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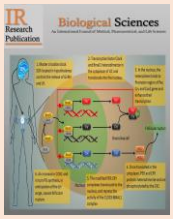

CO2 Plasma Activated Water (PAW), a Novel Weapon for Hepatocellular Carcinoma

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ABSTRACT

Cancer poses significant challenges to global health, necessitating the exploration of innovative therapies. CO2 plasma activated water (PAW) holds promise as a novel therapeutic agent due to its selective cytotoxicity against cancer cells while sparing healthy tissues. This study investigated the in-vitro cytotoxic activity of CO2 PAW against human hepatocarcinoma (HepG2) cells. The cytotoxic effect of CO2 PAW on HepG2 cells was evaluated using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. Cells were seeded in 96-well plates, treated with varying volumes of PAW. Morphological changes induced by PAW treatment were assessed using phase-contrast microscopy. The total RNA isolated from HepG2 cells, treated with PAW using the GF-1 total RNA extraction kit, qRT-PCR was performed using SYBR-green master mix with cDNA templates and synthesized primers. Data were analyzed using GraphPad Prism (ver. 9.5, USA, 1992) and presented as mean ± standard error of means. The results revealed dose-dependent cytotoxic effects, with higher concentrations inducing pronounced cell death. Morphological changes in HepG2 cells post-PAW treatment and gene expression analysis showed significant ($P<0.05$) alterations in Bax and Bcl-2 mRNA levels, indicative of intrinsic apoptotic pathway activation.

Introduction

Cancer continues to pose significant challenges to global health, exerting a profound impact on individuals, families, and healthcare systems globally [1]. Characterized by uncontrolled cell proliferation and dissemination, cancer manifests diversely, each form portraying distinct diagnostic and therapeutic challenges [2]. Notably, hepatocellular carcinoma (HCC) emerges as a formidable adversary, linked closely with chronic liver diseases and often evading discovery until advanced stages [3].

The liver, playing a critical role in various physiological functions such as metabolism, detoxification, and nutrient storage, is particularly susceptible to malignancies [4]. Hepatocellular carcinoma, the predominant form of primary liver cancer, frequently arises in the setting of underlying liver pathologies including viral hepatitis, alcoholic

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liver disease, and non-alcoholic fatty liver disease [5]. Despite advancements in medical science, the prognosis for HCC patients remains dismal, with limited therapeutic options and poor overall survival rates.

Conventional therapies for HCC, such as surgical resection, liver transplantation, chemotherapy, and radiation therapy, are faced with various limitations. Tumor recurrence, drug resistance, and compromised liver function among others pose significant challenges, thwarting effective disease management. Furthermore, the systemic toxicity associated with chemotherapy and the scarcity of donor organs for transplantation complicates the quagmire, necessitating innovative, targeted, and less invasive therapeutic approaches [6].

Plasma is generated by electrical discharges at atmospheric pressure and is capable of triggering different physical phenomena as well as chemical reactions [7,8]. The kernel of plasma production and its existence is the transformation of gas molecules into an approximately electrically neutral ionized gas consisting of a population of electrons and ions after they have acquired high energy under the effect of heating or strong electromagnetic fields.

In medical innovation, plasma-based therapies present a ray of hope in cancer treatment [9]. Plasma often referred to as the fourth state of matter, comprises a complex array of charged particles and reactive species with potential for selective cancer cell eradication [10]. Cold atmospheric pressure plasma has garnered attention for its ability to induce apoptosis in cancer cells while sparing normal tissues. Within this domain, CO2 plasma activated water (PAW) emerges as a promising agent for cancer therapy [11].

Derived from subjecting carbon dioxide to non-thermal plasma, CO2 plasma water is enriched with reactive oxygen species (ROS) and bioactive molecules. This unique composition enables CO2 plasma water to unleash cytotoxic effects on cancer cells, triggering oxidative stress and disrupting critical cellular processes 1[12]. The biocompatibility and versatility of CO2 plasma water offer exciting prospects for targeted cancer therapy, signaling a paradigm shift in the management of hepatocellular carcinoma [13].

Materials and Method

Preparation of CO2 Plasma Activated Water (PAW)

CO2 plasma activated water (PAW) was prepared using a non-thermal micro-hollow cathode discharge (MHCD) system, following a setup described by Chen et al. [14]. The MHCD operated at atmospheric pressure, employing air and oxygen as working gases. Gas flow and discharge power were controlled at 4 L per minute and 100 W, respectively. Deionized water served as the base for generating PAW. The discharge plasma was initiated by positioning the discharge probe approximately 5.0 mm away from the surfaces of deionized water contained in 50-mL conical tubes. PAW was generated for specified durations (10, 15, and 30 minutes) and promptly utilized or stored in 15-mL conical tubes at refrigerated temperatures.

Cell Line and Culture Conditions

Human liver cancer cell line (HepG2) obtained from the American Type Culture Collection (ATCC), USA, was revived and maintained in a CO2 incubator with 5% CO2 at relative humidity. Cells were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 10% fetal bovine serum (FBS), penicillin (100 U/ml), and streptomycin (100 µg/ml). Sub-culturing was performed at 70-80% confluence by detaching cells with trypsin and plating in new T-25 culture flasks.

Cell Viability Assay (MTT)

The cytotoxic effect of CO2 PAW on HepG2 cells was evaluated using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. Cells were seeded in 96-well plates, treated with varying volumes of PAW, and incubated for 48 hours. MTT solution was added, and formazan crystals were solubilized using DMSO. Absorbance was measured at 570 nm using a microplate spectrophotometer.

Assessment of Apoptotic Morphological Changes

Morphological changes induced by PAW treatment were assessed using phase-contrast microscopy. HepG2 cells were cultured and treated with PAW samples for 24 hours. Changes in cell morphology were observed and compared with the negative control.

RNA Isolation

Total RNA was isolated from HepG2 cells treated with PAW using the GF-1 total RNA extraction kit. Isolated RNA was stored at -20°C for further analysis.

cDNA Synthesis

Table 1. In-vitro Cytotoxicity Effect of CO2 PAW on HepG2 Cells.

Groups	Cancer Cell Lines	
	HepG2 (Liver)	**MCF7 (Breast)**
Concentrated CO_2 PAW (200µL)	57.88±1.18^{c}	56.65±1.18^{c}
Diluted CO_2 PAW (200µL)	59.14±1.14^{c}	15.41±10.12^{a}
Concentrated CO_2 PAW (100µL)	11.53±1.10^{a}	48.84±2.38^{e}
Diluted CO_2 PAW (100µL)	38.74±0.97^{b}	29.53±8.83^{d}

Results are presented as mean ± SD, and values with different superscript within a column indicate statistically significant difference at 95% confidence interval and probability value of 0.05.

Table 2. Fold Change in Bax and Bcl-2 Genes Treated with CO2 PAW.

Groups	**Fold Change in Gene ($2^{-\Delta\Delta Ct}$)**	
	Bax	**Bcl2**
Control (Untreated Cancer Cells)	1.00±0.00^{a}	1.00±0.00^{c}
Concentrated CO_2 PAW	33.13±1.18^{c}	0.05±0.01^{a}
Diluted CO_2 PAW	1.68±0.32^{b}	0.15±0.06^{b}

Results are presented as mean ± SD, and values with different superscript within a column indicate statistically significant difference at 95% confidence interval and probability value of 0.05.

Isolated RNA was reverse transcribed into cDNA using ReverTraAceTM qPCR RT Master Mix with gDNA Remover. cDNA was stored at -20°C for subsequent assays.

Primer Design

Primer sequences for apoptotic genes (BAX and Bcl-2) were designed using the NCBI website Primer Blast. Bax (F: 5'- GAG TGT CTC AAG CGC ATC GG -3' R: 5'- AGT AGA AAA GGG CGA CAA CCC -3') and bcl-2 (F: 5'-CGT CCG TGC CTG CAT TTA GC -3' R: 5'-GTA TCC ACC GGA CCG CTT CA-3').

Quantitative Real-Time PCR (qRT-PCR) Analysis

qRT-PCR was performed using SYBR-green master mix with cDNA templates and synthesized primers. β-Actin was used as the reference gene. Relative quantification was determined using the 2-ΔΔCT method.

Statistical Analysis

Data were analyzed using GraphPad Prism (ver. 9.5, USA, 1992) and presented as mean ± standard error of means. One-way analysis of variance (ANOVA) was used to determine the level of significance at a 95% confidence interval.

Results

Result of In-vitro Cytotoxicity Activity of CO2 PAW

The cytotoxic effect of CO2 plasma activated water (PAW) on liver (HepG2) and breast (MCF7) cancer cell lines after 48 hours of exposure is summarized in Table 1. Treatment with both concentrated and diluted PAW at different volumes resulted in measurable cytotoxicity on both cell lines. At 200 µL exposure, HepG2 cells exhibited insignificant cytotoxic effect ($p > 0.05$) of 57.88% ± 1.18 and 59.14% ± 1.14 for concentrated and diluted PAW, respectively. Conversely, at 100 µL exposures, significant low cytotoxic effect ($p < 0.05$) was observed with mean values of 11.53% ± 1.10 and 38.74% ± 0.97 for concentrated and diluted PAW, respectively. Notably, there was a significant difference in the cytotoxic effect with concentrated PAW at 200 µL PAW against MCF7 cells (56.65% ± 1.18), to that of the dilute (15.41±10.12). The same trend was observed at 100 µL for both concentrated and dilute PAW. However, diluted PAW at both concentrations, showed better cytotoxic effects on HepG2 cells to that of MCF7 cells; hence it was selected for gene expression analysis.

Effect of CO2 PAW on HepG2 Cell Morphology

Phase contrast micrographs of HepG2 cells treated with CO2 plasma activated water (PAW) are presented in Figure 1. This includes untreated HepG2 cells (negative control) showing typical cell morphology under normal conditions, HepG2 cells treated with diluted PAW, revealing slight distortion of HepG2 cell morphology and HepG2 cells treated with concentrated PAW, exhibiting gross distortion in cell morphology. Cellular aberrations observed include cell shrinkage, rounding, loss of adherence, and nuclear condensation, indicative of apoptosis.

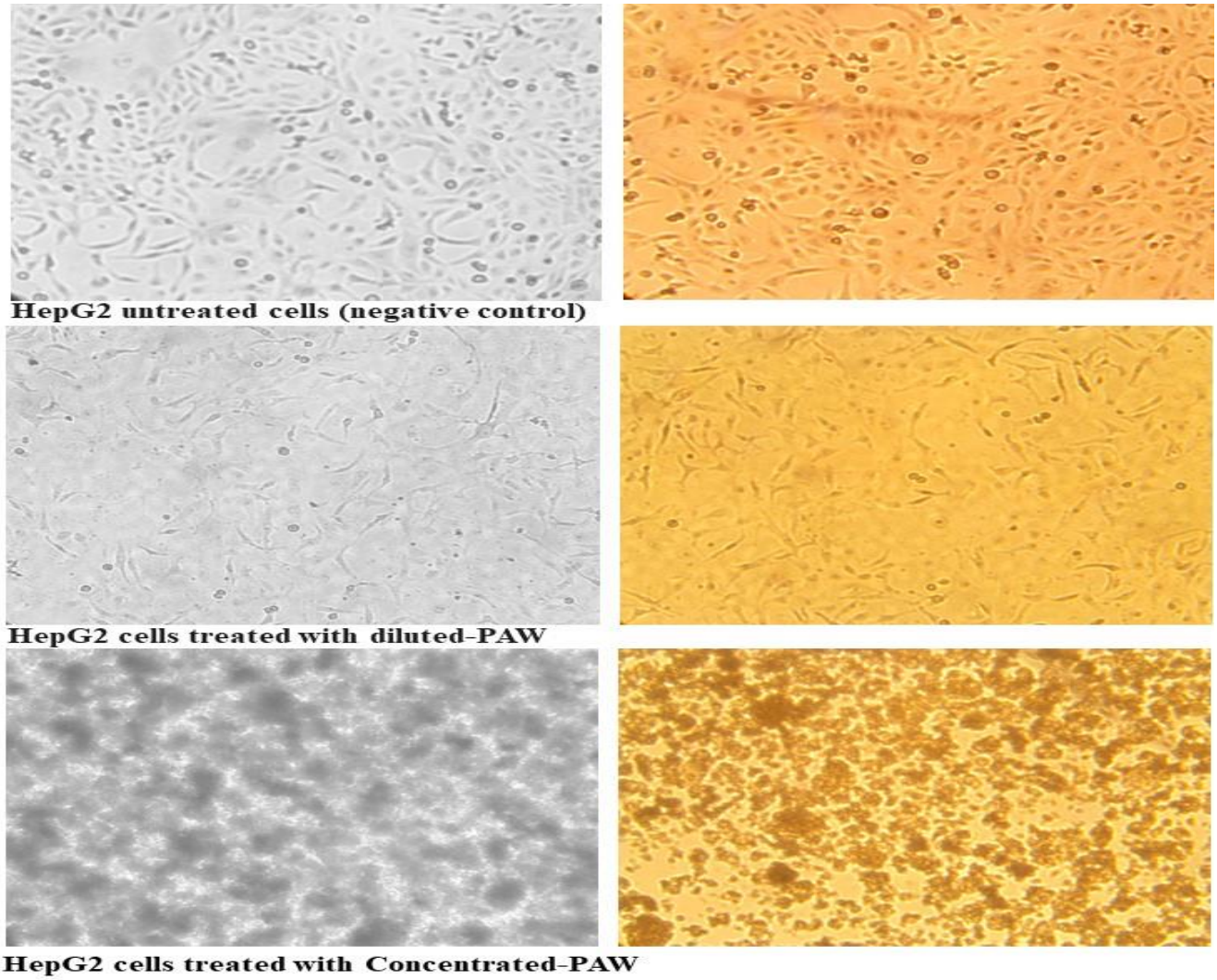

Figure 1. Phase contrast micrographs of HepG2 cells treated with PAW

Result of Gene Expression Study

Table 2 presents the mRNA levels of HepG2 cells treated with CO2 PAW. Treatment with PAW significantly ($p<0.05$) increased the expression of the Bax gene in HepG2 cells, with concentrated PAW exhibiting a higher fold change (33.13 ± 1.18) compared to control. Conversely, treatment caused a significant ($p < 0.05$) decrease in the expression of the Bcl2 gene, with concentrated PAW showing the lowest fold change (0.05 ± 0.01) compared to control.

Discussion

Cancer remains a formidable challenge in modern medicine, characterized by the uncontrolled proliferation and dissemination of abnormal cells within the body [2]. This devastating disease exerts a profound impact on individuals, families, and societies, exacting both physical and emotional tolls while straining healthcare systems globally[15, 16]. In the pursuit of effective cancer therapies, researchers have explored a myriad of approaches, including the investigation of novel agents derived from natural sources [17, 18]. Plasma-activated water (PAW) has emerged as a promising avenue in cancer research, leveraging the unique properties of plasma to generate biologically active species within water. CO2 plasma-activated water, in particular, has garnered attention for its potential anticancer properties, with studies suggesting its cytotoxic effects against various cancer cell lines [11, 19]. The underlying mechanisms of PAW-induced cytotoxicity remain under investigation, with proposed involvement of oxidative stress, DNA damage, and modulation of apoptotic pathways. This study aimed at investigating the cytotoxic effects and underlying molecular mechanisms of CO2 PAW in hepatocarcinoma.

The investigation into CO2 PAW's cytotoxicity on HepG2 and MCF7 cell lines unveiled a promising avenue for cancer therapy. The dose-dependent response observed, with higher concentrations inducing more pronounced cytotoxic effects, aligns with previous studies on plasma-activated water in

various cancer models [20]. Notably, the greater cytotoxicity observed in HepG2 cells compared to MCF7 cells underscores the importance of considering cell line specificity in evaluating PAW's efficacy. This phenomenon might be attributed to differences in genetic makeup, metabolic activity, and membrane properties between cancer cell lines [21].

The morphological changes observed in HepG2 cells following PAW treatment provide valuable insights into its mode of action. The apoptotic-like features, including cell shrinkage, rounding, loss of adherence, and nuclear condensation, suggest the activation of programmed cell death pathways. These findings corroborate previous research demonstrating the ability of plasma-activated water to induce apoptosis in cancer cells through various mechanisms, including oxidative stress, DNA damage, and mitochondrial dysfunction [22].

Comparatively, it is evident that CO2 PAW shares similar cytotoxic effects with other plasma-activated mediums against different cancer types [23]. Studies by Bai et al. [24] and Tanakaet al., [25] have reported comparable alterations in cell morphology and cytotoxicity in response to plasma-activated media treatment in hepatocellular carcinoma and glioblastoma models, respectively. This consistency across diverse cancer types highlights the broad applicability of plasma-activated water as a potential anti-cancer therapy.

The gene expression study revealed significant alterations in the mRNA levels of Bax and Bcl-2 genes- key regulators of apoptosis, in HepG2 cells. The up regulation of Bax and down regulation of Bcl-2 gene expression indicates the activation of intrinsic apoptotic pathways in response to PAW treatment. This molecular mechanism aligns with the observed morphological changes which are indicative of apoptosis, further supporting PAW's cytotoxic activity against HepG2 cells. This finding agrees with similar studies on anticancer activity of plasma activated medium and/or water on selected cancers [17, 26, 27]. This consistency underscores the potential of PAW as a modulator of apoptotic pathways across different cancer types.

Conclusion

The study demonstrates the potent cytotoxic activity of CO2 plasma-activated water (PAW) against human hepatocarcinoma (HepG2) cells, as evidenced by dose-dependent cytotoxicity and morphological changes indicative of apoptosis. Furthermore, the up regulation of Bax and down regulation of Bcl-2 gene expression highlights the activation of intrinsic apoptotic pathways by PAW treatment. These findings underscore the potential of CO2 PAW as a promising therapeutic modality for hepatocellular carcinoma.

Contribution of authors

This work was carried out in collaboration between all authors. Author FOU designed the study, performed the statistical analysis, wrote the protocol, and FOU, AAM wrote the first draft of the manuscript. Authors AAM and ATI managed the analyses of the study. Authors ANS, AAM, ATI and FOU managed the literature searches. All authors read and approved the final manuscript.

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Conflict of Interest

Authors have declared that no conflicting interests exist

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