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Thermotherapy for Harding-Passey melanoma: light and electron microscopic study

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Summary. The changes of implanted Harding-Passey melanoma in C57Bl/6J mice following treatment with wholebody hyperthermia were studied.

The treated tumours showed a progressive growth delay, cellular and architectural irregularities as well as cell injury characteristics. The presence of distended and irregular blood vessels, the peripheral localization of the melanosomes and the melanosome complexes were constant.

Key words: Harding - Passey melanoma - Hyperthermia - Thermotherapy - Ultrastructure

Introduction

The succesful treatment of melanoma is a challenge to oncology since its high metastasis rate and evolution is usually diagnosed at stages of development when its surgical remission is ineffective.

Hyperthemia had been used to treat varios tumours (Luk et al., 1981; Vora et al., 1982) and other sorts of disorders where cell proliferation takes place (Urano et al., 1980). The first experiment as antineoplastic therapy was carried out by Greech et al., (1959), who used the blood
stream as heat source. Later, many reports have been published where isolated or associated hyperthermia was applied with radiotherapy and chemotherapy (Didolkar et al., 1986; González et al., 1986; Silverman et al., 1986).

The mechanisms causing harmful effects of heat on neoplastic cells are not completely clear. Protein denaturation phenomena, disorders of the cell membrane with an increase of its permeability as well as an irreversible alteration of the intermediary metabolism are some of the possible answers (Streffer et al., 1978; Cozad, 1983).

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Research on the morphological changes that occur in the tumoral parenchyma and vascular-connective stroma after hyperthermia is scarce. Our aim is to fill this gap.

Materials and methods

80 C57B1/6J male mice, 6 weeks old and weighing 20 g were used. Harding-Passey melanoma supplied by the Institute Cancer, Research, Royal Cancer Hospital, London, was transplanted subcutaneously at a rate of 10^6 viable cells per 0,3 ml in the dorsal side of the femoral root of mice, which were further separated at random into control and treated groups (40/40).

One group was treated with whole-body hyperthermia introducing them into a ventilated hyperthermia chamber Unitemp at 40°C/45'/5 consecutive days from the implant day and at 65% relative humidity. The other were used as a control group and kept at ambient temperature (23±2°C). Rectal temperature was determined by a thermoselective pair connected to an Ellab thermometer and three RMG thermoselective sondes. Ketamine (3mg/K/day/i.p.) was administered to every mouse before hyperthermia.

Animals were killled on the 11th, 15th, 18th, 22nd and 25th days after the implant. Tumour weights and volumes were studied according to the method of Yamada et al. (1984). Samples of tumours and organs were fixed in 10% neutral formaldehyde for 24 hours, dehydrated, cleared, and embedded in paraplast. Six step sections 5μ thick were cut and stained with hematoxylin and eosin (H&E), Masson-Fontana and Gordon-Sweet.

The mitotic index was obtained by counting mitosis at random from every tumor in ten ground ×40 magnification.

On ultrastructural study, tumor tissue fragments of 1 cu mm were fixed in 2% glutaraldehyde buffer; post-fixed in 2% osmiun tetroxide for 2 hours; block-stained with 2% uranyl acetate, dehydrated through graded acetones; cleared with propylene oxide, and embedded in Epon. Semithin sections were stained with 1% toluidine blue. Viable areas of tumours were chosen, and thin sections were cut with a diamond knife and stained with

uranyl acetate and lead citrate and studied under a Zeiss EM 10 C transmission electron microscope.

Analysis of variance from factorial design with 2 factor was used to test the effect of different groups and different days on avarage weights. Also the evolutions of average weights for both groups, treated and control, by parabolic regression analysis were studied.

Results

Macroscopically, tumours from both groups showed similar characteristics during the first stage of the experiment (days 8, 11 and 15 after implant) appearing as irrregular masses with multiple projections in subcutaneous tissue and difficult to separate. During the second stage (days 18, 22 and 25), well defined nodular masses surrounded by a thin capsule were noted. During the last days of the experiment (22, 25) the capsule usually surrounded underlying muscular tissue as well as papilar dermis; so that the epidermis could be ulcerated as a consequence of compression. In those treated, it is important to emphasize the presence of irregular and depressed areas where fibrosis thickening of the capsule occurred.

The characteristic soft consistency became fluctuating and friable when sectioned, and the homogeneous blackish stain showed white or yellowish areas enlarging throughout the experiment.

Table 1 shows significant differences between group weights. Differences between both groups were highly significant depending on days (interaction) and weights on different days.

Table 2 shows the regression analysis corresponding to the study on mean weight evolution in both groups. Both evolutions adjusted themselves perfectly to a parabolic function of range of the studied days, although their curves were different (Table 3). Thus the parabole adjusted to the mean weight of the hyperthermia group which reached its maximum by the 23rd day.

The group treated with hyperthermia always obtained a mean weight below the control group. Its mean weight evolution was progressive per day up to the 23rd day when it became stable; thereby, its differences with the control group, with respect to mean weight, became higher and higher.

With regard to tumoral volumes, variance analysis did not show significant differences of mean volumes in either group.

Microscopically, in the control group the tumoral architecture did not show important changes throughout the experiment: melanocytes arranged in quite regular nests and surrounded by a thin reticulum and many narrow blood vessels. Adjoining cells were usually homogeneous, polygonal or rounded with eosinophilic cytoplasms and regularly bordered rounded or ovoid nuclei, chromatin in fine granules and usually, a single prominent eosinophilic nucleolus. (Fig. 1).

In those treated, the presence of blood vessel disorders was constant. During the first days blood vessels appeared distended and hyperemic. It made tumours present architectural irregularities since the beginning which increased during the last days. Nests showing characteristics similar to the controls, alternated with fusiform melanocyte areas, as well as others where a prevalence of globular cells occurred with clear cytoplasms and uni-or binucleate cells. (Cells also revealed higher pleomorfism and large multinucleated cells were frequent too). The nucleus borders were irregular, chromatin was arranged in thick clots strengtheining the nuclear membrane and there were refrigent vacuoles in many of them. Nucleoli were frequently multiple and voluminous. Mitosis was frequent, ranging between 7.5 and 10.5 per ground ×40 magnification. No significant differences with respect to

Table 1. Table of analysis of variance of the tumour weights.

ANALYSIS OF TWO-WAY VARIANCE					
SOURCES OF VARIATION	D.F.	S.S.	M.S.	F	P<
GROUP	1	Q1=27'71887	M1=27'71887	F1=59'15	P<0'001
DAYS	5	Q2=85'97555	M2=17'19511	F1=36'69	P<0'001
INTERACTION	5	Q3=16'48128	M12=3'29625	F12=7'03	P<0'001
ERROR	48	Qo=39'36731	Mo=0'468658		

Table 2. Regression analysis of the evolution of the average weights for data sets of control and hyperthermia.

GROUPS	EVOLUTION EQUATION	CORRELATION COEFFICIENT	P<	SIGNIFICANT DAY
CONTROL	$Y=0'12-0'09t+0'01t^2$	r=0'94	0'005	22
HYPERTHERMIA	Y=2'28+0'36t–0'01t	r=0'95	0'04	22

the mitosis rate between both groups were found (Figs. 2 - 4).

Tumoral necrosis in all groups was constant. During the first days it was arranged in small irregular foci, mainly central; whereas during the last days it filled up almost the whole tumoral volume, spreading along the periphery, reaching the capsule with many tumoral islands inside (Fig. 5).

Stroma was scarce and represented by a thin reticulated framework and the vessels already described. However, during the last days of the experiment, treated tumours showed numerous fibrosis areas, irregularly extended from the capsule to the tumour centre, lobulate and containing melanocytes with frequent involutive phenomena (Fig. 6).

Host's cellular answer was also constant, composed of many melanophages, polymorphonuclear leucocytes, lymphocytes and plasmocytes; and mainly arranged all around the necrosis areas and the tumoral periphery. In those treated, the tumoral periphery seemed to be gradually changed in such a way that during the last days, it was arranged in a wide band surrounding the tumoral circumference; while in the control group, it remained the same during the first days and then seemed to diminish to small, irregularly distributed foci.

Ultrastructurally, the low number of variations observed by the light microscope in the control group was verified. The architectural pattern corresponded to compact groups of polygonal melanocytes regularly surrounding rounded or ovoid nuclei. Heterochromatin formed thick clots which strengthened the nuclear membrane (Fig. 7).

In relation to cytoplasm, it is important to emphasize the richness of rough endoplasmic reticulum and free ribosomes, well developed Golgi complexes, many egg-shaped mitochondrias and many melanosomes in various stages of development.

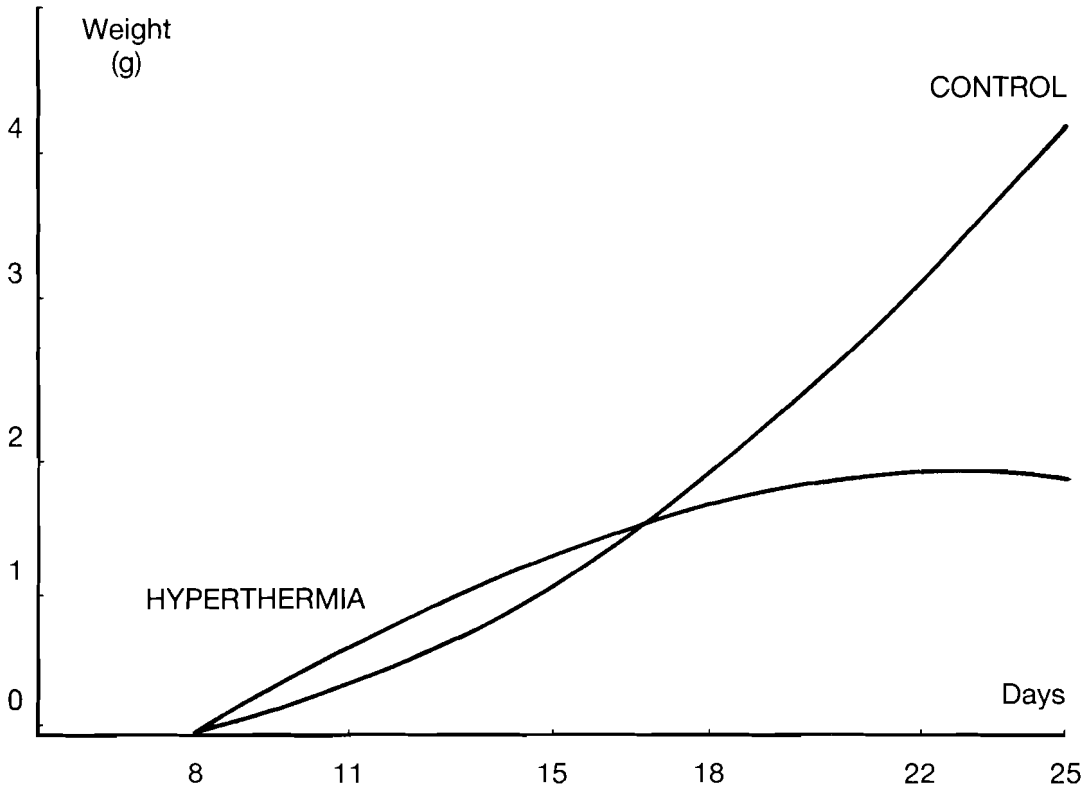
Treated tumours showed architectural and cytological irregularities, mainly during the last days of the experiment. Next to areas of similar characteristics already described, other areas with voluminous melanocytes, low electron-density cytoplasms as well as fusiform melanocyte areas with few cytoplasms were found. (Fig. 8).

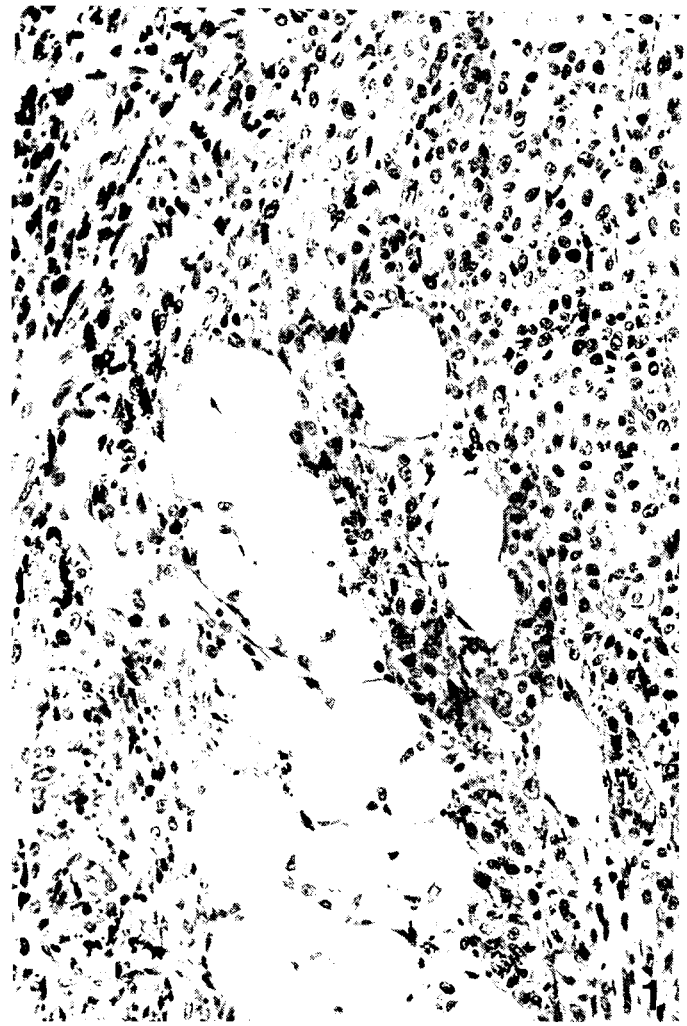
Nuclear borders were usually irregular with lobulate nuclei. Heterochromatin was located peripherally, strengtheining the nuclear membrane by vacuolization processes. In some areas it was reduced to a thin layer next to the nuclear membrane. Nucleoli were large and usually multiple; dilation of the nuclear envelope ocurred (Figs. 9, 11).

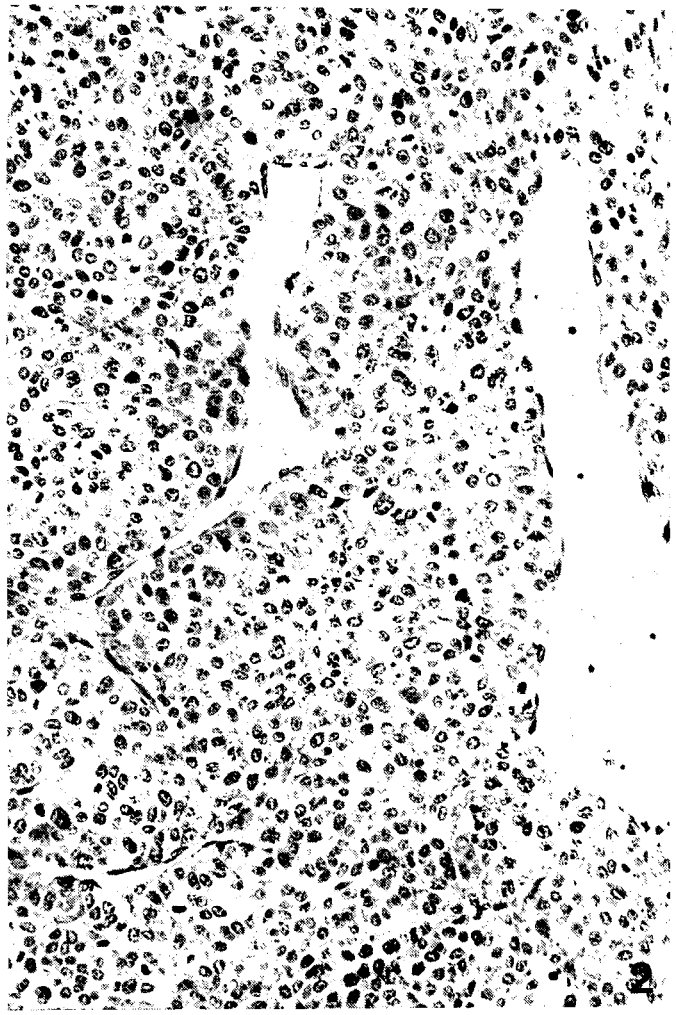
With respect to cytoplasms, the most important aspects were the swelling of the organelles, especially the mitochondria, frequent lipid vacuoles and the location of peripheral melanosomes and the important discovery in this group was melanosome complexes formed by several melanosomes in various stages of development and enveloped by membrane (Fig. 10).

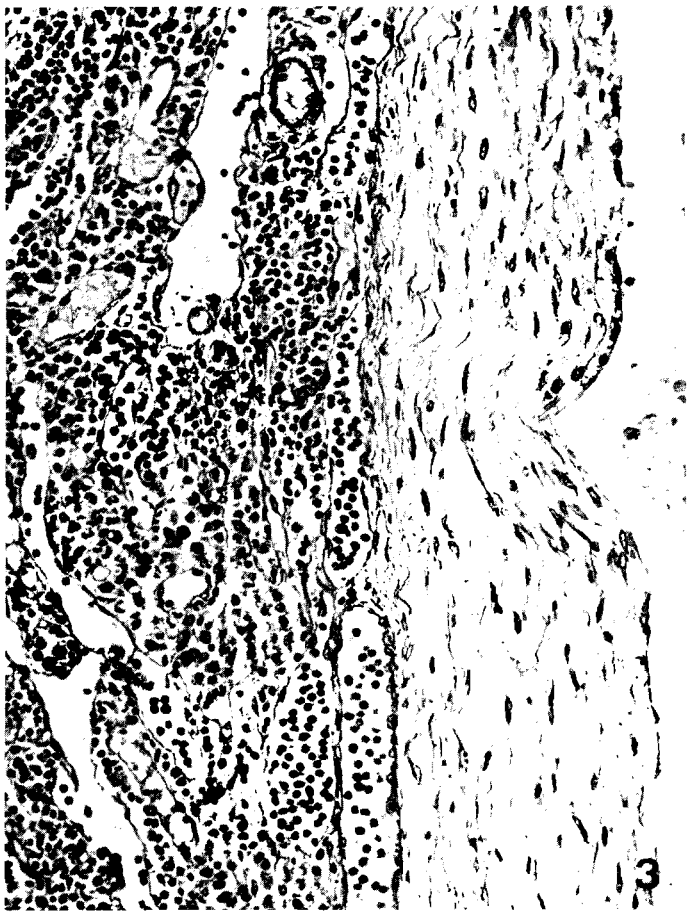
Blood vessels presented irregular cavities with prominent endothelial cells. Numerous pinocytosis vesicles in cytoplasms and basal membrane zones of differnet thicknesses showing rupture at times were also noticed (Fig. 12).

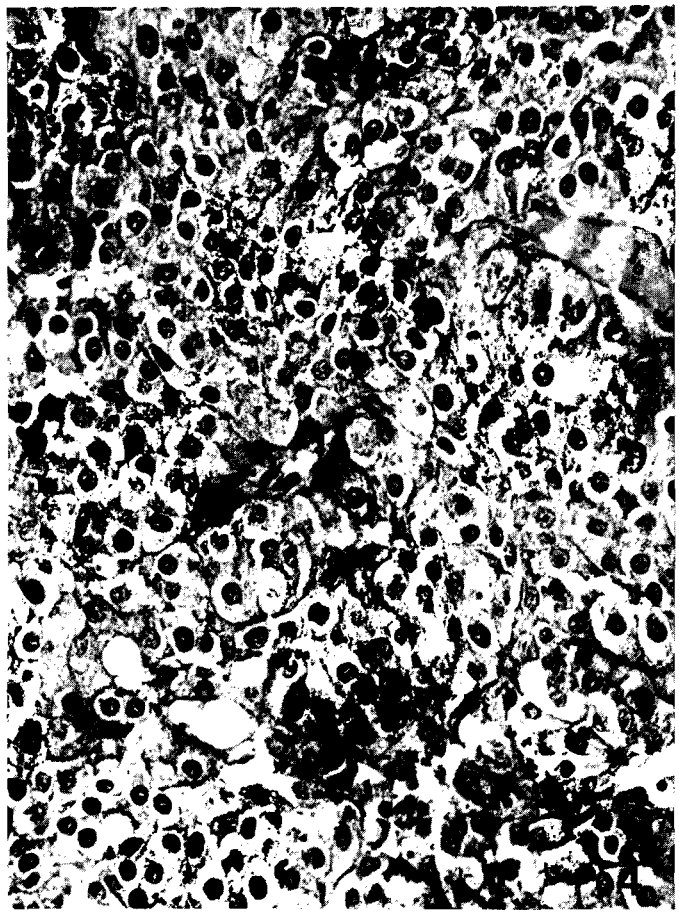
Table 3. Plot of the Fitting of regression curve for the relationship between average weight and days for data sets of control and hyperthermia.

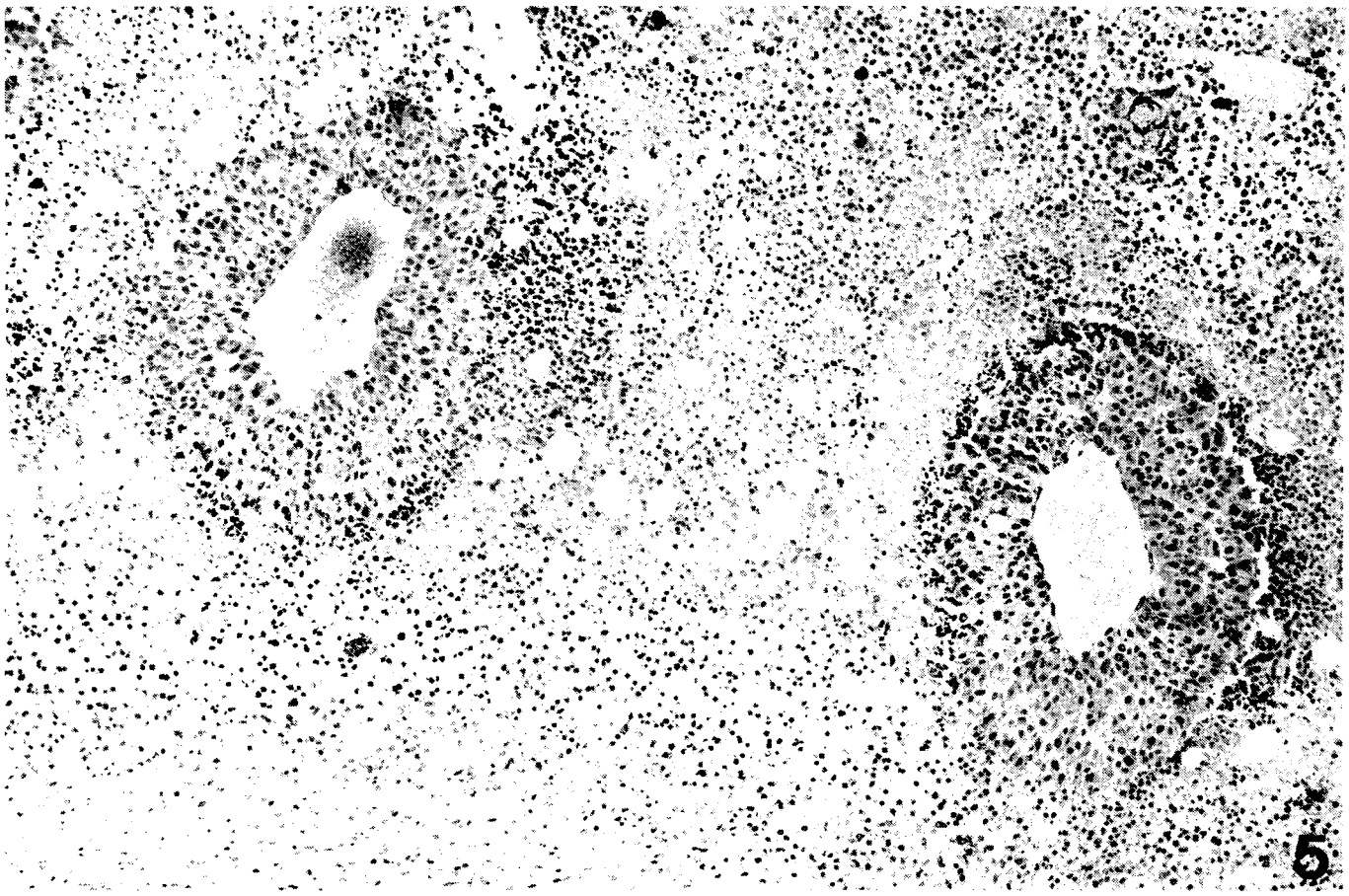












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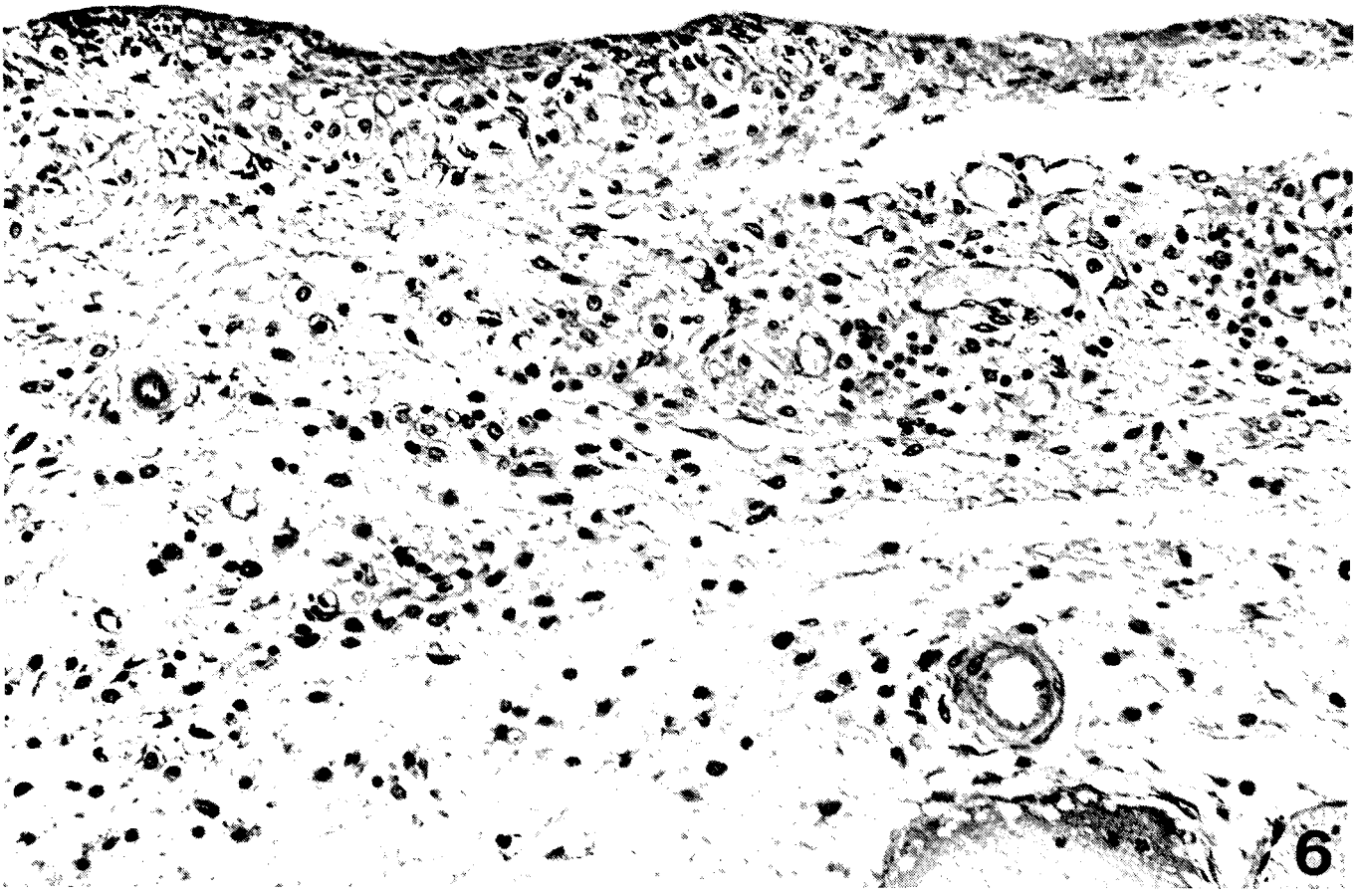

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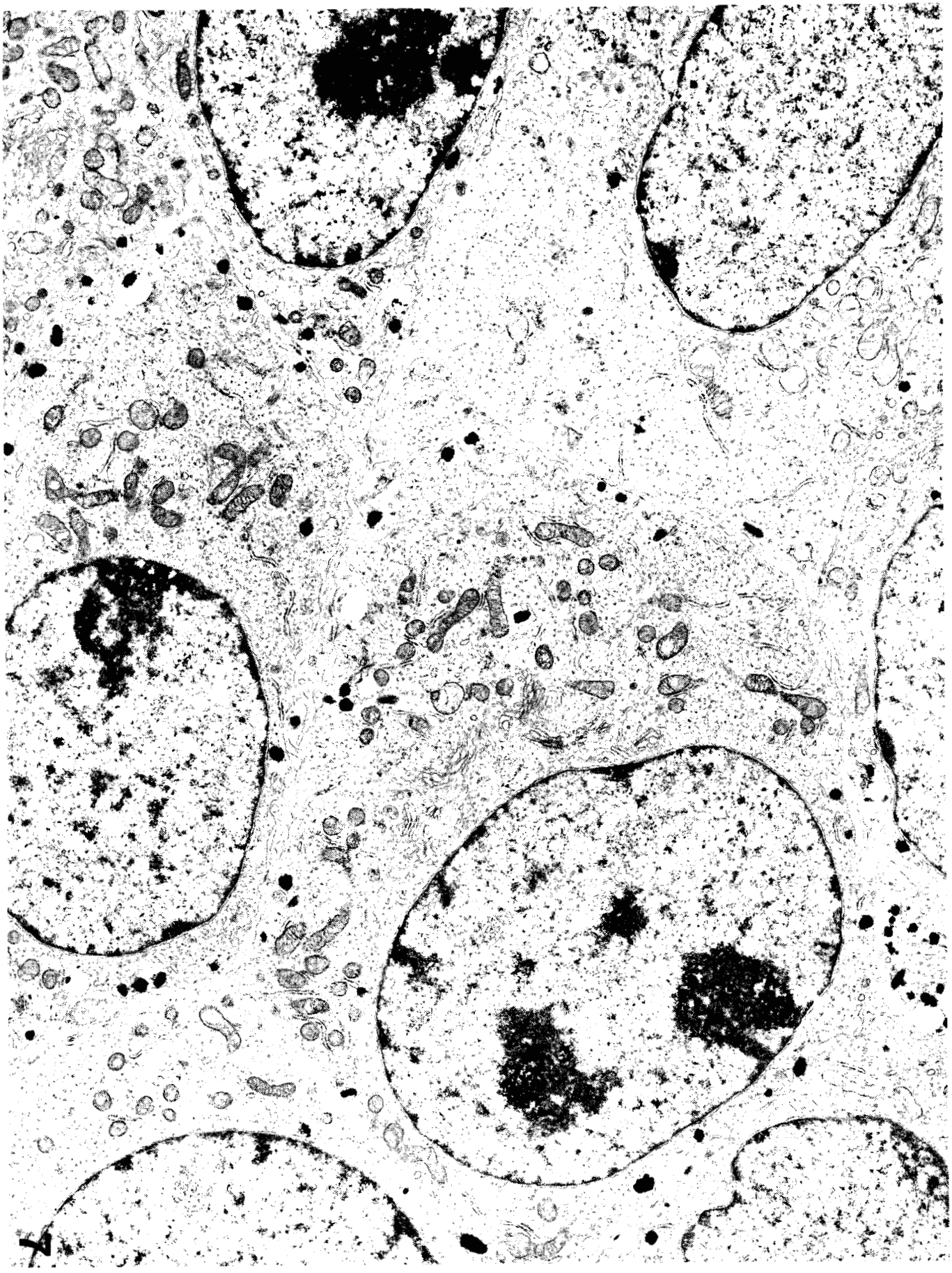

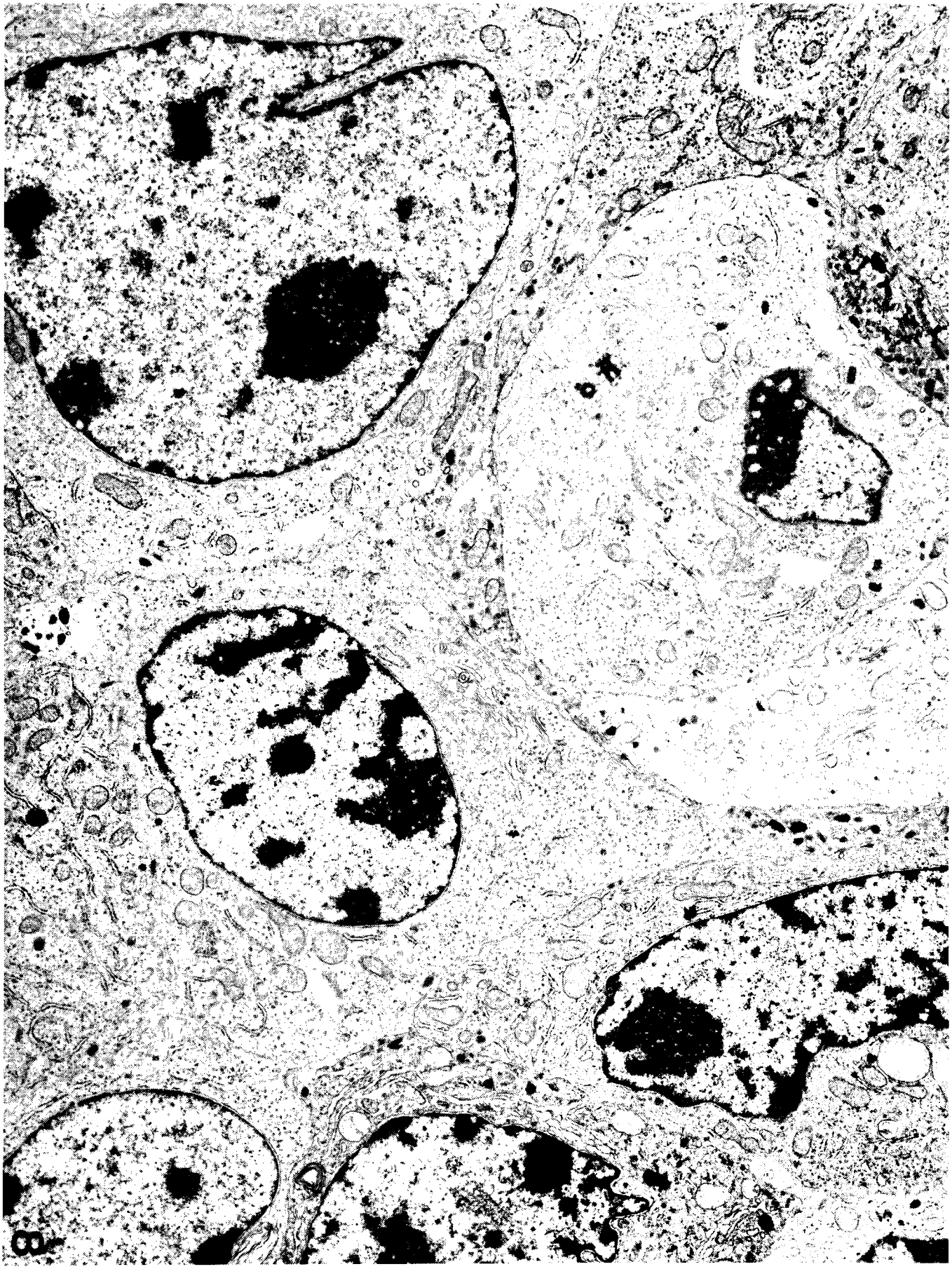

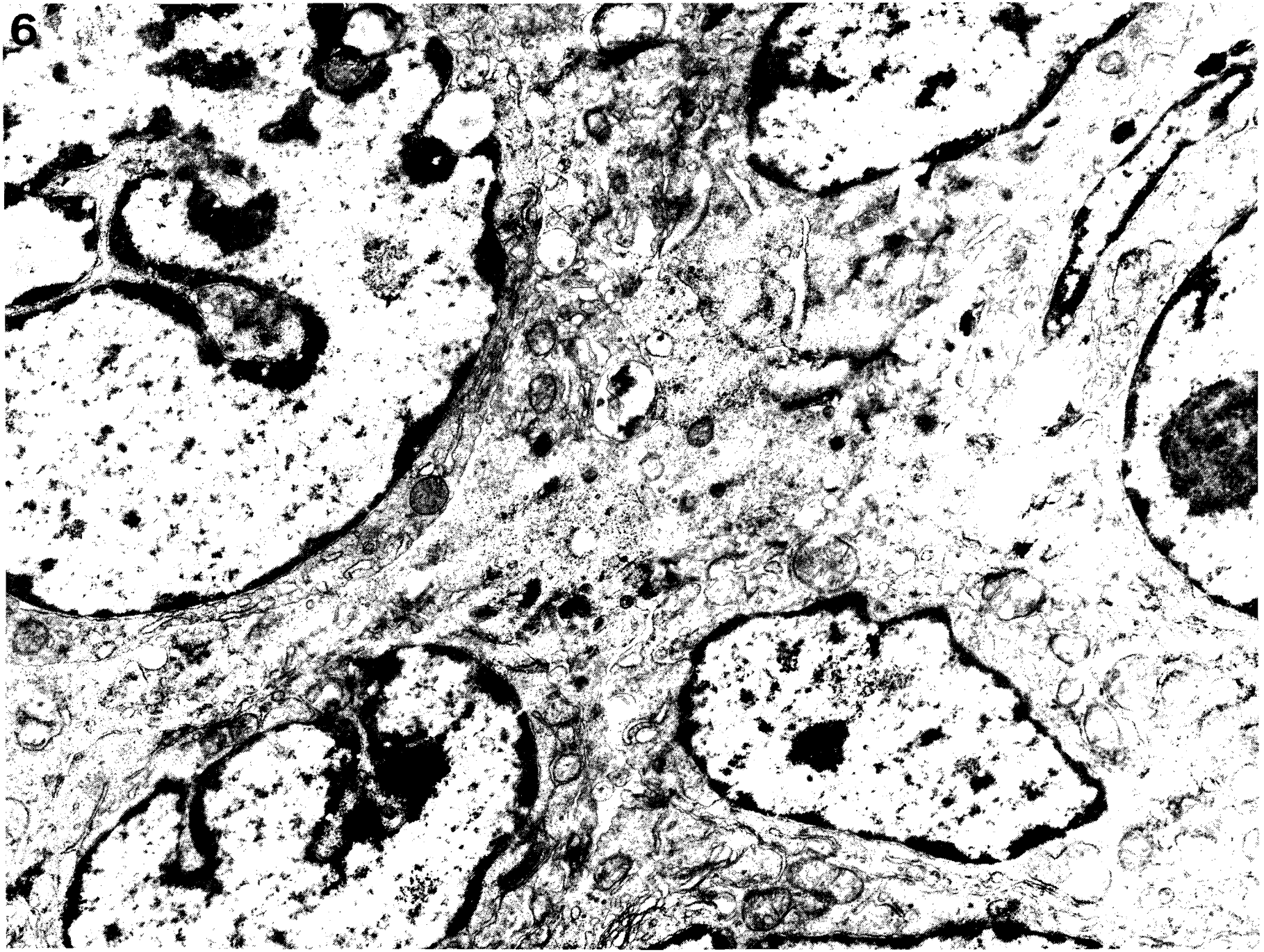
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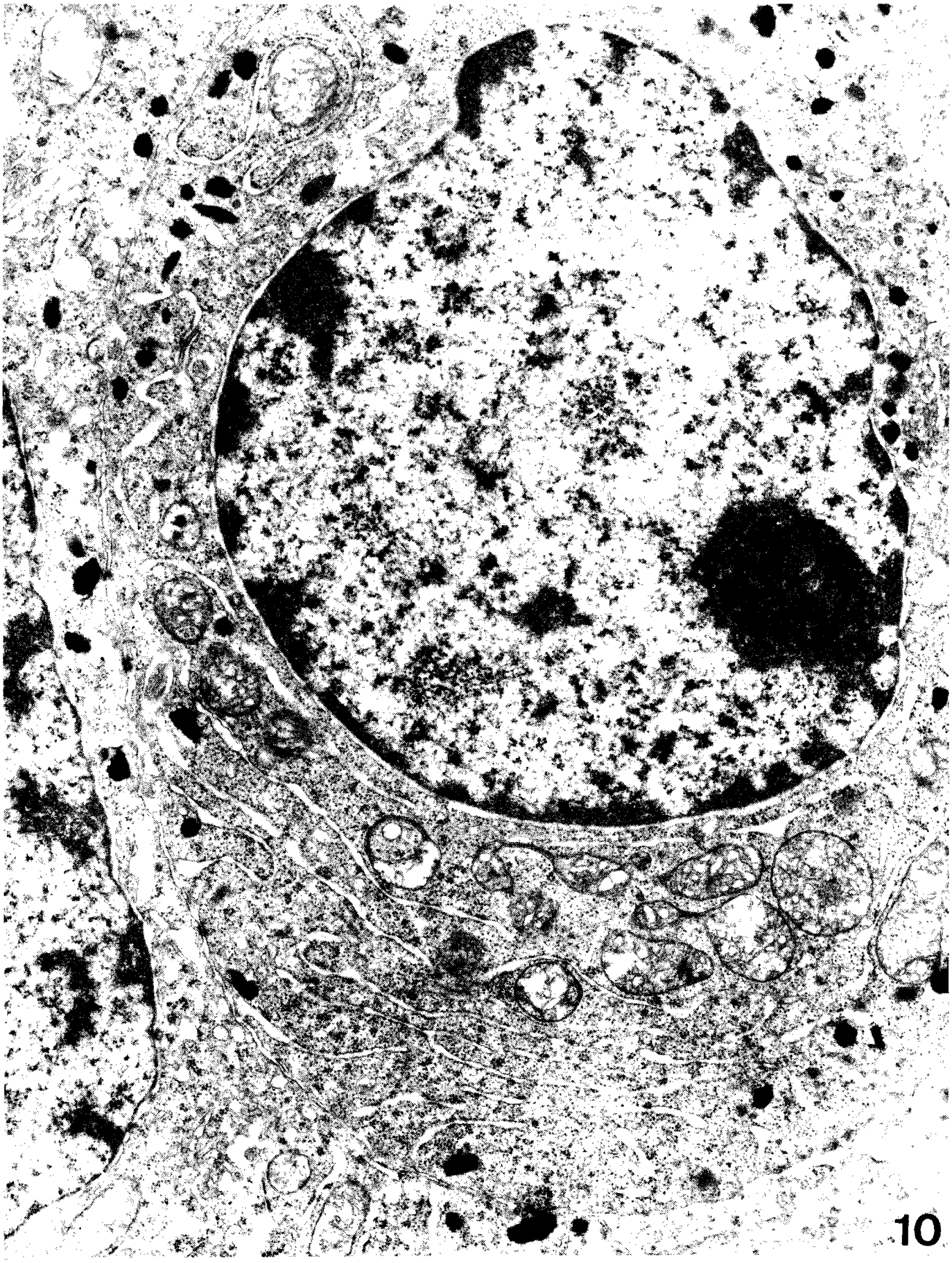

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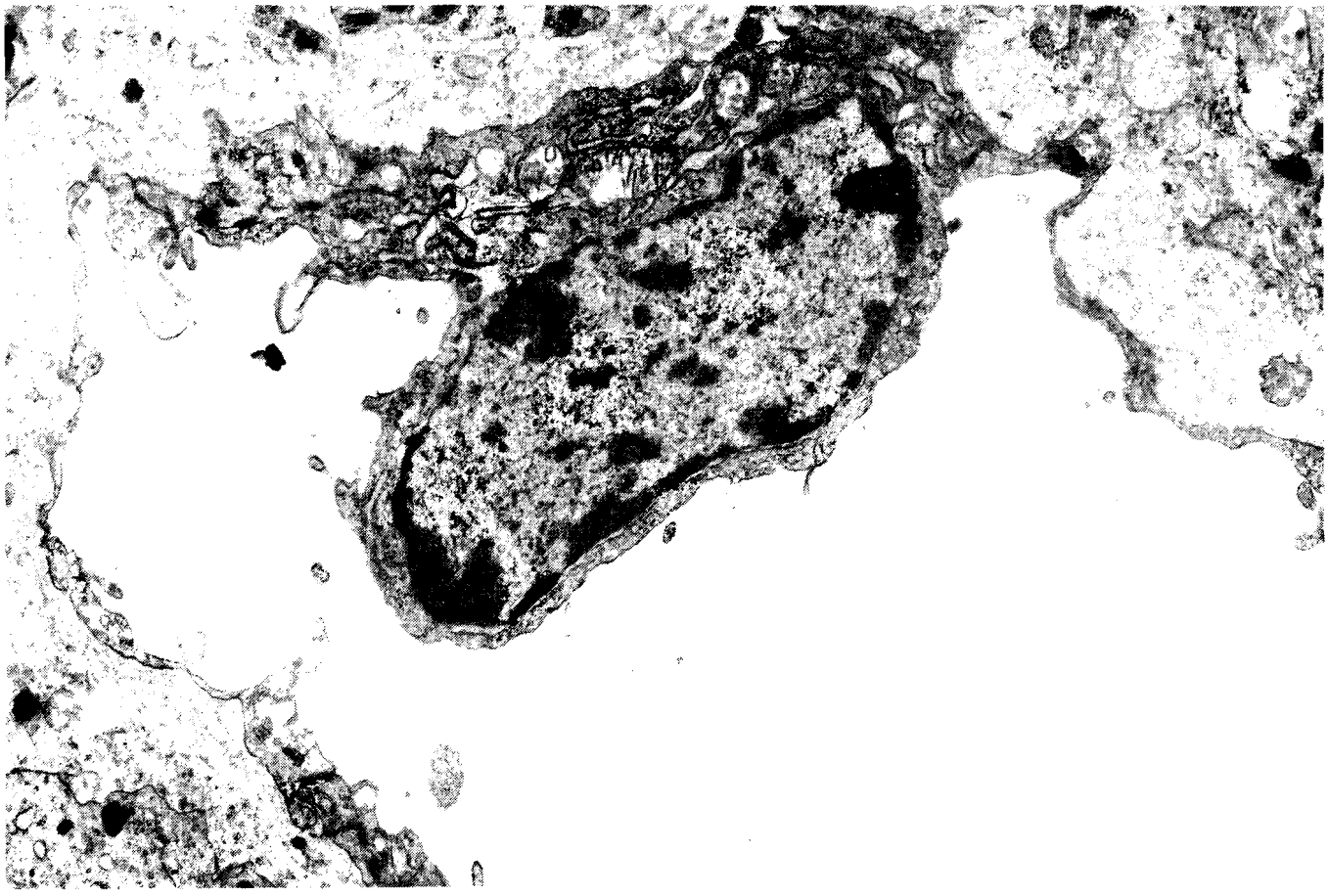

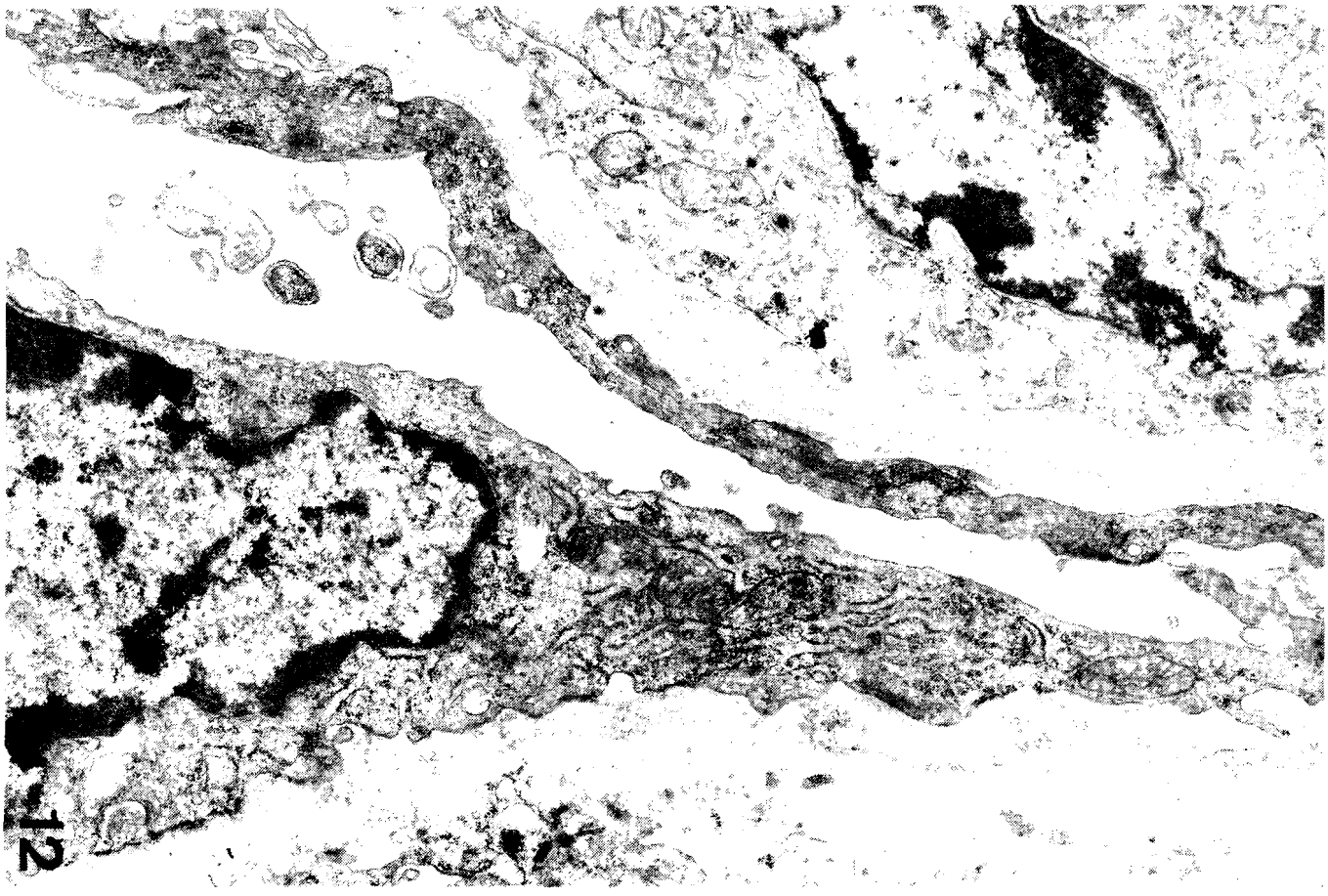

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Fig. 1. Control (1st week). Badly delimited tumour among adipose tissue. H.E. ×312'5

Fig. 2. Hyperthermia: Blood vessels with wide lumens H.E. ×312'5

Fig. 3. Hyperthermia: Numerous hyperemic vessels with irregular lumens near the capsula. H.E. ×312'5

Fig. 4. Hyperthermia: Groups of globular cells with melanic pigment in the periphery. H.E. ×500

Fig. 5. Necrotic tissue with viable tumor tissue around blood vessels. H.E. ×312'5

Fig. 6. Detail of the fibrous capsula, increased and infiltrated by neoplastic melanocytes. H.E. ×500

Fig. 7. Control: Densely grouped melanocytes with little pleomorphism. ×7,500

Fig. 8. Hyperthermia (1st week): Melanocytes with moderated nuclear pleomorphism and cytoplasm with different electron-density ×7,500

Fig. 9. Hyperthermia (3rd week): Nuclear lobulation. Swollen organelles and lipid vacuoles in cytoplasm. ×11,200

Fig. 10. Hyperthermia (3rd week). Peripheral location of the melanosomes. ×18,300

Fig. 11. Hyperthermia (2nd week). Melanocyte with involutive phenomena near a vessel with a narrow wall. ×18,00

Fig. 12. Hyperthermia (3rd week). Vessels with irregular lumens. Prominent endothelium, frequent pinocytosis vesicles and irregular basal membranes. ×14,500

Discussion

The experimental thermotherapy in Harding-Passey melanoma developed throughout this report was carried out according to tumoral line followed by successive transplants in C57Bl/6J mice, showing us the biological behaviour of the tumor.

Over the last few years, experiments with hyperthermia as an antineoplastic agent have been extensively published (Hideyuki et al., 1984). However, works reporting the disturbances hyperthermia cause in host or neoplastic cells and literature where heat is applied, as we report, are infrequent (Robins et al., 1984). Therefore, different experiments with various periods of heat application have been carried out in order to get maximum survival of animals and, thereby, improve our knowledge of these aspects.

On our line of research, the tumours taken from all the animals were palpable as irregular lumps 4-5 days after implant. The maximum diameter was reached 4-5 days after one month and death caused by cachexia or sepsis, happened about 1 1/2 months post-implant.

Macroscopically, tumours tended towards progressive encapsulation during the experiment in such a way that during the second week they looked like well defined edged nodules. Those treated presented irregular fibrous areas, lobulling and enlarging the capsules, and containing melanocytes with involutive phenomena. These findings seem to be caused by the treatment following neoplastic cell necrosis, described by Yamada et al. (1985) on different research lines of human melanomas transplanted into mice, following hyperthermia and nitrosureas treatment.

With regard to the consequences of treatment on tumoral growth, it is important to stress its effectiveness, as supported by weight evolution where the growth rate (acceleration) was lower in the hyperthermia group than the control one. However, when the effects of melphalan on this tumor were compared (Vicente et al., 1986) with hyperthermia this last treatment seemed to slow down and caused such an evolution that although at the beginning it showed superior values to both melphalan, and the control, it finally tended to be progressively lower, maintaining this line.

Taking into account tumoral volumes, although there was a tendency towards values below the control group, their differences were not significant, probably due to the measurement method, which presented a high dispersion caused either by different animals' responses or by measurement method gap (Yamada et al., 1984) and because our experiment started a week before the two above mentioned authors, our mean diameter between 0.05 and 0.1 cm resulted significantly lower than Vicente and Yamada 's diameter. So a relevant margin for error in the measurement occurred. On the other hand, when volumes from the last days were compared, significant differences between both groups were found, therefore we agree that this method may be useful from the second week on after implant when tumoral volumes are noticeable.

A constant finding in the treated group corresponded to blood vessels, characterised by different size, distended and hyperemic walls of varying thicknesses and prominent endothelium. In their study on tumoral microvasculature, Emami et al. (1981) reported alterations over 40°C, vasodilation and congestion over 42°C as well as necrosis, and wall rupture with consecutive haemorrhage over 44.5°C. In our opinion alterations at 40°C and 42°C could be due to heat applied for 5 consecutive days, and although it did not exceed 40°C. these support the hypothesis of Song et al. (1980) that the tumour deteriorated more than healthy tissue after hyperthermia because of: disturbed vascularization, lower blood irrigation, faulty heat dissipation as well as secondary intratumoral vascular occlusion to passive hyperemia according to Kang et al. (1980). All these features would decrease pH, the partial oxygen pressure and intratumoral nutrients damaging the tumoral tissue.

Another constant observation, this time in both groups, was that tumoral necrosis gradually grew during the experiment until it almost completely occupied the tumoral volume, therefore consistency ranged from elastically soft during the first days to fluctuating and friable on section during the last days. This contrasted with progressive necrosis replacement by fibrous and viable tumoral tissues from the 4th day on nitrosoureas treatment of B16 melanoma reported by Li et al. (1984) which we have not found.

Also characteristic of treated tumours, were cellular and architectural irregularities, mainly during the last days of the experiment where intranuclear vacuoles were

frequent, as well as the peripheral location of this pigment strengthening cellular limit that Yamada et al. (1984) considered as results of the combined hyperthermia and chemotherapia treatment.

Ultrastructurally, this group showed characteristics considered by Li et al. (1984) as cellular injury signs, such as heterochromatin margination and vacuolization with occasional nucleoli disappearance (observed by Love et al. (1970) in cells at an early stage of exponential growth at 45-46°C, reported in one of the first works on morphological changes caused by hyperthermia) nuclear envelope dilation, the peripheral location of melanosomes and frequent lipid vacuoles as well as swollen organelles, supporting Cozad's theory (1983) that heat affects enzymes releasing lysosomes responsible for neoplastic cell destruction.

The presence of melanosome complexes differing from those found in non-melanocytic cells, consequence of melanosomes in different stages of development, was described by Novikoff et al. (1968) as autophagocitosis phenomena, and subsequently by Bleehen (1974) and Seiji et al. (1971) as common findings in mice melanomas.

Mitotic activiy was high in both groups; no significant differences between them were observed as Yamada et al. (1984) also reported.

In relation to the host's cellular response, an inverse tendency as to its presentation between groups existed, whereas it predominated in the control during the first days without surrounding the tumoral periphery, a progressive delimitation seemed to happen in those treated during the last days, lymphoplasmocytes and macrophages formed a continuous band around the tumoral periphery. This piece of information was not found in the bibliography.

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References

Bleehen S.S. (1974). Ultrastructural studies on tumours and cell cultures of the H.P. mouse melanoma. Br. M. Dermatol. 90, 637-648

Cozad, J.R. (1983). Hyperthermic perfusion for melanoma. A.O.R.N. Journal 38, 974-978.

Didolkar M.S., Fitzpatrick J.L., Jackson A.J. and Jokwston G.S. (1986). Toxicity and complications of vascular isolation and hyperthermic perfusion with imidazolecarboxamide in melanoma. Cancer 57, 1961-1966.

Emami B., Nussbaum G.H., Hahn N., Piro A., Dritschild A. and Quimbi F. (1981). Histopathological study on the effects of hyperthermia on microvasculature. Int. M. Radiat. Oncol. Biol. Phys. 7, 343-348.

González G.D., Van Dijk J.D.P., Blank L.E.C.M. and Rumke P. (1986). Combined treatment with radiation and hyperthermia in metastatic malginant melanoma. Radiother. Oncol 6, 105-114.

Greech O., Ryan R.F. and Kremena E.T. (1959). Treatment of malignant melanoma by isolation perfusion tecnique. Jama 169, 339-352.

Hideyuki K., Odagiri S., Fujita H., Nagano T. and Murakami M. (1984). Hyperthermia in cancer therapy. J. Uoeh. 6, 307-315.

Kang M.S., Song C.W. and Levitt S.H. (1980). Role of vascular function in response of tumors in vivo to Hyperthermia. Cancer Res. 40, 1130-1135.

Little J. (1984). Animaux de laboratoire. Iffa Credo. Barcelona.

Love R., Soriano R.Z. and Walsh R.J. (1970). Effects of Hyperthermia on normal and neoplastic cells in vitro. Cancer Res. 30, 1525-1533.

Luk K.H., Purser P.R., Castro J.R., Meyler T.S. and Phillips T.L. (1981). Clinical experiences with local microware hyperthermic. Int. J. Radiot. Oncol. Biol. Phys. 6, 615-620.

Novikoff A.B., Albala A. and Biempica L. (1968). Ultrastructural and cytochemical observations on B16 and Harding-Passey mouse melanomas. J. Histochem. Cytochem. 16, 299-309.

Robins H.I., Steevens R.A., Shecterle L.H., Martín P.A., Miller K.A., Paliwal B., Neville A.J. and Warren D. (1984). Whole body Hyperthermia (41-42°C): A simple technique for unanesthetized mice. Med. Phys. 11, 833-839.

Seiji M. and Otaki N. (1971). Ultrastructural studies on Harding-Passey mouse melanoma. J. Invest. Dermatol. 56, 430-435.

Silverman R.H., Coleman D.J., Lizzi F.L., Torpey J.H., Driller J., Iwamoto T., Burgess S.E.P. and Rosado A. (1986). Ultrasonic tissue characterization and histopathology in tumor xenografts following ultrasonically induced hyperthermia. Ultrasound med. Biol. 12, 639-646.

Song C.W. Kang M.S., Rhee J.G. and Levitt S.H. (1980). Effects of Hyperthermia on vascular function in normal and neoplastic tissues. Ann. N.Y. Aca. Sci. 335, 35-47.

Streffer C., Van Beunigen D. and Dietzel F. (1978). Cancer therapy by Hyperthermia and radiation. Cancer Treat. Rev. 11, 179-185.

Urano M., Rice L., Kahn J. and Sedlacek F. (1980). Studies on fractionated hyperthermia in experimental animal systems, the foot reaction after equal doses: heart resistance and repopulation. Int. J. Radiat. Oncol. Biol. Phys. 6, 1519-1523.

Vicente V., Lázaro M.J., Gómez M., Gómez S., Canteras M. and Cremades A. (1986). Efectos del melfalan sobre el melanoma Harding-Passey. Patología. (in press).

Vora, N., Forrel B., Lipsett J., Archambeau J.O. (1982). Interstitial implant with interstitial Hyperthermia. Cancer 50, 2578-2580.

Yamada, K., Someya T., Shimada S., Ohara O. and Kukita A. (1984). Thermochemotherapy for malignant melanoma: Combination theraphy of ACNU and Hyperthermia in mice. J. Invest. Dermatol. 80, 180-184.

Yamada K., Someya T., Shimada S., Nakagama H., Kukita A., Tokita M. and Tanaka N. (1985). Thermochemotherapy for malignant melanoma: Overcoming heterogenity in drug sensitivity. J. Invest. Dermatol. 85, 43-46.

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