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Article

Evaluation of *in vitro* anti-inflammatory effect of PLAY® on UC-MSCs: A COX-2 expression study

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SUMMARY

Wound healing is an intricate process consisting of multiple phases, each of which is indispensable for requisite tissue repair. Timely initiation and resolution of each overlapping phase is critical in aiding the wound healing cascade. A prolonged inflammatory phase causes overexpression of proteolytic mediators, resulting in extracellular matrix (ECM) degradation and increased oxidative stress. This imbalance in inflammatory mediators, ECM degradation, and upregulated levels of reactive oxygen species retards cell proliferation and migration, resulting in delayed tissue repair and the development of chronic wounds. Cyclooxygenase-2 (COX-2) is a potent inflammatory mediator that plays a pivotal role in the wound healing process at different stages of the tissue repair cascade. Overexpression of COX-2 during the tissue repair process impairs the wound healing cascade and contributes to the formation of chronic wounds. PLAY® is an in-house product of International Stem Cell Services Limited (iCREST) that is a human blood-platelet cocktail of natural growth factors and bioactive modulators. In our study, we hypothesized that PLAY® decreases inflammation in phorbol myristate acetate (PMA)- and 5-fluorouracil (5-FU)- stimulated umbilical cord derived mesenchymal stem cells (UC-MSCs) by downregulating *COX-2* expression to its basal levels. We used an experimentally induced inflammatory *in vitro* model to produce enhanced *COX-2* expression in mesenchymal stem cells (MSCs) and PLAY® showed a significant decrease in *COX-2* levels in PMA induced UC-MSCs, providing evidence for an innate anti-inflammatory effect of PLAY® and a potential role in accelerated wound healing.

INTRODUCTION

Wound healing is a complex process of tissue repair and regeneration, a normal biological process that includes four different overlapping phases: homeostasis, inflammation, proliferation, and remodeling (1). This process is delayed in cancer patients undergoing chemotherapy, radiotherapy, and in chronic wound conditions (2,3). This is mainly due to prolonged inflammation, loss of repair mechanisms, formation of drug resistant microbial biofilms, and persistent infection (4–6).

At the site of injury, the platelets and clotting factors aggregate, forming a scaffold to stop the loss of blood. Activated platelets release growth factors and cytokines, which triggers the inflammatory phase, recruiting cells like neutrophils, macrophages, and eosinophils. Proinflammatory cytokines like tumor necrosis factor (TNF), interleukin-1 (IL-1), interleukin-6 (IL-6), and C-X-C motif chemokine ligands 2 and 8 (CXCL2/8) released by these activated leukocytes recruit T-cells and natural killer (NK) cells, further amplifying the Th1-mediated proinflammatory response, resulting in the production of cyclooxygenase-2 (COX-2), a proinflammatory marker. COX-2 or prostaglandin g/h synthase is an inducible enzyme that produces prostaglandins which are the key mediators of inflammation, where vascular permeability and inflammatory cells are stimulated causing symptoms including redness, swelling, and pain (7–10).

The downregulation of proinflammatory cytokines and the increase in anti-inflammatory mediators play an important role in the transition from the inflammatory phase to the proliferation phase, which is impaired in chronic wounds. Excessive infiltration of leukocytes, primarily macrophages, increases pro-inflammatory cytokine expression partly mediated by *COX-2*. This, in turn, activates metalloproteinases, which degrade the growth factors and structural proteins of the extracellular matrix (ECM) tissue, leading to unbalanced proteolytic activity and increased reactive oxygen species (ROS, e.g., H_2O_2, O_2^-). This oxidative stress damages the neighboring healthy cells and tissue, causing the wound to grow and leading to persistent chronic wounds (11–13).

Currently, there is no single effective treatment for chronic wounds, so treatment is aimed at relieving symptoms and preventing complications. Treatments may include wound dressings, antiseptics to prevent infection, antibiotics to treat infection, pain-relieving topical steroids to reduce inflammation, debridement, and skin grafts (14).

5-flurouracil (5-FU) is one of the most used chemo-drugs in treating breast, head and neck, and colon cancers (15). It induces cytotoxicity by inhibiting deoxythymidine monophosphate production, which interferes with DNA replication and repair. While this targets cancer cells, it also targets rapidly dividing cells like those found in the oral mucosal tissue lining, resulting in inflammation and ulcer formation in a condition called mucositis. Mucositis, one of the chronic wounds is the most common complication developed by cancer patients undergoing radiotherapy or chemotherapy, occurring in 80% patients undergoing 5-FU therapy (16–18). Increased levels of *COX-2* expression in early oncogenic events have been shown to develop resistance to these chemo drugs, reducing their therapeutic efficacy (19). Therefore, regulating the increased

COX-2 expression would be a potential therapeutic strategy to overcome resistance to chemo drugs.

PLAY®, an in-house product of International Stem Cell Services Limited (iCREST), is a human blood-platelet cocktail of natural growth factors and bioactive modulators. Platelet derivatives containing growth factors like vascular endothelial growth factor (VEGF), platelet derived growth factor (PDGF), transforming growth factor beta (TGF-β), and epidermal growth factor (EGF) and bioactive mediators have shown a significant role in tissue repair and regenerative properties (20). These growth factors play an important role in each phase of wound healing which aids in cell migration and regulate angiogenesis by providing balanced release of pro- and anti-angiogenic factors like VEGF, fibroblast growth factor (FGF), EGF, sphingosine-1-phosphate (S1P), angiopoietin-1 (Ang1), and PDGF (23, 25–27). Studies have shown that platelet-released growth factor (PRGF)-based formulations induce ECM-involved genes in human keratinocytes and in human fibroblasts, showing accelerated wound healing *in vitro* (21, 22).

Platelet-based formulations have gained interest clinically in treating non-healing chronic wounds due to their ease, cost effectiveness, regenerative properties, and potential to stimulate and accelerate wound healing (23). Our previous study has shown that PLAY® consistently outperformed FBS in terms of wound healing properties such as scratch assay and measuring wounding cytokines (24).

In our study, we developed an experimentally induced *in vitro* inflammatory model by inducing *COX-2* expression in Umbilical Cord-derived Mesenchymal Stem Cells (UC-MSCs) using PMA. PMA is a mitogen that induces the expression of *COX-2* by activating certain signaling pathways, such as the mitogen-activated protein kinase (MAPK) pathway. Additionally, PMA has been shown to upregulate the expression of certain transcription factors, such as NF-κB, that are known to regulate the expression of *COX-2* (24,25). UC-MSCs of passage 5 and 7 were chosen for studies as cellular characteristics of MSCs are impacted by passage number. Studies have shown as the passage number increases the senescence related genes, like *P21*, and oncogenes are upregulated (26). Henceforth, we aimed to study if aging of cells influences the effect of PLAY® in downregulating the inflammation through *COX-2* expression.

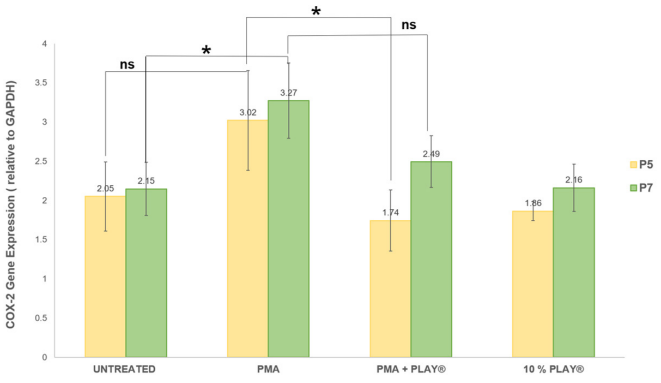

Figure 1: Effect of 10% PLAY® on COX-2 expression in PMA-treated P5 and P7 UC-MSCs (N=2). COX-2 levels shown as mean ± SD. UC-MSCs of P5 (yellow) and P7 (green) were treated as follows: (i) untreated control, (ii) 3.048 µg/ml PMA, (iii) PMA + 10% PLAY®, and (iv) 10% PLAY®, respectively. *p < 0.05, ns = p > 0.05.

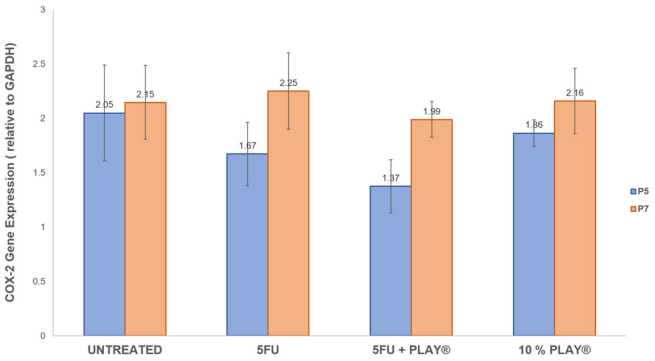

Figure 2: Effect of 10% PLAY® on COX-2 expression in 5-FU-treated P5 and P7 UC-MSCs (N=2). COX-2 levels shown as mean ± SD. UC-MSCs of P5 (blue) and P7 (orange) were treated as follows: (i) untreated control, (ii) 2 µg/ml 5-FU, (iii) 5-FU + 10% PLAY®, and (iv) 10% PLAY®, respectively. p > 0.05 for all comparisons.

In this study, we hypothesized that PLAY® lowers the inflammatory marker, *COX-2* mRNA expression in PMA and 5-FU induced UC-MSCs at passage 5 and 7. We also aimed to study if 5-FU induces the expression of *COX-2* in UC-MSCs and further study the effect of PLAY® in downregulating *COX-2*, implying its role in anti-inflammation. Our results demonstrate, in PMA-induced UC-MSCs, PLAY® significantly reduced the levels of *COX-2*, demonstrating an intrinsic anti-inflammatory effect of PLAY® and suggesting a potential role in accelerated wound healing.

RESULTS

Effect of PLAY® on UC-MSCs at P5 and P7 in *COX-2* expression induced by PMA

To examine the anti-inflammatory activity of PLAY®, we treated P5 and P7 UC-MSCs with PMA for 48 hours and assessed *COX-2* expression using semi-quantitative PCR. Under *in vitro* conditions the expression of *COX-2* was successfully induced using PMA. UC-MSCs treated with PMA showed a significant increase in *COX-2* levels at P7 (two-tailed t-test, $p = 0.029$), whereas at P5, induction was not significant (two-tailed t-test, $p > 0.05$) when compared to the respective control groups.

Further, cells treated with PMA +10% PLAY® downregulated the induced *COX-2* levels significantly in P5 (two-tailed t-test, $p = 0.04$), whereas in P7, downregulation was not significant (two-tailed t-test, $p > 0.05$). The untreated group and 10% PLAY® group showed similar levels of *COX-2* expression, which implies that 10% PLAY® downregulated only the induced *COX-2* levels **(Figure 1)**.

Effect of PLAY® on UC-MSCs at P5 and P7 in *COX-2* expression induced by 5-FU

After demonstrating that PLAY® was successful in downregulating COX-2 expression in PMA induced UC-MSCs, we evaluated the effect of PLAY® in 5-FU treated P5 and P7 UC-MSCs on *COX-2* expression. Since in both P5 and P7 passages, 5-FU treated cells did not demonstrate a substantial elevation of *COX-2* expression compared to untreated groups (two-tailed t-test, $p > 0.05$), further effect of PLAY® in downregulating the *COX-2* expression could not be assessed **(Figure 2)**.

DISCUSSION

In our study we investigated the anti-inflammatory properties of PLAY® through *COX-2* expression studies in PMA and 5-FU induced UC-MSCs. As prolonged inflammation is a common characteristic of non-healing chronic wounds, resolving it would accelerate the wound healing process (27).

Our results showed a significant induction of *COX-2* expression in P5 and P7 UC-MSCs when treated with PMA, which mimics an *in vitro* inflammatory model for our studies. Although non-significant, *COX-2* expression was increased in P7 UC-MSCs compared to that of P5 UC-MSCS. This was found intrinsically linked to the passage numbers of MSCs in culture.

For stem cell based clinical applications, *in vitro* cultured autologous MSCs till passage 5 has been authorized. Studies have shown as the passage number increases the expression of senescence-related genes are significantly upregulated in the late phase (26). Earlier passage numbers tend to result in more robust and functional cells, while later passage numbers may result in reduced functionality. A previous study demonstrated immunomodulatory properties of early and late passage of UC-MSCs. Their findings showed a higher level of *COX-2* expression in late-passage UC-MSCs, which is consistent with our findings as well (28).

PLAY® downregulated the extrinsically induced *COX-2* levels back to basal levels as seen in untreated groups in both P5 and P7 UC-MSCs, with P5 UC-MSCs being more effective in downregulating the *COX-2* levels. Thus, in our experimental context, we infer that PLAY® has an anti-inflammatory property.

Based on many *in vitro* and *in vivo* studies, basal levels of *COX-2* are essential for early phases of wound healing. Importantly, it is the overexpression of *COX-2* that impairs the tissue repair and wound healing cascade, resulting in chronic wounds (29-31). This supports interest of using PLAY®, a platelet derivative, for non-healing wounds as certain levels of inflammation are required to complete the process of healing in a systematic, timely manner.

Experimental and clinical studies have shown wide applications of platelet derivatives in treating chronic inflamed wounds and ulcers. *In vivo* studies with rat models have shown a decrease in pro-inflammatory (IL-6, TNF-α) and substantial increase in anti-inflammatory cytokines (IL-10, TGF-β) with the administration of platelet derivatives (32). Furthermore, several clinical studies have demonstrated the use of platelet-derived products (like platelet-rich plasma (PRP) and plasma lysate (PL) to reduce inflammation and enhance the wounds repair in different tissues in the human body (33). Thus, it may be inferred that platelet derivatives may possess a central role in tissue repair and wound healing by regulating inflammation and cellular physiologies like cell proliferation, differentiation, and migration (34).

Unlike with the PMA-treated group, there was not a significant induction of *COX-2* expression in the 5-FU-treated group. A possible future study would be to investigate the reason behind 5-FU not inducing *COX-2* expression in UC-MSCs, though our previous studies with 5-FU significantly induced *COX-2* expression in cervical cancer cell lines (35). Other studies have shown a correlation of *COX-2* stimulation and 5-FU resistance through the Akt pathway in gastric cancer cell lines (36).

To the best of our knowledge, this is the first study exploring the role of a blood derivative downregulating the inflammatory marker *COX-2*. To evaluate a potential role for PLAY® resolving the prolonged inflammation, further studies can be explored in accelerating the process of wound healing. Our future work would include investigating the *COX-2* expression levels and other inflammatory mediated markers like IL-6, etc. with a wide range of passages in UC-MSCs and other fibroblastic cell lines. Our study would extend to assess the protein expression levels with PLAY® treatment, as protein being the functional product of the cell physiology. We would further want to assess the role of other cytokines and signaling cross-talk involved in the process of wound healing in particular emphasis with PLAY® treatment *in vitro* and *in-vivo* developing a better wound healing model. This approach to hasten and improve the wound healing and tissue repair may be extended to clinical trials in the future to mitigate chronic inflamed wounds and diabetic wounds as well.

MATERIALS AND METHODS

PLAY® is an in-house product of iCREST, a human blood-platelet derivative which contains active biological concentrate that is a rich natural source of growth factors and bioactive modulators. Human blood was collected from healthy donors with informed consent from the subjects.

UC-MSCs isolation and cell culture

UC-MSCs were purchased from Lonza and cultured in 10% Fetal Bovine Serum (FBS) containing Dulbecco's Modified Eagle's Medium (DMEM, Gibco) and maintained at standard cell culture conditions (37°C and 5% CO_2). Adhered cells at 80% confluency were detached using 0.25% trypsin-EDTA (Hi Media) and were reseeded in T-25 Flask at a density of 3000 cells/cm^2 with DMEM (10% FBS) for further studies. UC-MSCs of P5 and P7 were used for experiments.

Drug treatment

UC-MSCs (P5 and P7) were seeded in a 6-well plate (150,000 cells per well) in DMEM supplemented with 10% FBS. Cells were stimulated with PMA (3.048 ng/ml), PMA + 10% PLAY®, 5-FU (2 µg/ml), 5-FU + 10% PLAY®, and 10% PLAY® for 48 hours. PMA and 5-FU of respective concentrations are prepared with DMEM (Gibco). Cells in the control group were cultured in DMEM supplemented with 10% FBS.

RNA extraction and cDNA synthesis

Total RNA of UC-MSCs (P5 and P7) of the groups: PMA, PMA + 10% PLAY®, 5-FU, 5-FU + 10% PLAY®, and 10% PLAY® was extracted using the Nucleospin® RNA Isolation Kit (Takara Bio Inc.) as per the manufacturer's instructions. The concentration and purity of RNA was quantified using a spectrophotometer (Thermo Scientific) and was determined using the formula: Concentration of RNA (µg/mL) = Absorbance@260nm × dilution factor × 40.

For cDNA synthesis, RNA sample of 1000 ng was first reverse transcribed into cDNA using the Prime Script™ cDNA Synthesis Kit (Cat. #6110A, Takara Bio Inc.) following the manufacturer's instructions.

Semi-quantitative polymerase chain reaction

For the amplification of glyceraldehyde-3-phosphate dehydrogenase (*GAPDH)* and *COX-2*, 55 ng of cDNA was used and PCR was carried out using JumpStartTM Taq DNA

Polymerase (6 µL) (Sigma-Aldrich) and 1 pmol (1 µL) of forward and reverse primer for each gene (Juniper Life Sciences). A final volume of 12.5 µL PCR reaction was obtained using molecular grade water.

Semiqualitative PCR was carried out to measure the *COX-2* expression using the following primer sequences (Applied Biosystems): Forward primer sequence for *COX-2*: 5'-CCACTTCAAGGGATTTTGGA-3'. Reverse Primer sequence for *COX-2*: 5'-GAGAAGGCTTCCAGCTTTT-3'. Forward primer sequence for *GAPDH: 5'*-GGTCGGAGTCAACGGATTTGGTCG-3'. The Reverse Primer sequence for *GAPDH*: 5'-CCTCCGACGCCTGCTTCACCAC-3'. The expression levels obtained were normalized using *GAPDH,* a housekeeping gene.

To resolve the PCR products, 10µl of PCR product was added with 2 µl of 2.5X gel loading buffer (Genei) and loaded along with 100 bp DNA ladder (Takara) on a 2% agarose gel and the gel was run at 100V for 30 minutes. Ethidium Bromide was used to observe the bands under a UV-transilluminator (Biobee Tech). ImageJ software was used to assess and quantify the target gene expression, and the results were normalized to *GADPH*.

Mycoplasma detection

Mycoplasma contamination for UC-MSCs P5 and P7 was done using semiquantiative PCR following the instruction guidelines of LookOut® Mycoplasma PCR Detection Kit (Sigma-Aldrich). The results were negative. UC-MSCs P5 and P7 were tested for absence of mycoplasma contamination, to make sure the cells used for experiments are contamination free and inflammation induced in PMA and 5FU groups is not due to presence of mycoplasma.

Statistical Analysis

Data in bar graphs represent mean +/- standard deviation. Significance was determined using an unpaired two-tailed t-test for two independent sets for all the groups using Microsoft Excel.

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