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RESEARCH ARTICLE

Alpha-lactalbumin Effect on Myo-inositol Intestinal Absorption: *In vivo* and *In vitro*

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Abstract: ***Background:*** Myo-inositol is a natural molecule with important therapeutic applications and an impaired oral absorption may result in a reduced clinical effect. Aim of this study was to determine if the combined oral administration of α-lactalbumin and myo-inositol in healthy subjects, could increase the plasma level of myo-inositol administered alone. *In vitro* studies on human differentiated intestinal Caco-2 cells were also conducted to identify the mechanisms involved in myo-inositol absorption.

Objective: The *in vivo* study was conducted on healthy volunteers in two phases. Subjects received a single oral myo-inositol dose. After 7 days washout, the same subjects were administered a single dose of myo-inositol and α-lactalbumin. Cmax, Tmax and AUC for myo-inositol in plasma were calculated from samples collected at different times. Transepithelial myo-inositol passage, with or without addition of digested α-lactalbumin, was measured *in vitro* in differentiated Caco-2 cells and compared to transepithelial electrical resistance and phenol red passage.

Results: The bioavailability of myo-inositol was modified by the concomitant administration of α-lactalbumin. Although peak concentration of myo-inositol at 180 min (Tmax) was similar for both treatments, administration of α-lactalbumin with myo-inositol in a single dose, significantly increased the plasma concentrations of myo-inositol compared to when administered alone. *In vitro*, myo-inositol absorption in Caco-2 cells was improved in the presence of digested α-lactalbumin, and this change was associated with an increase in tight junction permeability.

Conclusion: Better myo-inositol absorption when orally administered with α-lactalbumin can be beneficial in non-responder patients. Preliminary *in vitro* findings suggest that peptides deriving from α-lactalbumin digestion may modulate tight junction permeability allowing increased absorption of myo-inositol.

Keywords: Myo-inositol, α-lactalbumin, pharmacokinetics, healthy volunteers, bioactive peptides, bioavailability, absorption enhancers, intestinal permeability.

1. INTRODUCTION

In the last decades, myo-inositol (MI), the most abundantly represented isomer in all living forms among the nine possible isomers of inositols, has attracted scientific interest and has stimulated research in lots of therapeutic areas. MI is a cyclic polyol ($C_6H_{12}O_6$) with MW 180.16, present in all living forms, that is involved in several metabolic pathways, with significant therapeutic applications in pathologies such as Polycystic Ovary Syndrome (PCOS) [1-3], Gestational Diabetes Mellitus (GDM) [4-5], Metabolic Syndrome (MetSyn) [6], where insulin sensitizing agents play a key role, with the additional advantage of lacking significant adverse events [7]. Among its manifold physiological activities, in the ovary MI regulates glucose uptake and Follicle Stimulating Hormone (FSH) signalling, while D-chiro-inositol (DCI), a different isomer of the family, modulates insulin-induced androgen synthesis [2]. These activities are pivotal to counteract important endocrine and metabolic anomalies. Despite this, neither, MI, nor other pharmacological treatments (e.g. metformin, clomiphene citrate, etc.) are always completely effective. A strategy to overcome this problem is to enhance the absorption rate of molecules with therapeutic effects. Oral absorption can be influenced by a variety of factors such as physiochemical properties, formulation and dose of the compound, presence of carrier molecules, physiological and pathological conditions of the gastrointestinal tract. Our interest was focused on the search of a molecule that could increase MI uptake alongside with additional or synergistic contribution to its therapeutic activities.

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Our attention was drawn by α-lactalbumin (α-LA), a molecule belonging to the broader class of milk proteins in all mammals. α-LA is the second most important protein in whey (approximately 20% to 25% of whey proteins) [8]. It is a small, globular protein composed of 123 amino acid residues with MW of 14.07 kDa in human milk and 14.18 kDa in bovine milk. α-LA structure shows a large helical domain and a small beta sheet domain which have a deep cleft between them, linked by a loop [9]. α-LA is characterized by low immunogenicity in comparison to other allergens of cow's milk, such as casein or beta-lactoglobulin, and it is reputed that no dominant epitope exists in this protein [10]. Clearly, this feature gives a reassuring profile to it. We know that organisms generally do not respond to 'self' proteins and this might be the reason of the low immunogenicity exerted by bovine α-LA that has high homology (74%) with human α-LA [11, 12]. α-LA is characterized by exerting several effects, with relevant therapeutic uses. It is part of the group of proteins with roles beyond nutrition, including enzyme activities, enhancement of nutrient absorption, growth stimulation, modulation of the immune system and defence against pathogens [13]. α-LA was found to provide a gastroprotective activity in experimental gastric ulcer models induced by ethanol or stress [14]. Furthermore, it fortifies the mucus gel layer in gastric mucosa *in vivo*, increasing the mucin content of the mucus gel layer in rat gastric mucosa [15]. In addition, α-LA shows anti-inflammatory activities by means of its capacity to inhibit type 2 cyclooxygenase (COX 2), an isoenzyme of the prostaglandin endoperoxide synthase family, which depends on the synthesis of prostanoids. Additionally, it also significantly decreases Interleukin 6, an inflammatory cytokine [16]. Oral administration of α-LA (300 mg/kg, twice a day) for 10 weeks in Goto–Kakizaki (GK) rats, a model of type 2 diabetes, was able to significantly reduce blood glucose levels after glucose loading, probably by means of adiponectin enhancement [17]. α-LA can serve as an illustrative example of a "hybrid moonlighting protein" [18], the various functions of which depend on its location and micro-environment, and range from participation in lactose biosynthesis in the lactating mammary gland, to contribution to the antiviral, antimicrobial, and antitumor activity of milk, also including involvement in diverse transport functions [18]. Structure, conformational properties and functions of α-LA were also shown to be regulated by binding of metal ions [18]. With the aim of improving MI clinical efficacy, we planned a pharmacokinetics study in humans to test the effects of α-LA on MI bioavailability, taking advantage of the wide range of α-LA activities, including its reported effects on improved mineral bioavailability [9]. Furthermore, we carried out experiments with the human intestinal Caco-2 cell line in order to evaluate the effects of α-LA on MI at the cellular level and to get preliminary information on the mechanism of action underlying these effects. α-LA is relatively easily digested and there is no remnant of this protein in the stool of breastfed infants. However, during the passage along the stomach and upper small intestine, digestion of α-LA results in the formation of several peptides of various dimensions. Numerous studies have been reported on the functional properties of bioactive components in milk and dairy products, especially in human and bovine milk, exerting several physiological effects both in new-borns and in adults, ranging from opioid-like activity, angiotensin-converting enzyme (ACE) inhibition, stimulation of immune function, mineral and trace element absorption and defence against infection [9, 19-20]. In addition, evidence is rapidly emerging on the *in vivo* absorption of milk protein peptides, larger than the di- and tripeptides transported by well characterized intestinal carriers [21]. Other mechanisms of peptide absorption across intestinal cells that have been proposed are passive permeability, pore formation, endocytosis and transcytosis. These characteristics are being exploited to design peptide-drug conjugates to enhance penetration of biological barriers, increase cellular uptake, and avoid the immune system [21]. Alternative approaches using peptides to enhance transport across intestinal barriers have exploited the ability of certain peptides to bind tight junction (TJ)-forming proteins, transiently increasing paracellular passage of other molecules [22].

2. SUBJECTS, MATERIALS AND METHODS

2.1. Materials

MI (purity 99%) and α-LA from cow milk (purity 96%) were obtained from LoLiPharma srl (Rome, Italy).

All other reagents, unless otherwise stated, were from Sigma Aldrich (Milan, Italy).

2.2. Pharmacokinetic Study

2.2.1. Subject Eligibility

The *in vivo* study involved 18 healthy volunteers, 7 men and 11 women. Subjects were evaluated based on medical history, physical examination and laboratory screenings, with the exclusion of those with poor general health. Volunteers were aged between 18 and 35 years, with a body mass index (BMI) ranging between 21 and 25 kg/m^2. Before entering the trial, an informed consent was obtained from the volunteers, and the study was performed according to the Helsinki declaration and the Italian national law. The study was approved by the institutional review board of SIFIOG (Italian Society of Phytotherapy and Dietary Supplements in Obstetrics and Gynecology).

2.2.2. Study Design and Intervention

This pharmacokinetics study was aimed at determining whether the combination of MI and α-LA can increase the plasma levels of MI above that observed after administering MI alone. The intervention study was carried out at Altamedica Medical Center (Rome, Italy) and consisted of two phases:

Phase I: subjects, all in fasting for 12 hours, received orally 6 g MI in powder form administrated in single dose dissolved in 80 ml H_2O.

Phase II: after a washout period of 7 days, the same subjects, in fasting for 12 hours, were given an oral dose with a dose of 6 g MI and 150 mg α-LA in powder form, dissolved in 80 ml H_2O.

2.2.3. Sample Collection and Preparation

Blood samples were collected by venous puncture at pre-dose (time point 0), and at time points 60, 120, 180, 240 and

300 min post-administration, placed in heparinized tubes and kept on ice. Blood samples were centrifuged and stored at -80° until assayed.

2.2.4. MI Assay in Plasma

Quantification of MI levels (µmol/l) was performed by Mérieux NutriSciences Italia (Resana, Treviso, Italy) and was carried out with the same procedure adopted in a previous study [23]. After extraction with organic solvents and derivatization, sample analysis was made by gas chromatography-mass spectrometry with Agilent 6890 (Agilent, 5301 Stevens Creek Blvd, Santa Clara, CA 95051, USA). The injection (1.0 µl) was performed in a split-less mode at 270°C, using a capillary column Agilent 122-5532 DB-5 ms (0.25 mm x 30 m x 0.25 µm). The total run-time lasted 15 min: oven at 70°C from 0 to 1 min; 20°C/min to 150°C; 10°C/min to 240°C; 4 min at 320°C post-run. The flow rate was fixed at 1.2 ml/min, and the results analysed by a MS 5973 Network Series detector in sim mode.

2.2.5. Pharmacokinetic Parameters

Pharmacokinetic (PK) parameters were evaluated following oral administration of MI alone or in combination with α-LA, determining plasma MI concentration at various time points up to 300 min post-administration. Maximum plasma concentration (Cmax) and time to reach it (Tmax), were calculated directly from the plasma concentration. The area under the time-course curve (AUC 0-300) was calculated by the trapezoidal method over the time span of 0 to 300 min [24].

2.3. *In vitro* Experiments

2.3.1. Cell Culture and In vitro Transport Experiments

The human intestinal Caco-2 cell line, obtained from INSERM (Paris, France) was routinely sub-cultured at 50% density [25] and differentiated on polycarbonate filters (Transwell, Corning Inc. Lowell, MA, USA) for 21 days [26]. Transport experiments were performed as previously reported [27], in saline buffer solution (BSS: 137 mM NaCl, 5.36 mM KCl, 1.26 mM $CaCl_2$, 1.1 mM $MgCl_2$, 5 mM D-glucose) maintained at pH 6.0 in the apical (AP) compartment and pH 7.4 in basolateral (BL) chamber, in order to reproduce the pH conditions existing *in vivo*. To avoid possible transporter competition, the BSS solution in the AP compartment was deprived of glucose since MI transporters are known to be inhibited by physiologic concentrations of D-galactose, D-glucose and L-glucose, but not by mannitol, sorbitol, and fructose [28]. To deplete MI intracellular stores, before transport, cells were pre-equilibrated for 30 min in BSS, experimental media were then replaced with fresh BSS containing 5 mM MI in the absence and in the presence of 5 or 10 mg/ml of α-LA digest in the AP solution; alternatively, cells were incubated in BSS containing α-LA alone in the AP compartment. After 4 h, AP and BL media were collected and stored at -80 °C for further analysis. In order to monitor monolayer permeability, Trans-Epithelial Electrical Resistance (TEER) was measured throughout the transport experiments, using the voltmeter apparatus Millicell (Millipore Co, USA). TEER was calculated as $\Omega \cdot cm^2$ after subtracting the resistance value of the supporting filter [29]. As an additional measure of monolayer permeability, the passage of the paracellular marker phenol red, was assayed at the end of transport experiments [29].

2.3.2. In vitro Gastrointestinal Digestion of α-LA

α-LA gastrointestinal *in vitro* digestion was performed according to a previously described method [30], with minor modifications. Briefly, α-LA was dissolved in PBS (0.1 g/ml) and gastric digestion phase was performed after addition of pepsin (15000 U/g α-LA at pH pH 2.0) in a shaking water bath at 37°C for 2 h. The digest was then maintained on ice for 10 min to stop pepsin activity. For intestinal digestion phase, a freshly prepared pancreatin solution (1,000 U/g α-LA) was added to the digest, the pH was adjusted to 8.0, and incubation at 37°C was continued for 3 h. Samples were kept for 10 min at 100 °C to inhibit protease activities and subsequently centrifuged at 4,000 g for 100 min at 4°C in Centricon centrifugal filter devices (10,000 MW cut-off - Millipore Corporation, Bedford, MA, USA) to remove the digestive enzymes. The α-LA digest was subjected to SDS-PAGE electrophoresis to verify complete digestion of α-LA into peptides. Final concentration of α-LA peptides in digested sample was 55 mg/ml (Lowry determination).

2.3.3. MI Assay in Medium

Quantification of MI levels (µmol/l) was performed by LabAnalysis srl (Casanova Lonati, Pavia, Italy). MI levels (µmol/l) were detected in BSS samples diluted 1:4 in distilled water, without any extraction, by means of HPLC-MS analytical technique using a high-resolution mass spectrometer: UPLC-MS-Q/TOF, Agilent HPLC 1290, Agilent 6550. Analysis were performed at 20k resolution. 5 µl of the 1:4 diluted sample were directly injected in the instrument. The instrumental conditions were: HPLC Column Metacarb 87C 300x7.8 (Agilent); Column compartment temperature: 80°C; Flow: 0.65 ml/min. The monitored accurate mass for MI was 203.0526 a.m.u.

2.4. Statistics

All results are presented as mean ± standard deviation. Data sets were compared using a Student's t-test and a p value <0.05 was considered statistically significant. Analysis were performed using Intercooled Stata 8.2.

3. RESULTS

3.1. *In vivo* Study

All the enrolled subjects completed the trial. No adverse events were detected. The analysis of MI plasma concentrations measured with or without concomitant α-LA administration, yielded interesting outcomes regarding the PK parameters of the combined formulation (MI + α-LA), with respect to the resulting plasma concentration of MI alone. In both tests the curve profile was similar, with Tmax at 180 min. The time course of MI concentrations in the plasma of 18 volunteers before and after oral administration of MI alone or with α-LA is graphically depicted in Fig. (**1**). When MI was administered alone, its average peak plasma concentration (at 180 min) increased about threefold with respect to baseline, whereas in the presence of α-LA, it augmented fourfold (from 32.2 to 95.1 µmol/l, and from 31.2 to 125.9 µmol/l, respectively).

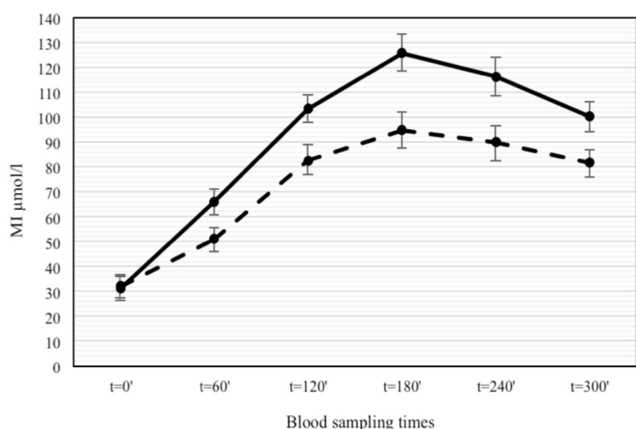

Fig. (1). Comparison of MI concentration (µmol/l) in plasma of 18 healthy volunteers at different time points after MI administration alone (dotted line), o with α-LA (solid line). For each point: mean ± SD.

Calculated pharmacokinetics parameters of MI absorption are shown in Table **1**. MI plasma concentrations after administration of powder containing 6 g MI and 150 mg α-LA were significantly higher than after administration of 6 g of MI powder alone: the increase of Cmax (µmol/l) was 32.4%, while AUC (0-300) increased by 27.5%.

3.2. *In vitro* Experiments

In order to evaluate the effects of α-LA on MI intestinal absorption, the protein α-LA was previously subjected to simulated gastrointestinal digestion and analysed by SDS PAGE to verify complete digestion into peptides <5kDa (data not shown). The AP to BL passage of MI was assayed in the absence and in the presence of α-LA peptides for 4 h. As shown in Fig. (**2**), a significant increase in MI passage (almost 4.5-fold) was observed in the presence of 10 mg/ml α-LA peptides, while the lower α-LA peptides concentration (5mg/ml) had no apparent effect compared to MI alone.

To monitor the permeability of the monolayer, two assays were employed, namely TEER (Fig. **3**) and the passage of the paracellular marker phenol red (Fig. **4**), were measured at the end of the MI transport experiment.

MI (5 mM) and 5 mg/ml α-LA when applied alone to the monolayer did not alter the permeability of the monolayer over the 4 h of the experiment. Conversely, the higher concentration of α-LA peptides (10 mg/ml) induced a decrease

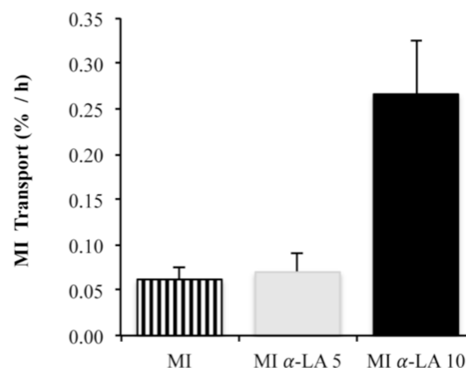

Fig. (2). MI trans-epithelial passage in Caco-2 cells was assayed at a concentration of 5 mM for 4 h in the absence (hatched bar) and in the presence of 5 (grey bar) and 10 mg/ml (solid bar) α-LA peptides. Transport rate was expressed as % of MI concentration in the AP compartment / h. Data represent the mean ± SD of 3 experiments performed in triplicate.

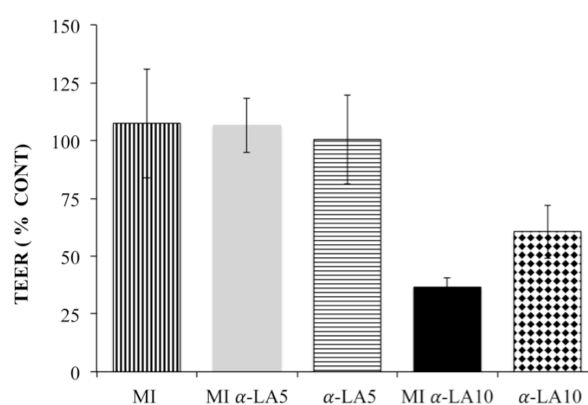

Fig. (3). Monolayer permeability of Caco-2 cells following different experimental treatments. After 4 h transport experiments, TEER (expressed as % of control cells maintained in saline) was measured. Histogram shows: 5 mM MI (lined vertical bar), 5 mM MI + α-LA 5 mg/ml (grey bar), α-LA 5 mg/ml (lined horizontal bar), 5 mM MI + α-LA 10 mg/ml (solid bar), α-LA 10 (pointed bar). Data represent the mean ± SD of 3 experiments performed in triplicate

in TEER not accompanied by an enhancement in phenol red passage. However, in the presence of MI and 10 mg/ml α-LA peptides, both permeability assays were markedly altered, producing a decrease in TEER and an increase in phenol red passage measured at the end of the transport experiment.

Table 1. Pharmacokinetic parameters after oral administration in healthy volunteers.

Parameter	Mean ± SD			
	MI 6 g Admin.	**MI 6 g + α-LA 150 mg Admin.**	**Δ %**	**p-value**
Cmax (µmol/l)	95.1 ± 7.8	125.9 ± 8.1	32.4%	p< 0.0001
Tmax (min)	180.0 ± 8	180.0 ± 5	0%	Not significant
AUC (0-300)	22676.7 ± 1549.7	28918.3 ± 1577.3	27.5%	p< 0.0001

Data are expressed as mean ± SD. t=180 min.
Cmax: (µmol/l) maximum observed plasma concentration during the 0 - 300 min dosing interval; Tmax: Time (min) to reach the peak concentration; AUC (0 - 300): (µmol·min/l) area under the time-course curve of plasma concentration, from baseline to 300 min.

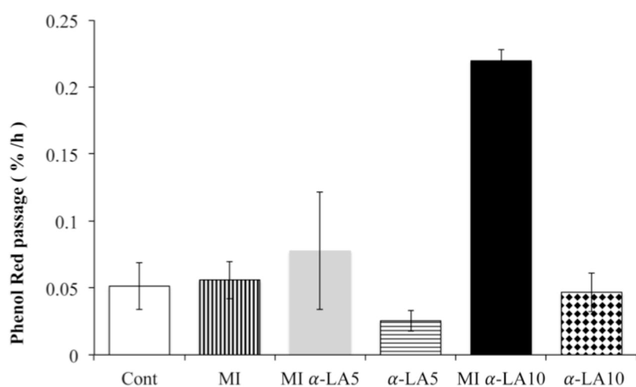

Fig. (4). Monolayer permeability of Caco-2 cells following different experimental treatments. After 4 h transport experiments paracellular phenol red passage was assayed for 1h and expressed as % of the AP phenol red concentration. Histogram shows: control cells (white bar), 5 mM MI (lined vertical bar), 5 mM MI + α-LA 5 mg/ml (grey bar), α-LA 5 mg/ml (lined horizontal bar), 5 mM MI + α-LA 10 mg/ml (solid bar), α-LA 10 (pointed bar). Data represent the mean ± SD of 3 experiments performed in triplicate.

4. DISCUSSION

This pharmacokinetics study, carried out on healthy volunteers, demonstrated that the simultaneous oral administration of MI and α-LA leads to a significantly higher plasma concentration of MI than observed when MI is given alone. In fasting subjects, the endogenous plasma concentration of MI is approximately 30 μmol/l (the same found in this study) with a half-life of 22 min [31]. The shape of the curve and Tmax found in the present study are very similar to those previously reported [32].

MI concentrations in cells and tissue are maintained by means of three mechanisms acting at different sites: gut uptake and kidney excretion, carrier-mediated passage from plasma into cells and endogenous synthesis and catabolism. MI transport in several cell types occurs through active processes against concentration gradient [28], leading to intracellular concentrations higher than in plasma or culture medium. The uptake of MI into cells is mediated by two kinds of carriers, sodium-coupled and proton-coupled. Two Sodium/Myo-Inositol Transporters, called SMIT1 and SMIT2, belong to the Solute Carrier Families 5 and 6-like Superfamily. Of the second type is the H^+/myo-inositol transporter (HMIT) [28, 33-34], belonging to the Major Facilitator Superfamily. However, across intestinal cells exposed to relatively high therapeutic concentrations of MI [18], additional passive transport mechanisms are likely to contribute to its bioavailability, given the low Km (50-300 μM) of both Na^+/linked myo-inositol transporters and the H^+/myo-inositol transporter [28, 33-34].

While *in vivo* testing on human volunteers is best suited for observing overall effects of oral drugs or health supplements administration, many factors affecting bioavailability of the active ingredient(s) cannot be controlled. *In vitro* experiments on suitable cell culture models can, on the other hand, contribute to unravel the molecular mechanisms underlying intestinal absorption, largely affecting the bioavailability of orally administered substances.

The human intestinal Caco-2 cell line differentiated on permeable filters still represents the best available *in vitro* model of the small intestinal mucosa. Upon differentiation, the cells form a monolayer of polarized epithelial cells, joined by functional TJ, expressing enzymatic, metabolic and transport activities similar to those encountered in enterocytes *in vivo*, albeit sometimes quantitatively under represented [35]. Small intestinal TJ are not static barriers but highly dynamic and plastic structures, rapidly responding to extracellular stimuli to maintain the integrity of the epithelial cell monolayer [36], and whose permeability can be transiently modulated by absorption enhancers that can be beneficial for promoting the oral absorption of poorly permeable drugs [37]. The Caco-2 intestinal model has often been utilized to investigate the mechanisms of nutrients and drugs transport and toxicity, and has recently been applied to the screening of drug absorption enhancers of different nature, including peptides [38-39]. In addition, published methods of *in vitro* simulated gastrointestinal digestion have allowed bioavailability studies in Caco-2 cells of proteins and more complex food matrices that do not reach the intestinal mucosa in their intact form due to digestive processes [30].

Data in the literature report that proteolytic digestion α-LA gives rise to several peptides of varying dimensions, produced from protein breakdown during passage along the gastrointestinal tract. Protein digestion is initiated in the stomach by pepsin under acidic pH conditions. Then, the digesta are further hydrolysed by pancreatic enzymes such as pepsin, trypsin and chymotrypsin and membrane peptidases resulting in amino acids and peptides of various lengths [40].

A characterized tetrapeptide of α-LA, α-lactorphin, was reported to have opioid agonist and ACE-inhibitor activities, others were shown to possess bactericidal properties [19]. In addition, bioactive peptides from α-LA have been shown *in vitro* to be involved in immune stimulation, to enhance mineral absorption and to have prebiotic activity. While some of the bioactive peptides exert their bioactivities directly in the gastrointestinal lumen, others function at peripheral organs after being absorbed at the intestinal mucosa. The resistance to additional degradation by digestive enzymes and the rate of intestinal assimilation of these peptides may positively contribute to their bioavailability. The presence of phosphatidylcholine, secreted in the stomach and contained in high amounts in milk, has been shown to slow down the process of α-LA demolition during gastric digestion, and may further preserve the resulting peptides from further degradation during duodenal digestion [41]. Therefore, partially degraded α-LA may in part reach the large intestine where it undergoes microbial enzyme hydrolysis [42], with the in loco release of potentially beneficial bioactive peptides.

The observation that *in vitro* digested α-LA improves the intestinal bioavailability of MI suggests a novel role of the yet uncharacterized α-LA peptides derived from *in vitro* simulated gastrointestinal digestion. Recently, peptides have been proposed as new intestinal absorption enhancers, and several studies have shown their potential in increasing transport across the intestinal barrier [22]. It is therefore possible that some naturally occurring peptides may possess similar activities. Based on the amino acid sequence and/or

structure of epithelial TJ and adherens junction proteins, peptides were designed as novel TJ modulators to specifically interact with the extracellular loops of these proteins, affecting their organization and thus increasing TJ permeability. In Caco-2 cells, these peptides increased the paracellular passage of extracellular markers, decreased TEER and produced disorganization of TJ proteins, as shown by their immunofluorescent localization [39]. Although the peptides arising from α-LA digestion were not characterized in the present study, the observed decrease in TEER after treatment with 10 mg/l α-LA, may suggests a similar mechanism of TJ modification, facilitating MI absorption. Alternatively, the mineral binding activity of α-LA peptides [8, 20, 40] may contribute to TJ opening by a mechanism of Ca^{++} sequestration. In addition, the observation that MI appeared to act synergistically with α-LA in promoting its own passage (Fig. **3**), may be related to a direct effect of MI on TJ permeability. The dietary phosphorylated form of MI, namely inositol 6-Phosphate (IP6), a potential absorption enhancer of flavonoids, was reported to increase TJ permeability in Caco-2 cells by downregulating the expression of TJ proteins occludin, ZO-1 and claudin-1, and also attenuating their correct membrane localization [43]. Interestingly, the observed effect of IP6 on TJ permeability was attenuated by the addition of divalent cations Ca^{++} e Mg^{++}. In addition, Caco-2 have been shown to express dephosphorylating activity towards IP6 and its isomers [44].

Long term administration of α-LA may promote additional effects linked to the modulation of the composition of the gut microflora as prebiotic effects have been reported for α-LA peptides. To the best of our knowledge, very few studies on the possible effects of α-LA in this field have been carried out on experimental animals or humans (only newborns, not adults). Among them, there is a patent on α-LA as prebiotic agent showing data obtained in rats [45]. Milk formula supplemented with two milk peptides, including α-LA, given for 6 months to healthy term infants, produced a prebiotic effect only in infants not initially breast-fed, that had a low starting population of beneficial microbiota [46].

It would therefore be interesting to investigate if long-term effects of α-LA, mediated by changes in the microbiota, could affect the bioavailability of MI or similar molecules.

CONCLUSIONS

The present *in vivo* and *in vitro* study demonstrated that the simultaneous administration of MI and α-LA leads to a significantly higher bioavailability of MI than observed when MI is given alone. These very promising findings deserve to be investigated in more depth and further research is necessary to clarify the mechanisms of action by which α-LA positively affects MI transport from the intestine to the blood and its specificity for other inositol stereoisomers. Additional studies are also needed to characterize the products of α-LA digestion, to determine their potential binding sites, bioavailability and modes of action, and to ascertain if these peptides retain the anti-inflammatory activity reported for α-LA. Combined bioactive properties of MI and α-LA may improve their beneficial effects on complex pathologies such as PCOS, GDM and MetSyn.

AUTHOR CONTRIBUTIONS

Giovanni Monastra designed, organized and supervised the pharmacokinetics study in humans; Yula Sambuy, Giulia Ranaldi and Simonetta Ferruzza designed, organized and supervised the *in vitro* research and experiments; Giulia Ranaldi, Simonetta Ferruzza and Daniela Ferrari carried out the *in vitro* experiments; Giovanni Monastra supervised the procedures of myo-inositol dosage in plasma and in saline; Yula Sambuy and Giovanni Monastra wrote the manuscript. The authors discussed and interpreted all the results, read the final manuscript and approved it.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

The pharmacokinetic study on healthy volunteers was approved by the institutional review board of SIFIOG (Italian Society of Phytotherapy and Dietary Supplements in Obstetrics and Gynecology) and before entering the trial, an informed consent was obtained from the volunteers.

HUMAN AND ANIMAL RIGHTS

Human studies were performed according to the Helsinki declaration and the Italian national law. No Animals were used for studies that are the basis of this research.

CONSENT FOR PUBLICATION

Not applicable.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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REFERENCES

[1] Monastra, G.; Unfer, V.; Harrath, A. H.; Bizzarri, M., Combining treatment with myo-inositol and D-chiro-inositol (40:1) is effective in restoring ovary function and metabolic balance in PCOS patients. *Gynecol. Endocrinol.* **2017**, *33* (1), 1-9.

[2] Nestler, J. E.; Unfer, V., Reflections on inositol(s) for PCOS therapy: steps toward success. *Gynecol. Endocrinol.* **2015**, *31* (7), 501-5.

[3] Unfer, V.; Facchinetti, F.; Orru, B.; Giordani, B.; Nestler, J., Myo-inositol effects in women with PCOS: a meta-analysis of randomized controlled trials. *Endocr Connect* **2017**, *6* (8), 647-658.

[4] Costabile, L.; Unfer, V., Treatment of gestational diabetes mellitus with myo-inositol: analyzing the cutting edge starting from a peculiar case. *Eur. Rev. Med. Pharmacol. Sci.* **2017**, *21* (2 Suppl), 73-76.

[5] Santamaria, A.; Di Benedetto, A.; Petrella, E.; Pintaudi, B.; Corrado, F.; D'Anna, R.; Neri, I.; Facchinetti, F., Myo-inositol may prevent gestational diabetes onset in overweight women: a randomized, controlled trial. *J. Matern. Fetal Med.* **2016**, *29* (19), 3234-7.

[6] Facchinetti, F.; Bizzarri, M.; Benvenga, S.; D'Anna, R.; Lanzone, A.; Soulage, C.; Di Renzo, G. C.; Hod, M.; Cavalli, P.; Chiu, T. T.; Kamenov, Z. A.; Bevilacqua, A.; Carlomagno, G.; Gerli, S.; Oliva, M. M.; Devroey, P., Results from the International Consensus Conference on Myo-inositol and d-chiro-inositol in Obstetrics and Gynecology: the link between metabolic syndrome and PCOS. *Eur. J. Obstet. Gynecol. Reprod. Biol.* **2015**, *195*, 72-6.

[7] Carlomagno, G.; Unfer, V., Inositol safety: clinical evidences. *Eur. Rev. Med. Pharmaco. Sci.* **2011**, *15* (8), 931-6.

[8] Kamau, S. M.; Cheison, S. C.; Chen, W.; Liu, X.-M.; Lu, R.-R., Alpha-Lactalbumin: Its Production Technologies and Bioactive Peptides. *Comp. Rev. Food Sci. Food Safety* **2010**, *9* (2), 197-212.

[9] Permyakov, E. A.; Berliner, L. J., alpha-Lactalbumin: structure and function. *FEBS Lett.* **2000**, *473* (3), 269-74.

[10] Meulenbroek, L. A.; den Hartog Jager, C. F.; Lebens, A. F.; Knulst, A. C.; Bruijnzeel-Koomen, C. A.; Garssen, J.; Knippels, L. M.; van Hoffen, E., Characterization of T cell epitopes in bovine alpha-lactalbumin. *Int. Arch. Allergy Immunol.* **2014**, *163* (4), 292-6.

[11] Fiocchi, A.; Schunemann, H. J.; Brozek, J.; Restani, P.; Beyer, K.; Troncone, R.; Martelli, A.; Terracciano, L.; Bahna, S. L.; Rance, F.; Ebisawa, M.; Heine, R. G.; Assa'ad, A.; Sampson, H.; Verduci, E.; Bouygue, G. R.; Baena-Cagnani, C.; Canonica, W.; Lockey, R. F., Diagnosis and Rationale for Action Against Cow's Milk Allergy (DRACMA): a summary report. *J. Allergy Clin. Immunol.* **2010**, *126* (6), 1119-28.e12.

[12] Hochwallner, H.; Schulmeister, U.; Swoboda, I.; Focke-Tejkl, M.; Civaj, V.; Balic, N.; Nystrand, M.; Harlin, A.; Thalhamer, J.; Scheiblhofer, S.; Keller, W.; Pavkov, T.; Zafred, D.; Niggemann, B.; Quirce, S.; Mari, A.; Pauli, G.; Ebner, C.; Papadopoulos, N. G.; Herz, U.; van Tol, E. A.; Valenta, R.; Spitzauer, S., Visualization of clustered IgE epitopes on alpha-lactalbumin. *J. Allergy Clin. Immunol.* **2010**, *125* (6), 1279-1285.e9.

[13] Lonnerdal, B., Human Milk: Bioactive Proteins/Peptides and Functional Properties. *Nestle Nutrition Institute Workshop Series* **2016**, *86*, 97-107.

[14] Matsumoto, H.; Shimokawa, Y.; Ushida, Y.; Toida, T.; Hayasawa, H., New biological function of bovine alpha-lactalbumin: protective effect against ethanol- and stress-induced gastric mucosal injury in rats. *Biosci. Biotech. Biochem.* **2001**, *65* (5), 1104-11.

[15] Ushida, Y.; Shimokawa, Y.; Toida, T.; Matsui, H.; Takase, M., Bovine alpha-lactalbumin stimulates mucus metabolism in gastric mucosa. *J. Dairy Sci.* **2007**, *90* (2), 541-6.

[16] Yamaguchi, M.; Yoshida, K.; Uchida, M., Novel functions of bovine milk-derived alpha-lactalbumin: anti-nociceptive and anti-inflammatory activity caused by inhibiting cyclooxygenase-2 and phospholipase A2. *Biol. Pharm. Bull.* **2009**, *32* (3), 366-71.

[17] Yamaguchi, M.; Takai, S., Chronic administration of bovine milk-derived alpha-lactalbumin improves glucose tolerance via enhancement of adiponectin in Goto-Kakizaki rats with type 2 diabetes. *Biol. Pharm. Bull.* **2014**, *37* (3), 404-8.

[18] Permyakov, E. A.; Permyakov, S. E.; Breydo, L.; Redwan, E. M.; Almehdar, H. A.; Uversky, V. N., Disorder in Milk Proteins: alpha-Lactalbumin. Part C. Peculiarities of Metal Binding. *Curr. Protein Peptide Sci.* **2016**, *17* (8), 735-745.

[19] Nagpal, R.; Behare, P.; Rana, R.; Kumar, A.; Kumar, M.; Arora, S.; Morotta, F.; Jain, S.; Yadav, H., Bioactive peptides derived from milk proteins and their health beneficial potentials: an update. *Food & function* **2011**, *2* (1), 18-27.

[20] Park, Y. W.; Nam, M. S., Bioactive Peptides in Milk and Dairy Products: A Review. *Korean J Food Sci. Anim. Resources* **2015**, *35* (6), 831-40.

[21] Komin, A.; Russell, L. M.; Hristova, K. A.; Searson, P. C., Peptide-based strategies for enhanced cell uptake, transcellular transport, and circulation: Mechanisms and challenges. *Adv. Drug Deliv. Rev.* **2017**, *110-111*, 52-64.

[22] Sanchez-Navarro, M.; Garcia, J.; Giralt, E.; Teixido, M., Using peptides to increase transport across the intestinal barrier. *Adv. Drug Deliv. Rev.* **2016**, *106* (Pt B), 355-366.

[23] Unfer, V.; Carlomagno, G.; Papaleo, E.; Vailati, S.; Candiani, M.; Baillargeon, J. P., Hyperinsulinemia Alters Myoinositol to d-chiroinositol Ratio in the Follicular Fluid of Patients With PCOS. *Reprod. Sci.* **2014**, *21* (7), 854-858.

[24] Purves, R. D., Optimum numerical integration methods for estimation of area-under-the-curve (AUC) and area-under-the-moment-curve (AUMC). *J. Pharmacokinet. Biopharm.* **1992**, *20* (3), 211-26.

[25] Natoli, M.; Leoni, B. D.; D'Agnano, I.; Zucco, F.; Felsani, A., Good Caco-2 cell culture practices. *Toxicol. In vitro* **2012**, *26* (8), 1243-6.

[26] Ferruzza, S.; Rossi, C.; Scarino, M. L.; Sambuy, Y., A protocol for differentiation of human intestinal Caco-2 cells in asymmetric serum-containing medium. *Toxicol. In vitro* **2012**, *26* (8), 1252-5.

[27] Lammi, C. A., G.; Vistoli, G.; Zanoni, C.; Arnoldi, A.; Sambuy, Y.; Ferruzza, S.; Ranaldi, G., A multidisciplinary investigation on the bioavailability and activity of peptides from lupin protein. *J. Funct. Foods* **2016**, *24*, 297-306.

[28] Schneider, S., Inositol transport proteins. *FEBS Lett.* **2015**, *589* (10), 1049-58.

[29] Ferruzza, S.; Scarino, M. L.; Gambling, L.; Natella, F.; Sambuy, Y., Biphasic effect of iron on human intestinal Caco-2 cells: early effect on tight junction permeability with delayed onset of oxidative cytotoxic damage. *Cell. Mol. Biol. (Noisy-le-Grand)* **2003**, *49* (1), 89-99.

[30] Frontela, C.; Scarino, M. L.; Ferruzza, S.; Ros, G.; Martinez, C., Effect of dephytinization on bioavailability of iron, calcium and zinc from infant cereals assessed in the Caco-2 cell model. *World J. Gastroenterol.* **2009**, *15* (16), 1977-84.

[31] Clements, R. S., Jr.; Reynertson, R., Myoinositol metabolism in diabetes mellitus. Effect of insulin treatment. *Diabetes* **1977**, *26* (3), 215-21.

[32] Carlomagno, G.; De Grazia, S.; Unfer, V.; Manna, F., Myo-inositol in a new pharmaceutical form: a step forward to a broader clinical use. *Expert Opin. Drug Deliv.* **2012**, *9* (3), 267-71.

[33] Bourgeois, F.; Coady, M. J.; Lapointe, J. Y., Determination of transport stoichiometry for two cation-coupled myo-inositol cotransporters: SMIT2 and HMIT. *J. Physiol.* **2005**, *563* (Pt 2), 333-43.

[34] Aouameur, R.; Da Cal, S.; Bissonnette, P.; Coady, M. J.; Lapointe, J. Y., SMIT2 mediates all myo-inositol uptake in apical membranes of rat small intestine. *Am. J. Physiol.* **2007**, *293* (6), G1300-7.

[35] Sambuy, Y.; De Angelis, I.; Ranaldi, G.; Scarino, M. L.; Stammati, A.; Zucco, F., The Caco-2 cell line as a model of the intestinal barrier: influence of cell and culture-related factors on Caco-2 cell functional characteristics. *Cell Biol. Toxicol.* **2005**, *21* (1), 1-26.

[36] Sambuy, Y., Cellular tight junctions as mediators of adverse effects. In: *General and applied toxicology*, Ballantyne B, M. T., Syversen T Ed. John Wiley & Sons, **2009**; pp. 345-365.

[37] Lemmer, H. J.; Hamman, J. H., Paracellular drug absorption enhancement through tight junction modulation. *Expert opinion on drug delivery* **2013**, *10* (1), 103-14.

[38] Sun, D.; Yu, L. X.; Hussain, M. A.; Wall, D. A.; Smith, R. L.; Amidon, G. L., *In vitro* testing of drug absorption for drug 'developability' assessment: forming an interface between *in vitro* preclinical data and clinical outcome. *Current opinion in drug discovery & development* **2004**, *7* (1), 75-85.

[39] Bocsik, A.; Walter, F. R.; Gyebrovszki, A.; Fulop, L.; Blasig, I.; Dabrowski, S.; Otvos, F.; Toth, A.; Rakhely, G.; Veszelka, S.; Vastag, M.; Szabo-Revesz, P.; Deli, M. A., Reversible Opening of Intercellular Junctions of Intestinal Epithelial and Brain Endothelial Cells With Tight Junction Modulator Peptides. *J. Pharm. Sci.* **2016**, *105* (2), 754-765.

[40] Wada, Y.; Lonnerdal, B., Bioactive peptides derived from human milk proteins-mechanisms of action. *J. Nutr. Biochem.* **2014**, *25* (5), 503-14.

[41] Moreno, F. J.; Mackie, A. R.; Mills, E. N., Phospholipid interactions protect the milk allergen alpha-lactalbumin from proteolysis during *in vitro* digestion. *J. Agr. Food Chem.* **2005**, *53* (25), 9810-6.

[42] Moller, N. P.; Scholz-Ahrens, K. E.; Roos, N.; Schrezenmeir, J., Bioactive peptides and proteins from foods: indication for health effects. *Eur. J. Nutr.* **2008**, *47* (4), 171-82.

[43] Fu, Q.; Wang, H.; Xia, M.; Deng, B.; Shen, H.; Ji, G.; Li, G.; Xie, Y., The effect of phytic acid on tight junctions in the human intestinal Caco-2 cell line and its mechanism. *Eur. J. Pharm. Sci.* **2015**, *80*, 1-8.

[44] Briviba, K.; Schollenberger, M.; Rodehutscord, M.; Greiner, R., Dephosphorylation of myo-inositol phosphates in the *in vitro* intestinal Caco-2 cell model. *Int. J. Food Sci. Nutr.* **2018**, *69* (1), 46-51.

[45] Maase K, S. J.; Jozef, J.M.; Steijns, M. Use of alpha-lactalbumin as prebiotic agent. Patent EP 1228701 A1, Aug 7, 2002.

[46] Bruck, W. M.; Redgrave, M.; Tuohy, K. M.; Lonnerdal, B.; Graverholt, G.; Hernell, O.; Gibson, G. R., Effects of bovine alpha-

lactalbumin and casein glycomacropeptide-enriched infant formulae on faecal microbiota in healthy term infants. *J. Pediatr. Gastroenterol. Nutr.* **2006**, *43* (5), 673-9.