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The Significance of a Smooth Muscle Component in Hemostasis.* (30325)

W. O. CRUZ
Laboratory of Hematology, Instituto Oswaldo Cruz, Rio de Janeiro, Brasil

Currently accepted theories on the control of bleeding in oozing blood postulate two main but quite distinct mechanisms: formation of blood clots and agglutination of platelets to form a plug in this region. In both instances a mechanical stopper is visualized and hemostasis is interpreted as a phenomenon entirely dependent on blood components, particularly the disintegrative processes concerned with these.

Several observations have been presented (1,2,3,4) which were extremely difficult to resolve in terms of the classical theories noted above. It was then suggested that hemostasis is a function of active metabolic events and that the local area of injury, rather than blood itself, plays the most important role in the control of bleeding.

Methods. The results presented here were carried out by a method developed in our laboratory. A small area of keratinized epidermis in a rat tail is removed with a razor blade and the ensuing bleeding stops in 1 to 2 minutes. When mechanical trauma (such as strong scouring with gauze) is subsequently inflicted on such scarified skin and the tail immersed, in a vertical position, in different fluids at 37°C, the bleeding in each vessel (arteriole or venule) can be observed through a dissecting microscope (30 to 50 × magnification) until hemorrhage completely ceases. Table I shows that this method is quite consistent, the range of bleeding time (B.T.) varying between 40 to 90 seconds (Table I).

Results. Direct visual observation. Bleeding from arterioles never stopped abruptly. There was always a progressive decrease in the diameter of the falling column of blood until a very thin stream was observed just before the end of bleeding.

Repeated mechanical stimulation. When mechanical trauma was applied at about 10-minute intervals, B.T. remained fairly constant for a period of about 2 hours (average for 12 determinations — 56″ with a range of 20″-93″). When, however, the trauma was applied as soon as the bleeding stopped, then, after from 5-10 such stimulations, the scarified skin failed to bleed even though subjected to the most violent mechanical stimulus.

Inhibitors of hemostasis. Inflence of anions and cations. When the saline in the bath was substituted with sodium or potassium salts of non-penetrating anions (HPO_4^{--}, SO_4^{--}, HCO_3^-) at isotonic concentrations, or by salts of slow moving anions (CNS^-, NO_3^-) it was observed that these solutions had a tendency to prolong bleeding. The anions SO_4^{--} and HPO_4^{--} had an especially strong anti-hemostatic action. A strong anti-hemostatic effect with cations was observed when $CaCl_2$ or $MgSO_4$ (10 mM or higher) was tested isotonically in saline.

Inhibitors of cell respiration. Inhibitors of

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TABLE I. Normal Bleeding Time in Rat Tail Preparation Immersed in Different Solutions.

Solution	Concentration (mM)	No. of determinations	Bleeding time (seconds) Maximum	Minimum	Avg	S.D.
NaCl	150	117	138	20	67	22
Krebs	150	117	150	40	87	29
KCl	150	22	82	31	49	—
Glucose	280	17	88	28	43	—
Distilled water	—	16	176	20	87	—
Veronal buffer pH 7.4	300 mg %	12	115	51	80	—

TABLE II. Cell Respiration Inhibitors Tested on Hemostasis (Rat Tail Preparation).

Inhibitor	Activity	Concentration (mM)	Bleeding time (min) Scarified skin	Mechanical stimulus	Saline aft. wash.
Dinitrophenol	Chain uncoupler	1	+10	+10	N
Cyanide	Unspecific, Fe	100	N	+30	N
Azide	Cytochrome oxidase	1	N	+10	N
Fluoride	Succinic deshidrogenase	200	—	+30	N
BAL	Metal inhibition reversal	1	—	+10	N
Iodoacetate	SH-Thiol alkilating	10	+10	+10	+10
P-Chloromercuribenzoate	SH-Mercaptide forming	1	—	+30	N
N-Ethylmaleimide	SH-Mercaptide forming	1	—	+10	N

cell respiration and uncouplers of oxidative phosphorylation (2,4 dinitrophenol, cyanide, azide) either increased B.T. or strongly interfered with the hemostatic mechanism. Inhibitors of sulphhydryl groups (iodoacetate, p-chloro-mercuribenzoate, N-Ethylmaleimide) were very powerful inhibitors of hemostasis (Table II).

Effect of pH. Acetate buffer (0.1 M) or succinate buffer (0.05 M) at acid pH (5.4 to 4.5) had strong inhibitory effects on hemostasis.

Anti-hemostatic after-effect. When most hemostasis inhibitors (at the concentrations studied) were removed from the bathing fluid the skin vessels regained their capacity to control bleeding. However, after iodoacetate (10^{-2} M), EDTA (10^{-2} to 15×10^{-2} M) or particularly heparin (1 to 25 mg/ml), the bleeding continued for more than one hour even after thorough washing of the tail with saline.

Effect of temperature. Bleeding from the rat tail was very much prolonged in low as well as in high temperatures (Fig. 1). The effect was reversible.

ATP activity in hemostasis. a. *Activity in normal bleeding time.* When the scarified rat tail is mechanically stimulated and quickly immersed in a test tube containing ATP in saline at optimum concentrations (10^{-3} to 5×10^{-3} M) the B.T. was drastically shortened to between 60 to 90% of its normal value found by previous determinations using saline alone as bathing fluid (Table III).

b. *Intermittent effect.* When an excess of ATP was added to the saline bath (10^{-2} to 10^{-1} M) a peculiar intermittent bleeding occurred. After mechanical stimulus all the bleeding vessels showed the same behavior: the bleeding flow suddenly stopped spontaneously and no bleeding was observed for some seconds; after this quiescent period renewed bleeding occurred for a few seconds, then

TABLE III. ATP Capacity to Lower Bleeding Time in Saline (Rat Tail Preparation).

ATP concentration (M)	Bleeding time (sec) Before	After ATP addition	Decrease from previous B.T. (%)
10^{-2}	72	17	76
10^{-2}	44	14	68
10^{-2}	55	28	49
$10^{-4} + 10^{-4}$ Mg^{++}	55	33	40
$5\times10^{-3} + 5\times10^{-3}$ Mg^{++}	132	19	86
$10^{-3} + 10^{-3}$ Mg^{++}	240	12	95

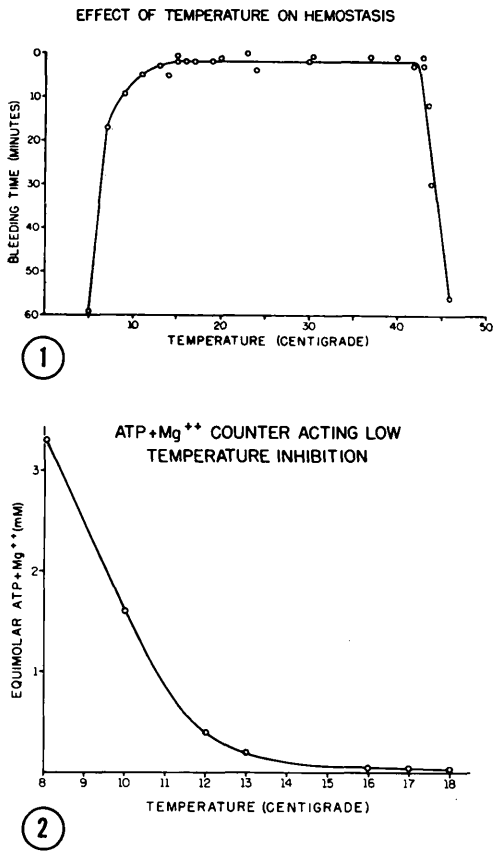

FIG. 1 and 2

stopped again. This on-and-off bleeding (the intermittent effect) is repeated for hours as long as the concentration of ATP in the bath stays sufficiently high. The microscopic observation of a single vessel, during a half-hour period, is shown in Table IV, as well as observations collected in 5 other animals. In these experiments the ratio between the time interval with no bleeding and the time during which the vessel bled varied between rather short limits (3.4 to 4.4). Many cations, especially Mg^{++}, when added to these excessive concentrations of ATP, corrected the intermittent effect so that bleeding stopped completely.

c. *Anti-inhibitory capacity.* Very small concentrations of ATP (20 to 150 micromolar) corrected the anti-hemostatic activity of some salts, respiration inhibitors and uncouplers as well as an anti-histaminic drug (Table V). ATP plus $MgSO_4$ in equimolar moderate concentrations (10^{-3} to 10^{-2} M) was able to overcome stronger inhibitors (Table VI). High concentrations of ATP and Mg (ATP, 10^{-2} M plus $MgSO_4$, 4×10^{-2} M) corrected the very strong inhibition of substances shown in Table VII.

d. *Correction of temperature effects.* ATP-Mg corrected the inhibition of hemostasis induced by low as well as by high temperatures. There was correlation between temperature and concentration of ATP-Mg required to correct bleeding (Fig. 2).

Discussion. A. *Muscle participation in hemostasis.* That a local factor should be involved in hemostasis is indicated by several facts observed in our laboratory(3). We described(4) that:

1. Mechanical trauma, exerted by a blunt instrument (as employed in dermographic studies by Lewis) previous to skin incision, modifies drastically the bleeding picture by decreasing the bleeding time, or, in some instances, prevents bleeding altogether. When a skin region is denervated and complete nerve degeneration occurs, the so-called "Lewis hemostatic effect" is no longer present.

TABLE IV. Intermittent Type of Bleeding in High Concentration of ATP (25 mM). 100 successive observations in a single arteriole, during 33 min.

Successive groups of determinations	Flux duration (sec)			Interval duration (sec)			Ratio
	Max	Min	Avg	Max	Min	Avg	Interval/Flux
25	5	1	3.5	23	7	13.6	3 9
25	6	1	3.1	25	6	11.4	3.7
25	11	3	5.9	53	12	22.3	3.8
25	8	3	5.0	42	7	17.0	3.4
100 (single rat) (ATP, 25 mM)	11	1	4.4	—	—	—	—
111 (5 rats) (ATP, 50 mM)	13	1	4.2	—	—	—	—

2. Faradic nerve stimulation on the skin area under the control of the stimulated nerve decreases the bleeding time.

3. Adrenalin and noradrenalin, when applied topically or intravenously, shorten the bleeding time in some dogs. Catecholamine antagonists like Dibenamine and Priscol, as well as substances that interfere with the liberation of catecholamine from tissues, such as Guanethidine and Bretylium, both have a strong anti-hemostatic activity.

4. EDTA, sodium citrate, oxalate and fluoride, as well as heparin, showed anti-hemostatic activity *in vivo* and a relaxing activity *in vitro* on smooth muscle contracted by addition of catecholamine to the surrounding fluid.

It seems useful to emphasize the fact that

TABLE V. Inhibitors Counteracted by Equimolar ATP + Mg^{++} in Concentrations Between 20 to 150 Micromolar.

Inhibitors	Concentration	Bleeding time (min)	ATP + Mg^{++} conc capable to keep B.T. lower than 3′ in presence of the inhibitors (micromolar)
K cyanide	10^{-1} M	+30	50
2,4 Dinitrophenol	1 mM	14	100
Na azide	1 mM	19	150
Na oxalate	10^{-2} M	10	20
Na sulfate	10^{-1} M	+30	30
Na citrate	2 mM	+30	80
Na fluoride	2×10^{-1}	+30	100
Neo-antergan	2.5 mg/ml	+10	50

TABLE VI. Inhibitors Counteracted by Equimolar ATP + Mg^{++} in Concentrations Between 10^{-3} to 10^{-1} M.

Inhibitors	Concentration (mM)	Bleeding time (min)	ATP + Mg^{++} conc capable to keep B.T. lower than 3′ in presence of the inhibitors (molar)
Iodoacetate	1	+15	10^{-3}
Ethanol	8 ml %	+10	10^{-3}
Priscol	10 mg/ml	+10	10^{-3}
BAL	10	+30	5×10^{-3}
Sucrose (hypertonic)	37 g %	+30	5×10^{-3}
Phosphate buffer, pH 7.4	.1 M	+10	10^{-2}
P-Chloromercuribenzoate	1	+30	10^{-2}
Heparin	5 mg/ml	+20	10^{-2}
KCl (hypertonic)	.5 M	+10	10^{-2}
N-Ethylmaleimide	1	+10	10^{-1}

TABLE VII. Inhibitors Counteracted by ATP (10^{-2} M) plus Mg^{++} (4 to 5×10^{-2} M).

Inhibitor	Concentration (molar)	Bleeding time (min)	ATP + Mg conc capable to keep B.T. lower than 3′ in presence of inhibitors
Acetate buffer, pH 4.8	.1	+10	ATP (10^{-2} M) + $MgSO_4$ (4×10^{-2} M)
Succinate buffer, pH 4.8	.05	+10	ATP (10^{-2} M) + $MgSO_4$ (4×10^{-2} M)
EDTA	5×10^{-2}	+15	ATP (10^{-2} M) + $MgSO_4$ (5×10^{-2} M)

in the rat tail preparation the bleeding occurs not through capillaries but through vessels of medium caliber as arterioles and venules. Probably most of the blood oozes from arterioles since the so-called capillary blood from human fingertip is blood that oozed mainly from arterioles. In fact, when cutaneous blood in humans is drawn under oil from a slit in the fingertip, it shows 96-97% saturation with oxygen, exactly as does arterial blood(5). Moreover, capillaries, at least in mammals, have no capacity to contract(6), therefore, the contractile small vessels that are often characterized as capillaries in the literature, as in the paper of MacFarlane(7), are, in fact, very tiny arterioles. These observations should be stressed in connecting smooth muscle activity and bleeding control.

B. *Mechanical plug and stoppage of bleeding.* After recording the observations presented here, a revision of the respective roles of muscular contraction, coagulation, and platelet plug (in different preparations used for studies on hemostasis *in vivo*) seems warranted. Hugues(8), comparing these factors in mesentery preparations, concluded that the platelet plug was of main importance but suggested that in other circumstances the relative significance between these factors could be different. In our preparations, the column of oozing blood decreases in diameter, suggesting that the vessels gradually taper toward a final occlusion. However, this observation could not be considered of decisive value, since Zucker(9) claimed that in her preparation bleeding also ceased gradually as the platelet plug was built up. Even in studies that emphasized exclusively the role of platelets, some observations cannot be explained by plug formation. For example, Honour and Mitchell(10), studying microscopically the vessels of the rabbit cortex, show that arrest of bleeding does occur before the formation of platelet masses. They claim that in major injury of arteries, the bleeding stops "usually within a few seconds," and only after the vessels have relaxed do white bodies form in a steady succession at the site of injury. We wonder if the successive formation, for hours, of white masses following an injury serious enough to cause bleeding in an artery(10,11) could be due to platelet aggregation induced by ADP. Some ADP could have escaped rephosphorylation by creatine phosphokinase during a sustained contraction of smooth muscle around the site of injury.

C. *Analogies between muscle physiology and hemostasis* are: 1. Hemostatic inhibitory effect *in vivo* of substances known as smooth muscle relaxing agents *in vitro* being counteracted by adequate concentrations of ATP or ATP-Mg in both instances.

2. Bleeding and spontaneous stoppage followed by renewed bleeding with repetition of this phenomenon for hours (so-called intermittent effect), obtained by high concentration of ATP in the rat tail preparation bath.

3. The temperature effect in hemostasis is similar to that observed in rabbit skeletal muscle.

D. *A theory on hemostasis.* Knowing that when a wound is inflicted in the skin the bulk of blood oozes through medium caliber vessels, mainly arterioles, and not through capillaries, mechanisms applicable to these larger vessels should be considered. We postulated that bleeding control is carried out by some special type of muscular contraction, that the smooth muscle once contracted remains in that state until the injured vessel is repaired.

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