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Investigation of the neurotrophic effect of dairy phospholipids on cortical neuron outgrowth and stimulation

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A B S T R A C T

A novel purified dairy phospholipid (PL) extract was generated from a buttermilk powder (BMP) substrate combining techniques of enzymatic hydrolysis, ultrafiltration (UF) and supercritical fluid extraction (SFE). This combined process yielded a purified lipid extract with 56.34 ± 0.15% total PL in dry matter, depleted of protein and lactose. Application of this PL enriched extract in vitro demonstrated a neurotrophic effect of dairy PLs on cortical neurite outgrowth. At a concentration of 150 $\mu g\ mL^{-1}$, a 43% stimulated increase in neurite outgrowth was observed compared with the control (0% stimulation). % stimulation decreased to 12% upon increasing the PL dose to 300 $\mu g\ mL^{-1}$. The results of this study demonstrate that a dairy PL extract from buttermilk promotes the development and outgrowth of cortical neurons and complements the outcomes of new research (Matsuo, 2016) demonstrating that PLs are further stimulated in this role by omega PUFAs present.

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1. Introduction

Phospholipids (PLs) represent a complex class of polar lipids, whose core structure is composed of a "water loving" hydrophilic head group and a "water hating" hydrophobic fatty acid tail and are differentiated by the organic group (choline, ethanolamine, serine, etc.) linked to the third hydroxyl of the phosphate residue (Contarini & Povolo, 2013; Dewettinck et al., 2008). PLs are amphiphilic molecules (Contarini & Povolo, 2013; Lopez, 2011) which balance the conflicting polar versus non-polar forces residing within their structures. PLs are divided into two distinctive groups, namely glycerophospholipids, where structurally the first two carbon atoms of glycerol are fatty acid esters and the third a phosphate ester, and sphingolipids - a class of lipids containing a backbone of sphingoid bases (aliphatic amino alcohols). It is important to note that not all sphingolipids are PLs, such as sphingosine due to the lack of the phosphate group (Avalli & Contarini, 2005; Rombaut, van Camp, & Dewettinck, 2005). The four major glycerophospholipids found in milk are phosphatidylethanolamine (PE), phosphatidylcholine (PC), phosphatidylserine (PS) and phosphatidylinositol (PI) (Donato, Cacciola, Cichello, Russo, Dugo, & Mondello, 2011; Rombaut, van Camp, & Dewettinck, 2006). Sphingomyelin (SM) is the major representative of sphingolipids in milk (Avalli & Contarini, 2005; Contarini & Povolo, 2013; Rombaut, Dejonckheere, & Dewettinck, 2007; Rombaut, Dewettinck, & van Camp, 2007). PLs constitute approximately 1–5% of the total lipid found in milk (Sánchez-Juanes, Alonso, Zancada, & Hueso, 2009) of which >60% are associated with the milk fat globule membrane (MFGM) that surrounds lipid droplets (Dewettinck et al., 2008; Mather & Keenan, 1998; Singh, 2006). Due to their distinctive amphiphilic nature and close association with the MFGM, PLs play a key role in the emulsification of fat in milk (Contarini & Povolo, 2013; Kanno, Shimomura, & Takano, 1991; Roesch, Rincon, & Corredig, 2004).

There is increasing appreciation of the nutritional and technological functionalities of dairy PLs in recent decades in light of their uniquely higher concentrations of PS and SM (Barry, Dinan, Murray, & Kelly, 2016; Burling & Gaverholt, 2008; Dewettinck et al., 2008; Gassi et al., 2016). Buttermilk (BM), a by-product produced during cream churning, is a unique source of dairy PLs. As the milk fat globules disrupt, the fragmented MFGM associates with the aqueous BM phase resulting in a higher concentration of PLs in BM compared with milk or skim milk (Ferreiro, Martinez, Gayoso, & Rodriguez-Otero, 2016; Morin, Britten, Jiménez-Flores, & Pouliot, 2007; Vanderghem et al., 2011). In fact, PL concentrations in BM have been reported to be as much as fifteen times that found in milk (Barry et al., 2016; Christie, Noble, & Davies, 1987; Morin et al., 2007; Sodini, Morin, Olabi, & Jimenéz-Flores, 2006). For this reason, BM is an ideal substrate for the enrichment of dairy PLs and much research has been

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focused on its processing to harness its naturally high content of these bioactive compounds, in particular through use of membrane technology.

Microfiltration (MF) has been the predominant form of membrane filtration investigated to enrich BM substrates in previous works (Astaire, Ward, German, & Jimenéz-Flores, 2003: Corredig, Roesch, & Dalgleish, 2003; Holzmüller, & Kulozik, 2016; Morin, Jimenéz-Flores, & Pouliot 2004; Morin, Pouliot, & Jiménez-Flores, 2006; Morin et al., 2007; Sachedeva & Bucheim, 1997; Surel & Famelart, 1995). However, technological challenges due to the similarity in size of casein micelles and PLs have been encountered and so isolation of MFGM components by means of MF has had limited success and been shown to be dependent on numerous factors including pH, membrane pore size and the processing temperature. More recently enzymatic hydrolysis when combined with ultrafiltration has succeeded in generating much higher levels of PL enrichment (Barry, Dinan, & Kelly, 2017a; Barry, Dinan, & Kelly, 2017b; Konrad, Kleinschmidt, & Lorenz, 2013). Barry et al. (2017a) generated a 50 kDa retentate with a 7.8-fold increase in PL material which was subsequently exceeded, 8.5-fold increase, during transfer of the original laboratory-based process to pilot scale (Barry, Dinan, & Kelly, 2017b). This represented a considerable advance on the 2.5% increase in PL previously obtained by Konrad et al. (2013) using UF post peptic hydrolysis. Selectively removing non-polar lipids using supercritical fluid extraction (SFE) (Astaire et al., 2003; Barry et al., 2017b; Costa, Elias-Argote, & Jiménez-Flores, 2010; Spence, Jimenéz-Flores, Qian, & Goddik, 2009) has also been combined with membrane separation (Astaire et al., 2003; Spence et al., 2009) to further enrich PL content. However, a PL enrichment level representing more than half the weight of the lipid extract in terms of dry matter achieved by Barry et al. (2017b) as a result of combining their enzymatic hydrolysis/UF process represents the highest known concentration obtained to date form a BM substrate using novel, sustainable and dairy-friendly process technologies.

PLs from a variety of sources are widely utilised in the dairy and food industry due to their emulsification and stabilisation properties e.g. positive effects of heat stability, viscosity and milk powder wettability (Corredig & Dalgleish 1997; Corredig & Dalgleish, 1998; Dewettinck et al., 2008; Roesch et al., 2004; Singh & Tokley, 1990). Furthermore, the numerous health and nutritional benefits associated with PLs have been extensively reviewed (Contarini & Povolo, 2013; Dewettinck et al., 2008; Spitsberg, 2005) along with both in vivo and in vitro investigations of associated effects of PLs on health. PLs have been demonstrated to exhibit anticancer effects, particularly suppression of colon tumours (Berra, Colombo, Scottocornola, & Giacosa, 2002; Hertervig, Nilsson, Cheng, & Duan, 2003; Kuchta-Noctor, Murray, Stanton, Devery, & Kelly, 2016; Parodi, 2003). Other health benefits associated with PLs include myelination of the central nervous system (Oshida et al., 2003), development, activation and regulation of the immune system (Cinque et al., 2003; Mills, Ross, Hill, Fitzgerald, & Stanton, 2011), and reduced absorption of cholesterol (Eckhart, Wang, & Donovan, 2002). PLs have also been reported to have associations with cognitive development and reduced incidence of cognitive decline (Astaire et al., 2003; Kidd, 2000). PS has been utilised for the treatment of age-related diseases such as Alzheimer's disease (Burling & Gaverholt, 2008). Sphingomyelin has been identified as a key PL involved in cognitive development. Recent studies in the field of infant nutrition have demonstrated the positive effects of PLs on the neurodevelopment of term and pre-term infants (Timby, Domellöf, Hernell, Lönnerdal, & Domellöf, 2014).

In vitro work utilising cell models to investigate the association of PLs on neurological development, survival and apoptosis have primarily focused on the effects attributed by docosahexaenoic acid (DHA), a fatty acid side chain linked to the PL structure. Cao, Xue, Xu, and Lie (2005) investigated the effects of DHA on the survival and neurite outgrowth of rat cortical neurons with the authors demonstrating that treatment of the neuronal cells with a 20–50 μM concentration of DHA significantly enhanced neuronal viability with 49% increase in neuritic length compared with 34.70% in the control (Cao et al., 2005). Nerve cells grow by projection in the form of axons and dendrites from the cell body of the neuron (Khodosevich & Monyer, 2011). The growth of axons and dendrites is carefully regulated during neuronal development and PLs, namely glucosylceramide, have been demonstrated to be required for axonal growth in hippocampal cells (Schwarz & Futerman, 1998). Harel and Futerman (1993) determined that inhibition of sphingolipid synthesis in hippocampal cells through treatment with funonisin B1, an inhibitor of dihydroceramide synthesis, reduced axon growth. Schwarz and Futerman (1998) demonstrated that inhibition of sphingolipid metabolism in hippocampal cells significantly reduced the rate of dendrite growth by 15–25% after 3–14 d incubation with fumonisin B1. More recently, Latifi et al. (2016) generated a nanoliposome containing a natural lecithin extracted from salmon heads and demonstrated that the metabolic activity of cortical neurons was significantly increased following a 3 d incubation. The authors also demonstrated that this natural lecithin, containing the major PL, phosphatidylcholine, also promoted neuronal outgrowth and aborization.

The objective of the present study was to evaluate the effect exhibited by a purified dairy lipid extract containing in excess of 50% milk PLs generated by the authors using novel technologies previously outlined (Barry et al., 2017b) in an in vitro assay to investigate the neurotrophic effect on cortical neurons, namely neurite stimulation and outgrowth.

2. Materials and methods

2.1. Materials

Dulbecco's Modified Eagles Medium (DMEM), L-glutamine (200 mM), penicillin–streptomycin, Fetal Calf serum, neurobasal medium, B-27 supplement (50 X), Dulbeccos' Phosphate Buffered Saline (DPBS 1 X), Alexa fluor 488 goat anti- rabbit IgG, Minimum Essential Medium (MEM) without phenol red, and Trypsin-EDTA (0.5%) were sourced from Thermo Fisher Scientific (Gibco, Gaithersburg, MD, USA). Poly-lysine pre-coated 96-well plates, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT), dimethyl sulfoxide and bisBenzimide H 33258 were sourced from Sigma Aldrich (Lyon, France). Polyclonal Beta III tubulin primary antibody was procured from Abcam (Cambridge, United Kingdom) and DNAase was sourced from Roche Diagnostics (Mannheim, Germany). All other chemicals were HPLC grade, >99.9%, and purchased from Sigma Aldrich (Arklow, Ireland).

2.2. Generation of a purified dairy phospholipid extract

A purified dairy phospholipid extract was generated according to the protocol previously outlined by the authors (Barry et al., 2017b). Briefly a buttermilk powder (Tipperary Co-operative Creamery, Tipperary, Ireland) substrate was hydrolysed following reconstitution with the serine endoprotease Alcalase (E.E. 3.4.21.62) (Novozymes, Bagsværd, Denmark) to a degree of hydrolysis of 19%. Conditions for the hydrolysis reaction were as follows: 10% total solids buttermilk powder (BMP) in reverse osmosis (RO) water, at 50 °C and pH 8.00 stat-control by titration with 4 M NaOH. Termination of the hydrolysis reaction was accomplished by means of pH inactivation by addition of 2 M citric acid until the pH had decreased from pH 8.00 to pH 6.50. Following the enzy-

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