Chemical modulation of apoptosis in molluscan cell cultures

Andrey Victorovich Boroda [Corresp., 1], **Yulia Olegovna Kipryushina** [1, 2], **Nelly Adolphovna Odintsova** [1, 2]

[1] Cytotechnology Laboratory, National Scientific Center of Marine Biology of the Far Eastern Branch of the Russian Academy of Sciences, Vladivostok, Russia
[2] Laboratory of marine invertebrate biology, Far Eastern Federal University, Vladivostok, Russia

Corresponding Author: Andrey Victorovich Boroda
Email address: borodandy@gmail.com

This study focused on the alterations that occur in larval molluscan cells during the induction or inhibition of apoptosis both in standard culture conditions and in response to cold injury during the induction of different death pathways. This is the first report on the modulation of apoptosis in molluscan cells using apoptotic inducers and inhibitors known to mammalian cells, which has been assessed by flow cytometry. The activity of mitochondria, general caspase activation, and the membrane integrity of intact molluscan cells were compared to those of cells frozen-thawed both prior to treatment and after incubation with apoptotic inducers or inhibitors, and to those of primary mouse embryonic fibroblasts and human colon tumor cells (HCT 116 cell line) treated with the same compounds. We tested three apoptotic inducers (staurosporine, camptothecin, and mitomycin C, routinely used for the chemical induction of apoptosis in different mammalian cells) and found that only staurosporine resulted in an evident increase of apoptosis in molluscan cells (6.6% in comparison with 2.9% in control unfrozen cells, and 9.1% in comparison with 5.6% in control frozen-thawed cells). Camptothecin did not significantly induce apoptosis of molluscan cells but did slightly increase the number of active cells after thawing. Mitomycin C showed similar results, but its effect was less pronounced. We suggest that some apoptotic inducers have hereto unknown effects on molluscan cells. In addition, we hypothesize that the use of the apoptotic inhibitors could reduce apoptosis, which is significant after cryopreservation in molluscan cells. Development of this direction is important for understanding the mechanisms of cold susceptibility of marine organisms.

Chemical modulation of apoptosis in molluscan cell cultures

Andrey Victorovich Boroda[1], Yulia Olegovna Kipryushina[1,2], Nelly Adolphovna Odintsova[1,2]

[1] Cytotechnology Laboratory, National Scientific Center of Marine Biology of the Far Eastern Branch of the Russian Academy of Sciences, Vladivostok, Russia

[2] Laboratory of marine invertebrate biology, Far Eastern Federal University, Vladivostok, Russia

Corresponding Author:

Andrey Boroda [1]

17 Palchevsky St, Vladivostok, Primorsky krai, 690041, Russia

Email address: borodandy@gmail.com

A B S T R A C T

This study focused on the alterations that occur in larval molluscan cells during the induction or inhibition of apoptosis both in standard culture conditions and in response to cold injury during the induction of different death pathways. This is the first report on the modulation of apoptosis in molluscan cells using apoptotic inducers and inhibitors known to mammalian cells, which has been assessed by flow cytometry. The activity of mitochondria, general caspase activation, and the membrane integrity of intact molluscan cells were compared to those of cells frozen-thawed both prior to treatment and after incubation with apoptotic inducers or inhibitors, and to those of primary mouse embryonic fibroblasts and human colon tumor cells (HCT 116 cell line) treated with the same compounds. We tested three apoptotic inducers (staurosporine, camptothecin, and mitomycin C, routinely used for the chemical induction of apoptosis in different mammalian cells) and found that only staurosporine resulted in an evident increase of apoptosis in molluscan cells (6.6% in comparison with 2.9% in control unfrozen cells, and 9.1% in comparison with 5.6% in control frozen-thawed cells). Camptothecin did not significantly induce apoptosis of molluscan cells but did slightly increase the number of active cells after thawing. Mitomycin C showed similar results, but its effect was less pronounced. We suggest that some apoptotic inducers have hereto unknown effects on molluscan cells. In addition, we hypothesize that the use of the apoptotic inhibitors could reduce apoptosis, which is significant after cryopreservation in molluscan cells. Development of this direction is important for understanding the mechanisms of cold susceptibility of marine organisms.

Keywords: Apoptotic inducers; Apoptotic inhibitors; Cell death pathways; Flow cytometry; Mussel; *Mytilus trossulus.*

Abbreviations: CAM, camptothecin; CMFSS, Ca^{+2} and Mg^{+2}-free salt solution; DAPI, 4'-6-diamidino-2-phenylindole; DPBS, Dulbecco's phosphate-buffered saline without Ca^{2+} and Mg^{2+}; FBS, fetal bovine serum; MEFs, mouse embryonic fibroblasts; Me_2SO, dimethyl sulfoxide; MMC, mitomycin C; ROCK, Rho-associated protein kinase; RT, room temperature; STS, staurosporine; SW, seawater.

Introduction

Apoptosis and necrosis have been detected in all eukaryotes and are induced by many stress factors. Although these major cell death pathways are highly conserved, they vary in morphology, biochemistry, and physiology (Kiss 2010; Zeiss 2003; Zhivotovsky 2004). Only a few studies have analyzed multiple stressors in marine mollusks (Lockwood et al. 2015; Przeslawski et al. 2015; Sokolova et al. 2004). The cell death programs can be activated by elevated levels of oxidative stress in intertidal organisms, including adult mollusks (Sokolova et al. 2004). The early life stages of marine organisms are marked by increased susceptibility to different stressors than affect adults (Przeslawski et al. 2015). As shown in our previous research, total number of live larval molluscan cells significantly decreased when exposed to oxidative stress in cell culture (Odintsova et al. 2017). An approximately equal number of cells in the early stages of apoptosis (6–7%) was found in unfrozen mussel cells treated with staurosporine (STS-induced apoptosis) or hydrogen peroxide (oxidative stress-induced apoptosis) (Odintsova et al. 2017).

Mussels of the genus *Mytilus* are sessile organisms that inhabit highly stressful intertidal ecosystems and, therefore, must possess mechanisms to withstand the stress effects (Halpin et al. 2002; Lockwood et al. 2015). Environmental pollutants and drastic temperature changes (Cheng 1988; Cherkasov et al. 2007; Kefaloyianni et al. 2005; Mičić et al. 2001; Odintsova et al. 2017;

Sokolova 2009; Sokolova et al. 2004) can lead to a variety of cellular disorders in molluscan cells including eventual apoptosis. This research focused on the modulation of apoptosis in larval molluscan cells specifically.

We have used flow cytometry to study the effects of some inducers and inhibitors on cell death pathways in cultivated molluscan cells, and for comparison, in primary mouse embryonic fibroblasts (MEFs), and human colon tumor cells (HCT 116 cell line), since apoptotic cells remain detectable for extended periods of time in culture (Wlodkowic et al. 2011). Flow cytometry has become a powerful tool for detecting apoptotic, live, and dead cells and is the most commonly used laboratory method for distinguishing apoptosis from necrosis in dissociated cells (Chen et al. 2000; Przeslawski et al. 2015). Our previous studies revealed that annexin V is an unreliable marker for apoptosis in molluscan primary cell cultures (Odintsova et al. 2017), which can lead to an increased false-positive identification of non-apoptotic annexin V-positive cells (Marión et al. 2009). We found that an analysis of cell samples conducted by two different staining combinations (FLICA® (fluorochrome-labeled inhibitors of caspases) and YO-PRO™-1 staining) more accurately reflects apoptosis in molluscan cells and avoids confusion from false-positive or -negative artifacts. Moreover, the time-window of apoptosis detected by this staining combination is much wider than that assessed only by the annexin V binding (Morris & Geller 1996).

In all animals, an impact on numerous branch points is required to prevent or induce intracellular programmed cell death (Elmore 2007; Zeiss 2003). The balance between inducers/inhibitors of apoptosis plays an important role in determining cell fate (Schultz & Harringto 2003). Here we have examined whether apoptotic inducers routinely used for chemical induction of apoptosis in mammalian cells (Mehlen et al. 1996; Morris & Geller 1996; Pirnia et al. 2002) can induce apoptosis in cultivated molluscan cells. We tested STS (a natural antibiotic),

79 camptothecin (a topoisomerase I inhibitor, CAM), and mitomycin C (a drug of
80 the camptothecin family, MMC). To reduce apoptosis after cryopreservation, which can reach
81 24% in molluscan cell cultures (Odintsova et al. 2017), we also tested three apoptotic inhibitors
82 known for mammalian cells: Y-27632, cyclic pifithrin-α, and CHIR99021. Y-27632 is a highly
83 permeable, potent, and selective inhibitor of the Rho-associated protein kinase (ROCK) signaling
84 pathway in mammalian cells. Human corneal endothelial cells treated with this inhibitor had a
85 decreased level of apoptosis, likely by affecting the expression and activity of caspase-3 (Peh et
86 al. 2015). Moreover, it has been previously found that a Rho-enzyme may be involved in
87 antiapoptotic mechanisms in oyster hemocytes (Lacoste et al. 2002), also as P35-sensitive caspases
88 and mitogen-activated protein kinases. In murine cell cultures, cyclic pifithrin-α reversibly
89 prevents p53-mediated-apoptosis developed in response to stressors such as ultraviolet or ionizing
90 radiation (Marión et al. 2009). Another specific apoptotic inhibitor, CHIR99021, also associated
91 with a p53-mediated-apoptosis, has been shown to block the acetylation of lysine 120 in the p53
92 protein and thereby prevent the initiation of apoptosis in human lymphoma cells exposed to
93 ionizing radiation (Ambroise et al. 2015).

94 Our previous results revealed a high number of molluscan cells in the early apoptotic stage
95 after freezing-thawing (Odintsova et al. 2017). Therefore, we hypothesize that the use of the
96 apoptotic inhibitors could promote a higher yield of viable cells after cryopreservation. The goal
97 of this study is two-pronged: to find apoptotic inducers, used for chemical induction of apoptosis
98 in mammalian cells, that can operate in non-mammalian systems; and to reduce apoptosis in
99 molluscan cells after a freeze-thaw cycle.

100

101 **Materials and methods**

Materials

Farmed marine bivalves, *Mytilus trossulus,* were collected from the Vostok Bay of the Sea of Japan (Russia) and maintained in tubs filled with running seawater (SW) for 10–20 days at 7–10°C. The spawning of sexually mature specimens was induced by a thermal shock, and developing embryos were harvested at the trochophore stage (22 h post-fertilization at 17°C) for isolating embryonic cell culture, as described previously (Odintsova et al. 2010). The resulting larval molluscan cells were treated with apoptotic inducers or inhibitors (control cells), and their state (the activity of mitochondria, general caspase activation, and the membrane integrity) was compared to that of cells that had been frozen-thawed (Fr cells) prior to treatment with the same compounds. An effective cryopreservation protocol developed for molluscan cells, involving a three-step slow freezing (1–2°C/min) to the temperature of liquid nitrogen with 5% dimethyl sulfoxide (Me_2SO) as a cryoprotectant, has been previously described (Odintsova et al. 2017). Control and Fr cells were cultivated at 120–150 × 10^3 cells/well in sterile SW supplemented with 2% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 µg/ml streptomycin in 6-well plates (TPP, Switzerland) at 17°C for 4–48 h. To induce apoptosis and identify the concentration or exposure time dependency, the control and Fr cells were either incubated with 1–5 µM STS, 1-10 µM CAM, or 1-10 µM MMC during 4–48 h. The apoptotic inducers were dissolved in Me_2SO at 1 mM (stock solution) and stored at -20°C. Hydrogen peroxide (H_2O_2) was added to the cells at the final concentration of 125 µM to induce oxidative stress and apoptosis for 6–48 h at 17°C, according to the previously published conditions for mammalian (Jurkat cells) (Hampton & Orrenius 1997) and molluscan (mussel larval) (Odintsova et al. 2017) cells. To decrease the level of apoptosis after a freeze-thaw cycle, the Fr molluscan cells were incubated with apoptotic inhibitors: Y-27632 (final concentration 10-50 µM, Y), cyclic pifithrin-α (5 µM, Alpha) or

CHIR99021 (1-5 μM, CHIR); all inhibitors were purchased from Sigma (USA). Specimens were examined using a CKX41 inverted microscope (Olympus, Japan) equipped with phase-contrast optics and imaged with an Axiocam 105 color digital camera (Carl Zeiss, Germany). Cell photos in two parallel samples were performed on ten randomly selected microscopic fields in each experiment for each sample.

Mammalian cells (MEFs and HCT 116 cells) were chosen as positive controls of apoptosis induction, inhibition and detection methods since the effects of the compounds tested are well described in the literature (Mehlen et al. 1996; Morris & Geller 1996; Pirnia et al. 2002). MEFs were obtained according to the protocol described in (Peterson et al. 2011). All the experiments on animals were reviewed and approved by the Ethics Committee of National Scientific Center of Marine Biology of the Far Eastern Branch of the Russian Academy of Sciences. HCT 116 cell line was purchased from Sigma (Germany). Mammalian cells were cultivated in DMEM supplemented with 10% FBS in 6-well plates (TPP) at 5% CO_2, 37°C. The cells were either incubated with apoptotic inducers (1–5 μM STS, 1-10 μM CAM, or 1-10 μM MMC) or with apoptotic inhibitors (10-50 μM Y, 5 μM Alpha, or 1-5 μM CHIR) or with apoptotic inhibitors in the presence of 1 μM STS for 6–48 h.

Flow cytometry

To estimate cell state, we used the following staining assays: 2',7'-dichlorodihydrofluorescein diacetate (H_2DCFDA) (Sigma), DAPI (Gerbu, Germany), FLICA® (Molecular Probes, USA) and YO-PRO™-1 (Molecular Probes). H_2DCFDA was used for detection of reactive oxygen species (a marker of active mitochondria in live cells); DAPI was used for staining the nuclei of dead cells with damaged membranes; FLICA® was used for estimating general caspase activation in cells, pointing to early apoptosis; and YO-PRO™-1

148 staining was used to detect the integrity of the plasma membrane indicating late apoptotic cells.
149 Flow cytometric analysis of apoptosis-associated fluorescence (H_2DCFDA-, FLICA®-, or YO-
150 PRO™-1-staining – excitation at 488 nm, detection in 525/40 BP channel) and necrosis-associated
151 fluorescence (DAPI – excitation at 405 nm, detection in 450/45 BP channel) was conducted within
152 20 min after staining with a CytoFLEX flow cytometer (Beckman-Coulter, USA) equipped with
153 three lasers (405, 488 and 638 nm) and connected to a computer running CytExpert software
154 (version 1.2.11.0, Beckman-Coulter). At least 20000 events were evaluated for each sample.

155 *Detection of cells with active mitochondria*

156 H_2DCFDA was added to 100 µl of suspended cells at the final concentration of 10 µM and
157 incubated at room temperature (RT) for 20 min in the dark. The cell suspension was then diluted
158 with 150 µl of CMFSS (molluscan cells) or DPBS (mammalian cells), centrifuged at 500 x *g* for
159 5 min and then re-suspended in 100 µl of fresh CMFSS or DPBS. The samples were stained with
160 DAPI at a final concentration of 1 µg/ml at RT for additional seven min in the dark and then diluted
161 with 150 µl of CMFSS or DPBS, respectively, followed by immediate analysis by flow cytometry.

162 *General caspase detection via FLICA® binding and plasma membrane integrity detection via*
163 *YO-PRO™-1 staining*

164 To estimate the number of apoptotic cells, we used two different staining combinations. First, a
165 50-µl cell suspension was stained at RT for 45 min in the dark with FAM-VAD-FMK FLICA®,
166 according to the manufacturer's recommendations. FLICA® provides an opportunity to detect
167 caspase activation in live cells (Peterson et al. 2011). Unbound FLICA® was removed from the
168 cells by rinsing with 150 µl CMFSS (molluscan cells) or DPBS (mammalian cells) followed by
169 centrifugation at 500 x *g* for 5 min and re-suspended in 95 µl of fresh CMFSS or DPBS (depending
170 on the type of cells). The samples were then stained with DAPI, as described above, and diluted

171 with 150 µl of CMFSS or DPBS (depending on the type of cells) just before the flow cytometric
172 analysis. Second, 1 µl of YO-PRO™-1 (15 µM) was added to a 100-µl cell suspension in CMFSS
173 or DPBS (depending on the type of cells). After a 10-min incubation with YO-PRO™-1 at RT in
174 the dark, the samples were stained with DAPI, as described above, and diluted with 150 µl of
175 CMFSS or DPBS (depending on the type of cells) just before the flow cytometric analysis.
176 *Data analysis*
177 Each experiment was performed independently at least three times, and all assays were
178 performed in triplicate. The files obtained with CytExpert Software were analyzed with Kaluza
179 Software v.1.5a (Beckman-Coulter). The results were subjected to a one-way analysis of variance
180 (ANOVA) followed by Tukey's multiple comparison test using Office Excel 2013 software
181 (Microsoft Corporation, USA) to test whether the values of the means from each experimental
182 group were significantly different. A p-value < 0.05 was considered statistically significant in all
183 data analyses.
184
185 **Results**
186 *Effects of apoptotic inducers/inhibitors and freezing-thawing in molluscan larval cells*
187 Figure 1 presents a time course of the induced alterations in mitochondrial activity of
188 molluscan cells after treatment with apoptotic inducers or inhibitors and after a freeze-thaw cycle.
189 Results revealed significant changes in the percentage of molluscan cells with active mitochondria
190 experiencing oxidative stress (incubated with H_2O_2) at all exposure times (distinctive apoptotic
191 changes were detectable after 6 h). The number of active cells 6 h after a freeze-thaw cycle was
192 slightly less than that in the intact unfrozen cells and relatively increased after 24–48 h cultivation
193 due to the destruction of some part of dead cells. CAM did not increase apoptosis in molluscan

cells (also as MMC) but did slightly improve their activity after thawing, whereas only STS resulted in about 10%-decrease in cell survival accompanied by a progressively increase of the percentage of apoptotic cells (Figs. 2 and 3). The apoptotic inhibitors tested did not increase the activity of control and Fr cells. The results revealed a time-dependent effect of the added compounds with the increasing duration of exposure: 6 h of exposition was insufficient for the development of compound effects but after 48 h many of cells were destroyed. Therefore, an optimal 24-h exposure period was selected for all following experiments.

2D-plots from flow cytometry of 24-h-old cultivated cells stained with apoptotic markers after a freeze-thaw cycle are illustrated in Figure 2. STS increased a portion of apoptotic cells (9.1% in comparison with 5.6% in control frozen-thawed cells). Of the apoptotic inhibitors, only the evident effect of CHIR99021 is presented, which reduces a number of early-apoptotic cells (with active caspases) (1.0% in comparison with 5.6% in control cells) but shows an increased number of dead molluscan cells (13.48% in comparison with 10.57% in frozen-thawed cells).

A pro-survival effect of camptothecin addition (Fr+Cam cells) was detected in all Fr molluscan cells (Figs. 2 and 3). MMC showed similar results, but its effect was less pronounced. CAM increased a number of live Fr cells and reduced a part of apoptotic cells both detected by FLICA® and YO-PRO™-1 staining (Fig. 3A and B). STS resulted in an increased number of apoptotic and dead cells after a freeze-thaw cycle (Fig. 3A and B).

The most typical photos of morphological alterations in all molluscan cells exposed to the compounds for 24 h (both in control and Fr cells) are presented in Fig. 4. These alterations include changes to cell shape and spreading, which might be dependent on cell attachment. Incubation of the Fr cells with STS always led to the mass appearance of round-shaped cells and was accompanied by a noticeable reduction in cell density. Fr cells treated with CAM or MMC

appeared healthier with no observable differences in cell morphology compared to control or Fr cells.

Effects of apoptotic inducers and inhibitors in mammalian cells

MEFs treated with the compounds were stained with either FLICA® or YO-PRO™-1 and DAPI followed by flow cytometric analysis (Fig. S1): a significant apoptosis and a lot of dead cells were detected after STS or CAM treatment, or after oxidative stress. In other cell model (HCT 116 cells), apoptotic inducers increased caspase activity, wherein the most evident effects were observed only after STS-treatment (Fig. S2). Apoptotic inhibitors decreased apoptosis in MEFs (Fig. S1), and more noticeably in HCT 116 cells previously treated with STS (Fig. S2).

Discussion

Examining the responses to external stressors in cells of simpler organisms, such as Protostomes (mollusks), can further elucidate the components of the programmed cell death pathways in mammals. In this study, we compared the effects of apoptotic inducers and inhibitors both in molluscan and mammalian cells. Some compounds and culture conditions may distort the lipid bilayer structure, leading to increased membrane permeability even in the absence of apoptosis (Wlodkowic et al. 2011). In most cases, the apoptotic pathway in vertebrates is characterized by increased mitochondrial outer membrane permeability (Elmore 2007). However, mollusks, similar to other marine hydrobionts, always maintain increased membrane permeability due to the specific lipid composition of their cell membrane (Loomis 1996; Odintsova & Boroda 2012). Thus, result interpretation may be difficult.

The activation time of apoptosis has been reported to vary by type of molluscan cells (Sokolova et al. 2004). In this study, we used a culture containing all larval molluscan cell types.

Only STS, one of the most potent and frequently used apoptotic inducers, demonstrated an evident apoptosis processes in molluscan cells. The little to lack of response to other known mammalian apoptotic inducers in molluscan cells is probably connected to the existence of a powerful antiapoptotic system in bivalve mollusks. An antiapoptotic regulatory network in the Pacific oyster *Crassostrea gigas* has been previously described as having a crucial role in protecting cells against heat and other stresses (Zhang et al. 2012).

The sensitivity of molluscan and mammalian cells to apoptosis is different. The oyster genome contains a high abundance of genes related to cellular defense pathways including protein folding, oxidation, apoptosis, and the immune response. Eighty-eight genes encoding heat shock proteins 70 (HSP70) have been detected in oysters, compared to only 17 in humans and 39 in sea urchins (Zhang et al. 2012). As the authors suggest, HSP70 genes are likely crucial to the oyster's cellular defense system. Environmental stressors, such as thermal or chemical exposures, induce the synthesis of HSP proteins (Snyder et al. 2001). Furthermore, the oyster genome includes 48 genes encoding apoptotic inhibitors, compared to eight in humans and seven in sea urchins (Zhang et al. 2012). Evidence of caspase-specific responses to pathogens and pollutants has been presented for bivalve mollusks (Romero et al. 2011). However, no significant caspase-3-like activity has been detected in oyster hemocytes undergoing cadmium-induced apoptosis, which suggests that different stressors exert different effects on caspase activity in marine mollusks (Sokolova & Pörtner 2001).

The lack of effect of Y-27632 on the number of apoptotic cells in mollusks found in this study questions both its use in apoptotic inhibition after a freeze-thaw cycle and the role of ROCK signaling pathway, associated with serine-threonine kinases, in apoptosis of molluscan cells. Other mammalian apoptotic inhibitors, cyclic pifithrin-α, and CHIR99021, had a slight positive (or even

negative) effect on the number of apoptotic and active cells in molluscan cultures. These results could indicate non-involvement of the p53 mitochondria-dependent signaling pathway in activating apoptosis in mollusks. Now we do not know the real signaling pathways leading to activating apoptosis in mollusks, and appear nobody in the world knows.

So far, nothing evident about the mechanisms through which apoptosis develops in molluscan cells is present. Even for mammals, there are the conflicting data on the effects of apoptotic inducers: apoptotic inducers may lead to apoptotic death in some cells although other cells are unaffected or even stimulated (Schultz & Harringto 2003). The mechanism of action of STS and its analogs on mammalian cells is poorly understood. They exert antiproliferative activity in certain cancer cell lines (Meyer et al. 1989), when possessing a poor or no effect on apoptosis of normal cells (Chen et al. 2000). This drug not only triggers the classical mitochondrial apoptosis pathway in a variety of mammalian tumor cells but activates additional apoptosis pathway, such as activation of caspase-9 in the absence of apoptotic protease activating factor 1 (Manns et al. 2011). STS was initially discovered by Omura and colleagues (Omura et al. 1977) in some marine actinomycetes, and then it has been isolated from several taxonomically diverse marine invertebrates such as ascidians and a prosobranch mollusk (Cantrell et al. 1999; Horton et al. 1994; Kinnel & Scheuer 1992; Schupp et al. 2002). The presence of substances like STS in marine invertebrates, including the tissues of some mollusks, can explain the significant apoptotic effect of this apoptotic inducer on molluscan cells. However, STS had not a significant effect on inducing the apoptosis of hemocytes in the oyster *C. gigas* (Lacoste et al. 2002). The authors suggest that mitogen-activated protein kinases and Rho, a member of the Ras GTPase family, may be involved in antiapoptotic mechanisms that modulate the apoptotic effect of noradrenaline (often referred to as one of the 'stress hormones') in these hemocytes.

Using flow cytometry alone, it is difficult to obtain true numbers of apoptotic cells because mechanically disrupted cells and isolated nuclei have reduced light scatter properties and may be mistakenly counted as small apoptotic cells. Nevertheless, important conclusions can be drawn from this comparative study since it includes both molluscan and mammalian cells. It is possible that the differences observed in drug activities between molluscan and mammalian cells could be due to differences in sequence of the drug protein targets. However, this assumption requires further research.

Apoptosis is a vital phenomenon in all eukaryotes. MEFs is a heterogenic model system, similar to larval molluscan cell culture; the effect of apoptotic modulators appears to be expressed in this system not explicitly, in contrast to HCT 116 cells. All tested inducers of mammalian apoptosis increased the number of apoptotic cells in MEFs and in the HCT 116 cell line in different extents. Significant apoptotic activation in control and Fr molluscan cells was detected only after treatment with STS. Another apoptotic inducer, CAM, did not result in a higher yield of apoptotic molluscan cells but reliably increased the number of active cells after a freeze-thaw cycle. A pro-survival effect of CAM addition in Fr molluscan cells appeared to be connected with a hormetic response when a cell or organism try to survive in an unfavorable environment and activate adaptive mechanisms (Zhang et al. 2015). CAM has been shown to reliably stimulate the cell growth of rat pheochromocytoma cells by as much as 39% at low doses and even protect the cells from H_2O_2-induced cell death (Zhang et al. 2015). CAM activates many downstream signaling pathways by reversible binding and stabilizing cleavable complexes formed between DNA and topoisomerase I (Ding et al. 2009). Therefore, the protecting effects of CAM may be resulted from upregulating phosphoinositide 3-kinase/Akt and nuclear factor-E2-related factor 2/heme

oxygenase-1 pathways in cells under the oxidative stress (Zhang et al. 2015). We cannot exclude the unknown effects of tested apoptotic inducers on molluscan cells.

Conclusion

Our findings indicate that the apoptosis in molluscan and mammalian cells is determined by the specific features of cells, that it is coincided with the previously obtained data about a strong dependence of the induction of apoptosis on the cell type (Wlodkowic et al. 2011). We have found apoptotic inducers (STS and the shock after a freeze-thaw cycle) that operate in molluscan cell cultures. Unfortunately, we did not reveal apoptotic inhibitors (among tested mammalian inhibitors) that could significantly reduce apoptosis in larval molluscan primary cell cultures after a freeze-thaw cycle. Further research on the cell death pathways in mollusks is needed to fully establish the optimal experimental approaches. Overall, this research will lead to more effective cell protection of economically and environmentally important organisms such as the bivalve mollusks.

Declaration of interest

The authors have declared that no competing interests exist.

Acknowledgements This study was supported in part by the Russian Science Foundation of Far Eastern Federal University (grant № 14-50-00034). Flow cytometric analysis was performed in the National Scientific Center of Marine Biology, FEB RAS (Vladivostok, Russia). We would like to express a special thanks to Dr. I.V. Kudryavtsev for his help in interpreting flow cytometric data and Dr. Mariia Miorova for technical assistance.

Figure captions

Fig. 1. A time course of alterations in the number of active, non-active and dead molluscan cells after treatment with apoptotic inducers or inhibitors before and after a freeze-thaw cycle.

Cells were cultivated for 6 h, 24 h and 48 h and assessed by H_2DCFDA and DAPI staining. Treatment key: control unfrozen cells (C); unfrozen cells undergoing staurosporine-induced apoptosis (STS), unfrozen cells undergoing camptothecin-induced apoptosis (CAM), unfrozen cells undergoing mitomycin C-induced apoptosis (MMC); unfrozen cells cultivated with apoptotic inhibitors – cyclic pifithrin-α (Alpha), CHIR99021 (CHIR), Y-27632 (Y); unfrozen cells undergoing oxidative stress (H_2O_2); cells frozen with 5% Me_2SO (Fr). Standard deviations were less than 5.0%.

Fig. 2. 2D-plots from flow cytometry of frozen-thawed cells cultivated for a 24-h recovery period. The samples were analyzed with a CytoFLEX flow cytometer. Cells were stained with FLICA® and DAPI to identify early apoptotic and dead cells, respectively (A) or YO-PRO™-1 and DAPI to identify late apoptotic and dead cells, respectively (B).

Treatment key: frozen-thawed cells (Fr); frozen-thawed cells cultivated with apoptotic inducers: frozen-thawed cells undergoing STS-induced apoptosis (Fr+STS), frozen-thawed cells undergoing CAM-induced apoptosis (Fr+CAM); frozen-thawed cells incubated with apoptotic inhibitor CHIR99021 (Fr+CHIR).

Fig. 3. Flow cytometric analysis of apoptosis-associated fluorescence and necrosis-associated fluorescence in molluscan cells before and after a freeze-thaw cycle. Cells were cultivated for 24 h and stained with FLICA® in conjunction with DAPI (A) or YO-PRO™-1 in conjunction with DAPI (B).

353 Treatment key: control unfrozen cells (C); cells frozen with 5% Me_2SO and then cultivated for 24
354 h (Fr); frozen-thawed cells undergoing STS-induced apoptosis (Fr+STS), frozen-thawed cells
355 undergoing CAM-induced apoptosis (Fr+CAM), frozen-thawed cells undergoing MMC-induced
356 apoptosis (Fr+MMC); frozen-thawed cells cultivated with apoptotic inhibitors: cyclic pifithrin-α
357 (Fr+Alpha), CHIR99021 (Fr+CHIR), Y-27632 (Fr+Y). The significance levels are *P < 0.05 and
358 **P < 0.01.

359 **Fig. 4.** Morphology of control unfrozen and frozen-thawed molluscan cells cultivated in different
360 conditions for 24 h. Treatment key: control unfrozen cells (C); unfrozen cells undergoing STS-
361 induced apoptosis (C+STS); frozen-thawed cells (Fr); frozen-thawed cells undergoing STS-
362 induced apoptosis (Fr+STS); frozen-thawed cells undergoing CAM-induced apoptosis (Fr+CAM);
363 frozen-thawed cells incubated with an apoptotic inhibitor CHIR99021 (Fr+CHIR).
364 Specimens were examined in a CKX41 inverted microscope (Olympus, Japan) equipped with
365 phase-contrast optics and imaged with an Axiocam 105 color digital camera (Carl Zeiss,
366 Germany). Scale bar – 100 μm.
367
368 **Supplementary material:**
369 **Fig. S1.** The results of flow cytometric detection of apoptotic and dead MEFs after a 24 h-treatment
370 with inducers or inhibitors of apoptosis. Cells were stained with FLICA® in conjunction with
371 DAPI (A) or YO-PRO™-1 in conjunction with DAPI (B). The samples were analyzed with a
372 CytoFLEX flow cytometer.
373 Treatment key: control cells (C); cells undergoing STS-induced apoptosis (STS), cells undergoing
374 CAM-induced apoptosis (CAM), cells undergoing MMC-induced apoptosis (MMC); cells

cultivated with apoptotic inhibitors: cyclic pifithrin-α (Alpha), CHIR99021 (CHIR), Y-27632 (Y); cells undergoing oxidative stress (H_2O_2). The significance levels are *P < 0.05 and **P < 0.01.

Fig. S2. The results of flow cytometric detection of apoptotic and dead HCT 116 cells after a 24 h-treatment with inducers or inhibitors of apoptosis. Cells were stained with FLICA® in conjunction with DAPI. The samples were analyzed in a CytoFLEX flow cytometer.

Treatment key: control cells (C); cells cultivated with apoptotic inducers: cells undergoing STS-induced apoptosis (STS); cells undergoing CAM-induced apoptosis (CAM); cells undergoing MMC-induced apoptosis (MMC); cells cultivated with apoptotic inhibitors: cyclic pifithrin-α (Alpha), CHIR99021 (CHIR), Y-27632 (Y); STS-treated cells incubated with apoptotic inhibitors: STS+Alpha, STS+CHIR, STS+Y; cells undergoing oxidative stress (H_2O_2). The significance levels are *P < 0.05 and **P < 0.01.

References

Ambroise G, Portier A, Roders N, Arnoult D, and Vazquez A. 2015. Subcellular localization of PUMA regulates its pro-apoptotic activity in Burkitt's lymphoma B cells. *Oncotarget* 6:38181–38194. http://doi.org/10.18632/oncotarget.5901

Cantrell CL, Groweiss A, Gustafson KR, and Boyd MR. 1999. A New Staurosporine Analog from the Prosobranch Mollusk *Coriocella Nigra*. *Natural Product Letters* 14:39–46. http://doi.org/10.1080/10575639908045433

Chen X, Lowe M, Herliczek T, Hall MJ, Danes C, Lawrence DA, and Keyomarsi K. 2000. Protection of Normal Proliferating Cells Against Chemotherapy by Staurosporine-Mediated, Selective, and Reversible G1 Arrest. *JNCI: Journal of the National Cancer Institute* 92:1999–2008. http://doi.org/10.1093/jnci/92.24.1999

Cheng TC. 1988. *In vivo* effects of heavy metals on cellular defense mechanisms of *Crassostrea virginica*: Total and differential cell counts. *Journal of Invertebrate Pathology* 51:207–214. http://doi.org/10.1016/0022-2011(88)90027-4

Cherkasov AS, Grewal S, and Sokolova IM. 2007. Combined effects of temperature and cadmium exposure on haemocyte apoptosis and cadmium accumulation in the eastern oyster *Crassostrea virginica* (Gmelin). *Journal of Thermal Biology* 32:162–170. http://doi.org/10.1016/j.jtherbio.2007.01.005

Ding L, Qiu L, Zhang J, and Guo B. 2009. Camptothecin-induced cell proliferation inhibition and apoptosis enhanced by DNA methyltransferase inhibitor, 5-aza-2'-deoxycytidine. *Biological & pharmaceutical bulletin* 32:1105–1108. http://doi.org/10.1248/bpb.32.1105

Elmore S. 2007. Apoptosis: A review of programmed cell death. *Toxicologic Pathology* 35:495–516. http://doi.org/10.1080/01926230701320337

Halpin PM, Sorte CJ, Hofmann GE, and Menge BA. 2002. Patterns of Variation in Levels of Hsp70 in Natural Rocky Shore Populations from Microscales to Mesoscales. *Integrative and Comparative Biology* 42:815–824. http://doi.org/10.1093/icb/42.4.815

Hampton MB, and Orrenius S. 1997. Dual regulation of caspase activity by hydrogen peroxide: implications for apoptosis. *FEBS Letters* 414:552–556. http://doi.org/10.1016/S0014-5793(97)01068-5

Horton PA, Longley RE, McConnell OJ, and Ballas LM. 1994. Staurosporine aglycone (K252-c) and arcyriaflavin A from the marine ascidian, *Eudistoma sp*. *Experientia* 50:843–845. http://doi.org/10.1007/bf01956468

Kefaloyianni E, Gourgou E, Ferle V, Kotsakis E, Gaitanaki C, and Beis I. 2005. Acute thermal stress and various heavy metals induce tissue-specific pro-or anti-apoptotic events via the p38-MAPK signal transduction pathway in *Mytilus galloprovincialis* (Lam.). *Journal of Experimental Biology* 208:4427–4436. http://doi.org/10.1242/jeb.01924

Kinnel RB, and Scheuer PJ. 1992. 11-Hydroxystaurosporine: a highly cytotoxic, powerful protein kinase C inhibitor from a tunicate. *The Journal of Organic Chemistry* 57:6327–6329. http://doi.org/10.1021/jo00049a049

Kiss T. 2010. Apoptosis and its functional significance in molluscs. *Apoptosis* 15:313–321. http://doi.org/10.1007/s10495-009-0446-3

Lacoste A, Cueff A, and Poulet SA. 2002. P35-sensitive caspases, MAP kinases and Rho modulate β-adrenergic induction of apoptosis in mollusc immune cells. *Journal of Cell Science* 115:761–768.

Lockwood BL, Connor KM, and Gracey AY. 2015. The environmentally tuned transcriptomes of *Mytilus* mussels. *The Journal of Experimental Biology* 218:1822–1833. http://doi.org/10.1242/jeb.118190

Loomis SH. 1996. Freezing tolerance of marine invertebrates. *Oceanographic Literature Review* 43:337–350.

Manns J, Daubrawa M, Driessen S, Paasch F, Hoffmann N, Löffler A, Lauber K, Dieterle A, Alers S, Iftner T, Schulze-Osthoff K, Stork B, and Wesselborg S. 2011. Triggering of a novel intrinsic apoptosis pathway by the kinase inhibitor staurosporine: activation of caspase-9 in the absence of Apaf-1. *The FASEB Journal* 25:3250–3261. http://doi.org/10.1096/fj.10-177527

Marión RM, Strati K, Li H, Murga M, Blanco R, Ortega S, Fernandez-Capetillo O, Serrano M, and Blasco MA. 2009. A p53-mediated DNA damage response limits reprogramming to ensure iPS cell genomic integrity. *Nature* 460:1149–1153. http://doi.org/10.1038/nature08287

Mehlen P, Schulze-Osthoff K, and Arrigo A-P. 1996. Small Stress Proteins as Novel Regulators of Apoptosis: Heat Shock Protein 27 Blocks Fas/Apo-1- And Staurosporine-Induced Cell

Death. *Journal of Biological Chemistry* 271:16510–16514. http://doi.org/10.1074/jbc.271.28.16510

Meyer T, Regenass U, Fabbro D, Alteri E, Rösel J, Müller M, Caravatti G, and Matter A. 1989. A derivative of staurosporine (CGP 41:251) shows selectivity for PKC inhibition and in vitro antiproliferative effects as well as in vivo antitumor activity. *International Journal of Cancer* 43:851–866. http://doi.org/10.1002/(SICI)1097-0215(19990517)81:4<669::AID-IJC26>3.0.CO;2-F

Mičić M, Bihari N, Labura Ž, Müller WEG, and Batel R. 2001. Induction of apoptosis in the blue mussel *Mytilus galloprovincialis* by tri-n-butyltin chloride. *Aquatic Toxicology* 55:61–73. http://doi.org/10.1016/S0166-445X(01)00156-4

Morris EJ, and Geller HM. 1996. Induction of neuronal apoptosis by camptothecin, an inhibitor of DNA topoisomerase-I: evidence for cell cycle-independent toxicity. *The Journal of Cell Biology* 134:757–770. http://doi.org/10.1083/jcb.134.3.757

Odintsova N, Dyachuk V, and Nezlin L. 2010. Muscle and neuronal differentiation in primary cell culture of larval *Mytilus trossulus* (Mollusca: Bivalvia). *Cell and tissue research* 339:625-637.

Odintsova NA, and Boroda AV. 2012. Cryopreservation of the cells and larvae of marine organisms. *Russian Journal of Marine Biology* 38:101–111. http://doi.org/10.1134/s1063074012020083

Odintsova NA, Boroda AV, Maiorova MA, and Yakovlev KV. 2017. The death pathways in mussel larval cells after a freeze-thaw cycle. *Cryobiology* 77:41–49. http://doi.org/10.1016/j.cryobiol.2017.05.009

Omura S, Iwai Y, Hirano A, Nakagawa A, Awaya J, TSUCHIYA H, Takahashi Y, and ASUMA R. 1977. A new alkaloid AM-2282 of Streptomyces origin taxonomy, fermentation, isolation and preliminary characterization. *The Journal of antibiotics* 30:275–282. http://doi.org/10.7164/antibiotics.30.275

Peh GSL, Adnan K, George BL, Ang H-P, Seah X-Y, Tan DT, and Mehta JS. 2015. The effects of Rho-associated kinase inhibitor Y-27632 on primary human corneal endothelial cells propagated using a dual media approach. *Scientific Reports* 5:9167. http://doi.org/10.1038/srep09167

Peterson S, Loring JF, Wesselschmidt RL, and Schwartz PH. 2011. *Human Stem Cell Manual: A Laboratory Guide*: Elsevier Science.

Pirnia F, Schneider E, Betticher DC, and Borner MM. 2002. Mitomycin C induces apoptosis and caspase-8 and -9 processing through a caspase-3 and Fas-independent pathway. *Cell Death And Differentiation* 9:905–914. http://doi.org/10.1038/sj.cdd.4401062

Przeslawski R, Byrne M, and Mellin C. 2015. A review and meta-analysis of the effects of multiple abiotic stressors on marine embryos and larvae. *Global Change Biology* 21:2122–2140. http://doi.org/10.1111/gcb.12833

Romero A, Estévez-Calvar N, Dios S, Figueras A, and Novoa B. 2011. New insights into the apoptotic process in mollusks: Characterization of caspase genes in *Mytilus galloprovincialis*. *PLOS ONE* 6:e17003. http://doi.org/10.1371/journal.pone.0017003

Schultz DR, and Harringto WJ, Jr. 2003. Apoptosis: Programmed cell death at a molecular level. *Seminars in Arthritis and Rheumatism* 32:345–369. http://doi.org/10.1053/sarh.2003.50005

Schupp P, Proksch P, and Wray V. 2002. Further New Staurosporine Derivatives from the Ascidian *Eudistoma toealensis* and Its Predatory Flatworm *Pseudoceros sp*. *Journal of Natural Products* 65:295–298. http://doi.org/10.1021/np010259a

Snyder MJ, Girvetz E, and Mulder EP. 2001. Induction of marine mollusc stress proteins by chemical or physical stress. *Archives of Environmental Contamination and Toxicology* 41:22–29. http://doi.org/10.1007/s002440010217

Sokolova IM. 2009. Apoptosis in molluscan immune defense. *Invertebrate Survival Journal* 6:49–58.

Sokolova IM, Evans S, and Hughes FM. 2004. Cadmium-induced apoptosis in oyster hemocytes involves disturbance of cellular energy balance but no mitochondrial permeability transition. *Journal of Experimental Biology* 207:3369–3380. http://doi.org/10.1242/jeb.01152

Sokolova IM, and Pörtner HO. 2001. Physiological adaptations to high intertidal life involve improved water conservation abilities and metabolic rate depression in *Littorina saxatilis*. *Marine Ecology Progress Series* 224:171–186. http://doi.org/10.3354/meps224171

Wlodkowic D, Telford W, Skommer J, and Darzynkiewicz Z. 2011. Chapter 4 - Apoptosis and Beyond: Cytometry in Studies of Programmed Cell Death. In: Darzynkiewicz Z, Holden

E, Orfao A, Telford W, and Wlodkowic D, eds. *Methods in Cell Biology*: Academic Press, 55–98.

Zeiss CJ. 2003. The apoptosis-necrosis continuum: Insights from genetically altered mice. *Veterinary Pathology* 40:481–495. http://doi.org/10.1354/vp.40-5-481

Zhang C, Chen S, Bao J, Zhang Y, Huang B, Jia X, Chen M, Wan J-B, Su H, Wang Y, and He C. 2015. Low Doses of Camptothecin Induced Hormetic and Neuroprotective Effects in PC12 Cells. *Dose-Response* 13:1–7. http://doi.org/10.1177/1559325815592606

Zhang G, Fang X, Guo X, Li L, Luo R, Xu F, Yang P, Zhang L, Wang X, Qi H, Xiong Z, Que H, Xie Y, Holland PWH, Paps J, Zhu Y, Wu F, Chen Y, Wang J, Peng C, Meng J, Yang L, Liu J, Wen B, Zhang N, Huang Z, Zhu Q, Feng Y, Mount A, Hedgecock D, Xu Z, Liu Y, Domazet-Lošo T, Du Y, Sun X, Zhang S, Liu B, Cheng P, Jiang X, Li J, Fan D, Wang W, Fu W, Wang T, Wang B, Zhang J, Peng Z, Li Y, Li N, Wang J, Chen M, He Y, Tan F, Song X, Zheng Q, Huang R, Yang H, Du X, Chen L, Yang M, Gaffney PM, Wang S, Luo L, She Z, Ming Y, Huang W, Zhang S, Huang B, Zhang Y, Qu T, Ni P, Miao G, Wang J, Wang Q, Steinberg CEW, Wang H, Li N, Qian L, Zhang G, Li Y, Yang H, Liu X, Wang J, Yin Y, and Wang J. 2012. The oyster genome reveals stress adaptation and complexity of shell formation. *Nature* 490:49–54. http://doi.org/10.1038/nature11413

Zhivotovsky B. 2004. Apoptosis, necrosis and between. *Cell Cycle* 3:63–65. http://doi.org/10.4161/cc.3.1.606

Figure 1

Fig. 1. A time course of alterations in the number of active, non-active and dead molluscan cells after treatment with apoptotic inducers or inhibitors before and after a freeze-thaw cycle.

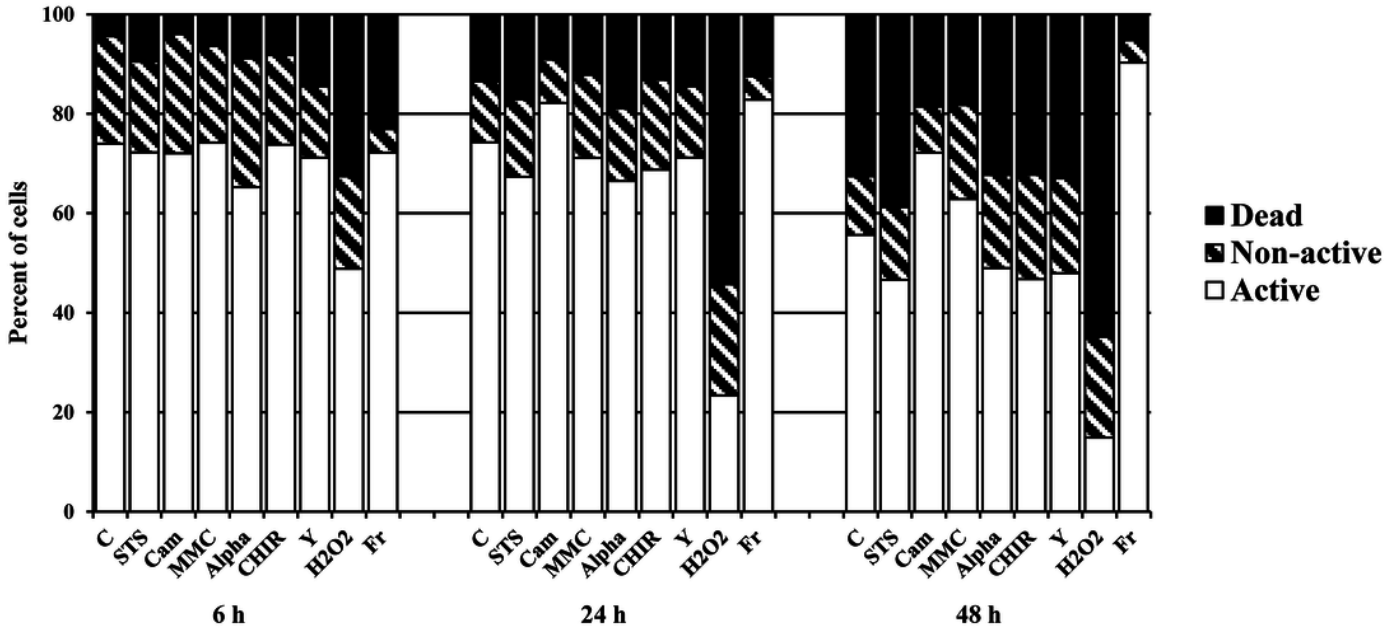


Figure 2

Fig. 2. 2D-plots from flow cytometry of frozen-thawed cells cultivated for a 24-h recovery period. The samples were analyzed with a CytoFLEX flow cytometer.

PeerJ Preprints | https://doi.org/10.7287/peerj.preprints.27100v1 | CC BY 4.0 Open Access | rec: 9 Aug 2018, publ: 9 Aug 2018

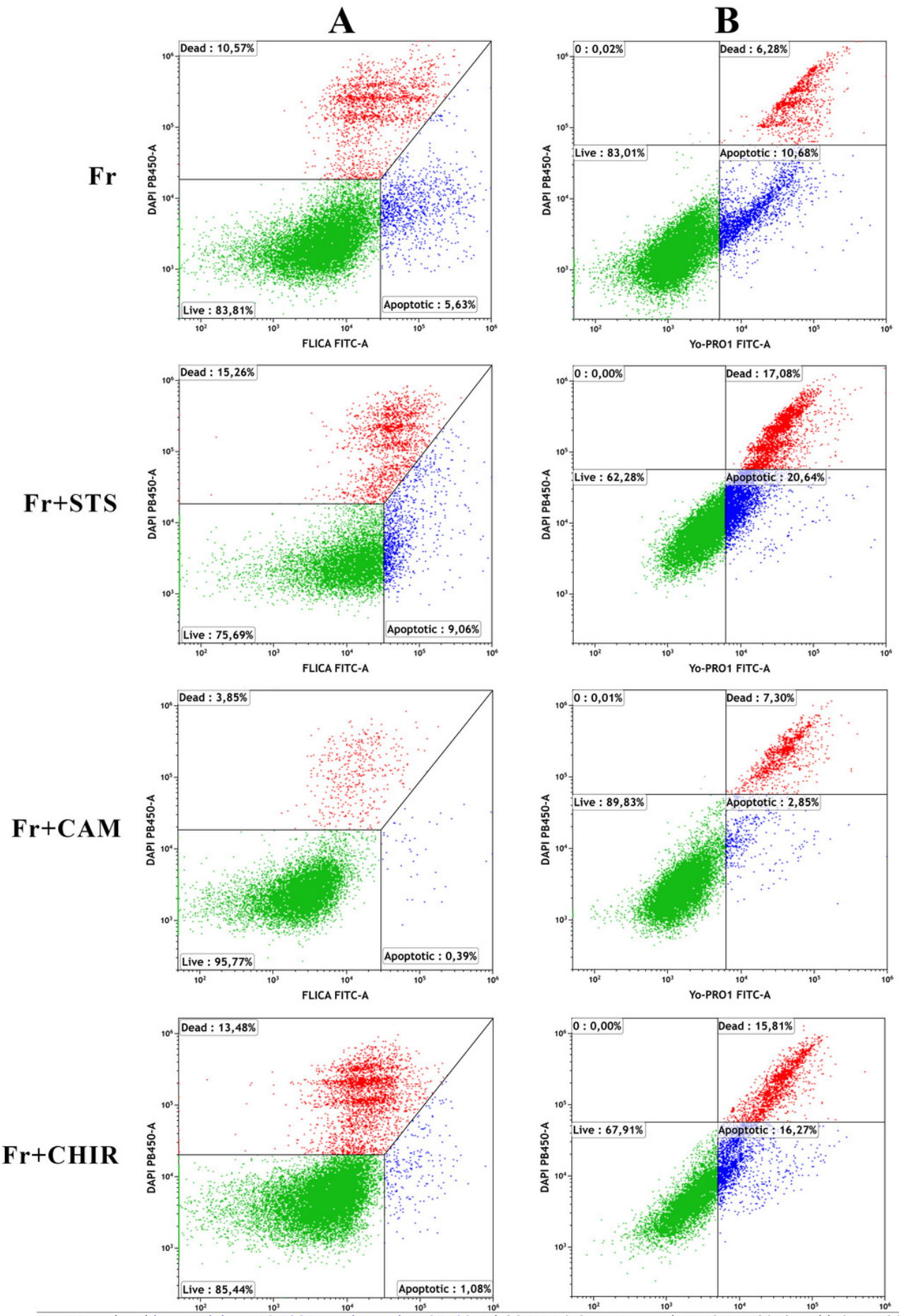


Figure 3

Fig. 3. Flow cytometric analysis of apoptosis-associated fluorescence and necrosis-associated fluorescence in molluscan cells before and after a freeze-thaw cycle.

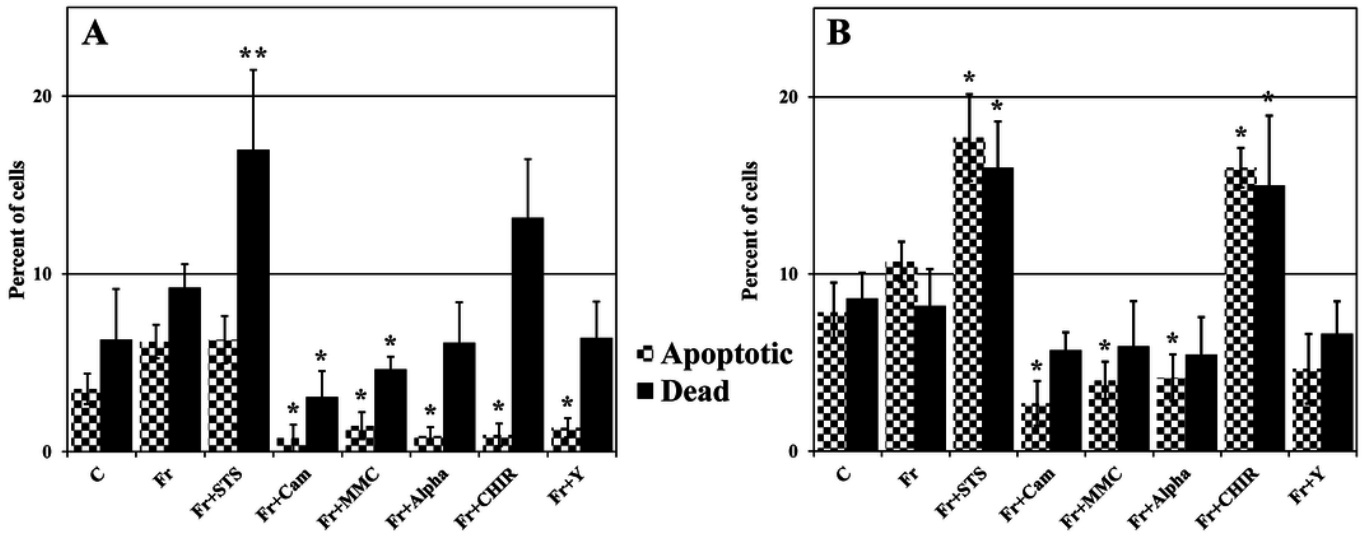
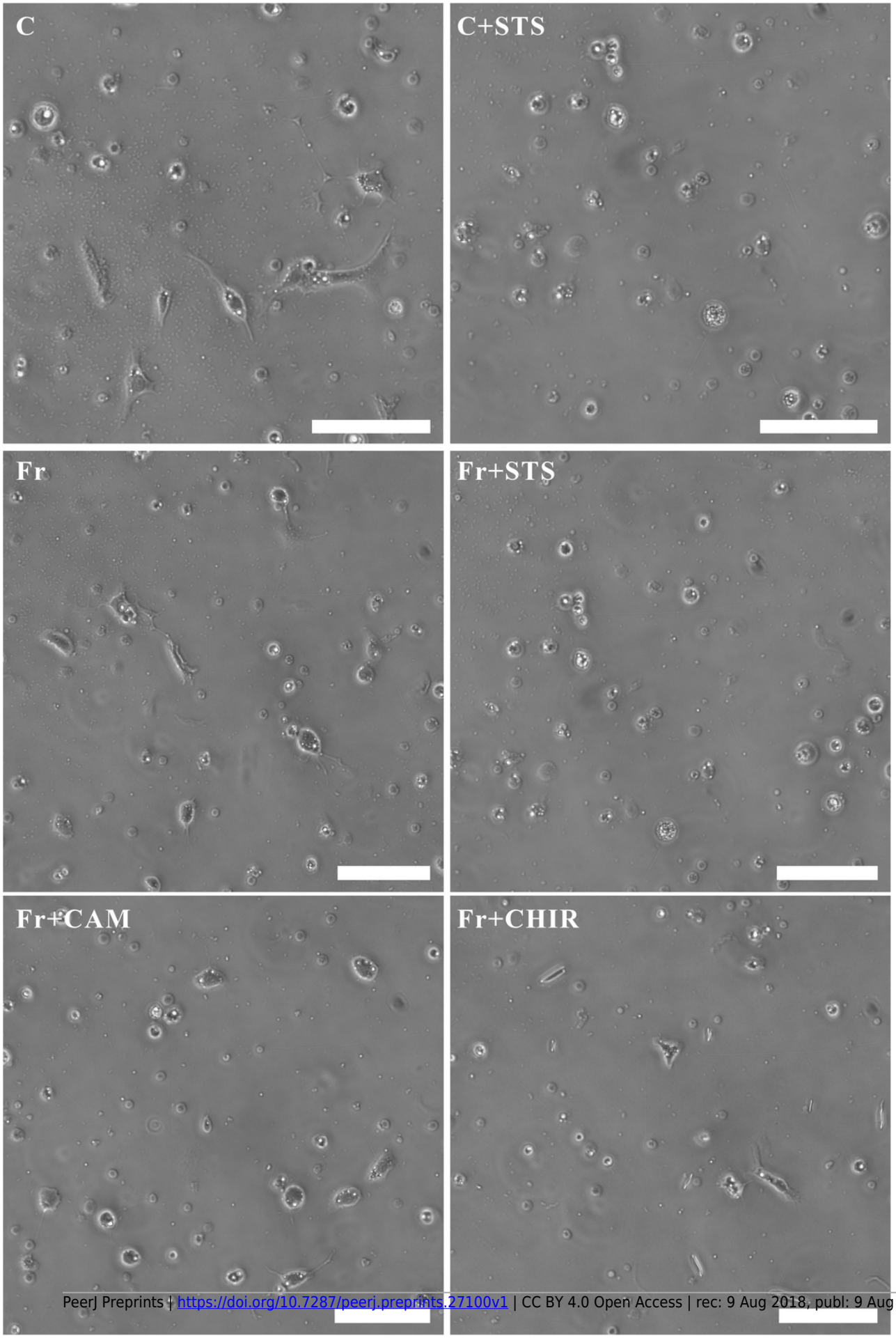


Figure 4

Fig. 4. Morphology of control unfrozen and frozen-thawed molluscan cells cultivated in different conditions for 24 h.

**Note: Auto Gamma Correction was used for the image. This only affects the reviewing manuscript. See original source image if needed for review.*



PeerJ Preprints | https://doi.org/10.7287/peerj.preprints.27100v1 | CC BY 4.0 Open Access | rec: 9 Aug 2018, publ: 9 Aug 2018