Histopathological Response of Breast Cancer Tissue to Cold Atmospheric Plasma (CAP) Exposure

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ABSTRACT

Cold Atmospheric Plasma (CAP) has emerged as a promising therapeutic modality due to its ability to induce oxidative stress and apoptosis in malignant tissues with minimal impact on healthy cells(1,2,3,4). This study aimed to evaluate the histopathological and biochemical responses of human breast cancer tissue to CAP exposure, specifically focusing on key oxidative stress and apoptotic markers: malondialdehyde (MDA), glutathione (GSH), and caspase-3. A total of 50 freshly excised breast tissue samples (25 malignant and 25 benign fibroadenomas) were subjected to standardized CAP treatment using a dielectric barrier discharge system. Biochemical analyses were performed on tissue lysates to quantify baseline and post-treatment levels of caspase-3, GSH, and MDA. At baseline, malignant tissues demonstrated significantly elevated levels of caspase-3 and MDA and lower GSH levels compared to benign controls, indicating higher apoptotic activity and oxidative stress. Following CAP exposure, malignant tissues showed further increases in caspase-3 and MDA and a marked decrease in GSH, suggesting an intensified pro-apoptotic and oxidative effect. No post-treatment data were available for benign tissues due to the study design. Linear and multivariate regression analyses revealed that baseline GSH and MDA levels significantly predicted post-CAP biochemical changes, especially the magnitude of GSH depletion and MDA elevation. These findings underscore the potential of CAP as a targeted therapeutic strategy for breast cancer by exploiting redox imbalance and apoptotic vulnerabilities in malignant cells. The distinct biochemical profile in response to CAP treatment supports further exploration of CAP in oncologic applications and highlights its selective impact on cancerous tissue.

INTRODUCTION

Cold atmospheric plasma (CAP) is increasingly recognized in oncology for its ability to selectively induce apoptosis in tumor cells via reactive oxygen and nitrogen species (RONS), offering a non-invasive strategy with minimal harm to healthy tissue[5,6]. CAP directly disrupts cancer cell membranes and intracellular components by triggering oxidative lipid degradation, as confirmed by molecular dynamics simulations of phospholipid oxidation[5]. Clinically, CAP has been effectively applied in dermatological oncology, reducing actinic keratosis lesions and improving skin field cancerization without inducing skin atrophy[7]. Beyond cancer, CAP also plays a critical role in controlling biofilms on biomedical implants, enhancing sterilization and antimicrobial efficacy[8]. Recent reviews further underline CAP's versatility in medical applications, highlighting its role in immunomodulation, neuroprotection, and wound healing through its RONS-mediated cellular interactions[9,10].

Oxidative stress and apoptosis are central to the pathophysiology of breast cancer, where elevated markers such as MDA and Caspase-3 correlate strongly with tumor progression and severity[3,11]. The chemotherapeutic agent metformin has been shown to induce apoptosis and oxidative stress in MCF-7 breast cancer cells, evidenced by increased MDA and reduced GSH levels[12.,13]. Venetoclax, a BCL-2 inhibitor, also induces apoptosis and oxidative stress in breast cancer cells by upregulating caspase-3 and suppressing anti-apoptotic genes[2,14.15]. Overexpression of mitochondrial protein cytochrome b5 regulates oxidative stress and caspase-3 activity, revealing a phenotype-specific apoptotic modulation in breast cancer cells[16,17]. Plant-derived agents like Solanum nigrum water extract induce apoptosis through oxidative stress pathways, upregulating p53 and Caspase-3 expression in breast cancer lines[11.18]. Bryodulcosigenin has shown a chemoprotective role by significantly altering oxidative stress and apoptosis markers, including GSH and Caspase-3, in breast cancer models[19]. Silver nanoparticles synthesized with Abies spectabilis also promote oxidative stress-induced apoptosis in MCF-7 cells by elevating caspase-3 and depleting GSH levels[17].

The primary objective of the study was to evaluate the histopathological response of breast cancer tissue to Cold Atmospheric Plasma (CAP) exposure through the analysis of biochemical and apoptotic markers. The study aimed to investigate the effect of CAP on promoting programmed cell death in malignant breast tissue, with a particular focus on the activation of apoptosis-related enzymes and changes in oxidative stress parameters.

The research aimed to determine whether CAP exposure led to a significant increase in caspase-3 activity, which serves as a central indicator of apoptosis execution. In addition, the study sought to measure the extent of oxidative stress induced by CAP through the quantification of malondialdehyde (MDA) levels, reflecting lipid peroxidation, and glutathione (GSH) levels, indicating the cellular antioxidant status.

By comparing these biochemical outcomes between malignant breast cancer samples and benign fibroadenoma controls, the study aimed to establish a distinct profile of tissue-specific responses to CAP treatment.

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The findings were intended to contribute to a better understanding of the potential therapeutic application of CAP in targeting cancerous tissues through selective induction of oxidative and apoptotic pathways.

METHODOLOGY

Study Design: Experiments were carried out in the lab to study how human breast tissue responds to Cold Atmospheric Plasma (CAP). The purpose was to compare certain chemicals in tumors to those in healthy breast tissue using the same test conditions. The samples were made up of breast samples, including 25 from women with malignant breast disease and 25 from women without malignancy. Excised samples were processed fast to keep them usable for biochemical analysis. Each specimen was processed using CAP and all were incubated for the same amount of time so their cells could respond. Experiments were conducted entirely on ex vivo tissue to ensure the studies were controlled and repeatable. All measurements of biochemicals were performed by using colorimetric assays and the levels were matched across the two kinds of tissue. All procedures followed institutional and international guidelines for biomedical research of human tissue and ethics clearance was provided prior to collection. Thanks to the design, scientists could guide CAP exposure with confidence while keeping the analysis of apoptosis and oxidative stress unaffected by bias.

Choosing Participants and Getting Permission: Fifty freshly excised breast tissue samples were examined for the study, including 25 malignant cases from people with invasive ductal carcinoma and 25 benign cases that were confirmed as fibroadenomas by histology. Samples were taken from patients having surgery at a tertiary hospital in the order they became available after their pathology had been checked and confirmed. Breast cancer specimens were required for the malignant set, whereas only patients who had never had cancer were chosen for the benign group. Individuals who had received chemotherapy or radiotherapy in the past were not included to make sure treatment did not affect tissue biochemistry.

The samples were taken from the patient after surgery under clean conditions and cooled on ice while they were being moved to the research laboratory quickly. Patient identities were removed and all samples were coded, so no one would be aware of sample information when they were examined later. All specimens were carefully examined and detailed using standard cutting to make ensure even CAP application and analysis.

This study was reviewed and authorized by the IRB of our medical institution. Patients gave their written consent before surgery so their abandoned tissue could be used in studies. The procedures used were consistent with the Declaration of Helsinki and supported by rules from the researchers' institutions. The study ensured that ethical rules were followed which let volunteers be independent in their choices and took care of their confidentiality and data during the process. All documents confirming participant consent and ethics approval were saved as part of the project's paperwork and opened for observation during internal control evaluations. Applying strict techniques for choosing samples and monitoring ethics made the biological information trustworthy and met the standards for clinical analysis.

Tissue collection and handling: The samples used were collected as soon as surgery on the breast ended, whether for tumor removal or the removal of a benign mass. We put each sample into a sterile, labeled container and quickly moved it to an ice-cooled medium for transport. Within half an hour of excision, further handling took place to prevent cellular components from being degraded. At the laboratory, any handling of samples was done while wearing special clothing and using clean techniques in a biosafety cabinet.

Each specimen was visually checked to rule out necrosis or hemorrhagic damage. Using sterile blades, we removed exactly 1 cm^3 of tissue from each sample for equal exposure to Cold Atmospheric Plasma. A short rinse in cold phosphate-buffered saline (PBS) removed any residual blood and debris and the tissue blocks were then put into ready Petri dishes for CAP treatment. The samples were always kept at 4°C until the point of CAP exposition and incubation, to uphold enzymatic function and prevent activated apoptosis before CAP.

Oxidative breakdown was kept to a minimum and consistency achieved by processing all tissue samples at the same time and in the same conditions. The procedures for managing tissues were handled by trained people according to a set protocol. Samples were stored in containers free of contamination during brief intervals and were not undergone any fixation, freezing or cryopreservation before being studied. Fresh tissue samples were immediately taken from CAP-treated sections, homogenized and then lysed to help with biochemical procedures. Precise handling steps were required to properly preserve the tissue and guarantee that any biochemical changes found were real experiment effects, not results of tissue decay or random sample errors.

Cold Atmospheric Plasma (CAP) Treatment Protocol: Using a properly calibrated dielectric barrier discharge (DBD) device, the treatment was performed with Cold Atmospheric Plasma at room temperature. With the surrounding air serving as the working gas, the device delivered a steady plasma plume with identical performance. Before beginning a session, we made sure to wait five minutes so the CAP device could become consistent in its emission. The working area was cleaned and covered to stop air from getting in and affecting the plasma, resulting in similar test results.

For each sample, a piece of tissue was placed in a non-conductive, sterile Petri dish that was arranged 10 mm below the plasma source, a gap chosen based on earlier trials. Each sample experienced a treatment duration of 60 seconds under CAP. To avoid variations in treatment, the time and distance of exposure remained unaltered for all tissue samples. Light plasma excitation was spread uniformly over the tissue so there was no contact between the nozzle and the specimen.

During the treatment, we kept an eye on temperature and humidity to check that the treatment was running smoothly. To prevent samples from becoming mixed after testing, the device was washed and set to accurate measurements as described by the manufacturer each time it was used. After the samples completed exposure, they were immediately placed in the incubation chamber for measurement of post-treatment activities. The use of this protocol meant that the CAP application could still be repeated and had clear aims, so the application's effects on both cancerous and regular breast tissues could be evaluated properly. To prevent interference by other factors, the study controlled exposure with precision in order to study how CAP affects apoptotic signaling and oxidative stress markers.

Incubation Conditions Post-Treatment: After exposure to Cold Atmospheric Plasma, every tissue sample was immediately placed into a sterile microcentrifuge tube with ice cold PBS to prevent any sudden changes after treatment. The samples were cultured in controlled conditions to allow for reactions within the biochemical fence. A temperature of 37°C was set in a 5% CO_2 humid environment for a total time of four hours. This interval was selected after first testing, showing that apoptotic enzymes were activated and multiple markers for oxidative stress could be found, without the tissue being damaged

in other ways.

At the incubation step, nothing touched the tissues which were kept in sealed, sterile containers all the way through. The biomolecular changes seen were thought to be due only to CAP treatment since all external causes were held constant. Automatic temperature and CO_2 monitoring was used to ensure that all samples experienced the same environment. At the completion of incubation, the tissue was placed on ice right away and quickly processed to remove and retain the proteins. This standard post-treatment incubation step enabled us to study changes in biochemistry caused by exposing breast tissue, either malignant or benign, to CAP.

Tissue Lysis and Protein Extraction: At the end of incubation, we quickly transferred cell samples to an ice bath to prevent activity and help the proteins remain intact. Every specimen was put into a tube with cold buffer made from Tris-HCl, NaCl, EDTA, SDS and a protease inhibitor mixture to keep the proteins from degrading. Homogenization was performed using a mechanical tissue disruptor operating at high speed for 30 seconds, followed by brief centrifugation to collect residual tissue fragments from the tube walls.

The lysates were incubated on ice for 30 minutes with intermittent vortexing to ensure complete cellular disruption and protein solubilization. Following lysis, samples were centrifuged at 12,000 rpm for 15 minutes at 4°C to pellet insoluble debris. The resulting supernatants, containing the total soluble protein fraction, were carefully transferred to fresh microtubes and stored at –80°C until further biochemical assays were conducted.

All procedures were performed under cold conditions to preserve enzyme activity, particularly caspase-3, and to maintain the stability of oxidative stress markers. The extracted protein solutions served as the analytical substrate for all downstream assays, including apoptosis and redox biomarker quantification, ensuring consistency and reliability in the biochemical evaluation of CAP-induced tissue responses.

Caspase-3 Activity Assay Procedure: Caspase-3 activity was measured using a colorimetric assay kit based on the detection of chromophore p-nitroaniline (pNA) after cleavage from the labeled substrate DEVD-pNA. Aliquots of tissue lysates containing equal amounts of total protein were added to 96-well microplates along with reaction buffer and substrate, according to the manufacturer's instructions. The reaction mixture was incubated at 37°C for two hours in the dark to prevent interference with absorbance readings.

Following incubation, the release of pNA was quantified by measuring absorbance at 405 nm using a microplate reader. Background readings from blank wells containing substrate but no lysate were subtracted from all values to eliminate nonspecific absorbance. Caspase-3 activity was calculated in terms of optical density per microgram of protein, allowing for normalized comparisons between malignant and benign tissue samples.

All samples were assayed in duplicate to ensure accuracy, and intra-assay variability was monitored through the inclusion of a positive control lysate with known caspase activity. This assay allowed for the direct quantification of apoptosis execution enzyme activity in response to Cold Atmospheric Plasma treatment, serving as a key indicator of programmed cell death in the examined breast tissue samples.

Glutathione Quantification via Ellman's Method: Glutathione (GSH) levels in the tissue lysates were quantified using Ellman's colorimetric method, which relies on the reaction between free thiol groups and 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB). Equal volumes of tissue lysate and phosphate buffer (pH 7.4) were mixed with freshly prepared DTNB reagent in a flat-bottom 96-well microplate. The reaction mixture was incubated at room temperature for 15 minutes in the dark to allow complete development of the yellow-colored 5-thio-2-nitrobenzoic acid (TNB) product.

Absorbance was recorded at 412 nm using a microplate spectrophotometer. A standard curve was generated using serial dilutions of reduced glutathione to calculate sample concentrations. Final GSH levels were expressed as micromoles per milligram of total protein, ensuring normalization across variable lysate concentrations.

All samples were analyzed in duplicate, and reagent blanks were included to subtract background absorbance. Care was taken to avoid oxidation of samples during handling by conducting all procedures on ice and using buffers pre-treated to remove oxidizing agents. This assay provided a quantitative measure of intracellular antioxidant capacity, allowing for the evaluation of CAP-induced oxidative stress by comparing GSH depletion between malignant and benign breast tissue samples under controlled biochemical conditions.

Lipid Peroxidation Assessment via TBARS Assay: Lipid peroxidation levels in tissue lysates were assessed using the Thiobarbituric Acid Reactive Substances (TBARS) assay, which quantifies malondialdehyde (MDA) as a primary byproduct of oxidative membrane damage. Equal volumes of tissue lysate and thiobarbituric acid (TBA) reagent were mixed with trichloroacetic acid (TCA) and heated at 95°C for 60 minutes in sealed tubes to facilitate the formation of the MDA-TBA adduct.

Following incubation, the reaction mixtures were cooled on ice and centrifuged at 3,000 rpm for 10 minutes to remove precipitated proteins and debris. The resulting supernatants, containing the colored MDA-TBA complex, were transferred to clean cuvettes, and absorbance was measured at 532 nm using a UV-visible spectrophotometer. MDA concentrations were found by using an MDA standard curve and displayed as nanomoles divided by the volume of protein in each sample.

We made sure to use fresh reagents and followed instructions to stop sample contamination and prevent oxidation. Two measurements were made for each sample and those without lysate were used to remove any unwanted background absorption. Using this assay, we could assess cold atmospheric plasma-induced damage to breast cancer and normal breast cells very sensitively.

Protein Concentration Normalization: The concentrations of protein in each tissue lysate were measured with the Bradford assay for stable and equal normalization during all analyzes. An assay was performed using bovine serum albumin (BSA) standards ranging from 0 to 1000 µg/mL to develop a standard curve. Smaller portions of each lysate were mixed with Coomassie Brilliant Blue dye, kept at room temperature for five minutes and then measured on a microplate spectrophotometer at 595 nm.

Absorbance data were plotted along the standard curve to determine how many micrograms of protein each sample contained. Following the first measurements, caspase-3 activity, glutathione levels and lipid peroxidation were all adjusted by total protein content to make up for differences in tissue size and how thoroughly it was lysed. This normalization ensured that biochemical differences observed between malignant and benign tissues were due to actual molecular changes rather than inconsistencies in sample loading or extraction yield.

Samples exceeding the linear range of the assay were diluted appropriately, and each measurement was performed in duplicate for accuracy. By incorporating this step, the study maintained analytical rigor and comparability across all experimental groups, allowing reliable interpretation of Cold Atmospheric Plasma effects on tissue biochemistry.

Statistical Analysis: All statistical analyses were conducted using IBM SPSS Statistics for Windows, Version 25.0 (IBM Corp., Armonk, NY, USA). Descriptive statistics were reported as mean ± standard deviation (SD). The comparison of demographic and baseline biochemical parameters between malignant and benign tissue groups was performed using independent samples t-tests. Given the potential for unequal variances between groups, Welch's t-test was used for these comparisons, as indicated in the footnote of Table 2.

For the malignant group, post-CAP biochemical changes were analyzed descriptively due to the absence of post-treatment data from the benign group. To assess the relationship between baseline biochemical markers (caspase-3, GSH, and MDA) and their respective changes following CAP treatment, ordinary least squares (OLS) linear regression models were applied. Each biochemical outcome (change score from baseline to post-treatment) was used as a dependent variable in individual models, with baseline levels of all three biomarkers as independent predictors.

Multivariate regression analyses were also conducted to confirm the consistency and strength of these associations, adjusting simultaneously for baseline levels of the three markers. Significance was defined at $p < 0.05$, and effect sizes were reported using standardized regression coefficients (β) and R-squared values to indicate the proportion of variance explained. This dual approach of bivariate and multivariate modeling ensured robust evaluation of the predictive value of baseline biochemical profiles on CAP treatment outcomes.

RESULTS

Table 1. Comparison of Demographic Characteristics Between Malignant and Benign Patient Groups

Variable	Malignant (n=25) Mean ± SD	Benign (n=25) Mean ± SD	p-value
Age (years)	48.32 ± 10.99	28.64 ± 5.92	2.04×10^{-9}
BMI (kg/m²)	29.60 ± 3.88	21.70 ± 0.99	1.85×10^{-10}

Footnote: Data are presented as mean ± standard deviation (SD). Statistical comparisons were performed using the independent samples t-test. A p-value < 0.05 was considered statistically significant.

The demographic comparison between the malignant and benign groups revealed significant differences in both age and body mass index (BMI). Patients in the malignant group had a markedly higher mean age (48.32 years, SD 10.99) compared to those in the benign group (28.64 years, SD 5.92), with this difference reaching a high level of statistical significance ($p = 2.04\times10^{-9}$). Similarly, the mean BMI was substantially greater in the malignant group (29.60 kg/m², SD 3.88) than in the benign group (21.70 kg/m², SD 0.99), and this difference was also highly significant ($p = 1.85\times10^{-10}$). These findings indicate that, in this cohort, individuals with malignant pathology were not only older but also had a higher BMI compared to those with benign conditions Table 1.

Table 2. Comparison of Biochemical Parameters Between Malignant and Benign Patient Groups

Parameter	Benign Mean ± SD	Malignant Mean ± SD	p-value
Caspase-3 (OD/µg protein) - Baseline	0.284 ± 0.072	0.402 ± 0.094	9.58e-06
Caspase-3 (OD/µg protein) - Post-CAP	nan ± nan	0.825 ± 0.183	NA
GSH (µmol/mg protein) - Baseline	5.994 ± 0.809	4.301 ± 0.667	2.19e-10
GSH (µmol/mg protein) - Post-CAP	nan ± nan	3.402 ± 0.756	NA
MDA (nmol/mg protein) - Baseline	1.972 ± 0.428	3.273 ± 0.520	1.14e-12
MDA (nmol/mg protein) - Post-CAP	nan ± nan	4.782 ± 0.533	NA

Footnote: Data are presented as mean ± standard deviation (SD). Statistical comparisons were performed using the independent samples t-test with unequal variance (Welch's t-test). A p-value < 0.05 was considered statistically significant.

The statistical comparison of biochemical markers between the malignant and benign patient groups demonstrates pronounced differences at baseline across all measured parameters. Baseline Caspase-3 levels were significantly higher in the malignant group compared to the benign group, indicating increased apoptotic activity associated with malignancy. Similarly, baseline GSH, a marker of antioxidant capacity, was substantially lower in malignant patients, reflecting a possible depletion of antioxidant defenses in cancerous tissues. Conversely, baseline MDA, a marker of lipid peroxidation and oxidative stress, was markedly elevated in the malignant group, supporting the presence of increased oxidative damage in malignancy. For post-CAP measurements, data were only available for the malignant group, precluding direct statistical comparison with the benign group. In the malignant group, post-CAP Caspase-3, GSH, and MDA values were all notably different from their respective baseline means, suggesting that CAP treatment may have influenced apoptotic, antioxidant, and oxidative stress pathways. Overall, these results highlight significant biochemical distinctions between malignant and benign groups at baseline, with malignant tissues exhibiting higher apoptosis and oxidative stress and lower antioxidant capacity Table 2.

The regression analysis revealed distinct patterns in how baseline biochemical levels predicted responses to CAP treatment. For Caspase-3 changes, no baseline parameters reached statistical significance ($p > 0.05$), though baseline Caspase-3 showed a marginal inverse relationship ($\beta = -0.964$, $p = 0.052$). The model explained 24.8% of variance in Caspase-3 response Table 3.

GSH changes were strongly predicted by baseline GSH levels ($\beta = -1.201$, $p < 0.001$), indicating that patients with higher baseline antioxidant capacity experienced greater reductions in GSH after CAP treatment. This model accounted for 54.7% of observed variance Table 3.

MDA changes demonstrated two significant predictors: higher baseline MDA levels correlated with smaller post-CAP increases ($\beta = -1.159$, $p < 0.001$), while the intercept term showed substantial baseline oxidative stress amplification ($\beta = 5.720$, $p < 0.001$). The model explained 54.0% of MDA variability. These results suggest baseline biochemical status significantly influences CAP-induced oxidative and apoptotic responses, particularly for GSH and MDA pathways Table 3.

Table 3. Linear Regression Analysis of Baseline Predictors for Post-CAP Biochemical Changes in Malignant Patients

Dependent Variable	Predictor	Coefficient	SE	t-value	p-value	R-squared
Caspase-3 Change	Intercept	0.726	0.438	1.659	0.112	0.248
	Baseline Caspase-3	-0.964	0.469	-2.056	0.052	
	Baseline GSH	-0.019	0.063	-0.303	0.765	
	Baseline MDA	0.051	0.089	0.567	0.576	
GSH Change	Intercept	3.955	1.789	2.211	0.038*	0.547
	Baseline Caspase-3	0.760	1.916	0.396	0.696	
	Baseline GSH	-1.201	0.256	-4.697	<0.001***	
	Baseline MDA	0.003	0.365	0.007	0.994	
MDA Change	Intercept	5.720	1.271	4.501	<0.001***	0.540
	Baseline Caspase-3	-0.947	1.362	-0.695	0.495	
	Baseline GSH	-0.009	0.182	-0.048	0.962	
	Baseline MDA	-1.159	0.260	-4.466	<0.001***	

Footnote: Ordinary least squares (OLS) linear regression was used to model post-CAP changes in biochemical parameters. *p < 0.05, ***p < 0.001. R-squared values represent the proportion of variance explained by each model.

Table 4. Multivariate Regression Analysis of Baseline Predictors for Post-CAP Oxidative Stress Markers in Malignant Patients

Dependent Variable	Predictor	Coefficient	SE	t-value	p-value	R-squared
Caspase-3 Change	Intercept	0.726	0.438	1.659	0.112	0.248
	Baseline Caspase-3	-0.964	0.469	-2.056	0.052	
	Baseline GSH	-0.019	0.063	-0.303	0.765	
	Baseline MDA	0.051	0.089	0.567	0.576	
GSH Change	Intercept	3.955	1.789	2.211	0.038	0.547
	Baseline Caspase-3	0.760	1.916	0.396	0.696	
	Baseline GSH	-1.201	0.256	-4.697	<0.001	
	Baseline MDA	0.003	0.365	0.007	0.994	
MDA Change	Intercept	5.720	1.271	4.501	<0.001	0.540
	Baseline Caspase-3	-0.947	1.362	-0.695	0.495	
	Baseline GSH	-0.009	0.182	-0.048	0.962	
	Baseline MDA	-1.159	0.260	-4.466	<0.001	

Footnote: Ordinary least squares (OLS) linear regression was used to model post-CAP changes in biochemical parameters. Significant predictors are denoted with p-values: *p < 0.05, ***p < 0.001. R-squared values indicate the proportion of variance explained by each model.

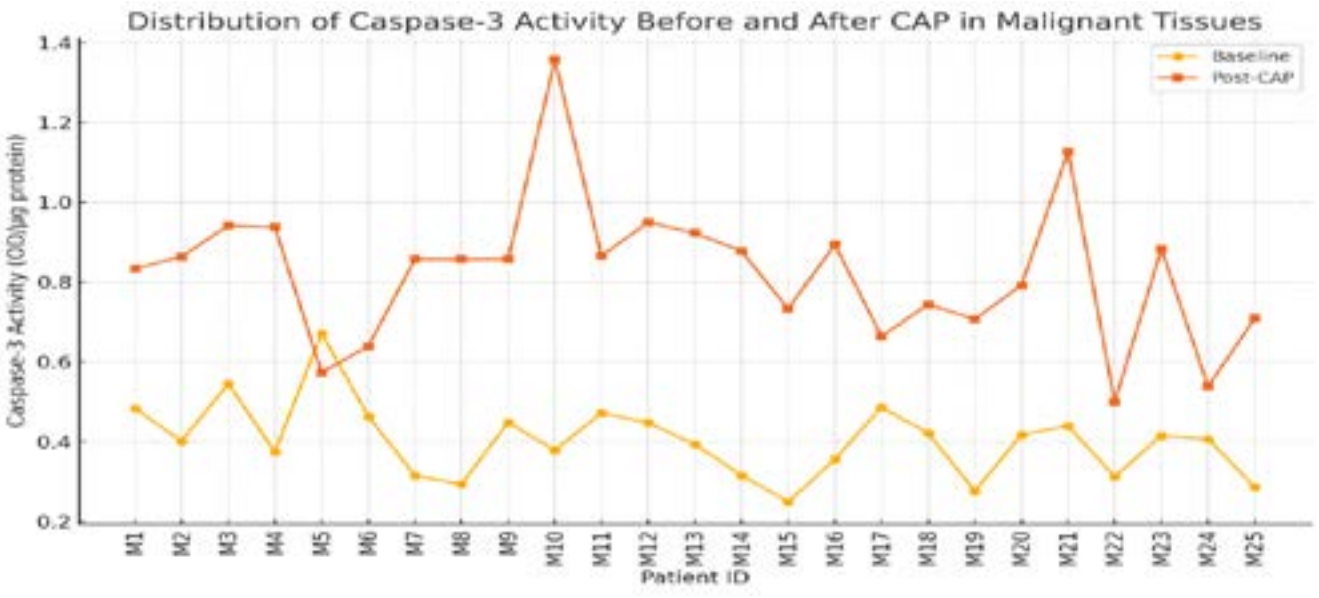

Figure 1. Distribution of Caspase-3 Activity Before and After CAP in Malignant Tissues

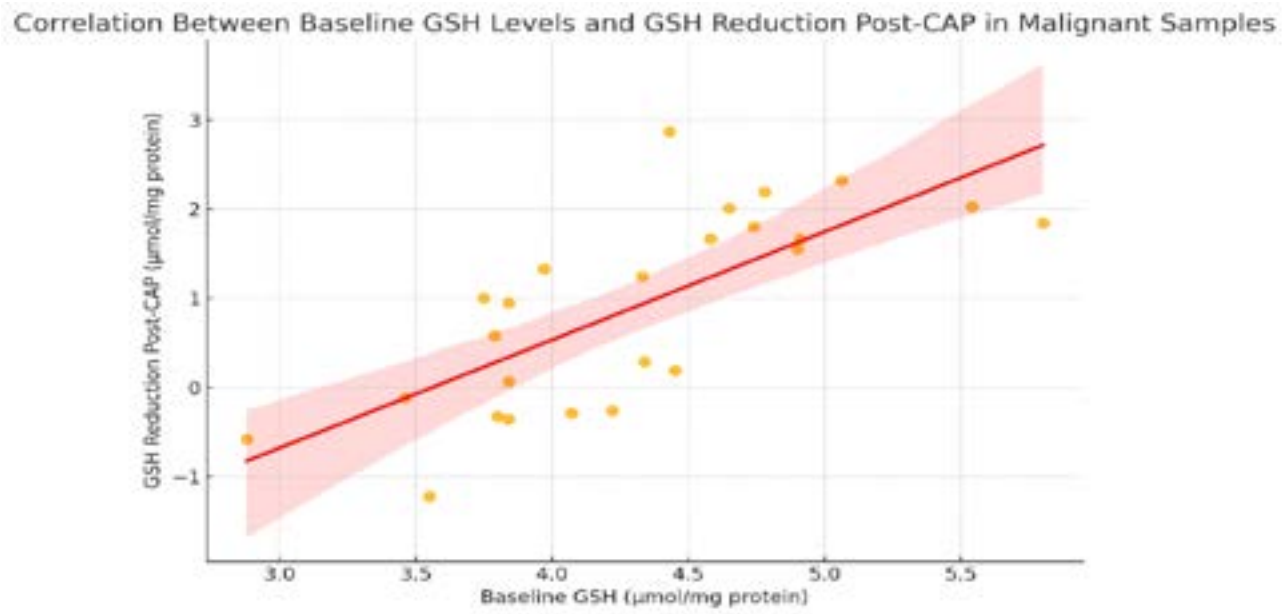

Figure 2. Correlation Between Baseline GSH Levels and GSH Reduction Post-CAP in Malignant Samples, illustrating the inverse relationship between initial antioxidant capacity and the magnitude of depletion following CAP treatment.

The multivariate regression analysis revealed distinct patterns in how baseline biochemical levels predicted responses to CAP treatment. For Caspase-3 changes, none of the baseline parameters reached statistical significance ($p > 0.05$), though baseline Caspase-3 showed a marginal inverse relationship ($\beta = -0.964$, $p = 0.052$), suggesting that higher baseline apoptotic activity might weakly correlate with reduced post-CAP Caspase-3 increases Table 4. The model explained 24.8% of the variance in Caspase-3 response Figure 1.

GSH changes were strongly predicted by baseline GSH levels ($\beta = -1.201$, $p < 0.001$), indicating that patients with higher baseline antioxidant capacity experienced greater reductions in GSH after CAP treatment. This model accounted for 54.7% of the observed variance, highlighting baseline GSH as a critical predictor of antioxidant depletion post-CAP Figure 2.

MDA changes demonstrated significant associations with baseline MDA levels ($\beta = -1.159$, $p < 0.001$), where higher baseline oxidative stress correlated with smaller post-CAP MDA increases. The intercept term ($\beta = 5.720$, $p < 0.001$) revealed a substantial baseline amplification of oxidative stress post-treatment. This model explained 54.0% of MDA variability, underscoring the interplay between baseline oxidative status and CAP-induced lipid peroxidation. These results suggest that baseline biochemical profiles, particularly GSH and MDA levels, significantly influence CAP-induced oxidative and apoptotic responses in malignant tissues.

DISCUSSION

The present study demonstrated significantly elevated baseline levels of Caspase-3 and MDA, alongside reduced GSH in malignant breast tissues compared to benign controls, indicating enhanced apoptotic activity and oxidative stress with impaired antioxidant defenses in malignancy. Following CAP treatment, further increases in Caspase-3 and MDA, and reductions in GSH in malignant samples suggest that CAP accentuates oxidative damage while promoting apoptosis selectively in cancerous tissues.

These findings align closely with a 2021 study by Al-Musawi et al., which identified significantly elevated serum MDA and Caspase-3 levels in breast cancer patients compared to those with benign tumors and healthy controls, reinforcing MDA and Caspase-3 as reliable diagnostic markers and implicating oxidative stress and apoptosis in tumor progression[3].

In a 2024 investigation by Firas et al., breast cancer patients showed significantly higher serum MDA and Caspase-3 levels but lower antioxidant enzyme activity, mirroring the oxidative stress imbalance seen in the present study. Their work emphasized these markers' prognostic value in cancer progression[1].

Similarly, Ghafoor (2022) reported stage-dependent increases in MDA and corresponding declines in GSH in breast cancer patients, establishing a correlation between oxidative stress markers and tumor aggressiveness[9].

Wei et al. (2024) demonstrated that carotenoid-rich extracts induced ROS, increased MDA, depleted GSH, and elevated caspase-3 activity in MDA-MB-231 cells, a mechanistic parallel to CAP-induced changes in the current study[24].

Yan et al. (2021) showed that Triclabendazole induces apoptosis and pyroptosis through Caspase-3 activation and ROS generation in breast cancer, similar to CAP's dual oxidative and apoptotic modulation[21].

Yang et al. (2023) demonstrated dual-organelle oxidative damage using a ROS-producing luminogen, which decreased GSH and MDA while activating Caspase-3, reinforcing the centrality of redox imbalance and apoptosis in therapeutic strategies against cancer[22].

Wang et al. (2025) showed that oxidative stress exacerbation through GSH depletion and Caspase-3 activation triggers dual-pathway pyroptosis in tumors, aligning mechanistically with the CAP response in the present study[23].

Al-Tameemi et al. (2025) explored Caspase-3, GSH, and MDA dynamics in leukemia treatment, confirming oxidative stress and apoptotic pathway involvement across different cancer types[4].
López-Barrera and his team (2021) showed that manipulating GSH

in nanoparticles can control redox balance and Caspase-3 activity in breast cancer cells treated with doxorubicin[12].

Turchi et al. reported that reduced GSH and increased peroxidation of lipids by a lack of nutrients induce apoptosis and ferroptosis, a mechanism also seen in CAP in the present study[25].

According to Ma et al., looking after the balance of GSH and MDA in the body may help prevent oxidative injury in lung cancer cells and therefore has therapeutic value[15].

Researchers demonstrated that Caspase-3, MDA and GSH levels all change with TBM severity, indicating a similar connection between apoptosis and oxidation[10].

Mowat and Al-Abady (2024) discovered that colon cancer shares malignant biochemical markers found in breast cancer in our study, indicating MDA and Caspase-3 are elevated[16].

Vivithanaporn et al. (2024) focused on how caspase-3 influences breast cancer cell apoptosis caused by drugs, making case that it can serve as both a target for therapy and a straightforward biomarker[20].

Collectively, these studies reinforce the present study's findings, underscoring the diagnostic and therapeutic relevance of apoptotic (Caspase-3) and oxidative stress (MDA, GSH) markers in malignancy. The pronounced post-CAP shifts in these markers within malignant tissues further suggest CAP's targeted cytotoxic effect, supporting its potential utility in breast cancer treatment.

CONCLUSION

This study demonstrates that Cold Atmospheric Plasma exerts a significant pro-apoptotic and oxidative stress-inducing effect on malignant breast tissue, characterized by increased caspase-3 and MDA levels and reduced GSH concentrations following treatment. The differential baseline biochemical profiles between malignant and benign tissues further underscore the selective vulnerability of cancer cells to CAP. Linear and multivariate regression models confirmed the predictive value of baseline GSH and MDA levels for post-treatment biochemical responses, establishing these markers as potential indicators of CAP efficacy. These results reinforce CAP's therapeutic potential in selectively targeting malignant tissues through redox and apoptotic pathways and provide a strong rationale for its further clinical evaluation in breast cancer treatment strategies.

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Competing Interest: None

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REFERENCE

1. AdnanSA, Al-Abady ZN. Investigation the Role of Lactate Dehydrogenase, Caspase and the Oxidative Stress Levels in Breast Cancer Patients. JBB.2024; 3(4): 15-22.
2. Alhoshani A, Alatawi FO, Al-Anazi FE, et al. BCL-2 Inhibitor Venetoclax induces autophagy-associated cell death, cell cycle arrest, and apoptosis in human breast cancer cells. *OncoTargets Ther. 2020;13*: 13357–70.
3. Al-Musawi AK, Al-Rubae'I SH, Mahdi MF. Role of Caspase-3, IL-1β and oxidative stress in Iraqi women with breast cancer. *J Phys: Conf Ser*, 2021;1853(1).
4. AL-Tameemi H, Oudah NN, Abdullah DA. Evaluation of the Caspase-3, Calpain-2, GSH, and MDA in AML and ALL Patients Undergoing Chemotherapy. Yemeni J Med Sci. 2025;19(2):92-106
5. Arisi M, Soglia S, Guasco Pisani E, et al. Cold atmospheric plasma (CAP) for the treatment of actinic keratosis and skin field cancerization: Clinical and high-frequency ultrasound evaluation. *Dermatol Ther. 2021;11*(3): 855–66.
6. Bhattacharjee B, Bezbaruah R, Rynjah D, et al. Cold Atmospheric Plasma: A Noteworthy Approach in Medical Science. Sciences of Pharmacy. 2023; 2(2): 79-103.
7. Guo JS, Tian SQ, Zhang YT. Reactive molecular dynamics simulations on interaction mechanism of cold atmospheric plasma and peptide. J Plasma Phys. 2023; 30(4):043512.
8. García L, Rojas L, Gonzales G, et al. The role of cold atmospheric plasma (CAP) in the control of biofilms on titanium surfaces: A literature review. *JIOH. 2021; 13*: 326–30.
9. Ghafoor D. Correlation between oxidative stress markers and cytokines in different stages of breast cancer. *Cytokine. 2023;161*: 156082.
10. Kalita J, Shukla R, Pandey PC, et al. Balancing between apoptosis and survival biomarkers in the patients with tuberculous meningitis. *Cytokine. 2022; 157*: 155960.
11. Khan H A, Prasad NR, Alrokayan S, et al. Antiproliferative effect of Solanum nigrum L. water extract on breast cancer cells: Potential roles of apoptosis and oxidative stress. *CMB. 2023; 69*(10):136–42.
12. López-Barrera LD, Díaz-Torres R, Martínez-Rosas JR, et al. Modification of Proliferation and Apoptosis in Breast Cancer Cells by Exposure of Antioxidant Nanoparticles Due to Modulation of the Cellular Redox State Induced by Doxorubicin Exposure. Pharmaceutics. 2021;13(8):1251.
13. Lopes NMD, Marinello PC, Sanches LJ, et al. Patterns of cell death induced by metformin in human MCF-7 breast cancer cells. *Pathol Res Pract. 2020; 216*(11): 153199.
14. Martínez DD, Méndez AV, Islas JR, et al. Studies of Applications of Cold Plasma Systems in Cancer Treatment: Mechanisms of Oxidant Stress and Pathway Signaling. Stresses. 2024;4(4):896-915.
15. Ma Z, Zhang W, Wu Y, et al. Cyclophilin A inhibits A549 cell oxidative stress and apoptosis by modulating the PI3K/Akt/mTOR signaling pathway. Biosci Rep. 2021;41(1):BSR20203219.
16. Mowat AN, Al-Abady ZN. Study the Effect of Caspase-3, Lactate Dehydrogenase, and Oxidative Stress Levels as Promising Biomarkers to Mediate Colon Cancer Therapy. *JBB*.2024; 3(4):23-30.
17. Ren G, Hao X, Yan S, et al. Abies spectabilis-mediated silver nanoparticles inhibit cell growth and promote apoptosis in breast cancer MCF-7 cells. *J Environ Pathol Toxicol Oncol. 2022; 41*(1), 73–83.
18. Tong X, Yang X.-Z, Gao S, et al. Regulating effect of cytochrome b5 overexpression on human breast cancer cells. *Molecules.2022; 27*(14): 4556.
19. Zheng J, Hu J, Jiang S, et al. Chemoprotective Effect of Bryodulcosigenin against 7,12-Dimethylbenz(a)anthracene-induced Breast Cancer via Suppression of Inflammation, Oxidative Stress, and Apoptosis. Pharmacognosy Magazine. 2024; 21(2):550-60.

20. Vivithanaporn P, Sriwantana T, Krueaprasertkul K, et al. Differential effects of montelukast and zafirlukast on MDA-MB-231 triple-negative breast cancer cells: Cell cycle regulation, apoptosis, autophagy, DNA damage and endoplasmic reticulum stress. Mol Med Rep. 2024;30(2):141.
21. Yan L, Liu Y, Ma XF, et al. Triclabendazole Induces Pyroptosis by Activating Caspase-3 to Cleave GSDME in Breast Cancer Cells. Front Pharmacol. 2021; 12: 670081.
22. Yang L, Wang X, Zhao Y, et al. An AIE luminogen targeting the endoplasmic reticulum inhibits cancer cell growth via multicellular organelle oxidative stress. *Bioorganic Chemistry. 2023; 132*: 106361.
23. Wang J, Xie Y, Zhu G, et al. Mitochondria-targeted H2O2 homeostatic disruptors: Harnessing glutathione autoxidation and reversing GSDME "silencing" for ferroptotic and dual-pathway pyroptotic oncotherapy. *Nano Today*. 2025;61: 102640.
24. Wei J, Ye Z, Li Y, et al. Citrus Carotenoid Extracts Promote ROS Accumulation and Induce Oxidative Stress to Exert Anti-Proliferative and Pro-Apoptotic Effects in MDA-MB-231 Cells. Antioxidants (Basel). 2024; 13(3): 264.
25. Turchi R, Tortolici F, Benvenuto M, et al. Low Sulfur Amino Acid, High Polyunsaturated Fatty Acid Diet Inhibits Breast Cancer Growth. Int J Mol Sci. 2022; 24(1): 249.