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# Effects of Certain Inducers of Leukocytes Migration into the Bovine Mammary Gland on Neutrophil Apoptosis Manifestation in a Subsequent *in Vitro* Cultivation

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## Summary

The aim of the study was to elucidate the effects of induced leukocyte migration into the bovine mammary gland on the manifestations of early and late apoptotic features of neutrophils cultivated *in vitro*. The Latin square design was used in two experiments, each involving four experimental repetitions in 4 clinically healthy virgin heifers. The neutrophil early apoptotic features were detected by flow cytometric detection (FCM) of phosphatidyl-serine translocation. Late neutrophil apoptotic features were detected by ELISA quantitation of histone-complexed DNA fragments. Leukocyte influx induction was accomplished by using four inducers: i) sterile buffered saline solution (PBS); ii) 5 % glucose solution (GLU); iii) synthetic muramyl dipeptide analogue (MDP); and iv) lipopolysaccharide (LPS), administered into the mammary gland lumen. Leukocytes from mammary glands were obtained by mammary gland lumen lavages after influx induction. The total cell counts in lavages increased after treatment by all inducers in comparison to the counts before influx induction ($P<0.001$). Cell counts were higher and differed significantly by MDP and LPS ($P<0.01$) in contrast to PBS. The highest proportion of neutrophils was induced by LPS ($P<0.01$). After three-hour incubation, light microscopy examination revealed the highest manifestation of neutrophil apoptosis after induction by GLU ($P<0.05$). The lowest apoptosis manifestation, though statistically non-significant, was detected after induction by MDP and LPS. Determination of early manifestation of neutrophil apoptosis revealed the lowest manifestation of neutrophil apoptosis after induction by LPS ($P<0.01$). The results of late manifestation of neutrophil apoptosis revealed the highest proportion of apoptotic neutrophils after induction by GLU ($P<0.05$). The manifestation of secondary necrosis of apoptotic neutrophils or neutrophil lysis after 3 h of incubation was low and not significant. In conclusion, certain inducers of neutrophil migration into the lumen of bovine mammary glands (GLU and LPS in the present experiments) significantly influence the manifestation of neutrophil apoptosis during their subsequent *in vitro* incubation.

**Key words**

Heifers • Virgin mammary gland • Leukocyte influx • Inducers • Neutrophils • Apoptosis



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## Introduction

Neutrophils play an important role in antibacterial defense mechanisms. They migrate from blood to infected tissues, where they eliminate pathogens either within cells by phagocytosis or outside the cells; in both cases by activating bactericidal substances, e.g. reactive oxygen species. This is associated with more or less collateral tissue damage. In the resolution of inflammation, the accumulated neutrophils need to be safely removed (Simon 2003). Apoptosis plays an important role in eliminating neutrophils from inflamed tissues. Apoptotic neutrophils are phagocytozed by macrophages as professional scavengers. There is growing evidence that the clearance of apoptotic cells by phagocytosis can result in powerful anti-inflammatory effects. Thus this process controls the duration and intensity of the inflammatory response (Haslett 1999, Savill and Fadok 2000, Savill *et al.* 2002).

Moreover, as large numbers of neutrophils are generated every day in normal adult individuals, apoptosis of neutrophils is an important mechanism that maintains appropriate neutrophil numbers under physiological conditions (Cotter *et al.* 1990). Inadequate apoptosis and elimination of neutrophils would consequently result in tissue damage (Simon 2001). Apoptosis of neutrophils also plays an important role *ex vivo*. Culturing cells in the absence of sufficient concentrations of nutritional substances leads to spontaneous apoptosis (Payne *et al.* 1994). All these facts must be taken into account in construction of *in vitro* models for studying neutrophil function and its moderation.

Mammary glands of ruminants have been recommended as a useful source of leukocytes for *in vitro* studies (Derbyshire and Berman 1968, McDowell *et al.* 1969, Wardley *et al.* 1976, Paape *et al.* 1977). A large variety of inducers have been used to induce migration of leukocytes into the mammary gland and for subsequent *in vitro* studies: apyrogenic distilled water, buffered saline solution, glucose solution, oyster glycogen solution, *E. coli* endotoxin-lipopolysaccharide and synthetic muramyl dipeptide analogue. Although induction of leukocyte influx into the mammary gland and subsequent *in vitro* studies of neutrophil function have been broadly used, no studies of the effects of influx inducers on the manifestation of neutrophil apoptosis in subsequent *in vitro* cultivations have been made.

Therefore, and because we also planned *in vitro* studies, we decided to investigate the influence of selected inducers of neutrophil migration into the mammary gland on the manifestation of neutrophil apoptosis**,** secondary necrosis of apoptotic neutrophils or on neutrophil lysis in a subsequent *in vitro* cultivation.

## Methods

*Animals and experimental design*

Two experiments were carried out in two groups consisting of 4 clinically normal, holstein x bohemian red pied crossbred virgin heifers aged 14-15 months, 8 animals in total. The heifers were free of intramammary infections, as demonstrated by a bacteriological examination of mammary lavages collected before every experimental repetition. The heifers were housed in an experimental stanchion barn and fed a standard ration.

Each experiment was organized by the Latin square design (four animals x four repetitions with four inducers). Experimental repetitions were made at two-week intervals.

The first experiment was targeted on early features of neutrophil apoptosis manifestation. Flow cytometric detection (FCM) of phosphatidyl-serine translocation (Annexin V neutrophil positivity) and cell wall permeability (propidium iodide neutrophil positivity) were used. The second experiment was targeted on late features of neutrophil apoptosis manifestation detected by ELISA quantitation of histone-complexed DNA fragments. Additionally, total cell counts, microscopic differential cell counts, and microscopic survey of apoptotic neutrophil percentages were determined.

*Inducers of leukocyte migration*

The four inducers used were: i) sterile phosphate-buffered saline solution – PBS (0.01 M; pH 7.4; laboratory prepared) – treatment dose 20 ml/quarter; ii) sterile glucose – GLU (5 % w/v infusion) (Bieffe Medital, Italy) – treatment dose 20 ml/quarter; iii) sterile solution of synthetic muramyl dipeptide analogue – MDP (MurNAc-L-Abu-D-IsoGln) (Institute of Organic Chemistry and Biochemistry, Czech Academy of Sciences, Prague, Czech Republic) – treatment dose 500 µg in 20 ml of PBS; iv) sterile solution of lipopolysaccharide – LPS from Escherichia coli serotype 0128:B12, (Sigma, MO, USA) – treatment dose 5 µg in 20 ml of PBS.

*Induction of leukocyte migration and cell suspension collection*

Modified urethral catheters (AC53061H06, Porges, France) were inserted into the teat canal after thorough disinfection of the teat orifice with 70 % ethanol. Through the catheter, each mammary quarter was rinsed with PBS, and the lavage was collected through the same catheter to a syringe as samples for bacteriological examination. The catheter was then used for administering the respective influx inducer. The same procedure was used 18 h after the administration of inducers for collecting samples of leukocyte suspensions used for the examination.

*Cell processing and in vitro cultivation*

The lavages obtained before induction were examined bacteriologically by culture on blood agar plates (5 % washed sheep erythrocytes) and aerobic incubation at 37 °C for 24 h. No bacteria were detected in all samples collected during the experiments. Cell lavages obtained before and after influx induction were used for total cell count determination. The cell lavages were then centrifuged at 4 °C and 200 *x* g for 10 min and washed with PBS. The resulting pellets were resuspended in RPMI-1640 medium (Sigma, MO, USA) and adjusted to $10^6$ neutrophils per 1 ml. A hemocytometer was used for the cell adjustment, neutrophil count determination and the Trypan Blue dye exclusion test. Neutrophil cell counts ranged from 4.5 to 5.5 x $10^6$/ml; live (trypan blue negative) neutrophil percentages ranged from 96.7 % to 100 %.

Cell suspensions adjusted to $10^6$/ml diluted in RPMI-1640 medium were used for FCM determination of apoptotic and secondary necrotic apoptotic neutrophils. Cell suspensions diluted in RPMI-1640 medium to $10^5$/ml were used for light microscopic determination of apoptotic neutrophils, and then diluted to $10^4$/ml for determination of neutrophil apoptosis and neutrophil lysis by the ELISA.

Cell suspensions for microscopic determination of apoptotic neutrophils and for the differential leukocyte count were smeared on slides (0.5 ml/slide) and incubated at 37 °C in a 5 % $CO_2$ atmosphere for 3 h in a moist chamber. Specimens of cell suspensions of 200 µl/well for ELISA were pipetted into wells of an 8 x 12 microtiter plate (GAMA, Czech Republic). Two specimens of each cell suspension were made in all the described procedures.

*Total cell counts*

Total cell counts in lavages before and after influx induction were determined using the FOSSOMATIC 90 (A/C N. Foss Electric, Denmark). The instrument and the procedure recommended by the International Dairy Federation (IDF Standard 148A, 1995) were used.

*Microscopic determination of differential leukocyte count of apoptotic neutrophils*

Immediately after cultivation, cells adhered on slides were stained by the Pappenheim method. To view the preparations, the JENAMED 250 CF light microscope with a 100x oil-immersion plan achromate objective (Carl Zeis, Germany) was used. Differential cell counts were determined by evaluating at least 200 cells. Morphological features used for mammary gland neutrophil apoptosis determination have been described elsewhere (Sládek and Ryšánek 2000, 2001).

*Flow cytometric (FCM) assessment of apoptosis and secondary necrosis of apoptotic neutrophils*

Apoptotic and secondary necrotic apoptotic neutrophils were enumerated by FCM after simultaneous staining with Annexin V labeled with fluorescein isothiocyanate (FITC) and propidium iodide (PI) as described by Vermes *et al.* (1995). The commercial Annexin-V-FLUOS Staining Kit (Boehringer Mannheim, Germany) was used according to the manufacturer's instructions. After staining the suspension was analyzed using FCM (FACS Calibur apparatus, Becton Dickinson, Mountain View, CA, USA) by recognition of at least 10 000 cells. Dot plots were evaluated qualitatively and quantitatively using the WinMDI 2.8 software (Trotter 2000).

*ELISA quantitation of histone-complexed DNA fragments*

In order to detect neutrophil apoptosis and neutrophil lysis after *in vitro* incubation, the histone-associated DNA fragments were examined in the cell lysate (neutrophil apoptotic material) and in cultivation media (neutrophil lysis). The Cell Death Detection ELISA Plus kit (Roche Diagnostics, Mannheim, Germany) was used. This assay was based on the quantitative sandwich enzyme immunoassay principle, with mouse monoclonal antibodies directed against DNA and histones. The manufacturer's laboratory protocol was used, with the exception of the calculation of the "enrichment factor," which was replaced by the

"apoptosis and cell lysis absorbance rate" introduced by the authors. The absorbances of the samples and the background at 405 nm were measured using the Sunrise Photometer (Tecan, Austria). To calculate the apoptosis and cell lysis absorbance rate, the sum of apoptotic and cell lysis absorbance values were considered as 100 %. The proportion of apoptotic neutrophils and lysed neutrophils was then expressed as a percentage of that total, and multiplied by the sum of the original absorbance values.

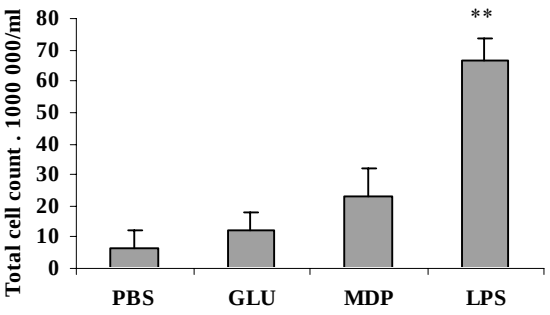


**Fig. 1. Total cell counts following leukocyte influx.** Data are means ± S.D. PBS – buffered saline solution, GLU – 5 % glucose solution, MDP – synthetic muramyl dipeptide analogue, LPS – lipopolysaccharide. Significant differences were observed following influx induction by MDP and LPS compared to cell count after PBS induction **P<0.01.

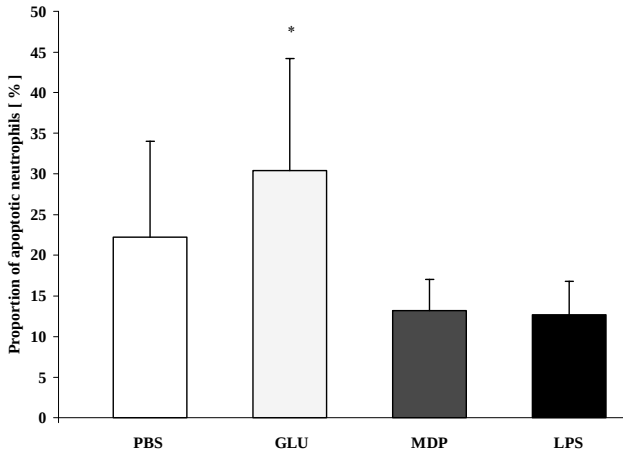


**Fig. 2. Percentages of apoptotic neutrophils after a 3-hour incubation *in vitro* following leukocyte influx induction.** Light microscopy, percentages of neutrophils count; data are means ± S.D. PBS – buffered saline solution, GLU – 5 % glucose solution, MDP – synthetic muramyl dipeptide analogue, LPS – lipopolysaccharide. The higher value of apoptotic neutrophil percentage after GLU induced influx was statistically significant. *P<0.05 compared to PBS induction.

*Statistical methods*

The results were processed by multifactorial analysis of variance. The significance of differences between arithmetic means of single influx inductors was tested by the Tukey method. Statistical analyses were carried out using the STAT plus software (Matoušková et al. 1992).

## Results

All the inducers selected for this study caused an influx of leukocytes into the mammary gland lumen. The rise of cell counts compared with cell counts before influx induction was highly significant (P<0.001). The cell counts following influx induction by MDP and LPS differed significantly as compared to the cell counts following PBS induction (P<0.01) (Fig. 1). The highest proportions of neutrophils were found in the differential count when the influx was induced by means of LPS. The difference compared with PBS was highly significant (P<0.01). Neutrophil percentages following the glucose-induced influx were the lowest and not significant. On the other hand, the macrophage proportion showed a completely opposite trend. The lowest macrophage proportion was observed in the LPS-induced influx. The difference was highly significant (P<0.01) compared to the percentages following glucose-induced influx. The proportions of lymphocytes were low and were not significantly different.

Light microscopic examination of neutrophils after 3 h incubation revealed the highest manifestation of proportion of apoptotic neutrophils following influx induction by glucose (Fig. 2). The difference compared with PBS was significant (P<0.05). The lowest apoptosis manifestation was detected following influx induction by MDP and LPS (not significant).

Determination of early manifestation of neutrophil apoptosis by FCM revealed the lowest manifestation of neutrophil apoptosis by LPS (P<0.01) (Fig. 3). The manifestation of secondary necrosis of apoptotic neutrophils after 3 h of incubation was low and non-significant. This described pattern is shown as dot-plot analyses in Figure 4. The proportions (arithmetic mean ± S.D.) of apoptotic neutrophils were as follows: PBS 22.2 ± 11.8 %, GLU 30.4 ± 13.8 %, MDP 13.2 ± 3.8 %, LPS 12.7 ± 4.1 %.

The results of late manifestation of neutrophil apoptotosis and lysis by the ELISA revealed the highest manifestation of apoptotic neutrophils after induction by

glucose (P<0.05) (Fig. 5). The manifestation of neutrophil lysis after 3 h of incubation was low and not significant.

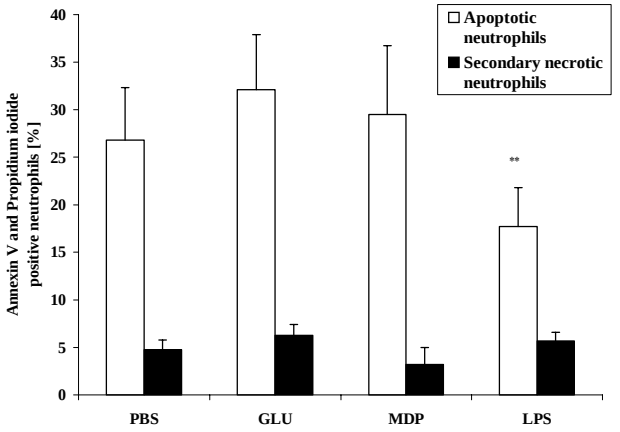


**Fig. 3. Early manifestation of apoptotic and secondary necrotic neutrophils after a 3-hour incubation *in vitro* following leukocyte influx induction.** FCM assay Annexin V positivity; percentages of neutrophils count; data are means ± S.D. PBS – buffered saline solution, GLU – 5 % glucose solution, MDP – synthetic muramyl dipeptide analogue, LPS – lipopolysaccharide. The lower value after LPS induced influx was highly significant. **P<0.01 compared to PBS induction.

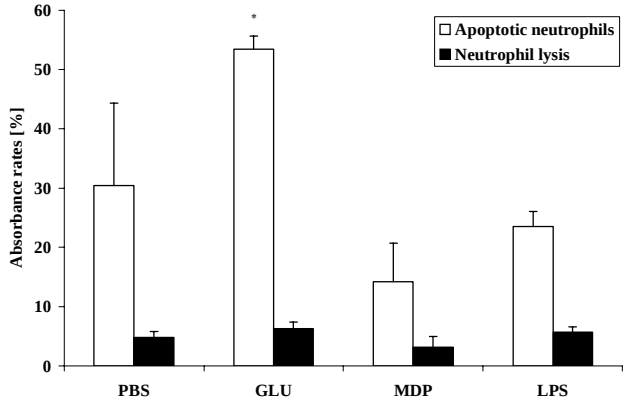


**Fig. 5. Late manifestation of neutrophil apoptosis and lysis after a 3-hour incubation *in vitro* following leukocyte influx induction. ELISA quantitation of histone-complexed DNA fragments**. Percentages of apoptosis and cell lysis absorbance rate; data are means ± S.D. PBS buffered saline solution, GLU 5 % glucose solution, MDP synthetic muramyl dipeptide analogue, LPS lipopolysaccharide. The higher value after glucose-induced influx was statistically significant. *P<0.05 compared to PBS induction.

## Discussion

As stated earlier, the experimental induction of leukocyte migration into the mammary gland lumen has been used as a source of leukocytes for *in vitro* studies. The authors had also used experimental influx induction for similar purposes in their previous studies (Ryšánek *et al.* 2001, Sládek *et al.* 2002), but according to their knowledge the effects of influx induction on the manifestations of neutrophil apoptosis and secondary necrosis of apoptotic neutrophils or on neutrophil lysis in subsequent *in vitro* incubations have never been studied.

A new finding, reported in the present study, was the observed difference in proportions of apoptotic neutrophils following influx induction by different inducers after a brief duration of incubation. The lowest proportions of apoptosis were observed after the LPS-induced influx. This also confirms the findings of Watson *et al.* (1997), who reported that neutrophils isolated from rat lungs, following a challenge by LPS, significantly delayed their spontaneous and accelerated apoptosis (by UV irradiation), and that LPS delayed apoptotic manifestations in neutrophil migration *in vitro.* Similar results were also obtained by other authors (Colotta 1992, Lee *et al.* 1993, Whyte *et al.* 1993, Yamamoto *et al.* 1993, Sweeney *et al.* 1997, Tennenberg *et al.* 1999).

It is interesting to note that the highest proportion of apoptotic neutrophils was observed after the glucose-induced influx. This is in agreement with findings that neutrophils of human diabetic patients undergo spontaneous apoptosis, but do not demonstrate LPS-induced inhibition of apoptosis, and that the LPS-induced inhibition of apoptosis in normal neutrophils is prevented under the conditions of high glucose concentration. This would cause decreased functional longevity of neutrophils and increased neutrophil clearance from infectious sites, possibly contributing to the increased susceptibility and severity of infections in diabetic patients (Tennenberg *et al.* 1999). It was also observed that neutrophil apoptosis is accelerated by a hypertonic glucose-containing peritoneal dialysis solution (Catalan *et al.* 2001). This is in contrast with the finding that spontaneous apoptosis of human neutrophils was reduced by increasing the extracellular concentration of glucose in *in vitro* experiments (Healy *et al.* 2002). It is feasible to assume that the protective effect of glucose against neutrophil apoptosis appears to correlate with glucose utilization and that glucose metabolism is essential for the protective effect against apoptosis (Healy *et al.* 2002). It may therefore be presumed that after influx induction by glucose, its protective effect against apoptosis *in vitro* is not operative.

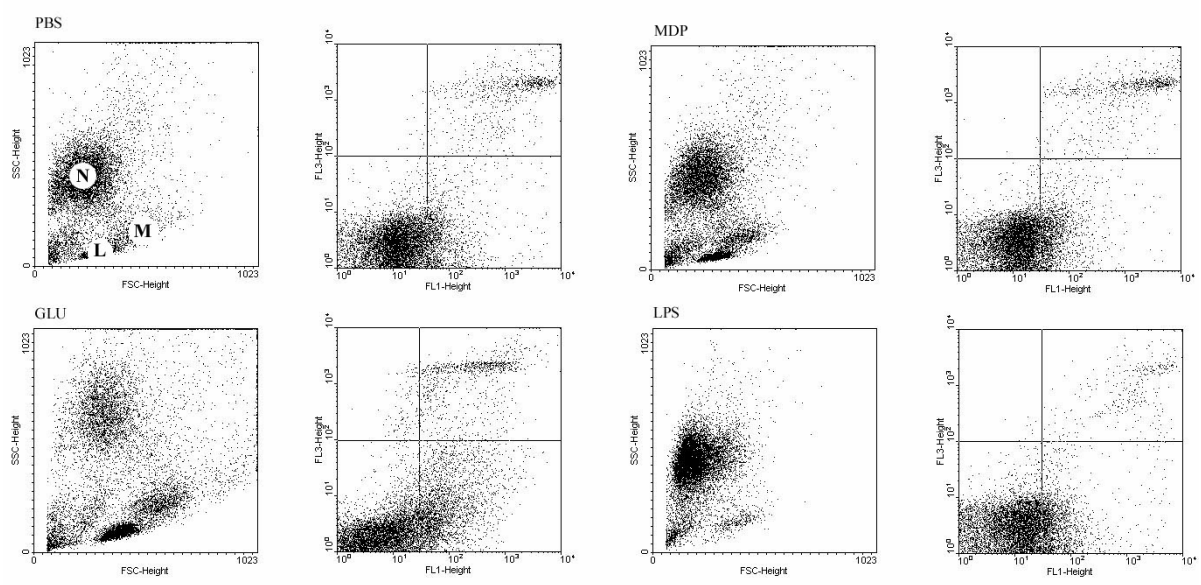


**Fig. 4. Flow cytometry analyses of viable, apoptotic and secondary necrotic neutrophils after a 3-hour incubation following leukocyte influx induction into mammary gland.**
Left column represents dot plots of leukocytes gated for scatter parameters. Clusters of neutrophils N, macrophages M, and lymphocytes L are shown. Right column represents dot plots of neutrophils only. Lower-left quadrant shows viable neutrophils Annexin V - / Propidium iodide -; lower-right, apoptotic neutrophils Annexin V + / Propidium iodide -; and upper-right, secondary necrotic neutrophils Annexin V + / Propidium iodide +. PBS buffered saline solution, GLU 5 % glucose solution, MDP synthetic muramyl dipeptide analogue, LPS lipopolysaccharide. The proportions arithmetic mean ± SD of apoptotic neutrophils were, respectively: PBS 22.2 ± 11.8 %, GLU 30.4 ± 13.8 %, MDP 13.2 ± 3.8 %, LPS 12.7 ± 4.1 %.

The proportion of apoptotic neutrophils was higher after the incubation of migrated cells as it has been elucidated in human neutrophils (Payne *et al.* 1994) and bovine neutrophils (Van Oostveldt *et al.* 2002). The higher proportion of apoptotic neutrophils was observed after incubation of cells particularly after glucose-induced influx in this study. Compared with incubation after PBS and glucose induction, incubation after MDP or LPS induction demonstrated that the proportion of apoptotic neutrophils was lower. It indicates that MDP and LPS influx inducers of bacterial origin continue to inhibit the process of apoptosis even later during incubation. This is in agreement with the report of a delayed apoptosis during an *in vitro* migration of human neutrophils through the endothelial monolayer which was observed only in cells that had previously been exposed to the effects of LPS (Colotta *et al.* 1992, Watson *et al.* 1997). The effect of MDP on neutrophil apoptosis *in vitro* has not yet been studied.

The delay of neutrophil apoptosis, after the MDP and LPS induced influx, had already been revealed during the detection of early signs of neutrophil apoptosis (Annexin V positivity). In contrast, the higher proportion of apoptotic neutrophils after the glucose-induced influx was detected by means of methods that reveal late signs of neutrophil apoptosis (apoptosis morphological signs and DNA fragmentation).

The present study allows the conclusion that inducers of leukocyte migration into the mammary gland, especially glucose and lipopoly-saccharide, affect the manifestation of neutrophil apoptosis in subsequent *in vitro* cultivations. These findings are important for a rational choice of the influx inducer for the harvesting of cells used in an *in vitro* study and for interpretation of the obtained results.

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