FAILURE OF CLINICAL CONCENTRATIONS OF HALOTHANE TO BLOCK ADENOSINE DIPHOSPHATE-INDUCED PLATELET AGGREGATION IN VITRO

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SUMMARY
A dose-related inhibition by halothane of adenosine diphosphate-induced platelet aggregation was measured for human platelets in plasma anticoagulated with sodium citrate 6.8 mmol litre\(^{-1}\) or 13.6 mmol litre\(^{-1}\), or heparin 2 u. ml\(^{-1}\) and for platelets separated from plasma by albumin density gradient centrifugation and requiring no anticoagulant. By maintaining exposure and assay temperature at 37 °C, maintaining physiological pH, avoiding age-related deterioration and using an atraumatic equilibration method, no inhibition was found at clinical concentrations of halothane and 50% inhibition required greater than halothane 10%. Sodium citrate, a calcium chelating anticoagulant, was found to enhance halothane inhibition.

Extensive interest in the clinical implications of platelet dysfunction has led to efforts to define the causes of acquired dysfunction. Since other inhibitors of platelet aggregation such as aspirin may enhance haemorrhage (Sutor, Bowie and Owen, 1971), the question of whether anaesthetic agents increase haemorrhage during operation by this method has been raised. Volatile general anaesthetics have been reported to be inhibitors of platelet aggregation induced by adenosine diphosphate (ADP) (Ueda, 1971).

In order for haemostasis to occur, it is necessary for a platelet–fibrin plug to form at the site of the vascular leak. Platelets must adhere to the damaged vessel, aggregate and release endogenous substances which cause further aggregation and release. ADP, an important inducer of in vivo aggregation when added to platelet-rich plasma (PRP) in vitro will cause primary aggregation and, in sufficient concentration, the release of endogenous platelet ADP.

When platelets, suspended in plasma in vitro, are induced to aggregate, the light transmission of the plasma increases (Born, 1962; O'Brien, 1962). Since this change in transmission may be displayed graphically and used to quantitate aggregation, prior exposure to drugs will indicate the effect of the drug on this aspect of platelet function. However, the volatility of halothane and the plasma handling necessary prohibit the study of in vivo exposure by subsequent in vitro assay. Exposure must also be in vitro if one is to be assured that the platelets being studied are still equilibrated with a known amount of anaesthetic.

Ueda (1971) has reported a dose-related suppression of ADP-induced platelet aggregation with aggregation inhibited by 50% at 1.0 kPa partial pressure of halothane. Since, clinically, there is an apparent absence of enhanced bleeding during halothane anaesthesia, this investigation reconsidered the effects of halothane on platelet aggregation with several experimental modifications. (1) Human, not canine, platelets were used. (2) All assay and anaesthetic exposure temperatures were at 37 °C. (3) Since plasma equilibrated with room air rapidly becomes alkalotic (pH usually greater than 8.0), pH was maintained in the physiological range. (4) To avoid deterioration with time, the study of all platelet suspensions was complete within 3.5 h of venepuncture. (5) The reversibility of the effect of halothane was investigated to assure the method of study alone did not result in diminished aggregation. (6) Equilibrium with the anaesthetic agent was verified. (7) The effect of calcium chelating anticoagulants was investigated also, by studying PRP with two concentrations of citrate, heparinized PRP and platelets separated from plasma by albumin density gradient centrifugation.

METHODS
Whole blood was obtained by venepuncture, using a two-syringe technique, from healthy male volunteers with no history of recent drug exposure. Nine parts blood were immediately mixed with one part anticoagulant solution: sodium citrate 68 mmol litre\(^{-1}\) or 136 mmol litre\(^{-1}\) or porcine heparin 20 u. ml\(^{-1}\) in

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isotonic saline, for the three preparations studied directly. PRP was prepared by centrifugation of whole blood for 12 min at 250 g. The platelet count was adjusted to 200 000 \( \mu l^{-1} \) by addition of platelet free plasma (PFP) prepared by 15 min centrifugation at 2200 g. Plasma was maintained for no longer than 3.5 h in a 5% carbon dioxide in air atmosphere at 37 °C to preserve physiological pH (Han and Ardlie, 1974). Plastic or siliconized glassware was used throughout the experiment.

The fourth preparation of platelets studied was made free of procoagulants and therefore did not require an anticoagulant. It was prepared by modification of the albumin density gradient technique of Walsh, Mills and White (1977). Following preparation of PRP with sodium citrate 12.9 mmol litre\(^{-1} \), 7.8 ml was layered over a 2-ml gradient of bovine serum albumin (BSA) (Sigma Chemical, fraction V) buffered with NaOH 2 mol litre\(^{-1} \) to pH 7.40 at 37 °C. The discontinuous gradient was composed of four 0.5-ml aliquots of approximately 40, 27, 20 and 16 g dl\(^{-1} \) BSA respectively. Following 15 min centrifugation at 1200 £ the lower 2.5 ml was resuspended in 5.5 ml of an isotonic electrolyte solution buffered to pH 7.40 at 37 °C. The solution composition was glucose 5.55 mmol litre\(^{-1} \), NaCl 140 mmol litre\(^{-1} \), KCl 5.4 mmol litre\(^{-1} \), MgCl\(_2\) \(6\)H\(_2\)O 1.0 mmol litre\(^{-1} \), Tris–HCl 10.5 mmol litre\(^{-1} \) and Tris base 4.0 mmol litre\(^{-1} \). The resulting suspension was relayered twice over a BSA gradient and centrifuged at 1000 £ for 15 min. The resulting suspension was adjusted to a platelet count of 200 000 \( \mu l^{-1} \) and \( CaCl_2 \) 100 mmol litre\(^{-1} \) was added to achieve an ionized calcium concentration of approximately 1.0 mmol litre\(^{-1} \) as measured by an Orion model SS-20 calcium electrode. In addition to BSA, the final suspension contained bovine fibrinogen 25 mg dl\(^{-1} \) (Calbiochem, fraction I). All solutions used in this separation contained apyrase 7 mg dl\(^{-1} \) (Sigma Chemical) to degrade any ADP released by damaged platelets (Ardlie et al., 1971). Because of the Tris buffer system, 5% carbon dioxide was not required to maintain a physiological pH.

After transferring 0.5 ml of the 200 000-\( \mu l^{-1} \) platelet suspension to a 16-mm round bottom tube in a 37 °C bath, halothane vapour was passed over the surface of the suspension at 100 ml min\(^{-1} \). Halothane was vaporized in a copper kettle with 5% carbon dioxide in air except for the albumin density gradient separated (ADGS) platelet study, in which air was used. Concentrations were verified by a Miran 1 A Infrared Analyzer at 3.30 \( \mu m \). Mixing of the plasma was by low amplitude oscillation at a frequency of 140 min\(^{-1} \), sufficient to achieve a maximal effect after 15 min.

Four-tenths millilitre of the equilibrated aliquot was then transferred to a siliconized, transmission-matched cuvette in a Sienco model DP-247E platelet aggregation meter and stirred at 1000 rev min\(^{-1} \). ADP 10 \( \mu l \) (Sigma Chemical) was added to induce aggregation, yielding a final ADP concentration of 50 \( \mu mol \) litre\(^{-1} \). For each assay the ratio of change in light transmission with aggregation to the difference in light transmission between PRP and PFP was calculated. The residual activity was the ratio found in an aliquot after exposure to halothane compared with the ratio calculated for the control aliquot of the same specimen incubated and handled in the same manner. Statistical evaluation of the halothane suppression data for the four preparations was made using two-way analysis of variance with appropriate contrasts.

**RESULTS**

It was found that halothane induced a dose-related suppression of ADP-induced platelet aggregation \((P< 0.001)\) (fig. 1). By orthogonal comparisons with coefficients corrected for unequal spacing of halothane concentrations, it was found that halothane suppressed ADP-induced aggregation in a dose-related manner. The albumin density gradient (ADG) separated platelets required no anticoagulant. The final concentrations of anticoagulant in the heparin, low citrate and high citrate preparations were sodium heparin 2 u. ml\(^{-1} \), sodium citrate 6.8 mmol litre\(^{-1} \) (2% w/v) and 13.6 mmol litre\(^{-1} \) (4% w/v) respectively; 100% indicates a response after halothane identical to that of the control. Each point is the mean of results from seven donors.
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concentrations, the suppression has a linear dependence on the dose of halothane \( (P<0.001) \). For the two preparations with no calcium chelation, no inhibition occurred at the two halothane concentrations in the clinical range \( (P>0.05) \). Fifty per cent inhibition was achieved only at very high concentrations of halothane, about 9%, with calcium chelation by sodium citrate 13.6 mmol litre\(^{-1}\). With half as much sodium citrate, 6.8 mmol litre\(^{-1}\), the 50% inhibitory dose increased to more than 10% halothane.

The halothane suppression in the heparin and ADGS preparations did not differ when compared by analysis of variance with contrasts \( (P>0.30) \). The ionized calcium concentration (mean ± SEM) of the heparinized preparations was in the normal range, 0.991 ± 0.014 mmol litre\(^{-1}\). This was not different from the concentration after adjustment with calcium chloride in the ADGS platelet suspension, 0.986 ± 0.008 mmol litre\(^{-1}\). The preparations with a normal calcium ion concentration were suppressed significantly less by halothane than either the 6.8-mmol litre\(^{-1}\) citrate preparation \( (P<0.001) \) or the 13.6-mmol litre\(^{-1}\) citrate preparation \( (P<0.001) \).

Other variables were verified also for the halothane effect studied. The pH of the determinations in figure 1 was well within the physiological range. The two preparations with normal calcium ion concentrations, heparin and ADGS platelets, had a pH of 7.465 ± 0.011 unit (mean ± SEM). Equilibrium with the methods used was achieved at 15 min (fig. 2), since no additional inhibition occurred with additional exposure. The concentration of ADP, 50 \( \mu \)mol litre\(^{-1}\), was selected in the same manner as by Ueda \((1971) \); that is, the minimum ADP concentration required to prevent deaggregation. However, when the ADP concentration was varied (fig. 3), the 50 \( \mu \)mol litre\(^{-1}\) concentration resulted in inhibition not significantly different from 25 \( \mu \)mol litre\(^{-1}\) which produced the maximum inhibition \( (P>0.20) \).

Figure 4 shows the levels of activity of PRP with sodium citrate 13.6 mmol litre\(^{-1}\) after 15 min exposure to halothane followed by 10 min exposure to the

![Fig. 2. Effect of exposure time on residual activity of a 13.6-mmol litre\(^{-1}\) citrated specimen exposed to halothane 10.1%. The sum of incubation time with halothane plus incubation time with carrier gas was 25 min for all aliquots. Each point represents seven preparations from a single donor.](image1)

![Fig. 3. Effect of altering the ADP concentration on residual activity after 6.1% halothane treatment of platelets in 13.6-mmol litre\(^{-1}\) citrated plasma. Each point represents the mean of five preparations from a single donor.](image2)

![Fig. 4. Reversal of the effect of halothane on platelets in 13.6-mmol litre\(^{-1}\) citrated plasma. Exposure to halothane for 15 min followed by 10 min flush with carrier gas. Controls were incubated in carrier gas for 25 min. Each point represents the mean of results from seven donors.](image3)
carrier gas alone. At the two clinical concentrations, reversal was complete ($P>0.05$). Minimal depression at high concentrations of halothane did remain after 10 min.

**DISCUSSION**

When methods for the study of *in vitro* platelet function were adjusted to simulate the physiological *in vivo* condition, an order of magnitude difference was found for the halothane concentration causing 50% inhibition of ADP-induced aggregation. The enhancement of anaesthetic potency at low temperatures (Eger, Saidman and Brandstater, 1965) may have been the primary cause of the failure to demonstrate inhibition at 37 °C at clinical concentrations while Ueda (1971) observed inhibition at 25 °C. Han and Ardlie (1974) predicted that platelets stored at room temperature were sensitized to ADP, and we used a greater concentration of ADP to induce aggregation in this study, since platelets were maintained at 37 °C. The reversibility of the equilibrated suspensions suggests a drug effect rather than destruction by handling and equilibration methods, although the remaining minimal suppression at high halothane concentrations may have represented insufficient removal of halothane or a component of irreversible change.

Platelet aggregation in response to ADP is dependent on the concentration of calcium and may be inhibited completely by high concentrations of citrate for anticoagulation (Skoza et al., 1967). Feinstein, Fiekers and Fraser (1976) have shown that inhibition of platelet aggregation by local anaesthetic agents is in part dependent on the calcium ion concentration. Although varying the citrate concentration confirmed the dependence of halothane suppression of platelet aggregation on a calcium chelating anticoagulant, the experiment did not distinguish between the effect of the citrate anion and that of the low calcium cation. Heparin does prevent coagulation and allow normal calcium concentrations, but may cause aggregation (Zucker, 1977). This problem was not apparent since a low concentration of heparin was used and aggregates present may have been broken up by mixing during incubation. Resuspension of platelets in a procoagulant-deficient solution required no anticoagulants, but the advantage of studying platelets in a minimally altered chemical environment was lost. The results, however, were equivalent to those obtained when heparin was present.

Our data represent only one aspect of platelet function and were obtained by an *in vitro* method. ADP-induced aggregation does not reveal directly the effect of halothane on the platelet release reaction. If a high concentration of halothane or halothane plus calcium chelation did suppress the release reaction, less suppression would have been expected when small amounts of ADP were used, since release would have been minimal (note, for example the assays with low ADP (fig. 3)). Perhaps halothane would have a significant role in collagen-induced aggregation which is more dependent on the release reaction. No benign *in vitro* assay of platelet function analogous to the method of Born (1962) has gained wide acceptance. The observation (Harker et al., 1976) that *in vitro* platelet function tests and *in vivo* antithrombotic effects of drugs have had a variable correlation may be a result of poor control of experimental conditions such as those considered in this investigation.

**REFERENCES**


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**ECHEC DES CONCENTRATIONS CLINIQUES D’HALOTHANE POUR BLOQUER L’AGREGATION DE GLOBULINS PROVOQUEE PAR LE DIPHOSPHATE D’ADENOSINE IN VITRO**

**RESUME**

On a mesure l’inhibition obtenue en fonction de la dose d’halothane, d’une agrégation de globulins provoquée par le diphosphate d’adénosine, pour des globulins humains se trouvant dans du plasma non coagulé, grâce à du citrate de soude, à raison de 6,8 mmol litre⁻¹ ou de 13,6 mmol litre⁻¹ de citrate de soude ou de 2 u. ml⁻¹ d’héparine, de même que pour les globulins séparés du plasma par centrifugation du gradient de densité de l’albumine, et sans anticoagulant. En maintenant la température de l’exposition et de l’essai de conformité à 37 °C, en maintenant le pH physiologique, en évitant la détérioration lié à l’âge et en utilisant une méthode d’équilibre atraumatique, on n’a trouvé aucune inhibition aux concentrations cliniques d’halothane, et 50% d’inhibition aux concentrations d’halothane supérieures à 10%. Le citrate de soude, qui est un agent complexant anticoagulant du calcium, augmente l’inhibition de l’halothane.

**ZUSAMMENFASSUNG**

Eine dosisbezogene Unterdrückung der durch Adenosin Diphosphat eingeleiteten Zusammenballung von Blutplättchen mittels Halothan wurde bei menschlichen Plättchen in Plasma gemessen, das durch 6,8 mmol Liter⁻¹ Natriumzitrat, oder durch 13,6 mmol Liter⁻¹ Natriumzitrat, oder durch 2 u. ml⁻¹ Heparin am Gerinnen gehindert wurde, sowie bei vom Plasma getrennten Blutplättchen durch eine Albumindichte-Zentrifugierung, wobei kein Anti-Gerinnungsmittel erforderlich war. Bei gleichbleibender Aussetzung und Testtemperatur von 37 °C, gleichbleibendem physiologischem pH und bei Vermeidung eines altersbezogenen Verfalls sowie unter Verwendung einer atraumatischen Gleichgewichtsmethode wurde bei klinischen Halothan-Dosen keine Unterdrückung festgestellt, und eine Unterdrückung von 50% erforderte mehr als 10% Halothan. Natriumzitrat, ein Kalzium Chelate-Anti-Gerinnungsmittel, erhöhte die Halothan-Unterdrückung.

**SUMARIO**

Se midió la inhibición relacionada con dosis de halotano en la agregación de plaquetas, inducida por difosfato de adenosina, para plaquetas humanas en plasma anticoagulado con 6,8 mmol litro⁻¹ de citrato sódico, 13,6 mmol litro⁻¹ de heparina y para plaquetas separadas de la plasma por centrifugación de gradiente a densidad de albúmina, que no requieren anticoagulante. Manteniendo la temperatura de exposición y prueba a 37 °C, conservando el pH fisiológico, evitando el deterioro relacionado con la edad y aplicando un método de equilibrio atraumático, no se descubrió inhibición alguna a concentraciones clínicas de halotano, y un 50% de inhibición requirió más de un 10% de halotano. Se descubrió que el citrato sódico, un anti-coagulante quelador de calcio, aumenta la inhibición de halotano.