

# Cyclosporine Effects on Chick Limb Bud Mesenchyme and DNA Content

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## Abstract

**Background:** Cyclosporine A (CsA) is a powerful immunosuppressive agent, which is used for the prevention of allograft rejection and for the treatment of autoimmune diseases. Many transplant recipients must take this medicine for the rest of their lives. Females in reproductive age group on prolonged CsA therapy have legitimate concerns about drug effects on pregnancy.

**Objective:** To explore CsA's teratogenicity in embryonic limb development.

**Methods:** Mesenchymal cells obtained from stage 23-24 chick embryo limb buds were grown in 96-well plates using chemically defined tissue culture medium. Cultures were treated with a range of CsA concentrations and incubated (at 37 °C, 5% CO<sub>2</sub>) with daily medium changes for 4 days. After incubation, each well received Hoechst 33342 and DNA content was assayed using a 96-well fluorometer.

**Results:** It was found that high concentrations of CsA caused cell loss and intermediate concentrations decreased DNA content. Low CsA concentrations however had no significant effect on DNA content in these cultures. Thus, the decrease in DNA content was dose-dependent.

**Conclusion:** CsA teratogenicity may involve reducing the number of dividing cells or reducing the proliferation rate in developing structures.

**Iran J Med Sci 2003; 28(4):180-184.**

**Keywords** • Cyclosporin • Hoechst 33342 • DNA Cytofluorometry • chick limb bud

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## Introduction

**C**yclosporin A (CsA) is an immunosuppressive drug used to prevent graft rejection by suppressing T-lymphocyte function.<sup>1, 2</sup> Since immune cells also send regulatory signals to the connective tissue, there may be an adverse impact on the metabolism of connective tissue or mesenchymal cells as well.<sup>3</sup> Auto-immune disease patients are also candidates for CsA pharmacotherapy.<sup>4</sup> Reproductive-age females on prolonged cyclosporine therapy have legitimate concerns about drug effects on pregnancy and

development.<sup>5</sup> Many studies have been carried out on CsA side effects such as nephrotoxicity, hepatic dysfunction, neurological disturbances and hypertension. However, few *in vitro* studies of CsA teratogenic effects have been conducted in this area.<sup>6</sup> Previous studies have focused on describing gross abnormalities in mouse embryos and have only researched the examination of cleft palate in CsA-treated embryos.<sup>7</sup>

Only a limited number of studies have described CsA effects on bone resorption and osteoclast formation, most of which focusing on responses in mature bone.<sup>8, 9, 10</sup> Published case studies indicate that some women treated with CsA during pregnancy give birth to normal infants, but others have newborns with congenital abnormalities, including those in the limbs.<sup>11</sup> Chondrogenic differentiation of clonal mouse embryonic carcinoma cells revealed that CsA did not induce chondrogenesis at concentrations up to 1000 ng/ml.<sup>12</sup> Tissue-culture analysis of the mechanisms underlying CsA developmental effects have rarely been attempted, and detailed studies of this sort have not been carried out. The aim of this study was to explore *in vitro* CsA teratogenicity in embryonic limb development, using tissue culture and fluorometric assays of DNA content.

## Materials and Methods

In the present study, the chick limb bud was used as a model for evaluating cell proliferation. Limb-bud mesenchymal cells have proven to be useful for examining specific cell behaviors during complex morphogenetic processes. Finch and Zwilling were the first to study chick limb-bud mesenchyme growing in tissue culture.<sup>13</sup> Ahrens et al.<sup>14</sup> developed micromass culture methodology to reduce the number of cells required for each culture. Paulsen and Solursh adapted the micromass cultures to 96-well microtiter plates to reduce the amount of expensive media, additives and treatment, and to facilitate assays of their effects.<sup>15, 16</sup>

The current study was carried out using the microtiter culture method by preparing tissue culturing from limb bud mesenchyme of chick embryo. The cells were grown for 4 days and treated with different CsA concentration. At the end of the experiment the fluorometric DNA determination was done by adding Hoechst 33342 to the culture.<sup>17</sup>

### *Tissue preparation*

Fertile white leghorn chicken eggs were obtained from Hy-Line International Poultry (Newborn, GA) and incubated in a Peterisme rotating incubator to provide embryonic limb buds. Fertile eggs were incubated for 103 hours to obtain stage 23-24 em-

bryos.<sup>18</sup> Embryos were dissected under a stereomicroscope. After removing the amniotic membrane, the wing buds were excised and collected in Tyrodes buffered saline solution (Sigma) on ice. Limb buds were dissociated in Trypsin-collagenase (0.1% Trypsin, 0.1% Collagenase, Worthington, Freehold, New Jersey) in Ca<sup>++</sup>, Mg<sup>++</sup>-free Tyrodes solution containing 10% heat-inactivated chicken serum (Hyclone) by rotating at 70 rpm in a 37°C water bath for 3 minutes. The tissue was then triturated with a Pasteur pipet to suspend the cells. The resulting suspension was passed through a 20µm pore size nylon mesh filter (Nitex) to remove any aggregated cells or ectoderm. The pelleted cells were resuspended in serum-free medium,<sup>19-22</sup> washed twice with unsupplemented F12/DMEM (Cellgro, Fisher Scientific), pelleted at 600xg for 6 minute in a table-top clinical centrifuge and resuspended in defined medium [(DM; F12/DMEM) supplemented with 100nM hydrocortisone (Sigma), 5µg/ml insulin (Collab. Res. MA), 5µg/ml chicken transferrin conalbumine, Sigma), 1mg/ml L-ascorbic acid (Sigma) and penicillin-streptomycin and fungizone) ,(Gibco)]. Final suspension was prepared in 2ml DM and cells were counted on a hemocytometer. The cell density was adjusted to 1×10<sup>6</sup> cells/ml.

The isolated mesenchymal cells were plated in 96 well microtiter plates (Costar) at 2.5×10<sup>5</sup> cells in 250 µl/well.<sup>14</sup> The period of cell growth was 4 days, with daily complete medium changes. Fresh CsA was added to the well with each medium change. The control culture received ethanol vehicle equal to the highest concentration in the treatments (0.01%) or DM only.

### *CsA preparation*

CsA was obtained in purified powder form, through Sigma chemical Company (St Louis, Missouri). Fresh solutions were made for each experiment. 1 mg of pure CsA was dissolved in 200 µl of 100% ethanol in small glass bottle as stock A (final concentration, 5mg/ml). Stock B was prepared by adding 10 µl of stock A to 990 µl of DM. Stock B was the highest concentration (final concentration after adding to the 250 µl in well was 5µg/ml). The other concentrations were made by diluting stock B with DM. The final treatment concentrations in the wells were 0.01, 0.1, 0.5, 1, 5 µg/ml. The same volume of ethanol used for the stock A was used as control for ethanol. All treatments were stored in light-tight, glass containers. Each treatment was used for one time and one experiment only (i.e., stocks and treatments were kept for no longer than 4 days).

#### Preparation of DNA stock for standard curves

Lyophilized chicken blood DNA (Pharmacia, Piscataway, NJ) was used to prepare stock A (1 mg DNA/ml deionized water). The preparation was made in no less than one day and no longer than 2 weeks before the assay. Because DNA that belongs to each species has unique A-T: C-G ratio, chicken DNA was used to generate the standard curves. Care was taken to prevent denaturing the DNA by mechanical shearing or progressive degradation that normally accompanies freezing, thawing or other manipulations. On the day of the DNA assay, a 100 $\mu$ l aliquot of stock A was transferred to a centrifuge tube containing 1.9 ml of deionized water (50  $\mu$ g DNA/ml). This solution was analyzed for absorbance at 260 nm in a UV spectrophotometer, adjusting the concentration to achieve an absorbance of 1 (50 $\mu$ g/ml as stock B). Stock B was used to prepare the DNA standards.

#### Preparing Hoechst 33342

1 mg of Hoechst 33342 (Polysciences) was dissolved in 1 ml deionized water, using glove and mask precautions in a darkened room. A second stock was made by diluting the first stock with TBSS 1:10 to a concentration of 100  $\mu$ g/ml and stored in a light-tight tube at 4°C.

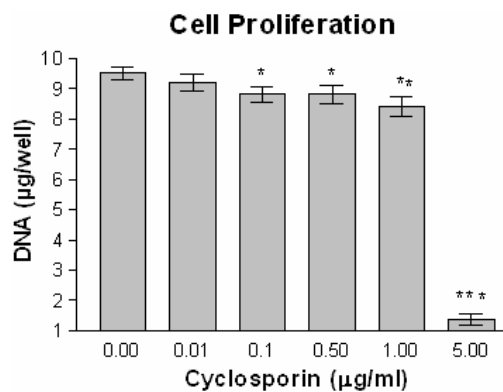
#### DNA standards

DNA standards were prepared in DM, in triplicate, in 96 well plates (Costar).<sup>14</sup> Finally the Hoechst 33342 was added to a final concentration of 10 $\mu$ g/ml. The plate was then covered and incubated for 45 minutes at 37°C and 5% CO<sub>2</sub> incubator.

#### Tissue culture assessment

Tissue cultures were prepared in 96- microwell plates for DNA assay to study mesenchymal cell numbers after 4 days of incubation. At the end of the incubation period, 10 $\mu$ g/ml Hoechst was added to each of the wells. The culture plate and that containing the standards were then incubated simultaneously for 45 minutes in tissue culture incubator. Plates were removed from incubator at the end of incubation in the dark and transferred to a Cyto FLUOR (Millipore) 96-well fluorescence spectrophotometer for analysis. Readings took less than one minute and two readings were taken for each plate. Readings were taken in the darkened room, first for the standard and then for the experiment plates.

#### Statistical Analysis



**Fig 1:** DNA fluorometric determination in micro-cultures of stages 23-24 chick limb-bud mesenchymal cells grown for four days in presence of different concentration of cyclosporine (CsA). Hoechst 33342 – associated fluorescence in treated culture from limb-bud mesenchyme. CsA has a dose related effect on DNA accumulation in culture. Values are mean  $\pm$  Standard error. Significantly different from control: \* $p$ <0.05, \*\* $P$ <0.01, \*\*\* $P$ <0.001. Spearman rank correlation:  $r = -0.98$ ,  $P < 0.001$ .

Data was analyzed by Student's t-test, one-way ANOVA and Spearman rank correlation.

#### Results

The results revealed that after 4 days in culture, CsA had dose-dependent effects on chick limb-bud mesenchymal cells (Fig 1). The effects were on DNA accumulation and hence cell proliferation in this normally expanding population by decreasing DNA accumulation during the incubation period. The reduction was detectable in cultures treated with CsA concentration as low as 0.1  $\mu$ g/ml. Treatment with 5 $\mu$ g/ml concentration was cytotoxic and the cells started to die after 2 days of treatment. Growth decreases occurred gradually toward the 4<sup>th</sup> day when most of the cells were dead. The lowest concentration of CsA used in this experiment was 0.01  $\mu$ g/ml which did not affect DNA content and cell proliferation. The reduction of DNA content was statistically significant for all effective doses (Fig 1). The resulting P values were <0.05 for 0.1 and 0.5  $\mu$ g/ml, <0.01 for 1 $\mu$ g/ml and <0.001 for 5 $\mu$ g/ml concentration of CsA.

#### Discussion

Teratogenicity is dependent on several factors including teratogen concentration, duration of exposure, developmental period of exposure, and cell sensitivity. Many of these factors reflect pharma-

cokinetic parameters such as protein binding, placental transfer, serum drug concentration and clearance of drug, all of which vary among species.<sup>23</sup> In vitro studies using limb bud mesenchyme are alternatives to in vivo experiments. In vitro systems eliminate many of the variables in pharmacokinetics and homeostatic regulation observed with in vivo animal models. Moreover, in vitro investigation allows greater control over concentration and duration of exposure and also help provide information concerning the mechanism of drug action which is pivotal for adequate risk assessment.<sup>15,24</sup> Several studies have used animal models, but the results were controversial. With regards to the limited information that is available on the effects of CsA on human fetus, in vitro studies and micromass culturing may prove to be useful. The results of the present study showed concentration-dependent reductions in DNA accumulation. These data are consistent with those of other studies reporting that the CsA decreased osteoblast number in a dose-dependent manner. Increasing matrix metalloproteinases-9 (MMP-9) and unchanged tissue inhibitors of MMP-1, reduced the osteoid formation.<sup>25</sup> In the present study, 5µg/ml of CsA revealed the same effects by reducing matrix synthesis and cells proliferation. A decrease in matrix synthesis occurs subsequent to reduction in cell proliferation. The data from the present study also show that the CsA concentration capable of reducing cell accumulation in these cultures (0.5-1.0 µg/ml) is within the normal range of blood levels at which cyclosporin circulates in transplant patients receiving immunosuppressive therapy.<sup>26,27</sup> Additional studies comparing these results with human samples needed.

A retrospective study revealed stillbirth, preterm deliveries and low birth weight in mothers receiving CsA treatment.<sup>4</sup> The DNA determinations in our experiment were dose-dependent and CsA's apparent antimitotic activity reduced DNA accumulation. These results show significant DNA reduction at CsA concentrations from 0.1µg/ml to 5µg/ml. Other reports show that CsA reduces immune cell secretions, such as IL-1, a degradative agent.<sup>28</sup> Previous studies have shown that uptake and intracellular concentrations of CsA are mediated by the cytoplasmic protein, cyclophilin. CsA-Cyclophilin complex could inhibit calcium-calmodulin-dependent phosphatase (calcineurin).<sup>29, 30</sup> Inactivation of the calcineurin results in a transcriptional blockade of CsA-induced cytokine production.<sup>31-34</sup> Mohri reported that inhibiting calcineurin might regulate integrin-dependent cell adhesion and the spread of Colo201 cells in metastasis.<sup>35</sup> Cells attachment and therefore attachment-dependent matrix production is also affected by CsA inhibiting calcineurin. Tomono et al<sup>36</sup> found that CsA markedly

inhibited the DNA synthesis in swiss 3T3 fibroblasts normally stimulated by PDGF, FGF or thrombin. This finding implies CsA interference in physiological processes such as connective tissue remodeling and repair, or mesenchymal cell proliferation during organogenesis. Therefore, CsA-mediated reductions in cell proliferation could reduce the population of cells able to synthesize new matrix that form the critical mass of tissue necessary to carry out normal organogenesis

The results obtained suggest that additional studies examining CsA effects on the synthesis of DNA, integrins, and extracellular matrix components may prove especially useful in understanding the mechanisms underlying congenital abnormalities associated with prolonged CsA treatment during pregnancy.

### Acknowledgements

*This work was supported by NIH grants GM08248 and RR03036 to the Morehouse School of Medicine in Atlanta, Georgia. Authors thank Dr. Mary Scanlon and Andrew Shaw for kind advice and access to the Morehouse School of Medicine Image Analysis Facility. We also thank Ms. Candace Marshall for technical assistance.*

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