Preparation of Chitosan Nanoparticles Loaded with Glutathione for Diminishing Tissue Ischemia-Reperfusion Injury

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Abstract- Nanoparticles composed of chitosan or chitosan plus cyclodextrin-beta comlex for tissue delivery of the glutathione were prepared. Mean size of nanoparticle systems were 100-150 nm in both groups. Encapsulation efficiency for glutathione of chitosan/cyclodextrin nanoparticles was 2,5 time higher than simple chitosan system thus leaded to improvement delivery of glutathione to mucosal layer of small intestine and diminishing tissue ischemia-reperfusion injury.

Index Terms — glutathione, nanoparticles, chitosan, cyclodextrin.

I. INTRODUCTION

Glutathione (GSH) has emerged to be one of the most fascinating molecules virtually present in all animal cells often in quite high (mM) concentrations [1]-[2]. It is known to have multifaceted physiological functions including modulation of redox regulated signal transduction, storage and transport of cysteine, regulation of cell proliferation, synthesis of deoxyribonucleotide synthesis, regulation of immune response, and regulation of leukotriene and prostaglandin metabolism. Perhaps most importantly, GSH and GSH-associated metabolism provide the major line of defense for the protection of cells from oxidative and other forms of stress [3]-[4]. However, the application of GSH as a functional food ingredient has been limited because of its low bioavailability due to its poor cellular uptake and its instability. Under conditions of oxidative stress, the GSH thiol group can be easily oxidized to glutathione disulfide, which loses its antioxidant activity based on its radical scavenging activity [5]. To achieve oral delivery of GSH, drug carrier systems are required to protect this drug from the gastrointestinal environment and from enzymatic degradation. Several nanoparticle (NP) prototypes from biodegradable polymers have been proposed for transmucosal drug delivery [6]. Among them, NPs based on the polysaccharide chitosan (CS) have shown particularly promising results due to their intrinsic properties including biocompatibility,

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mucoadhesion and ability to transiently open the tight junctions of the intestinal barrier.

II. MATERIAL AND METHODS

A. Materials

Chitosan (CS, Mw 600 kDa, degree of deacetylation 90%) is a commercial product of Bioprogress (Russian Federation). Sodium tripolyphosphate (TPP) and fluorescein isothiocyanate (FITC) was purchased from Sigma (USA). Reduced glutathione, dimethyl sulfoxide (DMSO) were purchased from Applichem (Germany), cyclodextrin-beta (CD) – from Roquette (France). All other chemicals were of analytical grade and used as received.

B. Preparation of NPs

Chitosan nanoparticles were prepared spontaneously based on ionic gelation by addition of TPP anions aqueous solution to CS solution according to the procedure first reported by Calvo et al. [7]. Different amounts of CS were dissolved in acetic aqueous solution to give concentrations of 0.1, 0.3, 0.5, 0.7, 1.0, and 2.0 mg/mL. The concentration of acetic acid in aqueous solution was 1.75 times bigger that of CS. The pH of the CS solution was adjusted to 5.4 using 0.1 N NaOH. Under magnetic stirring at room temperature, TPP aqueous solution with various concentrations (0.2, 0.4, 0.6, 0.8, and 1.0 mg/mL) was added into CS solution respectively at room temperature. In all cases CS:TPP ratio was 3:1. Three kinds of phenomena were observed: solution, aggregates and opalescent suspension. The zone of opalescent suspension was further examined as nanoparticles. For preparation of GSH loaded NPs to 9 ml of CS 2 ml aqueous solution of 0,5% w/v of GSH (1-st group) or 2 ml of mixture of GSH and CD in molar ratio 1:1 as 3.5% w/v in bidiatilled water or NaCl solution (2-nd group) were added and mixed 45 min under magnetic stirring before administration of TPP solution.

C. Morphological Examination of the NPs

The morphological examination of the NP prototypes was performed by transmission electron microscopy (TEM). For sample preparation, NPs were resuspended in water, stained with 2% (w/v) phosphotungstic acid, placed on copper grids with Formvar films and dried overnight at room temperature.

D. Yield and Entrapment Efficiency CS NP

The yield of nanoparticles fabrication process was calculated by weighing dried samples of the isolated nanoparticles and referring them to the initial amounts of

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CS, tripolyphosphate and CD [8]. The nanoparticles were separated from the aqueous dispersion medium by centrifugation at 9000 rpm for 1,5 h.The yield was calculated as follows:

yeild = 100× weight of nanoparticles / total amount of the components.

E. Determination of GSH Encapsulation Efficiency

The encapsulation efficiency (EE) of the tripeptide to the particles was calculated by an indirect method. CS or CS/CD NPs were isolated from free GSH by centrifugation (9000 rpm for 1,5 h) and free GSH in the supernatant was quantified. Experiments were performed in triplicate and the encapsulation efficiency was calculated as follows:

EE = 100× (total GSH - free GSH)/total GSH.

F. Determination of CD Content in NP

CD was quantified in the supernatants of unloaded and GSH-loaded CS/CD NPs by spectrophotometric analysis of the fading of phenolphthalein alkaline solutions [9]. Briefly, a 3 mM phenolphthalein stock solution in methanol was diluted 1:100 in 0.05 M carbonate buffer (pH 10.5). 1 ml of CS/CD NPs were added to 2.6 ml of diluted phenolphthalein solution prepared as described above and were centrifuged at 9000 rpm for 20 min. The absorbance at 553 nm of resulting solution was measured by spectrophotometer.

G. Preparation of FITC-labeled CS

0.5 mg of chitosan nanoparticles was redispersed in 5 ml DMSO solution followed by the addition of 0.5 ml 0.1 M NaOH solution [10]. Then FITC was dissolved in DMSO at 10.0 mg ml –1 concentration, and was slowly added to the suspension of chitosan nanoparticles. The labeling ratio of the amino group in total glucosamine unit was controlled with a final concentration of FITC in the reaction medium. The reaction between the isothiocyanate group of FITC and the amino group of the D-glucosamine residue was allowed to proceed for 12 hours in the dark at room temperature. FITC-labeled chitosan nanoparticles were centrifugated and washed with DMSO several times until the free FITC could not be detected in the supernatant.

H. In Vitro Release Study

In vitro release of GSH from loaded CS and CS/CD NPs was car ried out for 3 h in simulated gastric (pH 1.2) and intestinal (pH 6.8) medium without enzymes and performed according to USP XXVI recommendations. Freshly prepared CS and CS/CD NPs were isolated in Eppendorf tubes with a 10 mcl glycerol bed laid in the bottom of the tube, set to help on NP resuspension. In screw-capped test tubes, each formulation was resuspended in 0.4 ml of distilled water and the resulting resuspended formulation was mixed with 1 ml of release medium (simulated gastric or intestinal medium) and incubated at 37 C under agitation. At appropriate time intervals, an aliquot (0.4 ml) was withdrawn and centrifuged. The initial volume of release medium was maintained by refilling 0.4 ml of the same medium after each withdrawal. The supernatant was analyzed for GSH content. GSH in solution was dissolved in the same media and analyzed at the same time points, as a control for GSH degradation. Each experiment was performed in triplicate.

I. In Vivo Determination of Ability of NPs for Tissue GSH Delivery

To determine the ability of NPs deliver GSH to tissue intestinal rat model of gut ishemia-reperfusion injury was used [11]. Rats (weight 250-300 gr) were anesthetized with ketamine (80 mg/kg, im) and xylazine (10 mg/kg, im). Intestinal ischemia was induced by 60 min occlusion, followed by 240 min reperfusion11 . During the 5 h of the surgical procedure, animals were kept at room temperature, and given intraperitoneal fuid as 0.9% NaCl (10 mL/kg). The superior mesenteric artery was exposed through a midline abdominal incision, and both this artery and the collateral branches coming from the celiac axis, and the inferior mesenteric artery were occluded with atraumatic vascular clamps for 1 h, followed by 4 h of reperfusion. Existence of pallor and absence of pulsation ensured mesenteric occlusion during the ischemic period. Recovery of pulsation and pink color were controlled in each animal when the clamps were removed. The existence of intestinal I/R in this model was also confrmed in our laboratory by the appearance of pulses at the marginal arteries (direct vision of mesenteric circulation by microscopy). CS or CS/CD NP loaded with 3 mg of GSH were infused into jejunum just before ischemia period in two groups of animals and appropriate volume of normal saline in control animals (n=15 in every group). FITC-labaled CS or CS/CD NPs were introduced into jejunum to 8 animals of each groups for determination mucoadhesive activity of prepared NPs. After reperfusion period animals were killed by sodium thiopental overdosing.

Concentration of GSH [12], malone dialdehyde (MDA) [13], diene conjugates (DC) [14] and catalase (CAT) [15] in mucosal layer of small intestine were determined using spectrophotometric methods. For estimation of mucoadhesive properties of NPs jejunum and ileum were removed for hystological investigation. Briefly, parts of intestine 1-2 sm in lenth were fixed in 4% phosphate-buffered formalin for 2 to 3d and embedded in paraffin. The blocks were then sectioned in transversal orientation and stained with hematoxylin-eosin. The evaluation of the histological grade of injury was based on 3 parameters: the percentage of injured villi, ratio of villus height within the mucosa, and total mucosal thickness. Fluorescence of FITC-labelled NPs, which adhesed to mucosal surface, were determined by luminescent microscope.

III. RESULTS

CD solubility increased from 8 to 35 mg/ml when it was dissolved in 1-5% NaCl solution in front of distilled water, so 1% NaCl was choosed for futher prepaprations. Opalascent solution occured in both NPs when concetnration of CS and TPP were 1 mg/ml. Regular and spherical morphology was revealed by TEM both for CS and CS/CD NPs, and this was observed irrespective of the presence or the absence of GSH. CS/CD NPs morphology was similar to that of CS NPs, but with some

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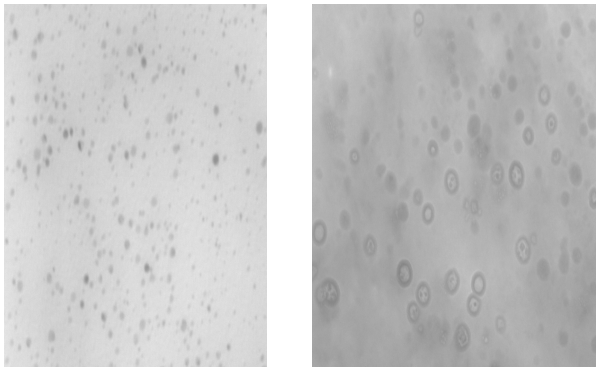
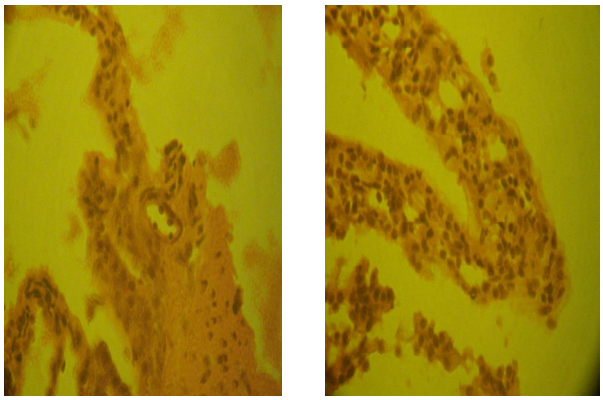
patterns (Fig. 1). A tight structure is typically observed for CS NPs, while a more loose inner structure were observed in CS/CD NPs. Mean size of both NPs were not different and situated in range of 100-150 nm.



A B

Fig.1. TEM image of CS (A, 20000 magnification) and CS/CD (B, 40000 magnification) NPs loaded with GSH

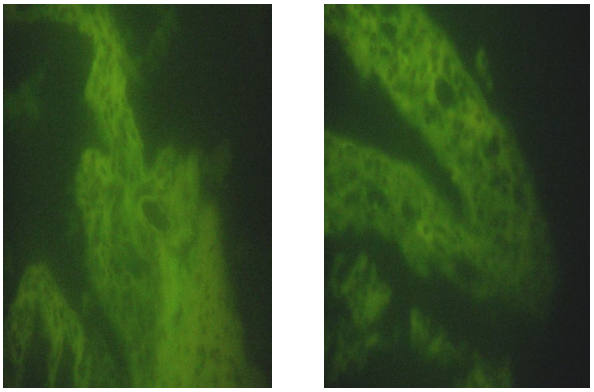
Hystological investigation show marked mucosal injury in CS group with ulceration, denuded villi, decellularization of lamina propria and crypt (fig. 2).



A B

Fig.2. Light microscopic hematoxylin-eosin stained image of hystologic section of ileum after ischemia-reperfusion injury and intraluminal infusion of CS (A) and CS/CD (B) NPs loaded with GTH

In CS/CD group changes represented II-III grade of injury and included shortened villi, epithelial lifting, and partial separation of the lamina propria. Fluorescent microscopy revealed high mucoadhesive properties of both NP system regardless of presence of GSH (fig. 3).



A B

Fig.3. Luminescent microscopy image of ileum section after ischemia-reperfusion injury and intraluminal infusion of FITC labeled CS (A) and CS/CD (B) NPs

Yield and entrapment efficacy of both NP systems were not different and consisted 55-60%. The capacity of the different NP systems to load GSH was determined: CS NPs presented encapsulation efficiency near 9% and CS/CD NPs – 24%.

In this work, we have confirmed CD inclusion values by analyzing the amount of non-incorporated CD in the NP supernatant through a colorimetric method. The fading of phenolphthalein solutions confirmed that 85% of the CD molecules are incorporated to unloaded CS/ NPs, while 65% of the CD molecules are incorporated to GSH-loaded CS/CD NPs.

In vitro release tests of NPs were carried out in simulated gastric d intestinal media without enzymes (USP XXVI), and very different behaviours were observed for CS and CS/CD NPs. In gastric medium, CS NPs released 100% of the loaded GSH over 3 h, whereas CS/CD NPs reached a plateau corresponding to approximately 30% of the loaded GSH. In intestinal medium, these trends were inverted: CS/CD NPs reached 100% release in less than 1 h, whereas CS NPs were only able to release 50% of the loaded GSH in 3h.

GSH was delivered to mucosal tissue in rat model of intestinal ischemia-reperfusion injury by CS/CD NPs on 84,9% ($p<0,05$) higher level than CS NPs (Table 1), which was followed by decreasing of concentration of oxidant injury markers.

Table 1 – Concentration of Markers of Oxidant Injury and Antioxidants In Mucosal Layer of Small Intestine, M±m

	Groups			
	CS NPs, (n=15)	CS/CD NPs, (n=15)	Control, (n=15)	Sham-operated, (n=15)
GSH, μmol/g	1,22±0,16 [c]	2,32±0,14 [ab]	0,92±0,17 [c]	2,62±0,08
MDA, nmol/g	7,61±0,44	6,11±0,31 [a]	8,88±0,29 [c]	5,61±0,21
DC, nmol/g	10,14±0,31 [c]	9,04±0,31 [a]	11,43±0,41 [c]	8,13±0,21
CAT, μmol/min /g	375±21,4 [c]	445±21,4	305±31,4 [c]	542±11,4

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a- p<0,05 with control group; b - p<0,05 between CS and CS/CD NPs; c- p<0,05 with control group

IV. Discussion

At concentrations between 0.5 and 10 mM, the tripeptide glutathione (g-L-glutamyl- L-cysteinyl-glycine) is the most abundant, low molecular weight thiol in plant and animal cells [16]-[19]. As such, it plays an important role in a number of critical cellular processes including the synthesis of the deoxyribonucleotide precursors of DNA, the metabolic processing of certain endogenous compounds such as estrogens, prostaglandins, and leukotrienes and the inactivation of drugs modulates protein structure through both direct and indirect effects on protein sulfhydryl groups. Perhaps most importantly, GSH and GSH-associated metabolism provide the major line of defense for the protection of cells from oxidative and other forms of stress [1]-[3]. It can react non-enzymatically with carbon-centered radicals and is also the electron donor in the enzymatic reduction of both H2O2 and organic peroxides catalyzed by the glutathione peroxidases. The product of the oxidation of GSH by glutathione peroxidase is glutathione disulfide (GSSG). GSSG can be converted back to GSH by glutathione reductase in a reaction that requires NADPH as a reductant. Free GSSG can also be eliminated from cells by either direct conjugation to proteins (glutathionylation) or export by specific transporters. Intracellular GSH is also depleted when it is used by the glutathione transferases to detoxify electrophilic compounds.

Unfortunately, this peptide cannot be administered by the most acceptable route of administration (i.e. orally) because it undergoes enzymatic degradation by the enzyme c-glutamyltranspeptidase, a process characterized by the cleavage of the peptide bond between L-glutamate and L-cysteinylglycine by c-glutamyltranspeptidase

Oral route is most common and non-invasive way to deliver drugs or nutrients into the body. There are some limitations, however, such as the harsh condition in gastrointestinal tract or poor intestinal absorption, which can in turn, lowers the bioavailability of bioactive materials. Over the past two decades, many researchers have developed various polymeric NPs as a strategy for oral delivery to overcome these limitations and deliver proteins, peptides, vaccines, DNA, and/or nutrients efficiently [6]. Nanoparticles are colloidal particles varying in size from 10 nm to 1000 nm. Nanoparticles have been explored as drug delivery systems for both small drug molecules and macro- molecules. Either direct nanosizing of drug or incorporation into lipidic and polymeric particles can help deliver drugs with poor aqueous solubility and permeability. Nanoparticles have shown to be absorbed in systemic circulation from GIT through Peyer's patches via M cells in lymphatic systems. In recent years, the focus is on developing biodegradable polymeric nanoparticles for drug delivery. These particles apart from increasing the bioavailability provide sustained release of drug. The drug is dissolved, adsorbed, attached or encapsulated in the polymeric matrix of nanometer size. Depending upon the method of preparation nanospheres or nanocapsules are obtained with different release and surface properties [7]-[8]. Nanoparticles are also being explored for targeted drug delivery. There are reports suggesting those drugs which are encapsulated in high molecular weight polymeric nanoparticles which passively target the tumor tissue through enhanced permeation and retention effect. The nanoparticulate delivery can prevent the degradation of these agents in the GIT, which will help in improving the bioavailability of the antioxidants which degrade in the gastric environment.

In particular, CS, a cationic polysaccharide, has been broadly studied as a carrier material for oral delivery. CS has shown favorable biocompatibility characteristics [19]-[20], as well as the ability to increase membrane permeability, both in vitro and in vivo, and can be degraded by lysozyme in serum. CS is similar in structure to cellulose; both are made from linear monosaccharides. However, unlike cellulose, CS is composed of 2-amino-2-deoxy-D-glucan combined with glycosidic linkages. The primary amine groups give CS special properties that make it very useful in pharmaceutical applica- tions. Compared to many other natural polymers, CS has a positive charge and is mucoadhesive [20]. As CS is a cationic polysaccharide in neutral or basic pH conditions, it contains free amino groups and, hence, is insoluble in water. At acidic pH, amino groups can undergo protonation, thus making them soluble in water [21]. Solubility of CS depends on the distribution of free amino and N-acetyl groups. Protonation of the amino group allows the polymer to interact with negatively charged materials. It is this functional group that enables the formation of CS nanoparticles cross-linked with anion materials, such as TPP, by the ionic gelation method [19]. CS nanoparticles have been synthesized as constituent non- viral carriers for the delivery of peptides, proteins, oligonucle- otides, vitamins, plasmids, and drugs. They have the capacity to protect sensitive bioactive macromolecules from enzymatic and chemical degradation in vivo and during storage and facilitate the transport of charged macromolecules across absorptive epithelial cells.

CS and CS/CD NPs were prepared by ionic gelation in the presence of TPP, as described in the methodology section. The low affinity of GSH to CS NPs matrix might be explained by the low molecular weight of the peptide and to the presence of only one net negative charge in its chemical structure. Formation of CD/GSH complex before it incorporation in CS matrix increased encapsulation effeiciency of NPs system thus enhancing GSH delivery to tissue.

CD are cyclic compounds consisting of six, seven, or eight α-D- glucopyranose units connected by α-(1-4) linkages which are commonly referred to as α-, β-, and γ-CD, respectively [22]-[24]. CD possess a characteristic toroidal shape with a well-defined hydrophobic cavity and lipophilic exterior that is suitable for inclusion and binding of appropriate sized guest compounds. CD are of interest because of their ability to form stable inclusion complexes in aqueous solution as well as in solvents. These macromolecules (CD), which can be spatially represented as a torus with wide and narrow openings corresponding to secondary and primary hydroxyl groups, respectively, can

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encapsulate a large variety of compounds due to the hydrophobic characteristic of their internal cavity [23]. This outstanding property has long been utilized in pharmaceutical, food, cosmetic, and textile industries and has also found its applications in the field of catalysis, environmental remediation, chemical sensing, and enantiomeric separations [24].

CD is poor soluble in water so it content in CS matrix is limitted [22]. To increased it entrapment to NPs we disolved CD in NaCl solution which is frequently applied in clinics. CS/CD NPs were only 25% loaded with GTH but their oral administration diminished ischemia-reperfusion injury of small inteatinal mucosa: concentration of MDA decreased on 31,2% ($p<0,05$), DC – on 20,9% ($p<0,05$), but CAT increased on 45,9 ($p>0,05$).

V. Conclusion

CS NPs could be effectively loaded with GTH after preparation of inclusion complex with CD. GTH inclusion complexes content in CH matrix may be increased due to enhanced solubility of CD. Oral administration of CS/CD NPs loaded with GTH diminish ischemia-reperfusion tissue injury in mucosal layer of small intestine.

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