Anticancer Activity of Mineral-Supplemented Organically Cultivated Carrot on HT-29 Cells and Its Anti-Inflammatory Effect on Mice Splenocytes

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Abstract: Carrot (Daucus carota) is one of the world’s most significant root vegetables, with various bioactivities. This study aimed to investigate the anticancer activity and anti-inflammatory effects of natural dream cultivation carrot (NC). Natural dream cultivation is a cultivation method based on organic farming incorporating minerals. An MTT assay was used to evaluate the inhibitory rate of carrot samples on HT-29 human colon cancer cells, and qPCR was used to assess the mRNA expression of the cell cycle and apoptosis-related genes in the cancer cells. The nitrite oxide (NO) concentration was determined using the Griess method. The levels of inflammatory cytokines in LPS-induced mouse splenocytes were determined using an enzyme-linked immunosorbent assay, and the activity of NK cells was determined using LDH analysis. The results revealed that NC effectively inhibited cancer cell growth rate. Moreover, NC upregulated the mRNA expression of cell-cycle-arrest-related genes (p53 and p21) and apoptosis-related genes (Bim, Bad, Bak, caspase-9, and caspase-3) in cancer cells while downregulating the expression of anti-apoptotic genes, Bcl-2 and Bcl-xL. NC inhibited NO production and the release of inflammatory cytokines (TNF-α, IL-6, IL-1β, IFN-γ, and IL-12) in LPS-induced mouse splenocytes. NC also demonstrated the ability to stimulate NK cell activation. This study explored the potential mechanisms underlying carrots’ anticancer and anti-inflammatory properties by investigating their inhibitory effects on cancer cells and regulating the inflammatory response. The innovative mineral-supplemented organic cultivation method, as explored in this study, opens new avenues for harnessing the potential of carrots as a functional food source with promising applications in cancer and inflammation management. This research not only provides insights into the bioactive potential of carrots but also contributes to the future development of novel dietary interventions and therapeutics.

Keywords: anti-inflammatory; anticancer; minerals; natural dream cultivation carrot; organic cultivation

1. Introduction

Carrot (Daucus carota) is one of the most significant root vegetables in the Apiaceae family, and its fleshy root is widely consumed [1]. Carrot roots are high in nutrients, such as carotenoids, anthocyanins, dietary fiber, and vitamins, which act as antioxidants and have important roles in immune function, heart health, digestive health, and cancer prevention [2,3]. The mineral and phytochemical composition of plants is influenced by various factors, including cultivation methods, fertilizers, and environmental conditions [4]. Organic foods free of chemical pesticide residues and meeting organic farming standards...
have gained popularity as the emphasis on a healthy diet has increased [5]. Organic cultivation, as opposed to conventional cultivation, places a greater focus on ecosystem health by using natural organic fertilizers and organic pesticides, such as animal and plant-based fertilizers and natural insecticides, reducing reliance on chemical substances, promoting soil health and ecological balance, and providing healthier and environmentally friendly agricultural products [6]. The iCOOP Natural Dream Co. (Goesan, Chungcheongbuk-do, Republic of Korea) has developed a cultivation method based on organic farming and includes adding natural minerals. The use of minerals in cultivation may directly affect the growth of plants and the content of phytochemical components. To investigate whether this newly developed cultivation method can improve crop bioactivity, this study compares carrot antioxidant, anticancer, and anti-inflammatory abilities using traditional, organic, and natural dream cultivation methods.

Colorectal cancer is a common malignant tumor that develops in a multifactorial, multistep process. It often begins with abnormal colonic or rectal mucosal proliferation, develops via adenoma development and proliferation, and, finally, becomes cancer [7]. Colorectal cancer has a high incidence and mortality rate, showing an increasing trend globally [8]. It is often diagnosed at an advanced stage, posing patient survival and treatment challenges [8]. Therefore, research into colorectal cancer treatment has received increasing attention in recent years [9]. HT-29 cells are a human colorectal cancer cell line with strong proliferative ability, demonstrating typical colorectal cancer cell characteristics, such as active proliferation, apoptosis suppression, abnormal cell cycle, increased invasion, and metastatic capability [10]. As a result, they are commonly used as a colorectal cancer cell model and are widely used in researching colorectal cancer mechanisms and treatment methods [11]. In contrast to costly medications with accompanying side effects, eating nutrient-rich natural foods provides a sustainable approach to disease prevention or treatment [12]. The HT-29 cell model was used in this study to investigate the potential role of organically cultivated carrots enriched with minerals in treating colorectal cancer and the underlying mechanisms involved.

Inflammation is the body's nonspecific immune response to external causes, including injury, infection, or stimulation. Its primary function is to attract and activate immune cells to eliminate infections, repair damaged tissues, and restore homeostasis [13]. The inflammatory response comprises several complex biological processes involving interactions between various cell types and molecular signaling pathways [14]. One of the most useful cellular models for investigating inflammatory responses is murine splenocytes [15]. As a vital immune organ in the body, the spleen includes a diverse population of immune cells, including macrophages, dendritic cells, and lymphocytes. Splenocytes are often used in trials in inflammation research to investigate the regulatory effects of various drugs on characteristics such as cytokine production during the inflammatory process, activity, and function of immune cells [16,17]. A commonly used experimental approach is to stimulate splenocytes with inflammatory stimuli, such as lipopolysaccharide (LPS), then measure the cells' production of inflammatory cytokines, such as tumor necrosis factor-alpha (TNF-α), interleukin-6 (IL-6), and interleukin-1beta (IL-1β) [18,19]. Splenocytes can also be used to assess the activity of immune cells, such as natural killer (NK) cells, and the regulatory effects of cytokines on immune cells [20]. There is currently limited research on carrots' anti-inflammatory effects, particularly traditionally cultivated and organically cultivated carrots. This study evaluates the potential role of organically cultivated carrots enriched with minerals in treating LPS-induced inflammation in splenocytes and the underlying mechanisms involved.

The present study aims to assess the anticancer and anti-inflammatory effects of three varieties of carrots grown under different cultivation methods using HT-29 colon cancer cells and an LPS-induced mouse splenocyte inflammation model. The evaluation will be performed by examining the inhibitory effect of carrots on HT-29 cell growth, the mRNA expression levels of cell cycle arrest and apoptosis-related genes in HT-29 cells, as well as the viability of RAW 264.7 cells, nitric oxide (NO) production in RAW 264.7 cells and mouse
splenocytes, the levels of inflammatory cytokines released by mouse splenocytes, and the activity of natural killer (NK) cells. These assessments aim to investigate the anticancer and anti-inflammatory mechanisms of carrots, providing a theoretical basis and evidence for the development of healthy anticancer and anti-inflammatory agricultural products.

2. Methods

2.1. Sample Preparation

The samples used in this experiment, including conventional cultivation carrot (CC), organic cultivation carrot (OC), and natural dream cultivation carrot (NC), were provided by iCOOP Natural Dream Co. (Goesan, Chungcheongbuk-do, Republic of Korea). OCs were grown without artificial fertilizers or pesticides and were certified by the Republic of Korea’s Ministry of Agriculture, Food, and Rural Affairs. The cultivation method of NC was 15 times of foliar fertilization with 500 times diluted deep sea water minerals (DSWMs, Gurye, Jeollanam-do, Republic of Korea), based on OC. The mineral content of DSWM was tested by the Korea Quality Testing Institute (Suwon, Gyeonggi-do, Republic of Korea), and showed the following concentrations: Cl at 147,000 mg/L, SO$_4$ at 65,000 mg/L, Mg at 60,000 mg/L, K at 11,588 mg/L, Na at 9565.36 mg/L, B at 179 mg/L, Ca at 49.5 mg/L, NO$_3$ at 27 mg/L, Si at 0.87 mg/L, Cu at 0.19 mg/L, Mn at 0.01 mg/L, and Se at 0.01 mg/L. Freeze-dried powder samples (20 g) were mixed with 400 mL of methanol (20-fold dilution) and extracted twice for 24 h, using a stirrer. The filtrates from both extractions were collected, powdered using a vacuum concentrator (EYELA, Tokyo Rikakikai Co., Tokyo, Japan), and dissolved in dimethyl sulfoxide to prepare a concentration of 250 mg/mL for CC, OC, and NC samples to be used in subsequent experiments [21].

2.2. In Vitro Antioxidant and Anticancer Effects

2.2.1. Determination of Total Phenolic (TP) Content

The total phenolic (TP) compound content was determined using our previous research method [22]. A volume of 25 µL of each sample (CC, OC, and NC) was mixed with 75 µL of Folin–Ciocalteau reagent (Sigma-Aldrich Co., St. Louis, MO, USA) and allowed to react at room temperature for 5 min. Subsequently, 200 µL of 7.5% Na$_2$CO$_3$ solution and 700 µL of distilled water were added. After 40 min at room temperature in the dark, the absorbance at 765 nm was measured using a Wallac Victor3 1420 Multilabel Counter. Gallic acid (Sigma-Aldrich Co.) was used as a standard, and the TP content of CC, OC, and NC was determined using the derived standard calibration curve.

2.2.2. Determination of Total Flavonoid Content

The total flavonoid (TF) content was determined using the color change principle caused by the interaction of NaOH and flavonoid compounds. A volume of 100 µL of each sample (CC, OC, and NC) was mixed with 1 mL of diethylene glycol, followed by gentle mixing and 5 min incubation at room temperature. Subsequently, 100 µL of 7.5% Na$_2$CO$_3$ solution and 700 µL of distilled water were added. After 40 min at room temperature in the dark, the absorbance at 520 nm was measured using a Wallac Victor3 1420 Multilabel Counter. Quercetin (Sigma-Aldrich Co.) was used as a standard, and the TF content of CC, OC, and NC was determined using the derived standard calibration curve [22].

2.2.3. 2,2-Diphenyl-1-picrylhydrazyl (DPPH) Radical Scavenging Assay

A volume of 100 µL of a diluted 150 µM 1,1-diphenyl-2-picrylhydrazyl (DPPH) solution in methanol or 100 µL of methanol was added at a particular concentration to 100 µL of the respective sample (CC, OC, and NC). The mixture was combined in a 96-well plate, and 100 µL of a 150 µM DPPH solution or 100 µL of methanol to 100 µL of methanol was added as a control. The reaction was conducted in the dark for 30 min at room temperature.
The absorbance at 517 nm was measured using a Wallac Victor3 1420 Multilabel Counter. The DPPH inhibition rate (%) was calculated using the following formula [22]:

$$\text{DPPH inhibition rate (\%)} = \left(1 - \frac{(\text{sample} + \text{DPPH}) - (\text{sample} + \text{methanol})}{(\text{methanol} + \text{DPPH}) - (\text{methanol} + \text{methanol})}\right) \times 100.$$  

**2.2.4. HT-29 Cell Culture**

The HT-29 cells were obtained from the Korean Cell Line Bank (KCLB, Seoul, Republic of Korea). The cells were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium (Welgene Inc., Daegu, Republic of Korea), which was supplemented with 10% heat-inactivated fetal bovine serum (FBS, GIBCO, Grand Island, NY, USA) and 1% penicillin-streptomycin (PS) solution (Welgene, Gyeongsan-si, Gyeongsangbuk-do, Republic of Korea). The cells were kept in a humidified incubator with 5% CO$_2$ at 37 °C. Passaging was performed three to four times each week. The culture medium was removed during passaging, and the cells were washed with phosphate-buffered saline. A 0.05% trypsin-0.02% ethylenediaminetetraacetic acid solution was used to remove the cells. The supernatant was removed after centrifugation, and the cells were resuspended in a complete culture medium for subculturing [23].

**2.2.5. Cytotoxicity Assessment of Samples on HT-29 Cells**

The number of HT-29 cells was measured using a cell counter (Luna automated cell counter, Logos Biosystems, Anyang, Gyunggi-do, Republic of Korea). The cells were seeded in a 96-well plate at a density of $5 \times 10^4$ cells/mL per well and incubated for 24 h in a CO$_2$ incubator at 37 °C. The culture medium was then removed, and the samples were prepared in different concentrations using the culture medium. Each sample was put into the wells at a volume of 100 µL. After 48 h of incubation, the culture medium was removed, and 100 µL of MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) solution (500 µg/mL) was added to each well. The cells were then incubated for another 3.5–4 h. Then, the formazan crystals were dissolved by adding DMSO, and the absorbance at 550 nm was measured after 15–30 min using a Wallac Victor3 1420 Multilabel Counter [23].

**2.2.6. Measurement of mRNA Levels of Cell Cycle Arrest and Apoptosis-Related Genes in HT-29 Cells Using RT-qPCR**

HT-29 cells were cultured in a 6-well plate at $1 \times 10^5$ cells/mL concentration in a CO$_2$ incubator at 37 °C for 24 h. After removing the culture medium, the samples were diluted in the medium and added to each well. The culture medium was released after 48 h, and RNA was extracted by treating the cells with 1 mL of Trizol (Invitrogen, Carlsbad, CA, USA) per plate. RNA was quantified using a NanoDrop ND-1000 (NanoDrop Technologies Inc., Wilmington, DE, USA), and the quantified RNA was reverse-transcribed and synthesized into cDNA using Superscript II reverse transcriptase (Invitrogen). The gene expression of the synthesized cDNA was assessed using a BioRad CFX-96 Real-Time System (Bio-Rad, Hercules, CA, USA), and the relative transcription levels of mRNA were determined using the $2^{-\Delta\Delta Ct}$ technique. The particular experimental procedures were conducted in accordance with previous research protocols [22]. The expression levels of the p53, p21, Bim, Bad, Bcl-2, Bcl-xL, Bax, Bak, caspase-9, caspase-3, and GAPDH genes in HT-29 cells were detected, and the primer sequences used are provided in Table 1.

**2.3. Ex Vivo Anti-Inflammatory Effect**

**2.3.1. RAW 264.7 Cell Culture**

RAW 264.7 cells were obtained from the Korean Cell Line Bank (KCLB, Seoul, Republic of Korea). The cells were cultured in Dulbecco’s Modified Eagle’s Media (GIBCO) supplemented with 10% heat-inactivated FBS and 1% PS solution. The cells were kept in a humidified incubator with 5% CO$_2$ at 37 °C. Passaging was performed three to four times each week [21].
Table 1. Primer sequences of RT-qPCR assay.

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Primer Sequence</th>
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| p53       | F: 5’-ATGGAGGAGCGCAGTCAGA-3’  
R: 5’-GCCCAGGCCGCAGTTGTAG-3’ |
| p21       | F: 5’-ATGTCAAGACGGCTGGG-3’  
R: 5’-GCCCAGGCCGCAGTTGTAG-3’ |
| Bcl-2     | F: 5’-AAAGATTGATGGATCGTTCGTGC-3’  
R: 5’-GCCCAGGCCGCAGTTGTAG-3’ |
| Bcl-xL    | F: 5’-GCTGGGACACTTTTGTGGAT-3’  
R: 5’-GCCCAGGCCGCAGTTGTAG-3’ |
| Bim       | F: 5’-CAATGACCCTTCTCAGGACC-3’  
R: 5’-GCCCAGGCCGCAGTTGTAG-3’ |
| Bad       | F: 5’-TGCTTTCAAGGGTTTCTCATCCT-3’  
R: 5’-GCCCAGGCCGCAGTTGTAG-3’ |
| Bax       | F: 5’-CTTCCAGGTTTCATCCAG-3’  
R: 5’-GCCCAGGCCGCAGTTGTAG-3’ |
| Bak       | F: 5’-CTTGGGCTACAGTCTACCC-3’  
R: 5’-GCCCAGGCCGCAGTTGTAG-3’ |
| Caspase-9 | F: 5’-CTTCCAGGTTTCATCCAG-3’  
R: 5’-GCCCAGGCCGCAGTTGTAG-3’ |
| Caspase-3 | F: 5’-CTTCCAGGTTTCATCCAG-3’  
R: 5’-GCCCAGGCCGCAGTTGTAG-3’ |
| GAPDH     | F: 5’-AGGTCTGGTGGAACCCGATTGGG-3’  
R: 5’-GCCCAGGCCGCAGTTGTAG-3’ |

2.3.2. Isolation and Culture of Mice Splenocytes

Ten 6-week-old male C57BL/6 mice (body weight: 20 ± 2 g, Orient Bio, Seongnam, Republic of Korea) were kept in a specific pathogen-free room with a controlled temperature of room temperature and a relative humidity of 55 ± 5% under a 12:12 h light-dark cycle. After 1 week of acclimation, the mice were sacrificed, using sterile surgical scissors to isolate the spleens and prepare a splenocyte suspension. The splenocytes were isolated using a mesh filtered and treated with red blood cell lysis buffer. The suspension was centrifuged at 1200 rpm for 3 min, and the pellet containing the dissociated cells was resuspended in RPMI 1640 medium (Welgene Inc.) supplemented with 10% FBS and 1% PS. The cells were cultured in preparation for future experiments [24].

2.3.3. YAC-1 Cell Culture

YAC-1 cells used in this study were obtained from the American Type Culture Collection (Manassas, VA, USA). The cells were cultured in RPMI 1640 medium supplemented with 10% FBS and 1% PS. Passaging was performed three to four times weekly, and the cells were used for future experiments [25].

2.3.4. Cytotoxicity Assessment of Samples on RAW 264.7 Cells

RAW 264.7 cell count was determined using a cell counter. The cells were seeded at a density of 1 × 10^5 cells/mL per well in a 96-well plate and incubated for 24 h at 37 °C with 5% CO₂ in a humidified incubator. The culture medium was then removed, and the samples were prepared at different concentrations using the culture medium. Each well received 100 μL of the sample solution. After 48 h of incubation, the culture medium was removed, and 100 μL of 500 μg/mL MTT solution was added to each well. The cells were then incubated for another 3.5–4 h. The formazan crystals were dissolved in DMSO, and the absorbance at 550 nm was measured after 15–30 min [21].
2.3.5. Measurement of Nitrite (NO) Production

To measure nitrite, we followed the protocol of agreement using the Griess method [21]. RAW 267.4 cells and spleen cells were seeded in 6-well plates at $2 \times 10^5$ and $1 \times 10^6$ cells/mL concentration, respectively, and kept in a humified incubator at 5% CO$_2$ and 37 °C for 24 h. The culture medium was removed, and the wells were refilled with medium containing the samples at specific concentrations along with LPS (1 µg/mL). The cells were incubated for 48 h. After this, the cell culture supernatant was collected and placed on a 96-well plate. Equal amounts of Griess reagent were added to each well, and absorbance at 550 nm was measured using a Wallac Victor3 1420 Multilabel Counter.

2.3.6. Measurement of Inflammatory Cytokines Using Enzyme-Linked Immunosorbent Assay

Spleen cells were seeded in a 6-well plate at $1 \times 10^6$ cells/mL concentration and incubated for 24 h at 37 °C with 5% CO$_2$ in a humified incubator. After removing the culture medium, the wells were treated with specific concentrations of samples and LPS (1 µg/mL). The cells were incubated for 48 h. TNF-α, IL-6, IL-1β, IFN-γ, IL-12, and IL-10 levels in cell culture supernatant were determined using enzyme-linked immunosorbent assay kits (BioLegend, San Diego, CA, USA). The specific testing procedures were performed in accordance with the manufacturer’s instructions.

2.3.7. Measurement of NK Cell Activity

Spleen cells were dispersed in a 96-well plate at $1 \times 10^6$ cells/well concentration to be used as effector cells. YAC-1 cells were used as target cells, with a 5:1 ratio of effector cells to target cells in each well. The samples were treated at specific concentrations. The lactate dehydrogenase (LDH) secreted by YAC-1 cells was measured at 450 nm absorbance after 4 h of incubation at 37 °C with 5% CO$_2$ in a humified incubator using the EZ-LDH assay kit (DoGenBio, Geumcheon-gu, Seoul, Republic of Korea) according to the manufacturer’s instructions. The cytotoxicity of the samples against YAC-1 cells was calculated using LDH measurements [24].

3. Results

3.1. In Vitro Antioxidant and Anticancer Effects

3.1.1. TP and TF Contents and DPPH Inhibition Rate of the Samples

The TP and TF contents and the DPPH inhibition rates of the samples are shown in Figure 1. The NC group had the highest TP among different concentrations (1–4 mg/mL) and was significantly different from the CC and OC groups ($p < 0.05$). The TF content in the NC group was higher than in the CC and OC groups within the concentration range of 1–4 mg/mL, but there was no significant difference between the groups ($p > 0.05$). Additionally, the NC group had the highest inhibition rate at different sample concentrations, followed by the OC group, whereas the CC group had the lowest inhibition rate. Except for the 1 mg/mL concentration, there were significant differences between the NC, CC, and OC groups ($p < 0.05$). These results suggest NC has higher antioxidant bioactivity than CC and OC, which may contribute to its higher anticancer and anti-inflammatory effects.

3.1.2. Effect of the Samples on HT-29 Cell Growth

The results of the growth inhibition of HT-29 cancer cells by carrots (Figure 2) showed that as the sample concentration increased, the cancer cell growth inhibition rate improved in a concentration-dependent manner. There were no significant differences between groups at 1 mg/mL ($p > 0.05$). At 1.5 mg/mL, the NC group showed the highest growth inhibition rate compared with the other groups, and there was a significant difference ($p < 0.05$). At 2 mg/mL, the growth inhibition rate of cancer cells was classified as CC, OC, and NC, with significant differences between groups ($p < 0.05$). At a 2.5 mg/mL concentration, OC and NC exhibited growth inhibition rates higher than 40% for HT-29 cancer cells. When the apoptotic rate of HT-29 cells exceeds 40%, it is thought to affect
normal cells. Therefore, a 2 mg/mL carrot extract concentration was used for subsequent experiments.

![Graph showing total phenol, total flavonoid contents, and DPPH free radical scavenging by the concentration of carrot.](image1)

**Figure 1.** Total phenol, total flavonoid contents, and DPPH free radical scavenging by the concentration of carrot. CC—conventional cultivation carrot; OC—organic cultivation carrot; NC—natural dream cultivation carrot. According to two-way ANOVA, * p < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001 (significantly different).

![Graph showing MTT assay results.](image2)

**Figure 2.** Inhibition rate of HT-29 colon cancer cells according to the carrot. CC—conventional cultivation carrot; OC—organic cultivation carrot; NC—natural dream cultivation carrot. According to two-way ANOVA, *** p < 0.001 and **** p < 0.0001 (significantly different).

### 3.1.3. Effect of the Samples on mRNA Expression of Cell-Cycle-Arrest-Related Genes in HT-29 Cells

Figure 3 shows the mRNA expression levels of cell-cycle-arrest-related genes, p53 and p21, in HT-29 cells. The mRNA expression levels of p53 and p21 after NC treatment were significantly higher than in the CON, CC, and OC groups (p < 0.05). The expression level of p53 in the NC group was 1.5 times that of the CC group, whereas the expression level of p21 in the NC group was twice that of the CC group, with no significant difference between the CC and OC groups (p > 0.05). This suggests that NC effectively upregulates the expression of cell-cycle-arrest-related genes, thereby inhibiting cancer cell growth.

### 3.1.4. Effect of the Samples on mRNA Expression of Apoptosis-Related Genes in HT-29 Cells

The mRNA expression levels of apoptosis-related genes, such as Bim, Bad, Bcl-2, Bcl-xL, Bax, and Bak, in HT-29 cells are presented in Figure 4. The CON group had the lowest Bim, Bad, and Bax expression levels and the highest Bcl-2 and Bcl-xL expression levels. In contrast, NC-treated cells had the highest Bim, Bad, Bax, and Bak expression levels and the lowest levels of Bcl-2 expression. Additionally, Bad, Bcl-2, and Bax expression levels in the
NC group differed significantly from the OC, CC, and CON groups ($p < 0.05$). However, there were no significant differences in the Bim, Bcl-xL, and Bak expression levels between the NC and OC groups ($p > 0.05$). These results suggest that NC treatment promotes the mRNA expression of pro-apoptotic genes (Bim, Bad, Bax, and Bak) while downregulating the expression of anti-apoptotic genes (Bcl-2 and Bcl-xL), thus increasing apoptosis and having an anticancer effect.

**Figure 3.** Effects of carrot on mRNA expression levels of p53 and p21 in HT-29 cancer cells. Con—no treatment; CC—conventional cultivation carrot, 2 mg/mL; OC—organic cultivation carrot, 2 mg/mL; NC—natural dream cultivation carrot, 2 mg/mL. Means with the different letters (a–c) above the bars are significantly different ($p < 0.05$) using Duncan’s multiple range test.

**Figure 4.** Effects of carrot on mRNA expression levels of Bim, Bad, Bcl-2, Bcl-xL, Bax, and Bak and in HT-29 cancer cells. Con: no treatment; CC: conventional cultivation carrot, 2 mg/mL; OC: organic cultivation carrot, 2 mg/mL; NC: natural dream cultivation carrot, 2 mg/mL. Means with the different letters (a–c) above the bars are significantly different ($p < 0.05$) using Duncan’s multiple range test.
3.1.5. Effect of the Samples on mRNA Expression of Caspase-Family-Related Genes in HT-29 Cells

Figure 5 shows that caspase-9 and caspase-3 mRNA expression levels in HT-29 cells were highest after NC treatment, with significant differences from the other groups ($p < 0.05$). There were no significant differences in caspase-9 levels between the CON, CC, and OC groups ($p > 0.05$). The mRNA expression level of caspase-3 after OC treatment was lower than that of NC but higher than that of CC, and there was a significant difference ($p < 0.05$), whereas CC treatment did not show a significant difference compared with the CON group ($p > 0.05$). These results indicate that compared with CC and OC, NC effectively regulates the mRNA expression of caspase-family-related genes, increasing apoptosis in cancer cells.

![Figure 5. Effects of carrot on mRNA expression levels of caspase-9 and caspase-3 and in HT-29 cancer cells. Con: no treatment; CC: conventional cultivation carrot, 2 mg/mL; OC: organic cultivation carrot, 2 mg/mL; NC: natural dream cultivation carrot, 2 mg/mL. Means with the different letters (a–c) above the bars are significantly different ($p < 0.05$) using Duncan’s multiple range test.](image)

3.2. Ex Vivo Anti-Inflammatory Effects

3.2.1. Effects of the Samples on RAW 264.7 Cells

We initially evaluated the cytotoxicity of the samples using RAW 264.7 cells, which have similar characteristics to mouse splenocytes, to perform ex vivo experiments using mouse splenocytes. The effects of the three carrot samples on RAW 264.7 cells are shown in Figure 6. Cell viability was more than 90% for all three samples at a concentration of 2 mg/mL, with no significant differences ($p > 0.05$), showing that the three carrot samples were nontoxic to the cells. However, cell viability reduced as the concentration increased. Therefore, a 2 mg/mL concentration was used for subsequent experiments.

3.2.2. Effects of the Samples on NO Production in RAW 264.7 Cells and Mice Splenocytes

As shown in Figure 7, the expression of NO production in RAW 264.7 cells and splenocytes was the lowest in the CON group. RAW 264.7 cells and splenocytes exhibited the highest levels of NO production after LPS treatment, and there were significant differences compared with the other groups ($p < 0.05$). Carrot treatment significantly reduced LPS-induced NO production in RAW 264.7 cells. The CC group produced half as much NO as the LPS group, whereas NO production in the OC and NC groups was a quarter of that in the LPS group. There was no significant difference between the OC and NC groups ($p > 0.05$). Furthermore, NO production in splenocytes was higher in the NC group than in the CON group but lower in the CC and OC groups, with significant differences across all groups ($p < 0.05$). These results show that NC inhibits NO production better than the CC group in RAW 264.7 cells and better than CC and OC in splenocytes, suggesting that NC treatment effectively suppresses LPS-induced NO production.
Effects of the Samples on NO Production in RAW 264.7 Cells and Mice Splenocytes

3.2.1. Effects of the Samples on RAW 264.7 Cells

After LPS stimulation, cytokines, such as TNF-α, IL-6, IL-1β, IFN-γ, IL-12, and IL-10, were significantly increased in splenocytes compared with the CON group (Figure 8, \( p < 0.05 \)). However, treatment with carrot samples significantly reduced the release of these cytokines, with levels decreasing in the order of CC, OC, and NC. The cytokine levels in the NC group were closest to those of the CON group. There were no significant differences in TNF-α and IL-12 detection between the CC and OC groups (\( p > 0.05 \)), but the levels in the NC group were significantly lower than those in the CC and OC groups (\( p < 0.05 \)). Additionally, there were no significant differences in IL-1β and IFN-γ levels between the OC and NC groups (\( p > 0.05 \)), but they were significantly lower than those in the CC group (\( p < 0.05 \)). Furthermore, in terms of IL-6 and IL-10 levels, CC, OC, and NC showed a significant decrease (\( p < 0.05 \)). These results suggest that compared with CC and OC, NC effectively suppresses inflammatory cytokines to relieve LPS-induced inflammation.

Figure 6. Viability of RAW 264.7 cells according to carrot type. CC—conventional cultivation carrot; OC—organic cultivation carrot; NC—natural dream cultivation carrot. According to two-way ANOVA, ** \( p < 0.01 \), **** \( p < 0.0001 \) (significantly different).

Figure 7. Nitric oxide production in RAW 264.7 cells and C57BL/6 mice splenocytes after exposure to LPS and carrot. Con: no treatment; LPS: 1 \( \mu \)g/mL lipopolysaccharide (LPS); CC: conventional cultivation carrot, 2 mg/mL + 1 \( \mu \)g/mL LPS; OC: organic cultivation carrot, 2 mg/mL + 1 \( \mu \)g/mL LPS; NC: natural dream cultivation carrot, 2 mg/mL + 1 \( \mu \)g/mL LPS. Means with the different letters (a–e) above the bars are significantly different (\( p < 0.05 \)) using Duncan’s multiple range test.

3.2.3. Effects of the Samples on Cytokine Release from Splenocytes

After LPS stimulation, cytokines, such as TNF-α, IL-6, IL-1β, IFN-γ, IL-12, and IL-10, were significantly increased in splenocytes compared with the CON group (Figure 8, \( p < 0.05 \)). However, treatment with carrot samples significantly reduced the release of these cytokines, with levels decreasing in the order of CC, OC, and NC. The cytokine levels in the NC group were closest to those of the CON group. There were no significant differences in TNF-α and IL-12 detection between the CC and OC groups (\( p > 0.05 \)), but the levels in the NC group were significantly lower than those in the CC and OC groups (\( p < 0.05 \)). Additionally, there were no significant differences in IL-1β and IFN-γ levels between the OC and NC groups (\( p > 0.05 \)), but they were significantly lower than those in the CC group (\( p < 0.05 \)). Furthermore, in terms of IL-6 and IL-10 levels, CC, OC, and NC showed a significant decrease (\( p < 0.05 \)). These results suggest that compared with CC and OC, NC effectively suppresses inflammatory cytokines to relieve LPS-induced inflammation.

Figure 5. Effects of carrot on mRNA expression levels of caspase -9, -10, and IFN-γ. The effects of the three carrot samples on mRNA expression levels of caspase -9, -10, and IFN-γ in RAW 264.7 cells are shown. Means with the different letters (a–e) above the bars are significantly different (\( p < 0.05 \)) using Duncan’s multiple range test. ** \( p < 0.01 \), **** \( p < 0.0001 \) (significantly different).
3.2.4. Effects of the Samples on NK Cell Activity in Splenocytes Isolated from Mice

The effects of carrot samples on NK cell activity in splenocytes are shown in Figure 9. The NK cell activity was highest in the NC group (39.14%), followed by the OC group (24.72%), and the CC group had the lowest activity (6.31%). In statistical analysis, there were significant differences between the groups ($p < 0.05$). Therefore, the NC group exhibited the most significant impact on NK cell activity, indicating that NC has the greatest potential for improving immune function.

![Figure 9](image-url)

**Figure 9.** Activity of NK cells in C57BL/6 mice splenocytes after carrot. CC, conventional cultivation carrot 2 mg/mL; OC, organic cultivation carrot, 2 mg/mL; NC, natural dream cultivation carrot, 2 mg/mL. Splenocyte:YAC-1 cell processed in a ratio of 5:1. According to two-way ANOVA, $**** p < 0.0001$ (significantly different).
4. Discussion

Carrots contain antioxidants such as phenols and flavonoids, exerting a regulatory role in intracellular oxidative stress [3]. Oxidative stress denotes an imbalance in cellular redox homeostasis, leading to an excessive generation of reactive oxygen species (ROS), encompassing both free radicals (such as superoxide anions and hydroxyl radicals) and non-radical oxygenated species (like hydrogen peroxide and nitric oxide) [26]. When cellular antioxidant defense systems are insufficient to counteract external or internal oxidative pressures, ROS generation surpasses clearance capacity, thereby inducing oxidative damage to intracellular molecules, including oxidation modifications of proteins, lipids, and DNA, consequently perturbing cellular structure and function [27]. Oxidative stress is considered a contributing mechanism to numerous diseases, including cancer, neurodegenerative disorders, and cardiovascular ailments [28]. Total phenols are a group of polyphenolic compounds that include flavonoids, flavonols, phenolic acids, and other subclasses, with flavonoids referred to as TFs [29]. They have strong antioxidant properties, neutralize free radicals, affect cellular signaling pathways, and influence gene expression, reducing oxidative stress and inflammation and preventing cardiovascular diseases, cancers, and inflammatory diseases [28]. Research has found that organically grown onions have higher levels of phenolic substances, such as myricetin and quercetin-3-rhamnoside, than conventionally grown onions, but there is no significant difference in TF content. Additionally, organic carrots have higher phenolic acid 5-O-Caffeoylquinic acid (5-CQA) levels than conventionally cultivated carrots [30]. Similarly, in this study, organic cultivation carrots (OCs) had higher levels of TFs and total phenols than conventional cultivation carrots (CCs); however, the difference was only significant at 4 mg/mL ($p < 0.0001$). However, natural dream cultivation carrots (NCs) consistently revealed significantly higher levels of total phenols than OC and CC at all concentrations ($p < 0.05$). Furthermore, in the analysis of TFs, NC showed higher levels than OC and CC; however, the difference was not statistically significant. In this study, we observed that the contents of total phenols and total flavonoids did not increase proportionally with the escalation of carrot extract concentration. This phenomenon could be attributed to various factors, such as saturation effect, biological utilization efficiency, and chemical interactions. Further investigations into component content, utilizing techniques like LC-MS and Fourier-transform infrared spectroscopy (FTIR), are warranted to elucidate the underlying mechanisms and influential factors.

The DPPH radical scavenging activity is commonly used to evaluate antioxidant activity. When antioxidants react with the DPPH radical, it is neutralized, changing colors from purple to pale yellow. The DPPH radical scavenging rate of antioxidants may be measured by evaluating the level of color change, thereby indirectly assessing their antioxidant capacity. It has been demonstrated that organically grown tomatoes and Batavia lettuce exhibit higher DPPH radical scavenging activity than conventionally grown crops [31,32]. NC showed the highest DPPH radical scavenging activity among the three types of carrots, followed by OC. This suggests that natural dream cultivation enhances carrot phytochemical content more effectively than organic cultivation, thereby increasing carrot antioxidant activity.

Carrots have been suggested as an alternative therapy for the traditional medical treatment of leukemia, leading to more research into their potential anticancer properties [33]. As widely recognized, carrots are renowned for their abundant content of β-carotene. However, investigations have revealed that other carotenoids found in carrots are alphacarotene, lutein, zeaxanthin, and lycopene [34]. Besides its rich alfa- and beta-carotene contents, the root contains a wide range of bioactive compounds, including chlorogenic acids [35], quercetin, luteolin, kaempferol, myricetin [36], cyaniding, pelargonidin, and peonidin [37] as phenolic compounds; falcarinol, falcarindiol, and falcarindiol-3-acetate [38] as polyacetylenes; and vitamin C [34]. According to research, carotenoids have been clinically studied as an anticancer agent and found effective in animal models and humans. Beta-carotene is a very strong antioxidant compound because of its 11 conjugated double
bonds and a $\beta$-ring at each end of the chain, which may neutralize free radicals in lipophilic environments, including membranes [35]. Extracts obtained through hexane from red carrots have been found to have cytotoxic activity against human breast cancer cell lines (MCF-7) [39]. Similarly, ethanol or acetone extracts from black carrots have been shown to have cytotoxic activity against the same cancerous cell line. Therefore, the carrot samples in this study could potentially serve as a source for the extraction of carotenoids, which might be developed as targeted agents for cancer therapy in the future.

The inhibition of cancer cell growth is one of the key mechanisms underlying anticancer activity. Cancer cell control often involves regulating the cell cycle and promoting apoptosis [40]. p53, a tumor suppressor protein, is essential for preserving genomic stability and initiating cell apoptosis. p53 is activated in response to DNA damage or other stress conditions and initiates a series of responses, such as cell cycle arrest, DNA repair, and apoptosis induction [41]. p21, a cell cycle regulatory protein, is a downstream target gene of p53. p21 can inhibit cell cycle progression, prevent cell proliferation and DNA replication, and guide cells to apoptosis [42]. Bcl-2 family proteins associated with apoptosis include Bim, Bad, Bcl-2, Bcl-xL, Bax, and Bak. Bim and Bad belong to the BH3-only protein group, which is important in regulating cell apoptosis. Bim and Bad inhibit anti-apoptotic members of the Bcl-2 family, such as Bcl-2 and Bcl-xL, while releasing or activating pro-apoptotic proteins, such as Bax and Bak, within the cell. These proteins operate directly on the outer mitochondrial membrane, increasing the permeability of the mitochondrial outer membrane and the release of apoptotic factors, such as cytochrome C, eventually triggering cell apoptosis [43]. Caspase-9 and caspase-3 are key cysteine aspartate-specific protease family members that act as apoptosis executioners. Caspase-9 activation can activate caspase-3, resulting in a cascade of apoptosis-related reactions that eventually lead to cell apoptosis [44]. According to the study in [45], the water extract of purple carrot anthocyanins effectively inhibited the growth of HT-29 cells [46], whereas the extract of black carrot exhibited inhibitory effects on the proliferation of various human cancer cell lines, including MDA-MB-231 (human breast cancer), HT-29, and PC-3 (human prostate cancer) [47,48]. In this study, carrots demonstrated growth inhibitory effects on HT-29 cancer cells, particularly OC and NC. Moreover, NC exhibited improved cell cycle and apoptosis-related gene regulation in HT-29 cells compared with CC and OC. These results suggest that mineral-enriched organic cultivation improves carrot control over cancer cells and that a diet rich in NC may potentially prevent cancer growth.

Inflammation is the body’s immune response to injury, infection, or other stimuli, and it involves the production and interaction of various inflammatory mediators. Nitric oxide (NO) is a signaling molecule that plays a vital role in the pathogenesis of inflammation, and abnormal NO production is an important marker of the inflammatory response to LPS treatment [49,50]. Tumor necrosis factor-alpha (TNF-$\alpha$) is a central cytokine in the inflammatory response, and neutralizing TNF has been one of the most effective treatments for chronic inflammation and autoimmune diseases [51]. In mouse splenocytes, TNF-$\alpha$ promotes the synthesis and release of inflammatory mediators, such as IL-6 and IL-1$\beta$, which regulate immune responses. By binding to its receptor complex, IL-6 activates downstream signaling pathways, such as the Janus kinase/signal transducer and activator of the transcription (JAK/STAT) pathway and the mitogen-activated protein kinase (MAPK) pathway, thereby modulating the extent of the inflammatory response [52]. Similarly, when IL-1$\beta$ binds to its receptor (IL-1R), it activates myeloid differentiation primary response gene 88 (MyD88), which activates the NF-$\kappa$B and MAPK signaling pathways and the IFN response [53]. IFN-$\gamma$ regulates immune cell activation, cell proliferation, and antiviral responses in mouse splenocytes, and its production can be stimulated by the activation of immune cells, such as T cells and NK cells [54]. Additionally, IFN-$\gamma$ stimulates the production of IL-12 by activating dendritic cells and macrophages. IL-12 promotes Th1 cell development and IFN-$\gamma$ production [55]. IFN-$\gamma$ and IL-12 mutually regulate each other in mouse splenocytes, forming a positive feedback loop that enhances immune responses and inflammation [54]. Purple carrots inhibited the expression of pro-inflammatory cytokines.
TNF-α, IL-6, and IL-1β in mice in a colitis animal model [56]. Moreover, in vitro studies revealed that purple carrot extract reduces NO production and the expression of pro-inflammatory cytokines (IL-6, IL-1β), and TNF-α in LPS-induced macrophages [57]. Carrots also inhibited NO production and the release of inflammatory factors TNF-α, IL-6, IL-1β, IFN-γ, and IL-12 in LPS-induced mouse splenocytes, showing carrot’s ability to control inflammation. NC showed higher anti-inflammatory capability than CC and OC, making it a promising dietary therapy for treating and controlling chronic diseases.

Natural killer cells are important immune cells that perform multiple functions in mouse splenocytes. Cancer cells, particularly metastatic tumor cells, can be recognized and eliminated by NK cells. They release cytotoxic granules that trigger apoptosis in tumor cells when they detect abnormal cell surface signals, such as those lacking MHC-I molecule expression [58]. Additionally, NK cells regulate the balance of immune responses in the spleen [20]. They can modulate the function of other immune cells by secreting cytokines such as IFN-γ and IL-12 [54]. Furthermore, NK cells can regulate the intensity and duration of inflammatory responses. They can alleviate inflammation in certain situations by producing anti-inflammatory factors, thus protecting tissues from excessive inflammatory damage. Carrot pomace polysaccharide has been found in studies to increase the frequency of NK cells in the spleens of immunosuppressed mouse models [59]. This study shows that compared with CC and OC, NC significantly increases the activity of NK cells, which may be a reason for its anti-proliferative effects on cancer cells and its regulation of inflammatory responses.

In this study, our primary focus was on elucidating the positive attributes of organically cultivated and mineral-supplemented organically cultivated carrots; yet, latent limitations or confounding factors persist. Firstly, the significance of observed disparities in phenolic compound levels among carrots cultivated using different growth methodologies may not possess universal applicability, as additional variables, such as soil composition, climate, and cultivation practices, could also contribute to variations in plant chemical content. Subsequent investigations will strive to encompass a broader spectrum of species to achieve enhanced generalizability. Secondly, the potential anticancer and anti-inflammatory properties of carrots, as deduced from in vitro research outcomes, warrant further exploration through animal experiments to comprehensively investigate any plausible adverse effects or contraindications associated with their consumption. Furthermore, the specific bioactive substances within carrots and their impact on cellular conditions and behaviors were not comprehensively analyzed, and subsequent investigations will involve a more in-depth exploration utilizing instruments such as LC-MS, FTIR, and Confocal Laser Scanning Microscopy to address these aspects. Lastly, to prudently extrapolate implications for human health and disease management, further clinical trials are imperative.

5. Conclusions

Our study provides the first description of the anticancer and anti-inflammatory activities of conventionally cultivated, organically cultivated, and mineral-supplemented organically cultivated carrots, revealing the beneficial potential of mineral-supplemented organically cultivated carrots in the prevention and treatment of cancer and chronic inflammatory diseases. The inhibitory effects of carrots on cancer cell growth were validated using the HT-29 colon cancer cell model, which revealed that carrots regulate cell-cycle-arrest-related genes (p53 and p21) and cell-apoptosis-related genes (Bim, Bad, Bcl-2, Bcl-xL, Bax, Bak, caspase-9, and caspase-3). Additionally, carrots inhibited inflammation-controlled nitric oxide (NO) production and released inflammatory cytokines (TNF-α, IL-6, IL-1β, IFN-γ, and IL-12) in LPS-stimulated mouse splenocytes. Furthermore, compared with conventionally and organically cultivated carrots, natural dream carrot (NC) significantly increased the activation of NK cells. In conclusion, our study shows that NC is a high-quality vegetable with anticancer and anti-inflammatory properties, making it a viable, functional food, and the summarized mechanisms are depicted in Figure 10; however,
further testing of the mineral content of agricultural crops as well as animal and clinical data are required to support these findings.

**Figure 10.** Mechanistic summary of this study.

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