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Bovine colostrum promotes growth and migration of the human keratinocyte HaCaT cell line

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Abstract

Bovine colostrum represents a rich source of growth factors, which are known to play a central role in wound healing. The aim of our study was to investigate the possible mitogenic and motogenic effects induced by colostrum on human keratinocytes. Cell proliferation evaluated by 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide test and 5-Bromo-2′-deoxyuridine incorporation revealed that colostrum exerts a growth promoting activity. Scratch assay and immunofluorescence of actin cytoskeleton showed its effectiveness also in inducing cell migration. Furthermore, colostrum treatment increases the levels of tyrosine phosphorylated proteins and the activated forms of the extracellular signal-regulated kinases 1 and 2 and such effects appear to be repressed by the tyrosine kinase inhibitor genistein. Our results indicate that the biological activities of colostrum are specifically mediated by the growth factor-induced activation of tyrosine kinase receptors and underline the relevance of the synergistic action exerted by the growth factors in stimulating keratinocyte proliferation and migration essential for tissue repair.

Keywords: *bovine colostrum, growth factors, wound healing*

Introduction

Cutaneous wound healing is a complex process characterized by strictly regulated cellular and molecular events and includes an inflammatory response, cell migration and proliferation, neoangiogenesis and tissue remodelling with the aim to restore tissue integrity in the wounded area (Singer and Clark 1999; Santoro and Gaudino 2005). Several molecules and growth factors released by the different skin cell types play a central role in this process facilitating wound closure (Werner and Grose 2003; Barrientos et al. 2008). Particularly, the binding of these growth factors to their specific receptors on the cell membrane, results in the activation of intracellular signalling pathways, which control cell functions such as motility, proliferation and differentiation essential for promoting tissue repair. It is well known that altered release of such molecules is often responsible for impaired wound healing (Cooper et al. 1994; Mast and Schultz 1996; Beer et al. 1997) and it has been reported that the treatment with exogenous growth factors improves the healing process both *in vitro* and *in vivo* (Brown et al. 1989; Robson et al. 1992; Jaschke et al. 1999; Smiell et al. 1999). Since there is a coordinated modulation of several growth factors, which act synergistically in the wounded area, it has been shown that the application of a mixture of growth factors appears more effective than the single factor (Lynch et al. 1989; Hennessey et al. 1990; Greenhalgh et al. 1993; Brown et al. 1994; Gope 2002).

Bovine colostrum is the milk produced for few days after the birth and represents a natural source of biologically active substrates and molecules such as vitamins, immunoglobulins, cytokines, hormones and growth factors (Hironaka et al. 1997; Pakkanen and

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Aalto 1997; Playford et al. 2000). Previous studies have shown that bovine colostrum promotes growth and migration of rat intestinal epithelial cells, human colonic carcinoma cell lines and human foetal small intestinal cell line (Playford et al. 1999; Purup et al. 2007; An et al. 2009). It has been reported that it is effective in stimulating the growth of NIH3T3 and normal canine dermal fibroblasts (Hironaka et al. 1997; Torre et al. 2006) and the migration of human fibroblasts in type I collagen gels (Takayama et al. 2001). The ability of colostrum to affect such cellular activities appears mainly correlated to the high concentration of several growth factors including insulin like growth factors (IGFs), epidermal growth factor (EGF), basic fibroblast growth factor-like growth factor, transforming growth factor β (TGF-β) and platelet-derived growth factor (PDGF).

The aim of the present study was to evaluate the biological effects of bovine colostrum collected within the first hour after parturition, on keratinocytes using the human HaCaT cell line as a model system. We first analysed the growth-promoting activity of increasing concentrations of colostrum by both the MTT and the BrdU incorporation assays. We also determined cell migration induced by colostrum using the scratch assay and the modifications of the actin cytoskeleton by immunofluorescence analysis. In addition, to study if the effects exerted by colostrum could be primarily ascribed to the growth factor-mediated activation of tyrosine kinase receptors (TKRs), we analysed the overall levels of tyrosine phosphorylated proteins and the specific activation of extracellular signal-regulated kinase (ERK1/2) by western blot. Finally, we used the tyrosine kinase inhibitor genistein to better determine the specific role of the growth factor receptor activation. Our results underline the main role of the combination of growth factors contained in colostrum to stimulate keratinocyte growth and migration confirming its beneficial effects to improve wound healing.

Material and methods

Cell line and treatments

The human keratinocyte HaCaT cell line, spontaneously immortalized from a primary culture of keratinocytes, was cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% foetal bovine serum (FBS) plus antibiotics. Bovine colostrum used for the experimental procedures was colostrum H1™ (provided by Sirton Pharmaceuticals, Como, Italy), which is collected within the first hour after parturition according to specific procedures to assure its integrity and activity and immediately frozen. For all the experiments, it was diluted in cultured medium plus antibiotics and passed through a microfilter of 0.2 μm pore. For the treatments lasting 24 and 48 h, cells were serum starved for 24 h and then incubated with different concentrations of colostrum (ranging from 0.5 to 20%) diluted in culture medium without FBS. For the short treatment experiments, cells were serum starved as above and then incubated with 10% colostrum for 15′, 30′, 1 or 3 h and maintained at 37°C in medium without FBS until the 24th hour. Cells grown in the presence or absence of 10% FBS were used as positive and negative controls, respectively. For tyrosine kinase inhibition experiments, cells were pre-incubated with genistein (Sigma Chemicals, St Louis, MO, USA; 100 μM) for 30 min before the treatment with colostrum for the indicated time points.

Cell growth

Cell growth was evaluated by both the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) test and the 5-Bromo-2′-deoxyuridine (BrdU; Sigma) incorporation assay. For MTT test, HaCaT cells, seeded onto 24 well plates, were treated as described above and maintained for additional 24 h at 37°C. Cells were then incubated with MTT (1 mg/ml) for 2 h at 37°C and lysed in dimethyl sulfoxide. The absorbance at 570 nm was measured by a μQuant™ Microplate Spectrophotometer (BioTek Instruments, Inc., VT, USA). The results represent the mean value ± standard deviation (SD) of three different experiments performed in triplicates. Statistical significative differences were evaluated using the Student's *t*-test. For BrdU incorporation assay, cells grown on coverslips previously coated with 2% gelatin onto 24-well plates were treated with 5–10% colostrum and 10% FBS for 24 h and incubated with 100 μM BrdU for 1 h at 37°C. Cells were then fixed in 4% paraformaldehyde in Phosphate buffered saline (PBS) for 30 min at 25°C followed by a treatment of 0.1 M glycine for 20 min and with 0.5% HCl, 0.1% Triton X-100 for an additional 45 min to allow permeabilization. After washes in PBS, cells were buffered with 0.1 M $Na_2B_4O_7$ and incubated with anti-BrdU monoclonal antibody (1:50 in PBS; Sigma) for 30 min at 25°C followed by goat anti-mouse IgG-FITC (1:40 in PBS). The percentage of BrdU positive HaCaT cells was analysed counting for each condition a total of 500 cells randomly observed from 10 microscopic fields in three different experiments and expressed as mean value ± SD.

Scratch wound assay

HaCaT cells were seeded on 35 mm plates and allowed to grow until confluence. Cells were then serum starved for 24 h and scratched to create a standardized cell-free area by scraping the monolayer with a 200 μl pipette tip, as previously described (Cha et al. 1996). After extensive washes, cells were incubated with 5–10% colostrum and 10% FBS for 24 h. Cells were then fixed with 4% paraformaldehyde

for 30 min at 25°C at different time points (T0 immediately after the scratch corresponding to the T0 control and after 6, 12 and 24 h) and images were recorded using an Axiovert 25 inverted microscope (Carl Zeiss, Oberkochen, Germany) and a Power Shot G5 digital camera (Canon, Inc., Tokyo, Japan). Migration was quantitated by measuring the recovered scratch area. The data presented are a mean of triplicate experiments ± SD.

Immunofluorescence

For immunofluorescence staining of actin cytoskeleton, HaCaT cells fixed in 4% paraformaldehyde in PBS for 30 min at 25°C and permeabilized with 0.1% Triton X-100 in PBS for 5 min were incubated with TRITC-phalloidin (1:100 in PBS; Sigma Chemicals). Nuclei were visualized using 4′,6-diamido-2-phenylindole dihydrochloride (DAPI; 1:10,000 in PBS) (Sigma Chemicals). Fluorescence signals were analysed by recording stained images using a CCD camera (Zeiss).

Western blot

Cells were lysed in RIPA buffer (10 mM Tris pH 7.4, 50 mM NaCl, 1 mM EDTA, 10 mM KCl, 1% NP40, 0.1% SDS, 0.05% Tween 20) supplemented with protease inhibitors (10 μg/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin) and phosphatase inhibitors (25 mM sodium orthovanadate, 20 mM sodium pyrophosphate, 0.5 M NaF). Fifty micrograms of total proteins were resolved under reducing conditions by 10% SDS-PAGE and transferred to reinforced nitrocellulose (BA-S 83, Scheleider & Schuell, Keene, NH, USA). The membrane was blocked with 10% bovine serum albumin (BSA) in Tris-buffered saline (TBS) 0.05% Tween-20 (TBS-T) for 1 h at room temperature and incubated overnight at 4°C with anti-phosphotyrosine monoclonal antibody diluted 1:500 (4G10, Upstate Biotechnology, Lake Placid, NY, USA) or p-ERK monoclonal antibody (E-4, Santa Cruz Biotechology, Santa Cruz, CA, USA) diluted 1:500 in TBS-T with 5% BSA, followed by ECL detection. Densitometric analyis was performed using the GS-800 Calibrated Image Densitometer (Bio-Rad Laboratories Srl, Milan, Italy).

Results and discussion

Cell proliferation induced by bovine colostrum

The treatment with 5 and 10% of colostrum for 24 h induced a significant ($p < 0.01$) promotion of keratinocytes proliferation evaluated by MTT test, in comparison to that observed for the cells grown in serum free medium. The mitogenic effect was similar to that of cells grown in 10% FBS up to 24 h, whereas it was not detected after 48 h (Figure 1). At lower

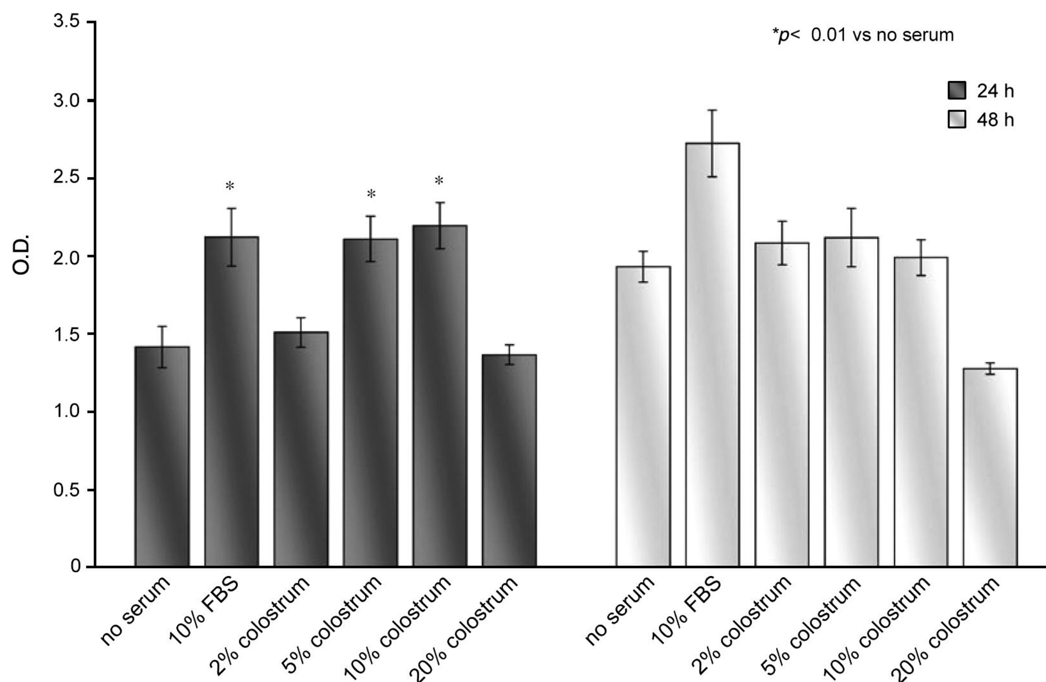

Figure 1. Cell growth evaluated by MTT assay on HaCaT cells treated with increasing concentrations of colostrum for 24 and 48 h. Compared to serum-starved cells, colostrum promotes growth activity similarly to 10% FBS when added at the concentration of 5 and 10% for 24 h, whereas lower or higher concentrations do not show any significative effects. The treatment with colostrum prolonged to 48 h does not induce any significant variations in cell growth compared to serum-starved cells. Results represent the mean values ± SD from three different experiments in triplicate. Student's t-test was performed to evaluate significative differences. (*, $p < 0.01$ vs. no serum).

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concentrations (0.5–1%, data not shown, and 2%) no significant modification of the growth rate was observed at either 24 or at 48 h. On the contrary, at the highest concentration tested (20%), cell growth was comparable to that of serum-starved condition at 24 h and an inhibitory effect appeared at 48 h (Figure 1). The cell growth promoting activity of bovine colostrum has been previously described (Klagsbrun 1980; Steimer et al. 1981; Ramirez et al. 1990; Pakkanen et al. 1992; Playford et al. 1999; Purup et al. 2007; An et al. 2009), although the range of concentrations able to stimulate cell proliferation are variable among the studies, likely due to the different cell models and/or the colostrum preparations used. In addition, our results are consistent with previous data on Madin–Darby canine kidney epithelial cells (MDCK) and on mouse hybridoma cells reporting a poor cell adhesion and growth at high concentrations of colostrum (Klagsbrun 1980; Steimer et al. 1981; Ramirez et al. 1990; Pakkanen et al. 1992).

To better analyse the growth promoting effect induced by 5 and 10% colostrum for 24 h, we performed the BrdU incorporation assay. The immunofluorescence analysis and the parallel phase contrast microscopy showed that colostrum induced an increase in the number of cells with nuclei positively stained for BrdU (Figure 2(c)–(f)) compared to serum-starved ones (Figure 2(a),(b)) and similar to those observed in cells grown in 10% FBS (Figure 2(g),(h)). Quantitative analysis performed counting the percentage of BrdU positive nuclei confirmed a marked increase of proliferating cells after treatment with both 5% (59.8 ± 2%) and 10% colostrum (59.2 ± 3.4%) compared to no serum (38.4 ± 4.8%) and comparable to that observed with 10% FBS (66 ± 9.2%). The growth promoting activity of bovine colostrum, already shown in different cell types, has been mainly correlated to its high content of bioactive molecules, including growth factors (Hironaka et al. 1997; Playford et al. 1999; Torre et al. 2006; Purup et al. 2007; An et al. 2009). In addition, the synergistic action of the numerous growth factors present in the colostrum preparation is probably responsible for decreasing non-steroidal anti-inflammatory drug (NSAID)-induced gut injury in animal models and humans and for promoting mucosal healing in patients with distal colitis (Playford et al. 1999; Playford et al. 2001; Khan et al. 2002).

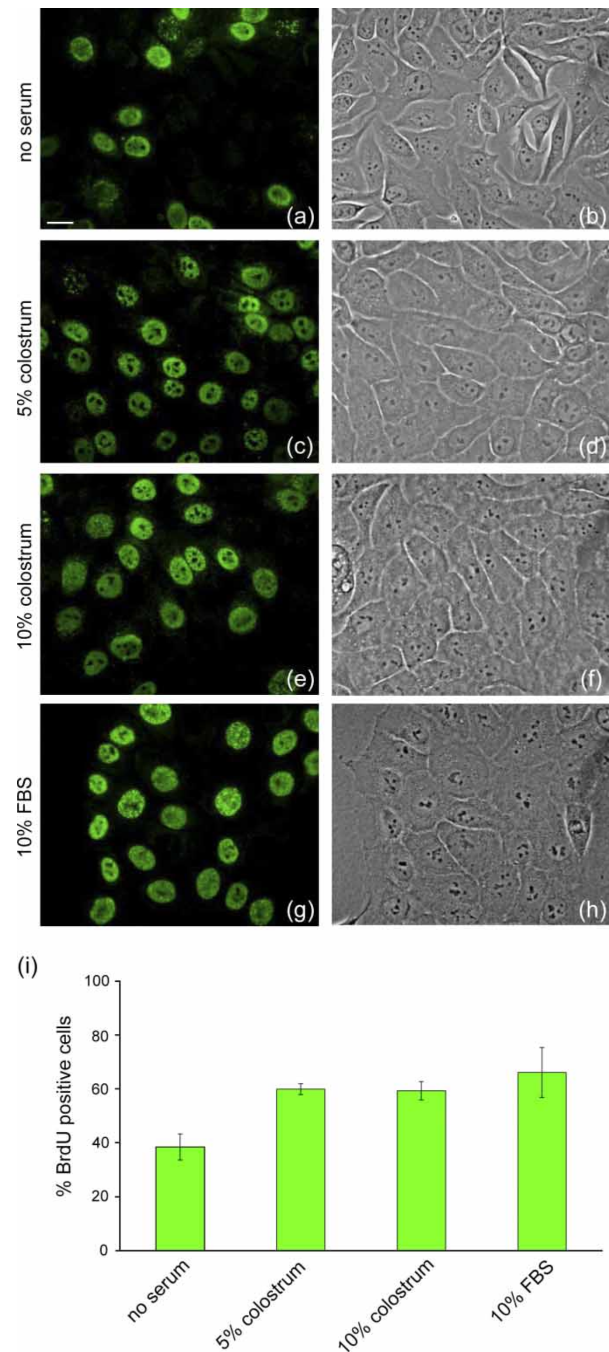

Figure 2. (a–h) BrdU incorporation assay (a, c, e, g) and parallel phase contrast microscopy (b, d, f, h) of HaCaT cells grown for 24 h in serum-free condition (a, b), in the presence of 5% (c, d) and 10% colostrum (e, f) or 10% FBS (g, h). Immunofluorescence analysis with anti-BrdU antibody reveals a number of BrdU-positive nuclei in colostrum-treated cells markedly higher respect to serum-starved cells and comparable to that observed in cells grown with 10% FBS. Scale bars: 20 μm. (i) Percentage of BrdU positive HaCaT cells maintained without serum, kept in the presence of 5 and 10% colostrum or 10% FBS. Results represent the mean value ± SD. For each condition, a total of 500 cells randomly observed from 10 microscopic fields in three different experiments were analysed.

Motogenic effect and actin cytoskeleton reorganization in response to bovine colostrum

We next evaluated whether colostrum could also affect keratinocytes migration, an other essential step contributing to the healing process (Singer and Clark 1999; Santoro and Gaudino 2005), by performing the wound "scratch assay". HaCaT kept in medium containing colostrum started to cover the scratched area within 6 h and, compared to T0 control (Figure 3(a)), showed a more elongated shape, typical

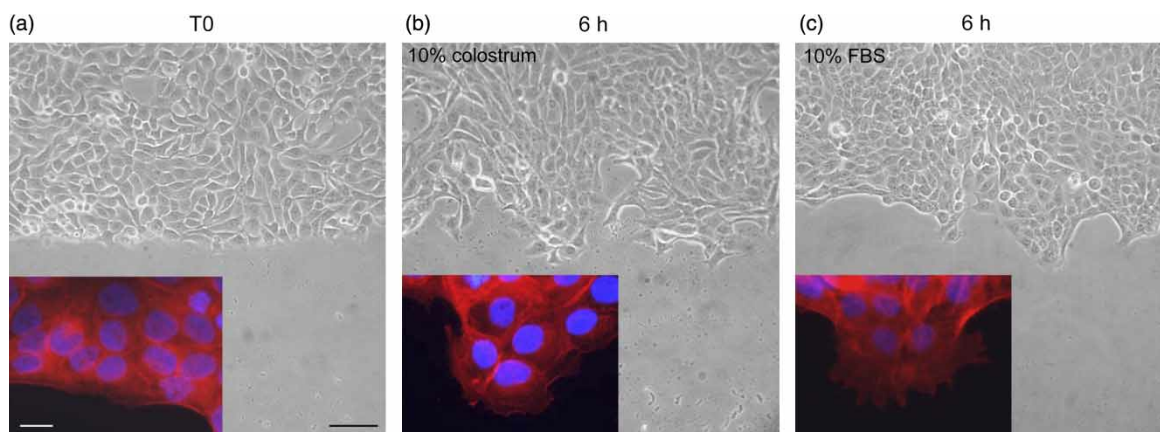

Figure 3. (a–c) Scratch assay on HaCaT cells in response to treatment with 10% colostrum and 10% FBS. Confluent starved cells were scratched as described in "Material and methods" section and were allowed to migrate monitoring the repopulation of the cell free area. Treatment with colostrum (b) was able to induce cell migration similar to that observed for cells maintained in the presence of FBS (c). (a) Represents the edge at time 0 (T0) recorded immediately after the scratch. Insert in (a–c): Parallel immunofluorescence analysis of actin cytoskeleton reorganization stained with TRITC-phalloidin to reveal filamentous actin. Cells recorded at T0 (insert in (a)) displayed regular edges and defined stress fibres in the cytoplasm. Similarly to FBS-treated cells (insert in (c)), HaCaT maintained for 6 h with colostrum showed a clear migratory phenotype with ruffles and lamellipodia extensions (insert in (b)). Scale bars (a–c): 10 μm, insert in (a–c): 20 μm.

of a migratory phenotype (Figure 3(b)). A similar motogenic induction was evident in the positive control cells maintained in medium with FBS (Figure 3(c)). A marked covered area, evaluated measuring the reduction of the distance between the scratch edges compared to T0, was observed after 24 h (37 ± 3.4 and 48 ± 3% for colostrum and FBS, respectively), indicating a contribution in the repopulation of the scratched area ascribed to cell migration and also to proliferation, as expected from the results obtained on growth promoting activity.

Migration requires actin cytoskeleton remodelling characterized by cell polarization and formation of protrusions such as lamellipodia and membrane ruffles towards the direction of the movement (Ridley et al. 2003; Mattila and Lappalainen 2008). The analysis of cell morphology and actin reorganization showed that, similarly to the cells maintained in FBS (Figure 3(c), insert), HaCaT grown in the presence of colostrum displayed an elongated cellular shape towards the direction of the movement with the cortical actin reorganized in membrane ruffles and short lamellipodia (Figure 3(b), insert), typical

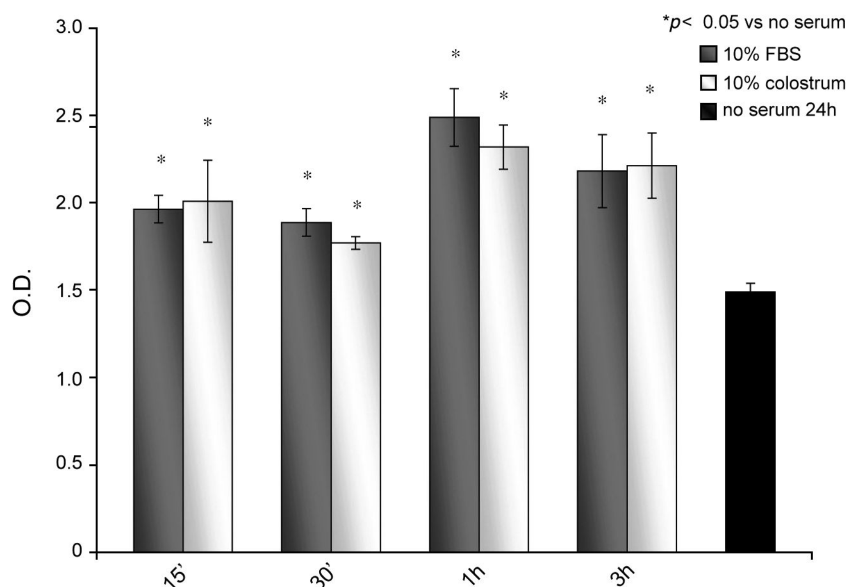

Figure 4. Growth-promoting activity evaluated by MTT assay on HaCaT cells treated with 10% FBS or 10% colostrum for the indicated time points and maintained for additional 24 h in serum-free culture medium: in comparison to serum-starved cells, colostrum stimulates the growth at all time points tested similarly to FBS. Results represent the mean values ± SD from three different experiments performed in triplicate. Student's t-test was performed to evaluate significative differences. (*, $p < 0.05$ vs. no serum).

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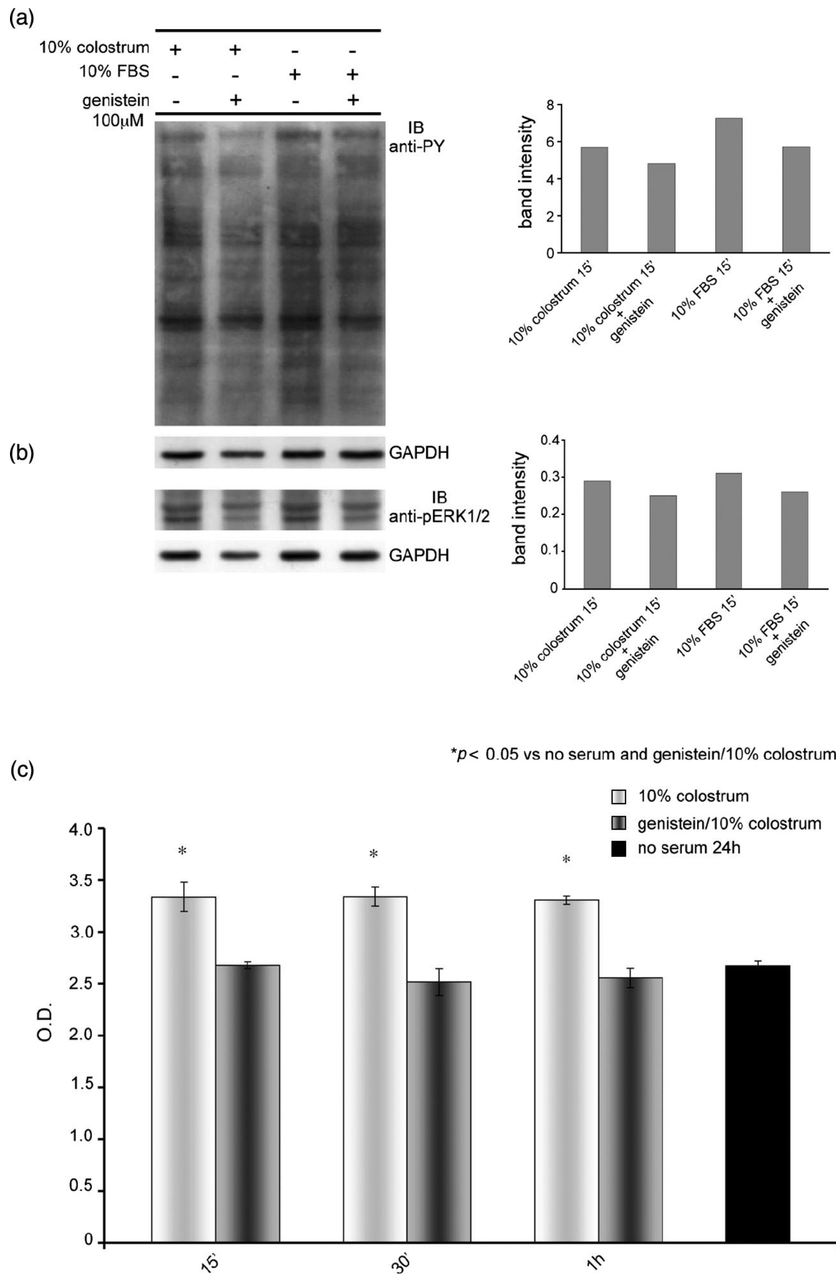

Figure 5. (a) Western blot and the corresponding densitometric analysis of tyrosine phosphorylation on serum-starved HaCaT cells: the treatment for 15′ with 10% colostrum reveals a pattern of phosphorylated proteins similar to 10% FBS. An overall reduction of phosphorylation is evident upon treatment with the tyrosine kinase inhibitor genistein (100 μM). A representative experiment is shown. (b) Western blot and the corresponding densitometric analysis of phospho-ERK1/2 on the same samples. HaCaT cells maintained with 10% colostrum show levels of phosphorylated ERK1/2 comparable to 10% FBS. Such phosphorylation appears reduced in the presence of genistein treatment (100 μM). A representative experiment is shown. (c) Growth-promoting activity evaluated by MTT assay on HaCaT cells treated with 10% colostrum in the presence or absence of genistein and maintained for additional 24 h in serum-free culture medium. Respect to serum-starved cells, the proliferation induced by colostrum at all the time points tested appears clearly inhibited by the treatment with genistein. Results represent the mean value ± SD from three different experiments performed in triplicate. Student's *t*-test was performed to evaluate significative differences. (*, $p < 0.05$ vs. no serum and genistein/10% colostrum).

hallmarks of a migratory phenotype. In contrast, cells at T0 showed regular edges (Figure 3(a), insert) with the actin cytoskeleton presenting more defined stress fibres (Figure 3(a), insert).

During wound healing, the reepithelization step is essential to reestablish the integrity of the damaged epithelium (Singer and Clark 1999; Santoro and Gaudino 2005). This process is characterized by both keratinocytes migration and proliferation at the wound edges and is regulated by the action of several growth factors and cytokines (Werner and Grose 2003; Barrientos et al. 2008). Our results are consistent with previous studies demonstrating that bovine colostrum increases the migration of rat

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intestinal epithelial cells, human colonic carcinoma cells and normal human fibroblast in *in vitro* models of repair (Playford et al. 1999; Takayama et al. 2001) and further suggest that, through the action of its biologic factors, bovine colostrum may promote cutaneous wound healing.

Activation of tyrosine kinase pathways exerted by bovine colostrum

Receptors for several growth factors contained in bovine colostrum such as EGF, IGF, PDGF are characterized by tyrosine kinase activity (Schlessinger 2000; Werner and Grose 2003). Upon ligand binding, these receptors undergo activation followed by phosphorylation of downstream target proteins, and triggering of signalling cascades which ultimately activate transcription factors regulating the expression of genes involved in the control of cell cycle, proliferation, motility and survival (Schlessinger 2000; Hubbard and Miller 2007; Pastore et al. 2008). Since the activation of these TKRs and the downstream signalling cascades occur rapidly (Sturani et al. 1988), we evaluated the mitogenic cellular response to short treatments of colostrum. Compared to serum-starved cells, the addition of 10% colostrum resulted in an evident and significant increase of the proliferative rate at each time point tested ($p < 0.05$) similarly to 10% FBS (Figure 4), strongly suggesting that the growth-promoting effect is likely a direct consequence of the activation of growth factor receptors and their signalling pathways. The studies to analyse the molecular mechanism induced by the treatment with colostrum, demonstrated that, similarly to FBS, the treatment with colostrum for 15′ induced an overall increase in tyrosine phosphorylation of several proteins (Figure 5(a)), further supporting the specific involvement of the growth factors. Pre-treatment with the tyrosine kinase inhibitor genistein (100 μM) resulted to be effective in decreasing the induction of the phosphorylation due to both colostrum and FBS (Figure 5(a)).

Several mitogen-activated protein kinases (MAPKs) are downstream effectors in the signal cascades triggered by TKRs. Particularly, among the three pathways of the MAPKs c-Jun N-terminal kinases (JNK), p38 and ERK1/2, the latter are known to be mostly activated in response to growth factors and play a central role in mediating cell proliferation and migration (Pearson et al. 2001; Huang et al. 2004; Roux and Blenis 2004). We analysed their activation to evaluate in more detail the downstream molecules of the tyrosine kinase pathways involved. The results showed the phosphorylation of ERK1/2 in response to both colostrum and FBS and the suppression of such activation in the presence of genistein (Figure 5(b)). Finally, to support the functional involvement of the signal transduction activated by the TKRs observed in our cell model, we evaluated the growth-promoting activity in response to short time treatments with colostrum in the presence of the tyrosine kinase inhibitor. Genistein reduced the increase in the growth rate induced by colostrum ($p < 0.05$) at all time points tested (Figure 5(c)), indicating that the TKRs and their signalling pathways are specifically involved in the increased proliferative response of keratinocytes to colostrum.

Conclusions

In this study, we demonstrate that bovine colostrum promotes the proliferation rate and enhances the migratory phenotype of epidermal keratinocytes, suggesting its positive effects in the reepithelization at the wound site. Our data on the biological effects of bovine colostrum are in agreement with previous studies on other cell types (Hironaka et al. 1997; Playford et al. 1999; Takayama et al. 2001; Torre et al. 2006; Purup et al. 2007; An et al. 2009) and show its ability to act *in vitro* also on keratinocytes, one of the cell types mainly involved in the skin restoration after wounds. Furthermore, our findings on the molecular mechanisms regulating cell responses to colostrum underline the fundamental link between the growth factor-mediated phosphorylation of TKRs and the signal transduction events associated with cell growth and migration. Overall, our results indicate that colostrum has pro-healing effects in our *in vitro* model of wound repair and that this effects specifically result from the combined and synergistic action of the growth factors.

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Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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