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Effect of extract of Hirudo Medicinalis L. against adherence of calcium oxalate crystals to acid-injured bladder mucosa

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Summary. The effect of extract of Hirudo Medicinalis L. on preventing the adhesion of calcium oxalate crystals to 0.1 M hydrochloric acid-injured bladder urothelium of the rat was studied. It was found that in this species the extract coated to the bladder mucosa after it was instilled into the chemically injured bladder; and the adhesion of calcium oxalate crystals was prevented. In regard to the anti-adhesion property the Hirudo extract appears more effective than heparin, a documented glycosaminoglycan.

Key words: Extract of Hirudo Medicinalis L. – Heparin – Bladder Mucosa – Adhesion of calcium oxalate crystals

Introduction

Transitional cells on the surface of the urinary bladder secrete one or more glycosaminoglycans [1], which bind to their surface and act as a defence mechanism, i.e. to prevent adherence of urinary crystals, bacteria and even carcinogens, to the urothelium [1, 4, 5, 7]. The layer of glycosaminoglycans lining the bladder can be removed by acid, and detergents. Once injured, the bladder epithelium will increase the adherence of urinary crystals, bacteria and carcinogens [5]. But when an exogenous glycosaminoglycan, such as heparin, is added to the bladder exhibiting mucin deficiency, the adherence of those particles declined to the control level [2].

It has been recognised that Hirudo Medicinalis L (Hirudo) exerts effects similar to those of heparin. Therefore, we tested the ability of the extract of Hirudo (E-Hirudo), which is a traditional Chinese medication, with regard to its intravesical anti-adhesion property, and compared its effects with those of heparin. We found that E-Hirudo protects the mucin-deficient bladder from attracting calcium oxalate crystals.

Materials and methods

Preparation of E-Hirudo

2,000 g of dry crude Hirudo were washed and immersed in distilled water for 2 days in the refrigerator (2–5°C). After swelling, it was minced with scissors. Then it was immersed for 48 h in distilled water containing 0.5% phenol as bacteriostat. After filtration through gauze, the residue was again immersed in distilled water for 24 h; thereafter the two solutions were pooled. The pH was adjusted to 3.9–4.0 with 0.1 M HCl, and kept at 4°C for 2 days. Filtration through filter paper yields a grey-brown crude E-Hirudo.

This material was dissolved in 4,000 ml of saline to which 5% activated charcoal was added after stirring it stood overnight. Then it was filtrated to remove charcoal, and the pH was brought to 3.9–4.0 with 0.1 M HCl once more. A more refined E-Hirudo was obtained by centrifugation at 4°C, 12,000 × g.

At this stage E-Hirudo was dissolved in 2,000 ml of distilled water, and the pH brought to 5.5–6.0 with 0.1 M NaOH. After complete dissolution a transparent colloid-like liquid was obtained. This stock solution was designed to contain the anti-adherent capacity of 1 g crude Hirudo in 1 ml water, and allowed appropriate dilution.

Preparation of calcium oxalate crystals

Equal volumes of 10 mM calcium chloride and 10 mM sodium oxalate solutions, both chemicals being of analytical grade, were mixed and stirred at room temperature. After 30 min crystals of calcium oxalate monohydrate had been formed (identified by polarization microscopy). Thereafter, the suspension could be used for bladder instillation.

Surgery

Male rats (strain KUNMING; $n = 80$), weighing 200–250 g, were anesthetized with ether. After the bladder was exposed via a median lower abdominal incision, the proximal urethra was clamped with a mosquito forceps and the bladder was grasped at its dome and punctured with a 5 gauge needle.

Table 1. Calcium content of rat bladder (μg per mg dry weight), after various kinds of treatment of the urothelium (for details see sections Materials and Methods, and Results). Data are median/range of individual values. Number of tissue samples studied per group is given in parentheses

Group 1	Group 2	Group 4	Group 6	Group 8
Controls	CaOx	HCl + CaOx	HCl + heparin + CaOx	HCl + E-Hirudo + CaOx
0/0–0 (5)	0/0–0.78 (5)	2.78/1.20–7.86 (5)	3.46/0.87–8.88 (5)	0/0–0 (5)

CaOx = Calcium oxalate crystal suspension

Injury to urothelium

Chemical injuries of the bladder urothelium were produced by intravesical instillation of 0.1 M HCl for 2 min; then the acid was drained and the bladder was washed four times with saline [2].

Experimental design

Rats were subdivided into 8 groups, with 10 rats per group. From each group five rats were used for examination by scanning electron microscopy; in the remaining five rats in each of groups 1, 2, 4, 6, 8, tissue calcium was measured (see below). In group 1 the bladder mucosa was left untreated and was considered as the reference control. In group 2, 0.25 ml of calcium oxalate crystal suspension was instilled intravesically and left in situ for 30 min, then the content was drained. In groups 3–8 the bladder was injured by 0.1 M HCl (see above). Group 3 was left untreated (control of injury). In group 4, 0.25 ml of calcium oxalate suspension was instilled for 30 min. In group5, 0.25 ml of a heparin solution (10 mg/ml) was instilled for 2 min; thereafter the bladder was drained. In group 6, following injury by acid and subsequent instillation of heparin, the suspension of calcium oxalate crystals was instilled for 30 min. In group 7 and 8, we proceeded as in groups 5 and 6, except that instead of heparin the E-Hirudo was instilled.

Preparation of bladder mucosa for scanning electron microscopy

After termination of the instillation experiment the bladder was resected, incised and its inner surface rinsed with saline; thereafter it was fixed with glutaraldehyde.

Tissue calcium content

This variable was considered to reflect to some degree the amount of calcium sticking to tissue in form of calcium oxalate crystals. Tissue was dried in an oven at 50–60°C until constant weight was reached. The dry weight was recorded, thereafter the material was grounded to powder and dissolved in concentrated nitric acid. After appropriate dilution with distilled water calcium was measured by atomic absorption spectrophotometry: concentrations below 0.5 μg per mg dry weight are given as zero (=0; Table 1).

Results

Ultramorphology of bladder urothelium (Fig. 1a–e)

For group 1 the normal bladder urothelium is shown; it resembles closely the situation in human bladder, with irregularity in cell shape and with folds and 1–3 pits on the surface (Fig. 1a). In group 2, after the suspension of calcium oxalate was instilled without prior damaging the urothelium, only a few crystals adhered to its surface (not shown). In group 3, after 0.1 M HCl treatment, the normal morphology of the urothelium had completely disappeared, with shrinking of superficial cells and with wide gaps between cells (Fig. 1b). In group 4, with the suspension of the calcium oxalate instilled into the acid-injured bladder, there were many crystals adherent to its surface, especially in the shrunken areas (Fig. 1c). In group 5, after instillation of heparin, there was only a milky film visible covering the injured bladder mucosa (not shown). In group 6, despite the heparin-pretreatment there were a few crystals adherent to the injured mucosa (Fig. 1d). In groups 7–8, aimed at showing the effects of E-Hirudo upon restoration of the normal antiadhesion properties of urothelium to crystals, there was a film similar to the one present after heparin instillation (not shown), which covered the urothelium, but calcium oxalate crystals were not detected (Fig. 1e); however, the ultrastructure of the epithelium was still clearly abnormal, especially with regard to the wide gaps between cells compared with those shown in Fig. 1a (normal urothelium). Similar results were observed irrespective of whether a two- to threefold dilution of E-Hirudo was used.

Calcium content of bladder tissue (Table 1)

Calcium in the normal bladder is usually below 0.5 μg/mg dry tissue (group 1). After intravesical instillation of calcium oxalate suspension only, i.e. without prior

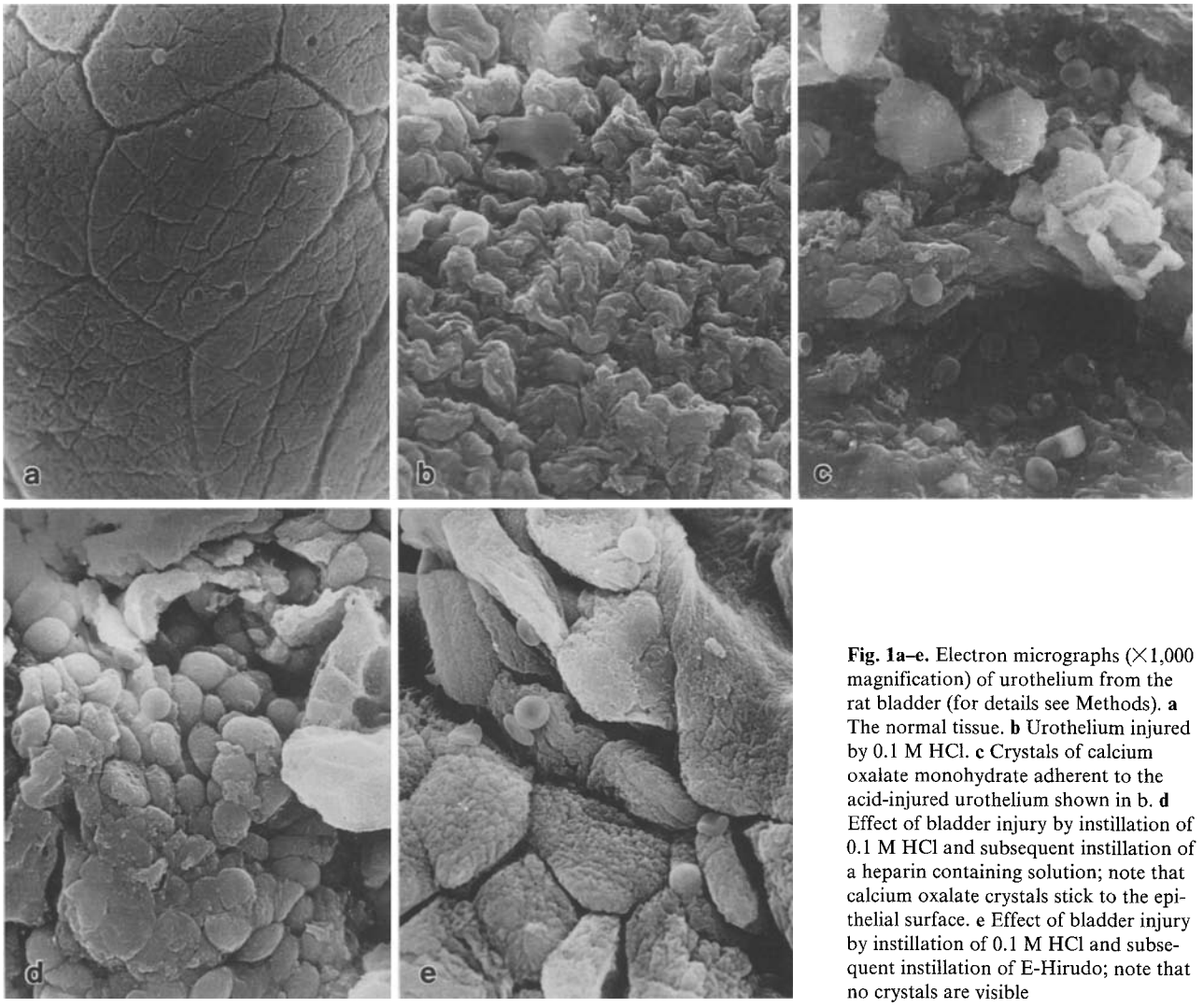

Fig. 1a–e. Electron micrographs (×1,000 magnification) of urothelium from the rat bladder (for details see Methods). **a** The normal tissue. **b** Urothelium injured by 0.1 M HCl. **c** Crystals of calcium oxalate monohydrate adherent to the acid-injured urothelium shown in b. **d** Effect of bladder injury by instillation of 0.1 M HCl and subsequent instillation of a heparin containing solution; note that calcium oxalate crystals stick to the epithelial surface. **e** Effect of bladder injury by instillation of 0.1 M HCl and subsequent instillation of E-Hirudo; note that no crystals are visible

instillation 0.1 M HCl, calcium was increased in one out of five rats (group 2). In contrast, after subsequent instillation of acid and calcium oxalate crystals, calcium was increased in all rats (group 4). Calcium was also increased in all animals in group 6, which were pretreated with acid, heparin and calcium oxalate crystals. However, when E-Hirudo was instilled instead of heparin, tissue calcium stayed at the low level of controls (group 1). Although these differences in tissue calcium appear impressive, no statistical examination was carried out owing to the small number of animals studied.

Discussion

The normal urothelium exhibits a unique protective property against the nucleation and adhesion of calcium oxalate crystals. Gill [1] demonstrated an elevation of the upper limit of metastability, i.e. retardation of nucleation and absence of adherence of calcium oxalate crystals in an urothelium-lined system (female rat bladder); this observation contrasted markedly with the lowered limit of metastability and with significant crystal adhesion to the surface of glass containers. Parson [4] has shown that the surface of normal bladder was lined with glycosaminoglycans. He [7] also suggested that the endogenous bladder glycosaminoglycans may bind a molecule of water to the bladder surface thereby creating a barrier between the transitional cells and the luminal environment. Particles and substances in urine, including crystals, bacteria, proteins, carcinogens, may be prevented from interaction with urothelium [5]. An intact surface of bladder mucosa containing glycosaminoglycans not only prevents adhesion of calcium oxalat crystals, but

also of struvite and urate crystals [3, 8]. Our study also demonstrated that, when the suspension of calcium oxalate was instilled into the normal bladder, there were only a few or even no crystals adhering to its surface.

In contrast, the anti-crystal-adhesion properties of normal urothelium were destroyed by a variety of injurious agents, such as 0.1 M HCl, 5% Triton X 100, a non-ionic detergent, and the same holds for papain, a proteolytic enzyme, all when instilled intravesically for 2 min; similarly, deleterious effects were seen upon systemic administration of cyclophosphamide [2]. In our study the normal bladder mucosa was injured by 0.1 M HCl. After the suspension of calcium oxalate crystals was instilled into the acid-injured bladder many crystals adhered to its inner surface. In line with other authors [2, 6] we demonstrated that heparin can coat the injured urothelium and act as a barrier between the cells of the transitional epithelium and urine, and this means that it almost restores the anti-crystal-adhesion property of previously injured urothelium. While the nature of the superficial film layer produced by both heparin and E-Hirudo is unknown the layer may account for rejection of particles otherwise sticking to the damaged urothelium. Heparin is a highly charged glycosaminoglycan, and its potency as an in vitro inhibitor of processes involved in stone formation, adhesion of crystals to surfaces included, may reside in this characteristic. However, heparin is not present in human urine, and therefore cannot account for in vivo inhibitory activity found in urine of normals and stone patients [9].

Hirudo Medicinalis L. is an annelid. According to the theory of traditional Chinese medicine extracts like E-Hirudo can promote the absorption of exsudates and improve the microcirculation. Its active component is a polypeptide which inhibits the conversion of prothrombin to thrombin. Our present data demonstrate that in the presence of E-Hirudo the anti-adherent potential of bladder urothelium can be restored, despite the fact that the latter was acid-injured; moreover, this beneficial effect of E-Hirudo was probably greater than the one shown by heparin (Fig. 1; comparison d and e). The mechanism underlying restoration of the anti-crystal-adhesion effect of E-Hirudo has not yet been elucidated. As outlined above for heparin it appears that there is a binding between some charged compound in E-Hirudo and ionised structural elements of injured urothelium, in the form of a hydrogen bond. As a result of the altered electrochemical conditions on the epithelial surface calcium oxalate crystals cannot stick to the tissue. In this regard E-Hirudo, heparin, and possibly other glycosaminoglycans with heparin-like or inhibitor activity toward crystal- and stone-forming processes, may share common pathways of action.

In regard to tissue the calcium content was normalized by E-Hirudo, contrasting with heparin pre-treatment (Table 1). We cannot explain why bladder tissue calcium is not normalized by heparin despite the fact that in its presence only a few calcium containing crystals were present on the inner surface. Thus, additional factors dependent on heparin may be operative. Probably these different findings in tissue calcium constitute an interesting advantage of E-Hirudo over heparin that deserves more specific investigations. The source of E-Hirudo (Hirudo Medicinalis L.) is abundant, the costs of its preparation are low and, as shown in this work, it is convenient for efficient application in the rat. Further studies are needed to clarify its possible usefulness in the clinical setting.

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