


# Anti-Inflammatory and Antioxidant Effects of Liposoluble C60 at the Cellular, Molecular, and Whole-Animal Levels

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**Introduction:** Liposoluble carbon-60 (C60) has potential applications in many fields, including cosmetics, medical devices, and medicine, but its specific mechanism of action remains unclear. This study explored whether liposoluble C60 could be delivered to human organs, tissues, and cells through blood, extracellular fluid, and cell culture fluid and whether it exerts anti-inflammatory and antioxidant effects at the molecular, cellular, and whole-animal levels.

**Methods:** At the cellular level, we mixed C60 dissolved in grape seed oil with cell culture medium containing 10% serum and investigated its effects on tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) release, migration, phagocytosis, respiratory burst, and apoptosis in freshly isolated human neutrophils. At the molecular level, we mixed a trace amount of C60 dissolved in grape seed oil with aqueous and ethanolic solutions and studied its antioxidant effect. At the animal level, we investigated the inhibitory effect of C60 on the serum inflammatory marker C-reactive protein (CRP) in beagle dogs after oral administration of C60 dissolved in grape seed oil.

**Results:** The results showed that the trace amount of C60 dissolved in grape seed oil significantly inhibited TNF- $\alpha$  release, cell migration, phagocytosis, and respiratory burst in freshly isolated human neutrophils. In addition, the trace amount of C60 dissolved in grape seed oil had a significant scavenging effect on superoxide free radicals and 1,1-diphenyl-2-trinitrophenylhydrazine free radicals. Oral administration of C60 dissolved in grape seed oil markedly reduced the level of the serum inflammatory marker CRP in beagle dogs.

**Conclusion:** In summary, a trace amount of hydrophobic C60 in hydrophilic media effectively produced anti-inflammatory and antioxidant effects in cells and animals. C60 dissolved in grape seed oil is a novel anti-inflammatory and antioxidant drug candidate.

**Keywords:** Carbon 60, anti-inflammation, anti-oxidation, C-reactive protein

## Introduction

Carbon-60 (C60 or fullerene) is a spherically structured molecule composed of 60 carbon atoms. It has a molecular weight of 720.<sup>1-3</sup> In 1985, astronomers used high-energy lasers to sputter graphite placed in a vacuum chamber environment in the laboratory, and accidentally discovered the soccer-ball-like C60.<sup>1-3</sup> In 1996, three scientists won the Nobel Prize in Chemistry for the discovery of this new material. C60 is a liposoluble substance. It can be dissolved in vegetable oils (including olive oil and grape seed oil) and is highly soluble in toluene and xylene. Although C60 is insoluble in water, it becomes water-soluble when more than 20 hydroxyl groups are introduced in it.<sup>1-3</sup>

C60 strongly absorbs free radicals, inhibits the toxicity of chemical toxicants, resists radiation, prevents ultraviolet damage, and prevents heavy metal-induced cell damage. It has potential applications in the fields of cosmetics, medical devices, and medicine.<sup>4,5</sup> C60, as an extremely potent antioxidant,<sup>6,7</sup> had shown important effects in the fields of skin beauty, hair follicle growth, and anti-aging.<sup>8-12</sup> Therefore, C60 has joined the list of cosmetics in Japan, Taiwan, the United States, and China. C60 is an antioxidant “genius” that has officially entered China’s beauty industry.<sup>6,7</sup> French scientists dissolved C60 in olive oil and fed

it to mice (4 mg of C60 per kg body weight) once every two weeks for 10–17 months. The results showed that the mice in the control group died in 17–38 months, while the life span of the mice in the experimental group was extended to 59–66 months. During the period, the experimental group showed no abnormalities in body weight or physiological manifestations.<sup>13</sup> Therefore, C60 is informally sold as a “health food” ingredient in the United States and the European Union.<sup>14</sup>

C60 dissolved in olive oil functions as a very strong antioxidant and has a therapeutic effect on inflammatory skin diseases, cancers, and intestinal diseases.<sup>11–18</sup> The authors of this study discovered that cosmetic-grade C60 dissolved in grape seed oil could quickly and noticeably relieve skin itching and pain in muscles, tendons, and joints when applied and massaged on the skin (patent applied for<sup>19</sup>). In this study, C60 dissolved in grape seed oil (3 mg/mL) was used as the research object, and its anti-inflammatory and antioxidant effects were investigated at the cellular, molecular, and whole-animal levels. Grape seed oil droplets containing a trace amount of C60 were mixed with culture medium supplemented with 10% bovine serum, and a series of cell-level experiments were conducted to measure the release of tumor necrosis factor  $\alpha$  (TNF- $\alpha$ ), migration, phagocytosis, respiratory burst, and apoptosis in freshly isolated human neutrophils. We next mixed the grape seed oil droplets containing a trace amount of C60 with aqueous and ethanolic solutions and investigated the antioxidant effect of the C60–grape seed oil at the molecular level. We mainly explored whether the trace amount of water-insoluble, liposoluble C60 could be delivered in water-soluble liquids and produce an anti-inflammatory effect at the cellular level and an antioxidant effect at the molecular level. Finally, we explored the effect of oral administration of C60–grape seed oil on C-reactive protein (CRP), a marker of inflammation, in the blood of dogs.

## Materials and Methods

### Materials

#### Experimental Samples

Human blood samples were obtained from the forearm venous blood of 18 healthy volunteers aged  $26 \pm 5$  years. The venous blood samples were obtained from total of six beagles (three males and three females, weighing  $7 \pm 2$  kg) under the same feeding conditions. The documented review and approval from a formally constituted review board (Ethics committee of Qingdao Agriculture University) as well as the written informed consent of all the 18 volunteers in accordance with the Declaration of Helsinki were obtained for collection of all the human blood samples and experimentation of the six beagles.

### Experimental Reagents

The following reagents were used in this study: C60 dry powder (Suzhou Dade Carbon Nano Technology Co., Ltd., China), grape seed oil (Pinli Food Co., Ltd., China), fetal bovine serum (FBS, Tianhang Biotechnology, China), penicillin (HyClone, USA), phorbol ester (PMA), lipopolysaccharide (LPS), agarose, molecular probe DHR123, vitamin E (Solarbio life sciences, China), ascorbic acid (Guangcheng Chemical, China), N-formyl-methionyl-leucyl-phenylalanine (fMLP, Shanghai PrimeGene Bio-Tech Company Ltd., China), carboxylate-modified polystyrene (latex beads) (Sigma-Aldrich, USA), Human Neutrophil Isolation Kit (Tianjin Haoyang Huake Biotechnology Co., Ltd., China), TNF- $\alpha$  ELISA kit (R&D Systems, USA), the Annexin V–FITC Cell Apoptosis Detection Kit (Beyotime Biotechnology, China), and RPMI1640 medium.

### Methods

#### Preparation of C60 Stock Solution

C60 dry powder with a purity of  $>0.99$  was dissolved in grape seed oil (hereinafter referred to as C60-Oil). To prepare C60-Oil stock solution with a concentration of 3 mg/mL, C60-Oil was placed in a magnetic stirrer and mixed thoroughly for 9 days at room temperature. The stock solution was sterilized by passing through a 0.22- $\mu$ M filter and then stored.

#### Dilution of the C60-Oil Stock Solution

The wrapping method. This method was used to investigate the release of TNF- $\alpha$ , phagocytosis of foreign-body material, and apoptosis in human neutrophils. The C60-Oil stock solution was mixed with RPMI1640 (10% FBS, 1% S/P),

resulting in a diluted C60-Oil suspension with a concentration of 20 µg/mL. The C60-Oil suspension was added to cell culture plates (100 µL each well). After repeated pipetting, 80 µL of the C60-Oil suspension was aspirated out, leaving 20 µL of C60-Oil on the bottom of the cell culture plate. Next, 180 µL of human venous blood neutrophils were added to each well. The final concentration of C60-Oil was 2 µg/mL in the culture medium ([Figure S1](#)).

The direct dilution method. This method was used to investigate the migration, cellular respiratory burst, and foreign-body material-induced respiratory burst of human neutrophils. The C60-Oil stock solution was mixed with RPMI1640 (10% FBS, 1% S/P) and human venous blood neutrophils into a C60-Oil suspension with a final concentration of 2–10 µg/mL.

## Isolation of Neutrophils from Human Venous Blood

The neutrophils were isolated using the Human Neutrophil Isolation Kit in accordance with the manufacturer's instructions. Cell morphology was examined after staining with a leukocyte classification stain. After counting, the neutrophils were adjusted to the desired density. In each experiment described below, blood was collected from three different volunteers.

## Quantification of the Release of TNF-α by Human Neutrophils

In this experiment, the wrapping method was used to treat the bottom surface of a 96-well cell culture plate. The density of human neutrophils was adjusted to  $5 \times 10^5$  cells/mL, and 180 µL of cell suspension was added to each well of the plate. The final concentration of the samples in the C60-Oil group was 2 µg/mL. After 24 h of culture, the cell supernatants were collected. The concentration of TNF-α was determined using a human enzyme-linked immunosorbent assay kit following the manufacturer's instructions.

## Examination of the Migration of Human Neutrophils

Five hundred microliters of 0.8% sterile agarose gel was mixed with 500 µL RPMI1640 (20% FBS, 1% S/P). The neutrophils were adjusted to a density of  $3 \times 10^8$  cells/mL, mixed thoroughly with the above mixture at a ratio of 1:1, and placed in a 37°C water bath. The neutrophil/agarose mixture was added to a precooled 96-well plate (2 µL each well), which formed droplets with a diameter of 2 mm on the bottom of the well. The droplets were kept at 4°C for 15 min. Cell migration was observed after treatment with either 1 ng/mL LPS in grape seed oil, 1 nM fMLP in grape seed oil, or 4 µg/mL C60-Oil for 3 h. The migration distance and area were calculated in ImageJ.

## Examination of the Phagocytosis of Foreign Bodies in Human Neutrophils

The wrapping method was used to treat the bottom surface of the 24-well cell culture plate. The human neutrophils were adjusted to a density of  $2 \times 10^6$  cells/mL, and 180 µL of the cell suspension was added to each well of the plate. C60-Oil and LPS were then added at the final concentrations of 2 µg/mL and 1 ng/mL, respectively. Then 3.5 µL of latex beads with a diameter of 2 µm were added to stimulate the neutrophils to achieve a fluorescent particle density of  $2 \times 10^7$ /mL, thereby establishing the optimal phagocytosis model of neutrophils and fluorescent particles. After 1 h at 37°C, the phagocytosis rate of the neutrophils was measured by flow cytometry.

## Respiratory Burst Assay of Human Neutrophils (Examination of the Production of Reactive Oxygen Species (ROS))

Human neutrophils were resuspended at a density of  $3 \times 10^6$  cells/mL, and 177 µL of the cell suspension was added to each well of a 24-well plate. Then, 3 µL of molecular probe DHR123 was added to the cells at a final concentration of 5 µM. After incubation of cells at 37°C for 15 min in the dark, C60-Oil was added at a final concentration of 2 µg/mL, 5 µg/mL, or 10 µg/mL. After 30 min at 37°C, 24 µL of PMA (final concentration, 50 nM) was added to stimulate cellular oxidative respiration. The cells were then incubated at 37°C for 30 min, rinsed once with phosphate-buffered saline (PBS), and resuspended in 800 µL of PBS. The production of ROS was examined by flow cytometry.

In the assay of the respiratory burst induced by foreign-body particles, cells were treated with C60-Oil and LPS at final concentrations of 2 µg/mL and 1 ng/mL, respectively. Finally, 3.5 µL of latex beads with a diameter of 2 µm were

added to the cells. After incubation at 37°C for 40 min in the dark, the cells were washed and resuspended in PBS. Again the amount of ROS was measured.

## Examination of the Apoptosis of Human Neutrophils

The wrapping method was used to treat the bottom of a 24-well cell culture plate, leaving 20  $\mu$ L of C60-Oil suspension in each well. Human neutrophils were resuspended and their cell density adjusted to  $1 \times 10^6$  cells/mL. Next, 180  $\mu$ L of the cell suspension was added to each well of the plate. C60-Oil and LPS were then added at respective final concentrations of 2  $\mu$ g/mL and 1 ng/mL. After 3 h, the cells were stained using the Annexin V-FITC Apoptosis Detection Kit following the manufacturer's instructions.

## Determination of the Superoxide Radical Scavenging Activity

C60-Oil was suspended in an aqueous solution containing a small amount of PBS, and the scavenging effect of C60-Oil on the superoxide anion radicals in the aqueous solution was examined. The total reaction volume was 5.14 mL (see [Table S1](#) for details). The control groups in this experiment included Oil, Vitamin E+Oil, and Vitamin C groups. The concentration of vitamin E dissolved in grape seed oil and the concentration of vitamin C dissolved in PBS were the same as that of C60.

## Determination of the Activity of 1,1-Diphenyl-2-Trinitrophenylhydrazine (DPPH) Free Radical Scavenging

C60-Oil was suspended in an ethanolic solution with PBS, and the scavenging effect induced by C60-Oil on the DPPH free radical in the PBS-containing ethanolic solution was examined. The total reaction volume in this experiment was 4 mL (see [Table S2](#) for details). The control groups of this experiment included Oil, Vitamin E+Oil, and Vitamin C groups. Vitamin E was soluble in grape seed oil, ethanol, and PBS-containing ethanolic solutions. Vitamin C was soluble in both PBS and ethanolic solutions with PBS. The concentrations of vitamin E and vitamin C in the solutions were the same as that of C60.

## Investigation of the Effect of Oral Administration of C60-Oil on CRP, a Marker of Inflammation, in Dog Blood

Three male and three female beagle dogs with similar spirit, appetite, body temperature, and other clinical manifestations were selected. The CRP levels in their venous blood were determined (Day 0). Every day for 9 days, 50 mL of C60-Oil at a concentration of 3 mg/mL was mixed with the dog food that the dogs were fed. The CRP levels were measured on the 3rd, 6th, and 9th days.

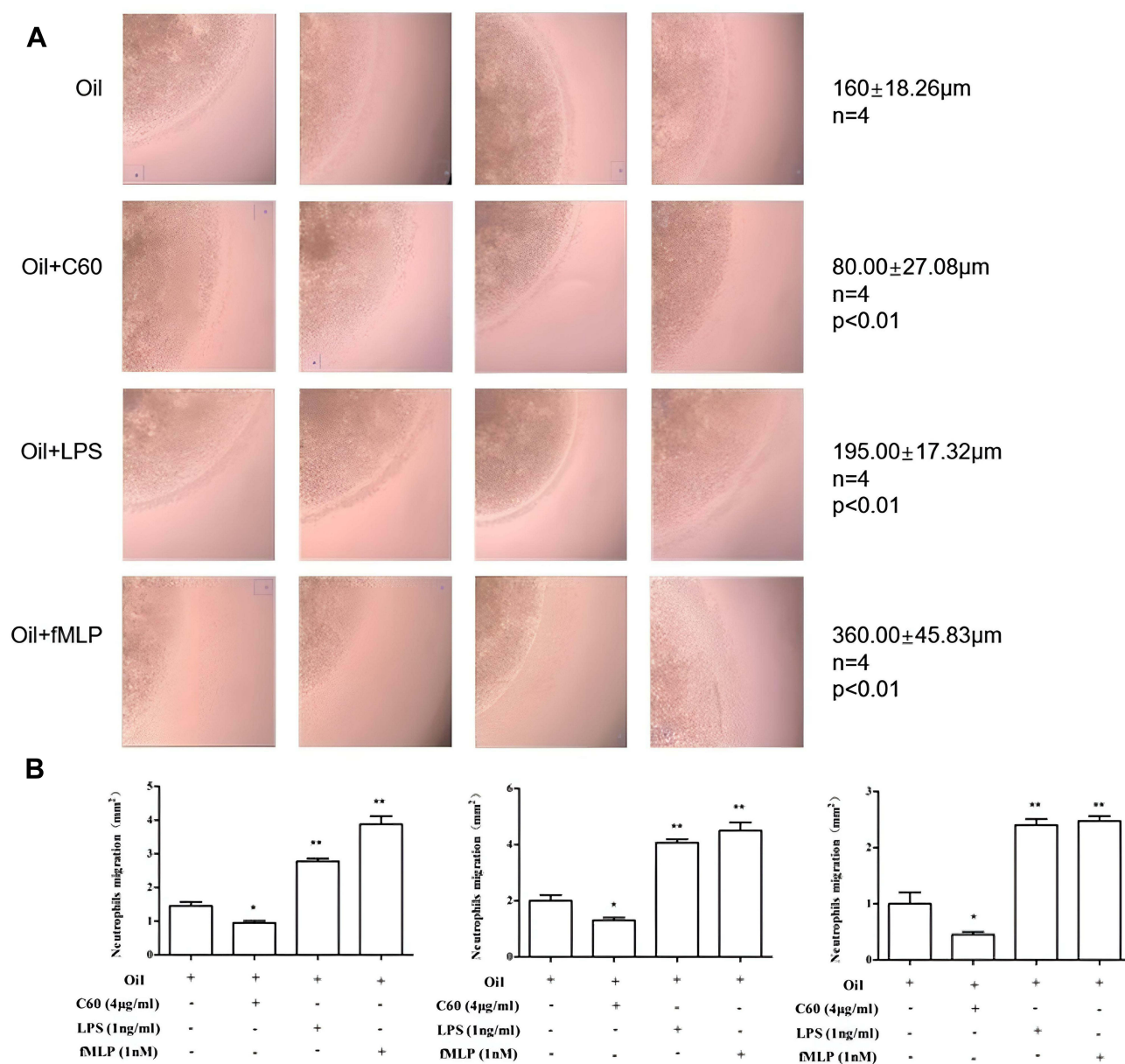
## Statistical Analysis

All data are expressed as  $\bar{x} \pm s$ . Two groups of data were compared by the paired-sample Student's *t*-test. \*  $P < 0.05$  and \*\*  $P < 0.01$  indicated significant differences and highly significant differences, respectively. Data analysis was conducted in GraphPad Prism 6.0, which was also used to output the tables and graphs.

## Results

### The Anti-Inflammatory Activity of C60-Oil at the Cellular Level

Neutrophils participate in tissue inflammatory responses by migrating to the inflammatory tissues. The migration of neutrophils is also involved in the tissue damage process in inflammatory diseases. This study employed a method of mixing neutrophils with agarose gel to investigate the effect of C60-Oil on the migration of freshly isolated human neutrophils. The results showed that the area and distance of neutrophil migration from the inside to the outside of the agarose gel droplets were lower in the C60 group than the Oil group ( $P < 0.05$ ,  $n=4$ ). In contrast, the migration area and distance of neutrophils were significantly increased in the LPS and the fMLP positive control groups ( $P < 0.01$ ,  $n=4$ ) ([Figure 1](#)). These results indicate that the C60 dissolved in grape seed oil entered the agarose gel droplets through cell

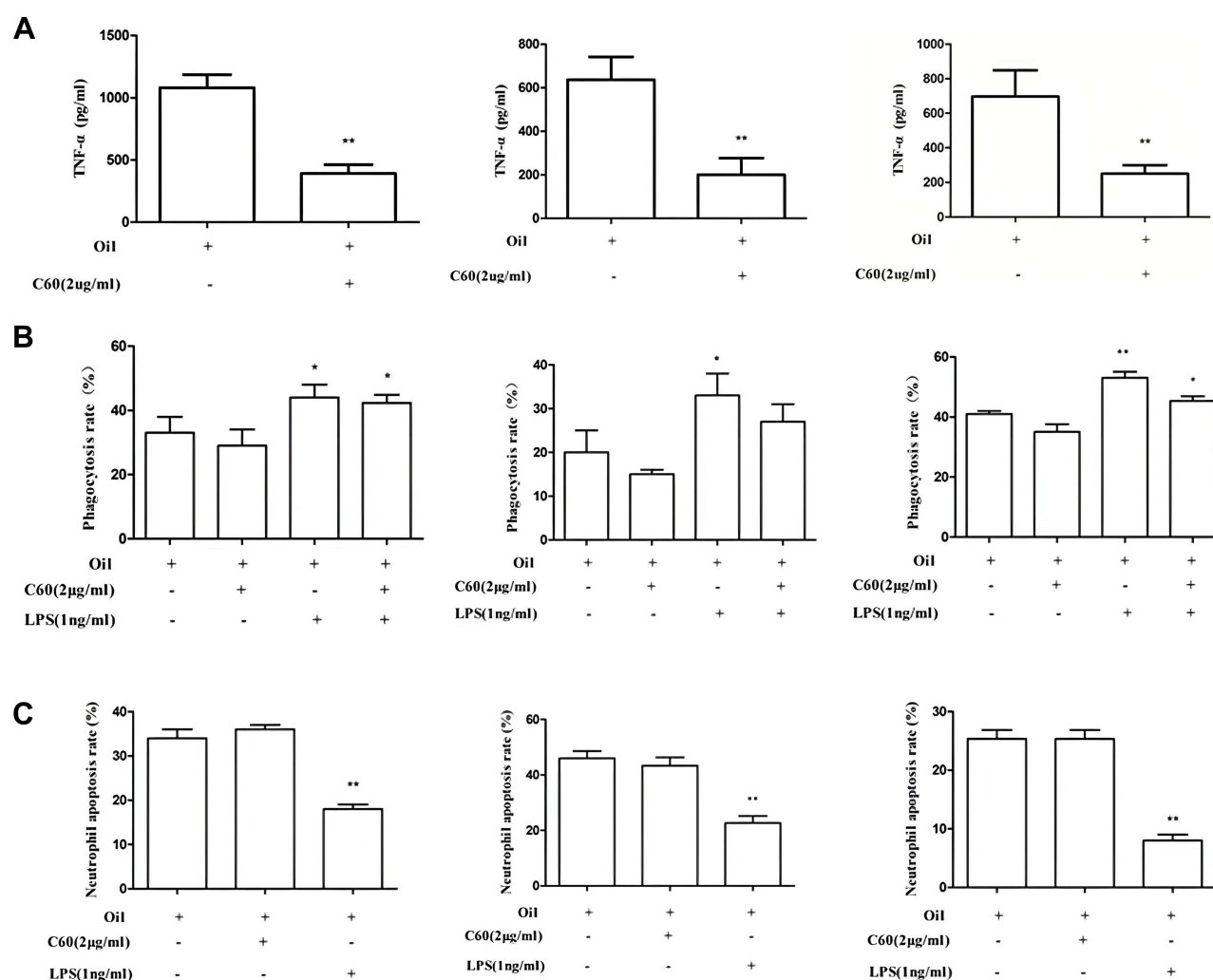


**Figure 1** The effect of C60-Oil on the migration of human neutrophils. **(A)** Examination of the inhibitory effect of C60 dissolved in grape seed oil on the migration of human neutrophils under a microscope (4 $\times$ ) ( $\bar{x} \pm s$ ,  $n=4$ ). Migration distance is in  $\mu\text{m}$ . **(B)** The effect of C60-Oil on the migration of human neutrophils ( $\bar{x} \pm s$ ,  $n=3$ ). The three groups represent the results of examining the blood samples derived from different volunteers. \*  $P < 0.05$  vs the Oil group, while \*\*  $P < 0.01$  vs the Oil group.

culture medium and inhibited the migration of freshly isolated human neutrophils. In addition, compared with the Oil group, the C60-Oil group had less  $\text{TNF-}\alpha$  release by neutrophils (Figure 2A). These results demonstrate that C60 significantly inhibited the release of  $\text{TNF-}\alpha$  by freshly isolated neutrophils.

Human neutrophils can be activated by LPS to phagocytize damaged tissue fragments, foreign microorganisms, and foreign bodies. This study examined the ability of the freshly isolated human neutrophils to phagocytize exogenous fluorescent particles. C60-Oil reduced the phagocytic rate of neutrophils regardless of whether LPS was present (Figure 2B). This finding indicated that C60-Oil could enter neutrophils and inhibit their phagocytic function whether or not they were activated. In addition, when C60-Oil was cocultured with the freshly isolated neutrophils, it had no effect on neutrophil apoptosis (Figure 2C).

The inflammatory cell activator PMA induces respiratory burst in neutrophils, which produces a large amount of ROS. This study explored the effect of C60-Oil on respiratory burst in freshly isolated human neutrophils. C60 dissolved



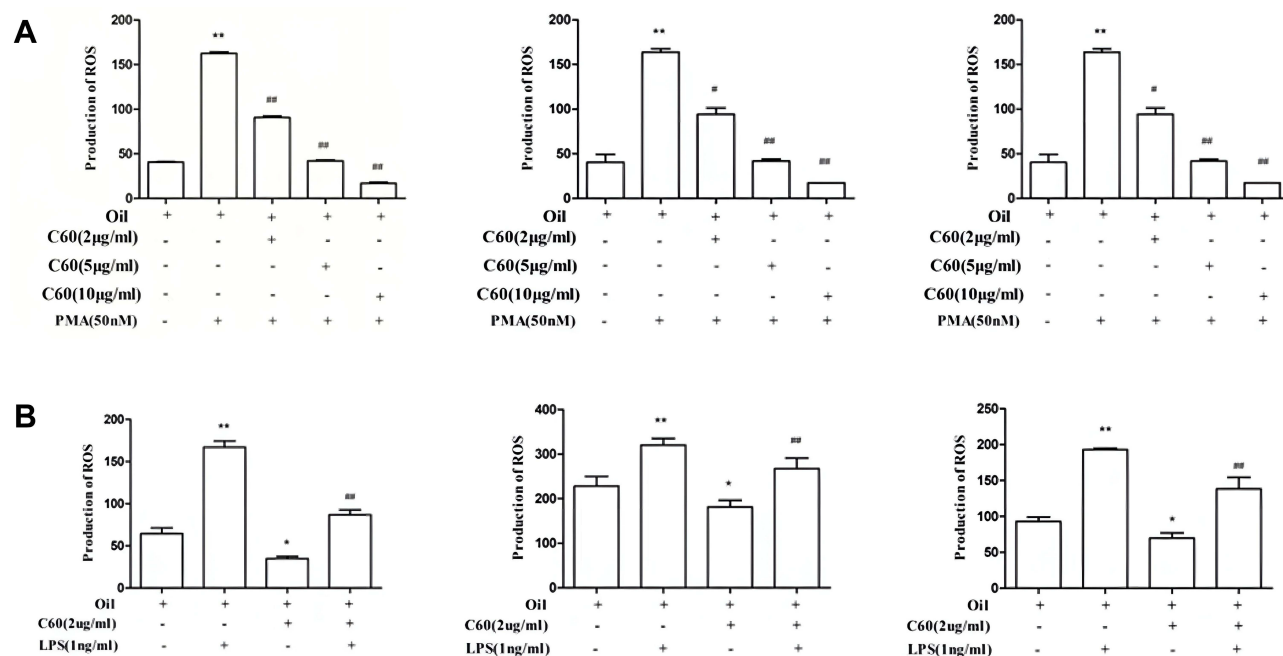
**Figure 2** Effect of C60-Oil on the release of TNF- $\alpha$ , phagocytosis, and apoptosis by human neutrophils. **(A)** Effect of C60-Oil on the release of TNF- $\alpha$  by human neutrophils ( $\bar{x} \pm s$ ,  $n=3$ ). The results were obtained by examining blood samples from three different volunteers. \*  $P < 0.05$  vs the Oil group, \*\*  $P < 0.01$  vs the Oil group. **(B)** Effect of C60-Oil on the phagocytic activity of human neutrophils ( $\bar{x} \pm s$ ,  $n=3$ ). \*  $P < 0.05$  vs the Oil group, \*\*  $P < 0.01$  vs the Oil group. **(C)** Effect of C60-Oil on the apoptosis of human neutrophils ( $\bar{x} \pm s$ ,  $n=3$ ). \*  $P < 0.05$  vs the Oil group, \*\*  $P < 0.01$  vs the Oil group.

in grape seed oil (2–10  $\mu\text{g/mL}$ ) inhibited the PMA-induced ROS production by neutrophils in a dose-dependent manner (Figure 3A). Since foreign particles such as latex beads stimulate respiratory burst in freshly isolated neutrophils, we also explored the effect of C60-Oil on the foreign particle-induced respiratory burst in freshly isolated human neutrophils. The results showed that C60-Oil not only inhibited the foreign particle-induced ROS production in neutrophils but also inhibited their LPS-stimulated ROS production (Figure 3B). These results demonstrate that the C60 dissolved in grape seed oil entered neutrophils through cell culture fluid and inhibited the respiratory burst in neutrophils induced by two foreign particles, latex beads and LPS.

In summary, C60 in grape seed oil can enter neutrophils through cell culture fluid to inhibit the ability of neutrophils to migrate, phagocytize, and release TNF- $\alpha$ . In addition, C60 inhibits the ability of neutrophils to generate ROS and induce respiratory burst after stimulation. However, C60 does not affect the apoptosis of neutrophils.

## The Antioxidant Activity of C60-Oil at the Molecular Level

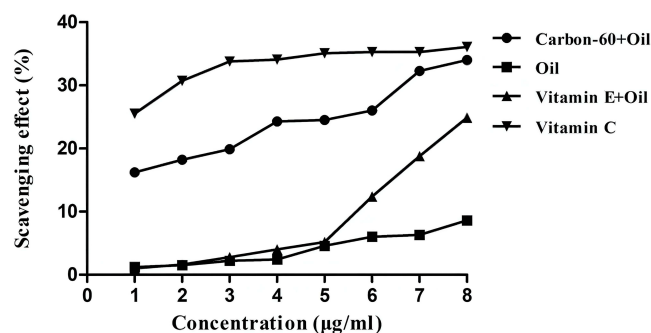
C60 is insoluble in water and PBS. However, the scavenging effect of C60-Oil suspended in PBS on superoxide radicals was dose dependent (Table S3). This result demonstrated that C60 entered the PBS solution through an unknown mode that did not involve dissolution and exerted its scavenging effect on the superoxide free radical ( $\text{O}_2^-$ ). Furthermore, this



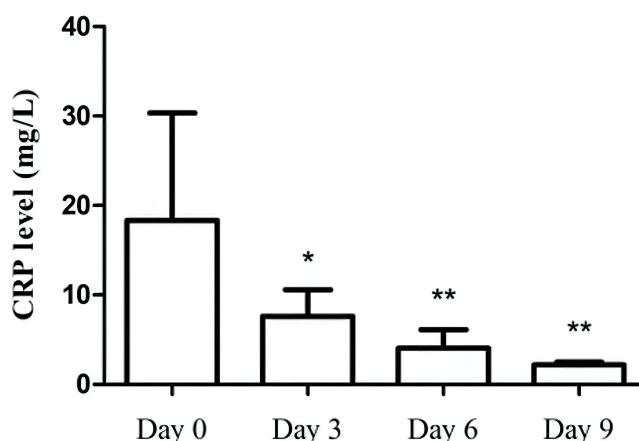
**Figure 3** Effect of C60-Oil on respiratory burst in human neutrophils. **(A)** Effect of C60-Oil on respiratory burst in human neutrophils ( $\bar{x} \pm s$ ,  $n=3$ ). \*\*  $P < 0.01$  vs the Oil group; #  $P < 0.05$  vs the PMA group; ##  $P < 0.01$  vs the PMA group. **(B)** Effect of C60-Oil on the foreign particle-induced respiratory burst in human neutrophils ( $\bar{x} \pm s$ ,  $n=3$ ). \*  $P < 0.05$  vs the Oil group; \*\*  $P < 0.01$  vs the Oil group; #  $P < 0.05$  vs the LPS group; ##  $P < 0.01$  vs the LPS group.

result suggested that the cytobiological effect of C60-Oil on the freshly isolated human neutrophils was related to its antioxidant activity. The grape seed oil droplets alone had no ability to scavenge superoxide. Vitamin E is insoluble in water and PBS. Therefore, vitamin E-grape seed oil droplets suspended in PBS had no ability to scavenge superoxide. In contrast, the water-soluble vitamin C did scavenge superoxide.

C60 is insoluble in ethanolic reaction solution containing PBS. The experimental results of this study showed that, in ethanol reaction solution containing PBS, C60 dissolved in grape seed oil exerted a scavenging effect on DPPH free radicals in a dose-dependent manner (Figure 4). In other words, C60 entered the PBS-containing ethanolic reaction solution through an unknown transfer mode that did not involve dissolution and exerted a scavenging effect on DPPH free radicals, indicating that the molecular mechanism of the anti-inflammatory biological effect of C60-Oil on the freshly isolated human neutrophils was related to its antioxidant effect. Grape seed oil droplets alone had no ability to scavenge DPPH. Vitamin E is soluble in ethanol. Therefore, vitamin E-grape seed oil droplets had the ability to scavenge DPPH at the high concentrations of 6–8  $\mu\text{g/mL}$ . Vitamin C can be dissolved by PBS. Therefore, in an ethanolic solution containing PBS, vitamin C also exerted a scavenging effect on DPPH in a dose-dependent manner.



**Figure 4** Scavenging effect of C60-Oil on DPPH free radicals.



**Figure 5** Oral administration of C60-Oil reduces the level of the blood inflammation marker CRP in beagle dogs. Day 0, Day 3, Day 6, and Day 9 represent before treatment, treatment for 3 days, treatment for 6 days, and treatment for 9 days, respectively ( $\bar{x} \pm s$ ,  $n=6$ ). \*  $P < 0.05$  vs Day 0, \*\*  $P < 0.01$  vs Day 0.

## The Effect of Oral Administration of C60-Oil on the Inflammatory Marker CRP in Canine Blood

After 3 days of oral administration of C60-Oil, the dogs' serum CRP level was decreased drastically and continued to decline in the following 6 days (Figure 5). These results show that the C60 dissolved in grape seed oil entered the Beagle's body and significantly reduced the serum level of the inflammatory marker CRP. That is, C60-Oil had a therapeutic effect on inflammation.

## Discussion

Under normal conditions, human neutrophils are inactive in blood vessels and do not participate in immune or inflammatory responses. Once activated, human neutrophils migrate to the sites of microbial invasion and inflammation, where their effect is a double-edged sword. Neutrophils not only eliminate the invading pathogenic microorganisms but also participate in the pathological process of inflammatory diseases. For example, ROS released by the activated neutrophils are involved in inflammatory atherosclerosis and brain tissue injury.<sup>20</sup> Activated neutrophils migrate to the skin and participate in the pathological processes of inflammatory diseases such as papules and ulcers.<sup>21</sup> The above studies suggest that inhibition of the activation and inflammation-promoting effect of neutrophils is a potential treatment for inflammatory diseases.

C60 is insoluble in aqueous solutions, including blood, interstitial fluid, and cell culture fluid. Therefore, we dissolved C60 in grape seed oil and diluted this with serum-containing cell culture medium and wrapped it on the surface of the cell culture medium, forming oil droplets (Figure S1). We also directly diluted the C60-Oil with R1640 cell culture containing 10% bovine serum to form a cell culture liquid containing oil droplets.

The experimental results showed that C60-Oil inhibited TNF- $\alpha$  release, migration, phagocytosis, and ROS production by human neutrophils (Figures 1–3). However, C60-Oil had no significant effect on neutrophil apoptosis or death (Figure 2C). Such results indicate that C60-Oil exerts an inhibitory effect on the immune activity of neutrophils and has no cytotoxic activity. The amount of C60 dissolved in grape seed oil was 2–10  $\mu\text{g/mL}$ , while the concentration of C60 that entered the cultured neutrophils was even lower. This finding suggests that a trace amount of the water-insoluble, liposoluble C60 was effectively delivered in an unknown way and produced an anti-inflammatory effect at the cellular level. This study suggests that liposoluble C60 might be administered through the mucous membrane and the skin to treat the inflammatory diseases related to the release of TNF- $\alpha$ , migration, phagocytosis, and production and release of ROS in human neutrophils. This study used freshly isolated human blood neutrophils, which eliminated the species differences caused by the use of small animals in drug studies and makes our study one step closer to a human clinical trial. At present, there is no report that the liposoluble inorganic C60 used in cosmetics has side effects on the human body.

The water-soluble derivatives of C60 with introduced hydroxyl groups represent a promising therapeutic agent to control ROS-dependent inflammation (including allergic diseases).<sup>22</sup> The water-soluble derivatives of C60 may enhance the enzyme activity of superoxide dismutase in microglia, which is involved in inflammatory pain.<sup>23</sup> The results of this study showed that C60-Oil had a significant scavenging effect on superoxide free radicals and DPPH free radicals, which effects were dose dependent (Table S3, Figure 4). These results indicate that C60 can be effectively transferred at the molecular level in water-soluble liquids, and the free radical-scavenging effect of C60-Oil is related to the anti-inflammatory mechanism of C60-Oil at the cellular level.<sup>24</sup> Clinical drug studies suggest that at the molecular level, transfer of water-insoluble corticosteroids in aqueous liquids is similar to the transfer of C60 dissolved in grape seed oil. In cellular-level studies, corticosteroids are generally dissolved in ethanol first. The corticosteroids dissolved in ethanol will precipitate out tiny crystals after dilution with cell culture fluid, which dissolve into the cell membrane and produce cytological effects. In contrast, C60-Oil does not precipitate out tiny crystals. After entering the human body, C60 binds to blood albumin and is transported in the body, as corticosteroids are.<sup>25–27</sup> In addition, the C60 dissolved in vegetable oil can directly enter cell membranes<sup>28–30</sup> and the human body.<sup>15,31,32</sup> The C60 dissolved in vegetable oil can also enter the human blood circulation after being phagocytized by cells.<sup>32</sup> One study has reported on one of the anti-inflammatory mechanisms of C60: C60 may attract human neutrophils and induce them to phagocytize it after entering the human body, rendering the neutrophils unable to damage human tissues and cause diseases.<sup>15</sup> The interaction between liposoluble C60 and neutrophils in water-soluble liquids is a new scientific link between physics and cell biology. This study found, through the clear inflammatory cell-regulating function of liposoluble C60 at the cellular level and the antioxidant effect of C60 at the molecular level, that the water-insoluble liposoluble C60 is effectively transferred to cells in water-soluble liquids through an unknown mode.

CRP is a serum marker of inflammation. CRP is synthesized in the liver and released into the blood. Inflammatory cells release inflammatory factors to stimulate the synthesis of CRP and the release of CRP into the blood.<sup>33,34</sup> Plasma CRP is significantly increased during acute inflammation (such as that caused by infection, trauma, tissue necrosis, malignant tumors, skin infection, cystitis, bronchitis, and allergic reactions). Plasma CRP is slightly increased in chronic inflammation (such as atherosclerosis, type 2 diabetes, obesity, periodontal disease, colitis, and rheumatoid arthritis).<sup>35–37</sup> However, the level of CRP will decrease rapidly after the inflammation subsides.<sup>38</sup> This study explored the effect of oral administration of C60-Oil on the blood CRP level in beagle dogs. Their serum CRP level showed a continuous declining trend after treatment (Figure 5). Elevation of serum CRP is common in cardiovascular and cerebrovascular diseases.<sup>39</sup> Oral administration of C60-Oil strongly reduced the serum CRP of the dogs, suggesting that C60 may have a therapeutic effect on cardiovascular and cerebrovascular diseases related to elevated CRP.

## Conclusion

In summary, a trace amount of hydrophobic C60 in hydrophilic media effectively produced anti-inflammatory and antioxidant effects in cells and animals. C60 dissolved in grape seed oil is a novel anti-inflammatory and antioxidant drug candidate. How water-insoluble C60 was effectively transferred at the molecular level in water-soluble liquids should be further studied.

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## Disclosure

The authors report no conflicts of interest in this work.

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