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Effects of Rizoma Arisaematis, a Traditional Chinese Natural Medicine, on In Vitro Development of Mouse In Vivo Zygotes and Embryos Produced by Intracytoplasmic Sperm Injection and Somatic Cell Nuclear Transfer

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Abstract: *After screening 408 crude drugs, we found that Rizoma Arisaematis increased the cell numbers of mouse blastocysts developed from in vivo zygotes. We examined the effects of Rizoma Arisaematis on the in vitro development of mouse zygotes and embryos produced by ICSI and SCNT, as well as on fetal development. Mouse zygotes were cultured in media containing a water-soluble extract of Rizoma Arisaematis at various concentrations, and the potential of zygotes to develop into blastocysts and the cell numbers of blastocysts were examined. In addition, the effects of Rizoma Arisaematis on the in vitro and in vivo developmental potential of embryos produced by ICSI and nuclear transfer were examined. In vitro treatment of zygotes with Rizoma Arisaematis increased the cell numbers of blastocysts. The proportions of the blastocysts that implanted and developed into fetuses were slightly higher in the blastocysts which were developed from zygotes treated with Rizoma Arisaematis than those of the control. The Rizoma Arisaematis treatment of mouse fetal fibroblast cells or embryos produced by ICSI and SCNT did not affect the growth of the cells, or the in vitro development of the zygotes. The present study demonstrated that Rizoma Arisaematis improved the quality of embryos developed from in-vivo zygotes, but not that of embryos produced by ICSI and nuclear transfer.*

Key words: *Rizoma Arisaematis, Zygotes, ICSI, SCNT*

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Introduction

Since McLaren and Biggers [1] successfully obtained live mice after transfer of blastocysts developed from eight-cell stage embryos *in vitro*, a large number of studies have examined embryo transfer of *in vitro* manipulated and *in vitro* cultured embryos in various mammals [2]. One of the important factors affecting the potential of embryos developed *in vitro* to develop into live offspring is considered to be the culture medium for zygotes and preimplantation embryos. The culture system including culture medium has been most fully studied for the mouse [3]. The proportion of mouse zygotes that develop into blastocysts *in vitro* is high when zygotes recovered from hybrid mice are used; however, the potential of blastocysts developed *in vitro* to develop into offspring after transfer to recipients is low compared with that of blastocysts developed *in vivo*(40% vs 75%) [4]. The low potential of blastocysts developed *in vitro* after transfer to recipients has been attributed not only to the small number of cells in the blastocysts [5], but also to the expression levels of developmentally important genes [6]. Although suitable culture media for mouse zygotes such as KSOM/AA [7] have been developed recently, the proportion of blastocysts developing from zygotes into full-term fetuses (40%) [4] is not different from that obtained with M16 (31%) [8], reported in 1971 [9]. This fact indicates that the culture medium for mouse zygotes is still required improvement.

Herbal medicines have been used for the treatment of human infertility [10]. Yu *et al.* [11] reported that gastric perfusion of herbal medicines consisting of four natural products into superovulated and implantation-dysfunc-

tional mice increased pregnant and embryonic implantation rates. There are also a few reports of a single natural product that enhances the developmental potential of preimplantation embryos. Wang *et al.* [12] reported that the supplementation with catechin derivatives at a low concentration (15 μM) improved the developmental competence of bovine oocytes. While Tu *et al.* [13] demonstrated that epicatechin gallate decreased the viability and embryonic development of mouse blastocysts. In our preliminary studies, we examined the *in vitro* development of mouse zygotes treated with crude drugs at a concentration of 10 μg/ml [14]. None of the 408 crude drugs examined increased the potential of zygotes to develop into blastocysts. However, *Rizoma Arisaematis*, significantly increased the cell numbers of blastocysts. *Rizoma Arisaematis* is a traditional Chinese medicine used in the treatment of convulsions, inflammation, and cancer [15, 16].

In the present study, we demonstrated for the first time that extract of *Rizoma Arisaematis* significantly or slightly increased the cell number of mouse blastocysts developed from *in vivo* zygotes, and slightly increased the implantation and fetus rates after transfer of the resulting blastocysts into recipients.

Materials and Methods

All experiments and protocols were performed in strict accordance with the Guiding Principles for the Care and Use of Research Animals adopted by the Kinki University Committee on Animal Research and Bioethics. All chemicals were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO), unless otherwise stated.

Extracts of Rizoma Arisaematis

Twelve grams of *Rizoma Arisaematis* (Uchida Wakennyaku, Tokyo, Japan) was boiled in 40 ml distilled water for 15 min. The extract was centrifuged at 900 x *g* for 5 min at 4°C. The supernatant was decanted, freeze-dried, and stored at 4°C until use. *Rizoma Arisaematis* extract was added to KSOM/AA [7] or KSOM [17] at a concentration of 1 mg/ml, incubated at 37°C for 15 min, and centrifuged at 500 x *g* for 5 min. The supernatant was used as stock solution. The stock solution was diluted with each medium before use.

Embryo culture

To study the drug's effect on the development of zygotes, zygotes were recovered from superovulated and mated F1 female mice (C57BL/6 x DBA) 20 h after hCG injection as previously reported [14]. Five to ten zygotes were cultured in 10 μl KSOM/AA or KSOM with 0, 10, 1 or 0.1 μg/ml *Rizoma Arisaematis* extract under 5% CO_2 in air at 37°C for 5 days. The numbers of cells in the inner cell mass (ICM) and trophectoderm (TE) of blastocysts that developed in each group were individually counted at 114 h or 118 h after hCG injection using previously described double staining methods [18].

To study the effect of *Rizoma Arisaematis* on the development of zygotes produced by intracytoplasmic sperm injection (ICSI), ICSI was performed utilizing a piezo micromanipulator as previously described [18]. Briefly, sperm samples were mixed with an equal volume of 12% PVP solution, and the sperm head was separated using piezo pulses before injection into the cytoplasm of MII oocytes at 20 to 23°C. Sperm-injected oocytes were cultured in KSOM/AA for 4 to 5 h, and oocytes with two pronuclei and a second polar body, zygotes, were further cultured for 4 days with or without *Rizoma Arisaematis*. The cell numbers of blastocysts were determined 114 h after hCG injection.

To examine the development of somatic cell nuclear-transferred (SCNT) oocytes, nuclear transfer was performed as previously described [19]. Chromosomes at the second metaphase oocytes were mechanically removed and a single cumulus cell was injected directly into the enucleated oocyte. After nuclear transfer, the reconstructed oocytes were cultured in KSOM containing 100 nM Trichostatin A (TSA) for 2 h, and activated in 10 mM $SrCl_2$ and 5 μg/ml cytochalasin B supplemented with calcium-free KSOM and 100 nM TSA for 6 h, and then cultured in KSOM with or without 1 μg/ml *Rizoma Arisaematis* extract. At 64 h after the start of activation, the embryos were transferred into KSOM supplemented with a 1:200 stock solution of essential and nonessential amino acids and 3.5 mg/ml glucose, and further cultured for 32 h with or without *Rizoma Arisaematis*. The cell numbers of blastocysts were counted 118 h after hCG injection.

Embryo transfer

To examine the potential of embryos to develop into fetuses, 5 to 10 blastocysts developed from *in vivo* zygotes or SCNT embryos in the medium with or without *Rizoma Arisaematis* were transferred to each oviduct of day 1.0 pseudopregnant females (afternoon of the day a vaginal plug was observed) of outbred ICR strain. Following the method of a previous report [20], the recipients received 10 IU hCG daily from day 3.5 to 6.5 to enhance the pregnancy rate. The recipients were sacrified on day 18.5 to examine the numbers of implantation sites, fetuses, and the weights of fetuses and placentae.

Effect of Rizoma Arisaematis on proliferation of mouse embryonic fibroblasts (MEFs)

MEFs were prepared following the procedures reported by Tokunaga and Tsunoda [21]. Briefly, 13.5–15.5 d fetuses obtained from ICR females were minced using a pair of scissors and then treated with 0.25% trypsin/0.04% EDTA solution for 20 min at room temperature. The supernatant was centrifuged at 300 x *g* for 5 min at 4°C, and the precipitate was resuspended in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS). Primary MEFs were cultured in 10 cm tissue culture dishes at 37°C with 5% CO_2, and were used within two to three passages. MEFs at a concentration of 2.8×10^5 cells/ml were cultured in 500 µl DMEM supplemented with 10% FBS containing *Rizoma Arisaematis* at concentrations ranging from 0.1 µg/ml to 10 µg/ml in gelatin-coated dishes for 24, 48, 72, 96 and 120 h. The viable cells were counted by an eosinophil counter after staining with 0.5% trypan blue.

Statistical analysis

Data from experiments with more than three groups were statistically compared the multivariate Tukey-Kramer test. Data from the other experiments on embryo development, and the cell number of blastocysts and the numbers of cells in ICM and TE were compared using the chi-square test or Student's *t*-test as appropriate. A *P*-value of less than 0.05 was considered statistically significant.

Results

Effect of Rizoma Arisaematis on the development of in vivo zygotes, ICSI and SCNT embryos

Table 1 shows the *in vitro* development of zygotes treated with different concentrations of *Rizoma Arisaematis*. Zygotes were cultured with KSOM/AA (series 1 to 3) or KSOM (series 4) supplemented with or without *Rizoma Arisaematis*, and cell numbers of blastocysts were determined at 114 h (series 1 to 3) or 118 h (series 4) after hCG injection. In all series of experiments, the proportions of zygotes developing to two-cell, four- to eight-cell, morula and blastocyst stages did not differ between the control and *Rizoma Arisaematis* groups or among different concentrations of *Rizoma Arisaematis* groups. When zygotes were cultured in the presence of 10 µg/ml *Rizoma Arisaematis*, cell numbers of ICM were significantly or slightly higher than those of the controls in series 1, 2 and 4, but not in series 3. However, cell numbers of TE in the presence of 0.1 to 10 µg/ml *Rizoma Arisaematis* in series 3 were significantly or slightly higher than these of the controls. The proportion of ICM cells as a percentage of total cell numbers of blastocysts did not differ except for the 0.1 µg/ml group in series 3.

The *in vivo* developmental potential after embryo transfer of blastocysts developed from zygotes cultured in 1 µg/ml *Rizoma Arisaematis* is shown in Table 2. The proportion of embryos implanted after transfer to recipients was slightly higher in the *Rizoma Arisaematis* group (49%) than the control (43%). The proportion of embryos developed to live fetuses on day 18.5 was also slightly higher in the *Rizoma Arisaematis* group (38%) than the control (33%). However, no significant differences were observed in implantation and fetus rates between the *Rizoma Arisaematis* and control groups. The weights of the fetuses and placentae did not differ between the *Rizoma Arisaematis* and control groups.

Table 3 shows the effect of *Rizoma Arisaematis* on the *in vitro* developmental potential of zygotes produced by ICSI. The proportions of zygotes that developed into the two-cell, four- to eight-cell, morula and blastocyst stages did not differ between the *Rizoma Arisaematis* and control groups. The cell numbers of ICM, those of TE, the total cell numbers of blastocysts and the proportion of ICM cells as a percentage of the total cell number of blastocysts also did not differ.

Table 4 shows the effect of *Rizoma Arisaematis* on the *in vitro* developmental potential of embryos produced by SCNT. The proportions of SCNT embryos that developed into two-cell, four- to eight-cell, and morula stages did not differ among the groups, but the developmental potential of SCNT embryos cultured in 1 µg/ml *Rizoma Arisaematis* to blastosysts (32%) was significantly lower than that of the control (62%). The cell numbers of ICM, those of TE, and the total cell numbers of blastocysts did not differ among the groups.

Table 5 shows the effect of *Rizoma Arisaematis* on the *in vivo* developmental potential of SCNT embryos. The proportions of embryos implanting and developing to live fetuses did not differ between the groups.

Effect of Rizoma Arisaematis on proliferation of mouse embryonic fibroblasts (MEFs)

The numbers of fetal fibroblast cells after culture with *Rizoma Arisaematis* at the different concentrations for 24 to 120 h were compared with those of the respective control groups. The number of viable cells in the control increased from 2.8×10^5 to 9.7×10^5 cells/ml during culture for 120 h. The growth of fetal fibroblast cells was neither stimulated nor inhibited after the treatment with 0.1, 1 and 10 µg/ml *Rizoma Arisaematis* up to 120 h, compared with the control (9.4 to 9.7×10^5 cells/ml vs 9.7×10^5 cells/ml).

Table 1. Effect of *Rizoma Arisaematis* on the *in vitro* development of mouse *in vivo* zygotes

Series	Concentration (μg/ml)	No.of zygotes cultured	No.of zygotes developed to (%)				Cell numbers of blastocysts (mean ± SD) (n)			ICM ratio (Mean ± SD)
			2-cell	4~8-cell	morulae	blastocysts	ICM	TE	Total	
1	0	81	80 (99)	66 (81)	62 (77)	43 (53)	22.4 ± 9.0a	38.4 ± 10.4	60.8 ± 13.9 (43)	36 ± 10.9
	10	83	82 (99)	61 (73)	60 (72)	44 (53)	28.5 ± 14.7b	37.4 ± 13.8	65.9 ± 16.9 (44)	42 ± 16.8
2	0	42	42 (100)	42 (100)	42 (100)	39 (93)	15.7 ± 5.6a	40.1 ± 13.8	55.7 ± 16.6 (38)	28 ± 8.2
	10	42	42 (100)	42 (100)	42 (100)	37 (88)	18.6 ± 6.5b	43.5 ± 11.5	62.1 ± 14.5 (36)	30 ± 8.1
3	0	46	46 (100)	46 (100)	45 (98)	43 (93)	17.5 ± 5.1	40.5 ± 11.5a	58.0 ± 12.6a (43)	31 ± 9.6a
	10	46	46 (100)	46 (100)	46 (100)	46 (100)	16.8 ± 5.1	44.8 ± 11.6ab	61.6 ± 13.4ab (45)	27 ± 7.5ab
	1	46	46 (100)	46 (100)	46 (100)	44 (96)	17.6 ± 6.1	48.1 ± 11.7b	65.7 ± 13.5b (41)	27 ± 7.9ab
	0.1	46	46 (100)	46 (100)	46 (100)	45 (98)	15.6 ± 4.1	45.8 ± 10.5ab	61.4 ± 12.4ab (45)	26 ± 5.4b
4	0	51	51 (100)	51 (100)	51 (100)	49 (96)	21.2 ± 4.5	54.1 ± 14.5	75.4 ± 16.3a (37)	29 ± 6.4
	10	52	52 (100)	52 (100)	52 (100)	50 (96)	24.6 ± 8.6	55.2 ± 14.4	79.8 ± 16.3ab (39)	31 ± 9.2
	1	52	52 (100)	52 (100)	52 (100)	51 (98)	24.8 ± 7.1	60.5 ± 16.9	85.3 ± 15.3b (44)	30 ± 10.9
	0.1	52	52 (100)	52 (100)	52 (100)	50 (96)	21.2 ± 5.5	53.7 ± 15.8	74.9 ± 16.6a (40)	29 ± 9.1

a-b Values with different superscripts in the same culumn and same series differ significantly ($P < 0.05$).

Table 2. Effect of *Rizoma Arisaematis* on the *in vivo* development of mouse blastocysts developed from *in-vivo* zygotes

Concentration (μg/ml)	No. of zygotes cultured	No. of blastocysts developed (%)	No. of embryos transferred	No. of recipients	No. of pregnant (%)	No.of implantations (%)	No.of live fetuses (%)	Size (Mean ± SD) (g)	
								live fetuses	placenta
0	231	223 (97)	196	20	19 (95)	85 (43)	65 (33)	1.55 ± 0.25	0.15 ± 0.04
1	230	218 (95)	195			95 (49)	74 (38)	1.52 ± 0.27	0.15 ± 0.03

Control embryos and embryos treated with *Rizoma Arisaematis* were transferred into a different oviduct of the same recipient, respectively.

Table 3. Effect of *Rizoma Arisaematis* on the *in vitro* development of mouse embryos produced by ICSI

No. of oocytes injected	No. of oocytes survived	No. of oocytes with pro-nuclei (%)	Concen-tration (μg/ml)	No. of zygotes treated	No. of zygotes developed to (%)				Cell numbers of blastocysts (Mean ± SD) (n)			ICM ratio (Mean ± SD)
					2-cell	4~8-cell	morulae	blasto-cysts	ICM	TE	Total	
314	268 (85)	244 (78)	0	61	58 (95)	58 (95)	55 (90)	41 (67)	13.6 ± 4.8	36.2 ± 11.1	49.9 ± 13.5 (40)	28 ± 9.1
			10	62	58 (94)	58 (94)	53 (85)	41 (66)	12.8 ± 4.6	37.9 ± 10.0	50.6 ± 12.6 (40)	25 ± 8.0
			1	61	61 (100)	61 (100)	54 (89)	41 (67)	12.5 ± 3.9	38.5 ± 10.5	51.0 ± 12.3 (40)	25 ± 6.9
			0.1	60	58 (97)	58 (97)	52 (87)	40 (67)	12.3 ± 3.9	39.9 ± 9.0	52.1 ± 10.6 (38)	24 ± 7.1

Table 4. Effect of *Rizoma Arisaematis* on the *in vitro* development of SCNT embryos

Concen-tration (μg/ml)	No. of oocytes		No. of embryos cultured	No. of embryos developed to (%)				Cell numbers of blastocysts (Mean ± SD) (n)		
	enucleated /used (%)	with pronucleus (ei) (%)		2-cell	4~8-cell	morulae	blastocysts	ICM	TE	Total
0	308/328 (94)	250/308 (81)	61	61 (100)	57 (93)	53 (87)	38 (62)a	12.1 ± 4.3	31.8 ± 13.3	49.9 ± 16.2 (34)
10			63	60 (95)	52 (83)	48 (76)	30 (48)ab	12.3 ± 4.8	30.4 ± 10.7	42.8 ± 14.6 (29)
1			63	60 (95)	52 (83)	44 (70)	20 (32)b	11.4 ± 4.0	32.7 ± 14.0	44.1 ± 17.5 (15)
0.1			63	61 (97)	55 (87)	53 (84)	32 (51)ab	12.0 ± 3.6	31.3 ± 10.4	43.3 ± 12.9 (31)

a-b Significantly different ($P < 0.05$).

Table 5. Effect of *Rizoma Arisaematis* on the *in vivo* development of mouse embryos produced by SCNT

Concentration (μg/ml)	No. of embryos transferred	No. of recipients	No. of pregnant (%)	No. of implantations (%)	No.of live fetuses (%)
0	137	14	11 (79)	50 (36)	2 (1)
1	133			49 (37)	2 (1)

Control embryos and embryos treated with *Rizoma Arisaematis* were transferred into a different oviduct of the same recipient, respectively.

Discussion

Rizoma Arisaematis is a Chinese natural medicine used widely as a sedative, and as anti-inflammatory or anti-stagnation agent [16]. More than 30 compounds have been isolated from *Rizoma Arisaematis,* but the active components and effective mechanisms have not been identified [15]. Recently, it was reported that a lipid-soluble fraction of *Rizoma Arisaematis* inhibited the growth of human cervical cancer cells [15]. The precise mechanism underlying this inhibition is not known, but it might be attributable to an apoptosis-inducing property of *Rizoma Arisaematis* [15].

To date, although there have been a number of pharmacologic and therapeutic studies of *Rizoma Arisaematis* [15, 16, 22], its effect on fertility has not been reported. In the present study, we demonstrated that a water-soluble extract of *Rizoma Arisaematis* increased the cell numbers, a marker of embryo quality of blastocysts developing *in vitro* from zygotes. Moreover, we found that the proportions of blastocysts implanting and developing into live fetuses after transfer to recipients were increased slightly in the *Rizoma Arisaematis* groups. This is the first report to show that *Rizoma Arisaematis* enhanced the quality of mouse zygotes during *in vitro* culture. However, the mechanism underlying this enhancement is not clear. Since the proportion of cell numbers of ICM as a percentage of total cell number of blastocysts did not differ between the *Rizoma Arisaematis* and control groups, it seems likely that *Rizoma Arisaematis* does not affect the direction of differentiation into ICM or TE. Also, *Rizoma Arisaematis* did not affect the cell numbers of blastocysts developed from embryos produced by ICSI and SCNT, nor did it affect the implantation and fetus rates after transfer of SCNT blastocysts to recipients.

It is known that the crude *Rizoma Arisaematis* has toxic properties, such as mucous membrane inflammation, necrosis and paralysis of motor nerve terminals, but the refinement process of *Rizoma Arisaematis* reduces its toxic properties (see Huang *et al.* [16]). Huang *et al.* [16] reported that the neurotoxic effects observed in mice after administration of crude *Rizoma Arisaematis* were accompanied by an increase in plasma lipid peroxidation, an indicator of ROS formation, a decrease in whole blood nitric oxide levels, and an inhibition of Na^+/K^+-ATPase activities in the membrane fraction of erythrocytes. The reasons for the different effect of *Rizoma Arisaematis* between zygotes fertilized *in vivo* and embryos produced by ICSI or nuclear transfer are not clear. However, a different effect was also observed in the cell growth of human cells. Li *et al.* (2010) reported that *Rizoma Arisaematis* extract inhibited the cell growth of human cancer cells but did not affect normal cells.

Embryos developed from ICSI oocytes [23] and SCNT oocytes [4, 6] showed different expression levels of various genes compared with zygotes fertilized *in vivo*. Considering the different effect of *Rizoma Arisaematis* on human cancer and normal cells, it is especially noteworthy that *Rizoma Arisaematis* showed different effects on the development of zygotes of different origin. Since ICSI and nuclear transfer were performed using a piezo-drill which damages the cytoplasm of MII oocytes, especially in the case of SCNT oocytes, it is possible that unknown toxic components of crude *Rizoma Arisaematis* adversely affected the development of SCNT embryos. Since Na^+/K^+-ATPase activities in the membrane are important for the formation of blastocysts [24], an extract of crude *Rizoma Arisaematis* might more severely inhibit the activity of SCNT embryos and lead to the significantly low potential of SCNT oocytes treated with 1 μg/ml to develop into blastocysts.

Assisted reproductive technologies are widely used for the genetic improvement of livestock animals, for the rescue of infertility in humans, and for basic medicine and biology. However, the proportions of manipulated and *in vitro* cultured embryos that develop into live young is low compared with *in vivo*-developed embryos even for the mouse, for which the *in vitro* culture systems of preimplantation embryos have been most fully studied. The present findings that a crude extract of *Rizoma Arisaematis* increased the quality of mouse embryos developed from zygotes fertilized *in vivo* during *in vitro* culture encourages us to test the effects of processed *Rizoma Arisaematis* on the developmental potential of mammalian preimplantation embryos cultured *in vitro*.

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