Intense exercise induces increased levels of pro-inflammatory and anti-inflammatory cytokines. Thus, the purpose of this study was to examine the effects of a special cake (consisting of carbohydrate to whey protein 3.5:1) vs. an isocaloric carbohydrate cake on inflammatory markers after exhaustive cycling in humans. Nine subjects received either the experimental or placebo cake in a counterbalanced fashion using a crossover, double-blind, repeated-measures design. They performed one trial involving a 2 h exercise on a cycle ergometer at 60–65% VO2max which was increased at 95% VO2max. Blood samples were collected pre-exercise, 30 min and 4 h post-exercise, post-time Trial and 48 h post-time Trial. Cakes were consumed immediately post-exercise and every 1 h for the next 3 h. The results showed that consumption of the experimental cake reduced significantly (p < 0.05), 4 h post-exercise, the pro-inflammatory protein TNF-a, IL-10, IL-8 and IL-6 levels increase in response to exercise (Febbraio and Pedersen, 2002, 2005; Gleeson and Bishop, 2005; Pedersen et al., 2004; Petersen and Pedersen, 2005). Toward the end of the inflammatory cascade, C-reactive protein (CRP), an acute-phase protein, is induced by inflammatory cytokines, particularly IL-6. The magnitude of the inflammatory response depends on the intensity, duration and chronicity of the exercise. If pathological inflammation occurs, then excessive, irreparable damage to host tissues can occur (Calder et al., 2009). To avoid these detrimental effects, investigators have tried to suppress inflammation via supplementation of carbohydrate or carbohydrate and protein beverages (Afrondeh et al., 2010; Miles et al., 2007; Robson-Ansley et al., 2011; Rowlands et al., 2008; Starke et al., 2000, 2001). Carbohydrate supplementation during prolonged endurance exercise has been associated with higher blood glucose and lower cortisol, epinephrine and growth hormone responses (Murray et al., 1991). During intense exercise due to the low levels of blood glucose, the HPA axis is activated leading to increased levels of cortisol and epinephrine, which have anti-inflammatory action. The example, plasma interleukin (IL)-1α, tumour necrosis factor-a (TNF-a), IL-10, IL-8 and IL-6 levels increase in response to exercise (Febbraio and Pedersen, 2002, 2005; Gleeson and Bishop, 2005; Pedersen et al., 2004; Petersen and Pedersen, 2005). Toward the end of the inflammatory cascade, C-reactive protein (CRP), an acute-phase protein, is induced by inflammatory cytokines, particularly IL-6. The magnitude of the inflammatory response depends on the intensity, duration and chronicity of the exercise. If pathological inflammation occurs, then excessive, irreparable damage to host tissues can occur (Calder et al., 2009). To avoid these detrimental effects, investigators have tried to suppress inflammation via supplementation of carbohydrate or carbohydrate and protein beverages (Afrondeh et al., 2010; Miles et al., 2007; Robson-Ansley et al., 2011; Rowlands et al., 2008; Starke et al., 2000, 2001). Carbohydrate supplementation during prolonged endurance exercise has been associated with higher blood glucose and lower cortisol, epinephrine and growth hormone responses (Murray et al., 1991). During intense exercise due to the low levels of blood glucose, the HPA axis is activated leading to increased levels of cortisol and epinephrine, which have anti-inflammatory action. The...
organism in order to balance this anti-inflammatory activity produces pro-inflammatory cytokines. Given the potential link between stress hormones and cytokine production it is hypothesized that carbohydrate vs. placebo supplementation would keep plasma glucose levels at a higher level, attenuating the rise in epinephrine and cortisol and both pro- and anti-inflammatory cytokines.

In recent years, milk constituents as proteins have been recognized as functional foods suggesting that their use has a direct and measurable effect on health outcomes (Ainutis, 2004; Nagendrak, 2000). The main sources of milk proteins are the casein and the whey. Specifically, whey is a by-product of cheese manufacturing that remains in an aqueous liquid after milk has been curdled and strained. The components of whey including beta-lactoglobulin, alpha-lactalbumin, bovine serum albumin, lactoferrin, immunoglobulins, lactoperoxidase enzymes, glycomacropeptides and lactose demonstrate a range of immune-enhancing properties (Madureira et al., 2007; Walzem et al., 2002). Therefore, whey is considered a functional food, as it is involved in preventing or improving several pathological conditions (Marshall, 2004; Smithers, 2008). Thus, whey is currently a popular dietary protein supplement purported to provide antimicrobial activity, immune modulation, improved muscle strength and body composition, and to prevent cardiovascular disease and osteoporosis (Marshall, 2004). Relative to other protein sources, whey has a high concentration of branched-chain amino acids (BCAAs) – leucine, isoleucine and valine. BCAAs, particularly leucine, are important factors in tissue growth and repair. Moreover, leucine has been identified as a key amino acid in protein metabolism during the translation-initiation pathway of protein synthesis (Anthony et al., 2001; Balage and Darvevet, 2010). Whey is also rich in sulphur-containing cysteine and methionine amino acids that enhance immune system function due to their intracellular conversion to glutathione (Marshall, 2004).

The aim of the present study was to examine the effects of a special cake consisted of a specific ratio of carbohydrates and whey protein on inflammatory markers in athletes after exhaustive exercise. Supplementation of carbohydrates and proteins in a cake form is innovative, since previous studies used mostly liquid supplements.

2. Materials and methods
2.1. Subjects
Nine physically active men (age, 28 ± 2 years; height, 184 ± 3 cm; weight, 77 ± 2 kg; body fat, 13 ± 2%; body mass index, 23 ± 1 kg/m²; VO2max, 41 ± 0.2 L/min mean ± SEM) participated in the present study. The subjects were training at least three times per week for at least 3 h and had a training history of at least 2 years. They were nonsmokers and were not receiving anti-inflammatory medication or nutritional supplements. VO2max measurement ensured that the subjects exercised at similar intensities. A written informed consent to participate in the study was provided by all participants after they had been informed of all risks, discomforts and benefits involved in the study. The procedures were in accordance with the Helsinki declaration of 1975 and approval was received by the human subjects committee of the University of Thesissey.

The subjects visited the laboratory for the first time for a screening of anthropometric parameters and they completed a health and activity questionnaire. Each participant reported to the laboratory in the morning after an overnight fast and abstained from alcohol and caffeine for 24 h. Body mass was measured to the nearest 0.5 kg (Beam Balance 710, Seca, UK) with the subjects lightly dressed and barefoot. Standing height was measured to the nearest 0.5 cm (Stadiometer 208, Seca). Percentage body fat was calculated from seven skinfold measures (average of two measurements at each site), using a Harpenden caliper (John Bull, UK), according to the procedures published (American College of Sports Medicine, 2000). Body mass index was calculated as the ratio of body weight (kg)/height (m²). VO2max was determined after a maximal consumption test on a cycle ergometer (Monark 834E, Sweden) was performed. The protocol began at 1.5 km (≈ 70RPM) for 1 min and was increased by 0.5 km every 2 min until VO2max was reached. Respiratory gas variables were measured using a metabolic cart (Vmax29; SensorMedics, USA), which was calibrated before each test using standard gases of known concentration. Exercise heart rate was monitored by telemetry (Polar Tester, S610TM, Electro Oy, Finland).

2.2. Diet and activity before the experiment
The subjects were instructed to follow their usual eating habits during the days before the experiment. They were also asked to record on a dietary record sheet their diet 3 days before the first exercise bout and for 2 days after it. All volunteers were instructed to stay away from physical exercise for 48 h preceding and 2 days following the experiment. The subjects received a copy of their dietary record sheets and were asked to follow exactly the same food intake patterns (as recorded in their dietary record sheets) before their second experimental session.

2.3. Design
Each subject participated in two trials in a counterbalanced fashion (same subjects received both the experimental cake and placebo cake in a random order) using a crossover, double-blind, repeated-measures design. The subjects visited the laboratory for a second time 5 days after their VO2max determination (08:00–09:00 h in the morning) and were participated either in the experimental or placebo trial. Each subject participated in two experimental sessions separated by a wash out period of 1 week. During each session the subjects consumed either an experimental cake providing 0.9 g carbohydrate/kg body weight/h and 0.26 g protein/kg body weight/h, providing a ratio between carbohydrates and protein of 3:5:1, or a placebo cake providing 1.1 g carbohydrate/kg body weight/h and 0.1 g protein/kg body weight/h. The experimental protocol consisted of the following phases: (I) 2 h of continuous cycling on cycle ergometer (Monark 834E, Sweden) at an intensity corresponding to 60–65% of their established VO2max, (II) 4 h of recovery, (III) 1 h of continuous cycling at 60–65% of their VO2max, (IV) cycling speed was increased to 95% of their VO2max until exhaustion (time Trial), (V) 1 h of recovery (Fig. 1). Exercise was performed at a temperature of 21 ± 2 °C and 45 ± 4% relative humidity. To attenuate subjects’ discomfort, water was available ad libitum throughout the experiment and its consumption was recorded. Expired gas samples were collected every 15 min to ensure the prescribed exercise intensity. Perceived fatigue of the subjects was recorded every 15 min using Borg scale during phases I and III and at the end of phase IV. Blood samples were collected pre-exercise (T1), 30 min post-exercise (T2), 4 h post-exercise (T3), immediately post-time Trial (T4) and 4 h post-time Trial (T5) (Fig. 1). During phase I (2 h cycling at 60–65% VO2max), muscle glycogen stores are depleted and the organism is stressed and as a response produces cytokines. During phase II (4 h recovery), the cake was administered in order to find out how it affects inflammatory markers after intense exercise. It was followed 1 h cycling at 60–65% VO2max in order to see the magnitude of the increase in the inflammatory markers after the first bout of exercise and the cake administration. The intensity of exercise was increased at 95% VO2max until exhaustion in order to determine if the cake administration affects performance. One experimental or placebo cake was consumed by the subjects immediately post-exercise and three more experimental or placebo cakes were consumed every 1 h after the first one. Exercise testing, cake administration and blood sampling was repeated at the same time of day and in the same order before and after the first trial (experimental, Fig. 1A) as well as before and after the second trial (placebo, Fig. 1B).

2.4. Blood collection and handling
Blood samples (10 mL) were drawn from a forearm vein with subjects in a seated position. Blood was collected in ethylenediamine tetraacetic acid (EDTA) tubes, centrifuged immediately at 1370 g for 10 min at 4 °C and the plasma was collected and used for the determination of IL-6, IL-10 and CRP. Plasma lysate was then stored at −80 °C until analyses.

2.5. Plasma IL-6 and IL-10 measurement
For IL-6 and IL-10 determination, a quantitative sandwich enzyme immunoassay technique (R & D systems, Minneapolis, MN, USA) was used. Briefly, a monoclonal antibody specific for IL-6 or IL-10 has been pre-coated onto a microplate. Afterwards, 100 µl and 200 µl of standards and samples were added into the wells for IL-6 and IL-10 assay respectively, and any IL-6 or IL-10 present in the samples was bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for IL-6 or IL-10 was added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution was added to the wells. After an incubation period, an amplifier solution was added to the wells and color develops in proportion to the amount of IL-6 or IL-10 bound in the initial step. The color development was stopped and the intensity of the color was measured at 490 nm and also at 650 nm as a reference wavelength in a Bio-Tek ELx800 ELISA microplate reader (Winoiski, VT, USA). The minimum detectable dose (MDD) of IL-6 and IL-10 was 0.039 pg/mL and 0.09 pg/mL, respectively. The intra-assay coefficients of variation (CV) for IL-6 and IL-10 were 7.4% and 6.6%, respectively.

Please cite this article in press as: Kerasioti, E. et al. Anti-inflammatory effects of a special carbohydrate–whey protein cake after exhaustive cycling in humans. Food Chem. Toxicol. (2013), http://dx.doi.org/10.1016/j.fct.2013.01.023
2.6. Plasma CRP measurement

The immunoturbidimetric assay for CRP was carried out using Olympus System CRP reagent, with an Olympus AU2700 apparatus (Rungis, France). The lower detection limit for CRP was 1.57 mg/L. The assay was linear within a 5–300 mg/L concentration range.

2.7. Statistical analysis

Inflammation data were analyzed by two-way (treatment × time) analysis of variance (ANOVA) with repeated measures on time. Pairwise comparisons were performed through simple main-effect analysis. The level of statistical significance was set at $p < 0.05$. For all statistical analyses SPSS, version 13.0 (SPSS Inc., Chicago, Ill.) was used. Data are presented as mean ± SEM.

3. Results

In plasma concentration of IL-6 (Fig. 2A), main effect of time and treatment ($p < 0.05$) was found. In particular, there was a statistical significant increase in plasma IL-6 concentration at both 30 min post-exercise (T2) and post-tT (T4) compared to pre-exercise (T1) (Fig. 2A). Moreover, administration of the experimental cake caused a significant ($p < 0.05$) reduction in plasma concentrations of IL-6, at 4 h post-exercise (T3), by 50% compared to the placebo group (Fig. 2A). On the contrary, in plasma IL-10 concentration (Fig. 2B), there was not observed significant main effect of time or treatment. However, plasma IL-10 levels, at 4 h post-exercise (T3), were higher by 118%, in the experimental group compared to the placebo group, but this difference was not statistically significant (Fig. 2A). In plasma CRP concentration (Fig. 2C), main effect of time and treatment ($p < 0.05$) was found. In particular, there was a significant ($p < 0.05$) increase in plasma CRP concentration at 4 h post-exercise (T3) compared to pre-exercise (T1). Moreover, similar to IL-6, plasma concentrations of CRP in the subjects receiving the experimental cake were statistically significant ($p < 0.05$) lower by 46%, at 4 h post-exercise (T3), than the subjects receiving the placebo (Fig. 2C).

4. Discussion

The aim of the present study was to investigate the effects of a cake containing carbohydrates and whey protein in a specific ratio (3.5:1) on inflammatory markers after exhaustive cycling in humans. Specifically, the inflammatory markers IL-6, IL-10 and CRP were measured in plasma samples of the subjects participated in the experiment. IL-6 is produced in larger amounts (up to 100-fold) than any other cytokine in response to exercise (Fischer, 2006). During physical exercise IL-6 is predominantly produced within the working skeletal muscles (Jonsdottir et al., 2000; Starkie et al., 2001; Wood et al., 2009) and this production, in turn, accounts for the exercise-induced IL-6 increase in plasma (Pedersen and Edward, 2009; Steensberg et al., 2000). The magnitude of the exercise-induced IL-6 response is dependent on intensity and especially duration of the exercise, while the mode of exercise has little effect. IL-6 has been classified as both an anti-inflammatory and a pro-inflammatory cytokine (Gleeson et al., 2011). It exerts its anti-inflammatory properties only during exercise, but at the end of exercise it exhibits pro-inflammatory activity through involvement in the generation of the acute phase response, the local and systemic events accompanying inflammatory local response (Mastorakos et al., 2005). IL-6 increases basal and insulin-stimulated glucose uptake which is facilitated by translocation of GLUT4 to the plasma membrane (Carey et al., 2006). Exercise training may reduce basal IL-6 production as well as the magnitude of IL-6 response in the acute exercise by counteracting several potential
stimuli of IL-6. Accordingly, a decreased plasma IL-6 concentration in response to exercise characterizes normal training adaptation (Fischer, 2006). IL-10 downregulates or completely inhibits the expression of several pro-inflammatory cytokines and other soluble mediators, thereby further compromising the capacity of effector T cells to sustain inflammatory responses (Maynard and Weaver, 2008; Moore et al., 2001). Thus, IL-10 is a potent promoter of an anti-inflammatory state. CRP is an acute phase protein which reflects a measure of the acute phase response. IL-6 and other cytokines trigger the CRP synthesis in liver.

The key finding of the present study is that the consumption of the experimental cake attenuated post-cycling inflammatory response. Our findings are in accordance with the majority of previous studies, which have shown that carbohydrate ingestion attenuated increase in pro-inflammatory cytokines, especially IL-6. For example it has been reported that consumption of about 1 g of carbohydrate per kilogram of body mass per hour attenuates the IL-6 response to prolonged endurance exercise (Nieman et al., 2000). Scharhag et al. (2006) showed that carbohydrate supplementation reduced significantly total plasma IL-6 after cycling for 4 h in humans. Moreover, in another study, carbohydrate supplementation attenuated the increase in plasma IL-6 during both running and cycling compared to placebo beverage ingestion (Nieman et al., 2003). Furthermore, Robson-Ansley et al. (2011), who examined the effect of carbohydrate ingestion on IL-6 during a 90 min self-paced time trial in seven trained male runners, reported that carbohydrate ingestion attenuated IL-6 response to exercise. Also, the results from a study involved 7 moderately trained males performing both running and cycling, demonstrated that carbohydrate ingestion decreases the increase in IL-6 (Starkie et al., 2001).

Concerning the anti-inflammatory IL-10, its levels were elevated by 118% at 4 h post-exercise compared to the placebo group, although not statistically significant. However, in a previous study, after 2 h of intensive resistance training, there were not differences in the plasma IL-10 level between carbohydrate and placebo ingestion (Nieman et al., 2003).

CRP is an inflammatory protein made by the liver in response to increases in IL-6 and other inflammatory mediators (Edward, 2005). Rises in CRP indicate that the IL-6 produced at the tissue level is triggering an acute-phase, systemic inflammatory response. Thus, the reduced levels of CRP in the experimental group, at 4 h post-exercise, compared to the placebo group are attributed to the reduced levels of IL-6 at the same time point. However, Henson et al. (2000) demonstrated that after 2 h of rowing there were no differences in CRP levels between the carbohydrate and the placebo group. Similarly, in another study, after eccentric elbow flexion there was also no significant difference in CRP between carbohydrate and placebo group (Afroundeh et al., 2010).

The above differences in the results between the present and the previous studies may be due to the different type of exercise and to the protein that our supplement contains.

In the literature there are a limited number of studies concerning the effects of a carbohydrate-protein supplement on inflammatory markers. Cosio-Lima et al. (2012) demonstrated that feedings of a carbohydrate–protein drink during long periods of cycling did not greatly attenuate inflammatory responses in cyclists when compared to feedings of a carbohydrate-alone solution. Moreover, in another study, the effects of a carbohydrate–protein ingestion on inflammatory markers in 12 cyclists were inconclusive or trivial (Rowlands et al., 2008). These results are in contrast with ours, which showed that the consumption of a carbohydrate–protein supplement in a cake form resulted in attenuated levels of inflammatory markers after exhaustive cycling. Since, in the above studies, the protein source used was also whey protein and a similar type of exercise was used, the differences in the results may be due to the specific peptide composition of our whey protein and/or to the fact that our supplement was in a cake form, while the other studies used liquid beverages. Interestingly, a mouse study demonstrated that lactoferrin, a component of whey protein, had anti-inflammatory properties by reducing the levels of TNF-a and increasing IL-10, thus decreasing inflammation (Kobayashi et al., 2011).

Moreover, since increased formation of reactive oxygen species (ROSs) occurring during exercise is capable of activating transcription factors known to regulate IL-6 synthesis (Fischer, 2006), the anti-inflammatory activity of the experimental cake may also be attributed to its antioxidant effects (Kerasioti et al., 2012). In

![Fig. 2. The effects of experimental and placebo cake administration on (A) plasma interleukin (IL)-6, (B) plasma interleukin (IL)-10 and (C) plasma C-reactive protein (CRP). T1 = pre-exercise, T2 = 30 min post-exercise, T3 = 4 h post-exercise, T4 = immediately post-time Trial, T5 = 48 h post-time Trial. *Significantly different between experimental and placebo groups at the same time point (p < 0.05). †Significantly different compared to pre-exercise (p < 0.05).](http://dx.doi.org/10.1016/j.fct.2013.01.023)
particular, in a previous study using the same type of exercise, we have shown that the administration of the experimental cake reduced thiobarbituric acid reactive substances (TBARS) plasma levels at 30 min post-exercise (T2) (Kerasioti et al., 2012). Thus, by exerting its antioxidant effects the carbohydrate–protein supplement may attenuate the post-exercise inflammatory response.

Moreover, elevated stress and inflammation is related to a higher probability of developing the overtraining syndrome (Margonis et al., 2007; Tanskanen et al., 2010). Previous research (Kerasioti et al., 2012) has shown that this nutritional intervention results in reduced oxidative stress and the results from this study indicated lower inflammatory following ingestion of this cake. Therefore, long term ingestion of this food could result in lower oxidative stress and inflammation and therefore preventing unwanted situations such as overtraining.

Conflict of Interest

The authors declare that there are no conflicts of interest.

Acknowledgments

This work was co-financed by the European Union (European Social Fund – ESF) and Greek national funds through the Operational Program “Education and Lifelong Learning” of the National Strategic Reference Framework (NSRF) – Research Funding Program: Heraclitus II. Investing in knowledge society through the European Social Fund.

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