

Cold Air Plasma Inhibiting Tumor-Like Biological Behavior of Rheumatoid Arthritis Fibroblast-Like Synovial Cells via G2/M Cell Cycle Arrest

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Background: Rheumatoid arthritis fibroblast-like synovial cells (RA-FLS) have become the core effector cells for the progression of rheumatoid arthritis due to their “tumor-like cell” characteristics, such as being able to break free from growth restrictions caused by contact inhibition, promoting angiogenesis, invading surrounding tissues, and leading to uncontrolled synovial growth. In recent years, cold air plasma (CAP) has been widely recognized for its clear anticancer effect. Inspired by this, this study investigated the inhibitory effect of CAP on the tumor-like biological behavior of RA-FLS through in vitro experiments.

Methods: Treatment of RA-FLS with CAP at different time doses (0s, 30s, 60s, 120s). 5-ethynyl-2'-deoxyuridine (EdU) proliferation assay was used to determine the cell viability. Analysis of cell migration and invasion was performed by wound-healing assay, transwell assay and immunofluorescent staining for f-actin, respectively. Flow cytometry technique was used for analysis of cell cycle and determination of reactive oxygen species (ROS). Hoechst staining was used for analysis of cell apoptosis. Protein expression was analyzed by Western blot analysis.

Results: Molecular and cellular level mechanisms have revealed that CAP blocks RA-FLS in the G2/M phase by increasing intracellular reactive oxygen species (ROS), leading to increased apoptosis and significantly reduced migration and invasion ability of RA-FLS.

Conclusion: Overall, CAP has significant anti proliferative, migratory, and invasive effects on RA-FLS. This study reveals a new targeted treatment strategy for RA.

Keywords: cold air plasma, rheumatoid arthritis fibroblast-like synovial cells, proliferation, apoptosis, migration

Introduction

Rheumatoid arthritis (RA) is a common chronic inflammatory and progressive autoimmune disease with an unknown etiology.^{1,2} Although the onset of RA involves systemic immune dysregulation, the clinical manifestations are primarily synovial inflammation and joint injury, which usually begin with small joints affecting symmetrical hands and feet, followed by the involvement of large joints. RA eventually causes joint injury and dysfunction.³ Today, the global prevalence of RA is about 0.5–1%, with more women suffering from the disease, and the incidence rate and prevalence may be increasing.^{4,5} At the moment, the joint targeted treatment for RA is anti-inflammatory drugs and immune-targeted disease-modified anti-rheumatic drugs, which have numerous side effects and a poor prognosis.⁶ To improve treatment efficiency, RA now requires more targeted, specific, and minimally invasive therapies.

RA is distinguished by persistent synovial hyperplasia and progressive articular cartilage erosion. RA-FLS exhibits distinct aggressive behavior and is important in disease pathogenesis and progression.⁷ In chronic inflammatory environments, the number of RA-FLS activated increases significantly and becomes the major component of destructive pannus, exhibiting anti-apoptotic and aggressive characteristics similar to tumor cells.⁸ The pathological basis of RA is generally thought to be excessive proliferation and insufficient apoptosis of RA-FLS.⁹ Furthermore, RA-FLS mediates the overexpression of matrix metalloproteinase (MMPs), which can be “metastasized” to distant joints in vivo, potentially spreading the disease from one joint to the next and exacerbating joint damage.¹⁰ Finding pathways and targets to inhibit the proliferation and migration of RA-FLS is therefore of great scientific importance.

Cold air plasma (CAP) is an ionized neutral gas that is primarily made up of reactive oxygen species (ROS), reactive nitrogen species (RNS), electrons, ions, and ultraviolet photons.¹¹ CAP is used in the medical field because it is close to room temperature. CAP has demonstrated clear anticancer potential in vitro and in vivo in a large number of studies over the last ten years.¹² According to current research, CAP’s anti-cancer effect is primarily based on the production of ROS in cells. CAP destroys cells in cancer cells, and many basic cellular reactions can be observed in treated cancer cells, such as growth inhibition, cytoskeleton damage, cell cycle arrest, nuclear and DNA damage, mitochondrial damage, cell apoptosis, selective increase of intracellular ROS and autophagy, etc.^{13–15} As a result, CAP may be a viable treatment option for RA.

CAP has been used in human medicine for cancer treatment, but not yet for RA. Although some studies have looked into the effects of CAP on RA, the underlying molecular mechanisms remain unknown. In this study, we looked at the role of CAP in the tumor-like biological behavior of RA-FLS, as well as the potential mechanisms. This research could pave the way for the use of CAP in the treatment of RA.

Materials and Methods

Dielectric Barrier Discharge (DBD) Device

The classic dielectric barrier discharge along the surface is used as the cell intervention device in this study.¹⁶ As shown in Figure 1A, the design process is as follows: the insulating dielectric layer and discharge electrode on one side of the dielectric barrier discharge are created using micro-arc oxidation technology, and then the discharge electrode on the other side of the created insulating dielectric layer is created using film coating technology, resulting in the creation of a tightly fitting and integrated dielectric barrier discharge electrode. The plasma generated spreads uniformly along the dielectric surface.

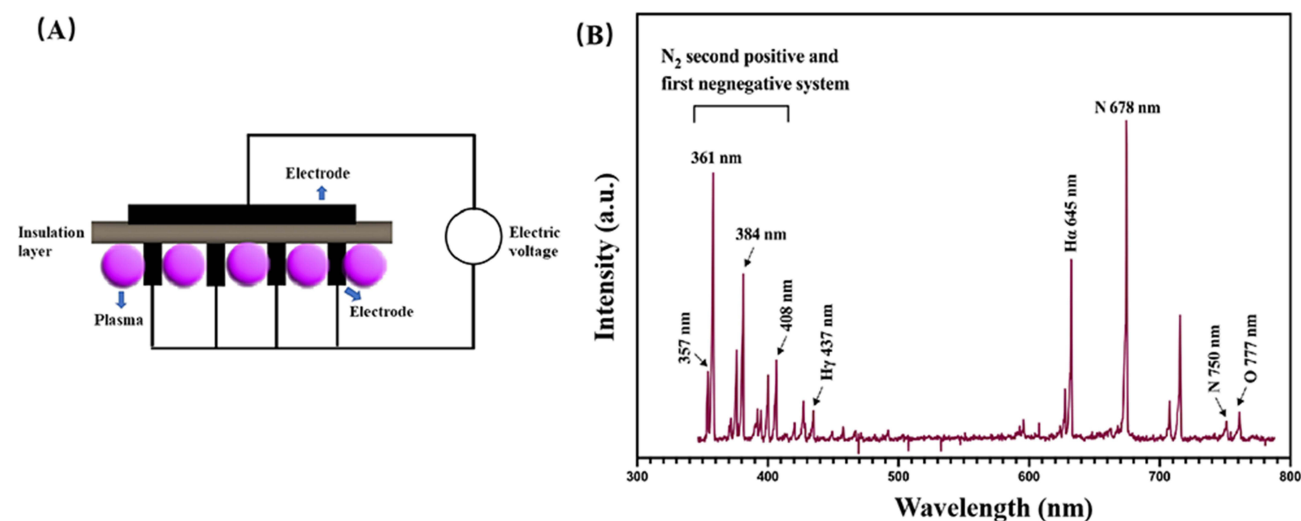


Figure 1 Illustration of dielectric barrier discharge (DBD) device.

Notes: (A) Illustration of dielectric barrier discharge (DBD) device. (B) Optical emission spectroscopy spectrum of DBD plasma at wavelengths ranging from 350 to 800 nm.

Cell Culture and Treatment

Shanghai Biyuntian Biotechnology Co., Ltd. provided the RA-FLS.¹⁶ According to the experimental protocol, cells were divided into four groups: control group, CAP30 group, CAP60 group, and CAP120 group (CAP treatment time of 0s, 30s, 60s or 120s). The cells were inoculated in a 6-well culture plate. After the cells were cultured overnight, the CAP treatment was carried out (the action height was 5 mm higher than the upper part of the culture plate, the voltage was 6 kV~8 kV, and the current was 3 mA). After the treatment, the cells continued to be cultured for 24 h, and then the subsequent experiments were carried out.

5-Ethynyl-2'-Deoxyuridine (EdU) Proliferation Assay

RA-FLS was seeded in a 6-well plate medium and cultured to 90%. The cells were grouped together and subjected to passaging, and cells were transfected after cell wall attachment. After transfection, each group of cells received a pre-warmed EdU staining solution (Kane Biologicals, China, KGA331) at a final concentration of 10 M and were incubated at 37°C in a 5% CO₂ cell incubator for 2 h. The medium was discarded, and the cells were fixed in 4% paraformaldehyde for 15 min at room temperature, then the cells were washed twice with PBS containing 3% BSA. After removing the washing solution, 0.1 mL 0.5% Triton X-100 in PBS was added to each well and incubated at room temperature for 20 min. After washing the cells twice, the Click-iT reaction solution was added. The cells were then incubated at room temperature in the dark for 30 min. The cells were washed twice and stained for 5 min with 4', 6-diamino-2-phenylindole (DAPI). Two washes were performed on the cells. The fluorescence microscope was used to examine cell morphology.¹⁷

Hoechst Staining

After each cell group was treated, the culture fluid was aspirated. 0.5 mL of fixative was added, and the cells were fixed for 10 min overnight at 4 °C. After removing the fixative, the cells were washed twice with PBS for 3 min each time, with the goal of aspirating the liquid. The cells were stained with 0.5 mL Hoechst 33,342 staining solution (Wanleibio, China, WLA042) for 5 min. The cells were washed twice in PBS for three min each time before being sealed with an anti-quenching blocking solution. The morphology of the cells was observed using a fluorescent microscope.¹⁸

Wound-Healing Assay

Groups of cells were cultured until the density was fused. The medium was changed to serum-free medium and treated with 1 µg/mL mitomycin C (Sigma, USA, M0503) for 1 h before the experiment. 200 µL pipette tip was used to cause cell scratching for each group of cells, and the cell surface was washed once with serum-free medium to remove cell debris. The cells of each group were incubated in an incubator at 37 °C and 5% CO₂ for 0h and 24 h, respectively, and then photographed and recorded. The migration area of each experimental group was calculated.¹⁹

Transwell Assay

The transwell chamber method was used to assess the invasive ability of CAP-treated RA-FLS. First, the transwell invasion experiment small chamber model was established. The Matrigel gel (Corning, USA, 354,234) was thawed overnight at 4 °C. The Matrigel gel was placed on ice and diluted 1:3 with serum-free medium. Transwell chamber was taken out and put into a 24-hole plate. 40 µL Pre-diluted Matrigel gel was coated on the small chamber membrane and placed in a 37 °C incubator for 2 h to coagulate the gel. A transwell invasion test was immediately performed, and the cells in each group grew to approximately 90% of their original size. The cells were washed with PBS, digested with 0.25% trypsin, and then placed in serum-free medium. Each group's cells were blown off the culture plate and dispersed in a single cell suspension. The cells were counted and then diluted into a standby cell suspension. The encapsulated transwell chambers were placed in a 24-well plate, and 800 µL of culture medium containing 10% FBS was added to the lower chamber; 200 µL of cell suspension was added to the upper chamber, with a total of 3×10^4 cells/well. The 24-well plates were incubated in a cell culture incubator at 37 °C, 5% CO₂, and saturated humidity for 24 h. Then, the transwell chambers were fixed and stained by washing them twice with PBS. Four percent paraformaldehyde (aladdin, China, C104188) was fixed at room temperature for 25 min. About 0.4% crystalline violet staining solution (Amresco, USA,

0528) was stained for 5 min and rinsed with distilled water. Finally, the cells that invaded the lower layer of the microporous membrane were counted under an inverted microscope and the mean number was taken.¹⁹

Immunofluorescent Staining for F-Actin

Each cell crawl was fixed in 4% paraformaldehyde for 15min. To remove the 4% paraformaldehyde, crawl pieces were immersed in PBS for 5 min × three times. About 0.1% triton X-100 (Beyotime, China, ST795) was added to the cells until the cells were completely covered and incubated at room temperature for 30 min. To remove 0.1% triton X-100, cell crawl pieces were submerged in PBS for 5 min × three times. Anti-stain™488 Fluorescent Phalloidin (Solarbio, China, CA1610) was dripped, diluted with PBS at 1:100, and then dripped until the cells were completely covered for incubation. The cell crawl pieces were immersed in PBS for 5 min × three times to washout primary antibody. DAPI (Aladdin, China, d106471-5 mg) was added dropwise to completely cover the cells to counterstain the nuclei. The cell crawl pieces were immersed in PBS for 5 min × 3 times to remove DAPI. Adhesive tip drops were added half a drop of antifluorescence quencher (Solarbio, China, s2100) on the slide, cell crawl pieces were buckled onto the slide with antifluorescence quencher drop. The staining effect was observed under a fluorescence microscope.²⁰

Cell Cycle

Groups of cells were collected and then centrifuged. PBS was used to wash the cells, and the supernatant was carefully aspirated. Pre-chilled 70% ethanol was added to the cells and fixed overnight at 4 °C. The fixed cells were centrifuged at 106 g for 3 min. The supernatant was discarded, and the cells were washed twice with PBS. Following that, 100 µL of RNase A was added, and the cells were bathed in 37°C water for 30 min. 500 µL of Propidium Iodide (PI) (Wanleibio, China, WLA010a) staining solution was added, and the cells were protected from light for 30 min at 4°C. Flow cytometry (Aceabio, USA, NovoCyte) was then used to analyze the distribution of cell cycle phases.²¹

ROS

The cells were inoculated in 6-well plate medium. Cells were incubated overnight before being treated with CAP. After treatment, the cells were continued to be cultured in a cell incubator at 37 °C and 5% CO₂ saturated humidity for 24 h. After reaching the time, each group of cells was collected and washed twice with PBS. The cells were incubated in an incubator at 37 °C for 20 min, inverting and mixing every 5 min, with 1 mL of DCFH-DA (Wanleibio, China, WLA131) dilution (1:1000 in medium). Cells were washed 3 times with PBS to fully remove any DCFH-DA that had not entered the cells. 500 µL of PBS was used to resuspend the cells, and then flow cytometry (Aceabio, USA, NovoCyte) was used for flow detection.¹⁹

Western Blot Analysis

Ripa buffer was used to lyse the cells, which were then centrifuged (4 °C, 12,000 rpm, 10 min) and the supernatant was absorbed to obtain cellular proteins. The total proteins were then separated by SDS-PAGE and transferred to PVDF membranes. The PVDF membranes were immersed in milk solution and sealed for 2 h at room temperature. Next, PVDF membranes were completely immersed in MMP3 (Abclonal, China, A11418), MMP9 (Wanleibio, China, WL03096), P21 (Wanleibio, China, WL0362), CyclinB1 (Wanleibio, China, WL01760), CDK1 (Wanleibio, China, WL02373) in primary antibody working solution, incubated overnight at 4°C, and then washed with TBST for 15 min × 3 times. The PVDF membranes were then immersed for 1 h in sheep anti-rabbit IgG-HRP working solution (secondary antibody: sealed solution = 1:5000), followed by 3 washes with TBST. Finally, the ECL kit was used to perform the assay, and -actin was used as an internal reference for quantitative analysis. ImageJ software was used to calculate protein content.¹⁹

Statistical Analysis

All experimental data were recorded in the form of mean standard deviation (SD). SPSS 23.0 software was used for statistical analysis of all data. The differences between the experimental groups were assessed using univariate analysis of variance (ANOVA). GraphPad Prism 8.0 is used to generate graphics. P < 0.05 was considered statistically significant.¹⁹

Results

Optical Emission Spectroscopy (OES) Analysis

OES was used to identify the composition of reactive species produced by DBD. The emission spectrum characteristics of the DBD plasma system from 350 to 800 nm were showed in Figure 1B. There are some obvious peaks in the range of 350–410 nm, including $\lambda=357, 361, 384,$ and 408 nm, which are attributed to the emission of the second positive system of molecular N₂. The characteristic emission peaks of the nitrogen atom (N) appear at 678 nm and 750 nm, respectively, while the peak at 777 nm corresponds to the oxygen atom (O). They are the primary constituents of air.

CAP Inhibits the Proliferation of RA-FLS and Induces Apoptosis

To determine whether CAP inhibits cell proliferation, we chemically labeled new and synthesized DNA from CAP-treated RA-FLS by adding EdU and counterstained nuclei with DAPI. As shown in Figure 2A and B), EdU assay results showed that the proportion of edU-positive cells in the control group, CAP30, CAP60, and CAP120 was $80.1\% \pm 7.2\%$, $70.0\% \pm 6.3\%$, $63.7\% \pm 5.1\%$, and $46.2\% \pm 6.1\%$, respectively. Compared with the control group, the ratio of EdU positive cells in CAP treatment group was lower. EdU-stained cells are incorporated into newly synthesized DNA, and CAP reduces the proportion of edU-positive cells and DNA synthesis in a time-dependent manner. CAP30 was not statistically significant compared with the control group. RA-FLS cell proliferation can be inhibited by CAP, especially after 120 s of action.

The results of Hoechst experiment showed the formation of apoptotic cells. As shown in Figure 2C, intact nuclei could be observed for the control group and CAP30 group, showing weak fluorescent staining. However, the CAP60 group and CAP120 group showed typical morphological changes of apoptotic nuclei, such as nuclear condensation, increased brightness, and nuclear shrinkage, which were more frequent in the CAP120 group. These results indicate that treatment with a certain temporal dose of CAP can induce apoptosis in RA-FLS.

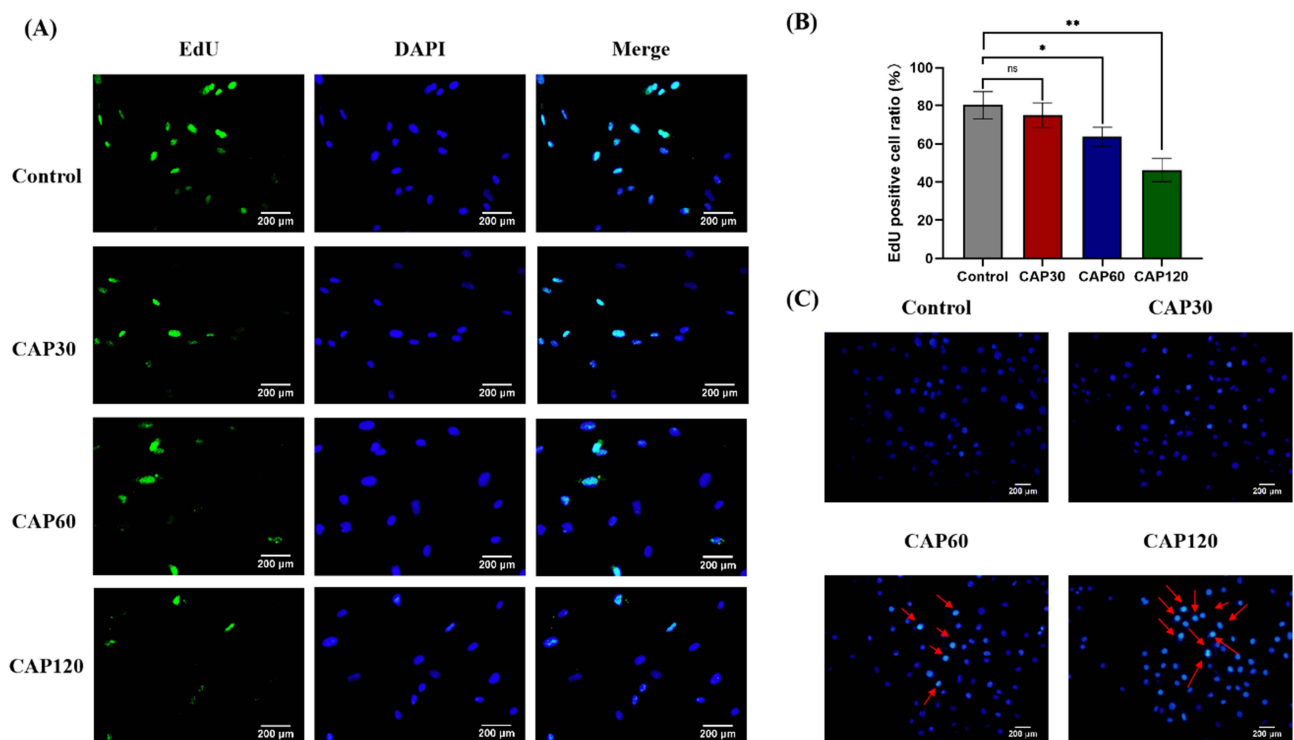


Figure 2 CAP inhibited the proliferation of RA-FLS.

Notes: CAP inhibited the proliferation of RA-FLS. **(A)** EdU staining is shown in green fluorescence, and DAPI staining is shown in blue fluorescence. A representative micrograph (400x) of three independent experiments is shown. **(B)** The percentage of EdU positive cells and the error bar represent the average value \pm SD of the three tests*. Compared with the relative control group, * $p < 0.05$, ** $p < 0.01$. **(C)** Hoechst staining analysis showed nuclear fragmentation and condensation during apoptosis, and the cells showed typical apoptotic nuclear morphological changes, as indicated by arrows. Photographs were taken under a 200x fluorescence microscope ($\times 200$).

CAP Inhibits RA-FLS Invasion and Migration

To explore the effect of CAP on the invasion ability of RA-FLS, different time and dose of CAP were used to treat RA-FLS. Wound-healing assay and Transwell assay showed that CAP treatment of RA-FLS could inhibit the migration and invasion of RA-FLS in a time and dose-dependent manner. As shown in Figure 3A and B, the wound-healing assay showed that 24 h after CAP treatment, compared with the control group, the migration rate of CAP30 group was slightly reduced (not statistically significant), and the

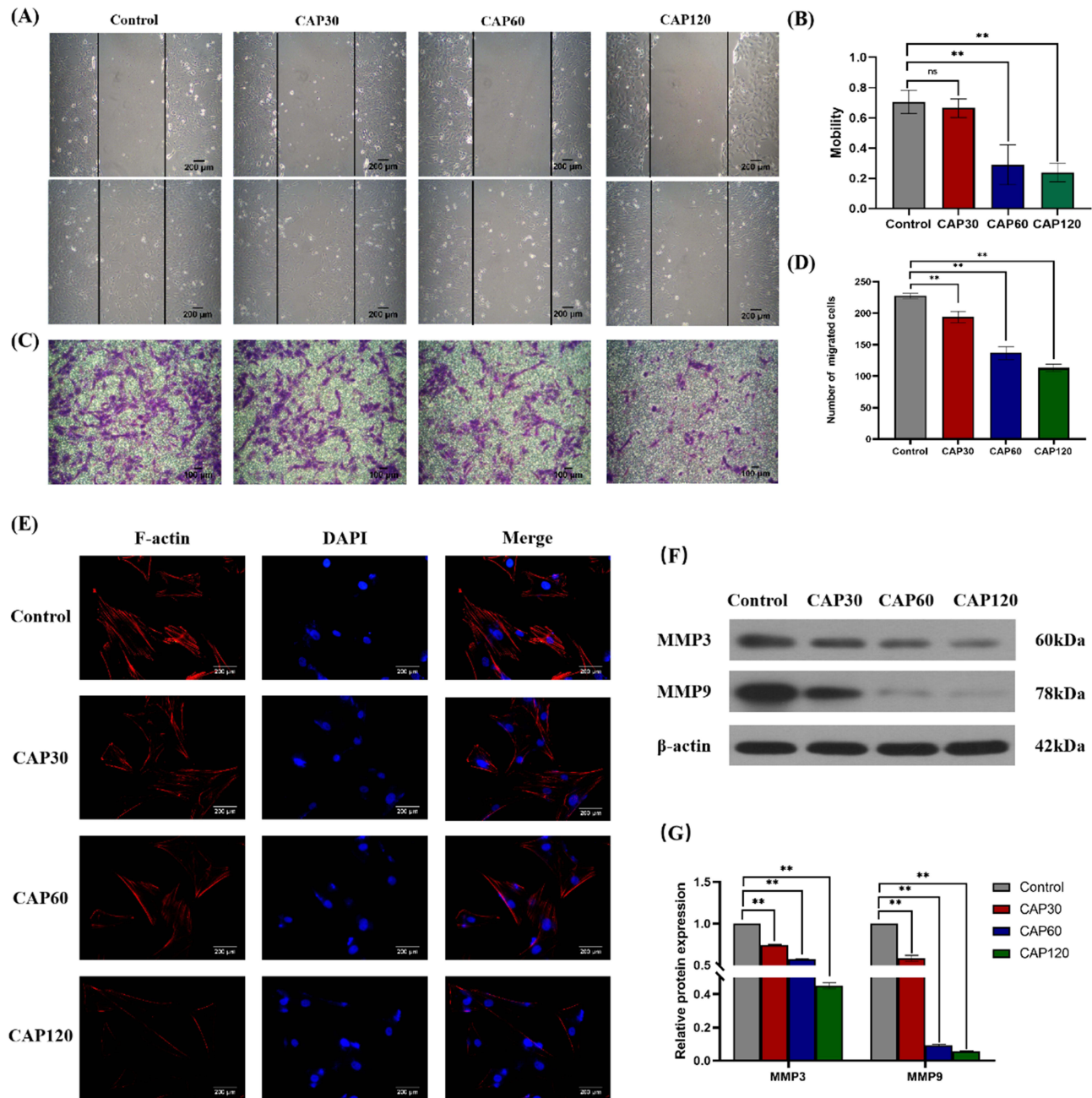


Figure 3 CAP inhibit the ability of migration and invasion on RA-FLS.

Notes: CAP inhibit the ability of migration and invasion on RA-FLS. (A) The effect of CAP on cell migration was detected using wound-healing assay. The scratched area was photographed at 0 h and 24 h ($\times 100$). (B) Mobility = (number of cells at 24h – number of cells at 0h)/number of cells at 24h. (C) Transwell analysis was performed using a Transwell chamber, and the invaded cells were photographed. Three areas were randomly selected for cell counting ($\times 200$). (D) Migrating cells were counted to quantify the degree of chemotaxis. (E) Typical photographs ($\times 400$) of fluorescent phalloidin staining from different groups. Fluorescent phalloidin was used to stain cells to make F-actin visible (red), and DAPI was used to stain nuclei (blue). (F) Immunofluorescence morphology of RA FLS was scored for actin cytoskeleton characteristics. (G) Representative images of MMP3 and MMP9 expression levels and density quantification in RA FLS after CAP treatment measured by Western blot. Data are expressed as mean \pm SD ($n = 3$), ** $P < 0.01$ compared with control group.

migration rate of CAP60 group and CAP120 group was significantly reduced. As shown in Figure 3C and D, the percentage of invasive cells in the CAP treatment group was significantly lower than that in the control group ($P < 0.01$), showing a time-dependent manner. Cell migration and invasion are usually accompanied by dynamic reorganization of the actin cytoskeleton, and to further confirm the role of CAP in RA-FLS regulation of cytoskeletal reorganization, immunofluorescence staining was used to visualize cytoskeletal reorganization. As shown in Figure 3E, treatment with CAP reduced the formation of actin stress fibers compared with the control group. As shown in Figure 3(F and G), the expression of MMPs protein was detected by Western blot. MMPs played an important role in the proliferation, migration and invasion of RA-FLSs and even the erosion of cartilage joints. After CAP treatment of RA-FLS, MMP-3 and MMP-9 were significantly decreased, and the expression of CAP120 group was very little and almost undetectable, which indicated that CAP treatment inhibited the expression of MMP-3 and MMP-9 in RA-FLS.

CAP Induces RA-FLS G2/M Cell Cycle Arrest

To deduce the role experienced by CAP in regulating the mitotic cycle of RA-FLS. The analysis of cell cycle was done using flow cytometric examination. Flow cytometry in Figure 4A–E showed a decrease in the S phase and an increase in the G2/M phase in the CAP-treated group compared with the control group, which eventually resulted in cell cycle arrest at the G2/M phase. In addition, Cyclin B1 and CDK1 expressions were down-regulated, and P21 expression was up-regulated in a time-dependent manner by Western blotting (Figure 4F and G). Thus, flow cytometry and Western blot analysis demonstrated that the anti-proliferative effect of CAP was induced by a time-dependent G2/M phase cell cycle arrest.

CAP Increases ROS Content in RA-FLS

CAP mainly produces ROS to play a role in disease. To check whether CAP produces ROS in this study, we measured intracellular ROS content by flow cytometry after CAP treatment of RA-FLS. As shown in Figure 5A–D), flow cytometry showed that the median fluorescence intensity (MFI) shifted to the right as proof that CAP treatment increased ROS content in RA-FLS. Comparing the MFI, which represents the intracellular ROS content, the MFI was significantly higher in the CAP-treated group than in the control group ($P < 0.01$) (Figure 5E).

Discussion

With the continuous research and improvement of plasma, plasma medicine still has great research prospects in medical applications, and the anti-tumor effect of plasma is becoming a research hotspot. Plasma has been shown to have significant anti-cancer properties, spawning a new field of medicine known as “plasma oncology”²² In modern medicine, high air plasma is used for sterilization of medical devices and implants.²³ In contrast, CAP can be used in the field of heat-sensitive organisms, such as living tissues, with a certain degree of safety.²⁴ RA is prone to recurrent attacks, and the progression of the disease leads to joint bone destruction and loss of function. The cell that mainly plays the role of synovial proliferation and invasion is RA-FLS, which has similar characteristics with tumor cell.²⁵ Therefore, we investigated the “anti-tumor” effect of CAP on RA-FLS. Over-proliferation of FLS is a typical pathological feature of RA. Inhibiting the proliferation of RA-FLS or promoting the apoptosis of RA-FLS may be a potential approach for the treatment of RA.²⁶ The results of this study showed that the proliferation of RA-FLS in CAP60 group and CAP120 group was significantly lower than that in the control group, indicating that CAP was an effective lead factor against the oncological behavior of RA-FLS, and its direct effect on RA-FLS could reduce the proliferation of RA-FLS. However, previous researches have shown that the antiproliferation effect of cold plasma on normal cells is very low,²⁷ so it is considered that CAP can target “abnormal synovial tissue” without adverse effects on surrounding normal tissues and synovium. Studies have shown that CAP exerts its anti-tumor effect mainly through cell death in a time-dependent manner, and a large number of studies have found that the treatment of tumor cell lines with CAP is related to cell apoptosis. Our study is supported by previously published studies which demonstrating the pro-apoptotic effect of CAP on RA-FLS via the mitochondrial pathway.^{16,19} Similarly, Hoechst staining again demonstrated the pro-apoptotic effect of cold plasma on RA-FLS.

The strong invasion and migration ability of RA-FLS accelerates the destruction of articular cartilage and bone, which is an important reason for the progression of RA. Studies have shown that CAP can inhibit the migration and invasion of cancer cells. Therefore, we suspected that CAP can inhibit the migration and invasion of RA-FLS. As we expected, we observed that the number of migrated and invasive cells decreased significantly after CAP treatment through wound-

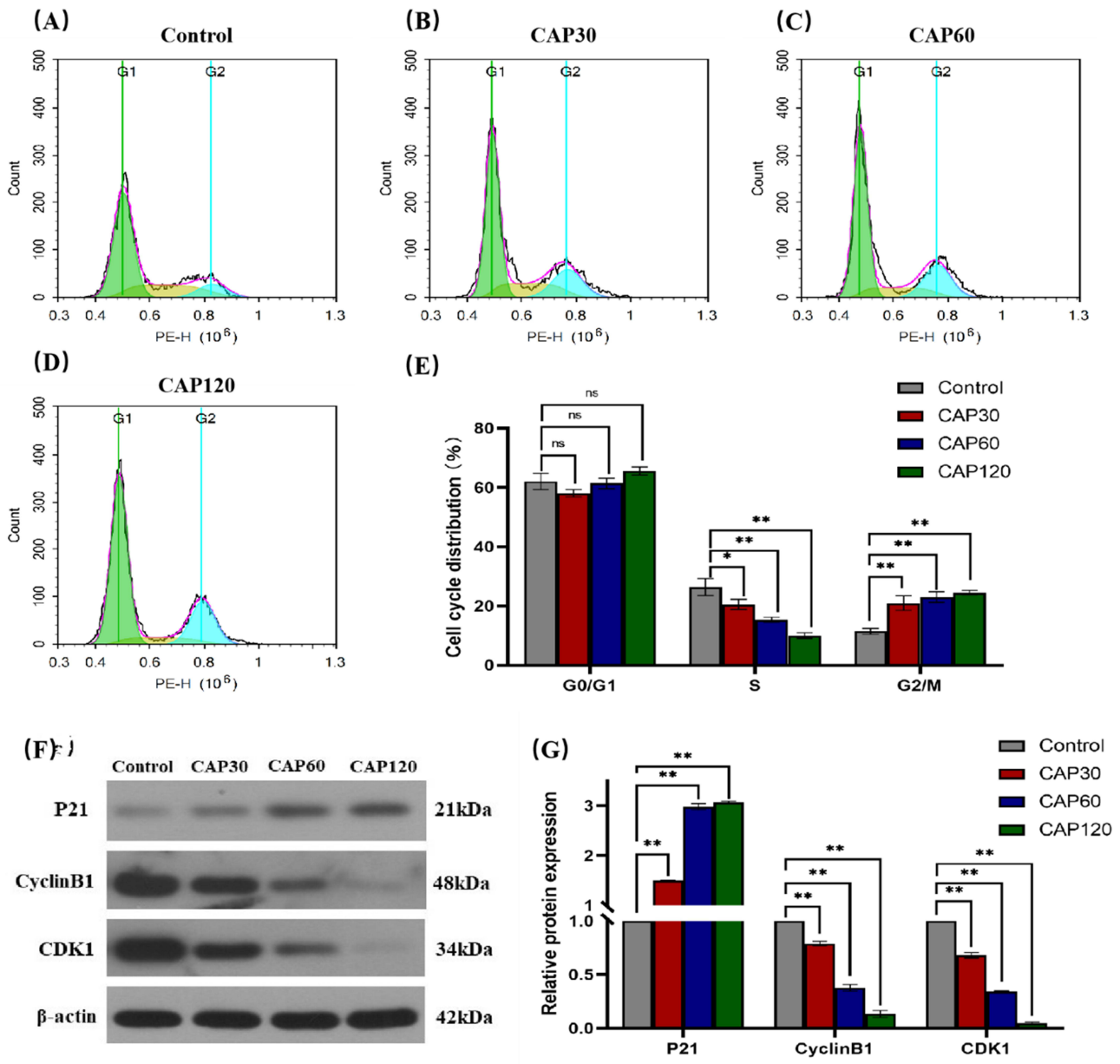


Figure 4 Effect of CAP treatment on the cell cycle of RA-FLS. **Note:** Effect of CAP treatment on the cell cycle of RA-FLS. **(A–D)** Cell cycle distribution of RA-FLS in each group was examined by flow cytometry analysis. **(E)** Statistical analysis showing the percentage of cell cycle distribution of RA FLS treated with CAP. Expression levels of P21, CyclinB1 and CDK1 in RA FLS treated with CAP were measured by Western blot **(F)** and quantitative statistical results of protein relative values **(G)** of P21, CyclinB1, and CDK1 data are expressed as mean \pm SD (n = 3) *P < 0.05, **P < 0.01 compared with control group.

healing Assay and Transwell experiment. A reduction in the formation of actin stress fibers can be seen in immunofluorescent staining for F-actin. RA-FLS secretes MMPs, a class of proteolytic enzymes closely related to invasion and metastasis, which further aggravate cartilage matrix degradation and eventually lead to bone erosion.²⁸ MMP-3 is a reliable marker of RA activity, radiological monitoring, predictability of disease outcome, and response to therapy.²⁹ Li Wei et al reported that non-thermal plasma inhibited the invasiveness of human cervical cancer HeLa cells by inhibiting the MAPK pathway and reducing the expression of MMP-9.³⁰ Similarly, the down-regulation of MMP3 and MMP9 levels after CAP treatment of RA-FLS further confirmed that CAP inhibited the migration and invasion ability of RA-FLS. The characteristic of weakening RA-FLS migration makes CAP different from other treatments of RA.

It is generally believed that the loss of Cell cycle checkpoint control is a sign of human cancer. The active progression of cell cycle is one of the important characteristic of human cancer, and cell cycle arrest is a major way to inhibit cell growth.³¹ To elucidate

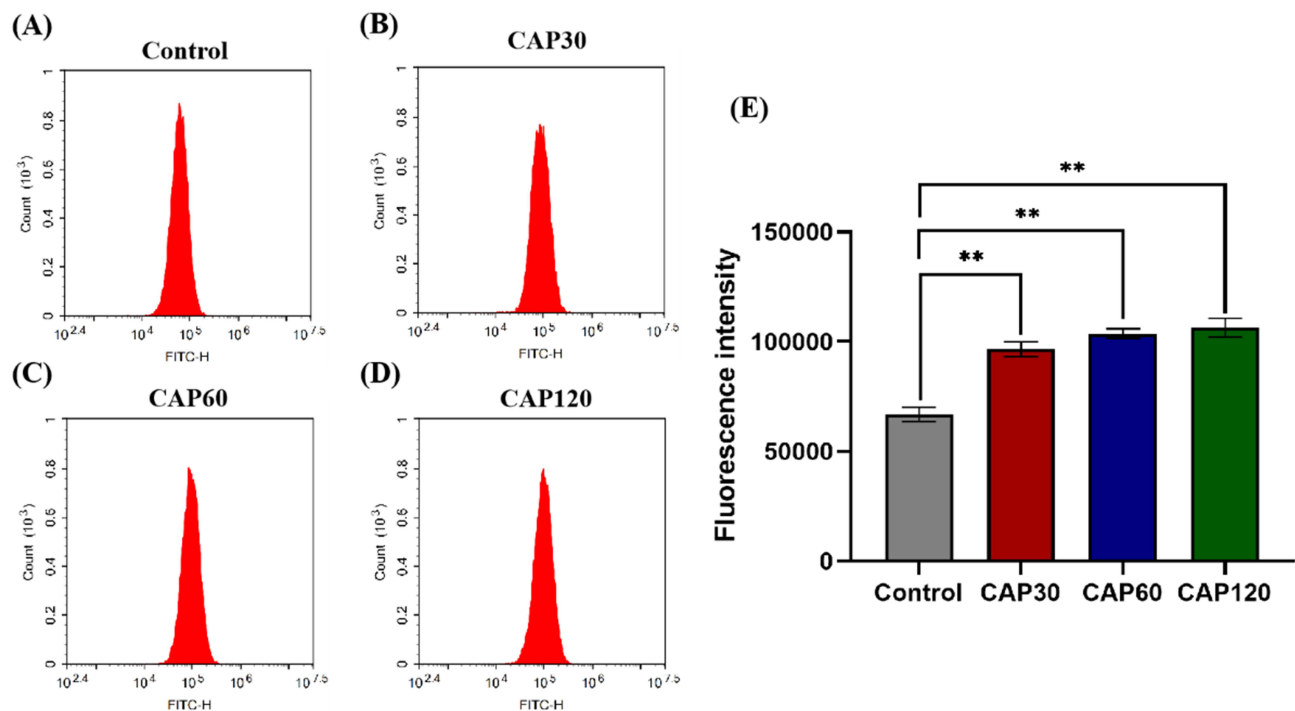


Figure 5 Changes of intracellular reactive oxygen species and apoptosis of RA-FLS after CAP treatment.

Note: Changes of intracellular reactive oxygen species and apoptosis of RA-FLS after CAP treatment. **(A–D)** Intracellular reactive oxygen species (ROS) were detected by flow cytometry. **(E)** The fluorescence intensity values of each group after CAP treatment of RA-FLS were statistically analyzed. Expressed as mean \pm standard deviation (** $P < 0.01$).

the mechanism of the anti-proliferative effect of CAP on RA-FLS, we compared the cell cycle progression of RA-FLS with and without CAP treatment. Compared with the untreated control group, the percentage of CAP-treated RA-FLS in the G2/M phase increased, while the percentage of RA-FLS in the S phase decreased, which may indicate that CAP is directly or indirectly involved in cell cycle checkpoint regulation and blocking of RA-FLS in the G2/M phase. Almeida Ferreira C et al studied that CAP acting on human breast cancer cells may induce cell cycle arrest and reduce long-term survival rate in G2/M phase, which is consistent with our research results.³² CDK1 and cyclin B1 kinase are the major regulators that advance the cell cycle from G2 to M phase, regulating the transport of cells into the G2/M phase. P21, a common inhibitor of CDKs that blocks cell cycle G2/M phase progression in various types of cancer cells. It has been identified as a key mediator of cell cycle G2 arrest via cell cyclin B1 degradation to inactivate cyclin B1/CDK1 kinase in response to genetic damage.^{33–35} Therefore, the expression of CDK1, cyclin B1 and P21 protein were evaluated to clarify the CAP-mediated cell cycle arrest. Western blot results in the present study suggested that CDK1 and cyclin B1 kinase were down-regulated and P21 was up-regulated with the increase of CAP, providing further evidence that CAP treatment of RA-FLS caused G2/M phase cell cycle arrest. As a result, it is reasonable to conclude that the decrease in CAP-treated RA-FLS cell proliferation is due to G2/M cell cycle arrest during RA-FLS proliferation. The study of Volotskova O et al proved that CAP induced about 2-fold increase in G2/M in two different types of cancer cells with different tumorigenicity,³⁶ which may mean that CAP can not only act on cancer cells but also be applied to other cells similar to tumor cells.

ROS has a dual role. On the one hand, the basic ROS level in cells increases, which may promote cell growth, such as abnormal proliferation of cancer cells and CAP promoting wound healing are also based on this principle. On the other hand, when the concentration of ROS rises beyond the threshold, tumor suppression will occur, including cell apoptosis and autophagy. For example, many anticancer drugs up-regulate ROS to the toxic level, and the anticancer effect of CAP.^{37–39} Similarly, CAP treatment induced a dose-dependent increase of intracellular ROS in RA-FLS. In tumor cells with high levels of ROS, the additional ROS produced by CAP may overburden the system and shift the role of ROS from promoting to inhibiting tumor formation, comparing differences in CAP-derived ROS uptake and clearance between tumor and normal cells may explain the mechanism of selective CAP effects on tumor cells.⁴⁰

In conclusion, our study demonstrated that intracellular ROS was increased after CAP treatment, causing cell cycle arrest and thus providing a new therapeutic strategy for RA. CAP can inhibit the tumor-like behavior of RA-FLS in

a short period of time, and some herbal medicines such as Shikonin⁴¹ and Guizhi-Shaoyao-Zhimu decoction⁴² that have been reported to inhibit the tumor-like behavior of RA-FLS, but the treatment time cycle is long and requires repeated interventions. CAP induces physical effects (generation of ultraviolet (UV), heat and electromagnetic fields) and chemical effects (generation of RONS). In the future experiments, it is necessary to eliminate the interference of other active components of CAP, such as UV, which also plays a role in tumor treatment. It is required to explore the dosage and toxicity of CAP treatment to determine its safety. In this study, we only observed the inhibitory effect of CAP on RA-FLS, but how to translate it into in vivo therapy still requires a lot of exploration.

Conclusion

In this study, we evaluated the anti-tumor effect of CAP on RA-FLS through in vitro experiments and discovered that CAP could promote apoptosis and inhibit the proliferation, migration and invasion of RA-FLS, which possibly related to the increase of intracellular ROS that arrested the cell cycle. The inhibitory effect of CAP on the proliferation of RA-FLS is clearly due to the enhanced cell cycle arrest at G2/M. However, further research is needed to examine whether CAP-induced G2/M arrest is related to cell apoptosis, and to elucidate the mechanism of CAP's inhibitory effect on RA-FLS migration and invasion progression. Overall, CAP can target RA-FLS, making it a more effective way to explore, invent and use in the treatment of RA and improve the quality of life.

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Disclosure

The authors report no conflicts of interest in this work.

References

1. Smolen JS, Aletaha D, McInnes IB. Rheumatoid arthritis. *Lancet*. 2016;388(10055):2023–2038. doi:10.1016/S0140-6736(16)30173-8
2. Radu AF, Bungau SG. Management of rheumatoid arthritis: an overview. *Cells*. 2021;10(11):2857. doi:10.3390/cells10112857
3. Smith MH, Berman JR. What is rheumatoid arthritis? *JAMA*. 2022;327(12):1194. doi:10.1001/jama.2022.0786
4. van Delft MAM, Huizinga TWJ. An overview of autoantibodies in rheumatoid arthritis. *J Autoimmun*. 2020;110:102392. doi:10.1016/j.jaut.2019.102392
5. Finckh A, Gilbert B, Hodkinson B, et al. Global epidemiology of rheumatoid arthritis. *Nat Rev Rheumatol*. 2022;18(10):591–602. doi:10.1038/s41584-022-00827-y
6. Kumar LD, Karthik R, Gayathri N, Sivasudha T. Advancement in contemporary diagnostic and therapeutic approaches for rheumatoid arthritis. *Biomed Pharmacother*. 2016;79:52–61. doi:10.1016/j.biopha.2016.02.001
7. Németh T, Nagy G, Pap T. Synovial fibroblasts as potential drug targets in rheumatoid arthritis, where do we stand and where shall we go? *Ann Rheum Dis*. 2022;81(8):1055–1064. doi:10.1136/annrheumdis-2021-222021
8. Wu Z, Ma D, Yang H, et al. Fibroblast-like synoviocytes in rheumatoid arthritis: surface markers and phenotypes. *Int Immunopharmacol*. 2021;93:107392. doi:10.1016/j.intimp.2021.107392
9. Aihaiti Y, Song Cai Y, Tuerhong X, et al. Therapeutic effects of naringin in rheumatoid arthritis: network pharmacology and experimental validation. *Front Pharmacol*. 2021;12:672054. doi:10.3389/fphar.2021.672054
10. Tsaltskan V, Firestein GS. Targeting fibroblast-like synoviocytes in rheumatoid arthritis. *Curr Opin Pharmacol*. 2022;67:102304. doi:10.1016/j.coph.2022.102304
11. Gangemi S, Petrarca C, Tonacci A, Di Gioacchino M, Musolino C, Allegra A. Cold atmospheric plasma targeting hematological malignancies: potentials and problems of clinical translation. *Antioxidants*. 2022;11(8):1592. doi:10.3390/antiox11081592
12. Limanowski R, Yan D, Li L, Keidar M. Preclinical cold atmospheric plasma cancer treatment. *Cancers*. 2022;14(14):3461. doi:10.3390/cancers14143461
13. Motaln H, Recek N, Rogelj B. Intracellular responses triggered by cold atmospheric plasma and plasma-activated media in cancer cells. *Molecules*. 2021;26(5):1336. doi:10.3390/molecules26051336
14. Dubuc A, Monsarrat P, Virard F, et al. Use of cold-atmospheric plasma in oncology: a concise systematic review. *Ther Adv Med Oncol*. 2018;10:1758835918786475. doi:10.1177/1758835918786475
15. Mitra S, Nguyen LN, Akter M, Park G, Choi EH, Kaushik NK. Impact of ROS generated by chemical, physical, and plasma techniques on cancer attenuation. *Cancers*. 2019;11(7):1030. doi:10.3390/cancers11071030
16. Ding C, Chen C, Ouyang W, Liu Q, Lin L, Wu Z. Cold air plasma: a potential strategy for inducing apoptosis of rheumatoid arthritis fibroblast-like synoviocytes. *High Volt*. 2021;7(1):106–116. doi:10.1049/hve2.12132

17. Hao Z, Liu Y. IL-38 and IL-36 target autophagy for regulating synoviocyte proliferation, migration, and invasion in rheumatoid arthritis. *Dis Markers*. 2021;20:7933453.
18. Wang H, Jia XZ, Sui CJ, et al. Effects of thapsigargin on the proliferation and survival of human rheumatoid arthritis synovial cells. *Sci World J*. 2014;9:605416.
19. Ding C, Ni L, Liu Q, Zhou C, Wang G, Chu P. Cold air plasma improving rheumatoid arthritis via mitochondrial apoptosis pathway. *Bioeng Transl Med*. 2022;8(1):e10366. doi:10.1002/btm2.10366
20. Li N, Gong Z, Li X, et al. Betulinic acid inhibits the migration and invasion of fibroblast-like synoviocytes from patients with rheumatoid arthritis. *Int Immunopharmacol*. 2019;67:186–193. doi:10.1016/j.intimp.2018.11.042
21. Yan Y, Zou H, Lian X, Yang L. Cyclic mechanical stimulation inhibits rheumatoid arthritis fibroblast-like synoviocytes proliferation via cell cycle arrest. *J Biomech*. 2021;121:110424. doi:10.1016/j.jbiomech.2021.110424
22. Schlegel J, Körtzner J, Boxhammer V. Plasma in cancer treatment. *Clin Plasma Med*. 2013;1(2):2–7. doi:10.1016/j.cpme.2013.08.001
23. Bernhardt T, Semmler ML, Schäfer M, Bekeschus S, Emmert S, Boeckmann L. Plasma medicine: applications of cold atmospheric pressure plasma in dermatology. *Oxid Med Cell Longev*. 2019;2019:3873928. doi:10.1155/2019/3873928
24. Perrotti V, Caponio VCA, Muzio LL, et al. Open questions in cold atmospheric plasma treatment in head and neck cancer: a systematic review. *Int J Mol Sci*. 2022;23(18):10238. doi:10.3390/ijms231810238
25. Falconer J, Murphy AN, Young SP, et al. Review: synovial cell metabolism and chronic inflammation in rheumatoid arthritis. *Arthritis Rheumatol*. 2018;70(7):984–999. doi:10.1002/art.40504
26. Zhao J, Jiang P, Guo S, Schrodi SJ, He D. Apoptosis, Autophagy, NETosis, necroptosis, and pyroptosis mediated programmed cell death as targets for innovative therapy in rheumatoid arthritis. *Front Immunol*. 2021;12:809806. doi:10.3389/fimmu.2021.809806
27. Kim SJ, Chung TH. Cold atmospheric plasma jet-generated RONS and their selective effects on normal and carcinoma cells. *Sci Rep*. 2016;6(1):20332. doi:10.1038/srep20332
28. Mousavi MJ, Karami J, Aslani S, et al. Transformation of fibroblast-like synoviocytes in rheumatoid arthritis; from a friend to foe. *Auto Immun Highlights*. 2021;12(1):3. doi:10.1186/s13317-020-00145-x
29. Lerner A, Neidhöfer S, Reuter S, Matthias T. MMP3 is a reliable marker for disease activity, radiological monitoring, disease outcome predictability, and therapeutic response in rheumatoid arthritis. *Best Pract Res Clin Rheumatol*. 2018;32(4):550–562. doi:10.1016/j.berh.2019.01.006
30. Li W, Yu KN, Bao L, Shen J, Cheng C, Han W. Non-thermal plasma inhibits human cervical cancer HeLa cells invasiveness by suppressing the MAPK pathway and decreasing matrix metalloproteinase-9 expression. *Sci Rep*. 2016;6:1972.
31. Almeida-Ferreira C, Silva-Teixeira R, Gonçalves AC, et al. Cold Atmospheric Plasma Apoptotic and Oxidative Effects on MCF7 and HCC1806 Human Breast Cancer Cells. *Int J Mol Sci*. 2022;23(3):1698. doi:10.3390/ijms23031698
32. Golias CH, Charalabopoulos A, Charalabopoulos K. Cell proliferation and cell cycle control: a mini review. *Int J Clin Pract*. 2004;58(12):1134–1141. doi:10.1111/j.1742-1241.2004.00284.x
33. Porter LA, Donoghue DJ. Cyclin B1 and CDK1: nuclear localization and upstream regulators. *Prog Cell Cycle Res*. 2003;5:335–347.
34. Jang SH, Kim AR, Park NH, Park JW, Han IS. DRG2 regulates G2/M progression via the Cyclin B1-Cdk1 complex. *Mol Cells*. 2016;39(9):699–704. doi:10.1016/molcells.2016.0149
35. Chen SM, Lin TK, Tseng YY, et al. Targeting inhibitors of apoptosis proteins suppresses medulloblastoma cell proliferation via G2/M phase arrest and attenuated neddylation of p21. *Cancer Med*. 2018;7(8):3988–4003. doi:10.1002/cam4.1658
36. Hawley TS, Stepp MA, Keidar M, Keidar M. Targeting the cancer cell cycle by cold atmospheric plasma. *Sci Rep*. 2012;2(1):636. doi:10.1038/srep00636
37. Mittler R. ROS are good. *Trends Plant Sci*. 2017;22(1):11–19. doi:10.1016/j.tplants.2016.08.002
38. Cheung EC, Vousden KH. The role of ROS in tumour development and progression. *Nat Rev Cancer*. 2022;22(5):280–297. doi:10.1038/s41568-021-00435-0
39. Bauer G, Sersenová D, Graves DB, Machala Z. Cold atmospheric plasma and plasma-activated medium trigger RONS-Based tumor cell apoptosis. *Sci Rep*. 2019;9(1):14210. doi:10.1038/s41598-019-50291-0
40. Min T, Xie X, Ren K, et al. Therapeutic effects of cold atmospheric plasma on solid tumor. *Front Med*. 2022;9:884887. doi:10.3389/fmed.2022.884887
41. Liu FY, Wang MQ, Liu MM, et al. Therapeutic effects of shikonin on adjuvant-induced arthritis in rats and cellular inflammation, migration and invasion of rheumatoid fibroblast-like synoviocytes via blocking the activation of Wnt/ β -catenin pathway. *Phytomedicine*. 2023;116:154857. doi:10.1016/j.phymed.2023.154857
42. Zhang Q, Peng W, Wei S, et al. Guizhi-Shaoyao-Zhimu decoction possesses anti-arthritis effects on type II collagen-induced arthritis in rats via suppression of inflammatory reactions, inhibition of invasion & migration and induction of apoptosis in synovial fibroblasts. *Biomed Pharmacother*. 2019;118:109367. doi:10.1016/j.biopha.2019.109367

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