Autologous Fibroblast Culture in the Repair of Aging Skin

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BACKGROUND Human cell cultures are being developed to replace various body tissues.

OBJECTIVE To assess the safety and efficacy of dermal regeneration with the injection of young autologous fibroblasts obtained from culture containing serum from patients themselves.

MATERIALS AND METHODS Dermal tissue from the groin of five patients was cultivated in M199 medium supplemented with 10% human serum. Four population doublings were obtained. The fibroblasts were injected intradermally into forehead wrinkles and periorbital and paranasal areas.

RESULT At the fourth population doubling, a mean of 3.85×10^6 cells/mL was obtained; viability was 98%. Sixty days after completing treatment, with four injections given at 15-day intervals, periorbital tonicity had improved significantly, although the quantity of fibroblasts used resulted in little improvement to surface lines and no improvement at all in deeper wrinkles. After 6 months, no further changes were found beyond the initial results obtained.

CONCLUSION Injection of skin fibroblasts cultivated in medium supplemented with human serum is a viable technique and provokes no side effects. Four injections given at 15-day intervals containing a total of 6.4×10^6 fibroblasts/mL resulted in significant improvement in periorbital skin flaccidity. Further studies should be conducted with larger sample sizes.

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Skin aging is the consequence of a reduction in the number of fibroblasts and extracellular matrix and a decrease in skin elasticity and tonus,[1] resulting in the formation of wrinkles.

Implants of bovine collagen, hyaluronic acid, polymethylmethacrylate, liquid silicone, and polylactic acid may provoke adverse events including misplacement, allergy, nodules, necrosis, abscesses, and rejection.[2–4]

Injections of cultured autologous fibroblasts have recently been proposed for the treatment of wrinkles, scars,[5] wounds,[6] and subcutaneous atrophy,[7] but the use of fetal bovine serum (FBS) in fibroblast culture medium may increase the risk of infection from bovine diseases or of a reaction to foreign proteins.[8] Cell division, and consequently the number of fibroblasts, has been shown to be greater in autologous dermal fibroblasts cultivated in human serum than in FBS.[8]

The objective of this study was to assess the safety and efficacy of the injection of autologous fibroblasts cultivated in serum from patients themselves for dermal repair of skin flaccidity and wrinkles.

Materials and Methods

This study conformed to the ethical guidelines described in the 1975 Declaration of Helsinki. The Internal Review Board of the University of Santo

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Amaro approved the protocol under approval letter # 030/2009 before initiation of the study. All subjects signed an informed consent form before being enrolled.

Eight nonsmoking women aged 45 to 65 with flaccid periorbital skin and wrinkles were selected, five composing the study group and three a control group. A biopsy was performed on the patients in the study group on 1 cm^3 of skin in the groin region, an area that is protected from external injury. Lidocaine without a vasoconstrictor agent was injected before removal of the tissue sample. Immediately after removal, while still in a surgical environment, the tissue sample was washed with phosphate buffered saline (PBS) supplemented with 1% penicillin/streptomycin (LGC, Cotia, SP, Brazil). The sample was then transported to the cell culture laboratory in a tube containing Dulbecco's phosphate buffered saline (Cotia), ethylenediaminetetraacetic acid (UT), and enzymatic solution (Hyqtase—Hyclone, Logan, UT), in which it remained for 3 to 4 hours at 37°C. Next, the dermis was mechanically separated from the epidermis and the hair follicles and then fragmented and transferred into 25-cm^2 culture flasks and incubated for 30 minutes at 37°C with 5% carbon dioxide (CO_2) in air. To obtain the autologous serum, a 45-mL sample of blood was withdrawn from each patient into a dry tube containing a serum separator gel with clot activator (Becton Dickinson , Franklin Lakes, NJ). Serum separation was completed after centrifugation at 2,000 rpm for 10 minutes. After this procedure, 5 mL of culture medium containing L-amino acids, Earle's salts, and sodium bicarbonate (M199 LGC), supplemented with 10% human serum from the patients themselves, were added to the culture flasks containing the tissue fragments. Cell culture was performed at a temperature of 37°C with 5% CO_2 in humidified air.[9]

The culture medium was changed every 2 days until cell expansion and every 4 days thereafter. Fibroblast expansion up to the fourth passage ensured that no genetic alterations occurred to the cells.[10] After the primary culture reached 70% confluence, the cells were treated with 0.25% trypsin solution (LGC), washed three times with PBS, and centrifuged at 1,500 rpm for 5 minutes. The cell pellet was resuspended in 2 mL of PBS and divided into two aliquots: 1 mL for expansion and 1 mL for injection. The aliquot for expansion was cultivated in a 75-mL cell culture flask containing 10 mL of M199 and 20% human serum, constituting the first cell passage (first population doubling). The culture medium containing the cells in expansion was changed every 4 days, and when confluence of 70% was reached, the cells at first population doubling were once again submitted to the process of trypsinization, with 50% of the cells being used for injection and the remainder for expansion until completion of the second population doubling. This process was repeated up to the fourth population doubling, when the entire cell content was used for injection.[11]

The periorbital region was swabbed with 70% alcohol, after which a topical anesthetic cream containing 4% lidocaine (Dermonax Biossintética, São Paulo, SP, Brazil) was applied 30 minutes before the injection of fibroblasts into the skin. A 1-mL syringe and a 30-G 0.5-inch needle were used. Injections into the superficial dermis were performed using a retrograde linear threading technique. Injections were given in the forehead wrinkles, perioral wrinkles, nasolabial fold, chin, and periorbital skin over a total of four sessions, with a minimal interval of 15 days between each session. In the control group, only PBS, the vehicle used for the cultured fibroblasts, was injected.

The results were analyzed using a previously described image method.[12] A digital camera (Sony Steady Shot DSC-H2, Atsugi-shi, Kanagawa, Japan) was used to obtain the images. Two physicians and the patients themselves evaluated and classified improvement in flaccidity, surface lines, and deep wrinkles as no improvement, mild, good, or excellent.

The Newman–Keuls test was used in the statistical analysis.

Data were collected 15 days after each application and 60 and 180 days after the end of treatment on the immediate and late occurrence of edema, erythema, and pain at the site of application, as well as the occurrence of any complications such as allergy, rejection, ischemia, necrosis, nodules, abscesses, and tumors.

Results

After 6 days in primary culture, the tissue fragment in the 25-mL culture flask had acquired a monolayer of fibroblast cells (Figure 1).

The first injection was given after the first passage (first population doubling), with a mean of 0.16×10^6 cells/mL. Injections were given every 15 days at the second, third, and fourth population doublings. The cell population increased progressively, with a mean of 0.70×10^6 cells/mL at the second, 1.70×10^6 cells/mL at the third, and 3.85×10^6 cells/mL at the fourth population doubling. A statistically significant difference was found in the number of fibroblasts between the fourth and third population doublings (Figure 2).

The cells stained with trypan blue solution and counted in a Neubauer chamber resulted in 98% viable cells at the fourth population doubling (Figure 3).

Sixty days after completing the four intradermal injections at 15-day intervals, significant improvement was found in periorbital tonicity in two cases (Figures 4 and 5), with slight improvement in surface lines in one case and no improvement at all in deeper wrinkles. Six months after completion of treatment, no further changes were found. In the control group, no improvement was seen in

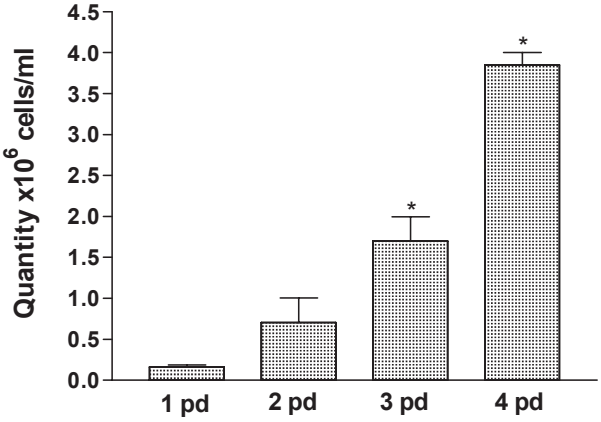


Figure 2. Histogram showing cell expansion of fibroblasts of human skin (mean ± standard error) in five women after the first (1 pd), second (2 pd), third (3 pd), and fourth (4 pd) passage. * $p \leq .05$ (3 pd vs 4 pd) (Newman–Keuls test).

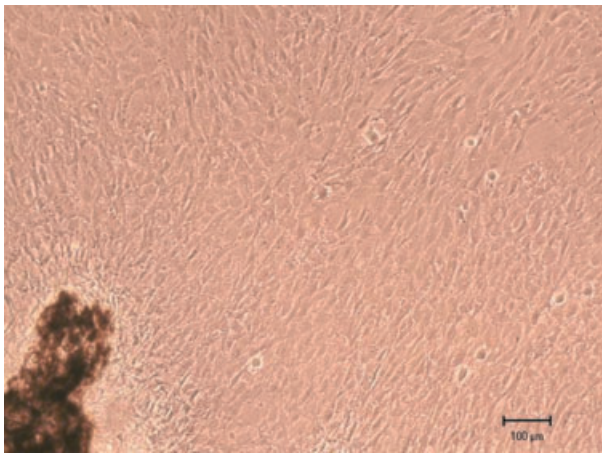


Figure 1. Primary culture of human skin after 6 days of culture (no stain, 100×).

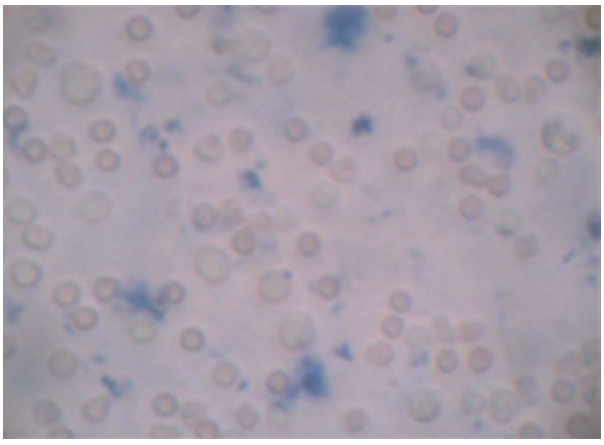
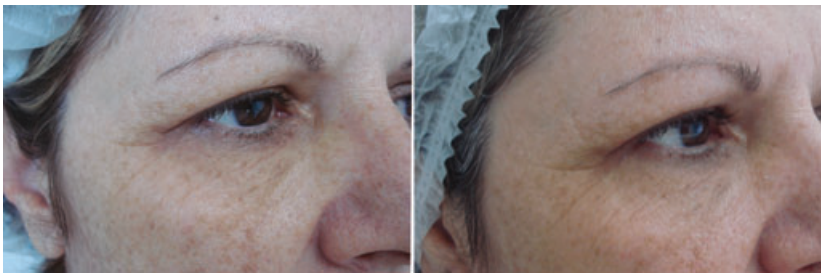


Figure 3. Test of cell viability (trypan blue, 400×).

Figures 4 and 5. Test group: Before and 60 days after the end of treatment consisting of four injections of autologous cultured fibroblasts applied at 15-day intervals into the lax skin of the lower eyelid.



Figures 6 and 7. Control group: Before and 60 days after the end of phosphate buffered saline solution injections.

tonicity, superficial lines, in deep wrinkles (Figures 6 and 7).

Immediate side effects in the study and control groups consisted of pain at the moment of injection, erythema, and edema proportional to the volume of liquid injected, which took from 24 to 48 hours to disappear. These side effects did not become chronic in any cases. None of the patients who were injected with the cultivated autologous fibroblasts experienced any complications.

Discussion

In 2002, Catherine Verfaillie demonstrated the presence of adult stem cells in all tissues of the human body.[13]

Since the first transplants of adult stem cells from bone marrow in the 1960s[14] and the transplant of adult stem cells from umbilical cord blood in 1988,[15] there is no record in the scientific literature of any case of tumor formation resulting from the injection of these cells, the technique being considered safe at an expansion of up to the fourth population doubling.[10]

Cell expansion was performed using a tissue sample measuring 1 cm^3. Fibroblasts and adult stem cells divide through asymmetric division[16] (i.e., the replicating cell gives rise to one adult stem cell and one specialized stem cell [fibroblast]), suggesting the continuity of the process of cell division until complete differentiation of the stem cells.

The rate of fibroblast multiplication in a controlled in vitro environment is high, being stimulated by growth factors, vitamins, and amino acids[17] until the desired number of cells is reached. For this reason, there was a higher number of mother flasks at the beginning of the procedure in the present study than the number of those that reached the fourth population doubling.

These cultures resulted in rapid cell expansion and a higher percentage of live cells with the human serum technique than with the use of fetal bovine serum.

A total of 6.4×10^6 cells/mL was injected, resulting in a good response in the periorbital region, although surface wrinkles and deeper wrinkles may require a greater number of fibroblasts, as shown in a study[18] in which 20 million cells/mL were injected, resulting in intradermal proliferation for periods that ranged from 12 to 48 months. This effect was more significant than the results achieved with the injection of placebo. The effect produced mechanically by the prick of the injection or subcision by the liquid injected failed to improve tonus or wrinkles in the control group.

Conclusion

Injections of skin fibroblast culture supplemented with human serum were shown to represent a viable option for the repair of aging skin and were found to cause no side effects. Four injections administered at 15-day intervals containing a total of 6.4×10^6 fibroblasts/mL led to significant improvement in periorbital skin flaccidity but failed to improve the appearance of surface lines or deeper wrinkles during the period evaluated. Further studies with larger sample sizes are required.

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