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The protective action of carnosine against 4-hne and 3-nt in glucolipotoxic cellular model of insulin resistance

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Abstract

A major pathological defect in diabetes is insulin resistance, which is characterized by the impaired capacity of peripheral tissues to utilize glucose effectively in the face of hyperinsulinemia. Peripheral insulin resistance is the central pathogenesis of major metabolic disorders, and thus insulin resistance in skeletal muscle impacts whole-body glucose homeostasis. There are currently a limited number of options to treat type 2 diabetes mellitus (T2DM), and oral and injectable medications often become less effective over time. Thus, there is an urgent need to identify new targets for the development of novel treatment strategies. Carnosine (β-alanyl-L-histidine) is an endogenously synthesized dipeptide that is widely and abundantly distributed in skeletal muscles. A diabetic model of glucolipotoxicity was generated by incubating myotubes in standard tissue culture media supplemented with 28mM glucose, 200μM palmitic acid, and 200μM oleic acid. Intracellular reactive species content was assayed using 2, 7-dichlorofluorescein diacetate dye (DCFDA), whereas 3-nitrotyrosine (3-NT) and 4-hydroxynonenal (4-HNE) content, were assayed and quantified using respective ELISA assays. Glucose uptake was determined through 2-deoxy glucose-6-phosphate (2-DG6P) luminescence. Carnosine supplementation resulted in the protection of cells against GLT-mediated generation of reactive species, and thereby enhanced glucose uptake into skeletal muscle. Thus, this dipeptide could offer potential therapeutic benefits to individuals that are insulin-resistant or diabetic.

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Introduction

The accumulation of excess ROS because of oxidant production, when higher than the available antioxidant defenses in the cell, can induce damage to biological molecules, such as unsaturated fatty acids in membranes, thiol groups in proteins, and nucleic acids in DNA (Valko *et al.*, 2007). Therefore, oxidative stress may lead to the development and progression of various chronic diseases. One particular group of compounds that have a crucial role in the progression of metabolic disorders, such as diabetes, are the reactive carbonyl species – products that result from the oxidation of polyunsaturated fatty acids and sugars (Hwang *et al.*, 2016). The electrophilic nature of these carbonyl compounds allows them to favorably react with the nucleophilic regions of amino acids, such as lysine, histidine, and cysteine. This, in turn, leads to the formation of protein adducts that can cause irreversible cellular dysfunction (Dalle-Donne *et al.*, 2006).

In addition to 4-HNE, another biomarker of oxidative stress that is also capable of forming damaging adducts with protein is 3-nitrotyrosine. A combination of superoxide radical anion (O2.-) and nitrogen monoxide (NO**.**) drives the formation of 3-nitrotyrosine through the generation of the intermediate, peroxynitrite. In addition to this, the peroxidase enzyme-catalyzed reaction using hydrogen peroxide and nitrite is another possible route for the formation of 3-NT (Radi, 2013). L-tyrosine and protein- bound tyrosine are prone to attack by reactive nitrogen species (RNS), including peroxynitrite, and form either free or protein-3-NT adducts. Thus, the formation of nitrotyrosine and the detection of this molecule in proteins may not only signify RNS-mediated protein modifications but could also be an important indicator of endogenous peroxynitrite activity, which can lead to the development of diverse pathologic conditions (Stadler, 2011). Elevated circulating levels of 3-nitrotyrosine and other cellular oxidative stress markers are shown in patients with metabolic syndrome (Ruiz-Ojeda *et al.*, 2018) and diabetes (Stankova *et al.*, 2019).

L- Carnosine is a histidine-containing dipeptide (HCD) that is expressed in different tissues, such as skeletal muscle, cardiac muscle, kidneys, and some regions of the brain. Reported roles of carnosine include neutralization of reactive species, detoxification, and acid-base regulation, and these properties attract attention relating to its potential to improve normal functions or help treat conditions where oxidative stress and glycation play a critical role. In this paper, we have demonstrated that the naturally occurring dipeptide, carnosine, could sequester the reactive species, and thus could potentially prevent damaging adduction events which may lead to T2DM pathogenesis and its associated complications.

Materials and methods

Cell Culture

Mouse C2C12 and human skeletal myoblasts (HSkM) were maintained in their respective growth media (C212: DMEM-high glucose, 10% hi Fetal Bovine Serum, 10% Newborn Calf Serum, 1% Pen-Strep,1%L-glutamine; HSkM: ready to use Human Skeletal Muscle Growth Media with supplement pack containing fetal calf serum, fetuin, dexamethasone, and epidermal growth factor, insulin, basic fibroblast growth factor) in a humidified atmosphere with 5% CO2 at 37oC. At ~ 80% confluency, cells were washed twice with PBS and thereafter, the medium was then switched to differentiation medium (C2C12: DMEM-high glucose, 2% hi Horse Serum; HSkM: DMEM-F12 HEPES, 2% hi Horse Serum) to facilitate the myocytic differentiation prior to performing the relevant assay and was replaced after 2, 4, and 6 days of culture.

Glucolipotoxicity (GLT) Treatment

For C2C12, the media for the control or normal condition (“healthy”) are composed of DMEM (11mM of glucose) with 2% heat-inactivated horse serum whilst for HSkM healthy control is DMEM-F12 (5mM glucose media) with 2% horse serum. Each prepared healthy control media was then sterilely filtered using a 0.20 µm syringe filter. On the other hand, to mimic diabetic extracellular glucolipotoxic conditions or GLT (high glucose and high fatty acids) in vitro, cells

were incubated for 5-days in similar media components as healthy except that glucose concentration is 28mM for mouse muscle cells, 17mM for HSkM and all were supplemented with 200µM palmitic acid and 200µM oleic acid in combination. GLT media contains 2% of fatty-acid free BSA, this mixture was placed at 37°C for a minimum of 1 hour to allow the fatty-acid and BSA conjugation and thereafter this was then sterilely filtered. All experimental condition media were freshly prepared as required and were used only within the treatment period.

Cell Viability

HSkM and C2C12 cells were cultured in DMEM or GLT media for 5 days before media was removed and cells washed 3 times in KREBS. A final concentration of 5 µM Calcein AM Cell Viability Dye (ThermoFisher) in KREBS was then added for 1 hour before washing again with KREBS. Cell viability was measured via fluorescence, with excitation and emission at 490 nm and 520 nm respectively.

DCFDA Assay

The level of intracellular reactive species was estimated using 2',7'-Dichlorofluorescin diacetate (DCFDA) (Sigma, UK). Old media of cells that have been previously incubated in either control or experimental conditioned growth media for the desired period of time were removed and cells were then washed 3 times in KREBS to ensure that carry-over media were completely removed. Afterwards, the cells were then incubated for 1 hour at 370C under 5% CO2 with 20µM of light-protected DCFDA solution freshly prepared using KREBS. The intracellular reactive species or reactive oxygen and nitrogen species (RONS) activity was then measured via fluorescence, with excitation and emission at 490nm and 530nm respectively and quantifications are presented as percentage change compared to standard control conditions.

3-NT and 4-HNE Species Detection

A 3-nitrotyrosine Enzyme-Linked Immunosorbent Assay (ELISA) (Abcam, UK) was used for the quantitative measurement of 3-nitrotyrosine and the 4-HNE ELISA kit (Universal Biologicals, UK) was used for the quantitative measurement of 4-HNE. The cellular protein content of the sample that was previously quantified using BCA protein assay (PierceTM BCA Protein Assay Kit – ThermoFisher Scientific, UK) was then used to normalize the corresponding 3-NT and 4-HNE data.

Glucose-Uptake Assay

A non-radioactive bioluminescent Glucose Uptake-Glo™ Assay (Promega, UK) was employed to measure the glucose transport in cells. Following the desired treatment, cells (in 12-well format) were serum-starved overnight in DMEM supplemented with 5 mM glucose, the media were then removed and washed twice with DPBS before incubating this for 1 hour at 370C under 5% CO2 in glucose-free DMEM +/− 100 nM insulin to stimulate GLUT4 translocation. Immediately after, the medium was then replaced with PBS + 2-deoxy glucose (2-DG) and allowed for the uptake reactions to take place for 30 min at 37°C under 5% CO2. Afterwards, the reaction was then terminated by addition of Stop Buffer provided in the kit containing 0.4 M HCl + 2% dodecyl trimethyl ammonium bromide, and added with the Neutralisation Buffer (1M Trizma, pH>10). A 100µL mixture of the above was added with 100µL of 2-DG6P Detection Reagent and after 1 hour reaction, luminescence data were then acquired using a CLARIOStar luminometer (BMG Labtech, Ortenberg, Germany).

Statistical analysis

Results are expressed as mean ± standard error of the mean (n = 3 or more independent experiments). Parameters were compared using a two-tailed unpaired t-test, with statistical significance determined using Tukey's test or Dunnett's test critical values at $\alpha = 0.05$. A *p-value* below 0.05 was considered to be statistically significant.

Results and discussion

The following data indicated here present the scavenging action of carnosine against reactive 4-

HNE and 3-NT and its beneficial effects upon glucose uptake in skeletal muscle cells under metabolic stress. Intracellular reactive oxygen and nitrogen species (RONS) are postulated to contribute to muscle tissue dysfunction and are, therefore, linked to the negative regulation of the insulin signalling pathway. We have previously reported (Cripps *et al.*, 2017) that carnosine was effective in quenching reactive species in GLT-mediated insulin-resistant mouse C2C12 muscle cells. Here, we have shown that carnosine did a similar effect in human HSkM muscle myotubes. In order to determine the level of cellular reactive species generated from each treatment condition, a fluorescence-based assay was employed using a cell-permeant fluorogenic dye called 2'7'-dichlorofluorescein diacetate (DCFDA) which will be deacetylated by cellular esterases and upon the presence of oxidizing species, is converted into 2', 7' – dichlorofluorescein (DCF) – a highly fluorescent compound, which can be detected by fluorescence spectroscopy. As indicated in Fig. 1, incubation of cells in GLT media also showed a significantly increased level of reactive species to 179.25± 6.88%. Supplementation with 10mM carnosine significantly reduced this level to 123.13 ± 7.44%, with there being no significant difference in the level of cellular reactive species between control conditions with carnosine added.

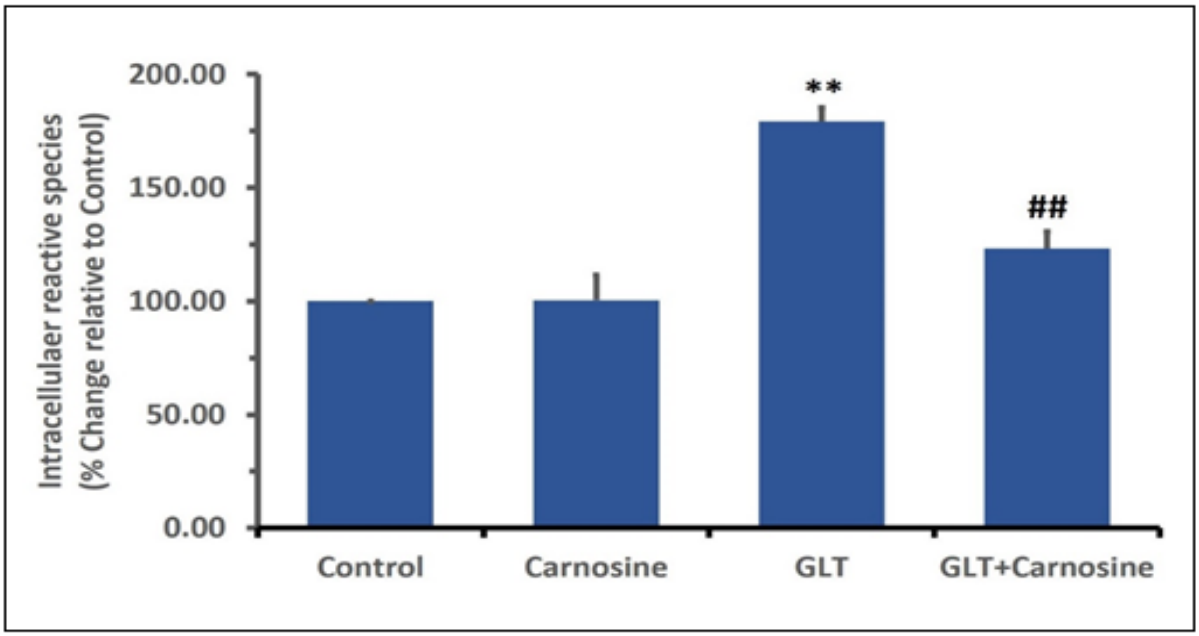

Fig. 1. Carnosine effectively scavenges reactive species in human skeletal muscle cells (HSkM). HSkM myotubes were cultured in control or GLT media for 5 days. Corresponding media were then replaced supplemented ± 10mM carnosine for 1h. A 20μM DCFDA was loaded in KREBS buffer for 1h and reactive species detected via fluorescence with excitation and emission of 495nm and 530nm. Reactive species are expressed as a percentage change in comparison to control from 4 independent experiments ± SEM. (** versus control $p<0.01$; ## $p<0.01$ versus GLT; Tukey's test).

In this DCFDA assay, the detection of ROS activity or cellular reactive species detection was not normalized to either cell number or protein content, and so it was necessary to investigate the effect of GLT on cell number and viability to ensure that the response shown in GLT was not an artifact of either glucolipotoxic-driven cell death or glucose-driven cell proliferation. Using a cell-permeant dye called Calcein AM, which can only fluoresce upon the action of intracellular esterases through acetoxymethyl ester hydrolysis, it is possible to determine the cell viability of most eukaryotic cells.

All viable eukaryotic cells will have the esterases necessary to drive the hydrolysis reaction forward and the use of Calcein AM is thus an accepted model for

cell viability and number measurement (Bratosin *et al.*, 2005). Since there was a non-statistically significant change observed in the cell viability and number of cells exposed to GLT in 5 days with respect to control Fig. 2, it can be inferred that the significant changed detected in the level of reactive species in chronic exposure of GLT conditions were not due to muscle cell viability.

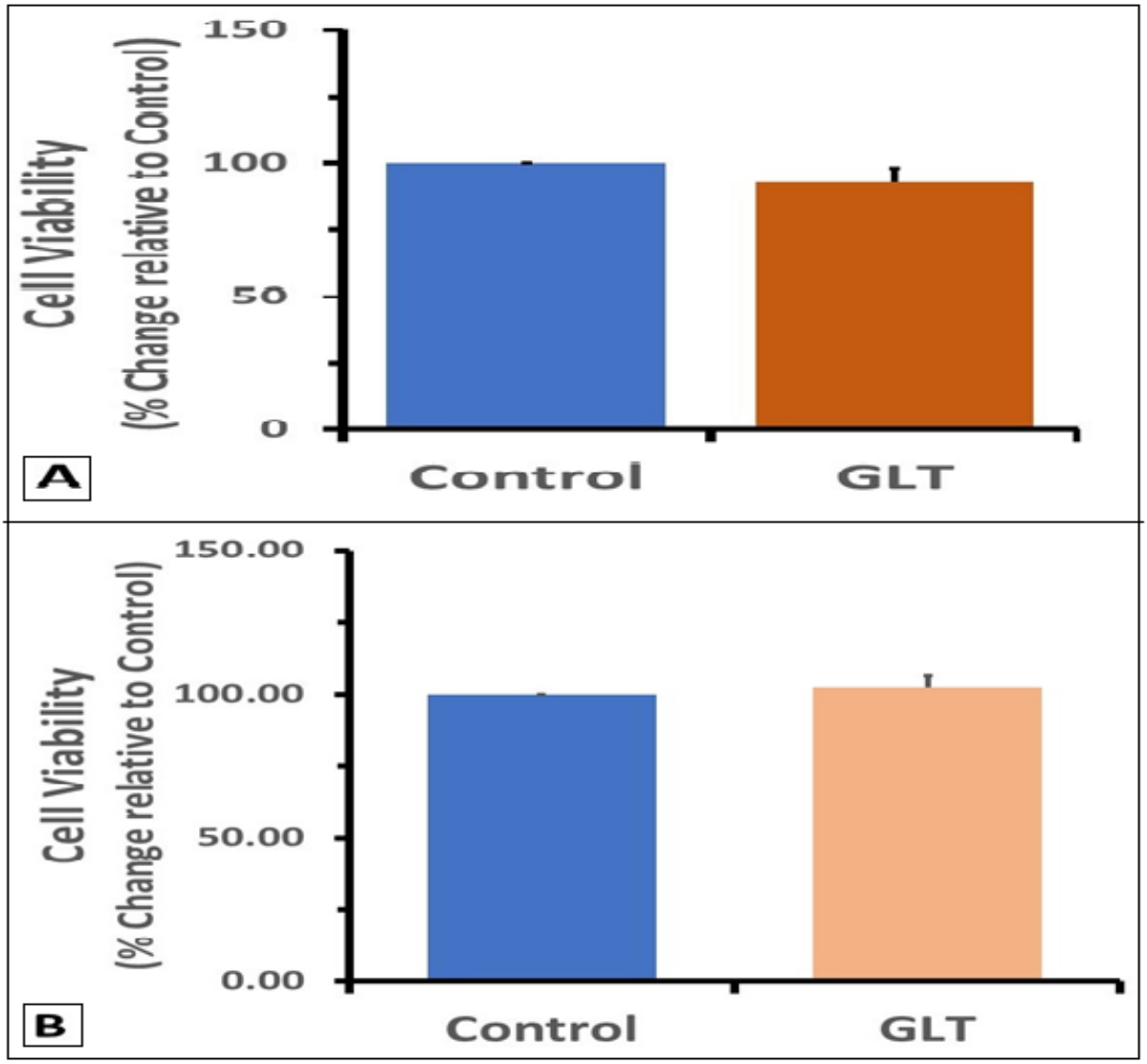

Fig. 2. GLT treatment does not significantly affect cell viability. (A) C2C12 and (B) HSkM myotubes were cultured in control or GLT media for 5 days. After 1h incubation with 5µM solution of Calcein AM, fluorescence intensity was measured using excitation and emission of 490nm and 520nm. Results shown are expressed as percentage change compared to control from 4 independent experiments ± SEM. ($p>0.05$; t-test).

A simultaneous flux of nitric oxide and superoxide anion overproduction leads to the formation of a potent oxidant in the biological system called peroxynitrite, which has been implicated in several important diseases, including but not limited to, cancer, neurodegeneration, stroke, inflammatory conditions, cardiovascular problems, and diabetes mellitus (Reiter *et al.*, 2000; Stadler, 2011). The oxidative reaction through nitration of key functional parts in active sites of enzymes, receptors and other proteins by peroxynitrite produces 3-nitrotyrosine. This 3-nitrotyrosine is an established biomarker of cell, tissue, and systemic nitroxidative stress that again resulted from incorporating the nitro group into the tyrosine residue of the protein which caused to modify its functional and structural properties and

thus contribute to altered cell and tissue homeostasis (Radi, 2012). This product is indicative of the level of peroxynitrite that is present in the cell and can also be used to assess the degree of reactive nitrogen species generated; the formation of 3-nitrotyrosine can be quantified using ELISA.

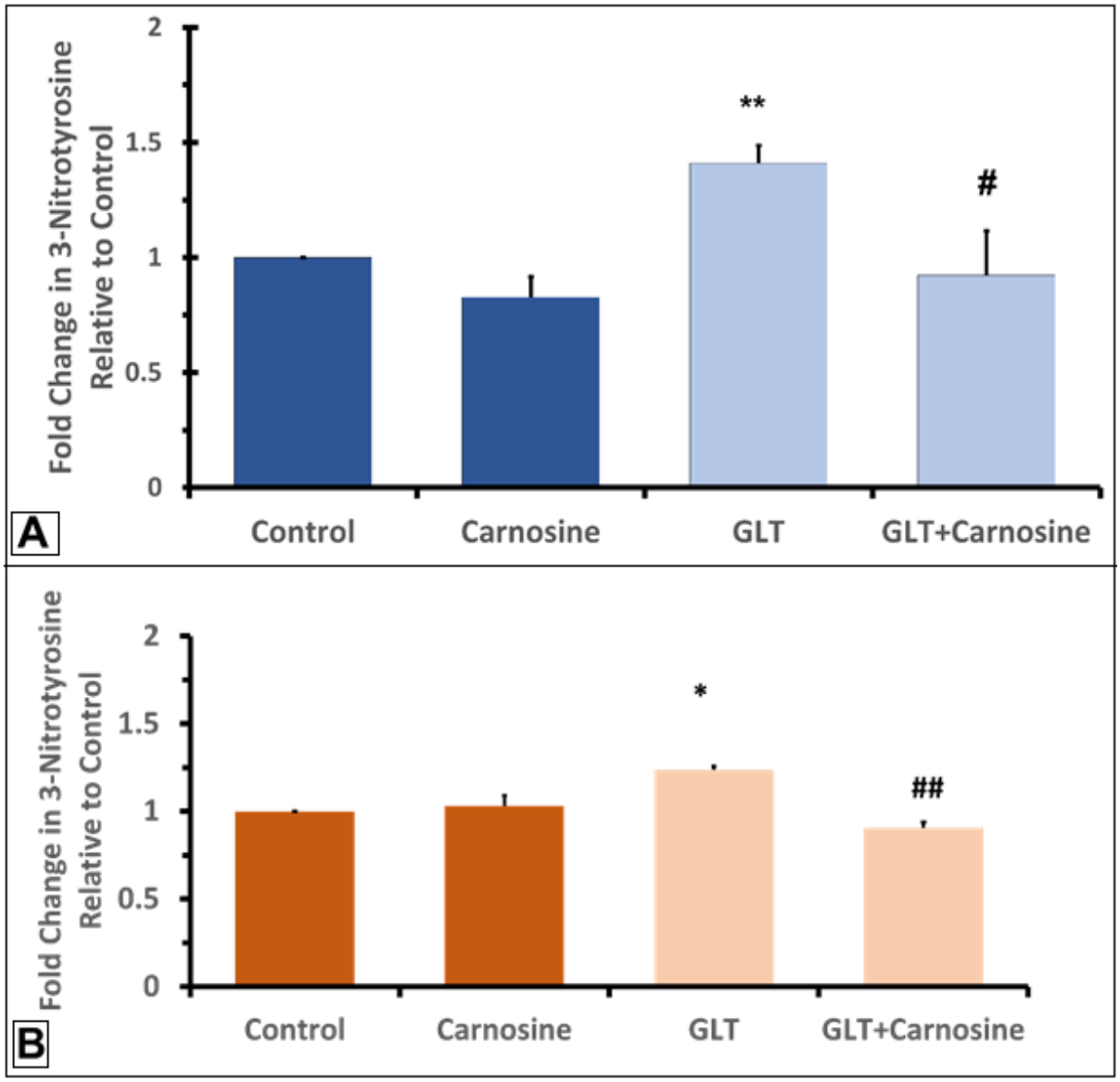

Fig. 3. Carnosine is an effective antioxidant against nitroxidative stress markers. (A) C2C12 and (B) HSkM myotubes were incubated in control or GLT media supplemented ± 10mM carnosine for 5 days. Cells lysates were then determined for 3-NT using ELISA with absorbance measured at 450nm. Concentrations detected were normalised to protein content. Results are expressed as fold change compared to control from 3 independent experiments ± SEM. (*p<0.05 **p<0.01 vs Control, #p< 0.05 ##p<0.01 vs GLT; Tukey's test).

In order to determine the level of this marker of cell damage and inflammation, C2C12 and HSkM myotubes were incubated in control or GLT media supplemented with or without 10mM carnosine for 5 days. The sample lysates were then obtained using the extraction buffer supplied in the kit, and the extracts were then analyzed according to the protocol. As shown in Fig.3, 5-day treatment of GLT media resulted in a significant increase of 3-NT species formation (40.91 ± 7.73% and 30.34 ± 4.64%) in C2C12 and HSkM cells compared to their respective controls. Importantly, carnosine was able to prevent this 3-NT adduct formation and for C2C12 and HSkM, respectively.

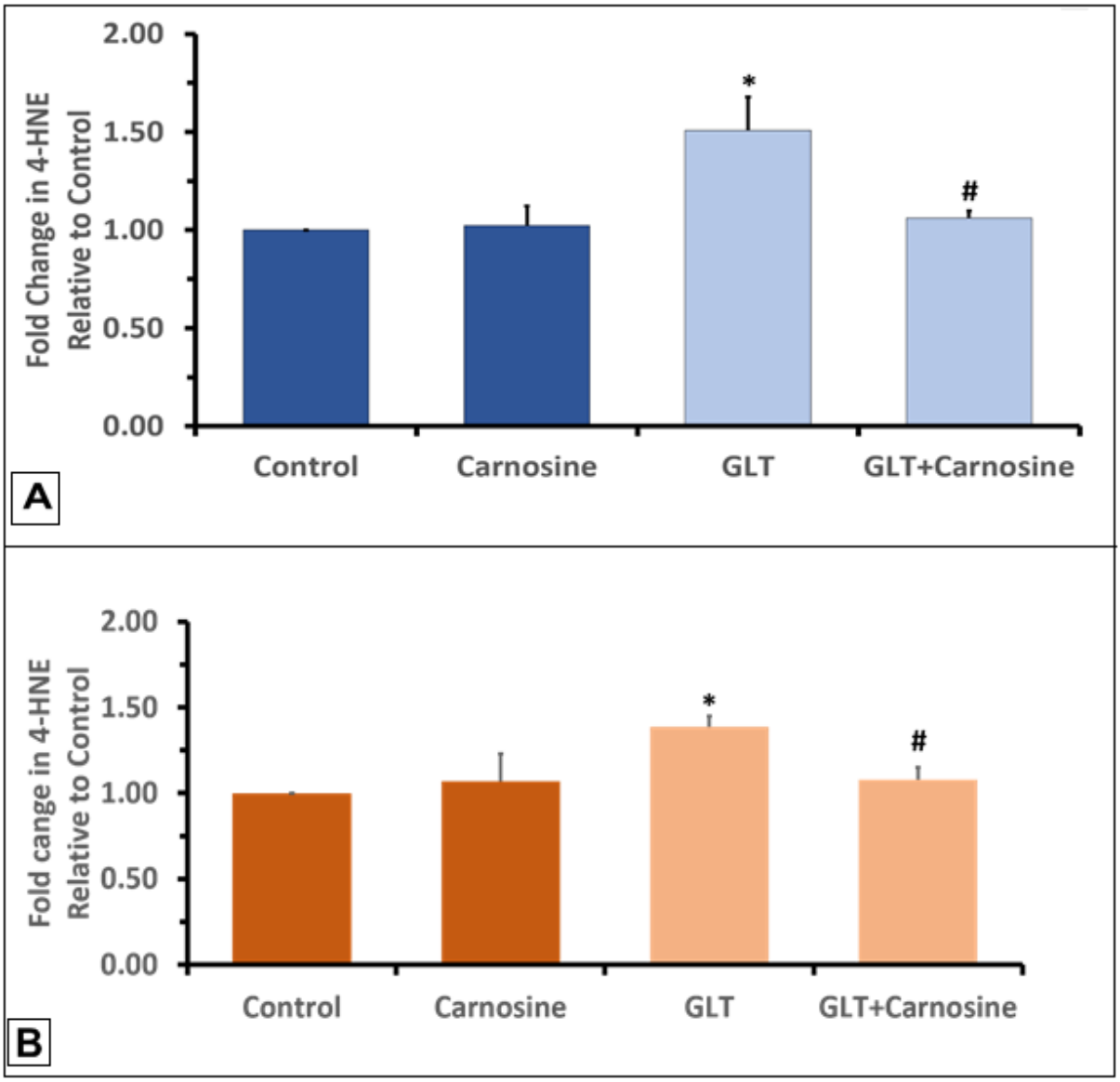

Fig. 4. Carnosine is effective in sequestering reactive aldehyde (4-HNE) generated in GLT-exposed muscle cells. (A) C2C12 and (B) HSkM myotubes were incubated in control or GLT media supplemented ± 10mM carnosine for 5 days. Cell lysates were then determined for 4-HNE using ELISA. Concentrations detected were normalised to protein content. Results are expressed as fold change compared to control from 3 independent experiments ± SEM. (* versus control $p<0.05$; # $p<0.05$ versus GLT; Tukey's test).

A consequence of established oxidative stress is the formation of reactive carbonyl species or aldehydes, including 4-hydroxynonenal (4-HNE), which is formed through enhanced ROS-induced lipid peroxidation (Yang *et al.*, 2003). Excessive levels of 4-HNE are believed to induce insulin resistance and desensitization of insulin signaling pathways and have been associated with metabolic defects present in obesity (Pillon *et al.*, 2007; Ingram *et al.*, 2012). After C2C12 and HSkM myotubes were incubated in control or GLT media in the presence or absence of 10mM carnosine treatment, sample lysates were collected using RIPA buffer and immediately assayed for 4-HNE using ELISA kits. Indicated in Figure 4, an increase of (50.77 ± 17.02%) and (38.56 ± 6.38%) in 4-HNE were detected in GLT-treated cells of C2C12 and HSkM, respectively as compared to their control. However, in the presence of carnosine supplementation at 10mM concentration, a significant amount of this generated oxidant molecule was being significantly neutralized by this dipeptide in both types of myotubes used.

As shown earlier, carnosine has a protective effect on muscle cells against GLT-mediated reactive species. Therefore, it was next sought to determine whether this, as a result, could have a beneficial action upon glucose uptake, and to answer this a luminescence-based glucose uptake assay was conducted. We have reported previously (Cripps *et al.*, 2017) that GLT-exposed C2C12 muscle cells have reduced capacity to uptake glucose both in basal and in insulin-stimulated conditions. Importantly, carnosine scavenging the glucolipotoxic reactive species resulted in a significant enhancement of glucose uptake. This finding is not due to significantly altered C2C12 cell viability, as in C2C12 myotubes treated with GLT media for 5 days, cells are still viable Fig. 2. In order to validate the findings obtained through C2C12 in vitro experiments, it was essential to conduct similar studies using human skeletal muscle cell-line employing the same treatment conditions and methodology. Results obtained in these experiments Fig. 5 showed a similar response as found previously with C2C12. When comparing all values to control basal, a significant reduction of glucose uptake was shown from 170.30% to 102.20% under insulin-stimulated conditions, and importantly was enhanced when supplemented with 10mM carnosine by about 57%.

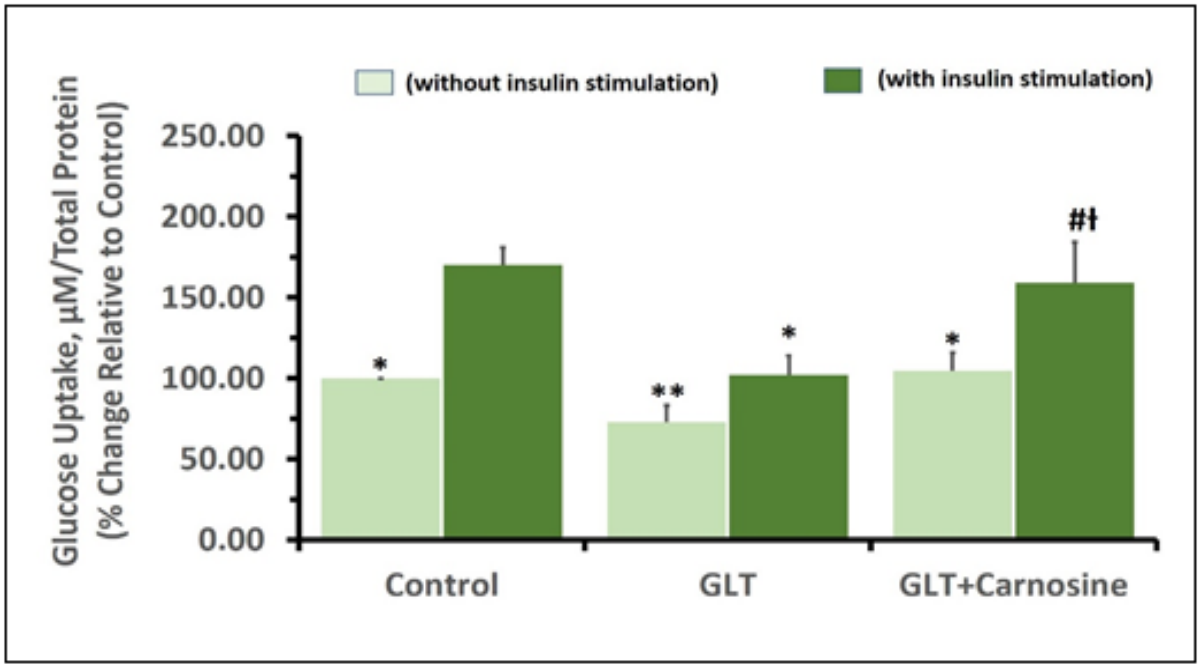

Fig. 5. Carnosine enhances glucose uptake in GLT-exposed human skeletal muscle cells. Human skeletal myotubes (HSkM) were cultured in DMEM-F12 media, or DMEM-F12 GLT media (17mM glucose, 200 µM Palmitic acid, 200 µM Oleic acid) for 5 days ± 10mM Carnosine. Cells were serum-starved overnight in DMEM supplemented with 5 mM glucose, then incubated for 1 h in glucose-free DMEM +/− 1µM insulin. Medium was replaced with PBS + 0.150 mM 2-deoxy glucose (2-DG). Glucose uptake reactions were conducted for 30 min. 2DG6P was detected using a luminometer. Data are expressed as means ± SEM of 3 independent experiments. (*$p < 0.05$,**$p<0.005$, vs Control with insulin stimulation; $^{\#}p<0.05$ vs GLT with stimulation, $^{ł}p<0.01$ vs GLT without stimulation; Tukey's test).

There is growing evidence to indicate that oxidative stress (OS) is a common denominator for the pathogenesis of several diseases, including cancer, diabetes, obesity, neurodegenerative disorders, among others. When production of both ROS and RNS are carefully regulated, they participate and have functional effects in normal physiology such as hormone action, immune response, cell growth, and cell adhesion. By contrast, ROS and RNS can also become toxic agents and thereby participate in

pathophysiological processes, causing irreversible modifications via inducing damage to cellular components (lipids, DNA, carbohydrates) and thereby altering their normal function (Chiarugi *et al.*, 2003; Yang *et al.*, 2013; Sisein, 2014).

There are two specific reactive species focused on this study, namely 3-nitrotyrosine (3-NT) and 4-hydroxynonenal (4-HNE). The former is a useful biomarker of peroxynitrite-driven OS and has been implicated in diabetic neuropathy and nephropathy (Thuraisingham, 2000; El-Remessy *et al.*, 2003). The presence of 3-NT suggests that peroxynitrite is just one of the reactive species in GLT-exposed cells. On the other hand, 4-HNE, an α-β-unsaturated alkenal, is a reactive carbonyl specie that could easily react with the nucleophilic sites of proteins such as Lys, His, and Cys, and DNA, thereby causing cellular dysfunction, is the reactive carbonyl species (RCS). HNE is the most intensively investigated and quantitatively most important product of lipid peroxidation due to its highly cytotoxic role in inhibiting gene expression. It also enhances the development and progression of several pathological states, including diabetes, Alzheimer's diseases, cancer, cardiovascular diseases, liver diseases, and Parkinson's disease (Ayala, Muńoz and Arguelles, 2014).

Conclusion and recommendation

In this work it has been demonstrated that excessive production of reactive species mediated by glucolipotoxicity has deleterious effects on skeletal muscle cells. For instance, in the skeletal muscles, the GLT-induced OS might have contributed to the impairment of the main signaling pathways involved in insulin action and thus reduced glucose uptake capacity. Given the implication of oxidative stress in the onset of the disease, it is possible that antioxidant strategies would be effective in the prevention or treatment of diabetes – as these are molecules that have the ability to scavenge, quench or even neutralize these excess reactive species, thereby preventing cellular damage and preserving function. This work has shown that a natural dipeptide, carnosine, has the ability to offset the negative effects these reactive species have upon muscle cells, and by so doing offered a significant improvement on glucose uptake by these cells exposed to glucolipotoxic conditions. The scavenging action and the use of carnosine as a blocking agent against those deleterious species could therefore potentially offer a novel treatment and therapeutic perspective for T2DM patients. Despite the promising role of carnosine as a supplement, its action would likely require regular administration at high dosage, due to the presence of tissue and serum carnosinase enzymes that catalyze carnosine turnover. One possible strategy to offset this limitation would be to design or synthesize carnosinase inhibitors and carnosine mimetics that are stable against the hydrolytic action of carnosinases.

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