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Original article

Induction of sublingual immunotherapy to cow's milk (raw, pasteurized and sterilized) in Balb/c mice sensitized to beta-lactoglobulin

Induction d'une immunothérapie au lait de vache (cru, pasteurisé et stérilisé) chez des souris Balb/c sensibilisées à la bêta-lactoglobuline

S. Benhatchi *, S. Addou , H. Grar , Y. Benaïssa , O. Kheroua , D. Saidi

Laboratory of Physiology of Nutrition and Food Safety, Department of Biology, Faculty of Natural and Life Science, University of Oran1 Ahmed Ben Bella, 31000 Oran, Algeria

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Abstract

Objective. – Our study was conducted to improve the efficacy of cow's milk (CM), reducing the duration of treatment by using different types of cow's milk, including raw milk (RM), pasteurized milk (PM) and sterilized milk (SM).

Material and methods. – SDS-PAGE was performed to compare the protein composition of the 3 types of cow's milk (raw, pasteurized and sterilized). Allergenicity was evaluated in vitro in an Ussing chamber using intestinal mucosa of Balb/c mice immunized with bovine protein beta-lactoglobulin (β -Lg) and treated with these three different milks for 2 months. Histological examination of the intestinal lamina propria was performed to assess gut inflammation.

Results. – The electrophoretic profile clearly shows the absence of a BSA band in pasteurized and sterilized milk. A secretory response reflecting local intestinal anaphylaxis was evident in sensitized mice, as indicated by an increase in Isc ($P < 0.0001$). Sensitized mice also exhibited enhanced epithelial permeability as measured by conductance (G) ($P < 0.0001$). Histological study of the intestine revealed that after 95 days of treatment with SM, the villous structure was not affected; on the contrary, it presented a well-serrated villus with normal intraepithelial lymphocytes (IEL).

Conclusion. – After two months of treatment, the efficacy of ITSL with SM was confirmed while maintaining the same dose of allergen administered. However, these results remain to be confirmed in patients with cow's milk protein allergy.

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Keywords: Allergy; Sublingual immunotherapy; Raw milk; Pasteurized milk; Sterilized milk; Beta-lactoglobulin

Résumé

Objectif. – Notre travail vise à évaluer l'efficacité du lait de vache (CM), en réduisant la durée du traitement tout en utilisant différentes qualités de lait de vache dont le lait cru (RM), le lait pasteurisé (PM) et le lait stérilisé (SM).

Matériel et méthodes. – SDS-PAGE est faite pour comparer la composition protéique des 3 qualités du lait de vache (cru, pasteurisé et stérilisé). L'allergénicité est évaluée in vitro dans la chambre de Ussing sur la muqueuse intestinale de souris Balb/c immunisées avec des bêta-lactoglobuline de protéines bovines (β -Lg) et traitées avec ces trois laits différents pendant 2 mois. Un examen histologique de la lamina propria intestinale a été réalisé pour évaluer l'inflammation intestinale.

Résultats. – Le profil électrophorétique montre clairement l'absence de bande de BSA dans le lait pasteurisé et stérilisé. Une réponse sécrétoire reflétant l'anaphylaxie intestinale locale était évidente chez les souris sensibilisées, comme indiqué par une augmentation de l'Isc ($p < 0,0001$). Les souris sensibilisées ont également démontré une perméabilité épithéliale accrue mesurée par la conductance (G) ($p < 0,0001$). L'étude histologique de l'intestin révélée après 95 jours de traitement avec SM, la structure villositaire n'est pas affectée ; au contraire, il présente des villosités bien dentelées avec des lymphocytes intraépithéliaux normaux (LEL).

* Corresponding author.

E-mail address: benhatchi@hotmail.com (S. Benhatchi).

Conclusion. – En deux mois de traitement, l'efficacité de l'ITSL avec SM a été confirmée tout en gardant la même dose d'allergène administrée, il reste à confirmer ces résultats chez les patients présentant une allergie aux protéines du lait de vache.

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Mots clés : Allergie ; Immunothérapie sublinguale ; Lait cru ; Lait pasteurisé ; Lait stérilisé ; Bêta-lactoglobuline

1. Introduction

Cow's milk is ubiquitous in diets around the world and can constitute an important source of protein for young children.

Unfortunately, milk allergy is also one of the most common food allergies in childhood [1]. It affects from 2 to 6% of children, with the highest prevalence during the first year of life [2]. Onset of symptoms occurs most often during the first year of life when up to 7.5% of infants become affected by milk allergy [3]. Although in most cases the milk allergy is outgrown by adulthood [4], recent epidemiological data suggest slower rates of resolution and a higher rate of children with persistence of the disease into adolescence and even into adulthood [4–6]. Avoidance is currently the only approved therapy, and, although effective, avoidance diets can be difficult and may also put children at risk of nutritional deficiencies and impaired growth [5,6].

Nowak-Węgrzyn et al. have shown that these milks are less allergenic in baked goods than the semi-cooked or uncooked form due to the conformational changes in the allergen epitope associated with cooking at high temperatures.

In fact, most children allergic to milk in its raw form are able to tolerate milk in baked goods. There is emerging evidence that children with cow's milk allergy who are able to tolerate these allergens when cooked in baked foods are more likely to develop tolerance than those who are reactive to the allergen in its baked form [2,3]. As a result, a hypothesis has arisen that exposure to milk in baked goods may hasten the development of tolerance.

Several allergen-specific approaches, including oral immunotherapy (OIT), sublingual immunotherapy (SLIT), and epicutaneous immunotherapy (EPIT) with food allergens, have been pursued to treat food allergy [7]. Specific immunotherapy has been used for almost a century in allergic patients to redirect inappropriate immune responses [8].

Among the noninvasive routes used for the treatment of allergic diseases, Sublingual Immunotherapy (SLIT) is the most widely used. SLIT is an efficient desensitization method [9]. Recent data provide an explanation for its multiple mechanisms inducing both rapid and specific long-term allergen desensitization and immune tolerance with suppression of allergic inflammation in affected tissues [10].

The setting point of sublingual immunotherapy has opened up new horizons in terms of efficacy and tolerance. Our study aimed to improve the efficacy of cow's milk (CM), reducing the duration of treatment by using different types of cow's milk, including raw milk (RM), pasteurized milk (PM) and milk sterilized (SM) in β -Lg sensitized mice.

2. Materials and methods

2.1. Cow's milk preparation

Fresh cow's milk was collected from a local dairy farm located in Messerghin (Oran, north of Algeria). Milk was then transported in an icebox to our laboratory. For sample preparation, the milk was divided into: raw milk (RM), pasteurized milk [obtained by heat treatment at 85 °C for 5 minutes (min) then cooled with cold water] and sterilized milk (obtained by heat treatment at 110 °C for 10 min in an autoclave) [11].

2.2. SDS-PAGE

Twelve percent SDS-PAGE was performed under denaturing conditions using the Leammli method (1970). Proteins from the different milk samples (RM, PM, SM) were separated in an acrylamide gel and stained with a Coomassie Blue R250 kit containing reference proteins: Bovine Serum Albumin (BSA) (68 KDa "A-7638 5g Lot 79H7614"), Casein (24 KDa "Sigma, 9000-71-9, USA"), and β -Lg (18 KDa "Sigma, L-O130, USA").

2.3. Animals

All animal experiments described in this study complied with the current Algerian legislation covering the protection of animals. Three- to four-week-old female Balb/c mice purchased from the Pasteur Institute of Algiers and weighing (15.2 ± 0.25) were divided into 3 groups: a sensitized group ($n = 12$), a treated group ($n = 18$) and a control group ($n = 6$) (not sensitized and not treated). The sensitized group was divided into two subgroups: (1) sensitized mice sacrificed on day 35, (2) sensitized mice sacrificed on day 60, whereas the treated group was divided into three subgroups for sublingual treatment: (1) raw milk-treated mice, (2) pasteurized milk-treated mice, (3) sterilized milk-treated mice. The mice were fed a standard diet (local production in Bouzareah, Algiers) and given water ad libitum.

2.4. Immunization protocol

The mice were immunized on days 0, 14, 21 and 28 by intraperitoneal injection of 10 μ g β -Lg adsorbed on 2 mg of alum $Al(OH)_3$ in 0.1 mL of PBS [12].

2.5. Sublingual immunotherapy (SLIT)

At day 35, the mice received sublingual desensitization with 1 mL of cow's milk (raw, pasteurized and sterilized) under the tongue twice a week for 2 months [13].

The treatment was not to be given in a single dose but drop-wise during the day to ensure that each animal had fully absorbed the entire dose of 1 mL.

2.6. Ex vivo experiments (Ussing chamber assay)

The efficacy of sublingual immunotherapy (SLIT) with different qualities of milk (RM, PM, SM) was analyzed ex vivo in an Ussing chamber according to previously published protocols (Ussing and Zerahn, 1951). Briefly, the mice were anesthetized with sodium pentothal (5 mg/kg) and intestinal segments were removed. The small intestine was then opened along the mesenteric border and mounted as sheets in Ussing chambers. Both sides were filled with 5 mL of Ringer solution. After an equilibration period of 20 min, 60 µg/mL of β -Lg was added to the serosal side of the tissue. Changes in transepithelial potential difference (PD) and impulse amplitude of short-circuit current (Isc) induced by β -Lg were used for the calculation of tissue conductance (G) based on Ohm's law.

In order to evaluate tissue viability, glucose was added to the luminal bathing medium at a concentration of 10 mM at the end of each experiment.

2.7. Histology

Segments of jejunum adjacent to those mounted were processed for histological analysis. Tissues were fixed in 10 mM formalin, embedded in paraffin, cut into 3- to 5-µm-thick sections and subsequently stained with hematoxylin (Hould, 1984). As a marker of inflammation, villous length was measured using an optical microscope equipped with a micrometer (Optica Axiom 5000, Beijing, China).

2.8. Statistical analysis

Data are presented as mean $\pm$ standard error of mean (SEM). Comparisons between groups were performed using analysis of variance (ANOVA) or unpaired Student's *t*-test where appropriate. A *P*-value of <0.05 was considered statistically significant.

3. Results and discussion

This technique was carried out, in a first stage to ensure the presence of the main proteins constituting the cow's milk used (RM, PM, SM), and in a second stage to evaluate possible modification of the protein composition of the cow's milk following heat treatment.

Analysis of the deposit of raw cow's milk in well 2 and well 6 clearly showed the presence of intact protein bands comparable to reference proteins. These were as follows, in order of migration speed: BSA, caseins, β -Lg and α -Lac. Moreover, we noted the absence of BSA bands in pasteurized and sterilized milk (Fig. 1).

While caseins are very heat-resistant, serum proteins are less resistant to heat (O'Connell and Fox, 2000). The different serum proteins do not have the same resistance to heat treatment. The order of heat sensitivity is Ig > BSA > β -Lg > α -Lac [14].

The heating of serum proteins leads to the opening of secondary and tertiary structures that allow the aggregation of these proteins with each other or with casein micelles [15]. Denaturation of serum proteins results in the depletion of their polypeptides, exposing residues usually hidden within the globular structure [16].

In the case of β -Lg, this intramolecular process exposes the hydrophobic bond and exposes the free thiol group at the same time, as well as highly reactive disulfide bridges [17]. Denaturation of β -Lg is reversible until aggregation takes place, while denaturation of α -La is reversible up to 85 °C [18].

Heat changes the spatial configuration of the proteins without harming the polypeptide sequence (primary structure). This denaturation begins at a temperature of 80 °C and is partially

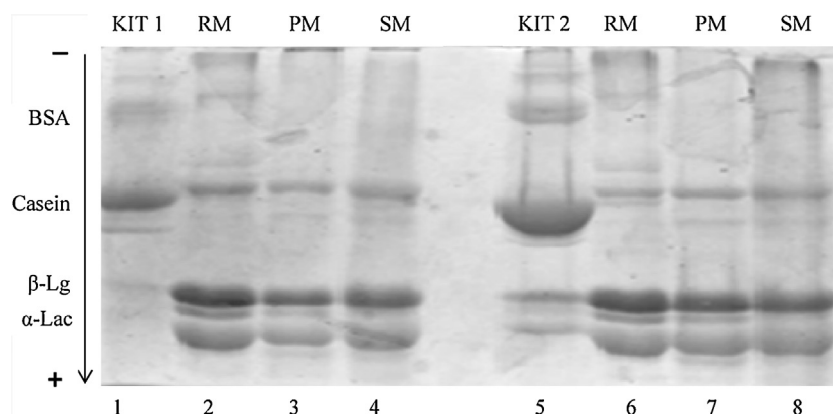


Fig. 1. Electrophoresis on polyacrylamide-SDS gel (12%) of the cow's milk. Well 1: proteic kit beta mercaptoethanol. Well 5: protein kit without beta mercaptoethanol. Well 2, Well 3 and Well 4: cow's milk (RM, PM, SM) respectively with beta mercaptoethanol. Well 6, Well 7 and Well 8: cow's milk (RM, PM, SM) respectively without beta mercaptoethanol. The first well corresponded to a marker kit constituted by a mixture of pure bovine proteins, containing BSA (68,000 Da), casein (24,000 Da), β -Lg (18,000 Da) and α -Lac (14,000 Da).

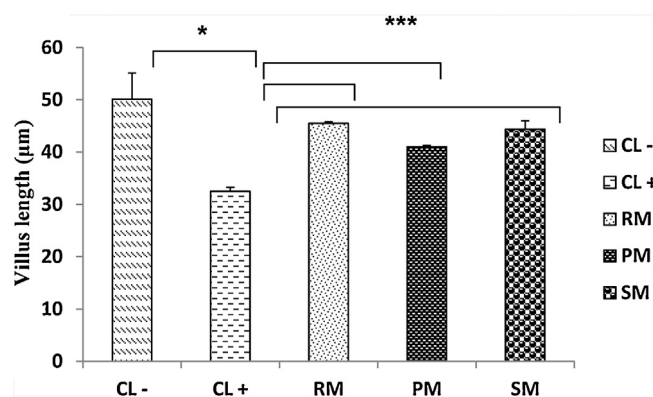


Fig. 2. Evaluation of the villous height in mice immunized with β -Lg and treated with cow's milk (RM, PM, SM), CL–: negative control; T+: positive control.

Table 1
Villous height in mice immunized with β -Lg and treated with cow's milk (RM, PM, SM).

Mouse treatment	n	Villus length (µm)	Significance
CL–	10	50.12 ± 12.16	–
CL+	10	32.50 ± 1.81	P<0.06
RM	10	45.5 ± 0.82	P<0.0001
PM	10	41.02 ± 0.52	P<0.002
SM	10	44.36 ± 3.90	P<0.004

CL–: negative control; T+: positive control.

reversible. Casein is resistant to thermal effects: it coagulates only after heating for one hour at 125 °C.

Pasteurization denatures 10 to 20% of whey proteins. Finally, sterilization denatures them too, but not totally. The quality of serum proteins therefore varies according to the heat treatment undergone, and pasteurized and sterilized milks are not equivalent in this respect.

There was a significant decrease in villous height in the immunized mice (positive controls), while in immunized and treated mice, there was regeneration of the intestinal mucosa, hence the increase in villous height (Fig. 2, Table 1).

The microscopic images revealed regular villi with unstratified epithelium and polymorphous lamina propria in the negative group. However, the β -Lg sensitized group demonstrated clear atrophy with distortion of epithelium architecture and severe intraepithelial lymphocyte (LIE) infiltration. Treatment with RM and SM clearly reduced these microscopic lesions caused by β -Lg (Fig. 3).

Morphological changes in the epithelium due to the immunologic reaction were underscored by histological results demonstrating villi atrophy and a greater increase of mucosal inflammatory cells in jejunum. Several published studies have shown that food allergy in mice is characterized by villous atrophy and goblet-cell hyperplasia, as well as infiltration of IgE-positive mast cells causing degranulation in the jejunum and increased histamine release [19].

However, mice sensitized by β -Lg and treated with RM and SM gave a better result than those treated with PM: food processing alters proteins and can make them more or less allergenic.

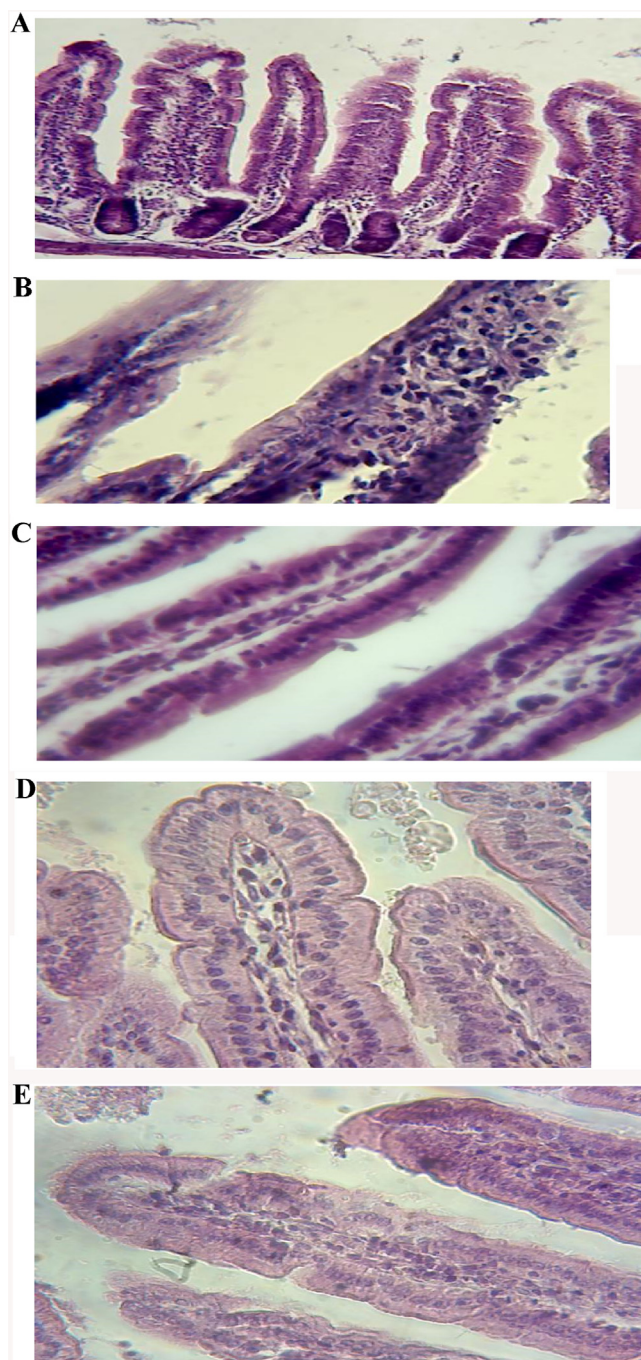


Fig. 3. Light microscopy showing intestinal villi stained with hematoxylin-eosin. Jejunal tissues were obtained from different group of mice (G $\times$ 10).

For example, conformational epitopes can be denatured by heat, while linear epitopes are likely to stay intact [20].

According to the studies by Nowak-Węgrzyn et al., after heating milk at 100 °C for 10 minutes, a substantial reduction of allergenicity was noted. Their studies evaluating the effects of heating (time and temperature) and food matrix on allergenicity of casein and whey proteins are ongoing.

The typical responses of the intestinal epithelium to allergens are an increase in watery diarrhea due to stimulation of chloride secretion, and increased protein inward permeability from lumen to blood [21].

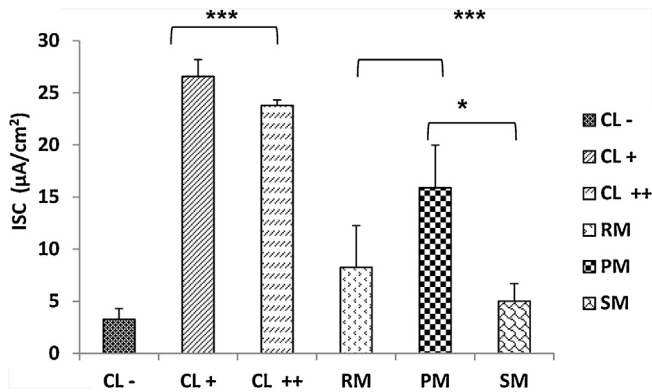


Fig. 4. Effect of β -Lg on the short-circuit current (Isc) in Ussing chamber measured in mouse jejunal fragments sensitized intraperitoneally to the β -Lg.

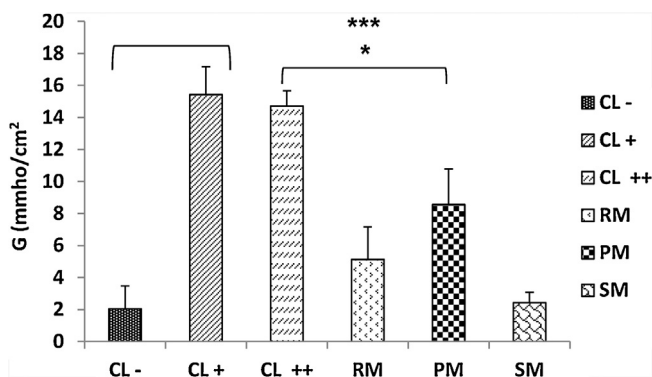


Fig. 5. Effect of β -Lg on the conductance (G) in Ussing chamber measured in mouse jejuna fragments sensitized intraperitoneally to the β -Lg.

Isc, transepithelial voltage potential (Vt) and conductance (G) measurements in Ussing chamber studies have shown that electrogenic chloride (Cl) secretion is not only important for normal digestive physiology but is also a marker of enterotoxic and inflammation-mediated secretory diarrhea [22].

Compared with naive mice, Ussing chambered-intestinal segments from sensitized mice (CL+) responded to serosal β -Lg challenge with an increase in Isc (Δ Isc: 26.56 ± 3.98 mA/cm²) ($P < 0.0001$) (Fig. 4).

These results confirm our previous studies on jejunal responses to bovine b-Lg in infants during the active phase of cow milk allergy [23] and in animal models [24] showing that challenge with β -Lg of jejunal fragments induced a significant increase in Isc due to electrogenic chloride secretion.

In sensitized mice, the increased Isc response was correlated with a gradual time-dependent increase in tissue conductance, which is a measure of the integrity of tight junctions (Fig. 5). This suggested an impairment of intercellular junctions of intestinal epithelium and implies the occurrence of a local anaphylactic reaction when β -Lg interacts as an allergen with the intestinal immune system of β -Lg sensitized mice.

Isc tissue of control animals did not vary after stimulation of the latter with sensitizing protein (β -Lg). These results therefore suggest the existence of a local anaphylactic reaction due to the direct interaction of sensitizing antibodies with the immune system associated with the digestive tract.

There was a significant increase in Isc after deposition of the antigen on intestinal mucosa sensitized and treated with pasteurized milk (Δ Isc: 15.88 ± 10.01 mA/cm²; $P < 0.01$). As shown in the figure, Isc in mice treated with raw milk (Δ Isc: 8.24 ± 9.79 mA/cm²; $P < 0.08$) and sterilized milk (5.01 ± 4.09 mA/cm²; $P < 0.0001$) was decreased after the deposition of β -Lg compared to the group of sensitized mice (CL+) (Fig. 4).

The increased Isc response was correlated with gradual time-dependent increased tissue conductance, which is a measure of the integrity of tight junctions.

This suggested impairment of the intercellular junctions of intestinal epithelium and implies the occurrence of a local anaphylactic reaction when β -Lg interacts as an allergen with the intestinal immune system of β -Lg sensitized mice.

Several studies have reached similar conclusions regarding the effect of β -Lg on epithelium conductance, concluding that immunization alters the tight junction and increases the paracellular permeability of the intestinal epithelium [19,25].

However, elevated conductance decreased in mice treated with raw and sterilized milk, indicating inhibition of non-specific epithelial permeability. Casein in milk is heat-stable, while α -lactalbumin and β -lactoglobulin in milk is heat-labile [26].

Studies have shown that milk-allergic patients with predominant IgE binding to linear epitopes of casein are more likely to have persistent milk allergy [27].

Using serum from milk-allergic patients, Bloom et al. demonstrated that heating decreased IgE binding to milk proteins, and that all baked milk-reactive patients retained IgE binding to casein, regardless of heating time [26]. Bloom et al. used SDS-PAGE to compare IgE binding of baked milk and regular milk heated under the same conditions and found that allergenicity decreased in the presence of wheat, even to heat-stable casein proteins [26].

Exposing milk proteins to high temperatures through baking reduces allergenicity by destroying the conformational epitopes [12,13]. Analysis of serum samples of children with persistent cow's milk allergy demonstrates significantly higher ratios of IgE to linear versus conformational epitopes compared to children who have achieved tolerance [14]. In addition to heating, milk protein allergenicity may also be decreased through interaction with other food proteins in a complex food, such as in the wheat matrix in a baked muffin or cupcake [9].

The effect of cooking milk proteins in a food matrix was highlighted recently when proteins extracted from muffins and waffles showed that Bos D 8 was still present after cooking in the wheat and rice matrix [28]. In contrast, whey proteins such as Bos d 4 and Bos d 5 were denatured within 30 min of heat treatment and were no longer present in the waffle and muffin.

New methodologies are needed to define milk allergy phenotypes and to assist in determining the likelihood of resolution of the allergy. The allergenicity of major proteins has been difficult to determine due to the complexities surrounding heat treatment and matrix modification on many of the proteins. Further understanding of the molecular basis of the different milk allergy phenotypes may provide valuable opportunities to better differentiate prognosis for allergic infants and children. Given

that dietary restrictions are potentially nutrient-deficient and can adversely impact the quality of life of children and their families, it is reasonable to consider liberalizing inclusion of baked milk in the diet of milk-allergic children respectively following confirmation of tolerance to these foods in baked form. Milk allergy has a good prognosis and we have presented observational evidence to support the idea that children who are able to tolerate these foods in their baked form are more likely to develop tolerance. However, there is insufficient evidence to conclude that the natural history of milk allergy will be hastened by dietary inclusion of these foods in the baked form [29].

4. Conclusion

Studies have shown the effectiveness of sublingual immunotherapy in introducing cow's milk into the diet of children allergic to cow's milk. Published data indicate that heating decreases but does not completely eliminate milk allergenicity.

However, more research is needed to conclude that heated milk speeds up and reduces treatment time, but above all, to define the humoral and cellular mechanisms associated with non-reactivity to heated milk and the impact of heated milk ingestion on the development of milk tolerance.

Disclosure of interest

The authors declare that they have no competing interest.

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