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The Study of Cyclic Analogues of Carnosine Peptide on Isolated Mitochondria of A549 Cells

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Abstract: *Bachground & Aims:* In this work a various range of toxicity on A549 cells was resulted by the cyclic Carnosine analogues using MTT assay. The purpose of this research was to study the effects of analogues Carnosine peptide on A549 cells using several experiments. Also, applying peptides on the mitochondria of A549 cells, a raise of mitochondrial reactive oxygen species (ROS) level, mitochondrial swelling, mitochondrial membrane potential (Δψm) collapse, release of cytochrome c of the affected mitochondria were detected. *Methods:* For determination of Cytotoxicity, Normal calls and A549 cancerous cells (1×107/well) were transferred into 96-well plates and treated with 10 µg/mL concentration of Carnosine analogues for 12h. The effect of peptides on the activity of SDH was assayed by MTT test. 100 µL mitochondrial suspensions from A549 and normal groups were incubated with applied concentration of peptides (10µg/mL) at 37°C for 30 min. The fluorescence intensity of DCFH which is an indicator of ROS concentration was then assayed by a Shimadzu RF-5000U fluorescence spectrophotometer. Mitochondrial accumulation and also redistribution of the cationic fluorescent dye, Rhoda mine 123 (Rh 123, concentration, 10 µM), from mitochondria into the cytosol have been used for the determination of MMP collapse. Mitochondria from A549 and normal groups were suspended in corresponding assay buffer and incubated at 37°C with 10µg/mL peptides. The release of Cytochrome c by peptides was assayed by the Quantikine Cytochrome c. Results: The results showed that all the synthesized cyclic peptides increased ROS in various levels in comparison with unaffected mitochondria isolated from A549 group. A significant increase of ROS was resulted by 2c, 4c and 6c analogues at 30 min. Rh 123 fluorescence staining indicated that the integrity of the mitochondria was damaged by the cyclic peptides. Compounds 2c, 4c and 6c significantly increased the collapse of MMP among the Carnosine analogues in comparison with mitochondria isolated from A549 group. Peptides 2c, 4c and 6c significantly increased mitochondrial swelling in comparison with untreated mitochondria isolated from the A549 group. The result was that cyclic peptides 2c, 4c and 6c significantly increased the release of cytochrome c in comparison with unaffected mitochondria isolated from the A549 group. *Conclusion:* Based on the overall results, cyclic analogues of Carnosine peptide, especially compounds 2c [*Cyclo-(Pro-β-alanine-His-β-alanine-His)*], 4c [*Cyclo-(Pro-His-β-alanine-β-alanine-His)*] and 6c [*Cyclo-(Pro-β-alanine-His-His-β-alanine)*], showed more toxic activity than other Carnosine analogues, which would be supporting to develop these peptide analogues as new anticancer and complementary therapeutic agents for the treatment of lung cancer.

Keywords: Cyclic Analogues, Linear Analogues, Cytotoxicity, Mitochondria

1. Introduction

Cancerous cells are introduced by uncontrolled growth into nearby tissues. The cell division and growth are caused by genetic mutations that either turn on oncogenes or silent tumor suppressor genes. The continuation of this type of

genetic destruction over time can lead to the progressive transformation of the cells and survival of uncontrolled cells populations that can form tumors. The diagnosis is typically detected with a combination of CT scan, MRI scan, and tissue biopsy [1]. The medication temozolomide is frequently used as a part of chemotherapy [2, 3]. Antineoplastic drugs, however, have side effects on normal cells. Possible functions of the dipeptide Carnosine (β-alanyl-L-histidine) include buffer, anti-oxidant, antiglycator, aldehyde and carbonyl scavenger, chelator of metal ion, immuno-stimulant, wound healing and neurotransmitter agent [4-13]. In other years, it was reported that Carnosine can inhibit growth of tumour cells [14]. In the recent years, Carnosine was shown to inhibit growth of cultured glioblastoma cells [15], most probably via effects on glycolysis [16, 17]. Other works investigated that Carnosine can suppress tumor growth in animals [18, 19]. Analogues of Carnosine peptide designed and studied in this work were six cyclic peptides.

The cyclization of Carnosine analogues, the linear (*N-Trt*) derivatives were used first, and then the whole deprotection on the cyclic peptides was implemented by treating with TFA 95% containing scavengers. In this study, some of the cyclic Carnosine analogues leads for increasing ROS formation, reduction of collapse of MMP, reduction of ATP generation and mitochondria swelling that finally produce cytochrome c release that signals apoptosis. The functions and the cytotoxicity mechanisms of cyclic Carnosine analogues on mitochondria isolated from the A549 cells were reported. This study focused on the effects of analogues of Carnosine peptide on mitochondria obtained from the human lung cancer. It suggests that therapeutic methods to inhibit anti-apoptotic signals in A549 cells might have the potential to provide powerful tools in the future to treat in the patients with lung cancer.

2. Materials and Methods

Trifluoroacetic acid (TFA), Triisopropylsilane (TIS), Fmoc amino acids and coupling reagents O-(7-Azabenzotriazol-1-yl)-*N, N, N, N,*-tetramethyluronium hexafluorophosphate (HATU), (benzotriazol-1-yloxy) - tripyrrolidinophosphonium hexafluorophosphate (PyBop), were supplied by Merck. Solvents like acetonitrile (MeCN), Piperazine*, N, N*-diisopropylethylamine (DIPEA), Diethylether, Dichloromethane (DCM), *N, N*-dimethylformamide (DMF), and methanol (MeOH) were purchased from Merck. 2-Chlorotritylchloride resin (1% DVB, 200-400 mesh, 1mmol/g) was purchased from Aldrich, UK. Flash column chromatography were carried out using silica Gel 60 (particle size 0.04–0.06 mm / 230–400 mesh). The mass spectral measurements were performed on a 6410 Agilent LCMS triple quadrupole mass spectrometer (LCMS) with an electrospray ionization (ESI) interface.

Experimental

Isolation of mitochondria

The nuclei and broken cell debris were sedimented through centrifuging at 1500 ×g for 10 min at 4 °C and the pellet was discarded. The supernatant was subjected to a further centrifugation at 10,000 ×g for 10 min and the superior layer was carefully discarded. The mitochondrial pellet was washed by gently suspending in the isolation medium and centrifuged again at 10,000 ×g for 10 min [20].

Determination of Cytotoxicity

Normal calls and A549 cancerous cells (1×10^7/well) were transferred into 96-well plates and treated with 10 µg/mL concentration of Carnosine analogues for 12h (cells were maintained in RPMI 1640, supplemented with 10% FBS and antibiotics (50 U/mL of penicillin and 50µg/mL streptomycin)). After treatment, MTT (5 mg/mL in RPMI 1640) reagent was added to each well. After 4 h, the reaction was stopped by addition of 50 µL DMSO. The absorbance at 570 nm of the solubilized MTT products was measured with an ELISA reader. The process was repeated in triplicate to confirm accuracy.

Succinate Dehydrogenase (SDH) activity assay

The effect of peptides on the activity of SDH was assayed by MTT test. Briefly, to carry out this assay, 100 µL mitochondrial suspensions from A549 and normal groups were incubated with applied concentration of peptides (10µg/mL) at 37°C for 30 min. Then, 50 µL of MTT was added to the medium and incubated at 37°C for 30 min.

ROS formation assay

The fluorescent dye dichloro-dihydro-fluorescein diacetate as DCFH-DA was used for ROS measurement. Normal and cancerous cells isolated from tumor tissue of lung were suspended in respiration buffer (0.32mM sucrose, 10 mM Tris, 20 mM Mops, 50µM EGTA, 0.5Mm $MgCl_2$, 0.1mM KH_2PO_4, and 5 mM sodium succinate). Then, DCFH (final concentration, 10 µM) was added to cells of both groups and incubated at 37°C for 15 min. The fluorescence intensity of DCFH which is an indicator of ROS concentration was then assayed by a Shimadzu RF-5000U fluorescence spectrophotometer at EXλ = 488 nm and EMλ = 527 nm [21, 22].

Mitochondria membrane potential (MMP) assay

Mitochondrial accumulation and also redistribution of the cationic fluorescent dye, Rhoda mine 123 (Rh 123), from mitochondria into the cytosol have been used for the determination of MMP collapse. Rh 123 (10 µM) was added to the normal and A549 cells suspensions (1000 µg mitochondrial protein/mL) in MMP assay buffer (220 mM sucrose, 68 mM, D-mannitol, 10 mM KCl, 5 mM KH_2PO_4, 2 mM $MgCl_2$, 50 µM EGTA, 5 mM sodium succinate, 10 mM HEPES, 2 µM rotenone). The cytosolic Rh 123 fluorescence intensity which represents the redistribution of the dye from mitochondria into the cytoplasm was determined using Shimadzu RF-5000U fluorescence spectrophotometer at the EXλ = 490 nm and EMλ = 535 nm [22].

Mitochondrial swelling assay

Mitochondria from A549 and normal groups were suspended in corresponding assay buffer (70 mM sucrose, 230 mM mannitol, 3 mM HEPES, 2 mM Tris-phosphate, 5 mM succinate, and 1 µM rotenone) and incubated at 37°C with 10µg/mL peptides. The absorbance was measured at

540 nm on 15-min intervals using an ELISA reader (Tecan, Rainbow Thermo). A decrease in the absorbance indicates an increase in mitochondrial swelling [21].

Cytochrome c release

The release of Cytochrome c by peptides was assayed by the Quantikine Cytochrome c Immunoassay kit provided by R & D Systems, Inc. (Minneapolis, MN, USA).

3. Results

3.1. The Deprotected Carnosine and Deprotected Cyclic Carnosine Analogues

S*ynthesis of β-alanine-His* (1b)

LC-MS (ESI) *m/z* Calcd for (1b) 226.11, Found *m/z* = 227.2$[M+H]^+$.

Synthesis of Cyclo-(*β-alanine-His-Pro-β-alanine-His*) (1c)

LC-MS (ESI) *m/z* Calcd for (1c) 513.26, Found *m/z* = 514.3$[M+H]^+$.

Synthesis of Cyclo-(*Pro-β-alanine-His-β-alanine-His)* (2c)

LC-MS (ESI) *m/z* Calcd for (2c) 513.26, Found *m/z* = 514.3$[M+H]^+$.

Synthesis of Cyclo-(*His-β-alanine-Pro-β-alanine-His*) (3c)

LC-MS (ESI) *m/z* Calcd for (3c) 513.26, Found *m/z* = 514.3$[M+H]^+$.

Synthesis of Cyclo-(*Pro-His-β-alanine-β-alanine-His*) (4c)

LC-MS (ESI) *m/z* Calcd for (4c) 513.26, Found *m/z* = 514.3$[M+H]^+$.

Synthesis of Cyclo-(*β-alanine-His-Pro-His-β-alanine*) (5c)

LC-MS (ESI) *m/z* Calcd for (5c) 513.26, Found *m/z* = 514.3$[M+H]^+$.

Synthesis of Cyclo-(*Pro-β-alanine-His-His-β-alanine*) (6c)

LC-MS (ESI) *m/z* Calcd for (6c) 513.26, Found *m/z* = 514.3$[M+H]^+$.

3.2. The Effect of Carnosine Analogues in Cytotoxicity

The cytotoxicity was done using the MTT test after 12h incubation of cells with different concentrations of Carnosine analogues. The IC_{50} value obtained with the mean of the three independent tests for analogues of Carnosine peptide was 10µg/mL. Figure 1 shows the human lung cancer viability decreased by Carnosine analogues after 12 hr treatment.

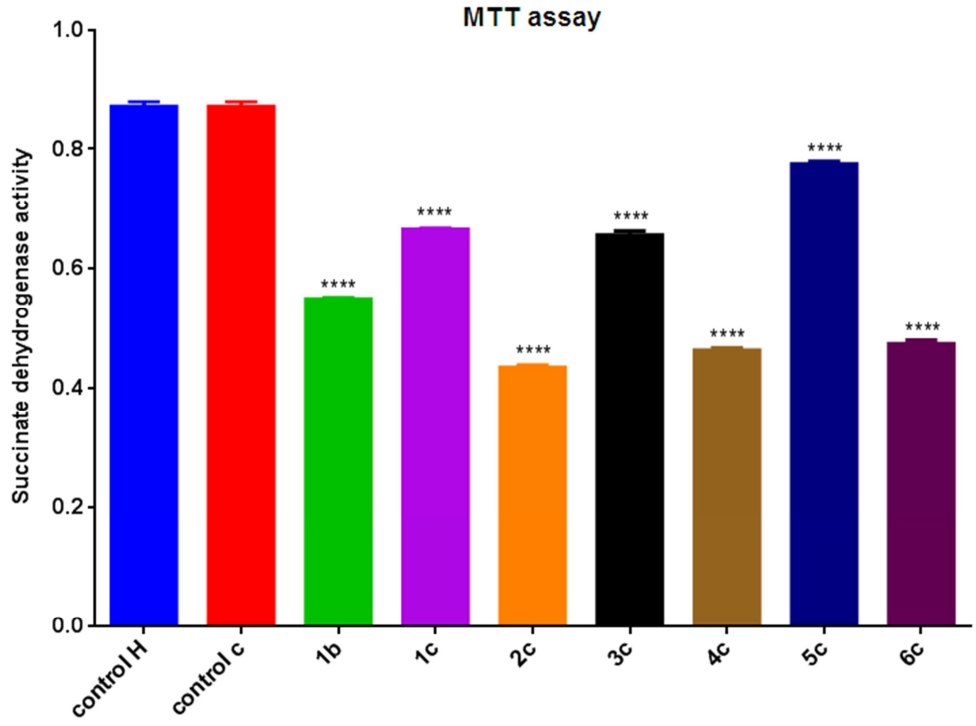

Figure 1. *Shows cytotoxic effects of cyclic Carnosine analogues on lung normal cells and human lung cancer cells (A549). The cells were treated with peptides for 12 h, and cytotoxic effects were determined by MTT assay. Control H represents normal cells and control c shows A549. Data represent the mean ± SD of three replicates in three independent experiments (n=3). The stars show that values were significantly different from the corresponding control (****p < 0.0001).*

3.3. The Effect of Carnosine Analogues in Producing ROS

Cyclic Carnosine analogues 2b, 4b and 6b at 30 min and 60 min showed significant difference in ROS production compared with the cancerous control group (Figure 2). ROS generation for all the cyclic Carnosine analogues (1-6c) was significant in comparison with the cancerous group at 60 min.

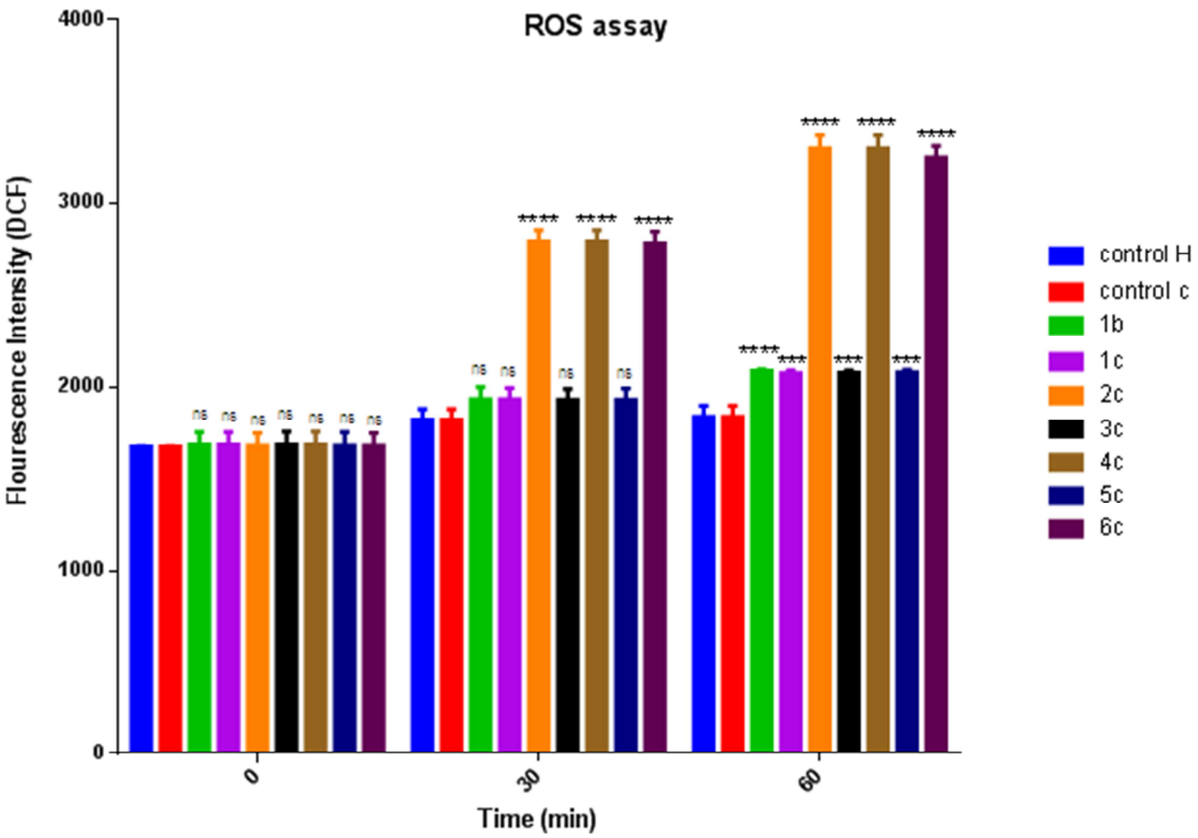

Figure 2. *Shows the effect of cyclic Carnosine analogues on ROS generation at different times (0, 30, and 60 min) in lung normal cells and A549 cells isolated from tumor tissue of lung. Data represent the mean ± SD of three replicates in three independent experiments (n=3). The two-way ANOVA test was performed. *** and **** significantly different from the corresponding control (p<0.001 and p<0.0001, respectively).*

3.4. The Effects of Carnosine Analogues in Collapsing MMP

Results in Figure 3 show that, all Carnosine analogues have significant difference in comparison with the cancerous group at two different times. Cyclic peptides 2b, 4b and 6b have significant effect on increasing fluorescein intensity (i.e., MMP reduction) at 30 and 60 min in comparison with the cancerous group.

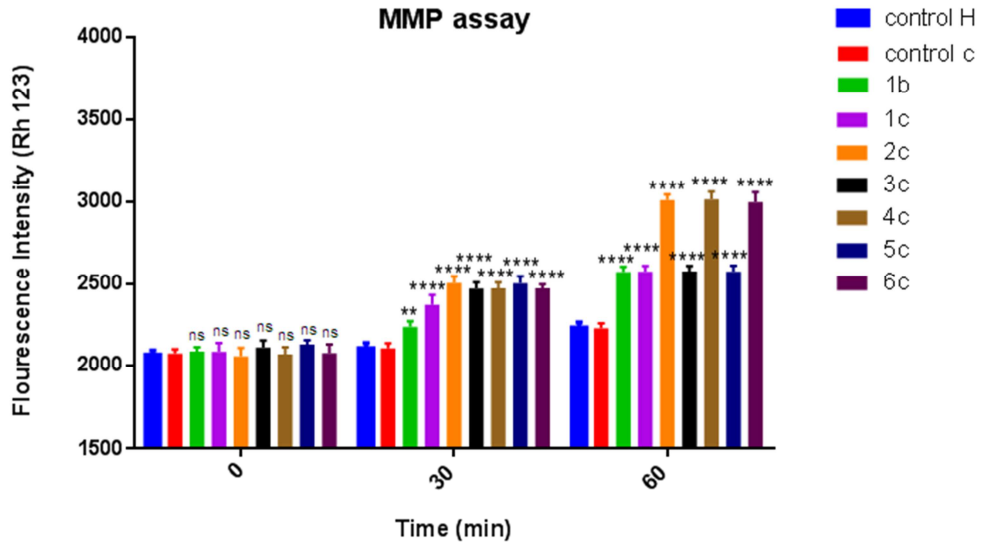

Figure 3. *Shows Mitochondrial membrane potential (MMP) assay. Figure shows the effect of cyclic Carnosine analogues on collapse MMP at different times (0, 30 and 60 min) in A549 cells isolated from tumor tissue of lung. Data represent the mean ± SD of three replicates in three independent experiments (n=3). The two-way ANOVA test was performed. ** and **** significantly different from the corresponding control (p<0.01 and p<0.0001, respectively).*

3.5. The Effect of Carnosine Analogues on Mitochondrial Swelling

Figure 4 shows, compounds 2b, 4b and 6b at 60 min have mitochondrial swelling effects with significant difference in comparison with the cancerous group.

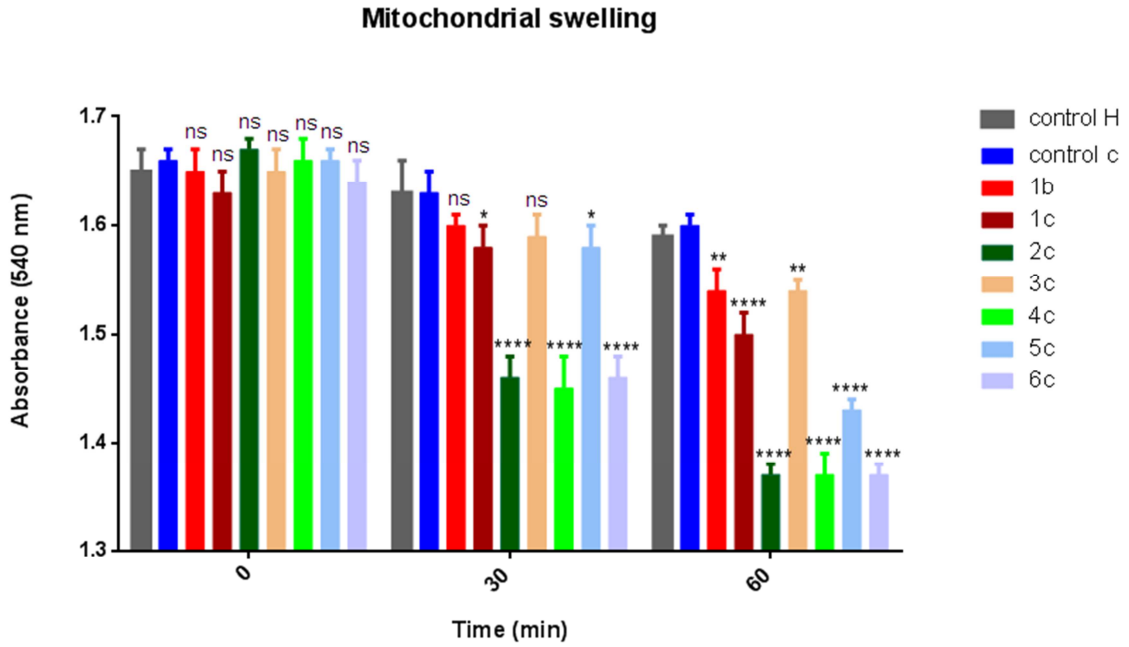

Figure 4. *Shows Mitochondrial swelling assay. Figure shows the effect of cyclic Carnosine analogues on mitochondria swelling at different times (0, 30 and 60 min) in A549 cells isolated from tumor tissue of lung. Data represent the mean ± SD of three replicates in three independent experiments (n=3). The two-way ANOVA test was performed *, ** and **** show a significant difference in comparison with the corresponding control (P<0.05, P<0.01 and P<0.0001, respectively).*

3.6. The Effect of Carnosine Analogues on Cytochrome c Release

Figure 5 shows, compounds 2b, 4b and 6b have significant in comparison with the cancerous group.

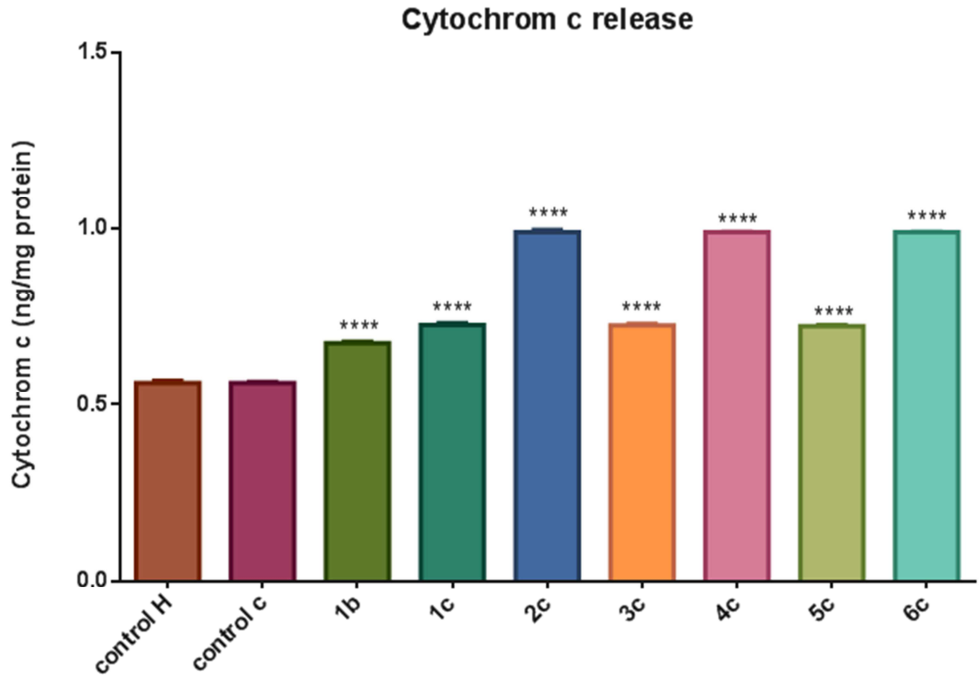

Figure 5. *Shows Cytochrome c release assay. Figure shows the amount of expelled cytochrome c from normal cells and A549. Fractions into the suspension buffer was determined using cytochrome c ELISA kit. Data represent the mean ± SD of three replicates in three independent experiments (n=3). The one-way ANOVA test was performed. **** show significant difference in comparison with the corresponding control (p<0.0001).*

4. Discussion

The cancer is one of the main diseases in the world leading to human death, therefore, there is a highly demand for discovering a new and effective treatment to overcome this aggressive disease. [23]. Recently, other studies on structural differences between normal and cancerous cells have been used to design new anticancer drugs [21, 24]. The purpose of this research was to study the effects of analogues Carnosine peptide on A549 cells using several experiments. First, primarily results in MTT assay indicated that the percentage of cell viability (enzyme succinate dehydrogenase activity) was more decreased by the synthesized cyclic peptides compared with together using cancerous group as control cells (Figure 1). The results showed that all the synthesized cyclic peptides increased ROS in various levels in comparison with unaffected mitochondria isolated from A549 group. A significant increase of ROS was resulted by 2c, 4c and 6c analogues at 30 min (Figure 2) [25]. Here, Rh 123 fluorescence staining (see Figure 3) indicated that the integrity of the mitochondria was damaged (i.e., reduction of the mitochondrial membrane potential was occurred by increasing Rh 123 fluorescence intensity) by the cyclic peptides. In this regard, compounds 2c, 4c and 6c significantly increased the collapse of MMP among the Carnosine analogues in comparison with mitochondria isolated from A549 group. Thus, it could be deduced that the synthesized peptides caused apoptosis by the mechanism of MMP collapsing. The cyclic peptides 2c, 4c and 6c (among the cyclic peptides) significantly increased mitochondrial swelling (reduction of absorbance in the experiment) in comparison with untreated mitochondria isolated from the A549 group (see Figure 4). The result was that cyclic peptides 2c, 4c and 6c significantly increased the release of cytochrome c in comparison with unaffected mitochondria isolated from the A549 group. These peptides showed more release of cytochrome c compared with the cyclic ones (see Figure 5). In overall, the results of our study showed that Carnosine peptide analogues, especially cyclic peptides 2c, 4c and 6c, raise the mitochondrial ROS level via the disruption of mitochondrial respiratory chain in comparison with the untreated mitochondria isolated from the human lung cancer. 2c, 4c and 6c as the cyclic Carnosine peptides showed more pronounced effect on A549 mitochondria, compared with the cyclic ones. These phenomena can be interpreted that these cyclic peptides generally have better permeability properties, it may be due to having dimer building blocks of Carnosine in its structure. This finding can confirm the results of the previous works [26] on demonstrating the activity of Carnosine on lung cancer. Moreover, Carnosine dimer and inverse Carnosine dimer with a proline amino acid at end of chain, constructed in a cyclic structure, gives better compound to show anticancer activity in lung.

5. Conclusion

The study showed the effects of synthesized cyclic Carnosine analogues on cancerous cells of human lung. Based on the increase in mitochondrial reactive oxygen species (ROS) level, swelling in mitochondria, mitochondrial membrane potential ($\Delta\psi m$) collapse and release of cytochrome c after exposure of mitochondria of the lung carcinoma by the synthesized peptides, cyclic Carnosine analogues 2c, 4c and 6c rather than other Carnosine analogues would be supporting to develop new anticancer agents and they may be considered as a promising complementary therapeutic agents for the treatment of lung cancer.

Compliance with Ethical Standards

Conflict of interest: The authors declare that there is no conflict of interest on this research work.

Ethical approval This work did not involve any studies on human or animal experiment undertaken by any of these authors.

Informed consent Informed consent was obtained from all individual participants included in this study.

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