

Micromass culture: a recent *in vitro* system for testing embryotoxic potential of chemicals

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Abstract: Drugs and other chemicals must be evaluated for their teratological potential before releasing them in the market. One of the model test system that is used in screening of embryotoxic chemicals *in vitro*, is the micromass culture test. The test involves exposing the rat embryo limb cells or cells of central nervous system (CNS) to various test agents for different time intervals and estimating the inhibition of cell differentiation in these cell cultures. The technique can be best used for identifying strong embryotoxic chemicals due to its sensitivity to a number of classes of potent teratogens and insensitivity to non-teratogens.

Keywords: Embryotoxic, *in vitro*, micromass, teratogen, teratogenicity

Introduction

In vitro methods for screening and ranking of chemicals are widely used. Information from these test systems can be used to predict risks or hazards that can be caused by certain chemicals and can be efficiently used for prescreening purposes instead of being an alternative to *in vivo*. Occurrence of Thalidomide tragedy in early 1960s alarmed that the developing embryos and fetuses are vulnerable to the drugs exposed *in utero*. After this tragic episode, among risk assessment processes pertaining to the reproduction performance, chemically induced teratogenicity has received greater attention in order to save the developing embryo at risk due to chemical exposure. Since then, testing teratogenicity has been the subject of immense interest. Initially only *in vivo* systems were available but from the last two decades testing teratogenicity using *in vitro* system, has shown an impressive progress. In the past few years several culture systems have been proposed and developed as possible screening tests for developmental toxicity with special emphasis on teratogenicity and embryotoxicity. These systems include invertebrate species like Hydra (Johnson & Gabel, 1983), drosophilla (Bournias- Vardiabasis & Teplitz, 1982), cricket (Waltan, 1983), Dugesia (Best & Morita, 1982); vertebrate embryos or embryonic cell aggregates such as fish (Guiney et. al., 1980), amphibian and birds; embryonic stem cell lines and mammalian cell lines for example mesenchymal cells, human embryonic palate mesenchymal cells (HEPM) (Prall et. el., 1982), mouse ovarian tumour (MOT) cells (Braun et al., 1982) and neuroblastic cells (Mummery et al, 1984); whole mammalian embryo (New, 1978) and Micromass culture involving culture of differentiating rat embryo limb bud and mid brain cells (Flint, 1993). This article emphasizes on the micromass cultures which represents robust test systems for studying potential teratogens. Various researchers agree to the point that the micromass

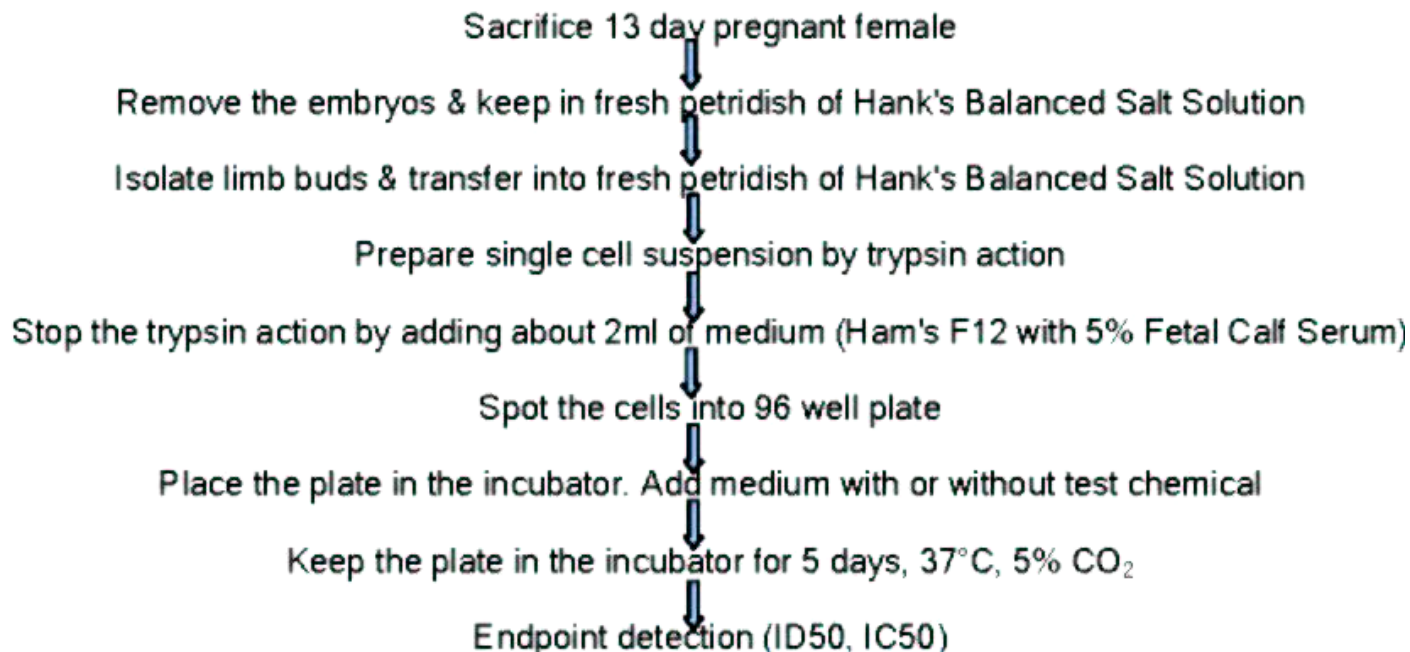
cultures that support a wider range of cellular processes are inherently superior to isolated cell systems. Further this technique can be excellently exploited to prepare large numbers of homogeneously responding cultures from very small amounts of embryo tissue (Flint, 1993).

Micromass cultures

The *in vitro* micromass teratogen test is intended to identify those chemical substances which can induce malformations resulting in embryotoxicity. The test can be employed on a number of compounds such as pharmaceuticals, agricultural and industrial chemicals, consumer products, contaminants and food additives. It can detect various test agents that interfere with some of the normal processes of cell differentiation observed in the developing embryo resulting in embryotoxicity (Flint, 1993). The Micromass culture technique, devised by Umansky (1966), reported that when cells from the undifferentiated mesenchyme of early chick embryo limbs cultured in small volumes at high density, they formed numerous small foci of differentiating chondrocytes within a background of apparently undifferentiated cells. It has been well documented by now that the characteristics such as cell proliferation, adhesion, movement, division, differentiation and cell to cell extracellular matrix interactions all occur in micromass cultures (Flint, 1983; Umansky, 1966). The test involves exposing the rat embryo limb cells or cells of central nervous system (CNS) to various test agents for different time intervals and estimating the inhibition of cell differentiation in these cultures (Flint, 1984; Tsuchiya et al., 1991). In principle, the micromass teratogen test is based on detecting the ability of a particular chemical to inhibit the formation of foci. Thus the positive chemicals will reduce the number of foci or number of cells within foci. Any interference with these basic cell developmental functions may provide primordial endpoints for detecting teratogenicity *in vitro*. The technique has subsequently been modified for use with 96 well microtitre plates (Flint, 1993). Flint (1983) also introduced the use of central nervous system cell culture.

The test procedure is based on obtaining the embryos from 13day pregnant Sprague-Dawley or Wistar rats and isolating the limb buds. The main criterion is to generate embryos of 45± 5 somites which is equivalent to 13 days embryonic age. However 40-45 somites are also recommended. All operations should be undertaken under highly sterile conditions. Instruments should either be autoclaved or oven sterilized before use. The selection of animal strain is not critical. There are reports that the potency of chemicals may vary without affecting the overall performance of the culture test (Ward & Newall, 1990). Limb buds are used in this test as other

Flow chart showing Methodology of Micromass Culture Test In Brief



tissues detect some additional chemicals which in turn may affect the overall test performance. Studies on neural cultures also showed them to be too variable for data analysis. Both fore and hind limbs can be used to maximize the information per animal. Metabolic activation is not preferred. Single cell suspension of the limb buds is prepared by trypsin action. After 20 min. of incubation period, trypsin action is stopped by adding appropriate quantity of the medium (filter sterile solution of 5% v/v Fetal Calf Serum in Ham's F-12 medium & 10ml 100X pen/strep per litre). This cell suspension is then passed through 10 μ m nylon mesh. Volume of cell suspension is measured and recorded. The cell sample is diluted so as to achieve approximately 2×10^7 cells/ml in the case of limb buds (5×10^7 cells/ml for midbrain). Next step is the most critical one and involves spotting of the cell suspension in the center of 96well plate. Care should be taken at this step otherwise the drop will be pulled by surface tension up the walls of the well. The volume and number of cells within the spot is kept as consistent as possible. Spots that spread or displaced onto the side wall are marked and excluded from analysis. After spotting the plate is kept in the incubator for 2-3hr at 37°C, to allow the cells to get attached to the plate. After 2-3hr, medium with or without test chemical is added into these wells. The 96 well-plate is then placed in the incubator at 37°C, 5% CO₂ for 5 days, At the end the spots are stained with appropriate dyes (neutral red for cell viability and alcian blue for number of differentiated cells and foci) and total number of viable cells (IC50) and differentiated cells and foci (ID50) are determined. Based on the results the test chemicals can be classified into

three classes of *in vitro* embryotoxicity i.e. non embryotoxic, weak embryotoxic and strong embryotoxic chemicals. Validation studies comparing chemicals from different classes suggests that the percentage of teratogens detected with micromass culture test may vary between 60 and 90% and that of non- teratogens may differ between 89 and 100% (Flint, 1983; Brown & Wiger, 1992; Koelman, 1991). Thus the micromass culture technique can be used for identifying strong embryotoxic chemicals instead of representing a complete replacement for animal testing.

Sensitivity of the test system

Several studies using micromass culture technique have been reported so far. Examples include various structure- activity related research and suggest that micromass test can differentiate between teratogens and non-teratogens within certain classes of test agent such as retinoids (Kistler *et al.*, 1990), triazole antifungals (Flint & Boyle, 1986). Organ-specific, species specific and strain specific toxicities have also been modeled in micromass cultures (Tsuchiya *et al.*, 1991a &b). The metabolites such as phenytoin and albendazole have been studied using micromass culture test and suggest that metabolism can affect teratogenicity (Brown *et al.*, 1986; Whittaker *et al.*, 1991). Uphill *et al.* (1990) reported the results from blind trial of 25 compounds tested with or without S-9 mix. The data assessed was based on either concentration or specific inhibition of cell differentiation at relatively non-cytotoxic concentrations. The effect of some environmental contaminants on differentiation in micromass culture of rat embryo limb bud cells has been studied by Minta & Wlodarczyk (2003). Among the three

test compounds (Cadmium chloride, Lead acetate & Sodium fluoride) evaluated in this study Cadmium chloride was found to be strong embryotoxic whereas lead acetate and sodium fluoride were found to be weakly embryotoxic. Laboratory values of rat embryo midbrain using micromass culture test with Penicillin G and 5-Fluorouracil has been also studied (Minta, 2003). Viability was directly related to the number of cells plated in the control cultures and the viability ranged from 80 to 95%. In nine runs IC50 values of 5-Fluorouracil ranged from 148-257ng/ml. Embryotoxicity of carbendazim in rat and hamster micromass cultures has also been recently reported by Minta et. al (2004) and suggest that both models can detect the same potential of dismorphogenic effects. Based on the results carbendazim was classified as strong embryotoxic compound.

Use of aggregating brain cell cultures to study developmental effects of organophosphorous insecticides was studied by Zurich et. al. (2000). Tissue engineering of bone and cartilage tissue is of growing interest in cranio-and maxillofacial surgery. Recently there was a controversy concerning use of artificial scaffolds as compared to the use of a natural matrix. New approaches such as micromass technology have been invented to overcome these problems by avoiding the need for scaffolds. The cells are dissociated and reaggregated into cellular spheres. Thus the micromass technology enables the investigators to follow tissue formation from single cell sources to organized spheres in a controlled environment (Handschel et. al. 2007).

In our laboratory, standardization of micromass teratogen test using rat embryo limb bud cells with two model compounds, penicillin G (negative control, 500µg/ml) and 5-fluorouracil (positive control, 15.625-1000ng/ml) is in progress.

Limitations

Attempts to validate any *in vitro* method is based on the premise that the results from *in vitro* systems can be related directly to *in vivo* systems. However, it is generally inapplicable due to various factors. Similar to any other *in vitro* methods, micromass culture test also shares the possibility of generating false negative or false positive data. Firstly, pharmacokinetics can be considered as one of the important factor in this regard. The rat embryo cells may not be exposed *in vivo* to the concentrations which can impart toxicity *in vitro* thus leading to false interpretation of data. Secondly *in vitro* systems may lack those biological mechanisms which cause teratogenicity *in vivo* (Flint, 1993). However it does not limit its usefulness where the question of prioritization is concerned among a huge list of test substances for teratogenecity testing.

Validation studies comparing chemicals from different classes suggests that the percentage of teratogens detected with micromass culture test may vary between 60 and 90% and that of non- teratogens may differ between 89- 100%.

Conclusion

There are several *in vitro* tests available for evaluating teratogenic potential of a drug or compound. Among them, micromass culture technique is a robust test system for evaluating effects of potential teratogens. It is a scientifically validated test for studying strong embryotoxic chemicals. Much has been known regarding the working of this test system in context to its limitations and the correct interpretation of the data. The test excels in its application because of their sensitivity to a number of classes of potent teratogens as well as their insensitivity to non teratogens. Another advantage is its rapidity with which results can be obtained from very small quantities of test compounds (Flint, 1993). Based on the test performance and various validation studies, the European Scientific Advisory Committee (ESAC) has recommended this test to be used for identifying embryotoxic chemicals considered for regulatory purpose.

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