

Development of an *in vivo* Evaluation System of Antioxidants for their Vascular Protective Activities using the Chick Embryonic Chorioallantoic Membrane

by

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Abstract

The drug discovery research of clinical-use antioxidants, which may control various vascular disorders caused by the oxidative stress, is extremely important. We present the development of an *in vivo* evaluation system of antioxidants for their vascular protective activities using the chick embryonic chorioallantoic membrane (CAM). In the case of 12-days chick embryos, the topical administration of 2,2'-azobis(2-methylpropionamide) dihydrochloride (AAPH) induced their vascular injuries against the CAM veins and venous capillaries but without substantial fatal damage. Artepillin C, a potent natural antioxidant, did not show the chick embryo's venous injury, and pre-treatment with artepillin C would tend to protect the CAM veins injuries induced by the post-administration of AAPH. These results suggest that artepillin C might be able to protect the AAPH-induced vascular injury in *in vivo*. In conclusion, we showed the possibility of *in vivo* evaluation system of antioxidants for their vascular protective activities using the chick embryo.

Key words: Antioxidant, Artepillin C, Chick embryo chorioallantoic membrane, vascular injury

1. Introduction

Agents with anti-inflammatory and antioxidant actions have been demonstrated to exert beneficial effects on cardiovascular diseases ⁽¹⁾. In fact, the *in vitro* evaluation system of antioxidants is being mainly used now. It, however, has a problem of no appreciable *in vivo* redox status of the antioxidants.

Preclinical assays generally using mammalian models such as mouse and rat are time-consuming, expensive, and require special facilities and limitation from ethical and legal points of view. Therefore, the developments of an alternative method of the mammalian models are necessary.

The chick embryo is frequently used as an alternative experimental animal for angiogenic investigations ⁽²⁾. Current laws regulating animal experimentation in Japan allows us to use chick embryo without authorization from our animal experimentation committee. The development of an *in vivo* model using the chick embryo has more advantages, such as, handy-size, quickness, less expensiveness, and possible building in

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high-throughput screening than a general animal model.

We adopted artemillin C as an antioxidant to evaluate the utility of our designed *in vivo* model using the chick embryo. Artemillin C is a diprenyl-p-hydroxycinnamic acid first isolated from *Baccharis* species as a major constituent (>5%) in Brazilian propolis⁽³⁾. Artemillin C showed a potent antioxidative activity for lipid peroxidation⁽⁴⁾. We succeeded in the first total synthesis of artemillin C⁽⁵⁾, and then we termed “isoprenomics” for medicinal chemistry of isoprenoids involved in their structural analyses, biosyntheses, biological functions, and chemotypes⁽⁶⁾.

In this paper, we present the development of an *in vivo* evaluation system of antioxidants for their vascular protective activities using the chick embryo.

2. Materials and Methods

2.1. *In vivo* model for the evaluation of antioxidants using the 6- or 12-days chick embryo

Fertilized chicken eggs were purchased from Goto Hatchery, Inc. (Gifu, Japan). The pro- or anti-oxidative effects of AAPH and artemillin C on the chick embryonic chorioallantoic membrane (CAM) vascular were evaluated according to the CAM method⁽⁷⁾. CAM vascular injury was judged by ratio to total number of egg of inhibited one; digestion (Figure 1B) or disappearance (Figure 1C) of CAM blood vessel within the silicon ring. Figure 1A shows the control (no injury).

Fertilized chicken eggs (Ishii co-ltd, Inc., Tokushima, Japan) were incubated in a humidified incubator at 37.6 °C for 9 days. At day 10, air cell region and a part of the CAM blood vessel region (2 x 2 cm) on the egg shell were cut off with a grinder. A shell membrane was peeled off from CAM and the shell membrane was removed. Opened shell window was sealed with oposite and then the egg was incubated at 37.6°C in a humidified atmosphere until day 12. At day 12, a silicon ring was placed on the CAM blood vessel and various concentrations of antioxidant solution including 1% methyl cellulose/0.9% NaCl (5% DMSO) were dropped into the silicon ring.

Opened shell window was sealed again with tegaderm and the egg was incubated at 37.6°C in a humidified atmosphere for 24 hours. CAM veins (size: ca. 0.5 mm) and venous capillaries (size: < 0.1 mm) injury were judged by according to the above-mentioned method. The significance of difference between different groups was analyzed by the multiple numbers of eggs.

2.2. Protection effect of artemillin C for AAPH-induced oxidative vascular injury

At day 12, a silicon ring was placed on the CAM blood vessel, and various concentrations of artemillin C including 1% methyl cellulose/0.9% NaCl (5% DMSO) were dropped into the silicon ring. Opened shell window was sealed again with tegaderm and the egg was incubated at 37.6°C in a humidified atmosphere for 1 hour. The topical administration of AAPH (3 mg) into the silicon ring and egg was incubated at 37.6°C in a humidified atmosphere for 24 hours. CAM veins and venous capillaries injury were judged according to the description of 2.1.

3. Results

3.1. CAM vascular toxicity of AAPH or artemillin C for 6- and 12-days chick embryo

The topical administration of AAPH (a dose from 100 to 300 µg), a water-soluble prooxidant, to the CAM of 6-days chick embryos, resulted in their vascular injuries of percentages from 0 to 100% dose dependently, which were correlated with their increased fatal rate of percentages from 0 to 71% (Table 1). Artemillin C (a dose from 0.1 to 10 µg), a potent natural antioxidant, tends to show a weak vascular injury and fatal damage (each 33% at 1.0 µg) (Table 2).

In the case of 12-days chick embryos, AAPH (doses from 0.3 to 7.0 mg) induced their vascular injuries up to 80% against the CAM veins and venous capillaries without substantial fatal damage (Table 3). Artemillin C, with its dose of up to 30 µg, did not show the CAM veins injury and fatality of chick embryo (Table 4).

3.2. Protection effect of artepillin C for AAPH-induced oxidative vascular injury

In the case of 12-days chick embryos, pre-treatment with artepillin C (a dose of 1 and 10 μg)

tends to protect the CAM veins injuries, and the injury rate has de-creased up to 20%, induced by the post-administration of 3 mg of AAPH (Table 5).

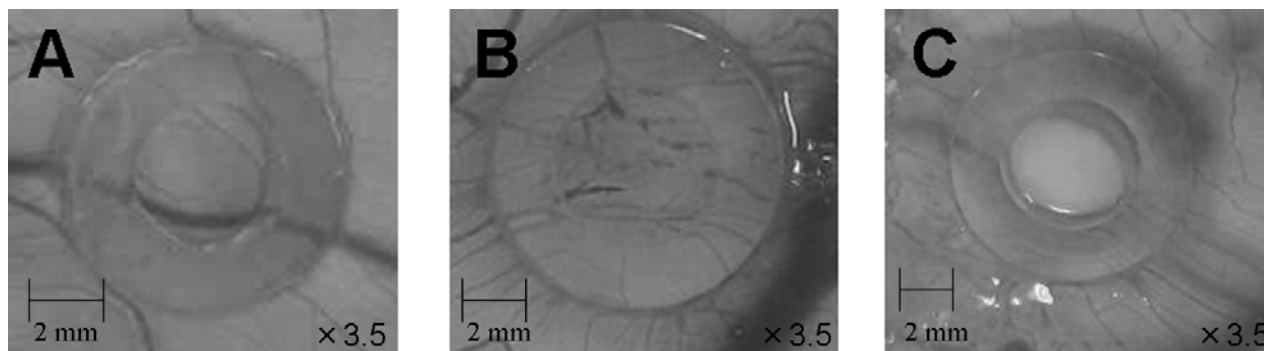


Figure 1. CAM images of no injury (A), digestion (B), and disappearance (C) of CAM blood vessel

Table 1. Embryo (6-days) and CAM vascular toxicity of AAPH

AAPH (μg)	CAM vascular injury rate (%)	Embryo fatal rate (%)	Number of eggs
0	14	14	7
100	0	0	5
150	33	11	9
200	86	43	7
250	86	71	7
300	100	71	7

Table 2. Embryo (6-days) and CAM vascular toxicity of artepillin C

Artepillin C (μg)	CAM vascular injury rate (%)	Embryo fatal rate (%)	Number of eggs
0	0	0	7
0.1	17	0	6
1.0	33	33	6
10	17	0	6

Table 3. Embryo (12-days) and CAM vascular toxicity of AAPH

AAPH (mg)	Veins injury rate (%)	Venous capillary injury rate (%)	Embryo fatal rate (%)	Number of eggs
0	0	20	0	5
0.3	0	20	20	5
1.0	0	20	0	5
3.0	40	60	0	5
7.0	80	80	0	5

Table 4. Embryo (12-days) and CAM vascular toxicity of artepillin C

Artepillin C (μg)	Veins injury rate (%)	Venous capillary injury rate (%)	Embryo fatal rate (%)	Number of eggs
0	0	0	0	5
0.1	20	60	0	5
1.0	0	20	0	5
10	0	25	0	4
30	0	0	0	2

Table 5. Protection effect of artepillin C for AAPH-induced vascular injury

Artepillin C (μg)	AAPH (mg)	Veins injury rate (%)	Venous capillary injury rate (%)	Embryo fatal rate (%)	Number of eggs
0	0	0	20	0	5
0	3	80	100	40	5
0.1	3	40	100	0	5
1	3	20	80	0	5
10	3	20	60	0	5

4. Discussion

In this paper, we presented the development of the new in vivo evaluation system of antioxidants for their vascular protective activities using the chick embryo. In the case of 6-days chick embryo, not only AAPH but also artepillin C showed the CAM vascular injury and embryo's fatality. These results suggest that immature developing CAM vasculature might have low tolerance to oxidative injury. Besides, artepillin C did not show the CAM veins injury without their fatal damage while AAPH had shown vascular injuries as observed in the case of 12-days chick embryo. The CAM blood vessels of chick embryo formed during the day 4 or 5 and its rapid capillary proliferation continued until day 11⁽⁸⁾, therefore we thought that this different result depends on the maturity of the CAM blood vessels. We presumed that artepillin C would tend to protect the CAM veins injuries induced by AAPH because of the amphiphilic property of artepillin C having both vascular penetrativeness and still water solubility. Our developed in vivo evaluation system of antioxidants for their vascular protective activities using the chick embryo might be useful for the drug discovery research of clinical-use antioxidants.

5. Conclusion

In our developed in vivo evaluation system of antioxidants for their vascular protective activities using the chick embryo, we demonstrated that artepillin C would tend to protect the AAPH-induced vascular injury of 12-days chick embryonic CAM.

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References

1. D. L. Bhatt : Anti-inflammatory agents and antioxidants as a possible "third great wave" in cardiovascular secondary prevention, *Am. J. Cardiol.*, 101(10A), 4D-13D (2008).
2. R. Auerbach, L. Kubai, D. Knighton and J. Folkman : A simple procedure for the long-term cultivation of chicken embryos, *Dev. Biol.*, 41(2), 391-394 (1974).
3. H. Aga, T. Shibuya, T. Sugimoto, M. Kurimoto and S. Nakajima : Isolation and identification of antimicrobial compounds in Brazilian propolis, *Biosci. Biotech. Biochem.*, 58, 945-946 (1994).
4. K. Hayashi, S. Komura, N. Isaji, N. Ohishi and K.

- Yagi : Isolation of antioxidative compounds from Brazilian propolis: 3,4-Dihydroxy-5-prenylcinnamic acid, a novel potent antioxidant, *Chem. Pharm. Bull.*, 47(11), 1521-1524 (1999).
5. Y. Uto, A. Hirata, T. Fujita, S. Takubo, H. Nagasawa and H. Hori : First total synthesis of artemisinin established by *o,o'*-diprenylation of *p*-halophenols in water, *J. Org. Chem.*, 67(7), 2355-2357 (2002).
6. Y. Uto, S. Ae, D. Koyama, M. Sakakibara, N. Otomo, M. Otsuki, H. Nagasawa, K. L. Kirk and H. Hori : Artemisinin C isoprenomics: design and synthesis of artemisinin C isoprene analogues as lipid peroxidation inhibitor having low mitochondrial toxicity, *Bioorg. Med. Chem.*, 14(16), 5721-5728 (2006).
7. S. Nakayama, Y. Uto, K. Tanimoto, Y. Okuno, Y. Sasaki, H. Nagasawa, E. Nakata, K. Arai, K. Momose, T. Fujita, T. Hashimoto, Y. Okamoto, Y. Asakawa, S. Goto and H. Hori : TX-2152: a conformationally rigid and electron-rich diyne analogue of FTY720 with *in vivo* antiangiogenic activity, *Bioorg. Med. Chem.*, 16(16), 7705-7714 (2008).
8. D. Ribatti, B. Nico, A. Vacca, L. Roncali, P. H. Burri and V. Djonov : Chorioallantoic membrane capillary bed: A useful target for studying angiogenesis and anti-angiogenesis *in vivo*, *Anat. Rec.*, 264, 317-324 (2001).