THE STUDY OF TERATOGENIC EFFECT OF CYCLOSPORINE IN VITRO

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ABSTRACT

The use of immunosuppressive medication such as azathioprine, methoterxate and mercaptopurine in treatment of rheumatic disease in women at childbearing age has some risks of teratogeniesis. Cyclosporine is one of the newer medicines, which has been introduced for this disease but little is known about its teratogenicity. This study was designed to investigate the possible teratogenicity of this drug by using cultured rat limb bud cells, which were obtained from rat embryos 13 days after conception. Cells were incubated in trypsin-EDTA solution for 30 min at 37^0C and then filtered through 50 μm nylon filters. The resultant cell suspension was cultivated in 1 ml Dulbecco modified Eagle medium (DMEM) containing 10% fetal bovine serum and 445 μg/L L -glutamine at 37 °C with 5% CO_2. After 8 days of culture the differentiated foci extract were measured by staining with 1% alcian blue. To assess the teratogenic effects of cyclosporine, it was placed in the culture well together with the cells. Results showed that the decrease in the expression of the extracellular matrix at dose of 0.01 molar of cyclosporine is due to limb bud cell toxicity rather than inhibition of cell differentiation.

Key words: Cyclosporine, Teratogenicity, Rat limb bud, Micromass culture

INTRODUCTION

Many rheumatic diseases affect women of childbearing age, and the medications used to treat these diseases may have influences on conception, pregnancy, fetal development, and lactation. Physicians who care for these women need to be aware of the potential adverse effects of these medications, and to know which medications can be used safely prior to conception or during pregnancy and lactation. Although the combination of corticosteroids and intermittent pulse doses of cyclosporine have considerably improved the prognosis of lupus nephritis, there are still some unresolved questions about this regimen, in particular its use in pregnancy. Since cyclosporine appeared to be effective in experimental models of lupus nephritis, some studies have been performed for its use in patients suffering from this disease (1). However, there are no reports on the use of this drug in patients during pregnancy. In view of the reports about the safe use of cyclosporine during pregnancy in renal transplant recipients it appeared of interest to employ recently established prescreen *in vitro* method to examine possible teratogenicity of cyclosporine. This assay method has been successfully used as a reliable method for this purpose (2,3,4).

The embryo, during its phase of organogenesis, may be uniquely sensitive to toxic insult from certain drugs and other xenobiotics. The mammalian embryo itself (normally the rat embryo) can be cultured for limited periods of time and it is possible to observe the effects of teratogens. The use of the culture of some sensitive parts of embryo like midbrain or limb bud is preferable. Limb bud mesenchymal cells, when are grown in high-density cultures, can be differentiate into a num-ber of cell types, including cartilage and muscle (5), and have been used extensively for *in vitro* studies of chondrogenesis (6, 7, 4).

Chondrogenesis is expre-ssed morphologically by the formation of cartilage nodules and biochemically by the synthesis and accumulation of an extracellular matrix product, i.e. the cartilage proteoglycan. In this study by the use of alcian blue, which is a stain specific for cartilage proteoglycan, different concentration of cyclosporine were determined and 50% inhibition of differentiation was considered as an indication of teratogenicity.

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MATERIALS AND METHODS

Chemicals:

Cyclosporine was obtained from Sahami Darui Keshvar Co., IPA Co. Ltd. Dulbecco modified eagle medium (DMEM) and fetal calf serum (FCS) were obtained from Gibco Laboratories (Gibco, UK). . Nylon mesh were obtained from Portex, UK and all chemicals used were of the highest quality available.

Culture method:

Pregnant female Wistar-Albino rats (10-12 weeks) were obtained from Pasteur Institute (Iran). On day 13 of gestation (day of discovery of copulatory plug = Day 0) the uteri were removed and the embryos (corresponding to 36-41 pairs of somites) were pooled from animals. The forelimb buds were removed according to the method described previously (2,8). Limb bud tissues were dissociated into individual cells by successive washings in calcium and magnesium-free balanced salt solution (CMF) and trypsin (1% in CMF) digestion (15 min at 37°C) and filtrated through 50 μm nylon mesh (Portex Co, UK). Cell suspensions were prepared in culture medium consisting of DMEM and 10% FCS supplemented by 445 μg/ml L-glutamine and was adjusted to give $2x10^5$ LB cells/ml (Viability of LB cells >95%). Twenty-microliter aliquots of the cell suspensions were delivered to each well of 24-well tissue culture plates (Nunclone, Denmark). The cells were allowed to settle and to adhere to the dish for 2 hrs at 37°C. The plate was then flooded with 1 ml of culture medium for the *in vitro* tests. The adhering cells, which formed separate micromass islands, were cultured for 8 days at 37°C in 5% CO_2/95% air. In the test groups, cyclosporine in different concentration was then added to cells on the day after culture. The plates were incubated for 5 days and after washing with PBS replaced with fresh medium. Each dose of drug was tested, in triplicate, on 3 separate occasions. The alcian blue stain of the differentiated foci was extracted by guanidine (4 M) and the absorptions were measured at 630 nm. The viability of cells was measured by using lysosomal and Golgi body activity (neutral red assay) in which cells were incubated in 200 μl of neutral red for 90 minutes at 37°C. Then dye was extracted with 100 μl of solvent (absolute ethanol: 0.1 M citrate buffer, pH=4.2) and the optical activity of the solution was measured at 540 nm on a standard ELISA reader.

Data analysis:

The results presented are the means ± SEM of at least 3 separate experiments. The effects of the increase in the concentrations of cyclosporine were assessed by one-way analysis of variance. The extent of the reduction in the staining of differentiated limb bud foci were examined using Student's independent t-test.

RESULTS

The results presented in figure 1 indicate that in comparison with control, cyclosporine at concentrations as low as 6 mg/ml (0.01 M) caused a significant (approximately 50%) reduction in staining of differentiated limb bud foci ($P<0.01$). Higher doses also significantly reduced differentiation, but there was no significant effect at the highest dose of 60 mg/ml (0.1 M). There was also no difference in the number of differentiated foci between the control group (no treatment) and the group that were given distilled water. Addition of 50 μl of dimethyl sulfoxide (DMSO as vehicle) to the culture medium of the limb bud cells had no significant effect on viability, as it was shown by neutral red assay. The results from figure 2 show that addition of cyclosporine directly to the culture medium significantly reduced the limb bud cell viability, which was determined by neutral red assay ($p<0.005$) with cell survival being reduced to 50% of control at a concentration of approximately 6 mg/ml (0.01 M). The results presented in figure 3 demonstrate the ratio of the differentiation to the viability and it seems that increased in cyclosporine concentration dose not decrease the extent of differentiation when the viability of the limb bud cells are taken into account.

DISCUSSION

Limb bud assay is a long established test for studying teratogenicity. Classification of compounds as teratogen and non-teratogen based on this tests in most cases is valid and by using this method, 93% teratogens and 89% non-teratogens have been correctly identified (9). Up to present no other *in vitro* assay for teratogens has tested sufficient compounds in order to permit this kind of conclusion. However *in vivo* tests are still necessary for formal conclusion. On the basis of reliability and sensitivity, in this study micromass culture of the day of 13 of rat embryos was employed to examine the potential toxicity and teratogenicity of cyclosporine. Reproducibility of the method is acceptable, but it is sensitive to change of nutritional materials (e.g. L-glutamine). The data in the figure 1 show that cyclosporine caused a significant reduction in limb bud differentiation. The ability of a drug to inhibit limb bud differentiation by more than fifty percent has been shown in fig 1. The effect of cyclosporine on the rat embryo limb bud differentiation using alcian blue staining (n=9, three separate occasion) significantly correlated with *in vivo* teratogenicity (9,10). The results of the limb bud assays show a reduction in limb bud differentiation, indicating a possible teratogenic effect. These results were, however, confounded by the cytotoxic action of

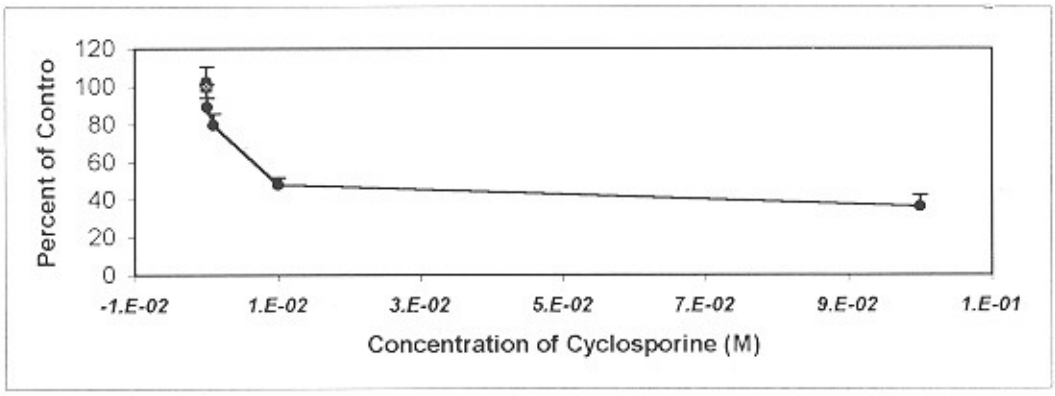

Fig 1. The effect of cyclosporine on the rat emberyo limb bud differentiation using alciin blue staining (n=6, three separate occasion

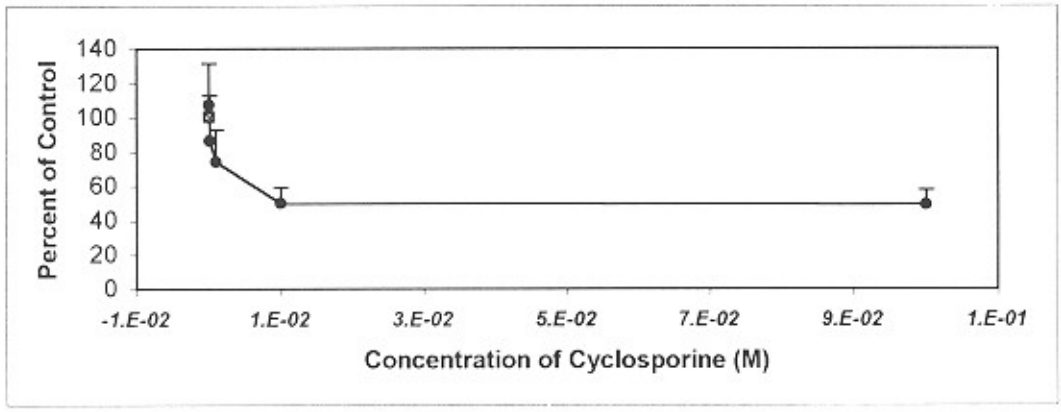

Fig 2. The cytotoxicity effect of cyclosporine on the rat embryo limb bud using neutral red assay (n=9, three separate occasion).

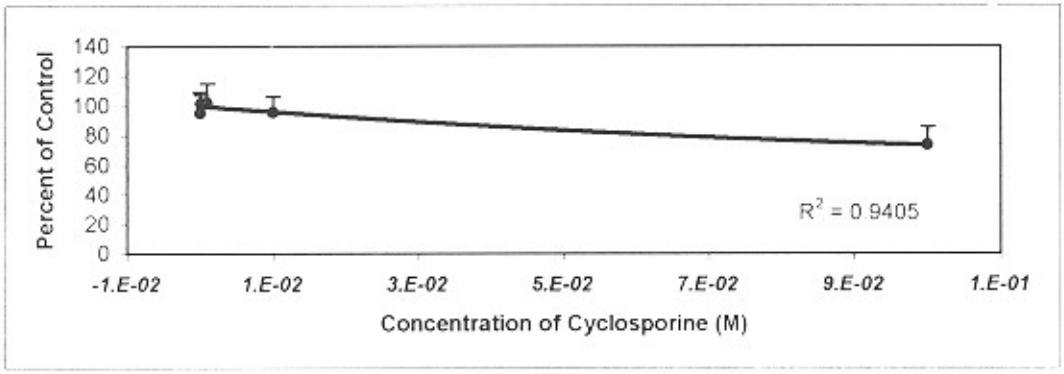

Fig 3. The ratio of teratogenicity and cytotoxicity of cyclosporine on the rat embryo limb buds using flint method (Mean ± SEM, n=9).

cyclosporine. The foetal cells appeared to be more susceptible to the effects of the drug, and a 50% reduction in viability occurred at concentrations as low as 1.2×10^{-2} M. However result of viability test showed that at the same time viability decreased due to cell toxicity. It seems that the results of the limb bud assay reflect a cytotoxic effect of cyclosporine on foetal cells rather than an inhibition of differentiation. These findings suggest that the cyclosporine may have a toxic effect on foetal cells, but there was no evidence of teratogenicity. While several reports including Gasser et al (11) results about the effect of cyclosporine on isolated cleft palates in specific mouse strain during pregnancy support our finding, there are some conflicting results in several clinical trials (12-14). Although some concentrations that were used in this investigation, have never been employed in clinical practice but it can help to find the safety margin of cyclosporine.

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