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ORIGINAL ARTICLE

Stability of Tenogenic Secretomes Optical Density, Colour, Weight, TGF-β Concentration and Bacterial Growth After 30-Day Storage Under Room or Refrigerator Temperature

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Abstract

Background/Aim: The effectiveness and efficiency of tenogenic secretomes products are crucial to assess. Storage conditions affect both the distribution process and the stability of the tenogenic secretomes and their components. This study aimed to examine how different storage temperatures impact the stability of tenogenic secretomes chemically, physically and biologically.

Methods: This *in vitro* experimental, comparative study was conducted in a laboratory setting. The effect of room temperature (R) at 20-25 °C was compared with a refrigerator (K) at 4 °C on tenogenic secretome stability over 30 days. The sample size was 24 liquid tenogenic secretomes determined using the Federer Formula (1963). The samples were divided into four groups: (R1, K1) for day-1 evaluation and (R30, K30) for day-30 evaluation. The parameters used in this study were physical (optical density (OD); weight differences (Δ weight) and colour changes); chemical (transforming growth factor beta (TGF-β) concentration); and biological (bacterial growth) stability. The main outcome measures were TGF-β concentration, colour, weight differences, optical density changes and bacterial growth.

Results: There were no significant differences between each parameter evaluation in the R1-K1 group ((OD, p-value = 0.729); (TGF-β, p-value = 0.656); (Δ weight, p-value = 0.262)) or R30-K30 group ((OD, p-value = 0.505); (TGF-β p-value = 0.182); (Δ weight, p-value = 0.214)). There was no significant correlation between the storage temperature and bacterial growth in either the R1-K1 group (p-value = 0.439) or R30-K30 group (p-value = 0.439). The R30 group samples, however, displayed more noticeable colour changes compared to the initial colour and the K30 group.

Conclusion: The OD, TGF-β, weight, colour and bacterial growth were not significantly affected at either fridge or room temperature. The components of the tenogenic secretome remained stable after 30 days of storage without experiencing substantial deterioration despite the temperature condition.

Key words: Secretome; Stability; Transforming growth factor beta; Physical phenomena; Bacterial growth; Temperature; Storage.

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Introduction

Tendon injury still remains a challenging case for orthopaedic surgeons in the world due to decreased of function results on the patients, whether after conservative or operative therapy. Tissue damage requires at least one year to reach the final remodelling stage with reduced tendon function due to adhesions due to the formation of fibrotic tissue. After reconstruction surgery, the injured tendon must delay its movement to avoid re-tearing, but on the other hand resulting joint stiffness and muscle atrophy due to prolong immobilisation. Poor compliance on the patient's post-operative rehabilitation and physiotherapy programs can also worsen outcomes.[1, 2]

The use of adipose-derived mesenchymal stem cell secretomes (ADMSCs) products, known as tenogenic secretomes, has been increasingly used as free-cell therapy in tendon injuries in recent decades. Apart from being safer and more economical, the growth factor content in a tenogenic secretomes is able to support the formation of tendon progenitor cells, increase and accelerate healing regeneration thereby supporting better recovery in cases of injury.[3]

However, like proteins in general, the structure and components contained in a secretomes product will be affected by temperature, humidity and the surrounding environment. The stability of a tenogenic secretomes both physically, chemically and microbiologically must be tested and proven, ensured that it is efficient for distribution and storage and has good product efficacy before being mass produced so that it can be used easily in all health facilities.[4,5]

Based on these factors, the stability of a tenogenic secretomes product physically, chemically and microbiologically needs to be analysed to ensure the efficiency of storage methods and its efficacy as a pharmaceutical product before it is mass-produced and widely used by all healthcare facilities. This study aimed to examine how different storage temperatures impact the stability of tenogenic secretomes chemically, physically and biologically.

Methods

This experimental *in vitro* laboratory research utilised a comparative study design. Study was conducted from February to March 2024 and was done in Tropical Disease Centre Universitas Airlangga, Surabaya. The study population comprised liquid tenogenic secretomes sourced from Tissue Bank and Stem Cells Dr Soetomo Academic General Hospital, Surabaya. It's harvested from ADMSC after went into seeding process with fresh frozen tendon scaffold after 4 passages under 2 % hypoxia condition in the conditioned *in vitro* medium and augmented into approximately 35 mL total.

The sample size, determined using the Federer Formula (1963) which is typically written as $(t-1)\ (n-1) \geq 15$, where "t" represents the number of treatment groups; "n" is the sample size per group; and the number "15" represents a minimum threshold value used to ensure sufficient statistical power in research, therefore based on the formula, 24 samples were decided sufficient for this study.[6]

A vial contained 0.3 mL liquid tenogenic secretomes is a sample for TGF-β and optical density (OD) as a chemical stability analysis, whereas 1 mL vial is a sample used for microbacterial culture, colour and weight as a physical and biological stability analysis. Twenty-four samples prepared and divided into four groups (R1, R30, K1, K30), each group consists of 6 samples. K1 and K30 groups were put into refrigerator (4 °C), while R1 and R30 were put into room temperature (25 °C) in a sterile condition.

All stability analysis performed on day-1 after storage for R1 and K1 groups, while R30 and K30 performed on day-30 after storage.

For physical stability, physical colour changes were clinically documented, weight differences (Δ weight) measurement using analytical weight balances. Initial measurement was also performed prior to storage.

For chemical stability, TGF-β concentration and OD analysed by enzyme-linked immunosorbent assay (ELISA) reading using Bioassay Technology Laboratory (BT Lab) human TGF-β kit, which is capable for accurate quantitative detection of TGF-β in serum, plasma, cell culture supernates (ie secretomes), ascites, tissue homogenates, or

other biological fluids. By adding sample and ELISA reagent into each well, the samples were incubated for 1 hour at 37 °C, then the plate was washed 5 times. Next, substrate solution A and B was added, then incubated 10 minutes at 37 °C. Stop solution was added and the colour develops afterwards. The OD was shown of each well using microplate reader set to 450 nm within 10 minutes after adding the stop solution. Finally, the TGF-β concentration on each sample was analysed.

For biological stability, bacterial growth assessment evaluation was performed using Mueller–Hinton (MH) agar sample culturing and incubation at 37 °C condition.

Statistical analysis was performed by using SPSS 27.0 (IBM Corporation, USA) and included normality tests (Shapiro-Wilk), parametric Independent T-tests, non-parametric Mann-Whitney tests, ANOVA and Fisher's test for non-categorical data.

Results

After 24 hours of storages, R1 and K1 groups was evaluated with results as follows. All the samples colour changed into a slightly darker shade on both R1 and K1 with no significant differences between both groups. OD on the refrigerator group was slightly larger than the room tempera-

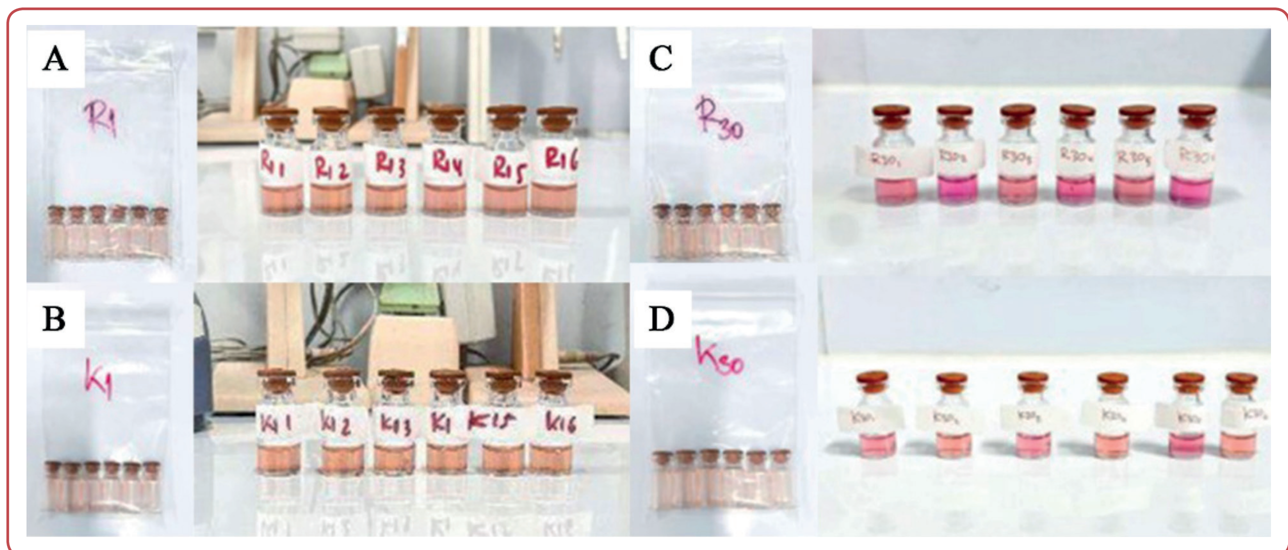

Figure 1: Colour changes of the samples on room temperature or refrigerator after storages on day 1 (A, B) and day 30 (C, D)

Table 1: Analysis of optical density, transforming growth factor beta (TGF-β) concentration and weight differences at room temperature (R) or refrigerator (K) on day 1 and 30

Indicator	Group	N	Mean (SD)	Median	Min value	Max value	p-value
Optical density	R1	6	0.57 (0.11)	0.58	0.39	0.71	0.729*
	K1	6	0.61 (0.14)	0.57	0.49	1.00	
	R30	6	0.69 (0.10)	0.67	0.56	0.86	0.505*
	K30	6	0.60 (0.11)	0.57	0.44	0.84	
TGF-B (ng/L)	R1	6	196.75 (52.73)	195.51	124.32	258.89	0.656*
	K1	6	221.35 (69.09)	193.34	172.65	352.00	
	R30	6	360.75 (87.34)	349.35	273.39	511.13	0.182*
	K30	6	287.50 (89.73)	269.29	187.26	442.49	
Weight differences (g)	R1	6	1.04 (0.50)	1.02	0.99	1.14	0.262*
	K1	6	0.85 (0.38)	0.98	0.08	1.10	
	R30	6	1.02 (0.05)	0.99	0.97	1.09	0.214*
	K30	6	1.04 (0.03)	1.03	1.01	1.11	

**Independent T-test;*

ture (R1 = 0.568 ± 0.112; K1 = 0.608 ± 0.139), as well as the TGF-β concentration (R1 = 196.75 ± 52.73; K1 = 221.35 ± 69.09). The Δ weight on room temperature group were slightly larger than the refrigerator (R1 = 1.036 ± 0.5; K1 = 0.85 ± 0.38). Bacterial growth was found on both groups (R1 1colony on 2 samples; K1 1colony on 1 sample) (Figure 1) (Table 1 and 2).

After 30 days of storages, R30 and K30 groups was evaluated with results as follows. All the samples colour gradually changed from a lucent pink into a darker purplish shade, the R30 samples showed a darker colour compared to the K30 group. OD on the room temperature group was slightly larger than the refrigerator group (R30 = 0.687 ± 0.097; K30 = 0.60 ± 0.116), as well as the TGF-β concentration (R30 = 360.75 ± 87.34; K30 = 287.50 ± 89.73). The Δ weight on the refrigerator group were slightly larger than the room temperature group (R30 = 1.02 ± 0.048; K30 = 1.043 ± 0.034). Bacterial growth was found on both groups (R30 1 colony on 1 sample; K30 3 colony on 1 sample) (Figure 1) (Table 1 and 2).

Table 2: Analysis of bacterial colony growth at room temperature (R) or refrigerator (K) on day 1 and 30

Group	Colony (n)						p-value
	1	2	3	4	5	6	
R1	0	0	1	0	1	0	0.439*
K1	0	0	0	0	0	1	
R30	0	0	1	0	0	0	0.439*
K30	0	3	0	0	0	-	

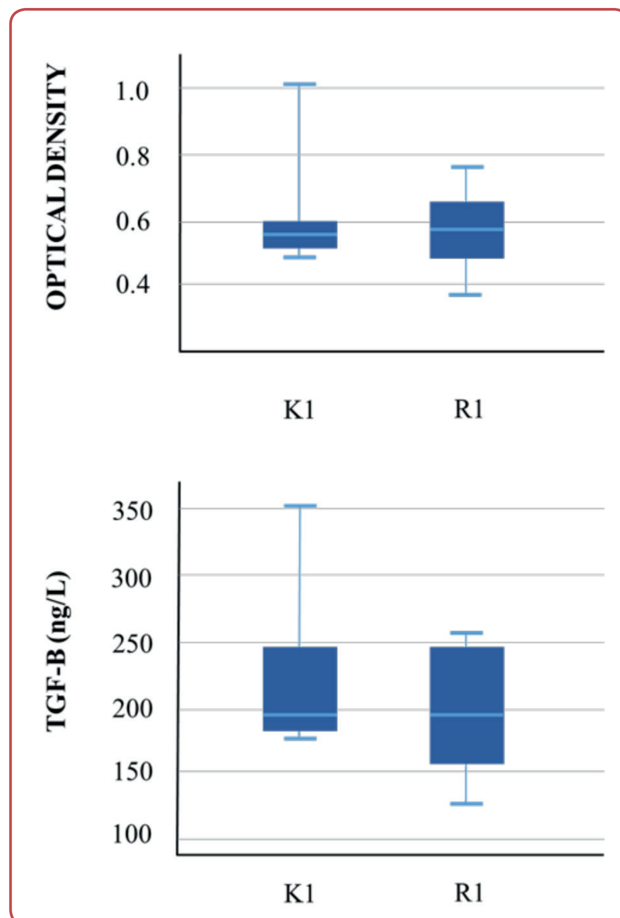

Figure 2: Boxplot of optical density and transforming growth factor beta (TGF-β) concentration at room temperature (R1) or refrigerator (K1) on day 1

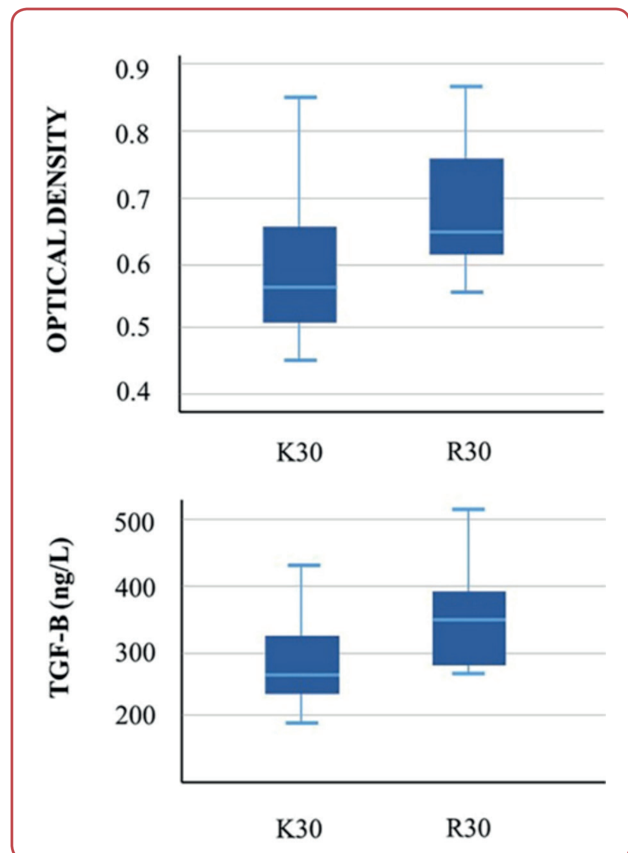

Figure 3: Boxplot of optical density and transforming growth factor beta (TGF-β) concentration at room temperature (R30) or refrigerator (K30) on day 30

There were no significant differences between each parameter evaluation on R1-K1 group ((OD, p-value = 0.729); (TGF-β, p-value = 0.656); (Δ weight, p-value = 0.262)) or R30-K30 group ((OD, p-value = 0.505); (TGF-β p-value = 0.182); (Δ weight, p-value = 0.214)). There were no significant corelation between the storage temperature and bacterial growth on either R1-K1 group (p-value = 0.439) or R30-K30 group (p-value = 0.439). The R30 group samples, however, displayed more noticeable colour changes compared to the initial colour and the K30 group (Figure 2 and 3) (Table 1 and 2).

Discussions

The application of secretomes in cell-free therapy offers distinct advantages compared to conventional cell-based approaches. In stem cell-based therapy, only a small fraction (< 1 %) of transplanted MSCs survive after one week, leading to limited therapeutic efficacy. The secretomes, on the other hand, can overcome this issue as its therapeutic effects do not rely on cell survival. Additionally, the secretomes has lower immunogenicity compared to living and proliferating cells. This reduced immunogenicity is due to the lower protein expression secretion of the secretomes, making it less likely to trigger an immune response. Moreover, the secretomes offers a practical and cost-effective approach for clinical applications. Despite its great potential, the clinical efficiency and efficacy of tenogenic secretome products need to be demonstrated before clinical trials, particularly regarding their stability in different environments, which will affect the distribution process and the product's potential when applied directly to clinical cases of tendon injuries.[7]

In this research, the physical stability of the tenogenic secretomes showed significantly different sample colour changes compared to the initial colour on both groups after storages on day 30, which was more visible on the room temperature group. The OD and Δ weight, however, showed no significant differences between both groups, as well as the chemical stability evaluation from the TGF-β concentration. The findings on this study showed although the temperature slightly affected the secretomes physically, the stable temperatures on both refrigerator and room condition were not enough for causing protein denaturation, therefore the GF concentration could be maintained, especially in short-term storage period.[8] As for the of biological stability of both storage group on day 1 and 30, the bacterial growth might be caused by contamination on the packaging process or on the mobilisation process of the samples. Humidity, light exposure and other environmental elements of the storage area which not be included as a variable on this study may also affecting the sterility, hence the insignificance results of corelation between the temperature and the bacterial growth on either day 1 nor day 30.

The validity of ELISA reading to determine the concentration of growth factor in a respective sample such as tenogenic secretomes has been proven in some of the previous study. In 2017, Kalinia et al used Quantikine enzyme-linked ELISA reading to analyse the accumulation of granulocyte-colony stimulating factor (G-CSF) on ADMSCs with results of concentration of G-CSF in secretomes of normoxic ADSC was 13.4 ± 8.5 fmol, while in hypoxia condition, G-CSF mRNA and protein were up-regulated 1.7 ± 0.3 and 1.9 ± 0.5-fold, respectively.[9] In 2021, Putra, RA used ELISA reading on the on the ADMSCs sample, performed on day-2, 4 and 6 after the secretomes harvested to evaluate the concentration of Insulin-like Growth factor 1 (IGF-1) and transcripting factor scleraxis (Scx), while comparing the concentration on the sample harvested with and without tendon scaffold seeding process on normoxic and 2 % hypoxic condition. The results shows both secretion of Scx and IGF-1 in four groups were different significantly ($p \leq 0.001$).[10] In 2024, Kresnadi compared the interleukin 6 (IL-6) and interleukin 10 (IL-10) concentration analysis from ADMSCs with crude secretom using ELISA reading with results of the average IL-10 level in the tenogenic secretome group (309.2 ± 50.973 pg/mL) was higher compared to the crude secretome group (200.1 ± 32.030 pg/mL). On the other side, the IL-6 level in the tenogenic group (10.2 ± 2.432 pg/mL) was lower than the crude group (18.3 ± 3.175 pg/mL).[11] ELISA reading such used in this study as well is valid for analysing the growth factor concentration.

In the study by Leemasawatdigul et al the stability of the chemotactic cytokine MCP-1 concentration was analysed at different storage temperatures. It was found that there were no significant changes in MCP-1 concentration after 7 days of storage at 37 °C, 4 °C, -20 °C, or -81 °C, but it started to lose its concentration by about 50 % after undergoing freeze-thaw cycles.[8] Rogulska et al also analysed the stability of several GFs in the secretomes. Their study showed that the duration of storage directly affects the stability of the secretomes, depending on the associated GFs. Of all the GFs tested; brain derived neurotrophic factor (BDNF), hepatocytes growth factor (HGF), vascular endothelial growth factor (VEGF), interleukin-6 (IL-6), monocyte chemotactic protein-1 (MCP-1), 60 % of the GF concentrations remained stable during short-term storage (3 months). Long-term storage (3-30 months) was proven to be more stable after lyophilisation at -80 °C, with a risk of protein denaturation after more than one thawing process.[7] All the previous study and

this research has been proving that as long as the temperature is not extremely changes, the protein inside the secretomes would remain stable.

One disadvantage of this work is that the evaluation period was extremely brief, lasting only 30 days. This may not provide a complete understanding of the long-term stability of the tenogenic secretome. The frequency of evaluations was insufficient, indicating a necessity for more regular assessments to acquire more precise data on growth factor concentrations. The sample size was relatively small, consisting of just 24 samples separated into four groups, which could potentially restrict the strength and reliability of the findings. In addition, the study solely examined two temperature situations, namely room temperature and refrigerator temperature, without investigating other environmental elements such as humidity or other temperature ranges that could potentially affect stability.

Conclusion

The study found that the tenogenic secretome components, such as optical density, TGF-β concentration, weight and bacterial growth, did not change after 30 days of storage at both ambient temperature and in the refrigerator. While the samples held at room temperature exhibited visible colour changes, the other components did not display any notable variations. The study indicates that the tenogenic secretome can be stored efficiently at both room temperature and refrigerator temperature for a maximum of 30 days without experiencing substantial deterioration. It is recommended to conduct more evaluations with more frequent assessments and longer storage periods in order to gain more precise data on the ideal storage conditions and durations for tenogenic secretome products.

Ethics

This is an *in vitro* study with an approved ethical clearance number 0845/KEPK/XII/2023 approved by the Health Research Ethics Committee, Dr Soetomo General Academic Hospital, Surabaya, Indonesia, dated December 2023.

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Conflicts of interest

The authors declare that there is no conflict of interest.

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Data access

The data that support the findings of this study are available from the corresponding author upon reasonable individual request.

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