rial and by the complexity of its processing and storage. However, these limitations should not be the determining factors in such situations as insufficient supply of donor skin, treatment of patients suffering from polyvalent allergy, elderly and senile patients with severe somatic pathology hampering autodermal plasty, treatment of children, and cases of incurable infections. This study does not deal with the mechanisms underlying the reparative effects of human fetal tissues on deep burns. We hope that the data collected during our further observations and the use of immunological and histological methods will allow us to characterize the positive effect of human fetal tissues on burn healing and the whole body.

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Opioid Peptides Improve Human Fetal Nervous Tissue Survival after Cryopreservation in Culture

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The study was carried out on the 18-20-week human fetal brain by culturing organotypic and dissociated cells of the cerebral cortex and the corpora quadrigemina area. Tissue viability was assessed from the formation of growth cones and neuroglial bundles, as well as of glial cell formation. The study showed that after tissue freezing in organotypic cultures, viable cells were detected in not more than 10% of grafts. The addition of opioid peptides noticeably (by 1.3-2 times) increased the number of surviving cells. This regularity was not observed in dissociated cultures.

Key Words: *nervous tissue culture; human fetus; cryopreservation; opioid peptides*

Problems of fetal tissue transplantations for therapeutic purposes are no longer an object solely of basic research, but a procedure attracting the most active attention of clinicians. Human fetal nervous tissue transplantations are now quite often resorted to in some neurological diseases. Obviously, both the potentials and efficacy of this method when used clinically depend on a number of factors, and primarily on the choice of the optimal method for donor tissue preservation to maximally retain its viability. According to previous reports, freezing and storage of the material in liquid nitrogen is the most convenient and effective method [10,13]. This technique ensures a high share of surviving cells, whose value, however, varies within a wide range and depends on the procedure used [5].

We have developed in the last two years and are implementing at present a research program aimed at the creation of a bank of frozen fetal tissues; this program has helped introduce fetal

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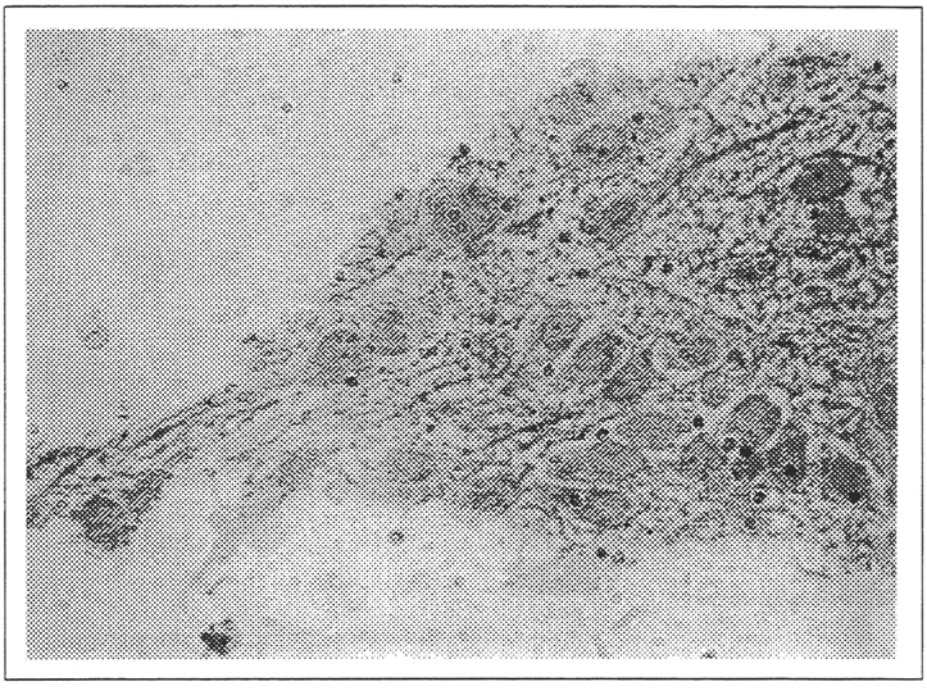

Fig. 1. Organotypic culture of human fetal brain tissue from corpora quadrigemina. Neuritoglial bundles and migration of cells along periphery of explant. Cellular elements with clearly defined structure of nucleus and perikaryon. Staining with hematoxylin eosin. ×500.

transplantation methods into clinical practice. It goes without saying that minimization of the traumatic effect of cryopreservation on donor tissue becomes one of the principal prerequisites of a high efficacy of this method. Use of standard nonspecific cryoprotectors for this purpose may be, on the one hand, limited under clinical conditions, but, on the other, supplemented with or replaced by neurotropic agents selectively affecting nervous tissue development and regeneration. At present we know quite a number of agents stimulating growth processes in nervous tissue: fibroblast growth factor [14], epidermal growth factor [9], etc. Of special interest is the large class of opioid peptides and dalargin, a synthetic analog of leu-enkephalin, whose neuroprotective properties were demonstrated by M. V. Kozlova *et al.* They showed that some opioid peptides and dalargin appreciably improved neuron survival rate and adhesive properties in a culture [3,4]. A study of the possibility of using dalargin and leu-enkephalin as agents conducive to the recovery of nervous tissue cell viability after tissue freezing in liquid nitrogen was the purpose of this research.

MATERIALS AND METHODS

Experiments were carried out with the 18-20-week human fetal brain. The samples were taken from the upper corpora quadrigemina area and frontal lobes of the cortex under sterile conditions within 30 min postpartum. Experiments were made with organotypic and dissociated cultures. Leu-enkephalin (Sigma) and dalargin (tyr-D-Ala-Gly-Phe-Leu-Arg) were used in a concentration of 10×10^{-9} M. A brain cell suspension was prepared as described previously [2]. The brain samples obtained were put in Hanks solution with antibiotics. A portion of material was sealed in polypropylene capsules and immediately frozen in liquid nitrogen. After an hour the tissue was thawed at room temperature, the brain membranes were removed, and the samples washed in phosphate buffered saline. Each sample was cut into small fragments. For the preparation of dissociated cultures the fragments were put in 0.25% trypsin solution (Serva) in a CO_2 incubator at 37°C for 15 min. The trypsin solution was discarded and the tissue washed several times with culture media. The brain fragments were shaken in a tube. The supernatant of the resultant suspension was used for culturing. The cell suspension and tissue fragments were inoculated in 6- and 24-well plates on glass covered with polylysine (Serva) or collagen (Serva) at 37°C in ambient air with 5% CO_2. The cells were grown in a medium of the following composition: 50 ml MEM medium (Flow), 30 ml fetal calf serum (Flow), 11.5 ml Hanks saline (Flow), 5 ml of a 20% glucose solution, 0.5 ml insulin, 40 U/ml, 5000 U/ml penicillin (Flow), 5000 mg/ml streptomycin (Flow), and 1 mmole of glutamine (200 mmole). Opioid peptides were added to the growth medium simultaneously with cell and tissue inoculation into the culture and then every 3-4 days during every change of medium in a concentration of 10^{-9} M. For control a culture of fro-

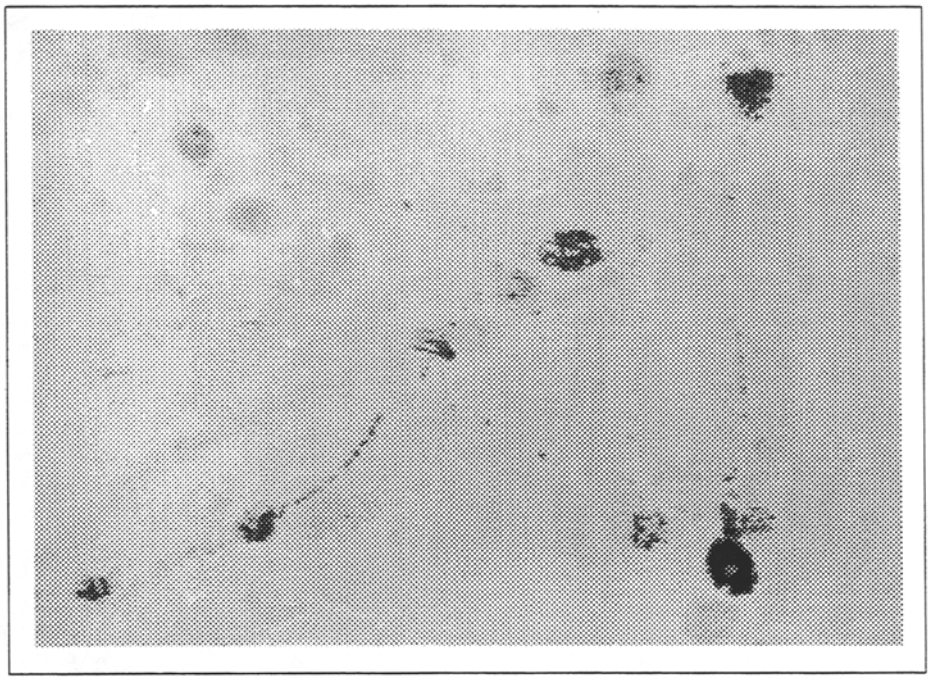

Fig. 2. Dissociated culture of human fetal brain tissue from corpora quadrigemina. Growth cones and formation of neurites by individual cells (5 days in culture). Life–time microscopy. ×300.

zen tissue from similar brain portions grown in a medium without opioid peptides was examined. The cells were cultured for 15 days. The growth pattern and status of the cultures were assessed in live cultures. The cultures were then fixed with paraformaldehyde in 0.1 M phosphate buffer, pH 7.2, and postfixed in osmic acid solution, detached from slides with liquid nitrogen, and embedded in araldite. Semithin slices were stained with hematoxylin eosin.

RESULTS

Development of intact explants of both brain areas in the control was standard for the said culturing conditions: the primary formation of a growth area at the expense of glial cells and fibroblasts was replaced by the formation of radially directed neuritoglial bundles. During the second week of culturing the majority (up to 95-100%) of cells were concentrated along the periphery of fragments and had a uniformly oval shape with poles of neurite growth cones in neurons (Fig. 1). In dissociated cultures some cultured cells formed during the first week neuroglial aggregations surrounded by neuroglial bundles. Growth cones of the rest of the neurons were scarce as well (Fig. 2). Freezing of tissue samples from both brain areas noticeably decreased the degree of morphological expression of neuronal viability during 2 weeks of their culturing. In 10% of fragments of organotypic cultures of corpora quadrigemina tissue signs of formation of neuroglial processes were detected. At the same time, in at least 35% of these fragments rather well-expressed dissemination of glial cells from some sites of the surface beyond the explant was observed. Photooptic examination of the cultures demonstrated as a rule disaggregation of fragments, partial or complete destruction of neuropil structures, and an intact perikaryon in some neurons which, however, did not form growth cones. Cerebrocortical explants demonstrated a much lower capacity to survive freezing. No neuroglial bundles were detected here, and glial growth was observed in not more than 10% of fragments. In dissociated cultures of both brain hemispheres no signs of cellular viability were detected. Culturing of frozen tissue fragments in medium with leu-enkephalin and dalargin noticeably increased the share of surviving neurons. Signs of neuroglial bundle formation were detected in 3-7% of cortical fragments and in 16-20% of corpora quadrigemina fragments. Photooptically the majority of cellular elements underwent changes similar to those observed in the control, but some cells retained a regular or unipolar shape of the perikaryon. No significant differences in the effects of opioid peptides were seen. In dissociated cultures peptide effects on the brain cell survival rate were negligible as well, although in cultures of both sites individual astrocytes were seen which formed a well-developed dendritic tree and contained large osmiophilic lysosome-like inclusions. These data on the search for optimal methods for nervous tissue recovery after cryopreservation reflect the results of one of the initial stages in our stud-

ies of this problem. Relevant studies of many authors are mainly devoted to the possibility and efficacy of transplantation of some frozen tissues, assessment of the share of intact cells by a number of morphohistochemical tests, and effects of substances of some groups on nervous tissue viability in a culture under various conditions [6-8,11,12]. On the basis of these data, one may speak of a rather low count of brain cells which survived freezing in our experiments. One of the possible explanations for this is the rather long period of culturing, after which not all the surviving cells met the stringent viability criteria we selected, but only the cells capable of active repair processes over the course of a long period. The neuroprotector properties of opioid peptides are sufficiently well known [1]. Our data indicate that these agents may be effectively used to reduce the negative aftereffects of freezing on intact glial cells and neurons. It is still unclear what specific degree of cellular damage permits the cells to realize the neuroprotector properties of these agents; whether it is possible to use them for this purpose when preparing cultures for cryopreservation; and how the administration of these agents with donor tissue may influence neurotransplantation efficacy. Obviously, solving these problems will help assess the efficacy of opioid peptides as cryoprotectors. On the whole, the results indicate that under conditions of organotypic culturing leu-enkephalin derivatives effectively stimulate repair processes in human fetal brain tissue cells after deep freezing.

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