Title: All trans retinoic acid modulates peripheral nerve fibroblasts viability and apoptosis

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- ATRA modulated viability of peripheral nerve fibroblasts.
- ATRA induced apoptosis in peripheral nerve fibroblasts.
- The higher level of caspase 3/7 activity was measured following ATRA treatment
- ATRA may help regeneration of axons via moderating scare tissue formation.

All trans retinoic acid modulates peripheral nerve fibroblasts viability and apoptosis

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Running title: ATRA treatment of peripheral nerve fibroblasts

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Abstract

Objective: Following peripheral nerve injury, residing fibroblasts start to proliferate and accumulate at the injury site and may participate in neuroma tissue evolution. Retinoic acid has been shown to regulate many cellular processes and to display anti-proliferative and anti-fibrotic properties. The aim of this study was to investigate the impact of all trans retinoic acid (ATRA) on rat peripheral nerve fibroblasts.

Materials and Methods: Peripheral nerve fibroblasts and C166 cells were treated with increasing doses of ATRA (0.05 nM to 1µM). The viability of cells was determined with 3-(4,5-dimethlthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. In addition, the number of peripheral nerve fibroblasts were counted after two days of ATRA treatment and alternatively up to the end of next week. Acridine orange/ethidium bromide double staining was implemented to morphologically visualize the possible mechanism of cell death. For apoptosis, caspase 3/7 activity was measured using Caspase-Glo 3/7 assay kit.

Results: MTT assay revealed that 0.05 to 1nM of ATRA reduces fibroblasts viabilities. Then, almost a plateau state was observed from 1nM to 1µM of ATRA exposure. Additionally, a deceleration in peripheral nerve fibroblasts growth was confirmed via cell counting. Quantification of acridine orange/ethidium bromide staining displayed highly increased number of early apoptotic cells following ATRA administration. Amplified activation of caspase 3/7 was in favor of apoptosis in ATRA treated peripheral nerve fibroblasts.

Conclusion: The data from the present study demonstrate that ATRA could interfere in peripheral nerve fibroblasts viabilities and induce apoptosis. Although more investigations are needed to be implemented, our in vitro results indicate that retinoic acid can probably help the regeneration of injured axon via reducing of fibroblasts growth.

Keywords: ATRA; Peripheral nerve fibroblasts; Viability; Apoptosis.

1. Introduction

Axons of the peripheral nervous system (PNS) have the ability to grow beyond the lesion area (Yiu and He, 2006). Compelling evidence suggests the presence of permissive environment allowing axonal regeneration in PNS (Chen et al., 2007; Vargas and Barres, 2007). Indeed, a complex of cellular and molecular changes referred to Wallerian degeneration takes place within the distal stump of the damaged nerve as the natural mechanism for healing (Dubovy, 2011). Following loss of contact with axon, Schwann cells initiate proliferation and migration (Rotshenker, 2011) and participate actively with accumulated fibroblasts at the injury site, establishing guidance channel (i.e. Bands of Bungner) in a specialized cell sorting manner (Parrinello et al., 2010). The accomplishment or failure of peripheral nerve regeneration, to the most part, depends on the integrity of connective tissue which surrounds the nerve unit (Dreesmann et al., 2009). Disruption of nerve structure reduces the functional recovery since the regenerating axons gets stuck within a scar tissue (i.e. neuroma) made of fibroblasts originating from surrounding connective tissues and Schwann cells as well (Parrinello et al., 2010). Post traumatic neuroma not only produces neurite growth repellent factors (Tannemaat et al., 2007), such as semaphorin3A produced by fibroblasts (Berger et al., 2011) but also plays a major role in chronic neuropathic pain development (Kotulska et al., 2006). On the other hand, it has been shown that fibroblastic cells may be as a contaminator agent in the Schwann cell cultures. This predominantly happens once implemented peripheral nerves do not lapse the duration of Wallerian degeneration or elapse over ten days. Thus, it is recommended implementing Wallerian degeneration with one week duration for attaining more and activated Schwann cells in culture (Kraus et al., 2010).

All trans retinoic acid (ATRA) is a functional metabolite of vitamin A that participates in multiple biological processes from embryogenesis, reproduction, inflammation, and proliferation to differentiation and apoptosis (Noy, 2010; Wolf, 2008). Interestingly, retinoic acid signaling involves in response to peripheral nerve injury (Zhelyaznik and Mey, 2006). It has been shown that retinaldehyde dehydrogenase II enzyme (RALDH II), which synthesizes retinoic acid, attains its maximum activity in seven days of

peripheral nerve insult (Maden, 2007 Oct.). Moreover, retinoic acid exert an influence on initiation and progression of fibrotic disease(Zhou et al., 2012). In this regard, Dong and colleagues displayed that ATRA could ameliorate bleomycin-induced pulmonary fibrosis through inhibition of interleukin-6 (IL-6) and transforming growth factor β (TGF-β) gene expression which both are considered as fibroblasts growth enhancers (Dong et al., 2012). The same mechanism was verified in case of ATRA administration for epidural fibrosis relief after laminectomy (Zhang et al., 2013). Therefore, the aim of the current study was to evaluate the possible effects of ATRA on peripheral nerve fibroblasts in culture condition.

2. Materials and Methods

2.1. Cell culture

Human gingival fibroblast cell line (HGF2, NCBI: C166) was purchased from National Cell Bank of Iran, Pasteur institute in Tehran, Iran (Khosravi et al., 2004). Peripheral nerve fibroblasts were isolated from rats sciatic nerve. Adult Sprague Dawley rats (150-200g; Pasteur Institute, Tehran, Iran) were deeply anesthetized with intraperitoneal injection of ketamine (30 mg/kg) and xylazine (3mg/kg). Animal experiments were carried out in strict compliance with the approval of Institutional Animal Ethics Committee of the Ardabil University of Medical Sciences, which follows the NIH guidelines for care and use of experimental animals. Sciatic nerves were excised bilaterally and transferred to cell culture laboratory. Under stereomicroscope, epineurium was stripped off and cut into small pieces. Epineural explants maintained in petri dishes for 10 minutes at room temperature to adhere more efficiently onto culture vessels. Then, explants were incubated in medium containing Dulbecco's modified eagles medium (DMEM, Gibco: 12800116) and 10% fetal bovine serum (FBS, Gibco: 10270) supplemented with, 2mM L-glutamine (Gibco: 25030), 100U/ml penicillin/streptomycin (Gibco: 15140) at 37°C and 5% CO_2. After 7-10 days, fibroblasts which have migrated out from explants onto tissue culture plates were collected and considered as passage zero (Dreesmann et al., 2009; Van neerven et al., 2013). Peripheral nerve fibroblasts and C166 cells at passages 3-5 were used in this study. Fibroblasts were seeded in 96 well plates at a density of 1×10^3 cells per well. A day after plating, fibroblasts were treated with increasing concentrations of ATRA (Sigma: R2625) for 2 days; 0.05, 0.1, 1, 10, 100 nM and 1µM. Control group received an equal amount of ethanol, as a solvent for ATRA.

2.2. Cell viability assay

Cell viability was determined with 3-(4,5)-dimethylthiahiazo-(-z-y1)-3,5-di-phenytetrazoliumromide dye reduction (Jafari et al., 2012 Sep; Mosmann, 1983 Dec 16). MTT (Sigma: M2128) was dissolved in PBS

at 5 mg/ml. The stock solution was then added to the culture medium at a dilution 1:10 and incubated for 4 hours at 37°C. After discarding medium, MTT formazan was dissolved in dimethyl solfoxide (DMSO). The absorbance was measured at 540 nm by using a microplate reader (Synergy HT, BioTek). All tests were done in triplicate.

2.3. Cell counting using trypan blue exclusion dye

For cell counting, peripheral nerve fibroblasts were treated with different concentrations of ATRA for two days and continued one more week in normal culture condition. Cells were detached and an equal volumes of 0.4% trypan blue stain (Sigma: T6146) and cell suspension were mixed. Ten microliter of cell mixture was counted under a microscope using haemocytometer. Trypan blue negative cells were counted at days 1, 3, 5, 7 and 9. All tests were done in triplicate.

2.4. Acridine orange/ethidium bromide double staining

Acridine orange permeates both viable and dead cells and fluoresce green when bound to double stranded DNA in living cells. Ethidium bromide is excluded from living cells. However, in late apoptotic or necrotic cells, which their membrane integrity is impaired, ethidium bromide intercalate into DNA and fluorescence red (Ho et al., 2009 Aug). After incubation with different concentrations of ATRA, tissue culture plates were centrifuged and washed with ice cold PBS. Then, a mixture containing 5µl of ethidium bromide (Merck: 4310) (100 µg/ml) and 5µl of acridine Orange (Merck: 113000) (100 µg/ml) were added on peripheral nerve fibroblasts and kept for 5minutes at room temperature. After rinsing with PBS, cells were observed under an inverted fluorescence microscope (Olympus IX71) equipped with a digital camera (Olympus, DP71). In each experiment, more than 100 cells/per concentration were counted.

2.5. Caspase 3/7 activity assay

For quantitative evaluation, caspase 3/7 activity was measured using the Caspase-3/7 Glo apoptosis assay kit (Promega: G8090). Based on manufacturer's protocol, 1×10^4 cells of control and 1nM of ATRA treated peripheral nerve fibroblasts were incubated at room temperature with caspase 3/7 kit reagents for 3 hours. The cleavage of tetra peptide sequence DEVD was measured by a plate reader (Synergy HT, BioTek) at 570 nm luminescent emission. For positive control, peripheral nerve fibroblasts were treated with 15µg/ml of Cisplatin (Sigma: P4394). Tests were carried out in triplicate.

2.6. Statistical analysis

All data were expressed as mean ± SEM. Data were analyzed using one way ANOVA following post hoc Tukey's test for comparing means among groups. P-value of less than 0.05 was considered significant. Statistical analyses were performed using computer software; SPSS 15 for Windows.

3. Results

The effect of different doses of ATRA on the viability rate of C166 cell line and peripheral nerve fibroblasts was shown in Figure 1A. The 0.05 nM of ATRA significantly decreased mean peripheral nerve fibroblasts viability (19.4%) and continued up to 42.8 % at 10 nM (P□ 0.001) while no significant change in nerve fibroblasts viability was observed from 1nM to 1μM using MTT assay. Moreover, cumulative amount of peripheral nerve fibroblasts in successive days of counting demonstrated the growth rate declination in ATRA treated groups in comparison to control (Fig 1B). In the same way, the viability of C166 cells reduced in all concentrations of ATRA compared to control (P□ 0.001). ATRA concentrations from 0.05 to 1nM lowered C166 cells viability rate as detected by MTT assay (Fig 1A). The maximum effect of ATRA observed at 1nM in which the mean viability of C166 cells diminished up to 48% of control (P□ 0.001) The inhibitory effect of ATRA was moderated from 1nM to 100nM and C166 cells viability recovered up to 69.2% of control. No major alterations in viability of C166 cells were detected by the end of assayed condition (Fig 1A).

Peripheral nerve fibroblasts were subjected to acridine orange/ethidium bromide fluorescent staining to morphologically determine the possible mechanism of cell death following ATRA treatment (Fig 2A-D). The observed cells were divided as defined permutations: living cells (normal green nucleus), early apoptotic (bright green nucleus with fragmented/pyknotic appearance), late apoptotic (orange-stained nuclei with fragmented/pyknotic appearance) and necrotic cells (uniformly orange-stained cell nucleus) (Attari et al., 2009). Our results indicated that the higher number of cells in control group were alive (Fig 2A, E). The rise in ATRA concentration caused the more number of early apoptotic cell while no significant number of late apoptotic or necrotic cells were detected in two days of treatment (Fig 2B-E). Further, some typical characteristics of apoptotic cells like plasma membrane blebbing were observed (Fig 2D).

Given the observed effect of ATRA on peripheral nerve fibroblasts, the caspase-3 and caspase-7 activity was measured via a luminescent kit. The caspases proteins are known for the execution of apoptosis. Caspase-3 and caspase-7 are key effector molecules that amplify the signal from initiator caspases, such as caspase-8 or caspase-10 and induce apoptosis in variety of cells. We detected significantly higher level of effector caspase-3 and caspase-7 activity at 1nM treated peripheral nerve fibroblasts rather than negative control which suggests induction of apoptosis (P□ 0.001)(Fig 3A).

4. Discussion

Retinoic acid signaling elements including its synthesizing enzymes, cytosolic binding protein and nuclear receptors are expressed in PNS (Latasa and Cosgaya, 2011). The amplification and activation of retinoic acid signaling imply a potential role for retinoic acid during PNS regeneration and repair (Latasa and Cosgaya, 2011). It is well known that retinoic acid helps axonal regeneration in nervous system, at least, through three mechanisms; a) directly stimulation of axonal elongation (Wong et al., 2006), b) inhibition of myelin formation and promotion of different cytokines production by SCs (Latasa and Cosgaya, 2011; Latasa et al., 2010; Maden, 2007 Oct.) and c) inhibition of macrophage invasion into damaged area (Maden, 2007 Oct.; Mey, 2006).

ATRA can interfere with cell growth via controls over cellular differentiation, cell cycle arrest, and induction of apoptosis or by a combination of these events (Noy, 2010). Our results demonstrated that ATRA administration not only reduces peripheral nerve fibroblasts survival but also induces apoptosis in comparison with untreated cells. The finding of the current study could be especially important in human beings since functional restoration after nerve injury is often less successful due to tendency to strong fibrosis than it is in mice and rats (Rotshenker, 2011). These findings are in line with previous study demonstrating the declination of dermal fibroblasts survival after ATRA treatment (Daly and Weston, 1986) and support retinoic acid inhibitory effect on initiation and progression of fibrotic disease (Zhou et al., 2012). ATRA has been shown to ameliorate bleomycin-induced pulmonary fibrosis through inhibition of IL-6 and TGF-β gene expression (Dong et al., 2012). Moreover, ATRA administration could alleviate laminectomy induced epidural fibrosis in rats. Zhang and colleagues displayed that ATRA was able to suppress IL-6, TGF-β gene expression, down-regulate nuclear factor kB (NF-kB) in fibroblastic cells and prohibit epidural scar adhesion (Zhang et al., 2013).

On the other hand, penetrating traumas to the CNS (such as total transection or dorsal/lateral hemisection models of spinal cord injury) cause the invasion of fibroblasts, which originate from meninges, into the injured area. These cells start to deposit collagens and participate in collagenous basement membrane formation around the damaged region (Hermanns et al., 2001; Stichel et al., 1999 Feb). Thus, findings of the current study may point a new role for retinoic acid in axonal regeneration beyond nervous system injuries.

5. Conclusion

Taken together, although more in vivo studies need to be clarified, our findings provide a preliminary in vitro base that retinoic acid may modify nerve fibroblasts viability following peripheral nerve damage and modulate scare tissue (i.e. neuroma) formation.

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Figure legends

Fig 1: Fibroblastic cells viability assessment and counting. ATRA administration resulted in a significant decrease in fibroblastic cells viability as shown via MTT assay (A). Peripheral nerve fibroblasts counted two days after ATRA administration and continued alternatively for one more week (B). * $p<0.001$ vs. control, $ $p<0.05$ in comparison with its previous dosage, # $p<0.001$ in comparison with its previous dosage.

Fig 2: Acridine orange and ethidium bromide fluorescent staining. After treatment with ATRA, peripheral nerve fibroblasts were double stained with acridine orange/ethidium bromide. Part A, B, and C are representative panels for control, 1nM and 1μM of ATRA receiving groups, respectively. Block arrows indicate live cells, arrow heads represent early apoptotic cell and asterisk shows late apoptotic cell. A cell with characteristics of blebbing are shown in part D applying concomitant normal and fluorescence light. The line chart in part (E) shows the percentage of alive, early and late apoptotic and necrotic peripheral nerve fibroblasts. * $p<0.001$ vs. control.

Fig 3: Caspase activity measurement in peripheral nerve fibroblasts. The amount of Caspase-3/7 activity was illustrated following treatment with 1nM of ATRA, 15μg/ml of cisplatin (positive control) and negative control (A). * $p<0.001$ vs. negative control.

Fig 1.

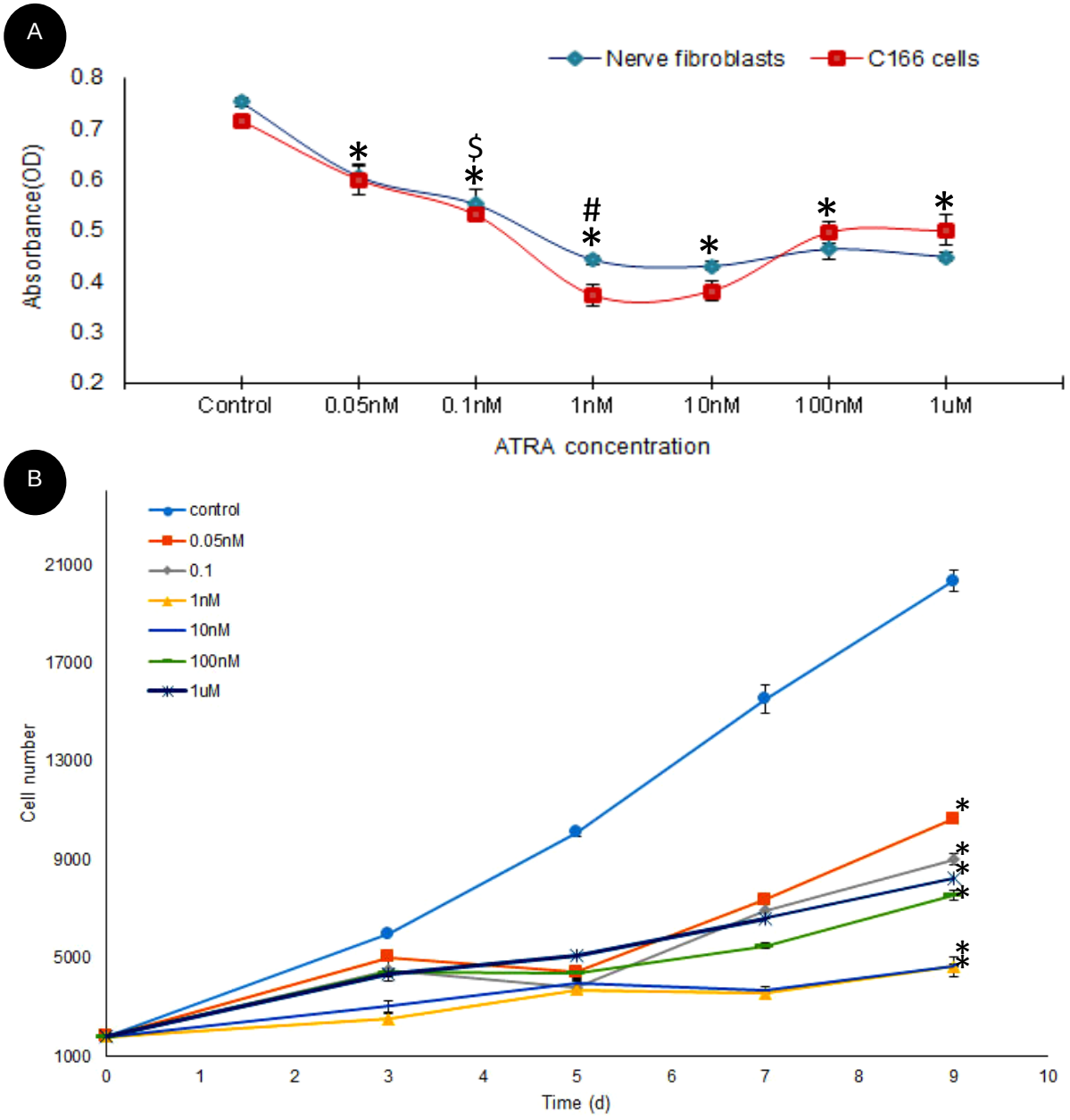
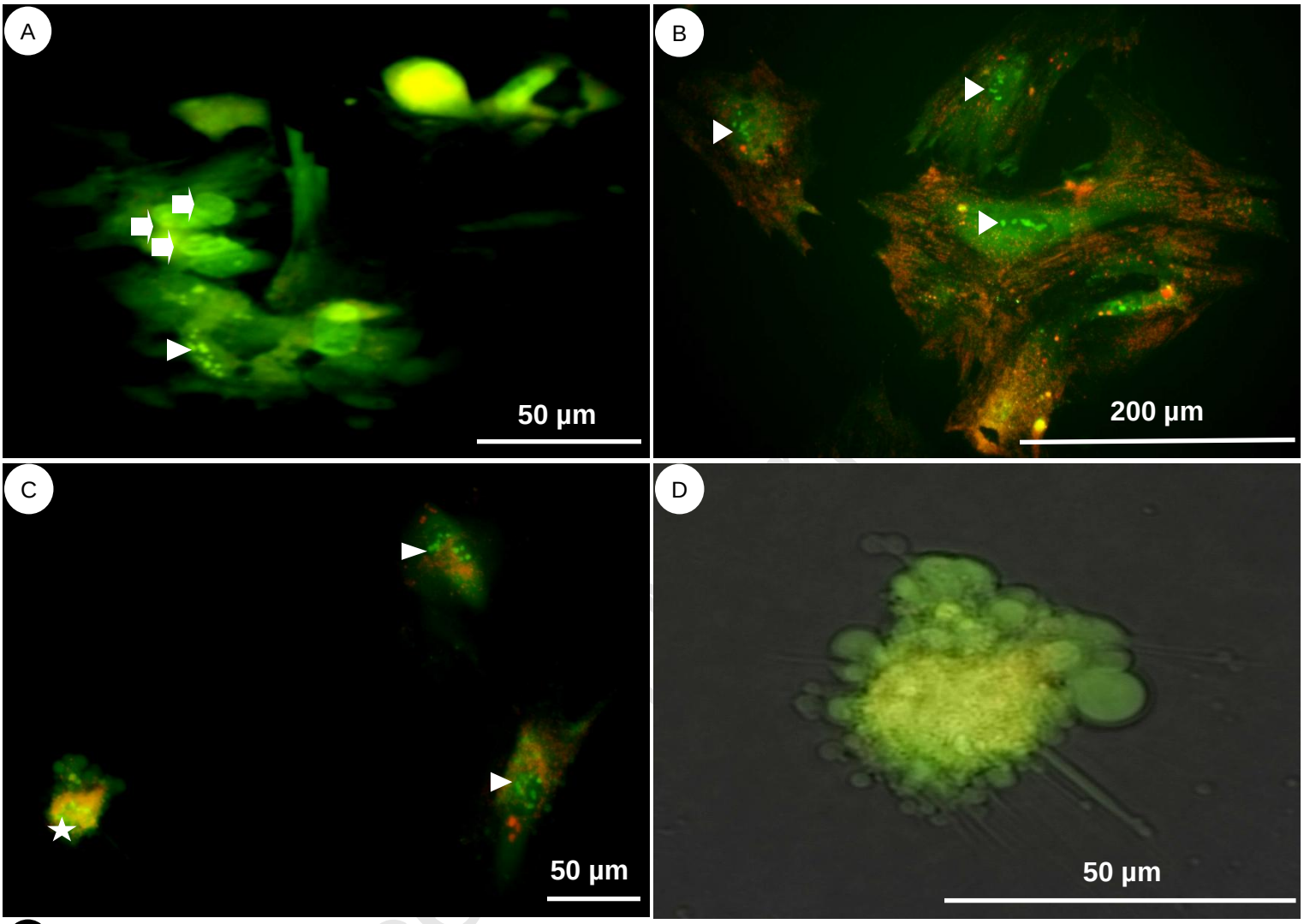


Fig 2.



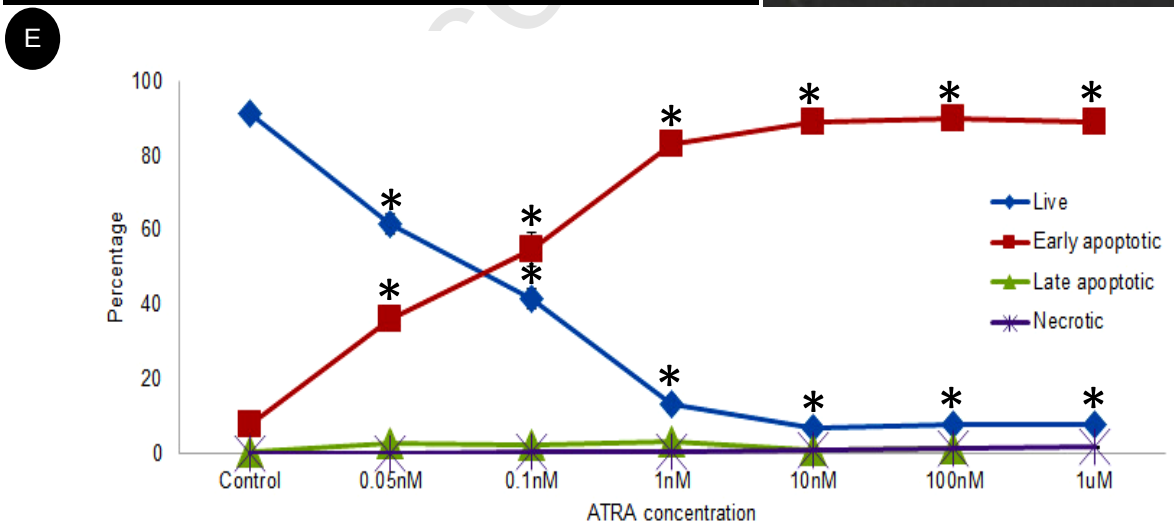


Fig 3.