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Letter

Paraphenylene diamine exacerbates platelet aggregation and thrombus formation in response to a low dose of collagen

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ABSTRACT — Paraphenylene daimine (PPD) is an aromatic amine that is widely used in several industrial products; however, its toxicity has been reported in several cases of cardiac arrests. As platelets play a key role in cardiovascular diseases, we aimed to determine the impact of PPD *in vitro* and *in vivo* on platelet function. Our findings demonstrated that platelet activation and aggregation were strongly enhanced by PPD. Treatment with PPD primed human platelets that became more reactive in response to low doses of collagen. Furthermore, PPD exacerbated thrombus formation in rats in comparison with those untreated. Our results suggest that PPD is an important platelet primer predisposing platelets to promote thrombus formation in response to vascular injury. This should prompt the authorities to consider controlling the marketing of this product.

Key words: Paraphenylene diamine, Toxicity, Platelet, Aggregation, Thrombus formation

INTRODUCTION

Para-phenylenediamine (PPD) is an aromatic amine widely used in the pharmaceutical, chemical, rubber, dye, textile, and photographic industries (Dressler and Appelqvist, 2006; Hueber-Becker *et al*., 2007). Many cases of toxicity and mortality either due to its accidental or deliberate ingestion were reported in Egypt, Sudan, Israel, Morocco, Saudi Arabia, India and Tunisia (Sir Hashim *et al*., 1992). PPD is well known as a skin irritant and sensitizer, and allergic contact dermatitis may occur after exposure to PPD in hair dye (Picardo *et al*., 1996; McFadden *et al*., 2011). For example, The North American Contact Dermatitis Group studied *in vivo* positive patch test results in more than 34,000 patients and found about 5% PPD responsive patients in 2002 (Nguyen *et al*., 2008).

To assess the significance of PPD allergy, a ten year review at the Ottawa Patch Test Clinic was conducted. PPD was found to be an important source of contact allergy (LaBerge *et al*., 2011).

Moreover, data from Scandinavian, central and southern European patch test centers between 2003 and 2007 revealed among 21,515 patients a weighted average prevalence for PPD sensitization of 4.6% with PPD sensitization being more frequent in southern Europe (Thyssen *et al*., 2009).

In vitro, data from Germany demonstrated that lymphocyte activation test with PPD can be used to detect PPD sensitization as a possible alternative to patch testing

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at least in patients with severe allergic reactions to PPD (Kneilling *et al*., 2010).

Other studies have demonstrated that at non cytotoxic concentration PPD induced intracellular adhesion molecule 1 (ICAM 1) expression on keratinocytes (Piguet *et al*., 1991).

Platelets are one of the cellular elements of blood which have widely established function in hemostasis. Lower counts in peripheral blood can lead to bleeding episodes, ranging from minor bleeds to life threatening one. A normal human platelet count ranges from 150,000 to 400,000 per microliter of blood. The exact mechanisms involved in thrombocytopenia due to PPD has not been studied or reported but appears to be the combination of direct toxic effect to platelets leading to their accelerated killing and effect on bone marrow leading to ineffective or suppressed production (Burnett *et al*., 1977; Hopkins and Manoharan, 1985). To this end we first evaluated *in vitro* the effect of PPD on human platelets and then we investigated to assess its effect on platelets and formation of pulmonary micro-emboli in rat in response to collagen.

MATERIALS AND METHODS

Reagents and antibodies

Native type I collagen were from Chronolog Corp. (Havertown, PA, USA), PPD and thrombin were purchased from Sigma-Aldrich (Oaskville, ON, USA), P-PLCγ-2 Tyr759 and β-actin antibodies were obtained from Cell Signaling Technology (Beverly, MA, USA).

Experimental animals and care

Male and female Sprague-Dawley rats, 9-10 weeks old (240-260 g) were purchased and housed in the animal facility of the faculty of Sciences Semlalia, Marrakesh, Morocco. Handling and care of rats were conducted in conformity with approved institutional protocols and in accordance with the provisions for animal care and use described in the Scientific Procedures on Living Animals ACT 1986 (European Council directive: 86/609 EEC). All efforts were made to minimize animal suffering.

Preparation of human platelets

Venous blood was drawn from healthy volunteers, free from medication known to interfere with platelet function for at least 10 days before the experiment, in accordance with the guideline of the ethical committee of the Montreal Heart Institute. The platelets were prepared as previously described (Théorêt *et al*., 2001; Caron *et al*., 2002). Briefly, the platelets were obtained by series of differential centrifugations and adjusted to a final concentration of 250×10^6/mL using an automated cell counter in which purity exceeded 99%. The platelets were allowed to stand at 37°C for 30 min before further experiments.

Platelet aggregation

Aggregation of washed platelets was monitored on a four-channel optical aggregometer (Chronolog Corp.) (Théorêt *et al*., 2001; Caron *et al*., 2002). The platelets were preincubated with a dose of 20 μg/mL of PPD for 5 min at 37°C. Several doses of PPD (5, 20 and 50 μg/mL) were used prior to the aggregation experiment in order to find an appropriate dose (data not shown). The samples were then stimulated with collagen under continuous stirring (1,000 rpm) at 37°C. Platelet aggregation was monitored until stabilization of aggregation traces.

SDS-PAGE and immunoblotting

For protein quantification, platelets were homogenized in Laemmli and lysis buffer. In brief, protein extracts were assayed, loaded (15 to 30 μg) in 10% SDS-polyacrylamide gels, separated by electrophoresis and transferred to nitrocellulose membranes. Membranes were incubated (1 hr) in a blocking buffer containing 5% non-fat milk in Tris-buffered saline (TBS) + Tween 20 (TBS-T, 0.1%), then blotted overnight at 4°C with anti-p-PLCγ2 Tyr^{759}(Cell Signaling Technology) (or anti-β-actin to assess equal protein loading). Following washing steps, membranes were labeled with horseradish peroxidase-conjugated secondary antibody for 1 hr, washed and bound peroxidase activity was then detected by enhanced chemiluminescence (PerkinElmer Life Sciences, Akron, OH, USA). Band intensities were assessed by densitometry quantification.

Tail-bleeding time and platelet count

Collagen (2.5 μL/g of animal weight) at low (0.25 μg/mL) and high (2 μg/mL) doses was injected, after 5 min of preincubation or not with PPD (1 mg/kg or approximately 200 μg/rat to obtain a final concentration of 20 μg/mL), into the jugular vein of anesthetized rats. Rat tails were transected from the tip and then immediately immersed into a 0.9% isotonic saline solution. The bleeding time was defined as the time required for cessation of blood flow. Blood was then collected and platelet count was determined with a Coulter counter (Beckman Coulter).

Pulmonary emboli

Following tail-bleeding time measurements, lungs were excised, fixed in 1% formalin and analyzed for

the presence of micro-emboli. Briefly, sections were stained with Hematoxylin-Eosin, visualized using an Olympus BX61 microscope (Olympus Imaging America Inc., Richmond Hill, ON, Canada) and images were captured with a digital camera (DP 71), and analyzed by the Image Pro Plus 6.2 software (Media Cybernetics, Rockville, Warrendale, USA). Microscopic evaluations were done randomly and supervised by a pathologist.

Statistical analysis

The experiments were performed at least three independent times. The results are presented as the means ± S.E. Statistical comparisons were done using a one-way ANOVA, followed by a Dunnett's test for comparison against a single group. Data with $P \leq 0.05$ were considered statistically significant.

RESULTS

PPD triggers platelet activation and aggregation in response to a priming dose of collagen

We first evaluated the functional effects of PPD on platelet activation and aggregation, as it remains poorly characterized. Incubation of platelets with PPD alone had no effect on platelet aggregation but led to a significant increase of platelet aggregation induced by a subthreshold or priming concentration of collagen (Fig. 1A), indicating that this is a broad platelet phenomenon and not agonist-specific. As PPD showed a significant impact on platelet aggregation, we sought to determine its effect on platelet activation. As expected, PPD induced a significant increase PLCγ-2 phosphorylation in Tyr^{759} in response to a low dose of collagen (Fig. 1B).

Effect of PPD on platelet function *in vivo* in response to a priming dose of collagen

In view of the important potential role in pathophysiological conditions, the effect of PPD on platelets has yet to be explored *in vivo*. In the present study, we demonstrate that rats pretreated with PPD prior to injection of a low dose of collagen show a significant reduction in the tail bleeding times, confirming the *in vitro* potentiation of platelet function due to PPD activity in response to a priming dose of collagen (Fig. 2). In contrast, separate injection of collagen or PPD in a low dose (1 mg/kg or approximately 200 μg/rat) in rats was without any significant effect on bleeding times. A high dose of collagen (2 μg/mL) was used as a positive control.

This reduction also correlates with a significant reduction in platelet counts in rats injected with PPD following a priming dose of collagen (Fig. 3). These results highlight the *in vivo* effect of PPD in platelet function in response to suboptimal concentrations of collagen.

PPD exacerbates thrombus formation

To date, no direct correlation between PPD and thrombosis has been established, although this could be of important clinical and physiopathological relevance. To explore this aspect, we assessed thrombo-emboli formation in rats in response to collagen, as it has previously been documented that a decrease in platelet counts cor-

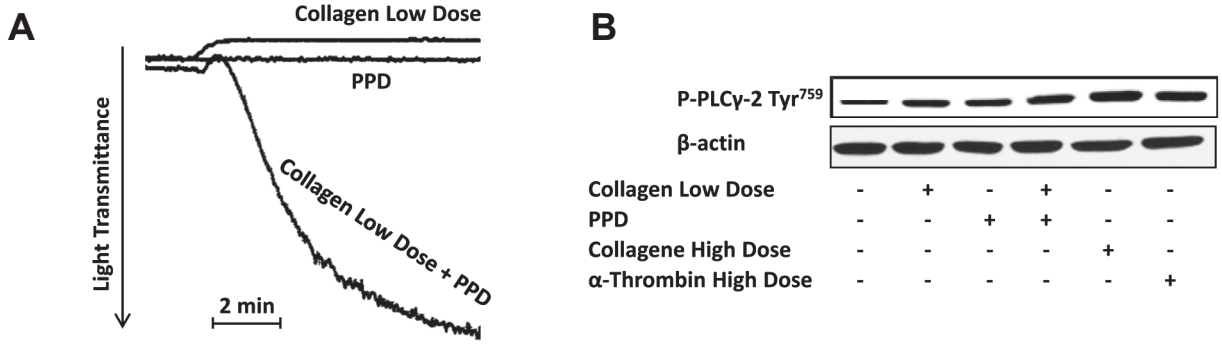

Fig. 1. PPD enhances platelet activation and aggregation in response to a priming dose of collagen. A, Effect of PPD on human platelet aggregation. Platelets were incubated with 20 μg/mL of PPD for 5 min at 37°C and aggregation was induced by a priming dose of collagen (0.25 μg/mL). Aggregation traces are representative of 3 independent experiments. B, PLCγ-2 phosphorylation is increased following treatment with PPD in response to a priming dose of collagen. PLCγ-2 phosphorylation was detected from platelets untreated (control) or preincubated with PPD (20 μg/mL) for 5 min at 37°C. Platelets were then stimulated by a priming dose of collagen (0.25 μg/mL). Platelets were also stimulated by a high doses of collagen (2 μg/mL) or α-thrombin (0.5 U/mL), which were used as a positive control. Platelet lysates were then analyzed by SDS-PAGE for p-PLCγ-2 Tyr^{759}. Blots are representative of 5 independent experiments.

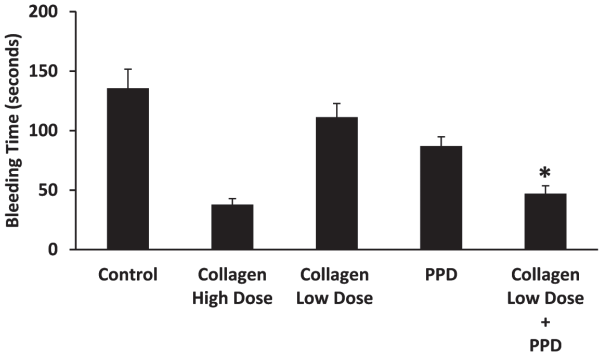

Fig. 2. PPD reduces bleeding time in response to a priming dose of collagen. Rats were injected into the jugular vein with 20 µg/mL of PPD or its vehicle (control) for 5 min prior to injection of a priming dose of collagen (0.25 µg/mL). A high dose of collagen (2 µg/mL) was used as a positive control. The bleeding time was then assessed and defined as the time required for cessation of blood flow. Histogram represents the mean of data ± S.E.M. of plots for bleeding time (n = 8); *P < 0.05 *versus* Control.

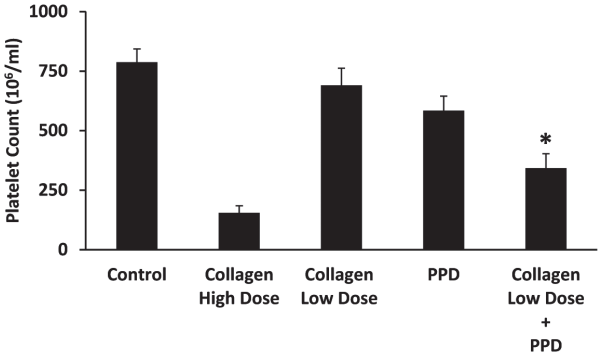

Fig. 3. PPD reduces platelet count in response to a priming dose of collagen. Rats were injected into the jugular vein with PPD or its vehicle (control) for 5 min prior to injecting a priming dose of collagen (0.25 µg/mL). A high dose of collagen (2 µg/mL) was used as a positive control. Whole blood was then retrieved by heart puncture and assessed with a Coulter counter. Histogram represents the mean of data ± S.E.M. of plots for bleeding time (n = 8); *P < 0.05 *versus* Control.

relates with an increase in emboli formation (Mustafa *et al*., 1989). As shown in Fig. 4, injection of PPD (1 mg/kg or approximately 200 µg/rat) prior injection of a low dose of collagen was associated with a significant increase in thrombus mass in the lung vessels of in comparison with untreated ones. A high dose of collagen (2 µg/mL) was used as a positive control. These results establish a direct *in vivo* correlation between PPD and arterial thrombosis.

DISCUSSION

PPD is an aromatic amine which undergoes autoxidation with the generation of shortlived free radicals as well as reactive oxygen species such as superoxide and hydrogen peroxide (Picardo *et al*., 1990). However, the major product formed is Bondrowski's base which is allergenic, mutagenic and highly toxic (Ashraf *et al*., 1994).

PPD has gained much attention over the years for its involvement in cardiac toxicity. Moreover, in numerous clinical reports of PPD toxicity, cardiac arrest was the main cause of death, which was attributed to arrhythmia. Most notably ventricular tachyarrhythmia including ventricular fibrillation has been the major feature of PPD cardiac toxicity (Lifshits *et al*., 1993), (Ashraf *et al*., 1994). However, no studies have addressed the effect of PPD on platelet function.

This study provides the first experimental evidence that PPD could cause platelet thrombus formation. In fact, we show that PPD primes platelets and enhances aggregation in response to a priming dose of collagen.

Phospholipase C gamma-2 (PLCγ-2) has been implicated in collagen-induced signal transduction in platelets and antigen-dependent signaling in B-lymphocytes (Ozdener *et al*., 2002)

Our results show an increase in the phosphorylation of PLCγ-2 on Tyr^{759} site, following treatment with PPD and stimulation with low doses of collagen, suggesting the involvement of PPD in platelet activation.

The present study suggests that PPD positively influences thrombosis and hemostasis in an *in vivo* setting. Rats pretreated with PPD and that received a priming dose of collagen had decreased tail bleeding times and platelet counts as compared to untreated rats.

Our data also provide novel evidence demonstrating a direct correlation between PPD and thrombosis. Rats that received PPD before collagen stimulation showed increased thrombus formation, indicating that they were predisposed to thrombotic stimulus (platelets became more reactive).

Likewise, reactive platelets are reported in many pathological conditions, such as late-stage metastatic cancer, stable coronary artery disease, critical limb ischemia or type 2 of diabetes (Furman *et al*., 1998; Cooke *et al*., 2013; Wisman *et al*., 2015).

Our study adds new insights to previous investigations showing toxicity of PPD, (Davis, 1946; Ram *et al*., 2007;

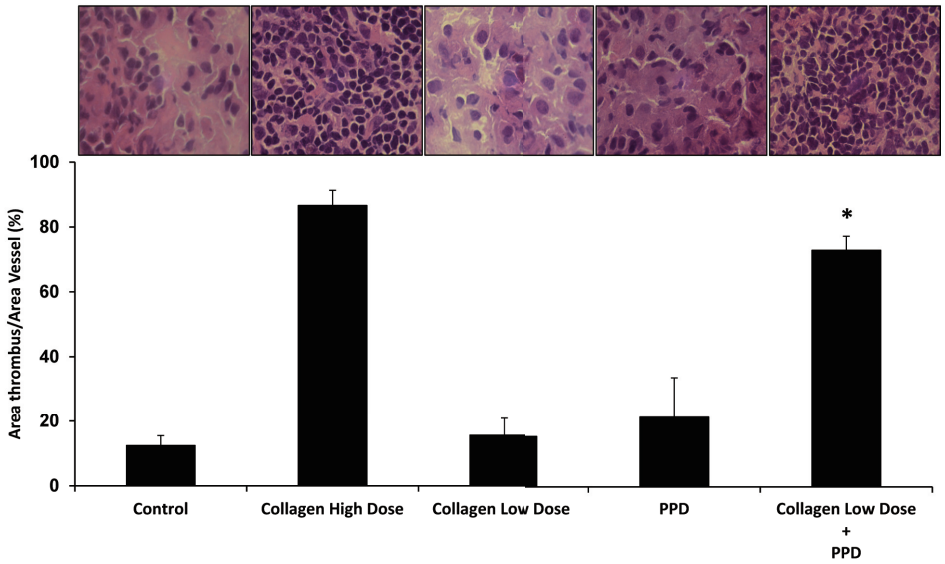

Fig. 4. PPD exacerbates pulmonary thrombo-emboli formation. (Top) Rats were initially injected with PPD (20 µg/mL) or vehicle (control) and then injected with a priming dose (0.25 µg/mL) and a high dose (2 µg/mL) of collagen into the jugular vein. Lung sections from these rats were stained with Hematoxylin-Eosin and observed by optical microscopy (magnification, × 10). Images are representative of 8 rats/group. (Bottom) Morphometrical quantification of pulmonary thrombo-emboli in rats, represented as the ratio of thrombus area over vessel area ($n = 8$); *$P < 0.05$ *versus* Control.

Abdelraheem *et al*., 2009). We therefore suggest that PPD-primed platelets are predisposed to form a platelet thrombus. Since there is no specific antidote for PPD and treatment is only supportive, we recommend that PPD is a product that should be controlled and regulatory commercialized.

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Conflict of interest---- The authors declare that there is no conflict of interest.

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