12-1-1994

Morphological Evidence of Apoptosis During Early Structural Luteolysis in the Golden Hamster

Matthew G. Friederichs

MORPHOLOGICAL EVIDENCE OF APOPTOSIS DURING EARLY STRUCTURAL LUTEOLYSIS IN THE GOLDEN HAMSTER

by

Matthew G. Friederichs
Bachelor of Science, University of North Dakota, 1992

A Thesis

Submitted to the Graduate Faculty

of the

University of North Dakota

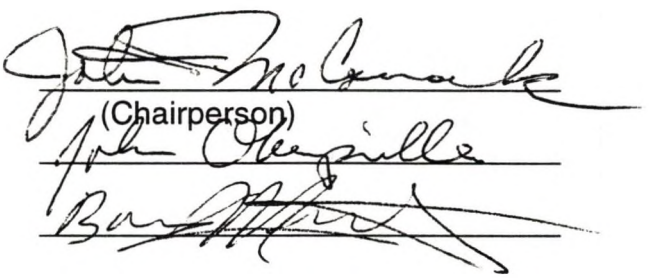
in partial fulfillment of the requirements

for the degree of

Master of Science

Grand Forks, North Dakota
December
1994

This Thesis, submitted by Matthew G. Friederichs in partial fulfillment of the requirements for the Degree of Master of Science from the University of North Dakota, has been read by the Faculty Advisory Committee under whom the work has been done and is hereby approved.



(Chairperson)

This thesis meets the standards for appearance, conforms to the style and format requirements of the Graduate School of the University of North Dakota, and is hereby approved.

Harvey Knull

Dean of the Graduate School 12-14-94

PERMISSION

Title MORPHOLOGICAL EVIDENCE OF APOPTOSIS DURING EARLY STRUCTURAL LUTEOLYSIS IN THE GOLDEN HAMSTER

Department Anatomy and Cell Biology

Degree Master of Science

In presenting this thesis in partial fulfillment of the requirements for a graduate degree from the University of North Dakota, I agree that the library of this University shall make it freely available for inspection. I further agree that permission for extensive copying for scholarly purposes may be granted by the professor who supervised my thesis work or, in his absence, by the chairperson of the department or the dean of the Graduate School. It is understood that any copying or publication or any other use of this thesis or part thereof for financial gain shall not be allowed without my written permission. It is also understood that due recognition shall be given to me and to the University of North Dakota in any scholarly use which may be made of any material in my thesis.

Signature Matthew G. Friederichs

Date 11/30/94

TABLE OF CONTENTS

LIST OF PLATES AND FIGURES

ACKNOWLEDGMENTS

I would like to thank the members of my faculty advisory committee, Doctors John McCormack, John Oberpriller, and Barry Milavetz for their guidance and critical review of this study. I want to give a special thanks to Dr. McCormack and his wife Carolyn for their kindness and generosity. I will always be grateful.

I would also like to thank the faculty members of the Department of Anatomy and Cell Biology for their support throughout graduate school.

Thanks also to my fellow graduate students for their support and friendships over the last two years. A special thanks to Mark Rodacker for his unending help in the laboratory both as a fellow graduate student and friend.

I would also like to thank my parents for their support both emotionally and financially throughout all of my college years. Thanks Mom and Dad. I would like to especially thank my wife, Jules, for all of her support and friendship through this endeavor.

I want to thank all who helped to make my years as a graduate student both enjoyable and unforgettable.

ABSTRACT

Structural luteolysis (SL) in the golden hamster (Mesocricetus auratus) is a remarkable event. The corpus luteum (CL) virtually disappears during a time span of 42 hours. SL begins around 0600 hours on day three of the cycle and is essentially complete by 2400 hours on day four of the cycle.

The purpose of this study was to determine when SL in the golden hamster actually begins and secondly to determine the way in which the three cell types involved in SL are depleted. The three cell types in question include luteal cells, endothelial cells, and neutrophils.

This study employed two separate *In Situ* techniques. The first technique incorporates biotinylated nucleotides onto the ends of fragmented genomic DNA. Since the fragmentation of DNA is the biochemical hallmark of apoptosis, labeling the fragmented DNA greatly enhances the detection of apoptotic cells in paraffin embedded tissue sections. Apoptag™ which utilizes digoxigenin conjugated dUTP is also used in this study. This system proves to be highly valuable in the detection of apoptotic cells due to low background staining and extremely consistent results. In both of these systems nuclei which contain fragmented DNA stain brown.

Ovaries were collected at nine different time frames throughout the four

day estrus cycle. Ovaries from a minimum of three animals were harvested for each time frame. Serial sections of three to four microns were then subjected to the two separate techniques.

The results of this study indicate that SL begins at 0600 hours on day three of the cycle, and is marked by a large infiltration of neutrophils. Following this neutrophilic infiltration there is a marked increase in apoptotic activity throughout the CL. Interestingly, in this model of study all three cell types involved, die by the process of apoptosis. The fact that apoptosis is the mode of cell death is important, since it does not elicit an intense inflammatory response

INTRODUCTION

There is a long history of the study of the corpus luteum dating back to the first twenty-five years of this century. The formation and maintenance of this organ in the mouse, hamster, rat, guinea pig and rabbit were reviewed in-depth by Greenwald and Rothchild in 1968. The corpus luteum (CL) is an endocrine organ with an extensive vasculature. The CL forms from the collapsed graffian follicle following ovulation. It develops primarily from the follicular granulosa cells with a smaller contribution from the endocrine cells of the surrounding theca interna. After ovulation the basement membrane between the theca and granulosa cells is disrupted allowing developing blood vessels to invade, ultimately producing an extensive vascular network (G.D. Niswender et al., 1984, Forsman and McCormack, 1992). During this time of vascularization, thecal cells undergo hyperplasia and hypertrophy and migrate into the follicular space along with the newly formed vasculature (Niswender et al., 1994). The CL of the hamster produces the steroid hormone, progesterone, needed to prepare the uterus for the implantation of the conceptus (Greenwald, 1968). Toward the end of an infertile estrus cycle the CL must regress structurally and functionally in order to make way for the maturation of follicles of the next estrus cycle. Furthermore, progesterone secretion must decline in order to remove its blocking

effect on the pre-ovulatory surge of luteinizing hormone (LH) required to release the next grouping of ova (Niswender et al., 1994).

There are two separate phases to the estrus cycle in the vast majority of mammals. The luteal phase is characterized by the presence of an active, progesterone secreting, CL. The follicular phase coincides with a marked increase in estrogen synthesis and secretion by developing follicles (Terranova and Greenwald, 1978).

The golden hamster, (Mesocricetus auratus), was utilized in this study of structural luteolysis for several reasons. The hamster differs from other laboratory animals in the regularity of its estrus cycle. The four day estrus cycle of the hamster is very regular in contrast to other laboratory species that may have estrus cycles of varying length. Early morning of day one of the estrus cycle in the golden hamster coincides with ovulation. Shortly thereafter corpora lutea are formed and begin functioning.

In an infertile estrus cycle, the corpora lutea under-go inevitable luteolysis. For convenience, luteolysis is divided into two separate processes; functional luteolysis (FL) and structural luteolysis (SL). Functional luteolysis begins around 2000 hours on day two and is essentially completed within a four hour time span. Progesterone reaches a maximum production level at 2000 hours on day two, followed by a rapid decline in production and secretion to undetectable serum levels by 0400 hours on day three. It is during this time that the luteal-follicular

shift is occurring. Estrogen levels begin rising and reach maximum levels by 0400 hours on day three. It has been suggested that this enormous increase in estrogen levels is largely due to developing antral follicles (Terranova and Greenwald, 1978). In a particularly astute observation, based strictly on a light microscopic investigation, Terranova and Greenwald, 1978 speculated that structural luteolysis, heralded by an invasion of "leukocytes" begins around 0600 hours on day three and is completed by 2400 hours on day four. Interestingly, leukocytic infiltration, margination, and emigration is followed by a decline in blood flow and subsequent complete avascularity by day four (Terranova and Greenwald, 1978).

Most significantly, Azmi and O'Shea 1984, using guinea pigs as a model, described an endothelial cell death that becomes apparent with the onset of leukocytic cells invading the extravascular space. This coincides with a significant decrease of and a concomitant rapid decline of endothelial cell populations. Extended reduction in tissue function is correlated with a reduction of blood flow and vasculature (T.I. Azmi and J.D. O'Shea, 1984). They also noted that the structural changes associated with endothelial cell depletion has the morphologic characteristics of apoptosis as described by Kerr in 1972. The depletion of endothelial cells appears to happen in a random fashion and is often observed next to viable cells. Initially, endothelial cells are observed bulging into the vascular lumen, while still maintaining contact with neighboring cells and the

basal lamina. Electron micrographic work has revealed that these cells develop extensive adherens junctions with neighboring cells (T.I. Azmi and J.D. O'Shea, 1994). Also, the possibility of apoptosis is evidenced by cytoplasmic and nuclear condensation observed in endothelial cells. Endothelial cell membranes soon lose contact with the basal lamina and show signs of cytoplasmic blebbing. There is, however, no evidence at this time indicating damage to the basal lamina or an aggregation of platelets at the site of endothelial sloughing which would be indicative of mechanical damage to the vessels (T.I. Azmi and J.D. O'Shea, 1994). Intact basal lamina and surrounding endothelial cells form a complete barrier around the luteal cells, isolating them from formed elements of the blood. Further degeneration of endothelial cells and their fragments happens completely within the lumen. There is little evidence of endothelial cells possessing a great deal of phagocytic activity. Therefore, the possibility exists that endothelial cell deletion by apoptosis may play a significant role in the process of structural luteolysis.

Loss of luteal cells in a random fashion is another noteworthy event of structural luteolysis as noted in cattle (J.L. Juengel et al., 1984). Based on nuclear and cytoplasmic morphologic changes, a number of reports indicate that luteal cells are depleted by the process of apoptosis (Zeleznik et al, 1989; Sawer et al, 1990). However, none of these earlier studies made an attempt to identify The exact mechanism of cell death, that is, whether the cells being depleted die

by apoptosis or necrosis. In the hamster, this spontaneous luteal regression continues throughout days three and four until the CL is completely annihilated (Greenwald, 1968). This process is so well regulated in the hamster that only one generation of corpora lutea is present during a particular estrus cycle. This is in sharp contrast to rats, mice and other laboratory animals which may have up to three generations of corpora lutea present at any one given time.

Structural luteolysis (SL) in the hamster is an impressive phenomenon in large part due to its highly regulated and limited inflammatory reaction. Luteal cell depletion by apoptosis has extremely significant consequences, since cell death by necrosis is known to elicit a rapid and intense inflammatory response. Such a severe inflammatory response may result in extensive damage and scarring, which would be detrimental to cyclic ovarian activity. In contrast, apoptosis likely plays a protective role in SL by limiting the exposure of cellular contents and debris to the formed elements of the blood. Thereby, limiting the opportunity for an extensive inflammatory response.

The term apoptosis was originally coined by Kerr, Wyllie, and Currie to describe the process of cellular death long recognized by embryologists and previously described as shrinkage necrosis or programmed cell death. In its original form the Greek word “apoptosis”, (pronounced apo-tosis), described the act of leaves falling off of trees (J.J. Cohen, 1993). Apoptosis appears to play a major role in tissue kinetics acting as the antithesis of mitosis (J.F.R. Kerr, A.H.

Wyllie and A.R. Currie, 1972). Normal tissue size, growth and regression is dependent on the balance between apoptosis and mitosis in several organ systems. Apoptosis occurs in a multitude of locations during development and adulthood. During ontogeny, apoptosis is noted in the regression of interdigital webs of the limbs, development and shaping of organs, lumenization of the vascular system and intestinal tract, and loss of primordial appendages. In adulthood, apoptosis is prevalent in neoplasms as well as during neoplastic regression, and also in the atrophy of organs due largely to loss of trophic support (W. Burch, F. Oberhammer and R. Schulte-Hermann, 1992). Examples of the control of trophic support include the apoptotic involution of the adrenal cortex following hypophysectomy and subsequent loss of Adrenal cortical trophic hormone (ACTH) and degeneration of rat ventral prostate following castration. In addition, excessive levels of certain compounds may induce apoptosis in certain cell populations as seen in apoptotic involution of the thymus following treatment with excessive amounts of glucocorticoids. Certainly a small number of apoptotic cells may be found in almost all healthy tissue at any given time, since it is a normal part of tissue kinetics.

Necrotic cell death has several characteristics that separate it from the phenomenon of apoptotic cell death. The loss of large cell populations is observed in necrosis. Necrotic death results from an extreme insulting stimulus of a non-physiologic nature. Subsequent to this insult, the cell loses control of

ion-flux. Calcium flows into the cell and in turn will then enter the mitochondria, and subsequently lead to an elevated metabolism. High metabolic rates tend to produce increasingly hypertonic cells. This hypertonic condition causes water to enter the cell in abundance and the cell along with its organelles swells to the point of lysis. One significant feature of necrosis is uncontrolled swelling and dilatation of mitochondria (S. Sen, 1992).

Apoptosis can be conveniently subdivided into two separate mechanisms; morphological and biochemical. Furthermore, morphological changes can be broken down into three or more different aspects including decrease in volume, nuclear changes and plasma membrane changes.

Apoptotic cells undergo an increase in buoyant density (Ohyyama, Yamada, and Watanabe, 1981; Thomas and Bell, 1981; Wyllie and Morris, 1982). This characteristic has been utilized for the collection of purified apoptotic cells by flow cytometry. Apoptotic cells lose a considerable amount of water without a coinciding increase in cellular permeability. This has been shown in cells using vital dye uptake techniques. Cellular shrinkage coincides with the biochemical onset of the apoptotic process (S. Sen, 1992).

Following the biochemical events, nuclear changes begin and are characterized as follows. Within the nucleus, chromatin starts to appear in a granular form. The chromatin will condense and aggregate at the perimeter of the nuclear membrane. The nucleolus disintegrates into densely osmiophilic

granules. Other portions of chromatin show distinct argyophilia and demonstrate high protein concentrations. Within apoptotic cells chromatin is anchored to the protein skeleton of the nucleus (S. Sen, 1992), resulting in condensed granular fragments strongly adherent to the inner lamina of the nuclear membrane. The nuclear membrane remains intact while exhibiting extensive convolutions. Along with the cytoplasmic shrinking there is a concomitant reduction in nuclear size.

Plasma membranes of apoptotic cells exhibit morphological and biochemical changes. These changes allow phagocytes and neighboring cells to recognize them as apoptotic. Pseudopodia and other membrane structures are lost. There is also a distinct change in surface charge. Membrane carbohydrates undergo changes which facilitate recognition and binding by phagocytic cells (S. Sen, 1992). The endoplasmic reticulum swells and fuses with the cell membrane (Yamada and Ohyama, 1980; Galili et al, 1984; Morris et al,, 1984). Characteristic membrane convolutions or blebbing are then observed. The plasma membrane buds off encasing the entire cellular contents into membrane bound apoptotic bodies. This serves to limit the exposure of cytoplasmic contents as opposed to cell lysis as observed in necrosis.

Biochemical events include the activation of an endogenous endonuclease (Wyllie and Morris, 1982). A calcium-magnesium dependent endonuclease has long been assumed to be the nature of this enzyme, although the presence of this enzyme is confirmed in the cells of only a few tissues.

Enhanced endonuclease activity may result from direct activation of this enzyme by increased calcium levels. Subsequently, genomic DNA is cleaved at regular intervals in the internucleosomal spaces (A.H. Wyllie, 1992). Extracted DNA fragments exhibit the classic "ladder" pattern upon agarose gel electrophoresis. These fragments of 180-200 base pairs correspond to the size of a single nucleosome plus short bits of linker DNA. DNA fragments exist in two separate classes; one class bound to the nucleus and one unbound. The bound class represents 70% of the chromatin consisting of oligonucleosomes rich in the histone H_1 The unbound or remaining 30% represents short oligonucleosomes and mononucleosomes low in H_1.

Apoptosis is an active form of cell death; therefore, RNA and protein synthesis are prerequisites for apoptosis. Inhibitors of protein and RNA synthesis have in many instances blocked apoptotic cell death in vitro (Sen, 1992).

These aforementioned features of apoptosis were sequenced into three phases by M.J. Arends, R.G. Morris, and A.H. Wyllie (1990). Condensation of chromatin, nuclear capping, and disintegration of the nucleolus characterize nuclear changes of the first phase. Concomitant with nuclear changes are cytoplasmic events consisting of cell shrinkage, swelling of the endoplasmic reticulum, and compaction of cellular organelles with the exception of the mitochondria. During the second phase, cellular components are subdivided into

membrane bound bodies of varying size, with subsequent phagocytosis by neighboring parenchymal cells and resident phagocytes. In the third phase apoptotic bodies begin to undergo what appears to be autolysis and final degradation within the phagocytic cells.

In light of the escalating interest in apoptosis, a multitude of techniques have been developed to detect apoptotic cells. Original work using agarose gel electrophoresis has yielded a great deal of insight into the fragmentation of genomic DNA, a basic biochemical phenomenon of apoptosis. Gel techniques have proven useful in the detection of apoptosis; however, these lack the sensitivity of more recently developed techniques that detect apoptotic cells *In Situ*. The use of gel techniques, unfortunately, is not valuable in identifying apoptotic cells on a cell by cell basis or in whole organs composed of a number of sub-populations of cells. Within cell cultures apoptotic cells may be identified by a decrease in buoyant density or by flow cytometric detection of cell shrinkage (Swat et al., 1991). Over the last four years huge advances have been made in the detection of apoptotic cells in tissue sections. Kishimoto et al., 1990, used In Situ Nick Translation (ISNT) to identify apoptotic cells. An In Situ End Labeling (ISEL) technique was developed by Wijsman et al., 1993. Recently, there has been growing use of Terminal Deoxynucleotydal Transferase (TdT) as the enzymatic agent to label fragmented DNA in a modified ISEL. TdT catalyzes the reaction whereby digoxigenin-dUTP polynucleotide chains are added onto the

3'end of DNA fragments. Anti-digoxigenin-peroxidase conjugate then binds to the digoxigenin-dUTP of the DNA fragments. A chromogenic agent is utilized to visualize fragmented DNA.

Agarose gel electrophoresis of extracts of hamster corpora lutea at regular intervals throughout the estrous cycle show the classic "ladder" pattern observed during apoptosis (McCormack; submitted, Biology of Reproduction). Definitive ladder patterns, however, are not apparent until 1200 hours on day three in this gel system. Since the corpus luteum is an organ composed of at least four cell types the issue of specificity in respect to cell types is extremely important. Our purpose is to determine which cell or cells actually die by apoptosis, and to determine the initiation of this process of structural luteolysis. These two questions are beyond the scope of agarose gel techniques. It is the purpose of this study to extend the results of the agarose gel studies of apoptosis using the more sensitive In Situ techniques. In addition, this study will show that structural luteolysis begins between 0600 to 0900 hours on day three of the estrus cycle.

MATERIALS AND METHODS

Twenty-seven female golden hamsters (Mesocrietus auratus) were used in this study of structural luteolysis. Hamsters used weighed between 100-145 grams. Hamsters were housed individually, to reduce stress on the animals. Hamsters were subject to a 14:10 hour light:dark cycle, with lights on at 0500 hours. All hamsters used had a minimum of three consecutive four day estrus cycles. A thick vaginal discharge signified day one of the estrus cycle. Day one is the day of ovulation. Animals were checked for vaginal discharge between 0800 to 1100 hours each day to verify cyclicity. Ovaries were collected at nine separate time frames. Time frames included in this study consisted of: day one; 1200h, on day two; 1200h, 2100h, 2400, on day three; 0300h, 0600h, 0900h, 1200h, on day four; 1200hrs.

At the time of ovary collection, the hamsters were anesthetized by intraperitoneal injection of 0.30-0.34 ml Sodium Pentobarbital, Veterinary Laboratories; Lenexa, Kansas. Upon verification of deep anesthesia, a midline incision was made to gain access to the thoracic and abdominal cavities. The left ventricle was punctured using an 18 gauge intravenous catheter, followed by an incision of the right atrium producing an open flow of perfusate. A Harvard infusion pump was used for the perfusion at a rate of 15.3 ml/min. Hamsters

were first perfused with 35ml of saline solution to clear the vasculature of blood, followed by three 45 ml volumes of 4% neutral buffered formalin. Following the perfusion, the ovaries were removed from the abdominal cavity and placed in 4% formalin solution. The ovaries were cleaned of the surrounding fat pat, trimmed, and placed in 10 ml of 4% formalin for sixteen to twenty-four hours.

All ovaries were dehydrated in increasing concentrations of ethanol, cleared in Hemo-De and embedded in paraffin blocks according to standard histological procedures. Serial sections were cut at 3-4 microns using a rotary microtome. Sections were affixed to pre-cleaned glass microscope slides using ovalbumin.

An In Situ end labeling technique (ISEL), adapted from Wijsman et. al, was used to identify apoptotic cells. After deparaffinizing in Xylene and rehydrating in decreasing concentrations of ethanol and distilled water, the sections were pretreated with pepsin (Sigma, St. Louis, Mo.) 1μg/ml in PBS (0.14 M NaCl, 2.7 mM KCL, 0.01 M Na_2HPO_4, 1.0 mM KH_2PO_4) for 15 minutes at 37°C in a humidified chamber. Pepsin treatment was stopped by washing slides in running tap water for 2 minutes. Sections were then treated with 1% TritonX-100 (Sigma) for 20 minutes at 37°C and rinsed with two changes of PBS for two minutes each wash. Nucleotides were then incorporated onto the ends of fragmented DNA by the following method. Sections were incubated in Buffer A (50mM Tris-HCL, 5mM $MgCl_2$, 10mM B-Mercaptoethanol, and 0.005% BSA, pH

7.4) for 5 minutes at 15°C. Sections were allowed to air dry and were subjected to incubation in a modified Buffer A containing 0.01mM dATP, 0.01mM dCTP, 0.01mM dGTP, 0.01mM biotin-11-dUTP, and 13U/ml DNA polymerase I. 75ul sufficed to flood an area of 3 cm^2. All chemicals were purchased from Sigma, St. Louis, MO.

To block endogenous peroxidases, sections were treated with PBS containing 0.1% H_2O_2 in a coplin staining jar for 15 minutes at room temperature and washed in two changes of PBS for 5 minutes each wash. Sections were then incubated with Extra-Avidin™ (Sigma) conjugated Horseradish peroxidase diluted 1:100 in PBS containing 1% BSA and 0.5% Tween 20 for 30 minutes at RT. Staining was achieved by developing in 0.5% 3-3'diaminobenzidine and 0.02% H_2O_2 for 5 minutes at 37°C and counterstaining with 0.2% methyl green in sodium acetate (pH 4). Sections were dipped in distilled water, dehydrated in two changes of acetone for 3 seconds each change and cleared in two changes of xylene for 3 minutes per wash. Coverslips were applied using permount as a mounting medium.

Negative controls were completed during each ISEL procedure. The negative controls consisted of the removal of the DNA polymerase I from Buffer A containing the nucleotides. The controls were kept separate during the step of ashing in PBS containing 1% H_2O_2 to avoid possible exposure to DNA polymerase I.

The Apoptag™ Peroxidase *In Situ* apoptosis detection kit (ONCOR, Gaithersburg, MD.) was also utilized to identify apoptotic cells. This technique represents an immunohistochemical modification of standard ISEL, to improve the specificity and reduce background.

Sections were deparaffinized by washing in two changes of xylene for five minutes. The sections were then rehydrated by washing in two changes of absolute ethanol for five minutes each wash followed by one change each of 95% and 70% ethanol for three minutes each wash. Sections were rinsed in PBS for five minutes.

Oncor Protein Digesting Enzyme (Protienase K, 20 μg/ml in PBS) was applied directly to the slide for 10 minutes at RT. Sections were washed in four changes of distilled water, 2 minutes each change. Excess liquid was tapped off and two drops of Equilibrium Buffer were applied to sections and covered with a plastic cover slip. Sections were incubated with Equilibrium Buffer for 15 minutes at RT. Following this incubation, excess liquid was tapped off, and 36 μl of Working Strength TdT Enzyme which consisted of 32 μl TdT enzyme for every 76 μl of reaction buffer was applied to the sections, covered with a flexible plastic coverslip and incubated in a humidified chamber for one hour at 37°C. Following TdT incubation, slides were placed in a coplin staining jar containing 35 ml pre-warmed Working Strength Stop/Wash Buffer (1 ml Stop/Wash buffer and 34 ml dH_2O) and incubated for thirty minutes, with agitation every ten minutes.

Sections were subsequently washed in three five minute changes of PBS. Two drops of Anti-Digoxigenin conjugated Peroxidase were added to the sections. Plastic coverslips were reapplied and sections were incubated for thirty minutes in a humidified chamber at RT. Following the previous incubation, the sections were rinsed with four changes of PBS for five minutes each wash at RT. Staining was developed with 0.05% 3-3'Diaminobenzidine and 0.02% H_2O_2 for 3 minutes at RT. Specimens were subjected to three washes of distilled water for 1 minute and one wash for 5 minutes. Sections were counterstained in 0.5% methyl green in sodium acetate (pH 4) for 12 minutes followed by two washes in distilled water and dehydrated by two changes of N-Butanol for 30 seconds each wash. Specimens were cleared in two washes of xylene for three minutes each. Coverslips were applied using permount as a mounting medium.

Negative controls were run by substituting distilled water for TdT enzyme when preparing the Working Strength TdT Enzyme.

All sections were viewed on an Olympus BHTU light microscope. Kodak Gold 100 film was used and developed by Ritz/Black's Photo; Grand Forks, ND.

RESULTS

Ovaries harvested at 1200 hours on day one exhibited incomplete vasculogenesis. The periphery of corpora lutea demonstrated a healthy vasculature; however, the most internal aspects of the corpora lutea remained avascular at this time (Figure 1). One to two apoptotic cells were present per section of corpora lutea (Figure 1). The remaining portions of the corpus luteum appeared very healthy. A few neutrophils were observed in the center of the corpora lutea (Figure 2).

Ovaries collected at 1200 hours on day two were structurally sound. Two to three apoptotic cells were observed to be present in each section of corpora lutea (Figure 3). Vasculogenesis of the corpora lutea was essentially complete. A few corpora lutea presented an avascular center (Figure 3). There were no neutrophils present in these corpora lutea (Figure 4).

Day two 2100 hours ovaries contained corpora lutea with a healthy vascular network(Figure 5) Avascular centers were noted in some CL (Figures 5 and 6). Apoptotic cells could be identified in the avascular centers(Figures 5 and 6). Remaining luteal cells had intact morphology.

Day two 2400 hour ovaries contained corpora lutea with a healthy, intact vascular system. In some corpora lutea with avascular centers a high number of

apoptotic cells and bodies were present (Figure 7). There were no neutrophils identified in the corpora lutea. A few apoptotic cells were observed scattered throughout each section of corpora lutea (Figures 7 and 8). The luteal cells of the remaining portions of the corpora lutea appeared healthy.

Ovaries harvested at 0300 hours of day three exhibited corpora lutea with an extensive vascular network (Figures 9 and 10). There was no apparent damage to the vasculature. All endothelial cells were morphologically sound (Figure 10). No neutrophils were observed in the corpora lutea. A few apoptotic bodies were located in each section of corpora lutea (Figure 10). The luteal cells as a whole appeared to be healthy.

Corpora lutea from ovaries harvested at 0600 hours on day three show a minimum of eight to ten apoptotic cells per section of corpora lutea (Figure 11). Many apoptotic bodies were also present at this time (Figure 12). Also observed was an extensive infiltration of neutrophils, with many located in the extravascular space (Figure 12). For the most part, the vascular system appeared to be healthy. There were some signs of endothelial cells staining positive for apoptosis (Figure 13). Remaining luteal cells appeared morphologically intact.

Ovaries harvested at 0900 hours on day three contained corpora lutea that exhibited several apoptotic cells and bodies scattered throughout (Figure 14). Positive staining of apoptosis was observed in neutrophils (Figure 15). A

large number of neutrophils were also present within the extravascular space (Figures 15 and 16). Several endothelial cells stained positive for apoptosis in each section of corpora lutea (Figure 16). Luteal cells as a whole remained morphologically healthy. There was also signs of vascular degeneration (Figure 16).

Ovaries collected at 1200 hours on day three contained corpora lutea that demonstrated wide spread apoptotic activity of luteal cells (Figure 17). Many apoptotic cells and bodies were identified (Figure 17). A significant amount of vascular degeneration was also apparent (Figures 18 and 19). Many endothelial cells stained positive for apoptosis(Figures 18, 19, 20, 21, and 22). The lumen of some vessels were partially clogged with sloughed endothelial cells and apoptotic bodies (Figures 21 and 22). A large population of neutrophils was present (Figures 18, 19, and 20).

Day four ovaries collected at 1200 hours contained avascular corpora lutea (Figures 23 and 24). There was a noticeable decrease in the size of the corpora lutea. Considerable apoptotic activity was apparent (Figures 23 and 24). Numerous cells stained positive for apoptosis, and many apoptotic bodies were present. Relatively few neutrophils were distinguished at this time.

Ovaries stained with the In Situ End Labeling technique mimic the results obtained through the use of Apoptag™-peroxidase kit. The results from these slides did however exhibit less reliability, and somewhat higher background.

DISCUSSION

The golden hamster was the animal of choice for this study of structural luteolysis due to its extremely regular four day estrus cycle. This rapid development and regression of the corpus luteum provides a tremendous advantage over other laboratory animals that may have a number of generations of corpora lutea present at one given time. The thick vaginal discharge released upon gentle pressure around the vaginal orifice that signifies the first day of the estrus is characteristic of the golden hamster. The regularity of the hamster estrus cycle and the easily verifiable ovulation encouraged the use of the golden hamster over other laboratory animals.

Normal cyclic female hamsters have the dilemma of ridding themselves of corpora lutea both functionally and structurally in a short period of time. Functional luteolysis (FL) is necessary to reduce progesterone levels and enable the preovulatory luteinizing hormone (LH) surge required to release the ova of the next estrus cycle. Functional luteolysis has been studied in-depth by Terranova and Greenwald, 1978 and Niswender et al., 1994. Coinciding with FL, structural luteolysis (SL) must occur to make way for the development of the follicles of the next estrus cycle.

Recent evidence indicates that luteal cells are depleted through the process of apoptosis (McCormack et al.; submitted, Biology of Reproduction). In this study, using agarose gel electrophoresis of luteal extracts McCormack demonstrated a ladder pattern characteristic of apoptotic cells. This data revealed that significant DNA fragments were detectable by 1200 hours on day three of the cycle. To further substantiate this evidence of luteal cell apoptosis two separate In Situ techniques were utilized. In Situ techniques have significant advantages over gel techniques. The techniques utilized in this study allow for the study of structural luteolysis on a cell to cell basis in an entire organ. In Situ techniques allow for a more accurate determination of the initiation of apoptosis, and therefore, structural luteolysis.

All results reported in the present study were generated using the Apoptag™-peroxidase kit because of its superior performance. This technique had considerably less background than the In Situ technique which utilized biotinylated nucleotides and DNA polymerase I. The Apoptag™ kit is an immunohistochemical modification of ISEL in which a plant protein, digoxigenin, is conjugated to the nucleotide dUTP. Digoxigenin is only found in the digitalis plant, thereby limiting background. High background staining visualized with the biotin technique could be due to endogenous biotin located in luteal cells. Ovaries collected at 1200 hours on day one and on day two that were stained with the Apoptag™ kit contain luteal cells whose nuclei demonstrate some

background staining. One possible explanation for this background is that at this time there is intense steroid production and secretion. It is possible during enhanced periods of transcription to have some staining of the naked DNA and RNA due to the elevated amounts of euchromatin.

This study of structural luteolysis using two separate In Situ techniques to label apoptotic cells allowed the recognition of apoptotic cells by 0600 hours on day three, fully six hours earlier than previously possible using agarose gel electrophoresis. Specific labeling of apoptotic cells is very important since this study has shown that all three cell types involved with the structural demise of the corpus luteum including: endothelial cells, luteal cells, and neutrophils die via the process of apoptosis. The fact that endothelial cells die by the process of apoptosis is important due to the vascular nature of the corpus luteum. Neutrophils being depleted by apoptosis could also have a value in the control of the limited inflammatory response observed during structural luteolysis.

Endothelial cell regression during luteolysis was reviewed by Azmi and O'Shea in 1984. During structural luteolysis there is a sharp reduction in blood flow and a corresponding loss of endothelial cells. The fact that endothelial cells are depleted by the process of apoptosis could be due to a wide range of factors. Endothelial cells are exposed to mild ischemia with the beginning stages of SL through the reduction of blood flow. Azmi and O'Shea postulated that vascular damage is the result of reduced blood flow, rather than a causative agent. It has

been shown that apoptosis and necrosis can both occur during ischemia, with apoptosis occurring at the periphery of the lesions where the ischemia is not as intense; however, necrosis is not observed in structural luteolysis. Endothelial cells are also subjected to chemical mediators released from the neutrophils that invade the corpus luteum at the onset of structural luteolyis. TNFα, a cytokine present in neutrophils, has been shown to induce apoptosis in certain tissues including corpora lutea (Bagavondoss et al, 1990). McCormack et al. demonstrated that hamster luteal neutrophils contained TNFα. It has been postulated that TNFα works in a synergistic fashion with other cytokines released from neutrophils. It is our belief that endothelial cell apoptosis is more prevalent than shown in this study. Since apoptotic endothelial cells remain in the vascular lumen, it is reasonable to assume that some apoptotic debris is carried away by blood flow and the flushing effects of the perfusion fixation technique employed in this study. Endothelial cell regression is an area that has yet to be thoroughly examined.

Neutrophil infiltration must play a certain role in structural luteolysis; however, the exact and total role remains unclear. Neutrophil presence, by definition, indicates an inflammatory response is in progress. Inflammation in the degenerating corpora lutea is a limited process due to cell depletion by apoptosis. Cell depletion via necrosis would, in theory, result in an acute inflammatory response due to the leakage of cellular debris, including hydrolytic

enzymes into the extracellular space. Necrosis would also result in formation of scar tissue. This would have an adverse affect, since one of the prime responsibilities of structural luteolysis is to make way for the developing follicles of the next estrus cycle. There have been reports of macrophages located in the CL of hamsters, (Leavitt et al, 1971). However, in the present study, we failed to observe any mononuclear phagocytes within the CL at any point during the estrus cycle. This supports the idea of an extremely well regulated inflammatory response. As shown by the results obtained from the Apoptag™-peroxidase procedure, neutrophils within degenerating corpora lutea also die by the process of apoptosis. This is one possible method the corpora lutea may utilize to prevent an intense inflammatory response. Although it is possible to find neutrophils in day four corpora lutea, the number is greatly reduced. The reduction of neutrophils corresponds with vascular degeneration. A decline of neutrophil populations is noticed by 1500h on day three when the vascular system has undergone a great deal of regression (McCormack, submitted Biology of Reproduction). At this time reduced vasculature inhibits further neutrophilic infiltration.

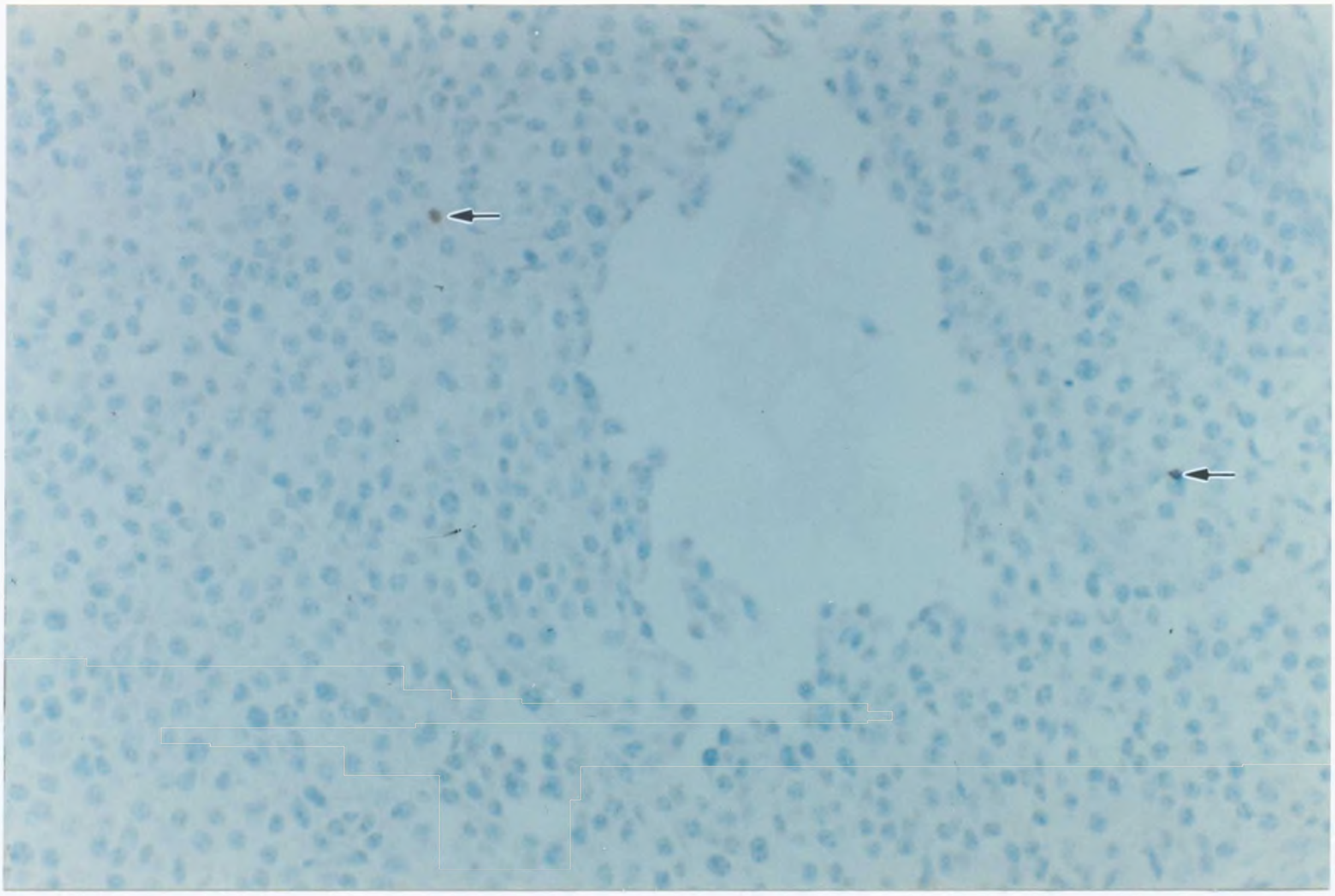
At all times during the estrus cycle it is possible to find one to two apoptotic cells present per section. Apoptotosis is present in small numbers in many normal tissues, and plays a role in the removal of malformed or improperly functioning cells. As alluded to previously, apoptosis has an active part in tissue

kinetics. It is therefore reasonable to assume that the presence of apoptotic cells in corpora lutea during all phases of the estrous cycle does not signify SL. The increase in apoptotic activity around the time of neutrophil infiltration coincides with the first signs of significant luteal cell depletion. Luteal cells along with the endothelial cells are subject to the various cytokines released from neutrophils. The onset of luteal cell depletion may, as in the case of endothelial cells, have a large variety of causative agents. Luteal cells of the estrus cycle may also be hormonally dependent. The onset of structural luteolysis occurs shortly after peak estrogen concentrations on day three of the cycle.

Results from this study indicate that structural luteolsysis begins at 0600 hour on day three of the cycle. The onset of structural luteolysis is marked by the infiltration of neutrophils. Neutrophilic emigration is followed shortly thereafter by endothelial cell regression. Coinciding with endothelial cell regression is a marked increase of apoptotic activity of the luteal cells. Staining procedures showed that all three cell types discussed are depleted by apoptosis.

Figure 1. Day 1 1200 hours, Corpus luteum demonstrating an avascular center. Two apoptotic cells are identified (Arrows). (200X)

Figure 2: Day 1 1200 hours, CL with two neutrophils located near avascular center (Arrows). (400X)



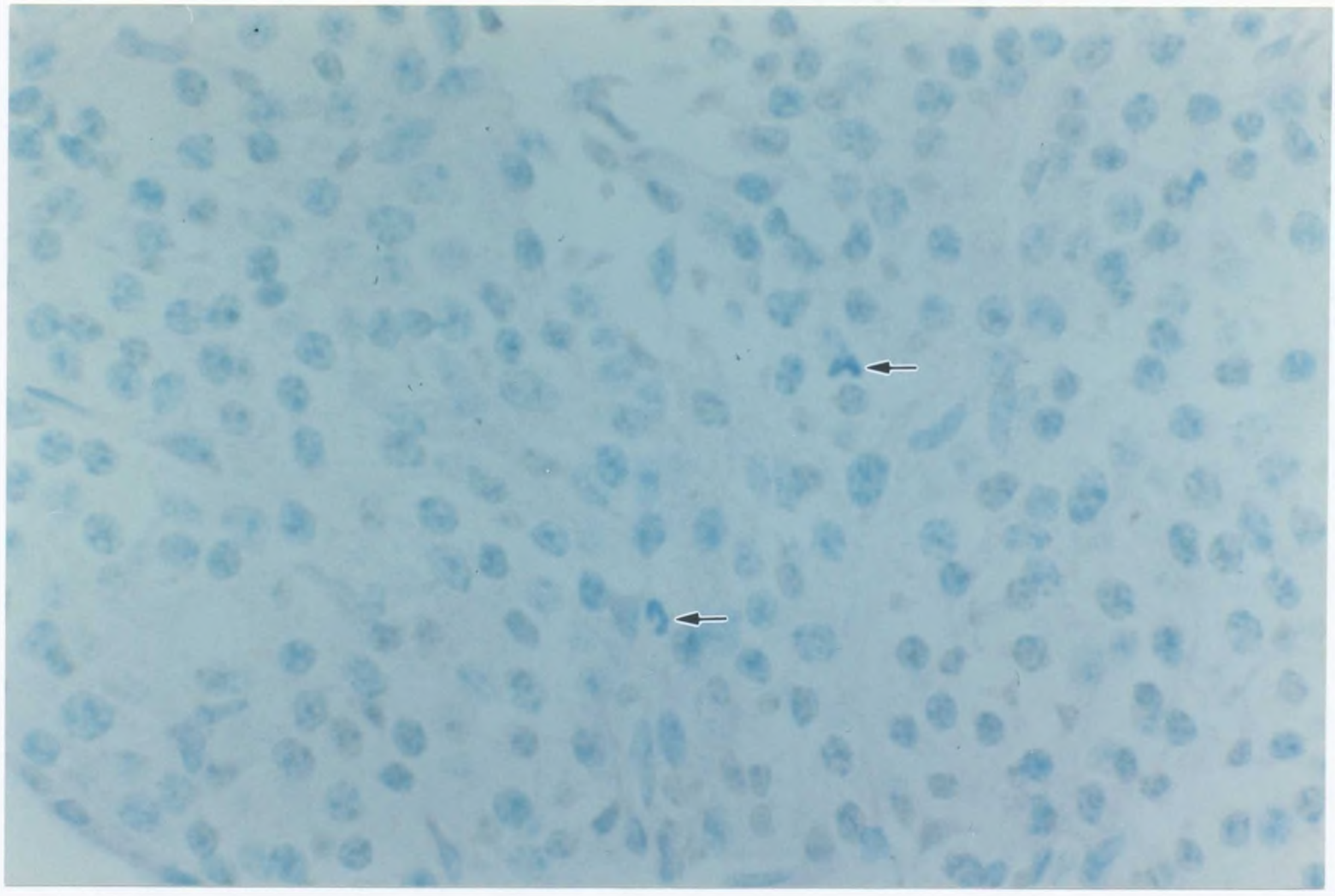
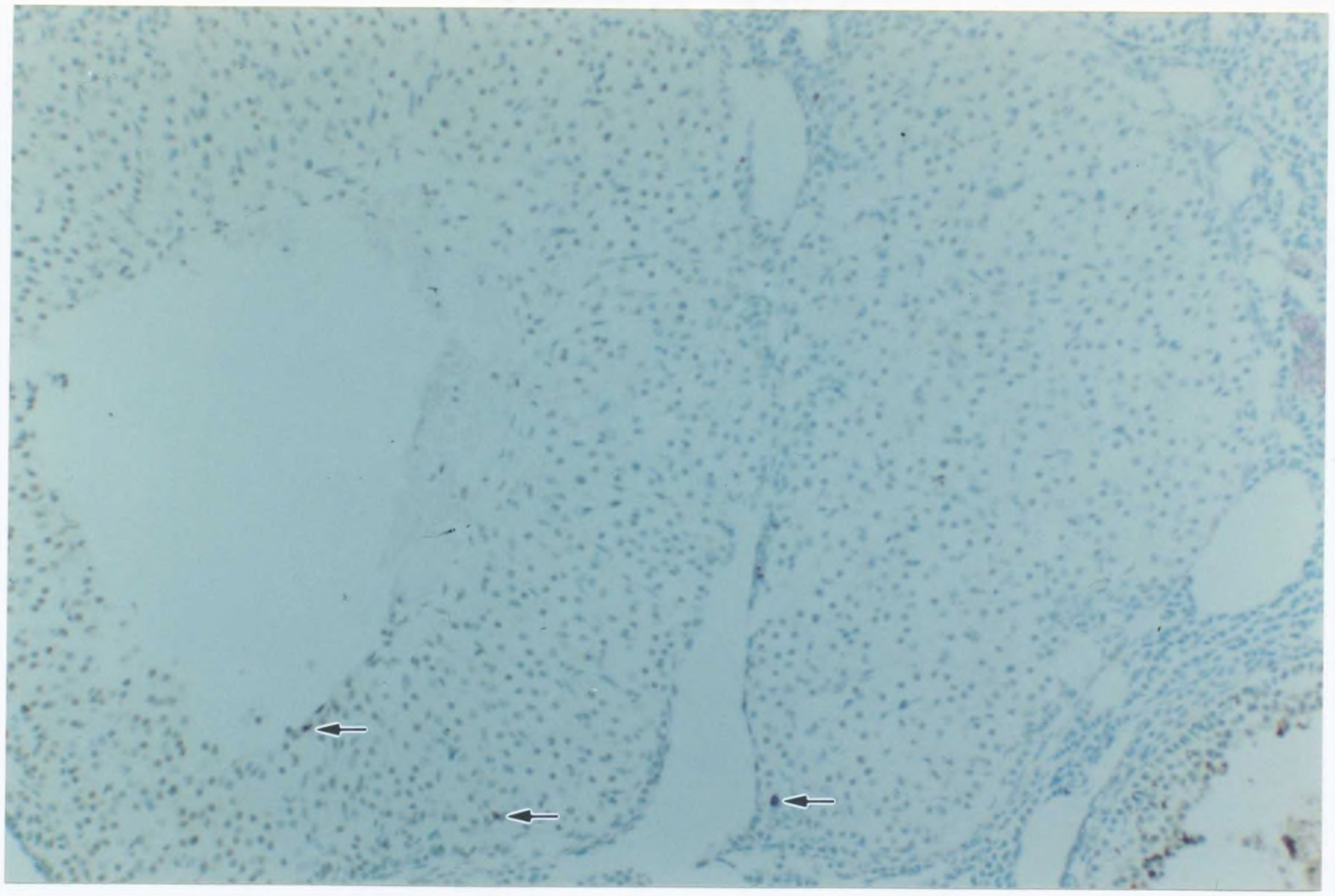


Figure 3: Day 2 1200 hours, CL demonstrating avascular center. A few apoptotic cells are identified (Arrows). (100X)

Figure 4: Three apoptotic cells can be identified in this section of day 2 1200 hours CL (Arrows). (200X)



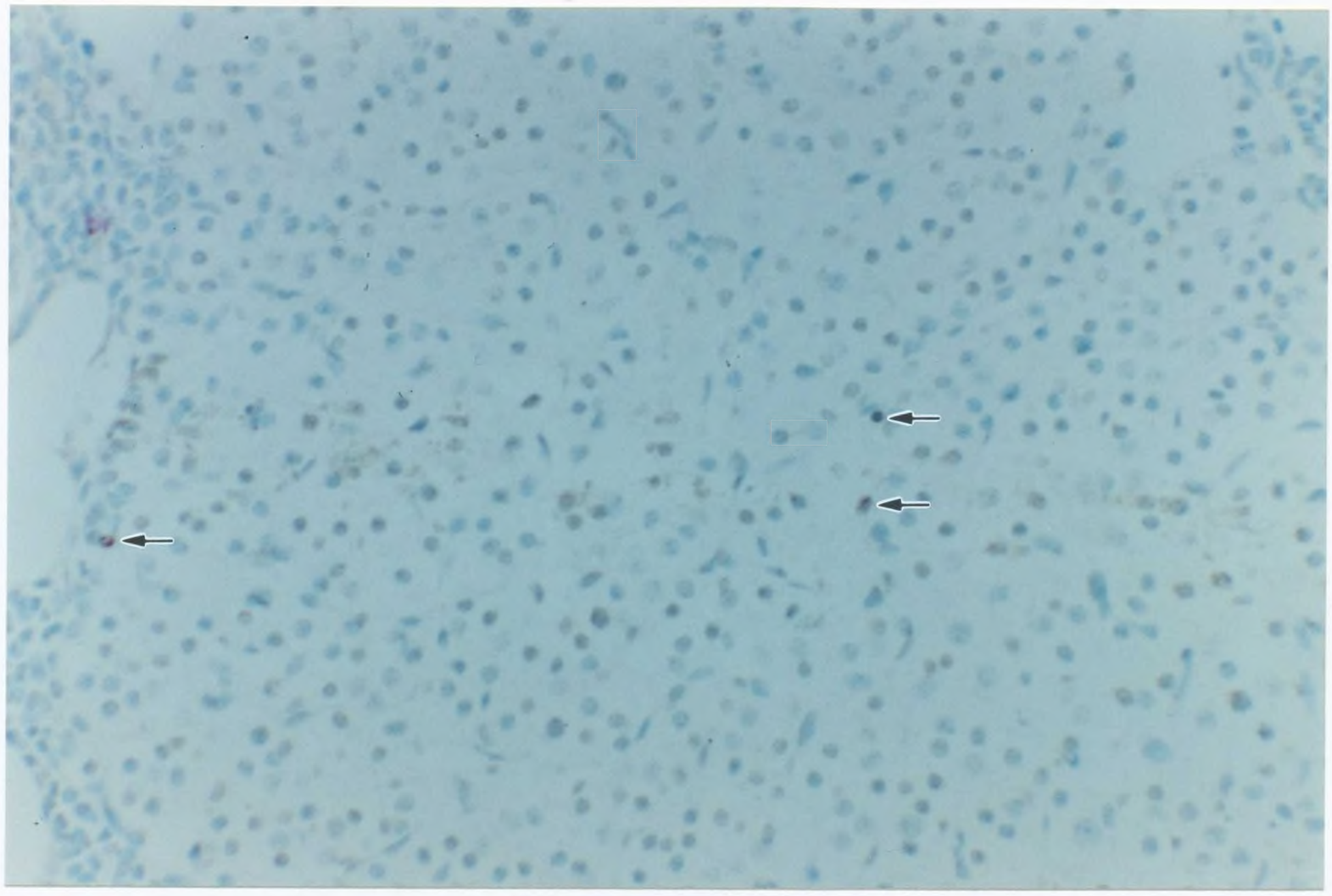
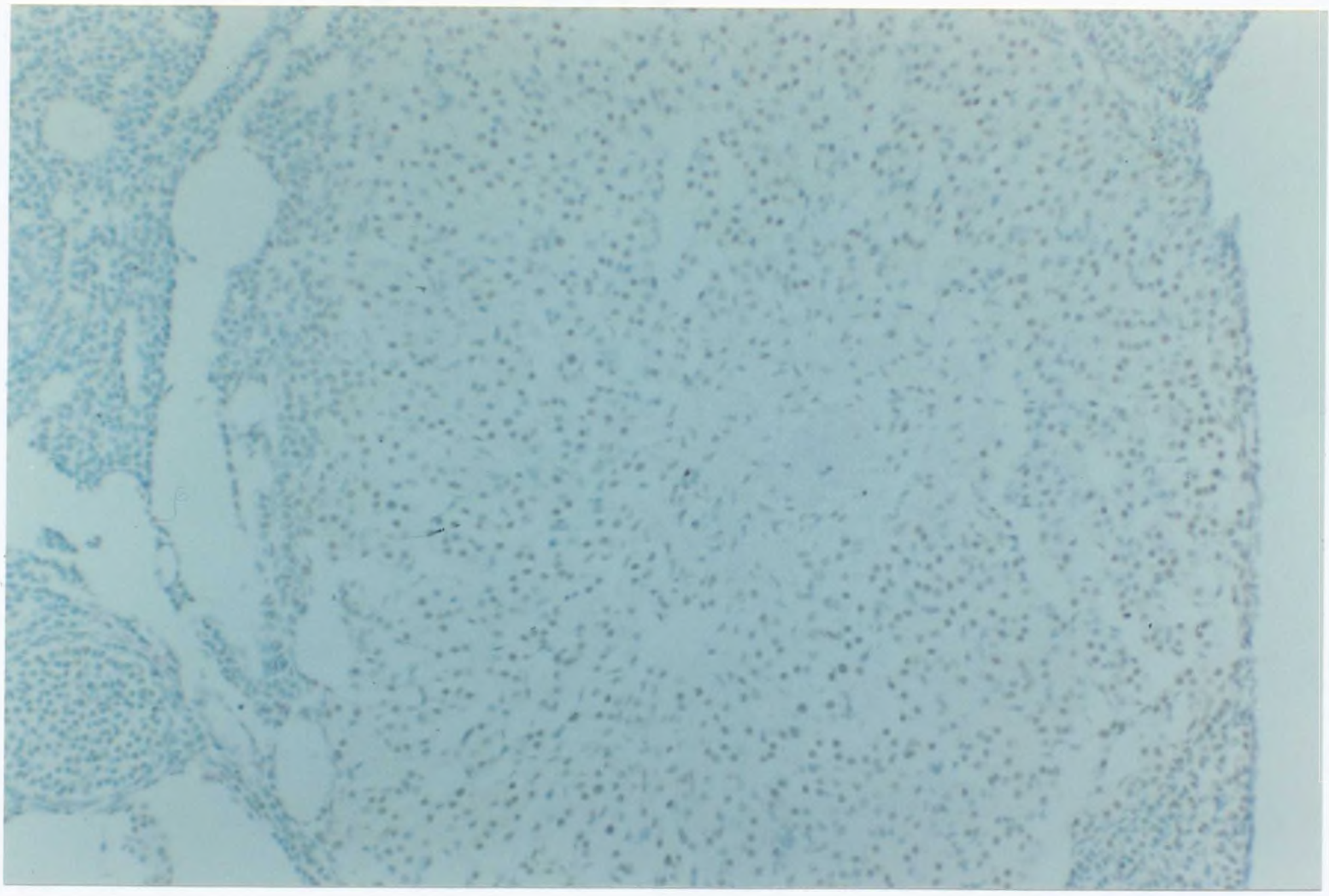


Figure 5: Day 2 2100 hours, CL demonstrates healthy vasculature with the exception of an avascular center. (100X)

Figure 6: Magnified view of figure 5. The avascular center contains three apoptotic cells (Arrows). (200X)



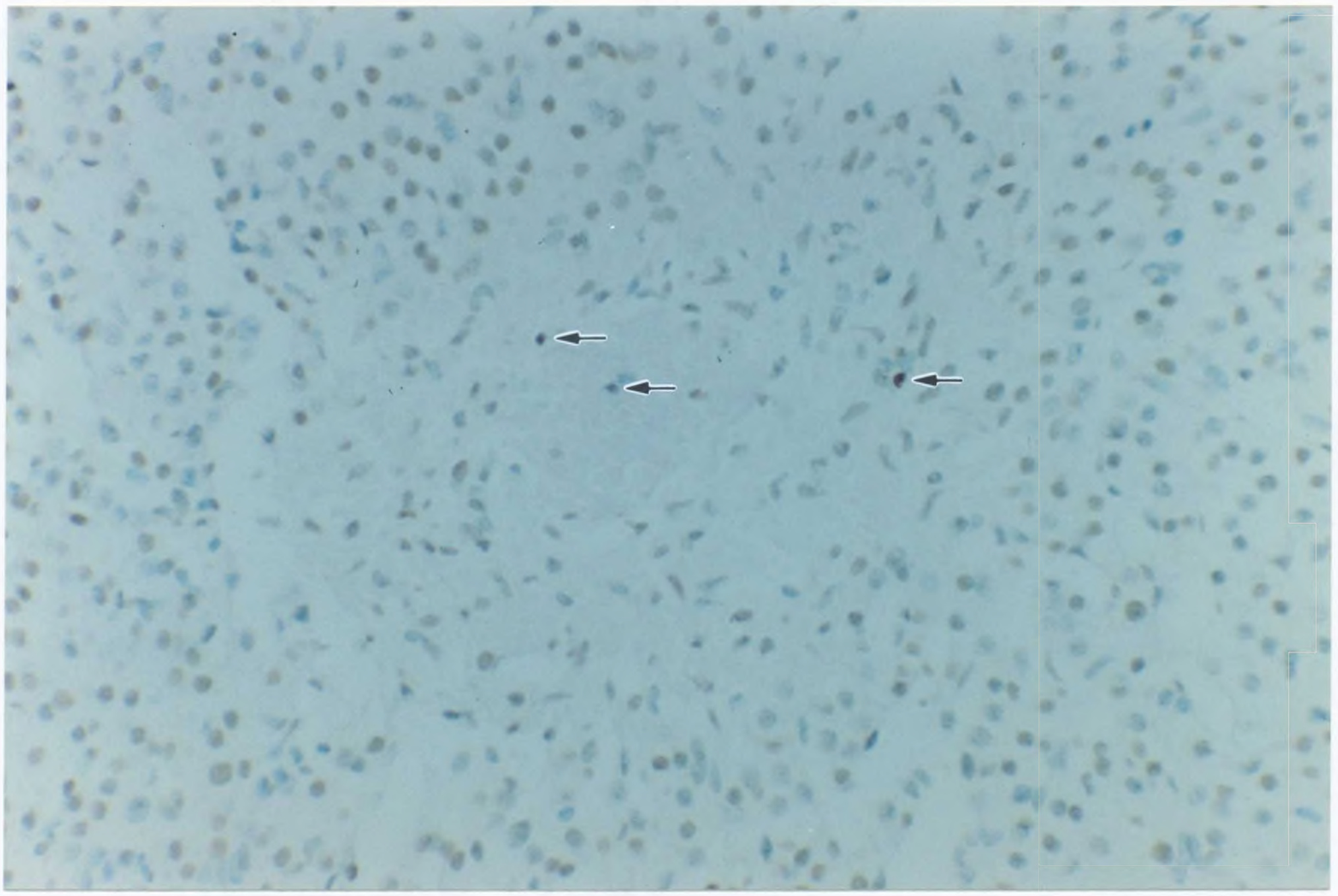
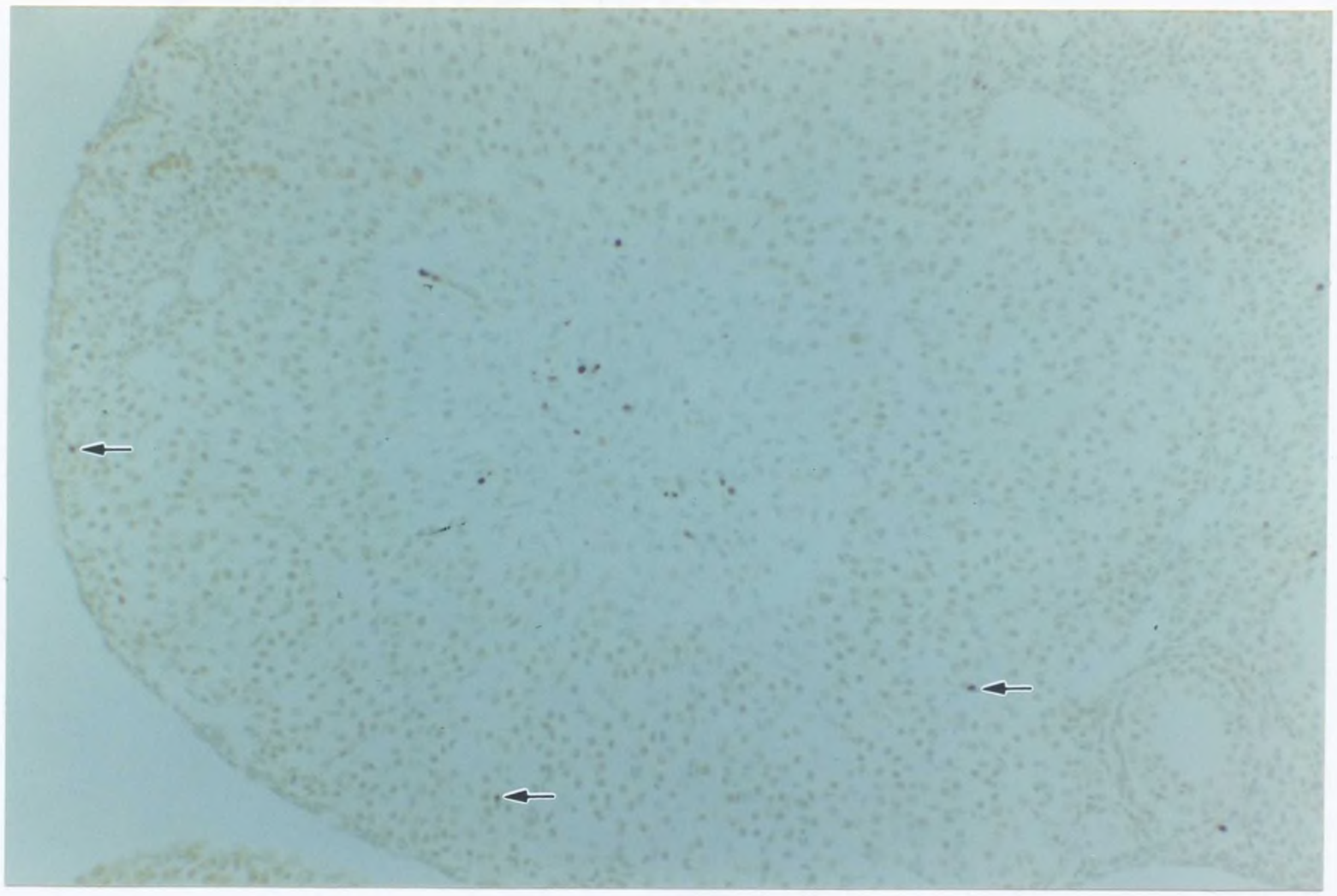


Figure 7: Apoptotic activity is apparent in the center of this day 2 2400 hour CL. Three apoptotic cells are identified in the periphery of this CL (Arrows). (100X)

Figure 8: Day 2 2400 hours, A few apoptotic cells can be identified scattered throughout the CL. The avascular center is located in the lower right of this field. (200X)



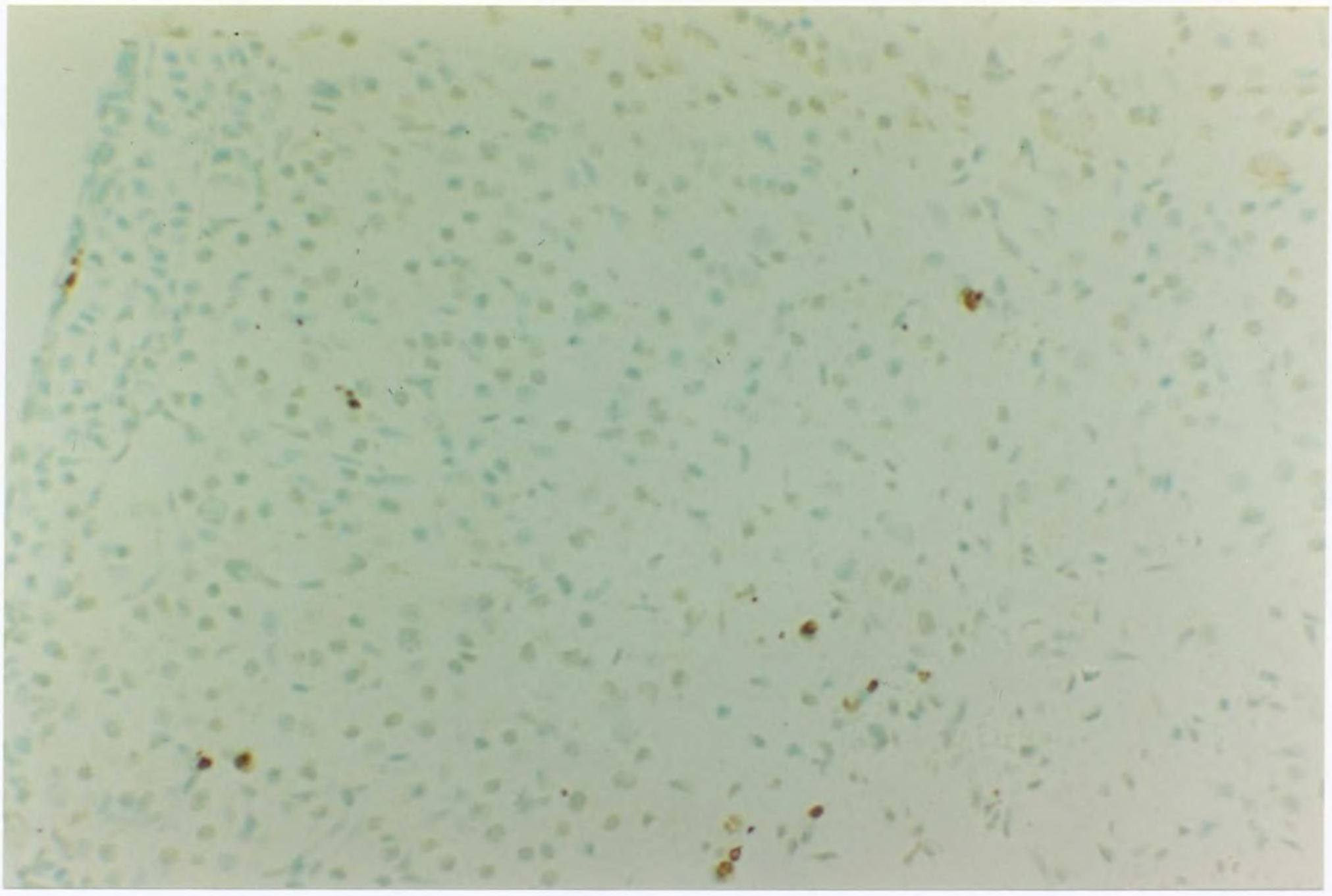
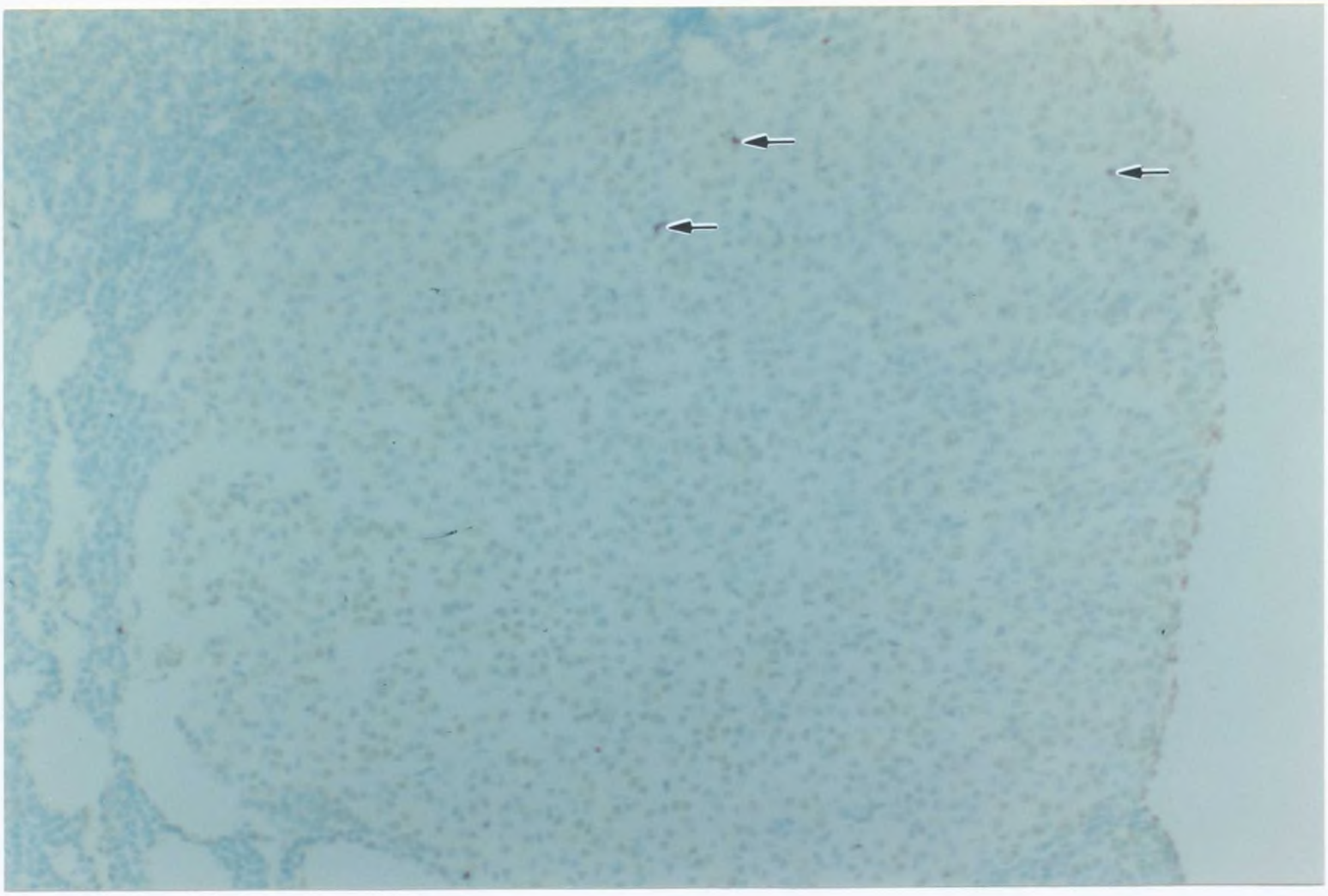


Figure 9: Day 3 0300 hours, CL showing a healthy vascular system. Three apoptotic cells can be identified (Arrows). (100X)

Figure 10: Day 3 0300 hours, CL demonstrating healthy morphology. Three apoptotic cells can be identified (Arrows). (200X)



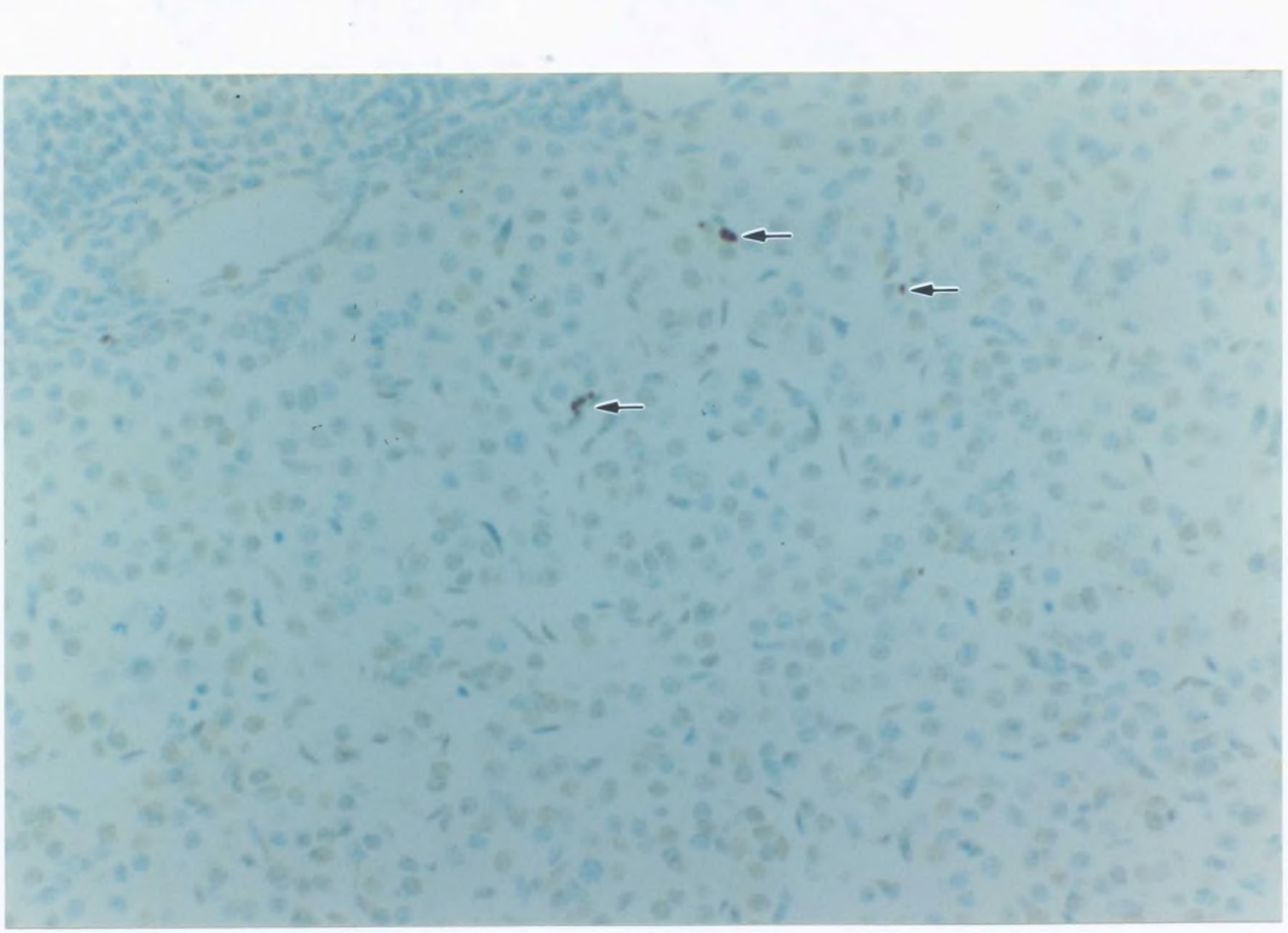
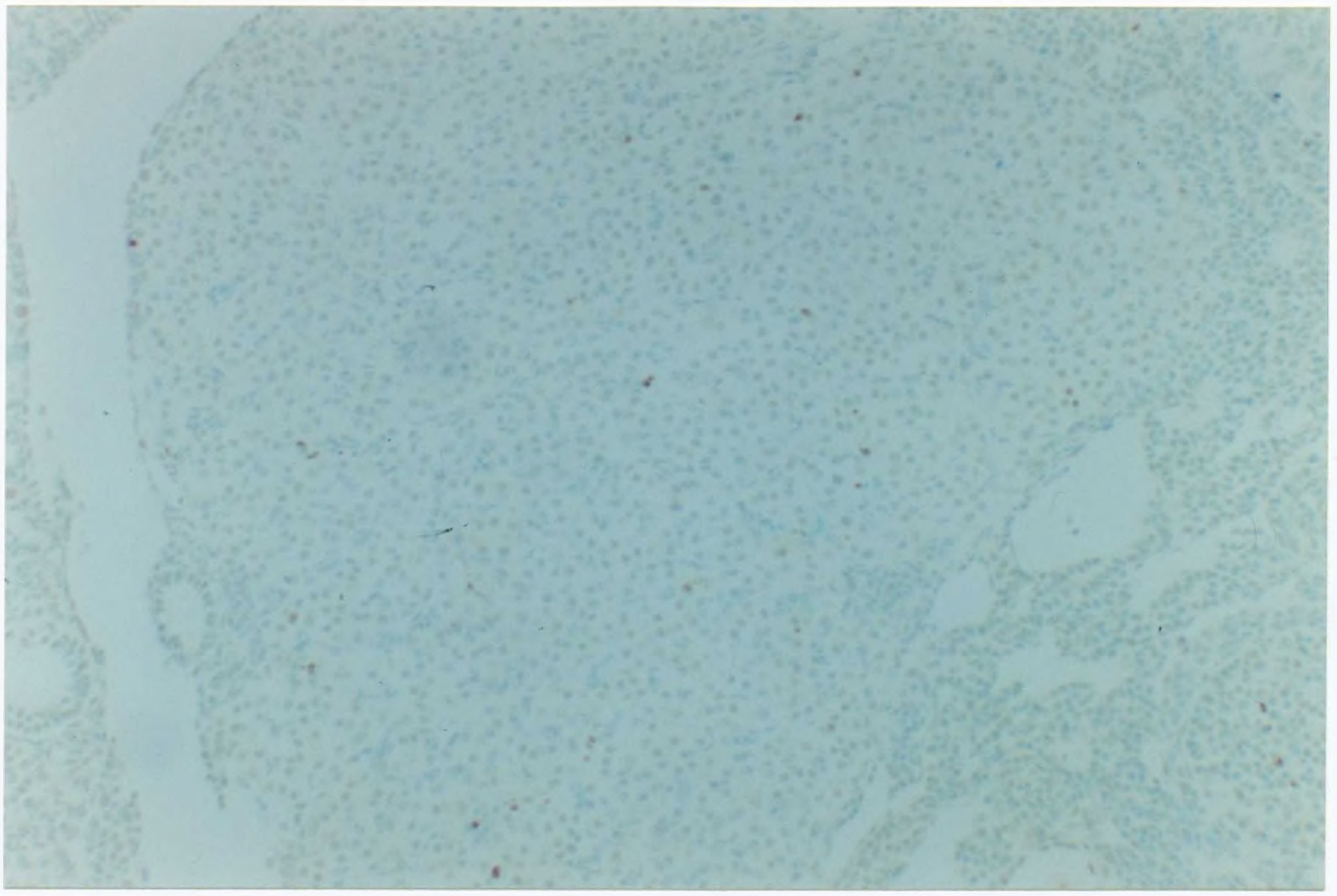


Figure 11: Day 3 0600 hours, Several apoptotic cells can be identified. (100X)

Figure 12: Day 3 0600 hours, CL demonstrates neutrophilic infiltration and invasion into extravascular space. Several cells stain positive for apoptosis. (400X)



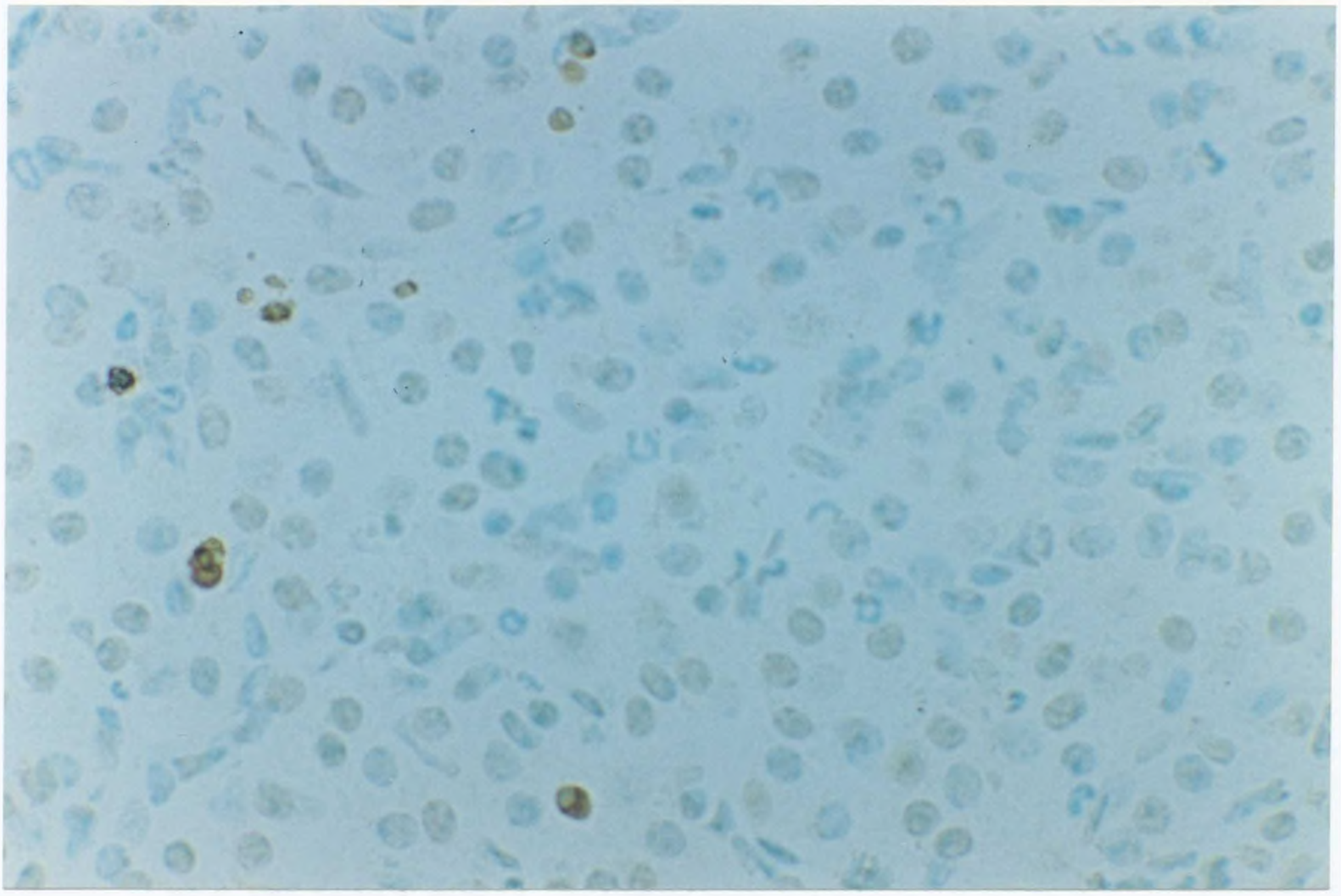
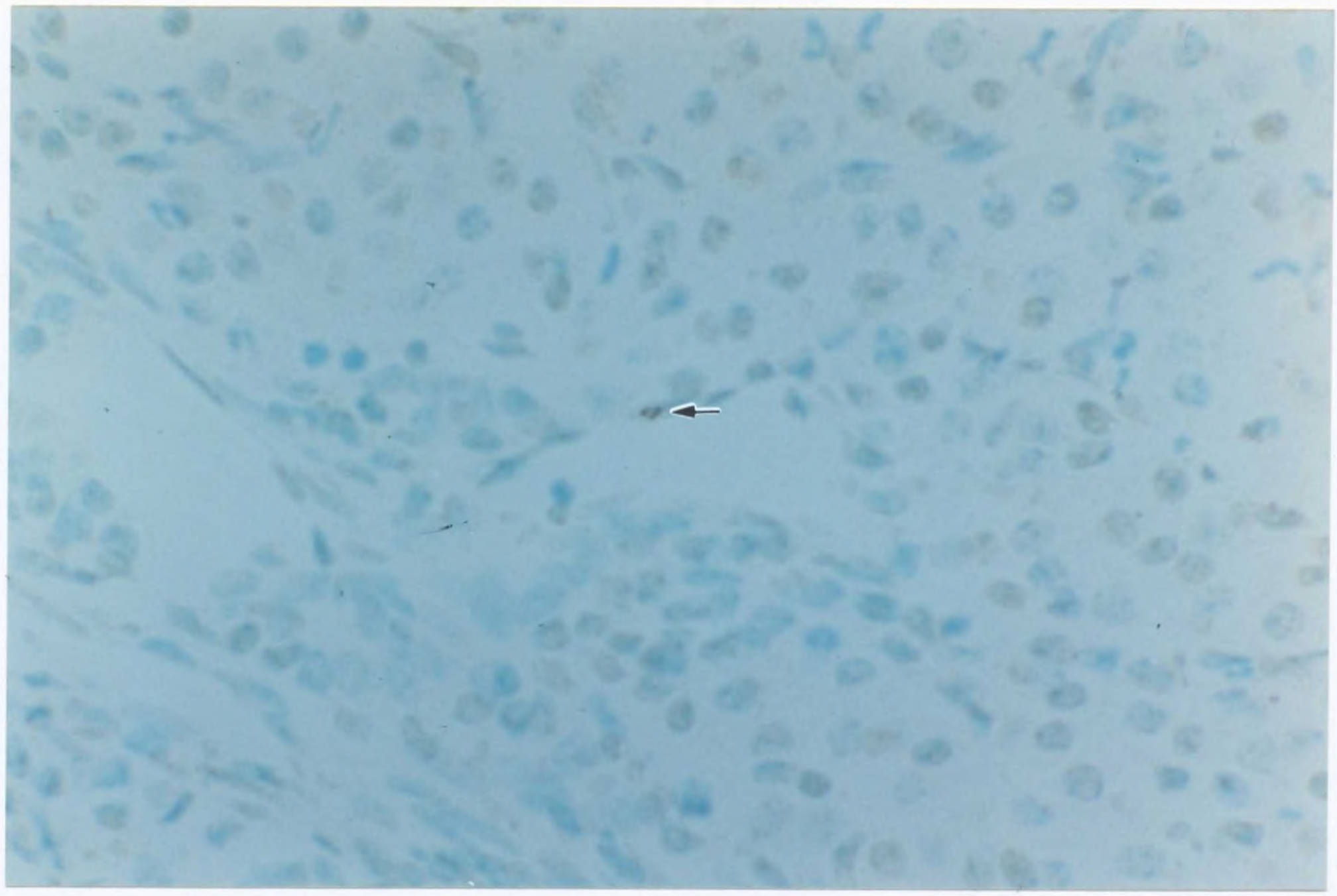


Figure 13: Day 3 0600 hours, Endothelial cell displaying apoptotic activity (Arrow). (400X)

Figure 14: Day 3 0900 hours, Two adjacent Corpora Lutea demonstrating extensive apoptotic activity .(100X)



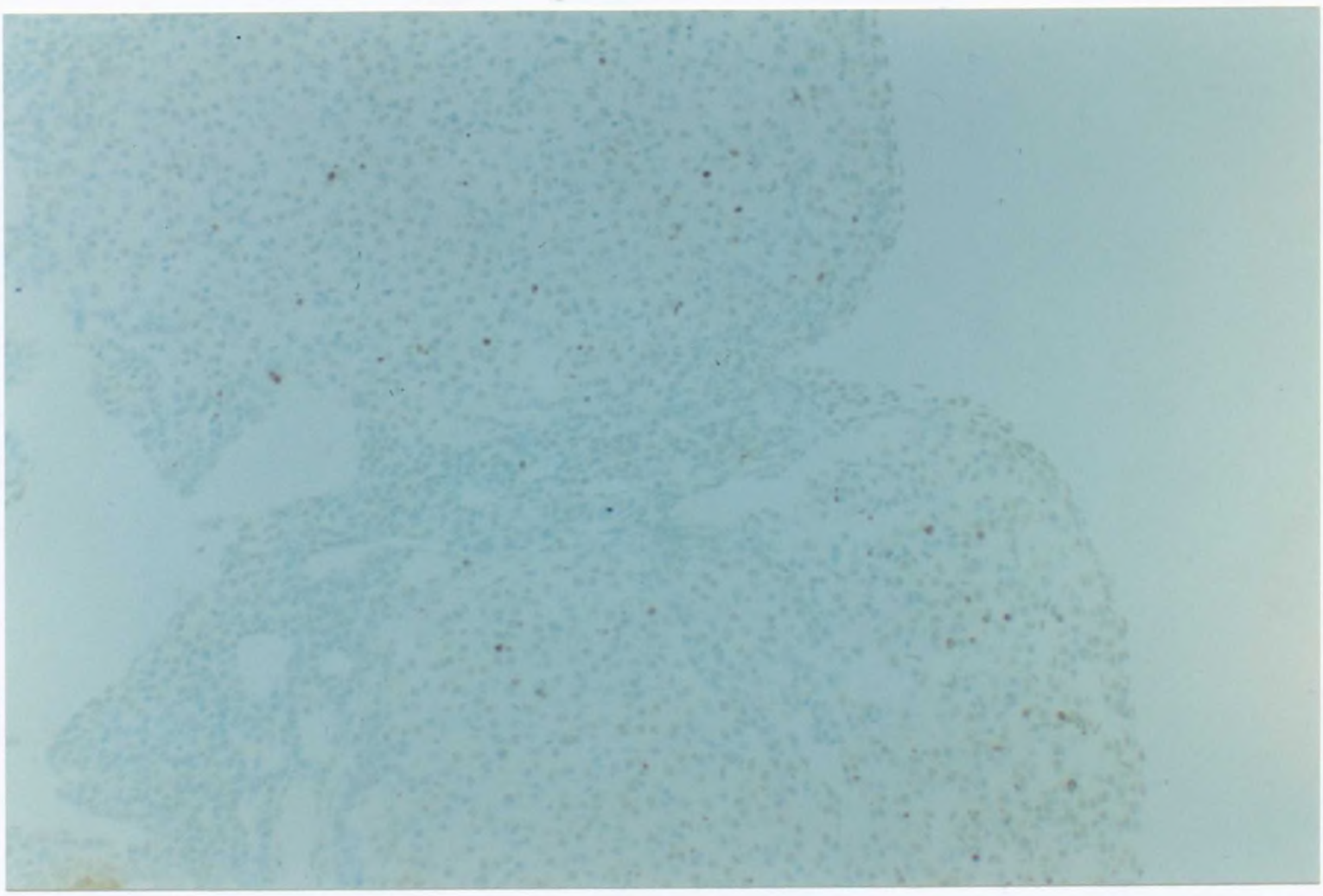
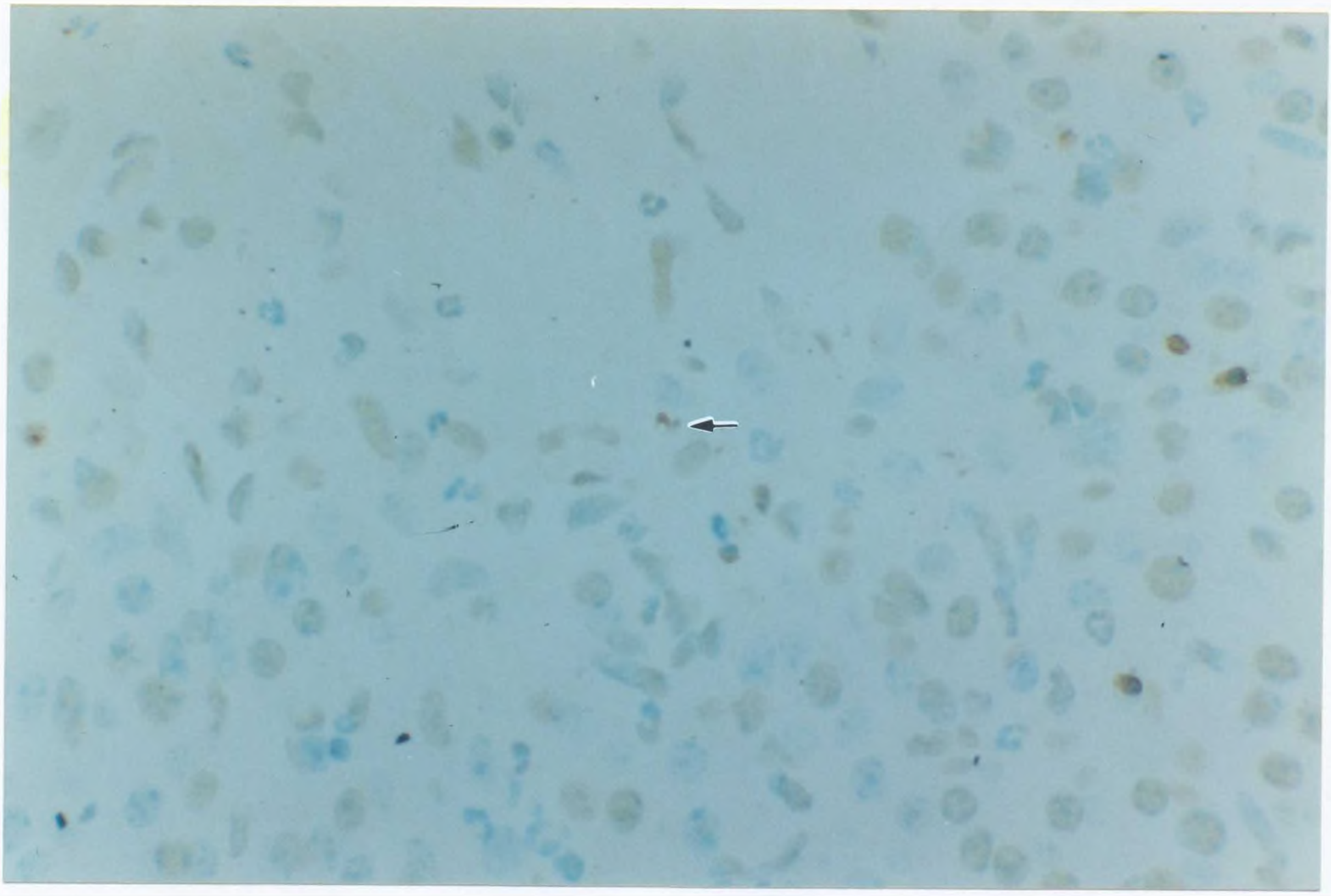


Figure 15: Day 3 0900 hours, CL with an apoptotic neutrophil cell in the center of the field (Arrow). Several neutrophils are identified as well as several apoptotic luteal cells. (400X)

Figure 16: Day 3 0900 hours, Apoptotic endothelial cells are identified in the center and right side of this field (Arrows). (400X)



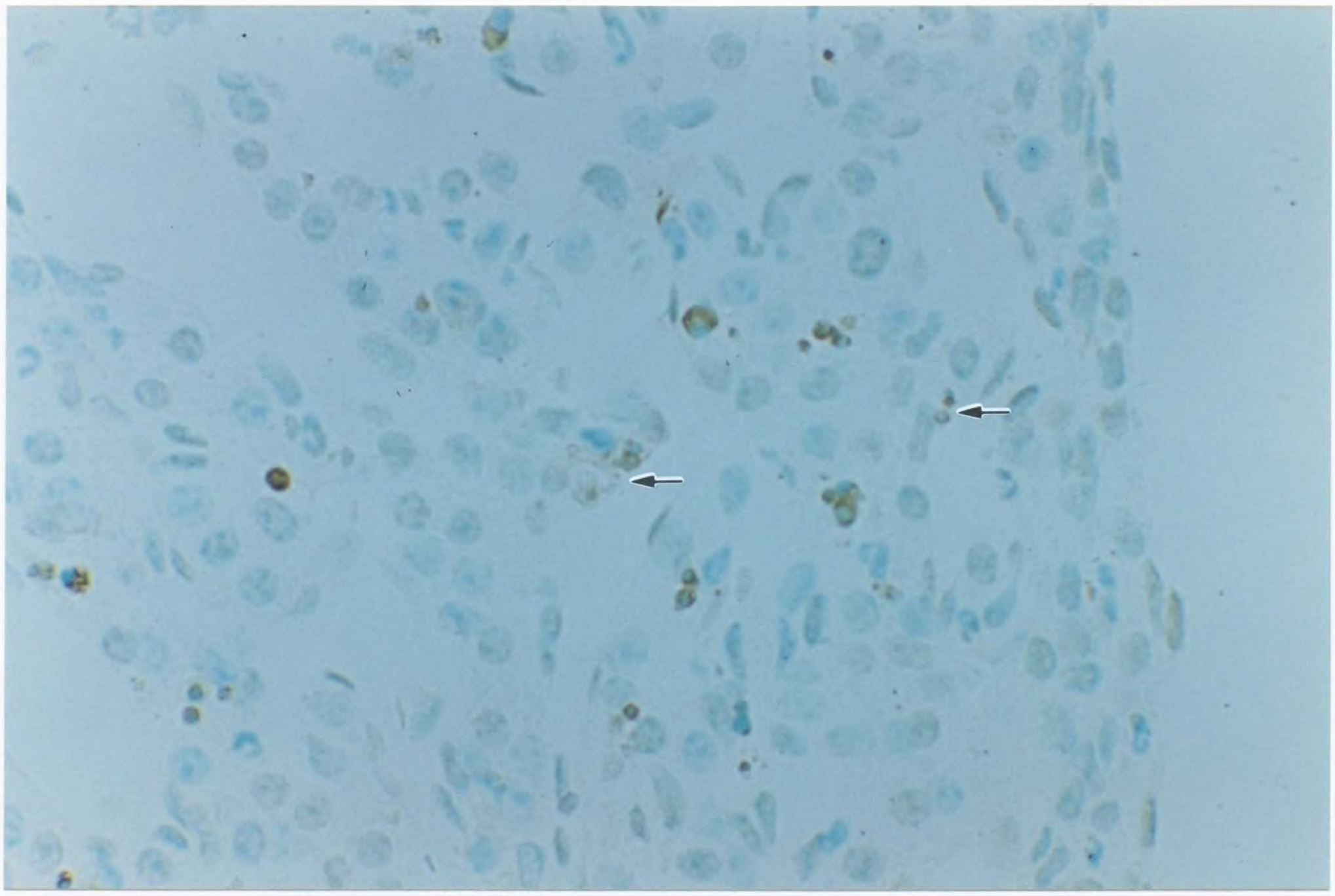
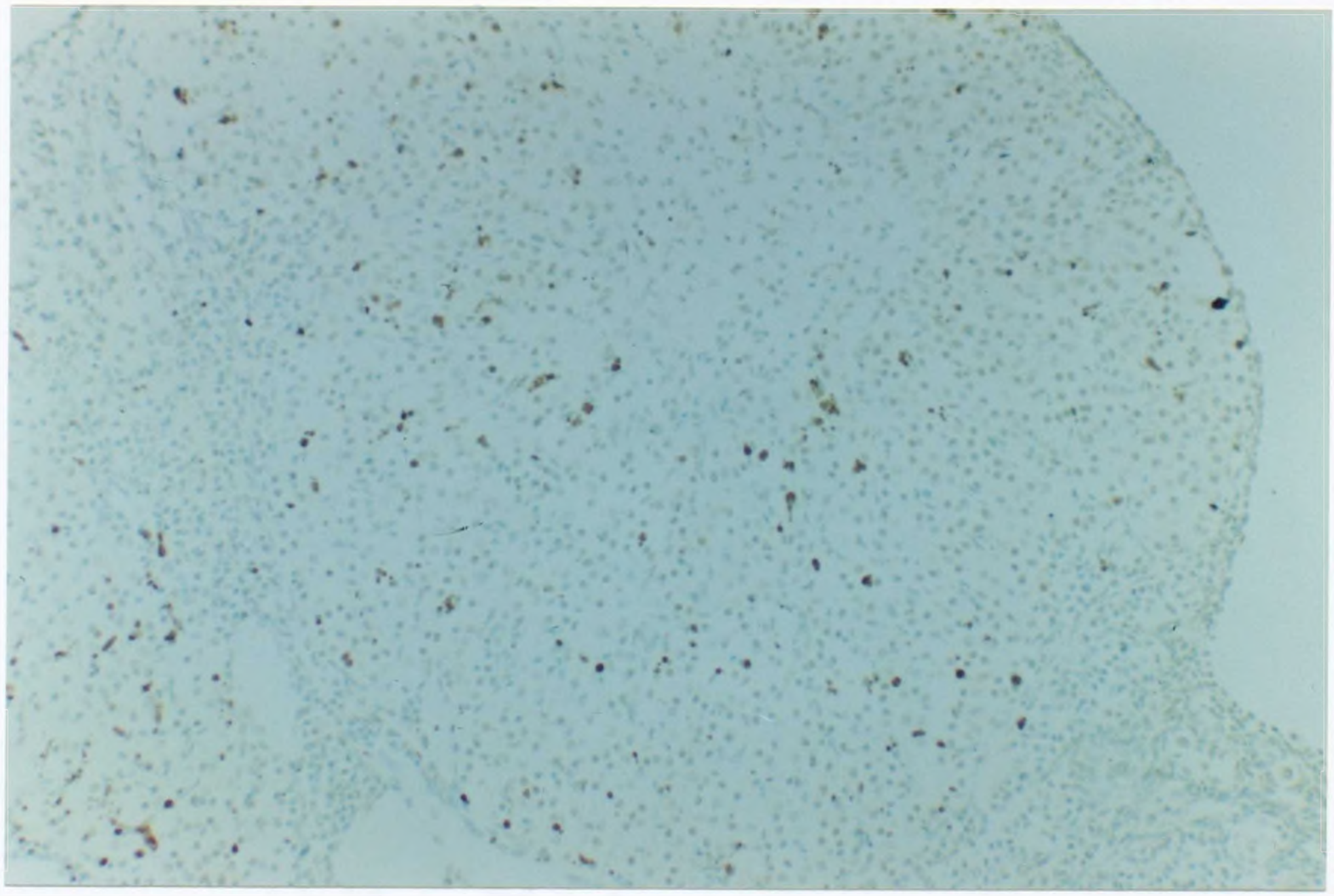


Figure 17: Day 3 1200 hours, CL demonstrating wide spread apoptotic activity. (100X)

Figure 18: Day 3 1200 hours, Apoptotic endothelial cells can be identified in the center of this field (Arrows). (200X)



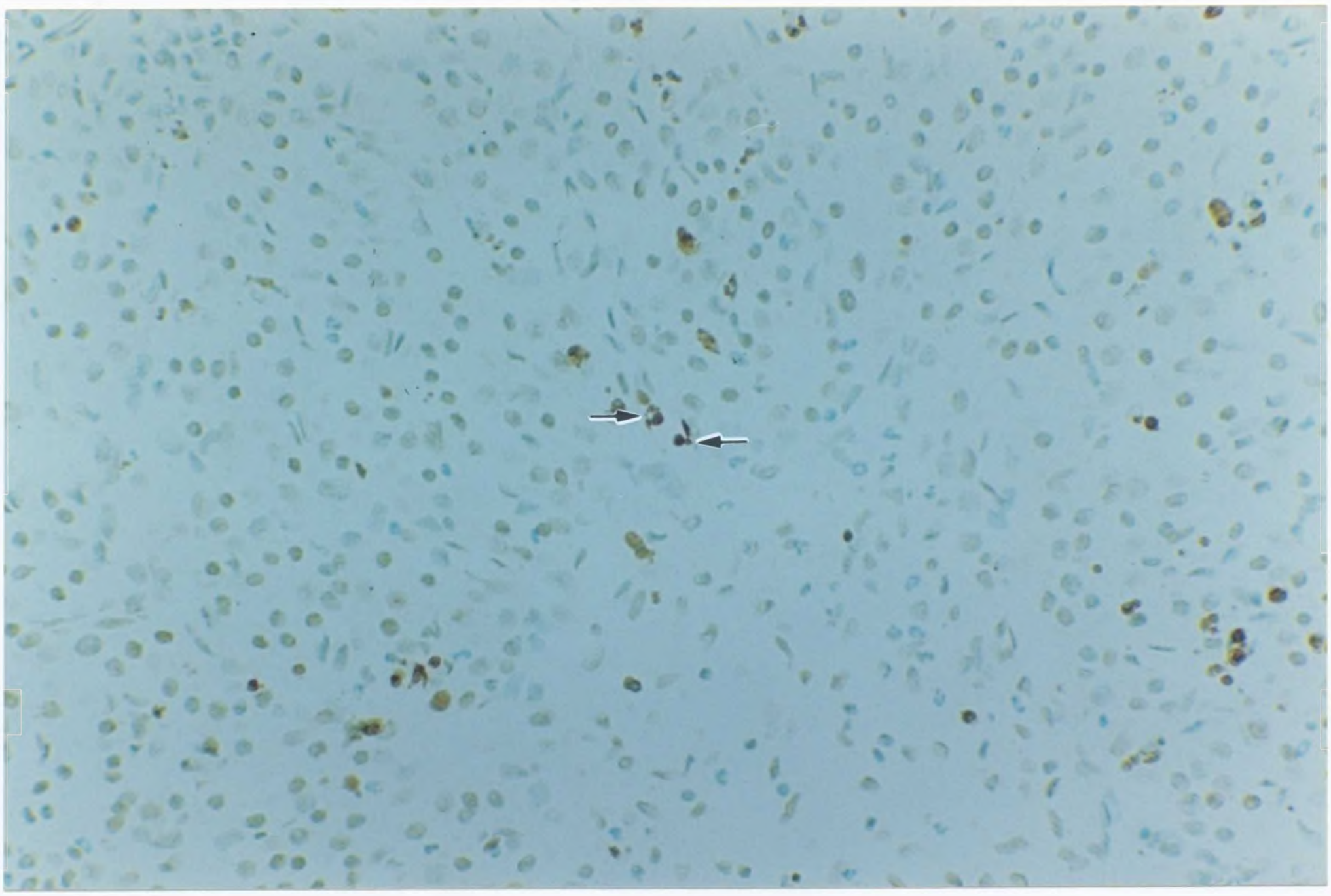
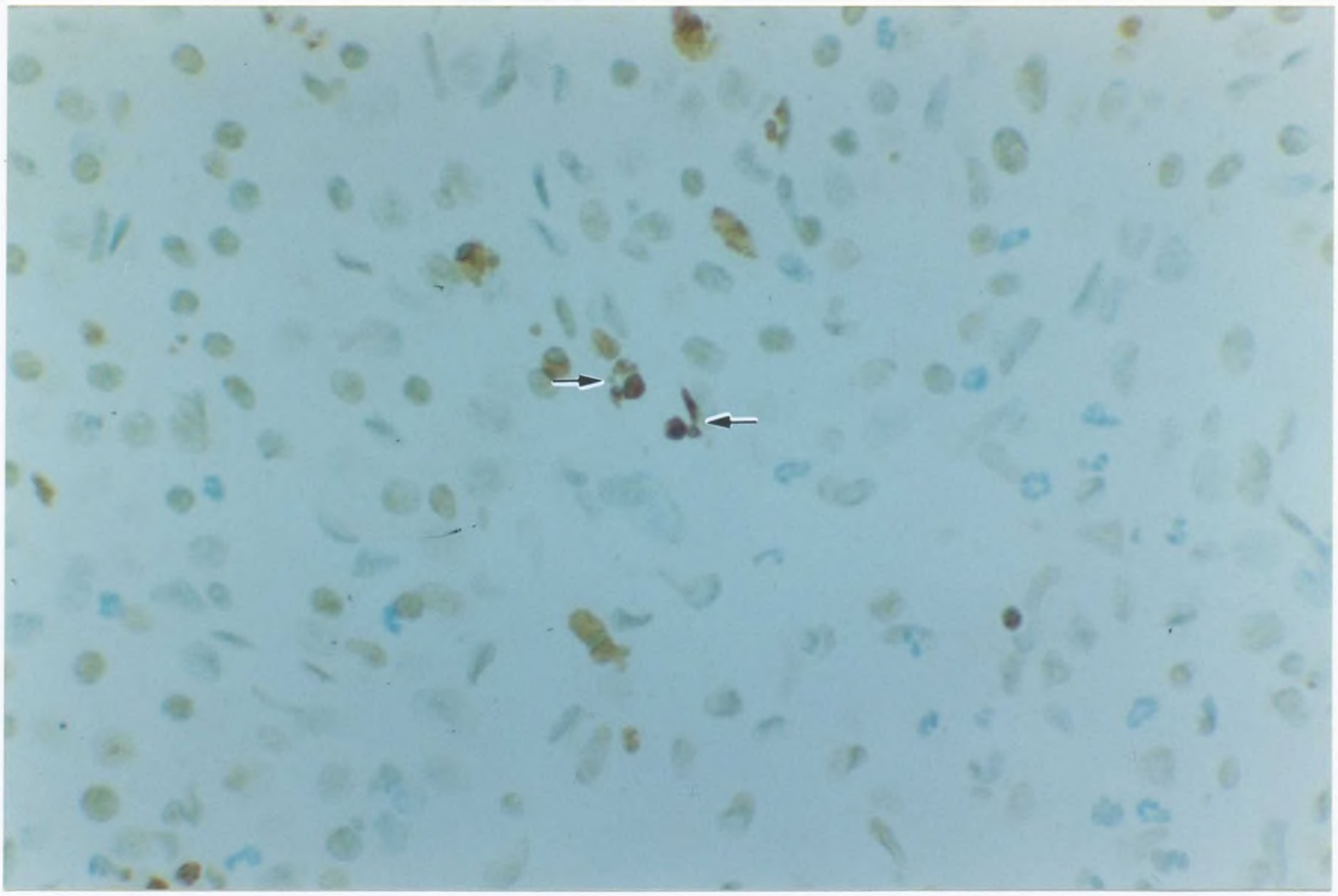


Figure 19: High power view of figure 18 demonstrating endothelial cells apoptosis in the center of the field (Arrows). (400X)

Figure 20: Day 3 1200 hours, CL demonstrating neutrophilic infiltration. An apoptotic endothelial cell can be identified in the upper left (Arrow). (400X)



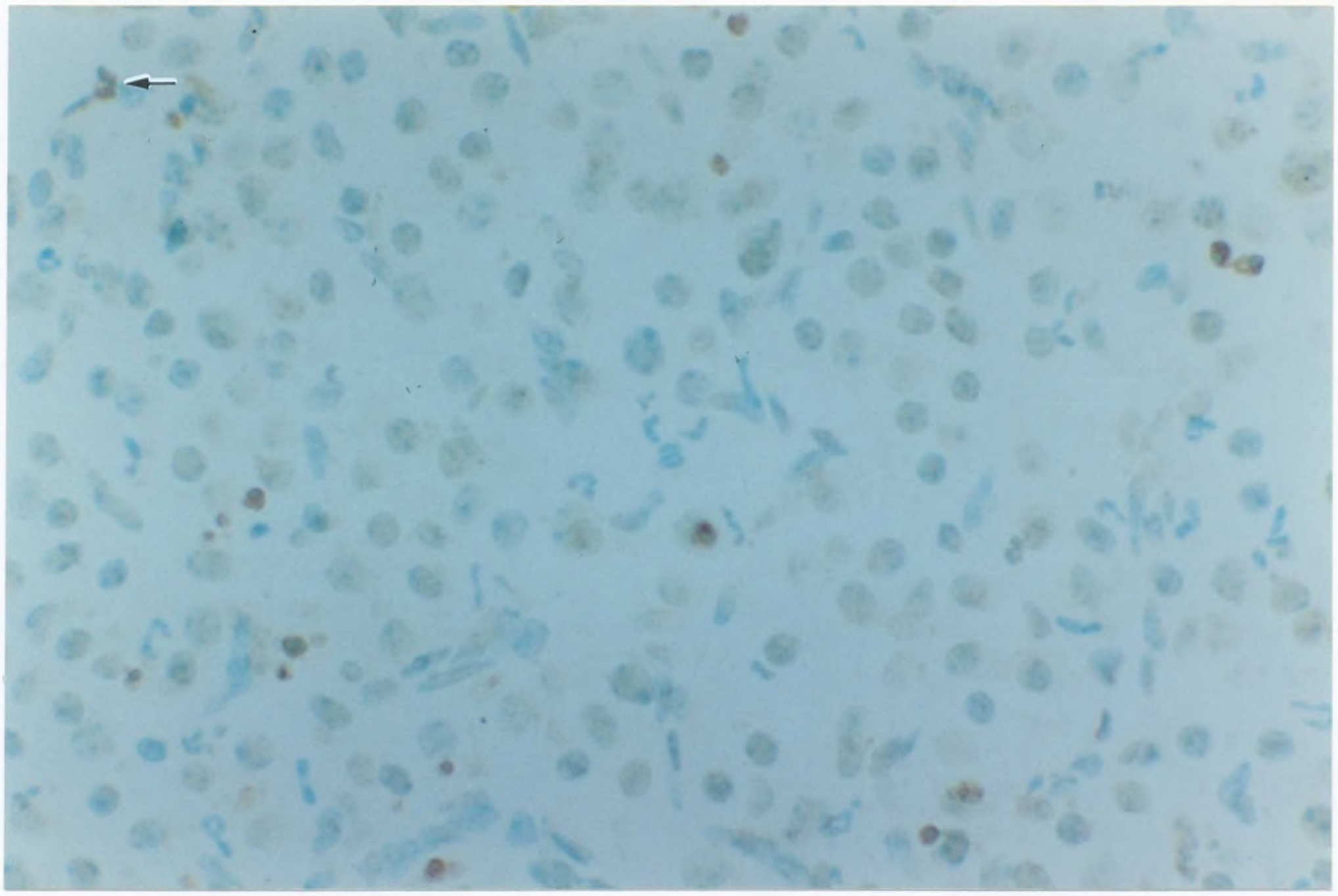
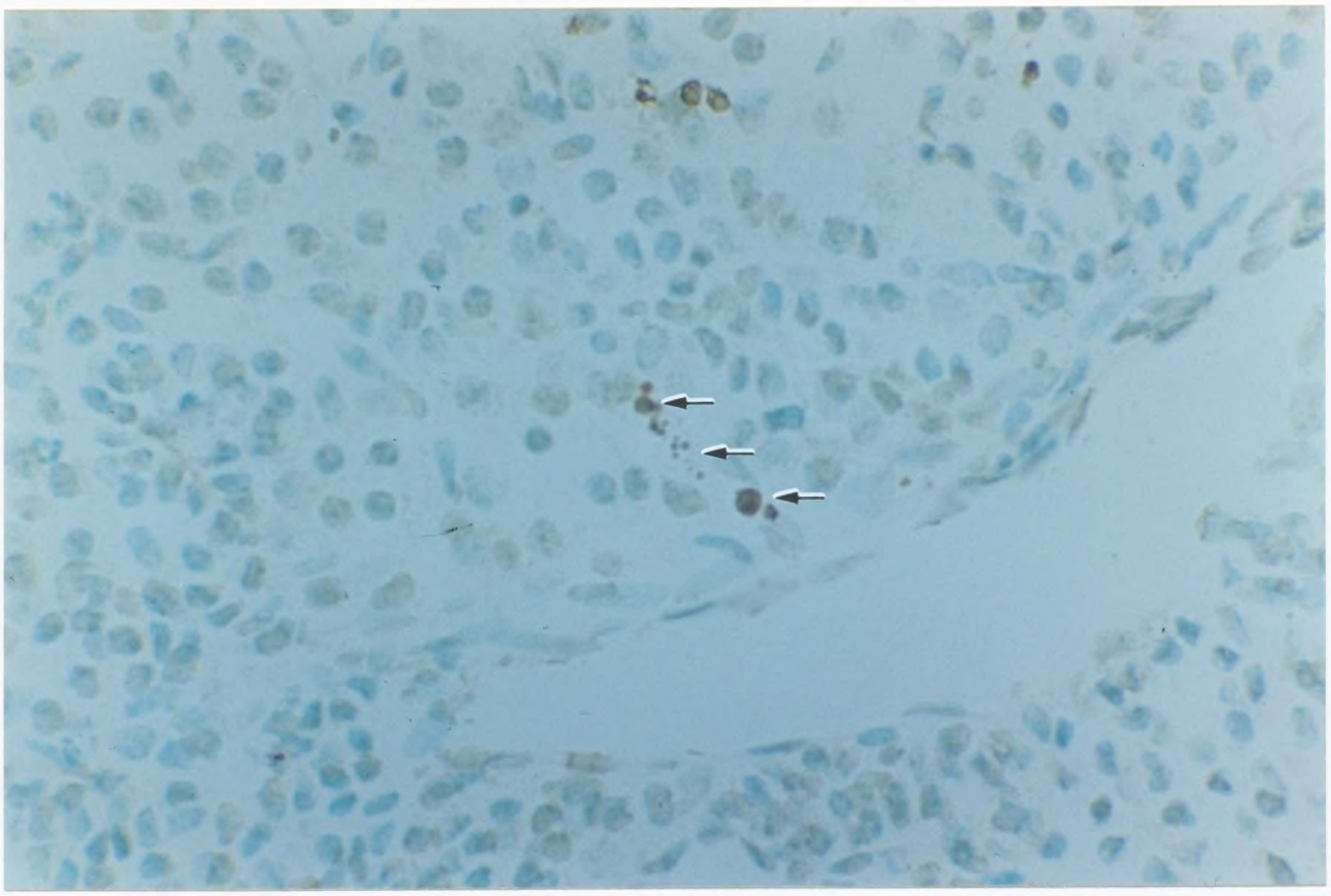


Figure 21: Day 3 1200 hours, CL with apoptotic debris filling vasculature (Arrow). (400X)

Figure 22: Oil immersion view of figure 21 demonstration vasculature plugged with apoptotic debris.(1000X)



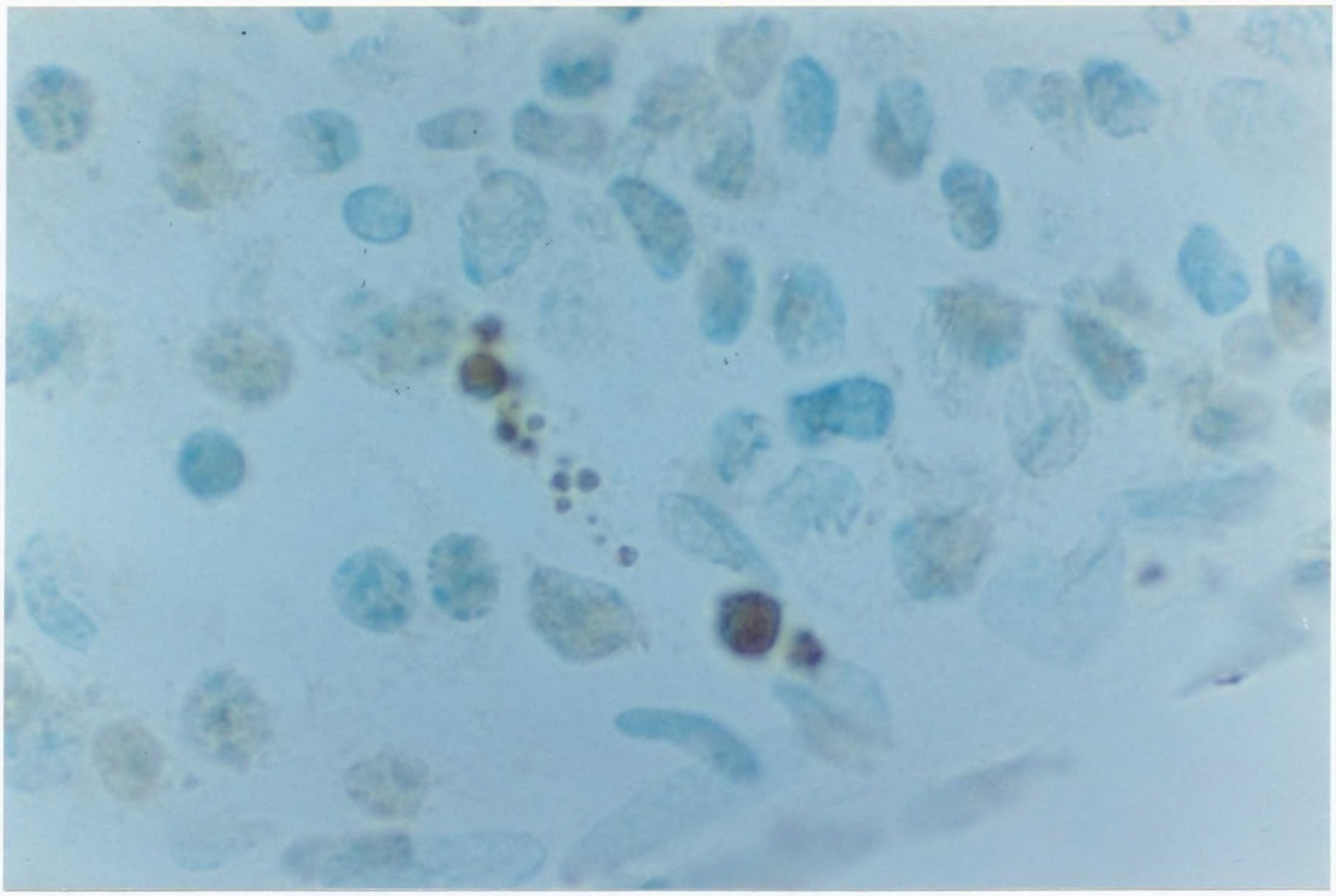
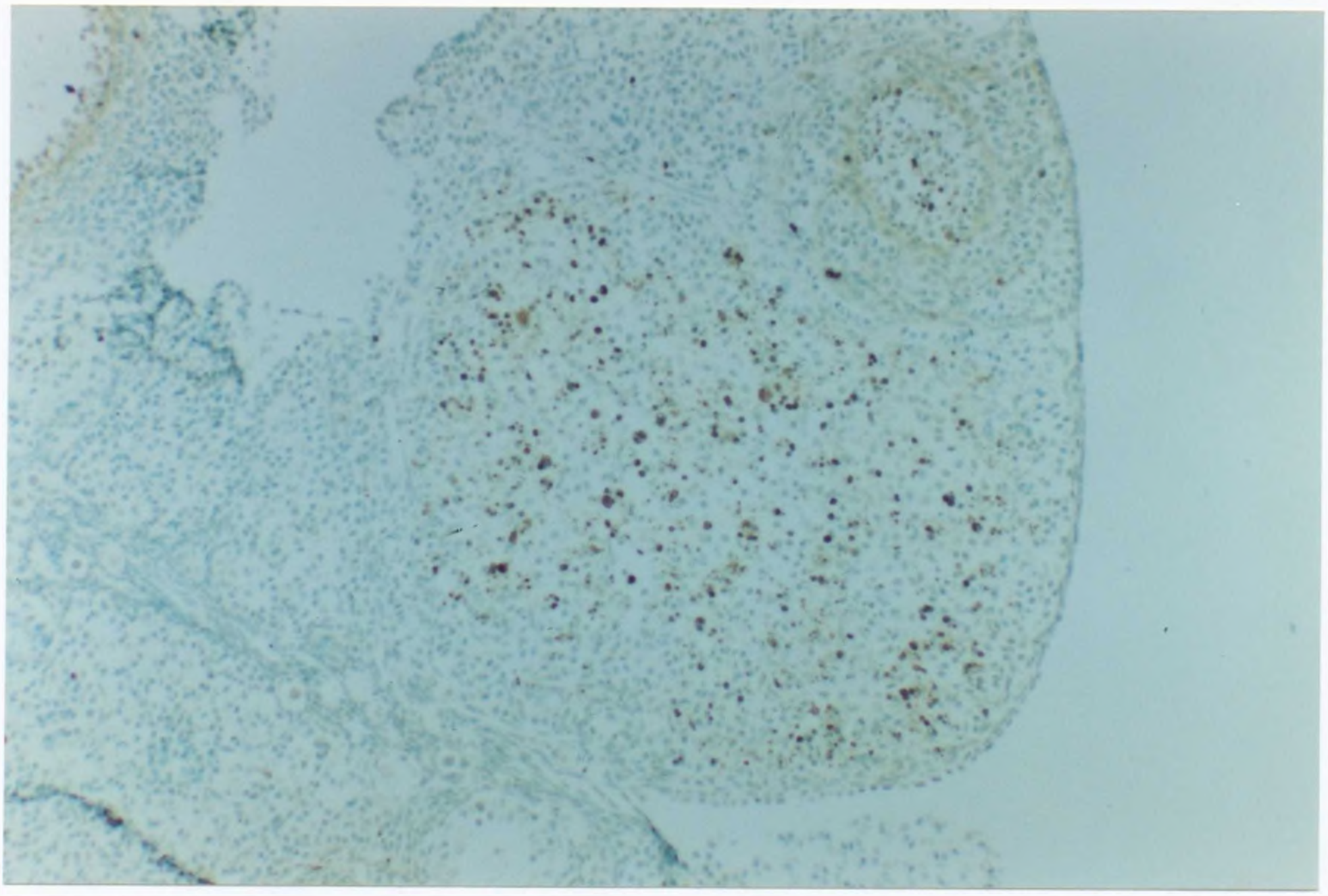


Figure 23: Day 4 1200 hours, CL demonstrating extensive apoptotic activity.(100X)

Figure 24: Day 4 1200 hours, Avascular CL with advanced apoptosis of luteal cells.



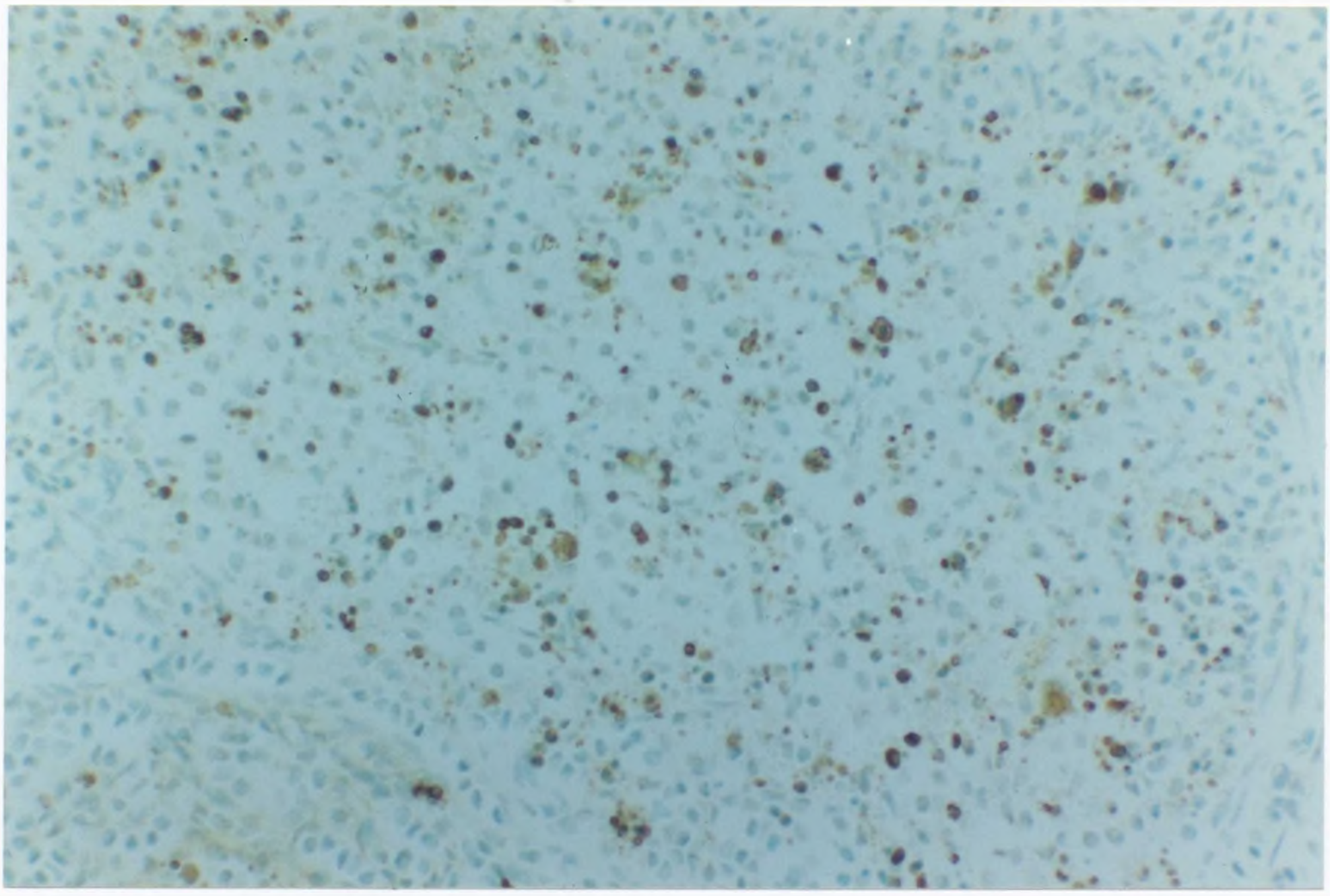
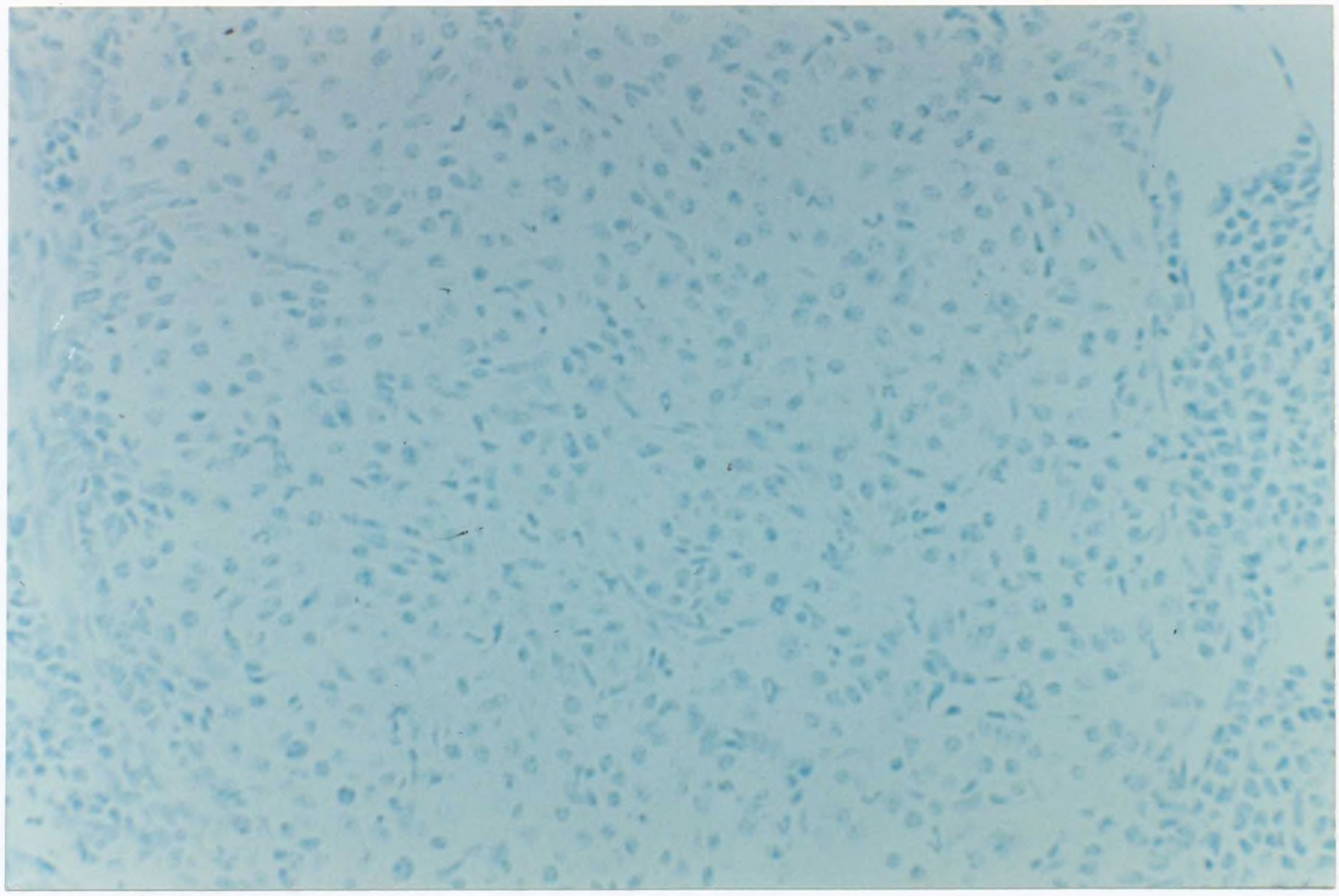


Figure 25: Day 3 0600 hours, TdT(-) control, no staining of apoptotic cells is observed.(200X)

Figure 26: Day 4 1200 hours, TdT(-) control demonstrating absence of staining of apoptotic cells.(100X)



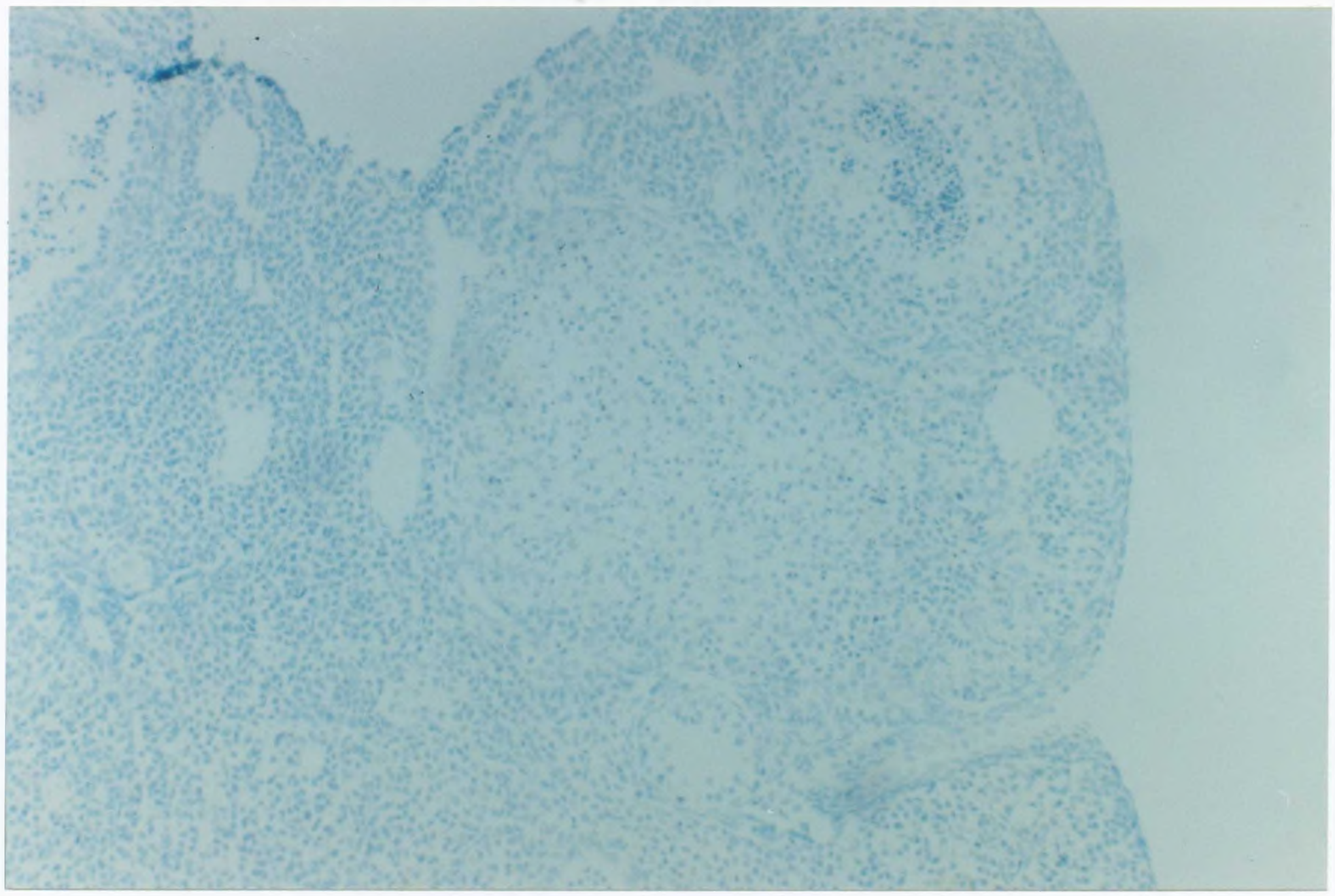
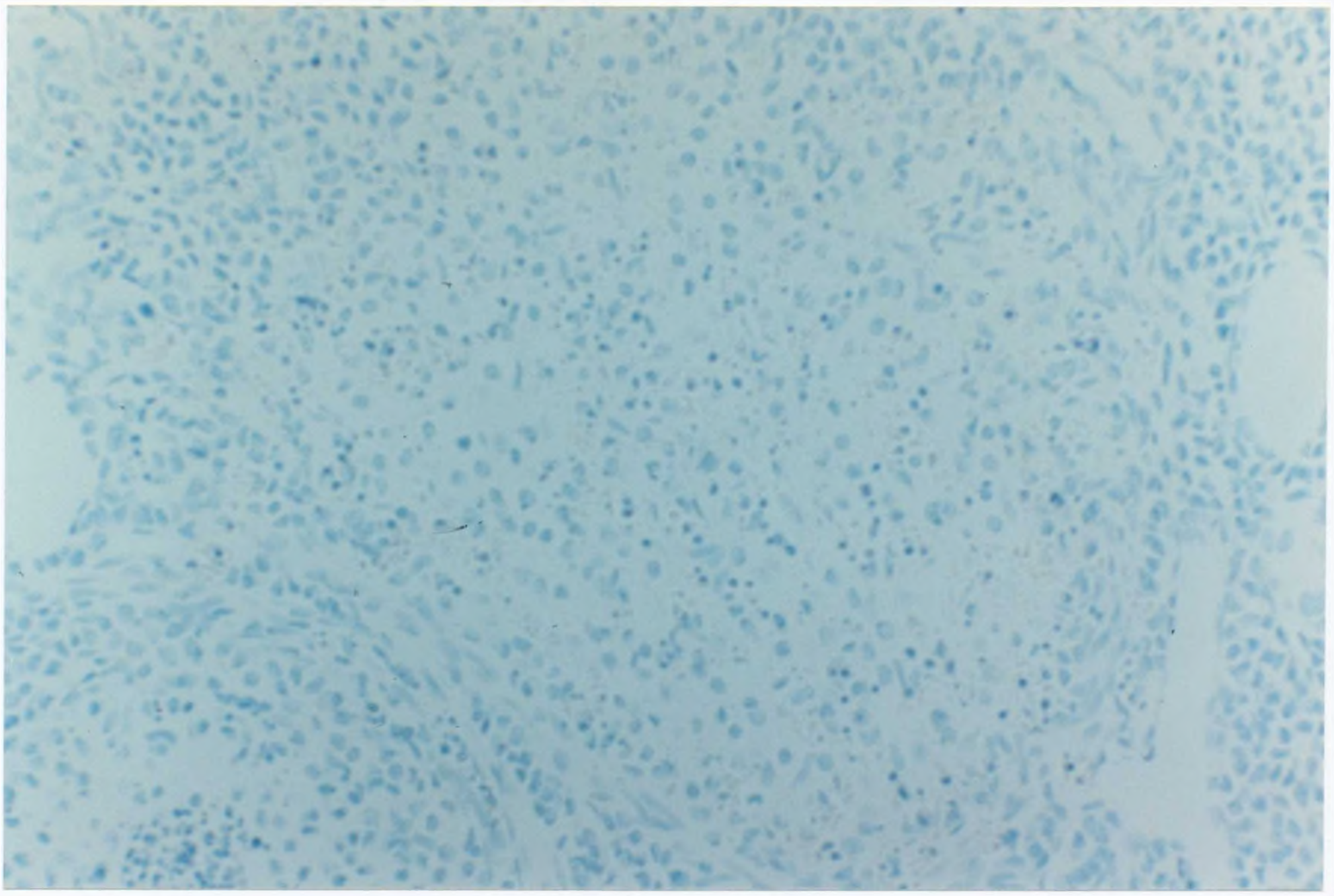


Figure 27: Day 4 1200 hours ,high power view of figure 26.(200X)



Araki,S.,Simada,Y.,Kaji,K.,Hayashi,H. 1990 Apoptosis of vascular endothelial cells by fibroblast growth factor deprivation. Biochem. and Biophys. Research Communication. 168(3):1194-1199.

Araki,S.,Ishida,T.,Yamamoto,T.,Kaji,K.,Hayashi,H. 1993 Induction of apoptosis by hemorrhagic snake venom in vascular endothelial cells. Biochem. and Biophys. Research Communications. 190(1):148-153.

Araki,S.,Simada,Y.,Kaji,K., and Hayashi,H. 1990 Role of protein kinase C in the inhibition by fibroblast growth factor of apoptosis in serum-depleted endothelial cells. Biochem. And Biophys. Research Communications. 172(3):1081-1085.

Arends,M.J.,Morris,R.J.,and Wyllie,A.H. 1990 Apoptosis-the role of the endonuclease,American J. of Pathology,136:593-608.

Azmi,T.I. and O'Shea,J.D. 1984 Mechanism of deletion of endothelial cells during regression of the corpus luteum, Laboratory investigations 51:206-217.

Benyo,D.F. and Pate,J.L. 1992 Tumor necrosis factor-α alters bovine luteal cell synthetic capacity and viability. Endocrinology 130(2):854-860.

Bagavandoss,p.,Wiggins,R.C.,Kunkel,D.G.,Remick,D.G.,Keyes,P.L. 1990 Tumor necrosis factor production and accumulation of inflammatory cells in the corpus luteum of pseudopregnancy and pregnancy in rabbits. Biology of Reproduction 42:367-376.

Bajaj,M.S.,Kew,R.R.,Webster,R.O.,Hyers,T.M. 1992 Priming of human neutrophil functions by tumor necrosis factor. Inflammation 16(3)241-250.

Burns,P.D.,Spitzer,J.C.,Burns,G.L., and Plyler,B.B. 1993 Inhibition of estrus and corpora lutea function with norgestomet. Therionology 39:863-873.

Bursch,W.,Oberhammer,F.,Schulte-Hermann,R. 1992 Cell death by apoptosis and its protective role against disease. 15:245-251.

Cohen,J.J. 1993 Apoptosis:The physiologic pathway of cell death. Hospital Practice Dec 15:35-43.

Compton,M.M. 1992Abiochemical hallmark of apoptosis: internucleosomal degradation of the genome. Cancer and Metastasis Reviews 11:105-119.

Duby,R.T.,McDaniel,J.W.,Spilman,C.H.,Black,D.L. 1969 Utero-ovarian relationships in the golden hamster. ACTA Endocrinologica 60:595-620.

Elliot,S.J.,Doan,T.N. 1993Oxidant stress inhibits the store-dependant Ca^{2+}-influx pathway of vascular endothelial cells. Biochem. J. 292:385-393.

Endo,T.,Aten,R.F.,Leykin,L., and Behrman,H.R. 1993 Hydrogen peroxide evokes antisteroidogenic and antigonadotropic actions in human granulosa luteal cells. J. Of Clinical Endocrinology and Metabolism 76(2):337-342.

Fesus,L. 1993 Biochemical events in naturally occuring forms of cell death. Federation of European Biochemical Societies 328(1,2):1-5.

Fukumatsu,Y.,Katabutchi,H.,Naito,M.,Takeya,M.,Takahashi,K., And Okamura,H. 1992 J. Repoductive Fert. 96:241-249.

Gavrieli,Y.,Sherman,Y.,Ben-Sasson,S.A. 1992 Identification of pragrammed cell death In Situ via specific labeling of Nuclear DNA fragmentation. J. of Cell Biology 119(3):493-501.

Gold,R.,Schmied,M.,Rothe,G.,Zischler,H.,Breitschopf,H.,WekerL,H.,Lassmann,H. 1993 Detection of DNA fragmentation in apoptosis: Aplicationof In Situ Nick Translation to cell culture systems and tissue sections. J. of Histochemistry and Cytochemistry 41(7):1023-1030.

Greenwald,G.S. 1968 Formation and maintainance of corpora lutea in labratory animals. J. of Animal Science 27:139-162.

Han,Z.C., and Caen,J.P. 1993 Are megokaryocytes and endothelial cells sisters? J. Lab. Clin. 121:821-825.

Hennig,B.,Wang,Y.,Ramasamy,S., and McClain,C.J. 1993 Zinc protects against tumor necrosis factor-induced disruption of porcine endothelial cell monolayer integrety. J.of Nutrition 123:1003-1009.

Hubbard,C.J.,Greenwald,G.S. 1982 Cyclic nucleotides,DNA,and steroid levels in ovarian follicles and corpora lutea of the cyclic hamster. Biology of Reproduction 26:230-240.

Juengel,J.L.,Garverick,H.A.,Johnson,A.L.,Youngquist,R.S.,and Smith,M.F. 1993 Apoptosis during luteal regression in cattle. Endocrinology 132(1):249-254.

Krishna,A., and Terranova,P.F. 1985 Alterations in mast cell degranulation and ovarian histamine in the proestrous hamster. Biology of Reproduction 32:1211-1217.

Nawroth,P.P.,and Stern,D.M. 1986 Modulation of endothelial cell hemostatic properties by tumor necrosis factor. J. experimental Medicine 163:740-745.

Niswender,G.D.,Juengel,J.L.,Mcguire,W.J.,Belfiore,C.J.,and Wiltbank,M.C. 1994 Luteal function: The estrous cycle and early pregnancy. Biology of Reproduction 50:239-247.

Oberhammer,F.,Fritsch,G.,Schmied,M.,Pavelka,M.,Printz,D.,Purchio,T.,Lassman n,H.,and Schulte-Hermann,R. 1993 Condensation of the chromatin at the membrane of an apoptotic nucleus is not associated with activation of an endonuclease. J. of Cell Science 104:317-326.

Ohyama,H., Yamada,T. And Watanabe,I. 1981 Cell volume reduction associated with interphase death in rat thymocytes. Radiation Research 85:333-339.

Oxberry,B.A.,Greenwald,G.S. 1982 An autoradiographic study of the binding of ^{125}I-labeled follicle-stimulating hormone,human chorionic gonadotropin and prolactin to the hamster ovary throughout the estrous cycle. Biology of Reproduction 27:505-516.

Ridley,K. andGreenwald,G.S. 1975 Progesterone levels measured every two hours in the cyclic hamster. Proceeding of the Society for Experimental Biology and Medicine 149:10-12.

Robaye,B.,Mosselmans,R.,Fiers,W.,Dumont,J.E.,Galand,P. 1991 Tumor necrosis factor induces apoptosis(Programmed cell death) in normal endothelial cell In Vitro. American J. of Pathology 138(2): 447-453.

Saidapur,S.K.,and Greenwald,G.S. 1978 Sites of steroid synthesis in the ovary of the cyclic hamster: A histochemical study. American J.of Anatomy 151:71-86.

Sawada,M.,Carlson,J.C. 1989 Superoxide radical production in plasma membrane samples from regressing rat corpora lutea. Can. J. Physiol. Pharmacol. 67:465-471.

Sawyer,H.R.,Niswender,K.D.,Braden,T.D.,and Niswender,G.D. 1990 Nuclear changes in ovine luteal cells in response to $PGF_{2\alpha}$. Domestic Animal Endocrinology 7(2):229-238.

Segerson,E.C. and Gunsett,F.C. 1993 interference with the cytolytic activity of interluleukin-2-treated lymphocytes by bovine uterine luminal protein. Biology of Reproduction 48:1036-1041.

Self,D.A.,Schroeder,P.C., and Gown,A.M. 1988 Hamster thecal cells express muscle characteristics. Biology of Reproduction 39:119-130.

Sen,S. 1992 Programmed cell death: Concept, mechanism and control. Biol. Rev. 67:287-319.

Schmied,M.,Breitschopf,H.,Gold,R.,Zischler,H.,Rothe,G.,Werkerle,H.,Lassmann, H. 1993 Apoptosis of T lymphocytes in experimental autoimmune encephalomyelitis. American J. Pathology 143(2):446-452.

Schulte-Hermann,R.,Bursch,W.,Kraupp-Grasl,B.,Oberhammer,F., and Wagner,A. 1992 Programmed cell death and its protective role with particular reference to apoptosis. Toxicology Letters 64-65:569-574.

Shinohara,H.,Nakatani,T.,Morisawa,S.,Matsuda,T.,Naruse,Y. 1987 Mast cells in the ovarian bursa of the golden hamster. Biology of Reproduction 36:445-450.

Speel,E.J.M.,Schutte,B.,Ramaekers,F.C.S.,Hopman,A.H.N. 1992 The effect of avidin-biotin interactions in detection systems for In Situ Hybridization. J. of Histochemistry and Cytochemistry 40(1):135-141.

Stern,J.,Coulam,C.B. 1992 New concepts in ovarian regulation: an immune insight. American Journal of Reproductive Immunology 27:136-144.

Swat,W.,Ignatowicz,L. and Kisielow,P. 1991 Detection of apoptosis of immature $CD4^{+}8^{+}$ thymocytes by flow cytometry. J. Of Immunol. Methods 137:79.

Tamura,H.,Greenwald,G.S. 1987 Role of luteinizing hormone in luteotropic complex of pregnant hamster. American Physiological Society E500-E504.

Takeda,Y.,Watanabe,H.,Yonehara,S.,Yamashita,T.,Saito,S.,and Sendo,F. 1993 Rapid acceleration of neutrophil apoptosis by tumor necrosis factor-α. International Immunology 5(6):691-694.

Terranova,P.F., and Greenwald,G.S. 1978 Steroid and gonadotropin levels during the luteal-follicular shift of the cyclic hamster. Biology of Reproduction 18:170-175.

Thomas,N. And Bell,P.A. 1981 Glucocorticoid-induced cell size changes and nuclear fragility in rat thymocytes. Molecular and Cellular Endocrinology 22:71-84.

Vassalli,P. 1992 The pathophysiology of tumor necrosis factors. Annu. Rev. Immunology 10:411-452.

Walker,P.R.,Kokileva,L.,LeBlanc,J., and Sikorska,M. 1993 Detection of the initial stages of DNA fragmentation in apoptosis. Biotechniques 15(6).

Wang,S.,Wu,J.,Lue,C.,Tsai,A.,Chen,W.,Lin,H. 1992 Effects of light deprivation on prolactin cells in golden hamsters:an immunoelectron microscopic study. Histology and Histopathology. 7:307-313.

Ward,P.A., and Varani,J. 1990 Mechanisms of neutrophil-mediated killing of endothelial cells. Journal of Leukocyte Biology 48:97-102.

Wijsman,J.H.,Jonker,R.R.,Keijzer,R.,Van De Velde,C.J.H., and Van Dierendonck,J.H. 1993 A new method to detect apoptosis in paraffin sections: In Situ End-Labeling of fragmented DNA. Journal of Histochemistry and Cytochemistry 41(1):7-12.

Wyllie,A.H. 1993 Apoptosis (The 1992 Frank Rose Memorial Lecture). Br.J. Cancer 67:205-208.

Wyllie,A.H. and Morris,R.G. 1982 Hormone induced cell death. Purification and properties of thymocytes undergoing apoptosis after glucocorticoid treatment. Am. J. Of Pathology 109:78-87.

Yamada,T. And Ohyama,H. 1980 Changes in surface morphology of rat thymocytes accompanying interphase death. J. Of Radiation Research 21:190-196.

Zeleznik, A.J., Ihrig,L.L., Basset,S.G. 1989 Developmental expression of Ca^{++}/Mg^{++}-dependant endonuclease activity in rat granulosa and luteal cells. Endocrinology 124(4):2218-2220.