This study evaluated the effects of platelet gel released supernatant at different concentrations on angiogenesis (process involved in tissue reparation mechanism)

**Methods**

- Human umbilical vein endothelial cells (HUVECs) were isolated from umbilical cord veins and grown on 1% gelatin-coated flasks in DMEM supplement
- Eight (8) male donors donated 450 mL of whole blood for evaluation
- In vitro assays included
  - Proliferation – determined by XTT assay
  - Motility (chemotaxis) – evaluates cell’s ability to move towards a soluble attractant
  - Invasion – measures the invasive capacity of cells the degradation of Matrigel (a compound that mimics the composition of the extracellular matrix
  - Cord Formation – measures the ability of endothelial cells to invade, migrate, organize, and differentiate into capillary-like tubular structures within a three-dimensional matrix
  - Wound Healing – evaluates directional cell migration into a wound created on a cell monolayer
- The assays were exposed to platelet gel supernatant concentrations ranging from $0.3 - 3.0 \times 10^6$ platelets per µl

**Results**

- The concentration of platelet gel released supernatant had a significant influence on the angiogenic potential of endothelial cells in a dose and time dependent manner
- In vitro assays results were as follows
  - Proliferation – Induction of cell proliferation peaked at 72 hours and the most effective concentration was $1.5 \times 10^6$ platelets per µl
  - Motility (chemotaxis) – a significant effect was evident for concentrations ranging between $1.5 \times 10^6 - 2.0 \times 10^6$ platelets per µl, motility of treated cells was 3.5x higher than that of untreated cells (negative control)
  - Invasion – a significant effect was evident for concentrations ranging between $1.5 \times 10^6 - 2.0 \times 10^6$ platelets per µl, motility of treated cells was 6.5x higher than that of untreated cells
  - Cord Formation – platelet concentrations up to $2.0 \times 10^6$ platelets per µl resulted in cordlike structures
  - Wound Healing – $1.5 \times 10^6$ platelets per µl showed a significant wound healing effect

**Conclusions/Key Points**

- The optimal concentration for stimulation of angiogenesis ranged between $1.5 \times 10^6 - 2.0 \times 10^6$ platelets per µl
- Lower or higher platelet concentrations of platelet gel displayed lower angiogenic potential
Identification of an optimal concentration of platelet gel for promoting angiogenesis in human endothelial cells

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BACKGROUND: Numerous studies have supported the use of topical blood components to improve wound healing and tissue regeneration. Platelet gel (PG), a hemocomponent obtained from mix of activated platelets (PLTs) and cryoprecipitate, is currently being used clinically in an attempt to improve tissue healing. The present study sought to define the most effective PG concentration to promote angiogenesis in vitro.

STUDY DESIGN AND METHODS: The effects of PG-released supernatant at different concentrations on human endothelial cells were studied using different in vitro assays (proliferation, migration, invasion, cord formation, and wound healing).

RESULTS: The concentration of PG-released supernatant had a significant influence on the angiogenic potential of endothelial cells. The optimal concentration for the stimulation of angiogenesis was $1.5 \times 10^6$ PLTs per $\mu$L in most of the in vitro experiments used in this study. Lower or higher concentrations of PG displayed a lower angiogenic potential.

CONCLUSION: An optimal concentration of PG to promote angiogenesis in human endothelial cells was identified. Excessively high PG concentrations may inhibit the angiogenic process, thereby being counterproductive for wound healing in a clinical setting.

In recent years, numerous studies have supported the use of topical blood components to improve wound healing and tissue regeneration. Platelet gel (PG), a hemocomponent obtained from mix of activated platelets (PLTs) and cryoprecipitate, is currently being used clinically in reconstructive, cosmetic, orthopedic, cardiovascular, oral maxillofacial, and dermatologic surgery in an attempt to improve tissue healing. PLTs have concentrated levels of naturally occurring growth factors and other substances that have the potential to accelerate tissue repair by influencing several cellular processes including chemotaxis, cell proliferation, cell differentiation, angiogenesis, matrix deposition, and tissue remodeling.

Previous studies have demonstrated that the healing process might be accelerated by the use of PG. Compared with lesions of patients treated by conventional methods, the sternal dehiscent wounds and skin necrotic ulcers of...
patients treated with PG recovered more rapidly. Moreover, patients treated by partial mandibulectomies for malignant tumors experienced satisfactory mandibular reconstructions when PLT-rich plasma was combined with autologous bone grafts.

Despite the promising clinical results, PG use has proceeded in a totally empirical way, mainly grounding on experience of individual doctors, and the most effective PG concentration to promote wound healing has not yet been determined. In this study, the effects of PG-released supernatant at different concentrations on angiogenesis (process involved in tissue reparation mechanism) were investigated using different in vitro assays (proliferation, migration, invasion, cord formation, and wound healing).

MATERIALS AND METHODS

Preparation of PG-released supernatants

A total of eight male donors participated in the study. Whole blood (450 mL) was collected using a triple-bag system (Teruflex with CPD/SAGM, Terumo Corp., Rome, Italy). Each donor provided informed consent according to national laws (Ministerial Decree of 3 March 2005; Law number 219 of 21 October 2005).

Fractionation was carried out by a first centrifugation of the bag at 462 × g for 10 minutes at 22°C in a centrifuge (Cryofuge 6006i, Heraeus Instruments, AHSI SpA, Massa Martana, Italy) to obtain PLT-rich plasma and red blood cells (RBCs). PLT-rich plasma was subjected to a second centrifugation at 3932 × g for 6 minutes at 22°C to provide PLT concentrate and PLT-poor plasma. PLTs were finally collected as hyperconcentrated in 10 to 15 mL of plasma.

The PG was produced by mixing the PLT concentrate in tubes (Vacutainer Plus, 367817, Becton Dickinson, Plymouth, UK) with 5 NIH units of thrombin and calcium gluconate 1:20 (Bioindustria Laboratorio Italiano Medicinali SpA, Novi Ligure, Italy). The mixture was allowed to clot for 5 minutes at 37°C and then centrifuged for 10 minutes at 153 × g to obtain a supernatant enriched of growth factors released from activated PLTs.

The supernatant was further subjected to a succession of centrifugations (10 min each, 153-1700 × g) to remove RBCs, debris, or cellular stroma and immediately used in the experiments. The initial concentration of PLTs in PG, obtained from blood count in starting PLT concentrate, differed in each preparation (from 3.0 × 10⁶ to 7.5 × 10⁶ PLTs/μL). Since the concentration of growth factors released from PLTs is proportional to the initial PLT concentration (expressed as PLTs/μL), we assumed that supernatants obtained from each preparation had the same concentration of PLTs per μL. To obtain PG-released supernatant at different concentrations, each supernatant was diluted with either Dulbecco’s modified Eagle’s medium (DMEM) or DMEM plus fetal calf serum (FCS).

Cell culture

Human umbilical vein endothelial cells (HUVECs) were isolated from umbilical cord veins and grown on 1 percent gelatin-coated flasks in DMEM supplemented with 10 percent FCS, 10 percent newborn calf serum, 20 mmol per L N-(2-hydroxyethyl)perazine-N’-(2-ethanesulfonic acid), 6 U per mL heparin, 2 mmol per L glutamine, 50 μg per mL endothelial cell growth factor (crude extract from bovine brain), penicillin, and streptomycin. Cells from the third to fifth passages of culture were used.

Proliferation assay

Cell proliferation was determined by using the 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT) assay (Sigma, St Louis, MO). The XTT assay is based on the cleavage of the yellow sulfonated tetrazolium salt to form an orange formazan dye by metabolic active cells; the amount of produced formazan is directly proportional to the number of viable cells. Briefly, cells were seeded in a 96-well plate (1500 cells/well) and incubated overnight at 37°C in a 5 percent CO₂ humidified incubator to allow cell adhesion.

Two different treatments were performed: in a preliminary test PG was diluted with DMEM plus 2.5 percent FCS to obtain the desired concentrations (0.3 × 10⁶, 1.5 × 10⁶, or 3.0 × 10⁶ PLTs/μL) and placed onto a transwell. In a subsequent test, cells were treated with PG-released supernatant diluting the original preparation with DMEM plus 2.5 percent FCS to obtain different PLT concentrations (0.7–7 × 10⁶ PLTs/μL).

HUVECs grown in DMEM plus 2.5 percent FCS were used as the negative control. Cells were incubated at 37°C in a humidified atmosphere containing 5 percent CO₂. The XTT assay was performed for the following 3 days after the treatment and optical density was evaluated at 450 nm. Each experiment was performed in triplicate and repeated at least twice. All values are expressed as mean ± standard deviation (SD).

Motility and invasion assay

Motility assay enables to evaluate, in vitro, cells’ ability to move toward a soluble attractant; invasion assay measures, in vitro, the invasive capacity of cells through degradation of Matrigel, a compound that mimics the composition of the extracellular matrix. HUVEC motility and invasion were assayed using modified Boyden chambers with polycarbonate polyvinylpyrrolidone-free nucleopore filters (pore size, 8 μm).
PG-released supernatant at different concentrations was used as the attractant and was added to the lower compartment of the chamber. Different concentrations were obtained by dilution of PG-released supernatant with variable amounts of DMEM. The medium alone or the conditioned medium of NIH-3T3 cells was used as negative and positive controls, respectively.

For motility (chemotaxis) assays, filters were coated with 0.1 percent gelatin. For invasion assays, filters were coated with a thick layer of the reconstituted basement membrane Matrigel growth factor reduced (Becton Dickinson, Bedford, MA; 0.5 mg/mL) that cells must degrade to reach and migrate through the filter. HUVECs were detached, washed by DMEM plus 0.1 percent bovine serum albumin, resuspended in the same medium at a concentration of $5 \times 10^5$ per mL, and added to the upper compartment of the chamber. After 4 hours (motility) or 6 hours (invasion), filters were stained with 1 percent crystal violet in methanol, and migrated cells in five high-power fields were counted. Each experiment was performed in triplicate and repeated at least twice. All values were expressed as mean $\pm$ SD.

Cord formation assay

This assay measures the ability of endothelial cells to invade, migrate, organize, and differentiate into capillary-like tubular structures within a three-dimensional matrix. HUVECs (70,000/well) were seeded onto Matrigel-coated 24-well plates in endothelial growth medium containing 2.5 percent serum (negative control), complete HUVEC medium (positive control), or PG-released supernatant diluted with DMEM plus 2.5 percent FCS to reach the desired concentration. After 4 hours, the formation of cords was photographed and independently scored by two blinded observers.

Wound-healing assay

The wound-healing assay is one of the earliest developed tests to study directional cell migration in vitro. This method is based on observation of cell migration into a wound created on a cell monolayer.

HUVECs were cultured in 24-well microplates in normal culture conditions and allowed to reach maximum confluence. A round-tip steel needle, previously sterilized, was used to draw several wounds of approximately 0.2 mm in the cellular stratum. Microplates were washed three times with DMEM. Cells were subsequently cultured in complete HUVEC medium (positive control), medium plus 1 percent FCS (negative control), or PG-released supernatant diluted with DMEM plus 1 percent FCS to reach the desired concentration ($0.3 \times 10^6$-$5 \times 10^6$ PLTs/$\mu$L). The status of wounds was monitored by contrast phase microscopy and representative images (0, 2, and 4 hr) were collected.

RESULTS

PLT releasate stimulates endothelial cell proliferation

We performed a series of in vitro experiments using HUVECs to evaluate the effects of PLT-associated growth factors on endothelial cell proliferation. PG was first diluted to obtain the desired concentrations ($0.3 \times 10^6$, $1.5 \times 10^6$, or $3.0 \times 10^6$ PLTs/$\mu$L) and placed onto a transwell. Exposure to PG induced HUVEC proliferation in a dose- and time-dependent fashion (Fig. 1). While no effects were seen after a 24-hour incubation, a significant induction of cell proliferation was observed at 48 hours, peaking at 72 hours; the most effective concentration was $1.5 \times 10^6$ PLTs per $\mu$L. The use of higher concentrations obtained a less evident stimulation of HUVEC proliferation. Morphologic analysis (Fig. 2) showed that the optimal PG concentration for the stimulation of HUVEC proliferation was $1.5 \times 10^6$ PLTs per $\mu$L (Fig. 2C). Notably, lower concentrations of PG displayed a reduced proliferative response in HUVECs (Fig. 2B), whereas higher
concentrations had an inhibitory effect (Fig. 2D). Exposure to suboptimal concentrations (higher or lower) of PG resulted in an unusual morphology of HUVECs.

In subsequent experiments, cells were treated with PG-released supernatants recovered after centrifugation and dilution with DMEM plus 2.5 percent FCS to obtain different concentrations (range, 0 to 7 × 10^6 PLTs/μL). Cells were incubated for 24, 48, and 72 hours. Also in this case, the optimal concentration for the stimulation of cell proliferation was 1.5 × 10^6 PLTs per μL at 72 hours (Fig. 3). Under these conditions, the proliferation rate was approximately sixfold higher than in basal conditions (2.5% FCS). Concentrations higher than 1.5 × 10^6 PLTs per μL exerted lower proliferative effects on human endothelial cells (Fig. 3).

PG induces HUVEC motility and invasiveness

To investigate whether PG may exert an angiogenic effect, the supernatant collected from PG was used as chemoattractant for HUVEC motility and invasion. Results showed that PG-released supernatant stimulated HUVEC motility and invasiveness in a dose-dependent fashion (Fig. 4). A significant effect was evident for concentrations ranging between 1.5 × 10^6 and 2 × 10^6 PLTs per μL. The motility of cells exposed to PG-released supernatant at this concentration was 3.5-fold higher than that of untreated cells (negative control) and approximately 2-fold higher than that of HUVECs stimulated with NIH-3T3 supernatant (positive control; Fig. 4A). Similarly, the invasive capability of HUVEC exposed to PG-released supernatant was 6.5-fold higher than that of untreated cells and 3-fold higher than that of positive control cells (Fig. 4B). Exposure of HUVECs to higher concentrations of PG-released supernatant resulted in a lower mobility and invasive capacity.

The ability of PG-released supernatant to stimulated HUVEC migration was subsequently assessed using the wound-healing assay (Fig. 5). HUVECs treated with the complete medium (positive control) were able to heal the wound by migrating into it as a sheet of cells. Approximately 4 hours were required for a complete healing. In contrast, endothelial cells treated with DMEM plus 1 percent FCS (negative control) did not have any wound-healing activity in the same time frame. PG-released supernatant at a concentration of 0.3 × 10^6 PLTs per μL was able to induce wound healing in a shorter time (2 hr) that the positive control. At a concentration of 1.5 × 10^6 PLTs per μL, a significant wound-healing effect of
PG-released supernatant was observed. However, a longer time was required to obtain the wound-healing effect compared with the positive control. Higher concentrations totally inhibited wound healing and resulted in significant morphologic alterations of HUVECs.

Effects of PG on HUVEC cord formation

To investigate whether PG may stimulate the formation of capillary-like network structures, a cord formation assay was performed. After 4 hours in complete medium containing 30 percent serum, HUVECs were organized into capillary-like structures (Fig. 6A). In contrast, cells grown in low-serum medium (2.5% FCS) were unable to form a tubular network (Fig. 6B). The HUVECs maintained in low-serum medium with PG-released supernatant at concentrations lower than $2 \times 10^6$ PLTs per μL (Figs. 6C and D) resulted in the formation of cord-like structures similar to those observed after treatment with complete medium (Fig. 6A). Concentrations higher than $2 \times 10^6$ PLTs per μL totally inhibited cord formation (Fig. 6E).

DISCUSSION

Normal wound healing is a complex and finely tuned process that is mediated by growth factors, cytokines, and angiogenic mediators. PLTs contain many growth factors that have been postulated to play an important role in the wound-healing process and reepithelialization, including PLT-derived growth factor, epidermal growth factor, transforming growth factor (TGF)-α and β, vascular endothelial growth factor, insulin-like growth factor, fibroblast growth factor (FGF)-1 and -2, keratinocyte growth factor, and tumor necrosis factor (TNF)-α. PLT-derived growth factor is a potent mitogen and chemotaxic agent for myofibroblasts and epithelial and endothelial cells and stimulates fibroblast recruitment and proliferation. Epidermal growth factor stimulates the proliferation of epidermal and epithelial cells including fibroblasts and promotes keratinocytes migration to the wound. TGF-α stimulates epithelial and endothelial chemotaxis and cell growth. TGF-α is mitogenic for fibroblasts and promotes the expression of fibronectin and collagen and their incorporation into the extracellular matrix. Vascular endothelial growth factor, the first endothelial cell-specific mitogen to be identified, is a potent endothelial chemoattractant and angiogenesis inducer. Insulin-like growth factor is involved in the regulation of cell growth and tissue regeneration. FGF-1 participates in various cellular functions including proliferation, differentiation, angiogenesis, and cell migration. FGF-2 stimulates endothelial cell proliferation and migration and promotes tube formation. It also acts as a mitogen for several cell types involved in wound healing, including fibroblasts, keratinocytes, and endothelial cells. Keratinocyte growth factor promotes wound healing through enhanced proliferation, differentiation, angiogenesis, and cell migration. Finally, TNF-α stimulates fibroblast proliferation and angiogenesis.

Clinical trials have been reported detailing improvement of wound healing after treatment with PG or PLT-rich plasma. PG was initially developed as a by-product of multicomponent pheresis. When PLT concentrate is combined with thrombin and calcium, a viscous coagulum (gel) is rapidly formed. PG contains supraphysiologic amounts of multiple growth factors acting synergistically. PG has been shown to enhance wound healing in several animal models and nonhealing wounds in humans. Unfortunately, the most effective concentration of PG to promote wound healing has not been determined yet. To address this issue, in this study we sought to investigate the effects of PG-released supernatant at different concentrations on human endothelial cells using different in vitro assays (proliferation,
migration, invasion, cord formation, and wound healing). Angiogenesis, the formation of new blood vessels, is indeed necessary for wound repair since the new vessels provide nutrients to support the active cells, promote granulation tissue formation, and facilitate the clearance of debris.\(^{25}\)

We previously showed that the concentration of PG-released supernatant had a significant influence on the angiogenic potential of endothelial cells.\(^ {26}\) This study was conceived to thoroughly evaluate effect of different concentrations on endothelial cell activity and further confirm that the optimal concentration for the stimulation of angiogenesis was \(1.5 \times 10^6\) PLTs per \(\mu\)L in most of the in vitro assays performed. Lower or higher concentrations of PG displayed a lower angiogenic potential. Notably, exposition to suboptimal concentrations (higher or lower results) of PG resulted in an unusual endothelial cell morphology of HUVECs.

In the first experiments investigating endothelial cell proliferation, PG was directly used in the in vitro assays. We subsequently focused on PG-released supernatant at different concentrations. Since the concentration of growth factors released from PLTs is proportional to the initial PLT concentration (expressed as PLTs/\(\mu\)L), we assumed that supernatants obtained from each preparation had the same concentration of PLTs per \(\mu\)L. Our results showed that the optimal concentration of PG-released supernatant for the induction of endothelial cells was \(1.5 \times 10^6\) PLTs per \(\mu\)L, with a maximum effect observed at 72 hours. Since PG and PG-released supernatants yielded similar results, only PG-released supernatants were used in all other experiments. In keeping with the results obtained in the proliferation assay, concentrations of PG-released supernatants ranging between \(1.5 \times 10^6\) and \(2 \times 10^6\) PLTs per \(\mu\)L were the most effective in promoting HUVEC motility and invasiveness. In contrast, higher concentrations significantly inhibited this response. Results obtained in the cord formation and wound-healing assays were also in line with the possibility that excessively high concentrations may inhibit the cellular effects of PG-derived supernatant.

Taken together, our current data clearly indicate that both PG and PG-derived supernatants are capable of inducing a functional angiogenic response in vitro, thereby functioning as a stimulator of the angiogenic stage of tissue repair. More importantly, we have identified an optimal concentration of PG to promote angiogenesis in human endothelial cells. Excessively high PG concentrations may inhibit the angiogenic process, thereby being counterproductive for wound healing in a clinical setting. Further in vitro studies into the mechanisms by which PG works to improve wound healing are warranted. Our current data might set the stage to optimize the use of PG or PG-released supernatant in the clinical setting. Obviously, these in vitro tests require further studies to best understand the mechanisms underlying PG effects in vivo; moreover, clinical

![Fig. 5. Effect of PG-released supernatant at different concentrations on wound healing. The space between wound edge is approximately 0.2 mm (magnification 100\(\times\)).](image)
studies are needed to validate the results obtained in this study.

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