Original Article

ABATEMENT OF DOXORUBICININDUCED TOXICITY BY WHEY PROTEIN CONCENTRATE

Bistra L. Alexieva,
Stoyanka R. Stoitsova[1],
Velichka G. Pavlova,
Tsvetelina S. Paunova[1]
Elena B. Nikolova

Institute of Experimental Morphology and Anthropology with Museum, Bulgarian Academy of Sciences, Sofia

Corresponding Author:
Bistra L. Alexieva
Institute of Experimental Morphology and Anthropology with Museum, Bulgarian Academy of Sciences,
Acad. Georgi Bonchev, str., Bl. 25
Sofia, 1113
Bulgaria

Acknowledgements
This work was supported by grant TKL 1609 by Ministry of Education and Science, Bulgaria

Summary
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. Whey proteins are considered as functional food ingredients which have high cysteine content and promote the biosynthesis of glutathione the primary intracellular antioxidant.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 a significant decrease of tissue glutathione level in the heart, kidney, liver and small intestine and visible histopathological changes, examined by light and transmission electron microscopy. These biochemical and histological alterations were effectively attenuated on pretreatment with whey protein concentrate for three weeks prior to DOX administration. We concluded that the beneficial effect of whey is due to the enhancement of tissue glutathione level which might have important cytoprotective effects on oxidative stress, induced by DOX treatment.
Key words: doxorubicin, glutathione, whey proteins, oxidative stress

Introduction
Doxorubicin (DOX) is an anthracycline antibiotic introduced in 1969 for the treatment of cancer. Since then it 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. DOX use in chemotherapy has however been limited due to its diverse toxicities. The most widely accepted mechanism to account for DOX toxicity involves reactive oxygen species (ROS) generation [1, 2]. 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. The protective effect of different agents in an attempt to prevent or attenuate DOX toxicity has been emerged. Amongst all conducted antioxidant strategies low-molecular-mass agents that scavenge reactive oxygen species such as melatonin, uric acid, lipoic acid, , as well as dietary antioxidant supplements as vitamin A, coenzyme Q10, garlic (*S*-allylcysteine) and grape seed proanthocyaniclins [3, 4, 5, 6] have been addressed.

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 [7]. A wide range of antioxidant, antitumoral, anticarcinogenic and immunity-enhancing actions of whey proteins have been observed in human and animal studies [8, 9, 10]. The majority of whey proteins are cysteine-rich, including α-lactalbumin, β-lactoglobulin, and bovine serum albumin [11]. 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. This ability makes GSH central to defense mechanisms against intra- and extra-cellular oxidative stress. The concept supported by Bounous et al. [12], that whey proteins promote GSH biosynthesis, and thus improve protection against oxidant-induced cell damage, has prompted us to investigate the possible protective 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. 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. Mice in group 1 was fed a whey supplemented diet for 25 consecutive days. The animals in groups 2 and 3 were fed a standard chow diet. DOX (Doxorubicin hydrochloride) was obtained from Pharmacia&Upjohn, Milan, Italy. Mice from group 1 (DOX+Whey)) and group 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 mice of group 3 were injected with saline intraperitoneally only (Controls). All animals were sacrificed on day 25. Samples from heart, kidney, liver, spleen, small intestine and lung were taken and proceeded to the routine histological examination by light and transmission electron microscopy and for biochemical measurement of GSH. Animal procedures were done after the rules of the Animal Ethics Committee.

Histological evaluation

Tissue samples from heart, kidney, small intestine, spleen, liver and lung 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 heart tissues were examined under transmission electron microscope (Opton EM 109).

Measurement of GSH

For GSH assay tissue homogenates (10%w/v) were prepared in 10% trichloroacetic acid and centrifuged at 3000x for 10 min. GSH was determined by the Ellman's procedure [13]. The level of GSH was defined from the standard curve with commercially available GSH (Sigma chemicals) and the results are expressed as μmol GSH/g tissue.

Statistical analyses

All data of GSH content are expressed as mean values ±standard deviation (SD) for six animals per group. The significance of differences was evaluated using Student's t test. The level $P<0.05$ was used as the criterion for significance.

Results

Tissue GSH level

The effects of DOX and DOX combined with whey supplementation on GSH content in heart, kidney, small intestine, spleen, liver and lung are summarized in Table 1. DOX treatment caused significant reduction in GSH content, compared to controls in all investigated organs ($P<0.05$). Whey supplementation, however, restored in part GSH level, but it did not reach those of the control group.

Table 1. Tissue GSH level (μmol/g)

Group	Heart	Kidney	Intestine	Spleen	Lungs	Liver
Control	0.50±0.93	2.56±0.16	1.98±0.09	0.91±0.08	0.53±0.07	3.30±0.21
DOX	0.30±0.02[a]	0.65±0.09[b]	1.09±0.15[b]	0.31±0.15[b]	0.25±0.04[a]	1.51±0.72[b]
DOX +Whey	0.38±0.14[c]	1.36±0.12[d]	1.88±0.40[d]	0.64±0.36[c]	0.44±0.15[c]	2.66±0.67[d]

[a] $P<0.05$ vs. control group; [c] $P<0.05$ vs. DOX group
[b] $P<0.01$ vs. control group; [d] $P<0.01$ vs. DOX+ Whey group.

Histology

Ultrastructural study of cardiac myocytes is shown in Fig 1. The tissues from control mice had normal appearance (Fig. 1a). Pathological changes to the myocardium were observed in DOX-treated mice: frequent areas of myofillament loss, cytoplasmic edema with vacuolization, deformation and disruption of mitochondrial membrane and cristae disappearance (Fig. 1b). In DOX treated and whey supplemented mice some minimal edematous spaces and vacuolization in the intramyofibrillary areas were seen, but no mitochondrial and myofibrillar damage was observed (Fig. 1c).

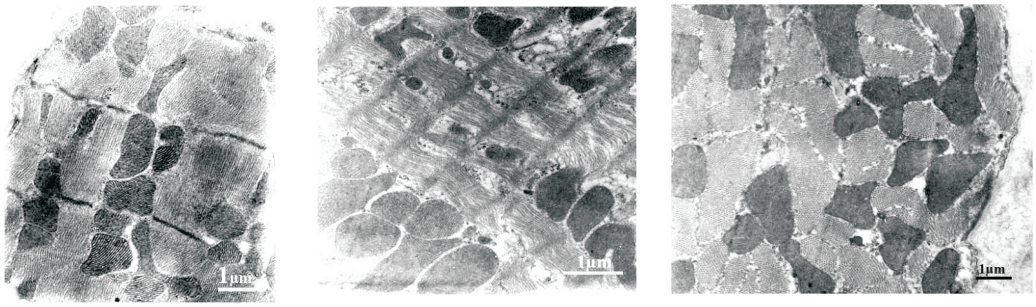

Fig.1. Electron photomicrographs of cardiac tissues of Control (a), DOX (b) and DOX+Whey treated mice (c)

Photomicrographs of H&E stained kidney sections are presented in Fig.2. In comparison to normal glomerular structure of kidney of control mice (Fig 2a), DOX treatment caused evident morphological alterations, as disorganization of glomerular structure, glomerular atrophy, widening of the Bawman's space and perivascular lymphocytic infiltrates.(Fig. 2b, 2c).Administration of whey with DOX significantly ameliorated the glomerular injury and the morphological pattern was similar to that of controls (Fig. 2d) (Original magnification x200).

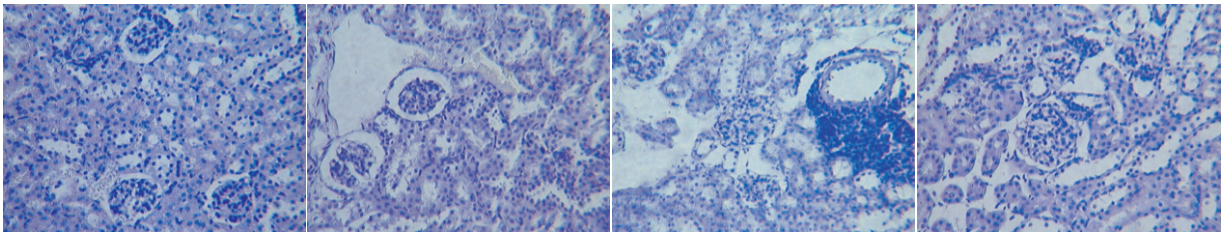

Fig.2. H&E stained kidney sections: Control (a), DOX (b, c) and DOX+Whey mice (d), x200

Representative photomicrographs of H&E stained transverse sections of small intestine are presented in Fig.3. Compared to Controls (Fig. 3a), the administration of DOX induced focal areas of denudation of the epithelial cell layer and visible shortening of the villi (Fig. 3b). Whey pretreatment significantly ameliorated the toxic effects of DOX (Original magnification x40).

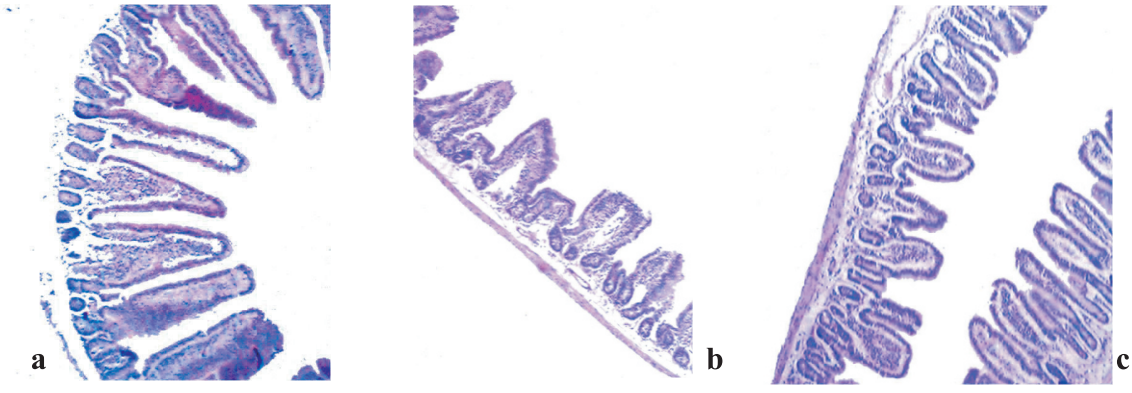

Fig. 3. H&E stained transverse sections of small intestine of Control (a), DOX (b) and DOX+Whey (c) mice, x40

No pathological changes attributable to the toxic DOX-induced effects were observed in liver, lung and spleen of the mice treated with DOX with, or without whey supplementation.

Discussion

Dietary GSH sources are few and its excess does not enhance the maximum hepatic GSH amount beyond the normal physiological level obtained with adequate dietary components due to the feedback regulation of GSH level. The liver, the key organ for xenobiotic

detoxification and elimination, is the major site of GSH synthesis. [14]. Almost 95% of GSH synthesized in the liver is released in the blood stream, which supplies the extra-hepatic tissues, and bile. The latter is the main GSH source for the intestinal mucosa, where its concentration is relatively high (50-60% of liver content), but its distribution is not uniform. The colorectal mucosa seems to have lower GSH transferase activity in comparison to other parts of the intestinal mucosa, and this could partially explain the susceptibility to cancerogenesis of this anatomic district [15]. GSH, GSH-peroxidase and GSH-transferases are not as abundant in the heart as they are in the liver, which is reflected by the greater resistance of liver to DOX induced toxicity from free radicals. It is of value to remember that heart tissue is very sensitive to free radical injury not only because of the lower amount of endogenous antioxidants in this organ, but also due to its highly oxidative metabolism

Intake of cysteine-containing foods like whey contributes to the increase in GSH synthesis in the state of oxidative stress [16]. In our experiment the GSH depletion reflects the extent of oxidant burden, 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 restored in part GSH content in DOX+Whey group. Thus whey supplementation promotes detoxification of DOX-oxygen metabolites and ROS.

Conclusions

We have shown in this study that the elevated GSH in DOX+whey group compared to only DOX-treated group could be an important contributory factor to the preventing activity of whey proteins. The protective properties of whey might be attributed to its antioxidant capacity in connection with promoting GSH synthesis.

References

1. Kalivendi S, Kotamraju VS, Zhao H, Joseph J, Kalyanaraman B. Doxorubicin-induced apoptosis is associated with increased transcription of endothelial nitric-oxide synthase. Effect of antiapoptotic antioxidants and calcium. J Biol Chem. 2001;276:47266-47276.
2. Dziegiel P, Surowiak P, Zabel M. Correlation of histopathological and biochemical appraisal of anthracyclin-induced myocardium damage. Folia Histochem Cytobiol. 2002;40:1278.
3. Quiles JL, Huertas JR, Battino M, Mataix J, Ramerez-Tortosa MC. (2002) Antioxidant nutrients and adriamycin toxicity. Toxicology 2002;180:79-95.
4. Conklin KA. Coenzyme Q10 for prevention of anthracycline-induced cardiotoxicity. Cancer Ther. 2005;4:1110-30
5. Das RN, Poudel N. Could garlic be an useful adjuvant therapy in adriamycin failure. Kathmandu University Medical Journal 2006;4:337-9.
6. Zhang XY, Li WG, Wu YJ, Gao MT. Amelioration of doxorubicin-induced myocardial oxidative stress and immunosuppression by grape seed proanthocyaniclins in tumour-bearing mice. J Pharm Pharmacol. 2005;57:104351.
7. Mclntosh GH, Royle PJ, LeLeu RK, Regester GO, Johnson MA, Grinsted RL. Whey proteins as functional food ingredients. Intern Dairy J. 1998;8:42534.
8. Bounous G, Batist G, Gold P. Whey proteins in cancer prevention. Cancer Letters, 1991;57:91-4.
9. Kent KD, Harpe WJ, Bomser JA. Effect of whey protein isolate on intracellular glutathione and oxidant-induced cell death in human prostate epithelial cells. Toxicol In Vitro 2003;17:27-33.
10. Alexieva B, Markova Tz, Nikolova E. Therapeutic potential of whey proteins in antioxidant defense. J Animal Sci 2008;XLV:86-9.
11. Morr CV, Ha EYW. Whey protein concentrates and isolate processing and functional properties. Critical Reviews in Food Science and Nutrition 1993; 33:43176.
12. Bounous G, Gervais F, Amer V, Batist G, Gold P.. (1989) The influence of dietary whey protein on tissue glutathione and disease of aging. Clin Invest Med .1989;12: 3439.
13. Ellman GL. Tissue sulfhydryl groups. Arch Biochem Biophys. 1959;82:70-7.
14. Kaplowitz N, Aw TY, Ookhtens M. The regulation of hepatic glutathione. Ann Rev Pharmacol Toxicol. 1985; 25:715-44.
15. Siegers CP, Riemann D, Thies E, Younes M. Glutathione and GSH dependent enzymes in the gastrointestinal mucosa of the rat. Cancer Lett. 1988; 40:71-6.
16. Oner OZ, Ogung AV, Cingi A, Uyar SB, Yalcin AS, Aktan AO. Whey feeding suppresses the measurement of oxidative stress in experimental burn injury. Surg Today, 2006;36:376-81