proposed that antioxidative activity of his containing peptides relates to the hydrogen donating lipid peroxyl radical trapping and/or the ion chelating ability of the imidazole group. Differentially, the SH group of cysteine has its specific direct interaction with radicals. The hydrolysate of whey protein by alcalase enhanced the antioxidant activity in liposome model system. On another hand, whey protein derived peptides obtained from proteolytic digestion showed considerable binding activity with cations such as Ca^{2+}, Fe^{2+} and Zn^{2+}. A tripeptide Tyr-Asp-Thr was identified in the hydrolysate where the major binding sites included the oxygen atom of the carboxyl group and the nitrogen of the amino or the imino groups.

In this submitted work, the predicted prophylacting NAC may acted as an exogenous provider of derived intracellular cysteine for renewal of GSH that increased significantly the cell anti-oxidative Nrf2. On the same level of action it was in *vivo AW*P within cellular physiological requirements for synthesizing the intracellular GSH from cysteine or even other metabolized amino acids have performed for such pathway of action.

Aim of the work

This work has been designed in order to use an afforded whey supplement as a natural product to be an efficient alternative competitively potent component exhibiting anti-oxidative and probable variable metal chelating capacity like NAC or more in iron overloaded living model without harmful or probable threatening bi side effects.

Materials and Methods

Experimental animals and design of the experiments

Forty eight rats (100 g-150 g B.W.) under standard laboratory conditions were incubated and thoroughly selected for this study. They were equally divided into four categories; G_0 for healthy normal negative control, G_1 for induced iron overloading positive control, G_2 induced iron overloading simultaneously prophylacted with N-Acetyl Cysteine (NAC) drug and G_3 induced iron overloading with simultaneously tested prophylacting supplemented Acetylated Bovine Whey (ABW). Route of administered scheduled doses for iron overloading by iron dextran as well as treating NAC or prophylacting tested bovine whey ABW in our laboratory have been recently published.

Tissue sampling

Livers of all rats tested groups were singly characterized and excited to be washed with distilled water to get rid of remaining blood, then thoroughly dried by blotting filter paper. The dissected liver samples of each rat were assigned into four patterns of tissue samples.

- Part to be homogenized in Phosphate Buffer Saline (PBS) and stored in -80°C for biochemical ELISA assay.
- Part to be directly used in electrophorytically tested Western blotting assay.
- Part to be fixed in 10% neutral buffered formalin for histopathological and immunohistochemical investigations.
- Part to be in glutaraldehyde fixation form for electronic microscope examination.

Biochemical determinations

Enzyme linked immunosorbent assay of hepatic tissue; ROS, IL-6 and ferritin: In the aliquot of hepatic tissue homogenate that was considered for determining hepatic reactive oxygen species (O_2- and H_2O_2), macrophagic interleukin-6 and the synthesized reducing ferritin by ELISA kits (of INNOVA Biotech Company, China, Cat. NO.; In-Ra 1426, In-Ra 0688 and In-Ra 1156 respectively) according to their protocols.

Western blot for detection of hepatic Nrf2: Tissues from the rats under experiment (3 mm^3) were lysed with ice-cold RIPA buffer (50 mM Tris-Cl (pH 7.6), 5 mM EDTA, 150 mM NaCl, 0.5% NP-40 and 0.5% Triton -X-100) containing 1 µg/ ml leupeptin and aprotinin, and 0.5 mM PMSF. Lysates were centrifuged at 2,500 rpm for 10 min at 4°C.

Protein concentrations were measured by Bradford assay. Thirty µg protein aliquots were separated by SDS PAGE using 10% gels then transferred to nitrocellulose membranes. Membranes were blocked with 5% skim milk and probed with primary antibodies (anti Nrf2 IgG and β-actin 1:1000) overnight at 4°C.

Membranes were then incubated with HRP-conjugated secondary antibody (1:5000) for 1 h at room temperature. Detection was performed using the ECL substrate. Band densities were measured by Imag j software and normalized to the control then equalized to the corresponding actin band.

Histopathological investigation

Light Microscope (LM): Deposited iron in hepatic tissue was performed by fixating dissected tissues in 10% buffered formaldehyde solution for one day. The specimens were embedded in paraffin wax and 5 µm sections were prepared for histological staining. Prussian Blue (PPB) staining was achieved for detection of iron deposition in the hepatic tissue specifically non heam iron "hemosiderin and investigated under LM at 100x magnification. Hœ E stained hepatic slides were examined at 100x, 400x and 1000x magnifications. Sections from rats liver Portal Area (PA) were stained by van grieson red colour stain for detection of hepatic collagen formations, visualized at 400x magnification.

Electronic Microscope (EM): Full thickness hepatic tissue slices were fixed in 4% glutareldehyde for 4 h at 4°C, cut into semithin sections (0.5 m–1 m thick) and stained with toluidine blue. Using the transmission electron microscope, ultrathin sections (50 nm-80 nm) were cut from selected portions of semithin sections, contrasted with uranyl acetate and lead citrate, analysed using (JEM-100 CXII AKishima, Tokyo Japan) and photographed at 80 Kv in E.M. unit of Assiut university.

Immunohistochemical investigation: The primary antibodies for the macrophagic heamoglobin–hoptoglobin complex scavenger, anti CD163 (Mouse monoclonal antibody Abcam Cat.

No; ab 156769) and the kupher cell marker anti CD68, Dako Agilent Cat. No; IR 609) as well as anti-caspase. Paraffin embedded tissue sections of 3 µm-5 µm thickness were first deparaffinized with with xylene and then hydrated in graded ethanol solution and heated in citrate buffer (pH 6.0) for 5 min. Next, the sections were blocked with 5% bovine serum in PBS for 2 h. the incubated slides overnight at 4°C with primary antibodies at dilution 1:100. The slices were rinsed with PBS for 10 min. in solution of 2% Diaminobenzidine (DAP). Sections were counterstained with hematoxylin, dehydrated and cleared in xylene and then cover slipped for LM examination.

Statistical analysis

Data were presented as mean ± S.D. the assayed markers; ROS, IL-6, ferritin concentration and Nrf2 were compared between the four different studied groups G0, G1, G2 and G3 using One Way Analysis (ANOVA).

Results

Biochemical results

Effect of tested prophylacting agents NAC and AWP on the hepatic tissue concentration of reactive oxygen species in the iron overloaded rats: Where the iron overload induction on the exposed rats, showed the hepatic tissue concentration of ROS was significantly elevated. The prophylacting agents NAC and AWP restored the levels similarly to the normal healthy attitude.

Effect of NAC and AWP on the hepatic tissue concentrations of inflammatory response marker IL-6 in iron overloaded rats: The results denoted that Iron intoxication (Fe) significantly increased the inflammatory response compared to the healthy control group G_0 (++P<0.01). While the concentration was relatively sustained as a pro-inflammatory marker in association of either NAC or AWP in iron overloading induction.

Effect of NAC and AWP on the hepatic tissue ferritin concentration in the iron overloaded rats: The results showed that iron overloading in the animal under experiment (G_1) significantly elevated the heap tic tissue ferritin concentration (++P<0.01) compared to the healthy control group G_0. The association of NAC enhanced such elevation while orally supplemented AWP was same what reduced than that of NAC.

Effect of the tested prophylacting agents NAC and AWP on Nrf2 gene expression protein matched with the internal standard protein β actin in iron overloaded rats: Results showed *Nrf2* anti-oxidative gene was dramatically reduced by iron overloading. The normal healthy levels were restored back by the action of NAC or AWP tested prophylacting agents.

Histopathological results

Effect of iron overloading on the hepatic tissue content of iron and the prophylacting action of the tested NAC and AWP supplement was visualized by E.M

The iron distribution in the overloaded rats without treatment (G_1) compared to the normal healthy (G_0). The lowering prophylacting efficacy on such enriched iron intoxication was obviously detected in NAC treated group (G_2) and AWP (G_3) treated group *via* Prussian blue staining sections. Moreover, toluidine blue staining of other sections has verified the same features of hepatocytic iron intoxication of nuclei and blood sinusoids lined by Kupffer cells and the reduced iron intoxication by active tested NAC or AWP supplement.

Effect of iron overloading on histopathological changes and treated ones in the hepatic tissue stained with H and E

The normal rat liver (G_0) showed normal structure in the Central Vein (CV) and Portal Area (PA) regions. The iron overloading (G_1) presented enriched Kupffer Cells (KCs) of blood sinusoids engorged with the iron. The prophylacted NAC group (G_2) and the supplemented AWP (G_3) showed lesser distribution.

Effect of iron overloading on the changes in liver Portal Area (PA) stained collagen by van Gieson stain and the tested prophylacting agents NAC and AWP supplement

The stained collagen in the iron overloaded animals (G_1) was prominently increased compared to the healthy normal ones. The prophylacting NAC or AWP supplement reduced such synthesis. The AWP seemed more improving one comparing to that of NAC.

Effect of iron overloading on the hepatic immunohistochemical staining CD68 and CD 163 markers: The hepatic markers CD 68 and CD 163 were increasing by iron intoxication (G_1) when compared to normal healthy group (G_0). Such an increase was somewhat reduced by pretreatment of NAC (G_2) or AWP supplement (G_3) when compared to iron overloaded rats.

Effect of iron overloading on the hepatic immunostaining of propoptotic marker Caspase-3: The healthy normal (G0) slightly responded cases showed intensive reaction. Pretreatment with NAC (G2) slightly reduced the response while AWP pretreatment (G3) dramatically reduced the staining.

Effect of iron overloading on the hepatic structure using E.M. investigation and pretreatment with the tested NAC or AWP supplement: Electron micrographs of healthy normal control rats (G_0) under examination showed that normal hepatocyte with euchromatic nucleus, well developed rER and mitochondria, lipid droplets and KCs lining the blood sinusoids. The iron overloading (G_1) showed hepatocytes with irregular heterochromatic nuclei surrounded by many vacuoles, KCs enriched with heavly iron overload and irregular secondary lysosomes. Plenty of amalgamated mitochondria with each other and ill defined cristae and electron-dense matrix.

The overloaded pretreated with NAC (G2) group of rats under experimentation showed maitanance of crowded KCs with precipitated iron in the hepatic tissue. Also presence of increased secondary lysosomes and smaller in size and amalgamated mitochondria were preminantly characterized. The overloaded pretreated with the AWP supplemented rats (G3) showed smaller in size of KCs in the hepatic tissue with lower sectional distribution of secondary lysosomes. The mitochondria appearance was appearing in more improved and healthy appearance in structure.

Discussion

Basically our health and diet is one of the considered pathways to prevent diseases, one of which is the iron overloading that could be prevented by whey, due to its high organic beneficial chemical nutritional and biological properities. The putative biological and physiological effects of α-La, β-Lg, Lf, lactoperoxidase, immunologlobins, Glycomacropeptide (GMP), protease peptone, and variety of growth factors as well as cysteine the building block of GSH, as well as the dietary antioxidant. In addition some digested whey proteins exhibited inhibitors as natural products acting against a wide array of tumors *via* an apoptosis like event. The induced iron overloading in the experimented rats has been executed in this submitted work.

The observed heavily stained iron of widely distributed in the liver slices of the overload G1, was locally predicted in hepatocytes, sinusoidal KCs and endothelial cells as it was observed by. Such tissue iron enrichment especially in KCs may be attributed to provoked macrophagic immunological response against induced iron intoxication as Nairz Manfred et al., have stated before. This G_1 enriched hepatic tissue iron compared to the healthy normal (control group G0) was associated with significant elevated levels of assayed ROS as O_2- and H_2O_2 that should be attributed to considerable increase in elevated percentage of free iron in such iron overloaded sections.

These threating indecies of oxidative stress promoted the predicted pro-inflammatory cytokine IL-6 biosynthesis. Noticeably, these iron intoxication biomarkers were going side by side with those of frankly detected histological features of characteristic hepatic injury as well as cellular apoptosis. The detected reduction in iron stain distribution in the treated groups; (G_2 and G_3) was mostly denoting a similar powerful metal chelating activity where the powerful metal chelating binding SH and neighboring OH residues in NAC structure or SH and NH_2 in different digested whey oligopeptides that were afforded.

The macrophagic immune respond that was prominently elevated by NAC, resulted in the assayed increased antioxidant ferritin synthesis in the chronically iron intoxicated livers of G_2. In this aspect Wallace has stated that chronically used NAC may enhance macrophagic receptor formation for further iron-importing from the surroundings and consequently this imported iron was rapidly complexed with the antioxidant ferritin apoprotein. Furthermore, Masayoshi, et al., had previously recorded that IL-6 as an acute phase protein enhances ferritin synthesis after receptor mediated endocytosis of Transferrin Binding Iron (TBI) and other iron concomitantly imported. In this area of interest, Sebastiani, et al., claimed that increments of macrophagic iron may be enhanced by IL-6-hepcidin pathway. Noticeably, the tested whey supplement in this work has resulted in a closely related effect looks like that of NAC whatever for assayed IL-6 or for the hepatic ferritin content, denoting that the whey peptidomic action may participate the preparation of the macrophagic receptors and other cellular immune response in hepatic iron overload (the task that should be approached in another work). Interestingly, the hydrolysis of α-Lactalbumin and β-Lactoglobulin by digestive enzymes may promoted specific immune response through modulation of splenocyte proliferation and cytokine section as it was observed by Brandelli, et al., and recently, by supplemented ABW to the induced iron intoxicated animals. In addition, the trypsin action resulted in peptidomic fractions with immune enhancing effects on proliferation and phagocytic activities on macrophage like cell. The increased free radicals may promoted the accelerated reduction of cellular endogenous GSH especially in mitochondria, the action that efficiently requires renewal of GSH *via* genetic Nrf2 pathway. The predicted cascade reaction that has been verified by the herein applied Western blot identification of Nrf2, where in previous work the NAC hepatoprotection against intoxicating agents was possibly performed *via* the Nrf2-HO-1 pathway. The tested competitively supplemented ABW to that of NAC treatment, deduced similar antioxidative Nrf2 activities in the iron overloaded livers were rationally accepted because some protein hydrolysates *via* regulation of GSH and other thiol modification have been thoroughly studied and confirmed. Generally the cytotoxic hepatoprotection *via* Nrf2 mechanism prominently promotes genes of the anti-inflammatory factors including the effector PPAR-ɣ that enhances the activation of M2-macrophagic gene which herein immunologically detected as CD163 and specifically CD68 of KCs. This peroxisome Proliferator Activated gamma (PPAR-ɣ) that we have previously assayed as improving effect in hepatic aflatoxicated injured tissue through Nrf2 axis of interactions. PPAR which is a lipid sensor in the affected tissue *via* regulating the transcriptional gene expression involved in hepatic inflammations considering the apoptotic immunostained caspase-3 identified in these induced iron overload hepatic macrophagic tissue cells appeared as intrinsic mitochondrial associating cellular pathway respond to the initiated oxidative stress.

In this aspect, the susceptible tested NAC as well as the ABW mostly acted as antioxidant and thiol reductant prevented such apoptotic cellular death.

In this approach, Zhang, et al., have recently investigated the effects of exogenous antioxidant NAC on tumor formation and growth using transcription analysis, found that the Transmembrane Bax Inhibitor Motif containing-1 (TMBIM1) significantly upregulated even in low NAC concentrations. Noticeably, the Van Gieson stain in the tested sections for collagen showed in portal or periportal area considerable sustained deposition on treatment with NAC. The authors have differentiated between exogenous and endogenous renewal of intracellular GSH where periportal collagen type 1 and 3 and matured elastic fibers initiated by acute or chronically exogenous GSH supplements probably promote cirrhosis or even tumors. Basically, the used peptidomic ABW or even amino acids probably appreciated antioxidants against liver injury, the suggestion of the endogenous physiologic regulatory agents for physiological renewal of intracellular GSH.

The digested whey protein fractions that had been characterized from α-lactalbumin or from β-lactoglobulin exhibiting antioxidative effects were suggested to involve inhibitory characteristic actions against hepatic lipid

peroxidation and scavenging of free radicals *via* specific cellular genes.

In this aspect, potent antioxidative amino acids that could be involved in the digested whey fractions especially those with aromatic residues that can donate protons to electron deficient radical scavenging characteristics of the amino acid residues. For instance, the antioxidative activity of His-containing peptides relates to the hydrogen donating lipid peroxyl radical trapping and/or metal ion chelating ability of the imidazole group. Moreover, the Thiol (-SH) of cysteine exhibits specific antioxidant action related to its direct interaction with radicals.

Formerly, the antioxidant capacity assay technique by using 2,2'-diphenyl-1-picrlyhydrazyl for detection of both electron transferrin and hydrogen atom transfer concomitant with ferric ion reduction has been rationally considered.

Practically, specified enzymatic orally digested whey supplement in laboratory tested animals resulted in the decrease of creatine kinase, and lactate dehydrogenase in vivo biomarkers of oxidative stress and tissue damage. Moreover the efficacy of whey protein derived peptides for binding cations is well known as one of the best tested iron peptide complexes. The net charge, the side chain length, and functional groups of the amino acids and peptides are directly determinants of the extent of complex formation with iron. The authors recognized; the primarly carboxyl groups, ξ-amino nitrogen of lysine, the guanidine nitrogen of arginine are involved in iron-peptide bonding as well as glycine and proline. Others observed that glutamic and aspartic and their carboxylic groups were amongst the main iron binding complexes.

Conclusion

Conclusively, because efficient oral therapeutic NAC should be more than 1200 mg/day in humans and stays for about 6 hours of clearance in renal and non-renal associating nausea, vomiting and diarrhea. The concentration and duration that can modulate hepatic cysteine fibrosis or even carcinoma and tumor growth *via* induction of TMBIM1 as exogenous GSH cellular renewal in injured liver tissue. The edible digested natural product of whey showed efficient chelating activity against induced iron overload similar to that of NAC as well as the antioxidative gene *Nrf2* up-regulating pathway. Such activated Nrf2 predicted chemoprotective effects against mitochondrial damage (as it was observed in the electro scanned photos). In addition, such activated Nrf2 regulator mostly balanced the mitochondrial hepatic ROS by promoting detoxification of accumulated peroxides associated induction of iron overloading *via* replenishment the depleted GSH.

Concomitantly, this predicted AWP promoted the induced highly toxic iron to be more complexed as reducing ferritin in hepatic tissue and macrophages especially KCs *via* receptor signaling. Moreover, oral supplemented whey which is mostly predicted as best provider for the hepatic tissue precursors for physiological pathway to endogenous renewal of GSH without enhancement or deposited fibrosis. In addition the probable whey derived amino acids Tyr, Trp, Met, Lys, Cys and His are predicted examples of amino acids that exhibit antioxidant activity could be provided. Hence, this work may appreciate the use of oral whey supplement to be the more preferable subsidizing hepatoprotective instead of the chronically risk old NAC drug, acting against iron overloading features in intact living animals.

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