Introduction

Hydrofluoric acid (HF) is used in a vast array such as semiconductor industry, mineral processing, electropolishing metals, petrochemical production, etching, frosting and polishing glass [5, 13, 24]. In spite of relatively high incidence of HF induced ocular burns, there is little information, regarding the optimal treatment modalities [4, 5]. Efforts have been continued to find an ideal chemical agent, some of which like Alkanna tinctoria Tausch [28, 29] and hyaluronic acid [2, 30] play an important role for both corneal and dermal burns to promote healing.

The importance of natural biopolymers like chitin and chitosan within the pharmaceutical industry and biotechnology has been growing. Apart from these polymers, natural polymers like alginate, fucoidan and laminarin derived from seaweeds and algae are also used extensively [7, 19, 27].

Chitosan is a cationic biopolymer achieved as a result of the deacetylation of chitin obtained by decalcification of shells of crustaceans such as berry, shrimp and crab. Chitosan is the long linear chain polymeric molecules of the glycans linked to each other with β(1→4) bond [15]. Although acceleration of wound healing was reported with chitosan [6, 18, 20], topical application of a 1% formulation had no any positive effects on the healing of corneal wound [25].

Laminarin obtained from brown marine algae (Phaeophyceae) is a biopolymer of polysaccharide structure SSUUMMMMAARRYY

Corneal burns were induced in 36 New Zealand white rabbits by instillation in both eyes of 0.05 mL of 2% hydrofluoric acid (HF) for 60 seconds. Following this, the eyes were irrigated with 500 mL isotonic saline (ISOT) and then the rabbits were divided into 4 treatment groups (n = 9 rabbits each) including: laminarin solution (LS), chitosan hydrogel (CHG), chitosan hydrogel containing laminarin (CHG+L) and ISOT as the control. For each treatment group, one drop of each regimen was instilled 2 times a day for periods of 2, 7 and 14-days respectively. Thus, 3 rabbits were used for each treatment period in each treatment group. The eyes were clinically examined immediately after the chemical burning and at days 1, 2, 7 and 14 of the follow up periods. The animals were euthanatized at the end of the follow-up periods and eyes were processed for histopathological examination. Clinical and histopathological results revealed that while LS or ISOT was effective, CHG and CHG+L were not effective in the treatment of HF corneal burns. LS had a better therapeutic effect than ISOT. CHG and CHG+L treatment had no accelerating effect on the healing of corneal erosion throughout the experimental procedures.

Key-words: Chitosan, laminarin, isonotic saline, HF induced corneal burn, rabbit.
FIGURE 1: Clinical appearance of the corneal erosion after fluorescein staining at day 2 following treatment. A. Isotonic saline, B. Chitosan hydrogel, C. Chitosan hydrogel+laminarin, D. Laminarin solution.

FIGURE 2: Clinical appearance of the corneal erosion after fluorescein staining at day 7 following treatment. A. Isotonic saline, B. Chitosan hydrogel, C. Chitosan hydrogel+laminarin, D. Laminarin solution.

FIGURE 3: Clinical appearance of the corneal erosion after fluorescein staining at day 14 following treatment. A. Isotonic saline, B. Chitosan hydrogel, C. Chitosan hydrogel+laminarin, D. Laminarin solution.


FIGURE 5: Day 7 of treatment. Note the increased oedema formation in the B. Chitosan hydrogel and C. Chitosan hydrogel+laminarin compared to A. Isotonic saline and D. Laminarin solution. H&E, Bar: 300µm.

Laminarin and Chitosan on Hydrofluoric Acid Corneal Burns in the Rabbits

with high solubility in water. It is obtained by glucose units binding to the main chain by 1,6-β linkage, although 20 to 30 glucose units are linked with a 1,3-β bridge [21]. The best example for this structure is the commercially manufactured Laminaria digitata. The physiological effect of laminarin appears to be multifactorial since it may act as anticoagulant [10], antilipaemic [1, 17], suppressor of apoptotic death [12] and stimulator of humoral immunity [3].

Considering the multiple biological functions of laminarin, this study investigated its the therapeutic effects as well as those of chitosan in pharmaceutical gel form and isotonic saline (ISOT) in HF-induced corneal burns in rabbits.

Material and Methods

CHEMICALS

Laminarin (L 9634, Sigma-Aldrich Corp. St. Louis, MO, USA), Chitosan (C 3646, Sigma-Aldrich Corp. St. Louis,
PREPARATION OF LAMINARIN SOLUTION (LS)

Laminarin was dissolved in bidistilled water (2% w/v) then the solution was sterilized by using a 0.22 micrometer filter.

PREPARATION OF CHITOSAN HYDROGEL (CHG)

Chitosan 2% (w/v) was dissolved in lactic acid (1% v/v) solution then the gel pH was adjusted to the neutral pH and sonicated 30 min to remove the air bubbles. The resulting mixture was sterilized by UV and the sample was stored at +4°C for in vivo usage.

PREPARATION OF CHITOSAN HYDROGEL CONTAINING LAMINARIN (CHG+L)

According to the gel preparation method [9], chitosan (2% w/v) was dissolved in lactic acid solution (1%) then, laminarin was added into the polymer solution and stirred overnight at room temperature then the gel pH was adjusted to the neutral pH and sonicated 30 min to remove the air bubbles. The resulting mixture was sterilized by UV and the sample was stored at +4°C for in vivo usage.

ANIMALS AND EXPERIMENTAL DESIGN

This experimental project was approved by the Animal Use Committee of University of Selcuk by which and all animals were treated in accordance with national or local animal welfare legislation which is based on European Council Directive.

A total of 36 male New Zealand White rabbits were used, housed individually and fed ad libitum. In order to keep the number of animals to a minimum, both left and right eyes were burned on the directives of the Animal Use Committee. Rabbits were anesthetized with xylazine hydrochlorure 2% (50 mg/kg) and 0.2 mL acepromazine, followed immediately by 14 mg/kg (15 mg/kg), ketamine hydrochlorure 10% (35 mg/kg) and 0.2 mL acepromazine, followed immediately by 14 mg/kg dipyrone sodium intramuscularly (IM) prior to corneal burn.

Both eyes were burned by instillation of 0.05 mL 2% HF Dipyrone (7 mg/kg) was given IM every 6 h thereafter for 48 h. Immediately after burning the ocular surface was irrigated with 500 mL ISOT 2 times a day as for the treatment protocol. The prepared formulations were applied to the burned eyes 2 times a day for 2, 7 and 14 days. Controls were treated with 500 mL of ISOT 2 times a day as for the treatment protocol.

Corneal erosion at day 7 as for the treatment day 2, being smaller in the latest (Table 1) and (Fig. 2).

Corneal erosion was significantly smaller (p < 0.05) at days 7 and 14 than at days 2 and 7 in the CHG+L treated eyes with no observed statistically significant difference (p > 0.05) between days 7 and 14 than at day 2 with no significant difference (p > 0.05) between days 7 and 14 in the ISOT treated eyes. The difference was significant (p < 0.05) in the LS treated eyes with no observed statistically significant difference (p > 0.05) between the CHG and CHG+L and between the LS and ISOT. The difference was significant (p < 0.05) in the LS and ISOT compared with that in the CHG and CHG+L. The CHG and CHG+L treated eyes had almost identical levels of corneal erosion at day 7 as for the treatment day 2, being smaller in the latest (Table 1) and (Fig. 2).

No corneal erosion was present immediately after HF eye burn. Corneal defects appeared in all eyes at day 1. At day 2, complete erosion was observed in all groups with no statistically significant difference (p > 0.05) among groups (Table 1 and Fig. 1).

At day 7, corneal erosion was less marked in the LS and ISOT treated eyes with no observed statistically significant difference (p > 0.05) between the CHG and CHG+L and between the LS and ISOT. The difference was significant (p < 0.05) in the LS and ISOT compared with that in the CHG and CHG+L. The CHG and CHG+L treated eyes had almost identical levels of corneal erosion at day 7 as for the treatment day 2, being smaller in the latest (Table 1) and (Fig. 2).

At day 14, although no statistically significant difference (p > 0.05) was seen among groups for the corneal erosion, no complete healing was observed in all treated eyes in the CHG and CHG+L. By contrast, complete healing was observed in the LS and ISOT treated eyes and was associated with corneal vascularization in the later treatment group (Table 1 and Fig. 3).

Corneal erosion was significantly smaller (p < 0.05) at days 7 and 14 than at day 2 with no significant difference (p > 0.05) between days 7 and 14 in the ISOT treated eyes. Corneal erosion was significantly smaller (p < 0.05) at days 14 than at days 2 and 7 in the CHG+L treated eyes with no significant difference (p > 0.05) between days 2 and 7. There was a statistically significant difference (p < 0.05) in the decreasing order among days 2, 7 and 14 for the corneal erosion observed in the CHG. In the LS treated eyes, there was a statistically significant difference (p < 0.05) between the
corneal erosion observed at days 7 and 14 compared to that observed at day 2 (Table 1). Although no statistically significant difference (p > 0.05) was observed between days 7 and 14, there was a complete clinical healing at day 14. No corneal neovascularization was observed at day 2 in all treated eyes. Corneal vessels were observed in all CHG- and CHG+L treated eyes, while only 2 and 4 corneas were vascularized at day 7 in the LS and ISOT treated groups respectively. No corneal vascularization was observed in the LS treated eyes, while it occurred only in 1 eye in the ISOT and CHG treated eyes. At day 14, corneal blood vessels were found in 4 eyes in the CHG+L treated eyes.

**MICROSCOPIC FINDINGS**

**Day 2 Following Treatment**

Corneal epithelial necrosis was found with stromal thickening due to stromal oedema in all groups (Figs. 4 A, B, C, D). While there was no statistically significant difference for the corneal thickness between the LS and CHG+L treated eyes, this parameter was found to be statistically significant (p < 0.05) for the LS when compared to ISOT and CHG treated eyes (Table 2). Polymorphonuclear (PMN) infiltration was seen in the upper part of the stroma in all groups with remarkable difference in the CHG and CHG+L treated eyes (Figs. 4 B, C). While PMN accumulation was observed in the anterior chamber in the CHG (Fig. 7) and CHG+L treated eyes, there was none in the LS and ISOT treated eyes. PMN infiltration was observed in the limbus of all CHG and CHG+L treated eyes (Fig. 7), but was only present in 3 eyes of the ISOT and LS treatment groups (Table 3).

**Day 7 Following Treatment**

Thickening of stroma was observed due to the oedema in all groups. Thickening was significantly (p < 0.05) smaller in the LS and ISOT treated eyes than in the CHG and CHG+L treated eyes (Table 2) and (Figs. 5 A, B, C, D). Necrosis was observed in the corneal epithelium and upper part of the stroma in all eyes of CHG and CHG+L treatment. On the other hand, less necrosis and more reepithelialization were observed in the ISOT and LS treated eyes (Table 3) and (Figs. 5 A, B, C, D).

Stromal haemorrhages (Table 3 and Fig. 8) were found in all groups being more frequent in the CHG+L (4/6) treated eyes.

While PMN infiltrations was seen in all groups, it was remarkably higher in the CHG and CHG+L treated eyes (Figs. 5 B, C) and lower in the LS and ISOT treated eyes (Figs. 5 A, D and Table 3).

Increases of collagen and fibroblasts were observed in the stroma where reepithelialization occurred with no statistically significant differences among groups (Table 3). While no PMN accumulation was observed in the anterior chamber in the LS and ISOT treated eyes, it was found in the CHG (4/6) and in the CHG+L (2/6) treated eyes. While no PMN infiltration was observed in the limbus of ISOT treated eyes, it was found in the CHG (3/6), CHG+L (4/6) and LS (1/6) treated eyes (Table 3).

**Day 14 Following Treatment**

Although thickening of stroma was observed in all groups, it was smaller in the LS and ISOT treated eyes (Figs. 6 A, B, C, D) and the difference between the two groups was not statistically significant. On the other hand, it was significantly (p < 0.05) different in the LS and ISOT treated eyes compared to the CHG and CHG+L treated eyes (Table 2). Corneal thickening did not reach the levels of normal corneal thickness (467±17.8 µm) in all groups. However it was closer in the LS (684±38.2 µm) and ISOT treated eyes (811±52.2 µm) followed by CHG+L (1059±46.5 µm) and CHG (1188±19.5 µm) treated eyes. No statistically significant difference (p > 0.05) was found between days 2, 7 and 14 in the ISOT treated eyes for the corneal thickening. On the other hand, progressive thickening of the stroma was observed with statistically significant difference (p < 0.05) between days 2, 7 and 14 in the CHG treated eyes. Stromal thickening was significantly larger (p < 0.05) at days 7 and 14 than at day 2 with no significant difference (p > 0.05) between days 7 and 14 in the CHG+L treated eyes. While stromal thickening was significantly smaller (p < 0.05) at day 14 than at day 7, no significant difference (p > 0.05) was found between days 2 and 14 and days 2 and 7 in the LS treated eyes (Table 2).

No necrosis was observed in the corneal epithelium and upper part of the stroma in the LS treated eyes. However, it was observed in the ISOT (1/6) and CHG (6/6) treated eyes, respectively (Table 3). While complete reepithelialization was found in and in the LS (4/6) and ISOT (3/6) treated eyes complete reepithelialization was observed in none of the CHG and CHG+L treated eyes. Epithelium returned to normal appearance in places where complete reepithelialization occurred in the LS treated eyes (Fig. 11). Slight PMN infiltration was seen in the stroma of eyes (2/6) in the LS and ISOT treated eyes but dense infiltration was present in the all of the CHG (Fig. 9.) and CHG+L (Figs. 6 B, C) treated eyes (Table 3). There was no statistically significant difference (p > 0.05) for the PMN infiltration in the stroma between the LS and ISOT treated eyes and between the CHG and CHG+L treated eyes. It was statistically significant (p < 0.05) in the LS and ISOT treated eyes compared with CHG and CHG+L treated eyes (Table 3).

Mononuclear cell infiltrations were lower in the LS (1/6) and ISOT (3/6) treated eyes compared to other treatment modalities in the CHG (5/6) (Fig. 10) and CHG+L (6/6) treated eyes in the periphery of cornea where reepithelialization occurred. New capillary formation was noted in the peripheral stroma of all eyes of the CHG (Fig. 10) and CHG+L treated eyes, and 4/6 and 1/6 in the ISOT and LS treated eyes, respectively. Stromal haemorrhage was observed with no statistically significant difference (p > 0.05) among the groups (Table 3). No statistically significant difference was observed for the fibroblast and collagen in the reepithelialized stroma among the groups (Table 3) with the increase seen in all the eyes in the CHG (Fig. 10) and CHG+L treated groups and that increase was lower in the LS (2/6) and ISOT (4/6) treated eyes.

While no PMN accumulation was observed in the anterior chamber in all groups, PMN infiltration was observed only in 1 eye in the ISOT group (Table 3).

Discussion

Because the severity of HF corneal burns depends on the physical and chemical characteristics and that clinical outcome is not stereotypic, it is difficult to establish optimal mode of clinical therapy.

Buffering is an exothermic reaction and therefore chemical neutralization is of limited value [26]. Although the use of an amphoteric solution, binding both base and acid, has been found beneficial compared to conventional buffers and electrolyte solution, it still has some exothermic reactivity [8] which would be harmful to the tissues. Irrigation of the eye is the most important factor to avoid progression of chemical burn damage following HF eye injuries [8, 14, 26]. It acts by removing mediators from the corneal and conjunctival surface [22]. Using low osmolarity fluids increases corneal oedema by continued uptake of water and diffusion of the diluted...
agent into the cornea. Therefore, in order to prevent water influx into the cornea, high osmolarity fluid use was recommended [26]. It was reported that following HF eye burns, irrigation of the eye with 500 mL ISOT 3 times a day for 2 days provided some accelerated healing by day 7 although cessation of irrigation after day 2 resulted in deterioration of clinical and histopathological signs at day 14 [28]. In the current study, on the other hand, continued use of 500 mL of ISOT 2 times a day as long as 2, 7 and 14 days caused progressive corneal healing. These results show the importance of irrigation in accordance with other studies [16] but its amount, duration and frequency needs to be established in order to have complete resolution as quickly and efficiently as possible. In addition to this, it would be wise to try and see the effects of different osmolarities of saline higher than ISOT, taking also the advantages of being not a buffer preventing an exothermic reaction to hamper the beneficial effects.

Although epithelial necrosis was seen in all groups at day 2 of treatment, its persistence at days 7 and 14 with heavy PMN infiltration especially in CHG and CHG+L treated eyes with incomplete reepithelialization was attributed to the gel structure of the CHG and CHG+L formulations. They might prevent the corneal surface from contact oxygenation, thus hampering the healing. Presence of epithelial necrosis less in the LS and ISOT treated eyes but better in the LS group at days 7 and 14 shows the effectiveness of LS and ISOT to the CHG and CHG+L. No accelerating effect of 1% chitosan was reported on the incisional corneal wound model [25]. We also found unsatisfactory results using CHG and CHG+L although in the latest it was better at days 7 and 14 due to inclusion of laminarin.

Laminarin sulphates with different degrees of sulphation and the commercial form of laminarin without sulphate had different effects [11]. Therefore it would be also interesting to assess the effects of different fractions of laminarin sulphates on the treatment of HF eye burns.

The finding of significant difference in corneal thickness in the LS and ISOT treated eyes compared to those treated with CHG and CHG+L with no significant difference between the LS and ISOT, shows the continuation of inflammatory reaction despite the use of treatment agents with superiority of LS followed by ISOT.

Conjunctiva may prevent more satisfactory healing of corneal [23, 28]. Therefore debridement of necrotic conjunctiva was reported to reduce the fluoride level for better healing [23] and a film between the two organs may contribute to the improved effect of corneal healing.

Based on the superiority of laminarin to CHG and CHG+L with no statistically significant difference between the ISOT in the corneal HF eye burns, the use of ISOT by itself with different osmolarities as well as an adjuvant prior to main drug use and/or together would be advocated for further investigation. In addition to this, better healing effect of CHG+L because of the additive effect of Laminarin compared to only CHG use which had no effect at the end of 14 days, would also urge one to use different treatment alternatives as a combination of more than one drug to have more satisfied results.

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References

1. - ADAMS S. S., HEATHCOTE B. V., WALKER D.: Sulphated degrada-
2. - ASARI A., MORETA M., SEKIUCHI T., OKAMURA K., HORIE K., MIYACHI S.: Hylaronuran, CD44 and fibronectin in rabbit cor-
3. - AWAD E. M., OSMAN O. A.: Laminarin enhanced immunological disorders of septicemic albino rats infected with aerosomus hydro-
4. - BEIRAN I., MILLER B., BENTUR Y.: The efficacy of calcium glu-
5. - BENTUR Y., TANNENBAUM S., YAFFE Y., HALPERT M.: The role of calcium glu-
6. - CHOU T.C., FU E., SHEN E.C.: Chitosan inhibits prostaglandin E2 formation and cyclooxygenase-2 induction in lipopolysaccharide-
7. - GEN L., FAGERHOLM P., KIM H.J.: Effect of leukocytes on cor-
Physiol., 1955, 33, 545-552.
12. - KIM K.H., KIM Y.W., KIM H.B., LEE B.J., LEE D.S.: Anti-apop-
totic activity of laminarin polysaccharides and their enzymatically hydrolyzed oligosaccharides from Laminaria japonica. Biotechnol.
tzungen der Hornhaut und deren therapeutische BeeinuÈfbarkeit.
15. - LIN Y., CHEN Q., LUO H.: Preparation and characterization of N-


