IDENTIFICATION OF TERATOGENS IN CELL CULTURE. II. LABORATORY VALUES OF RAT EMBRYO MIDBRAIN STUDIED BY MICROMASS TEST WITH PENICILLIN G AND 5-FLUOROURACIL

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One of the model systems that is included in the battery of tests for in vitro embryotoxicity studies is Micromass test. However, this test is based on the rat embryo limb bud cell culture and disregards other tissues. The aim of this work was to optimize culture condition and to check the influence of initial cell density and spot volume plated on the evaluation of embryotoxicity. Two model compounds (penicillin G at the concentration of 500 µg/ml and 5-fluorouracil at 7 concentrations ranging from 15.625 to 1000 ng/ml) were used. It was confirmed in control cultures that viability was directly related to the number of cells plated, however, it had no significant influence on the evaluation of the compounds. In nine runs IC50 values of 5-FU were similar for proliferation and differentiation and ranged from 148 to 257 ng/ml. In PEN-G cultures the viability ranged from 80 to 95% of controls.

Key words: rat embryo, brain, micromass test, embryotoxicity, model compounds.

Embryo cells when cultured at high cell density (micromass culture, MM) retain some aspects of cell behaviour that are most critical to embryogenesis, i.e. adhesion, movement, communication, division and differentiation. Since these cultures exhibit extensive histotypic cellular reorganization and maturation they offer unique possibilities for developmental studies. Up to now the technique with using limb buds and midbrain cells from rat embryos was most frequently used to screen chemicals as potential teratogens (4, 5, 7, 11). In spite of that Micromass test validated last time is based only on the culture of rat embryo limb bud cells (8, 12). This is because neural cultures are more difficult and too variable. From the other site it is well known that this tissue offer some advantages in many fields of studies (3, 9, 10, 13, 16-19, 23) including toxicity of neurotoxic compounds (2, 20-22, 24).

The aim of this work was to introduce rat embryo midbrain cell culture as additional model in embryotoxicity studies in our laboratory. As it was done previously for micromass culture of rat embryo limb bud cells (14, 15), systematic series of
experiments with rat embryo midbrain cell cultures were undertaken. Interrelationships between different variables were checked.

**Material and Methods**

This study was carried out according to Compliance with Bioethical Principles: Statement of Bioethical Commission and Permission of the Director of the National Veterinary Research Institute. The cultures and their assessment were carried out according to *INVITTOX PROTOCOL No. 114 (11)* with some own modifications to follow the most critical steps of the procedure.

**Animals.** Adult virgin albino Wistar rats were mated over the night. Next day at the morning vaginal smears were examined to confirm copulation. This day was designed as the 1st gestation day (GD). On day 13 after conception dams were sacrificed by CO$_2$ and cervical dislocation. The uterus was obtained by abdominal resection and removed into a dry sterile Petri dish containing EBSS.

**Embryos.** Embryos were removed in sterile environment (laminar flow cabinet) under a dissection microscope and using watchmaker’s forceps. Embryos (34 - 36 somite stage) were collected and submerged in a warm (37°C) mixture of horse serum (HS), GIBCO and Earle’s balanced salt solution (EBSS), GIBCO in the proportion of 1:1 (v/v).

**Isolation of tissue.** Using microdissection scissors and watchmaker’s forceps midbrain was removed from each embryo, transferred to sterile test tube and washed three times with calcium and magnesium–free EBSS (CMF), GIBCO. After incubation in CMF (37°C for 20 min.) CMF was exchanged for 1% trypsin (DIFCO, 1:250) in CMF and incubated (at 37°C for 20 min). The trypsin action was stopped by adding and twice washing with medium: Ham’s F-12:foetal bovine serum:L-glutamine:Pen/Strep (88:10:1:1, v/v), GIBCO.

**Preparation of single cell suspension.** Sticky and stringy midbrains were resuspended in small amount of culture medium. Mechanical dissociation was carried out by repeated flushing of the suspension through pipette (200 µl tip). A single cell suspension was ensured by passing through 10 µm sterile nylon mesh (circles of nylon mesh setting to Swynex 1 cm filter holder) into a sterile 6 ml tube.

**Plating cell spots and culture conditions.** Cells were counted in Bürker’s haemocytometer. High-cell-density cultures (5 x 10$^6$/ml or 10 x 10$^6$/ml) were set up carefully as a discrete (5 or 10 µl) drop in the centre of each well (96-well Nunclon dish). The time between mesh - filtering the single cell suspension and plating spots was kept to a minimum, to prevent clumping of the cells. The cultures were placed in humidified CO$_2$ incubator at 37°C for 2 h, to allow the cells to attach before the cultures were flooded with 200 µl of medium (without or with test compound) and incubated (37°C, 5% CO$_2$) for 5 days.

**Exposure to compounds.** Two model (reference) compounds were used:
- non teratogen – penicillin G (PEN-G), SIGMA PEN-NA, at the concentration of 500 µg/ml;
- strong teratogen - 5-fluorouracil (5-FU), SIGMA F-6627, at seven concentrations ranged from 15.625 ng/ml to 1000 ng/ml.
Assessment of viability. Medium (without or with test compound) was removed from the wells. Cultures were fixed for 20 min with 4.5% v/v glutaraldehyde solution, washed 3 times with 0.9% saline and exposed to 0.05% neutral red (NR) for 30 min at room temperature. After washing with saline, acid alcohol (1% acetic acid in 50% ethanol) was added to extract NR from living cells. After at least 2 h the eluted stain intensity (optical density, OD) was measured spectrophotometrically (at 540 nm) directly in wells with cells. Data (OD readings) were put into Excel. Mean absorbance and standard deviation as well as viability (% of control) were calculated at each level. Surviving cell number was directly related to the absorbance of the eluted stain.

Assessment of differentiation. After staining (approximately 10 s) with Gill’s haematoxylin foci of differentiated cells were evaluated using image analysis (LUCIA Measurement System). The mean number of differentiated foci (± standard deviation) and % of the controls at each level were calculated.

Calculation of endpoints. Concentration response curves for 5-FU were analysed and concentration which reduced cell number by 50% (IC50) and differentiation (ID50) were calculated.

Results

Preliminary studies. To estimate laboratory values 240 vehicle cultures which initial cell density was 5 x 10⁶/ml and volume plated - 5 µl/well (2.5 x 10⁴/well) were evaluated.

Mean NR reading was 0.483 ± 0.197 (ranged from 0.1028 to 0.7892) and the number of differentiated foci 100 ± 20 (ranged from 70 to 110).

Optimisation of culture conditions. Two variables (initial cell density and volume plated per well) were taken into account. Three experiments were undertaken in which 3 independent runs of cultures were carried out. Initial cell density was 5 x 10⁶/ml or 10 x 10⁶/ml and volume used 5 or 10 µl/well (Table 1).

It was found that NR readings were relating to the number of cells plated on the wells. The morphology of cultures depended on both, the number of cells seeded and on the volume used.

Experiment I. At the lowest number of cells (2.5 x 10⁴) seeded in 5 µl per well OD readings were similar to the mentioned in preliminary studies i.e. 0.4197 ± 0.0660. The aggregating cell bodies were of regular size and numerous (113 ± 10) (Table 1).

Experiment II. At two times higher initial concentration used at 5 µl/well (5 x 10² cells/well) the mean NR value was almost 2-times higher 0.7210 ± 0.1140 (Table 1). The number of aggregating cell bodies was very similar (mean 97 ± 18.5) to that in experiment I.

Experiment III. In opposite to the previous experiments, at the highest amount of cells (10 x 10⁴/well), plated in a volume of 10 µl NR readings were very high (2.1419 ± 0.0615). The aggregates were of larger size but the number of aggregates was twice lower (48 ± 23). It is of interest that such cultures were very often spreading or displacing onto the side wall, their adhesion was disturbed so they have to be excluded from the experiment.

Negative and positive controls. To follow the influence of variabilities mentioned above on the assessment of chemicals, two model compounds (PEN-G and 5-FU) were used in this study (Table 1).
Table 1

Relationship between initial cell density, spot volume plated and effect of model compounds (PEN-G and 5-FU) on proliferation and differentiation in embryo midbrain cell cultures (values represents mean of 3 determinations in each experiment)

<table>
<thead>
<tr>
<th>Experiments</th>
<th>Initial cell density No/ml vol/well (No/well)</th>
<th>Controls Viability NR, OD 540nm** Mean ± SD</th>
<th>Controls Number of differentiated foci** Mean ± SD</th>
<th>PEN-G Viability % of control**</th>
<th>PEN-G Differentiation foci** % of control</th>
<th>5-FU IP_{50}/ID_{50} ng/ml ***</th>
<th>5-FU IP_{50}/ID_{50} ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>5 x 10^6 5 µl (2.5x10^5)</td>
<td>0.4197 ± 0.0660</td>
<td>113 ± 10</td>
<td>82 ± 6.0</td>
<td>89 ± 19</td>
<td>170/159</td>
<td>1.06</td>
</tr>
<tr>
<td>II</td>
<td>10 x 10^6 5 µl (5x10^5)</td>
<td>0.7210 ± 0.1149</td>
<td>97 ± 18.5</td>
<td>87 ± 6.3</td>
<td>86 ± 16</td>
<td>178/188</td>
<td>0.95</td>
</tr>
<tr>
<td>III</td>
<td>10 x 10^6 10 µl (10x10^5)</td>
<td>2.1419 ± 0.0615</td>
<td>48 ± 23*</td>
<td>95 ± 7.3</td>
<td>119 ± 15</td>
<td>252/243</td>
<td>1.02</td>
</tr>
</tbody>
</table>

* Cultures were partly detached; **mean ± SD; ***IC_{50} – inhibition concentration which reduced cell number by 50% for proliferation (IP_{50}) and differentiation (ID_{50})

In PEN-G cultures the values in experiments I and II were very closed. Viability for proliferation covered 82% and 87% of respective control values, for differentiation 89% and 86% (Table 1). In experiment III the number of differentiated foci was twice higher than that in respective control.

In general, all 9 plates (experiments I, II, III) exposed to 5-FU (7 concentrations of 15.625, 31.25, 62.5, 125, 250, 500 and 1000 ng/ml) demonstrated a concentration dependent reduction in the viability and differentiation of cells at day 5 of culture (Fig. 1).

Fig. 1. Photographs of fixed and haematoxylin-stained midbrain cell micromass cultures (10 x 10^6/ml, 5 µl/well) treated with:

M – medium only; P – PEN-G (500 µg/ml); F1 to F7 - 7 concentrations of 5-FU ranged from 15.625 to 1000 ng/ml. The photograph demonstrates a concentration – dependent reduction in the number of differentiated foci.
When compared with the control (Fig. 2B) toxic effects in experiments I and II were first observed at the concentration of 125 ng/ml (Fig. 1- F4). At the concentrations of 250 and 500 ng/ml destruction was more and more visible (Fig. 2 C, D). IP<sub>50</sub> values were 170 and 178 ng/ml and ID<sub>50</sub> 159 and 188 ng/ml, respectively. In experiment III these values were higher i.e. 252 and 243 ng/ml (Table 1).

Fig. 2. Appearance of rat embryo midbrain cell cultures (x 100) on day 0 (A) and on day 5: control, medium only (B); treated with 5-FU at 250 ng/ml (C) and 500 ng/ml (D). Initial cell density was 10 x 10<sup>6</sup>/ml, volume plated 5 µl/well.

Discussion

It is well known that the test system that use primary cell cultures can be affected by different variables (1). In this study the time of tissue collection was in compliance with the requirements of the official method (11). Only embryos aged of 34-36 somite number were used. The length of time from the removal of the uterus to the start of cell incubation was not longer than 2.5 h, as it was suggested by Bacon (1).

According to functioning opinion the most critical step in the whole procedure is spotting cells into wells i.e. correct density, an optimum ratio of cell number to medium volume and precise cell seeding on proper plate (12). For this reason, in the present study much more attention was paid on two variables: initial cell density and spot volume setting on the plate. This was to check the interrelationships between these variables and their influence on the final results in vehicle, negative and positive control cultures.

According to the official method (11) the optimal number of rat embryo midbrain cells plated is 2.5 x 10<sup>4</sup>/well. However, it is necessary to stress that in the
literature there is no statement dealing with NR reading values for vehicle controls as well as concerning IC\textsubscript{50} for reference compound (5-FU) in CNS culture. This was well elaborated (calculation accepted in international validation studies) for limb buds cell cultures in which 95% confidence interval for OD, NR is 0.55-1.85 and for IC\textsubscript{50} 0.041 to 0.15 µg/ml (8).

Because NR readings obtained in this study for control cultures seemed to be rather low (mean 0.483 ± 0.197) so, to increase this value, suspensions of higher cell density (10 x 10\textsuperscript{6}/ml) were prepared and two volumes plated per well (5 µl and 10 µl) were verified (Table 1).

The most proper for start the culture seems to be: initial cell concentration of 10 x 10\textsuperscript{6}/ml and spot volume plated of 5 µl/well (experiment II). In spite of receiving higher NR readings it allowed to set the spots centrally without spreading or displacing onto the side wall as well as to keep them attached to the end of culture.

From the other side, the evaluation of differentiation in experiment II pointed out that it was similar to the observed in experiment I. The mean numbers of foci were 97 and 113, respectively (Table 1). Additionally this values corresponded with the observations of Flint \textit{et al.} (6). According to the authors the mean numbers of differentiated foci per CNS cell island were for different vehicle controls as follows: 113 ± 39 (saline), 110 ± 33 (Tween 80), 115 ± 60 (EtOH).

Moreover, the response to reference compounds in both experiments was the same and closed to the values suggested for LB cells cultures (12). For every individual plate exposure to 7 concentrations of 5-FU covered full range from 100 to 0% of controls and exposure to PEN-G did not differ from untreated cultures by more than 50%.

When the number of cells seeded was very high (experiment III) viability (OD, NR) was 3-5 times higher than in experiments I and II, such cultures were very often detached at the end of handling, exactly on the step of staining with haematoxylin. Following this, the number of differentiated foci was decreased (Table 1).

It is of interest, however, that in spite of these differences in all experiments of this study the final evaluation of embryotoxicity was proper: penicillin- G (usually used as negative control) was not embryotoxic and 5-fluorouracil (used as positive control) - strongly teratogenic. Even then much afford have to be done to achieve higher intralaboratory reproducibility which leads to reliability of the test in the assessment of new compounds.

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**References**