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HIF-1*α* & NF-*κ*B Downregulation by *S*-Carvone Mitigate Hypoxia and Inflammation in $CoCl_2$ Treated Cells

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ABSTRACT

Background: S-CAV is a natural monoterpene that possesses antioxidant, anti-inflammatory, and anticancer activities. Both HIF and NF-κB pathways play a role in the hypoxia and inflammation during cancer progression. **Objectives:** In this study, we determine the effect of S-CAV against hypoxia-induced by $CoCl_2$ on A549 cells through the relationship between HIF-1α and NF-κB as well as inflammatory cytokines and oxidative stress. **Methods:** A549 cells were treated with S-CVN and $CoCl_2$ either alone or in combination for 24 hours. Afterward, cytotoxicity, cell viability, and mode of cell death were determined. Different markers were measured to detect the effect of S-CVN on $CoCl_2$ induced oxidative damage (MDA and TAC), hypoxia associated inflammation (IL-6 and TNF-α), as well as HIF-1α and NF-κB gene expression. **Results:** The results showed that S-CAV and $CoCl_2$ induced cytotoxicity at concentrations 4 and 6.2 mM. S-CAV and $CoCl_2$ combination therapy showed apoptotic cell death and a reduction in oxidative stress markers. Moreover, S-CAV in combination therapy downregulates the mRNA HIF-1α and NF-κB expression that parallels the reduction in IL-6 and TNF-α levels. **Conclusion:** Therefore, S-CAV possesses an antihypoxic effect on A549 $CoCl_2$ treated cells by reducing the oxidative stress, HIF-1α, and NF-κB gene expression as well as proinflammatory cytokines.

Keywords: *Hypoxia, S-CAV, HIF-1α, CoCl2, TNF-α, NF-κB.*

INTRODUCTION

Hypoxia and inflammation are two major characteristics that occur during cancer development and are intimately linked[1].The transcription factor families of HIF (mediated hypoxia signaling) and NF-κB (mediated inflammatory signaling) are responsible for several cellular responses in cancer growth[1].

Hypoxia is a pathological state that develops from insufficient oxygen supply to tumor tissue[2]. The cellular hypoxic regulator is mediated by hypoxia inducible factor-1 that is composed of oxygen regulated HIF-1 α subunit as well as the constitutively expressed HIF-1 β subunit[3,4].Also, hypoxic areas of tumor result in necrosis cells in some distal regions, with the release of alarmins that binds to their receptors, resulting in activation of NF-κB followed by subsequent proinflammatory gene expression in tumor cells[5]. Hypoxia-induced activation of HIF-1 α in surviving tumor cells induces expression of several genes (VEGF, c-Myc, cyclin D1) that provoke carcinogenesis. Alarmin receptors are also activated, resulting in the activation of

NF-κB[5]. Van Uden and his colleagues showed that HIF-1α gene is efficiently regulated, transcribed, stabilized, and activated by NF-κB[6].On the other hand, both HIF and NF-κB trigger transcription of various proteins that are by involved in carcinogenesis likeIL-6, MMP9, COX2, and Bcl-2[7].Hypoxia produces proinflammatory mediators that provoke the tumor-associated inflammatory response in cancer. This is reflected in the activation of NF-κB with a chronic inflammatory response in the tumor tissue[8–10]. There is a complex interplay between the responses of HIF and NF-κB signal pathways during hypoxia that makes it difficult to study.

$CoCl_2$ is a hypoxic mimic agent that widely used *in-vivo* or *in-vitro* studies since cobaltous ions can replace ferrous ions in heme, inducing structural modification in the heme protein O_2 sensor that results in hypoxia, DNA damage, ROS generation as well as potentiates oxidative stress[29].

S-CAV is a natural hydrocarbon that belongs to the monoterpenoids family that exists in essential oils of caraway, spearmint, and dill[11].S isomer of carvone is the main form that represents 60–70% of the caraway seed oil[12]. Previous studies documented the antioxidant, antimicrobial, anticancer, immunomodulatory, and anti-inflammatory activities of S-CAV[13–17].

This study was designed to detect the protective effect of S-CAV against $CoCl_2$ induced hypoxia on the A549 cell line focusing on HIF-1 α and NF-κB gene expression in response to hypoxia-associated inflammation.

MATERIAL AND METHODS

Media and Reagents

All of the used chemicals and reagents were obtained from Sigma Aldrich Chemical Co. (St. Louis, MO, USA) unless otherwise stated. This study was performed at the Cell Culture Research Unit on October 6 University (Cairo, Egypt).

Cell line and Cell culture

Human lung adenocarcinoma epithelial A549 cells (ATCC, USA) were cultured according to the American Type Culture Collection protocol. A549 was grown in RPMI-1640 medium supplemented with 10% fetal bovine serum, 1% (v/v) 100 U/ml penicillin/ streptomycin and 2 mM L-glutamine [18]. Cells were maintained in a humidified air incubator at 37 ◦C and 5% CO2. All experiments were repeated three times independently, and the data were represented as the mean ± SD.

MTT assay

To assess the cytotoxicity of S-CVN alone or its combination with $CoCl_2$, the MTT method was used according to Hansen and his colleagues [19]. Briefly, A549 cells were treated with various concentrations of S-CVN (0.162 to 6.6 mM) and $CoCl_2$ (0.193 to 7.75mM) either alone or in combination then incubated for 24 hours [20,21]. After that, the MTT reagent of 100 µg/mL final concentration was added to cells and incubated for 4 h. Afterward, dimethylsufloxide (200 µl) dissolved the colored formazan metabolites of MTT and then measured the absorbance at 570 nm against the control group using ELISA reader (BioRad, Germany) [19]. We used S-CAV IC_{50} value either alone or in combination with IC_{50} of $CoCl_2$ for 24 h in the followed bioassays. The mean percentages of cell viability were detailed as mean ± SD.

Cell Viability by the Trypan Blue Dye Exclusion Assay

The loss of cell membrane integrity of A549 was determined by the trypan blue dye exclusion assay that indicates of the cell viability of different treated and untreated groups. Briefly, A549 cells either untreated or treated with S-CVN (4 mM), $CoCl_2$ (6.2 mM), alone or in combination were seeded in 6 well culture plates and kept for 24 h. Then 10µL of cell suspension was mixed with 10µL trypan blue dye (0.4% solution) and was put on a hemocytometer to count unstained live and blue stained dead cells [22].The mean percentages of live and dead cells per experiment were chronicled as mean ± SD of three autonomous experiments.

Apoptosis and Necrosis Staining

AO/EB was used to shed light on the mode of cell death induced by S-CVN and CoCl2 either alone or in combination [23]. Briefly, A549 cells were treated with S-CVN (4 mM) and $CoCl_2$ (6.2 mM) either alone or in combination for 24 h then were collected and stained using the nucleic acid-binding dye, a mixture of 100 g/ml AO and 100 g/ml EB in phosphate buffer saline. The percentage of apoptotic or necrotic cells was determined under the fluorescence microscopy (AxioImager Z2, Zeiss, Germany). The mean percentages of live, apoptotic or necrotic cells per experiment were chronicled as mean ± SD [24].

Cell Lysate preparation

The preparation of cell lysate was done according to Zakaria and her colleagues to be used in the analysis of oxidative stress and inflammatory markers [24]. The protein content of total cell lysate was determined by bicinchoninic acid and using bovine serum albumin as a standard, data not cited [25].

Estimation of Oxidative Stress Markers (MDA and TAC)

Malondialdehyde is the final product of lipid peroxidation that was determined using thiobarbituric acid by a commercially supplied kit (Biodiagnostic) [26].

Also, the antioxidant capacity of the cell lysate was determined through its reaction with an exogenous amount of hydrogen peroxide, then the residual hydrogen peroxide was determined colorimetrically by a Biodiagnostic supplied kit [27].

Determination of inflammatory mediators (TNF-α and IL-6)

Using ELISA Kit supplied by Ray Biotech did the determination of both TNF-α and IL-6 in the cell lysate. Products number ELH-TNFa-1 and ELH-IL6-CL-1 were used correspondingly for this evaluation according to the manufacturer's instructions.

Gene Expression Analysis using Quantitative Real-time Polymerase Chain Reaction (qRT-PCR)

qRT-PCR was used to estimate quantitatively the changes in HIF-1α and NF-κB mRNA expression in A549 cells either untreated or treated. Total RNA was isolated using a QIAamp RNeasy Mini kit (Qiagen, Germany, GmbH). RNA was reverse-transcribed into complementary DNA (cDNA) using a First strand cDNA synthesis kit. SYBR Green PCR Master Mix (Qiagen, Germany, GmbH) was used to perform qPCR. The variation in the data of gene expression of HIF-1α and NF-κB in all groups was analyzed by the ΔΔCT method using GAPDH mRNA as the internal control.[28] All primer sequences used in amplifying the HIF-1α and NF-κB genes are listed in **Table 1**.

Table 1. Primer sequences used in qRT-PCR analysis

Target Gene	Primer Sequences
GAPDH	**F:** CTCTGATTTGGTCGTATTGGG **R:** TGGAAGATGGTGATGGGATT
NF-κB	**F:** TGGTGCCTCACTGCTAACT **R:** GGATGCACTTCAGCTTCTGT
HIF-1α	**F:** ATCCATGTGACCATGAGGAAATG **R:** TCGGCTAGTTAGGGTACACTTC

Statistical analysis

Graph- Pad Prism (ISI®, USA) software (version 5) was used to analyze the experimental data that expressed as mean ± standard deviation (SD). The statistical variation between groups was determined by one-way ANOVA followed by Tukey Kramer as a post hoc test. A P-value of less than 0.05 was considered to indicate a statistically significant difference. (a) is significant comparing with the A549 cells and (b) is significant comparing with the $CoCl_2$ treated group, (c) is significantly comparing with the4mMS-CAV treated group.

RESULTS

Effect of *S*-CAV on $CoCl_2$ induced cytotoxicity in A549 cells

Cytotoxicity results showed that $CoCl_2$ reduced the viability of A549 cells and the calculated IC_{50} was 6.2 mM (**Figure 1a**). The high dose (5.3 mM) of S-CAV reduced the viability of A549 cells and calculated IC_{50} was 4 mM (**Figure 1b**). The combination therapy of 6.2 mM of $CoCl_2$ + 4mM of S-CAV showed a cytotoxic effect and expressed viability of 31 % ± 2.2 **(Figure 1c**). Also, trypan blue dye exclusion results of treated cells with 4 mM S-CAV for 24 h showed a viable cell count of 54% ± 1 compared to untreated cells (98% ± 0.6) (**Figure 2**). On the other hand, cells treated with 6.2 m$MCoCl_2$ either alone or in combination with 4 mM S-CAV for 24 h showed a significant decline in the percent of viable cells of 60% ± 2.6 and 29% ± 1.5 respectively regarding that noticed in untreated cells (98% ± 0.6) as shown in **Figure 2**.

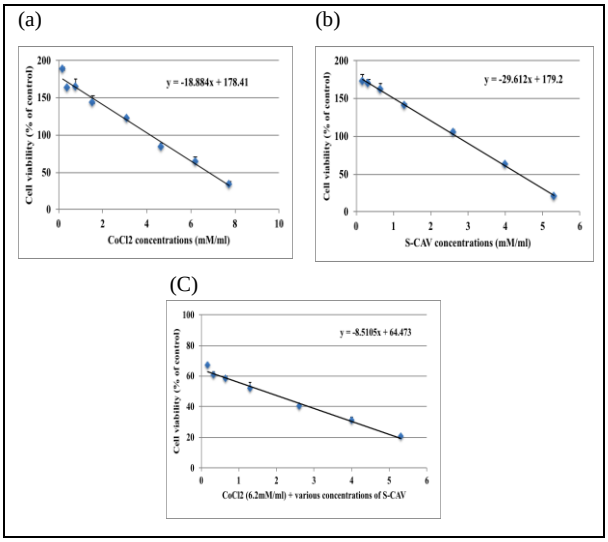

Figure 1. The cytotoxic effect of $CoCl_2$ (a) and S-CAV (b) on A549 cells after 24 h incubation using MTT assay. (c) The effect of individual treatment with IC_{50} of $CoCl_2$ and with various concentrations of S-CAV on A549 cells viability. Data expressed as a percentage of control untreated cells. The figure shows averages from triplicate data of three separate experiments and expressed as mean ± SD.

Effect of S-CAV on apoptosis and necrotic cell death

The combined DNA dyes AO/EB was used to differentiate between viable, apoptotic, and necrotic cell death. From fluorescence microscopy examination, cells treated with 4 mM of S-CAV alone showed bright yellowish-green fluorescence indicating early apoptotic cells, and the mean percentage of apoptotic cells was 35% ± 5.1 in S-CAV groups regarding that observed in

untreated cells as presented in **Figure 3a** and **3d**. On the other hand, cells treated with 6.2 mM of $CoCl_2$ alone showed a significant proportion of orange-stained cells with the morphology of late apoptotic cells (**Figure 3c),** the mean percentage of apoptotic cell death was 44%± 1.5. However, combined therapy of 6.2 mM of $CoCl_2$ + 4mM of S-CAV showed an increase in the number of apoptotic cells with a mean percentage was 52% ± 2 compared to the group treated with $CoCl_2$ alone as presented in **Figure 3a** and **3e**.

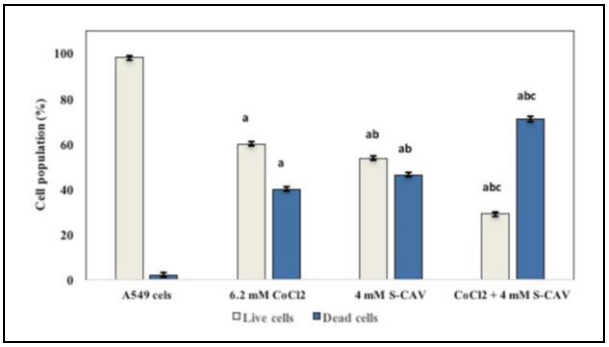

Figure 2. The cell viability assessment on A549 cells for S-CAV and $CoCl_2$ either alone or in combination after 24 h incubation, stained with trypan blue dye under a phase-contrast microscope; data showed mean (%) ± SD; n = 3; (light bars) demonstrates cell population percentages of viable cells; (dark bars) demonstrated cell population percentages of dead cells. (a) significantly different from A549 group ($P < 0.05$), (b) significantly different from 6.2 mM $CoCl_2$ group ($P < 0.05$), (c) significantly different from 4mMS-CAVgroup ($P < 0.05$).

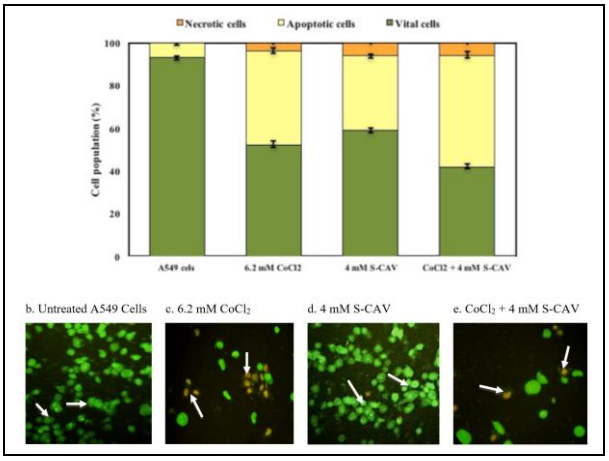

Figure 3. Representative photos for apoptotic and necrotic cell death in A549 cells treated with S-CAV and $CoCl_2$ either alone or in combination, stained with AO/EB under fluorescence microscope (x200); data showed mean (%) ± SD; n = 3; (a) demonstrates cell population percentages of apoptotic-necrotic cells; (b) vital cells (green), (c,e) apoptotic cells (yellow), (d) early apoptotic bodies (bright yellowish-green).

Effect of S-CAV on oxidative stress markers

As shown in **Figure 4a**, A549 cells treated with 6.2 mM of $CoCl_2$ generated a significant increase in the MDA level compared to that observed in untreated cells. While A549 cells receiving a combination therapy of S-CAV (4 mM) and $CoCl_2$ (6.2 mM) showed a significant reduction in MDA level compared with $CoCl_2$ treated cells with no changes compared to untreated cells. Whether the generation of ROS leads to activation of the antioxidant system or not, we measured the total antioxidant capacity in untreated and treated A549 cells. Compared to A549 cells, $CoCl_2$ (6.2 mM) treated group showed a significant reduction in the TAC level. Moreover, groups receiving a combination therapy of 6.2 mM of $CoCl_2$ + 4mM of S-CAV showed a significant increase in the TAC level compared to untreated and $CoCl_2$ (6.2 mM) groups. Also, S-CAV treated groups showed no significant changes in the TAC level compared to those observed in untreated cells as presented in **Figure 4b**.

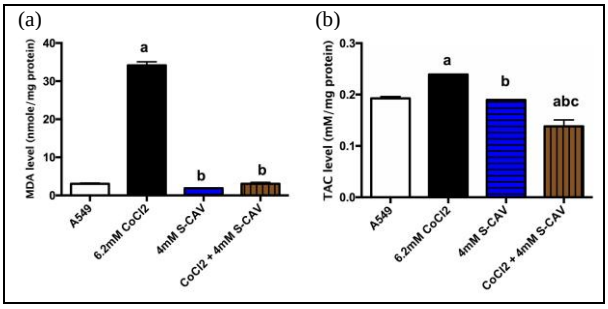

Figure 4. The effect of S-CAV (4 and 2.6 mM) and $CoCl_2$ (6.2 mM) either alone or in combination on (a) MDA and (b) TAC levels in A549 cells after 24 h exposure. Data expressed as nM/mg protein. (a) significantly different from A549 group ($P < 0.05$), (b) significantly different from 6.2 mM CoCl2 group ($P < 0.05$), (c) significantly different from 4mMS-CAVgroup ($P < 0.05$). The figure shows the mean ± SD of averages from triplicate data of three separate experiments.

Effect of S-CAV on inflammatory markers

A549 cells treated with 6.2mM of $CoCl_2$ showed a significant elevation in the levels of inflammatory cytokines TNF-α (1.82 ± 0.06) and IL-6 (2.1 ± 0.04) compared to untreated cells (0.40 ± 0.03 and 0.45 ± 0.03 respectively) after 24 h exposure, as presented in Figs. 5a and 5b. Compared to the $CoCl_2$ (6.2 mM) treated group, the levels of TNF-α and IL-6 were significantly reduced in A549 cells treated with 6.2 mM of $CoCl_2$ + 4mM of S-CAV for 24 h.

S-CAV attenuates gene expression of HIF-1α and NF-κB induced by CoCl2 on A549:

We noticed a marked increase in mRNA expression levels of HIF-1α and NF-κB (9.4 fold and

11.7 fold respectively) in A549 cells treated with 6.2 mM of $CoCl_2$ compared to untreated cells, as presented in Figs. 6a and 6b. The expression of HIF-1α mRNA was decreased significantly in A549 cells treated with 6.2 mM of $CoCl_2$ + 4mM of S-CAV (8.26 fold) compared to that observed in 6.2 mM $CoCl_2$ treated group. On the other hand, the expression of NF-κB mRNA in A549 cells treated with 6.2 mM of $CoCl_2$ + 4mM of S-CAV was significantly reduced by 6.02 fold compared to that noted in the $CoCl_2$ treated group (11.7 fold).

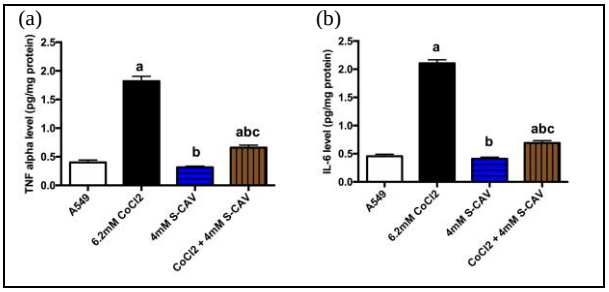

Figure 5. The effect of S-CAV (4 and 2.6 mM) and $CoCl_2$ (6.2 mM) either alone or in combination on (a) TNF-α and (b) IL-6 levels in A549 cells after 24 h exposure. Data expressed as pg/mg protein. (a) significantly different from A549 group ($P < 0.05$), (b) significantly different from 6.2 mM CoCl2 group ($P < 0.05$), (c) significantly different from 4mMS-CAVgroup ($P < 0.05$). The figure shows the mean ± SD of averages from triplicate data of three separate experiments.

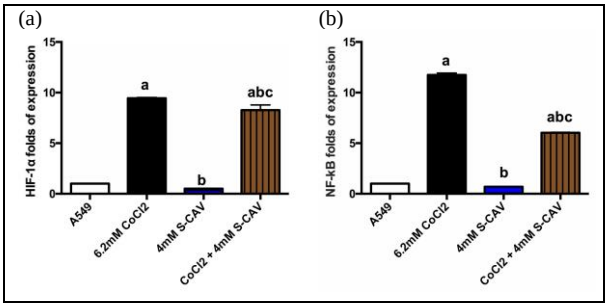

Figure 6. Changes in HIF-1α and NF-κB mRNA folds of expression in A549 cells treated with S-CAV (4 and 2.6 mM) and $CoCl_2$ (6.2 mM) either alone or in combination for 24 h, using qRT-PCR. Data expressed as the mean ± SD of relative expression. (a) significantly different from A549 group ($P < 0.05$), (b) significantly different from 6.2 mM CoCl2 group ($P < 0.05$), (c) significantly different from 4mMS-CAVgroup ($P < 0.05$).

DISCUSSION

$CoCl_2$ is a hypoxic imitator agent that induces structural modification in the heme protein O_2 sensor that results in the generation of reactive oxygen species, which provokes oxidative stress resulting in hypoxia-induced cytotoxicity [29,30]. From MTT results, IC_{50} value of $CoCl_2$ was 6.2 mM. Our finding was not similar to that observed by Mahey et al [31]. On the other hand, S-CAV is a natural monoterpenoid that was documented in earlier studies for its *in vitro* antitumor effect [32–34]. We demonstrate herein for the first time, that S-CAV induced cell death in A549 at a concentration greater than 4.6mM and IC_{50} value was 4mM. Patel and his colleagues reported that MCF 10A cells showed 51.23% viability after exposure to 20 mM L-carvone and this is differ from our findings[20].

$CoCl_2$ treatment resulted in a significantly higher percentage of apoptotic cells than that of necrotic cells. Cobalt ion possesses a cytocidal activity and induces hypoxia that is followed by the upregulation and downregulation of apoptotic and anti-apoptotic proteins respectively, which leads to apoptotic cell death [22,35]. These reports and our current results confirmed the link between $CoCl_2$, hypoxia signaling, and apoptotic cell death. Previously, L-carvone showed nuclei fragmentation in MCF-7 and HepG2 cancer cells indicating the induction of apoptosis [20,36]. Also, L-carvone arrested the S phase of the cell cycle in MCF-7, which inhibited further development of MCF-7 and subsequently induced apoptosis [20]. These previous studies confirm the results of this study, which showed that the combination of 6.2 mM of $CoCl_2$ + 4mM of S-CAV provoke the apoptotic cell death compared to $CoCl_2$ (6.2 mM) treated cells and to individual therapy.

Reactive oxygen species formation during hypoxia provokes the oxidative damage and stimulates the production of inflammatory proteins [37]. This study showed that the IC_{50} value of $CoCl_2$ (6.2 mM) disrupted the oxidant/antioxidant balance in A549 cells, which resulted in the increase of lipid peroxidation with a subsequent reduction in the TAC level. This finding further corroborates earlier observations of Tripathi et al,[22] as well as Mohamed et al [38]. S-CAV (4 mM) possesses antioxidant capacity when combined with $CoCl_2$ by reducing the level of MDA that parallels with the elevation in TAC level. This finding is in agreement with Aydin et al, and Saghir et al [17,39]. S-CAV had a strong radical scavenging activity by inducing glutathione S-transferase as well as restoring vitamin C, vitamin E, and glutathione levels as well as reduced pro-oxidants induced stress [40–42].

HIF-1α is released under hypoxic conditions to maintain oxygen homeostasis as well as modulate tumor-associated inflammation through the expression of transcriptional factor NF-κB [7,8]. NF-κB stimulates the release of pro-inflammatory cytokines like IL-6 and TNF-α, indicating the tight functional connection between hypoxia and inflammation [43]. Earlier studies reported that TNF-α induced NF-κB expression that subsequently increased the expression of HIF-1α mRNA

because several NF-κB subunits are bound to the HIF-1α promoter [6]. Together, these findings further support the results of our study. $CoCl_2$ treated cells showed a rapid increase of mRNA HIF-1α and NF-*κ*B expression as well as an elevation in the levels of IL-6 and TNF-α. These results indicated the rapid induction of hypoxia with concomitant inflammatory response and this is in agreement with other researchers using a similar approach [22,38].

This combination therapy showed a significant reduction in the mRNA HIF-1α and NF-*κ*B expression that results in the reduction of IL-6 and TNF-α levels. Earlier studies reported the anti-inflammatory effect of S-CAV by the alteration of TNF- α gene expression in high-fat diet-induced obesity in mice [44]. Also, Marques et al reported that carvone is a natural inhibitor of NF-*κ*B signaling with a successive reduction in the pro- and anti-inflammatory cytokine like TNF-α, IL-1α, and IL-10 [45]. Therefore, we could hypothesize that the anti-inflammatory effect of S-CAV originated as a secondary result of its role in the reduction of mRNA HIF-1α and NF-*κ*B expression.

CONCLUSION

The findings of the present study provide a narrative protective role for S-CAV (4 mM) against hypoxia-induced by $CoCl_2$ on the A549 cell line. S-CAV possesses a cytotoxic activity and promotes the apoptotic cell death induced by $CoCl_2$ in A549 treated cells. Also, S-CAV maintains the pro-oxidant / antioxidant balance as well as the anti-inflammatory effect that originated as a secondary result of a reduction in gene expression of HIF-1α and NF-*κ*B. Further studies are needed to explore the mechanism of S-CAV in the regulation of specific hypoxia-associated inflammation through an NF-*κ*B signaling pathway.

Abbreviation

S-CVN, S-carvone; MDA, malondialdehyde; TAC, total antioxidant capacity; NF-kB, nuclear factor kappa-light-chain-enhancer of activated B cells; $CoCl_2$, cobalt chloride; HIF-1α, hypoxia- inducible factor-1 alpha; IL-6, interleukin-6; TNF-α, tumor necrosis factor-alpha; AO/EB, Acridine orange/ethidium bromide staining; MTT, 3,4,5-dimethylthiazol-2,5- diphenyl tetrazolium bromide.

Conflict of interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

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