• Clinical Research •

Effect of human autologous serum and fetal bovine serum on human corneal epithelial cell viability, migration and proliferation *in vitro*

Ming-Feng Wu[1], Tanja Stachon[1], Berthold Seitz[1], Achim Langenbucher[2], Nóra Szentmáry[1,3]

[1]Department of Ophthalmology, Saarland University Medical Center, Homburg/Saar 66424, Germany
[2]Experimental Ophthalmology, Saarland University, Homburg/Saar 66424, Germany
[3]Department of Ophthalmology, Semmelweis University, Budapest 1085, Hungary
Correspondence to: Ming-Feng Wu. Department of Ophthalmology, Saarland Medical University, Kirrberger Street, Homburg/Saar 66424, Germany. enmengfong@hotmail.com
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Abstract

• AIM: To analyze the concentration-dependent effects of autologous serum (AS) and fetal bovine serum (FBS) on human corneal epithelial cell (HCEC) viability, migration and proliferation.

• METHODS: AS was prepared from 13 patients with non-healing epithelial defects Dulbecco's modified eagle medium/Ham's F12 (DMEM/F12) with 5% FBS, 0.5% dimethyl sulphoxide (DMSO), 10 ng/mL human epidermal growth factor, 1% insulin-transferrin-selenium, then were incubated in serum media: DMEM/F12 supplemented by 5%, 10%, 15% or 30% AS or FBS. HCEC viability was analyzed using cell proliferation kit XTT, migration using a wound healing assay, proliferation by the cell proliferation enzyme-linked immunosorbent assay (ELISA) BrdU kit. Statistical analysis was performed using the generalized linear model, the values at 30% AS or 30% FBS were used as the baselines.

• RESULTS: HCEC viability was the highest at 30% AS or 15% FBS and the lowest at 10% AS or 30% FBS application. HCEC migration was the quickest through 30% AS or 30% FBS and the slowest through 5% AS or 5% FBS concentrations. Proliferation was the most increased through 15% AS or 5% FBS and the least increased through 30% AS or 30% FBS concentrations. HCEC viability at 10% and 15% AS was significantly worse (P=0.001, P=0.023) compared to baseline and significantly better at 15% FBS (P=0.003) concentrations. HCEC migration was significantly worse (P≤0.007) and HCEC proliferation significantly better (P<0.001) in all concentration groups compared to baseline.

• CONCLUSION: For the best viability of HCEC 30% AS or 15% FBS, for HCEC migration 30% AS or 30% FBS, for proliferation 15% AS or 5% FBS should be used. Therefore, we suggest the use of 30% AS in clinical practice.

• KEYWORDS: autologous serum; eye drops; serum concentration; migration; proliferation; viability; human corneal epithelial cells

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INTRODUCTION

Tear film is a fluid layer essential for ocular surface lubrication, nutrition and immunology[1]. Abnormal tear film can result in keratoconjunctivitis sicca (KCS), which is still most commonly treated by lubricating artificial tears[2]. However, the components of tear film, including electrolytes, proteins, lipids, mucins, are hardly compensated by the only use of lubricants[1].

Human peripheral blood serum is a natural substitute of tears. Serum has similar pH and osmotic pressure to tears. Furthermore, it contains many identical components to tears, such as epidermal growth factor (EGF), nerve growth factor, insulin-like growth factor, platelet-derived growth factor, transforming growth factor (TGF)-β, lysozyme, IgA, albumin, vitamin A, substance P, *etc*[3]. In 1984, the beneficial effect of autologous serum (AS) eye drops, as "artificial tear" for KCS patients, was firstly reported by Fox *et al*[4]. Thereafter, serum eye drops, mostly autologous and sometimes allogenic, were used in various ocular surface diseases such as KCS, Sjögren's syndrome, persistent corneal epithelial defects, chemical eye burn and neurotrophic keratitis[5-10].

Like other materials, AS is only optimal and safe for the human corneal epithelial cells (HCECs) in a certain concentration range. In 2001, using an *in vitro* cell culture model, Geerling *et al*[11] found that 50% and 100% AS were toxic to HCECs; they either decreased cellular ATP level or increased cell membrane permeability. The 100% AS was less toxic compared to 50%

as. A few years later, Liu *et al*[12] found that 100% human serum was supporting more HCEC migration than 25% human serum (diluted with isotonic saline). Later it was also described that the relative cell growth of HCECs was best supported with human serum diluted to 12%[13]. Beside these *in vitro* studies, Akyol-Salman[14] has shown that 100% AS accelerates rabbit corneal wound healing more than 20% AS. Nowadays, 20% AS concentration seems to be the most commonly used concentration of AS in clinical and experimental studies[9,15-20]. However, there is still no consensus on AS preparation and application; AS concentration can vary from 20% to 100% among different institutions[3,21].

Recently, AS has also been recommended as an alternative of fetal bovine serum (FBS) use in cell cultures, in order to devoid animal-derived products during culturing and expansion of human corneal limbal epithelial cells, *in vitro,* with the aim of transplantation[22]. In some previous studies, corneal epithelial cells cultivated in AS and FBS supplemented media have shown similar morphology and expression pattern of intercellular junction proteins, basement membrane proteins and tissue-specific keratins. Likewise, BrdU enzyme-linked immunosorbent assay (ELISA) cell proliferation assay and colony-forming efficiency analysis did not demonstrate significant differences between corneal epithelial cells of both cultures[23-24]. Considering the complicated effects of different concentrations of AS on HCECs and the inconsistency of AS application, the dose-dependent effects of AS and the growth factors which may play key role in effects of AS, should be studied in detail.

The aim of the current study was to analyze the effects of AS and FBS on HCEC healing function *in vitro*, and to determine the impact of growth factors in AS on HCEC healing function.

MATERIALS AND METHODS

The study was performed in accordance with the tenets of the Declaration of Helsinki. No ethical committee approval was needed for this study.

Materials Cell proliferation kit XTT (AppliChem, Darmstadt, Germany), cell proliferation ELISA BrdU (colorimetric) kit (Roche, Mannheim, Germany), sulfuric acid (Titrisol, Darmstadt, Germany), phosphate-buffered saline (PBS) (Sigma-Aldrich, Steinheim, Germany), Dulbecco's modified eagle medium/Ham's F12 (DMEM/F12) (Life technologies, Paisley, UK), FBS (Life technologies, Paisley, UK), penicillin-streptomycin (P/S) (Sigma-Aldrich, USA), dimethylsulfoxide (DMSO) (Central Chemical Storage of Saarland University, Saarbruecken, Germany), human epidermal growth factor (hEGF) (Biochrom GmbH, Berlin, Germany), insulin-transferrin-selenium (Life technologies, Paisley, UK), trypsin-EDTA solution (Sigma-Aldrich, USA). Human fibroblast growth factor (FGF) basic DuoSet, human hepatocyte growth factor (HGF) DuoSet, human TGF-β1 DuoSet and human keratinocyte growth factor (KGF)/FGF-7 DuoSet (R&D systems, Minneapolis, USA).

Preparation of Autologous Serum AS was obtained from 13 patients with non-healing epithelial defects [38% females, age 69±16 (41 to 92)y] with the diagnosis non-healing corneal ulcer (6 patients) or corneal erosion (4 patients), Salzmann's nodular degeneration (1 patient), Sjögren's syndrome (1 patient), systemic lupus erythematosus (1 patient). Five of the patients had previous penetrating keratoplasty. All patients had non-healing epithelial defects and AS was used for their clinical treatment (details not described in this study). The rests of AS, not used in clinical treatment of our patients was available for our experiments. No other specific inclusion criteria were applied. Serological tests for hepatitis B, hepatitis C, HIV, cytomegalovirus and syphilis were all negative. To prepare the AS, peripheral blood was obtained by vein puncture, was stored for 1 to 3h at room temperature, then centrifuged at 3000 rpm (855 g) for 15min. Thereafter, under laminar flow, serum was pipetted into a sterile container and 1.5 to 2 mL aliquots of serum were filtered and injected into 5 mL sterile dropper bottles *via* a disposable filter connected to a syringe. The serum was stored at -20℃ for maximal 3mo.

Determination of Human Corneal Epithelial Cell Viability SV40-Adeno vector transformed HCECs (cell no. RCB2280), which obtained from RIKEN BioResource Center, Ibaraki, Japan, were cultured in DMEM/F12 culture medium containing 5% FBS, 5 μg/mL Insulin, 10 ng/mL hEGF and 0.5% DMSO. HCEC viability was determined as previous description using cell proliferation kit XTT[25]. Briefly, HCECs in 96-well plates was incubated in the 5%, 10%, 15% or 30% AS or FBS containing medium (serum media) for 24h, XTT-containing solution was then added to react with HCECs at 37℃ for 2h. Finally, the absorbance of the reaction mixture in each well, which representing the HCEC viability, were measured by a 96-well microplate reader.

Wound Healing Assay of Human Corneal Epithelial Cells HCECs grew in DMEM/F12 culture medium with 5% FBS, 5 μg/mL Insulin, 10 ng/mL hEGF and 0.5% DMSO until confluence in 6-well plate. Then the culture medium was replaced by serum media after rinsing the well twice by PBS, followed by incubation at 37 ℃ for 20min. The HCEC monolayers were scratched by 200 μL yellow pipette tips, then 3 photos of each scratch were taken at the beginning and after 9h incubation (Figure 1). The pixel areas of the scratch on the photos were measured by the software GNU Image Manipulation Program. Then, the average widths of the scratches were calculated and converted into micrometers.

Determination of Human Corneal Epithelial Cell Proliferation HCEC proliferation was determined by cell proliferation ELISA BrdU (colorimetric) kit as previous description[25].

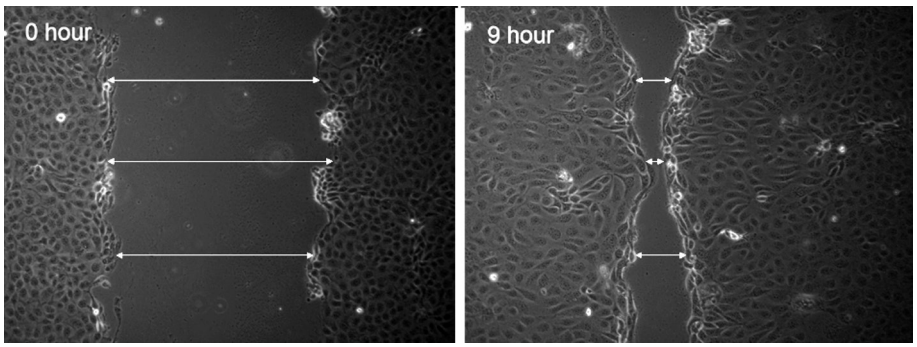

Figure 1 Photos of the scratch were taken at the beginning and after 9h incubation.

Briefly, HCEC monolayer was incubated in the serum media for 24h. BrdU labeling solution was added and incubated at 37℃ for 3h (BrdU incorporation). After removing the serum media, the cells underwent a series of reactions for photometric detection. The 96-well plates were analyzed by the microplate reader.

Measurement of Growth Factors in Autologous Serum KGF, FGFb, HGF and TGF-β1 concentrations in AS were measured by taking a 100 μL aliquot of the AS, as previous description[25].

Statistical Analysis Statistical analysis was done using the SPSS Statistics 22.0. Mann-Whitney *U* test was used to compare viability, migration and proliferation of AS and FBS groups with the same concentration. We used a generalized linear model to determine the effect of different concentrations of AS and the impact of growth factor concentrations in AS on HCEC viability, migration and proliferation. We calculated the concentration of the growth factors from the concentration measurement results of 100% AS. $P<0.05$ was considered statistically significant.

RESULTS

Effect of Different Concentrations of Autologous Serum and Fetal Bovine Serum on Human Corneal Epithelial Cell Viability, Migration and Proliferation Viability, migration and proliferation of HCECs using different concentrations of AS and FBS are displayed at Figures 2-4. Effect of different concentrations of AS or FBS on HCEC viability, migration and proliferation using a generalized linear model are shown in Table 1. HCEC viability was the highest when 30% AS or 15% FBS was applied, and lowest when 10% AS or 30% FBS applied. HCEC migration rate was the highest when 30% AS or 30% FBS applied, and lowest when 5% AS or 5% FBS applied. Proliferation was the highest when 15% AS or 5% FBS applied, and lowest when 30% AS or 30% FBS applied. HCEC viability at 10% and 15% AS was significantly worse (P=0.001, 0.023) compared to baseline and significantly better at 15% FBS (P=0.003) concentrations. HCEC migration was significantly worse ($P\leq0.007$) and HCEC proliferation significantly better ($P<0.001$) in all concentration groups compared to baseline.

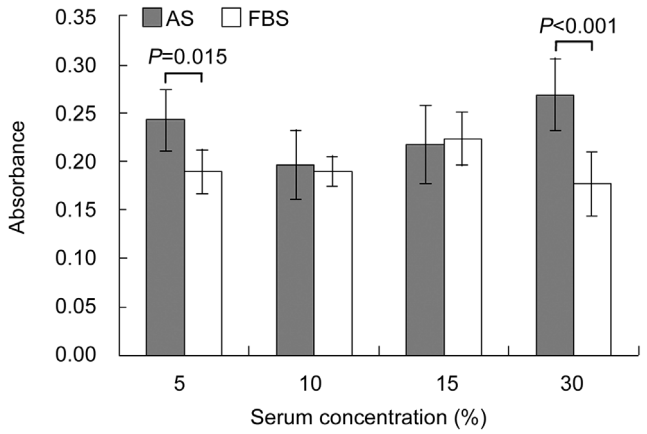

Figure 2 HCEC viability using different concentrations of AS and FBS Mann-Whitney *U* test was used to compare AS and FBS groups with the same concentration. Viability was significantly higher using 5% AS than 5% FBS (P=0.015) or using 30% AS than 30% FBS ($P<0.001$).

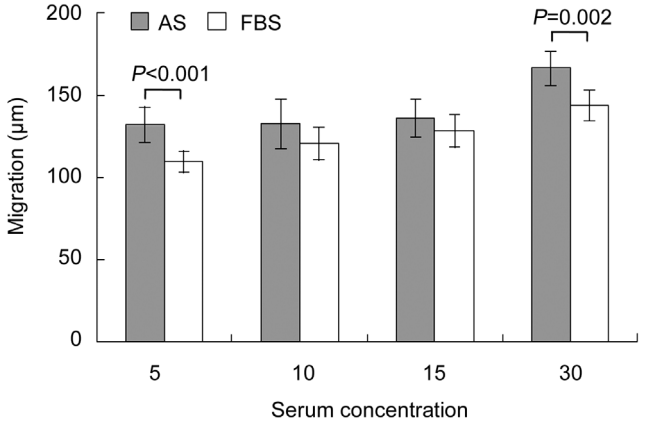

Figure 3 HCEC migration using different concentrations of AS and FBS Mann-Whitney *U* test was used to compare AS and FBS groups with the same concentration. Migration was significantly higher using 5% AS than 5% FBS ($P<0.001$) or using 30% AS than 30% FBS (P=0.002).

Effect of Autologous Serum and Fetal Bovine Serum with the Same Concentration on Human Corneal Epithelial Cell Viability, Migration and Proliferation Results of HCEC viability, migration and proliferation responding to AS and FBS are shown at Figures 2-4. Viability and migration was significantly higher using 5% AS than 5% FBS (P=0.015, $P<0.001$). Viability and migration were also significantly

Table 1 Effect of different concentrations of AS or FBS on HCEC viability, migration and proliferation using a generalized linear model

Serum	Concentration (%)	Viability		Migration		Proliferation	
		RC	*P*	RC	*P*	RC	*P*
AS	5	-0.026	0.244	-34.319	<0.001	0.164	<0.001
	10	-0.072	0.001	-33.595	<0.001	0.170	<0.001
	15	-0.052	0.023	-30.583	<0.001	0.187	<0.001
	30	0	-	0	-	0	-
FBS	5	0.013	0.428	-34.154	<0.001	0.150	<0.001
	10	0.013	0.423	-23.084	<0.001	0.118	<0.001
	15	0.047	0.003	-15.365	0.007	0.129	<0.001
	30	0	-	0	-	0	-

RC: Regression coefficient. $P<0.05$ was considered statistically significant, compared to baseline. The values at 30% AS and 30% FBS were used as baselines.

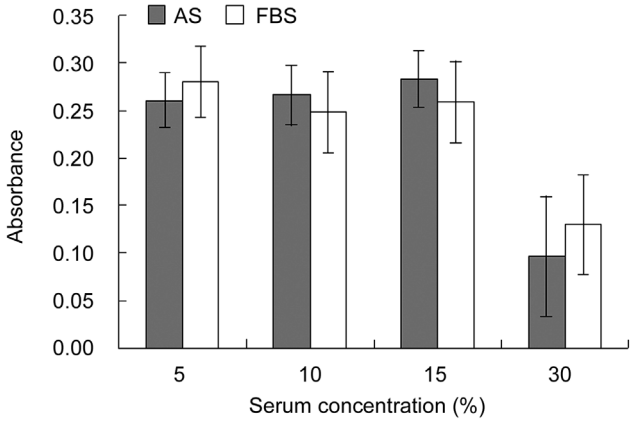

Figure 4 HCEC proliferation using different concentrations of AS and FBS Mann-Whitney *U* test was used to compare AS and FBS groups with the same concentration. Proliferation did not differ significantly between AS and FBS groups with the same concentration ($P>0.096$).

higher using 30% AS than 30% FBS ($P<0.001$, $P=0.002$). However, viability and migration did not differ significantly using 10% AS *vs* 10% FBS or 15% AS *vs* 15% FBS ($P>0.077$). Proliferation did not differ significantly between AS and FBS groups with the same concentration ($P>0.096$).

Effect of Growth Factors in Autologous Serum on Human Corneal Epithelial Cells Concentrations of KGF, FGFb, HGF and TGF-β1 in AS of 13 patients are shown in Table 2. The effect of FGFb, HGF and TGF-β1 concentrations in AS on HCEC viability, migration and proliferation using a generalized linear model is displayed in Table 3. Effect of KGF on HCEC viability, migration and proliferation was not considered in Table 3, since KGF was only measurable in one AS sample. Concentration of the measured growth factors did not affect HCEC viability ($P>0.590$). However, FGFb and HGF concentrations had a positive effect ($P<0.001$ for both) on HCEC migration and FGFb and TGF-β1 concentrations a negative effect ($P=0.006$, 0.008) on HCEC proliferation.

Table 2 Growth factor concentrations in AS of 13 patients

Patient No.	Growth factors (pg/mL)			
	KGF	FGFb	HGF	TGF-β1
1	0	202	139	2928
2	0	204	1115	2928
3	0	130	1884	2313
4	0	122	2888	3426
5	0	56	1272	3955
6	0	0	2784	16767
7	0	300	628	28906
8	106	136	5964	28218
9	0	58	1426	28873
10	0	0	1182	26046
11	0	130	308	25075
12	0	96	661	25781
13	0	131	1715	35045
Median	0	130	1272	25075

KGF: Keratinocyte growth factor; FGFb: Basic fibroblast growth factor; HGF: Hepatocyte growth factor; TGF-β1: Transforming growth factor-β1.

DISCUSSION

From the clinical point of view, migration and proliferation are the most important functions of HCECs during corneal epithelial regeneration and wound healing, in order to reach closure of a corneal epithelial defect/erosion. The 20% AS concentration seems to be the most commonly used concentration of AS in clinical and experimental studies, but the most important component of AS with beneficial effect on corneal epithelial regeneration could not be defined yet[9,15-20]. Appropriate function of the corneal limbal stem cells (proliferation, differentiation and centripetally migration) is also indispensable in renewal of the corneal epithelial layer[26]. AS has been used as a substitute for FBS in cultures of various cells, and supported better cell confluence, enhanced

Table 3 Effect of FGFb, HGF and TGF-β1 concentrations in AS on HCEC

Growth factor	Viability		Migration		Proliferation	
	RC	*P*	RC	*P*	RC	*P*
FGFb	3.3×10^{-4}	0.590	0.623	<0.001	-0.002	0.006
HGF	1.74×10^{-6}	0.945	0.024	<0.001	-8.02×10^{-5}	0.086
TGF-β1	2.30×10^{-6}	0.666	3.14×10^{-4}	0.808	-1.50×10^{-5}	0.008

Effect of KGF was not considered since it was only measurable in one AS sample; RC: Regression coefficient. $P<0.05$ was considered statistically significant, compared to baseline.

differentiation of bone marrow mesenchymal cells and increased cell proliferation rate, more compared to FBS[27-33]. However, as mentioned above, only a few studies tried to compare differences between AS and FBS in HCEC culture, and until now, no significant differences between both has been determined. In addition, the use of animal-derived material is not allowed for humans, or human studies, in Germany.

In our present study, HCEC viability and migration was better using AS than FBS. However, concerning proliferation, no difference could be shown between both groups. HCEC viability and migration were the highest at 30% AS, but this group increased HCEC proliferation the least. 15% AS concentration led to lower HCEC viability and migration than 30% AS, but 15% AS resulted in the best proliferation of the HCECs.

Based on our results, we suggest the clinical use of 30% AS, since the most important *in vivo* corneal epithelial functions are migration and viability. In our opinion, this concentration could be reached through dilution of AS in the remaining tear film of the patients. To the best of our knowledge, up to now there is no study analyzing the impact of tear volume on efficacy of AS therapy. Intrestingly, AS improves ocular surface disease index (OSDI), but does not have an impact on tear osmolarity[34]. Further *in vivo* studies should analyse the impact of tears on AS efficacy on corneal wound healing.

The high variability of growth factor concentrations in AS of different patients, should also be taken into account. It is already known that FGFb is a beneficial factor in corneal epithelial cell growth or corneal wound healing[35-36], and it is also required for corneal epithelial cell proliferation and differentiation during embryonic development[37]. HGF and FGFb are both major factors initiating proliferation and migration in the cornea, while TGF-β in the tear film suppresses the proliferation at the migrating cell front[38].

In the present study we analyzed the effects of FGFb, HGF and TGF-β1 concentrations on HCEC viability, migration and proliferation. FGFb and HGF concentrations had a positive effect on HCEC migration, but FGFb and TGF-β1 concentrations had negative effect on HCEC proliferation. The interactions of growth factors in human serum and their impact on human epithelial cell viability, migration and proliferation should be further analyzed.

In conclusion, HCEC viability is most supported by 30% AS or 15% FBS, migration by 30% AS or 30% FBS, and proliferation by 15% AS or 5% FBS. In addition, AS better supports HCECs viability and migration than FBS. Therefore, 30% may be an appropriate AS concentration in clinical practice. Based on our experiments, we also suggest the use of AS instead of FBS for *in vitro* HCEC cultures, especially for *ex vivo* expansion of limbal stem cells.

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