Interpretive Summary

New opportunities of cancer prevention through changes in diet regimens can be developed from the increasing knowledge of food components effects on health. Consumption of milk and dairy products, as well as lactoferrin enriched food may prevent the development of breast cancer, by the decrease of cancer cell viability and proliferation, increase of apoptosis levels and decrease of cancer cell migration.

Running title: Lactoferrin & Breast Cancer

The effect of bovine milk lactoferrin on human breast cancer cell lines

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ABSTRACT

The evidence that biologically active food components are key environmental factors affecting the incidence of many chronic diseases is overwhelming. However, the full extent of such components in our diet is unknown, as well as our understanding of their mechanisms of action. Beyond their interaction with the gut and intestinal immune functions, more benefits are being tested for whey proteins such as lactoferrin, namely as anti-cancer agents. Lactoferrin is an iron-binding protein that has been reported to inhibit several types of cancer. In the present work, the effects of bovine milk lactoferrin on human breast cancer HS578T and T47D cells were studied. The cells were either untreated or submitted to lactoferrin concentrations ranging from 0.125 to 125 μM. Lactoferrin decreased 47% and 54% the cell viability of HS578T and T47D, respectively, and increased apoptosis about twofold for both cell lines. Proliferation rates decreased between 40.3 and 63.9% for HS578T and T47D, respectively. T47D cell migration decreased in the presence of the protein. Although the mechanisms of action have still not been unrevealed, the results gathered in this work suggest that lactoferrin interferes with some of the most important steps involved in cancer development.

*Key words:* lactoferrin, breast cancer, viability and proliferation, apoptosis and migration
Milk and dairy products have become recognized as functional foods, suggesting their use has a direct and measurable effect on health outcomes, namely that their consumption has been related with a reduced risk of numerous cancers (Marshall, 2004). Cancer is the second leading cause of mortality worldwide, with an expected 1.4 million persons being diagnosed with breast cancer in 2010. Hence, this is an enormously important health risk, and progress leading to enhanced survival is a global priority (Jemal et al., 2009; Schiff and Osborne, 2005).

Breast cancer, like all the other cancer types, is a result of six essential alterations in normal cells that represent good targets for treatment; the so-called cancer hallmarks (Hanahan and Weinberg, 2000). These include the ability to be self-sufficient in growth signals (tumor cells present reduced dependence on exogenous growth stimulation); to evade anti-growth signs and apoptosis; to achieve endless replicative potential; to sustain angiogenesis; to evade tissues, and to form metastasis. Several strategies have been pursued in the last years in order to hamper the development of tumors or treat existing ones (Hanahan and Weinberg, 2000).

An increasing interest has been reported on the use of biologically active substances from food (Ferguson 2009; Gill et al., 2000; Jacobs et al., 2009; McCabe-Sellers et al., 2009; Perdigon et al., 2002; Tsuda et al., 2004). However, the full set of active components in our diet is unknown and the knowledge on their mechanisms of action is scarce. Proteins and peptides existing in milk have been reported to be cancer preventive agents (Korhonen and Pihlanto, 2006; Rodrigues et al., 2009; Tsuda et al., 2000; Wakabayashi et al., 2006), such as lactoferrin (LF), that is also known for its inhibitory action on cell proliferation, as well as for its anti-inflammatory and antioxidant abilities (Iigo et al., 2009; Legrand et al., 2005; Pan et al., 2007; Rodrigues et al., 2009; Tomita et al., 2002; Tsuda et al., 2002, 2010; Ward et al., 2005).

LF is an iron-binding glycoprotein from the transferrin family that can be found in many different tissues or secretions, such as tears, saliva, blood, secondary granules of neutrophils and milk (Rodrigues et al., 2009; Pan et al., 2007; Tsuda et al., 2002). In vivo studies showed that oral administration of bovine LF to rodents significantly reduces chemically induced tumorigenesis in different organs (breast, esophagus,
tongue, lung, liver, colon and bladder) and inhibits angiogenesis (Iigo et al., 2009; Tsuda et al., 2002). It has been demonstrated that more than 60% of administrated bovine LF survived passage through the adult human stomach and entered the small intestine in an intact form (Troost et al., 2001). Intact and partly intact bovine LF are likely to exert various physiological effects in the digestive tract. Moreover, subcutaneously administration of LF inhibited the growth of implanted solid tumors and exerted preventive effects on metastasis (Bezault et al., 1994).

These activities of LF have been attributed to its immunomodulatory potential and ability to activate T and NK cells (Bezault et al., 1994; Damiens et al., 1999). LF is able to limit the growth of tumor cells and it was shown that addition of exogenous LF to MDA-MB-231 breast cancer cell lines culture media induced the cell cycle arrest at the G1/S transition (Damiens et al., 1999). Also, LF was found to induce growth arrest and nuclear accumulation of Smad-2 in HeLa cells (Zemann et al., 2010). According to Babina and coworkers (2005), LF possesses DNase activity and is cytotoxic, suppressing the growth of several human and mouse cell lines. Recent studies demonstrated that LF has an estrogen element of response (ERE) (Teng, 2006) that confers the protein the ability to interfere with the genetic expression of several molecules that are vital for cell survival. Furthermore, LF was found to induce apoptosis in several human cell lines, as for example A459 lung cells, CaCO-2 intestine cells and HTB9 kidney cells (Hakansson et al., 1995), and to inhibit Akt activation and modulate its downstream proteins phosphorylation in apoptosis of SGC-7901 human stomach cancer cells (Xu et al., 2010). Moreover, LF was effective against melanoma cells (Pan et al., 2007), decreasing the proliferation rates and increasing apoptosis levels. Xiao and coworkers (2004) also reported LF’s inhibitory effects on head and neck cancer cells, down-regulating G1 cyclin-dependent kinases, and therefore influencing the cell cycle. Finally, LF has been reported to suppress cell-induced angiogenesis in mice, and to increase the IL-18 production (Xiao et al., 2004).

Knowledge on the effect of diet components on health will put forward new opportunities for cancer prevention through profound alterations in the diet regimens. Over time, small but recurrent doses of bioactive proteins may prevent the carcinogenic process by decreasing the rate of cell proliferation and growth of cancer cells. In the current work, the effect of a range of LF concentrations at different exposure times on cell viability, proliferation, apoptosis and migration using two model human breast cancer cell lines, HS578S and T47D, was studied. The rationale of using two cell lines
is due to their differences in the estrogen receptor. The cells lacking the estrogen receptor (e.g. HS578T) usually correspond to more aggressive type of tumors for which the existent therapy is not very efficient. Therefore, for these type cells, if LF shows an effect it would represent an important scientific advance. On the other hand, for cells that possess the estrogen receptor (e.g. T47D), if an effect is proved for LF, then a combination with estrogen could be further explored for breast cancer therapeutics.

MATERIALS AND METHODS

Lactoferrin

Lactoferrin (LF) (Sigma-Aldrich Co., St. Louis, MO) was dissolved in phosphate saline buffer (PBS) (PBS: 1g/L NaCl, 0.2g/L KCl, 0.24g/L Na2HPO4.2H2O, 1.805g/L KH2PO4, pH 7.4) in order to obtain different protein solution concentrations (0.125, 6.25, 12.5 and 125 μM). All LF solutions were passed through a 0.2-μm filter prior to use, and stored at 4°C.

Breast Cancer Cell Lines

Breast cancer cell lines, HS578T and T47D, were kindly provided by IPATIMUP (Portugal). The HS578T cell strain was derived from a carcinoma of the breast and is negative for estrogen receptor (ER-). The T47D line was isolated from a pleural effusion obtained from a 54 year old female patient with an infiltrating ductal carcinoma of the breast and is positive for estrogen receptor (ER+). The cells were maintained in an incubator with a 5% CO2 atmosphere and at 37°C. The culture medium used was the Dulbecco’s modified Eagle medium (DMEM) (GIBCO®, Invitrogen, Barcelona, Spain) supplemented with 10% of Fetal Bovine Serum (FBS) (GIBCO®, Invitrogen, Barcelona, Spain) and 1% of penicillin/streptomycin (Invitrogen, Barcelona, Spain).

Cell Viability

Two distinct methods were used to evaluate the cell viability to LF exposure as described below.
**Trypan blue method**

Cells were grown in 6-well plates until a concentration of $1 \times 10^5$ cells per well was achieved. Adequate volumes of the previously prepared LF solutions were added to each well, in order to obtain the required LF concentrations (0.125, 6.25, 12.5 and 125 $\mu$M). Additionally, control wells were included consisting of DMEM medium and PBS (no LF added). The plates were incubated for 24, 48 and 72 hours. Subsequently, the supernatant of each well was collected to a falcon tube. The adhered cells were washed with PBS, trypsinized, and then collected to the respective falcon. All falcons were centrifuged (700 x g, 5 minutes) and the supernatant was discharged. The remaining pellet was resuspended in 100 $\mu$l of PBS. The collected cell volumes were diluted 1:1 with Trypan Blue (TB) (Sigma-Aldrich Co., St. Louis, MO), and the viable and non-viable cells were counted in a Neubauer chamber, using an inverted optical microscope equipped with a 20X objective. The results are expressed as percentage of viable cells as compared to the control and represent an average of 3 independent cultures with 8 wells per concentration in each experiment.

**MTS method**

The cell viability to LF exposure was also determined using the MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) method. A commercial kit was used according to the manufacturer instructions (Promega, PROM G35800001, Lisbon, Portugal). In these experiments, 100 $\mu$l of cell suspension was added to each well of a 96-well plate. Additionally, control wells were included consisting of DMEM medium and PBS. When a cell concentration of $1 \times 10^5$ cells/ml was obtained, adequate volumes of LF solutions were added to the wells and incubated for different exposure times (24, 48 and 72 hours). Afterwards, 20 $\mu$l of the CellTitter 96 AQueous One Solution Cell Proliferation Assay reagent (MTS) was added to each well and left in the incubator (37°C, 5% CO₂) for 3 hours after which the cell viability was quantified by recording the absorbance at 490 nm. The results are expressed as percentage of viable cells compared to the control and represent an average of 3 independent cultures with 8 wells per concentration in each experiment.
Apoptosis

The effect of different LF concentrations (0.125, 6.25, 12.5 and 125 μM) and exposure times (24, 48 and 72 h) in cell apoptosis was accessed using a commercial kit (Promega, PROM G 8091, Lisbon, Portugal) with the same experimental setup as described above. After exposing the cells, in a concentration of 1×10⁴ cells/ml, to LF (37°C, 5% CO₂), 100 μl of Caspase-Glo 3/7 reagent was added to the wells, and after 3 hours standing at room temperature and in the dark, the apoptosis levels were determined by luminescence recording. The average blank value (DMEM medium alone) was 2845 Relative Luminescence Units (RLU) and 642 RLU for the experiments conducted with T47D and HS578T, respectively. The blank value was subtracted from all the other values. The luminescence of the cells in medium (control) lay above this value and is given by 0 μM LF. The results represent an average of 3 independent cultures with 8 wells per concentration in each experiment.

Cell Proliferation

Cell proliferation was determined using the bromodeoxyuridine (5-bromo-2-deoxyuridine, BrdU) (Sigma-Aldrich Co., St. Louis, MO) assay. Briefly, a lamella was placed in each well of a 24-well plate and cells were grown until a cell concentration of 1×10⁵ cells per well was achieved. These experiments were conducted for 12.5 μM LF. After 48 h of exposure to LF, 50 μl of BrdU per ml of culture medium was added to each well and the plates were incubated (37°C, 5% CO₂) for 3 hours. Afterwards, the cells were washed with PBS and left at 4°C in 1ml of paraformaldehyde 1% w/v (prepared in PBS). Subsequently, the fixed cells were treated with HCl 2M at room temperature for 30 minutes. Next, the lamellas were washed with PBS-0.5% Tween20-0.05% bovine serum albumin (BSA), and incubated with anti-BrdU (mouse) (DAKO Denmark, Denmark) at a 1:1 dilution with PBS-0.5% Tween20-0.05% BSA. After 1 h, lamellas were washed twice with PBS-0.5% Tween20-0.05% BSA, and subsequently incubated with anti-mouse Ig fluorescein isothiocyanate (FITC) (DAKO Denmark, Denmark) at a 1:100 dilution in PBS-0.5% Tween20-0.05% BSA for 30 minutes. Finally, the lamellas were washed with PBS-0.5% Tween20-0.05% BSA. The nuclei were counted in a fluorescence microscope (Zeiss HBO-50) using a 40X magnification. The total number
of nuclei was marked with 4',6-diamidino-2-phenylindole (DAPI) (blue) and the ones in proliferation were marked with FITC (green). Proliferation rates were determined as the percentage ratio between the number of nuclei in proliferation and total number of nuclei. The results represent an average of 3 independent cultures and 10 measures for each culture.

**Migration**

A suspension containing $1 \times 10^5$ cells was left to grow in 6-well plates, until 90% confluence was achieved. Next, adhered cells were washed twice with PBS. Afterwards, a wound was inflicted in the center of each well, with the help of a needle, and 3 spots were marked on the wound as reference points. Then, fresh DMEM medium was added to the wells, and also the adequate volume of LF solution to obtain a 12.5 μM concentration. During the 48 h of exposure, pictures were taken at the pre-defined measuring spots using an inverted microscope (Nikon Diaphot 300, Sony, Portugal) equipped with a 20X objective. The images were captured by a video camera (Sony CCD, Sony, Portugal) and processed using the Image Pro-Plus® version 7.0 software (Media Cybernetics, Bethesda, MD) which allowed the measurement of the distance (in micrometers (μm)) over time between the front cells in each edge of the wound. These distances correspond to an average of 3 independent cultures and 9 measures for each culture.

**Statistical Analysis**

Two-tailed unpaired Student’s t-test was used for the statistical evaluation of significant differences among the tested LF concentrations and exposure times as compared to the control. Statistical analyses were performed in Microsoft Office Excel 2007 (Seattle, WA).

**RESULTS**

**Cell Viability**
Trypan blue method

The trypan blue (TB) method enables distinguishing viable cells from dead cells, since dead cells are permeable to the dye and will be colored blue. The results obtained are compiled in Table 1. A decrease over 50% in the number of viable cells was obtained for increasing LF concentrations and exposure times, for both cell lines studied. Based on these experiments, 12.5 μM LF was found to be effective in decreasing T47D cell viability as compared to the control (set at 100%) between 28 and 54%, depending on the exposure time. The differences obtained for the range of LF concentrations studied, as compared to the control, were found to be statistically significant, except for 0.125 μM. Furthermore, the p-values obtained when comparing the different exposure times, namely 24 and 48 h, 48 and 72 h, 24 and 72 h, were 0.06, 0.08 and 0.05, respectively. For the HS578T cell line, the conditions that promoted a more pronounced decrease of cell viability were 72 h exposure to 125 μM LF. Nevertheless, at this exposure time and LF concentration, cellular lysis was observed (data not shown). Comparing to the control, 12.5 μM LF decreased HS578T cell viability between 18 and 47%, depending on the exposure time. The differences obtained for all the LF concentrations studied were found to be statistically significant. Also, comparing the different exposure times, the differences obtained between 24 and 48 h were not statistically significant (p-value = 0.14), while the p-values obtained for 48 and 72 h, and 24 and 72 h comparisons were 0.02 and 0.03, respectively.

MTS method

The MTS assay is assumed to reflect cell number and is used to determine exposure-response relationships. Table 1 presents the results obtained in the MTS assays. Although slightly higher reductions on cell viability were found with this method, especially for 72 h, the tendencies were similar, thus these results were found to be in agreement with the ones obtained with the TB dye method. Statistical significance was found for the comparisons between the different exposure times and the different LF concentrations used.

Apoptosis
LF influence in apoptosis was assessed by measuring caspase 3 and 7 activities, since these are the effectors of the apoptosis machinery. LF increasing concentrations were found to increase such activities for both cell lines, as can be observed in Fig. 1.

Generally, it was found that for a 72 h exposure a decrease in apoptosis occurs. This result coincides with the cellular lysis observed in the cell viability assays as described above. Although cell death was higher for a 72 h exposure to LF, necrosis was found to be the principal cellular death process occurring for this exposure time. For both cell lines, the caspases 3/7 activity increased about 1.5-2 times for the lower LF concentrations studied (except for the concentration 0.125 μM LF in the HS578T assays), being the differences observed, as compared to the control, statistically significant. An increase of about 3-5 times in the apoptotic levels was obtained in the experiments conducted with 125 μM LF. It is important to notice that the differences observed between the different exposure times were in most of the cases not statistically significant.

**Cell Proliferation**

Based on the results from MTT, TB and apoptosis assays, a 48 h exposure to 12.5 μM LF was chosen for the cell proliferation experiments. The effect of LF on cell proliferation was studied using BrdU, a synthetic thymidine analogue that binds to newly formed DNA in S-phase, thus allowing the determination of the nuclei in proliferation. As expected from the previous results, LF was found to decrease the proliferation rates for both cell lines (Fig. 2). Proliferation rate decreases of 35.5% and 52.5% were obtained for HS578T and T47D, respectively. The differences observed for the proliferation rates as compared to the control experiments were found to be statistically significant for both cell lines ($p<0.005$ for HS578T and $p<0.0005$ for T47D).

**Migration**

The LF concentration and exposure time used in the migration experiments were the same as the ones used to study cell proliferation. LF was found to decrease the migration of both cell lines used (Fig. 3 and Fig. 4) being this effect more pronounced for T47D. From the statistical analysis, the differences observed for HS578T cells treated and untreated were not significant ($p = 0.116$). Nevertheless, the differences...
observed for T47D were statistically significant ($p = 0.001$). The distance ($\mu$m) between the front cells in each edge of the wound was higher for the cells exposed to 12.5 $\mu$M LF during 48 h, as compared to the control (with no LF added).

**DISCUSSION**

The role of milk proteins and peptides as physiologically active factors in the diet is being increasingly acknowledged (Marshall, 2004). Environmental factors, such as food and physical activity, have an important influence on the risk of cancer, thus delayed cancer development may be obtained by changing diet regimens. A large amount of data regarding the bioactivity of food components have been obtained using cell lines, and an important question regarding much of this work is the relevance of the concentrations used (Petricoin and Liotta, 2003).

In the current work, the influence of LF on cell viability for both cell lines was studied using two complementary tests, namely the TB dye method and the bio-reduction of the MTS reagent (Table 1). Some cytotoxicity assays allow the immediate interpretation of the results, such as the incorporation of a dye by dead cells, release of $^{51}$Cr or fluorescein from pre-labeled cells. These assays are viability assays and intend to predict survival and not directly assessing it, since they are good for identifying dead cells but can overestimate survival in the long term. Most of these assays involve the cell membrane rupture and cell death. Cell viability is a measure of the metabolic status of a population and gives an indication of its growth potential. One of the simplest methods to evaluate cell viability is the dye exclusion approach, indicating the cellular membrane capacity to exclude a dye. The most commonly used dye is TB, and the method is based on the concept that viable cells do not absorb certain dyes while dead cells are permeable to them. Nevertheless, this method presents some disadvantages, such as also dying soluble proteins; causing stress to the cells, as well as being influenced by the presence of serum. Therefore, in this work a complementary test for assessing cell viability was included. The results obtained showed a decrease over 50% in the number of viable cells for increasing LF concentrations and exposure times, for both cell lines studied. Although the results pointed to 72 hours of exposure to 125 $\mu$M LF to be the most effective conditions regarding a decrease in cell viability, at these conditions cellular lysis was observed. For that reason, and since cellular lysis is
frequently associated with necrosis, such information was taken into consideration for
the choice of the conditions to use (12.5 μM LF was fixed at 48 h) in the following
experiments (proliferation and migration). Once necrosis occurs, the cellular contempt
is spilled into the surrounding tissues, causing inflammation and contamination of the
neighbor cells with carcinogenic products. Therefore, the occurrence of necrosis is a
nonfavorable factor when the goal is treating cancer (Werner et al., 2005).

The results obtained in these experiments are in accordance with data previously
reported for LF effects on other human cancer cell lines (Hakansson et al., 1995; Tsuda
et al., 2004; Xiao et al., 2004). As described above, there are other viability assays, such
as the bio-reduction of the MTS reagent, that measure the metabolic events occurring in
the cell, allowing a more precise quantification since they are more sensitive. These
reductions take place only when reductase enzymes are active, and therefore conversion
is often used as a measure of viable cells. However, these colorimetric assays also
present disadvantages regarding interferences from the culture medium, reversible
metabolic inhibitions and different colorimetric responses for different types of cells.
Also, it is important to keep in mind that other viability tests sometimes give completely
different results, as many different conditions can increase or decrease metabolic
activity. Changes in metabolic activity can give large changes in MTS results while the
number of viable cells is constant. When the amount of purple formazan produced by
cells treated with an agent, such as LF, is compared with the amount of formazan
produced by untreated control cells, the effectiveness of the agent causing death, or
changing metabolism of cells, can be deduced through a dose-response curve. Results
gathered from these experiments were similar to the ones obtained with the TB method.
Furthermore, it is important to notice that, although the higher LF concentration studied
(125 μM) was the most effective, this concentration, considering that a major part of
ingested LF survives passage through the gut (Troost et al., 2001), would represent an
unreasonable daily intake of milk or dairy products. Therefore, 12.5 μM LF was chosen
for the subsequent experiments.

Cell viability usually results from the balance between cell growth/proliferation
and cell death. Also, cell death can occur by one of two different mechanisms, namely
necrosis and/or apoptosis (Werner et al., 2005). Cells undergoing apoptosis are
characterized by cell shrinkage, chromatin condensation, DNA fragmentation and
membrane disassembling (Hakansson et al., 1995; Mader et al., 2005). Two main
pathways have been reported to mediate apoptosis (Mader et al., 2005). One is the death receptor pathway which is triggered by ligand-inducing aggregation of death receptors, such as the Fas protein (Fujita et al, 2004). The second pathway is related with the activation of mitochondria response to cytotoxic drug-induced cellular stress (Mader et al., 2005). The results clearly showed an effect of LF in the apoptotic levels based on the activities of caspase 3 and 7 (effectors of the apoptosis machinery), and confirmed the cellular lysis occurring for the 72 hours of exposure to the higher protein concentration used. According to Sakai and coworkers (2005), pepsin-digested bovine LF influences the apoptosis machinery by activating the JK-SAPK signalling pathway in human oral squamous cell carcinoma cell line SAS. The authors found that treatment with pepsin-digested bovine LF induced cell death with apoptotic nuclear changes preceded by the cleavage of caspase-3 and poly (ADP-ribose) polymerase (PARP) in the apoptotic cells. Moreover, the peptide induced phosphorylation of extracellular signal-regulated kinase (ERK1/2), a member of the MAP kinase family, at early stages of apoptosis. Another MAP kinase, c-Jun N-terminal kinase/stress-activated protein kinase (JNK/SAPK), was also phosphorylated by treatment with pepsin-digested LF. Also, other authors (Hakansson et al., 1995; Roy et al., 2002) reported that LF and related compounds induce the genetic expression of Fas (Fujita et al., 2004), which is involved in apoptosis triggering. Roy and collaborators determined the effects of LF and several protein hydrolysates on the growth of human myeloid leukemic cells (HL-60). In their studies they showed that bovine milk contains biochemical factors with potent cytotoxic properties against some tumor cells and, that the key factors are mostly inactive within the structure of the precursor compounds, but can be released by enzymatic proteolysis. Also, a report from Tomita and collaborators (1994) provided evidence of in vivo production of lactoferricin B by isolating the peptide from the gastrointestinal contents of rats fed a diet containing bovine LF. Based on these evidences, it is likely that the activity of LF in apoptosis and cell growth is due mostly to its major peptide (lactoferricin) and therefore, further studies to clarify the possible mechanisms involved, as well as if LF could be degraded and to what extent in the cells culture medium, are required.

As mentioned previously, cell proliferation is also an important factor affecting cell viability. As expected from the cell viability tests and apoptosis study, proliferation rates were found to decrease for both cell lines when exposed to LF, being this effect more pronounced for T47D. According to Xiao and coworkers (2004), LF has an effect
in the cell cycle machinery, by influencing the genetic expression of some of its key components. LF is known to apprehend cancer cells in the G1-phase or alternatively forcing them to enter the latency phase G0-phase (Ward et al., 2005). The cell cycle machinery is mainly regulated by cyclin-dependent kinases (cdks) that are responsible for the progression through the cell cycle phases (Damiens et al., 1999). In addition, the retinoblastoma protein (pRb) is known to inhibit the cycle progression, although it is frequently phosphorylated by cdks, causing its loss of functionality (Damiens et al., 1999). The mechanism by which LF inhibits cell proliferation may be related with the fact that it inhibits cdks and cyclin E genetic expression (Xiao et al., 2004). Furthermore, LF has been reported to increase the expression of pRb, p21 and p27, known as inhibitors of the cell cycle (Rodrigues et al., 2009; Damiens et al., 1999). Also, it is important to notice that LF possesses an estrogen response element (ERE) in its gene. The ERE is probably a key component for the anti-proliferation effect of LF, as it confers LF the ability to interfere in genetic expression (Stokes et al., 2004).

According to Baumrucker and collaborators (2000), LF also affects the cell growth by interacting with epidermal growth factor binding protein 3 (EGFBP3). EGFBP3 usually binds with the epidermal growth factor (EGF), exerting a protective effect, thus helping cellular growth. Competing with EGF by EGFBP3, LF prevents the stimulation of cell growth. In spite of the information gathered in this preliminary study, further research should be conducted to determine the expression of cell cycle regulatory proteins as a starting point to disclose the mechanisms by which LF exerts its effect in cell proliferation. When a cancer has developed, it is beneficial that food components can slow the rate of tumor growth; therefore LF effect on cell growth rates should be assessed as well as a long-term treatment, since the daily intake of milk and dairy products might not be enough to produce an immediate physiological relevant dose (Freiburghaus et al., 2009).

Cell migration is a key mechanism in the development of metastasis and tissue invasion. Therefore, any agent that can impair cell migration will yield a positive response in cancer treatment and recurrence. Fig. 3 and 4 showed that LF decreases the migration of both cell lines used, even for HS578T cells (Fig. 3) that have a higher migration potential (mainly due to the lack of estrogen receptor expression (ER-)) as compared to T47D (Fig. 4) for which the effect was more pronounced. This may be related with the fact that LF interacts with cadherins and integrins (Von Schlippe et al., 2000), which in part can explain some cell detachment that occurred for T47D cells.
Although these results would be more relevant if a greater effect had been observed for HS578T cells, as these are ER- and correspond to more aggressive type of tumors for which the existent therapy is not very efficient, this study is one of the first assessments on the effect of LF in migration (Giaconetti, 2006; Neve et al., 2006; Ariazi et al., 2002). Moreover, the results obtained for T47D suggest that a combination of estrogen and LF could be further explored for breast cancer therapeutics.

Although further research on the LF effects on breast cancer is required, the findings of this study are very promising, and of particular relevance for the food industry in general. The intake of milk and dairy products, or even food products enriched with LF, may be in the future a natural way of preventing breast cancer or enhancing patients’ treatment.

CONCLUSIONS

In conclusion, from the results gathered in this work it was found that a 48 h exposure to 12.5 \( \mu \)M LF decreases cell viability and proliferation for both cell lines studied. Furthermore, these conditions promoted an increase in apoptosis. Although the mechanisms of LF action are still not fully understood, there is evidence pointing to its ability to interact with some receptors, as well as to modulate genetic expression of several molecules that are vital to the cell cycle and apoptosis machinery. Moreover, LF was shown to decrease the ability of both cell lines to migrate. LF has a great potential to be used in breast cancer treatment.

ACKNOWLEDGMENTS

The authors acknowledge IPATIMUP for kindly providing the breast cancer cell lines used in this work.

REFERENCES


Table 1. Effect of LF treatment (0.125, 6.25, 12.5 and 125 μM) on HS578T and T47D cells for different exposure times (24, 48 and 72 h), by two complementary tests: TB dye method and the bio-reduction of the MTS reagent. For both cell lines and tests, the number of viable cells after treatment with PBS (no LF added) as control for each exposure time was set at 100%. Results represent the percentage of viable cells for each condition. Means (± standard deviation) were calculated from 3 independent cultures and 8 measures for each culture. Significant changes to control: *: \( p < 0.05 \); **: \( p < 0.005 \); ***: \( p < 0.0005 \) (two-tailed unpaired Student’s t-test).

### Trypan Blue Method

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<th>[LF] ( \mu \text{M} )</th>
<th>24 h</th>
<th>48 h</th>
<th>72 h</th>
<th>24 h</th>
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<td>6.25</td>
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<td>75.5 ± 4.0</td>
<td>54.1 ± 5.9</td>
<td>78.5 ± 2.4</td>
<td>69.5 ± 2.4</td>
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<td>12.5</td>
<td>69.1 ± 3.7</td>
<td>81.9 ± 9.2</td>
<td>53.1 ± 6.3</td>
<td>72.4 ± 7.3</td>
<td>59.1 ± 7.3</td>
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<td>125</td>
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<td>45.4 ± 4.9</td>
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### MTS Method

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<td>0.125</td>
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<td>77.1 ± 3.7</td>
<td>30.2 ± 4.1</td>
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<td>47.3 ± 2.6</td>
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FIGURE CAPTIONS

**Figure 1.** Apoptosis: effect of LF treatment (0.125, 6.25, 12.5 and 125 μM) on: (A) HS578T and (B) T47D apoptosis levels for different exposure times (24, 48 and 72 h). Results represent an average of 3 independent cultures and 8 measures for each culture. Significant changes to control: *: p < 0.05; **: p < 0.005; ***: p < 0.0005 (two-tailed unpaired Student’s t-test).

**Figure 2.** Cell proliferation: effect of LF treatment (12.5 μM; 48 h exposure). Control samples (with no LF added) were included for both cell lines. T47D: Control samples: (A) - total nuclei, (B) - nuclei in proliferation; Treated samples: (C) - total nuclei, (D) - nuclei in proliferation. HS578T: Control samples: (E) - total nuclei, (F) - nuclei in proliferation; Treated samples: (G) - total nuclei, (H) - nuclei in proliferation. Magnification used: 40X. Scale bar 10 μm.

**Figure 3.** Migration: effect of LF treatment (12.5 μM; 48 h exposure) on HS578T cells. Exposure time to LF: 0 h - (A); exposure time to LF: 48 h – (B). Table summarizes the distances (μm) over time between the front cells in each edge of the wound and the results represent an average of 3 independent cultures and 9 measures for each culture. Control samples (with no LF added) were included. Magnification used: 40X. Scale bar 10 μm.

**Figure 4.** Migration: effect of LF treatment (12.5 μM; 48 h exposure) on T47D cells. Exposure time to LF: 0 h - (A); exposure time to LF: 48 h – (B). Table summarizes the distances (μm) over time between the front cells in each edge of the wound and the results represent an average of 3 independent cultures and 9 measures for each culture. Control samples (with no LF added) were included. Magnification used: 40X. Scale bar 10 μm.
Figure 1.
Figure 2.
<table>
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Figure 3.
<table>
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<td>87 ± 5</td>
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Figure 4.