JPP JOURNAL OF Pharmacy and Pharmacology
ROYAL PHARMACEUTICAL SOCIETY
JPP Journal of Pharmacy And Pharmacology
Research Paper

The effect of two drug delivery systems in ropivacaine cytotoxicity and cytokine release by human keratinocytes and fibroblasts

Luiz Eduardo Nunes Ferreira[a], Bruno Vilela Muniz[a], Jonny Burga-Sánchez[a], Maria Cristina Volpato[a], Eneida de Paula[b], Edvaldo Antonio Ribeiro Rosa[c] and Francisco Carlos Groppo[a]

[a]Department of Physiological Sciences, Piracicaba Dental School, University of Campinas – UNICAMP – Piracicaba, [b]Department of Biochemistry, Biology Institute, University of Campinas – UNICAMP – Campinas, São Paulo and [c]Xenobiotics Research Unit, Laboratory of Stomatology, Biological and Health Sciences Center, The Pontifical Catholic University of Paraná – Curitiba, Paraná, Brazil

Keywords
2-hydroxypropyl-β-cyclodextrin; cell viability; cytokines; multilamellar liposome; ropivacaine

Correspondence
Luiz Eduardo Nunes Ferreira, Department of Physiological Sciences, Piracicaba Dental School, University of Campinas – UNICAMP – Piracicaba, Avenida Limeira, 901, São Paulo, Brazil.
E-mail: luiz.enferreira@gmail.com

Received July 5, 2016
Accepted November 12, 2016

doi: 10.1111/jphp.12680

Abstract

Objectives Modified drug delivery systems have been developed to improve pharmacological properties of local anaesthetics. However, the inflammatory potential of these formulations was not investigated. This study compared the *in-vitro* effects of ropivacaine (ropi) in plain, liposomal (MLV) or 2-hydroxypropyl-β-cyclodextrin (HP-β-CD) formulations on cell viability, apoptosis and cytokine (IL-1α, TNF-α, IL-6 and IL-10) release.

Methods Human immortalized keratinocytes (HaCaT) and human immortalized gingival fibroblasts (HGF) were exposed to 1–100 μM ropi concentrations. The cell viability was measured by XTT and LIVE/DEAD assay. Apoptosis was performed by flow cytometry, and cytokine release was measured by ELISA assay.

Key findings Human immortalized keratinocyte viability was reduced by ropi and both drug delivery systems. However, none of the formulations induced apoptosis. Results showed a differential regulation of IL-1α TNF-α, IL-6 and IL-10 by HaCaT and HGF. Ropi-HP-β-CD increased twofold the IL-6 release by HGF in comparison with the control, while 100 μM ropi-MLV led to an increased release of all pro-inflammatory cytokines by HGF.

Conclusion The loss in cell viability was not related to cellular apoptosis. Ropi complexed with HP-β-CD showed a similar cytokine release pattern when compared to the plain formulation. Thus, the HP-β-CD form was a better drug carrier than the MLV form for ropivacaine drug delivery.

Introduction

Ropivacaine (Ropi) is an amido-amide enantiomerically pure (S-isomer) local anaesthetic belonging to the pipecoloxylidide group, with a large use in surgical procedures, regional anaesthesia and for the management of postoperative and labour pain.[1]

Ropivacaine elicits nerve block by a reversible inhibition of the influx of sodium ions in the nerve fibres. In addition to the action on pain transmission mechanisms, the literature has reported a wide variety effects on different signalling pathways by local anaesthetics.[2,3]

The therapeutic benefits of local anaesthetic, under certain circumstances, are limited due to a fast-dispersing, toxicological properties or physiological barriers.[4] In an attempt to prolong anaesthesia and analgesia with low toxicity, many sustained drug release systems for local anaesthetics have been developed and made possible the manipulation of pharmaceutical and biopharmaceutical features.[5–7]

Liposomes are versatile carriers that can be applied to a wide variety of drugs. Liposomes are vesicles consisting of one or more membrane of phospholipid bilayer forming an internal aqueous compartment. Thus, hydrosoluble molecules are concentrated in the inner aqueous portion, while liposoluble drugs can be transported through the association with the liposome phospholipid bilayer.[4,8,9] Ropi loaded in large unilamellar liposomes modified the rate of

drug release, extending the intensity of anaesthetic effect after the infraorbital nerve block, and increased the analgesia duration in the sciatic nerve blockade in rats.[10]

Cyclodextrins are oligosaccharides produced by enzymatic hydrolysis of the starch. They form a conical molecule composed by a hydrophilic hydroxyl group on the outer surface and a hydrophobic central cavity. These carriers are able to form inclusion complexes with a wide variety of drugs.[5,11,12] Ropi complexed in 2-hydroxypropyl-β-cyclodextrin (HP-β-CD) also led to an increase in the intensity and duration of analgesia after sciatic nerve block *in vivo*.[13]

However, the effects of these new formulations of local anaesthetics on cell signalling pathways, as those involved in inflammatory responses that are important in postsurgical processes, remain poorly explored. Cytokines, especially the pro-inflammatory ones, such as IL-1α and TNF-α, and chemokines are directly related to acute inflammation process. They stimulate production of acute-phase proteins, cell adhesion through the release of intracellular adhesion molecules (ICAM), and vascular cell adhesion molecules (VCAM), and neutrophil activation/chemotaxis.[14,15]

A delivery system more suitable for ropivacaine should provide less cytotoxicity, and a reduced acute inflammatory response when compared to its plain formulations. Thus, this study aimed to compare the cytotoxic effects of ropi when loaded in liposomes or complexed in 2-hydroxypropyl-β-cyclodextrin and to test the effects of these formulations in the cytokine release *in vitro*.

Methods

Reagents

Ropivacaine hydrochloride was kindly donated by Cristália Ind. Quím. Farm (Itapira, SP, Brazil). Alpha-tocopherol acetate, egg phosphatidylcholine and cholesterol were purchased (Sigma-Aldrich, St. Louis, MO, USA) and used for liposomal vesicle preparation. 2-Hydroxypropyl-β-cyclodextrin (Roquettes Serv. Tech. Lab, Lestrem, France) was dissolved in 20 mM HEPES buffer (Sigma-Aldrich). DMEM (Vitrocell Embriolife, Campinas, SP, Brazil), foetal bovine serum, trypsin with EDTA (Life Technologies, Carlsbad, CA, USA), and the antibiotics penicillin (100 U/ml) with streptomycin 100 μg/ml (Cultilab, Campinas, SP, Brazil) were used for the cell culture assays. Cell viability was assessed by a colorimetric assay (XTT, TOX-2 kit; Sigma-Aldrich) and by a fluorescence LIVE/DEAD assay (Life Technologies). Both annexin V-FITC and actinomycin D from BD Pharmigen™ (Franklin Lakes, NJ, USA) were used to target cellular apoptosis. ELISA kits (Peprotech Inc., Rocky Hill, NJ, USA) were used to assay cytokines IL-1α, TNF-α, IL-6 and IL-10.

Ropivacaine loaded in multilamellar liposomes

Multilamellar vesicles (MLVs) were prepared using the previously described dry film hydration method. Briefly, a chloroform stock solution containing egg phosphatidylcholine, cholesterol and α-tocopherol (4 : 3 : 0.07 molar ratio) was evaporated under N_2 flow, followed by vacuum for 2 h at room temperature.[16,17] Films were suspended in 20 mM HEPES buffer, pH 7.4 (containing 154 mM NaCl). MLVs were obtained by vortexing the suspension for 5 min at ambient temperature (25°C). Total lipid concentration in the MLV final suspension was 15 mM, and 1 mM ropi hydrochloride was loaded into MLV. The formulation was autoclaved and stored at 4 °C protected from light. The physicochemical properties of the lidocaine loaded in MLV have previously reported.[10]

Ropivacaine complexation into 2-hydroxypropyl-β-cyclodextrin

Inclusion complexes were obtained by adding HP-β-CD and ropi hydrochloride in a 1 : 1 molar ratio in deionized water at room temperature. The solution was shaken for 24 h, freeze-dried (lyophilized) and stored at −20 °C until further use.[18,19] The HP-β-CD ropi complex was resuspended in 20 mM HEPES buffer pH 7.4 (containing 154 mM NaCl), to obtain 1 mM ropivacaine. The final solution was sterilized by filtration with 0.22-μm membranes and stored at 4 °C protected from light. The physicochemical characterization of the lidocaine inclusion complex in HP-β-CD has already been reported.[13]

Cell culture

Immortalized human keratinocytes (HaCaT) were kindly donated by Dr. Rodrigo Augusto da Silva from The Signal Transduction Laboratory of Biology Institute (UNICAMP). Immortalized human gingival fibroblasts (HGF, BCRJ 0089) were obtained from Paul Ehrlich Scientific Technical Association of Rio de Janeiro.

Human immortalized keratinocyte and HGF were cultured in DMEM supplemented with 10% foetal bovine serum, penicillin (100 U/ml) and streptomycin (100 μg/ml) and maintained in a humidified incubator with 5% CO_2 at 37 °C. Cells were grown in tissue culture flasks, being treated with 0.25% trypsin and 0.5 mM EDTA for transfer to tissue culture plate with 24 or 96 wells. Experiments were performed after reaching approximately 80% of confluence per well.

Experimental conditions

Human immortalized keratinocyte and HFG were exposed to ropi-plain, ropi-MLV and ropi-HP-β-CD formulations, considering 1, 10 and 100 μM concentrations[20] for 6 and 24 h. Cells treated with 1× PBS (pH 7.4) were used as control. The effects of ropi formulations on cell viability and apoptosis induction were observed. In addition, culture media from each sample were collected, and the release of cytokines was quantified.

Cell viability assay

Human immortalized keratinocyte and HGF at 7×10^4 cells/ml were placed in 96-well plates and incubated for 18 h in 5% CO_2 at 37 °C. Ropi formulations were added at the concentrations previously described. Cell viability was measured by the XTT reduction assay. After incubation with ropi formulations, the wells were washed with PBS, and XTT solution (0.3 mg/ml) was added. The plates were then incubated for 4 h to complete XTT reduction. Optical density was measured using a microplate reader at 450 nm with a reference wavelength set at 690 nm.

Cell viability by fluorescent labelling

Cell viability results performed by XTT assay were confirmed with a qualitative analysis by fluorescence labelling using the LIVE/DEAD assay. The test consists of two fluorescent markers; the calcein/AM is converted by ubiquitous intracellular esterase activity in living cells leading to an intensely green fluorescence. The ethidium homodimer-1 (EthD-1) enters into the cells with damaged membranes and binds to nucleic acids resulting in a red fluorescence labelling for dead cells.

Human immortalized keratinocyte and HGF cells were seeded in 24-well tissue cultures plates and exposed to ropi formulations. The cells were washed with PBS, and 300 μl of LIVE/DEAD reagent (2 μM calcein/AM and 4 μM EthD-1) was added. The plates were incubated for 30 min in the dark, at room temperature. Images were captured in a fluorescence microscope Zeiss Axiovert 40 CFL coupled with MEC AxioCam camera (Carl Zeiss, Oberkochen, Germany). Calcein was visualized using the filter in the 450–490 nm (Ex)/515–565 nm (Em) wavelength, and EthD-1 labelled cells were visualized using the filter in the 528–546 nm (ex)/590–617 nm (Em) wavelength.

Detection of early and delayed apoptosis by flow cytometry

Human immortalized keratinocyte cells (approximately 4×10^5 cells/well) on 6-well tissue cultured plates were treated with 100 μM ropi for 6 h. Apoptosis was determined by double staining with annexin V-FITC, which identifies the early stages of apoptosis, and 7-amino-actinomycin D (7-AAD) that marks the cells in late apoptosis or necrosis.

Cells were trypsinized and resuspended in binding buffer. Then, 1 μl of annexin V-FITC and 0.5 μl of 7-AAD were add in 100 μl of cellular suspension. Samples were vortexed and incubated at room temperature in the dark for 15 min. Binding buffer (400 μl) was added, and the results were analysed in the flow cytometer (BD FACS Calibur™). Cells without treatment were used as negative control, and cells treated with staurosporine were the positive control.

Quantification of cytokine release by enzyme-linked immunosorbent assay

Human immortalized keratinocyte and HGF cells were exposed to ropivacaine formulations for 24 h. Culture supernatant was collected and tested for the presence of pro-inflammatory cytokines IL-1α, TNF-α, IL-6 and anti-inflammatory cytokine IL-10 by the enzyme-linked immunosorbent assay (ELISA).

Briefly, 96-well plates were coated with the capture antibody 100 μl/well and incubated overnight at room temperature. Then, the wells were washed five times with 0.05% Tween-20 in PBS, this process was repeated before each step, and 300 μl/well of block buffer (1% BSA in PBS) was added and incubated for 1 h. The samples or standards were added, and the plates were incubated for 2 h until 100 μl of the detection antibody was added in each well. After 2 h, 100 μl of avidin solution and ABTS substrate were added. The colorimetric reaction was measured in 405-nm-wavelength microplate reader with wavelength correction set at 650 nm. The concentration of IL-1α, TNF-α, IL-6 and IL-10 was expressed in pg/ml.

Statistical analysis

All data were tested for normal distribution by Shapiro–Wilks' test, and the equivalency of variances was tested by Levene's test. Data from cell viability were analysed by ANOVA and Tukey's (*post hoc*) tests. The *G* test was applied for cell apoptosis, and Kruskal–Wallis' (Student–Newman–Keuls) test was used to analyse the cytokines concentrations. Significance level was set at 5% ($\alpha = 0.05$), and all analyses were performed using Biostat® 5.0 and GraphPad Prism 6.0.

Results

Effects of ropivacaine formulations on cell viability

Figure 1 presents the results of the XTT assay for cell viability. The different concentrations of ropi formulations were

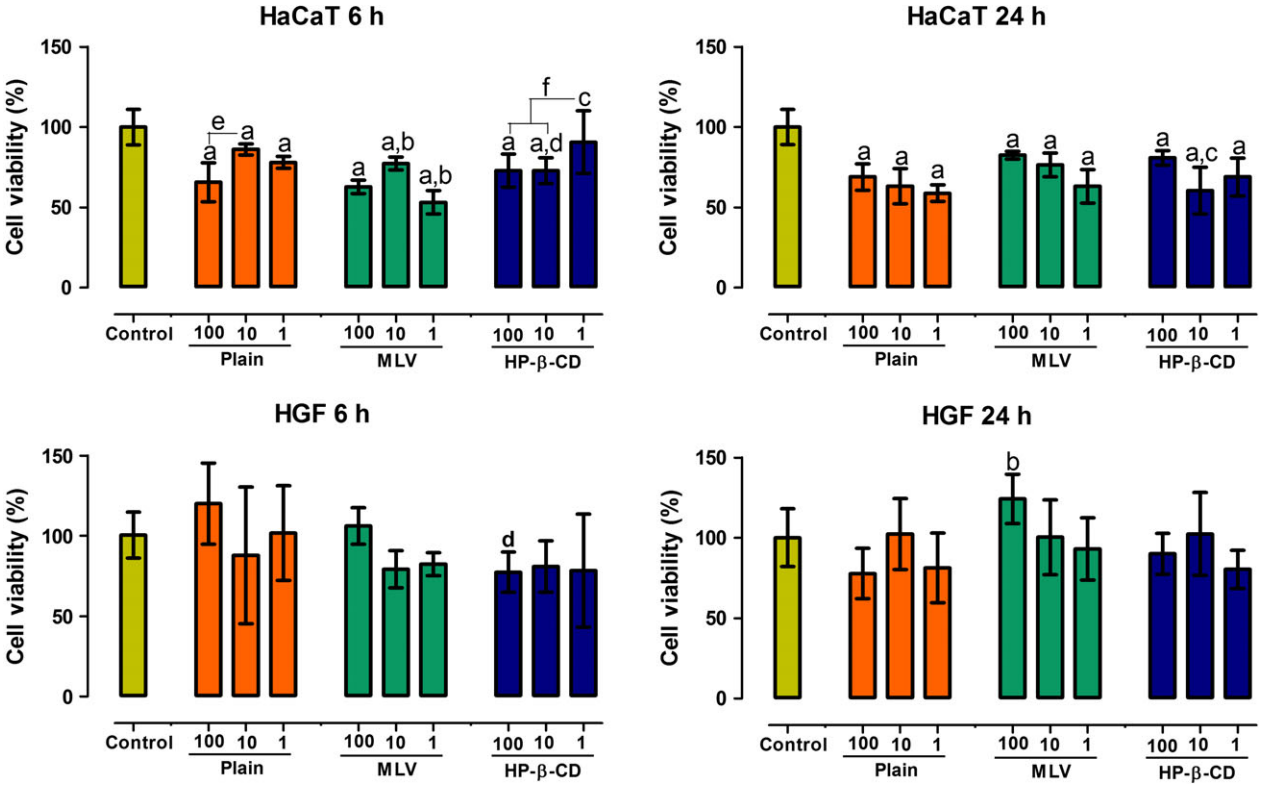


Figure 1 Mean and standard deviation of human immortalized keratinocyte and human gingival fibroblast cell viability after exposure to ropivacaine formulations for 6 and 24 h in different concentrations (100–1 μM) measured by XTT assay. *Y*-axis = cell viability in % relative to the control group. *X*-axis = treatments with plain lidocaine, multilamellar vesicle and 2-hydroxypropyl-β-cyclodextrin formulations. a – Ropivacaine formulations × control; b – ropivacaine × ropivacaine-multilamellar vesicle, both at the same concentration; c – ropivacaine-2-hydroxypropyl-β-cyclodextrin × ropivacaine-multilamellar vesicle, both at the same concentration; d – ropivacaine-2-hydroxypropyl-β-cyclodextrin × ropivacaine, both at the same concentration; e – significant differences between the 100 μM ropivacaine × 10 μM ropivacaine; f – significant differences between the 100 and 10 μM ropivacaine-2-hydroxypropyl-β-cyclodextrin × 1 μM ropivacaine-2-hydroxypropyl-β-cyclodextrin, $P < 0.05$ ANOVA, Tukey test. [Colour figure can be viewed at wileyonlinelibrary.com]

not able to affect HGF viability throughout time, and no statistical differences were observed in comparison with the control group.

However, HaCaT cells were susceptible to ropi formulation exposure. The different concentrations of ropi-plain induced a significant cell viability loss throughout time. The tested drug delivery systems were not able to modify the *in-vitro* effect of ropi on HaCaT cells.

At 6 h, only the treatment with 1 μM ropi-HP-β-CD did not reduce ($P = 0.228$) the cell viability when compared to the control group. The 10 μM ropi-HP-β-CD, 10 μM ropi-MLV and 1 μM ropi-MLV significantly reduced cell viability compared with the ropi-plain at the same concentrations. After 24 h, all MLV and HP-β-CD formulations showed a significant cell viability loss when compared to the control group.

The results on the cell viability obtained by the XTT assay were confirmed by fluorescence labelling using the LIVE/DEAD reagents. Figure 2 shows representative images of cell viability of both HaCaT and HGF after 24 h of exposure to 1 and 100 μM ropi formulations, respectively.

Loss of cell viability can be observed in the HaCaT cells exposed to ropi in the LIVE/DEAD assay. The HaCaT cell images in this assay showed more gaps between groups of cells, characterizing cell death by detached cells or reduced proliferation rate due to the local anaesthetic formulation. Moreover, both treatments with 1 μM ropi and 1 μM ropi-MLV increased the number of cells stained by EthD-1. The effects of ropi formulations in the HGF cells were less evident when compared to control, even after 24 h of exposure to the highest ropi concentration.

Induction of apoptosis in human immortalized keratinocyte cells by ropivacaine formulations

To observe the role of apoptosis induction in the cell viability loss induced by ropi formulations, HaCaT cells were

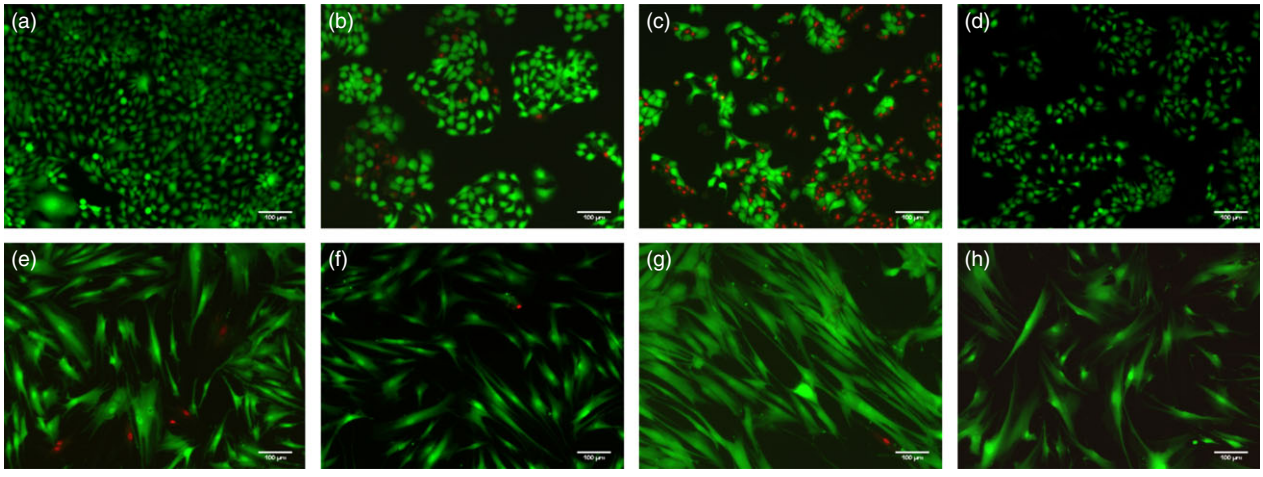


Figure 2 Representative images of the cell viability in human immortalized keratinocyte and human gingival fibroblast after 24-h exposure to ropivacaine. Human immortalized keratinocyte cells were treated with PBS for the control group (a) and 1 μM ropivacaine at plain formulation (b), multilamellar vesicle (c) and 2-hydroxypropyl-β-cyclodextrin (d). Human gingival fibroblast were treated with PBS (e) and 100 μM ropivacaine at plain formulation (f), multilamellar vesicle (g) and 2-hydroxypropyl-β-cyclodextrin (h). Fluorescent labelling was performed by LIVE/DEAD reagents. The images were obtained with a fluorescence microscope at 10× magnification and superposition of green and red images; the scale bar represents 100 μm. The nucleus of dead or damage cells is marked in red by EthD-1, and viable cells exhibit green fluorescence due to the intracellular distribution of calcein AM. The results on the cell viability. [Colour figure can be viewed at wileyonlinelibrary.com]

exposed to the highest ropi concentration for 6 h. The results for early and late apoptosis are shown in Figure 3a. Figure 3b compares the treatments regarding the percentage of living and early apoptotic cells. Ropivacaine treatments showed a similar apoptosis rate to the control group, and it induced significantly lower apoptosis rate than staurosporine ($P < 0.01$).

Cytokine release after exposure to ropivacaine formulations

The modulatory effects of ropi formulations on some inflammatory mediators were observed by measuring pro- (IL-1α, TNF-α, IL-6) and anti-inflammatory (IL-10) cytokines (Figure 4). HaCaT and HGF cells showed different patterns in the cytokine release. In general, HaCaT cells released higher volumes of cytokines than HGF cells, in all groups.

Ropivacaine-plain formulations led to a significant increase (approximately 12-fold) in IL-6 release by HaCaT cells when compared to the control. However, these plain formulations significantly reduced both IL-1α and TNF-α release. The IL-10 release did not show significant differences among the plain formulations and control. Ropi-plain also increased IL-10 release by HGF cells in approximately 3.6-fold when compared to control. The plain formulations did not affect the IL-1α, TNF-α and IL-6 released by HGF cells.

Ropivacaine-HP-β-CD formulations did not affect the IL-1α, TNF-α and IL-10 released by HGF cells when compared to control. A significant twofold increase in IL-6 released by HGF cells was induced by ropi-HP-β-CD when compared to control. In general, no significant differences were observed among ropi-plain and ropi-HP-β-CD formulations in most of the concentrations used.

Ropivacaine-HP-β-CD also induced an approximately twofold increase in the IL-10 release by HaCaT in comparison with control and 3.2-fold when compared to ropi-plain formulations. As verified in plain formulations, IL-6 release was also increased by ropi-HP-β-CD when compared to control. These formulations significantly decreased the TNF-α released by HaCaT cells when compared to control. However, they increased TNF-α release in comparison with the plain formulations.

The 100 μM ropi-MLV significantly increased IL-1α (2.16-fold), TNF-α (6.34-fold) and IL-6 (4.21-fold) concentrations by HGF when compared to the control group. This formulation also induced higher levels of the pro-inflammatory cytokines when compared to the other ropi-plain and ropi-HP-β-CD formulations. Other ropi-MLV concentrations did not affect pro-inflammatory cytokine release in comparison with the control group. The changes in pro-inflammatory cytokines balance were reflected in IL-10 release. The 100 μM ropi-MLV induced the highest IL-10 released (ninefold when compared to the control) by HGF cells. The treatments with 10 and 1 μM MLV ropi also significantly increased the release of this mediator.

The effect of ropi-MLV formulations on HaCaT cells varied among the cytokines. These formulations did not affect the IL-1α and IL-10 released when compared to

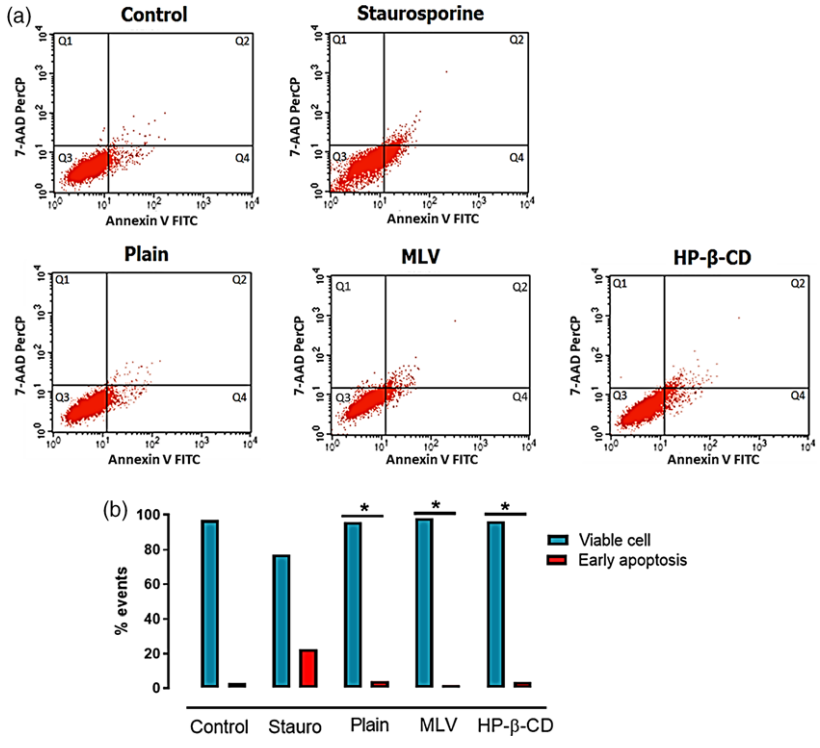


Figure 3 (a) Scatter charts of the viable cells, early apoptosis, late apoptosis or necrosis measured by flow cytometry in human immortalized keratinocyte cells after exposure for 6 h to PBS (control), staurosporine and 100 μM ropivacaine at plain, multilamellar vesicle and 2-hydroxypropyl-β-cyclodextrin formulation. The *y*-axis shows the fluorescent labelling cells for 7-AAD and on the *x*-axis the cells labelled with annexin V. Q1 shows the cells in necrosis, Q2 the late apoptosis cells, Q3 the viable cells and Q4 the cells in early apoptosis. (b) The percentage of viable and early apoptotic cells, * indicates a statistically significant difference compared with staurosporine ($P < 0.01$, *G* test), and the total number of events evaluated for each treatment was 20 000. The results for early and late. [Colour figure can be viewed at wileyonlinelibrary.com]

control, but they reduced TNF-α. IL-6 release was increased by ropi-MLV formulations in a similar fashion when compared to the other plain and ropi-HP-β-CD formulations.

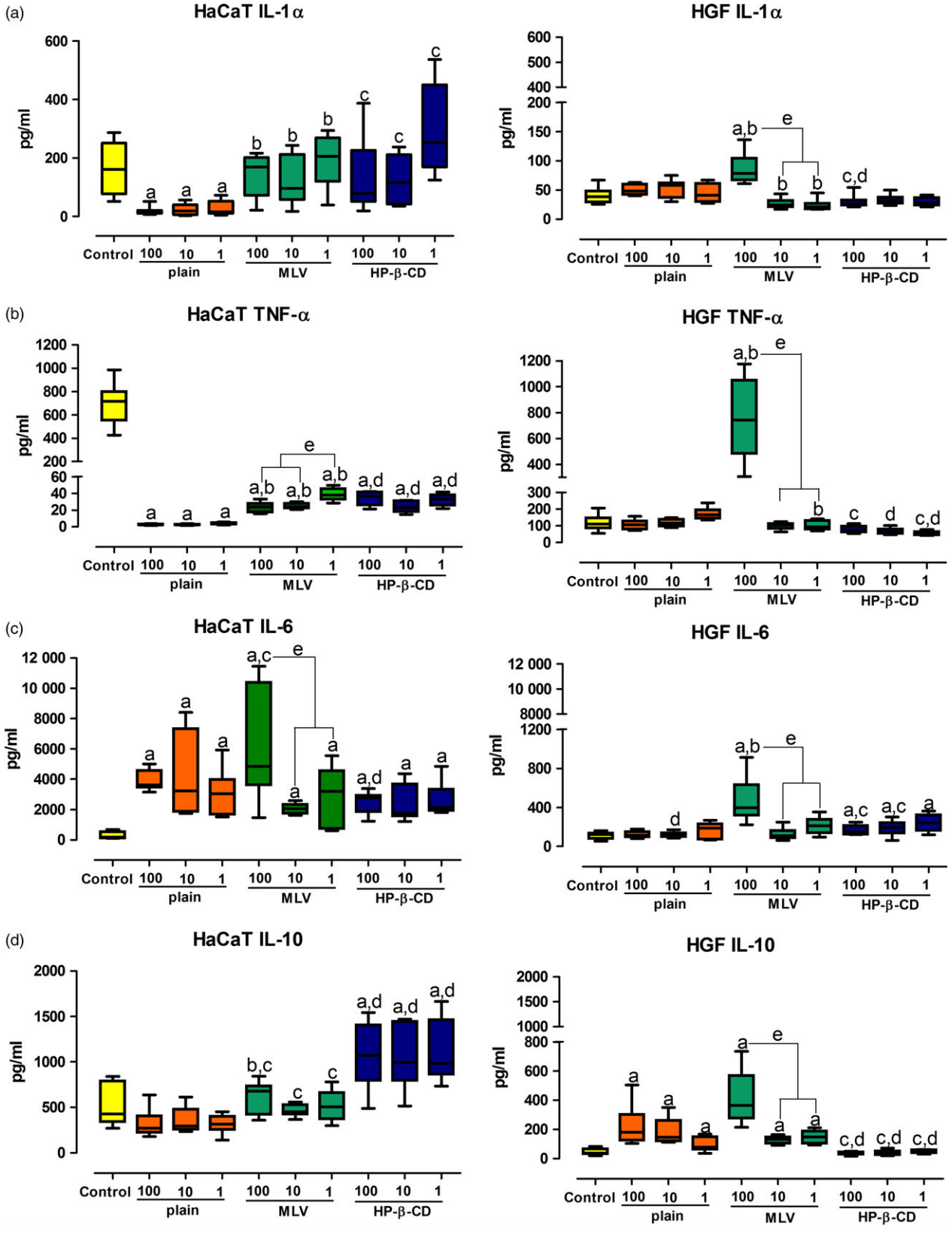
Discussion

Local anaesthetics are often used in dental practice, exposing oral epithelial and gingival fibroblasts cells to these drugs. Thus, both epithelial (HaCaT) and fibroblasts (HGF) were used as *in-vitro* experimental model in the present study. HaCaT are a proper cell type to replace primary human keratinocytes. The keratinocytes play a major role in the immune skin response by releasing pro-inflammatory cytokines, chemokines, growth factors, proteases and matrix metalloproteinases.[21,22]

Fibroblasts are essential cells to tissue repair being one of the first cells to appear in injured sites. The HGF culture is a suitable model to replace *in-vivo* tests, as *in-vitro* cells have morphologies and spatial distribution similar to the *in-vivo* system.[23,24] These two cell lineages have been used in well-defined experimental models in several pharmacology studies, which were designed to investigate intracellular signalling pathways and responses to different stimulation.[25,26]

The ropi concentrations used in the present study were based on kinetic studies after infiltrative anaesthesia in rat maxilla. The concentration peak of ropi in the palatal mucosa reached 204 μM after 30 s of an injection of 200 μl of 0.5% ropivacaine. The local anaesthetic concentration was reduced by 60.5%, 78.9% and 57.1% of the maximum peak after 2, 5 and 10 min, respectively.[27]

Figure 4 IL-1α (a), TNF-α (b), IL-6 (c) e IL-10 (d) release by human immortalized keratinocyte and human gingival fibroblast cells after exposure for 24 h to plain ropivacaine, multilamellar vesicle and 2-hydroxypropyl-β-cyclodextrin at different concentrations. All values presented the median, first and third quartile and the whiskers indicate the maximum and minimum values in each group. a – Ropivacaine formulations × control ($P < 0.05$, Kruskal–Wallis); b – plain ropivacaine × multilamellar vesicle, both at the same concentration ($P < 0.05$, Kruskal–Wallis); c – 2-hydroxypropyl-β-cyclodextrin × multilamellar vesicle, both at the same concentration ($P < 0.05$, Kruskal–Wallis); d – 2-hydroxypropyl-β-cyclodextrin × plain ropivacaine, both at the same concentration ($P < 0.05$, Kruskal–Wallis); e – significant differences between the 100 μM and both 10 and 1 μM for multilamellar vesicle and plain lidocaine formulations ($P < 0.05$, Kruskal–Wallis). The modulatory effects of ropivacaine. [Colour figure can be viewed at wileyonlinelibrary.com]


(a)
HaCaT IL-1α
HGF IL-1α
(b)
HaCaT TNF-α
HGF TNF-α
(c)
HaCaT IL-6
HGF IL-6
(d)
HaCaT IL-10
HGF IL-10
pg/ml
Control
100 10 1
plain
MLV
HP-β-CD

The effects of ropi associated with drug delivery systems on cell viability were previously described in the literature. The viability of Schwann cells from sciatic nerve of rats was not affected after exposure to 4.02 mM ropivacaine loaded into large unilamellar liposomes for 2 h.[8] In addition, the exposition to 2 mM HP-β-CD ropivacaine for 24 h showed no changes in the viability of the same cells.[28]

However, in the present study, ropi formulations, even in low concentrations, induced a significant reduction in HaCaT viability, without changing the cell apoptosis rate. The differences among results observed with Schwann, HGF and HaCaT cells could be related to the cell proliferation rate. HaCaT are a basal keratinocyte cell lineage with high proliferative ability (higher than both Schwann and HGF cells) and autocrine secretion of growth factors from the EGF family, which are essential for the epidermal functions.[29–31]

The effects of ropivacaine on cell proliferation have been shown in many cell types. Ropivacaine ranging between 10 and 100 μM led to antiproliferative effects on mesenchymal stem cells (MSC) that were arrested at the G0/1-S phase transition. The time necessary to double MSC population increased threefold in cultures treated with ropivacaine 100 μM.[32] Exposure to 250 μM ropivacaine for 24 h reduced the HaCaT proliferation rate. Apoptosis was evident only with 1000 μM ropivacaine, being the cell viability reduced by 50% with 2000 μM ropivacaine.[33] Similar results were observed in the present study, as HaCaT viability was reduced by all ropi formulations at 6 h of exposition, remaining low at the 24-hour period. In addition, the used ropi concentrations did not change the apoptosis rate.

Fedder *et al.*[34] studied the ropivacaine antiproliferative effects on human fibroblasts (osteosarcoma cells, osteoblast-like cell types with the morphology of human fibroblasts). In these cells, only after an incubation period longer than 3 days with ropi concentrations of 0.3–0.6 mg/ml, the cell proliferation rate was able to change. Surprisingly, the HGF cells were not affected by any of the ropi formulations. The lack of ropivacaine anti-proliferative effects on HGF in the present study may be related to the experimental model which used exposure time less than 3 days and ropi concentrations lower than 0.3 mg/ml. Ropivacaine associated with MLV and HP-β-CD did not alter the cytotoxic properties presented by ropi-plain. Moreover, 100 μM ropi-MLV showed cell viability significantly higher than 100 μM plain ropi.

The amphiphilic nature of local anaesthetics allows their distribution across the cellular membranes, which affect the membrane dynamics and interfere with protein function. Local anaesthetics, even in lower concentrations than those required to block sodium channels, also interfere with other cellular functions.[35–37]

Amide local anaesthetics have shown anti-inflammatory properties, and they influence several stages of the inflammatory cascade.[38] Ropivacaine can block TNF-α signalling in endothelial cells, reducing the *in-vitro* expression of adhesion molecules from human T-lymphocyte.[39,40] They also decreased inflammation in arthritis, leading to a significant reduction in tissue cytokine levels.[41]

Cytokines are important mediators of local and systemic inflammatory response and are also involved in nociception and in the development of hyperalgesia.[42–44] TNF-α is directly involved in the inflammatory cascade activation by interacting with endothelial cells, which induces the production of adhesion molecules (ICAM, VCAM and E-selectin), allowing the penetration of granulocytes in the inflammation site. TNF-α also leads to neutrophil activation resulting in the degranulation and oxidative stress and stimulates the secretion of other cytokines such as IL-6.[45,46]

IL-6 is a multifunctional cytokine with an important role in acute inflammatory response acting on the activation and differentiation of T cells. IL-1 share multiple biological activities with TNF-α including the stimulation of endothelial cells to produce adhesion molecules for leucocytes adhesion.[15,47]

In contrast, the cytokine IL-10 has a protective role in inflammation by inhibiting the antigen-presenting activity, cytokine secretion by others cells and suppression of the macrophages activation.[48] The inflammatory response mediated by cytokines directly depends on the balance in the release of pro-inflammatory and anti-inflammatory mediators. Therefore, it is important to also evaluate the IL-10 release to better understand the effects of ropivacaine formulations on the inflammatory response.

The fact that the pattern of cytokine release was different between HaCaT and HGF was previously noticed regarding other pro-inflammatory mediators, such as TGF-β_1.[49,50] Keratinocytes are the major source of cytokines in the epidermis and produce a wide range of cytokines such as IL-1, IL-3, IL-6, IL-8, CSF, TNF-α, TGF-α, TGF-β and PDGF, serving as mediators for inflammatory and immunologic reactions in skin exposed to irritants.[51,52] Moreover, epidermal keratinocytes are a major source of IL-10 in skin.[49]

Ropivacaine-plain significantly reduced the release of TNF-α and IL-1α by HaCaT, but it increased the release of IL-6. Moreover, when in drug delivery systems, it led to increased IL-10 concentration. This result may be due to increased cAMP intracellular levels. Grandjean-Laquerriere *et al.*[53] showed cAMP-elevating agents decreasing TNF-α concentration at the protein and mRNA levels and enhancing IL-10 and IL-6 in human keratinocytes.

At the concentrations and conditions tested, ropi-plain formulations did not affect the release of pro-inflammatory cytokines by HGF, but it stimulated the release of IL-10

when compared to control. Ropi-HP-β-CD showed similar pattern with the plain formulation. The release of pro-inflammatory cytokines was increased after exposing HGF to the highest concentration of ropi loaded into MLVs, and it could cause acute inflammatory response. Thus, HP-β-CD is a better carrier for complexing with the ropi than MLV.

With the development of medical polymeric materials, sustained-release formulations for ropivacaine have been produced in order to improve the intracellular delivery, mitigate local tissue site reactions and systemic toxicity, prolong half-lives and explore others drug administration routes to replace the traditional anaesthesia puncture. In this way, carriers such as polyethylene glycol-co-polylactic acid (PELA), polylactic-co-glycolic acid (PLGA), magnetic nanoparticles, chitosan thermogels and other kinds of lipid nanocapsules have been associated with ropivacaine delivery.[54–57] These biomaterials have potentiated the ropivacaine analgesia *in vivo*; however, further studies on the induction of the inflammatory response and other effects at the cellular level are required.

In conclusion, the use of MLVs and HP-β-CD did not produce changes on cell viability compared to the plain formulation. However, ropivacaine loaded in liposomal vesicles stimulated a larger release of cytokines. Thus, the *in-vitro* results demonstrated that complexation in HP-β-CD was the most appropriate carrier for ropivacaine in relation to immune cell response. Further, *in-vivo* and clinical studies are necessary in order to observe the inflammatory effect of the two drug carriers.

Declarations

Conflict of interest

The Authors report no conflict of interests in this work.

Acknowledgements

This work was supported by the FAPESP (Fundação de Amparo a Pesquisa do Estado de São Paulo), grants FAPESP #2011/12666-8 and #2012/07310-2.

References

1. Simpson D *et al.* Ropivacaine: a review of its use in regional anaesthesia and acute pain management. *Drugs* 2005; 65: 2675–2717.
2. Hollmann MW, Durieux ME. Local anesthetics and the inflammatory response: a new therapeutic indication? *Anesthesiology* 2000; 93: 858–875.
3. Zhou Y *et al.* Single-dose intra-articular ropivacaine after arthroscopic knee surgery decreases post-operative pain without increasing side effects: a systematic review and meta-analysis. *Knee Surg Sports Traumatol Arthrosc* 2016; 24: 1651–1659.
4. Grant AS. The Holy Grail: long-acting local anesthetics and liposomes. *Best Pract Res Clin Anesthesiol* 2002; 16: 345–352.
5. de Paula E *et al.* Drug delivery systems for local anesthetics. *Recent Pat Drug Deliv Formul* 2010; 4: 23–34.
6. Shikanov A *et al.* Long acting local anesthetic-polymer formulation to prolong the effect of analgesia. *J Control Release* 2007; 117: 97–103.
7. Suzuki R *et al.* Complex of branched cyclodextrin and lidocaine prolonged the duration of peripheral nerve block. *J Anesth* 2009; 23: 295–297.
8. Yamaguchi T, Mizushima Y. Lipid microspheres for drug delivery from the pharmaceutical viewpoint. *Crit Rev Ther Drug Carrier Syst* 1994; 11: 215–229.
9. Lian T, Ho RJ. Trends and developments in liposome drug delivery systems. *J Pharm Sci* 2001; 90: 667–680.
10. de Araujo DR *et al.* Pharmacological and local toxicity studies of a liposomal formulation for the novel local anaesthetic ropivacaine. *J Pharm Pharmacol* 2008; 60: 1449–1457.
11. Duchêne D, Wouessidjewe D. The current state of β-cyclodextrin in pharmaceutics. *Acta Pharm Technol* 1990; 36: 1–6.
12. Loftsson T, Duchêne D. Cyclodextrins and their pharmaceutical applications. *Int J Pharm* 2007; 329: 1–11.
13. de Araujo DR *et al.* Development and pharmacological evaluation of ropivacaine-2-hydroxypropyl-beta-cyclodextrin inclusion complex. *Eur J Pharm Sci* 2008; 33: 60–71.
14. Licinio J. Central nervous system cytokines and their relevance for neurotoxicity. *J Neural Transm* 1997; 49 (Suppl.): 169–175.
15. Borish LC, Steinke JW. 2. Cytokines and chemokines. *J Allergy Clin Immunol* 2003; 111 (2 Suppl.): 460–475.
16. Boogaerts JG *et al.* Epidural administration of liposome-associated bupivacaine for the management of postsurgical pain: a first study. *J Clin Anesth* 1994; 6: 315–320.
17. Boogaerts JG *et al.* Toxicity of bupivacaine encapsulated into liposomes and injected intravenously: comparison with plain solutions. *Anesth Analg* 1993; 76: 553–555.
18. Dollo G *et al.* Inclusion complexation of amide-type local anesthetics with b-cyclodextrin and derivates. III. Biopharmaceutics of bupivacaine-SBE7-b-CD complex following percutaneous sciatic nerve administration in rabbits. *Int J Pharm* 1998; 164: 11–19.
19. Loftsson T, Masson M. Cyclodextrins in topical drug formulations: theory and practice. *Int J Pharm* 2001; 225: 15–30.
20. Huang YH *et al.* Bupivacaine inhibits COX-2 expression, PGE_2, and cytokines production in endotoxin-activated macrophages. *Acta Anaesthesiol Scand* 2008; 52: 530–535.
21. Nestle FO *et al.* Skin immune sentinels in health and disease. *Nat Rev Immunol* 2009; 9: 679–691.

22. Di Caprio R *et al.* Anti-inflammatory properties of low and high doxycycline doses: an in vitro study. *Mediators Inflamm* 2015; 2015: 329418.
23. Hillmann G *et al.* Matrix expression and proliferation of primary gingival fibroblasts in a three-dimensional cell culture model. *J Cell Sci* 1999; 112: 2823–2832.
24. Pelegrini CB *et al.* Morphological, functional and biochemical characterization of canine gingival fibroblasts. *Braz Dent J* 2013; 24: 128–135.
25. Boukamp P *et al.* Normal keratinization in a spontaneously immortalized aneuploid human keratinocyte cell line. *J Cell Biol* 1988; 106: 761–771.
26. Ravid A *et al.* Vitamin D inhibits the activation of stress-activated protein kinases by physiological and environmental stresses in keratinocytes. *J Endocrinol* 2002; 173: 525–532.
27. Kimi H *et al.* The local pharmacokinetics of ^{3}H-ropivacaine and ^{14}C-lidocaine after maxillary infiltration anesthesia in rats. *Anesth Prog* 2012; 59: 75–81.
28. Cereda CM. Local neurotoxicity and myotoxicity evaluation of cyclodextrin complexes of bupivacaine and ropivacaine. *Anesth Analg* 2012; 115: 1234–1241.
29. Denning MF. Epidermal keratinocytes: regulation of multiple cell phenotypes by multiple protein kinase C isoforms. *Int J Biochem Cell Biol* 2004; 36: 1141–1146.
30. Garach-Jehoshua O *et al.* 1,25-Dihydroxyvitamin D3 increases the growth-promoting activity of autocrine epidermal growth factor receptor ligands in keratinocytes. *Endocrinology* 1999; 140: 713–721.
31. Piepkorn M *et al.* Autocrine regulation of keratinocytes: the emerging role of heparin-binding, epidermal growth factor-related growth factors. *J Invest Dermatol* 1998; 111: 715–721.
32. Lucchinetti E *et al.* Antiproliferative effects of local anesthetics on mesenchymal stem cells: potential implications for tumor spreading and wound healing. *Anesthesiology* 2012; 116: 841–856.
33. Kontargiris E *et al.* Ectopic expression of clusterin/apolipoprotein J or Bcl-2 decreases the sensitivity of HaCaT cells to toxic effects of ropivacaine. *Cell Res* 2004; 14: 415–422.
34. Fedder C *et al.* *In vitro* exposure of human fibroblasts to local anaesthetics impairs cell growth. *Clin Exp Immunol* 2010; 162: 280–288.
35. de Paula E, Schreier S. Molecular and physicochemical aspects of local anesthetic-membrane interaction. *Braz J Med Biol Res* 1996; 29: 877–894.
36. Martinsson T *et al.* Ropivacaine inhibits leukocyte rolling, adhesion and CD11b/CD18 expression. *J Pharmacol Exp Ther* 1997; 283: 59–65.
37. Piegeler T *et al.* Endothelial barrier protection by local anesthetics: ropivacaine and lidocaine block tumor necrosis factor-α-induced endothelial cell Src activation. *Anesthesiology* 2014; 120: 1414–1428.
38. Brocco MC *et al.* A study of interleukin 6 (IL-6) and tumor necrosis factor alpha (TNF-α) serum levels in rats subjected to fecal peritonitis and treated with intraperitoneal ropivacaine. *Acta Cir Bras* 2012; 27: 494–498.
39. De Iuliis A *et al.* Differences of ropivacaine and bupivacaine relevant to antiinflammatory activity, platelet aggregation and antioxidant activity in vitro. *Farmaco* 2001; 56: 153–157.
40. Zhang XW, Thorlacius H. Inhibitory actions of ropivacaine on tumor necrosis factor-alpha-induced leukocyte adhesion and tissue accumulation in vivo. *Eur J Pharmacol* 2000; 392: R1–R3.
41. Rabinow B *et al.* Intra-articular (IA) ropivacaine microparticle suspensions reduce pain, inflammation, cytokine, and substance p levels significantly more than oral or IA celecoxib in a rat model of arthritis. *Inflammation* 2015; 38: 40–60.
42. Martin F *et al.* Antiinflammatory effect of peripheral nerve blocks after knee surgery: clinical and biologic evaluation. *Anesthesiology* 2008; 109: 484–490.
43. Watkins LR *et al.* Characterization of cytokine-induced hyperalgesia. *Brain Res* 1994; 654: 15–26.
44. Watkins LR *et al.* Immune activation: the role of pro-inflammatory cytokines in inflammation, illness responses and pathological pain states. *Pain* 1995; 63: 289–302.
45. Beutler B, Cerami A. The biology of cachectin/TNF – a primary mediator of the host response. *Annu Rev Immunol* 1989; 7: 625–655.
46. Dinarello CA. Proinflammatory cytokines. *Chest* 2000; 118: 503–508.
47. Monfrecola G *et al.* Nicotinamide downregulates gene expression of interleukin-6, interleukin-10, monocyte chemoattractant protein-1, and tumour necrosis factor-α gene expression in HaCaT keratinocytes after ultraviolet B irradiation. *Clin Exp Dermatol* 2013; 38: 185–188.
48. Sheeran P, Hall GM. Cytokines in anaesthesia. *Br J Anaesth* 1997; 78: 201–219.
49. Byun JY *et al.* Expression of IL-10, TGF-beta(1) and TNF-alpha in cultured keratinocytes (HaCaT cells) after IPL treatment or ALA-IPL photodynamic treatment. *Ann Dermatol* 2009; 21: 12–17.
50. Byun JY *et al.* The expressions of TGF-β(1) and IL-10 in cultured fibroblasts after ALA-IPL photodynamic treatment. *Ann Dermatol* 2011; 23: 19–22.
51. Ansel J *et al.* Cytokine modulation of keratinocyte cytokines. *J Invest Dermatol* 1990; 94 (6 Suppl.): 101S–107S.
52. Samberg ME *et al.* Evaluation of silver nanoparticle toxicity in skin *in vivo* and keratinocytes *in vitro*. *Environ Health Perspect* 2010; 118: 407–413.
53. Grandjean-Laquerriere A *et al.* Differential regulation of TNF-alpha, IL-6 and IL-10 in UVB-irradiated human keratinocytes via cyclic AMP/protein kinase A pathway. *Cytokine* 2003; 23: 138–149.
54. Foley PL *et al.* A chitosan thermogel for delivery of ropivacaine in regional musculoskeletal anesthesia. *Biomaterials* 2013; 34: 2539–2546.
55. Mantha VR *et al.* Nanoanesthesia: a novel, intravenous approach to ankle

block in the rat by magnet-directed concentration of ropivacaine-associated nanoparticles. *Anesth Analg* 2014; 118: 1355–1362.

56. Zhai Y *et al.* Lipid nanocapsules for transdermal delivery of ropivacaine: in vitro and in vivo evaluation. *Int J Pharm* 2014; 471: 103–111.

57. Wang Z *et al.* Long-term effect of ropivacaine nanoparticles for sciatic nerve block on postoperative pain in rats. *Int J Nanomedicine* 2016; 11: 2081–2090.