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The Effect of *O*-(β-Hydroxyethyl)-Rutoside (HR) on Macromolecular Leakage, Thrombosis and Haemostasis in Experimental Animals

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ABSTRACT

O-(β-hydroxyethyl)-rutoside (HR) (Venoruton®, Zyma AS, Nyon, Switzerland) has been investigated experimentally to evaluate the effect on microvascular permeability and thromboembolism. Permeability to macromolecules is diminished in a hamster cheek-pouch model. Haemostatic plug formation is impaired whereas laser-induced intravascular platelet aggregation is uninfluenced. There is a small but insignificant protection against sodium morrhuate (Eli Lilly and Co., Indianapolis, Indiana) induced femoral vein thrombosis.

INTRODUCTION

O-(β-hydroxyethyl)-rutoside (HR), Venoruton® is currently used for treatment of venous disorders like varicose veins and it has been suggested to reduce capillary permeability (25). It has been shown to affect platelet activity in different in vitro tests (15). As platelets are fundamental in thrombosis and haemostasis we found it of importance to investigate the effect of HR on some various in vivo models where normal platelet activity is necessary. HR has also been shown to decrease the transvascular transport of macromolecules in burned areas (7). This effect was suggested to depend on inhibitory effect on prostaglandin synthesis. We have shown that prostaglandins increase the microvascular permeability (22). Consequently, we studied the effect of HR on permeability of macromolecules in microvessels.

MATERIALS AND METHODS

Microvascular permeability

18 male golden hamsters weighing 65–110 g were prepared as described by Svensjö et al. (23). Briefly, hamsters were anaesthetised with 6 mg/100 g b.w. pentobarbital i.p. supplemented with pentobarbital i.v. The trachea and femoral vein were cannulated. The cheek pouches were everted, pinned out on a microscope stage and opened by a small incision for viewing as a single layer preparation. This was suffused with a Tris-buffered electrolyte solution with a pH of 7.35, and a PO_2 around 25 mmHg. The temperatures of both the pouch and the hamster were maintained independently at 37°C. Observations were made with a Leitz Ortholux microscope with a long working distance objective ×3.5. A 100 W A.C. mercury lamp was used with appropriate filters for observations in ultraviolet light. HR was given to 8 hamsters in a dose of 500 mg/kg b.w. s.c. for 2 days before and a further 500 mg/kg was given i.v. just before preparing the cheek pouch. 10 hamsters were used as controls.

An i.v. infusion of FITC-dextran 150 (Dextran, M_w=150000, tagged with fluorescein isothiocyanate, Pharmacia Fine Chemicals, Uppsala, Sweden) was given slowly in a dose of 500 mg/kg b.w. The dissected area of the cheek pouch (~1.5 cm^2) was scanned at 1/2 hour intervals for 5 hours and the number of FITC-leakage sites counted. At the end of each experiment the observed cheek pouches and the contralateral pouches of the experimental groups were analysed for prostaglandin E_2 (PGE_2)-activity as described by Arturson et al. (8).

Haemostatic plug formation

The method is described in detail elsewhere (3, 11). In short, microvessels of diameter 20–<40 μm and 40–<60 μm (four arterioles and five venules in every size group) in the mesentery of urethane-anaesthetized rabbits are transected. The haemostatic plug formation time is measured and the time and frequency of rebleeding recorded. The time in seconds between transection and the first arrest of bleeding is called the primary haemostatic plug formation time (PHT), and the sum of this and all the rebleeding times in seconds is called the total haemostatic plug formation time (THT). Five control and five HR-treated New Zealand white rabbits (2.8±0.6 kg bodyweight) were studied. The rabbits were given 500 mg/kg b.w. of HR i.v. daily for three days, the last injection being given ½ hour before anaesthesia.

Laser-induced microvascular thrombus formation

This method has been studied in detail using a titanium rabbit ear chamber technique (2). We have earlier shown that comparable results are obtained when the laser injuries are made in the mesenteric arterioles (4). The New Zealand white rabbits (2.2±0.2 kg bodyweight) were studied, five control and five HR-treated. The same dose as for the haemostatic plug formation studies were given.

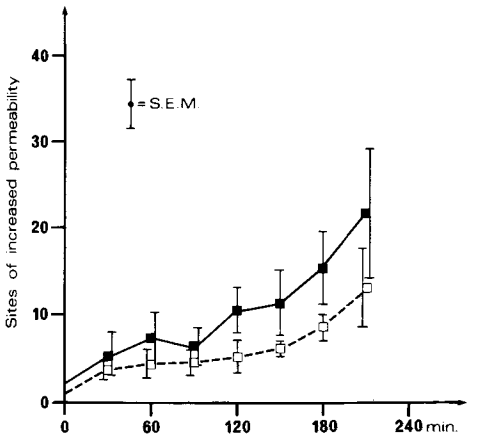

Fig. 1. Sites of increased permeability over time. ■=control; □=HR.

After anaesthesia (urethane) the mesenteric microcirculation was observed through a Leitz Biomed intravital microscope using a ×23 water immersion objective. Laser injuries were inflicted an arterioles measuring 15–25 μm using a 200 J single pulse ruby laser discharge. Platelets from the passing blood adhere immediately at the site of injury to form a microthrombus which grows by accretion of platelets. The thrombus or part of it embolises and further accretion of platelets with embolisation continues. The number of platelet microemboli leaving the site of laser injury was counted and the cumulative number of emboli in 10 min used as a measure of platelet activity (19). Four laser injuries were made in every rabbit (4).

Experimental venous thrombosis

For this study the femoral veins were exposed and thrombus induction performed with sodium morrhuate (Eli Lilly and Co., Indianapolis, Indiana) in combination with venous stasis according to Peterson & Zucker (20) as modified by Ah-See et al. (1). Twenty control and twenty HR-treated New Zealand white rabbits (3.2±0.4 kg bodyweight) were studied with a HR dose of the same size as in the above mentioned experiments. Saline was given in a dose of 5 ml/kg. In another series of experiments only 500 mg/kg of HR was given immediately before the experiment (11 rabbits) or 20 ml/kg of saline (12 rabbits). The sodium morrhuate was in contact with the vessel wall for 5 min and after 45 min of stasis the veins were cut distal to the constricting ligature and the vessels inspected for the presence of macroscopically visible thrombi. No distinction was made between occlusive and non-occlusive thrombi.

Serum concentration of HR

Serum concentration of HR (μg/ml) was measured in the group where venous thrombus formation was studied and the animals treated for three days, using a spectrophotometric method described by Barrow & Griffiths (9). The samples were taken before injection and 15 min, 3 h, 6 h, 12 h, 24 h, 48 h and 49 h after the first HR injection.

All experiments were made blindly.

Statistical analysis

In haemostatic experiments the skew distribution of the haemostatic plug formation time was taken into consideration (10). For details see Arfors et al. (3). In assessing the significance of the frequency of rebleeding the rank-sum test was used (15). The difference in number of laser emboli was calculated according to the Students *t*-test. The difference in venous thrombosis was calculated with Fischer's exact test.

RESULTS

The PGE_2-activity in the pooled samples of observed pouches was 25.7 ng/g tissue for untreated controls and 6.7 ng/g tissue in HR-treated hamsters. In the contralateral pouches the corresponding values were 1.3 ng/g and 6.6 ng/g tissue. Number of leakage sites up to 3½ hours are plotted in Fig. 1. At this time FITC-dextran from some leakage sites had diffused so widely that it became impossible to count accurately even if most of the preparation was not leaking. Number of FITC-dextran leakage sites were lower in HR-treated hamsters than control hamsters although this difference was not statistically significant. *t*-Test for a difference between HR and control at 120, 150, 180 and 210 min after start of observation resulted in *P*-values 0.06, 0.12, 0.07 and 0.22.

In Fig. 2 is shown the haemostatic plug formation times. As can be seen there is a tendency to pro-

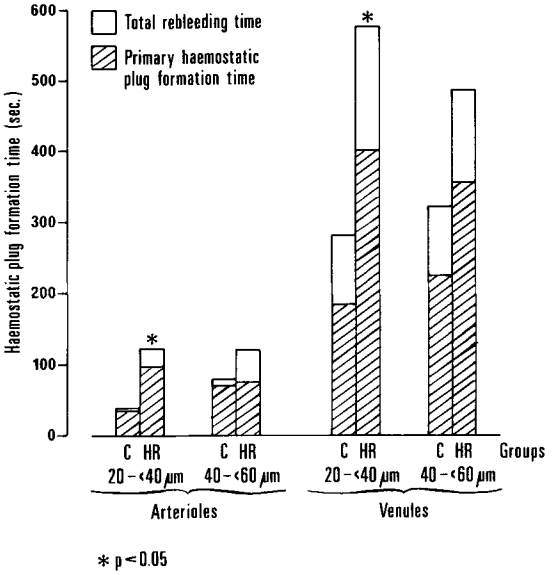

Fig. 2. Bar-chart showing total and primary haemostatic plug formation times. C (control) and HR.

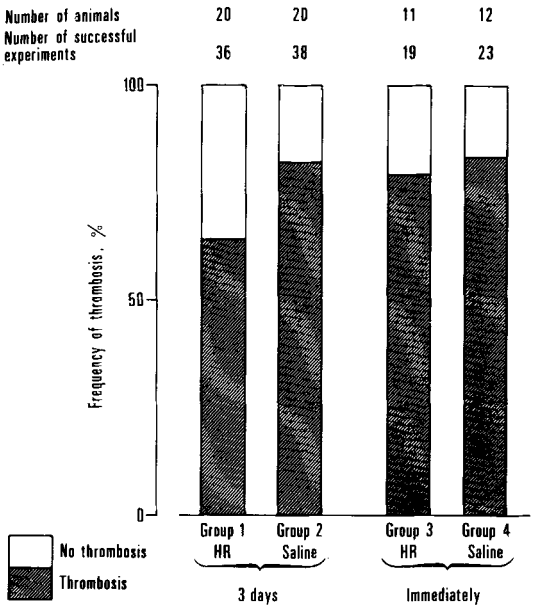

Fig. 3. Histogram showing the frequency of venous thrombosis in the different experimental groups.

longation of the times in all vessel groups in HR-treated rabbits although the prolongation is only significantly longer for THT in the smaller arterioles and THT and PHT in the smaller venules ($p<0.05$). The frequency of rebleeding is significantly higher in arterioles in HR-treated animals ($p<0.05$) whereas it is not affected in venules (Table I). There is no difference between the two groups in the frequency of cumulative number of emboli after laser-induced intravascular injury (control 6.5±2.2, HR 6.7±2.6; $p>0.05$).

The frequency of thrombosis is presented graphically in Fig. 3. HR treatment for three days diminished the frequency although not to a significant level (64 and 82% respectively) ($p=0.07$). Acute administration of one dose of HR shows no effect (HR 79%, control 83%).

In Fig. 4 is shown the plasma levels of HR over time. At 6 h, 12 h, 24 h, and 28 h after the first injection the levels were zero.

DISCUSSION

In untreated hamsters there is a continuous increase in the number of postcapillary leakage sites over time. This increase is to some extent inhibited by HR, thus verifying the observations made by Arturson (7) after experimental skin burn injury.

Table I. *The frequency of rebleeding*

The frequency of rebleeding is given by dividing the total number of rebleeding episodes by the number of transected vessels

	Arterioles		Venules	
	20–<40μm	40–<60 μm	20–<40 μm	40–<60 μm
Control	0.10	0.30	1.04	1.32
HR	0.45*	0.85*	1.04	1.40

*=$p<0.05$.

Although this diminished permeability after burn injury was suggested to depend on inhibition of the prostaglandin formation this could not be confirmed. Indomethacin, which is a potent prostaglandin synthesis inhibitor (24), completely abolishes the prostaglandin content in microvascular preparations (6). Although indomethacin diminishes microvascular permeability it is not totally blocked (6) and thus also other substances than prostaglandins must be responsible for the leakage of macromolecules in slightly traumatised tissue.

Activation of platelets in haemostatic plug formation has been suggested dependent on collagen, thrombin and adenosine diphosphate (ADP). After many years of work with the haemostatic plug formation problems we have come to the conclusion that the main initiator for primary haemostatic plug formation is ADP, red cells being absolutely necessary (13), and that the stability of the haemostatic

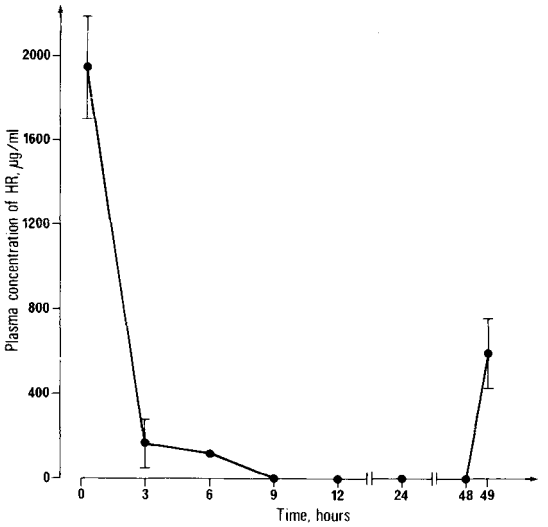

Fig. 4. The plasma levels of HR over time. Mean and S.D. are given.

plugs is a function of the fibrin formation (12). We have found a tendency to prolongation of the haemostatic plug formation times after HR treatment, which possibly is explained by an inhibition of the platelet activity. The in vitro studies made have required very high doses of HR (15), which probably explains the slight effect in our experiments. The plasma concentrations were only part of that having effect in vitro at the time for experiment. Born et al. (14) have shown that stabilisation of the cell membrane with chlorpromazine prolongs bleeding time in an *in vitro* system. This stabilisation takes place when doses of chlorpromazine are used which have no effect on platelet aggregation in vitro. As Schmidt-Schönbein et al. (21) has shown HR to stabilise the red cell membrane this gives another possibility for explaining the prolongation of the haemostatic plug formation time. This later explanation is the most likely as HR does not modify the platelet behaviour using the laser method. This model has been suggested to be an ADP-induced platelet aggregation in vivo (17, 19).

Deep vein thrombosis is not only dependent on platelets but also coagulation, fibrinolysis and flow. In the sodium morrhuate model we have earlier shown dextran 70 to diminish the thrombosis frequency, and this effect could be ascribed to a combined effect of platelet inhibition and fibrin structure alteration (1). On the other hand some different platelet function inhibitors did not diminish the thrombosis frequency in this model (5). In the sodium morrhuate model HR decreased the frequency of thrombosis although not significant ($p=0.07$) if the animals were treated three days. An effect on the vessel wall can not be excluded, as assumption which is supported by some clinical results (18).

In conclusion, using our models for in vivo platelet studies we found a very small effect of HR. Using a venous thrombosis model the effect of HR was of borderline significance. An inhibitory effect of the inflammatory response of the vessel wall could not be excluded which is also supported by the recorded effect on microvascular permeability.

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