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Prevention of intra-abdominal adhesions by a hyaluronic acid gel; an experimental study in rats

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Abstract

Background: In 80% to 90% of the patients intra-abdominal adhesions occur after abdominal surgery, which can cause small-bowel obstruction, chronic abdominal pain, female infertility and difficulty during reoperation. A novel crosslinked hyaluronic acid gel is evaluated regarding its anti-adhesive capacities in an ischemic button model in rats.

Method: 51 adult, male Wistar rats from a registered breeder, received eight ischemic buttons each and were treated with hyaluronic acid gel (HA, HyaRegen©), hyaluronic acid carboxymethylcellulose (HA-CMC, Seprafilm©) or no anti-adhesive barrier. After 14 days, the animals were sacrificed and adhesions were scored macroscopically. The number of buttons and organs involved in adhesions were recorded. Per animal, one button with adhesions and one without adhesions was explanted for qPCR analysis. Mann-Whitney U, Fisher's exact and Wilcoxon signed rank test were used for data analysis. A p-value of 0.05 was considered significant.

Results: Macroscopic evaluation of adhesion formation did not differ between the groups. The number of organs involved in adhesions in the HA gel group was significantly lower compared to HA-CMC ($p = .041$) and the control group ($p = .012$). A significantly, 1.36-fold higher *clec*10a ($p = 0.25$), 1.80-fold higher *cd*163 ($p = 0.003$) and 5.14-fold higher *mmp1* expression ($p = 0.028$) was found in ischemic buttons with adhesions compared to buttons without adhesions.

Conclusion: HA gel application reduces the number of organs involved in adhesions in an ischemic button model, but no overall reduction in adhesion formation was encountered. Macrophage subtype 2 polarization and high *mmp1* expression are associated with adhesion formation. Further investigation is needed in the exact pathophysiologic process of adhesion formation and the role of macrophage polarization.

Keywords

Adhesions, hyaluronic acid, prevention, ischemic button model, rat model, abdominal wall, macrophage polarization

Introduction

Intra-abdominal adhesion formation is the most frequent occurring complication following surgery of the abdomen, with an incidence reported of 80% to 90%.[1,2] The risk of morbidity, which is directly related to adhesions, is 3.8% after abdominal surgery accompanied by an average rate of readmission of 2.1.[3,4] Of small-bowel obstructions, 56% is caused by adhesions. In 57% of patients with chronic abdominal pain, adhesions are considered responsible, and 23% of female patients seek fertility treatment after abdominal surgery.[5] Apart from these complications, future surgery is hampered by adhesions and adhesiolysis is needed with an increased risk of iatrogenic bowel injury.[5,6] Consequently, adhesiolysis leads to an increased

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incidence of sepsis, intra-abdominal complications and wound infections resulting in longer hospital stay and higher hospital costs.[6]

Laparoscopic surgery is associated with less intra-abdominal adhesions than conventional surgery, although 37.7% of the laparoscopic operated patients still develop postoperative adhesions. A surgical intervention to treat adhesions is therefore not preferable and prevention of adhesions using anti-adhesive barriers might offer a better strategy. Two such barriers, oxidised regenerated cellulose (Interceed©, Ethicon, US, NJ) and hyaluronate carboxymethylcellulose (HA-CMC, Seprafilm©, Sanofi, US, NJ) are suggested to be effective in reducing adhesion formation. Also, in case of HA-CMC, a reduction of reoperations for small bowel obstruction is reported.[7,8] The only available liquid anti-adhesive barrier, icodextrin 4% (Adept, Baxter Healthcare, Deerfield, IL) is afflicted by limited research and does not show a beneficial effect on the reoperation rate for small bowel obstruction.[8]

HA-CMC has a beneficial effect on the severity of the adhesions, but the extent of adhesion formation is merely reduced by 25.9%. This translates in a reduction of the reoperation rate, although the incidence of small-bowel obstruction is not altered after HA-CMC application.[7,8] These results are reflected by the opinion of Dutch surgeons in a National survey, where responders were reluctant in using anti-adhesive barriers.[9] Furthermore, is Seprafilm, when wrapped around an anastomosis of the bowel, associated with a higher occurrence of anastomotic leakage and its consequences, such as fistula, peritonitis, abscess and sepsis.[10]

The pathophysiological process of the formation of adhesions is characterized by a complex interaction between different factors, such as fibrin deposition and failure of the fibrinolytic system, macrophages and fibroblasts.[11–15] Isolated pathways are described, but the interaction between these components is poorly understood. A continuum between two polarized states of macrophage activation is described in the macrophage M1/M2 paradigm. Macrophage subtype 1 (M1) is recognized as a pro-inflammatory macrophage or classical macrophage and is associated with tissue injury. Macrophage subtype 2 (M2) defines the other side of the spectrum and is characterized by more anti-inflammatory properties including extracellular matrix remodeling and fibroblast regulation.[16–18] In this animal experiment the secondary aim is to investigate the pathophysiological process of adhesion formation. Genes of interest in regard to adhesion formation consist of genes associated with macrophage subtype 1 and 2, fibroblast activation and adhesion specific genes.

The quest for an optimal anti-adhesive barrier continues. The primary aim of this study is to evaluate the effect of a hyaluronic acid-based gel on the intra-abdominal adhesion formation in the ischemic button model in rats and to compare its effect to an already commercially available anti-adhesive barrier (HA-CMC). To be of added value in clinical practise, the novel anti-adhesive barrier should be superior to what is currently available for clinicians in the prevention of adhesions.

Materials and methods

The ethical committee of animal experiments approved the study protocol (AVD107002016720), which complied to the Dutch Animal Experimental Act and the European Directive 2010/63/EU.

Materials

Crosslinked hyaluronic acid gel, HyaRegen© (HA) is produced by BioRegen Biomedical Co., Ltd. (Changzhou, China) and showed promising results regarding adhesion prevention in the gynaecological patient.[19] The product was delivered in 20 cc sterile syringes and a 3 cc volume was applied intra-abdominally in the rat. Hyaluronate carboxymethylcellulose, Seprafilm© (HA-CMC) was acquired commercially and under sterile conditions, cut to a size of 5x5 cm after which it was placed on top of the intestines.

Animals

51 adult, male Wistar rats with a body weight between 200–250 g were obtained from a registered breeding company (Envigo, Horst, the Netherlands). All animals were accommodated at the central animal facilities of Maastricht University and cared for according to local protocol, with free access to food and water, a 12-hour dark/light cycle and social housing.

Study design

Due to the obvious difference between HA-CMC and HA gel, blinding of the surgeon was not possible at the time of surgery. The animals were randomly placed in one of three groups (n = 17 per group); HA gel, HA-CMC or control group. The animals in the control group did receive 8 ischemic buttons but did not receive any anti-adhesive barrier. It was made sure that in each cage every group was equally represented, with three animal per cage. After a 14-day observation period the animals were sacrificed.

Surgical procedure

Pre-operative pain medication was administered via subcutaneous injection of buprenorphine 0.05 mg/kg and carprofen 4 mg/kg. The animal was placed in an

induction chamber to induce anaesthesia via inhalation with 3–4% isoflurane. Anaesthesia was maintained with 2% isoflurane. Before surgery, the abdomen was shaved and disinfected with chlorhexidine. Access to the abdomen was gained through a 6 cm midline incision and on either side of the midline incision four ischemic buttons were created.[20–22] In short, 5 mm of parietal peritoneum was lifted and ligated with a non-absorbable suture (Prolene, Ethicon, Johnson & Johnson, Somerville, NJ) to create the ischemic button. All animals, regardless of group allocation, received eight ischemic buttons. The button was placed 1 cm lateral of the incision and at least 1 cm apart from one another. Thereafter, HA-CMC or 3 ml of HA gel, was left behind according to group allocation and in case of the control group, no product was used. No suturing nor fixation took place, because HA-CMC is self-adherent. A continuous 4–0 polyglactin suture (Vicryl, Ethicon, Johnson & Johnson, Somerville, NJ) was used for closure of the abdominal wall after which the skin was intracutaneously closed using an absorbable Monocryl 4–0 suture (Ethicon, Johnson & Johnson, Somerville, NJ). The animals received fluid resuscitation postoperatively and were allowed to recover under a heat lamp. Careful welfare assessment was conducted using welfare score sheets during follow-up.

After 14 days of follow-up a second operation took place for the evaluation of intra-abdominal adhesion formation. The previously described protocol was followed regarding preoperative pain management and the anaesthesia. The abdomen was accessed through the midline scar and any complications or unexpected observations were recorded. Next, the intra-abdominal adhesions were scored macroscopically. If present, one ischemic button with attached adhesions and one ischemic button which failed to induce adhesions was collected per animal and snap frozen for qPCR analysis. The animals were sacrificed by cardiac puncture while still sedated after completion of the procedure.

Adhesion scoring

Adhesions were scored macroscopically regarding the quantity and quality of the adhesions. The quantity of adhesions was assessed using the Nair score,[23] both the number of ischemic buttons involved in adhesions and the number of intra-abdominal organs involved in adhesions were scored. The tenacity (Zühlke score[24]) and vascularisation of the present adhesions were a measure for the quality of adhesions. See Table 1 for the scoring system.

Evaluation by expert panel

In order to directly link this animal experiment to clinical application, three clinical experts were asked to evaluate adhesion formation in the animals. During the macroscopic scoring digital photographs were taken from either side of the midline. Three abdominal surgeons (SB, LS, NB) were blinded to the allocation of the animals and asked to score both the quantity and the quality of adhesions (see Table 2). Both sides of the midline incision were scored separately, generating two scores regarding quantity and quality of adhesions per

Table 2. Scoring of adhesions by experts.[22,25–28]

Quantity score:	What percentage of the surface of the abdominal wall is involved in adhesions?
0	0%
1	<25%
2	25–49%
3	50–74%
4	75–100%
Quality score:	**How would you describe the severity of the adhesions?**
0	No adhesions visible
1	Filmy, avascular adhesions
2	Moderate thickness, limited vascularity
3	Dense thickness, well vascularised

Table 1. Adhesion scoring systems.[24]

Nair score	Grade 0	No adhesions/ insignificant adhesions
	Grade 1	Only one adhesions band between the organs or between one organ and abdominal wall
	Grade 2	Two adhesions bands between organs or between one organ and abdominal band
	Grade 3	More than two adhesion bands between the organs or between one organ and abdominal wall or adhesions of intestinal loops without any adhesion to the abdominal wall
	Grade 4	Adhesions of all viscera to the abdominal wall
Tenacity score (Zühlke score)	Grade 0	No adhesions
	Grade 1	Filmy adhesions, blunt dissection
	Grade 2	Strong adhesions, sharp dissection
	Grade 3	Very strong vascularized adhesions, sharp dissection, damage hardly preventable
Vascularisation	Yes/no	

animal. Per expert, the scores were added and the total scores from the three experts were averaged and reported. The used scoring systems are regularly used in other animal adhesion models.[22,25–28]

RNA isolation and quantitative real time PCR

Samples from animals from which a button with adhesions and a button without adhesions was available, were selected for qPCR analysis. Abdominal wall tissue specimen was collected and snap frozen, from which total RNA was isolated using TRI reagent (Sigma, NL). 750 ng DNAse-treated RNA was used to synthesize cDNA (SensiFAST^{TM}, cDNA synthesis kit, Bioline, London, UK). A volume of 10 µl consisting of the cDNA equivalent of 2.5 ng total RNA, 1× Absolute qPCR SYBR Green Fluorescein Mix (SensiFAST^{TM} SYBR® Hi-ROX Kit, Bioline, London, UK) and 0.15 µM of gene-specific primers (Sigma, NL) was used for qPCR reactions. The LightCycler® 480 Instrument II (Roche Molecular Systems, Inc) was used to perform the qPCR. Gene expression levels were established with LinRegPCR software. The geometric mean of two internal control genes (*Rplp0* and beta-actin (*Actb*)) was calculated and used as normalization factor. Relevant primers were identified from the literature and build using a primer design tool (Primer-blast),[29] the sequences are reported in Table 3. All primers were tested for transcription of the intended gene.

Statistical analysis

Sample-size calculation was based on previous research with an ischemic button model within our research group. An effect size of 1.11 with a reduction of 30% of involved ischemic buttons in adhesions was considered clinically relevant. This resulted in a sample-size of 15 animals per group with a power of 0.8 and an alpha

Table 3. Primers used for qPCR analysis.

Gene symbol (name)	Product length		GC%	sequence
rplp0 (ribosomal protein lateral stalk subunit P0)	190	f	55.00	CCTCACCGAGATTAGGGACA
		r	45.00	ATCGCTCAGGATTTCAATGG
actb (actin, beta)	297	f	55.00	CCGCGAGTACAACCTTCTTG
		r	55.00	CAGTTGGTGACAATGCCGTG
il6 (Interleukin 6)	246	f	57.14	CTCTCCGCAAGAGACTTCCAG
		r	47.62	TTCTGACAGTGCATCATCGCT
nos2 (iNOS)	234	f	52.38	TAGTCAACTACAAGCCCCACG
		r	60	GTGAGGAACTGGGGGAAACC
cd86 (CD86)	164	f	45.45	AGACATGTGTAACCTGCACCAT
		r	55	TACGAGCTCACTCGGGCTTA
il10 (Interleukin 10)	186	f	52.38	CGACGCTGTCATCGATTTCTC
		r	60.00	CAGTAGATGCCGGGTGGTTC
clec10a (C-type lectin domain containing 10a)	164	f	60.00	GAGGCTTGAGCCAGAAGGTG
		r	52.38	TGCTGAGCCGTTGTTCTTGAG
mrc1 (mannose receptor C typ 1)	212	f	60.00	CCCGCTCCTCAAGACAATCC
		r	55.00	AAATACGGTGACTGCCCACC
cd163 (CD163)	131	f	60	CTCTGAAGCGACGACAGACC
		r	50	ATGCCAACCCGAGGATTTCA
tgfb1 (transforming growth factor-β)	115	f	60.00	GGCTGAACCAAGGAGACGGA
		r	55.00	CCTCGACGTTTGGGACTGAT
Infg (Interferon gamma)	128	f	50	CAACCAGGCCATCAGCAACAACAT
		r	50	TCTGTGGGTTGTTCACCTCGAACT
Serpine1 (pai-1)	123	f	55.00	CGTCTTCCTCCACAGCCATT
		r	55.00	GCTGGCCCATGAAGAGGATT
Serpineb2 (pai-2)	223	f	52.38	AGCCGCTCAGAAGATAACGAG
		r	39.13	CAAAATTCAGCACTTTGGCCATT
col1a1 (collagen type I alpha I chain)	237	f	60	CTGACTGGAAGAGCGGAGAG
		r	55.00	CAGGATCGGAACCTTCGCTT
mmp1 (matrix metallopeptidase 1)	144	f	55.00	AAGGCCACTGGTGATCTTGC
		r	43.48	GGTATTTCCAGACTGTTTCCACA
fn1 (Fibronectin 1)	165	f	63.16	TCCCCTCCCAGAGAAGTGG
		r	43.48	TTGGGGAAGCTCATCTGTCTTTT

of 0.05. A 10% drop-out was taken into account resulting in 17 animals per group.

Values were expressed as median with interquartile ranges or in proportions. Given the small sample size, non-parametric testing was performed using the Mann-Whitney U test for continues and ordinal variables. Nominal variables were analysed with a Fisher's exact test. In case of dependent samples, a Wilcoxon signed rank test was performed. A p-value of 0.05 was considered significant. Because only the intervention group was compared to either the control group or HA-CMC group no correction for multiple testing was applied. Data-analysis was performed with SPSS 23.0 for Mac (SPSS Inc, Chicago, IL).

Results

Of the 51 animals, one animal in the HA-CMC group died during the operation related to anaesthesia. The remaining 50 animals completed a follow-up of 14 days as planned. The mean weight of the animals prior to surgery was 232.9 gr (SD 12.3 gr), this was not significantly different between the groups (p=.816).

Macroscopic evaluation

Results of macroscopic evaluation are described in Table 4. Quantity of adhesions was assessed based on the Nair score, number of buttons and number of organs involved in adhesions. The Nair score was equal between all groups with a median of 3.0 (IQR 1.0). Median number of involved buttons was not different when comparing HA gel (median 7.0, IQR 3.0) to the control group (median 7.0, IQR 1.0, p=.067) or to the HA-CMC group (median 7.0, IQR 1.5, p = 0.451). Regarding the number of organs involved in adhesions the median was 2.0 in all groups, but the interquartile range was 0.0 in the HA group compared to 1.0 in the HA-CMC and control group. Number of organs involved in adhesions in the HA group was significantly different compared to the HA-CMC (p=.041) and the control group (p=.012). The greater omentum was involved in adhesions in all the animals and scrotal fat in 82.4% of the animals in the HA gel group, 93.8% of the animals in the HA-CMC group and 94.1% of the animals in the control group (p = 0.600). Organs other than the greater omentum or scrotal fat were involved in 11.8%, 37.5% and 47.1% of the animals respectively (p = 0.069).

Tenacity and the vascularisation of the adhesions define the quality of the adhesions. The tenacity was not different between HA gel and the other two groups with a median of 2.0 (IQR 1.0). There was no vascularisation of adhesions observed in the HA gel group, in one animal in the HA-CMC group (6.3%) and in two animals in the control group (11.8%). The difference was not significant between the groups.

Expert panel

Three abdominal surgeons, blinded to the allocation of the animals scored the photographs taken after sacrifice, (See Table 5). The total adhesion score was highest when HA-CMC was used (median 8.0, IQR 4.8, p=.179) followed by the rats in the control group (median 7.0, IQR 2.0, p=.734). HA gel group showed

Table 4. Results of macroscopic adhesion scoring presented as median with IQR or percentage of occurrence in comparison to HA gel.

	HA gel	HA-CMC	Control
Nair score Median (IQR)	3.0 (1.0)	3.0 (1.0) p = .950	3.0 (1.0) p = .138
Tenacity (Zühlke score) Median (IQR)	2.0 (1.0)	2.0 (1.0) p = .878	2.0 (1.0) p = .918
Vascularisation yes/total (%)	0/17 (0.0%)	1/16 (6.3%)	2/17 (11.8%)
Number of buttons Median (IQR)	7.0 (3.0)	7.0 (1.5) p = .451	7.0 (1.0) p = .051
Number of organs involved in adhesions Median (IQR)	2.0 (0.0)	2.0 (1.0) **p = .041**	2.0 (1.0) **p = .012**

P values are related to the comparison of the HA group to one of the control groups (Mann-Whitney U test).

Table 5. Results of adhesions scoring by three experts, both sides of the midline incision were scored separately leading to a maximum score of 8 regarding quantity, 6 regarding quality and a maximum total score of 14 per animal. Median with IQR are presented.

	HA gel	HA-CMC	Control
Quantity of adhesions Median (IQR)	3.0 (2.7)	4.0 (2.8) p = .146	3.33 (1.5) p = .708
Quality of adhesions Median (IQR)	3.3 (1.2)	4.0 (2.2) p = .276	3.7 (0.7) p = .946
Total score Median (IQR)	6.3 (3.7)	8.0 (4.8) p = .179	7.0 (2.0) p = .734

Comparison of HA gel to HA-CMC and to the control group, p-values are expressed in relation to the HA group.

the lowest score with a median of 6.3 (IQR 3.7); this did not reach significance compared to the other groups.

Quantity of adhesions was scored lowest in the HA animals with a median of 3.0 (IQR 2.7). This was not significantly lower compared to HA-CMC (median 4.0, IQR 2.8, p=.146) and the control group (median 3.33, IQR 1.5, p=.708). Quality of adhesions was also scored lowest after the use of HA gel (median 3.3, IQR 1.2) compared to HA-CMC (median 4.0, IQR 2.2, p=.276) and the control group (median 3.7, IQR 0.7, p=.946).

qPCR

The qPCR was used to determine the role of macrophage polarisation in the formation of adhesions. Gene expression characteristic for subtype one (M1: *il6*, *nos2*,

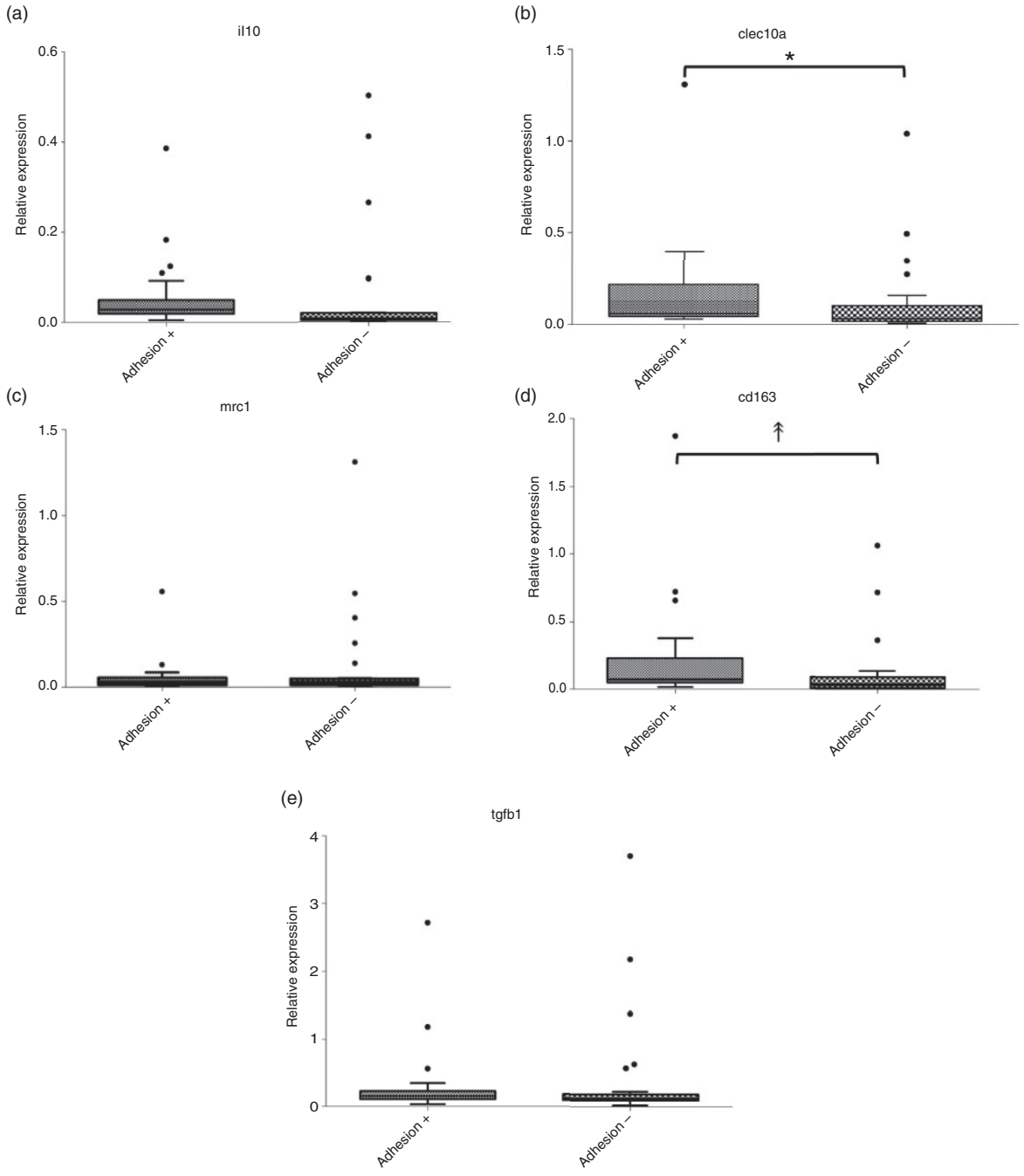

Figure 1. Relative expression of genes associated with M2 ((a) il10, (b) clec10a, (c) mrc1, (d) cd163, (e) tgfb1) comparing tissue with adhesions to tissue without adhesions depicting medians with IQR.
*p-value = 0.025.
↟p-value = 0.003.

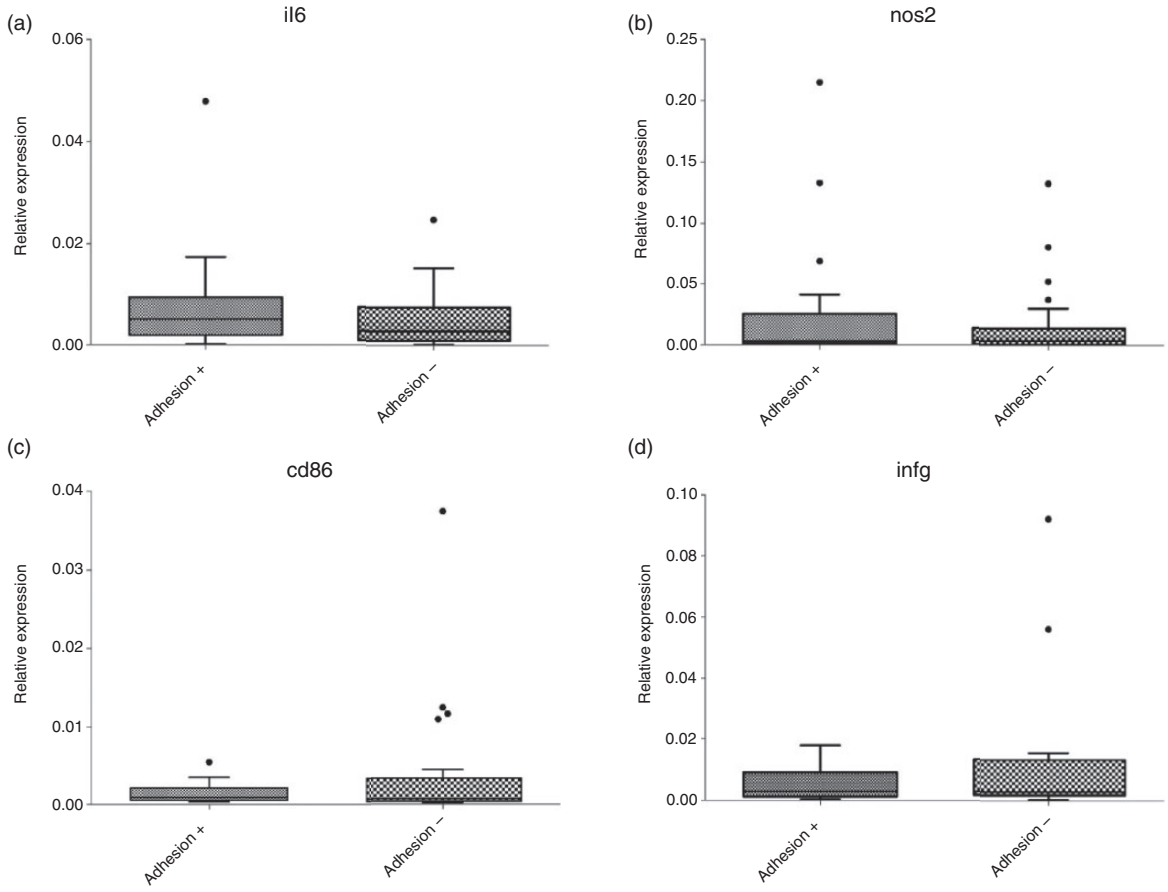

Figure 2. Relative expression of genes associated with M1 ((a) il6, (b) nos2, (c) cd86, (d) infg) comparing tissue with adhesions to tissue without adhesions depicting medians with IQR.

cd86 and *infg*) and subtype two (M2: *il10, clec10a, mrc1, cd163 and tgfb1)* were both investigated to provide information regarding dominant subtype presence in the tissue. M1 is associated with pro-inflammatory properties in contrast to M2, which plays a role in extra-cellular matrix remodelling and regulation of fibroblast.[16–18] In this experiment, it is hypothesised that a dominant presence of M2 is involved in the formation of adhesions. In addition, the expression of genes typically active in fibroblasts (*col1a1*, *mmp1* and *fn1*) and specifically involved in adhesion formation (*pai-1* and *pai-2*) was evaluated.

From the 50 animals that completed the follow-up, 32 animals were identified to have both an ischemic button with an adhesion and an ischemic button that failed to induce adhesion formation. In one sample, the RNA concentration was too low and in one sample no expression was detected for housekeeping genes, making normalisation impossible. Both samples contained an ischemic button with an adhesion and were together with their corresponding control sample excluded from the analysis. Of the 30 animals available for qPCR analysis, 12 were allocated to the HA group, 10 to the HA-CMC group and 8 to the control group.

Clec10a, a M2-associated gene, was 1.36-fold higher expressed in tissue with adhesions compared to tissue without adhesions ($p = 0.025$). Another gene typically expressed in M2, *cd163*, showed a higher expression in presence of adhesions compared to absence of adhesions (1.80-fold, $p = 0.003$) (Figure 1). Genes specific for M1 polarisation (*il6*, *nos2*, *cd86* and *infg)* did not reach a significant difference between samples with and without adhesions (Figure 2). Regarding fibroblast activation, a 5.14-fold higher expression of *mmp1* was encountered in samples with adhesion in comparison to samples with no adhesions ($p = 0.028$) (Figure 3). Other markers for fibroblast activation (*col1a1* and *fn1*) showed no differences. *Pai-1* and *pai-2*, known to be involved in adhesion formation, did not differ between groups (Figure 4).

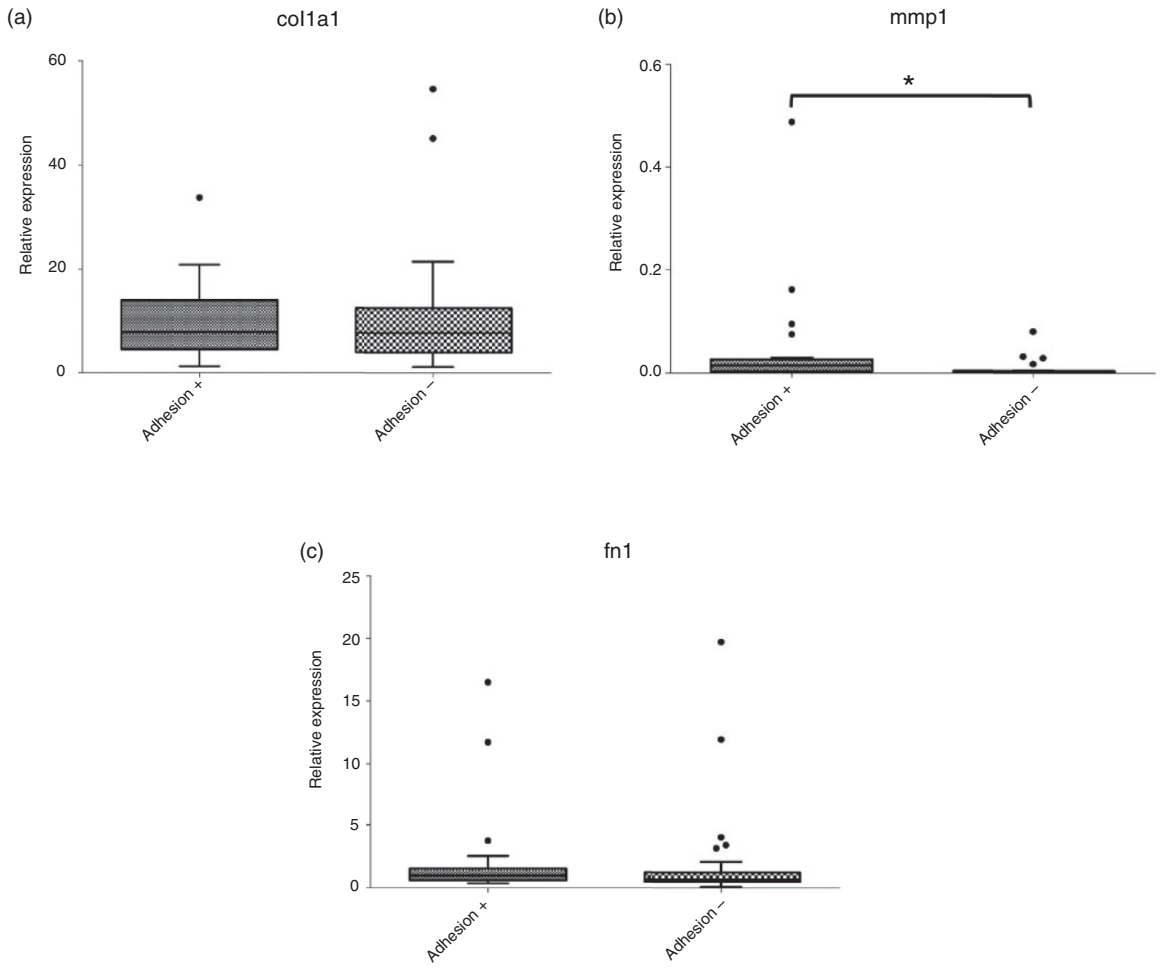

Figure 3. Relative expression of genes associated with fibroblast activation ((a) col1a1, (b) mmp1, (c) fn1) comparing tissue with adhesions to tissue without adhesions depicting medians with IQR.
*p-value= 0.028.

Discussion

Intra-abdominal formation of adhesions continues to be a burdening complication for patients who have had surgery.[3,4] Preventive measures with satisfying results are not available.[7,8] HA is proposed in this experiment for the prevention of adhesion formation and has the same main component as HA-CMC, hyaluronic acid. The HA gel was crosslinked and differed from HA-CMC by its viscous properties. Due to these viscous properties, it can be used during laparoscopic procedures in contrast to anti-adhesive barrier sheets, such as HA-CMC. Instead of an anti-adhesive sheet (HA-CMC) that only provides a barrier between the intra-abdominal organs and the incision in the abdominal wall, HA spreads throughout the abdominal cavity acting as a barrier.

In this study, no reduction in macroscopic adhesion formation was established by the application of HA gel compared to HA-CMC and the control group. A significant lower organ involvement in adhesion formation was found in the HA group (median 2.0, IQR 0.0) compared to the HA-CMC (median 2.0, IQR 1.0) and control group (median 2.0, IQR 1.0). Furthermore, a higher gene expression of *clec10a* and *cd163* was encountered, both typical markers for M2, so an association between adhesion formation and M2 expression was concluded.

Regarding the lower organ involvement after application of HA gel, interestingly the effect was most profound when evaluating organ involvement without the greater omentum and scrotal fat. However, this subgroup analysis did not reach significance. This result emphasizes the difficulty in preventing adhesions to viscera which move relatively free in the abdominal cavity, such as the greater omentum and scrotal fat.

The Nair and tenacity score were unable to distinct in extent and severity of adhesion formation. The number of buttons involved appears to be a more

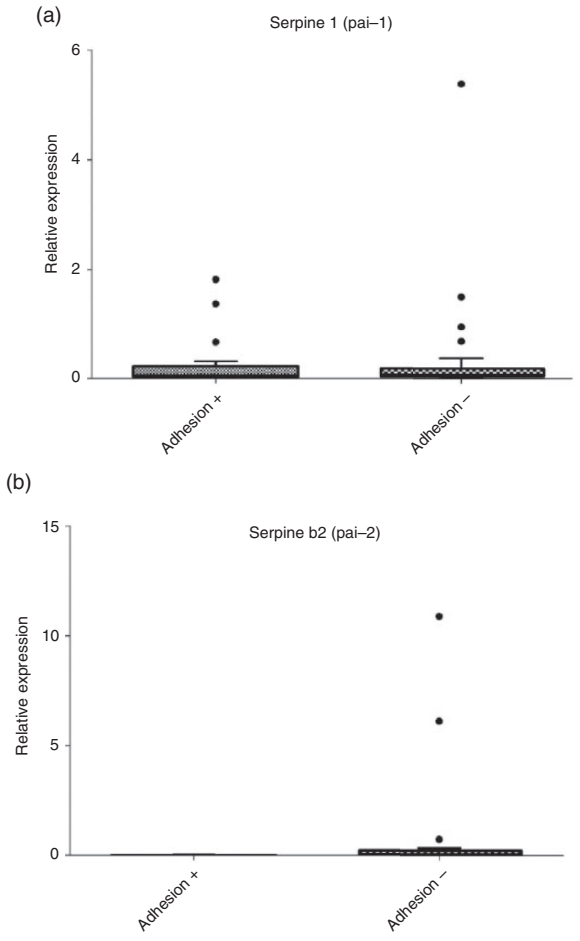

Figure 4. Relative expression of genes associated with adhesions formation ((a) Serpine1, (b) Serpineb2) comparing tissue with adhesions to tissue without adhesions depicting medians with IQR.

adequate measure for evaluating the extent of adhesion formation. The Nair score ranges from 0–4, with grade 3 accounting for multiple adhesions formed. The number of buttons involved in adhesions measures all the individual adhesions formed, which constitutes a more detailed measure of adhesion formation. In this experiment no significant differences were found between the groups. An expert panel evaluated the extent and severity of the adhesions in order to bridge the translational gap between animal studies and clinical practice, which unfortunately did not add new insights in distinction in adhesion formation between HA and the other two groups.

In order to gain more insight in the pathophysiological process of adhesion development and the role of macrophage polarization in adhesion formation, gene expression analysis was performed to compare buttons with adhesions and buttons without adhesions within the same animal and irrespective of treatment group. Macrophages play a pivotal role in the pathophysiologic process of adhesion formation; it has been shown that depletion of macrophages results in lower adhesion scores.[14] Additionally, the macrophage M1/M2 paradigm was identified, describing a continuum between two functionally polarized states of activated macrophages. On one side M1 is known as the classical or pro-inflammatory macrophage associated with tissue injury and M2 on the other side of the spectrum has more anti-inflammatory properties, such as extracellular matrix remodeling and fibroblast regulation.[16–18]

In this study, we demonstrated that buttons with adhesions showed significantly more M2-related gene expression then buttons without adhesion formation. This is based on a pattern of significantly higher expression of *clec10a* and *cd163* (two markers typical for M2) in buttons with adhesions. Genes specific for M1 did not show any significant expression differences. The role of macrophage polarization has been described earlier in a mouse model, but the opposite effect was observed after seven days of follow-up in an ischemic button model in mice. Higher expression of M2 markers were encountered in tissue without adhesion formation, suggesting a protective effect from M2 polarization.[30] This contradicts the known function of M2, which is extra-cellular matrix remodeling and fibroblast regulation.[16–18] In our experiment, adhesion formation is associated with increased M2 polarization. Subsequently, it is agreed that mesothelial regeneration takes place 5 to 8 days post injury,[31] the question remains why after 14 days still a high M2 expression is encountered in buttons with adhesions. This could be explained by ongoing tissue remodeling, but could also be a prolonged activation of M2 macrophages as part of the pathophysiologic process of adhesion formation.

Subsequently, a significantly higher *mmp1* expression was found in buttons with adhesions compared to buttons without adhesions. This is consistent with findings from *in-vitro* studies, where fibroblasts from adhesions expressed higher *mmp1* levels compared to normal peritoneum fibroblasts.[15] The high expression of *mmp1* in adhesive tissue is linked to continued, prolonged tissue remodeling in adhesions after development. Also wound healing of the peritoneum is altered by unregulated production of *mmp1*.[13]

The inhibition of the fibrinolytic system is thought to play an important role in the pathophysiology of adhesion formation. High levels of *pai-1* and *pai-2* are associated with an decreased fibrinolytic activity and are therefore associated with adhesion formation.[12] In this experiment, although *pai-1* is a stronger inhibitor than *pai-2*, both were analyzed as specific markers for adhesion formation.[12,32] No differences in *pai-1* or *pai-2* expression between tissue with and without adhesions were found. In animal studies, *pai-1* levels peaked 1 day

after surgery,[14] but in human studies also differences were found years after the first operation, during reoperation.[33] However, in these studies adhesive tissue was compared to normal peritoneum.[14,33] It appears that *pai-1* and *pai-2* influence adhesion formation by interacting with the fibrinolytic system in the acute phase and early postoperative period, which may account for the absence of expression after 14 days in our experiment.

The animals in this experiment act as their own control by taking two tissue samples, one ischemic button with adhesions and one without adhesions. The differences that were found between both conditions suggest polarization towards M2 and higher fibroblast activation in adhesional tissue and suggest that adhesion formation is a rather local process. In this experiment the dynamic process of macrophage polarization is investigated at one time-point. The exact pathophysiologic process of adhesion formation and the role of macrophage polarization are not clear yet. The focus should be on multiple time-points to evaluate the course in time and on the interaction between the fibrinolytic system, macrophage polarization and fibroblast activation.

Some limitations of this animal experiment need to be discussed. Starting with the impossibility of blinding due to the obvious difference of the investigated materials, which could be a possible source of bias. However, the animals were randomly allocated to a group and equally divided over the cages to minimize the occurrence of possible bias. Data processing and analysis was performed blind. Subsequently, an evaluation of photographs of the adhesions by three experts was performed blind. In this study a gel barrier is compared to a sheet barrier, which might perform their barrier function differently. However, is the HA-CMC barrier frequently used in clinical practice and most extensively researched, which makes it the obvious candidate for comparison. The Nair and tenacity score appear to be inaccurate for adhesion assessment in the ischemic button model, for this model induces a great deal of adhesions throughout the abdomen. The number of buttons appears to be more suitable in this case.

Conclusion

HA gel application reduces the number of organs involved in adhesion formation in an ischemic button model, but shows no significant overall reduction in the formation of adhesions. Macrophage polarization towards M2 and high *mmp1* expression are associated with adhesion formation.

Authors' note

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Declaration of conflicting interests

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References

1. Menzies D and Ellis H. Intestinal obstruction from adhesions–how big is the problem? *Ann R Coll Surg Engl* 1990; 72: 60–63.
2. Stommel MW, Ten Broek RP, Strik C, et al. Multicenter observational study of adhesion formation after open- and laparoscopic surgery for colorectal cancer. *Ann Surg* 2017; 267: 743–748.
3. Parker MC, Ellis H, Moran BJ, et al. Postoperative adhesions: ten-year follow-up of 12,584 patients undergoing lower abdominal surgery. *Dis Colon Rectum* 2001; 44: 822–829; discussion 9–30.
4. Parker MC, Wilson MS, Menzies D, et al. The SCAR-3 study: 5-year adhesion-related readmission risk following lower abdominal surgical procedures. *Colorect Dis* 2005; 7: 551–558.
5. ten Broek RP, Issa Y, van Santbrink EJ, et al. Burden of adhesions in abdominal and pelvic surgery: systematic review and met-analysis. *BMJ* 2013; 347: f5588.
6. ten Broek RP, Strik C, Issa Y, et al. Adhesiolysis-related morbidity in abdominal surgery. *Ann Surg* 2013; 258: 98–106.
7. Kumar S, Wong PF and Leaper DJ. Intra-peritoneal prophylactic agents for preventing adhesions and adhesive intestinal obstruction after non-gynaecological abdominal surgery. *Cochrane Database Syst Rev* 2009; DOI: 10.1002/14651858.CD005080.

8. ten Broek RP, Stommel MW, Strik C, et al. Benefits and harms of adhesion barriers for abdominal surgery: a systematic review and meta-analysis. *Lancet* 2014; 383: 48–59.
9. van Steensel S, van den Hil LCL, Schreinemacher MHF, et al. Adhesion awareness in 2016: an update of the national survey of surgeons. *PLoS One* 2018; 13: e0202418.
10. Beck DE, Cohen Z, Fleshman JW, et al. A prospective, randomized, multicenter, controlled study of the safety of seprafilm adhesion barrier in abdominopelvic surgery of the intestine. *Dis Colon Rectum* 2003; 46: 1310–1319.
11. Maciver AH, McCall M and James Shapiro AM. Intra-abdominal adhesions: cellular mechanisms and strategies for prevention. *Int J Surg* 2011; 9: 589–594.
12. Arung W, Meurisse M and Detry O. Pathophysiology and prevention of postoperative peritoneal adhesions. *World J Gastroenterol* 2011; 17: 4545–4553.
13. Chegini N, Kotseos K, Zhao Y, et al. Expression of matrix metalloproteinase (MMP-1) and tissue inhibitor of MMP in serosal tissue of intraperitoneal organs and adhesions. *Fertil Steril* 2001; 76: 1212–1219.
14. Honjo K, Munakata S, Tashiro Y, et al. Plasminogen activator inhibitor-1 regulates macrophage-dependent postoperative adhesion by enhancing EGF-HER1 signaling in mice. *FASEB J* 2017; 31: 2625–2637.
15. Saed GM, Kruger M and Diamond MP. Enhanced matrix metalloproteinase expression by Tisseel in mesothelial cells, normal peritoneal fibroblasts, and adhesion fibroblasts. *Eur J Plast Surg* 2006; 28: 472–479.
16. Martin P and Leibovich SJ. Inflammatory cells during wound repair: the good, the bad and the ugly. *Trends Cell Biol* 2005; 15: 599–607.
17. Wynn TA and Barron L. Macrophages: master regulators of inflammation and fibrosis. *Semin Liver Dis* 2010; 30: 245–257.
18. Martinez FO and Gordon S. The M1 and M2 paradigm of macrophage activation: time for reassessment. *F1000Prime Rep* 2014; 6: 13.
19. Liu C, Lu Q, Zhang Z, et al. A randomized controlled trial on the efficacy and safety of a new crosslinked hyaluronan gel in reducing adhesions after gynecologic laparoscopic surgeries. *J Minim Invasive Gynecol* 2015; 22: 853–863.
20. Kraemer B, Wallwiener C, Rajab TK, et al. Standardised models for inducing experimental peritoneal adhesions in female rats. *Biomed Res Int* 2014; 2014: 1–8.
21. Rajab TK, Wauschkuhn CA, Smaxwil L, et al. An improved model for the induction of experimental adhesions. *J Invest Surg* 2010; 23: 35–39.
22. Whang SH, Astudillo JA, Sporn E, et al. In search of the best peritoneal adhesion model: comparison of different techniques in a rat model. *J Surg Res* 2011; 167: 245–250.
23. Nair SK, Bhat IK and Aurora AL. Role of proteolytic enzyme in the prevention of postoperative intraperitoneal adhesions. *Arch Surg* 1974; 108: 849–853.
24. Zuhlke HV, Lorenz EM, Straub EM, et al. Pathophysiology and classification of adhesions. *Langenbecks Arch Chir Suppl II Verh Dtsch Ges Chir* 1990; 1009–1016.
25. Lucas PA, Warejcka DJ, Young HE, et al. Formation of abdominal adhesions is inhibited by antibodies to transforming growth factor-beta1. *J Surg Res* 1996; 65: 135–138.
26. Peyton CC, Keys T, Tomblyn S, et al. Halofuginone infused keratin hydrogel attenuates adhesions in a rodent cecal abrasion model. *J Surg Res* 2012; 178: 545–552.
27. Schreinemacher MH, Emans PJ, Gijbels MJ, et al. Degradation of mesh coatings and intraperitoneal adhesion formation in an experimental model. *Br J Surg* 2009; 96: 305–313.
28. van den Hil LCL, Vogels RRM, van Barneveld KWY, et al. Comparability of histological outcomes in rats and humans in a hernia model. *J Surg Res* 2018; 229: 271–276.
29. Ye J, Coulouris G, Zaretskaya I, et al. Primer-BLAST: a tool to design target-specific primers for polymerase chain reaction. *BMC Bioinformatics* 2012; 13: 134.
30. Hong GS, Schwandt T, Stein K, et al. Effects of macrophage-dependent peroxisome proliferator-activated receptor gamma signalling on adhesion formation after abdominal surgery in an experimental model. *Br J Surg* 2015; 102: 1506–1516.
31. diZerega GS and Campeau JD. Peritoneal repair and post-surgical adhesion formation. *Hum Reprod Update* 2001; 7: 547–555.
32. Ivarsson ML, Bergstrom M, Eriksson E, et al. Tissue markers as predictors of postoperative adhesions. *Br J Surg* 1998; 85: 1549–1554.
33. Holmdahl L, Kotseos K, Bergstrom M, et al. Overproduction of transforming growth factor-beta1 (TGF-beta1) is associated with adhesion formation and peritoneal fibrinolytic impairment. *Surgery* 2001; 129: 626–632.