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# Anticancer effect of Copper Activated Plasma Water on MCF7 Breast Cancer Cells

**Isaac John Umaru[1], Iseko Iyoko Kingsly[2], Edom Favour Ogochukwu[3], Tensaba Andes Akafa[4], Jackson Soyinka Malantso[5], Bilyaminu Habibu[6], Kerenhappuch Isaac Umaru[7]**

[1,4,5,6,7]Federal University Wukari, Taraba State, Nigeria;
[2]Limi Hospital Abuja, Nigeria; [3]University of Port Harcourt, Nigeria
umaruisaac@gmail.com

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## Abstract

Broad biological activities of "plasma-activated water" (PAW) have drawn great attentions recently. Treatment of water using gas discharge plasma led to acidic solutions with excellent and broad antibacterial activity. Because PAW caused severe membrane damages in bacteria and diffused freely in extracellular matrix, PAW also demonstrated good anti-biofilm activity. However, further studies revealed that trace amounts of metal ions (mainly copper) in PAW brought by plasma treatment played key roles in bacteria inactivation. The contribution of metal ions to the antibacterial activity varied among PAWs from different working gases. However, solution acidification caused by reactive species in plasma was essential. The experimental results demonstrated that potential artifacts in reported biological activities of PAWs should be considered. Therefore, Copper has important redox activity and can participate in various biochemical reactions by accepting and donating electrons. As a trace element, thus, Anticancer effect of Copper Activated Plasma Water on MCF7 Breast Cancer Cells. Materials and method, used are a non-thermal micro-hollow cathode discharge (MHCD) was used to generate

plasma-activated waters (CU-PAWs), The MCF-7 human breast adenocarcinoma cell line (IBRC C10082), and 3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was acquired. Cell Viability Measurements; After 48 h of incubation 0.5 mg/ml MTT (20 µl) was added to the control and experimental cells and the cells were incubated for 3 h in a $CO_2$ incubator at 37 °C, Measurement of Reactive Oxygen Species (ROS) and Flow Cytometry was conducted. Results: The 3- and 4-min CU-PAW reduced MCF-7 cells viability to approximately 62% and 56% of control ($p< 0.01$), respectively. However, in the cases of 1- and 2-min CU-PAW cell proliferation did not diminish significantly as compared with the control group ($p> 0.05$). This observation is consistent with earlier studies, which illustrated that plasma irradiation reduced cell viability in a time-dependent manner. Thus, in this research, DOX (0.45 µM) combined with 3- or 4-min CU-PAW killed MCF-7 cell efficiently (44% and 39% cell viability, respectively; $p< 0.01$) than DOX (54% cell viability) or 3- or 4-min CU-PAW alone (63% and 56% cell viability, respectively). These was in line with a that PAW plus cisplatin at low doses reduced viability of human endometrial carcinoma more effectively than cisplatin or PAW alone. Conclusion: Although further investigations are crucial, CU-PAW combined with DOX could be a promising cancer treatment strategy, contributing to a more positive therapeutic agent.

**Keywords:** Anticancer, Copper, Activated, Plasma, Water on MCF7 Breast Cancer Cells

## INTRODUCTION

Cancer is a group of diseases in which genetically damaged cells proliferate autonomously. Such cells cannot respond to normal regulatory mechanisms that ensure the intercellular cooperation required in multicellular organisms. Consequently, they continue to proliferate, thereby robbing nearby normal cells of nutrients and eventually crowding surrounding healthy tissue [1].

There is a steady increase in incidence of cancers as observed in most developed and developing countries. Apart from incidence, cancer related deaths are also increasing. In 2008 alone, up to 7.6 million people died from cancers all over the world with about 70% of these deaths occurring in developing countries [2].

In the whole world, there were 14 million new cases and 8 million deaths in 2012, and in 2018, there were 18.1 million new cases and 9.6 million cancer-related deaths [3]. This value is projected to rise by at least 70% by the year 2030 [4]. It is estimated that more than

250,000 new cases of cancers are diagnosed in Nigeria every year and up to 10,000 Nigerians die each year from cancer related causes (Baba and Hincal, 2016). These estimates are based largely on hospital generated data without provision for the many cases that do not present themselves in hospitals as well as the many cases of misdiagnosis in our numerous peripheral hospitals ([2].

Breast cancer is the most widespread malignancy among females worldwide, accounting for the greatest percentage of deaths (15.5%) from all cancers in women. It was estimated the number of new cases in 2020 would exceed 2.2 million [1, 2]. Subsequently, it seems obvious that more effective treatments than those currently in use are sorely needed. Current cancer treatments include hormone therapy, chemotherapy, radiotherapy, immunotherapy, and surgery. Some of these treatments have serious deleterious side effects in patients [3-5].

Copper is an essential element of life, from bacteria and fungi to plants and animals. In humans, copper is an indispensable trace metal element in the body and a necessary cofactor for the organism [6]. It binds to enzymes to assist blood clotting, hormone maturation, and cellular energy processing. The unique redox properties of copper make it both beneficial and detrimental to cells. When the copper content exceeds the everyday needs of the cells, it will produce cytotoxicity and kill the cells; when the copper is deficient, the absorption and transport of copper will be hindered, resulting in an abnormal distribution of copper in each cell [7]. It can be seen that excessive or insufficient copper accumulation will affect the regular physiological activity of cells. Because the growth and metastasis of tumors have a higher demand for copper, a metal nutrient, copper-related diagnosis and treatment methods are very suitable for tumors. According to the unique mechanism of copper, researchers have applied it to tumor cells and found that it can effectively induce tumor cell death. They have designed a variety of copper complexes, which have achieved good results in tumor treatment [8]. The current research progress on the induction of autophagy and apoptosis in tumor cells by copper and its complexes and their mechanisms (activation of stress pathways, arrest of cell cycle, and inhibition of angiogenesis) are summarized as follows (Figure 1). diagnosis and treatment methods are very suitable for tumors. According to the unique mechanism of copper, researchers have applied it to tumor cells and found that it can effectively induce tumor cell death. They have designed a variety of copper complexes, which have achieved good results in tumor

treatment [9]. The current research progress on the induction of autophagy and apoptosis in tumor cells by copper and its complexes and their mechanisms

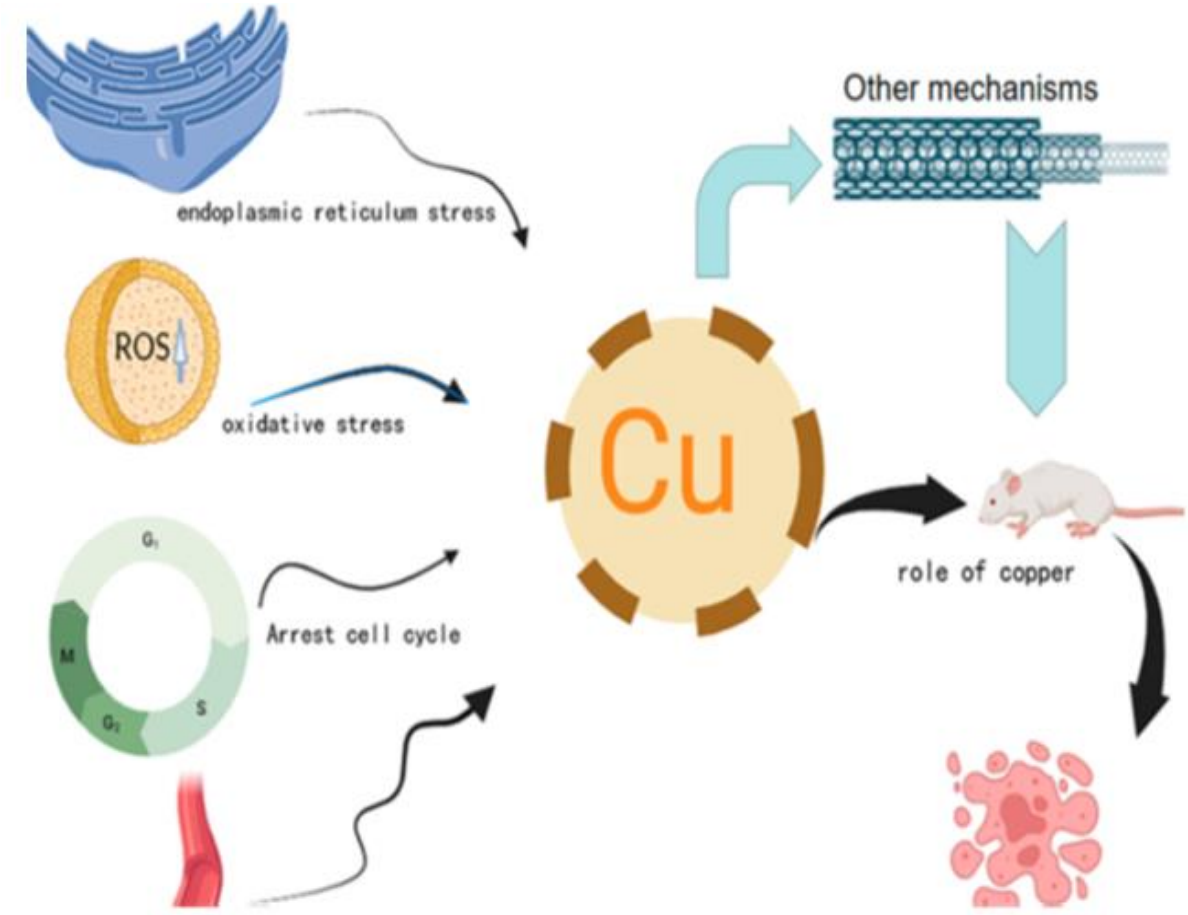


Figure 1: The primary mechanism of copper-induced cell death. Activating stress pathways (endoplasmic reticulum stress and oxidative stress), blocking the cell cycle, and anti-angiogenesis are all used to treat cancer by controlling copper levels and utilizing the cytotoxicity produced by copper excess to kill tumor cells. There are other therapeutic mechanisms to treat cancer by reaching the tumor's exact location in mice suffering from the tumor and causing tumor cell death under the action of copper.

## Distribution and Importance of Copper

It is well known that the copper ion is an essential micronutrient for the human body. A copper intake of 0.8 mg/d is sufficient to maintain and regulate the copper balance in the human body [10]. Most of the copper ingested by the human body from food is Cu2+, which cells cannot absorb and utilize. Generally, it needs to be processed by digestive tract

cells. There is a variety of reductases on the surface of these cells. With the assistance of divalent metal transporter 1 (DMT1), Cu2+ can be reduced to Cu+ and combined with copper transporter 1 (CTR1) to enter the cells. The Cu+ that enters the cell is delivered to the copper chaperone protein antioxidant 1 (Atox1), which combines with the copper transport ATPase (ATP7 ) through the Golgi complex pathway and preceruloplasmin to produce ceruloplasmin (CP) [11], which further acts on organs and tissues throughout the body.

Copper has important redox activity and can participate in various biochemical reactions by accepting and donating electrons. As a trace element, copper is widely distributed in biological tissues, participates in the formation of proteins and related physiological regulation, and plays a vital role in the human body [11]. The function of some proteins requires the activation of copper, such as cytochrome c oxidase (COX), NADH deoxygenase-2 (ND2), and other essential proteins in the body; the role of copper is irreplaceable [12]. Copper, combined with various proteins or enzymes, can also participate in crucial life processes in the body, such as energy metabolism, oxidative detoxification, and mitochondrial respiration. The absorption, transport, storage, and excretion of copper ions in the body are the basic processes for stabilizing copper metabolism. Intracellular copper is strictly regulated, on the one hand, to supply sufficient copper proteins and, on the other hand, to prevent toxic oxidative stress. Studies have shown that an excessive accumulation of intracellular copper can trigger the accumulation of mitochondrial lipid-acylated proteins and interfere with the role of Fe-S cluster proteins [13]. The reasonable control of the toxicity of copper can maximize its function.

In addition, there is a strong link between copper and cancer cells. Studies have shown that copper is cytotoxic to cancer cells, has anti-angiogenic, cell cycle growth-inhibiting, and energy metabolism-regulating effects in anticancer therapy, and its copper complexes can also produce antibacterial, antiviral, anti-inflammatory, antitumor, and enzyme-inhibiting activities, which can play an essential role in cancer therapy [14-17]. Based on this feature, copper has been continuously introduced into anticancer drugs. It is of great importance for cancer treatment.

**Plasma**

Plasma is an ionized gas, fourth state of matter. Ionized refers that at least one electron is not bound to an atom or molecule, converting the atoms or molecules into positively

charged ions from neutral state. As temperature increases, molecules become more energetic as they receive the thermal energy and transform matter in the sequence from solid to liquid, from liquid to gas, from gas to plasma. The presence of free charges make the plasma electrically conductive, it is reported that the conduction can be higher than the highly conductive gold and copper. Plasma is internally interactive, and strongly responsive to electromagnetic fields. Ionized gas is called plasma when it is electrically neutral (the electrons and positive ions are in the same number) and contains a significant number of electrically charged particles, enough to affect its electrical properties and its behavior. In addition to being important in many aspects of our daily lives through products based on plasma, plasmas are estimated to constitute more than 99% of the visible universe [18].

**Thermal and non-thermal plasmas**

As in the case of gases, temperature of plasma is determined by average energies of the plasma particles which are neutral and charged particles and their applicable degrees of freedom -translational, rotational, vibrational, and those associated to electronic excitation. Thus, plasmas, being multi-component systems, are able to present multiple temperatures. In electric discharges, the common method for generating plasma in laboratory, energy from electric field is accumulated by electrons between collisions, the energy is transferred from the electrons to the heavy particles afterwards. Electrons receive energy from the electric field during their mean free path and, during the following collision with a heavy particle, lose only a small portion of that energy (because electrons are much lighter than the heavy particles). This is why electron temperature in plasma is initially higher than that of heavy particles. The collisions of electrons with heavy particles (Joule heating) can equilibrate their temperatures, unless either time or energy are not sufficient for equilibration or there is an intensive cooling mechanism preventing heating of the entire gas. The temperature difference between electrons and heavy neutral particles because of Joule heating in the collisional weakly ionized plasma is conventionally proportional to the square of ratio of electric field (E) to pressure (p). Only in small values of E/p, the temperatures of electrons and heavy particles approach each other.

This is a basic requirement for local thermodynamic equilibrium (LTE) in plasma. LTE conditions also require chemical equilibrium as well as restrictions on the gradients. LTE plasma follows the major laws of equilibrium thermodynamics, and it can be characterized using a single temperature at each point of space. Hence the Ionization and chemical

processes in these plasmas are determined by temperature and indirectly by electric fields through Joule heating. Quasiequilibrium plasma of such kind is called thermal plasma.

Many plasmas exist very far from thermodynamic equilibrium and are characterized by multiple temperatures related to its different plasma particles and their different degrees of freedom. The electron temperature significantly exceeds that of heavy particles. Ionization and chemical processes in these non-equilibrium plasmas are directly determined by the temperatures of electron and, therefore, not sensitive to thermal processes and temperature of the gas. Non-equilibrium plasma of this kind is called non-thermal plasma [18]. Thus, Plasmas can be classified into two main groups based on the thermodynamic temperature equilibrium of the constituents, thermal plasma and nonthermal plasma. The thermal plasma has electrons at temperatures similar to that of heavier species.

**Plasma Discharge in Liquids**

During the plasma discharge between two electrodes, the medium between the electrodes is ionized, creating a plasma channel. When plasma discharge occurs in water, the plasma discharge generates UV radiation and converts the water to active radical species because of the high energy level produced by the discharge. Various active species can be considered to be generated in the plasma discharge in water, the production of these species can be affected by a number of parameters such as applied voltage, rise time, pulse duration, total energy, polarity, electrical conductivity etc. Among the active species hydroxyl radical, atomic oxygen, ozone and hydrogen peroxide are the most important ones for sterilization and removal of unwanted organic compounds in water. The discharge of plasma in water produces an abundance of reactive species which have unique applications to a variety of fields from seed germination to cancer treatment owing to their chemical properties [19].

**Plasma Activated Water**

Plasma activated water (PAW) produced by interaction of non-thermal plasma and water possess outstanding biological applications in agricultural and biomedical applications. PAW possess highly potent biological applications in fields of agriculture and biomedicine. In the field of agricultural applications, owing to its biochemical activity, PAW is used to

increase the rate of germination of seeds and subsequent growth of the seedlings and plants, inactivate plant-based pathogenic organisms and

cure fungus-infected plants. In biomedicine, applications of PAW span from biofilm removal, wound healing, deactivation of bacteria and viruses, dentistry (for teeth disinfection and whitening), and cancer therapy. The biochemical activity of PAW is derived from synergistic effects of the highly reactive species, specifically reactive oxygen and nitrogen species (RONS). The RONS in PAW include long-lived species as nitrates ($NO_3$ - ), nitrites ($NO_2$ - ), hydrogen peroxide ($H_2O_2$) and ozone (O3), with

corresponding half-lifetime of years, several days, ~104 s, and several to dozens of minutes, and short-lived ones: hydroxyl radicals (OH•), nitric oxide (NO•), superoxide (O2 - ), peroxynitrate ($OONO_2$ - ) and peroxynitrites (ONOO- ), the half-lifetimes of which have been proved as 1 ns, few seconds, 1.5 s and less than l s, respectively.

Furthermore, PAW is considered a biofriendly and prospective solution for biotechnology applications due to the time dependent nature of its biochemical activity because of the active species, and its economic and environmental benefits of using air rather than toxic chemicals as the raw material [20].

It has been confirmed that the biological activity of PAW is directly related to the chemistry and relative concentration of RONS, which are produced in the aqueous medium or at the interface between the gas and liquid phases. The production of plasma induced RONS depends on different typical parameters such as types of plasma power supply, type of carrier gas, electrode configuration, applied voltage, voltage polarity, treatment time, gas flow, the volume of solution, the distance between electrode and

liquid surface [20].

From the previous report, a micro-hollow cathode discharge (MHCD) was used to create PAW and demonstrated that PAW had excellent antibacterial and anti-biofilm activity [21]. Numerous reports have shown that the efficient antimicrobial agents in the plasma-activated solutions are free radicals' species [22-28], Some reports have also shown that the principal species are superoxide anion radicals (O2 −·), hydroxyl radicals (OH·), and nitric oxide ($NO^{\cdot}$) when the plasma generated uses air as the working gas at atmospheric pressure. When these reactive species are dissolved in the liquids, the long-lived products, such as $H_2O_2$ (hydrogen peroxide), NO3 − (nitrate), $NO_2$ − (nitrite), and ONOO−

(peroxynitrites) anions, as well as other species, have been effective in killing or inactivating bacteria and can be used as a disinfectant to contaminated solid surfaces and liquids. Many research groups reported the pH and concentrations of $HNO_2$, $HNO_3$, and $H_2O_2$ in the solutions after plasma treatment. Naïtali and colleagues found that PAW can kill more than 99% of bacteria after water was treated with glidarc plasma for 5 min. The pH of PAW was around 3 and contained 0.01 ± 0.01 mmol/L of $H_2O_2$, 0.13 ± 0.02 mmol/L of $NO_3$ −, and 1.6 ± 0.2 mmol/L of $NO_2$ – [27]. It was also reported that the used gliding electric plasma to create PAW, and approximately 80% of Hafnia alvei was killed. The pH of PAW was around 3, and 10−5 mol/L of $H_2O_2$, 5 × 10−5 mol/L of $NO_3$ −, and 3 × 10−3 mol/L of NO2 − were measured of PAW [29]. Oehmigen's group used the surface dielectric barrier discharge (DBD) to investigate water disinfection.

They determined the kinetics of NOx and subsequent nitrous $HNO_2$ and nitric acid $HNO_3$ formation responsible for Ph changes. Simultaneously, $H_2O_2$ was generated up to concentrations of 3~18 mg/L. Escherichia coli was completely inactivated[30]. Recent results have also shown that the antimicrobial capacity of PAW can persist bactericidal activity for couple days or more but artificial prepared solutions were less powerful than adequate solutions prepared by plasma (i.e., PAW) ([31,32,28]

In this research study, we found that the electrically conductive tube (cathode) made of brass material was sensitive to corrosion when the plasma was produced by the micro-hollow cathode discharge. There is a significant amount of copper ions found in the PAW after each experiment. According to Abushelaibi's research, they showed that copper ions are released from the brass into the surrounding environment, and copper can completely kill bacteria. [33]. Zhang et al. reported copper ions near the surface of polyethylene modified by plasma immersion ion implantation (PIII) have excellent anti-healing ability [34, 35]. The copper ions released from copper oxide nanoflakes also have ability of the inactivation of bacteria [36,37]. Therefore, we suggested that copper and zinc ions may play important roles healing in PAWs.

It was also reported that plasma is the fourth state of matter (after solid, liquid and gas). The macroscopic temperature of non-thermal equilibrium in plasma can be as low as 300 K, which is close to room temperature (non-thermal equilibrium plasma is also called cold plasma). As the generation of cold plasma does not require a special vacuum environment and can occur under atmospheric pressure, it is also referred to as cold atmospheric plasma

(CAP). The main chemically active groups in CAP are ions, free electrons, neutral particles, free radicals, and electromagnetic waves (UV, electric field, and visible light) [39-40]. The active species produced by CAP mainly include reactive oxygen species (ROS) and reactive nitrogen species (RNS), including $H_2O_2$, OH, $O_2$ , $O_3$, $NO_2$, $NO_3$, NO, and ONOO [41-46].

In recent years, due to the high activity levels of CAP particles, they have received much attention in the field of biomedicine, forming a new discipline: plasma medicine. Plasma medicine is a new and innovative field combining plasma physics, life sciences, and clinical medicine. The first application of CAP in the biomedical field was in 1996. Dr. Laroussi used glow discharge plasma at atmospheric pressure to sterilize instruments and food, proving that plasma can effectively inactivate bacteria [47]. In the past 20 years, due to the physical and chemical advantages of atmospheric pressure cold plasma, its applicability in biomedicine has increased, especially in the areas of sterilization, dental diseases, skin care, skin disease treatment, cancer treatment, wound healing, etc. [48–60]. Infection is a major factor that hinders wound healing, so CAP, a new technology that can sterilize and painlessly treat wounds, offers unique advantages in the field of wound healing. Studies have shown that the direct treatment of CAP can not only promote wound healing in terms of wound anti-infection, but it also promotes the proliferation and migration of two key skin cells (keratinocytes and fibroblasts) involved in wound healing, thereby accelerating the healing of the wound [61,62]. In addition, CAP has been shown to accelerate wound healing by producing relevant growth factors, recruiting granulocytes, and promoting angiogenesis around the wound [63–65].

The objectives of this study is to evaluate the anticancer effect of Copper Activated Plasma Water on MCF7 Breast Cancer Cells. Using two working gases (air and oxygen) to generate plasma and treated D. I. water for specified times to create PAWs, perform anti-cancer and measure each PAW's play key factors to inactivate the cell proliferation.

## MATERIAL AND METHODS

### Preparation of Copper Plasma Activated Water (CU-PAW)

Preparation of plasma-activated water a non-thermal micro-hollow cathode discharge (MHCD) was used to generate plasma-activated waters (CU-PAWs) and the experimental set-up was similar to that reported previously [66]. In this study, the MHCD was operated

at atmospheric pressure by using two different gases (air and oxygen) as working gases. The gas flow and discharge power were controlled at 4 l per minute and 100 W, respectively.

During this study, deionized water (D. I. water) was used to generate PAW. Discharge plasma was generated by fixing the discharge probe 5.0 mm away from the surfaces of D. I. water in 50-mL conical tubes. After exposure to plasma for specified treatment time (10, 15, and 30 min), those plasma-activated waters were immediately used or collected in 15-mL conical tubes and stored in the refrigerator.

**Detection of Copper**

Detection of copper concentration within plasma-activated water, the concentration of copper and zinc within PAWs was detected by inductively coupled plasma optical emission spectrometry (ICP-OES). ICP-OES measure light emitted at element-specific wavelength from thermally excited analyte ions and atoms. The intensity of this emitted electromagnetic radiation can be converted into an elemental concentration by comparison to calibrated reference standards.

**Delay Time Experiments**

Delay time is the time that starts from when CU-PAW was created by plasma until CU-PAW was exposed to cancer suspensions. To evaluate the effect of delay time, three different time points were selected (0 min, 1 day, and 14 days after plasma treatment). For 0-min delay time, CU-PAW was exposed to cancer suspensions immediately after plasma treatment. For 1- and 14-day delay time points, CU-PAW was stored at 4 °C (in the refrigerator) before anticancer activity assay.

**MCF-7 Human Breast Adenocarcinoma Cell Line**

The MCF-7 human breast adenocarcinoma cell line (IBRC C10082), and 3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was acquired from Sigma Aldrich Co., USA. Doxorubicin hydrochloride (a bright red crystalline powder with the empirical formula $C_{27}H_{29}NO_{11}$ under the trade name Adriamycin) was obtained from IQ product, Netherlands. The fetal bovine serum (FBS) and trypsin-EDTA were obtained from Gibco, USA. Dimethylsulfoxide (DMSO) and N-acetyl-L-cysteine (NAC) were obtained from Merck, Germany. Modified Eagle Medium without sodium pyruvate (DMEM/F12), streptomycin, and penicillin were obtained from Bio-idea, USA. The Annexin FITC kit was obtained from IQ product, Netherlands.

Anthracycline drugs are one of the most important drug classes in chemotherapy. Doxorubicin (DOX), an anthracycline component derived from Streptomyces peucetius, has been used to treat various cancers including those of breast, head and neck, lung, liver, and ovaries. Numerous mechanisms have been proposed to describe DOX antitumor activity; these include disruption of topoisomerase IIα function, protein and nucleic acid synthesis inhibition, DNA intercalation or alkylation, reactive oxygen species (ROS) production, and interaction with the cell membrane [67, 68]. However, DOX initiates several critical adverse effects including congestive heart failure (CHF), myelosuppression, and nausea [69,70]. Additionally, multidrug resistance (MDR) of malignant cells to DOX remains the most significant barrier to effective treatment.

In 1928, plasma was introduced as a fourth phase of matter [71]. This ionized gas is comprised of photons, electrons, ions, and neutrals, which include molecules, excited atoms, and radicals [72-74]. Cold atmospheric plasma (CAP) is a plasma in which the temperature of the heavy particles is between 25 and 45 °C [75]. Applications of plasma for acne, aesthetics, dental caries, and endoscopic applications are well known [72, 76-78]. Recently, indirect and direct CAP treatment became a new subject in plasma medicine. According to the literature, non-thermal atmospheric plasma (NTAP) can be utilized for cancer treatment with few side effects [71, 79-82]. In indirect CAP therapy, the CAP-stimulated solutions (PSS) are applied to affect malignant cell growth in vivo or in vitro by injecting PSS into the malignant tissue. One of the advantages of PSS treatment is that it can be stored for a long time at −80 °C (by optimizing the composition of the solution), so, it can be utilized without dependence on the CAP-producing device [83]. Current studies reported that plasma can affect malignant cells indirectly with water irradiated by plasma or previously prepared medium, termed plasma-activated water (PAW) [84]. The antitumor properties of PAW are due to its ability to eliminate cell survival elements and generate cell death factors such as singlet oxygen, nitric oxide (NO), superoxide, and the hydroxyl radical. These relatively short-lived death factors can be switched to other relatively long-lived species; for example, nitrate/nitrite (NOx) and hydrogen peroxide ($H_2O_2$) [80, 85]. Thus, CU-PAW treatments may represent valuable and innovative tools for malignant therapy [85, 86].

Due to resistance to Doxorubicin (DOX), and its harmful adverse effects, the scientific community is obliged to design novel cancer treatments. Therefore, we aimed to determine the effect of DOX in the presence of CU-PAW prepared at various time points on MCF-7

cell proliferation by MTT and N-acetyl-L-cysteine (NAC) assays, and flow cytometry. The results of this research could offer advantageous and innovative clinical strategies for cancer therapy in the future.

**Samples Preparation**

Log-phase MCF-7 cells were treated with trypsin-EDTA and seeded in 96-well plates. The cells were washed with PBS buffer, 1×105 cells/well were seeded into 96-well plates, and 180 μl of growth medium were added to each well. The cells were randomly divided into one control and four experimental groups. The experimental groups were [86] cells incubated with 80 μl of PAM (plasma irradiated for 1, 2, 3, or 4 min) for 48 h, [87] cells incubated with sterilized DOX at 0.025, 0.1, 0.2, 0.4, 0.62, and 1 μM for 48 h, [88], cells incubated with 3-min CU-PAW plus sterilized DOX (0.45 μM) for 48 h, and (4) cells incubated with 4-min CU-PAW plus sterilized DOX (0.45 μM) for 48 h. Control cells received no CU-PAW or DOX. In this study, the variations in temperature and pH of the medium before and after plasma irradiation were negligible.

**Cell Viability Measurements**

After 48 h of incubation 0.5 mg/ml MTT (20 μl) was added to the control and experimental cells and the cells were incubated for 3 h in a $CO_2$ incubator at 37 °C. Later, the insoluble formazan formed was dissolved in 100 μl of DMSO and mixed (25, 26). The optical density (OD) of each well was measured at 570 nm against a reagent blank on an ELISA reader (ELx808, BioTek Instruments, Inc., USA). Each trial was repeated 4 times.

**Morphology Alterations Research**

After 48 hrs of incubation, the medium was removed from the wells and the cells were washed once with 2 ml of cold PBS buffer (0.01 M, pH 7.4). The cells were photographed on an inverted microscope (INV100; BEL Engineering, Italy), and morphological alterations were determined as previously described [89,90].

**Measurement of Reactive Oxygen Species (ROS)**

The effect of DOX plus CU-PAW on ROS generation in the absence or presence of NAC was investigated. NAC powder was dissolved in DMEM, then the cells were incubated with 0-, 3-, 4-, or 5-mM NAC for 3 h in a $CO_2$ incubator at 37 °C. The cells were then

incubated with CU-PAW. After 48 h, 20 µl of 0.5 mg/ml of MTT were added to each well and cell viability was determined by MTT assay.

**Flow Cytometry**

Annexin V binding was determined via the Annexin FITC kit. Cells were seeded into 6-well plates at 106 cells/well for 48 h, then treated with CU-PAW. The cells were collected by centrifugation for 5 min at 1000 × g and washed twice with PBS buffer (0.01 M, pH 7.4), suspended in 100 µL of Annexin V binding buffer (10 mM HEPES/NaOH, pH 7.4, 140 mM NaCl, and 2.5 mM $CaCl_2$), and double-stained with 5 µL of propidium iodide and 5 µL of FITC-labelled Annexin V. All samples were incubated for 30 min in the dark at room temperature and analysed by flow cytometry on a BD FACS Calibur™ (BD Biosciences Inc., USA).

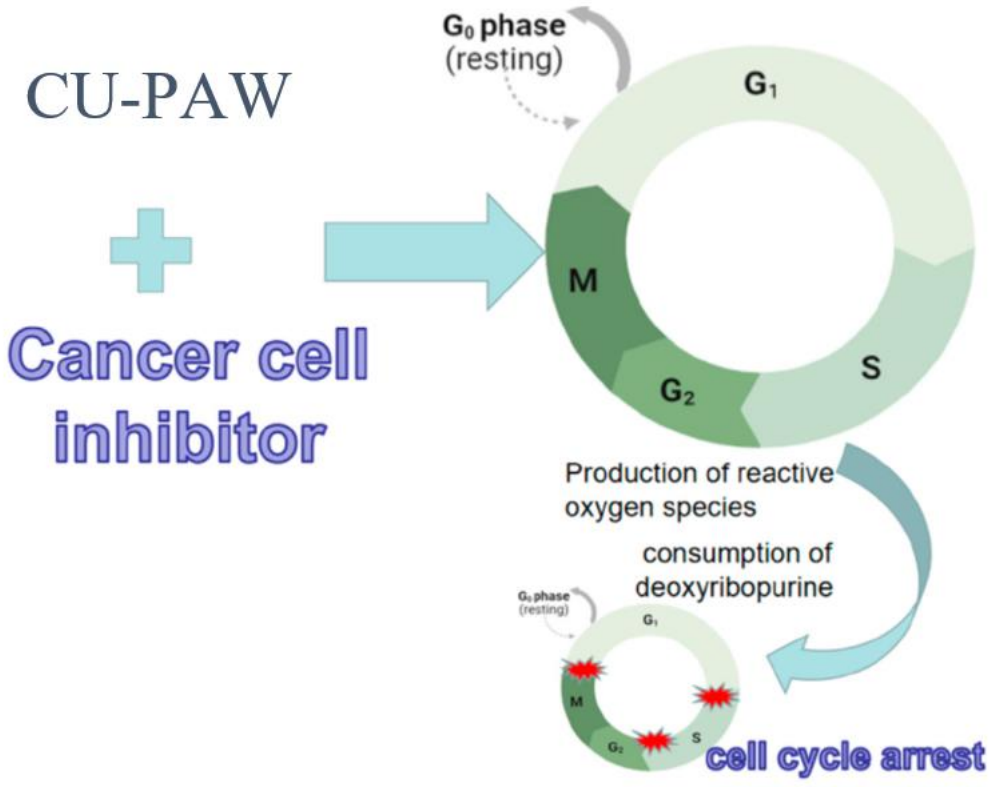


**Figure 2:** Cu-PAW binding inhibitors induce reactive oxygen species production and deoxy ribosyl purine depletion. Treatment of cancer cells with copper in combination with NSC319726 enhances the inhibitory effect of this compound on cancer cells, leading to the intracellular production of reactive oxygen species and the depletion of

deoxyribonucleopurines (e.g., 20-deoxyguanosine), inducing cellular G-phase arrest and apoptosis.

### Statistical Analysis

Significant differences were evaluated by t-test using GraphPad Prism Software (Version 8.4.3, GraphPad Software Inc., San Diego, USA). The flow cytometry data were analysed using Flow Jo software (Version 7.6.1). All data were reported as means±standard deviations.

## RESULTS

### Growth Rates of MCF-7 Cells

We assessed whether Doxorubicin (DOX), or CU- PAW affected cell viability. As described in materials and methods, MCF-7 cells were first examined after incubation with increasing DOX concentrations for 48 h. Cell viability decreased in a dose-dependent manner (Figure. 3). The IC50 (50% inhibition concentration) of DOX was determined to be 0.49 μM. The MTT assay also demonstrated that CU-PAW prepared with increasing irradiation periods reduced cell viability in a time-dependent manner (Figure 4). The addition of 1- or 2-min CU-PAW did not affect cell viability. In the follow-up experiments, cells were incubated in 0.46 μM DOX (less than the IC50) plus 3- or 4-min CU-PAW for 48 h to attain the optimum combination condition that impacted the majority of tumor cells. Cell viability was significantly less in the DOX plus 3-min CU-PAW than in the DOX or 3-min CU-PAW, and in the DOX plus 4-min CU-PAW than in the DOX or 4-min CU-PAW (Figure 3 $p< 0.01$ for both). The addition of 1- or 2-min CU-PAW to DOX did not significantly affect cell viability compared to DOX alone (Figure. 5).

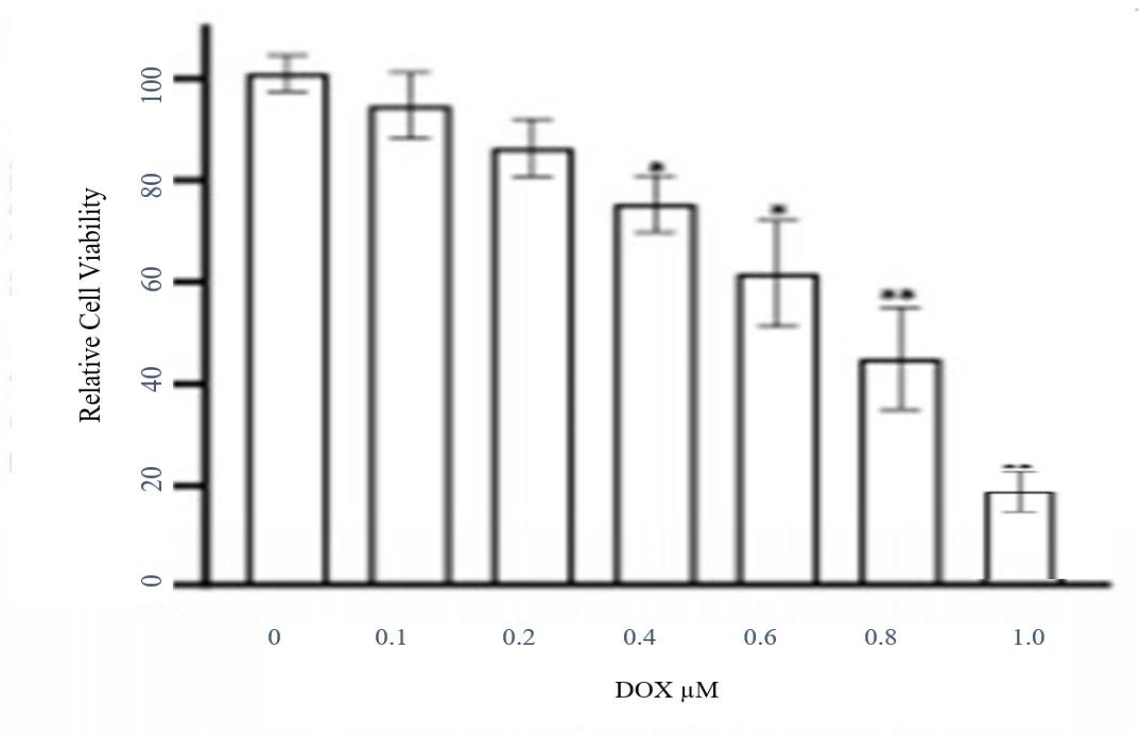


**Figure 3:** Cell viability decreased in a dose-dependent manner

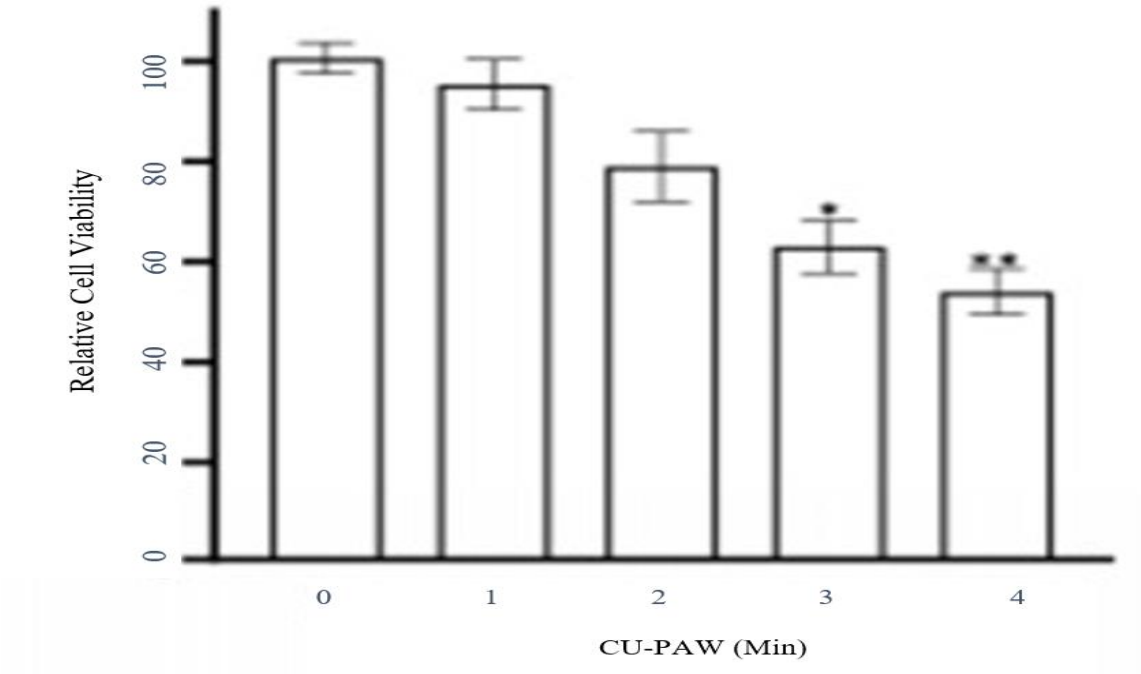


**Figure 4:** The MTT assay also demonstrated that CU-PAW prepared with increasing irradiation periods reduced cell viability in a time-dependent manner

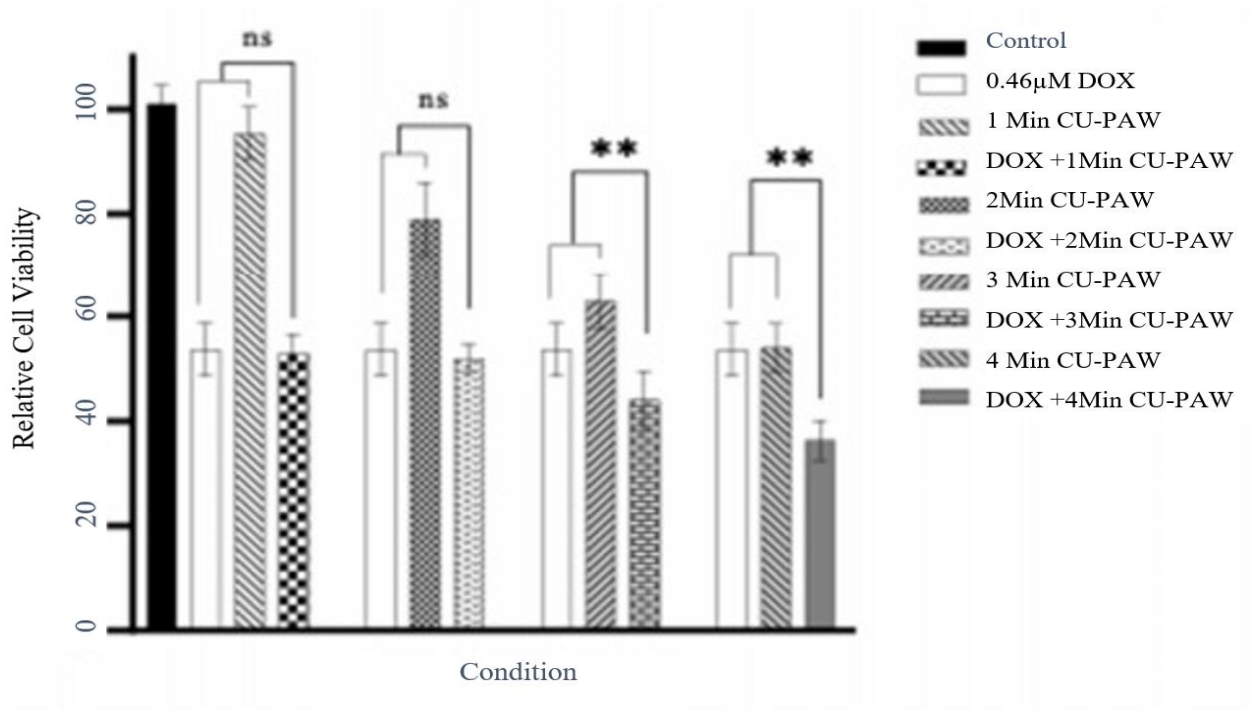


**Figure 5:** The addition of 1- or 2-min CU-PAW to DOX did not significantly affect cell viability compared to DOX alone.

**ROS Measurements**

To explore whether oxidative stress contributed to cytotoxicity, cells were treated with DOX, 3- or 4-min CU-PAW, or DOX plus CU-PAW in the presence of 0-, 3-, 4-, or 5-mM NAC. Pre-treatment with NAC inhibited DOX-induced cytotoxicity at all NAC concentrations in a dose-dependent manner ($p< 0.05$ (for 3-mM NAC) and $p< 0.01$ (for 4- and 5-mM NAC)) (Figure. 6). The results were slightly different with 3- and 4-min CU-PAW; 3-mM NAC had almost no effect; however, 4- and 5-mM NAC inhibited CU-PAW-induced cytotoxicity in a dose-dependent manner ($p< 0.05$ for 4-mM NAC and $p< 0.01$ for 5-mM NAC) (Figure. 7). In the follow-up experiments, cells were treated with DOX plus 3-min CU-PAW and DOX plus 4-min CU-PAW in the presence of 5-mM NAC. It was observed that 5-mM NAC significantly inhibited DOX plus 3-min CU-PAW and DOX plus 4-min CU-PAW cytotoxicity (Figure 8, $p< 0.01$ for both).

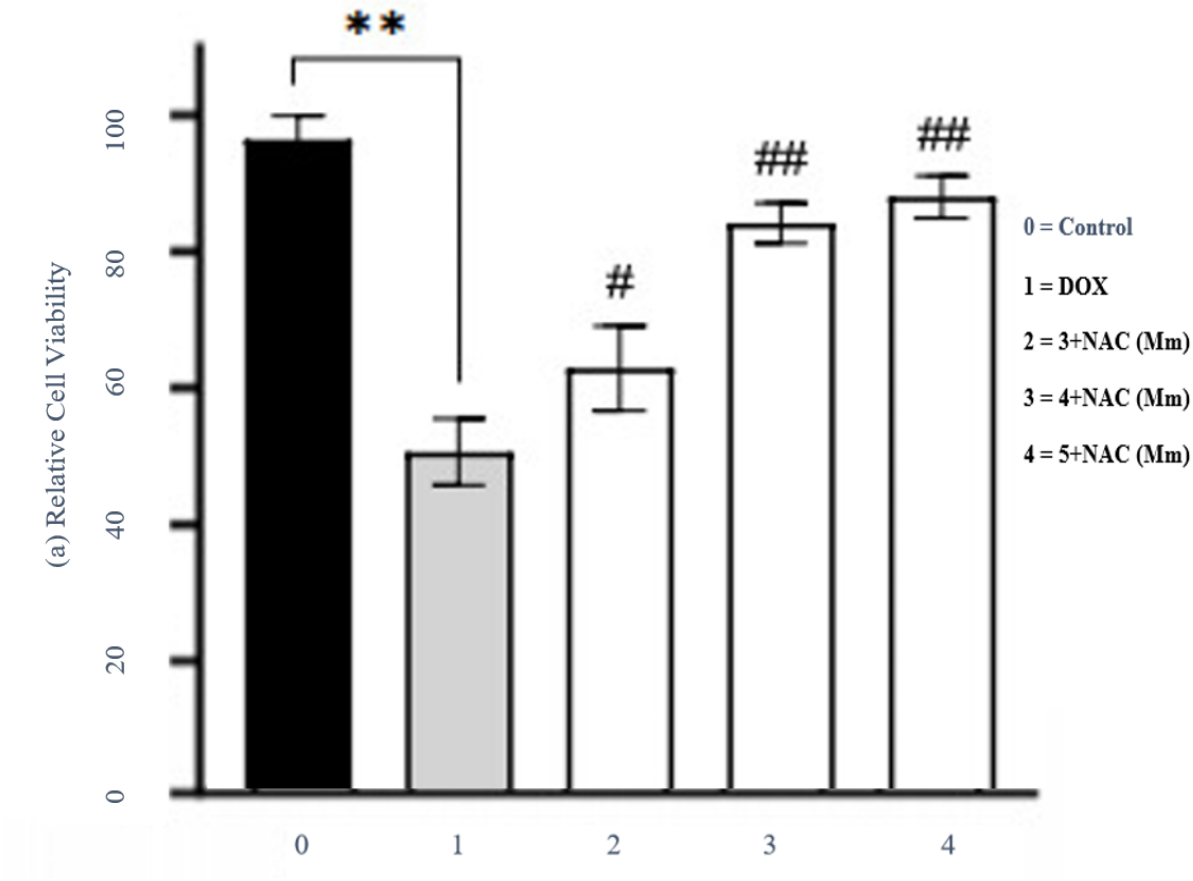


**Figure 6**: Oxidative stress contributed to cytotoxicity, cells were treated with DOX, 3- or 4-min CU-PAW, or DOX plus CU-PAW in the presence of 0-, 3-, 4-, or 5-mM NAC

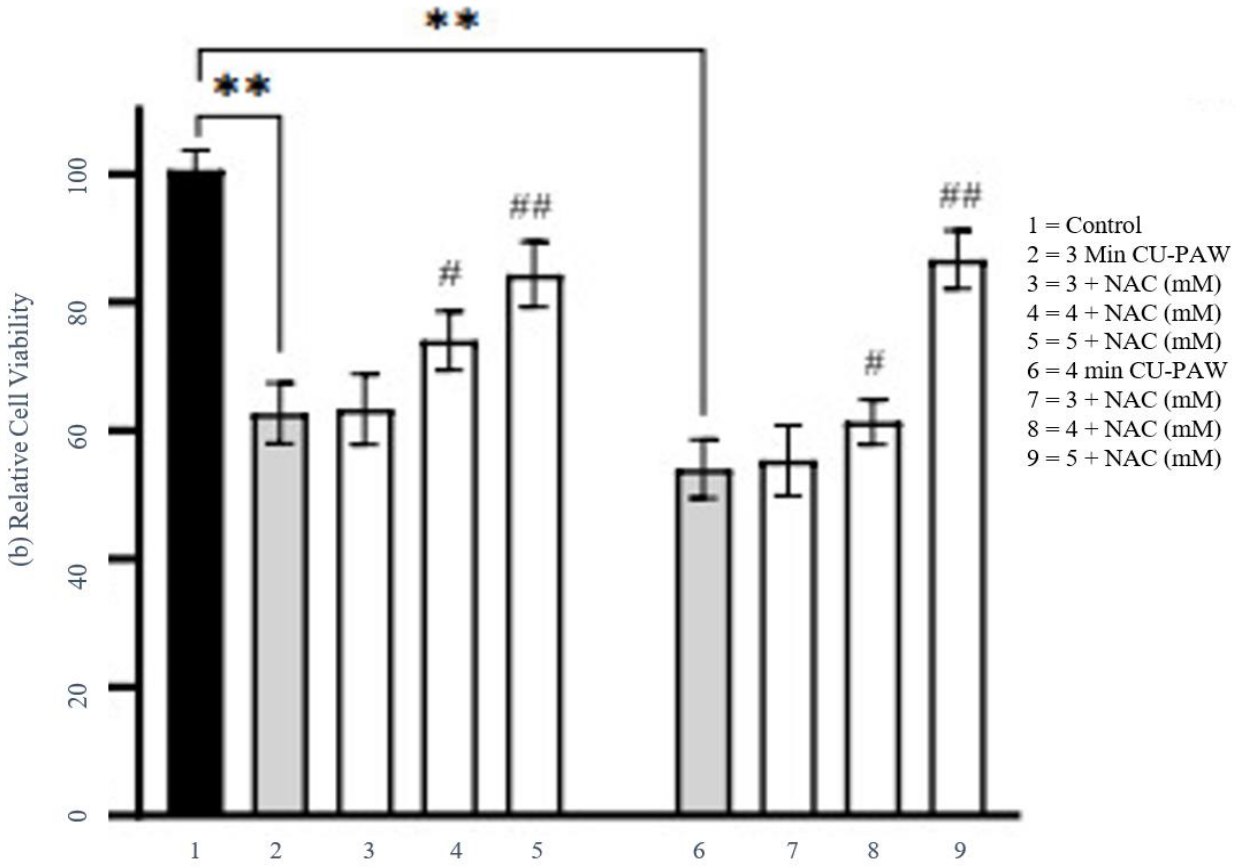


**Figure 7:** 4- and 5-mM NAC inhibited CU-PAW-induced cytotoxicity in a dose-dependent manner ($p < 0.05$ for 4-mM NAC and $p < 0.01$ for 5-mM NAC)

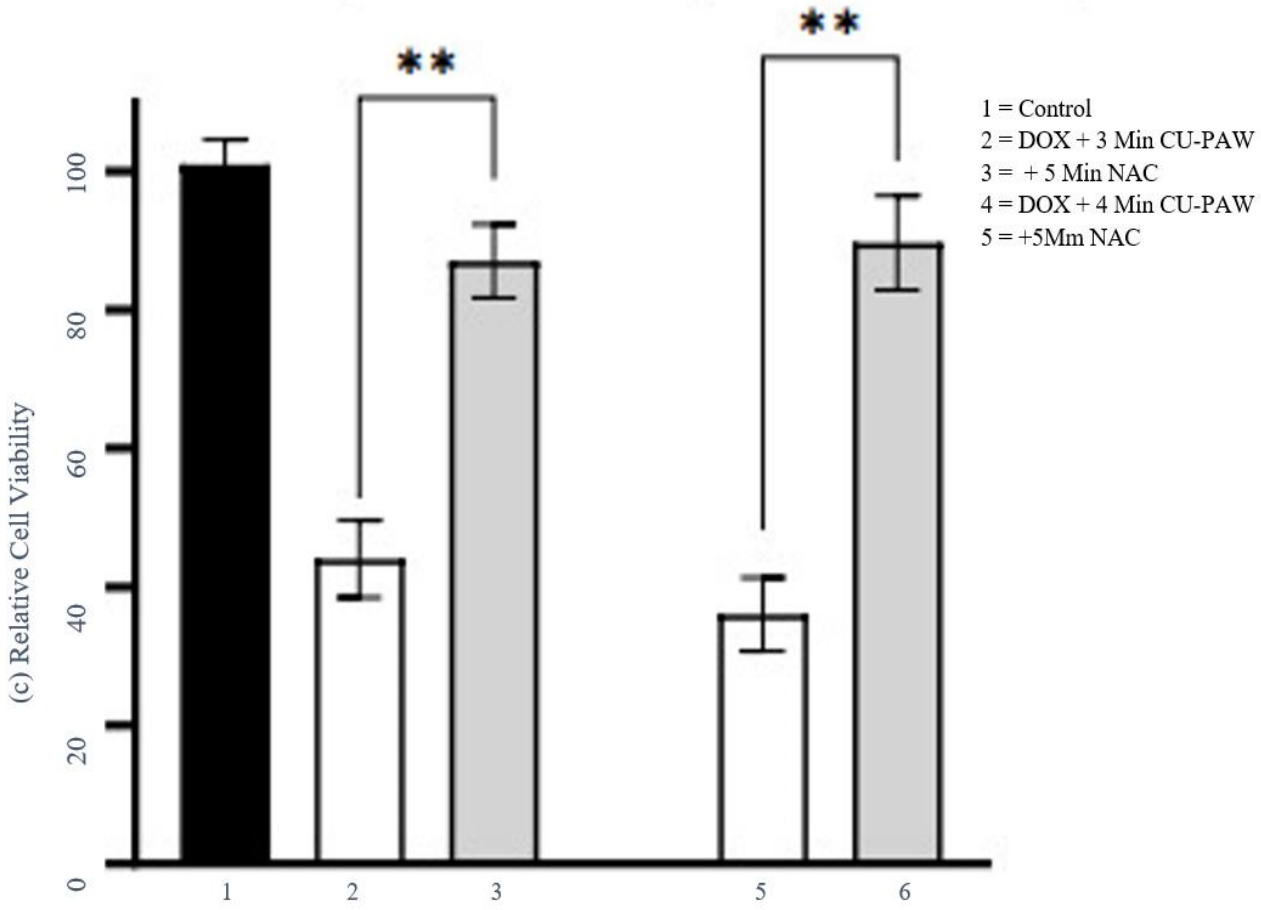


**Figure 8:** 5-mM NAC significantly inhibited DOX plus 3-min CU-PAW and DOX plus 4-min CU-PAW cytotoxicity

Measurement of the reactive oxygen species (ROS) generation by MTT assay. MCF-7 cells were incubated for 3 hrs at 37 °C with 0-, 3-, 4-, or 5-mM NAC; then incubated for 48 hrs with: (a) 0.45 μM DOX (**p< 0.01 vs. the control group; #p< 0.05 and ##p< 0.01 vs. the DOX-treated group) and (b) 3- or 4-min CU-PAW (**p< 0.01 vs. the control group; #p< 0.05 and ##p< 0.01 vs. the CU-PAW-treated group). In (c) two sets of cells were first incubated for 3 h at 37 °C with 5 mM NAC; then one set was incubated for 48 hrs with 45 μM DOX plus 3-min CU-PAW and another set with 45 μM DOX plus 4-min CU-PAW. One other set of cells received 45 μM DOX plus 3-min CU-PAW and another set received 45 μM DOX plus 4-min CU-PAW. These cells received no NAC. Values are means±standard deviations; ** indicates p< 0.01.

### Effects of DOX Plus CU-PAM on Cell Apoptosis and Necrosis

The cells were incubated with 0.45 μM DOX, 3- and 4-min CU-PAW, or DOX plus CU-PAW, and analysed by flow cytometry (Table 1.). The early apoptotic cell percentages were dramatically greater in the 0.45 μM DOX, 4-min CU-PAW, and DOX plus 3-min CU-PAW-treated cells (33.12Q4b, 30.19Q4d, 43.20Q4e, respectively) compared to control cells (8.63Q4a). The late apoptotic cells percentages were also greater in the 0.45 μM DOX, 4-min CU-PAW, and DOX plus 3-min CU-PAW-treated cells (15.13Q2b, 15.45Q2d, 16.25Q2e, respectively) compared to control cells (2.55Q2a). However, the early apoptotic cell percentages did not change in the DOX plus 4-min CU-PAW-treated cells compared to control cells (8.23Q4f % vs 8.63Q4a). Surprisingly, the percentage of necrotic cells was dramatically greater in the DOX plus 4- min CU-PAW-treated cells than in controls (46.02Q1f vs 0.28Q1a%, $p < 0.01$), but necrosis was similar to controls for all other incubated cells (Figure.9).

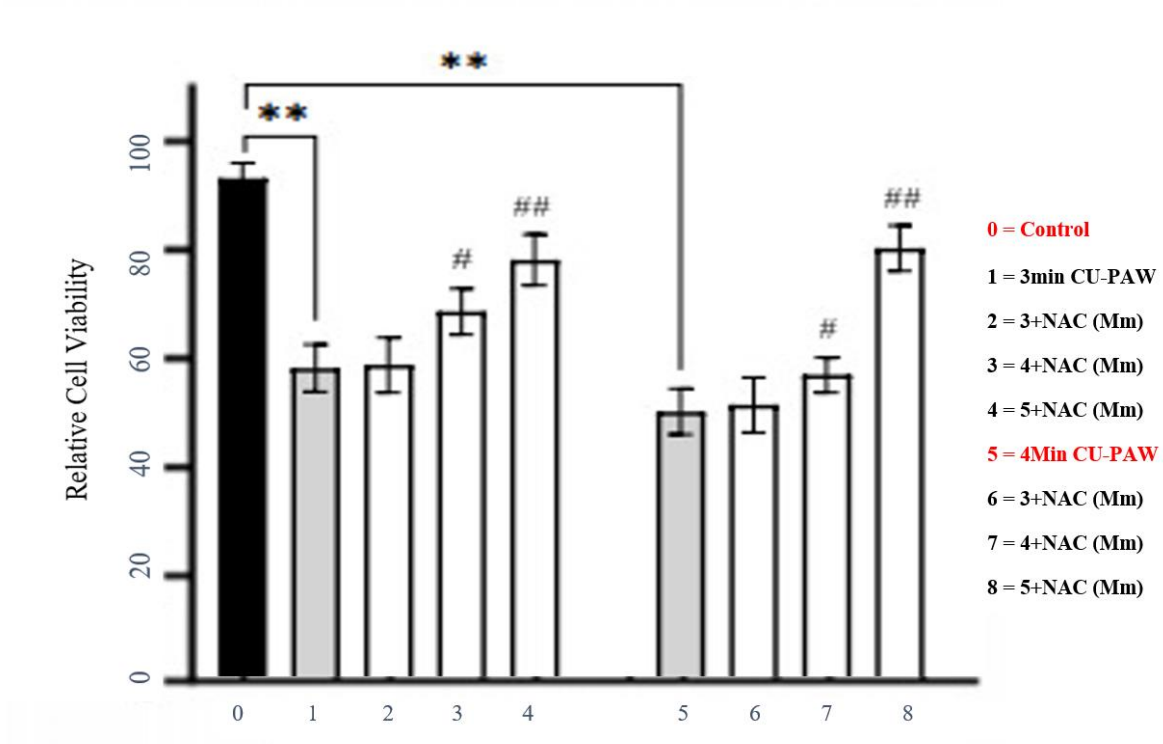


Figure 9: Effects of DOX plus CU-PAW on cell apoptosis and necrosis

**Table 1:** Flow Cytometry analysis of MCF-7 Cells (Annexin V-FITC+/PI)

| | a | b | c | d | e | f |
|---|---|---|---|---|---|---|
| Q1 | 0.28 | 0.23 | 0.47 | 0.35 | 0.27 | 46.02 |
| Q2 | 2.55 | 15.13 | 25.68 | 15.45 | 16.25 | 12.20 |
| Q3 | 89.21 | 63.11 | 66.15 | 57.13 | 44.28 | 37.50 |
| Q4 | 8.63 | 33.12 | 10.05 | 30.19 | 43.20 | 8.23 |

NB: Flow cytometry analyses of MCF-7 cells: (a) control cells, (b) cells incubated for 48 h with 0.45 μM DOX, (c) cells incubated for 48 h with 3-min CU-PAW, (d) cells incubated for 48 h with 4-min CU-PAW, (e) cells incubated for 48 h with 0.45 μM DOX plus 3-min CU-PAW, (f) cells incubated for 48 h with 0.45 μM DOX plus 4-min CU-PAW. (g) percentage of cell populations by flow cytometry analysis (@p< 0.05 and @@p< 0.01 vs. the control group; *p< 0.05 and **p< 0.01 vs. the control group; #p< 0.05 and ##p< 0.01 vs. the control group; ++p< 0.01 vs. the control group).

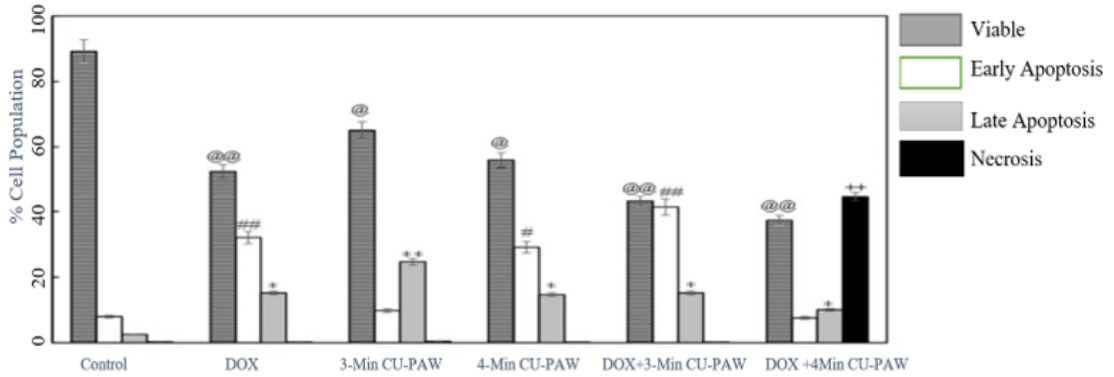


**Figure 10:** Percentage of necrotic cells was dramatically greater in the DOX plus 4- min CU-PAW-treated cells than in controls (44.8 vs 0.28%, p< 0.01), but necrosis was similar to controls for all other incubated cells

## DISCUSSION

Cancer is currently the first or second most common cause of premature death in most countries. The incidence of all cancers combined is projected to double by 2070 compared to 2020 [91]. There is still a lack of effective treatments for many types of cancer, including breast cancer and melanoma, so the search for new compounds with potential selective

efficacy against tumor cells seems reasonable. Therefore, the Non-thermal plasma therapy has been investigated as a useful but challenging cancer treatment. Previous results indicated that the target of direct plasma treatment is limited to superficial lesions. More recently, CU-PAW has been utilized as an indirect plasma therapy, which is more practical than the previous method, because tumor cells inside the peritoneal cavity are wholly and directly subjected to cytotoxic fluids [92,92]. On the other hand, despite decades of good results in the application of Doxorubicin (DOX), as a chemotherapeutic agent, this anticancer agent generates cumulative, dose-dependent adverse reactions in patients. Thus, this study focused on elucidating the effects of CU-PAW and DOX combinations on MCF-7 cell proliferation. Chemically active CU-PAW can be generated by NTAP in a culture medium [93]. Thus, we first prepared CU-PAW with various irradiation periods by plasma jet. Then, we assessed whether DOX in the presence of CU-PAW could change cell viability. MTT identifies cell mortality at the advanced stage of apoptosis, wherein tetrazolium salt metabolization is diminished. The MTT results demonstrated that CU-PAW prepared with different irradiation periods affected MCF-7 cell viability in a time-dependent manner. The 3- and 4-min CU-PAW reduced MCF-7 cells viability to approximately 62% and 56% of control ($p< 0.01$), respectively. However, in the cases of 1- and 2-min CU-PAW cell proliferation did not diminish significantly as compared with the control group ($p> 0.05$). This observation is consistent with earlier studies, which illustrated that plasma irradiation reduced cell viability in a time-dependent manner [94, 95-99]. Thus, in this research, DOX (0.45 μM) combined with 3- or 4-min CU-PAW killed MCF-7 cell efficiently (44% and 39% cell viability, respectively; $p< 0.01$) than DOX (54% cell viability) or 3- or 4-min CU-PAW alone (63% and 56% cell viability, respectively). These was in line with a that PAW plus cisplatin at low doses reduced viability of human endometrial carcinoma more effectively than cisplatin or PAW alone [100]

As mentioned above, previous research confirmed that plasma irradiation can induce ROS production (101, 102, 103). Generally, NAC has been utilized as an antioxidant and can scavenge hypochlorous acid (HClO), $H_2O_2$, and hydroxyl radicals (·OH) directly but not superoxide radicals ($O_2$-). Our results showed that 4- and 5-mM of NAC reduced statistically significant DOX- and CU-PAM-induced cytotoxicity. Interestingly, 5-mM NAC pretreatment significantly reduced the cytotoxic effect of DOX plus 3- or 4-min CU-PAW ($p< 0.01$). Several studies showed that plasma irradiation can induce ROS production, including $H_2O_2$, $NO^{2}-$, and $NO_3-$ within cancer cells, and in the liquid phase (extracellular

culture medium), and subsequently induce cell death in cell lines, including breast cancer cells [104, 105, 106]. Conway et al., [107] demonstrated that NAC could not alleviate the cytotoxic effects of NTAP in relatively NTAP-resistant cells, but was protective against toxicity caused by NTAP in NTAP-sensitive cancer cells.

Consequently, even though the exact components of CU-PAW remain unclear, CU-PAW-induced cellular ROS play a crucial role in anti-proliferative effects against cancer cells (18, 35). Thus, our results suggest that ROS is one of the effectors in CU-PAW in combination with DOX to abolish MCF-7 cell viability, i.e., MCF-7 cells undergo ROS-dependent cell death in response to CU-PAW plus DOX. It could be hypothesized from our results and previous investigations [1-08] that due to MCF-7 cells' high metabolic rate, the cells were under high oxidative stress; consequently, they could not tolerate an increase in ROS; thus, cell death occurred after 48 hrs of incubation with DOX plus CU-PAW.

When cells were treated with DOX plus 3-min CU-PAW, the percentage of apoptotic cells increased considerably (43.20% of cells were Annexin V-FITC+/PI-, early apoptotic cells ($p< 0.01$)) and 16.25% of cells were Annexin V-FITC+/PI+ (late apoptotic cells ($p< 0.05$). Surprisingly, after treatment with DOX plus 4-min CU-PAW, 46.02% of the cells were Annexin V-FITC-/PI+($p< 0.01$), which recognizes primary necrotic cells. Thus, our results demonstrated that DOX plus CU-PAW effectively generates cell death in MCF-7 cells; however, this process is irradiation time-dependent. Hence, based on our results, although the relative mechanisms need to be explored further, DOX combined with CU-PAW represents an alternative strategy for cancer treatment while having minimal harmful side effects on normal adjacent cells or tissue.

## CONCLUSION

we demonstrated that 3-min CU-PAW plus DOX, which induced apoptosis through intracellular ROS generation, had greater antiproliferative effects on MCF-7 cells than did DOX alone. We also showed that in 4-min CU-PAW plus DOX the percentage of necrotic cells increased dramatically. Hence, DOX combined with 4-min CU-PAW has cytotoxic effects with different mechanisms than 4-min CU-PAW alone, in which the number of apoptotic cells increases. Although further investigations are crucial, CU-PAW combined with DOX could be a promising cancer treatment strategy, contributing to a more positive therapeutic Proposal.

**Conflicts of Interest:**

The authors declare no conflicts of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

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