

Hyaluronic Acid Induces the Release of Growth Factors from Platelet-Rich Plasma

(ヒアルロン酸は多血小板血漿からの成長因子の放出を促進する)

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ABSTRACT

Objective: To investigate the influence of HA on platelet in PRP by measuring releasing growth factors.

Methods: PRP was produced from 9 healthy adult volunteers (mean age: 32.8 ± 2.9 years; range: 29–37) with commercial separation system (Arthrex ACP, Arthrex, Naples, FL, USA). HA of weight-average molecular weight of 50-120 kDa (Artz-dispo, Seikagaku, Tokyo, Jpana) was used as HA. PRP group (PRP 1ml + PBS 0.2ml) and PRP + HA group (PRP 1ml + HA 0.2ml) were incubated. The amount of transforming growth factor $\beta 1$ (TGF- $\beta 1$) and platelet-derived growth factor (PDGF-AA) released from the PRP and PRP + HA samples were measured on days 0, 3, and 5. In addition, same growth factors on day 5 were measured for PRP + high HA group (PRP 1ml + HA 0.6ml) with 5 donors. After collecting all of the samples on day 5, the remaining gels with Giemsa stain were observed. The statistical analyses were performed using paired t-tests to compare the PRP and HA groups at each time point, and a one way ANOVA with Tukey post-hoc tests was used to compare the PRP, PRP + HA, and PRP + high HA groups.

Results: The TGF- $\beta 1$ concentrations in the PRP and PRP + HA were 24.3 ± 7.2 $\mu\text{g/mL}$ and 22.4 ± 1.8 $\mu\text{g/mL}$ ($p=0.689$) on day 0, 17.2 ± 13.9 $\mu\text{g/mL}$ and 25.4 ± 7.1 $\mu\text{g/mL}$ ($p=0.331$) on day 3, and 12.7 ± 10.5 $\mu\text{g/mL}$ and 33.7 ± 8.3 $\mu\text{g/mL}$ ($p=0.034$) on day 5. The TGF- $\beta 1$ concentrations on day 5 were 24.1 ± 5.2 $\mu\text{g/mL}$ (PRP group), 28.3 ± 2.4 $\mu\text{g/mL}$ (PRP + HA), and 31.9 ± 4.8 $\mu\text{g/mL}$ (PRP + high HA) (ANOVA: $p=0.003$; post-hoc PRP vs. PRP + HA: $p=0.016$). The PDGF-AA concentrations in the PRP and PRP + HA groups were 2.30 ± 1.21 $\mu\text{g/mL}$ and 2.32 ± 0.79 $\mu\text{g/mL}$ ($p=0.931$) on day 0, 2.03 ± 0.53 $\mu\text{g/mL}$ and 2.13 ± 0.73 $\mu\text{g/mL}$ ($p=0.500$) on day 3, and 1.51 ± 0.40 $\mu\text{g/mL}$ and 2.00 ± 0.52 $\mu\text{g/mL}$ ($p=0.003$) on day 5. The PDGF-AA concentrations were 1.48 ± 0.46 $\mu\text{g/mL}$ (PRP group), 1.94 ± 0.57 $\mu\text{g/mL}$ (PRP + HA), and 2.69 ± 0.70 $\mu\text{g/mL}$ (PRP + high HA) (ANOVA: $p=0.0002$; PRP vs. PRP + high HA: $p=0.002$; PRP + HA vs. PRP + high HA: $p=0.011$) on day 5. The PRP showed larger coagulated masses than the PRP + HA. The high concentrate HA group had the smallest coagulated mass of all of the group.

Conclusion: The levels of growth factors released by PRP on day 5 were increased by the addition of HA. A mixture of PRP and HA may be a more effective therapy than PRP or HA alone for osteoarthritis and tendinopathy.

INTRODUCTION

The use of platelet-rich plasma (PRP) to treat musculoskeletal soft tissue injuries [1], bone grafts [2], osteoarthritis (OA) [3, 4], and even skin ulcers [5] is increasing. Although the long-term effects of PRP remain controversial, the high concentration of autologous growth factors in PRP is expected to reduce the time needed for healing based on the accumulated basic and clinical research. Therefore, assessment of the levels of growth factors released from PRP is important.

Hyaluronic acid (HA) is widely used to treat OA of the knee [6]. The beneficial effects of HA are attributed to its function as a viscosupplement and its anti-inflammatory activity. HA injection is also used to treat tendon and ligament injuries and after surgery [7, 8].

Several reports that compare the clinical outcomes achieved with HA and PRP for OA have been published [3, 4]. However, the clinical results of simultaneous HA and PRP injections have not yet been reported.

Recently, Chen et al. published an in vitro study of the synergistic anabolic actions of HA and PRP on cartilage regeneration in OA [9]. In that report, the combination of HA and PRP reduced the levels of pro-inflammatory cytokines and increased articular chondrocyte proliferation and chondrogenic differentiation. The authors concluded that the observed synergistic effects were the result of different molecular mechanisms: the HA-dependent Erk1/2 pathway and the PRP-dependent Smad2/3 pathway. However, the direct influence of HA on the platelets in PRP was not discussed. In the present study, we tested the hypothesis that the addition of HA increases the levels of growth factors released by PRP.

MATERIALS AND METHODS

The protocol for this study was approved by the Ethics Committee of Hirosaki University Graduate School of Medicine.

Preparation of the platelet-rich plasma

Nine healthy adult volunteers (2 females and 7 males) with an average age of 32.8 ± 2.9 years (range: 29–37) were included in this study. Only one patient was taking medication of any kind, and that person was taking purgative medicine. No impairment of liver or kidney functions was detected in the patient blood samples.

Forty-five mL of peripheral blood for PRP preparation and an additional 1 mL of blood for the whole blood cell count were collected from the median cubital veins of each donor using a 21-gauge needle. No anticoagulant or activation materials, such as calcium chloride, were used. The PRP was produced using a commercial PRP separation system (Arthrex ACP, Arthrex, Naples, FL, USA) using a double syringe system according to the manufacturer's instructions. From each donor, 10–12 mL of PRP was prepared. Blood counts for the PRP preparations were measured using 1 mL of PRP.

PRP culture and harvest of the released growth factors

ARTZ-Dispo HA (Seikagaku, Tokyo, Japan) with a weight-average molecular weight of 50–120 kDa was used as the HA. Three replicate wells of 1 mL of PRP and 0.2 mL of phosphate buffered saline (PBS) (PRP group), three replicate wells of 1 mL of PRP and 0.2 mL of HA (PRP + HA group), and one well of 1 mL of PRP and 0.6 mL of HA (PRP + high HA group) were incubated on non-coating 6-well dishes (Nunc, Shanghai, China) in a cell culture incubator at 37°C with 5% of CO₂ immediately after PRP preparation. After 2 hours of incubation (defined as day 0), all the specimens had formed gels. At that time, 8.8 mL of PBS was added to one well from the PRP and PRP + HA groups to a 10-fold dilution, and all the liquid was collected 1 hour later. Any remaining platelets were removed with gentle centrifugation for 15 minutes at 200×g

and then another centrifugation for 15 minutes at 10,000×g. The samples were immediately frozen with liquid nitrogen and stored at -80°C until the growth factors were assessed. In the same way, samples from the PRP and PRP + HA groups were obtained on days 3 and 5 after PRP preparation.

For five of the donor PRPs (n=5 donors), the PRP + high HA group samples obtained on day 5 were diluted with 8.4 mL of PBS because of the higher dose of 0.6 mL of HA. In addition, to confirm that the growth factors were continuously released from the PRP, 0.2 mL of PBS for the PRP group and 0.2 mL of HA for the HA group was added to the remaining gels (n=5 per group) after sample collection on days 0 and 3. The released growth factors were collected on day 3 (days 0–3) and day 5 (days 3–5) in a similar way as was done for the PRP and PRP + HA groups.

Gross appearance on day 5

After collecting all of the samples, the remaining gels were fixed with absolute methanol for 5 minutes and stained with Giemsa stain for 5 minutes. Microscopic images (Olympus IMT-2-21 RFM, Olympus Corp., Tokyo, Japan) were taken using a digital camera (Canon DS 126181, Canon Inc., Tokyo, Japan).

Hematological analysis

The platelet, white blood cell (WBC), neutrophil, lymphatic cell, and red blood cell (RBC) counts in the peripheral blood and PRP were determined using an automated cell count analyzer (Sysmex XE-5000, Sysmex Corp., Kobe, Japan).

Transforming growth factor-β1 and platelet-derived growth factor-AA levels

After thawing the stored samples, quantitative determinations of the transforming growth factor-β1 (TGF-β1) and platelet-derived growth factor-AA (PDGF-AA) levels were performed using a commercially available enzyme-linked immunosorbent assay kit (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's

instructions. The color intensity of each well was measured using a spectrophotometer (Multiskan FC, Thermo Fisher Scientific, Yokohama, Japan) at 450 nm with a wavelength correction of 570 nm. The final calculations were made using 10-fold sample dilutions.

Statistical analysis

All data are expressed as mean \pm SD. The statistical analyses were performed using paired t-tests to compare the PRP and HA groups at each time point, and a one way ANOVA with Tukey post-hoc tests was used to compare the PRP, PRP + HA, and PRP + high HA groups. P-values less than 0.05 were considered statistically significant. All statistical analyses were performed in GraphPad Prism 6.0 (GraphPad Software, San Diego, CA, USA).

RESULTS

Blood cell counts

The concentrations of platelets and WBCs in the PRP preparations were 2.35 ± 0.30 times and 0.305 ± 0.152 times those found in the peripheral blood. The percentage of lymphocytes was higher in the PRP preparations (Table).

Gross appearance at day 5

The PRP group showed larger coagulated masses than the PRP + HA group. The PRP + high HA group had the smallest coagulated mass of all of the groups (Fig. 1).

Growth factor concentrations

The TGF- β 1 concentrations in the PRP and PRP + HA were 24.3 ± 7.2 $\mu\text{g/mL}$ and 22.4 ± 1.8 $\mu\text{g/mL}$ ($p=0.689$) on day 0, 17.2 ± 13.9 $\mu\text{g/mL}$ and 25.4 ± 7.1 $\mu\text{g/mL}$ ($p=0.331$) on day 3, and 12.7 ± 10.5 $\mu\text{g/mL}$ and 33.7 ± 8.3 $\mu\text{g/mL}$ ($p=0.034$) on day 5 (Fig. 2a). The TGF- β 1 concentrations on day 5 were 24.1 ± 5.2 $\mu\text{g/mL}$ (PRP group), 28.3 ± 2.4 $\mu\text{g/mL}$ (PRP + HA), and 31.9 ± 4.8 $\mu\text{g/mL}$ (PRP + high HA) (ANOVA: $p=0.003$; post-hoc PRP vs. PRP + HA: $p=0.016$) (Fig. 3a).

The PDGF-AA concentrations in the PRP and PRP + HA groups were 2.30 ± 1.21 $\mu\text{g/mL}$ and 2.32 ± 0.79 $\mu\text{g/mL}$ ($p=0.931$) on day 0, 2.03 ± 0.53 $\mu\text{g/mL}$ and 2.13 ± 0.73 $\mu\text{g/mL}$ ($p=0.500$) on day 3, and 1.51 ± 0.40 $\mu\text{g/mL}$ and 2.00 ± 0.52 $\mu\text{g/mL}$ ($p=0.003$) on day 5 (Fig. 2b). The PDGF-AA concentrations were 1.48 ± 0.46 $\mu\text{g/mL}$ (PRP group), 1.94 ± 0.57 $\mu\text{g/mL}$ (PRP + HA), and 2.69 ± 0.70 $\mu\text{g/mL}$ (PRP + high HA) (ANOVA: $p=0.0002$; PRP vs. PRP + high HA: $p=0.002$; PRP + HA vs. PRP + high HA: $p=0.011$) on day 5 (Fig. 3b).

The TGF- β 1 and PDGF-AA levels on day 3 after the wells were washed on day 0 (PRP days 0–3, PRP + HA days 0–3) were more than half of that found on day 3 without washing. Those on day 5 after being washed on day 3 (PRP days 3–5, PRP + HA days 3–5) were also more than half of those on day 5 without washing out (Fig. 4), which suggests that the TGF- β 1 and PDGF-AA were continuously released from the PRP gels through at least 5 days after incubation.

DISCUSSION

PRP can stimulate the healing process of different tissues by delivering various growth factors and cytokines that are released by platelets. In the present study, we hypothesized that adding HA to the PRP would increase the concentration of growth factors released. This hypothesis was correct on day 5, but not on days 0 or 3. These findings suggest that stimulatory effect of HA on growth factor release seems to appear slowly. Frelinger III et al. [10] suggest that pulse electric field may cause a selective permeabilization of specific granules or populations of alpha granules. In this study, after removed growth factors involved in supernatant, both TGF β 1 and PDGF-AA releasing from platelet was greater than that without removing supernatant and close to Day 0 released growth factors. Platelet may detect the surrounding growth factor concentrate and release growth factors depending on the concentrate. Surrounding HA possibly affect the selective permeabilization and population of alpha granules. Activated platelets also form CD41 microparticles, which function as a transport and delivery system for bioactive molecules, participating in hemostasis and thrombosis, inflammation, malignancy infection transfer, angiogenesis, and immunity [11]. Hu Y et al. showed the expression of P-selectin dramatically increased after PRP interacted with biomacromolecule complex film (HA-collagen (I)/chitosan) [12]. HA engagement of CD44 leads to MAP kinase-dependent increased trafficking of TGF-beta receptors to lipid raft-associated pools, which facilitates increased receptor turnover and attenuation of TGF-beta1-dependent alteration in proximal tubular cell function. Further investigate is need to elucidate the mechanism on growth factor delivery.

Fibrin networks are formed by the conversion of fibrinogen. Different fiber diameters, mass/length ratios, densities, porosities, and permeabilities of the fibrin networks can alter cell adhesion and migration [13]. Perez et al. [14] found that different PRP preparations made different fibrin networks. In the present study, smaller fibrin clots were observed in the HA group than in the other groups. Srinivasan et al. reported that heparin sulfate proteoglycan (Perlecan/HSPG2) protects bone morphogenetic protein 2 (BMP2) from proteolytic cleavage through storing and controlling the release kinetics

of BMP2, which reduced knee OA in mice [15]. Viscosupplementation with HA may inhibit the aggregation of platelets and may affect the delivery of growth factors.

HA has been widely used to treat OA, especially in Japan [6]. HA provides viscoelastic properties to the synovial fluid and contributes to boundary lubrication [16]. HA demonstrates several pleiotropic signaling properties, including anti-inflammatory, anti-apoptotic, anti-angiogenic, and anti-fibrotic effects on animal models of OA [17]. HA also has analgesic properties with a specific activity on opioid receptors [18, 19]. Chen et al. showed in an in vitro study the synergistic anabolic actions of HA and PRP on cartilage regeneration in OA [9]. In that report, a combination of HA and PRP reduced the pro-inflammatory cytokines and increased articular chondrocyte proliferation and chondrogenic differentiation via the HA-dependent Erk1/2 pathway and the PRP-dependent Smad2/3 pathway. Together, those reports and our results suggest that the clinical application of a PRP and HA mixture may be more effective than either PRP or HA alone for certain tissues.

There were several limitations to the present study. First, we only examined two of the many potential growth factors, such as epidermal growth factor, fibroblast growth factor, vascular endothelial growth factor, or insulin-like growth factor. We selected TGF- β 1 and PDGF for analysis because those are two of the most widely studied growth factors that play central roles in tissue regeneration. However, the effects of HA on the release of other growth factors may be different than the results obtained for those two. Second, only two female donors were included in this study. Sex differences may have influenced the results, although Weibrich et al. [20] found no effect of sex on growth factor concentration. Finally, the time of day when the PRP samples were produced was not exactly the same for all donors, and circadian rhythms may have influenced the data. However, Aoto et al. [21] reported that there were no significant diurnal variations in the release of TGF- β 1 and PDGF-BB.

In conclusion, HA increased the release of TGF- β 1 and PDGF-AA from PRP on day 5. Thus, a mixture of PRP and HA may result in an enhanced healing effect on certain tissues.

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CONFLICT OF INTEREST

Arthrex Japan G. K. (Tokyo, Japan) provided special syringes for the platelet-rich plasma production (the ACP double syringe system). The authors certify that they have NO affiliations with or involvement in any organization or entity with any financial interest (such as honoraria; educational grants; participation in speakers' bureaus; membership, employment, consultancies, stock ownership, or other equity interest; and expert testimony or patent-licensing arrangements), or non-financial interest (such as personal or professional relationships, affiliations, knowledge or beliefs) in the subject matter or materials discussed in this manuscript.

AUTHORS' ROLES: Study design: KI. Study conduct: KI, KF and YI. Data collection: KI, SM, TN and YK. Data analysis: KI. Data interpretation: KI, YY, and KF. Drafting manuscript: KI. Revising manuscript: ET, YU, KF, and YI. Approving final version of manuscript: KI, YY, KF and YI.

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Table Cell counts from the platelet-rich plasma and peripheral blood.

	Platelet (cells/ μ L)	WBC (cells/ μ L)	Neutrophil (% of WBC)	Lymphocyte (% of WBC)	Monocyte (% of WBC)	Eosinophil (% of WBC)	Basophil (% of WBC)	RBC (cells/ μ L)
PRP	4.68 ± 0.95 $\times 10^6$	1.53 ± 0.64 $\times 10^3$	15.2 ± 7.8	77.3 ± 8.2	6.8 ± 2.7	0.1 ± 0.4	0.5 ± 0.4	3.1 ± 1.2 $\times 10^5$
PB	2.13 ± 0.53 $\times 10^6$	5.15 ± 0.97 $\times 10^3$	55.6 ± 22.1	27.1 ± 12.9	4.2 ± 1.0	2.4 ± 1.7	0.4 ± 0.2	561 ± 102 $\times 10^5$

n=9,

WBC: white blood cell; RBC: red blood cell; Hb: hemoglobin, Ht hematocrit, PRP: platelet-rich plasma; PB: peripheral blood.

FIGURE LEGENDS

Fig. 1. Morphology of the PRP gels on day 5. The gels placed in wells were stained after all measurements with Giemsa stain (n=5). A: The PRP group, B: the PRP + HA group, and C: the PRP + high HA group. The size of the stained clots inside the gels was decreased in the samples with higher HA concentrations.

Fig 2. Comparison of the concentration of growth factors between the PRP alone and HA groups (n=9).

a: The TGF- β 1 level in the PRP + HA group was larger than that in the PRP group on day 5. b: The PDGF-AA level in the PRP + HA group was larger than that in the PRP group on day 5. *: $p < 0.005$.

Fig 3. Comparison of the concentration of growth factors with HA concentration on day 5 (n=5).

a: The TGF- β 1 level in the PRP + high HA group was higher than that in the PRP group. b: The PDGF-AA level in the PRP + high HA group was higher than that in the PRP and PRP + HA groups. *: $p < 0.005$.

Fig. 4. Growth factor release between the observation time points (n=5).

Days 0–3 samples were assessed on day 3 after being washed on day 0, and days 3–5 samples were assessed on day 5 after being washed on day 3. TGF- β 1 (A) and PDGF-AA (B) levels were assayed, and all the values were more than half of that of the same condition when assessed without being washed out.

Fig. 1

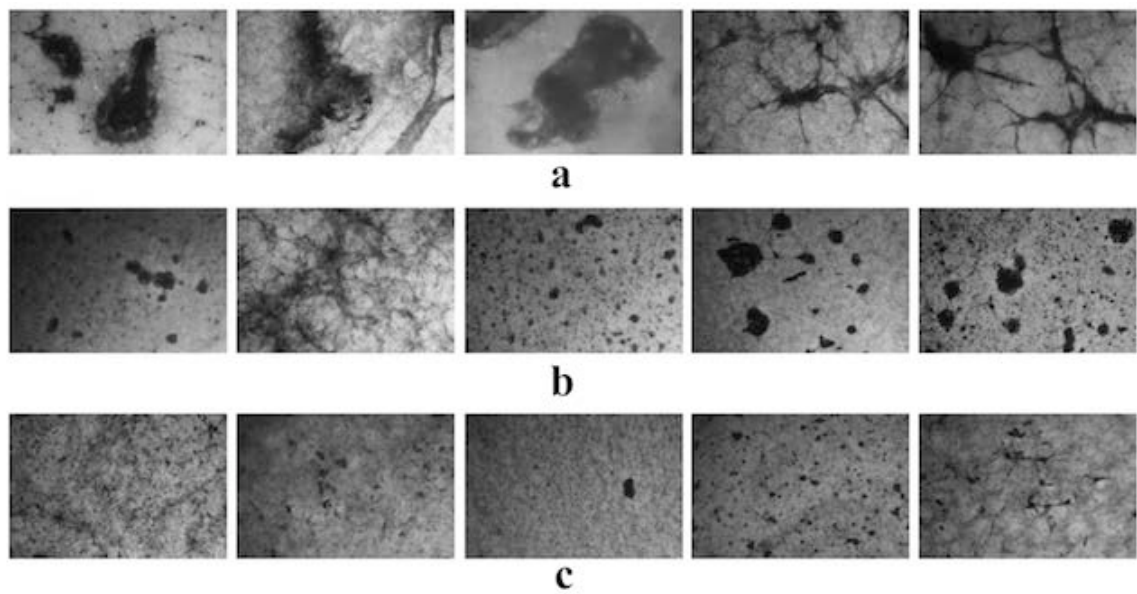


Fig. 2

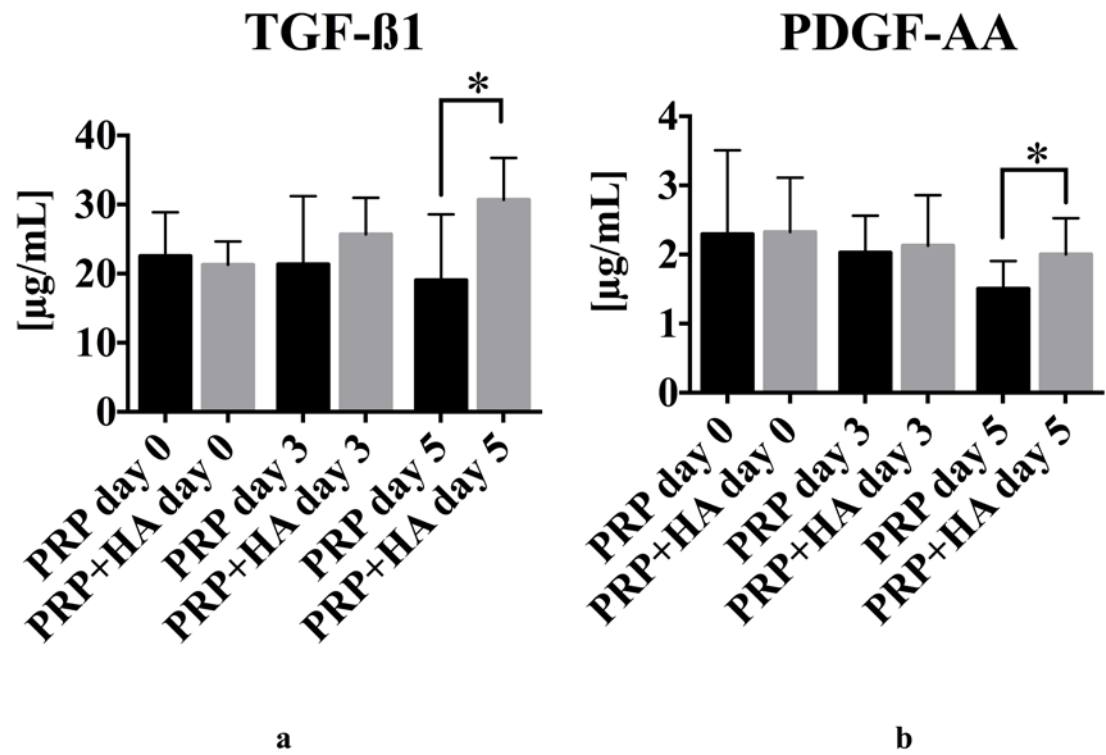


Fig. 3

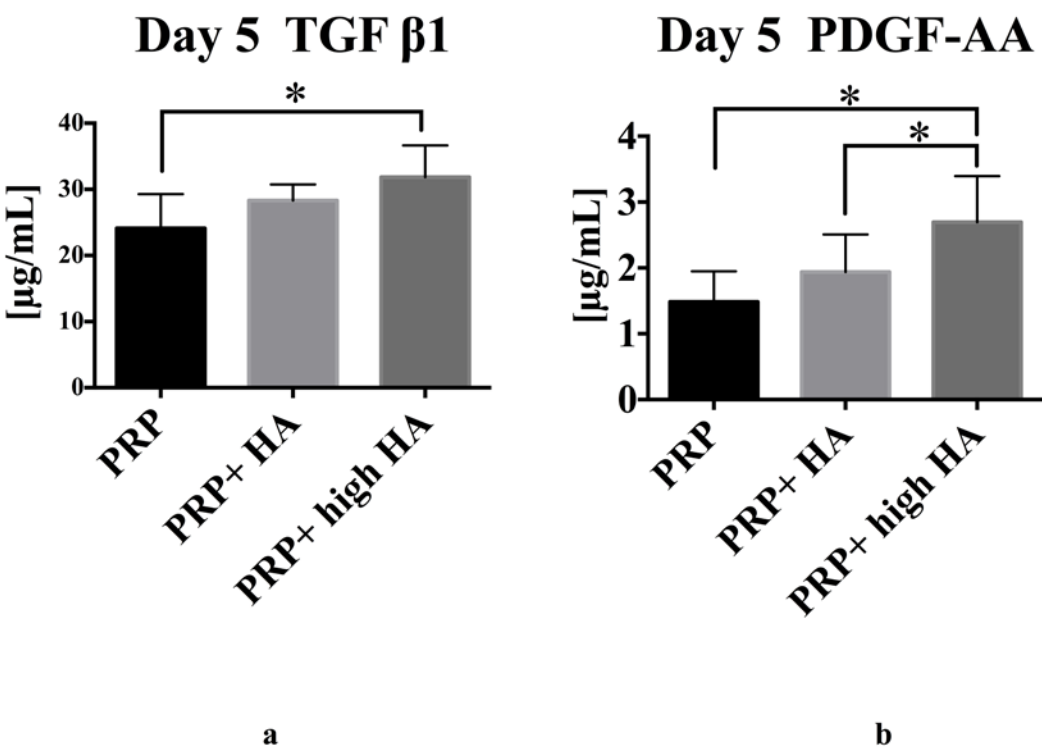


Fig. 4

