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HISTOFLUORESCENCE, IMMUNOHISTOCHEMICAL AND ELECTRON MICROSCOPICAL STUDIES ON THE INNERVATION OF BOVINE SEMINAL AND AMPULLARY GLANDS

(With 15 figures)

By

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دراسات نسيجية فلورية ونسيجية كيميائية مناعية ومجهرية دقيقة للأعصاب المغذية للغدة المنوية والأمبولية فى الابقار

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أجريت هذه الدراسة على عينات للغدة المنوية والغدة الأمبولية. وقد جمعت هذه العينات من ١٥ عجلا بقريا بالغا، حيث تم دراسة نمط المدد العصبي لكلا الغدتين باستخدام ثلاث تقنيات هي: نسيجية فلورية باستخدام مادة SPG ونسيجية كيميائية مناعية باستخدام مادة PGP 9.5 إضافة إلى الميكروسكوب الإلكتروني. هذا وكان توزيع المدد العصبي متشابها بشكل عام فى كلا الغدتين حيث وجدت شبكة كثيفة من الألياف العصبية فيما بين خلايا الطبقة العضلية. أما فى طبقة النسيج الضام الخلالى وتحت الغشاء المخاطي فقد لوحظ وجود حزم عصبية سميكة ومتموجة. ومن ناحية أخرى فلقد وجد بالقرب من الجدار القاعدي للنسيج الطلائى نهايات عصبية ناشئة من الحزم العصبية الخلالية ، حيث كانت هذه النهايات اكثر كثافة فى الغدة المنوية عنها فى الغدة الأمبولية. أما بالنسبة للفحص بالميكروسكوب الإلكتروني النافذ، فلقد أظهرت الدراسة وجود حزم ألياف عصبية غير نخاعية تحت النسيج الطلائى وكانت النهايات العصبية منغمسة كليا أو جزئيا فى زوائد خلايا " شوان " ومفصولة عن النسيج الطلائى الغدى بمسافة صغيرة. تحتوى هذه النهايات العصبية على نوعين من الحويصلات ، نوع صغير لا يحتوى على المادة الإلكترونية الكثيفة ونوع كبير يحتوى على هذه المادة. هذا ولم يتم مشاهدة أي اتصال مباشر بين النهايات العصبية والنسيج الطلائى الغدى.

SUMMARY

Samples of seminal and ampullary glands collected from 15 mature healthy bovine bulls were used in this study. The pattern of innervation of the two glands was investigated using a combination of SPG-histofluorescence, PGP 9.5 -immunohistochemical and electron microscopical techniques. For fluorescence microscopical examination, cryostat sections (10-25 um thick) were prepared and stained with SPG - technique for detection of pre- and terminal axonal nerve fibers containing positive monoamine transmitters. For immunohistochemical reaction, antibodies against PGP 9.5, as a very sensitive nerve marker, were used. Both glands revealed nearly similar distribution of nerve fibers. In the muscular coat, a dense network of fine nerve fibers was observed in between the muscle fibers. In the submucosa and interlobular connective tissue, thick wavy bundles of nerve fibers were observed. In close position to the basal border of the glandular epithelium, fine nerve terminals were seen arising from the interstitial axonal bundles. These terminals formed a dense nerve plexus around the glandular end-pieces and appeared highly dense in the seminal gland than that in the ampullary gland. Ultrastructural examination of the glandular epithelium-terminal nerve fibers relationship revealed subepithelial bundles of unmylinated nerve fibers. The terminal axonal nerve fibers were embedded either completely within Schwann's cell processes or appeared partially free toward the glandular epithelium. They were separated from the epithelium by a thin layer of extracellular matrix. Within these nerve terminals two types of synaptic vesicles were demonstrated; small agranular vesicles and large granular vesicles containing electron dense core. Nerve terminals that forming a direct neuro-glandular contact with the epithelial cells were not identified.

Key Words: *Bovine-Seminal gland-Ampullary gland- Immunofluorescence-Immunohistochemistry- Ultrastructure*

INTRODUCTION

The seminal and ampullary glands form, with the prostate and bulbourethral glands, the accessory male genital glands in bovine bull. They are responsible for the secretion of the seminal plasma, which is

very important to induce a successful fertilization (Aumüller, 1979; Wrobel & Sinowatz,1985 and Amselgrüber and Feder, 1986).

Several studies dealing with the morphology, physiology and biochemistry of the accessory male genital glands of laboratory animals have been recorded. Concerning the seminal and ampullary glands of bull, beside the light microscopical studies (Hendrich, 1905; Mann et al., 1949; Egli,1956; Hay et al.,1961 and Kainer et al., 1969) and the biochemical investigations (Cons, 1956; Mosimann, 1959; Künzel & Tanyolac, 1968a&b; Rama et al., 1971; Wrobel & Mercsék, 1976 and Wrobel & Inczédy-Mercsék, 1977), electron microscopical examinations have been performed by several authors (Künzel & Tanyolac, 1968a and Amselgrüber & Feder, 1986). In addition, immunohistochemical and ultrastructural studies concerning both cholinergic and adrenergic innervation of the seminal vesicle and vas deferens glands have been recorded; in buffalo (Abou-Elmagd et al., 1992), laboratory animals (Sjöstrand & Hammrström, 1995; Falck et al., 1965; Owman & Sjöstrand 1965; Norberg et al., 1967; Al-Zuhair et al., 1975 &1977; Ventura & Bürnstock, 1996; Pinho et al., 1996 and Kempinas et al., 1995) and in human being (Tainio, 1995).

In the last few years, the accessory male genital gland have been the object of few physiological and pharmacological studies with respect to secretomotor and secreto-inhibitory innervation, in order to better understand the synchronization between the innervation and both secretory substance synthesis and its excretion. These recent studies indicate the need to more knowledge of immunohistochemical and ultrastructural investigations of the bull accessory genital glands. Therefore, this investigation was undertaken to clarify the innervation of the seminal and ampullary glands using a combination of histofluorecence, immunohistochemical and ultrastructural techniques.

MATERIALS and METHODS

The seminal and ampullary glands were collected from 15 mature healthy bovine bulls immediately after slaughtering. They were completely removed together with their local supplying arteries. For ultrastructural examination, the local supplying arteries were canulated and a rinsing fluid (for composition see Wrobel et al., 1977) was injected, followed by perfusion fixation at

room temperature using a glutaraldehyde-paraformaldehyde mixture as described by Karnovesky (1965). After fixation small tissue blocks were cut and washed in 0.1 M cacodylate buffer and osmicated by 1% OsO_4. The blocks were dehydrated in up-graded ethanol and embedded in ERL4206 (Spurr, 1969). Ultrathin sections were mounted on copper grids, stained with urinyl acetate and lead citrate (Reynolds, 1963) and examined by electron microscope.

For histofluorescence demonstration of the innervation of the seminal and ampullary glands, sacrose-potassium phosphate-glyoxylic acid (SPG) technique was used for monoamines localization. Fresh tissue blocks were cut into 5–10 mm slabs and immediately frozen on a pre-cooled chuck in cryostat. After tissues were frozen (about 10 min), cryostat sections 8–25μ thick were cut and mounted on glass slides. The sections were stained with SPG – solution as described by Lindvoll et al. (1974) and examined with fluorescence microscope.

For immunohistochemical examination of the general pattern of innervation, reaction against protein gene product 9.5 (PGP 9.5), as a general nerve marker, was performed. The material of seminal and ampullary glands from six animals were fixed by perfusion. Fixative I was injected with slow pressure for 5 min, followed by fixative II for 10 min (For composition see Abou-Elmagd et al., 1992). After fixation, tissue blocks of 1.0 cm^3 were cut and postfixed in ice-cold fixative II for 45 min. Tissue blocks were then washed frequently in ice-cold mixture of 0.1 M phosphate buffer (pH 7.4) and sacrose (the sacrose percent in phosphate buffer was increased gradually from 10 % to 40%). For immunohistochemical reaction against PGP 9.5, 12 μ thick mounted cryostat sections were incubated in blocking buffer containing goat serum and fetal calf serum for 60 min, then incubated in primary antibody (dilution 1:5000) for 18h at room temperature and in secondary antibody (biotinylated goat-antirabbit) in dilution (1: 200). Blocking of endogenous peroxidase with phenylhydrazine and H_2O_2 (6%), followed by incubation with the avidin-biotinylated peroxidase complex (ABC) for 60 min and

staining with DAB solution for 15 min at room temperature, washing, dehydration and mounting in Depex were performed.

RESULTS

The SPG –histofluorescence demonstrated clearly the general pattern of distribution of pre- and terminal axonal nerve fibers in both seminal and ampullary glands of bovine bull. Fluorescence microscopical observations revealed nerve fibers containing positively reacted monoamines transmitters. They were of different diameters and scattered in the muscular coat, submucosal connective tissue and around the glandular end-pieces. They appeared in form of variable fluorescent varicosities (Fig.1). The muscular coat of both glands showed a fine dense network of nerve fibres surrounding the muscle cells (Figs. 1 & 5). In the submucosa and interlobular connective tissue septa, relatively thick wavy bundles of axonal aminergic nerve fibers were observed (Figs. 2 & 4). In the interstitial connective tissue and near the basal border of the glandular epithelium, thin bundles of nerve fibres were easily distinguished (Fig.2). Fine nerve terminals arising from the interstitial and subepithelial nerve bundles could be seen (Figs. 3). These terminals surrounded the glandular end-pieces and formed a dense nerve plexus of very fine nerve fibres in close relation to the basal border of the glandular epithelium. Comparatively, the fluorescent fine terminal nerve plexus in the interstitial tissue and around the glandular end-pieces appeared well developed in the seminal gland than that in the ampullary gland (Figs. 4 & 5).

Immunohistochemical reaction against PGP 9.5, as a very sensitive nerve marker, demonstrated similar observations as those described by using the SPG -histofluorescence technique. In addition, a very clear branching of the nerve bundles were demonstrated forming fine nerve terminals that run in between and parallel to the longitudinal axis of muscle fibers (Fig.6). Some of them were observed crossing the muscle bundles. In the submucosa and interlobular supporting connective tissue, wavy or zigzag-like preterminal nerve fibres as well as thick bundles of axonal nerve

fibers were clearly seen (Figs.7-9). In addition, a dense plexus of terminal nerve fibers around the glandular end pieces were relatively more prominant in the seminal gland as compared with that in the ampullary gland (Figs. 8 & 9).

Electron microscopical investigation demonstrated clearly the relation between the subepithelial nerve terminals and the glandular epithelium. The terminal nerve fibers which were located very close to the glandular cells in histofluorescence and immunohistochemical observations, appeared as bundles of variable number of unmyelinated axonal nerve fibers (Fig.10 & 13). These axonal nerve fibres were observed near the blood vessels or scattered in the subepithelial connective tissue. They were embedded within the cytoplasmic processes of the Schwann cells. Nerve terminals penetrating the basal lamina of glandular epithelium or located within the intercellular spaces of the glandular cells were not observed with electron microscopical examination. Terminal nerve fibers, located in a position close to the basal lamina of the glandular epithelium of both seminal and ampullary glands, were partially devoid of a covering neurolemmal cell cytoplasm toward the epithelial basal lamina. They were separated from the extracellular matrix only by a basal lamina (Figs. 11, 14 & 15). Within the axoplasma of the nerve terminals, in addition to the neurofilaments, neurotubules and small mitochondria, two types of synaptic vesicles were observed (Fig.12). The first type was agranular containing unstained core and measured 25 – 50 nm in diameter. The other type contained electron dense core and measured 60- 120 nm in diameter. Some of the nerve terminals contained predominantly dense vesicles (Fig.11), others had more unstained vesicles than the dense ones (Fig.12). Nerve terminals containing very fine electron-dense granules were also demonstrated (Fig.11).

DISCUSSION

Using SPG-histoflurescence of monoamine transmitters in combination with immunohistochemical techniques against PGP-

9.5 showed nearly a similar distribution of pre- and terminal nerve fibers in both seminal and ampullary glands of bovine bull. This similarity could be attributed to the fact that both glands are derived from the same premordium. In addition, both glands are morphologically identical where their end-pieces are lined by secretory cells and their glandular tissue is surrounded by a muscular coat.

The present study revealed the presence of a dense network of aminergic nerve fibers around the muscle fibers in the muscular coat. Similar observations have been recorded in the seminal gland of buffalo (Abou-Elmagd et al., 1992), guinea pig and rat (Al-Zuhair et al. 1975, and Kepper & Keast, 1995; Sjöstrand and Hammarström, 1995) as well as in human-being (Tainio, 1995). In agreement with Abou-Elmagd & Wrobel (1989), Abou-Elmagd et al. (1992), Sjöstrand & Hammarström (1995) and Tainio (1995), these nerve terminals may be responsible for determination of the exact timing of evacuation of the glandular end-pieces and the central duct lumen during ejaculation. Using immunohistochemical investigation, Abou-Elmagd et al. (1992), recorded dopamine-β-hydroxylase positive terminal nerve fibers in the muscular coat of the buffalo seminal gland. Moreover, immunohistochemical and expermintal studies on the seminal gland and vas deferens in guinea pig (Al-Zuhair et al., 1975; Sjöstrand & Hammarström, 1995) and in human-being (Tainio, 1995) revealed the presence of excitatory adrenergic nerve fibers supplying the muscular coat. Ultrastructurally and as stated by Furness & Iwayama (1971,1972); Al-Zuhair et al. (1975), these nerve terminals are unmyelinated, partially or totally devoid of a covering neurolemmal cell cytoplasm and in close contact with the muscle cells.

Concerning the glandular epithelium-subepithelial nerve terminals relationship in both glands, a heavy terminal nerve plexus surrounding the end-pieces was demonstrated. They were frequently well developed in the seminal than in the ampullary gland. The seminal gland contributes the largest volume to the seminal fluid (Peters and Ball, 1987). It can be postulated that the dense innervation of the seminal gland is due to its high secretory

activity as compared with the ampullary gland and that there is a relation between the glandular secretion and the density of its innervation.

The subepithelial connective tissue displayed terminal nerves in close contact with the basal border of glandular epithelium. These terminals were separated by narrow distance from the basal lamina of the glandular epithelium when examined by electron microscope. Although nerve terminals were not observed within the intercellular spaces in electron microscopy of bovine seminal and ampullary glands, some organs such as seminal and prostate glands of buffalo (Abou-Elmagd and Wrobel 1989; Abou-Elmagd et al., 1992), seminal and sweat glands of guinea pig (Al-Zuhair et al., 1975) showed a great number of naked axons penetrating the epithelial basal lamina and situated within the intercellular spaces of the glandular epithelium. After expermintal studies on the seminal gland in guinea pig, Sjöstrand & Hammartröm (1995) mentioned that, the distance between the nerve terminals and the effector cells is presumably one reason for the rather high stimulation frequencies needed in order to obtain significant neurogenic responses.

The present ultrastructural picture of the nerve terminal contents, concerning the presence of granular and agranular vesicles, simulates the findings of Richardson (1964&1966), Hökfelt (1968), Ehinger et al. (1970), Saxena (1970), Sjöstrand (1965) and Sjöstrand & Swedin (1970) in laboratory animals, Abou-Elmagd & Wrobel (1989) and Abou-Elmagd et al. (1992) in water buffalo. The previously mentioned authors reported that these nerve terminals are adrenergic and cholinergic in nature. In addition, using immunohistochemical and ultrastructural studies, Al-Zuhair et al. (1975) reported that the terminal regions containing small agranular vesicles represent cholinergic terminals. All these findings point to the presence of cholinergic and adrenergic innervation in bovine seminal and ampullary glands.

In recent experimental study on the guinea pig, Sjöstrand and Hammerström (1995) did not found a convincing evidence of the presence of adrenergic innervation and mentioned that the

cholinergic secretomotor terminals stimulate the secretory cells directly via acetyl choline release and excitation of innervated muscarinic receptors. According to them, the adrenergic suppression of the secretory cells takes place indirectly through inhibition of the secretomotor fibers by noradrenaline release and activation of innervated neuronal receptors. The released noradrenaline may also togethar with catecholamine from blood activate humoral inhibitory adrenoreceptors on glandular cells. In addition, Kepper and Keast (1995) stated that the activity of the gland is enhanced by sympathetic outflow where as the role of the parasympathetic nervous system in the organ is unclear.

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LEGENDS

Figs. 1-5: Histofluorescence reaction of aminergic nerve fibers in seminal and ampullary glands of bovine bull.

Fig. 1: Dense nerve terminals (arrow) are seen in the muscular coat of the ampullary gland. Arrowhead indicates nerve terminals in the subepithelial connective tissue and lamina propria. x63.

Fig. 2: Thick bundles of nerve fibers (arrow) in the interlobular connective tissue and subepithelial thin bundles of nerve fibers (arrowhead) in the ampullary gland. Notice the presence of fine nerve fibers in close relation to the epithelial cells. x160.

Fig. 3: Higher magnification of the subepithelial nerve terminals in the form of bundles (arrow) and fine nerve terminals (arrowhead) arising from it in the ampullary gland. x400.

Fig. 4: Seminal gland showing dense nerve plexus (arrow) in the interstitial tissue and around the secretory end pieces. x160.

Fig. 5: Ampullary gland showing low density of nerve plexus (arrow) in the interstitial connective tissue and around the secretory end pieces. x160.

Figs. 6-9: Immunohistochemical reaction against PGP 9.5 in the seminal and ampullary glands of bovine bull. x250.

Fig. 6: Muscular coat of seminal vesicle showing nerve bundles (arrow) and fine nerve terminals (arrowhead).

Fig. 7: Nerve bundle (arrow) in lamina propria and subepithelial nerve terminals (arrowhead) in the ampullary gland.

Fig. 8: Seminal gland showing dense nerve plexus (arrowhead) in the interstitial tissue and around the secretory end pieces.

Fig. 9: Ampullary gland subepithelial nerve terminals. Arrow head indicates wavy nerve bundles.

Figs. 10-15: Electron micrographs of the subepithelial innervation of the seminal and ampullary glands of bovine bull.

Fig. 10: Bundles of variably numbered nerve fibers (arrow) embedded within cytoplasmic processes of Schwann cells (arrowhead) in the interstitial tissue of ampullary gland. x11900.

Fig. 11: Few unmyelinated nerve terminals in the subepithelial connective tissue of the seminal gland showing electron-dense granules (empty arrowhead) and agranular clear vesicles (arrowhead).

Small arrow indicates partially uncovered nerve terminal containing very small electron-dense granule with the uncovered surface opposing the epithelial basal lamina. Large arrow indicates the dilated basal intercellular spaces. x11900.

Fig. 12: Interstitial nerve terminals in the ampullary gland containing large amount of agranular vesicles (arrow) and small mitochondria (arrowhead). Empty arrowheads indicate granular vesicles with electron-dense core. x19000.

Fig. 13: Subepithelial connective tissue of ampullary gland showing unmyelinated nerve fibers (empty arrow) partially without neurolemma and separated from the extracellular matrix only by a basal lamina (arrowhead). Arrow indicates Schwann cell process. x11900.

Fig. 14 & 15: Subepithelial connective tissue of ampullary (Fig.14) and seminal (Fig.15) glands showing a narrow distance (empty arrow) separating an unmyelinated nerve terminal without neurolemma (arrow) and the basal lamina (arrowhead) of the epithelial cells. x9600 in fig. (14) and x 14900 in fig. (15).

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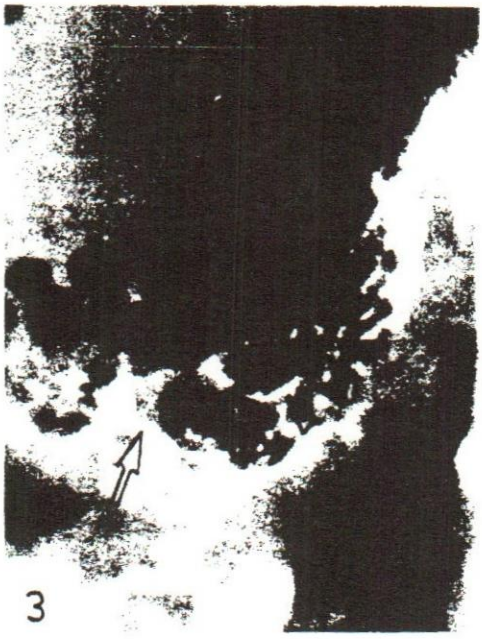

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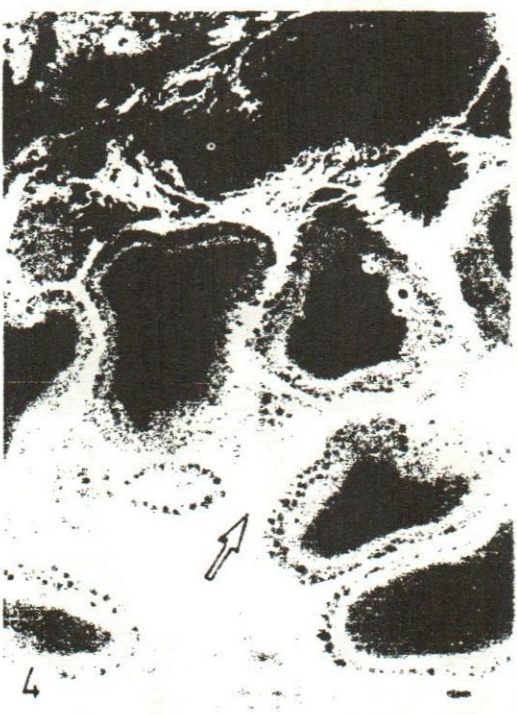

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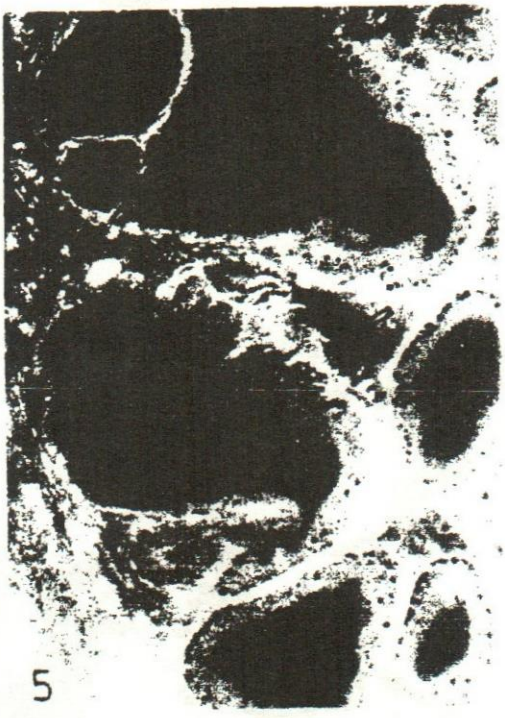

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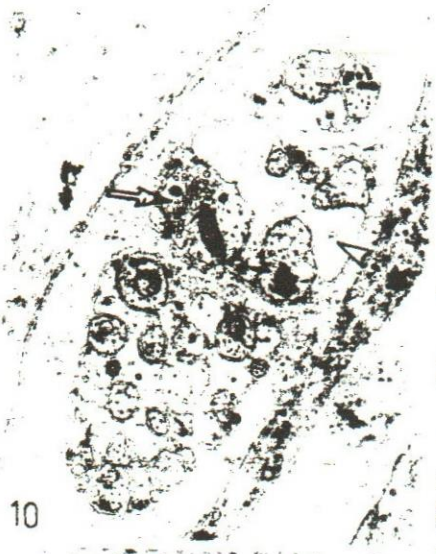

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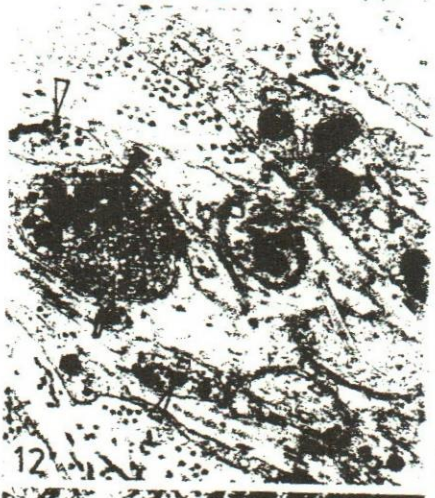

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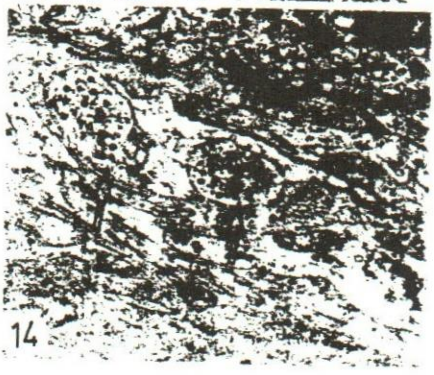

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