ELSEVIER

Brain Research 773 (1997) 213–216

BRAIN RESEARCH

Short communication

Caffeine-induced disturbances of early neurogenesis in whole mouse embryo cultures

Stéphane Marret [a,b], Pierre Gressens [b], Geneviève Van-Maele-Fabry [c], Jacques Picard [c], Philippe Evrard [b,*]

[a] *Service de Médecine Néonatale, Centre Hospitalier Universitaire de Rouen, Rouen, France*
[b] *INSERM CRI 96-03, Faculté de Médecine Xavier-Bichat and Hôpital Robert-Debré, Paris, France*
[c] *Laboratoire de Génétique du Développement, Université de Louvain, Louvain-la-Neuve, Belgium*

Accepted 22 July 1997

Abstract

In toto mouse embryos were cultivated at embryonic day 8.5 for 26 h with 105, 310 or 620 μM caffeine; 105–310 μM correspond to concentrations transferred by the placenta of heavy caffeine consumers. Failure of neural tube closure, excessive proliferation of neuroepithelial cells and premature evagination of telencephalic vesicles were present in 50% of treated embryos. When reaching the embryonic neural tube before neuronal migration, caffeine regionally modifies the schedule and/or rate of neural cell proliferation.

Keywords: Neural tube closure; Pregnancy; Development; Environment

Caffeine is a widely used neuroactive drug present in coffee, tea, chocolate and cola drinks. During pregnancy, caffeine crosses the placenta and some of its metabolites (theophylline, theobromine, paraxanthine) seem to be concentrated in the fetal brain [17]. In vitro, caffeine has some deleterious effects on metabolic pathways which play a crucial role in neural development: inhibition of cholesterol synthesis [15], stimulation of extracellular matrix synthesis [4] and antagonism of adenosin [6]. The purpose of the present study is to investigate in whole cultured mouse embryos the effects of caffeine on brain development at the pre-migratory steps of neurulation and encephalization.

NMRI mouse embryos displaying 6–8 somites were cultivated, for 26 h, in propylene tubes containing culture medium (80% adult human and 20% rat sera) in an incubator at 37°C with 5% CO_2 and 95% air [3,14]. Caffeine (Sigma, St. Louis, MO) was added to the culture medium at the beginning of the culture period to obtain final concentrations of 105, 310 and 620 μM caffeine. At the end of the culture period, embryos were examined for macroscopic abnormalities under a microscope. Absence of heartbeat and/or lack of yolk-sac circulation were used as criteria for death. Growth of the surviving embryos was assessed by counting the number of pairs of somites and by using size measurements: yolk sac diameter, crown-rum length and head length [3,14]. After glutaraldehyde and osmium tetroxide fixation, embryos were embedded in resin. Serial coronal sections were stained with Toluidine blue for light-microscopic observations. In each embryo, the pyknotic (PI) and mitotic (MI) indices were determined on five non-adjacent sections (taken at 10-μm intervals) at the level of the optic vesicles. A Kruskal-Wallis non-parametric ANOVA test was used to compare MI and PI in control group and each caffeine group. When group interaction was significant, Dunn's multiple comparison test was performed. Measurements are reported as mean $\pm$ S.D.

In caffeine-treated embryos, three types of disorders of neurulation were observed (Table 1, Fig. 1): (1) thickening of the neuroepithelium; (2) premature evagination of the ventral prosencephalic neuroepithelium corresponding to the encephalization level reached 36 h later in non-treated in vivo animals [12]; (3) absence of neural tube closure. A significant dose-dependent increase of the MI and of the PI in neural tube was observed between controls and embryos treated with different doses of caffeine. No significant differences could be detected between the MI and the PI of

* Corresponding author. Neurologie Pédiatrique, Hôpital Robert-Debré, 48 boulevard Sérurier, F-75019 Paris, France. Fax: +33 (1) 40 03 47 74; E-mail: 101323.501@compuserve.com

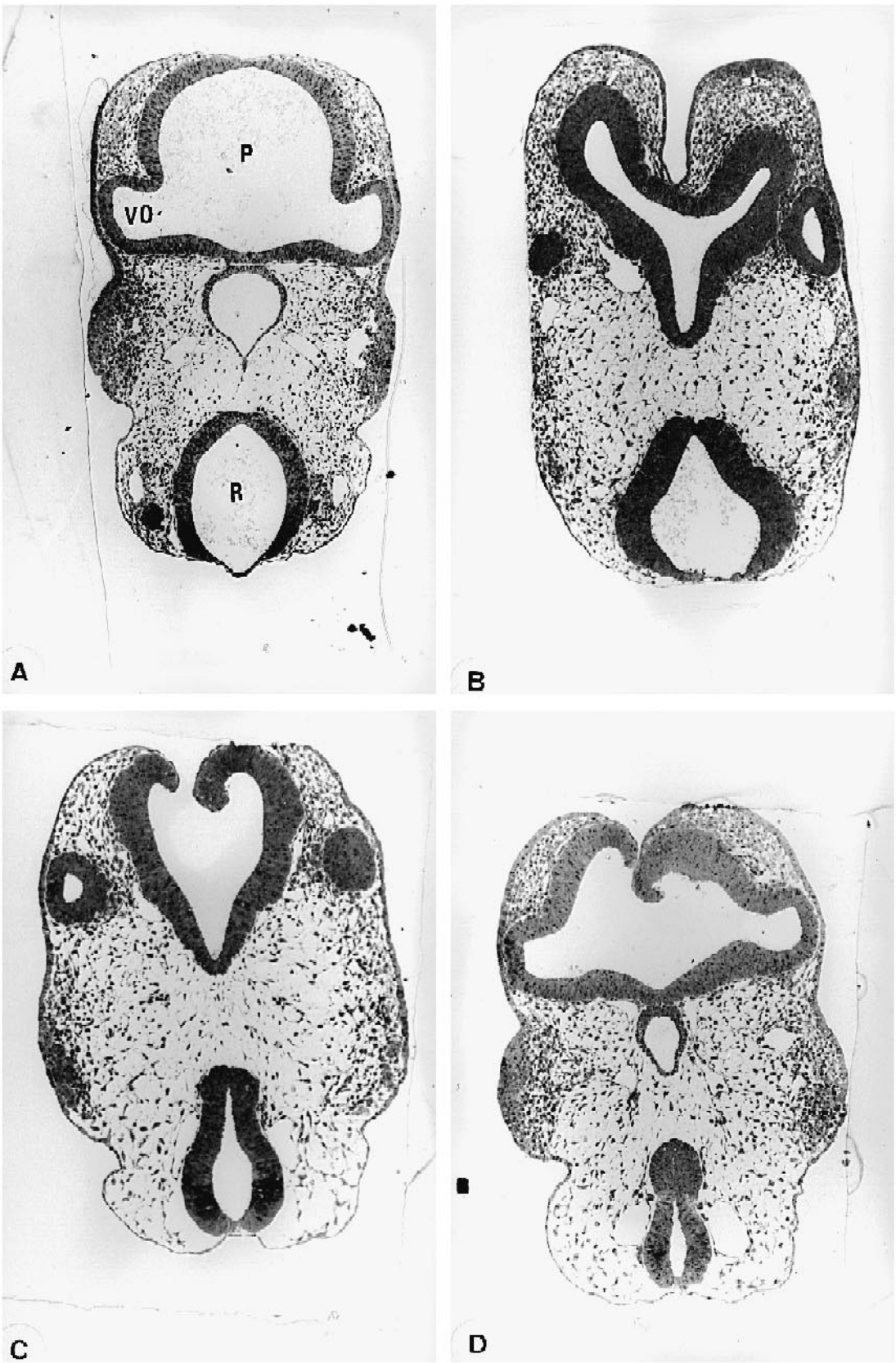

Fig. 1. Photographic representation of coronal 2-μm-semi-thin sections of mouse embryos. A: control. P, prosencephalon; VO, optic vesicles; R, rhombencephalon. B: caffeine-treated embryo displaying premature evagination of the ventral prosencephalic neuroepithelium. C: caffeine-treated embryo displaying absence of neural tube closure. D: caffeine-treated embryo displaying thickening of the neuroepithelium. Magnification $\times 80$.

Table 1
Caffeine-induced dysmorphogenesis in 8.5-day-old mouse embryo cultures

Caffeine dose (μM)	Number of embryos	Number of malformations (%)	Normal neural tube	Premature evagination/thickening of neuro epithelium	Open neural tube	Abnormal eye and/or arch	Mitotic index in prosencephalon	Pycnotic index in prosencephalon
0	7	0	7	0	0	0	4.5 ± 1.5	1.5 ± 0.6
105	9	22	6	2	1	1	5.0 ± 1.4 NS	1.8 ± 0.5 NS
310	9	44	5	3	1	1	8.3 ± 1.4 [a]	2.6 ± 1.1 [a]
620	5	88	2	3	0	3	12 ± 3 [a]	3.9 ± 1.5 [a]

Dunn's multiple comparison test. NS, non-significant difference; [a] $P < 0.001$.

ventral and dorsal parts of the prosencephalic neuroepithelium. Regardless of caffeine dosage, increased MI and PI were still present when all caffeine-treated embryos were divided in two subgroups: (1) embryos displaying telencephalic evagination or neuroepithelial thickening (MI = 9.4 ± 3.9; PI = 3.1 ± 1.6; $n = 8$; statistically different from controls, $P < 0.001$); (2) treated embryos without macroscopic neuroepithelial abnormalities (MI = 7.1 ± 2.7; PI = 2.4 ± 1.3; $n = 13$; statistically different from controls, $P < 0.001$). The number of somites and the growth of the embryos were not significantly affected by caffeine exposure as compared to the control group (data not shown).

The present study indicates that a short exposure to caffeine induces disturbances of the early neurogenesis in whole cultured embryos. Caffeine was shown to be a teratogen in several species including rabbits, mice and rats. In most previous studies, placental blood flow and embryonic growth were severely reduced [2,6–8]. In our study, embryonic growth and placental circulation do not seem disturbed by caffeine, suggesting that caffeine does not act primarily on the placental vascular bed. The lowest equivalent dosage used in the present study (105 μM) corresponds to 25 mg/kg, one-fourth of the dose used in teratological studies [2,6]. Our data confirm the severe influence of caffeine on neural tube closure and, to our knowledge, provide the first demonstration of defective neural tube closure induced by low doses of caffeine. In addition, we report a dramatic increase of the MI and the PI, with thickening of the neuroepithelium and premature evagination of the ventral prosencephalic vesicles, which suggests a focal acceleration of neural maturation. Cumulative labelling of S-phase cells with bromodeoxyuridine could approach the effects of caffeine on mitotic cell cycle [11].

At the molecular level, several hypotheses could be proposed to explain our data. Caffeine interferes with cell division before DNA replication is completed [9]. It also leads to a release of calcium from intracellular stores through a caffeine-sensitive ryanodine receptor [13]. Caffeine induces expression of immediate-early genes which are key regulators of cellular transcription [10]. In glial cell cultures, caffeine decreases cholesterol synthesis [15], which could lead to cell membrane disturbances, and increases hyaluronan secretion [4], which is a key component of extracellular matrix during brain development [1]. Caffeine antagonizes the action of adenosine in the metabolism of neurotransmitters [6]; however, mRNAs for adenosine A2a receptor were not detected by in situ hybridization before E14 in rat brains [16].

The reported defects of early neurogenesis induced by caffeine raised concerns about the possibility of side-effects in humans. In in vivo animal studies, ≈ 25 mg/kg/day of caffeine are necessary to obtain a caffeine blood level of 100 μM which is the smallest concentration of caffeine used in the present study. It could correspond to a heavy human consumption of 8–10 cups of coffee or caffeine-containing beverages [5]. In rats, caffeine crosses the placenta and is accumulated in fetal brain at higher concentration than in serum after maternal consumption of coffee [17]. To date, teratogenic associations with caffeine consumption in humans are anecdotal [5,6]. However, little data are available on consumers of very high amounts of caffeine during pregnancy. Human studies only focused on evident birth defects but did not analyze less conspicuous alterations of brain development induced by caffeine, which could be suggested by the present study and which could alter brain function.

In the present model, caffeine during early brain development accelerates encephalization. It could carry positive and/or negative consequences which will be tested in in vivo experiments.

Acknowledgements

We thank Dr. Astrid Nehlig for critical reading of the manuscript, Ms. A.-M. Rona for technical assistance and Mr. J.-F. Ménard for statistical evaluations. This work was supported by INSERM, Fondation de France, Fonds National de la Recherche Scientifique (Belgium) and Centre Hospitalier Universitaire of Rouen.

References

[1] A. Delpech, B. Delpech, Expression of hyaluronic acid-binding glycoprotein, hyaluronectin, in the developing rat embryo, Dev. Biol. 101 (1984) 391–400.

[2] R.A.R. Fadel, T.V.N. Persaud, Effects of alcohol and caffeine on cultured whole rat embryos, Acta Anat. 144 (1992) 114–119.
[3] P. Gressens, F. Gofflot, G. Van-Maele-Fabry, J.-P. Misson, J.-F. Gadisseux, P. Evrard, J. Picard, Early neurogenesis and teratogenesis in whole mouse embryo cultures, J. Neuropathol. Exp. Neurol. 51 (1992) 206–219.
[4] S. Marret, B. Delpech, N. Girard, A. Leroy, C. Maingonnat, J.F. Ménard, C. Fessard, Caffeine decreases glial cell number and increases hyaluronan secretion in newborn rat brain cultures, Pediatr. Res. 34 (1993) 716–719.
[5] S.A. Narod, S. De Sanjosé, C. Victora, Coffee during pregnancy: a reproductive hazard?, Am. J. Obstet. Gynecol. 164 (1991) 1109–1114.
[6] A. Nehlig, J.L. Daval, G. Derby, Caffeine and the central nervous system: mechanisms of action, biochemical, metabolic and psychostimulant effects, Brain Res. Rev. 17 (1992) 139–170.
[7] H. Nishimura, K. Nakai, Congenital malformations in offspring of mice treated with caffeine, Proc. Soc. Exp. Biol. Med. 104 (1960) 140–142.
[8] E.J. Ritter, W.J. Scott, J.G. Wilson, P.R. Mathinos, J.L. Randall, Potentiative interactions between caffeine and various teratogenic agents, Teratology 25 (1982) 95–100.
[9] R. Schlegel, A.B. Pardee, Caffeine-induced uncoupling of mitosis from the completion of DNA replication in mammalian cells, Science 232 (1986) 1264–1266.
[10] M. Sheng, M.E. Greenberg, The regulation and function of c-*fos* and other immediate early genes in the nervous system, Neuron 4 (1990) 477–485.
[11] T. Takahashi, R.S. Nowakowski, V.S. Caviness, BUdR as an S-phase marker for quantitatives studies of cytokinetic behaviour in the murine cerebral ventricular zone, J. Neurocytol. 21 (1992) 185–197.
[12] K. Theiler, The House Mouse. Development and Normal Stages From Fertilization to 4 Weeks of Age, Springer, Berlin, Germany, 1975.
[13] T.D. Tsai, M.E. Barish, Imaging of caffeine-inducible release of intracellular calcium in cultured embryonic mouse telencephalic neurons, J. Neurobiol. 27 (1995) 252–265.
[14] G. Van Maele-Fabry, F. Delhaise, J.J. Picard, Morphogenesis and quantification of the development of postimplantation mouse embryos, Toxicol. In Vitro 99 (1989) 431–451.
[15] J.J. Volpe, Effects of methylxanthines on lipid synthesis in developing neural systems, in: T.K. Oliver, T.H. Kirschbaun (Eds.), Seminars in Perinatalogy, Grune and Straton, New York, NY, 1981, pp. 395–403.
[16] D.R. Weaver, A1-Adenosine receptor gene expression in fetal rat brain, Dev. Brain Res. 94 (1996) 205–223.
[17] J.M. Wilkinson, I. Pollard, Acumulation of theophylline, theobromine and paraxanthine in the fetal rat brain following a single dose of caffeine, Dev. Brain Res. 75 (1993) 193–199.