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Human Embryo Neuronal Culture *in Vitro*: A Model to Study Cellular Physiology, Receptors, Power and Toxicity of Cytostatic Drugs for Human Use

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ABSTRACT

Neural cells cultures from human embryo brain of 9˚ - 11˚W gestational age have been used to study ER*α* (Estrogens Receptor *α*) and to perform toxicity test for Mitomycin C and Methotrexate. Histochemical confirmation of cellular neuronal phenotype was based on histochemical evidence of NSE (Neuron Specific Enolase).The detection of ER*α* in neuronal cells was performed with a rabbit Monoclonal Antibody. ER*α* was absent both on neurons grown *in vitro* and on tissue brain specimens. This finding is apparently in contrast with the positive immunoreactivity of ER*α* and ER*β* reported by other Authors on foetal and adult CNS (Central Nervous System). The absence of nuclear ER*α* on neurons in culture and in brain tissue specimens in our experiment is not in contrast with the relevant physiologic role of estrogens on nervous central system, but it could be correlated to the embryonic period of life and could represent a protection of male brain from an undue estrogens imprinting. The mitomycin C, alkylation agent, has shown in our experiment a major neurotoxic and cytostatic power in comparison with methotrexate. Our conclusion is that human embryo neuronal culture *in vitro* is a powerful instrument for physiology and human therapy for cancer and neurodegenerative diseases.

Keywords: Human Embryo Neuronal Culture; ER*α* on Embryonic Brain; Mitomycin C Toxicity Test *in Vitro*; Methotrexate Toxicity Test *in Vitro*

1. Introduction

Neural cells culture has been successfully accomplished from neural precursors from marine embryonic stem cell [1] and from human ones [2].

The aim of the above mentioned researches was to obtain neural progenitors fit to integrate *in vivo* into a host tissue in order to replace damaged or absent neurons [3-6].

Our *in vitro* model, that is the culture of well differentiated neurons and not stem cells, from human embryo brain, is important for the research on hormones, drugs, neuromediators receptors with the aim to understand their role in morphogenesis and a potential therapeutic use or neurotoxicity.

In addition, an original contribution of our paper is the study of estrogens receptor ER*α* on neurons in culture and toxicity test for Mitomycin C and Methotrexate on neurons *in vitro*.

2. Materials and Methods

2.1. Embryos Collection

We have obtained the positive judgement by the ethic committee (Prot. n. 47519/I3D7 Asl Sa 2 the 25.08.2005) for use of embryonic material from voluntary abortion.

The gestational age of the pregnant women were between 9˚ - 11˚W; an expert pathologist was in operating room in order to collect the embryo just delivered, enucleate brain and spinal medulla and, after washing, place the material in medium culture. Fragmented embryos were not selected for experiments.

2.2. Neurons *in Vitro* Culture

Brain and spinal medulla were gently cutted in a Petri dish, put in a sterile solution of Collagenase Type I 176.0 units/mg (Gibco) at a concentration of 1 mg/100 ml. After 2 hours of incubation at 37˚C in atmosphere of CO_2

5%, the material was collected in conical tube and centrifuge at 1500 rpm for 10 m'. Thereafter, the sediment was suspended in culture medium B-27 Electrophysiology Kit (Invitrogen) specially formulated to promote an increased density of synapses and neurotransmitter receptors; 1 ml/100 of antibiotic antimycotic solution (100×) (Sigma) was added to the culture medium. The cellular suspension was plated in glass Petri dishes having in the centre a glass slide Superfrost plus (Thermo Scientific Menzel-Glaser) 25 × 75 × 1 mm, in order to have a neuronal growth directly on glass slide fit to histochemical analysis; Superfrost plus has an electrostatically surface which binds better the cells.

Contrast phase microscope observation at 2, 4, 6 and 8 days from setting up the primary culture evidenced the growth of typically shaped neuronal cells with prolongations and synapses between cellular processes (**Figure 1**).

Only three out of the six originally embryo culture were successfully performed for a period of about 30 days; the other 3 cultures failed because bacterial and yeast contamination (too high the initial microbial load).

The embryo sex was established by FISH on interphase neurons culture; the sex was female for all three. In addition the complete karyotype was successfully obtained through treatment for cytogenetic investigation of neuron cultures.

2.3. Histochemical Confirmation of Neuronal Phenotype

High concentration of Neuron Specific Enolase (NSE) are present in neurons and in neuroendocrine lineage. For this reason we have used the Mouse Monoclonal Antibody anti-NSE (Neuron Specific Enolase-Ventana Medical System, Inc.) that reacts with NSE localized in the neuronal cytoplasm cells (**Figure 2**).

3. Detection of ER*α* in Neurons *in Vitro* and in Brain Specimens

Detection of Estrogen Receptor (ER*α*) in neuronal cells was performed with a rabbit Monoclonal Antibody (IgG) (CONFIRM Estrogen Receptor-Ventana Medical System, Inc.), that is intended for laboratory use for the qualitative detection of ER antigen. It is directed against an epitope present on human ER protein.

The cells that have in their nucleus the Estrogen Receptor stain brown and result positive. Absence of nuclear histochemical stain of ER*α* is shown in **Figure 3(a)** and absence of immunoreactivity ER*α* of embryonic brain specimens fixed in formalin in **Figure 3(b)**.

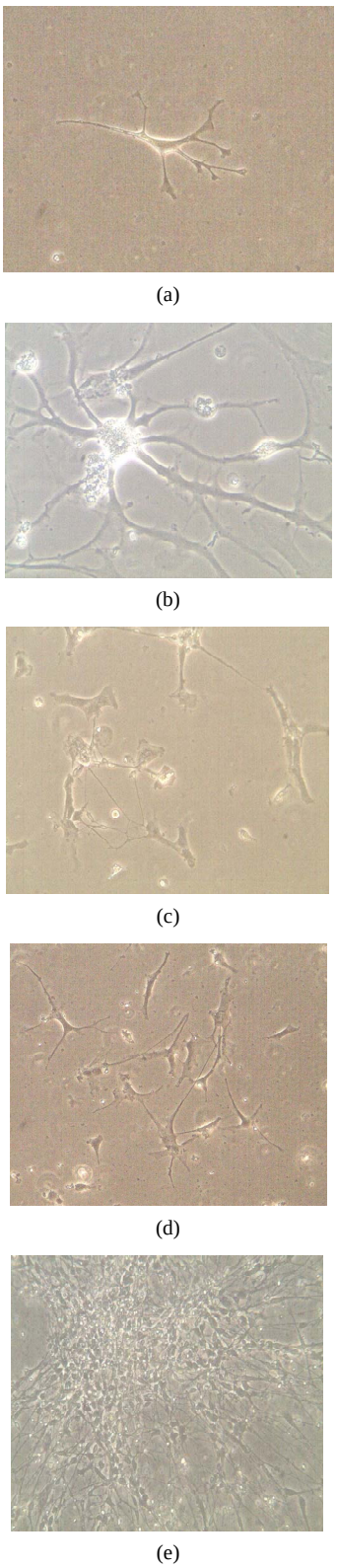


Figure 1. Neuronal culture: (a) After 2 days; a1 typical neuron; (b) Protoplasmic astrocyte; (c) After 4 days; (d) After 6 days; (e) After 8 days. Neuronal synapses are yet evident by the 4 days.

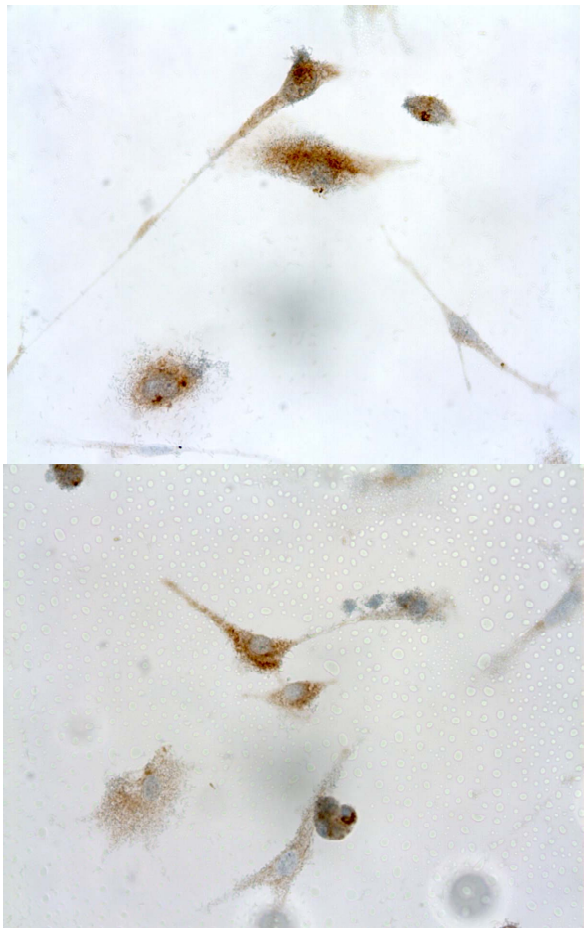
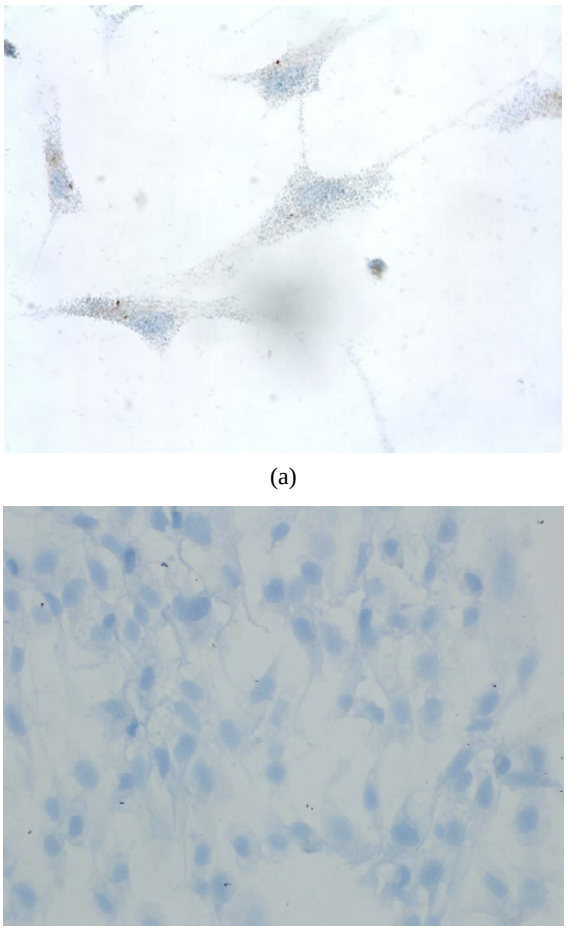


Figure 2. Immunohistochemical positivity for NSE (Neuronal Specific Enolase) of the neuron grown on glass slide: staining with anti-NSE antibody is uniformly intense on the cytosol of the cells confirming the neuronal phenotype.



(a)

(b)

Figure 3. Histochemical stain of ER*α* with a rabbit Monoclonal Antibody (IgG) (CONFIRM Estrogen Receptor-Ventana Medical System, Inc.): (a) Absence of brown coloration indicating absence of ER*α* protein, in neurons *in vitro*; (b) In brain tissue.

4. Toxicity Test *in Vitro* for Two Antineoplastic Drugs: Mitomycin C and Methotrexate

The toxicity of the two drugs has been evaluated on two distinct subcultures in flasks from a primary culture of embryonic neurons.

Both has been added to the culture medium with a final concentration of 50 µg/ml, comparable to blood concentrations used in human cancer therapy [7].

After 48 hours from the administration of Mitomycin C (Mitomycin C 10 mg, Kyowa Pharmaceuticals), the culture showed clear signs of cellular suffering with most of the cells in suspension (**Figure 4**).

Instead, the culture with Methotrexate (Metotrexate 5 mg, Lederle Pharmaceuticals) did not show, after 48 hours, any sign of cytopathy or detachment from the surface monolayer and proliferation was non apparently inhibited (**Figure 5**).

5. Discussion

Human embryonic neurons from spinal cord in culture have been obtained by Kato A. C. *et al.* 1985 [8]: biochemical studies demonstrate a prevalence of cholinergic and GABAergic neurons.

Sah D. W. 1995 [9] has studied voltage and ligand-gated currents in human foetal central neurons in culture.

Human embryonic neuronal cultures have been established in our laboratory without particular difficulty. The more critical step of the procedure is the collection of material: an expert operator must be present in the surgical room and has to select embryonic material immediately after the expulsion. A further careful micro-dissection of the material has been performed in laboratory; it is better to initiate the culture with a reduced quantity (5 - 10 mg) of embryonic nervous tissue material instead of a contamination with other cellular type. More than 90% of

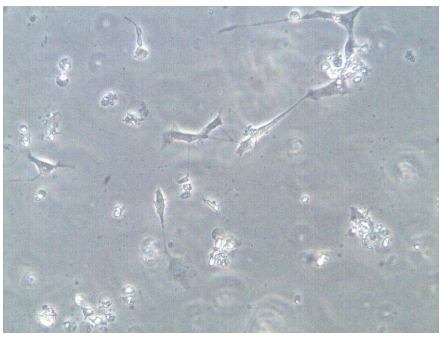


Figure 4. Mitomycin C added neuronal culture: reduction of cellular prolongations and many cells are in suspension was yet evident at 2 days from subcultures.

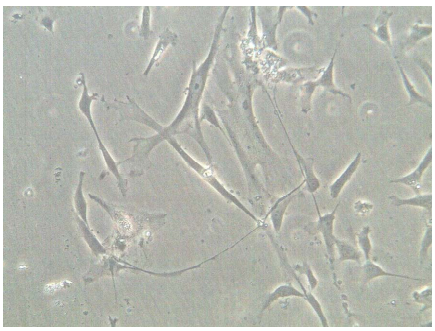


Figure 5. Metotrexate added neuronal culture: no cytotoxic effect of metotrexate was apparent at 2, 4, 6 days from subculture.

cells *in vitro* in our experiment are neurons, astrocytes, oligodendrocytes and Neural Stem Cell on the basis of morphological aspect and histochemical study.

A protective rule of estrogens in neurodegenerative diseases has been postulated [10,11].

The two types of Estrogens Receptors (ERs), ERα and ERβ, belong to the nuclear receptor superfamily, a family of ligand-regulated transcription factor. Both receptors are coexpressed in a number of tissue and form homodimers (α/α, β/β) and heterodimers (α/β) [12]. When coexpressed, ERβ inhibits the ERα mediated gene expression. In mice ERα is expressed primarily in the uterus, liver, kidney, and heart, whereas ERβ is expressed primarily in the ovary, prostate, lung, gastrointestinal tract, bladder, hematopoietic and central nervous systems [13-15]. Cells containing immunoreactive estrogen receptor-alpha have been found in the human basal forebrain [16,17]. Differential Expression of Estrogen Receptor α and β immunoreactivity has been found in the Human Supraoptic Nucleus (dl-SON) in Relation to Sex and Aging [18]. Significant correlations between the percentage of ERβ- and ERα-positive and -negative AVP (Plasma Arginine Vasopressin) neurons and age were found in women, but not in men: a strong decrease of ER β and an increase of ERα immunoreactivity in AVP neurons of the dl-SON of postmenopausal women. Both receptor changes could participate in the activation of the AVP neurons in postmenopausal women.

Estrogen receptors localization in the human spinal trigeminal nucleus has been evidenced by Fenzi e Rizzuto [19].

Although ER subtypes may be expressed in the same tissue, they may not be expressed in the same cell type. Nonetheless, ERα and ERβ proteins have been simultaneously detected in many cell types including neurons. The absence of nuclear ERα on neurons in culture and in brain tissue specimens in our experiment is not in contrast with the relevant physiologic role of estrogens on nervous central system, but it could be correlated to the embryonic period of life and could represent a protection of male brain from an undue estrogens imprinting. A role of progesterone in inducing human embryonic stem cell proliferation and differentiation into neuroectodermal rosettes has been established [20].Considering that the two hormones, estrogens and progesterone, often have an antagonistic effect, the absence of ERα receptor in embryonic brain could have its significance in non-antagonize the morphogenetic effect of progesterone.

Mitomycin C and metotrexate are antineoplastic drugs present in many polichemiotherapic schedules, including brain cancer.

The mitomycin C, alkylating agent, has shown in our experiment a major neurotoxic and cytostatic power in comparison with methotrexate.

Metotrexate, competitive antagonist of the folic acid on catalytic site of the dihydrofolate reductase (DHFR), has an action which can be weakened by three factors: the reduction of polyglutamate inside the cell, increase of DHFR by genetic amplification, reduction of intracellular transport [7]. Therefore the apparent absence of neurotoxic effect in our model need a more complex experimental design to be explained. Nevertheless, the therapeutic index of Metotrexate is better than Mitomycin C on the basis of our experimental finding.

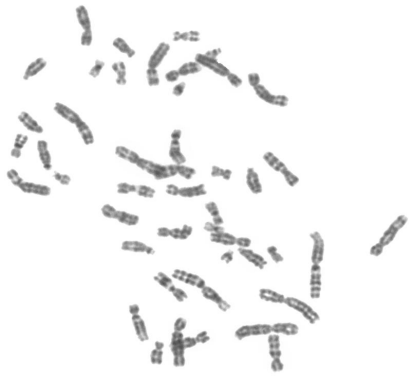


Figure 6. Metaphase with GTL banding from human neurons in culture.

The cytogenetic investigation has easily been performed on our human embryonic neurons (**Figure 6**); the analysis of chromosomal fragility and SCE (Sister Chromatide Exchange) is another powerful method to test the clastogenic activity of many substances for human therapy [21,22].

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