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REFLEX RESPONSIVENESS OF CF-1 MOUSE NEONATES FOLLOWING MATERNAL ASPARTAME EXPOSURE

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ABSTRACT

Brain dysfunction may be a sign of neurotoxic effects observed following late-gestational administration of a suspected teratogenic agent. Mice exposed to teratogenic insults during early gestation exhibit anomalies of structures that develop during this part of gestation, including ocular and craniofacial malformations. Likewise, exposure to a teratogen late in gestation would affect other specific areas, most notably brain myelination and development. Because the end-products of aspartame (APM) metabolism (methanol, aspartic acid and phenylalanine) can affect neuronal myelination and produce hypothalamic lesions in laboratory animals, the possibility exists that neonates born to mothers exposed to APM during the period of ontogenesis critical for brain development could display poor performance in behavioral and reflex testing routines.

APM was given by intubation to gravid mice on days 15-18 of gestation in doses of 1 and 4 g APM/kg body weight. There were no significant differences among the untreated, saline, 1 or 4 g/kg APM test groups in three of the four procedures used to evaluate responsiveness, including negative geotaxis, or surface and air righting. The achievement age for visual placing, however, a barometer of neurosensory development, was significantly later in both the 1 and 4 g/kg APM groups with respect to either the untreated or saline control groups. In addition, the achievement age for this parameter with the higher APM dose was significantly later than that for the 1 g/kg APM dose.

Failure of the neonates to perform this basic physiologic task within normal limits may indicate diminished functional ability. Therefore, although pathoanatomic alterations in brains of APM-treated animals and their offspring may not be histologically apparent, the possibility of brain dysfunction appears to be a viable sequela to excessive APM exposure.

INTRODUCTION

Aspartame (APM), a synthetic combination of two amino acids (L-aspartyl-L-phenylalanine methyl ester), has a sweetening power 200 times that of sucrose (Cloninger and Baldwin, 1970). This dipeptide is currently available in the United States as a sweetener in various food preparations and low-calorie beverages.

Methanol, aspartic acid and phenylalanine are the endproducts of APM metabolism in the gastrointestinal tract (Ranney, et al., 1976) and some studies have suggested that high levels of these agents may lead to neurotoxic results. For example, central nervous system (CNS) damage (Stegnik, et al., 1981), cleft lips and cleft palates (Tocci and Beber, 1973) in people with phenylketonuria (PKU) or hyperphenylalanemia, and hypothalamic lesions in mice given aspartate or glutamate (Olney, 1979), have been attributed to high levels of each respective amino acid. Although APM has been found to promote glutamate-similar hypothalamic lesions, the amount of APM required to produce these defects was very high (Federal Register, 1981). In a related study, the peak plasma phenylalanine levels produced by excessive doses of APM, although at least double the proposed normal consumption doses of APM, were nevertheless below phenylalanine levels normally associated with toxic effects, even in humans with PKU

(Stegnik, et al., 1980). This information, together with reports of minimal or no teratogenicity, implies relative safety for both the adult and fetus if consumption of APM is moderate in various species.

One area that has not been thoroughly investigated, however, is the effect of APM and its breakdown products on pre- and post-natal intellectual and reflex development. Brain dysfunction may be a sign of neurotoxic effects observed following late-gestational administration of a suspected teratogen. It has been shown that mice exposed to specific teratogenic insults during the early part of gestation (i.e., from days 7 through 9) display gross defects such as craniofacial and ocular anomalies, whereas exposure during later gestational periods (i.e., from days 15 through 17) can produce severe brain necrosis (Wilson, 1972). Therefore, neonates born to mothers exposed to APM during late gestation may perform poorly in behavioral and reflex testing routines without the concurrent appearance of gross physical abnormalities. In fact, one of the endproducts of APM metabolism, methanol, has been linked to the fetal alcohol syndrome and diminished intellectual capacity in rat neonates if their mothers ingested alcohol during pregnancy (Brown et al., 1979). In addition, elevated levels of another APM endproduct, phenylalanine, may lead to mental retardation in humans similar to that observed in untreated PKU patients

(Dobson, et al., 1977; Bessman, et al., 1978).

The high plasma phenylalanine levels observed in PKU patients are due to the genetic deficiency of phenylalanine 4-hydroxylase (Jervis, 1953). Due to the importance of ketone bodies in the developing brain, this enzyme deficiency appears to be the primary pathogenic basis for the diminished intellectual ability of patients with PKU. During brain development, kerone bodies are needed for respiration (Page, et al., 1971) and myelination (via acetyl Coenzyme A) (Klee and Sokoloff, 1967), and any abnormalities of ketone-body utilization during the stages of brain development when myelination is at a maximum could lead to brain dysfunction, as in PKU. Phenylketonurics are essentially normal at birth (with respect to mental ability) but display a progressive intellectual decline immediately postpartum because it is during this early neonatal period that neuronal myelination (Davison and Dobbing, 1968) and the concomitant ketone-body metabolism (Williamson and Buckley, 1973) predominate. The observations that a low-phenylalanine diet can prevent mental retardation in PKU patients (Dobson, et al., 1977), and that untreated PKU patients display progressive mental deterioration (Bessman, et al. 1978; Stegnik, et al., 1980), support the above.

Considering that aspartame is metabolized to methanol, aspartate and phenylalanine, and that these agents can produce CNS defects and mental retardation in certain

species under appropriate conditions, the possibility of adverse effects on the developing fetus due to maternal consumption of large amounts of this sweetener exists and should be investigated. Therefore, the purpose of this study was to assess the effect of APM on brain development and function in CF-1 mouse neonates by evaluating their responsiveness to reflex testing of surface and air righting, negative geotaxis and visual placing, all parameters of neuronal (brain) functional ability.

MATERIALS AND METHODS

Animals

CF-1 albino mice, weight 20 to 25 grams, were obtained from Charles River Breeding Laboratories, Wilmington, Massachusetts. Females were caged in aggregates of 10 each and no attempt to breed these animals was made for at least two weeks after their arrival, when each weighed at least 25 g. The male mice were placed in individual cages measuring 12.5 x 15 x 10 cm having wire mesh fronts and bottoms. These cages were held in a 120 unit RD-T cage rack manufactured by the Norwich Wire Works, Norwich, New York, and maintained in a room protected from exposure to natural sunlight. This breeding room was equipped with an electrical lighting system controlled by an Intermatic Time All (Model V-45073 astronomic dial time switch with "skipper" manufactured by International Register Co., Spring Grove, Illinois) which allowed 14 hours of light and 10 hours of darkness.

The temperature was kept at 22-26°. All animals were maintained on Purina Laboratory Mouse Chow C, No. 5001, from the Ralston Purina Co., St. Louis, Missouri, and tap water ad libitum.

Breeding Procedure

On designated breeding dates, two females were placed in the cage of a male at 3:00 P.M. and allowed to remain until 7:00 A.M. the following morning. At that time, the females were examined for the presence of vaginal plugs, the last part of the male ejaculate. Females exhibiting these plugs were considered as being gravid, and the morning that the plug appeared designated as day 0 of gestation. These gravid mice were weighed on an Ohaus triple-beam balance to the nearest tenth of a gram, with this being recorded as the maternal starting weight. The pregnant females were then placed into individual cages, identical to those of the males, and permitted to remain undisturbed until the morning of day 18, one day prior to expected delivery. Each mouse was then weighed (maternal terminal weight) and subsequently placed into individual cages, each containing food and water ad libitum. These cages, 15 x 30 x 15 cm, have a bottom layer of sawdust bedding that was left undisturbed until termination of the experiment. The gravid mice gave birth in these cages the next day and were left undisturbed until the respective treatment regimen. Total numbers of neonates born to each maternal

animal, as well as any overt developmental abnormalities, were also recorded.

Drug Preparation and Treatment

The route of administration, solution preparation and dosages employed in this investigation were the same as those in previous studies that have found no detrimental effects on adult mice even after chronic exposure to high doses of aspartame. In one such study, aspartame was administered in the diet at levels of 1, 2 and 4 g/kg/day for 104 weeks with no significant histological evidence of neoplasia in bladder and brain sections (Reno, et al., 1975). The effects of such doses on the developing fetus might be dramatically different than those on adults, however, especially with respect to postnatal intellectual ability.

The solution of aspartame[1] employed in this study was prepared immediately before administration in double-distilled water. The aspartame solutions were administered by standard intubation (oral) techniques. This is a relatively simple procedure and affords the luxury of mimicking the major route of human consumption.

There were two solutions of aspartame providing 1 and 4 g aspartame/kg body weight. These are the same doses employed in a previous study (Reno, et al., 1975). Each dose was administered to gravid CF-1 mice on each day of gestation from days 15 through 18 of the normal 19-day mouse gestational period (day 0 being the day

that vaginal plug appears). This period was chosen because it appears to be critical for brain development. Aspartame was given on each day of gestation from days 15 through 18 to mimic chronic exposure (consumption) as performed in previous studies (Reno, et al., 1975) and to encompass the period most critical for development of the brain and intelligence.

The sodium chloride[2] solution was prepared commercially. Each piece of reflex testing equipment was handmade according to the specifications outlined in respective procedural routines.

Visual Placing

The control experiment group was comprised of 10 litters with a total of 94 fetuses obtained from 3 different breeding dates. All animals in this group were left undisturbed in their individual cages until testing began on postnatal day 17. These animals were evaluated each day thereafter until termination of the experiment on postnatal day 25, when each neonate in every litter displayed positive reflexes. The criterion for a positive reflex was the achievement of the following routine for 2 out of 2 consecutive trials per neonate in an entire litter. The neonate was held by the base of the tail and removed from its litter-mates and mother, unless it was nursing. This animal was held approximately 15 cm above the testing apparatus, which consisted of a taut, light brown rope, 0.6 cm O.D., 14

cm in length with a 6 cm vertical rise from the base, a wooden board with a white background. The animal was then held briefly at this 15 cm high starting position and, facing forward and parallel to the rope, the neonate was slowly lowered straight down. A positive response was evidenced by the extension of both forelimbs in an attempt to grab the rope when tested on two consecutive trials. A criterion was established, and utilized for the other three regimens, that if one-third of a specific litter did not display positive responses, then the remainder of that litter could be disregarded until the next testing date. In this way, the possibility of bias in testing a single defective neonate and getting a false response was averted, along with the fact that this procedure would save a great deal of time and make testing more efficient. The animals in the other treatment groups were evaluated similarly.

The results of this experiment (Table 1) show the achievement ages for visual placing in CF-1 mice, as specified by two consecutive positive responses for each neonate in a complete litter. Each animal displaying a positive response on the first trial did so again on the second trial. Also, there was an increase in neonatal mortality as postnatal life progressed.

Surface Righting

The control experimental group was comprised of 10 litters with a total of 127 fetuses obtained from 2

different breeding dates. All animals in this group were left undisturbed in their individual cages until testing began on postnatal day 4. These animals were evaluated each day thereafter until termination of the experiment on postnatal day 12, when each neonate in every litter displayed positive responses. The criterion for a positive reflex was the achievement of the following routine for 3 out of 3 consecutive trials per neonate in an entire litter. The experimental routine was as follows: the neonate was gingerly removed from its littermates and mother, unless it was nursing. This animal was placed on its back on a padded surface. The testing apparatus was a flat surface 18 x 23 cm made of foam rubber to ensure a suitable surface for easy mobility with minimal slippage. The neonate was placed on its back in the center of this foam-padded surface and held momentarily. Upon releasing the animal, the time (in seconds) for the neonate to rotate to a fully prone position was recorded. The criterion for a positive reflex was the movement of the neonate to the fully prone position, with all four limbs touching the surface, within 2 seconds, as counted verbally "one thousand one, one thousand two, ..." (same counting procedure is used in other regimens where a time element is involved); this must be observed for each neonate on 3 consecutive trials for a positive response to be recorded. The animals in the other treatment groups were evaluated similarly. The results of this experiment

(Table 2) show the achievement ages for surface righting in CF-1 mice, as specified by 3 consecutive positive responses for each neonate in a complete litter.

Air Righting

The control experimental group was comprised of 11 litters from 4 different breeding dates. All animals in this group were left undisturbed in their individual cages until testing began on postnatal day 17. These animals were evaluated daily thereafter until termination of the experiment on postnatal day 21, when each neonate in every litter displayed positive responses.

Initiating the testing on day 17 postpartum, neonates were grasped by loose skin on the dorsal surface of the cervical area and held in an inverted position 60 cm above a padded board. The testing apparatus was a flat surface 18 x 23 cm made of foam rubber to cushion the fall of the neonate. After quickly releasing the neonate, the landing position was observed. It should be noted that the neonates were held dorsally instead of ventrally because of their increasing ability to grasp and bite, thereby disrupting the uniformity of the drop. A positive trial was noted when the neonate landed in a fully prone position with all four paws fully extended away from the body or immediately underneath. This criterion was used to differentiate when neonates would land partially on their sides or hindquarters and those appendages would appear to be tucked under the body. A positive test was

noted upon the successful completion of three out of three trials. Each litter was tested daily until the entire litter displayed a positive reflex.

The results of this experiment (Table 3) show the achievement ages for air righting in CF-1 mice, as specified by three consecutive positive responses for each neonate in a complete litter.

Negative Geotaxis

The control experimental group was comprised of 10 litters with a total of 102 fetuses from 4 different breeding dates. All animals in this group were left undisturbed until testing began on postnatal day 4. These animals, caged individually, were evaluated each day thereafter until termination of the experiment on postnatal day 11, when each neonate in every litter displayed positive reflexes. Beginning on day 4 postpartum, each neonate was placed face down on a foam-padded board slanted at a 45° angle. The time was recorded from release of the neonate until it made a 180° turn. A positive test was obtained when each neonate achieved 3 out of 3 turns within 15 seconds. One-third negative responses voided that litter for that testing day.

The results (Table 4) show the achievement ages for negative geotaxis in CF-1 mice, as specified by 3 consecutive positive responses for each neonate in a complete litter.

Table 1. Positive Litter Responses for Visual Placing on Each Day Postpartum (Postnatal Day) and Mean Achievement Age per Respective Treatment.

Treatment	Positive Litters per Postnatal Day								Achievement Age (Days)
	18	19	20	21	22	23	24	25	
Untreated	1	1	5	3	0	0	0	0	20.0 ± 0.9
Saline	0	2	3	3	0	0	0	0	20.1 ± 0.8
1 g/kg APM	0	0	0	2	4	2	0	0	22.0 ± 0.7[a]
4 g/kg APM	0	0	0	0	0	1	3	5	24.4 ± 0.7[a,b]

[a]significant ($p < 0.05$) when compared with either saline or untreated control groups.

[b]significant ($p < 0.05$) when compared with 1g/kg APM group.

Table 2. Positive Litter Responses for Surface Righting on Each Day Postpartum (Postnatal Day) and Mean Achievement Age per Respective Treatment.

Treatment	Positive Litters per Postnatal Day			Achievement Age (Days)
	10	11	12	
Untreated	2	4	4	11.2 ± 0.8
Saline	0	4	2	11.3 ± 0.5
1 g/kg APM	0	3	3	11.5 ± 0.5
4 g/kg APM	0	2	4	11.7 ± 0.5

Table 3. Positive Litter Responses for Air Righting on Each Day Postpartum (Postnatal Day) and Mean Achievement Age per Respective Treatment.

Treatment	Positive Litters per Postnatal Day					Achievement Age (Days)
	17	18	19	20	21	
Untreated	2	1	0	4	4	19.6 ± 1.6
Saline	0	0	3	3	0	19.5 ± 0.5
1 g/kg APM	0	0	2	3	1	19.8 ± 0.8
4 g/kg APM	0	0	1	3	2	20.2 ± 0.8

Table 4. Positive Litter Responses for Negative Geotaxis on Each Day Postpartum (Postnatal Day) and Mean Achievement Age per Respective Treatment

Treatment	Positive Litters per Postnatal Day				Achievement Age(Days)
	8	9	10	11	
Untreated	2	2	5	1	9.5 ± 1.3
Saline	1	1	4	0	9.5 ± 0.8
1 g/kg APM	0	2	4	0	9.7 ± 0.5
4 g/kg APM	0	0	4	2	10.3 ± 0.5

NOTE: There were no significant differences among any of the treatment groups with respect to maternal weight gain during gestation or litter size, and maternal/neonatal/fetal survival was unaffected by the treatments. The trauma of the intubation procedure did cause 3 fatalities out of the 78 treated animals, but this was a negligible factor because it was constant and there were no significant differenges between the untreated and saline controls in any of the procedures. Statistical evaluation of the data for all parts was accomplished by an analysis of variance among the groups and Student's "t" test for paired data. Significance was indicated by p-values of 0.05 or less (F-values corrected accordingly).

CONCLUSIONS

The potential of the sweetening agent aspartame (APM) to produce brain dysfunction in mouse neonates whose mothers were exposed to APM late in gestation was evaluated. Impaired mental ability is a possibility in these neonates because of their potential exposure in utero to the breakdown products of APM metabolism (methanol, aspartic acid and phenylalanine), each possessing ability to affect brain neuronal myelination and, subsequently, intellectual capacity. Although high doses of APM have not produced significant gross teratogenic results or severe morphologic/histologic anomalies in laboratory animals, lingering postpartum effects, such as learning disabilities, hyperkinesis, mental retardation and similar intellectual abnormalities, may not be easily evaluated or discerned. In fact, it is interesting to note that one study has found that there is a permanent shortage of brain neurons, despite neuroepithelial reconstitution, in mice exposed to certain teratogenic agents (Langman and Cardell, 1977). This residual lesion is of a type that is usually very difficult to detect, but is nevertheless present in the animal. Therefore, potential abnormalities in brains of neonates born to APM-treated pregnant mice might not present themselves physically but, rather, could appear later in life as brain dysfunctions. This is even more important because some investigations have found behavioral anomalies at subteratogenic doses

(Poppe, et al.,1983). Altered performance in various teratologic behavioral and reflex testing procedures could be indicative of these possible neurotoxic effects.

APM was given by intubation to gravid mice during the period of gestation most critical for brain development. The doses administered were the same as those utilized in a study where no gross physical or histologic anomalies were observed (Reno, et al., 1975). The parameters evaluated were all indicative, to varying degrees, of neurosensory development. In only one of the four experiments, visual placing, was there any significant delay in achievement age. This means that the mean achievement age of visual placing for neonates exposed to APM was significantly delayed and did not occur until several days after the normal time postpartum for performance of this task. The delay in positive performance also appeared to be dose-related because the achievement age for visual placing with the higher APM dose was significantly later than that for the 1g/kg APM dose. Similar delays were also observed in all other APM-treated groups in the other experiments, but these were not statistically significant.

The failure of the neonates to perform this basic physiologic task (visual placing) within normal limits may indicate diminished functional ability. Therefore, although pathoanatomic alterations in brains of APM-treated animals and their offspring may not be histologically

apparent, the possibility of brain dysfunction appears to be a viable sequela to excessive aspartame exposure. Considering the large doses required to produce these effects, however, the question of aspartame safety cannot be adequately addressed at this time without additional research.

FOOTNOTES

[1]Aspartame, L-Aspartyl-L-Phenylalanine Methyl Ester, Lot 122F-0884, Sigma Chemical Company, St. Louis, MO.

[2]Sodium chloride USP (code 2A1302), Travenol Laboratories, Deerfield, IL.

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