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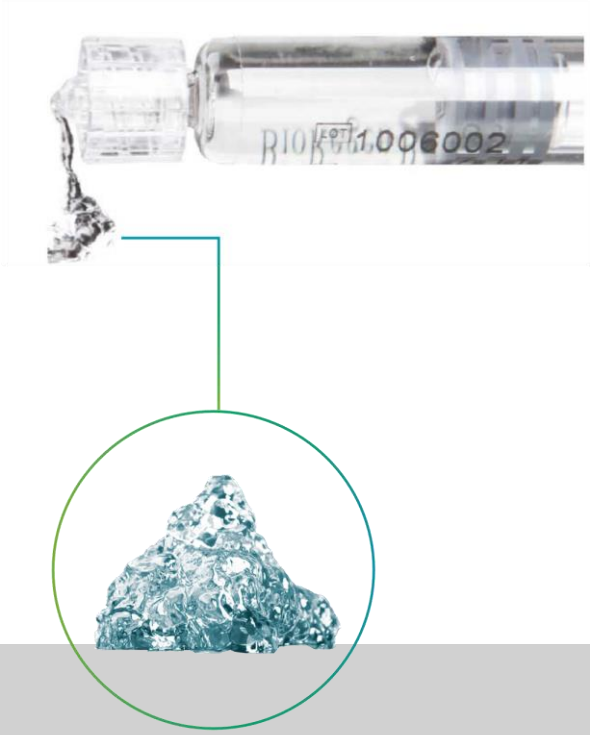

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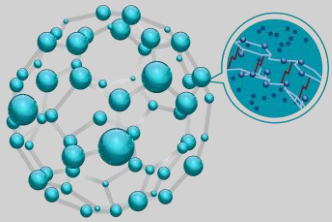

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The Future of Adhesion Prophylaxis Trials in Abdominal Surgery: An Expert Global Consensus †

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Abstract: Postoperative adhesions represent a frequent complication of abdominal surgery. Adhesions can result from infection, ischemia, and foreign body reaction, but commonly develop after any surgical procedure. The morbidity caused by adhesions affects quality of life and, therefore, it is paramount to continue to raise awareness and scientific recognition of the burden of adhesions in healthcare and clinical research. This 2021 Global Expert Consensus Group worked together to produce consented statements to guide future clinical research trials and advise regulatory authorities. It is critical to harmonize the expectations of research, to both develop and bring to market improved anti-adhesion therapies, with the ultimate, shared goal of improved patient outcomes.

Keywords: adhesion; antiadhesion agent; minimally invasive surgery; consensus

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1. Introduction

Postoperative adhesions represent a frequent complication of abdominal surgery. Adhesions can result from infection, ischemia, and foreign body reaction, but commonly develop after any surgical procedure. Indeed, abdominal and pelvic surgeries are the most common cause of peritoneal adhesions and remain a source of considerable morbidity. Among these patients, 66–79% develop adhesions following abdominal and pelvic surgeries [1,2]. The most common complications of postoperative adhesions are difficulty at reoperations, small bowel obstruction, pelvic pain, and female infertility [3,4] The economic consequences of morbidity caused by adhesions are well-documented: longer hospitalization or rehospitalization and patients' reduced quality of life. The data from the 2020-SCAR-update study demonstrate that 1 in 4 patients in whom abdominal or pelvic open surgery was performed were readmitted to hospital within 5 years of the initial procedure for adhesion-related causes or subsequent surgery were complicated by adhesions [3]. The data suggest that laparoscopic procedures decrease readmission to the hospital by 30%, but the morbidity and associated factors remain substantial. As a result of pre-existing adhesions, following surgeries can be more time consuming and challenging, posing increased risk to the patient [5]. Up to 60% of surgeries performed today are repeat surgeries and up to 20% of patients undergoing operative adhesiolysis suffer an inadvertent enterotomy [6]. The morbidity caused by adhesions affects quality of life and, therefore, it is paramount to continue to raise awareness and scientific recognition of the burden of adhesions in healthcare and clinical research [3,4].

The objective of this paper is to set the stage for the next frontier of adhesion prevention, moving beyond barriers and into pharmaceuticals that begin to address the key cellular targets implicated in adhesion prevention. This paradigm shift requires rethinking on how trials are conducted; regulatory agencies', particularly the drug division; perspectives and expectations as to incorporating clinical trial endpoints that take into account the patients' voice and perspective (as per the Patient Focused Drug Development initiative launched by the U.S. Food and Drug Administration (FDA)); and how clinical researchers may look at advancing the field in assessing how reduction or complete prevention translates to clinically relevant outcomes. This consensus document, as an expert opinion paper, offers recommendations on how to conduct clinical drug trial research and defines the components of a strong clinical study design, including relevant primary and secondary endpoints that can be measured in the population within a reasonable period.

2. History of Adhesion Treatments

Currently, several medical products are commercially available for reducing postoperative adhesions [7]. Some of the mechanisms aim to prevent fibrin deposition in the peritoneal exudate, reduce local tissue inflammation or remove fibrin deposits [8]. Most of the existing methods inhibit only one mechanism, however, and have produced limited scientific evidence (Table 1). Physical barriers used to prevent adhesion formation are utilized most frequently. Physical barriers do not interact with the process of adhesion formation. Instead, barriers act as a spacer separating wound surfaces during the initial phase of wound regeneration, thus reducing the risk of adhesion formation in the process. The barrier products INTERCEED (Ethicon, Somerville, NJ, USA), SEPRAFILM (Baxter, Deerfield, IL, USA), and ADEPT (Baxter, Dearfield, IL, USA) are currently approved by the FDA to prevent postoperative adhesion formation. INTERCEED and SEPRAFILM are approved for use during laparotomy and have demonstrated an approximately 32–55% efficacy rate in pre- and post-market clinical trials [8–11]. ADEPT (Baxter, Dearfield, IL, USA), an adhesion barrier solution composed of 4% icodextrin, is a colloidal osmotic agent commonly used in the form of aqueous solution. ADEPT is FDA-approved for use in gynecological laparoscopic procedures; it temporarily separates peritoneal surfaces by hydroflotation, maintaining a fluid reservoir within the peritoneal cavity for 3 to 4 days [12]. Icodextrin has a safety profile similar to that of Ringers Lactate Solution and was previously used as a vehicle for peritoneal dialysis at a 7% concentration [13,14]. Data support the

efficacy of ADEPT in preventing postoperative adhesions, and surgeons have reported that ADEPT is easy to administer and well-tolerated [15]. Other barrier products approved outside of the United States include OXIPLEX (Nordic, Tonsberg, Norway), HYALOBARRIER (Nordic Pharma, Paris, France), and HYAREGEN (Bioregen, Changzhou, China), barrier gels that aim to reduce postoperative peritoneal adhesions by separating the tissues traumatized by surgery from the healthy peritoneum. 4DryField PH (PlantTecMedical, Luneburg, Germany) is a starch-based polysaccharide powder that, when mixed with saline, aims to reduce adhesions according to the same barrier concept as the aforementioned gels [16]. Clinical trial data in anti-adhesion products have been unable to yield strong support for widespread use and, therefore, there is currently no widely accepted standard of care [4].

Table 1. Adhesion-preventing products, mechanisms of action and drug/device stage of development.

Product Category	Product	Mechanism or Strategies	Stage of Development
Medical Device-Mechanical Barriers (Fabric, film, gels, polymers, liquids)	Seprafilm®(Baxter, Deerfield, IL, USA), Interceed®(Ethicon, Somerville, NJ, USA), Adept®(Ethicon, Somerville, NJ, USA), SprayShield®8Coviden; Dublin, Irland), Hyalobarrier®(Nordic Pharma, Paris, France), Repel-CV®((SyntheMed Inc, Iselin, NJ, USA), Adcon®, (Leader Biomedical, Amsterdam, The Netherlands), Coseal®(Baxter Healthcare Inc, Deerfield, IL, USA), etc.	Physical separation of tissues Site specific	FDA, CE mark, approved.
Anti-adhesive Agents	Ibuprofen, celecoxib, resveratrol or pirfenidone, myomycin C, heparin.	ECM Fibrinolytic Inflammation Cell proliferation Anticoagulant	Serious side effects, delivery problems and/or moderate to low efficacy.
Gene Therapy	tPA gene siRNA HGF gene	Promote fibrinolysis HIF1a PAI-1 Mesothelial regeneration	Serious side effects, low efficacy, expensive.

3. From Anti-Adhesion Barriers to Drugs

Morbidity associated with postoperative abdominal and pelvic adhesions is well reported by patients and research. However, only insufficient progress has been made in prevention and treatment (Figure 1).

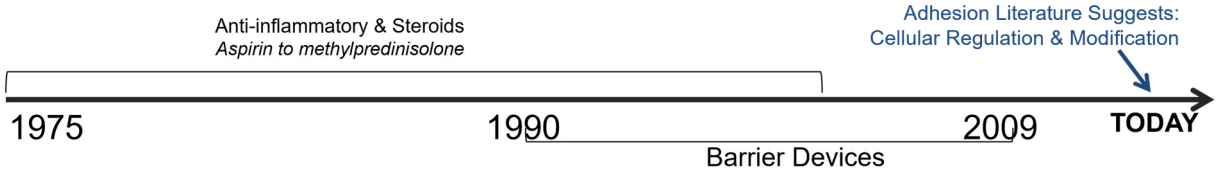

Figure 1. History of adhesion preventing drugs and device development.

Important advances in understanding of normal peritoneal healing and the pathophysiology of adhesion formation (Figure 2) have raised the prospect of targeting molecular pathways and key fibrotic mediators involved in adhesiogenesis to further reduce postsurgical adhesions [4,17–22].

As discussed, the current barrier therapies aim to isolate the surgical tissue, in an effort to promote proper wound healing in the initial postoperative phase, yet do not address the underlying mechanism of adhesiogenesis. In 2020, a Cochrane review did not support the routine clinical use of the currently available, FDA-approved products, citing insufficient evidence for clinically relevant outcomes [4]. The limitations in the evidence for barriers, in combination with the limited use in surgical practice, provide the opportunity to develop

other solutions. Of interest are new therapies able to affect the underlying pathophysiology of adhesion formation.

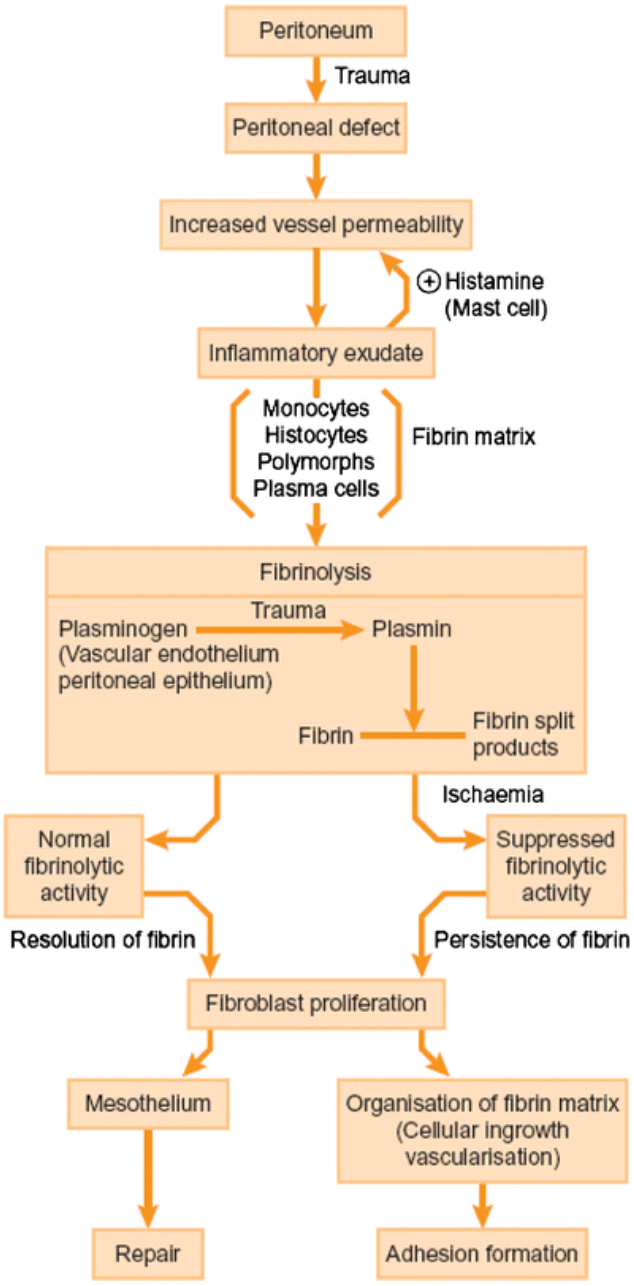

Figure 2. Pathogenesis of adhesion formation. Reproduced with permission from: De Wilde, R.L., Trew, G. & on behalf of the Expert Adhesions Working Party of the European Society of Gynaecological Endoscopy (ESGE) [5].

4. Guidance for Clinical Research Design in Anti-Adhesion Research

Despite the realization that scar tissue reduces patients' quality of life and can lead to complications, there is no standard of care for the prevention and treatment of post-operative adhesions in the abdomen, pelvis or other surgical sites. Targeting novel molecular mechanisms not only provides opportunities to develop new therapies, but will certainly result in a shift in the FDA regulatory expectations of anti-adhesion drug research. It is therefore important to reach a consensus on how to prevent and treat adhesions, how to design and conduct clinical research trials, and ultimately reduce adhesion-related morbidity, improving patient's quality of life. This consensus document, as an expert opinion paper,

offers recommendations on how to conduct clinical drug trial research and defines the components of a strong clinical study design, including relevant primary and secondary endpoints that can be measured in the population within a reasonable period. The present global consensus document is critical in lighting two key 2020 reviews published in the *Lancet* and Cochrane Library, which cite the rising burden of adhesions, the lack of standard of care and the paucity of scientifically proven effective therapies, in order to advance innovation for the primary benefit of patients [3,4].

5. Assessment and Diagnosis of Adhesions

Clinical trial research with a strong design to yield high-quality evidence requires standardized assessment and diagnostic tools to determine efficacy of an investigational drug or adhesion prophylaxis agent. Traditionally, in clinical practice, adhesions have been diagnosed based primarily on symptoms rather than on diagnostic evidence of adhesions. Adhesions need to be visualized for assessing, scoring, and diagnosing them. A medically necessary, scheduled second-look laparoscopy (SLL) provides the ethical basis for visualizing the extent or absence of adhesions; therefore, SLL is currently the gold standard for the assessment and diagnosis of adhesions.

However, SLL is usually indicated after a limited number of surgical procedures for which the possibility of adhesion formation is high and adhesiolysis would be necessary to prevent such adhesion-related complications or morbidities. Indeed, the indication for SLL is widely debated in both the clinical and research realms. In clinical trial research, adhesions must be visualized in order to assess them and to evaluate adhesion prophylactic agents, such as investigational drugs. Performing an SLL for research purposes alone carries ethical uncertainties. Indeed, the number of surgical procedures that medically warrant an SLL is limited and therefore poses a challenge to clinical trial researchers. How should research into known adhesiogenic complexes for which SLL is not traditionally indicated be conducted? Non-invasive diagnostic approaches for abdominal and pelvic adhesions may provide the opportunity to investigate a wider group of procedures and patients concerning the effectiveness of adhesion prevention and reduction therapies.

CineMRI is a sequencing of MRI images that captures movement within the area of the body under evaluation (Figure 3). Emerging research has demonstrated that CineMRI can be used to detect and visualize adhesions [23]. This method of adhesion evaluation is non-invasive and, therefore, removes the restrictions currently in place for surgical models of SLL and allows for standardized follow-up after initial surgery. CineMRI has already been implemented in clinical practice to diagnose and map adhesions in patients with chronic pain, showing good results in reducing the risk of both negative laparoscopies and inadvertent enterotomies [23]. A limitation of this technique, however, is the limited number of radiologists with experience in this area. Integration of artificial intelligence (AI) into computer-aided detection systems is expected to improve usage and accuracy for wide-scale applications. The principles of visceral slide that are used to detect adhesions on the CineMRI, are also applicable to other dynamic imaging methods, such as transvaginal or transrectal ultrasound (US). Echography in combination with cineMRI could help to accurately diagnose adhesions in regions that eventually are difficult to assess with cineMRI alone.

The American Fertility Society classification (AFSC) of adnexal adhesions is a validated tool used in both research and clinical practice at the time of surgery [24]. The AFSC evaluates the extent and aspect of adhesions at four anatomical sites, right and left ovaries and tubes, requiring the examiner to distinguish between filmy and dense adhesions. This AFSC tool could be applied in clinical trial research for adhesion-reducing agents at the time of initial surgery to obtain baseline measurement and again at the time of SLL. Due to the focus of this tool on the morphology and extent of adhesions and the subjectivity of the examiner, the AFSC has limitations in the prognosis of adhesion-related morbidity. Aside from the above, the AFSC is the most widely used and accepted adhesion scoring system in both research and clinical settings.

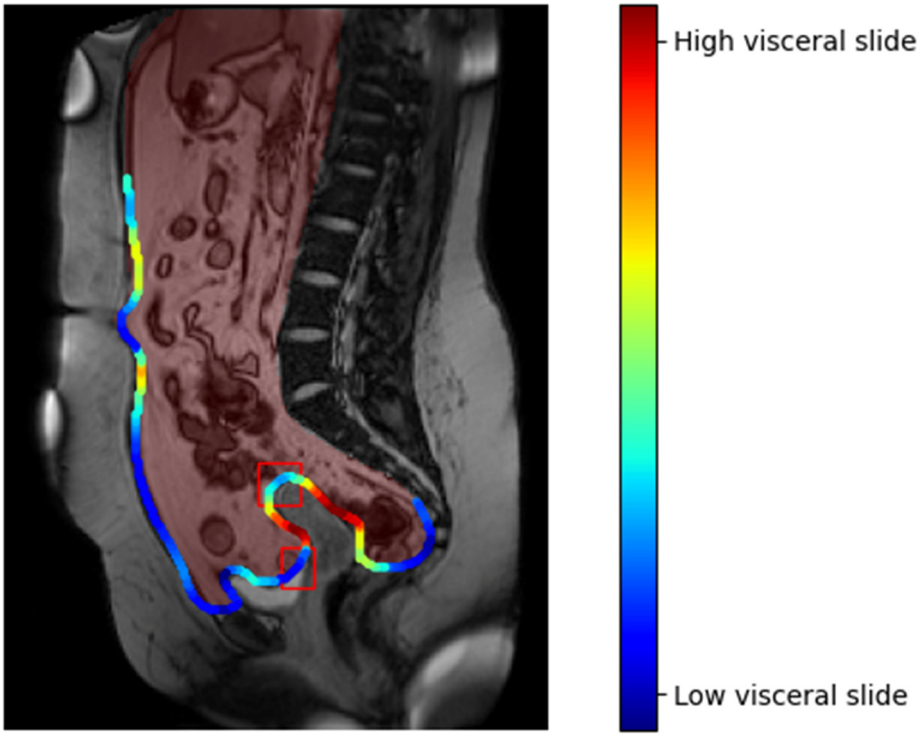

Figure 3. Computationally estimated visceral slide on CineMRI along the contour of the peritoneal cavity. The red mask is the output of a deep learning system that segments the peritoneal cavity, the red boxes show the reference annotations by a radiologist. Low visceral slide (blue) corresponds to locations suspicious for adhesions. Figure adapted from https://github.com/DIAGNijmegen/adhesion_detection, accessed on 8 February 2022.

The clinical adhesion score (CLAS) is a novel clinical score, developed using the Delphi method. The CLAS aims to measure and monitor clinical morbidity of adhesion-related complications, with a minimum of 24 months to follow-up [25]. The CLAS includes outcomes, which describe the morbidity or clinical consequences, and a weight factor, which corrects the outcome for the likelihood that it was caused by adhesions. The integrative score evaluates four major components of adhesion-related morbidity: small bowel obstruction, female infertility, difficulties at reoperation, and chronic abdominal pain. The CLAS could be used to evaluate research endpoints to determine the effectiveness of treatment or decision making, which is particularly useful in post-marketing studies. As a result, the CLAS could also provide valuable long-term data to emphasize the burden of adhesions and the need for effective adhesion prevention and agents specializing in adhesion reduction; further research is required to validate the practical utility of this tool.

There is currently no regulatory guidance from the FDA's Center for Drug Research and Evaluation for the industry on how to design and execute clinical trial research for the investigation of anti-adhesion agents or adhesion-reducing pharmacological agents. Through this consensus meeting, the medical community has taken steps to generate a global key opinion-based perspective, which includes the U.S., EU, Middle East, and Asia, to make recommendations on clinical design and to move towards collegial harmonization for the future of anti-adhesion research and advancement of innovation for patients. The selection of a good surgical model is critical for the validity and reliability of a clinical research trial. When investigating adhesion-reducing therapies, it is important to select a surgical model that is known to be adhesiogenic (endometriosis, pelvic inflammatory disease, myomectomy, etc.) so as to optimize the opportunity for the investigational drug to demonstrate efficacy. Ideally, surgical groups should not be mixed; as an example, the nonoperated abdomen versus conditions after previous surgeries or the mixing of surgery types. As currently, SLL is the gold standard for evaluating the extent of adhesions,

necessary for determining the efficacy of an anti-adhesion agent. Therefore, a surgical model that clinically warrants an SLL might be selected, where applicable. Promising non-invasive evaluation and scoring techniques have the potential to change measuring outcomes in clinical trial research, and studies to validate and standardize these techniques should be prioritized.

The endpoints of clinical trial research aimed at reducing or preventing adhesions should ultimately measure the incidence of adhesions and the burden that is associated with them. The primary endpoint should clearly and objectively outline the investigational drug's ability to prevent adhesion formation. The percentage of adhesion-free patients postoperatively represents the most objective predictive clinical parameter. Quite simply, if a patient is adhesion-free, then there are no associated morbidities or consequences or any disturbances to quality of life to be expected. Therefore, the ideal goal would be a zero-adhesion state. Secondary endpoints should subsequently examine the effects on quality of life and clinical morbidities or consequences. This would require both short- and long-term monitoring and follow-up. Once validated, the CLAS may be a valuable tool in evaluating secondary endpoints by measuring the clinical, quality of life and economic impact that an investigational drug may carry. Again, if the patient achieves a zero-adhesion state, there should be no associated morbidity or effect on quality of life related to adhesions.

6. Conclusions

Adhesions are anticipated sequelae of most pelvic and abdominal surgeries. Current FDA-approved adhesion prevention products do not target the pathophysiology of adhesion formation and could therefore be associated with reduced efficacy. The burdens and consequences of adhesions are well-studied; yet clinical trial research on preventing adhesions has been woeful and stagnant, partly due to regulatory challenges. In the 2010 Adhesion Prevention and Reduction Consensus statement, the panel strongly prioritized improvements in "validated and clinically relevant scale(s) to assess intra-abdominal adhesions" and development of safe and effective anti-adhesion methods [26]. This 2021 Global Expert Consensus Group agrees with these statements and worked together to produce consented statements to guide future clinical research trials and advise regulatory authorities. It is critical for researchers and regulatory authorities to harmonize the expectations of research, to both develop and bring to market improved anti-adhesion therapies, with the ultimate, shared goal of improved patient outcomes.

7. Further Outlooks Brought Up by The Global Consensus Meeting

- Adhesions are a common surgical sequela and health problem. Lack of awareness and acceptance of these problems needs to be rectified. Patients' voices concerning the burden of adhesions on their daily quality of life need to be heard. Patients need to be informed about the risks that are associated with postoperative adhesions, as part of surgical informed consent.
- Surgeons face medicolegal implications related to the morbidities associated with postoperative adhesions and should be informed of this risk.
- The cost of adhesions is considerable. Health care authorities, insurance providers, scientific societies, and governments should think in longer time frames when tackling adhesion-related disease.
- Secondary endpoints such as fertility, pain, bowel obstruction, and quality of life are important, but difficult to scientifically study and evaluate.
- Second-look laparoscopy has remained the gold standard for adhesion assessment and diagnosis, until now. New diagnostic tools such as CineMRI and US require further clinical evaluation.
- A patient with no adhesions assumes no risk of the related complications: pursuit of a zero-adhesion state should be the goal.
- More research regarding new antiadhesion options is necessary, by means of prospective, randomized and blinded designs, when possible.

- A surgery- and disease-related risk score should be constructed, as long as a genetic biobank evaluation is not established to clearly define adhesion-prone populations.

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PERSPECTIVE

Prevention of adhesions in gynaecological surgery: the 2012 European field guideline

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Abstract Postoperative adhesions have become the most common complication of open or laparoscopic abdominal surgery and a source of major concern because of their potentially dramatic consequences. The proposed guideline is the beginning of a major campaign to enhance the awareness of adhesions and to provide surgeons with a reference guide to adhesion prevention adapted to the conditions of their daily practice. The risk of postoperative adhesions should be systematically discussed with any patient scheduled for open or laparoscopic abdominal surgery prior to obtaining her informed consent. Surgeons should adopt a routine adhesion reduction strategy with good surgical technique. Anti-adhesion agents are an additional option, especially in procedures with a high risk of adhesion formation, such as ovarian, endometriosis and tubal surgery and myomectomy. We conclude that good surgical practice is paramount to reduce adhesion formation and that anti-adhesion agents may contribute to adhesion prevention in certain cases.

Keywords Adhesions · Adhesiolysis · Adhesion prevention · Treatment guideline · Anti-adhesion agents

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Postoperative adhesions—fibrous connections developing between tissues and organs as a sequel to surgical trauma—have become the commonest complication of open or laparoscopic abdominal surgery and a source of major concern because of their potentially dramatic consequences.

Only a few specialists are aware of the extent of the adhesions problem. Adhesions are a complication of surgery and the problems they are causing can be severe. The lack of awareness about adhesions and adhesion-related disease makes many doctors unable to take care, insurance companies unwilling to pay, and patients left with their complaints.

Regarding the fact that nearly every abdominal surgery causes adhesions, bowel obstructions due to the adhesions can cause death and many patients have persistent pain, dyspareunia, infertility or bowel complaints after operations, it is amazing that there is such a lack of interest and scientific investigations.

Adhesiolysis, the most common treatment of postoperative adhesions, is too often followed by adhesion reformation. To ensure that their patients receive the best standard of care and avoid adhesion-related litigation claims, surgeons should routinely adopt effective measures to prevent postoperative adhesions.

Several consensus statements on adhesion prevention give similar recommendations based on available evidence [1–5]. However, the format of these academic documents may be less practical for the busy gynaecological surgeon.

The proposed guideline is the beginning of a major concept and work in order to enhance the awareness of adhesions in general, make the scientific research grow and at the end reduce the adhesion-related disease in our patients.

This "field guideline" written by a panel of European Experts aims to provide surgeons with a quick reference guide to adhesion prevention adapted to the conditions of their daily practice. Postoperative adhesions—fibrous connections developing between tissues and organs as a sequel to surgical trauma—have become the commonest complication of open or laparoscopic abdominal surgery and a source of major concern because of their potentially dramatic consequences.

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This "field guideline" written by a panel of European Experts aims to provide surgeons with a quick reference guide to adhesion prevention adapted to the conditions of their daily practice.

What you should know about postoperative adhesions and their consequences

Adhesions have become the most frequent complications of abdominal surgery—93 % of patients undergoing any abdominal/pelvic surgery are affected [5]—and an important source of postoperative problems

- The overall risk of adhesion-related readmission following either laparoscopic or open surgery is comparable [8]
- Over one third of patients who undergo extensive open surgery seem to be readmitted with adhesion-related complications within 10 years [9]
- Adhesions are involved in 56 % of reintervention complications [10]
- Seventy-four percent of cases of bowel obstruction are due to post-surgical adhesions [11]
- Adhesions are associated with a marked risk of enterotomy jeopardising 19 % and 10–25 % of patients undergoing open and laparoscopic surgery, respectively [12, 13]
- Adhesions are responsible for 20–40 % of secondary infertility cases in women [14, 15]

In addition, adhesions generate a high number of reinterventions, increase hospital stays, extend reintervention times and can make it impossible to apply minimally invasive surgery. Last but not least, managing adhesions and their related complications impose an enormous economic burden. In the UK, the cost of adhesion-related readmissions was estimated at £24.2 and £95.2 million at 2 and 5 years after surgery, respectively [16]

The six basic rules of postoperative adhesion prevention in gynaecological surgery [2]

1. The risk of postoperative adhesions should be systematically discussed with any patient scheduled for open or laparoscopic abdominal surgery prior to obtaining his/her informed consent
2. Surgeons need to act to reduce postoperative adhesions in order to fulfill their duty of care towards patients undergoing abdominal surgery
3. Surgeons should adopt a routine adhesion reduction strategy at least for patients undergoing high-risk surgery, including:

(a) Ovarian surgery
(b) Endometriosis surgery
(c) Tubal surgery
(d) Myomectomy
(e) Adhesiolysis

4. Good surgical technique is fundamental to any adhesion reduction strategy
   (a) Carefully handle tissue with field enhancement (magnification) techniques
   (b) Focus on planned surgery and, if any secondary pathology is identified, question the risk: benefit ratio of surgical treatment before proceeding
   (c) Perform diligent haemostasis and ensure diligent use of cautery
   (d) Reduce cautery time and frequency and aspirate aerosolised tissue following cautery
   (e) Excise tissue—reduce fulguration
   (f) Reduce duration of surgery
   (g) Reduce pressure and duration of pneumoperitoneum in laparoscopic surgery
   (h) Reduce risk of infection
   (i) Reduce drying of tissues
   (j) Use frequent irrigation and aspiration in laparoscopic and laparotomic surgery when needed
   (k) Limit use of sutures and choose fine non-reactive sutures
   (l) Avoid foreign bodies when possible—such as materials with loose fibres
   (m) Avoid non-peritonised implants and meshes
   (n) Minimal use of dry towels or sponges in laparotomy
   (o) Use starch- and latex-free gloves in laparotomy

5. Surgeons should consider the use of adhesion reduction agents as part of the adhesion reduction strategy
   (a) Give special consideration to agents with data supporting safety in routine surgery and efficacy in adhesion prevention
   (b) Practicality, ease of use, and cost of agents should influence their selection for routine practice

6. Good medical practice implies that any serious or frequently occurring risks be discussed before obtaining the patient's informed consent prior to surgery

For women undergoing gynaecological surgery, and particularly those undergoing tubal and ovarian surgery procedures, who wish to conceive, the implementation of good surgical practice, together with the adoption of adhesion-reduction agents, is paramount to reduce adhesion formation. As all healthcare providers, surgeons have the duty to protect patients by providing the best standards of care—this includes taking steps to reduce adhesion formation.

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Original Article

A Randomized Controlled Trial on the Efficacy and Safety of a New Crosslinked Hyaluronan Gel in Reducing Adhesions after Gynecologic Laparoscopic Surgeries

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ABSTRACT **Study Objective:** To evaluate the safety and efficacy of a new crosslinked hyaluronan (NCH) gel in reducing postoperative adhesions.

Design: Randomized controlled trial (Canadian Task Force classification I).

Settings: Seven departments of obstetrics and gynecology in China.

Patients: A total of 216 women scheduled for gynecologic laparoscopic surgery for primary removal of adhesions, myomas, ovarian cysts, or endometriotic cysts.

Interventions: Patients were randomized to receive either NCH gel or saline with 1:1 allocation.

Measurements and Main Results: All patients were evaluated using a modified American Fertility Society (mAFS) scoring system for the incidence, extent, and severity of pre-existing and postoperative adhesions at the 10 anatomic sites of ovaries/tubes and at the expanded 23 or 24 anatomic sites throughout the abdominopelvic cavity by laparoscopy. A total of 215 randomized patients were treated with either saline solution (108 of 108) or NCH gel (107 of 108), composing the full analysis set (FAS), and 196 patients (94 of 108 in the saline control group and 102 of 108 in the NCH gel group) completed the entire study, composing the per protocol set (PPS). The postoperative incidence of moderate or severe adhesions evaluated at the 10 sites (the primary endpoint for efficacy) was 27.7% in the control group and 9.8% in the NCH gel group, a difference of 14.4% (95% confidence interval [CI], 2.6%–20.6%) in the PPS, and 37.0% in the control group and 14.0% in the NCH gel group, a difference of 20.0% (95% CI, 8.9%–26.8%) in the FAS. The postoperative incidence of moderate or severe adhesions evaluated at the 24 sites was also significantly lower in the NCH gel group compared with the control group (5.9% vs 14.9%; $p = .036$) in the PPS. Also in the PPS, the NCH gel group had significantly lower postoperative adhesion scores of severity, extent, and mAFS: 60.0%, 50.8%, and 76.9%, respectively (median scores of the 10 sites; $p = .002$) and 48.5%, 50.0%, and 72.2% (median scores of the 24 sites; $p = .001$) lower than those recorded in the control group. No serious adverse events were observed, and the safety profile of NCH gel was comparable to that of saline control.

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Conclusion: This study demonstrates that NCH gel is safe and significantly reduces adnexal adhesion formation and global adhesion formation throughout the abdominopelvic cavity after gynecologic laparoscopic surgery. Journal of Minimally Invasive Gynecology (2015) 22, 853–863 © 2015 AAGL. All rights reserved.

Keywords: Adhesion prevention; Crosslinked hyaluronan gel; Laparoscopic surgery; Randomized controlled trial

DISCUSS You can discuss this article with its authors and with other AAGL members at http://www.AAGL.org/jmig-22-5-JMIG-D-15-00045.

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Although various preventive techniques have been implemented, postoperative adhesion formation after gynecologic surgery remains inevitable [1–15]. Using physical barriers to separate healing peritoneal injuries is believed to be a promising strategy for adhesion reduction during the critical repair phase postsurgery. Ideal barriers are absorbable, safe, deliverable by either laparotomy or laparoscopic approaches, and broadly efficacious for the reduction of de novo as well as reformed adhesions throughout abdominopelvic cavity. Although some new adhesion barriers have been developed, only some of the foregoing issues have been addressed, and there remains great potential for improvement [4,5].

Hyaluronan, a nonsulfated glycosaminoglycan consisting of repeating disaccharide units (α-1,4-D-glucuronic acid and β-1,3-*N*-acetyl-D-glucosamine) and presenting in all connective tissues as a major constituent of the extracellular matrix, has unique physicochemical properties as well as distinctive biological functions in wound healing [16–18]. Although one of hyaluronan's major applications is expected to reduce postoperative adhesions, it failed to demonstrate convincing efficacy in 2 pivotal clinical studies [19,20]. Owing to its fluid nature and rapid in vivo degradation, hyaluronan could not persist long enough to keep the healing injuries separated during the critical phase of peritoneal reepithelialization (5–7 days) [20].

Crosslinking modification is an effective way to improve in vivo persistence by increasing material viscosity and retarding degradation [21–24]. Thus, crosslinked hyaluronan may yield a desired level of postoperative adhesion reduction. One such crosslinked hyaluronan is Seprafilm (Genzyme, Cambridge, MA), a film consisting of hyaluronan complexed with carboxymethylcellulose (CMC) that has proven efficacious in reducing adhesions [25–27]. Disadvantages of Seprafilm are that it is difficult to handle, site-specific only, and very challenging to apply via laparoscopy [7,9,15,20,28]. Hyalobarrier (Anika Therapeutics, Abano Terme, Italy) is another currently available crosslinked hyaluronan product for adhesion reduction. Several studies with a limited number of cases have shown that Hyalobarrier may have a site-specific antiadhesive function following laparoscopic myomectomy [29,30].

Recently, a new crosslinked hyaluronan (NCH) gel has been developed to serve as an absorbable adhesion barrier by BioRegen Biomedical (Changzhou, Jiangsu, China). This NCH gel has a much higher viscosity than natural hyaluronan and is gradually absorbed within 1 to 2 weeks in vivo. Once applied, the NCH gel creates an antiadhesion barrier to keep the healing tissues separated during the critical repair phase. Animal studies have shown favorable safety and significant efficacy in adhesion reduction. Therefore, a pivotal, randomized controlled study was conducted to evaluate the safety and efficacy of this NCH gel in reducing de novo, as well as reformed, adhesion formations throughout the abdominopelvic cavity, with a specific focus on the adnexa region after gynecologic laparoscopic surgeries.

Methods

This prospective study had a randomized, reviewer-blinded, placebo-controlled, parallel-group design and was conducted at 7 departments of obstetrics and gynecology in China. The study protocol was approved by the Ethics Committee of each hospital. Investigators were qualified surgeons experienced in gynecologic laparoscopic surgery. The surgeries were video recorded according to protocol, to enable all assessments to be made through a blinded review of video recordings.

HyaRegen NCH gel is a sterile, transparent, viscoelastic, and nonpyrogenic gel composed of highly purified crosslinked hyaluronan molecules. The placebo control was saline solution purchased from commercial sources.

Participants

The inclusion criteria for this study were female, aged 18 to 45 years, and undergoing laparoscopic surgery for the primary removal of adhesion, myoma, ovarian cyst, or endometriotic cyst. All patients had a negative pregnancy test before entering the study and agreed to use adequate forms of contraception throughout the study period. Patients underwent a scheduled second-look laparoscopy (2LL) at 9 weeks after the first-look laparoscopy (1LL). All patients were required to provide written, signed informed consent before participating.

The exclusion criteria for the study included acute or severe infection; autoimmune disease; abnormal liver/renal function (alanine aminotransferase or creatine 50% above the upper normal range); abnormal cardiovascular function (grade $\geq$III from clinical evaluation); abnormal blood

coagulation; medical history of peripheral vascular disease, alcohol/drug abuse, or mental illness; known/suspected intolerance or hypersensitivity to hyaluronan or its derivatives; concurrent use of a systemic antiinflammatory drug; clinical evidence of cancer; use of anticoagulant, fibrin glue, other thrombogenic agents, or any other antiadhesion agent during the procedure; and concurrent peritoneal grafting or tubal implantation.

Participants were allowed to voluntarily withdraw from the trial for any reason at any time, and could be terminated by investigators owing to safety concerns, violations of inclusion/exclusion criteria, or pregnancy.

Study Schedule

The study duration was 12 weeks. All patients were required to make a minimum of 5 visits to the study site, including screening/baseline checking within 2 weeks before 1LL, on the day of 1LL, and at 3 (±1) days, 30 (±5) days, and 9 (±5 days) weeks after 1LL for physical examination and/or laboratory tests. The 2LL was conducted at the 9-week follow-up visit.

Treatments and Allocation

Patients were assigned at random to either the NCH gel or control group in a 1:1 ratio through a central web-based program, which the investigators in study departments contacted immediately after the completion of 1LL. The program system was administered by the Statistics Center of Medical Research at the National Center for Cardiovascular Diseases China in Beijing. A total 216 random sequences were generated by the SAS PROC PLAN procedure (stratified randomization with block size 4).

Patients in the NCH gel group had 160 mL of NCH gel instilled into the peritoneal cavity through a large-bore cannula following standard laparoscopic procedures, to coat the organ and tissue surfaces that sustained surgical trauma, as well as the adjacent and suspected adhesiogenic surfaces. Conversely, patients in the control group had 160 mL of saline instilled instead. Operators could not be blinded to treatment allocation, because NCH gel is much more viscous than saline; thus, only the patients were blinded to treatment allocation.

Surgical Technique

Standardized laparoscopic techniques were followed by the investigators in the 2 study groups. After video recording, all preexisting adhesions during 1LL and the de novo and/or reformed adhesions during 2LL were surgically removed.

Video Recording and Review

During 1LL, video recordings were made of 23 anatomic sites designated by the mAFS scoring system and the surgical sites according to protocol before any surgical intervention [31–36]. This allowed for a blinded review of the videos and scoring of the number, extent, and severity of adhesions at any of the 23 sites throughout the abdominopelvic cavity (Table S1). During 2LL, video recordings were repeated before any possible surgical interventions. A 24th site, the anterior peritoneum incision from 1LL, was observed and recorded as well.

To avoid the potential bias of operating surgeons, participant randomization was done only after the surgical procedure was completed. All videos were provided to 2 qualified reviewers for blinded assessment. To ensure minimal interobserver variability, adhesion scoring in the blinded reviewer assessments was compared, and any discrepancies were settled by the principal investigator.

Efficacy and Safety Assessment

In accordance with the mAFS scoring system, the following definitions were applied for each anatomic site: adhesion incidence was classified as presence or absence; adhesion severity was classified as mild (i.e., filmy, avascular) or severe (i.e., organized, cohesive, vascular, dense) and scored on a 3-point scale (0, none; 1, mild; 2, severe); and adhesion extent was classified according to the site covered with adhesions as localized (<1/3), moderate (1/3–2/3), or extensive (>2/3) and scored on a 4-point scale (0, none; 1, localized; 2, moderate; 3, extensive) [31–36]. Based on their severity and extent, adhesions occurring at each of the 23 or 24 sites were scored as 0 (no adhesion), 1 (severity, mild; extent, localized), 2 (severity, mild; extent, moderate), 4 (severity, mild; extent, extensive or severity, severe; extent, localized), 8 (severity, severe; extent, moderate), or 16 (severity, severe; extent, extensive) [31–36].

Scores from all 23 or 24 sites, excluding those no longer existing, were averaged to yield a mAFS score throughout the abdominopelvic cavity for each patient. Similarly, scores from the 10 sites (ovaries and tubes) were averaged to yield an mAFS score for adnexa [32–36]. Based on mAFS scores (0–16), the adhesion degree was classified into 5 categories: none (0), minimal (>0 and ≤1), mild (>1 and ≤4), moderate (>4 and ≤8), and severe (>8 and ≤16) [32,33]. In addition, the adhesion degree of each site was classified into the same 5 categories in the same manner.

Because moderate/severe adhesions are the major concern after abdominopelvic surgery [9,32,33], in this study, the incidence of moderate/severe adhesions, evaluated at 10 sites (ovaries and tubes), was defined as the primary endpoint of efficacy. Secondary endpoints of efficacy included the incidence of moderate/severe adhesions evaluated at 24 sites, as well as the mAFS score, severity, and extent of adhesions evaluated at both 10 sites and 24 sites.

Safety evaluation was based on vital signs, physical examination, clinical signs and symptoms, electrocardiography findings, clinical laboratory tests, concomitant medications, and the type and severity of adverse events

recorded throughout the study. Laboratory tests included hematology, blood chemistries, urinalysis, C-reactive protein, and urine pregnancy test. Hematologic evaluations consisted of red blood cell (RBC), white blood cell (WBC) and blood platelet counts, neutrophil, and hemoglobin. Blood chemistry test comprised alanine aminotransferase, aspartate aminotransferase, albumin, globin, total protein, total bilirubin, glucose, urea nitrogen, creatinine, potassium, sodium, and chloride. Urinalysis consisted of protein, WBC, RBC, and glucose. The number of events and numbers of patients reporting at least 1 event were recorded. Clinically significant abnormal values from laboratory tests were also assessed as adverse events, as were any clinically significant changes from baseline.

Statistical Analysis

The primary objective of this study was to demonstrate the superiority of NCH gel over saline placebo with respect to the incidence of moderate/severe adhesions. The primary assumption was that that the estimated incidence was 60% in the control group and would be reduced to 40% by application of the NCH gel. With a 2-sided .05 significance level and 10% rate of loss to follow-up, 216 patients with a 1:1 allocation would yield 80% power to detect the superiority.

The statistical analysis was based on a predefined plan. All randomized patients who started treatment were included in the analysis according to the intent-to-treat (ITT) principle. Continuous variables were expressed as mean ± standard deviation (SD); categorical variables, as count and percentage. The Student t test and χ^2 test/Fisher's exact test were used to check the homogeneity of baseline characteristics. The Wilcoxon rank-sum nonparametric test was used for nonnormally distributed variables, and the results were expressed as median (interquartile range [IQR]). The Cochran-Mantel-Haenszel (CMH) χ^2 test with center effect adjustment was performed to estimate the difference in incidence between groups with a 95% confidence interval (CI). All analyses were performed with SAS 9.13 (SAS Institute, Cary, NC), and a p value $\leq$.05 (2-tailed; $\alpha = 0.05$) was considered significant.

Results

Figure 1 shows a flow chart of the study participants. A total of 216 patients who had undergone primary surgery of adhesiolysis, ovary cystectomy, myomectomy and/or endometriosis were enrolled and randomized. The recruitment ran from June 2011 to February 2013, with the last patient completing follow-up in April 2013. In the NCH gel group, 1 patient was mistakenly randomized and then withdrawn before receiving treatment. Therefore, during 1LL, a total of 215 randomized patients were treated with either saline (108 of 108) or NCH gel (107 of 108). According to the ITT principle, these 215 patients constituted the FAS, as well as the safety population. No patients were withdrawn because of adverse events. Nineteen patients did not undergo 2LL because they did not return within the stipulated time period. As a result, postoperative efficacy data were available for 196 patients (94 of 108 in the control group, 102 of 108 in the NCH gel group), and these patients constituted the PPS.

The 2 groups were generally comparable with respect to patient demographics and surgical history (Table S2); however, 10 more patients in the NCH gel group had undergone previous abdominopelvic surgery (36 of 108 in the control group vs 46 of 107 in the NCH gel group; p = .145).

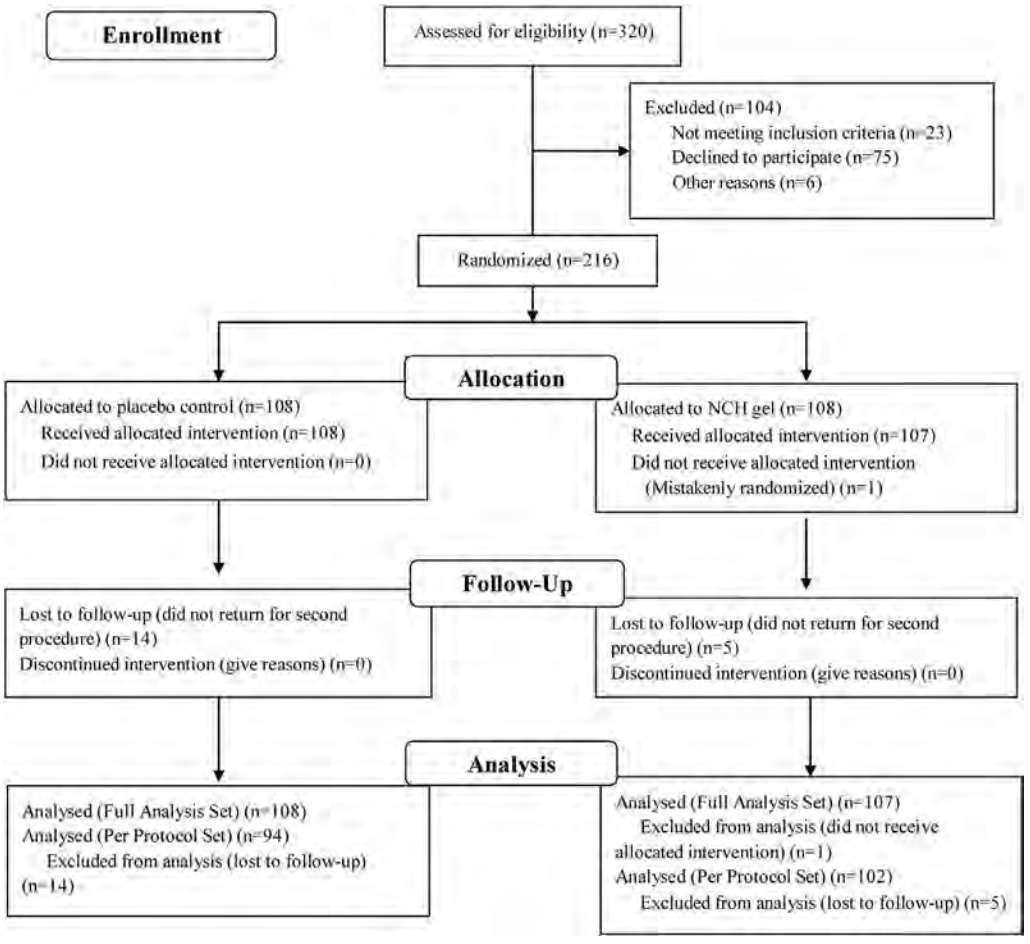
Surgical procedures and occurrence of 1LL for the patients in both groups (summarized in Table S3) were comparable for the 4 primary procedures (adhesiolysis, ovary cystectomy, myomectomy, and endometriosis), as well as the concurrent procedures. Adhesiolysis was the major procedure in both study groups (105 of 108 in the control group, 102 of 107 in the NCH gel group; p = .499), and the techniques used to lyse adhesions (blunt dissection, cautery, and sharp dissection) were comparable in the 2 groups (p = .914, .621, and .837, respectively). The etiology for most of these preexisting adhesions is previous abdominopelvic surgery and/or chronic infection, but some adhesions were not associated with a clear etiology. The duration of surgery was slightly longer in the NCH gel group compared with the control group (93.01 ± 50.13 minutes vs 82.98 ± 39.68 minutes; p = .106), and the associated blood loss was greater in the NCH group (50.67 ± 70.98 mL vs 37.69 ± 43.45 mL; p = .108).

The occurrence of surgeries and adhesiolysis alone performed at each of the 23 anatomic sites was comparable in the 2 groups for most sites. Generally, there were slightly more operative sites in the NCH gel group than in the control group (1160 sites total; 10.84 sites/patient vs 1066 sites total; 9.87 sites/patient). Similarly, slightly more sites underwent adhesiolysis in the NCH gel group (1131 sites total; 10.57 sites/patient vs 1036 sites total; 9.59 sites/patient).

Before surgery, the preexisting adhesions in both groups were first evaluated at the 10 sites (ovaries and tubes) (Table 1). The distribution of various degrees of adhesion (none, minimal, mild, moderate, or severe) did not differ significantly between the 2 groups (p = .169), and the percentage of moderate/severe adhesions was also comparable in the 2 groups (49.1% in the control group vs 57.0% in the NCH gel group; p = .244). However, compared with the control group, the severity of preexisting adhesions was significantly greater in the NCH gel group, with 12 more patients with severe adhesions (20 of 108 in the control group vs 32 of 107 in the NCH gel group), when further examining the sites with moderate/severe adhesions (p = .046) and the scores for severity, extent, and mAFS (p = .041, .031, and .025, respectively). The worse preexisting adhesions in the NCH gel group were even more evident when evaluated at 23 sites (Table 2). The between-group differences were statistically significant (p = .015–.038) in terms of distribution of degrees of adhesion, percentage

Fig. 1

CONSORT flow diagram of the study participants.



and number of sites with moderate/severe adhesions, and scores of severity, extent, and mAFS.

The postoperative adhesions evaluated at 10 sites (ovaries and tubes) are summarized in Table 3. The incidence of moderate/severe adhesions at 2LL (the primary endpoint for efficacy) was significantly lower in the NCH group compared with the control group. In FAS analysis, moderate/severe adhesions were present in 15 of 107 patients (14.0%) in the NCH gel group and in 40 of 108 patients (37.0%) in the control group, a difference of 20.0% (95% CI, 8.9%–26.8%). A similar result was obtained in PPS analysis, with moderate/severe adhesions in 10 of 102 patients (9.8%) in the NCH gel group and in 26 of 94 patients (27.7%) in the control group, a difference of 14.4% (95% CI, 2.6%–20.6%). After bias adjustment of the baseline mAFS score, this incidence difference was even more significant (FAS: 34.3%; 95% CI, 20.5%–48.2% vs PPS: 30.5%; 95% CI, 16.3%–44.7%). In the subgroup of patients with preexisting adhesions (105 of 108 in the control group and 102 of 107 in the NCH gel group), the between-group difference in incidence was significant as well (Table 3).

Details of the postoperative adhesions evaluated at 10 sites in the PPS are presented in Table 4. There were more patients with mild, moderate, or severe adhesions in the control group than in the NCH gel group (51 of 94; 54.3% vs 27 of 104; 26.0%). There were 64.6% fewer patients with moderate/severe adhesions in the NCH gel group compared with the control group (10 of 102; 9.8% vs 26 of 94; 27.7%; $p < .001$). The mean number of sites with moderate/severe adhesions per patient was 57.5% lower in the NCH gel group (0.82 ± 1.91 vs 1.93 ± 2.67; $p = .001$). The median adhesion scores of severity, extent and mAFS were 60.0%, 50.8%, and 76.9% lower, respectively, in the NCH gel group compared with the control group ($p = .002$).

Similar results were obtained when the postoperative adhesions were evaluated at the expanded 24 sites (Table 5; PPS). There were more patients with mild, moderate, or severe adhesions in the control group than in the NCH gel group (43 of 94; 45.7% vs 19 of 102; 18.6%). The percentage of patients with moderate/severe adhesions was 66.4% lower in the NCH gel group (6 of 102; 5.0% vs 14 of 94; 14.9%; $p = .036$). The mean number of sites with moderate/severe

Table 1

Preexisting adhesions at baseline: 10 anatomic sites of ovaries and tubes, FAS analysis

Variable	Control (n = 108)	NCH gel (n = 107)	p value
Adhesion degree, n (%)			.169
None	10 (9.3)	8 (7.5)	
Minimal	22 (20.4)	12 (11.2)	
Mild	23 (21.3)	26 (24.3)	
Moderate	33 (30.6)	29 (27.1)	
Severe	20 (18.5)	32 (29.9)	
Moderate/severe adhesions, n (%)	53 (49.1)	61 (57.0)	.244
Sites with moderate/severe adhesions, mean ± SD	2.81 ± 2.90	3.66 ± 3.27	.046
Adhesion score, median (IQR)			
Severity	0.80 (0.40–1.40)	1.10 (0.60–1.50)	.041
Extent	1.10 (0.50–1.95)	1.60 (0.70–2.30)	.031
mAFS	3.80 (0.78–6.55)	4.90 (1.70–8.90)	.025

adhesions per patient was 52.5% lower in the NCH gel group (1.26 ± 3.02 vs 2.65 ± 3.69; p = .004). The median adhesion scores of severity, extent, and mAFS were 48.5%, 50.0%, and 72.2% lower, respectively, in the NCH gel group (p = .001).

In both study groups, the incidences of moderate/severe adhesions at 2LL were reduced when compared with the baseline, as shown in Figure 2 (PPS). The absolute incidence reduction (baseline - 2LL) was greater in the NCH gel group compared with the control group at the 10 sites (106% greater; 48.20% vs 23.40%) and at the 23 of 24 sites (528% greater; 33.30% vs 5.30%) (Fig. 2A). Moreover, the relative incidence reduction compared with baseline was greater in the NCH gel group compared with the control group at the 10 sites (81% greater; 83.10% vs 45.79%) and at the 23 of 24 sites (224% greater; 84.95% vs 26.24%) (Fig. 2B).

Table 2

Preexisting adhesions at baseline: expanded 23 anatomic sites throughout the abdominopelvic cavity, FAS analysis

Variable	Control (n = 108)	NCH gel (n = 107)	p value
Adhesion degree, n (%)			.035
None	3 (2.8)	5 (4.7)	
Minimal	31 (28.7)	15 (14.0)	
Mild	50 (46.3)	47 (43.9)	
Moderate	19 (17.6)	31 (29.0)	
Severe	5 (4.6)	9 (8.4)	
Moderate/severe adhesions, n (%)	24 (22.2)	40 (37.4)	.015
Sites with moderate/severe adhesions, mean ± SD	3.96 ± 3.91	5.36 ± 4.95	.023
Adhesion score, median (IQR)			
Severity	0.57 (0.37–0.85)	0.74 (0.43–1.00)	.038
Extent	0.72 (0.43–1.22)	1.00 (0.48–1.39)	.026
mAFS	2.33 (0.80–3.80)	2.83 (1.39–5.43)	.028

During the study period, no adverse events were attributed to the NCH gel treatment. No serious adverse events were observed. The adverse events were mostly mild, spontaneously resolved, and comparable in the 2 groups. Two adverse events from laboratory tests, defined as clinically significant changes from baseline (WBC count and blood glucose level), were reported at 9 weeks after surgery in the control group, whereas there were no clinical significantly changes from baseline in the NCH gel group. There were no prolonged hospitalizations or surgeries related to the adverse events.

Discussion

Meticulous surgical technique with less trauma has been considered particularly important for adhesion prevention. Laparoscopy is believed to cause fewer peritoneal injuries and thus is expected to cause fewer adhesions, although an unequivocal consensus has not yet been reached [37]. The reduction in adhesion formation by laparoscopic surgery alone remains unsatisfactory, however. In this study, postoperative adhesions were still formed/reformed in a high proportion of patients: 77.7% total and 27.7 % with moderate/severe adhesions at 10 sites in ovaries and tubes (Table 4), and 88.3% total and 14.9% with moderate/severe adhesions at 24 sites throughout the abdominopelvic cavity (Table 5). This high incidence is consistent with those reported in the literature: 75.4% and 86.1% of patients with de novo adhesion formation after laparoscopic myomectomy and ovarian cystectomy, respectively [36] and 55% to 100% of patients (mean, 85%) with reformed adhesion formation after adhesiolysis irrespective of whether laparotomy or laparoscopy was performed [38].

This pivotal randomized controlled study demonstrates that NCH gel application during laparoscopic surgery significantly reduced postoperative adhesion formation compared with laparoscopic surgery alone (saline control group), as indicated by the lower incidence of moderate/severe adhesions, fewer sites with moderate/severe adhesions, and lower scores for adhesion severity, extent, and mAFS in the NCH gel group at the 10 sites and the expanded 24 sites (Tables 3–5 and Fig. 2). These results confirm that NCH gel is efficacious in reducing postoperative adhesion formation at the adnexa and throughout the abdominopelvic cavity.

Adhesion-reducing agents generally fall within 2 main categories: physical barriers (e.g., films, gels) and solutions (intraperitoneal instillates) [4–15]. Despite the biochemical

Table 3

Incidence of moderate or severe adhesions at 9 weeks after surgery: 10 anatomic sites of ovaries and tubes

	FAS analysis		PPS analysis	
	Control (n = 108)	NCH gel (n = 107)	Control (n = 94)	NCH gel (n = 102)
Incidence, % (n)	37.0 (40)	14.0 (15)	27.7 (26)	9.8 (10)
Difference, % (95% CI)*	20.0 (8.9–26.8)		14.4 (2.6–20.6)	
	34.3 (20.5–48.2)†		30.5 (16.3–44.7)†	
	19.0 (7.4–26.0)‡		13.8 (1.4–20.4)‡	
	35.0 (20.8–49.1)§		31.5 (17.0–46.0)§	

* Incidence difference (%) was calculated by point estimation, and 95% CI was calculated by CMH χ^2 test after the adjustment of center effect; difference = control - NCH gel.
† Sensitivity analysis after bias adjustment of the preexisting adhesion mAFS score.
‡ Sensitivity analysis, subgroup of patients with preexisting adhesions (105 of 108 in the control group and 102 of 107 in the NCH gel group).
§ Sensitivity analysis, subgroup of patients with preexisting adhesions after bias adjustment of the preexisting adhesion mAFS score.

differences, all of these agents have a common primary mode of action as a physical barrier to separate the healing tissues from other tissue surfaces during the critical period of peritoneal reepithelialization [7,9,15]. In general, physical barriers are site-specific (i.e., reducing adhesions where they are placed), but have no effect on the global reduction of adhesions throughout the entire abdominopelvic cavity. Conversely, solutions typically have the advantage of providing broad coverage throughout the cavity [4–15].

Currently, the Food and Drug Administration has approved only 2 physical barriers for adhesion reduction after laparotomy: oxidized regenerated cellulose (Interceed; Ethicon, Somerville, NJ) and Seprafilm. However, a number of other site-specific barriers have been approved for use in Europe, including polyethylene oxide/CMC gel (Intercoat; Ethicon), polyethylene glycol hydrogel (CoSeal; Baxter International, Deerfield, IL), and Hyalobarrier [7,9,12,15]. These physical barriers have demonstrated variable efficacy for reducing site-specific adhesion formation, although most of them, except Interceed and Seprafilm, have not yet been evaluated through pivotal randomized controlled trials [7,9,12,15,39].

To date, Adept (4% icodextrin solution; Baxter BioSurgery, Deerfield, IL), approved in Europe for abdominal surgery and in US for laparoscopic gynecologic adhesiolysis, is the only broad-coverage solution agent shown to be safe and to have some efficacy in global adhesion reduction throughout the abdominopelvic cavity [7,9,12,15]. This agent has not demonstrated sufficient performance, however. In a pivotal randomized controlled study in the US, Adept did not reduce the extent and severity of

Table 4

Postoperative adhesions at 9 weeks: 10 anatomic sites of ovaries and tubes, PPS analysis

Variable	Control (n = 94)	NCH gel (n = 102)	p value
Adhesion degree, n (%)			<.001
None	21 (22.3)	22 (21.6)	
Minimal	22 (23.4)	53 (52.0)	
Mild	25 (26.6)	17 (16.7)	
Moderate	15 (16.0)	4 (3.9)	
Severe	11 (11.7)	6 (5.9)	
Moderate/severe adhesions, n (%)	26 (27.7)	10 (9.8)	<.001
Sites with moderate/severe adhesions, mean ± SD	1.93 ± 2.67	0.82 ± 1.91	.001
Adhesion score, median (IQR)			
Severity	0.50 (0.10–1.10)	0.20 (0.10–0.50)	.002
Extent	0.61 (0.10–1.50)	0.30 (0.10–0.60)	.002
mAFS	1.30 (0.10–4.80)	0.30 (0.10–1.10)	.002

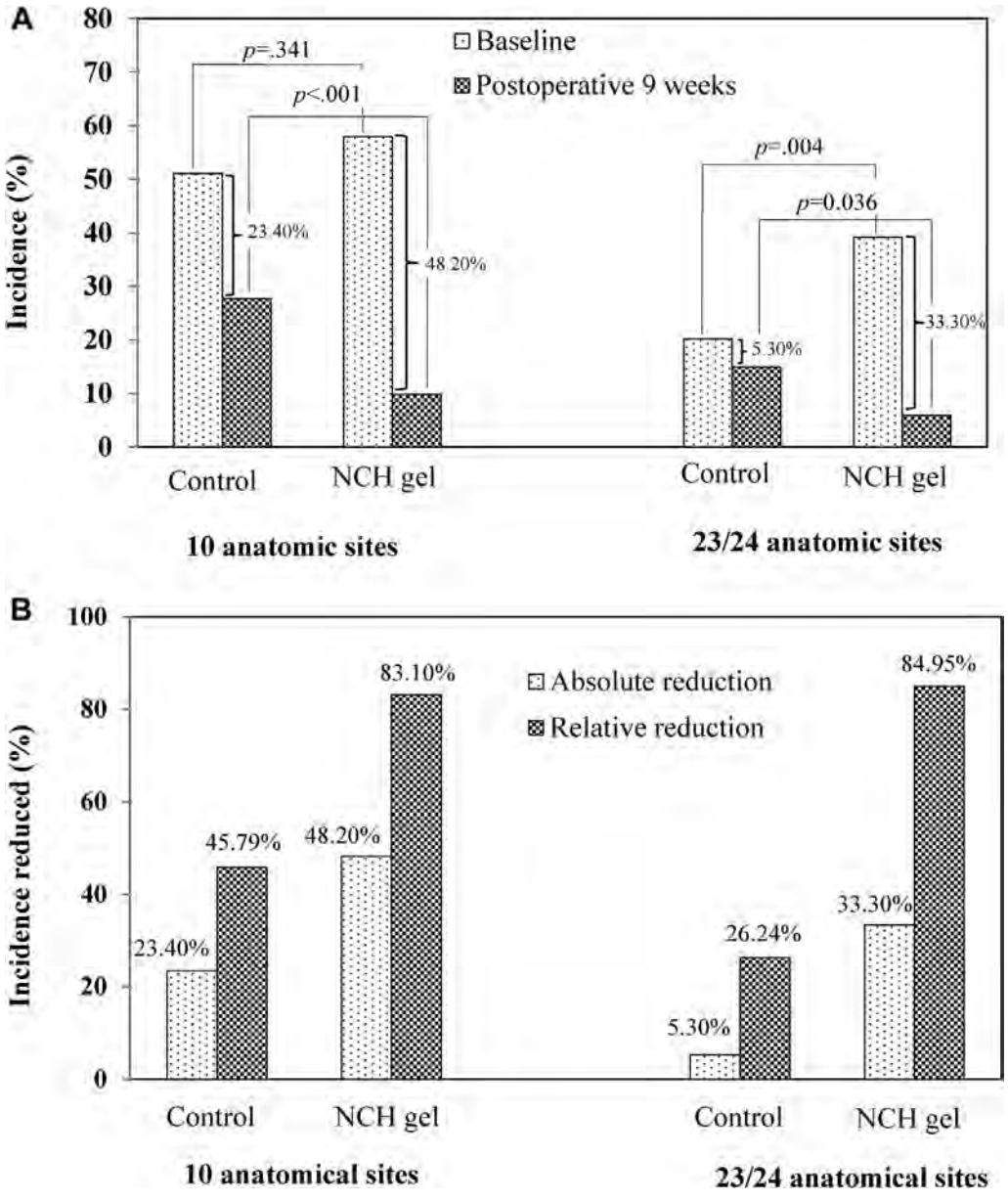
Table 5

Postoperative adhesion at 9 weeks: expanded 24 anatomic sites throughout the abdominopelvic cavity, PPS analysis

Variable	Control (n = 94)	NCH gel (n = 102)	p value
Adhesion degree, n (%)			.001
None	11 (11.7)	12 (11.8)	
Minimal	40 (42.6)	71 (69.6)	
Mild	29 (30.9)	13 (12.7)	
Moderate	13 (13.8)	5 (4.9)	
Severe	1 (1.1)	1 (1.0)	
Moderate/severe adhesions, n (%)	14 (14.9)	6 (5.9)	.036
Sites with moderate/severe adhesions, mean ± SD	2.65 ± 3.69	1.26 ± 3.02	.004
Adhesion score, median (IQR)			
Severity	0.33 (0.08–0.63)	0.17 (0.08–0.33)	.001
Extent	0.42 (0.08–0.83)	0.21 (0.08–0.42)	.001
mAFS	0.90 (0.13–2.79)	0.25 (0.08–0.71)	.001

Fig. 2

Moderate/severe adhesions at baseline and 2LL (PPS): (**A**) incidence and (**B**) incidence reduction (baseline−2LL). Absolute incidence reduction = incidence at baseline−incidence at 2LL; relative incidence reduction = (incidence at baseline−incidence at 2LL)/incidence at baseline × 100%.



adhesions, and there was only an 11% between-group difference (49% in the Adept group vs 38% in the control lactated Ringer's solution group; p = .018) in terms of clinical success, defined as a reduction in adhesions in at least 3 sites or in 30% of the sites lysed [33]. More recently, another pivotal randomized controlled study in Europe showed that Adept lacked a global effect in reducing de novo adhesions after laparoscopic gynecologic surgery [36]. The authors concluded that for the purposes of future research on this agent, focusing on site-specific (e.g., posterior uterus) changes rather than on a global effect is likely to provide more important data on clinical efficacy [36].

The protocol in this pivotal study was similar to that for the Adept studies [33,36]. The results show that the NCH gel significantly reduced both adnexal adhesion formation and global adhesion formation throughout the abdominopelvic cavity, in terms of the incidence of moderate/severe adhesions, mAFS score, and adhesion severity and extent. To the best of our knowledge, NCH gel is the sole barrier currently able to significantly reduce

global adhesion formation throughout the abdominopelvic cavity, as supported by the data from this pivotal randomized control trial. The efficacy of the NCH gel in reducing de novo or reformed adhesions, as well as the adhesions at each site, was not determined in this study. Well-designed future studies might be necessary to demonstrate this efficacy.

When applied to the surgical site, natural hyaluronan quickly enters the systemic circulation via the lymph and is then rapidly cleared by catabolic pathways. Its reported elimination half-life ($t_{1/2}$) from the peritoneum is approximately 25.5 hours [21]. Crosslinking is believed to delay metabolic clearance and allow the material to persist for the time window of adhesiogenesis [21–23,40]. Along with Seprafilm and Hyalobarrier, another example of crosslinked hyaluronan is ionically crosslinked ferric hyaluronan (Intergel; Lifecore Biomedical, Chaska, MN). Intergel was developed with increased viscosity and prolonged in vivo persistence, and both animal and clinical studies have shown desired levels of efficacy in global adhesion reduction throughout the abdominopelvic cavity [21,32,40]. Unfortunately, this gel was withdrawn from the US market in 2003 owing to a serious possible Intergel reaction syndrome caused by the component of iron (Fe^{3+}) and ammonia [41–46].

The physical properties of the NCH gel are similar to those of Intergel; however, unlike Intergel, NCH gel was developed via a new crosslinking technology and contains no toxic agents. In a preclinical animal study, the maximum volume of NCH gel administered without any adverse effects was at least 15-fold higher than that applied in this study (unpublished data). The favorable safety profile of NCH gel is further confirmed by the present study, in which no adverse events associated with NCH gel treatment were observed.

Owing to the high safety threshold, 160 mL of NCH gel was applied to the abdominopelvic cavity to provide broad coverage on the organ and tissue surfaces that sustained surgical trauma as well as their adjacent and suspected adhesiogenic surfaces, and thus a global effect on reduced adhesion formation throughout the cavity. In the contrast, the doses for most site-specific gels are within 40 mL because of safety and/or cost concerns [29,30,34,35,47–49]. Furthermore, the widespread distribution of the gel also possibly could be achieved via intestinal peristalsis and eventually reach broader coverage, similar to that for Intergel [32]. Numerous factors may contribute to postoperative adhesions, and it is difficult to predict which locations and organs will be involved [1]; therefore, broad prophylactic coverage and comprehensive application of antiadhesive gel represent an effective approach to adhesion reduction.

Adhesion formation is inherently a defect of the peritoneal healing process; thus, any factors that theoretically aid the normal healing process may reduce adhesion formation [1,7,9,15]. Hyaluronan has been reported to have distinctive functions in scar-free wound healing by reducing inflammation and improving peritoneal reepithelialization [16–18]. In a bowel anastomotic rat model, application of NCH gel significantly improved tissue healing (unpublished data). A gel similar to NCH also has been reported to significantly improve wound healing (i.e., mucosa reepithelialization) after endoscopic sinus surgery in both animal and clinical studies [22,23,50].

Adhesions may result in infertility, pain, or bowel obstruction and may increase operating time and the risk of bowel injury during subsequent surgeries [1–15]. The present study has demonstrated the effectiveness of the NCH gel in reducing postoperative adhesion formation; however, the clinical significance of associated improved fertility, decreased pain, or reduced incidence of postoperative bowel obstruction remains to be evaluated in future studies.

Conclusion

NCH gel proved to be safe and significantly reduced postoperative adhesion formation both at the 10 anatomic sites of ovaries and tubes and at the expanded 24 anatomic sites throughout the abdominopelvic cavity. NCH gel provides a new, easy-to-use, and effective intraperitoneal barrier for adhesion reduction throughout the abdominopelvic cavity after surgery.

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Supplementary Data

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ORIGINAL ARTICLE

The effect of new cross linked hyaluronan gel on quality of life of patients after deep infiltrating endometriosis surgery: a randomized controlled pilot study

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ABSTRACT

In this prospective randomised placebo-controlled study, we aimed to evaluate the effect of New Cross linked Hyaluronan Gel (NCH gel) on the quality of life of patients who underwent laparoscopic surgery due to Deep Infiltrating Endometriosis (DIE). The intervention group received 40 mL of NCH gel, and the control group had a 40 mL sterile saline solution instilled into the peritoneal cavity following standard laparoscopic procedures. The patients were called in the third and sixth postoperative months and requested to fill the Visual Analogue Scale (VAS), Endometriosis Health Profile (EHP-5), and Short Form for Mental and Physical Health (SF-12) questionnaires. There was a significant reduction in dysmenorrhoea, dyschezia, dyspareunia VAS scores at 3rd, and 6th-month visits in NCH gel group. The postoperative 6th-month EHP-5 scores were significantly lower (1.16 ± 1.51, p-value: .02) in NCH gel group. Besides, NCH gel group had higher SF-12 mental and SF-12 physical scores.

Clinical Trials registration number: NCT04023383

IMPACT STATEMENT

- **What is already known on this subject?** Application of solid or liquid physical barriers is believed to be a promising strategy to reduce adhesions after laparoscopic endometriosis surgery. However, comparable data regarding the effects of adhesion barriers are still lacking.
- **What the results of this study add?** We revealed that there was a significantly higher decrease in VAS and EHP-5 scores and an increase in SF-12 physical-mental ratings after surgery in NCH gel group.
- **What are the implications of these findings for clinical practice and/or further research?** Using NHC gel in addition to standard surgical procedure improves postoperative VAS scores, and provides better quality of life scores.

KEYWORDS

Endometriosis; hyaluronic acid; quality of life; tissue adhesions; adhesion barriers

Introduction

Endometriosis is defined as the presence of endometrial glands and stroma outside of the uterine cavity. Endometriosis is one of the most common diseases of women during their reproductive period, with a prevalence of 7–10% (Laufer et al. 1997; Eskenazi and Warner 1997). The lesions are typically observed in the peritoneal cavity, ovaries, and tubes. Still, it can also be found in the rectum, rectosigmoid colon, bladder, ureter, and other pelvic structures such as the uterine ligaments and vagina (Jansen and Russell 1986; Vercellini et al. 2014). The patients with endometriosis have a low quality of life (QoL) due to dysmenorrhoea, dyspareunia, chronic pelvic pain, and infertility as a result of inflammation and adhesion formation.

Surgery is required for patients who failed to respond to medical therapy and those who develop acute abdominal pain or suspicion of malignant adnexal mass (Singh and Suen 2017). The surgical technique may vary from simple laparoscopic excision of endometrial foci to complex procedures including extensive adheziolysis, ureterolysis, partial resection of bladder, ureter, and bowel to treat deep infiltrative endometriosis (DIE) (Arcoverde et al. 2019).

One of the significant postoperative concerns is the high recurrence rate of the symptoms due to de novo pelvic adhesions that are associated with endometriosis-related pain (Al-Jabri and Tulandi 2011). Immunohistochemical analyses also confirmed that there were nerve fibres in the adhesions that had been removed from patients with pelvic pain (Hammoud et al. 2004). Administrating solid or liquid physical barriers is believed to be a promising strategy to reduce postoperative adhesions and to separate peritoneal injuries from each other (Ahmad et al. 2008). Ideal barriers should be absorbable, safe, deliverable by either laparotomy or laparoscopic approaches, and broadly efficacious to reduce both de novo and reformed adhesions in the abdominopelvic cavity (Chen and Abatangelo 1999).

Hyaluronan, a glycosaminoglycan found in connective tissues and extracellular matrix, has been thought to reduce

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postoperative adhesions, because of its biological functions in tissue repair. However, its fluid nature causes rapid degradation, and it cannot affect long enough to work as an adhesion barrier (Chen and Abatangelo 1999; Wiseman et al. 2000). For this reason, a new cross-linked hyaluronan (NCH) gel, with a higher viscosity than natural hyaluronan has been developed. It is gradually absorbed within 1–2 weeks *in vivo*, which is the required period for tissue repair and adhesion formation. Although it has already known that endometriosis has a serious impact on the quality of life of women; comparable data regarding the effects of adhesion barriers on patients who have had laparoscopic DIE surgery is lacking.

Therefore, in this study, we aimed to conduct a pilot randomised controlled study to evaluate the effect of NCH gel on short term quality of life in patients who had undergone laparoscopic surgery due to DIE.

Materials and methods

A prospective, 1:1, randomised, placebo-controlled study was conducted in University of Health Sciences Bakirkoy Dr. Sadi Konuk Hospital, Istanbul, which serves as a tertiary referral hospital between January 2017 and January 2019. The study protocol was approved by the local ethics committee of our hospital (approval number: 2017/04/35).

The inclusion criteria for this study were as follows; women aged 18–45 years, undergoing laparoscopic surgery for suspicion of DIE, and having persistent pain unresponsive to any medical treatment.

The exclusion criteria for the study were as follows; the presence of an acute or severe infection, autoimmune disease, use of a systemic anti-inflammatory or hormonal drug at least three months before the planned surgery, having clinical evidence of cancer. Also, patients with known/suspected hypersensitivity against hyaluronan or its derivatives, use of any anticoagulant agents, fibrin glue, or other anti-adhesion agents, patients with bowel involvement proven via ultrasound or MRI, and those who want to receive postoperative hormonal treatment were excluded.

One hundred twenty-four patients who were admitted to the endometriosis outpatient clinic, corresponding inclusion/exclusion criteria were informed and asked to anticipate to the study. The patients who were scheduled to undergone laparoscopic surgery for the treatment of deep infiltrating endometriosis without bowel involvement were enrolled. Sixty patients were enrolled in the study after written and signed informed consent was obtained. The same surgical team who experienced in minimally invasive DIE surgery performed all of the procedures. The patients were called for follow-up visits in the 3rd and 6th postoperative months.

Participants were allowed to withdraw from the study for any reason at any time, and the study was decided could be terminated by investigators in case of safety concerns, violations of inclusion/exclusion criteria, or presence of pregnancy. None of the participants lost during the study period, and the study was completed with the planned number of patients.

Surgical procedure

Patients were randomly assigned to either the NCH gel or control group in a 1:1 ratio through a computer-based programme. A standardised laparoscopic approach was conducted consisting of removal of all the endometriotic foci. After hemostasis, 40 mL of NCH gel (HyaRegen, BioRegen Biomedical, Changzhou, China) was administered into the peritoneal cavity of the intervention group. In contrast, the control group received a 40 cc of sterile saline solution. The operators were not blind to the type of treatment due to the nature of the study, but the questionnaire assessors and the patients were blinded to the kind of treatment applied.

Preoperative and postoperative assessment of pain and quality of life (QoL)

A research assistant recorded the scores of Visual Analogue Scale (VAS), Endometriosis Health Profile (EHP-5), and Short Form for Mental and Physical Health (SF-12) of patients before the day of the surgery.

A validated, Likert fashion, VAS score form was used. The scoring varied between 0 and 10 that 0 was referred to as no pain, and ten was the worst pain that they had ever experienced. Then, the VAS and QoL questions were asked in the 3rd and 6th months of their follow up visits. A QoL form SF-12, which assesses physical functioning, bodily pain, general health, vitality, social functioning, role limitations due to physical and mental health problems, was used. The scores were analysed to obtain both physical and mental scores.

A validated form of EHP-5 questionnaire was used to determine endometriosis-related QoL (Selcuk et al. 2015). EHP-5 consists of two parts. The first part evaluates parameters, namely; pain, control and powerlessness, emotional well-being, social support, and self-image; and the second part measures the sexual life, work, relationship with children, feelings about medical professional, treatment, and infertility.

Statistical analysis

Data analysis was performed by using SPSS (IBM SPSS Statistics for Windows, version 20.0. Armonk, NY: IBM Corp.). One-sample Kolmogorov–Smirnov test was performed to analyse the distribution of clinical variables. The frequency and percentage of the categorical variables and the mean, standard deviation, median, and range values of the continuous and ordinal variables were presented. The study groups were compared using Student *t*-test for parametric variables and Mann Whitney *U* test for the non-parametric variables. A post-hoc sample size calculation was performed via a two-sided *Z* test ($\alpha = 0.05$, $\beta = 0.20$) for each study group to obtain VAS scores as the primary outcome. A *p*-value of $<.05$ was considered statistically significant for all calculations.

Results

There was no significant difference between the study groups in terms of age, BMI, gravidity, parity, size of adnexal

Table 1. Baseline characteristics of the study population.

	Control group	NCH† gel group	*p* Value	95% Confidence interval(CI)
Age (years)	36.43 ± 8	34.36 ± 7.58	0.23	0.23–0.25
Gravidity	1.33 ± 1.1	0.86 ± 0.97	0.1	0.1–0.11
Parity	1.16 ± 0.9	0.76 ± 0.85	0.09	0.09–0.11
BMI (kg/m^2)	26.27 ± 3.94	24.39 ± 3.4	0.06	0.060–0.069
Size of adnexal mass (mm)	61 ± 20.56	66 ± 22.06	0.28	0.26–0.30
Laterality				
Unilateral	14 (46.7%)	8 (26.7%)	0.09	0.17–0.18
Bilateral	16 (53.3%)	22 (73.3%)		
Number of intra/postoperative complication	2 (6.7%)	0	0.49	0.48–0.51
Number of recurrence	2	4	0.67	0.66–0.68
Type of operation				
Cystectomy	16	22	0.11	0.17–0.18
Oophorectomy	14	8		
Preoperative Hb g/dL	12.46 ± 1.59	12.16 ± 1.17	0.38	0.38–0.4
Preoperative Hct %	38.06 ± 3.96	37.86 ± 2.94	0.8	0.79–0.81
Postoperative Hb g/dL	10.46 ± 1.53	10.43 ± 1.47	0.99	0.99–1
Postoperative Hct %	33.03 ± 4.27	33.13 ± 3.96	0.97	0.97–0.98
Decrease in Hb	2 ± 1.28	1.73 ± 0.98	0.31	0.316–0.334
Decrease in Hct %	5.03 ± 3.63	4.73 ± 2.81	0.82	0.819–0.834
Duration of surgery (min.)	135.83 ± 56.32	166.5 ± 58.28	0.06	0.059–0.069
Duration of hospital stay (days)	2.83 ± 0.91	2.83 ± 0.64	0.66	0.64–0.66

†NCH: New cross linked hyaluronan.

Table 2. Preoperative and postoperative VAS‡ scores of the study population.

	Control group	NCH† gel group	*p* Value	95% Confidence interval(CI)
Preoperative VAS dysmenorrhea dysmenorrhoea	8.06 ± 0.9	8.13 ± 1.63	0.71	0.71–0.73
3rd month VAS dysmenorrhea dysmenorrhoea	3.1 ± 2.39	1.26 ± 1.99	**0.001**	**0–0.002**
6th month VAS dysmenorrhea dysmenorrhoea	2.46 ± 1.75	1 ± 1.33	**0.001**	**0–0.001**
Decrease in VAS dysmenorrhea dysmenorrhoea	5.6 ± 1.92	7.13 ± 2.17	**0.007**	**0.005–0.008**
Preoperative VAS dyschezia	2.9 ± 2.1	2.43 ± 2.29	0.33	0.333–0.352
3rd month VAS dyschezia	2.2 ± 1.5	1.13 ± 1.27	**0.006**	**0.004–0.007**
6th month VAS dyschezia	2.13 ± 1.54	0.76 ± 1.01	**<0.001**	**0–0.001**
Decrease in VAS dyschezia	0.76 ± 0.93	1.66 ± 1.71	0.067	0.062–0.071
Preoperative VAS dyspareunia	3.23 ± 2.25	3.26 ± 2.59	0.91	0.919–0.929
3rd month VAS dyspareunia	2.43 ± 1.69	1.03 ± 1.42	**0.001**	**0–0.002**
6th month VAS dyspareunia	2.2 ± 1.56	0.8 ± 1.06	**<0.001**	**0.000–0.000**
Decrease in VAS dyspareunia	1.03 ± 1.27	2.46 ± 2.08	**0.01**	**0.009–0.01**
Pre-op VAS dysuria	2.33 ± 1.6	2.6 ± 2.38	0.6	0.601–0.620
3rd month VAS dysuria	1.7 ± 1.14	1.4 ± 1.32	0.39	0.391–0.410
6th month VAS dysuria	1.26 ± 0.94	0.93 ± 0.94	0.18	0.165–0.180
Decrease in VAS dysuria	1.06 ± 1.11	1.66 ± 1.82	0.38	0.379–0.398
Preoperative VAS pelvic pain	2.93 ± 2.16	3.03 ± 3.02	0.85	0.853–0.867
3rd month VAS pelvic pain	2.26 ± 1.61	1.6 ± 1.84	0.21	0.211–0.227
6th month VAS pelvic pain	1.5 ± 1.16	0.8 ± 1.03	**0.01**	**0.010–0.014**
Decrease in VAS pelvic pain	1.43 ± 1.47	2.23 ± 2.41	0.36	0.351–0.370

‡VAS: visual analogue scale.
†NCH: new cross linked hyaluronan.
Bold values indicates statistically significant ($p < 0.05$).

cyst, laterality of the mass, peri/postoperative complications and recurrence rates, type of operation, pre and postoperative Hb/Hct levels, duration of surgery, and duration of postoperative hospital stay (Table 1). There was a statistically significant reduction in dysmenorrhoea, dyschezia, dyspareunia at 3rd and 6th month, and in VAS scores at 6th month in NCH gel group compared to the control group (Table 2). No significant difference for preoperative EHP-5 and postoperative 3rd-month EHP-5 results between the study groups was noted. However, postoperative 6th-month EHP-5 scores were significantly lower (1.16 ± 1.51, *p*-value: .02) in NCH gel group. Preoperative SF-12 mental and physical parameters and postoperative 3rd-month physical scores were not statistically different between the study groups. On the other hand, significantly higher scores of the postoperative 3rd month mental SF-12 and postoperative 6th month SF-12 physical and mental ratings (50.43 ± 11.09, 51. 92 ± 11.21, and 51.55 ± 10.93, respectively) found in NCH gel group (Table 3).

Discussion

It is a well-known fact that endometriosis itself progresses with the formation of postoperative adhesions despite definitive radical excisional surgeries and hormonal therapies. So far, the studies had focussed on the effect of surgery alone on QoL of patients, recurrence, and postoperative adhesion formation rates. However, there is still a lack of evidence on the impact of adhesion barriers on postoperative pain and QoL (Chen and Abatangelo 1999; Garry et al. 2000).

To enlighten the relation between adhesion barriers and postoperative de novo adhesions, diZerega et al. compared patients who had surgery only (*n*: 17, control patients with

Table 3. Preoperative and Postoperative SF-12[§], EHP5[¶] ,QoL[°] assesment scores of the study population.

	Control group	NCH gel group	*p* Value	95% Confidence interval(CI)
Preoperative EHP-5	5.46 ± 3.13	7.83 ± 4.98	0.06	0.065–0.075
3rd month EHP-5	2.66 ± 1.56	1.93 ± 1.74	0.07	0.066–0.076
6th month EHP-5	2.16 ± 1.91	1.16 ± 1.51	**0.02**	**0.017–0.022**
Decrease in EHP-5	3.3 ± 3.19	6.6 ± 5.12	**0.009**	**0.007–0.011**
Preoperative SF 12 physical score	43.15 ± 10.11	39.99 ± 9.45	0.21	0.216–0.233
Preoperative SF 12 mental score	44.89 ± 9.31	40.38 ± 11.41	0.06	0.066–0.076
3rd month SF 12 physical score	49.34 ± 7.43	50.11 ± 11.16	0.18	0.182–0.197
3rd month SF 12 mental score	46.41 ± 7.93	50.43 ± 11.09	**0.005**	**0.004–0.006**
6th month SF 12 physical score	49.24 ± 7.89	51.92 ± 11.21	**0.004**	**0.002–0.004**
6th month SF 12 mental score	49.21 ± 7.07	51.55 ± 10.93	**0.01**	**0.010–0.014**
Decrease in SF 12 physical score	6.09 ± 10.02	11.93 ± 14.61	**0.03**	**0.027–0.034**
Decrease in SF 12 mental score	4.32 ± 11.29	11.17 ± 14.76	**0.01**	**0.015–0.020**

[§]SF 12: Short form-12 health survey for mental-physical health.
[¶]EHP-5: Endometriosis health profile-5.
[°]QoL: Quality of life.
Bold values indicates statistically significant ($p < 0.05$).

total 30 adnexal involvement) and the other group received Oxiplex/AP gel (approximately 12 mL; range, 4–60 mL in 90 s) in addition to standard surgery (*n*: 20 patients with total 35 adnexal involvement) (diZerega et al. 2007). They concluded that Oxiplex/AP gel was effective in the reduction of adhesion formation scores obtained in second-look surgery since increased scores were observed in patients with stage I and stage II disease in the control group. They also indicated that, especially in cases of red endometriotic lesions, which are indicative of early endometriosis, intraoperative administration of gel might provide additional benefits in reducing endometriotic lesions (diZerega et al. 2007).

To date, Adept (4% icodextrin solution; Baxter Bio-Surgery, Deerfield, IL) has been approved in Europe for abdominal surgery and in the US for laparoscopic gynaecologic adhesiolysis (Darai et al. 2010; Mabrouk et al. 2012). However, in a pivotal randomised controlled study in the US, Adept was found to be ineffective in reducing the extent and severity of adhesions. There was only an 11% difference (49% in the Adept group vs. 38% in the control group which lactated Ringer's solution was used.) in terms of clinical success, which was basically considered as the reduction in adhesions (Tanmahasamut et al. 2012). To evaluate the anti-adhesion effect of barriers, in a randomised study with 215 patients by Liu et al. 160 mL of NCH gel (107 of 108) was instilled into the peritoneal cavity following standard laparoscopic procedures, and the patients in control group had 160 mL of saline (108 of 108) instillation (Liu et al. 2015). They concluded that NCH gel could significantly reduce postoperative adhesion formation, severity, and modified American Fertility Society (mAFS) scores compared to the control group (Liu et al. 2015). In contrast to our study, patients in this trial underwent a laparoscopic surgery due to a variety of indications such as pelvic adhesions, uterine fibroids, simple ovarian cysts, and endometriotic cysts.

These results confirm that NCH gel is efficient in reducing postoperative adhesion formation in the abdominopelvic cavity. A 160 mL of NCH gel can be applied to the abdominopelvic cavity to provide broad coverage on the surfaces of organs and tissues with a high safety threshold. However, like the previous studies, we also used 40 mL of gel in our research. (Garry et al. 2000; Sintonen 2001). We believe that 40 mL NCH gel is adequate to cover the ovaries, fallopian tubes, and pouch of Douglas as it has a cross-linked molecular structure. Its high viscosity allows expansion and slow degradation, which provides enough time interval to prevent adhesions at the surgical area.

Recently, Pang et al. have demonstrated that NCH gel inhibits migration, invasion, and proliferation of ovarian cancer cells *in vitro*. It can also suppress implantation *in vivo* by blocking the activation of epidermal growth factor receptor (EGFR) in implantation nude mice model of ovarian cancer (Pang et al. 2018). Also, a marked increase of epidermal growth factor (EGF) concentrations has been previously demonstrated in the peritoneal fluid of women with endometriosis (Rakhila et al. 2016). Therefore, one of the reasons why NCH gel may have a positive effect on the quality of life scores can be explained by its anti-proliferative effects on endometriotic implants.

Hyaluronan has distinctive functions on scar-free healing by reducing inflammation and improving peritoneal re-epithelialization (Ribeiro et al. 2014). We believe that adhesion formation is an inherited defect of the peritoneal healing process; thus, better tissue hemostasis and enhancement of routine healing may reduce adhesion formation. In our study, we used the EHP-5 questionnaire, which is a condition-specific scale and health-related QoL questionnaire, which is less burdensome for respondents to complete (Jones et al. 2004). There are few studies that assessed the efficacy of laparoscopic surgery on improving the QoL of women with endometriosis (Minas and Dada 2014). In a study by Minas and Dada, 40 out of the 49 women (81.6%) completed the EHP-5 questionnaires. They reported that there was an improvement in the QoL after surgery, as lower scores were seen in the post-surgery EHP-5 scale. Specifically, the mean score before surgery was 46.9, and after surgery was 27.5 with a reduction by 41.3% (Minas and Dada 2014). In our study, we also used the EHP-5 questionnaire to evaluate disease-specific QoL scores. Although a significant reduction was observed regarding postoperative scores compared with preoperative scores in both groups, a significantly higher decrease was observed in NCH gel group.

Garry et al. conducted a study on 57 patients who underwent laparoscopic excision of invasive endometriosis and

determined QoL by using the SF-12 questionnaire (Garry et al. 2000). They reported a significant improvement in SF-12 physical score (44.8 vs. 51.9) four months after radical laparoscopic excision of deep endometriosis. Still, the mental health score of SF-12 failed to show any statistical improvement (47.1:48.4) (Garry et al. 2000). In our study, we observed no difference in preoperative SF-12 mental and physical parameters and postoperative 3rd-month physical scores between the study groups. On the other hand, significantly higher scores were found in favour of NCH gel group in the postoperative 3rd month SF-12 mental scores and postoperative 6th month SF-12 physical and mental scores. According to the preoperative VAS assessment, Garry et al. showed that the patients were experiencing a significant degree of pain (Garry et al. 2000). The mean VAS score of 53 patients who complained of dysmenorrhoea was eight preoperatively and four postoperatively. Non-menstrual pelvic pain improved 7–2 in 4 months. A similar reduction in the level of dyspareunia and rectal symptoms were reported as VAS scores decreased from 6 to 0 and 4 to 0, respectively (Garry et al. 2000).

In a recent review, dysmenorrhoea, chronic pelvic pain (CPP), and dyspareunia scores were evaluated preoperatively and in postoperative 3rd and 12th months by using VAS (Marqui 2015). The results show a significant improvement in pain symptoms after three months, and this remained significant throughout 12 months postoperatively. Furthermore, at the end of the study, 79% of the patients reported general satisfaction regarding pain relief. In our study, there was a significant reduction in dysmenorrhoea, dyschezia, dyspareunia at 3rd, and 6th months after surgery. Pelvic pain parameters in the 6th month were observed significantly lower in NHC gel group compared to the control group (Marqui 2015).

Limitations of the study include that we could not perform a second-look laparoscopy to evaluate the postoperative adhesions, and we should have increased the size of the study.

In conclusion, laparoscopic surgery alone or with saline instillation may provide improvement in postoperative pain scores and QoL. However, using NHC gel improves postoperative VAS scores. It provides better QoL scores by preventing possible adhesions that occurred after surgical denudation, ischaemia, desiccation, abrasion, and peritoneal trauma during laparoscopic DIE surgery.

Disclosure statement

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RESEARCH Open Access

Cross-linked hyaluronan gel inhibits the growth and metastasis of ovarian carcinoma

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Abstract

Background: The recurrence, metastasis and poor prognosis are important characteristics of ovarian carcinoma (OC), which are associated with exfoliation of cells from the primary tumor and colonization of the cells in pelvic cavity. On the other hand, the life quality of the patients undergoing surgical resection of OC was influenced by postoperative adhesions. Therefore, preventing postoperative implant tumor and adhesion may be effective methods to improve OC treatment. HyaRegen Gel, a cross-linked hyaluronan gel (CHAG), has been widely used as an anti-adhesive agent following pelvic operation in clinic. However, whether it can affect the implantation and growth of OC cells or not is still not clear.

Methods: Migration and invasion assays were applied to detect the effect of CHAG on migration and invasion of OC cells. Western blotting was performed to detect the phosphorylation/activation of EGFR and ERK, and the expression of PCNA and MMP7. Pull down assay was used to analyze the effect of CHAG on the activation of small G protein Rac1. Nude mice implantation tumor model was applied to observe the effect of CHAG on implantation tumor of OC cells.

Results: The results of in vitro experiments showed that CHAG suppressed both basic and EGF-induced migration and invasion of OC cells, blocked the activation of EGF-initiated EGFR activation, inhibited downstream signal transduction of EGFR, and decreased expression of proliferation and migration/invasion related proteins. Meanwhile, results of in vivo experiments showed that CHAG not only inhibited the formation of implantation tumor of OC cells but also delayed the of the growth of the tumors.

Conclusions: CHAG inhibited migration, invasion and proliferation of OC cells in vitro, and suppressed development of implantation tumor of OC in vivo. This made it as both anti-tumor and anti-adhesion agents.

Keywords: HyaRegen gel, Ovarian carcinoma, Migration, Invasion, Growth

Background

Ovarian cancer (OC) represents the most lethal malignancy of female reproductive system and the poor prognosis of OC is often attributable to late diagnosis, postoperative metastasis and recurrence [1, 2]. Many factors affect the prognosis, for example, post-operative adhesion [3, 4], malignant cells dropping off from the primary during surgical resection, and then implanting in abdominopelvic cavity. Therefore, taking measures to prevent **abscission of tumour cells** and choosing suitable anti-adhesion produces to reduce postoperative adhesions are equally important for favorable prognosis [5–7].

Hyaluronic Acid (HA) is made up of glucuronic acid and N-acetylglucosamine disaccharide units. It is abundant in the skin and connective tissues, with a turnover time from several hours to a few days according to tissues [8]. HA plays crucial roles in cell motility, cell adhesion, organization of tissue architecture, and cell proliferation processes [9, 10]. Owing to its biological properties, HA has several clinical applications in

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aesthetic surgery, dentistry, dermatology, ophthalmology and orthopedics [8]. **Clinical** research showed that injection of hydrogel containing HA and chitosan could serve as an ideal barrier to prevent postoperative tissue adhesion [11]. **A** randomly-controlled trial reported that a new crosslinked gel (CHAG) was efficient and safe in reducing adhesions after gynecologic laparoscopic surgeries in clinical practice [12]. Additionally, absorbable autocrosslinked hyaluronan gel could prevent intrauterine adhesion (IUA), decrease adhesion severity, and improve menopause postoperatively, indicating that this absorbable gel could be proposed as a barrier for preventing IUA after intrauterine procedures [13]. In a word, hyaluronan might be applied in resection of pelvic tumor for anti-adhesion, but it was still not clear whether the gel might be a potential stimulus for tumor metastasis and growth or not? In other words, is it safe for HA gel to be applied in tylectomy of pelvic tumor? Our previous study showed that CHAG suppressed colonization, growth and metastasis of gastric cancer cells [14]. However, it is still worthy to investigate whether CHAG has the similar effect on female pelvic tumor, such as OC, and whether CHAG is safe enough to be applied in OC operation for preventing post-operatic adhesion. Therefore, the present work was designed to address the above question. Our results showed that CHAG suppressed the migration and invasion of ovarian cancer cells, and delayed the development OC implantation tumor through blocking EGF-stimulated activation of EGFR and its downstream signal transduction.

Methods

Cell lines and mice

Human ovarian cancer cell line A2780 was purchased from CHI Scientific Inc. (Maynard, MA, USA) and human ovarian cancer cell line SKOV3 was purchased from Institute of Cell Biology (Shanghai, China). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% heat-inactivated fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA), 100 U/ml penicillin and 100 μg/ml streptomycin (HyClone; GE Healthcare Life Sciences, Logan, UT, USA), in a humidified incubator at 37 °C and 5% CO_2. Female nude BALB/c mice (with the age of 6 weeks) were purchased from the Animal Center of Yangzhou University (Yangzhou, Jiangsu Province, China) and maintained in the Animal Center of Jiangsu University in compliance with the Guide for the Care and Use of Laboratory Animals (NIH,76 FR 91; May 11, 2011).

Reagents

CHAG was provided by BioRegen Biomedical (Changzhou) Co., Ltd. (Changzhou, Jiangsu Province, China). Antibodies against β-actin (cat. no. sc-4778; dilution, 1:1000), Rac 1 (cat. no. sc-24,567; dilution, 1:500), and matrix metalloproteinases (MMP) 7 (cat. no. sc-80,205; dilution, 1:500) were purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). The antibodies against proliferating cell nuclear antigen (PCNA; cat. no. #13110; dilution, 1:1000), phosphorylation (p)-EGFR (Tyr1068) (cat. no. #3777; dilution, 1:1000), p-EGFR (Tyr1173) (cat. no. #4407; dilution, 1:1000), p-Akt (Ser473) (cat. no. #4060; dilution, 1:1000) and p-Erk1/2 (Thr202/Tyr204) (cat. no. #4370; dilution, 1:1000) were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). Horseradish peroxidase (HRP)-conjugated goat anti-rabbit and goat anti-mouse secondary antibodies (cat. nos. 111–035-003 and 115–035-003; dilution, 1:10,000) were purchased from Jackson Immuno Research Laboratories, Inc. (West Grove, PA, USA). Transwell plates were purchased from Corning Incorporated (Corning, NY, USA). ECM Gel was purchased from Sigma-Aldrich, Inc. (St. Louis, MO, USA). Recombinant Human EGF (rEGF) was purchased from PeproTech, Inc. (Rocky Hill, NJ, USA). Enhanced chemiluminescence (ECL) reagents were from EMD Millipore (Billerica, MA, USA).

Cell migration assay

Cells in logarithmic growth phase were cultured in FBS-free DMEM for 12 h. After trypsin digestion and centrifugation, 5×10^5/mL cells were re-suspended in different concentrations of CHAG in FBS-free DMEM medium. EGF (100 ng/mL) was added to stimulate the migration of the cells. **Three hundred μL** of the above cell suspension was added to the upper chamber of Transwell plate and 500 μL of DMEM containing 10% FBS was added to the lower chamber. The migration time for the cells was 12 h. At the end of the migration, the cells retained on the upper surface the membrane were swapped off and the cells migrated onto the lower surface of the membrane were stained with Giemsa and then counted under inverted microscopy.

Cell invasion assay

Cell invasion assays were same as described in cell migration assay except that the membrane of the upper chamber was coated with 60 μL of 1.125 μg/μL ECM Gel before adding cell suspension and the invasion time for the cells was 24 h.

The model of transplantation tumor of ovarian cancer cells in nude mice and treatment

Specific Pathogen Free (SPF) grade female BALB/c nude mice with weights of 8.76 ± 1.34 g were maintained in a SPF barrier system. In order to increase the rate of tumor formation, 1×10^7 cells suspended in 400 μL of PBS were implanted into one mouse by subcutaneous injection. After 2 weeks, the tumors were collected and ground into cell suspension with glass homogenizer. The

dispersed cells were continually cultured to an adequate number. After trypsin digestion, 1×10^7 cells suspended in 400 μL of PBS were implanted into each mouse by intra-peritoneal cavity injection. After 2 h, 400 μL PBS or 400 μL PBS containing **20 μg CHAG** were injected into the peritoneal cavity and the injection was repeated once every week until the 4th weeks. At the end of the experiment, the animals were euthanized, and the tumors were collected and weighed.

Western blotting

The A2780 and SKOV3 cells were accordingly treated and the whole cell lysates were harvested. All procedures of Western blotting were performed following the manufacturer's protocol (Bio-Rad, Hercules, CA). The primary antibodies were incubated over night at 4 °C, and the corresponding secondary antibodies were incubated for 1 h at room temperature. Protein bands were showed by ECL reagents.

"Pull-down" analysis of active small G protein Rac1

The activity of Rac1 was detected by Pull-down method. Cells (3×10^6/mL) were cultured in 10 cm dishes with serum-free DMEM overnight. Then, the cells were treated with 500 μg/mL or 1000 μg/mL CHAG for 1 h and then stimulated with 100 ng/ml EGF for 5 min. Finally, the cells were harvested with lysis buffer. After centrifugation at 12000 g, 4 °C for 10 min, 20 μL of supernatant was used as the control of the loading. The remaining supernatants were incubated with 100 μL of glutathione glucan beads with GST-Pak1 protein binding domain (GST-PBD) at 4 °C for 1 h. Finally, the activated Rac1 bound to the beads and total Rac1 in cell extracts was detected by Western blotting.

Statistical analysis

All experiments were performed in triplicate. Data are expressed as means ± standard deviation (SD). Statistical analysis was performed using a two-tailed ANOVA with SPSS statistical software. Student's t test was performed if equal variance was ascertained in two groups by F test. $P < 0.05$ was considered significant.

Results

CHAG inhibits basic and EGF-induced migration and invasion of ovarian cancer cells

The results of Transwell migration assay showed that compared to the control group, both 500 and 1000 μg/mL CHAG significantly inhibited the migration of A2780 and SKOV3 cells without stimulation of growth factors (Fig. 1a-d, $P < 0.05$). Furthermore, CHAG also inhibited the EGF-induced migration, and especially, 1000 μg/mL CHAG had a significant inhibitory effect (Fig. 1a-d, $P < 0.05$). Similar results were observed in invasion assay (Fig. 1e-h, $P < 0.05$). The above results indicated that CHAG had an inhibitory function on migration and invasion activities of ovarian cancer cells.

CHAG inhibits the growth of implantation tumor originated from ovarian cancer cells

To investigate the effect of CHAG on the in vivo growth of cancer cells, ovarian cancer cells were implanted into the pelvic cavity of nude mouse. CHAG was administrated into pelvic cavity 2 h later, and then the administration was repeated once per week for 4 weeks. At the end of the experiment, the mice were executed and the weights of the transplantation tumors were measured. The results showed that CHAG treatment significantly decreased the weight of transplantation tumor of A2780 and SKOV3 cells (Fig. 2), demonstrating that CHAG had an inhibitory effect on the growth of transplanted ovarian cancer cells.

CHAG inhibits the activation of EGFR and the EGF/EGFR-initiated signalings in ovarian cancer cells

Epidermal growth factor receptor (EGFR) is a key signaling molecule that drives cellular proliferation, migration, and invasion [15]. Some reports showed that high EGFR expression found in ovarian tumors [16] and EGFR signaling was involved in promoting ovarian cancer cell proliferation [17]. However, there is still some conflicting reports. For example, some data favored EGFR as a reliable marker of survival or responsiveness to therapy [18, 19] but others did not [20, 21], and some reports indicated that using EGFR inhibitors in ovarian cancer patient had not shown favorable clinical outcomes [15, 22, 23]. Thus, in this paper, **it was investigated** whether CHAG inhibited the development of ovarian cancer cells via abrogation of activation of EGFR and EGF/EGFR mediated signaling cascades. The result demonstrated both A2780 and SKOV3 expressed EGFR (Additional file 1: Figure S1), and EGF treatment (100 ng/mL, 5 min) led to an increase of Tyr1068 and Tyr1173 phosphorylation of EGFR, and pretreatment with CHAG (500 and 1000 μg/mL) efficiently inhibited the EGF-induced phosphorylation of EGFR (Fig. 3a, b), indicating that CHAG inhibited EGF-induced activation of EGFR. Additionally, Western blotting results showed that EGF treatment (100 ng/mL, 5 min) caused significant increase of phosphorylation/activation of Akt and ERK, which were main signaling components downstream of EGFR. Treatment with CHAG blocked EGF-induced phosphorylation/activation of Akt and ERK (Fig. 3a, b). These results confirmed that CHAG could inhibit OC cells through blocking activation of EGFR and its downstream signalings.

CHAG inhibits the activation of small G protein Rac1

Small G protein Rac1 is the **chief** member of Rho family which play important role in regulating migration of

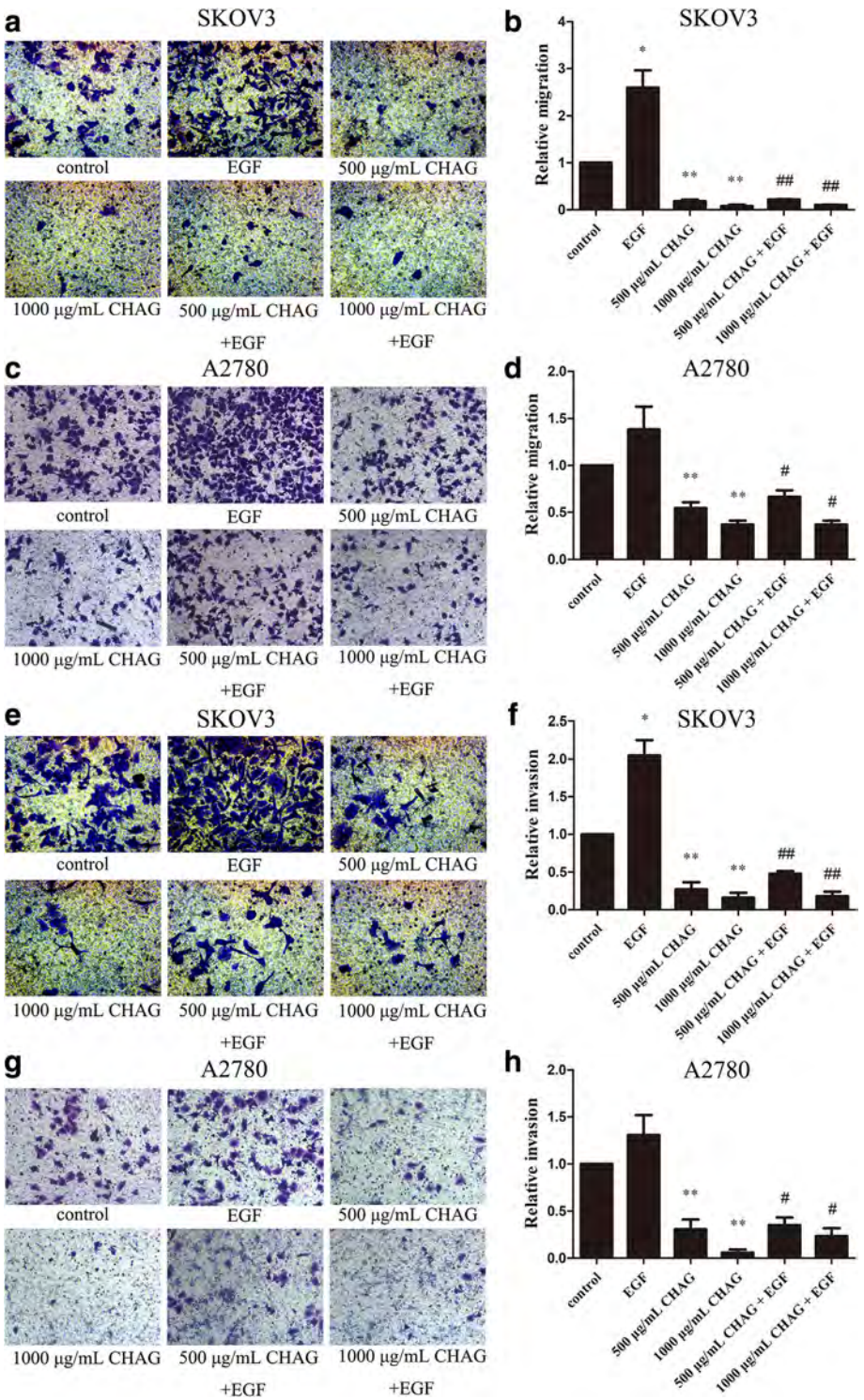

Fig. 1 CHAG inhibits EGF-induced migration and invasion activities of ovarian cancer cells. **a**-**d** Migration activity of A2780 and SKOV3 cells. **e**-**h** Invasion activity of A2780 and SKOV3 cells. **a**, **c**, **e** and **g** were representative photomicrographs of cells stained by Giemsa (200×). **b**, **d**, **f** and **h** represent the folds of cells' migration or invasion in the corresponding groups. Data are showed as means ± SD from 3 independent experiments. (*$P < 0.05$, **$P < 0.01$, compared with control group; $^{\#\#}P < 0.01$, compared with EGF group)

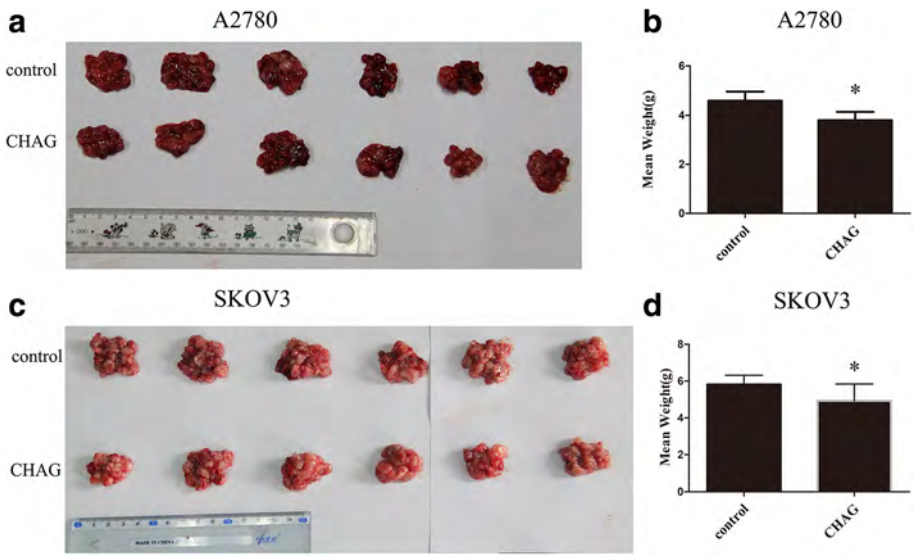

Fig. 2 CHAG inhibits growth of ovarian cancer cell in pelvic cavity. **a** and **c** The tumors from the control and CHAG groups were shown. **b** and **d** The weight of the tumors in corresponding group. The data were shown as means ± SD. (*$P < 0.05$, compared with control group)

cancer cells [24]. Some of EGFR-mediated signal transduction can activate them and thereafter stimulate cell migration [25–27]. Therefore, it is worthy to investigate the inhibitory effect of CHAG on activation of Rac1. In this study, the "Pull-down" assay was performed to detect the level of active (GTP-bound) Rac1. The results showed that treatment with EGF (100 ng/mL, 5 min) increased the amount of GTP-bound/active Rac1. Pretreatment with CHAG efficiently restrained the stimulating effects of EGF on the activation of the small G protein in both A2780 and SKVO3 (Fig. 3g, h).

CHAG inhibits the expression of metastasis and proliferation related proteins.

To get more evidence for the inhibition of CHAG on the migration, invasion and proliferation of OC cells, the proliferation marker, proliferating cell nuclear antigen (PCNA) [28] and tumor-metastasis associated proteins, Matrix metalloproteinase-7 (MMP-7) [29] were detected by Western blotting. The results showed that the expressions of PCNA and MMP7 were increased by EGF treatment (100 ng/mL, 24 h). Applying CHAG (500 or 1000 μg/mL) with EGF at the same time efficiently inhibited EGF-induced expressions of MMP-7 and PCNA (Fig. 3c–f). These results indicated that CHAG could inhibit the expression of migration and proliferation related proteins.

Discussion

OC is one of the major causes of gynecologic cancer-related death, with an overall five-year survival rate of ~ 45% and an overall 10-year survival rate of 35% in the USA [30]. Currently, the mainstay of OC treatment includes cytoreductive surgery and platinum-based chemotherapy [30]. It is important to perform complete surgery for OC patients, and platinum resistance is the crucial problem in the treatment of these patients. In addition, postoperative tumor metastasis and recurrence cannot be ignored. There are four ways for OC cells to metastasize: direct spreading to the adjacent parts, lymph node metastasizing, blood metastasizing, and planting to other location. During the operation of tumor resection, it is easy to cause tumor cells to fall off and plant into the abdominal cavity or pelvic cavity. In addition to the above problems, adhesion is the most important complication of abdominopelvic surgery, causing some short- and long-term problems, **such as** infertility, small bowel obstruction and chronic pelvic pain [31]. The main strategies for adhesion prevention in gynecological surgery are focused on improvement of surgical technique and use of anti-adhesive agents, which fall into two major categories: pharmacological agents and barriers [32], and HA is one of them. HA is a naturally component of many body tissues and fluids, where it provides physically supportive and mechanically protective roles [33]. Various combinations of HA have been used for adhesion prevention. However, there were still some unsatisfactory results owing to rapid degradation of native HA. So, modification might be one of the effective ways to prolong the half-life of HA, such as crosslinking modification. In fact, CHAG applied in this paper had been reported to significantly reduce adhesion in abdominopelvic cavity after gynecological laparoscopic surgeries [12, 34]. However, since there was no data elucidating the effect of CHAG on the cells of gynecologic tumor, it is still unclear whether CHAG is safe enough for preventing postoperative adhesion in pelvic surgery of this kind of tumor.

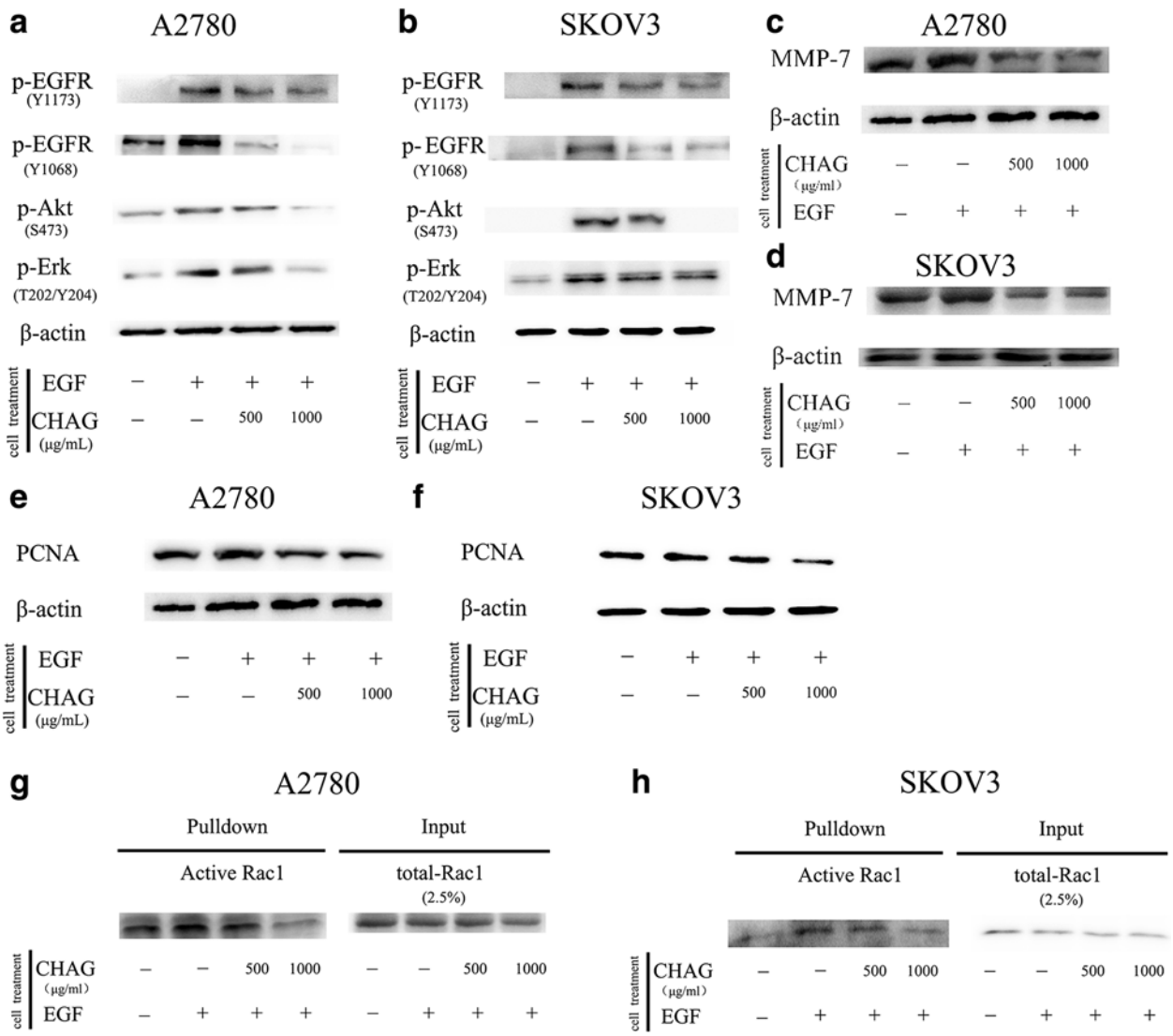

Fig. 3 CHAG blocks activation of EGFR and its downstream signaling molecules, and inhibits EGF-induced expression of MMPs and PCNA in OC cells. **a** and **b** CHAG inhibited phosphorylation/activation of EGFR, Akt and ERK in A2780 and SKOV3 cells. The cells were cultured in serum free DMEM overnight and treated with 500 or 1000 μg/mL CHAG solutions for 1 h and then treated with 100 ng/ml EGF for 5 min. The cellular lysates were subjected to Western blotting. **c** and **d** Detection of the expression of MMP7 in A2780 and SKOV3 cells by Western blotting. In EGF group, the cells were treated with 100 ng/ml EGF for 24 h. In the CHAG + EGF groups, the cells were treated with 500 or 1000 μg/mL CHAG and 100 ng/mL EGF for 24 h. The cells were harvested and the lysates were subjected to Western blotting with anti-MMP7 antibodies. **e** and **f** Western blotting detection of the expression of PCNA in A2780 and SKOV3 cells. The cells were treated same as described in panel **c** and **d**. **g** and **h** CHAG blocked the activation of Rac1. The pull-down results were representatives of three independent experiments

In the in vitro study of this paper, we found that CHAG could inhibit the migration and invasion activities of ovarian cancer cells. And in the in vivo study of this paper, CHAG was administrated 2 h after inoculating ovarian cancer cells into pelvic cavity, which simulated the period that the inoculated cancer cells had been implanted and begun to proliferate in peritoneal cavity. After the first administration, CHAG was administrated weekly until the 4th week, which could simulate the period of mid-term growth of the implantation tumors. The exciting results was that the growth of the transplantation tumors was also efficiently inhibited, indicating that CHAG might be safe to be applied for preventing postoperative adhesion in surgical resection of OC.

In clinical, the effects of HA on tumor are different according to diverse molecular weights. HA synthase (HAS) generates predominantly high molecular weight HA (HMW-HA) with molecular weight between 200 and 2000 kDa, while the degradation generates different-sized

HA polymers (or fragments), such as low molecular weight HA (LMW-HA; < 200 kDa) and HA oligomers [9]. In general, LMW-HA has pro-cancerous effect [35], whereas HMW-HA controls normal homeostasis and displays anti-cancerous activity [34, 35]. Furthermore, there are many controversial findings, which are related to the lack of consensus on size definition, the polydispersity of HA commercial products, and the use of HA from different animal or different tissues. And all the notices should be taken into account whenever a new study on HA is undertaken. The research results of this paper showed that crosslinked hyaluronan, as a HA polymer with boundless molecular weight, had an anti-tumor effects, which was in line with most previous data.

Next, it is worthy to investigate how does CHAG affect proliferation and metastasis activity of cancer cells. Owing to its physical adhesive characteristic, CHAG is speculated to cover the cells and block the interaction between ligands and their receptors. So, the effect of CHAG on the activation of the key growth factor receptor EGFR and its associated signaling molecules were detected. The result indicated that CHAG effectively blocked the EGF-induced phosphorylation/activation of EGFR, inhibited the EGF/EGFR-initiated activation of ERK, Akt and Rac1, and decreased the EGF-induced expression of PCNA and MMP7, suggesting that CHAG inhibited the growth and metastasis of OC cells via blocking the activation of EGFR and subsequent signaling. Our results are similar to the previous research that EGFR is reported to overexpressed in most ovarian cancer [36], and the activation of the EGFR pathway has impact on invasion and metastasis as well as cell survival through the MAPK/ERK, PI3K/AKT and **Rac1** pathways [37–41].

Conclusion

In conclusion, our results demonstrated that CHAG could suppress the development of OC though blocking the activation of EGF-induced activation of EGFR and its downstream signal transduction. This provides evidence for safe application of CHAG in preventing post-operative adhesion of surgical resection of OC.

Additional file

Additional file 1: Figure S1. The expression of EGFR in A2780 and SKVO3 cells. The celluar lysates were subjected to Western blotting with antibody against EGFR. Expression of β-actin was used at the same time as loading control. (TIFF 676 kb)

Abbreviations

CHAG: Cross-linked hyaluronan gel; DMEM: Dulbecco's modified Eagle's medium; EGFR: Epidermal Growth Factor Receptor; GST-PBD: GST-Pak1 protein binding domain; HA: Hyaluronic Acid; HAS: HA synthase; HMW-HA: High molecular weight HA; IUA: Intrauterine adhesion; LMW-HA: Low molecular weight HA; MMP-7: Matrix metalloproteinase-7; OC: Ovarian carcinoma; PCNA: Proliferating cell nuclear antigen; SD: Standard deviation; SPF: Specific Pathogen Free

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Availability of data and materials

None.

Authors' contributions

YCC, JZG and YW: designed the project; JP performed experiments; JP, HQ, YT analyzed the data; YW and YCC wrote the manuscript; LJ, YT, RXS and PCJ contributed to performing data analysis and assisted with manuscript preparation. All authors read and approved the final manuscript.

Ethics approval and consent to participate

All animal procedures were approved by the guidelines of the Jiangsu University Administrative Panel on Laboratory Animal Care. The study does not include any human samples.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Research Paper

Cross-linked hyaluronic acid gel inhibits metastasis and growth of gastric and hepatic cancer cells: *in vitro* and *in vivo* studies

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ABSTRACT

Cross-linked hyaluronic acid gel (CHAG) has been used to prevent postoperative adhesion of abdominal tumorectomy. However, its effect on tumor cells is still unknown. This paper was designed to investigate the effect of CHAG on metastasis and growth of tumor cells. Migration and invasion assays, Western blotting, pull down assay, siRNA interference, and nude mice implantation tumor model were applied in this study. The results of *in vitro* experiments with gastric cancer cell line AGS and hepatic cancer cell line HepG2 showed that CHAG inhibited the migration and invasion activities, the MAPK and PI3K/Akt mediated signaling, the activation of small G proteins Rac1 and RhoA, and the expression of MMPs and PCNA initiated by EGF, through blocking the activation of EGFR. CHAG also had inhibitory effect on activation of other membrane receptors, including integrin and VEGFR. When the expression of hyaluronic acid receptors (CD44 or RHAMM) was interfered, the above inhibitory effects of CHAG still existed. *In vivo* experimental results showed that CHAG suppressed colonization, growth and metastasis of gastric cancer cell line SGC-7901 in peritoneal cavity of nude mice. In conclusion, CHAG had inhibitory effect on tumor cells, through covering cell surface and blocking the interaction between extracellular stimulative factors and their receptors.

INTRODUCTION

Tumorectomy is one of the effective therapies for tumors with suitable stages. However, there are primarily two challenges which needed to be solved for performing tumorectomy. Post-operational adhesion is the first one. The patients with adhesion may have chronic pain and bowel abstraction, and the life quantity of them will be made worse. The second one is tumor metastasis, which is one of the main causative factors for poor prognosis and short survival time of the patients undergoing tumorectomy [1]. Postoperative adhesion in peritoneal cavity happens in more than 93% patients received abdominal tumorectomy [2]. The peritoneal cavity is also a well-known metastatic site for intra-abdominal malignancies of several organs, such as stomach, liver, colon, pancreas and rectum [3]. Therefore, prophylactic application of anti-adhesion agentia has been proposed. Moreover, if the adhesion preventive material has anti-tumor effect, it will be more suitable to the application in these patients.

Hyaluronic acid (HA), either native or crosslinking modified, has been broadly used to prevent postoperative adhesion with varies level of successes [4–6]. HA is a non-sulfated glycosaminoglycan consisting of repeated disaccharide units (a-1,4-D-glucuronic acid and β-1,3-*N*-acetyl-D-glucosamine) and presents in all connective tissues as a major constituent of extracellular matrix. HA has been reported with unique role in wound healing [7, 8]. Cluster designation 44 (CD44) and receptor for hyaluronic acid mediated motility (RHAMM) are the receptors of HA. Through binding with these receptors, HA could regulate cell biological activities by activating several signaling pathways, including the transforming growth factor β (TGF-β) mediated, Rho GTPase mediated, and focal adhesion kinase (FAK) mediated pathways [9–11]. In some cancers, HA levels were correlated well

with malignancy and poor prognosis. Hence, HA is often identified as a tumor marker for some cancers and used to monitor the progression of the diseases [12, 13].

Research data have shown that HA with different molecular weight/size had different functions. Most studies indicated that HA with low molecular weight promoted tumor development while HA with high molecular weight had opposite effect [14–16]. In clinical, native HA has been used for anti-adhesion after surgery with unsatisfactory results. The fluid feature and rapid degradation of native HA (usually within 48 hours *in vivo*) may contribute to the primary reasons for the failure. However, crosslinking modification is an effective way to enhance the viscosity of HA and reduce the degradation of it, causing the formation of HA hydrogel. This gel can cover the traumatized tissue surface during the critical period of wound healing and prevent adhesion [17]. Clinical study showed that cross-linked HA gel (CHAG) could significantly reduce adhesion in abdominopelvic cavity after gynecological laparoscopic surgeries [18]. However, there is still a lack of information about whether CHAG is safe enough for preventing postoperative adhesion of peritoneal tumorectomy. Or in other words, the effect of this gel on tumor metastasis and growth is not clear while it is applied in preventing post-operative adhesions of tumorectomy. The main aim of this study was to evaluate the effect of CHAG on cancer cell growth and metastasis and to explore the related action mechanism via *in vitro* and *in vivo* experiments.

RESULTS

CHAG inhibits basic and EGF-induced migration and invasion activities of gastric and hepatic cancer cells

The results of Trans-well migration and invasion assays showed that CHAG with concentrations of 50 μg/ml, 125 μg/ml, 250 μg/ml, 500 μg/ml and 1000 μg/ml inhibited the basic migration and invasion activities of both AGS and HepG2 cells, with a dosage-dependent pattern (Figure S1). Furthermore, when the migration and invasion activities of AGS and HepG2 cells were stimulated by EGF treatment (100 ng/ml, 12 h), CHAG at the concentrations of 500 μg/ml and 1000 μg/ml significantly inhibited the increase of migration and invasion activities induced by EGF treatment (Figure 1). These results indicated that CHAG had inhibitory effect on both the basic and the EGF-induced migration and invasion activities of AGS and HepG2 cells.

CHAG inhibits colonization and growth of gastric and hepatic cancer cells in peritoneal cavity of nude mice

In nude mouse transplantation tumor model, co-injection of CHAG (500 μg/ml) together with transplanted cancer cells completely inhibited the formation of transplantation tumor of SGC-7901 gastric cancer cells (Figure 2A and 2B) and dramatically decreased the weight of transplantation tumor of HepG2 hepatic cancer cells (Figure S2A and S2B). These results indicated that CHAG had inhibitory effect on the attachment/colonization of the cancer cells in peritoneal cavity.

To investigate the effect of CHAG on the early growth of cancer cells, the nude mice were given a one-time peritoneal cavity injection of CHAG (200 μg per mouse, diluted in 400 μl PBS, with a concentration of 500 μg/ml) 2 hours after intra-peritoneal implantation of SGC-7901 gastric cancer cells. To investigate the effect of CHAG on the mid-term growth of transplanted cancer cells, the nude mice were given the first intra-peritoneal cavity injection of CHAG (200 μg per mouse, 500 μg/ml) at the 7th day after the cancer cell implantation and then the injection was repeated weekly for 7 weeks. Both injections significantly decreased the weight of transplantation tumors of SGC-7901 cells (Figure 2C, 2D, 2E and 2F). With HepG2 cells, the experiment of inhibition on early growth was performed and the result was similar to those of SGC-7901 cells (Figure S2C and S2D). These results demonstrated that CHAG inhibited both early growth and mid-term growth of transplanted cancer cells.

CHAG inhibits the activation of cell membrane receptors of gastric and hepatic cancer cells

Integrin is the transmembrane receptor associated with cell movement through bridging cell-cell and cell-extracellular matrix (ECM) interactions. One integrin molecule consists of one α subunit and one β subunit and integrin α5β1 is fibronectin receptor [19]. To investigate the effect of CHAG on the activity of integrin, the cells were treated with fibronectin and CHAG, and the change of phosphorylation of integrin β1 was detected by Western blotting. The results showed that treatment with fibronectin (1 μg/ml, 15 min) caused obvious increase of phosphorylation of integrin β1. Pre-treatment with CHAG (1000 μg/ml, 1 h) effectively inhibited fibronectin-induced phosphorylation of integrin β1 (Figure 3A and 3B). These results indicated that CHAG could inhibit fibronectin-induced activation of integrin α5β1.

Other cell surface receptors studied in this experiment included EGFR and VEGFR, which were receptor tyrosine kinases (RTKs) associated with tumor progression. Western blotting with antibodies against Tyrosine 1068 (Tyr1068) or Tyrosine 1173 (Tyr1173) phosphorylated EGFR was applied to detect the phosphorylation/activation of EGFR. The result demonstrated that EGF treatment (100 ng/ml, 5 min) led to significant increase of Tyr1068 and Tyr1173 phosphorylation of EGFR, and pre-treatment with CHAG (1000 μg/ml, 1 h) efficiently hindered the EGF-induced phosphorylation of EGFR (Figure 3C–3F), indicating

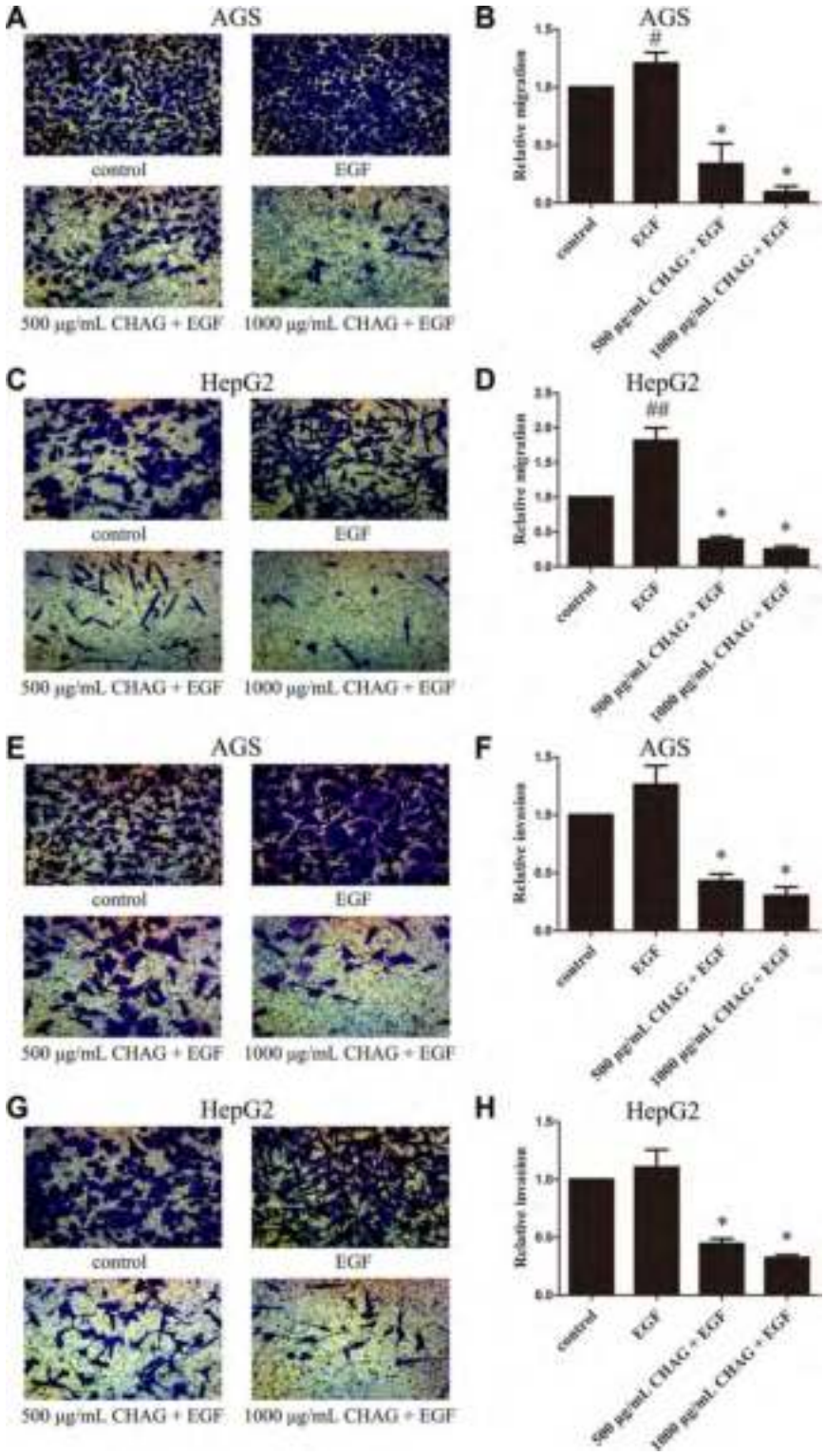

Figure 1: CHAG inhibits migration and invasion activities of gastric and hepatic cancer cells. (**A**–**D**) Migration activity of AGS and HepG2 cells. The cells were serum starved overnight, and then divided into Control, EGF, 500 μg/ml CHAG + EGF, and 1000 μg/ml CHAG + EGF groups. In the EGF group, the cells were treated with EGF (100 ng/ml). In the CHAG+ EGF groups, the cells were treated with CHAG (500 μg/ml and 1000 μg/ml respectively) and EGF (100 ng/ml). The migration time was 12 h. (**E**–**H**) Invasion activity of AGS and HepG2 cells. Cell treatments were same to migration assay, except the invasion time was 24 h. A, C, E, and G were representative images of migrated or invaded cells stained by Giemsa (×200). B, D, F, and H were the relative migration or invasion activities of the cells in the corresponding groups. The data shown were the means ± SD from 5 independent experiments, each performed in duplicate. ($^{\#}P < 0.05$, $^{\#\#}P < 0.01$, compared with control group; $^{*}P < 0.01$, compared with EGF group).

that CHAG inhibited EGF-induced activation of EGFR. Furthermore, CHAG could also inhibit VEGF-induced phosphorylation/activation of VEGFR-2 (Figure S3).

CHAG inhibits cellular activities downstream of membrane receptors

Western blotting results showed that EGF treatment (100 ng/ml, 5 min) caused significant increase of phosphorylation/activation of Akt and ERK, which were main signaling components downstream of EGFR. Treatment with CHAG (1000 μg/ml, 1 h) inhibited the stimulating effect of EGF on the activation of these signaling components, confirming the inhibition of CHAG on EGF/EGFR initiated signal transductions (Figure 4A, 4B, 4C, and 4D).

"Pull-down" assay was performed to detect the inhibitory effect of CHAG on activation of small G protein Rac1 and RhoA. The results showed that treatment with EGF (100 ng/ml, 5 min) or LPA (1 μM, 5 min) increased the

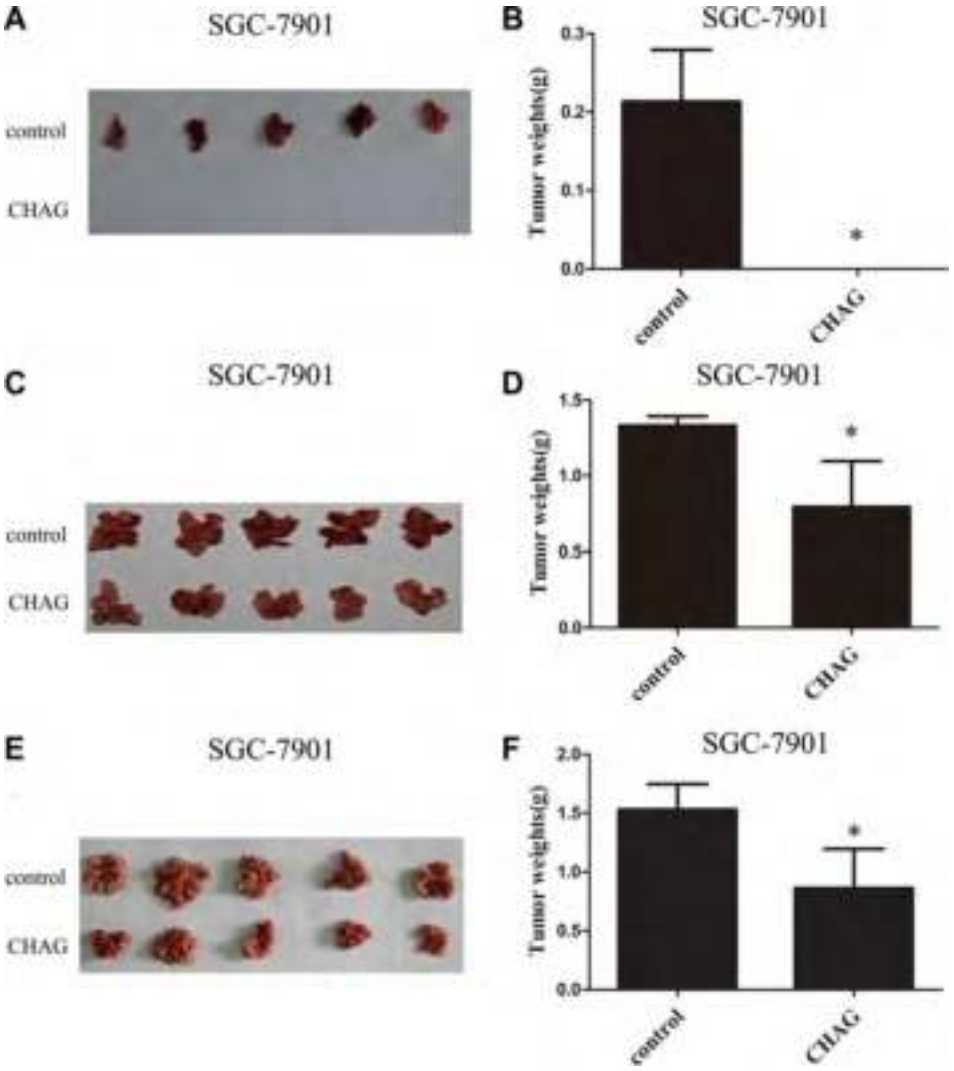

Figure 2: CHAG inhibits colonization and growth of gastric cancer cells in peritoneal cavity. (**A** and **B**) The inhibition of CHAG on the colonization of gastric cancer cells in peritoneal cavity. Ten million SGC-7901 cells (suspended in 400 μl PBS) with or without CHAG (500 μg/ml) were injected into peritoneal cavity of nude mouse. Twenty five days later, the mice were executed, the tumors were excised, and the weights of the tumors of the different groups were calculated. (**C** and **D**) The inhibition of CHAG on the early growth of gastric cancer cells. Ten million SGC-7901 cells suspended in 400 μl PBS were injected into peritoneal cavity of nude mice. Two hours late, 400 μl CHAG solution (at concentration of 500 μg/ml) was injected into the cavity once only. The mice were fed normally for 8 weeks and then were executed and the tumors were collected and weighed. (**E** and **F**) The inhibition of CHAG on mid-term growth of gastric cancer cells. The cells were given to the mouse same as described in (C and D) Seven days later, 400 μl CHAG solution (at concentration of 500 μg/ml) was injected into peritoneal cavity of the mouse and the injection was repeated once a week. After 7 weeks, the mice were executed, the tumors were collected and weighed. A, C, and E were images of tumors from the mice in control and CHAG groups. B, D, and F were results of weight analysis of the tumors in corresponding group. The data shown were means ± SD. (*$P < 0.01$, compared with the control group).

amount of GTP-bound/active Rac1 or RhoA respectively. Pre-treatment with CHAG (1000 μg/ml, 1 h) efficiently restrained the stimulating effects of EGF and LPA on the activation of the small G proteins (Figure 4E and 4F).

In addition, Western blotting results showed that the expressions of matrix metalloproteinase 2 (MMP2) and metalloproteinase 7 (MMP7) and proliferating cell nuclear antigen (PCNA) were increased by EGF treatment (100 ng/ml, 24 h). Applying CHAG (500 or 1000 μg/ml) with EGF at the same time efficiently inhibited the stimulating effect of EGF on the expressions of MMPs and PCNA (Figure 4G–4J). These results indicated that CHAG could inhibit the expression of migration and proliferation related proteins.

The tumor-inhibitory effect of CHAG is not related to its binding with HA receptors

siRNA interference technology was applied to down-regulate the expression of HA receptors, including CD44 and RHAMM. When the expressions of CD44 and RHAMM in AGS and HepG2 cells were decreased by

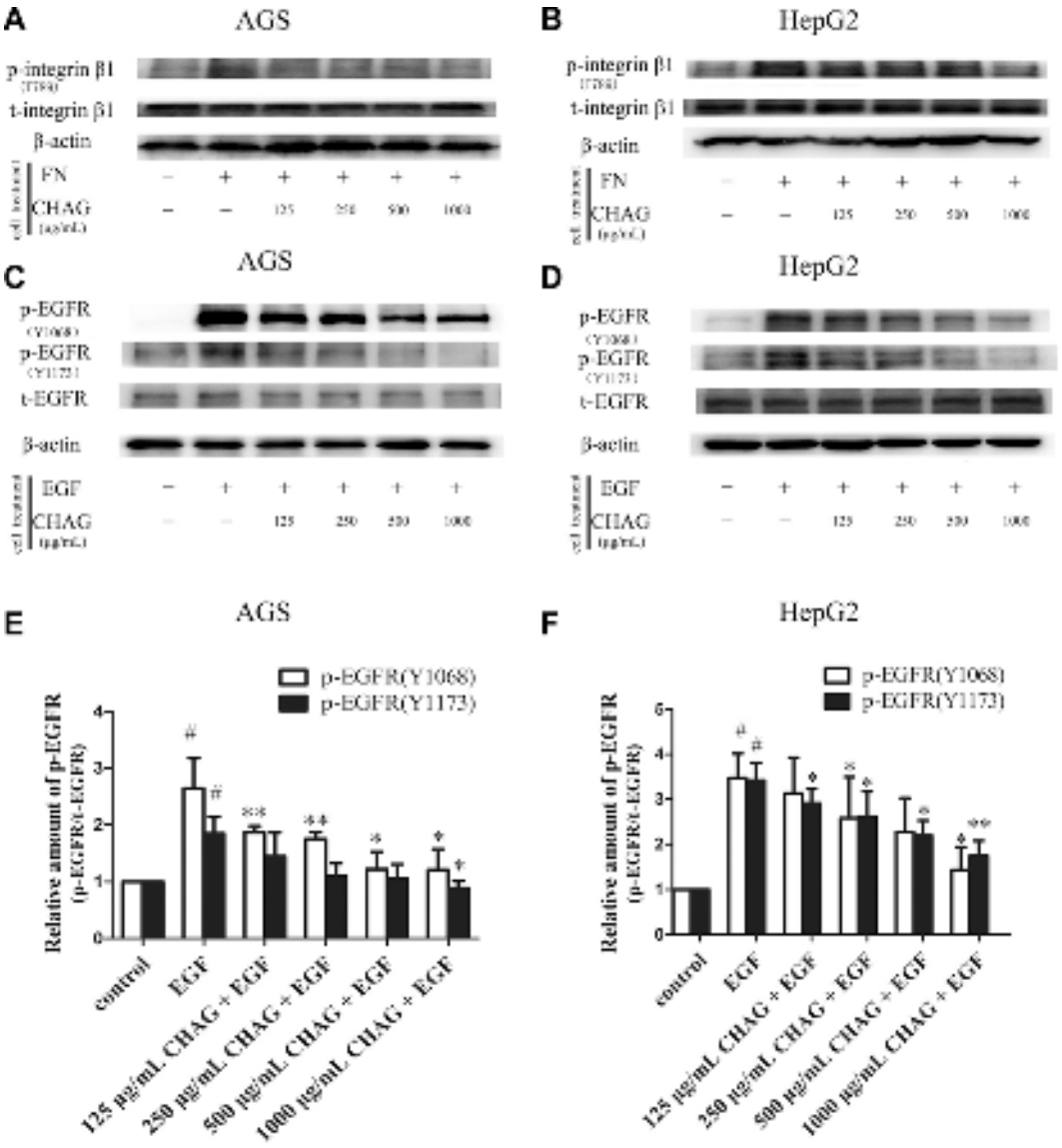

Figure 3: CHAG inhibits activation of membrane receptors in gastric and hepatic cancer cells. (**A** and **B**) The inhibition of CHAG on phosphorylation/activation of Integrin β1 in AGS cells and HepG2 cells. The cells were serum starved overnight and treated with fibronectin (FN, 1 μg/ml) for 15 min, or with various CHAG solutions (at concentrations of 125, 250, 500, 1000 μg/ml respectively) for 1 h and then with FN (1 μg/ml) for 15 min. (**C** and **D**) The inhibition of CHAG on phosphorylation/activation of EGFR in AGS cells and HepG2 cells. The cells were serum starved overnight and treated with EGF (100 ng/ml) for 5 min, or with various CHAG solutions (at concentrations of 125, 250, 500, 1000 μg/ml respectively) for 1 h and then with EGF (100 ng/ml) for 5 min. A–D were representative Western blotting results of three independent experiments. (**E** and **F**) were results of densitometry analysis of Western blotting results. ($^{\#}P < 0.05$, compared with control group; $^{*}P < 0.05$, $^{**}P < 0.01$, compared with the EGF group).

siRNA, the inhibitory effect of CHAG on phosphorylation/activation of EGFR still existed (Figure 5). These results suggested that CHAG did not actualize its inhibitory effect through binding with CD44 or RHAMM.

DISCUSSION

In this study, we carried out both *in vitro* and *in vivo* experiments to investigate the effect of CHAG on migration, invasion, growth and implantation of gastric and hepatic cancer cells. In *in vitro* experiment, the results showed that CHAG was able to inhibit the migration and the invasion activities of gastric and hepatic cancer cells. In *in vivo* study, we investigated whether CHAG might affect colonization and growth of gastric and hepatic cancer cells, using a well-defined intra-peritoneal tumor implantation model. The results showed that CHAG, when administrated through injection with the cancer cells at the same time, effectively suppressed the colonization of the cancer cells in peritoneal cavity. When the cancer cells were inoculated into peritoneal cavity first and CHAG was injected into the cavity 2 hours or 7 days later, the administrations simulated the application of CHAG in early growth and mid-term growth of the transplanted cells respectively, the growth of the transplantation tumors was also efficiently inhibited. These results confirmed that CHAG had a definite anti-tumor effect when applied both *in vitro* and *in vivo*.

The effect of HA and polymerized HA on tumor cells had been a disputable topic. Some reported data favored the application of them in prevention of adhesion. For example, Sikkink *et al.* found that bio-absorbable HA membrane resulted in a significant reduction of adhesions, but had no obvious impact on the intra-peritoneal tumor implantation and growth in mice and rats [20]. Haverlag *et al.* also reported that HA-based coating solution had no appreciable effect on intra-abdominal tumor growth in rats and mice [21]. The results from Tian *et al.* suggested that high-molecular-mass HA could induce cancer resistance in naked mole rat [22]. However, other reports warned pernicious effect of the materials. For example, Tan *et al.* reported that sodium hyaluronate enhanced tumor metastatic potential *in vitro* and *in vivo*, suggesting that application of sodium hyaluronate to avoid adhesions might potentiate intra-peritoneal tumor growth after colorectal cancer surgery [23]. The above difference of conclusions may be due to that the biological responses triggered by HA depend on the HA polymer length. It was reported that lower molecular weight HA promoted tumor growth [24], while high molecular weight HA (>1,000 kDa) had inhibitory effect on the tumor [22, 25]. Based on the above data, we speculated that as a HA polymer with boundless molecular weight, CHAG might have an anti-tumor effects similar to the high molecular weight HA. In this paper, our speculation has been proved.

As extracellular substance, how does CHAG affect proliferation and metastasis activity of cancer cells? To answer this question, we investigated whether CHAG affected membrane receptor-initiated cell biological activities. The results demonstrated that CHAG treatment efficiently blocked the phosphorylation/activation of EGFR, integrin and VEGFR, inhibited the EGF-induced signaling of MAPK/ERK, PI3K/Akt and Rac1 mediated pathways, and diminished the EGF-induced expression of proliferation and migration related proteins. LPA-induced RhoA activation was also inhibited by CHAG. These results confirmed that CHAG blocked the activation of some cell membrane receptors, inhibited the downstream signal transduction, and finally down-regulated the expression of related proteins, suggesting that blocking the activation of the receptors was the mechanism for CHAG to inhibit the activities of cancer cells.

The next worthy question was "why CHAG had such a wide-range inhibitory effect on the receptors?". We put forward two assumptions for answering this question. One was that CHAG might specifically bind and cause the activation of HA receptors and then exert its inhibitory effect on other membrane receptors. The other one was that CHAG with sticky property might prevent all of the interactions between the stimulating factors and their receptors by wrapping around the cells. To clarify whether the anticancer effect of CHAG was via binding/activating HA receptors, the cells were transfected with siRNA to decrease the expression of CD44 or RHAMM and the change of inhibitory effect of CHAG was investigated. The results showed that when the expression of CD44 or RHAMM was significantly decreased, the inhibitory effect of CHAG on the activation of EGFR still existed, indicating that the inhibitory effect of CHAG on the EGFR activation was not through binding with and activating HA receptor CD44 and RHAMM. Furthermore, there was no research data indicating the connection between CD44/RHAMM and other membrane receptors such as EGFR, integrin, VEGFR and LPA receptor. Therefore, it was likely that CHAG, owing to its physical sticky property, wrapped the cells and prevented the interaction between stimulating factors and their corresponding receptors, and therefore blocked the activation of the receptors, contributing to the inhibition on migration, invasion and proliferation activities of the cancer cells.

The most noteworthy results of this study came from the *in vivo* experiment with implantation tumor model. The results indicated that when CHAG was administrated together with gastric cancer cells through intra-peritoneal injection, the formation of implantation tumors were completely abolished in gastric cancer cells and dramatically decreased in hepatic cancer cells, indicating the CHAG could efficiently hinder the attachment/colonization of disseminated tumor cells in peritoneal cavity. To investigate the effect of CHAG on the growth

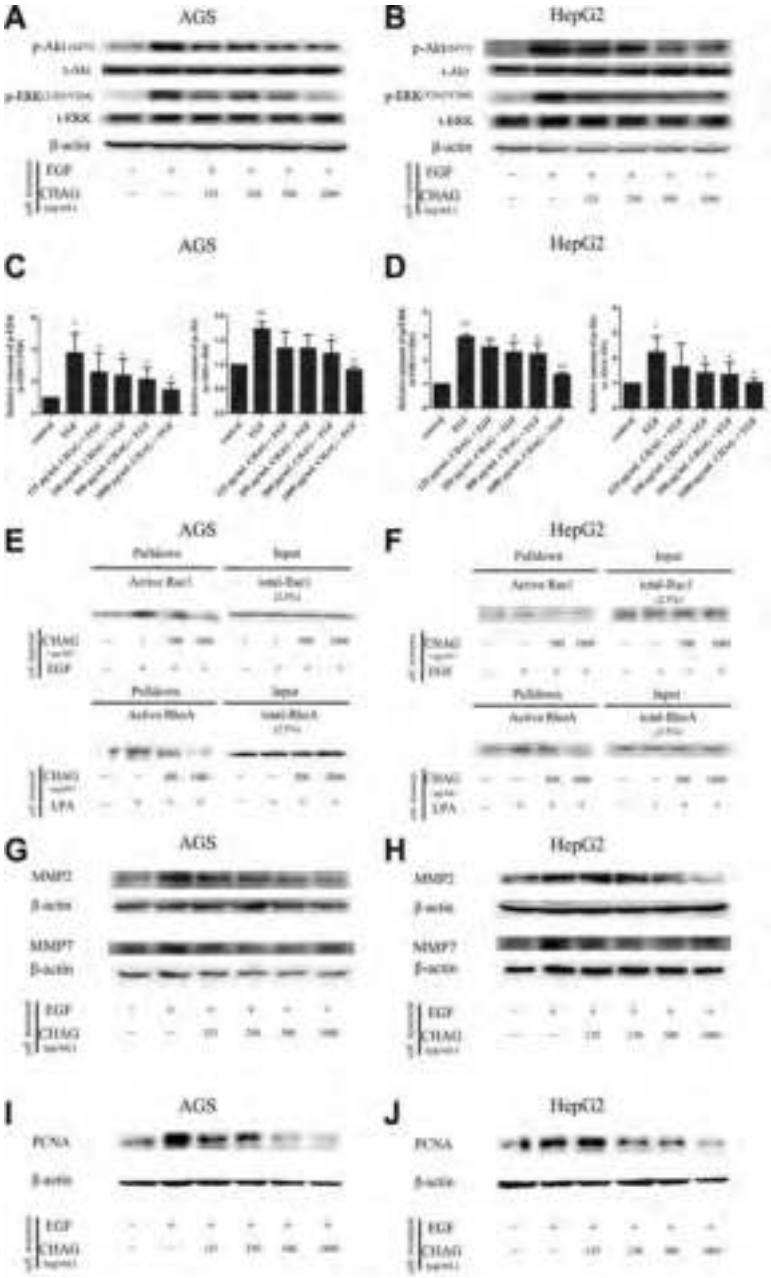

Figure 4: CHAG blocks the activation of downstream signaling molecules of EGFR and inhibits EGF- induced expression of MMPs and PCNA. (**A**–**D**) The inhibition of CHAG on the phosphorylation/activation of Akt and ERK in AGS cells and HepG2 cells. The cells were treated same as in Figure 3 (Panel C). The cellular lysates were subjected to Western blotting with antibodies against phosphorylated Akt (p-Akt) or phosphorylated ERK (p-ERK). Total Akt (t-Akt), total ERK (t-ERK) and β-actin were detected as loading control. A and B were the representative Western blotting results of three independent experiments. C and D were results of densitometry analysis of the corresponding Western blotting results. ($^{\#}P < 0.05$, $^{\#\#}P < 0.01$, compared with control group; $^{*}P < 0.05$, $^{**}P < 0.01$ compared with EGF group). (**E** and **F**) CHAG blocked the activation of Rac1 and RhoA in AGS and HepG2 cells. For detection of Rac1 activation, the cells were serum starved overnight, treated with EGF (100 ng/ml, 5 min), or with CHAG solutions (at concentrations of 500, 1000 μg/ml respectively) for 1 h and then with EGF (100 ng/ml, 5 min); For detection of RhoA activation, the cells were serum starved overnight, treated with LPA (1 μM, 5 min), or with CHAG solutions (at concentrations of 500, 1000 μg/ml respectively) for 1 h and then with LPA (1 μM, 5 min). The level of active Rac1 or RhoA was analyzed by ''Pull-down'' method. The results were representatives of three independent experiments. (**G** and **H**) Detection of the expression of MMP2 and MMP7 in AGS and HepG2 cells by Western blotting. In EGF group, the cells were treated with EGF (100 ng/ml, 24 h). In the CHAG + EGF groups, the cells were treated with CHAG at various concentrations (125, 250, 500, 1000 μg/ml respectively) and EGF (100 ng/ml) for 24 h. The cells were harvested and the lysates were subjected to Western blotting with anti-MMP2 and anti-MMP7 antibodies. (**I** and **J**) Western blotting detection of the expression of PCNA in AGS and HepG2 cells. The cells were treated same as described in panel G and H, and the lysates were probed by Western blotting with anti-PCNA antibody. The results were representatives of three independent experiments.

of implantation tumors, the gel was administrated 2 hours or 7 days after tumor cell inoculation in peritoneal cavity. Application of CHAG 2 hours after cancer cell inoculation could simulate the period that the detached tumor cells had been implanted and begun to grow in peritoneal cavity while application of CHAG 7 days after cancer cell inoculation could simulate the period of mid-term growth of the implantation tumors. The results showed that CHAG had inhibitory effect on tumor growth in both situations. This confirmed that, except the anti-metastasis effect, CHAG also had anti-proliferation effect on tumor cells. All of these results will have very important clinical significance, and make it safe to use CHAG in clinical tumorectomy for preventing postoperative adhesion.

In summary, our results demonstrated that CHAG could prevent the interaction between stimulating factors and their receptors, block the downstream signal transduction and inhibit tumor progress by wrapping

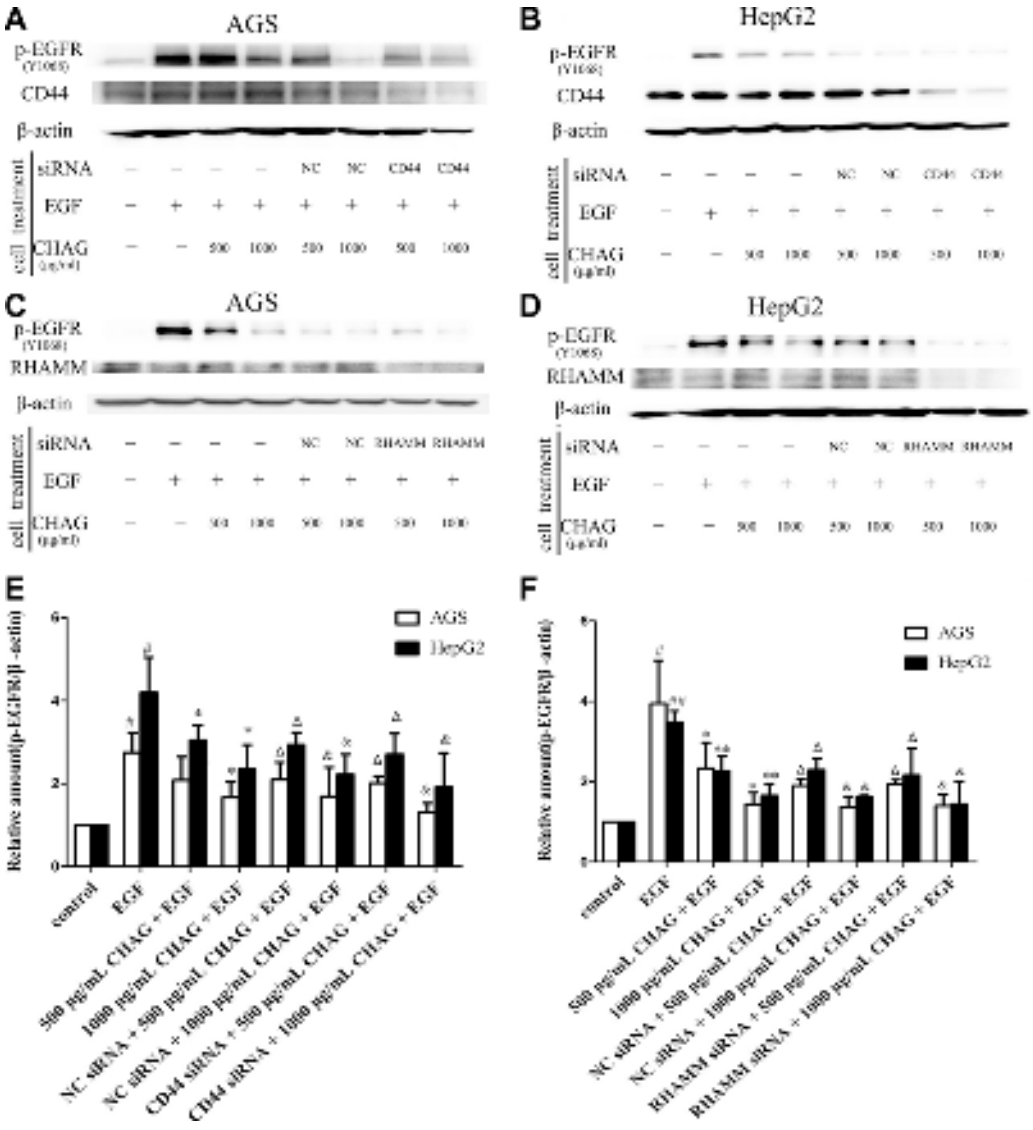

Figure 5: Interference of expression of HA receptors does not affect the inhibition of CHAG on cancer cells. (**A** and **B**) The influence of expression interference of CD44 on the inhibitory effect of CHAG. AGS and HepG2 cells were transfected with CD44 siRNA or control siRNA (negative control, NC) for 36 h. And then in the EGF group, the cells were treated with EGF (100 ng/ml) for 5 min. In the CHAG + EGF and CD44 siRNA + CHAG + EGF groups, the cells were treated with two concentrations of CHAG (500, 1000 μg/ml) for 1 h and then with EGF (100 ng/ml) for 5 min. The cells were harvested and the lysates were subjected to Western blotting with corresponding antibodies. (**C** and **D**) The influence of expression interference of RHAMM on the inhibitory effect of CHAG. The cells were transfected with RHAMM siRNA or control siRNA (negative control, NC) for 36 h, and following treatments were same to panel A and B. The results were representatives of three independent experiments. (**E** and **F**): The results of densitometry analysis of Western blotting results of the corresponding groups. ($^{\#}P < 0.05$, compared with control group; $^{*}P < 0.05$, $^{**}P < 0.01$, compared with the EGF group; $\Delta P < 0.01$, compared with the EGF group and $P > 0.05$, compared with 500 μg/ml CHAG + EGF group; & $P < 0.01$, compared with the EGF group and $P > 0.05$, compared with 1000 μg/ml CHAG + EGF group).

around the cells. This suggests that application of CHAG to prevent post-operative adhesion of tumorectomy may also hinder tumor implantation, growth and metastasis in peritoneal cavity, possessing the effect of killing two birds with one stone.

MATERIALS AND METHODS

Cell migration assay

Trans-well plates (Costar, Corning, USA) were used to analyze migration activity of human gastric cancer cell line AGS and human hepatic cancer cell line HepG2 (from Institute of Cell Biology, Shanghai, China), according to the manufacturer's instruction. Briefly, after trypsinization, the cells were suspended in DMEM culture medium (GIBCO, Grand Island, USA) at a concentration of 5×10^5/mL in control groups. In CHAG groups, the cells were suspended in DMEM containing CHAG (From BioRegen Biomedical Co. Ltd, Changzhou, Jiangsu, China) at the same concentration of control group. In epidermal growth factor (EGF, from Sigma, St. Louis, USA) group, EGF (100 ng/ml) was added to the cell suspension to stimulate the migration of the cells. In the upper chamber of the well, 300 µl cell suspension was added. Cell migration to the bottom side of the membrane was induced by 500 µl of DMEM with 10% FBS (GIBCO, Grand Island, USA) in the lower chamber. The migration time was 12 h. At the end of the migration, the cells migrated onto the bottom side of the membrane were stained with Giemsa and then observed and counted under light microscopy.

Cell invasion assay

Cell invasion assays were performed using the trans-well plates same as described in cell migration assay except that the membrane of the upper chamber was coated with 60 µl of Extracellular Matrix (ECM, 0.125 µg/µl, from Sigma, St. Louis, USA). The cells were treated with CHAG and EGF and seeded into the upper chamber in the same way as for the migration assay. After incubation for 24 h at 37°C, the cells migrated onto the bottom side of the membrane were stained and counted. Subsequent operation was same as for the migration assay.

In vivo study on the tumor inhibition effects of CHAG in a nude mouse tumor transplantation model

This experimental study received full approval from the Institutional Animal Case and Use Committee (IACUC). Specific Pathogen Free (SPF) grade BALB/c nude mice with weights of 8.76 ± 1.34 g were maintained in a SPF barrier system. In the colonization inhibition experiment, 1×10^7 cells of SGC-7901 gastric cancer cell line (from Institute of Cell Biology, Shanghai, China) suspended in 400 µl of PBS or PBS containing CHAG (20 µg per mouse, at a concentration of 500 µg/ml) were implanted into each mouse by intra-peritoneal cavity injection. The mice were bred for 25 days under standard conditions. In the proliferation/growth inhibition experiment, each mouse was given the same amount of cancer cells suspended in PBS. For experiment of early growth inhibition, 400 µl PBS or 400 µl PBS containing CHAG (20 µg per mouse, at concentration of 500 µg/ml) were injected into the peritoneal cavity of the mouse two hours after cancer cell implantation. The animals were normally fed for 8 weeks. For experiment of mid-term growth inhibition, the weekly injection of PBS or PBS containing CHAG were started at the 7th day after cancer cell implantation and repeated for 7 weeks. At the end of the experiment, the animals were euthanized, and the tumors were collected and weighed.

Western blotting

The differently treated AGS and HepG2 cells were harvested. Protein samples were subjected to SDS-PAGE and membrane transfer was performed following the manufacturer's protocol (Bio-Rad, Hercules, CA). The primary antibodies were incubated over night at 4˚C, and the corresponding secondary antibodies (West Grove, PA, USA) were incubated for 1 h at RT, with three washes after each incubation. ECL reagents (Billerica, MA, USA) were used to show the positive bands on the membrane.

Cell transfection and RNA interference

For transfection, the AGS and HepG2 cells were seeded in six-well plates at a density of 80% confluence and transfected at the following day. Transfection of cells with siRNA for CD44 or RHAMM was performed using Lipofectamin 2000 (Invitrogen, Carlsbad, CA), following the manufacturer's instruction. After 36 h, the cells were treated with CHAG (500, 1000 µg/ml) for 1 h and then with EGF (100 ng/ml) for 5 min. The protein was extracted and detected by Western blotting.

"Pull-down" analysis of active small G protein RhoA and Rac1

The activity of RhoA was detected with Pull-down method. The cells were treated with CHAG (500, 1000 µg/ml) for 1 h and EGF (100 ng/ml) for 5 min, and then lysed in lysis buffer (25 mM HEPES pH 7.5, 150 mM NaCl, 1% NP40, 10% glycerol, 25 mM NaF, 10 mM $MgCl_2$, 0.25% sodium deoxycholate, 1 mM EDTA,1 mM Na_3VO_4, 10 mg/ml aprotinin and 10 mg/ml leupeptin). The protein extracts were incubated with Rhotekin-RBD bound to glutathione-agarose beads. The activated RhoA bound to the beads or total RhoA in cell extracts was detected by Western blotting with antibody against RhoA (Santa Cruz Biotechnology,

Dallas, USA). The active Rac1 was detected with similar method but with GST-Pak1 protein binding domain (GST-PBD) and antibody against Rac1 (Cell Signaling Technology, Danvers, USA).

Statistical analysis

All *in vitro* experiments were performed in triplicate for each cancer cell type and each treatment setting. Data are expressed as means ± standard deviation (SD). Statistical significance was performed using a two-tailed ANOVA with SPSS statistical software. Student's *t* test was performed if equal variance was ascertained in two groups by *F* test. A *P*-value of less than 0.05 was considered significant.

ACKNOWLEDGMENTS

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CONFLICTS OF INTEREST

The authors have no conflicts of interest to declare.

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Effect of a new cross-linked hyaluronan gel on the staple line after sleeve gastrectomy in a rat model[1]

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Abstract

Purpose: To evaluate the effect of a new cross-linked hyaluronan (NCHA) gel on healing of the staple line in an experimental sleeve gastrectomy.
Methods: Eighteen rats were randomly divided into three groups. The control group (n = 6) received no medication. In the saline group (n = 6) and NCHA gel group (n = 6), saline and NCHA gel were respectively administered onto the staple line and intraperitoneally into the abdominal cavity after the standard stapling procedure.
Results: The fibroblast activity and collagen deposition were significantly higher in the NCHA gel group than in the control group (p = 0.00, p = 0.017) and saline group (p = 0.004, p = 0.015). The tissue hydroxyproline protein level was significantly higher in the NCHA gel group than in the control group (p = 0.041). Adhesion formation was significantly lower in the NCHA gel group than in the control and saline groups (p = 0.015, p = 0.041).
Conclusions: New cross-linked hyaluronan gel could be an effective approach to improve staple line wound healing and prevent potential leakage after sleeve gastrectomy. Moreover, NCHA gel helps to prevent adhesion formation without compromising healing of the staple line.
Key words: Tissue Adhesions. Wound Healing. Bariatric Surgery. Gastrectomy. Hyaluronan Gel. Rats.

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■ Introduction

The significantly increased mortality rate in patients with obesity contributes to the development of diseases such as cardiovascular and type II diabetes. This is an urgent public health problem. Bariatric surgical procedures are the most effective treatments for long-term weight loss[1]. Vertical sleeve gastrectomy restricts the stomach volume to reduce the level of ghrelin and effectively achieve weight loss. More recently, sleeve gastrectomy has been considered an effective and low-risk bariatric solution to the increasingly severe obesity problem[2].

Sleeve gastrectomy complications associated with the staple line are one of the major challenges facing general surgeons. Complications such as leakage at the staple line often require surgical intervention and result in a prolonged hospital stay and significant increase in the financial burden. Several different techniques have been adopted to strengthen the staple line, such as reducing bleeding and/or using glycolide–trimethylene carbonate copolymer, bovine pericardial strips, fibrin glue, hemostatic agents, and protein-rich plasma[3–6].

Hyaluronan is a non-sulfated glycosaminoglycan containing recurrent disaccharide units (a-1,4-D- glucuronic acid and b-1,3-N-acetyl-D-glucosamine). As a major extracellular matrix (ECM) building block, hyaluronan has unique physiochemical properties such as marked biological function in all connective tissues and enhancement of wound healing[7]. Tissue repair is a complex function of hyaluronan and cannot be based on only one of its many features. Based on these unique physiochemical properties of hyaluronan, many products have been developed for tissue repair, anti-adhesion, tissue implants, and moisturizers. Many studies have shown beneficial results with respect to wound healing after exogenous hyaluronan application, including in patients with skin damage, chronic venous leg ulcers, tympanic membrane perforation, and pelviureteral anastomoses[8–11]. Hyaluronan is used to prevent postoperative adhesions because it creates a physiological barrier between the healing tissue and other tissue surfaces during peritoneal reepithelialization[12]. Moreover, Lan *et al*.[13] reported that hyaluronan suppressed colonization, growth, and metastasis of a gastric cancer cell line in the peritoneal cavity of a mouse model.

The aim of our study was to evaluate the efficacy of a new cross-linked hyaluronan (NCHA) gel on staple line healing in an experimental sleeve gastrectomy model with rats. To the best of our knowledge, it is the first study to investigate the influence of hyaluronic acid in sleeve gastrectomy.

■ Methods

The procedures were reviewed and approved by the Akdeniz University Local Committee on Animal Research Ethics (approval number/date 49/06.05.2016). This study conformed to the Guidelines for the Care and Use of Laboratory Animals as published by the US National Institutes of Health (NIH Publication No. 85–23, revised 1985).

Eighteen female Sprague–Dawley rats at 3 to 4 months of age and weighing 300 to 400 g were supplied by the Experimental Animal Care and Production Unit of Akdeniz University. The animals were fasted for 18 h before the procedures, with free access to water.

All rats were randomly divided into three experimental groups: Control group (n = 6): standard sleeve gastrectomy was performed. Saline group (n = 6): upon completion of standard sleeve gastrectomy, 2 ml of physiological saline (Pro-flex 0.9%, Çetinkaya İlaç, Bolu, Turkey) was applied; half of the physiological saline was administered

over the staple line, and the remaining half was applied to the bottom of the abdominal cavity. NCHA gel group (n = 6): upon completion of standard sleeve gastrectomy, the NCHA gel (HyaRegen® gel; BioRegen Biomedical, Changzhou, China) was applied at 2.0 ml/kg; half of the gel was administered over the staple line, and the remaining half was applied to the bottom of the abdominal cavity (Figure 1).

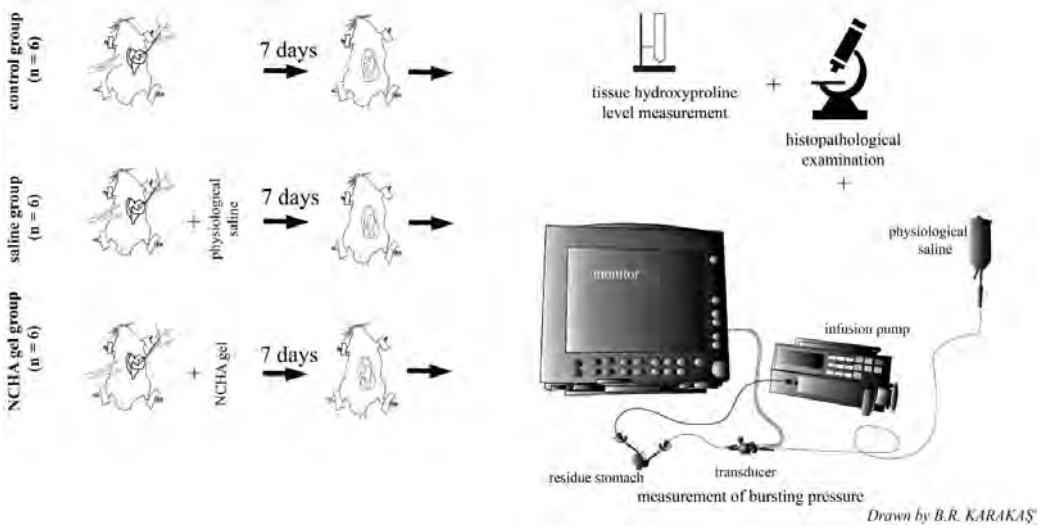

Figure 1 - Schematic drawing of experimental design.

Surgical procedure

Anesthesia was achieved by intraperitoneal administration of ketamine (75–100 mg/kg) (Alfamine 10%; Alfasan International B.V., Woerden, Holland) and xylazine hydrochloride (10 mg/kg) (Alfazyne 2%; Alfasan International B.V.). After shaving the operation site on the abdominal wall and disinfecting the skin with povidone–iodine (Poviiodeks 10%; Kim-Pa, Istanbul, Turkey), a 4 cm midline abdominal incision was made. The stomach was released by dissection and covered with gauze moistened with physiological saline. The stomach was released from the abdomen, and approximately 70% to 80% of the fundus between the esophagus and pylorus was resected using a stapler (Echelon Flex 45 Endopath; Johnson & Johnson, Guaynabo, Puerto Rico, USA) and staples (six rows, 2.5 to 1.0 mm, ECR45W white reloads; Johnson & Johnson) (Figure 2a, b).

The abdominal cavity was rinsed with normal saline. Correspondent treatment was applied separately for the saline and NCHA groups as described above. The abdomen was closed with continuous 3-0 polyglycolic acid suture (Pegesorb®; Doğsan, Istanbul, Turkey). All surgical procedures were performed by two surgeons (B.R.K., V.V.). On the first postoperative day, the rats were given only water and then were given standard laboratory chow. On the 7th postoperative day, re-laparotomy was performed for analytical procedures under the above-described anesthesia protocol. Adhesions between the organs and the wall were separated. The adhesions were scored according to the method as previously described: grade 0, no adhesions; grade 1, filmy adhesions that spontaneously separate; grade 2, firm adhesions that separate by traction; and grade 3, dense adhesions requiring sharp dissection [14]. The residual stomach was resected at the level of the esophagus and pylorus. Burst pressure was measured to evaluate the staple line in vitro

(Figure 1), (Figure 2c, d). The healing staple line was removed for tissue hydroxyproline level measurement and histopathological examination (Figure 1). All rats were killed by cardiac exsanguination under deep anesthesia at the end of the experiment.

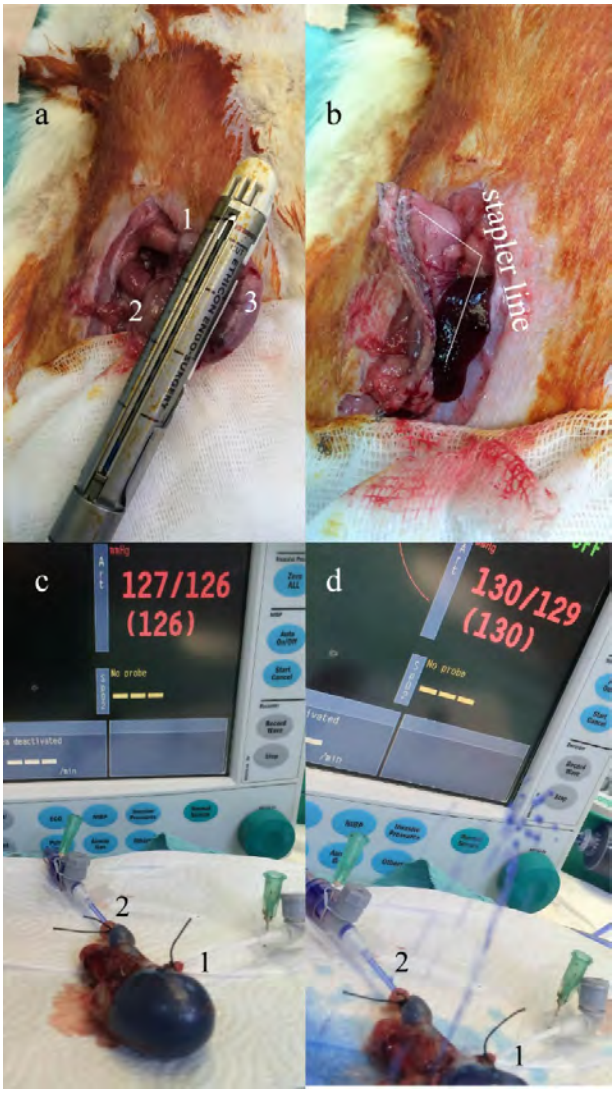

Figure 2 - Pictures of the sleeve gastrectomy procedure and measurement of the burst pressure. (**a**) Closing the stapler just before firing. (**b**) View of the staple line after sleeve gastrectomy. (**c**) Infusion of the methylene blue solution into residue stomach just before leaking. (**d**) View of leakage of the methylene blue solution from the staple line. (1) Esophagus. (2) Pylorus. (3) Gastric fundus.

Measurement of bursting pressure

The residual stomach was resected at the level of the esophagus and pylorus. A 16-gauge plastic angiocatheter was advanced from the open end to the proximal (esophagus) and distal (pylorus) lumen. When the ends of the catheters were palpable in the residual stomach, the catheters were attached in a watertight fashion with 2-0 silk suture. The catheters were fixed to the operation table so that they could not move during the measurement. The proximal catheter connected to the infusion pump (Perfusor Compact; B. Braun®, Melsungen, Germany) with fixed-length tubing. A bedside pressure monitor (Datex-Ohmeda S/5; GE Healthcare, Little Chalfont, UK) was used to connect the distal catheter with the pressure transducer (Monitoring Kit, Transpac IV; Abbott Critical Care Systems, Abbott, Ireland) (Figure 1). Colored liquid (methylene blue solution) was infused into the infusion pump via a 50-ml syringe at a constant rate of 2 ml/s until the methylene blue solution was observed leaking from the staple line. When a sudden pressure drop was seen and leakage of the methylene blue solution from the staple line was noted, it was recorded as the burst pressure for the given sample (Figure 2c, d). All infusion procedures and pressure measurements were performed by the same person (A.K.) without knowledge of the rats' group assignments. The systems were calibrated to zero before each measurement.

Tissue hydroxyproline levels

Tissue specimens were stored at −80°C after weighing. At the time of analysis, the samples were dissolved and homogenized using a sonicator. Tissue hydroxyproline measurement was performed with a commercial kit using the colorimetric method (Rat Hydroxyproline, Cat. No: CSB-E08838r; Cusabio Biotech Co., Ltd.).

The amount of hydroxyproline in the samples was calculated using the standard curve. The results per unit wet tissue weight were given. In addition, tissue protein levels were measured with a commercial kit using the Lowry method (Modified Lowry Protein Assay Kit, PI-23240; Thermopierce). Hydroxyproline levels were compared with those obtained by ratios to tissue protein as µcg/mg protein.

Histopathological examination

For histopathologic analytical evaluation, tissue specimens were fixed in 10% formalin solution and then embedded in paraffin. Thin sections (5–7 µm) were cut perpendicular to the anastomosis line and stained with hematoxylin and eosin and Masson's trichrome stain. Under a light microscope, a single pathologist who was blinded to the treatment method of the samples histopathologically graded the staple line according to the Ehrlich and Hunt numerical scale as modified by Phillips *et al*.[15]. Inflammatory cell infiltration, fibroblastic activity, neoangiogenesis, and collagen deposition were graded from 0 to 4 as follows: 0, no evidence; 1, occasional evidence; 2, light scattering; 3, abundant evidence; and 4, confluent cells or fibers. The characteristic photographs of the different grades are shown in Figure 3a, b.

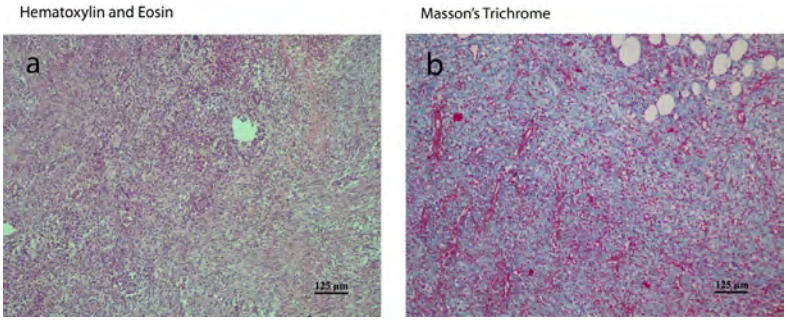

Figure 3 - Microscopic appearance of anastomosis site in the (**a**) control group and (**b**) NCHA gel group. (**a**) Neutrophil-rich inflammation on the anastomosis line (the section stained with hematoxylin and eosin, original magnification ×100, scale bars: 125 µm). (**b**) Mild fibrosis on the anastomosis line (the section stained with Masson's trichrome, original magnification ×100, scale bars: 125 µm).

Statistical analysis

Data were analyzed using Statistical Package for Social Sciences 19.0 (IBM Corp., Armonk, NY, USA). All values are given as the median (minimum–maximum). Kruskal–Wallis variance analysis and the Mann–Whitney U-test were used to compare the groups. A p value of <0.05 was considered statistically significant.

■ Results

The adhesion score, burst pressure, histopathological score, and tissue hydroxyproline level are summarized in Table 1 and shown in Figures 4 to 7 for each group.

Significantly lower adhesion scores were found in the NCHA gel group than in the control and saline groups (p = 0.015, p = 0.041) (Figure 4). During the burst measurement, all leaks were monitored on the staple recovery line. No significant difference in burst pressures was identified among the groups (Figure 5).

Table 1 - Adhesion scores, burst pressure, tissue hydroxyproline level, and histopathological grading in the three study groups

Parameters	Control Group	Saline Group	NCHA gel Group
Adhesion Scores	2.5(2–3)	2(2–3)	1(1–2) *¶
Bursting Pressure (mm Hg)	37(22–154)	118(35–157)	100(71–130)
Tissue Hydroxyproline Level Hydroxyproline/Protein (mcg/Mg Protein)	44.49 (3.20–216.03)	166.04 (11.83–253.70)	240.06 (56.52–500.52) ▲
Histopathological Grading			
Inflammatory Cell Infiltration	4(3–4)	3(2–4)	2(1–2) Δ■
Neoangiogenesis	3(2–4)	2.5(2–3)	3(3–4)
Fibroblast Activity	2.5(2–3)	2.5(2–3)	4(3–4) □●
Collagen Deposition	1(1–2)	1.5(1–2)	3(2–3) ○♦

The values represent the median (min–max). Significance was defined as $p < 0.05$. Differences between the two groups were compared using the Mann–Whitney U-test. *p = 0.015 compared with control group. ¶p = 0.041 compared with saline group. ▲p = 0.041 compared with control group. Δp = 0.002 compared with control group. ■p = 0.009 compared with saline group. □p = 0.004 compared with control group. ●p = 0.004 compared with saline group. ○p = 0.017 compared with control group. ♦p = 0.015 compared with saline group.

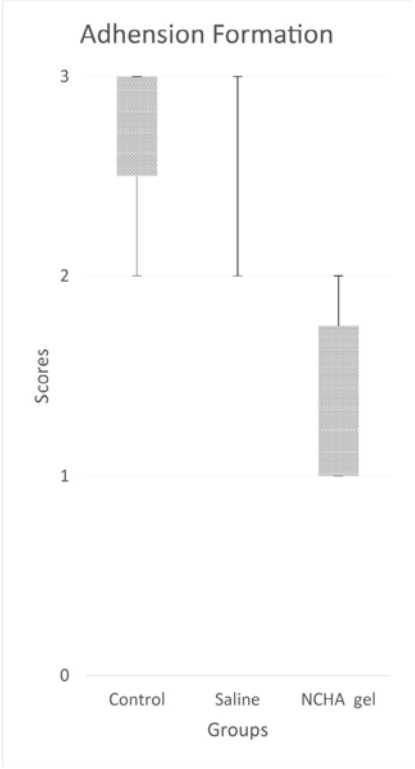

Figure 4 - Box plots showing adhesion formation according to Evans *et al*.[14]. The boxes represent the median, 25th, and 75th percentiles, and the whisker lines show the maximum and minimum levels.

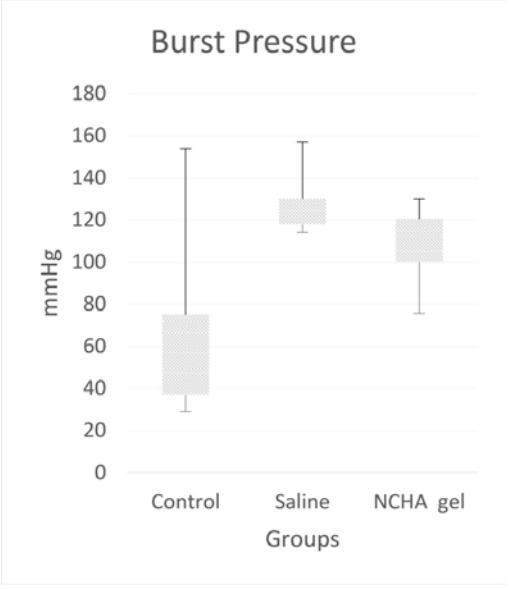

Figure 5 - Box plots showing burst pressures (mmHg). The boxes represent the median, 25th, and 75th percentiles, and the whisker lines show the maximum and minimum levels.

Inflammatory cell infiltration was significantly lower in the NCHA gel group than in the control and saline groups (p = 0.002, p

= 0.009). The fibroblast activity and collagen deposition were significantly higher in the NCHA gel group than in the control group (p = 0.004, p = 0.004) and saline group (p = 0.017, p = 0.015) (Figure 6). The tissue hydroxide protein level was significantly higher in the NCHA gel group than in the control group (p = 0.041) (Figure 7).

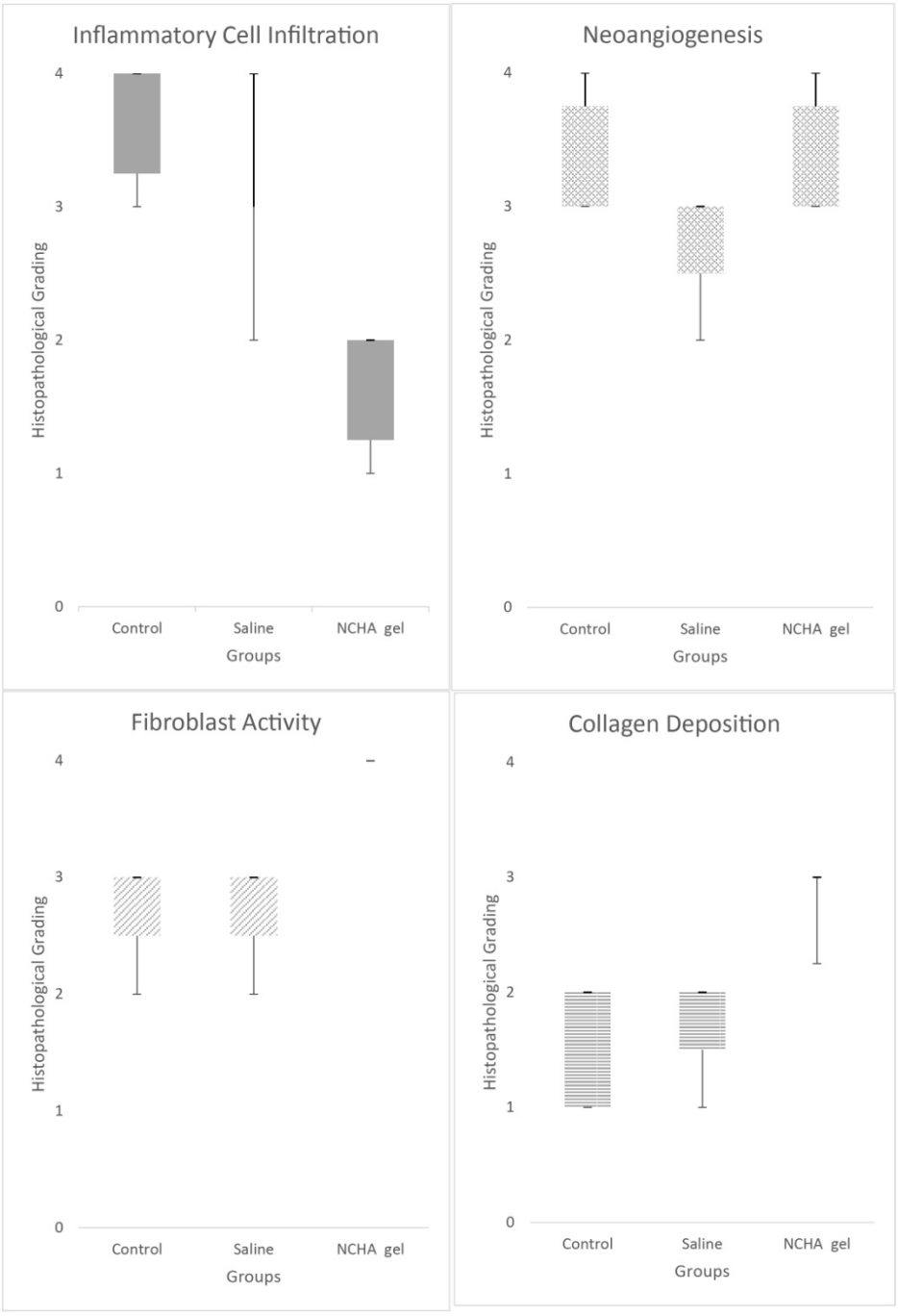

Figure 6 - The box plots showing the changes in the histopathological grade. The boxes represent the median, 25th, and 75th percentiles, and the whisker lines show the maximum and minimum levels.

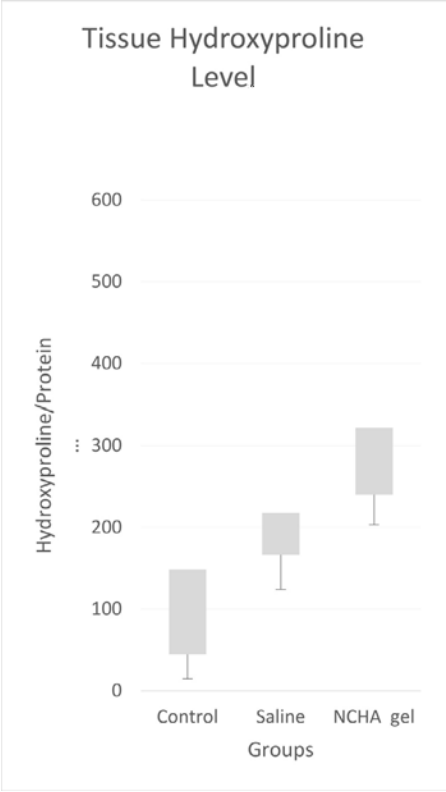

Figure 7 - Box plots showing the tissue hydroxyproline levels [hydroxyproline/protein (µcg/mg protein)]. The boxes represent the median, 25th, and 75th percentiles, and the whisker lines show the maximum and minimum levels.

■ Discussion

Morbid obesity is a serious public health problem, and the most effective treatment is bariatric surgery. Sleeve gastrectomy is one of the most accepted methods among bariatric surgical procedures. One of the most consequential complications of sleeve gastrectomy is leakage. Leakage from the staple line is the second most frequent cause of death associated with bariatric surgery[16]. The present study is the first to show that NCHA gel administration as a reinforcement agent increases crucial wound-healing parameters such as fibroblastic activity, neoangiogenesis, collagen deposition, and the tissue hydroxide protein level and reduces inflammatory reactions such as cell infiltration in the staple line after sleeve gastrectomy in rats.

Reinforcement of the staple line with buttress material is among the technical recommendations for preventing this type of leakage. Gentileschi et al. found no differences among oversewing, buttressed transection with polyglycolic acid and trimethylene carbonate, and staple-line roofing with a gelatin fibrin matrix[17]. Wang *et al*.[18] showed that oversewing of the staple line increased the operation time, but no evidence demonstrated that it reduced leakage. Al Hajj and Haddad reported that staple-line buttressing with bovine pericardium reduced staple line leakage[4]. Gagner and Buchwald[19] compared no reinforcement, oversewing, nonabsorbable bovine pericardial strips, and absorbable polymer membrane application and reported that the most successful method among these procedures was absorbable polymer membrane application. Karakoyun *et al*.[20] reported that oversewing with a continuous suture on the staple line was more successful than reinforcement of the staple line with fibrin sealant. Shikora *et al*.[21] compared the effectiveness of different methods including oversewing, a biocompatible glycolide copolymer, bovine pericardium, and no reinforcement and found that the most successful method was buttressing with bovine pericardium. Çoşkum *et al*.[22] used fibrin sealant for reinforcement of the staple line in their study and found no leakage. Sepulveda *et al*.[23] reported that the staple line was strengthened with imbricated oversewing in their study and reported no leakage. Overall, however, there is still no consensus regarding which reinforcement procedure can significantly and reliably prevent leakage from the staple line.

There is a clinical need to identify a consistently reliable method for reinforcement of the staple line.

Ortonne *et al*.[9] reported that hyaluronan decreased ulcer dimensions in patients with venous leg ulcers. Hellstrom *et al*.[10] showed that hyaluronan-treated perforations closed more rapidly than did untreated perforations in a model of induced tympanic membrane perforations in rats. Yurtçu *et al*.[11] reported that hyaluronan facilitated wound healing by increasing re-epithelialization and neovascularization in an experimental model of pelviureteral anastomosis in rabbits. Lan *et al*.[13] reported that hyaluronan suppressed colonization, growth, and metastasis of a gastric cancer cell line in an experimental model. Taken together, these studies indicate that hyaluronan gel is a potential reinforcement agent for the staple line by improving the tissue repair process.

Hyaluronan has been shown to support acute and chronic wound healing in many models. Hyaluronan binds to three main cell surface receptor classes: CD44, receptor for hyaluronan mediated motility (RHAMM), and intracellular adhesion molecule-1 (ICAM-1). CD44 receptors play a role in hyaluronan uptake and degradation, cell–cell and cell–substrate adhesion, and cell migration and activation. One of the major biological effects of hyaluronan when it associates with the CD44 receptor is stimulation of the ECM; it also provides localized balance of the hyaluronan level. Upregulation of the expression of several cytokines, such as interleukin 1β (IL-1β), tumor necrosis factor-α, and insulin-like growth factor-1 induce several inflammatory gene expressions through a CD44-directed mechanism in macrophages; this in turn increases collagen production in endothelial cells. RHAMM is present in most mobile cells, including migrating fibroblasts and highly metastatic tumor cells, and is involved in cell locomotion. ICAM-1 is a widely distributed cell adhesion molecule in macrophages and other cells, including endothelial cells. The ICAM-1 receptor allows hyaluronan to be taken up by the cells, and destruction of the intracellular area then occurs. By binding with hyaluronan and ICAM-1, leukocyte integrins are affected by other receptors such as lymphocyte function associated-1 and macrophage antigen complex-1, potentially contributing to inflammatory activation[7].

Hyaluronan plays multiple roles in healing. Although inflammation is crucial for the formation of granulation tissue, stabilization of the granulation tissue matrix is necessary by alleviating inflammation to maintain normal tissue repair. The free-radical scavenging role of hyaluronan may influence inflammatory activation. In this regard, hyaluronan provides protection against free radical and proteolytic damage in the cell and ECM[24]. Campo *et al*.[25] showed that hyaluronan reduced inflammatory mediators such as tumor necrosis factor-α, IL-1β, IL-17, MMP-13, and inducible nitric oxide synthase, which play active roles in inflammation and cartilage destruction in an experimental arthritis mouse model. Hyaluronan is one of the main components of the ECM. It is the most important support structure of proteoglycans. It is also associated with collagen, fibrin, and other matrix molecules. Hyaluronan promotes the early tissue damage response and the formation of fibrin-rich transient matrix. Accordingly, it promotes fibroblast and endothelial cell movement to the wound area and subsequent formation of granulation tissue in the early stage of tissue repair. If the hyaluronan binds to the ECM components by its hydrophilic structure, it creates an environment that

allows cell migration to the newly formed tissue region[26].

In the present study, neutrophil infiltration was significantly lower in the NCHA gel group. Fibroblast infiltration, fibrosis formation, and the hydroxyproline level were significantly higher in the NCHA gel group. Although there were no statistically significant differences in burst pressure among the groups, the burst pressure showed a tendency to increase in the NCHA gel group compared with the control group. These results suggest that NCHA gel administration had a positive effect on tissue healing.

Wong *et al*.[27] reported that the level of cross-link modification of hyaluronic acid is positively correlated with resistance to enzymatic degradation of substrates. NCHA is a cross-linked hyaluronan gel with high viscosity and stickiness; it can stay on the surface of abdominal organs for up to 2 weeks and therefore prevent intra-abdominal adhesions. A recent study showed that NCHA gel significantly reduced adhesion formation throughout the abdominal cavity[12]. Menzies and Ellis[28] showed that postoperative adhesions formed in 10.4% of patients after first abdominal surgery. Postoperative adhesions may cause a life-long risk of different complications such as chronic abdominal pain, bowel obstruction, and infertility in women. Therefore, preventing postoperative adhesions in the abdominal cavity should be one of the treatment focuses of sleeve gastrectomy.

■ Conclusions

New cross-linked hyaluronan gel, a potent wound healing agent, can effectively improve reinforcement of the staple line of sleeve gastrectomy in rats. Moreover, the NCHA gel contributes to preventing adhesion formation without compromising healing of the staple line. All of these results have important clinical implications and relevance.

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Established in 2008, BioRegen Biomedical (Changzhou) Co.,Ltd., with its original proprietary self-crosslinking technology prepared sodium hyaluronate gel, which has been protected by 44 international patents, has been widely used in gynecology, otolaryngology, ophthalmology, medical beauty and other fields in China and abroad.

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