FABAD J. Pharm. Sci., 46, 3, 271-278, 2021

RESEARCH ARTICLE

Serum Type Hyaluronic Acid Formulations: *In vitro* Characterization and Patch Test Study

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SUMMARY

Hyaluronic acid is a natural polymer that provides moisture to the skin and supports the skin's elasticity by helping to keep it supple. Hyaluronic acid-containing serum, semi-solid and injectable formulations are available commercially. In this study, serum type formulations containing hyaluronic acid were prepared. The final formulation containing 1% hyaluronic acid was selected from the prepared formulations and stability tests, protective efficacy tests (ISO 11930), and in vivo allergic irritation tests of this formulation were performed. The pH of the 1% hyaluronic acid formulation was adjusted to 5.5. Microbial analysis using Pseudomonas aeruginosa, Staphylococcus aureus, Escherichia coli, and Candida albicans strains showed that the final formulation does not pose a contamination risk. In addition, it has been proven in the protective efficacy test of the final formulation that it has an antimicrobial effect of up to 28 days. According to the patch-shaped irritation test results in 15 subjects between the ages of 22-70, no allergic reaction was observed in the subjects for one week. No change was observed in the physicochemical properties of the final formulation at 25°C 65% relative humidity. In conclusion, the hyaluronic acid serum formulation has been evaluated as a product that can be used safely for moisturizing the skin.

Key Words: *Hyaluronic acid, skin moisturizing, serum type formulation, in vivo allergy test, cosmetic product*

Hyaluronik Asit Serum Formülasyonları: In vitro Karakterizasyon ve Yama Testi Çalışması

ÖZ

Hyaluronik asit, cildin nemli ve esnek kalmasına yardımcı olarak cildin elastikiyetini destekleyen doğal bir polimerdir. Hyaluronik asit içeren serum, yarı katı ve enjektabl formülasyonlar ticari olarak bulunmaktadır. Bu çalışmada, hyaluronik asit içeren serum tipi formülasyonlar hazırlanmıştır. Hazırlanan formülasyonlardan %1 hyaluronik asit içeren formülasyon sonuç formülasyon olarak seçilerek, stabilite testeri, korucuyu etkinlik testleri (ISO 11930) ve in vivo alerjik irritasyon testleri yapılmıştır. Sonuç formüsyonun pH'sı 5,5 olarak ayarlanmıştır. Pseudomonas aeruginosa, Staphylococcus aureus, Escherichia coli ve Candida albicans suşlarının kullanıldığı mikrobiyal analiz, sonuç formülasyonun kontaminasyon riski oluşturmadığını göstermiştir. Ayrıca sonuç formülasyonun koruyucu etkinlik testinde 28 güne kadar antimikrobiyal etkiye sahip olduğunu kanıtlamıştır. 22-70 yaş arası 15 kişide yama şeklindeki alerjik irritasyon testi sonuçlarına göre, deneklerde 1 hafta süreyle herhangi bir alerjik reaksiyon görülmemiştir. 25°C %65 bağıl nemde sonuç formülasyonun fizikokimyasal özelliklerinde bir değişiklik olmamıştır. Sonuç olarak hyaluronik asit serum formülasyonu cildi nemlendirmede güvenle kullanılabilecek bir ürün olarak değerlendirilmiştir.

Anahtar Kelimeler: *Hyaluronik asit, cilt nemlendirici, serum tipi formülasyon, in vivo alerji testi, kozmetik ürün*

Received: 23.03.2021
Revised: 01.05.2021
Accepted: 18.05.2021

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INTRODUCTION

Loss of epidermal polarity by chronic sun exposure, keratinocyte atypia, and reduction of collagen are the main reasons for skin aging (Lee, 2015). Crow's feet wrinkles are associated with skin aging. One of the reasons for the appearance of them is the reduction in the epidermal content of hyaluronic acid (Y. J. Lee, 2019). Hyaluronic acid (HA) is a natural polysaccharide that is a primary component of the extracellular matrix of human tissues and the endogenous component of human skin. It has a vital water-binding capacity (6 L water in 1 g) (Jegasothy, 2014). It is used the overcome skin wrinkles, provide skin elasticity and moisture (Choi, 2017). Because of the biocompatibility, biodegradability, non-immunogenicity, and viscoelasticity properties of HA, it is ideal to use in cosmetic, medical, or pharmaceutical purposes, especially to fabricate injectable fillers (Brown and Jones 2005; J. S. Lee, 2019). HA is commercially produced by synovial fluid, umbilical cord, or skin of the animals, from rooster comb, or bacteria by fermentation or isolation (Brown and Jones, 2005).

There are various forms of HA such as hydrogels, scaffolds, creams, films, foams, gels, serum, lotion, implants, etc. (Bukhari, 2018). The general approach to delivering HA through the skin is the injection of HA filler; however, this method is invasive, painful, and usually have side effects (J. S. Lee, 2019). Therefore, it may be the more promising approach to deliver HA into the skin with serum type formulation to provide skin moisture. However, because the HA has a hydrophilic characteristic, the lipophilic stratum corneum roles as a natural barrier against HA. Nevertheless, HA in serum or cream formulations plays a transitory water holding and smoothing effect on the skin (Wu, 2020).

HA formulations can be classified as low molecular weight (500–1000 kDa), medium molecular weight (1200–4500 kDa), and high molecular weight (6000-7000 kDa) (Iturriaga, 2021). The water absorption capacity and skin moisturizing properties of HA depend on the molecular weight of HA. The higher molecular weight provides a more robust water absorption capacity and also has more resistance for degradation by hyaluronidase (Jang, 2020). However, HA with low molecular weight (20-300 kDa) passes more effectively through the skin in comparison with high molecular weight HA (1000-1400 kDa) (Essendoubi, 2016).

The primary purpose of this study is to develop low molecular weight (400 kDa) HA serum for topical application. Viscosity, surface tension, and pH measurements were evaluated as in vitro characterization studies. Physical and microbiological stability tests and protective efficacy tests were carried out. In vivo allergic irritation test was performed with 15 subjects regarding patch-shaped irritation test.

According to the Cosmetics Regulation, the final product was declared by the Republic of Turkey Ministry of Health has been commercially launched by Fiber Farma Drug and Cosmetics Co. with the brand of ResCare® Hyaluronic Acid Serum since 2017.

MATERIALS AND METHODS

Materials

HA (Mw. 400 kDa) was purchased from Kadıoğlu Medical Ltd. (Ankara, Turkey). 2-Phenoxyethanol and lactic acid were purchased from Tekkim (Istanbul, Turkey). All other chemicals were of analytical grade.

Preparation of serum formulations

HA has a gelling property related to its molecular weight. Therefore, three different concentrations (0.5, 1, and 2 %) of HA were selected for preparing serum formulations. HA was added to purified water and mixed until completely dissolved at room temperature. Then, 1% of 2-phenoxyethanol was added and mixed. Finally, the pH of the solution was adjusted to 5.5 with lactic acid.

Viscosity and surface tension measurements

The viscosity of serum formulations was measured with a cone-plate rheometer (Brookfield Rheometer DV-III) using a CP-52 spindle at room temperature. Shear rate (1/s) -viscosity (cP.s) curves were evaluated in terms of viscosity measurements.

Surface tension is a critical value for cosmetic products. Therefore, surface tension values of formulations were analyzed using an optical tensiometer (ThetaLite Optical Tensiometer). The pendant drop method was used with 10 µL of sample solution (Özden and Avcı, 2017).

Protective efficacy tests (Challenge tests)

The protective effectiveness of 2-phenoxyethanol was evaluated with standard challenge tests (ISO 11930). For this purpose, five different microorganisms (*Pseudomonas aeruginosa* (ATCC 9027), *Escherichia coli* (ATCC 8739), *Staphylococcus aureus* (ATCC 6538), *Candida albicans* (ATCC 10231), *Aspergillus niger* (ATCC 16404)) were used. The final serum formulation was held at room temperature and evaluated on the 7th, 14th, and 28th days after inoculation. At each time point, colonies were counted, and the log reduction of each microorganism was calculated.

Stability tests

The physical stability test of the final serum formulation was made at 25°C 65% relative humidity (RH) for six months. The appearance, weight variation, and pH values of test samples were determined at 30-day intervals.

In vivo allergy testing

In vivo allergy tests were conducted according to the Regulation of the European Parliament and Council Regulation (EC) No 1223/2009 of November 30, 2009; Cosmetics Europe- The Personal Care Association Guidelines, Product test Guidelines for the Assessment of Human Skin Compatibility 1997; and WMA Declaration of Helsinki – Ethical Principles for Medical Research Involving Human Subjects. Dermatological tests were performed following the COLIPA Guidelines for the Assessment of Human Skin Compatibility. The patch test was performed at Skin Lab International, Cracow, Poland, with a test number of 20/12/17/D/7. Fifteen women aged 22 – 70 years were selected for the dermatological tests of the product. All of the patch samples selected for testing met the requirements for inclusion in the study, signed an agreement to participate in the study, and were informed about the study's purpose, how it is carried out, and the possible side effects. The test was conducted using the Jodassohn-Bloch model (Lachapelle and Maibach, 2009) by the International Contact Dermatitis Research Group (ICDRG). Patch testing was made with standard IQ chambers. A small drop of serum formulation was applied to the patients' forearm for 48 hours and then removed (Figure 2). After 30 min, 72 h, 96 h, and one-week readings were recorded and evaluated according to a graphic scale consistent with the generally accepted clinical dermatological scale.

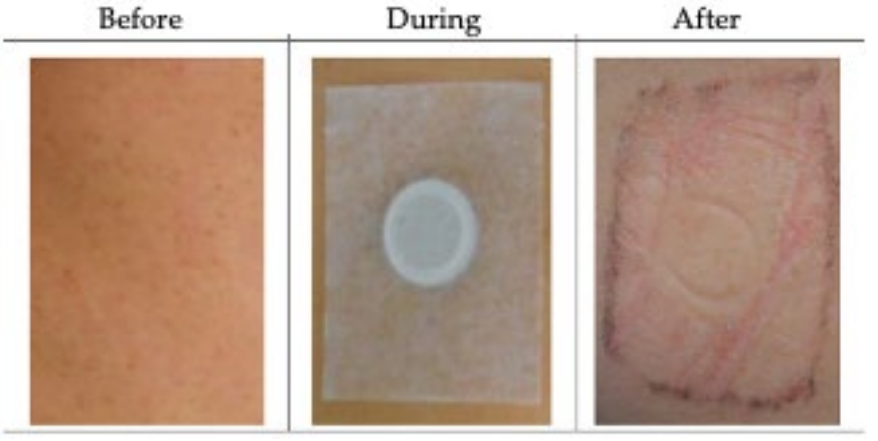

Figure 1. Application of test HA gel formulations to human skin

Statistical analysis

Statistical differences for viscosity and surface tension were analyzed with one- way ANOVA test following Tukey HSD post-hoc test using GraphPad Prism Version 8. Results were expressed as the mean ± standard deviation, and evaluation was carried out with a significance level of 0.05.

RESULTS AND DISCUSSION

Preparation of HA serum

HA serum formulations were prepared by dissolving different concentrations of HA in distilled water in the existence of 1% 2-phenoxyethanol as a stabilizer (Dreno, 2019). A minimum number of excipients were added to avoid facial skin irritation, where some fragranced ingredients were reported to cause allergy or sensitizations (Panico, 2019). A high concentration of HA in serum allows that the formulation remains on the skin surface to provide long-acting skin moisturization. In this way, topical HA helps skin regeneration and shows the anti-wrinkle effect (Choi, 2017). Similar to this study, the anti-wrinkle efficacy of HA-based topical cream has investigated by Poetschke et al., and the researchers found that daily application of HA cream resulted in a significant reduction in the depth of wrinkles improvement of skin elasticity and tightness (Poetschke, 2016). Jegasothy et al. applied topical lotion, serum, and cream of HA for 8-weeks to 33 women and found a significant effect on improving moisturizing, skin elasticity, and skin roughness (Jegasothy, 2014).

Viscosity and surface tension measurements

Viscosity is a critical parameter for topical formulations (Karakucuk, 2021). Topical solutions with low viscosity have faster clearance than viscous solutions. In addition, highly viscous solutions can have an undesirable effect on the skin. In terms of penetration, solutions with lower viscosity should penetrate better than a thicker control serum (Surini, 2018). The viscosity of solutions affected directly by polymer concentration and molecular weight of the polymer. In this study, HA with 400 kDa, which could be classified as intermediate, was used. Although solutions with 2% HA showed shear thinning behavior, solutions with 1% and 0.5 % concentration showed Newtonian flow (Figure 2). When the concentration of HA increased from 0.5 to 1 and 2 %, the viscosity solution increased significantly four times and 20 times at 50 rpm ($p<0.05$). A similar result can be seen in the literature. Budiasih et al., prepared argan oil containing serum formulations with 214-351 pa (Budiasih, 2018).

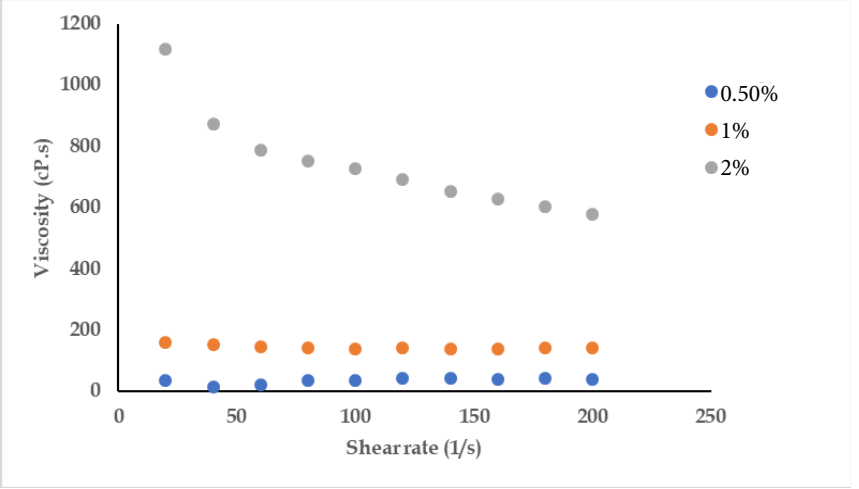

Figure 2. Shear rate – viscosity curve of HA gel serum formulations

The surface tension of formulation can play a critical role in wetting the skin surface. The surface tension of 1% HA containing solution was 67.35 ± 0.12 mN/m, and it was a significantly higher value than 0.5% HA serum which had a surface tension of 66.83 ± 0.15 mN/m ($p<0.05$). Increasing HA concentration to 2% decreased the surface tension significantly to 63.88 ± 0.33 mN/m ($p<0.05$). Although cleaning cosmetic products have very low surface tension values, cosmetic products for hydration should not have low surface tension values. The main effect that provides moistening is related to the material's structure rather than the surface tension. HA can create hydration film on the skin to moisturize the stratum corneum (Xie, J. 2018). The surface tension of solutions could be decreased with surfactant addition. However, surfactants have skin irritation in cosmetic products according to the amounts (Seweryn, A. 2018).

Protective efficacy tests (Challenge tests)

For cosmetic products, at least one preservative agent should be added to the formulation. Preservative agents have a critical effect on product quality and also on consumer health. In addition, microorganisms can affect the product quality after opening the product. Therefore, the effectiveness of protective agents should be performed with challenge tests. For this purpose, cosmetic challenge test standards are based on five different microorganisms. Products without water or alcohol-based products (more than 20%) do not need this test procedure. Phenoxyethanol is a safe and commonly used preservative for cosmetic products. This preservative can be used between 0.1 to 1 % concentrations (Dreno, 2019). An essential advantage of Phenoxyethanol is that it does not change the appearance and smell of the formulations. However, the possibility of skin irritation is a disadvantage (Farage 2019). In this study, phenoxyethanol was added to formulations at a 1% concentration. It was shown that this concentration successfully protected the product from selected bacteria and fungus in Table 1. Phenoxyethanol provided more than 5 log and 4 log reduction towards bacteria and fungi, respectively, for 28 days. It was shown that phenoxyethanol has an efficient bactericidal and fungicidal effect in the final product.

Table 1. Challenge tests result for 2-Phenoxyethanol

Microorganism	Inoculation (CFU)	Number of CFU/g				Log reduction		
		Day 0	Day 7	Day 14	Day 28	Day 7	Day 14	Day 28
P. aeruginosa	$2.7x10^7$	$2.7x10^7$	<10	<10	<10	5.18	5.18	5.18
E. coli	$2.3x10^7$	$2.3x10^7$	<10	<10	<10	5.18	5.18	5.18
S. aureus	$2.5x10^7$	$2.5x10^7$	<10	<10	<10	5.48	5.48	5.48
C. albicans	$2.2x10^8$	$2.2x10^8$	<10	<10	<10	4.48	4.48	4.48
A. niger	$3.0x10^8$	$3.0x10^8$	<10	<10	<10	4.48	4.48	4.48

Stability tests

Physical stability tests of the final product were made at 25°C, 65% RH. Final products were determined as transparent, colorless and no color change was observed during storage time. The samples showed the same results as the first weighing (approximately 12 g ± 0.1) at the end of the study, and no weight variation was observed. The pH of solutions was found stable at 5.5 during the test period. At the end of the study, microbiological and physical stability were protected.

In vivo allergy testing

Patch testing for in vivo allergy is the gold standard to identify skin compatibility for cosmetic products and cause allergic contact dermatitis and diagnose

a delayed type of hypersensitivity (Kasemsarn and Boonchai 2012). Patch testing allows test substance application on skin surface and causes of reaction of the immune system by a characteristic pattern of erythema and edema develops from 6 h and reaches a maximum by 36- 48 h (Friedmann and Ardern-Jones 2010). Patch testing was performed for HA serum formulations, and the results were evaluated according to a graphic scale that was consistent with the generally accepted clinical dermatological scale. Results were evaluated by the recommendations of the International Contact Dermatitis Research Group (ICDRG) (Lachapelle and Maibach, 2009) (Table 2).

Table 2. Identification of the patch test reactions

Record	Diagnosis	Interpretation
-	Negative reaction	No skin lesions
?	Doubtful reaction	Faint erythema only
+	Weak positive reaction	Palpable erythema, infiltration, possibly papules
++	Strong positive reaction	Erythema, infiltration, papules, vesicles
+++	Extreme positive reaction	Intense erythema, infiltration, and coalescing vesicles, bullous or ulcerative reaction
IR	The irritant reaction of different types	Discrete patchy erythema without infiltration.

There were no allergic reactions or hypersensitivity of the formulation on female skin during the study (Table 3). Similarly, Kong et al., (2011) reported that HA-based nanoemulsions did not cause skin irritation in the dermis and skin surface. Choi et al., (2017) also reported the non-irritant effect of HA-containing microneedle patches.

Table 3. The clinical dermatological scale of patch testing on female subjects

No	Identification number	Sex (Female – F)	Age	Test result			
				48 h	72 h	96 h	One week
1	20/12/17/D/7-1	F	22	(-)	(-)	(-)	(-)
2	20/12/17/D/7-2	F	24	(-)	(-)	(-)	(-)
3	20/12/17/D/7-3	F	23	(-)	(-)	(-)	(-)
4	20/12/17/D/7-4	F	62	(-)	(-)	(-)	(-)
5	20/12/17/D/7-5	F	70	(-)	(-)	(-)	(-)
6	20/12/17/D/7-6	F	27	(-)	(-)	(-)	(-)
7	20/12/17/D/7-7	F	44	(-)	(-)	(-)	(-)
8	20/12/17/D/7-8	F	26	(-)	(-)	(-)	(-)
9	20/12/17/D/7-9	F	23	(-)	(-)	(-)	(-)
10	20/12/17/D/7-10	F	23	(-)	(-)	(-)	(-)
11	20/12/17/D/7-11	F	61	(-)	(-)	(-)	(-)
12	20/12/17/D/7-12	F	49	(-)	(-)	(-)	(-)
13	20/12/17/D/7-13	F	23	(-)	(-)	(-)	(-)
14	20/12/17/D/7-14	F	24	(-)	(-)	(-)	(-)
15	20/12/17/D/7-15	F	48	(-)	(-)	(-)	(-)

CONCLUSION

HA is one of the most successful cosmetic ingredients using in anti-aging products because of its compatibility with skin and capacity for hydration. HA serum formulations were prepared for a topical application to provide skin moisture in this study. The in vitro characterization studies showed that the formulation is smooth and easy for application, increasing consumers' compliance. The final formulation stayed physically and microbiologically stable during the storage time. Patch testing was performed to evaluate any skin reactions of the products, and there were no adverse effects such as hypersensitivity or erythema. The benefits of using HA and the safety of the final formulation promise that the product, which is available commercially as a cosmetic product in Turkey, can be safely used as skin moisturizing.

ACKNOWLEDGMENTS

Fiber Farma Drugs Cosmetics Co. was established in 2015 by a grant from The Ministry of Industry and Technology (0104.TGSD.2015).

CONFLICT OF INTEREST

The authors declared no conflict of interest.

AUTHOR CONTRIBUTION STATEMENT

Concept (Tort, S., Karaküçük, A.), Design (Tort, S., Karaküçük, A.), Supervision (Karaküçük, A.), Resources (Tort, S., Karaküçük, A.), Materials (Tort, S., Karaküçük, A.), Data Collection and Processing (Tort, S., Karaküçük, A.), Analysis, and Interpretation (Tort, S., Karaküçük, A.), Literature Search (Tort, S., Karaküçük, A.), Writing (Tort, S., Karaküçük, A.), Critical Reviews (Tort, S., Karaküçük, A.).

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