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The dynamics of morphological changes during *in vitro* aging of bovine virgin mammary gland neutrophils

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Abstract: The present study was an *in vitro* analysis of the dynamics of bovine mammary gland neutrophil apoptosis based on the detection of morphological changes. The neutrophils were isolated from mammary glands of five virgin heifers. The mammary glands were lavaged, the suspensions were then bacteriologically examined, and total and differential cell counts were made. The cells were cultivated *in vitro* for 4 hours. After 2, 3 and 4 hours of cultivation, they were panoptically stained, and the proportions of apoptotic neutrophils and trypan blue positive neutrophils were determined. Neutrophil apoptosis and impaired cytoplasmic membrane integrity of neutrophils were already observed in the mammary gland lavages (11.9% and 0.8%, respectively). During the cultivation, a progressive increase in the number of apoptotic neutrophils in various stages of apoptosis – karyopyknosis, zeiosis and apoptotic bodies – was observed. Karyopyknotic neutrophils represented a dominant part of the apoptotic neutrophil population in the course of the whole cultivation. The most intensive increase was observed in zeiosis, whereas the levels of apoptotic bodies remained the same. After 4 hours of cultivation, 31.7% apoptotic neutrophils and 9.8% trypan blue positive neutrophils (i.e. Secondary necrotic cells) were found. The results of this work show that spontaneous apoptosis and secondary neutrophil necrosis must be taken into account during *in vitro* cultivations of bovine mammary gland neutrophils.

Keywords: neutrophil; apoptosis; aging; bovine virgin mammary gland

INTRODUCTION:

Neutrophils form the first line of immunological defence against bacteria invading the bovine mammary gland (Sordillo *et al*., 1997). They are produced in the bone marrow, enter the peripheral blood and migrate through the walls of capillaries into the lumen of the mammary glands, where they phagocytize invading bacteria (Paape *et al*., 1991).

Neutrophils are postmitotic, terminally differentiated elements with a limited lifespan of 1–2 days (Paape and Wergin, 1977). Neutrophils cannot return from tissues to blood, and eventually meet their fate in the mammary glands. There they rapidly die by apoptosis; apoptotic neutrophils are recognised and phagocytized by macrophages (Sládek and Ryšánek, 2000a,b).

Apoptosis of neutrophils is a well-regulated process that in general is accompanied by characteristic phenomena: cell shrinkage, chromatin condensation (karyopyknosis), DNA fragmentation, membrane blebbing (zeiosis), and finallythe fragmentation into apoptotic bodies (Squier*et al*., 1995).

Apoptosis of neutrophils, characterised by specific morphological and biochemical properties, is accompanied by the loss of a number of fundamental functions. The most important of these include a reduced capability to quickly change shape, a reduced capability of random migration and chemotaxis, a reduced capability to respond to stimulation, a reduced intensity of phagocytosis (ranging to a total inability to phagocytize),

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and a lowered degree of degranulation and respiratory burst (Haslett *et al*., 1991; Whyte *et al*., 1993; Squier *et al*., 1995; Narayanan *et al*., 1997; Tanji-Matsuba *et al*., 1998; Yagi *et al*., 2002; Van Oostveldt *et al*., 2002b).

In vitro neutrophil cultivations will therefore be accompanied by an incidence of partially or fully non-functional cells with typical morphological alterations representing various stages of apoptosis (Payne *et al*., 1994). In this respect, the length of cultivation time will certainly be a very important factor, because a direct relationship has been demonstrated between the neutrophil cultivation time and the expression of apoptosis (Payne *et al*., 1994).

Short cultivations lasting up to an hour maximum are usually used in *in vitro* studies of functional aspects of bovine mammary gland neutrophils (Smith and Romel, 1977; Ryšánek *et al*., 2001). Some techniques, however, (e.g. in interaction studies of neutrophils and prokaryotae or antibiotics) require cultivation periods of 2 to 8 hours, sometimes even as long as 24 hours (Sanchez *et al*., 1988; Stevens and Czuprynski, 1996; Yang *et al*., 1998; Van Oostveldt *et al*., 1999).

Thus far, no reports are available about senescence of bovine mammary gland neutrophils during their *in vitro* cultivation. It is therefore not possible to get a realistic idea of the dynamics of apoptosis; nor of the potential impact of the dynamics of apoptosis on the proportion of non-functional cells in a population of cultivated neutrophils.

For this reason, the aim of the study was to analyse the dynamics of *in vitro* neutrophil apoptosis of the bovine virgin mammary gland based on the detection of morphological changes of neutrophils.

MATERIAL AND METHODS

Animals and experimental design

The experiments were carried out in five clinically healthy Holstein × Czech Red Pied crossbred heifers aged 14 to 16 months. All heifers were free of intramammary infections, as demonstrated by a bacteriological examination of cell suspensions obtained by mammary lavage. The virgin heifers were used as mammary gland cell donors for *in vitro* studies of senescence. Cell suspension was incubated *in vitro* to induce senescence of neutrophils. Their morphological features were detected by light

microscopy after incubation. Fresh mammary gland neutrophils were used as a control.

Neutrophil isolation and processing

The neutrophils from mammary glands were isolated using the procedure of induced influx as described elsewhere (Wardley *et al*., 1976; Sanchez *et al*., 1988; Sládek and Ryšánek, 2000a).

Briefly, modified urethral catheters (AC5306CH06,Porges S.A., France) were inserted into teat canals after a thorough disinfection of the teat orifice with 70% ethanol. Through the catheter, each mam- mary quarter was injected with 20 ml of phosphatebuffer solution (PBS), pH 7.4, and lavages were immediately collected in the syringe, through thesame catheter, to remove resident cell populations.The lavage was followed by administration of 20 ml PBS. Samples of cell populations were obtained by mammary lavage 24 h after treatment.

Bacteriological examination of all the lavages, by culture on blood agar plates (5% washed sheep erythrocytes) and aerobic incubation at 37°C for 24 h, yielded invariably negative results. No bacteria were detected in any of the tested mammary lavages.

Total mammary cell counts were determined using a haemocytometer. After the induced influx by PBS, total cell counts averaged 52.6×10^6 cells/ml. On average there were 82.6% neutrophils in cell suspension. Trypan blue dye exclusion test demonstrated their 98.5% viability. The cell suspensions were centrifuged at 4°C and 200 × g for 10 min. One millilitre of supernatant was retained for resuspension of the pellet.

Cultivation of neutrophils

The fresh mammary gland neutrophils were adjusted (5×10^6 cells/ml) and resuspended in a cultivation mixture [RPMI 1640 medium and 10% fetal bovine serum (Sigma Chemical CO, Prague, Czech Republic)]. For light microscopy analysis, adjusted suspensions were spread on slides (0.5 ml/slide) and placed in a moist chamber. Two specimens of each cell suspension were made. Slides were incubated at 37°C in a 5% CO_2 atmosphere for up to 4 h in accordance with Savill *et al*. (1989). At the time points described in the text, the cells were assessed by trypan blue exclusion test and stained with May-Grünwald Giemsa stain.

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Light microscopy assessment of neutrophil senescence

The viability of cultured neutrophils was assessed by trypan blue dye exclusion test by enumeration of at least 200 neutrophils. In oil immersion light microscopy (Olympus BH2, Olympus Optical Co., LTD, Tokyo, Japan), the dynamics of neutrophil senescence were assessed by enumeration of at least 200 apoptotic neutrophils in three structurally different stages: karyopyknosis, zeiosis and fragmentation into apoptotic bodies in accordance with described morphological features (Sládek and Ryšánek, 2000a).

Statistics

The proportions of apoptotic neutrophils and trypan blue positive neutrophils are presented as statistical means and standard deviations of the mammary glands of the five heifers examined. Significant differences in the proportion of mammary neutrophils in each stage of apoptosis during cultivation, compared to fresh neutrophils as a control, were determined by a paired *t*-test. The data were processed by STAT Plus software (Matoušková *et al*., 1992).

RESULTS

Identification of three distinct populations of neutrophils

Light microscopy analysis revealed three distinct populations in suspensions of freshly isolated neutrophils, and in suspensions of neutrophils aged for up to 4 hours *in vitro*: structurally normal neutrophils (Figure 1a), apoptotic neutrophils (Figu-

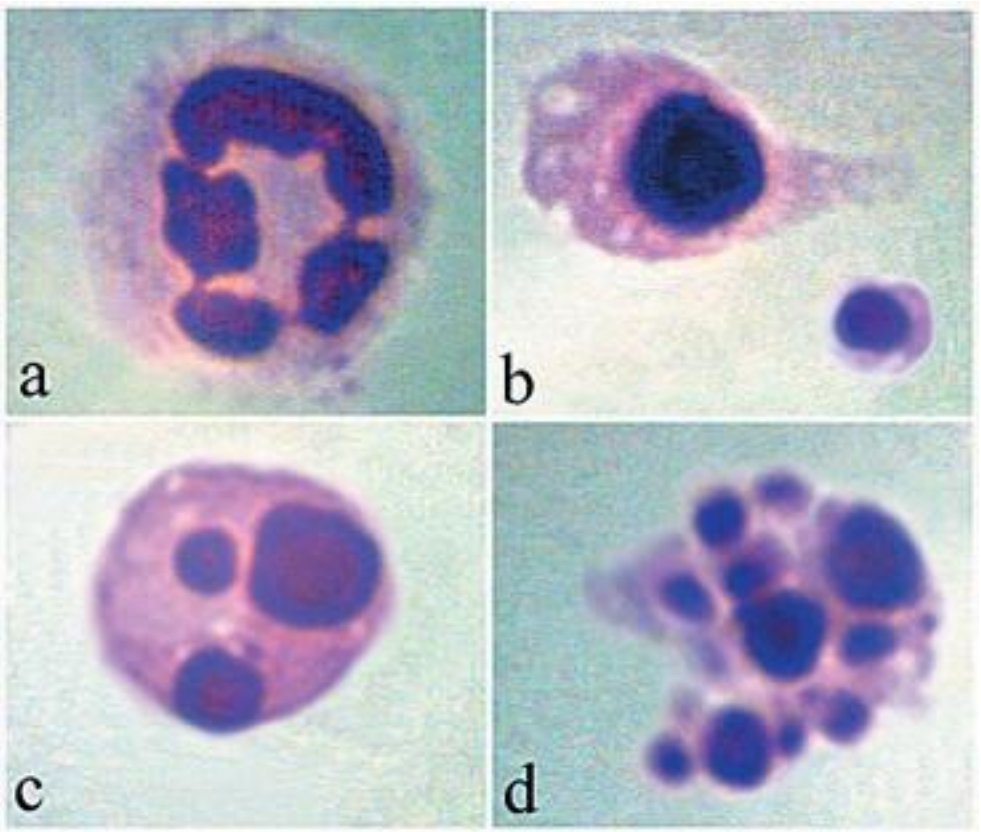

Figure 1. Light microscopy of neutrophils cultivated *in vitro* for 4 hours at 37°C. Picture 1a shows a structurally normal neutrophil with lobular polymorphous nucleus, pink cytoplasm, and pseudopodia. Also seen are karyopyknotic neutrophils with smooth surfaces, spherical nuclei containing densely condensed chromatin, intracytoplasmic vacuoles (1b) and fragmented nuclei (1c). Neutrophils with surface protuberances and fragmented nuclei were typical for zeiosis stage (1d). A small fragmented apoptotic body is shown in picture 1b (lower right). Magnification 1 000×

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res 1b–d) and necrotic neutrophils. Sorting these populations and evaluation by light microscopy revealed distinct morphological features.

Apoptotic neutrophils included three morphologically distinct stages: karyopyknosis, zeiosis and apoptotic bodies.

In the karyopyknosis stage, small spherical apoptotic cells formed, which displayed smooth surfaces, spherical nuclei containing densely condensed chromatin, and coalescence of nuclear lobes into a single body. Karyopyknotic cells from the population of neutrophils cultured *in vitro* were characterised by a presence of intracytoplasmic vacuoles (Figure 1b), which were absent in the karyopyknotic cells of freshly obtained neutrophils from lavages. Karyopyknotic nuclei were fragmented into small spherical particles as the initial phase of the next stage (Figure 1c).

Neutrophils with protuberances on the surface were typical for the zeiosis stage (Figure 1d). Finally, apoptotic neutrophils in the form of small apoptotic bodies about 3 µm long were distinguished (Figure 1b lower right).

Necrotic neutrophils were the trypan blue positive cells and were characterised by ruptures of their plasma and nuclear membranes. They represented primary necrotic cells in suspensions of freshly isolated neutrophils, and secondary necrotic cells in suspensions of neutrophils aged for up to 4 hours *in vitro*.

Dynamics of neutrophils senescence with cultured

Suspensions of freshly isolated neutrophils contained populations of apoptotic cells (11.9%) and necrotic cells (0.8%). The apoptotic cell population consisted of 9.3% karyopyknotic cells, 1.7% cells in the zeiosis stage and 0.9% cells with apoptotic bodies.

After 2 hours of cultivation, the proportions of all three neutrophil apoptosis stages increased. The highest relative growth was observed in the zeiosis stage, where the proportion almost doubled, increasing from 1.7% to 6.9% after 4 hours. The same trend in growing proportions was observed in the karyopyknosis stage during the whole cultivation, where 22.9% of karyopyknotic cells were recorded after 4 hours. Conversely, the number of apoptotic bodies increased only slightly, to 1.7%, after 4 hours of cultivation.

The dynamics of neutrophil apoptosis during *in vitro* cultivations expressed by the proportion of individual apoptosis stages is given in Figure 2.

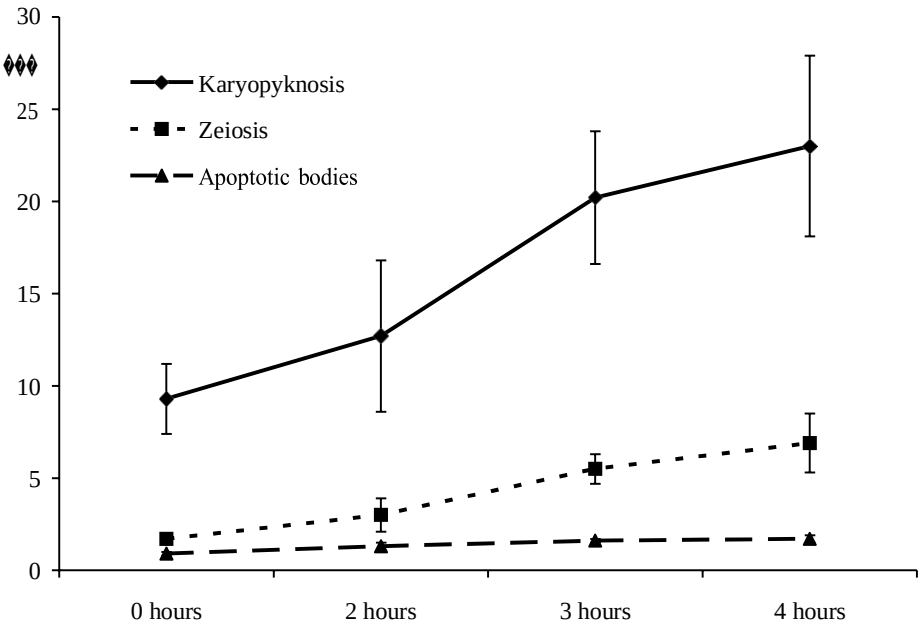

Figure 2. Percentages of neutrophils in individual stages of apoptosis (karyopyknosis, zeiosis and apoptotic bodies) during cultivation *in vitro* at 37°C. Significant differences between control (0 hours) and incubation (2, 3 and 4 hours) are marked with asterisks

($P < 0.05$)

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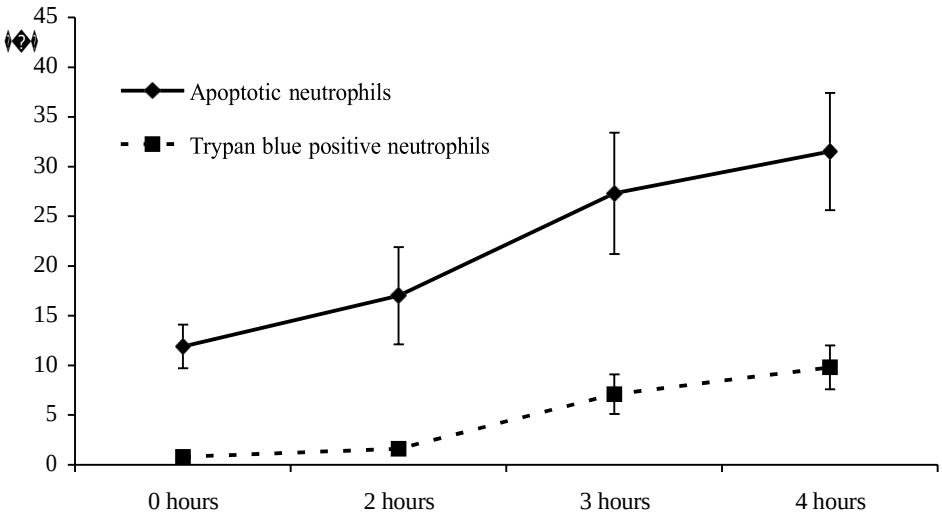

Figure 3. Percentages of apoptotic neutrophils and trypan blue positive neutrophils in control and during cultivation *in vitro* at 37°C. Significant differences between control (0 hours) and incubation (2, 3 and 4 hours) are marked with asterisks

($P < 0.05$)

In addition to apoptosis, a tenfold increase in the number of neutrophils with an impaired integrity of their cytoplasmic membranes was observed during cultivation (Figure 3). After 4 hours, 9.8% of trypan blue positive cells were found compared with 0.9% in the freshly isolated neutrophils.

DISCUSSION

The production and the destruction of neutrophils are tightly regulated processes that help maintain a constant number of these cells in blood and body tissues. Neutrophils migrate into the mammary gland from blood and, because they have a very short lifespan, undergo apoptosisthere (Sládek and Ryšánek, 2000a,b). This is the reason why apoptotic neutrophils were found in this study when an induced cell influx, by the intramammary application of PBS, was performed, or when muramyl dipetide (MDP) (Sládek and Ryšánek, 2000a,b) and lipopolysaccharide (LPS) (Sládek and Ryšánek, 2001) were used in previous studies. The induced cell influx in this study (PBS) produced about 10% apoptotic neutrophils in the lavage, which is much less than the numbers found in lactating mammary glands of dairy cows. Van Oostveldt *et al.* (2001) reported more than 40%

apoptotic neutrophils in milk during early lactation. This difference, however, is understandable because once neutrophils are in the lumen of the alveoli, the ingestion of fat and casein causes a loss in phagocytotic and bactericidal functions and leads to the death of neutrophils (Paape *et al*., 1975).

Although the lifespan of neutrophils ranges from 1 to 2 days, apoptotic neutrophils can be observed as early as 4 hours after induced cell influx (data in preparation). Their presence is a result of two processes. Firstly, the intramammary application of PBS, and also LPS or MDP, all of which are inflammation inducing agents, causes an inflammatory reaction characterised by a massive influx of neutrophils into the lumen of the virgin mammary gland and their accumulation there (Wardley *et al*., 1976; Sládek and Ryšánek, 2000a,b, 2001; Sládek *et al*., 2001; Ryšánek *et al*., 2001). Neutrophils are recruited for migration at different ages (Cox *et al*., 1995); furthermore, the influx is continuous. Neutrophil populations, therefore, are a mixture of cells of different ages. Secondly, the resolution of the inflammatory reaction is initiated by apoptosis of neutrophils and their subsequent phagocytosis by macrophages (Sládek and Ryšánek, 2000b, 2001). These facts represent a very important aspect and need to be taken into consideration when virgin mammary gland neutrophil populations acquired

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in this way are being subsequently used for *in vitro* studies.

Cell apoptosis is characterised by several typical morphological features including cell shrinkage, chromatin condensation (karyopyknosis), blebbing (zeiosis) and fragmentation to apoptotic bodies (Kerr *et al*., 1972). In the present study, these features were used as the criteria in an analysis of the dynamics of bovine mammary gland neutrophil apoptosis during *in vitro* neutrophil cultivation. It was found that neutrophils underwent apoptosis during cultivation, i.e. the longer the cultivation, the greater the number of apoptotic cells, and also the greater the number of cells with impaired cytoplasmic membrane integrity.

These findings are not very surprising. Spontaneous apoptosis resulting from natural aging of these cells has been earlier demonstrated in *in vitro* cultivations of human blood neutrophils (Savill *et al*., 1989), and also bovine blood neutrophils (Van Oostveldt *et al*., 1999). It has also been previously found that apoptosis expression is directly proportional to the length of neutrophil cultivation (Savill *et al*., 1989; Payne *et al*., 1994; Van Oostveldt *et al*., 1999). This is probably due to the fact that neutrophils represent postmitotic, terminally differentiated elements with a limited lifespan and a predisposition to apoptosis (Raff, 1992).

There is data on bovine blood neutrophils that show that 3–5% of cells undergo apoptosis *in vitro* after 2-hour cultivation, and up to 15% of cells after a 4-hour cultivation (Van Oostveldt *et al*., 1999; Chin *et al*., 2000; Van Oostveldt *et al*., 2001). If we compare these data with our results, we may say that the dynamics of both neutrophil populations (i.e. blood and mammary), *in vitro*, are almost the same. This is certainly quite surprising because, in theory, the tissue pool neutrophils may be expected to show a different incidence of apoptotic cells than blood neutrophils. Normal blood neutrophils are short-lived cells and they die as a consequence of the constitutive activation of apoptotic programmed cell death (Haslett, 1992). Expression of this program can be altered in mammary gland neutrophils. The reason for that lies in the possible effects of migration on neutrophil apoptosis and also in the presence of cytokines or pro-inflammatory agents in mammary glands.

On the one hand, it was established that endothelial transmigration delayed apoptosis of neutrophils (Watson *et al*., 1997). Contrarily, diapedesis through the blood-milk barrier induces apoptosis

in bovine blood neutrophils *in vitro* (Van Oostveldt *et al*., 2002a).

At the same time, many factors with anti-apoptotic activities exist in mammary glands. Cytokines, especially the pro-inflammatory ones (e.g. IL-1, IL-2, IL-8, IFN-γ), released mainly in the initial phase of the inflammatory reaction of mammary glands(Rainard and Paape, 1997; Shuster *et al*., 1997; Caswell *et al*., 1999), and some growth factors (G- CSF, GM-CSF), may have an equally important impact on the lifespan (i.e. the point of apoptosis)of neutrophils (for details see in Akgul *et al*., 2001). These cytokines enter into the blood circulation andaffect apoptosis of neutrophils before their migration. Bacterial toxins (e.g. LPS) delay apoptosis of neu- trophils (Watson *et al*., 1997; Sládek and Ryšánek, 2001) and also prolong clearance of apoptotic neu- trophils by macrophages during resolution of acute mammary gland injury (Sládek and Ryšánek, 2001). The reported effects of LPS on neutrophil apoptosisare variable, because Van Oostveldt *et al*. (2002b) demonstrated *in vitro* that LPS induces apoptosis in bovine blood neutrophils.

It should be emphasised that the lumen of the bovine mammary gland contains an extensive network of biological influences that have not yet been adequately explained (Sordillo *et al*., 1997; Paape *et al*., 2000). Therefore, further research should focus on the modulatory effect of these factors on apoptosis of bovine neutrophils.

During the cultivations, the increase in the proportion of apoptotic cells corresponded with an increase in the proportion of cells with an impaired cytoplasmic membrane integrity. After 4 hours of cultivation, the proportion of the latter increased from less than one percent to ten percent. According to Payne *et al*. (1994), trypan blue positive neutrophils are secondarily necrotic apoptotic cells because, unlike under *in vivo* conditions, no phagocytosis of apoptotic cells by macrophages takes place under *in vitro* conditions. After apoptotic cells have exhausted their energy supplies, they lose the ability to maintain the integrity of their membranes and undergo necrosis. Apoptotic neu-trophils undergoing secondary necrosis are mor- phologically characterised by a total destruction of their nuclear and cytoplasmic components, andexhibit all the manifestations and signs of necrosis (Payne *et al*., 1994). The consequences of second- ary necrosis of apoptotic neutrophils, due to their potentially negative effect on their environment (under *in vitro* conditions on the culture medium),

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are similar to those of a discharge of the histotoxic content of granules associated with neutrophil necrosis (Payne *et al*., 1994).

The above findings on the numbers of apoptotic cells and trypan blue positive cells during cultivations should not be taken only as a quantitative expression of intensity of the aging process of bovine virgin mammary gland neutrophils *in vitro*. They are the primary data that might play a very important role in the interpretation of the results of functional analyses of these cells, especially when rather long periods of cultivation are used. Each cultivation is, in fact, a model of cell aging, in which the occurrence of functional heterogeneity during longer cultivations may be expected.

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