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Note

Ex vivo study of bevacizumab transport through porcine nasal mucosa

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ABSTRACT

Introduction: Hereditary hemorrhagic telangiectasia (HHT) is a genetic disorder associated with abnormal angiogenesis and disabling epistaxis, for which bevacizumab is reported to be a new therapeutic option. In the present study, bevacizumab transport in porcine nasal mucosa was investigated to determine antibody bioavailability.

Material and methods: Transmucosal absorption of bevacizumab was examined by using nasal mucosa specimens mounted onto static vertical diffusion cells then treated with bevacizumab solution (25 mg mL^{-1}, 500 μg) for 2.5 h. Bevacizumab concentrations were measured by enzyme-linked immunosorbent assays. Mucosal integrity was examined by histological examination of treated mucosa.

Results: Transmucosal transport of bevacizumab followed a Fickian diffusion process (permeability coefficient: $[0.63 \pm 22] \times 10^{-6}$ cm s^{-1}; and steady-state flux: 56.4 ± 19.6 μg cm^{-2} h^{-1}). Total recovery of bevacizumab throughout the 2.5 h experiment was 83% of the initial dose distributed (i) at the mucosal surface (263 ± 73 μg; ~53%) and (ii) into (95 ± 14 μg; ~19%) and through (56 ± 26 μg; ~11%) the mucosa. There was no evidence of any noticeable histological effects, confirming the harmlessness of nasal bevacizumab delivery.

Conclusion: In the present study, absorption of bevacizumab into nasal mucosa was demonstrated, providing new fundamentals that are mandatory for further clinical trials in HHT patients.

1. Introduction

Hereditary Hemorrhagic Telangectasia (HHT) or Rendu–Osler disease, is a rare but ubiquitous disease, that affects at least 1 in 6000 people. HHT is clinically characterized by cutaneous and muco-cutaneous telangiectasias leading to epistaxis and gastrointestinal bleeding. Patients also have arteriovenous malformations (AVM) that affect many organs including the lungs, liver, and brain. Epistaxis is the most life-threatening manifestation of the disease and often causes chronic anemia. Episodes of epistaxis are spontaneous, repeated, frequent, sometimes abundant, justifying continuous martial treatment and sometimes multiple red blood cell transfusions. The local surgical treatments proposed are often aggressive (repeated packing, electrocoagulation, instillation of a sclerosing agent, embolization, or surgically closing the nasal fossae), but their efficacy is highly variable and has not been proven.

HHT is a genetic disease with autosomal dominant inheritance associated with abnormal angiogenesis. The two major genes that are known to be implicated in HHT encode endoglin (*ENG*) and activin-receptor-like kinase (*ALK1*). Both genes are involved in the

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transforming growth factor beta (TGF-β) signaling pathway. Recently, it was reported that angiogenesis is implicated in the pathogenesis of HHT. Patients with HHT have significantly increased plasma levels of both TGF-β and vascular endothelial growth factor (VEGF).

Inhibition of angiogenic factors could be a new therapeutic strategy as VEGF is involved in the growth of both normal and abnormal blood vessels throughout the body. Bevacizumab (Avastin®), currently used intravenously in combination with chemotherapy for the treatment of cancer, is a recombinant, humanized, IgG1 monoclonal antibody of 149 kDa. It binds to VEGF, a key factor for vasculogenesis and angiogenesis and thereby inhibits the binding of VEGF to its receptors, Flt 1 (VEGFR-1) and KDR (VEGFR-2), on the surface of endothelial cells. Neutralizing the biological activity of VEGF thus inhibits angiogenesis.

Systemic administration of bevacizumab in patients with severe HHT has already been used off-label in case reports and has led to improvement to liver damage-related symptoms and epistaxis [1]. One study, conducted retrospectively in 32 patients presenting recurrent HHT epistaxis, showed that submucosal injection (associated or not with laser cautery) or topical spray (25–100 mg) significantly improved the epistaxis severity score [2]. Consequently, local nasal delivery of bevacizumab may constitute a new and challenging strategy for treating severe epistaxis in HHT as it may minimize systemic side-effects. Furthermore, the intranasal route is widely used for either local or systemic delivery of generic or innovative drugs.

The main objective of the present *ex vivo* study was to assess the transport of bevacizumab, a high molecular weight drug (149 kDa), through porcine nasal mucosa, a model previously used to study intranasal drug delivery [3,4]. Local instillation of bevacizumab onto the nasal mucosa was carried out in order to characterize antibody bioavailability as a prerequisite for further clinical trials in HHT patients.

2. Materials and methods

2.1. Porcine nasal specimens and experimental setup

Nasal mucosa specimens were isolated by an ear, nose, and throat surgeon from porcine snouts obtained from a local slaughterhouse. The snouts were opened up to expose the septum and conchae; then, using forceps and scalpels, two anatomical subtypes of mucosa were isolated from the cavity and the septum. Mucosal specimens were cut into appropriate-sized pieces and were mounted in vertical static diffusion cells with an effective surface area of 0.77 cm^2. The donor compartment was in contact with the anterior surface of the mucosa while the receptor compartment faced the posterior mucosal surface. The receptor compartment was filled, avoiding bubbles, with 10 mL of Ringer lactate solution (Aguettant, Lyon, France), then maintained at 37 °C by means of a thermostated water bath. Receptor solution, magnetically stirred to insure medium homogeneity for the duration of the experiment, was gassed by means of oxygen bubbling [4]. In order to reach water and ionic physiological status within mucosa heated at 33 °C, the vertical static diffusion cells were left for one hour to equilibrate [3].

2.2. Intranasal bevacizumab delivery

Ex vivo penetration and permeation of bevacizumab into and through the nasal mucosa were investigated after a single topical exposure of 2.5 h. The surface of the nasal septum and cavity specimens was treated with 500 μg (649 μg cm^{-2}) of bevacizumab (20 μL of Avastin® 25 mg mL^{-1}, Roche, Basel, Switzerland: bevacizumab, trehalose dihydrate, sodium phosphate, polysorbate 20, and water for injections). This dose was similar to that tested previously in local injections in HHT patients receiving 100 mg [2] per 150 cm^2 of total nasal surface [5]. Aliquots (1 mL) were collected from the receptor compartment at regular time intervals (every 30 min) over 2.5 h. The volume withdrawn was replaced by the same volume of preheated Ringer lactate solution [4]. After 2.5 h, the mucosal surface was washed with 1 mL of Ringer lactate solution to recover unabsorbed bevacizumab. The mucosal tissues were weighed, then homogenized in tubes containing glass beads and filled with 500 μL of Ringer lactate solution by using Mini-BeadBeater® (Biospect Products), and finally centrifuged (1250*g*). The aliquots and supernatants were kept at −18 °C before content analysis. Earlier studies confirmed the stability of bevacizumab in serum stored at −20 °C for 25 weeks as well as at room temperature for 14 days [6].

2.3. Histological examination

This intranasal bevacizumab delivery experiment was completed by histological studies of the treated and untreated mucosa after the 2.5 h of the experiment, performed in order to determine whether or not the administration of bevacizumab causes mucosal damage. Negative controls (normal mucosa) and positive controls (100 μL of 37% nitric acid-treated mucosa) were also done immediately after isolation. Mucosal specimens were fixed in formalin, embedded in paraffin, and processed according to standard techniques in which 4-μm thick sections were stained with hematoxylin and eosin. The sections were analyzed by a pathologist blinded to the experimental conditions.

2.4. Bevacizumab content analysis

Bevacizumab content in the receptor fluids, washing solutions, and supernatants was analyzed by means of a validated enzyme-linked immunosorbent assay (ELISA) method [6]. Bevacizumab was captured by $VEGF_{165}$ and detected by peroxidase-conjugated goat anti-human IgG (specific for the γ chain Fc fragment). Calibrators and quality controls were prepared in phosphate-buffered saline containing 1% bovine serum albumin. The detection limit was 0.033 mg L^{-1}. Lower and upper quantitation limits were 5 and 75 mg L^{-1}, respectively. Samples with concentrations above the upper limit of quantitation were appropriately diluted, with the variation coefficient of the 1:10 dilution less than 15%.

2.5. Calculations and mathematical model

The experimental permeation profile of bevacizumab through porcine nasal mucosa was fitted to the non-steady-state solution to Fick's second law for a single-layer membrane:

$$Q_t = C_d \cdot (KL)\left[\frac{D}{L^2}\cdot t - \frac{1}{6} - \frac{2}{\pi^2}\sum_{n=1}^{\infty}\frac{(-1)^n}{n^2}\cdot \exp\left(-\frac{D}{L^2}\cdot n^2\cdot \pi^2 \cdot t\right)\right] \quad (1)$$

making it possible to determine the permeability parameters of bevacizumab. Q_t is the cumulative quantity of bevacizumab absorbed through the mucosa as a function of time; C_d is the concentration in the donor compartment; K is the partition coefficient of bevacizumab between the mucosa surface and the bevacizumab vehicle; L is the diffusion path length and D is the diffusion constant through the mucosa for bevacizumab [7].

The permeability coefficient (P), the steady-state flux (J_{ss}), and the time needed to reach the steady-state conditions (T_{lag}) were calculated as follows:

$$P = KL \times \frac{D}{L^2} \quad (2)$$

Table 1
Permeability characteristics of bevacizumab after a single topical application (donor concentration: 25 mg mL^{-1}; initial applied amount: 500 μg) onto both porcine nasal septum and cavity mucosa. All data are the mean ± standard deviation of 3–4 experimental determinations.

Nasal mucosa specimens	Amount of bevacizumab (μg)			log $K^{\dagger}$	Mucosal bevacizumab concentration (μg g^{-1})
	Washing solution	Mucosa	Receptor solution		
Septum	75 ± 82	211 ± 43	29 ± 37	−1.00 ± 0.10	2325 ± 554
Cavity	263 ± 73	95 ± 14	56 ± 26	−0.80 ± 0.05	3927 ± 452

† Logarithm of apparent partition coefficient determined as the ratio of mucosal bevacizumab concentration to donor concentration.

$$J_{ss} = P \times C_d \quad (3)$$

$$T_{lag} = \frac{1}{6} \times \left(\frac{D}{L^2}\right)^{-1} \quad (4)$$

3. Results and discussion

3.1. Intranasal bevacizumab delivery

The transport of bevacizumab through porcine nasal mucosa was investigated by using five mucosal specimens (septum and cavity). However, in a few samples, minimal recovery of bevacizumab from mucosa and receptor compartments excluded further permeability data processing. Table 1 shows the permeability characteristics of bevacizumab through both the nasal septum ($n = 4$) and cavity mucosa ($n = 3$). Poor recovery of certain experimental determinations (one of a septum mucosa and two of cavity mucosa) may be explained by artefactual adsorption of antibodies onto glass material. Different patterns of bevacizumab tissue distribution might be evidenced from septum and cavity mucosa, suggesting a relationship between tissue thickness (i.e., diffusion pathlength) and apparent permeability. Bevacizumab quantities in the septum mucosa were higher than in cavity mucosa, whereas an inverse tendency was found for mucosal bevacizumab concentrations. After 2.5 h of topical exposure to bevacizumab, the amount recovered from the septum and cavity mucosa accounted for 42% and 19% of the initial dose applied, respectively, whereas the total amount permeated through the mucosa ranged between 6% (septum) and 11% (cavity) (receptor solution). The anatomical specificities of both the septum and cavity mucosa were of paramount importance in the tissue pharmacokinetics of bevacizumab. Differences between the unabsorbed quantities found in the septum and cavity (washing solution), i.e., 15% and 53% of the initial dose, were observed respectively. However, a similar partition coefficient for bevacizumab between the septum/cavity mucosa and vehicle was also found ($\log K \sim -1$), underlying comparable biophysical properties in both media. The total recovery of bevacizumab in the septum and cavity mucosa experiments following 2.5 h of topical exposure was 63% and 83%, respectively. The low recovery of bevacizumab in the septum mucosa experiment and subsequent high variability in the permeation data may be explained by the hand-made removal of cartilage, which resulted in inconsistent mucosa thickness and the persistence of connective tissue affecting nasal permeability. Moreover, the greater thickness of the septum mucosa may have decreased the efficiency of antibody extraction from the tissue by mechanical homogenization. Chemical extraction using alkali, acids, or organic solvents was not privileged due to possible antibody aggregation. Cavity mucosa also showed more reproducible and less variable results in the study by Wadell et al. [3].

Therefore, as a result of the low recovery of bevacizumab in the septum mucosa experiment, and due to probably higher *in vivo* bevacizumab absorption in the cavity mucosa, only the permeation profile of bevacizumab through cavity mucosa was established as a function of time of exposure (Fig. 1). Plotting antibody permeability through the nasal mucosa first showed an exponential increase followed by a linear trend corresponding to unsteady and steady-state conditions, respectively. The permeability parameters of bevacizumab through the cavity mucosa calculated with Eq. (1) are shown in Table 2. The permeability coefficient (P) and the steady-state flux (J_{ss}) of bevacizumab through the mucosa were $[0.63 \pm 22] \times 10^{-6}$ cm s^{-1} and 56.4 ± 19.6 μg cm^{-2} h^{-1}, respectively. The lag-time (T_{lag}) was 1.30 ± 0.88 h. The diffusion path length for

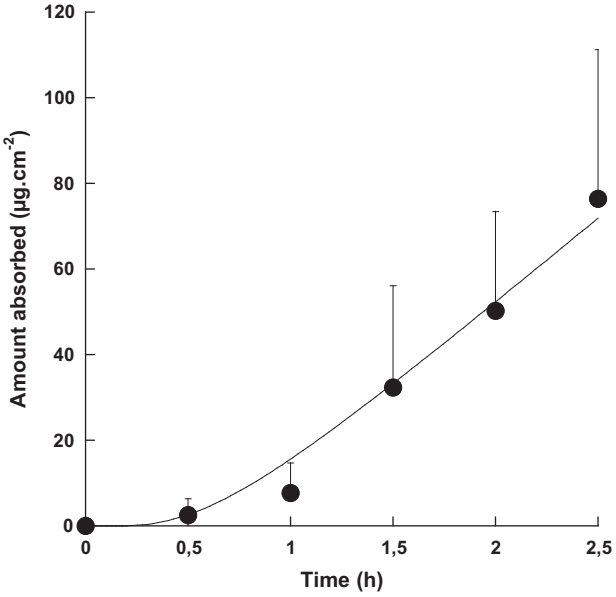

Fig. 1. Profile of transmucosal absorption of bevacizumab through porcine nasal cavity mucosa mounted onto a static vertical diffusion cell as a function of time. *Ex vivo* data were fitted to a mathematical solution of Fick's second law for a single membrane ($R = 0.991$; $p < 0.01$; see Section 2.5 in the text). All data are the mean ± standard deviation of three experimental determinations.

Table 2
Permeation characteristics of bevacizumab after a single topical application (donor concentration C_d: 25 mg mL^{-1}; initial applied amount: 500 μg) through porcine nasal cavity mucosa. Permeability parameters (K, D/L^2, T_{lag}, P) and steady-state flux (J_{ss}) were determined from a mathematical solution of Fick's second law for a single membrane $\left(Q_t = C_d \cdot (KL)\left[\frac{D}{L^2} \cdot t - \frac{1}{6} - \frac{2}{\pi^2}\sum_{n=1}^{\infty}\frac{(-1)^n}{n^2} \cdot \exp\left(-\frac{D}{L^2} \cdot n^2 \cdot \pi^2 \cdot t\right)\right]\right)$ fitting experimental permeation data (Fig. 1) in which Q_t is the cumulative quantity of bevacizumab absorbed through the mucosa as a function of time, K is the apparent partition coefficient, D is the diffusion constant, L is the diffusion path length, P is the permeability coefficient, and T_{lag} is the lag-time. All data are the mean ± standard deviation of three experimental determinations. P, J_{ss}, and T_{lag} were calculated as follows: $P = KL \times \frac{D}{L^2}$ $J_{ss} = P \times C_d$ $T_{lag} = \frac{1}{6} \times \left(\frac{D}{L^2}\right)^{-1}$.

Permeability of bevacizumab through porcine nasal cavity mucosa				
KL (cm)	$10^5 \cdot D/L^2$ (s^{-1})	$10^6 \cdot P$ (cm s^{-1})	J_{ss} (μg cm^{-2} h^{-1})	T_{lag} (h)
0.019 ± 0.013	5.5 ± 4.7	0.63 ± 0.22	56.4 ± 19.6	1.30 ± 0.88

bevacizumab (L) through the mucosa determined from $\log K$ (-0.80) and KL (0.019 cm) was 1.2 mm, corresponding approximately to the thickness of the cavity mucosa.

3.2. Hypothesis of different mechanisms of intranasal transport of bevacizumab

To our knowledge, the nasal mucosal bioavailability of bevacizumab has not been reported in either animals or humans. As reported previously, the physical and chemical parameters of drugs and vehicles (e.g., partition coefficient, molecular weight, drug concentration, and pH), nasal mucociliary clearance, and nasal enhancers (e.g., microspheres, liposomes, and gels) must be taken into account in nasal drug absorption [8].

Interestingly, in spite of its molecular weight (149 kDa), marked hydrophilia ($\log K \sim -1$), and size (300 Å), all *a priori* against significant transport through biological membranes, a large amount of antibody was found in and through the porcine cavity mucosa. Although rabbit nasal mucosa has been widely used in the past, porcine and human nasal mucosa have been reported to be comparable [3]. Moreover, the large available surface of porcine mucosa is an advantage for intranasal drug delivery experiments. As reviewed by Costantino et al. [5], polar drugs were reported to be transported via the paracellular route. On the contrary, limited or negligible transport of molecules larger than 15 Å or bigger than 1000 Da was explained by the efficiency of tight junctions between nasal epithelial cells acting as an impermeable diffusion barrier.

Different mechanisms of intranasal transport of bevacizumab may thus be suggested. First, the negatively charged nasal mucosa surface (pH 5.5–6.5) would reinforce the adsorption of positively charged antibodies (isoelectric point ~ 8) and would consequently improve antibody partition onto the nasal surface, which is a key element in the next step of permeation. Secondly, IgG may bind to FcRn, a receptor expressed on many epithelial surfaces including those in the intestine and lung and localized within acidic endosomal vesicles in cytoplasm [9]. Transcellular transfer of IgG from apical to basolateral and finally a release of IgG into intercellular spaces (transcytosis) [9] might also explain bevacizumab's permeability through the nasal epithelium, although no FcRn receptor has yet been detected on the nasal mucosa. In addition, the viability of nasal mucosa specimens, not ascertained in the present experimental setup, is mandatory if this means of transport is to be validated. Finally, the direct impact of IgG on the opening of tight junctions in the nasal epithelium would promote further bevacizumab transport into underlying tissue. A recent patent for new pharmaceutical composition methods, including monoclonal antibodies, antibody fragments, and chain antibody delivery, claims to open the tight junctions in the nose. These permeabilizing agents may bind to either junctional adhesion molecule-1 (JAM-1), occludin, or claudin-4 and may enhance mucosal epithelial paracellular transport by changing the structure of the tight junctions [10]. Although the tight junctions may be modulated by anti-VEGF therapies in numerous tissues (e.g., retinal pigment epithelium; ovarian tissues), such modulation was not clearly evidenced in the present study.

The pharmacokinetic data for the nasal tissue reported here should be weighted by the absence of mucociliary clearance, which is likely to decrease *in vivo* bevacizumab nasal absorption. Physiological factors such as posture during drug administration may affect mucociliary clearance [8]. Pharmacokinetics may also be

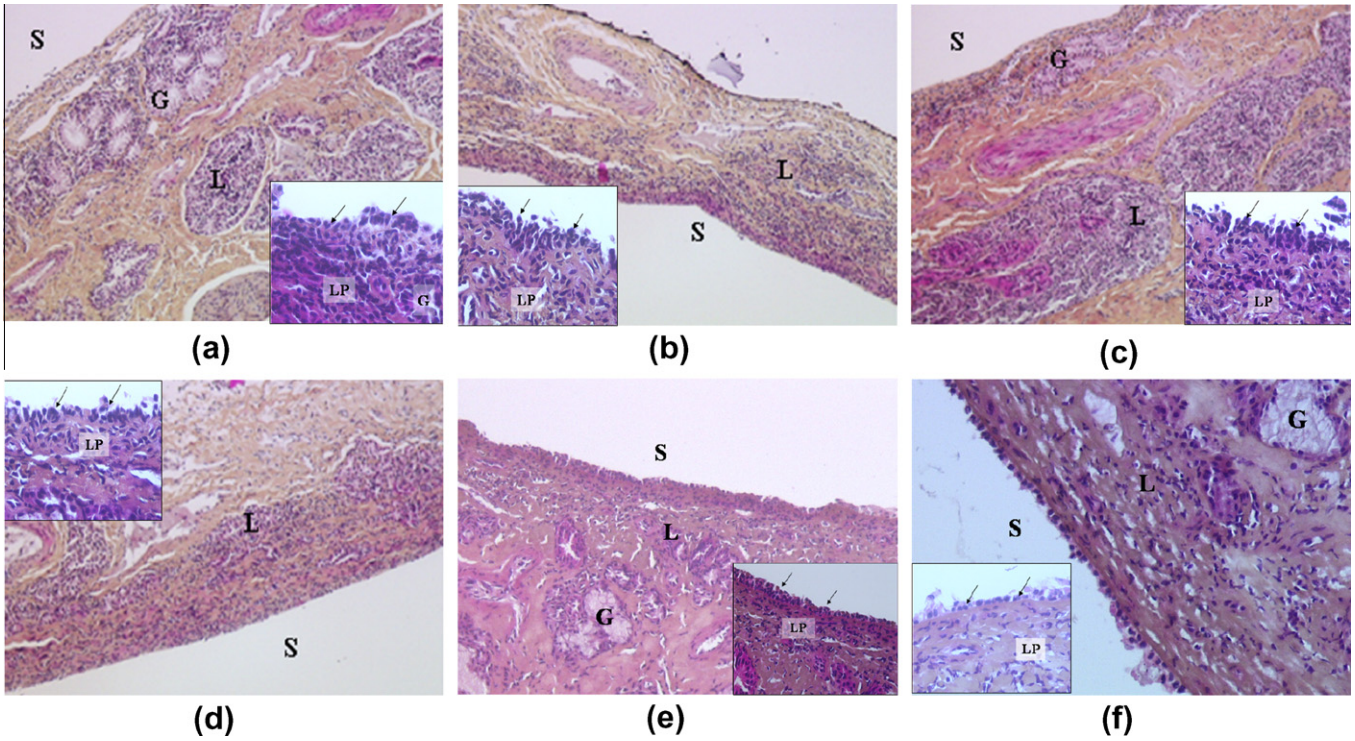

Fig. 2. Histological sections of different porcine nasal mucosa (Hematoxylin eosin saffron staining). Untreated septum and cavity mucosa after 2.5 h (original magnifications: ×120 and ×80, respectively) (a, b); bevacizumab treated septum and cavity mucosa after 2.5-h (original magnifications: ×120 and ×80, respectively) (c, d). Inserts: higher magnifications of the superficial layers of epithelial cells (indicated by arrows) (original magnification: ×520). After incubation with bevacizumab, the overall structure of the mucosa was well preserved, without evidence of necrosis, ulceration, or hemorrhage. Negative control: normal cavity mucosa immediately after isolation (original magnification: ×110) (e). The mucosa was lined by a thick, well-preserved epithelial lining. Glands were numerous in the lamina propria. Insert: higher magnification of the epithelial layers (indicated by arrows) (original magnification: ×520). Positive control: 100 μL of 37% nitric acid treated cavity mucosa immediately after isolation (original magnification: ×180) (f). Epithelial cells were altered: the epithelial lining was reduced to one layer. Insert: in higher magnification of the superficial layers, epithelial cells (indicated by arrows) display a rounded appearance; they were often dissociated and vacuolated; some shedding was focally visible (original magnification: ×520). Surface of the mucosa (S); serous glands (G); lymphoid aggregates (L); lamina propria (LP).

different in HHT patients compared to healthy nasal mucosa. Telangectasias in HHT patients are effectively responsible for severe nose bleeds, and the vessels are dilated.

Nasal absorption enhancers such as chitin and chitosan gels with bioadhesive properties are reported to control the release of drugs [8]. A modified preparation of Avastin® solution with such a compound may make it possible to increase the residence time of bevacizumab in the nasal cavity by slowing down the mucociliary clearance, thus increasing its absorption. Further research is needed to determine the usefulness of such a preparation.

3.3. Integrity of the nasal mucosa: histological examination

Histological sections of treated and untreated septum and cavity mucosa were found to be similar after 2.5 h of exposure (Fig. 2a–d). Cavity mucosa specimens were thin; they contained the surface epithelium, the underlying connective tissue, and a layer of serous glands, admixed with lymphoid aggregates of variable size. The overall structure of the mucosa was preserved. However, some detachment of parts of the surface epithelium could be observed. In addition, epithelial layers were sometimes disorganized but epithelial cells retained their cylindrical appearance and their overall polarity. There was no evidence of necrosis, ulceration, or hemorrhage in any treated and untreated cavity mucosa. Septum mucosa specimens were comparatively thicker due to an abundance of connective tissue presenting numerous mucosal and submucosal glands as well as lymphoid aggregates. Again, no necrosis, ulceration, or hemorrhage in any treated and untreated septum mucosa was evidenced.

In normal mucosa (negative control) (Fig. 2e), the structure of the mucosa was well preserved; the surface epithelium displayed normal characteristics.

After treatment with nitric acid (positive control) (Fig. 2f), marked alterations in the surface epithelium were visible: the epithelial lining was detached from large surfaces; in areas with residual epithelium, only one layer of cells was usually present: they showed a rounded appearance, with loss of their polarity; they were loosely cohesive, sometimes detached, and often vacuolated.

During this experiment, although the viability of mucosa specimens was ascertained, mucosal barrier function was unaffected by bevacizumab treatment as evidenced by the results of the histological study. A topical nasal spray of bevacizumab was recently used as a safe treatment regimen in a retrospective chart review, although no nasal bioavailability data was detailed [2].

This characterization of bevacizumab transport makes it possible to consider future local applications of this antibody for the treatment of epistaxis in HHT. The nasal route may minimize the risk of systemic adverse events. The next steps will be to evaluate the safety and efficacy of administration of bevacizumab by nasal spray in HHT patients.

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