# A study on the growth of continuous culture cell lines embedded in Mebiol gel

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**Mebiol gel is an aqueous solution of newly developed thermo-reversible gelation polymer that turns into hydrogel upon warming and liquefies upon cooling. A preliminary experimental study of the growth pattern of Vero, HEp-2, HeLa, BHK-21, McCoy and CHO cell lines embedded in Mebiol gel was performed in 24-well tissue-culture plates with controls without the gel. The migration rate of cells through the gel, the rate of monolayer formation outside the gel and change in pH of the medium were determined. Rate of migration of cells to the margin of the gel varied from 4 to 8 h and the rate of monolayer formation outside the gel varied from 24 to 48 h between the cell lines. Cells grown outside the gel were healthy, without showing any signs of degeneration or cytotoxicity in the presence of Mebiol gel and the pH was maintained during the seven-day study period compared to the controls. The results of the experiments designed in this preliminary study may be useful to develop methods to harvest single cells or groups of cells outside the gel for specifically planned procedures and develop techniques to preserve viable cells within the gel at room temperature.**

MANY cell types *in vivo* secrete extracellular matrix made of glycoproteins and proteoglycans on the substrate they attach to and grow. Major components of extracellular matrix are fibronectin, laminin and collagen which are rich in electrocharges, allowing growth factors, cytokines, etc. to be stored in them. These are either synthetic or biomatrices useful in the field of tissue engineering for improved cell functioning and cell viability. Porcine heptocytes were grown on polyurethane-based nonwoven three-dimensional matrix and were shown to be a biocompatible matrix structure for functionally active high density cultures[1,2]. Human neuroblastoma cells were grown over collagen hydrogel matrices for improved cell stability[3]. The use of biomatrices for tissue engineering and heart valve is well discussed[4]. Mebiol gel is an aqueous solution of newly developed thermo-reversible gelation polymer (TGP), which is a block copolymer composed of temperature-responsive polymer blocks and hydrophilic polymer blocks[5]. The TGP is in fluid state at temperatures lower than the gel–sol transition temperature because both TGP blocks and hydrophilic polymer blocks are water-soluble. The transparent Mebiol gel shows a fluid–solid property, because cross-linkages in it contain several hydrophobic domains in spite of high water content[5]. Cells or tissues cultivated in Mebiol gel have been harmlessly recovered from the same[6]. Since only a few anecdotal reports are available in the literature on the use of Mebiol gel in the cultivation of animal cells, this preliminary study was undertaken to determine the growth pattern of a few laboratory-maintained, continuous cell lines of animal origin embedded in the gel.

Under a clean-air, laminar, hood workbench, a flask containing sterile, 10 ml of Mebiol gel (supplied by Mebiol Gel Inc., Ltd, Tokyo, Japan through Nichi In Drugs & Devices (Pvt) Ltd, Chennai, India) was opened and 10 ml of tissue culture (TC) Eagle's Minimum Essential Medium (MEM) containing 10% foetal calf serum, gentamicin 50 μg/ml, ciprofloxacin 10 μg/ml (all reagents from HiMedia, Mumbai, India) was added. The gel dissolved in the medium at 4 to 8°C in 3 days.

Six continuous cell lines (supplied by National Centre for Cell Science, Pune, India) used in the experiments were Vero (African green monkey cell line), HEp-2 (human larynx carcinoma cell line), HeLa (human cervical carcinoma cell line), BHK-21 (baby hamster kidney cell line), McCoy (of unknown origin) and CHO (Chinese hamster ovary cell line). The trypsinized cells were suspended in 10 ml of fresh growth medium and enumerated by trypan blue exclusion test. The cell count of this suspension was adjusted to $2 \times 10^5$ cells/ml with growth medium and mixed in the proportion of 1 : 1 of the liquid gel at 4°C in a precooled, sterile, 15 ml screw-capped glass bottle placed in ice bath with the aid of a sterile pipette without formation of air bubbles. Using a large bore tip sterile Pasteur pipette, 50 μl (approximate) of gel–cells mixture was placed at the centre of the designated pre-warmed (at 37°C) 24-well (100 mm diameter) TC plate. The TC plate was left undisturbed inside (at 37°C) the $CO_2$ incubator until the gel solidified (approximately 60 min). The cells embedded in the gel were checked for their individual dispersal under phase contrast inverted microscope (Nikon, Eclipse, TS 100, Japan). Once the gel solidified, 500 μl of Dulbecco's MEM growth medium was added covering the solidified gel–cells mixture and the plate was incubated at 37°C in 10% $CO_2$ atmosphere, which is a routine laboratory procedure. The temperature of the incubator was maintained at 37°C throughout the experiment with generator supply. Dulbecco's MEM medium contains HEPES, which is a zwitterionic buffer that ensures good pH stability with phenol red as indicator. For each cell line, four wells were used and the experiments were repeated three times to confirm the results. The growth of the cell lines in the 24-well plates without the gel was used as controls. The test and the control TC plates were examined under phase contrast-inverted microscope at varying periods of 3–6 h everyday for 7 days, when experiments were terminated. The rates

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of migration of the cells to the border of the gel drop and formation of the monolayer on the surface of the well surrounding the gel drop were documented by photomicrography. The average time period for these was calculated. The change in colour in the medium indicative of the metabolic activity of these cell lines and of change in pH was monitored and compared with the corresponding cell cultures in the controls.

At the beginning of the experiment, the cells were well embedded inside the gel. They were spherical in shape, appeared healthy with clear cytoplasm and without any granularity (Figure 1 *a*). At the end of 4–8 h of incubation, many cells showed enlargement (Figure 1 *b*) and subsequently migrated towards the periphery of the drop of the gel (Figure 1 *c*). They showed margination outside of the edge of the gel (Figure 1 *d*) and spread over the surface of the well surrounding the gel (Figure 1 *e*). The rate of this migration and movement onto the surface of the well outside the gel varied according to the type of the cell line. As shown in Figure 1, Vero cells showed migration to the border of the gel at an average of 5 h of incubation (Figure 1 *c*) and showed slower growth outside the margin of the gel during the next 24–30 (Figure 2 *a* and *b*), with the formation of a monolayer only at the end of 48 h of incubation (Figure 2 *c* and *d*). McCoy cells reached the margin of the gel at an average period of 7 h of incubation. A monolayer of this cell line was formed outside the gel at the end of 22–24 h of incubation. BHK-21 and CHO cells showed migration from the centre of the gel to its margin at an average period of 4 h. Both these cell lines rapidly multiplied to form a monolayer surrounding the gel in 24 h. HEp-2 cell line invariably was the last to show movement of cells outside the gel at the end of 8 h of incubation.

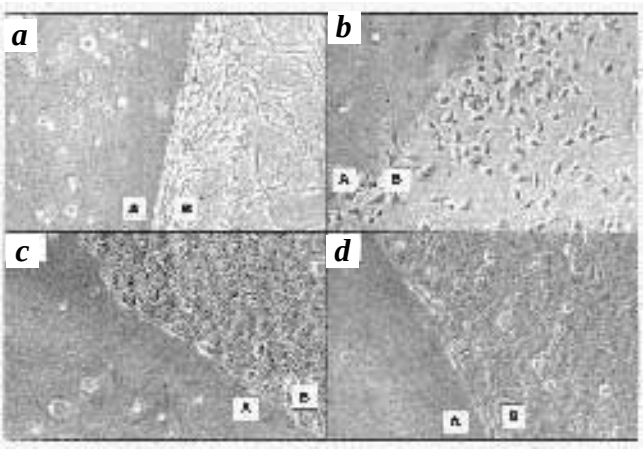


**Figure 2.** Photomicrograph taken at varied time intervals showing growth of Vero cell line in Mebiol gel in tissue culture wells. Area of inside Mebiol gel is indicated as A, with a clear margin of the same separating the area B which is outside the Mebiol gel. ***a***, Vero cells showing growth outside the gel margin at the end of 26 h of incubation (500X magnification). ***b***, Vero cells showing growth outside the gel margin at the end of 30 h of incubation (500X magnification). ***c***, Monolayer of Vero cells showing growth outside the gel margin at the end of 48 h of incubation (500X magnification). ***d***, Monolayer of Vero cells showing growth outside the gel margin at the end of 48 h of incubation (another area of the well; 500X magnification).

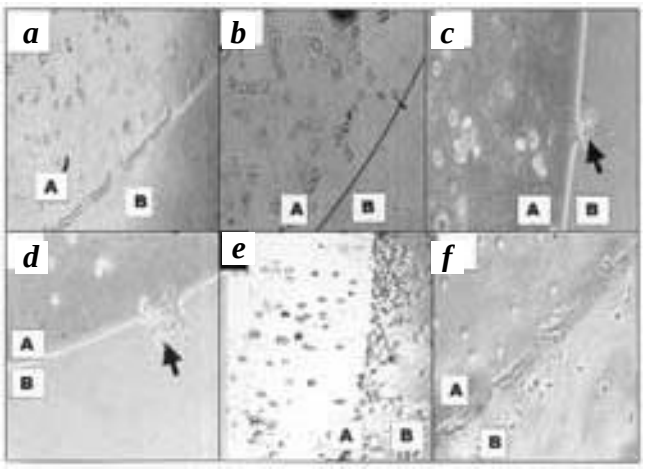


**Figure 1.** Photomicrograph taken at varied time intervals showing growth of Vero cell line in Mebiol gel in tissue culture wells. Area inside Mebiol gel is indicated as A, with a clear margin of the same separating the area B which is outside the Mebiol gel. ***a***, Vero cells enclosed in Mebiol gel at 1 h (500X magnification). ***b***, Vero cells in Mebiol gel at 3 h; some of the cells enlarged with nucleus are clearly are seen, indicating growth of cell (500X magnification). ***c***, Vero cells breaking through the margin of Mebiol gel at the end of 5 h (see arrow; 500X magnification); ***d***, Vero cells showing multiplication outside Mebiol gel at 12 h (500X magnification); ***e***, Vero cells showing multiplying cells outside Mebiol gel at 24 h (100X magnification); ***f***, Vero cells showing multiplying cells outside Mebiol gel at 24 h (500X magnification).

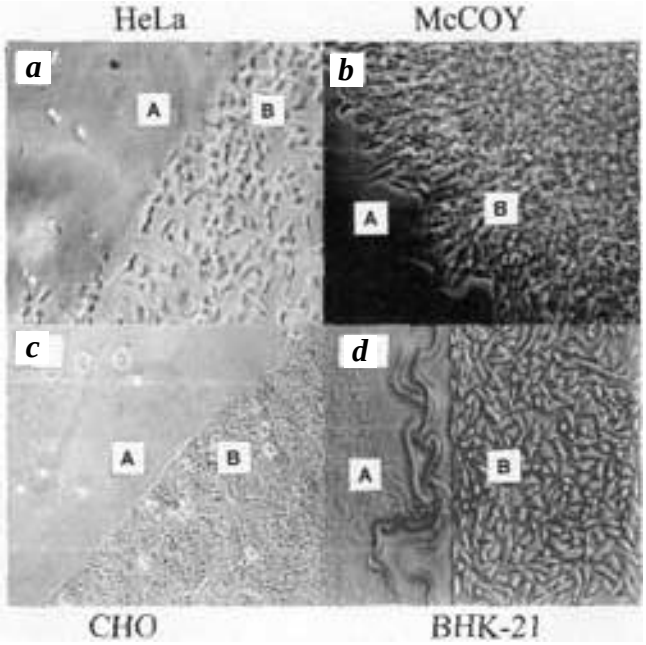


**Figure 3.** Photomicrograph of growth of HeLa, McCoy, CHO and BHK-21 cell lines outside the margin of Mebiol gel (500X magnification). Area inside Mebiol gel is indicated as A, with a clear margin of the same separating the area B which is outside the Mebiol gel. ***a***, HeLa cell line; ***b***, McCoy cell line; ***c***, CHO cell line and ***d***, BHK-21 cell line.

Growth of this cell line outside the gel was noticed at the end of 20 h of incubation and a monolayer was formed at the end of 48 h of incubation. Growth of BHK-21, CHO, MCcoy and HeLa outside the gel is shown in Figure 3.

An average of only 5–10 rounded cells remained inside the gel without migration and the rest migrated out to form a monolayer, whereas many (not countable) floating cells were present in controls indicating that the gel preserved the viability of cells better than in the controls. The viability of the cells appeared to have been unaffected by temperature variations. The colour of the medium in the tests did not change, whereas in the controls it became acidic in a period of 3 to 5 days, indicating the ability of the gel to maintain the pH up to 7 days when the experiments were terminated. The cells did not get detached from the substrate during the experimental period unlike in the controls, indicating the ability of the gel to preserve the cells for any type of prolonged cell culture experiments. The present experiments showed that the gel exerted influence on the growth pattern of some of the standard, continuous cell cultures. These cell lines were selected for the variability of their origin in relation to species, organ tissue and tissue type such as normal or malignant and also because their growth and metabolic rate have been uniformly equal in our day-to-day routine subcultures both in plastic and glass TC flasks.

The repeated experiments performed showed that the gel–cells mixture could be placed on the surface in the well of the TC plate as a drop, which solidified without the cells extruding out of it. This factor was important because all the cells utilized in the experiment were fully embedded in the gel from the beginning of the experiment. Our results indicated that Mebiol gel does have varying influence on the growth pattern of the continuous cell lines. The migration rate of cells from the inner part of the gel to its margin and growth rate to form monolayer outside the gel varied considerably among the types of cell lines. Though the time difference between McCoy and Vero for migration to the margin of the gel was 2 h, McCoy cell line formed monolayer within 24 h of incubation, whereas Vero needed 48 h to form the monolayer. Similarly, BHK-21 and CHO cell lines reached the margin of the gel at an average period of 4 h, but rapidly multiplied to form a monolayer surrounding the gel in 24 h. Migration of HEp-2 cell line to the margin was slow, needing 8 h of incubation and formed a monolayer only after 48 h. Emphasis is made at this point that the number of cells embedded in the gel was the same for all cell lines. The gel was not toxic to any of the cell lines studied, since nearly 90% of cells in each cell line migrated out of the gel, whereas large a number of cells (not countable and dead) were floating in their respective control wells. It is well known that nearly 20–30% of cells are killed during subculture of a cell line using trypsin. Whether Mebiol gel has any protective effect from the action of residual trypsin on the cells is not known. Another study that could be comparable was by Tsukikawa *et al.*[7], who utilized the TGP to grow human cancer cells. These cells formed multicellular spheroids in three-dimensional form in the aqueous solution of this polymer and the cells could be easily harvested without loss by simply cooling. In the present study multiplication of cells in the gel probably did occur but was not observed since most cells migrated by 4 to 8 h.

The experiments designed in our study could be performed on cells growing out of trypsinized or non-trypsinised biopsy tissues embedded in the gel and further for selectively harvesting different types of viable cells growing outside the Mebiol gel for characterization. Technically, it is possible to select out viable cells from within and outside the gel for further characterization. In such experiments, single cells or groups of cells may be harvested for specifically planned procedures. Since the pH of medium is maintained for 7 days, techniques may be developed to preserve viable cells within the gel at room temperature for specific periods of time and recovered for other experimental purposes. The future use of Mebiol gel in tissue and cell cultures appears to be enormous.

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