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Effects of Chymotrypsin Therapy on Alpha 1-Anti Trypsin and Glutathione Peroxidase in Facial Skin of Rabbits Injected by Hyaluronic Acid

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ABSTRACT

THE USE of hyaluronic acid in the treatment of several skin and cosmetic illnesses is very common, and this is frequently followed by the appearance of some side effects. Doctors are looking for chemicals that can reduce the occurrence of these adverse effects. The goal of this study is to look into the effect of using chymotrypsin in alpha-1-antitrypsin and glutathione peroxidase when using hyaluronic acid in skin of rabbits. Materials and procedures: There were four groups of 30 rabbits. The first group served as a negative control group and received no therapy. The second group was a positive control group that received only hyaluronic acid injection. The third group received hyaluronic acid injections together with chymotrypsin. Blood and skin samples were collected 24 and 72 hours following treatment, respectively. This was done after anesthetizing the rabbits and dissecting them in order to obtain blood samples and serum, as well as histology section from the skin in order to do histopathological examination. The results revealed decreasing inflammation, as reduced the infiltration of inflammatory cells in the skin, increased the levels of glutathione peroxidase and alpha-1-antitrypsin in the third group of combination of chymotrypsin with hyaluronic acid when compared to with groups that were not treated with chymotrypsin. **Conclusion**: chymotrypsin when combined with Hyaluronic acid, exhibits beneficial effects while reducing the adverse effects of hyaluronic acid.

Keywords: Hyaluronic Acid, Chymotrypsin, Alpha 1-Anti Trypsin, Glutathione Peroxidase, Rabbits

Introduction

Hyaluronic acid (HA) is a natural polymer found in the bodies of humans and animals. It is frequently used to treat a wide range of human and animal ailments. HA is a necessary component of connective tissue, skin, umbilical cord, and synovial fluid, and it contributes to the extracellular matrix's elasticity and viscosity [1,2].

HA contributes to the suppleness and viscosity of synovial fluid as well as the integrity of connective tissues such as joints. HA fillers are an alternate treatment option for treating facial aging, facial wrinkles, and facial scars. These chemicals may have an effect on the skin. Because of the exact nature of the reaction, time-delayed responses in skin testing are required [2, 4] .The topic of skin testing requires further investigation. These substances certainly have the potential to cause delayed inflammatory skin reactions, albeit in a smaller percentage of patients, According to certain research, the usage of chymotrypsin can help to reduce the appearance of inflammatory reactions in various skin lesions.

Chymotrypsin prevents the formation of inflammatory edema and hematoma by removing necrotic tissue in skin and corneal wounds [3, 4]. Our research focuses on the examination of two pivotal biomarkers, Alpha 1-Anti Trypsin and Glutathione Peroxidase. These biomarkers have emerged as essential components in the intricate regulatory network that manages inflammation and counters the detrimental effects of oxidative stress within skin tissues [4-6]. By examining the interplay between HA fillers, chymotrypsin therapy, and the dynamic role of these biomarkers, We investigated the effect of using chymotrypsin on alpha-1-antitrypsin and glutathione peroxidase when using hyaluronic acid in skin of rabbits.

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Material and Methods

Ethical Approval

This study was carried out adhering to ethical guidelines and obtained approval from both the Research Ethics Committee and Scientific Committee within the Department of Dental Basic Science at the College of Dentistry, University of Mosul. The approval reference number is UOM. Dent/A.L.23/15, dated 05.02.2023.

Animal Housing and Pre-Experiment Procedures

The study involved locally sourced adult male rabbits with an average weight of 1.5 ± 0.25 kg. These rabbits were permanently kept indoors, following established international, national, and institutional protocols for the care and use of animals. Before the commencement of the study, a seven-day adaptation phase was implemented under standard housing conditions, maintaining a consistent room temperature of 25 ± 2°C. Throughout this period, the rabbits had unrestricted access to a standard diet and water.

Drug Administration

Based on the rabbits' age and body weight, each rabbit in the study underwent a 0.5 ml injection of HA (5) filler sourced from PROMOITALIA. The rabbits were randomly assigned to four main groups:

Group I: This serves as the control negative group, with 1 rabbits providing baseline measurements without any filler injection or treatment, encompassing blood and tissue components.

Group II: This functions as the control positive group, with a total of 10 animals receiving a topical 0.5 ml HA injection on the first day of the study period. The rabbits were then divided into two subgroups:

Subgroup II a: 5 animals were sacrificed 24 hours post-treatment.

Subgroup II b: 5 animals were sacrificed 72 hours post-treatment.

Group III: In this group, 10 animals were treated with chymotrypsin after receiving a 0.5 ml HA injection. Chymotrypsin was intramuscularly injected at a dose of 8.1 units/kg [6], two hours after the HA injection, on the first day of the study period. This group was further subdivided into two subgroups:

Subgroup IIIa: 5 animals were sacrificed 24 hours post-treatment.

Subgroup IIIb: 5 animals were sacrificed 72 hours post-treatment.

Histopathological Assessment

The soft tissue from the forehead of each rabbit was separated and immersed in a 10% formalin solution for 48 hours. Following this, the tissue was embedded in paraffin wax, longitudinally sectioned into 5µm slices, stained with hematoxylin and eosin (H & E), and then analyzed using a light microscope.

Biochemical Assessments

Blood Sample Collection and Analysis

For biochemical analysis, a tube of fresh blood was collected from the jugular vein of each rabbit at the time of sacrifice. The tube was centrifuged at 3000 rpm for 15 minutes to obtain serum, that was later collected using a micropipette and transferred to Eppendorf tubes. The tubes were stored at -20°C. The primary blood parameters analyzed in this study included α1-antitrypsin and Glutathione Peroxidase , and were measured in the serum using the following specialized kits:

1. Rabbit Glutathione peroxidase (GPX) ELISA Kit from Sunlong Biotech Co., Ltd
2. Rabbit Alpha 1-Antitrypsin (a1-AT) Elisa Kit from eiyue

The same procedure was followed for both kits.

Statistical Analysis

The information was presented in the form of mean values along with their corresponding standard deviations (SD). Statistical significance, set at a level below 0.05, was determined for all tests conducted using the SPSS program. The statistical methodologies applied encompassed an independent two-sample t-test for comparing treatment and control groups, a two-sample t-test for intra-group comparisons, post hoc Waller Duncan's test, and analysis of variances (ANOVA) to identify differences in various parameters across the study groups. Furthermore, a correlation test was employed to investigate the relationships between parameters within each specific study group.

Results

Histological Evidence

The histology of the rabbit skin observed changes in their keratin layers, epidermis, and dermis, due to the HA injection and/or Chymotrypsin treatment. Congestion in blood vessels and related changes in glands and hair follicles were also observed. This is shown in Figures 1, 2, 3, 4, and 5.

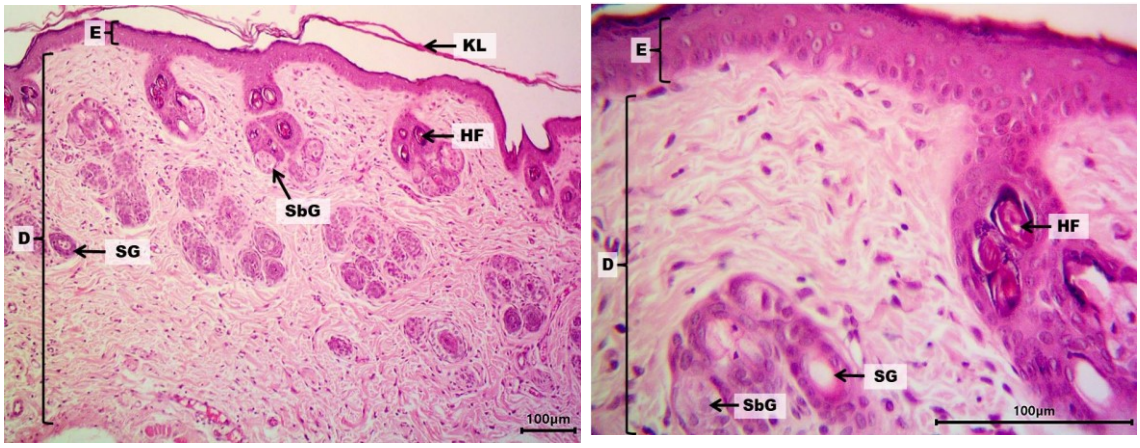

Fig. 1. Photomicrograph of rabbit skin of the negative control (without filler) group showing normal architecture representing by the keratin layer (KL), epidermis (E), dermis (D) with hair follicles (HF), sweat glands (SG) and sebaceous glands (SbG). H&E stain at 100X (left figure) and 400X (right figure).

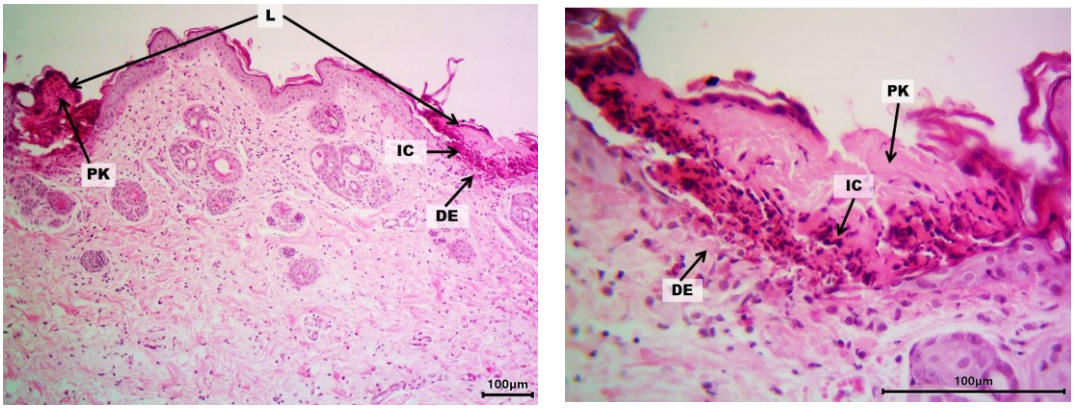

Fig. 2. Photomicrograph section of rabbit skin of the positive control (with filler) group after 24 hours showing sub-corneal lesions (L) as inflammatory exudate containing inflammatory cells (IC), with para-keratosis (PK), and damaged epithelial cells in the epidermis (DE), without hair follicles in the dermis. H&E stain, at 100X, revealing 2 L (left figure) and 400X, thereby revealing one L (right figure).

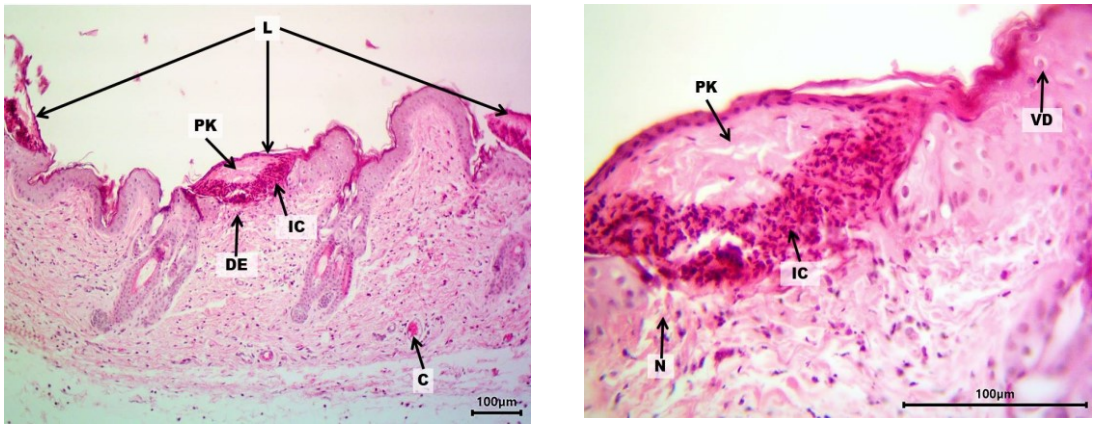

Fig. 3. Photomicrograph section of rabbit skin of the positive control (with filler) group after 72 hours. The right figure shows 3 sub-corneal lesions (L) as inflammatory exudate containing inflammatory cells (IC), with para-keratosis (PK), and damaged epithelial cells in the epidermis (DE), with congestion of blood vessels (C). H&E stain, 100X. The right figure shows inflammatory exudate containing IC, with PK, and DE as necrosis (N) and vacuolar degeneration (VD). H&E stain, 400X.

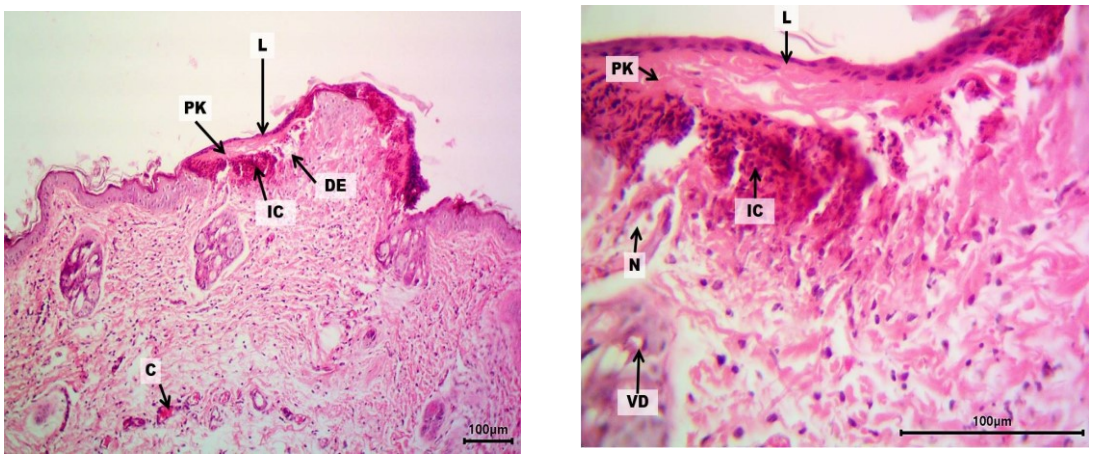

Fig. 4. Photomicrograph section of rabbit skin of the Chymotrypsin (with filler) group after 24 hours. The left figure shows sub-corneal lesions (L) as inflammatory exudate containing inflammatory cells (IC), with para-keratosis (PK), and damaged epithelial cells in the epidermis (DE), with congestion of blood vessels (C). H&E stain, 100X. Right figure shows sub-corneal lesions (L) as inflammatory exudate containing inflammatory cells (IC), with para-keratosis (PK), and damaged epithelial cells as necrosis (N) and vacuolar degeneration (VD). H&E stain, 400X.

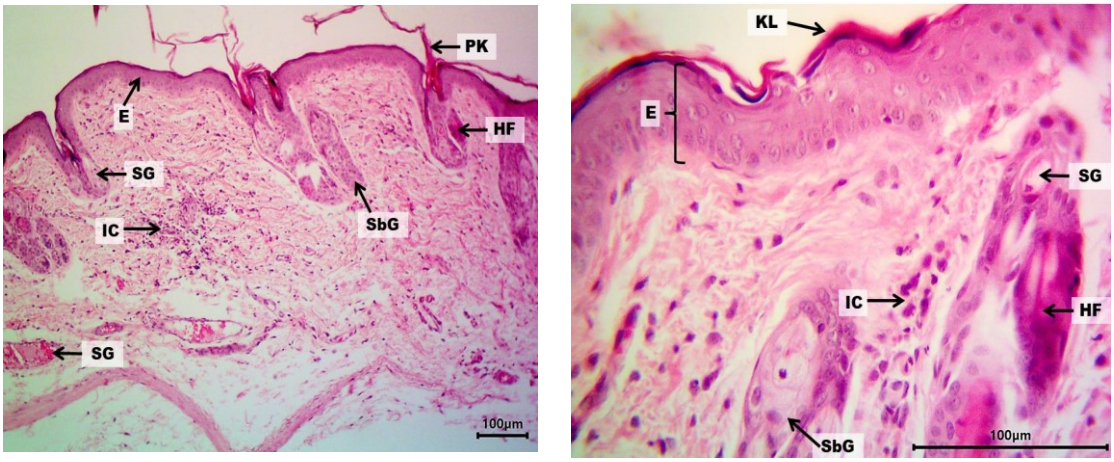

Fig. 5. Photomicrograph of rabbit skin of the Chymotrypsin (with filler) group after 72 hours group. The left figure shows mild para-keratosis (PK), intact epidermis (E), mild infiltration of inflammatory cells in the dermis (D) with intact hair follicles (HF), sweat glands (SG) and sebaceous glands (SbG). H&E stain, 100X. The right figure shows the intact architecture of the keratin layer (KL), intact epidermis (E), mild infiltration of inflammatory cells in the dermis (D) with intact hair follicles (HF), sweat glands (SG) and sebaceous glands (SbG). H&E stain, 400X.

Glutathione peroxidase and Alpha 1-Antitrypsin

Results after first 24 hours

1. Glutathione Peroxidase:

Between Groups Analysis:

The ANOVA results for Glutathione Peroxidase show a significant difference between the study groups ($F(2, 12) = 5.396$, $p = 0.021$). The calculated F-statistic (5.396) is higher than the critical value, indicating that the means of Glutathione Peroxidase levels are significantly different among the groups. The p-value (0.021) is less than the commonly used significance level (e.g., 0.05), providing evidence to reject the null hypothesis of no difference between the groups.

Within Groups Analysis:

The within-groups variability (Mean Square = 1.033) represents the random variation of Glutathione Peroxidase levels within each group. The total within-groups sum of squares is 12.392.

2. Alpha 1-Antitrypsin:

Between Groups Analysis:

The ANOVA results for Alpha 1-Antitrypsin indicate a highly significant difference between the

study groups (F(2, 12) = 27.836, p = 0.000). The F-statistic (27.836) is substantially higher than the critical value, indicating a significant variation in Alpha 1-Antitrypsin levels among the groups. The p-value (0.000) is well below the common significance threshold, providing strong evidence to reject the null hypothesis.

Within Groups Analysis:

The within-groups variability (Mean Square = 0.029) represents the random variation of Alpha 1-Antitrypsin levels within each group. The total within-groups sum of squares is 0.344 (Table 1).

The changes in these components' levels are shown in Figures 6 and 7.

TABLE 1. Comparison of serum Glutathione peroxidase and Alpha 1-Antitrypsin among study groups at the end of the 24 hours

ANOVA						
Blood Sample	**S.O.V.**	**Sum of Squares**	**df**	**Mean Square**	**F**	**Sig.**
Glutathione peroxidase	**Between Groups**	11.145	2	5.573	5.396	0.021
	Within Groups	12.392	12	1.033		
	Total	23.537	14			
Alpha 1-Antitrypsin	**Between Groups**	1.597	2	0.799	27.836	0.000
	Within Groups	0.344	12	0.029		
	Total	1.941	14			

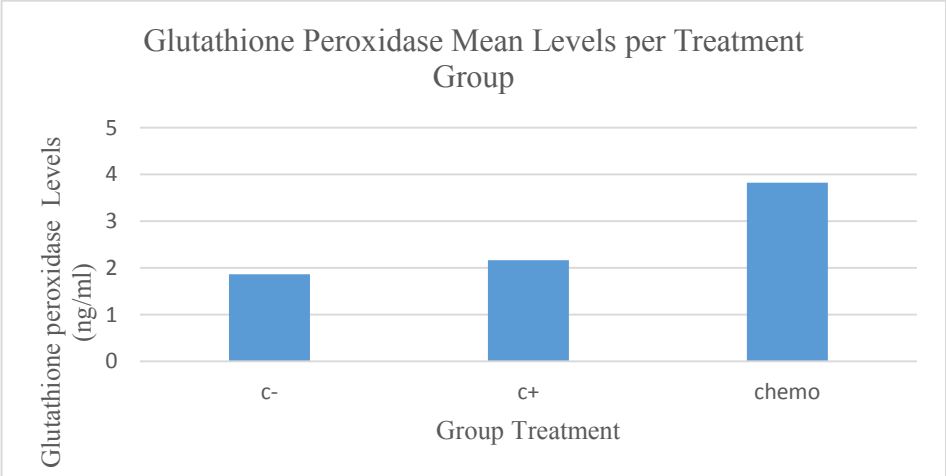

Fig. 6. The Comparison between Glutathione peroxidase mean during first 24h in all groups.

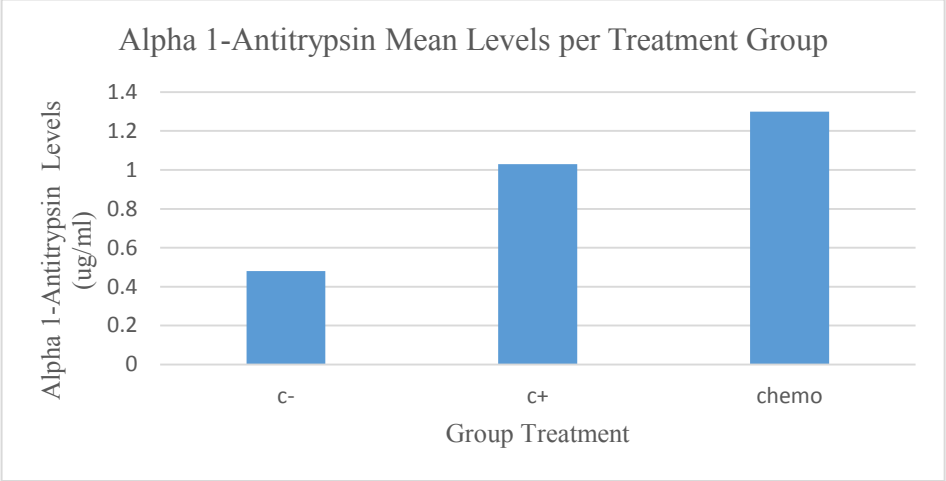

Fig. 7. The Comparison between Alpha 1-Antitrypsin mean during first 24h in all groups.

Results after first 72 hours

1. Glutathione Peroxidase:

Between Groups Analysis:

The ANOVA results for Glutathione Peroxidase at 72 hours show no significant difference between the study groups ($F(2, 12) = 3.412$, $p = 0.067$). The calculated F-statistic (3.412) is below the critical value, suggesting that the means of Glutathione Peroxidase levels are not significantly different among the groups at a 72-hour time point. The p-value (0.067) is greater than the common significance level of 0.05, indicating that the observed difference may be due to random chance.

Within Groups Analysis:

The within-groups variability (Mean Square = 2.751) represents the random variation of Glutathione Peroxidase levels within each group. The total within-groups sum of squares is 33.008.

2. Alpha 1-Antitrypsin:

Between Groups Analysis:

The ANOVA results for Alpha 1-Antitrypsin at 72 hours indicate a highly significant difference between the study groups ($F(2, 12) = 16.499$, $p = 0.000$). The F-statistic (16.499) is substantially higher than the critical value, indicating a significant variation in Alpha 1-Antitrypsin levels among the groups at the 72-hour time point. The p-value (0.000) is much less than the common significance threshold, providing strong evidence to reject the null hypothesis.

Within Groups Analysis:

The within-groups variability (Mean Square = 0.023) represents the random variation of Alpha 1-Antitrypsin levels within each group. The total within-groups sum of squares is 0.274.

TABLE 2. Comparison of serum Glutathione peroxidase and Alpha 1-Antitrypsin among study groups at the end of the 72 hours

ANOVA						
Blood Sample	**S.O.V.**	**Sum of Squares**	**df**	**Mean Square**	**F**	**Sig.**
Glutathione peroxidase	Between Groups	18.772	2	9.386	3.412	0.067
	Within Groups	33.008	12	2.751		
	Total	51.780	14			
Alpha 1-Antitrypsin	Between Groups	0.752	2	0.376	16.499	0.000
	Within Groups	0.274	12	0.023		
	Total	1.026	14			

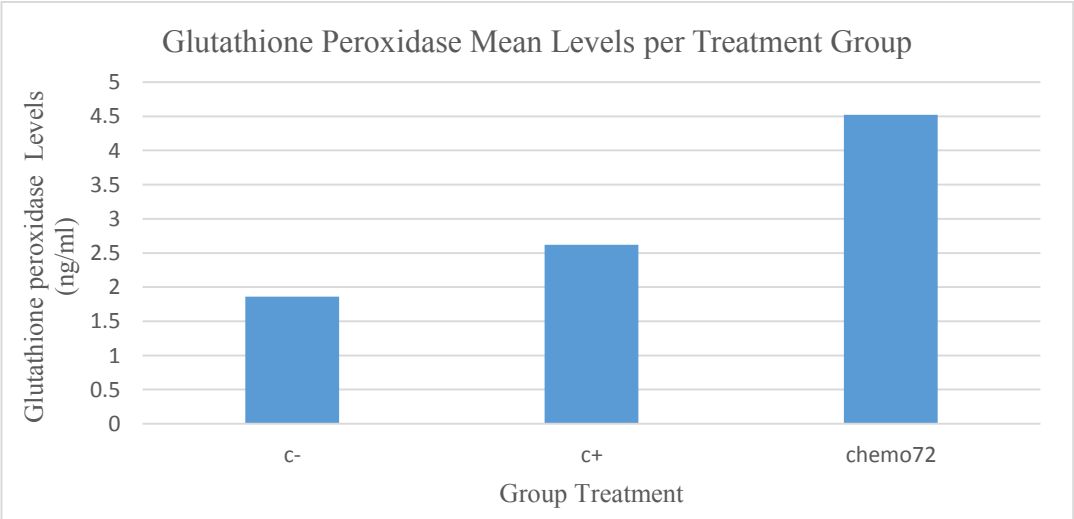

Fig. 8. The Comparison between Glutathione peroxidase mean during first 72h in all groups.

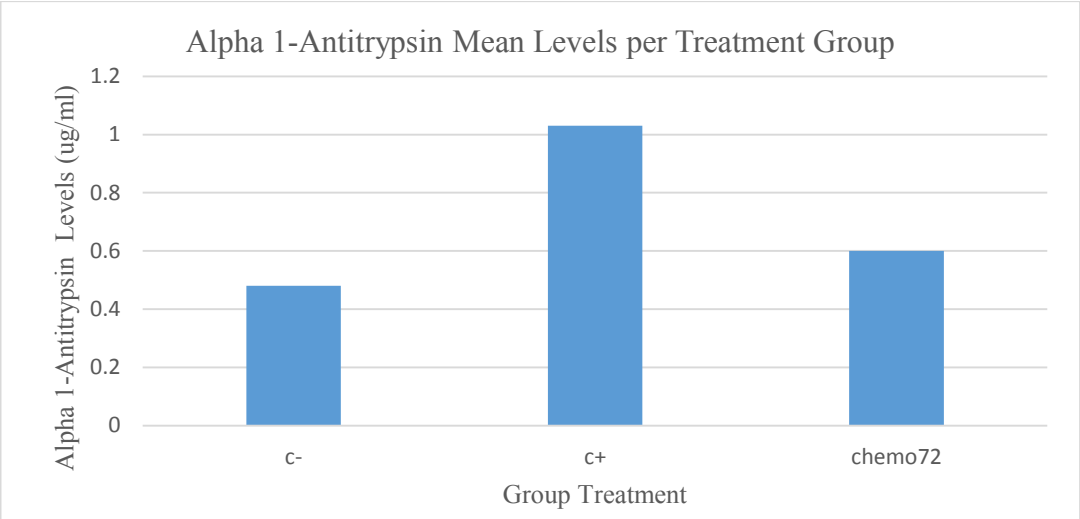

Fig. 9. The Comparison between Alpha 1-Antitrypsin mean during first 72h in all groups.

Discussion

Different inflammatory responses were elicited by hyaluronic acid injections (Figure 6). The consequences manifested as keratotic and semi-keratotic lesions, with epithelial cell breakdown in the skin. Hair follicles were also severely impacted, particularly after 24 and 72 hours (Figure 3).

After 72 hours, the use of chymotrypsin reduced the negative effects of inflammation in the dermis, and the severity of keratinization in the dermis decreased, as did the infiltration of inflammatory cells, preserving the hair follicles, dermis, sweat and sebaceous glands.

Thus, chymotrypsin appears to have good effects in lowering inflammation and maintaining the integrity of the skin's tissue structure following hyaluronic acid injection, which is important in the field of skin care and cosmetology (7).

Glutathione peroxidase is an antioxidant enzyme that helps the body fight oxidative damage (7).

Glutathione peroxidase works to reduce hydrogen peroxide and other harmful organic hydroperoxides, thereby reducing the oxidative damage caused by oxidative stress in the cells, which appeared in our study by injecting hyaluronic acid into the skin, whereas the use of chymotrypsin led to a reduction in oxidative stress and an increase in glutathione peroxidase levels in skin cells after 24 and 72 days. An hour of therapy

The enzymes trypsin and elastin, which break down tissue proteins, are inhibited by the protein alpha-1 antitrypsin, which is produced into the bloodstream by the liver (9). The study found a rise in alpha-1 antitrypsin levels 24 hours after chymotrypsin administration, which could be owing to proteolytic enzyme activity being altered (10).

To maintain protein balance, the body is thought to enhance the production of alpha-1 antitrypsin as a defensive reaction against chymotrypsin. However, the study discovered a drop in its rate after 72 hours, which could be attributed to the body's physiological systems to cope with chymotrypsin treatment (11). Alpha-1 antitrypsin levels may fall as chymotrypsin levels fall in the body.

Conclusion

The study found that chymotrypsin reduces the rate of inflammatory reaction generated by hyaluronic acid injections into the skin, influencing the different layers of the skin as well as hair follicles, perspiration and sebaceous glands. It also helps to reduce the degree of oxidative stress in the body by boosting the antioxidant status and increasing the levels of glutathione peroxidase and alpha-1 antitrypsin. This finding has promising implications for dermatological and cosmetic skin therapies.

***Conflicted interest**:*

None.

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آثار علاج الكيموتربسين على ألفا 1-مضاد التربسين والجلوتاثيون بيروكسيداز في وجه جلد الارانب المحقون بحامض الهيالورونيك

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الخلاصة

يعد استخدام حامض الهيالورونيك في علاج العديد من الأمراض الجلدية والتجميلية أمرًا شائعًا جدًا، وغالبًا ما يتبعه ظهور بعض الآثار الجانبية، ويبحث الأطباء عن مواد كيميائية يمكن أن تقلل من حدوث هذه الآثار الضارة. الهدف من هذه الدراسة هو النظر في تأثير حامض الهيالورونيك على جلد الأرانب عند استخدامه بالتزامن مع ألفا -1 أنتيتريبسين و لمحاولة تقليل آثاره الضارة وحالة الإجهاد التأكسدي في الجسم. تم تقسيم30 ارنبا الى 3 مجاميع، أعتبرت المجموعة الأولى بمثابة مجموعة سيطرة سالبة ولم تتلق أي علاج، وكانت المجموعة الثانية عبارة عن مجموعة سيطرة موجبة تلقت حقن حامض الهيالورونيك فقط. تلقت المجموعة الثالثة حقن حامض الهيالورونيك مع الكيموتربسين. تم جمع عينات الدم والجلد بعد 24 و 72 ساعة من العلاج على التوالي. تم ذلك بعد تخدير الأرانب وتشريحها للحصول على عينات الدم والمصل وكذلك عينات أنسجة من الجلد لإجراء الفحص النسيجي المرضي عليها. اظهرت النتائج قلة الالتهاب وارتشاح الخلايا الالتهابية في الجلد، وزيادة مستويات الجلوتاثيون بيروكسيداز وألفا -1 أنتيتريبسين في المجموعة الثالثة التي تم فيها اعطاء الكيموتربسين مع حامض الهيالورونيك مقارنة بالمجموعات التي لم تعالج بالكيموتريبسين. نستنتج من هذه الدراسة انه عندما يقترن الكيموتربسين بحامض الهيالورونيك فإنه يظهر تأثيرات مفيدة إذ يقلل الالتهاب و الآثار الضارة لحامض الهيالورونيك.

الكلمات المفتاحية: حمض الهيالورونيك، كيموتربسين، ألفا 1-مضاد التربسين، الجلوتاثيون بيروكسيداز، الأرانب.