Erythrocyte-mediated Delivery of Pravastatin: *In Vitro* Study of Effect of Hypotonic Lysis on Biochemical Parameters and Loading Efficiency

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Exposure of erythrocytes to hypotonic lysis creates pores in the cell membrane, through which pravastatin can enter and become trapped, after resealing them with a suitable buffer. We investigated the effects of tonicity, incubation time and drug concentration on drug loading into erythrocytes. Furthermore, we investigate the effects of pravastatin on erythrocyte oxidative stress markers and osmotic fragility behavior. Encapsulation was achieved using buffer solutions of different tonicities (0.5, 0.6 and 0.7% NaCl) and different drug concentrations (2, 4, 8 and 10 mg/mL) for a range of incubation times (15, 30, 60 and 120 min). The results demonstrated that controlled hypotonic lysis could entrap pravastatin in human erythrocytes, with acceptable loading parameters. The highest loading (34%) was achieved at 0.6% NaCl and 10 mg/mL pravastatin for 60 min incubation. At this pravastatin concentration, oxidative stress markers were similar to those seen in controls, and fragility and hematological parameters were unaffected in drug-loaded erythrocytes. These results indicate that the loading process and pravastatin concentration had no deleterious effects on the structure of pravastatin-loaded erythrocytes, suggesting that they may therefore have a similar life span to normal cells. Pravastatin-loaded erythrocytes may thus provide an effective extended-release-delivery system for pravastatin.

**Key words:** Carrier erythrocytes, Hypotonic dilution, Pravastatin, Protein carbonyl, Oxidative stress, Osmotic fragility

INTRODUCTION

The efficient delivery of drugs to diseased organs requires that maximal therapeutic levels are maintained to ensure effective treatment (Gopal et al., 2007; Shavi et al., 2010). Currently available delivery systems utilize carriers including small molecules and macromolecules, as well as multi-component structures such as cells (Hamidi et al., 2007a). Erythrocytes represent one of the most promising biological drug delivery systems investigated to date (Millan, 2004). These cells are biodegradable and biocompatible, and have the ability to circulate throughout the body. Furthermore, their degradation products are reusable (Pierigé et al., 2008; Gupta et al., 2010). According to the preferred therapeutic approach, erythrocytes are used either as a carrier for sustained release of the drugs, or for targeting the drugs to specific organs (Hamidi et al., 2007b). Preservation of the normal oxidant/antioxidant balance in erythrocytes during drug encapsulation may help to produce loaded cells with similar characteristics to normal erythrocytes (Alanazi, 2010). In this case, such drug-loaded cells can be used as slow-release carriers for the entrapped drugs (Hamidi et al., 2007b). In contrast, modification of loaded erythrocytes results in the accelerated removal and targeting of loaded cells to the reticuloendothelial system (RES) (Alanazi et al., 2011).

Exposure of erythrocytes to physical tensions, and osmotic changes, as well as oxidative stress, can cause...
alterations in their morphological characters. This may accelerate their removal from the circulation by the RES (Maurizio et al., 2007). The major difficulty associated with the use of red blood cells as extended drug carriers thus involves their uptake in vivo by the RES (Hamidi et al., 2007a). This accelerated uptake may be attributed to a decreased antioxidant capacity, as well as the oxidation of structural lipids and proteins in the erythrocyte membrane (Zwaal and Schroit, 1997). An increase in the amount of oxidatively-modified proteins is a feature of erythrocyte ageing (Robaszkiewicz et al., 2008).

Therapeutic agents can be loaded into erythrocytes either by physical methods, such as endocytosis and osmosis-based systems, or by chemical perturbation of the membrane (Gopal et al., 2007). Controlled lysis (hypotonic stress) creates pores in the erythrocyte membrane allowing drugs to pass through the pores and become permanently entrapped after resealing with a resealing buffer solution. Hypotonic dilution has been widely studied as a technique for drug loading of erythrocytes. This method has previously been used for the entrapment of anticancer drugs (Mishra and Jain, 2002), angiotensin-converting enzyme inhibitors (Tajerzadeh and Hamidi, 2000; Hamidi et al., 2001) and corticosteroids (Rossi et al., 2004; Shavi et al., 2010).

Statins reduce intracellular cholesterol biosynthesis by inhibiting hydroxymethyl glutaryl coenzyme A reductase. Pravastatin is a statin that undergoes degradation under the acidic conditions in the stomach, as well as pre-systemic metabolism by cytochrome P450 (Hatanaka, 2000). Pravastatin is incompletely absorbed from the gastrointestinal tract because of the efflux action of phosphorylated glycoproteins (Chen et al., 2007), resulting in decreased bioavailability (Hatanaka, 2000). Alternative administration routes are thus required to enhance the bioavailability of pravastatin, or protective carriers are needed to reduce the destruction and efflux action of phosphorylated glycoproteins and cytochrome P450. Pravastatin elicits antioxidant actions to maintain the normal oxidant/antioxidant balance of erythrocytes, thus protecting them from oxidative stress. Pravastatin can also preserve erythrocyte fragility and morphology under the effect of drug induced oxidative stress (Alanazi, 2010).

Thiols, including glutathione (GSH), play a role in cellular protection against oxidative stress (Lajos et al., 2007). Exposure of erythrocytes to excess physical, osmotic or oxidative stress may deplete glutathione and induce lipid peroxidation (Becker et al., 2004). Moreover, the decline in glutathione levels induces protein oxidation and modification of the erythrocyte cytoskeleton and morphology (Robaszkiewicz et al., 2008).

This study therefore aimed to encapsulate pravastatin into human erythrocytes by controlled lysis, and examined the effects of pravastatin concentrations and incubation times on entrapment efficiency at different concentrations of NaCl. The effects of pravastatin on oxidative status, osmotic fragility and hematological indices were also determined.

**MATERIALS AND METHODS**

**Materials**

Pravastatin sodium was obtained from SPIMACO (Riyadh, KSA). HPLC-grade formic acid, acetonitrile, 2,4-dinitrophenylhydrazine, and methanol were acquired from BDH. Polytetrafluoroethylene membrane disposable syringe filters were from Macherey-Nagel GmbH. Magnesium chloride hexahydrate was obtained from Avonchem Limited. Magnesium sulfate heptahydrate, O-phthalaldehyde (OPT), N-ethyl maleimide (NEM), disodium ethylene diamine tetra acetic acid (Na2EDTA), reduced glutathione (GSH), oxidized glutathione (GSSG), Ellman’s reagent, tetraethoxypropane, and thiobarbituric acid (TBA) were purchased from Sigma Chemical Co., adenosine 5-triphosphate (ATP) was from Spectrum Chemical Mfg. Corp. Guanidine hydrochloride was obtained from Winlab. All other chemicals used were high analytical grade.

**Equipment**

An ultra-performance liquid chromatography Acquity® (UPLC) system equipped with binary solvent manager, automatic sample manager, column heater, photodiode array detector and Acquity® UPLC BEH C18 column (2.1 × 50 mm, 1.7 µm) was obtained from Waters Inc. An analytical balance AJ150 was from Mettler and a water bath SW22 was from Julabo. Centrifuges EBA 20 and MIKRO20 were supplied by Hettich, a vortex mixer VWR was from Scientific Industries Inc., and a Coulter® ACT Diff™ hematology analyzer was from Beckman Coulter, Inc. A Spectro UV-Vis Split Beam PC, model UVS-2800 was from Labomed, Inc.

**Pravastatin loading into erythrocytes**

Blood samples from apparently healthy donors with no acute or chronic diseases were collected in heparinized tubes. Informed consent was obtained from all blood donors. The plasma and the buffy layer were detached by aspiration. The erythrocytes were then washed three times in cold isotonic phosphate-buffered saline (PBS) with frequent centrifugation for 5 min at 5000 rpm and the hematocrit was adjusted to 45% with PBS to form the erythrocyte suspension.