WHEY PROTEIN CONCENTRATE AS A PROTECTIVE AGENT AGAINST DOXORUBICIN-INDUCED CARDIOTOXICITY IN MICE

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ABSTRACT

Whey proteins are considered as functional food ingredients which have high cysteine content and promote the biosynthesis of glutathione—the primary intracellular antioxidant. Doxorubicin (DOX) is one of the most active antitumor antibiotics in current use. The therapeutic value of DOX, however, is limited by its toxicity. Oxidative stress is one of the underlying mechanisms of DOX toxicity in noncancerous (nontargeted) tissues. We investigated the protective effect of whey protein concentrate against DOX toxicity and oxidative stress. The administration of DOX (20 mg/kg i.p.) to BALB/c mice caused significant decrease of tissue glutathione level in the heart and severe histopathological changes, examined by light and transmission electron microscopy. These biochemical and histological alterations were effectively attenuated on pretreatment with whey protein concentrate. We therefore concluded that the protective action of whey is due to the enhancement of tissue glutathione level which might have important cytoprotective effects on oxidative stress, induced by DOX treatment.

Keywords: cardiotoxicity, doxorubicin, glutathione, whey proteins,

Introduction

First isolated in the early 1960s, the anthracycline doxorubicin (DOX) remains among the most effective anticancer drugs ever developed, with high antitumor efficacy in breast cancer, aggressive lymphomas, childhood solid tumors and soft tissue sarcomas. Although the dose dependent chronic cardiomyopathy of DOX is well documented (16, 12, 18), it remains a major anticancer agent. The most widely accepted mechanism to account for anthracycline–induced cardiotoxicity involves reactive oxygen species (ROS) generation (10, 8). When the formation of ROS exceeds cellular adaptive and repair capacities, a condition that is referred to as oxidative stress occurs, in which biological molecules such as nucleic acids, proteins, and membrane phospholipids become damaged through oxidative reactions. Oxidative stress results from an imbalance between the generation of ROS and their removal by the cellular antioxidant system.

A wealth of studies has been emerged using antioxidants in an attempt to prevent or attenuate DOX cardiac toxicity. Amongst all conducted antioxidant strategies metal ion chelators like transferrins, metallothionein, desferoxamine and ceruloplasmin have been investigated. Also low-molecular-mass agents that scavenge reactive oxygen species such as melatonin, uric acid, lipoic acid, vitamin A, coenzyme Q10, selenium, vitamin C and vitamin E have also been addressed (17, 5). Dietary antioxidant supplements as garlic (S-allylcysteine) and grape seed proanthocyanidins have also been indicated to enhance the anti-tumor activity of DOX and ameliorate myocardial oxidative stress in mice (6, 19).

Whey proteins are a heterogeneous group of proteins, obtained in milk after casein precipitation. They are considered as functional food ingredients of important nutritional and health effects (13). A wide range of antioxidant, antitumoral, anticarcinogenic and immunity-enhancing actions of whey proteins have been observed in human and animal studies (3, 11, 1).

The majority of whey proteins are cysteine-rich, including α-lactalbumin, β-lactoglobulin, and bovine serum albumin (15). Cysteine is known as an amino acid that regulates the in vivo concentrations of glutathione (GSH)—an ubiquitous thiol-containing tripeptide (L-γ-glutamyl-L-cysteinyl-glycine). GSH is the primary intracellular antioxidant that neutralizes oxidative stress, detoxifies...
toxins, and scavenges ROS formed during normal metabolic processes or as a result of trauma, infection or medication. GSH-dependent enzymes, such as glutathione-peroxidase, reductase and S-transferase utilize GSH to neutralize ROS. This ability makes GSH central to defense mechanisms against intra- and extra-cellular oxidative stress. GSH is also able to regenerate the most important antioxidants, vitamins C and E, back to their active forms. In a healthy organism GSH usage and synthesis are balanced. Increased GSH usage is observed after exposure to free radicals, toxin metabolism, inflammation, cancer, HIV, diabetes, excessive physical activity and UV. Decrease in GSH synthesis is common in ageing and nutrition shortage of cysteine—the limiting building block for GSH (14, 7). The ROS that are produced in response to the chemotherapeutic agent DOX decrease cellular GSH reserves in nontargeted tissues and thereby escalate oxidative stress.

The concept that whey proteins promote GSH biosynthesis (2) and thus improve protection against oxidant-induced cell damage has prompted us to investigate the possible cardioprotective effect of whey proteins in Balb/c mice challenged with a single cumulative dose of DOX.

Materials and methods

Animals and Experimental design

Male and female Balb/c mice aged 3 months and weighing 25-30 g came from Slivnitza animal breeding house, Sofia. They were randomized into 3 experimental groups of 6 animals. Group 1 was fed a whey supplemented diet for 25 consecutive days. Whey supplementation was made by mixing whey protein powder (Eligo, Czech Republic) with standard chow formula in powdered form in proportion 1:3. The mixture was made semisolid by adding 15% water to the powder, which was then easily shaped in the form of pellets and dehydrated at 40°C. The animals in groups 2 and 3 were fed a standard formula chow diet. DOX (Doxorubicin hydrochloride, Adriamycin®) was obtained from Pharmacia&Upjohn, Milan, Italy. It was freshly prepared in saline solution, protected from light. Mice from group 1 (DOX+WHEY)) and 2 (DOX) received a single intraperitoneal dose of DOX (20mg/kg b.wt.) on the 21-st day after the beginning of whey supplementation (1st day). Untreated controls of group 3 were injected with saline intraperitoneally only (CONTROLS). All mice were sacrificed on day 25. Heart samples were taken and proceeded for the routine histological examination by light and transmission electron microscopy and for biochemical measurement of GSH.

Histological evaluation

Since it has been ascertained that DOX induced myocardial lesions are focal and uniformly scattered throughout the whole organ, isolated trial preparations were used instead of whole organs. Samples were fixed in 10% neutral phosphate buffered formalin, dehydrated in ethanol series, embedded in paraffin 5µm sections, stained with Hematoxylin&Eosin and examined under light microscope (Karl Zeiss Jena).

Heart tissue for ultrastructural analysis was fixed with 2.5% glutaraldehyde in 0.1M cacodilate buffer, post-fixed in 1% OsO4, dehydrated through an ascending ethanol series and embedded in Durcopan resin. Ultrathin sections of the myocardium were examined under transmission electron microscope (Opton EM 109).

Measurement of GSH

For GSH assay heart homogenates (10%w/v) were prepared in 10% trichloroacetic acid and centrifuged at 3000x for 10 min. GSH was determined by the Ellman procedure (9) using 2,4-dithionitrobenzoic acid. Absorbances were measured at 412 nm. The level of GSH was determined from the standard curve with commercially available GSH (Sigma chemicals) and the results are expressed as µmol GSH/g tissue.

Statistical analyses

All data are expressed as mean values ±standard deviation (SD) for six animals per group. The significance of differences in GSH content in the cardiac tissue of control and experimental mice was evaluated using Student’s t test. The level P≤0.05 was used as the criterion for significance.

Results and Discussion

Tissue GSH levels

The effects of DOX and DOX combined with whey supplementation on cardiac GSH content are shown in Fig. 1. DOX treatment caused significant reduction (about 40%) in GSH cardiac content, compared to controls (0.30±0.02 vs. 0.50±0.21µmol/g wet weight, P≤0.05). Whey supplementation, however, restored GSH level near to normal value and animals in DOX+whey group showed only about 14% reduction of cardiac GSH level compared to controls (0.43±0.14 vs. 0.50±0.21µmol/g wet weight)

Histology

The results from the light microscopy examinations are shown in Fig. 2. The light micrographs of the heart of control mice revealed normal myofibrillar structure with striations and branched appearance (a). In DOX treated mice (b) fragmented and destructed muscle fibers and widening of...
intercellular spaces were observed. Animals pretreated with whey proteins (c) showed better preserved appearance of cardiac muscle fibers with slightly widened intercellular spaces.

![Fig. 1](image1)

**Fig. 1.** Effect of whey proteins on GSH cardiac content (μmol/g); a: Mean values were significantly different from those for the control group (P≤0.001); b: Mean values were significantly different from those of the DOX treated group (P≤0.05)

When examined by electron microscopy, (Fig 3) cardiac myocytes from control group showed normal appearance (a). Ultrastructural pathological changes to the myocardium were observed in DOX-treated mice: frequent areas of myofilament loss, cytoplasmic edema with vacuolization, mitochondrial swelling, deformation and disruption of mitochondrial membrane and cristae disappearance (b). In DOX treated and whey supplemented mice this histological changes were significantly reduced. Some minimal edematous spaces in the intramyofibrillary areas were seen, but no mitochondrial and myofibrillar damage was observed.

Cardiac myocytes contain the highest volume density of mitochondria in the body, occupying about 40% of the total intracellular volume. DOX has a high affinity for cardiolipin, a negatively charged phospholipid abundant in the mitochondrial inner membrane, leading to mitochondrial accumulation of DOX. Under clinically relevant plasma DOX concentrations of 0.5–1 μM, intramitochondrial concentrations can reach approximately 50–100 μM, resulting in high redox reactivity and severe mitochondrial damage in cardiomyocytes (4). It is of value to remember that heart tissue is very sensitive to free radical injury because of its highly oxidative metabolism and the lower amount of endogenous antioxidants in this organ.

![Fig. 2](image2)

**Fig. 2.** Representative photomicrographs of the heart of control (a), DOX-treated (b) and DOX-treated and whey supplemented mice (c), H&E.

Intake of cysteine-containing foods contributes to the increase in GSH synthesis in the state of DOX induced oxidative stress. However, rich sources of this GSH-precursor are rare in animal and plant edible proteins and glutamyl-cysteine group with a disulfide link is indeed limited to some of the whey proteins (mainly serum albumin fraction) and to the ovomucoid faraction of egg white.

In our experiment GSH content was measured in order to evaluate the extent of oxidant burden, resulted in GSH depletion in cardiomyocytes following DOX injection.

Since GSH plays an important role as an oxygen radical scavenger, the decreased GSH level in DOX treated mice suggests consumption of intracellular GSH due to the influx of DOX and its toxic metabolites. Pre-feeding with whey proteins prior to and after DOX administration restored GSH
content in DOX+WHEY group almost up to the control level. Thus whey supplementation promotes detoxification of intracellular and possibly, extracellular DOX-oxygen metabolites and ROS. As DOX treatment seriously impairs GSH system in myocardial cells and the detoxification of DOX oxygen radicals might be delayed, this may contribute to the further advance of the damage. The feeding of whey proteins may also protect cardiomyocytes against DOX toxicity by improving DOX metabolism. In our study results from histological studies (cellular and mitochondrial pathological changes) correspond to the cardiac GSH content in the experimental and control groups.

In conclusion we have shown in this study that oxidative damage to the heart contributes to the myocardial toxicity induced by DOX in Balb/c mice. This effect might be limited by whey protein feeding. The protective properties of whey might be attributed to its antioxidant capacity in connection with promoting GSH synthesis.

REFERENCES
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