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Soyabean fortification and enrichment of regular and quality protein maize tortillas affects brain development and maze performance of rats

Carlos Amaya-Guerra[1], Sergio O. Serna Saldívar[2]* and Maria Guadalupe Alanis-Guzman[1]

[1]*Facultad de Ciencias Biológicas, Universidad Autónoma de Nuevo León, Av. Pedro de Alba s/n, Cd. Universitaria, San Nicolas de los Garza, Nuevo León, Mexico*

[2]*Department of Biotechnology and Food Engineering, ITESM-Campus Monterrey, Nuevo León, Mexico*

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The brain development and performance of rats fed throughout two generations with an indigenous maize tortilla-based diet was studied. The experiment compared casein control with five different diets produced from: regular fresh *masa*; regular, enriched dry *masa* flour containing thiamin, riboflavin, niacin, folic acid, Fe and Zn (REDMF); dry *masa* flour fortified with 60g/kg defatted soyabean meal and enriched (FEDMF); enriched quality protein maize (QPM) flour (EQPM); QPM flour fortified with 30g/kg defatted soyabean meal and enriched (FEQPM). In both generations, brain and cerebellum weights and myelin concentration were significantly higher ($P<0{\cdot}05$) in rats fed the FEDMF and FEQPM diets. There was no significant difference ($P>0{\cdot}05$) in brain DNA in first-generation rats; however, second-generation rats fed FEDMF, EQPM and FEQPM tortillas had higher cerebral DNA, neuron size and brain activity as estimated by the RNA:DNA ratio. Short-term and long-term memory performance in the Morris maze improved ($P<0{\cdot}05$) among rats fed the FEDMF, FEQPM and EQPM diets. Second-generation rats fed the FEDMF and FEQPM diets had a superior ($P<0{\cdot}05$) working memory and learning performance. The utilisation of regular or QPM tortillas enriched with selected micronutrients and fortified with soyabean is highly recommended to assure adequate brain development. The high lysine–tryptophan QPM made it possible to save half of the soyabean flour without sacrificing the nutritional value of soyabean-fortified tortillas.

Maize tortillas: Brain development: Quality protein maize: Soyabean meal: Enrichment and fortification: Morris maze

According to the Mexican National Nutrition Survey, protein–energy malnutrition affects approximately 20 % of the rural population (5 million people), whereas in urban areas the incidence is only 6 %. The survey does not consider temporary malnutrition, also named grade 1, which progressively affects the physical and intellectual development of growing infants of 3–20 months of age (Instituto Nacional de Salud Pública, 2000). This type of malnutrition is mainly caused by the lack of good-quality supplementary foods to complement maternal milk.

Nixtamalisation is the process of cooking maize kernels in a solution containing $Ca(OH)_2$. The cooked grain or nixtamal is stone-ground into a *masa* that is further shaped into thin discs and baked to obtain tortillas. During lime cooking, some of the pericarp and germ tissues are lost. The amount lost is related to the time–temperature cooking profile, lime concentration and extent of nixtamal washing (Serna Saldivar *et al.* 1990), and is also controlled by genetic traits (Serna Saldivar *et al.* 1991). Most people, however, agree that tortillas are considered more as a whole-grain than a refined product. Tortillas are the staple food for people inhabiting Mexico and Central America. In rural areas, people depend more on tortillas and related nixtamalised products. In some areas, these maize-based products supply more than 70 % of the total energy intake (Serna Saldivar *et al.* 1990).

Unfortunately, tortillas are not a perfect food because they lack the essential amino acids lysine and tryptophan and adequate levels of Fe, Zn and vitamins A, D, E and B_{12} (Serna Saldivar *et al.* 1990). Quality protein maize (QPM) can be an important way to upgrade the protein value of tortillas because it contains approximately twice as much lysine and tryptophan as regular maize. Therefore, QPM tortillas have improved protein quality and nutritional value (Mertz *et al.* 1965; Eggum *et al.* 1979; Sproule *et al.* 1988). Bressani (1990) has suggested that QPM is a practical solution for children recovering from malnutrition.

It has been observed that early malnutrition reduces both the brain and cerebellum size (Winick & Nobel, 1966; Chase *et al.* 1967; Culley & Lineberger, 1968; Zamenhof *et al.* 1971; Smart *et al.* 1973). It has also been documented that brain size in early malnourished rats recovered after they were given an appropriate and well-balanced diet. In contrast, it has been determined that cerebellum size never recovers from an early episode of malnutrition, even after the animal is exposed to a complete diet and adequate stimulation (Warren & Bedi, 1984).

Abbreviations: EQPM, enriched quality protein maize flour; FEDMF, fortified enriched dry *masa* flour; FEQPM, fortified enriched QPM flour; FM, fresh *masa*; QPM, quality protein maize; REDMF, regular enriched dry *masa* flour.

* **Corresponding author:** Professor Sergio O. Serna Saldívar, fax +52 81 83284136, email sserna@itesm.mx

The neuron myelination process is altered in infants suffering from malnutrition, mainly owing to the lower synthesis of proteolipids, cerebrosides, sulphatides and the plasmalogen of the white substance. Animal studies have clearly shown that malnutrition severely reduces the concentration of cerebral myelin (Krigman & Hogan, 1976; Wiggins, 1982; Fuller *et al.* 1984; Reddy & Horrocks, 1986; Yeh, 1988). This phenomenon resists alimentary rehabilitation, and rats with early malnutrition will never achieve myelin levels similar to those of their well-nourished counterparts (Fuller *et al.* 1984; Royland *et al.* 1992). Functionally speaking, this reduction is very important because myelinated axons transmit information faster than non-myelinated fibres (Wiggins, 1982).

Malnutrition also reduces the number of neurons and synapses (Winick & Nobel, 1966; Zamenhof *et al.* 1968; Winick, 1969; Zamenhof, 1985; Stylianopoulos *et al.* 2002). When the brain is deprived of an optimal supply of nutrients, there are no discrete lesions. Instead, generalised distortion occurs in those areas that were maturing at the time of nutrient deficit. In other areas of the brain, where the cells have differentiated during prenatal life, malnutrition in infancy reduces the formation of synapses and the branching of dendrites (Zamenhof, 1985). A post-mortem inspection of children who were severely affected by marasmus found significantly lower levels of brain cholesterol, phospholipids, RNA and DNA (Rosso *et al.* 1970; Winick *et al.* 1970).

In Mexico, the dry *masa* flour industry started to enrich flours with 5 mg thiamin/kg, 3·0 mg riboflavin/kg, 35 mg niacin/kg, 0·5 mg folic acid/kg, 30 mg Fe/kg and 20 mg Zn/kg in 1998. The enrichment and fortification of fresh *masa* is difficult and impractical because the premix and/or flours are difficult to incorporate and distribute homogeneously into the *masa* at the concentration desired, and tortilla factories lack the technology to perform this task. The nutritional value of enriched tortillas can be further improved by the utilisation of QPM or the addition of oilseed flours such as soyabean meal (Stylianopoulos *et al.* 2002).

In previous research, Amaya-Guerra *et al.* (2004) determined the physiological development of rats fed tortilla-based diets for two generations. The growth and reproductive performance of rats fed soyabean-fortified tortillas based on regular and QPM tortillas were significantly higher than those of their counterparts fed regular tortillas. Soyabean fortification improved the biological value, net protein utilisation value and protein digestibility-corrected essential amino acid scores, whereas the use of QPM halved the amount of supplemented soyabean to be used without affecting protein quality, animal growth and reproductive performance. The objective of the present research was to study further the effects of soyabean fortification and enrichment with selected minerals and vitamins of regular and QPM tortillas on brain development and maze performance of rats fed throughout two generations.

Materials and methods

Treatments

Six different diets were tested. Five consisted of experimental diets based on tortillas made from enriched regular dry *masa* flour (REDMF), fresh *masa* (FM), soyabean fortified enriched dry *masa* flour (FEDMF), enriched QPM flour (EQPM) and soyabean fortified enriched QPM flour (FEQPM). The other treatment consisted of a control group fed a balanced casein-based diet (Association of Official Analytical Chemists, 1990) that contained all necessary nutrients for optimum rat growth (Amaya-Guerra *et al.* 2004).

Diet preparation

The experimental tortilla-based diets, which contained 73·6 % of dry tortillas on an as-fed basis, were formulated and elaborated according to previous work (Table 1; Amaya-Guerra *et al.* 2004). During the growth periods of the test animals, diets were offered as powder, whereas during mating, pregnancy and lactation, they were offered as pellets. The proximate composition, digestible energy, amino acid, vitamins and mineral compositions of formulated diets were determined by Amaya-Guerra *et al.* (2004).

Rat bioassays

Fifty-four female weanling Wistar rats (25 d old) with an average initial body weight of 48·4 g were randomly assigned to the six treatments. Rats were acquired from the animal feeding facilities of ITESM-Campus Monterrey, Mexico. Female rats were housed in individual stainless steel cages under controlled environmental conditions (20–22°C with alternating periods of 12 h light and darkness). When female rats reached an average of 64 d of age, they were tested with the Morris maze tests. Simultaneously, thirty-six male weanling rats were randomly assigned to the six treatments. The six rats

Table 1. Composition of diets used during the first- and second-generation rat bioassays (Amaya-Guerra *et al.* 2004)*†

Food item	%
Maize tortillas	73·6
Beans	3·46
Cactus	2·09
Pasta	1·10
Bread	1·36
Potatoes	0·24
Rice	0·08
Dry whole milk	1·12
Meat	1·13
Dry whole egg solids	0·61
Tomatoes	0·26
Peppers	0·39
Other vegetables‡	0·14
Fruits§	0·11
Oil/shortening‖	2·86
Sugar	11·45
Total	100·00

* Source of variation between diets was the type of tortilla. The percentage composition of the control casein-based diet was 50 % corn starch, 20 % casein (AIN purified high N), 15·2 % sucrose, 5 % vegetable oil with 0·005 % butylated hydroxytoluene, 5 % cellulose (Alphacel; ICN Biomedicals, Irving, CA, USA), 3·5% mineral mix (AIN 76; ICN Biomedicals), 1 % vitamin mix (AIN 76; ICN Biomedicals) and 0·3 % DL-methionine.

† Quantities are expressed on an as-fed basis. All ingredients were dehydrated and ground before diet formulation.

‡ Onion and carrot (1:1w/w).

§ Banana and squash (1:1w/w).

‖ Vegetal oil and pork lard (2:1w/w).

belonging to the same treatment were divided into two groups of three rats each and housed in collective acrylic cages. Females were fed the diet in a powdered form, and males the diet in pellets. Food and water were provided *ad libitum* (Amaya-Guerra *et al.* 2004).

After females finished the 60 d post-weaning feeding period, three rats of the same treatment were placed with one male of the same treatment in collective acrylic cages for a 6 d mating period. After the first mating stage ended, other males belonging to the same treatment replaced the first male for other 6 d mating, and finally the third male also had the opportunity to breed the dams. In order to avoid confusion, rats were identified beforehand by ear piercing. Dams had at least four chances to get pregnant as they were usually in oestrus every 4 d. After the 18 d mating period, the females were separated from the males. Males were anaesthetised with chloroform and blood was withdrawn via intracardiac puncture.

Pregnant rats were individually placed in maternity cages and fed *ad libitum* with pellets. On the date of parturition, the number of newborn pups and the total litter weight were registered (Amaya-Guerra *et al.* 2004). The litter was subjected to a 28d-lactation period, and the sex and weight of each living pup were registered. Dams were anaesthetised in a hermetically sealed chamber containing chloroform and killed via intracardiac puncture; the brain and cerebellum were then surgically removed. These tissues were immediately immersed in a buffered neutral formalin solution (100 ml 40 % formalin (v/v), 4 g NaH_2PO_4, 6·5 g Na_2HPO_4 brought to 1 litre with distilled water).

Three males and three females belonging to the second generation with weights closest to the average weight of the weaned litter were individually housed for a 28 d period as described before in preparation for the maze tests. Female rats with an average age of 158 d were then sedated with chloroform and killed via intracardiac puncture with the aim of obtaining cerebral and cerebellar tissues. All brains and cerebellums were weighed and sagittally cut to obtain the two hemispheres. One of the hemispheres was used for histological studies and determination of myelin; the other was used to measure protein, RNA and DNA.

Determination of cerebral and cerebellar myelin

Myelin was extracted following the modified procedure of Folch *et al.* (1957). The cerebral and cerebellar tissues were extracted with a mixture of chloroform–methanol–water (8:4:3, by vol.) containing butylated hydroxytoluene (0·02 g/l) added to prevent lipid oxidation. Samples were homogenised with 10 volumes of 0·32 M-sucrose solution. The resulting mixture was diluted with 14 volumes of 0·8 M-sucrose solution and then centrifuged for 70 min at 12 000 rpm.

The low-density myelin present in the top part of the solution was separated and suspended with 30 ml distilled water previously tempered on crushed ice. The solution was continuously agitated for 20 min and centrifuged again. Myelin was resuspended with 0·32 M-sucrose solution, washed twice with distilled water and centrifuged after the resuspension and washing steps. The extracted myelin was dried in a vacuum oven (Model 1400 E; VWR, West Chester, PA, USA) set at 60°C, and the dried material was weighed.

Histological analysis of hypothalamus

The modified method of Nelson and Silverstein (1994) was used to calculate the density of neuronal synapses in the hypothalamus. One of the cerebral hemispheres was frozen at −25°C. Three or four 15 μm-thick histological cuts were obtained with a Cryostat (Bright Instruments, Huntingdon, UK). The cuts were first soaked with diaminobenzidine at 37°C for 2 h in darkness before being immersed three times for 5 min in a phosphate buffer at ambient temperature and then placed on poly-L-lysine microscope slides (Ploysceinces Inc., Warrington, PA, USA). The resulting histological cuts were covered with a cover glass and allowed to dry at room temperature. The stained tissues were observed through a 40% objective lens on a microscope (Model BH-2; Olympus America, Melvilla, NY, USA) equipped with a green filter (wavelength 510–550 nm) positioned to improve the contrast.

Photographs of the histological cuts were taken with a camera (Cohu 4915; Cohu, San Diego, CA, USA) and the resulting image digitised and transformed into grey scale with the National Institutes of Health Image program on a Macintosh Apple 8500 computer (Cupertino, CA, USA). This program assigned values of 1 to 256 depending on the intensity of the black colour on the grey scale. The areas that were completely black were assigned the highest value of 256, whereas the completely white areas were given the lowest value of 1. The computer program added the total values of the image and divided the resulting number by the value of a completely black image. This percentage value correlated with the density of the synapses among the neurons. The higher the number of synapses, the higher the program's percentage value.

Determination of cerebral protein, RNA and DNA

One of the brain and cerebellum halves was homogenised with distilled water and brought to a volume of 25 ml. Two 5 ml aliquots were vacuum dried at 60°C to determine the dehydrated weights of the brain and cerebellum.

Cerebral protein was determined in duplicate by taking a 2 ml aliquot. The brain tissue was digested with 3 ml H_2SO_4 and 1 g K_2SO_4:$CuSO_4$ (25:1w/w) in a micro-Kjeldahl flask (Labconco Corporation, Kansas City, MO, USA). We then measured N_2 gas colorimetrically after adjusting the volume to 25 ml with distilled water using an Orion 901 microprocessor ion-analyser (Orion Research, Inc. Cambridge, MA, USA). Bovine albumin serum and ammonium sulphate were used to standardise the assay, and N was converted to protein using the factor 6·25 (Association of Official Analytical Chemists, 1990).

RNA and DNA were determined in 2 ml aliquots with the ARN/ADN BDtract Kit (Maxim Biotech Inc., San Francisco, CA, USA). The isolation procedure followed was previously described by Chattopadhyay *et al.* (1993) and the RNA/DNA determination protocol by Schmidt & Thannhauser (1945) and Burton (1956). The calibration curves (absorbance at 260 nm/280 nm) for DNA and RNA were constructed using bovine thymus (Sigma Aldrich, St. Louis, MO, USA) and yeast (Schwartz, Mann, Orangeburg, NY, USA), respectively. The purity of RNA was determined by the absorbance at

$(A_{260})/(A_{280})$ being 1·8–2·0 and that of DNA with $(A_{260})/(A_{280})$ being 1·6–1·8. The following equation was used to measure RNA and DNA:

$$\text{Concentration} = (A_{260})\,(200)\,(50)/(A_{280})\,(10).$$

Morris water maze tests

A modified Morris water maze was used to determine short-term, long-term and work memory and learning performance (Morris, 1981). The water maze consisted of a circular tank (1·6 m diameter by 60 cm height) painted on the inside with white paint. The pool was filled with 30 cm water stained with cornstarch and regulated to a temperature of 26 ± 2°C. The stained water concealed a 10 cm × 10 cm platform located 1 cm beneath the water level. The water maze was placed among three visible and recognisable objects so that rats could triangulate or position themselves. The tank was divided into four imaginary quadrants: north east, north west, south east and south west.

The tests consisted of measuring the time required for rats to find the hidden platform (latency) and the number of errors measured as the number of occasions on which the rats entered an incorrect quadrant.

Short-term memory. This test was also named the constant point of entry. The platform was located in the south-east quadrant, and the rat was placed next to the rim of the west quadrant. Rats were allowed to swim until finding the platform. If the rat could not find the platform in less than 120 s, it was placed onto the platform for 20 s so that it could recognise its position. Twenty-five consecutive tests were performed within 1 day in which the latency and number of errors were recorded (Griffith *et al.* 1998).

Long-term memory. Three days after the termination of short-term memory tests, rats were assessed for long-term memory. The procedure was similar to the one described earlier with the difference that four daily tests were performed. Tests were conducted until the latency per treatment was equal to or lower than 10 s in three of the four daily tests for two consecutive days (Griffith *et al.* 1998).

Working memory. Working memory was determined by performing six daily tests for three consecutive days, changing the place of entry. The point of entry when conducting tests 1, 2, 4 and 5 was in the west quadrant, whereas the initial entry point was randomly changed in tests 3 and 6. Latency and number of errors were determined by averaging all the observations (Griffith *et al.* 1998).

Learning performance. Learning performance was estimated by conducting four tests for three consecutive days. In this case, the platform was moved by 180° from its initial position only once and the point of entry changed in the order north, south, east and west. Latency and number of errors were determined by averaging all the observations (Griffith *et al.* 1998).

Statistical analyses

Data were analysed as a randomised design using ANOVA procedures. Minimum significant differences and Duncan's test were applied to determine statistical differences ($P<0·05$) between means. The standard deviation was calculated for all means. All analyses were performed using the StatView software (SAS Institute, 1999).

Results

First- and second-generation rats fed regular tortilla diets (FM, REDMF) had significantly lower ($P<0·05$) cerebral and cerebellar weights when compared with their counterparts fed fortified diets (Table 2). First-generation rats fed the control, FEDMF, FEQPM or EQPM diet (Table 2) had cerebral myelin levels that were similar and that were higher ($P<0·05$) than those of counterparts fed the unfortified diets (REDMF, FM). Second-generation rats fed the control diet had significantly higher ($P<0·05$) levels of myelin than the animals fed a soyabean-fortified diet. Interestingly, rats fed the FM diet that was not enriched with thiamin, riboflavin, niacin, folic acid, Fe and Zn had approximately half the myelin compared with the rest of the animals fed the vitamin/mineral-enriched diets.

A highly significant difference ($P<0·05$) in cerebral synapse density was observed in first-generation rats fed QPM and/or soyabean-fortified tortillas (Table 2). This positive trend was not observed in second-generation rats, probably because they were killed at a younger age.

Among first-generation rats, there were no significant differences in cerebral DNA, probably because the experiment started with well-nourished weaned animals of 25 d of age. Significant differences in brain DNA were, however, observed in second-generation rats. Animals fed the soyabean-fortified diets had significantly higher amounts of brain DNA compared with their counterparts fed regular tortilla diets (Table 3). The improper balance of essential amino acids during gestation and lactation affected both the nourishment of the pregnant mother and the condition of the offspring, as has clearly been shown by previous research (Stylianopoulos *et al.* 2002; Amaya-Guerra *et al.* 2004).

We also observed lower amounts of cerebral RNA in second-generation animals fed the low-quality-protein tortilla-based diets (FM, REDMF). When, however, cerebral activity was evaluated (RNA/DNA), there were no significant differences ($P>0·05$) between treatments. The lower amount of RNA observed was due to the lower DNA concentration and was not attributed to the lower synthesis of RNA.

First- and second-generation rats fed the soyabean-fortified tortilla-based diets had significantly larger neurons as estimated by the ratio protein:DNA when compared with their counterparts fed regular tortilla diets. First-generation rats fed the FM and REDMF tortilla-based diets had significantly lower ($P<0·05$) cerebral activity (RNA/DNA) when compared with animals fed the better-quality-protein diets (Table 3). This positive effect on cerebral activity was not observed in second-generation rats.

Short-term memory tests in first-generation rats indicated that there were no differences ($P>0·05$) in the time required to find the hidden platform. However, rats fed the control and soyabean-fortified tortilla and QPM diets had a lower number of errors compared with their counterparts fed the regular tortilla diets (Table 4). Second-generation rats assigned to the FM tortilla-based diet could not complete the short-term memory Morris test because they did not have enough strength to swim and complete the trials.

Table 2. Effect of tortilla fortification and enrichment with minerals and vitamins on brain and cerebellar weights, myelin concentration and density of synapses in first- and second-generation rats

(Values are means and standard deviations)

	Brain weight (mg)		Cerebellar weight (mg)		Myelin (mg/g)		Density of neuronal synapses (%)	
Diet	Mean	SD	Mean	SD	Mean	SD	Mean	SD
First generation (average age 158 d)								
FM*	1024·3[a]	21·3	174·4[a]	6·8	86·6[a]	4·6	26·4[a]	3·1
REDMF†	1130·9[b]	36·3	205·8[a]	13·1	124·2[b]	11·8	29·8[a]	9·6
FEDMF†	1460·4[d]	36·8	340·0[c]	6·3	187·4[c]	4·6	41·5[b]	3·4
EQPM†	1324·5[c]	42·3	302·6[b]	7·2	181·0[c]	7·2	43·6[b]	1·6
FEQPM†	1503·4[d]	24·8	354·2[c,d]	8·6	193·0[c]	6·3	42·3[b]	2·6
Control†	1671·1[e]	29·6	368·3[d]	5·0	190·7[c]	5·3	47·5[c]	2·2
Second generation (average age 62 d)								
FM‡	394·6[a]	4·6	61·2[a]	4·7	68·8[a]	6·8	32·5[a]	5·8
REDMF‡	412·2[b]	8·3	64·9[a]	5·2	126·8[b]	3·9	34·6[a]	3·7
FEDMF‡	656·0[d]	21·6	99·0[c]	2·6	136·7[c]	3·4	36·4[a]	5·2
EQPM‡	578·6[c]	19·7	91·7[b]	3·4	128·6[b]	4·2	36·5[a]	5·2
FEQPM‡	694·3[d]	22·3	99·3[c]	2·3	131·3[b,c]	5·5	32·8[a]	5·9
Control‡	803·4[e]	18·6	104·3[d]	2·6	142·6[d]	2·3	38·6[a]	4·3

FM, tortilla-based diet from fresh *masa*; RDMF, tortilla-based diet from regular dry *masa* flour; FEDMF, tortilla-based diet from soyabean-fortified and enriched dry *masa* flour; EQPM, tortilla-based diet from enriched dry *masa* flour from quality protein maize; FEQPM, tortilla-based diet from soyabean-fortified and enriched QPM dry *masa* flour; Control, casein-based diet.

[a,b,c,d,e] Mean values within a column with unlike superscript letters were significantly different ($P<0·05$).

* n 8.

† n 9.

‡ n 6.

Amaya-Guerra *et al.* (2004) determined that these rats weighed less than half that of their counterparts fed the QPM or soyabean-fortified tortilla-based diets (EQPM, FEDMF, FEQPM). Second-generation rats fed the control and soyabean-fortified diets performed better or had lower latencies and errors than the group fed the REDMF diet.

In the long-term memory test in first-generation rats fed the control and soyabean-fortified tortillas, the animals successfully completed the test in 3·4 d, whereas their counterparts fed the REDMF or FM diet took 5·1 d (Table 4). Animals fed the low-quality-protein diets took approximately 35 % more time to solve the test. The effects on the second-generation rats were exacerbated because it took 9·4 d for animals fed the REDMF diet to complete the test, whereas their counterparts fed the control, EQPM, FEDMF and FEQPM diets completed the test in 5·2 d. The clear difference in favour of rats fed the soyabean-fortified and enriched tortillas

Table 3. Effect of tortilla fortification and enrichment with minerals and vitamins on brain protein, RNA and DNA in first and second-generation rats

(Values are means and standard deviations)

	Protein/brain weight (mg/g)		RNA/brain weight (mg/g)		DNA/brain weight (mg/g)		Protein:DNA		RNA:DNA	
Diet	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
First generation (average age 158 d)										
FM*	96·76[a]	4·33	3·66[a]	0·12	6·96[a]	0·12	13·90[a]	0·27	0·55[a]	0·03
REDMF†	96·72[a]	5·15	4·05[b]	0·09	6·82[a]	0·13	14·18[a]	0·25	0·59[a]	0·02
FEDMF†	108·67[b]	5·43	4·41[c]	0·18	6·84[a]	0·22	15·88[b]	0·37	0·64[b]	0·02
EQPM†	105·43[b]	3·20	4·52[c]	0·15	6·75[a]	0·22	15·61[b]	0·46	0·66[b]	0·03
FEQPM†	106·86[b]	4·45	4·43[c]	0·17	6·82[a]	0·17	15·66[b]	0·39	0·65[b]	0·03
Control†	123·74[c]	7·32	4·61[c]	0·11	6·78[a]	0·12	18·25[c]	0·20	0·67[b]	0·02
Second generation (average age 62 d)										
FM‡	86·76[a]	3·34	3·86[a]	0·04	5·79[a]	0·06	14·98[a]	0·33	0·66[a]	0·02
REDMF‡	91·43[a]	2·61	4·01[b]	0·04	6·11[b]	0·08	14·96[a]	0·29	0·66[a]	0·03
FEDMF‡	101·45[b]	2·16	4·30[c]	0·06	6·25[c]	0·04	16·23[b]	0·22	0·69[a]	0·02
EQPM‡	99·64[b]	2·80	4·23[c]	0·04	6·28[c]	0·04	15·86[b]	0·32	0·67[a]	0·03
FEQPM‡	102·63[b]	3·43	4·27[c]	0·06	6·25[c]	0·04	16·42[b]	0·40	0·68[a]	0·03
Control‡	109·61[c]	2·62	4·31[c]	0·06	6·36[d]	0·06	17·23[c]	0·24	0·68[a]	0·02

FM, tortilla-based diet from fresh *masa*; RDMF, tortilla-based diet from regular dry *masa* flour; FEDMF, tortilla-based diet from soyabean-fortified and enriched dry *masa* flour; EQPM, tortilla-based diet from enriched dry *masa* flour from quality protein maize; FEQPM, tortilla-based diet from soyabean-fortified and enriched QPM dry *masa* flour; Control, casein-based diet.

[a,b,c,d] Mean values within a column with unlike superscript letters were significantly different ($P<0·05$).

* n 8

† n 9.

‡ n 6.

Table 4. Effect of tortilla fortification and enrichment with minerals and vitamins on short-term, long-term and working memory and learning performance in first- and second-generation rats

(Values are means and standard deviations)

	Short-term memory				Long-term memory		Working memory				Learning performance			
	Latency (s)		No. of errors		Days to obtain a positive result		Latency (s)		No. of errors		Latency (s)		No. of errors	
Diet	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
First generation (average age 64 d)														
FM*	15·1^{a}	2·32	4·5^{c}	0·23	4·9^{b}	0·32	16·2^{a}	0·53	4·2^{a}	0·21	§		§	
REDMF†	14·3^{a}	3·21	4·3^{c}	0·12	5·3^{b}	0·28	16·7^{a}	0·43	5·7^{b}	0·32	32·1^{c}	2·54	18·4^{c}	1·58
FEDMF†	13·9^{a}	1·23	3·9^{b}	0·23	3·3^{a}	0·52	17·1^{a}	0·32	6·8^{c}	0·22	21·3^{a}	1·24	9·5^{a}	1·46
EQPM†	14·4^{a}	1·68	3·7^{b}	0·23	3·1^{a}	0·47	16·4^{a}	0·51	5·8^{b}	0·20	26·4^{b}	1·41	12·6^{b}	0·55
FEQPM†	13·6^{a}	2·36	3·6^{b}	0·38	3·9^{a}	0·34	16·8^{a}	0·44	7·9^{d}	0·38	21·4^{a}	1·54	11·4^{a}	0·60
Control†	13·6^{a}	1·62	2·1^{a}	0·42	3·6^{a}	0·11	17·2^{a}	0·51	5·7^{b}	0·34	19·6^{a}	1·12	12·3^{a,b}	0·32
Second generation (average age 62 d)														
FM‡	§		§		§		§		§		§		§	
REDMF‡	18·6^{b}	2·10	4·3^{b}	0·2	9·4^{b}	2·3	28·9^{c}	0·6	8·6^{c}	0·4	§		§	
FEDMF‡	13·4^{a}	2·07	3·6^{a}	0·2	5·1^{a}	1·6	20·2^{a}	1·2	5·1^{a}	0·4	34·4^{b}	2·0	15·4^{b}	1·80
EQPM‡	13·8^{a}	2·32	4·1^{b}	0·2	6·2^{a}	0·8	23·6^{b}	0·8	7·6^{b,c}	0·4	42·4^{c}	2·4	16·7^{b}	1·62
FEQPM‡	13·1^{a}	1·64	3·2^{a}	0·2	5·2^{a}	1·1	21·1^{a}	1·2	6·1^{b}	0·4	32·9^{b}	2·6	14·8^{b}	2·78
Control‡	12·7^{a}	1·66	2·9^{a}	0·4	4·1^{a}	1·6	18·9^{a}	2·3	6·8^{b}	0·8	19·3^{a}	3·6	8·4^{a}	0·56

FM, tortilla-based diet from fresh *masa*; RDMF, tortilla-based diet from regular dry *masa* flour; FEDMF, tortilla-based diet from soyabean-fortified and enriched dry *masa* flour; EQPM, tortilla-based diet from enriched dry *masa* flour from quality protein maize; FEQPM, tortilla-based diet from soyabean-fortified and enriched QPM dry *masa* flour; Control, casein-based diet.

^{a,b,c,d} Mean values within a column with unlike superscript letters were significantly different ($P<0·05$).

* *n* 8.

† *n* 9.

‡ *n* 6.

§ Rats could not complete the test.

was due to the better protein quality and higher intake of Fe, Zn, thiamin, riboflavin, niacin and folic acid (Amaya-Guerra *et al.* 2004).

There were no significant differences ($P>0·05$) between treatments on the latency required to solve the working memory tests. In this particular assay, first-generation animals fed the FM diet had a significantly lower number of errors compared with rats fed the other treatments. However, working memory performance differed considerably in second-generation rats. Rats fed the FEQPM or FEDMF diet had the lowest latency, followed by animals fed the EQPM diet. The worst performance was again observed in rats fed REDMF, and once more the FM rats could not complete the test.

Among the experimental treatments, first-generation rats fed the FEDMF or FEQPM diet had the best performance in the learning performance tests, followed by animals fed EQPM. Second-generation rats fed the FM or REDMF diet could not complete the learning performance tests. As expected, the control casein-fed animals had the best performance and lower latency, followed closely by animals fed the FEDMF, FEQPM and EQPM diets. Except for the control animals, second-generation rats fed all the experimental treatments had a lower learning performance compared with their first-generation counterparts. The lack of essential nutrients and protein quality during gestation and lactation exacerbated the negative effects observed in second-generation rats. Even rats fed the REDMF diet could not complete the second-generation learning performance tests.

Discussion

The significantly lower cerebral and cerebellar weights observed in first- and second-generation rats fed regular tortilla diets (FM, REDMF) is mainly attributed to the deficient quality of dietary protein or improper essential amino acid balance. Previous investigations conducted by Chase *et al.* (1967), Winick & Nobel (1966), Culley & Lineberger (1968), Zamenhof *et al.* (1971) and Smart *et al.* (1973) showed that early malnutrition significantly reduced the brain and cerebellar size. Gramsbergen & Westerga (1992) determined that these negative changes in the cerebellum were related to lower performance on motor and working memory tests in laboratory animals. Pollit *et al.* (1993) established a relationship between these motor deficiencies and the reading and writing abilities of malnourished infants. In a previous study, Amaya-Guerra *et al.* (2004) observed that the same rats fed the FM or REDMF diet also had lower body weight gains and lower reproductive performance.

Rats fed the FM diet that was not supplemented with B vitamins and trace minerals had approximately half the myelin when compared with the rest of the animals fed the enriched diets. Several authors have determined that Fe and Zn play an important role in cerebral myelin synthesis (Yeh, 1988; Oloyede *et al.* 1992; Golub *et al.* 1995). De los Monteros *et al.* (2000) found that Fe deficiency in well-balanced diets does not result in extensive growth deficits in body and brain weight. However, the Fe deficit profoundly delayed the development of myelination. Rahmannifar *et al.* (1993) reported that the supplemental intake of riboflavin, niacin

and pyridoxine by pregnant women positively affected the alertness of their infants.

First-generation rats fed QPM and/or soyabean-fortified tortillas developed a greater density of cerebral synapses than did their counterparts fed the regular tortilla diets. Warren & Bedi (1984) concluded that laboratory animals that had excellent brain activity possessed an increased number of synapses between their neurons. It is normally considered that the formation of these new synapses, called plasticity, is related to the external stimulation to which the animal is subjected (repetitions, experiences, etc.) and not to its nourishment. It is also known that when animals are fed low-protein diets, the number of blood vessels that irrigate the brain cortex is reduced, and as a result the brain cells obtain less energy and do not benefit as much from external stimulation when compared with well-nourished animals (Taleb *et al.* 1999).

Second-generation rats fed the soyabean-fortified diets had significantly higher amounts of brain DNA compared with their counterparts fed regular tortilla diets. This agrees with the findings of other authors who have also related malnutrition to a significant reduction in brain DNA in laboratory animals (Winick & Nobel, 1966; Zamenhof *et al.* 1968; Winick, 1969; Zamenhof, 1985; Warren & Bedi, 1988). Winick & Nobel (1966) proposed that cerebral DNA content was related to the number of brain neurons, that the DNA:protein ratio could be used as an indicator of neuron size and that the RNA:DNA ratio was a good indicator of the metabolic activity of the brain cells. Both first- and second-generation rats fed QPM and/or soyabean-fortified tortillas had significantly larger neurons than animals fed the lower-quality-protein diets. However, only the first-generation rats fed the good-protein-quality diets had higher cerebral activity ($P<0{\cdot}05$). Second-generation rats had similar cerebral activity ($P>0{\cdot}05$), probably because these animals were terminated approximately 1 month after weaning (Table 3). Serra *et al.* (1982) observed that the delay in DNA and RNA synthesis caused by undernutrition was most evident in the cerebellum, probably owing to its intense postnatal cell proliferation.

With the exception of short-term memory, first-generation rats fed QPM and/or soyabean-fortified diets had a better performance in the Morris maze tests (long-term memory, working memory and learning performance). Second-generation rats fed the good-quality-protein diets had better performance in the four Morris maze tests, including the short-term memory test. Surprisingly, in the working memory test, first-generation rats fed FM had a significantly lower number of errors compared with rats fed the other treatments. One possible explanation is that, in the working memory test, the place of entry was changed and the well-nourished animals swam along their old path and then circumnavigated the pool until finding the platform. On the other hand, rats fed the FM diet generally navigated in a random fashion and therefore found the platform with a lower number of errors.

Tonkiss *et al.* (1994) studied the working memory performance of malnourished young rats whose mothers were severely malnourished during gestation or lactation with a similar procedure to that used in this study. The authors observed that young rats from malnourished pregnant dams had a significantly lower performance than their counterparts fed a control diet. There were, however, no significant differences when young rats from malnourished lactating dams were compared.

In summary, the utilisation of QPM instead of regular maize tortillas, and tortillas fortified with defatted soyabean meal, improved the brain development and maze performance of rats fed with a tortilla-based diet throughout two generations. The effect was more marked for the second-generation rats. Animals had higher brain and cerebellar weights, cerebral myelin DNA concentration, neurone size, brain activity and short-term and long-term memory performance in the Morris maze.

The utilisation of regular or QPM tortillas enriched with selected micronutrients and fortified with soyabean is highly recommended to assure adequate brain growth and brain performance. The performance of rats fed QPM tortillas that contained 30g/kg soyabean meal was similar to that of counterparts fed regular tortillas fortified with 6 % soyabean meal; therefore, using QPM can save half 60g/kg the soyabean flour without sacrificing the nutritional value. The present study clearly shows the beneficial effects of QPM, soyabean fortification and the enrichment of tortillas with Fe, Zn, thiamin, riboflavin, niacin and folic acid. Despite the meritorious effects of the QPM and soyabean-fortified tortillas, the results from the control diet were in most instances superior, indicating that there is still opportunity for improvement. The utilisation of QPM and/or soyabean-fortified tortillas is highly recommended because this staple food is most deficient in nutrients for the low-income and rural people who inhabit Mexico and Central America.

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