J. Pharm. Pharmacol. 1998, 50: 1275–1279
Received April 14, 1998
Accepted June 30, 1998

Effects of Copper–aspirin Complex on Platelet Aggregation and Thrombosis in Rabbits and Mice

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Abstract

The effects of intragastric and intraduodenal copper–aspirin complex on rabbit platelet aggregation were observed by Born's method. Myers's method was used to evaluate the antithrombotic effect of copper–aspirin complex in mice.

In-vitro copper–aspirin complex selectively inhibited arachidonic acid-induced platelet aggregation with an IC50 value (concentration resulting in 50% inhibition) of 13·2 μM (95% confidence limits 9·1–16·8 μM). Copper–aspirin complex (10 mg kg^{-1} given intragastrically or intraduodenally) was more potent than aspirin in inhibiting arachidonic acid-induced platelet aggregation. Copper–aspirin complex (10 mg kg^{-1}) had a stronger inhibitory effect and a longer duration of action when given intragastrically than when given intraduodenally. It was shown by radioimmunoassay that copper–aspirin complex significantly reduced the level of thromboxane B_2 in plasma while markedly increasing that of 6-ketoprostaglandin $F_{1\alpha}$ (6keto-$PGF_{1\alpha}$). Copper–aspirin complex (10 mg kg^{-1} given intragastrically for 7 days) significantly reduced mouse mortality caused by intravenous injection of arachidonic acid.

The results suggest that both in-vitro and in-vivo copper–aspirin complex is more potent in selectively inhibiting arachidonic acid-induced platelet aggregation than aspirin. When given intragastrically the complex has a more potent antiplatelet effect and a longer duration of action than when given intraduodenally. The antithrombotic effect of the complex was more potent than that of aspirin.

Copper–aspirin complex [Cu_2(aspirinate)$_4$; Figures 1 and 2] has more potent anti-inflammatory effects (Roch-Arveiller et al 1990) but fewer gastrointestinal side-effects than aspirin because of the Cu^{2+}-catalysed action (Li et al 1996). This investigation focused on the antiplatelet effects of copper–aspirin complex given intragastrically and intraduodenally, and its antithrombotic effect in mice.

Materials and Methods

Animals

Rabbits of either sex, 2·0–3·0 kg, and male ICR mice, approximately 25 g, were obtained from Yunnan Pharmacological Laboratories of Natural Products.

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Reagents and drugs

Copper–aspirin complex (Cu 14·99%, C 51·21%, H 3·32%; purity > 98%) was synthesized by the Kunming Institute of Precious Metals. It was dissolved in water containing 5% propylene glycol and 1·4% polyvinyl alcohol (pH 6·5). Crystalline aspirin was dissolved in 1% Na_2CO_3 before use. Arachidonic acid was from Sigma.

Preparation of platelet-rich and platelet-poor plasma

Blood from the rabbit carotid artery was anticoagulated with 3·8% sodium citrate solution (9 : 1, v/v). Platelet-rich plasma (PRP) and platelet-poor plasma (PPP) was obtained by centrifuging the blood at 1000 or 3000 rev min^{-1}, respectively, for 10 min. PPP was used as the reference for platelet aggregation or to adjust the platelet count in PRP, which was kept 5×10^8 cell L^{-1} in all experiments.

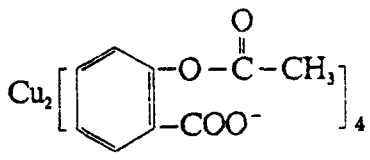

Figure 1. The molecular formula of copper–aspirin complex.

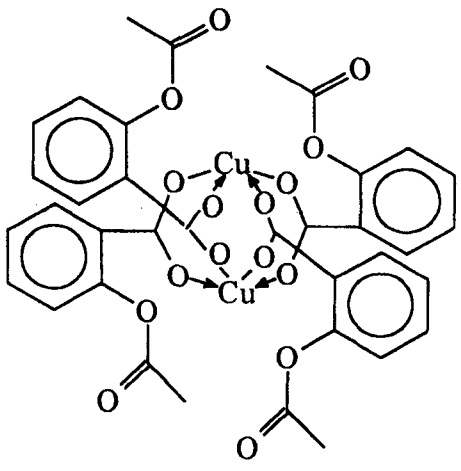

Figure 2. The structure of copper–aspirin complex.

Platelet aggregation test in-vitro

Platelet aggregation was measured as described by Born (1962). The maximum aggregation was recorded (final concentration of arachidonic acid 0·35 mM). Percentage inhibition by drugs was calculated by use of equation 1:

$$\text{Inhibition of aggregation (\%)} = (A - B)/A \times 100 \quad (1)$$

where A is the maximum change of turbidity when the control is added and B is the maximum change of turbidity when the sample is added.

Platelet aggregation in-vivo

Intragastric administration. Rabbits were randomly divided into 9 groups of six. Group A was given the same volume of solvent (water containing 1·4% polyvinyl alcohol and 5% propylene glycol) as was used in the drug preparations. Groups B and C were given 5 and 10 mg kg^{-1} $CuSO_4$, groups D–F, respectively, were given 5, 10 and 75 mg kg^{-1} aspirin, and groups G–I, respectively, were given 5, 10 and 75 mg kg^{-1} copper–aspirin complex. PRP and PPP were prepared before administration and 0·5, 1, 2, 3, 4, and 6 h after administration. Platelet aggregation induced by arachidonic acid was measured.

Intraduodenal administration. Four groups of six rabbits were given, intraduodenally, 10 mg kg^{-1} copper–aspirin complex, aspirin, $CuSO_4$ or the same volume of the solvent used to prepare the drugs. Platelet aggregation was again measured.

Determination of thromboxane B_2 and 6-ketoprostaglandin $F_{1\alpha}$ in plasma

Blood from the carotid artery was anticoagulated with heparin–indomethacin–saline and spun at 3000 rev min^{-1} in an ice bath for 10 min to obtain plasma. Thromboxane B_2 and 6-ketoprostaglandin $F_{1\alpha}$ (6keto-$PGF_{1\alpha}$) in plasma were extracted from the supernatant and assayed by use of radioimmunoassay kits (Terashita et al 1995).

Thrombosis

Mice were divided into three groups of fifteen. Groups A and B, respectively, were given 10 mg kg^{-1} day^{-1} copper–aspirin complex or aspirin for 7 days, intragastrically, and group C was given the appropriate solvent intragastrically, as a control. The mortality was estimated 15 min after injection of arachidonic acid (80 mg kg^{-1}) into the mouse tail vein, as described by Myers et al (1986).

Difference between data from treated and control groups was analysed by means of the χ^2 test.

Results

Effect of copper–aspirin complex on platelet aggregation in-vitro

In-vitro copper–aspirin complex and aspirin concentration-dependently inhibited arachidonic acid-induced aggregation; IC50 values (concentrations resulting in 50% inhibition) were 13·2 and 27·6 μM (95% confidence limits 9·1–16·8 and 15·6–49·2 μM), respectively. $CuSO_4$ had no effect (Table 1).

Effect of intragastric copper–aspirin complex on platelet aggregation

Copper–aspirin complex at 5 mg kg^{-1} markedly inhibited arachidonic acid-induced aggregation 2, 3 and 4 h after intragastric administration ($P < 0.05$ compared with 0 h). Copper–aspirin complex at 10 and 75 mg kg^{-1} significantly ($P < 0.01$ compared with 0 h) suppressed arachidonic acid-induced aggregation; the inhibitory effect of 10 mg kg^{-1} copper–aspirin complex was nearly equivalent to that of 75 mg kg^{-1} aspirin (Table 2). $CuSO_4$ at 10 mg kg^{-1} had a significant ($P < 0.05$ compared with 0 h) inhibitory effect on arachidonic acid-induced platelet aggregation 1, 2, and 3 h after administration but $CuSO_4$ at 5 mg kg^{-1} had no effect (Table 3).

Table 1. In-vitro effect of copper–aspirin complex on rabbit platelet aggregation induced by arachidonic acid.

Concn (μM)	Inhibition of platelet aggregation (%)		
	Copper–aspirin complex	Aspirin	Copper sulphate
3·75	16·4 ± 3·2	3·1 ± 2·2	3·1 ± 1·1
7·5	35·2 ± 1·4*	12·5 ± 2·8	0·9 ± 0·6
15	51·3 ± 1·5*	31·7 ± 2·2*	0·8 ± 0·2
30	60·1 ± 2·3*	53·7 ± 5·2*	1·5 ± 1·2
60	78·6 ± 2·5*	68·7 ± 2·4*	1·9 ± 1·1
120	96·3 ± 2·1*	91·8 ± 3·4*	2·5 ± 1·3

Values are means ± s.d. (n = 6 rabbits). *$P < 0.05$, significantly different from result for control group (for which platelet aggregation was 65·7 ± 4·2%).

Table 2. Effect of intragastric copper–aspirin complex on rabbit platelet aggregation induced by arachidonic acid.

Drug	Concn (mg kg^{-1})	Inhibition of platelet aggregation (%)					
		0·5 h	1 h	2 h	3 h	4 h	6 h
Copper–aspirin complex	5	5·7 ± 2·6	12·3 ± 1·7	32·3 ± 4·6*	58·8 ± 2·8†	48·9 ± 2·9*	9·6 ± 2·6
	10	92·1 ± 3·9†	94·6 ± 2·3†	95·1 ± 1·8†	91·2 ± 3·3†	85·4 ± 1·8†	71·0 ± 3·3†
	75	97·4 ± 1·5†	98·1 ± 1·2†	98·5 ± 1·1†	92·0 ± 2·5†	91·5 ± 2·9†	87·7 ± 2·0†
Aspirin	5	2·2 ± 0·9	5·5 ± 1·9	13·0 ± 2·4	37·4 ± 2·2*	24·1 ± 4·1*	4·7 ± 1·7
	10	48·3 ± 1·4*	51·6 ± 1·8*	86·6 ± 2·5†	87·4 ± 2·9†	77·3 ± 1·5†	51·0 ± 3·3†
	75	91·3 ± 2·9†	92·0 ± 2·3†	93·3 ± 5·3†	90·2 ± 2·4†	78·3 ± 1·1†	65·7 ± 1·0†

Values are means ± s.d. (n = 6 rabbits). *$P < 0.05$, †$P < 0.01$, significantly different from result at time 0 (when platelet aggregation was 67·4 ± 2·6%).

Table 3. Effect of intragastric copper sulphate on rabbit platelet aggregation induced by arachidonic acid.

Concn (mg kg^{-1})	Inhibition of platelet aggregation (%)					
	0·5 h	1 h	2 h	3 h	4 h	6 h
5	3·3 ± 2·0	5·6 ± 1·7	8·3 ± 2·3	4·1 ± 1·3	5·5 ± 1·9	6·3 ± 2·1
10	12·2 ± 2·4	31·4 ± 5·7*	57·4 ± 4·1*	40·4 ± 3·5*	7·4 ± 1·9	4·9 ± 2·4

Values are means ± s.d. (n = 6 rabbits). *$P < 0.05$, significantly different from result at time 0 (when platelet aggregation was 65·5 ± 3·4%).

Effect of intraduodenal copper–aspirin complex on platelet aggregation

When copper–aspirin complex was given intraduodenally, inhibition was greater than that by aspirin, and the antiplatelet aggregation action lasted 3 h only. $CuSO_4$ at 10 mg kg^{-1} given intraduodenally had no inhibitory effect on arachidonic acid-induced platelet aggregation (Table 4).

Effects of copper–aspirin complex on plasma levels of thromboxane B_2 and 6-keto-$PGF_{1\alpha}$

Copper–aspirin complex at 10 mg kg^{-1} significantly suppressed plasma thromboxane B_2 levels and markedly elevated 6-keto-$PGF_{1\alpha}$ levels 2 and 6 h after oral administration; aspirin at 10 mg kg^{-1} significantly reduced plasma levels of both thromboxane B_2 and 6-keto-$PGF_{1\alpha}$ (Table 5).

Effect of copper–aspirin complex on thrombosis

Pretreatment with copper–aspirin complex had a potent inhibitory effect on mouse death as a result of pulmonary thrombi induced by arachidonic acid injection into the tail vein. The level of inhibition by copper–aspirin complex (86·7%) 15 min after

Table 4. Effect of intraduodenal copper–aspirin complex (10 mg kg^{-1}) on rabbit platelet aggregation induced by arachidonic acid.

Drug	Inhibition of platelet aggregation (%)					
	0·5 h	1 h	2 h	3 h	4 h	6 h
Copper–aspirin complex	77·9±5·1*	77·3±5·7*	67·1±4·1*	48·8±6·1*	12·4±5·4	4·2±2·1
Aspirin	3·9±2·0	56·3±4·6*	45·7±5·3*	14·0±4·3	6·2±3·4	3·5±2·1
Copper sulphate	4·1±1·3	2·5±1·7	5·7±2·2	4·3±2·3	5·6±1·7	6·5±3·2

Values are means±s.d. (n=6 rabbits). *$P < 0.05$, significantly different from result at time 0 (when platelet aggregation was 68·4±4·1%).

Table 5. Effects of intragastric copper–aspirin complex on levels of thromboxane B_2 and 6-ketoprostaglandin $F_{1\alpha}$ in rabbit plasma.

Time	Saline		Copper–aspirin complex (10 mg kg^{-1})		Aspirin (10 mg kg^{-1})	
	Thromboxane B_2	6-Ketoprostaglandin $F_{1\alpha}$	Thromboxane B_2	6-Ketoprostaglandin $F_{1\alpha}$	Thromboxane B_2	6-Ketoprostaglandin $F_{1\alpha}$
Before administration	1·53±0·61	0·52±0·31	1·52±0·62	0·49±0·31	1·53±0·51	0·48±0·31
2 h after administration	1·41±0·71	0·54±0·31	0·13±0·06*	2·41±1·61*	0·51±0·08*	0·22±0·08*
6 h after administration	1·51±0·42	0·52±0·23	0·22±0·02*	4·13±1·21*	0·73±0·06*	0·31±0·09*

Table 6. Preventive effect of copper–aspirin complex against mouse sudden death caused by injection of 80 mg kg^{-1} arachidonic acid in the tail vein.

Drug	Dose (mg kg^{-1})	Died/total	Mortality (%)
Control	–	12/15	80·0
Copper–aspirin complex	10	4/15	13·3*
Aspirin	10	2/15	26·6*

The drugs were given orally to mice once daily for 7 days. 'Died' denotes the number of animals that died 15 min after injection of arachidonic acid; 'total' denotes the number of animals used in the study. *$P < 0.05$, significantly different from result for control group (the same volume of water containing 5% propylene glycol and 1·4% polyvinyl alcohol).

injection of arachidonic acid was greater than that by aspirin (73·4%) (Table 6).

Discussion

This study has confirmed that copper–aspirin complex selectively inhibits arachidonic acid-induced platelet aggregation. In-vitro, the IC50 of copper–aspirin complex was lower than that of aspirin, indicating that copper–aspirin complex inhibited platelet aggregation more potently than did aspirin. The antiplatelet activity of 10 mg kg^{-1} copper–aspirin complex given intragastrically was nearly equivalent to that of 75 mg kg^{-1} aspirin. This suggested that within a certain range of doses given orally the antiplatelet aggregation effect of copper–aspirin complex was stronger than that of aspirin. Interestingly, 10 mg kg^{-1} $CuSO_4$ given intragastrically had a significant inhibitory effect on arachidonic acid-induced aggregation 1–3 h after administration, suggesting that Cu^{2+} might contribute to the more potent antiplatelet aggregation of copper–aspirin complex. Given intraduodenally, however, 10 mg kg^{-1} $CuSO_4$ failed to inhibit platelet aggregation. Copper–aspirin complex and aspirin had antiplatelet aggregation activity 0·5 and 1 h after being given intraduodenally, with their duration of action lasting 3 and 2 h, respectively; this demonstrates that the duration of antiplatelet aggregation activity of copper–

aspirin complex and aspirin given intragastrically is longer than when given intraduodenally.

An increase of thromboxane A_2 or a decrease of prostacyclin will result in the adhesion, aggregation and release of platelets (Masakado et al 1994). This study showed that copper–aspirin complex differed from aspirin in that it significantly elevated plasma levels of 6-keto-$PGF_{1\alpha}$ while markedly reducing those of thromboxane B_2. This led us to speculate that this characteristic of copper–aspirin complex makes it different from aspirin both in potency and quality of action.

Platelet hyperfunction and abnormality in thromboxane A_2 and prostacyclin equilibrium are closely related to thrombosis (Myers et al 1983) and thromboxane A_2 is a mediator of the sudden death induced by intravenous arachidonate in mice (Ogletree 1987). Copper–aspirin complex markedly reduced arachidonic acid-induced mortality of mice and resulted in more potent antithrombotic effect. We infer that the antithrombitic action of the copper–aspirin complex is related to its antiplatelet aggregation activity, its reduction of thromboxane A_2 formation and its increasing of prostacyclin levels. Aspirin was inferior to the copper complex in suppressing mouse sudden death; this might be because aspirin reduced both prostacyclin levels and thromboxane A_2 synthesis.

These results indicate that the copper–aspirin complex was more potent than aspirin in inhibiting platelet aggregation both in-vitro and in-vivo. When given intragastrically rather than intraduodenally the copper–aspirin complex had stronger aggregation-inhibiting action and its duration of action was longer. The complex also had a more potent antithrombotic effect than aspirin.

Taken together these results suggest that the copper–aspirin complex has the potential to become a useful antiplatelet and antithrombotic drug.

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