Original Article

Time and Temperature Stability of TGF-β1, EGF and IGF-1 in 20% and 100% Human Serum

(autologous serum / autologous serum eye drops / storage / growth factors / dry eye disease)

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Abstract. Autologous serum eye drops (ASEDs) are used as a treatment for severe dry eye disease. The concentration and stability of various growth factors in ASEDs is determinative for their efficiency. We therefore assessed the concentrations of transforming growth factor beta 1 (TGF-β1), epidermal growth factor (EGF) and insulin-like growth factor 1 (IGF-1) in ASEDs following storage at 4–8, −20, −80 and −156 °C. Twenty % and 100% sera from eight healthy volunteers were analysed by the sandwich enzyme immunoassay at different time intervals up to seven months. The mean levels of TGF-β1 and EGF in undiluted and 20% serum did not differ significantly from the baseline levels in fresh serum for any storage conditions after 7 days at 4–8 °C, as well as after 4- and 7-month preservation at sub-zero temperatures. In 20% serum, no IGF-1 concentration decrease was found following 7 days of preservation at 4–8 °C. However, a decrease to 78 % and 81 % (P < 0.01) of baseline values was found in 20% serum after 4-month storage at −20 °C and 7-month storage at −156 °C, respectively. A more pronounced decrease in IGF-1 was observed in undiluted serum. All assessed growth factors present in 20% frozen serum remained stable for up to 7 months. The highest stability was achieved at −80 °C. At −20 and −156 °C, some decrease in IGF-1 occurred. Our results indicate that 20% ASEDs can be stored frozen up to 7 months under proper conditions.

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Abbreviations: ASEDs – autologous serum eye drops, CV – coefficient of variation, DED – dry eye disease, EGF – epidermal growth factor, GFs – growth factors, IGF-1 – insulin-like growth factor 1, NGF – nerve growth factor, TGF-β1 – transforming growth factor beta 1.

Introduction

The presence of growth factors (GFs) in tears is a prerequisite for maintaining the normal function of the ocular surface. Loss of homeostasis of the tear film is a characteristic feature of the dry eye disease (DED) (Murube and Rivas, 2003; Jirsova et al., 2006).

A natural way to supplement GFs in DED is to use autologous serum eye drops (ASEDs) (Tsubota et al., 1999a). The complex activity of growth factors, vitamins, anti-inflammatory and anti-oxidative compounds in ASEDs accelerate wound healing and promote homeostasis on the ocular surface. As a non-immunogenic treatment, ASEDs naturally dilute hyperosmolar conditions typical of DED. They moisten the ocular surface without causing an allergic reaction. In contrast to many

artificial tear products, ASEDs do not impair the integrity of cell membranes; thus, their use is particularly gentle (Tsubota et al., 1999a; Geerling et al., 2004; Jirsova et al., 2014).

The main GFs present both in tears and in the serum are transforming growth factor beta 1 and 2 (TGF-β1 and -β2), epidermal growth factor (EGF), nerve growth factor (NGF), insulin-like growth factor 1 (IGF-1), fibroblast growth factor, keratinocyte growth factor, hepatocyte growth factor, vascular endothelial growth factor, platelet-derived growth factor, brain-derived neurotrophic factor and neurotrophin 3 (Tsubota et al., 1999a; Bradley et al., 2008).

TGF-β1 is accepted as one of the most important growth factors participating in ocular surface homeostasis. A decrease in TGF-β levels in tears is associated with autoimmune diseases, and its elevated concentration accompanies several inflammatory proliferative and degenerative ocular pathologies, such as pterygium, or vernal and atopic keratoconjunctivitis (Benito et al., 2013). It has been shown that the concentration of TGF-β in the serum is approximately five times higher compared to tears (Tsubota et al., 1999a). Due to the concerns about the potential anti-proliferative effect and suppression of wound healing of the ocular surface epithelium when using concentrated serum, Tsubota et al. (1999a) diluted serum 1 : 5 to equilibrate the concentration of TGF-β in the tears. Since then, 20% ASEDs have been used in most clinical studies (Kojima et al., 2005; Na and Kim, 2012; Jirsova et al., 2014).

Serum dilution also brings the level of EGF closer to its native levels in tears. EGF enhances migration and proliferation of epithelial cells and inhibits their terminal differentiation and apoptosis (Grant et al., 1992; Klenkler et al., 2007). Besides these two growth factors, fibronectin also plays an essential role in epithelium wound healing, promoting migration and attachment of cells to the defect (Nishida et al., 1983). TGF-β and EGF are considered the two main GFs attributed to the qualitative parameters of ASEDs (Geerling et al., 2004; Bradley et al., 2009), and therefore their concentration and stability are frequently monitored (Tsubota et al., 1999a, b; Bradley et al., 2009; Phasukkijwatana et al., 2011; Lopez-Garcia et al., 2014, 2016). IGF-1, NGF and other neurotrophic factors also influence epithelial proliferation, migration and differentiation (Lambiase et al., 2009; Wirostko et al., 2015).

The efficacy (healing effect) throughout the period in which ASEDs are applied can be maintained only if the stability of growth factors is preserved (Geerling et al., 2004; Fischer et al., 2012). The total storage period of ASEDs is typically 3 to 4 months (one month for microbiology examination and three months during which the patients use the serum) (Jirsova et al., 2014). However, an even more extended storage period was documented without ASED quality disruption (Geerling et al., 2004; Fischer et al., 2012).

Most of the studies show that patients are instructed to store unopened storage containers with ASEDs in the freezer at −20 °C (for 3 months at maximum), and the vial currently in use in the refrigerator at 4–8 °C for 7 days at maximum (Geerling et al., 2004; Yoon et al., 2007). If the ASEDs are planned for the treatment of neurotrophic keratitis and other pathological processes in which the content of neurotrophic factors plays an important role, it is necessary to ensure the stability of these factors during the entire treatment period (Bradley et al., 2009).

Although the effect of storage conditions on the stability of various GFs in ASEDs has been studied frequently, data from all storage periods and temperatures used in practice are not complete. In particular, the results concerning the stability of TGF-β after 6 months are contradictory; whereas Lopez-Garcia et al. (2016) did not detect any TGF-β level decrease, Phasukkijwatana et al. (2011) found a significant decline.

Our study aimed to assess the stability of both epitheliotropic and neurotrophic GFs within an 8-day storage period in a refrigerator and after 4- and 7-month storage in a freezer (−20 and −80 °C, respectively). Next, we stored ASEDs in liquid nitrogen vapours (−156 °C), because we presumed that this temperature would allow long-term serum storage without significant changes in GF concentrations.

Material and Methods

Specimens

Eight adult controls (4 males, 4 females) with a mean age of 54 (28–75) years were eligible for enrolment in the study, which followed the tenets outlined in the Helsinki Declaration and was approved by the institutional ethics committee. Informed consent was obtained from all participants. Serum was prepared from 40 ml of venous blood after 1 h of clotting at room temperature.

After centrifugation (3000 × *g* for 15 minutes), the supernatant (serum) was removed under sterile conditions in a biohazard hood. Part of the serum was kept in a refrigerator and part was diluted with sodium chloride, which was 0.9–20% (w/v). The aliquots (0.3–0.5 ml) of concentrated and 20% serum were placed in cryogenic tubes (Nunc, Thermo Fisher Scientific, Waltham, MA) for consequent use or storage. Aliquots of undiluted and 20% fresh serum were immediately analysed for baseline levels by ELISA (see below).

Serum aliquots were stored at 4–8 °C for up to 8 days to determine the concentration of GFs during a period in which patients typically store ASEDs in a refrigerator (approximately one week) after bottle opening. Other aliquots were stored in a freezer at −20 and −80 °C and in vapours of liquid nitrogen at −156 °C for up to 7 months to store specimens for a period longer than ASEDs are standardly preserved (4 weeks from preparation to delivery to patients with space for microbial control, then 3-month application) (Jirsova et al., 2014).

Measurement of growth factor concentrations

The levels of GFs were measured after storage at 4–8 °C (nominal refrigerator temperature) for 24 hours, 72 hours, 7 and 8 days, and at sub-zero temperatures −20 °C, −80 °C and liquid nitrogen (LN) vapours (−156 °C) for 4 and 7 months. The sandwich enzyme immunoassay technique (Quantikine® ELISA, R&D Systems) measured the levels of human TGF-β1, EGF and IGF-1. All assays proceeded according to the manufacturer's instructions; each GF was measured by an experienced laboratory technician. All measurements of 100% and 20% sera were performed in doublets.

The assay sensitivities determined by the manufacturer were 0.0046 ng/ml for TGF-β1, 0.026 ng/ml for IGF-1 and 0.0007 ng/ml for EGF. We used assays from the same product batch for our experiments, and all measurements were performed under the same conditions to minimize the variations among the individual determinations. Aliquots were stored at −80 °C and −156 °C, respectively, and freshly thawed before each analysis. The determined concentrations were used to calculate the inter-assay variability for each parameter.

Statistical analysis

The results are expressed as mean ± standard deviation. The inter-assay variability for each tested parameter was calculated using a coefficient of variation (CV, %). The GF levels of aliquots stored for the studied storage conditions were compared to the baseline levels determined from the aliquots of the fresh serum 3 hours after preparation. A Wilcoxon signed ranked test was used to determine the statistical significance of the differences. A P value < 0.05 was considered statistically significant. Data were analysed using GraphPad Instat and RStudio software (RStudio Team, 2020).

Results

A summary of the mean concentrations of TGF-β1, EGF and IGF-1 in 20% and 100% serum under all tested conditions is given in Table 1. No statistically significant differences for TGF-β1 in diluted serum were observed compared to the baseline for all used periods and storage conditions. In concentrated serum, the only statistically significant change was the decrease to 76 % observed at 4–8 °C after 8-day storage.

EGF showed higher resistance to storage, because its concentration did not decrease in any of the tested conditions. On the contrary, a slight but not statistically significant rise in concentration was observed (101–123 % for 20% serum, 101–110 for 100% serum). The inter-assay variability of TGF-β1 and EGF methods (CV = 19 % for both assays) was higher than the average variability of samples in all tested times and conditions (CV = 11 % and 4 %, respectively).

Relative to the baseline, the levels of IGF-1 during 8-day storage in a refrigerator reached 100–126 % (100% serum) and 103–133 % (20% serum). The increases in concentration relative to the baseline after 7 (100% serum) and 8 (20% and 100% serum, respectively) days were statistically significant. In the case of IGF-1, the CV values among all the determinations were 16.3 % and 19 % for the tested samples, indicating that the differences in values were not caused by the storage conditions, but originated from the method's variability.

Discussion

The stability of GFs in their active form is a major prerequisite for the healing effect of ASEDs in the treatment of DED and other ocular surface-related diseases (Geerling et al., 2004; Matsumoto et al., 2004; Bradley et al., 2009).

Table 1. Stability of growth factors in 20% and 100% serum in several intervals and temperatures

Storage		TGF-β1 (ng/ml)		EGF (ng/ml)		IGF-1 (ng/ml)	
Time	**Temper.**	**20% serum** (% of baseline)	**100% serum** (% of baseline)	**20% serum** (% of baseline)	**100% serum** (% of baseline)	**20% serum** (% of baseline)	**100% serum** (% of baseline)
3 h	4–8 °C	9.90 ± 2.69 (100)	51.89 ± 12.59 (100)	0.081 ± 0.035 (100)	0.36 ± 0.17 (100)	24.55 ± 8.24 (100)	73.86 ± 21.89 (100)
24 h		8.72 ± 2.33 (88)	39.58 ± 9.15 (76)	0.091 ± 0.036 (112)	0.38 ± 0.19 (106)	25.17 ± 8.23 (103)	73.64 ± 21.56 (100)
72 h		9.19 ± 2.21 (93)	40.65 ± 8.98 (78)	0.086 ± 0.040 (106)	0.38 ± 0.19 (107)	26.55 ± 8.73 (108)	76.29 ± 20.04 (103)
7 days		9.59 ± 2.43 (97)	44.83 ± 12.14 (86)	0.085 ± 0.039 (106)	0.36 ± 0.17 (101)	28.77 ± 9.84 (117)	89.91 ± 23.56* (122)
8 days		8.93 ± 2.10 (90)	39.31 ± 9.40* (76)	0.082 ± 0.034 (101)	0.38 ± 0.19 (106)	32.76 ± 10.92* (133)	93.38 ± 20.50* (126)
4 months	–20 °C	11.25 ± 2.55 (114)	48.99 ± 13.59 (94)	0.089 ± 0.042 (111)	0.38 ± 0.18 (106)	19.12 ± 6.43* (78)	63.54 ± 20.06 (86)
	–80 °C	10.82 ± 2.46 (109)	48.85 ± 12.66 (94)	0.088 ± 0.040 (109)	0.38 ± 0.18 (107)	20.43 ± 7.21 (83)	61.40 ± 22.25 (83)
	–156 °C	10.46 ± 2.31 (106)	48.86 ± 12.96 (94)	0.099 ± 0.047 (123)	0.39 ± 0.19 (110)	20.97 ± 7.48 (85)	59.18 ± 20.15* (80)
7 months	–20 °C	10.58 ± 2.50 (107)	40.87 ± 11.36 (79)	0.092 ± 0.039 (114)	0.39 ± 0.19 (110)	21.54 ± 6.80 (88)	52.90 ± 18.97** (72)
	–80 °C	10.06 ± 2.35 (102)	43.34 ± 11.27 (84)	0.090 ± 0.040 (112)	0.39 ± 0.19 (100)	21.31 ± 6.61 (87)	51.14 ± 16.40* (69)
	–156 °C	10.19 ± 2.59 (103)	41.53 ± 11.06 (80)	0.087 ± 0.034 (108)	0.39 ± 0.20 (100)	19.94 ± 6.70* (81)	56.12 ± 18.29* (76)

Results are expressed as mean concentration ± standard deviation. Concentrations measured 3 hours after preparation are presented as a baseline (100 %), *P < 0.05, **P < 0.01.

Our study demonstrates stable concentrations of TGF-β1, EGF and IGF-1 in 20% human serum up to one week of storage in the refrigerator (4–8 °C). The decreased TGF-β1 and IGF-1 levels after 8-day storage support the previously stated recommendation to store ASEDs in the refrigerator for a maximum of 7 days (Geerling et al., 2004; Jirsova et al., 2014). Although the stability of EGF and TGF-β at 4 °C for up to 7 days has been documented in some studies (Tsubota et al., 1999a; Phasukkijwatana et al., 2011; Lopez-Garcia et al., 2014), and one month in others (Tsubota et al., 1999a; Phasukkijwatana et al., 2011), it should be taken into consideration that the average temperature of domestic refrigerators in everyday use may normally be higher than this value. Up to now, only the study of Fischer et al. (2012) evaluated the stability of EGF, fibronectin, vitamins A and E and albumin in 20, 50 and 100% serum after 7 days at 6 °C, a temperature more typical in a patient's refrigerator.

Concerning the neurotrophic factors, no significant decrease in IGF-1 was documented after 24 h at 4 °C and after 6 weeks at −15 °C (Bradley et al., 2009). This study also confirmed that the neurotrophic factors, represented here by IGF-1, are less stable compared to TGF-β1 or EGF (Bradley et al., 2009), but to our best knowledge, no comprehensive analysis for more extended storage periods has been published to date.

The TGF-β and EGF levels in 20% serum were shown to be stable at −20 °C for 3 months (Tsubota et al., 1999a; Fischer et al., 2012; Lopez-Garcia et al., 2014), but another study found a significant decrease in TGB-β1 and TGB-β2 from 3- and 1-month storage, respectively (Phasukkijwatana et al., 2011). Our results agree with the first three studies mentioned above because TGF-β1 and EGF in frozen 20% serum were stable not only after 4 months, but also after 7 months of storage in all experimental conditions, i.e., at −20, −80, and −156 °C. Regarding IGF-1, our results indicate that its stability at sub-zero temperatures is inferior to TGF-β1 and EGF, but still high in diluted autologous serum when stored at −80 °C.

The demonstrated stability TGF-β1 and EGF at −80 °C and −156 °C suggests that it is possible to store the autologous serum for periods of up to 7 months under these conditions without significant degradation. This long-term storage may, thus, allow patients to complete the ASED therapy when the application of ASEDs has been interrupted for some reason (e.g., hospitalization). Longer preservation can also be profitable if larger ASED volumes are prepared and stored institutionally and repeatedly distributed to patients (Geerling et al., 2004). Finally, long-term storage can also be used to preserve allogeneic serum eye drops. The allogeneic products prepared from the blood of healthy donors (Espinosa et al., 2015; Giannaccare et al., 2017) offer a certain degree of standardization, because the variations in the tear composition (GF composition and concentrations) may influence the results obtained after ASED application for patients suffering from different clinical entities (Jirsova et al., 2020; Ripa et al., 2020).

To prepare ASEDs, we applied saline, the most commonly used solvent (Tsubota et al., 1999a; Lopez-Garcia et al., 2014). Other solvents, such as sodium hyaluronate (Lopez-Garcia et al., 2014, 2016) or balanced salt solution (Bradley et al., 2009; Phasukkijwatana et al., 2011), have also been used with no apparent impact on the GF stability (Tsubota et al., 1999a; Bradley et al., 2009; Phasukkijwatana et al., 2011; Lopez-Garcia et al., 2014).

We demonstrated that no significant difference was detectable in the concentrations of TGF-β1, EGF and IGF in ASEDs when stored in a refrigerator for up to 7 days. All studied GFs were stable for up to 7 months of storage in 20% serum at −80 °C. Thus, ASEDs appear to be able to be successfully stored in a frozen state for up to 7 months at −80 °C and for one week in a refrigerator.

Conflict of interest

The authors state that there are no conflicts of interest regarding the publication of this article.

Ethics approval

Informed consent was obtained from all participants.

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