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Behavioral and Biochemical Consequences of Perinatal Exposure of Mice to Instant Coffee: A Correlative Evaluation

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AJAREM, J. S. AND M. AHMAD. *Behavioral and biochemical consequences of perinatal exposure of mice to instant coffee: A correlative evaluation.* PHARMACOL BIOCHEM BEHAV **40**(4) 847–852, 1991.—In the present study, the lasting effects of prepartum and perinatally consumed instant coffee by female mice on the behavior as well as on the level of activities of certain enzymes in the tissues of their male offspring have been investigated. The behavioral observations of nonsocial investigation, defense, displacement, latency to threat and naso-nasal contact has decreased significantly in offspring of treated mothers, while the threat, attack, latency to threat and attack and number of fights have increased significantly. Hence, coffee has proven to be an inducer of hyperactive behavior in these offspring. Such effects are both dose dependent and duration-of-treatment dependent. Moreover, variations were detected in the level of AChE activity in the brain tissues of these offspring together with variations in the levels of AcPase and AlPase activities in their liver, kidneys and testes. Such variations in these organs have developed in utero, making these enzymes convenient markers in teratological studies.

Coffee Caffeine Methylxanthine Perinatal Prepartum Ethological analysis Enzyme analysis Mice

THERE is growing evidence now that perinatal consumption of drugs may cause several disturbances to the brain maturation and behavior in mammalian offspring (16). Several reports are available for the association of early exposure of pregnant mice to a wide range of compounds with the essential changes in the subsequent behavior of their male offspring (2, 5, 8, 27).

Instant coffee, a widely consumed beverage, may contain several chemical substances like caffeine, theophylline and theobromine, etc., among which caffeine, an alkaloid methylxanthine, forms its major component (44,57). The structural configuration of caffeine is similar to that of purine molecules in DNA and thus possesses the ability to interfere with the metabolism and cell division (1). West et al. (54) have recently reviewed the caffeine effects on various physiological, metabolic, immune, endocrine, neurochemical and behavioral functions. Moreover, the pharmacological, endocrinological, neurotoxic and direct toxicological effects of caffeine on developing fetuses have been briefly reviewed by Pollard et al. (41).

It has been established beyond doubt in many animal studies that high doses of caffeine cause acute structural teratogenesis (34,57) and exposure of rodents during pregnancy results in behavioral, developmental and biochemical changes in offspring (12, 13, 24, 41, 46, 47, 54). Human studies have also reported increased fetal loss and fetal growth retardation after excessive coffee intake during pregnancy (53). Hence, these studies point to the real and potential perinatologic risk of maternal caffeine ingestion during pregnancy (19).

The objectives of the present study were to examine the influence of prepartum and perinatal coffee intake on mice offspring behavior together with the possible mode of transfer of coffee (caffeine) from dams to offspring. Further, the role of some enzymes like acetylcholinesterase (AChE) in brain tissues and levels of acid and alkaline phosphomonoesterases (AcPase and AlPase) in liver, kidneys and testes of the male offspring, as possible teratological markers has been explored.

METHOD

Swiss-Webster albino mice (*Mus musculus*) were bred and housed under highly controlled conditions (2) in the animal facility of the Zoology Department, College of Science, King Saud University, Riyadh.

Coffee Administration and Experimental Design

Instant coffee dissolved in tap water formed the only source of drinking fluid for the experimental group of mice. On the basis of average consumable volume of water per adult mouse per day, coffee from a 2 g sachet was dissolved accordingly so as to produce the required doses per kg body weight per day. Coffee in one sachet is usually equivalent to 1 cup for an average 60 kg person. Thus the doses of 1, 2 and 4 mg used herein are respectively equivalent to 1, 2 and 4 cups of coffee per kg body weight per day in mice. No effort was made to analyse the actual caffeine content in 1 cup of coffee, however, it is worth mentioning that 4 cups of coffee per day amount to approximately 5 mg of caffeine per kg body weight per day (43).

Thirty-two pregnant mice (pregnancy estimated from the date of finding vaginal plug) were divided into four groups of eight

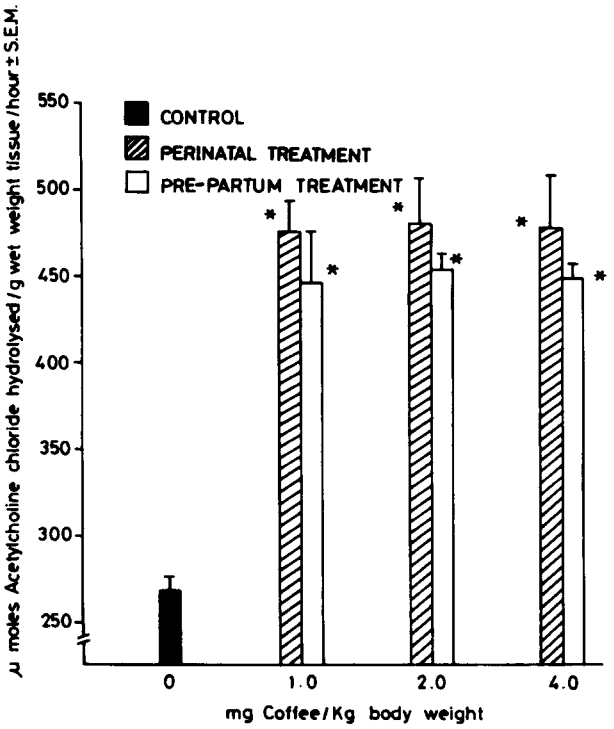

FIG. 1. Effects of coffee consumption by mothers on the level of acetylcholinesterase in the brain of their male offspring. *Indicates significance ($p<0.02$) by Mann-Whitney U-test. Number of mice in each group were the same as given in tables.

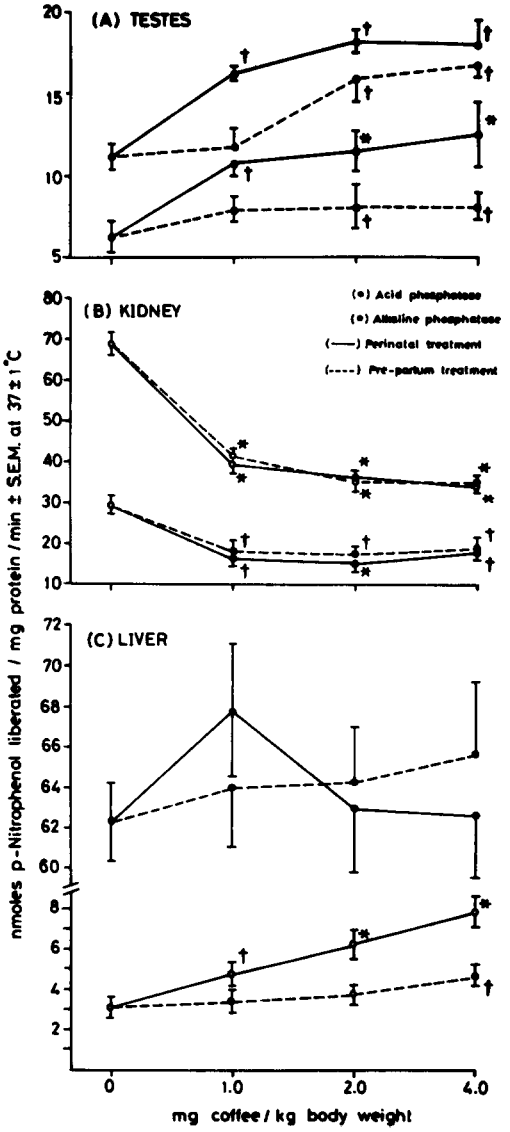

FIG. 2. Effects of coffee consumption by mothers on activity of phosphomonoesterases in testes (A), kidney (B) and liver (C) of their male offspring. *Indicates significance ($p<0.02$) and † indicates significance ($p<0.05$) by Mann-Whitney U-test. Number of mice in each group were the same as given in tables.

in each. Group 1 received plain water only and was used as control. Groups 2, 3 and 4 were treated with 1, 2 and 4 mg coffee per kg body weight per day, respectively.

The pregnant mice were housed individually in opaque plastic cages measuring 30 × 12 × 11 cm. Initial studies (7, 8, 22) have suggested that the period around parturition is most suitable for assessing the lasting effects of drug treatments on subsequent behavior in developing rodents. Consequently, half the number of each treated group received coffee during prepartum period only and were then switched to plain water on the day their litters were born. The remaining halves were continued on coffee doses for perinatal treatment until the weaning of their litters. However, the litters were reduced to eight in all groups on the day of birth and left undisturbed with their mothers until 22 days of age when the male offspring were housed individually for 14 days, and thereafter were subjected to each of the following:

'Standard Opponent' Tests

The anosmic 'standard opponent' intruders were introduced singly in the home cages of test animals and the 'standard opponent' tests were carried out under dim red lighting (ca. 8 lux) as described elsewhere (9). The opponents were used only once and the behavioral tests were carried out visually for 500 seconds.

Behavioral Tests

The selected "elements" of behavior were based on the descriptions of Brain et al. (7,8). The following behavioral postures/measures were observed: 1) The latency to threat; 2) The latency to attack; 3) The total time allocated by subjects to the broad categories of: a) Nonsocial behavior; b) Social investigations; c) Defensive behavior; d) Attack behavior; e) Threatening behavior; f) Displacement behavior; 4) Total number of fights; 5) The frequencies of various acts or postures, such as "rears," "wall-rears," "naso-nasal contact," "naso-genital contact."

Enzyme Assays

The day after finishing behavioral tests, the experimental mice were killed by decapitation. The brain, liver, testes and kidneys were immediately removed, rinsed gently in physiological saline (0.9%), blotted on a Whatman filter paper, weighed and frozen.

TABLE 1

EFFECTS OF PERINATAL AND PREPARTUM CONSUMPTION OF COFFEE BY THE MOTHERS ON BEHAVIOR OF THEIR MALE OFFSPRING IN A "STANDARD OPPONENT" TEST

Coffee Dose	Treatment of Mother	Number of Replicates (n)	Median (with ranges) Number of Seconds Allocated to Behaviors Like: Nonsocial Investigation	Social Investigation	Defense	Threat	Attack	Displacement
% (Control)	—	6	233 (158–284)	144 (21–264)	66 (0–119)	12 (0–20)	9 (0–25)	49 (11–70)
1 mg/kg								
	Perinatal	6	247 (141–290)	142 (32–244)	22‡ (4–56)	39* (5–68)	37* (10–67)	33* (6–49)
	Prepartum	5	212 (70–255)	124 (28–252)	48* (2–68)	23* (12–39)	13* (9–36)	76* (42–172)
2 mg/kg								
	Perinatal	6	164* (142–238)	189 (76–271)	38† (1–56)	49* (39–65)	30* (2–45)	38* (19–126)
	Prepartum	4	309* (292–326)	117 (41–193)	29† (9–69)	9 (0–18)	7 (0–22)	44 (4–84)
4 mg/kg								
	Perinatal	4	244 (204–260)	201 (196–224)	14‡ (7–20)	28* (2–42)	9* (4–45)	23* (11–41)
	Prepartum	8	239 (116–254)	125 (18–291)	33† (0–67)	35* (18–58)	5 (0–59)	78* (59–116)

*$p<0.05$; †$p<0.02$; ‡$p<0.002$ (by Mann-Whitney U-test).

A 10% (w/v) homogenate of each tissue was prepared in teflon-glass homogenizer at 4 ± 1°C, centrifuged at 1000 × g for 10 min to remove cell debris and the supernatant was used for enzyme assays. The brain homogenate was prepared in ice-cold phosphate buffer, 0.067 M, pH 7.2, and its AChE activity was estimated using acetylcholine chloride as the substrate (23). The liver, kidneys and testes were homogenized in chilled 0.25 M sucrose solution, their protein content was estimated according to the method of Lowry et al. (35) and the levels of AcPase and AlPase using sodium p-nitrophenol phosphate as the substrate (6).

The specific activity of AChE is expressed as μmoles acetyl-

TABLE 2

EFFECTS OF PERINATAL AND PREPARTUM CONSUMPTION OF COFFEE BY THE MOTHERS ON THE INCIDENCES OF ACTS AND POSTURES (MEDIAN WITH RANGES) SHOWN BY THEIR MALE OFFSPRING IN A "STANDARD OPPONENT" TEST

Coffee Dose	Treatment of Mother	No. of Replicates (n)	Acts and Postures: Latency to Threat (Seconds)	Latency to Attack (Seconds)	Number of Fights	No. of Naso-Nasal Contact	No. of Naso-Genital Contact	Wall Rears	Rears
% (Control)	—	6	96 (0–301)	262 (0–419)	9 (0–19)	14 (4–19)	7 (2–21)	9 (2–24)	3 (2–10)
1 mg/kg									
	Perinatal	6	64* (17–261)	291 (30–489)	33† (11–48)	3* (2–15)	8 (1–36)	10 (1–17)	5 (0–16)
	Prepartum	5	82 (37–276)	186 (74–432)	18* (2–83)	7† (1–9)	12 (4–14)	27* (17–35)	10† (4–29)
2 mg/kg									
	Perinatal	6	20† (31–73)	346* (151–476)	14* (2–24)	13 (6–16)	14 (4–18)	14 (10–31)	3 (2–8)
	Prepartum	4	47* (0–94)	243 (0–486)	6 (0–12)	9 (6–12)	8 (1–15)	13 (0–26)	10* (1–20)
4 mg/kg									
	Perinatal	4	297* (220–358)	393† (317–475)	7 (2–16)	7 (4–18)	11 (2–19)	12 (6–26)	3 (1–15)
	Prepartum	8	198* (40–392)	222 (0–477)	3* (0–9)	4* (1–11)	9 (2–15)	23† (13–31)	10† (2–14)

*$p<0.05$; †$p<0.02$ (by Mann-Whitney U-test).

choline chloride hydrolysed per mg tissue wet weight per hour at 37 ± 1°C, and AcPase and AlPase as nmoles p-nitrophenol liberated per mg protein per minute at 37 ± 1°C.

Statistical Analysis

The data for behavioral measures were initially subjected to the Mann-Whitney U-test (45), thereafter, the elements of behavior found significantly different from the control groups, were compared (within the experimental groups) with respect to the factors of dose and duration-of-treatment (perinatal and prepartum) by the analysis of variance (ANOVA) (48). Similarly, the data for the enzyme estimations were first analysed by the Student's *t*-test (56) and the significant results were subsequently subjected to ANOVA testings within the experimental groups.

RESULTS

Behavioral data for the standard opponent test are given in Table 1. From the Mann-Whitney U-test, it is found that the median time allocated to broad categories of behavior like non-social investigation, defense, threat, attack and displacement are generally significantly affected. ANOVA testings reveal that while the overall defensive behavior has significantly decreased and is dose dependent, especially in the perinatally treated groups, $F(6) = 3.08$, $p<0.05$, the behavior of threat, though significantly increased in the perinatally treated groups, it is not dose dependent. The attack behavior has also increased in the perinatally treated groups but the effect is inversely dose dependent. The displacement behavior has shown a significant decrease in activity of perinatally treated groups which is also dose dependent, $F(6) = 3.70$, $p<0.05$, while in the prepartum-treated groups the activity has increased.

Table 2 summarizes the incidences of acts and postures shown by male offspring in the 'standard-opponent' test. The ANOVA testings of the significant experimental groups reveal that the latency to threat has significantly decreased with coffee doses of 1 and 2 mg/kg but at 4 mg/kg, the effect is reversed and the latency to threat has significantly increased, $F(6) = 4.86$, $p<0.05$. On the other hand, the latency to attack has increased in the perinatally treated groups only and the effect is also dose dependent, $F(6) = 4.37$, $p<0.05$. The number of fights has also significantly increased in the perinatal groups, $F(6) = 3.36$, $p<0.05$, but only at the lower dose rates, while the naso-nasal contact has significantly decreased in both groups, $F(6) = 3.11$, $p<0.05$, but only at low dose of 1 mg/kg. The number of wall-rears and rears has increased only in the prepartum-treated groups ($F = 3.55$ and $F = 2.99$, $df = 6$, $p<0.05$), but the increase is not dose dependent.

AChE activity has significantly increased in the brains of offspring whose mothers had been exposed to coffee perinatally, $F(6) = 5.36$, $p<0.01$, compared to those whose mothers have received it prepartly (Fig. 1). However, the level of stimulation is almost similar, irrespective of the dose rate.

The effect of coffee on the level of AcPase and AlPase activity in liver, kidney and testes of the male offspring are presented in Fig. 2. In testes and kidney the AlPase activity is higher than that of AcPase, whereas in liver the opposite is true.

The activity of phosphomonoesterases in the testes (Fig. 2A) is significantly higher in the perinatally treated groups compared to the groups which received prepartum treatment [$F(6) = 3.77$ and $F(6) = 4.31$, $p<0.05$] and this level of stimulation is both dose dependent and treatment-duration dependent.

In kidney (Fig. 2B) the AcPase and AlPase are significantly inhibited, $F(6) = 2.98$, $p<0.05$ and $F(6) = 5.31$, $p<0.01$, respectively, in both perinatal- and prepartum-treated groups, irrespective to the dose rate. While in liver, only the AlPase is significantly stimulated and the effect is dose dependent and is much pronounced in the offspring of mothers treated perinatally, $F(6) = 4.39$, $p<0.05$ (Fig. 2C).

DISCUSSION

It appears from the present study that coffee treatment of dams has a lasting effect on their male offspring. The alteration in the normal activity is at a higher level in offspring whose mothers were treated perinatally compared to those whose mothers were treated prepartum. Such effects of coffee, however, are not always dose dependent. The major component of coffee, instant or otherwise, is caffeine which has undoubtedly been studied more than any of the other compounds, both psychopharmacologically and biochemically (17). Hence, not ignoring the other components of instant coffee, the detected effects could well be largely due to caffeine. Even though the half-life of caffeine in rodents is less than 3 h (11,34), the continuous availability of coffee as the only drinking fluid to the experimental mice in the present study is more likely to cause a build-up of caffeine in their plasma. Thus it appears that besides the ready passage of coffee (caffeine) into the embryo through the placenta (21) and its penetration through the preimplantation blastocyte (20) during prepartum treatment, it is likely that coffee can also be passed to the offspring through milk during lactation. This possible combination might have led to the greater degree of the effects on the offspring whose mothers were treated perinatally compared to those whose mothers were treated prepartum only.

During pregnancy, the females are known to have reduced capability for biotransformation and consequently for the elimination of consumed caffeine (18, 31, 39), while the developing fetuses possess an extremely limited capacity of metabolizing caffeine since the necessary enzymes responsible for demethylating it are completely absent (52). Hence, these factors might lead to the accumulation of coffee (caffeine) in the embryo during prepartum period which has been accentuated by suckling in the case of offspring of perinatally treated groups. According to Ordy et al., significant quantities of compounds that are given in late pregnancy may be transmitted to the offspring during lactation (38).

The behavioral changes detected in male offspring in the present study could well be indicators to teratogenic effects incited by caffeine and other components of instant coffee. This is because teratogenic defects are not necessarily structural, but biochemically related behavioral changes are more sensitive indicators of such actions (28). Moreover, abnormalities in response to teratogens may be due to several factors that lead to aberrations in normal cell metabolism especially in enzymes and their substrates (55) or due to a combination of factors (15). Hence, in present study, the activities of several enzymes in various organs whose malfunctions might have led to the detected instant coffee induced behavioral changes. One of these is AChE, an enzyme required for hydrolyzing acetylcholine that has been implicated as an important neurotransmitter in aggressive behavior (3). It is often maintained that changes in the behavior by drugs are presumably due to the alterations in the availability of neurotransmitters (29, 32, 33). An increase in the level of AChE was detected in the brain tissues of treated groups. Increases in brain enzymes have been reported among the factors responsible for long-lasting disturbances in behavioral activities of affected animals (10,30). Changes in such neurochemicals due to caffeine have been reported and correlated with changes in aggressive behavior (51). A major portion of brain cells (70%) of the closely related rats are known to be formed

after birth (40). Hence, coffee (caffeine) due to its possible cumulative effects could have well produced developmental abnormalities, in the brain of offspring of treated mice as well as in its enzymes which might have brought about the observed abnormal behavior, as has been suggested for other compounds (26,30).

The other enzymes tested are AcPase and AlPase which are known indicators of the well-being of eukaryotic cell membranes (25, 36, 42), and are frequently associated with the transport of nutrients across the cell membranes (49,50). Alterations in the levels of these enzymes might have led to variations in the phosphate pool of the animal which might lead to disturbed energy sources available to the animal with the consequent disturbances in its metabolism (55).

Coffee administered to mice in the present study has led to the detection of variations in the level of activities of these enzymes in liver, kidneys and testes. Some of the variations in the levels of these enzymes had been attributed to treatment with cadmium (4, 14, 37), while others to the effect of coffee (1). Hence, the enzymes AChE, AlPase and AcPase could be used as convenient markers in teratological studies.

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REFERENCES

1. Adler, I. D. The problem of caffeine mutagenicity. In: Vogel, F.; Rohrborn, G., eds. Chemical mutagenesis in mammals and man. chapter 24. New York: Springer-Verlag; 1970.
2. Ajarem, J. S. Lasting behavioural consequences of perinatal exposure of laboratory mice to a variety of drugs. Ph.D. Dissertation, University of Wales, UK; 1985.
3. Allikmets, L. H. Cholinergic mechanisms in aggressive behaviour. Med. Biol. 52:19–30; 1974.
4. Axelsson, B.; Piscator, M. Renal damage after prolonged exposure to cadmium. Arch. Environ. Health 12:360; 1966.
5. Benton, D.; Ajarem, J. S.; Brain, P. F. The role of opiate receptors in social behaviour. In: Brain, P. F.; Ramirez, J. M., eds. Cross-disciplinary studies on aggression. Publicaciones de la Universidad de Sevilla; 1986:113–134.
6. Bergmeyer, H. U.; Gawehn, K.; Grassl, M. Enzymes as biochemical reagents. In: Bergmeyer, H. U., ed. Methods of enzymatic analysis. 2nd ed. vol. I. New York: Academic Press; 1974:425–522.
7. Brain, P. F.; Ajarem, J. S.; Petkov, V. V. The application of ethopharmacological techniques to behavioural teratology: Preliminary investigations. Acta Physiol. Pharmacol. Bulg. 12:3011; 1986.
8. Brain, P. F.; Ajarem, J. S.; Petkov, V. V. The utility of ethological assessments of murine agonistic interactions in behavioural teratology: the foetal alcohol syndrome. In: Olivier, B.; Mos, J.; Brain, P. F., eds. Ethopharmacology of agonistic behaviour in animals and humans. Dordrecht: Martinus Nijhoff Publishers; 1987:110–121.
9. Brain, P. F.; Jones, S. E.; Brain, S.; Benton, D. Ethological analysis of the effects of naloxone and the opiate antagonist ICI 154, 129 on social interactions in male house mice. Behav. Proc. 10:341–354; 1985.
10. Branchey, L.; Friedhoff, A. J. Biochemical and behavioural changes in rats exposed to ethanol in utero. Ann. NY Acad. Sci. 273:328–330; 1976.
11. Burg, A. W. Physiological disposition of caffeine. Drug Metab. Rev. 4:199–228; 1975.
12. Butcher, R. E.; Vorhees, C. V.; Wooffen, V. Behavioral and physical development of rats chronically exposed to caffeinated fluids. Fundam. Appl. Toxicol. 4:1–13; 1984.
13. Concannon, J. T.; Braughler J. M.; Schechter, M. D. Pre- and postnatal effects of caffeine on brain biogenic amines, cyclic nucleotides and behavior in developing rats. J. Pharmacol. Exp. Ther. 226:673–679; 1983.
14. Copius, J. H. J.; Melet, J.; Copius, J. W.; Hooghwinkel, G. J. M. Influence of chronic Cd intoxication on the alkaline phosphatase activity of liver and kidney, biological and histological investigations. Toxicology 14:67; 1979.
15. Coyle, I.; Wayner, M. J.; Singer, C. Behavioural teratogenesis: A critical evaluation. Pharmacol. Biochem. Behav. 4:191–200; 1976.
16. Cuomo, V. Perinatal neurotoxicology of psychotropic drugs. Trends Pharmacol. Sci. 8:348–350; 1987.
17. Dews, B. Caffeine: Perspectives from recent research. Berlin: Springer-Verlag; 1984.
18. Dvorchik, B. H. Drug disposition during pregnancy. Biol. Res. Pregnancy Perinatol. 3:129–137; 1982.
19. Emory, E. K.; Konopka, S.; Hronsky, S.; Tuggey, R.; Dave, R. Salivary caffeine and neonatal behavior: assay modification and functional significance. Psychopharmacology (Berlin) 94:64–68; 1988.
20. Fabro, S.; Sieber, S. M. Caffeine and nicotine penetrates the preimplantation blastocyte. Nature 223:410–411; 1969.
21. Fujii, T.; Nishimura, H. Adverse effects of prolonged administration of caffeine on rat fetus. Toxicol. Appl. Pharmacol. 22:449–457; 1972.
22. Grimm, V. E. A review of diazepam and other benzodiazepines in pregnancy. In: Yanai, J., ed. Neurobehavioural teratology. Amsterdam: Elsevier/North-Holland; 1984:153–162.
23. Hestrin, S. The reaction of acetylcholine and other carboxylic acid derivatives with hydroxylamine and its analytical application. J. Biol. Chem. 180:249–261; 1949.
24. Holloway, W. R., Jr. Caffeine: Effects of acute and chronic exposure on the behavior of neonatal rats. Neurobehav. Toxicol. Teratol. 4:21–32; 1982.
25. Hopfer, U. Transport in isolated plasma membranes. Am. J. Physiol. 234:89–96; 1978.
26. Johnson, J. W. C.; Mitzner, W.; Beck J. C.; London, W. I.; Sly, D. L.; Lee, P. A.; Khonzami, V. A.; Calvalieri, R. L. Long-term effects of betamethasone on fetal development. Am. J. Obstet. Gynecol. 141:1053–1064; 1981.
27. Jones, S. E.; Brain, P. F. An illustration of simple sequence analysis with reference to the agonistic behaviour of four strains of laboratory mouse. Behav. Proc. 11:365–388; 1985.
28. Karnofsky, P. A. Drugs as teratogens in animals and man. Annu. Rev. Pharmacol. 5:447–472; 1965.
29. Kellog, C.; Tervo, D.; Ison, J.; Parisi, T.; Miller, R. K. Prenatal exposure to diazepam alters behavioral development in rats. Science 207:205–207; 1980.
30. Kellog, C.; Chisholum, J.; Simmons, R. D.; Ison, J. R.; Miller, R. K. Neural and behavioural consequences of prenatal exposure to diazepam. Mongr. Neural Sci. 9:119–129; 1983.
31. Knutti, R.; Rothweiler, H.; Schlatter, C. The effect of pregnancy on the pharmacokinetics of caffeine. Arch. Toxicol. (Suppl.) 5:187–192; 1982.
32. Kruck, Z. L.; Pycock, C. J. Neurotransmitters and drugs. London: Croom-Helm; 1979.
33. Lee, M. L. In: McCormack, M. K., ed. Aminoacidopathy and mental retardation and other developmental disabilities. New York: Marcel Dekker; 1980:135–150.
34. Legator, M. S.; Zimmering, S. Review of the genetic effects of caffeine. J. Environ. Sci. Health C13:135–188; 1979.
35. Lowry, O. H.; Rosebrough, N. J.; Farr, A. L.; Randall, R. J. Protein measurement with folin phenol reagent. J. Biol. Chem. 193: 266–275; 1951.
36. Oaks, J. A.; Knowles, J.; Cain, G. D. A. Simple method of obtaining an enriched fraction of tegumental brush border from *Hymenolepis diminuta*. J. Parasitol. 63:476–485; 1977.
37. O'Brien, I. G.; King, L. J. The effect of chronic parenteral administration of cadmium on isoenzyme levels of alkaline phosphatase in intestinal mucosa. Toxicology 56:87–94; 1989.

38. Ordy, J. M.; Samorajski, J.; Collins, R. L.; Rolsten G. Pre-natal chlorpromazine effects on liver, survival and behaviour of mice offspring. J. Pharmacol. Exp. Ther. 151:110–125; 1966.
39. Parsons, W. D.; Pelletier, J. G. Delayed elimination of caffeine by women in the last 2 weeks of pregnancy. Can. Med. Assoc. J. 127: 377–380; 1982.
40. Patel, A. J. Undernutrition and brain development. Trends Nat. Sci. 6:151–154; 1983.
41. Pollard, I.; Jabbour, H.; Meharabani, P. A. Effects of caffeine administered during pregnancy on fetal development and subsequent function in the adult rat: prolonged effects on a second generation. J. Toxicol. Environ. Health 22:1–15; 1987.
42. Rahman, M. S.; Mettrick, D. F.; Podesta, R. B. Use of saponin in the preparation of brush border from parasitic flatworm. Can. J. Zool. 59:911–917; 1981.
43. SCOGs-89: Select committee on grass substances. Evaluation of the health aspects of caffeine as food ingredient. Fed. Am. Soc. Exptol. Biol. (FASEB). Springfield, VA: NTIS, PB-283-441/AS; 1978.
44. Seely, S.; Freed, D. L. J.; Silverstone, G. A.; Rippere, V. Diet-related diseases the modern epidemic. London: Croom-Helm; 1985.
45. Siegel, S. Nonparametric statistics for the behaviour sciences. London: McGraw-Hill; 1966:116–127.
46. Sinton, C. M.; Valatx, J. L.; Jouvet, M. Gestational caffeine modifies offspring behaviour in mice. Psychopharmacology (Berlin) 75: 69–74; 1981.
47. Sobotka, T. J.; Spaid, S. L.; Brodie, R. E. Neurobehavioral teratology of caffeine exposure in rats. Neurotoxicology 1:403–416; 1979.
48. Sokal, R. R.; Rohlfe, F. J. Biometry: The principles and practice of statistics in biological research. San Francisco: W. H. Freeman; 1981.
49. Starling, J. A. Tegumental carbohydrate transport in intestinal helminths: correlation between mechanisms of membrane transport and the biochemical environment of absorptive surfaces. Trans. Am. Microsc. Soc. 94:508–523; 1975.
50. Ussing, H. H.; Erly, D.; Lassen, U. Transport pathways in biological membranes. Annu. Rev. Physiol. 36:17–49; 1974.
51. Valzelli, L.; Bernasconi, S. Behavioral and neurochemical effects of caffeine in normal and aggressive mice. Pharmacol. Biochem. Behav. 1:251–254; 1973.
52. Warszawski, D.; Ben-Zvi, Z.; Gorodischer, R. Caffeine metabolism in liver slices during postnatal development in rat. Biochem. Pharmacol. 30:3145–3150; 1981.
53. Weathersbee, P. S.; Lodge, J. R. Caffeine: Its direct and indirect influence on reproduction. J. Reprod. Med. 19:55–63; 1977.
54. West, G. L.; Sobotka, T. J.; Brodie, R. E.; Beier, J. M.; O'Donnell, M. W., Jr. Postnatal neurobehavioral development in rats exposed in utero to caffeine. Neurobehav. Toxicol. Teratol. 8:29–43; 1986.
55. Wilson, J. G. Mechanism of teratogenesis. Am. J. Psychiatry 136: 129–132; 1973.
56. Yamane, T. The t distribution. In: Yamane, T., ed. Statistics, an introductory analysis. 3rd ed. London: Harper and Row Publishers; 1973:647–650.
57. Yanai, J. Neurobehavioural teratology. Amsterdam: Elsevier; 1984.