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Apoptosis in the myocardium of the adult dromedary camel: Ultrastructural characterization

Abdel-Hamid K. Osman[1]*, Shigeaki Sato[2], Sakan Maeda[2], and Thomas Caceci[3]

1. Department of Cytology and Histology, Faculty of Veterinary Medicine, Suez Canal University, Ismailia, Egypt
2. Department of Molecular Epidemiology, Kobe University School of Medicine, Kobe 650-0017, Japan
3. Department of Biomedical Sciences, Virginia-Maryland Regional College of Veterinary Medicine, Blacksburg Virginia 24061-0442, USA. E-mail: ahkosman@hotmail.com

ABSTRACT

Apoptosis is a highly regulated mode of cell death that occurs in the absence of inflammation. Light microscopic examination of the myocardium of apparently healthy camels did not reveal evidence of apoptosis in any of the samples. The most common features observed with the transmission electron microscope included: (1) an intact sarcolemma with some bleb formation; (2) nuclear chromatin condensation and margination with nucleolar disruption; (3) mitochondrial swelling and disorganization, accompanied by degeneration or hypercondensation of cristae; and (4) an intercalated disc region with a higher-than-normal mitochondrion/myofibril ratio, or surrounded from both sides by asymmetrically contracted sarcomeres. Apoptotic alterations among the endotheliocytes lining the microvasculature comprised: (1) marked nuclear chromatin condensation and margination; (2) villous blebs on the adluminal plasmalemma, which projected into the lumen; (3) cytoplasmic vacuolation; (4) the presence of intraluminal membrane-bounded vesicles; and (5) occasional pericapillary edema and accumulations of cellular debris. The results of this study indicate that myocardial apoptosis can occur in apparently healthy camels, in the absence of a clear-cut etiology.

Keywords: apoptosis; myocardium; myocardial; microvessels; ultrastructure; camel

INTRODUCTION

Apoptosis is a distinct form of cell death. It displays characteristic alterations in cell morphology and cell fate which are markedly different from those seen in cell death due to necrosis (for review, see Hacker 2000). Necrosis is a passive form of cell death, characterized by depletion of ATP, damage to intracellular organelles, cell swelling, rupture of the cell's plasma membrane, and release of materials that cause local inflammation (Goping *et al*. 1999). Apoptosis, on the other hand, is an active and presumably pre-programmed form of cell death (Buja *et al.* 1993; Henderson 1996). Participation of the cell on the pathway to death is a process requiring energy.

With respect to the heart, there is accumulating evidence from both animal and human studies strongly suggesting that apoptosis occurs in several situations. It can be a feature of prenatal myocardial development, a normal mechanism involved in overall cardiac growth and differentiation (for review, see Poelmann *et al*. 2000). In the adult apoptosis can be the result of various cardiovascular diseases (Haunstetter & Izumo 1998). Adult myocardial apoptosis has been described in numerous situations: *e.g.*, hypoxia and

* Address for Correspondence

reoxygenation (Kang *et al*. 2000); heart failure (De Windt *et al*. 2000); myocardial infarction (Sam et al. 2000); oxidative stress (Sastre et al. 2000); mechanical stretch (Cheng *et al*. 1995); and transplantation rejection (Koglin *et al*. 1999).

Ultrastructural features of apoptosis in myocardiocytes include compaction and segregation of nuclear chromatin into sharply delineated masses that abut the nuclear envelope, nuclear deformities and fragmentation, sarcolemmal blebbing formation, disorganization of organelles, glycogen depletion and myofibrillar degenerations (Sharov *et al*. 1996; Kang *et al*. 2000; Su *et al*. 2000).

While mitochondria have largely been neglected in apoptosis research, probably because of their minor morphological changes in comparison to those seen during necrosis (Stadelmann & Lassmann 2000), they have recently gained attention as key regulators of the apoptotic process (Bossy-Wetzel 1999). Apoptotic changes in mitochondria include swelling, ultra-condensed, distorted and degenerated cristae, accumulation of electron-dense matrix granules and rupture of the outer membrane (Buja & Entman 1998; Sanchez-Alcazar *et al*. 2000).

The aim of the present study is to determine whether myocardial apoptosis occurs in apparently healthy adult camels. The camel is a unique animal, whose whole physiology, even more than in other species, is geared around an exquisite level of control over body volume, water balance, and ionic composition of body fluids; it is to be expected that the demands on the heart of such an animal will differ from that of other ruminants, and some degree of apoptotic change might well be expected as a result, to keep the myocardium in peak condition. Furthermore, characterization of any such alterations and understanding of their effects on myocardial performance, maintenance, or remodeling would be of key importance to understanding, and perhaps implementing, apoptosis in the diagnosis and therapy of heart disease in other species.

MATERIALS AND METHODS

The hearts of 6 adult, apparently healthy camels were collected from commercial abattoirs in Cairo and Zagazig, Egypt. Egyptian camel slaughterhouses routinely perform clinical health assessments on animals brought in for slaughter, and the camels awaiting it are allowed to drink water *ad libitum* during the minimum 24 hour rest period that precedes slaughtering. The hearts were removed within approximately one half-hour of death. Small pieces of the left ventricular myocardium were removed with a scalpel and used for light microscopy (LM) and transmission and scanning electron microscopy (TEM and SEM).

For LM preparations, the myocardium was minced into 5.0 mm cubes and fixed in 10% formol-saline solution. After fixation for 2 weeks, the specimens were washed in tap water, dehydrated in a graded series of ethanol from 30% to absolute, infiltrated with and embedded in Paraplast medium and sectioned on a rotary microtome. Sections were stained with hematoxylin/eosin (H&E) for routine observations and with PAS (McManus 1946) for the detection of polysaccharides.

For TEM preparations, cubes of myocardium ≤ 3.0 mm^3 in size were fixed at room temperature (RT) in 4% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) for 10 hours. After this primary fixation, the tissue pieces were further minced to approximately 1.0 mm^3 volume, washed in buffer, and postfixed in 1.0% buffered osmium tetroxide for two hours at RT and pH 7.4. After postfixation the specimens were washed in the same buffer, dehydrated in an ethanol series, and cleared in propylene oxide; then infiltrated and embedded in Polybed 812 resin. Orientation sections 1.0 micron thick were cut with glass knives and stained with 1.0% toluidine blue in 1.0% sodium borate for LM observation; ultrathin sections (80 nm) suitable for TEM were cut with a diamond knife and double

stained with lead citrate and uranyl acetate. They were examined on a JEOL 100CX-II microscope at 80 kV.

For SEM preparations, cubes of myocardium approximately 5.0 mm on a side were fixed in 10% formol saline as for LM. Following fixation, the pieces were washed and immersed in a solution of 1.0% tannic acid in 0.1 M sodium cacodylate for 1.0 hour at RT, according to the method of Caceci and Frankum (1987). This was followed by washing in buffer and post-fixation in 2.0% osmium tetroxide in 0.1 M cacodylate buffer for 2 hours at RT and pH 7.4. The postfixed specimens were dehydrated in ethanol, critical-point dried through CO_2, and mounted on aluminum stubs with silver paint. The specimens were sputter-coated with 60:40 gold:palladium for 2.0 minutes and examined in a JEOL JSM-35 scanning electron microscope at acceleration voltage between 10 and 25 kV.

RESULTS

Light Microscopy:

Most of the myocardial architecture was normal in appearance. Morphological evidence of inflammatory or retrogressive changes were completely absent. The contractile myocardiocytes (CM's) were branched and anastomosed, crossly striated, and connected end-to-end by intercalated discs, as expected. The centrally-located nuclei were euchromatic, and the eosinophilic sarcoplasm exhibited occasional unstained vacuolation (Fig.1A). The myocytes were surrounded by numerous thin-walled vessels and scanty fibrous interstitial tissue. Large polymorphic Purkinje fibers (PF's) were clustered among the CM's, in a more appreciably fibrovascular interstitium (Fig.1B). These cells displayed a comparatively small euchromatic nucleus surrounded by faintly eosinophilic, PAS-positive sarcoplasm. The latter showed peripheral myofibrillar condensation.

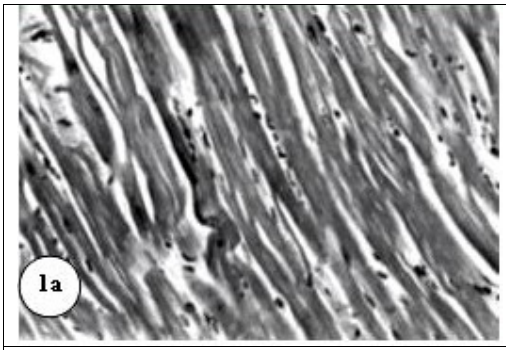


Fig 1a: Light microscopic morphology of normal myocardium in the camel (H&E Stain, 250x).

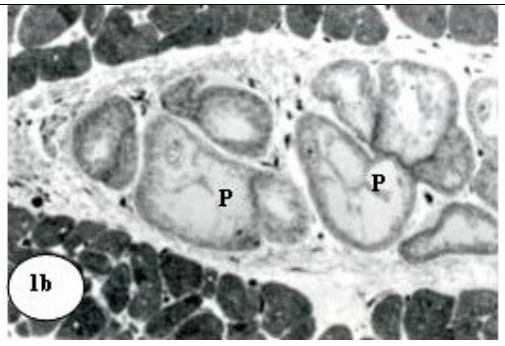


Fig 1b: Purkinje fibers (P) in the normal myocardium (1.5µ plastic section, toulidine blue stain, 250x).

Electron Microscopy:

Typical ultrastructural features of myocardial apoptosis were identified in the left ventricular tissue obtained from 2 out of the 6 examined camels. CM's showing apoptotic alterations were difficult to identify because they occurred sporadically as individual cells, or in small clusters among the predominating normal myocytes. Moreover, they always demonstrated intact sarcolemmae and were never associated with any inflammatory reaction. Neither the orientation sections prepared for the light microscope nor the ultrathin sections cut from had any of the typical features of an inflammatory lesion.

The most common apoptotic features observed were nuclear, mitochondrial, myofibrillar, and sarcolemmal alterations (Fig 2). All of the affected CM's showed one or more of such changes.

Nuclear deformities in the form of peripheral infoldings of various depths were very distinct and easy to identify in the EM. The chromatin in such nuclei tended to be compacted and segregated into sharply delineated peripheral masses abutting the nuclear envelope (Fig.2). Sharply defined chromatin bodies were randomly distributed within the nucleoplasm. Nucleolar disruption or dispersion into osmiophilic fragments was demonstrated, but nuclear fragmentation *per* se was not observed in any of the specimens.

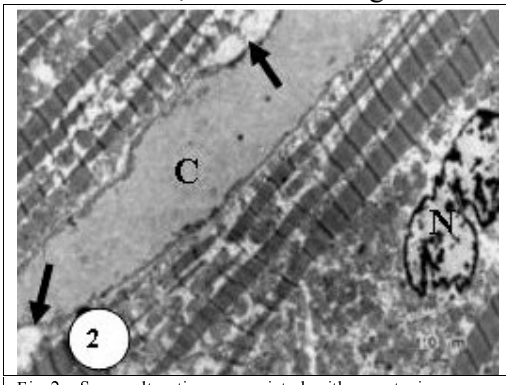


Fig 2. Some alterations associated with apoptosis are seen here. The capillary (C) between two adjacent cardiac myocytes shows localized pericapillary edema, pressing on the capillary wall (arrows). Irregular outlines of the apoptotic nucleus (N) that surrounded by normal and degenerated mitochondria.

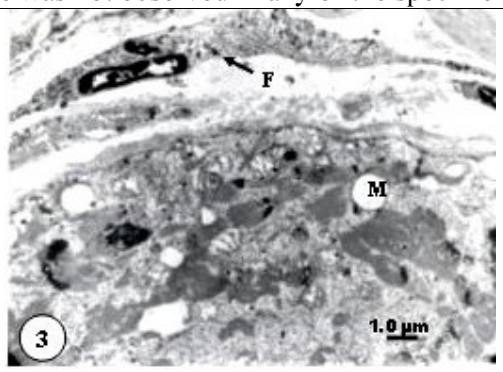
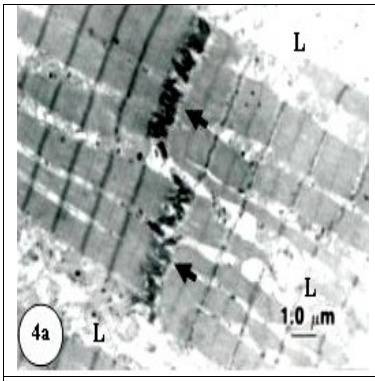
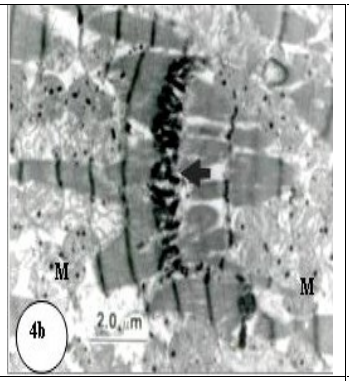


Fig 3. A Purkinje fiber shows doughnut-shaped mitochondria (M), and the adjacent fibroblast (F) displays marginal condensation of the chrmoatin in the nucleus.

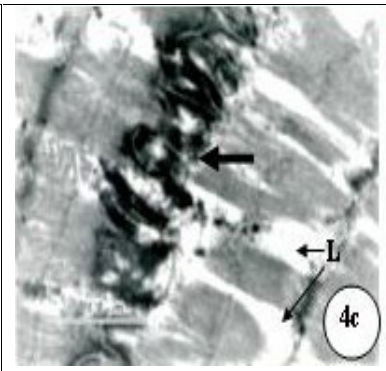
Alterations of mitochondrial morphology took a variety of forms. Swollen mitochondria were observed in the perinuclear and subsarcolemmal regions. Prominent electron-dense matrix granules were found in the majority of mitochondria (Fig 4B). Mitochondria with deformed, hypercondensed, and/or partially or completely degenerated cristae were not uncommon (Fig.2, 4B). Mitochondria that completely had lost their cristae and acquired a "doughnut" appearance were found in the Purkinje fibers (Fig.3).



Two contiguous cardiomyocytes at the intercalated disc (arrow) region. 4a: unequal sarcomere lengths on both sides of the disc and focal myofibrillar lysis (L).



4b: the disc is surrounded by plenty of mitochondria with electron-dense matrix granules.

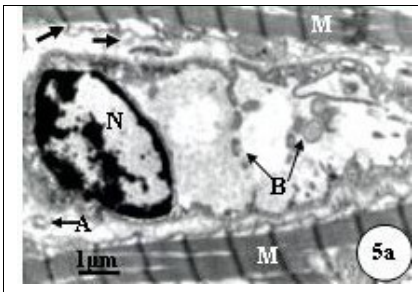


4c: the hyperdense intercalated disc is surrounded by asymmetrically contracted sarcomeres and areas of myofibrillar lysis (L).

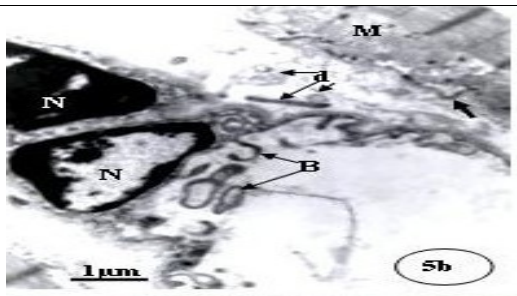
Within the cytoplasm of apoptotic cells, most of the myofibrils were well-maintained and organized into sarcomeres. In few cases myofibrils demonstrated out-of-register contraction bands with unequal sarcomere lengths among the same myofibril. Some intercalated discs were surrounded by relaxed sarcomeres at one side and contracted ones at the other side (Fig. 4A). Occasionally the intercalated discs were surrounded by a region of high mitochondrial content (Fig. 4B) in contrast to the ramifying, interrupted myofibrillar masses (high mitochondria-myofibrillar ratio). Focal areas of myofibrillar

lysis were also encountered (Fig 4C). A few glycogen agglomerations were observed in the inter-myofibrillar spaces.

The sarcolemmae of the myocytes exhibiting the aforementioned alterations were always intact, and always associated with intact amorphous basal laminae. The most striking sarcolemmal alteration was the formation of circumscribed protrusions (blebs) into the extracellular space (Fig. 5A,B). A torn-out mitochondrion could be detected within one of such blebs. What we took to be detached blebs were observed as free, membrane-bounded vesicles (apoptotic bodies) in the extracellular space, particularly in the pericapillary areas (Fig. 5B).

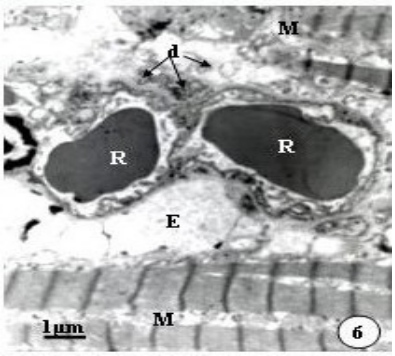


5a: A sarcolemmal bleb (**A**) contains a torn-out mitochondrion. Endolthelial blebbing (B) has occurred as well, and the nucleus (N) of the endothelial cell displays compaction and marginalization of the chromatin.

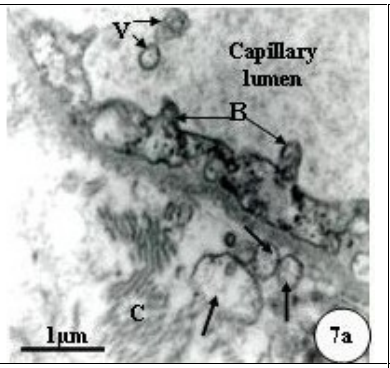


5b: Membrane-bound apoptotic bodies and cellular debris (d) are located in the interstitium; endothelial blebs (B) and compact, marginated chromatin in the endothelial cell nucleus (N) are visible; and there is also sarcolemmal blebbing (arrow) and formation of apoptotic bodies.

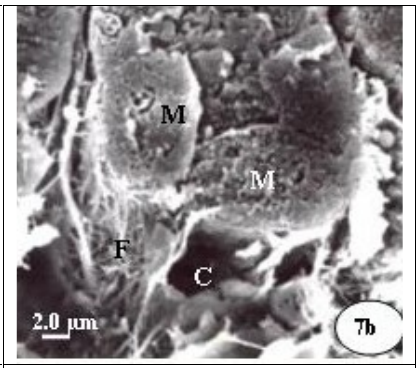
The myocardial microvasculature showed also alterations, these affecting the endothelial cells lining the vessels. The nuclei of the latter showed massive chromatin condensation and margination, in addition to nucleolar disruption. The endothelial adluminal plasmalemma was thrown into a number of villous blebs projecting into the capillary lumen. The latter contained free circulating membrane-bound vesicles (Fig. 5A,B). Some endothelial cells contained cytoplasmic vacuolar structures. The abluminal plasmalemma was always entirely devoid of blebbing and always associated with an amorphous basal lamina. Pericapillary edema and cell debris were encountered in some areas (Fig. 6). In some regions, localized fibrous condensation was observed in the interstitium between a myocardiocyte and the adjacent capillary (Fig. 7A,B). Fibroblast-like cells with chromatin condensation and margination were also visible (Fig. 3).



Two cardiomyocytes (M) surrounding a capillary containing erythrocytes (R).The pericapillary spaces show a circumscribed edematous area (E) and cellular debris (D).



7a: There is blebbing (B) of the adluminal plasma membrane, and membrane-bound vesicles (V) associated with the luminal membrane of the capillary; pericapillary fibrous condensation (C) and the formation of apoptotic bodies (arrows) are also visible.



7b: Scanning EM shows the localized fibrous condensation (**F**) in close apposition to both cardiomyocytes (M) and microvessels (C).

DISCUSSION

Prior to slaughter, the camels were all individually judged to be healthy by experienced clinicians; furthermore, their carcasses and hearts, subjected to close post-death examination and certification, were devoid of any pathological lesions. The brief period of time that is involved in the slaughter process is insufficient to cause significant post-mortem alteration. The camels were killed by exsanguination while completely conscious (this is required by Egyptian law; as a codification of the Islamic practice of *halal* slaughter, it is universally used in Egypt). Their hearts were therefore under the same hypoxic stress caused by a rapid tracheotomy accompanied by convulsions and profuse bleeding. While the actual duration from beginning (which is a single knife slash that severs the ventral neck region, including the major blood vessels and the trachea) to end (the complete cessation of the heartbeat) differs from one animal to another, thanks to variation in age, strength, and size, it is never more than a few minutes. Since LM examination demonstrated that the myocardium retained its normal architecture and was devoid of any inflammatory or retrogressive changes, we conclude that the potential to undergo apoptotic changes was likely present in some fraction of the normal myocardium before slaughter.

Despite recent advances in techniques for detecting fragmented DNA, ultrastructural characterization of myocardial apoptosis is considered to be the most reliable criterion for demonstrating that it has taken place (Bonfoco *et al*. 1995; Ying-Shiung & Yun-Ying 1998; Goping *et al*. 1999). One significant use of the survey sections from the EM blocks was to confirm that tissue which looked normal and intact at the light microscope level did in fact show morphological alterations consistent with apoptosis *in situ*, when seen at higher levels of magnification and resolution. In our investigation we found ultrastructural alterations characteristic of apoptosis in only two of the examined animals. This is to be expected if only a small percentage of the total volume of myocardium is undergoing the process at any given moment. Our results in this respect are consistent with the observations of Kavantzas *et al*. (2000) in the human heart. We conclude therefore that apoptosis of myocytes is difficult to recognize because it is quite restricted in occurrence, either temporally or spatially. The implications of this statement are that while apoptosis may be a normal event, is not common; and that while morphological methods can demonstrate its occurrence, they cannot provide a quantitative assessment of how often it occurs, nor where it is most easily visualized.

In this model of adult myocardiocyte apoptosis, we demonstrated nuclear, mitochondrial, myofibrillar, and sarcolemmal alterations. Nuclear changes comprised profile deformity, nucleolar disruption or dissolution and chromatin condensation and margination. Similar findings were reported from human and animal hearts exposed to hypoxia and/or oxygenation (Cheng *et al*. 1996; Sharov *et al*. 1996; Buja and Entman 1998; Ohno *et al*. 1998; Kang *et al*. 2000; Su *et al*. 2000). Nuclear changes can be attributed to cleavage of nuclear laminins, nuclear proteins which are attached to the inner side of the nuclear envelope (Fraser *et al*. 1997), and activation of caspases (Wilhelm & Hacker 1999).

The mitochondrial alterations we observed comprised swelling, presence of electron-dense matrix granules, and disorganization or even disintegration of cristae. These regressive features are usually present in the myocardiocytes exposed to ischemic stresses or reoxygenation injury (Cheng *et al*. 1996; Sharov *et a*l. 1996; Buja *et a*l. 1998; Kang *et al*. 2000). Similar findings have also been shown following the administration of tumor necrosis factor α (TNFα) (Sanchez-Alkazar *et al*. 2000). Recently, mitochondria have come under scrutiny as apoptosis regulators, where pro- and anti-apoptotic signaling

pathways converge (Green & Kroemer 1998; Bossy-Wetzel & Green 1999). Mitochondrial swelling, hyperdense cristae, and finally rupture of the outer membrane have been proposed a mechanism for the release of cytochrome c into the cytosol (Vander-Heidin *et al*. 1997).

We observed sarcolemmal protrusions, or blebs, some of which contained a mitochondrion. Sarcolemmal blebbing has been reported by Barr & Tomei (1994) in early stages of myocardial ischemia in the rat. Blebbing has been found to depend to some extent on actin polymerization and on myosin phosphorylation (Milles *et al.* 1998b). A number of aspects of this process have been discussed in a recent review article by Hacker (2000). Thus there seems also to be a further connection between oxygen deprivation and morphological changes characteristic of apoptosis.

We noticed marked glycogen depletion in the myocytes showing these alterations. A significant depletion of glycogen in the early stages of myocardial infarction has also been reported (Su *et al*. 2000), but in chronically ischemic myocardium, in contrast to the acute situation, there exists glycogen accumulation due to low consumption (Dusek *et al.* 1971).We suggest that cells potentially at risk of death by apoptosis may require a high energy level to survive, and that it is those myocytes whose glycogen reserves are depleted that are most likely to undergo apoptotic death under conditions of acute hypoxia. Since utilization of glycogen reserves is a longer-term process that cannot in a few seconds, this observation supports the idea that a certain sub-population of myocytes is predisposed to undergo apoptosis, and that acute hypoxic conditions bring it about in those cells but not in others.

Myofibrillar alterations consisting of disorganization, focal lysis and asymmetry of the contraction bands of a given myofibril were also prominent features of the cells we judged to be apoptotic. The myofibril to mitochondrion ratio at the intercalated disc region of chronically ischemic myocardiocytes is known to undergo a significant increase (Su *et al*. 2000), and consistent with this prior work, we noted a striking increase in the number of mitochondria in comparison to the myofibrils near the intercalated disks as well.

Typical apoptotic alterations were observed among the endotheliocytes of the myocardial microvasculature, including nuclear chromatin condensation and margination, blebbing of the adluminal plasmalemma, presence of luminal plasmalemmal vesicles, and partly occurrence of pericapillary edema and cellular debris. Similar ultrastructural findings have been previously recorded in ischemic myocardial microvessels (Welt *et al*. 2000). The very characteristic blebbing formation is regarded as a typical feature of ischemic reaction of endothelium (Hearse *et al*. 1993; Ward & Scoote 1997).

The extent of pericapillary edema is significantly increased after ischemia/reperfusion (Welt *et al*. 2000). This may be of special clinical interest since interstitial edema is regarded as the main cause of the post-ischemic "no reflux phenomenon" (Lindal *et al*. 1988). Pericapillary cellular debris was probably caused by the destruction of interstitial cells by free radicals, since the free-radical defense capacity is known to be very low in myocardial interstitium (Gerber & Siems 1987).

In conclusion, to the best of our knowledge, we describe for the first time a camel model of myocardial apoptosis. In discussing our observations with those of the above-cited authors, we have come to the conclusion that our observations of apoptotic alterations in the camel are most likely attributable to hypoxic stresses, we must also consider the mechanical stretch model that has been shown to cause myocardial apoptosis in other species (Cheng *et a*l. 1995; Sam *et al.* 2000). It is not, of course, out of the question that both mechanisms could act in concert: overstretch can easily lead to

localized compression of capillaries, with consequent localized anoxic conditions triggering apoptosis.

We also conclude that routine LM examination is insufficient to detect myocardial apoptosis, and it should be accompanied by EM examination which is more reliable, if more difficult to use. Moreover, since our work was confined to the ventricular myocardium, we suggest that further studies on the extent and features of tissue from different heart compartments be done, and that in doing such studies it would be profitable to combine EM techniques with those for the detection of DNA fragmentation.

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الملخص العربى

الموت المبرمج للخلايا العضلية فى قلب الجمل

عبد الحميد كامل عثمان[١] ، شجياكى ساتو[٢] ، ساكان مائيدا[٢] ، توماس كاشيشى[٣]
١ــ قسم الخلية والأنسجة- كلية الطب البيطرى- جامعة قناة السويس ــ مصر
٢ــ قسم الوبائيات الجزيئية- كلية الطب البشرى- جامعة كوبية ــ اليابان
٣ــ قسم العلوم الطبية الأساسية- كلية الطب البيطرى- جامعة ولاية فرجينيا ــ الولايات المتحدة الأمريكية

يعتبر الموت المبرمج أحد أنواع الموت الخلوى الغير مصحوب بمظاهر الألتهابات . أهتمت هذة الدراسة بأستقصاء هذا النوع من الموت الخلوى فى النسيج العضلى لقلوب مجموعة من الجمال الخالية من المظاهر المرضية الأكلينيكية وذلك بأستخدام المجهر الضوئى والأليكترونى . أوضحت الدراسة ولأول مرة أن الخلايا العضلية التى تذوق الموت المبرمج تتصف بالمظاهر التالية: (١) ظهور بروزات حلمية الشكل فى الغشاء الخلوى ، (٢) تخثر الكروماتين النووى وتحلل النويات ، (٣) تضخم أحجام الميتوكوندريا وتحطم أعرافها ، (٤) زيادة ملحوظة فى عدد الميتوكوندريا على كلا جانبى القرص الضام للخلايا العضلية المتجاورة على حساب كثافة الليفات السيتوبلازمية . وتصاب الخلايا المبطنة لجدران الشعيرات الدموية المجاورة للخلايا العضلية تلك بمظاهر مشابهةبلأضافة لبعض الأرتشاحات حولها .
نستنتج من هذة الدراسة أمكانية اصابة الخلايا العضلية لقلب الجمل بمظاهر الموت المبرمج دون ظهور أعراض مرضية .