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ELECTRICAL INDUCTION OF CORONARY ARTERY THROMBOSIS IN THE AMBULATORY CANINE: A MODEL FOR IN VIVO EVALUATION OF ANTI-THROMBOTIC AGENTS[1]

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ABSTRACT

A simple technique for the reliable induction of coronary artery thrombosis in a conscious dog by delivery of low amperage electric current to the intimal surface of the artery is described. Ibuprofen, an agent known to inhibit platelet function and prostaglandin synthesis is evaluated in this model. Comparison of myocardial infarct size, thrombus weight, arrhythmia development and scanning electron micrographs of drug treated and control animals indicate that Ibuprofen is capable of protecting against the deleterious effects of coronary artery thrombosis produced by this technique. This method holds considerable potential as an in vivo model of coronary artery thrombosis and one in which evaluation of anti-thrombotic agents is possible.

INTRODUCTION

The association between thrombotic coronary occlusion and transmural myocardial infarction underscores the need for better understanding of the events involved in coronary artery thombus development (1,2). Considerable attention is being turned to the interactions between platelets and the vascular endothelium as these may significantly alter the development of the thrombotic process (3). Significant progress has been made in elucidating the role of platelets in initiating and promoting this process. However, much of this work has been done in vitro and it is clear that even the most sophisticated of these techniques cannot duplicate the complex interactions which occur in vivo. Therefore, complete pharmacologic characterization of drugs capable of influencing the development of coronary artery thrombosis requires in vivo evaluation. Only with an appropriate animal model is it

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possible to thoroughly evaluate agents which, on the basis of *in vitro* data and theoretical considerations, are potentially useful in a clinical setting to prevent the development of coronary artery thrombi.

A number of *in vivo* models of coronary artery thrombosis have been proposed (4,5). Salazar (4) introduced a novel technique of inducing coronary artery thrombosis by the application of anodal current to the intravascular lumen *via* a stainless steel electrode positioned under fluoroscopic control. While this technique reliably induced coronary artery thrombus formation, its use is restricted by fluoroscope availability and the necessity of general anesthesia during the stimulation period. Presented here is a technique by which coronary artery thrombosis is reliably induced *via* electric current delivered through a silver wire inserted into the left circumflex coronary artery (LCX). This technique eliminates the need for costly fluoroscopic instrumentation and induces coronary artery thrombosis in a conscious, ambulatory animal which better models the clinical condition. Ibuprofen, a non-steroidal anti-inflammatory agent known to inhibit platelet function and prostaglandin synthesis, is evaluated in this model to demonstrate that appropriate pharmacologic intervention protects against the detrimental effects of experimentally induced coronary artery thrombosis. It is proposed that this *in vivo* model will aid in characterizing anti-thrombotic agents which have potential clinical value in preventing coronary artery thrombosis and acute myocardial infarction.

METHODS

Day 1 - Surgical Procedure

Male mongrel dogs, 12 to 18 kilograms, were anesthetized with sodium pentobarbital (30 mg/kg, I.V.), intubated and subjected to positive pressure ventilation with a Harvard respirator. Surgery was performed under aseptic conditions beginning with the insertion of cannulae into the common carotid artery and jugular vein by way of a neck incision. The heart was exposed *via* a left thoracotomy through the 4th intercostal space. Incision of the pericardium allowed access to the LCX of which approximately 5 mm was isolated from surrounding tissue by blunt dissection. A 28 gauge teflon coated silver wire, with 3 mm of the tip of a 25 gauge hypodermic needle secured on the wire's leading end to aid penetration of the arterial wall, was inserted through the wall of the circumflex artery so that approximately 2-3 mm of the bare wire-needle tip assembly was within the vessel lumen and in contact with the intimal lining. The wire was then secured to the epicardium with two sutures. An electromagnetic flow probe (Carolina Medical Electronics, Inc.) was affixed to the LCX approximately 1-2 cm proximal to the point of entry of the wire (Figure 1). The thoracotomy incision was closed in layers and a Grass disc electrode was secured to a subcutaneous muscle layer to complete the electrical circuit. LEAD II electrocardiographic (ECG) data were obtained by securing subcutaneous electrodes at appropriate sites. All wires and cannulae were tunneled subcutaneously and exteriorized from the back of the neck. The animal was allowed to recover from surgery before being returned to the laboratory for further study.

Day 2 - Initiation of Stimulation of LCX

Twenty-four hours post-surgery, the animal was placed in a quiet laboratory and monitored for any surgically induced aberrant electrocardiographic

rhythms. Only animals free of electrocardiographic evidence of myocardial injury, Q-waves, ST segment changes or arrhythmias, were subjected to further study. Ibuprofen (Upjohn Co., Kalamazoo, Michigan) dissolved in 0.2 M

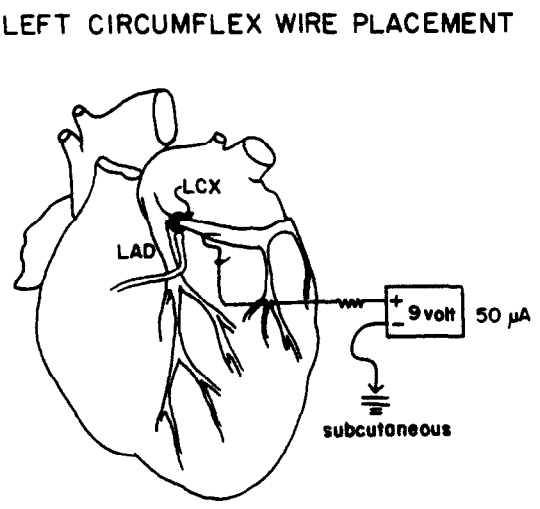


FIG. 1

A schematic drawing indicating the position of the wire electrode in the left circumflex coronary artery (LCX). An electromagnetic flow probe is affixed to the LCX just proximal to the site of insertion of the electrode. (LAD: Left anterior descending coronary artery).

Na_2CO_3 (final pH: 8) was given *via* esophageal cannula according to the following treatment regimen: 50 mg/kg as the first dose, repeated 12 hours later, and at 4, 8, 20 and 24 hours after the initial dose, 25 mg/kg. All doses were prepared just prior to administration. One hour after the first dose, anodal current from a 9 volt battery was delivered to the intimal surface of the LCX *via* the indwelling silver wire. A 250,000 ohm potentiometer, placed in series, allowed complete control of the current output which was adjusted to 50 microamperes. This circuit as well as an ECG telemetry transmitter (Narco Bio-Systems, Inc.) were held in a lightweight nylon jacket which allowed unrestricted movement by the dog. The ECG data were received *via* telemetry and recorded with a tape recorder programmed to record 28 seconds of tracing every 15 minutes. A permanent record was later obtained by replaying the tape into a Grass polygraph.

Day 3 - Sacrifice

After delivery of 50 microamperes of current to the LCX for 24 hours, the current was interrupted, the animal reanesthetized with sodium pentobarbital, placed on positive pressure respiration and the original thoracotomy incision reopened to expose the heart. At sacrifice, a 20% patent blue violet solution (1 ml per 5 kilograms body weight) was injected into the left atrial appendage. Introduction of this flow dependent dye into the myocardial circulation allowed visualization of perfusion defects in the area of myocardium dependent on the thrombosed LCX as indicated by the absence of dye

in these areas. After removal of the heart, the LCX in the region of the inserted wire was dissected free of surrounding tissue and opened lengthwise. The thrombus was scraped free from the intimal wall and a wet weight gravimetric determination was performed. The heart was then sectioned transversely from apex to base into roughly 1.5 cm thick slices and areas of nonperfusion were noted. The area of infarcted myocardium was determined by incubation of the slices in 2,3,5-Triphenyltetrazolium chloride. This vital dye gives a positive reaction in the presence of myocardial cellular dehydrogenases, therefore revealing areas of viable tissue (6). The non-stained, infarcted regions were excised and analyzed by gravimetric methods.

Scanning Electron Microscopy

LCX coronary artery samples to be examined by scanning electron microscopy were fixed for 2 hours at room temperature in a solution of 2.5% glutaraldehyde (V/V) and 2% paraformaldehyde (W/V) in 0.1 M cacodylate buffer (pH 7.4). The samples were then transferred to 10% sucrose in 0.1 M cacodylate buffer (pH 7.4) and stored at 4° C. Next, the samples were postfixed for 1 hour in 1% osmium tetroxide in 0.1 M cacodylate buffer (pH 7.4), dehydrated in a graded series of ethanol solutions and desiccated by critical point drying. After the samples had been coated with gold, they were examined with an AMR 1200 scanning electron microscope.

In Vitro Platelet Aggregation

In vitro assessment of platelet function was accomplished by established spectrophotometric methods (7) utilizing a Chronolog platelet aggregometer. Aggregation was initiated by the addition of 50 µl of collagen (1:160 dilution of Ethicon Collagen Dispersion-TD 150) to 400 µl of diluted platelet rich plasma (PRP). PRP was prepared by collecting venous blood in 1.5 ml of 3.8% sodium citrate to a total volume of 15 ml. This was centrifuged at 310 x g for 3 minutes to obtain the PRP fraction and then at 2,200 x g for 10 minutes to obtain the platelet poor fraction (PPP). PRP was diluted with PPP to a platelet count of 200,000 per mm^3 before use in the aggregation assays. All platelet samples were assayed immediately, less than 2 hours from the time of collection. Values are expressed as percent light transmission standardized to the PRP and PPP samples yielding 0 and 100% light transmission, respectively.

Statistics

All data are expressed as the mean ± standard error of the mean (SEM). Student's t test was used in evaluating the level of significance.

RESULTS

Presented in Figure 2 is a representative sample of the progressive ECG changes seen in an untreated dog receiving continuous stimulation at 50 microamperes for 24 hours. Constant monitoring of the animal allowed for documentation of the increasing severity of myocardial injury, as witnessed by the ST segment depression (6 hours into stimulation) as well as episodes of ventricular tachycardia (12 hours into stimulation). The absolute degree of ECG disturbance shows some variability between individual animals, however in preliminary experiments each of 20 dogs receiving LCX coronary artery stimulation showed recurrent bouts of ventricular tachycardia at the end of 24

hours of stimulation. If stimulated at higher amperage or for longer periods, these runs of ventricular tachycardia were observed to degenerate into ventricular fibrillation.

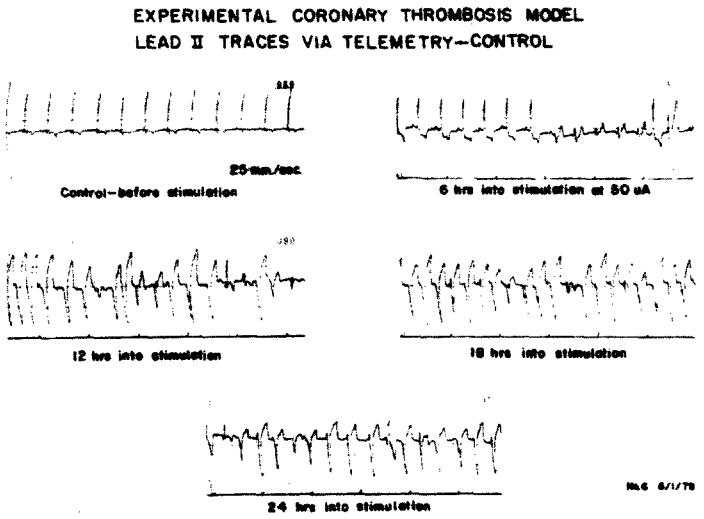


FIG. 2

A representative sample of electrocardiographic traces received via telemetry from an untreated control dog receiving 50 microamperes of current for 24 hours.

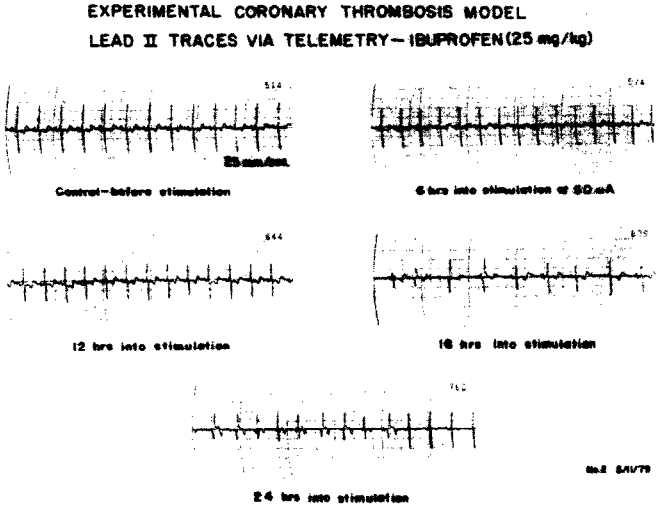


FIG. 3

A representative sample of electrocadiographic traces received via telemetry from an animal treated with Ibuprofen during electrical stimulation of the LCX.

The corresponding trace from a dog treated with Ibuprofen during the course of the stimulation is shown in Figure 3. As can be seen, no significant alteration of the ECG pattern occurred over the 24 hours of stimulation. This striking effect of Ibuprofen on the development on ventricular arrhythmias is summarized for seven control and six drug treated animals in Figure 4. These data were obtained by determining the percent ectopic beats in a 28 second segment of ECG trace received by telemetry, sampled 4 times per hour (every 15 minutes). Figure 4 clearly shows the beneficial effects of Ibuprofen in reducing the severity of ventricular arrhythmias produced by this technique of inducing coronary artery thrombosis.

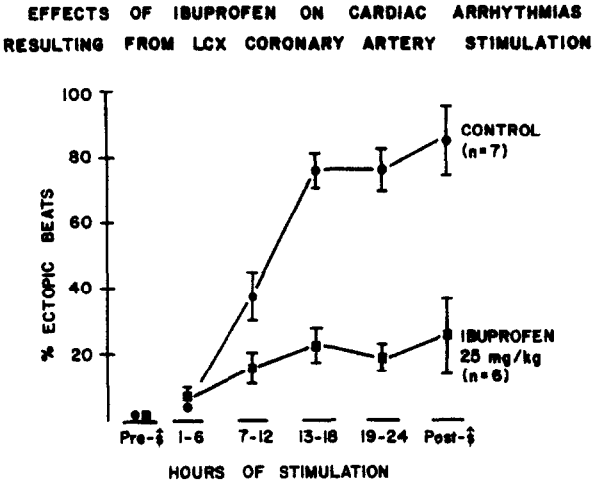


FIG. 4

The percent of ectopic beats in a 28 second segment of ECG trace was determined and averaged over 6 hour time periods beginning with the initiation of stimulation. Pre-$ and Post-$ refer to determinations made before and after 24 hours of stimulation at 50 microamperes, respectively. All points for the Ibuprofen treated animals after the 1-6 hour segment are significantly different from control ($p < .05$).

Further evidence of Ibuprofen's ability to protect the myocardium from the deleterious effects of an experimentally induced thrombus comes from the quantification of thrombus size and left ventricular infarct mass. Ibuprofen treated animals had a marked reduction in the percent of infarcted left ventricle compared to control animals ($1 \pm .3\%$ versus $38 \pm 4\%$, $p < 0.001$). In addition, a significant decrease in thrombus wet weight was seen in the drug treated dogs in comparison to control animals (10 ± 2 mg versus 19 ± 2 mg, $p < .01$).

<u>In vitro</u> assessment of platelet aggregation has indicated that animals treated with Ibuprofen show a progressive inhibition of platelet responsiveness to the addition of collagen, as the aggregating stimulus (Figure 5). The decline in platelet function over the course of the experiment

may be a significant factor in producing the beneficial effects of Ibuprofen treatment.

Scanning electron micrographs of selected samples of LCX coronary arteries near the insertion site of the wire electrode are shown in Figures 6, 7 and 8. An artery from a sham operated dog in which the electrode was inserted into the vessel six days before sacrifice was the source of the sample used for the micrographs seen in Figure 6. No current was delivered to the artery during this time. Figure 6A is a low magnification view of the portion of

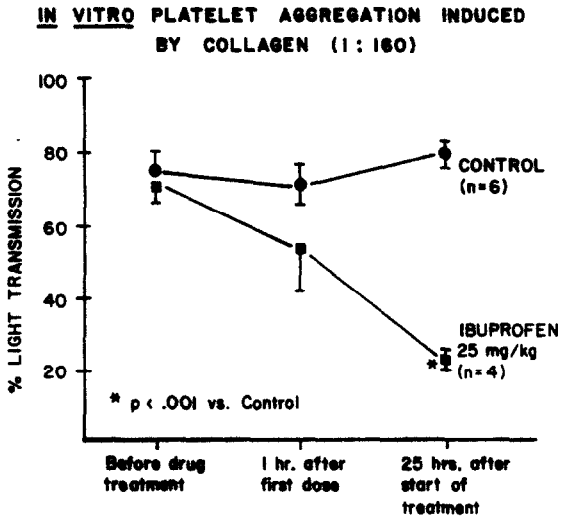


FIG. 5

In vitro platelet aggregation induced by Ethicon collagen dispersion-TD150 (diluted 1:160 with H_2O). Stimulation of the LCX at 50 microamperes was initiated 1 hour after the 1st dose of Ibuprofen and ended 25 hours after the 1st dose. Percent light transmission is directly proportional to the extent of platelet aggregation. Platelet samples were prepared as stated in the methods section.

the LCX containing the electrode (E). The only indication of thrombus formation is at the point where the electrode entered the vessel lumen (□) (88X). A higher magnification view of the area indicated by (□) in Figure 6A is shown in Figure 6B. This material is fibrous in appearance (arrows), and contains many erythrocytes (RBC) and platelets (P) (963X). Figure 6C is a micrograph of the area indicated by the arrow in Figure 6A. The luminal surface in this region appears to be covered by torn and disrupted endothelial cells (arrow heads). Only a few erythrocytes and platelets are evident in this area (437X). A high magnification view of the luminal surface proximal to the point of insertion of the electrode into the vessel lumen is shown in Figure 6D. The surface is covered by a continuous layer of endothelial cells. A few microvilli (M) are present on the surface of the cells and the cell borders are demarcated by a series of interconnecting ridges (arrows) (2750X). Even with the degree of endothelial trauma seen in these micrographs, fibrin deposition was limited to the site of the electrode

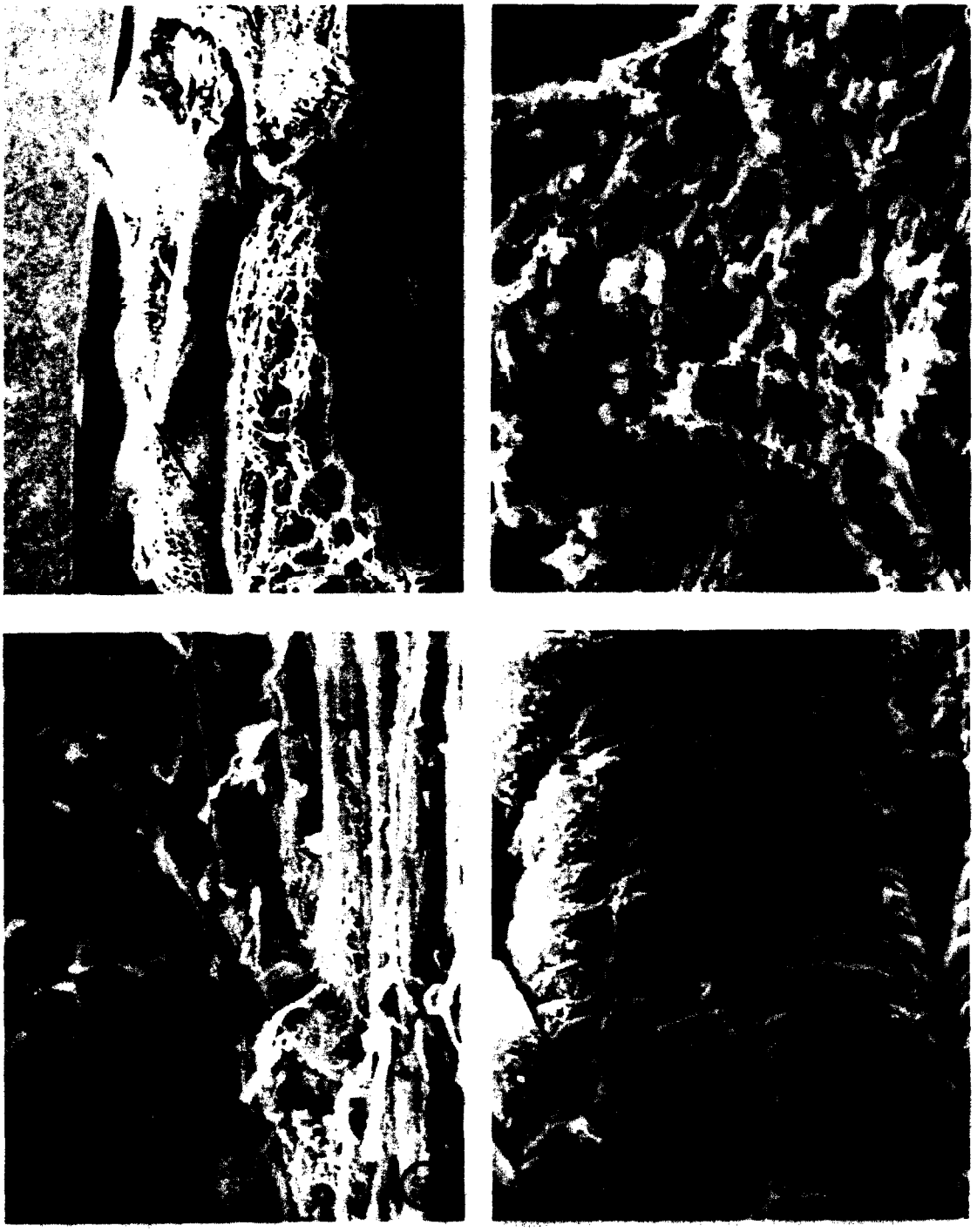
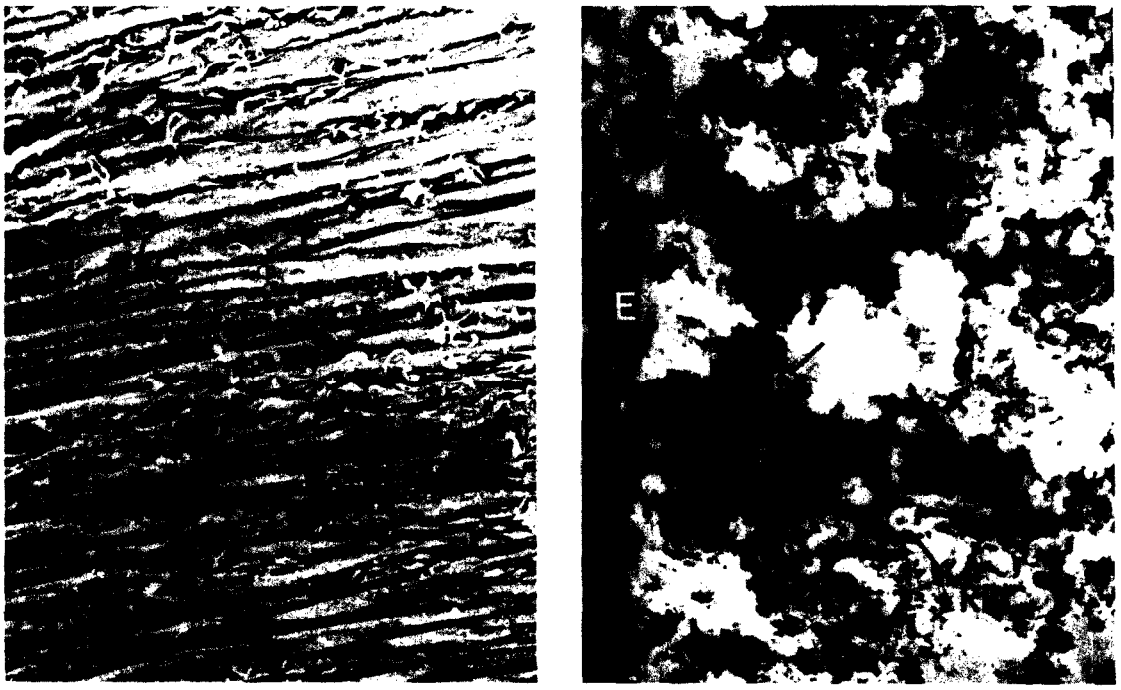


FIG. 6

Scanning electron micrographs of an LCX from a sham operated animal. See text for details.



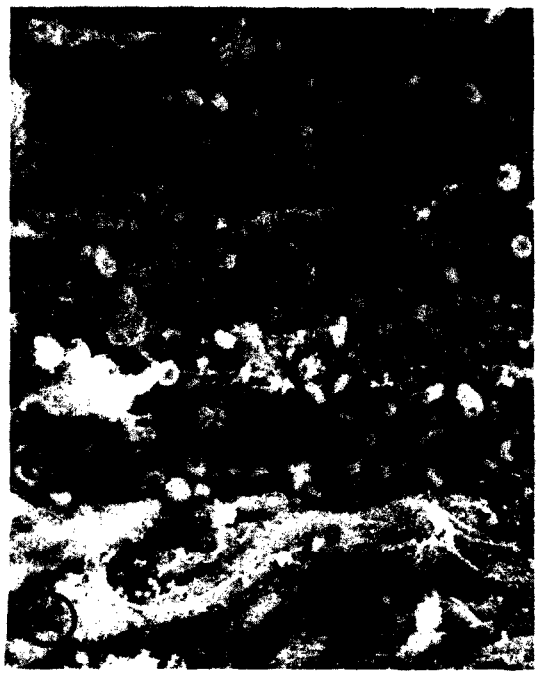


FIG. 7

Scanning electron micrographs of the luminal surface of the LCX from an animal stimulated for 24 hours at 50 μA. 7A. A region of the luminal surface proximal to the site of insertion of the electrode into the LCX. Removal of the distending blood pressure prior to fixation resulted in the surface exhibiting many infoldings. The intimal surface is covered by a smooth layer of endothelium with a few erythrocytes (RBC) and platelets (P) adhering (125X). 7B. A view of the luminal surface adjacent to the indwelling electrode (E). The surface is covered by a rough, fibrous coating (F), erythrocytes (RBC) and large aggregates of platelets (P) (575X). 7C. Distal to the electrode, the luminal surface is covered by a smooth layer of endothelial cells upon which strands of fibrous material (F), erythrocytes (RBC), platelets (P) and non-identifiable clumps of material have been deposited (575X).

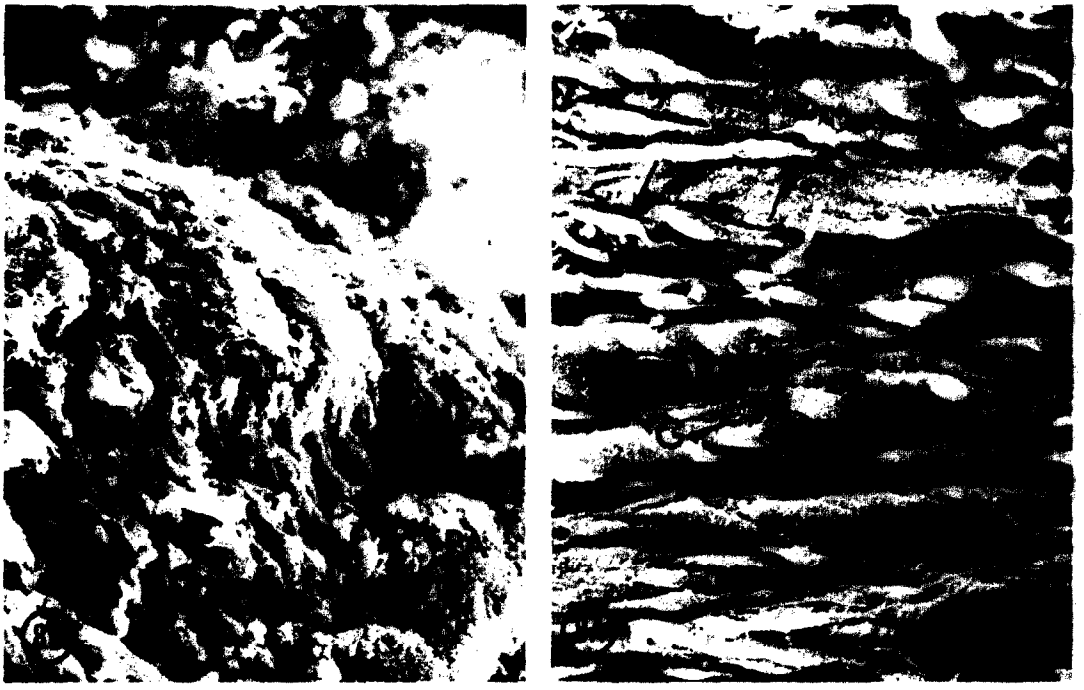


FIG. 8

Scanning electron micrographs of the luminal surface of the LCX from a dog that had been stimulated for 24 hours at 50 μA and received oral Ibuprofen treatment. 8A. Proximal to the electrode, some gaps (G) appear between endothelial cells and a few cells (arrows) appear to have pulled away from the underlying subintima (575X). 8B. In the area of the wall adjacent to the electrode, the endothelium appears to have been completely removed. Some platelet aggregation has occurred (PA) but for the most part the cells and connective tissue components of the tunica media remain free of adhering material (575X). 8E. Distal to the electrode, the intimal surface appears virtually intact. Very few platelets or erythrocytes are present and none of the fibrous material seen in Fig. 7C is evident (575X).

penetration and only a few erythrocytes and platelets were found adhering to the intimal surface (Figure 6C). This indicates that the trauma to the vessel resulting from insertion of the wire is minimal and even after six days, no significant platelet adhesion or spontaneous thrombosis is seen.

Figure 7 shows a series of micrographs of segments of LCX coronary artery taken from an animal which received 50 microamperes of current for 24 hours. Immediately upstream from the point of insertion of the electrode into the vessel lumen, some disruption of the endothelial lining was found (Fig. 7A). However, there was little evidence of platelet adherence in this area. Intracoronary stimulation produced a thrombus, limited to the area of vessel wall in contact with the electrode, consisting of dense aggregates of platelets, erythrocytes and fibrous material. Distal to the electrode, the endothelium appeared intact although greater numbers of erythrocytes and platelets adhered to the intima than in the corresponding area from the sham operated or Ibuprofen treated animals (Figs. 6C and 8C, respectively).

Scanning electron micrographs of an LCX sample from a dog receiving Ibuprofen treatment during the 24 hours of electrical stimulation are shown in Fig. 8. Proximal to the electrode, the endothelium forms a smooth surface layer with occasional gaps between cells. Closer examination of this area reveals a few endothelial cells have pulled away from the subintima with minimal platelet or erythrocyte adhesion to the artery wall. The endothelium was completely removed in the region of the electrode, revealing fibrous connective tissue and smooth muscle cells of the media. Little thrombotic material was evident (Fig. 8B). In contrast to the region shown in Fig. 7C, the intima distal to the electrode was smooth with few platelets adhering to this surface. Thus, even with complete denuding of the endothelium and exposure of subintimal structures, Ibuprofen treatment prevents all but limited platelet and erythrocyte adherence and fibrous material deposition.

DISCUSSION

The model of coronary artery thrombosis described in this report permits in vivo investigation of anti-thrombotic agents in laboratories which were previously unable to do so due to the prohibitive costs of procedures requiring fluoroscopic placement of intra-coronary catheters. The primary advantage of the fluoroscopic technique is that it avoids the surgical trauma involved in the initiation of thrombogenesis by other techniques. However, in order to use fluoroscopic guidance as a means of placing intra-coronary electrodes, the experimental animal must be anesthetized. Although the impact of general anesthesia on platelet-vascular endothelium interactions has not been fully eludicated, the work of Vatner (8) emphasizes the need for recognizing the limitations of cardiovascular data obtained from such studies. The limitations of these approaches led to the development of the present model in which acute trauma from the thoracotomy is minimized by allowing the animal to recover from the surgical intervention before the initiation of thrombus formation. Thus, this protocol approaches the ideals of closed chest models using fluoroscopic techniques by minimizing the impact of surgical trauma as well as eliminating the need for expensive and often difficult to obtain equipment. In addition, it would seem prudent to investigate potentially useful anti-thrombotic agents under conditions which replicate the clinical setting as closely as possible. The use of conscious, ambulatory dogs is an attempt at replicating these conditions.

Unlike other models which induce coronary artery thrombosis by high amperage current of short duration, the present model utilizes continuous low amperage current over a prolonged period, thus producing gradual injury to the intima of the artery. With continued stimulation, the animals are seen to go through a typical pattern of ECG changes generally leading to sustained runs of ventricular tachycardia. An occasional animal will succumb to an episode of ventricular fibrillation. Visual examination of the LCX after 24 hours of stimulation reveals an intra-vascular thrombus with morphology similar to that of naturally occurring thrombi. Histologic techniques indicate that on the average, 25-30% of the left ventricle will infarct as a result of the thrombus development. Finally, scanning elecron microscopy reveals that the mere insertion of the electrode into the lumen of the vessel does not precipitate spontaneous thrombosis. In short, when coronary artery thrombosis is induced in this fashion, the myocardium responds in a manner similar to that seen after naturally occurring coronary stenosis or occlusion. Because the thrombus develops in response to injury occurring over several hours, this experimental model may more closely imitate the naturally occurring thrombotic process. Considering the complex sequence of events involved in platelet-endothelial interactions and thrombus formation, it appears this would be a better model in which to evaluate antithrombotic agents than protocols in which thrombosis is initiated by brief, intense electrical current. The results presented here demonstrate the validity of this technique as a model of coronary artery thrombosis.

The effectiveness of oral Ibuprofen administration in protecting against the deleterious effects of low intensity LCX stimulation emphasizes two points. First, the potential of this model as one in which anti-thrombotic agents may be investigated is underscored by the notable success of altering the thrombotic process with a drug known to inhibit platelet function and thromboxane synthesis. Conversely, the marked beneficial effects of Ibuprofen in this model should stimulate further investigation of this and other proprionic acid derivatives as potentially useful anti-thrombotic agents.

This method is also potentially useful as a model of sudden cardiac death resulting from coronary vasospasm and/or thrombosis. While the incidence of ventricular fibrillation after stimulation of the LCX for 24 hours at 50 microamperes is low, preliminary data suggest that stimulating for longer periods or with higher currents leads more frequently to this fatal event. Consequently, the technique may be useful for investigations of the mechanisms producing, and agents preventing, ventricular fibrillation.

In summary, the method described in this report reliably induces coronary artery thrombosis in conscious dogs by the delivery of low amperage electric current to the intimal surface of the LCX. It has the advantages of eliminating the need for fluoroscopic instrumentation and thrombosis occurring under clinically relevant conditions. It is felt that this type of in vivo model is essential to the thorough evaluation of drugs capable of altering the progression of coronary artery thrombosis.

REFERENCES

1. Coronary Thrombosis in Myocardial Infarction: Report of a Workshop on the Role of Coronary Thrombosis in the Pathogenesis of Acute Myocardial Infarction. Am. J. of Cardiology, 34, 823-833, 1974.

2. HAFT, J.I. Role of Blood Platelets in Coronary Artery Disease. Am. J. of Cardiology, 43, 1197-1206, 1979.

3. SIVOKOFF, M., PURE, E., HSUEH, W., and NEEDLEMAN, P. Prostaglandins and the Heart. Federation Proceedings, 38, 78-82, 1979.

4. SALAZAR, A.E. Experimental Myocardial Infarction, Induction of Coronary Thrombosis in the Intact Closed-Chest Dog. Circulation Research, 9, 1351-1356, 1961.

5. KODENAT, R. K., KEZDI, P., STANLES, E.L. A New Catheter Technique for Producing Experimental Coronary Thrombosis and Selective Coronary Visualization. Am. Heart J., 83, 360-364, 1972.

6. NACHLAS, M. and SHNITKA, T. Macroscopic Identification of Early Myocardial Infarcts by Alterations in Dehydrogenase Activity. Am. J. of Pathology, 42, 379-405, 1963.

7. MILLS, D. and ROBERTS, G. Membrane Active Drugs and the Aggregation of Human Blood Platelets. Nature, 213, 35-38, 1967.

8. VATNER, S. Effects of Anesthesia on Cardiovascular Control Mechanisms. Environmental Health Perspectives, 26, 193-206, 1978.