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Early embryos of dams of heat stress

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EARLY EMBRYOS OF DAMS OF HEAT STRESS

by

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ABSTRACT

Increased environmental heat causes early embryonic death before implantation. This study was designed to examine tissues of dams exposed to environmental temperatures of 36°C and to examine 72 hour old embryos from these dams. Results showed adult mice exposed to heat stress had significant changes in liver morphology with hepatocyte swelling and vacuolization of the cytoplasm, organelles in the hepatocytes were displaced next to the cell membrane. After 48 hours of recovery from heat stress, liver morphology appeared normal.

Embryos from heat stressed dams had delayed development indicated by increased fibrous 2α helix inclusions. Embryos responded differently to different fixation techniques indicated permeability changes in either the zona pellucida or cellular membranes. Litter size or pup survivability from heat stressed dams allowed to recover indicated changes seen at this point were reversible.

INTRODUCTION

Hot arid lands occupy one third of the world land mass, contain 15% of the worlds human population, and are politically and economically underdeveloped countries (Gradus 1986). These lands are characterized by high temperatures, intense solar radiation, strong winds, low rainfall and low humidity. The livestock of arid lands include 1/2 of the world's cattle, 1/3 of the world's sheep, and 2/3 of the world's goats. Arid regions with little water (and that is usually of poor quality) are ordinarily stocked with breeds that are poor converters of feed to protein and have low reproductive rates. Consequently arid lands have a low capacity for livestock production (McGinnies 1968; Schechter 1985). In hot environments fertility ranges between 10-50% as compared to reproductive rates of 40-80% in mild temperatures (Gwazdauskas 1985). Holstein cows may have live birth rates of 20% from artificial inseminations during the hot months of July, August, and September in Arizona (Monty 1983).

Animals exposed to intense summer heat have alterations in hormone levels, metabolic activity, and water and mineral balance which help the animal to maintain homeothermy. As homeothermy is threatened the ability of that animal to

reproduce is also adversely affected. Intense heat (solar radiation) cause heavily lactating dairy cows to become hyperthermic (Wolff-Vaught et al. 1977). Heat stressed animals experience reduced sexual activity, fertility, and milk production. Ovulation, corpus luteal development, and the length of the non-fertile estrous cycle are not significantly altered in Holstein cows during hot weather. The incidence of cleaving embryos found in artificially inseminated heat stressed cows does not differ significantly from the incidence of cleaving embryos found in non-stressed cows (Monty 1983). However, fertility is consistently lower when maximum temperatures were greater than 33°C on the day of breeding (Cavestany et al. 1985). Lactating cows have a lower conception rate at 30°C due to their greater internal heat production. The maximum air temperature and the amount of rain on the first day post breeding have been shown to be the most critical factors in lowering the conception rate (Badinga et al. 1985).

High environmental temperatures also adversely affect the reproductive rates of pigs (Hennessy and Williamson 1984), and sheep (Hafez 1965) as well as many laboratory animals,including the rat (Hendrickx and Houston 1970), mouse (Bellve 1972), and rabbit (Shah 1956 and Burfening et al.

1969). Marked fetal resorption occurs in rats exposed to temperatures of 35°C between day 6 and day 12 of gestation (Hendrickx and Houston 1970). Severe prenatal mortality and embryo absorption following the exposure of the pregnant female rabbits to 35°C are thought to be due to the effect of heat on the maternal tissues (Shah 1956). Burfening et al. (1969) recovered a significantly lower proportion of normal embryos from rabbits subjected to a short term stress of 32°C than from rabbits kept at 21°C. Ulberg (1973) claimed that the harmful effect of high ambient temperatures on reproduction is due to an increase in core temperatures of the dam. Unfertilized ova and spermatozoa in the uterus appear unaffected by high temperatures. Embryos are most affected during the first cell division by a temperature increase and as the number of embryonic cells increase the embryos become more heat resistant. Alterations of the metabolic rate in embryos from heat-stressed mothers results in retarded development of the embryo (Ulberg 1973). These findings suggest that reproductive failure may be due to embryonic death before implantation in the uterus.

The loss of homeothermy and the increase in core temperature in heat stressed animals results in altered hormone levels. Animals under hyperthermic conditions usually have an increase in growth hormone, cortisol, prolactin, epinephrine and norepinephrine (Johnson, H.D. 1981).

Hyperthermic cattle show a decrease in aldosterone (Johnson, H.D. 1981). Decreases in triiodothyronine (T3) and L-thyroxin (T4) levels in cattle are thought to be the result of a lower food intake and the rise in core temperature, rather than to a decrease in thyrotropin releasing hormone (Johnson, H.D. 1981).

Thyroid hormones have a modulating effect on gonadotropic hormones and enhance follicle stimulating hormone and luteinizing hormone secretions (LaRochelle and Freeman 1974). Thyroid hormones also play an important role in regulating cell function, replication, differentiation and development, and in regulating caloric and oxygen consumption. Enzyme activities are modulated by the regulation of RNA protein synthesis and this same mechanism regulates the activity of the Na^+ and K^+-ATP enzyme systems (Samuels 1983, and Guernsey and Eldelman 1983).

Progesterone serum levels increase and the estrogen levels decrease in heat stressed cattle (Monty 1983). This alteration of the progesterone/estrogen ratio appears to be an important cause of early embryonic death in heat stressed animals. High levels of progesterone in the pre-implantation stage may contribute to early embryonic mortality by delaying egg transport (McCarthy et al. 1977). This slower egg transport results in the arrival of an embryo at a uterus that is not in synchrony with it. Rabbit blastocysts transferred

into a uterus that is asynchronous to the stage of development of the blastocyst will die (Chang 1950, Adams 1974). Guilbert-Blanchette and Lambert (1978) found that there is a toxic substance in the uterine fluids when the uterus is more advanced than the embryo.

Early embryonic death during heat stress may involve several factors including hormonal changes that alter the proteins in uterine fluids. High levels of progesterone cause the production of a uterine milk protein (molecular weight 14-15,000) called "uteroglobin" (Beier 1968, Bullock and Willen 1974, Bullock et al. 1981). Abnormal levels of uteroglobin during early embryonic development create a hostile uterine environment and may be embryocidal (Maurer and Beier 1976, McCarthy et al. 1977).

Uteroglobin may not be the only component in the uterine fluid of heat stressed animals that is detrimental to the early embryo. Clark (1979) found electrolyte (calcium, magnesium, sodium, and potassium) levels decreased and amino acid levels in uterine fluids of heat stressed rabbits are different from the levels in unstressed animals. He found a decrease in amino acids but an increase in o-phosphoserine, α aminoadipic acid, ammonia and citrulline.

Heat stress may play a role at the cellular level of the embryo. Heat shock has been studied in plants, non-mammalian and invertebrate species, and mammals, and has been found to

cause changes in protein synthesis in cells. Tissiers (1974) found gene puffing in heat shocked Drosophila and de novo synthesis of 74k molecular weight proteins (appropriately termed heat shock proteins and abbreviated HSPs). The role of HSPs in the recovery or repair of the Na^+,K^+ ATPase activity is been described by Burdon (1982). HSPs are found at different locations in the cell indicating that these proteins have more than one role in cellular function (Tanguay 1983). Heat shock protein gene activity occurs in various eukaryotic cells and is thought to be a normal part of the metabolic response to heat shock in both plant and animal tissues (Tanguay 1983, Atkinson 1983).

Chromosome function is altered in heat stressed embryos. Heikkila et al. (1985) reported degradation of chromatin regions following heat shock and a decrease in actin mRNA in the cleavage stage but not in the neurula stage of Xenopus laevis embryos. Xenopus laevis ova store mRNA for HSPs but it cannot be demonstrated after fertilization. HSP production starts in the blastocyst stage (Heikkila et al. 1985). Heat stress appears to cause a repression of normal gene protein synthesis and a rapid activation of the HSP gene (Atkinson 1983).

Brown et al. (1982) reported that HSPs are produced in both the doe and the fetus of heat stressed pregnant rabbits. HSP's are found in the fetal brain, liver, kidney, and heart

after raising the doe's body temperature 3 degrees Celsius. Witting (1983) demonstrated production of the HSP protein in the early mouse embryo. In the 36-48 hour embryo there is no HSP production but these proteins do occur when the embryo reaches the morula/blastocyst stage at 84-96 hours (Witting 1983). The effect of age on the ability to respond to heat stress by becoming able to synthesize HSPs may be related to the increased thermal resistance found by Ulberg (1973) as the embryos age. If HSPs serve as protection against the effects of heat stress, their lack of production would be an important factor in heat related early embryonic death.

Ulberg (1973) felt that development of the early embryo is delayed when the dam is heat stressed. A way to access embryonic development is necessary. The ultrastructure of early embryos is helpful in staging development, and may be useful to evaluate delayed development. This evaluation of embryo stages can be done through ultrastructure of cellular organelles and inclusions.

Stern et al. (1971) observed that mitochondria are pleomorphic during oogenesis and early embryogenesis. They are variable in size and shape, and have large areas of electron density. The morphology of the mitochondria is altered during the 8 cell stage and again during the morula stage. These changes are a common feature of post-fertilization development and appear to be real and not

artifacts of fixation.

The four blastocyst substages of development described by Nadijcka and Hillman (1974) are used as staging criteria for this study. These substages are based mainly on cell types and membrane contacts. Tight junctions and desmosomes occur between adjacent apical trophoblast cells in the blastocyst from the time of cavitation to early implantation. The contacts between basal trophoblast cells vary with the substage of development as do the contacts of the cells of the inner cell mass.

During substage one the basal trophoblast cells have no junctions with other trophoblast cells but the cells of the inner cell mass are connected together and to the adjacent trophoblast cells by tight junctions and desmosomes. In substage one the apical and lateral trophoblast cells are elongate but the embryonic trophoblast cells are squamous and resemble the cells of the inner cell mass. Organelles of this substage include two types of mitochondria (filamentous and vacuolated), rough endoplasmic reticulum, crystalline inclusions, both free ribosomes and polyribosomal clusters, degenerate bodies, lipid droplets, multivesicular bodies, and fibrous material. These organelles are present in all cells in approximately equal numbers.

During substage two cells of the inner cell mass are smooth but the trophoblast cells have microvilli on the

luminal and coelomic surfaces. Interdigitation of the basal trophoblast cells increase during substage two. The inner cell mass cells are divided into two layers but these cells are indistinguishable from each other. The trophoblast cells of substage two have their rough endoplasmic reticulum oriented toward the inner embryonic regions. Crystalline inclusions are less common in the embryo but are still abundant in the trophoblast cells.

Substage three occurs just prior to and after hatching from the zona pellucida. Trophoblast cells of this substage have no microvilli on their luminal surfaces but cytoplasmic projections occur on the coelomic surfaces. All trophoblast cells at this stage are elongate, flattened and have tight junctions and desmosomes, and the remainder of their apposing cell surfaces interdigitate. The cells of the inner cell mass at this stage are of two morphologically different types. Epiblast cells are in two layers and may be arranged in regular stacks or irregular clusters. Proximal endodermal cells form a single continuous layer beneath the epiblast and are spindle or cuboidal. Organelle occurrence and arrangements in this substage are similar to those of substage two.

Substage four trophoblast cells have tight junctions and desmosomes and the lateral and abembryonic trophoblast cells also have interdigitations. There is a basement membrane

present on the coelomic surfaces and cytoplasmic projections occur on the juxtaluminal surfaces. Only the embryonic trophoblast cells are elongated; the others are randomly shaped. The cells of the inner cell mass are characterized by the development of the distal entoderm. The epiblast and the endodermal cells are separated by intercellular spaces, although there are interdigitations by means of cytoplasmic projections. Tight junctions occur among the endodermal cells and among the epiblast cells but not between the two cell types.

Calarco and Brown (1969) described cytoplasm inclusions of vesicle aggregates in two celled embryos that they referred to as "jigsaw bodies." These inclusions also occur in older embryos. Other cytoplasmic structures occurring in pre-implantation blastocysts include degenerate bodies, type A inclusions, lipid inclusions, membrane whorls, and fibrous 2α helix inclusions (Chavez 1984).

Different methods have been used for fixing embryos for electron microscopy. Nadijcka and Hillman (1974) fixed mouse embryos with 3% glutaraldehyde and flushed the uterine horns with Brinster's medium but it was difficult to recover the embryos after they have settled into the uterine crypts when using this method. The use of 3% glutaraldehyde to flush the uterine horns improved embryo recovery at the stage after the embryos settled into the uterine crypts. One percent

glutaraldehyde was used by Brackett et al. (1980) for fixation of cow ova.

The current study compares the ultrastructure of blastocysts from heat stressed mice with those from normal mice in order to evaluate delayed development of the blastocyst and other possible anomalies in the embryo that are associated with early embryonic death. Evaluation of fixation methods for embryos are also studied.

The effects of heat stress on maternal tissues is examined for their possible effects on the fetus. The tissues of recovered adult heat stressed mice are compared with normal and heat stressed mice for possible differences.

The significance of the changes in the early embryos from heat stressed dams, is determined in bred females heat stressed then removed from the heated environment, and allowed to give birth.

MATERIALS AND METHODS

Breeding Procedure

Mice were divided into groups of four 7-week old virgin female CF1 mice and a single 11-week old male CF1 mouse and housed in 30x20x12.7 centimeter metal cages in a room maintained at 22°C with a 12 hr light/12 hr dark cycle for breeding. Food and water were provided ad libitum.

Females were checked at 7 a.m. and 5 p.m. for the presence of a vaginal plug as evidence of successful breeding. The time of discovery of the plug was considered as time zero and the females were separated into individual cages at this time.

Weight, Temperature, Food and Water Intake

Bred females were weighed to 0.1 gm. at time zero and daily thereafter using a Sartorius (Brinkmann Instrument Co. Westbury, N.Y.) 1406 MP scale. Rectal temperatures were measured at the beginning of the experiment and every 24 hours thereafter using a YSI Tele-thermometer model 47TS with a 2 mm diameter YSI flexible probe no. 402 (Yellow Springs Instrument Co. Yellow Springs, Ohio) inserted through the rectum into the descending colon.

Experimental female mice were placed in individual 30x20x12.7 cm metal cages and housed in a controlled environmental chamber approximately 2.7x1.6x2.4 meters.

Temperature was maintained at 36±1°C and monitored by a mercury laboratory thermometer that met SAMA specifications. The light cycle was a 12 hour cycle. Humidity was controlled at 40% as measured by an Abbeon Relative Humidity Indicator, model 62 (Abbeon Inc., Jamaica, N.Y.).

Control mice were housed in separate cages and maintained in a room with a controlled temperature of 22°C and a light cycle of 12 hours. Humidity could not be controlled.

Tap water was supplied to all mice by burettes fitted with a drinking tube and was available ad libitum. Every 24 hours from time zero the level of water in the burettes was recorded and the burettes were refilled. A known weight of Wayne Rodent Blox (Wayne Pet Food Division Continental Grain Company Chicago, Ill) was supplied at the start of the experiment. Food was weighed with a Sartorius scale every 24 hours to estimate consumption.

Embryo and Histologic Methods

Seventy two hours post breeding, mice were removed from the chamber or room and killed with an overdose of ethyl ether anesthesia. Immediately after death the uteri were removed. In order to determine whether different treatments might produce artifacts that would disguise the effects of heat stress four separate techniques were used to collect and fix embryos.

Group 1 uteri were flushed with 3% glutaraldehyde in

Millonig's phosphate buffer, pH 7.3, as described by Nadijcka and Hillman (1974) and allowed to fix for 30 minutes, removed from the flushing fluid, and attached to 1x3 inch glass microscope slides previously coated with poly-L-lysine hydrobromide 1mg/ml distilled H_2O (Sigma no. p-1524 Sigma Chemical Co.).

Group 2 uteri were flushed with 1% glutaraldehyde in Millonig's phosphate buffer and then treated as in group 1.

Group 3 uteri were flushed with Millonig's phosphate buffer pH 7.3 (see Appendix 1). Embryos were removed from the flushing fluid, fixed to 1x3 inch glass slides previously coated with poly-L-lysine hydrobromide 1mg/ml distilled H_2O (Sigma no. p-1524 Sigma Chemical Co.), and placed in 3% glutaraldehyde in Millonig's buffer, pH 7.3 for 30 minutes.

Group 4 embryos were flushed in a manner similar to group 3 except 1% glutaraldehyde in Millonig's phosphate buffer was used for fixation as described by Brackett et al. (1980) .

After fixation embryos were rinsed with one change of Millonig's phosphate buffer, pH 7.3, and stored for 4 to 6 days in fresh Millonig's phosphate buffer, pH 7.3. Embryos were post fixed in Millonig's fixative (1% OsO_4 in phosphate buffer, pH 7.3), dehydrated in ascending series of ethyl alcohol and propylene oxide then embedded in epon/araldite blocks (Appendix 2).

Thin sections, 90-150 nanometers thick selected using

silver interference color, were made with glass knives using an American Optical/Reichert Ultracut Microtome. The thin sections were placed on 300 mesh naked copper grids (Ted Pella Electron microscope supply Co. Tustin, Ca.) and stained with uranyl acetate and lead citrate (Appendix 3). Sections were observed with an Hitachi HU 12 transmission electron microscope operating at 75 kv.

The criteria used for staging the embryos were those described by Nadijcka and Hillman (1974). Other criteria used for appraisal of the embryos were evaluation of the cellular inclusions as described by Calarco and Brown (1969) and Chavez (1984).

Kidney, liver, lung, and ovaries were removed post-mortem from the mothers and placed in 10% formaldehyde for 48 hours before sectioning into 2 mm. thick blocks. The tissues were then dehydrated and embedded in paraffin blocks according to standard histological techniques. Tissues were sectioned with a microtome to a thickness of 5 microns, placed on 1x3 inch glass slides, and stained with hematoxylin-eosin. Other stains used on the liver sections were 2% osmium for fat and mucicarmine for glycogen. The glass slides were studied using a Nikon Labophot microscope.

Pieces of liver were cut into 1mm cubes, dehydrated in ascending series of ethyl alcohol and propylene oxide and embedded in epon/araldite (Appendix 2). Thin sections were

placed on grids and, stained in the same manner as above. The sections were examined with an Hitachi HU 12 transmission electron microscope.

Recovery Experiments

To study the ability of the mice to recover from heat stress twelve female CF1 mice were placed in the environmental chamber described above with the temperature and humidity set as above. They were housed in groups of six in 30x20x12.7 cm metal cages. The mice were transferred from the chamber after 72 hours into a 22°C controlled temperature room with uncontrolled humidity. All mice received food and water ad libitum.

After 24 hours, six adult females were sacrificed by anesthetic over dose. Their livers were removed, placed in 10% formalin, and prepared as above for histological examination. After 48 hours the remaining six mice were sacrificed in the same manner and treated as before. The sections were stained with hematoxylin-eosin.

Photographic Techniques

Electron micrographs were taken using Kodak electron microscope film 4489. The film was developed using Kodak D-19 developer diluted 1 part developer plus 2 parts water. The negatives were printed on Kodak extra hard resin coated Kodabrome paper. The prints were developed using Kodak Dektol in a 1:1 dilution with water.

Light micrographs were taken using Kodak Plus-Pan X film. The negatives were developed in Kodak Microdol-X developer. Prints were made using the same procedure as above.

Litter Size and Young Viability

To determine litter size and young viability of heat stressed mice eleven CF1 7-week old virgin female mice were bred as described before and checked for vaginal plugs as previously described. When the vaginal plug was found time was started as time zero. Six of the bred female mice were placed into individual cages in the previously described controlled environmental chamber where temperature was maintained at 36±1°C with relative humidity of 40%. Five of the bred females were placed in a room with a controlled temperature of 22°C. The humidity could not be controlled. The light cycle in both areas was a 12 hour cycle. Food and water was provided ad libitum to both groups. After 72 hours the mice in the 36±1°C chamber were moved in the same room as the control mice. Both groups were maintained in this room until parturition. Neonates were counted at birth and weighed on a Sartorius 1406 MP Scale. The pups were reweighed every 24 hours for 7 days and deaths were recorded.

RESULTS

Adult Mice Weight, Temperature, Food and Water Intake

Body weights, temperatures, food and water intake for control and pregnant mice subjected to heat stress are presented in Tables 1 and 2. There were no significant differences between the body weights of control and heat stressed mice at 72 hours of heat stress. The average rectal temperatures of the heat stressed mice were significantly higher than those of the non-stressed mice (see Table 1). Food consumption during the experimental period was significantly decreased in the heat stressed mice but water intake was not affected (see Table 2).

Morphology of Tissues from Dams

The lungs, kidneys, uteri, and ovaries were morphologically similar in heat stressed and control groups. Morphological differences were apparent in the livers of the heat stressed mice. Hepatocytes were larger and there was an increase in cytoplasmic vacuolation in the livers of heat stressed mice (fig. 2). The organization of the sinusoids in the livers of the heat stressed mice was obscured and lobular structure was less well defined than in the livers of control animals (fig. 2). Osmium staining indicated a lack of cytoplasmic lipid accumulation in the hepatocytes (figs. 3a

& b). Mucicarmine staining showed no accumulation of glycogen in the hepatocytes.

The hepatocytes of heat stressed animals had fewer cytoplasmic organelles, and those organelles that were present were eccentrically located in close association with the plasmalemma but they were not morphologically different from those of unstressed mice (figs. 4a & b). The hepatocyte cytoplasm of heat stressed mice had a homogenous granular appearance resembling proteinaceous fluid. The hepatocytes of heat stressed mice had a condensation of nuclear material around their nucleoli and their intracellular spaces were wider and filled with an amorphous material resembling cellular debris (fig. 4).

Recovery Studies

The livers of the mice allowed to recover from the heat stress for 24 hours had swollen peri-portal hepatocytes with vacuolated cytoplasm, but there was less vacuolation than in the livers from non-recovered mice. There was a loss of lobular organization, and the sinusoids were obscured in the portal area. The central lobular areas were similar to those from control animals. Sinusoids of stress-recovered mice were slightly smaller than those of control mice but there was a good lobular organization (figs. 5 & 6).

The livers of the mice allowed to recover from heat stress for 48 hours were not morphologically different from

livers from non-stressed mice (figs. 6a & b).

Fixation Methods for Embryos of Unstressed Dams

All groups of embryos had elongated but not flattened trophoblast cells with many microvilli and pinocytotic vesicles. Lateral and apical trophoblast cells were connected by tight junctions and desmosomes. The nearly squamous cells of the inner cell mass were tightly bound together and to the trophoblast cells by tight junctions, desmosomes and the interdigitation of microvilli.

Group one embryos (flushed from the uterus with 3% glutaraldehyde in Millonig's phosphate buffer; fixed in the same solution)(figs. 7a & b): The trophoblast cell cytoplasm was homogeneous and closely resembles that of the cells in the inner cell mass. The most plentiful cytoplasmic inclusions were fibrous 2α helix figures, but crystalloid structures were also very numerous. Other inclusions were lipid bodies, membrane whorls, jigsaw inclusion bodies, degenerate bodies, and type A inclusions. Two populations of mitochondria were present; one population was composed of filamentous mitochondria, and the other type of mitochondria were vacuolated. Rough endoplasmic reticulum was found in close association with the mitochondria and crystalloid inclusions. Polyribosomes were also present.

The nuclei had double membranes with ribosomes attached to the outer membrane. The nucleoplasm was homogenous except

for some electron dense granular clusters that appeared to be ribosomes. The nucleoli had two structural elements; 1) a net-like nucleolonema, and 2) a spherical dense area of the partes amorphae. Intranuclear annulate lamellae were sometimes present.

Group two embryos (uterus flushed with 1% glutaraldehyde in Millonig's phosphate buffer; fixed in the same solution)(figs. 8 a & b): The cells of the inner cell mass were not as tightly bound together as were those of group one and there were fewer interdigitations of the microvilli between these cells. Cellular inclusions were similar to those in group one but there were no jigsaw bodies or filamentous mitochondria. Both vacuolated and degenerating mitochondria were present. The rough endoplasmic reticulum was dilated and the intranuclear membrane space was widened. The nucleoplasm was relatively homogenous but the nuclear ribosomes are clustered. The net-like nucleolonema was not found, but the dense areas of the partes amorphae were evident.

Group three embryos (uterus flushed with Millonig's phosphate buffer; fixed in 3% glutaraldehyde in Millonig's phosphate buffer)(figs. 9a & b): The blastocysts of this group had very little intercellular or perivitelline space. The cytoplasm of the trophoblast and inner cell mass cells appeared to be randomly perforated. The fibrous inclusions

had less banding than those of the other groups and there were no jigsaw bodies. Both vacuolated and filamentous mitochondria types were present. The perinuclear space is wider and the nuclear membrane had fewer associated ribosomes than in the first two groups. The nucleoplasm and nucleoli were similar to those in group one.

Group 4 embryos (uterus flushed with Millonig's phosphate buffer; then fixed in 1% glutaraldehyde in Millonig's phosphate buffer)(figs. 10 a&b): The intercellular space of the inner cell mass was the same as or slightly greater than that in group one. The trophoblast cytoplasm was discontinuous with large spaces. The cytoplasm of the inner cell mass appeared perforated but did not resemble the cytoplasm of the trophoblast cells. Both vacuolated and degenerating mitochondria were present. There were fewer fibrous bands in the cytoplasm of both the trophoblast and inner cell mass cells and there were no jigsaw bodies. The other cytoplasmic inclusions were typical of those in group one. The nucleus had an irregular shape and contained ribosomal clusters and intranuclear annulate lamellae.

Embryos of Heat Stressed Dams

Group one embryos (figs. 11 a&b): The cells of the inner cell mass were widely spaced and the cells appeared to be stretched apart. The cytoplasm of all cells in embryos from heat stressed dams had decreased continuity and was more

homogenous than in group one embryos from unstressed dams. There were numerous fibrous and lipid inclusions present but there were fewer bands on the fibrous inclusions than occurred in the cells of control group one embryos. Jigsaw bodies and both types of mitochondria were present and the rough endoplasmic reticulum was similar to that of the embryos from unstressed dams.

Group two embryos (figs 12a &b): The cytoplasm of trophoblast cells was discontinuous. There were fewer fibrous inclusions but more vacuoles in these cells. The cells of the inner cell mass had an homogenous cytoplasm but their organelles were more apparent. There was a greater number of fibrous inclusions but they had less banding than those of the inner cell mass cells from controls.

Group three embryos (Figs 13a & b): the trophoblast cells of these heat stressed embryos had a less dense cytoplasm than did group three control embryos. The cytoplasm of the cells of the inner cell mass more closely resembled that of the control embryo inner cell mass cells. The number of fibrous inclusions in the cells of the inner cell mass and in the trophoblast cells was greater than in group two embryos.

Group four embryos (figs. 14a & b): the cytoplasm of both the trophoblast cells and the cells of the inner cell mass was discontinuous. The intercellular spaces in the inner cell mass were much greater than control embryos of the same group.

There were many cytoplasmic vacuoles in both the trophoblast cells and the cells of the inner cell mass. Fibrous inclusions were not as dense as in the control embryos but occurred in high numbers. These cells had fewer vacuolated mitochondria than were present in the control group. Some nuclei had a very discontinuous nucleoplasm, while others had a more homogeneous nucleoplasm.

Study of Young Born to Heat Stressed Dams

The litter size, survivability and body weight of the pups (at seven days) was similar for heat stressed and control dams.

DISCUSSION

Body Temperature and Food Intake

The significant hyperthermia seen indicated that the mice studied were not able to maintain homeothermy when exposed to environmental temperatures of 36°C. A 2-3°C rise in core temperatures cause the production of heat shock proteins in mice (Witting et al. 1983). The 1°C rise in body temperature in the present study didn't provide evidence for the production of heat shock proteins.

Food intake for the experimental group of mice was lower that the food intake of the control mice. This was an expected response of animals experiencing heat stress (Wolff-Vaught et al. 1977). The rise in body temperature and the decreased food intake were good evidence of heat stress and show that this model allowed study of heat stress.

Morphology of Livers from Heat Stressed Mice

The liver of humans (Kew et al. 1978) and dogs (Brauer et al. 1963) undergo morphological changes during heatstroke that are similar to those in rat livers perfused with 41°C oxygenated Krebs-Ringer bicarbonate solution for 90 minutes (Bower et al. 1981). These changes are similar to those reported here for heat stressed mice. The cellular organelles of heat stressed mice did not show evidence of damage but were

concentrated near the hepatic cell membrane. Heat stressed mice allowed to recover for 24 hours had more normal dispersement of organelles and the hepatic cells of mice allowed to recover from heat stress for 48 hours were similar to those of control mice. Consequently, these morphological changes were reversible. Although not studied here it has been reported that bile production decreases and AST levels increase in rats with livers perfused at 42°C for one hour (Wynne et al. 1984).

Fixation Studies of Non-Heat Stressed Embryos

Morphologic characteristics of the tissues of group one embryos were most consistent with those described by others (Nadijacka and Hillman, 1974; Stern et al. 1971). This was the only group to demonstrate the jigsaw bodies. The rough endoplasmic reticulum appeared normal as did the mitochondria. The mitochondria population consisted of two types, one being a filamentous type and the other being a vacuolated type. The majority of the vacuolated type of mitochondria had vacuoles that were membrane bound and the matrix was uniformly electron dense. The mitochondria and rough endoplasmic reticulum were consistent with those described by Stern et al. (1971) and Nadijcka and Hillman (1974). The nucleus and perinuclear space was within normal limits showing a double nuclear membrane that is intact essentially parallel to each other and showing nuclear pores. This fixation method gave results that

were consistent with satisfactory preservation (Hayat 1981) and cellular inclusions described by other authors were present (Calarco and Brown 1969, Nadijcka and Hillman 1974, and Chavez 1984).

Group two showed cell membranes and cellular contacts similar to those in group one. No filamentous mitochondria were found. Most mitochondria found appeared degenerate rather than vacuolated. The mitochondrial matrix had an uneven loss of density that was not membrane bound. The rough endoplasmic reticulum was swollen. The appearance of the mitochondria and the rough endoplasmic reticulum was consistent with cellular degeneration (Cheville 1976). Cells with cytoplasm that appears dark has been reported in the center of blocks of liver and was attributed to slower penetration of glutaraldehyde in to the deeper cells, or to osmotic phenomena found in the choroid plexus cells in a decreased hydration state (Hayat 1981). The embryos in group two did not meet the criteria for satisfactory fixation (Hayat 1981) and inclusions in the cells were not consistent with those found by other authors (Calarco and Brown 1969, Nadijcka and Hillman 1974, and Chavez 1984).

In group three embryos, the decrease in cytoplasmic density of the inner cellular mass cells and in intercellular and perivitelline space suggested cellular swelling which was probably due to mild cellular degeneration. The nuclear

membranes were intact but the perinuclear space had areas that were wider than expected. These unsatisfactory results (Hayat 1981, Calarco and Brown 1969, Nadijcka and Hillman 1974, Chavez 1984) may have been due to flushing the uterus with Millonig's phosphate buffer prior to fixing the embryos with 3% glutaraldehyde. The ionic concentrations of the buffer or inappropriate temperature during the flush may have induced cellular degeneration before fixation occurred.

Group four embryos were very poorly fixed when compared to acceptable fixation criteria of other tissues (Hayat 1981). Membrane characteristics were like those of other groups. The cytoplasm of both trophoblast and inner cell mass cells was discontinuous and had areas of very low density. Most of the mitochondria were degenerate and only a few vacuolated mitochondria were present. The nucleus was degenerate as shown by the lack of continuity of the nucleoplasm. These unsatisfactory results (Calarco and Brown 1969, Nadijcka and Hillman 1974, Hayat 1981, Chavez 1984) were probably due to a combination of the detrimental effects of the poor penetration and/or fixative properties of 1% glutaraldehyde and the ineffective buffering ability of Millonig's phosphate buffer used as a uterine flush.

Studies of Heat Stressed Embryos

The shape of the trophoblast cells and their intercellular connections in the embryos from both control and

heat stressed dams indicated these embryos were in the second blastocyst substage (Nadijcka and Hillman 1974).

All embryos from the heat stressed dams had similar differences when compared to the corresponding control groups. The intercellular spaces were widened, and there were increased numbers of 2α helical fibrous inclusion bodies with decreased banding and density of these inclusions. The stage two blastocyst should have fewer 2α helical fibrous strands than stage one embryos (Nadijcka and Hillman 1974). The abnormal accumulation of fibrous strands may indicate retarded maturation due to metabolic changes in the heat stressed embryo or an altered rate of degeneration of the fibrous strands. The cytoplasm of the trophoblast cells also demonstrated differences. There was a loss of the homogenous nature of the cytoplasm with non-electron dense areas apparent. Hayat (1981) describes the cytoplasm of normal, well fixed tissues as having a fine granular precipitate showing no empty spaces. The appearance of the cytoplasmic ground substance in the trophoblast cells in embryos from heat stressed dams indicated some cellular degeneration. The differences between the heat stressed and the control groups was greater in the embryos from groups three and four which were fixed with what were determined to be poorer fixation techniques. Fixation with the poorer fixation methods also caused more degenerative changes in the cells of the inner

cell mass than those embryos flushed from the uterus with 3% glutaraldehyde and fixed with 3% glutaraldehyde. This may be due to altered permeability of either the zona pellucida or the cell membranes in the embryos from the heat stressed dams, leading to a decreased rate of diffusion of glutaraldehyde in to the cells and a poorer fixation.

Studies of Young Born to Heat Stressed Dams

Study of the number of pups born to heat stressed dams compared to control dams show no statistical difference. This signifies that the changes seen in the ultrastructure of the heat stressed embryos are reversible or not significant at this stage.

Table 1
Effects of 72 hour heat stress on mouse weight and temperature

	Average weights (gm)			Average temperatures (C°)		
	starting	ending	change	starting	ending	change
control n=29	25.33 ±2.39	24.69 ±2.16	.64 ±1.2	37.3 ±.41	37.1 ±.42	.2±.54 decrease
heat n=27	24.99 ±2.00	24.52 ±1.68	.47 ±1.1	37.3 ±.27	*38.0 ±.34	*.7±.65 increase

* values of significant difference p <0.05

Table 2

Effects of 72 hour heat stress on mouse food and water intake

	Average intake	
	food (gm)	water (ml)
control	11.44±2.3 n=25	16.22±2.67 n=28
heat	* 6.95±1.5 n=27	14.04±2.02 n=25

* values of significant difference $p < 0.05$

Table 3
Effects of 72 hour heat stress on mouse litter size, young weight, and seven day survivability

	Average litter size	Average weight (gm) birth	Average weight (gm) seven days	Seven day mortality
control n=5	6.3	1.5	4.2	4
heat n=6	6.8	1.6	4.2	2

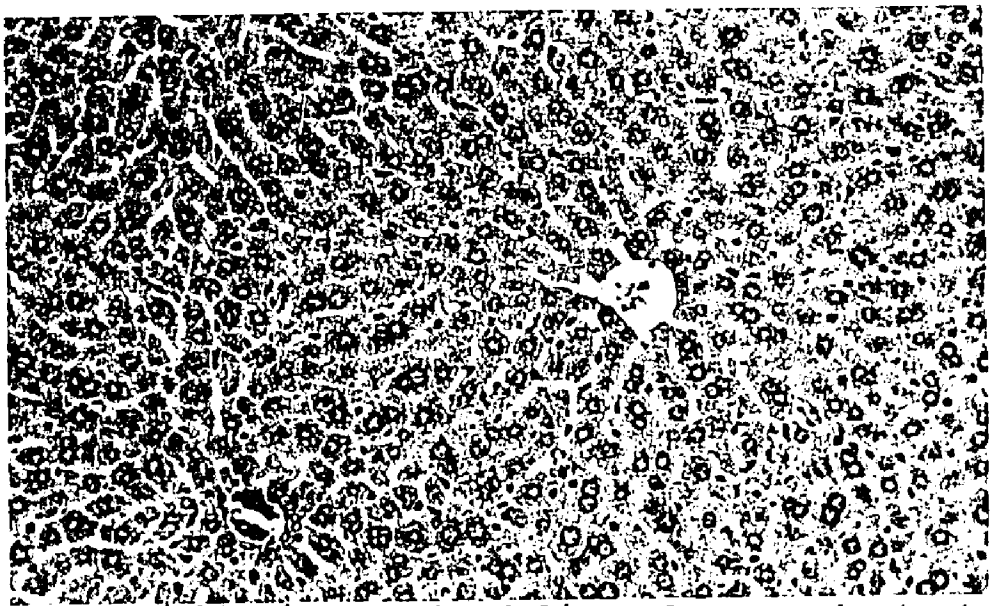

Figure 1a Light micrograph of liver from non-heat stressed mouse. Magnification: 200x.

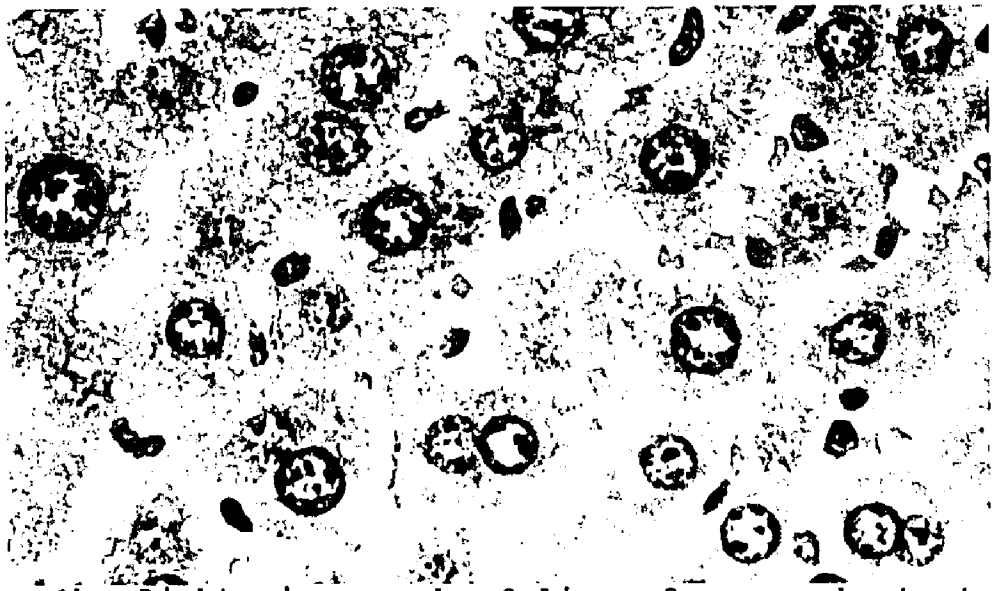

Figure 1b Light micrograph of liver from non-heat stressed mouse. Magnification: 800x.

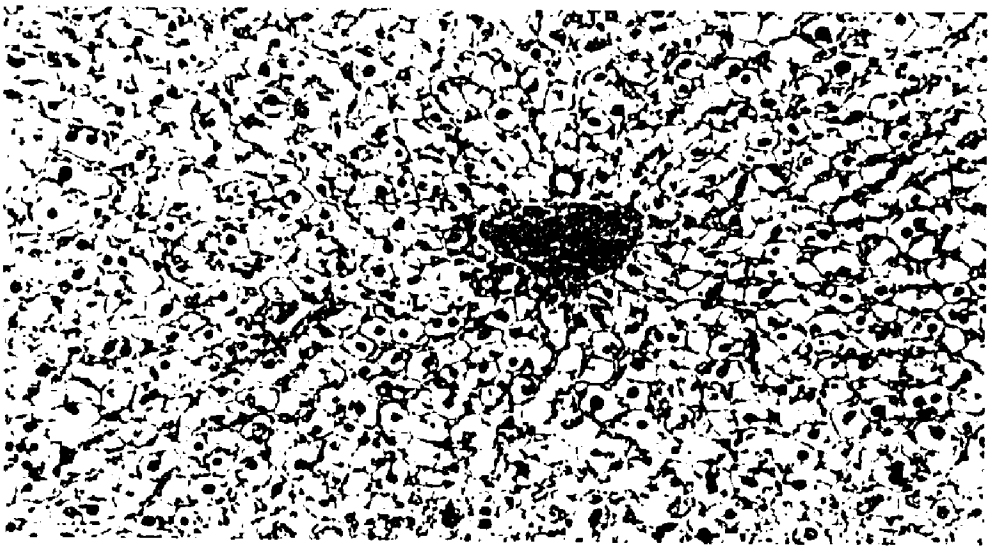

Figure 2a Light micrograph of the liver showing effects of 72 hour heat stress on hepatic architecture. Magnification: 190x.

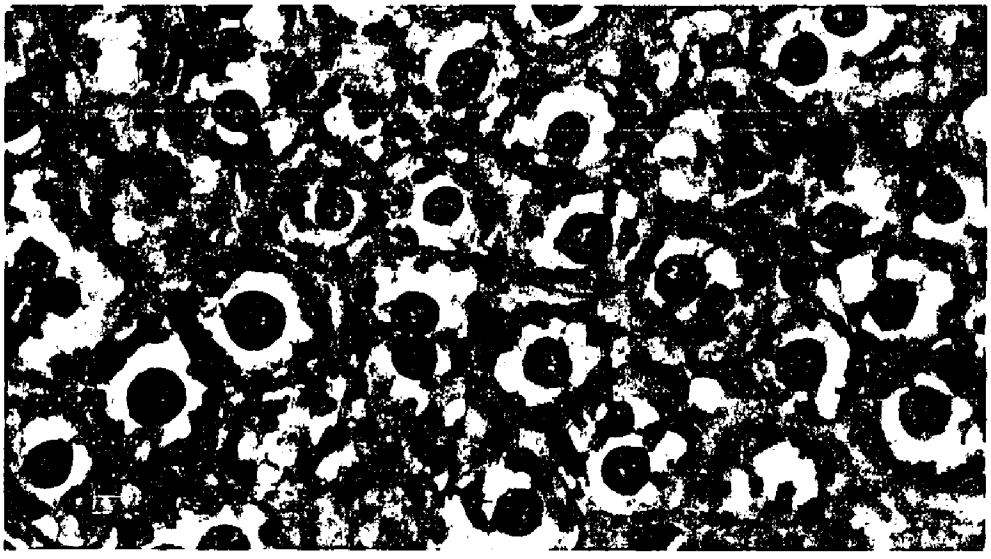

Figure 2b Light micrograph of the liver showing effects of 72 hour heat stress on hepatocytes. Magnification: 780x.

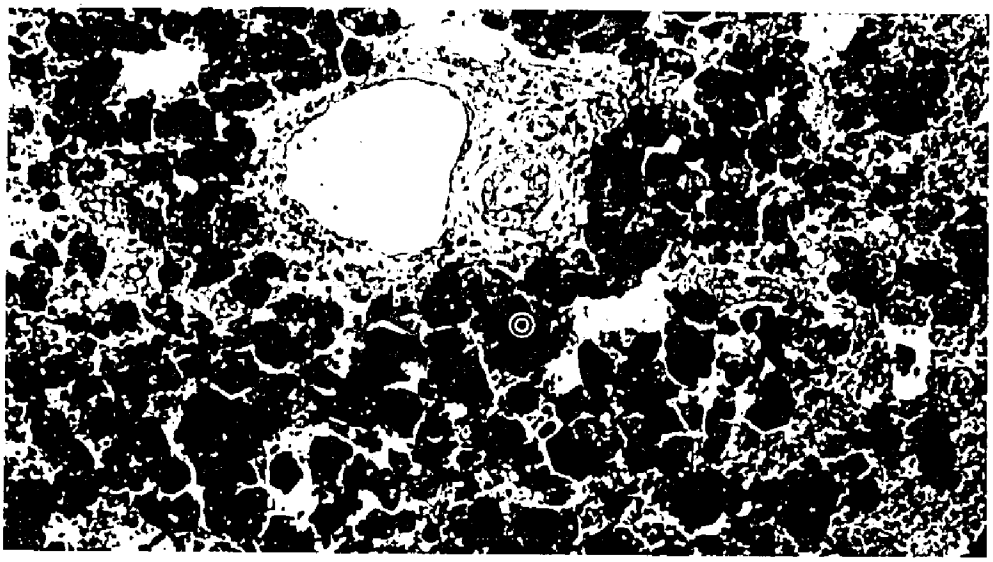

Figure 3a Light micrograph of a fatty liver stained with 2% osmium tetroxide to show fat accumulation in the hepatocytes. O=osmium stained fat. Magnification: 200x.

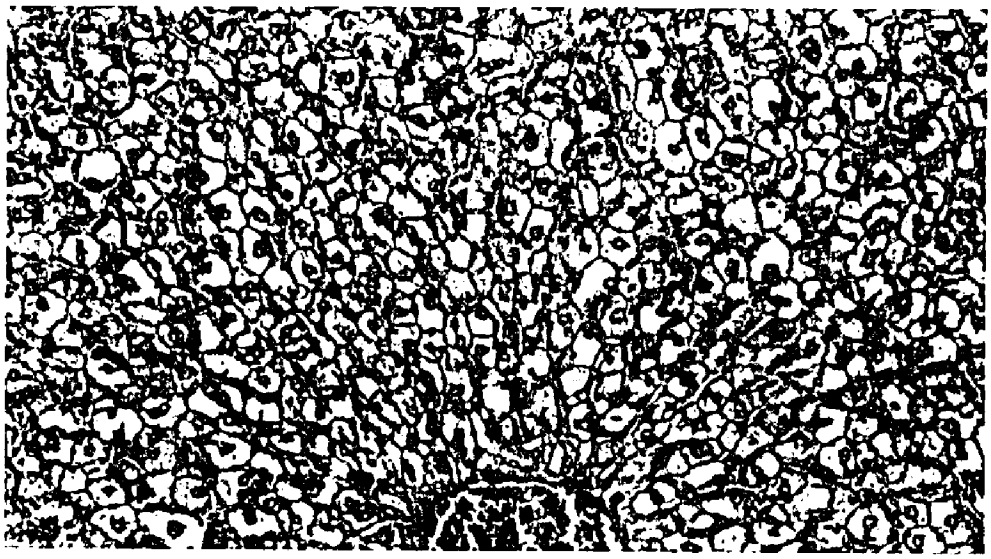

Figure 3b Light micrograph of liver from a heat stressed mouse stained with 2% osmium tetroxide to show absence of fat in hepatocytes. Magnification: 200x.

Figure 4a Electron micrograph of normal mouse hepatocyte. N=nucleus, Nu=nucleolus, R=rough endoplasmic reticulum, M=mitochondria, P=plasmalemma, B=bile cuniculi, G=glycogen granules. Magnification: 7400x.

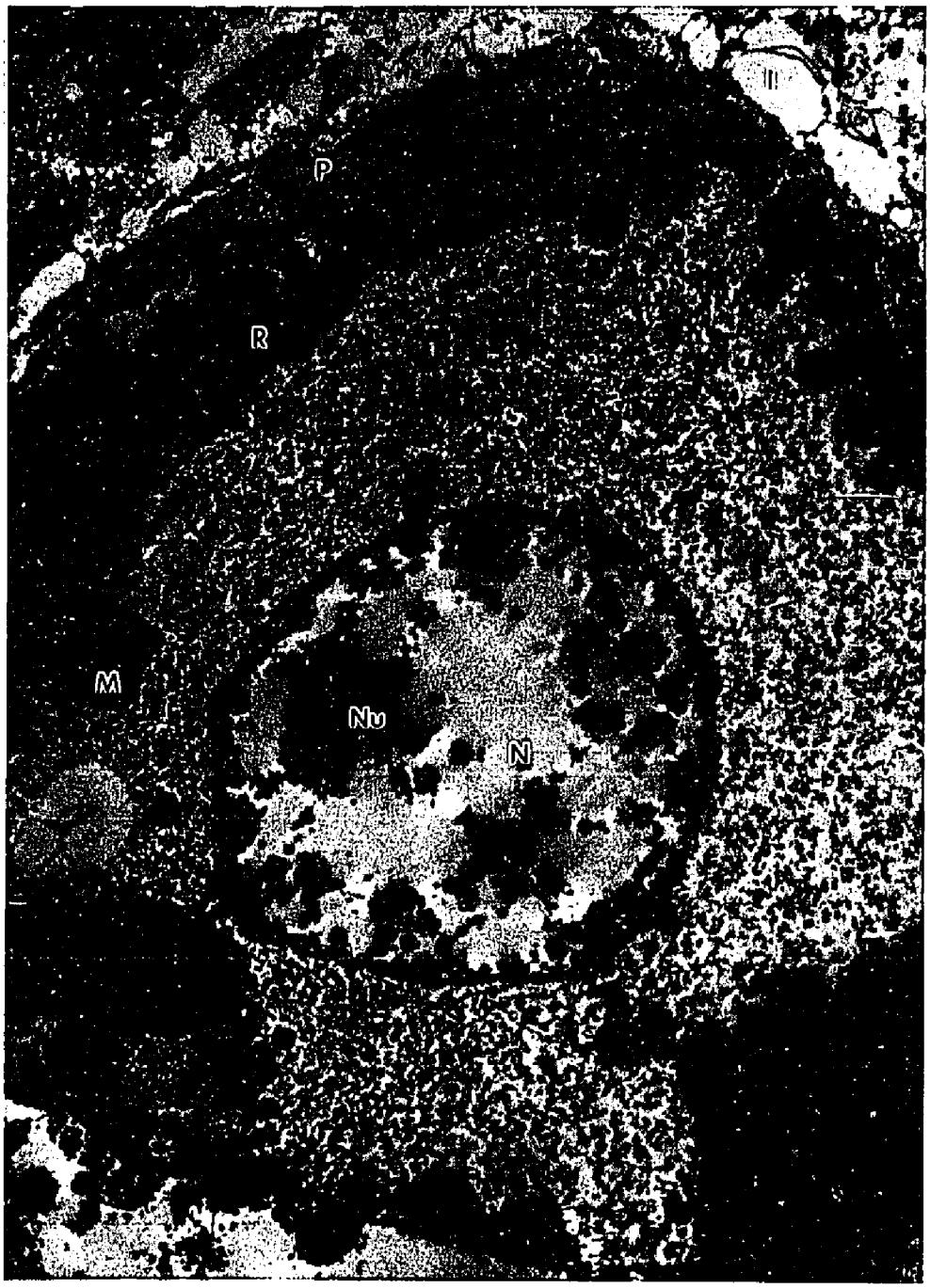

Figure 4b Electron micrograph of hepatocyte from mouse exposed to 72 hours of heat stress. N=nucleus, Nu=nucleolus, R=rough endoplasmic reticulum, M=mitochondria, P=plasmalemma, I=intracellular space. Magnification: 7000x.

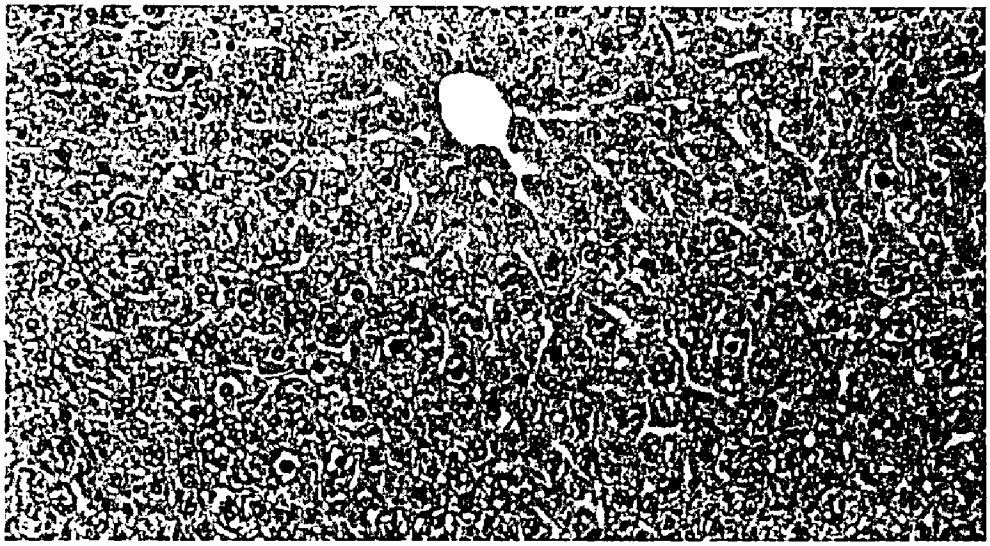

Figure 5a Light micrograph showing central lobular area of liver from mouse allowed to recover for 24 hours. Magnification: 200x.

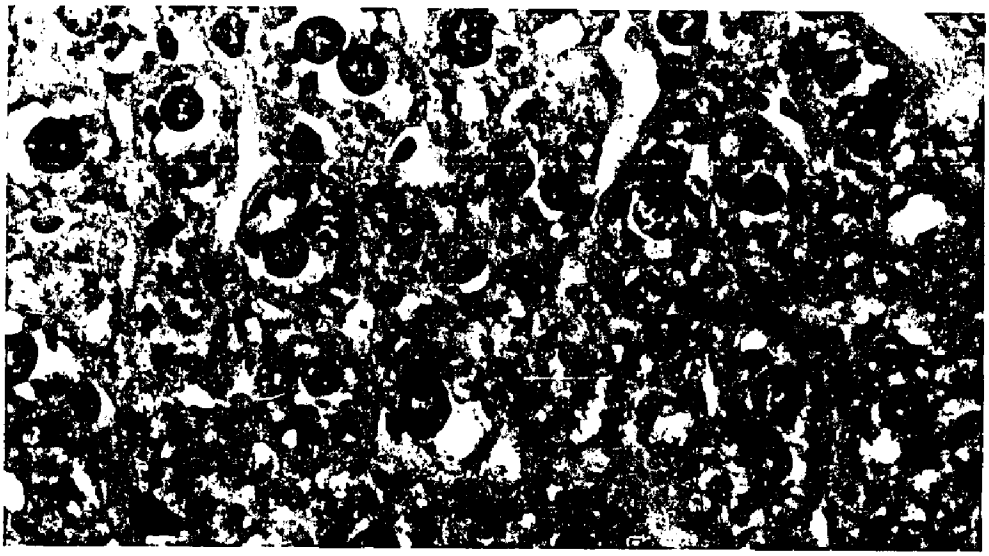

Figure 5b Light micrograph showing effects of 24 hour recovery of hepatocytes after heat stress. Magnification: 760x.

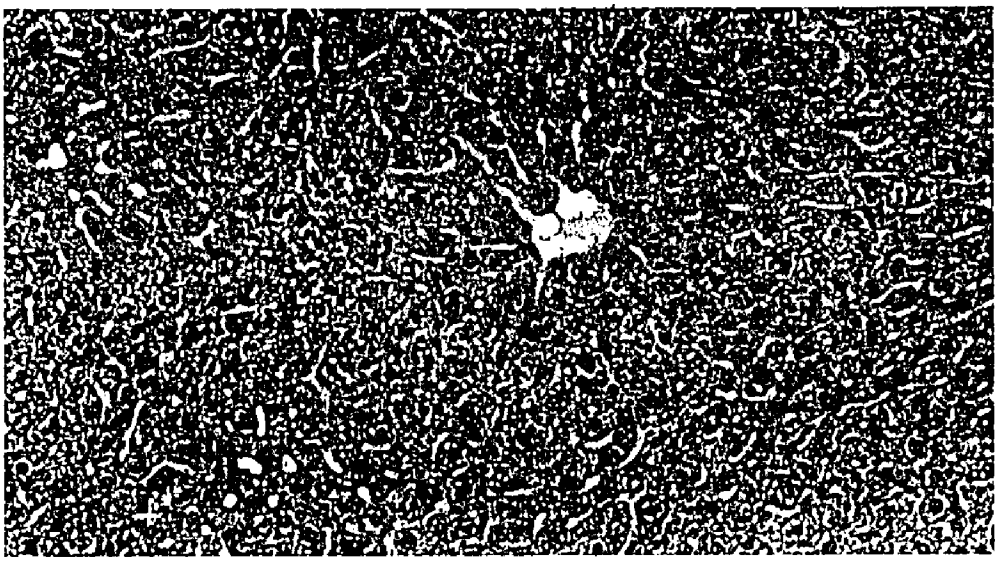

Figure 6a Light micrograph showing effects of 48 hour recovery on lobular architecture in liver of mouse that had been heat stressed for 72 hours. Magnification: 190x.

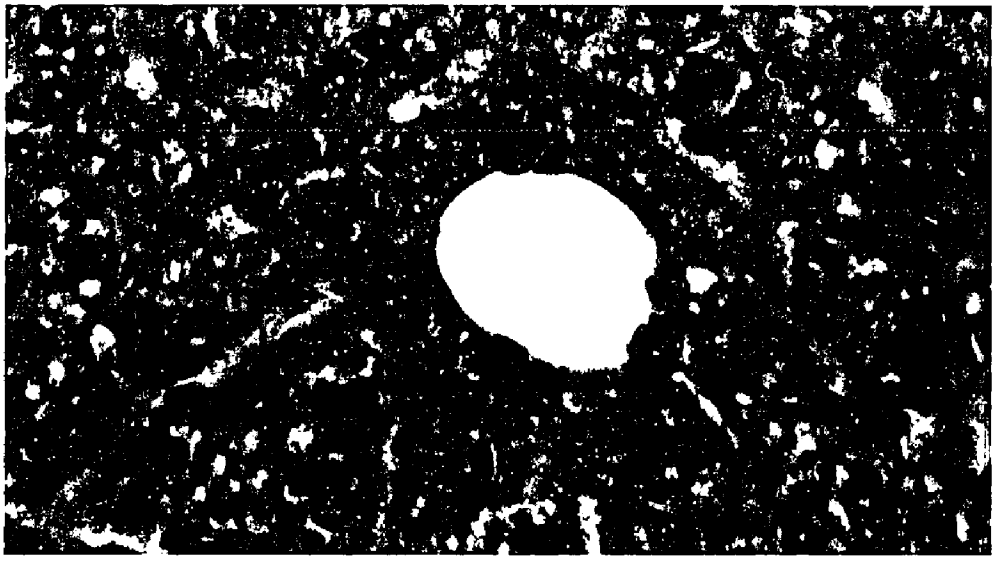

Figure 6b Light micrograph showing hepatocytes after 48 hour recovery after 72 hour heat stress. Magnification: 760x.

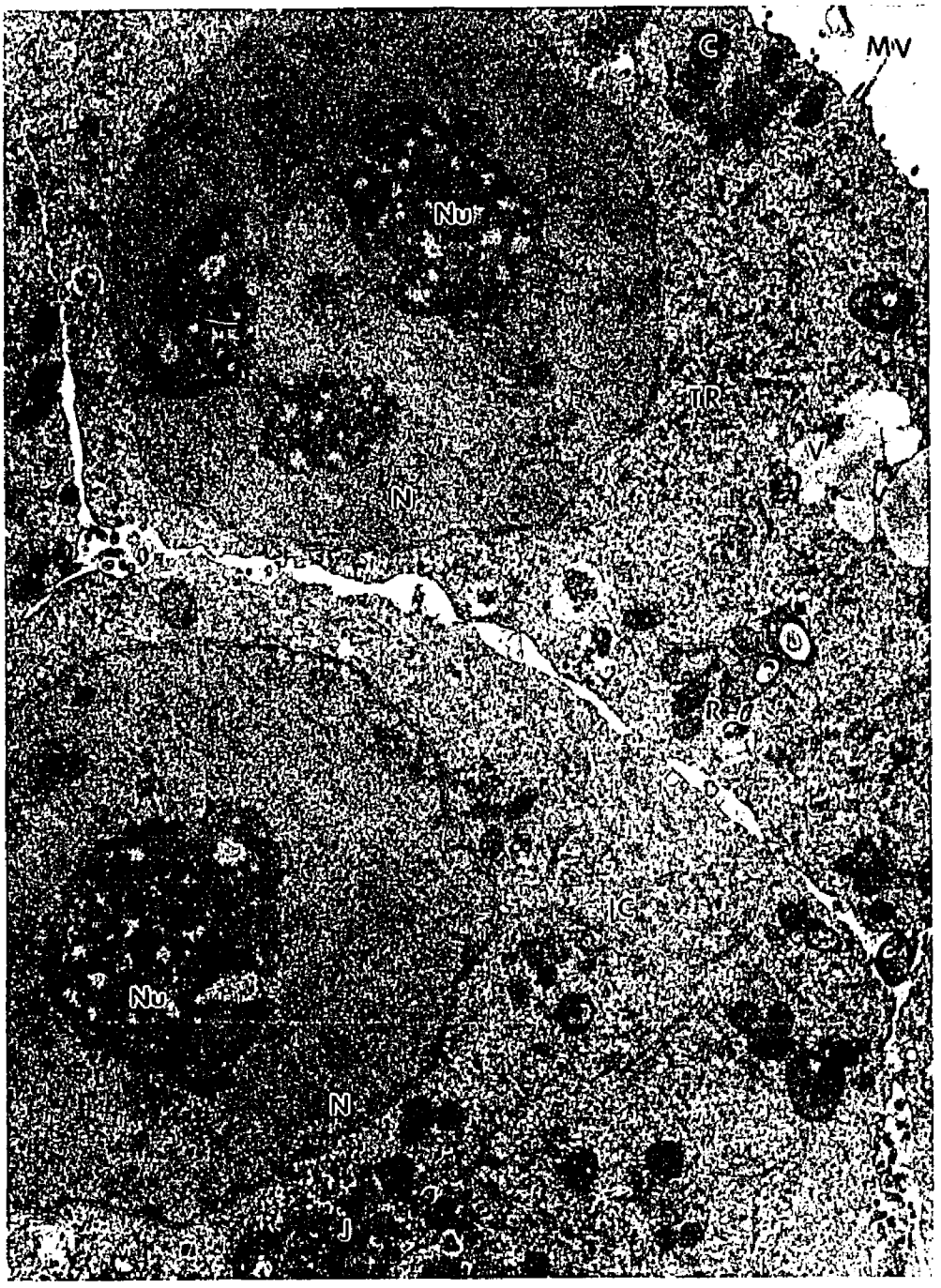

Figure 7a Electron micrograph of embryo flushed from uterus and fixed with 3% glutaraldehyde (group 1). TR=trophoblast cytoplasm, IC=inner cell mass cytoplasm, MV=microvilli, C=crystalloid inclusions, J=jigsaw body inclusions, V=lipid-like cytoplasmic vesicles, R=rough endoplasmic reticulum, N=nucleus, Nu=nucleolus. Magnification: 10000x.

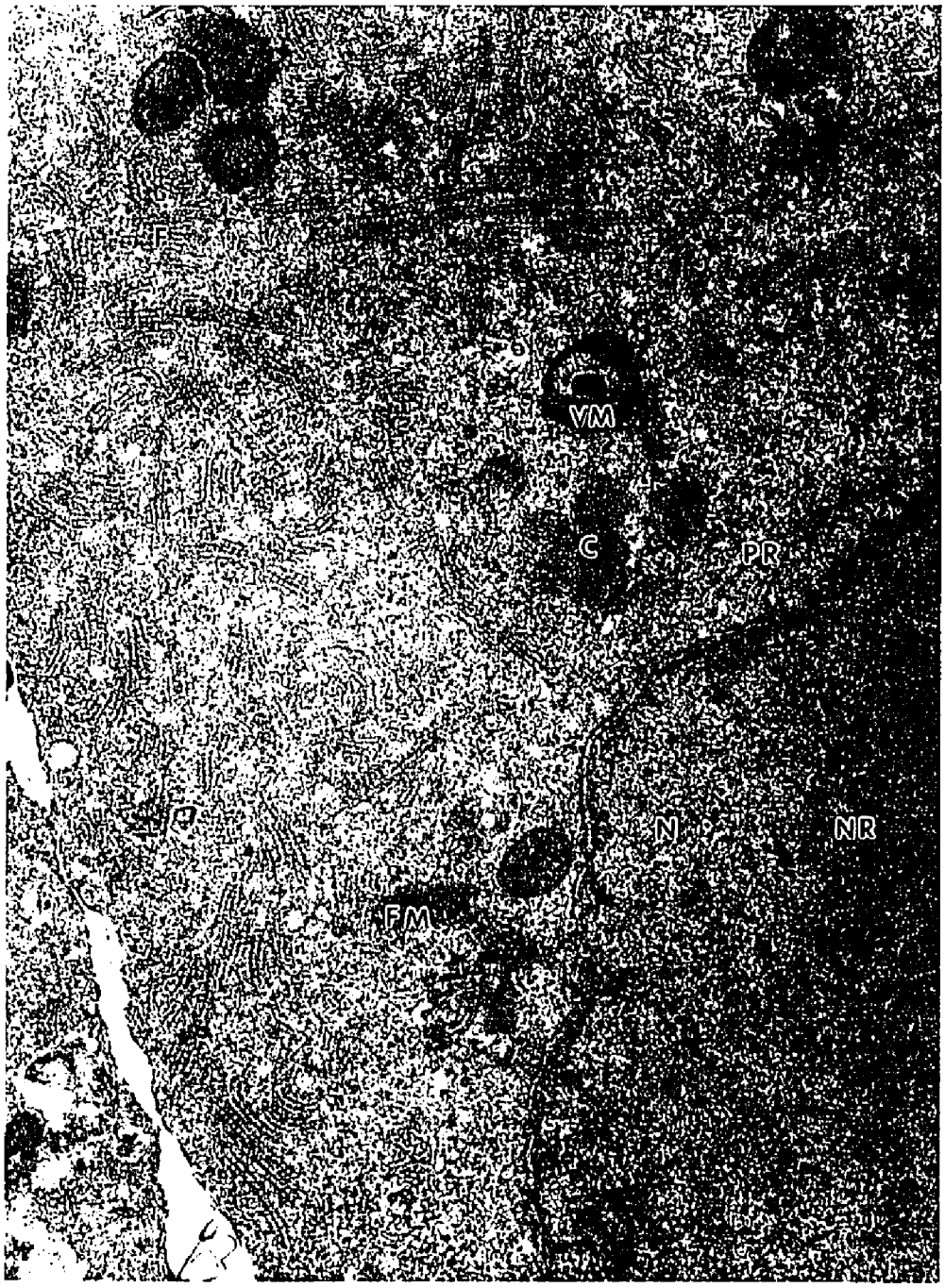

Figure 7b Electron micrograph of a cell of inner cell mass from embryo flushed from uterus and fixed with 3% glutaraldehyde (group 1). C=crystalloids, F=fibrous 2α helix inclusions, VM=vacuolated mitochondria, FM=filamentous mitochondria, PR=polyribosomal clusters, N=nucleus, NR=nuclear ribosomes. Magnification: 30000x.

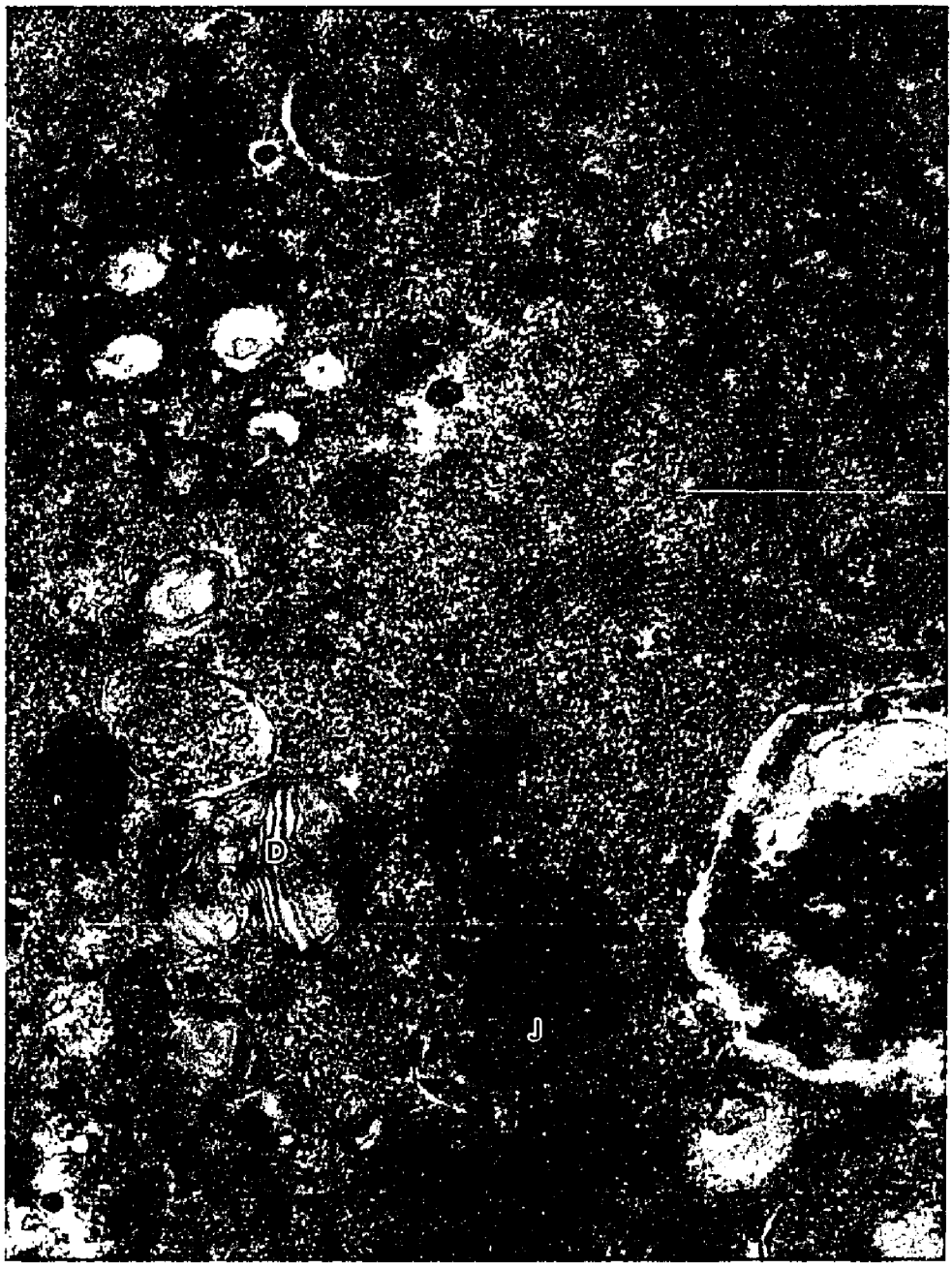

Figure 7c Electron micrograph of cell from inner cell mass of embryo flushed from uterus and fixed using 3% glutaraldehyde (group 1). D=degenerate body, J=jigsaw body inclusion. Magnification: 37000x.

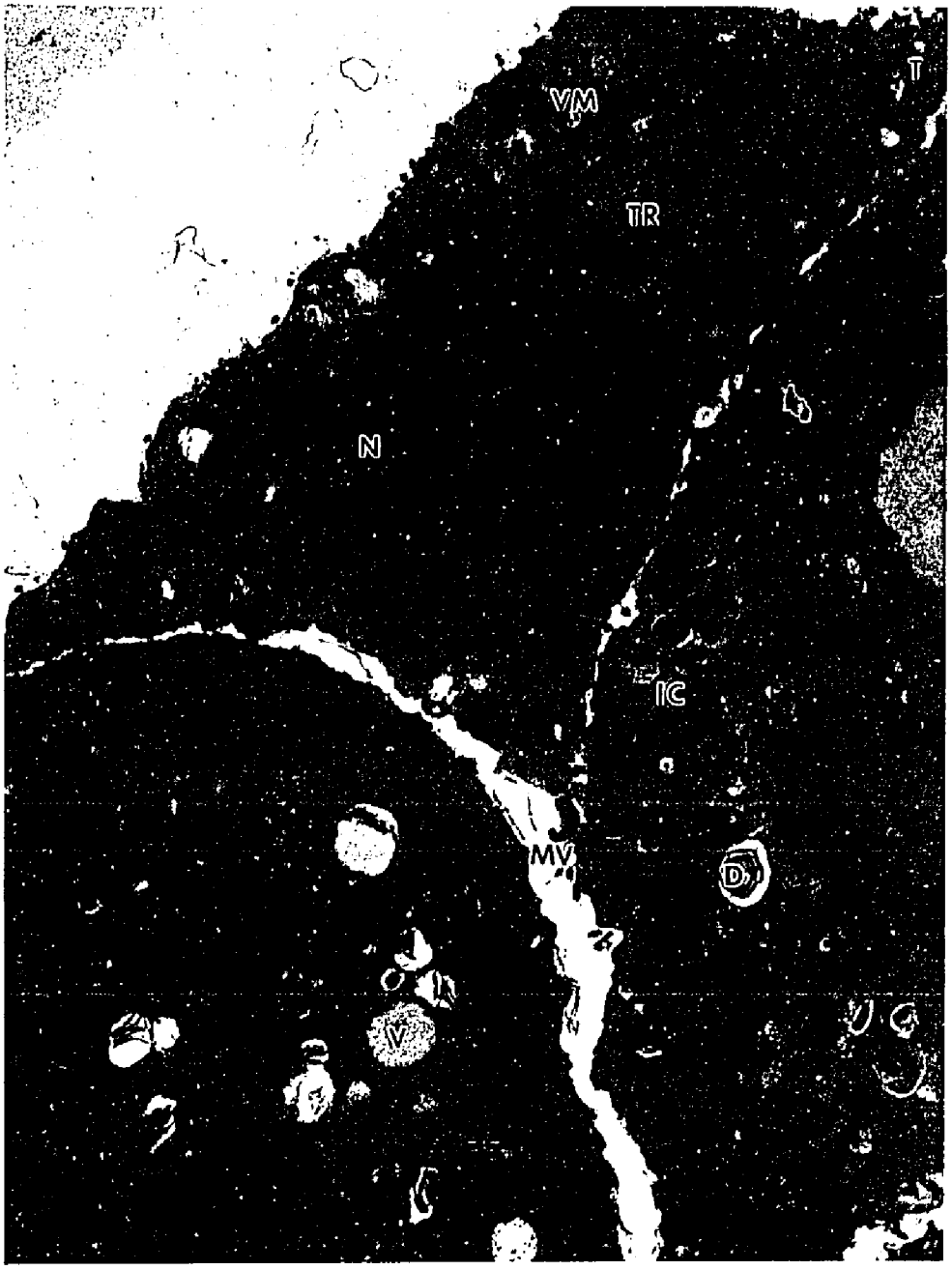

Figure 8a Electron micrograph of embryo flushed from uterus and fixed with 1% glutaraldehyde (group 2). TR=trophoblast cytoplasm, IC=inner cell mass cytoplasm, MV=microvilli, T=tight junction, V=lipid-like cytoplasmic vesicles, VM=vacuolated mitochondria, D=degenerate body, N=nucleus. Magnification: 12000x.

Figure 8b Electron micrograph of trophoblast cell of embryo flushed from uterus and fixed with 1% glutaraldehyde (group 2). C=crystalloid inclusions, PR=polyribosomal clusters, R=rough endoplasmic reticulum, A=inclusion type A, D=degenerate body. Magnification: 48000x.

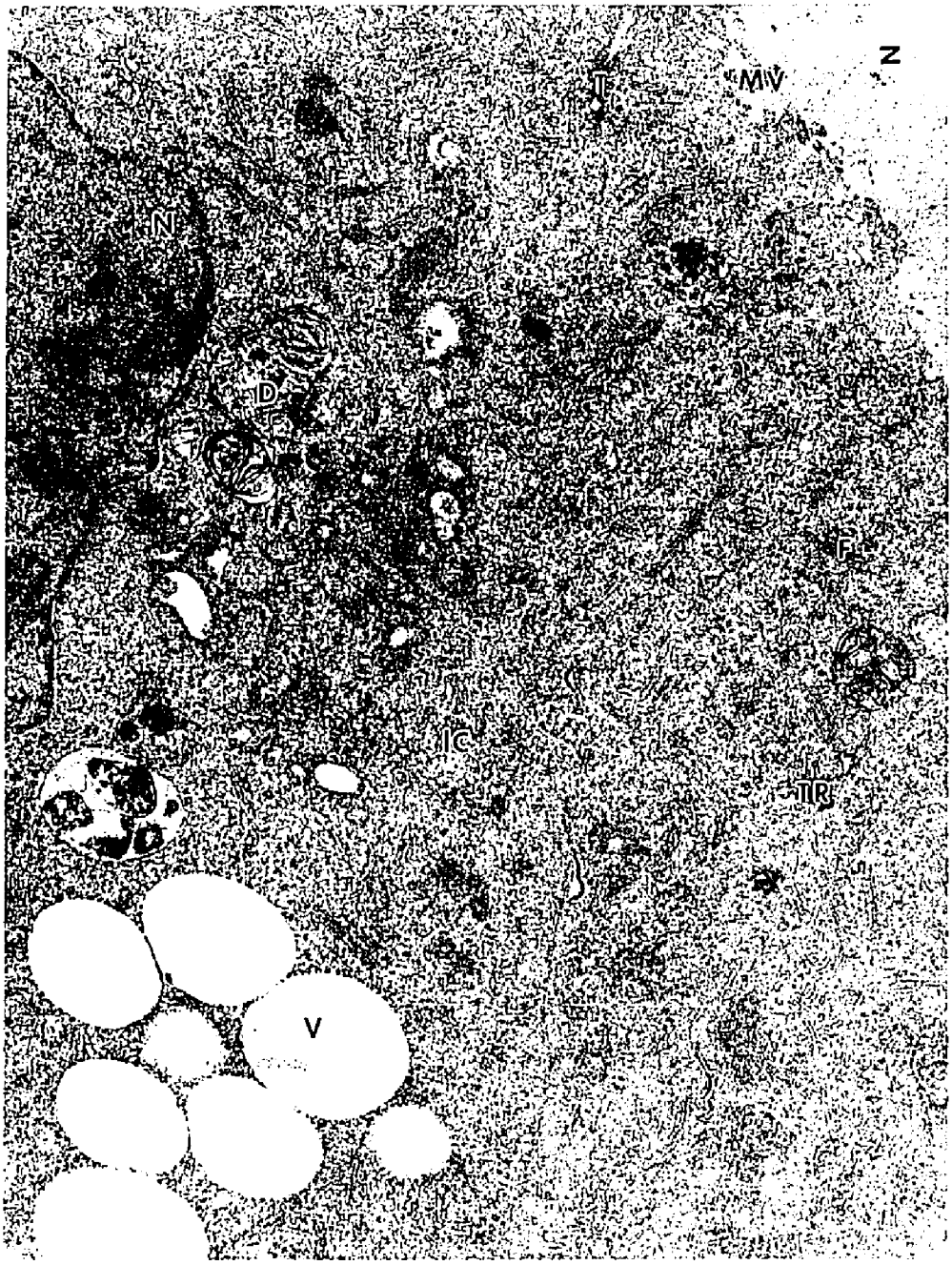

Figure 9a Electron micrograph of embryo flushed from uterus with Millonig's phosphate buffer then fixed with 3% glutaraldehyde (group 3). Z=zona pellucida, TR=cytoplasm of trophoblast cell, IC=cytoplasm of a cell of the inner cell mass, MV=microvilli, T=tight junction, F=fibrous 2α helix inclusions, D=degenerate bodies, V=lipid-like cytoplasmic vesicles, N=nucleus. Magnification: 17000x.

Figure 9b Electron micrograph of trophoblast and cell of inner cell mass of embryo flushed from uterus with Millonig's phosphate buffer and fixed with 3% glutaraldehyde (group 3). Z=zona pellucida, TR=cytoplasm of a trophoblast cell, IC=cytoplasm of a cell of the inner cell mass, VM=vacuolated mitochondria, R=rough endoplasmic reticulum, D=degenerate body, C=crystalloids. Magnification: 16000x.

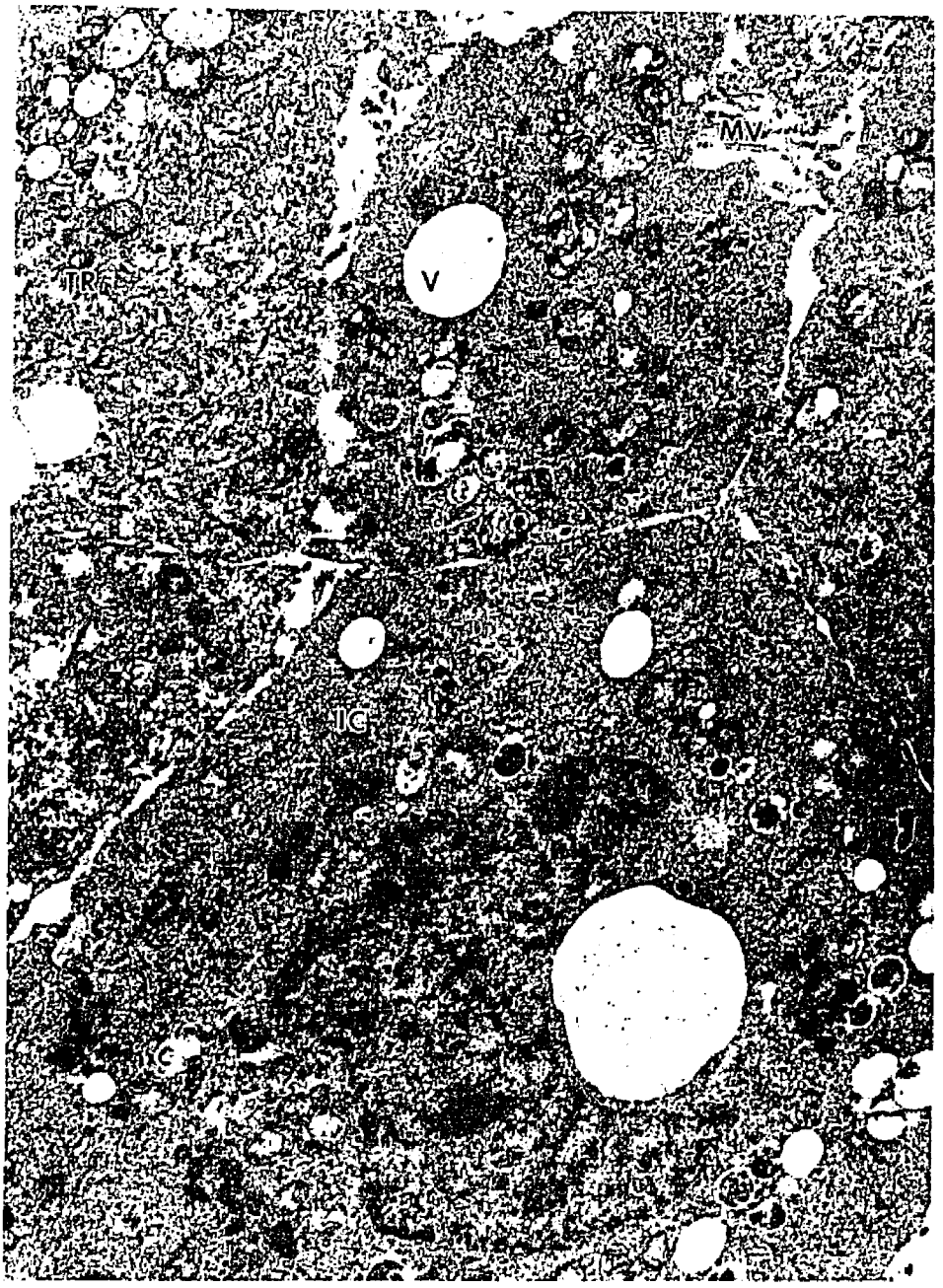

Figure 10a Electron micrograph of embryo flushed from uterus with Millonig's phosphate buffer and fixed with 1% glutaraldehyde (group 4). TR=cytoplasm of a trophoblast cell, IC=cytoplasm of a cell from the inner cell mass, MV=microvilli, V=lipid-like cytoplasmic vesicles. Magnification: 8000x.

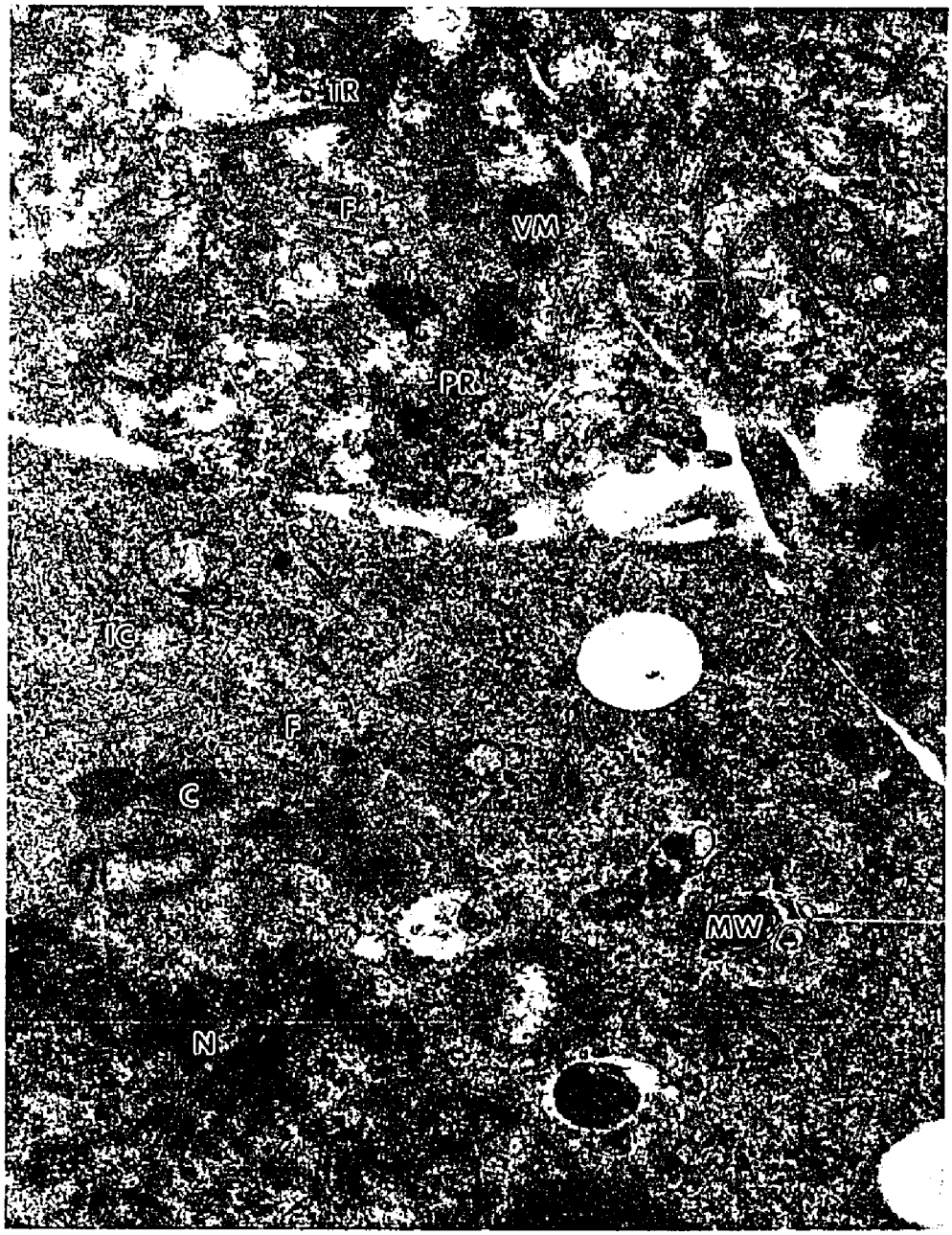

Figure 10b Electron micrograph of embryo flushed from uterus using Millonig's phosphate buffer then fixed with 1% glutaraldehyde (group 4). TR=cytoplasm of the trophoblast cell, IC=cytoplasm of a cell of the inner cell mass, VM=vacuolated mitochondria, PR=polyribosomal clusters, F=Fibrous 2α inclusions, C=crystalloids, MW=membrane whorls, N=nucleus of a cell of the inner cell mass. Magnification: 21000x.

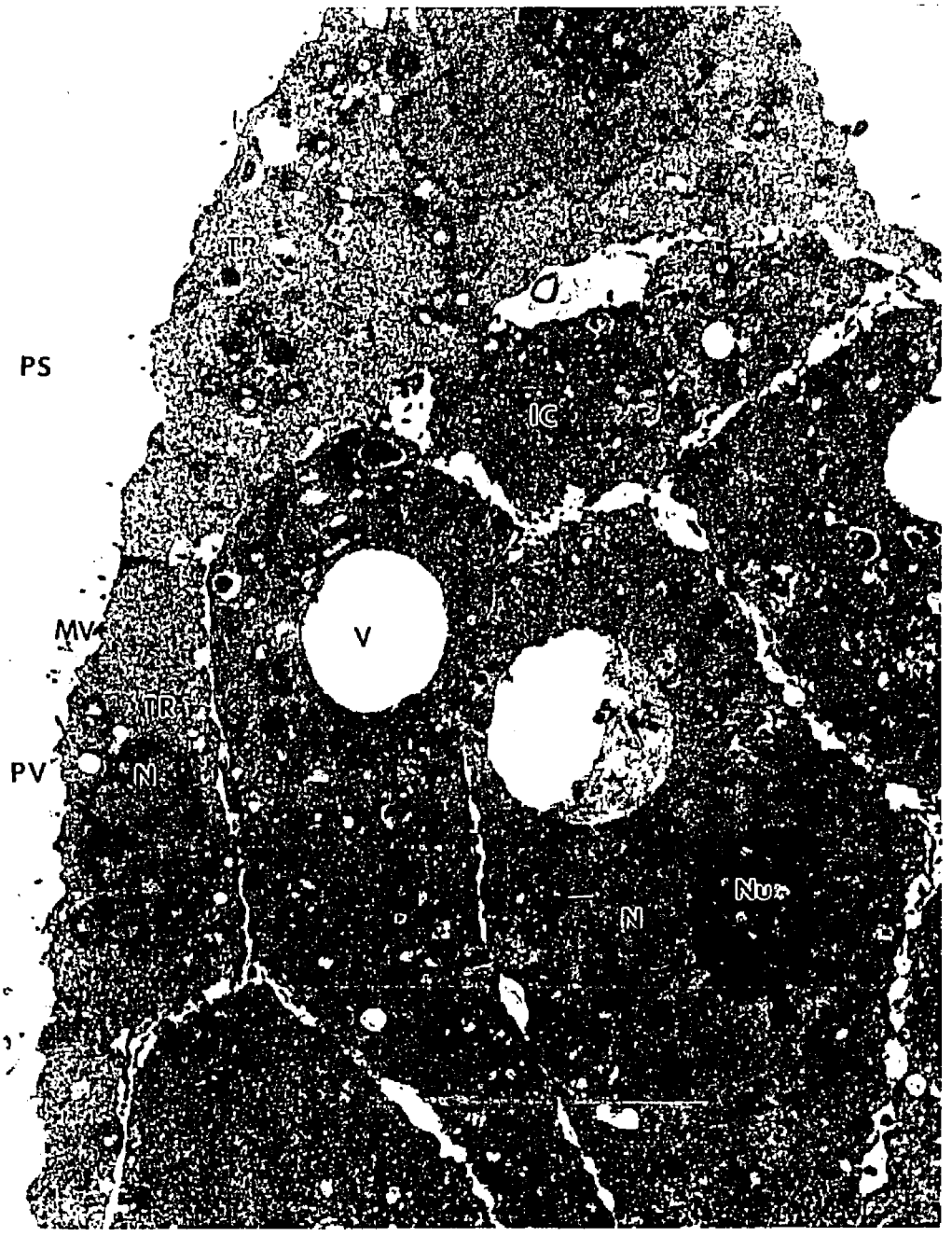

Figure 11a Electron micrograph of a embryo exposed to 72 hour heat stress. Embryo was flushed from uterus and fixed using 3% glutaraldehyde (group 1). TR=cytoplasm of the trophoblast cell, IC=cytoplasm of a cell of the inner cell mass, MV=microvilli, PV=pinocytotic vesicle, PS=perivitelline space, V=lipid-like cytoplasmic vesicle, N=nucleus, Nu=nucleolus. Magnification: 8500x.

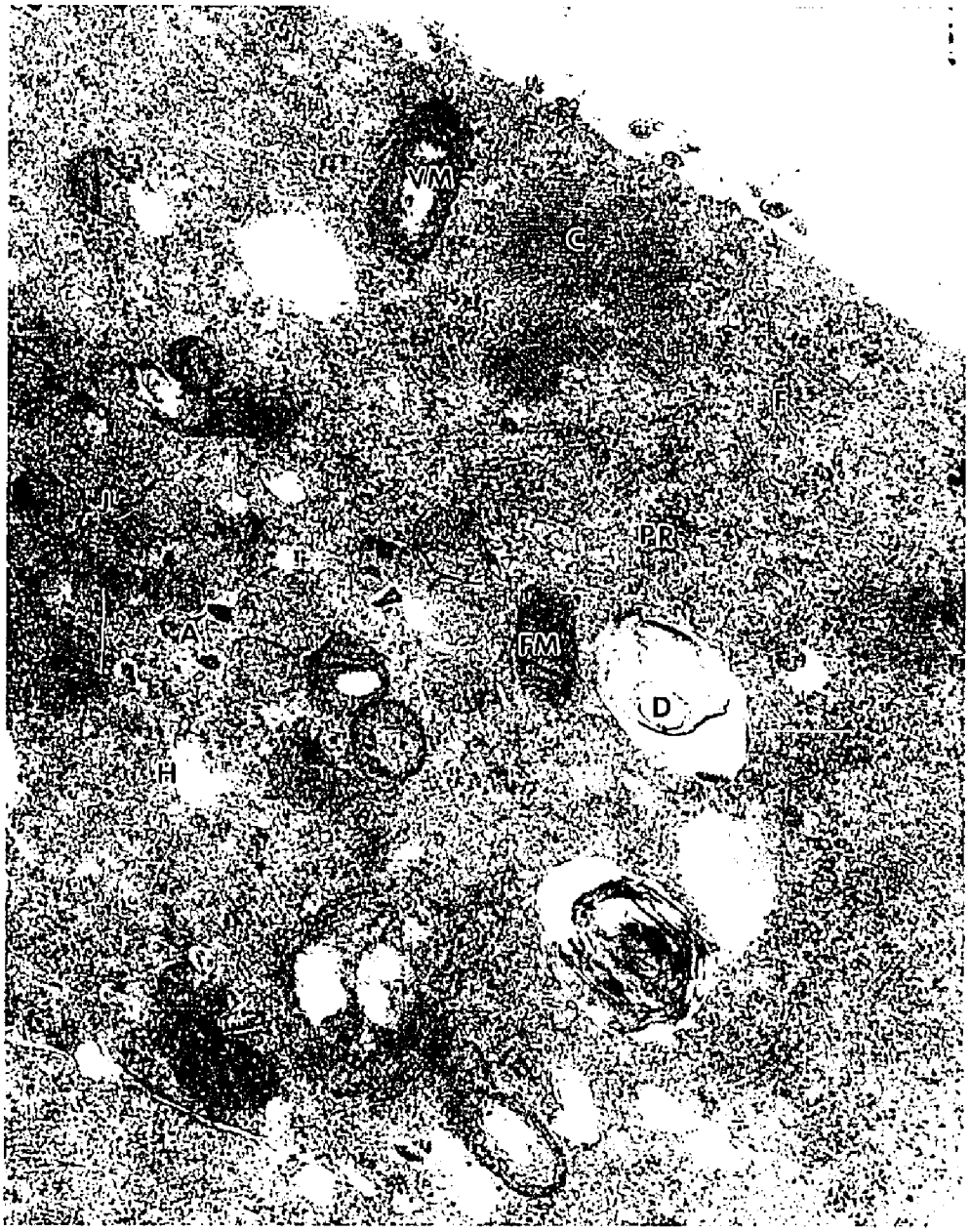

Figure 11b Electron micrograph of trophoblast cell from embryo exposed to 72 hour heat stress. Embryo was flushed from uterus and fixed using 3% glutaraldehyde (group 1). H=area of non-continuous cytoplasm, C=crystalloids, F=fibrous 2α helix inclusions, D=degenerate body, FM=filamentous mitochondria, VM=vacuolated mitochondria, J=jigsaw bodies, A=type A inclusions, PR=polyribosomal clusters. Magnification: 36000x.

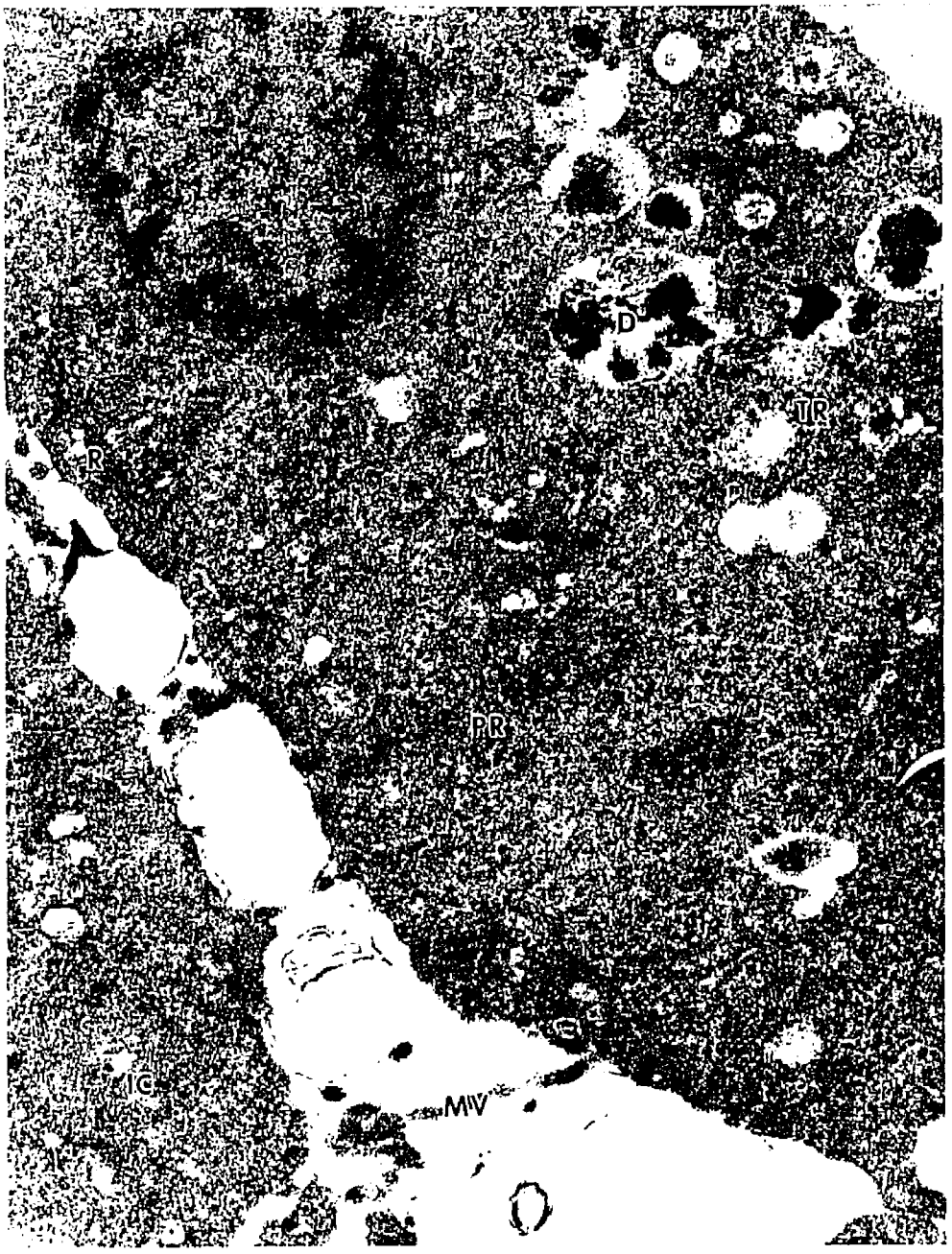

Figure 11c Electron micrograph of trophoblast cell and cell of inner cell mass of embryo exposed to 72 hour heat stress showing increased intercellular space. Embryo was flushed from uterus and fixed using 3% glutaraldehyde (group 1). TR=cytoplasm of the trophoblast cell, IC=cytoplasm of a cell of the inner cell mass, R=rough endoplasmic reticulum, PR=polyribosomal clusters, MV=microvilli, D=degenerate bodies. Magnification: 19000x.

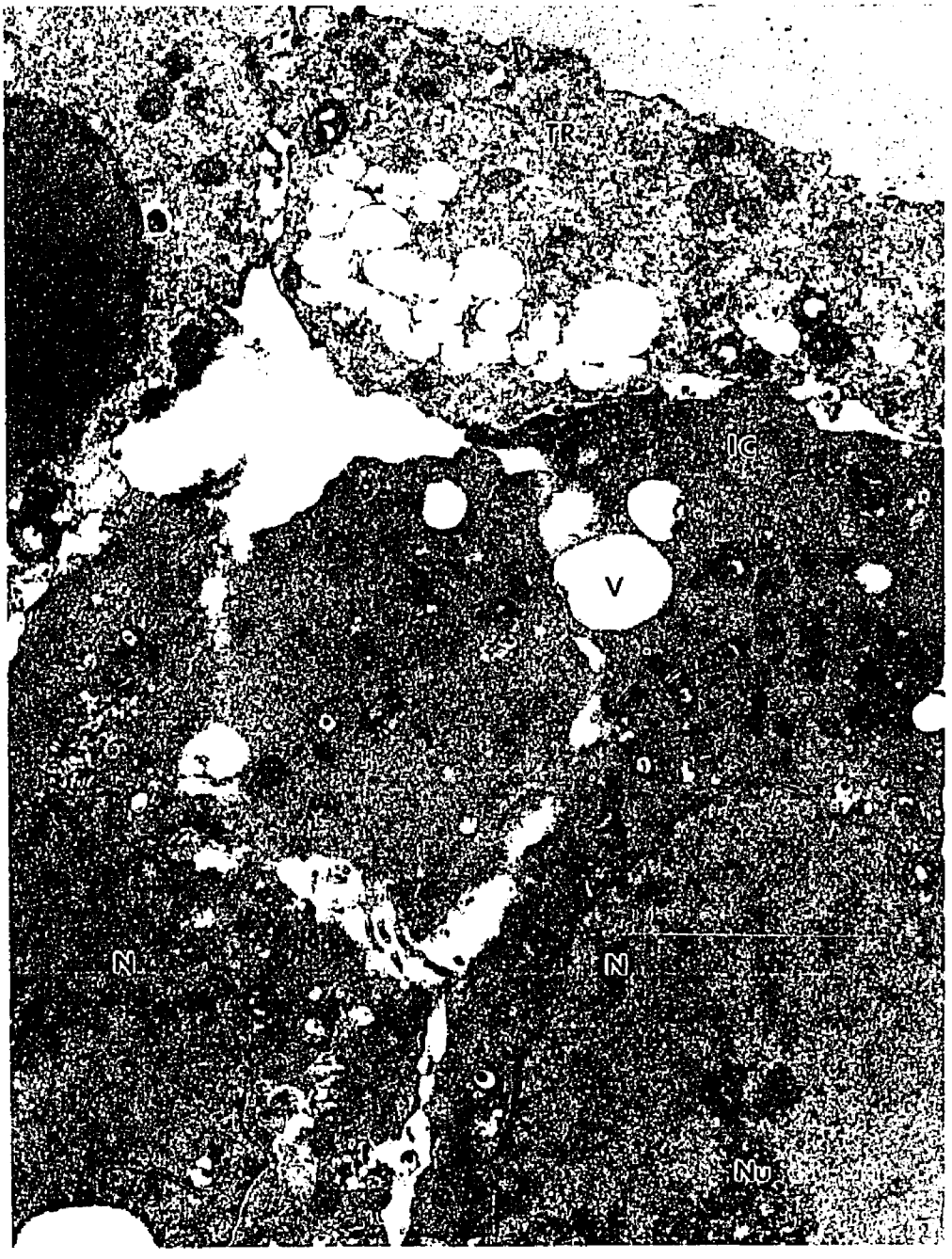

Figure 12a Electron micrograph of embryo from dam that was heat stressed for 72 hours. Embryo was flushed from uterus and fixed using 1% glutaraldehyde (group 2). TR=cytoplasm of a trophoblast cell, IC=cytoplasm of a cell of the inner cell mass, V=lipid-like cytoplasmic vesicles, N=nucleus, Nu=nucleolus. Magnification: 11000x.

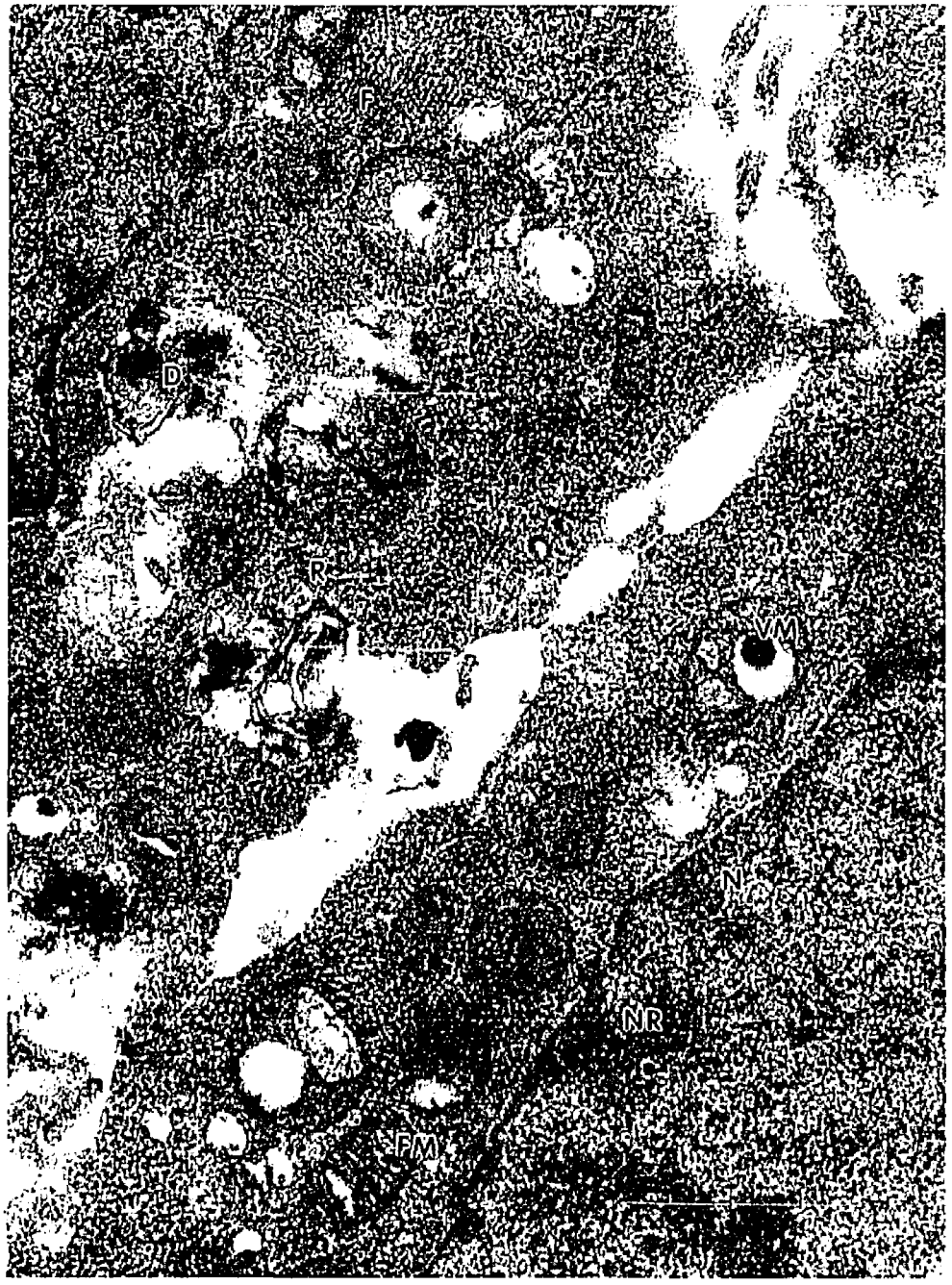

Figure 12b Electron micrograph of cells of inner cell mass of an embryo from dam exposed to 72 hour heat stress. Embryo was flushed from uterus and fixed with 1% glutaraldehyde (group 2). VM=vacuolated mitochondria, FM=filamentous mitochondria, D=degenerate body, F=fibrous 2α helix inclusions, R=rough endoplasmic reticulum, N=nucleus, NR=nuclear ribosomes. Magnification: 33000x.

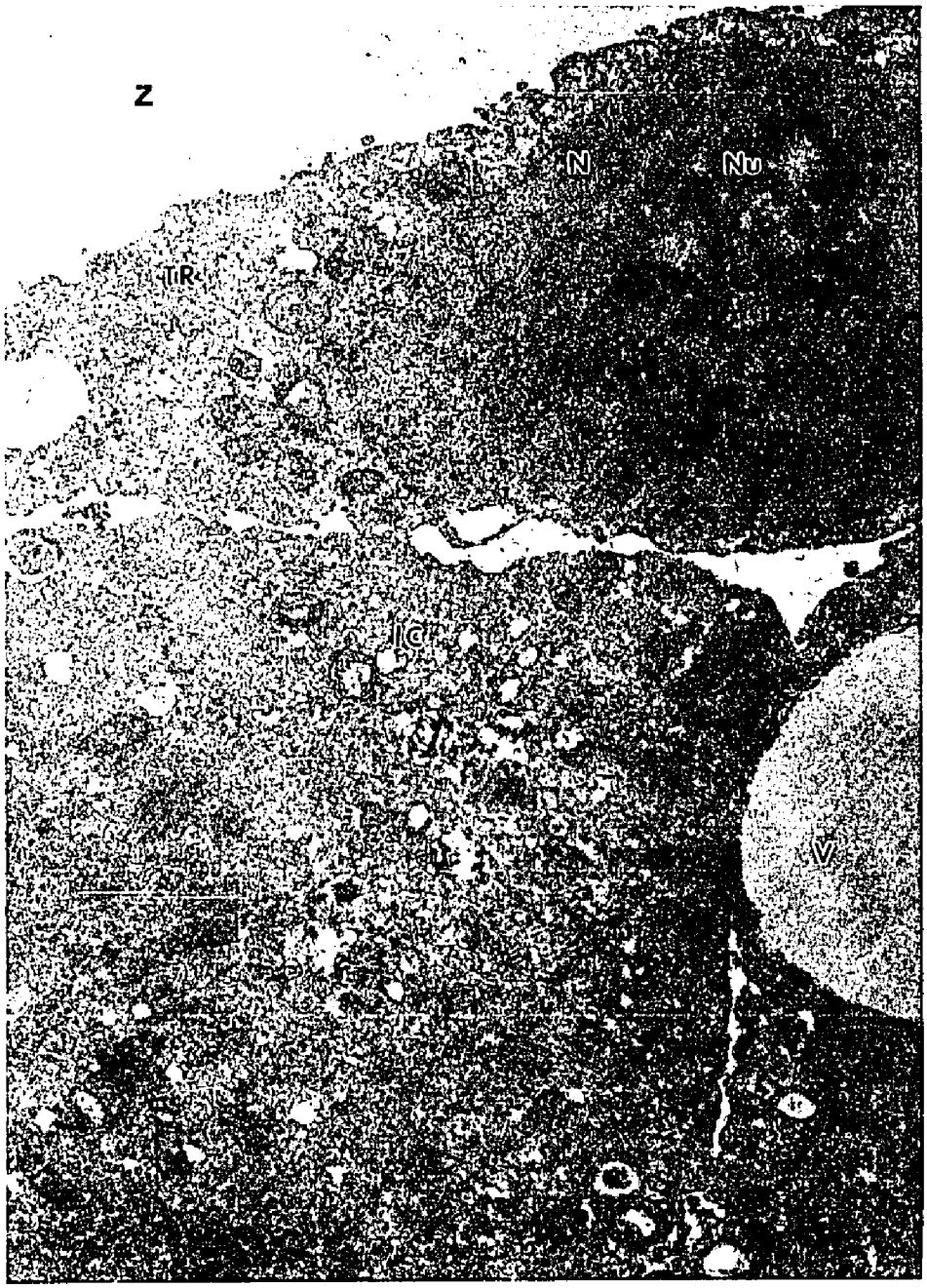

Figure 13a Electron micrograph of embryo from dam exposed to 72 hour heat stress. Uterus was flushed with Millonig's phosphate buffer and embryo fixed with 3% glutaraldehyde (group 3). Z=zona pellucida, TR=cytoplasm of a trophoblastic cell, IC= cytoplasm of a cell of the inner cell mass, V=lipid-like cytoplasmic vesicle, N=nucleus, Nu=nucleolus. Magnification: 14000x.

Figure 13b Electron micrograph of trophoblastic cell of embryo from heat stressed dam. Uterus was flushed with Millonig's phosphate buffer and fixed with 3% glutaraldehyde (group 3). MV=microvilli, F=fibrous 2α inclusions, D=degenerate body, C=crystalloids, VM=vacuolated mitochondria, R=rough endoplasmic reticulum, N=nucleus, Nu=nucleolus. Magnification: 11000x.

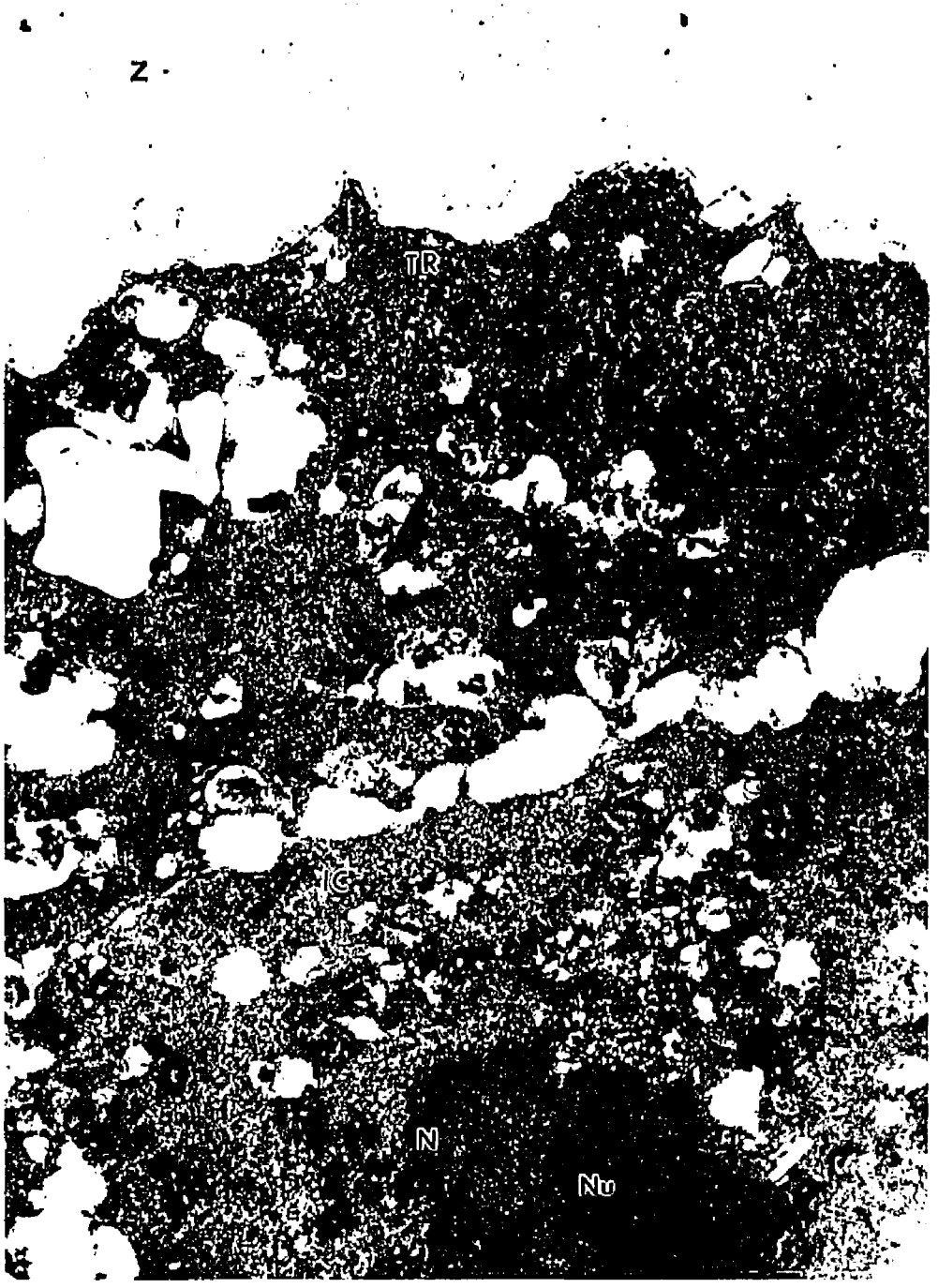

Figure 14a Electron micrograph of embryo from dam exposed to 72 hour heat stress. Millonig's phosphate buffer was used to flush uterus and embryos were fixed with 1% glutaraldehyde (group 4). Z=zona pellucida, TR=cytoplasm of the trophoblast cell, IC=cytoplasm of a cell of the inner cell mass, N=nucleus, Nu=nucleolus. Magnification: 8000x.

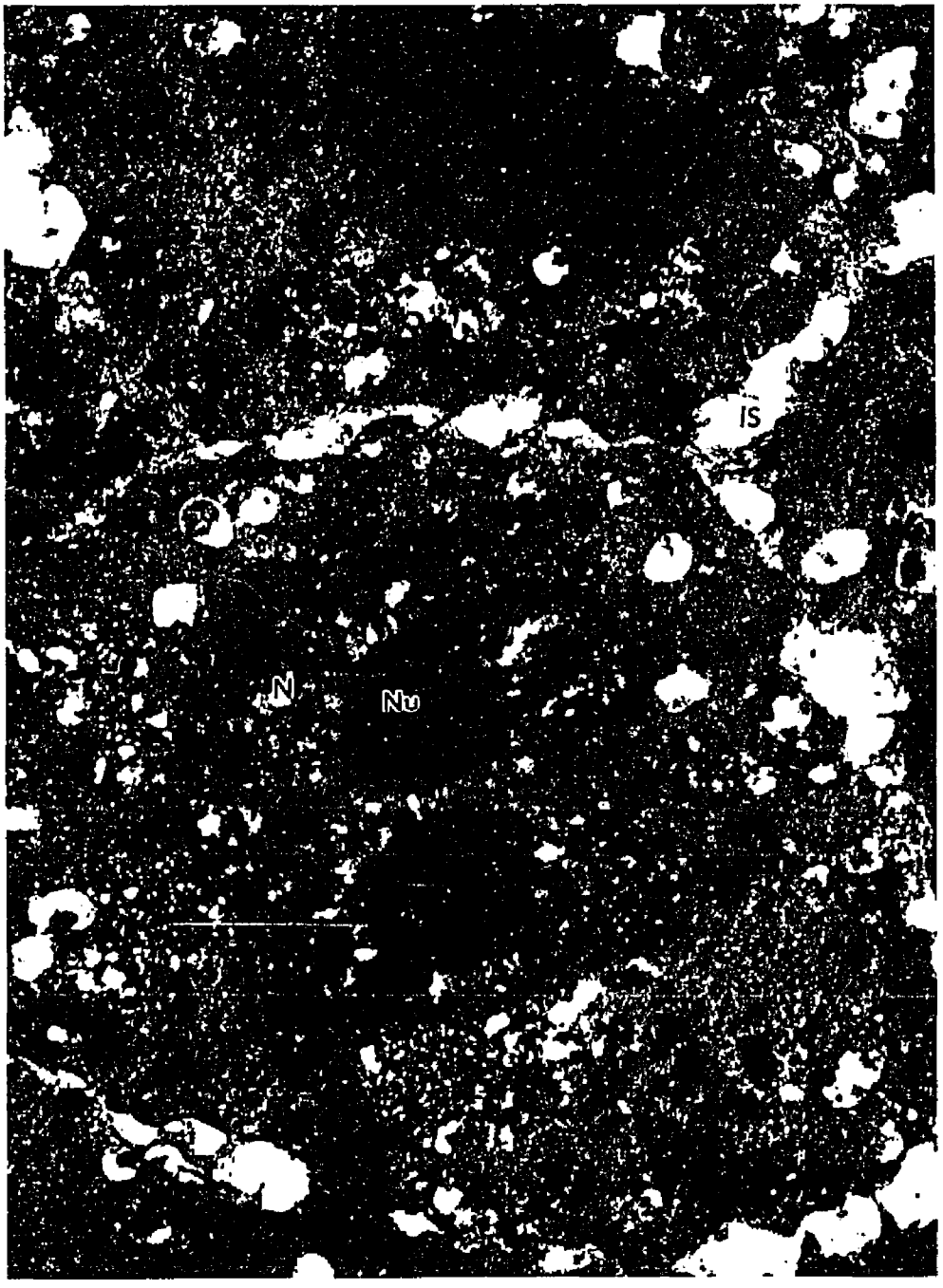

Figure 14b Electron micrograph of cells of inner cell mass of embryo from dam exposed to heat stress for 72 hours showing increased intercellular space. Embryo was flushed from the uterus using Millonig's phosphate buffer then fixed using 1% glutaraldehyde. IS=intercellular space, N=nucleus, Nu=nucleolus. Magnification: 7400x.

Figure 14c Electron micrograph of cells of inner cell mass from embryo from dam exposed to 72 hour heat stress. Uterus was flushed using Millonig's phosphate buffer and embryo fixed with 1% glutaraldehyde. C=crystalloids, R=rough endoplasmic reticulum, N=nucleus. Magnification: 19000x.

APPENDIX ONE

MILLONIG'S PHOSPHATE BUFFER

83 ml of 2.26% monosodium phosphate solution
17 ml of 2.52% sodium hydroxide solution
10 ml distilled water
adjust pH to 7.3

MILLONIG'S FIXATIVE

99 ml warm Millonig's phosphate buffer
1 gram osmium tetroxide
1 ml 5.4% glucose added to each 10 ml 1% osmium tetroxide solution before use

APPENDIX TWO

PROCEDURE FOR DEHYDRATION AND EMBEDDING

ELECTRON MICROSCOPY

1. Sections of liver were cut into 1 mm square sections and washed three times with Millonig's phosphate buffer.
2. Tissues were placed in Millonig's fixative for 90 minutes.
3. The samples were washed three times with distilled water.
4. Samples were placed in 35% ethyl alcohol for a few minutes.
5. The samples were then placed in 70% ethyl alcohol for 15 minutes.
6. The samples were then washed with 90% ethyl alcohol and allowed to sit in 90% ethyl alcohol for 30 minutes. This step was repeated.
7. The samples were then washed with 100% ethyl alcohol and left in 100% ethyl alcohol for 30 minutes. This step was repeated.
8. The samples were then washed with propylene oxide and left in the propylene oxide for 30 minutes. This step was repeated
9. A mixture of 2:1 propylene oxide to Epon-Araldite* was made and the samples were placed in the mix for 1 hour.
10. The mixture was then replaced with a mix of 1:2 propylene oxide to Epon-Araldite and allowed to sit for 1 hour.
11. The samples were then placed in a 100% Epon-Araldite solution and left uncovered over night.
12. The samples were then placed into Beem capsules and fresh Epon-Araldite was placed into the capsules.
13. The capsules were placed into a 60^{0} C oven for 72 hours to cure.

The embryos used in this study were adhered to glass slides with poly-l-lysine and processed in the above manner for embedding.

*Epon-Araldite (Ted Pella inc. Tustin, Ca.)
75 ml Epon 812
165 ml Dodecenyl succinic anhydride
45 ml Araldite 502
9 ml Dibutyl phthalate

APPENDIX THREE

SAMPLE STAINING FOR ELECTRON MICROSCOPY

1. Place a drop of 5% saturated uranyl acetate on to dental wax.
2. The grid was then placed in the drop with the section side up for 20 minutes.
3. The grid was then removed and placed into a drop of distilled water.
5. The grid was then rinsed with 150 ml of distilled water dispensed from a laboratory wash bottle.
6. The grid was then placed on clean, lint free paper until staining with lead citrate.
7. Sodium hydroxide was placed on to dental wax and covered with a petri dish. A drop of Reynold's lead citrate* was placed onto the wax adjacent to the sodium hydroxide pellets.
8. The grid was then placed into the drop specimen side up, covered with a petri dish, and allowed to stand for 10 minutes.
9. The grid was then placed in to a drop of distilled water.
10. The grid was then rinsed with 150 ml of distilled water dispensed by a 250 ml laboratory wash bottle.
11. The grid was dried on clean, lint free paper, then placed in a grid box until examined.

* Reynold's lead citrate stain
1.33 g lead nitrate (PbNO3)
1.76 g sodium nitrate (Na3(C6H5O7) 2H2O)
30 ml distilled water
shake for 1 minute
add 8.0 ml 1N NaOH
Dilute to 50 ml with distilled water

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