Assessing the role of selected growth factors and cytostatic agents in an *in vitro* model of human dura mater healing

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Objectives: Cerebrospinal fluid (CSF) leaks are a common concern in skull base surgery. Appropriate dural healing is crucial to prevent CSF leaks but the entire process has been barely understood so far. Here, we review the impact of growth factors and chemotherapeutic agents on an explant culture of human dural fibroblasts and a 3D subculture grown in a collagen mesh scaffold.
Methods: Human dural specimens were harvested during surgical procedures where they would not be further used therapeutically or diagnostically. Explant cultures were grown in Petri dishes, and subcultures were grown in collagen mesh scaffolds. Insulin, fibroblast growth factor type 2 (FGF-2), and human serum were analyzed for their effect as growth factors, whereas mitomycin C, vincristine, and colchicine were analyzed for their role as inhibitors. Cell count was used as a parameter to assess the effects of these factors. In addition, the effects of human serum were assessed using collagen mesh scaffolds.
Results: Insulin, FGF-2, and human serum increased culture cell count; human serum also achieved an increased number of viable fibroblasts embedded in a collagen mesh. Mitomycin C, which is a mitosis inhibitor, showed no significant effect on cell count, whereas colchicine and vincristine, which inhibit both mitosis and migration, resulted in cell growth suppression.
Discussion: In our model, dural defect closure is achieved through cell migration rather than through cell growth. Adding growth factors to the dural suture line or into a collagen mesh might prove useful to stimulate dural closure.

Keywords: Dura mater, CSF leaks, Chemotherapy, Growth factors

Introduction

Cerebrospinal fluid (CSF) leaks represent a common and severe complication in neurosurgery, with an incidence rising to 10% in posterior fossa surgery even in health care sites where procedures are performed in great numbers.[1,2] Cerebrospinal fluid leaks are associated with meningitis, pneumocephalus, longer hospital stay, and even death. In many patients, reoperation is needed to achieve closure of the dural gap.[3–5]

Understanding of dural healing and tissue neoformation over the dural grafts, which are usually used for duraplasty, is still partial. Fibroblast growth factor type 2 (FGF-2) and transforming growth factor beta (TGF-beta) have been shown to mediate fibroblast growth in skin healing models; additionally, there is evidence that porcine dural fibroblasts respond to insulin whereas murine fibroblasts respond to FGF-2.[6–9] The role and potential effect of these molecules on human dural fibroblasts remains unknown. The effect of these molecules is relevant when it is defining normal dural closure and the potential utilization as closure stimulating factors.

Human serum is a source of growth factors and hormones.[10] It has been used extensively in the management of skin ulcerations because it is inexpensive and easily obtainable.[11] Its effect on dural closure is unknown; however, based on the previously described features, it can be regarded as a promising option to stimulate the dural closure process.

Chemotherapy is known to have deleterious effects on the closure of surgical wounds.[12,13] Many

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cytostatic drugs inhibit cell growth by preventing DNA synthesis at some points, whereas some other drugs prevent cytoskeleton formation and plasticity. Taxanes and vinca alkaloids inhibit microtubule formation or produce excessive microtubule stabilization; as a result, cell migration is suppressed and cell growth inhibited. The potentially differential effect that vinca alkaloids and taxanes have on dural closure versus exclusively mitotic inhibitors is unknown.

We have recently reported on an *in vitro* model of human dural closure and described the deleterious effect of steroids in this process.[14] Here, we will show how various growth factors, such as FGF-2, insulin, and human serum, as well as cytostatic or chemotaxis-inhibiting agents (mitomycin C, vincristine, and colchicine), impact on the healing process we modeled in the laboratory.

We will also describe how human serum can stimulate dural fibroblast growth in a 3D culture with a collagen polymer – commonly used for closure – serving as scaffold.

Our hypothesis is that migration plays a major role in dural healing and, in our model, growth factors can promote cell migration. Cytostatic agents that impair the function of the cytoskeleton may have a deeper impact in dural healing than those that only affect mitosis.

Materials and Methods

Materials

Fibroblast growth factor, insulin, and human serum were obtained from Invitrogen™ (Carlsbad, California, USA); vincristine, colchicine, and mytomicine C were acquired from MerckMillipore™ (Darmstadt, Germany).

Methods

Specimen collection

Human dural specimens were obtained in decompressive craniectomies, where a small fragment of dura could be resected; the duraplasty was closed using a collagen mesh, and the small dural excision did not change the closing technique. Informed consent was obtained in every case and the protocol was approved by the Institutional Review Board. Specimens were kept at 4°C in sterile saline solution overnight to be processed at the laboratory the following morning.

Explant culture

The tissue was processed as previously described.[14] Specimens were washed with Dulbecco's modified Eagle's medium (D-MEM, Gibco Life Sciences, Langley, OK, USA) and debrided with a sterilized swab to remove blood and tissue. After debridement, specimens were fragmented into 4×4 mm squares. A central circular section (3 mm in diameter) was cut to allow cells to migrate from the periphery inwards to the center (Fig. 1). Fragments with the circular defect were used to prepare the explant culture, and circular fragments were used to prepare subcultures.

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), 200 mM of alpha-glutamine (Gibco), and 10% of bovine fetal serum (Bioser, Reston, Virginia, USA). Simple plates (Gibco) were used. Cultures were performed in a humidified atmosphere with 5% CO_2 at 37°C.

Growth curves were elaborated using growth factors and migration- and growth-inhibiting factors as described below.

Subcultures in collagen mesh

The circular fragments were used to perform subcultures in 30-mm plates (Nunc) under the above-mentioned 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 trypan blue exclusion method and 10 000 cells were seeded on 1-cm^2 fragments of synthetic dura mater consisting of bovine collagen (Duragen™, Integra Life Sciences, Plainsboro, NJ, USA). Twenty-four hours later, the matrix fragments were washed with medium; a control group of specimens was kept in 1% D-MEM + SFB, whereas a second group was treated with D-MEM + 20% adult human serum (Sigma). Specimens were analyzed at 48 and 96 hours time points, when the cell count found in the mesh and the viability of cells were assessed at each time point, as described below.

Anti-actin or anti-vimentin antibody immunofluorescence was performed (as described in our model) in order to check for cells in the mesh; then, the mesh was observed under a confocal microscope.

Cell count and viability

In explant cultures, the total amount of cells that have migrated toward the defect were quantified on a daily basis using an inverted microscope (Nikon TMS) for 5 consecutive days or until the cells completed the defect (with a surface of 3.95 cm^2). Vital staining with acridine orange and ethidium bromide (AO/EB) was performed to count the cells that had grown in the collagen meshes and to assess viability. The total cell count was thus established and assessed for viability, with viable cells staining green and non-viable cells staining red. Cell count in the subculture was expressed as the average of five fields at $10\times$ power. The cells were mounted and

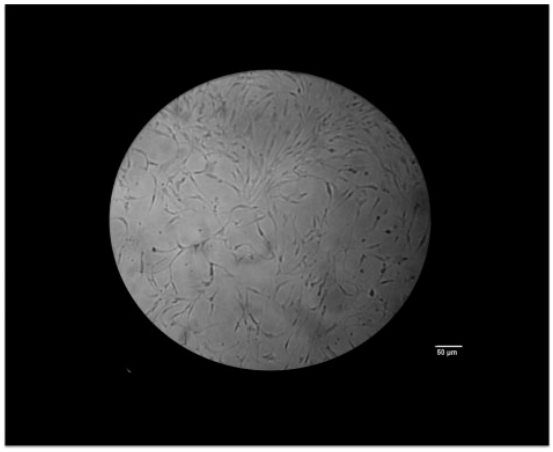

Figure 1 Explant culture of dural fibroblasts. A circular cut made on a dura mater sample is occupied by multiple fibroblasts in a Petri dish containing D-MEM and 10% bovine fetal serum (day 5).

observed under a Nikon Eclipse E400 fluorescence microscope. Photographs were taken to facilitate the counting process.

Treatment with growth factors

Fibroblast growth factor type 2 (10 ng/ml), insulin (1.2 μg/ml), and 20% human adult serum were assessed for their effects on the development of fibroblasts within the explant culture. A control curve was performed with D-MEM + 1% SFB, and a parallel curve was performed with the same medium plus growth factors. We always used paired specimens from the same patient. Both the medium and growth factors were replaced every 48 hours.

Treatment with cytostatic agents

Mitomycin C (10 μg/ml) was used for mitosis inhibition, vincristine (25 ng/ml), and colchicine (10^{-7} M) for migration and mitosis inhibition. Mitomycin was added for 2 hours; then the plate was washed with the control medium to prevent free-radical-induced cellular death. Several curves were performed: a control curve with D-MEM + 10% bovine fetal serum, a curve with each inhibitor, and a curve with both colchicine and mitomicine C in the same medium. The effect of each of the treatments was assessed separately and in combination. The effects of both mitomycin and colchicine were controlled by means of an explant subculture with cells growing in a plate without tissue. The effects of different drugs on cell count and viability in the subculture were assessed. Medium and drug replacement was performed every 48 hours.

Statistical analysis

An ANOVA model for repeated measures was used. Cell count was taken as a response variable. Factors used as explanatory variables included treatment (two levels: growth factor treatment and control, or cytostatic treatment and control). The five measurements used were chronologically ordered along a 5-consecutive-day sequence for explants, and two separate measures throughout 48 hours for cultures in collagen mesh. 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 or treatment combinations) were estimated. Statistical analysis was carried out with SPSS software, v. 20. $P < 0.05$ values were considered statistically significant.

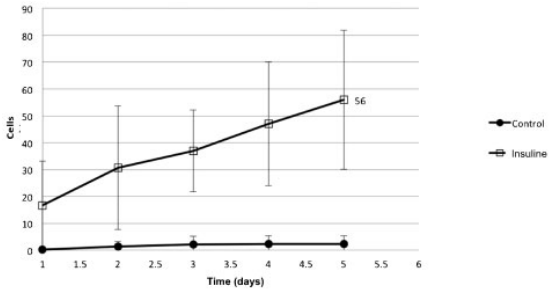

Figure 2 Cell growth curve for insulin versus control. The *y*-axis shows the total cell count; the *x*-axis shows the time, expressed in days. The addition of insulin is seen to increase cell count significantly. Dots represent the mean and bars represent the 95% CI, $n = 4$.

Results

The dura used for the study was obtained from 10 subjects, who underwent decompressive craniectomies. Cell growth was seen in 100% of the specimens.

Effect of growth factors on the explant culture

Cell growth was very scant in plates with D-MEM + 1% SFB, the mean being 1.62 (95% CI, −7.47 to 10.72); with the addition of FGF-2, insulin, and 20% human serum, cell growth increased markedly by comparison with the control, but no significant difference was seen between the treatments, the means being 54.33 (95% CI, 39.46–69.19), 37.46 (95% CI, 22.6–52.33), and 47.73 (95% CI, 30.42–65.04), in each case; $P < 0.05$ ($n = 4$) (Figs. 2–4, Table 1).

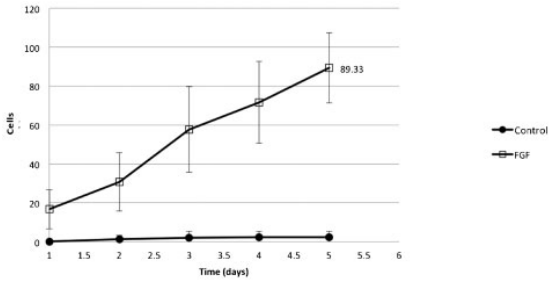

Figure 3 Cell growth curve for fibroblast growth factor (FGF) versus control. The *y*-axis shows the total cell count; the *x*-axis shows the time, expressed in days. The addition of FGF is seen to increase cell count significantly. Dots represent the mean and bars represent the 95% CI, $n = 4$.

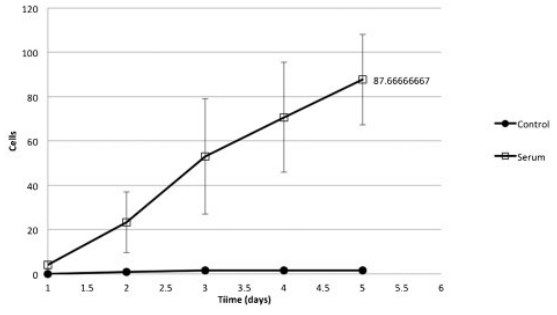

Figure 4 Cell growth curve for human serum versus control. The *y*-axis shows the total cell count; the *x*-axis shows the time, expressed in days. The addition of 20% human adult serum is seen to increase cell count significantly. Dots represent the mean and bars represent the 95% CI, $n = 4$.

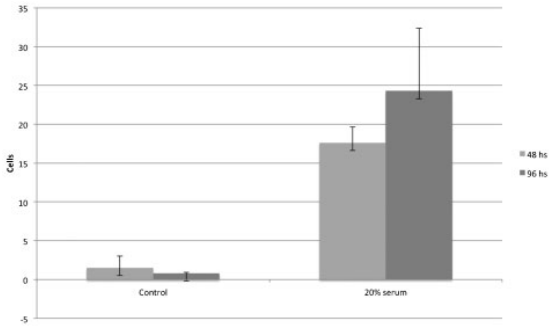

Figure 5 Cell viability in a collagen mesh. The *x*-axis shows the viable cell count; the addition of human serum increased viable cell count significantly.

Effect of 20% human serum on the viability of dural fibroblast seeded in a collagen mesh

The control experiment showed a low viable cell count at 48- and 96-hour time points (1.5, 95% CI, −0.09 to 3.09 and 0.08, 95% CI, 0.07–0.09, respectively). The addition of human serum increased viable cell count significantly (17.63, 95% CI, 15.58–19.68 and 24.3, 95% CI, 16.6–32.44) at 48- and 96-hour time points; $p < 0.001$, $n = 3$. Confocal microscopic observation of the cells marked with anti-vimentin antibodies showed cells distributed throughout the whole width of the mesh rather than on the mesh surface exclusively (Figs. 5 and 6). The mesh had no autofluorescence.

Effect of mitosis and migration inhibitors on the explant culture

The mean cell count in control specimens for 5 days (D-MEM + 10% bovine fetal serum) was 94.88 (95% CI, 70.01–119.35); the addition of mitomycin C lowered cell count, with a mean of 77.52 (95% CI, 52.45–102.59), which was not statistically significant ($p = 0.64$, $n = 3$). The addition of colchicine or vincristine actually lowered cell count at a mean of 1.13 (95% CI, −1.2 to 3.45) and 0.7 (95% CI −1.4 to 2.8), $p < 0.05$ ($n = 3$). Combined treatment with colchicine and mitomycin C showed no differences compared to the treatment with colchicine alone (Figs. 7–9).

Discussion

Appropriate dural closure and a consequent dural healing are paramount to reduce the incidence of CSF leaks. Despite the variety of materials available for use to close the dura, little is known about the basic mechanisms involved in the dural healing process.

In this study, we tested stimulating and inhibiting factors of dural healing on an *in vitro* model. In summary, the cell count was improved by adding insulin, FGF, and human serum. On the other hand, only chemotherapy agents inhibiting cellular migration had a significant effect on the numbers of cells appearing on the plate.

In vitro studies like ours characteristically entail a series of limitations; however, our findings can be used to formulate hypotheses, but their confirmation logically requires progression into *in vivo* models. On the other hand, ours is the first model that describes the process of dural healing using human dural cells at a cellular level, which can only be assessed by using these kinds of models. This is also a very good

Table 1 Effect of growth factors and cytostatics.

Treatment with growth factors			
	Drug	**Control**	***P***
FGF-2	54.33 (95% CI, 39.46–69.19)	1.62 (95% CI, −7.47 to 10.72)	<0.05
Insulin	37.46 (95% CI, 22.6–52.33)	1.62 (95% CI, −7.47 to 10.72)	<0.05
Serum	47.73 (95% CI, 30.42–65.04)	1.62 (95% CI, −7.47 to 10.72)	<0.05
Treatment with cytostatics			
	Drug	**Control**	***P***
Mytomicine	77.52 (95% CI, 52.45–102.59)	94.88 (95% CI, 70.01–119.35)	=0.64
Colchicine	1.13 (95% CI, −1.2 to 3.45)	94.88 (95% CI, 70.01–119.35)	<0.05
Vincristine	0.7 (95% CI −1.4 to 2.8)	94.88 (95% CI, 70.01–119.35)	<0.05

The cell count is expressed as mean and 95% CI for each treatment.
The influence of adding FGF, insulin. and 20% human serum factors in a medium containing 1% of FCS is shown. In experiments testing colchicine, vincristine, and mytomicine C. we used medium with 10% of FCS as control.
FGF: fibroblast growth factor and FCS: fetal calf serum.

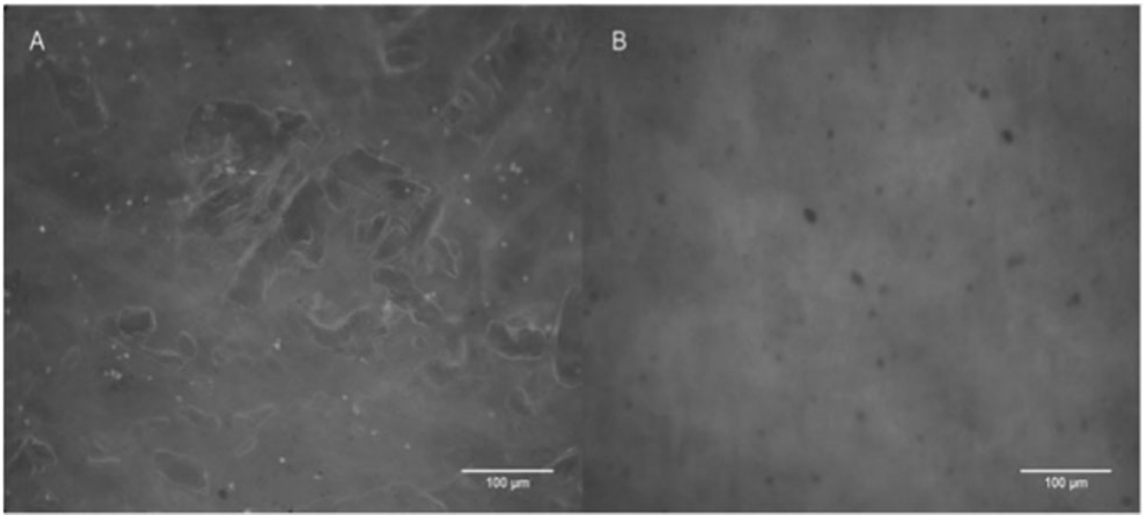

Figure 6 Cell viability in a collagen mesh. At the 48-hour time point, numerous viable cells were observed (green) in the mesh containing 20% human serum (A), but no cells were found in the plate containing only maintenance medium (B). Staining with acridine orange and ethidium bromide (AO/EB) (10 ×) was performed.

scenario to test individual variables in a very control environment with accurate controls.

The effect of growth factors

The role of insulin as a dural fibroblast growth stimulator was established in a rabbit model. In our model, as in Zhou *et al.*'s, insulin increased fibroblast migration from the dural margins.[8] Insulin is a good option for fibroblast growth stimulation because it is easily obtainable, its effect has a limited duration and its toxicity is restricted to hypoglycemia (possibly almost non-existent if applied locally).

Fibroblast growth factor type 2 is one of the main fibroblast growth signaling factors in several models of organ healing.[15,16] Its effect on dural cells has been assessed in *in vitro* rabbit models and *in vivo* rat models. Fibroblast growth factor type 2 was as effective in increasing fibroblast count as was insulin in Zhou *et al.*'s model.[8] Fibroblast growth factor type 2 was tested in rats for the treatment of surgically created spinal dural leaks.[7] Where FGF-2 doses were similar to the ones we used in our model at a local level, the dura healed more quickly compared to saline

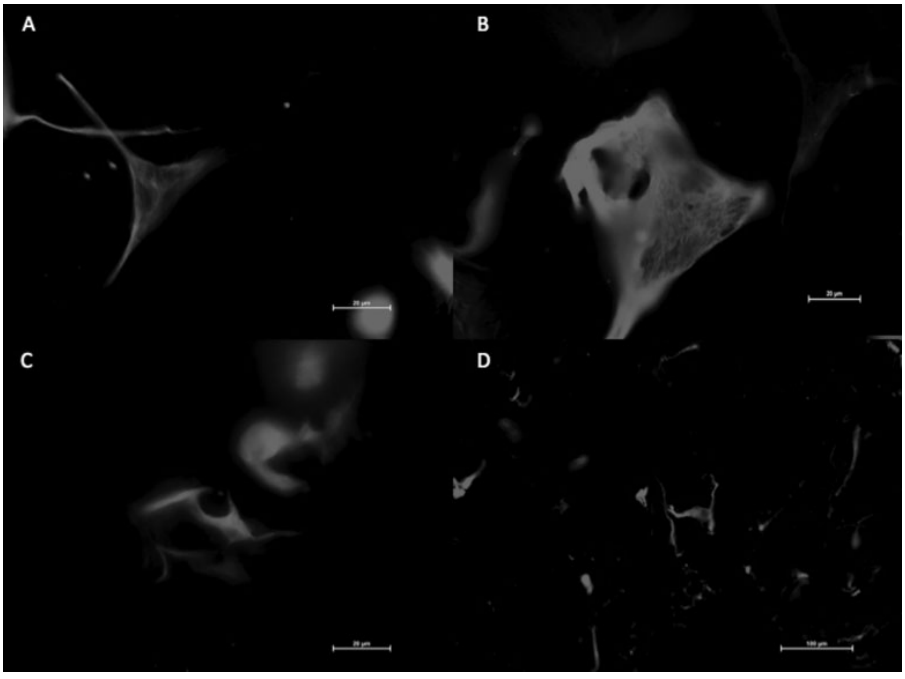

Figure 7 Normal shaped fibroblasts are seen throughout the mesh. Cells are marked by anti-vimentin antibodies.

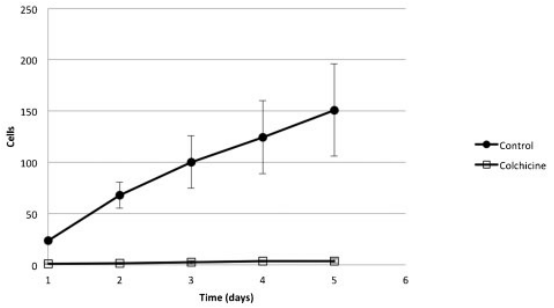

Figure 8 Effect of colchicine. The *y*-axis shows the cell count; the *x*-axis shows the time, expressed in days. Colchicine induced an almost complete inhibition of cell growth in the culture plate, $n = 6$.

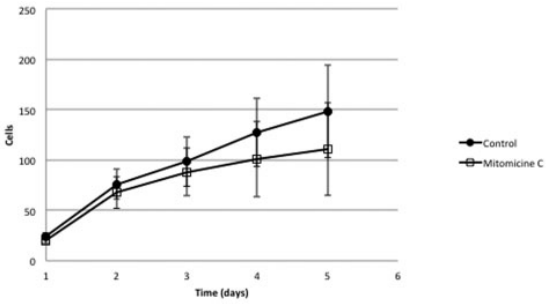

Figure 9 Effect of mitomycin. The *y*-axis shows the cell count; the *x*-axis shows the time, expressed in days. Mitomycin induced no statistically significant reduction in cell count, $n = 6$.

doses. Fibroblast growth factor type 2 is not used therapeutically, so it is less easily obtainable than insulin. Additionally, its safety profile still needs to be established. However, given its key role in dural healing, FGF-2 could be a good option for *in vivo* tests.

Human serum contains numerous growth factors.[11] In addition, it can be easily obtained, represents minimal risks for the patient, and is quite inexpensive. In this work, the performance of human serum has been assessed on human dural fibroblasts. The addition of 20% human serum increased cell count significantly in the explant culture and raised the viable cell count in the mesh of the 3D subculture. We worked with a collagen membrane that is commonly used to repair dural gaps; confocal microscopy showed that there were cells distributed throughout the collagen scaffold depth. In this sense, our model can prove useful to analyze different types of available scaffolds and the ability of human dural fibroblast to grow and proliferate within these scaffolds, which so far has only been shown at cell level in animals.

In a patient, the dural graft is adjacent to the subarachnoid space on one side and to the bone flap on the other, so it is slowly exposed to the extracellular medium, which looks like serum. The addition of human serum at the moment of replacing the flap in contact with the patient's dural margins could provide cells with a better medium for extracellular matrix migration, growth, and synthesis.

Growth factors have traditionally been used therapeutically; examples of such factors are the granulocyte-colony stimulating factors for neutropenia and the epidermal growth factor (EGF) for chemotherapy-induced mucositis.[17,18] Serum and derivatives such as platelet-rich plasma have also been used topically to treat skin wounds. Nevertheless, their use for dural closure has been scantily explored.

The use of growth factors may lead to excessive tissue growth, a risk of special concern within the intracranial space or of brain adhesion formation. Neoplasm formation or growth with these agents is always a concern, so they should be assessed for safety in the long term, although short half-life agents can help to prevent this effect. Despite these limitations, however, growth factors used to stimulate closure in *in vivo* models can potentially begin a new generation of dural defect repair options.

The effect of chemotherapy

Chemotherapy is known to be deleterious for wound closure; indeed, chemotherapy is seldom administered postoperatively for some days or weeks in order to enable appropriate healing of the surgical wound. Cytostatic agents have different action mechanisms; most cytostatic agents interfere with DNA synthesis, either by affecting DNA structure or by inhibiting the synthesis of its components. Taxanes and vinca alkaloids, however, inhibit mitosis by altering microtubule polymerization—they inhibit both cell replication and migration, as colchicine does.

Chemotherapy dose and treatment duration are actually known to be relevant; also, the wound has been shown to be most sensitive during the first 4 days. These data come mainly from abdominal surgery studies and are related to cytostatic agents that do not inhibit microtubule formation.[13,19]

According to our findings, microtubule inhibiting agents may be more detrimental to dural closure than exclusively antimitotic agents. This differential effect on dural wound or other wound-type closure has not been reported elsewhere. If these findings were confirmed in *in vivo* models, it would be possible to establish that chemotherapy can have a differential effect on wound closure depending on the agent's mechanism of action.

Taxanes are indicated after metastases removal (breast, lung, ovary, and melanoma). Removal in these cases is followed by whole-brain radiation therapy combined with taxanes. Knowing exactly which of the agents is responsible for the healing anomaly becomes difficult in such scenario.[20]

As for vinca alkaloid therapy, however, relevance may be more significant. Patients with oligoastrocytomas or anaplastic astrocytomas are commonly

treated with temozolomide, an alkylating agent that inhibits mitosis, or the combined regime procarbazine, CCNU, and vincristine (PCV).[21,22] If the findings in our model are confirmed, PCV could be considered stronger than temozolomide as a dural closure inhibitor. This should also be considered particularly in patients with skull base lesions with higher risk of CSF leaks.

The differential effect of chemotherapy depending on the agent's mechanism of action could be considered a new contribution. Wound closure does not depend on cell growth exclusively.

The results of our study could be applied to *in vivo* models to examine the effects of these factors when used as healing-stimulating agents and to assess whether certain types of chemotherapeutic agents are better suited for patients with a higher risk of CSF leaks.

Conclusion

Stimulating factors improve cell counting in an *in vitro* dural healing model and thus could potentially be either embedded in closure materials or applied over the dural suture line to prevent CSF leaks. On the other hand, chemotherapeutic agents that affect cellular migration have a much deeper effect on cell counting that drugs that only impair mitosis; this differential effect may change how we understand the way in which chemotherapy affects wound closure.

Disclaimer Statements

Contributors Ezequiel Goldschmidt designed the protocol, wrote the manuscript, and performed most of the experiments; Marcelo Ielpi, Monica Loresi, and Maximiliano Dadamo performed some of the experiments, analyzed the results, and reviewed the manuscript; Diego Giunta helped in the design of the protocol, performed the statistical analysis, and reviewed the manuscript; Antonio Carrizo, Pablo Ajler, and Claudio Yampolsky help in the developing of the protocol, contributed in the discussion, and revised the manuscript; Pablo Argibay directed the entire project, revising every stage of the process, and he contributed in the writing process and interpreted the results with the first author.

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Conflicts of interest The authors have no conflict of interest.

Ethics approval The protocol was approved by the Institutional Review Board.

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