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The effects of age and aspirin on porcine platelet function

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The effects of age and aspirin on porcine platelet function

Matthews, James A., Ph.D.

Iowa State University, 1987

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The effects of age and aspirin on porcine platelet function

by

James A. Matthews

A Dissertation Submitted to the

Graduate Faculty in Partial Fulfillment of the

Requirements for the Degree of

DOCTOR OF PHILOSOPHY

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              (Veterinary Clinical Pathology)

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For the Graduate College

Iowa State University
Ames, Iowa

1987

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GENERAL INTRODUCTION

Platelets are small cytoplasmic fragments released from megakaryocytes and found in relatively large numbers in the peripheral blood. Platelets function in hemostasis, help maintain vascular integrity, enhance coagulation and participate in the inflammatory response.[1]

Platelet disorders can be classified as inherited or acquired and include thrombocytopenia, thrombocytosis, thrombasthenia, idiopathic thrombopathies and thromboembolic disease. Von Willebrand disease (vWD), which is a clinical syndrome secondary to a deficiency in functional von Willebrand factor (vWf), has the clinical presentation of a platelet disorder.[2]

The domestic pig *(Sus scrofa domesticus)* is a well recognized model of cardiovascular and bleeding disorders[3,4] including immune-mediated neonatal[5] and viral-induced thrombocytopenias,[6-8] bacterial-induced thrombotic disease,[9-12] hereditary thrombopathy[13] and von Willebrand disease.[14,15]

The objectives of these studies were 1) to determine selected age-related normal values for platelet numbers and platelet aggregation curves in pigs, 2) to examine the effects of oral aspirin, a potent inhibitor of arachidonic acid metabolism, on porcine platelet function and 3) to

determine if aspirin pretreatment would modify the effects of intravenous collagen infusions in anesthetized piglets.

Explanation of the dissertation format

This dissertation is presented in the alternative format as 3 manuscripts written in the style of the American Journal of Veterinary Research. A review of the literature precedes the first manuscript. The first manuscript has been submitted to the American Journal of Veterinary Research. A general summary follows the last manuscript. Each manuscript has a list of references while literature cited in the general introduction, literature review and general summary appears at the end of the dissertation. In addition, three appendices offer additional research information pertinent to the dissertation but not included in the manuscripts.

The Ph.D. candidate, James A. Matthews, was the principal investigator in each of the experiments and is listed as the first author on each of the manuscripts. Dr. Arlo E. Ledet was instrumental in the planning and review of each of the experiments. He also facilitated the use of the veterinary clinical pathology laboratory at the Iowa State University Veterinary College. Research pigs were generously provided by Dr. Lawrence E. Evans. Dr. Evans also was generous with his time and provided invaluable

instruction concerning animal handling and blood sampling techniques. The last experiment was performed in Dr. Richard L. Engen's research laboratory under his direct supervision and with his help.

LITERATURE REVIEW

There is a large body of information concerning platelets but only a relatively small number of studies using porcine platelets. Several explanations for this discrepancy can be advanced. The most obvious reason is that research opportunities follow funding and human diseases have priority over diseases of swine. Furthermore, human platelets are readily available from both volunteers and blood banks. Platelet-rich samples can be drawn repeatedly from the same donor with only a minimum of discomfort and at very small risk. Additionally human subjects apparently readily agree to ingest drugs (i.e., aspirin, indomethacin and alcohol) that affect platelet function.

Pigs are large malodorous vociferous animals that can violently resist blood collection. As research animals they require feed, water, housing and occasional veterinary care. In addition pigs are generally more expensive than other common laboratory animals (rabbits, rats, cats, guinea pigs and even mongrel dogs).

Pigs, however, share many functional and anatomical similarities with man. Atherosclerosis and von Willebrand disease are two naturally occurring diseases in pigs which parallel human diseases.[3,4]

The understanding of porcine platelet physiology and its role in the pathogenesis of swine diseases has intrinsic as well as possible economic value. The United States Department of Agriculture, in *Agriculture Statistics 1986*, estimated the total value of 1985 U.S. hog production at 8.86 billion dollars. This represented a total U.S. herd size of 52.3 million hogs and pigs: less than 8% of world hog numbers.

Platelet numbers

Megakaryocytes, large granular cells containing a single multilobed nucleus, are found primarily in the bone marrow although they also occur in the lung and less frequently in the spleen, liver and lymph nodes. Platelets are cytoplasmic fragments of megakaryocytes. Thrombopoiesis is regulated by thrombopoietin which in turn is controlled by the circulating platelet mass. In health it is estimated that approximately one-third of an animal's platelet mass is sequestered in the spleen where they can be rapidly mobilized.[16]

Reported blood platelet counts in pigs vary markedly.[17-22] A portion of the difference may be breed related differences. The breeds represented in these reports include the White/Wessex cross,[17] Chester White,[18] Berkshire,[18] Hampshires[19] and Duroc[19] breeds, Norwegian[20] and Nigerian[21] pigs and miniature swine.[22]

Platelet counts in the pig may also be age dependent. While the cited reports represent pigs between 1 day[19-21] and 42 months of age[22] a general pattern is evident. Mean blood platelet counts rise from birth, peak at 10 days to 2 weeks of age and then gradually decrease from 6 weeks of age down to stable adult values by 9 months.[20-23]

Some properties of porcine platelets

Porcine platelets are very similar to platelets in other species including man. Porcine platelets are small (1 to 4 micron, 6.1 to 8.7 fl)[24,25] nonnucleated cell fragments that appear granular in Romanowsky stained blood smears examined by light microscopy.

Transmission electron microscopic examination of platelets has revealed two major types of granules: dense granules and α granules. Porcine platelet dense granules contain adenosine diphosphate (ADP) and adenosine triphosphate (ATP), serotonin (5-hydroxytryptamine) and the bivalent cations magnesium and calcium.[26,27] The blood platelet nucleotide content and the ATP:ADP ratio of porcine platelets is very close to that of man.[28,29] The α granule is generally more numerous than the dense granule and contains proteins with antiheparin activity, von Willebrand factor, fibrinogen, fibronectin and platelet derived growth factor.[26,30,31] The heparin neutralizing ability of porcine

platelets is reportedly greater than that of human platelets.[26] Two porcine platelet heparin-binding proteins, porcine platelet factor 4 and porcine platelet basic protein have been identified. The amino acid composition of porcine platelet factor 4 is similar to human platelet factor 4 and the two proteins have significant immunologic cross reactivity. Porcine platelet basic protein shares immunologic reactivity with two human platelet proteins that bind to heparin with low affinity: β-thromboglobulin and low affinity platelet factor 4.[32] The porcine platelet membrane contains 3 major glycoproteins which are similar to the 3 major glycoproteins of the human platelet membrane.[33]

Platelets, in all mammalian species, respond sequentially to various stimuli, or agonists, by first changing shape and then aggregating.[26] Aggregation can be monitored in the laboratory by turbidometric methods as a way of testing platelet function.[34] Comparative studies into platelet aggregation have found that the extent and pattern of aggregation induced by an agonist varies markedly between species.[26,35-41] This variance is further confounded by variations within species,[42,43] by variations due to age[44-46] and, most remarkably, by variations in the type of response from a single platelet rich plasma (PRP) sample to different concentrations of a single agonist.[47]

Quantitative turbidometric studies of platelet aggregation began with a 1962 report by Born using "pig's plasma...containing 3.6 x 10^8 platelets/ml". Born also noted that platelet aggregation could occur without clot formation.[48] Later it was shown that porcine platelet aggregation is dependent on the presence of divalent cations and fibrinogen.[49,50]

Platelet aggregation in man may occur through the activation of 2 or 3 related pathways which are triggered by a variety of stimuli.[51,52] The mechanism of platelet aggregation in animals is not as well defined. Porcine platelets will aggregate when exposed to many of the same agonists that trigger human platelet aggregation but, as previously suggested, there are some major differences.

ADP stimulates either reversible monophasic or, with higher concentrations, irreversible biphasic platelet aggregation in human PRP.[47] ADP stimulates only reversible monophasic platelet aggregation in porcine PRP.[38-40] Meyers incorrectly states that this aggregation is irreversible.[26] Platelets from both humans and pigs are inhibited by prior incubation with adenosine, adenosine monophosphate (AMP) or prostaglandin E$_1$.[26,37,39,40] Adult human platelets will respond with biphasic irreversible aggregation to epinephrine.[26,39] Porcine platelets neither respond to

epinephrine nor does epinephrine potentiate their response to other agonists the way it does in human and canine PRP.[26,39] Serotonin, however, potentiates ADP-induced aggregation in man and pigs.[39] Collagen and low concentration thrombin induce irreversible platelet aggregation in both man and pigs.[37,49,50] Arachidonic acid concentrations adequate to induce irreversible platelet aggregation in human PRP only induce shape change in porcine platelets.[26] The pig platelet, in this instance, more resembles the rat than the human platelet.[53] Finally, snake venom coagglutinin causes von Willebrand factor dependent platelet aggregation in man and in pigs.[54,55]

Platelet related disorders in pigs

Von Willebrand disease (vWD) is a spontaneously occurring disease which affects dogs, man and pigs.[14,15,56-58] In man, vWD occurs as either a quantitative decrease or an abnormality in von Willebrand factor (vWf)[59-61], a large multimeric molecule which is also known as factor VIII:related antigen (VIIIR:Ag).[62] This factor is synthesized by endothelial cells[63-66] and megakaryocytes[67] and can be immunologically localized to plasma, platelets, megakaryocytes and endothelial cells.[59,68-70] Although vWD is not a primary defect of platelets vWf is necessary for platelet adherence to the subendothelium and the formation

of the primary hemostatic plug.[71-73] Porcine vWD is
transmitted as an autosomal recessive trait.[58] The major
focus of porcine vWD research has been directed at the role
of the platelet-blood vessel interaction in the development
of atherosclerosis.[74-80] Pigs with severe vWD are resistant
to diet induced and spontaneous atherosclerosis due to a
reduced interaction between platelets and damaged
endothelial surfaces. In other words, the normal platelet
mediated vessel repair process may be a "trigger" for the
development of atherosclerosis.[80] Born has stated, in no
uncertain terms, that platelets do not contribute to
atherosclerosis by damaging normal arterial endothelial
surfaces.[81]

An unusual bleeding disease in pigs has recently been
described. These affected pigs are part of a colony of vWD
research pigs at the Mayo clinic research facilities. The
clinical signs (bleeding times markedly increased), reduced
collagen stimulated platelet aggregation response and a
decrease in platelet dense granule numbers and contents
(ADP, ATP and serotonin) suggest a platelet storage pool
deficiency. This thrombopathy is believed to be an
inherited platelet defect transmitted in an autosomal
recessive manner.[13]

Neonatal thrombocytopenic purpura occurs in piglets of multiparous sows who have been immunized by fetal antigens from the boar. The primary clinical signs are petechial hemorrhages, excessive bleeding times and extensive bruising.[5]

The thrombocytopenia associated with African swine fever may also be immune related. While the exact mechanism is still unclear, pigs innoculated with an African swine fever isolate became thrombocytopenic on days 6 to 8 post innoculation concurrent with the appearance in serum of antiviral antibodies.[8]

Iron deficiency in piglets is reported to cause a thrombocytosis. Copper deficiency apparently does not affect platelet numbers.[18,21,23]

Thrombotic disease in connection with various bacterial infections is very probably the most common platelet related disorder found in pigs. Both renal and pulmonary thrombi are described with induced *Salmonella, Hemophilus* and *Erysipelothrix* infections in pigs.[9-12]

Morphologic and functional platelet changes have been described in porcine stress syndrome susceptible pigs. The significance of these changes, a dilation of the open canalicular system and a decreased aspirin inhibition of in vitro collagen-induced platelet aggregation, and their relationship to the syndrome are not known.[82,83]

Thromboembolic pulmonary disease, resembling adult respiratory distress syndrome (ARDS) in people, has been induced in anesthetized pigs by shooting them in the hind leg with a rifle. Pulmonary trapping of platelets and fibrin was monitored by external detection of radioactivity.[84]

Platelet function is impaired in alcoholic patients.[85] Anesthetized pigs given ethanol through a gastric tube, however, had increased numbers of circulating microaggregates in the vena cava and in the portal vein both before and after standardized trauma (200 blows with a rubber hammer on the inside of each thigh). This trauma was not lethal to control pigs while 6 of 7 pigs pretreated with alcohol died. The authors of this report felt that the mortality followed an alcohol suppressed ability to maintain circulating volume rather than increased pulmonary microaggregate trapping.[86]

Aspirin, arachidonic acid and pigs

Aspirin, acetylsalicylic acid, is a nonsteroidal anti-inflammatory drug that inhibits the cyclo-oxygenase enzyme in platelets.[87,88] This inhibits the formation of thromboxane from membrane derived arachidonic acid for the life time of the platelet.[89-91] An excellent review of the effects of aspirin on platelet physiology has been recently published.[92]

Aspirin also inhibits the formation of prostacyclin from arachidonic acid by endothelial cells.[91] Although endothelial cyclo-oxygenase activity is rapidly restored after withdrawal of aspirin[93] there has been concern that high dose aspirin therapy may be thrombogenic. One study in man reported that a single low dose oral aspirin treatment (0.3 g) increased the bleeding time when compared against both the control and the high dose (3.9 g) treatment groups.[94]

The concept that high dose aspirin may be thrombogenic has been attacked from two directions. First, the role of prostacyclin in the maintenance of vascular patency has not been established. Arthritis patients at the Mayo Clinic on prolonged high dose aspirin therapy did not have an increased rate of thrombosis. On the contrary, the aspirin treated male group had a decreased incidence of thrombotic events.[95] The second stronger argument suggests that salicylic acid, the breakdown product of acetylsalicylic acid, interferes with the platelet inhibitory effects of aspirin. In man salicylate has a longer plasma half-life (4-5 hours) than aspirin (0.25-0.33 hours) so a dose of aspirin may partially inhibit the effects of a subsequent aspirin dose.[96]

Interestingly, while salicylate does not suppress arachidonic acid-induced platelet aggregation[96] it is still a potent anti-inflammatory drug.[97] Apparently salicylate, like aspirin, exhibits its anti-inflammatory action by inhibiting prostaglandin E_2 synthesis.[98]

While aspirin has been used in a wide variety of inflammatory and thrombotic diseases in man and animals[91-93,99-101] it has had very limited use in the pig. Aspirin has been reported to reduce the incidence and severity of atherosclerotic lesions in pigs following induced intravascular trauma (repeated balloonings) and in pigs fed atherogenic hyperlipidemic diets.[102,103] Similarly platelet deposition and mural thrombus formation following balloon angioplasty was reduced in pigs pretreated with aspirin alone or in combination with another drug.[104,105]

Aspirin, 20 mg/kg, administered intravenously 15 minutes before challenge with heterologous erythrocytes prevented severe shock reactions in 5 sensitized pigs while severe shock reactions, usually fatal, occurred in 9 of 14 untreated controls.[106]

Unfortunately oral aspirin has been shown to cause severe gastric mucosal damage in pigs given 30 mg/kg for 10 days.[107] The development of the mucosal lesions coincided with the inhibition of mucosal prostaglandin and prostacyclin production.[108]

EXPERIMENT I

The effect of age on in vitro porcine platelet aggregation

James A. Matthews, D.V.M., M.S.

Arlo E. Ledet, D.V.M., Ph.D.

Lawrence E. Evans, D.V.M., Ph.D.

SUMMARY

Platelet counts in whole blood and platelet aggregation responses to adenosine diphosphate (ADP), collagen and sodium arachidonate were measured in three age groups of pigs: neonates, 3 to 6 week old piglets and adult gilts and sows. Mean platelet counts were significantly ($P<0.05$) higher in the 3 to 6 week old group than in the neonates which were also significantly higher than the mean adult platelet count. Neonatal platelet response to ADP and collagen was similar to the adult platelet response. Sodium arachidonate induced reversible platelet aggregation in 8 of 20 neonates tested but induced only irreversible aggregation in the adult pig platelet samples. The 3 to 6 week old piglet platelet showed the greatest aggregation response to ADP and collagen but a slightly decreased aggregation with sodium arachidonate when compared with the adult pigs.

INTRODUCTION

Platelet aggregation can be induced in the laboratory by specific platelet aggregation agents and recorded with a platelet aggregometer. The aggregation tracing represents an increasing transmittance of light as a cloudy suspension of platelets in plasma becomes a clear plasma containing a few large platelet aggregates. Analysis of this tracing can be use to quantitate the delay between the addition of an aggregating agent and the first measurable increase in transmittance (the lag phase), the rate of change in transmittance as aggregation progresses (the slope) and the maximal percent transmittance (maximal aggregation). The overall pattern of the tracing can indicate if the induced aggregation represents primary aggregation, secondary aggregation or a combined diphasic aggregation.[1]

The purpose of the present study was to examine the response of the porcine platelet at three different ages which are potentially associated with increased hemostatic stress: neonatal (birth), 3 to 6 weeks of age (castration) and adulthood (parturition).

MATERIALS AND METHODS

Pigs-Sixty healthy Hampshire-Landrace-Duroc cross pigs, 20 in each age group, were used. Neonates, 10 of each sex, were bled at 2 days of age. Twenty male piglets were bled between 3 and 6 weeks of age. Twenty mature gilts and sows formed the adult group. All pigs were pseudorabies and rhinitis free and maintained indoors at Iowa State University. The pigs were drug free prior to and during sampling. Piglets had received a single subcutaneous injection of iron dextran at 3 days of age.

Platelet aggregation-Blood from the anterior vena cava (sows) or the periorbital venous sheath (neonates and piglets) was collected from physically restrained unanesthetized pigs into plastic syringes or plastic tubes containing 3.8% sodium citrate at a ratio of 9 volumes of blood to 1 volume of anticoagulant.

Platelets were counted manually using a dilution system[a] and light microscopy.

Undiluted citrated blood was transferred to capped polypropylene centrifuge tubes and centrifuged (600 x g for 2.0 min at 20° C) to obtain platelet rich plasma (PRP). The PRP was diluted with platelet poor plasma (PPP:1300 x g for 20 min at 20° C) to obtain a platelet concentration of

approximately 200,000 platelets/μl. This adjusted PRP was held at room temperature in tightly capped polystyrene tubes until testing. All tests were completed within 3 hours of sample collection.

Platelet aggregations were performed in adjusted PRP according to the turbidometric method of Born.[2] Samples were maintained at 37° C for 3 min before the addition of the aggregating agent. Adenosine diphosphate[b] (ADP), collagen[c] and sodium arachidonate[b] were used as the aggregating agents. Adenosine diphosphate was used as a 200 μM stock solution giving a final concentration of ADP in the PRP of 20 μM. Collagen from bovine tendon was used in a final concentration of 10.0 μg/ml. Sodium arachidonate was diluted with normal saline to a final concentration of 6.9 mM. Platelet aggregation was recorded over a 5 minute period using a multichannel platelet aggregometer[c].

Measured parameters were the maximum % aggregation where PRP was set as 0% transmission and PPP was 100% transmission and the slope as the initial % change in transmittance per minute. The lag time between the addition of collagen and the point in which measurable aggregation occurred was also measured. In addition, aggregation curves were examined for overall pattern.

Plasma cholesterol values were determined on 5 pigs from each age group using a routine spectrophotometric technique[d].

All aggregations were performed in duplicate and the mean result was calculated. Group data are expressed as the mean ± the standard error of the mean (SEM). An analysis of variance (ANOVA)[e] was performed to detect age differences in the measured aggregation parameters for all three aggregating agents. When the ANOVA indicated that an age effect was present least significant differences were calculated to determine which means were significantly different from one another. A significance level of 0.05 was maintained throughout the study.

RESULTS

There was considerable overlap in the range of the platelet counts between the 3 age groups however mean platelet counts rose significantly from 2 days of age to 3 to 6 weeks of age then subsided to the adult values (Table 1).

Adult pig platelets aggregated with sodium arachidonate, collagen and, to a lesser degree, ADP. Addition of sodium arachidonate resulted in either a single or diphasic curve representing an irreversible aggregation. Collagen-induced a single wave of irreversible aggregation while ADP caused a spontaneously reversible aggregation (Fig 1).

Neonatal piglet platelet maximum aggregations induced by collagen and ADP were similar to adult porcine platelet responses (Table 2). Sodium arachidonate, however, induced an attenuated response in 8 (5 females and 3 males) of the 20 neonatal porcine samples with a single wave of reversible aggregation (Fig 2). A biphasic response to sodium arachidonate was not observed in this age group.

Platelet samples obtained from the 3 to 6 week old piglets responded at or above adult values with the single exception of a decreased lag phase following the addition of

TABLE 1. Normal porcine platelet numbers in sodium citrate
          anticoagulated whole blood (9 parts blood:1 part
          3.8% sodium citrate)

Group[a]	n	Platelets x $10^3/\mu l$ [*] (mean±SEM[b])	Range (platelets x $10^3/\mu l$)
Adult	12	275.2±26.2	160-474
Piglet	18	565.3±21.4	263-740
Neonate	21	408.4±19.8	264-562

[a]Adult gilts and sows, 3 to 6 week old piglets and
2 day old (neonatal) piglets.

[b]SEM = Standard error of the mean.

[*]Each age group differs significantly ($P<0.05$) from
each other group.

FIGURE 1. Typical adult pig aggregation tracings
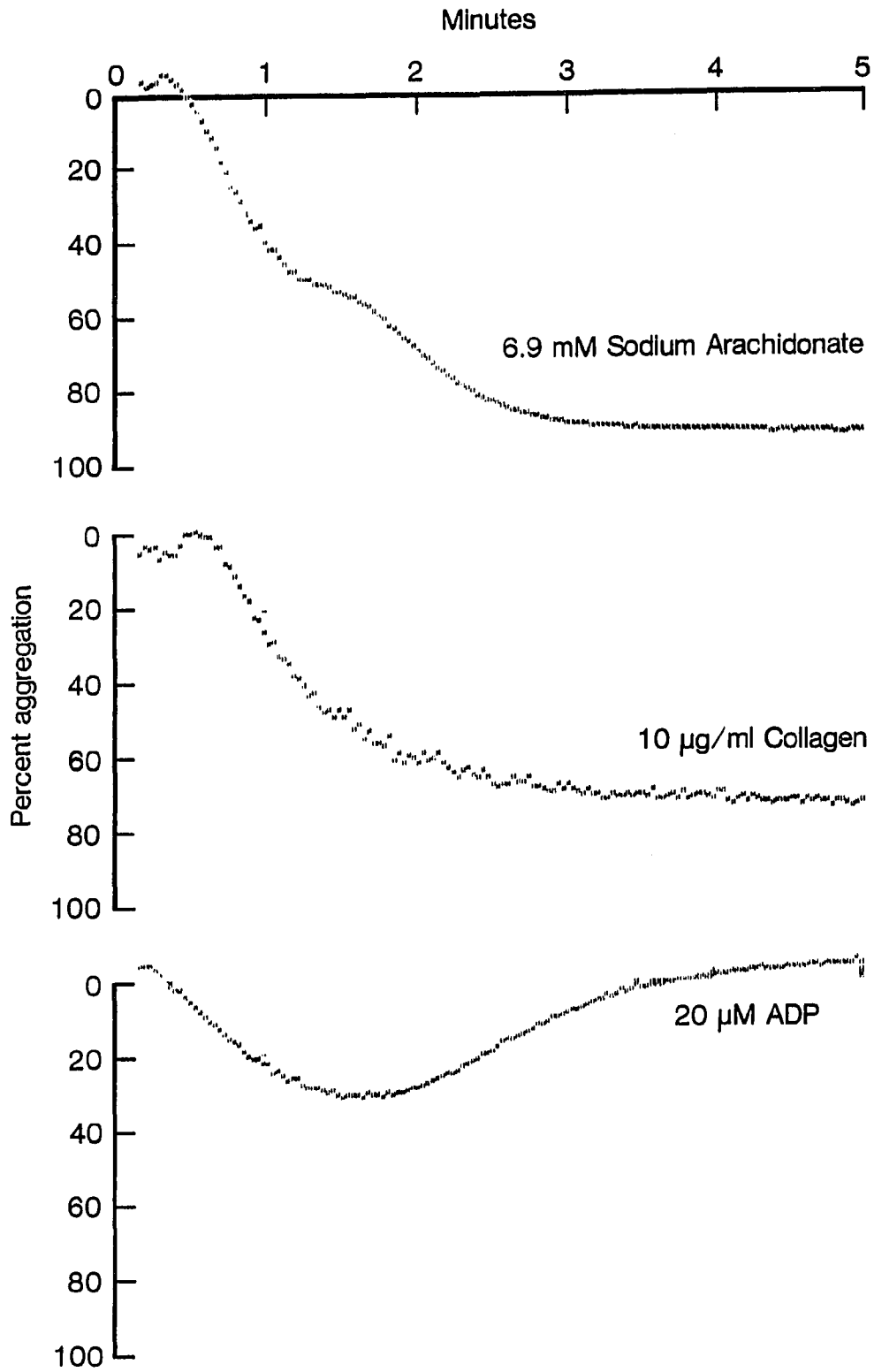
TABLE 2.   Platelet aggregation parameters in adult gilts and sows, 3 to 6 week old piglets and 2 day old (neonatal) piglets

Group	Aggregation Reagent		
	Sodium arachidonate (6.9 mM)	ADP (20 μM)	Collagen (10 μg/ml)
Adult			
Maximum aggregation (%)	89.4±3.6[a]	32.4±2.9	75.7±1.5
Slope (% change/min)	79.6±18.6	36.4±3.8	71.6±4.1
Lag (seconds)	....	....	41.6±1.2
Piglet			
Maximum aggregation (%)	80.7±3.6	44.4±2.9[*]	81.5±1.5[*]
Slope (% change/min)	185.9±18.6[*]	63.0±3.8[*]	105.7±4.1[*]
Lag (seconds)	....	....	37.7±1.2[*]
Neonate			
Maximum aggregation (%)	58.1±3.6[*]	37.4±2.9	73.0±1.5
Slope (% change/min)	111.9±18.6	55.5 ±3.8[*]	84.2±4.1[*]
Lag (seconds)	....	....	39.1±1.2

[a]Data are expressed as mean values ± the standard error of the mean.   Each group consisted of 20 pigs.

[*]The value differs significantly ($P<0.05$) from the adult value.

FIGURE 2.   Atypical neonatal piglet platelet aggregation
            tracing induced by 6.9 mM sodium arachidonate
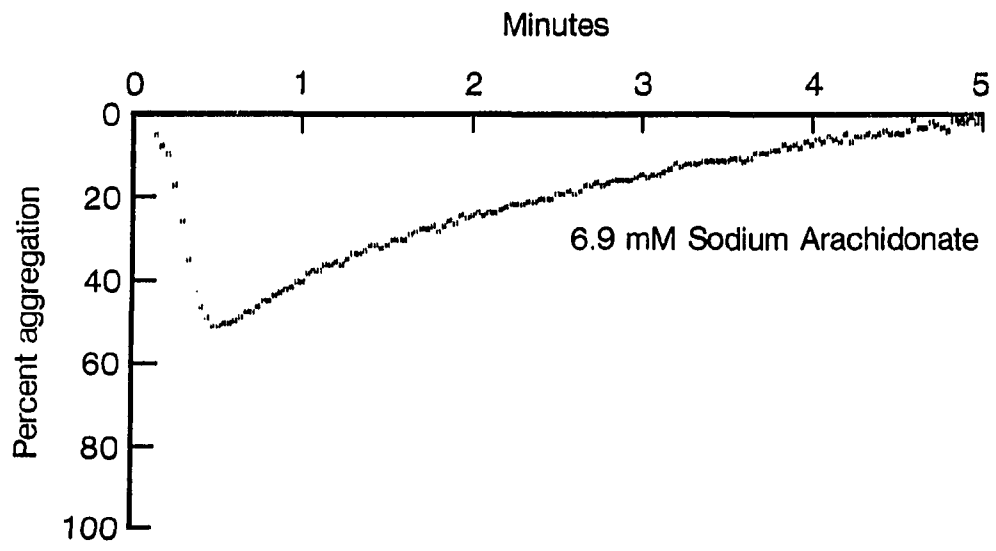
collagen (Table 2). Only 2 of 20 piglets in this age group exhibited a reversible platelet aggregation response to the addition of sodium arachidonate.

Mean cholesterol concentrations were higher in the neonatal serum (mean=101 mg/dl) than in either the adult gilt and sow (mean=80 mg/dl) or the 3 to 6 week old serums (mean=85 mg/dl). There were, however, no significant differences among the 3 groups.

DISCUSSION

Reported porcine whole blood platelet numbers vary markedly.[3-5] Platelet numbers reported here correspond with reported values in miniature and Nigerian pigs.[6,7] Platelet numbers increased from the relatively thrombocythemic levels shortly after birth to the very high levels at weaning before decreasing to adult values.

Newborn human infant platelets exhibit decreased responsiveness to ADP and collagen and no response to epinephrine. Arachidonic acid-induced platelet aggregation in the human infant is comparable with that in adults.[8,9] Similarly when the newborn foal is compared to the mare the newborn foal has reduced platelet responsiveness to ADP and collagen and a normal response to arachidonic acid.[10] Epinephrine does not induce platelet aggregation in the foal, mare or pig.[11]

In the present study the neonatal porcine platelet maximal aggregation response to ADP and collagen was not significantly different from that of the adult platelet. This suggests that the neonatal pig platelet is relatively more mature than either the human infant or the newborn foal platelet. The 3 to 6 week old porcine platelet appears to be relatively hyperaggregable when induced with ADP and

collagen. Diet has been reported to affect thrombus formation in pigs. In that study serum cholesterol had a significant positive correlation with thrombus formation in an extracorporeal shunt.[12] Diet was not controlled in this study but a retrospective evaluation of blood cholesterol values among the 3 age groups failed to reveal a significant difference.

Platelet aggregation responses may be dependent on the plasma concentration of the anticoagulant which varies with the packed cell volume.[13] None of the pigs used in this study showed clinical evidence of anemia. Packed cell volumes were not measured in this study.

Human platelet aggregation is apparently mediated by 2 or 3 interrelated pathways.[14] In one of these pathways arachidonic acid, a cell membrane fatty acid, is mobilized in response to the action of a variety of agonists and rapidly converted by the cyclo-oxygenase enzyme complex to intermediate cyclic endoperoxides and then to thromboxane by thromboxane synthetase. Arachidonic acid metabolism in the human neonatal platelet differs from that of adults in that thromboxane formation is decreased while leukotriene formation through the alternative lipoxygenase pathway is increased. Increased release of substrate arachidonic acid from human neonatal platelet membranes counterbalances this

relative decrease in thromboxane formation so that the overall conversion of arachidonic acid to thromboxane is similar in adult and neonatal platelets.[15] In this study neonatal porcine platelets reacted one of two ways to sodium arachidonate. One group reacted similarly to adult gilts and sows with irreversible aggregation. The second group, however, exhibited a primary reversible aggregation response. This suggests that either thromboxane synthesis is imperfectly developed in neonatal pigs and that the aggregation response may be a direct action of sodium arachidonate (minimal nonspecific platelet lysis with the release of ADP) or due to an intermediate aggregatory prostaglandin (PGG_2 and PGH_2).[16]

LETTERED FOOTNOTES

a. Unopettes, Becton-Dickson and Co., Rutherford, NJ.

b. Sigma Chemical Co., St. Louis, MO.

c. Helena Laboratories, Beaumont, TX.

d. Gilford Systems, Oberlin, OH.

e. ANOVA, SAS Institute Inc., Cary, NC, 1986.

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EXPERIMENT II

The effect of aspirin on porcine platelet function

James A. Matthews, D.V.M., M.S.

Arlo E. Ledet, D.V.M., Ph.D.

SUMMARY

Four levels of aspirin treatment (0, 1, 20, 100 mg/kg) were given daily for 2 days to four groups of 5 piglets. Complete blood counts, clot retraction, factor VIII:related antigen concentration, bleeding time and platelet aggregation were measured 24 hours before the first and 24 hours after the last aspirin treatment.

Aspirin treatment did not significantly alter the cell counts, the degree of clot retraction, the concentration of factor VIII:related antigen or the bleeding time. Platelet aggregation induced by collagen was decreased with maximal suppression of platelet aggregation being evident in the 20 mg/kg dosage group. Aggregation induced by adenosine diphosphate and high dose sodium arachidonate was not significantly changed by aspirin treatment. These observations suggest that aspirin does suppress platelet function in the pig but that the degree of suppression is mild.

INTRODUCTION

Aspirin (acetylsalicylic acid) has been recommended as an anti-inflammatory agent in animals including the pig.[1,2]

In addition to its analgesic and antipyretic properties aspirin irreversibly inhibits platelet function by acetylation of the cyclo-oxygenase complex thereby inhibiting the conversion of arachidonic acid to the prostaglandins and thromboxane.[3] Aspirin inhibition of platelet function has been utilized in malignant hyperthermia and comparative vascular research in pigs.[4-8]

It has been demonstrated that the porcine platelet generates less thromboxane than equal numbers of human or canine platelets under the same stimulation.[9,10] This suggests that aspirin may not be as potent an inhibitor of platelet function in pigs as in other species.

This project will examine the effects of various dosages of oral aspirin on porcine platelet function.

MATERIALS AND METHODS

Pigs-Twenty piglets (4 to 7 weeks old, 6 to 15 kgs) of either sex were used. These pigs are part of a pseudorabies and rhinitis free herd maintained indoors at Iowa State University.

The piglets were evenly divided into 4 groups: a control group (no aspirin), a low dose group (1 mg/kg body weight for 2 consecutive days), a high dose group (20 mg/kg for 2 consecutive days) and a super-high dose group (100 mg/kg for 2 consecutive days).

Sample Collection-Piglets were anesthetized to allow for ease of venipunctures and bleeding time measurements. An intramuscular (IM) injection of azaperone[a] (5 mg/kg) was followed 15-20 minutes later by an IM injection of ketamine[b] (10 mg/kg). This combination provided 20-30 min of complete relaxation.

Blood samples were obtained at 24 hours before oral aspirin administration[c] (day 0) and again 24 hours after the second oral aspirin dose (day 3). Samples were collected from either the jugular vein or the carotid artery into evacuated tubes without anticoagulant (clot retraction) and into tubes containing EDTA (total and differential white blood cell counts, hemoglobin, packed cell volume and plasma protein, and platelet count). Blood for platelet

aggregation was collected from the same site into siliconized glass tubes containing 3.8% trisodium citrate at a ratio of 9 volumes of blood to 1 volume of anticoagulant. Platelet poor plasma (PPP) from this sample was stored frozen at $-70°$ C until submission to the reference laboratory on dry ice for rocket immunoelectrophoretic determination of relative amounts of Factor VIII:related antigen.[d]

Following blood collection, a bleeding time was performed on anesthetized piglets using a commercial bleeding time device.[e] The bleeding time site used was the relatively hairless portion of the thoracic wall medial to the forelimb.

An additional EDTA blood sample was collected from the cephalic vein of unanesthetized piglets 4 hours after ingestion of the second aspirin treatment. Plasma salicylate concentrations were determined by a colorimetric technique.[f]

Clot retraction-The degree of clot retraction was quantitatively evaluated by a modification of Rowsell's method.[11] Whole blood was allowed to clot undisturbed in a corked siliconized glass tube at $37°$ C for 24 hours. The whole blood volume and the poured serum volume were measured in a graduated cylinder. The clot volume is the difference between these two measurements. The degree of clot

retraction was expressed as the per cent volume of clot to the total blood volume originally in the tube.

Platelet Aggregation-This technique has been described elsewhere.[g] In short, citrated whole blood was centrifuged (600 x g for 3.0 min at 20° C) to obtain platelet rich plasma (PRP). The PRP was diluted with autogenous PPP to obtain a working PRP with approximately 200,000 platelets/μl. Adenosine diphosphate[f] (ADP, 20 μM), collagen[h] (5 and 10 μg/ml) and sodium arachidonate[f] (6.9 mM) were used as the aggregating agents. Platelet aggregation was recorded over a 5 min period using a multichannel analyzer.[h]

Parameters measured were the maximum % aggregation where PRP was set as 0% transmission and PPP was 100% transmission of light and the slope as the initial % change in transmittance per minute. The lag time between the addition of collagen and the point in which measurable aggregation had occurred was also measured. In addition, aggregation curves were examined for overall pattern.

Statistics-All aggregations were performed in duplicate and the mean result calculated. Group data are expressed as the mean ± the standard error of the mean. An analysis of variance (ANOVA)[i] or, where appropriate, an analysis of covariance (ANCOVA)[i] was performed to detect group

differences. A significance level of 0.05 was maintained
throughout the study.

RESULTS

Overall hematologic values did not change significantly between day 0 and day 3 (Table 3). There was a significant difference in adjusted mean lymphocyte counts among the four treatment groups after aspirin treatment (P=0.046) but no clear trend was evident.

Bleeding times, clot retraction and relative concentrations of Factor VIII:related antigen did not vary significantly among the 4 treatment groups either before or after aspirin treatment (Table 4). Mean plasma salicylate concentrations, four hours after the second oral dose of aspirin, varied significantly among the 4 groups. The high dose (mean plasma salicylate=3.27 mg/dl) and the super-high dose (mean=14.73 mg/dl) aspirin treatment groups were significantly different from the control group (mean=0.90 mg/dl) and each other. The plasma salicylate concentration of the low dose aspirin group (mean=1.01 mg/dl) was not significantly different from that of the control group.

Reversible ADP-induced platelet aggregation was not significantly altered by aspirin treatment (data not shown).

The maximum % platelet aggregation induced by collagen was reduced by increasing doses of aspirin (Table 5). Maximal suppression of platelet aggregation was achieved

TABLE 3. Pooled hematologic data for 20 piglets before (day 0) and after (day 3) oral aspirin treatment

	Day 0	Day 3
PCV (%)	27.8±0.92[a]	27.7±0.85
Hemoglobin (g/dl)	8.6±0.31	8.4±0.36
MCHC (g/dl)	31.0±0.31	30.7±0.29
WBC (numbers/μl)	9005±453	7965±404
Band Neutrophils (numbers/μl)	60±19	65±14
Neutrophils (numbers/μl)	2673±258	2112±194
Lymphocytes (numbers/μl)	6065±310	5686±260
Monocytes (numbers/μl)	115±22	43±11
Eosinophils (numbers/μl)	62±17	38±12
Basophils (numbers/μl)	27±10	21±9
nRBC (numbers/100 WBC)	0.15±0.11	0.15±0.08
Plasma Prot (g/dl)	4.4±0.09	4.4±0.07
Fibrinogen (mg/dl)	200±14	205±15
Platelets (numbers x 10^3/μl)	412±33	462±23

[a]Values expressed here as mean ± SEM, n=20. There were no significant differences (P<0.05) between the day 0 and the day 3 values.

TABLE 4. Bleeding times, clot retractions and plasma Factor
VIII:related antigen concentrations in piglets
before (day 0) and after (day 3) oral aspirin
treatments

Measurement	Day 0	Day 3
Bleeding Time[a] (minutes)		
Control (0 mg/kg)	1.6±0.5[b]	2.2±0.5
Low dose (1 mg/kg)	1.5±0.2	2.5±0.5
High dose (20 mg/kg)	2.1±0.5	3.0±0.4
Super-high dose (100 mg/kg)	1.9±0.3	2.9±0.5[c]
Clot Retraction[d] (% clot/total blood volume)		
Control	25.5±3.1	24.0±2.9
Low dose	24.0±2.5	24.2±3.0
High dose	24.2±1.3	25.2±2.7
Super-high dose	23.8±2.6	24.5±4.4
Factor VIII:related antigen[a] (% normal reference pool)		
Control	86.0±10.5	79.6±7.2
Low dose	93.4±5.1	83.2±4.7
High dose	92.6±10.6	84.8±10.2
Super-high dose	91.6±10.0	87.6±9.4

[a] $n = 5$.

[b] The adjusted mean ± the standard error of the
adjusted mean. P>0.05 for all tests.

[c] One bleeding time was technically unsatisfactory and
was excluded from the statistical analysis.

[d] $n = 4$.

with the 20 mg/kg high dose treatment. Further suppression was not observed in the super-high dose treatment group. The lag phase preceding collagen-induced platelet aggregation and the initial rate of platelet aggregation (the slope) were not significantly altered by the aspirin treatments although a trend toward an increasing lag phase and a decreased rate of initial aggregation was evident. Aspirin treatment did not alter the monophasic irreversible nature of collagen-induced platelet aggregation.

Sodium arachidonate induced either monophasic and biphasic waves of irreversible platelet aggregation which were not suppressed by aspirin treatment (data not shown).

TABLE 5. Maximum % platelet aggregation induced by 2 doses
          of collagen in piglets having received various
          amounts of oral aspirin

Treatment group	Final collagen concentration	
	5 μg/ml[a]	10 μg/ml[b]
Control (0 mg/kg)	55.9±6.5[c]	72.7±2.5
Low dose (1 mg/kg)	48.9±6.9	69.2±3.0
High dose (20 mg/kg)	29.9±6.7[*]	60.7±2.6[*]
Super-high dose (100 mg/kg)	29.6±6.4[*]	60.4±2.6[*]

[a] $n=4$.

[b] $n=5$.

[c] Values are expressed as the adjusted mean ± the
standard error of the adjusted mean.

[*] The mean treatment value is significantly different
($p < 0.05$) from the corresponding mean control value.

DISCUSSION

Oral aspirin treatment in piglets did not alter the numbers of circulating platelets or decrease the ability of clotted blood to retract.

Aspirin has been shown to increase the bleeding time in man[12] and horses[13,14]. Increased bleeding times were not observed in this study. A possible explanation is that the test, as it was performed, was not sensitive to detect the possible increase in bleeding time. An alternative explanation is based on the observation that collagen induced maximum platelet aggregation, while suppressed, was not less than 50% of the control value. This suggests that substantial platelet function was maintained.

ADP induces only primary reversible platelet aggregation in the pig. Primary aggregation is reported to not be inhibited by cyclo-oxygenase inhibitors.[15] The results of this study support that observation.

Aspirin has been shown to reduce arachidonic acid-induced platelet aggregation in man[16-18], dogs[18] and cats[19]. It has been indicated, however, that platelet aggregation induced by high concentrations of arachidonic acid is not mediated by intermediates of cyclo-oxygenase derived products.[15] The results described in this report are consistent with the later observation.

This study suggests that aspirin does reduce porcine platelet function but this effect is much less pronounced in pigs when compared to other species including man. This is consistent with the relatively low amounts of thromboxane generated by stimulated porcine platelets.[9,10]

LETTERED FOOTNOTES

a. Pitman-Moore Inc., Washington Crossing, NJ.

b. Parke-Davis, Morris Plains, NJ.

c. Saint Joseph Aspirin for Children, Plough Inc., Memphis, TN.

d. Veterinary Hematology Laboratory, New York State Department of Health, Albany, NY.

e. Simplate Bleeding Time Device, General Diagnostics, Morris Plains, NJ.

f. Sigma Chemical Co., St Louis, MO.

g. Matthews JA, Ledet AE, Evans LE. The effect of age on in vitro porcine platelet aggregation. (In preparation.) 1987.

h. Helena Laboratories, Beaumont, TX.

i. SAS Institute Inc., Cary, NC, 1986.

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EXPERIMENT III

The effects of intravascular collagen infusion on aspirin

pretreated piglets

James A. Matthews, D.V.M., M.S.

Richard L. Engen, Ph.D.

Arlo E. Ledet DVM, Ph.D.

SUMMARY

Aspirin reduced the in vitro porcine platelet aggregation response to collagen. Two groups of piglets, an untreated control group and an oral aspirin pretreated group, received intravascular infusions of bovine tendon collagen. Although both groups had increased pulmonary vascular resistance and increased pulmonary arterial pressure following collagen infusions aspirin pretreatment significantly reduced the degree of response.

The control piglet group became neutropenic following collagen infusion while the aspirin pretreated piglets showed no change in numbers of circulating neutrophils. Aspirin pretreatment, therefore, reduced the cardiovascular effects of intravascular collagen infusion but it is not clear if the cause of this reduction is platelet inhibition or decreased platelet-neutrophil interaction.

INTRODUCTION

Collagen suspensions are commonly used to induce platelet aggregation in the laboratory. Aspirin (acetylsalicylic acid), an arachidonic pathway inhibitor, has been shown to reduce collagen-induced platelet aggregation in man[1] and pigs.[a]

Upon aggregation platelets release a variety of biologically active substances that affect subsequently arriving cells, alter blood flow and affect the coagulation cascade.[2,3] The platelet response is further modified by intact vascular endothelium.[4] Drugs that affect platelet aggregation in vitro therefore may not be effective in vivo.[5]

The purpose of this experiment was to evaluate and compare the hematologic, respiratory and cardiovascular responses of aspirin treated and untreated control piglets subjected to an intravascular infusion of collagen.

MATERIALS AND METHODS

Pigs-Eight piglets (6 to 7 weeks old, 13 to 17 kg) of either sex were used. These pigs are part of a pseudorabies and rhinitis free herd maintained indoors at the College of Veterinary Medicine, Iowa State University.

Experimental design-The piglets were evenly divided into two groups: a control group (no aspirin) and an aspirin treatment group. The aspirin treatment group received oral aspirin, 20 mg/kg, once a day for 2 days with collagen challenge occurring approximately 24 hours after the second aspirin dosage.

Collagen preparation-Bovine tendon collagen[b] was suspended in sterile, endotoxin free Tyrode's salt solution[b] as previously described.[6] This suspension contained approximately 60 μg collagen/ml and was stored frozen at $-70°$ C until used. Aliquots of the same collagen suspension were used in all piglets.

Animal preparation-Each piglet was tranquilized with an intramuscular injection of acepromazine[c] (5 mg/piglet) and allowed to rest undisturbed for 30-45 minutes. Anesthesia was induced by slow intravenous injection of thiamylal sodium[d] to effect (approximately 8 mg/kg). Anesthesia was maintained by intermittent thiamylal sodium injections.

Cardiovascular and respiratory responses were collected using a multichannel physiograph recorder connected to a microcomputer for data analysis. This laboratory is described in detail elsewhere.[7]

The anesthetized piglets were placed in dorsal recumbency, a tracheostomy performed and a cuffed endotracheal tube tied in place. The endotracheal tube was attached to a pneumotach which in turn was attached to a differential transducer allowing the measurement of inspiratory and expiratory flow rates. The electronic integration of the pneumotach measurements gave a measure of air volume per respiratory cycle.

Both femoral arteries and one femoral vein were isolated for cannulation. Two Swan-Ganz double-lumen thermodilution catheters[e] were used to cannulate a femoral vein and artery. The femoral vein catheter was advanced through the heart and into the pulmonary artery. This catheter was used for recording pressure changes in the pulmonary artery and for thermodilution determinations of cardiac output. The second catheter was advanced up the femoral artery to the aortic valve. The proximal port (30 cm from the tip) was used to measure aortic pressure and heart (pulse) rate. The contralateral femoral artery was also cannulated and the cannula was stoppered with a 3-way valve providing ready access for arterial blood collection.

The pigs were heparinized (100 units/kg, intravenous injection) and allowed to lie undisturbed for at least 5 minutes before baseline (time period 1) sampling.

Measured respiratory parameters were tidal volume (ml/breath), respiratory rate (breaths/min) and minute volume (ml/min). Measured cardiovascular parameters were mean pulmonary arterial pressure (mmHg), mean systemic pressure (mmHg) and heart rate (pulses/min). Additionally thermodilution curves[8] were obtained allowing the measurement of cardiac output (CO; ml/min), right ventricular stroke volume (ml/pulse), pulmonary vascular resistance (mmHg/ml-sec^{-1}) and systemic vascular resistance (mmHg/ml-sec^{-1}).

Blood samples—Blood samples were collected into a variety of anticoagulants. Citrated plasma (9 volumes of blood to one volume of 3.8% citrate) was used for prothrombin time (PT), activated partial thromboplastin time (PTT) and factor VIII:related antigen (VIIIR:Ag) determinations.[f] EDTA anticoagulated whole blood was used for complete blood counts and platelet counts. Arterial blood gas and acid-base samples were collected anaerobically into heparinized syringes, corked, and stored under ice water until processed.[g] All blood gas and acid-base determinations were made within an hour and a half of

collection. Additionally, blood was collected into a plastic syringe containing 3.8% trisodium citrate volume adjusted against the individual piglet's packed cell volume to give a final plasma citrate concentration of 15 mM. These samples were used in platelet aggregation studies as previously described.[a] In this study platelets were challenged with commercial collagen[h] (10 μg/ml final concentration), an aliquot of the bovine tendon collagen used as an infusate (6 μg/ml), and adenosine diphosphate (ADP; 20 μM).[b]

Collagen infusions-The collagen suspension was slowly infused over 30 seconds into the femoral vein (0.44 ml/kg). This infusion was repeated twice more (see below).

Sampling protocol-A citrated blood sample was collected before the piglet was heparinized and was used to determine preheparinization PT, PTT and VIIIR:Ag concentration.

Cardio-pulmonary measurements were made at time 1 (baseline), time 2 (maximal systemic pressure response to collagen infusion I), time 3 (collagen I recovery, approximately 10 minutes after collagen infusion I), time 4 (maximal response to collagen infusion II) and time 5 (collagen II recovery, again approximately 10 minutes after collagen infusion). Notice that time 3 acts as a baseline measurement for comparison to time 4. Blood samples were

drawn simultaneously with the cardio-pulmonary measurements but not all possible blood samples were collected at each time period (Table 6).

After the time 5 samples were collected the pig was given a final collagen infusion (collagen infusion III) and then euthanized with an intravenous injection of barbiturates at the maximal systemic vascular pressure response. A routine post mortem examination was performed. The lungs were collected within 15 minutes following euthanasia and perfused, intratracheally, with 10% neutral buffered formalin at 30 cm water pressure. The lungs were allowed to fix in the formalin solution for a minimum of 1 day. Lung samples from the dorsal aspect of the cranial and caudal lung lobes, both left and right sides, were dehydrated in graded alcohols, embedded in paraffin, sectioned at 5 μm and stained with hematoxylin and eosin.

Statistical analysis-The piglets were divided into 4 blocks. Each block contained a pair of piglets, one from each of the two treatment groups. The data were evaluated by the use of the analysis of variance using a general linear model (PROC GLM).[i] The overall experimental design was considered a split plot design where aspirin treatment formed the whole block factor and time was the split plot factor. In addition selected least significant differences

TABLE 6. Blood sample collection protocol

Time period[a]	Platelet count	CBC	Blood gas & acid-base	Platelet aggregation	PT, PTT & VIIIR:Ag
1	+	+	+	+	+
2	+	-	+	-	-
3	+	-	+	-	-
4	+	-	+	-	-
5	+	+	+	-	+

[a]See text for explanation.

between groups within a time period (LSD_1) and between time periods for a group (LSD_2) were calculated and are included in the appropriate figure titles. Platelet aggregations were performed in duplicate and the mean result was calculated. Since aggregation profiles were determined once for each animal the analysis was a simple randomized block design.

Data are expressed as the mean and the mean ± the standard error of the mean. A significance level of $P < 0.05$ was maintained throughout the study.

RESULTS

Platelet aggregation-Aspirin treatment significantly reduced the maximum platelet aggregation response to both 10 and 6 μg/ml collagen suspensions. The initial rate of aggregation (slope) and the lag time between the addition of collagen and detectable platelet aggregation were not affected by aspirin treatment. Platelet response to ADP was not significantly affected by aspirin treatment (Table 7).

Hematology-Mean platelet counts decreased after both collagen infusions for both aspirin treated piglets and controls but, due to the wide variation of the standard error of the mean platelet count, this decrease was not statistically significant (P=0.09, 1 and 24 degrees of freedom: Figure 3).

Total leukocyte numbers decreased significantly for both groups across the entire experiment. Lymphocyte numbers decreased while band neutrophil numbers increased slightly. The most dramatic change was the decrease in circulating numbers of mature segmented neutrophils. The control group's mean neutrophil count dropped from 3,953±538/μl (time 1) to 1,710±199/μl (time 5). Aspirin treated piglets, however, showed only a slight, statistically insignificant, decrease from a mean segmented neutrophil count of 4,023±1,449/μl to 3,738±1,954/μl.

TABLE 7. The effect of aspirin (20 mg/kg) on porcine
         platelet aggregation

Aggregation parameter	Commercial collagen (10 μg/ml)	Collagen infusate (6 μg/ml)	ADP (20 μM)
Maximal aggregation (% aggregation)			
Control group	84.0[a]	68.8	50.5
Aspirin treated	68.3*	55.3*	48.8
Slope (initial % aggregation/min)			
Control group	85.8	59.0	61.2
Aspirin treated	88.5	54.8	67.2
Lag phase (seconds)			
Control group	35	60	NA[b]
Aspirin treated	34	59	NA

[a]Each value represents the mean result of four animals.

[b]NA=not applicable.

*The value differs significantly (P<0.05) from the control group's corresponding value.

Packed cell volumes and hemoglobin, plasma protein and fibrinogen concentrations did not change significantly during the course of the experiment.

Acid-base and blood gases-There was no significant change in the blood pH or the partial pressure of carbon

FIGURE 3.   Mean arterial platelet counts, 4 piglets per
            treatment group. Samples were taken at time
            1 (baseline), time 2 (maximal systemic pressure
            response to collagen infusion I), time 3
            (collagen I recovery, approximately 10 minutes
            after collagen infusion), time 4 (maximal
            response to collagen infusion II) and time 5
            (collagen II recovery, again approximately 10
            minutes after collagen infusion). Least
            significant difference (P<0.05) between
            treatment groups within a time period (LSD_1)
            is 149. Least significant difference (P<0.05)
            between time periods for a treatment group
            (LSD_2) is 132
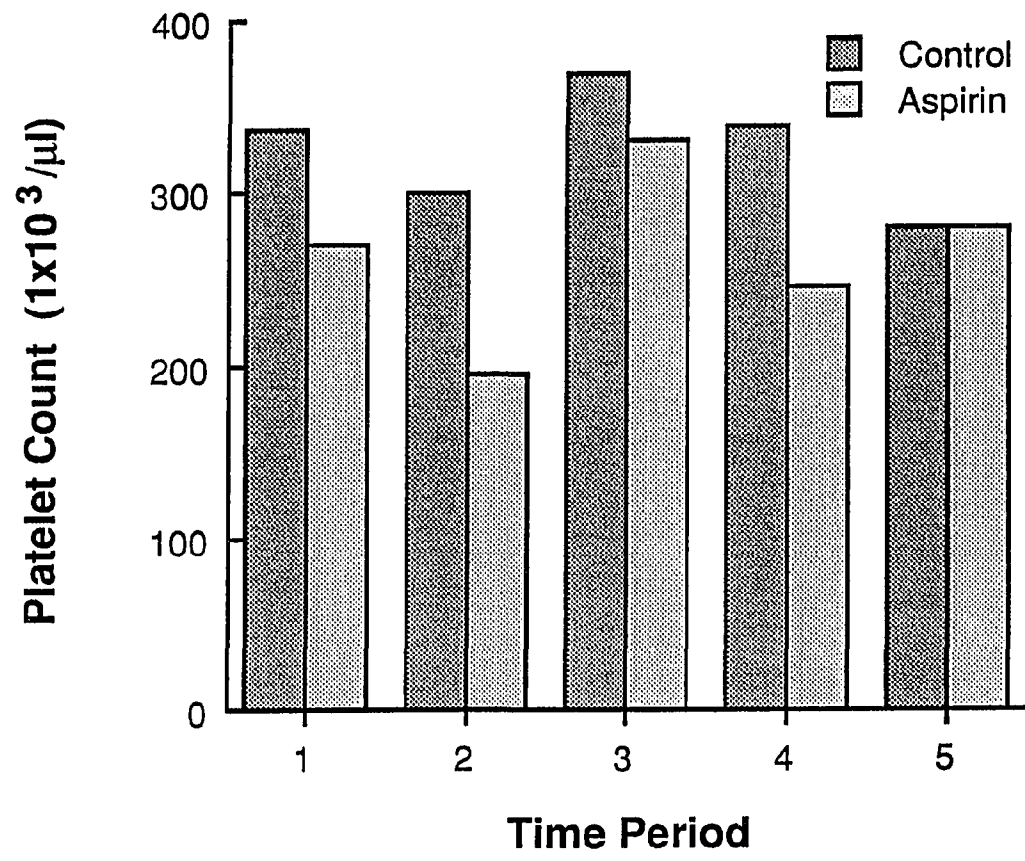
dioxide either between groups or across the duration of the experiment. The arterial pO_2, however, dropped significantly for both groups following collagen infusions and showed an overall significant downward trend across the duration of the experiment. There was no significant difference between aspirin treated and untreated control piglets (Figure 4).

Respiratory parameters-There were no significant changes in tidal volume, respiratory rate or minute volume between groups or due to collagen infusion.

Cardiovascular parameters- The most obvious response to repeated collagen infusions was a marked transient decrease in systemic vascular pressure. Baseline mean systemic pressures were significantly greater in aspirin treated piglets than in control animals. There was, however, no significant difference between the two groups in their response to collagen infusion (Figure 5).

Mean systemic vascular resistance rose significantly in both of the treatment groups following the first collagen infusion (time 2) and never returned to baseline values.

Mean pulmonary arterial pressure in the control piglets also increased significantly following the first collagen infusion and also never returned to baseline pressures. Aspirin treated piglets also had a rise in pulmonary

FIGURE 4.   Mean arterial partial pressure of oxygen, 4
            piglets per treatment group. LSD_1=16.4.
            LSD_2=9.6. See figure 3 for an
            explanation of the time periods and the
            abbreviations

Mean Arterial Partial Pressure of Oxygen (mm Hg)
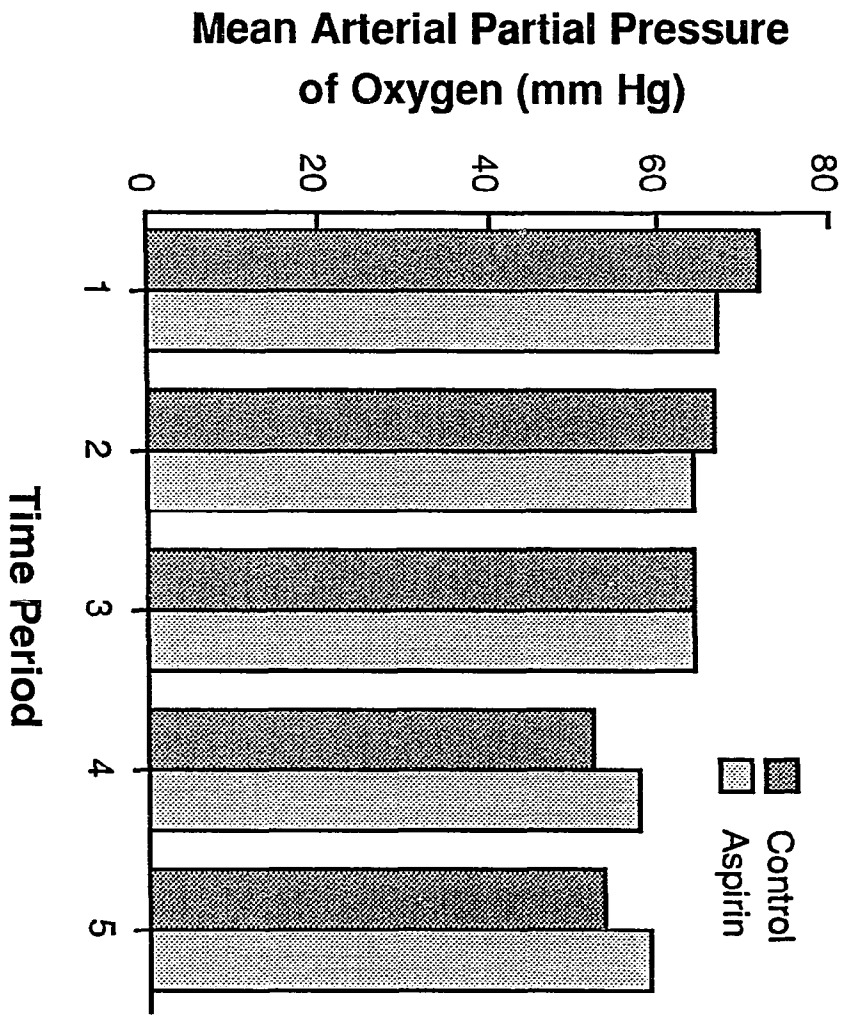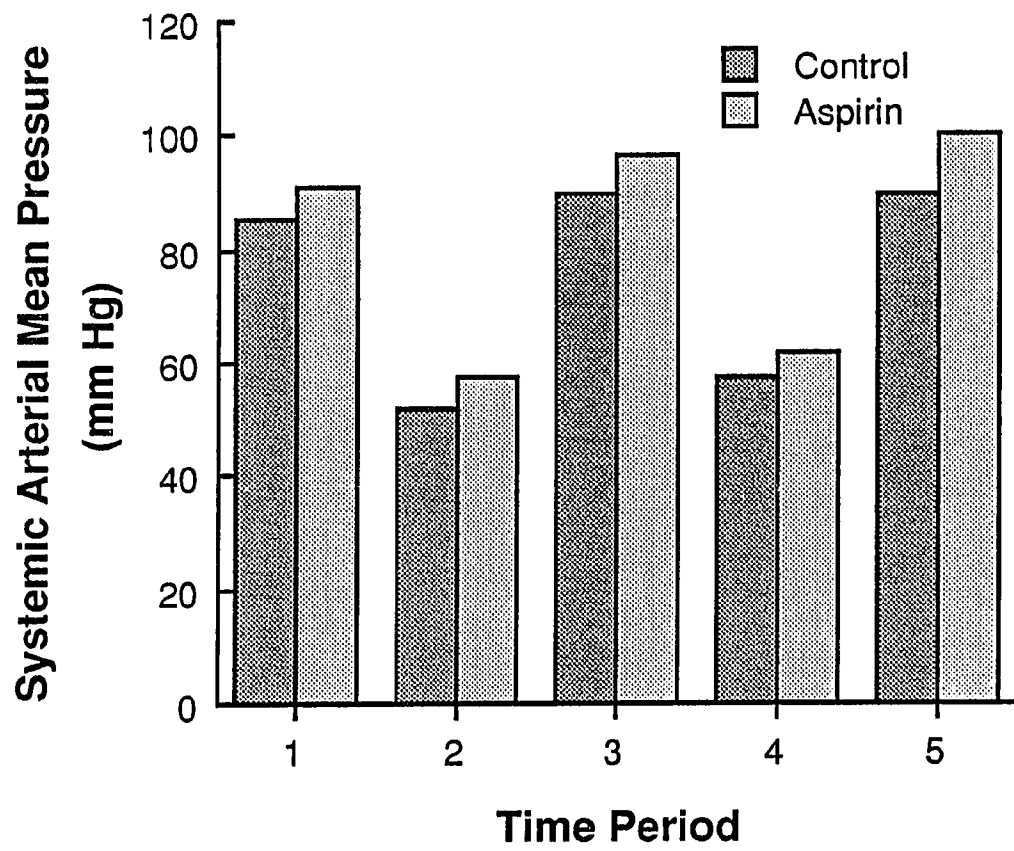
FIGURE 5.   Systemic arterial mean pressure, 4 piglets per
            treatment group. LSD_1=10.3. LSD_2=11.0.
            See figure 3 for an explanation of the time
            periods and abbreviations

arterial pressure but this rise was significantly less than in the controls (Figure 6).

Pulmonary vascular resistance and heart rate increased significantly in control piglets following the first collagen infusion while aspirin treated piglets showed a lesser change. Pulmonary vascular resistance in aspirin treated piglets rose slightly (Figure 7) while heart rate did not change significantly.

There were no significant changes in stroke volume or cardiac output between the groups or following collagen infusion.

Coagulation times and factor VIII:related antigen concentration-Heparin infusion did not alter the PT or the VIIIR:Ag concentration. The overall mean PTT for both groups increased from 17.9 to 29.4 sec five minutes after intravenous heparin injection (time 1). Neither the PT nor the PTT changed over the course of the experiment (from time 1 to time 5). Factor VIII:related antigen, however, showed a significant 31% increase during the course of the experiment.

Post mortem examinations-All piglets showed a diffuse purplish discoloration of the dorsal aspects of all lung lobes. This discoloration was markedly decreased when the lungs were expanded with formalin. No gross lesions were identified in the remainder of the carcasses.

FIGURE 6. Pulmonary arterial mean pressure, 4 piglets per
treatment group. LSD_1=12.7 LSD_2=5.5.
See figure 3 for an explanation of the time
periods and the abbreviations

Pulmonary Arterial Mean Pressure (mm Hg)
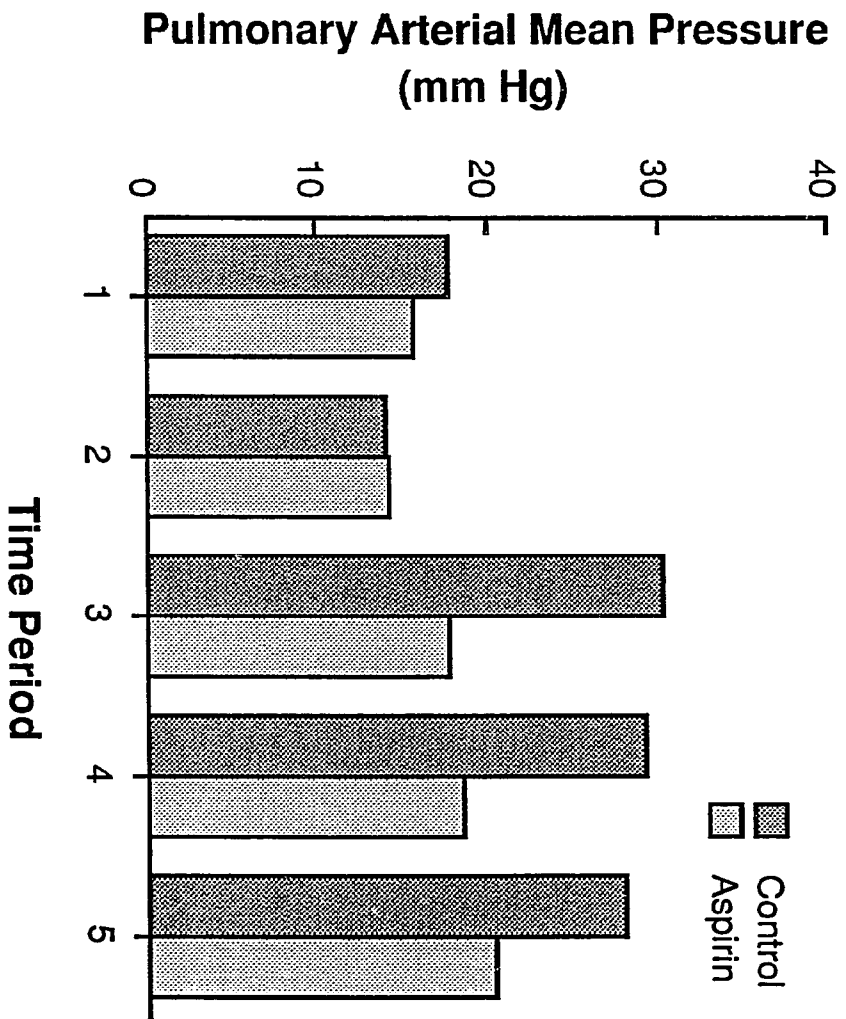
FIGURE 7. Pulmonary vascular resistance, 4 piglets per
treatment group. $LSD_1=0.48$. $LSD_2=0.16$.
See figure 3 for an explanation of the time
periods and the abbreviations

Pulmonary Vascular Resistance
(mm Hg/ml/second)
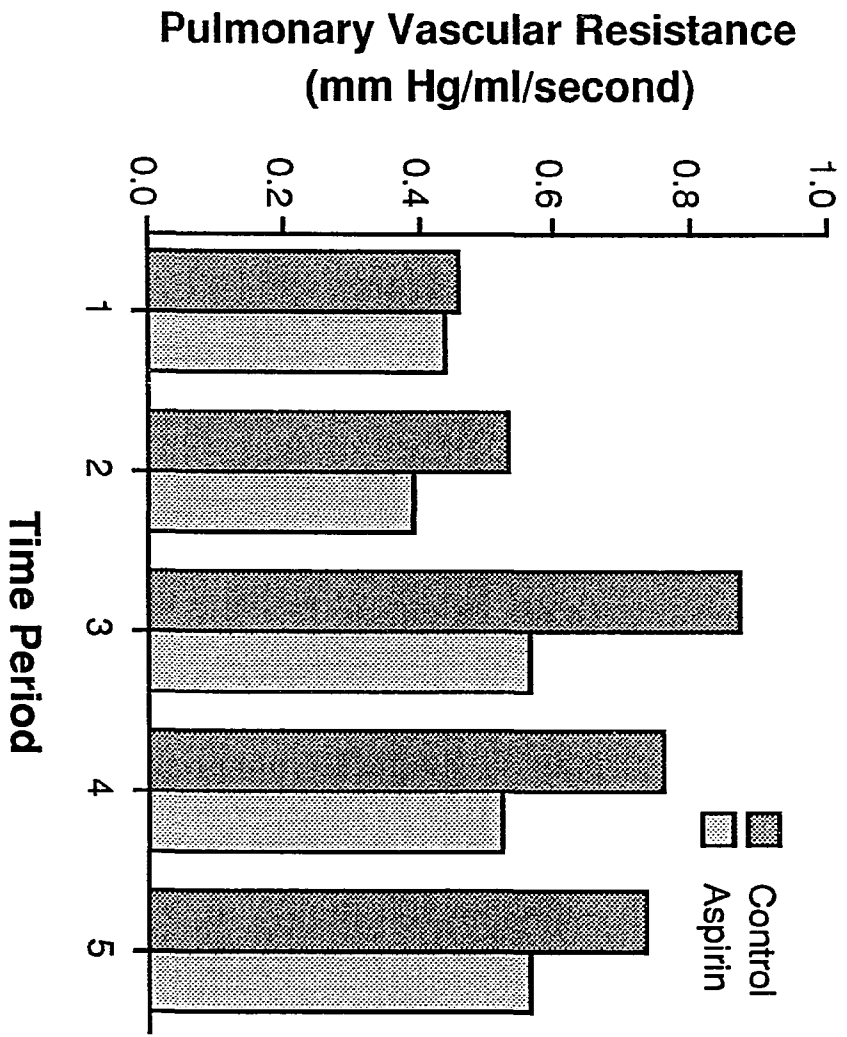
The microscopic appearance of the lungs from the
aspirin treatment group was compared to the appearance of
the lungs from the untreated control piglet group. The
control group had a diffuse, primarily neutrophilic,
cellular infiltrate in the walls of the pulmonary alveoli
(Figure 8) and increased numbers of intravascular platelet
microaggregates (Figure 9). These intravascular platelet
aggregates contained relatively large numbers of
neutrophils; many more than expected in proportion to the
numbers of erythrocytes. One piglet in the control group
who had suffered iatrogenic laryngeal hemorrhage before the
tracheostomy, had small numbers of erythrocytes free in the
alveolar lumina.

FIGURE 8.   (Top) Lung, from an aspirin pretreated
            piglet following 3 intravascular infusions
            of bovine tendon collagen. Hematoxylin
            and eosin; x 158

FIGURE 8.   (Bottom) Lung, from an untreated control
            piglet following 3 intravascular infusions
            of bovine tendon collagen. There are
            increased numbers of neutrophils and mono-
            nuclear cells in the walls of the alveoli
            throughout the section and a moderately
            sized platelet aggregate in the lumen of the
            vein in the center of the photograph.
            Hematoxylin and eosin; x 158
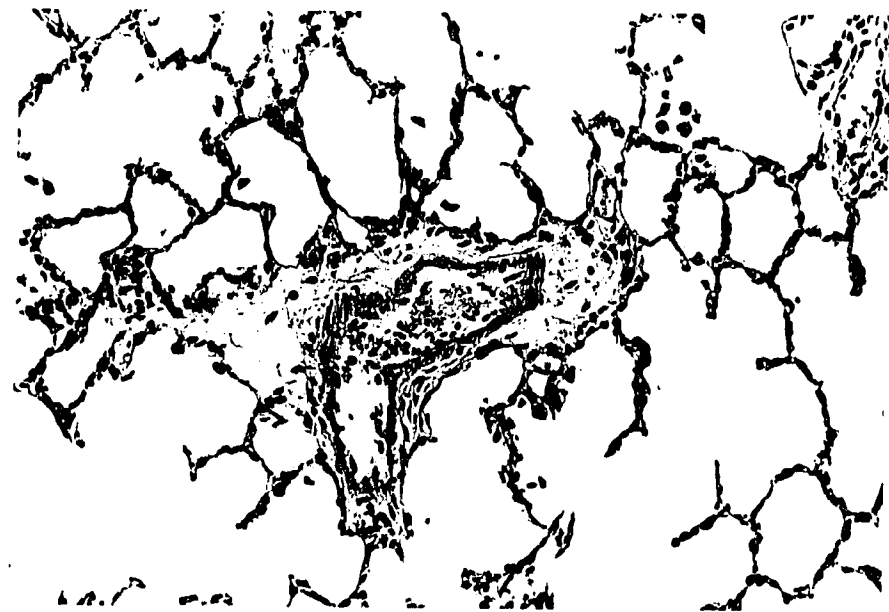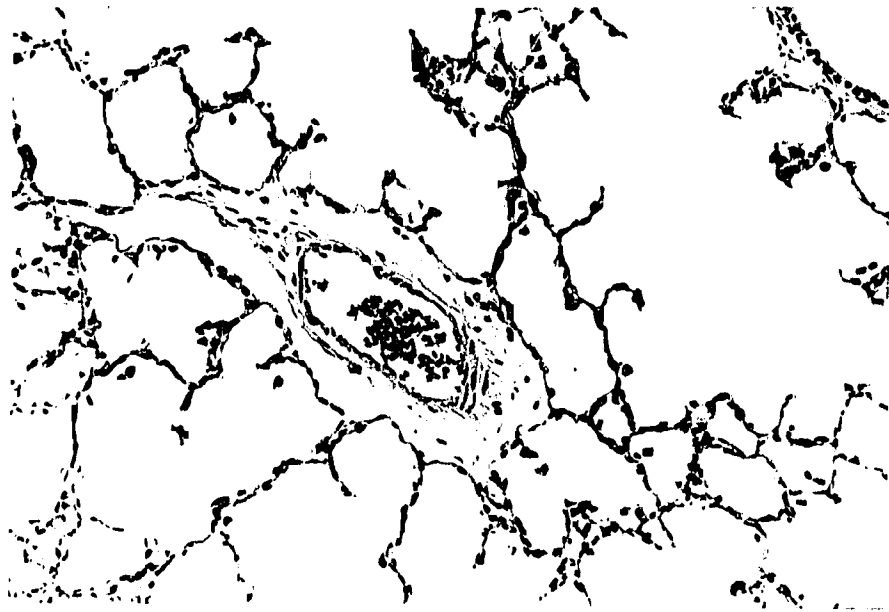
FIGURE 9.   Lung, from an untreated control piglet
            following 3 intravascular infusions of
            bovine tendon collagen.   There is a
            large platelet aggregate with moderate
            numbers of erythrocytes and neutrophils
            in the lumen of the large vein in the
            center of the photograph.   The
            surrounding alveoli are hypercellular.
            (See figure 8.)   Hematoxylin and
            eosin; x 62
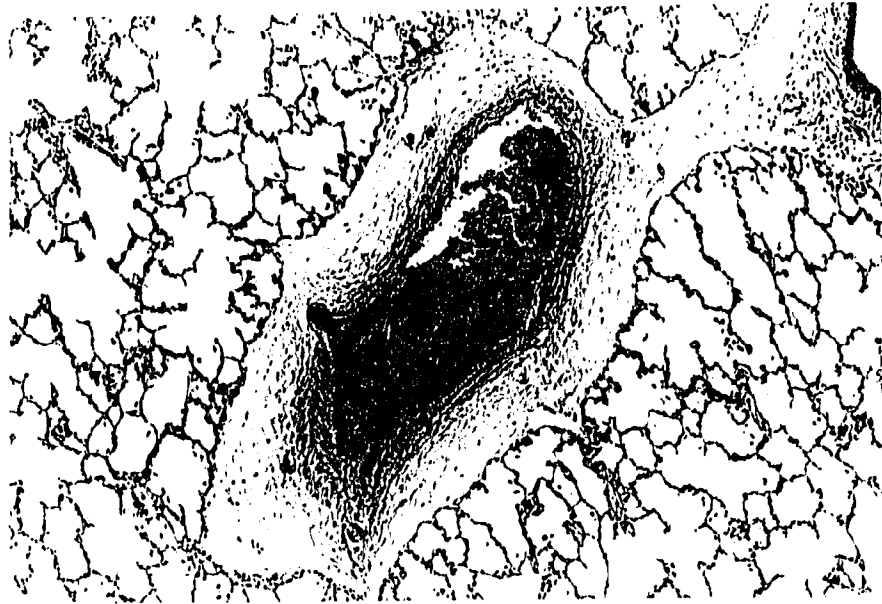
DISCUSSION

Aspirin inhibits platelet aggregation by irreversibly acetylating the cyclo-oxygenase enzyme thereby inhibiting the conversion of arachidonic acid to thromboxane. Aspirin also inhibits the formation of prostacyclin from arachidonic acid by vascular endothelial cells.

In this study aspirin decreased collagen-induced ex vivo maximum % platelet aggregation. This correlates well with previous work in piglets[a] and suggests that arachidonic acid metabolites have a role in porcine platelet aggregation.

Intravascular collagen injection causes platelet aggregation and a decrease in circulating platelet counts in cats, dogs, rabbits, guinea-pigs and rats.[9-16] In the guinea-pig the decrease in platelet count is collagen dose dependent with only a transient decrease in platelet counts being seen in the 30 μg/kg treatment group.[14]

In this study platelet counts decreased following collagen infusion but this decrease was not statistically significant. Part of the reason for this may be due to the very low concentrations of collagen used in this experiment (26 μg/kg). A low collagen dose was used for a variety of reasons. The most important reason is that high dose

collagen-induced aggregation is independent of arachidonic acid metabolism and aspirin inhibition.[17] Additional causes for the lack of a significant decrease in platelet numbers may be small sample size and occasional marked variations in platelet counts in the same piglet. This individual variation may be the result of splenic contraction with the mobilization of large stores of platelets.[18]

Collagen-induced intravascular platelet aggregation has been used to induce cardio-pulmonary injury.[10] Expected cardiovascular changes are increased pulmonary arterial pressure, decreased or normal cardiac output, increased pulmonary vascular resistance and decreased systemic arterial pressure.[9,19] These changes also occurred in this study. Systemic vascular resistance increased suggesting that not all of the vasoactive platelet microaggregates localized in the lung but that some occured in the systemic vasculature. This combination of pulmonic and systemic platelet microaggregates following collagen infusion has been demonstrated in the guinea-pig.[14]

There is no ready explanation why baseline systemic arterial pressures should be greater in aspirin treated piglets than in untreated controls.

Leukopenia, decreased fibrinogen concentrations and decreased complement activity have been found in dogs

following intravascular collagen injection.[19] In this study we also noted a significant leukopenia. Both groups became lymphopenic. Lymphopenias are commonly associated with a stress response in animals. More importantly, control piglets had a marked drop in numbers of circulating mature neutrophils. Neutrophil numbers did not significantly decrease in aspirin treated animals. Since aspirin is reported to have no effect on complement-induced granulocyte aggregation in cats[20] it is doubtful that aspirin inhibited the neutropenia by interfering with that mechanism. Platelets can modify neutrophil function in other ways. Thromboxane, a potent constrictor of arteriolar smooth muscle induces platelet aggregation and is the major active metabolite of platelet arachidonic acid metabolism. Thromboxane also mediates increased polymorphonuclear leukocyte adhesiveness. Indomethacin, an inhibitor of arachidonic acid metabolism, reduced augmented human polymorphonuclear leukocyte adherence to nylon.[21] Platelet derived growth factor, a constituent of the platelet *a* granule, acts as a strong chemoattractant for neutrophils, increases neutrophil aggregation and has been shown to increase neutrophil adherence to plastic surfaces.[22,23] Aspirin will inhibit platelet *a* granule release[24] thereby theoretically decreasing platelet derived growth factor release and neutrophil function.

The gross discoloration of the dorsal aspects of the lungs in all of the piglets may be attributed to both atelectasis and hypostatic congestion. These changes are most likely secondary to the animals being placed in a supine position for the duration of the experiment and they provide a reasonable explanation for the slow decrease in the arterial partial pressure of oxygen observed in both treatment groups. The neutropenic untreated control group had increased numbers of neutrophils in the walls of the pulmonary alveoli strongly suggesting that circulating neutrophils were sequestered in the lungs. The observation that relatively increased numbers of neutrophils are present in platelet aggregates lends morphologic support to a possible platelet-neutrophil interaction.

VIIIR:Ag can be found in plasma, platelets, megakaryocytes and endothelial cells. Increased concentration of this antigen in the plasma has been associated with vascular diseases in man and is reported to have originated with increased release from endothelial cells. The origin of the increase in VIIIR:Ag was not identified in this study.

In summary, aspirin pretreatment moderated the increases in mean pulmonary arterial pressure, pulmonary vascular resistance and heart rate as well as decreases in

circulating segmented neutrophil numbers. It was beyond the scope of this study to investigate whether the protectant effect was a direct action of aspirin on platelet function or a result of decreased platelet-neutrophil interactions.

LETTERED FOOTNOTES

a. Matthews JA, Ledet AE. The effect of aspirin on porcine platelet function. (In preparation.) 1987.

b. Sigma Chemical Co., St. Louis, MO.

c. TechAmerica Group Inc., Elwood, KS.

d. Parke-Davis, Morris Plains, NJ.

e. Edwards Laboratories Inc., Anasco, Puerto Rico.

f. Veterinary Hematology Laboratories Inc., New York State Department of Health, Albany, NY.

g. ABL3, Radiometer America Inc., West Lake, OH.

h. Helena Laboratories, Beaumont TX.

i. SAS Institute Inc., Cary, NC, 1986.

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GENERAL SUMMARY AND DISCUSSION

When a blood vessel is damaged a complex series of events called the hemostatic mechanism is initiated. Platelets interact with plasma von Willebrand factor and exposed subendothelial structures to form the initial hemostatic plug. Following shape change and primary aggregation platelets release their granule contents. The dense granules contain adenine nucleotides, amines and divalent cations which together amplify platelet responses and cause vasoconstriction. The more numerous a granules contain antiheparin and coagulant proteins, fibronectin, growth factor(s) and von Willebrand factor. Platelet membrane arachidonic acid is liberated and rapidly metabolized to a variety of prostaglandins and the potent aggrgating agent thromboxane A_2. Platelet recruitment and aggregation continues until an adequate platelet mass has formed over the site of damage.[1,26,31,71]

Platelet dysfunction can be quantitative or qualitative, primary or secondary, inherited or acquired.

In man and the horse normal neonatal platelets exhibit reduced responsiveness to collagen and adenosine diphosphate (ADP).[44-46] The cause of this normal age related variation is not known but it is thought to be a relative defect in

either a membrane receptor or phospholipase A_2.[45] Neonatal piglet platelets, however, respond to collagen and ADP as well as or even more vigorously than sow and mature gilt platelets suggesting that the neonatal porcine platelet is relatively more mature at birth than human or equine platelets.

Porcine platelets do not aggregate at concentrations of arachidonic acid that cause irreversible platelet aggregation in man. The suggested explanation is that alternative arachidonic-independent platelet activation pathways are relatively more important in the basic porcine platelet response.[109] Higher doses of arachidonic acid cause platelet aggregation in rats[53] and pigs (Matthews, Department of Veterinary Pathology, ISU, unpublished data) but this aggregation is apparently independent of cyclo-oxygenase derived products. Interestingly, high dose sodium arachidonate did not cause irreversible aggregation in 8 of 20 neonatal piglets. The reason for this is not known but it appears to be a defect in the direct action of arachidonic acid on platelet function. It has been shown that arachidonic acid will covalently bind to intact human platelet proteins independent from both cyclo-oxygenase and lipoxygenase activity.[110]

Aspirin inhibits human platelet function by inhibiting the enzyme cyclo-oxygenase. Pig platelets have a relatively poor ability to generate thromboxane when stimulated by either thrombin or collagen.[109,111] This would suggest that cyclo-oxygenase inhibition by aspirin would have little physiological consequence in the pig. In spite of this aspirin was one of the best platelet function inhibitors tested in pigs following balloon angioplasty.[104,105]

Our results indicate that aspirin does affect porcine platelet function, particularly the aggregation response initiated by low dose collagen. This in vitro inhibition appears to be relatively mild and can be partially overcome by increasing the concentration of the collagen used to initiate aggregation. These findings are consistent with the observations made on aspirin treated human platelets.[112]

The in vivo response to intravascular collagen infusion is consistent with the in vitro findings. Aspirin pretreatment did not prevent the vascular response to the infusion but the response was attenuated. More surprising was the fact that aspirin prevented the neutropenia found in the untreated control animals. Perhaps the clinical importance of aspirin therapy in the pig will lie not with the ability of aspirin to inhibit platelet cyclo-oxygenase but with the ability of aspirin to decrease platelet interactions with other mediators of inflammation.

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APPENDIX A

Sodium arachidonate-induced porcine platelet aggregation

Introduction

Human, feline, canine, equine, bovine and porcine platelets all possess an arachidonate pathway and generate thromboxane, however, bovine and porcine platelets do not aggregate when stimulated by 0.5 mM arachidonate.[1]

Porcine platelet aggregation apparently is induced by 6.9mM sodium arachidonate.[2] It could be argued that platelet aggregation tracings following high dose arachidonate stimulation represent platelet lysis rather than platelet aggregation.

Gross examination of aggregation cuvettes after 5 minutes of 6.9 mM sodium arachidonate-induced aggregation revealed macroaggregates of platelets suggesting that at least some platelets were still intact. Stronger evidence that 6.9 mM sodium arachidonate does not cause significant platelet lysis during the 5 minutes that platelet aggregation is being monitored can be found in careful examination of reversible sodium arachidonate-induced platelet aggregation tracings. The tracings show that the final turbidity (5 min) is back to the baseline platelet

rich plasma value. Platelet lysis would produce an irreversible change in turbidity.

This project was designed to further investigate the possibility that high dose arachidonate causes platelet lysis rather than aggregation by examining the concentration of lactate dehydrogenase (LDH) found in the plasma after platelet aggregation.

Materials and methods

Part A-Seven sets of aggregation cuvettes were prepared in duplicate with 450 μl of platelet rich plasma (PRP:200,000 platelets/μl) being placed into each of 4 sets of cuvettes and 450 μl of platelet poor plasma (PPP) being added to the remaining 3 sets. Routine platelet aggregation was initiated in one set of cuvettes with 50 μl of collagen[a] (10 μg/ml). A collagen control was prepared by adding an identical amount of the same collagen suspension to a set of PPP cuvettes. Similarly platelet aggregation was induced with 6.9 mM sodium arachidonate[b] in PRP. An arachidonate control in PPP was also prepared. One set of PRP and one set of PPP cuvettes were left undisturbed. Finally, in an attempt to lyse platelets, one set of PRP cuvettes were frozen (-20° C) and thawed 3 times in succession.

All of the cuvettes were simultaneously centrifuged, the supernatant citrated plasmas collected, labeled with a

code number and submitted to the veterinary clinical
pathology laboratory for routine determination of LDH
activity. The time lapse from the addition of arachidonate
to the PRP cuvettes and the collection of plasma was
approximately 40 minutes.

Part B-Four additional 450 μl cuvettes were prepared.
These cuvettes were placed into a centrifuge and collagen
was added to one set of cuvettes while arachidonate was
added to the other set. The samples were spun for 5 minutes
and the plasma was then harvested. These plasma samples
were labeled with a code number and submitted to the
clinical pathology laboratory for LDH activity
determination. This submission was separate from the Part A
submission and was analyzed by the lab the following work
day.

Results

The results of the LDH assays are in Table 8.

Part A-The addition of collagen and sodium arachidonate
did not increase the plasma LDH activity of the PPP over the
basal PPP LDH activity. This shows that there was no
significant LDH activity in either one of the platelet
agonists.

LDH activity did not differ between the untreated PRP
sample and the PPP sample.

Both collagen aggregation and 3 cycle freeze-thawing did not increase the plasma LDH activity. A 40 minute exposure of PRP to 6.9 mM sodium arachidonate did result in a marked increase in plasma LDH activity.

Part B-A 5 minute incubation of PRP with sodium arachidonate did not significantly increase the plasma LDH activity over parallel collagen and PRP incubation.

Discussion

Lactate dehydrogenase is found in the cytoplasm of cells.[3] Cell membrane damage and cell necrosis cause leakage of this enzyme from the cell into the surrounding plasma. It appears that prolonged incubation of porcine platelets with relatively high concentrations of sodium arachidonate will cause cell membrane damage but little to no detectable platelet damage occurs during the first 5 minutes after the addition of sodium arachidonate to the PRP, the time period when platelet aggregation is evaluated.

Based on physical examination, turbidity measurements and enzyme assays it appears that 6.9 mM sodium arachidonate does not lyse porcine platelets during the first 5 minutes of platelet aggregation.

TABLE 8. LDH activity following platelet aggregation

Cuvette	LDH activity[a]
Part A:	
PPP[b]	154
PPP+collagen	140
PPP+arachidonate	144
PRP[c]	160
PRP+collagen	153
PRP+arachidonate	328
PRP freeze-thawed	143
Part B:	
PRP+collagen	111
PRP+arachidonate	117

[a]Lactate dehydrogenase activities (international units/liter) are expressed as the mean of 2 determinations.

[b]Platelet poor plasma.

[c]Platelet rich plasma.

Lettered footnotes

a. Helena Laboratories, Beaumont, TX.

b. Sigma Chemical Co., St. Louis, MO.

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APPENDIX B

Salicylic acid concentrations in porcine plasma

Introduction

A commercial kit for the quantitative, colorimetric determination of serum and plasma salicylic acid concentration was purchased for use in a porcine research project. Before it was used, however, the ability of this test to accurately determine salicylic acid concentrations in porcine plasma was evaluated.

Materials and methods

Sample preparation-A commercial kit used to determine salicylic acid concentrations in human serum and plasma[a] and a series of salicylic acid standards[a] (12, 25, 50 and 100 mg/dl) were purchased. Five piglets were bled from the jugular vein or carotid artery into evacuated tubes containing EDTA. Plasma was obtained by centrifugation of the anticoagulated blood samples and pooled.

Experimental design-The design of this experiment follows the original work in human serum and urine by Trinder.[1] A series of plasma samples with known salicylate concentrations was obtained by mixing equal volumes of pooled plasma with each of the salicylate standards. A

plasma blank was prepared by mixing equal volumes of pooled plasma with distilled water. These prepared samples were then routinely processed for determination of salicylate concentrations following the manufacturer's directions. The % recovery was calculated as the mean of observed concentrations minus the plasma blank concentration and then divided by the expected value.

% recovery=[(mean observed - blank) ÷ expected] x 100%

Results

The results are presented in Table 9. The coefficient of variation at the 7.5 and the 12.5 mg/dl salicylate concentrations were 2.4 and 2.7% respectively.

Discussion

This colorimetric test for the determination of salicylate concentrations in plasma is simple, rapid (5 minutes/sample) and provides reproducible results. This test appears applicable to salicylic acid determinations in porcine EDTA plasma.

Lettered footnote

a. Sigma Chemical Co., St. Louis, Mo., USA.

TABLE 9. Recovery of salicylic acid added to porcine plasma

Salicylic acid added (mg/dl)	Number of samples	Mean (mg/dl)	Recovery[a] (%)
0	2	0.35	....
7.5	4	7.80	99.3
12.5	4	12.70	98.8
25.0	2	25.51	100.6
50.0	2	48.26	95.8

[a] $r=0.999$.

Reference

1. Trinder P. Rapid determination of salicylate in biological fluids. *Biochem J* 1954;57:301-303.

APPENDIX C

The in vitro effects of aspirin and sodium salicylate on
porcine platelet aggregation

Introduction

Aspirin (acetylsalicylic acid) in a humid environment
gradually hydrolyzes to salicylic and acetic acids.[1] The
half-life of aspirin in the blood of man is 15-20 minutes
while the half-life of the metabolite salicylic acid is 4-5
hours.[2]

Pigs receiving oral aspirin have a depressed collagen-
induced maximal platelet aggregation response.[3]

The purpose of this study was to examine the effects of
aspirin and sodium salicylate on maximal % platelet
aggregation induced by various doses of collagen and high
dose (6.9 mM) sodium arachidonate.

Materials and methods

Sample preparation-Blood was collected from two mature
gilts belonging to a herd maintained indoors at the College
of Veterinary Medicine, Iowa State University. The blood
was collected into plastic syringes containing 3.8%
trisodium citrate as 9 volumes of blood to 1 volume of
citrate. Platelet rich plasma (PRP) and platelet poor

plasma was prepared as described previously.[3] Platelet aggregations were performed in duplicate and the mean maximal % aggregation was calculated. Aspirin[a] (molecular weight=180.15) was dissolved in sterile normal saline (pH=5.5) to provide a 4.5 mM stock solution. (This provided a final aspirin concentration of 500 μM in the PRP). Dilutions were made of the aspirin stock to provide PRP final aspirin concentrations of 250, 100 and 50 μM. Salicylic acid, sodium salt[a] (molecular weight=160.1) was also dissolved in sterile normal saline to provide a 2.25 μM stock solution which then provided a 250 μM final sodium salicylate concentration in the PRP. Both salicylate stock solutions were prepared the morning of the experiment and used within 2-5 hours of preparation. The PRP (400 μl; approximately 220,000 platelets/μl) was incubated with 50 μl of saline or one of the concentrations of salicylate for 3 minutes at 37° C before aggregation was begun. Platelet aggregating agents were collagen[b], 5 or 10 μg/ml or 6.9 mM sodium arachidonate.[a] Platelet aggregation was monitored and maximal platelet aggregation within the first 5 minutes after the addition of the aggregating agent was recorded.

Results

Aspirin, at all levels, markedly reduced 5 μg/ml collagen-induced platelet aggregation. Aspirin suppression of 10 μg/ml collagen-induced platelet aggregation was obvious but less dramatic. Sodium salicylate did not suppress collagen-induced platelet aggregation (Table 10).

Neither aspirin or sodium salicylate suppressed sodium arachidonate induced platelet aggregation (Table 11).

TABLE 10. Collagen-induced platelet aggregation

Inhibitor	5 μg/ml collagen		10 μg/ml collagen	
	Gilt A	Gilt B	Gilt A	Gilt B
Normal saline				
	64.6[a]	62.6	75.8	74.4
Aspirin (μM)				
50	20.5	22.6	68.8	53.8
100	19.0	21.2	55.9	53.8
250	16.1	18.8	53.8	43.9
500	22.8	18.4	54.8	47.2
Sodium salicylate (μM)				
250	73.3	61.4	77.2	77.2

[a]Maximal % aggregation, mean of 2 runs.

TABLE 11. Sodium arachidonate-induced platelet aggregation

Inhibitor	6.9 mM sodium arachidonate	
	Gilt A	Gilt B
Normal saline		
	23.4^a	87.8
Aspirin (μM)		
250	13.4	87.3
Sodium salicylate (μM)		
250	15.4	87.7

[a]Maximal % aggregation, mean of 2 runs.

Discussion

These results have an excellent correlation with previously described effects of aspirin on porcine platelet aggregation.[3] The degree of suppression of collagen-induced aggregation is greater in vitro than in vivo. This was not unexpected.

Aspirin treatment, in vivo, would rapidly produce a mixture of acetylsalicylic acid and salicylic acid. In rats oral aspirin dosing resulted in peak aspirin concentrations immediately after administration. After only 5 minutes plasma salicylic acid concentrations were 20 times greater

than aspirin concentrations.[4] In man, approximately 60% of absorbed ingested aspirin is converted to salicylic acid in the first pass through the liver.[5] Salicylic acid then interferes with the platelet inhibitory effects of aspirin.[2]

The difference in the timing of the aspirin treatments between the in vivo and in vitro experiments may have played a role in the difference in degree of inhibition but this is unlikely. In man inhibition of platelet prostaglandin synthesis persists for approximately 48 hours after a single dose of aspirin[6,7] suggesting that oral aspirin inactivates megakaryocyte cyclo-oxygenase as well as irreversibly inhibits platelet cyclo-oxygenase.[7]

Suppression of collagen-induced platelet aggregation was more evident at the lower concentration of collagen. In man collagen concentrations greater than 2 μg/ml can bring about the platelet reaction without activation of the arachidonic acid pathway.[8] The results presented here would suggest that this is also true for the pig.

I do not know why gilt A was hyporesponsive to arachidonic acid-induced platelet aggregation. One possibility is that these aggregations were performed last and some loss of platelet function may have occurred. This seems unlikely since gilt B, run in parallel, did not exhibit this decrease in reactivity. Another possibility is

that I inadvertently contaminated gilt A's PRP pool with a small amount of an aggregation agent after the collagen-induced platelet aggregation study. This also seems unlikely since the pipette tips for the PRP were different than those used for the aggregating agents and I could not have easily misused one in place of the other.

High dose arachidonic acid-induced platelet aggregation was not inhibited in pigs given oral aspirin.[3] The results of this limited study would support this in that neither aspirin or sodium salicylate appeared to inhibit "normal" sodium arachidonate-induced platelet aggregation. Indomethacin, another common cyclo-oxygenase inhibitor, did not inhibit 6 mM arachidonic acid-induced platelet aggregation in rats.[9] The author of this study concluded that arachidonic acid, at this high level, directly triggered the platelet release reaction.

In summary, in vitro platelet inhibition by aspirin strongly supports the previous report on the effects of oral aspirin on porcine platelet aggregation.

Lettered footnotes

a. Sigma Chemical Co., St. Louis, Mo.

b. Helena Laboratories, Beaumont, Tx.

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