In Vitro Study of Teratogenic Effects of Caffeine on Cultured Rat Embryos and Embryonic Cells

Takayuki IWASE, Kazuyoshi ARISHIMA[1)], Naoki OHYAMA, Keiko INAZAWA, Yumiko IWASE, Yasuo IKEDA, Mitsuyuki SHIRAI[2)], Masako YAMAMOTO[1)], Hiroaki SOMIYA[3)], and Yasunobu EGUCHI[1)]

Toxicology Laboratory, Research Center, Mitsubishi Kasei Co., Midori-ku, Yokohama, Kanagawa 227, and Departments of [1)]Anatomy, [2)]Pharmacology and [3)]Developmental Biotechnology, School of Veterinary Medicine, Azabu University, Fuchinobe, Sagamihara, Kanagawa 229, Japan

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ABSTRACT. The teratogenic potential of caffeine was examined *in vitro* by a whole embryo culture system (WECS) and an embryonic cell culture system (micromas teratogen assay: MTA) in the rat. In the WECS, hyperemia of the tail, and a reducion of the placental size was induced by caffeine at concentrations higher than 50 μg/m*l*; hypoplasia of the forelimb bud was induced at concentrations higher than 100 μg/m*l*; hematoma in the yolk sac and dysmorphogenesis of the fore- and hind-limb buds, prosencephalon and tail were induced by 200 μg/m*l* caffeine. In the MTA, even with 200 μg/m*l* caffeine, the toxicological parameters obtained by proliferation and differentiation assays of the midbrain and limb bud cells were almost the same as in the control. In conclusion, caffeine induced various morphological anomalies, but did not affect proliferation or differentiation of cells in these experimental systems.—KEY WORDS: caffeine, rat, whole embryo culture.

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Since it has been reported that caffeine exerts teratogenesis in mice [9], many studies have examined the teratogenicity of caffeine in various experimental animals. For example, caffeine induced cleft palate, limb malformations and skeletal abnormalities in mice and rats [4, 5, 10, 11, 14]. It has also been reported that caffeine decreased the blood supply to the uterus in rats [7], and that the decreased oxygen supply to pregnant rats had teratogenic effects in the fetuses [2, 16]. Recently, the embryonic cell culture system has been used for detection of teratogenic activity of various agents. In this system, particular cells such as forelimb bud cells or mid-brain cells of rodent embryos having a high sensitivity to chemicals are cultured. The present study was designed to investigate how caffeine affects cultured rat embryos and embryonic cells, using a whole embryo culture system and an embryonic cell culture system.

Female Wistar Imamichi rats were placed with males overnight, and the day of plug detection was considered as day 0 of gestation. The whole embryo culture was performed by the method of New [8] with modification. On day 11 of gestation, intact rat embryos were isolated from the uteri of pregnant rats under ether anesthesia. The culture medium was 3.8 m*l*/embryo/vial of immediately centrifuged rat serum including 2 mg/m*l* glucose, 5 IU/m*l* penicillin G and 5 mg/m*l* streptomycin. The vials were rotated at 20 rpm in an incubator (rotated whole embryo apparatus; Ikemoto Rika Co.) at 38°C and were continuously supplied with a mixed gas of 95% O_2 and 5% CO_2. After 2-hr-preincubation, 20 embryos were exposed for 46 hr to caffeine, the concentrations of which were 25, 50, 100 and 200 μg/m*l*. The yolk sacs were opened after 22-hr incubation, and the embryos were further cultured. The heart beats were counted at the time of 0.5, 1, 2, 16, 22, 26 and 46-hr exposure. At the completion of the culture, the embryos were examined to determine morphological changes. The diameters of placentae were measured as well.

The embryonic cell culture was performed by Flint and Orton's micromass teratogen assay method [3] using forelimb bud cells (LBC) and midbrain cells (MBC) isolated from rat embryos on day 12 of gestation. Using a CO_2 incubator, 1×10^5 LBC/well and 5×10^4 MBC/well were inoculated on 96 well plates and cultured at 37°C. After 2-hr preincubation, 200 μl/well of Ham's F12 medium with 10% fetal calf serum containing caffeine at concentrations of 52, 73, 102, 143, and 200 μg/m*l*, was added to 6 wells each. After 5-day incubation, the cultured cells were fixed with 2.5% glutaraldehyde and subjected to a cell proliferation assay, using a microplate reader of neutral red absorption at 540 nm [1]. The cultures formed foci as micromass islands consisting of high cell densities of chondrocytes differentiated from LBC or neural cells differentiated from MBC. The foci of LBC were stained with 0.1% alcian blue. Those of MBC were stained with Delafield's hematoxyline. The cell differentiation assay was performed by measuring the surface areas of the foci with an image analyzer [3].

The incidence of morphological abnormalities were statistically analyzed by 2×2 χ^2 test. Placental diameters and assay values were statistically analyzed by one-way analysis of variance, and then analyzed by Dunnett's test.

The cultured embryos were all alive. Table 1 shows types and incidences of various morphological abnormalities in cultured embryos. In embryos exposed to 50 μg/m*l* caffeine, hyperemia of the tail and a significantly reduced diameter of the placenta were observed. The embryos exposed to 100 μg/m*l* caffeine had hypoplasia of the forelimb bud as well (Fig. 1). In addition, 200 μg/m*l* caffeine induced hematoma in the yolk sac, hypoplasia of the prosencephalon, edema in both the forelimb and hindlimb buds, hypoplasia of the hindlimb buds and shortened tails. The incidences of these morphological anomalies were correlated with the concentrations of ceffeine. However, the heart rate remained within 140 to 180 beats/min during the time-course of culture, with no significant differences between different concentrations of caffeine.

Figure 2 shows the values obtained by proliferation and differentiation assays in the LBC and MBC, expressed as

Table 1. Effects of caffeine on the morphogenesis of rat embryos cultured for 46 hr

Type of dysmorphogenesis	Concentration of caffeine (μg/ml)				
	0 (control)	25	50	100	200
No. of live embryos	20	20	20	20	20
Embryos with dysmorphogenesis (%)	0	0	95*	100*	100*
Yolk sac hematoma (%)	0	0	0	0	45*
Prosencephalon hypoplasia (%)	0	0	0	5	30*
Forelimb bud hypoplasia (%)	0	0	20	45*	90*
Forelimb bud edema (%)	0	0	0	15	40*
Hindlimb bud hypoplasia (%)	0	0	0	20	50*
Tail hyperemia (%)	0	5	90*	95*	100*
Tail shortened (%)	0	0	0	5	60*
Placental diameter (mean±SEM, mm)	2.9±0.04	2.7±0.07	2.6±0.04*	2.7±0.04*	2.5±0.04*

*: Significantly different from the control ($P<0.05$).

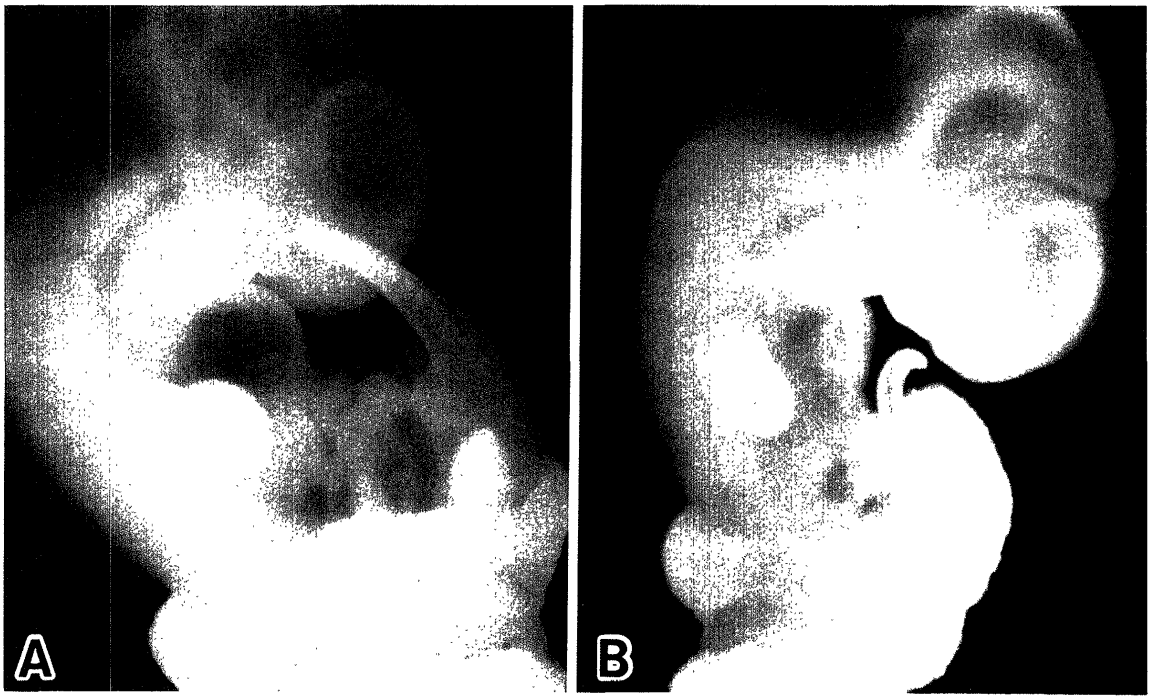

Fig. 1. Morphological changes in cultured embryos exposed to 100 μg/ml caffeine. A: A day-11 embryo cultured in the medium without caffeine *in vitro* for 48 hr showing normal morphogenesis. B: A day-11 embryo exposed to caffeine showing dysmorphogenesis, including hypoplasia of the prosencephalon, hypoplasia of the fore- and hind-limbs, and hyperemia in the tail which was shortened.

percents of the control values. The values in both the LBC and MBC were almost constant regardless of caffeine concentrations, with no statistically significant differences.

In the cultured embryos, caffeine induced hyperemia and/or dysmorphogenesis of the tail, forelimb and hindlimb buds and the prosencephalon. It also reduced the diameter of the placenta and produced hematoma in the yolk sac. The morphological anomalies in the forelimb and hindlimb buds were considered to result in deleted fingers or abnormalities of the limb, as previously reported in mouse and rat fetuses exposed to caffeine *in vivo* [4, 5, 9, 11]. In relation to this, the teratogenicity of caffeine can be reduced *in vivo* by treatment with propranolol, an adrenergic β-blocker, which reduces the level of cyclic AMP in cells [6], and that caffeine exerts its pharmacological action by raising the level of cyclic AMP through inhibition of phosphodiesterase activity and thereby dilating the peripheral blood vessels [11]. In particular, the dilation of peripheral blood vessels by caffeine is thought to induce hyperemia of the tail and

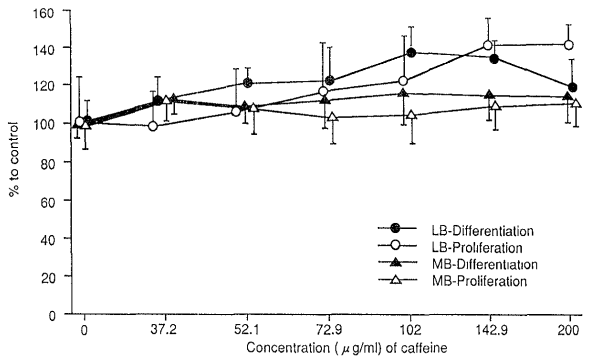

Fig. 2. The values of proliferation and differentiation assays using LBC and MBC.

hematoma in the yolk sac [12]. The reduced diameter of the placenta suggests that caffeine also injures the placenta either directly or indirectly.

Although it has been reported that caffeine increases the level of cyclic AMP in the limb bud and palate of mouse embryos *in vitro* [13], neither the LBC or the MBC in the present embryonic cell culture system showed any cell proliferation impairment or cell differentiation impairment at any concentration of caffeine up to 200 μg/ml, in agreement with the result by Uphill *et al.* [15]. Therefore, it is speculated that morphological anomalies induced by caffeine are partly due to an effect on the peripheral blood vessels, but not due to a direct effect on the embryonic cells, at least in the experimental systems used in the present study.

REFERENCES

1. Borenfreund, E. and Puerner, J. A. 1985. *Toxicol. Lett.* 24: 119–124.
2. Fantel, A. G., Barber, C. V., and Mackler, B. 1992. *Teratology* 46: 285–292.
3. Flint, O. P. and Orton, T. C. 1984. *Toxicol. Appl. Pharmacol.* 76: 383–395.
4. Fujii, T., Sasaki, H., and Nishimura, H. 1969. *Jpn. J. Pharmacol.* 19: 134–138.
5. Fujii, T. and Nishimura, H. 1972. *Toxicol. Appl. Pharmacol.* 22: 449–457.
6. Fujii, T. and Nishimura, H. 1974. *Teratology* 10: 149–152.
7. Kimmel, C. A., Kimmel, G. L., White, C. G., Grafton, T. F., Young, J. F., and Nelson, C. J. 1984. *Fund. Appl. Toxicol.* 4: 240–247.
8. New, D. A. T. 1978. *Biol. Rev.* 53: 81–122.
9. Nishimura, H. and Nakai, K. 1960. *Proc. Soc. Exp. Biol. Med.* 104: 140–142.
10. Nolen, G. A. 1981. *Toxicol. Appl. Pharmacol.* 58: 171–183.
11. Pitel, M. and Lerman, S. 1964. *Am. I. Ophth.* 58: 464–470.
12. Rall, T. W. 1985. pp. 589–603. *In*: The Pharmacological Basis of Therapeutics, 7th ed. (Gilman, A. G., Goodman, L. S., Rall, T. W., and Murad, F. eds.), Macmillan, New Yolk.
13. Schreiner, C. M., Zimmerman, E. F., Wee, E. L., and Scott JR, W. J. 1986. *Teratology* 34: 21–27.
14. Scott, W. J. 1983. *Teratology* 28: 427–435.
15. Uphill. P. F., Wilkins, S. R., and Allen, J. A. 1990. *Toxic. in Vitro* 4: 623–626.
16. Webster, W. S., Lipson, A. H., and Brown-Woodman, P. D. C. 1987. *Teratology* 35: 253–260.