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ATRIAL NATRIURETIC FACTOR SYNTHESIS AND STORAGE DURING ONTOGENY OF THE CHICKEN EMBRYO HEART: AN IMMUNOCYTOCHEMICAL AND ULTRASTRUCTURAL STUDY USING TANNIC ACID AS INHIBITOR OF THE EXOCYTOSIS PROCESSES[1]

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1. INTRODUCTION

In the last decate, the chicken embryo has gained growing interest for its physiological, morphological, as well as molecular biology aspects, as a model organism for the study of physiology, organogenesis and development (*4*, *6*). Differently from the mammals, the avian system allows the study of growth and developmental patterns independently of the maternal organism, particularly for endocrine studies of hormones that are known to pass the placental barrier and to have influence on embryonic and fetal development (*15*, *23*).

After initial reports on atrial natruiretic factor (ANF) in heart atria of rats (*5*), many studies have shown that the heart of vertebrates acts as an

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endocrine organ synthesizing and processing a family of peptides with an important vasorelaxing, diuretic and natriuretic action. The existence of a hormone system in adult birds has begun to become evident due to different investigations (*10, 16, 20*). The administration of Atriopeptin produces diuretic and natriuretic effects in chicks (*11*).

A 29-aminoacid natriuretic peptide in the chick has been recently isolated and identified (*16*), being considered as a homologue molecule of 28-aminoacids of mammals.

Later studies carried out on chick ANF have shown its capacity of modifying the duck angiotensin-aldosterone system (*10*), showing a close correlation between the plasmatic ANF and the vascular volume (*10*).

Using radio receptor and autoradiography assays in adult Peking ducks, a modulation in water and salt renal elimination due to ANP-interaction on glomerular receptors was determined by SCHÜTS *et alii* (*20*). These receptors established an important role of heart peptides in adult bird salts and liquid homeostasis.

On the other hand, we have recenty reported initial evidence that a peptide of the family of natriuretic peptides is present in the myoblast of the myotome of chick embryos. This occurrence has led us to suggest the hypothesis that this ANP-like factor could participate in a paracrine and/ or autocrine function (*8*).

The previous immunofluorescence studies of the click embryo heart CASCO *et al* (*3*) indicated the presence of natriuretic peptides. But contrasting to what occurs in mammal early ontogeny, where numerous morphological and immunohistochemical studies have shown ANP-immunoreactivity in embryonic cardiac cells (*3, 7, 9, 14, 17, 18, 24, 25*,), in chick embryos the heart cell population with endocrine characteristics is scarce, and the secretory granules are small and reduced in number. This has led us to establish that instead of a constitutive pathway which gets around the packaging in the typical secretory granules, there may be a regulated pathway in which the processing of these granules is so quick that it becomes impossible to pick up these structures with the classic transmission electron-microscopy techniques.

In order to elucidate this problem, a protocol was applied in which embryonic hearts were incubated in a tannic acid solution before primary fixation. This technique was first applied on the study of neurosecretory granules (*1*) and has been successfully used to study processes such as ANP granule exocytosis in mammals (*19*).

Tannic acid is considered to act because of its interaction with membrane components or blocking processes that otherwise are of difficult sighting (ANP secretory granules or neurotransmitters exocytosis) (*19*).

Applying this protocol allowed us to observe a significant increase in number as well as in size of the secretory granules. This condition is specially evident in 12-16-day old embryo hearts.

2. MATERIALS AND METHODS

2.1. *Immunofluorescence*

Chick embryos at 4, 6, 8, 12 and 16 days of age (*12*) were removed from the yolk mass and immediately submerged in Bouin fixative at room temperature or in acetone cooled to -20°C. The hearts were dissected, dehydrated and embedded in paraffin. The, sections of 6-8 μm were stored at -20°C until labelling.

Sections were briefly exposed to a Phosphate Buffer Saline (PBS) containing 0.5% Triton X-100 and 1% Bovine Serum-Albumin (BSA) followed by incubation in antiserum anti-human ANP (ARG 101-TYR 126, 1:200) at 4°C, during 18 h. They were then rinsed in PBS and in the secondary antibody fluorescein conjugate 1:4. Control section were made following similar protocols but the antiserum was replaced with non-immune serum.

Some 6- and 8-day samples were processed with Avidin-biotin. The samples were introduced in PBS during ten minutes.

In order to inhibit endogenous peroxidase, an H_2O_2 solution was used. The elimination of unspecific agents was carried out with PBS-BSA during thirty minutes, then, the samples were incubated with the primary antibody in refrigerator (anti ANP 1/50). After washing with PBS they were incubated with the secondary antibody at room temperature during thirty minutes.

- Incubation with Avidin- Peroxidase solution.
- Incubation with Dicemine-bencidin.

2.2. *Transmission Electron- Microscopy*

Embryos 4-, 6- and 8-day old were obtained complete with 12-and 16- day old ones presenting the right and the left atria and right and left ventricles dissected.

For ultrastructural studies and localization of the secretory granules, hearts were submitted to two protocols during the fixation process.

2.2.1. *Protocol 1*

Hearts were fixed in a solution containing 3% glutaraldehyde, 3% formaldehyde, 1% picric acid satured solution, in 0.1M cacodilate buffer pH 7.4.

After fixation, hearts were treated with 1% osmium tetroxide and stained *en bloc* with 2% uranyl-acetate. Then the hearts were dehydrated with acetone and embedded in Durcupan ACM (FLUKA). Ultrathin sections were mounted on copper grids and counterstained with uranyl acetate and lead citrate.

2.2.2. *Protocol 2*

Embryos at the same stage of those used in protocol 1 were removed and incubated in 2% tannic acid in a cacodilate buffer pH 7.4 for 2 h, and then in a fixative containing 3% glutaraldehyde, 3% formaldehyde, 1% picric acid saturated solution in cacodilate buffer.

From this stage onwards the postfixation in osmium tetroxide, dehydration and embedding process, indicated in protocol 1, is to be followed.

3. RESULTS AND DISCUSSION

3.1. *Immunofluorescence*

At four days a specific reaction is observed at atrial and atrial-ventricular canal level, in the ventricle subpericardial and trabecule sub-endocardial myocardiocytes (Figs. 1-3).

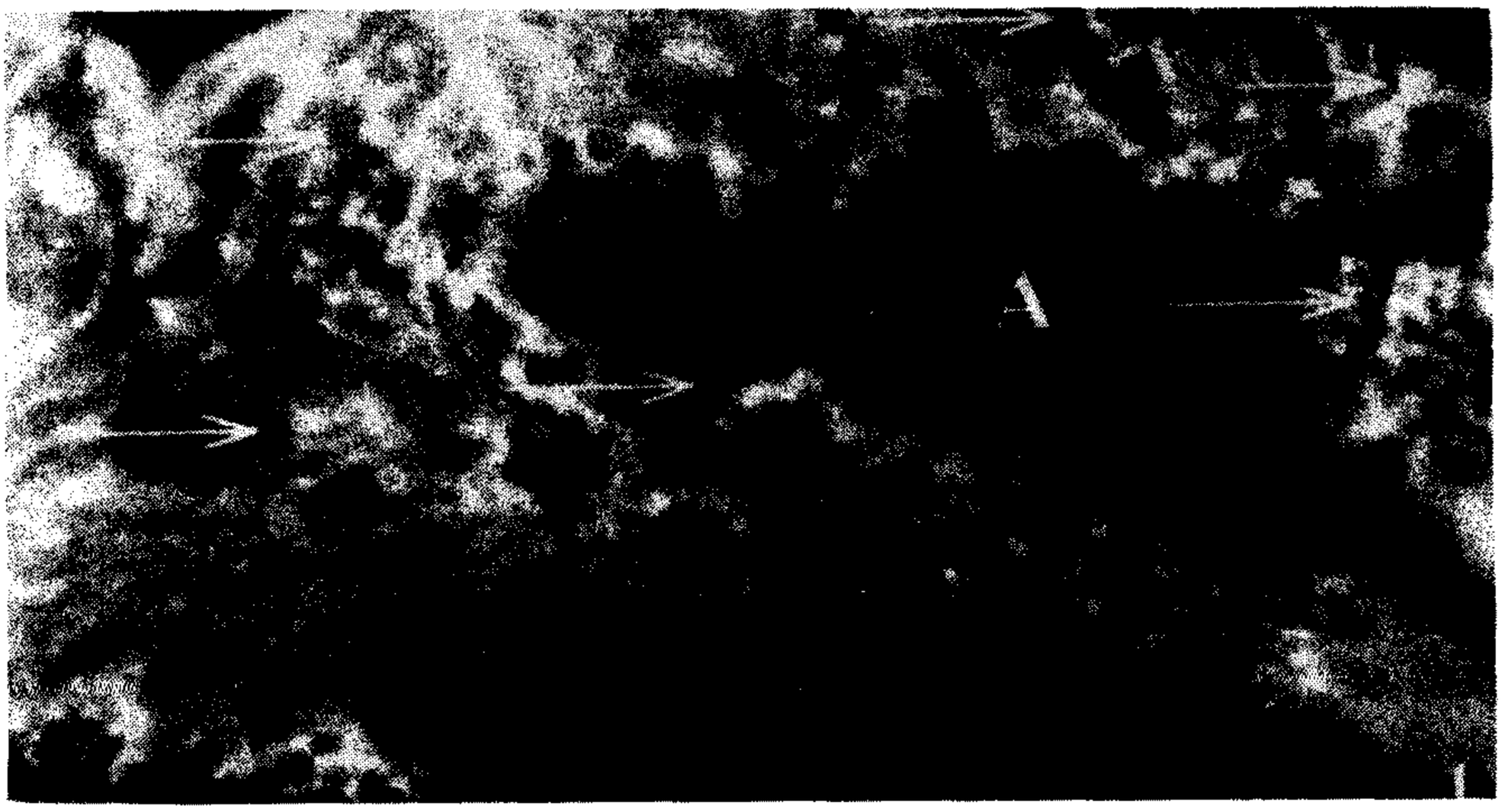

FIGURA 1 - Immunofluorescence localization of ANP (arrows) in a section of a 4-day chick embryo atrial myocardiocytes. A; atrium. 400 X.

In 6- and 8-day embryos, immunofluorescence shows a labelling increase in the atria and evident labelling decrease in the auricular-ventricular canal as well as in the ventricles (Fig. 4).

At 12 days, labelling in the auricular-ventricular canal is lost, but a specific labelling in the *arteriosus truncus* is observed in the aorta middle layer as well as in the lung layers, while the reaction is kept at auricular level (Fig. 5).

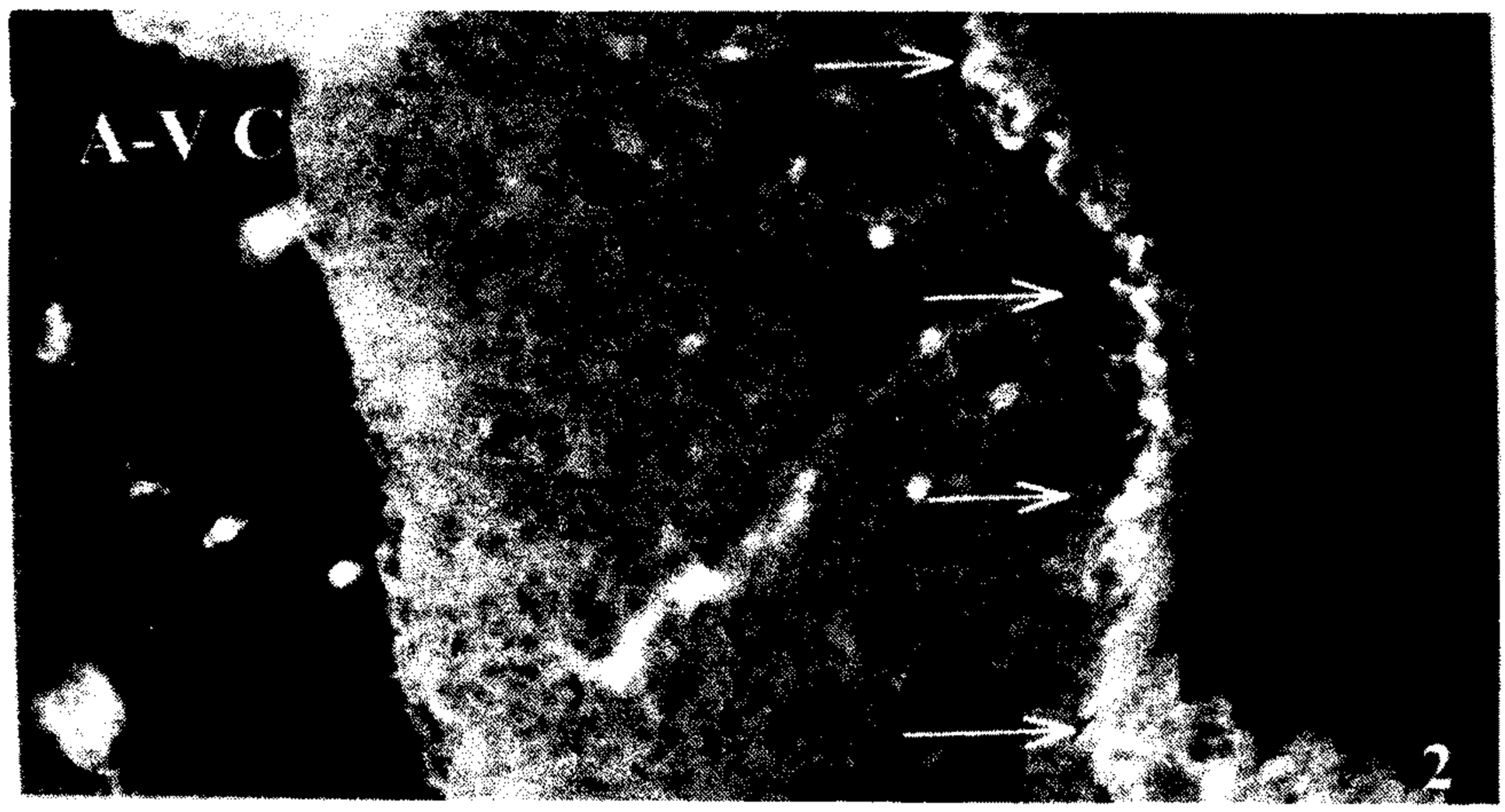

FIGURA 2 - Photomicrographs showing an enlarged area of the atrial-ventricular canal showing specific immunofluorescence labeling (arrows) in 4-day chick embryo subpericardial myocardiocytes. 474 X.

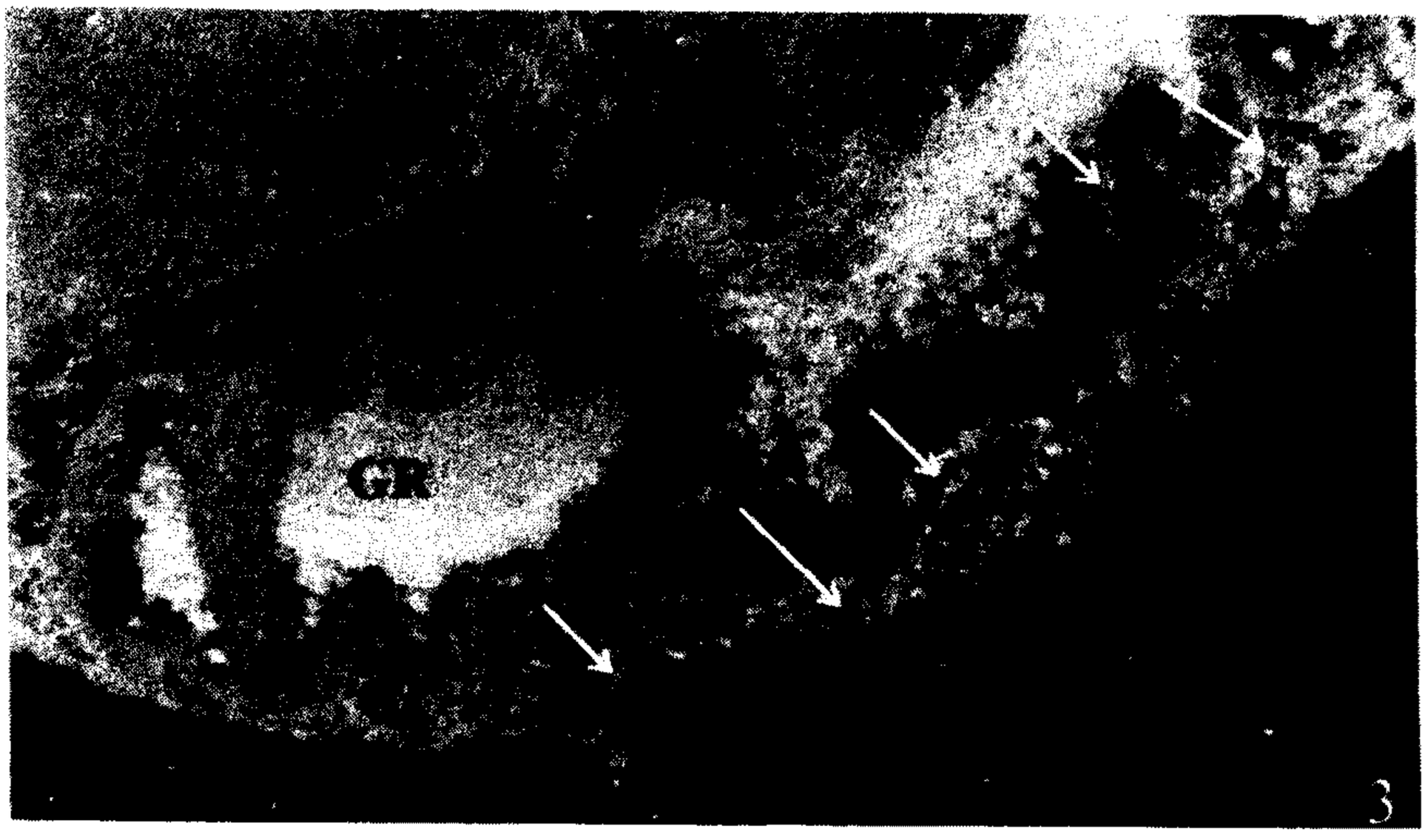

FIGURA 3 - Immunofluorescence localization of ANP in a section of a 4-day chicken embryo ventricle, showing specific labeling in trabeculae subpericardial and subendocardial myocardiocites (arrows). GR: self-fluorescent red cells. 400 X.

FIGURA 4 - Avidin-biotin labelling of ANP (Arrows) in auricular region (A) and decrease of ANP labelling at the auricular-ventricular canal (AVC), of an 8-day chick embryo. GR: self-fluorescent red cells. 200 X.

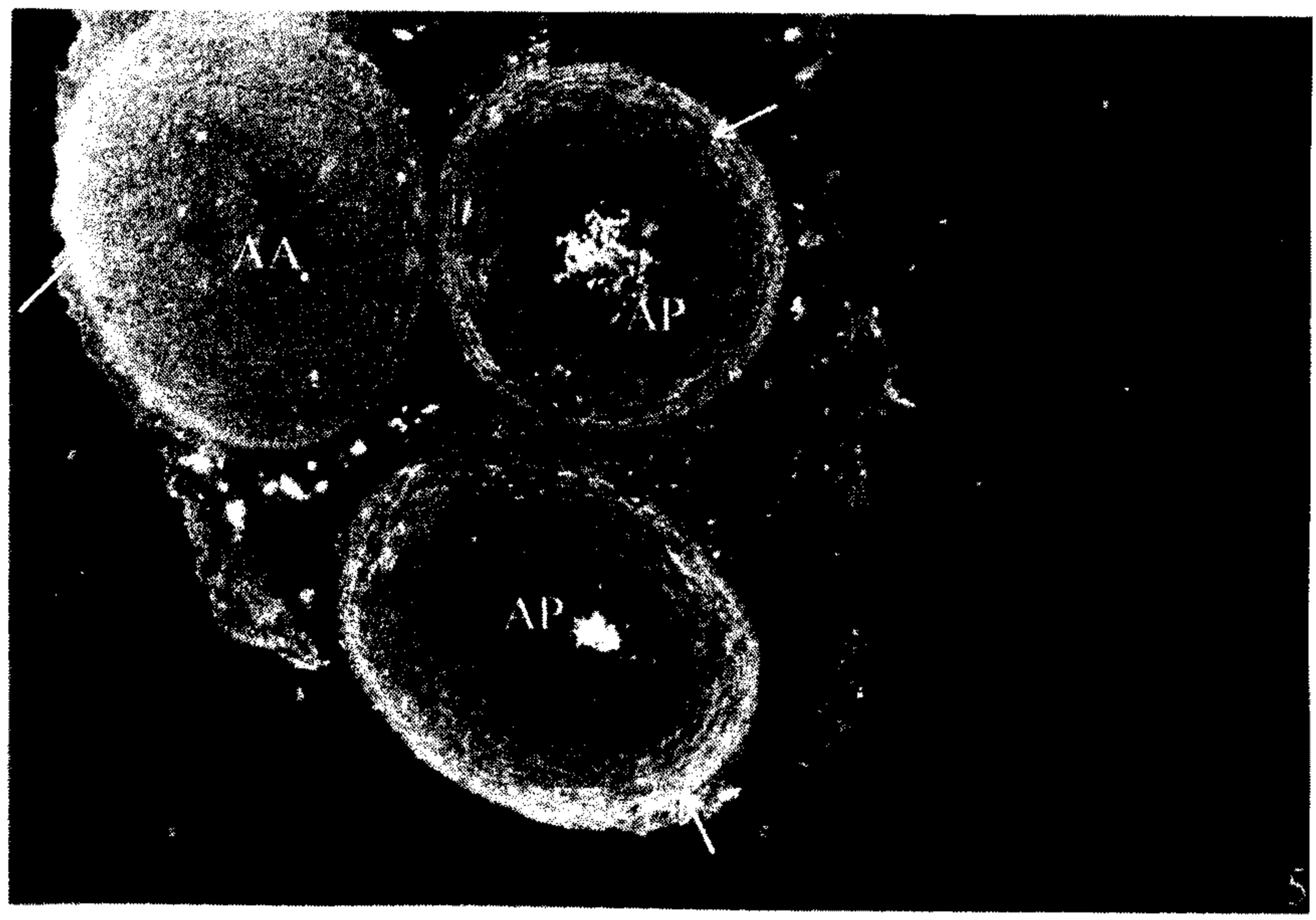

FIGURA 5 - Immunofluorescence localization of ANP (arrows) in a transversal section of a 12-day chick embryo aorta (AA) and pulmonary arteries (AP). 300 X.

3.2. *Transmission Electron-Microscopy*

3.2.1. *Protocol 1*

At ultrastructural level, differentiation degrees of the different areas of the heart in early embryonary stages show remarkable variations. The sarcoplasmic reticulum and the mitochondria develop earlier than the contractile apparatus since
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321 *Protocol 1*]

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The scarce myofibrillar development of the cytoskeleton presented by 3- or 4-day old cells, is shown by primitive and disordered fibrils.

At 4 days, atrial cells present an irregular membrane nucleus with some indentations which are gradually reduced compared with earlier stages. Mitochondria present crests more prominent and numerouns than in earlier stages. As the number of crests increase, the content of the matrix is more dense than the cytoplasm. The myofibrillar apparatus is not yet well developed, showing myofibrils in all stages of development, with a prevalence of those with a disorganized aspect. Some striated myofibrils and organized packs with neat Z-lines become observable. The Golgi apparatus is in juxtanuclear position with some cistemae and great number of vesicles. Among the different elements in the Golgi apparatus, short profiles of RER can be observed. Small dense granules are generally observed near the nucleus and mainly in atrial cells. The intercellular spaces, full of a dense substance called cardiac jelly, seem to diminish, as intercalated disks and desmosomes (associated or not to indifferentiated fibrils) increase.

Six and eight-day old cells still present a high indifferentiation degree, a sensible decrease of secretory granules and great development of the Golgi apparatus. The fibrils are aligned parallel and show a more observable transversal striation. The mitochondrial matrix density

increases. In general, the sarcoplasmic reticulum undergoes the greatest development degree (Figs. 6 and 7).

At 12 days, cells show an indented nucleus, mitochondria with an electron-dense content and a considerable increase of fibrillar apparatus in myofibrils packs, similar to those observed in adult myocardiocytes; and the sarcomer shows a high differentiation degree.

No increase in number or size of electron-dense secretory granules is observed. Multivesicular bodies and great development of the Golgi apparatus can be seen (Fig. 8).

At 16 days, three kinds of coexisting cells, with a disparity in the differentiation degree can be observed, specially in the atrial wall. Some of them, indifferentiated and with no granules, others, showing myoendocrine characteristics which have begun their myofibrillogenesis presenting considerable increase in granule number and not in granule size (Fig. 9), and a third group more developed but granuleless cells with abundant myofibrils organized in sarcomeres and with dense mitochondria.

FIGURA 6 - Electron micrograph of the perinuclear area of an 8-day chick embryo atrial cell. Note the high indifferentiation degree and the absence of secretory granules despite the great development shown by the Golgi apparatus (Gl); N: nucleus; Nu: nucleolus; Z: Z-lines: iMy: immature myofibrils; My: mature myofibrils; M: mitochondria; Li: lipidic droplets; V: electro-lucent vesicles; IF: intermediate filaments. 8.000 X.

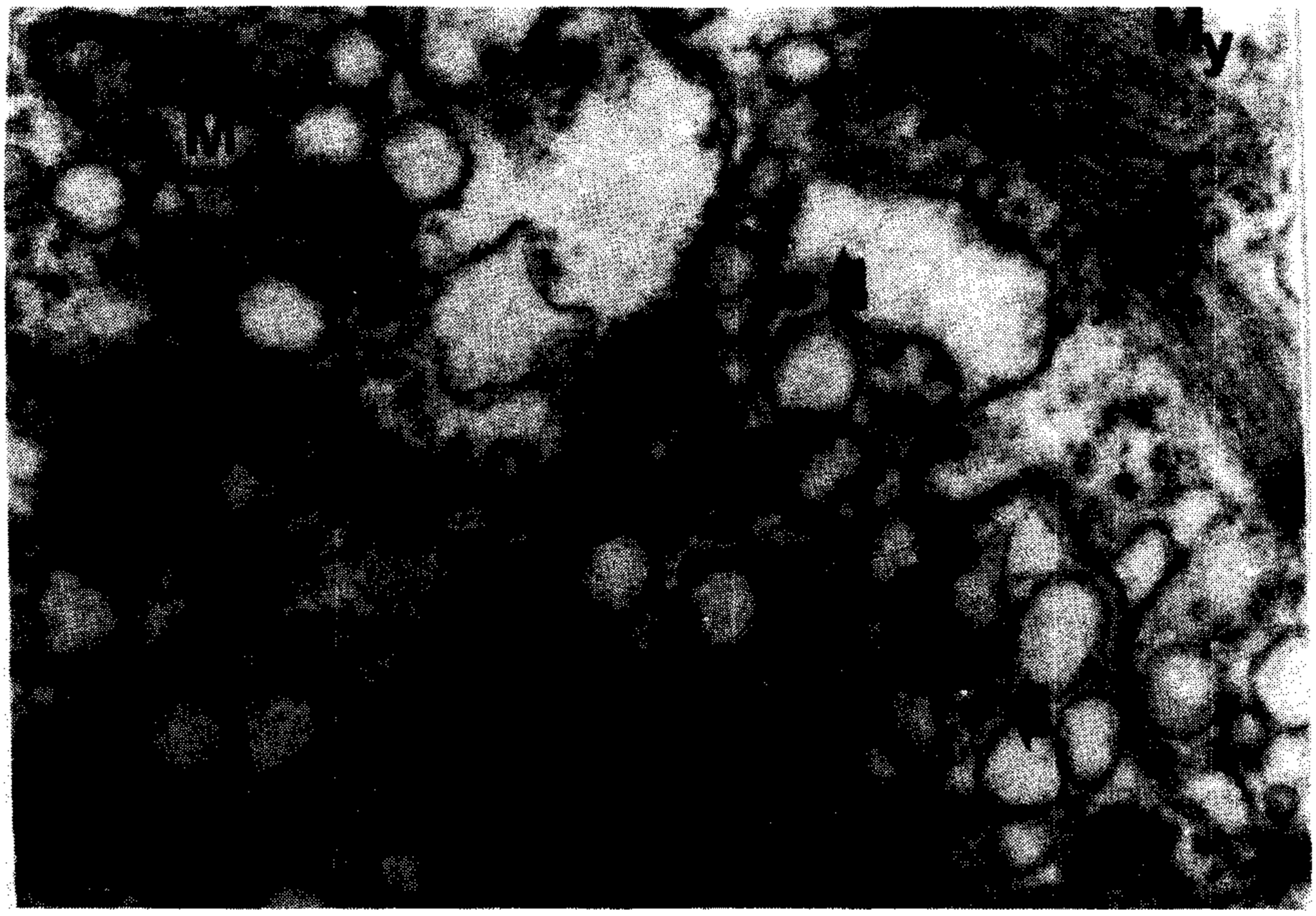

FIGURA 7 - Electron micrograph showing secretory granules (arrows) in the interfibrillar sarcoplasm of an 8-day chick embryo myoendrocrine cell. My: immature myofibrils; Z: Z-lines; M: mitochondria. 15.000X.

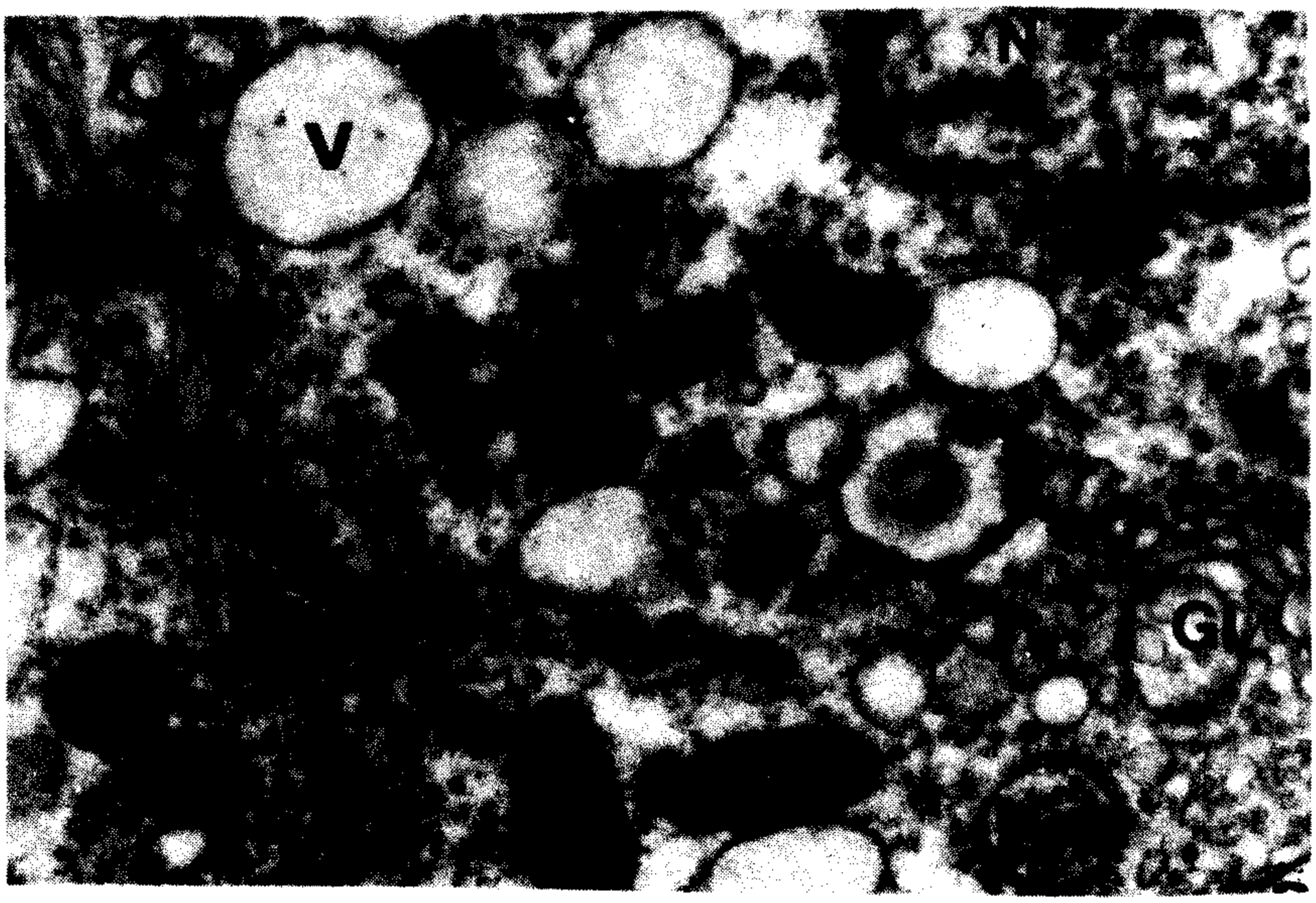

FIGURA 8 – Electron-micrograph of the perinuclear area of a 12-day chick embryo atrial myocardiocyte. Note the great development of the Golgi apparatus (Gl) with a great number of electro-lucent vesicles (V) and the multivesicular bodies (cmV); M: mitochondria; My: immature myofibrils; Li: lipid droplets. 9.000 X.

FIGURA 9 – Electron micrograph of the perinuclear area of a 16-day embryo atrial cell with myoendocrine characteristics. Note the increase of the perinuclear secretory granules (arrow). N: nucleus; Nu: nucleulus; G: Golgi apparatus; M: mitochondria; My: myofibrils. 6.000 X.

3.2.2. *Protocol 2*

Plasmatic membrane, actin filaments, microfilaments and microtubules contrasts are remarkably intensified, compared to protocol 1. Tannic acid binds to glycoproteins and other proteins associated to the plasma membrane. This protocol allows the observation of certain very contrasted structures against a light background even when they have not been stained with lead citrate. Most novel results have been achieved in 12-and 16-day-old embryo atrium. At 12 days atrial cells present already explained developmental characteristics but with a considerable increase in big electron-dense perinuclear as well as subsarcoplasmatic granules. The high contrast of membranes makes the electron-dense content of the mitochondria not clearly observed, with the mitochondrial crests neatly standing out (Fig. 10).

FIGURA 10 - Electron micrograph of a 12-day embryo atrial cell, showing electro-dense granules (arrows), size increased due to tannic acid (arrows). RER: rough endoplasmic reticulum; M: mitochondria, My: myofibrils. 14.000 X.

At 16 days the myoendocrine atrial cells show a remarkable increase in granule population and size, while keeping the ultrastructural characteristics described in protocol 1 (Figs. 11-12).

The results achieved demonstrate that ANP is present in chick embryo hearts since early developmental stages. A specific and constant immunofluorescence labelling is found in atrium, although classic ultrastructural analysis does not reveal large secretory granules population.

A gradual specialization in endocrine function might exist in the atrial tissues. While in 48- and 96-hour stages immunofluorescence appeared homogeneously weak, labelling diminishes in the ventricles and shows a great increase in the atrial cells (*2*).

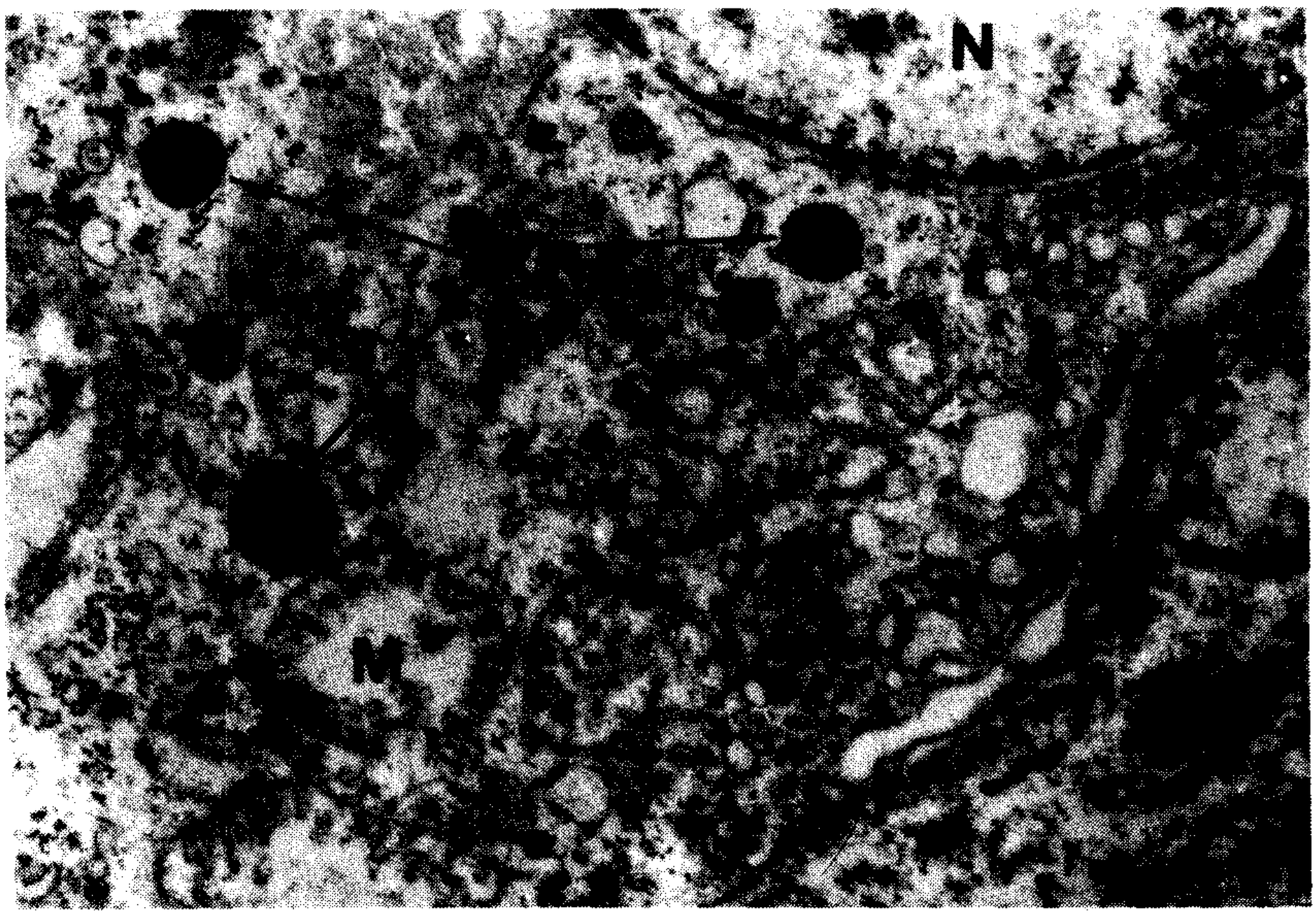

FIGURA 11 - Electron micrograph of the perinuclear area of a 16-day embryo atrial cell, showing secretory granules (SG) size increased due to tannic acid (arrow). N: nucleus; M: mitochondria. 14.000 X.

The labelling obtained in the subpericardial zone at 6-day incubation appears interesting, since some authors have suggested that these cells in rat precede the conductor system (*21, 24, 25*). In the chick embryos heart, labelling is evident in the subpericardial zone at that moment of development when contraction stops being myogenic and is performed by the conductor system.

Ultrastructural analysis shows reduced secretory granule populations when compared to cardiac myoendocrine cells of rat embryos. However, the relatively scarce secretory granule population does not allow us to rule out the possibility that ANF synthesis and secretion be also processed by a nonregulated pathway where the hormone would be liberated without secretory granules storage or formation. This hypothesis is supported by present theories on pluricellular organisms secretory system ontogeny (*2, 13*).

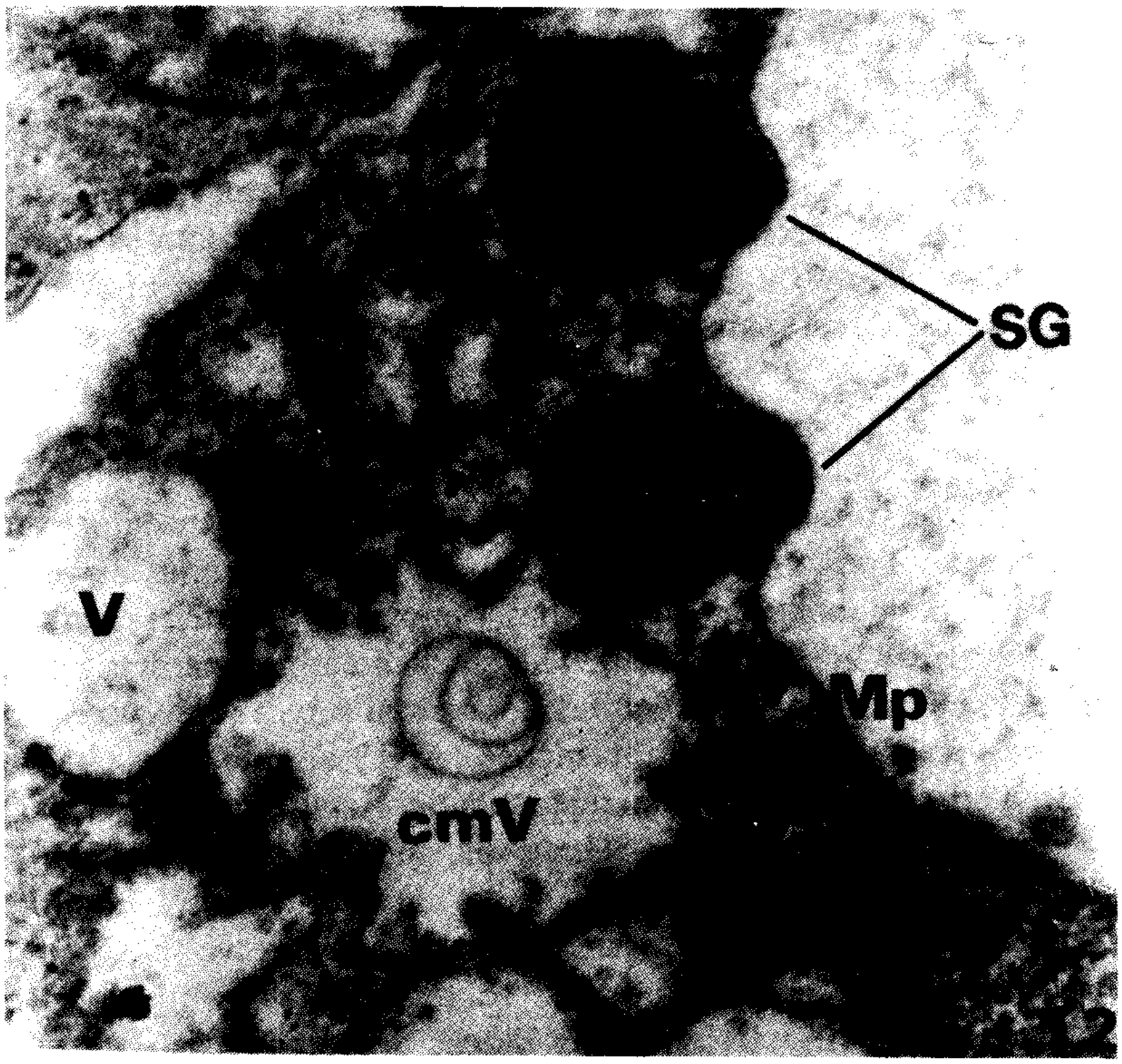

FIGURA 12 - Atrial secretory granules arrested by the action of tannic acid as they fuse with the plasma membrane. SG: secretory granules; V: electro-lucent vesicles; cmV: multivesicular bodies; Mp: plasma membrane. 45.000 X.

Undifferentiated cells would synthesize and release their products by a quick pathway, and as cell differentiation takes place, they gradually acquire a second intracellular processing pathway known as regulated, where ANF would be concentrated and stored in secretory granules (*8*). This might explain the increase in granules population in auricular cells with an evident development process observed at 16 days and lack of them in undifferentiated cells of the same stage.

The important increase in size and number of the granules observed in the tannic acid treated samples, shows that not little ANF is produced during chicken heart embryonic development.

Studies carried out by SCOTT and JENNES (*22, 23*), where ANF specific receptors distribution in rat embryos was analyzed, led to postulate that this peptide could be a factor involved in different processes such as the regulation of the brain microcirculation (as an angiogenic factor), the regulation of the production of the surfactant substances in the embryonic

lung, the glial growth and the formation of axonal pathways. This evidence could also explain its presence in chicken heart and myotome ontogeny, despite being scarce in the adult stage.

The present results allow us to state that cardiac peptides are of great importance for avian embryogenesis, and lack of secretory granules must be imputed to the fast intracellular processing that peptides undergo. Further studies should be carried out to clarify the function of this ANP-like factor during development.

4. SUMMARY

In addition to the contractile function, the cardiac muscular cells of most vertebrates synthetize and secrete, on normal conditions, a family of biologically active peptides, altogether denominated Atrial Natriuretic Peptides or cardiac hormones (*5. 7*). The chicken embryo cardiac hormone system was monitored at different developmental stages (embryonic day 4 to 16). The presence of cardiac hormones (ANF) in chick embryo hearts was examined combining immunocytochemical and classic transmission electron microscopy techniques. However, in chick embryo heart, the secretory granules were difficult to detect ultrastructurally. In order to study the ANP synthesis, store and release events, we have applied incubation of tissues in a tannic acid solution which, while keeping a certain degreee of cell activity, partly blocks the exocytosis processes of the endocrine cells. Immunofluorescence study revealed the presence of immunoreactive Atrial Natriuretic Factor (ANF) in atrial cardiocytes as well as in atrial-ventricular canal level and in the ventricle subpericardial and trabecule sub-endocardial myocardiocytes since the 4th day of embryonic life. At electron-microscopy level, tannic acid treated hearts showed a significative increase in number as well as in size in the secretory granules, specially in 12- to 16-day-old embryos. Our data suggest that, during chicken embryos early development, ANF seems to be required in the different functions that are now known.

5. RESUMO

(ASPECTOS ULTRAESTRUTURAIS E IMUNOCITOQUÍMICOS DA ONTOGENIA DO CORAÇÃO DA GALINHA UTILIZANDO ÁCIDO TÂNICO COMO INIBIDOR DOS PROCESSOS DE EXOCITOSE)

Além da função contrátil, as células musculares cardíacas da maioria dos vertebrados sintetizam e secretam, sub condições normais, uma família de peptídeos biologicamente ativos, denominados Peptídeos Natriuréticos

Atriais ou Hormônios Cardíacos (*5, 7*). O sistema de hormônios cardíacos (ANF) de embriões de galinha foi monitorado em diferentes estádios de desenvolvimento (do 4° ao 16° dia de vida embrionária), através de técnicas imunocitoquímicas e de microscopia eletrônica de transmissão. Entretanto, no coração destas aves, os grânulos secretores dessas células endócrinas são de difícil detecção ultraestrutural. Visando estudar a síntese, o armazenamento e a liberação do ANF, utilizou-se a incubação dos tecidos em solução de ácido tânico, o qual além de manter certo grau de atividade celular, bloqueia parcialmente o processo de exocitose das células endócrinas. A técnica de imunofluorescência revelou a presença de imunorreatividade ao ANF, a partir do 4° dia de vida embriônica, em cardiócitos atriais e do canal átrio-ventricular como também nos miocardiócitos ventriculares subpericardiais e trabeculares subendocardiais. A microscopia eletrônica revelou que os corações tratados com ácido tânico apresentaram aumento numérico significativo, além de aumento do tamanho dos grânulos secretores, principalmente nos embriões de 12 a 16 dias de idade. Nossos dados sugerem que durante o desenvolvimento inicial dos embriões de galinha, o ANF parece ser requisitado em diferentes funções, até então desconhecidas.

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