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EFFECT OF AIR PLASMA ON HUMAN HEPATOCELLULAR CARCINOMA (HEPG2) AND NORMAL LIVER (THLE2) CELLS

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ABSTRACT

Background, hepatocellular carcinoma is the fourth cause of cancer related death, with increasing morbidity and mortality over the last decades. Plasma Activated Water (PAW) has received a great attention due to its potency in cancer treatments. **Aims**, the current study aims at investigating long-term effects of PAW on the viability of HepG2, as well as THLE2 to assess their potential antitumor activity *in vitro*. **Methods**, PAW was generated by exposing the tap water to three different doses of plasma. Following each exposure, PAW was divided into two aliquots; one used for treatment one day after being generated and the other aliquot was kept at room temperature for 60 days before using for treatment. HepG2 and THLE2 cells were treated using different concentration of PAW, and their viability were assessed utilizing MTT assay. **Results**, a significant increase in both pH and dissolved oxygen was observed after incubation for 60 days compared with incubation for one day. Meanwhile, the level of NO_2 showed a slight increase. Treatment with PAW significantly reduced the viability of HepG2 cells in an exposure time dependent manner. Moreover, keeping PAW for (60 days) before treatment increased its efficiency in reducing viability of HepG2 compared to the freshly prepared PAW in the presence of cisplatin as reference. Interestingly, PAW, does not have harmful impact on normal cell lines. **In conclusions**, these results suggest that some plasma active species in tap water remains effective up to 60 days. PAW demonstrated a selective inhibitory effect on theHepG2 cells rather than the THLE2 cells. Furthermore, PAW, besides having significant selective lethal impact against HepG2, is economic as well impact when compared with cisplatin.

KEYWORDS: Plasma activated water(PAW), HepG2, THLE2, MTT assay, cisplatin. Incubation of PAW, Long term effects of PAW.

1 INTRODUCTION

Hepatocellular carcinoma (HCC) is the most frequent type of primary liver cancer and is one of the leading causes of cancer death worldwide in recent years.[1] HCC is nearly always associated with persistent liver damage caused by viral infections or other causes. Among the numerous etiologies of HCC, viral aetiology is the most common, with Hepatitis B virus infection being the most common. Viral infections, along with alcoholic liver disease and nonalcoholic hepatitis, are the leading causes of cirrhosis, which can lead to death. About 90% of all HCC cases is associated with chronic liver diseases and cirrhosis.[2] In the majority of cases, HCC is detected at a late stage, which limits therapeutic options. Chemotherapy, conventional, liver transplantation, surgical resection, and radio frequency ablation[3] are currently accessible treatments that are only effective in a tiny percentage of patients. However, as evidenced by recurrent recurrence and low response rates, these interventions are ineffective.[4] In order to improve the success of hepatocellular carcinoma treatment, it is critical to find new effective and safe therapeutic agents.

A new and promising method in this field is non- thermal (cold) atmospheric plasma. Plasma is classified into a fully or partially ionized plasma that contains negative and positive ions, free radicals, free electrons, and ultraviolet and visible radiation.[5,6] Cold atmospheric plasma (CAP) has been produced for a variety of biomedical purposes. Actually, CAP has been developed for different medical applications. Cold plasma, have been investigated in blood coagulation[7], sterilization, inactivation of several microorganisms, skin regeneration, wound healing, cancer therapy and tooth bleaching.[8] It has been shown repeatedly in literature that CAP induces apoptotic pathway in cancer cells.[9] Several cancerous cell lines were investigated in the last decade, such as melanoma[10], and lung carcinoma cells.[11] The production of reactive nitrogen species (RNS) and reactive oxygen species (ROS) by plasma is to blame for this.[12] There are different CAP devices that can directly be applied to biomedical applications. One

of these devices is plasma jet under the present study. In plasma jet device, the system is fed by a constant flow of air, in which discharge occurs between the powered electrodes configuration. As the ionized gas is transported outside in propagating ionization waves, it forms a stream of active species extending in the ambient air as a jet of length up to millimeters away from the cathode surface.[13] Some plasma species produced in the gaseous phase above the liquid are carried through the plasma–liquid contact into the water when air plasma is a water surface. As a result, this water becomes activated and is referred to as plasma-treated or plasma-activated water (PAW)[14,15] PAW contains a variety of chemically active species formed in gaseous plasma and at the plasma–liquid interface, particularly RNS and ROS, which are referred to as RONS collectively. OH, O, NO, NO2, H, H2O2, O3, NO2, NO3, and ONOOH are some of the most important species detected in the bulk liquid of PAW and may be involved in numerous cell mechanisms.[16] Generation of PAW will be the connecting link between the unique properties of the non-thermal plasma and the way it will be used in different biomedical applications.

Plasma activated water (PAW) has different applications in medicine.[17] The device under the present study was used for tap water treatment to generate PAW. PAW was used, afterwards, for oral administration of rats according to[18], who clearly revealed that treatment of tap water with plasma jet significantly increased its content of dissolved oxygen (DO) and NO_2 as well as pH; this increase in DO, NO_2 and pH occurred in a dose-dependent manner. Moreover, Shaimaa et al. postulated that oral administration of plasma treated water for long period (60 days) in albino rats does not have destructive effects. In addition, no mortality or changes in the behavior were observed in the PAW treated rats. Hence, this preliminary data supports the aims of the current study in using (PAW) on a large scale in biomedical applications. Over the past few years, the anticancer capacity of non-thermal plasma has been investigated in different cancer cell lines such as skin[19], breast, pancreas[20], lung[21], cervix[22] and brain[23], yet, it is hardly investigated in hepatocellular carcinoma as well as in using aging PAW against hepatocellular carcinoma. As consequence, the present study will focus on the effects of plasma activated water at different plasma doses and after incubation of PAW for a day as well as 60 days against the viability of both HepG2 and THLE2 cells. Furthermore, the present study provides a solution to the unanswered question, that has long been confusing about whether the effect of plasma active species in water temporary or prolonged?

2. MATERIALS AND METHODS

2-1-Chemicals

All the materials employed in this experiment were of a high analytical grade and obtained from a variety of commercial sources, including.

Plate, 96-well plate (Falcon, Franklin Likes, NJ), High‑ glucose Dulbecco's Modified Eagle's Medium (DMEM) (GIBCO, Grand Island, New York, USA; Cat.no. A1049101). Cisplatin was purchased from Bristol-Myers, Squibb. [3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a yellow tetrazole, (Molecular probes, Eugene, Oregon, USA; Cat.no.V-13154)], microplate reader (ELX800) was obtained from Biokit, Spain, isopropanol was purshased from BDH, Poole, England.

Phosphoric acid, sulfanilamide, N-(1-naphthyl)-ethylenediamine dihydrochloride, sodium oxalate, ferrous ammonium sulfate, sodium nitrite, permangante solution, all kept from commerical reagent grade(El gomohory componey Zagzig,Egypt).

2-2 Cell line

Human liver cancer cells (HepG2) and normal liver cells (THLE2) were obtained from, American type Culture Collection (ATCC), Manassas, Virginia, USA.

2-3- The atmospheric non-thermal plasma jet (ANPJ) device used in this study

The device (ANPJ) under consideration was designed, developed and operated at the Plasma and Nuclear Fusion Department, Nuclear Research Center, Egyptian Atomic Energy Authority to conduct researches in cold plasma biomedical applications.[24]

2-4- Tap water

In the current study, tap water, obtained from Plasma and Nuclear Fusion Department**,** Nuclear Research Center (NRC), Egyptian Atomic Energy Authority (EAEA) was expossed to plasma jet, except for tap water samples**,** in all cases of discharges operated non-thermal plasma in contact with or in close vicinity to water, acidification and nitration was observed.[25] Therefore, in the current study, tap water was chosen for exposure to plasma jet to rule out any probability of acidification**.**

2-5- Plasma activated water (PAW)

PAW, was generated using an experimental setup similar to the one previously described by **Ahmed et al.**[24], using air as the feeding gas. The distance between the cathode surface and the treated water surface was fixed at 2 cm using a special holder. PAW was obtained by exposure of tap water (100 ml) to direct non-thermal plasma jet for (5, 10 and 15) minutes with corresponding exposure plasma doses of (0.212, 0.425 & 0.637 J/cm2), respectively, according to a previous investigation.[18] The generated PAW samples were kept in closed containers filled up to few mm below cap, at room temperature (25–30°C), for incubation, and, then, analyzed one day after treatment, as well as after 60 days of the incubation. The incubated PAW samples are used against (HepG2 and THLE2) as shown in Fig. (1). During laboratory trials, the authors of the present work found that the exposure dose (5 minutes) of tap water remarks the beginning of a significant difference in pH,

NO_2 and DO levels as compared with the tap water control samples. Moreover, the incubation of PAW was conducted in room temperature to test the sterilizing activity of plasma jet. PAW at 5 minutes exposure continued to retain its properties (color, odor and purity) for 65 days of incubation, rather than (10,15 minutes) which still for 6 and 9 months respectively. Therefore PAW was used at exposure time of 5 minutes and its replicates (10 and 15 minutes) as well as using PAW after 60 days only of incubation to decrease any risks.

2-6- Quantification of NO_2 and dissolved oxygen (DO) and pH in plasma activated water after one and 60 days of incubation

The RONS in PAW typically contain long-lived species including nitrates (NO3), nitrites (NO2), hydrogen peroxide (H2O2), and ozone (O3), with half-lives of years, days, 104 seconds, and several to dozens of minutes, and (relatively) short-lived radicals such as hydroxyl radicals (•OH), nitric oxide (NO•), superoxide (O2), peroxynitrate (OONO2), and peroxynitrites (ONOO), with half-livetimes of 1 ns, a few seconds, 1.5 s, and less than l s, respectively (26&27).DO concentration is also an important metric for characterizing natural and wastewaters, as well as assessing the overall status of water quality.[28] Therefore, current study evaluates only (NO_2) as RNS, and DO in PAW after one and sixty days of incubation rather than ROS.

2-6-1- The majority of NO was generated in the gas phase during the afterglow a few milliseconds after the discharge pulse, while NO2- was mostly created as NO. The principal breakdown product of NO (of life time in milliseconds) in water is known as NO2 (29), and through the following pathways (30). Thus, current study infers levels of NO via measuring the levels of NO_2

$$N_2 + e \rightarrow 2N + e \quad 1$$

$$N + O_2 \rightarrow NO + O \quad 2$$

$$4NO + O_2 + 2H_2O \rightarrow 4NO_2^- + 4H^+ \quad 3$$

Measuring of No_2 value

Levels of NO_2 were estimated using a colorimetric method according to (31). Ntrite (NO2) is determined by combining diazotized sulfanilamide with N-(1-naphthyl)-ethylene diamine dihydrochloride at pH 2 to 2.5 to generate a reddish-purple azo dye (NED dihydrochloride). The absorbance of the product was then measured using absorbance spectroscopy at 543nm of a 1 cm light path (Spectramax M2).

Calculations

A standard curve was prepared by plotting absorbance of standards against $N0_2$ –N concentrations, and computing the sample concentrations directly from curve.

2-6-2- pH Value: pH electrode is used to measure the pH value within a liquid according to a previous publication.[32]

2-6-3- Dissolved oxygen (DO)

According to earlier investigations (33 and 34) membrane electrodes method (SM.no 4500- o. G 23St Ed: 2017) provide an excellent method for dissolved oxygen (DO) analysis in polluted waters, highly colored waters, and strong waste effluents. The apparatus consists of oxygen-sensitive membrane electrode, with appropriate meter, which has accuracy of ±0.1 mg DO/L and a precision of ±0.05 mg DO/L.

2-7- Experimental design

In the current study, the potential inhibitory effect of PAW against both the hepatocellular carcinoma cell line HepG2 and the normal hepatic cell line THLE2 was assessed (Figure 1). This is in addition to assessing the effect of plasma doses and the elapsed time after plasma exposure on the efficiency of PAW inhibitory effect. The following treatments were assessed in the present design. To conduct these assessments, PAW was generated by exposing the tap water to three different doses of plasma by varying the exposure time; namely five minutes to give a dose of 0.212 J/cm^2, 10 minutes to give of 0.425 J/cm^2 and 15 minutes to give a of 0.637 J/cm^2.

Following each exposure, PAW was divided into two aliquots; one used for treatment after one day and the other aliquot was kept at room temperature for 60 days before using for treatment. The treatment of cells was then carried out according to the following scheme (Figure 1).

1- **Negative control treatment (tap water),** different dilutions of tap water (6.25- 200 µL /mL) via serial dilutions as (1/160, 1/80, 1/40, 1/20, 1/10 and 1/5) were used for "treatment" of both HepG2 and THLE2.
2- **Positive control treatment (Cisplatin used commonly for HCC treatment),** different concentrations of cisplatin (6.25-200 µL /mL) were used against both HepG2 and THLE2.
3- **Plasma activated water (PAW), (**6.25-200 µL /mL), of varying exposure time periods (5, 10 and 15 min.) and after one day of incubation were used against HepG2.
4- **Plasma activated water (PAW),** (6.25-200 µL /mL), of varying exposure time periods (5, 10 and 15 min.) and after 60 days of incubation were used against HepG2.
5- **Plasma activated water (PAW), (**6.25-200 µL /mL),of exposure time (15 min.) and after 60 days of incubation were used against THLE2, according to the data (Fig.2) it contains the highest levels of No_2, disloved oxygen and pH, so it was important to show its impact on the normal liver cells.

2-8- Cell culture and Measurment of cytotoxic activity by Methyl Thiazol Tetrazolium MTT assay

Human hepatocellular carcinoma (HepG2) cells and normal hepatic (THLE2) cells were seeded at a density of 1 x 10^4 cells per well (100 µl/well) onto 96-well plate

at the exponential growth phase and sustained in DMEM medium at 37 °C, 5% CO_2 and 95% humidity incubator for 24 hours. All the treatments including PAW (different plasma exposure time periods and diferent longevities), tap water (negative control) and cisplatin (positive control) was prepared at the desiered concentration(s) using DMEM medium and replaced the media of the cells seeded at the 96 well plates and kept for 48 Hours before assaying. The medium was washed gently twice with ice-cold PBS and a volume of 200 µL MTT -2,5-diphenyltetrazolium bromide, a yellow tetrazole was added to each well. The microplate was incubated at 37 °C for another 4 hours in CO_2 incubator. About 180 µL medium/MTT was removed and 100 µL of acidified isopropanol were added per well to solubilize the formazan produced. Finally, the microplate was incubated with shaking for 15 minute. The absorbance of each well was measured at 630 nm using a microplate reader (ELX800, Biokit, spain). Assays were performed in triplicate on three independent experiments. Percentage of cell viability was detected according to calculation equation.[35]

Calculations

Percentage of cell viability (%) = treated sample absorption / control sample absorption x 100

3. RESULTS

3.1 pH and Levels of dissolved oxygen and NO_2 in PAW after one day of exposure to ANPJ

Water pH, dissolved oxygen and NO_2 were measured one day after plasma exposure. There were significant increases in the pH value as well as in the levels of dissolved oxygen and NO_2 PAW compared to regular tap water (Figure2).

3.2 levels of (pH, DO and NO_2) in water after 60 days of exposure to ANPJ

To assess the effect of the elapsed time after plasma exposure on the pH value and levels of dissolved oxygen and nitrogen species in PAW, the pH value and the levels of dissolved oxygen and NO_2 one day and sixty days following plasma exposure were compared. This comparison demonstrated significant increases in both pH and DO after 60 days incubation compared with incubation for only one day. However, the level of NO_2 showed only a slight increase after 60 days compared to one day incubation (Figure 2).

3.3. Effect of PAW on viability of HepG2 cells

In the first set of experiments, HEPG2 cells were treated with PAW generated by exposing tap water to 0.105 J/cm2, 0.210 J/cm2 and 0.315 J/cm2 doses of plasma only one day after production alongside with regular tap water as negative control and cisplatin as positive control. PAW treatment inhibited growth of HEPG2 cells in dose dependent fashion having the maximum inhibition (viability of 24.2%) with plasma dose of 0.315 J/cm2 in a concentration of 200 µL /mL. This inhibition was very competitive to the 21.43% viability obtained after cisplatin treatment at 200 µL /mL (Figure 3).

In the second set of experiments, HEPG2 cells were treated following the same experimental setup used in the first set except for incubating PAW for 60 days before treatment. Interestingly, PAW efficiency in inhibiting the growth of HEPG2 cells was persistent over 60 days after generation and was even more potent compared to the freshly prepared reducing the viability of HEPG2 cells to 19.27% at concentration 200 µL /mL (Figure 4).

3.4. Impact of 60 days of incubation PAW (15) and Cisplatin on THLE2 viability

The same experimental set up was used for HEPG2 cells to treat the normal liver cell line THLE2 with PAW. The highest concentrations of PAW (200 µL /mL) generated using plasma exposure time of 15 minutes and after 60 days of incubation showed only a slight inhibitory effect against THLE2 compared with the same concentration with HEPG2 cells.

Meanwhile, there is, invariably, non-significant difference achieved for treating THLE2with PAW compared to cisplatin at the same concentration of cisplatin as chemotherapy with viability 72.8 and 72.9 % for PAW and cisplatin respectively (Figure 5).

As a bare outline, throughout the current results, the effective induced potential of PAW occasioned by its exposure to plasma prior to treatment of HepG2 cell line became obvious, especially after 60 days of incubation and with exposure time of 15 min. This treatment has the advantage of selective inhibitory effect over cisplatin as standard chemotherapy, i.e. PAW has non-harmful effects against normal liver cells compared with tap water as negative control.

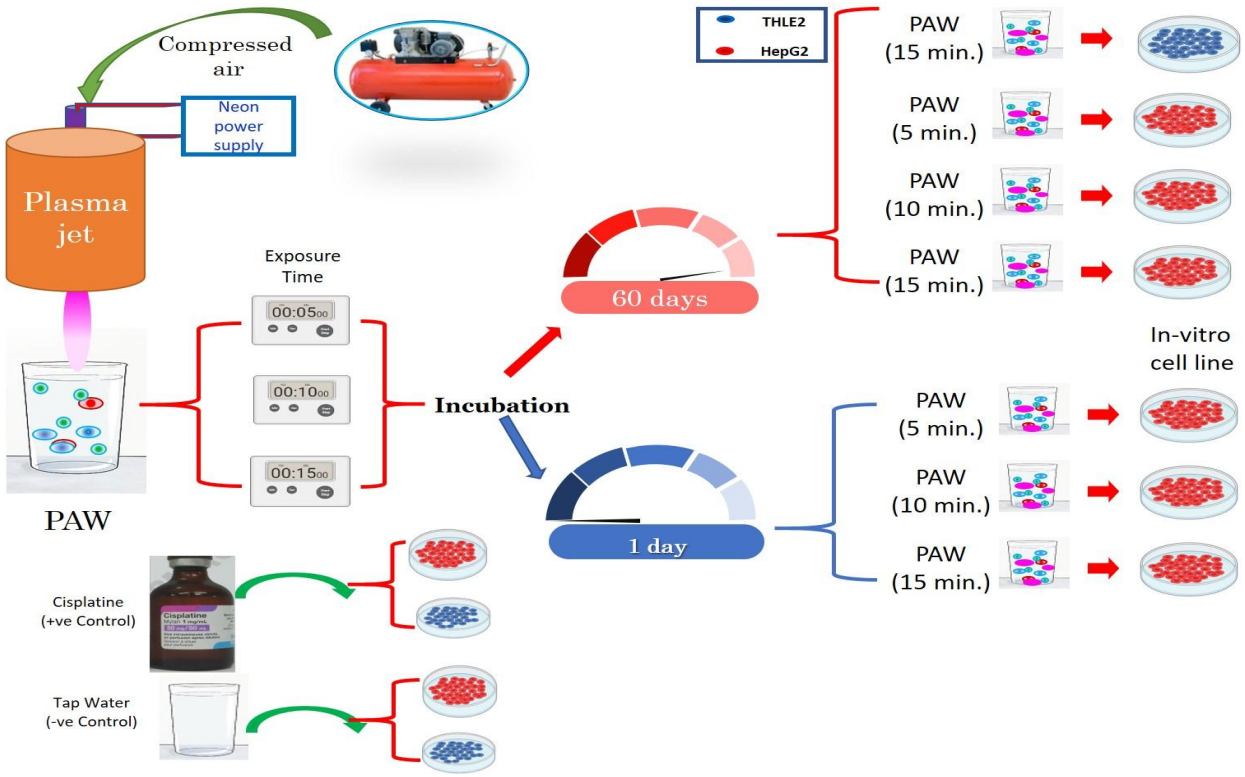

Figure 1: An illustration for experimental design and experimental procedures followed in assessing the potential inhibitory effect of PAW against HepG2 and THLE2 in two separate sets: PAW (1 day) and PAW (60 days) incubated for one day and 60 days, respectively, and each set contains different plasma exposure times (5, 10 and 15min.) As well as using both of tap water as negative control and cisplatin as positive control against HepG2 and THLE2.

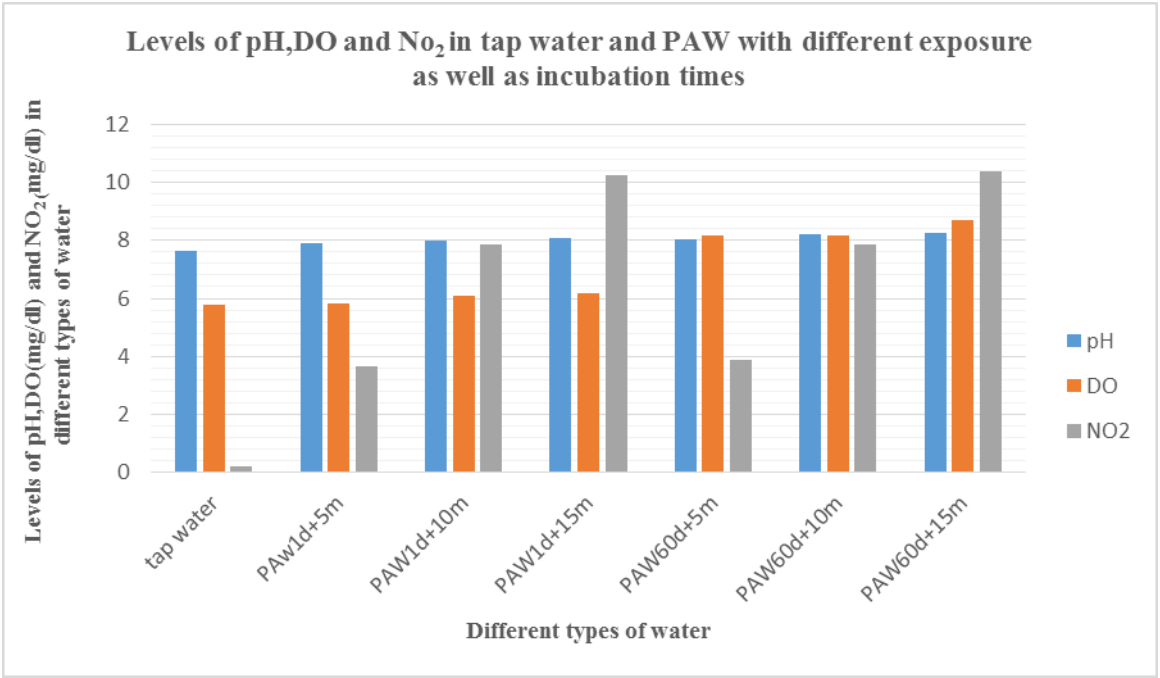

Figure 2: The levels of pH, DO and NO_2 in tap water compared with PAW with different exposure time periods (5, 10 and15 minutes) and after one day of incubation and PAW with different exposure time periods (5, 10 and 15 minutes) after 60 days of incubation.

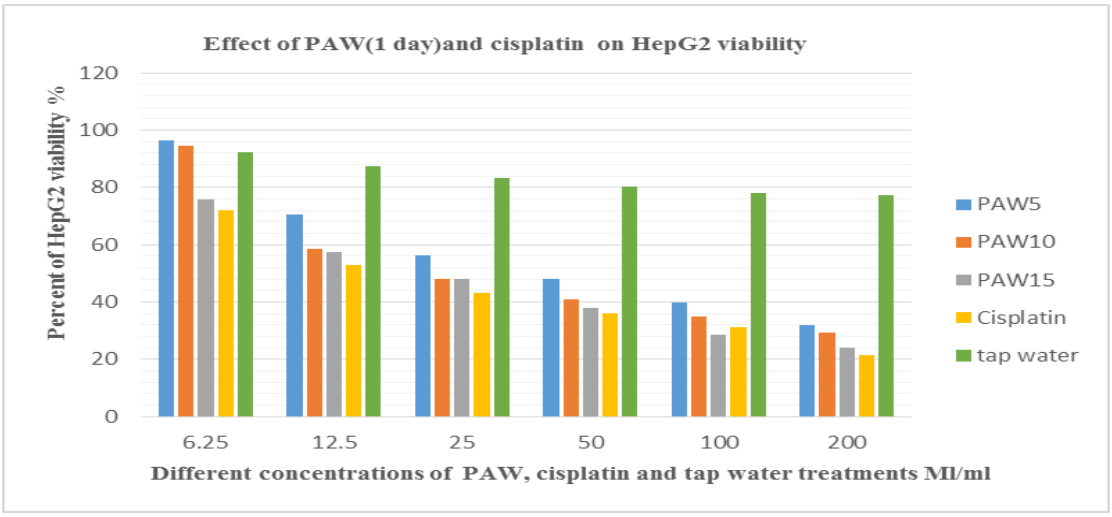

Fig. 3: Comparison between the cytotoxic effects of different concentrations of PAW (1 day) with exposure times (5, 10 and 15 minutes) and cisplatin against HepG2 viability.

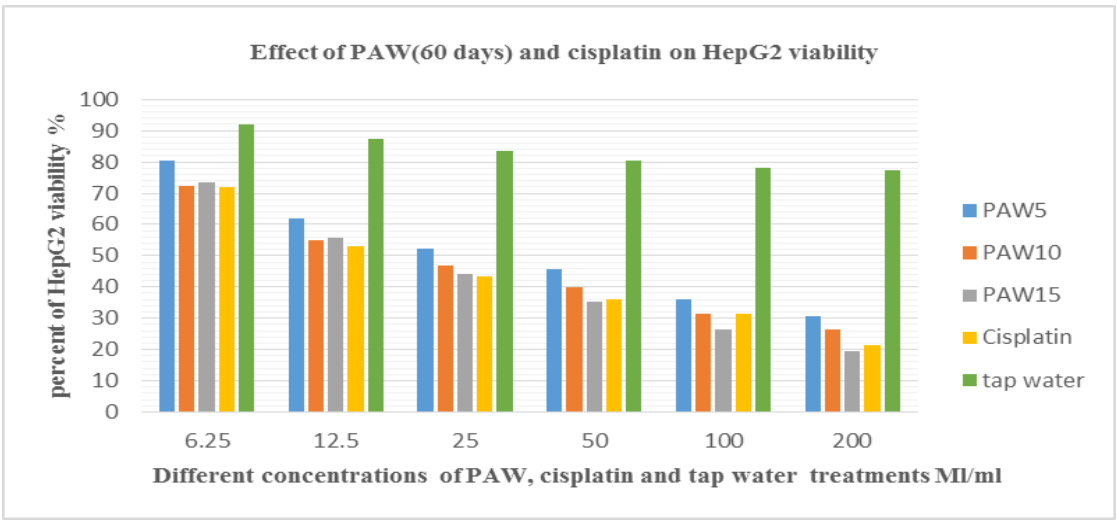

Fig. 4: Comparison between the cytotoxic effects of different concentrations of PAW (60days) with exposure times (5, 10 and 15 minutes) and cisplatin against HepG2 viability.

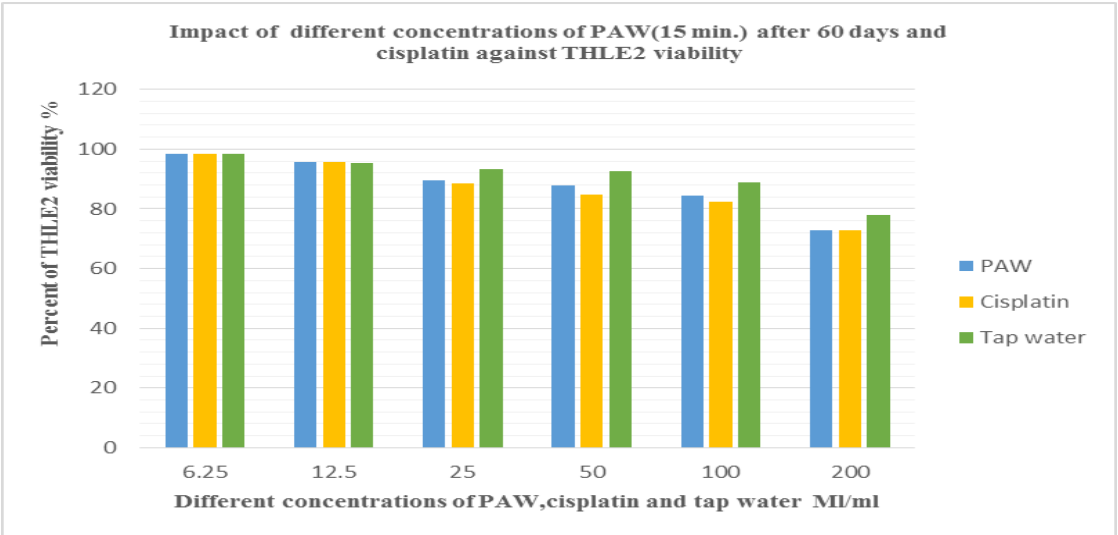

Fig. 5: Impact of cytotoxic effects of both PAW (60days) and cisplatin on THLE2 viability compared with tap water.

4. DISCUSSION

In current study, it was hypothesized that plasma-activated water (PAW) has prolonged effects (of longer life time) via long-lived secondary products, such as nitrite, nitrate and H_2O_2 as well as pH which may be responsible for the aging or extended biological effects of PAW after plasma treatment. To test this hypothesis, both the quantity/activity and the endurance reactive oxygen and nitrogen species in PAW over time (up to two months) were investigated via assessing the pH, NO_2 as well as dissolved oxygen one day and sixty days following PAW production. In addition, assessment was conducted for the potential anti-tumor activity of PAW against HCC cells and how aging could affect its potential anti-tumor activity. The amount/activity of the dissolved oxygen and NO_2 in PAW was able to persist for as long as two months when compared to the freshly prepared PAW (one day after generation) according to the current design. This may be attributed to the long lifetime of PAW which has been found to persist for varying periods from days to months depending on source of plasma and gas used for the plasma discharge (36-38). Several studies showed that the major long lived RONS produced in PAW have been identified to be the NO_2^-, NO_3^-, and H_2O_2.[39] Moreover, according to a previous work[40] Ozone, (O_3) is one of the long-lived species of plasma. These long-lived species participate in increasing levels of pH, NO_2 and oxygen in PAW, according to equations 1, 2, 3, 4, and 5.[18] Hence, it might be contributed to increasing PAW potential after 60 days.

$H2O2 + h\nu \rightarrow OH\bullet + OH\bullet$ (1)

$2\ O_3 + H2O \rightarrow OH\ OH\cdot + O_2 + HO_2$ (2)

$N_2 + e \rightarrow 2N + e$ (3)

$N + O_2 \rightarrow NO + O$ (4)

$4NO + O_2 + 2H_2O \rightarrow 4NO_2\text{-} + 4H$ (5)

In addition, **Lukes et al.**, have investigated the levels of concentrations of species and their lifetime related to the pH value of the treated water. For example, at acidic conditions (pH 3.3), lower concentrations of H_2O_2 and NO_2 were detected compared to alkaline conditions (pH of 6.9 and 10.1). This is in line with the present study as it was shown that significant increases occurs in both the pH value and the NO_2 levels after incubation for 60 days.

The present data clearly demonstrated that PAW treatment inhibits proliferation of HEPG2 cells. To illustrate, PAW treatment substantially inhibited the growth of HEPG2 cells in a dose dependent manner. This inhibition was pronounced even when PAW prepared by exposure to only 0.212 J/cm2 of plasma (5 minutes exposure). Interestingly, this inhibitory effect was persistent over two months and this effect was even more potent compared to the freshly prepared PAW. The anti-cancer effect of cold plasma is well documented and most likely attributed to the wealth of reactive nitrogen and oxygen species, which play important role in reduction of cancerous cells via multiple mechanisms of action.[41] However, the mechanism how the interactions occur between plasma and cancer cells is still largely obscure. According to **Ramin et al.**, the biological effects of non-thermal plasma are based on two properties. The first one is that plasma impelled changes in the liquid environment of cells. The second property is the reactive nitrogen and oxygen species (RNS, ROS) that are produced in or transferred into water phases. These two characteristics play a potential role in plasma-induced biological responses. In fact, an excess of oxidative stress via increase of RONS concentration causes a disturbance of the cell oxidative balance.[43] Plasma-derived ROS have been found to oxidize lipids in the cell membrane, diminish membrane fluidity, and encourage pore formation[44-46], as detailed in a previous work.[47] As seen in necrotic cells, permeabilization of the cell membrane enhances the entry of ROS into the intracellular compartment as well as the discharge of cell contents into the extracellular matrix (ECM).[48] Additionally, plasma can cause oxidative stress in intracellular organelle membranes.[49] Plasma can also cause double-strand DNA breaks.[50&51], which can lead to cell death if they are irreversible.[52] Moreover, Adachi et al. claimed that NO has a short life span and that it quickly converts to NO2 and NO3 in PAW, resulting in species that had a synergistic effect with H_2O_2, as evidenced by the addition of H2O2 to a medium that had a weaker killing effect than PAW when exposed to lung adenocarcinoma cells.[53] This could be due to the fact that when NO2 and H2O2 combine, peroxynitrite (ONOO) is formed, which is poisonous and attacks key macromolecules in various cells. However, the RONS in PAW usually include such long-lived species. These include nitrates (NO_3^-), nitrites (NO_2^-), hydrogen peroxide (H2O2) and ozone (O_3), with the corresponding typical half-lifetime of years, several days, ~104 s, and several to dozens of minutes. This is in addition to relatively short-lived ones such as hydroxyl radicals (•OH), nitric oxide (NO•), superoxide (O_2^-), peroxynitrate (OONO2 −) and peroxynitrites (ONOO−), with half-lifetimes 1 ns, a few seconds, 1.5 s and less than l s, respectively (26 and 27). Since the current study showed that PAW has potential effect against HepG2 viability after incubations for 60 days, the authors believe that No_2 along with unknown factors as well as a specific mechanism are the main reasons for giving PAW unique properties against cancer cells. In addition, alkalinity of PAW may be involved in plasma-mediated HepG2 growth inhibition. For example, alkalinity of water was found to induce cyclin D1 expression.[54] Moreover, according to Lee et al.[55], alkaline reduced water has substantial antioxidant activity as well as an anticancer effect in normal mice. Furthermore, many studies have shown an association between cancer development and pH. For example, it was documented that cancer cells thrive when they were exposed to low pH medium but cannot survive in alkaline medium.[56 and 57]

The importance of dissolved oxygen (DO) against cancer cells, while cancer is thought to occur due to DNA

mutations, the high genetic variety of tumor cells makes it challenging to use these alterations to treat cancer. Non-genetic factors are increasingly recognized as having an impact on cancer, and a growing body of research suggests that hypoxia (a decrease in normal oxygen levels) could play a crucial part in the disease's progression.

The fact that tumors often have hypoxic areas, that hypoxia activates the hypoxia-inducible factor 1 (HIF-1), and that HIF-1 activation plays a vital role in cancer development support this line of research.[58] Furthermore, according to **Al-Dosoki et al**.,[59], oxygenated water is a promising treatment for induced hamster buccal pouch (HBP) carcinogenesis prevention (epithelial dysplasia & invasive carcinoma). According to their findings, oxygenated water improves apoptosis by lowering the risk of tissue hypoxia and maintaining a natural oxygenation level in the body. This can also be explained **by Greijer and Van der Wall's**[60], who found that antiapoptotic proteins could be overexpressed during hypoxia, whereas proapoptotic proteins are down regulated. Thus, when cells are treated with a powerful apoptosis inducer, they become closer to apoptosis. Moreover, oxygenated water acts as an antioxidant, reducing oxidative stress and restoring the cell's normal state. Therefore, DO in PAW could play a potential role against HepG2 viability in the current study. The present study also, showed the selectivity lethal impact of PAW against HepG2 rather than the normal cells (THLE2). The excessive production of oxidative stress molecules may be related to exceeding the cellular anti-oxidative capacity that can lead to cell death in both normal and aberrant cells via activating intracellular signaling pathway.[61] Tumor cells, on the other hand, have greater steady-state RONS concentrations and have dysfunctional antioxidant systems, which leads to cell death. As a result, increasing RONS concentration can be used as a dose-dependent, effective, and selective cancer treatment.[62] Due to the novelty of this field of study, there is a scarcity of data on the mechanism by which the plasmas interact with cells and modifying their biology.

CONCLUSIONS

PAW may be deemed as green prospective alternative for biomedical applications. The current findings support the efficacy of using PAW to kill cancer cells. These findings emphasize one of the benefits of this indirect treatment with ANPJ-treated medium: it avoids the impacts of UV radiation, electric fields, and other factors that could have potentially harmful effects on the normal cells over time if left uncontrolled. It has been shown in current study that dissolved oxygen, NO_2 as well as high pH generated in water are the major players in aging PAW, by promoting selective oxidative stress and triggering different signaling pathways against the hepatocellular carcinoma cells versus the normal hepatocytes. The PAW treated cell culture media is cytotoxic to liver cancer cells, producing a cell viability reduction between 68.06 and 80.73% in a dose-dependent manner. As a result, PAW could be a promising anticancer treatment whose effects are unaffected by tumor heterogeneity and, more crucially, it has some advantages over traditional chemotherapy and radiotherapy in a way that it can be produced safely and in low cost. The described advantages of PAW against cancer cells compared with chemotherapy and radiotherapy, can be summarized as follows. (1) PAW has significant potential effects against cancer cells. (2) It is considered environmentally friendly. (3) It is also energy efficient and inexpensive especially when air is used as plasma ignited gas. (4) Aging or retention time of PAW is 60 days as postulated in current study. Meanwhile, detailed investigations of the mechanisms of reaction of atmospheric plasma jet with flow water processes is needed. It is to be hoped that this would pave the way for further sophisticated developments.

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Ethical Approval and Consent to participate
This article does not contain any studies involving human or animals participants performed by any of the authors. All procedures performed in studies were in accordance with the ethical standards of the institutional and national research committee.

Competing interests.
All authors declare that they have no conflict of Interests.

Consent for publication
All authors declare that they have consent for publications.

Availability of supporting data
This article have supporting data.

Authors' contributions
This article contain authors' contributions and all authors have no conflict of interest.

5 REFERENCES

1. Chen W, Zheng R, Baade PD, Zhang S, Zeng H, Bray F, Jemal A, Yu XQ and He J. Cancer statistics in China, CA Cancer J Clin, 2015; 66: 115-132.
2. Ratna Kumari, Manoj Kumar Sahu, Anindita Tripathy1, Kanishka Uthansingh and Manas Behera. Hepatocellular carcinoma treatment: hurdles, advances and prospects, Hepat. Oncol, HEP08, 2018; 5(2): 1-14.
3. Forner A, Reig ME, de Lope CR and Bruix J. Current strategy for staging and treatment: The BCLC update and future prospects. Semin Liver Dis, 2010; 30: 61-74.

4. Peters GJ and Honeywell RJ. Drug transport and metabolism of novel anticancer drugs. Expert Opin Drug Metab Toxicol, 2015; 11: 661-663.
5. Schutze, A., James Y., Jeong and Steven E. The atmospheric-pressure plasma jet: a review and comparison to other plasma sources. IEEE Trans. Plasma Sci, 1999; 26: 1685–1694.
6. Hamoouda, J. Guillem_Marti, M.P. Ginebra and C. Canal. Liquids treated by Atmospheric Pressure Plasma Jet for bone cancer therapy Biomecánica, 2017; 25: 7-15.
7. Ahmed M. Kamal, Shaimaa. M. Eldeighdy T. M. Allam, W. F.Hassanin. Power Density Measurements to Optimize AC Plasma Jet Operation in Blood Coagulation, j. Australasian physical & Engineering Sciences in Medicine, 2018; 41(3): 621-632.
8. Laroussi, M. Low-Temperature Plasma Jet for Biomedical Applications: A Review. IEEE Trans. Plasma Sci, 2015; 43: 703–712.
9. Kim Rouven Liedtke, Sander Bekeschus, André Kaeding and Christine Hackbarth. Non-thermal plasma-treated solution demonstrates antitumor activity against pancreatic cancer cells *in vitro* and *in vivo*. Scientific Reports, 2017; 7: 1-12.
10. Ishaq, M. Atmospheric gas plasma-induced ROS production activates TNF-ASK1 pathway for the induction of melanoma cancer cell apoptosis. *Mol Biol Cell, 2014;* 25: 1523–1531.
11. Hou, J. Non-thermal plasma treatment altered gene expression profiling in non-small-cell lung cancer A549 cells. *BMC Genomics, 2015;* 16: 1644-8.
12. Zhang, X. Ablation of liver cancer cells in vitro by a plasma needle. *Applied Physics Letters, 2008;* 93: 021502-3.
13. Ahmed K. Design and experimental investigations of electrical breakdown in a plasma jet device and applications. Faculty of Engineering at Shoubra Electrical Engineering Dep. Benha University-Egypt, 2014; 130-140.
14. Bruggeman, P.J.; Kushner, M.J.; Locke, B.R.; Gardeniers, J.G.E.; Graham, W.G. and Gravesa D.B. Plasma-liquid interactions: A review and roadmap. Plasma Sources Sci. Technol, 2016; 25: 053002.
15. Kutasi, K.; Popovi´c, D.; Krstulovi´c, N.; Miloševi´c, S. Tuning the composition of plasma-activated water by a surface-wave microwave discharge and a kHz plasma jet. Plasma Sources Sci. Technol, 2019; 28: 095010.
16. Lukes, P.; Dolezalova, E.; Sisrova, I.; Clupek, M. Aqueous-phase chemistry and bactericidal effects from an air discharge plasma in contact with water: Evidence for the formation of peroxynitrite through a pseudo- second-order post-discharge reaction of H2O2 and HNO2. Plasma Sources Sci. Technol, 2014; 23: 015019.
17. Hensel, K.; Kuˇcerová, K.; Tarabová, B.; Janda, M.; Machala, Z.; Sano, K.; Mihai, C.T.; Ciorpac, M.; Gorgan, L.D.; Jijie, R. Effects of air transient spark discharge and helium plasma jet on water, bacteria, cells, and Biomolecules. Biointerphases, 2015; 10: 029515.
18. Shaimaa M. Eldeighdye, Tarek M.Allam and Hanaa A. Elsayed. Non-thermal plasma applications: Impact of non-thermal plasma treated water on some bilogical functions in albino rats. World journal of pharmacy and pharmaceutical sciences, 2020; 9: 114-130.
19. Lee HJ, Shon CH, Kim YS, Kim S, Kim GC and Kong MG. Degradation of adhesion molecules of G361 melanoma cells by a non-thermal atmospheric pressure microplasma. New Journal of Physics, 2009; 11(11): 115026.
20. Kim SJ, Chung TH, Bae SH and Leem SH. Induction of apoptosis in human breast cancer cells by a pulsed atmospheric pressure plasma jet. Applied Physics Letters, 2010; 97(2): 023702.
21. Kim JY, Ballato J, Foy P, Hawkins T, Wei Y, Li J and Kim SO. Apoptosis of lung carcinoma cells induced by a flexible optical fiber-based cold microplasma. Biosensors & bioelectronics, 2011; 28(1): 333-338.
22. Ahn HJ, Kim KI, Hoan NN, Kim CH, Moon E, Choi KS, Yang SS and Lee JS. Targeting cancer cells with reactive oxygen and nitrogen species generated by atmosphericpressure air plasma. PloS one, 2014; 9(1): e86173.
23. Vandamme M, Robert E, Lerondel S, Sarron V, Ries D, Dozias S, Sobilo J, Gosset D, Kieda C, Legrain B, Pouvesle JM and Pape AL. ROS implication in a new antitumor strategy based on non-thermal plasma. International journal of cancer Journal international du cancer, 2012; 130(9): 2185-2194.
24. Ahmed K., Allam T., El-sayed H., Soliman H., Ward S. And Saied E. Design, Construction and Characterization of AC Atmospheric Pressure Air Non-thermal Plasma Jet. J Fusion Energ, 2014; 33: 627–633.
25. Michael Schmidt, Veronika Hahn 1, Beke Altrock 1, Torsten Gerling. Plasma-Activation of Larger Liquid Volumes by anInductively-Limited Discharge for Antimicrobial Purposes. Appl. Sci, 2019; 9: 2150.
26. Thirumdas R, Kothakota A, Annapure U, Siliveru K, Blundell R, Gatt R and Valdramidis V P. plasma activated water (PAW), chemistry physic-chemical properties, applications in food and agriculture *Trends Food Sci. Technol, 2018;* 77: 21–31.
27. Slutsker Y Z, Semenov V, Krasik Y E, Ryzhkov M, Felsteiner J, Binenbaum Y, Gil Z, Shtrichman R and Cohen J J. Electrical model of cold atmospheric plasma gun, Phys. *Plasmas, 2017;* 24: 103510.
28. Keeling, R.F.; Garcia, H.E. The change in oceanic O2 inventory associated with recent global warming. Proc. Natl. Acad. Sci. USA, 2002; 99: 7848–7853.
29. Yan X., Z. Xiong, F. Zou, S. Zhao, X. Lu, G. Yang, G. He, and K. K. Ostrikov. Plasma- Induced Death of HepG2 Cancer Cells: Intracellular Effects of Reactive Species Plasma Processes and Polymers, 2012; 9: 59-66.

30. N. Popov. Associative ionization reactions involving excited atoms in nitrogen plasma. Plasma physics reports, 2009; 35: 436-449.
31. U.S. Environmental protection agency. Methods for chemical analysis of water and wastes. Methods 353.3.U.S. Environmental protection agency, Washington, D.C., 1979.
32. Public Health England. Determination of pH in Food and Water Samples. National Infection Service. Food, Water & Environmental Microbiology Standard Method FNES63 [P2]2017; Version 2.
33. 4500-O oxygen (dissolved). Standard Methods for the Examination of Water and Waste water, 2017; DOI: 10.2105/SMWW.2882.091.
34. MCKeown, J.J., L.C. Brown & G.W. Gove. Comparative studies of dissolved oxygen analysis methods. J. Water Pollut. Control Fed, 1967; 39: 1323.
35. Elgazar, A.A., Selim, N.M., Abdel-Hamid, N.M., El-maggd, M.A., El Henawy, H.M. Isolates from Alpinia officinarum hance attenuate LPS induced inflammation in HepG2:Evidence from in Silico and in Vitro studies. Phytotherapy research, 2018; 32: 1273-1288.
36. Brisset J-L and Pawlat J. Chemical effects of air plasma species on aqueous solutes in direct and delayed exposure modes: Discharde, post-discharde and plasma activated water, Plasma Chem. Plasma Process, 2016; 36: 355-381.
37. Laurita R, Barbieri D, Gherardi M, Colombo V and Lukes P. Chemicalanalysis of reactive species and antimicrobial activity of water treated by nanosecond pulsed DBD air plasma, Clin. Plasma Med, 2015; 3: 53-61.
38. Shen J, Tian Y, Li Y, Ma R, Zhang Q, Zhang J and Fang J. Bactericidal effects against S. Aureus and Physicochemical properties of plasma activated water stored at different temperatures. Sci. Rep, 2016; 6: 1-10.
39. Lukes P, Dolezalova E, Sisrova I and Clupek M. Aqueous-phase chemistery and bactericidal effects from an air discharge plasma in contact with water:evidence for the formation of peroxnitrite through apseudo-second-order post – discharge reactin of H_2O_2 and HNO_2. Plasma Sources Sci. Technol, 2014; 23: 015019.
40. Tian W. and Kushner MJ. Atmospheric pressure dielectric barrier discharges interacting with liquid covered tissue J. phys. D: Appll. phys, 2014; 47: 165201.
41. Dayun yan, JonathanH. Sherman and Michael Keidar. Cold atmospheric plasma, a novel promising anti-cancer treatment modality, oncotarget, 2016; 8: 15977-15995.
42. Ramin Mehrabifard1*, Hassan Mehdian1, Mahdi Bakhshzad Mahmoud. Effect of non-thermal atmospheric pressure plasma on MDA-MB-231 breast cancer cells, Pharm Biomed Res, 2017; 3(3): 12-16.
43. Trachootham, D., Alexandre, J. and Huang, P. Targeting cancer cells by ROS-mediated mechanisms: a radical therapeutic approach? Nat. Rev. Drug Discov, 2009; (8): 579–591.
44. Van der Paal, J.; Neyts, E.C.; Verlackt, C.C.W.; Bogaerts, A. Effect of lipid peroxidation on membrane permeability of cancer and normal cells subjected to oxidative stress. Chem. Sci., 2016; (7): 489–498.
45. 45. Van der Paal, J.; Verheyen, C.; Neyts, E.C.; Bogaerts, A. Hampering Effect of Cholesterol on the Permeation of Reactive Oxygen Species through Phospholipids Bilayer: Possible Explanation for Plasma Cancer Selectivity. Sci. Rep., 2017; (7): 1-11.
46. Shaw, P.; Kumar, N.; Hammerschmid, D.; Privat-Maldonado, A.; Dewilde, S.; Bogaerts, A. Synergistic effects of melittin and plasma treatment: A promising approach for cancer therapy. Cancers, 2019; 11: 1109.
47. Privat-Maldonado, A.; Schmidt, A.; Lin, A.; Weltmann, K.-D.; Wende, K.; Bogaerts, A.; Bekeschus, S. ROS from physical plasmas: Redox chemistry for biomedical therapy. Oxid. Med. Cell. Longev, 2019; 29.
48. Zhang, Y.; Chen, X.; Gueydan, C.; Han, J. Plasma membrane changes during programmed cell deaths. Cell Res., 2018; (28): 9–21.
49. Dezest, M.; Chavatte, L.; Bourdens, M.; Quinton, D.; Camus, M.; Garrigues, L.; Descargues, P.; Arbault, S.; Burlet-Schiltz, O.; Casteilla, L. Mechanistic insights into the impact of cold atmospheric pressure plasma on human epithelial cell lines. Sci. Rep., 2017; (7): 41163.
50. Hirst, A.M.; Simms, M.S.; Mann, V.M.; Maitland, N.J.; O'Connell, D.; Frame, F.M. Low-temperature plasma treatment induces DNA damage leading to necrotic cell death in primary prostate epithelial cells. Br. J. Cancer, 2015; 112: 1536–1545.
51. Han, X.; Klas, M.; Liu, Y.Y.; Stack, M.S.; Ptasinska, S. DNA damage in oral cancer cells induced by nitrogen atmospheric pressure plasma jets. Appl. Phys. Lett., 2013; 102.
52. Dickey, J.S.; Redon, C.E.; Nakamura, A.J.; Baird, B.J.; Sedelnikova, O.A.; Bonner, W.M.H2AX: Functional roles and potential applications. Chromosoma, 2009; (118): 683–692.
53. Adachi, T.; Nonomura, S.; Horiba, M.; Hirayama, T.; Kamiya, T.; Nagasawa, H.; Hara, H. Iron stimulates plasma-activated medium-induced A549 cell injury. Sci. Rep., 2016; 6: 1-12.
54. Huda A. Al Doghaither1, Ayat B. Al-Ghafari1, Sawsan A. Rahimulddin1, Shiekhah M. Al Zahrani1, Abdulkader M. Shaikh Omar2, Ulfat M. Omar. Evaluation of the Potential Anticancer Activity of Zamzam Water in Human Colon Cancer Cell Line, Cancer and Oncology Research, 2016; 4(3): 33-41.
55. Lee KJ, Park SK, Him JW, Kim GY and Kim GH. anticancer effect of alkaline reduced water, J Int soc life inf. sci, 2004; (22): 302-305.

56. Estrella, V., Chen, T., Lloyd, M., Wojtkowiak, J., Cornnell, H.H., Ibrahim-Hashim, A., Bailey, K., Balagurunathan, Y., Rothberg, J.M., Sloane, B.F., Johnson. J. Acidity generated by the tumor microenvironment drives local invasion. Cancer Research, 2013; 73(5): 1524-1535.
57. Robey, I.F., Baggett, B.K., Kirkpatrick, N.D., Roe, D.J., Dosescu, J., Sloane, B.F., Gillies, R.J. Bicarbonate increases tumor pH and inhibits spontaneous metastases. Cancer Research, 2009; 69(6): 2260-2268.
58. Miguel López-Lázaro. Role of Oxygen in Cancer: Looking Beyond Hypoxia, Anti-Cancer Agents in Medicinal Chemistry, 2009; (9): 517-52.
59. Al-Dosoki Mohamed A., Ahmed M. Mansour and Mohamed M. Ahmed. Effect of oxygenated water as a new treatment modality on experimentally induced hamaster buccal pouch carcinogenesis. Al-Azhar Journal of Dental Science, 2018; 21: 261-273.
60. Greijer A, Van der Wall E. The role of hypoxia inducible factor 1 (HIF-1) in hypoxia induced apoptosis. J Clin Pathol, 2004; (10): 1009-14.
61. Movahed, Z.G.; Rastegari-Pouyani, M.; Mohammadi, M.H.; Mansouri, K. Cancer cells change their glucose metabolism to overcome increased ROS: One step from cancer cell to cancer stem cell? Biomed. Pharmacother, 2019; (112): 1-15.
62. Trachootham, D.; Alexandre, J.; Huang, P. Targeting cancer cells by ROS-mediated mechanisms: A radicaltherapeutic approach? Nat. Rev. Drug Discov, 2009; 8: 579–591.