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VIABILITY ASSESSMENT OF FRESH CHICKEN BLASTODERMAL CELLS BY FLOW CYTOMETRY

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ABSTRACT

This study describes the viability assessment of fresh chicken blastodermal cells (BCs) by flow cytometry using a novel DNA-detecting far-red-fluorescing dye, DRAQ5 and LIVE/DEAD (LD) Fixable Green Dead Cell Stain Kit. Overall, the technique provides both quantitative and qualitative evaluation of BCs. Particular attention was put on the determination of a portion of nucleated ($DRAQ5^+$) and dead ($DRAQ5^+/LD^+$) BCs from freshly isolated samples. The results showed that the proportion of nucleated cells from a sample was ranged from 50.39 to 72.58 % and dead cells from 0.40 to 11.38 % in all repetitions. Furthermore, our study suggests that flow cytometry allows precise examination of fresh BCs viability in a very short time and that a combination of DRAQ5/LD staining is suitable for the detection of nuclear BCs and their viability evaluation, respectively. Moreover, this technique might also be applied for the evaluation of frozen-thawed BCs after cryopreservation process for animal gene bank.

Key words: chicken; blastodermal cells; viability; flow cytometry

INTRODUCTION

Chick embryos are an important model for developmental biology research and transgenesis. At stage X (Eyal-Giladi and Kochav, 1976), avian embryos already contain 40-60,000 BCs, and at this time the different tissues can be established. A small group of cells (less than 100), the primordial germ cells (PGCs), are clearly committed to form the germline (Carsience *et al.,*1993; Etches *et al.*, 1997; Kagami *et al.*, 1997; Pettite *et al.*, 1990; Thoraval *et al.*, 1994). These cells will be of the interest for germplasm preservation programs based on germline technologies, where germ cells of endangered breeds and species can be cryopreserved and later propagated through germline chimeras (Petitte, 2006).

Flow cytometry technique has been extensively used to investigate the cell viability. This technique is very popular for the analysis of cell suspensions containing live, apoptotic and necrotic cells because of their higher sensitivity (Liegler *et al.*, 1992), in comparison with other methods, such as Trypan blue assay. An accurate assessment of the cell viability necessitates the maintenance of the cell integrity and viability during the isolation procedures. DRAQ5 (1.5-bis {[2-(methylamino) ethyl] amino}-4, 8-dihydroxy anthracene-9,10-dione) is a specific dye with high DNA affinity which can be used to detect nucleated cells.

To determine viability of cells, „live-dead" kits are often used to detect necrotic cells. These dyes stain both viable and dead cells, since they specifically bind

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to the proteins. However, they stain dead cells much more brightly, making them easily distinguishable during analysis (Perfetto *et al.*, 2006).

The aim of our work was to assess the viability of freshly isolated BCs of Oravka breed using DRAQ5/LD staining by flow cytometry.

MATERIAL AND METHODS

Hatching eggs obtained from sexually mature (≥ 60 week old) and health ROSS 908 breeding hens (n = 10.000) reared in a private breeding facility (Liaharenský podnik Nitra a.s., Párovské Háje, Slovak Republic) were used in this experiment. Hens were maintained under artificial conditions (14 h of light at 10 lux and 10 h of dark) and were fed with a commercial diet (KV; TEKRO Nitra, s.r.o., Slovakia), and water was given *ad libitum*. The eggs were stored at 14-16 °C and 80 % of humidity for 4 days. The experiment with BCs samples was repeated six times (from February to May 2017).

To obtain blastoderms, each egg was broken and the yolk was separated from the albumen. The blastoderm was carefully covered with a filter paper ring (Petitte *et al.*, 1990). The yolk membrane was cut around the filter paper ring and blastoderm was washed several times in a calcium-magnesium free PBS (CMF-PBS, Applichem, Darmstadt, Germany) to remove excessive yolk. After cleaning, *area pellucida* was separated from *area opaca*, mechanically dispersed and single cells were stored for further analysis.

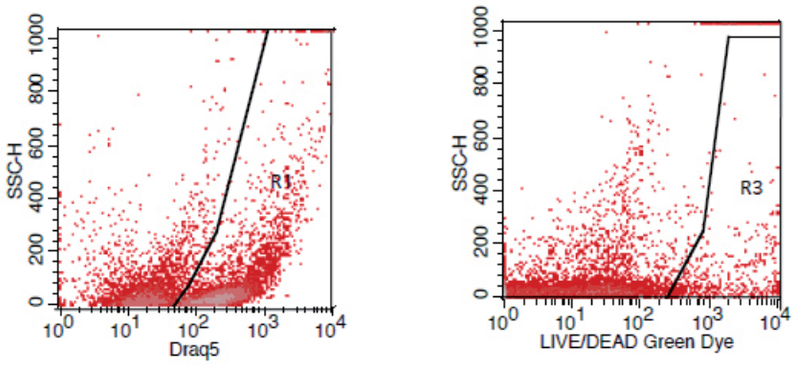

Fig. 1: Representative flow cytometry dot plots of fresh BCs. Region R1 (A) represents DRAQ5⁺ BCs (nucleated cells) and region R3 (B) represents DRAQ5⁺/LD⁺ dead (necrotic) BCs

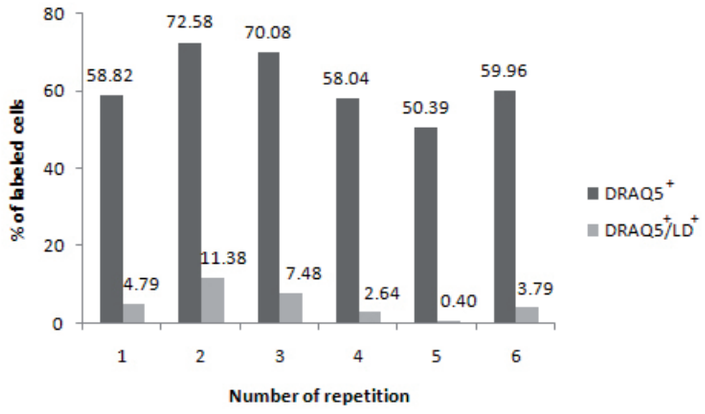

Fig. 2: The incidence of DRAQ5⁺ (nucleated) and DRAQ5⁺/LD⁺ (necrotic) BCs in cell suspension in different replications

The cell suspension was subdivided and placed into prepared tubes intended for flow cytometric assessment of nucleated and necrotic cells. Cells were stained with DRAQ5 (Biolegend, Germany) and LIVE/DEAD Fixable Green Dead Cell Stain Kit (Molecular Probes, Eugene, USA) according to the producer manual. DRAQ5 nuclear dye identifies the nucleated BCs, and the proportion of necrotic cells within the population was determined using the specific dye LIVE/DEAD Fixable Green Dead Cell Stain Kit (Figure 1). Samples were analysed by a FACS Calibur flow cytometer (BD Biosciences, San Jose, CA, USA).

RESULTS AND DISCUSSION

The different labelling patterns in the DRAQ5/LD analysis identify two different cell populations (Figure 2). The DRAQ5 nuclear dye was used for the determination of the nucleated cells in fresh samples. Using the LD dye, we identified two different cell subpopulations within nucleated cells, live ($DRAQ5^+/LD^-$) and necrotic ($DRAQ5^+/LD^+$).

Due to the high content of cellular debris in a freshly isolated cell suspension and different cell size it is very difficult to distinguish the BCs from other cells. However, Trypan blue method is widely used for detection of frozen-thawed BCs viability (Sawicka *et al.*, 2015; Chełmónska *et al.*, 1997; Thereshenko *et al.*, 1994), though it has certain limitations. The dye can be incorporated into live cells after a short exposure time, and personal reliability can affect the results (Avelar-Freitas *et al*., 2014). Thus, the viability of BCs cannot be properly discerned by the Trypan blue exclusion method. In our study, DRAQ5 dye was used to determine portion of nucleated cells and LD dye for the detection of dead BCs in freshly isolated samples. This allowed examination of the isolated cell population by a flow cytometry and characterization of cell viability through the necrosis-based pathways.

In previous study, flow cytometry protocol was also used to analyse viability of fresh chicken BCs (Hamidu *et al.*, 2010). In this study, viability of fresh BCs was evaluated basing on a propidium iodide staining and an annexin V assay.

CONSLUSION

In conclusion, we showed in this work that flow cytometry could become useful technique for evaluation of BCs quality. DRAQ5 staining allows determination of nucleated cells from the cell suspension of freshly isolated sample, and in combination with LD kit also dead cells can be detected. These findings might also be applied to the field of cryopreservation to determine the frozen-thawed cell viability.

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