Inhibitory Effect of Protamine on Action of Intravenous Thromboplastin in Dogs.* (22168)

ELEAZAR SHAFRIR, YECHEZKEL STEIN, AND AHARON BRZEZINSKI.
(Introduced by M. Wolman.)

Department of Clinical Research, Hebrew University-Hadassah Medical School, Jerusalem, Israel.

Slow intravenous infusion of thromboplastin in dogs and other animals produced intravascular clotting resulting in deposition of fibrin on walls of blood vessels(1-7). With prolonged infusion, fibrinogen disappears from blood, while other clotting factors such as prothrombin and proaccelerin (plasma-Ac-globulin, labile factor) are only partially reduced(4,6). Animals recover and regain a normal fibrinogen level in 2-6 days(5-7). Rapid intravenous injection of thromboplastin induces formation of large thrombi near site of injection and causes prompt death of the animals(1,5,6). Attempts have been made with varying success to counteract the effects of thromboplastin *in vivo* with clotting inhibitors such as heparin(1,2), Congo Red, serum(2), soy bean trypsin inhibitor(3), alpha tocopherol phosphate and inositol phosphatide(8), and with thromboplastin splitting enzyme, thromboplastinase(9). Natural basic polypeptide protamine and some synthetic basic poly-amino acids were found recently to have antithromboplastic activity (10-12). They also accelerate clotting of fibrinogen by thrombin(12-14). However, when added to whole blood, coagulation is retarded as their antithromboplastic activity predominates.

The present communication deals with the influence of protamine on defibrinating and lethal effects of intravenous thromboplastin.

Materials and methods. Thromboplastin. This term refers to an extract in 0.9% NaCl of homologous, acetone dehydrated brain prepared according to Ratnoff and Conley(6). The fresh extract was diluted to give clotting time of 13-14 seconds in one stage prothrombin assay of Quick using plasma of dog to be infused. Protamine was obtained from Nutritional Biochemicals Corp. Solutions of 5 mg/ml were made in 0.9% NaCl, pH 7.4. Healthy mongrel dogs of either sex weighing 5 to 17 kg were used. Sodium pentobarbital (3% solution in 0.9% NaCl), was injected intraperitoneally, 1 ml/kg. Anesthesia was maintained during experiments by further doses as required. *Administration of thromboplastin and protamine.* Surgical sterility was not maintained. Femoral artery and vein were exposed on both sides. In slow infusion thromboplastin was introduced into femoral vein from calibrated burette. The first 50 ml were infused at 2-3 ml/min and thereafter at 4-5 ml/min. Rapid injection of thromboplastin was given at approximately 1 ml/sec. At end of experiments, incision sites were sutured and 500000 units penicillin and 0.5 g streptomycin administered intramuscularly daily for 3 consecutive days. Protamine, when given simultaneously with thromboplastin, was infused into opposite exposed femoral vein at 1 ml/min. When protamine was given prior to infusion of thromboplastin it was injected at the rate of 2-3 ml/min. Whole blood clotting time. Blood samples were taken from femoral artery. Two aliquots of 1 ml were put in glass tubes at 37°C. Clotting time of blood in the second tube was recorded. Thrombin clotting time was employed as a quick indication of presence of fibrinogen. One ml of blood was added to glass tube containing 5 units of Thrombin (Upjohn) in 0.1 ml 0.9% NaCl. After careful mixing the tube was put into water bath at 37°C. Blood was considered depleted of fibrinogen if no clot formed during 3 minutes. *Determination of fibrinogen.* One ml blood was collected into solid oxalates(15) and plasma separated by centrifugation. The method of Ware *et al.*(16) was adapted for analysis of fibringen in 0.1 ml of plasma.

Results. Inhibition by protamine of defibrinating effect of thromboplastin. Eight

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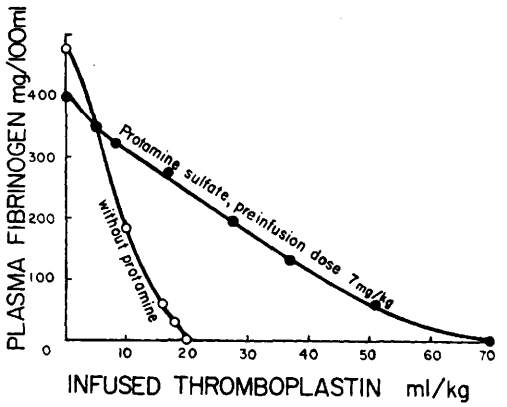

FIG. 1. Representative experiment showing inhibitory effect of protamine administered intravenously prior to infusion of thromboplastin on the decrease of plasma fibrinogen. Mean rate of thromboplastin infusion was 3.5 ml/min. An 8 kg dog was used.

normal dogs were given a slow infusion of thromboplastin, which resulted in a gradual decrease of their plasma fibrinogen level. Whole blood clotting times were elevated to 20-30 min soon after beginning of infusion as compared with 6-8 min in preinfusion samples. When the quantity of infused thromboplastin was near 20 ml/kg, blood became incoagulable. In some dogs a sudden drop in clotting time occurred in samples taken just before total defibrination, presumably due to presence of thrombin in circulation. Some of these samples clotted although they were mixed immediately with oxalate.

Ten dogs received protamine and were divided into 2 groups. The first group of 5 dogs was given 7-10 mg/kg protamine by slow injection; no untoward reactions or changes in plasma fibrinogen level or blood clotting time were observed with amounts and rates of injection employed. Thromboplastin infusion was started 5 min. after injection of protamine. Decrease in plasma fibrinogen level was markedly slowed (Fig. 1) and total defibrination required more than 3 times as much thromboplastin per kg as compared with dogs which did not receive protamine. Blood clotting times were similar to control dogs. Fibrinolysis could not be detected in spontaneously clotted blood at various intervals during thromboplastin infusion into protamine treated and control animals. Nor was lytic activity observed on addition of fibrinogen free blood, obtained at the end of infusion, to a clotted preinfusion sample of the same dog.

In the second group of 5 dogs the fibrinogen level was lowered to nearly half normal value by slow infusion of thromboplastin. Protamine was then infused. Fig. 2 shows, that plasma fibrinogen remained fairly constant during simultaneous infusion of both thromboplastin and protamine. There was no significant decrease in fibrinogen level even after infusion of protamine was terminated and infusion of thromboplastin was continued to a total of 70 ml/kg.

Effect of repeated infusions of thromboplastin and protamine. All defibrinated dogs recovered from the effects of slow infusion of thromboplastin (20 ml/kg) and their plasma fibrinogen returned to normal within a week. Several of these dogs were twice reinfused with thromboplastin at monthly intervals and approximately the same doses were needed for repeated defibrinations.

In another group of previously defibrinated dogs, protamine followed by thromboplastin was given one month later. The amount of thromboplastin (60 ml/kg), required for the second defibrination, was similar to that needed to produce first defibrination of protamine pretreated dogs (Fig. 1). After an-

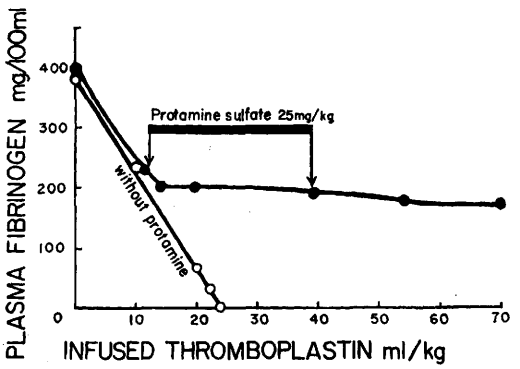

FIG. 2. Representative experiment showing arrest of plasma fibrinogen decrease resulting from administration of protamine to thromboplastin infused dog. Protamine infusion was started when 12 ml/kg thromboplastin had been given and terminated when the quantity of simultaneously infused thromboplastin reached 39 ml/kg. Mean rate of thromboplastin infusion was 4 ml/min. A 10 kg dog was used.

other month when only thromboplastin was infused, the usual dose of 20 ml/kg was again sufficient to render them fibrinogen free. These experiments indicate that repeated infusions did not cause development of either refractoriness or hypersensitivity to thromboplastin and that treatment with protamine did not produce any lasting resistance to the defibrinating action of thromboplastin.

Protecting action of protamine against lethal effect of rapid injection of thromboplastin. Rapid injection of thromboplastin of 5 ml/kg caused almost immediate apnea and death in 10 out of 14 dogs; 2 dogs died during the next 4 hours without recovering from anesthesia, while 2 more survived the injection. An illustrative experiment is described below: Respiration rate of 7 kg female dog under pentobarbital anesthesia was 18/min and pulse 120/min, clotting time 8 min and plasma fibrinogen 380 mg/100 ml. 35 ml of thromboplastin were injected into exposed femoral vein in 40 seconds. At end of injection breathing was rapid and shallow (60/min) then became sporadic and laboured. 75 seconds later breathing ceased and pulse rate was 15/min. At this time, a blood sample showed: clotting time 10 min, fibrinogen 285 mg/100 ml. 135 sec. after injection the heart stopped beating. Pathological examination revealed a blood clot in femoral vein, inferior vena cava, right atrium and ventricle and pulmonary artery, with marked dilatation of right heart. Protamine in doses of 7-10 mg/kg was slowly injected into 14 dogs. Five minutes later the dogs received a rapid injection of thromboplastin as described above. Soon afterwards some showed transient dyspnea, but none died. There was negligible decrease in plasma fibrinogen and recovery of dogs seemed complete. In one of 2 dogs sacrificed 10 days after injection, fibrin clots were found on wall of the right atrium. *Comment.* The present results show that protamine has the ability to counteract the effects of intravenous thromboplastin in dogs. It increases the amount of thromboplastin required for defibrination when injected prior to thromboplastin; it arrests the decrease of fibrinogen when administered simultaneously with thromboplastin and it protects animals against lethal effect of rapid injection of thromboplastin.

Inactivation of thromboplastin by protamine or by synthetic poly-arginine(17) *in vitro* is associated with the formation of a precipitate. This results from a salt-like interaction between basic guanidine residues of numerous arginine groups(18) of protamine and the acidic phospholipid components of thromboplastic protein(19). It may be assumed that a similar interaction of protamine with thromboplastin takes place *in vivo*.

Inhibitory effect of protamine infusion on intravenously administered thromboplastin suggests its application in clotting disorders which involve entry of thromboplastic material into the blood stream. Such a mechanism has been supposed to be the cause of afibrinogenemia in abruptio placentae(1,20), in amniotic fluid embolism(21,22) and in some cases of intrauterine death(23,24).

Summary. 1. Intravenous administration of protamine markedly increases the amount of slowly infused thromboplastin required for defibrination of dogs. Protamine given during infusion of thromboplastin, to a dog already partially depleted of fibrinogen, arrests further decrease in plasma fibrinogen level. 2. Intravenous administration of protamine protects dogs from the lethal effect of subsequent rapid injection of thromboplastin. 3. Mode of action of protamine and possible clinical utilization of its antithromboplastic properties are discussed.

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Variation in Reduced Glutathione in Bloods of Inbred Lines of Chickens.* (22169)

ENSEL C. STUTTS, WARREN JOHNSON, W. E. BRILES, AND H. O. KUNKEL.

Departments of Biochemistry and Nutrition, Genetics and Poultry Husbandry, Texas Agricultural Experiment Station, College Station.

There can be no doubt that the reduced glutathione (GSH) levels of blood and other tissues are influenced by the adrenal, thyroid, pituitary, pancreatic, and parathyroid hormones(1). There is a paucity of data, however, which evaluate the extent that activities of endocrine glands are under hereditary control and, therefore, the extent that variations in genetically controlled activities of endocrine glands regulate chemical activities of the cells.

Proof of genetic control of vital biochemical reactions in living organisms has been demonstrated principally by results of studies conducted on monoploid microorganisms(2,3). In higher animals, such evidence has resulted from studies of physiological abnormalities, primarily in the human being, from studies of pigment development and body coloration, or the presence or absence of a chemical constituent such as atropinase or a β-globulin in the sera(4-8). Williams *et al.*(9) have suggested a quantitative individuality in metabolism of humans and rats on the basis of variation in patterns of excretion of metabolites in urine. Breed differences in plasma or serum alkaline phosphatase in chickens(10), in thyroxine secretion rate of chickens(11,12), and in total body glutathione concentration in 2- to 14-day-old chicks(13) lay a broad basis for the study of biochemical genetics in chickens, but the majority of such studies has not dealt with these biochemical factors as bases for biological individuality.

Genetically controlled individual quantitative differences in biochemical constituents should tend to become fixed by inbreeding and, as a result of this increase in intra-line genetic uniformity, differences between inbred lines become more easily demonstrable. With availability of inbred lines of chickens within a single breed (White Leghorn) in our

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