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TRANSPLACENTAL CARDIOTOXICITY OF COCAINE: ATRIAL DAMAGE FOLLOWING TREATMENT IN EARLY PREGNANCY

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Abstract

Using light, transmission (TEM) and scanning (SEM) electron microscopy, cocaine-induced defects were observed in hamster atria. Compared with controls, the treated atria from neonates show endocardial and myocardial damages as the atrial walls thicken. SEM micrographs show intensive blebbing, damage and incomplete coverage of myocardium by the endocardial endothelium. TEM data demonstrate blebs, thinning, and other endothelial cell injuries and complement the SEM findings. Areas of endothelial sloughing may facilitate the formation of luminal and mural thrombi as noticed in many neonatal atria. Adjacent subendocardial myocardial cells display contraction bands, swellings, and vacuolizations. Local and large areas of damaged myocardial cells are observed in the subendothelial spaces; they contact fibroblasts squeezed or intercalated between the subendocardial spaces and the basal side of damaged endothelial cells. Many of these defects correspond to well-known ischemic changes. One can hypothesize that cocaine-induced defects appear to be linked to membranous alterations, including those associated with the endothelial cells of the endocardium.

KEY WORDS: Hamster, cocaine, atrium, endocardium, thrombus, myocardium, cell injury, fetal heart defects, heart development, transmission electron microscopy, scanning electron microscopy.

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Introduction

Forensic and clinical reports have documented several cardiovascular defects associated with adult cocaine overdose or abuse. Commonly described histopathological and functional alterations can be summarized as follows: **(a)** decreased coronary blood flow due to vasoconstriction or spasm (Avakian et al., 1990; Billman, 1990; Kossowsky and Lyon, 1984; Gradman, 1988; Isner and Chokshi, 1989; Lange et al., 1989; Little et al., 1989; Schachne et al., 1984; Tazelaar et al., 1987; Wilkerson, 1988; Zimmerman et al., 1987); **(b)** arrhythmia or angina (Billman, 1990; Isner et al., 1986; Nanji and Filipenko, 1984; Wetli and Wright, 1979); **(c)** acute myocardial infarction (Ascher et al., 1988; Cregler and Mark, 1985; Gradman, 1988; Howard et al., 1985; Kossowsky and Lyon, 1984; Rod and Zucker, 1987; Schachne et al., 1984; Tazelaar et al., 1987; Welder et al., 1988; Zimmerman et al., 1987); and **(d)** subendocardial necrosis (Chokshi et al., 1985). In addition, the formation of coronary thrombi (Hadjimiltiades et al., 1988; Isner and Chokshi, 1989; Zimmerman et al., 1987) can be associated with these defects.

Cocaine use by people of all ages, races, and socioeconomic groups has increased in recent years with a concurrent increase of cocaine-related deaths. The number of female cocaine users has been estimated in the U.S. alone to be at least 2.5 million (NIDA, 1989). The prevalence of cocaine abuse among pregnant women has also increased. More addicted mothers are giving birth to babies whose health is at risk before and at birth; drug users have complicated pregnancies (Chasnoff et al., 1985) with increased incidence of abruptio placentae and fetal death. There has been an increase in babies stillborn (Critchley et al., 1988), and in offspring crippled by low birth weight (Chouteau et al., 1988; Zuckerman et al., 1989), and other physical, mental and behavioral disabilities (Chasnoff and Schnoll, 1987; Chasnoff et al., 1985) as a result of drug use during pregnancy. These damages probably originate from the ischemic fetal conditions caused by a reduction in the uterine blood flow and modifications of cardiovascular activity in the addicted mother (Woods et

al., 1987) or in the fetus (van de Bor et al., 1990). Only a small number of reports have dealt with the study of animal models (e.g., Catravas and Waters, 1981; Church et al., 1988; Geaty, 1987; Nunez and Klein, 1989).

Previous findings concerning the morphology of development and aging of the endocardial tissues in normal hamster atria (Gilloteaux, 1989; Gilloteaux and Linz, 1989) and in the cardiomyopathic Syrian hamster atria have been described (Gilloteaux et al., 1990).

The toxic effects of transplacental cocaine exposure upon the endocardium are described in this report in the hamster neonates born from mothers that received cocaine during an early stage of heart development. Comparisons will be made with the findings described in matching control atria and with previous studies. The effects of cocaine on adult heart and offspring were reported elsewhere (Gilloteaux and Dalbec, 1990) and reports about the cardiotoxic effect on other age groups are in preparation.

Material and Methods

Twelve (12) pregnant female (F1B strain, 11-week-old) Syrian hamsters (Mesocricetus auratus Waterhouse) sired by 15-week-old males; all were obtained from BioBreeders Co. (Watertown, MA). Kept one to a cage under constant temperature (20-21°C) and with rodent chow and water ad libitum in the Accredited Comparative Medicine facility of the College of Medicine, pregnant females were then randomly distributed into two groups of 6: in the first group each female received at the 6th, 7th, and 9th day of gestation i.p. injections of 0.5 ml buffered (phosphate, pH 7.0) saline containing cocaine-HCl (Sigma, St. Louis, MO) while the control, second group was injected i.p. by 0.5 ml buffered saline. These injections, respectively, contained 3, 5, and 7 mg of cocaine/100 gm body weight. After delivery neonates (1-day-old) were anesthetized by Na barbital (7 mg/100 gm b.w.) and hearts were excised. After rapid washing in saline containing 2 mM EGTA (to prevent blood coagulation), right and left atria were excised and were immersed in 2.5% glutaraldehyde solution buffered by 0.1 M Na cacodylate (pH 7.35) to avoid distension and other damages due to perfusion. Not more than 20 sec of delay occurred from the time of excision to immerse atria in fixative. There the atria were split in two halves. Following 30 min fixation at room temperature, all specimens were fixed during a 2 h period at 4°C and washed in the buffered solution, then postfixed 1-1.5 h in 2% aqueous OsO_4 solution. The specimens were again washed in the same buffer and processed for transmission (TEM) or scanning (SEM) electron microscopy. One-half of each specimen destined for TEM analysis was dehydrated through graded ethanols and propylene oxide before they were embedded in Polybed (Polysciences, Warrington, PA). One-micron-thick sections were stained by Toluidine blue and atrial appendage areas showing damages were sectioned. Ultrathin sections were collected on 100-mesh hexagonal copper grids (SPI, Inc., West Chester, PA.). The sections were contrasted by uranyl acetate and lead citrate and examined in a JEOL 100 S electron microscope. SEM was used to study the other halves of each specimen. SEM specimens were dehydrated in graded alcohols (30-100%). Critical-point drying was performed in a Polaron E 3000 apparatus (Polaron/Biorad, Cambridge, MA) using liquid CO_2 as transitional medium. All the SEM samples were coated with 250-275 Angstrom-thick gold layer before study.

Results

Litters from treated or control hamsters did not differ from each other in terms of number of stillbirths. The average litter size was in both cases 7 ± 3. However, treated offspring were usually born 1/2 to 1 day earlier than control hamsters without significant difference in body weight.

For ease of comparison between control and treated samples, data were grouped together on the first plate (consisting of Figs. 1 to 6, Figs. 1, 3 and 5 versus Figs. 2, 4 and 6) and second plate of Figs. 7 and 8 (Fig. 7 versus Fig. 8). Other ultrastructural observations from cocaine-treated atria (Figs. 9-18) are followed by a view of control atria (Fig. 19).

Light microscopy: (Figs. 1-2 and 9)

The newborn atria of cocaine-treated hamsters reveal a myocardial musculature with a 2-5 myocyte-thick wall with typical trabeculae similar to the control atria (Gilloteaux and Linz, 1989). A preliminary examination indicates that the atrial structure is completed as trabeculae are organized in the appendages, but the atrial myocytes appear less contrasted than in the controls; even though the section thickness were always 1 μm thick. The cocaine-treated atria show typical trabeculae covered by the endocardial endothelium but possess paler nuclei than in controls (Figs. 1 versus 2). Subendocardial regions show pale and large expanded myoplasmic evaginations with vacuoles. Frequent mural or intertrabecular thrombi are observed in these cocaine-treated atria (Fig. 9). The atrial myocytes of cocaine-treated hearts show less contrast than the control ones. They show less differentiated myocytes, with myofibrils still loosely organized in a vacuolated sarcoplasm surrounded by aggregated mitochondria. With toluidine blue these are usually more pale than control atria but not as swollen as could be expected from fixation artifact. In addition, the chamber walls of the main atrial chamber adjacent to the large vessels and the subepicardial regions, show focal to large, vacuolated damages (Fig. 9).

Ultrastructural Changes

SEM examination of the endocardial surfaces demonstrate endothelial cells with bulging endothelial cells in both control and treated atria. The trabecular endocardium shows that endothelial cells have a flat

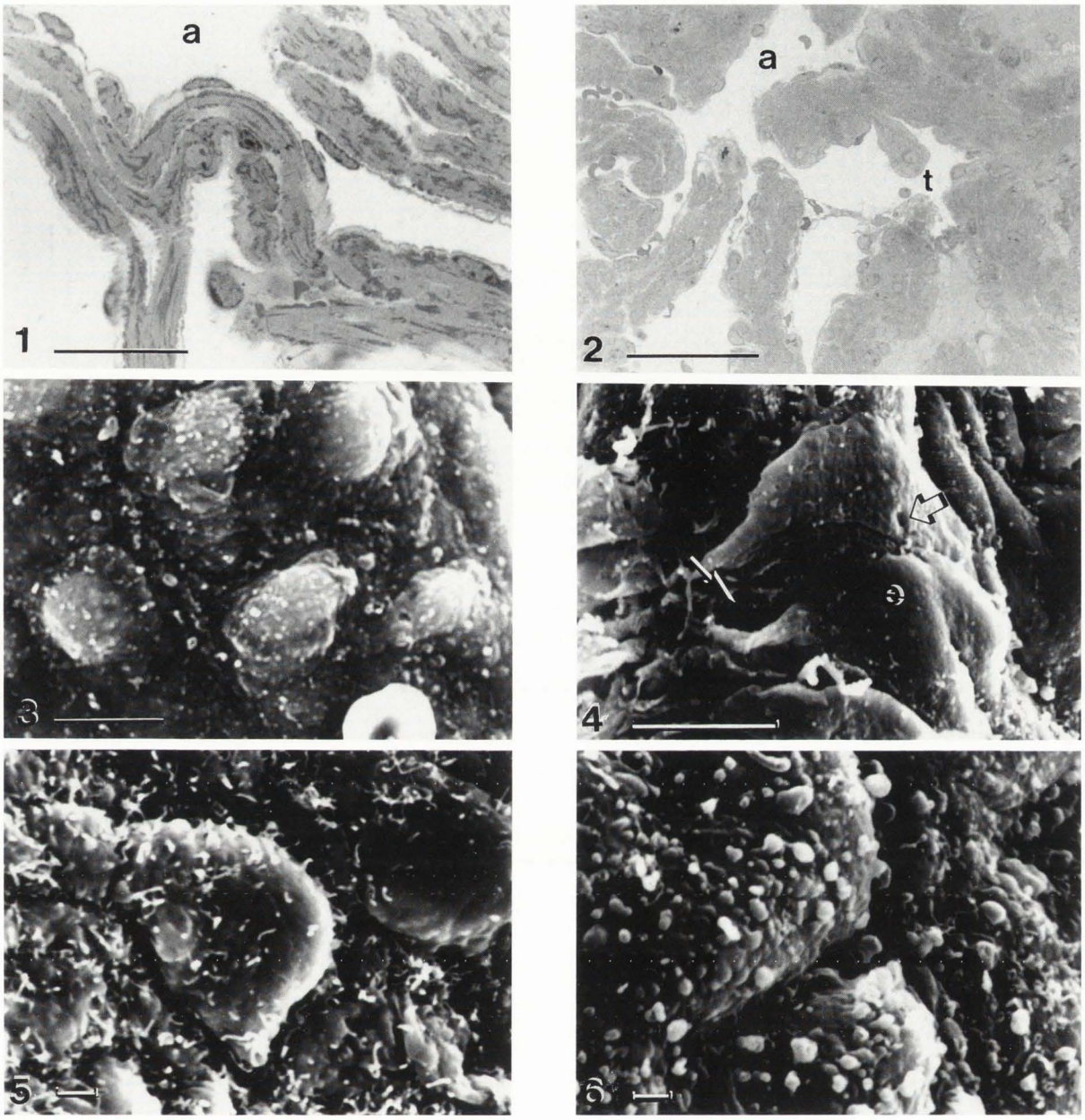
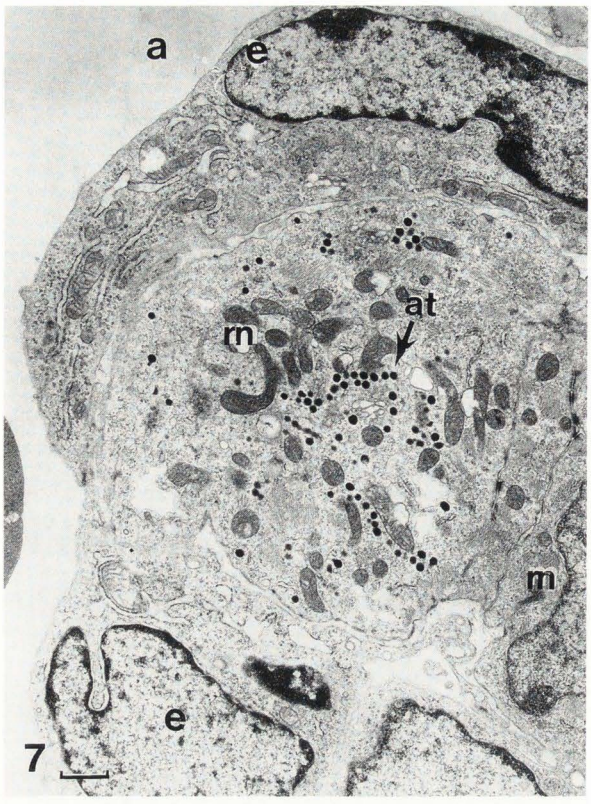
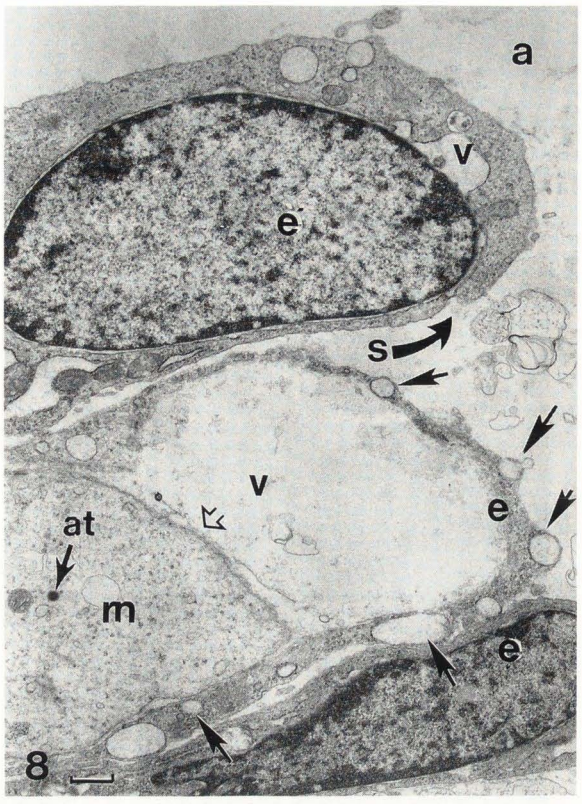


Figure 1: LM view of 1-μm-thick section from control right atrium showing trabeculae and a small part of the atrial chamber (a) delineated by endothelial endocardial cells. **Figure 2**: Comparable field of a cocaine-treated neonate right atrium. a: atrial chamber. Notice the loose coverage of atrial trabeculae and wall by the endocardial endothelium and the pale myocytes. Vacuolated and swollen myocytes can be detected as subendocardial damages; t: thrombus. Toluidine blue stained. Scale bars are 25 μm.

Control atria: **Figures 3 and 5**: **Fig. 3**: SEM view showing rounded, bulging profile of endocardial cells covering inter-trabecular and trabecular structures of the right atrium. Convex, apical surfaces suggestive of nuclear regions are covered by small projections and cell margins are detected by encircling blebs and/or projections in the groove-like spaces. Scale bar is 10 μm. **Fig. 5**: Enlarged view of a bulging apex covered by small projections and microvilli. Scale bar is 1 μm.

Cocaine-treated atria: **Figures 4 and 6**: From a cocaine-treated neonate right atrium. **Fig. 4**: Comparable field as shown in Fig. 3. Notice the more smooth surface of the endocardial endothelium (e) with perforation (opened arrow). The irregularly-shaped, narrow extensions create incomplete covering for subjacent myocardial cells (arrow). Scale bar is 10 μm. **Fig. 6**: Bulging apices of endocardial surfaces are covered by numerous small to large blebs. Scale bar is 1 μm.





Figures 7 and 8: TEM of control atria (**Fig. 7**) and atria of neonates from cocaine-treated mothers (**Fig. 8**).

Figure 7: Endothelial cells show prominent nucleus surrounded by rough endoplasmic reticulum and mitochondria. The cytoplasm, attenuated with peripheral extensions, covers subjacent myocytes containing numerous atrial granules (at) and incubated mitochondria. The primitive, subendocardial extracellular matrix is not clearly detected.

Figure 8: Damaged bulging endothelial (e) cells, covered by abundant membranous blebs extruded from the apical surfaces, as they originate from the cytosol (arrows). Vacuolated spaces (v) surround the nuclear envelope, and hyperchromatic nuclei are detected. a: atrial chamber; at: atrial granule; m: myocyte; s arrowed: sucker-like structure; opened arrow is directed toward damaged basal membrane of vacuolated endothelial cell. Scale bars are 1 μm.

Figures 9 to 12 (on the facing page): Views of 1-day-old atrial structures from transplacentally cocaine-treated hamster: **Fig. 9**: LM view of the main right atrium wall (W) and trabeculae (T). Endocardial damages (e arrowed) and subendocardial swellings and injuries can be detected by their vacuolated appearance; this includes the myocytes where large intracellular vacuoles can be found as well as an endothelialized thrombus (t). Scale bar is 10 μm. **Fig. 10**: Small and large marginal blebs on the surface of endothelial cells. Scale bar is 1 μm. **Fig. 11**: Small area of thrombus (t); a: atrial chamber; e: endothelium; m: myocyte. Scale bar is 10 μm. **Fig. 12**: TEM view of damaged endocardial region. Notice flattened endothelial cells (e), vacuolated and pyknotic, and eventually sloughed away from the endothelium. a: atrial chamber; at and unmarked arrows: atrial granule; f: fibrocyte; m: myocyte; se: subendocardial space; v: blebs and vacuoles originating from endothelial cells; opened arrows are directed toward membrane or endothelial discontinuities. Scale bar is 10 μm.

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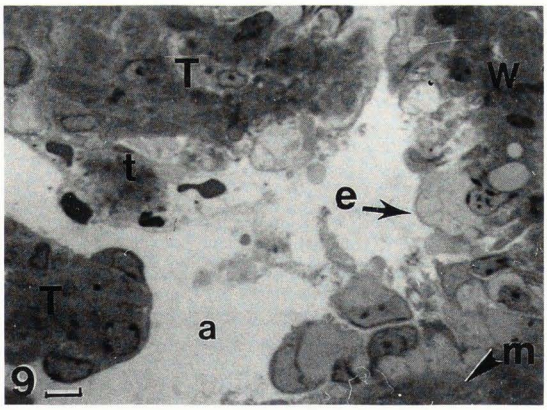
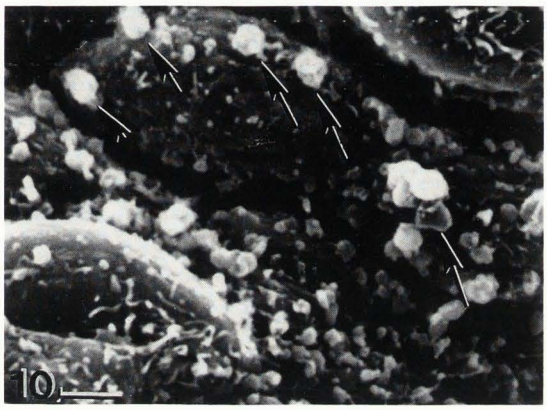
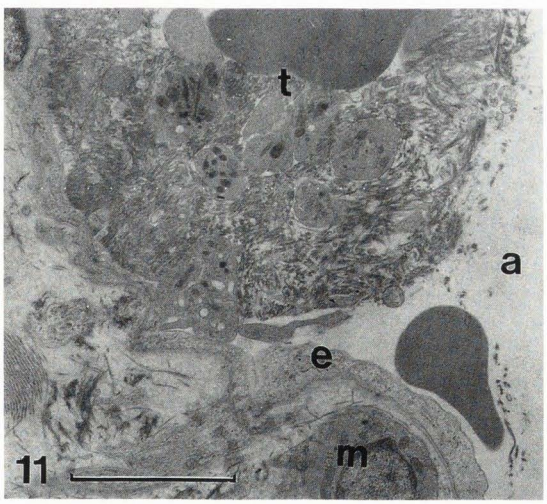
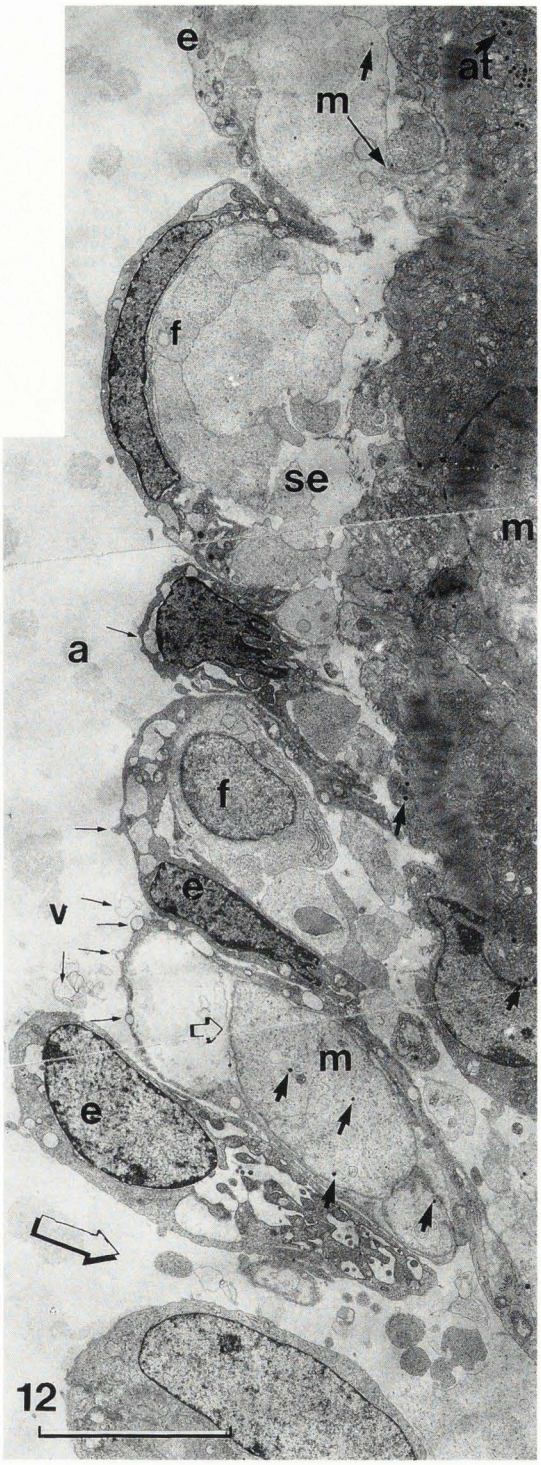
cytoplasm with typical marginal folds (Fig. 3). In control endocardial endothelium the bulging nuclear region is usually smooth or covered by small blebs (less than 0.25 μm) and scattered microvilli (about 1 μm in length). These microvilli are usually displayed on the flattened, peripheral protoplasmic surfaces (Figs. 3 and 5). In contrast, in cocaine-treated atria of neonates, many areas of trabeculae show the same cells with a smoother surface perforated by depressions and with suggestive discontinuities between their extended, trailing cytoplasm extensions and the subjacent myocardium (Fig. 4). The endocardial endothelium often demonstrate abundant blebs of heterogenous size which can attain 0.5 to 3.0 μm in diameter. Blebs are displayed as



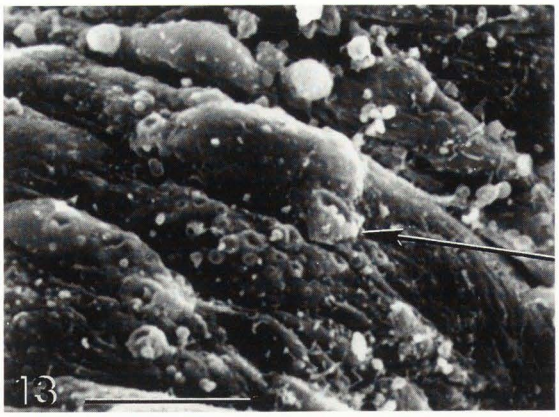


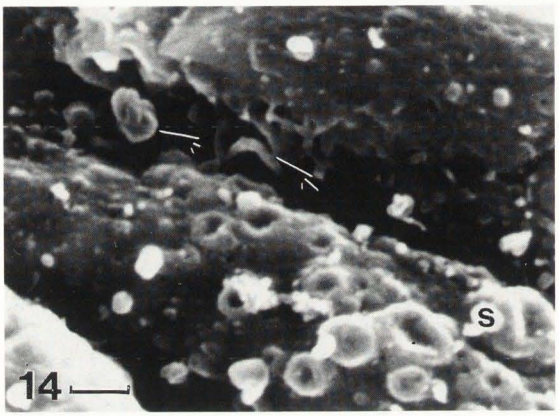


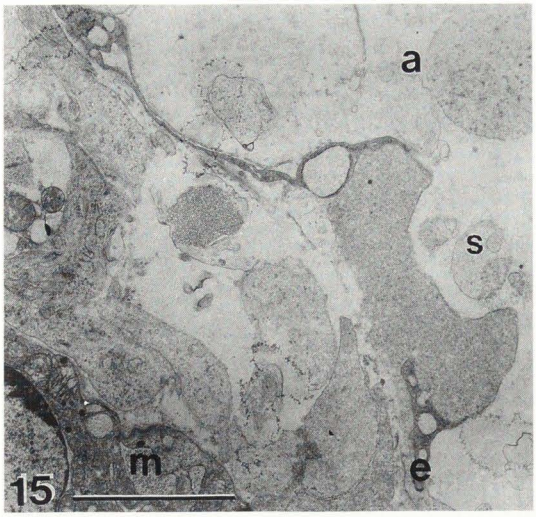


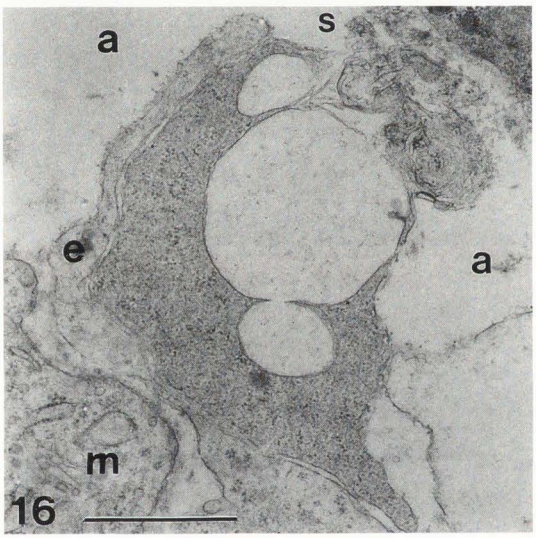
somewhat spherical to oblong profiles (Figs. 6, 10, 13, 14). The abundant blebbing is not necessarily noticed throughout the atrial luminal surfaces but is detected in large areas of the endocardium. Membranous and cytoplasmic expulsions could form crater- (Fig. 13; 0.4-1.5 μm in diam.) or sucker-like structures (Fig. 13; 1.0-1.7 μm in diam.) which are noticed on some apical surfaces of elongated endothelial cells of the appendicular trabeculae as if material had already been expelled from these cells and has left these peculiar surface damages.

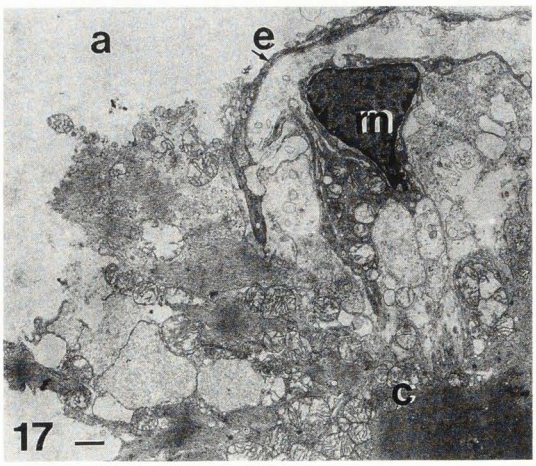
TEM aspects of comparable fields of observation detected by light and scanning microscopic studies confirm the described endothelial morphology and damage and the morphology of the adjacent subendocardial, myocardial cells.









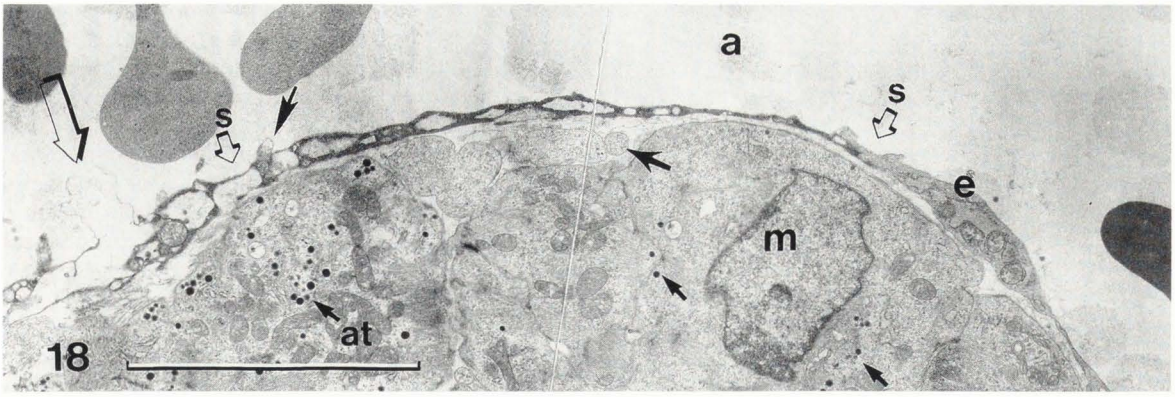


Figures 13-17: Peculiar membrane damages in cocaine-treated atria. Scale bar in 13 is 10 μm; scale bars in 14-17 are 1 μm.

Figs. 13-14: SEM aspects of blebs (arrows) and in some areas, sucker- or crater-shaped damages (s) are detected (long arrow).

Figs. 15-17: TEM aspects of endothelial perforation (15) extrusion of material from (16) and pycnosis (17) of adjacent myocytes (m). a: atrial chamber; c: contraction band; e: endothelium or endothelial cell; s: sucker-like structure produced by endothelial damage.

The typical bulging and flat endothelial surfaces detected in normal, untreated atria (e.g., Fig. 7, and in Gilloteaux and Linz, 1989) are replaced by areas of elevated or accordion-like folds covered by flattened and damaged endothelial cells showing minor to marked perinuclear and cytoplasmic vacuolizations and nuclear pyknosis (Fig. 8). Endothelial cells are also abutted by adjacent expanded cytoplasm of swollen fibroblasts/fibrocytes. The fibrocytes sometimes appear intercalated between the endothelial cells and the swollen, damaged myocardial cells (Figs. 8 and 12). The subendocardial, myocytic swellings can be recognized by the presence of atrial granules. Their cytoplasm is more pale than that of damaged fibroblasts which are eventually interposed between the endothelial cells and the



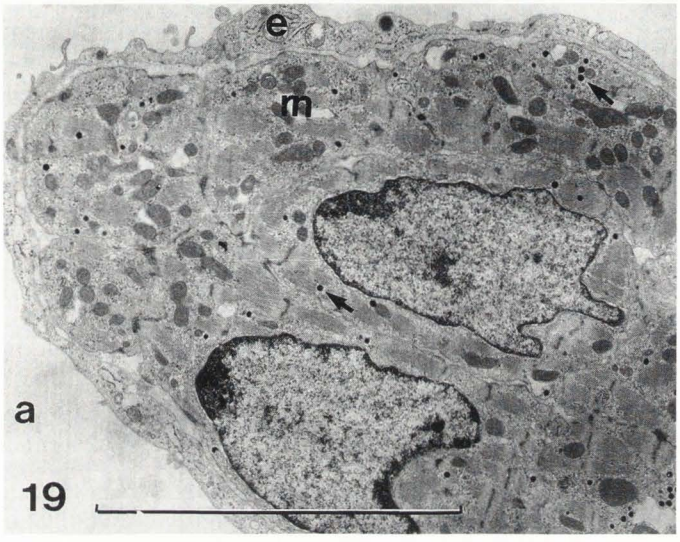


Figure 18: TEM view of trabecular structure from cocaine-treated neonates complementing Figs. 8 and 12 showing endocardial cells vacuolizations and membrane expulsion. Cytoplasmic electron-density is increased whereas cytosol and membranous material are wasted (arrows) creating a very thin endocardial endothelial barrier. Crater-like or sucker-shaped profiles (s opened arrows) can be detected. Notice an intact endothelial cell (e) on the right. at or arrows: atrial granule; m: myocyte. Scale bar is 10 μm.

Figure 19: Atrial trabecular structure from untreated neonate show the narrow endocardial endothelial (e) barrier between the atrial chamber (a) and the myocytes (m) without vacuolization or damage. Scale bar is 10 μm.

myocytes (Figs. 12 and 15). Membranous blebs (0.5-2 μm), oozing from most of the endothelial cells were shown (Figs. 8, 12, 15-18) and suggestive stages of expulsion of rimmed vesicles can be shown (Figs. 8, 12). Their size is comparable to those found in SEM illustrations (Figs. 6, 10, 13, and 14). Expulsion of material from these endothelia could create depressions or crater-like aspects at the surface of the endocardial endothelial cells. This series of events can be reconstituted by choosing a sequence of micrographs 15 to 17. A section through a sucker- or crater-like structure is observed in Figure 15. There the thinned, endothelial cell extensions are pushed away from the basal lamina and are retaining a flocculent material. The overall aspect in profile suggests similar structures as found in Figures 13 and 14. Figure 16 illustrates a further damage of the broken endothelial lining exuding the flocculent material into the atrial chamber. Figure 17 illustrates the ultimate damage in which the interrupted endothelial "barrier" is incapable of containing the growing myocytes. These myocytes, incompletely covered by a physiological and protective endothelial layer, are locally swollen and are extruding some myofibrillar material, mitochondria and other sarcoplasmic content into the atrial chamber. These events can be observed in many areas of the wall and trabecular apical epithelial surfaces. In many areas of the atrial appendages, the endocardial endothelium is thinned by abundant blebs expelling membrane components to the circulation and can be adjacent to normal endothelial cells (Fig. 18). Adjacent to these damaged areas the atrial granules contained in the myocytes are more numerous than in myocytes facing intact endocardial endothelium (Fig. 18). Control atria always demonstrate a continuous, intact endothelial layer (Fig. 19 and Gilloteaux and Linz, 1989 and 1990).

Considering these endothelial cell damages and dramatic dissections of the endothelial lining it is not surprising to detect many thrombi (Figs. 9 and 11) in the atria of neonates from cocaine-treated mothers. In many places along the atrial endothelium one can detect fibrin deposits, even in the 1-μm-thick sections (Fig. 9). These are obviously suggestive sites of forming clots which could have been detached during the procedures of tissue collection and fixation. These are detected by light and ultrastructural surveys (Figs. 2 and 9).

Subendocardial myocytes can show contracting bands, sarcoplasmic vacuolizations and mitochondrial aggregates. Where there is extensive damage to the endothelial cells (e.g., Fig. 17), pyknotic nuclei of

irreversibly injured myocytes can be found. A poorly developed collagenic matrix is filling the subendocardial spaces. Remarkably, the atrial granules appear well preserved throughout the damaged myocytes; they can be used as markers to delineate these atrial myocytes.

Discussion

Cardiac lesions in hamster neonates, including atrial ischemia and thrombi are exceptional anomalies that are usually found in adult or old hamster hearts (McMartin and Dodds, 1982) or in cardiomyopathic hamsters (Gilloteaux, 1990; Kondolios et al., 1989). Only a small number of reports have described abnormal cardiorespiratory patterns (Bush, 1988; Karch and Billingham, 1988; Cregler and Mark, 1985; Wang, et al., 1988) and coronary (Zimmerman et al., 1987) histopathologic damages following cocaine abuse. The atrial damages observed in this age group are similar to those observed in some adult human pathologies, including the subendocardial damages described in several forensic and histopathological reports comparable to ischemia and acute myocardial infarction obtained after coronary atherosclerosis (Mittleman and Wetli, 1984; Peng et al., 1989; Tazelaar et al., 1987; Wang et al., 1988).

Many of the structural changes are similar to those found following myocardial infarction (crater-like structures: Gertz et al., 1981), cocaine-induced cardiotoxicity in vitro (myocytic vacuolizations: Welder et al., 1988), septic shock (blebbing endothelial cells and thinning: Pretorius et al., 1987). Global ischemia of the rat heart produces blebs and cocaine is able to induce congenital vascular defects (Webster and Brown-Woodman, 1990). Three to four hours of ischemia produces endocardial craters, sloughing and subendocardial vacuolization of myocytes (Carter and Gavin, 1986) similar to the endothelial alterations we have detected. In many instances the cellular ultrastructure of the endocardial and subendocardial myocytes typified acute (hydropic swelling) and persistent (definite cell damages) cell injury suggestive of increased permeability of the plasma membrane to sodium. In addition, endothelial vacuolizations and expulsion resemble rimmed vacuoles observed during endocardial development (Markwald et al., 1975). Finally, control atria do not show the changes observed in the cocaine-treated hamsters. Gilloteaux and Linz (1989) have already discussed the surface specializations, including the blebs found in fetuses and neonates atrial endothelia. It is likely that the endocardial endothelium, rendered tenuous by excessive losses in membrane, can easily be further injured. Because of their size and randomness the blebs observed in cocaine-treated offspring resemble the cytosegresomes described and discussed by Pexieder (1981). Pexieder suggests that there are signs of physiological cell death in and beneath the endocardial endothelium. Exfoliation or damage of endothelial cells would trigger interaction of prothrombin with basal membranes and, with other elements, then trigger the cascade events favoring blood clotting and thrombi formation (Osterud, 1986). Thrombi could also develop from these thinned endothelial regions that would be easily damaged by mechanical stresses occurring during hypertrophic cardiac growth, during the last fetal week and the first postnatal week. These events and a focal defect of endothelium could also create an abnormal physiological milieu for the atrial myocardium which then would be ischemically damaged, especially in the neonatal atria, as these regions are not yet supplied by their own vascular beds. In our report, the vasoconstrictive action of cocaine on placental blood supply (Mahalik et al., 1984; Sherman and Gautieri, 1972; Woods and Plessinger, 1990; Woods et al., 1987) are additional factors that could have created ischemic changes in susceptible, organizing endocardial and myocardial tissues. In fact, the overall, pale nuclei and cytoplasm observed at birth suggest immaturity of atrial structure. In the human adult, cocaine abuse can be associated with coronary spasm, mural thrombi and probably emboli carried to vital organs. In addition, endocardial and subendocardial damages of adult hearts could be affected directly or indirectly via the sympathetic actions on the subjacent myocardial cells. In fact, cocaine is cardiotoxic by acting as an anesthetic but also as an arrhythmic agent by perturbing the action potential of myocytes, by decreasing Purkinje automaticity and promoting K^+ efflux and Na^+ entry by overloading cells with Ca^{2+} (Billman, 1990).

Our observations suggest that the Syrian hamster can be used as a model to study transplacental cocaine-induced damages in the cardiovascular system. Cocaine toxic effects are certainly more pronounced on fetal tissues since drug detoxification is decreased during pregnancy (Neale and Parke, 1973). Moreover, the highest level of the drug has been detected in the placenta (DeVane et al., 1989) and it is possible that the harmful effects of the drug would favor defects according to the relative critical timing and sensitivity of the organizing tissues. It is likely that the developing heart will be more affected than others due to its depressed incoming blood supply. In support of this contention Woods and Plessinger (1990) showed that the cardiovascular response to cocaine is greater in pregnant than non-pregnant ewe. Results of these authors and others indicated that cocaine abuse increased risk for offspring cocaine-related cardiac or vascular complications due to the vasoconstrictive properties of cocaine. In addition, cocaine or its metabolite benzoylecgonine are amphipathic. This property should facilitate intracellular Ca^{2+} overload to trigger mitochondrial poisoning worsened by ischemic events and to affect Na^+/K^+ channels and favor the arrhythmic effects of the drug (Billman, 1990). Normal pregnancy is characterized by a hypercoagulable state (Crowley, 1989) which can be exacerbated following cocaine abuse (e.g., Howard et al., 1985) as one can detect atrial thrombi. Further developmental study have shown that only vacuolization of endocardial endothelium and of subendocardial

cardiomyocytes are detected if treatment was started by day 11 of development (Gilloteaux, 1990). Similar damages of endothelial cells were detected previously by others as vascular lesions in intestinal ischemia (Garfia et al., 1990) using in vitro cytotoxicity tests (Welder et al., 1988) or isolated rat hearts (Vitullo et al., 1989) and aging studies are under way in our laboratory to evaluate the survival rate of these offspring.

From our observations, we hypothesize that cocaine (or its metabolite benzoylecgonine) directly or indirectly causes damages in Syrian hamster fetuses by depleting the normal blood supply to the placenta resulting in defects of the endothelial lining by damaging the endothelial lining of the endocardium of the developing heart. These alterations of the endothelial barrier are detected as endothelial vacuoles and contraction bands of the myocardial cells. Increased sympathetic tone and high levels of circulating catecholamines may have contributed to affect also the atrial endocardial endothelium (Rosenblum et al., 1965) and the myocardial contractility (Pilati et al., 1990) in the adult heart. Similar endothelial defects were observed in vitro by Vitullo et al. (1989). In addition, the observed endocardial damages resemble the injuries caused by 5-HT on the endocardial epithelial layer of papillary muscles in the ventricles (Shah et al., 1989).

Other studies have already shown that the integrity of the endothelial cells is *sine qua non* condition to prevent a vasospastic response to platelet products (Van Houtte, 1989). This report provides another argument in favor of the complexity of functions already assigned to the endothelial cells (Fajardo, 1989) which can be affected by cocaine abuse.

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Discussion with Reviewers

W.H. Wilborn: Please name other components of the cardiovascular system of the fetal hamsters used for the present study where you observed damage caused by cocaine during pregnancy.

Authors: Hemorrhages were detected in the atrial chambers (6/47) and in the heart apex of 3/47 neonates. Lung ultrastructure showed immature Clara cells and type II pneumocytes (2 neonates only were studied). Lung alveoli displayed edema and hemorrhages in a small number of surviving hamster pups but the study about these tissues awaits further funding support.

W.H. Wilborn: What evidence do you have that the passage of material in the "membranous blebs" is only in the direction of the atrial cavities? Is it not possible that the passage of material is bi-directional or in the direction opposite to that you proposed?

Authors: Passage of material can be bi-directional as well as in the direction opposite to what we proposed. However, we have observed that apparently intact endothelial cells show developed Golgi apparatus (Fig. 20). Numerous membranous blebs appear to be produced at the level of these Golgi saccules in the form of small vesicles and many other micrographs suggest that their fusion contribute to the formation of large sized vesicles. Another illustration (Fig. 21a,b) show that membranes can be recycled via clathrin-coated vesicles in endocardial epithelium even though these cells are undergoing vacuolization necrosis.

W.H. Wilborn: Did the authors try other fixatives and buffers? If so, did they obtain the same or comparable results.

Authors: No, we did not try to use other fixatives or buffers.

T. Pexieder: What is pregnancy duration in hamster?

Authors: In the Syrian hamster, F1B strain, pregnancy is 18 to 18.5 days long in our conditions of 14 h light and 10 h dark cycle.

T. Pexieder: Do you believe that it is appropriate in your discussion to compare the effects of cocaine on the adult heart (more less instant) with the changes in the neonate (after 7 days of delay)? The delay between the treatment (6th, 7th and 9th day of gestation) and the neonatal period (7-10 days) is too long to make a direct cocaine effect on the atria plausible. Unless you examine the embryonic hearts 24 to 72 hours after cocaine administration you cannot differentiate a potential direct effect of cocaine from indirect, placenta or general fetotoxicity mediated changes. Please comment.

Authors: We fully agree with Dr. Pexieder. However, both fetal and neonate mammalian heart tissues are probably damaged as changes associated with ischemia are detected postnatally. As a result of our treatment, mothers' hearts also show ischemic myocardial defects, including thrombi (not reported in this manuscript). In addition, other damages could have been produced during fetal development and already repaired in neonates.

T. Pexieder: How can you tell the ultrastructural

changes are due to direct cocaine effect and are not simply consequences of circulation failing for any other reasons such as e.g., placental insufficiency?

Authors: As stated above, it is possible that cardiovascular insufficiency of the pregnant mothers and/or of the fetuses could provide cardiac defects. These damages and those observed in other organs show these offspring to be premature (lung with edema and poor differentiation of pneumocytes II, for example [not submitted in this report]).

T. Pexieder: As we have demonstrated in 1981 discontinuity in the atrium is a normal phenomenon in prenatal cardiac development. How did you differentiate the "cocaine-induced" dehiscences from the physiological ones?

Authors: Dehiscences in the hamster atria are not detected after the 14th embryonic day (Gilloteaux, 1989). These dehiscences were all by damaged and sloughed endothelial cells and damages were then covered by thrombi.

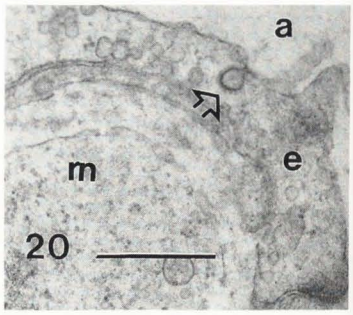
T. Pexieder: Within the same heart the endocardial morphology, as seen in SEM, may vary largely, as we have shown in 1981. How did you sample the atrial wall? How many SEM micrographs did you take from each heart? How many hearts were studied in TEM and how many with SEM? How was the tissue sampling for thin sectioning organized?

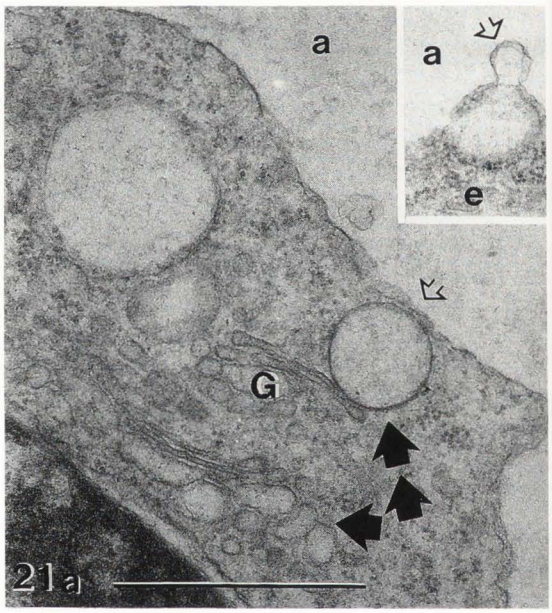
Authors: Twelve half neonate right atria from each litter were fixed, and 5 atria were cross-sectioned perpendicularly to the heart long axis and 1-μm thick sections were studied under light microscopy for the presence of anomalies, including thrombi. Thrombi were often detected in the appendage region and on the endocardium, at the branching sites of trabeculae; these sites were studied by TEM (at least 50 micrographs were taken per atria). A similar number of atria were studied by SEM after the atria were split open and each half examined; at least 10-low magnification field micrographs were taken per atria. Samplings appear

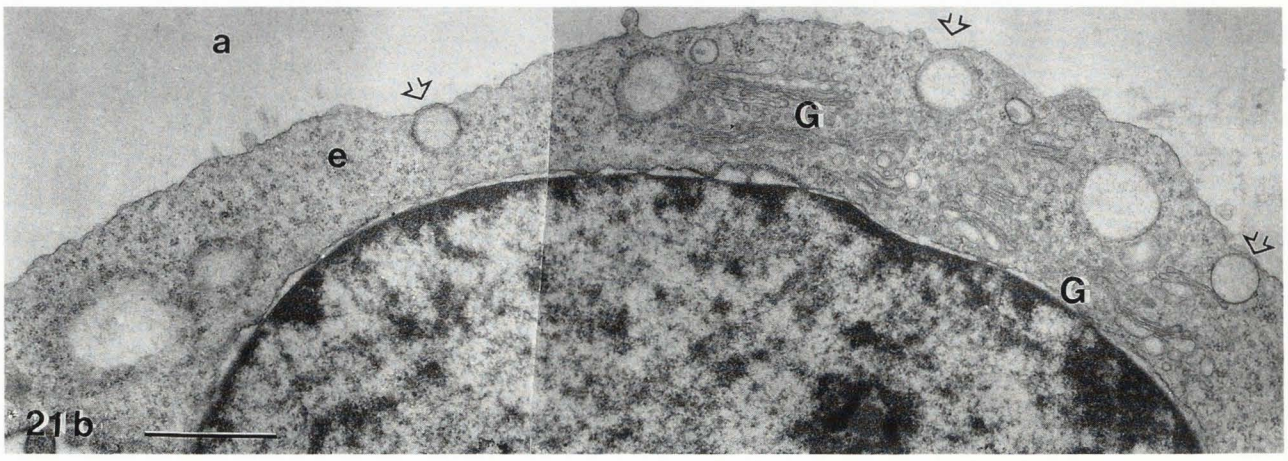
Figures 20 and 21: One-day-old atria of transplacentally cocaine-treated hamster. a: atrial chamber; m: myocyte. Scales are 1 μm.

Figure 20: Recycling by endocytotic-coated vesicle (opened arrow) of membrane into endocardial endothelial cell (e).

Figures 21 a-b: Blebs and vacuoles (opened arrows) formed by the prominent Golgi complex (G) reaching (thick arrows) the epithelial surface and exocytosed like air bubbles (**Fig. 21a** insert) from the endocardial endothelial cells (e).







sufficient according to Jakstys B.P. (Artifacts in Sampling Specimens for Biological Electron Microscopy. In: Biological Electron Microscopy, R.F.E. Crang, K.L. Klomparens, eds., Plenum Press, New York, 1988, pp. 6-7).

J.N. Skepper: Was the administration of three injections of cocaine intended to mimic dependency, or acute cocaine toxicity during a specified period of pregnancy and how does this model fit in with existing human studies?

Authors: This study was intended to show the transplacental toxic effect of cocaine on developing heart structures. In this case, study is limited to the atrium, although other organs are affected and their study is in progress. As the title indicated, this report is intended not to mimic dependency, but to verify if cocaine abuse during the early developmental stage of fetal organization of the heart can leave sequelae of this abuse in Syrian hamster neonates. Other results originating from our laboratory are submitted elsewhere and deal with cocaine abuse during late development and in mothers. In all cases, cardiac defects resemble observations described in a small number of forensic reports, including a recent report of Rezhalla et al. (Rezhalla, S.M., Hale, S. and Kloner, R.A., Cocaine-induced heart diseases, Am. Heart J. 120: 1403-1408, 1991).

J.N. Skepper: Do the authors feel that the existing data could be improved by the use of perfusion fixation and lipid cytochemistry?

Authors: Immersion fixation is usually inferior to perfusion fixation but in this case the study of a thin layer of cells and tissues can be adequately fixed by immersion as demonstrated by control illustrations from atria fixed simultaneously and as discussed in a previous publication (Gilloteaux and Linz, 1989). It is also possible that cocaine (or benzoylecgonine), via its anesthetic and membrane poisoning effects (Billman, 1990), modifies the membrane fluidity and changes membrane permeability by facilitating cell swelling and further membranous damages. In this study we have elected to use an immersion fixation technique to examine the endocardium. This technique has also been used by other researchers (cfr. several references critically cited in Pexieder, 1981; Morse, 1978). High quality preservation of large samples may be obtained through perfusion (Hayat, 1981; Roberts et al., 1990). Immersion fixation was used for several reasons: (a) we are fixing within a short delay of dissection at room temperature; (b) we are investigating a thin layer of cells directly in contact with the fixative, which is slightly hypertonic. Moreover, (c) the atrial wall of neonate hamsters is comprised between 25 and 70 μm in thickness; no blood supply is present in the atrial walls and the trabeculae are not more than 15 μm in width (Figs. 1 and 2 and Gilloteaux and Linz, 1989 [Figs. 7-15]) and (d) the fixing solution is in contact with both the epi- and endocardial sides. Figure 7 versus Figs. 8 and 19 represent only a sample of numerous illustrations collected and demonstrate that immersion fixation provides interesting data to report. In addition, damaged endothelial cells neighbor intact cells in treated pups (Fig. 18). This indicates that only some cells are damaged but not others even though they were fixed simultaneously. Membrane lipid and lipid cytochemistry could be studied but the purpose of this work was to investigate if topographical damages could be detected in neonatal atria. Histochemistry studies will be realized in the near future as soon as we have access to supplemental research funds.