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IN VITRO CYTOMODULATORY AND IMMUNOMODULATORY EFFECTS OF BOVINE COLOSTRUM WHEY PROTEIN HYDROLYSATES

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ABSTRACT

Bovine colostrum possesses biological compounds involved in the development of the new-born. Among them, the proteins have drawn attention as a source of bioactive peptides. This study shows the *in vitro* cytotoxic and immunostimulatory potential of two colostrum whey protein (CWP) hydrolysates obtained by *in vitro* digestion with pepsin and pancreatin. MTT cell viability, apoptosis induction, polymorphonuclear proliferation and phagocytic activity assays were performed. Treatment with the hydrolysates induced a significant decrease in the viability of MDA-MB-231 cell lines due to apoptosis and also a significant increase in the proliferation of blood mononuclear cells. It could also be observed that for the RM-1 and PC-3 prostate cancer cell lines and for the two times of exposure (24 and 48 h), the hydrolysate H1 is significantly more cytotoxic than CWP. These results showed the potential of bovine CWP and its hydrolysates for the treatment of chronic diseases such as cancer.

KEYWORDS

Bovine colostrum, bioactive peptides, cytotoxic, immunomodulatory, hydrolysates

INTRODUCTION

Colostrum is a nutrient-rich fluid produced by the cow during the first 4 days after the calf delivery (Dzik *et al.*, 2017). Proteins, peptides, lipids, vitamins, minerals, hormones, growth factors, cytokines and nucleotides, in a higher concentration than in mature milk, are among its main components (McGrath *et al.*, 2016). Colostrum also contains a higher proportion of bioactive proteins and peptides than milk and a very high concentration of immunoglobulins (they make up to 70-80% of the total colostrum proteins) to protect the new-born who has not yet fully developed its immune system. All these components decrease after subsequent milkings, being almost absent in mature milk (Borad and Singh, 2018).

The whey fraction of bovine colostrum (BC) represents 12.7% of the total protein. There are globular proteins, such as lactoferrin (0.75 mg/mL), that has shown iron-binding, anticancer and immunomodulatory capacities both *in vitro* and *in vivo* (Zhang *et al*., 2014). Preventive effects of carcinogenesis and cancer metastasis by bovine lactoferrin and its derived peptide lactoferricin have been shown in rats and mice (Tsuda *et al.*, 2006; Agarwal and Gupta, 2016). It has also been observed that proteins, such as α-lactalbumin (13.82 mg/mL), are able to form complexes with fatty acids with anticancer activity (Rodríguez-Carrio *et al.*, 2014; Athira *et al.*, 2015). The structural characteristics and amino acid sequences of the proteins contained in BC whey are attractive for their use as ingredients in functional food development (Sah *et al.*, 2015; Mohanty *et al.*, 2016).

There are many studies on *in vitro* digestion using commercial enzymatic preparations, such as pepsin, chymotrypsin, and trypsin, to release bioactive peptides from whey proteins (Inglingstad *et al.*, 2010) with important antioxidant capacity (Corrochano *et al.*, 2018). Some of these peptides have been reported as cytotoxic agents or cytomodulators (Gaspar *et al*., 2013). In the case of α-lactalbumin and β-lactoglobulin, anti-tumor, apoptosis-induction, immunomodulatory, antihypertensive, and many other bioactivities have been found (Hernández-Ledesma *et al*., 2008; Kamau *et al.*, 2010).

Since cancer remains one of the chronic degenerative diseases with the highest morbidity and mortality rates worldwide, there is a need for new, selective, and more effective medications. It is expected that colostrum-derived peptides with cytomodulatory or anticancer activity can be selective towards tumorous cells (Chalamaiah *et al*., 2018).

In this study, we tested the effect of BC whey and its hydrolysates with digestive enzymes on the cell proliferation of the MDA-MB-231, RM-1 and PC3 tumorous cell lines, as well as the immunomodulatory effect on the proliferation of mononuclear cells and induction of phagocytosis in polymorphonuclear cells, which could be potentially useful in the development of functional foods with therapeutic potential in the treatment of cancer.

MATERIALS AND METHODS

Chemicals

Bradford Reagent, TNBS (0.1% w/v 2,4,6-trinitrobenzenesulfonic acid solution), pepsin 1:2500 (620 U/mg), pancreatin 8x USP, Histopaque-1119, RPMI 1640 medium, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), and carboxylate-modified polystyrene latex beads with red fluorescent dye were obtained from Sigma-Aldrich; DMEM (Dulbecco's Modified Eagle Medium – Gibco) and Alexa Fluor 488 Annexin V/Dead Cell Apoptosis Kit were acquired from Thermo Fisher Scientific, and Lymphosep (Lymphocyte Separation Media) was purchased from BioWest.

BC whey separation, protein analysis and *in vitro* gastrointestinal digestion.

BC whey from the first and second milkings following calving of the Holstein cows was obtained by centrifugation at 10,000 RFC to remove fat, and by adjusting the pH to 4.6 and centrifuging again at 10,000 RFC to remove caseins.

Protein determination was carried out following the Bradford methodology adapted for Nanodrop 2000c (Thermo) following the manufacturer protocol for micro assay.

In vitro gastrointestinal digestion of BC whey was performed using the procedure described by Fajardo-Espinoza *et al.* (2020). Briefly, the pH of the samples was adjusted to 2 with concentrated HCl and then pepsin, previously dissolved in HCl-KCl (0.1M pH 2) buffer, was added at a 1:20 enzyme:substrate ratio. The sample was incubated with constant orbital agitation (150 rpm), at 37°C for 60 min to obtain a first hydrolysate containing higher molecular weight (MW) peptides (H2). Samples of these H2 pepsin hydrolysates were frozen at -50°C for further experimentation.

Subsequently, the pH was adjusted to 7.5 with 0.5M NH_4HCO_3 and pancreatin dissolved in 0.1M phosphate buffer pH 7.5 was added until a 1:25 enzyme: substrate ratio was reached. It was incubated under 150 rpm constant orbital agitation at 37°C for 3 h. To stop the hydrolysis, the temperature was raised to 75°C for 15 min using a controlled-temperature bath. From this sequential hydrolysis the pepsin-pancreatin hydrolysates (H1) were obtained. The samples were centrifuged at 10,000 RFC and the supernatants were frozen (-50°C) for further testing.

Degree of hydrolysis (DH)

The method of Adler-Nissen (1979) was used. Briefly, 125 µL of the CB whey sample or its hydrolysates were mixed with 1 mL of 0.2125M phosphate buffer, pH 8.2 and 100 µL of the 0.1% TNBS solution. The reaction mixture was incubated under constant stirring at 50°C for 60 min. The reaction was stopped by addition of 2 mL of 0.1N HCl to each tube which was allowed to cool and read at 340 nm using a Thermo Scientific GENESYS 10S Vis spectrophotometer. L-Leucine (0-2 mM) was used to build a standard curve and calculate the concentration of free amino groups in the samples. The DH obtained for the CWP, the hydrolysate with pepsin-pancreatin (H1), pepsin at pH 2 (H2), were expressed as concentration of mEq Leucine.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

The methodologies reported by Laemmli (1970) and Schägger (2006) for long and small peptides were used. For the Laemmli protocol, a 13% total percentage concentration of both, acrylamide and the crosslinker

bisacrylamide (%T), 3% concentration of the crosslinker relative to total of both monomers (%C) polyacrylamide stacking gel and a 5% separating gel were prepared with standard Tris-Glycine buffer solutions. Sample was diluted in 1 mL of the sample buffer, heated in a water bath at 90°C for 5 min, allowed to cool and 10 µL were applied to each lane. The stacking and separating gels were subjected to a successive constant voltage (100V) for 90-120 min with Tris-Glycine buffer. The gels were stained with Coomassie R250 bright blue 0.05%(w/v) in 50% methanol (v/v) and 10% acetic acid (v/v) with constant agitation for 30 min, and then washed with constant stirring for 90 min in a solution of acetic acid:methanol:water in a 1:4:5 ratio. For the Schägger protocol, a gradient gel (10 to 16%T, 2%C) was prepared and electrophoresis was carried out with Tris-Tricine catode buffer and Tris-HCl anode buffer with an initial voltage of 30V during 20 min, and 150V for 3h or until samples reached the lower end of the gel. Gels were stained and washed as previously described. MW markers for proteins (Sigma-Aldrich- 70 to 10 kDa) and peptides (BioRad- 26.6 to 1.4 kDa) were used to determine the approximate MW of the peptides in the electrophoresis gel.

Sample preparation for cell culture use

The colostrum and the hydrolysates from whey samples were kept frozen at -70°C and lyophilized at -48°C and 0.280 mBar vacuum pressure in a Labconco FreeZone 2.5 Liter Cascade Benchtop Freeze-Dry System.

Separation of Different Molecular Weight Fractions

Four peptide fractions were obtained as follows: Fraction 1 with MW>30 kDa, Fraction 2 with MW between 10-30 kDa, Fraction 3 with MW between 3-10 kDa, and Fraction 4 with MW<3 kDa. These peptide fractions were obtained using a centrifugation-assisted ultrafiltration system AMICON (Millipore, Billerica, USA) with cellulose membranes, following the manufacturer instructions.

MTT assay to determine cell viability.

The MDA-MB-231 and RM-1 cell lines were cultured in Dulbecco's Modified Eagle's Medium (DMEM) and the PC-3 cell line was cultured in RPMI-1640 medium, both supplemented with 10% foetal bovine serum (FBS) and 1% penicillin/streptomycin (10,000 U/µg/mL) at 37°C with 5% CO_2. Once the confluence stage was reached, the cells were trypsinized and resuspended in complete medium for counting. They were seeded at a density of 5000 cells/well in 100 µL of medium in 96-well plates and incubated 24 h. After this time the medium was removed and the cells exposed to different concentrations (0.03 to 30 mg/mL) of the colostrum whey and the hydrolysates dissolved in medium, a negative 100% viability control was used with complete medium and a positive control with 50 µg/mL paclitaxel (Zurich Pharma) for the MDA-MB-231 cell line and 50 µM Etoposide (Sigma E-1383) for the PC-3 and RM-1 cell lines. The plates were incubated 24 and 48 h and the medium was removed with the treatments. An aliquot of 100 µL of a 5 µg/mL MTT (Methylthiazolyldiphenyl-tetrazolium bromide) solution was added and the plates incubated 4 h at 37°C with 5% CO_2, then the MTT was carefully removed and 100 µL of dimethyl sulfoxide (DMSO) were added to solubilize the formazan crystals. The absorbance at 570 nm was read and the viability percentage was calculated as follows:

% Viability = (Absorbance treated cells/Absorbance cells without treatment) X 100

The assay was performed in triplicate on the above-mentioned cell lines. Comparison values were made on a 50% growth inhibition basis (IC_{50}) in cells treated with colostrum whey hydrolysates (Mada *et al.*, 2017). The NIH-3T3 cell line of fibroblasts was used as a healthy control, in which no significant decrease in cell viability was observed when applying the treatments at the same time points.

Apoptosis Induction Assay

The cells of the MDA-MB-231 cell line were seeded at a density of 100,000 cells/well in a 6-well plate with 1 mL of complete medium and incubated 24 h, then the cells were synchronized with serum-free medium and treated with the hydrolysates (10mg/mL in medium) for 6, 12, and 24 h, as well as an untreated cells control. Apoptosis was assessed with the Alexa Fluor® 488 Annexin V/Dead Cell Apoptosis Kit (Thermo Fisher) for flow cytometry following the manufacturer instructions.

Immunomodulatory activity of colostrum whey hydrolysates.

Effect of Colostrum Whey Hydrolysates on PBMC Proliferation.

Peripheral blood mononuclear cells (PBMC) are critical in breast cancer because their number has been reported to be decreased in this population (Dewan *et al.*, 2009). PBMC proliferation is also important since they are part of the mononuclear phagocyte system in tissues along with the polymorphonuclear (PMN) cells (Ginhoux *et al.*, 2016). Blood samples were obtained from a healthy adult in tubes with heparin in a sterile environment. They were diluted 1:1 with Phosphate Buffered Saline (PBS), and layered over the separation medium (Lymphosep®) at a 1:2 ratio and centrifuged at 800 RCF for 20 min at 20ºC. Subsequently, the intermediate layer (between plasma and erythrocytes), rich in PBMC, mainly lymphocytes, was removed and placed in a new sterile tube and washed twice with PBS. Afterward, they were resuspended in complete RPMI 1640 medium to be counted and seeded in 96-well plates and incubated 24 h.

Samples were added in different concentrations (0.1 to 30 mg/mL) and incubated 24, 48 and 72 h along with an untreated control with complete medium. After the exposure time to the treatments, 10 µL of the medium was removed again and 10 µL of MTT (50 µg/mL) were added to each well. The plates were incubated 4 h, the medium was removed and 100 µL of DMSO was added to solubilize the formazan crystals and the absorbance was read at 570 nm. Proliferation was assessed by comparing with an untreated control (Benson *et al.*, 2011).

Phagocytosis assay

The methodology of Benson *et al.* (2011) was used as follows: PMN cells were isolated by placing 2 mL of Lymphosep® (density 1.077) over 2 mL of Histopaque® (density 1.119) in a sterile conical centrifuge tube, 2 mL of blood (mixed 1:1 with sterile PBS) was added, centrifuged at 340 RCF for 30 min. The PMN-rich layer was isolated, washed in PBS and the PMN cells resuspended in 7.5 mL of RPMI-1640 to place in 24-well plates. 10 µL of the BC, H1 and H2 samples (1mg/mL) were taken to treat the PMN cells in triplicate, PBS and

untreated cells were used as control and incubated 1, 6 and 24 h. They were centrifuged and the supernatant removed. The cells were resuspended in RPMI-1640 with the fluorescent beads and incubated for two hours. The reaction was stopped by adding PBS with 0.02% sodium azide. Flow cytometry counting was performed (excitation wavelength 575 nm and emission wavelength 610 nm) with the allophycocyanin spectrum (APC) as a reference.

For the analysis, the electronic selection of the PMN population was performed using frontal and lateral dispersion properties. The percentage of PMN involved in phagocytic activity was determined as the mean fluorescence intensity (MFI), reflecting the number of fluorescent beads that each cell had engulfed. The MFI of the untreated samples indicates the reference level of phagocytosis that occurs in the absence of treatments. All tests were performed in triplicate.

Statistical analysis

All the experiments were performed in triplicate and the results expressed as the mean±standard deviation. The Shapiro-Wilk test (α=0.05) was used to determine if the data sets in this study were adequately fitted by a normal distribution. Differences among samples were determined by One-way ANOVA and Tukey HSD test (α=0.02) using the SPSS software (IBM SPSS Statistics v24) and Graph Pad Prism 7 for the determination of the IC_{50} of the hydrolysates in the MDA-MB-231 cell lines. Paired one tailed Student's t test (α=0.05) was used to evaluate significant differences in the case of the experiments with the RM-1 and PC-3 cell lines.

RESULTS AND DISCUSSION

Determination of the DH

The highest DH was obtained in H1 (386.66 µg eq. Leucine), followed by H2 (331.18 µg eq. Leucine) and CWP (171.18 µg eq. Leucine). In general, the greater the hydrolysis time, the higher the concentration of free amino groups, the lower the protein concentration and the higher the number of lower MW bands on the SDS-PAGE.

A greater effect was observed with respect to the hydrolysis time of the proteins by the action of pepsin, considering that the incubation time with this enzyme in the digestion model is shorter than the one used for pancreatin. The DH obtained at the end of the *in vitro* digestion was higher than that obtained by Megías *et al.* (2008) using a similar model with sunflower protein. These researchers observed that the action of pepsin is stronger in the first 10 min of incubation and then decreases, whereas pancreatin has a constant hydrolysis rate throughout the 3 h of incubation.

SDS-PAGE

Figure 1 shows that the pepsin-pancreatin hydrolysate (H1) has the highest number of bands with low MWs (less than 17 kDa). In both hydrolysates the bigger proteins were almost completely hydrolysed. This indicates

that the consumption of BC whey proteins will lead to the release of several bioactive peptides after the gastrointestinal digestion.

Morgan *et al.* (2014) found that BC proteins and peptides (colostrum treated with pepsin, chymosin, or trypsin) had a beneficial effect on human intestinal epithelial cell proliferation (T84 cell line). This proliferation is important since this tissue has a crucial role on the immune function, mainly linked to the gut-associated lymphoid tissue. These results confirm that BC is a good source of growth promoting agents, similar to foetal calf serum (FCS) control. FCS is known to content EGF, IGF-1, HGF and TGF-β as growth factors, some of these have been reported in colostrum as well.

Normality test

All the data sets in this study were adequately fitted by a normal distribution according to the Shapiro-Wilk test (p values were in the range of 0.9 to 0.94).

MTT assay to determine cell viability in breast and prostate tumorous lines

In the MDA-MB-231 line, a higher cytotoxicity of the hydrolysates was observed at concentrations of 1, 3, 10, and 30 mg/mL after 24 h in relation to the lower concentrations (Figure 2). These results are similar to those observed by González-Montoya *et al.* (2016), who reported a similar behaviour at concentrations between 10 and 30 mg/mL of germinated soybean peptides for the same incubation time points. The IC_{50} of the samples in our study were 5.52 mg/mL for H1, and 4.15 mg/mL for H2 after 24 h and decreasing to 2.48 mg/mL for H1, and 1.69 mg/mL for H2 after 48 h, which were lower values than those reported in the above mentioned study using the same cell line (15.19 mg/mL).

A greater inhibition by H1, compared to H2, was observed in the MDA-MB-231 line which can be attributed to the higher DH and the presence of lower MW peptides, which can access the intracellular environment (Li and Yu, 2015), however, this difference was only significant ($p \leq 0.05$) for the 30 mg/mL concentration. The CWP sample did not show a significant decrease in viability ($p > 0.05$), which confirms that hydrolysis increases the cytotoxic effect by releasing bioactive peptides. On the other hand, we can conclude that the concentration of lactoferrin present in the CWP was not enough to show this effect and that, as some previous studies report (Duarte *et al.*, 2011; Zhang *et al.*, 2014), concentrations higher than 6.25μM are necessary. It has also been reported that high MW complexes associated with bovine lactoferrin have a greater cytotoxic effect on the same cell line at concentrations of 800 μg/mL (Ebrahim *et al.*, 2014). A similar behaviour was observed after 48 h of incubation, where the highest concentrations were the more cytotoxic ones (see Figure 2). However, these differences were significant only for the CWP and H2 samples at concentrations in the range of 0.1 and 0.3 mg/mL, as well as in higher concentrations (3 to 30 mg/mL) of both hydrolysates compared to CWP ($p \leq 0.05$).

The different MW fractions or incubation times did not show significantly different effects on this cell line (see Figure 3), however, the pool of the different MW fractions in the colostrum hydrolysate samples showed a better cytotoxic effect on the MDA-MB-231 cell line. This effect has been reported previously for milk protein hydrolysates, suggesting a synergic effect among the different fractions (Chan-Remillard and Ozimek, 2008).

In the case of RM-1 prostate cancer cell lines (Figure 4), it was observed that both hydrolysates and CWP had a cytotoxic effect which is dependent on their concentration and exposure time. After applying a paired one tailed Student's t test ($\alpha=0.05$) to these data, it could be shown that for the two cell lines and the two times (24 and 48 h), the decrease in viability is significantly higher ($p\leq0.05$) for the hydrolysate H1 than for CWP. This indicate that H1 is significantly more cytotoxic than CWP for the RM-1 and PC-3 cell lines. A similar effect could only be observed for the hydrolysate H2 on the RM-1 cell line after 48 h. There are few antecedents regarding the treatment of prostate cancer with protein hydrolysates. However, many casomorphin peptides derived from α- and β-caseins decrease the proliferation of prostate carcinoma cell lines by a process partly involving opioid receptors which is, as in the case of our study, concentration dependent (Sah *et al.*, 2015). Chi *et al.* (2015) reported that blood clam (*Scapharca broughtonii*) protein hydrolysates had a dose-dependent anticancer effect on PC-3 cell line.

Apoptosis Induction Assay

When analysing the type of cell death, it was observed that the cytotoxicity observed in H1 and H2 is mainly due to apoptosis induction (Table 1, Figure 5). This is relevant because other types of cell death (necrosis, for example), are not desirable in the treatment of cancer due to the inflammatory and immune response triggered (Zhang *et al.*, 2014). This effect has been previously reported in the case of casein-derived antitumor peptides, where apoptotic mechanisms are involved in their anticancer activity (Sah *et al.*, 2015).

There are numerous reports on the cytotoxic effect on tumorous cell lines treated with lactoferrin. It has been shown that the action mechanisms of this protein depend on the conditions of the treatment and are mainly due to the formation of pores in the cell membrane, apoptosis induction, cell cycle arrest or modulation of immune pathways in cells. All these types of cell death have numerous potential advantages in the treatment of cancer since they are less aggressive than most of the chemotherapeutic agents currently employed (Zhang *et al.*, 2014).

An increase in apoptosis close to 50% was observed for both hydrolysates. It has been reported that pepsin is a very efficient enzyme for the production of peptides with cytomodulatory properties, due to the fact that it hydrolyses the peptide bonds between hydrophobic or aromatic amino acids leaving them exposed and with the possibility of interacting with the cells through the cell membrane, which explains the effect observed in Table 1 and Figure 4, where H1 and H2 significantly increased cell death by apoptosis (Chalamaiah *et al.*, 2018). It is not yet possible to predict the anticancer mechanism of peptides based on their structures.

Bioavailability of peptides derived from food proteins could be a limitation, since intact peptides have to be transported across the intestinal barrier and into the circulatory system, so they can reach their targets in their active form. Many properties of the peptides, such as hydrophobicity, electric charge, MW, primary structure, stability, and enzymatic degradability have a strong influence on the transport process, which in turn will determine the bioavailability of the compound and therefore must be studied further (Xu *et al.*, 2019).

Effect of Colostrum Whey and Hydrolysates on PBMC Proliferation.

A significant increase in PBMC proliferation was observed with the 3 mg/mL concentration of both colostrum and whey protein hydrolysates ($p \leq 0.05$) as shown in Figure 6. There is a previous study where white blood cell proliferation was observed after oral supplementation with a low MW fraction of colostrum (150 mg) in healthy human subjects. This effect confirms that gastrointestinal digestion of colostrum proteins might have a beneficial effect on immune cells proliferation on *in vivo* models (Jensen *et al.*, 2012). The aforementioned increase in PBMC is important, since a decrease in their population has been observed in some cancer patients (Dewan *et al.*, 2009). Therefore, we consider that the results derived from our study with the colostrum hydrolysates could lead to the development of a complementary treatment for these patients.

INDUCTION OF PHAGOCYTOSIS IN POLYMORFONUCLEAR CELLS

The effect on phagocytosis of polymorphonuclear cells (PMN), previously isolated from peripheral blood, measured by flow cytometry was observed, finding a slight increase in the percentage of phagocyte cells in particular for the H1 sample after one hour of incubation (Table 2, Figure 7). However, the differences were not significant ($p > 0.05$) unlike those reported by Benson *et al.* (2011), who found a 15% increase in phagocytic activity; however, in this study the incubation times were different in the PMN cells treated with a fraction of low MW of BC. The differences in these results could be due to the number of events analysed, as well as the incubation times and the characteristics of the sample fraction used.

Recent studies show that both casein- and whey-derived peptides have immunomodulatory capacity which is related to the increase in human lymphocyte proliferation, as well as macrophage activation, antibody synthesis and cytokine expression (Mohanty *et al.*, 2016).

Although the increase in phagocytosis in the present analysis was not significantly different ($p > 0.05$) between CWP and its hydrolysates, a trend is observed confirming that the released peptides, because of its size and hydrophobic characteristics, interact more efficiently with the cells of the immune system than intact proteins (Gaspar *et al.*, 2013). Even though the percentage of phagocyte cells decreased with time, we can observe a similar tendency in the control of untreated cells that might be explained by the natural cell lifetime. Nevertheless, the PMN cells treated with the hydrolysates, showed a greater phagocytic capacity at any time when compared with the control, even after 24h. Some reports confirm the stability of these bioactivities even

in food matrices or in combination with other beneficial components, such as probiotics, showing synergistic effects (Wei *et al.*, 2007).

CONCLUSIONS

An apoptosis-related cytotoxic effect of the hydrolysates was detected in the tumorous cell line MDA-MB-231, confirming the importance of the DH and peptide size for the anticancer effect observed in this cell line. It could also be observed that for the RM-1 and PC-3 prostate cancer cell lines and for the two times of exposure (24 and 48 h), the hydrolysate H1 is significantly more cytotoxic than CWP. A positive significant effect on immune cells treated with the hydrolysates was observed in the case of PBMC proliferation. In phagocytosis induction, a tendency to increase this capacity of PMN cells was also observed. The results obtained in this study proved the cell selectivity of the cytototoxic effect in tumorous cell lines, as well as the enhanced immune cells proliferation and activation with the colostrum hydrolysates. Since BC and its hydrolysates are a safe and available product, these results suggest they could be used for functional food development.

DATA AVAILABILITY

Data available on request from the authors.

ETHICAL GUIDELINES

Ethic approval was not required for this research.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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FIGURE CAPTIONS

Figure 1. 13%T Tris-Tricine SDS PAGE of colostrum whey protein (CWP), CWP pepsin-pancreatin hydrolysates (H1), and CWP pepsin hydrolysates (H2) using a 10-16% gradient separating gel. The molecular weights of peptide molecular marker electrophoresis standards (L) are shown on the left. The bands corresponding to the highest molecular weight proteins in CWP are fully digested in both, H1 and H2, there are several bands below 17kDa that represent the peptides generated during hydrolysis, this is clearer in H1.

Figure 2. Determination of cytotoxicity in the MDA-MB-231 line of the CWP and its hydrolysates. Test in triplicate, * p≤0.05, ** p≤0.005, *** p≤0.0005, the results are expressed as the mean ± SD. There is a significant reduction of viability when MDA-MB-231 cells are treated with both H1 and H2 at concentrations above 1 mg/ml, being consistent at 24 and 48 h time points.

Figure 3. Determination of the cytotoxicity in tumorous cell lines of the CWP fractions and their hydrolysates (10 mg / mL) in the MDA-MB-231 line. Test performed in triplicate, *** p≤0,0005, the results are shown as the mean ± SD. The low molecular weight peptides (<3kDa) of H1 sample showed a significant decrease in cell viability, comparable to the peptides with molecular weight above 30 kDa of sample H2, however the greater cytotoxic effect was observed in the complete hydrolysates as shown in Figure 2.

Figure 4. Determination of the cytotoxic effect on prostate cancer lines RM1 and PC3 of the CWP and its hydrolysates. Test in triplicate, * p≤0.05, the results are shown as the mean ± SD. A paired one-tailed Student's t test showed that for the two cell lines and the two times (24 and 48 h), the decrease in viability is significantly higher for the hydrolysate H1 than for CW indicating that H1 is significantly more cytotoxic than CWP for the RM-1 and PC-3 cell lines.

Figure 5. Representative images of the determination of the effect on the induction of apoptosis in MDA-MB-231 cell line in the presence of CWP hydrolysates. Test performed in duplicate. A: Untreated, B: H1, C: H2; at 24 h.

Figure 6. Determination of the effect on lymphocyte proliferation (PBMNC) of CWP and its hydrolysates. Test performed in triplicate, (* p≤0.05); the results are expressed as the mean ± SD. There is an induction on lymphocyte proliferation by the three samples, with concentrations above 1 mg/mL, being H2 the sample with higher effect.

Figure 7. Representative images of the determination of the effect on the induction of phagocytosis in PMN cells in the presence of CWP and its hydrolysates. Test performed in duplicate. A: Control, B: CWP, C: H1, D: H2; at 1-hour incubation. H1 and H2 showed phagocytosis induction in PMN cells compared to the untreated control and CWP treated sample, we can attribute this effect to the presence of peptides in H1 and H2.

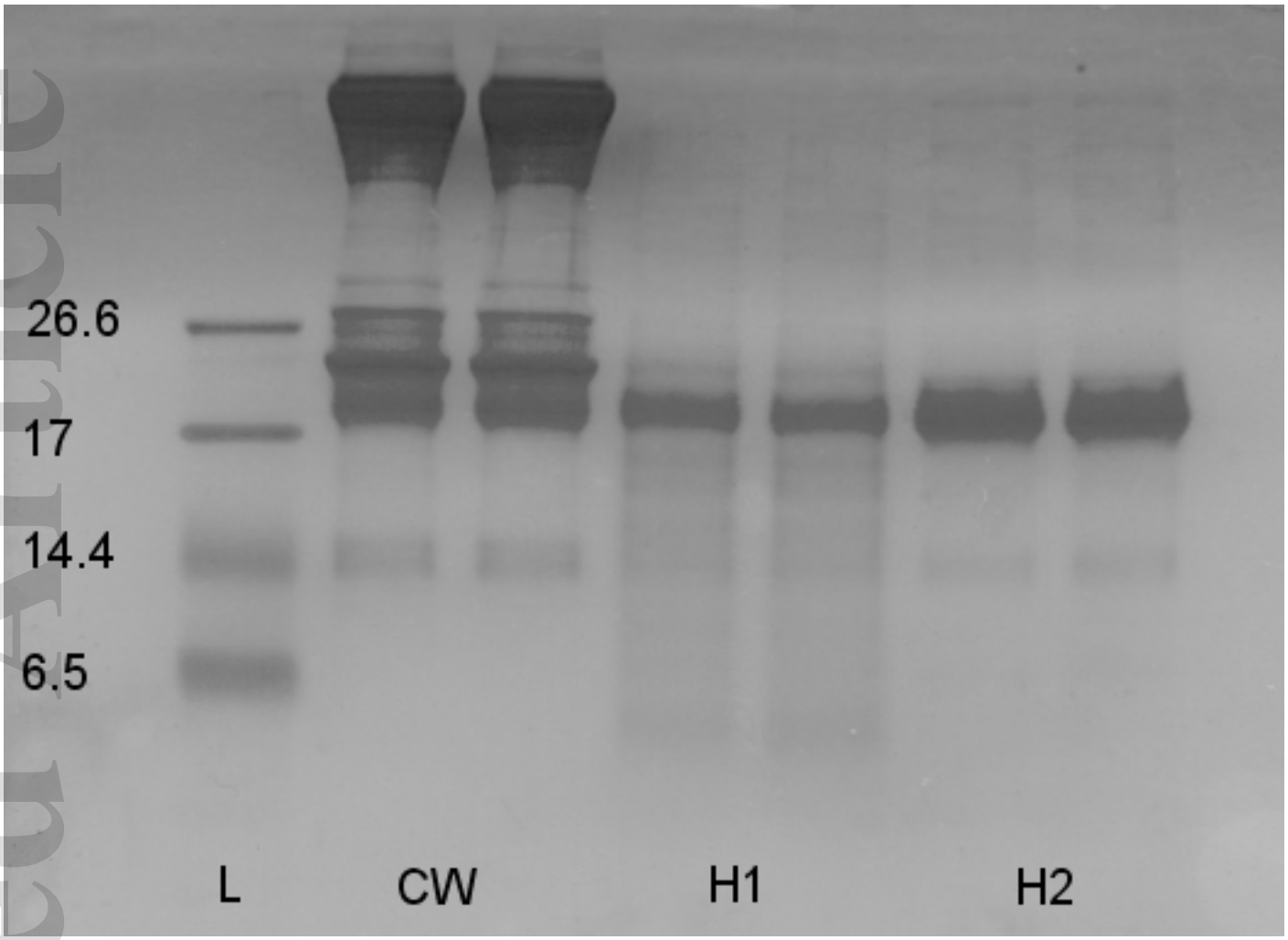
TABLES

Table 1. Apoptosis induction on MDA-MB-231 cells (%). Cells were stained with Alexa® Fluor 488 annexin V and propidium iodide (PI) for flow cytometry to distinguish apoptotic and necrotic cells. (%). Test performed in duplicate with an untreated control and the CWP hydrolysates (H1 and H2)

SAMPLE	EARLY APOPTOSIS (%)			LATE APOPTOSIS (%)			VIABLE (%)			NECROTIC (%)		
TIME	**6 h**	**12 h**	**24 h**	**6 h**	**12 h**	**24 h**	**6 h**	**12 h**	**24 h**	**6 h**	**12 h**	**24 h**
UNTREATED	7.1±0.35	6.77±1.03	10.82±1.96	3.07±0.63	4.21±0.03	2.37±0.06	89.5±1.13	87.6±0.71	66.1±1.5	0.33±0.08	1.4±0.27	0.75±0.14
H1	7.33±0.85	15.2±0.99	21.85±7.7	3.77±0.52	10.22±2.66	26.25±19.72	88.25±0.35	72.75±4.17	50.3±19.6	0.68±0.03	1.89±0.35	1.56±0.28
H2	8.64±0.4	12.35±0.49	17.7±9.05	7.21±1.05	9.92±3.22	37.25±28.35	82.95±0.21	76.15±3.04	43.25±25.5	1.18±0.33	1.57±0.21	1.84±0.97

Table 2. Phagocytosis induction on polymorphonuclear (PMN) cells (%). The PMN cells were incubated with carboxylate-modified polystyrene latex beads with red fluorescent dye for flow cytometry (excitation wavelength 575 nm and emission wavelength 610 nm). Test performed in duplicate in an untreated control and the CWP, H1, and H2 samples

SAMPLE (1mg/mL)	**1 h**	**6 h**	**24 h**
UNTREATED	3.59±0.17	3.21±1.26	2.77±0.53
CWP	4.9±0.54	6.12±3.12	4.77±0.08
H1	7.93±0.49	5.34±1.53	4.65±0.59
H2	6.49±0.54	5.59±0.74	5.86±0.02



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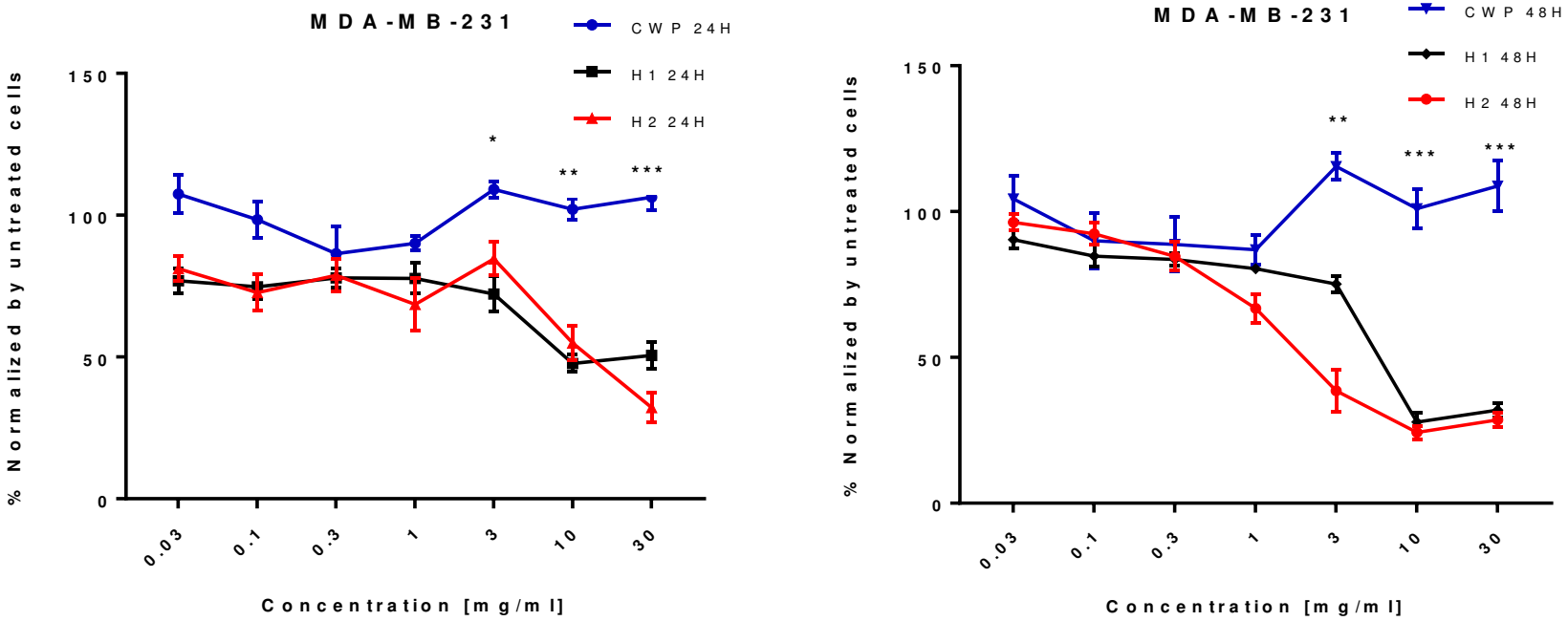

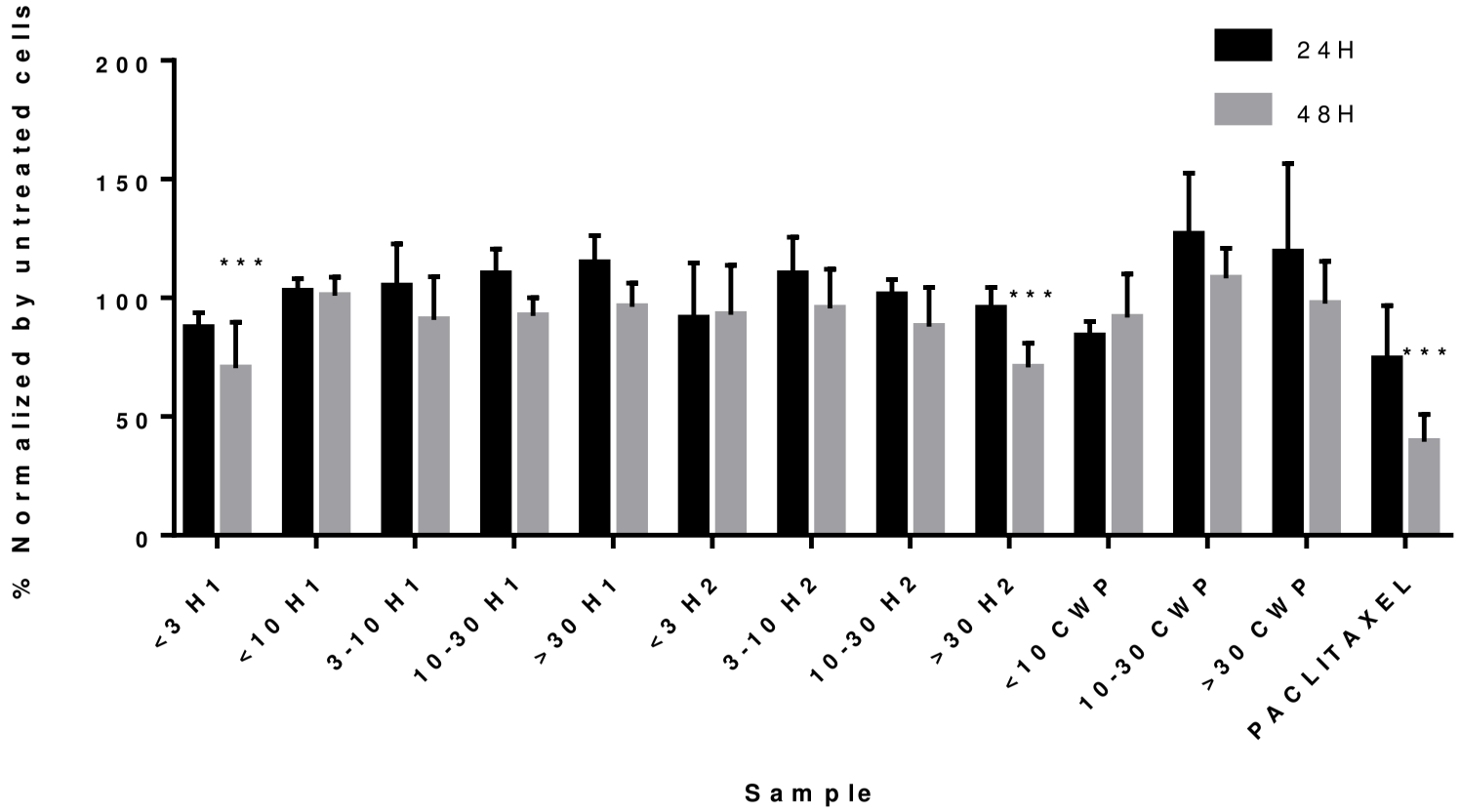

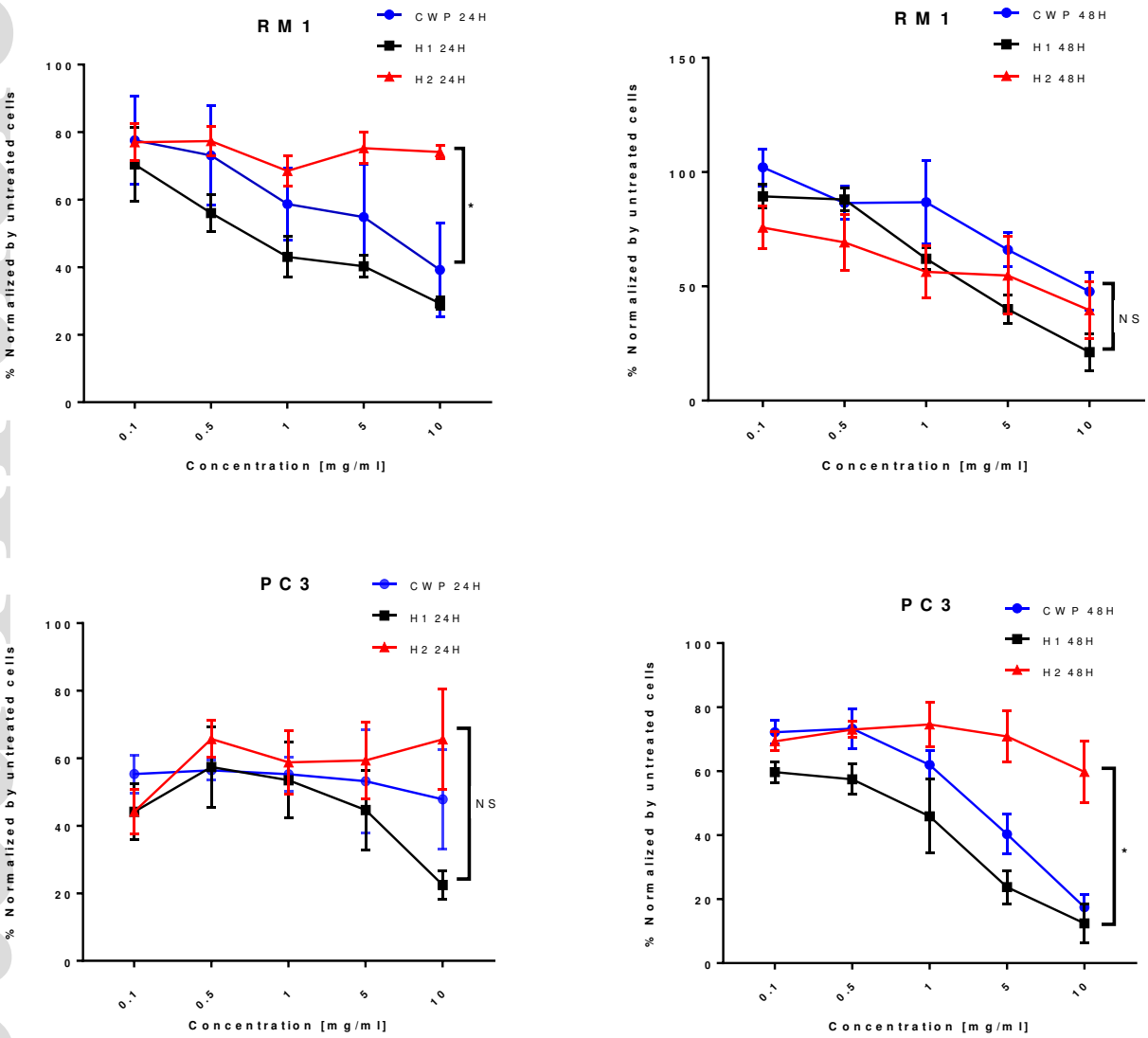

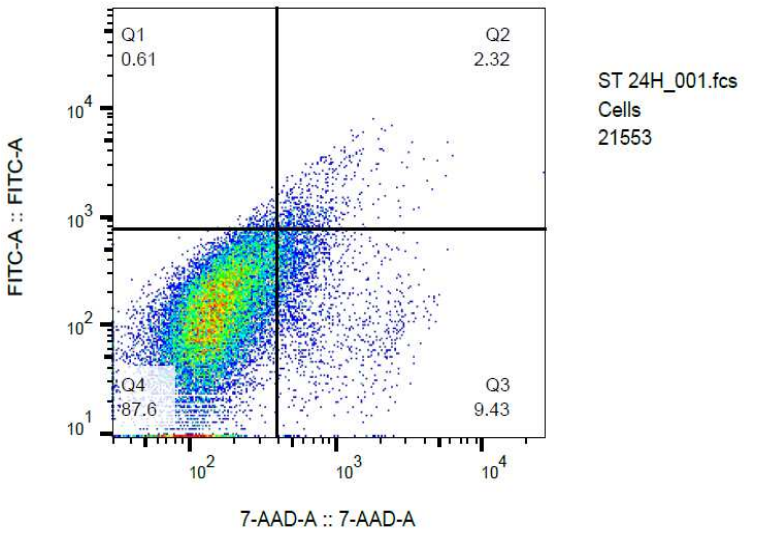

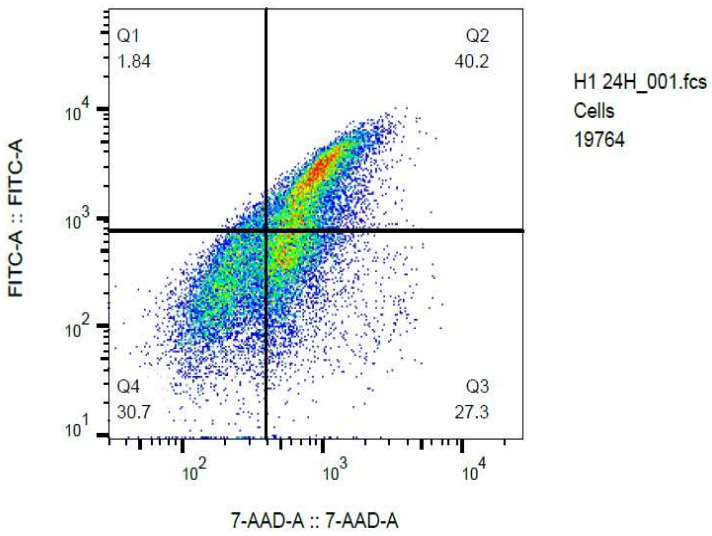

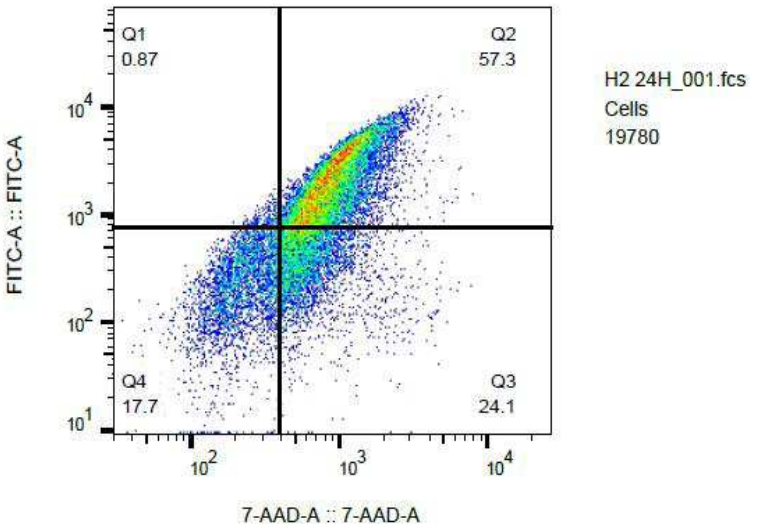

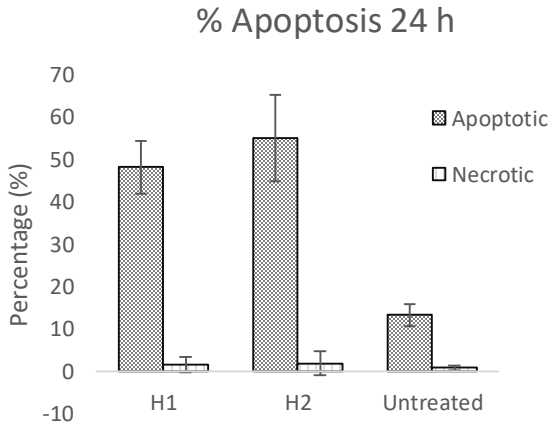

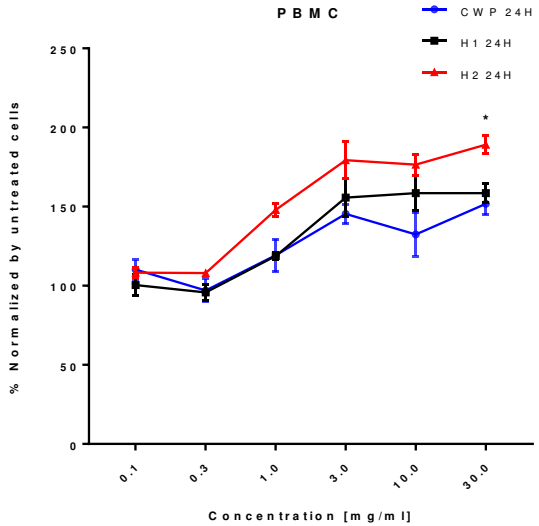

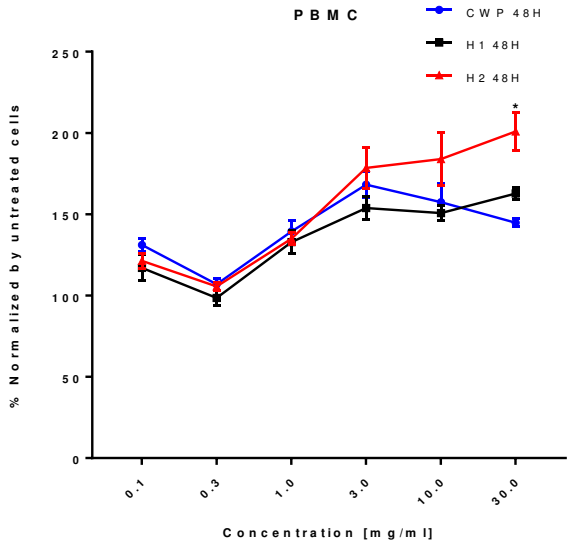

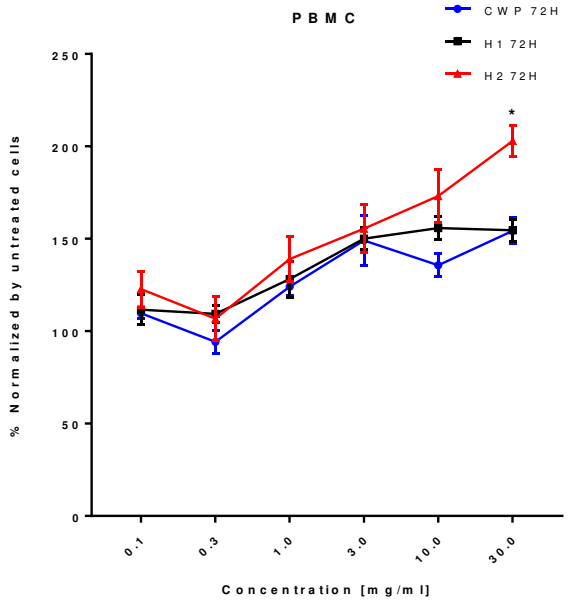

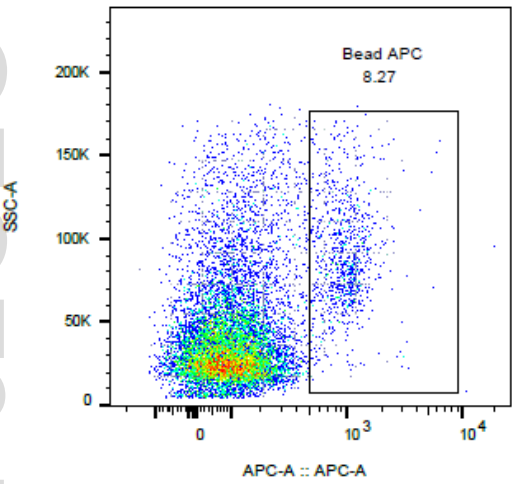

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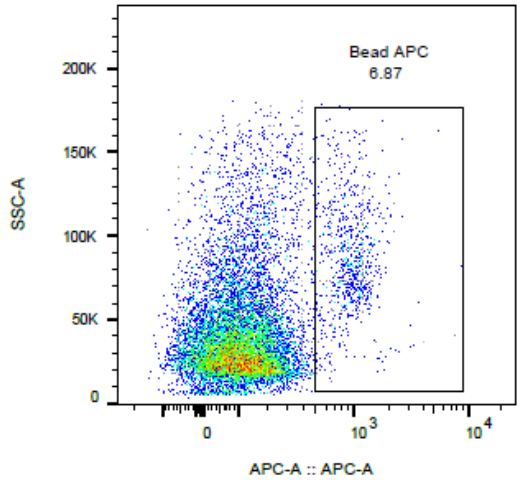

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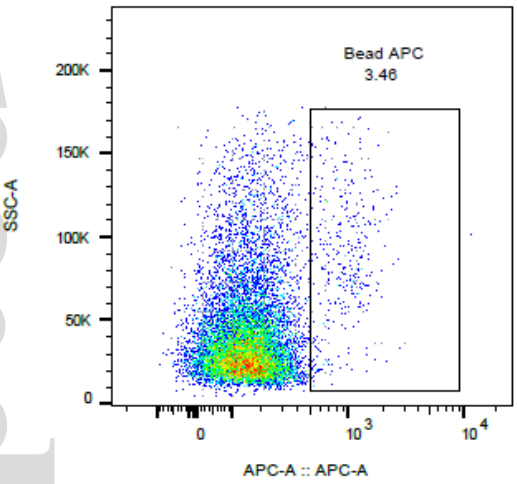

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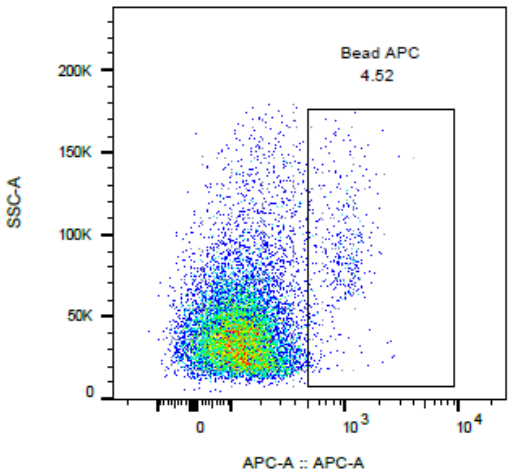

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