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Original article

Injectable chitosan hydrogel effectively controls lesion growth in a venous malformation murine model

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ABSTRACT

Purpose: The purpose of this study was to evaluate the safety and efficacy of intralesional injection of chitosan hydrogel (CH) combined with sodium tetradecyl sulfate (STS) to sclerose and embolize venous malformations (VMs) by comparison with 3% STS foam and placebo in a mouse model.
Materials and methods: Subcutaneous VMs were created by injecting HUVEC_TIE2-L914F cells, mixed with matrigel, into the back of athymic mice (Day [D] 0). After VM-like lesions were established at D10, 70 lesions were randomly assigned to one of six treatment groups (untreated, saline, 3% STS-foam, CH, 1% STS-CH, 3% STS-CH). For 3% STS-foam, the standard Tessari technique was performed. VMs were regularly evaluated every 2–3 days to measure lesion size until the time of collection at D30 (primary endpoint). At D30, VM lesions including the matrigel plugs were culled and evaluated by histological analysis to assess vessel size, chitosan distribution and endothelial expression. One-way analysis of variance (ANOVA) test was performed to compare quantitative variables with normal distribution, otherwise Kruskal-Wallis test followed by pairwise comparisons by a Wilcoxon rank sum test was performed.
Results: All VMs were successfully punctured and injected. Six VMs injected with 3% STS-CH showed early skin ulceration with an extrusion of the matrigel plug and were excluded from final analysis. In the remaining 64 VMs, skin ulceration occurred on 26 plugs, resulting in the loss of three 3% STS-foam and one 1% STS-CH plugs. Both chitosan formulations effectively controlled growth of VMs by the end of follow-up compared to untreated or 3% STS-foam groups ($P < 0.05$). Vessel sizes were smaller with both CH formulations compared to untreated and saline groups ($P < 0.05$). Additionally, there were smaller vascular channels within the 1% STS-CH group compared to the 3% STS-foam group ($P < 0.05$).
Conclusion: Chitosan's ability to control the growth of VMs suggests a promising therapeutic effect that outperforms the gold standard (STS-foam) on several variables.
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1. Introduction

Venous malformations (VMs) are characterized by low-flow dysplastic or cavitary venous structures [1]. Symptomatic VMs are often treated with percutaneous sclerotherapy using a chemical agent or detergent [1-4]. Sclerotherapy typically requires multiple treatments which can be distressing for patients [5]. Sodium tetradecyl sulfate (STS), ethanol, ethanolamine oleate, bleomycin, polidocanol and bleomycin are the most commonly used sclerosing agents and are

Abbreviations: CH, Chitosan hydrogel; EC, Endothelial cells; HUVEC, Human umbilical vein endothelial cells; IsoB4, IsolectinB4 antibody; STS, Sodium tetradecyl sulfate; TIE2, Endothelial-specific receptor, tyrosine kinase with immunoglobulin-like loops and epidermal growth factor homology domains-2; UEA1, Ulex europaeus agglutinin 1; VM, Venous malformation

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typically injected as a foam or liquid for STS and as a foam for polidocanol [6-9]. A retrospective study revealed that STS was safe but less effective than other popular sclerosants such as ethanol, polidocanol or bleomycin [6,7]. However, it is less painful and safer than ethanol, ethanolamine and less expensive than polidocanol [6,8]. The lower efficacy of STS may be related to a lower active concentration, as the original 3% STS concentration is reduced to less than 1% after mixing with air [10]. Foam stability is another concern and may be better using polidocanol foam made of carbon dioxide. In addition, STS foam can be rapidly washed away from the target site. Sclerosants can be inactivated by plasma proteins, membrane lipids, and circulating blood cells [11,12]. It has also been reported that more successful treatments combine venous outflow occlusion of the defective vessel [13]. Therefore, we hypothesize that a method to improve the efficacy of STS-based treatment would be to embolize the lesion and progressively elute the sclerosant locally.

Recently, hydrogels have gained momentum as an attractive drug delivery system [14]. Chitosan is a polysaccharide derived from chitin. It is a thermogel that remains in the form of an injectable viscous solution form at room temperature but rapidly solidifies into a gel at body temperature, allowing for rapid flow blockage. Chitosan can become radiopaque and sclerosing when mixed with iodinated contrast material and STS [15,16].

The purpose of this study was to evaluate the safety and efficacy of sclerotherapy/embolization using chitosan hydrogels without (CH) or with STS (STS-CH) by comparison with STS foam and placebo using an established endothelial-specific receptor, tyrosine kinase with immunoglobulin-like loops and epidermal growth factor homology domain-2 (TIE2)-associated VM xenograft mouse model [17].

2. Materials and methods

2.1. Generation of TIE2-L914F-associated VM mice

All animal procedures were approved by the Ethical Committee for Animal Experimentation at the Centre Hospitalier de l'Université de Montréal Research Center (CRCHUM) in accordance with the ARRIVE guidelines and EU Directive 2010/63/EU for animal experiments. Generation of TIE2-associated VM xenograft mice was performed as previously described [17]. Human umbilical vein endothelial cells (HUVECs) expressing high levels of the TIE2-L914F mutation were cultured, detached, mixed with matrigel and injected subcutaneously into the backs of athymic male mice (Crl:Nu(NCr)-*Foxn1*^{nu}; Charles River). Two to three implantation sites per mice were performed on day (D)0. This model develops VM-like lesions in a rapid and consistent manner. It produced engorged, blood-filled vascular channels visible as purple-blue lesions within one week. The VM-like lesions were allowed to develop until D10 to ensure sufficient development prior to injection (Fig. 1).

A total of 27 mice were enrolled. Between two and three VM lesions were created in each mouse and treatment allocation for each lesion was assigned randomly per lesion for a total of 70 lesions. Treatment lesion groups included an untreated group (n = 8), saline (n = 9), 3% STS-foam (n = 15), CH alone (n = 15) and two groups in which CH was mixed with two different concentrations of STS (1% STS-CH [n = 17] and 3% STS-CH [n = 6]) (Table 1).

2.2. Preparation of chitosan (CH) hydrogel

A chitosan solution (solution A) was produced by dissolving Chito-Clear chitosan (Primex) in 0.1 M HCl containing a 30% v/v iopamidol solution (Isovue 370®, 370 mg/mL iodine, Bracco Imaging). The solution autoclaved and sterilized. A gelling solution (solution B) was made by dissolving β-glycerophosphate disodium salt hydrate (BGP, Sigma-Aldrich) in a phosphate buffer (pH 7.0); for STS-CH formulations, STS (Niaproof, Sigma-Aldrich) was added to the phosphate buffer [15]. Solution B was sterilized by filtration through a 0.2 μm membrane filter.

To prepare the hydrogel, solution A and solution B were aspirated into separate 1-mL male luer-lock syringes, at a volumetric ratio of 3:2. The two syringes were connected to a luer-lock connector and the two solutions were mixed by pumping them well. The final concentration of CH in the hydrogel was 3% w/v, while the concentrations of STS were either 1 or 3% w/v (Fig. 1). 1% STS-CH would be similar to the usual 3% STS foam, while 3% STS-CH is 3 × the concentration typically used.

2.3. Preparation of STS foam

STS foam was prepared by the standard Tessari method, using a 1:3 volume ratio of 3% STS to air [18]). Of note, 3% STS foam is the standard formulation used clinically to treat VM in patients; however, the final effective concentration of STS in the foam was closer to 1% STS.

2.4. Injection treatment and follow-up of VMs within matrigel plugs

At D10, after anesthesia, each matrigel plug was evaluated for the darkest purple spot, which indicated an enlarged, blood-filled vascular channel ready for injection. The plug was lightly pinched between two fingers and punctured with a 26-gauge needle, bevel up, at the center of the darkest purple area; the depth of the puncture was sufficient to cover the needle orifice. Once blood return was obtained, a volume of 50–150 μL of the appropriate treatment was slowly and carefully injected into each plug (max 150 μLfor saline and STS foam; 50–100 μL for CH and CH-STS). This volume range was determined to allow a filling of the matrigel plug without stretching or discoloration of the skin. Due to the risk of leakage and bleeding, only one puncture site within the lesion was made. The injection of the solutions was done slowly, and several points were monitored carefully: the solutions are being expelled from the syringe as the plunger is pressed, and the added solutions remain within the borders of the plug. These points are sufficient indicators that enough solution has been injected. Care must be taken not to inject too much chitosan as this can cause ulceration. Following these key points allowed for successful injection of lesions within all assigned groups.

After injection, the vascular lesions were measured and evaluated every 2–3 days until point of collection at D30, or earlier when a lesion became too large (> 450 mm^2) [17]. Beyond this threshold the lesion might affect mice the vitality of the mouse and would require sacrifice and earlier collection. Macroscopic photographs of lesion progression over the 20 days of treatment and at D30 were taken with a 5MP camera of a Samsung Galaxy Tab E (Samsung) and after lesion and matrigel plug excision at D30 with a 12MP camera of a Samsung Galaxy S10 to measure overall mean area size (primary endpoint). This quantification method has been used previously and showed a good reproducibility [17].

2.5. Histology and immunohistochemical staining

Matrigel plugs and skin-attached lesions were excised and fixed in formalin. Lesions including the plugs and skins were embedded in paraffin and then processed through a series of degraded alcohol then embedded in paraffin. 5-μm sections were processed for hematoxylin and eosin (H&E) or immunohistochemical staining. For evaluation of inflammation, H&E stained slides (n = 6 per treatment group) were blindly examined and scored (range: 0–3) as previously described [19]. For immunohistochemical staining, specimens were incubated with the endothelial markers (ulex europaeus agglutinin I [UEA1] or isolectinB4 [IsoB4] antibodies), followed by a horseradish

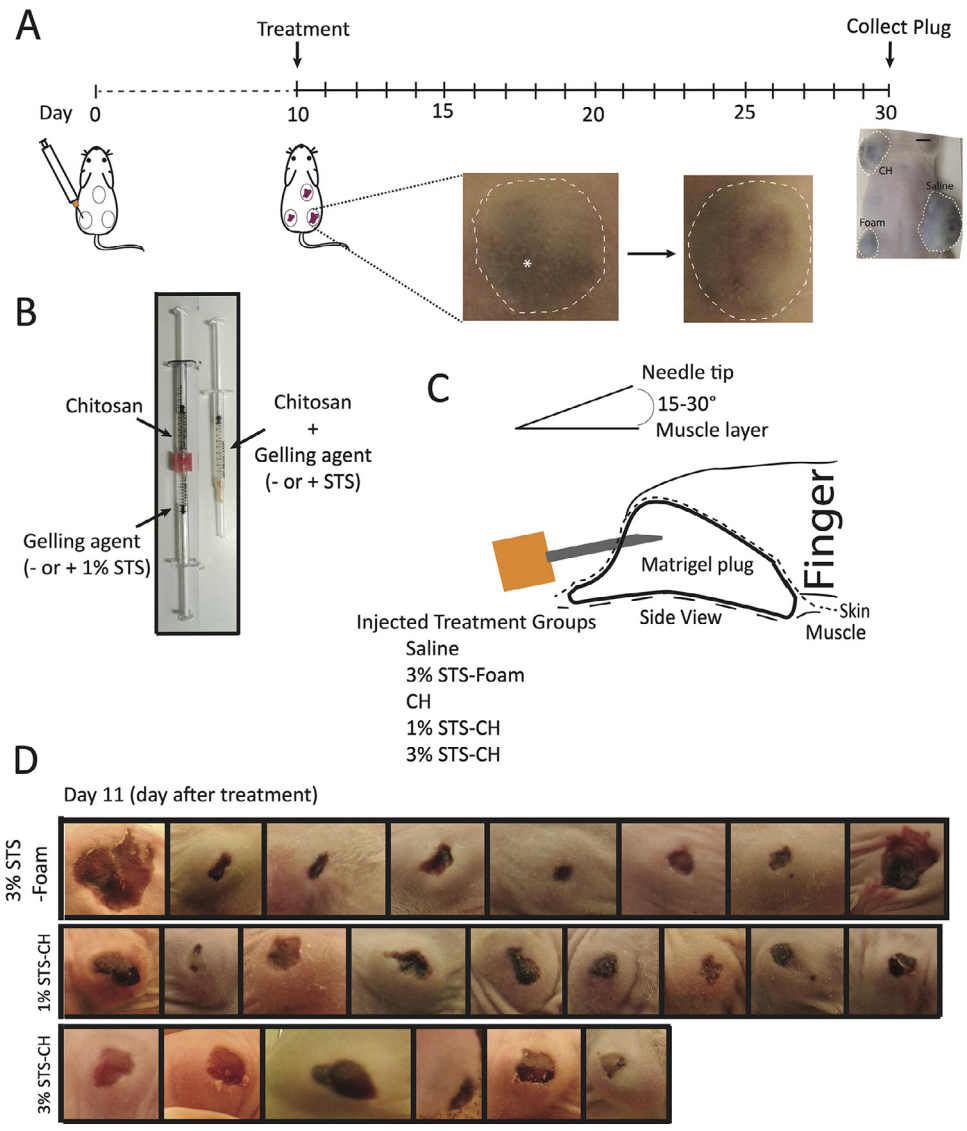

Fig. 1. Schematic representation of the injection treatment of vascular malformations (VMs). A) 2 to 2.5 × 10^6 human umbilical vein endothelial cells with the endothelial-specific receptor, tyrosine kinase with immunoglobulin-like loops and epidermal growth factor homology domains-2 (TIE2-L914F) cells were subcutaneously injected at two to three sites on the dorsal back of athymic male mice at day (D)0. At D10, VMs were injected with either saline, 3% sodium tetradecyl sulfate (STS) foam, chitosan (CH), 1% sodium tetradecyl sulfate chitosan gel (1% STS-CH), or 3% sodium tetradecyl sulfate chitosan gel (3% STS-CH). The area of each lesion was measured every 2 to 3 days. On D30 (20 days post-injection treatment), lesions with matrigel plugs were collected. (Representative photo scale bar, 0.5 cm). B) CH was added into one syringe and the gelling agent (in which STS can be added) in a second syringe, at a 3:2 ratio. The two syringes were connected via a luer lock-to-luer lock connector (red) then the solutions were mixed until becoming homogenous. C) To inject solutions (saline, 3% STS foam, chitosan gels (CH) with or without STS into the VMs), the matrigel plug was punched and held between two fingers. The needle was inserted into the middle of the darkest purple portion of the lesion (*, for example) at a slightly upward angle, no more than 30°, away from the mouse's body. Needle puncture should be shallow, ideally just enough to cover the needle's beveled tip. D) Several lesions injected with solutions containing STS led to skin ulceration by D11, the first day following treatment injection (see Table 1). Due to the 100% rate of skin ulceration with 3% STS-CH, this treatment was discontinued.

peroxidase-conjugated streptavidin secondary antibody. After treatment with a 3,3′-diaminobenzidine-tetrahydrochloride-dihydrate substrate (Labconsult), sections were counterstained with hematoxylin and dehydrated.

Histology slide scans were acquired at 20 × magnification and then non-overlapping snapshots of entire plugs were taken at different planes (n = 8–15 total). For quantification of luminal areas, the lumens along UEA1-positive endothelial cells (ECs) were traced and measured using ImageJ Fiji (National Institute of Health). Quantification of the optimal density of UEA1 and IsoB4 expression was performed as described with a good accuracy and reproducibility using the same software [20]. All histological and immunostaining endpoints were considered as secondary endpoints.

2.6. Statistical analysis

Normality of quantitative variables was checked using Shapiro-Wilk test, followed by a Bartlett test for the homogeneity of variances. When both normality and homogeneity were confirmed, a one-way analysis of variance (ANOVA) test was performed. Otherwise, the Kruskal-Wallis test was used to compare treatment groups, followed by pairwise comparisons with a Wilcoxon rank-sum test (*i.e.*, when the Kruskal-Wallis test revealed a significant difference) [21]. Qualitative variables were compared using Fisher exact test. An in-house R script was used for statistical analysis (all functions came from the stats R package). Graphs were generated using JMP Pro software (v. 16.0, SAS Institute). A P value < 0.05 was considered to indicate a significant difference.

Table 1
Evolution of skin ulceration and plug destruction at days 11, 20 and 30.

Number of vascular malformations	70	70	64	64	64
Treatment group	Ulcers formed D11	Plugs destroyed D11	Ulcers formed D20	Ulcers healed D30	Plugs destroyed D30
Untreated 8 (11%)	0 (0%)	0 (0%)	1 (2%)	1 (2%)	0 (0%)
Saline 9 (13%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
3% STS-foam 15 (21%)	8 (11%)	0 (0%)	8 (13%)	6 (9%)	2 (3%)
CH alone 15 (21%)	0 (0%)	0 (0%)	6 (9%)	6 (9%)	0 (0%)
1% STS-CH 17 (24%)	8 (11%)	0 (0%)	6 (9%)	5 (8%)	1 (2%)
3% STS-CH 6 (9%)	6 (9%)	6 (9%)	NA	NA	NA
Total	22 (31%)	6 (9%)	21 (33%)	18 (28%)	3 (5%)

At day (D) 11, several plugs injected with solutions containing sodium tetradecyl sulfate (STS) led to skin ulceration by D11, the first day following treatment injection. Due to the 100% rate of skin ulceration with 3% STS-CH, this treatment was discontinued. At D21, several plugs developed skin ulcerations during this period, even without STS, as well as one non-injected plug. Most ulcers were able to heal. Three plugs injected with 3% STS-foam and one with 1% STS-CH were destroyed.
CH indicates chitosan; D indicates day; NA indicates not applicable; STS indicates sodium tetradecyl sulfate; VM indicates venous malformation. All percentages are given in proportion to the total number of lesions observed during the study period.

3. Results

3.1. Mice population and treatment allocation

Twenty-seven mice with 70 lesions were enrolled. All lesions not assigned to the untreated lesion group (n = 8) were successfully injected. Preliminary tests showed that all 3% STS-CH treated plugs (n = 6) developed ulcers by D11 (one day after treatment) and the matrigel plugs were completely destroyed, indicating that this concentration was too potent and toxic; therefore, this treatment group was removed from the study and not used in the remaining animals leaving 64 lesions for comparative analysis (Table 1).

3.2. Treatment follow-up

The overall mean area sizes between all treatments were indistinguishable by D25 (15 days after treatment). However, by D30, untreated, saline-treated, and 3% STS-foam plugs continued to grow rapidly, with no significant difference in size between the three groups. Two mice with untreated plugs required early removal on D28 and D29 because a lesion larger than 450 mm^2 was compromising their vitality.

Lesions injected with the chitosan hydrogel, regardless of whether it was mixed with STS, maintained a similar size at D30 as at the first injection (D10), indicating that the hydrogel helped control lesion growth. There were no differences between the CH and 1% STS-CH groups (P = 0.5111), but a significant reduction in size for VMs injected with CH or 1% STS-CH compared to the untreated and 3% STS foam groups (CH: untreated, P = 0.007; CH: 3% STS-foam, P = 0.001; 1% STS-CH: untreated, P = 0.018; 1% STS-CH: 3% STS foam, P = 0.047). There were no significant differences between the saline groups and any of the other treatment groups (saline: untreated, P = 0.601; 3% STS-foam, $P > 0.99$; CH alone, P = 0.133; 1% STS-CH, P = 0.204) (Fig. 2).

3.3. Complications following treatments

All lesions injected with 3% STS-CH developed ulcerations leading to plug extrusion within one day after treatment (D11) and were excluded from the final analysis. Regarding the other groups, several STS treatments resulted in skin necrosis, and even complete destruction of the plug after injection treatment (Fig. 1D). No significant differences in complication rates were found between 3% STS-foam (53%; 8/15) and 1% STS-CH (47%; 8/17) (P = 0.74) (Table 1). Volume limitation (*i.e.*, 150 μL for 3% STS-foam and 50–100 μL for 1% STS-CH) was found to help reduce the risk of ulceration development.

During the 20-day observation period, superficial ulcerations occurred spontaneously on plugs in all treatment groups, except for the saline group. In most VMs, the ulcerations healed. Ultimately, three 3% STS foam and only one 1% STS-CH plugs were completely destroyed (Fig. 2A).

3.4. Macroscopic results

Lesions within the untreated, saline-treated and 3% STS-foam-treated plugs were engorged with blood. The blood-filled sacs were fragile and prone to rupture during the collection of the plugs (Fig. 3). The chitosan remained within the plugs, appearing as a slightly yellow, solid lump that could be cut with a razor blade.

3.5. Histological results

Histological analysis of the plugs revealed enlarged, pseudovascular lumens within the untreated, saline-treated and 3% STS-foam-treated plugs. Matrigel persisted within the plugs, indicating multiple vascular channels. No traces of 3% STS foam were detected. Within the CH and 1% STS-CH injected lesions, the chitosan completely filled the space into which it was injected and was surrounded by residual matrigel (Fig. 3B). Moderate inflammation was observed around the vascular channels in all experimental groups, with no differences observed between the untreated/saline-treated groups and either chitosan-injected groups (Fig. 3B).

Staining for the human endothelial cell marker UEA1 revealed the VMs were lined by ECs. Since the EC lining was largely negative for the mouse EC marker, IsoB4, this indicated that the vascular channels were made from the HUVEC_TIE2-L914F and, consequently, were VM-like (Fig. 4). Measurement of the mean vascular lumen area of the UEA1+ lined vascular channels confirmed that the untreated and saline-treated vascular lumens were significantly larger than the two chitosan treatment groups (untreated: CH alone, P = 0.004; 1% STS-CH, P = 0.001; saline: CH alone, P = 0.020; 1% STS-CH, P = 0.009). The mean lumen area of 1% STS-CH was smaller than that of the 3% STS-foam (P = 0.004). No significant differences were observed between the other groups (3% STS-foam: untreated, P = 0.056; saline, P = 0.188; CH alone, P = 0.059).

To evaluate endothelial destruction induced by the STS and/or CH, the expression levels of UEA1+ and IsoB4+ were measured in ECs directly contacting the chitosan or lining a dilated channel. There was very low expression of IsoB4 within the vascular channels. Overall, there was no significant difference in the UEA1 expression levels among all experimental treatment groups seen (Fig. 5). However, as this includes a mixture of injected and non-injected vascular channels, a specifically evaluation of vascular areas in contact with CH was made. Quantification of vascular channels in direct contact with chitosan (with and without STS) showed low UEA1 expression and a significantly lower expression in the 1% STS-CH group compared to the 3% STS-foam (P = 0.016).

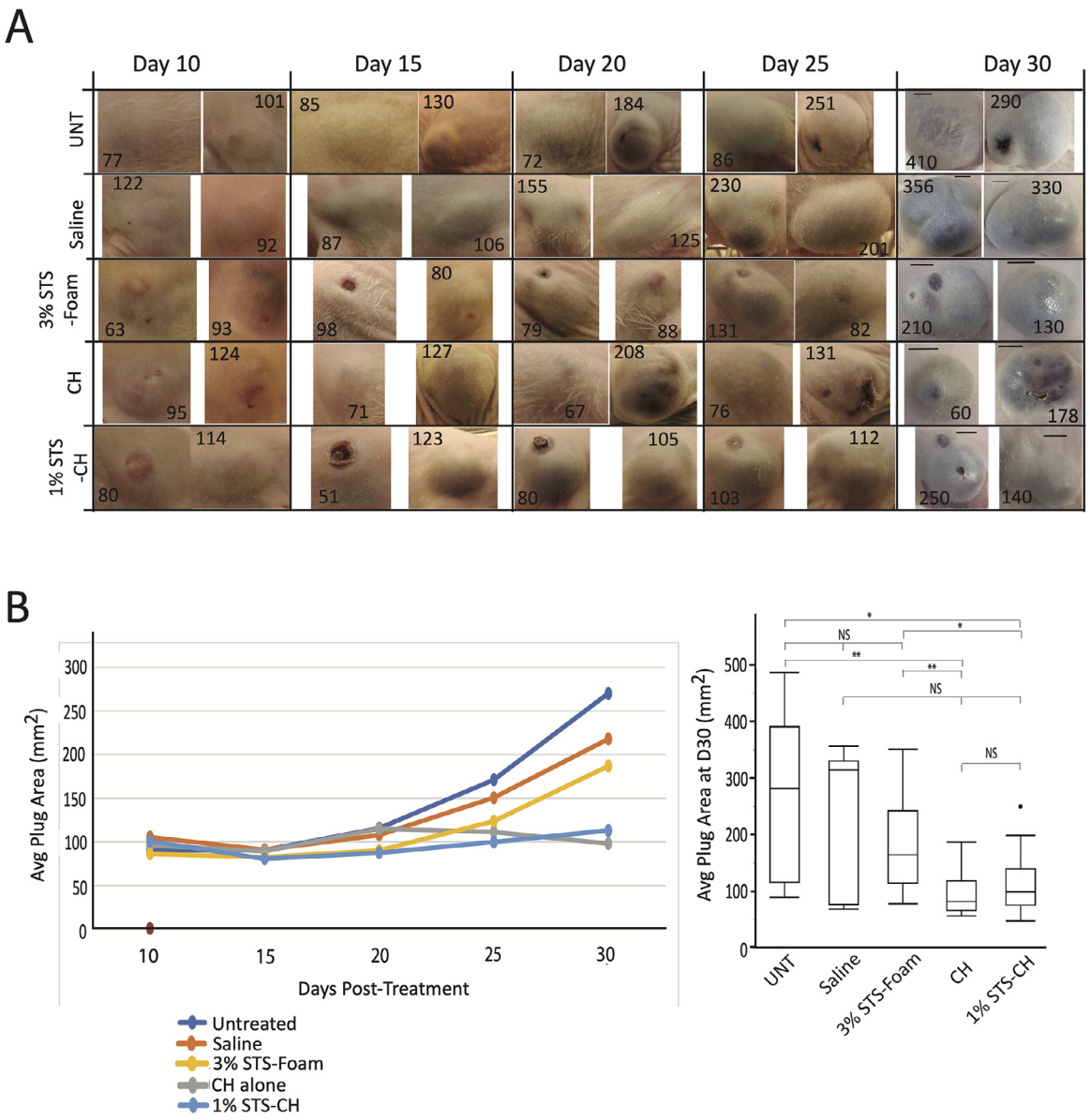

Fig. 2. Lesion progression in response to treatment. A) Representative images of lesions over the 20-day treatment period. Numbers indicate the particular lesion's size (area, in mm^2). Several lesions developed skin ulcerations during this period, even without sodium tetradecyl sulfate (STS), as well as one non-injected plug. Most ulcers were able to heal. Three lesions injected with 3% STS foam (3% STS-foam) and 1 with 1% STS chitosan gel (1% STS-CH) led to early ulceration with plug extrusion (see Table 1). UNT indicates untreated. Scale bar for day 30 plugs, 5 mm. B) Injecting venous malformations (VMs) with a chitosan hydrogel (CH) solution, regardless of the addition of sodium tetradecyl sulfate (STS), effectively controlled overall lesion size. Left: the evolution curves of the mean (average) areas of the injected VMs on the left was estimated around 100 mm^2 at D10 for all formulations and varied between 180 and 270 for the control VMs (untreated, saline) and the VMs injected with 3% STS. VMs injected with CH or 1% STS-CH had no growth (mean areas still around 100 mm^2). Right: the box plots represent the minimal and maximal values, the 25% lower and 75% upper quartiles and the median of mean average areas. NS indicates not significant; * indicates $P < 0.05$; ** indicates $P < 0.01$.

4. Discussion

The study was designed to test the efficacy of injecting CH to treat VMs, in an established VM xenograft mouse model [17]. We showed that local injection of CH effectively controlled its growth and progression. There was no significant difference between lesion size with CH alone and when CH was mixed with STS. This may be due to the limited time period during which the treatment effect was followed. Both CH treatments were more effective than 3% STS-foam (the reference treatment), and UEA1 expression was lower with 1% STS-CH by comparison with 3% STS-foam, when focusing on the endothelial lining of enlarged luminal channels. This suggests that a combination of vascular occlusion and endothelial sclerosis may be beneficial. There were no significant differences in total plug area amongst all treatment groups up to D25. The rapid growth of the untreated, saline-treated, and 3% STS-foam groups was observed between D20 and D30, but mainly between D25 and D30. For future studies, longer follow-up, beyond 20 days and perhaps evaluating the treatments up to two months may be useful.

In this study, we found no significant differences in total lesion size between saline and all other treatment groups. This may be explained by the wider variety of lesion severity in the saline group. In addition, the composition of each plug and the lesion created should be considered. The vascular channels in lesions from the saline-treated group are engorged with blood but free of other material. The majority of CH and 1% STS-CH treated lesions contained a hydrogel that occluded a major vascular channel. This limited further growth of peripheral vessels but the CH being still present may

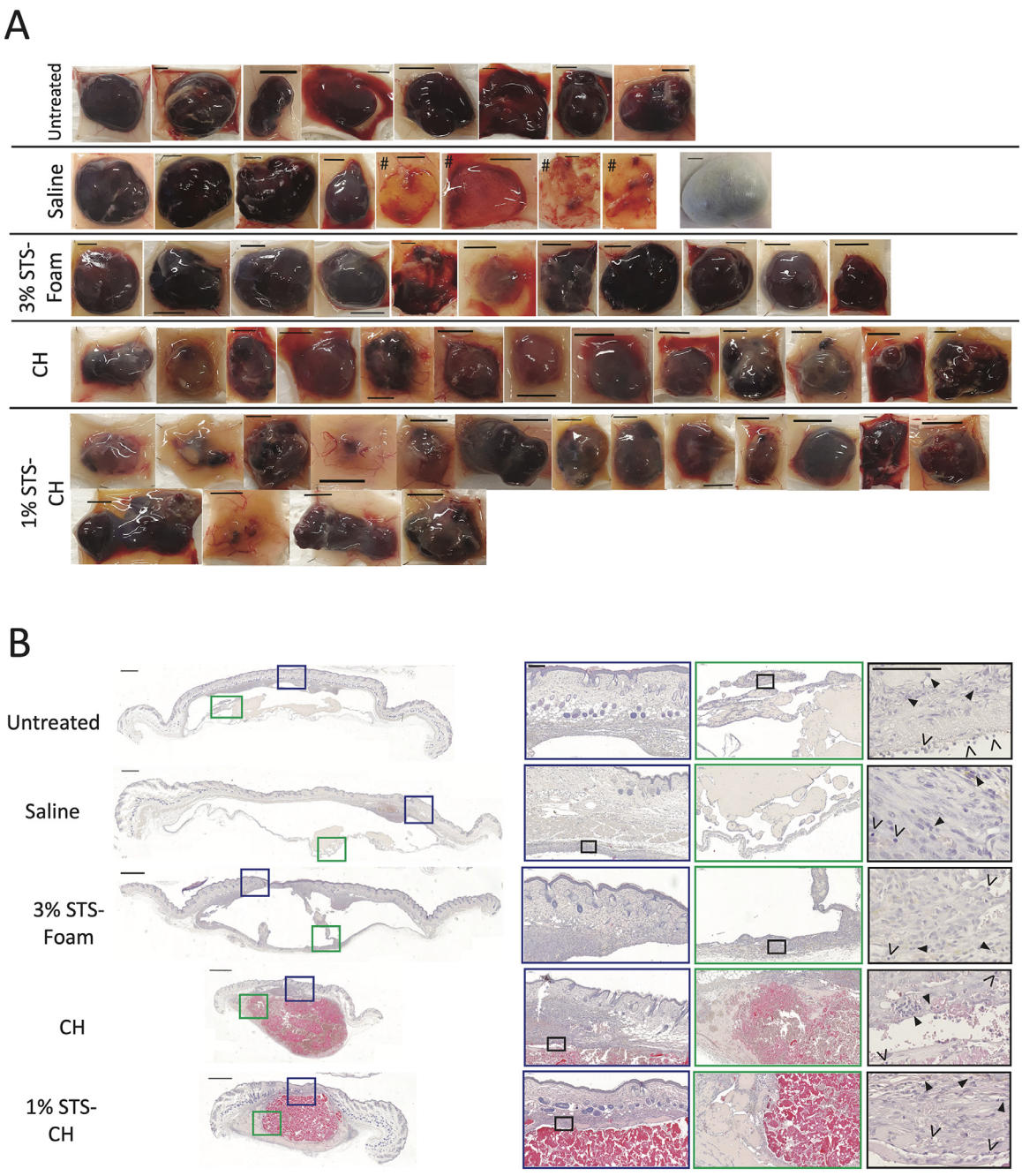

Fig. 3. Venous malformations (VMs) were collected at Day 30, 20 days post-treatment. A) Matrigel plugs containing VMs were excised, still attached to the skins. Excision of very large, blood-filled pseudo vascular areas (> 400 mm^2) was difficult, occasionally leading to draining of the VM during the process. An example of the phenotype of these deflated lesions is shown (#). (scale bar, 5 mm). B) Hematoxylin and eosin staining of the lesion reveals grossly enlarged vascular channels in the untreated, saline and 3% sodium tetradecyl sulfate foam (3% STS-foam) injected VMs. Chitosan hydrogel (CH) controlled lesions progression and (strong background eosin stain) remained in relevant lesions (CH and 1% sodium tetradecyl sulfate chitosan [1% STS-CH] gels. Blue box, representative close-up image of mouse skin. Green box, close-up of VM. Green box, close-up of VM. Black box, representative signs of inflammation (^, lymphoblasts; arrowhead, neutrophils). (Scale bar for overall images, 1 mm. Scale bar for boxes, 100 μm).

explain the lack of size difference with saline-injected lesions. We know from previous experiments with CH injection in endoleak models that CH degradation is observed after 20 weeks [16]. Thus, the deposition of CH was easily identified at D21 on histology allowing us to specifically analyze the vessel lumen area in areas injected with CH. We confirmed that the vessel lumen areas of hydrogel-injected treatment groups are significantly smaller than those of saline-treated plugs. In addition, the mean lesion size of both CH and 1% STS-CH treatment groups was significantly smaller than those of the 3% STS-foam treatment group. The lack of significant difference in mean vascular lumen area between the CH alone and 3% STS-foam groups may be explained by the limited observation time and the absence of STS. The absence of difference in inflammation scores between groups indicates that CH injection into MV does not appear to affect inflammatory responses around the MV in this immunocompromised mouse model.

In our study, larger volumes of 3% STS-foam were injected by comparison to the CH and 1% STS-CH gel formulations. This is likely due to the compressibility of the foam. The same endpoint was used for foam and CH formulation injections (filling of the VM lesion without evidence of skin discoloration). It can be concluded that CH gels with lower injection volumes may be more effective than 3% STS-foam.

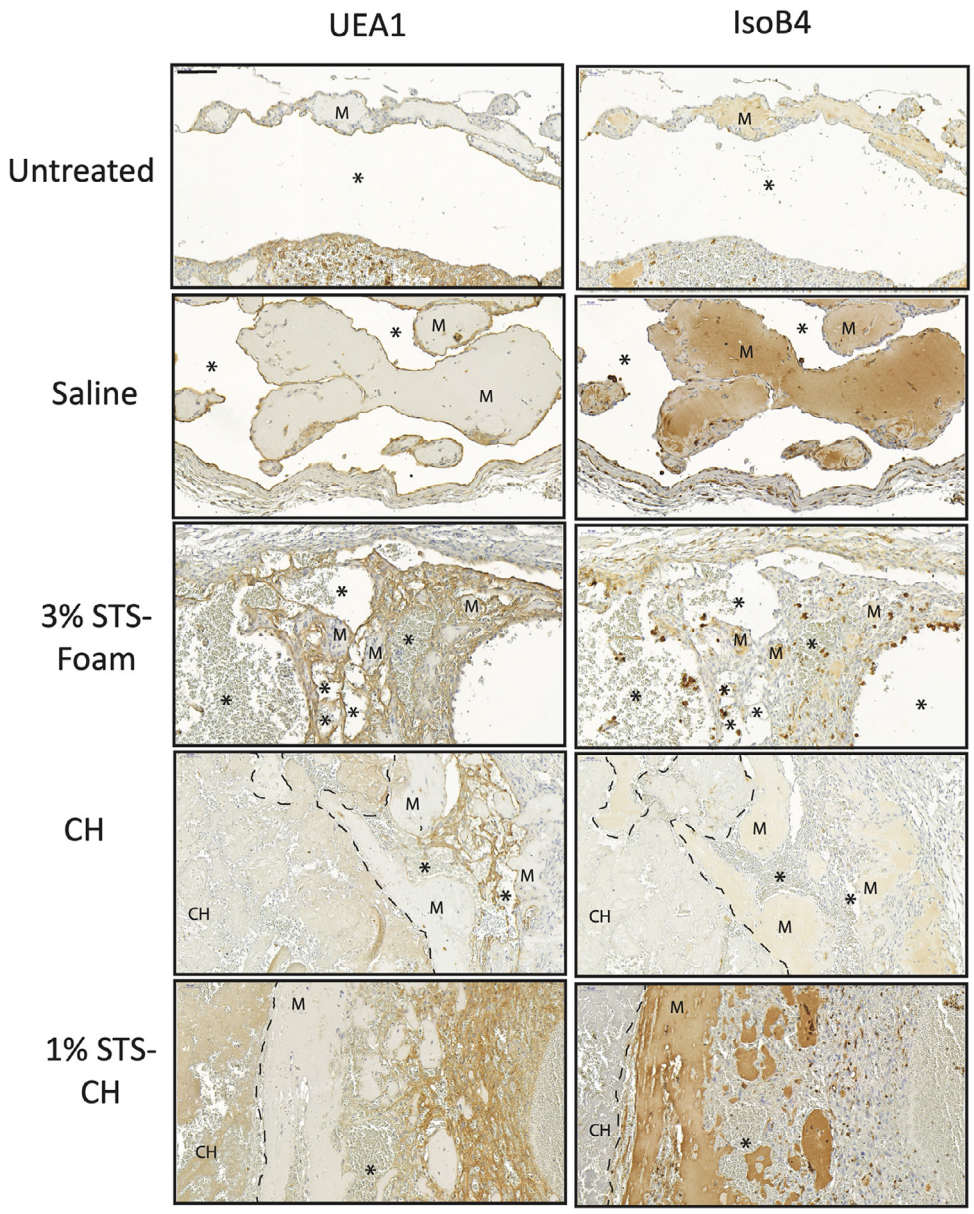

Fig. 4. Immunohistological staining of plugs with human and mouse endothelial markers. The endothelial cells lining of enlarged vascular channels were strongly positive for ulex europaeus agglutinin I (UEA1) but mostly negative for IsoB4, confirming the formation of lesions from the defective human umbilical vein endothelial cells (HUVEC) with the endothelial-specific receptor, tyrosine kinase with immunoglobulin-like loops and epidermal growth factor homology domains-2 (TIE2-L914F) cells. M indicates matrigel; * indicates vessel lumen; CH indicates chitosan.

Percutaneous sclerotherapy is the preferred primary option for treating VMs. Lower efficacy of STS compared to other agents like polidocanol has been reported by Qiu et al. but not by Horbach et al. [6,22]. All studies included in the meta-analysis by Qiu et al. used STS as a liquid mixed or not with contrast material whereas polidocanol was injected as a foam which can explain the lower efficacy of STS. Absolute ethanol is the most effective sclerosing agent, however, it can lead to more local and systemic adverse effects. To overcome these issues, gelified ethanol (by mixing with ethylcellulose) has recently been used clinically to localize treatment [23-25]. The semi-solid formulation resulted in better efficacy of VMs with reduced rate of side effects and pain in both children and adults. However, the studies had limited numbers of patients and lacked consistent follow-up, making it difficult to estimate the long-term safety of gelified ethanol [24,25]. STS-foam is the most commonly used application due to its higher safety profile, low cost and limited side effects [8]. However, it may have a lower efficacy and higher lesion recurrence rate compared to other sclerosing agents and can still seep into adjacent vessels, causing non-target vascular damage.

To overcome these limitations, creating a sclerosing embolic agent by mixing STS with CH to promote a simultaneous sclerosing effect and vascular occlusion may be a more attractive option. It has been shown in an endoleak model that the combination of flow occlusion with endothelial ablation can prevent vascular recanalization [26]. Chitosan has the benefit of being resorbable over time, which is not the case for ethylcellulose used in gelified ethanol formulations [27,28]. In addition, CH biodegradability rate can be tuned by changing its degree of deacetylation and molecular weight. Previous studies using STS-CH to embolize then ablate the endothelial lining to repair endoleaks in a canine aneurysm model support the efficacy of such an approach [16].

We found that it was sufficient and better to inject a small volume, instead of filling a vessel until the blood was displaced from the lesion and became colorless. When plugs were filled, there was a

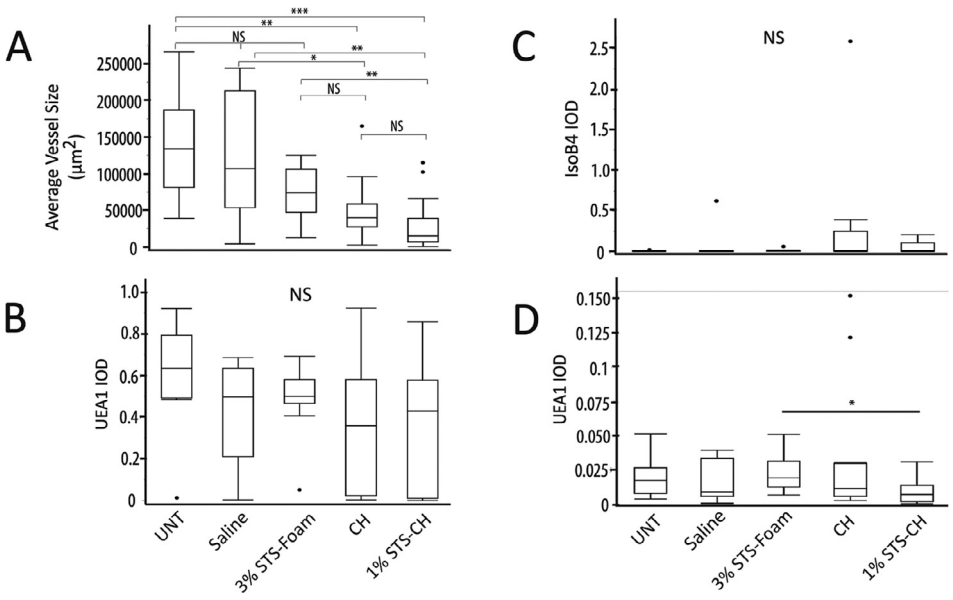

Fig. 5. Measurement of vessel size on histopathological analysis and quantification of endothelial markers. A) Box plots show the results of measurement of average vessel size in μm^2 shows lower vessel size in venous malformations injected with chitosan gels with or without sodium tetradecyl sulfate (STS) as compared to untreated (UNT) and saline injections. Lower vessel size was also observed when comparing venous malformation (VMs) injected with 1% sodium tetradecyl sulfate chitosan gel (1% STS-CH) and 3% sodium tetradecyl sulfate foam (3% STS-foam). B) Box plots show the results of quantification of ulex europaeus agglutinin 1 (UEA1) expression within the lesion. No significant differences were found between the five groups. C) Q Box plots show the results of quantification of IsoB4. No significant differences were detected between all groups. D) Box plots show the results of quantification of UEA1 expression of endothelial cells directly lining enlarged vessels and touching the chitosan showing lower UEA1 expression in VMs injected with 1% sodium tetradecyl sulfate chitosan gel (1% STS-CH) by comparison with VMs injected with 3% sodium tetradecyl sulfate foam (3% STS-foam). OD indicates optimal density. The box plots represent the minimal and maximal values, the 25% lower and 75% upper quartiles and the median. NS indicates not significant; * indicates $P < 0.05$; ** indicates $P < 0.01$; *** indicates $P < 0.001$.

higher risk of skin necrosis probably due to extravasation. The addition of a detergent appears to exacerbate the situation, as ulceration was observed only in the STS-containing treatment groups at D11. Overall, we found higher incidences of ulceration in plugs treated with 3% STS-foam and 1% STS-CH; those exposed to 3% STS-CH ended up being completely destroyed. This model creates superficial VMs close to the skin, which are more at risk for skin necrosis. Interestingly, in our study, only one untreated MV spontaneously developed an ulceration.

To our knowledge, this is the first instance that an injectable treatment has been tested in such a mouse model of VM. Because of our familiarity with the VM xenograft model, we were able to target a large vascular channel to inject the solutions. However, this is still a blinded approach, so the accuracy of the placement of the injection site could not be confirmed until histological analysis. Future studies could be improved by monitoring the injection in real time using high-resolution Doppler ultrasound or fluoroscopy. We used in this study CH gel formulations combined with radiopaque agent allowing fluoroscopy guided injections [16,29]. The gel was also well visible under ultrasound [30]. This could help better monitor gel injection under imaging guidance (fluoroscopy and or ultrasound). This could prevent gel extravasation into soft tissue and skin necrosis. Another limitation of our study is the limited time frame of observations. Lesions that develop with the VM xenograft mouse model can grow quite large. We have demonstrated the most rapid growth occurs after D25 after implementation of the HUVEC_TIE2-L914F cells. Since it is known that there is a significant difference in lesion size compared to untreated groups, future studies should focus on only the CH and STS-CH injections with longer follow-up. The time for chitosan degradation needs also to be determined. The VM-like lesions formed in the mouse model are confined to the area of the subcutaneously placed matrigel plug, without connection to the venous system as one would expect in patients. This may result in easier occlusion and lower risk of CH migration in the venous system. In addition, in the xenograft mouse model used, all ECs forming the VMs have the TIE2-L914F mutation, whereas VMs in humans (with the same mutation) are composed of a heterogeneous population of mutant and wild-type ECs [31]. Therefore, the VM-like lesions in the mice are more aggressive and progress more rapidly than those seen in patients. This feature may ultimately hamper the ability to perform long-term experiments should lesions become too large.

In conclusion, this study demonstrate the benefit of injecting CH hydrogel into experimental VMs, over the gold-standard STS foam sclerotherapy to prevent their growth. Our results suggest that the occlusion of the defective vessel may be sufficient to significantly control the growth of established VMs. However, longer-term studies are needed to determine whether the addition of a sclerosing agent to CH would improve the treatment efficacy. In a clinical setting, injection of CH, either with or without STS, may decrease the lesion recurrence rate and the need for repeated sclerotherapy sessions, as observed when using STS-foam.

Author contributions

All authors attest that they meet the current International Committee of Medical Journal Editors (ICMJE) criteria for Authorship.

Compliance with ethical standards

All animal procedures were approved by the Ethical Committee for Animal Experimentation at the Centre Hospitalier de l'Université de Montréal Research Center (CRCHUM) in accordance with the ARRIVE guidelines and EU Directive 2010/63/EU for animal experiments.

Declaration of competing interest

Gilles Soulez and Sophie Lerouge have patented the STS-CH hydrogel (A sclerosing and embolizing gel, US publication number: 8840867

B2, 23 Sept. 2014, Patent Appl. CA 2704,971, May 2010. Licensed to Cook Medical). They also act as consultants for Cook Medical.

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