**The Effect of Dietary Supplements (Creatine & Whey protein) in body weight of mice and Human Blood Lymphocytes**

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**Abstract:**

Specific nutritional strategy of ceartine revealed significant increasing in the body weight of mice after 3 weeks of treatment, the increasing in weight of animals that orally administrated by whey protein (Wp) were higher than the increasing obtained by animals administrated by creatine (CrM) . Determination of DNA in human blood lymphocytes recorded significant differences in apoptosis of lymphocytes treated with Creatine and whey protein, the obtained results showed significant increasing in percentages DNA fragmentation in human lymphocyte depending on time of exposure with creatine and whey protein and their concentrations. Furthermore the DNA fragmentation were illustrated in fluorescent sphere appears in slide of comet assay, whey protein groups(A),and creatine group(B), illustrated higher damage of DNA revealed in length of tail, tail moment and DNA fragmentation clarified in the intensity of fluorescent heads (Cyber green stain ),which determined more than two fold in length of tail for Creatine treatment and highest to more than six fold in tail moment and DNA fragmentation in the Whey protein treatment comparing to negative control.

**Introduction:**

Specific nutritional strategies that may enhance the acute anabolic response (i.e., stimulation of muscle protein synthesis) to resistance exercise (RE) which in turn may augment chronic physiological adaptations such as muscle hypertrophy and strength (Paddon, 2006).Whey protein (WP) and creatine monohydrate (CrM) are two dietary supplements commonly used to promote muscle strength and hypertrophy during resistance exercise (RE) (Rawson, 2003.).WP supplements generally contain a higher concentration of essential amino acids (EAA) than other protein sources and have rapid absorption kinetics. Protein supplementation appears to have at least three prominent roles in augmenting muscle accretion and promoting hypertrophy. Firstly, by supplementing close to RE to ensure a greater stimulation of muscle anabolism in response to this activity. Secondly, some data suggests that supplementation between meals may promote more frequent stimulation of muscle protein synthesis, thereby promoting a higher net gain in muscle protein on a daily basis. Finally, strategic supplementation with protein that is rich in EAA and in particular (dangin, 2003), leucine may help restore the acute anabolic response to meals which characteristically diminishes with aging(Rieu,2006).Creatine is a compound that can be made in our bodies or taken as a dietary supplement. The chemical name for Creatine is N-(aminoiminomethyl)-N-methyl glycine or methylglycocyamine. The most commonly used form is creatine monohydrate. Creatine is synthesized in the liver, pancreas, and kidney from the three amino acids - L-Arginine, Glycine and L-Methionine. Following its biosynthesis, creatine is transported to the skeletal muscles, heart, brain, and other tissues. Most of the creatine is metabolized in these tissues to creatine phosphate. Creatine phosphate is an essential part of energy utilization in the body. It is believed that about 95% of the creatine in our body is stored in our muscles. The remaining 5% is stored in various other parts of the body including the brain, heart and other tissues(Casey and Greenhaff, 2000).It has been established that certain types of protein affect whole body protein anabolism and accretion and therefore, have the potential to affect muscle and strength development during RE training(Phillips, 2006).The comet assay (single cell gel electrophoresis) is used for the detection of primary DNA damage induced in isolated cells or nuclei (Rothfuss et al., 2010).The principle of the alkaline comet assay is based upon the ability of denatured, cleaved DNA fragments to migrate out of the nucleotide under the influence of an electric field, whereas undamaged DNA migrates slower and remains within the confines of the nucleoid when a current is applied.

The study designed to examine the effects of oral administration of CrM and WP on body weight and their influence in DNA damage of human blood lymphocytes confirmed by comet assay.

**Material and methods:**

* **Animal experiment :**

Thirty male mice- Swiss albino (weight were 20 to 25.0 g) were used in this experiment, animals were divided into five groups each group included 6 individual, the groups administrated supplements orally in drinking bottle for five days and water for two days in a week by the following:

First group (1-1): orally treated with low dose of creatine 3.5g/120ml of water and second group ( 1-2): treated with high dose of creatine 14g/120ml water, two groups treated that dosages for one week then the dosages decreased to half for 14 days, after this period animals weight were recorded.

Third group (2-1): orally treated with low dose of Whey protein by dissolving 3.0 g/120ml water. Forth group (2-2): treated with high dose of Whey protein 12g/120ml ,two groups administrated the dosages ( for first week ) then decreased to half for 14 days then weighted. Fifth were Control group represented (negative control group without treatment) received water and diet without supplements.

**Effects of Creatine and whey protein in the viability of lymphocytes, and detection the apoptosisusing Quantitative method from healthy individuals**

**Collection of Blood samples**

**A-: Subjects**

The study included 15individuls, five ml of peripheral blood samples were obtained from healthy men, immediately transfer to sterile tubes for lymphocyte separation. While 0.8 ml of blood used for lymphocyte culturing in the presence of whey protein and creatine, 5 men were routinely administrated creatine and/or whey protein as supplemented drink for several months , and 5 of them didn’t exposed to any supplementation were considered as negative control.

**Isolation of blood lymphocytes-B**

* Cells Separate by using gradient centrifugation (Sykes *et al.,* 1970). The cells sedimentto an equilibrium position equivalent to their own density—a process called isopycnic sedimentation. The gradients can be formed by nontoxic, high-molecular-weight material such as ‘Ficoll’. This method is particularly effective for the isolation of certaincell types (lymphocytes from blood*).* . white Buffy coat of lymphocyte was aspirated (not disturbed) by Pasteur pipette and transferred into a ten ml tube, washed by PBS, centrifuged at 2500 rpm until a pellet was formed. Thesupernatant was discarded; this procedure was repeated for three times. Finally, the lymphocyte pellet was re-suspended in 0.5 ml of PBS.

The cell suspension used for determined the **viability of lymphocytes** and the DNA fragmentation.

**Detection of CrM and WP effects on the viability of lymphocytes isolated from healthy individuals**

* Serial concentrations (500, 1000, 2000 µg/ml) of CrM and WP were added to 4.5ml of RPMI-1640 (growth media) containing 1x106(0.5ml) of cell suspension and0.3 ml of PHA contents were mixed gradually by inversion and incubated at 37̊ C for 24, 48, and 72hrs.

One tube cultured without supplements additive as a negative control.

* After incubation periods a known volume of lymphocyte suspension (100µl) was mixed with an equal volume of trypan blue dye and examined immedatly under light microscope using Heamocytometer counting chamber to calculated of viable cell depending on viability formula:

% viability = viable cell count / total cell count×100.

**Preparation of different concentrations of supplements**

Serial concentrations of Creatine and Whey protein were prepared at different concentrations (500, 1000, 2000 µg/ml) with serum free media. These concentrations were prepared from stock solution of Creatine or Whey protein in 10000 µg/ml.

**C- Detection of apoptosis in lymphocytes using Quantitative method by DPA reagents:** (Siddique**,***et al*.2006)

Culture tubes were divided to three groups as the following:

**First group:**

Culturing tube containing 4 ml of  **co**mpleted RPMI-1640 medium, 0.5ml of cell suspension (1x106cell/ml), 0.3ml of PHA and 0.2 ml of **C**rM and /or WP at 500, 1000, 2000 µg/ml were added , the contents were mixed gradually by inversion and incubated at 37̊ C for **72hrs**

* Three tubes cultured without dietarysupplemented as a negative control.

**Second group**:

This group containing all components as first group, except the addition of CrM and WP after 24 h. of culturing , than reincubated at 37̊ C for **72h**.

**Third group:**

## This group similar to second group in contents and time of addition CrM and WP, but its incubation for 48 h.

Cultured cells were harvested after completed of incubation periods as following:

* Colcimied (0.1ml), was added to give a final concentration of 10µg/ml at 71.30 hrs to the tubes of the first , and second groups, well mixed and incubated at 37°C for another 30 min. While colcimied was added to third group at 47.35h then complete the incubation period.
* Cultured cells were harvested by centrifugation at 1500 rpm for 10 min, the supernatant was removed and the cell pellet was re- suspended by adding 5 ml of pre-warmed KCl solution, then incubated at 37°C for 30 min.
* The cells were centrifuged at 4Ċ at 1000 rpm for 10 min. to precipitate the cells, Supernatant was removed, and the remaining 1ml was transferred to another tube which takes **B** symbol. While first tube which contain pellet cells gave **A** symbol.
* A volume of 1.0ml TTE solution (Triton-x100 in the TE solution) was added to the pellet in tubes **A** and vortex vigorously. This procedure allowed the release of fragmented chromatin from nuclei, after cell lysis and disruption of the nuclear structure (following Mg++ chelating by EDTA in the TTE solution).
* To separate fragmented DNA from intact chromatin, tubes **A** were centrifuged at 14000 rpm for 10 min at 4̊ C.
* The supernatant were transferred carefully in new tubes labeled **C**.
* A volume of 1.0ml of TTE solution was added to the small pellet in tubes **A**.
* A volume of 1.0ml of 25% TCA was added to tubes **C, A**, and **B** and vortex vigorously.
* The precipitation was allowed to settle down **overnight at 4̊ C**.
* After incubation, precipitated DNA was recovered by pelleting for 10 min. at 14000 rpm at 4̊ C. The supernatants were discarded by aspiration.
* The DNA was hydrolyzed by addition 160µl (5%) TCA to each pellet then heated for 15 min at 90̊ C in water bath.To each tube 320µl of freshly prepared DPA solution was added, then vortex, allowing color to develop for about 4hrs at 37̊ C or overnight at 25̊ C. The optical densities of the tubes were measured with spectrophotometer on wave length of 600 nm. The percentage for DNA fragmentation which indicated the programmed cell death of cancer cells was calculated, according to the fallowing equation:

**% F= B+C  X 100**

**A+B+C**

%F: The percentage of fragmentation.

A: Reading of optical density of tube labeled with A.

B: Reading of optical density of tube labeled with B.

C: Reading of optical density of tube labeled with C.

**Detection of DNA fragmentation by Comet assay:**

The alkaline comet assay **(single cell gel electrophoresis)**  was used to investigate the possible DNA damage in peripheral blood lymphocytes treated with (C and W) in comparison with control.(Peggy and Banath2006).

**Preparation of samples and slides**

Agarose slides were prepared by dipping the slides into normal molten 1.5 %( w/v) agarose, allowed to air dry to a thin film. The cell suspension was centrifuged at 1500 rpm for 2 min. The supernatant was discarded and the pellet washed once with ice-cold PBS (without Mg2+ and Ca2+) and centrifuged at 1500 rpm for 2 min. Then supernatant was discarded(zainol,etal.2009).A cell sample was combined with low melting point agarose at 1:10 ratio (V/V) and the mixture (75μl/ well) immediately was added into slide comet by pipette. The slides transferred to 4ºC in a dark container for 30 min,then transferred to a small basin containing pre chilled lysis buffer, the slide was immersed in the buffer overnight (18-20 h) at 4ºC in the dark, the slides were immersed in an electrophoresis solution for 20 min, and then transferred to a horizontal electrophoresis chamber filled with a cold TBE electrophoresis solution, 24volt (V) /cm and 300 milliamps (mA) was applied to the chamber for 18 min. The TBE electrophoresis solution was aspirated from the chamber and replaced with neutralization buffer, 0.4M of Tris-HCl solution (pH 7.5) for 5 min in order to neutralize the cells. Diluted cyber green dye 50 μl was added to each well ofcomet assay slide and incubated at room temperature for 15 min.The slides were rinsed with distilled water to remove excess stain, finally examined by fluorescence microscopy image analysis software comet score, the analysis software will calculate different parameters for each comet, three parameters were estimated to indicate DNA migration, tail length (distance from the head center to the end of the tail), mean tail moment and % DNA in tail (Azqueta*et al.,* 2009).

**Results and Discussion**

**Effect of dietary supplements on mice body weight:**  -

The obtained result revealed significant increasing in the body weight of all animals after 3 weeks of treatment, the increasing in weight of animals in group 1-1 and 1-2 (creatine) were lower than that increasing in body weight of animals in group 2-2 and 2-1(whey protein) . The current results of the weights was summarized in Table 1

Table- 1 Body weight of mice drinking dietary supplements (Creatine and Whey protein) after three weeks of treatment. ***Before and***

|  |  |  |
| --- | --- | --- |
| Wt (gm)  After treatment | Wt (gm)  ment Before treat | Groups |
| 25.1 | 22.2 ±1.2 | Control |
| 27.6 ±1.42 | 21.7 ±0.97 | 1-1 |
| 26.8 ±2.08 | 20.13 ±2.12 | 1-2 |
| 30.4 ±1.79 | 22.9 ±3.05 | 2-1 |
| 32.9 ±2.22 | 23.8 ±2.76 | 2-2 |

**Determination of DNA fragmentation according to apoptosis in lymphocytes**:

The present study revealed the ability of two supplemented on DNA fragmentation in human lymphocytes after 24h, 48h and 72 h. The results of Percentage of DNA fragmentation of human lymphocytes treated by Creatine or Whey protein were shown in table -2 and table- 3 respectively.

Table- 2 Percentage of DNA fragmentation of human lymphocytes treated by Creatine at different exposure times

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Exposure Time  hr. | % of DNA fragmentation in lymphocytes treated with Creatine (µg∕ml) | | | |
| 0 | 500 | 1000 | 2000 |
| 24 | 21 ±2.1 | 24.31±1.3 | 24 .82±1.2 | 26.87 ±2.1 |
| 48 | 24±1.9 | 28.65±0.97 | 29.8±2.4 | 32.74 ±4.1 |
| 72 | 26±2.6 | 32.91±3.41 | 34.00±2.81 | 39.50 ±3.8 |

Table- 3 Percentage of DNA fragmentation of human lymphocytes treated by Whey protein at different exposure times

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Exposure Time  hr. | Whey% of DNA fragmentation in lymphocytes treated with whey protein (µg∕ml) | | | |
| 0 | 500 | 1000 | 2000 | |
| 24 | 22.6±2.4 | 23.5±1.8 | 25.8±2.1 | 27.12±0.98 | |
| 48 | 24.9±1.8 | 33.21±2.46 | 38.84±2.91 | 35.92±2.48 | |
| 72 | 27±2.2 | 36.67±3.84 | 40.3±3.21 | 43.8±4.2 | |

The obtained results showed significant difference (p ≤ 0.05) in percentages DNA fragmentation in human lymphocyte depending on time of exposure of creatine and whey protein and their concentrations. In addition, that significant difference also between DNA fragmentation of treated and untreated cells with supplemented during different periods of incubation, the current results concluded that supplements had highly toxic effect on the blood cells (lymphocytes) and that reflected on all the body activity because these cells represent important defense line of the body. Furthermore the more appearance of high percentage of DNA fragmentation considers bio mark for genotoxcity damage in blood lymphocyte (Naz, *etal.2012*).

**Fragmentation of DNA according to Comet assay:**

Comet assays are one of the most common tests for genotoxicity. The technique involves lysing cells using detergents and salts. The DNA released from the lysed cell was electrophoresed in an agarose gel under alkaline pH conditions. Cells containing DNA with an increased number of double-strand breaks will migrate more quickly to the anode compared to intact cells the present study showed the migration of DNA in Comet assay for human lymphocyte cells examined by florescent microscope (400X),the control cells showing fluorescent spheres without DNA damage and most cells with no tail (D), while whey protein groups(A),and creatine group(B), illustrated higher in length of tail more than two fold and highest tail moment and DNA fragmentation revealed in the intensity of fluorescent heads (cyber green stain ),more than six comparing to negative control .(C), Combination group showing a most fluorescent heads with tails indicating DNA damage figure -1.This technique is advantageous in that it detects low levels of DNA damage, requires only a very small number of cells, cheaper than many other techniques, easy to execute, and quickly displays results. However, it does not identify the mechanism underlying the genotoxic effect or the exact chemical or chemical component causing the breaks (Tice, 2000).DNA damages were quantified by measuring the displacement between the genetic material of the nucleus and the resulting tails using an image analysis system. Three parameters were used as an indicator of DNA damage: i.e. tail length, percentage of DNA in the tail and tail moment.

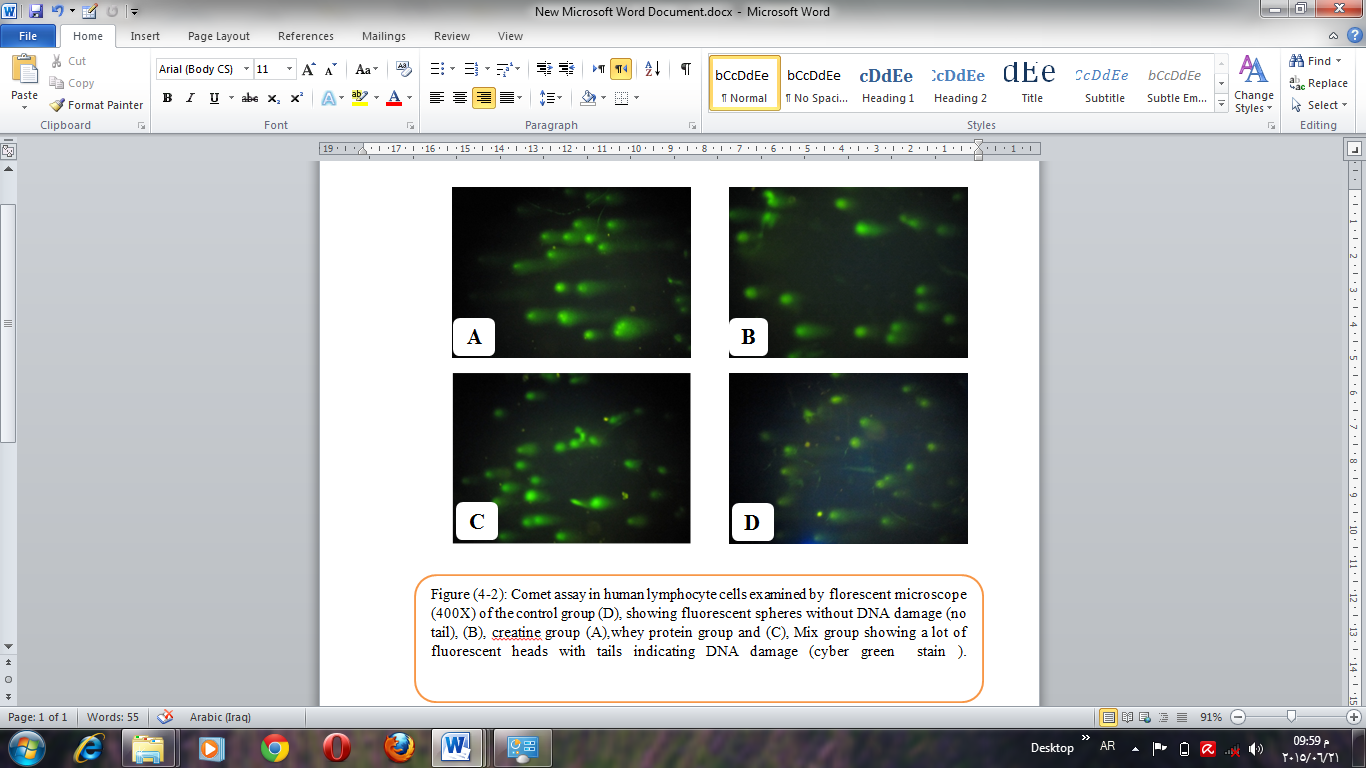


Figure (1): comet assay in human lymphocyte cells examined by florescent microscope (400X) (A): whey protein group , (B):creatine group, (C): combination group several fluorescent heads with tails indicating DNA damage (cyber green stain) and (D): control group.

The current results of an alkaline comet assay in lymphocyte handling with three groups studied were summarized in table (4).

Table -4 Tail length, tail moment and DNA% in the tail of lymphocytes (determined by Comet assay) treated with Creatine or Whey protein and combination of them (Mean±SD).

|  |  |  |  |
| --- | --- | --- | --- |
| **%DNA in tail** | **Tail moment** | **Tail length** | **Groups** |
| 1.09± 0.012 | 1.02 ± 0.004 | 3.9 ± 0.08 | **Negative control** |
| 20.14+0.07 | 29.9+0.23 | 18.5+0.57 | **Combination**(C and W) |
| 15.9±0.07 | 25.9 ± 0.17 | 13.18 ±0.21 | **Whey protein** |
| 6.2±0.04 | 6.09± 0.01 | 7.18± 0.06 | **Creatine** |

The results showed a significant difference (p ≤ 0.05) in tail length, moment of tail and % DNA fragmentation between all treatments and control. Statistical analysis by ANOVA revealed that whey protein had a moderate effect and the less affective in DNA fragmentation was creatine, while the combination treatment with ( C and W ) had a sever deleterious in DNA damage due to their components and its roles in variations of length and moment of DNA in tail. The comet assay (single cell gel electrophoresis) is used for the detection of primary DNA damage induced in isolated cells or nuclei from multiple tissues of animals usually rodents (Rothfuss *et al*., 2010).The genotoxic effects of environmental pollutants can be monitored using a broad range of both *in vitro* and *in-vivo* biomarker assays, and the comet assay is gaining popularity and acceptance over other assays since its advantages include its sensitivity for detecting low levels of DNA damage (0.1 DNA break/109 Daltons) (Ali *et al*., 2008 and Muid *et al*. 2012) . The principle of the alkaline comet assay is based upon the ability of denatured, cleaved DNA fragments to migrate out of the nucleotide under the influence of an electric field, whereas undamaged DNA migrates slower and remains within the confines of the nucleotide when a current is applied. Evaluation of the DNA “comet” tail shape and migration pattern allows for assessment of DNA damage (Lemay and Wood, 1999). There are two conditions used in comet assay, alkaline and neutral conditions, the alkaline comet assay can detect single and double stranded breaks resulting for example, from direct interactions with DNA, alkali labile sites or as a consequence of incomplete excision repair. Under certain modified conditions the assay can detect DNA-DNA and DNA-protein cross linking, and oxidized bases (Rothfuss *et al*., 2010). The fact that prolonged and extensive use of these drugs in our daily life may be hazardous and multifactorial risk factors (environmental, genetic and life style patterns) that may be responsible for additional DNA damage (Biri *et al*., 2002). The more versatile alkaline method of the comet assay was developed to measure low levels of strand breaks with high sensitivity. The electrophoresed DNA stained with a fluorescent dye. During electrophoresis, broken DNA fragments (damaged DNA) or relaxed chromatin migrates away from the nucleus. The extent of DNA liberated from the head of the comet is directly proportional to the DNA damage (Lovell and Omori, 2008).

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