



In vitro canine platelet aggregation caused by *Dirofilaria immitis* extract

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ABSTRACT. Platelet function hyper-activity has been reported in *Dirofilaria immitis* (heartworm, HW)-infected dogs. Although the mechanism of increased platelet hyper-activity has not yet been elucidated, it is suggested to be mediated by unknown factors, which may be related to adult HW components. This study aims to determine whether adult male HW whole body extract induces canine platelet aggregation *in vitro*. The results indicate that HW extract caused an aggregation of canine platelets in a concentration-dependent manner. This aggregation ability of the HW extract was not mediated by the adenosine diphosphate receptor. In addition, the mechanisms of aggregation did not require cyclooxygenase-dependent pathways, and the aggregating activity of substances contained in the HW extract was heat stable; therefore, the active substances may be different from collagen. Furthermore, the platelet aggregating activity remained within the molecular weight (MW) $\geq 100,000$ fraction obtained by ultrafiltrating the HW extract. In contrast, the MW $< 100,000$ fraction also had a platelet aggregation ability, but the aggregation pattern was reversible and the maximum extent decreased, compared with the MW $\geq 100,000$ fraction response. Our experiments have been conducted using a whole body extract from adult HWs to determine with certainty the aggregating activity of HW elements on canine platelets. More studies are necessary to evaluate the effects of the metabolic products released from live adult worms in pulmonary arteries and the symbiont bacterium *Wolbachia*-derived antigens on canine platelet aggregation.

KEY WORDS: *Dirofilaria immitis*, dog, heartworm extract, platelet aggregation

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Dirofilaria immitis (heartworm, HW) is the causal agent of canine cardiopulmonary dirofilariosis, a serious and potentially life-threatening disease. Adult HWs reside in pulmonary arteries and occasionally in the right heart chambers of dogs. Initially, the presence of adult worms in pulmonary arteries causes a proliferation of the intima with a subsequent narrowing of the vessels and perivascular inflammation. Moreover, the death of the worms, either spontaneously or by an adulticidal treatment, causes acute adverse events characterized by a thromboembolism [14, 21].

A thromboembolism is initiated as a result of platelet activation [24]. Although obvious thrombus formations have not been observed while HWs are alive [21], platelet function hyper-activity has been reported in HW-infected dogs [1, 2, 6, 17, 20]. The platelets of infected dogs showed an enhanced adherence to filarial surfaces, irrespective of the presence of circulating microfilariae [6], and an increased aggregation to adenosine diphosphate (ADP) or collagen *in vitro* [1, 2]. In addition, the number of platelet dense granules containing serotonin, calcium and non-metabolic pools of ADP was reduced [20]. Furthermore, the serum concentration of thromboxane B₂ (TXB₂, the stable hydrolysis product of TXA₂) was very high [17], and the plasma antithrombin (AT) activity was low [1] in HW-infected dogs compared with non-infected dogs. These findings indicate that HW-infected dogs had thrombophilia (i.e., a prothrombotic state), as reflected by their enhanced platelet reactivity and tendency toward both an increased serum TXB₂ level and decreased AT activity.

The mechanism of the increased platelet reactivity in HW-infected dogs has not yet been elucidated. However, it has been suggested to be mediated by unknown factors, which may be related to adult HW components but not to microfilaria that reside in the plasma of the infected dogs [2, 6]. Moreover, saline-soluble collagen-rich fractions of the equine filarial parasite *Onchocerca cervicalis* can induce platelet aggregation and serotonin release *in vitro* [15]. All these data are consistent with the hypothesis that adult HW-soluble components may possess platelet aggregation ability. Furthermore, adult HW-soluble components may be

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metabolic products of HWs and the substances released into the blood stream due to the death of HWs, because HW infection could exist in two conditions in the infected dogs: (1) a condition in which only live adult HWs reside in the pulmonary arteries; (2) a condition in which the live adult HWs coexist with some dead worms in the pulmonary arteries. Thus, this study aimed at determining whether HW whole body extract induces canine platelet aggregation *in vitro*.

MATERIALS AND METHODS

Preparation of the HW extract

The HW extract was prepared from adult male HWs, which did not comprise microfilariae components, obtained from HW-infected dogs. The collected HWs were washed several times with physiologic saline (0.9% sterilized NaCl solution) and kept at -30°C until used for the preparation. Five frozen uninjured HWs were thawed and cut into small fragments using scissors. They were then mashed with a mortar and sonicated at 30 sec \times 5 times in 1 ml of veronal buffer (5.88 g/l sodium diethylbarbiturate and 7.34 g/l sodium chloride, pH 7.4). The worm suspension was centrifuged at $10,000 \times g$ for 60 min, and the supernatant solution was collected. All steps were conducted at 4°C . The stock solution, of which 1 ml was equivalent to the volume extracted from 5 HWs, was stored in units of 0.1 ml at -80°C until used for the platelet aggregation assay (see below).

Since cyclooxygenase (COX) products, such as prostaglandins (PGs) and TXs, might be produced during the HW extract preparation [7], HWs were treated with 10 μM indomethacin, a COX inhibitor, in the veronal buffer at room temperature for 30 min [18] before preparation of the HW extracts in the same way as above. The indomethacin-treated HW extracts were stored at -80°C until they were compared with the non-treated HW extracts.

Preparation of canine platelets

Blood samples were collected from 12 healthy, HW-free beagles (6 sexually intact females and 6 sexually intact males, aged approximately 3–10 years, weighing 7.6–12.6 kg), who had not received any medications during the preceding 2 weeks, and mixed in a one-ten volume of 3.2% sodium citrate solution. Platelet-rich plasma (PRP) was obtained by centrifugation at $100 \times g$ for 5 min at room temperature. After collecting the PRP, the remaining samples were recentrifuged at $800 \times g$ for 10 min at room temperature, and the supernatant was collected as platelet-poor plasma (PPP). Platelet counts in the PRP were determined by the use of an automated hematology analyzer (PCE-170, ERMA, Tokyo, Japan) and adjusted to a concentration of $2\text{--}3 \times 10^8$ platelets/ml by the addition of autologous PPP. The PRP was allowed to sit undisturbed at room temperature until used for aggregation studies. The study was performed in compliance with the Gifu University Guidelines for Animal Experimentation.

Determination of platelet aggregation

Platelet aggregation was determined by a standard turbidimetric method [10, 22, 24] using an aggregometer (PAT-2M, Niko Bioscience, Tokyo, Japan). Platelet aggregation was expressed as an increase in light transmission, the levels of which were calibrated as 0% for PRP and 100% for PPP. PRP ($2\text{--}3 \times 10^8$ platelets/ml, 0.2 ml) in a cuvette was pre-incubated at 37°C for 3 min under continuous stirring at 1,000 rpm. After pre-incubation, platelet aggregation was initiated by the addition of appropriate concentrations of HW extract (22 μl of stock solution finally diluted 10 times by volume), ADP (final concentration 40 μM) or collagen (final concentration 2.5 $\mu\text{g}/\text{ml}$) and monitored for 5 min. The aggregation was expressed as the percent of maximal aggregation. This value was calculated by the following formula:

$$(\text{aggregation value for the sample} / \text{aggregation value obtained for } 40 \mu\text{M of ADP}) \times 100 (\%)$$

Effect of a heat treatment on platelet aggregation induced by a HW extract

To determine whether the platelet aggregating activity present in the HW extract was stable, the influence of a heat treatment on the HW extract and platelet aggregation was studied. A heat-treated HW extract was prepared as follows. The stock solution (non-treated HW extract) was incubated at 100°C for 10 min and centrifuged again at $10,000 \times g$ at 4°C for 60 min. The supernatant solution was collected and kept as a heat-treated HW extract at -80°C until it was compared with the non-treated HW extracts. As a control, platelet aggregation by collagen (final concentration of 2.5 $\mu\text{g}/\text{ml}$) with or without a heat-treatment was observed.

Involvement of COX-dependent pathways and the ADP receptor on canine platelet aggregation induced by a HW extract

To examine the involvement of COX-dependent pathways and the ADP receptor on canine platelet aggregation induced by a HW extract, the following tests were conducted. Firstly, after the pre-incubation of indomethacin (300 μM) [11] at 37°C for 3 min, platelet aggregation was initiated by the addition of 22 μl of HW extract stock solution or 10 $\mu\text{g}/\text{ml}$ (final concentration) of collagen (control) and monitored for 5 min. Next, ATP, an antagonist of P2Y (ADP) receptor, was pre-incubated with PRP at a final concentration of 1 mM for 3 min [9]. Then, 22 μl of the HW extract or 40 μM ADP (control) were added to the pre-mixed PRP.

Ultrafiltration of the HW extract

To estimate the relative size of the soluble HW-derived mediators involved in platelet aggregation, the HW extract was separated with a molecular weight (MW) limit of 100,000 by ultrafiltration using a Microcon YM-100 centrifugal filter device (Millipore, Billerica, MA, U.S.A.). Briefly, 100 μl of the HW extract was added to the device and centrifuged at $500 \times g$ for 15 min at room temperature. After centrifugation, the filtrated liquids (including substances with a $\text{MW} < 100,000$) and the remaining liquids (including substances with a $\text{MW} \geq 100,000$) were collected and subjected to a platelet aggregation assay, respectively.

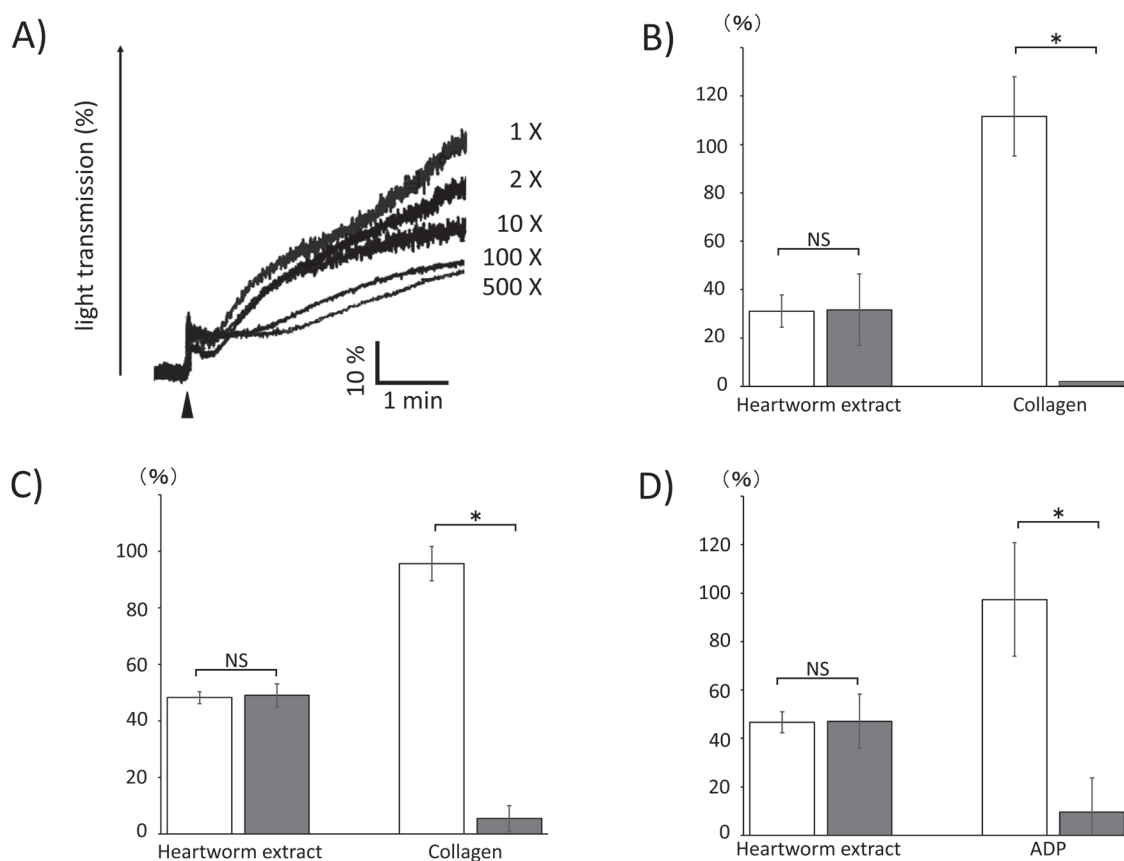


Fig. 1. Heartworm extract-induced platelet aggregation. A, canine platelets are stimulated with serially diluted heartworm (HW) extract at 37°C under stirring conditions. Platelet aggregation was measured using the conventional turbidimetric assay. The arrowhead indicates the time of the extract addition. The traces are representative data from 11 ($\times 1$, not diluted) or 3 ($\times 2$, 10, 100 and 500, serial dilutions) independent experiments. B–D, the aggregation value is expressed as (aggregation value for sample/aggregation value obtained for 40 μM of ADP) $\times 100$ (%). Graphs represent the mean \pm SD from at least 3 different experiments (*, $P < 0.05$). B, aggregation value of platelets induced by a heat-treated HW extract (22 μl) and collagen (final concentration 2.5 $\mu\text{g}/\text{ml}$). Open and solid bars indicate non-treated and heat-treated samples, respectively. C, aggregation level of platelets pretreated with indomethacin (300 μM) induced by a HW extract and collagen. Open and solid bars indicate PRP pretreated with a solvent control (ethanol) or indomethacin, respectively. D, aggregation value of PRP pretreated with ATP (1 mM) induced by the HW extract and ADP. Open and solid bars indicate PRP pretreated with a solvent control (deionized water) and with ATP, respectively.

Statistical analysis

Each experiment was repeated at least 3 times. Results are expressed as means \pm SD with the number of observations (n). Pair-wise comparisons were made using a Student's *t*-test where appropriate. A value of $P < 0.05$ was considered significant.

RESULTS

HW extract causes canine platelet aggregation

As shown in Fig. 1A, the HW extract was a potent inducer of irreversible platelet aggregation in citrated canine PRP. The aggregation response was observed immediately after the addition of the HW extract without the appearance of a lag phase (“shape change” period) and reached a maximum aggregation 5 min after the administration of the extract. All PRP samples collected from 11 healthy dogs also induced a similar aggregation of platelets. The maximum amplitude of platelet aggregation was $61.15 \pm 16.58\%$ (11).

When indomethacin-treated HW extracts were similarly assayed, they each elicited a substantially similar effect to that of the corresponding extract without an indomethacin treatment ($57.61 \pm 20.77\%$ (3) vs. $61.15 \pm 16.58\%$, not significant).

Then, having diluted the extract with the veronal buffer to make serial dilutions of 2, 10, 100 and 500 times, the maximum amplitudes of the platelet responses were decreased in a dilution ratio-dependent manner (Fig. 1A). Moreover, the aggregation proceeded more slowly, especially for the 100 times and 500 times diluted extracts.

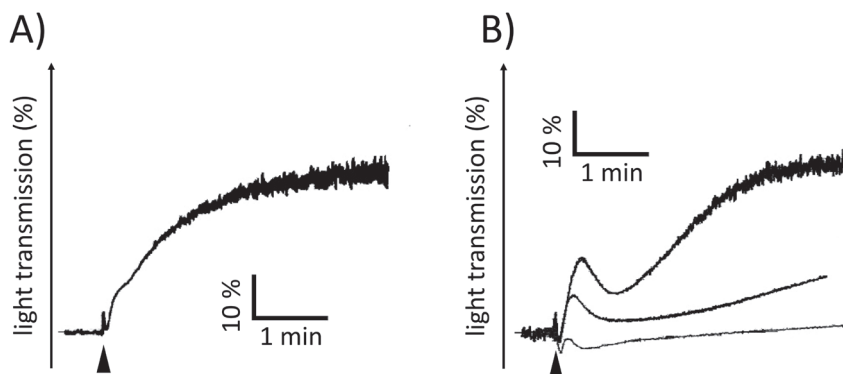


Fig. 2. Platelet aggregation induced by an ultrafiltrated HW extract. The arrowhead indicates the time of the extract addition. The traces are representative data from at least 3 independent experiments. A, typical aggregation patterns by the addition of a molecular weight (MW) $\geq 100,000$ fraction of HW extract. B, typical aggregation changes by a MW $< 100,000$ fraction.

Effect of a heat treatment on platelet aggregation induced by a HW extract

As shown in Fig. 1B, a heat-treated HW extract (100°C for 10 min) showed a similar activity to the non-treated HW extract. In contrast, heat-treated collagen completely lost the ability to induce platelet aggregation. These results indicate that the factors in the extract inducing platelet aggregation are heat-resistant and are other than collagen.

Involvement of COX-dependent pathways and the ADP receptor on canine platelet aggregation induced by a HW extract

Platelets are activated in either a COX-dependent or COX-independent pathway [3, 24]. To determine whether COX-dependent pathways may be involved in HW extract-induced platelet activation, we used the selective COX inhibitor, indomethacin [11, 18]. Canine PRP pretreated with indomethacin had no reduction in aggregation compared to full inhibition of aggregation in collagen (Fig. 1C). This suggests that COX-dependent pathways are not required for the activation of platelets induced by a HW extract, presumably through the feedback action of TXA₂.

ADP plays a pivotal role in hemostasis and thrombosis as an important physiological agonist which activates platelets through ADP receptors and induces platelet aggregation [19]. The involvement of P2Y (ADP) receptor on platelet aggregation induced by a HW extract was examined. Thus, ATP, an antagonist of P2Y receptor, was pre-treated with PRP at a final concentration of 1 mM. As shown in Fig. 1D, ATP inhibited platelet aggregation induced by ADP, but not platelet aggregation induced by a HW extract. These results indicate that platelet aggregation induced by a HW extract was ADP receptor-independent.

Separation of the soluble HW-derived mediators involved in platelet aggregation

When the HW extract was separated with a MW cutoff of 100,000, the MW $\geq 100,000$ fraction induced platelet aggregation (Fig. 2A). The pattern and the maximum extent of the aggregations were similar to those induced by the non-separated extract (Figs. 1A and 2A). Although the MW $< 100,000$ fraction also induced platelet aggregation, the aggregation pattern and the maximum extent were variable among the 3 dogs that were tested (Fig. 2B). Namely, the aggregation pattern was reversible, which was not observed with the non-separated PRP or with the MW $\geq 100,000$ fraction, and the maximum extent was decreased (Fig. 2B).

DISCUSSION

Our results showed that the whole body extract obtained from adult HWs caused an aggregation of canine platelets in a concentration-dependent manner. The platelet aggregating activities remained in an indomethacin-treated HW extract, indicating that the active substances differed from COX products that might be produced during HW extract preparation. This platelet aggregation ability of the HW extract was not mediated by the ADP receptor. The mechanisms of platelet aggregation did not require COX-dependent pathways, and the aggregating activity of substances contained in the HW extract was heat stable; therefore, the active substances may be different from collagen [3]. Furthermore, the platelet aggregating activity remained in the MW $\geq 100,000$ fraction. Conversely, the MW $< 100,000$ fraction also had platelet aggregation ability, but the aggregation pattern was reversible and the maximum extent was decreased compared with the MW $\geq 100,000$ fraction response. Thus, platelet aggregating substances in the HW extract might form homo- or hetero- polymers, and the macromolecular forms are stable. There is also a possibility that the HW extract contains more than one substance that induces platelet aggregation.

We used a HW extract, of which 1 ml was equivalent to the volume extracted from 5 HWs, that was finally diluted 10 times by volume. Although the platelet aggregating activities of the HW extract decreased gradually as its dilution with the buffer increased, it should be noted that the activity remained in the extract that was diluted 500 times by volume (finally diluted 5,000 times). If the platelet aggregating substances of HWs might be released into the circulation, these substances can diffuse and are diluted. As

a result, the diluted active substances in the circulation weakly stimulate platelets. When the stimulus is weak, platelets become hyper-responsive but do not aggregate. It can be said that the weak stimulation of the diluted active substances in the circulation resulted in the enhanced platelet reactivity/sensitivity to ADP and collagen *in vitro*, as previously reported by Boudreaux *et al.* [2]. In addition, it is possible that a more continuous or strong stimulation due to the death of a few worms causes degranulation of the platelets, resulting in the release of TXA₂, serotonin and ADP. Accordingly, the production of thrombin and the subsequent formation of thrombosis occur, resulting in a decrease in plasma AT activity [1] and an increase in the plasma D-dimer concentration [4, 5].

Our experiments have been performed using a whole body extract from adult HWs to determine with certainty the aggregating activity of HW elements on canine platelets. Although these results contribute to our understanding of the effects on canine platelets caused by the simultaneous death of a massive number of worms, either naturally or induced by a filaricide treatment, we cannot examine the effects of the metabolic products released from live adult worms in pulmonary arteries. In addition, the whole body extract prepared from adult male HWs did not comprise the microfilariae components, but the molecules of *Wolbachia* [16]. The symbiont bacterium *Wolbachia* is present in the HW body and has been implicated in the modulation of the host inflammatory and immune responses during infection [23]. Therefore, the results observed in this study might also be the effects of *Wolbachia* components on canine platelets.

In contrast to our findings in HWs, the microfilariae of *Brugia malayi*, a human filarial parasite, inhibited platelet aggregation [8, 12]. They showed that the microfilariae of *B. malayi* utilized both exogenous and endogenous arachidonic acid to generate COX-derived, anti-aggregatory eicosanoids, such as prostacyclin and PGE₂ [13]. To our knowledge, there have been no reports on the inhibition of platelet aggregation induced by the extract of adult worms or the microfilariae of HWs. A further study of whether the HW extract might inhibit platelet aggregation of the host should be conducted.

In conclusion, we have demonstrated for the first time that the whole body extract from adult male HWs induces canine platelet aggregation *in vitro*. More studies are necessary to determine whether the metabolic products, such as excretory/secretory products, excreted by live adult HWs or the *Wolbachia* components might cause platelet aggregation, respectively. Further studies are needed to elucidate the signaling mechanism involved in HW extract-induced platelet aggregation.

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