Antioxidant’s Cytoprotective Effects on Rotator Cuff Tenofibroblasts Exposed to Aminoamide Local Anesthetics

Ra Jeong Kim1, Young-Sool Hah2, Jae-Ran Kang3, Hyung Bin Park4.

1Department of Convergence Medical Science, Gyeongsang National University, Jinju-si, Korea, Republic of, 2Biomedical Research Institute, Gyeongsang National University Hospital, Jinju-si, Korea, Republic of, 3Namhae Garlic Research Institute, Namhae-gun, Korea, Republic of, 4Department of Orthopaedic Surgery and Institute of Health Sciences,, Jinju-si, Korea, Republic of.


Introduction: Local injection is a very important treatment modality for many types of tendinopathies. Local anesthetics (LA), such as Ropivacaine, Bupivacaine, and Lidocaine, are among the drugs most frequently used for musculoskeletal problems in procedures ranging from diagnosis to postoperative pain control1. Recently, these LA have been reported as possible causes of chondrolysis following arthroscopic surgery2. Additionally, in a recent in vitro study, these anesthetics have shown cytotoxic effects on human fibroblasts originating in human osteosarcoma; these fibroblasts play a crucial role in wound healing3. Other in vitro studies have reported that LA induce cytotoxicity in myocytes and Schwann cells4,5. These previous studies focused on demonstrating the adverse effects of local anesthetics. However, few suggestions were offered as to how to protect cells from those adverse effects6. The purpose of the current study was to determine whether cyanidin, a natural antioxidant, has cytoprotective effects on aminoamide local anesthetics induced tenofibroblasts cell death and to evaluate the cytoprotective mechanism of the cyanidin.

Methods: Tenofibroblasts were isolated from human supraspinatus tendon tissues harvested during arthroscopic rotator cuff repair. Third-passage cells were used throughout the current experiments. This study used a control group and LA and a cyanidin-LA groups. Cytotoxicity was induced in the LA group through exposure to ropivacaine (0.075%), bupivacaine (0.05%) and lidocaine (0.2%) for 24 hr. The cyanidin-LA group was exposed to 100 μg/mL cyanidin for 1 hr, before LA exposure. These study groups were evaluated for cell viability, cell-survival and death protease activity, caspase-3/7 activity, intracellular ROS production, and expression levels of phospho-ERK, phospho-p38, phospho-JNK, and cleaved PARP-1. All statistical analyses were performed via one-way ANOVA.

Results: Cell viability in all LA subgroups was significantly lower than that of the control group (p<0.001) (Fig. 1). Cell viability in the cyanidin-LA subgroups was significantly higher than that of their paired LA subgroups (p<0.001). The caspase 3/7 activity in the LA subgroups was significantly higher than that of the control group (p<0.001) (Fig. 2). The caspase 3/7 activity in all the cyanidin-LA subgroups was lower than that of their paired LA subgroups. The caspase 3/7 activity was significantly lower in the cyanidin-Bupivacaine subgroup than that of the Bupivacaine subgroup (p=0.004). According to the FACS analysis and confocal microscope (Fig. 3), the level of intracellular ROS production in the LA subgroups was significantly higher than that of the control group (Ropivacaine: p=0.001, Bupivacaine: p=0.04, Lidocaine: p=0.002). All of the cyanidin-LA subgroups showed significantly lower intracellular ROS production than the LA subgroups, except the cyanidin-Bupivacaine subgroup (p<0.05). According to the results of the western blot analysis (Fig. 4), the expression levels of phosphorylated ERK, p38 and JNK...
and cleaved PARP-1 in all the LA subgroups were higher than those of the control group. Their expression levels in the cyanidin-LA subgroups were significantly lower than those of the LA subgroups.

**Discussion:** The current study demonstrated that cyanidin had cytoprotective effects against LA-induced cytotoxicity on rotator cuff tenofibroblasts; these effects were achieved by reducing intracellular ROS production and by down-regulating ERK, p38, JNK, caspase 3/7, and cleaved PARP-1. Apoptosis is well-known to be induced by oxidative stress. Increased intracellular ROS induces MAPK (ERK, p38, and JNK) and Caspase-3. JNK and p38 are reported to have some roles in local anesthetic induced cell death. Caspase-3 is a recognized executioner that uses both the intrinsic and extrinsic apoptosis pathways. Anita et al.7 demonstrated that, after exposure to Bupivacaine, Ropivacaine, and Mepivacaine, caspase 3/7 activity increased in human chondrocytes. Fedder et al.8 reported that an increase in caspase-3 activity was observed during the 3 days of incubation in human fibroblasts exposed to Bupivacaine or Ropivacaine. PARP-1 has been accepted as the “death substrate”. PARP-1 plays a role in the two main pathways of cell death: apoptosis and necrosis. Cleaved PARP-1 is known to be a vital substrate for the appropriate function of the apoptotic cell-death machinery.

**Significance:** The current study suggests that cyanidin, an antioxidant, can be a candidate for preventing local anesthetic-induced rotator cuff tenofibroblasts cell death.
Figure 1. Cytoprotective effects of cyanidin against tenofovir-induced cell death induced by local anesthetic. The CCK-8 analysis demonstrated that cell viability in the LA subgroups was significantly lower than in the control. However, cell viability was significantly higher in the cyanidin-LA subgroups than in the paired LA subgroups (p<0.001).

Figure 2. (A) Intracellular ROS levels were significantly increased in the LA subgroups, as compared with the control. Intracellular ROS levels in the cyanidin-LA subgroups were all lower than in the LA subgroups. (B) According to the morphological analysis using a confocal microscope, intracellular ROS levels were higher in the LA subgroups than in the control; however, ROS levels were markedly lower in the cyanidin-LA subgroups than in the LA subgroups.

Figure 3. Caspase 3/7 activity levels were significantly increased in the LA subgroups, as compared with the control. Caspase 3/7 activity levels in the cyanidin-LA subgroups were lower than in the LA subgroups. The decrease in the caspase 3/7 activity level in the cyanidin-bupivacaine subgroup reached statistical significance (p<0.004).

Figure 5. Western blot analyses demonstrated that expressions of phosphor ERK, JNK, and p38, and of cleaved PARP 1 were increased in the LA subgroups, as compared with the control. Those expressions were lower in the cyanidin-LA subgroups than in the LA subgroups.

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