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Research Article

Study of Donkey Milk as A Possible Alternative for Patients Allergic to Cow's Milk

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Abstract

Background: Previous studies have determined a high degree of similarity in the biochemical composition of human and donkey milk. Our objective was to determine the allergenic response to donkey milk in patients sensitized to cow's milk attended during one year at our Allergy Department.

Methods: Samples of donkey milk were obtained from Zamora, Spain, and frozen at -72°C. *In vivo* (prick-prick and oral challenge test) and in vitro studies (specific IgE, SDS-PAGE and Western blot, and molecular analysis) were performed in four groups: two groups of patients sensitized to cow's milk (severe clinical allergy and eosinophilic esophagitis) and two control groups (healthy and allergic to grass pollens without digestive symptoms), after informed consent.

Results: During one year, 2032 patients presented hypersensitivity to some food, of whom 83 exhibited severe symptoms related to cow's milk intake (4%). Of these, 46 had positive IgE to alpha-lactoglobulin, 42 to beta-lactoglobulin and 47 to casein. Of these, 46 patients accepted donkey milk challenge, which was positive for 19. Of the 67 patients with eosinophilic esophagitis, 13 were challenged, being positive in 6 patients. The rest tolerated 100 ml of donkey milk without reactions. Western blot results showed an allergenic profile of donkey milk similar to that of cow milk in all patients analyzed, although clearly with less intensity in donkey milk.

Conclusion: We found a lower IgE-mediated allergic hypersensitivity to donkey milk in patients allergic to cow's milk. Therefore, given its accessibility, donkey milk could be a feasible alternative for these patients.

Abbreviations: SPT: Skin Prick Tests; sIgE: Specific Immunoglobulin E; FPIES: Food Induced Enterocolitis Syndrome; CRD: Component Resolved Diagnosis (molecular analysis by microarrays); SDS-PAGE: Electrophoresis In Polyacrylamide Gel With Sodium Dodecylsulfate

Introduction

The similarities in chemical and antigenic composition, immunological, metabolic, nutritional and functional properties between donkey (Equus africanus asinus) and human milk has been verified in several *In vivo* and in vitro studies [1], demonstrating well tolerance by children. The protein concentration of cow´s milk (2.5-4.2%) is twice that of human milk, and among its main allergens are beta-lactoglobulin, absent in human milk, and alpha-s1-casein, with anaphylactic power [2]. The most similar to human milk is donkey´s milk, which contains more whey protein in the serum (35-50%) than cow´s (20%). Beta-lactoglobulin from donkey's milk is a monomer and cow´s milk is a dimer, with a high lactose content like human milk. Goat's milk is better assimilated than cow´s milk, its main protein is beta-casein, but its allergenic power is similar to that of cow´s milk. In a study in 46 children allergic to cow's milk in which tolerance to donkey´s milk was tested, 38 of them achieved tolerance with a similar growth values. The cross-reactivity between IgE to cow´s and donkey´s milk was studied, which was very weak and not very specific [3]. The primary structure of donkey´s milk proteins (specially casein) is closer to that of human milk proteins. The linear amino acid sequence of the epitopes of cow´s milk is different from that of donkey´s, which may give rise to a lower allergenicity [4].

In a more recent study in 81 children with intolerance to cow´s milk, 70 due to an IgE-mediated mechanism and 11 with enterocolitis (foof induced enterocolitis syndrome or FPIES), tolerance to donkey´s milk was tested with *In vivo* techniques, including oral challenge, and in vitro. Of them, 77 tolerated it. In a subsequent nutritional study of 22 children who had tolerated cow´s milk, no changes in their growth were observed, concluding that donkey´s milk can be used in IgE-mediated allergy and in FPIES [5]. The interest of our work is focused on assessing whether donkey milk, and specifically that obtained from Arribes del Duero area, Zamora, Spain, (Equus africabus asinus), would be a useful and safe nutritional alternative in patients (children and adults) with severe allergic symptoms (anaphylaxis) and eosinophilic esophagitis due to IgE-mediated hypersensitivity to cow´s milk.

Methods

We obtain frozen, lyophilized and free samples of bacteria and other pathogenic microorganisms of donkey milk (Equus africanus asinus), a common European donkey, of the same family as other European donkeys. To carry out our tests in allergic patients and healthy and atopic controls. We will also test it patients with eosinophilic esophagitis with positive allergic test to milk who improved clinically and in their biopsies after avoiding milk and dairy products. The study used a cross-sectional case-control design. Patients diagnosed with hypersensitivity to cow's milk came from a database of patients with this possible etiology collected in 2022 in the Allergy, Digestive and Pediatric Service of the Rio Hortega University Hospital. Patients with eosinophilic esophagitis come from databases collected in previous years. Informed consent and the approval of the Rio Hortega Research Ethics Committee were obtained (Ref. CEIm: 23-PI044, Protocol version 1.0,)

The objective was to determine whether donkey milk might be a safe alternative in patients sensitized to cow's milk, using routine diagnostic allergy techniques, oral challenge and molecular techniques using Component Resolved Diagnosis (CRD) and immunodetection.

We analyzed four groups of patients and controls:

- Patients with severe clinical symptoms: urticaria, dermatitis, asthma, digestive symptoms, or anaphylaxis related to cow's milk ingestion diagnosed and treated in 2022
- Healthy blood donor controls
- Patients allergic to grass pollens without digestive symptoms related to milk or food
- Patients with eosinophilic esophagitis related to milk

Calculation of the sample size: Accepting an alpha risk of 0.05 and a beta risk of 0.2 in a bilateral contrast, 48 subjects in each group were needed to detect a minimum difference of 8 between the two groups, assuming there were 4 groups and a standard deviation of 10. A follow-up loss rate of 20% was estimated.

In vivo tests

Skin tests were carried out using conventional techniques in the case of commercialized allergens: Prick-test against common pneumoallergens (grass, tree and weed pollen), mites, animal epithelia, fungal allergens and food, and with commercial extracts of cow, sheep and goat milk (ALK-Abelló, Madrid, Spain).

Prick tests were performed with twice-boiled donkey milk using the prick-by-prick technique

Epicutaneous tests with donkey milk at 0.01% in petrolatum using a standard battery of True-Test contact allergens (ALK-Abelló, Madrid)) (Roxall, Bilbao); readings were made at 48 and 96 hours.

Provocation tests. We followed the amended method of Dunlop et al. [6] and the considerations of the Adverse Reactions to Foods Committee Work Group Report [7]. We used twice boiled donkey

milk. The starting dose was 0.1 ml for the first lip application and a sublingual test at one hour. The progressive introduction pattern is shown Figure 1. We considered that provocation could continue when the prescribed dose was consumed without symptoms or the need for medication. After tolerance of each dose, the patient or family (in pediatric patients) were told to continue the dose for 3-5 days: the subsequent dose was given in the clinic with surveillance of possible reactions for seven hours.

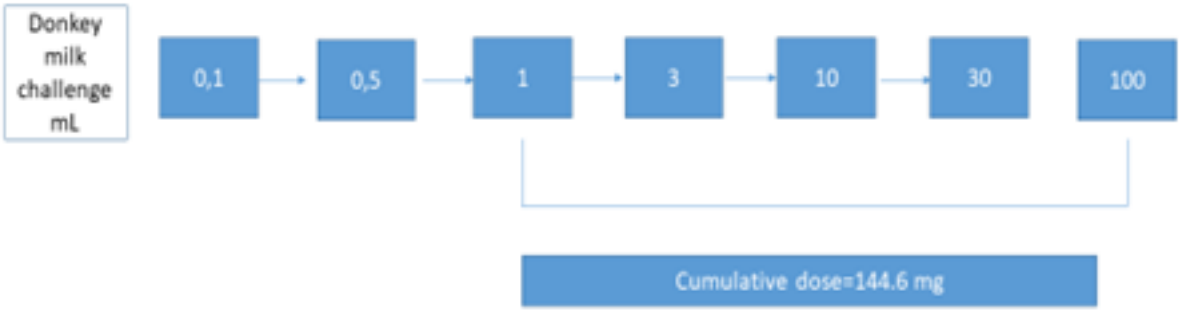

Figure 1: Open Challenge tests with donkey milk.

In vitro testing

Ten percent extracts were prepared with PBS and, after dialyzing, paper discs previously activated with BrCN were sensitized, as described by Ceska et al [8]. The quantity of proteins was determined according to Bradford's method [9] and was 7.96 mg/mL. Determination of specific IgE against ruminant milk (cow, sheep, goat) and camel milk was made using UniCAP, Thermofisher Uppsala, Sweden. Levels ≥0.35 kU/L were considered positive. Assessment of **specific IgE against donkey milk** was made using ImmunoCAP, Thermofisher, Uppsala, Sweden. The antigen binds to PDA by biotinylation, using Sander's method [10].

SDS PAGE/IgE-Western blot: Donkey and cow milk proteins were analyzed by electrophoresis in polyacrylamide gel with sodium dodecylsulfate (SDS-PAGE), according to Laemmli's method [11], in 15% polyacrylamide gels under reducing conditions. Proteins were visualized with bright blue staining of Coomassie R-250 and electrophoretically transferred to polyvinylidene difluoride (PVDF), Trans-Blot Turbo™ membranes, Bio-Rad, Hercules, CA, USA). Binding of the IgE antibody to allergens was analyzed by Western blot using sera from the groups of patients: A. typical allergic pathology, B. EoE. Human anti-IgE peroxidase conjugate (Southern Biotech, Birmingham, USA) and chemiluminescence detection reagents (Western Lightning® Plus-ECL, Perkin Elmer. Waltham, MA, USA) were added according to the manufacturer's instructions. IgE binding bands were identified using the Bio-Rad Diversity database program.

Molecular Analysis was made using CRD, ISAC Thermofisher, Uppsala, Sweden for 112 molecules of recombinant and native allergens, according to the manufacturer's instructions

Statistical Analysis

We collected clinical and demographic data from the 2032 patients with food hyper sensitivity detected in 2022. Of these, only the 83 patients who underwent cow milk hypersensitivity were included in the statistical analysis.

Between-group proportions of positives in the CRD tests and prick tests were compared using Fisher's exact test [12] followed by post-hoc pairwise test to determine which groups differed from the rest. To check if the proportions of positives in the provocation tests with donkey milk differed from those found with cow milk, an exact binomial test was used in each group. To compare IgE concentrations in the presence of different compounds (whole milk, α-lactalbumin, β-lactoglobulin, α-casein) between patient groups, Wilcoxon signed rank tests were performed [13]. Firstly, IgE concentration of patients allergic to cow's milk and patients with esophagitis were compared with IgE levels in healthy controls. Finally, IgE levels of both groups of patients were compared to check if the immunological response was different between them. All multiple comparisons were corrected using the Benjamini-Hochberg method [14]. Graphics were constructed using ggplot2 version 3.3.5 and reshape2 version 1.4.4 according of Wickham [15].

Results

In 2022, 7433 patients were diagnosed with hypersensitivity to allergens, of whom 2932 had proven food hypersensitivity. Of these, 83 (4.6%) had severe IgE-mediated symptoms after cow milk ingestion. The result analysis was also conducted in the 67 patients diagnosed with eosinophilic esophagitis, 50 patients with pollen allergy and 50 healthy controls.

Demographics

Milk sensitization mainly affected children (10.4±7.5 years) and males (60 males/23 females) (Table 1).

Results of skin tests and IgE

The results by patient groups for skin tests were significantly different (<0.001). The post-hoc test indicates that patients with milk allergy and eosinophilic esophagitis showed, in general, significantly-higher proportions of positives than the other groups. The proportion of positives between patients allergic to cow's milk and those with eosinophilic esophagitis also showed significant differences. Specific IgE in patients with eosinophilic esophagitis and in patients with cow's milk allergy are significantly positive in all cases. Overall, patients with anaphylaxis to cow's milk showed a higher concentration of specific IgE than patients with eosinophilic esophagitis, although no significant differences were found. The most relevant allergen in cow and donkey milk was casein <0.001. There was no positivity in the patch tests with donkey milk in whole patients.

Provocation with Cow and Donkey Milk

All clinical result including challenge test can see in Table 2. Patients 368 to 405 suffered from severe milk allergy, most of them anaphylaxis. Patients 1 to 406 suffered from eosinophilic esophagitis (in bold in Table 2). In six patients who had anaphylaxis after cow milk provocation, positivity was reached at 0,5 ml of donkey milk, with mild symptoms (ODS and/or urticaria). The remaining patients tolerated 100 ml of donkey milk without immediate or late reactions.

Table 1

	Esophagitis	**Healthy**	**Pollen allergy**	**Cow milk allergy**	**Sig.**
N	67	50	50	39	
Age	32.9 ± 17.5	31.4 ± 10.9	25.8 ± 10.3	10.4 ± 7.5	<0.001
Sex female (%)	21 (31.3%)	15 (30.0%)	23 (46.0%)	12 (30.8%)	0.271
Milk (%)	11 (16.4%)	1 (2.0%)	1 (2.0%)	38 (97.4%)	<0.001
Prick milk (%)	3 (4.5%)	0 (0.0%)	1 (2.0%)	34 (87.2%)	<0.001
Donkey milk challenge (%)	2 (3.0%)	0 (0.0%)	0 (0.0%)	6 (17.1%)	0.005
IgE alfalactoalbumin	2.4 ± 10.3	0.0 ± 0.0	0.02 ± 0.2	13.7 ± 27.6	<0.001
IgE betalactoglobulin	2.3 ± 11.3	0.0 ± 0.0	0.03 ± 0.2	18.0 ± 61.3	0.007
IgE caseíne	3.7 ± 15.2	0.0 ± 0.0	0.03 ± 0.2	33.6 ± 39.8	<0.001
IgE whole milk	4.0 ± 16.7	0.0 ± 0.0	0.03 ± 0.2	30.3 ± 35.0	<0.001

Table 2

P	sex	Age	Cli	Prick Cow milk	Prick Donkey milk	Cow milk challenge	Donkey milk challenge	IgE wholemilk	IgE alpha	IgE Beta	IgE casein	Ot
368	M	23	AN	+	-	+	ND	83.9	17.6	7.5	96.7	G
369	F	20	AN	+	+	+	+	100	19.5	8.5	100	G
370	M	8	AN	+	+	+	+	27.7	2.6	2.7	100	
371	F	20	AN	+	-	+	-	10.1	1.5	86	10.5	
372	F	14	AN	+	-	+	-	30.2	9.4	29	2.19	
373	F	16	D	+	-	+	-	59	6.8	1.3	59.6	
374	M	17	D	+	-	+	-	15	8.7	8.2	19	

375	M	13	AN	+	-	+	-	11	9	7.8	89	G
376	M	4	AN	+	-	+	-	10.6	1.4	1	4.68	
377	M	15	AN	+	+	+	+	38.4	38	42	63	
378	M	23	D	+	-	+	-	27	24	4	26	
379	F	13	AN	+	-	+	-	5.4	1.5	0	3.09	G
380	F	11	AN	+	-	+	ND	81	2	4	80	
381	M	2	AN	+	-	+	-	9	4	4	9	
382	M	5	D	+	+	+	+	100	48	3.81	100	
383	M	3	AN	-	-	+	-	7.6	59	0.4	0.76	
384	M	7	D	+	-	+	-	6.28	3.7	0.1	6.38	
385	M	2	AN	+	-	+	-	39.5	10.5	6.8	28.10	
386	M	2	AN	+	+	+	-	3.4	0.3	0.4	0.9	
387	M	7	AN	+	+	ND	ND	100	16.5	5.8	100	G
388	M	6	AN	+	-	+	-	5.7	2.3	0	5.4	
389	M	2	AN	+	-	+	-	4.9	0.9	4.6	0.7	
390	M	23	AN	+	+	+	-	10.5	1.4	1.1	10.1	
391	M	3	AN	+	-	+	-	0.5	0	0.3	0	
392	F	4	AN	+	+	+	+	29.8	22.6	1.9	7.6	G
393	F	15	AN	+	+	+	+	100	46.8	46	100	G
394	F	11	AN	+	-	+	-	1.6	0.3	0.2	0.81	
395	M	2	AN	+	+	+	-	4	3.5	3	2.7	
396	F	15	AN	+	+	+	-	6.4	3.5	3.4	4	G
397	F	23	AN	+	-	+	-	1.2	0.2	0.2	0.4	D
398	M	3	D	+	-	+	-	2.2	0.9	0.8	2.1	
399	M	10	A	+	-	+	-	0.9	1.2	1.1	1.9	
400	M	6	A	+	-	+	-	3	2.3	2.9	1	
402	M	2	A	+	-	+	-	7	5.8	8.6	0.3	
403	M	17	A	+	-	+	-	0.9	0.8	0.6	0.3	G
404	M	1	A	+	-	+	+	11	0.9	0.1	11.8	Egg
405	M	2	A	+	-	+	-	1	0.89	0.9	0.74	Egg
1	**F**	**20**	**EoE**	**+**	**-**	**+**	**-**	**4.49**	**6.65**	**0.9**	**0.86**	LTP
11	**F**	**19**	**EoE**	**+**	**-**	**+**	**-**	**10.1**	**1.50**	**0.86**	**10**	Bos G
12	**F**	**6**	**EoE**	**+**	**-**	**+**	**ND**	**100**	**76**	**89**	**92**	Egg
15	**F**	**70**	**EoE**	**+**	**-**	**+**	**-**	**4.5**	**6.2**	**2.5**	**4**	
29	**M**	**3**	**EoE**	**+**	**-**	**+**	**-**	**8.2**	**6.4**	**2.3**	**6.2**	

30	**M**	**13**	**AN**	+	**-**	+	**-**	**2.1**	**4.4**	**3**	**2.1**	GR
51	**F**	**19**	**EoE**	+	**-**	+	**-**	**8.9**	**2.6**	**2.6**	**3.8**	
55	**M**	**16**	**EoE**	+	**-**	+	**-**	**2.3**	**1.6**	**4.1**	**3**	LTP
57	**M**	**26**	**EoE**	+	**-**	+	**-**	**5.4**	**0.6**	**1.26**	**2**	
60	**F**	**26**	**EoE**	+	**-**	+	**-**	**1.2**	**1.2**	**2.1**	**4**	nuts
63	**F**	**30**	**EoE**	+	**-**	+	**-**	**0.9**	**0.8**	**2.1**	**3**	Prof
401	**F**	**21**	**EoE**	+	**-**	+	**-**	**3.88**	**5.61**	**-**	**3**	G
406	**M**	**16**	**EoE**	+	**-**	+	**-**	**3.4**	**2.3**	**2.5**	**3**	

In bold, patients suffered from eosinophilic esophagitis. +: positive test -: negative test ND: Not done. Symptoms: AN: anaphylaxis, D: severe digestive symptoms). EoE: Eosinophilic Esophagitis. Alpha: Bos d 4 Alpha-lactalbumin, Beta: Bos d 5 Beta-lactoglobulin, Casein: Bos d 8 Casein.Ot: others sensitization : GR: grass pollen. Prof: Profilin, LTP: lipid transfer proteins

Tables 1 and 2: Clinic and demographics data of included patients.

In total 33 allergic children introduced donkey milk into their diet without problems. Of the 67 patients with eosinophilic esophagitis, 13 accepted the provocation test. Finally, the donkey milk provocation test was completed in 12 patients with esophagitis, with no immediate symptoms. They were instructed to take 100 ml of donkey milk daily until the scheduled check-up in the digestive service, in which they underwent a clinical study and endoscopic study with biopsy, without observing any symptoms of worsening. According to the binomial tests there was a significant difference in the response to donkey milk provocation compared with the positive cow milk tests in patients allergic to milk and in patients with eosinophilic esophagitis (<0.003). In contrast, no differences in responses were found in pollen allergic or healthy patients (p=1).

CRD Molecular Results

The results by patient groups for the CRD molecular tests differed significantly ($p = 2.12 . 10^{-18}$). The molecular analysis detected more positive results to the different milk proteins than the prick (<0.007) and specific IgE by ImmunoCAP, and significantly ore I children<0.005. The most relevant allergen in both cow and donkey milk was casein <0.001. Other relevant allergens in patient allergic to milk were grass pollen and egg. The post-hoc test shows that patients with milk allergy and eosinophilic esophagitis had positive proportions to cow's milk molecules (Bos d 4 Alpha-lactalbumin, Bos d 5 Beta-lactoglobulin, Bos d 8 Casein and Bos d7 lactoferrin Transferrin) which were significantly greater than the other groups. However, the proportions of positives between patients allergic to cow's milk and those with eosinophilic esophagitis showed no significant differences.

Western Blot Results

Showed an allergenic profile of donkey milk similar to that of cow milk in all patients analyzed, being able to recognize proteins in common (caseins) in both types of milk; although clearly with less intensity in donkey milk See Figure 2.

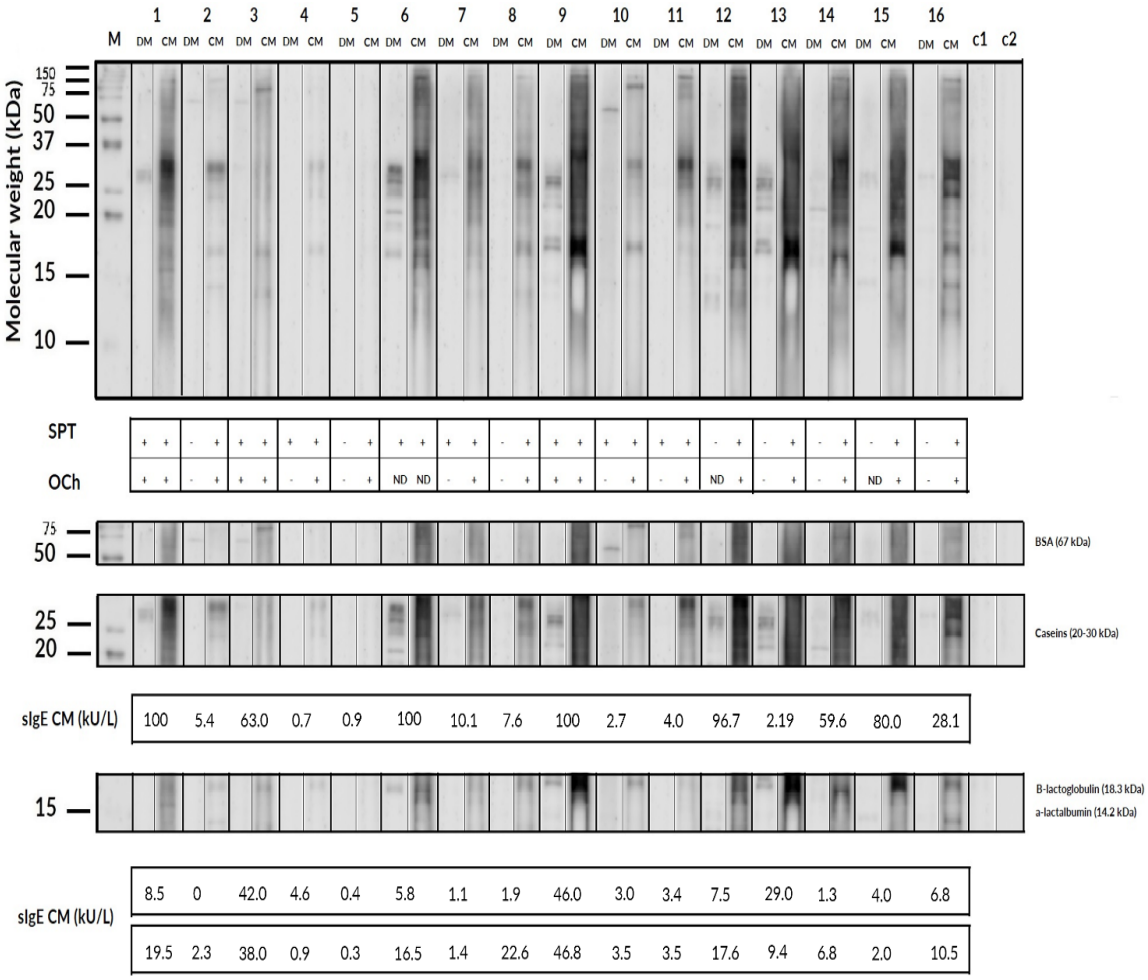

Figure 2: Analysis by Western blot of cow's milk extracts (bottom) and donkey milk (top), compared with patients with: A. typically allergic disease, B. EoE patients. Allergenic profile of donkey milk similar to that of cow milk in all patients analyzed, being able to recognize proteins in common (caseins) in both types of milk; although clearly with less intensity in donkey milk.

Discussion

Milk allergy is very common in children, both IgE-mediated and non-IgE mediated (including Food Protein-Induced Allergic Proctocolitis (FPIAP), food protein-induced enterocolitis syndrome, food protein-induced enteropathy, and Heiner syndrome (pulmonary hemosiderosis)) [16-21]. The most frequent manifestation is FPIAP but we only included patients that suffered from severe IgE-mediated milk allergy. We found that donkey milk was better tolerated than cow's milk, especially in patients with eosinophilic esophagitis. These results support the hypothesis that donkey milk could be a safe substitute for cow's milk in sensitive patients. The management of milk allergy has changed in recent years from an elimination diet to improve symptoms to active modulation of the immune system [18] with oral immunotherapy, which has been shown to be effective in many studies [19]. However, there is still no consensus on the different protocols of this technique [20]. Tolerance has been achieved with cow's milk baked in the form of cupcakes. However, adverse reactions are common, and positive provocation does not guarantee subsequent tolerance [21]. Specific IgE levels and casein testing have been found to be useful predictors of baked milk tolerance. Different cow's milk substitutes [21], plant drinks based on different formulas and plant derivatives have been tried, which do not necessarily address the nutritional requirements of infants and children.

Milk from other mammalian sources than the cow, such as goat, sheep, camel, donkey, and horse, has different protein composition profile, resulting in a potentially low cross-reactivity with cow's milk proteins. Recently, proteins from plant sources, such as potato, lentil, chickpeas, quinoa, in addition to soy and rice, have gained increased interest due to their climate friendly and vegan status as well as potential lower allergenicity [22]. Cow's milk allergy is the most prevalent type of food allergy among infants, affecting up to 3.8% of small children. In these cases, hypoallergenic infant formulas based on hydrolyzed cow's milk proteins commercially available for the management of cow´s milk allergy did not seem an alternative enduring for a lifetime. In contrast, donkey milk is a good source of all the nutrients, and it also contains enough vitamins to permit regular the growth of the growing child [23]. However, milk allergy does not only affect children, but can affect adults with eosinophilic esophagitis.

In our study, *In vivo* test showed that donkey milk was better tolerated than cow's milk, especially in patients with eosinophilic esophagitis. Furthermore, *in vitro* analysis manifested that although the protein profile of cow's milk and donkey's milk of the patients analyzed by SDS-PAGE was similar, allergic profile analyzed by Western blot did not identical. While the cow´s milk allergic profile showed intense IgE-recognition of different allergic proteins like BSA (67 kDa), caseins (20-30 kDa), β-lactoglobulin (18.3 kDa) and α-lactalbumin (14.2 kDa) in all the patients analyzed, when its compared with donkey´s milk allergic profile of the same patients, we observed clear differences: donkey milk caseins were recognized by majority of patients, but they did not recognized proteins in the lactoglobulin and lactalbumin areas. All this, added to the lower intensity of protein recognition in donkey milk by study patients, determinate the hypoallergenicity of donkey milk, making it a feasible and nutritive alternative for allergic patients. We suggest that donkey milk is safe and tolerable in patients aged >1 year with IgE-mediated milk allergy and in patients with eosinophilic esophagitis and a clinical response to milk. It also appears well tolerated in atopic patients. Donkey milk might be considered as a good alternative, given the benefit of its taste and properties compared with other formulas.

Conclusion

We found a lower IgE-mediated allergic hypersensitivity to donkey milk in patients allergic to cow's milk. Therefore, given its accessibility, donkey milk could be a feasible alternative for these patients. Donkey milk could be a feasible alternative for severe allergy and eosinophilic esophagitis due to IgE-mediated hypersensitivity to cow´s milk.

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