Enhancement of Wound Healing by Un-denatured Camel Whey Proteins in Protein Malnourished Mice

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ABSTRACT

According to the WHO, malnutrition is estimated to contribute to more than one third of all children deaths. This study aimed to investigate the impact of a diet containing whey protein (WP) on wound healing in malnourished mice. Diets comprised either 5 g/kg protein malnourished (MAL) or 150 g/kg protein (control) for 3 weeks. WP-supplemented animals received the MAL diet for 3 weeks, followed by a 1-week treatment with a WP supplemented diet. Thereafter, full thickness skin wounds were punched below the shoulder blades of each mouse. Results demonstrated that MAL-mice showed a very sharp increment in their malondialdehyde (MDA) level and a significant decrease in glutathione (GSH) level compared to the control during the first 24 h. In contrast, WP-supplemented MAL-mice showed a significant decrease in MDA level and displayed an improvement in GSH level compared to the MAL-mice. mRNA levels of IGF-1 and CCL22 (wound healing macrophages (WHM)) were significantly down-regulated in MAL-mice, while regulatory macrophage marker (RM) CCL-1 and the classically activated macrophages (CAM) marker, CCLX10, were higher than the control. WP was found to significantly restore the phagocytic activity in MAL mice closer to that of the control mice. Histological investigation of the skin revealed that epidermal cell proliferation and migration, and dermal reorganization was gradually improved in WP animals. Thus, the time required for wound healing was shorter in MAL-mice supplemented with a WP diet than in MAL-mice. Data of this study may recommend camel WP as a food additive for enhancing wound healing post-surgical operations.

INTRODUCTION

Despite great clinical efforts, malnutrition remains one of the most devastating problems worldwide, particularly in developing countries (Solis et al., 2002). Malnutrition affects multiple organs (Zanim et al., 2003) and its induced morphological alterations have been thoroughly investigated. Protein malnutrition leads to multiple detrimental alterations of host immune responses and cytokines (Dai and McMurray, 1998). Thus, prolonged wound healing resulting from the production of excessive reactive oxygen species (ROS) is a serious complication of protein malnutrition.

A multitude of cellular events, such as cell proliferation, cell migration, contraction, extracellular matrix degradation and synthesis, must occur to achieve wound closure and regeneration of the injured dermis (Singer and Clark, 1999). Macrophages play a crucial role in the inflammatory phase of wound healing, secreting cytokines and chemokines, and supporting innate immune responses to bacteria. Classically activated macrophages (CAMs) are induced by recognition of microbial patterns (Padgett et al., 1998) and cytokines, while wound-healing macrophages (WHMs) secrete components of the extracellular matrix and express numerous markers of tissue-remodelling (Rodero and Khosrotehrani, 2010). Regulatory macrophages (RMs), meanwhile, show anti-inflammatory properties (Sironi et al., 2006). Several chemokines have been identified and proved to be expressed constitutively on macrophages, function in the physiological traffic and targeting of leukocytes (Baggiolini and Loetscher, 2000).

Abbreviations: CAM, classically activated macrophages; CCL, chemokine; CXCL-10, chemokine interferon-γ inducible protein (10 kDa); C-C, motif ligand; GSH, glutathione; IGF-I, insulin-like growth factor; MAL, malnourished; MDA, malondialdehyde; RM, regulatory macrophages; WHM, wound healing macrophages; WP, whey protein.
The 10 kDa chemokine interferon-γ inducible protein (CXCL-10) is a member of the CXC chemokine family which binds to the CXCR3 receptor to exert its biological effects (Liu et al., 2011). It has been proved that CAMs express CXCL-10 and efficiently kill pathogens (Martinez-Gonzalez et al., 2008). CXCL-10 is involved in chemotaxis, induction of apoptosis, regulation of cell growth and mediation of angio-static effects. In the central nervous system, CCL-5 is a key regulator involved in inducing rabies encephalomyelitis (Huang et al., 2014). The chemokine (C-C motif) ligand 1 (CCL-1), is expressed in T-helper type-2 lymphocytes and peritoneal macrophages and is involved in various pathological conditions, including peritoneal adhesions (Oshio et al., 2014). The insulin (IGF-1) signalling pathway plays a critical role in stress resistance and longevity (Warnhoff et al., 2014).

Many studies have confirmed the role of glutathione, which is increased by the dietary whey protein (WP), as a powerful antioxidant system (Bounous, 2000; Ebaid et al., 2012). WP has been found to significantly suppress hydroperoxide and ROS levels in leukocytes as well as liver and cutaneous tissues in mice by restoring the glutathione to its normal level (Ebaid et al., 2012). Therefore, WP is able to stimulate the epithelization and proliferation of fibroblasts and increase the secretion of pre-inflammatory and post-inflammatory cytokines. Furthermore, un-denatured WP is a good source of the amino acid proline, which helps to produce collagen (Belokrylov et al., 1992). Hence it is accepted that the un-denatured WP can enhance the wound healing process in diabetics. Thus, we hypothesized that protein malnutrition alters the recruitment and functions of macrophages during wound healing, thereby impairing bacterial clearance and the healing process. The effect of protein malnutrition stress and dietary supplementation with WP on macrophage recruitment and its chemokine gene expression during wound healing was investigated in this study herein.

**MATERIALS AND METHODS**

**Preparation of whey proteins**

Camel milk from the Najd region (Alazeria farm; GPS: 300 02 47/300 02 27) in Saudi Arabia, was skimmed by centrifugation at 5000 g for 20 min using an IEC Model K centrifuge (Boston, USA) and un-denatured whey protein was extracted as previously performed (Ebaid et al., 2005).

**Experimental animals and ethical approval**

Regarding experimental animals, all procedures were conducted in accordance with the standards set forth in the guidelines for the care and use of experimental animals by the Committee for the Purpose of Control and Supervision of Experiments on Animals and the National Institutes of Health. The study protocol (care and handling of experimental animals) was approved by the Animal Ethics Committee of the Zoology Department in the College of Science at King Saud University.

Adult male mice were allocated into 3 groups of 15 animals each. Mice were housed in individual cages in a temperature- and humidity-controlled room (12-h light/dark cycle) with free access to tap water and diet. Mice were allowed for lab acclimatization for two days before initiation of dietary treatments.

**Animal diet**

Rodent powder diet was formulated to be isocaloric and contained either 5 g/kg protein diet (MAL) or 150 g/kg protein diet (control). The composition of the control and MAL diet has been published previously (Lim et al., 2006). The amount of WP added to the MAL diet was calculated so that the supplemented diet WP had the same amount of sulfur-containing amino acids as the control diet. Mice were fed control diet for 3 weeks. WP-supplemented animals received the MAL diet for 2 weeks, followed by a 1-week treatment with WP diet (Fig. 1).

![Fig. 1. Experimental design showing the three different mice groups according to the protein content in the experimental diet. MAL, malnourished: WP, MAL diet supplemented with whey protein.](image)

**Excisional wound preparation**

Mice were anesthetized, and the back was shaved and sterilized using an alcohol cotton swab. The wound biopsy model used in this experiment was performed as
previously described (Schwentker et al., 2002) with slight modification. The shaved skin was pinched and folded, and the wound was punched through the full thickness of the folded skin to form a 5 mm diameter circle below the shoulder blades of each mouse.

Blood and tissue sampling
Animals were sacrificed, and blood samples were obtained from the carotid artery. Then, animals were decapitated and dissected, and their livers and skins were rapidly excised and kept at -20°C for pending homogenization.

Estimation of glutathione and lipid peroxidation
Glutathione (GSH) assay was carried out on tissue as previously described (Clark, 1985). The peroxidation of the endogenous lipids in the liver homogenates was estimated spectrophotometrically following the method described by Okhawa et al. (1979) and expressed in nanomoles of malondialdehyde (MDA) per milligrams of homogenate (nmol/mg).

Histological analyses
Skin tissues were washed in PBS and immediately stored in a 10% formaldehyde solution in PBS, dehydrated in series of alcohol dilutions and embedded in paraffin. Micromate sections were cut vertically across the wound site and adhered to slides prior to staining with hematoxylin and eosin. Sections were examined under light microscopes and photographs of the sections in wound site were taken, and images were digitized using Adobe Photoshop (Adobe Systems, Mountain View, CA).

RNA extraction and reverse transcriptase PCR (RT-PCR)
RNA was extracted from the collected samples (RNAlater) using RNeasy Mini Kit (QIAGEN) according to the manufacturer instructions. RT-PCR was performed using QIAGEN One Step RT-PCR kit as directed by the manufacturer’s instruction manual. The targeted genes were amplified using specific primers (e-oligos, Hawthorne, USA) listed in Table I. For each sample, 25 µl reaction mixture was performed. PCR reaction was carried out using Gene-Amp 9700 thermal cycler. RT-PCR products were analyzed in 1.2 % agarose gel. The levels of the three mRNA and β-actin mRNA were quantified by gel electrophoresis and densitometry. mRNA levels were normalized versus β-actin and are expressed in arbitrary units.

Fluorescence microscopy
To detect the phagocytic activity in the inflammatory phase (4, 8, 24 h after wounding) of the skin tissues, fluorescence nano-particles were injected just one hour before sacrificing of mice. Skin pieces were embedded in OCT compound (Sakura, Zouterwede, The Netherlands) and snap frozen on dry ice before serial sections were mounted on superfrost plus slides (Menzel-Glaser, Braunschweig, Germany). Evaluation of fluorescence intensity was performed as previously described (Ebaid, 2014) using the Image J software.

Statistical analysis
The one-way ANOVA statistical measure was used because data were normally distributed with homogeneous variances. The results were expressed as mean (M) ± standard deviation (SD). Only statistically significant differences with P<0.05 were found between the treatment group and the control, and between the treatment group and the malnourished group considered.

RESULTS
Effect of whey on protein concentration
Because this work was designed on the basis of a protein-malnourished mouse model, it was necessary to estimate the total protein in the sera. Data showed that total protein concentration was significantly reduced in the protein-malnourished mice in comparison with the control mice (One way ANOVA, P<0.05). On the contrary, the protein concentration was normal in the sera of the protein-malnourished mice supplemented with WP comparing to the control mice (Fig. 2).

Oxidative stress
Prolonged oxidative stress impaired the inflammatory phase of wound healing and thus delayed this process. Therefore, the oxidative stress in the...
malnourished mice and oxidative stability after supplementation with WP in the inflammatory phase (at 4, 12 and 24 h) and at the end of the experiment were estimated. MDA is the first stable product of lipid peroxidation and is therefore a good parameter to assess the oxidative stress in treated animals. MAL-mice showed very sharp increments in their MDA level of 8%, 38% and 17% with respect to the control at 4, 12 and 24 h, respectively. WP-supplemented MAL-mice showed a significant decrease in MDA level by 23%, 28% and 30% with respect to the MAL-mice at 4, 12 and 24 h, respectively (Fig. 3).

MAL-mice showed a significant decrease in GSH level by 44%, 25% and 19% with respect to the control at 4, 12 and 24 h, respectively. In contrast, WP-supplemented MAL-mice displayed improvement in GSH level by 56%, 34% and 30% compared to the MAL-mice at the same time periods (Fig. 3).

Wound closure rate

External changes in the wound morphology were monitored daily during the experimental period. The percentage of the protein-malnourished mice exhibiting wound closure was found to be significantly lower than that of the control mice at day 6. WP was found to be significantly normalizing the wound closure rate in the protein-malnourished mice to a level closer to that of the normal mice (Fig. 4).

Histological examination

Histological examination demonstrated that wounded tissues from the MAL mice appeared disturbed at two days post-wounding (Fig. 5), while those of the MAL mice supplemented with WP seemed similar to the control mice tissues. Four days after wounding, wound areas in the MAL mice exhibited an increased extent of wound margin neoeptithelia, without obvious epidermal tongues. In contrast, the wound margin epithelia of MAL

Table I. List of primers used to amplify the desired genes.

<table>
<thead>
<tr>
<th>Chemokine</th>
<th>Sequence</th>
<th>No. of nucleotide</th>
<th>Size of PCR band</th>
</tr>
</thead>
<tbody>
<tr>
<td>CXCL-10</td>
<td>Forward</td>
<td>CCAAGTGCTGCGTCATTTC</td>
<td>21</td>
</tr>
<tr>
<td>Ccl-5</td>
<td>Forward</td>
<td>GCTGCTTTGCTACCTCTCC</td>
<td>20</td>
</tr>
<tr>
<td>Ccl-22</td>
<td>Forward</td>
<td>AGGTCCTATGCTGCAATTGT</td>
<td>21</td>
</tr>
<tr>
<td>Ccl-1</td>
<td>Forward</td>
<td>GCCGAGAATTGGAGGTCCA</td>
<td>20</td>
</tr>
<tr>
<td>IGF-1</td>
<td>Forward</td>
<td>AAATCAGCAGCTTTCTCATT</td>
<td>21</td>
</tr>
</tbody>
</table>

Fig. 3. Oxidative status in the early inflammatory stage of wound healing (4-24 hours) in malnourished and WP-supplemented malnourished mice. GSH and MDA were estimated in liver samples. D, days. * shows the significance in comparison to the control group. # shows the significance in comparison to the malnourished group. For other abbreviations, see Figure 2.
supplemented with WP, showed an increase in both size and migration (Fig. 5) with two epidermal tongues, directed inwards, clearly visible on both sides of the wound. The migration of the epithelial cells at the edge of the wound in MAL mice supplemented with WP, therefore, showed a moderate degree of wound closure on the fourth day post-wounding while, by the eighth day, the wounds of MAL mice supplemented with WP were completely re-epithelialized, whereas epithelialization was still incomplete in untreated MAL mice.

**Macrophage activity**

To investigate the effect of stress on the phenotype of activated macrophages present at the wound site, gene expression of markers of wound healing macrophages (WHMs) (IGF-1 and CCL22), regulatory macrophages (RMs) (CCL1) and classically activated macrophages (CAMs) (CXCL10 and CCL5) were measured by RT–PCR in wounded tissue commencing from day 2 post-treatment. mRNA levels of IGF-1 and CCL22 were significantly lower in malnourished stressed mice at day 2 post-wounding (Fig. 6A). Both malnourished and WP-supplemented malnourished mice revealed higher CCL1 gene expression than control (Fig. 6B). In mice supplemented with WP mRNA level of CCL1 was 1.5 folds in comparison with the malnourished mice animals. mRNA levels of CCL-5 were not significantly changed in malnourished stressed mice at day 2 post-wounding in comparison with the control wounded mice. In malnourished mice, CCLX10 gene expression was higher (Fig. 6C) than the control, whereas in mice supplemented with WP mRNA levels of CCLX10 were higher in comparison with the control and malnourished mice (Fig. 6C).

To confirm the above data from whole wounds, macrophages were generally tested for the presence of the phagocytosis process at the wound sites. To evaluate the phagocytic activity, fluorescent nano-particles were injected 1 h prior to animal scarification. Results revealed that phagocytic activity was significantly reduced in Mal- mice in comparison to control mice (One way ANOVA: P < 0.05) (Fig. 6D). Interestingly, WP was found to significantly restore the phagocytic activity in MAL mice to be similar to that of the control mice.

**DISCUSSION**

Strategies to reduce the physiological and cytological consequences of malnutrition is important to be investigated. The current study designed a protein malnutrition mice-model to determine whether dietary supplementation with WP could enhance normal wound
healing. WP was found to reduce oxygen radicals and increase the levels of the antioxidant glutathione (Ebaid, 2014a,b; Ebaid et al., 2015). Our data indicated an increased capacity of the un-denatured WP-fed animals to normally heal wounds in the protein malnutrition mice-model.

Wound healing is initiated by an inflammatory phase (recruitment of leukocytes that produce growth factors), followed by a proliferation phase. The last phase involves the production and reorganization of the extracellular matrix leading to wound healing (Graves et al., 2001). This is stimulated by a number of mitogens and chemotactic factors (Altavilla et al., 2001). Here we found that MDA in malnourished mice was significantly higher than in normal mice. During the inflammatory phase, increased numbers of recruited leukocytes in the dermis of mice are able to cause tissue damage by prolonged release of ROS. This impairs keratinocyte endothelial cells, fibroblasts, and collagen metabolism (Silhi, 1998) which in turn, causes healing impairment (delayed granulation tissue formation, decreased collagen, and its organization) (Niki et al., 1991). This is probably what has occurred in protein malnourished mice. In contrast, WP was found to significantly elevate the concentration of glutathione which scavenges ROS. WP contains cysteine which is necessary for the synthesis of glutathione (Middleton et al., 2004) and this may be a potential factor controlling the healing process.

Because of their capacity to produce inflammatory cytokines and growth factors, macrophages play a central role in wound repair. WP was also found to significantly enhance phagocytosis in malnourished mice. Similarly, Michee et al. (2013) found that macrophages exposed to LPS and TNF-α had increased phagocytosis. Chemokine expressions, which are necessary for normal migration and chemotaxis of macrophages, declined in the first two days post-wounding in malnourished mice. In contrast, WP was found to up-regulate these chemokines in malnourished mice in the inflammatory phase of wound healing. Peritoneal macrophages isolated from mice

Fig. 5. Histological structure of wounds from different mice groups (A, C, E; H & EX100) and the neutrophil infiltration in the wound site (B,D,F; H & EX200) on day 2 post wounding.
showed increased mRNA levels of several inflammatory mediators, including, CCL-5 (Manoharan et al., 2014).

Moreover, results indicated that wound healing macrophages (IGF-1 and CCL22) were inactivated under the protein malnutrition conditions, while both regulatory macrophages (CCL-1) and classically activated macrophages (CXCL-10 and CCL-5) were alternatively activated under this stress in comparison with the control mice. WP was found to activate all macrophage phenotypes even under malnutrition stress, while WP moderated levels of IGF-1 and CCL22 closer to the control, significantly upregulated CCL1 and CCLX10 levels. However, to explain why CCL1 and CCLX10 were higher than that of the MAL mice after WP administration, may be due to increasing defence mechanism under malnutrition stress. In disease situations, however, prolonged expression of inflammatory chemokines and cytokines induce chronic complications. We previously found that although WP encourages inflammatory mediators at the beginning of wound healing, it finally suppresses these mediators in order to permit the ensuing normal recovery phases (Ebaid et al., 2011, 2013). Thus, it is expected that high levels of inflammatory chemokines after 4 days were suppressed by WP treatment. This suggestion can be proved by normalizing wound healing in WP mice.

Wound contraction is a significant component of dermal excisional wound healing in mice and in humans (Singer and Clark, 1999). WP is a good donor of the amino acid proline which helps in production of collagen
(Belokrylov et al., 1992). This might assist the normal development of the collagen fibrils in the wounded area of the mice. WP was found to significantly normalize the wound closure rate in the protein-malnourished mice to a similar level to that of the normal mice.

In conclusion, the current study proved that camel un-denatured WP enhanced the wound healing process in malnourished mice. This could be probably because the efficiency of cysteine in increasing the glutathione level is greater when it is delivered in the WP (Bounous et al., 1989), and that the glutathione influenced the reduction of the oxidative stress and increased the macrophage inflammatory chemokines, which in turn enhanced wound healing.

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