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Efficacy of donkey's milk in treating highly problematic cow's milk allergic children: An *in vivo* and *in vitro* study

Giovanna Monti[1], Enrico Bertino[2], Maria Cristina Muratore[1], Alessandra Coscia[2], Francesco Cresi[1], Leandra Silvestro[1], Claudio Fabris[2], Donatella Fortunato[3], Maria Gabriella Giuffrida[3] and Amedeo Conti[3]

[1]Department of Paediatric and Adolescence Science, Regina Margherita Children's Hospital, Turin, Italy, [2]Neonatal Unit, Department of Paediatric and Adolescence Science, University of Turin, Italy, [3]CNR-Institute of Science of Food Production, Turin, Italy

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Successful therapy in cow's milk protein allergy rests on completely eliminating cow's milk proteins from the child's diet: it is thus necessary to provide a replacement food. This prospective study investigated tolerance of donkey's milk in a population of 46 selected children with cow's milk protein allergy, for whom it was not possible to use any cow's milk substitute. Thirty-eight children (82.6%) liked and tolerated donkey's milk at the challenge and for the entire duration of follow-up. Catch-up growth was observed in all subjects with growth deficit during cow's milk proteins challenge. The degree of cross-reactivity of immunoglobulin E (IgE) with donkey's milk proteins was very weak and aspecific. Donkey's milk was found to be a valid alternative to both IgE-mediated and non-IgE-mediated cow's milk proteins allergy, including in terms of palatability and weight-height gain.

Key words: donkey's milk; cow's milk allergy; alternative milk substitutes

Enrico Bertino, Department of Paediatric and Adolescence Science, University of Turin, Piazza Polonia 94, 10126 Turin, Italy
Tel.: +390 113 135775
E-mail: enrico.bertino@unito.it

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Cow's milk protein allergy (CMPA) chiefly occurs in childhood, involving approximately 3% of children below the age of 3 (1). Successful therapy depends on completely eliminating cow's milk proteins (CMP) from the child's diet. Ideally, the replacement food should be hypo- or anallergenic, non-cross-reactive with cow's milk (CM), nutritionally adequate and palatable – the latter being fundamental in view of the young age of these patients.

Extensively hydrolysed formulas (eHF), recommended as first choice for CMPA treatment by the European Society for Paediatric Allergology and Clinical Immunology (ESPACI), the European Society for Paediatric Gastroenterology, Hepatology and Nutrition (ESPGHAN) (2) and the American Academy of Pediatrics (AAP) (3), are not however tolerated by all these patients (4); may also contain residual allergenic epitopes (5, 6) and all present poor palatability. Only pure amino-acid formulas (AAF) are considered to be non-allergenic, but their use is hampered by their unpleasant bitter taste (4). Furthermore, studies on the nutritional adequacy of eHF and AAF have produced contradictory results (7–10).

Although the palatability of soy-protein-based formulas (SF) is moderate and they are safe and effective alternatives providing appropriate nutrition (11), they are not generally recommended for the initial treatment of infants and young children with CMPA, in whom soy protein allergy has been reported with frequencies between 17% and 47% (2, 4); SF are also contraindicated for the treatment of infants with some forms of non-immunoglobulin E (IgE)-associated gastrointestinal CMPA (3).

The possibility of using milk from other mammalian species for infants and young children with CMPA has been examined: goat's and sheep's milks are contraindicated as their proteins have shown extensive cross-reactivity with CMP both *in vitro* and *in vivo* (11–13). Mare's milk appears to be more promising, as in

composition it is much closer to human milk (HM) than to CM (11, 14) and it has been found to be tolerated by some children with severe IgE-mediated CMPA (15); however, its availability is limited and collection is difficult. Donkey's milk (DM) is equally similar to HM (11, 14, 16) and more readily available; it has successfully been used in two clinical studies, respectively on 9 (17) and on 21 (18) children with CMPA, and found to provide nutritional adequacy and good palatability.

As reported in a recent review by Muraro et al. (11), extensive clinical trials are needed on the safety profile of any alternative mammal-derived milk. The aim of this prospective study was to investigate tolerance *in vivo* and *in vitro*, palatability and nutritional adequacy of DM in a population of 46 selected infants and children with CMPA, for whom it was not possible to use any available CM substitute.

Materials and methods

Study subjects and design

Between 1 January 2003 and 31 January 2004, 46 children were recruited (24 boys; age range 12–149 months, median 24 months, mean 36 months) with proven CMPA, for whom maternal milk was not available and no available CM substitute (SF, eHF or AAF) could be used.

Age range at first observation was 1–146 months, median 12.5 months. Symptoms at first observation were cutaneous (atopic dermatitis, AD, 37/46; urticaria/angioedema, 2/46) and/or gastrointestinal (GI – 30/46); in particular, 19/46 had more severe GI symptoms (4/46 eosinophilic esophagitis or gastroenterocolitis, 7/46 dietary protein enteropathy, 7/46 gastroesophageal reflux disease and 1/46 dietary protein-induced enterocolitis); 11/46 children showed poor weight gain, in all cases except one associated with AD. Five children also had suffered prior anaphylaxis because of CMPA.

Unless contraindicated, the diagnosis of CMPA was made on the basis of a CMP elimination diet (2–4 weeks), followed by double-blind, placebo-controlled food challenge (DBPCFC). Before food challenge (FC), skin tests were also performed for CM, and CMP-specific IgEs were determined.

Between first observation and recruitment into the study, 35 of the 46 children (76%) presented a soy allergy, confirmed by positive DBPCFC. The remaining 11 already at first observation presented GI symptoms for which it was inappropriate to use SF as a replacement for CM. SF could therefore not be used as a replacement for CM for any of the 46 infants at the time of recruitment into the study, as likewise it was not possible to use either eHF or AAF: seven of the 46 children (15%) did not tolerate eHF and refused ingestion of AAF, and the remaining 39 categorically and systematically refused to take either eHF or AAF. Thirty-five children (76%) were also allergic to other foods (mainly wheat and egg). It was thus proposed to the parents of these children that they participate in the study using DM to replace CM. Ethical approval was obtained from the local Review Board, and informed parental consent was given. DM was from approximately 500 female donkeys raised outdoors and fed exclusively with organic foodstuffs.

Skin tests

CM or DM skin prick tests (SPTs) were performed before starting CM or DM challenge, respectively with fresh cow's milk containing 3.5% of fat or with fresh DM, as described elsewhere (19). Histamine 10 mg/ml was used as positive control and normal saline as negative control.

Skin wheal diameters (mm) in response to histamine and to CM/DM were measured after 15 min and the SPT was assessed as positive if the wheal was ≥3 mm without reaction of negative control.

Determination of specific IgE

Serum levels of alpha-lactalbumin, beta-lactoglobulin and casein-specific IgE (sIgE) were determined by the automated Pharmacia CAP system FEIA (Pharmacia & Upjohn Diagnostics, Sweden) on blood samples taken during the SPTs; the cut-off point for positivity was set at 0.35 KU/l.

Food challenge with CM

CMPA was diagnosed by DBPCFC (following Carroccio et al.) (20), performed in a day-hospital setting, administering fresh CM or CM formula (150–210 ml depending on age) mixed with pear juice in equal parts, starting with one drop placed on the inner side of the lower lip, and continuing with six increasing doses (1, 3, 5, 10, 25 ml, final dose) at 15-min intervals, unless symptoms occurred. Diluted pear juice was used as placebo. All patients were SPT-negative for pear juice. After the last administration, the children were kept under observation for at least

6 h and then discharged if no reactions had appeared. The patients continued the challenge at home, with opaque bottles coded A or B by a member of the hospital medical staff not involved in this study. The challenge period was 8 days for both fresh/formula CM and placebo. The parents were told how to recognize food allergy (FA) reactions; they were asked to record any clinical symptoms during this period and to call a FA Service doctor and return the child to hospital if they appeared. The challenge was stopped if a clinical reaction occurred. All children were re-examined in hospital in the case of any adverse reaction and/or at the end of the total challenge period.

Food challenge with DM

The DBPCFC to DM was performed as described for CM, using fresh DM after heating to 70 ° for 2 min. The FC for DM was performed during a period of 0.5–3 months after the last FC for CM. In case of clinical tolerance after the completed FC period, DM was included into the child's diet, which was appropriately balanced depending on requirements by age by our dietician.

Follow-up

The study design included follow-up in the form of clinical check-up and auxological evaluation, at DM food challenge and after 1 month (T_1), 2–3 months (T_2), 4–6 (T_3), 7–12 (T_4), 13–18 (T_5) and 18–24 months (T_6) of DM consumption. The last clinical and auxological evaluation of each patient was defined as T_{end} and corresponded to the moment in which the child stopped ingesting DM and thus left the study. Auxological evaluation considered weight, body length for children up to 2 years and stature thereafter. Weight was measured with electronic integrating scales (SECA 757; Hamburg, Germany: precision ± 1.0 g). Supine length was measured with a Harpenden infantometer and stature was measured with a Harpenden stadiometer. Measuring techniques were those suggested by Cameron (21). The parents were asked to keep a food diary, with particular reference to daily DM consumption. At each clinical and auxological check-up, compliance with DM ingestion was verified and the quantity of DM consumed by each child was noted.

Statistical analysis

Z-scores of weight (WA) and length/stature (LA) for age were calculated from the formula $Z = x - |X| / |$ s.d.$|$, taking the Gardner and Pearson growth curves as reference for children up to 24 months and the Tanner curves after 2 yr of age. Z-score values obtained between check-ups were analysed with the paired t-test. Significance was established with the paired t-tests, with $p < 0.05$ as cut-off. Statistical analysis was performed with the Statä 5.5 software (StatSoft, Inc, Tulsa, OK, USA).

Preparation of allergen extract

CM and DM samples were skimmed by centrifugation at 2000 g for 30 min at 4 °C. Protein concentration was determined using the bicinchoninic acid (BCA-Kit) (Pierce, Rockford, IL, USA).

1D electrophoresis

Skimmed milk was diluted in NuPage lithium dodecyl sulphate (LDS) Sample Buffer (Invitrogen, Carlsbad, CA, USA) to a concentration of 10 μg/cm and heated for 10′ at 70 °C. Proteins were then separated according to their molecular weights on NuPAGE ZOOM gels 12% (Invitrogen), using MOPS-SDS as running buffer, 200 V constant. Gels were either stained with Coomassie Colloidal Blue or electroblotted onto 0.2-μm nitrocellulose (Sigma, St. Louis, MO, USA) in a semi-wet blot module (Invitrogen) and reversibly stained with Red Ponceau.

Immunoblotting

In all subjects, the degree of cross-reactivity of the sIgE for CMP with DM proteins (DMP) was evaluated by immunoblotting as described elsewhere (22). Sera of the 46 patients were obtained when taking samples for radioallergosorbent test (RAST). Sera of children with AD without CMPA were used as controls.

Results

Twenty-nine children (63%) were found to be SPT positive to CM with wheal diameters ranging from 3 to 18 mm. Positive (≥0.35 KU/l) sIgE determinations for CMP were found in 30 children (in 27 for casein, in 22 for alpha-lactalbumin and in 26 for beta-lactoglobulin). Thirty-three children, with SPT- and/or RAST-positive for CMP, were found to have IgE-mediated CMPA and the remaining 13 non-IgE-mediated CMPA. These latter all presented GI symptoms (with or without AD) at the CMFC, as they had at the first observation.

As patients with a history of anaphylaxis should not be challenged, CMPA was confirmed with DBPCFC in 41 of the 46 children. Clinical symptoms at the last CMFC, performed before enrolment (age range 10–146 months, median 23 months, mean 35 months), were immediate (within 1 h) in 16/41 children, early (between 1 and 6 h) in 15/41 and late (after 6 h) in 18/41.

Among immediate symptoms, skin reactions prevailed (14/16, in the form of urticaria and/or angioedema), followed by respiratory (6/16, of which two were laryngeal edema) and GI symptoms (5/16, mostly vomiting). Among early reactions, GI clearly prevailed (15/15), followed by skin (5/15) and respiratory reactions (2/15, of which one was asthma). The late reactions were nearly always GI (17/18) and/or re-exacerbation of AD (10/18).

Eight children (17.4%) were found to be positive at the DM challenge, whereas the remaining 38 (82.6%) both liked and tolerated DM at the challenge and for the entire duration of follow-up. DM consumption in the 38 children was for 1–30 months (median 8 months, mean 8.8 months).

DM was tolerated by all 13 children with non-IgE-mediated CMPA and by 26 of the 33 children (78.8%) with IgE-mediated CMPA. In particular, only one of the five children (20%) with prior anaphylaxis to CM tolerated DM.

Of the 11 children who were intolerant to eHF, only one was also intolerant to DM. Only five (62.5%) of the eight children who were positive at the DM challenge were sensitized to DM (SPT positive, with wheal diameters of 7–35 mm) and all five presented immediate reactions to the challenge, none of which were life-threatening (5/5 positivity at the labial challenge, associated in 2/5 with urticaria/angioedema). The remaining three were SPT-negative to DM and two presented slightly early or immediate reactions (vomiting and/or diarrhoea) and one late reaction, consisting in re-exacerbation of eczema, accompanied by diarrhoea and abdominal pain, after 7 days from DBPCFC. Finally, in three cases the FC for DM was negative, despite the fact that the SPT to DM had been positive. In none of the three cases was the DM wheal diameter above 5 mm.

Growth-related data

Thirty-three children (18 boys) regularly and uninterruptedly ingested DM (200–500 ml/day depending on age) for periods of time varying between 2 and 30 months (mean 10 months, median 8 months); the duration of follow-up enabled statistical elaboration to be applied to growth figures for these cases.

At enrolment (T_0), the median age was 24 (12–149) months, the median weight was 11.250 (8–40.500) kg with a mean weight of 13.652 ± 6.500 and the median length/stature was 86 (72–157.4) cm with a mean length/stature of 92.48 ± 18.93 cm.

At T_0, the mean Z-score for weight was −0.85 ± 0.95; four patients (nos 2, 10, 43 and 46) had a weight Z-score between −2 and −3 s.d. At T_0 the mean length/stature Z-score was 0.04 ± 1.09; no patient had a Z-score for length/stature below −2 s.d.

At T_{end} the mean weight Z-score was −0.68 ± 0.85 and the mean length/stature Z-score was 0.28 ± 0.96. The mean Z-score was higher at T_{end} vs. T_0 for both weight ($\Delta T_0 - - T_{end}$ 0.17 ± 0.41, $p = 0.013$) and length/stature ($\Delta T_0 - - T_{end}$ 0.25 ± 0.53, $p = 0.006$).

The mean Z-score variations for weight and for length/stature between T_0 and each follow-up were evaluated. The Z-scores showed a growth trend with significant weight gain at T_1, T_3, T_4 and T_5 vs. T_0, whereas length/stature gains were significant at T_1 and at T_3 vs. T_0 (Table 1). All patients with Z-score for weight at T_0 below −2 s.d. showed a progressive increase in Z-score during the first three months of treatment, between T_0 and T_1 (−2.34 vs. −2.16, $p = 0.001$) and between T_1 and T_2 (−2.16 vs. −2.00, $p = 0.048$).

Table 1. Temporal Z-score variations in weight and length/stature

	T_1	T_2	T_3	T_4	T_5	T_6
Time from T_0 (months)	1	2–3	3–6	7–12	13–18	19–24
Cases (n)	30	27	23	19	7	4
Weight (ΔZ-score)	0.06 (0.16)*	0.07 (0.28)	0.18 (0.31)*	0.18 (0.42)*	0.37 (0.34)*	0.37 (0.79)
Length/stature (ΔZ-score)	0.11 (0.29)*	0.14 (0.46)	0.26 (0.52)*	0.17 (0.46)	0.13 (0.51)	0.25 (0.47)

Data presented as mean (s.d.).
*$p < 0.05$ compared with Z-score at T_0.

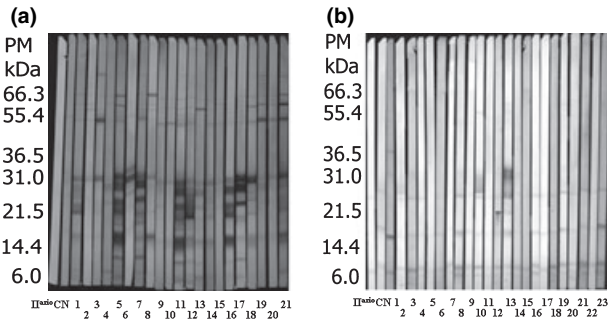

Fig. 1. Panel A: 1-D SDS-PAGE immunoblotting screening of 21 out of the 46 patients' sera for cow's milk protein-binding specific immunoglobulin E (sIgE). Panel B: 1-D SDS-PAGE immunoblotting screening of 23 out of the 46 patients' sera for donkey's milk protein-binding sIgE. Patients 17–21 did not tolerate donkey's milk. Same numbers in the two panels refer to the same patients' sera. II, secondary Ab alone; CN, negative control.

Immunoblotting

Fig. 1 – Panel A – shows the specific binding to CMP separated by 1D-SDS PAGE of IgE from the sera of 23 out of the 46 CMP allergic patients. Specific individual IgE-binding profile is visible, as expected. Fig. 1 – Panel B – shows a weak aspecific IgE binding to DMP separated in the same way as CMP for all the patients, regardless of positive or negative outcome of the oral DM challenge.

Discussion

This is the first prospective study investigating *in vivo* and *in vitro* efficacy of DM in children aged 12 months or above with problematic CMPA, for whom it is of vital importance to identify a replacement foodstuff for CM. All children in the study group (46 children) had multiple food allergies (MFA), and thus were subjected to very restrictive diets; maternal milk was not available and neither could use SF; some were intolerant to eHF and all systematically and categorically refused eHF and AAF, owing to their poor palatability.

In our patients, DM was found to be a valid alternative in terms of clinical tolerability, which was high at the FC (38/46, 82.6%), taking into account that these were all children allergic to numerous foods (mainly soy, wheat, egg and fish).

The AAP guidelines (3) establish that, in order to label an infant formula 'hypoallergenic for treatment' of CMPA, it must be tolerated clinically by at least 90% of subjects with CMPA. However, it must be remembered that the children in our study are not a representative sample of all children with CMPA, but are a selected sample.

The high tolerability of DM even in our patients intolerant to eHF (10/11, 91%) is of note, and in agreement with observations by Iacono et al. (17) and Carroccio et al. (18).

In a recent review (11), Muraro et al. suggest that DM can probably be considered a possible substitute for CM in children with severe IgE-mediated CMPA. In our study, DM was tolerated by 78.8% (26/33) of the children with IgE-mediated CMPA. However, of the patients with prior anaphylaxis to CM, only one tolerated DM at the challenge; the other four children all presented positivity already at the labial challenge (LC), which in two cases was followed by the onset of systemic reactions. Thus, although no definitive conclusions may be drawn with regard to the entire population of children with prior anaphylaxis to CM, the use of DM in these subjects should be contemplated with considerable caution.

DM was tolerated by all our patients with non-IgE-mediated CMPA. It is important to stress that this subgroup of patients consisted of children with GI symptoms (with or without AD) of CMPA, most of whom at first observation presented a malabsorption syndrome.

As to the predictivity of the SPT to DM with regard to the outcome of the DM oral challenge, a cut-off at and above which the challenge was always positive was identified. In our patients, when DM wheal diameter was ≥7 mm, the result of FC was always positive, with immediate reaction. Positive FC wes observed even in the absence of a wheal, but with reactions that were either late or immediate-early but of slight extent. The FC with DM should always be preceded by a SPT and might be superfluous for wheal diameters equal to or above the cut-off of 7 mm.

DM was found to be a valid alternative including from the standpoint of palatability: all our patients immediately found it acceptable. The patients recruited for this study were particularly demanding with regard to the palatability of foods, presumably because of their age. Some of them, who had reached our observation before 12 months of age, after ingesting eHF or AAF for months or years in the end rejected them. For others, on the contrary, the diagnosis of CMPA was made at an age such that the unpleasant taste of these formulas compromised their use right from the start. Most of the children already at enrolment were on restrictive and monotonous diets owing to MFA: this made it even more important to find a food that could replace CM, which was not only valid from the nutritional standpoint, but whose flavour and appearance were attractive to the child.

Our data show an adequate increase in length/stature and weight and an increase in *Z*-scores for weight and length/stature in the majority of the subjects treated; these were evident above all in the first month of treatment. In particular, in all subjects who presented a *Z*-score at enrolment below 2 s.d. the increase in weight *Z*-score continued until the third month of treatment. It is interesting to note that *Z*-scores for weight and length/stature, after the increase in the early months, tended to stabilize, suggesting that the effect of donkey's milk is not because of a better energy yield, but rather to its ability to fill some nutritional gaps present in the diet of the subjects treated.

Specific clinical studies on the nutritional validity of DM in infants and in children are not available. However, current knowledge on its composition indicates that DM is extremely similar in composition to HM (11). Its high lactose content, similar to that of HM, not only makes DM pleasant in taste but also stimulates intestinal absorption of calcium. The Ca/P ratio (1.48) is intermediate between those of HM and CM. Unlike the milk of ruminants, DM contains similar levels of casein and whey proteins, as in HM, as well as a high concentration of essential amino acids. Furthermore, DM and HM are substantially similar with regard to the low renal load of solutes (proteins and inorganic substances). Its long-chain polyunsaturated fatty acid content is as rich as that of HM (ω_6/ω_3 ratio similar to HM), with a high percentage of linoleic acid (11, 17). Samples of raw DM show low microbial counts, likely because of the high lysozyme content (16).

The weak aspecific reactivity vs. DMP revealed, by immunoblotting (Fig. 1 – Panel B), the IgE present both in subjects found to be allergic to DM and in those who tolerated it clinically, made it impossible to identify the cross-reactive proteins of CM and DM provoking the clinical symptoms in the DMP allergic group. Owing to the fact that immunoblotting only detects the linear epitopes, the *in vivo* cross-reactivity between CM and DM might thus be linked to conformational epitopes, common to the two types of milk. Further chemical investigation is needed on the structural features of DMP in order to detect any cross-reactive epitopes.

Our study found DM to be a valid feeding solution in a selected population of children with CMPA, for whom SF, eHF and AAF could not be used to replace CM and who, because of their concomitant MFA, required a substitute food that was palatable and tolerated, as well as being nutritionally valid.

These encouraging results should potentiate the production of this type of milk, which could lead to a reduction in cost. Its increased availability would enable studies to be performed to evaluate the possibility of utilizing it in the general population of subjects with CMPA.

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