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3 **Interpretive Summary**

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

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11 **Running title:** Lactoferrin & Breast Cancer

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15 # 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

INTRODUCTION

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,

76 tongue, lung, liver, colon and bladder) and inhibits angiogenesis (Iigo et al., 2009;
77 Tsuda et al., 2002). It has been demonstrated that more than 60% of administrated
78 bovine LF survived passage through the adult human stomach and entered the small
79 intestine in an intact form (Troost et al., 2001). Intact and partly intact bovine LF are
80 likely to exert various physiological effects in the digestive tract. Moreover,
81 subcutaneously administration of LF inhibited the growth of implanted solid tumors and
82 exerted preventive effects on metastasis (Bezault et al., 1994).
83 These activities of LF have been attributed to its immunomodulatory potential
84 and ability to activate T and NK cells (Bezault et al., 1994; Damiens et al., 1999). LF is
85 able to limit the growth of tumor cells and it was shown that addition of exogenous LF
86 to MDA-MB-231 breast cancer cell lines culture media induced the cell cycle arrest at
87 the G1/S transition (Damiens et al., 1999). Also, LF was found to induce growth arrest
88 and nuclear accumulation of Smad-2 in HeLa cells (Zemann et al., 2010). According to
89 Babina and coworkers (2005), LF possesses DNase activity and; is cytotoxic,
90 suppressing the growth of several human and mouse cell lines. Recent studies
91 demonstrated that LF has an estrogen element of response (ERE) (Teng, 2006) that
92 confers the protein the ability to interfere with the genetic expression of several
93 molecules that are vital for cell survival. Furthermore, LF was found to induce apoptosis
94 in several human cell lines, as for example A459 lung cells, CaCO-2 intestine cells and
95 HTB9 kidney cells (Hakansson et al., 1995), and to inhibit Akt activation and modulate
96 its downstream proteins phosphorylation in apoptosis of SGC-7901 human stomach
97 cancer cells (Xu et al., 2010). Moreover, LF was effective against melanoma cells (Pan
98 et al., 2007), decreasing the proliferation rates and increasing apoptosis levels. Xiao and
99 coworkers (2004) also reported LF's inhibitory effects on head and neck cancer cells,
100 down-regulating G1 cyclin-dependent kinases, and therefore influencing the cell cycle.
101 Finally, LF has been reported to suppress cell-induced angiogenesis in mice, and to
102 increase the IL-18 production (Xiao et al., 2004).
103 Knowledge on the effect of diet components on health will put forward new
104 opportunities for cancer prevention through profound alterations in the diet regimens.
105 Over time, small but recurrent doses of bioactive proteins may prevent the carcinogenic
106 process by decreasing the rate of cell proliferation and growth of cancer cells. In the
107 current work, the effect of a range of LF concentrations at different exposure times on
108 cell viability, proliferation, apoptosis and migration using two model human breast
109 cancer cell lines, HS578S and T47D, was studied. The rationale of using two cell lines

110 is due to their differences in the estrogen receptor. The cells lacking the estrogen
111 receptor (e.g. HS578T) usually correspond to more aggressive type of tumors for which the
112 existent therapy is not very efficient. Therefore, for these type cells, if LF shows an effect it
113 would represent an important scientific advance. On the other hand, for cells that possess the
114 estrogen receptor (e.g. T47D), if an effect is proved for LF, then a combination with
115 estrogen could be further explored for breast cancer therapeutics.
116

117 **MATERIALS AND METHODS**

118

119 ***Lactoferrin***

120 Lactoferrin (**LF**) (Sigma-Aldrich Co., St. Louis, MO) was dissolved in phosphate saline
121 buffer (**PBS**) (PBS: 1g/L NaCl, 0.2g/L KCl, 0.24g/L $Na_2HPO_4.2H_20$, 1.805g/L
122 KH_2PO_4, pH 7.4) in order to obtain different protein solution concentrations (0.125,
123 6.25, 12.5 and 125 μM). All LF solutions were passed through a 0.2-μm filter prior to
124 use, and stored at 4ºC.

125

126 ***Breast Cancer Cell Lines***

127 Breast cancer cell lines, HS578T and T47D, were kindly provided by IPATIMUP
128 (Portugal). The HS578T cell strain was derived from a carcinoma of the breast and is
129 negative for estrogen receptor (**ER-**). The T47D line was isolated from a pleural
130 effusion obtained from a 54 year old female patient with an infiltrating ductal carcinoma
131 of the breast and is positive for estrogen receptor (**ER**+). The cells were maintained in
132 an incubator with a 5% CO_2 atmosphere and at 37ºC. The culture medium used was the
133 Dulbecco's modified Eagle medium (**DMEM**) (GIBCO®, Invitrogen, Barcelona,
134 Spain) supplemented with 10% of Fetal Bovine Serum (**FBS**) (GIBCO®, Invitrogen,
135 Barcelona, Spain) and 1% of penicillin/streptomycin (Invitrogen, Barcelona, Spain).

136

137 ***Cell Viability***

138 Two distinct methods were used to evaluate the cell viability to LF exposure as
139 described below.

Trypan blue method

Cells were grown in 6-well plates until a concentration of 1×10^5 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 μM). 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 resuspensed in 100 μl 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 μl 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 1×10^5 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 μl of the CellTitter 96 AQueous One Solution Cell Proliferation Assay reagent (**MTS**) was added to each well and left in the incubator (37ºC, 5% CO_2) 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.

173 ### *Apoptosis*

174 The effect of different LF concentrations (0.125, 6.25, 12.5 and 125 μM) and exposure
175 times (24, 48 and 72 h) in cell apoptosis was accessed using a commercial kit (Promega,
176 PROM G 8091, Lisbon, Portugal) with the same experimental setup as described above.
177 After exposing the cells, in a concentration of 1×10^4 cells/ml, to LF (37ºC, 5% CO_2),
178 100 μl of Caspase-Glo 3/7 reagent was added to the wells, and after 3 hours standing at
179 room temperature and in the dark, the apoptosis levels were determined by
180 luminescence recording. The average blank value (DMEM medium alone) was 2845
181 Relative Luminescence Units (**RLU**) and 642 RLU for the experiments conducted with
182 T47D and HS578T, respectively. The blank value was subtracted from all the other
183 values. The luminescence of the cells in medium (control) lay above this value and is
184 given by 0 μM LF. The results represent an average of 3 independent cultures with 8
185 wells per concentration in each experiment.

186

187 ### *Cell Proliferation*

188 Cell proliferation was determined using the bromodeoxyuridine (5-bromo-2-
189 deoxyuridine, **BrdU**) (Sigma-Aldrich Co., St. Louis, MO) assay. Briefly, a lamella was
190 placed in each well of a 24-well plate and cells were grown until a cell concentration of
191 1×10^5 cells per well was achieved. These experiments were conducted for 12.5 μM LF.
192 After 48 h of exposure to LF, 50μl of BrdU per ml of culture medium was added to each
193 well and the plates were incubated (37ºC, 5% CO_2) for 3 hours. Afterwards, the cells
194 were washed with PBS and left at 4ºC in 1ml of paraformaldeid 1% w/v (prepared in
195 PBS). Subsequently, the fixed cells were treated with HCl 2M at room temperature for
196 30 minutes. Next, the lamellas were washed with PBS-0.5% Tween20-0.05% bovine
197 serum albumin (**BSA**), and incubated with anti-BrdU (mouse) (DAKO Denmark,
198 Denmark) at a 1:1 dilution with PBS-0.5% Tween20-0.05% BSA. After 1 h, lamellas
199 were washed twice with PBS-0.5% Tween20-0.05% BSA, and subsequently incubated
200 with anti-mouse Ig fluorescein isothiocyanate (**FITC**) (DAKO Denmark, Denmark) at a
201 1:100 dilution in PBS-0.5% Tween20-0.05% BSA for 30 minutes. Finally, the lamellas
202 were washed with PBS-0.5% Tween20-0.05% BSA. The nuclei were counted in a
203 fluorescence microscope (Zeiss HBO-50) using a 40X magnification. The total number

204 of nuclei was marked with 4',6-diamidino-2-phenylindole (**DAPI**) (blue) and the ones in
205 proliferation were marked with FITC (green). Proliferation rates were determined as the
206 percentage ratio between the number of nuclei in proliferation and total number of
207 nuclei. The results represent an average of 3 independent cultures and 10 measures for
208 each culture.

209

210 ***Migration***

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

224

225 ***Statistical Analysis***

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

230

231 **RESULTS**

232

233 ***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

265 LF influence in apoptosis was assessed by measuring caspase 3 and 7 activities, since
266 these are the effectors of the apoptosis machinery. LF increasing concentrations were
267 found to increase such activities for both cell lines, as can be observed in Fig. 1.
268 Generally, it was found that for a 72 h exposure a decrease in apoptosis occurs. This
269 result coincides with the cellular lysis observed in the cell viability assays as described
270 above. Although cell death was higher for a 72 h exposure to LF, necrosis was found to
271 be the principal cellular death process occurring for this exposure time. For both cell
272 lines, the caspases 3/7 activity increased about 1.5-2 times for the lower LF
273 concentrations studied (except for the concentration 0.125 μM LF in the HS578T
274 assays), being the differences observed, as compared to the control, statistically
275 significant. An increase of about 3-5 times in the apoptotic levels was obtained in the
276 experiments conducted with 125 μM LF. It is important to notice that the differences
277 observed between the different exposure times were in most of the cases not statistically
278 significant.
279

280 ***Cell Proliferation***

281 Based on the results from MTT, TB and apoptosis assays, a 48 h exposure to 12.5 μM
282 LF was chosen for the cell proliferation experiments. The effect of LF on cell
283 proliferation was studied using BrdU, a synthetic thymidine analogue that binds to new-
284 formed DNA in S-phase, thus allowing the determination of the nuclei in proliferation.
285 As expected from the previous results, LF was found to decrease the proliferation rates
286 for both cell lines (Fig. 2). Proliferation rate decreases of 35.5% and 52.5% were
287 obtained for HS578T and T47D, respectively. The differences observed for the
288 proliferation rates as compared to the control experiments were found to be statistically
289 significant for both cell lines ($p<0.005$ for HS578T and $p<0.0005$ for T47D).
290

291 ***Migration***

292 The LF concentration and exposure time used in the migration experiments were the
293 same as the ones used to study cell proliferation. LF was found to decrease the
294 migration of both cell lines used (Fig. 3 and Fig. 4) being this effect more pronounced
295 for T47D. From the statistical analysis, the differences observed for HS578T cells
296 treated and untreated were not significant ($p = 0.116$). Nevertheless, the differences

297 observed for T47D were statistically significant (p = 0.001). The distance (μm) between
298 the front cells in each edge of the wound was higher for the cells exposed to 12.5 μM
299 LF during 48 h, as compared to the control (with no LF added).
300

301 **DISCUSSION**

302

303 The role of milk proteins and peptides as physiologically active factors in the diet is
304 being increasingly acknowledged (Marshall, 2004). Environmental factors, such as food
305 and physical activity, have an important influence on the risk of cancer, thus delayed
306 cancer development may be obtained by changing diet regimens. A large amount of data
307 regarding the bioactivity of food components have been obtained using cell lines, and
308 an important question regarding much of this work is the relevance of the
309 concentrations used (Petricoin and Liotta, 2003).
310 In the current work, the influence of LF on cell viability for both cell lines was
311 studied using two complementary tests, namely the TB dye method and the bio-
312 reduction of the MTS reagent (Table 1). Some cytotoxicity assays allow the immediate
313 interpretation of the results, such as the incorporation of a dye by dead cells, release of
314 ^{51}Cr or fluorescein from pre-labeled cells. These assays are viability assays and intend
315 to predict survival and not directly assessing it, since they are good for identifying dead
316 cells but can overestimate survival in the long term. Most of these assays involve the
317 cell membrane rupture and cell death. Cell viability is a measure of the metabolic status
318 of a population and gives an indication of its growth potential. One of the simplest
319 methods to evaluate cell viability is the dye exclusion approach, indicating the cellular
320 membrane capacity to exclude a dye. The most commonly used dye is TB, and the
321 method is based on the concept that viable cells do not absorb certain dyes while dead
322 cells are permeable to them. Nevertheless, this method presents some disadvantages,
323 such as also dying soluble proteins; causing stress to the cells, as well as being
324 influenced by the presence of serum. Therefore, in this work a complementary test for
325 assessing cell viability was included. The results obtained showed a decrease over 50%
326 in the number of viable cells for increasing LF concentrations and exposure times, for
327 both cell lines studied. Although the results pointed to 72 hours of exposure to 125 μM
328 LF to be the most effective conditions regarding a decrease in cell viability, at these
329 conditions cellular lysis was observed. For that reason, and since cellular lysis is

330 frequently associated with necrosis, such information was taken into consideration for
331 the choice of the conditions to use (12.5 μM LF was fixed at 48 h) in the following
332 experiments (proliferation and migration). Once necrosis occurs, the cellular contempt
333 is spilled into the surrounding tissues, causing inflammation and contamination of the
334 neighbor cells with carcinogenic products. Therefore, the occurrence of necrosis is a
335 nonfavorable factor when the goal is treating cancer (Werner et al., 2005).

336 The results obtained in these experiments are in accordance with data previously
337 reported for LF effects on other human cancer cell lines (Hakansson et al., 1995; Tsuda
338 et al., 2004; Xiao et al., 2004). As described above, there are other viability assays, such
339 as the bio-reduction of the MTS reagent, that measure the metabolic events occurring in
340 the cell, allowing a more precise quantification since they are more sensitive. These
341 reductions take place only when reductase enzymes are active, and therefore conversion
342 is often used as a measure of viable cells. However, these colorimetric assays also
343 present disadvantages regarding interferences from the culture medium, reversible
344 metabolic inhibitions and different colorimetric responses for different types of cells.
345 Also, it is important to keep in mind that other viability tests sometimes give completely
346 different results, as many different conditions can increase or decrease metabolic
347 activity. Changes in metabolic activity can give large changes in MTS results while the
348 number of viable cells is constant. When the amount of purple formazan produced by
349 cells treated with an agent, such as LF, is compared with the amount of formazan
350 produced by untreated control cells, the effectiveness of the agent causing death, or
351 changing metabolism of cells, can be deduced through a dose-response curve. Results
352 gathered from these experiments were similar to the ones obtained with the TB method.
353 Furthermore, it is important to notice that, although the higher LF concentration studied
354 (125 μM) was the most effective, this concentration, considering that a major part of
355 ingested LF survives passage through the gut (Troost et al., 2001), would represent an
356 unreasonable daily intake of milk or dairy products. Therefore, 12.5 μM LF was chosen
357 for the subsequent experiments.

358 Cell viability usually results from the balance between cell growth/proliferation
359 and cell death. Also, cell death can occur by one of two different mechanisms, namely
360 necrosis and/or apoptosis (Werner et al., 2005). Cells undergoing apoptosis are
361 characterized by cell shrinkage, chromatin condensation, DNA fragmentation and
362 membrane disassembling (Hakansson et al., 1995; Mader et al., 2005). Two main

363 pathways have been reported to mediate apoptosis (Mader et al., 2005). One is the death
364 receptor pathway which is triggered by ligand-inducing aggregation of death receptors,
365 such as the Fas protein (Fujita et al, 2004). The second pathway is related with the
366 activation of mitochondria response to cytotoxic drug-induced cellular stress (Mader et
367 al., 2005). The results clearly showed an effect of LF in the apoptotic levels based on
368 the activities of caspase 3 and 7 (effectors of the apoptosis machinery), and confirmed
369 the cellular lysis occurring for the 72 hours of exposure to the higher protein
370 concentration used. According to Sakai and coworkers (2005), pepsin-digested bovine
371 LF influences the apoptosis machinery by activating the JK-SAPK signalling pathway
372 in human oral squamous cell carcinoma cell line SAS. The authors found that treatment
373 with pepsin-digested bovine LF induced cell death with apoptotic nuclear changes
374 preceded by the cleavage of caspase-3 and poly (ADP-ribose) polymerase (**PARP**) in
375 the apoptotic cells. Moreover, the peptide induced phosphorylation of extracellular
376 signal-regulated kinase (**ERK1/2**), a member of the MAP kinase family, at early stages
377 of apoptosis. Another MAP kinase, c-Jun N-terminal kinase/stress-activated protein
378 kinase (**JNK/SAPK**), was also phosphorylated by treatment with pepsin-digested LF.
379 Also, other authors (Hakansson et al., 1995; Roy et al., 2002) reported that LF and
380 related compounds induce the genetic expression of Fas (Fujita et al., 2004), which is
381 involved in apoptosis triggering. Roy and collaborators determined the effects of LF and
382 several protein hydrolysates on the growth of human myeloid leukemic cells (HL-60).
383 In their studies they showed that bovine milk contains biochemical factors with potent
384 cytotoxic properties against some tumor cells and, that the key factors are mostly
385 inactive within the structure of the precursor compounds, but can be released by
386 enzymatic proteolysis. Also, a report from Tomita and collaborators (1994) provided
387 evidence of *in vivo* production of lactoferricin B by isolating the peptide from the
388 gastrointestinal contents of rats fed a diet containing bovine LF. Based on these
389 evidences, it is likely that the activity of LF in apoptosis and cell growth is due mostly
390 to its major peptide (lactoferricin) and therefore, further studies to clarify the possible
391 mechanisms involved, as well as if LF could be degraded and to what extent in the cells
392 culture medium, are required.

393 As mentioned previously, cell proliferation is also an important factor affecting
394 cell viability. As expected from the cell viability tests and apoptosis study, proliferation
395 rates were found to decrease for both cell lines when exposed to LF, being this effect
396 more pronounced for T47D. According to Xiao and coworkers (2004), LF has an effect

397 in the cell cycle machinery, by influencing the genetic expression of some of its key
398 components. LF is known to apprehend cancer cells in the G1-phase or alternatively
399 forcing them to enter the latency phase G0-phase (Ward et al., 2005). The cell cycle
400 machinery is mainly regulated by cyclin-dependent kinases (**cdks**) that are responsible
401 for the progression through the cell cycle phases (Damiens et al., 1999). In addition, the
402 retinoblastoma protein (**pRb**) is known to inhibit the cycle progression, although it is
403 frequently phosphorylated by cdks, causing its loss of functionality (Damiens et al.,
404 1999). The mechanism by which LF inhibits cell proliferation may be related with the
405 fact that it inhibits cdks and cyclin E genetic expression (Xiao et al., 2004).
406 Furthermore, LF has been reported to increase the expression of pRb, p21 and p27,
407 known as inhibitors of the cell cycle (Rodrigues et al., 2009; Damiens et al., 1999).
408 Also, it is important to notice that LF possesses an estrogen response element (**ERE**) in
409 its gene. The ERE is probably a key component for the anti-proliferation effect of LF, as
410 it confers LF the ability to interfere in genetic expression (Stokes et al., 2004).
411 According to Baumrucker and collaborators (2000), LF also affects the cell growth by
412 interacting with epidermal growth factor binding protein 3 (**EGFBP3**). EGFBP3 usually
413 binds with the epidermal growth factor (**EGF**), exerting a protective effect, thus helping
414 cellular growth. Competing with EGF by EGFBP3, LF prevents the stimulation of cell
415 growth. In spite of the information gathered in this preliminary study, further research
416 should be conducted to determine the expression of cell cycle regulatory proteins as a
417 starting point to disclose the mechanisms by which LF exerts its effect in cell
418 proliferation. When a cancer has developed, it is beneficial that food components can
419 slow the rate of tumor growth; therefore LF effect on cell growth rates should be
420 assessed as well as a long-term treatment, since the daily intake of milk and dairy
421 products might not be enough to produce an immediate physiological relevant dose
422 (Freiburghaus et al., 2009).

423 Cell migration is a key mechanism in the development of metastasis and tissue
424 invasion. Therefore, any agent that can impair cell migration will yield a positive
425 response in cancer treatment and recurrence. Fig. 3 and 4 showed that LF decreases the
426 migration of both cell lines used, even for HS578T cells (Fig. 3) that have a higher
427 migration potential (mainly due to the lack of estrogen receptor expression (**ER-**)) as
428 compared to T47D (Fig. 4) for which the effect was more pronounced. This may be
429 related with the fact that LF interacts with cadherins and integrins (Von Schlippe et al.,
430 2000), which in part can explain some cell detachment that occurred for T47D cells.

431 Although these results would be more relevant if a greater effect had been observed for
432 HS578T cells, as these are ER- and correspond to more aggressive type of tumors for
433 which the existent therapy is not very efficient, this study is one of the first assessments
434 on the effect of LF in migration (Giacontti, 2006; Neve et al., 2006; Ariazi et al., 2002).
435 Moreover, the results obtained for T47D suggest that a combination of estrogen and LF
436 could be further explored for breast cancer therapeutics.

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

442

443 # CONCLUSIONS

444

445 In conclusion, from the results gathered in this work it was found that a 48 h exposure
446 to 12.5 μM LF decreases cell viability and proliferation for both cell lines studied.
447 Furthermore, these conditions promoted an increase in apoptosis. Although the
448 mechanisms of LF action are still not fully understood, there is evidence pointing to its
449 ability to interact with some receptors, as well as to modulate genetic expression of
450 several molecules that are vital to the cell cycle and apoptosis machinery. Moreover, LF
451 was shown to decrease the ability of both cell lines to migrate. LF has a great potential
452 to be used in breast cancer treatment.
453

454 # ACKNOWLEDGMENTS

455
456
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458

459 # REFERENCES
460

461 Ariazi, E. A., G. M. Clark, and J. E. Mertz. 2002 Estrogen-related receptor alfa and estrogen-
462 related receptor gama associate with unfavorable and favorable biomarkers, respectively, in
463 human breast cancer. Cancer Research 62: 6510-6518
464
465 Babina, E., D. Semenov, V. Buneva, and G. Nevisnkii. 2005. Human milk lactoferrin
hydrolyses nucleoside-5`-triphosphates. Mol. Biol. 39:513-520

Baumrucker, C. 2000. Mammary mechanisms for lactoferrin: interactions with IGFBP3. Biotechnol. Agron. Soc. Environ. 4:5-12

Bezault, J., R. Bhimani, J. Wiprovnick, and P. Furmanski. 1994. Human lactoferrin inhibits growth of solid tumors and development of experimental metastases in mice. Cancer Res. 54:2310 –2312

Damiens, E., I. Yazidi, J. Mazurier, I. Duthile, G. Spik, and Y. Boilly-Marer. 1999. Lactoferrin inhibits G1 cyclin-dependent kinases during growth arrest of human breast carcinoma cells. J. Cell. Biochem. 74:486-498

Ferguson, L. 2009. Nutrigenomics approaches to functional foods. J. Am. Diet. Assoc. 109:452-458

Freiburghaus, C., B. Janicke, H. Lindmark-Månsson, S. M. Oredsson, and M. A. Paulsson. 2009. Lactoferricin treatment decreases the rate of cell proliferation of a human colon cancer cell line. J. Dairy Sci. 92:2477–2484.

Fujita, K. -I, E. Matsuda, K. Sekine, M. Iigo, and H. Tsuda. 2004 Lactoferrin enhances Fas expression and apoptosis in the colon mucosa of azoxymethane-treated rats. Carcinogenesis. 25(10): 1961-1966

Giacontti V. 2006. Breast cancer markers. Cancer Lett. 243:145-159

Gill, H. S., F. Doull, K. J. Rutherfurd, and M. L. Cross. 2000. Immunoregulatory peptides in bovine milk. Br. J. Nutr. 84 (Suppl. 1):S111–S117

Hakansson, A., B. Zhivotovski, S. Orrenius, H. Sabharwal, and C. Svanbory. 1995. Apoptosis induced by a human milk protein. Proc. Natl. Ac. Sci. 92:8064-8068

Hanahan, D., and R. Weinberg. 2000. The hallmarks of cancer. Cell 100:57-70

Iigo, M., D. Alexander, N. Long, J. Xu, K. Fukamachi, M. Futakuchi, M. Takase, and H. Tsuda. 2009. Anticarcinogenesis pathways activated by bovine lactoferrin in the murine small intestine. Biochimie 91:86-101

Jacobs, D., M. Gross, and L. Tapsell. 2009. Food synergy: an operational concept for understanding nutrition. Am. J. Clin. Nutr. 89:1543S-1548S

Jemal, A., R. Siegel, E. Ward, Y. Hao, J. Xu, and M. J. Thun. 2009. Cancer Statistics, 2009. CA Cancer J. Clin. 59:225-249

Korhonen, H., and A. Pihlanto. 2006. Bioactive peptides: Production and functionality. Int. Dairy J. 16:945–960

Legrand, D., E. Elass, M. Carpentier, and J. Mazurier. 2005. Lactoferrin: a modulator of immune and inflammatory responses. Cell. Mol. Life Sci. 62:2549–2559

Mader, J., J. Salsman, D. Conrad, and D. Hoskin. 2005. Bovine lactoferricin selectively induces apoptosis in human leukaemia and carcinoma cell lines. Mol. Cancer Ther. 4:612-624

Marshall, K. 2004. Therapeutic applications of whey protein. Alt. Med. Rev. 9(2):136-156

McCabe-Sellers, B., C. Chenard, D. Lovera, C. Champagne, M. Bogle, and J. Kaput. 2009. Readiness of food composition databases and food component analysis systems for nutrigenomics. J. Food Comp. Anal. 22:S57-S62

Neve, R. M., K. Chin, J. Fridlyand, J. Yeh, F. L. Baehner, T. Fevr, L. Clark, N. Bayani, J. –P. Coppe, F. Tong, T. Speed, P. T. Spellman, S. DeVries, A. Lapuk, N. J. Wang, W. –L. Kuo, J. L. Stilwell, D. Pinkel, D. G. Albertson, F. M. Waldman, F. McCormick, R. B. Dickson, M. D. Johnson, M. Lippman, S. Ethier, A. Gazdar, and J. W. Gray. 2006. A collection of breast cancer cell lines for the study of functionally distinct cancer subtypes. Cancer Cell 10:515-527

511
512 Pan, Y., M. Rowney, P. Guo, and P. Hobman. 2007. Biological properties of lactoferrin: an overview. Aust. J. Dairy Technol. 62:31-42
513
514 Perdigon, G., A. De Moreno de LeBlanc, J. Valdez, and M. Rachid. 2002. Role of yoghurt in the prevention of colon cancer. Eur. J. Clin. Nutr. 56:65–68

515 Petricoin, E., and L. Liotta. 2003. Clinical applications of proteomics. J. Nutr. 133:2476–2484
516
517 Rodrigues, L.R., J. Teixeira, F. Schmitt, M. Paulsson, and H. Lindmark-Mansson. 2009. Lactoferrin and cancer prevention. Crit. Rev. Food Sci. Nutr. 49(3):203-217
518
519 Roy, M., Y. Kuwabara, K. Hara, Y. Watanabe, and Y. Tamai. 2002. Peptides from the N-
520 terminal end of bovine lactoferrin induces apoptosis in human leukemic (HL60) cells. J. Dairy Sci. 85:2065-2074
521
522 Sakai, T., Y. Banno, Y. Kato, Y. Nazawa, and M. Kawaguchi. 2005. Pepsin-digested bovine
523 lactoferrin induces apoptotic cell death with JNK/SAPK activation in oral cancer. J. Pharmacol. Sci. 98:41-48
524
525 Schiff, R., and C. Osborne. 2005. New insight into estrogen receptor α function and its implication for endocrine therapy resistance in breast cancer. Breast Cancer Res. 7:208-211
526
527 Stokes, K., B. Alston-Mills, and C. Teng. 2004. Estrogen response element and the promoter
528 context of the human and mouse lactoferrin genes influence estrogen receptor alpha-mediated transactivation activity in mammary gland cells. J. Mol. Endocrinol. 83:315-334
529
Teng, C. 2006. Factors regulating lactoferrin gene expression. Biochem. Cell Biol. 84:263-267
530
531 Tomita, M., H. Wakabayashi, K. Yamauchi, S. Teraguchi, and H. Hayasawa. 2002. Bovine
532 lactoferrin and lactoferricin derived from milk: production and applications. Biochem. Cell Biol. 80:109-112

533 Tomita, M., M. Takase, W. Bellamy, and S. Shimura. 1994. A review: the active peptide of
534 lactoferrin. Acta Paediatr. Jpn. 36:585

535 Troost, F. J., J. Steijns, W. H. M. Saris, and R.-J. M. Brummer. 2001. Gastric digestion of
536 bovine lactoferrin in vivo in adults. J. Nutr. 131:2101–2104
537
538 Tsuda, H., K. Sekine, K. Fujitaand, and M. Iigo. 2002. Cancer prevention by bovine lactoferrin
539 and underlying mechanisms - a review of experimental and clinical studies. Biochem. Cell Biol. 80:131-136
540
541 Tsuda, H., K. Sekine, Y. Ushida, T. Kuhara, N. Takasuka, M. Iigo, B. S. Hans, and M. A.
542 Moore. 2000. Milk and dairy products in cancer prevention: focus on bovine lactoferrin. Mutat. Res. 462:227-233

543 Tsuda, H., T. Kozu, G. Iinuma, Y. Ohashi, Y. Saito, D. Saito, T. Akasu, D. B. Alexander, M.
544 Futakuchi, K. Fukamachi, J. Xu, T. Kakizoe, and M. Iigo. 2010. Cancer prevention by bovine
545 lactoferrin: from animal studies to human trial. Biometals 23:399–409
546
547 Tsuda, H., Y. Ohshima, H. Nomoto, K. Fujita, E. Matsuda, M. Iigo, N. Takasuka, and M. A.
548 Moore. 2004. Cancer prevention by natural compounds. Drug Metab. Pharmacokinet. 19:245-263
549
550 Von Schlippe, M., J. F. Marshall, P. Perry, M. Stone, A. J. Zhu, and I. R. Hart. 2000. Functional
551 interaction between E-cadherin and alphav-containing integrins in carcinoma cells. J. Cell Sci. 113:425-437
552
553 Wakabayashi, H., K. Yamauchi, and M. Takase. 2006. Lactoferrin research, technology and applications. Int. Dairy J. 16:1241–1251.
554
555 Ward, P., E. Paz, and O. Conneely. 2005. Multifunctional roles of lactoferrin: a critical overview. Cell. Mol. Life Sci. 62:2540-2548

Werner, H., J. Franke, and R. Vermes. 2005. Apoptosis and proliferation in breast cancer cells cultured in vitro: effects on SERMs. Climacteric 83:294-299

Xiao, Y., C. Monitto, K. Minhas, and D. Sidranski. 2004. Lactoferrin down-regulates G1 cyclin-dependent kinases during growth arrest of head and neck cancer cells. Clin. Cancer Res. 10:8683-8686

Xu, X. X., H. R. Jiang, H. B. Li, T. N. Zhang, Q. Zhou, and Q. Liu. 2010. Apoptosis of stomach cancer cell SGC-7901 and regulation of Akt signaling way induced by bovine lactoferrin. J. Dairy Sci. (6):2344-2350

Zemann, N., P. Klein, E. Wetzel, F. Huettinger, and M. Huettinger. 2010. Lactoferrin induces growth arrest and nuclear accumulation of Smad-2 in HeLa cells. Biochimie 92:880-884

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

[LF]	HS578T			T47D		
μM	24 h	48 h	72 h	24 h	48 h	72 h
0.125	92.9 ± 6.0 *	91.6 ± 3.9 **	41.6 ± 8.6 **	95.6 ± 5.2 *	93.4 ± 5.2	97.9 ± 5.7
6.25	75.4 ± 7.3 *	75.5 ± 4.0 ***	54.1 ± 5.9 **	78.5 ± 2.4 **	69.5 ± 2.4 ***	59.3 ± 3.1 ***
12.5	69.1 ± 3.7 ***	81.9 ± 9.2 *	53.1 ± 6.3 **	72.4 ± 7.3 **	59.1 ± 7.3 ***	46.3 ± 1.7 ***
125	40.6 ± 5.8 ***	45.4 ± 4.9 ***	13.6 ± 2.5 ***	43.2 ± 8.6 **	41.6 ± 8.6 **	18.1 ± 3.6 ***

MTS Method

[LF]	HS578T			T47D		
μM	24 h	48 h	72 h	24 h	48 h	72 h
0.125	88.5 ± 5.3 *	77.1 ± 3.7 ***	30.2 ± 4.1 ***	82.3 ± 4.3 **	53.9 ± 2.7 **	28.4 ± 3.2 ***
6.25	60.6 ± 4.1 **	50.3 ± 1.9 ***	4.5 ± 3.3 ***	75.3 ± 3.2 ***	40.9 ± 4.3 ***	11.3 ± 2.9 ***
12.5	67.8 ± 2.2 ***	39.5 ± 2.2 ***	17.5 ± 7.1 ***	63.7 ± 5.1 **	32.4 ± 5.3 ***	8.2 ± 2.3 ***
125	25.7 ± 3.5 ***	13.3 ± 4.4 ***	0.1 ± 3.2 ***	47.3 ± 2.6 ***	21.3 ± 4.2 ***	7.3 ± 4.1 ***

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594 **FIGURE CAPTIONS**
595
596 **Figure 1.** Apoptosis: effect of LF treatment (0.125, 6.25, 12.5 and 125 μM) on: (A)
597 HS578T and (B) T47D apoptosis levels for different exposure times (24, 48 and 72 h).
598 Results represent an average of 3 independent cultures and 8 measures for each culture.
599 Significant changes to control: *: $p < 0.05$; **: $p < 0.005$; ***: $p < 0.0005$ (two-tailed
600 unpaired Student's t-test).
601
602 **Figure 2.** Cell proliferation: effect of LF treatment (12.5 μM; 48 h exposure). Control
603 samples (with no LF added) were included for both cell lines. T47D: Control samples:
604 (A) - total nuclei, (B) - nuclei in proliferation; Treated samples: (C) - total nuclei, (D) -
605 nuclei in proliferation. HS578T: Control samples: (E) - total nuclei, (F) - nuclei in
606 proliferation; Treated samples: (G) - total nuclei, (H) - nuclei in proliferation.
607 Magnification used: 40X. Scale bar 10 μm.
608
609 **Figure 3.** Migration: effect of LF treatment (12.5 μM; 48 h exposure) on HS578T cells.
610 Exposure time to LF: 0 h - (A); exposure time to LF: 48 h – (B). Table summarizes the
611 distances (μm) over time between the front cells in each edge of the wound and the
612 results represent an average of 3 independent cultures and 9 measures for each culture.
613 Control samples (with no LF added) were included. Magnification used: 40X. Scale bar
614 10 μm.
615
616 **Figure 4.** Migration: effect of LF treatment (12.5 μM; 48 h exposure) on T47D cells.
617 Exposure time to LF: 0 h - (A); exposure time to LF: 48 h – (B). Table summarizes the
618 distances (μm) over time between the front cells in each edge of the wound and the
619 results represent an average of 3 independent cultures and 9 measures for each culture.
620 Control samples (with no LF added) were included. Magnification used: 40X. Scale bar
621 10 μm.

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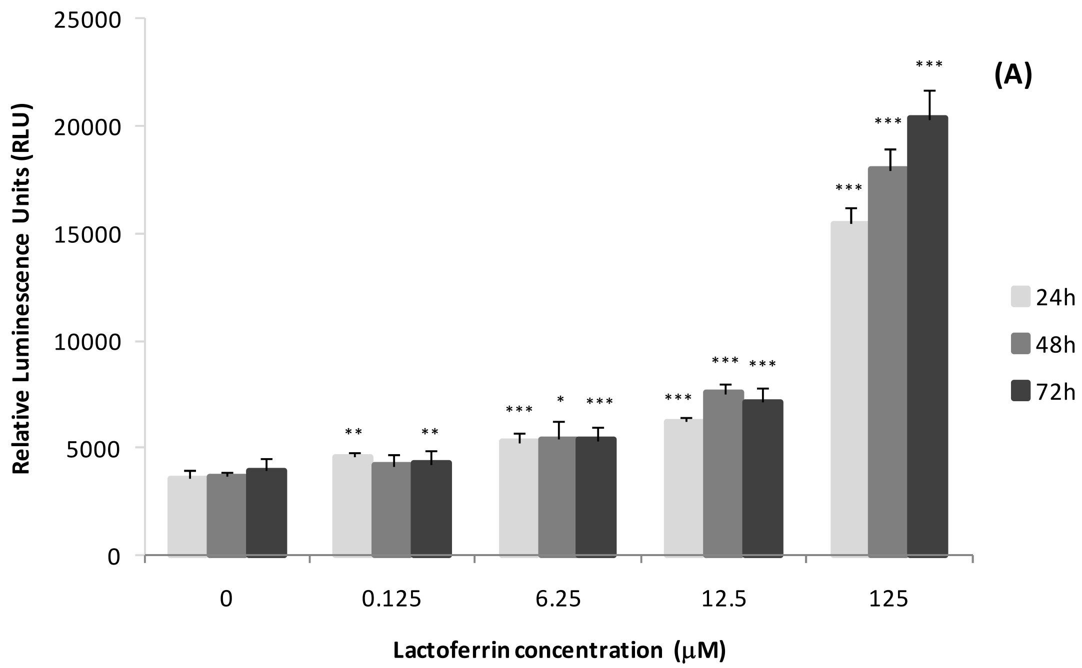

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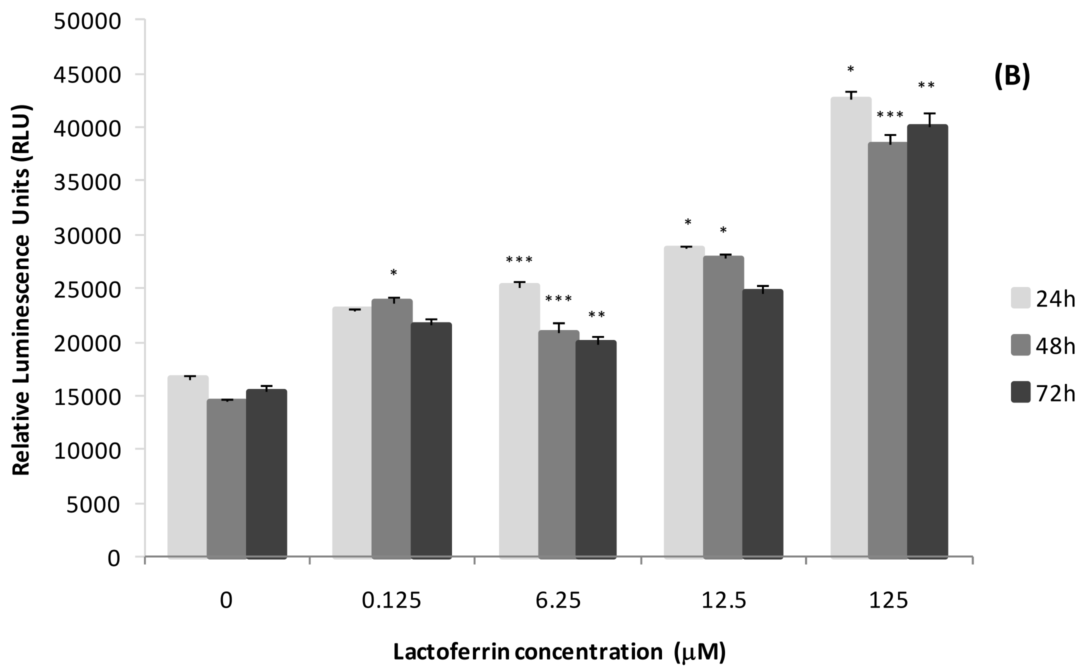

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Figure 1.

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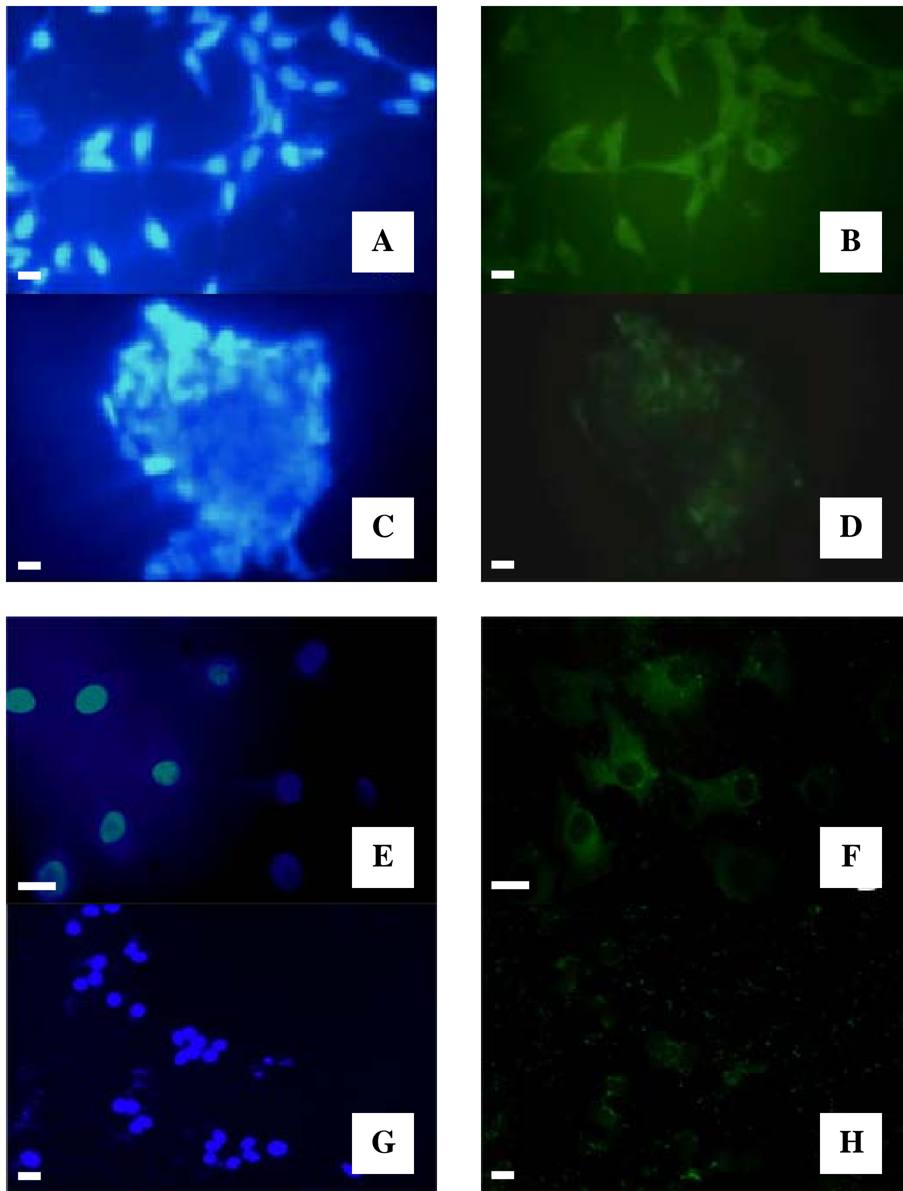

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Figure 2.

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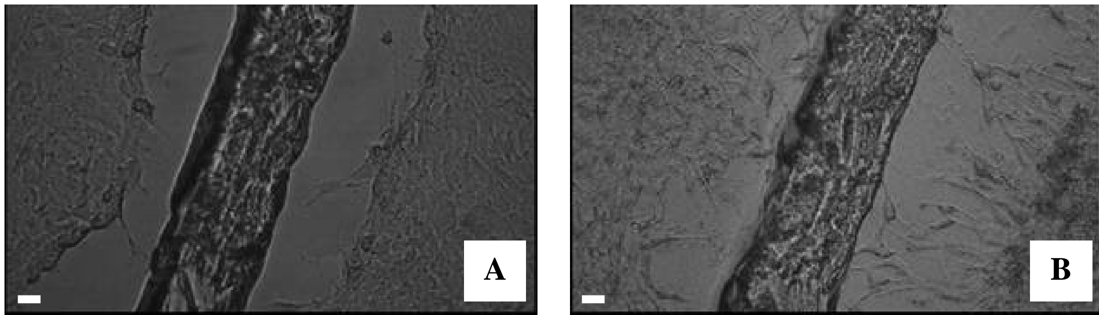

Time (h)	[Distance μm] 0 μM LF	12.5 μM LF
0	179 ± 2	167 ± 3
3	172 ± 1	162 ± 3
6	100 ± 3	116 ± 4
9	98 ± 2	109 ± 1
24	72 ± 3	82 ± 6
27	50 ± 12	48 ± 2
30	20 ± 10	27 ± 2
34	13 ± 6	26 ± 4
48	0 ± 1	6 ± 3

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Figure 3.

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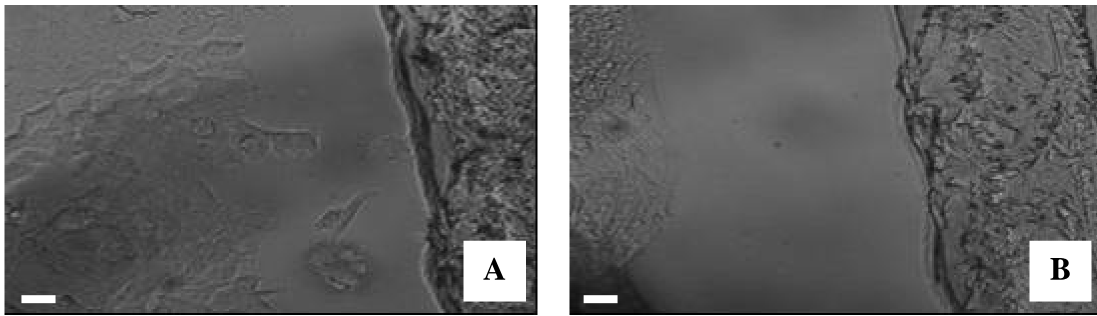

Time (h)	[Distance μm] 0 μM LF	12.5 μM LF
0	382 ± 8	380 ± 3
3	372 ± 19	379 ± 2
6	257 ± 14	366 ± 3
9	222 ± 12	373 ± 9
24	166 ± 12	380 ± 10
27	116 ± 13	390 ± 6
30	103 ± 9	377 ± 6
34	87 ± 5	377 ± 4
48	87 ± 5	386 ± 4

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Figure 4.