EVALUATION OF CHROMIUM TOXICITY IN CHICK EMBRYO FIBROBLAST MONOLAYERS

GAYATRI BISWAS AND S. DIVAKARAN.
Central Leather Research Institute, Adyar, Madras 600 020, India.

ABSTRACT

The cytotoxic changes induced by the addition of hexavalent chromium as potassium dichromate to the growth media of chick embryo cell monolayers were studied. The extent of toxic changes was directly dependent on the concentration of chromium. Chromium at 5 ppm level produced observable cytotoxic changes in the cell monolayers. The tissue culture lethal dose 50% ($TCLD_{50}$) was found to be in the range of 10 ppm. Severe necrotic changes were observed at 20 ppm. A gradual reduction in population of cells was noted commensurate with concentration of chromium salt from 5-20 ppm.

INTRODUCTION

TISSUE cultures have been commonly used in the past as a conveneint *in vitro* system for the study of virus infectivity[1,2] study of mechanisms of diseases and infectivity in cells[3] in veterinary medicine[4] in nutritional and deficiency studies[5] oncogenesis[6] and for the study of toxicity of compounds to assess the minimum dose required to inhibit growth[7].

Chromates have also been found to possess moderate mutagenic activity in bacteria[8,9] and yeast[10] and are capable of inducing abnormal morphological transformation[11,12] and chromosomal damage[13,14] in cultured mammalian cells.

In the present work, an attempt has been made to check the possible lethal effect of hexavalent chromium in chick embryo cell monolayers.

MATERIALS AND METHODS

Preparation of cell culture: The general methods of preparation of cell culture have been described earlier[15-17]. The method described here is a brief outline of the process followed in the present work.

Ten-day old embryonating chicken eggs were used to prepare whole embryo cell cultures. The experimental procedures were designed to ensure bacteriological sterility. The eggs after disinfecting with alcohol were cracked and the inner shell membrane and chorioallantoic memberane cut and the embryo removed. The yolk sac was severed from the body. After collecting a few embryos, they were washed in Hanks balanced salt solution (HBSS) pH 7.0, 37° C. Head, feet and viscera were detached from the embryo and the body was cut to small pieces and trypsinised with 0.2% trypsin in Earle's balanced salt solution (EBSS) pH 8.0-8.4, 37° C. About 200 ml of this solution were used for every 5 embryos. Trypsinization was continued for 30 min at 37° C with intermittent agitation after which the cell suspension was filtered through two layers of cheese cloth and then centrifuged in a 15 ml graduated centrifuge tube for 5 min at 1000 rpm. The supernatant fluid was decanted and centrifugation was repeated after resuspending in 10 ml of fresh HBSS containing 5% deactivated sheep serum to arrest the further action of residual trypsin.

After a cell count using a cytometer the cells were suspended in a predetermined volume of Hanks' growth medium with 10% sheep serum to contain approximately 1×10^7 cells/ml. The cell suspension in the nutrient media was then distributed into bottles and in latent tubes at 7 ml and 1.5 ml respectively. The cell cultures were incubated at 37° C. The cells sediment, adhere to the glass surface and form monolayer islands in 24 hr. These were found suitable for use in the present studies. Cultures allowed to grow for longer periods as 48 hr, turned confluent and produced necrotic areas, hence 24 hr cultures were found more suitable.

Inoculum: Potassium dichromate ($K_2Cr_2O_7$), the hexavalent salt of chromium was used to test its toxicity on chick embryo cell monolayers. Different concentrations namely 2.5, 5, 10, 20, and 30 ppm of chromium as solution of potassium dichromate in EBSS were used to find out tissue culture lethal dose 50% end point ($TCLD_{50}$) level. The amount of chromium used in these solutions does not in any way alter the osmolarity of the medium.

Treatment of monolayers with chromium: At the end of 24 hr of incubation of the cell culture, the Hanks growth medium pH of which has turned considerably acidic due to growth and metabolism of cells was discarded and replaced with Earle's maintenance medium without the serum containing the predetermined dose of chromium as described earlier. At the end of 24 hr of inoculation, the cell monolayers were observed for cytotoxic effects.

Cell count and slide preparation:

Cell count: Cells from control bottles and those treated with chromium were scraped with a rubber policeman after decanting the media and gentle wash-

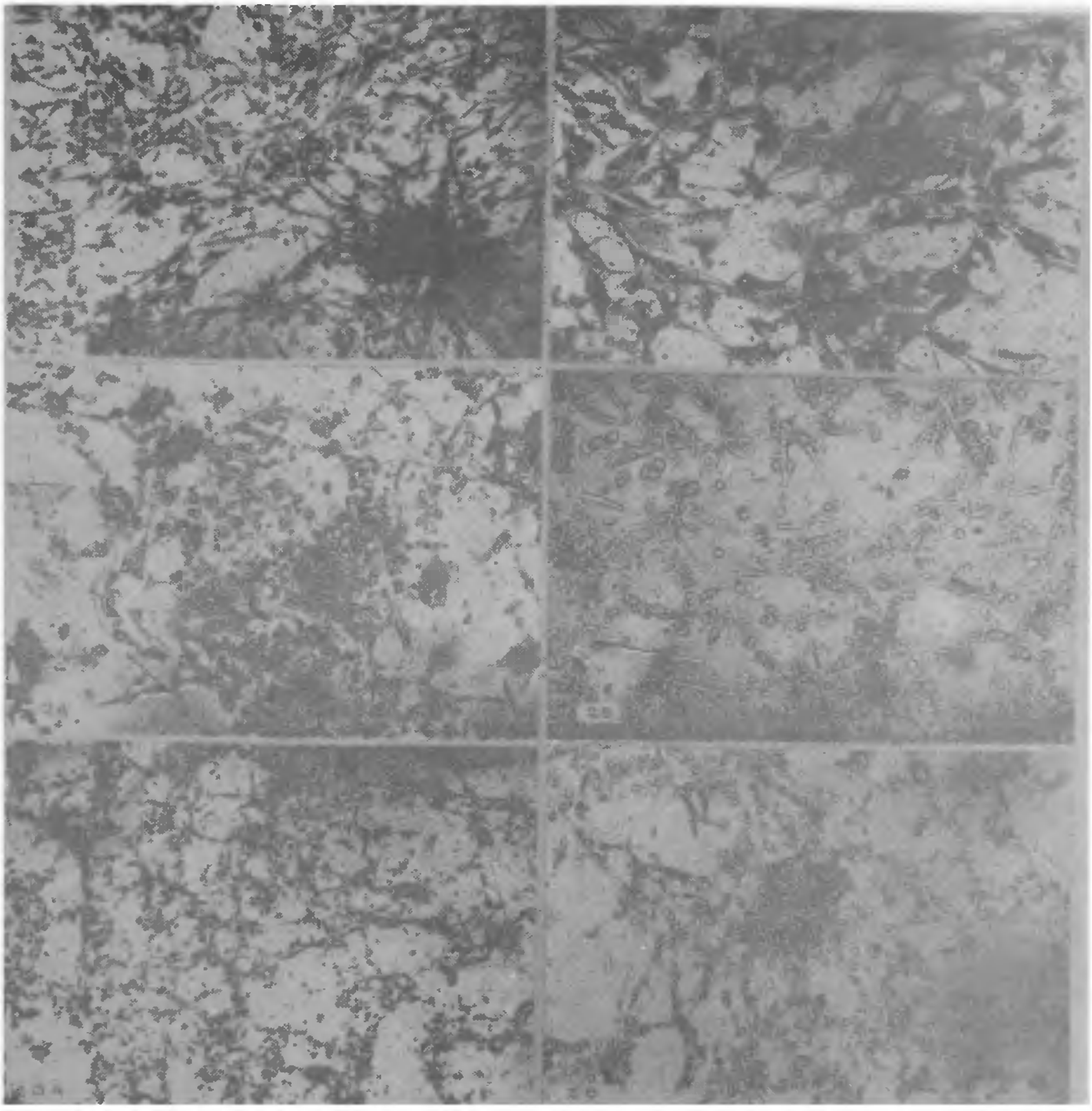

Figure 1a. Chick Embryo cell monolayer, normal (×1000), **1b.** Chick embryo cell monolayer, normal (×5000), **2a.** Cytotoxic effect of 10 ppm chromium in chick embryo cell monolayer (Ω1000), **b.** Cytotoxic effect at 10 ppm chromium in chick embryo cell monolayer (×1000), **3a.** Cytotoxic effect of 20 ppm chromium in chick embryo cell monolayer (×1000), **b.** Cytotoxic effect at 20 ppm chromium in chick embryo cell monolayer (×5000).

ing of the monolayer to remove cell cast. The cells resuspended in Earle's growth medium were sedimented by centrifuging at 1000 rpm for 10 min. The cell pack was made up to a known volume and counted in a cytometer.

Slide preparation: The monolayers from bottles were serially dehydrated in ascending grades of alcohol and covered with a thin layer of collodion solution and allowed to dry. The collodion film thus formed with the adherent monolayer were lifted and mountd on slides. The collodion film was washed off using a mixture of ether and alcohol (50:50) leaving the monolayer intact on the slide. These monolayers and those formed on cover slips in latent tubes were stained with Ehrlich haematoxylin and Eosin, serially dehydrated in ascending grades of alcohol, taken to xylol and mountd in DPX mountant.

Results and Discussion

Hexavalent chromium as potassium dichromate was found to be toxic to the chick embryo fibroblast cultures in the concentrations used. The extent of toxic changes was directly dependent on the increasing

concentrations of chromium. The cytotoxic effect observed at 2.5 ppm concentration was minimal. The tissue culture lethal dose 50 ($TCLD_{50}$) was found to be in the region of 10 ppm of chromium. The cytotoxic effect observed could be described as follows: At 2.5 ppm scattered islands of cells were found to have lost their charactristic confluence, separated into individual cells, some shrunk and necrosed. The same changes were more pronounced at 5 ppm. At 10 ppm the growth of the cell monolayer has been considerably attenuated. Single isolated cells had lost their characteristic spindle shape and extended into longer filaments. The nuclei of the cells having cytotoxic effect were somewhat hyperchromic as revealed by H and E stain. Some of the cells showed rounding which is usually an effect also revealed in viral cytopathogenicity[18].

Figures 1a (low power) and 1b (high power) show the normal cell monolayers and figures 2a and 2b show the typical cytopathic effect as revealed at 10 ppm concentration. Figures 3a and 3b show the cytotoxic effect produced at 20 ppm level. It is seen from these figures that the cells have undergone rounding and bear extensive necrotic foci. The distribution of cells is found to be scanty and there was considerable increase in cell debris in this concentration.

Cell count: In order to correlate the cytotoxic changes with variation in cell population, cell counts at 10 and 20 ppm were compared with controls. The change in reduction in cell population was gradual with increasing concentration of chromium. The average of ten cell counts made were 150×10^3 for control, 120×10^3 for 5 ppm, 75×10^3 for 10 ppm and 40×10^3 for 20 ppm.

The use of cell culture monolayers as a handy *in vitro* system for study of toxicity is not new. Tsuda and Kato[12] have used hamster embryonic cells to study the chromosomal aberrations caused by potassium dichromate. The cytotoxic effects described here clearly show that cell culture monolayers could serve as a very efficient, quick and direct method of determining toxic influences of not only chromium but other elements as well. The use of tissue culture monolayers offer several advantages in that besides direct visual observations the results could also be quantified by study of cell population index. The cell monolayers can also be lifted from bottles by using collodion or obtained as cover slips in latent tubes in order to provide permanent records after proper staining and mounting.

1. Syverton, T. T. in *Host parasite relationships in living cells* (ed. H. M. Felton), Thomas, C. C., Springfield Illinois 1957, pp. 47.
2. Dulbecoo, R. *Proc. Natl. Acad. Sci.*, 1952, **38**, 747.
3. Bankowski, R. A. *Avain diseases*, 1958, **2**, 197.
4. Livingood, C. S. and Hu, F. *Ann. N.Y. Acad. Sci.*, 1954, **58**, 1202.
5. Kieler, J. *Acta Pathol. Microb. Scand.*, 1954, **34**, 11.
6. Heidelberg, C., Reznikoff, C. A. and Krahn, D. F. *Malignant transformation of cells in culture. Methods and Applications*, Academic Press, New York, San Francisco, London, 1973, p. 644.
7. Romanoff, A. L. *Pathogensis of the Avain Embryo*, Wiley-Intersciences, New York, 1972, p. 243.
8. Nishioka, M. *Mutation Res.*, 1975, **31**, 185.
9. Venitt, S. and Levy, L. S. *Nature (London)*, 1975, **250**, 493.
10. Bonatti, S., Meini, M. and Abbondandola, A. *Mutation Res.*, 1976, **38**, 147.
11. Frandkin, A., Janoff, A., Lane, B. P. and Kushrer, M. *Cancer Res.*, 1975, **35**, 1058.
12. Tsuda, H. and Kato, K. *Mutation Res.*, 1977, **46**, 87.
13. Tsuda, H. and Kato, K. *Gann.*, 1976, **67**, 469.
14. Ponerat, C. M. and Leake, C. D. *Ann. N.Y. Acad. Sci.*, 1954, **58**, 1110.
15. Cameron, G. *Tissue culture techniques*, Edn. II Academic Press Incorporated, New York, 1950.
16. Krusse, P. F. Jr. and Patterson, M. K. Jr., (ed.), *Tissue culture. Methods and applications*, Academic Press, New York, San Francisco, London, 1973.
17. Paul, J., *Cells and tissue culture*, (IV ed.) E and S. Livingstone. Edinburgh and London.
18. Enders, J. F. *Annu. Rev. Microbiol.*, 1954, **8**, 473.