

Platelet Aggregation Test of Earthworm Powder on ADP Induced Rabbits.

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Objective of the study

Due to the fact that earthworm powder has fibrinolytic function, it has been used as dietary supplement ingredient not only in Japan, but also in many countries around the world. Most of the users suffer from hypercoagulability, hypertension, and high blood sugar and it is assumed that they take various medicines concurrently. In order to obtain an indicator of safety of concomitant use of earthworm powder and anti-platelet aggregation agent, effect of earthworm powder on blood platelet is studied by using ADP induced rabbit.

Overview of the study

①Location of the study site:

Department of Pharmacology I. School of Pharmacy and Pharmaceutical Sciences, Mukogawa Women's University (Examiner: Yuta Takahashi, Yoko Kimoto)

②Test sample : Earthworm powder (provided by Waki Pharmaceutical Co., Ltd)

③Test method : 18mL of blood sample is taken from auricular veins of rabbit per 2mL of 3.2% citric acid.

④Test animal : Japanese white rabbit

Subject No.	No.1	No.2	No.3	No.4	No.5	No.6	No.7	No.8	No.9	No.10	No.11
Weight	3.45kg	3.55kg	3.50kg	3.50kg	3.45kg	3.40kg	3.35kg	3.55kg	3.05kg	3.35kg	3.15kg

⑤Adjustment of platelet rich plasma and platelet poor plasma

Collected plasma is centrifuged at 200xg for 10 minutes at room temperature. Consider the isolated supernatant as platelet rich plasma (PRP).

The residue is centrifuged at 1600xg for 10 minutes at room temperature. Consider the isolated supernatant as platelet poor plasma (PPP).

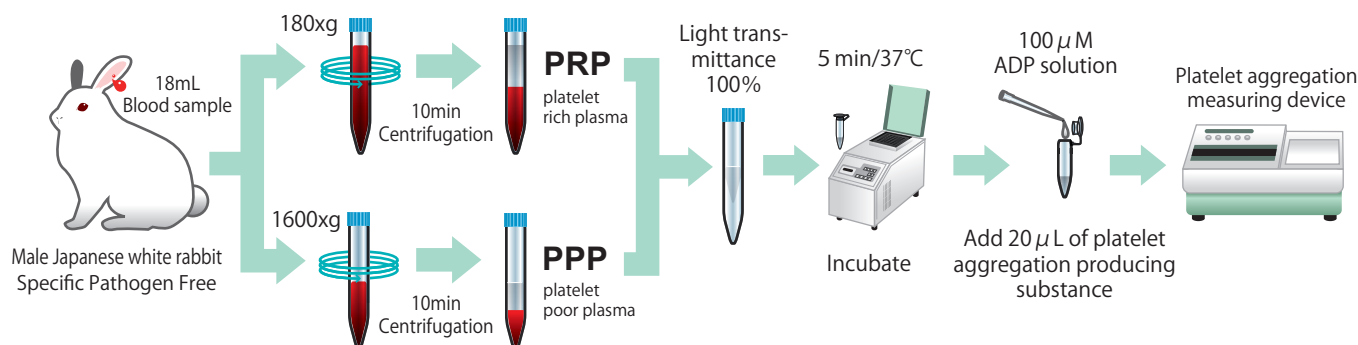
⑥Measuring equipment and principle

Assuming light transmission of PPP to be 100% and that of PRP to be 0%, aggregation producing substance is added and the light transmission of PRP is observed for 10 minutes by Platelet Aggregation Analyzer (Tokyo Koden). Based on the result, platelet aggregation curve is displayed and the maximum aggregation rate and the area under the curve of the platelet aggregation are automatically measured.

⑦ Measurement

As shown in the table on the right, add Cacl2 solution, reagent solution and PRP to cuvette (with stirrer) and set it on the device. Stir gently at 37 degrees Celsius and leave it for 5 minutes. Then add 20 μ L of ADP solution (100/ μ M concentration) as a platelet aggregation producing substance to measure the platelet aggregation activity.

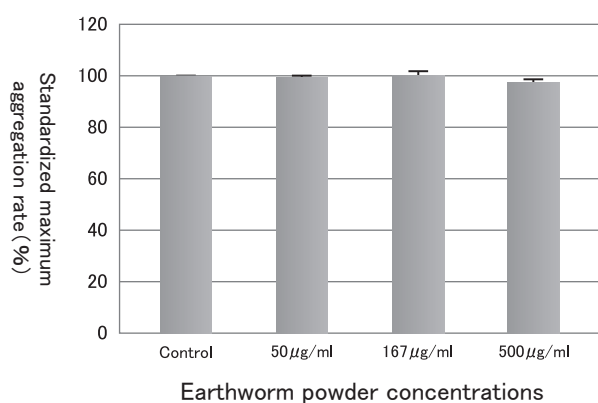
Individual reagent		Combination of reagents	
200mM Cacl2 solution	2 μ L	200mM Cacl2 solution	2 μ L
(Final concentration 2mM)		Reagent solution A or solvent	2 μ L
Reagent solution or solvent	2 μ L	Reagent solution B or solvent	2 μ L
PRP	196 μ L	PRP	194 μ L
Total	200 μ L	Total	200 μ L



Result ① (Effect of earthworm powder on blood platelet aggregation of ADP induced rabbit.)

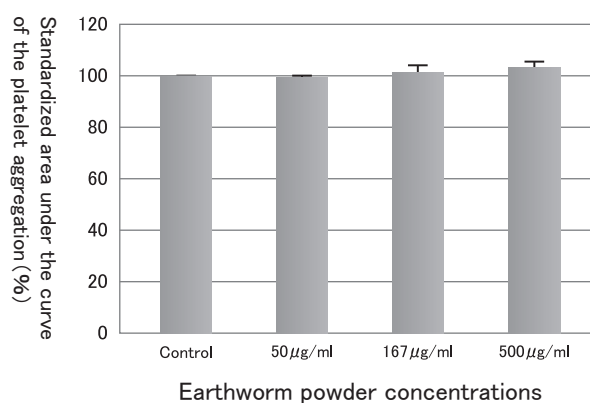
Assume that the earthworm concentration in blood to be 50 μ g/mL when the maximum daily dose of 240mg is taken at once by adult weigh 60kg and the whole quantity is absorbed into blood. Considering the difference in cellular sensitivity between test animal and human, the maximum concentration is set to 10 times the human. The result indicates that it does not affect the platelet aggregation of ADP induced rabbit.

Standardized maximum aggregation rate



	Control	50 μ g/mL	167 μ g/mL	500 μ g/mL
	100	101	99.3	99.0
	100	99.5	102	98.1
	100	98.5	100	97.8
Mean	100	99.6	100.5	98.3
SD	0	1.1	1.6	0.6
SE	0	0.6	0.9	0.4

Standardized area under the curve of the platelet aggregation



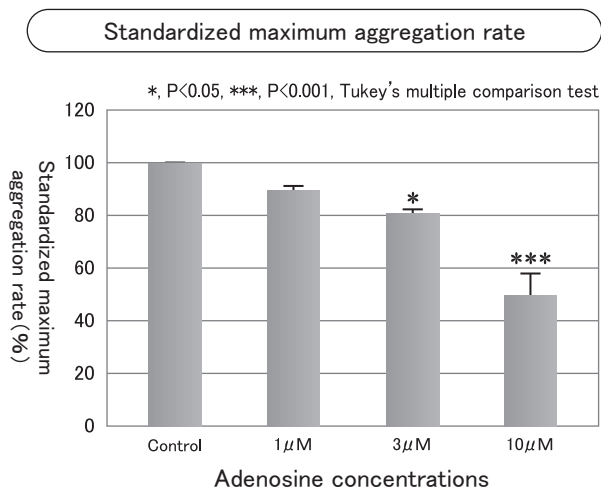
	Control	50 μ g/mL	167 μ g/mL	500 μ g/mL
	100	98.7	97.5	106
	100	103	107	106
	100	98.3	101	99.9
Mean	100	99.9	102	104
SD	0	2.4	5	3
SE	0	1.4	3	2

Maximum aggregation rate				
	Control	50 μ g/mL	167 μ g/mL	500 μ g/mL
	67.6	68.1	67.1	66.9
	65.9	65.5	67.4	64.6
	76.6	75.5	76.5	74.8
Mean	70.0	69.7	70.3	68.8
SD	5.8	5.2	5.3	5.4
SE	3.3	3.0	3.1	3.1

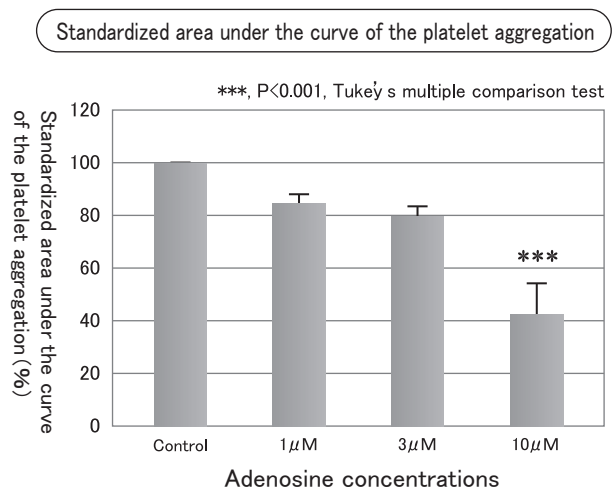
Area under the curve of the platelet aggregation				
	Control	50 μ g/mL	167 μ g/mL	500 μ g/mL
	22000	21611	21212	23116
	25679	26275	27287	27020
	38202	37486	38410	38086
Mean	28627	28457	28970	29407
SD	8494	8159	8722	7766
SE	4904	4711	5035	4484

Result ② (Effect of adenosine on blood platelet aggregation of ADP induced rabbit.)

Effect of 1 μ M, 3 μ M, and 10 μ M of adenosine on blood platelet aggregation of ADP induced rabbit is examined. Adenosine inhibits platelet aggregation in concentration dependent manner. The significant platelet aggregation inhibitory activity is observed in the maximum aggregation rate of more than 3 μ M and in area under the curve of the platelet aggregation of 10 μ M.



	100	89.7	82.3	39.3
	100	88.2	80.0	57.6
Mean	100	90.1	81.2	51.6
SD	0	2.1	1.1	10.7
SE	0	1.2	0.7	6.2



	100	86.3	84.2	23.9
	100	83.9	74.9	47.4
Mean	100	87.8	80.1	42.5
SD	0	4.8	5.0	16.7
SE	0	2.8	3.0	9.6

Maximum aggregation rate				
	Control	1 μ M	3 μ M	10 μ M
	64.5	59.5	52.3	37.4
	50.2	44.8	41.3	19.5
	59.8	52.9	48.0	34.7
Mean	58.1	52.4	47.2	30.5
SD	7.3	7.4	5.6	9.7
SE	4.2	4.3	3.2	5.6

Area under the curve of the platelet aggregation				
	Control	1 μ M	3 μ M	10 μ M
	32777	30549	26687	18460
	22047	18960	18759	5217
	29589	24953	22229	14160
Mean	28138	24820	22559	12612
SD	5510	5796	3974	6756
SE	3181	3346	2295	3900

Result ③ (Effect of concomitant use of earthworm powder and adenosine on blood platelet aggregation of ADP induced rabbit.)

Effect of single administration of $1 \mu\text{M}$ of adenosine, $50 \mu\text{g/mL}$ of earthworm powder, and combination of those two on blood platelet aggregation of ADP induced rabbit is examined. It indicates that concomitant use of $1 \mu\text{M}$ of adenosine and $50 \mu\text{g/mL}$ of earthworm powder do not significantly affect the platelet aggregation of ADP induced rabbit.

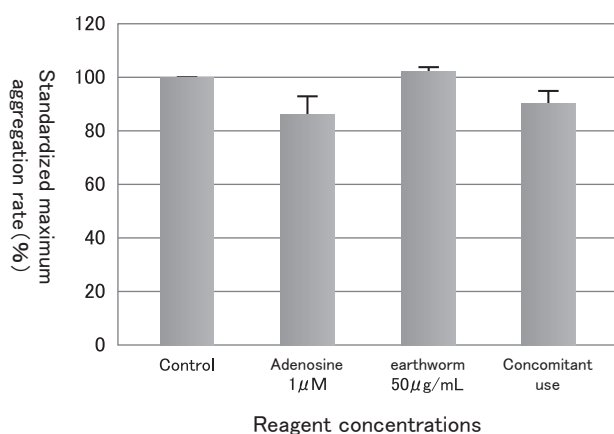
Maximum aggregation rate

	Control	Adenosine $1 \mu\text{M}$	earthworm $50 \mu\text{g/mL}$	Concomitant use
	66.3	61.3	67.0	60.2
	58.0	58.2	59.2	55.8
	56.2	41.2	57.1	42.3
	70.9	69.2	73.8	71.8
	44.3	36.6	48.5	40.5
Mean	59.1	53.3	61.1	54.1
SD	10.3	13.8	9.7	13.0
SE	4.6	6.2	4.3	5.8

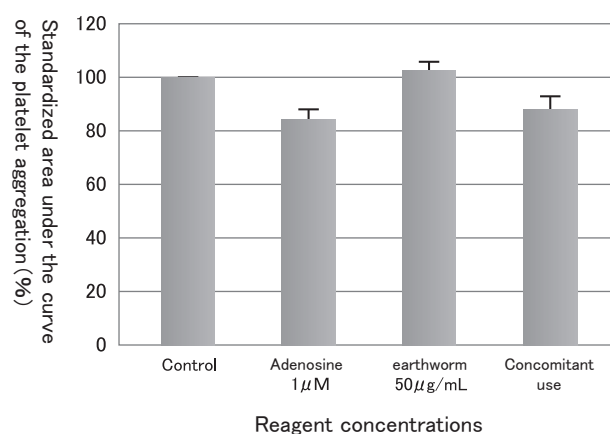
Area under the curve of the platelet aggregation

	Control	Adenosine $1 \mu\text{M}$	earthworm $50 \mu\text{g/mL}$	Concomitant use
	29189	25906	28810	23646
	25649	25560	26220	24252
	26410	16548	26608	17572
	35744	34269	37138	36285
	19745	15443	22668	18256
Mean	27347	23545	28289	24002
SD	5819	7734	5415	7507
SE	2602	3459	2422	3357

Standardized maximum aggregation rate

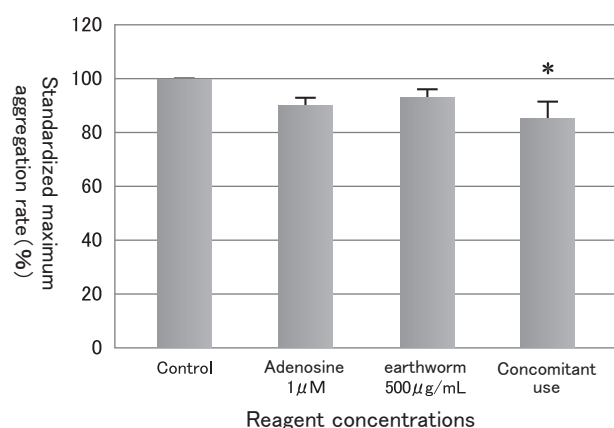


Standardized area under the curve of the platelet aggregation

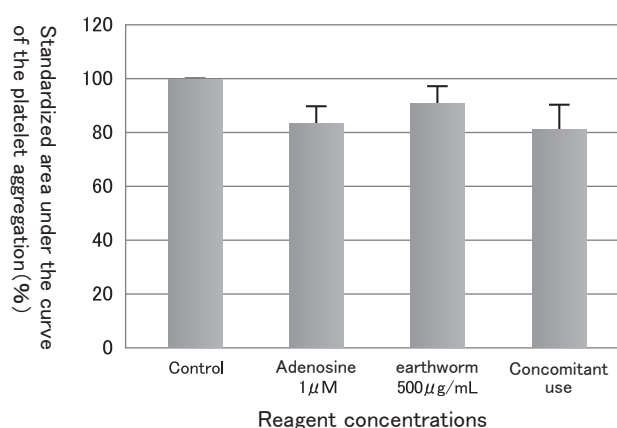


Result ④ (Effect of concomitant use of 10 day dose of earthworm powder and adenosine on blood platelet aggregation of ADP induced rabbit.)

Effect of single administration of $1 \mu\text{M}$ of adenosine, $50 \mu\text{g/mL}$ of earthworm powder, and combination of those two on blood platelet aggregation of ADP induced rabbit is examined. It indicates that concomitant use of $1 \mu\text{M}$ of adenosine and $50 \mu\text{g/mL}$ of earthworm powder do not significantly affect the platelet aggregation of ADP induced rabbit.

Standardized maximum aggregation rate


	Control	Adenosine 1 μ M	earthworm 500 μ g/mL	Concomitant use
	100	81.0	81.0	67.3
	100	97.1	97.0	84.7
	100	96.4	98.2	95.0
	100	95.9	93.5	85.4
	100	88.5	100.0	94.9
Mean	100	91.8	94.0	85.5
SD	0	7.0	7.6	11.3
SE	0	3.1	3.4	5.1

Standardized area under the curve of the platelet aggregation


	Control	Adenosine 1 μ M	earthworm 500 μ g/mL	Concomitant use
	100	57.0	63.0	46.0
	100	84.2	95.7	78.2
	100	95.5	99.2	96.5
	100	95.9	93.8	85.4
	100	82.0	100.0	102.0
Mean	100	82.9	90.3	81.6
SD	0	15.8	15.0	22.0
SE	0	7.1	7.0	9.8

Maximum aggregation rate

	Control	Adenosine 1 μ M	earthworm 500 μ g/mL	Concomitant use
	72.4	57.5	58.3	48.3
	57.1	55.4	55.4	48.4
	74.1	71.5	72.8	70.5
	68.8	65.9	64.3	58.7
	58.4	51.4	58.2	56.2
Mean	66.2	60.3	61.8	56.4
SD	7.9	8.2	7.0	9.1
SE	3.5	3.7	3.1	4.1

Area under the curve of the platelet aggregation

	Control	Adenosine 1 μ M	earthworm 500 μ g/mL	Concomitant use
	29531	15902	18197	13024
	19181	16152	18363	15000
	36938	35296	36629	35632
	32430	31050	30407	27666
	26072	21335	26011	27152
Mean	28830	23947	25921	23695
SD	6706	8826	7931	9483
SE	2999	3947	3547	4241

Conclusion

As an indicator for evaluating the effect of adenosine and earthworm powder on platelet aggregation, “maximum aggregation rate” and “Area under the curve of the platelet aggregation” are used. The former attaches importance to the absolute value of aggregation inhibitor without reflecting its durability. On the other hand, the latter indicates both absolute value of aggregation inhibitor and its durability.

Taking the maximum platelet aggregation rate as an indicator, the result of this study indicates that 500 μ g/mL of earthworm powder demonstrate a statistically significant increase in platelet aggregation inhibitory action of adenosine. However, even with the same amount, it shows no effect when considering the area under the curve of the platelet aggregation as an indicator. This is due to the fact that 500 μ g/mL of earthworm powder is an amount that 10 folds higher concentration than 50 μ g/mL (the concentration assuming an adult weigh 60kg taking a daily dose of 240mg all at once and fully absorbed into blood) and the statistical significance is relatively small due to the less variation in experimental data (the smaller the standard deviation, the clearer the significant difference). In conclusion, it is expected that concomitant use of the regular dose of earthworm powder and anti-platelet agents such as dipyridamole which increases the adenosine concentration in blood does not affect platelet aggregation of patients.