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FLUORESCEIN LABELLED ANNEXIN V STAINING LIMITATIONS FOR DETERMINATION OF BLASTODERMAL CELLS APOPTOSIS

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ABSTRACT

It is known that blastoderm contains not only blastodermal cells (BCs) but also cellular "debris". These particles include yolk granules and lipid droplets. Some authors used AnnexinV (AnV) staining technique for determination of cell apoptosis. However, AnV is a protein with high affinity to lipids. So the aim of our study was to demonstrate the AnV binding in the blastoderm and yolk cell suspensions. Cell populations were obtained from fertilized chicken eggs. The blastoderms were isolated using filter paper ring method and mechanically fragmented. After that, samples were stained with AnV-FITC and analyzed by fluorescent microscopy (FM) and flow cytometry (FC). FM showed non-specific binding of AnV-FITC on the yolk and lipid particles. Therefore, the FC method using specific nuclear dye DRAQ5 was used to separate BCs from cellular debris. Only 1.82 ± 0.27 % cells were DRAQ5 positive. On the other hand, increase of total events (21 ± 3.30 %) of AnV positive cells without DRAQ5 dye was recorded. Moreover, the affinity of yolk to AnV by FC was also observed (10.45 ± 2.59 %). Significant differences among the groups ($P < 0.001$) were recorded. This trend was caused by non-specific binding of AnV to the lipid and yolk particles. So it is necessary to stain suspension with nuclear dye to separate BCs than co-stained with some marker of apoptosis. Based on the results we can summarized that AnV staining is inappropriate technique for apoptosis of BCs, however, using nuclear dye we can prevent of non-specific binding.

Keywords: egg; development; blastodermal cells; viability; AnnexinV staining; flow cytometry

INTRODUCTION

Several studies have been investigated that the type of breed, age of flock, conditions of egg storage can influence quality and viability of chicken embryo (**Silversides and Scott, 2001; Roberts, 2004; *Samli et al., 2005*; *Jin et al., 2011*; Hamidu *et al.*, 2011; ; Dymond *et al.*, 2013**). The embryo quality also depends on the viability of blastodermal cells (BCs). The BCs are cells occured at egg oviposition (X stage) (**Eyal-Giladi, Kochav, 1976**) with the ability to contribute to the germline, ectodermal, mesodermal, and endodermal lines (**Carsience *et al.*, 1993; Fraser *et al.*, 1993; Thoraval *et al.*, 1994; Kagami *et al.*, 1995; Chelmonska *et al.*, 1997;**). The number of chicken BCs varies from 20 000 to 60 000 cells per one embryo, however, except of BCs, blastoderm also contains cellular "debris" (**Watanabe *et al.*, 1992; Chelmonska *et al.*, 1997; López-Díaz *et al.*, 2016; Svoradova *et al.*, 2018**).
Apoptosis is a physiological process of cell death with characteristics such as cell shrinkage, DNA fragmentation, chromatin condensation, blebbing of plasma membrane and also apoptotic bodies formation (**Elmore, 2007**). In mammal and chicken cells, apoptosis occurs in blastocyst cavitation and continues during gastrulation (**Coucouvanis and Martin, 1995; Sanders *et al.*, 1997**), therefore it is important to assess the extent of apoptosis beyond the viability of the embryo (Voss and Strasser, 2020). Viable cells exhibit phosphatidylserine (PS) primarily within the intracellular leaflet of the membrane. Phospholipids are organized asymmetrically in cell plasma membranes with neutral phospholipids on the outer leaflet (**Bevers and Wiliamson, 2016**). During apoptosis, lipid asymmetry is lost and PS is exposed on the outer leaflet of the plasma membrane (Davis et al. 2019). Annexin V (AnV) is a recombinant phosphatidylserine-binding protein that strongly interacts with PS and can be used for detection of apoptosis. The main inductor of this process is calcium which caused annexin-mediated aggregates mechanism of AnV (**Lizarbe *et al.*, 2013**). AnV binding is one of the most commonly used assays to measure apoptosis either by fluorescent microscopy (FM) or flow cytometry (FC). AnV can be conjugated to various fluorochromes and different combinations may be required depending on the cells used. Some authors used AnV staining for BCs viability (**Chelmonska *et al.*, 1997; Ko *et al.*, 2017**). FM and FC are often used to analyse cell characterization (viability, cluster of differentiation, gene expression, intracellular markers, etc. (**Saeys *et al.*, 2016**). Typically, cell viability measurement is based on the optical analysis of cells, which can localize place of dye biding to exclude non-specific binding. On the other hand, FC provides a fast, precise and reliable alternative to determine cell characteristics (Optitz et al. 2019). FC is based on the analysis of suspended particles based on their scatter light and/or fluoresce when passing through a laser beam. Subsequently, the detectors measure fluorescence emission based on the fluorochrome wavelength. The data are displayed as histograms or two-dimensional dot plots that visualize the intensity and frequency of signals received on different parameters (**Safford, Bischel, 2019**).
Blastodermal cells are composed of yolk granules and also blastoderm can contain lipid droplets (**Svoradova *et al.*, 2018**). Whereas, AnV is phospholipid-binding protein, how to stain BCs without the non-specific binding? Till now there is no information about the complete removal lipids from blastoderm. We investigated whether AnV could stain yolk or lipid granules in the blastoderm. We hypotetized that the yolk granules and lipid droplets can caused non-specific binding.

MATERIAL AND METHODS

Animals

Freshly laid chicken eggs (n = 40) obtained from sexually mature (55 wk old) and health ROSS 308 breeder hens reared in a private breeding facility (Lihen Chropyne Vykrm Trebic., s.r.o., Czech Republic) were used in this experiment. Hens were maintained and fed according to the dietary of breeding facility. All experiments with animals were approved by the Branch Commission for Animal Welfare of the Ministry of Education, Youth and Sports of the Czech Republic.

Experimental design

The eggs were stored at 4°C for 1 day. X stage blastoderms were isolated using filter paper method as previously described (**Petitte *et al.*, 1990**) and 10 µl of yolk was obtained from each egg. The thin and thick albumen were removed from the yolk surface and the blastoderm was carefully covered with a filter paper ring.

Then, vitelline membrane was cut around the filter ring with scissors and gently washed with calcium- and magnesium-free phosphate-buffered saline (PBS, Sigma, Saint Louis, Missouri, USA) to remove yolk as much as possible. After that, blastoderm was mechanically dispersed by gently pipetting and centrifuged at 300 g for 5 min at room temperature (RT). Supernatant was discarded and cells were used for the viability assessment.

Fluorescent microscopy and flow cytometry

The detection of apoptotic BCs was performed with the specific surface fluorochrome AnnexinV-FLUOS Staining Kit (Annexin-V-Fluos staining kit, Roche Diagnostics, Germany) and for FM and FC analysis. Moreover, DRAQ5 (Biolegend, Germany) as a marker of nucleated cells was used. Briefly, the freshly isolated BCs were washed and centrifugated in PBS at 300 g for 5 min. Samples of BCs were immediately stained with AnnexinV-FLUOS Staining Kit. BCs and also yolk aliquots were resuspended in 95 µl working solution (with Ca^{2+}) contained 5 µl of AnV-Fluos and incubated for 15 min at RT in the dark.
DRAQ5 staining was performed for a detection of BCs in the cell suspension. Briefly, cells were fixed with a formaldehyde based fixation buffer (eBioscience™ IC Fixation Buffer; Thermo Fisher Scientific, USA) for 15 min at RT according the producer's manual. After wash, cells were permeabilized using 0.1 % Cell Permeabilization Buffer (Triton™ X-100, Cell Signaling Technology, Inc., USA) for 15 min on ice. After final wash, cells were stained by DRAQ5 (Biolegend, Germany) according the producer's manual.
Afterwards aliquots of the BCs and yolk suspension were stained with 4′,6-Diamidine-2′-phenylindole dihydrochloride (DAPI, Invitrogen, USA) for 15 min at RT and after wash were placed between microslide and coverslip into 5 µl of the Aqua poly/Mount medium (Polysciences Icn., Germany). Stained samples were checked under the fluorescent microscope (Olympus BX51 with PROMICAM 3-5CP digital camera (5Mpx SONY PREGIUS CMOUS USB 3.0). Remaining suspensions were analysed by flow cytometer BriCyte E6 (Mindray, China) with the minimum of 10, 000 events (FL 1, FL 3) in each sample. A sample of the cells gated from FSC-H versus SSC-H was separated according to the fluorescent staining ability of AnV and DRAQ5.

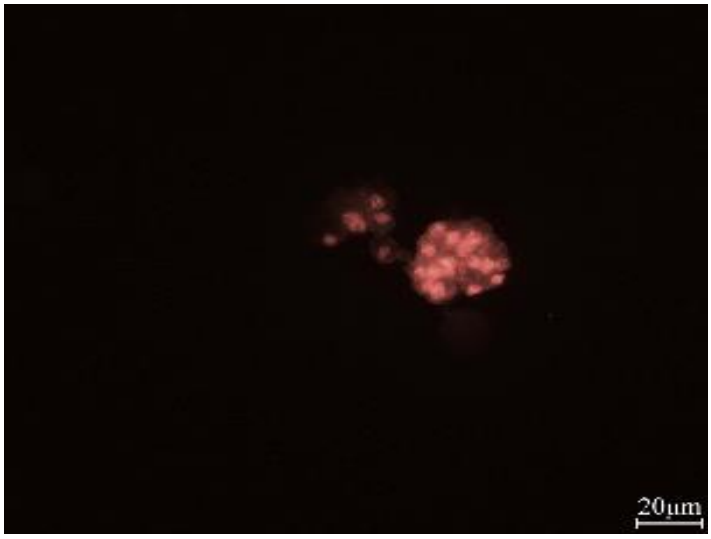
Statistical analysis

For the percentage of cell number assessment, arithmetic means and standard deviations were used. Normality distribution using Shapiro-Wilk W test was used. Statistically significant differences ($P < 0.001$) in the portions of the mentioned parameters were assessed using the paired t-test. The data were analyzed by the SigmaPlot software (Systat software Inc. Germany).

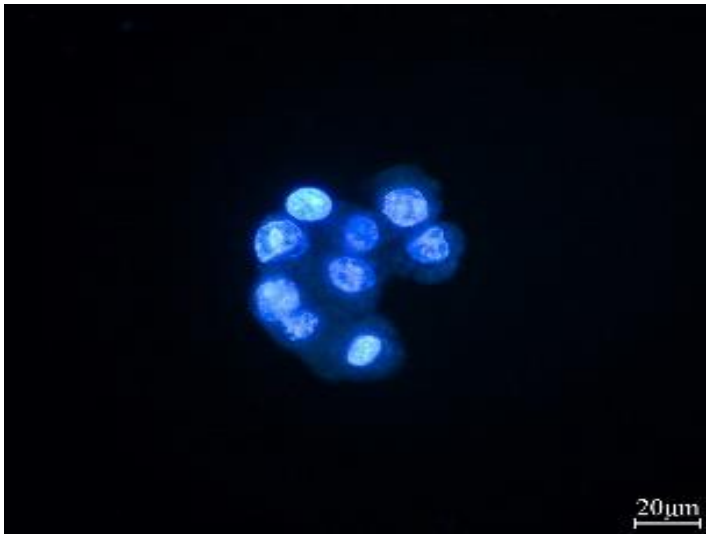
RESULTS

Fluorescent microscopy

Freshly isolated BCs were stained with nuclear dyes and analyzed by FM for localization and specifity of dyes. DRAQ5 and DAPI dyes were used for detection of BCs. Both of them stain DNA in the nucleus which allow the successful differentiation of BCs from other blastoderm particles (Figure 1).



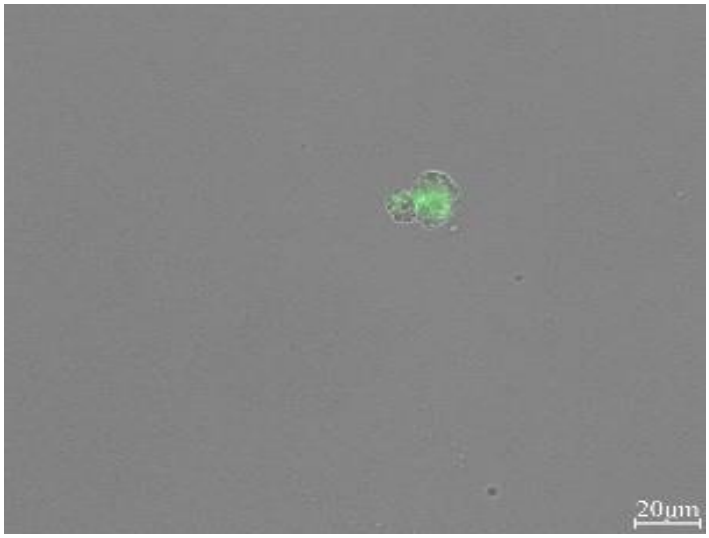
(a)



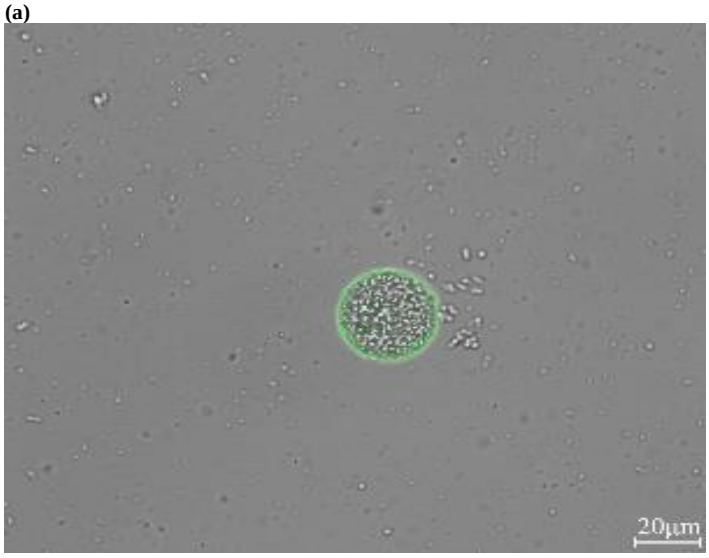
(b)

Figure 1 Representative figures of DRAQ5 binding of BCs (a), DAPI positive BCs (b).

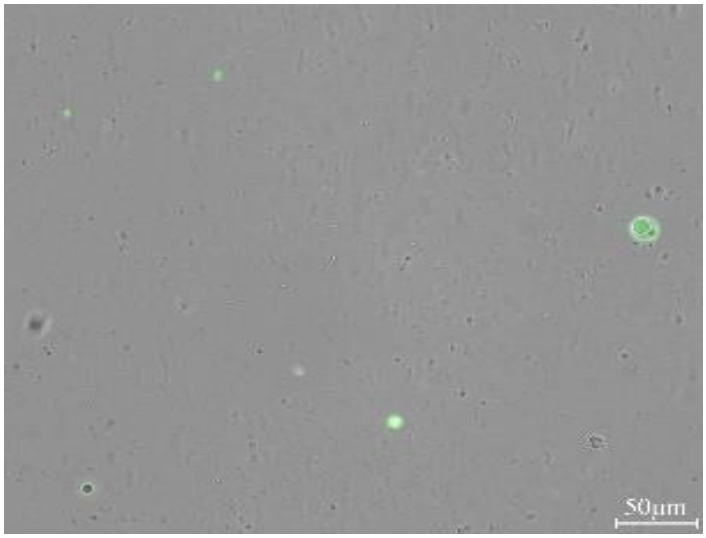
The apoptosis of BCs was analysed using AnV kit and also staining of yolk granules and lipid droplets was done. We observed that AnV stained membrane of lipid and yolk particles (a-d) and annexin-mediated aggregates (e). Moreover, damaged plasma membrane of BCs enabled binding of AnV on intracellular yolk granules in the cytoplasm (f). It was caused by interaction of AnV and yolk/lipids particles (Figure 2).



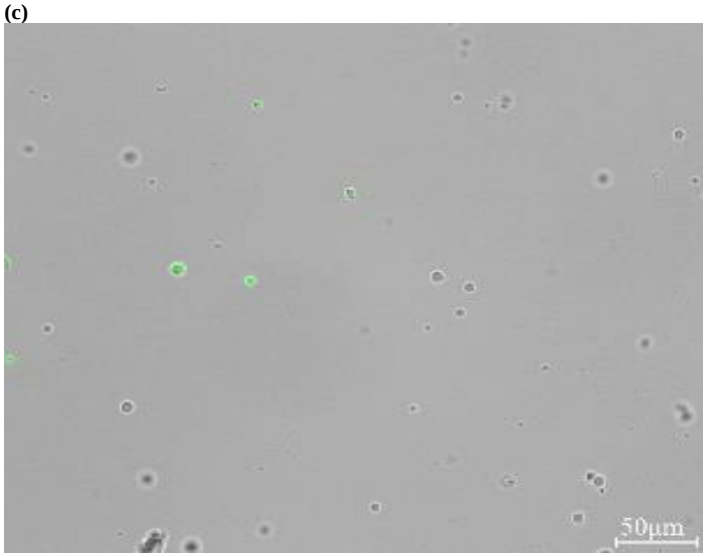
(a)



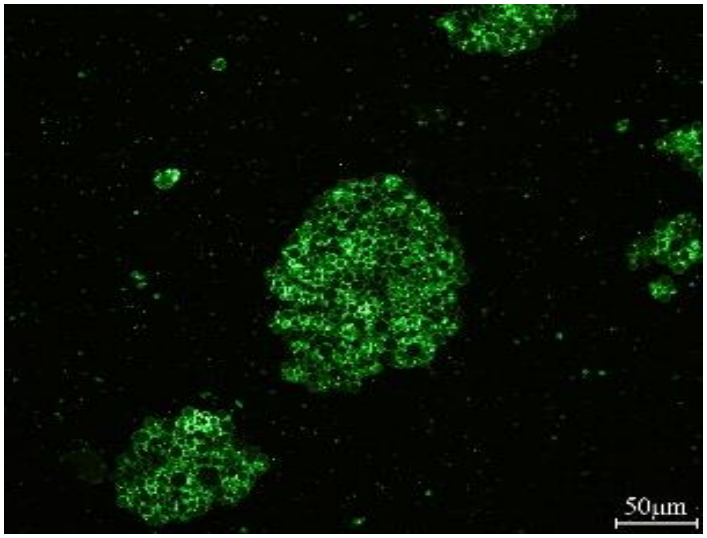
(b)



(c)



(d)

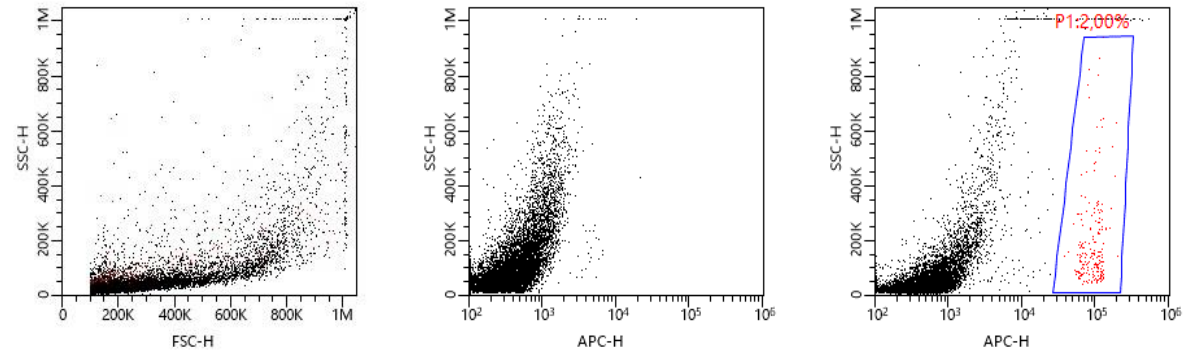


(e)

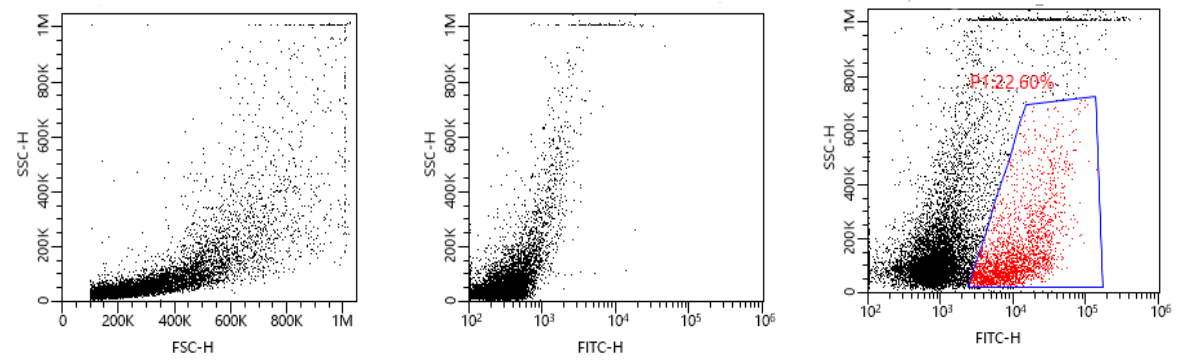
Figure 2 Representative figures of (a) AnV binding of BCs with damaged plasma membrane and (b-e) non-specific stained lipid droplets (droplets differ in size).

Flow cytometry

FC is more sensitive and precise method for cell characterization in comparison to FM. Due to blastoderm contains heterogenic cell suspension, FC was used. Flow cytometric dot plots represented fluorescent shift of DRAQ5 positive BCs (1.82 ± 0.27 %) what correlated with the number of BCs in one embryo. Number of events stained without DRAQ5 dye rapidly increased (21 ± 3.30 %). Comparison of percentage of DRAQ5 positive BCs to the AnV positive cells showed increase in the percentage of the events. The positivity for AnV of yolk granules (10.45 ± 2.59 %) was also recorded. This was caused by non-specific bind of AnV to phospholipids (Figure 3). Significant differences among the groups ($P < 0.001$) were recorded.



(a) (b) (c)



(d) (e) (f)

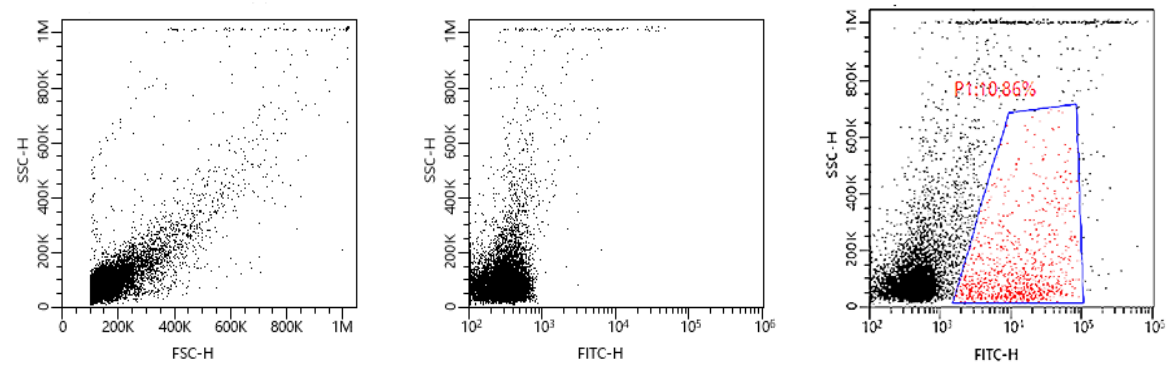


Figure 3 Flow cytometric representative dot plots of stained BCs.
(a,d,g) based dot plot FCS-H versus SCS-H, (b,e,h) non-stained controls, (c) DRAQ5 positive population of nucleated cells (f) AnV stained cell population, (i) AnV stained yolk granules. (a,b,c) are representative dot plots of fixed and permeabilized BCs number evaluation using DRAQ5. (d,e,f) are dot plots demonstrate non-fix and non-permeabilized BCs stained without DRAQ5, resulting in non-specific binding of AnV to the lipid and yolk particles, (g,h,i) are dot plots represent autofluorescence (non-specific binding) of yolk particles. APC-H is a pre-selected name of a channel used for DRAQ5 detection. FITC-H is a channel used for AnV detection.

DISCUSSION

The healthy chicken embryo (blastoderm) contains on average 20, 000 – 60, 000 embryonic cells after oviposition (**Petitte *et al*., 1990; Etches *et al*., 1996**). Therefore, it has been hypothesized that a viability of BCs is required for normal embryonic development (**Fasenko *et al*., 1992**). Many techniques have been used to determine BCs viability. The most common method is Trypan blue exclusion method. This method is based on the penetration of trypan blue dye through the damage plasma membrane of apoptotic cells and membrane of viable cells stay intact, non-stained (**Chelmonska *et al*., 1997; Svoradova *et al*., 2018**). This method is simply, fast but higher exposure of stained cells to trypan blue dye can induce apoptosis.

Previous studies have also shown that the viability of BCs can be measured using co-staining with AnV and propidium iodide (PI) (**Hamidu *et al*., 2010; Ko *et al*., 2017; Pokhrel *et al*., 2017**). In this study, we demonstrate mechanism of AnV staining in the case of BCs and lipid and yolk particles. Ultrastructure of BCs showed, that BCs contain a lot of yolk granules in the cytoplasm (**Svoradova *et al*., 2018**) and also dispersed blastoderms contain cellular "debris" (**Hamidu *et al*., 2011**). As we mentioned above, AnV is the protein that strongly interacts with phospholipids. Mechanism of AnV staining include binding of AnV to extracellular PS which is translocated from inner to outer leaflet of plasma membrane during apoptosis. The charge of the phospholipid's polar head seems to be very important point in the mechanism of binding. The interaction of the lipid head with the protein residues influences the affinity of lipid binding mechanism at the molecular level, which can explain the different binding of PS compared to phosphatidylcholine (PC) (**Lizarbe *et al*., 2013**). As we set up side scatter (SCS-H) and forwarded scatter (FCS-H) for BCs granularity and size is not relevant because yolk or lipid particles are similar in a size. BCs are cells with asymmetric nucleus, however lipid or yolk particles are non-nuclear substances. Hoechst 33342, DRAQ5 or DAPI are nuclear dyes interacting with the DNA in viable and non-viable cell nucleus (**Hamidu *et al*., 2010; Hwang *et al*., 2016; Svoradova *et al*., 2018**). Authors used AnV staining for the determination of cell apoptosis but without any nuclear dye, even though that AnV interacts with phospholipids (**Dymond *et al*., 2013**). For apoptosis or necrosis evaluation of BCs, Trypan blue or AnV-FITC, Live/Dead in co-staining with Hoechst 33342/DRAQ5/DAPI can be used (**Bloom *et al*., 1998; Hamidu *et al*., 2010; Svoradova *et al*., 2018;**). The most important is to separate nuclear (BCs) cells from cellular debris to exclude non-specific fluorescence. Moreover, several authors cannot identify or distinguish BCs from other component in blastoderm, where lipid droplets or cellular debris were considered for BCs (**Sawicka *et al*., 2011**) or not defined which cells are BCs (**Hamidu *et al*., 2010**). Study of (**Hamidu *et al*., 2010**) performed low resolution (40x or 20x objective) what is a limited factor of characterization of morphological features associated with apoptosis (**Vorobjev *et al*., 2017**).

Similarly, study of (**Pokhrel *et al*., 2017**) analyzed apoptosis of BCs using AnV in co-staining with PI and DAPI. Fluorescent figures also showed AnV binding to the lipid and yolk particles as non-specific binding as we recorded in our study by FM and FC.

Moreover, total BCs number for one embryo was measured in our study. Obtained results were in the agreement with the results of other authors where number of BCs in one embryo varied from 30, 000 – 60, 000 cells (**Petitte *et al*., 1990; Bloom *et al*., 1998; Bakst and Akuffo *et al*., 2002; Reijrink *et al*., 2010**). It is necessary to use fluorescent microscopy in the combination with the FC for BCs evaluation. FC is precise, objective and rapid method but FM can be used for the verification of specific marker binding. So researchers can exclude non-specific binding in the samples.

CONCLUSION

Finally, we can conclude that apoptosis evaluation of BCs using AnV kit has limitation. The cell-impermeant and phospholipid-binding protein, AnV, binds to the surfaced PS of apoptotic cells. It is known that blastoderm contains not only BCs but also cellular "debris" in the form of yolk granules or lipid droplets. Therefore, using AnV staining is an inappropriate because it is impossible to distinguish BCs from lipid and yolk particles by FC. Therefore, it is necessary to use some nuclear dyes such as DAPI or DRAQ5 for co-staining with other apoptotic markers (Yo-pro-1, TUNEL, caspases) to exclude non-specific binding.

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