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Preparation and *in vivo* toxicity study of allantoin incorporated hyaluronic acid-L-cysteine oral solution: A future treatment for mucositis

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Abstract

Background: Thiolated hyaluronic acid (HA) with interesting properties, such as muco-adhesiveness, enzyme inhibitory, permeation enhancing, and release controlling properties can be applied for drug delivery in various diseases like mucositis. The purpose of this study was to evaluate the stability and toxicity of thiol modified HA by the aid of L-cysteine ethyl ester hydrochloride (Cys) named (HA-Cys) and allantoin (Alla) incorporated HA-Cys (HA-Cys-Alla) to reveal their potential for the future treatment of mucositis.

Methods: The HA modification and drug incorporation were investigated using FTIR spectroscopy. The evaluation of *in vitro* cytotoxicity on Caco-2 cell line by means of MTT assay and *in vivo* toxicity by measuring the hematological and biochemical parameters in rats were performed. The appearance stability of HA-Cys and HA-Cys-Alla was evaluated at room and refrigerator temperatures over time. In addition, the stability of HA-Cys and HA-Cys-Alla subjected to heating and cooling, freeze-thaw, centrifugal forces, as well as the pH stability under the above-mentioned conditions were also investigated.

Results: The results indicated that the synthesized HA-Cys and HA-Cys-Alla with pseudo-plastic rheological behavior demonstrated excellent stability at refrigerator temperature. Although HA-Cys showed good stability, the HA-Cys-Alla revealed color change at room temperature. Moreover, despite no much resistance of HA-Cys and HA-Cys-Alla against the heating-cooling test, the samples exhibited good resistance against freeze-thaw and centrifugal forces. Also, convenient pH stability and high *in vitro* and *in vivo* biocompatibility were observed.

Conclusions: The low *in vitro* and *in vivo* toxicity and convenient stability of HA-Cys-Alla has introduced it as a proper candidate for future clinical applications.

Keywords: Allantoin; Hyaluronic acid; L-cysteine; Stability; Toxicity; Mucositis.

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Introduction

Oral mucositis is defined as a painful inflammation of oral mucosa restricting the efficiency of anticancer therapy.¹ Despite the preparation of different oral solutions for the prevention and treatment of oral mucositis, such as chlorhexidine and benzydamine, they were not entirely successful.¹ The low retention time and bioavailability due to the wet surface and constant movement of the mucosa is the main limitation of the routine formulations. Thus, designing new formulations with mucoadhesive property can be very convenient.² Thiol-bearing compounds through disulfide bond formation can interact with cysteine groups of mucin. Accordingly, through attaching sulfhydryl groups to the backbone of different polymers, a new generation of mucoadhesive polymers, called thiolated polymers or thiomers have been developed. The thiolation can improve muco-adhesiveness.³ This structural modification was conducted on different polymers, including alginate, chitosan (CS), polycarbophil, polyacrylic acid, xyloglucan, carboxy methylcellulose, poly-aspartamide, and hydroxyethyl to prompt their characteristics.³⁻¹⁰ Hyaluronic acid (HA) is another polymer that was studied for thiolation. HA, as a linear polysaccharide, is found throughout the body, from the vitreous of the eye to the extracellular matrix (ECM) of cartilage tissues. HA, as the main component of ECM, has crucial impacts on cell signaling, wound repair, morphogenesis, and matrix organization.¹¹ It was reported that a spray containing sodium hyaluronate (Mucosamin®) accelerated wound healing and pain relief of oral mucositis.¹² HA can be easily modified to alter its biological activity.¹¹ The presence of carboxylic acid in the structure of HA can facilitate the immobilization of the thiol groups and the formation of the amide bond in the presence of carbodiimides.³ Thiolation of HA through incorporating

sulfhydryl ligands onto its backbone and the ability to form disulfide bond within the polymer itself and with biological materials can endow the HA with exciting properties such as muco-adhesiveness,¹³ enzyme inhibitory,¹⁴ permeation enhancing,¹⁵ and release controlling properties.¹⁴ In contrast to unmodified mucoadhesive polymers interacting with mucus only through weak interactions, the formation of disulfide bonds between thiolated HA and mucus with cysteine-rich domains can improve the muco-adhesiveness. By improving mucoadhesion ability and high biocompatibility, thiolated HA can be applied for drug delivery through various pathways, including trans-mucosal, gastrointestinal, buccal, oral, nasal, ophthalmic, and vaginal routes.¹⁶⁻²⁰ Different agents such as aldehydes, epoxides, divinyl sulfone, and disulfide carbodiimide-mediated coupling of HA to primary amines can be applied to modify HA. According to the study of Krum Kafedjiiski et al. (2007) using a double catalytic system, in this study, the chemical modification of HA with L-cysteine ethyl ester hydrochloride (Cys) was conducted to prepare HA-Cys.¹³ Antioxidant capability of Cys along with positive effects of thiolated HA in the HA-Cys can be very advantageous to employ for curing the mucositis.²¹ In addition, the capacity of this formulation for incorporation of the model drug allantoin (Alla) as a popular anti-inflammatory agent to increase the therapeutic effects in the case of mucositis applications was investigated. Alla speeds up the growth of connective tissues, bones, and cartilage, while possessing antioxidant and anti-inflammatory activities.²² It provides restoration of the damaged skin and wound healing, which are regarded as a regulation of inflammatory responses through the prohibition of immune cell chemotaxis in the wound area, prevention of reactive oxygen intermediate release, stimulation of fibroblast proliferation, and matrix synthesis.²² Overall, in the present study, in addition to HA modification and drug loading, HA-Cys and HA-Cys-Alla were evaluated in terms of *in vitro* and *in vivo* toxicity, as well as stability at different storage conditions to demonstrate their potential for

the future application in oral mucositis. Besides, combined effects of thiolated HA, Cys and Alla in biocompatible HA-Cys-Alla offer a new option with a multifaceted mechanism of action to treat mucositis.

Materials and Methods

Materials

HA, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC), Cys, n-hydroxysuccinimide (NHS), thiazolyl blue tetrazolium bromide (MTT), Alla, sodium hydroxide, hydrochloric acid, dialysis bag (2000 Da), Hank's balanced salt solution-(4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) (HBSS-HEPES), Dulbecco's Modified Eagle's Medium (DMEM), fetal bovine serum (FBS), penicillin, L-glutamine, nonessential amino acids, streptomycin, dimethyl sulfoxide (DMSO) were purchased from Merck (Germany). The human epithelial cell line Caco-2 were from Pasteur Institute (Iran).

HA modification and drug incorporation

The attachment of the thiol group using Cys and in the presence of EDC and NHS as catalysts was performed according to the method developed by Krum Kafedjiiski et al. (2007).¹³ In this regard, first, the HA solution (0.4 % w/v) was prepared in deionized water under stirring, and the pH adjusted at 5.5 using HCL (1 M). Then, EDC (6 mM) and NHS (6 mM) were added to the solution under stirring, respectively. Following stirring the mixture reaction for 2 h in the dark using a glassware covered with aluminium foil, Cys (0.04 w/v) was added, and the pH of the mixture reaction was adjusted at 6.8 using NaOH (1M). Subsequently, the mixture reaction was incubated in the dark for 15 h under stirring. After the completion of the reaction, the excess of EDC and

NHS was removed from the final product using dialysis against deionized water for 12 h. To incorporate the drug, Alla as a drug model with a concentration of 0.4 % w/v was added to one part of the resulting conjugate and incubated for 15 min at room temperature under stirring. The samples with and without the drug were centrifuged at 7000 rpm for 10 min for purification.²³ The successful HA modification and incorporation of Alla as a drug model were evaluated using FTIR spectroscopy (Bruker, Germany). The ATR-FTIR spectra of Alla, HA, Cys, HA-Cys, chemically conjugated HA-Cys, HA-Cys-Alla, and chemically conjugated HA-Cys-Alla were recorded in the wavelength of 4000–400 cm^{-1} at room temperature.

Stability studies

Stability studies as per international conference and harmonization (ICH) guidelines

Stability studies of HA-Cys and HA-Cys-Alla were performed by keeping the samples at refrigerator (4 °C) and room temperature (25 °C) and then evaluated at 24, 48, 96 h, 1, 2, 3 weeks and 1, 3 and 5 months in terms of color, homogeneity, transparency and the presence of suspended particles.

Freeze-thaw test

To evaluate the stability of HA-Cys and HA-Cys-Alla under freeze-thaw conditions, HA-Cys and HA-Cys-Alla samples were subjected to the freezer of -21 °C for 48 h followed by thawing at 25 °C for 48 h during six cycles of freeze-thaw process, and they were evaluated in terms of the above-mentioned characteristics.

Heating and cooling test

In order to investigate the stability of HA-Cys and HA-Cys-Alla samples against temperature changes, six cycles between refrigerator (- 4 °C) and oven (40 °C) temperatures with storage of 48 h were performed, and the appearance alterations of samples were recorded.

The pH stability test

To evaluate the pH stability over time, the apparent pH of the HA-Cys and HA-Cys-Alla samples was measured by a pH meter (Metrohm, 827 pH Lab, Switzerland) at 0, 24, 48, 96 h, 1, 2, 3 weeks, and 1, 3 and 5 months at room (25 °C) and refrigerator (4 °C) temperatures. Furthermore, the pH stability of HA-Cys and HA-Cys-Alla after different cycles of freeze-thaw and heating-cooling tests was evaluated, too.

Centrifuge test

To investigate the stability of HA-Cys and HA-Cys-Alla samples against centrifugal force, the samples were subjected to centrifugation for 5, 15, 30, and 60 min at 2,000 rpm using a centrifuge (Eppendorf 5430, Germany), and their stability was evaluated. The stable formulations did not show any phase separation or turbidity.

Rheology studies

Rheological measurements were performed inside a rheometer (AMETEK Brookfield's rheometer, USA) equipped with a cone and plate geometry at 25 °C and flow curves were obtained, using a steady-state flow ramp at 0–300 s⁻¹ shear rate during 10 min.

Cell viability assay

The cell cytotoxicity assay of HA-Cys and HA-Cys-Ala samples was conducted on Caco-2 cells with three different concentrations (50, 100, and 250 µg/mL) compared with a negative control group ((Hank's balanced salt solution-(4-2-hydroxyethyl-1-piperazineethanesulfonic acid) (HBSS-HEPES, pH 7.4)) by means of MTT assay.¹⁵ For this purpose, prior to the analysis, Caco-2 cells were cultured in DMEM, which was enriched with 10 % v/v FBS, 100 IU mL⁻¹ penicillin, 1 % of L-glutamine, 1 % of nonessential amino acids, and 0.1 mg mL⁻¹ streptomycin. Following the preparation of the cell suspension in DMEM, the cell suspension was transferred to a 96 well plate and incubated overnight. After the attachment of the cells overnight, their media was removed, and different concentrations of HA-Cys and HA-Cys-Ala samples were added and incubated for 48 h at 37 °C in the incubator. Then, a MTT solution (5 mg/mL) diluted in PBS was added to each well and incubated for 2 hours at 37 °C. To solubilize the formazan crystals, DMSO was added to each well. Then the cell viability was measured using colorimetric measurements and a microplate reader (Infinite M 200, TECAN, Switzerland) at 570 nm wavelength as following equation (1):

$$\text{Cell viability (\%)} = \frac{\text{Average absorbance value of each sample}}{\text{Average absorbance value of negative control}} \times 100 \quad (1)$$

In vivo toxicity studies

In addition to cell cytotoxicity, *in vivo* toxicity was performed to evaluate the *in vivo* biocompatibility of HA/Cys, as well as the drug incorporated HA/Cys conjugate. For this reason, animals were kept in an animal room for a week for adaptation to the new environment. During the test period, 12 h light/dark daily cycle, an environmental temperature of 21–23 °C, and relative humidity of 50–60 % were established in the animal room. To perform the animal study, 9 male

Sprague-Dawley rats with a weight range of 230–250 g were divided into 3 groups, including control, HA-Cys, HA-Cys-Alla. At 1, 2, and 3 day-time periods, 1 mL of HA-Cys and HA-Cys-Alla formulations were administered to the animals using oral gavage, and in the control group, no treatment was employed. Seven days after administration, to monitor and compare blood biochemistry and hematological factors in different groups, 2 mL of blood was collected from animals in all groups.

Statistical analysis

Data sets are reported as mean $\pm$ SD (n=3). Statistical analysis was conducted by means of SPSS software and through one or two-way ANOVA as well as Dunnett's post-hoc test as part of ANOVA analysis at the probabilities of $*P < 0.05$ to demonstrate the levels of statistical significance.

Results and Discussion

HA thiolation and drug incorporation

In this study, modification of HA was performed through covalent attachment of Cys with thiol groups on the HA backbone with the aid of EDC and NHS as coupling catalysts. Firstly, EDC reacts with the carboxyl groups of HA to form an amine-reactive O-acylisourea intermediate. Since the above-mentioned intermediate is unstable in aqueous solutions and is sensitive to hydrolysis which may result in the decreasing the yield of thiol moieties, NHS was added to create amine-reactive intermediates for improving the coupling yield, and by adding Cys and through the formation of the amide bond, the thiol-bearing HA-Cys conjugate was prepared.²⁴ The IR spectra demonstrated the succeeded HA modification and drug incorporation. Figure 1 reveals the FTIR spectrum of Cys, HA, HA-Cys, and HA-Cys-Alla conjugates. The presence of a strong and wide

band at 3450 cm^{-1} in the HA IR spectrum is related to OH stretching and NH bending vibrations. The absorption band at 2925 cm^{-1} can be attributed to the C-H stretching vibration. Moreover, the bands at 1670 and 1599 cm^{-1} correspond to the stretching vibrations of carbonyl groups of the carboxylic acid moiety. The bands at 1420 and 1040 cm^{-1} can be attributed to the carboxylate symmetric stretching and C-O-C stretching vibration of HA skeleton. Furthermore, the absorption bands at $1020\text{-}1250\text{ cm}^{-1}$ and 1039 cm^{-1} are related to C-N and C-OH stretching vibrations, respectively.^{25,26} In the FT-IR spectrum of Cys, the absorption bands at $1550\text{-}1650\text{ cm}^{-1}$ are related to carbonyl groups symmetric and asymmetric stretching vibration, $1200\text{-}1250\text{ cm}^{-1}$ are related to stretching vibration of (C-O). Moreover, the absorption bands at 858 cm^{-1} and 1581 cm^{-1} are related to NH stretching vibrations. Furthermore, in the spectrum of Cys, the wide peaks are observed in the area of 3450 cm^{-1} (O-H), 2964 cm^{-1} (N-H), 1056 cm^{-1} (C-N), $600\text{-}800\text{ cm}^{-1}$ (C-S), and 2540 cm^{-1} (S-H).²⁷ In the HA-Cys and HA-Cys-Ala spectra, the presence of a sharper peak at 1567 cm^{-1} , which is corresponded to the formation of a new β bond of the NH group, the removal of the C-OH carboxylic acid band at 1039 cm^{-1} and the alteration in the absorption band at 1056 cm^{-1} related to stretching vibrations of (C-N) indicate the successful attachment of the Cys with thiol group on the HA backbone through amide bond formation.²⁸ Moreover, because of amide bond formation between amine groups of Cys and carboxylic groups of HA, the broad peaks related to OH groups around $3200\text{-}3550\text{ cm}^{-1}$ in HA-Cys and HA-Cys-Ala shifted to lower wavelengths with less broadening.²⁹

Furthermore, the presence of a new absorption band at 1720 cm^{-1} in HA-Cys-Ala compared with HA-Cys spectrum, which is related to C=O stretching vibration of Ala, indicates the incorporation of the drug within HA-Cys sample.³⁰

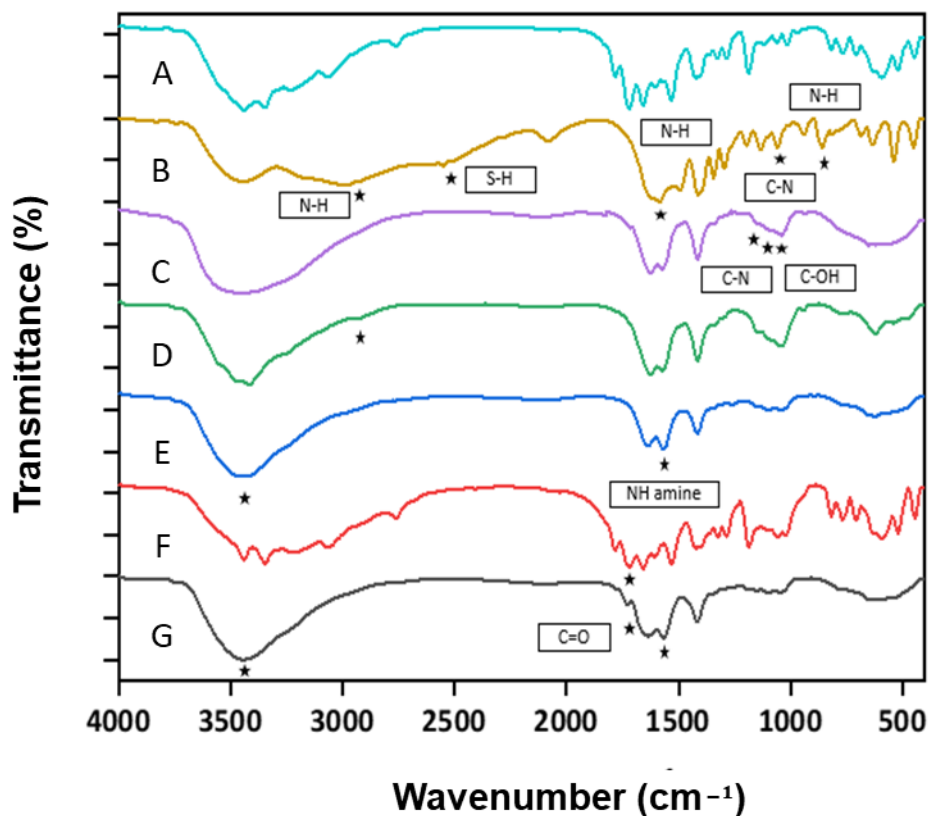


Figure 1. FTIR spectra of Alla (A), Cys (B), HA (C), HA-Cys (D), chemically conjugated HA-Cys (E), HA-Cys-Alla (F), and chemically conjugated HA-Cys-Alla (G).

Stability studies

The purpose of stability tests was to investigate the quality of formulations varying with time under the influence of thermal stress shocks such as freeze-thaw, heat, and cool and recommended storage conditions.³¹ To perform the stability tests, the samples under different temperature conditions were withdrawn at regular time intervals and then investigated in terms of color, homogeneity, transparency and the presence of suspended particles.³¹ In thermal stability tests, as shown in Figure 2, at 25 °C, the color of HA-Cys samples did not change for 48 h, while, after 96 h, it became a little cloudy; from the third week, the color of the sample was changed to pale

yellow. But, HA-Cys-All samples at 25 °C remained stable in terms of appearance and physical characteristics for up to 96 hours. After 96 hours, the color of the sample became bright yellow. In the second week, they turned to dark yellow, and after one month, the color of drug incorporated samples changed into bright red. The results demonstrated that the HA-Cys-Alla samples at room temperature showed more color changes, while these changes were less pronounced in HA-Cys samples. But, both HA-Cys and HA-Cys-Alla samples displayed excellent stability at refrigerator temperature (4 °C), and they did not show any color, homogeneity, and transparency changes over time.

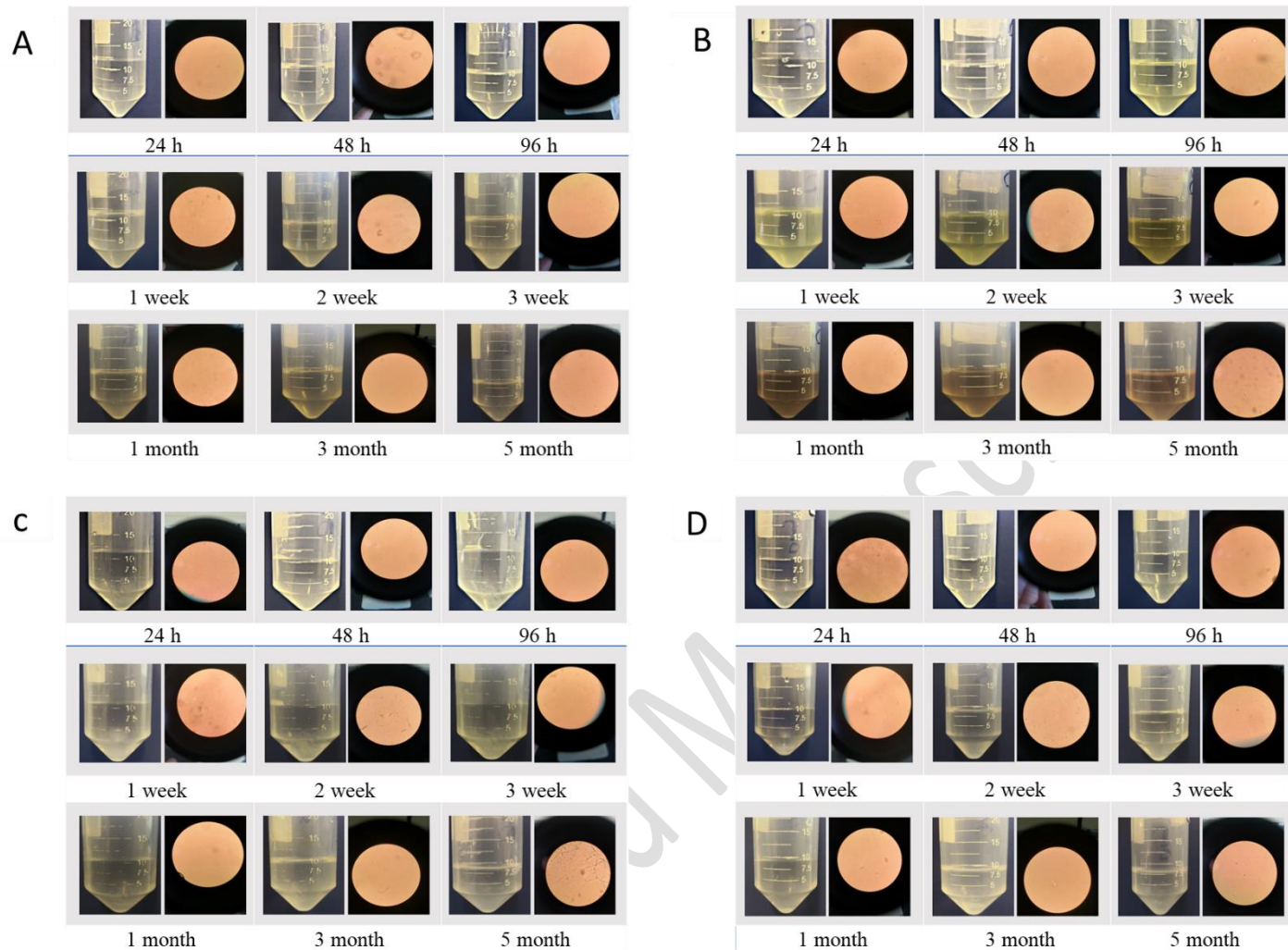


Figure 2. Stability studies: (A) HA-Cys samples at room temperature (25 °C). (B) HA-Cys-Alla samples at room temperature (25 °C). (C) HA-Cys samples at refrigerator temperature (4 °C). (D) HA-Cys-Alla samples at refrigerator temperature (4 °C).

The formulations remained stable against temperature changes (between 4 and 45 °C) until the end of the sixth cycle, and alteration in the physical stability of the samples (HA-Cys and HA-Cys-Alla) was not observed (Figure 3-A & 3-B). The formulations did not show much resistance to

temperature changes (between -21 & 25 ° C) and were able only to maintain their physical stability for up to 96 hours, and then their color and consistency changed. However, the color change was much more noticeable in drug-containing formulations. During this test, no significant differences were observed in the drug-free samples (HA-Cys) after one cycle, but after the third cycle, the samples became a little cloudy. Following the third cycle, the turbidity remained constant, but the color of the samples turned bright yellow. The color and turbidity did not change after the fourth cycle, but the consistency of the sample increased, and in some parts, it became clotted. In the samples with the drug (HA-Cys-Alla), after two cycles, no change in the physical properties of the samples was observed. But after the third cycle, the samples became cloudy. After the fourth cycle, the samples were slightly coagulated and clotted (Figure 3-C & 3-D). The results of the centrifuge test also demonstrated the convenient resistance of both samples against centrifugal forces (Figure 3-E & 3-F).

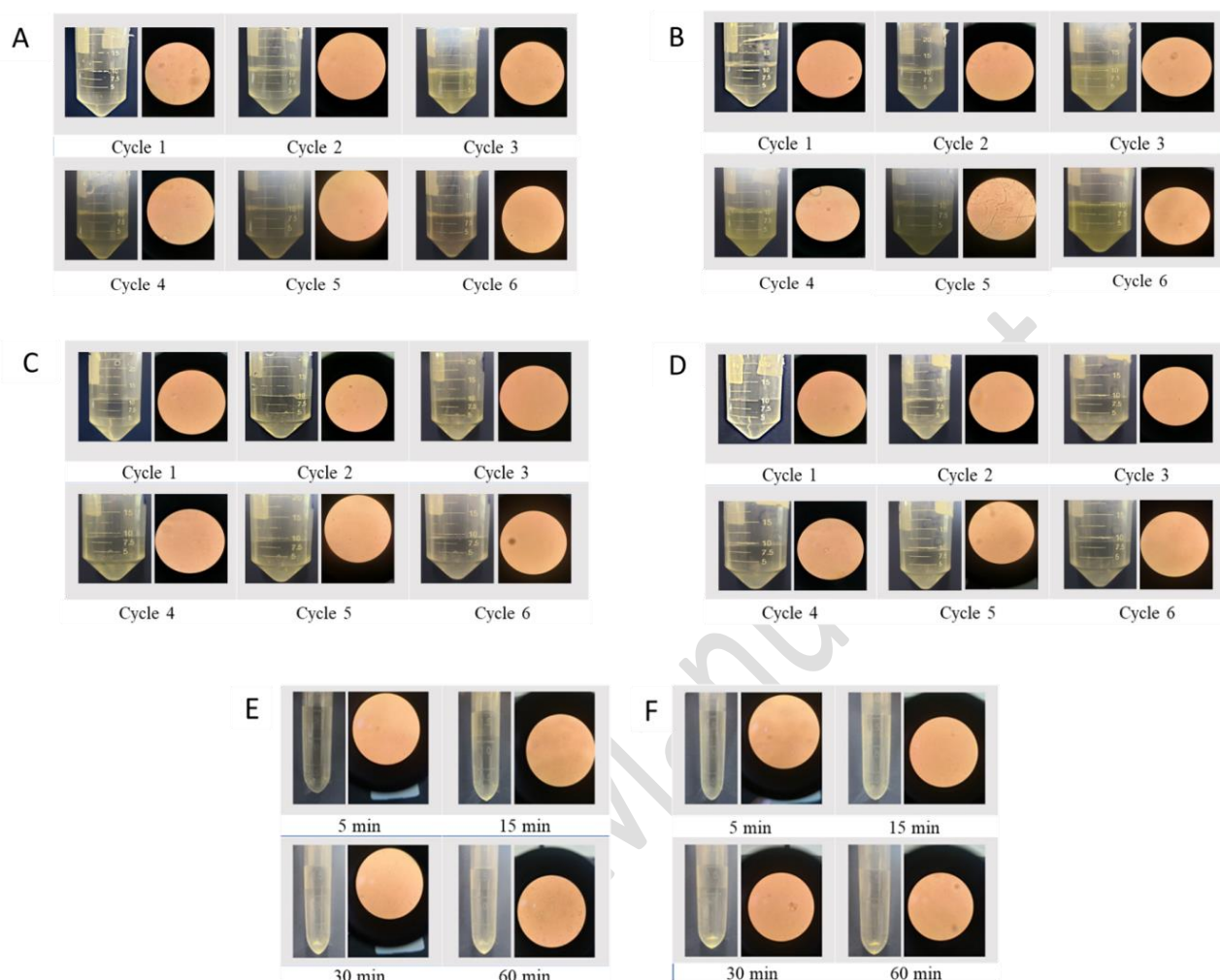


Figure 3. Stability studies: (A) HA-Cys subjected to the freeze-thawing test. (B) HA-Cys-Alla subjected to freeze-thawing test. (C) HA-Cys samples subjected to heating and cooling test. (D) HA-Cys-Alla samples subjected to heating and cooling test. (E) HA-Cys subjected to centrifuge test. (F) HA-Cys-Alla samples subjected to centrifuge test.

In terms of pH stability, HA-Cys and HA-Cys-Alla samples in the refrigerator during 5 months demonstrated no significant alteration. Only, HA-Cys-Alla sample at room temperature showed a slight change (Table 1). Moreover, the freeze-thaw and heating-cooling process during 6 cycles did not significantly change the pH of HA-Cys and HA-Cys-Alla samples (Table 2 & 3).

Table 1. pH of HA-Cys and HA-Cys-Alla at room and refrigerator temperatures during 5 months. Data are reported as the mean $\pm$ SD (n =3).

	25 °C (room temperature)		4 ° C (refrigerator)	
Time	HA-Cys	HA-Cys-Alla	HA-Cys	HA-Cys-Alla
0 h	6.8			
24 h	6.91 $\pm$ 0.01	6.83 $\pm$ 0.02	6.97 $\pm$ 0.3	6.88 $\pm$ 0.005
48 h	6.83 $\pm$ 0.02	6.85 $\pm$ 0.03	6.96 $\pm$ 0.06	6.88 $\pm$ 0.01
96 h	6.68 $\pm$ 0.03	6.87 $\pm$ 0.05	6.82 $\pm$ 0.01	6.80 $\pm$ 0.005
1 week	6.79 $\pm$ 0.14	7.02 $\pm$ 0.01	6.81 $\pm$ 0.02	6.90 $\pm$ 0.01
2 weeks	6.83 $\pm$ 0.07	7.44 $\pm$ 0.01	6.98 $\pm$ 0.01	6.94 $\pm$ 0.01
3 weeks	6.86 $\pm$ 0.03	7.64 $\pm$ 0.01	7.06 $\pm$ 0.05	6.98 $\pm$ 0.02
1 month	7 $\pm$ 0.05	7.9 $\pm$ 0.06	7.01 $\pm$ 0.03	7.01 $\pm$ 0.01
3 months	7.08 $\pm$ 0.07	8.35 $\pm$ 0.02	6.89 $\pm$ 0.08	7.01 $\pm$ 0.06
5 months	7.43 $\pm$ 0.08	8.72 $\pm$ 0.01	7.07 $\pm$ 0.01	7.1 $\pm$ 0.1

Table 2. pH of HA-Cys and HA-Cys-Alla investigated in consecutive freeze-thawing (48 h at -21° C and 48 h at 25 °C). Data are reported as the mean $\pm$ SD (n =3).

Time	HA-Cys	HA-Cys-Alla
Cycle 0	6.8	
Cycle 1	6.83 $\pm$ 0.01	6.78 $\pm$ 0.04
Cycle 2	6.71 $\pm$ 0.03	6.63 $\pm$ 0.03
Cycle 3	6.75 $\pm$ 0.1	6.87 $\pm$ 0.02
Cycle 4	6.98 $\pm$ 0.2	7.31 $\pm$ 0.04
Cycle 5	7.06 $\pm$ 0.17	7.26 $\pm$ 0.03
Cycle 6	7.17 $\pm$ 0.17	7.28 $\pm$ 0.02

Table 3. pH of HA-Cys and HA-Cys-Alla investigated in consecutive cooling and heating (48 h at 45 ° C and 48 h at 4 ° C). Data are reported as the mean $\pm$ SD (n =3).

Time	HA-Cys	HA-Cys-Alla
Cycle 0	6.8	
Cycle 1	6.7 $\pm$ 0.0	6.49 $\pm$ 0.05
Cycle 2	6.6 $\pm$ 0.02	6.25 $\pm$ 0.01
Cycle 3	6.54 $\pm$ 0.07	6.1 $\pm$ 0.06
Cycle 4	6.71 $\pm$ 0.11	6.02 $\pm$ 0.07
Cycle 5	6.4 $\pm$ 0.03	5.77 $\pm$ 0.07

Cycle 6	6.31±0.06	5.63±0.09
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Rheology studies

As shown in Figure 4, demonstrating the rheological behavior of HA-Cys and HA-Cys-Alla, the viscosities of HA-Cys and HA-Cys-Alla by increasing shear rate were significantly reduced all the shear rate range indicating their shear-thinning pseudo-plastic behavior. These rheological behaviors are due to entangled networks. The rate of entanglement formation and disruption can control the rheological responses. As the shear rate increases, the rate of disruption is highlighted, leading to the thinning behavior.³² The results demonstrated the presence of entanglements between polymer chains. Since the flow properties can influence the pharmaceutical processes, such as filling, mixing, packing, and removal from the container before the application as well as the *in vivo* behavior,³³ the change of the viscosity of pseudo-plastic systems with increasing shear rate can demonstrate their important applications in pharmaceutical formulations including hydrogel, ointment, suspensions, and emulsions through various routes such as oral, topical, ophthalmic and mucosal administration.³³ The results also indicate that the viscosity curve of HA-Cys samples did not change much after 5 months due to the high stability of HA-Cys at room temperature during 5 months. But, the viscosity curve of HA-Cys-Alla samples after 5 months revealed the decline of viscosity with a lower slope with increasing shear rate. Since Alla has hydrophilic functional groups which can interact with polymer chains and likely led to increasing the viscosity of the formulation over time. Also, the results of the thermal stability test of HA-Cys-Alla samples at room temperature demonstrated the more color change of this sample compared with HA-Cys samples.

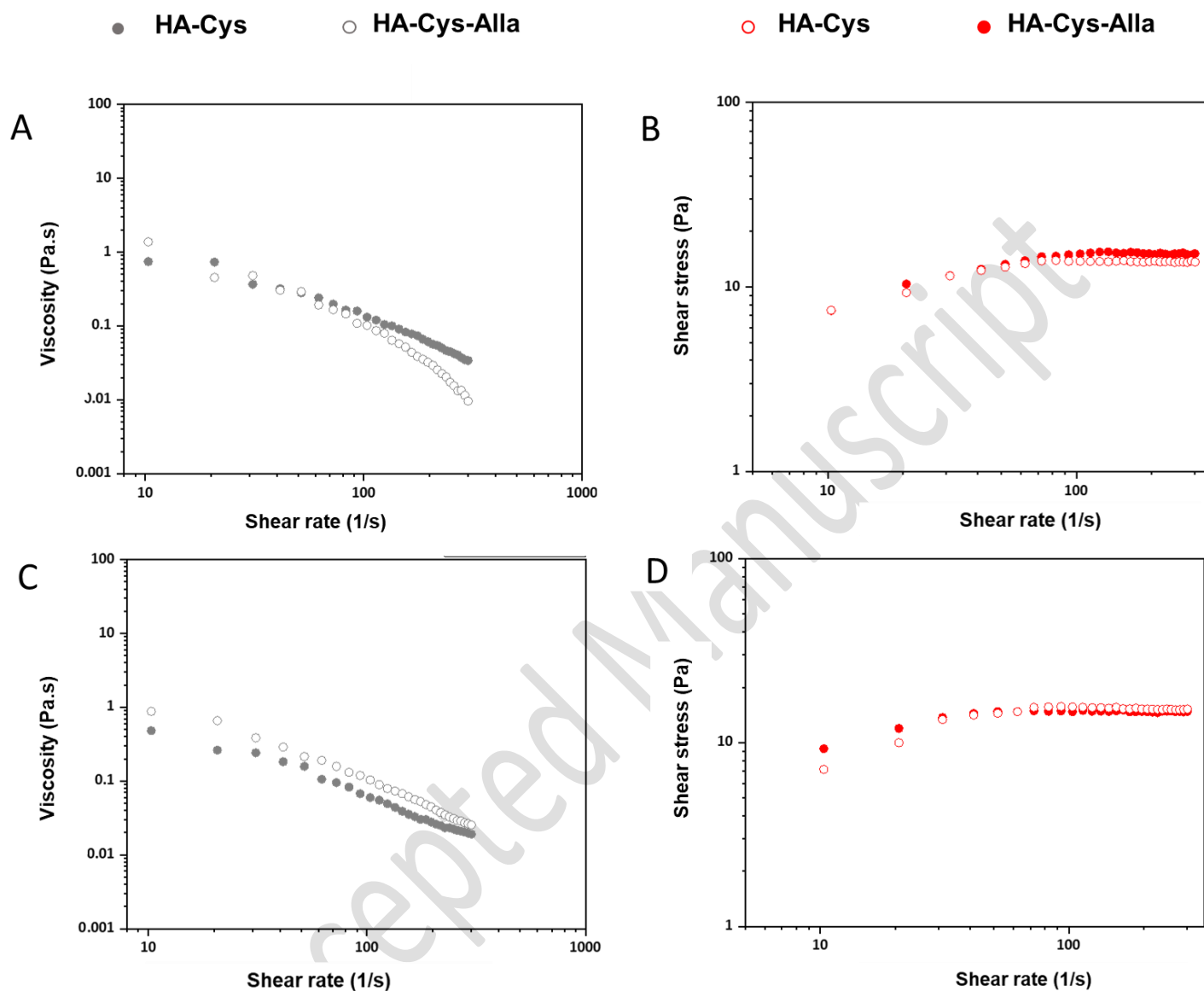


Figure 4. Rheological behavior studies. (A) Viscosity curve of HA-Cys and HA-Cys-Alla at room temperature. (B) The rheogram of HA-Cys and HA-Cys-Alla at room temperature. (C) Viscosity curve of HA-Cys and HA-Cys-Alla after storage at room temperature for 5 months. (D) The rheogram of HA-Cys and HA-Cys-Alla after storage at room temperature for 5 months.

In vitro cytotoxicity studies

The cell viability assay was conducted on Caco-2 cells by exposure to different concentrations of HA-Cys and HA-Cys-Ala for 48 h at 37 ° C. As shown in Figure 5, both HA-Cys and HA-Cys-Ala samples did not show significant toxicity on the cell viability, and the results indicated their high biocompatibility. HA as the essential component of ECM has been employed for tissue engineering and used as a possible cell carrier for tissue-engineered bone reconstruction. The low allergic and/or immunogenic reactions by HA application for tissue engineering and other applications was reported. Even in some studies demonstrated that a possible increase in cell viability associated with applying the HA as a matrix for tissue engineering was observed.³⁴ On the other hands, Ala, as a safe and non-toxic compound, induced cell proliferation and increased the percentage of cell viability.³⁰

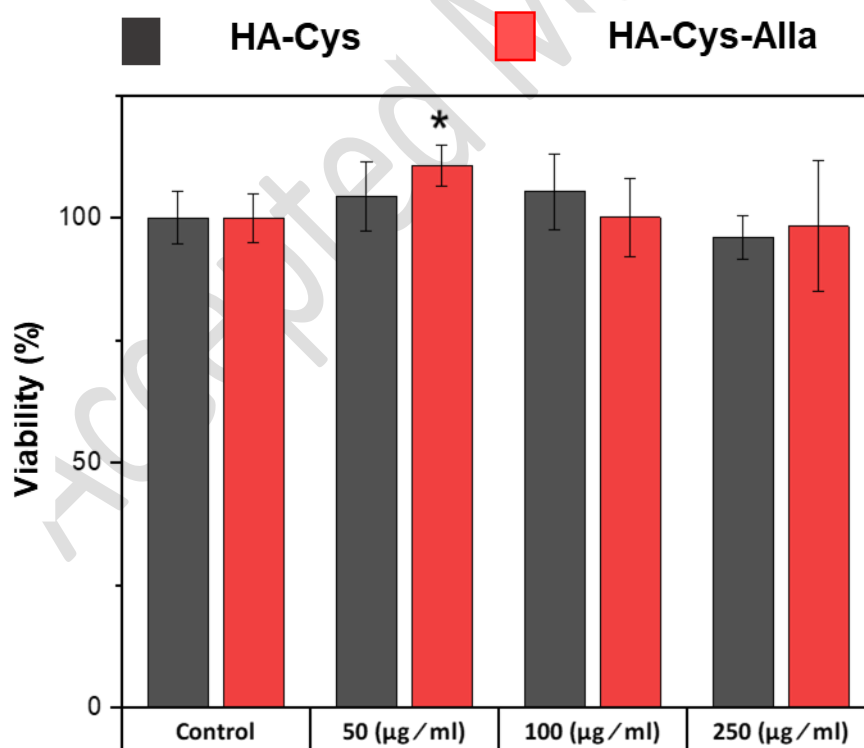


Figure 5. Cell viability assay on Caco-2 cell lines after exposure to different concentrations of HA-Cys and HA-Cys-Alla samples for 48 h at 37 ° C, quantified by means of MTT assay. Data are reported as mean $\pm$ SD (n = 3; *p < 0.05 vs HBSS-HEPES as negative control group)

In vivo toxicity studies

To investigate the *in vivo* toxicity, the levels of blood biochemical and hematological parameters were measured post administration of HA-Cys and HA-Cys-Alla formulations through the oral route. As shown in Figure 6, the hematological factors such as white blood cells (WBCs), neutrophils (NEUT), lymphocytes (LYMPH), monocytes (MONO), platelet (PLT), hemoglobin, eosinophil (EOS), red blood cells (RBCs), hematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular hemoglobin concentration (MCHC), red cell distribution weight (RDW), as well as, the levels of biochemical and minerals parameters (Figure 7), such as calcium (Ca), phosphor (ph), blood urea nitrogen (BUN), creatinine (CREA), alkaline phosphatase (ALP), lactate dehydrogenase (LDH), total protein (TP) demonstrated no significant changes in HA-Cys and HA-Cys-Alla treated groups compared with the control group and demonstrated their high *in vivo* biocompatibility.

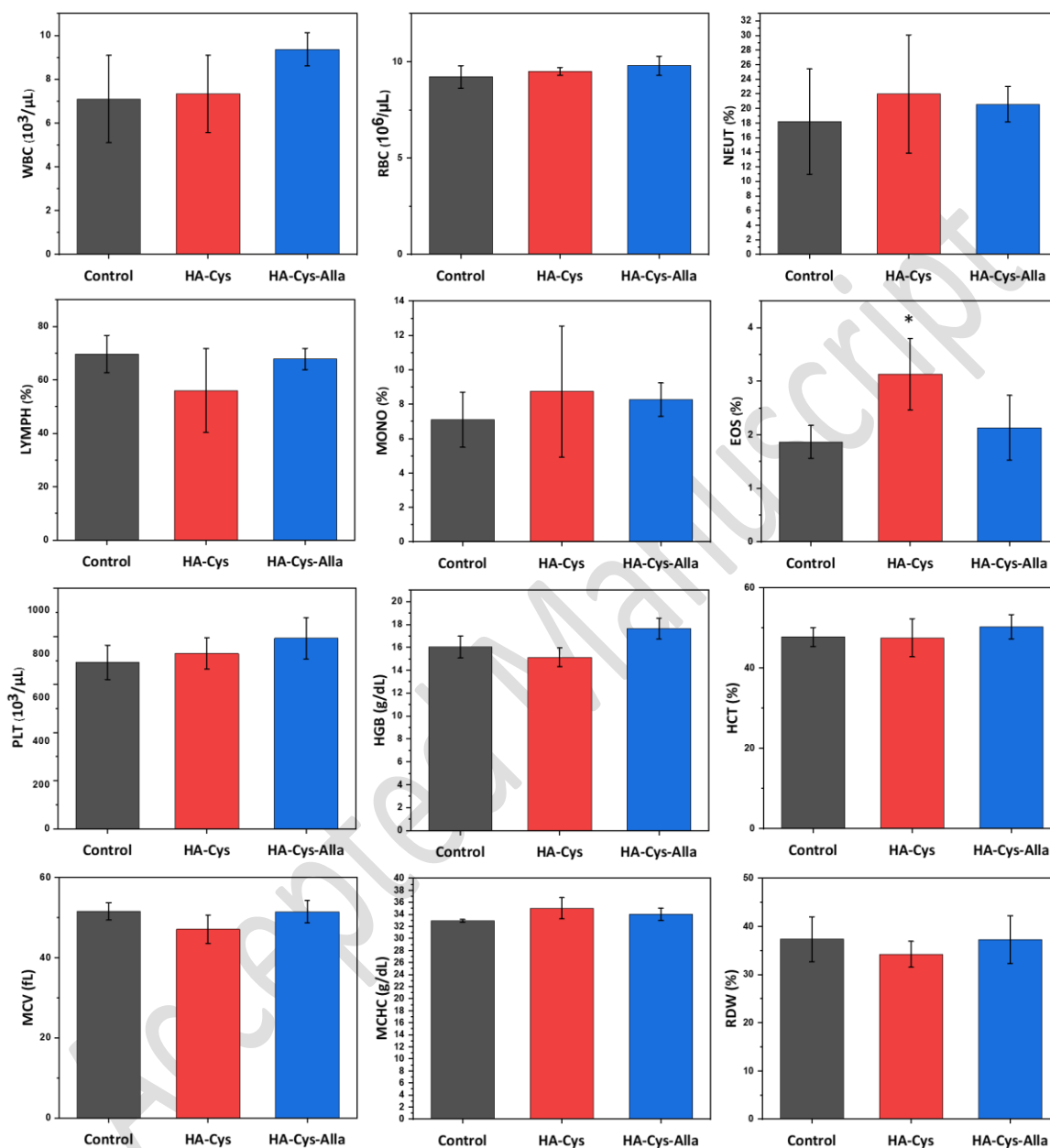


Figure 6. *In vivo* toxicity evaluation of HA-Cys, and HA-Cys-Alla samples through investigation of hematological factors after oral administration in the rat. Data are reported as the mean $\pm$ SD (n=3; *p < 0.05 vs. control group without any treatment).

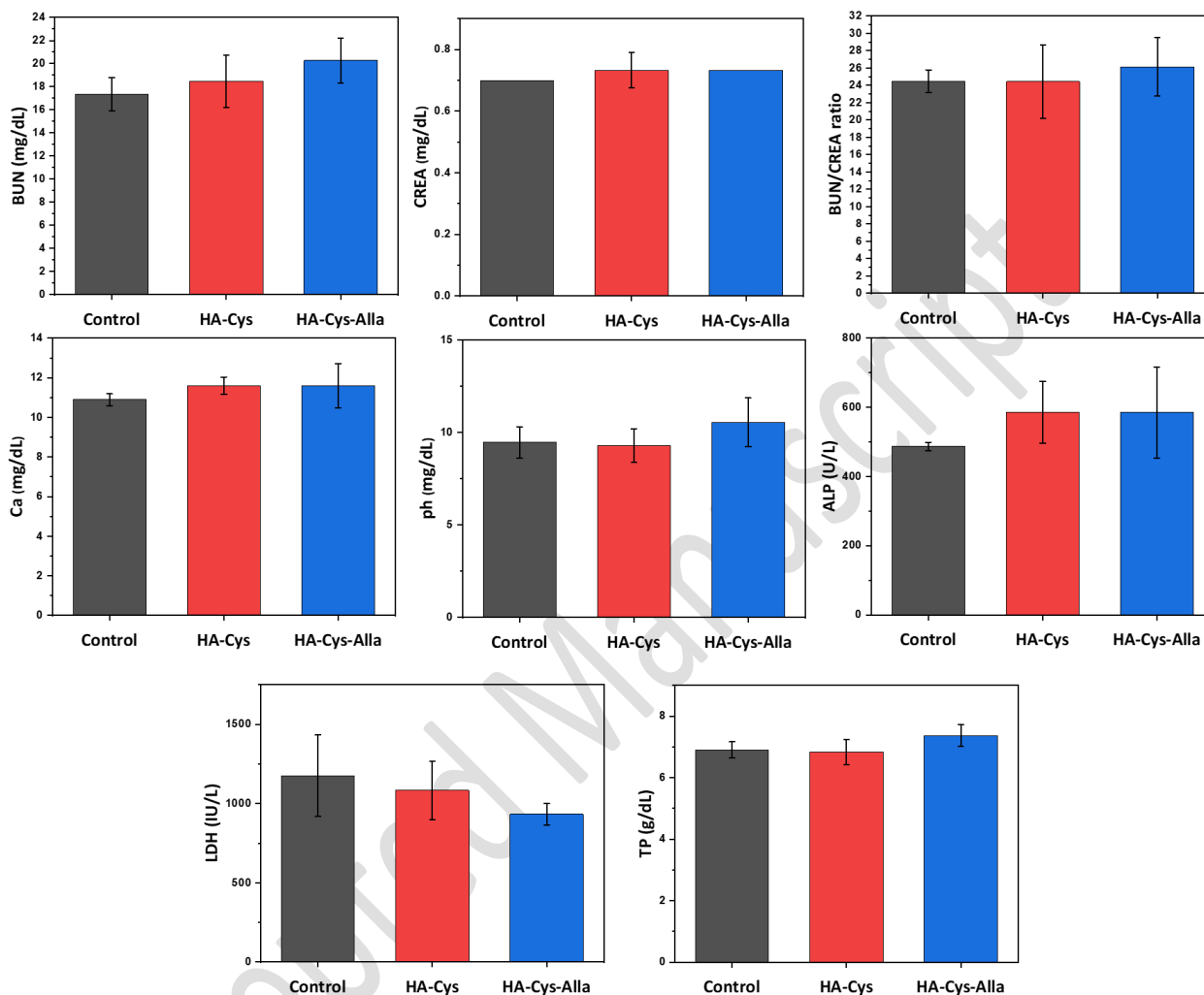


Figure 7. *In vivo* toxicity evaluation of HA-Cys, and HA-Cys-Alla samples through investigation of biochemical parameters after oral administration in the rat. Data are reported as the mean $\pm$ SD (n=3; *p < 0.05 vs. control group without any treatment).

Conclusion

In summary, in the present study using Cys as thiol bearing compound and in the presence of EDC and NHS as catalysts, HA was thiolated to obtain HA-Cys conjugate, and Alla as a popular anti-inflammatory agent was incorporated into HA-Cys to obtain HA-Cys-Alla. FTIR results indicated

successful HA modification and drug incorporation. Stability studies demonstrated the excellent stability of HA-Cys and HA-Cys-Alla at refrigerator temperature (4 °C), while HA-Cys demonstrated more stability at room temperature compared with HA-Cys-Alla. HA-Cys and HA-Cys-Alla showed no much resistance against the heating and cooling test. In contrast, both samples demonstrated high pH stability over time, as well as high stability against freeze-thaw and centrifugal forces. The results of rheology studies showed that both HA-Cys and HA-Cys-Alla have pseudo-plastic rheological behavior and indicated increased viscosity of HA-Cys-Alla over time. Moreover, HA-Cys and HA-Cys-Alla samples showed no significant toxicity effect on Caco-2 cells; also, they had no significant influence on blood biochemical and hematological parameters indicating the samples high *in vitro* and *in vivo* biocompatibility. This study demonstrates that HA-Cys-Alla solution with low toxicity and convenient stability is a promising candidate for future treatment of oral mucositis.

Author Contributions

Zainab Ahmadian: Formal analysis; data curation, methodology, writing – original draft and writing – review and editing and visualization. Ali Reza Dargahi: Investigation and validation. Kiyan Musaie: data curation and formal. Mohammad Reza Eskandari: Conceptualization, methodology, editing, supervision, and project administration. All authors read and gave approval of the final manuscript.

Conflict of Interest

Authors confirm that there are no conflicts of interest.

Ethical Issues

All experiments were conducted according to the ethical standards and protocols approved by the Committee of Animal Experimentation of Zanjan University of Medical Sciences, Zanjan, Iran (protocol approval number: (IR.ZUMS.REC.1399.090)).

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