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Effect of Banana Flour (*Musa paradisiaca* L.) on Pathological Changes in Mice with Induced Phenylketonuria

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Abstract

The effect of banana flour (BF) on the pathological changes caused by phenylketonuria (PKU) induced in mice was evaluated. We used 72 mice of the C57 strain, 60 of which, were induced PKU, administering daily 24 µmol/10g of live weight (LW) of α-methylphenylalanine intraperitoneally (ip) and 52 µmol/10g of PV ip of phenylalanine (PA), until weaning (d 16). Subsequently, the animals were divided into 3 treatments: Treatment 1: 30 PKU mice, which were kept the disease by administering daily α-methylphenylalanine and feeding on BF (60% crude banana flour and 40% pre-gelatinized BF), approximately 25 mg/mouse/d, for 21 d; Treatment 2: 30 PKU mice that received α-methylphenylalanine interdiarily, feeding on Ratarina® for 12 d; Treatment 3: 12 control mice treated daily with 0.9% w/v NaCl solution, administered ip, feeding on BF for 12 d. Blood samples were taken to measure PA concentrations, by fluorescent ultramicroassay (UMTEST® PKU). From day 4 post-treatment, with a 4-day interval, liver and brain samples were taken to evaluate histopathological changes and protein expression of phenylalanine hydroxylase (PAH) using the Western blot technique. The induction of PKU was obtained from day 8 post-treatment, when the blood levels of AF were higher than 2.8 mg/dL. During the delivery of BF to mice with PKU/(T1), it was possible to decrease blood levels of PA in a maintained manner, unlike the group of PKU mice fed with Ratarin (T2), which presented levels of PA greater than 2.8 mg/dL. There was higher protein expression of PHA in the liver of mice with PKU induced in the animals that had between 8 to 12 d post-treatment with the enzyme inhibitor and were supplemented with PA (H3 and H4, in the polyacrylamine gel). Hepatic damage, apoptosis and cytoplasmic vacuolization were evidenced, consistent with the blockade of PAH, completely altering the normal liver function, noting the great sensitivity of the liver to metabolic changes. Through the results it was possible to demonstrate the use of plantain flour as a nutritional alternative in phenylketonuric patients.

Keywords: *Banana Flour; Mice; α-Methylphenylalanine; Phenylketonuria; Liver; Brain Phenylalanine Hydroxylase*

Abbreviations

BF: Banana Flour; PKU: Phenylketonuria; PA: Phenylalanine; ip: Intraperitoneally; PAH: Phenylalanine Hydroxylase; BW: Body Weight; TIR: Tyrosine

Introduction

Phenylketonuria (PKU) is a progressive and severe metabolic disease of childhood, which can lead to mental retardation if not treated in time. This disease is caused by a deficiency of phenylalanine hydroxylase (PAH), an enzyme involved in the metabolism of phenylalanine (FA), causing an excessive accumulation of this amino acid in blood. Classic PKU occurs when the residual PAH activity is less than 1%, as expressed by blood levels of PA higher than 20 mg/100 mL, with an increase in phenyl ketones in urine.

In PKU, an excessive amount of PA prevents the uptake of vital amino acids by the central nervous system, an essential step for proper development, causing irreversible brain damage. For this reason, early detection of this disease is very important, as well as its appropriate treatment [1]. The supply of a diet low in FA delays disease development, reducing the extent of clinical signs [2]. Children with untreated PKU are often irritable and have behavioral problems. Both the urine and sweat of those infants can give off an unpleasant musty

odor. Dermatological disorders such as dry skin and rashes may occur. Seizures are also common. Typically, children with PKU have a good physical development and tend to have lighter hair than their siblings [1].

FA cannot be completely removed from the diet, because it is an essential component of food. Even in the same patient, the necessary amount of PA may vary with age, so that a low intake of PA can also be fatal [3,4]. Today, the development of banana based flour has become a trend for the development of different products that take maximum advantage of the fruit, adding value to their crop.

Rodriguez., *et al*. [5] analyzed the banana flour (*Musa paradisiaca* L.) and reported that both protein content and ash were found in minor proportions, so it is possible to diversify the use of green banana flour and use it in the industry of functional foods (phenylketonuria, celiac disease), whose availability is currently minimal in the country, being this sector of the population, especially children, completely neglected.

From the aforementioned disclosure, the main purpose of this investigation was to evaluate the effect of banana flour on pathological changes induced in mice with induced phenylketonuria.

Materials and Methods

Mice of the C57 strain, of both sexes, obtained at the animal facility of the Universidad Centro-Occidental Lisandro Alvarado (UCLA), the State of Lara, Venezuela, were used. Mice were kept and acclimatized at the Animal Facility of the Department of Biosciences, Faculty of Veterinary Medicine of the Universidad Central de Venezuela (FCV-UCV), the State of Aragua, Venezuela, and fed commercial feed concentrate (Ratarina; Protinal©, Valencia, Venezuela). The food composition was: crude protein (26%); crude fat (2%); crude fiber (6%); nitrogen-free extract (40%). The amount of food given was 25 mg/mouse/day. Water was given *ad libitum*. The animals were placed in cages and assigned by sex. Management of animals was done following the established guidelines for the management of laboratory animals of the FCV-UCV. Since the assay required very young mice with an age of 2 - 6 days of birth, and being transportation of newborn animals a source of stress, breeding of experimental animals was performed in the animal facility of the FCV-UCV. The breeding experimental unit was composed of 4 mice (3 females and 1 male). Likewise, each breeding unit was composed of 4 replicates, resulting in a total of 16 animals (12 females and 4 males). These animals produced litters, with an average of 6 live births. To induce the disease, two groups were used from the third day of birth, as follows: Group I (experimental): sixty healthy mice (n = 60) which were given 0.2 mL of a PAH inhibitor, α-methylphenylalanine (Sigma, St. Louis MO, USA), at a dose of 24 µmol/10g of body weight (BW) [6] and 0.2 mL of PA, at a dose of 52 µmol/10g/BW. This dose has been reported to cause classic PKU. Group 2 (control): twelve mice (n = 12) which were injected with 0.4 mL of saline (0.9% w/v of saline). All doses were administered ip, until weaning.

Determination of blood PA in mice with induced PKU

Blood levels of PA were measured using a fluorescent ultramicroassay test (UMTEST® PKU), for quantitative determination of PA in dried blood on filter paper. To obtain a reference value during the induction phase, the first sample was taken on day 1 of the experiment, and then, every 4 days after treatment, until weaning (approximately on day 16 post-treatment). Blood from mice was withdrawn through a cut in the tail and each drop was placed in a Guthier's paper filter. A blood pool was placed on each circle of the filter paper and PA readings were made in triplicate, taking different areas of each circle. After induction of PKU in the experimental animals, the groups were subdivided into three treatments, as follows: Treatment 1: thirty mice (n = 30) with PKU induced through the administration of 0.2 mL of α-methylphenylalanine (Sigma, St. Louis MO, USA) every other day, with the same dose as previously was described by Del Valle y Greegard [6]. This group was fed a mixture of 60% raw banana meal and 40% of pre-gelatinized banana meal, administering approximately 25 mg/mouse/day for 12 d. The banana meal mixture was obtained at the Institute of Science and Food Technology of the Faculty of Sciences of the UCV and its Chemical composition (Table 1) was determined as reported by Guzman (2001), being in terms of percentage, as follows: fat (2.45%); protein (3.32%); ash (2.10%); total starch (68.03%); digestible starch (47.07%); and resistant starch (21.06%). The experimental diet consisted of only one ingredient: banana meal, which has a low content of PA; treatment 2: thirty mice (n = 30) with PKU received 0.2 mL of α-methylphenylalanine (at a dose of 24 µmol/10 g BW ip, every other day), and were fed Ratarina in approximate amounts of 25 mg/mouse/day) for 12 days; treatment 3: twelve control mice (n = 12), which received 0.4 mL of saline (0.9% w/v of NaCl) ip, every other day. Mice were fed banana meal (approximately 25 mg/mouse/day) for 12 days. During the experimental period, mice behavior was assessed during manipulation. Mice were weighed daily. In addition, from day 4 post-treatment and with a 4-day interval, samples of liver and brain from different treatments were taken, and histopathological studies were conducted. Also, PAH protein expression was assessed, using the Western blot technique. Animals were euthanized with sodium pentobarbital (200 mg/kg BW), by intracardiac injection. Tissue samples were preserved in 10% v/v buffered formalin and placed in paraffin blocks for histological analysis; subsequently, they were examined under a Nikon optical microscope, with a 100X immersion objective. Samples were analyzed

in the laboratory of Pathology of the FCV-UCV. To evaluate PAH protein expression, samples were placed in cryovials and stored at -80°C until further processing, according to Ruiz and Kittok [7].

Parameter	Pre-mixture
Physicochemical	60:40
Moisture	8.8 ± 0.33
Fat	0.27 ± 0.02
Protein	3.64 ± 0.04
Ash	2.02 ± 0.07
Starch	78.09 ± 0.32
Amylose	--
Amylopectin	--
a_w	--
pH	6.40 ± 0.06
Acidity	0.41 ± 0.06
Color profile	
L*	85.01 ± 0.001
a*	1.88 ± 0.005
b*	13.66 ± 0.03
IB	79.63 ± 0.02
ΔE	86.12 ± 0.01

Table 1: *Nutritional composition of banana flour.*

Results and Discussion

Experimental induction of PKU in mice using α-methylphenylalanine

Table 2 shows that the induction of PKU in mice was achieved 8 days after the onset of treatment when blood levels of FA were above 2.8 mg/dl, remaining high until the end of the experiment. These results agree with those reported by Del Valle., *et al.* [6] who, using the same methodology, could evidence an increase in blood FA, 24h after injection of a PAH analogue. In contrast, FA values for mice of the control group were below 2 mg/dL during that period.

Phenylalanine Levels (mg/dL)				
Day/Treatment	**4**	**8**	**12**	**16**
0	1.87	1.40	1.94	1.82
1	2.31	2.94	2.92	3.00

Table 2: Phenylalanine concentration in mice during induction of phenylketonuria.

T_0: Samples of control animals

T_1: Samples of animals treated with PA and α-methylphenylalanine

N_0: Number of animals for T_0: 60

N_1: Number of animals for T_1: 12

^{a,b}: Values with different subscripts in the same column are statistically significant ($P < 0.05$).

The PAH analog (α-methylphenylalanine) used in this study did not affect the progressive growth of the mice (Table 3), or mortality during the experiment, as reported by Del Valle., *et al.* [6] in their investigation, demonstrating no loss of physical condition or high mortality due to this analog.

Mice Weight (g)									
Day/Treatment	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**
0	6.66	6.70	7.11	7.37	7.75	7.86	7.67	10.35	11.64
1	6.53	6.64	6.85	7.11	7.35	7.30	8.55	8.28	7.19

Table 3: Average weight of mice during the induct*ion of PKU.*

T_0: Control animal

T_1: Animals treated with values with different subscripts in the same column are statistically significant (P< 0.05) PA and α-methylphenylalanine

N_0: Number of animals under T_0

N_1: Number of animals under T_1

^{a,b}: Values with different subscripts in the same column are statistically significant (P < 0.05).

Regarding physical changes, mice injected with α-methylphenylalanine + PA showed reddening of the skin, alopecia of the neck, chest and flanks, foci of depigmentation on the tail, lower growth rates (Figures 1 and 2), and low weight (Table 3). These findings are consistent with what occurs in children with PKU, which have symptoms such as irritability, dry skin, frequent eruptions, tendency to have skin, eyes and hair lighter than their siblings [3]. Additionally, there are other characteristic clinical signs of PKU, such as seizures and a musty smell in skin and urine, which could not be evidence in mice with PKU in this investigation. This is in agreement with the findings reported by Marcos (2006) who notes that seizures caused by high concentrations of PA and low concentrations of tyrosine (TIR) in the brain, occurs only in 25% of cases. It is important to underscore that the maze test was not performed because the disease was induced to animals with a few days of birth (eyes closed and poor coordination when walking) and when they were placed in the maze, they did not walk, were disoriented, and only stayed walking around looking their mothers; therefore, it was not possible to assess whether there were changes in skill and ability to get food.

Figure 1: *Mouse with tail depigmentation*

Figure 2: *Size comparison between a sick animal (left) and a healthy animal (right).*

With regard to the supply of banana flour as the sole ingredient in the diet, since it did not provide all the nutritional requirements necessary for the normal development of the mouse, it caused the loss of clinical and physical condition of the mice, resulting in a progressive deterioration and even death. During the supply of banana flour to mice with PKU, these animals managed to lower and maintain blood levels of FA, unlike the PKU group of mice fed Ratarina that continued to show levels above 2.8 mg pA/dL, while control mice fed banana flour showed lower FA values in blood to 2.8 mg/dL (Table 4).

Phenylalanine Levels (mg/dL)			
Day/Treatment	**20**	**24**	**28**
T_1	1.42^{b}	1.66	1.72^{a}
T_2	4.84^{a}	3.22	3.00^{b}
T_3	2.46^{ab}	2.41	2.19^{a}

Table 4: Phenylalanine concentrations in blood during the administration of banana flour

T_1: Mice with PKU fed banana flour

T_2: Mice with PKU fed commercial diet

T_3: Control mice fed banana flour

$N_1 = 30$

$N_2 = 30$

$N_3 = 12$

^{a,b}: Values with different subscripts in the same column are statistically significant ($P < 0.05$).

These results suggest the possibility of using this ingredient in the development of national balanced food to feed children with PKU, complementing the research by Perez and Marin [8], who evaluated in our country other ingredients such as bananas, both the flour and its shell, promoting the use of it as an alternative feedstock for the production of foodstuffs. Including pastas for patients with PKU are developed; additionally, Suarez [9] developed a food made from rice flour, banana and beans are low in PA for child consumption. Table 5 and figure 4 depict the weight of mice during the administration of flour banana and commercial food, proving that mice with PKU and control mice which received as food flour banana, showed progressive loss of weight, while mice with PKU fed with commercial diet, maintained a steady weight gain.

Day Post-Treatment	Group Induced PKU (alterations in the liver)	Group Induced PKU (changes in the brain)
4	Cells with mild apoptosis, cells with several picnotic nuclei and with cytoplasmic microvacuolation (100x)	Neuronal area with a preserved appearance and neurons without apparent lesions
8	Moderate apoptosis and in 5/5, basilic, angular cells with pyknotic nuclei (100 x)	Acidophilic Purkinje cells with retracted nucleoli, pigmented axons, thickened acidophilic (100x), indicative of marked degeneration
12	Cariolysis and necrotic cells, hepatocytes with cytoplasmic microgranularity and pyknotic nuclei (100x)	Bleeding and axonal vacuolization and marked apoptosis in Purkinje cells

Table 5: *Pathological changes of liver and brain tissue in mice during the induction of phenylketonuria.*

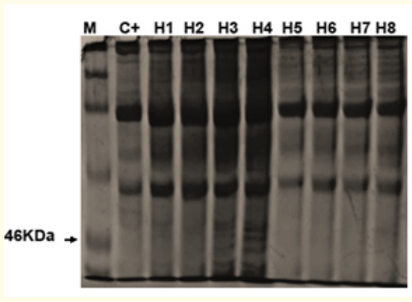

Figure 3: *Polyacrylamide-dodecyl sodium sulfate (10 - 5% w/v) gel of the discontinuous type (SDSPAGE) containing samples of livers of mice subjected to different diets. M: Molecular weight marker; C+: Positive control (liver of healthy mouse); H1: Control sample; H2: Samples with 4 days post-treatment; H3: Samples with 8 days post-treatment; H4: Samples with 12 days post-treatment; H5: Control samples at 21 days + banana flour; H6: Samples with 21 days post-treatment fed commercial diet; H7: Samples with 21 days post-treatment fed banana flour; H8: Samples with 26 days post-treatment fed commercial diet.*

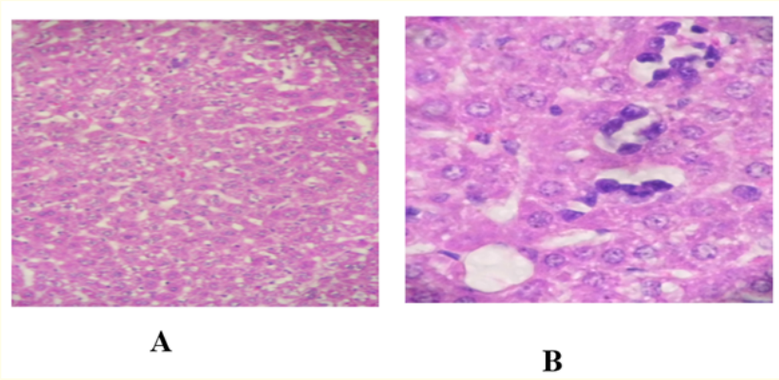

Figure 4: *A: Liver tissue of mouse with 4 days post-treatment (40x); B: Liver tissue of mouse with 4 days post-treatment (100x).*

Protein expression of phenylalanine hydroxylase in mice with induced PKU

The presence of a strong protein band was evident in the gel, with a molecular weight of about 40 - 50 kDa, suggesting that this corresponds to the phenylalanine hydroxylase, obtained from liver samples of mice of H3 and H4 treatments enzyme phenylalanine (Figure 3).

These samples correspond to animals that had been receiving injections of the enzyme inhibitor between 8 to 12 days, and additionally, doses of PA, which could be over-expressing PHA, as compensatory response to try to reduce increased levels of PA in blood.

The weight of the expressed protein band in the gel coincides with that reported by Gibbs., *et al*. [10], which evaluated the expression of PAH, using recombinant *E. coli* cells and Sf9 cells, being the expression of mouse liver PAH with a molecular weight of 45 kDa.

It was observed that the protein band intensity was lower in control animals (H1), of the induction phase of the disease which, were in the breastfeeding period and only received the placebo dose. In animals of group H2, the expression of the enzyme was fainter, since the animals had only four days of receiving the enzyme inhibitor. Additionally, the expression of PAH was not evident in H5 to H8 treatments, corresponding to controls or experimental animals with 20 and 26 d post-treatment, respectively (Figure 3).

Controls or experimental animals 20 d post-treatment, but they were consuming banana flour, did not express the PAHs as they had low levels of PA in blood and did not require the expression of this enzyme. This could show how the nutritional management helped to reduce the PA levels in animals with PKU.

Pathological changes of liver and brain tissues in mice with induced PKU

Pathological changes observed in liver and brain tissues in mice with induced PKU at 4, 8, and 12 days post-treatment, are summarized in table 5 and in figures 4-9. In the case of liver cells of mice with induced PKU fed banana flour at 21 days post-treatment, showed apoptosis (3/5 cells), lipidotic vacuolization, and picnotic nuclei (Figure 10). Brain tissue of mice with induced PKU fed banana flour 21 days after treatment, evidenced hemorrhagic foci and optic neurons, and Purkinje cells with a severe apoptotic index (Figure 11).

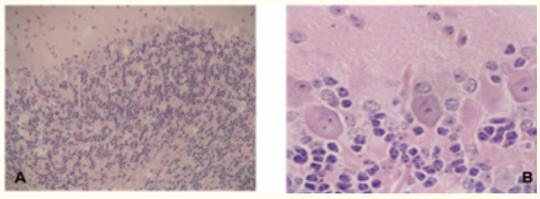

Figure 5: *A: Brain tissue of mice with 4 days post-treatment (40 x). B: Mouse neurons with 4 days post-treatment (100x).*

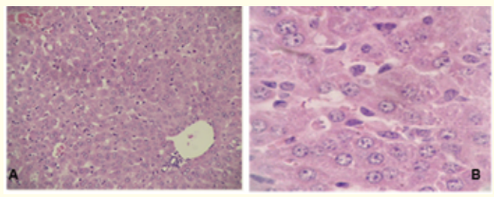

Figure 6: *A: Liver tissue of mouse with 8 days post-treatment (40x). B: Liver tissue of mouse with 8 days post- treatment (100x).*

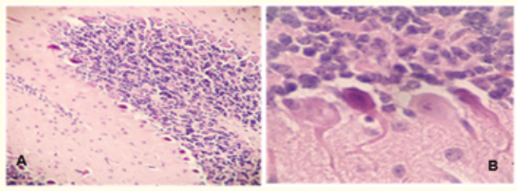

Figure 7: *A: Brain tissue of mouse with 8 days post-treatment (40x). B: Brain tissue of mouse with 8 days post-treatment (100x).*

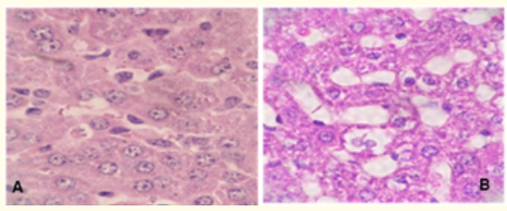

Figure 8: *A: Liver tissue of mouse with 12 days post-treatment (100x). B: Mouse hepatocytes with 12 days post-treatment showing cytoplasmic microgranularity and picnotic nuclei (100x).*

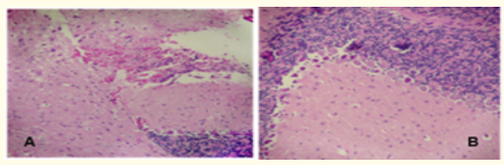

Figure 9: *Brain tissue of mouse with 12 days post-treatment. A: Hemorrhage and axonal vacuolization (100x). B: Marked apoptosis in Purkinje cells (100x).*

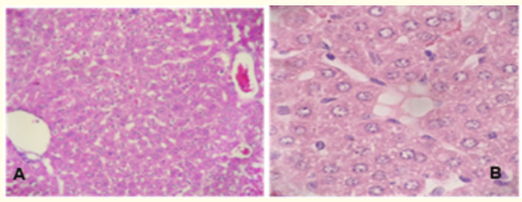

Figure 10: *A: Liver tissue of mouse with PKU fed banana flour (21 days post-treatment; 40x). B: Liver tissue of mouse with PKU fed banana flour (21 days post-treatment; 100x).*

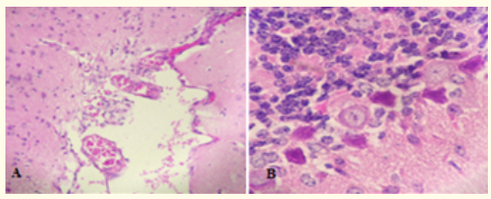

Figure 11: *A: Brain tissue of mouse with PKU fed banana flour (21 days post-treatment; 40x). B: Brain tissue of mouse with PKU fed banana flour (21 days post-treatment; 100x).*

In the control group of mice fed banana flour (21 d post-treatment), liver tissue did not show apparent lesions (Figure 12A), while the brain tissue showed moderate cerebellar apoptosis (Figure 12B). Liver tissue of mice fed PKU commercial diet (21 d post-treatment) revealed severe lesions with lipid accumulation of fatty material in hepatocytes (Figure 13). These findings found in the histopathological samples of liver tissue and brain studied, showed injuries that were more severe, as treatment days passed, and a severe lipidotic liver vacuolization was observed at 26 days post-treatment.

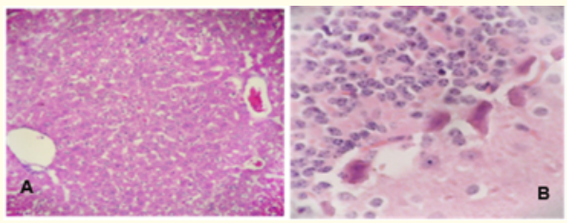

Figure 12: *A: Liver tissue of control mouse fed banana flour (21 days post-treatment; 40x. B: Liver tissue of control mouse fed banana flour (21 days post-treatment; 100x).*

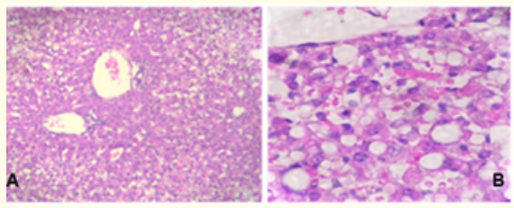

Figure 13: *A: Liver tissue of mouse with PKU fed commercial diet (21 d post-treatment; 40x. B: Liver tissue of mouse with PKU fed commercial diet (21 d post-treatment; 100x).*

Kumar., *et al.* [11] report that fatty degeneration represents an absolute increase of intracellular lipids. Steatosis often is preceded by cell swelling itself, indicating a nonlethal damage that often heralds cell death and is often seen in adjacent cells that have undergone necrosis. This is often seen in the liver, as this is the main organ involved in the metabolism of fats. Fatty change begins with the appearance of tiny inclusions limited by membranes. With the optical microscope, it is initially manifested by the appearance of lipidic small vacuoles in the cytoplasm around the nucleus, the vacuoles then fuse to create unique transparent spaces that displace the nucleus to the periphery of the cell; sometimes, neighboring cells and fat globules are break and fuse, producing the so-called fatty or lipid cysts [11].

A study by Pérez [12] reported the existence of only two works in humans where studies of brains affected by PKU were performed, in which a decrease in the number of dendritic spines at level of the cortical pyramidal neurons was demonstrated, as well as an increase in neuronal density, similar to that seen in early stages of neurologic development. Cordero., *et al*. [13] described findings similar to the ones found in the present investigation, in rats subjected to hyperphenylalaninemia, where pyramidal and Purkinje cells, and Hippocampus neurons are affected.

Moreover, Ruiz., *et al.* [14] conducted a histopathological examination of brain areas in neonatal rats intoxicated with *Crotaluss durissus terrifficus* isolated toxin, intracytoplasmic vacuoles, finding translucent and, with a less frequency, intranuclear localization.

The injury involved the cerebellum, the prefrontal cortex, and the striatum tissue. According to Becerra., *et al*. [15], programmed cellular death of neurons due to epilepsy, not only depends on the cellular type, but of other elements, as the metabolic state of neurons at the moment of injury. The existence of differential sensitivity of neurons to oxidative stress has been shown, after comparing slices of cerebellum regions of the hippocampus and cerebral cortex, being the most vulnerable the cells of the hippocampus and the granular cells, and the most tolerant, the cortical cells [16-19].

Conclusion

In this research, it was possible to experimentally induce phenylketonuria in mice of the strain C57, achieving values of PA above 2.8 mg/dL, from the 8th day of administration of α-methylphenylalanine, and these values were maintained until the day of weaning. After the induction of the disease, physical changes were observed in the mice. Such changes were: tail depigmentation, and skin redness. The banana flour ingredient is low phenylalanine, which was used for this study in mice with PKU. Once these animals reached values of 2.8 mg/dL with the, banana flour meal, values of 2.19 mg/dL were recorded at 8 d post feeding; demonstrating that it is a key ingredient to the regulation of phenylalanine levels in blood, helping to reduce the effects of the disease.

The physical changes of the disease observed in non-phenylketonuric mice, did not disappear with dietary experimental phase, which makes us think that possibly more days of experimentation are required for their evolution.

In liver samples of mice with 8 to 12 days post-treatment, a protein band was observed, suggesting that it corresponds with phenylalanine hydroxylase. This could coincide with the highest peak of blocking of the enzyme, which could be over-expressing, as a way to compensate and try to reduce the high levels of phenylalanine in blood. The changes observed in samples of liver and brains of mice with PKU were degenerative in varying degrees, such as cell apoptosis and axonal degeneration among others. These changes were caused by hyperphenylalaninaemia, although no seizures (a common symptom in children with phenylketonuria) were observed.

Bleeding and cell degeneration demonstrate that brain tissue is very sensitive to metabolic changes, being of paramount importance, for the early detection of phenylketonuria in suspect children.

The evident liver damaged, apoptosis and cytoplasmic vacuolization, are injuries that became more chronic with each day of treatment, and are consistent with the blocking of the phenylalanine hydroxylase, completely altering the normal functioning of this body, allowing himself to notice how sensitive to metabolic changes this organ is.

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