Acta Orthopaedica et Traumatologica Turcica xxx (2017) 1–7

Contents lists available at ScienceDirect

Acta Orthopaedica et Traumatologica Turcica

journal homepage: https://www.elsevier.com/locate/aott

# Cytotoxic effects of delphinidin in human osteosarcoma cells☆

Dong-Yeong Lee [a], Young-Jin Park [b], Sun-Chul Hwang [b], Kwang-Dong Kim [c], Dong-Kyu Moon [b], Dong-Hee Kim [b, *]

[a] The Armed Forces Daegu Hospital, Daegu, Republic of Korea
[b] Department of Orthopaedic Surgery, Gyeongsang National University School of Medicine and Gyeongsang National University Hospital, Jinju, Republic of Korea
[c] Division of Applied Life Science, Gyeongsang National University, Jinju, Republic of Korea

ARTICLE INFO

*Article history:*
Received 31 May 2017
Received in revised form
13 September 2017
Accepted 24 November 2017
Available online xxx

*Keywords:*
Delphinidin
Cytotoxicity
Autophagy
Apoptosis
Osteosarcoma

ABSTRACT

*Introduction:* The aim of this study was to evaluate whether delphinidin is cytoprotective or cytotoxic in osteosarcoma cell lines, and to elucidate the underlying mechanisms.
*Materials and methods:* The present study investigated whether apoptosis or autophagy is induced by delphinidin in human osteosarcoma cell lines. Delphinidin was used as the antioxidant, along with two autophagy inhibitors: 3-methyladenine and bafilomycin A1. Cell viability and known autophagic markers, such as LC3-II expression, were evaluated. Reactive oxygen species (ROS) formation and cell cycle analysis were also investigated.
*Results:* Delphinidin showed concentration-dependent cytotoxicity to osteosarcoma cell. Delphinidin is closely associated with apoptotic cell death mechanisms and pathways related to ROS accumulation. In addition, we observed delphinidin-induced autophagosome formation and increasing levels of LC3-II conversion. However, in spite of delphinidin induced autophagy, the cytotoxic effects induced in the osteosarcoma cells may not be operating via autophagic cell death mechanisms.
*Conclusions:* Delphinidin compromises the cellular protective mechanisms by inhibiting autophagy, permitting ROS to accumulate and finally enhance apoptotic cell death. Our results indicate that delphinidin may play a critical role as a chemotherapeutic agent by preventing the development and progression of osteosarcoma cells.


## Introduction

Osteosarcoma is one of the most common non-hematologic, primary, malignant bone tumors in children. Osteosarcoma is seen more frequently in younger individuals, and the incidence is slightly higher in males. Although the 5-year survival rate has increased from 10% to 70%, the prognosis for osteosarcoma is poor.[1]

Recently, natural compounds such as cyanidin, delphinidin, malvidin and pelargonidin have been used as therapeutics for several diseases.[2] These natural compounds promote the function of modern drug treatments, with few side effects. Many studies have shown that anthocyanins suppress the growth of certain cancers, because of its oxidative stress-based cytotoxic effect or antioxidant activity.[3,4] For example, Yun et al suggest that delphinidin could have potential in inhibiting colon cancer growth.[5] And Lim et al suggested that delphinidin plays a critical role as a new chemotherapeutic agent to prevent the development of human ovarian clear cell carcinoma.[4] Delphinidin (phytochemicals), a natural compound of anthocyanins, is a specific class of polyphenols. It has shown beneficial effects on cancer cell, such as potent antioxidant, anti-inflammatory, anti-mutation, and anti-neovascularization. Recent researches concerning cancer therapy revealed that delphinidin is correlated to apoptotic or autophagic cell death in several cancers.[3,5,6]

☆ This work was supported by Biomedical Research Institute Fund "GNUH CRF 2010-003" from the Gyeongsang National University Hospital.
* Corresponding author. Department of Orthopaedic Surgery, Gyeongsang National University School of Medicine and Gyeongsang National University Hospital, 15, Jinju-daero 816 beon-gil, Jinju-si, Gyeongsangnam-do, 660-751, Republic of Korea. Fax: +82 55 761 9477.
*E-mail address:* dhkim8311@gnu.ac.kr (D.-H. Kim).
Peer review under responsibility of Turkish Association of Orthopaedics and Traumatology.

https://doi.org/10.1016/j.aott.2017.11.011


Please cite this article in press as: Lee D-Y, et al., Cytotoxic effects of delphinidin in human osteosarcoma cells, Acta Orthop Traumatol Turc (2017), https://doi.org/10.1016/j.aott.2017.11.011

In general, cell death is most commonly associated with apoptosis, but it can also occur through other mechanisms, such as autophagy.[7,8] Apoptosis is a mechanism for self-destruction or suicide regulated by programmed cellular signaling pathways, and is characterized by stereotypical morphological changes.[9] Thus, it is important to induce cell apoptosis in osteosarcoma therapies. Contrary to apoptosis, autophagy is a homeostatic mechanism and an important process in all cells, for the removal of damaged mitochondria and misfolded proteins. Autophagy is considered to bypass apoptosis; however, excess autophagy induction is known to trigger autophagy-related apoptosis. Thus, understanding the role of apoptosis and autophagy in cancer treatment is critical, since many anti-cancer therapies have been shown to activate these mechanisms.

Reactive oxygen species (ROS) are highly reactive forms of molecular oxygen, and are generally derived during the normal metabolism of oxygen.[10] ROS acts as physiological regulators of normal cell proliferation and differentiation at low levels, but excessive levels of ROS damages the DNA and proteins, leading to cell apoptosis.[11] In addition, ROS is also closely associated with programmed cell death, such as autophagy. By regulating these mechanisms, ROS have the potential of either cytoprotective or cytotoxic effects. Some authors have reported that ROS can initiate autophagy, thus having a cytoprotective effect in several types of cancers.[11,12] Conversely, other studies have reported that the cytotoxic effect of anthocyanidins in cancer cells could result from ROS accumulation.[3,12,13] Thus, although it has not been determined if delphinidin has a therapeutic effects on human osteosarcoma, it is rationale to investigate the ability of ROS to act as a mediator of a cancer-blocking agent or cancer-suppressing agent.

In the current study, we investigated whether delphinidin triggers apoptotic or autophagic cell death in a human osteosarcoma cell line (U2OS). Cell death is associated with apoptosis or autophagy, however, it is not proven as to through which pathway delphinidin induces cell death of osteosarcoma cell in previous studies. The purpose is to evaluate whether delphinidin are cytoprotective or cytotoxic in osteosarcoma cell lines, and to further elucidate the associated mechanisms. We hypothesized that delphinidin may be helpful for improving osteosarcoma treatments via the apoptotic or autophagic cell death mechanism, and the combination of delphinidin may increase the incidence of therapeutic reactions.

## Materials and methods

### *Cell and chemicals*

The human osteosarcoma derived U2OS cell line was prepared (Korean Cell Line Bank, Seoul, Korea), and delphinidin was obtained from Extrasynthese (Genay, France). U2OS cells were cultured in DMEM/F-12 (Gibco, Grand Island, N.Y, USA) supplemented with 10% heat inactivated fetal bovine serum (FBS) (Gibco), 2 mM L-glutamine, and 1% Antibiotic-Antimycotic drug (Gibco); incubation was in humidified 5% $CO_2$ atmosphere at 37 °C. Delphinidin was dissolved in dimethyl sulfoxide (DMSO, Sigma Aldrich, St Quentin Fallavier, France) which was used for vehicle control in all assays. An enhanced chemiluminescence (ECL) detection system was purchased from Amersham (Arlington Heights, IL). All other chemicals that are not indicated here were procured from Sigma Chemical Co. (St. Louis, MO).

### *Determination of cell viability*

The effect of delphinidin on cell viability was determined by an MTT (3-[4, 5-dimethyldiazole-2-yl]-2,5-diphenyl tetrazolium-bromide) assay. The cells were plated in 96-well microtiter plates, at $1 \times 10^4$ cells per well in 200 μL of complete culture medium, and treated with the desired concentrations of delphinidin (0–200 μM) for 48 h at 37 °C in a humidified chamber. MTT (5 mg/ml in PBS; diluted in 10 ml of serum free media) was added to each well and incubated for further 2 h, after which the plate was centrifuged at 1000 rpm for 5 min at 4 °C. The culture medium was removed from the wells by aspiration, and the resultant formazan crystals were dissolved in 200 μL DMSO. The absorbance was recorded on automated microplate reader EL 311 (Bio-Tek Instruments, Winooski, VT) at a wave length of 540 nm. The effect of delphinidin on cell survival was assessed as percent cell viability, where vehicle-treated cells were considered as 100% viable.

### *ROS production using by FACS analysis*

The intracellular ROS generation induced by delphinidin was assessed using FACS and a confocal microscope. The measurement of intracellular ROS production using FACS (Cytomics FC 500, Beckman, CA, USA) was performed as follows. U2OS cells were seeded in each well of a 6-well cell culture plate. The cells were incubated with DCF-DA (2′, 7′-Dicholrofluorescin diacetate) solution (5uM, Sigma, St. Louis, MO, USA) for 15 min at 37 °C; they were then washed, and resuspended in PBS. Intracellular ROS products were measured using FACS. Data were analyzed using CXP software (Beckman, CA, USA.

### *Western blotting, flow cytometry*

To validate whether delphinidin can promote autophagy activation, we also observed autophagosome formation using western blotting analysis. In addition, to investigate the effects of delphinidin on the distribution of cells in the cell cycle, we performed DNA cell cycle analysis using flow cytometry. Detailed methods were indicated at Appendix 1.

### *Confocal microscopy*

In each well of a 24-well plate, 50 μL green fluorescent protein (GFP)-LC3-labeled transfected osteosarcoma cell lines were cultured to subconfluent density; the cells were maintained in a 37 °C humidified incubator for 16 h. The sample was cultured on a cover slide, washed with PBS, and fixed with 3% formaldehyde. It was then permeabilized with 0.5% triton-X100 and blocked in 1% BSA solution. Analysis under confocal microscopy (Olympus FV1000, OLYMPUS, Tokyo, Japan) was performed while cells were under reaction with primary and FITC or TRITC-labeled secondary antibody. GFP-fluorescence was analyzed by confocal microscopy, immediately after fixation of cell.

### *Statistical analyses*

Each experiment was performed at least three times, and representative data were reported. All statistical analyses were performed via one-way ANOVA with the exception of the cell-viability analysis for different concentrations of delphinidin. That cell-viability analysis was performed using repeated measure. Differences with a probability of less than 0.05 were considered statistically significant. All statistical analysis was done using SPSS 18.0 for Windows (SPSS, Chicago, IL, USA).

Please cite this article in press as: Lee D-Y, et al., Cytotoxic effects of delphinidin in human osteosarcoma cells, Acta Orthop Traumatol Turc (2017), https://doi.org/10.1016/j.aott.2017.11.011