ANTICANCER RESEARCH *40*: 689-694 (2020)
doi:10.21873/anticanres.13998

Delivery of Apoptosis-inducing Piperine to Triple-negative Breast Cancer Cells *via* Co-polymeric Nanoparticles

JAVAD GHASSEMI RAD[1] and DAVID W. HOSKIN[1,2,3]

[1]*Department of Pathology, Faculty of Medicine, Dalhousie University, Halifax, NS, Canada;*
[2]*Department of Microbiology and Immunology, Faculty of Medicine, Dalhousie University, Halifax, NS, Canada;*
[3]*Department of Surgery, Faculty of Medicine, Dalhousie University, Halifax, NS, Canada*

Abstract. *Background/Aim: Piperine, a major alkaloid of the fruit of black pepper plants, selectively inhibits the growth of triple-negative breast cancer cells but its lipophilicity restricts possible clinical application. This study therefore determined the feasibility of encapsulating piperine in nanoparticles (NPs) to increase its solubility in an aqueous environment. Materials and Methods: Piperine-loaded biodegradable methoxy poly(ethylene glycol)-poly(lactic-co-glycolic) acid copolymer-based NPs were produced by single emulsion solvent extraction and thin-film hydration. Growth and viability of triple-negative breast cancer (TNBC) cells were determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay and Annexin-V-FLUOS/propidium iodide staining, respectively. Results: Thin-film hydration was superior to single emulsion solvent extraction, yielding piperine-loaded NPs with an average size of 50 nm. Piperine-loaded NPs inhibited TNBC cell growth and induced apoptosis while sparing normal fibroblasts. Conclusion: It is feasible to deliver a cytotoxic concentration of piperine to TNBC cells via NPs with the potential for improved bioavailability and solubility in biological fluids.*

Bioactive phytochemicals have attracted considerable interest as a potential source of new and less toxic treatment modalities for cancer and other chronic diseases. Piperine, a pungent alkaloid that is the principle bioactive component of fruit from the black pepper plant (*Piper nigrum*), has anticancer properties, as well as other pharmacological activities (1). Numerous studies attest to the antiproliferative and cytotoxic effects of piperine on many types of cancer cells, including mammary carcinoma cells, both *in vitro* and *in vivo* (2-4). However, clinical application of piperine to cancer treatment is hindered by solubility challenges caused by its hydrophobicity (5).

The entrapment of hydrophobic anticancer drugs within nanoparticles (NP) markedly improves their solubility and *in vivo* stability (6). Synthetic polymeric NPs, in particular, have superior stability, payload capacity, and controlled drug release. Poly(lactic-co-glycolic) acid (PLGA) and poly(ethylene glycol) (PEG) are frequently used to construct co-polymer NPs because of their excellent biocompatibility (7). The use of hydrophilic PEG also guards against rapid opsonization of NPs and elimination by phagocytic cells. Passive targeting of NPs to the tumor microenvironment requires NPs with an average diameter of 40-100 nm, allowing selective passage across tumor-associated blood vessels (8).

In this study, we determined the feasibility of using NPs generated from a biodegradable methoxy poly(ethylene glycol)-poly(lactic-co-glycolic) acid (mPEG-PLGA) co-polymer to deliver a cytotoxic concentration of piperine to triple-negative breast cancer (TNBC) cells. Recurrent TNBC is in urgent need of new treatment strategies due to its inability to respond to estrogen receptor and human epidermal growth factor receptor 2 (HER2)-targeted second-line therapies (9).

Materials and Methods

Cell lines and tissue culture. MDA-MB-468 breast adenocarcinoma cells were obtained from Dr. P. Lee (Dalhousie University, Halifax, NS, Canada). BT-549 breast ductal carcinoma cells were from Dr. P. Marcato (Dalhousie University, Halifax, NS, Canada). Cell lines were authenticated using the short tandem repeat method. Human dermal fibroblasts (HDF) from Lonza Inc. (Walkersville, MD, USA) were cultured in Fibroblast Basal Medium supplemented with fetal bovine serum, insulin, gentamicin sulfate-amphotericin, and fibroblast growth factor (all from Lonza) and maintained at 37˚C in a humidified incubator with 5% CO_2. MDA-MB-468 cells and BT-549 cells were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% heat-inactivated fetal bovine

Correspondence to: Dr. David Hoskin, Department of Pathology, Dalhousie University, 5850 College Street, P.O. Box 15000, Halifax, Nova Scotia B3H 4R2, Canada. E-mail: d.w.hoskin@dal.ca

Key Words: Apoptosis, breast cancer, methoxy poly(ethylene glycol)-poly(lactic-co-glycolic) acid, piperine, thin-film hydration.

serum, 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine, and 5 mM HEPES (all from Invitrogen Canada, Burlington, ON, Canada), and maintained at 37˚C in a humidified incubator with 10% CO_2.

NP formulation. NPs were formulated by single emulsion solvent extraction, using a modification of the method developed by Fessi *et al*. (10). Briefly, 8.5 mg piperine (Sigma-Aldrich, Oakville, ON, Canada) and 100 mg mPEG-PLGA (5-10 kDa; Akina Inc., West Lafayette, IN, USA) were dissolved in 10 ml dichloromethane to generate the organic phase. The aqueous phase consisted of 150 ml distilled water. Lutrol® F68 (0.1%; Sigma-Aldrich) was used as a surfactant to stabilize the emulsion droplets. The organic phase was added drop-wise to the aqueous phase under constant high-speed stirring for 2 h. The emulsion was then heated to ~40˚C under vacuum using a rotary evaporator to remove the dichloromethane. NPs were pelleted by ultracentrifugation at 27,000 × *g* for 15 min, then dissolved in water containing 5% sucrose as a cryoprotectant, flash-frozen, lyophilized and stored at room temperature. Prior to use, NPs were reconstituted in sterile water.

To formulate NPs by thin-film hydration (11), 5 mg piperine and 45 mg mPEG-PLGA were dissolved in 5 ml dichloromethane. The solution was transferred to a round-bottom flask for evaporation under vacuum at 60˚C using a rotary evaporator. The resulting thin film was dissolved in saline solution and stirred at 60˚C to allow for the self-assembly of polymers into piperine-containing micelles. Dialysis across a membrane with a 14 kDa cut-off against saline solution at room temperature removed any unencapsulated piperine. NPs were then flash-frozen, lyophilized, and stored at room temperature. Prior to use, NPs were reconstituted in sterile water and sonicated for 5 min with an ultrasonic probe to generate NPs of the desired size. All NP preparations were passed through a 0.2 μm syringe filter to remove polymer aggregates and any crystals of piperine.

NP characterization. NPs were placed on a grid, stained with 0.2% uranyl acetate for 30 s, and dried. The grid was examined with a JEM 1230 transmission electron microscope (JEOL USA Inc., Peabody, MA, USA). Images were captured with an ORCA-HR digital camera (Hamamatsu Photonics, Hamamatsu, Japan). Particle size was measured using AMT Image Capture Engine (version 6.02; AMT Corp., Woburn, MA, USA). Measurements of 90 randomly selected NPs yielded an average NP size.

Encapsulation efficiency was determined by spectrophotometric analysis. The absorbance of piperine at 346 nm is proportional to its concentration over the range used in this study. Absorbance of piperine dissolved in dimethyl sulfoxide (DMSO) at a range of concentrations (10-80 μg/ml) was measured using an UVM 340 microplate reader (Biochrom, Cambridge, UK) to generate a standard curve. Absorbance of piperine-loaded NPs (5 mg/ml in DMSO) was also determined. Absorbance of empty NPs was subtracted from that of piperine-containing NPs to correct for NP polymer absorbance. The standard curve was used to determine the amount of piperine in the NP preparation. Encapsulation efficiency was calculated by comparing the amount of piperine contained within NPs with the amount of piperine used in NP preparation.

Cell growth and viability assays. All experiments used low passage cultures in which the passage number at 80-90% confluency did not exceed 25 in the case of MDA-MB-488 and BT-549 cell lines, or 12 in the case of HDFs.

TNBC growth in the absence and presence of piperine-loaded NPs or free piperine was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma-Aldrich) assay. TNBC cells were seeded in quadruplicate wells of a 96-well plate at a density of 5×10^3 cells/well and cultured overnight to allow for adherence. Cultures then received medium, vehicle or 50-150 μM of piperine (free or loaded into NPs), followed by culture for 48 h. The concentrations of piperine used were previously determined to be cytotoxic for TNBC cells (4).

Cell death was assessed by flow cytometric analysis of cells stained with Annexin-V-FLUOS (Roche Diagnostics, Laval, QC, Canada) and propidium iodide (PI; Sigma-Aldrich). TNBC cells were seeded in a 6-well plate at a density of 5×10^4 cells/well and cultured overnight to allow for adherence. Cultures then received medium, vehicle, or 150 μM piperine (free or loaded into NPs), followed by culture for 48 h. The MTT and the Annexin-V-FLUOS/PI staining assays were performed as described in detail by Greenshields *et al*. (4).

Statistical analysis. Microsoft Excel (Redmond, WA, USA) and GraphPad Prism (GraphPad Software, San Diego, CA, USA) were used for analysis of data from three independent experiments. Statistical significance was determined by two-tailed Student's *t*-test or analysis of variance (ANOVA) with the Tukey multiple comparisons post-test; differences were considered statistically significant at $p<0.05$.

Results

Thin-film hydration is superior to single emulsion solvent extraction for piperine encapsulation. Piperine was encapsulated in mPEG-PLGA co-polymer-based NPs using the single emulsion solvent extraction and thin-film hydration methods. The resulting NPs were subjected to negative staining and visualized at high magnification using transmission electron microscopy. As shown in Figure 1A, thin-film hydration resulted in NPs that were spherical in shape and uniform in size distribution, whereas NPs formed by the single emulsion solvent extraction showed considerable variability in both size and shape. In addition, NPs generated by thin-film hydration were significantly smaller than those generated by single emulsion solvent extraction (Figure 1B). The average diameter of NPs produced by single emulsion solvent extraction method was 165±5 nm (67-350 nm size range). The average diameter of NPs produced by thin-film hydration was 53±1 nm (32-82 nm size range). We focused on thin-film hydration for the production of NPs used in subsequent experiments. In our hands, the encapsulation efficiency of piperine by thin-film hydration was approximately 18%.

NP-encapsulated piperine inhibits TNBC cell growth. The effect of NP-encapsulated piperine with that of free piperine on the growth of TNBC cells was compared using MTT assays. Figure 2 shows that piperine-loaded NPs and free piperine had an equivalent dose-dependent inhibitory effect

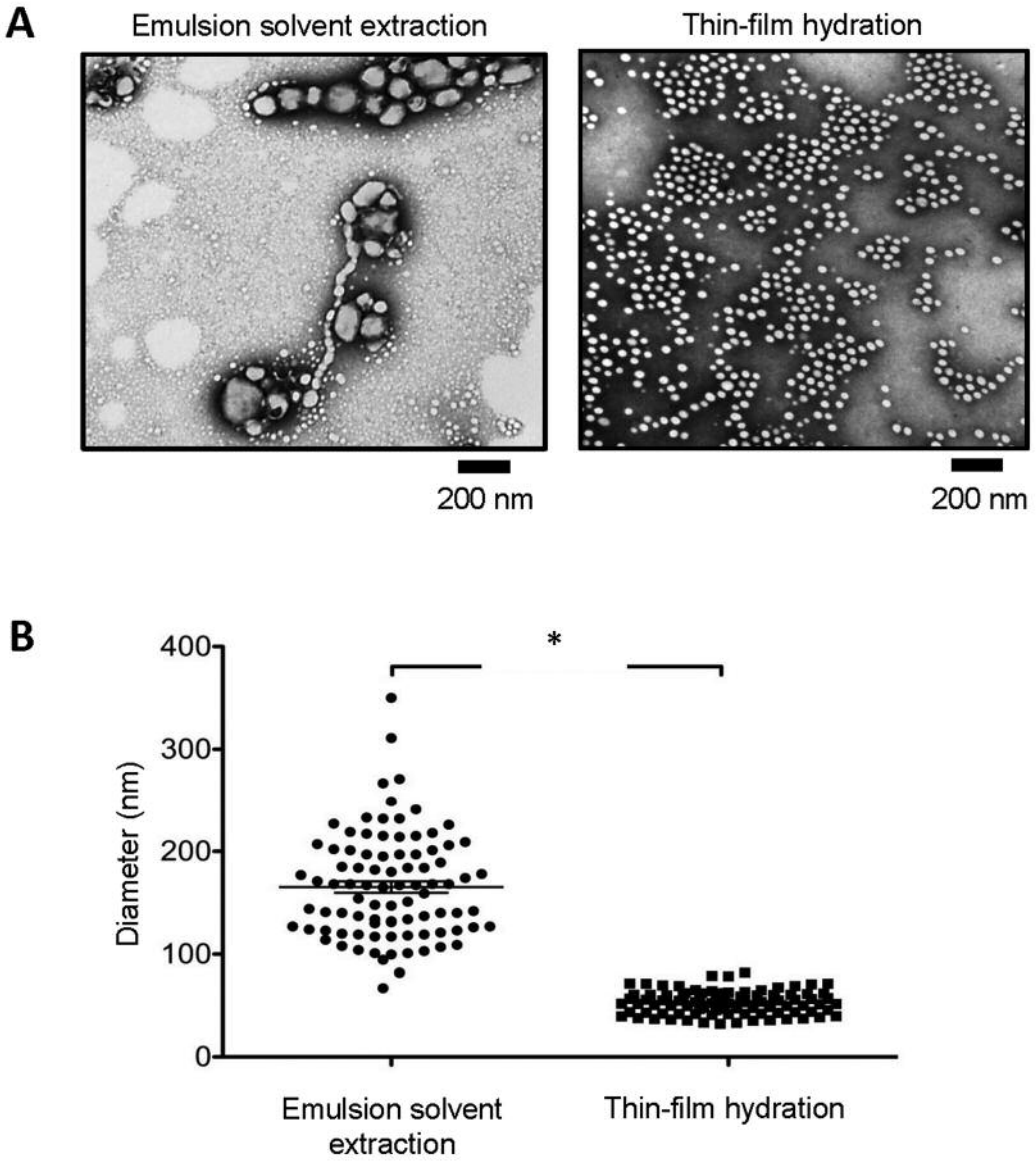

Figure 1. *Appearance and size distribution of piperine-containing nanoparticles (NPs). A: NPs prepared by emulsion solvent extraction and thin-film hydration were subjected to negative staining and visualized by transmission electron microscopy (×80,000). B: NP size was determined from a sample of 90 random particles using the AMT Image Capture Engine. *Significantly different at p<0.001 by two-tailed Students t-test.*

on the growth of MDA-MB-468 (Figure 2A) and BT-549 (Figure 2B) cells, indicating that encapsulation within NPs did not reduce the anticancer activity of piperine. Empty NPs, at the number required to achieve the delivery of 150 μM piperine, inhibited MDA-MB-468 cell growth, albeit to a lesser extent than the same number of piperine-loaded NPs. In contrast, the same number of empty NPs did not significantly affect the growth of BT-549 cells. MDA-MB-468 and BT-549 cell growth was not affected by lower numbers of empty NPs (not shown).

NP-encapsulated piperine causes apoptosis of TNBC cells. Flow cytometric analysis of MDA-MB-468 cells cultured for 48 h in the presence of high-dose piperine-loaded NPs revealed a significant increase in the number of early and late apoptotic cells relative to the minimal effect of empty NPs (Figure 3A). The number of apoptotic MDA-MB-468 cells, relative to the vehicle control, increased after 48-h culture in the presence of free piperine. Interestingly, in comparison to cultures treated with free piperine, there were more cells in the early stages of apoptosis in TNBC

cell cultures treated with NP-encapsulated piperine, suggesting a slow release of the cytotoxic agent from NPs. Although there was a slight, but not statistically significant, cytotoxic effect of free piperine on HDFs, there was no significant cytotoxic effect of piperine-loaded NPs on these cells (Figure 3B), suggesting a selective cytotoxic effect on TNBC cells.

Discussion

In this study, we determined the feasibility of using mPEG-PLGA co-polymers to produce piperine-loaded NPs *via* the single emulsion solvent extraction and thin-film hydration methods. Single emulsion solvent extraction produced larger NPs with irregular shape, whereas thin-film hydration yielded smaller spherical NPs (32-82 nm size range). NPs produced by thin-film hydration were chosen for further analysis since such NPs are an appropriate size for passive diffusion into the tumor microenvironment while avoiding potentially toxic NP retention in the kidney, liver, and spleen (8).

It is known that PEG-PLGA NPs enter cells *via* endocytosis and reside within lysosomes, releasing their cargo in response to the acidic environment of the lysosomal compartment (7). Inhibition of TNBC cell growth by piperine-loaded NPs was consistent with reports that free piperine suppressed the *in vitro* growth of breast cancer cells (3, 4). Although PGLA and PEG exhibit excellent biocompatibility (7), we observed that empty NPs, at the number required for the delivery of 150 μM piperine, also inhibited MDA-MB-468 cell growth, albeit to a minimal degree in comparison to the same number of piperine-loaded NPs. It may be that a large volume of endocytosed NPs perturbs the lysosomal membrane of MDA-MB-468 cells, resulting in the release of cytotoxicity-inducing cathepsins (12). For reasons that are not clear at this time, the viability of a second TNBC cell line (BT-549) was not affected by exposure to a large number of empty NPs.

We previously showed that treatment of MDA-MB-468 cells with a high micromolar concentration of free piperine resulted in cell death *via* apoptosis (4). The present finding that NP-encapsulated piperine also induced apoptosis of MDA-MB-468 cells was in line with our earlier finding. TNBC cell cultures treated with piperine-loaded NPs had more cells in the early stages of apoptosis in comparison to cultures treated with free piperine, suggesting a slow release of the cytotoxic agent from NPs. Piperine-induced apoptosis of breast cancer cells is characterized by caspase activation and inhibition of survival-promoting protein kinase B signaling (3, 4). It is reasonable to believe that these effects also account for the cytotoxic effect of NP-encapsulated piperine on breast cancer cells.

In contrast to TNBC cells, HDFs were able to withstand high concentrations of piperine-loaded NPs without any decrease in viability. This may be related to the finding that relative to healthy cells, there is preferential uptake of NPs by malignant cells (13). In addition, earlier work also showed that high-dose piperine treatment does not affect the viability of normal mammary epithelial cells (4). Taken together, these findings suggest that piperine is a biocompatible phytochemical, at least at the dose needed to kill breast cancer cells.

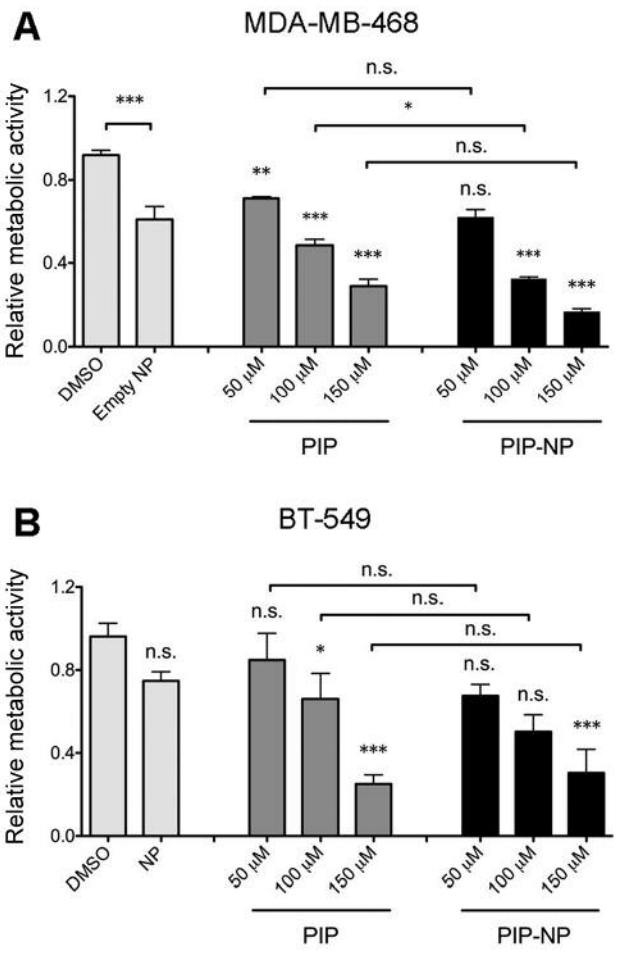

Figure 2. *Inhibition of triple-negative breast cancer (TNBC) cell growth by piperine-containing nanoparticles (NPs). (A) MDA-MB-468 or (B) BT-549 TNBC cells were cultured for 48 h in the presence of medium alone, vehicle (dimethyl sulfoxide; DMSO), empty NPs, the indicated concentration of free piperine (PIP) or PIP-NPs containing an equivalent concentration of piperine, as determined by the encapsulation efficiency of thin-film hydration. Colorimetric assays were used to determine relative metabolic activity as a measure of cell growth in comparison to the medium control. Data shown are the mean±SEM of three independent experiments. Significantly different at *p<0.05, **p<0.01, ***p<0.001 by ANOVA with the Tukey multiple comparisons post-test; n.s.: Not statistically significant.*

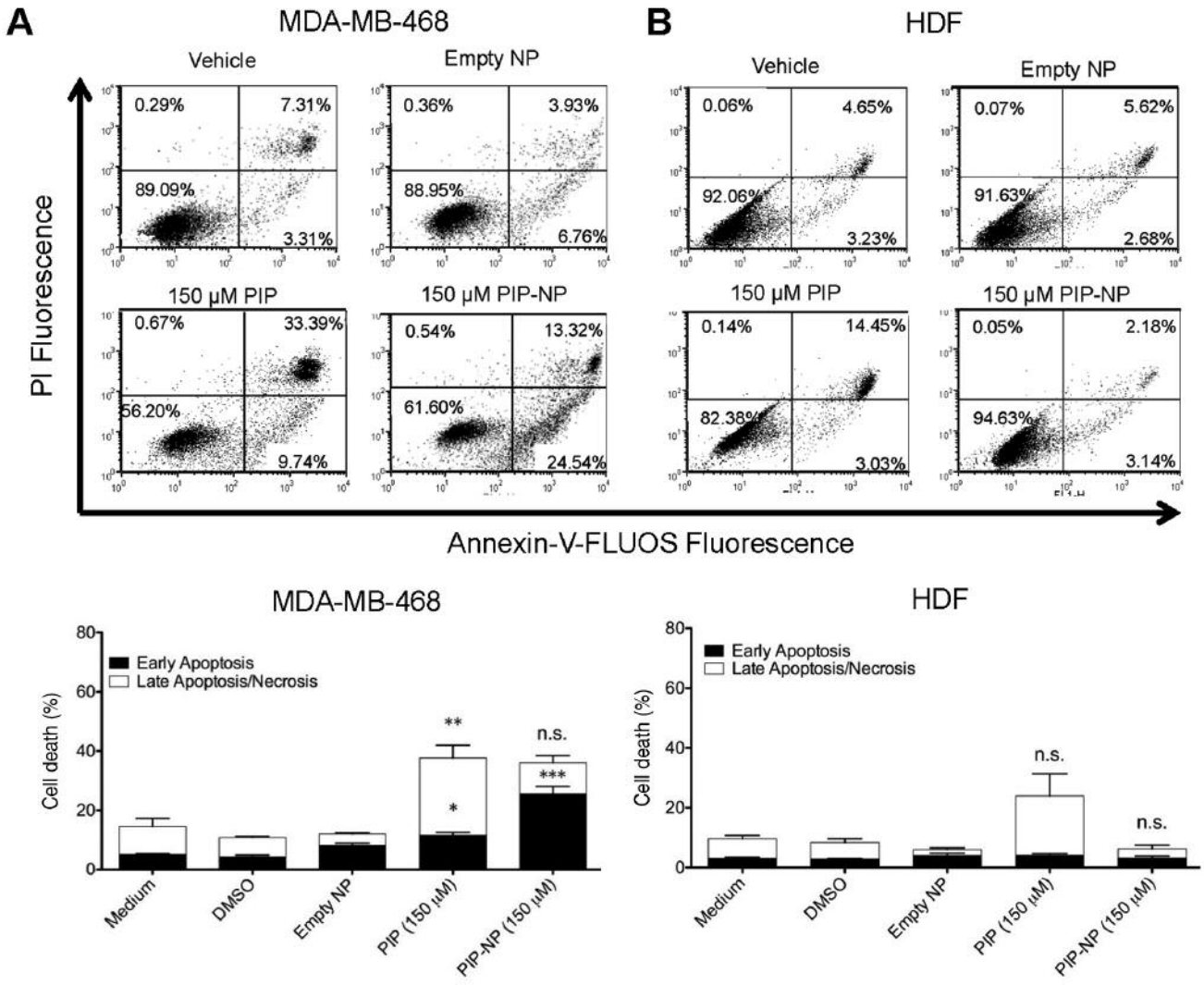

Figure 3. *Apoptosis induction in triple-negative breast cancer (TNBC) cells (A) but not normal fibroblasts (B) by piperine-loaded nanoparticles (NPs). MDA-MB-468 TNBC cells and normal human dermal fibroblasts (HDF) were cultured for 48 h in the presence of medium alone, vehicle (dimethyl sulfoxide; DMSO), empty NPs, 150 µM free piperine (PIP) or 150 µM PIP-NPs, then stained with Annexin-V-FLUOS/propidium iodide (PI) and analyzed by flow cytometry. Data are shown as flow cytometry dot plots from a representative experiment and as mean±SEM cell death from three independent experiments. Significantly different at *p<0.05, **p<0.01, and ***p<0.001 by ANOVA with the Tukey multiple comparisons post-test; n.s.: Not statistically significant.*

In conclusion, mPEG-PLGA NPs produced by thin-film hydration selectively delivered piperine at a concentration that was cytotoxic for TNBC cells but which did not have a significant effect on the viability of HDFs. The cytotoxic effect of piperine-loaded NPs was comparable to that of an equivalent concentration of free piperine. Since piperine acts as a chemosensitizer and radiosensitizer of breast cancer cells (3, 4), NP-mediated delivery of piperine to malignant tissues might be used to enhance the effectiveness of conventional treatments for triple-negative and other types of breast cancer.

Conflicts of Interest

None.

Authors' Contributions

Conceptualization: David Hoskin. Data curation: Javad Ghassemi Rad. Formal analysis: Javad Ghassemi Rad. Funding acquisition: David Hoskin. Methodology: Javad Ghassemi Rad. Project administration: David Hoskin. Supervision: David Hoskin. Writing – original draft: Javad Ghassemi Rad. Writing – review and editing: David Hoskin.

Acknowledgements

The study was funded by the Canadian Cancer Society/Canadian Breast Cancer Foundation Endowed Chair in Breast Cancer Research. JGR was the recipient of a Nova Scotia Graduate Scholarship and a Cancer Research Training Program Trainee Award.

References

1 Rather RA and Bhagat M: Cancer chemoprevention and piperine: molecular mechanisms and therapeutic opportunities. Frontiers Cell Dev Biol *6*: 10, 2018. PMID: 29497610. DOI: 10.3389/fcell.2018.00010

2 Lai LH, Fu QH, Liu Y, Jiang K, Guo QM, Chen QY, Yan B, Wang QQ and Shen JG: Piperine suppresses tumor growth and metastasis *in vitro* and *in vivo* in a 4T1 murine breast cancer model. Acta Pharmacol Sin *33(4)*: 523-530, 2012. PMID: 22388073. DOI: 10.1038/aps.2011.209

3 Do MT, Kim HG, Choi JH, Khanal T, Park BH, Tran TP, Jeong TC and Jeong HG: Antitumor efficacy of piperine in the treatment of human HER2-overexpressing breast cancer cells. Food Chem *141(3)*: 2591-2599, 2013. PMID: 23870999. DOI: 10.1016/j.foodchem.2013.04.125

4 Greenshields AL, Doucette CD, Sutton KM, Madera L, Annan H, Yaffe PB, Knickle AF, Dong Z and Hoskin DW: Piperine inhibits the growth and motility of triple-negative breast cancer cells. Cancer Lett *357(1)*: 129-140, 2015. PMID: 25444919. DOI: 10.1016/j.canlet.2014.11.017

5 Khajuria A, Zutshi U and Bedi KL: Permeability characteristics of piperine on oral absorption – an active alkaloid from peppers and a bioavailability enhancer. Indian J Exp Biol *36(1)*: 46-50, 1998. PMID: 9536651.

6 Jeong K, Kang CS, Kim Y, Lee YD, Kwon IC and Kim S: Development of highly efficient nanocarrier-mediated delivery approaches for cancer therapy. Cancer Lett *374(1)*: 31-43, 2016. PMID: 26854717. DOI: 10.1016/j.canlet.2016.01.050

7 Danhier F, Ansorena E, Silva JM, Coco R, Le Breton A and Préat V: PLGA-based nanoparticles: an overview of biomedical applications. J Control Release *161(2)*: 505-522, 2012. PMID: 22353619. DOI: 10.1016/j.jconrel.2012.01.043

8 Cabral H, Matsumoto Y, Mizuno K, Chen Q, Murakami M, Kimura M, Terada Y, Kano MR, Miyazono K, Uesaka M, Nishiyama N and Kataoka K: Accumulation of sub-100 nm polymeric micelles in poorly permeable tumours depends on size. Nature Nanotech *6(12)*: 815-823, 2011. PMID: 22020122. DOI: 10.1038/nnano.2011.166

9 Foulkes WD, Smith EE and Reis-Filho JS: Triple-negative breast cancer. N Engl J Med *363(20)*: 1938-1948, 2010. PMID: 21067385. DOI: 10.1056/NEJMra1001389

10 Fessi C, Devissaguet JP, Puisieux F and Thies C: Process for the preparation of dispersible colloidal systems of a substance in the form of nanoparticles. Patent US 5118528, 1992.

11 Lee H, Soo PL, Liu J, Butler M and Allen C: Polymeric micelles for formulation of anti-cancer drugs. *In*: Nanotechnology for Cancer Therapy. Amiji MM (ed.). Boca Raton, CRC Press, pp. 317-355, 2006.

12 Zhang B, Lung PS, Zhao X, Chu Z, Chrzanowski W and Li Q: Shape dependent cytotoxicity of PLGA-PEG nanoparticles on human cells. Sci Rep *7(1)*: 7315, 2017. PMID: 28779154. DOI: 10.1038/s41598-017-07588-9

13 Costa EC, Gaspar VM, Marques JG, Coutinho P and Correia IJ: Evaluation of nanoparticle uptake in co-culture cancer models. PLoS One *8(7)*: e70072, 2013. PMID: 23922909. DOI: 10.1371/journal.pone.0070072

Received December 4, 2019
Revised January 13, 2020
Accepted January 15, 2020