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Exogenous Reactive Oxygen Species Augments SMAD4 Expression And TGF-β Paradox in Human Breast Cancer

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ABSTRACT

Background: The Transforming growth factor-β (TGF-β) functions to induce apoptosis, cell cycle arrest, and differentiation is central to sustaining tissue homeostasis and maintaining genomic stability. TGF-β normally, an effective tumor-suppressor that restricts the uncontrolled division of cells augments the development and progression of human malignancies when cytostatic activities of TGF-β are resisted by genetic and epigenetic events caused by tumorigenesis. This dichotomic nature of TGF-β during oncogenesis termed as "TGF-β Paradox," persists to be the most crucial and puzzling query regarding its physio-pathological function and the role of cellular antioxidant status is highly interrelated which warrants more studies on the role of endogenous reactive oxygen species (ROS) in deciding epithelial-mesenchymal transition (EMT) process. The objective of the study was to check whether enhanced ROS augments the TGF-β pathway facilitating EMT.

Methods: In vitro toxicity assay was performed to assess the appropriate concentration of hydrogen peroxide (H2O2) imparting oxidative stress. Comet assay and 8-OHdG (8-hydroxy-2'-deoxyguanosine) enzyme-linked immunosorbent assay (ELISA) were performed to check the extent of DNA damage and adduct production respectively. Mitogen-activated protein kinase (MAPK) p38 ELISA and mRNA gene expression analysis of TGF-β and SMAD were done to verify the effect of H2O2 on these signaling.

Results: The objective of the study was to check whether enhanced ROS augments the TGF-β pathway facilitating EMT. Along with morphological alterations, a dose-dependent decrease in cell viability was seen at 300µM of H2O2 compared to 75µM. DCFDA labeling discovered the dose-dependent gradation of intracellular ROS generation and this was correlated to increased cellular DNA damage and DNA adduct production which was increased linearly with increasing H2O2 as evident with comet test and 8-OHdG ELISA. Significantly reduced MAPK p38 activity revealed by indirect ELISA analysis suggests lessened suppression of cell growth.

Conclusions: The study establishes that higher intracellular ROS will facilitate the TGF-β paradox leading to epithelial mesenchymal transition which can adversely affect therapeutic strategies targeting EMT.

INTRODUCTION

Breast cancer remains a global health challenge in society and is presently the most widespread cancer across the globe. It is a lethal disease and the foremost reason for fatality among females accounting for 23% of deaths of all malignancies, especially in post-menopausal women [1]. Studies focussing on reasons attributed to the development of breast cancer have directed amazing progress in our knowledge fostering new treatment avenues related to breast cancer [2]. The role of TGF-β during oncogenesis is ironic, revealing its competence to function as anti-oncogenic in cancers at early or normal stages, and as a promoter of tumor growth in their late stages [3–5].

Transforming growth factors or tumor growth factors (TGF) are polypeptide growth factors that comprise TGF-α and TGF-β. Though polypeptide growth factors, they are not genetically, structurally, or functionally

similar. TGF-α belongs to the Epidermal Growth factor (EGF) family and has a positive effect on some human cancers. TGF-β described by Roberts and Sporn, comprises 3 isoforms (TGFβ-1, TGFβ-2, and TGFβ-3 coded by unique genes on distinct chromosomes) in mammals. TGF-β is vital in regulating growth and development [6].

All fibrotic diseases showed characteristic increased expression of TGF-β, the strong pro-fibrogenic cytokine [7]. Cells either attain a mutation or lose anti-oncogenic gene function, cancer cells become resilient towards TGF-β stimulated growth arrest, subsequently TGF-β triggers cancer cells to undergo epithelial-mesenchymal transition (EMT) and functions to promote cancer and resistance to chemotherapy [8]. TGF-β further supports the growth and progression of cancer by activating fibroblasts associated with cancer and angiogenesis of the tumor, facilitating the tumor to elude the host immune responses [9]. At later stages of carcinogenesis, however, TGF-β promotes tumor progression through the induction of EMT [10]. TGF-β stimulates electron transport chain complex III which helps in mitochondrial ROS generation necessary for TGF-β-mediated gene transcription downstream of the phosphorylation and subsequent Smad3 nuclear translocation [11]. ROS controls TGF-β downstream signal transduction comprising SMADs, MAPKs, and NF-κB [12]. TGF-β1 augments ROS generation and restrains antioxidant enzymes, creating a redox imbalance [13]. TGF-β overexpression is controlled by ROS in cancer, through both SMAD and non-SMAD signaling pathways, directing to EMT [14,15].

TGF-β can activate MAPKs. A MAPK family member, p38 has strong implications in atherosclerosis progression and is vital in transmitting signals in reaction to different stress signs such as oxidative stress [16,17]. Many responses evoked by TGF-β are mediated by ROS and NADPH oxidase (Nox) enzymes but the mechanisms by which the Smad linker region (SmadL) phosphorylation happens remain ambiguous [18]. Hence, the present study was designed to validate the effect of increased ROS generation on SMAD4 and MAPK p38 signaling. In a quest to settle the persistent query of whether ROS contributes to the non-SMAD signaling pathway, the study evaluated the effect of ROS in determining the TGF-β paradox, and subsequent EMT was explored in detail.

METHODS

The study was focused on checking the role of ROS in activating TGF-β signaling thereby facilitating EMT. To begin with, an in vitro toxicity assay was performed to assess the appropriate concentration of hydrogen peroxide (H_2O_2) in imparting oxidative stress. The extent of DNA damage caused and adduct formation were assessed by Comet assay and 8-OHdG ELISA respectively. MAPK p38 ELISA and mRNA gene expression analysis of TGF-β and SMAD were done to verify the effect of H_2O_2 on these signaling.

MTT assay

Michigan Cancer Foundation-7 (MCF-7) cells were purchased from the National Centre for Cell Science (NCCS) in Pune, India, and were cultured in Dulbecco's modified Eagles medium (DMEM) (Sigma-Aldrich, USA). MTT((3-4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide) assay was performed as per the protocol described elsewhere [19,20]. The cells were washed with 1X Phosphate-buffered Saline (PBS) and 30µL of MTT solution (MTT-5mg\mL dissolved in PBS) was added to the culture and incubated at 37°C for 3 hours. Unreacted MTT was removed by washing with 1XPBS and 200µL of Dimethyl Sulphoxide (DMSO) was added to solubilize. Incubation was done at room temperature for 30 minutes until the cell was lysed and color was obtained. Optical density was read at 540nm using DMSO as blank.

Evaluation of ROS generation by dichlorodihydro-fluorescein diacetate (DCF-DA) staining method

After washing with PBS, the cells were treated with 50µL of DCF-DA and incubated at room temperature for 30 minutes. The excess dye was washed off with PBS and the cells were checked for fluorescence and imaged in a fluorescent microscope (Olympus CKX41 with Optika pro5 CCD camera) fluorescence was quantified using a fluori-meter with excitation-emission at 470nm–635nm (Qubit 3.0, Life Technologies, USA) and expressed in arbitrary units.

Comet assay

Comet assay was done as per the protocol explained by Ramachandran et al. [21]. Briefly, the cells cultured in 6 well plates after treatment with LC50 concentration of sample for 24 hours; were trypsinized, washed with fresh media, and used for comet assay. The cell suspension ($1*10^4$\5-30µL) mixed with low melting agarose was added onto slides pre-coated with 0.75% normal melting agarose. Cells were lysed by placing the slides in lysing solution (12.5µM $NaCl_2$, 100mM Na_2EDTA 1% SDS, 10mM Tris Base pH 10, 1% TritonX100 and 10% DMSO). The slides were then incubated in electrophoresis buffer (300mM NaOH and Na2EDTA pH 13) for 20 minutes to allow unwinding of DNA followed by electrophoresis at 0.8V\cm (300mA) for 20 minutes. Finally, slides were neutralized in buffer (0.4M Tris pH 7.5) thrice for 5 minutes each, followed by staining with 50µL ethidium bromide (20µL/mL). The images were captured using an Optika Pro5 (CI) camera of an inverted epifluorescent microscope Olympus CKX41. Tritek comet scoring software was used to score the comets and correlate them statistically.

Evaluation of MAPK activity using indirect ELISA

After incubation with the samples, the cells were collected and pelleted. The supernatant was collected

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and 100µL of each was added to the 96 well plate and incubated at 37°C overnight. The wells were washed with PBS twice and incubated with freshly prepared blocking buffer in PBS for an hour at RT. After blocking, the wells were washed twice with PBS-TWEEN followed by the addition of 100µL of primary antibody (MAPK) for 2 hours at room temperature. A secondary antibody was added to the wells for an hour after washing off the primary antibody with PBS-TWEEN. PBS TWEEN was used to wash off the unbound antibody. 2-O- dianizdine was added and kept at RT for 30 minutes for color development and the reaction was stopped by the addition of 5N HCL. Optical Density was measured at 415 nm in an ELISA reader. Protein Concentration was also determined.

Evaluation of 8-hydroxy-2'-deoxyguanosine (8-OHdG) using sandwich ELISA

The kit is a pre-coated ELISA plate with Universal 8-OHdG antibody. The samples were added and allowed to bind to antibodies coated on the wells and then biotinylated Universal 8-OHdG antibody was added followed by the addition of streptavidin-HorseRadish Peroxidase (HRP). A washing buffer was used to remove the unbound streptavidin-HRP followed by the addition of substrate solution. The color developed is comparable to the amount of universal 8-OHdG. Acidic stop solution was added to stop the reaction and absorbance was read at 450nm.

Reverse transcriptase polymerase chain reaction (RT PCR)

The RT PCR was done using an amplification kit from Thermo-scientific. The reaction mix was prepared as follows: for each 50µL reaction; 25µL of PCR master mix (2X), 2µL of forward primer (0.1–1.0µM), 2µL of reverse primer (0.1-1.0µM), 5µL of template DNA (10pg–1ug). The components were made up to 50µL, with nuclease-free sterile distilled water. PCR amplification conditions are as follows: Initial denaturation at 95°C for 3 minutes, followed by denaturation at 95°C for 30 seconds, annealing Tm for 30 seconds, and extension at 72°C for 1 minute which was repeated for 35 cycles, and the final extension at 72°C for 5 minutes. Amplification was validated by agarose gel electrophoresis. The sequence of the primers is shown in **Table 1**.

RESULTS

Assessment of in vitro cytotoxic effect of H_2O_2 towards MCF-7 using MTT assay

MCF-7 cells were exposed to H_2O_2 with varied concentrations and performed MTT assay to determine the cytotoxic effect of H_2O_2. Our results showed that when the cell line was exposed to H_2O_2, there was a decrease in the cell viability by 60 ± 1.09 % at a higher concentration of 300 µM when compared with the untreated control cells. The IC50 value was determined as 366.17 µM using ED50Plus V0.1 software. From the obtained results it can be confirmed that the H_2O_2 is exerting a cytotoxic effect on the breast cancer cells, MCF-7. The percentage of viable cells was depicted in **Table 1** and the morphological changes induced by various concentrations of H_2O_2 were photographed with the aid of an epi-fluorescent microscope (Olympus CKX41) connected with a Pro5 CCD camera **(Figure 1)**.

Evaluation of ROS generation by DCF-DA staining method

The intracellular ROS production in control cells and the effect of H_2O_2 in producing ROS were confirmed, visualized, and expressed as fluorescence intensity (AU). In the present study, 300 µM of H_2O_2 exposed cells showed maximum fluorescence of 8723.70 ± 242.2 AU in comparison with the untreated control cells (1037.67 ± 52.7 AU). It can be confirmed that the exposure of H_2O_2 towards the breast cancer cells, MCF-7 was able to exert oxidative stress. From the obtained results it can be confirmed that the ROS generation is increasing in a dose-dependent manner (**Figure 2**).

Table 1. List of primers used for RT PCR

Oligo name	Forward Sequence (5' → 3')	Tm	Reverse Sequence (5' → 3')	Tm
GAPDH	ACTCAGAAGACTGTGGATGG	57.3°C	GTCATCATACTTGGCAGGTT	55.3°C
SMAD 4	ATGGACAATATGTCTATTACGA	52.8°C	GGAATGCAAGCTCATTGTGA	55.3°C
TGF-β	TGGAGCAACATGTGGAACTC	57.3°C	TGCCGTACAACTCCAGGTGAC	59.4°C

Tm, melting temperature

Table 2. Determination of MAPK using indirect ELISA

Samples treated (µM)	MAPK activity (Enzyme units/mg protein)
Control	0.5577 ± 0.004
75	0.4745 ± 0.002
300	0.3143 ± 0.003

Table 3. Determination of 8-OHdG using sandwich ELISA

Samples treated (µM)	8-OHdG activity (ng/mL)
Control	117.66 ± 2.67
75	277.22 ± 4.61
300	502.25 ± 3.84

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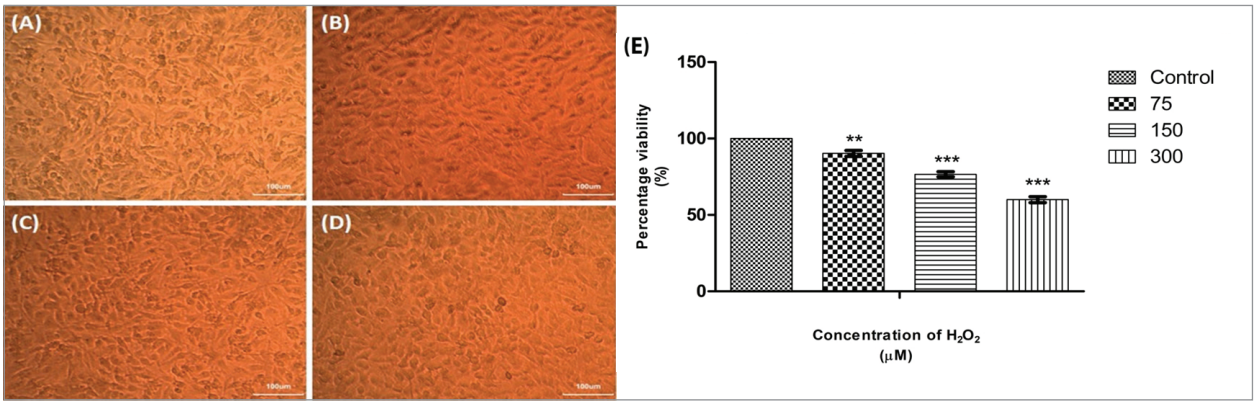

Figure 1: Photomicrographs illustrating the cytotoxic effect of H_2O_2 on cultured breast cancer cells (MCF-7). (A) Untreated control cells; (B) 75; (C) 150; (D) 300 μM; (E) Graphical representation depicting the *in vitro* cytotoxic effect of H_2O_2 in terms of percentage viability (%) on MCF-7 cells with varied concentrations. Y axis shows Percentage viability and the X axis shows a varied concentration of H_2O_2. The experiments were done thrice, and the data represented as Mean+/- SE. Statistical significance was analyzed using One-way ANOVA and the Dunnets test. ***p < 0.001 compared to untreated control group, **p < 0.01 compared to untreated control group. (MCF- Michigan Cancer Foundation; H_2O_2- hydrogen peroxide; ANOVA-Analysis of Variance)

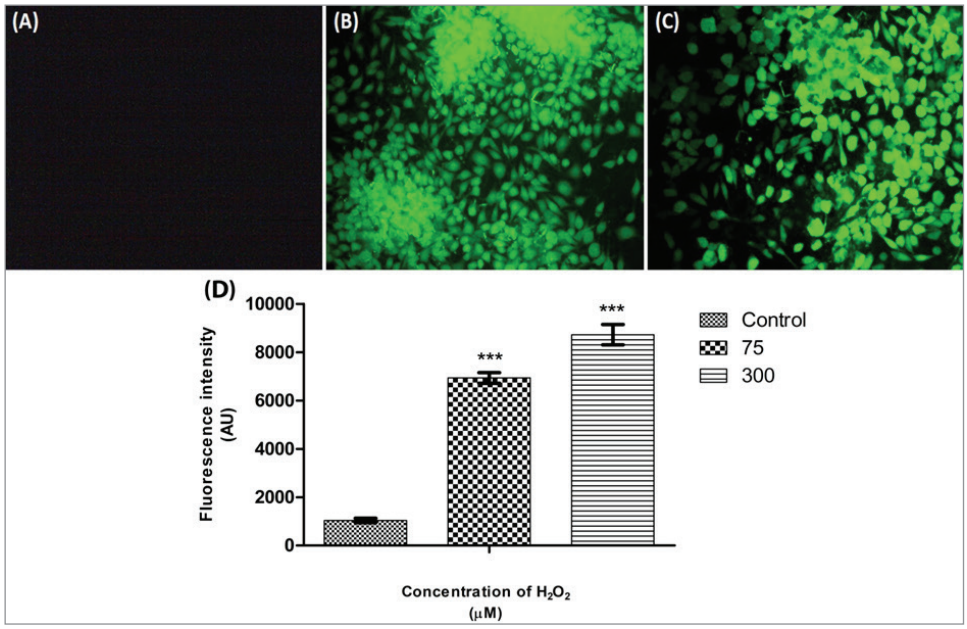

Figure 2: Determination of ROS generation using DCFD-HA staining method (A) untreated control cells; (B) 75 μM of H_2O_2; (C) 300 μM of H_2O_2; (D) Graphical representation depicting the ROS generation by DCFDA-HA method. The y-axis denotes fluorescence intensity, the varied concentration of H_2O_2 in the X-axis. All experiments were repeated thrice and Mean+/- SE is represented in the graph. Statistical analysis was done using One-way ANOVA and the Dunnets test. ***p < 0.001 compared to the untreated control group.

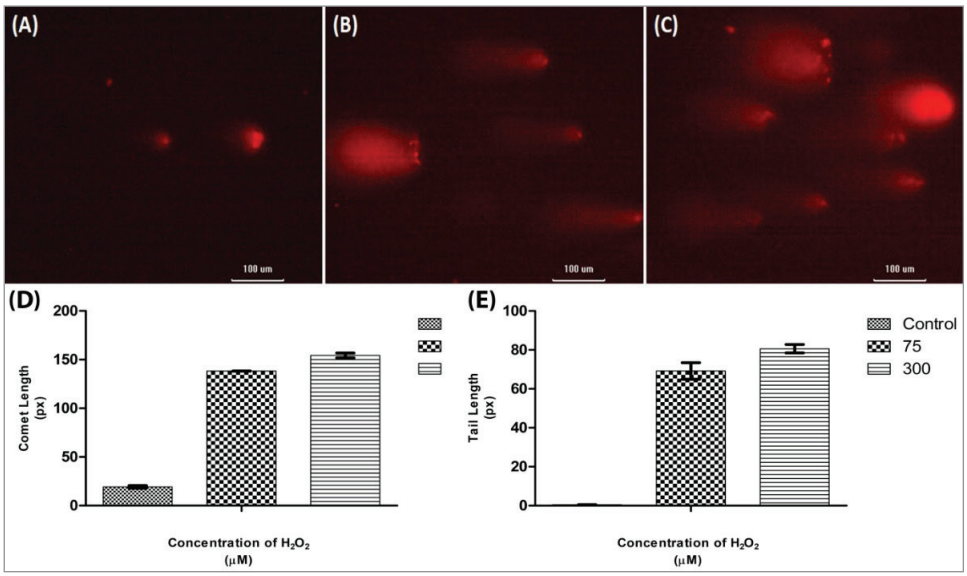

Figure 3: Single-cell gel electrophoresis for the determination of the extent of DNA damage. (A) untreated control cells; (B) MCF-7 cells exposed to 75 μM of H_2O_2; (C) MCF-7 cells exposed to 300 μM of H_2O_2; (D) Graphical representation depicting the comet length obtained upon the exposure of H_2O_2 by single cell gel electrophoresis. Along the y-axis comet length in px, Along the x-axis varied concentration of H_2O_2; (E) Graphical representation depicting the tail length obtained upon the exposure of H_2O_2 by single cell gel electrophoresis. Along the y-axis tail length in px, Along the x-axis varied concentrations of H_2O_2.

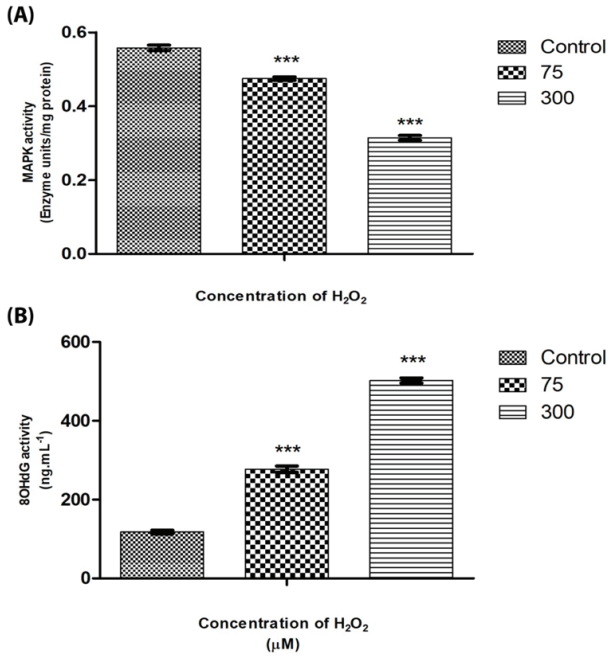

Figure 4. (A) Graphical representation depicting the MAPK activity by indirect ELISA. Along the y-axis MAPK activity in terms of Enzyme units/ mg protein, the x-axis represents a varied concentration of H_2O_2. The data presented is the Mean+/- SE value generated from triplicates. Statistical analysis was done using One-way ANOVA and the Dunnets test. ***p < 0.001 compared to untreated control group; (B) Graphical representation depicting the 8OHdG activity by sandwich ELISA. Along the y-axis 8OHdG activity in terms of ng/mL, and along the x-axis varied concentration of H_2O_2. All experiments were done in triplicates and results were represented as Mean+/- SE. One-way ANOVA and Dunnets test were performed to analyze data. ***p < 0.001 compared to the untreated control group

Table 4. Relative band expression

Samples treated (μM)	Relative band expression	
	SMAD 4	TGF-β
Control	0.8729	1.127
75	1.573	1.497
300	1.775	1.963

Assessment of genotoxicity induced by exposure of H_2O_2 to MCF-7 cells by comet assay

The level of nuclear DNA damage was determined by measuring the different parameters following the comet assay under alkaline conditions, which reflects the extent of DNA breakage in both the single and double-strand breaks. The slides were evaluated using an inverted epi-fluorescent microscope (Olympus CK41) connected to a CCD Pro5 camera. H_2O_2 exposed cells were showing an increased comet length in comparison with the untreated control cells. As the concentration of H_2O_2 increases, the DNA damage occurred also increases (**Figure 3**).

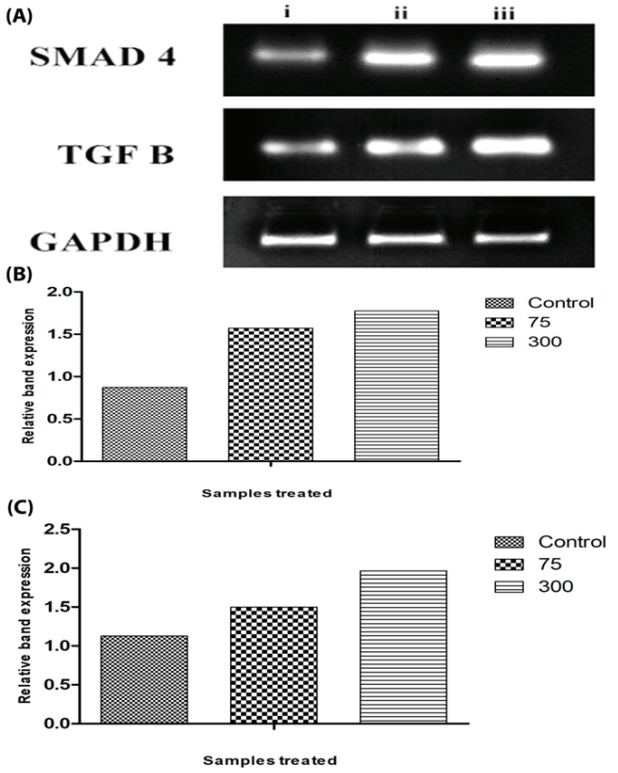

Figure 5. (A) Photomicrograph of a typical 1.5 % agarose gel depicting the gene expression pattern of SMAD-4 and TGF-β and housekeeping gene GAPDH, in the H_2O_2 exposed MCF-7 cells (i) untreated control (ii) MCF-7 cells exposed with 75 μM H_2O_2 and (iii) MCF-7 cells exposed with 300 μM H_2O_2.The gel image was captured by E-gel Image (Invitrogen, USA); (B) Graphical representation depicting the relative band expression of SMAD4 gene. Along the y-axis relative band expression, Along the x-axis varied concentration of H_2O_2 (C) Graphical representation depicting the relative band expression of TGF-β gene. Along the y-axis relative band expression, along the x-axis varied concentrations of H_2O_2.

Evaluation of MAPK activity using indirect ELISA

Deregulated MAPK signaling has been associated with several cancers the reason being aberrant expression of receptors of different pathways; mutations in critical genes that might activate receptors and downstream signaling molecules without proper stimuli and many other mechanisms. To check the activity of MAPK, indirect ELISA was performed. From the obtained results it can be inferred that the H_2O_2 (300 μM) exposure has significantly decreased the level of MAPK (0.3037 enzyme units/mg protein) when compared with the untreated control cells (**Table 2; Figure 4**). According to a dose-dependent manner, the MAPK activity was significantly decreased when compared with untreated control cells.

Evaluation of 8-hydroxy-2-deoxyguanosine (8-OHdG) using sandwich ELISA

Reactive oxygen species and the degree of oxidative stress generated by them have been crucial in tumor development and are normally better assessed by analyzing

stable metabolites of oxidative responses. The stable metabolite, 8-hydroxy-2'-deoxyguanosine (8-OHdG), a 2'-deoxyguanosine oxidative damage product, is used for evaluating oxidative DNA damage. 8-OHdG was determined using the ELISA method. From the obtained results it can be inferred that the H_2O_2 (300 µM) exposure has increased the level of 8-OHdG (367.22 ng/mL) when compared with the untreated control cells (**Table 3**; **Figure 4**). From the obtained results it can be confirmed the DNA damage exerted by H_2O_2 towards the cultured MCF-7 cells.

Relative expression study of TGF-β and SMAD on H_2O_2 exposed MCF-7 cell lines using RT PCR

mRNA from MCF-7 cells treated with H_2O_2 and untreated control was isolated using TRIzol method and cDNA was synthesized from it using Thermoscientific Verso cDNA synthesis Kit. TGF-β and SMAD genes were further amplified from the cDNA for analyzing the effect of H_2O_2 on the expression of TGF-β and SMAD genes using Thermoscientific amplification kit and were compared with untreated control. The results can be seen in **Table 4** and **Figure 5**.

DISCUSSION

TGF-β functions to maintain tissue homeostasis via cell cycle arrest, apoptosis, and differentiation and also to sustain genomic stability. TGF-β acts as an anti-cancer agent by prohibiting the uncontrolled proliferation of endothelial, epithelial, and hematopoietic cells. On the contrary, tumorigenesis can elicit aberrations in TGF-β signaling thereby developing resistance to cytostatic activities of TGF-β augmenting tumor progression. Additionally, these genetic and epigenetic events cooperate to transform TGF-β from a tumor suppressor to a promoter of their invasion, growth, and metastasis. This reciprocated nature of TGF-β during carcinogenesis termed as the "TGF-β Paradox," prevails as the most crucial and puzzling question regarding the pathophysiological role of this dual-hatted cytokine [22].

EMT associated with Smad protein loss- and angiogenesis contributes to tumor progression in many cancers [23]. Deliberations on the paradoxical roles of Smad and TGF-β signaling pathway in tumor suppression and progression of SCC, development of cancer therapies targeting TGF-β signaling is highly cautious and more studies have to be done to check the effect of SMAD and TGF-β paradox and underlying factors.

As per our review, it can be observed that both TGF-β and ROS play differential roles in tumor promotion as they have antitumor activity, through inducing senescence, cell cycle arrest, cell death, and pro-tumor effects by impacting cancer cell proliferation, survival, spreading, and metastasis. TGF-β controls ROS production either directly or by diminishing antioxidative systems. On the other hand, ROS can stimulate TGF-β expression and elevated expression activates it from the latent complex. This strong interplay between ROS and TGF-β is exploited by cancer cells to augment tumor growth. Furthermore, both TGF-β and ROS can foster cellular senescence, which either preserves impaired cells from malignant transformation or may cooperate in cancer advancement [24]. We kept the study to check the effect of lower to higher transition of the radical status of cells over the expression of TGF-β and SMAD proteins.

Breast cancer was selected as the cell line of our interest and we check the tolerability of ROS supplementation to MCF-7 cells by treating different concentrations of ROS. From our results, it can be observed that with an increase in the hydrogen peroxide concentration ie., free radicals the viability of the cancer cells decreased. These results confirm that there is a threshold for cancer cells to limit oxidative stress. Our findings as supported by the previous researcher who has shown that H_2O_2 in higher concentrations can generate mitochondrial and nuclear DNA lesions, elevate expression of genes related to cell adhesion, and augment the activity of p53 and other transcription factors coordinating tumor cell death [25]. From the results, we selected 75µM which showed little or no toxicity, and 300µM which decreased the cell viability to 60% for further studies. Higher concentrations were avoided as it imparted higher mortality.

Further studies were planned to compare the sub-lethal and nontoxic concentrations of hydrogen peroxide in MCF-7 cells and we started with studying the ROS generation in vitro by these concentrations. From the results, it was observed that the ROS generation increased from 1201 to 8147 arbitrary units which confirms an 85% increase in ROS generation which is a nearly 8-fold increase in intracellular ROS generation observed in 300µM H2O2 treated cells which were following previous findings.

A precise evaluation of DNA damage was quite required to support our findings and we employed comet assay and 8OHdG as parameters for assessing nuclear damages. Our findings report a more than four-fold increase in comet formation in cells treated with 300µM hydrogen peroxide when compared with lower concentrations. A similar pattern was observed in 8OHdG values which confirms that DNA damages followed a concentration-dependent pattern over cell death in MCF 7 cells exposed to higher concentrations of ROS. The DNA damages were further confirmed with comet assay analysis and our results depict an increased DNA damage with 300µM of H_2O_2 whereas 75µM produced only a negligible increase in DNA damage.

The major intention of the study was to check the effect of H_2O_2 administration in the increase of TGF-β, SMAD, and MAPK 28 signaling. We used indirect ELISA analysis to measure the extent of expression of the MAPK gene. As reviewed, MAPK pathways JNK and p38,

activated during stress can modulate the cancer cells' response to both chemotherapies and targeted therapies, as well as epigenetic and metabolic modulators. Our results suggest that the increase of intracellular ROS concentration is decreasing the expression of MAPK activity suggesting a critical role in EMT.

Our major objective was to check the effect of these higher concentrations of ROS in TGF-β expression and signaling and we employed reverse transcriptase PCR for the same. The inconclusive role of stress and TGF-β signaling has been studied several times and many authors have shown that sub-cytotoxic oxidative stress with H_2O_2 can induce Stress-induced premature senescence (SIPS) in human diploid fibroblasts after 3 days of exposure with development of numerous replicative senescence biomarkers [26]. From the ImageJ analysis, it was observed that the presence of higher concentrations of ROS produced a three-fold increase in TGF-β expression. In line with this, our results also confirmed that the cell overexpress TGF-β as a mechanism to overcome the oxidative stress-mediated cellular assault.

Similarly, RT PCR analysis of SMAD4 gene has shown a significant increase in expression when cells were treated with higher concentrations of hydrogen peroxide. This supports the findings that the SMAD/TGF-β signaling pathway demonstrates dichotomous roles of tumor-suppression and tumor-progression functions [24]. Even though the study provides valuable insights into the molecular effects of ROS exposure on cellular processes, its direct clinical relevance to cancer therapy or drug development remains speculative, and in context with this further studies, including in vivo experiments and clinical trials, would be necessary to authenticate the therapeutic potential of targeting TGF-β/SMAD4 signaling in cancer treatment. By building upon the findings of this commendable in vitro study, future research endeavors can further elucidate the therapeutic efficacy and safety profile of TGF-β/SMAD4 inhibitors in preclinical and clinical settings. Such investigations hold promise for the development of novel therapeutic strategies aimed at combating cancer progression by modulating ROS-mediated signaling pathways. Therefore, this study serves as a pivotal stepping stone toward the advancement of precision oncology and the improvement of targeted therapies specific to patients with cancer.

CONCLUSIONS

The effect of varied concentrations of ROS in terms of hydrogen peroxide was studied employing MCF-7 as the in vitro model. A dose-dependent decrease in cell viability was detected in 300μM of H_2O_2 when compared with 75 μM along with morphological changes. The intracellular ROS generation was found to follow a dose-dependent gradation when assessed by DCFDA staining. The lower to higher concentrations of hydrogen peroxide produced a linear increase in cellular DNA damage and DNA adduct formation when determined by comet assay and 8-OHdG ELISA analysis. Indirect ELISA analysis has shown a significant decrease in MAPK p38 activity suggesting decreased inhibition of cell proliferation. mRNA analysis by PCR has shown a shift in TGF-β expression where higher TGF-β levels were found in 300μM when compared with 75μM and similarly an increase in expression of SMAD 4 was also observed. Hence it can be concluded that novel attempts in developing drugs against TGF-β/SMAD4 should be centred on, or be knowledgeable about, the timing and mechanism of tumor suppressor- promoter swapping.

DECLARATIONS

Competing interest
The authors declare no competing interest in this study.

Ethics approval
N/A

Acknowledgment
N/A

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