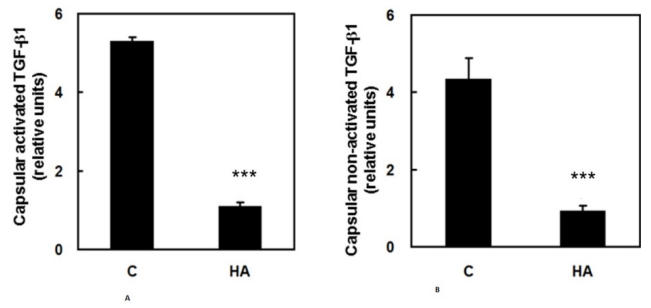

Figure 2: Effects of cHA on activated and non-activated TGF-β1 expressions in capsule tissue. Western blot was used to assess the changes of activated (A) and non-activated (B) TGF-β1 expressions in the capsule tissue from non-HA group (C group) and cHA-treated groups (HA group). Data were means ± standard deviation (SD) (n = 10). ***P<0.001 compared with C group.

The effect of cHA on inflammation

To investigate the role of inflammatory control on HA-associated protection, inflammatory mediators TNF-α, IL-6, NO, as well as iNOS expressions were detected in capsule tissue. ELISA results show that capsular TNF-α levels were 387 ± 57 pg/mg protein in C group and 289 ± 29 pg/mg protein in HA group (p value=0.018). Capsular IL-6 levels were 2040 ± 52 pg/mg protein in C group and 1654 ± 169 pg/mg protein in HA group (p value<0.0001). Griess assay results show that capsular nitrite levels were 21.3 ± 4.1 mmol/mg protein in C group and 10.2 ± 5.9 mmol/mg protein in HA group (p value= 0.0002). Western blotting results show that capsular iNOS expressions were 5.5 ± 4.2 relative units in C group and 1.5 ± 0.8 relative units in HA group(p value= 0.0096). These differences also reached statistical significances.

The effect of cHA on NF-κB activation

To investigate the role of NF-κB control on HA-associated anti-inflammation, the NF-κB activation was detected in capsule tissue. Western blotting results show that capsular nuclear NF-κB expressions were 0.94 ± 0.03 relative units in C group and 0.76 ± 0.13 relative units in HA group (p value=0.0337). The difference reached statistical significances.

Discussion

In this study, we have demonstrated the protective effect of cHA on BCC. cHA decreased fibrosis-regulated factors TGF-β1 and TIMP expressions and increased MMP-9 expression in capsule tissue. cHA also significantly decreased inflammatory mediators TNF-α, IL-6, NO, as well as iNOS expressions. In addition, cHA significantly decreased transcription factor NF-κB activation in silicone surrounded tissue. It is suggested that cHA may prevent BCC formation through inhibiting NF-κB-associated inflammation in rsats with silicone implant insertion.

Injectable fillers, such as autologous fat graft or HA, might offer women the advantage of deciding their desired size in breast enhancement. The infection rate of 0.08% in the large scale study was lower than prosthesis implants [11]. However, due to the concern of degradation of the volume, prosthesis implantation is still the main procedure of breast augmentation. Immune response of foreign objects to the human body might cause capsular contracture and distort the aesthetics of the breast. The cause of capsular contracture might be bacterial infection, implant shell rupture, and hematoma, but nevertheless interaction between implant and the surrounding injured tissue. Implant surface modification of biomaterials to reduce the capsular thickness is proved, which may be due to down regulation of macrophage adhesion and activation [12]. However, possible mechanism is still unknown.

Inhibiting fibrosis is crucial in preventing BCC after silicone implantation. Severe fibrotic reaction to foreign body such as silicone implant is an important cause of capsular contracture [13]. In addition, the stage of capsular fibrosis after breast augmentation is one of the main indicators of the severity of BCC [14]. TGF-β is a multifunctional cytokine that play a central role in tissue repair and fibrosis, which stimulates the production of various extracellular matrix proteins and inhibits the degradation of these matrix proteins [15]. TGF-β1 has been regarded as the dominant factor causing capsular contracture [16]. The balance between MMP and TIMP is also important in regulation of fibrosis [17] MMP plays an anti-fibrotic role through cleavage of the ECM [18]. TIMP is an inhibitor of metallo proteinases. TGF-β may also affect the balance through inhibiting MMP-9 expression [19] and increasing TIMP-1 expression [20]. The decrease in MMP-to-TIMP expression in capsular tissue of patients with silicone gel implants was observed with higher rates of capsular contracture [21]. In the present study, cHA decreased TGF-β and TIMP expressions and increased MMP-9 expression in capsule tissue. It is suggested that cHA may prevent BCC through inhibiting fibrosis in silicone surrounded tissue.

Inhibiting inflammation may be involved in the anti-fibrotic effect of cHA after silicone implantation. Inflammation is commonly involved in the pathogenesis of fibrosis in many diseases. During inflammation, fibroblasts and myofibroblasts activation, and macrophage infiltration has been implicated in the production of ECM and inflammation-induced organ fibrosis [22,23]. TGF-β produced or

released by infiltrating cells during inflammation plays an important role in activation of fibroblast [15]. Pro-inflammatory mediators including TNF-α, IL-6, and NO are important indicator of inflammation. In addition, these pro-inflammatory mediators are important in the activation of inflammation-related cells and in the pathogenesis of fibrosis [24,25]. In the present study, cHA treatment decreased TNF-α, IL-6, NO, as well as iNOS expressions in silicone surrounded tissue. It is likely that cHA may inhibit fibrosis via inhibiting pro-inflammatory mediators' production in silicone surrounded tissue.

NF-κB may play an important role in pro-inflammatory mediator's production in BCC. NF-κB is the dominant transcription factor on producing pro-inflammatory mediators [26] Inhibition of NF-κB activation decreased TNF-α, IL-6, and iNOS expressions during inflammatory stimulus [27-29]. In the present study, cHA significantly decreased nuclear NF-κB expression in silicone surrounded tissue. Therefore, cHA may decrease TNF-α, IL-6, and NO production by inhibiting NF-κB pathway activation. We concluded that cHA is able to prevent BCC fibrosis through inhibiting transcription factor NF-κB activation in rats. cHA has the potential in preventing BCC from patients with silicon implanted surgery. However, more investigation will be needed to confirm this.

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Conflict of Interest

None

Financial source

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