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A New Model for Dura Mater Healing—Human Dural Fibroblast Culture

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A New Model for Dura Mater Healing—Human Dural Fibroblast Culture

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*Corresponding author: J. D. Perón 4190 (C1181ACH), Ciudad Autónoma de Buenos Aires, Argentina; e-mail: pablo.argibay@hospitalitaliano.org.ar, Tel: +5411 4959 020**Abstract:**

Abstract

Objective: Dura mater healing is crucial to prevent cerebrospinal fluid (CSF) leaks after neurosurgical procedures. Biological mechanisms leading to dural closure are only partially understood and have been studied in animals exclusively. We studied an *in vitro* model of dural closure which uses human cells. **Materials and Methods**: We used human dura intended for disposal after surgery, rather than for reuse with diagnostic or therapeutic purposes. Explant primary cultures were performed. Cells were characterized through common staining and immunostaining with desmin, vimentin, glial fibrillary acid protein, neuronal nuclei and cytokeratin. A cell growth curve was elaborated and the effect of dexamethasone on cell count was assessed. **Results**: All specimens showed growth in fusiform cells, which project pseudopods and fuse into spindles. Cells showed desmin and vimentin positivity, and were negative for all the other stains, behaving phenotypically like fibroblasts. No collagen base was necessary for cell growth. Dexamethasone decreased cell count in the primary culture as well as in the explant, and reduced the cell proliferation marker Ki-67. **Discussion**: The first model of dural closure with human cells was successfully developed. According to the findings, unlike mini-pigs and rabbits, in humans, dural fibroblast sensitivity to collagen seems to be lower when it comes to migrating and growing. Dexamethasone inhibits fibroblast invasion, which is the biological base of wound dehiscence in cranial surgery. This model is currently being used at our laboratory to determine the biological basis of autologous and heterologous duraplasties, and the factors that can stimulate or inhibit dural closure.

Key words:

cerebrospinal fluid leak, dexamethasone, dura mater, fibroblast

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Disclosure Statement

No financial support was provided; the authors have no conflict of interest

Introduction:

Cerebrospinal fluid (CSF) leaks are a common and potentially severe complication associated with neurosurgery. Patients with CSF leaks show increased rates of meningitis, pneumocephalus, prolonged hospital stay and even death[1,2,3]. Although conservative management can be the choice of treatment, reoperation is often necessary to repair the dural gap[4].

Incidence rates reported for this complication vary from 2% to 20% in the case of cranial surgery, depending on the series and largely on the time, and from 1.4% to 6.4 % in the case of transsphenoidal surgery[5,6,7,8,9]. Dubey *et al.* have lately reported an incidence rate of 13% in 500 patients who required surgical intervention for posterior fossa conditions, CSF leaks being the most common complication[10].

Careful and water-tight dural closure with non-absorbable surgical suture is critical to prevent leaks, although this may not always be technically feasible[11].

From the 1930s onwards, there have emerged several dural substitutes including autologous and heterologous grafts, and synthetic materials which work as a matrix to hold the CSF and promote the migration of dural fibroblasts as well as extracellular matrix synthesis[12,13,14,15]. These implants, however, may not always be effective to prevent CSF leaks and may introduce a risk of infection and rejection[16,17,18,11].

Additionally, even when a tensionless, appropriate dural closure is achieved with grafts, adequate formation of scar tissue is essential to prevent CSF leaks and isolate the brain[19]. When this process fails to occur, or is slow, increased CSF pressure can lead to leaks and subsequently prevent appropriate healing of the dura and the underlying tissues, thereby forming a fistula.

So far, the processes leading to dura repair and the formation of new tissue over the drafts are incompletely described and understood, with data arising basically from a small number of animal models[20,21]. As a matter of fact, there are no records of healing models using human dura,

so the assimilation mechanism of dural drafts has been studied at a cellular level in animals only. To our knowledge, our study is the first attempt to approach the problem of CSF leaks based on an *in vitro* human cellular model.

Actually, there are several questions that need to be answered with respect to the existing models. One example is the case of glucocorticoids and their effect on dural healing, especially because these drugs are commonly administered postoperatively to neurosurgical patients and are known to inhibit the healing process of other tissues[22,23].

To address these questions we developed an explant-based dural fibroblast primary culture model. We consider that this model will help to understand dural healing mechanisms, the factors involved in the process and the effect of some commonly used drugs following cranial surgery. Additionally, it can help to assess several types of grafts (autologous and heterologous), and the different biomaterials available as compared to human cells.

Despite all the time elapsed since Dandy's first duraplasty, information about the biological mechanisms leading to dural healing is still largely lacking[24]. As far as we know, ours is the first human dural healing model to be developed, and we expect that it proves useful to understand and improve CSF leak treatment and prevention.

Materials and Methods

Specimen collection

Human dural specimens were obtained in neurosurgical procedures where dura was resected but not used for diagnosis or therapy, as is the case with duraplasty following decompressive craniectomies or in the resection of tumors infiltrating the dura with wide safety margins. For this latter case, a 1-cm-wide tumor-free margin was established for the tissue to be usable. Specimens were kept at 4^{o}C in sterile saline solution overnight to be processed at the laboratory the following morning.

Informed consent was obtained in every case. The protocol was approved by the ethics committee locally.

Explant culture

Specimens were washed with Dulbecco´s modified Eagle´s medium (D-mem, Gibco) and debrided with a sterilized swab to remove blood and tissue. After debridement, specimens were fragmented into 4 mm x 4 mm squares. Two types of explant culture were performed. In one case, a square was sectioned linearly to produce two fragments, which were placed 1 mm apart so as to analyze cell migration from one piece to the other. In the second case, a circular section (3 mm in diameter) was cut to allow cells to migrate from the periphery inwards to the center.

Specimens were cultured on 30-mm culture plates (Nunc) with 2 ml of D-MEM supplemented with 100 U/ml of penicillin, 100 µg/ml of streptomycin, 0.25 µg/ml of amphotericin B (Gibco), 200mM of α-glutamine (Gibco), and 10% of bovine fetal serum (Bioser).

We used 5 $\mu g/cm^2$ collagen (Gibco) pretreated plates and untreated plates. No significant differences were detected between both types of plate. For this reason, experiments continued to be performed on untreated plates. The results reported herein were obtained from simple plates. Cultures were performed in a humidified atmosphere with 5% CO_2 at 37^oC.

Subculture

The circular fragments were used to perform primary cultures in untreated 30-mm (Nunc) plates under the abovementioned conditions and in the same medium. Tissue specimens were seeded allowing the cells to migrate. When cell confluence was reached, the tissue specimens were removed from the plate and the cells were subcultured for magnification. The plate was treated with a 0.25% trypsin solution (Sigma) for 3 minutes. After neutralizing the trypsin with a culture medium, cells were

centrifuged at 1200 rpm for 10 minutes, resuspended, and plated onto T-75 flasks (Nunc). Magnified cells were quantified as per the triptan blue exclusion method and 10,000 cells were seeded on 30-mm plates. All the experiments were carried out with twice subcultured cells.

Cell count

In explant cultures, cells migrating to the created (linear or circular) defect were quantified on a daily basis with an inverted microscope (Nikon TMS) until the cells covered the defect completely, with no cell growth observed to continue after three days. The primary culture included hematoxylin and eosin staining for each time and treatment. Cell count was performed with a brightfield microscope (Nikon Eclipse E400). Cell count in the subculture was expressed as the average of 10 fields at 10X power. In explant cultures, cell count included the total cells invading the defect, which is 4 mm^2 in area (in the linear model) and 3.95 mm^2 (in the circular model). A growth curve was elaborated for each model. A control curve and a dexamethasone-treatment curve were produced for the subculture and the explant models, as explained below. Photographs were taken to simplify cell counting.

Dexamethasone treatment

To assess the effect of dexamethasone (Sidus) on cell count in the explant culture and the subculture, the drug concentrations used were equivalent to those estimated for a 70-kg subject administered with 8 mg TID (common postoperative dosing) on the basis of a 1-l/kg distribution volume, and a half-life of approximately 3 hours (dosing intervals of 3 half-lives). The concentration was estimated at 5.7×10^{-5} mg/ml. Explant (linear and circular models) and subculture were examined to determine the effect of dexamethasone. In the case of the subculture, effect on the cell proliferation marker Ki-67 was also analyzed 2, 4 and 6 days after seeding.

Immunohistochemistry

Cultured cells were examined using brightfield microscopy and hematoxylin-eosin and periodic acid Schiff (PAS) staining. Cells were

characterized by staining with anti-desmin primary antibodies, glial fibrillary acid protein (GFAP, Biogenex), vimentin, neuronal nuclei (Neu-N), and cytokeratin (CK, Millipore). Ki-67 was used as a cell proliferation marker. The protocol was conducted as follows: Cells were fixed in 4% paraformaldehyde for 10 minutes and then washed with phosphate-buffered saline (PBS) pH 7.2; membranes were treated with PBS with triton X-100 for 10 minutes, and incubated with Powerblock T.M. (Biogenex) for 5 minutes to block non-specific binding sites; the primary antibody was incubated overnight at 4°C in a moist chamber. The following day, they were washed with PBS and incubated with biotinylated (Multilink T.M., Biogenex) secondary antibody for 30 minutes at room temperature, and then with fluorescein streptavidin (FITC) (Vector Labs) for 60 minutes; finally they were washed with PBS pH 8.2. Lastly, they were mounted and analyzed under a Nikon Eclipse E400 microscope.

Statistical analysis

An ANOVA model for repeated measures was used. Cell count was taken as a response variable. Explanatory variables included treatment (two levels: dexamethasone treatment and control with no steroid treatment) and explant type (two levels: linear section and circular section), both of which are fixed factors. The five measurements used were chronologically ordered along a 5-consecutive-day sequence. A comparison was made of the individual (random) factor effect on cell count. Main effects (effects of fixed factors) and simple effects (effects of factor and treatment combinations) were estimated. The number of Ki-67 positive nuclei was regarded as a quantitative variable and expressed as a mean ± standard deviation. Differences between means were assessed with Student's t-test. Statistical analysis was carried out with SPSS software, v. 20. $P< 0.05$ values were considered statistically significant.

Results

The dura used for the study was obtained from 6 subjects in 2 decompressive craniectomies and 4 meningioma resections. Explant

culture or subculture procedures were performed according to the size of the tissue specimen. Cell growth was seen in 100% of the specimens.

Characterization of cultured cells

The first cells appeared on the edge of the explant, were elongated in shape, and migrated from the tissue towards the culture plate. They had a single nucleus and variable morphology, some of them being clearly elongated and some others star-shaped with multiple protruding pseudopods and progressively elongated cytoplasm as they grow and cluster radially forming spindles which eventually covered the whole culture plate (Figure 1).

The core was a single nucleus comprising 2 or 3 visible nucleoli; cytoplasm was acidophilic and fibrillary, and contained PAS-positive granules. Cytoplasms in cultured cells were positive for desmin and vimentin staining. Magnified views at high power showed the intermediate filaments stained by these antibodies, and the negative nucleus (Figure 1). Morphology, positive staining of intermediate filaments and negative staining for GFAP (glial marker), CK (epithelial marker) and Neu-N (neuronal marker) suggest a fibroblast phenotype.

Cell count and dexamethasone effect

Explant culture

Cell count was assessed to determine the occurrence of differences between the two explant models in both the control experiment and the steroid-treated one. The mean count estimate for the “linear section” explant was 25.17 cells (95% CI 16.06 – 34.27), and 20.97 (95% CI 11.86 – 30.07) for the “circular section” explant. The difference between the mean of the two models stood at 4.2 (95% CI -8.67 – 17.07) with no statistically significant differences between them (p 0.473; n=3) (Figure 2). Given the absence of differences, results to assess the effect of dexamethasone are reported in a unified manner for both models of explant.

The mean cell count estimate for the steroid-treated explant was 12.50 (95% CI 3.39 – 21.60), and 33.63 (95% CI 24.53 – 42.74) for the untreated control experiment. The difference between the two means was statistically significant, standing at 21.13 (95% CI 8.26 – 34.01, p 0.005; n=6). (Figure 3)

Subculture

In the primary culture, cells fused disorderly rather than in the spindle-like pattern of explants. Cell growth occurred in all specimens. In the control experiments, the 30-mm plates reached confluence by the fifth day.

The mean cell count estimate on the dexamethasone-treated plate was 370.5 (95% CI 302.65 – 438.35), and 497.05 (95% CI 429.2 – 564.9) on the control plate. The difference between the means was statistically significant, standing at 126.55(95% CI 30.595 – 222.505, p 0.022; n=3)(Figure 4 and 5).

Cell growth in explant and subculture

Ki-67 is a nuclear non-histone protein that is present during the active phases of cell proliferation (G1, S, G2, mitosis) but is absent from resting cells (G0). For this reason, it is a good marker of cell proliferation.

Both types of explant culture were negative for Ki-67 , i. e., no sign of cellular proliferation was found in any of the two cases. The subculture, however, was positive for this marker, with decreased expression occurring after dexamethasone treatment, as previously mentioned. The mean of positive nuclei per field (based on an average of 10 high-power fields) was 13.83±7.08 for the control subculture, and 6.78±3.92 for the dexamethasone-treated one, with a statistically significant difference (p=0.005).

Discussion

Dura mater biology has been extensively studied in animals, with a predominant focus on the dural role in skull embryogenesis and, consequently, in craniosynostosis pathogenesis[25,26]. The mechanism of dural healing, however, has been studied on animals basically with the aim of describing the use of duraplasty rather than the normal closure of the dura. To our knowledge, no studies so far have developed human models *in vitro* to focus on the normal mechanism of dural closure.

According to their morphology; arrangement; cytoplasmic positivity to desmin and vimentin; and negative staining for glia, epithelium and neuronal cell markers, the cells obtained for this study can be characterized as fibroblasts. This finding matches the *in vitro* models previously developed in animals.

There are two *in vitro* healing models –one in minipigs and one in rabbits. The model by Zhou and Schik *et al.* determines fibroblast cell migration towards the center of a defect created in the dura[20,21]. In both cases, the defect was covered in 7 days, and cells needed a collagen base to migrate. Conversely, untreated plates showed no growth whatsoever. Our study shows that human cells need no collagen cover to grow and reveals cell growth in both treated and untreated plates. Collagen is known to act as a chemotactic agent for fibroblasts, but its relative importance across species had never been studied. This is a significant finding because the value ascribed to collagen as a chemotactic agent is the main basis for the development of collagen mesh.

Dexamethasone is commonly used in the mediate and immediate neurosurgery postoperative period. Actually, many of its frequent uses (prevention of cranial nerve deficits, status post-decompressive craniectomy) have no empirical support; however, dexamethasone is widely administered, even despite the evidence that it can severely affect the neurosurgical outcome[27,28,22]. In a randomized prospective controlled study in 222 subjects following posterior fossa surgery, Marion *et al.* reported a higher incidence of CSF leaks but found none of the benefits

(prevention of cranial nerve deficits, reduction of headache) presumably obtained with dexamethasone[29].

In patients with primary or metastatic brain tumors, steroids improve quality of life and neurological deficits; however, the benefit is mainly seen in subjects with partial resections, or in patients having a biopsy or receiving radiation therapy. Conversely, in patients with complete resections, problems outnumber use-related benefits[28,23,28].

Dexamethasone is known to inhibit the growth of normal and transformed fibroblasts. Its potential deleterious effect on dural closure has been clinically established by Marion *et al*. [30,31,32,29]. Ours is the first study to show the effect of dexamethasone on human dural fibroblasts at a cell level. These findings highlight the importance of preventing dexamethasone overuse, administering minimal doses of the drug and discontinuing it as soon as possible.

At the laboratory we are currently studying the importance of fibroblast growth factor-2 (FGF-2) and transforming growth factor beta (TGF-β) as promoters of dural closure[25,26,33]. We are also using the model to determine dural fibroblast behavior with different autologous tissues (muscle, fat, fascia), and their interaction with some of the different synthetic elements that are commonly used for dural closure (synthetic dura mater, Spongostant TM, oxidized cellulose mesh).

We developed the first *in vitro* model of human dural healing, which we see as a possibility to learn about this repair mechanism and produce better strategies to prevent and manage CSF leaks. In line with several other reports, our initial conclusion concerning the effect of dexamethasone highlights the importance of carefully deciding which subjects should be treated postoperatively with this drug, giving them the smallest dose possible and discontinuing it promptly. This model is used at our laboratory to clarify the biological basis of different duraplasties commonly employed for the surgical closure of the dura.

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Figure captions

Figure 1. Explant culture, staining with hematoxylin and eosin: Spindle-shaped mononucleated cells fused in bundles, and acid and fibrillary cytoplasm (40X) (A), (B) subculture, star-shaped cells with multiple protruding pseudopods (100X). *Indirect immunofluorescence for desmin and vimentin:* Cells are strongly positive for desmin (C) and vimentin (D) in cytoplasm marking intermediate filaments, which are typical of fibroblasts (100X). E and F show positive staining for desmin and vimentin, nuclei are stained with acridine orange to show that all cultured cells are positive for these markers.

Figure 2. Comparison of two explant models. The y-axis shows the total number of cells invading the defect in the circular section model (•) and in the linear section model (❑). The x-axis represents the time expressed in days. No significant differences were observed between the two models. Bars represent the 95% CI.

Figure 3. Cell count in control explant cultures and dexamethasone-treated explant cultures. The y-axis shows the total cell count invading the defect. The x-axis represents the time expressed in days for the control culture (❑) and the dexamethasone-treated culture (•). The graph shows a statistically significant reduction in cell count. Bars represent the 95% CI.

Figure 4. Cell count in control subcultures and dexamethasone-treated subcultures. This expresses the cell count (as an average of 10 fields at 10x power), both on untreated (❑) and dexamethasone-treated plates(•). Cell count is significantly higher in untreated control plates. Bars represent the 95% CI.

Figure 5. Effects of dexamethasone treatment. A and B show the effect of dexamethasone treatment (B) versus the absence of treatment in the control experiment (A) in terms of cell count for the subculture, staining with hematoxylin and eosin, 4X.

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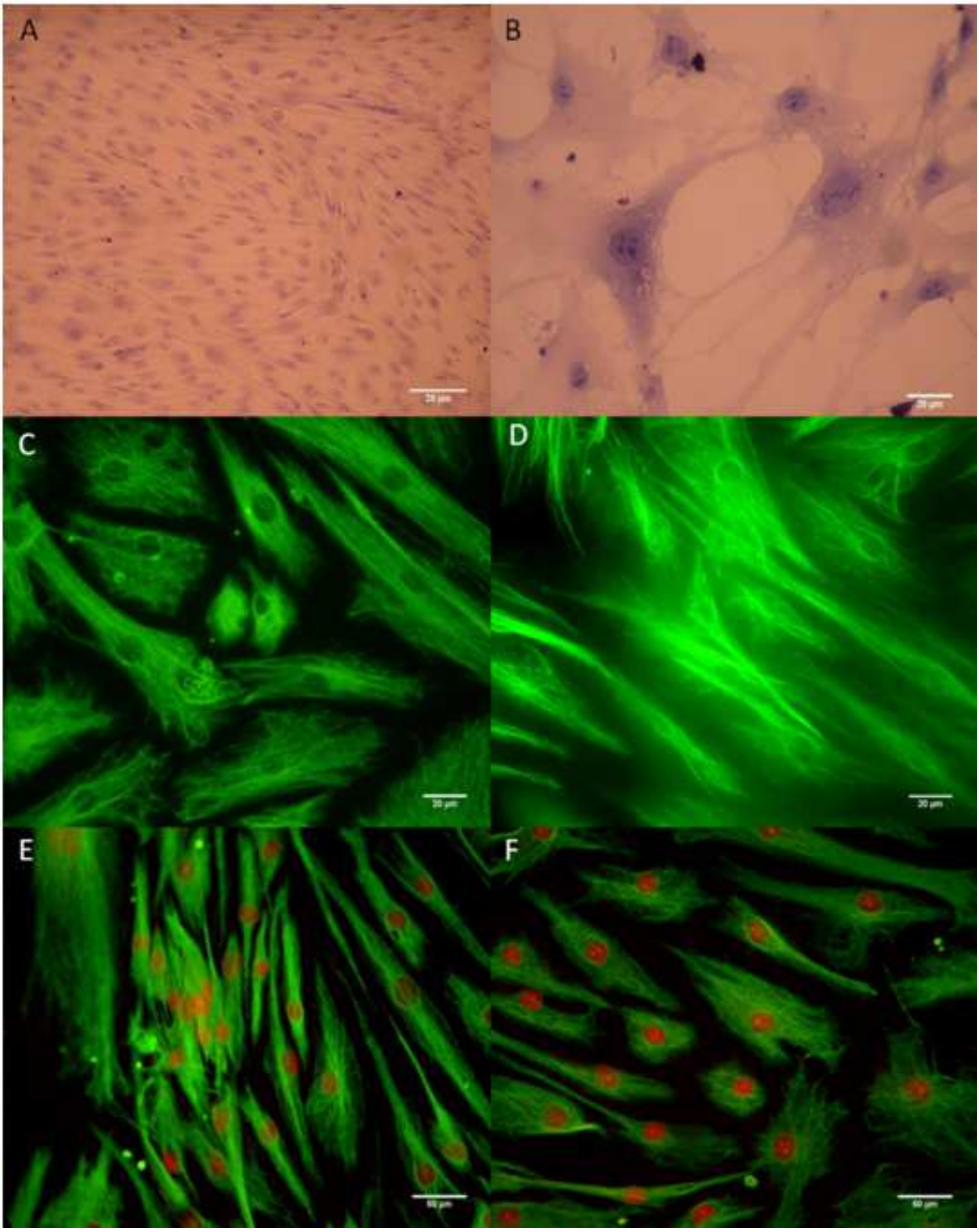

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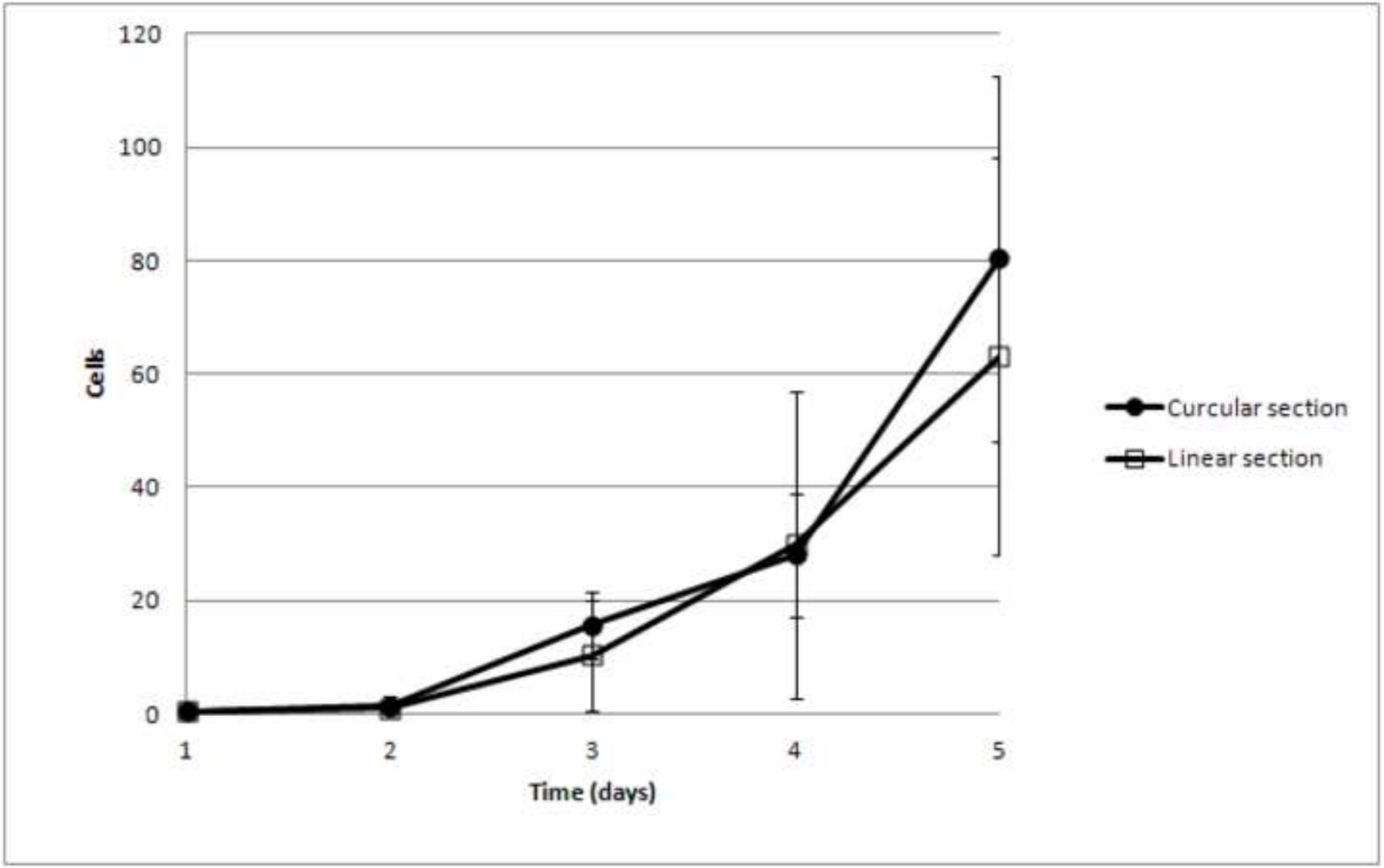

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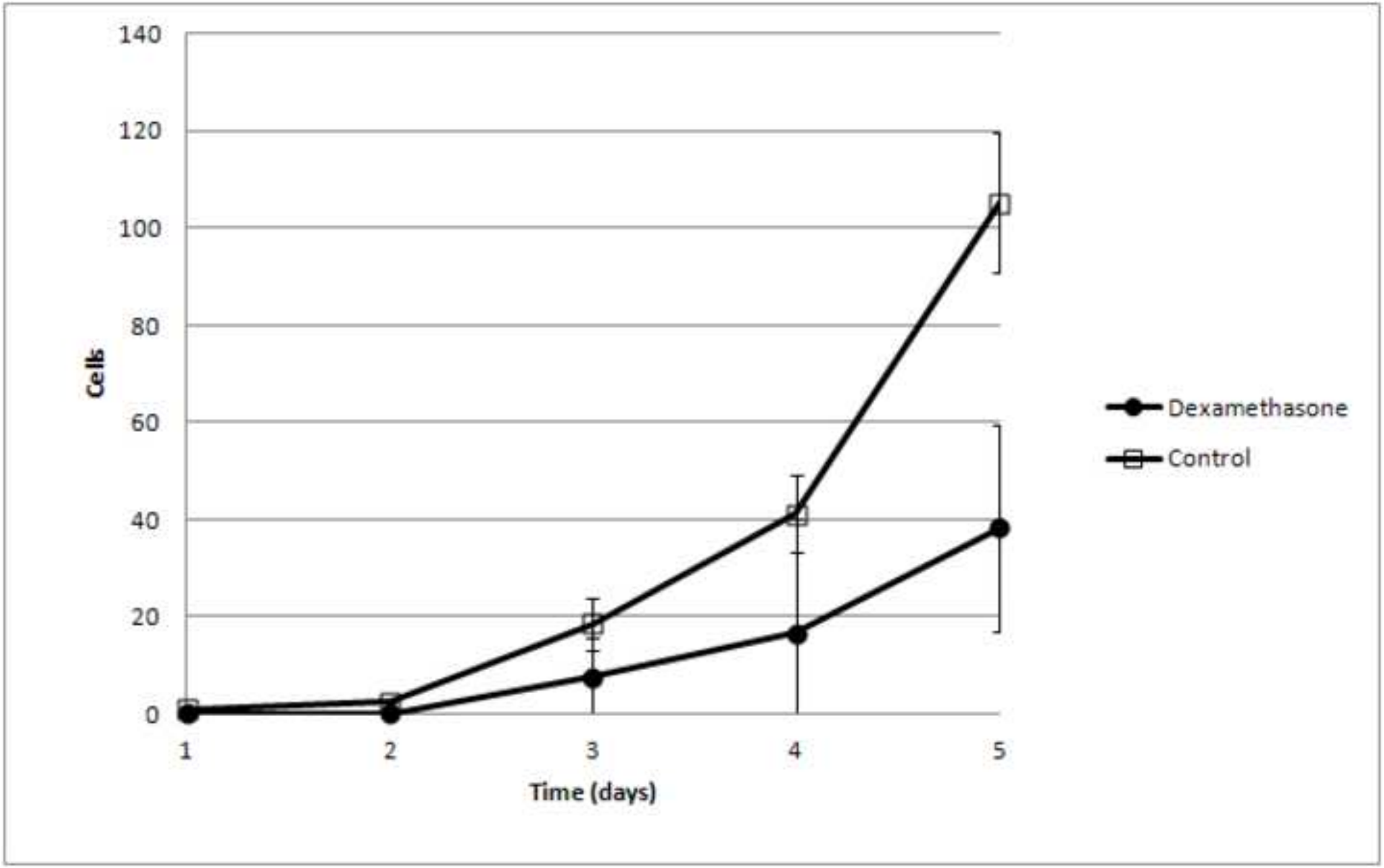

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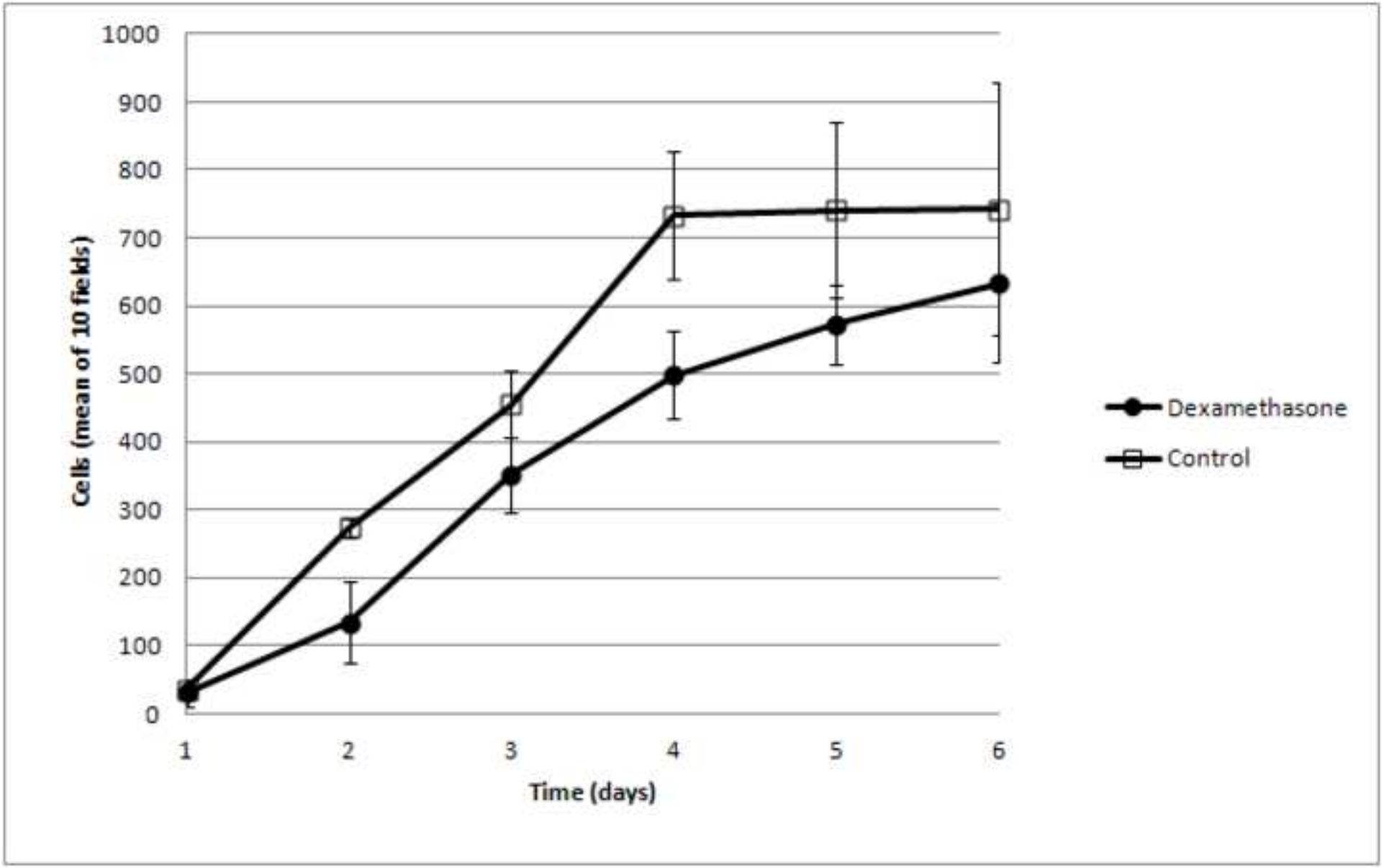

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