RAT MODEL OF ARTERIAL THROMBOSIS INDUCED BY FERRIC CHLORIDE

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ABSTRACT

The purposes of these studies were to produce a small animal model of arterial thrombosis for study of novel antithrombotic agents, to validate a simple temperature index of occlusive thrombosis, and to describe the composition of the thrombus. Small thermocouple transducers were fabricated from readily available materials. A thermocouple was inserted under a carotid artery of the anesthetized rat and vessel temperature was recorded continuously. Arterial injury was induced by FeCl₃ solution applied topically to the artery above the thermocouple. To validate the relationship between thrombotic occlusion and vessel temperature, blood flow velocity, proximal to the injury, and temperature were recorded simultaneously. Temperature decreased rapidly when velocity averaged 24 ± 12 percent of control and velocity did not differ from zero within 20 sec. In normal vessels, average flow velocity did not decrease significantly from control until a fixed stenosis decreased diameter by 78 percent. Average time to occlusion (TTO), signaled by the abrupt temperature inflection, ranged from 56 ± 4 min to 14 ± 1 min after 10 and 65 percent FeCl₃ application respectively. Vessel segments were fixed at various times after FeCl₃ exposure and examined by scanning electron microscopy. Endothelial damage was observed and was associated with thrombus composed of activated platelets, fibrin strands and entrapped erythrocytes. The results demonstrate that FeCl₃ dose-dependently induced formation of an occlusive mixed thrombus that was indexed by monitoring the time between FeCl₃ application and a rapid temperature decrease in the carotid artery of the rat.

KEYWORDS: arterial thrombosis, rat, ferric chloride
INTRODUCTION

The role of an animal model is essential to the discovery of new drugs effective in the prophylaxis and treatment of arterial thrombosis. The study of relatively large numbers of chemical entities, often available in limited quantity, typifies the drug discovery process and necessitates use of a simple inexpensive small animal model. The characteristics of the model should be common to those of the clinical disease. In the case of arterial thrombosis, such characteristics include injury of the vessel, dynamic blood flow and development of a thrombus with extensive platelet involvement in addition to fibrin.

An experimental model of arterial thrombosis in the rat was described by Hladovec (1) in which thrombus formation was induced in the carotid by electric current delivered by stainless steel electrodes. Formation of an occlusive thrombus was indicated by a rapid decrease in vessel temperature which was measured with a thermistor and telethermometer. Time to occlusion (TTO) was used as an index. Philp et al. (2) reported the thrombus was composed of platelets and fibrin.

The use of FeCl₃ in the present study evolved from initial use of electrically-induced injury. It was observed that iron-containing electrodes corroded quickly and a constant current generator was required to maintain uniform current (1). Other electrodes of platinum, copper, brass or carbon were not effective. It was hypothesized that iron was involved in injuring the vessel; therefore, application of a solution of FeCl₃ was substituted.

The purposes of these studies were: (1) to fabricate a simple temperature probe and validate its use to indicate thrombotic occlusion; (2) to demonstrate the thrombotic effectiveness of FeCl₃; and (3) to describe the composition of the arterial thrombus.

MATERIALS AND METHODS

Materials

Teflon jacketed T-type 30 gauge thermocouple wire (TT-T-30) was purchased from Omega Engineering, Stamford, CT. Polyurethane foam (Plast. No. 24) was obtained from Fibre-Glast Developments Corp; Dayton, OH. FeCl₃ hexahydrate was purchased from J. T. Baker Inc., Phillipsburg, NJ. FeCl₃ was dissolved in water and concentrations were expressed in terms of the actual weight of FeCl₃ only.

Thermocouple Construction and Testing

Changes in vessel temperature were monitored with a stainless steel thermocouple device. The inexpensive transducer consisted of hypodermic-type tubing (6 ga., 5.16 mm o.d. x 0.38 mm wall) cut in half (Dremel Moto-Tool) longitudinally and transversely to produce semicircular segments 3-4 mm in width. Teflon-insulated thermocouple wire was silver-soldered to the inside curvature. Heat transfer was limited from surrounding tissue to the device by polyurethane foam insulation deposited to a width of 5-6 mm on the inside semicircular volume. The opposite ends of the thermocouple wire were soldered to an electrical connector appropriate for the recording device.

A random selection of thermocouples was tested during immersion in water by recording output voltage of each probe on a strip chart recorder (Beckman R611). The water was warmed in a water bath over a range of 36-39°C. The response time of the thermocouple-recording system was determined in vivo. Noise was eliminated by a high frequency filter (0.08Hz) on the recorder. After a stable signal was recorded, thrombotic occlusion was emulated by occluding the carotid with a temperature equilibrated microvascular clamp and recording temperature at a fast paper speed.
Experimental Studies

Male Sprague-Dawley rats (375-450g) were anesthetized with xylazine (20 mg/kg, s.c.) followed by ketamine HCl (100 mg/kg, s.c.). Animals were laid on a water blanket in which the circulated water was maintained at 37°C. The carotid artery was approached through a midline cervical incision. Careful blunt dissection was used to expose and isolate the vessel from the carotid sheath. A silk suture was pulled underneath the artery to lift the vessel to provide clearance to insert a thermocouple underneath it. Vessel temperature changes were monitored on a strip chart recorder that had an ink-writing timer. Small forceps were used to dip discs (3 mm dia) of Whatman No. 1 filter paper into a FeCl₃ solution. The discs were cut to equal size using sharpened stainless steel tubing (3 mm i.d.) chucked in a drill press. A saturated disc was placed on each carotid artery above the thermocouple. The time between FeCl₃ application and the time at which temperature decreased abruptly was recorded as time to occlusion (TTO) of the vessel. For vessels that did not occlude within 60 mins, TTO was assigned a value of 60 min. The average time required for both vessels to occlude was used to represent TTO for each animal.

The association was established between vessel occlusion and the abrupt temperature decrease by simultaneous recording of temperature and blood flow on the same recorder. A pulsed Doppler flow probe (3) was placed around the carotid proximal to the thermocouple. The probe recorded changes in flow velocity; therefore, it was installed at a point where thrombosis did not occur and the internal vessel diameter remained constant due to distention with fluid blood (4). Baseline temperature and flow velocity (Directional Pulsed Doppler Flowmeter, Model 545-C, University of Iowa Bioengineering) were recorded before application of FeCl₃ (35 percent). Results were reported as percent change from initial baseline values (6 min before occlusion). The time at which vessel temperature decreased rapidly was arbitrarily established as zero and pre- and post-occlusion temperature and flow values were referenced from that point.

The extent to which the diameter of the carotid was stenosed by a developing thrombus before flow velocity decreased significantly was estimated. Silver bar stock (2 x 2 x 4 mm) was slotted using a dental grinding wheel to make vascular clips. Various slot widths were produced by inserting a strip of precision thickness stainless steel into the slot and compressing the bar stock onto the thickness gauge. The carotid was exposed as described and the outside diameter of the vessel was measured using a stereo microscope with an ocular micrometer. Only arteries measuring 1.4 mm ± 10 percent were used in this study. A flow probe and thermocouple were installed as described previously. After basal flow velocity was measured, a vascular clip was placed around the vessel distal to the thermocouple and steady state flow was recorded. The clip was removed and flow was allowed to return to baseline before the clip with the next smallest gap was applied. Stenosis was reported as a percent of the outside diameter of the vessel and flow velocity as percent change from the immediate pre-stenosis control.

Statistical Analysis

Results were expressed as means ± S.E.M. One-way analysis of variance was used to detect statistically significant differences and then the test for least significant difference was applied to determine which means were different. Significance level was set as P<0.05.

Histology

Carotid artery segments and thrombi were examined with a scanning electron microscope. The rats were perfused with a modified Karnovsky's fixative (at pH 7.2 for 5 min) before, 10 min or 20 min after application of FeCl₃ (35
percent). The 20 min period approximated the TTO in untreated animals. The carotid arteries were removed, cleaned, and immersion-fixed in modified Karnovsky's for a further 72 hr at 4°C. Following three 10-min phosphate buffer rinses, the arteries were post-fixed in 1 percent osmium tetroxide (OsO₄) in phosphate buffer (pH 7.2) for 6 hrs at room temperature. They were rinsed for two 15 min periods in buffer and dehydrated for 10 min each in 50, 75, 85, 95, and 100 percent ethanol. The samples were rinsed twice in 100 percent acetone for 10 min each prior to critical point drying in Freon-B. The dried samples were mounted with silver paint, coated with gold-palladium and examined at 25KV in a Phillips 501-B scanning electron microscope.

RESULTS

The thermocouple transducer, constructed as indicated, produced a linear voltage output ($r=0.992 \pm 0.005$, $n=5$) over the measured temperature range of 36-39°C. A representation of a typical vessel temperature recording and the temporal association with blood flow velocity is shown in Fig. 1. Thrombotic occlusion was designated by a sharp decrease in temperature at zero reference time. The abrupt decrease in temperature averaged 2.8 ± 0.2°C (18 vessels on 6 probes) and spanned 32 ± 4 sec/°C in this electronically highly damped system. Flow velocity was not significantly different from zero within 20 sec of the temperature inflection. The reference values for flow velocity and temperature were arbitrarily selected at 360 sec before vessel occlusion because these parameters were stable at that time and for some time thereafter. Average temperature declined relatively slowly and differed significantly from control 100 sec before vessel occlusion. Flow decreased more rapidly than temperature before time zero and was significantly different from control 120 sec before the inflection in temperature.

Fig. 1. Time course of the relationship between vessel temperature (•) and blood flow velocity (*) simultaneously recorded during thrombosis induced by topical application of FeCl₃ (35%) on the carotid artery. Double asterisks indicate the earliest time at which velocity was not significantly different from zero. Single asterisks signify the earliest time at which parameters differed from control ($P<0.05$, $n=8$).
The next series of experiments was designed to estimate the extent of thrombotic stenosis that developed within the carotid before blood flow decreased. The results summarized in Fig. 2 indicate that flow velocity did not decrease significantly until the fixed stenosis was greater than 77 percent of the outer diameter of the vessel.

![Graph showing % Decrease in Flow Velocity vs. % Stenosis](image)

Fig. 2. Effect on blood flow velocity of fixed stenosis of the carotid artery. (P<0.05, n=6 vessels).

The time between application of FeCl$_3$ on the carotid and occlusion of the vessel was inversely dependent upon the FeCl$_3$ concentration (Fig. 3).

![Graph showing Time to Occlusion vs. % FeCl$_3$](image)

Fig. 3. Dependence of time to occlusion of the carotid artery on the concentration of FeCl$_3$ applied topically to the vessel (n=rats).
None of 16 carotids (n=8 rats) occluded within 60 min after application of 5 percent FeCl\textsubscript{3}. After application of FeCl\textsubscript{3} concentrations of 10, 15, 25 and 35 percent, the proportion of vessels that did not occlude within 60 min were 86, 69, 30 and 4 percent respectively. All vessels occluded within 60 min after application of higher concentrations. A concentration of 35 percent was selected for subsequent studies.

**Histology**

Scanning electron microscopy revealed the endothelial surface of control carotid arteries was similar in appearance to the normal endothelium that has been characterized previously. The luminal surface was covered by a continuous sheet of endothelial cells. A few red blood cells (RBCs) were attached but no fibrin material was observed on the luminal surface (Fig. 4).

![Fig. 4. Scanning electron micrograph of endothelial surface of carotid artery from control rat (x 320).](image)

In vessels fixed 10 min after FeCl\textsubscript{3} application, separation of the endothelial cell junctions was observed and some segments were completely denuded of endothelium (Fig. 5). These segments had clumps of platelets adhered to the exposed subendothelium. Additionally, both leukocytes and RBCs were adhered to these clumps (Fig. 6). All vessels examined at this time had similar lesions. Total thrombotic occlusion of the carotid artery was observed 20 min after FeCl\textsubscript{3} application (Fig. 7). Thrombi consisted of masses of RBCs packed tightly in a fibrin mesh (Fig. 8). At the anterior end of the thrombus, numerous platelets with long projecting pseudopods were tightly adhered to each other with long fibrin strands (Fig. 9).
Fig. 5. Scanning electron micrograph of carotid artery 10 min after application of FeCl$_2$. The endothelial surface is disrupted and numerous platelets with a few red blood cells and leukocytes are attached (x 320).

Fig. 6. Higher magnification of platelet aggregation illustrating divided endothelium with attached clumps of platelets with a few scattered red blood cells and leukocytes (x 1250).
Fig. 7. Scanning electron micrograph of a cross-section of the carotid artery from a rat with an occlusive thrombus (x 160).

Fig. 8. Higher magnification of the thrombus shown in Fig. 7. The thrombus is formed of tightly packed RBC which have lost their original shape and are surrounded by fibrin (x 1250).
Fig. 9. Anterior end of the thrombus illustrating numerous platelets with long extending pseudopods adhered to each other (x 5000).

DISCUSSION

The primary objective of these studies was to develop a simple, rapid and inexpensive model of arterial thrombosis for potential use to evaluate novel antithrombotic agents. The characteristics of such a model should represent an analogy of the pathologic condition of arterial thrombosis. However, this is compromised somewhat by the practical reality that relatively large numbers of novel agents, available in very limited quantity, must be evaluated to select the optimal chemical structure for more detailed study. The model described in this report is a modification of a similar model first used by Herrmann et al. (5) which was an adaptation of a rat model of electrically-induced arterial injury described by Hladovec (1) and characterized by Philp et al. (2).

Continuous recording of carotid temperature was used in the original model (1). Temperature was recorded using a flat disc thermistor (5 mm) (5) interfaced with a telethermometer and a recorder. The present technique relies on a semicircular thermocouple to register temperature. The curved shape may create less disturbance of flow through the overlying carotid. Although rheologic disturbances may be more analogous to the pathology of arterial thrombosis, it may be difficult to reproduce consistent effects with a flat thermistor positioned under the vessel. The thermocouple transducer is inexpensive and easily constructed from readily available materials. A telethermometer is not necessary because the recorder has adequate amplification and the device can be calibrated easily. The transducer has a linear output over the relevant temperature range and the system response time is sufficient for the intended use.
The simple measurement of temperature changes to monitor thrombosis was quite adequate. In fact, relatively small changes in temperature reflected changes in blood flow. The temperature changes recorded before occlusion occur relatively slowly and were smaller than those at the time of occlusion. Consequently, the preocclusive changes were more difficult to interpret. The abrupt temperature decrease that corresponded to virtual occlusion was easily interpreted. Because of the relationship between the sharp temperature inflection and occlusion, it was not necessary to calibrate the thermocouple.

It was of interest to estimate the relationship between the extent of thrombotic stenosis that occurred before blood flow and temperature decreased. The results from the use of fixed external stenoses imply that deposits of thrombotic material encroached over approximately 80 percent of the internal diameter before carotid blood flow was compromised significantly. Ashton et al. (6) reported that 43 percent reduction in blood flow velocity corresponded roughly to 80-90 percent reduction in luminal cross-sectional area of the dog coronary artery constricted with an external constrictor. Our data from the rat carotid are in agreement.

Although in 1944 topical FeCl₃ was used to induce thrombosis in veins (7) and a variety of chemical agents have also been used as reviewed by Henry (8) and Didisheim (9), the use of FeCl₃ in the present studies evolved from the use of electric current to damage vessels. The mechanism of the electrically-induced endothelial injury is not known; however, it was noted that delivery of current to the external surface of the rat carotid induced thrombosis when iron-containing electrodes were used (1). Electrodes made of materials such as platinum, copper, brass or carbon were not effective. A similar observation was reported recently by Ubatuba (10). It seemed possible that iron was involved in the process of vessel injury. The present study demonstrates the thrombotic effect of FeCl₃. The dependence of time to thrombotic occlusion of the carotid artery on the concentration of applied FeCl₃ is probably due to the rate of diffusion of the FeCl₃ and to the extent of induced injury. The mechanism of injury caused by FeCl₃ and the similarity with that induced by electric current awaits further investigation.

The study of the dependence of TTO on the concentration of the applied FeCl₃ solution served to select the 35 percent concentration for use in the other experiments. The objective was to induce occlusion within a reasonable time period in control studies that would allow for anticipated increases in TTO after administration of antithrombotic agents. The selected concentration represents a submaximal effect. In preliminary studies, use of 50 percent FeCl₃ rapidly induced thrombosis that was difficult to inhibit with heparin. This observation was consistent with those reported by Hladovec (1) and Philp et al. (2) in which occlusive thrombosis of the rat carotid occurred 11 min after electric current induced injury. They reported high doses of heparin (300-1000 U/kg, i.v.), administered 1 min before stimulation, extended TTO. The extent of injury after electric stimulation was described as extensive and it was suggested that reduction in the strength of stimulus might improve sensitivity to antithrombotic agents (2). These observations served to justify use of a more moderate stimulus in the FeCl₃ model.

In this study, the damaged areas in the carotid artery, 10 min after application of FeCl₃, were characterized by areas of luminal surface denuded of endothelial cells and covered with adhered platelets mixed with a few red and white blood cells. In 20 min a total occlusive thrombus was formed characterized by numerous platelets, fibrin and red blood cells. This model has provided a precise evaluation of morphologic events which occur during the process of experimental thrombosis.

Hatsuoka (11) used scanning electron microscopy to study experimental thrombus formation in canine saphenous veins. Philp et al. (2), Haust et al. (12), Hermans and McDonagh (13) and French et al. (14) have described
ultrastructural characteristics of thrombi in small arteries while Jorgensen et al (15,16) and White and Gerrard (17) have focused on description of blood cellular elements such as platelets, red and white blood cells in thrombi. All these reports have described basically the same morphologic changes that we have observed in this FeCl$_3$ model.

In summary, the topical application of FeCl$_3$ solution offers a simple method to induce arterial injury and thrombosis in the rat. The fabrication of the thermocouple probe is easy, inexpensive, and it provides an adequate means to detect occlusive thrombosis. The composition of the thrombus involves platelets and red blood cells enmeshed in a fibrin network and appears to be characteristic of an arterial thrombus. The utility of this model for study of antithrombotic agents remains to be established.

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