Received: 12 March 2020 | Revised: 7 October 2020 | Accepted: 20 October 2020

DOI: 10.1111/wrr.12873

ORIGINAL RESEARCH-BASIC SCIENCE

Wound Repair and Regeneration — WILEY

Autologous thrombin preparations: Biocompatibility and growth factor release

Andrea M. Matuska PhD[1] | Marina L. Klimovich BS[2] | Adam W. Anz MD[3] | Luga Podesta MD[4] | John R. Chapman PhD[2]

[1]Arthrex, Inc., Naples, Florida

[2]Stem Cell Partners, LLC, Sacramento, California

[3]Andrews Institute, Gulf Breeze, Florida

[4]Bluetail Medical Group, Naples, Florida

Correspondence
Andrea M. Matuska, Arthrex Inc 1370 Creekside Blvd, Naples, FL 34108-1945.
Email: andrea.matuska@arthrex.com

Funding information
Arthrex

Abstract

Platelet-rich plasma (PRP) has been investigated to promote wound healing in a variety of tissues. Thrombin, another essential component of wound healing, is sometimes combined with PRP to generate a fibrin clot in order to retain the sample at the delivery site and to stimulate growth factor release. Using a fully autologous approach, autologous serum (AS) with thrombin activity can be prepared using a one-step procedure by supplementing with ethanol (E^{+}AS) to prolong room temperature stability or prepared ethanol free (E^{-}AS) by utilizing a two-step procedure to prolong stability. The objective of this study was to evaluate potential wound healing mechanisms of these two preparations using commercially available devices. A variety of tests were conducted to assess biocompatibility and growth factor release from PRP at various ratios. It was found that E^{-}AS contained greater leukocyte viability in the product (97.1 ± 2.0% compared to 41.8 ± 11.5%), supported greater bone marrow mesenchymal stem cell proliferation (3.7× vs 0.8× at a 1:4 ratio and 3.6× vs 1.6× at a 1:10 ratio), and stimulated release of growth factors and cytokines from PRP to a greater extent than E^{+}AS. Of the 36 growth factors and cytokines evaluated, release of 27 of them were significantly reduced by the presence of ethanol in at least one of the tested configurations. It is concluded that the high concentrations of ethanol needed to stabilize point of care autologous thrombin preparations could be detrimental to normal wound healing processes.

1 | INTRODUCTION

There has been much investigation toward understanding the mechanisms of platelet-rich plasma (PRP) to promote wound healing and pain reduction in hard and soft tissue injuries. In addition to platelet and leukocyte count, several recently proposed classification systems consider activation and/or the presence of fibrin as one factor that can influence the efficacy of PRP.[1-4] Activation of PRP is typically performed by the addition of calcium or exogenous thrombin.

Thrombin performs several essential functions in wound healing. It is the central enzyme involved in blood coagulation through conversion of fibrinogen to fibrin, as well as being a mitogen and secretagogue for a variety of cell types.[5] Proteomics has demonstrated that in response to activation by thrombin, human platelets release more than 300 different proteins including growth factors, cytokines, adhesion, and other molecules.[6] When thrombin is combined with PRP, soluble fibrinogen in the plasma is enzymatically converted to fibrin and cross-linked. The final result is the gelation of the platelet concentrate often for the purposes of improved handling or retention of cells and growth factors at the delivery site.[7,8] It may also be desired to use the resultant fibrin as a scaffold for cell growth and remodeling within a larger defect.[9]

Major sources of thrombin for clinical use are bovine thrombin, pooled plasma derived human thrombin, and recombinant derived thrombin. Due to safety concerns for these products including risks of immunogenicity and transmission of infectious agents[10,11] a desire for

TABLE 1 Commercial devices for preparing AS

Trade name	Manufacturer	Ethanol (EtOH) added to whole blood ratio (v/v)
*ActivAT	Cytomedix	2.4 mL of 100% EtOH to 12 mL of PPP (16.7% final)
*Harvest Autologous Thrombin (HAT) Kit	Harvest Terumo	2 mL of 100% EtOH to 10 mL of WB (16.7% final)
*Thrombin Processing Device (TPD)	ThermoGenesis	4 mL of 66% EtOH to 12 mL of WB (16.5% final)
Clotalyst	Zimmer Biomet	4 mL of 66% EtOH to 12 mL of WB (16.5% final)
Thrombinator System	Arthrex Inc	No EtOH added to 12 mL WB, PPP, or PRP
RegenATS Tube	RegenLab	No EtOH added to 8 mL WB

Note: Not all devices are still in commercial use (designated with an *).

a safer source of thrombin has led to the commercialization of medical devices that generate autologous thrombin from a patient's own blood. A fully autologous approach is an attractive option to employ in the regenerative medicine setting to avoid the aforementioned risks. Although there are more than 20 cell separation devices licensed by the FDA for PRP preparation, there are only a few devices being currently marketed for preparing autologous activated sera (AS) with thrombin activity at the point of care from a small sample of whole blood or blood fraction. AS is typically comprised of fluid derived from a clot that is formed within the device (serum), plasma proteins, and cytokines eluted from activated cells and platelets and depending on the source blood fraction used, may contain cells (red and/or white blood cells). AS can be categorized into two groups: ethanol supplemented (E^+AS) or ethanol free (E^-AS, Table 1).

Ethanol in substantial amounts (≥17% v/v) has been known to prolong the clotting activity of AS.[12] Ethanol containing devices are typically one step and allow room temperature storage to prolong AS thrombin activity within 60% of its original activity over the typical time frame of 4 hours for clinical reinjection. Alternatively, an ethanol free device needs to be used immediately or be stored on ice so as to maintain 25% of its original activity within a 4 hour time frame. Newer devices can use a two-step procedure (where the second step takes less than 2 minutes at the time of need) to maintain full activity over 4 hours.[13] Autologous devices typically produce more physiological concentrations of thrombin, which can be beneficial for a fibrin clot structure conducive to cell infiltration, viability, and wound closure.[14,15]

Ethanol in quantities obtainable by consumption of alcohol (<0.2%) has been well researched to inhibit various stages of wound healing both in vitro and in vivo models by blocking leukocyte recruitment and response, endothelial cell activation, and even platelet aggregation.[16-19] Minimal investigation into the effects of ethanol in autologous therapies have been performed, but reduced platelet function, altered coagulation characteristics, and cytotoxicity are all identified as potential concerns.[20,21]

Therefore, the hypothesis of this study was that the presence of ethanol in commercial preparations of autologous thrombin could affect wound healing through altered biocompatibility or growth factor release from PRP. Toward this, two commercially available devices that produce AS for wound healing applications were evaluated. An E^+AS device (Clotalyst, Zimmer-Biomet, Warsaw, Indiana) and an E^-AS device (Thrombinator System, Arthrex Inc, Naples, Florida) were used. Previous studies have evaluated thrombin activity of AS when using whole blood and both devices produce similar autologous thrombin activity when using whole blood (~20 U/mL).[13] Therefore, a variety of tests were conducted to assess biocompatibility and the potential of each AS to stimulate growth factor release from PRP (Figure 1).

2 | METHODS

2.1 | Blood donors and PRP preparation

Whole blood donations (n = 6) were acquired from Stem Express (Folsom, California) with informed consent and Institutional Review Board (IRB) approval (WIRB-Copernicus Group, Princeton, NJ, IRB No. 20152875). All testing was performed within 8 hours of phlebotomy. There were two males and four females in the study that ranged in age from 24 to 69 years. A unit of blood was collected at 8% ACD-A for use with the Zimmer Biomet Clotalyst System (recommended per instructions for use), and ACD-A was added back to the whole blood unit to create 13% ACD-A anticoagulated blood for use with the Arthrex Thrombinator System and Arthrex Angel Platelet Rich Plasma (cPRP) System. The Angel System was used to process 360 mL whole blood to produce 40 mL of PRP needed for elution experiments. Cell counts were performed on the whole blood and PRP using a Horiba Micro60 Analyzer Model M60CS (Irvine, California).

2.2 | Preparation of E^+AS and E^-AS

E^+AS was prepared using the Zimmer Biomet Clotalyst device according to the manufacturer's instructions. Briefly, the method consisted of adding 4 mL of the Clotalyst Reagent containing ethanol (66%) and calcium to 12 mL of 8% ACDA whole blood. This mixture was injected into the device and incubated for 15 minutes. At the end of the incubation, the E^+AS was harvested, with a theoretical final concentration of 16.5% ethanol. Ethanol concentration was assayed with a QuantiChrom Kit (BioAssay Systems) per manufacturer instructions. E^-AS was prepared using the Arthrex Thrombinator device according to the manufacturer's instructions. The method consisted of activating the device with 0.1 mL of 10% calcium chloride reagent and

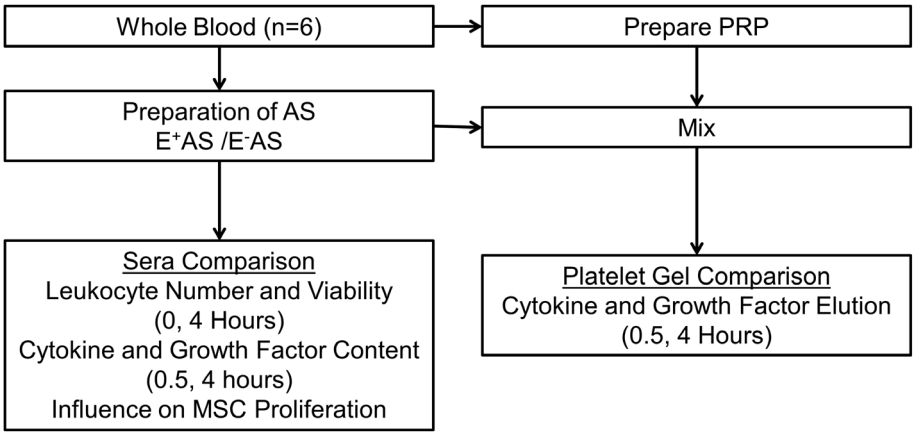

FIGURE 1 Both ethanol and nonethanol containing AS was prepared in two commercially available devices and systematically compared

4 mL of 13% ACD-A whole blood. Once a gelling reaction occurred, indicating successful activation, E^{-}AS was produced with an additional 0.2 mL of 10% calcium chloride reagent and 8 mL of 13% ACD-A whole blood.

2.3 | Cell count and viability of leukocytes in AS

Leukocyte absolute cell count and viability in E^{-}AS and E^{+}AS were compared at 0 and 4 hours of storage (n = 6 donors). The E^{-}AS was stored on ice (4°C) while the E^{+}AS was stored at room temperature (18-22°C), per the manufacturer instructions. The assessment of leukocyte number and viability was measured using Via1 Cassettes with a Chemometec NC-3000 Image Cytometer (Chemometec, Allerod, Denmark) wherein cells are stained with acridine orange (dead and alive cells) and DAPI (dead cells only). The stained cells are counted using an automated image cytometer. The AS were diluted 1:10 in PBS immediately before analysis to prevent red blood cell interference in cell counting.

2.4 | Bone marrow derived mesenchymal cell proliferation in AS

The effect of the E^{+}AS and E^{-}AS on bone marrow derived mesenchymal stem cell (MSC; BM-MSC, ATCC, Gaithersburg, Maryland) proliferation was determined by culturing the cells in serum free media (MSC Basal Media, ATCC) containing AS. To prepare serum for the assay, the AS (n = 4 donors) were centrifuged 1 hour after production at 1,500*g* for 10 minutes, and the supernatant was collected and stored at −80°C until testing. BM-MSC were seeded (15,000 cells/well) into 24-well plate and serum-starved for 2 hours at 37°C and 5% CO_2. Serum (E^{+}AS, E^{-}AS, or FBS) was added to the wells at 1:4 or 1:10 final dilution with some cultures remaining serum free as a control. The cells were allowed to proliferate at 37°C and 5% CO_2 for 2 days. After 2 days, the cells were removed from the wells using TrypLE (Gibco) and counted using the Chemometec NC3000 Image Cytometer as described previously to determine total cell number and viability.

Additional testing was performed to objectively quantify the effects of thrombin preparations on MSC proliferation. BM-MSC were stained with a CFSE dye (Sigma Aldrich) at a concentration of 0.125 μM CFSE per 0.5×10^{6} cells. CSFE stained and unstained (control) cells were seeded in 24 well plates at a seeded density of 15,000 cells per well. The MSC were incubated at 37°C and 5% CO_2 for 16 hours to ensure the MSC adherence and a small portion of cells were trypsinized and analyzed using a BD Accuri C6 flow cytometer to determine initial MFI values. Cells were serum starved for 2 hours and treated with serum (E^{+}AS, E^{-}AS, or FBS) at 1:4 or 1:10 final dilution (v/v) with some cultures remaining serum free as a control. The sera treated MSC cells were incubated at 37°C and 5% CO_2 for 4 days. The MSC were trypsinized and a total of 2,000 CFSE positive events were collected for each sample. Average MFI of the populations was calculated. MFI of the unstained, but treated, cells was also collected and subtracted from MFI of the stained treated cells. Theoretical population doublings were calculated by assuming MFI decreases by a factor of two for each doubling.

2.5 | Cytokine concentrations in AS and platelet gels

After production, the sera (n = 6) were aliquoted and stored for 0.5 and 4 hours per manufacturer instructions. After storage, the sera were centrifuged at 1500*g* for 10 minutes. The supernatant was collected and stored at −80°C for cytokine quantification. In addition, platelet gels were prepared by mixing PRP with AS at v/v ratios of 1:1, 1:4, and 1:10 AS:PRP. Gels were incubated 0.5 and 4 hours at 37°C. After incubation, the platelet gels were centrifuged at 1500*g* for 10 minutes. The supernatant was collected and stored at −80°C until it could be analyzed for cytokine content.

AS and platelet gel supernatants were analyzed for a broad spectrum of cytokines and chemokines. The Discovery Assay Human Cytokine Array/Chemokine Array 42-Plex (Eve Technologies Corp, Calgary, AB, Canada) was used. The 42-plex consisted of the following analytes; EGF, Eotaxin-1, FGF-2, Flt-3L, Fractalkine,

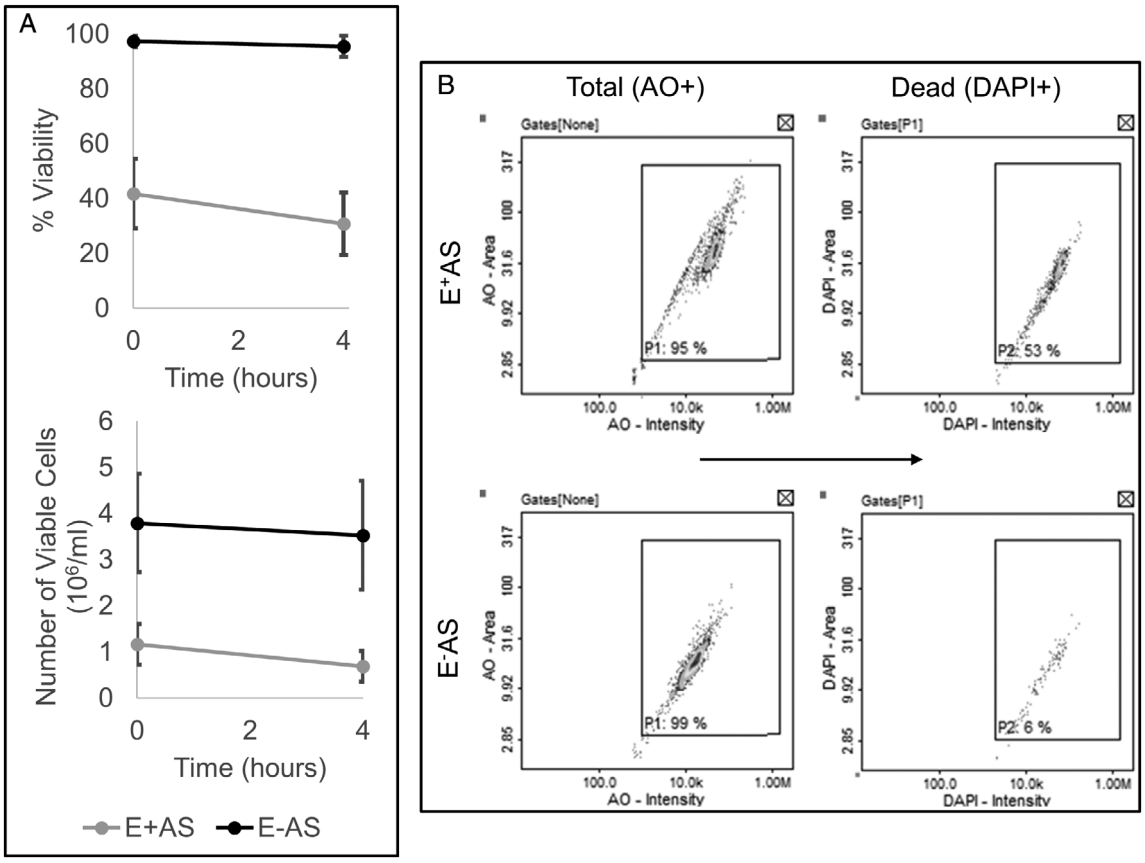

FIGURE 2 A, Average leukocyte number and viability in different AS preparations over time. B, Comparison of cell populations of the different preparations after 4 hours

G-CSF, GM-CSF, GRO(alpha), IFNα2, IFNγ, IL-1α, IL-1β, IL-1ra, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12 (p40), IL-12 (p70), IL-13, IL-15, IL-17A, IL-18, IP-10, MCP-1, MCP-3, MDC, MIP-1α, MIP-1β, PDGF-AA, PDGF-AB/BB, RANTES, sCD40L, TGFα, TNFα, TNFβ, and VEGF-A. An additional TGFβ 3-plex discovery panel was performed with the following analytes; TGFβ1, TGFβ2, and TGFβ3. A Bio-Plex 200 system (BioRad Laboratories, Inc., Hercules, California) was used. The kits were sourced from Milliplex (Millipore, St. Charles, Missouri).

2.6 | Data analysis and statistics

Where appropriate, mean and standard deviations were calculated, wherein each donor served as a replicate. Statistics were performed in Minitab 18.1 using General Liner Models (GLM). Donors were treated as random variables with other variables such as the presence of ethanol, time, or ratio treated as fixed factors. Main and interaction effects were evaluated. When significance was indicated, pairwise post hoc Tukey tests were performed to determine significant differences (α = .05). Of the 45 growth factors and cytokines evaluated, several were excluded from analysis including Flt-3L, Fractalkine, GRO(alpha), MCP-3, TGFα, TNFβ, IL-5, IL-13, and IL-15 due to more than 20% of the values being out of range of the assay.

3 | RESULTS

3.1 | Ethanol concentration, comparison of leukocyte viability and number in E^+AS and E^-AS

Concentration of ethanol in the E^+AS serum was found to be 17.5 ± 0.9% and ranged from 15.8% to 18.5%. Leukocyte viability in the AS was tested immediately after harvest from the devices and again after 4 hours of storage per manufacturer's instructions. Viability of the leukocytes was significantly lower in E^+AS as compared to E^-AS (P < .001, Figure 2). In addition, there were approximately 30% less total leukocytes detected in E^+AS relative to the E^-AS, as well as significantly less total viable leukocytes (P < .001, Figure 2). Immediately after harvest from the devices, an average of only 41.8 ± 11.5% of the leukocytes were viable in E^+AS. In contrast, the E^-AS had an average leukocyte viability of 97.1 ± 2.0%. This trend was maintained out to 4 hours.

3.2 | Comparison of bone marrow derived mesenchymal cell proliferation in AS

All BM-MSCs recovered from wells had greater than 90% viability, as expected, since processing to trypsinize adherent cells would results

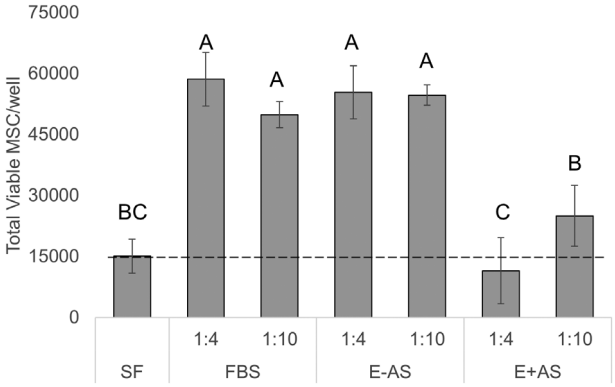

FIGURE 3 Effect of AS on mesenchymal stem cell proliferation over 48 hours. Dashed line indicates initial seeding density of mesenchymal cells. Fetal bovine serum (FBS) was the positive control and serum free culture media (SF) was the negative control. Groups sharing the same letter are not significantly different

in loss of on nonviable/non adherent cells. Bone marrow derived MSCs were evaluated in the presence of various sera which was shown to significantly affect proliferation and subsequently the number of viable cells recovered ($P < .001$, Figure 3). Cells cultured in serum free media did not proliferate over the two-day culture period (1.0 ± 0.3× of seeding density). Addition of E^+AS to culture media at both a 1:4 and 1:10 ratio failed to result in appreciable proliferation of MSC, although the overall average number of cells was increased when a 1:10 ratio was used (1.6 ± 0.5×) compared to a 1:4 ratio (0.8 ± 0.5×). In contrast, cells cultured in FBS proliferated 3.9 ± 0.4 (1:4 ratio) and 3.3 ± 0.2× (1:10 ratio) over baseline seeding density. Addition of E^-AS to culture media resulted in similar levels of BM-MSC proliferation as compared to FBS at both a 1:4 and 1:10 ratio (3.7 ± 0.4× and 3.6 ± 0.2×, respectively). Testing to quantify population doublings over 96 hours identified differences among serum groups, but not among different ratios ($P < .001$, Figure 4). Namely, E^-AS groups had the highest number of doubling, followed by FBS, E^+AS, and then serum free groups.

3.3 | Cytokines and growth factors in AS

Cytokine and GF content in AS was not significantly impacted by storage time (Table 2). Concentrations of EGF, Eotaxin-1, IP-10, MCP-1, MDC, MIP-1b, PDGF-AA, PDGF-BB, sCD40L, TGF-b1, TGF-b2, and TNFa were all significantly increased in E^-AS ($P < .05$) whereas IL-18, IL-1ra, and RANTES were significantly increased in E^+AS ($P < .05$).

3.4 | Growth factors released from platelet gels contacting AS

The PRP used to evaluate growth factor release was on average 6.8 ± 0.4× platelet and 2.5 ± 0.6× WBC over baseline with a 4.9 ± 0.8% hematocrit. The presence of ethanol, ratio of AS:PRP, duration of contact, and interaction thereof played a significant role in the release of many of the platelet- and hematopoietic-derived (Table S1) and immunomodulatory factors and chemokines (Table S2). Of the 36 factors, release of 27 of them were negatively affected by the presence of ethanol in at least one of the tested configurations (Figure 5). Only one factor, IP-10 was significantly increased with the addition of ethanol which was driven by a strong effect when used at a 1:1 ratio.

4 | DISCUSSION

The primary objective of this study was to compare the biocompatibility and functionality of two different AS formulations, with the predominant difference being the addition of ethanol (17.5%, final) into the milieu. This was investigated by characterizing AS derived from whole blood (cell health, growth factor content, effect on a BM-MSC line) and growth factor release from platelet gels. The most important finding of this study was that E^+AS negatively affected the viability of leukocytes and the release of the majority of growth factors from PRP gels while E^-AS did not. In addition, E^-AS presence in culture media resulted in higher proliferation of BM-MSC compared to E^+AS.

Viability of leukocytes in the different AS preparations was assessed. The average viability of leukocytes present in E^-AS were >95%, whereas the average leukocyte viability in the E^+ AS was less than 50% indicating cytotoxicity and cell lysis. Further, the average number of total leukocytes was 30% lower in the E^+AS than the E^-AS. High concentrations of alcohol primarily causes sudden cell death via the necrotic pathway instead of the physiologic pathway of aptotosis.[22] Apoptosis includes cellular shrinking, chromatin condensation, and margination at the nuclear periphery with the eventual formation of membrane-bound apoptotic bodies that contain organelles, cytosol, and nuclear fragments that are phagocytosed without triggering an inflammatory process. A necrotic cell swells, becomes leaky, and releases its contents into the surrounding tissue resulting in inflammation.[23] The clinical significance, if any, of the observed loss of cell viability is unknown, but the potential for increased amounts of necrotic cell debris in E^+AS would be expected to promote additional inflammation at the point of injection. In addition, the high ethanol content carries risk for cytotoxicity to directly contacted cells.

This was further investigated by the ability of AS supernatant to support bone marrow-derived MSC proliferation. MSC were selected because AS is frequently combined with bone marrow concentrate and autograft or allograft bone to form bone graft composites. AS were diluted 1:4 and 1:10 in tissue culture media before the MSC were contacted. Fetal bovine serum was employed as the positive control and culture media without any serum added was the negative control. Over 48 hours, E^-AS induced an MSC proliferation factor of >3.5× at both the 1:4 and 1:10 dilution after 2 days in culture, similar to the positive control (FBS). In contrast, the E^+AS did not support MSC proliferation, and cell concentrations were not significantly different than the negative control (serum free). To further explore this, CSFE staining was used to quantify population doublings over 96 hours in the same groups. This similarly trended with the

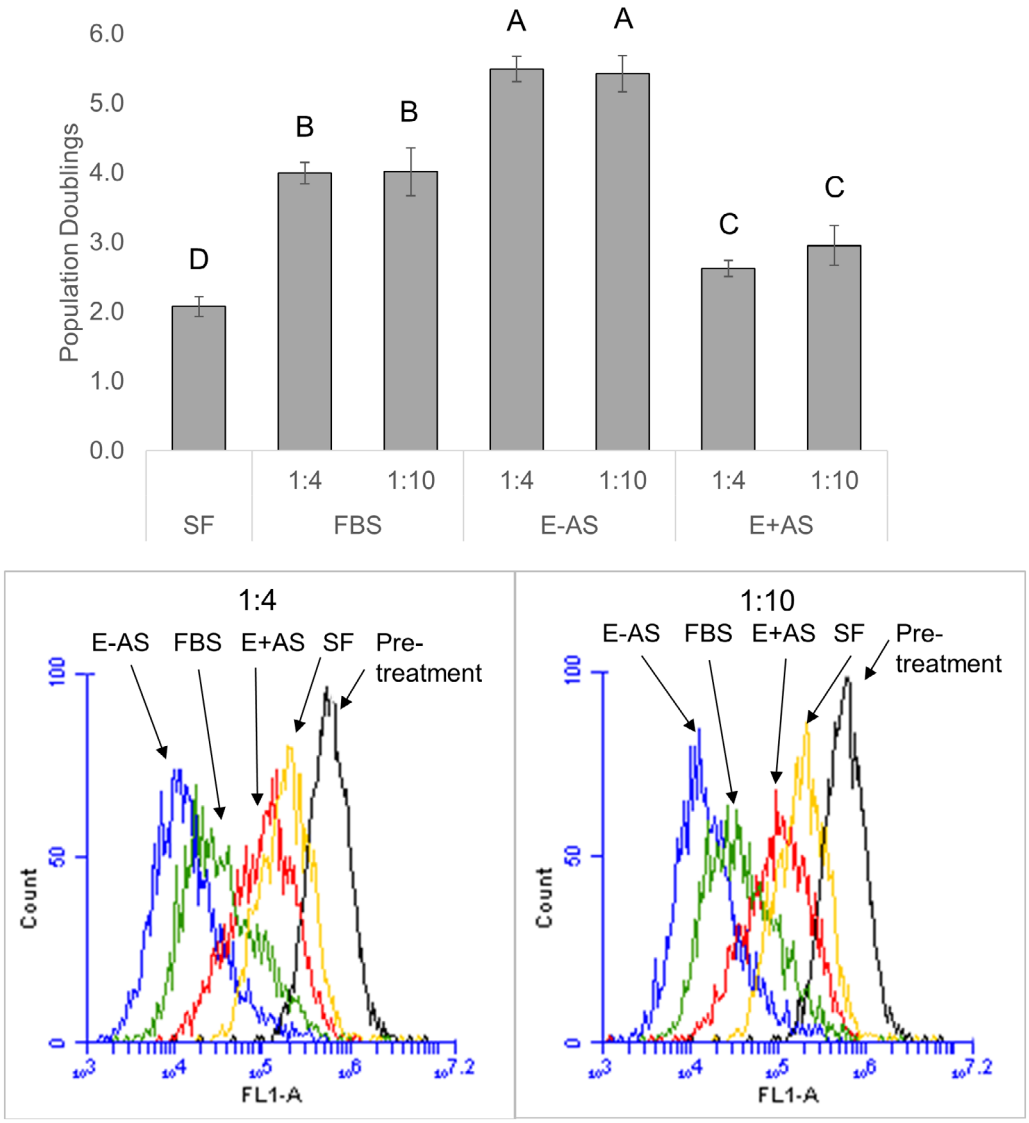

FIGURE 4 Effect of AS on mesenchymal stem cell population doublings over 96 hours. Fetal bovine serum (FBS) was the positive control and serum free culture media (SF) was the negative control. Groups sharing the same letter are not significantly different. Representative fluorescent intensity curves for CSFE staining are shown [Color figure can be viewed at wileyonlinelibrary.com]

proliferation results seen at 2 days, however similar doublings for the 1:10 and 1:4 E^+AS groups indicates some degree of cytotoxicity at the higher E^+AS ratio played a role in total number of cells recovered. Several cytokines and platelet derived factors were higher in E^-AS sera including PDGF and TGFβ which likely played a role in supporting the proliferation of MSC observed.

Generally, growth factors in the AS preparations were consistent over the storage period evaluated (4 hours). This is not surprising as the majority of cell stimulation would have occurred during production of the AS while the sample remained in contact with the materials of the device. The loss of leukocyte membrane integrity that occurred in E^+AS, likely led to a release of intracellular molecules into the supernatant. This could explain the increase of some cytokines in E^+AS preparations. For example, IL-18 is synthesized as an inactive precursor and stored intracellularly.[24] It is possible the ELISA detected an epitope on this not yet functional precursor which would have been released upon cell lysis. Similarly there are also intracellular IL-1ra (icIL-1ra) isoforms different from secreted IL-1ra (sIL-1ra) that act independently of IL-1 modulation.[25] Lower concentrations of growth factors could also be explained by degradation or denaturation of growth factors and the identifiable epitopes.

Wound healing is a complex series of orchestrated events generally divided into coagulation, inflammation, proliferation, and remodeling. Attempting to derive the effect that would occur with a difference of one specific factor is nearly impossible. However, it could be assumed that ethanol would likely be a detriment to the normal healing process due to the observed lesser release of around 70% of the growth factors and cytokines evaluated. For example, platelet derived factors released upon initial degranulation, such as PDGF's and TGF-β's, play a chemotactic role to attract cells to the wound bed

TABLE 2 Comparison of cytokines and growth factor concentrations in in AS (concentrations in pg/ml)

Factor	Storage time (H)	E^+AS	E^-AS	*P*-value
EGF	0.5	32.1 ± 20.0	96.6 ± 52.0	<.001
	4	54.5 ± 24.2	92.6 ± 53.5	
Eotaxin-1	0.5	21.8 ± 9.1	95.9 ± 47.2	<.001
	4	28.6 ± 13.8	90.3 ± 50.5	
FGF-2	0.5	157.9 ± 43.0	147.4 ± 99.3	.16
	4	179.9 ± 58.4	119.7 ± 41.2	
G-CSF	0.5	0.1 ± 0.1	8.0 ± 18.8	.26
	4	0.4 ± 0.9	1.3 ± 2.2	
GM-CSF	0.5	4.3 ± 4.3	27.6 ± 51.4	.19
	4	4.8 ± 3.9	9.3 ± 5.4	
IFNα2	0.5	11.2 ± 8.4	67.2 ± 129.5	.20
	4	10.2 ± 9.4	21.3 ± 17.6	
IFNy	0.5	0.3 ± 0.4	22.2 ± 46.6	.16
	4	0.7 ± 1.0	5.8 ± 9.4	
IL-10	0.5	7.3 ± 16.9	14.9 ± 32.9	.08
	4	4.5 ± 10.0	13.4 ± 29.7	
IL-12P40	0.5	7.7 ± 10.2	33.0 ± 61.4	.18
	4	7.5 ± 8.1	13.3 ± 16.2	
IL-12P70	0.5	2.3 ± 3.6	22.2 ± 47.6	.16
	4	2.0 ± 2.6	7.6 ± 12.4	
IL-17A	0.5	3.0 ± 5.0	12.0 ± 22.6	.14
	4	2.4 ± 3.5	5.1 ± 6.1	
IL-18	0.5	568.0 ± 167.2	196.2 ± 92.0	<.001
	4	590.8 ± 236.9	195.8 ± 74.0	
IL-1α	0.5	7.6 ± 12.3	51.6 ± 110.2	.21
	4	4.8 ± 6.8	15.0 ± 22.5	
IL-1β	0.5	0.7 ± 0.4	3.8 ± 6.8	.21
	4	0.9 ± 0.4	1.4 ± 1.4	
IL-1ra	0.5	1120.1 ± 530.0	84.3 ± 22.1	<.001
	4	1239.6 ± 586.5	79.5 ± 11.1	
IL-2	0.5	0.4 ± 0.4	4.7 ± 9.3	.17
	4	0.2 ± 0.2	1.1 ± 1.5	
IL-3	0.5	0.6 ± 0.3	0.6 ± 0.3	.047
	4	0.7 ± 0.3	0.5 ± 0.2	
IL-4	0.5	7.9 ± 2.0	27.5 ± 30.5	.08
	4	13.9 ± 9.5	18.0 ± 13.6	
IL-6	0.5	1.7 ± 2.0	4.2 ± 5.5	.04
	4	1.6 ± 1.5	3.3 ± 4.1	
IL-7	0.5	2.7 ± 5.4	6.4 ± 8.2	.01
	4	3.2 ± 4.7	5.5 ± 9.6	
IL-8	0.5	13.9 ± 10.1	13.2 ± 12.5	.24
	4	18.4 ± 12.4	9.8 ± 6.6	
IL-9	0.5	0.4 ± 0.2	1.3 ± 1.7	.11
	4	0.5 ± 0.2	0.7 ± 0.4	
IP-10	0.5	108.8 ± 85.1	149.1 ± 117.7	.01
	4	116.2 ± 63.2	150.4 ± 121.8	
MCP-1	0.5	185.9 ± 77.9	431.3 ± 260.7	<.001
	4	271.3 ± 119.6	437.1 ± 277.6	
MDC	0.5	82.7 ± 67.7	592.7 ± 495.5	<.001
	4	70.2 ± 38.1	448.6 ± 186.3	
MIP-1α	0.5	3.2 ± 3.3	8.5 ± 13.0	.09
	4	2.7 ± 3.5	5.6 ± 5.1	
MIP-1β	0.5	28.0 ± 23.6	67.0 ± 41.2	.01
	4	31.4 ± 20.7	56.0 ± 16.7	
PDGF-AA	0.5	306.6 ± 156.2	4301.0 ± 6928.7	.03
	4	385.9 ± 197.0	4036.6 ± 6254.1	
PDGF-BB	0.5	557.7 ± 343.1	4021.1 ± 1721.6	<.001
	4	354.2 ± 354.1	3677.6 ± 1409.1	
RANTES	0.5	2488.8 ± 3686.6	280.1 ± 74.9	.02
	4	2595.0 ± 3636.5	256.3 ± 71.5	
sCD40L	0.5	0.9 ± 1.5	835.5 ± 328.9	<.001
	4	0.6 ± 0.9	823.5 ± 349.0	
TGF-β1	0.5	13 013.4 ± 4646.3	37 515.9 ± 21 306.0	<.001
	4	12 587.3 ± 7038.3	44 062.4 ± 11 159.8	
TGF-β2	0.5	579.6 ± 292.5	1643.5 ± 972.4	<.001
	4	537.4 ± 332.2	1868.4 ± 578.1	
TGF-β3	0.5	95.8 ± 45.6	97.9 ± 44.0	.07
	4	58.9 ± 27.4	108.1 ± 64.3	
TNFα	0.5	4.4 ± 2.8	24.5 ± 13.5	<.001
	4	2.8 ± 3.0	19.9 ± 5.3	
VEGF	0.5	98.1 ± 31.5	118.6 ± 75.5	.78
	4	114.9 ± 31.0	102.4 ± 70.0	

along with stimulating proliferation.[26] Other factors, such as EGF, FGF, and VEGF, are known to stimulate migration, proliferation, and matrix deposition. Studies have shown decreased levels of these factors are associated with chronic wounds.[27] All of these factors were released to a lesser degree in platelet gels activated with E^+AS. IP-10 was the only factor with significantly more secretion in E^+AS platelet gels (specifically at a 1:1 ratio), and this factor is typically associated with inflammatory disease states.[28] Longer incubation (ie, 24 hours) could have detected additional differences in secreted factors that are part of later stages of wound healing. While a leukocyte rich PRP formulation was used to generate the platelet gels, growth factor release from other formulations, including utilizing AS prepared from PPP and leukocyte reduced PRP would be expected to alter growth factor release profiles.

There have already been many technological advancements in preparing and understanding the various types of autologous blood products that can be prepared at the point of care. In this study, two sources of AS containing thrombin activity were evaluated. It was found that E^-AS promoted greater cell viability, supported cell proliferation, and stimulated release of growth factors and cytokines from

FIGURE 5 Growth, immunomodulatory, and hematopoietic factors as well as chemokine release from PRP was significantly different when activated with E^+AS or E^-AS. When shaded, the first column indicates if there was an overall main effect on the release of a certain factor due to the presence of ethanol. The second column indicates an interaction of ethanol at a certain timepoint and the final columns indicate an interaction of ethanol at a certain ratio. There were no three-way interactions detected

KEY

Main Effect	EtOH*Time Interaction	EtOH*Ratio Interaction		
Factor	0.5H	1:1	1:4	1:10
	4H			
EtOH (-) Increased				
EtOH (-) Reduced				

GROWTH FACTORS

EGF	0.5H	1:1	1:4	1:10
	4H			
FGF-2	0.5H	1:1	1:4	1:10
	4H			
PDGF-AA	0.5H	1:1	1:4	1:10
	4H			
PDGF-BB	0.5H	1:1	1:4	1:10
	4H			
VEGF	0.5H	1:1	1:4	1:10
	4H			
TGF-b1	0.5H	1:1	1:4	1:10
	4H			
TGF-b2	0.5H	1:1	1:4	1:10
	4H			
TGF-b3	0.5H	1:1	1:4	1:10
	4H			
sCD40L	0.5H	1:1	1:4	1:10
	4H			

CHEMOKINES

RANTES	0.5H	1:1	1:4	1:10
	4H			
IL-8	0.5H	1:1	1:4	1:10
	4H			
IP-10	0.5H	1:1	1:4	1:10
	4H			
Fractalkine	0.5H	1:1	1:4	1:10
	4H			
Eotaxin-1	0.5H	1:1	1:4	1:10
	4H			
MIP-1a	0.5H	1:1	1:4	1:10
	4H			
MIP-1b	0.5H	1:1	1:4	1:10
	4H			
MDC	0.5H	1:1	1:4	1:10
	4H			
MCP-1	0.5H	1:1	1:4	1:10
	4H			

IMMUNOMODULATORY

IL-1b	0.5H	1:1	1:4	1:10
	4H			
IL-1a	0.5H	1:1	1:4	1:10
	4H			
TNFa	0.5H	1:1	1:4	1:10
	4H			
IL-17A	0.5H	1:1	1:4	1:10
	4H			
IL-18	0.5H	1:1	1:4	1:10
	4H			
IL-2	0.5H	1:1	1:4	1:10
	4H			
IL-4	0.5H	1:1	1:4	1:10
	4H			
IL-6	0.5H	1:1	1:4	1:10
	4H			
IL-9	0.5H	1:1	1:4	1:10
	4H			
IL-10	0.5H	1:1	1:4	1:10
	4H			
IL-1ra	0.5H	1:1	1:4	1:10
	4H			
IFNa2	0.5H	1:1	1:4	1:10
	4H			
IFNy	0.5H	1:1	1:4	1:10
	4H			

HEMATOPOETIC FACTORS

IL-12P40	0.5H	1:1	1:4	1:10
	4H			
IL-12P70	0.5H	1:1	1:4	1:10
	4H			
IL-3	0.5H	1:1	1:4	1:10
	4H			
IL-7	0.5H	1:1	1:4	1:10
	4H			
GM-CSF	0.5H	1:1	1:4	1:10
	4H			
G-CSF	0.5H	1:1	1:4	1:10
	4H			

PRP to a greater extent than E^+AS. The insert provided with E^+AS did not provide any warning statements related to the ethanol content of the AS produced or the need for its dilution to a certain percentage prior to the application to the body. The data supports the concept of developing autologous systems that do not employ ethanol reagents in order to prolong room temperature storage of the final product. Further clinical investigations would be needed to determine optimal ratios, proportions, and formulations for specific treatments.

ACKNOWLEDGMENTS

Funding for this study was provided by Arthrex Inc.

CONFLICT OF INTEREST

All authors are employees or contracted consultants of Arthrex Inc.

ORCID

Andrea M. Matuska https://orcid.org/0000-0001-9751-7986

REFERENCES

1. DeLong JM, Russell RP, Mazzocca AD. Platelet-rich plasma: the PAW classification system. *Art Ther*. 2012;28(7):998-1009.
2. Dohan Ehrenfest DM, Bielecki T, Mishra A, et al. In search of a consensus terminology in the field of platelet concentrates for surgical use: platelet-rich plasma (PRP), platelet-rich fibrin (PRF), fibrin gel polymerization and leukocytes. *Curr Pharm Biotechnol*. 2012;13(7):1131-1137.
3. Lana J, Purita J, Paulus C, et al. Contributions for classification of platelet rich plasma—proposal of a new classification: MARSPILL. *Regen Med*. 2017;12(5):565-574.
4. Magalon J, Chateau AL, Bertrand B, et al. DEPA classification: a proposal for standardising PRP use and a retrospective application of available devices. *BMJ Open Sport Exerc Med*. 2016;2(1):e000060.

5. Davie EW, Kulman JD. An overview of the structure and function of thrombin. *Semin Thromb Hemost*. 2006;32(Suppl 1):3-15.
6. Coppinger JA, Cagney G, Toomey S, et al. Characterization of the proteins released from activated platelets leads to localization of novel platelet proteins in human atherosclerotic lesions. *Blood*. 2004;103(6):2096-2104.
7. Piccin A, Di Pierro AM, Canzian L, et al. Platelet gel: a new therapeutic tool with great potential. *Blood Transfus*. 2017;15(4):333-340.
8. Everts PA, Knape JT, Weibrich G, et al. Platelet-rich plasma and platelet gel: a review. *J Extra Corpor Technol*. 2006;38(2):174-187.
9. Anitua E, Sanchez M, Nurden AT, Nurden P, Orive G, Andia I. New insights into and novel applications for platelet-rich fibrin therapies. *Trends Biotechnol*. 2006;24(5):227-234.
10. Diesen DL, Lawson JH. Bovine thrombin: history, use, and risk in the surgical patient. *Vascular*. 2008;16(Suppl 1):S29-S36.
11. Bishop PD, Lewis KB, Schultz J, Walker KM. Comparison of recombinant human thrombin and plasma-derived human alpha-thrombin. *Semin Thromb Hemost*. 2006;32(Suppl 1):86-97.
12. Sternberger LA. The stabilization of thrombin in plasma; development of a simple two-stage method for the determination of prothrombin. *Br J Exp Pathol*. 1947;28(3):168-177.
13. Matuska AM, Klimovich MK, Chapman JR. An ethanol-free autologous thrombin system. *J Extra Corpor Technol*. 2018;50(4):237-243.
14. Gugerell A, Pasteiner W, Nurnberger S, et al. Thrombin as important factor for cutaneous wound healing: comparison of fibrin biomatrices in vitro and in a rat excisional wound healing model. *Wound Repair Regen*. 2014;22(6):740-748.
15. Wolberg AS. Thrombin generation and fibrin clot structure. *Blood Rev*. 2007;21(3):131-142.
16. Saeed RW, Varma S, Peng T, Tracey KJ, Sherry B, Metz CN. Ethanol blocks leukocyte recruitment and endothelial cell activation in vivo and in vitro. *J Immunol*. 2004;173(10):6376-6383.
17. Goral J, Kovacs EJ. In vivo ethanol exposure down-regulates TLR2-, TLR4-, and TLR9-mediated macrophage inflammatory response by limiting p38 and ERK1/2 activation. *J Immunol*. 2005;174(1):456-463.
18. Fitzgerald DJ, Radek KA, Chaar M, Faunce DE, DiPietro LA, Kovacs EJ. Effects of acute ethanol exposure on the early inflammatory response after excisional injury. *Alcohol Clin Exp Res*. 2007;31(2):317-323.
19. Marumo M, Wakabayashi I. Sensitivity of thrombin-induced platelet aggregation to inhibition by ethanol. *Clin Chim Acta*. 2009;402(1–2):156-159.
20. Semple E, Speck ER, Aslam R, Kim M, Kumar V, Semple JW. Evaluation of platelet gel characteristics using thrombin produced by the thrombin processing device: a comparative study. *J Oral Maxillofac Surg*. 2008;66(4):632-638.
21. De Somer F, De Brauwer V, Vandekerckhove M, Ducatelle R, Uyttendaele D, Van Nooten G. Can autologous thrombin with a rest fraction of ethanol be used safely for activation of concentrated autologous platelets applied on nerves? *Eur Spine J*. 2006;15(4):501-505.
22. Castaneda F, Kinne RK. Ethanol treatment of hepatocellular carcinoma: high potentials of low concentrations. *Cancer Biol Ther*. 2004;3(5):430-433.
23. Rock KL, Kono H. The inflammatory response to cell death. *Annu Rev Pathol*. 2008;3:99-126.
24. Dinarello CA. Overview of the IL-1 family in innate inflammation and acquired immunity. *Immunol Rev*. 2018;281(1):8-27.
25. Higgins GC, Wu Y, Postlethwaite AE. Intracellular IL-1 receptor antagonist is elevated in human dermal fibroblasts that overexpress intracellular precursor IL-1 alpha. *J Immunol*. 1999;163(7):3969-3975.
26. Werner S, Grose R. Regulation of wound healing by growth factors and cytokines. *Physiol Rev*. 2003;83(3):835-870.
27. Barrientos S, Stojadinovic O, Golinko MS, Brem H, Tomic-Canic M. Growth factors and cytokines in wound healing. *Wound Repair Regen*. 2008;16(5):585-601.
28. Liu M, Guo S, Hibbert JM, et al. CXCL10/IP-10 in infectious diseases pathogenesis and potential therapeutic implications. *Cytokine Growth Factor Rev*. 2011;22(3):121-130.

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

How to cite this article: Matuska AM, Klimovich ML, Anz AW, Podesta L, Chapman JR. Autologous thrombin preparations: Biocompatibility and growth factor release. *Wound Rep Reg*. 2021;29:144–152. https://doi.org/10.1111/wrr.12873