

# Inhibition of platelet aggregation by transdermal glyceryl trinitrate

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## Abstract

**Objective**—To determine the optimum conditions for the demonstration of an antiplatelet effect of nitric oxide and to use these conditions to elucidate the effects of a transdermal glyceryl trinitrate patch on platelet aggregation in normal volunteers.

**Methods**—An open prospective crossover study. The effects of nitric oxide on platelet aggregation in whole blood and platelet rich plasma as stimulated by adenosine diphosphate and U46619 was assessed in the presence and absence of iloprost and MB22948. Optimum conditions for the demonstration of an antiplatelet effect of nitric oxide were then applied to whole blood from normal volunteers in the presence and absence of a transdermal glyceryl trinitrate patch.

**Setting**—University hospital.

**Subjects**—Eight normal volunteers.

**Main outcome measures**—Platelet aggregation in the presence and absence of transdermal glyceryl trinitrate.

**Results**—The optimum conditions for the demonstration of an antiplatelet effect of nitric oxide in whole blood were collecting blood into a tube containing MB22948 and citrate and stimulating platelet aggregation with adenosine diphosphate in the presence or absence of iloprost. Using this method a significant effect of transdermal glyceryl trinitrate on platelet aggregation was shown ( $P < 0.03$ ) in the presence and absence of iloprost.

**Conclusions**—These results are consistent with an inhibitory effect on platelet aggregation of nitric oxide liberated by transdermal glyceryl trinitrate. Optimum test conditions are needed to show this effect. The clinical significance of the antiplatelet effect of transdermal glyceryl trinitrate is unknown.

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Organic nitrates have been a mainstay of treatment for angina pectoris for more than a century. Their principal mode of action is through the reduction of myocardial oxygen consumption via venodilatation with a consequent reduction in preload and an increase in myocardial oxygen supply via dilatation of the large coronary arteries.

In 1967 it was shown that pharmacological doses of glyceryl trinitrate could produce

inhibition of platelet aggregation *in vitro*,<sup>1</sup> and over the last decade it has become clear that intravenous infusions of organic nitrates cause inhibition of platelet aggregation at therapeutic doses.<sup>2-6</sup> It is thought that the *in vivo* antiplatelet effects of organic nitrates are mediated by their metabolism to nitric oxide, which stimulates soluble guanylate cyclase in platelets with synthesis of cyclic guanosine monophosphate.<sup>7</sup> It has been suggested that this effect on platelets may be responsible for at least part of the clinical benefit seen with organic nitrates in acute myocardial infarction and unstable angina.<sup>6,8</sup>

Despite the effects of glyceryl trinitrate on platelets after intravenous infusion, attempts to show an antiplatelet effect after the administration of topical or oral organic nitrates have been unsuccessful.<sup>9,10</sup>

In this study we identified the optimum conditions for detecting inhibition of platelet aggregation by nitric oxide in whole blood and then used this method to show an effect of transdermal glyceryl trinitrate on platelet aggregation in normal volunteers.

## Methods

### EXPERIMENTAL

The initial phase of this study was to identify the optimum conditions to detect the inhibition of platelet aggregation by nitric oxide in whole blood. Samples of citrated whole blood from normal volunteers, or platelet rich plasma derived from it,<sup>11</sup> were stirred at 37°C in the presence of nitric oxide and also in the presence of various agents that could be expected to amplify the effect of nitric oxide on platelet aggregation. These were MB 22948 (a cyclic guanosine monophosphate cyclic phosphodiesterase inhibitor, a gift from Rhone-Poulenc, Rorer) and iloprost (a stimulator of adenylate cyclase, a gift from Shering AG, Berlin). Platelet aggregation was induced by adenosine diphosphate, collagen, or the thromboxane A<sub>2</sub> mimetic U46619. After stopping the reaction by adding fixative, the percentage decrease in the number of single platelets in response to the aggregating agent (percentage aggregation) was determined using the Ultra-Flo 100 Whole Blood Platelet Counter.<sup>12,13</sup>

DETERMINATION OF OPTIMUM CONDITIONS FOR DETECTING INHIBITION OF PLATELET AGGREGATION BY NITRIC OXIDE IN WHOLE BLOOD

In platelet rich plasma (0.5 ml), nitric oxide

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## Platelet aggregation (percentage aggregation) in whole blood. Results are median (25th quartile, 75th quartile) values

Hours after glyceryl trinitrate patch application	Iloprost added (ng/ml)	Adenosine diphosphate			
		(1 $\mu\text{mol/l}$ )	(3 $\mu\text{mol/l}$ )	(10 $\mu\text{mol/l}$ )	(30 $\mu\text{mol/l}$ )
0	0	50.5 (59, 40.8)	78.5 (75.5, 81)	84.5 (78.5, 87)	85 (80.5, 86)
1	0	40 (26.8, 50.8)	75 (70.3, 80.8)	83.5 (79.3, 85.8)	84 (82.3, 85.5)
2	0	32** (13, 38)	74* (70.5, 78)	81 (79, 85)	85.5 (81.3, 87.5)
0	0.5	22.5 (13.5, 30)	69.5 (62.8, 76.8)	80.5 (72.5, 85.3)	83 (76.3, 86)
1	0.5	13.5** (6.3, 19.3)	66** (57.5, 70)	80 (73.8, 82)	83 (78.3, 85)
2	0.5	8** (5.3, 10.5)	59.5** (54, 66.8)	78.5 (73, 81)	82 (74.8, 84)
0	1.0	8 (3.8, 11.5)	61 (48.8, 67.8)	81.5 (75.8, 83)	84 (79.8, 85.5)
1	0.5	5.5 (1.0, 12.5)	50.5* (38.5, 59.3)	76.5 (70.3, 82)	83.5 (79.3, 84.8)
2	1.0	3.5 (2.0, 7.5)	36.5** (32, 58.8)	76 (69.5, 82.5)	81 (77.5, 86.5)

\*\*P < 0.02, \*P < 0.03 with respect to 0 hours.

alone (5  $\mu\text{l}$  saturated solution of nitric oxide) and iloprost alone (0.5 ng/ml) inhibited platelet aggregation, but MB 22948 (100  $\mu\text{mol/l}$ ) alone had no effect. Nitric oxide synergised with MB 22948 and with iloprost. Nitric oxide, MB 22948, and iloprost produced even more inhibition of platelet aggregation. Although inhibition was observed for all three aggregating agents, adenosine diphosphate (unlike collagen and U46619) always produced smooth dose-response curves and this was the agent chosen for further investigation.

Further experiments were performed using citrated whole blood (instead of platelet rich plasma) containing 100  $\mu\text{mol/l}$  MB 22948 (0.5 ml). Here, a much greater volume of the saturated solution of nitric oxide (100  $\mu\text{l}$ ) produced only slight inhibition of aggregation; iloprost (0.5 ng/ml) still partially inhibited aggregation; nitric oxide in combination with iloprost produced a further inhibition of platelet aggregation.

In the second part of this study we chose and applied what we considered to be the optimum conditions to try to detect an effect of glyceryl trinitrate on platelet aggregation in whole blood after transdermal administration. The method chosen was as follows. Blood was withdrawn and collected into tubes that contained an anticoagulant (trisodium citrate) and MB 22948. The final concentrations of citrate and MB 22948 in the blood were 10.6 mmol/l and 100  $\mu\text{mol/l}$  respectively. Immediately after collection, aliquots of the blood were transferred to tubes that contained either 1, 3, or 10  $\mu\text{mol/l}$  adenosine diphosphate together with 0, 0.5, or 1 ng/ml iloprost. (The concentrations given are final concentrations in the blood.) Samples were stirred for exactly 60 seconds and the percentage aggregation was determined as before. Results were expressed as medians (25th and 75th quartiles) and those at one hour and two hours after patch administration were compared with those before administration using the Wilcoxon rank sum test.

## DRUG ADMINISTRATION

Glyceryl trinitrate was administered to eight healthy volunteers (age range 21–35 years) as a patch delivering 0.6 mg glyceryl trinitrate per hour (Nitro-dur, Schering-Plough Ltd). Platelet aggregation was measured before and one and two hours after patch application. Heart rate and blood pressure were also mea-

sured after 10 minutes of supine rest before each venepuncture. A parallel series of investigations was performed with the same volunteers on a different day without application of a glyceryl trinitrate patch. The local ethics committee approved the study and all the volunteers provided informed consent. Aspirin and other antiplatelet drugs were forbidden during 10 days before blood sampling.

## Results

## INHIBITION OF PLATELET AGGREGATION AFTER TRANSDERMAL ADMINISTRATION OF GLYCERYL TRINITRATE

The method chosen to try to detect an effect of transdermal glyceryl trinitrate on platelets was to measure platelet aggregation in whole blood in response to adenosine diphosphate in the presence of MB 22948. The effect of two concentrations of iloprost on the extent of aggregation were also investigated. The table gives the results obtained before and after application of the glyceryl trinitrate patch.

The results show that application of a glyceryl trinitrate patch leads to a small but significant reduction in platelet aggregation at one and two hours, in the absence and presence of iloprost. There is a trend towards more inhibition at two hours than at one hour (figs 1–3). There was no significant change in heart rate or blood pressure on either study day (results not shown).

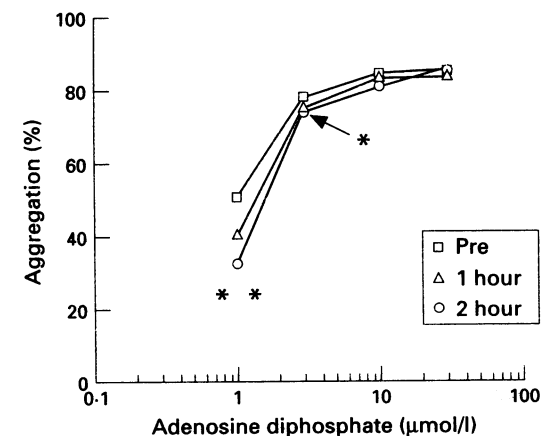


Figure 1 Platelet aggregation in whole blood in response to adenosine diphosphate before (0 hours) and one and two hours after the application of a glyceryl trinitrate patch. The blood contained MB 22948 only. \*\*P < 0.02; \*P < 0.03.

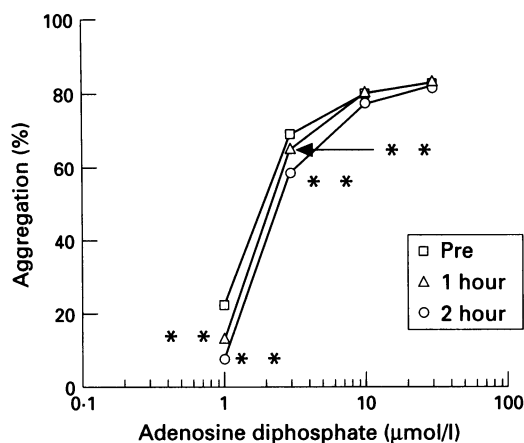


Figure 2 Platelet aggregation in whole blood in response to adenosine diphosphate before (0 hours) and one and two hours after the application of a glyceryl trinitrate patch. The blood contained MB 22948 and iloprost (0.5 ng/ml). \*\* $P < 0.02$ .

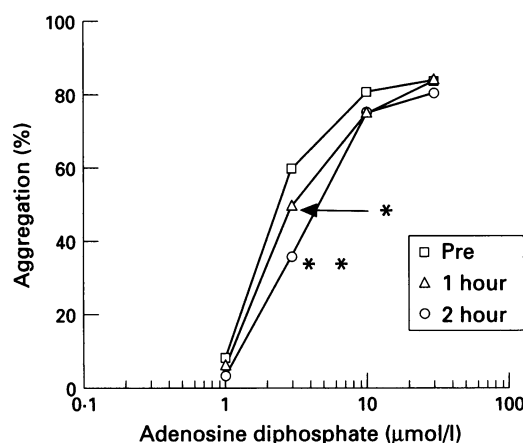


Figure 3 Platelet aggregation in whole blood in response to adenosine diphosphate before (0 hours) and one and two hours after the application of a glyceryl trinitrate patch. The blood contained MB 22948 and iloprost (1.0 ng/ml). \*\* $P < 0.02$ ; \* $P < 0.03$ .

No significant inhibition of platelet aggregation was observed on the control day at any of the concentrations of adenosine diphosphate used, either in the absence or presence of iloprost.

### Discussion

An inhibitory effect of glyceryl trinitrate on platelet aggregation *in vitro* was first shown by Hampton *et al* in 1967.<sup>1</sup> These findings were confirmed by subsequent workers,<sup>14,15</sup> and similar properties shown for other organic nitrates (isosorbide dinitrate and sodium nitroprusside).<sup>2,16,17</sup> The concentrations of organic nitrate required to produce a platelet inhibitory effect were far beyond those pharmacologically achievable *in vivo*, however, and the clinical relevance of these observations was questionable. Indeed, initial attempts to elicit an antiplatelet effect of intravenous glyceryl trinitrate were unsuccessful,<sup>18</sup> although this failure may have been due to methodological difficulties.<sup>19</sup>

Subsequently, the evidence for an inhibitory effect of intravenous glyceryl trinitrate on platelet aggregation has increased

substantially, with significant antiplatelet effects demonstrable at therapeutic doses in normal volunteers and patients with unstable angina.<sup>6,20</sup> Similar data are available supporting platelet actions of intravenous nitroprusside and isosorbide dinitrate.<sup>2,4</sup> There is no previous evidence supporting an antiplatelet effect of transdermal glyceryl trinitrate, however.

Hogan *et al* were unable to show a platelet inhibitory action of transdermal glyceryl trinitrate, although the regimen used was likely to have induced tolerance.<sup>9</sup>

Before starting the present study we were aware that the antiplatelet effects of nitric oxide and of nitric oxide donors such as sodium nitroprusside and SIN-1 (the active metabolite of molsidomine) are much less evident in whole blood than in platelet rich plasma, because the nitric oxide is largely neutralised by haemoglobin in red cells. We also knew, however, that the effects of agents that act via the stimulation of guanylate cyclase (such as nitric oxide) can be amplified by agents that inhibit cyclic guanosine monophosphate breakdown, and that nitric oxide and nitric oxide donors act in synergy with prostacyclin and prostacyclin analogues to inhibit platelet aggregation. Consequently, we suspected that it should be easier to detect the effects of nitric oxide in whole blood in the presence of an inhibitor of cyclic guanosine monophosphate phosphodiesterase (the enzyme responsible for the breakdown of cyclic guanosine monophosphate in platelets) and in the presence of iloprost (a stable analogue of prostacyclin).<sup>21-27</sup> Our preliminary experiments were carried out in platelet rich plasma to avoid the neutralising effects of haemoglobin, and these confirmed the anticipated effects of MB 22948 (the cyclic guanosine monophosphate phosphodiesterase inhibitor chosen for these investigations) and of iloprost in combination with nitric oxide. These experiments also indicated that adenosine diphosphate was a more suitable aggregating agent than collagen or U46619 for these investigations. In further experiments carried out in whole blood the reduced effectiveness of nitric oxide in whole blood compared with platelet rich plasma was confirmed, but again nitric oxide in combination with MB 22948 and with iloprost produced more inhibition of aggregation than nitric oxide alone. Consequently, it was decided to use this demonstrated synergism between nitric oxide, MB 22948, and iloprost to look for effects of transdermal glyceryl trinitrate. In addition to adding MB 22948 and combinations of MB 22948 and iloprost to samples of blood taken before and after glyceryl trinitrate administration, we also chose to measure platelet aggregation as close to venepuncture as possible to avoid any time dependent reduction in the inhibition of platelet aggregation that might occur.

The present study is, in fact, the first to show a significant inhibition of platelet aggregation by the transdermal application of an organic nitrate. We were able to show that



application of a glyceryl trinitrate patch leads to a small but significant reduction in platelet aggregation at one and two hours, with a trend towards more inhibition at two hours than at one hour. No such changes were demonstrable on a control day when no glyceryl trinitrate was administered. Although the demonstrable antiplatelet effect of glyceryl trinitrate was small, it is known that the inhibitory effects of glyceryl trinitrate on platelet aggregation attenuate rapidly *ex vivo*,<sup>6</sup> and it is possible that the *in vivo* effect is more marked. We tried to limit the extent of this attenuation by adding MB 22948 immediately after venepuncture and by carrying out the aggregation experiments as soon as possible thereafter, but even so some attenuation of the effects of the glyceryl trinitrate may have occurred.

It has been speculated that the beneficial effects of organic nitrates in unstable angina and myocardial infarction<sup>6,8</sup> are due in part to their antiplatelet properties. A meta-analysis found a 35% reduction in mortality when intravenous glyceryl trinitrate or nitroprusside were used during acute myocardial infarction, a finding not associated with other vasodilators.<sup>28</sup> Augmentation of the effects of intravenous glyceryl trinitrate by N-acetylcysteine substantially reduced myocardial infarction in unstable angina in one study, an effect compatible with an antiplatelet effect of glyceryl trinitrate.<sup>29</sup> The failure of intravenous nitrate and subsequent transdermal nitrate given alone to reduce mortality in the GISSI III study,<sup>30</sup> however, is against a clinically beneficial antiplatelet effect in acute myocardial infarction, though the apparent additive effect of glyceryl trinitrate and lisinopril in this study could conceivably be related to antiplatelet effects. The ISIS-4 study also did not show a beneficial effect of nitrate given by mouth on mortality after myocardial infarction,<sup>31</sup> though it has been suggested that nitrates may be of benefit in the reduction of infarct size in small rather than large infarcts, and the neutral results of ISIS-4 and GISSI-3 may be explained by the heterogeneity of effect.<sup>32</sup>

It may be argued that our findings obtained after glyceryl trinitrate administration to normal healthy volunteers may not be applicable in patients with ischaemic heart disease where there may be widespread endothelial dysfunction with limited metabolism of glyceryl trinitrate to nitric oxide, although the already demonstrated antiplatelet actions of intravenous glyceryl trinitrate in these patients is much against this viewpoint.<sup>6</sup>

If we accept that the antiplatelet effects of glyceryl trinitrate shown here in healthy volunteers would also be seen in patients with ischaemic heart disease, then it is appropriate to speculate about a role for glyceryl trinitrate in primary and secondary prevention of myocardial infarction. Potential limiting factors are the induction of tolerance to the platelet inhibitory actions of glyceryl trinitrate, an effect that is relevant to the haemodynamic actions of nitrates,<sup>33</sup> and the possible attenuation of platelet inhibition by the

increased release of plasma catecholamines.<sup>34</sup> These two possibilities would need to be investigated. Clearly, further studies are required, but our results suggest a possible exciting new application of an old treatment.

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