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—Original—

The Effect of Berberine Treatment on the Reversibility of the Development of Mouse Zygotes and Gametes, and on the Fertilization and Subsequent Development

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Abstract: *As reported previously, berberine, the main component extracted from Coptis rhizome and Phellodendron, has potential as a contraceptive for animals since berberine has a strong inhibitory effect on the in vitro development of mouse zygotes and on fetal development in vivo. The present study was undertaken to examine the effect of berberine treatment on the reversibility of the development of zygotes and gametes, and on the fertilization and subsequent development in the mouse. The reversibility of the berberine-induced inhibition of the development of mouse zygotes was dependent on the concentration used and treatment period. Berberine treatment did not inhibit the fertilizing capacity of epididymal spermatozoa and the fertilizability of oocytes at the second metaphase stage. The present study demonstrated that in vitro development of mouse zygotes is about 100 times more sensitive to berberine than the in vitro growth of mouse fetal fibroblast cells. The high stability of berberine at low temperatures for at least for 12 months and the high sensitivity of preimplantation embryos to berberine is useful information when considering the administration of berberine to females as a contraceptive.*
Key words: *Berberine, Antifertility*

Introduction

It is known that berberine, an isoquinoline plant alkaloid, inhibits the growth of various cancer cells originating from leukocytes, liver, lung, stroma, colon, skin, oral, esophagus, brain, bone, breast and sexual organs (for review; [1]). Serafirm *et al.* [2] reported that in mouse and human melanoma cells treated with berberine at low doses, berberine concentrates in the mitochondria and promotes G1 arrest, but at high doses concentrates in the cytoplasm and nuclei and then induces G2 arrest. Ehrlich ascites carcinoma cells treated with berberine showed cell death due to the formation of dsDNA breaks and inhibition of DNA synthesis, or induced apoptotic cell death due to inhibition of protein synthesis [3]. Besides its anti-cancer effect, berberine has various pharmacological effects such as anti-microbial, anti-pyretic, anti-inflammatory, anti- diarrhea, and cholesterol-lowering properties [4]. Hu *et al.* [5] demonstrated that the intra-articular injection of berberine provided protection against the development of osteoarthritis.

We previously reported that berberine has an inhibitory effect on the *in vitro* and *in vivo* development of mouse zygotes [6]. Although effective contraceptive pills for controlling human fertility have been developed [7], practical non-invasive methods for controlling fertility have not yet been developed in animals. We previously screened 269 natural products which have an anti-fertility effect by using a mouse zygote development assay, and found that Coptis rhizome and Phellodendron extracts inhibit the *in vitro* development of mouse zygotes to blastocysts. We also demonstrated that berberine, the main component of both natural products, has potential as a contraceptive for animals since berberine had a strong inhibitory effect on the *in vitro* development of mouse zygotes and on *in vivo* fetal development [6]. However, the detailed mechanisms by which berberine inhibits the development of mouse zygotes are not known.

The present study was undertaken to examine: 1) whether the effect of berberine treatment on the *in vitro* development of mouse zygotes is reversible; 2) the effect

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of berberine on the fertilizing capacity of mouse spermatozoa; 3) the effect of berberine on the fertilizability of mouse oocytes; and 4) the relationship between the concentrations of berberine necessary to inhibit the growth of somatic cells and the *in vitro* development of mouse zygotes.

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.

Embryo culture

To recover mouse zygotes, superovulation was induced in adult BDF1 females (C57BL/6 × DBA) by the injection of 5 IU equine chorionic gonadotropin (eCG) and 5 IU human chorionic gonadotropin (hCG) 48 h apart. These females were then mated with BDF1 or ICR males. Mated females were sacrificed 20 h after hCG injection, and eggs with cumulus cells were collected and treated with 300 NFU/ml hyaluronidase in M2 medium [8]. Denuded eggs with two pronuclei were used for the experiments. Ten zygotes were cultured in 10 µl KSOMaa [9, 10] containing 0.0001 µg/ml to 10 µg/ml berberine chloride (Wako Pure Chemical Industries, Ltd., Osaka, Japan) under 5% CO_2 in air at 37 °C for 4 days. The numbers of inner cell mass (ICM) and trophectoderm (TE) cells of blastocysts that developed in each group were individually counted using double staining methods as previously reported at 114 h after hCG injection (94 h after *in vitro* culture) except specially mentioned [11]. The addition of berberine chloride to the medium at 10 µg/ml had no effect on the pH (7.1) or osmolarity (250 to 251 mOsm). To examine the reversibility of berberine treatment, zygotes treated with 0.1 µg/ml berberine for 48 to 94 h were cultured for 6 days.

Treatment of spermatozoa with berberine and ICSI

The effect of berberine treatment on the fertilizing capacity of spermatozoa was examined using intracytoplasmic sperm injection (ICSI) instead of *in vitro* fertilization to avoid any effect on the fertilizability of oocytes. Spermatozoa were prepared according to the modified procedures reported by Wakayama *et al.* [12]. One cauda epididymis from mature ICR male was moved to 1 ml KSOM medium [9] supplemented with 1 or 10 µg/ml berberine; then, the dens mass of spermatozoa was released into the medium by squeezing the epididymis with fine forceps and a 25G needle. After incubation for 30 to 120 min in a CO_2 incubator at 37 °C, the upper 0.25ml medium from each sperm suspension was collected, moved to a micro centrifuge tube and centrifuged at 300 × g for 5 min at 4 °C. Sperm concentrations during incubation were 0.3 to 1.5 × 10^6 spermatozoa/ml. The supernatant was discarded and the tubes were centrifuged again after the addition of 0.25 ml KSOM. Finally, 0.25 ml KSOM was added to the precipitation, and mixed; and then, aliquots of 50 µl were dispensed into the centrifuge tubes and moved directly to a −80 °C freezer. The samples were stored for 1 day to 8 weeks before ICSI. For the control, a sperm suspension was prepared in KSOM supplemented with 1 or 10 µl distilled water (DW). Sperm samples were thawed by adding 200 µl M2 medium at room temperature and then used for ICSI utilizing a piezo micromanipulator as described by Kimura and Yanagimachi [13]. Briefly, sperm samples were mixed with an equal volume of 10% 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 KSOMaa for 4 to 5 h, and oocytes with two pronuclei and a second polar body were further cultured for 4 days. The numbers of ICM and TE cells of blastocysts were individually counted using double staining methods at 114 h after hCG injection.

To examine the effect of berberine on the mortality of spermatozoa, epididymal spermatozoa at a concentration of 3.0 × 10^5/ml were incubated in KSOM supplemented with 1 and 10 µg/ml berberine or 10 µl DW. After incubation for 2, 4 and 6 h in CO_2 incubator at 37 °C, the proportion of non-motile spermatozoa was calculated.

Treatment of oocytes with berberine and ICSI

Mature oocytes recovered from BDF1 females 15 h after hCG injection were incubated in KSOM supplemented with 1 µg/ml berberine or 1 µl DW for 60 min at 37 °C, rinsed with M2 medium several times, and then used for ICSI. The sperm samples which had been stored at −80 °C were thawed, and the sperm head was introduced into the cytoplasm of berberine-treated and control oocytes using the same procedures as described above. Sperm-injected oocytes with two pronuclei and a second polar body were cultured in KSOMaa for 4 days. The numbers of ICM and TE cells of blastocysts were individually counted using double staining methods at 114 h after hCG injection.

Effect of berberine on proliferation of mouse embryonic fibroblasts (MEFs)

MEFs were prepared according to the procedures reported by Tokunaga and Tsunoda [14]. Briefly, 13.5–

Table 1. Effect of berberine concentration on the *in vitro* development of mouse zygotes

Origin of embryos (♀×♂)	Concentrations of berberine (μg/ml)	No. of zygotes cultured	No. (%) of zygotes developed to				Cell number of blastocysts (Mean ± SD)		
			2-cell at day 1	4-8-cell at day 2	morulae at day 3	blastocysts at day 4	ICM	TE	Total
BDF1 × BDF1	–	46	46 (100)[a]	46 (100)[a]	46 (100)	45 (98)[a]	16.1 ± 4.6[a]	43.2 ± 12.9[a]	59.3 ± 14.4[a]
	0.0001	47	47 (100)[a]	47 (100)[a]	47 (100)	46 (98)[a]	16.0 ± 4.9[a]	41.3 ± 13.2[ab]	57.3 ± 15.9[ab]
	0.001	46	46 (100)[a]	46 (100)[a]	46 (100)	40 (87)[a]	13.7 ± 4.3[b]	36.9 ± 8.9[b]	50.6 ± 9.7[b]
	0.01	46	46 (100)[a]	45 (98)[a]	45 (98)	19 (41)[b]	11.8 ± 3.4[b]	19.6 ± 3.9[c]	31.4 ± 5.5[c]
	0.1	46	46 (100)[a]	26 (57)[b]	0 (0)	0 (0)	–	–	–
	1	46	28 (61)[b]	0 (0)	0 (0)	0 (0)	–	–	–
	10	46	0 (0)	0 (0)	0 (0)	0 (0)	–	–	–
BDF1 × ICR	–	34	34 (100)[a]	34 (100)[a]	34 (100)[a]	33 (97)[a]	14.5 ± 3.5[a]	40.8 ± 13.3[a]	55.2 ± 13.8[a]
	0.0001	35	34 (97)[a]	34 (97)[a]	34 (97)[a]	31 (89)[a]	14.2 ± 5.0[ab]	45.2 ± 10.9[a]	59.5 ± 12.3[a]
	0.001	35	35 (100)[a]	34 (97)[a]	34 (97)[a]	33 (94)[a]	15.1 ± 4.7[a]	39.5 ± 10.9[a]	54.6 ± 11.5[a]
	0.01	35	34 (97)[a]	34 (97)[a]	34 (97)[a]	15 (43)[b]	11.9 ± 3.2[b]	21.9 ± 6.1[b]	33.8 ± 6.6[b]
	0.1	33	33 (100)[a]	13 (39)[b]	2 (6)[b]	0 (0)	–	–	–
	1	34	19 (56)[b]	0 (0)	0 (0)	0 (0)	–	–	–
	10	34	0 (0)	0 (0)	0 (0)	0 (0)	–	–	–

[a–c] Values with different superscripts in the same column and same origin differ significantly ($P < 0.05$).

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 × g for 5 min at 4 °C, and the precipitate was resuspended in Dulbecco's modified eagle 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 2 to 3 passages. MEFs at a concentration of 1.15×10^5 cells/ml were cultured in DMEM supplemented with 10% FBS containing berberine at concentrations ranging from 0.001 μg/ml to 100 μg/ml in gelatin-coated dishes for 24, 48, 72, 96 and 120 h. The numbers of viable cells were counted by an eosinophil counter after staining with 0.5% trypan blue.

Statistical analysis

Data in experiments with more than 3 groups were statistically compared by the multivariate Tukey-Kramer test. Data in other experiments on embryo development, and the cell number of blastocysts and numbers of ICM and TE cells were compared using the chi-square test and the Student's *t*-test, respectively. A *P*-value of less than 0.05 was considered to be statistically significant.

Results

Effect of berberine concentration on the in vitro development of mouse zygotes

The *in vitro* development of mouse BDF1xBDF1 and BDF1xICR zygotes treated with berberine at the concentrations of 0.0001 to 10 μg/ml is shown in Table 1. The development of zygotes from both origins to blastocysts was completely inhibited at the concentrations of more than 0.1 μg/ml and was significantly inhibited at 0.01 μg/ml. Blastocysts developed from zygotes treated with 0.01 μg/ml berberine had significantly lower total numbers of cells, numbers of inner cell mass and trophectoderm cells at day 4 (94 h after *in vitro* culture). The responses to berberine were not different between the BDF1xBDF1 and BDF1xICR zygotes.

As shown in Table 2, treatment with 10 μg/ml berberine for 1 h did not inhibit the development of zygotes, but significantly reduced the cell numbers of blastocysts on day 4. When zygotes were treated with 10 μg/ml berberine for 3 h, the cleavage was completely inhibited. Treatment with 1 μg/ml berberine for 1 h and 3 h did not inhibit the development of mouse zygotes. But total cell and TE cell numbers of blastocysts on day 4 (94 h after *in vitro* culture) in the 3 h group were significantly decreased as compared with the control group. When zygotes were treated with 0.1 μg/ml berberine for 24 h and 48 h, the developmental potential to blastocysts was not different from the control. When the zygotes were treated for 48 h, 6 of 46 zygotes were arrested at the 2-cell stage. Although 4 of 6 embryos at the 2-cell stage developed to blastocysts 1 day behind time, total cell number and ICM and TE cell numbers were significantly low (data not shown). All zygotes in the control developed to blastocysts on day 4, but 12 (30%) and 26 (65%) embryos at

Table 2. The reversibility of berberine treatment on the *in vitro* development of mouse zygotes

Concentrations of berberine (μg/ml)	Treatment period of berberine (h)	Group	No. of zygotes cultured	No. of zygotes developed to (%)				Cell number of blastocysts (Mean ± SD)		
				2-cell at day 1	4-8-cell at day 2	morulae at day 3	blastocysts at day 4	ICM	TE	Total
10	1	control	34	34 (100)	34 (100)	34 (100)	34 (100)	16.0 ± 4.7^a	40.4 ± 9.8^a	56.4 ± 11.7^a
		berberine	34	33 (97)	33 (97)	33 (97)	31 (91)	12.5 ± 3.7^b	30.0 ± 10.1^b	42.5 ± 11.3^b
	3	control	34	34 (100)	34 (100)	34 (100)	34 (100)	15.0 ± 5.3	41.6 ± 10.6	56.6 ± 11.9
		berberine	35	0 (0)	0 (0)	0 (0)	0 (0)	–	–	–
1	1	control	37	37 (100)	37 (100)	37 (100)	37 (100)	14.3 ± 5.2	41.9 ± 11.3	56.1 ± 13.2
		berberine	35	35 (100)	35 (100)	35 (100)	34 (97)	14.1 ± 5.2	44.1 ± 11.3	58.1 ± 14.0
	3	control	36	36 (100)	36 (100)	36 (100)	36 (100)	15.1 ± 4.8	42.5 ± 11.3^a	57.6 ± 12.8^a
		berberine	38	37 (97)	37 (97)	37 (97)	34 (89)	13.9 ± 4.5	37.0 ± 10.6^b	50.9 ± 13.5^b
0.1	24	control	45	45 (100)	45 (100)	45 (100)	43 (96)	14.3 ± 4.3	37.9 ± 10.0^a	52.2 ± 11.7^a
		berberine	45	45 (100)	45 (100)	45 (100)	42 (93)	15.7 ± 5.2	42.7 ± 11.0^b	58.5 ± 12.5^b
0.1	48	control	46	46 (100)	46 (100)	46 (100)	46 (100)	14.7 ± 4.1	43.0 ± 9.1	57.7 ± 11.2^a
		berberine	40[1)]	40 (100)	40 (100)	40 (100)	38 (95)*	13.4 ± 5.3*	31.5 ± 10.6*	44.9 ± 11.5^b*
0.1	72	control	46	46 (100)	46 (100)	$46 (100)^a$	$44 (96)^a$	16.4 ± 5.2	42.4 ± 10.3	58.8 ± 15.5^a
		berberine	46	46 (100)	37 (80)	32 (70)[b2)]	$16 (35)^b$**	–	–	23.9 ± 11.2^b**
0.1	94	control	45	44 (98)	44 (98)	44 (98)	40 (89)	15.5 ± 4.9	41.8 ± 10.6	57.3 ± 13.6
		berberine	45	45 (100)	35 (78)	0 (0)	0 (0)	–	–	–

[a–b] Values with different superscripts in the same column differ significantly ($P < 0.05$). [1)]Six of 46 zygotes stopped at 2-cell stage after 48 h treatmentare excluded from the Table. [2)]Most embryos compacted at the 4- to 8-cell stage. *Number and cell numbers of blastocysts at days 4 and 5. ** Number and cell numbers of blastocysts at days 5 and 6.

Table 3. Effect of berberine treatment of spermatozoa on the fertilizing capacity and subsequent development in mouse zygotes

Series	Concentrations of berberine (μg/ml)	Culture period (min)	Groups	No. of oocytes (%)				No. of oocytes developed to (%)				Cell number of blastocysts (Mean ± SD)		
				injected	survived	with pronuclei	cultured	2-cell at day 1	4-8-cell at day 2	morulae at day 3	blastocysts at day 4	ICM	TE	Total
1	1	30	control	99	40 (40)	36 (90)	36	33 (92)	33 (92)	30 (83)	20 (56)	9.8 ± 2.0	31.0 ± 7.2	40.8 ± 8.0
			berberine	101	48 (48)	41 (85)	41	33 (80)	31 (76)	31 (76)	18 (44)	9.8 ± 4.0	28.8 ± 8.3	38.6 ± 10.9
		60	control	91	71 (78)	67 (94)	67	65 (97)	65 (97)	64 (96)	$47 (70)^a$	13.1 ± 3.1	27.7 ± 5.9	40.8 ± 7.9
			berberine	99	79 (80)	76 (96)	76	68 (89)	68 (89)	61 (80)	$36 (47)^b$	13.6 ± 3.2	27.5 ± 7.8	41.2 ± 9.5
		90	control	96	59 (61)	57 (97)	57	53 (93)	52 (91)	51 (89)	39 (68)	11.9 ± 3.8	26.2 ± 6.6	38.1 ± 8.3
			berberine	94	61 (65)	55 (90)	55	46 (84)	46 (84)	46 (84)	30 (55)	10.9 ± 3.0	26.1 ± 6.2	37.1 ± 7.3
		120	control	61	47 (77)	45 (96)	45	42 (93)	42 (93)	39 (87)	28 (62)	13.7 ± 3.4	29.5 ± 5.2	43.2 ± 6.7
			berberine	72	55 (76)	52 (95)	52	52 (100)	50 (96)	47 (90)	26 (50)	12.8 ± 3.6	26.8 ± 8.1	39.6 ± 9.1
2	10	60	control	101	79 (78)	68 (86)	68	66 (97)	64 (94)	61 (90)	45 (66)	14.2 ± 5.0	39.9 ± 11.0	54.1 ± 13.7
			berberine	104	82 (79)	75 (91)	75	74 (99)	72 (96)	69 (92)	47 (63)	15.5 ± 4.5	37.7 ± 11.0	53.2 ± 12.6
	1	60	control	100	84 (84)	74 (88)	74	74 (100)	73 (99)	71 (96)	59 (80)	16.6 ± 4.7	35.4 ± 9.7	52.0 ± 10.5
			berberine	107	86 (80)	76 (88)	76	76 (100)	76 (100)	76 (100)	60 (79)	14.4 ± 4.8	34.6 ± 10.4	49.0 ± 12.6

[a-b]Significantly different in the same concentration and the same period of berberine ($P < 0.05$).

the 4-cell stage after 48 h treatment developed to blastocysts on day 4 and day 5, respectively. Since the latter blastocysts had significantly low cell numbers compared with the control (data not shown), the combined data of the cell numbers of blastocysts treated with 0.1 µg/ml berberine for 48 h in Table 2 were significantly or slightly lower than those of the control. It is not appropriate in the strict sense, however, to compare data on the cell numbers of blastocysts in berberine-treated and control groups since the culture duration was different.

The developmental potential of zygotes treated with berberine for 72 h to develop into blastocysts was significantly lower than that of the control (35% vs 96%). Most embryos compacted at the 4-to 8-cell stage and the development to blastocysts was observed on days 5 (7/16, 44%) and 6 (16/16, 100%). It was difficult to perform separate cell counts of the ICM and TE of these blastocysts due to the poor morphology, and blastocysts' cell numbers were significantly lower than those of the control (23.9 vs 58.8). When zygotes were treated with berberine for 96 h, no zygotes developed to blastocysts and all embryos degenerated at the 4- to 8-cell stage.

Effect of berberine preserved at low temperature on the in vitro development of mouse zygotes to the blastocyst stage

The effect of berberine preserved at low temperatures for different periods on the development of mouse zygotes was examined. Berberine chloride solution could be preserved for at least 12 months at both 4 °C and −80 °C without reducing the inhibitory effect on the development of mouse zygotes (data not shown). The minimal concentrations of berberine to significantly and completely inhibit the developmental potential of zygotes to blastocysts were 0.01 and 0.1 µg/ml, respectively, except after 9 months preservation at −80 °C (0.01 µg/ml). The maximal concentration of berberine which did not inhibit the developmental potential to blastocysts was 0.001 µg/ml in all groups.

Effects of berberine treatment of mouse spermatozoa or oocytes on fertilization and the subsequent development

Table 3 shows the proportions of oocytes that survived and formed pronuclei, and the developmental potential of oocytes with pronuclei after the cytoplasmic injection of spermatozoa treated with berberine at different concentrations and different treatment times. In one experiment where spermatozoa treated with 1 µg/ml berberine for 60 min were used for ICSI, the proportion of oocytes with pronuclei to develop into blastocysts was significantly lower than that of the control. However, a repeat experiment did not confirm this result, and in the other all groups, the proportions of oocytes with pronuclei which developed *in vitro* after ICSI were not significantly different between the berberine-treated and control groups. Berberine treatment did not increase the sperm mortality. Mortality after treatment with 1 and 10 µg/ml berberine for 2, 4 and 6 h was not different from the controls: 30.7%, 46.0% and 65.2% in the control, 29.2%, 43.3% and 61.7% in 1 µg/ml berberine, and 27.5%, 46.7% and 63.8% in 10 µg/ml berberine.

The *in vitro* development of oocytes treated with 1 µg/ml berberine for 60 min after ICSI was not different from control oocytes. The proportions of surviving oocytes (65%), forming pronuclei (96%), the *in vitro* development to the 2-cell (96%), 4- to 8-cell (94%), morula (91%) and blastocyst (63%) stages in the berberine-treated group were not different from those of the respective control groups (68%, 91%, 94%, 91%, 89% and 63%, respectively). There were no significant differences in total (42.5 vs 41.7), ICM (12.6 vs 13.0), TE cell (29.9 vs 28.7) numbers of blastocysts between berberine-treated and control groups .

Effects of berberine treatment on the growth of mouse fetal fibroblast cells

The number of fetal fibroblast cells after culture with berberine at the different concentrations for 24 to 120 h were compared with the respective control groups. The number of viable cells in the control increased from 1.15 × 10^5 to 3.22 × 10^5 cells/ml in series 1 and from 1.15 × 10^5 to 4.57 × 10^5 cells/ml in series 2 during culture for 120 h. The effects of berberine on the growth of fetal fibroblast cells were dose- and time-dependent. The growth of fetal fibroblast cells was not inhibited after treatment with 0.001, 0.01 and 0.1 µg/ml berberine up to 120 h compared with controls (3.17 to 3.27 × 10^5 cells/ml vs 3.22 × 10^5 cells/ml). However, the cell numbers of fetal fibroblast cells after treatment with berberine at 1, 10 and 100 µg/ml were significantly lower than controls (0.05 to 0.62 × 10^5 cells/ml vs 4.51 × 10^5 cells/ml for 96 h culture and 0.01 to 0.36 × 10^5 cells/ml vs 4.57 × 10^5 cells/ml for 120 h culture). The minimum concentration of berberine to almost completely inhibit the growth of fibroblast cells after treatment for 96 h was 10 µg/ml.

Discussion

The present study demonstrated that the *in vitro* development of mouse zygotes with different genetic backgrounds to blastocysts was completely or significantly inhibited when zygotes were cultured in medium supple-

mented with 0.1 μg/ml or 0.01 μg/ml berberine chloride.

It has been reported that berberine, the main component extracted from Coptis rhizome and Phellodendron, has an inhibitory effect on the growth of various types of cancer cells [1]. Although berberine inhibits cell cycle progression, the stage at which berberine induces cell cycle arrest is different depending on the concentration, treatment period and cancer cell type. Berberine at the low concentrations (5 to 19 μg/ml for melanoma cells [2], 19 μg/ml for HONE 1 cells [15]) was accumulated in mitochondria and promoted G1 arrest, but at the high concentrations (over 19 μg/ml for melanoma cells [2], 111 μg/ml for HONE 1 cells [15]) it accumulated in cytoplasm and nuclei and promoted G2 arrest [2]. Berberine at high doses also induces apoptosis in cancer cells through the increase of caspase-3 following the release of cytochrome C [15, 16].

When mouse zygotes were cultured in medium supplemented with 0.1 μg/ml berberine, the developmental potential to the blastocyst was completely inhibited. However, when zygotes were cultured in medium without berberine after temporary treatment with 0.1 μg/ml berberine for 24 h or 48 h, the proportions of embryos developing to blastocysts were not significantly different from the controls. Also, the cell numbers of blastocysts obtained after treatment with 0.1 μg/ml berberine for 24 h were not different from the control. Although we did not examine the intracellular distribution of berberine after treatment, it might be located in the mitochondria of zygotes [2, 15], where it would not induce cell cycle arrest. However, the blastocyst cell number was significantly reduced due to a slowing of the cell cycle when zygotes were treated with 0.1 μg/ml berberine for 48 h, and then development to blastocysts was inhibited when the treatment was extended to over 72 h [2]. When mouse zygotes were treated with the higher concentration of berberine, 10 μg/ml, for 3 h, the development of zygotes was completely inhibited, possibly due to the cytotoxic effect of berberine [1], and / or to the translocation of berberine into the nucleus leading to G2 arrest and DNA damage [2, 3].

It has been reported that berberine is soon incorporated into bovine sperm heads [17] after treatment for 10 to 120 min. The present study demonstrated that the treatment of mouse epididymal spermatozoa with 1 and 10 μg/ml berberine did not inhibit the formation of the pronucleus, fertilization and the *in vitro* development after ICSI. Since the main mechanism of berberine for inhibiting the cell growth of cancer cells is cell cycle arrest and induction of apoptosis [2], spermatozoa with compacted nuclei might not be affected by berberine. The present study also demonstrated that treatment of MII oocytes with 1 μg/ml berberine for 60 min did not inhibit the fertilizability of the oocytes. However, the possibility that berberine inhibits the fertilizability of oocytes could not be excluded at higher concentrations and longer exposure times.

It has been reported that berberine chloride solution could be preserved with no significant degeneration at 25°C and 40°C for at least 6 months [18]. The present study demonstrated that berberine chloride solution could also be preserved without reduction of its inhibitory effect on the development of mouse zygotes at 4°C and −80°C for at least 12 months. The present study demonstrated that the *in vitro* development of mouse zygotes is about 100 times more sensitive to berberine than the *in vitro* growth of mouse fetal fibroblast cells. The minimum concentrations of berberine to almost completely inhibit the growth of fibroblast cells and the development of zygotes to blastocysts after treatment for 96 h or 94 h were 10 μg/ml and 0.1 μg/ml, respectively. Since, as shown in Table 2, treatment of zygotes with 0.01 μg/ml for 72 h, but not 24 h and 48 h, inhibited the development to blastocysts, berberine at more than 0.01 μg/ml need to be present in the genital tract during the zygote to morula stage if berberine is administered to females as a contraceptive.

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