PlateletsregulatescellviabilityandVEGF-AmRNAexpressioninHaCaTcellline

Abstract

PlateletRichPlasma(PRP)isanautologoustechniquethatusescentrifugedwholebloodtoconcentrateplateletsinplasma.TheregenerativeeffectofPRPisattributedtoplateletsduetotherelease ofgrowthfactorsinvolvedinhealing.ThisstudysoughttopromotetheisolationofplateletsfromPRP(PI-PRP)toidentifytheroleofplateletsinthemodulationof cell surviving and VEGF-A mRNA expression. The coculture protocol with PI-PRP/keratinocyte cell line HaCaT was established. Cellular viability by MTT, membraneintegrity by trypan blue, cell and cytoskeletal cell morphology by DAPI and phalloidinstainingandRNAextraction,forsubsequently,qRT-PCRVEGF-A,wereperformed.TheMTT test showed higher cell viability in PI-PRP group than CTRL. The trypan blue testshowed no difference between CTRL and PI-PRP groups. Fluorescence microscopyanalysis showed no changes in cellular morphology of the nucleus and cytoskeletonbetween groups. In the qRT-PCR the VEGF-A expression was higher in PI-PRP groupcompared to CTRL. The centrifugation proved to be effective for platelet enrichment.This protocol demonstrated efficiency in studying interaction between platelet and celllineage.

Keywords:PRP,keratinocyte,platelets,VEGF-A.

INTRODUCTION

Skin tissue contains cells called keratinocytes, which are responsible for cell renewal.When injured, the platelets initiate the healing process through the synthesis of growthfactors and the transfer of the molecular information to the target tissue. The most recentstudies have opened new paradigms for the functions of platelets, since they show thetransfer of messenger RNA (mRNA) and microRNA (miRNA) altering the function ofanother cell or even activating the production of proteins and the growth factors (Plé2012; Rowley et al. 2012; Nassa et al. 2017). The synthesis of growth factors and thetransfer of molecular information is the principle of cell therapies with stem cells, so theplatelets can be framed in this type of cell therapy. Therapies using platelets such asplatelet and platelet lysate transplantation have been used for decades (Apatzidou et al.2021). Autologous plasma treatments in regenerative medicine to optimize the healingprocesshavealower risk ofrejection(Draelos2019).

The Platelet Rich Plasma (PRP) is the new given to an autologous techniqueobtained by the process of centrifuging whole blood to obtain the ideal plasma fractionhas been proposed to be a therapeutical tool in regenerative medicine to treat somediseases and unaestheticism. The cellular response of proliferation and regeneration inthe injured tissue has been correlated with platelets, especially when they reach

valuesfourtofivetimeshigherthanthebasallevel,reachingupto1millionplatelets/µlinPRP(Marx 2004). Variations in the process of obtaining PRP directly affect the result, suchas:freshblood,amountofbloodcollected,needlegaugeforbloodcollectionandplasmatransfer, anticoagulants used, number and interval of centrifugations performed, G-forceandtemperatureofthecentrifuge(Akhundovetal.2012).Thevastmajorityofpublishedscientific articles attribute the regenerative effects of PRP to platelets due to the releaseofgrowthfactorsinvolvedinhealingvascularendotheliumgrowthfactor(VEGF),

epidermal growth factor (EGF), basic fibroblast growth factor (bFGF), platelet-derivedgrowthfactor(PDGF)andbetatransforminggrowthfactor(TGF-β)howevertheplasmacontains other components hypothetically able to regenerate the skin (Weibrich 2004;Hargrave&Li2015;Frykberget al.2010).

Basedontheabove,theaimofthisstudyisestablishedthatallowtheisolationofplatelets for laboratory tests that can assess the promotion of tissue changes made by theplatelets in the skin, especially on keratinocytes, epidermal cells responsible for cellrenewal and synthesis of growth factors (McRedmond 2004; Wrzyszcz 2017; Wagner2018; Akhundov 2012;Blanpain&Fuchs2009; Bae2015).

MATERIALSANDMETHODS

Plateletisolation

This study was approved by the ethics committee (CEP/UFRJ-Macaé 1.922.306). Fiftypatients between 18 and 70 years of both sexes, eligible for donation and who agreed tobevolunteers,signingtheFreeInformedConsentTerm(FICT)wereselected andexcluded patients with sexually transmitted infections (STIs), using antihypertensive oracetylsalicylic acid drugsand hadahistory of cancer.

The isolation of platelets from PRP protocol modified. 6 ml blood was collectedfrom patients with a 22G x 1” needle (25 x 0.7 mm) in a vacuum tube containingDisodium 2,2',2",2"'-(Ethane-1,2-diyldinitrilo)tetraacetic acid (EDTA/2Na). The plasmawas isolated by centrifugation of the fresh blood at 581x *g* for 5 minutes. Platelets wereisolated from the plasma by centrifugation at 908 x *g* for 10 minutes. The platelet pelletwassuspendedin6mlDMEM(PI-PRP)(Figure1).TheplateletcountsweredoneusingtheRees-EckermethodintheNeubauerchamber,usinglightmicroscopy(Aggeleretal.

1946). The number of platelets at coculture per well used was based on the averageacquiredinplateletcountingexperimentswiththePRPpreparation(312,000platelets/µl).

Cellculture

Theexperimentalmodelwasdesignedforcoculture ofPI-PRPwiththehumanimmortalized keratinocyte cell line - HaCaT, being two experimental groups control – CTRL(HaCaT)andcoculture(PI-PRP/HaCaT)(Figure2).HaCaTcellsweremaintainedin Dulbecco′s Modified Eagle′s Medium (DMEM - code: D5523, Sigma-Aldrich, Inc.)containing:lowglucose,4mML-glutamine,1mMsodiumpyruvate)supplementedwith1500mg/Lsodiumbicarbonate(Scientific Exodus),4500mg/L anhydrousglucose(Neon), 100 IU/ml penicillin (Sigma-Aldrich, Inc.), 100 µg/ml streptomycin (Sigma-Aldrich, Inc.), 250 µg/ml amphotericin B (Sigma-Aldrich, Inc.) and 10% Fetal BovineSerum(FBS–Cultilab).Thecellsweregrown,in25cm²flasks,withscrewcapandfilterand sterile (Techno Plastic - TPP, Switzerland) incubated in an incubator COM – 17A6(Sanyo-biomedical,Japan)at37ºCwithahumidifiedatmospherecontaining5%CO_2.

After approximately 5 to 7 days, they reached 100% confluence and were split insterile flat-bottom plates (6, 12 or 96 well -TPP, Switzerland). To split the cells, theDMEM were removed, and the cells were washed 2 times with Dulbecco's PhosphateBuffered Saline (D-PBS 1X) without calcium and magnesium and subsequently treatedwith 1 ml of Trypsin-EDTA solution (Sigma-Aldrich, Inc.) in a CO_2 incubator at 37 ºCfor 10 minutes. After this time, 4 ml of DMEM was added. Subsequently, the cellsuspension was transferred to a 15 ml sterile conical polypropylene tube. CentrifugationwasperformedinaCentrilab80-2Bcentrifuge(GlobalTrade

Technology,Brazil)at327x*g*for5minutes.AprecipitateofHaCaTcellswasformedandthesup
ernatantdiscarded.ThepelletHaCaTcellsweresuspendedin5mlDMEM.Afterthisstep,thecel
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suspensionwasdiluted1:5andplatedinsterileflat-bottomplates(6,12or96wells)andmaintained in a CO_2 incubator at 37 ºC until 90% of confluency. Subsequently, the cellswerewashedtwicewithD-PBS1Xwithcalciumandmagnesium(D-PBS-CM).Forcell-starving D-PBS-CM was replaced with DMEM without FBS for 12 to 15 hours in a CO_2incubator at 37 ºC. After a while, the cells were washed twice with D-PBS-CM and thecellsweregroupedintotwoexperimentalgroups:CTRLGroup:cellsculturedinDMEMwithoutFBS.Coculturegroup:cellsculturedwithDMEMwithoutFBS+PI-PRPcontaining 312,000 platelets/µl, for 24 hours in CO_2 incubator at 37 ºC. After that, thecellswerewashed withD-PBS-CMtwicefor future procedures.

Fluorescencemicroscopy

The cellmorphologywas analyzedby fluorescenceusing dihydrochloric salt,4',6-diamidino-2-phenylindole(DAPI-MolecularProbesInc.,USA)tostainthenucleusandphalloidin green to stain the cytoskeleton in cells cultured in 12-well plates. Aftercoculture and washing, 500 µl/well of 4% paraformaldehyde (PFA, VetecQuímica/Sigma-Aldrich, Inc.) was added for 10 minutes at room temperature (RT) (Shin et al.2021).Cellsfrom8wellswerewashedtwicewithD-PBS-CM.500µl/wellofthesolutioncontaining22.5µlDAPI(1mg/ml–Sigma-Aldrich,Inc.)+45µlphalloidingreen(0.165µM/ml – Invitrogen) + 4.43 ml D-PBS-CM were add in each well and incubated to 15minutes on ice protected from light. Cells from 4 wells were washed twice with D-PBS-CM. 500 µl/well of D-PBS-CM were add in each well and incubated for 15 minutes onice protected from light used for negative control for cell autofluorescence.The platewastakenforanalysisbyfluorescenceopticalmicroscopyinaninvertedmicroscopeDMI4

000B(Leica, Germany).

MTTandTrypanblue Cellviability

Cell viability tests were performed by trypan blue and MTT (Hagio-Izaki et al. 2018;Mosmann 1983). For the trypan blue experiment, was added to the plate 0.5 ml/well ofTrypsin-EDTA(Sigma-Aldrich,Inc.)containingadherentcellsfor 10minutesinanCO_2incubator at 37 ºC following mixing 0.5 ml/well of DMEM. The cell suspension wastransferred to sterile 15 ml polypropylene conical tubes and centrifuged at 327 x *g* for 5minutes. The supernatant was discarded, and the cell pellets were suspended in 1 ml ofDMEM, 100 µl of cell suspension was mixed to 300 µl of 0.2% trypan blue solution(Sigma-Aldrich,Inc.),andkindlymixedusingamicropipette.A10µlaliquotwasaddedinaNeubauerch amber. After2minutesthenumberof cellswascounted.

ForMTTassay(3-[4,5-dimethyl-thiazol-2-yl]-2,5-diphenyltetrazolium),after24hours of coculture, the cells were washed twice with D-PBS-CM supplemented with5mMofglucose(D-PBS-CMG).200µlofthe0.1%MTTsolution(Sigma-Aldrich,Inc.)inD-PBS-CMGwasaddedineachwellofthe96-wellplateandincubatedfor2hoursinCO_2incubatorat37^{o}C.Then,theplateswerekeptonicewh eretheywerewashedtwicewith D-PBS-CMG. 200 µl of dimethyl sulfoxide (DMSO, Sigma-Aldrich, Inc) wereaddedfor5minutesinKlineNT151shaker(Novatecnica,Brazil).Theresultswerereadin a microplate reader Thermo Plate (Thermo Fisher Scientific, USA) with a wavelengthof 540 nm.

RNAextractionandreal timePCR

The RNA was extracted from the cells using Trizol® (Invitrogen, Carlsbad, CA, USA)followingmanufacture'sinstructions.RNAquantificationwasperformedonaNanoDro p® 2000c spectrophotometer (Thermo Fisher Scientific, USA) with readings

at260nmand280nm.Whennecessaryduetothepresenceofgenomic DNA,RNAsamples

were treated with the DNAse (Turbo DNA-free® kit, Invitrogen, USA) following themanufacturer's instructions. VEGF-A gene expression was performed after washing theplateincoculture,byextractingtheRNAsusingthemethodwithadaptations.Theprimersfor VEGF-A and β-actin (used as gene reference) amplification were designed usingNCBI Primer Blast (http://www.ncbi.nlm.nih.gov) and Clustal omega (www.clustal.org)softwareusingthefollowingcriteria:18and24nucleotidesindifferentexon s,maintainingameltingtemperatureorTM(meltingtemperature)of60°C± 1°Candwitha guanine-cytosine (GC) content ratio of 45 to 60 % and product amplification size up to150 bp.

Complementary DNA (cDNA) synthesis was performed using the High-CapacitycDNAReverseTranscriptionkit(AppliedBiosystems,USA),followingthemanufac turer's instructions. The mRNA content was amplified using the conventionalPCR technique with the GoTaq® G2 Colorless Master Mix kit (Promega Corporation,USA),accordingtothemanufacturer'sguidelines,usingthespecificprimerpairs designedforthisstudy(Table1).Thisstepwasperformedtocertifythattheamplificationproduc t generated in the reaction was the same size as that predicted. After confirmingthe size of the amplification product, the mRNA content of VEGF-A and β-Actin wasdetermined by the real-time PCR technique (qRT-PCR) using the SYBR Green kit(AppliedBiosystems,USA)onaRealTimeStepOnePlus®(AppliedBiosystems,USA),acc ording to the manufacturer's instructions. Transcriptional levels were normalizedusing the β-actin gene. The relative expression was calculated from the mean ± SEMvaluesof $2^{-(\Delta\Delta Ct)}$(Livak &Schmittgen2021).

StatisticalAnalyses

The results were expressed as mean ± SEM. The Shapiro-Wilk test was used to assesswhethereachsamplegrouphadanormaldistribution.Confirmingthenormaldistributio n,significancewasdeterminedusingtheOne-WayANOVAunpairedtestwithBonferroni post-test for experiments with 3 or more experimental groups (Ghasemi &Zahediasl2012).TheStudentTtestwasusedforexperimentswithonlytwoexperimentalgrou ps.Differenceswereconsideredsignificantwithp<0.05.AnalyzeswereperformedusingtheGr aphPadPrism4 program (GraphPadSoftware, Inc).

RESULTS

Plateletisolationandenrichment

The PRP was prepared with adaptations made in this study to obtain the PI-PRP (Figure1). The precipitate of platelets was obtained (Figure 3A). The observation of PI-PRP inNeubauer chamber using optical microscopy shows significant enrichment of plateletsand rare red blood cells present (Figure 3B). The platelet count (platelet/µl) in PI-PRPwas higher than in whole blood (474,500±74,506 and 312,250±43,014; n = 4; p < 0.05;respectively)(Figure3C).Thetimesofplateletenrichmentwereapproximately2.3inPI-PRP compared to whole blood (2.28 ± 0.43 and 1.00±0.00; n = 6; p < 0.05; respectively)(Figure 3D).

Cellmorphologyunderfluorescencemicroscopy

Toascertainwhetherplateletscaninducechangesinthenuclearandcytoskeletalmorphologyof HaCaTcells,cellularlabelingwithDAPIandphalloidinfluorophoreswasconducted. Upon examination under fluorescence microscopy, there were no significantchangesobservedinnuclearmorphologyfollowingcoculturetreatment;however, distinctalterationsincytoskeletal organizationwerenoted.(Figure4).

Cellviability

To verify if platelets can induce changes in the viability of HaCaT cells, cell viabilitytestswereperformedbytrypanblueandMTT.TheTrypanbluetestshowednosignificantdifferenceincellnumber(cells/ml)betweencoculturegroup(540,000±89,019;n=9;p > 0.05) and CTRL group (531,667±76,879 n = 9) (Figure 5A). In the MTT test it waspossible to observe higher cell viability (times the CTRL) in the coculture group (1.18 ±0.09,n= 11)compared totheCTRLgroup (1.00± 0.00,n =15, p< 0,05) (Figure 5B).

VEGF-AmRNAexpression

In the qRT-PCR the VEGF-A expression (times the CTRL) was higher in the coculturegroup(4.61±1.85;n=6;p<0.05)comparedtotheCTRLgroup(1.00±0.00;n=9) (Figure 6).

DISCUSSION

This protocol performed in an analogic centrifuge, common in clinics and offices, usingalowervolumeofwholebloodwhencomparedtotheprotocolperformedbyMcRedmond et al.(2004) and Wryszcz et al.(2017) who used 50 ml and 10 ml,respectively, of blood and was effective in concentrating and purifying the plateletsobtained from PRP. Despite the red coloration of the formed precipitate, the presence ofred blood cells was minimally identified by optical microscopy in a neubauer chamber,demonstratingalevel of purityachieved in themethod.

Onceconcentratedandpurifiedin PI-PRP,theplateletscouldbeusedasanexperimental model in coculture studies with HaCaT cells. Engebretsen et al. (2010)advocatestheisolation of plateletsfor laboratorypurposes.

CellulardamagetoHaCaTwasnotobserved;wecanquestionwhetherthetherapeuticuse of purified platelets would be more appropriate due to the reduction of interferingfactors. No studies were found in the literature for the use of platelet precipitates or theirsuspensionfor therapeuticpurposes.

Theanalysisofcellularmorphologyofthenucleusandcytoskeletonthroughfluorescence microscopy revealed no evidence of morphological changes in the nucleusof HaCaT cells, but exhibited alterations in cytoskeletal organization. This data suggeststhat cells under co-culture with platelets adopt a different morphology, yet this change isnot associated with a decrease in cell viability (Han et al., 2007). Further supporting thishypothesis is the significant increase in mitochondrial activity observed in the coculture,as evidenced by the MTT assay. These findings imply an enhancement in cell viabilityfollowingtreatment with PI-PRP(Zheng et al., 2016).

FuturestudiesareneededtoverifywhetherPI-PRPincreasesthecellularoxygenationofkeratinocytes,amechanismtobeexploredfortheuseofPI-PRPinalopecia.Instudieson the use of PRP for the treatment of alopecia, vascular mechanisms are attributed, asbeingcausedbytheincreaseinvascularstructuresaroundhairfolliclesandangiogenesis,as a response to the anti-hair loss action found (El Taieb et al. 2017). Once the VEGF-Aisinvolvedinangiogenesis,thisgrowthfactorcanbeanimportantplayerintheendothelial growth promoting improvement in follicular oxygenation and reducing hairlossinalopecia.InthisstudyitwasobservedthatthePI-PRPwasabletoincreaseVEGF-AmRNAcontentinHaCaTcells,suggestingthatplateletscouldbeableincreasevascularproliferation and oxygenation of keratinocytes. This upregulation in VEGF-A mRNAmay be an effect of platelet adhesion to keratinocytes. Thus, it would be interesting forfuturestudieswithplateletstoidentifythecellularandmolecularmechanismsinvolved

in the improvement of cellular viability and upregulation of VEGF-A in keratinocytescells.

CONCLUSION

In this study was able to standardize a new protocol for platelet isolation from smallsamples of peripheral blood. Also, it was observed that PI-PRP seems to be harmless forHaCaT cell lines and promotes improvement in cell proliferation and VEGF-A mRNAexpression. For the first time, this study developed a feasible protocol to study theinteractionbetween plateletsand targetcells.

CONFLICTOFINTERESTS

The authors of this study do not have professional relationships with companies ormanufacturerswhowill benefitfrom theresultsof the presentstudy.

TheresultsofthepresentstudydonotconstituteanendorsementbytheACSM.

The authors of this study declare that the results are presented clearly, honestly, andwithoutfabrication,falsification, orinappropriate datamanipulation.

Consent

As per international standards or university standards, patient(s) written consent has been collected and preserved by the author(s).

Ethical approval: This study was approved by the ethics committee (CEP/UFRJ-Macaé 1.922.306).

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FIGURE'SLEGENDS

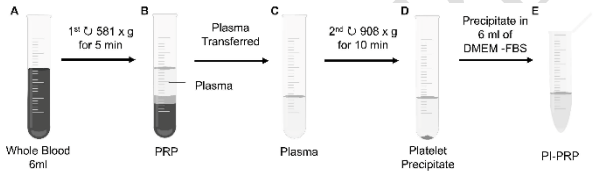

Figure 1

Experimental design of platelet isolation from PRP to obtain PI-PRP. (1a) 6 ml of bloodwere collected with a 22G x 1" needle (25 x 0.7 mm) in vacuum tube containing EDTANa+2. (1b) Whole blood was centrifuged at 581 x *g* for 5 minutes and the plasma wascollectedand(1c)transferredtoanewtube.(1d)Asecondcentrifugationwasperformedat9 08x*g*for10minutestoobtaintheplateletprecipitate.Thesupernatantwasdiscarded.(1e) The platelet precipitate was resuspended in 6 ml of DMEM without fetal bovineserum(DMEM-FBS),beingcalledisolatedplateletprecipitatesuspension(PI-PRP).

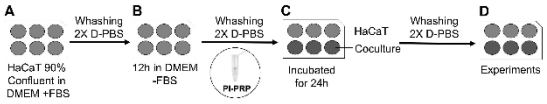

Figure 2

HaCaT/PI-PRPcoculture assay. (2a) After reaching confluence, around 48 to 72 hours in culture, the HaCaT cells were splitted in a 6-well plates in DMEM with 10% Fetal Bovine Serum (DMEM+FBS) and incubated in an oven with 5%CO2 at 37ºC until 90% confluence. (2b) The cells were washed twice with Dulbecco's Phosphate-Buffered Salinesolution (D-PBS 1X with calcium and magnesium) and 2 ml of DMEM without FBS(DMEM -FBS) was added to each well and incubated for 12 hours in incubator with 5%CO2 at 37ºC. (2c) The HaCaT was washed twice with D-PBS 1X with calcium and magnesium. Half of the wells (in light gray) received 2ml/wells of DMEM without FBS (DMEM-FBS) being HaCaT group. The other wells (in dark gray) were cocultured with isolated platelet precipitate suspension (PI-PRP 312,000 platelets/µl of DMEM -FBS final volume of 2 ml). being Coculture group. HaCaT and Coculture groups were incubated for 24 hours in 5% CO2 atmosphere incubator at 37ºC. (2d) The experimental groups were washed again twice with D-PBS 1X with calcium and magnesium to carryout the study experiments.

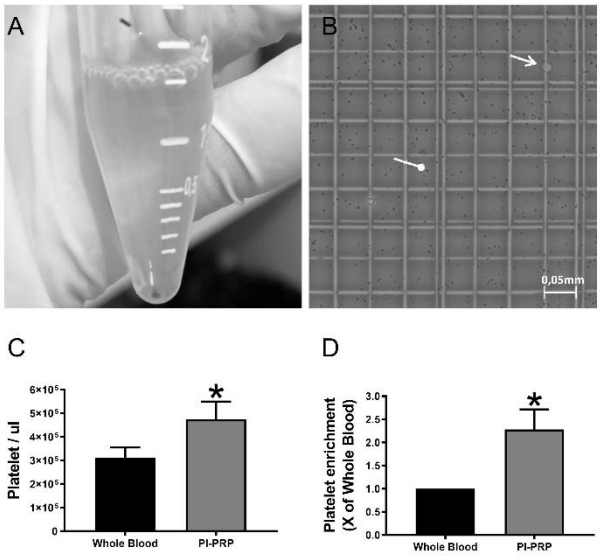

Figure 3

PlateletcountinginPI-PRP.(3a)representativeimageoftheprecipitateplateletsindicated by the black arrow. (3b) representative image showing PI-PRP in Neubauerchamber.Theplateletsareindicatedbyarrowwitharrowheadandtheredbloodcellsa reindicated by round tip arrow. The platelets were stained by Rees-Ecker method andanalyzedbyopticalmicroscopywithphasecontrastat200XmagnificationinaNeubauerch amber. (3c) Graphic representation of platelet counting in whole blood and PI-PRP.(3d) Graphic representation of the platelet enrichment in PI-PRP compared to wholeblood.Valuesarerepresented asMean ±SEM,n=5, (*) indicatesp < 0.05.

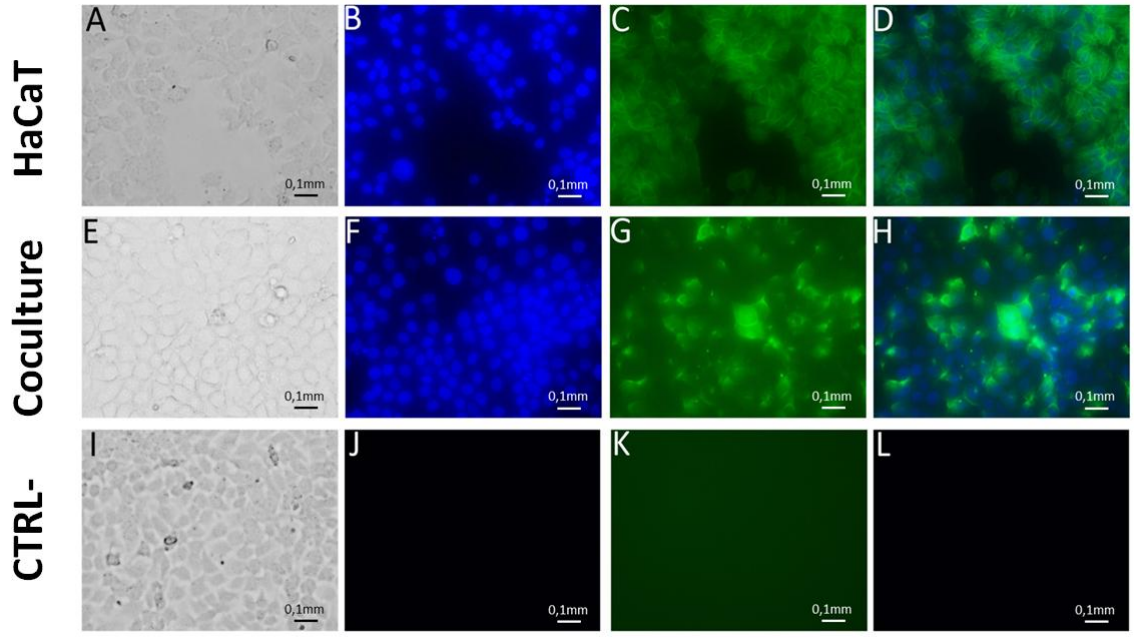

Figure 4

Fluorescence optical photomicrography of HaCaT and PI-PRP coculture. The imageswereobtainedusinganinvertedmicroscopewithamagnificationof400Xandascaleof 0.1mm.Where(4a)HaCaT.(4b)HaCaTshowingthenucleusstainedbyDAPI.(4c)

HaCaTshowingthecytoskeletonstainedbyphalloidingreen.(4d)HaCaTinoverlappingDAPI /Phalloidin. (4e) Coculture HaCaT and PI-PRP (HaCaT/PI-PRP). (4f) HaCaT/PI - PRPshowingthenucleusstainedbyDAPI.(4g)HaCaT/PI-PRPbyfluorescenceshowingthecytoskeletonstainedbyphalloidingreen.(4h)HaCaT/PI-PRPinoverlappingDAPI/Phalloidin. (4i) HaCaT without any staining. (4j) Cells of panel I visualized inDAPI filter showing no background fluorescence. (4k) Cells of panel I visualized inphalloidin green filter showing no background fluorescence. (4l) Overlapping of theimagensfrompanels4jand 4k.

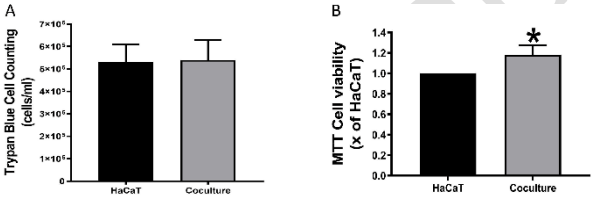

Figure 5

Cell viability accessed by trypan blue staining and MTT. (5a) Graphic representation oftrypan blue cell counting. The graph represents the cell counts of the HaCaT group andcoculture group showing no significant difference between both groups. Values arerepresented a Mean ± SEM, n=9. (5b) Graphic representation of MTT cell viability. Thegraph shows results from the two groups HaCaT and coculture showing a significantdifference between both groups. Values are represented as Mean ± SEM, n = 11, (*)indicatesp < 0.05.

	Forward 5'-3'	Reverse 5'-3'	Produ	Reference

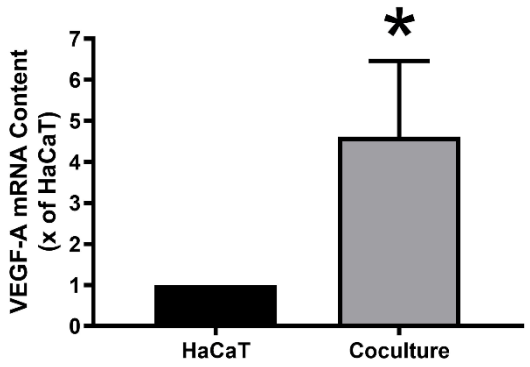

Figure 6

GraphicrepresentationofrelativeVEGF-AmRNAcontentininHaCaTandincoculturegroups. Real Time PCR showing that relative VEGF-A mRNA content in Coculture ishigherthanHaCaTgroup.ValuesarerepresentedasMean±SEM,n=8,(*)indicatesp<0.05.

			ct size	sequence
VEGF-A	ctctacctccaccatgccaag	tcgtgatgattctgccctcct	71 bp	NM_001025366.3
β-actin	cctcgcctttgccgatcc	cgcggcgatatcatcatccat	70 bp	NM_001101.5

Table 1. The primer pairs used for amplification of VEGF-A and β-actin mRNAs.