GMP-compliant Manufacturing Process of an Advanced Therapy Medicinal Product Based on Conjunctival Epithelial Cells

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Methodology

Keywords: Conjunctiva, stem cells, cell therapy, Advanced Therapy Medicinal Product, Good Manufacturing Practices

Posted Date: December 9th, 2020

DOI: https://doi.org/10.21203/rs.3.rs-122190/v1

Abstract

Background. Conjunctival epithelial stem cell therapy represents a potential and valuable therapeutic option for people suffering from conjunctival disorders. We recently developed a research protocol for the *ex vivo* cultivation of conjunctival epithelial cells. However, manufacturing and release of any Advanced Therapy Medicinal Product (ATMP) must be designed and planned according to the Good Manufacturing Practices (GMPs) guidelines. GMPs require the development and validation of properly defined manufacturing processes, analysis methods and process validations. Our previous experience with GMP-cultured corneal epithelial stem cells for clinical application on patients with limbal stem cell deficiency led us to set up a protocol for cultivation of conjunctival cells with standards complying with the requests for clinical studies. The major challenge for cell-based products is to develop manufacturing processes while maintaining the critical quality parameters in terms of safety, identity, purity and potency.

Results. The manufacturing process was re-designed in order to include all the quality control assays needed for the release of any ATMP, i.e., sterility, morphology, cell viability, dose, cell identity and impurities, potency, lack of pyrogens, mycoplasma and viral detection. Methods and acceptance values were set for all the assays. Quality control assays to evaluate safety and efficacy were also investigated.

Conclusion. Here, we describe the main phases of the manufacturing process of a conjunctival stem cell-based product to use in clinical applications. Such characterization is crucial for the preparation of documents and dossiers needed by the competent authorities to start a phase I clinical study on patients with conjunctival disorders. The procedure necessary to reach the *marketing authorization* of such a new cell-based product is still long, but, if reliable and validated, we believe that, in the near future, patients with conjunctival disorders might have a new treatment based on transplantation of autologous cultured conjunctival epithelial stem cells.

Introduction

According to the current European regulations, stem cell-based products are defined as Advanced Therapy Medicinal Products (ATMPs). Production, release and application of ATMPs are regulated by the Good Manufacturing Practices (GMP) guidelines and the manufacturing process has to be carefully defined in order to comply with the requests from the competent authorities [1, 2, 3]. As a consequence, the development and validation of properly defined manufacturing processes and analysis methods are of primary importance for successful translational research; the major challenge to commercialize cell-based products is developing manufacturing processes while maintaining the critical quality parameters [4]. The entire iter necessary to reach the *marketing authorization* of a new cell-based product is hard to realize. This probably explains why in Europe and USA few ATMPs have been approved [5].

In the last decades, progress in research and clinical applications of stem cells have led to revolutionary changes in medicine with substantial benefits to patients. Ophthalmology, in particular, has benefited

from stem cell-based treatments. Corneal epithelial stem cell transplantation is an example of the cell-based therapies that have been used successfully [6, 7, 8, 9].

Conjunctival epithelial stem cell therapy could also represent a potential and valuable therapeutic option for people suffering from conjunctival disorders [10, 11]. Recently, we developed a protocol for the *ex vivo* cultivation of conjunctival epithelial cells plated onto a GEL scaffold [10]. In vitro, conjunctival epithelial cells share similar features to corneal epithelial cells [10, 12, 13], and can be cultured using similar protocols. Our previous experience with the GMP-cultured corneal epithelial stem cells and their clinical application on patients with limbal stem cell deficiency [7] allowed us to set up a protocol for cultivation of conjunctival cells with standards that comply with the requests for clinical studies. In order to increase the safety of the final product, animal-free culturing conditions have been selected [10] and a new assay based on immunofluorescence set up to detect the potency of the cultures [14].

In this paper we describe the development and validation of the process needed to obtain a conjunctival stem cells-based product. The manufacturing process follows the GMP guidelines and additional assays have been set up in order to characterize the product in terms of safety, identity, purity and potency. Specifications for each analytical method have been defined. The final objective is the achievement of a marketing authorization for such ATPM and have finally a further treatment for the regeneration of diseased conjunctival ephitelia.

Methods

1. The process

1.1 Conjunctival epithelial cell grafts: the manufacturing process

The production protocol of conjunctival epithelial grafts was described previously [10]. Briefly, two processes are needed: (A) culturing of 3T3-J2 murine fibroblasts for preparation of feeder-layers used as support for conjunctival cell growth, and (B) culturing of conjunctival epithelial cells. Figure 1 is a schematic representation of both processes with all the steps required to achieve a transplantable graft. The protocols are described below.

The media used during the process are described in Table 1.

3T3-J2 cells (Fig. 1A) can be maintained in culture up to the 13th passage and are letally irradiated to obtain a feeder-layer (40,000 cells/cm^2). Conjunctival cells (Fig. 1B) are isolated from human conjunctival biopsies (starting material) after treatment with 0.05% trypsin/0.01% EDTA (TE) at 37 °C for 3–4 cycles of 30 minutes each. The collected cells are then counted in trypan blue and plated in one or more wells of a 24-well plate together with the feeder-layer in KM medium, until sub-confluence is reached (intermediate product). If the primary culture fails to reach confluence in the proper time (7–10 days), a

quick primary culture is carried out, by collecting the cells of the primary culture (TE enzymatic treatment), plating 1:1 with feeder layer and culturing in KM medium until confluence is reached. The secondary culture is then plated onto a fibrin glue gel scaffold (TISSEEL, Baxter, diluted at 3 UI/ml thrombin and at 44 mg/ml fibrin) without feeder layer and maintained in XF medium until confluence (final product). The cultures are incubated at 37 °C and 5% CO_2 in humidified atmosphere.

When the secondary culture onto the scaffold is confluent, the final product is ready for packaging and shipment. Before the shipmemt, quality control (QC) assays are performed, as shown in Fig. 2. Briefly, the exausted culture medium (XF) is collected and inoculated in the Bactec media to assess any microbial contamination. The graft is cut in two parts by using a punch of defined size. One part of the graft is washed, packed with TTL medium in a lens-holder and shipped for surgery, while the other part is inoculated in 4 ml of TTL medium for 2 hours, before performing the following analyses: (I) EP 2.6. sterility assay on TTL medium, (II) bacterial endotoxins assay (LAL test), (III) virus detection and (IV) immunostaining analyses for the assessment of identity, potency and impurity (after enzymatic dissociation of cells from the scaffold). The last QC assays require the preparation of the following samples: 15–20.000 cells are plated onto a slide using a cytospin centrifuge and fixed with 4% paraformaldehyde for 10 minutes before immunostaining is performed.

1.2 Supplying of materials

Reagents used throughout the *in vitro* expansion of conjunctival stem cells, if of animal origin, could introduce a risk of transmission of diseases or adventitious agents (e.g., prions). For this reason, the use of animal-derived reagents should be limited as much as possible or totally avoided. When these reagents need to be included in the manufacturing protocol, it is mandatory to certify that they are free from any contaminants or adventitious viruses. Our protocol was set up in order to comply with GMP requirements and include animal-free and GMP-grade reagents, as far as possible [10]. In Table 1, the media used in both processes (cultivation of 3T3-J2 and conjunctival cells) are described. Our current culturing protocol include two reagents of animal origin: sera (from calf and fetal bovine) and TE.

The European Medicines Agency (EMA) published specific guidelines for using bovine sera and porcine TE. The supplied sera must be sterile, suitable for cell culture for use in human and provided with certificate of Transmissible Spongiform Encephalopathy risk (characterized by the absence of pathogenic viruses because it originates from a bovine spongiform encephalopathy-free zone, like, for example, Australia). Our protocol involves the use of sera only during the first phase of the process, but not in the last step, and therefore serum is not present in the final product. The same holds true for TE, as not used in the final step of the process.

The presence of XF medium in the final step of the culture allows avoiding the use of both serum and feeder-layer [10]. Such modifications did not lead to reduced performances of the final product (on the contrary, an increasing performance was obtained, as shown in Bertolin 2019 [10]) while increasing its safety. However, residual feeder layer cells may be detected in the final product, as described afterwards.

2. Quality control assays used in the manufacturing process

Table 2 shows the "*in process controls*" performed according to GMP requirements [15] for (A) 3T3-J2 murine fibroblasts and (B) epithelial conjunctival cell cultures. The epithelial conjunctival cell manufacturing process includes controls on both the "starting material" (Step 1 and 2) and the "final product" (Step 3).

The QC assays for determination of viability, potency, impurity and identity are performed only after the graft has been packaged and shipped to the transplantation centre (Fig. 2). The final product is split into two parts, one of which is used to perform the analyses. The latter undergoes two enzymatic treatments [10] to isolate the cells forming the graft. To perform the vialibility assay, cells are diluited 1:1 in trypan blue solution and counted by means of a Neubauer counting chamber. Potency, impurity and identity tests are performed through immunofluorescence analysis, as previously described [10].

A. Sterility test on in-house prepared cell culture media

The assay we used is the EP 2.6.1, as indicated by the European Pharmacopeia [16]. Apseptic manufacture of the product (GMP grade) is supposed to prevent the risk of microbical contamination, moreover the complex of the manufacturing process makes this risk not impossible: bacteria, fungus, andventitious virus and mycoplasma contaminations could potentially be caused by starting materials, the facility, the staff, raw materials or the enviroment. The sterily tests are performed on each batch of homemade cell culture media.

B. Sterility test on exhausted cell culture media

The assay we used is the EP 2.6.27, as indicated by the European Pharmacopeia [16]. The media and protocols used to manipulate and culture the cells are by definition able to allow survival, if not proliferation, of living organism, so potentially supporting a rapid growth of microbial contamination. For this reason exhausted media sterility must be checked and must be performed in both processes of cell culturing included in the protocol (3T3-J2 and epithelial conjunctival cells).

C. Morphology

Epithelial conjunctival cells have specific features in terms of size, morphology and growth time. When cultured, conjunctival cells typically grow as round colonies with very regular edges, each of them formed by small cells with reduced cytoplasm and regular polygonal morphology. During cell cultivation, these paramethers may undergo changes that must be promptly assessed, in order to evaluate any problems that could affect the quality of the product.

D. Cell viability

The assessment of cell viability is crucial since the functionality of the final product relies on the percentage of living cells. The assay is performed throughout the process, namely phase 1 (cell extraction), phase 2 (intermediate product) and phase 3 (final product). Cell count is performed by using a trypan blue solution and a Neubauer counting chamber. Only dead or damaged cells absorb the trypan blue solution so that the exclusion test distinguishes between non-viable (Bl, blue cells) and alive cells (Br,

bright cells). Cell viability (V) is expressed as the percentage of alive cells respect to the total number of cells and is calculated using formula: V = Br / (Br + Bl) * 100. When we evaluated cell viability on our samples (N = 9), the average percentage of viable cells was 76.5 ± 5.0%. As a consequence, we set our acceptance value for viability at ≥ 76,5%.

E. Dose

The efficacy of a drug is normally correlated with the amount of active principle that is administered. In case of cell therapy products, the dose (D) is expressed as the total number of cells forming the final product. The dose was expressed as the number of cells/mm^2 following observation with a microscope (Axiovert 25 Zeiss, 20x magnification): 3 different fields (f1, f2, f3) were acquired, for each fields two squares of 0.05 mm^2 were selected (q1 and q2) using a Paint program (a square of 0.05 mm^2 corresponds to 340 × 340 paint unites). The cells included in the squares were counted (Fig. 3).

The dose of the final product was therefore calculated as the average of 6 fields x 20, that is: D = (f1q1 + f1q2 + f2q1 + f2q2 + f3q1 + f3q2) / 3 × 20. When we evaluated the dose on our samples (N = 9), the average number of cells in the final product was found to be 2843 ± 784 cells/mm^2. As a consequence, we set our acceptance value for the dose in the final product at ≥ 2.000 cells / mm^2.

F. Cell identity and impurities

The identity assay is performed in order to guarantee that the cells in the final product are conjunctival epithelial cells. We previously showed that cytokeratin 19 (cK19) is definitely expressed (100% of the cells) by conjunctival cells [10]. However, cK19 does not represent a specific marker for conjunctival cells (corneal cells can also express cK19). In order to reduce any potential source of contamination with cells other than of conjunctival origin (for example corneal cells), we harvested our biopsies from the inferior fornix.

Impurities are represented by autologous stromal fibroblasts (derived from the starting material) and residual feeder-layer cells. Previous validation studies (data not shown) showed that human and murine fibroblast cells (both irradiated and not irradiated 3T3-J2 cells) do not express cK19. Consequently, the immunostaining for cK19 allows to obtain information on both identity and impurity.

In order to minimize the presence of feeder-layer in the final product, we set up culturing conditions in which the feeder-layer is only used in phases 1 and 2 of the manufacturing process, thus avoiding its presence in the final product (phase 3).

The amount of impurities and cell identity are determined as follows: cells isolated from the final product are cytospinned on 1–2 slides (15.000–20.000 cells for each slide) and immunostaining against cK19 performed. The cell-count was performed on samples using a fluorescence microscope [10]. The cK19-positive cells were identified as conjunctival cells (K) while the cK19-negative cells as fibroblasts (F), including both feeder-layer residual cells and autologous stromal fibroblasts coming from the starting material. Impurity (Im) was expressed as the percentage of contaminant cells on the total number of cells

and is calculated using the formula: Im = F / (F + K) * 100. Three different fields of the same sample were analysed. Consequently, the assessment of identity (Id), was calculated as follows: Id = K / (F + K) *100 (average of three different fields of the same sample).

When we evaluated the percentage of impurities in our samples (N = 8), the average value in the final product was found to be 1.1 ± 0.8%. As a consequence, we set our acceptance value for the percentage of impurities in the final dose at ≤ 3%.

G. Potency

Critical for the release of ATMPs is the measurement of the activity, or potency [17, 18]. The EMA defines the potency as “the quantitative measure of biological activity based on the attribute of the product, which is linked to the relevant biological properties” [15]. Consequently, the way that the potency of a new ATMP is measured is of critical importance for its release. Determination of the stem cell content is the assay normally used to evaluate the potency of any ATMPs similar to ours [6, 7, 8, 9]. We [14] previously described a new method to quantify the potency (= stem cell content) of human conjunctival cell cultures-based ATMPs by means of immunofluorescence and with the limbal hTERT cells as a reference standard. Conjunctival stem cells were defined as those cells having a diameter of less than 15 μm and ΔNp63α expression values ≥ 3%.

Potency (P) is expressed as the percentage of stem cells respect to the total number of cells (included cK19 positive and negative cells) and is calculated using the formula: $P = SC / (CK19^{+} + CK19^{-}) * 100$. When we evaluated the potency in our samples (N = 7), the average percentage in the final product was found to be 11.4 ± 8.1%. As a consequence, we set our acceptance value for the potency in the final product at ≥ 3%.

H. Presence of pyrogens

It is mandatory to determine the presence of any pyrogen (endotoxins produced by Gram-negative bacteria) in the final product before transplantation of the graft, as it may cause toxic and inflammatory reactions and compromise the result of surgery. The main method (according to European Pharmacopeia) used to detect the endotoxins is the Limulus Amoebocyte Lysate (LAL) test. Based on literature search, we have set the acceptance limit to ≤ 1 EU/graft.

I. Mycoplasma detection

The presence of microbiological contaminants like human mycoplasma could lead to pathogen transmission and appearance of localized or systemic infections in the recipient. According to the European Pharmacopeia, Real-Time PCR is one of the assays indicated to detect human mycoplasma and the acceptance criterion is absence.

L. Viral contaminant detection

As for mycoplasma, the presence of viral contaminants could lead to pathogen transmissions and the appearance of localized or systemic infections in the recipient. According to the European Pharmacopeia,

the adopted method to detect viruses (Herpes virus-HSV1, Citomegalovirus-CMV and Varicella Zoster virus-VZV) is Real-Time PCR and the acceptance criterion is absence.

3. Quality control assays to determine safety and efficacy

3.1 Stability

The purpose of a stability test is to provide evidence on potential changes/variation on the quality of final product over time. In our case, the stability of the final product was carried out on one conjunctival graft maintained in the selected transport carrier at 25 ± 1 °C for up to 48 hours. Quality and safety parameters were evaluated at t = 0 and after 24 and 48 hours from the preparation of the final product. Results showed a decrease in potency over time, thus indicating a maximum limit of 24 hours (Table 8) for the stability of the final product.

3.2 Karyotype analysis

Chromosomal analysis (or karyotyping) is a test aiming to evaluate whether culturing and manipulation of cells during the manufacturing process induce damage or chromosomal abnormalities. We therefore performed the analysis of 3 different cultures of human conjunctival epithelial cells (Cong 200.12, Cong 200.15 and Cong 200.25), obtained after expansion of three biopsies extracted from the inferior fornix of conjunctival tissue from cadaveric donors. The cells collected from the biopsies were expanded and finally shipped to a certified laboratory (Cell Guidance Systems, Cambridge, UK) for karyotyping according to the indications set by the European Pharmacopeia. As shown in Fig. 4, no chromosome alterations, rearrangements, breakages and inversions were detected, thus answering the specifics requested for production of ATMPs for clinical use.

3.3 Tumorigenicity

One of the major concerns related to the use of stem cells in regenerative medicine is their potential to become tumorigenic.

The Soft Agar Growth Test is reccomended by the European Pharmacopoeia for the investigation of the ability of cells to form colonies in the absence of adhesion to the substrate. Growth in semi-solid medium is generally used as an index of neoplastic transformation related to tumorigenicity. The test requires that the cells (including both positive and negative controls) are grown in a semi-solid culture medium on 96-well plates. Once the incubation time is over, the semi-solid media containing the cells are solubilized and lysed and the samples read by a fluorescence reader by means of a patented dye that provides a quantitative and exhaustive method for accurately measuring cells transformation based on the fluorescence emission nucleic acid dye complexes correlated linearly with the number of cells. The results must be obtained in triplicate and comply with the following specifications: absence of growing colonies, OD reading < 0.003.

Three samples (Cong 200.12, Cong 200.14 and Cong 200.25) were subjected to the soft agar test and did not show any type of growth (absence of colonies, reading OD < 0.003), thus confirming the safety of the

final product. Data are reported in Fig. 5 The cultures showed no colony growth except for the positive control (Hela cells). 3T3-NIH cells were used as negative controls.

3.4 Efficacy

Efficacy of cellular products is a major concern in cell therapy studies. The efficacy of a cell therapy product is evaluated only after follow-up results of clinical studies are examined. During the pre-clinical phase, the only parameter that can potentially be associated with clinical success is the potency (= stem cell content), as reported by several cell therapy protocols [9]. Therefore, in order to maximize the efficacy of the conjunctival cell grafts when transplanted, the presence of a minimal percentage of ΔNp63α positive cells in cell cultures (in our case ≥ 3%) needs to be obtained. These data agree with our previous work [10], where we found percentages of ΔNp63α positive cells ranging between 1.67 ± 0.36% (inferior bulbar areas) and 6.79 ± 1.18% (inferior fornix) in healthy ocular surfaces.

Discussion And Conclusions

The present work describes the main phases of the manufacturing process of conjunctival stem cell-based products to be used in clinical applications. In order to comply with GMP regulations, we set up validated methods to characterize the final product in terms of safety, identity, purity and potency. Specifications for each analytical method have been defined. Such characterization is crucial for the preparation of documents and dossiers needed by the competent authorities to start a phase I clinical study on patients with conjunctival disorders. Phase II and III and request to EMA for a marketing authorization will require additional and more relevant funds and maybe the involvement of industrial partners, very much in line with what happened with Holoclar. The marketing authorization for an ATMP release is a challenging process. However, the therapeutic applications are fascinating and cell therapy represents a great promise for the treatment of many diseases. If the same path of authorization needed for Holoclar was repeated for this ATMP, in approximately 5–10 years patients with conjunctival disorders might have a new treatment based on autologous cells.

List Of Abbreviations

ATMPs, Advanced Therapy Medicinal Products.

GMP, Good Manufacturing Practices guidelines

EMA, The European Medicines Agency

CK19, cytokeratin 19

LAL, Limulus Amoebocyte Lysate test

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

Not applicable.

Compending interests

The authors have no conflict of interest or financial interests to disclose.

Funding

This study was partly supported by the 5x1000 funds to Fondazione Banca degli Occhi del Veneto from the Italian Ministry of Health and the Italian Ministry of Research and University.

Authors' contributions

All authors were involved in the final approval of the article submitted and are in agreement with the contents. Bertolin M, Breda C and Ferrari B performed the experiments and collected the data. Bertolin M, Ferrari S, Barbaro V and Ponzin D wrote the manuscript, contributed to the critical evaluation and revision of the paper and gave their expert opinion

Acknowledgements

Not applicable.

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Tables

Tables 1 and 2 are not available with this version.

Figures

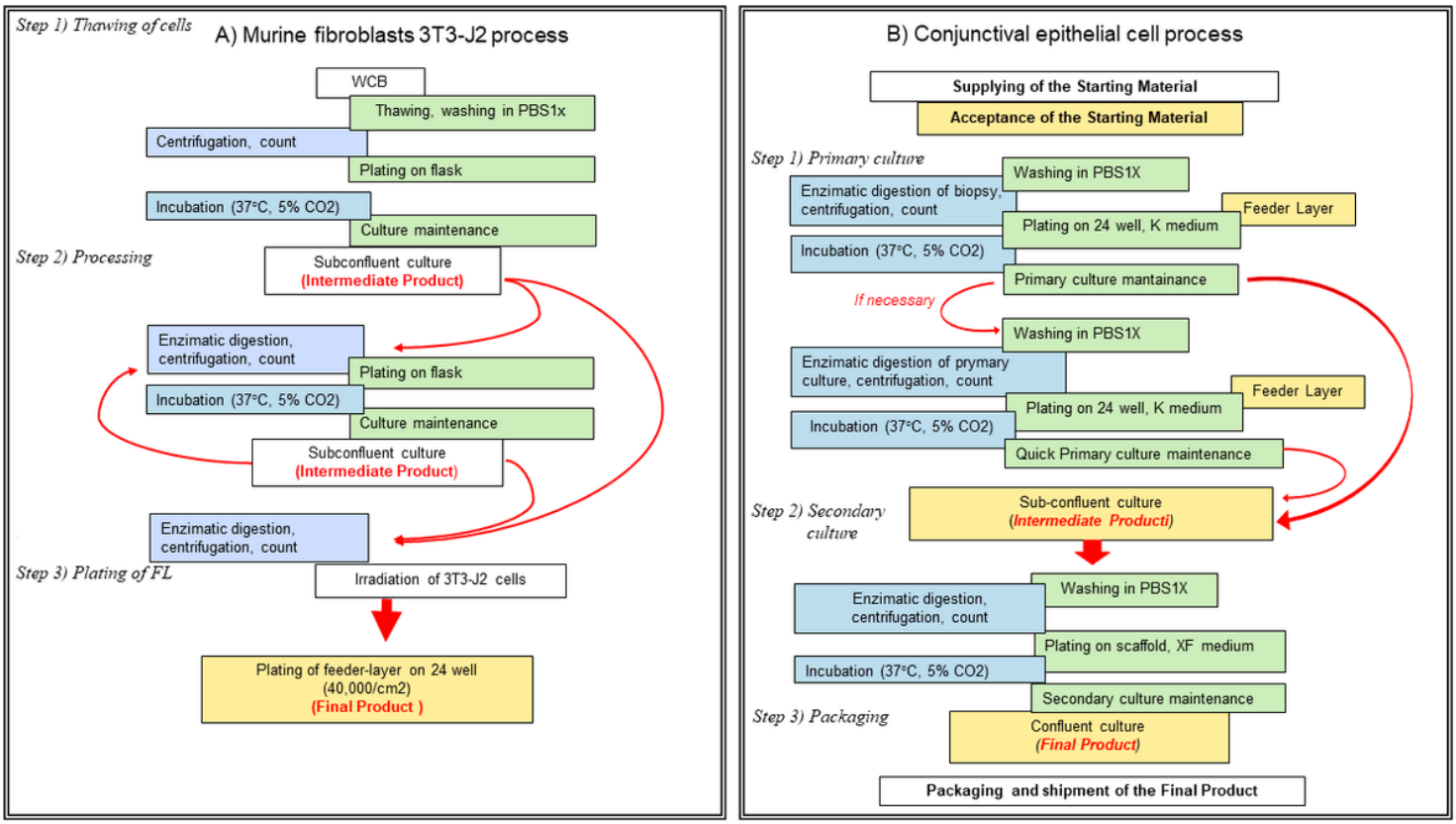

Figure 1

Schematic description of the production protocol. A schematic representation of the two processes involved in the production of conjunctival epithelial grafts: (A) the murine fibroblasts 3T3-J2 process (used as support for conjunctival cells growth) and (B) the conjunctival epithelial cell process.

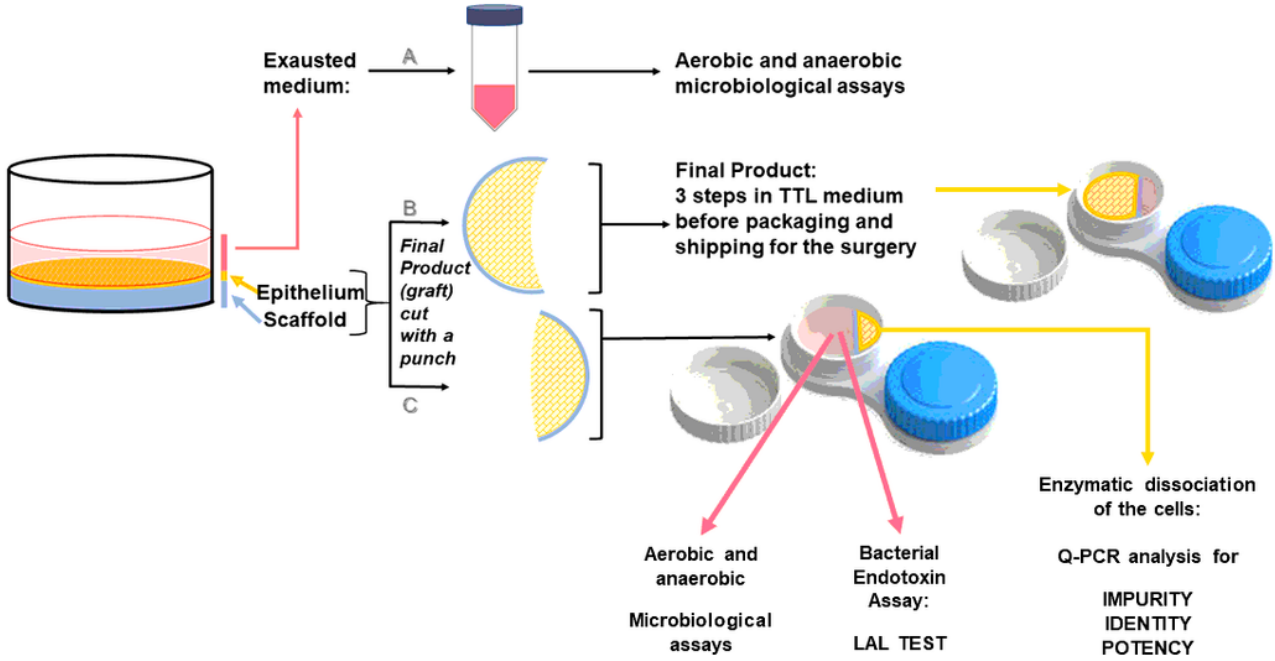

Figure 2

Schematic description of the quality control assays performed on the final product. The exhausted medium is collected and inoculated in the Bactec media to assess any microbial contamination (A). One part of the graft is washed, packed with TTL medium in a lens-holder and shipped for the surgery (B), while the other part is left in the presence of 4 ml of TTL medium for 2 hours (C), before performing QC analyses. These include aerobic and anaerobic microbiological controls, bacterial endotoxins assay (LAL test) and after a double enzymatic dissociation of cells from the scaffold, immunostaining analysis for the assessment of identity, potency, impurity, virus e mycoplasma.

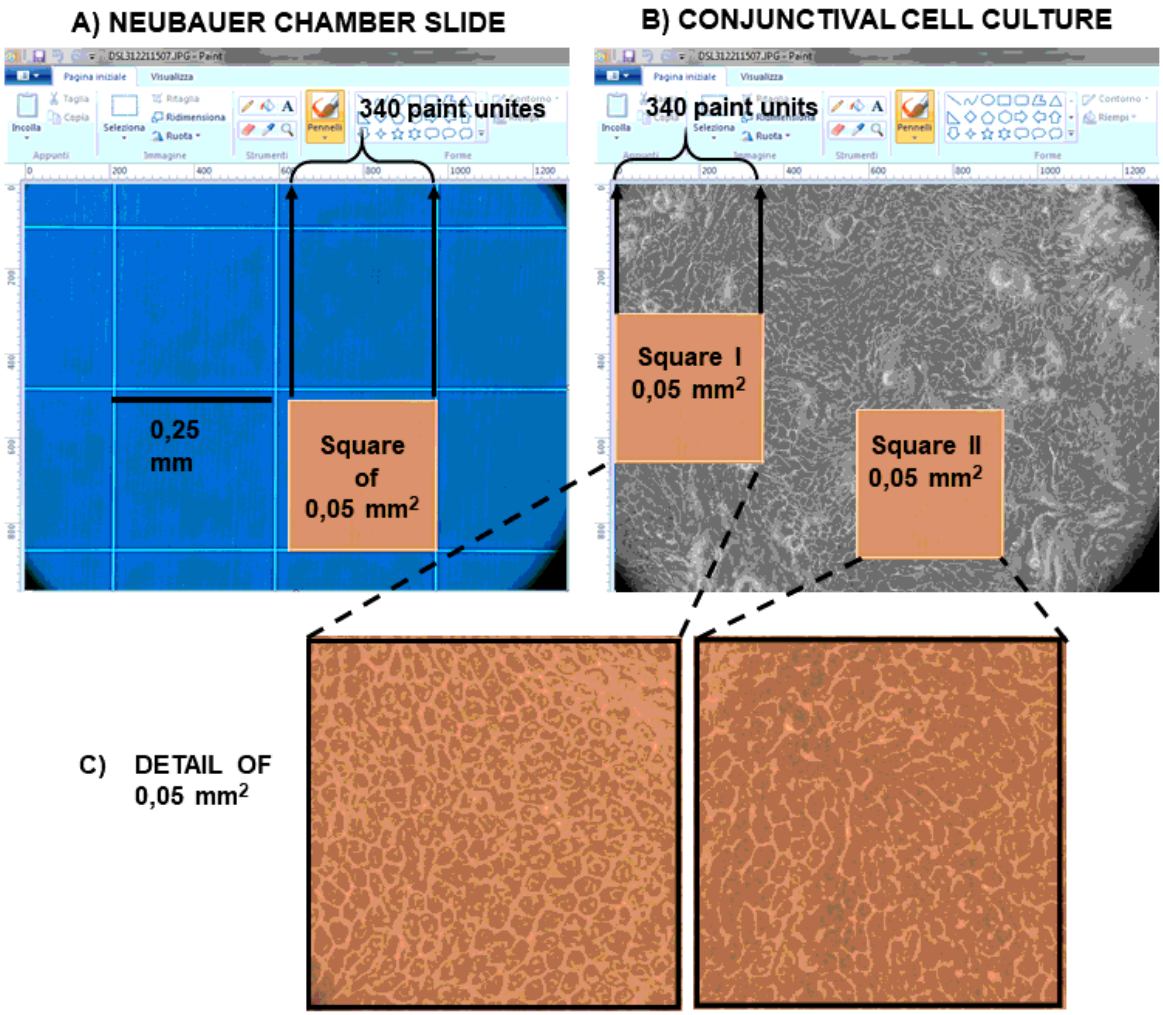

Figure 3

Dose quantification. (A) By using a Neubauer chamber slide, an inverted microscope and the paint image program, squares of 0.05 mm2 may be drawn by selecting an area of 340 * 340 paint units (20x magnification); (B) the same squares may be drawn on the picture of the cell culture representing the final product (20x magnification). (C) Detail of the selected squares (0.05 mm2) used for the assessment of the dose.

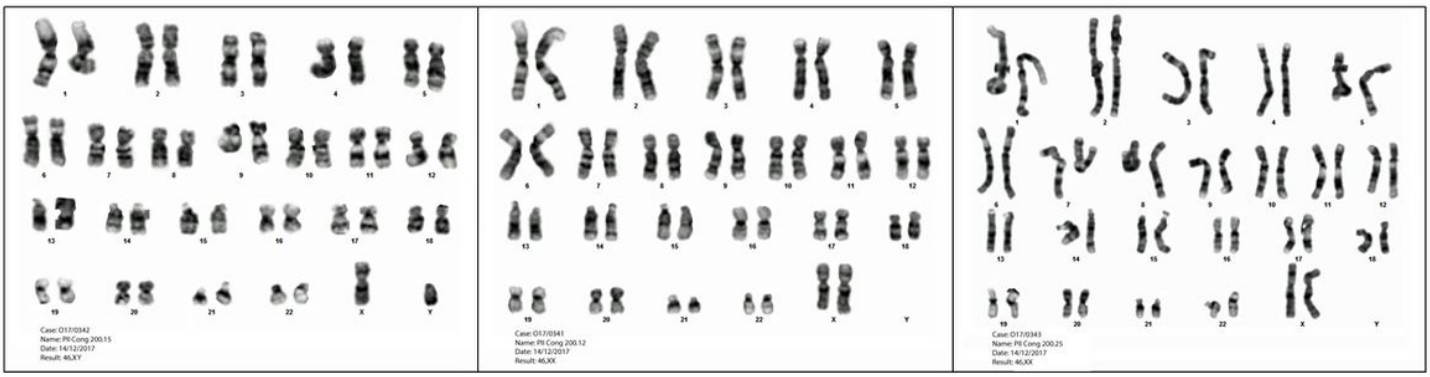

Figure 4

Karyotype analysis. Cromosomal analysis performed on three different final products in order to ascertain the absence of any damage or chromosomal abnormality in conjunctival cells during the manufacturing protocol.

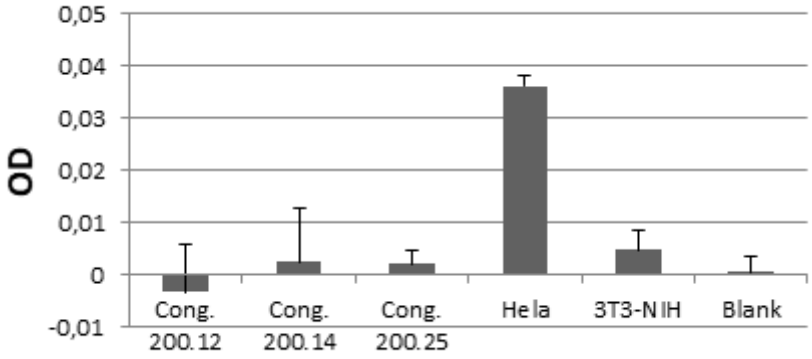

Figure 5

Soft Agar growth test. The soft agar assay showed that both the conjunctival samples (Cong 200.12, Cong 200.14 and Cong 200.25) and 3T3-J2 cultures had no colony growth, unlike the HeLa cells used as positive control. OD = Optical Density.