Original Article

The Effects of Human Amniotic Fluid on Periodontal Ligament Fibroblast Cell Viability, Proliferation, and Cytokine/Growth Factor Expression

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Abstract

Background: The importance of the amniotic fluid (AF) to the fetus is clear. However, very few studies have been published to examine the potential uses of this fluid in various areas such as tissue regeneration. AF contains epidermal growth factor, transforming growth factor-alpha, transforming growth factor beta-1, insulin-like growth factor-I, erythropoietin and granulocyte colony-stimulating factor, as well as hyaluronic acid and hyaluronic acid-stimulating factor. Previous studies suggest that AF can increase fibroblast proliferation and chemotaxis, and decrease apoptosis as well as promote wound healing. Furthermore, evidence showed that human AF inhibits hyaluronidase, elastase, and cathepsin. The current study examined the effects of human AF on periodontal ligament fibroblasts (PDLF) in terms of cell toxicity, cell proliferation, and cytokine/growth factor expression. **Materials and Methods:** Cytotoxicity of AF on PDLF was determined using lactate dehydrogenase assays. PDLF proliferation was determined using water-soluble tetrazolium-1 assays. Cytokine/growth factor expression was determined on AF-treated PDLF, AF alone, and PDLF alone utilizing protein arrays. **Results:** Human AF at 10% and below did not affect cell growth and was not toxic. AF-treated PDLF cells showed a decrease in cytokine/growth factor levels compared to the sum of cytokine/growth factor levels in AF only and cells only for 39 of the 80 proteins examined (48.8%). Of the 39 examined cytokines, 20 inflammatory cytokines, 11 cell cycle cytokines, 1 anti-inflammatory cytokine, and 7 other cytokines were decreased. **Conclusion:** Human AF at the examined concentrations was not toxic to PDLF cells and did not influence their proliferation. In addition, AF (10%) caused a decrease in the total protein levels of cytokines/growth factors expressed in 39 of the 80 proteins examined (48.8%). Of the 39 examined cytokines, 20 inflammatory cytokines, 11 cell cycle cytokines, 1 anti-inflammatory cytokine, and 7 other cytokines were decreased.

Keywords: Amniotic fluid, cellular proliferation, cytokines, cytotoxicity, fibroblasts, periodontal ligament

Introduction

Amniotic fluid (AF) is essential for fetal development, nutrition, and protection. Human AF is complex in nature, and very few studies have been published to identify other potential uses outside the mother's womb. Shimberg[1] in 1938 reported using AF in the treatment of joint disease in 68 patients with various orthopedic conditions and reported that AF accelerates a defense-repair mechanism within the joints.

Maternal plasma contributes the water and solutes to the AF by first passing across the amnion through transmembranous flow, across the fetal vessels on the placental surface through intramembranous flow, and across fetal skin. Fetal kidneys develop after 8 weeks of gestation, which allows the fetus to contribute to the contents and volume of the AF. However, this contribution through fetal urination and oral, nasal, tracheal, and pulmonary fluids secretion becomes significant only during the second half of the pregnancy as the fetus' skin keratinizes and acts as a barrier that blocks the early transfer of fluids.[2,3] The AF volume peaks by 28 weeks of gestation reaching approximately 800 mL where it plateaus to then decline by week 42 to approximately 400 mL.[4] Underwood examined the contents of the AF for cytokines

Access this article online

Quick Response Code:

Website: www.cellsindentistry.org

DOI: 10.4103/GFSC.GFSC_10_19

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How to cite this article: Ibraheem AG, Blanchard SB, Al-Hijji SM, Al-Nasr-Allah K, Windsor LJ. The effects of human amniotic fluid on periodontal ligament fibroblast cell viability, proliferation, and cytokine/growth factor expression. Int J Growth Factors Stem Cells Dent 2019;2:23-9.

and growth factors.[5] These included epidermal growth factor, transforming growth factor-alpha (TGF-α), TGF-β1, and insulin-like growth factor-I (IGF-1). AF also contains erythropoietin and granulocyte colony-stimulating factor, as well as hyaluronic acid and hyaluronic acid-stimulating factor.

Periodontal ligament fibroblasts (PDLF) predominate in the periodontal ligament connective tissues. Given the major role that they play in the development and function of the periodontal ligaments, they have been described by Ten Cate as the "architect, builder, and caretaker of connective tissue."[6] Therefore, the focus of this study was to examine the effects of AF on the viability and proliferation of human PDLFs. In addition, the cytokine/growth factor expression from AF-treated PDLF cells was examined to determine the potential of AF on periodontal regeneration.

Materials and Methods

Cell culture

Human PDLFs were purchased from ScienCell Research Laboratories (Carlsbad, San Diego, CA, USA). PDLFs were grown at 37°C in 5% CO_2 in low glucose (1 g/L) Dulbecco's Modified Eagle's Media (DMEM) (Hyclone Logan, Utah) supplemented with 10% fetal bovine serum, 200 mM L-glutamine, 100 U/mL penicillin, 50 μg/mL gentamycin, and 250 μg/mL fungizone.

Human AF collected from a single donor was a gift from MiMedx (MiMedx© Marietta, GA, USA). It was collected from a single consenting mother having cesarian surgery and then lyophilized. DMEM without serum was used to reconstitute the freeze-dried AF to its original volume. A previous study[7] showed that the effects of human AF on human skin fibroblast proliferation were similar when pooled AF donors samples or individual AF donors samples were used. Based on the concentration range published in Chrissouli's paper,[7] concentrations of 1.25%, 2.5%, 5%, and 10% human AF were utilized in the water-soluble tetrazolium (WST) and lactate dehydrogenase (LDH) assays to determine cytotoxicity and cell proliferation.

Cell toxicity

Cellular membrane integrity was monitored by the permeability assay based on the determination of the release of LDH into the media (Cytotoxicity Detection KitPLUS Roche Applied Science, Mannheim, Germany).

Human PDLFs were seeded in 6-well plates (100,000 cells/well) in DMEM plus serum and incubated overnight to allow the cells to attach. The media was then removed and concentrations of AF (1.25, 2.5, 5 and 10%) in serum-free DMEM were added. After 72 h, the assays were performed per manufacturer's instructions (Roche Applied Science, Mannheim, Germany). Absorbance was recorded at 490 nm in a microplate reader (Titertek, Multiskan MCC, Flow Laboratories, McLean, VA, USA). The high controls (total cell death) were generated by the addition of lysis mix to the control wells per the manufacturer.

The experiments were repeated three times, and the mean values were calculated. The percentage release of LDH was calculated from the treated cells by comparing it with the maximum release of LDH (total cell death). To determine the cytotoxicity, the absorbance value of the background was subtracted from the experimented samples. The cytotoxicity was calculated as follows:

Cytotoxicity (%) = (experiment value-low control)/(high control-low control) × 100%.

Proliferation

Human PDLFs were seeded in 6-well plates (100,000 cells/well) in DMEM plus serum and incubated overnight to allow the cells to attach. The media was then removed, and the human PDLFs were exposed to concentrations of AF (1.25%, 2.5%, 5%, and 10%) diluted in serum-free DMEM After 72 h, assays were performed per manufacturer's instructions (WST, Roche Applied Science, Penzberg, Germany). Briefly, the media in the six-well plates was removed, and the cells washed 3 times with 2 mL of serum-free DMEM. The cell proliferation reagent WST-1 was added, and the plate was incubated per manufacturer's instructions.

A 100 μL sample from each well of the six-well plates was placed in a 96-well plate, and the absorbance of the samples against the negative control as the blank was measured using a microplate reader (Titertek) at 450 nm. The experiment was repeated three times, and the mean values calculated.

The absorbance values of each sample were compared with the untreated cell control, by percentage, in the following equation:

Cell proliferation (%) = Absorbance value of AF condensate treatment/absorbance value of no AF treatment × 100%.

Cytokine and growth factor arrays

Protein arrays [Table 1] (RayBio Human Cytokine Antibody Array 5, RayBiotech, Norcross, GA, USA) were used to detect cytokine/growth factor expression from the human PDLFs after exposure to human AF (MiMedx) as described by the manufacturer (RayBio). The highest concentration of AF (10%) that was not toxic and did not affect cell growth was used to treat the cells for 3 days in serum-free DMEM.

Media (1 mL) from three repeated experiments were analyzed by protein arrays. The membranes were blocked for 30 min, incubated for 3 h with the collected samples, washed, incubated for 2 h with biotin-conjugated antibodies, washed, and incubated with horseradish peroxidase-conjugated streptavidin for 2 h as per the manufacturer. Detection agents supplied in the array kits were mixed and applied to each membrane for 2 min. The cytokine/growth factors on the membranes were then visualized by autoradiography on X-ray film. Signal intensities were quantified with a Bio-Rad Gel Doc XR imaging system and analyzed with Quantity One software (Bio-Rad,

Table 1: Cytokine/growth factor array

	A	B	C	D	E	F	G	H	I	J	K
1	POS	POS	POS	POS	NEG	NEG	ENA-78 (CXCL5)	G-CSF	GM-CSF	GRO a/b/g	GRO alpha (CXCL1)
2	I-309 (CCL1)	IL-1 alpha (IL-1 F1)	IL-1 beta (IL-1 F2)	IL-2	IL-3	IL-4	IL-5	IL-6	IL-7	IL-8 (CXCL8)	IL-10
3	IL-12 P40/p70	IL-13	IL-15	IFN-gamma	MCP-1 (CCL2)	MCP-2 (CCL8)	MCP-3 (CCL7)	M-CSF	MDC (CCL22)	MIG (CXCL9)	MIP-1 beta (CCL4)
4	MIP-1 delta (CCL15)	RANTES (CCL5)	SCF	SDF-1 alpha	TARC (CCL17)	TGF beta 1	TNF alpha	TNF beta (TNFSF1B)	EGF	IGF-1	Angiogenin
5	OSM	TPO	VEGF-A	PDGF-BB	Leptin	BDNF	BLC (CXCL13)	Ck beta8-1 (CCL23)	Eotaxin-1 (CCL11)	Eotaxin-2 (CCL24)	Eotaxin-3 (CCL26)
6	FGF-4	FGF-6	FGF-7 (KGF)	FGF-9	FLT-3 Ligand	Fractalkine (CX3CL1)	GCP-2 (CXCL6)	GDNF	HGF	IGFBP-1	IGFBP-2
7	IGFBP-3	IGFBP-4	IL-16	IP-10 (CXCL10)	LIF	LIGHT (TNFSF14)	MCP-4 (CCL13)	MIF	MIP-3 alpha (CCL20)	NAP-2 (CXCL7)	NT-3
8	NT-4	OPN (SPP1)	OPG (TNFRSF11B)	PARC	PLGF	TGF beta 2	TGF beta 3	TIMP-1	TIMP-2	POS	POS

Cytokine abbreviations: BDNF: Brain-derived neurotrophic factor, EGF: Epidermal growth factor, FGF: Fibroblast growth factor, FLT-3-Ligand: Fms-related tyrosine kinase 3 ligand, G-CSF: Granulocyte colony-stimulating factor, GDNF: Glial cell line-derived neurotrophic factor, GM-CSF: Granulocyte-macrophage colony-stimulating factor, TNFSF: Tumor necrosis factor ligand superfamily member, HGF: Hepatocyte growth factor, IFN-gamma: Interferon gamma, IGFBP: Insulin-like growth factor-binding protein, GRO alpha: Growth-regulated alpha protein, LIF: Leukemia inhibitory factor, M-CSF: Macrophage colony-stimulating factor, MIF: Macrophage migration inhibitory factor, PPBP: Pro-Platelet basic protein, NT: Neurotrophin, PDGF: Platelet-derived growth factor, PLGF: Placenta growth factor, SDF: Stromal cell-derived factor, TGF: Transforming growth factor, TIMP: Tissue inhibitor of metalloproteinase, TNF: Tumor necrosis factor, VEGF: Vascular endothelial growth factor, TARC: Thymus and activation regulated chemokine, PARC: Pulmonary and activation-regulated cytokine, SCF: Stem-cell factor, RANTES: Regulated on activation, normal T cell expressed and secreted, OPG: Osteoprotegerin, OSM: Oncostatin M, BLC: B lymphocyte chemoattractant, OPN: Osteopontin, IL: Interleukin, IGF: Insulin-like growth factor, GCP-2: Granulocyte chemotactic protein-2, ENA: Epithelial neutrophil- activating protein, CCL: Chemokine (C-C motif) ligand, CXCL: Chemokine (C-X-C motif) ligand, MCP: Monocyte chemoattractant protein, MDC: Macrophage-derived chemokine, MIP: Macrophage inflammatory protein, MIG: Monokine induced by gamma interferon, TPO: Thrombopoietin, Ck: Cytokine, IP: Interferon gamma-induced protein, NAP: Neutrophil activating peptide, GRO a/b/g: Growth-regulated oncogene -α/β/γ

Laboratories, Hercules, CA, USA). The experiments were repeated three times and the mean values calculated.

Statistical methods

The WST and LDH data were subjected to one-way analysis of variance (ANOVA) followed by the Tukey's honestly significant difference test for pair-wise comparisons among the groups ($P < 0.05$). To determine the level of cytokine/growth factor expression, the optical densities of the visible dots on the membrane were measured. For each membrane, the densities were adjusted for the background by subtracting the average value of the negative controls and then normalized by dividing by the average of the positive controls. The data were then converted back to the original scale by multiplying by the average of the positive controls for the first membrane. Three membranes were used for each of the three groups (AF + cells, AF only, and cells only). Group comparisons were made using one-way ANOVA, followed by pair-wise tests using Fisher's protected least significant differences to control the overall significance level at 5%. In addition to the direct comparisons among the three groups, the AF + cells group was compared against the sum of the AF only and cells only groups to evaluate nonadditive effects of the AF and cells; a nonsignificant test for this effect indicates the effect could be additive, whereas a significant test could indicate the effect is either synergistic (AF + cells is significantly greater than the sum of AF only and cells only) or inhibitory (AF + cells is significantly less than the sum of AF only and cells only). Tests of normality were performed before performing the ANOVAs. Statistical analyses were performed using SPSS 23.0 for Windows software system (SPSS Inc., Chicago, IL, USA) and SAS version 9.4 (SAS Institute Inc., Cary, NC, USA).

Results

Cytotoxicity and cell proliferation

Cell proliferation results [Figure 1a] showed that AF at concentrations of 1.25% (86.1 ± 1.31, 0.073), 2.5% (95.5 ± 4.25, 0.858), 5% (95.8 ± 2.04, 0.886), and 10% (90.4 ± 4.02, 0.286) (mean ± standard error [SE], *P* value) did not affect PDLF cell proliferation. Cytotoxicity [Figures 1b] showed that AF at concentrations of 1.25% (21 ± 0.53, 0.636), 2.5% (21.5 ± 1.31, 0.467), 5% (19.2 ± 0.73, 0.998), and 10% (17.2 ± 1.85, 0.892) (mean ± SE, *P* value) were not toxic to PDLF cells. Concentrations of more than 10% AF were not tested.

Cytokine array

AF-treated PDLF cells (AFC) showed a significant decrease ($P < 0.05$) in cytokine/growth factor levels compared to the sum of cytokine/growth factor levels in AF only and cells only for 39 of the 80 cytokine/growth factor (48.8%) examined [Table 2]. Of

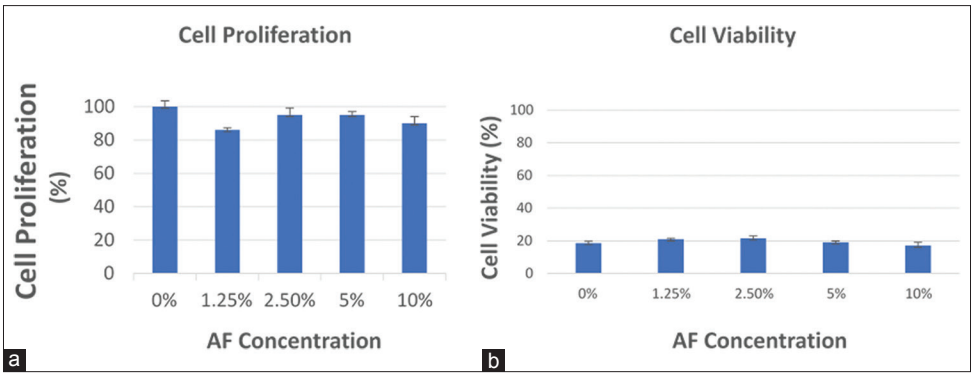

Figure 1: (a) Cell proliferation versus amniotic fluid concentration showing no significant difference ($P < 0.05$) in cell proliferation compared to 0% amniotic fluid, (b) cell viability versus amniotic fluid concentration showing no significant difference ($P < 0.05$) in viability compared to 0% amniotic fluid

Table 2: Inflammatory cytokines/growth factors that showed significant decrease $P<0.05$

Cytokine	Mean±SE			*P*			
	AFC	AF	C	AFC versus AF + C	AFC versus AF	AFC versus C	AF versus C
BLC	7.59±0.63	7.87±0.57	6.3±0.77	0.002	0.766	0.269	0.186
GRO a/b/g	6.36±0.32	5.54±0.57	3.61±0.67	0.033	0.298	0.038	0.097
IFN-gamma	2.03±0.75	1.76±0.99	4.83±0.68	0.021	0.842	0.052	0.072
IL-1 beta	2.64±0.6	2.33±0.67	4.62±0.77	0.012	0.754	0.119	0.093
IL-12	3.56±0.22	2.65±0.53	4.62±0.54	0.007	0.23	0.179	0.062
IP-10	4.04±0.85	4.77±0.72	5.45±0.56	0.004	0.557	0.255	0.503
OPN	7.89±0.59	7.8±0.71	7.16±0.35	0.001	0.926	0.363	0.482
RANTES	2.95±0.69	3.51±0.66	4.77±0.79	0.005	0.591	0.162	0.293
TNF alpha	1.87±1.07	3.06±0.59	3.34±0.51	0.026	0.4	0.307	0.741
TNF beta	2.36±0.75	3.37±0.67	2.62±0.51	0.021	0.378	0.795	0.429
MCP-4	7.47±0.72	7.74±0.57	5.67±1.04	0.009	0.787	0.238	0.177
Fractalkine	3.21±1.08	4.21±0.55	4.84±0.99	0.014	0.474	0.332	0.617
IL-15	1.84±0.91	2.37±0.17	4.53±0.57	0.013	0.625	0.08	0.054
IL-16	3.11±0.85	3.6±0.65	4.66±0.43	0.007	0.674	0.204	0.256
IL-3	2.91±0.84	3.19±0.6	4.96±0.46	0.006	0.802	0.119	0.084
Light	3.51±0.99	4.35±0.48	4.37±0.98	0.018	0.505	0.57	0.982
Leptin	1.75±1.22	2.89±0.41	4.25±0.69	0.025	0.458	0.169	0.181
TARC	2.93±1.16	3.3±0.47	5.42±0.58	0.017	0.795	0.155	0.049
M-CSF	3.17±0.75	4.51±0.42	2.71±0.97	0.03	0.215	0.731	0.197
GCP-2	3.3±0.95	3.83±0.72	3.58±0.82	0.031	0.68	0.833	0.83

AF: Amniotic fluid, SE: Standard error, BLC: B lymphocyte chemoattractant, IFN-gamma: Interferon gamma, IL: Interleukin, OPN: Osteopontin, RANTES: Regulated on activation, normal T cell expressed and secreted, TNF: Tumor necrosis factor, TARC: Thymus and activation regulated chemokine, M-CSF: Macrophage colony-stimulating factor, GCP-2: Granulocyte chemotactic protein-2, GRO a/b/g: Growth-regulated oncogene -α/β/γ, IP: Interferon gamma-induced protein, MCP: Monocyte chemoattractant protein

the 39 examined cytokines, 20 inflammatory cytokines [Table 2], 11-cell cycle cytokines [Table 3], 1 anti-inflammatory cytokine [Table 4], and 7 other cytokines [Table 4] were decreased. AFC showed inhibitory effects compared to the sum of AF only and cells only for BDNF, BLC, fibroblast growth factor (FGF)-6, FGF-7, FGF-9, FGF-4, FLT-3 Ligand, Fractalkine, GCP-2, GRO a/b/g, IFN-gamma, IGFBP-2, IGFBP-4, IL-1 beta, IL-15, IL-16, IL-3, IL-4, IL-12, IP-10, LIF, LIGHT, Leptin, M-CSF, MCP-4, NT-4, osteoprotegerin (OPG), OPN, OSM, PARC, PDGF-BB, POS, RANTES, SCF, SDF-1 alpha, TARC, TIMP-2, TNF alpha, TNF beta, and VEGF-A.

AFC demonstrated a significant increase in GRO a/b/g ($P = 0.038$), GRO alpha ($P = 0.025$), IGFBP-1 ($P = 0.026$), and IL-7 ($P = 0.039$) compared to their levels in cells only. IL-6 showed significantly higher levels ($P = 0.004$) in AFC compared to its levels in AF only. OPG levels were significantly lower ($P = 0.015$) in AF only versus in cells only and showed a significant increase ($P = 0.01$) in AFC compared to its levels in AF only. OPG levels in AFC were, however, significantly lower ($P = 0.009$) than the sum of its values in AF only and in cells only. The AF only levels of IGFBP-1 were significantly higher (0.019) than in cells only while the AF only levels of SDF-1 alpha and TARC were significantly less ($P = 0.045$ and $P = 0.049$) than in cells only. The AFC levels of SDF-1 alpha and TARC, however, were significantly less ($P = 0.016$ and $P = 0.017$) than the sum of their values in AF only and cells

Table 3: Cell cycle cytokines/growth factors that showed significant decrease $P<0.05$

Cytokine	Mean±SE			*P*			
	AFC	AF	C	AFC versus AF + C	AFC versus AF	AFC versus C	AF versus C
FGF-6	6.71±0.65	6.41±0.66	6.04±0.6	0.002	0.765	0.496	0.703
FGF-7	0.9±0.82	1.86±0.8	3.38±0.44	0.016	0.45	0.075	0.194
FGF-9	3.1±0.75	4.23±0.64	5.38±0.76	0.002	0.32	0.102	0.316
FGF-4	3.68±0.72	3.58±0.7	4.69±0.82	0.013	0.925	0.414	0.367
IGFBP-2	5.58±1	6±0.93	3.82±1.03	0.048	0.772	0.291	0.193
IGFBP-4	3.02±0.78	2.65±1.11	4.59±0.59	0.038	0.801	0.192	0.221
PDGF-BB	0.95±0.93	2.05±0.6	3.37±0.35	0.018	0.388	0.108	0.148
VEGF-A	1.73±0.58	1.99±0.61	3.71±0.46	0.007	0.78	0.06	0.094
LIF	3.49±0.94	4.4±0.62	4.8±0.75	0.007	0.473	0.341	0.704
SCF	1.97±0.84	2.53±0.56	3.97±0.27	0.013	0.615	0.131	0.109
SDF-1 alpha	2.58±0.92	2.82±0.11	4.82±0.47	0.016	0.825	0.122	0.045

AF: Amniotic fluid, SE: Standard error, FGF: Fibroblast growth factor, IGFBP: Insulin-like growth factor-binding protein, PDGF: Platelet-derived growth factor, VEGF: Vascular endothelial growth factor, LIF: Leukemia inhibitory factor, SCF: Stem-cell factor, SDF: Stromal cell-derived factor

Table 4: Anti-Inflammatory and other cytokines/growth factors that showed significant decrease $P<0.05$

Cytokine	Mean±SE			*P*			
	AFC	AF	C	AFC versus AF + C	AFC versus AF	AFC versus C	AF versus C
Anti-inflammatory							
IL-4	1.62±0.65	2.26±0.54	3.18±0.42	0.009	0.493	0.127	0.26
Others							
FLT-3 Ligand	1.91±1.04	2.74±0.52	3.82±0.83	0.024	0.533	0.23	0.343
TIMP-2	7.82±0.6	5.92±0.68	6.42±0.94	0.017	0.108	0.289	0.694
OPG	7.55±0.52	3.93±0.57	7.73±0.7	0.009	0.01	0.85	0.015
BDNF	4.65±0.94	5.13±0.63	6.05±0.85	0.005	0.7	0.333	0.436
NT-4	4.79±0.48	4.51±0.95	5.64±0.68	0.01	0.811	0.377	0.399
OSM	4.38±0.57	4.49±0.87	5.7±0.75	0.005	0.919	0.241	0.358
PARC	6.79±0.69	7.12±0.54	6.05±0.42	0.001	0.734	0.42	0.199

AF: Amniotic fluid, SE: Standard error, IL: Interleukin, FLT-3-Ligand: Fms-related tyrosine kinase 3 ligand, TIMP: Tissue inhibitor of metalloproteinase, OPG: Osteoprotegerin, BDNF: Brain-derived neurotrophic factor, NT: Neurotrophin, OSM: Oncostatin M, PARC: Pulmonary and activation-regulated cytokine

only. The cytokines that were increased in AFC versus C or AFC versus AF was increased due to an additive effect of their values in AF only and C only. No significant increase ($P < 0.05$) was reported in cytokines levels in AFC versus AF + C.

Images taken of X-rays of the cytokine/growth factor arrays for different groups are shown in Figure 2. Forty-one cytokines were not altered [Supplemental Table 1].

Discussion

The current study is the first to explore the effects of AF on PDLF cells. This study examined cytotoxicity, cell proliferation, and cytokine/growth factor expression from PDLF cells exposed to AF. The results showed that AF at concentrations of 1.25%, 2.5%, 5%, and 10% had no effects on cytotoxicity and cell proliferation of the PDLF cells. Concentrations of more than 10% AF were not tested.

A study by Chrissouli showed significant cell proliferation of human skin fibroblasts treated with amniotic fluid at concentrations 1%–50% (v/v) and 0.1% v/v fetal bovine serum.[7] Their study used a different fibroblast type, and fetal bovine serum (0.1% v/v) was included in the media with the AF. These differences could account for the differences seen between their study and the current study that showed no PLDF cell proliferation. Also, in a recent study,[8] the effects of AF on neonatal foreskin keratinocytes and fibroblasts were examined in terms of cytotoxicity and cell proliferation. The study showed that cell viability did not differ for the keratinocytes and fibroblasts. AF caused a decrease in keratinocyte proliferation and showed no difference in neonatal foreskin fibroblast proliferation.[8] The lack of cellular proliferation in their study is consistent with results from the current study.

The results of the current study show a downregulation in 20 inflammatory cytokines, including pro-inflammatory cytokines IL-1 beta, TNF-alpha, and IFN-gamma. These cytokines were found to be elevated in newborns with evidence of perinatal brain damage when compared to controls.[9] Infants who developed white matter brain damage had elevated postnatal levels of circulating IFN-gamma in umbilical cord blood and neonatal blood compared to those

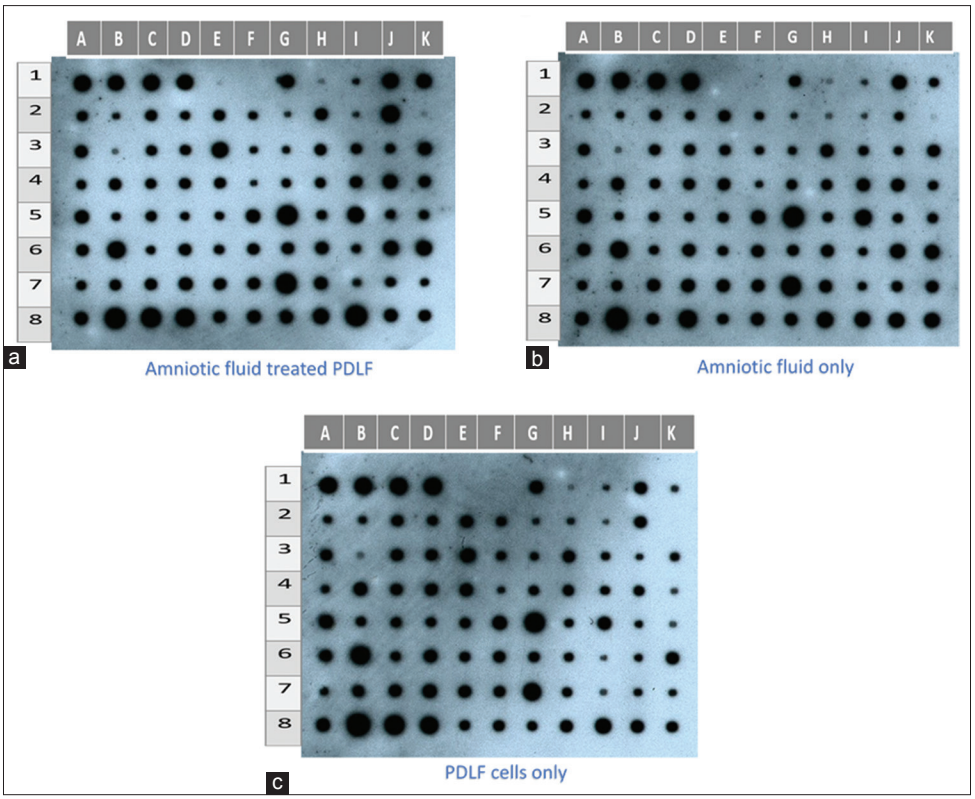

Figure 2: (a) X-ray of cytokine/growth factor array of amniotic fluid treated periodontal ligament fibroblasts cells, (b) X-ray of cytokine/growth factor array of amniotic fluid only, (c) X-ray of cytokine/growth factor array of periodontal ligament fibroblasts cells only

who did not develop brain damage.[10] A study undertaken to explore the association between the mean neonatal concentration of inflammatory mediators and chemokines of normal children compared to children with congenital cerebral palsy found that the concentrations of IL-1, IL-8, IL-9, TNF-α, and RANTES were higher.[11] IL-8 levels were not increased in the current study. However, IL-1, TNF-α, and RANTES levels were decreased. IL-9 levels were not examined in the present study.

The current study shows significant inhibition of TNF-α (P = 0.026) levels in AFC compared to the sum of the growth factor levels in AF only and cell only. TNF-α is a well-known mediator of bone resorption.[12] TNF-α may act independently or along with IL-1 to synergistically initiate the resorptive process.[12,13] Therefore, the ability of AF to downregulate IL-1 beta and TNF-α may suggest a protective function of AF. OPG was also downregulated in the current study. Its levels in AFC were significantly less (P = 0.009) than the sum of its values in AF only and in cells only. OPG is a decoy receptor that binds to receptor activator of nuclear factor-kB receptor and downregulates osteoclast formation and therefore, inhibits bone resorption.[14] Given the developmental stage of the fetus, OPG might not be needed since osteoclastic activity would not be expected.

In the current study, IL-6 expression showed a trend toward increasing (P = 0.082) on treating PDLF cells with AF. Certain cytokines are known to overlap in function and are sometimes present in antagonistic groups. IL-6 is one of those overlapping cytokines in that it can function as an anti-inflammatory or pro-inflammatory cytokine.[15] In its pro-inflammatory role, it causes B-cell activation, which leads to the production of IL-1, a pro-inflammatory cytokine.[16] IL-1 enhances bone resorption, stimulation of matrix metalloproteinase production, and prostaglandin synthesis.[16] Interestingly, in the current study, IL-1 beta was significantly downregulated (P = 0.012) in AFC compared to the sum of the growth factor levels in AF only and cells only. In its anti-inflammatory role, IL-6 inhibits TNF, and IL-1 production from macrophages.[15] In an animal study, the anti-inflammatory function exhibited by IL-6 could not be compensated for by IL-10, which is another anti-inflammatory cytokine.[17] IL-10 levels were unchanged in the current study. IL-6 in its anti-inflammatory role is considered crucial in regulating the levels of pro-inflammatory cytokines thereby sometimes playing a crucial anti-inflammatory role.[17]

In the current study, numerous cytokine/growth factors related to cell cycle regulation were downregulated. FGFs control a broad range of biological cell functions such as proliferation, survival, migration, and differentiation. Multiple FGF isoforms were downregulated in AFC, which may explain why PDL fibroblast cell proliferation was not observed when treated with AF in the current study. Other cell cycle cytokines/growth factors that were downregulated included IGFBP 2 and 4, PDGF-BB, VEGF-A, LIF, SCF, and SDF-1 alpha. IL-4, an anti-inflammatory cytokine, was

downregulated in the current study. It is unclear why it might be downregulated.

The current data suggest that AF promotes an anti-inflammatory state that provides a nonreactive state that is crucial for the developing fetus. This study suggests that AF may be useful in downregulating inflammation but may not induce cell proliferation. Therefore, AF may be valuable in treating inflammation but appears unlikely to be of any benefit for the regeneration of periodontal tissues involving PDLFs.

Regardless of these interesting findings, some limitations of the present study need to be addressed. For example, the AF used was from one individual. AF from different individuals should be examined to observe the variations between individuals. In addition, it would be valuable to do ELISA on some of the major cytokines in the different groups such as IL-1, GRO a/b/g, VEGF, PDGF, IL-4, TIMP 2, and OPG.

Conclusion

The study found that AF had no effects on cell viability and was not toxic at the concentrations examined. AF (10%) downregulated numerous inflammatory cytokines/growth factors.

Financial support and sponsorship

Nil.

Conflicts of interest

There are no conflicts of interest.

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