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Article

Synergistic induction of colorectal cancer cell proliferation by ox-LDL and TNF-α.

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ABSTRACT

Colorectal cancer (CRC) is the third most commonly diagnosed malignancy and the second leading cause of cancer-related deaths worldwide. Its incidence continues to rise, particularly in association with modifiable risk factors such as obesity, which is closely linked to chronic inflammation and metabolic disturbances, including dyslipidemia. These conditions contribute to the formation of a pro-inflammatory tumor microenvironment, characterized by high levels of TNF-α and ox-LDL. This study aimed to analyze the synergistic effects of ox-LDL and TNF-α on ROS production and cell proliferation via the WNT/β-catenin and PI3K/AKT pathways in CRC cells. COLO320 and SW620 cells were treated with various concentrations of ox-LDL, TNF-α, and their combinations. The proliferation induced was assessed using the IncuCyte® Real-Time Assay. ROS generation was measured using the 2′,7′-dichlorodihydrofluorescein diacetate (H2DCFDA) probe. Cell viability was evaluated using the MTT assay under conditions of pathway inhibition. Co-treatment with ox-LDL and TNF-α significantly increased proliferation in COLO320 cells, and was accompanied by a marked increase in ROS generation in both cell lines. Inhibiting the WNT/β-catenin and PI3K/AKT pathways revealed differential responses, suggesting a heterogeneous activation pattern dependent on the molecular context. To our knowledge, this is the first study to demonstrate the synergistic effect of ox-LDL and TNF-α in colorectal cancer cell models. These findings highlight the importance of considering both the molecular and redox context of the tumor microenvironment when designing personalized therapeutic strategies.
Keywords: Colorectal cancer; proliferation; ROS; TNF-α; ox-LDL.

INTRODUCTION

Colorectal cancer (CRC) is one of the leading causes of morbidity and mortality worldwide. According to the World Health Organization (WHO), cancer is characterized by the uncontrolled proliferation of abnormal cells that can invade tissues and form metastases; the latter is one of the main causes of death associated with the disease [1, 2]. In 2022, nearly 1.9 million new cases of CRC and approximately 904,000 cancer-related deaths were reported worldwide, ranking it as the third-highest cancer in terms of incidence and second-highest in terms of mortality[3].

CRC primarily originates from adenomatous polyps of the colorectal epithelium[4]. The progression of these polyps to invasive adenocarcinomas involves cumulative genetic and epigenetic alterations[5]. CRC is sporadic in approximately 60–65% of cases, while 25–30% have a hereditary component. The most common are [6, 7].

The main non-genetic risk factors for CRC include obesity, dyslipidemia, and inflammatory bowel disease. Obesity generates a state of low-grade chronic inflammation, characterized by the sustained production of pro-inflammatory cytokines, such as tumor necrosis factor-alpha (TNF-α), as well as increased reactive

oxygen species (ROS), which damage cellular macromolecules and promote tumor progression. In addition, 60–70% of obese individuals have dyslipidemia, which increases the presence of oxidized low-density lipoprotein (ox-LDL). This pro-inflammatory lipoprotein binds to scavenger receptors, such as LOX-1, promoting the ROS generation[8, 9].

An imbalance between ROS production and the body's antioxidant capacity leads to oxidative stress. This phenomenon causes cellular damage and modulates intracellular signaling pathways involved in proliferation, survival, and inflammation. In this context, two pathways stand out for their sensitivity to redox signals: PI3K/AKT and WNT/β-catenin[10]. The PI3K/AKT pathway is involved in transducing signals that promote cell proliferation and inhibit apoptosis[11]. AKT can then increase NOX1 activity, establishing a positive feedback loop[12]. On the other hand, the WNT/β-catenin pathway regulates the transcription of genes associated with cell proliferation. Under normal conditions, β-catenin is degraded by a complex that includes APC and GSK3β. However, mutations in APC or redox modulation of proteins, such as nucleoredoxin (NRX), can activate β-catenin[13]. NOX1 produces ROS, which can oxidize NRX and release Dishevelled (Dvl), activating WNT signaling[14].

Although both TNF-α and ox-LDL have been implicated in these processes, there are no conclusive studies evaluating their combined effect on the synergistic activation of these pathways in CRC[15-17]. This is a particularly relevant gap considering that both factors are elevated in patients with obesity[18, 19].

This study aimed to evaluate the synergistic effects of ox-LDL and TNF-α on activating the WNT/β-catenin and PI3K/AKT signaling pathways, as well as their relationship with cell proliferation in colorectal cancer cell lines. Understanding these mechanisms could lead to the identification of new CRC biomarkers and therapeutic strategies within the context of its inflammatory and oxidative microenvironment.

MATERIAL AND METHODS

Cell culture.

The human colorectal cancer cell lines HT-29 (ATCC; HTB-38) and SW620 (ATCC; CCL-227) were cultured in Dulbecco's Modified Eagle's medium (DMEM) (Gibco), while COLO320 (ATCC; CCL-220) was cultured in Roswell Park Memorial Institute Medium (RPMI) 1640 (Gibco). Each medium was supplemented with 10% heat-inactivated fetal bovine serum (FBS) (HycloneTM), 2 mM L-glutamine (HycloneTM), and penicillin/streptomycin (100 U/mL/0.1 mg/mL) (Gibco). Cells were incubated in a Nuaire incubator at 37°C with 5% CO2 and 95% relative humidity.

IncuCyte® Real-Time Assay.

HT-29, SW620, and COLO320 cells were seeded at a density of 5,000 cells/well in 96-well plates and incubated with 2% FBS. After 24h, cells were treated with ox-LDL (0, 6.25, 12.5, 25, and 50 µg/mL), TNF-α (0, 10, and 15, ng/mL), or their combination. Real-time proliferation experiments were conducted during 24, 48, 72, and 94 h using the IncuCyte® S3 live-cell analysis system (Bohemia) from the Advanced Microscopy Center (CMA Biobio, Concepción, Chile) of the University of Concepción.

ROS measurement.

Cells were seeded at a density of $5.5x10^4$ cells/well in a black, flat-bottom 96-well plate in phenol red–free medium supplemented with 10% FBS and incubated for 24 h. When applicable, cells were pretreated with NOX1 inhibitor ML171 (10 µM) (Sigma-Aldrich) for 1 h. Subsequently, cells were incubated with 5 µM of the ROS-sensitive probe 2′,7′-dichlorodihydrofluorescein diacetate (H2DCFDA, Tocris Bioscience) for 30

min according to the manufacturer's instructions. Finally, cells were treated with ox-LDL, TNF-α, or their combinations. Fluorescence was measured using a Synergy HTX multimode reader (Biotek) at excitation/emission wavelengths of 495/520 nm at 10, 30, 45, 60, 90, and 120 min post-treatment.

Cell viability.

Cell viability was assessed using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay (ThermoFisher Scientific). Cells were seeded at a density of 7.5 x 10^3 cells/well in 96-well plates and treated for 94 h with 30 nM of Copanlisib (PI3K/AKT inhibitor, Cayman), 10 µM of LF3 (WNT/β-catenin inhibitor, Abcam), ox-LDL, TNF-α, or combinations thereof. Following treatment, cells were incubated with 5 mg/mL of MTT for 3 h. Formazan crystals were solubilized in 100% isopropanol for 15 min. Absorbance was measured at 570 nm using a Synergy HTX multimode reader (Biotek).

Statistical Analysis.

Data are presented as mean ± SD. All statistical analyses were performed using GraphPad Prism 8.0 software. Depending on the dataset, multiple comparisons were carried out using one-way ANOVA or two-way ANOVA followed by Tukey post hoc test, or the Kruskal–Wallis test followed by Dunn's post test. A p-value < 0.05 was considered statistically significant.

RESULTS

Effects of ox-LDL and TNF-α on CRC cell proliferation.

To evaluate the impact of ox-LDL and TNF-α on the real-time proliferation of CRC cell lines, the IncuCyte® S3 live-cell analysis system was employed. Cells were treated for up to 94 h with increasing concentrations of ox-LDL and TNF-α, either individually or in combination, as described above.

In the COLO320 cell line, treatment with 10 ng/mL of TNF-α resulted in increased cell proliferation after 48 h of incubation (Figure 1A, 1C, 1E, and 1G); however, this difference was not statistically significant compared to the untreated control group. TNF-α induced an increase in proliferation at 72 h of incubation at 15 ng/mL (Figure 1B); however, this effect was not consistently observed under other experimental conditions with the same concentration (Figure 1D, 1F, and 1H). Cells treated with 12.5 µg/mL of ox-LDL exhibited an increased proliferation trend at 72 h (Figure 1C and 1D), but this was not statistically significant. No changes in proliferation were detected at 6.25, 25, or 50 µg/mL. However, a statistically significant increase in proliferation was observed only under the following combined treatment conditions: 6.25 µg/mL ox-LDL + 15 ng/mL TNF-α at 48 h (Figure 1B); 12.5 µg/mL ox-LDL + 15 ng/mL TNF-α at 94 h (Figure 3D); and 25 µg/mL ox-LDL + 15 ng/mL TNF-α at 72 h (Figure 1F). These results suggest that TNF-α and ox-LDL can induce proliferative responses under particular experimental conditions, yet not all combinations were statistically significant.

Notably, a significant increase in proliferation was consistently observed in COLO320 cells at low ox-LDL concentrations (6.25–25 µg/mL) when combined with 15 ng/mL TNF-α, which supports a potential synergistic effect between the two stimuli.

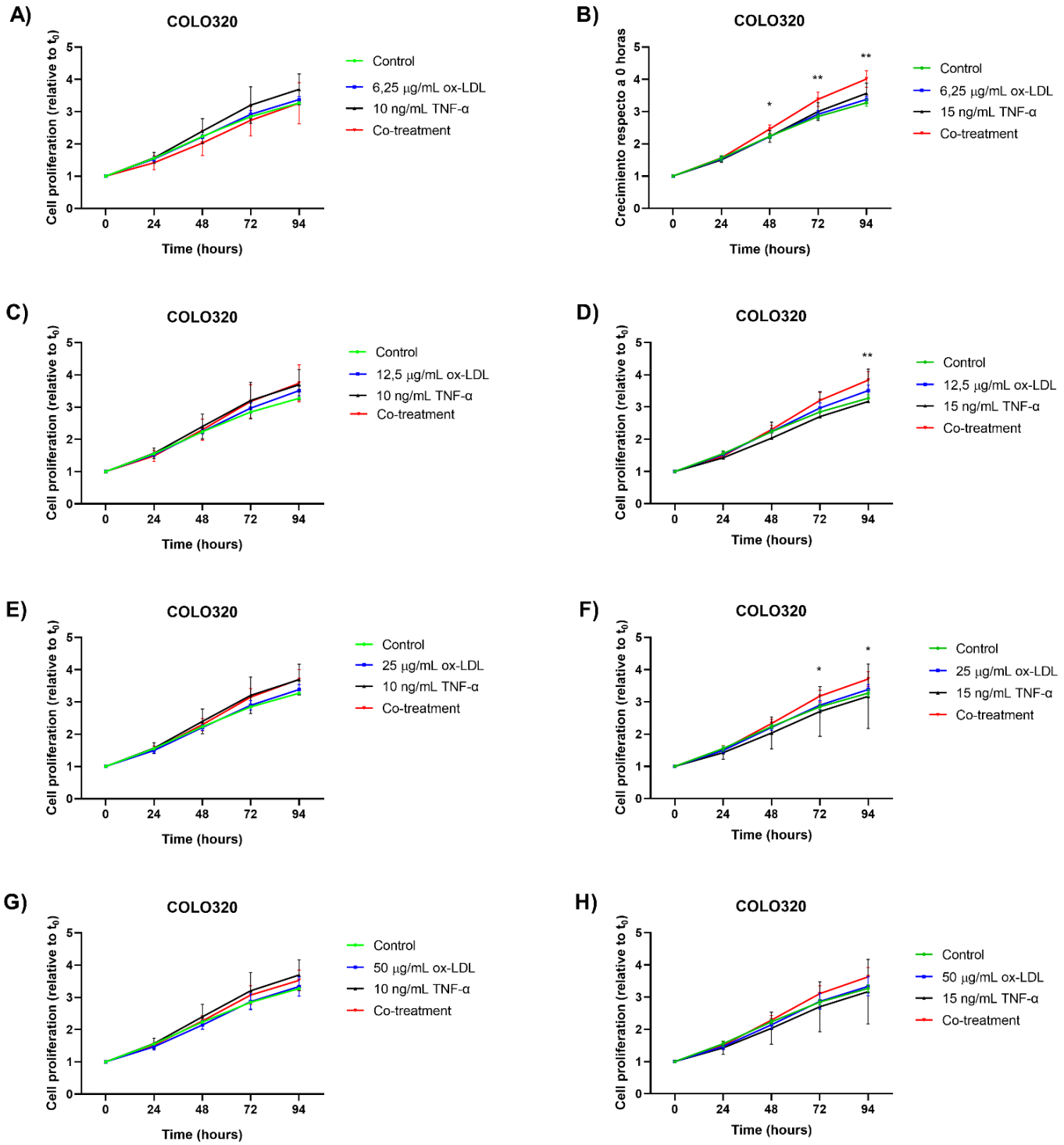


Figure 1. Proliferation of COLO320 colorectal cancer cells. Cells were treated with ox-LDL, TNF-α, or co-treatments for 24, 48, 72, and 94 h. (A–H) correspond to the different treatment conditions tested. Data are presented as mean ± SD (n = 6). Statistical analysis was performed using a two-way ANOVA followed by a Tukey's multiple comparisons test, *$p < 0.05$, **$p < 0.01$.

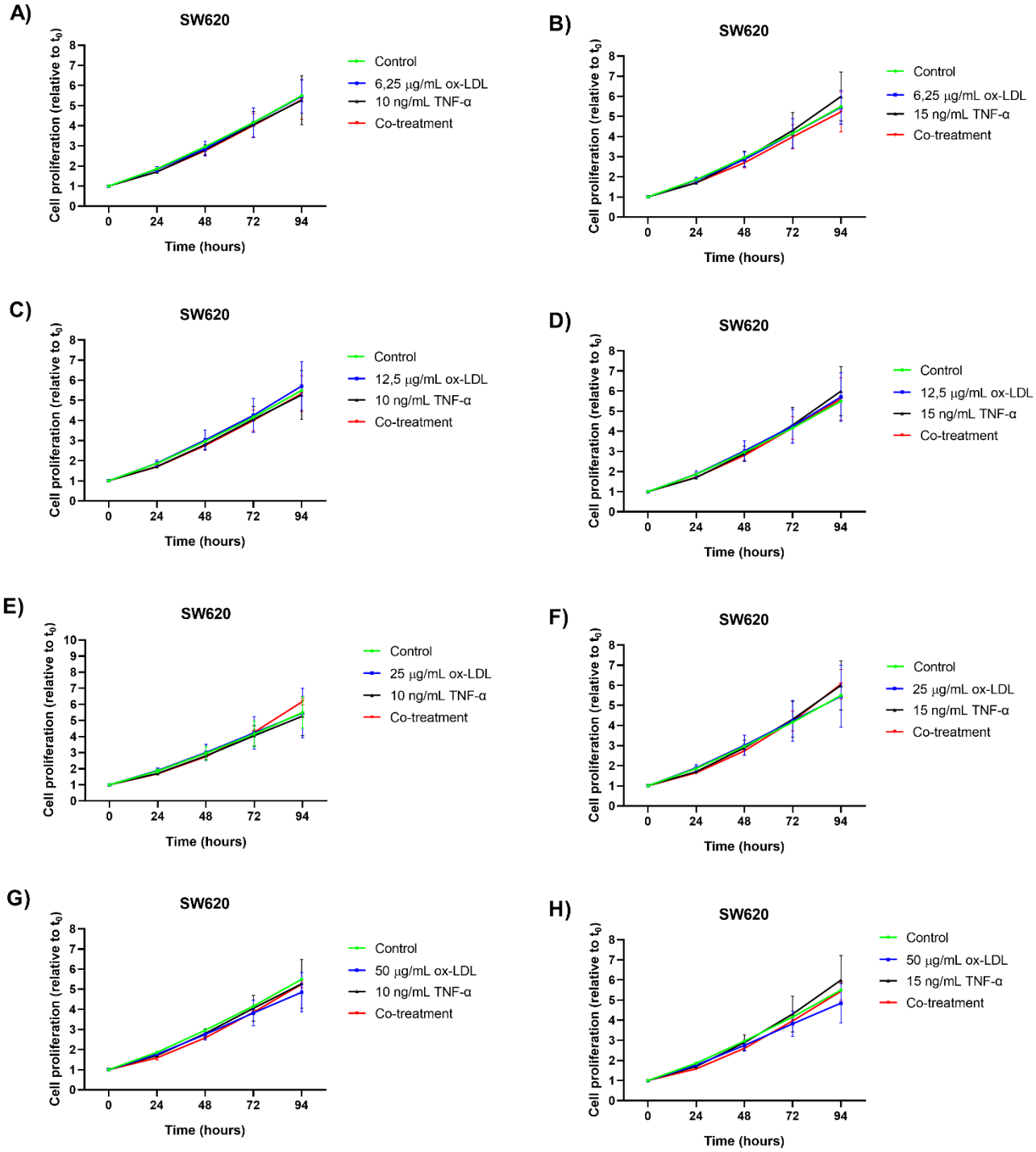


Figure 2. Proliferation of SW620 colorectal cancer cells. Cells were treated with ox-LDL, TNF-α, and co-treatments for 24, 48, 72, and 94 h. (A-H) correspond to the different treatment conditions tested. Data are presented as mean ± SD (n = 6). Statistical analysis was performed using two-way ANOVA followed by Tukey's multiple comparisons test.

In the SW620 cell line, treatment with TNF-α at 10 or 15 ng/mL did not result in a statistically significant increase in proliferation compared to the control group (Figure 2A–H). Similarly, individual treatments with ox-LDL at 6.25, 25, or 50 µg/mL showed no significant differences in proliferation relative to the control (Figure 2A, 2B, 2E, 2F, 2G, and 2H). However, treatment with 12.5 µg/mL ox-LDL exhibited a slight trend

toward increased proliferation after 72 h. Nevertheless, this difference was not statistically significant (Figure 2D).

Regarding co-treatments, no additional proliferative effect was observed in seven of the eight tested conditions. Only the combination of 25 µg/mL ox-LDL and 10 ng/mL TNF-α showed a modest trend toward increased cell proliferation after 72 h; however, this difference was not statistically significant compared to the control.

Overall, results from the SW620 cell line indicate a tendency toward increased proliferation under certain treatments. However, none of these changes reached statistical significance in this analysis.

Representative images were acquired for each cell line to provide a detailed visualization of the effects of treatment on cell proliferation. In COLO320 cells, a marked change in confluence was observed between 0 h and 94 h across treatment conditions (Figure 2A). In contrast, in SW620 cells, the variation in confluence among treatments was less pronounced than in COLO320. However, the treated groups still exhibited a visible increase in proliferation compared with the control group (Figure 2B).

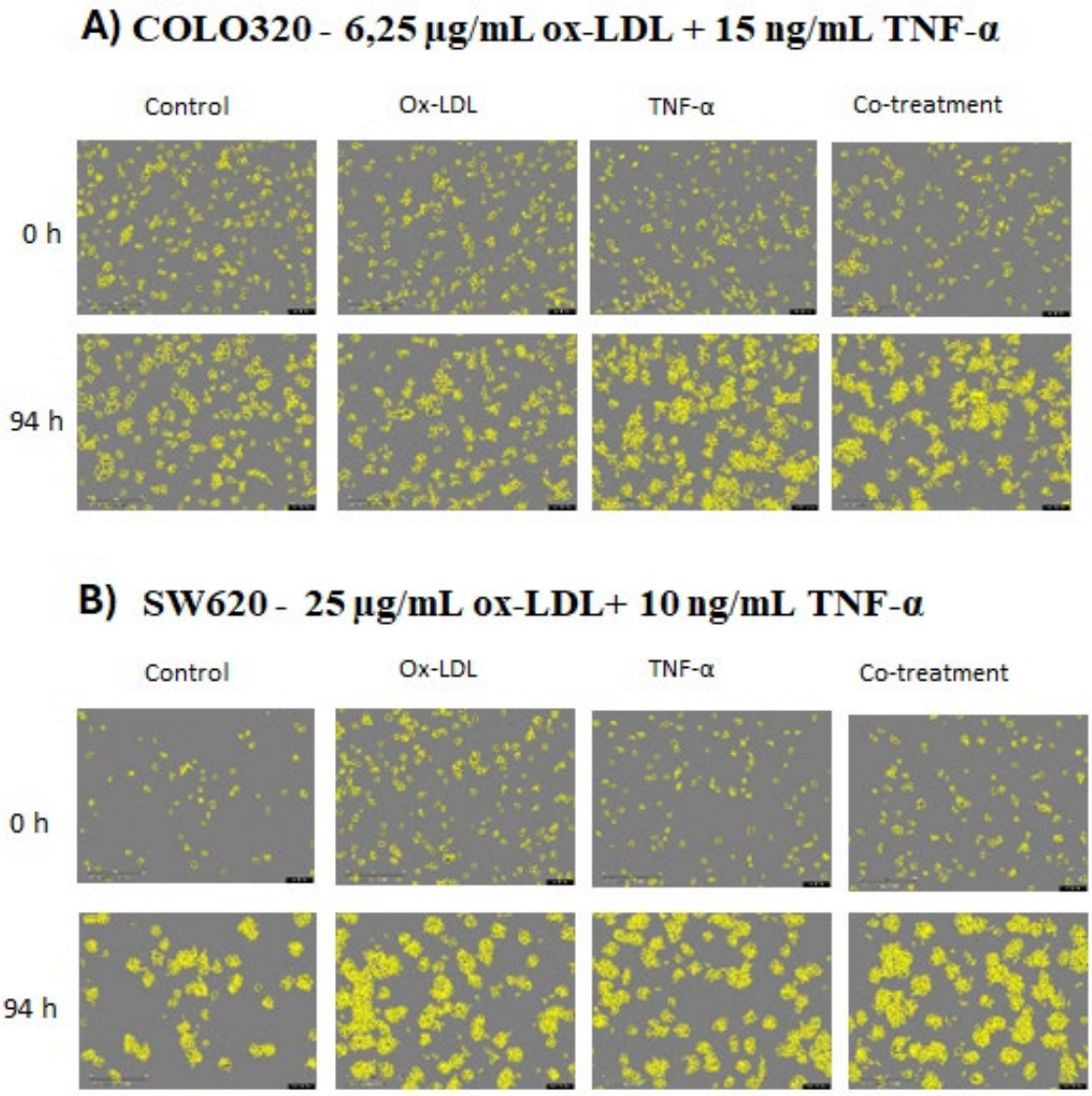


Figure 3. Cell growth of colorectal cancer cell lines monitored with the IncuCyte® live-cell imaging system. Representative micrographs of CRC cell lines at 10× magnification taken at 0 h and 94 h. Cells were treated with oxidized LDL, TNF-α, or their combination. Scale bar: 1.25 µm.

ROS induction by ox-LDL and TNF-α and its modulation by NOX1 inhibition.

ROS levels were assessed using the H2DCFDA probe. To evaluate the contribution of NOX1, cells were pretreated with the NOX1 inhibitor ML171 before incubation with the selected treatments, and ROS production was monitored over a 2 h period. In the COLO320 cell line, co-treatment significantly increased ROS levels at 30 min (2.7-fold), 45 min (2.0-fold), and 60 min (2.2-fold) compared to the control (Figure 3B–D). A trend toward increased ROS generation was observed at 10 min (Figure 3A), 90 min (Figure 3E), and 120 min (Figure 3F); however, these changes did not reach statistical significance. Pretreatment with ML171 led to a modest, non-significant reduction in ROS at 10 min (Figure 3A), but this effect was transient, as ROS levels subsequently increased over time.

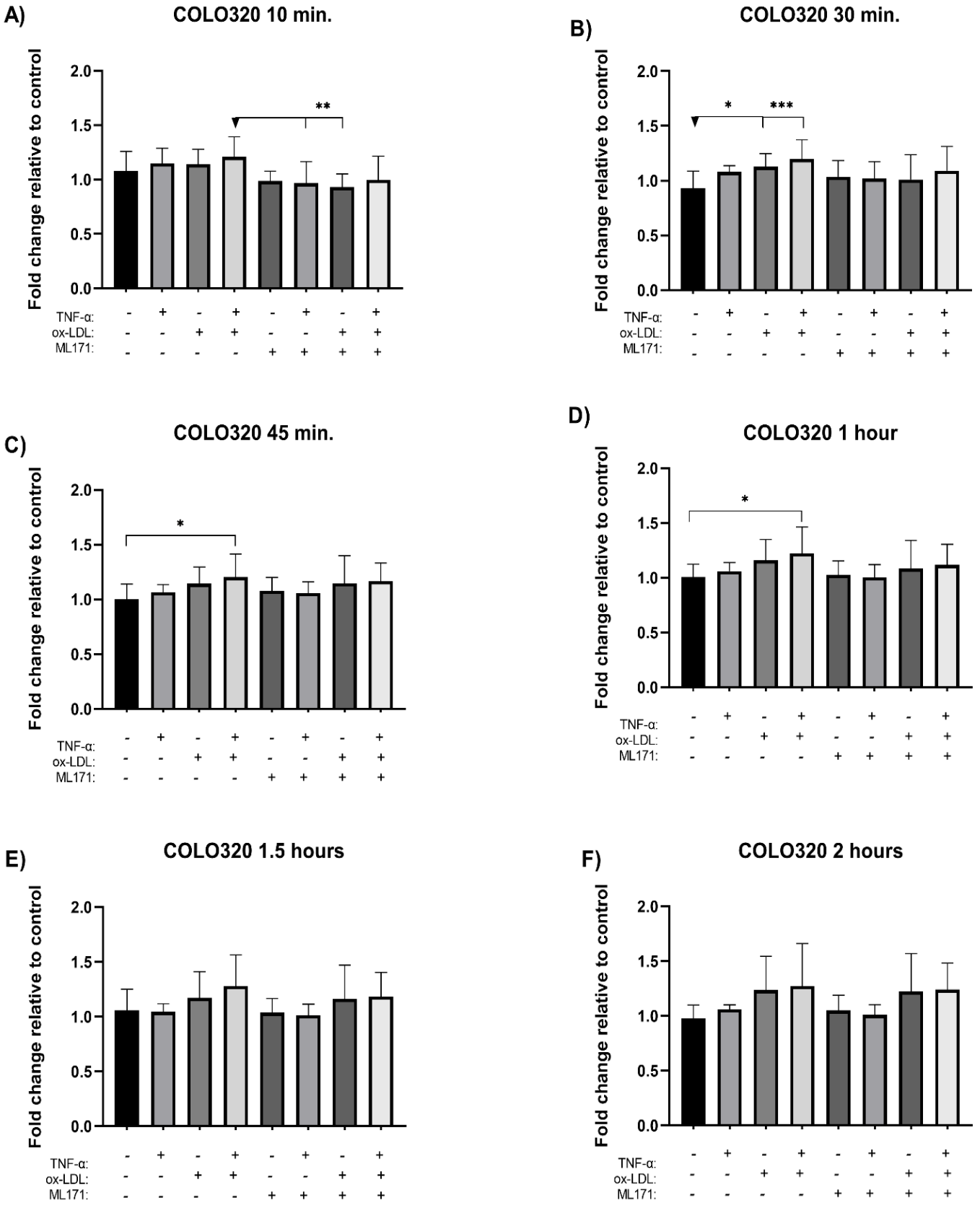
In the SW620 cell line, co-treatment significantly increased ROS levels at 45 min (2.8-fold), 60 min (2.4-fold), and 90 min (3.1-fold) compared with the control group (Figure 3I–K). Pretreatment with the NOX1 inhibitor ML171 markedly reduced ROS levels at 10 min (Figure 3G), 30 min (Figure 3H), and 45 min (Figure 3I), as well as at 60, 90, and 120 min post-incubation, compared with the co-treatment alone (Figure 3J–L). Similarly, co-incubation of ML171 with ox-LDL significantly reduced ROS generation at 45, 60, and 90 min compared with ox-LDL treatment alone (Figure 4 I–K).

Modulation of cell viability by PI3K/AKT and WNT/β-catenin pathway inhibition under ox-LDL and TNF-α co-treatment.

An MTT viability assay was performed in COLO320 and SW620 cells to evaluate the effect of co-treatment and its modulation by the PI3K/AKT and WNT/β-catenin signaling pathways. The inhibitors LF3 (10 μM) and Copanlisib (30 nM) were applied individually or in combination with co-treatment for 72 hours.

In COLO320 cells, co-treatment significantly reduced viability in the presence of inhibitors. LF3 did not produce significant changes when used individually compared with co-treatment alone; however, its combination with co-treatment reduced viability by 1.51-fold (Figure 5A). Copanlisib produced a stronger effect, decreasing viability 2.35-fold compared to co-treatment alone, and this effect was maintained when combined with co-treatment (1.65-fold decrease). The greatest reduction was achieved with double inhibition (LF3 + Copanlisib) and co-treatment with ox-LDL and TNF-α, resulting in a 2.48-fold decrease compared with co-treatment alone (Figure 5A). Although co-treatment alone did not significantly increase viability in the MTT assay, the enhanced sensitivity to pharmacological inhibition suggests that ox-LDL and TNF-α act synergistically to activate proliferative pathways, with a functional contribution from PI3K/AKT and WNT/β-catenin signaling.

In contrast, SW620 cells exhibited limited sensitivity to inhibition. A significant reduction in viability was observed only with the triple combination (LF3 + Copanlisib + co-treatment), which decreased viability by approximately 1.32-fold compared with co-treatment alone. This indicates that SW620 cells moderately depend on these pathways under the combined stimulus (Figure 5B).


A)
COLO320 10 min.
B)
COLO320 30 min.
C)
COLO320 45 min.
D)
COLO320 1 hour
E)
COLO320 1.5 hours
F)
COLO320 2 hours
Fold change relative to control
2.0
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TNF-α:
ox-LDL:
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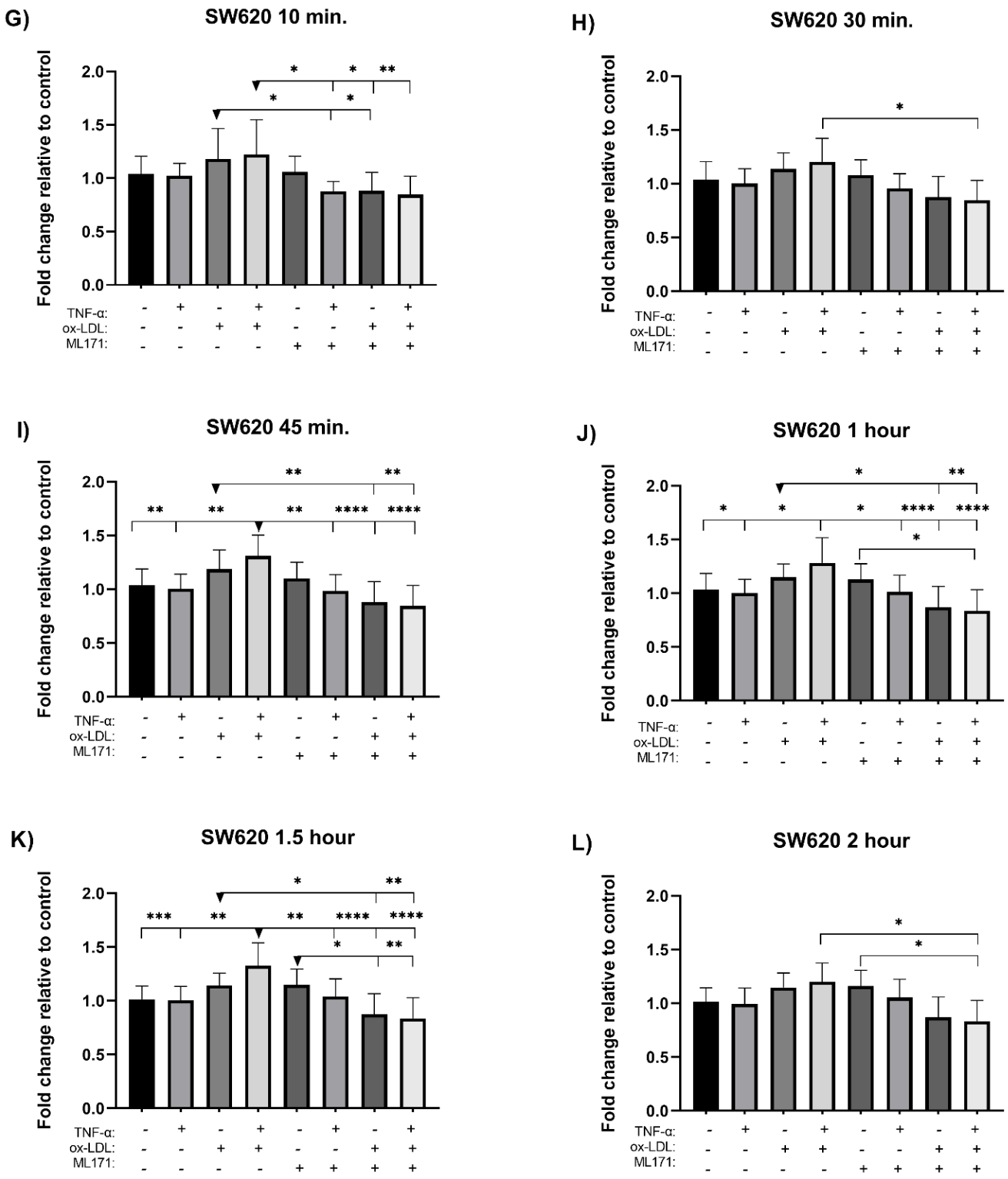

Figure 4. Measurement of ROS in colorectal cancer cell lines. COLO320 and SW620 cells were treated with 6.25 µg/mL ox-LDL and 15 ng/mL TNF-α, or with 25 µg/mL ox-LDL and 10 ng/mL TNF-α, respectively, either individually or in combination, and ROS levels were measured. Panels (A–F) correspond to COLO320 cells, and panels (G–L) correspond to SW620 cells. Data are presented as mean ± SD (n = 9). Statistical analyses were performed using one-way ANOVA followed by Tukey's multiple comparisons test or the Kruskal–Wallis test followed by Dunn's multiple comparisons test, as appropriate; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

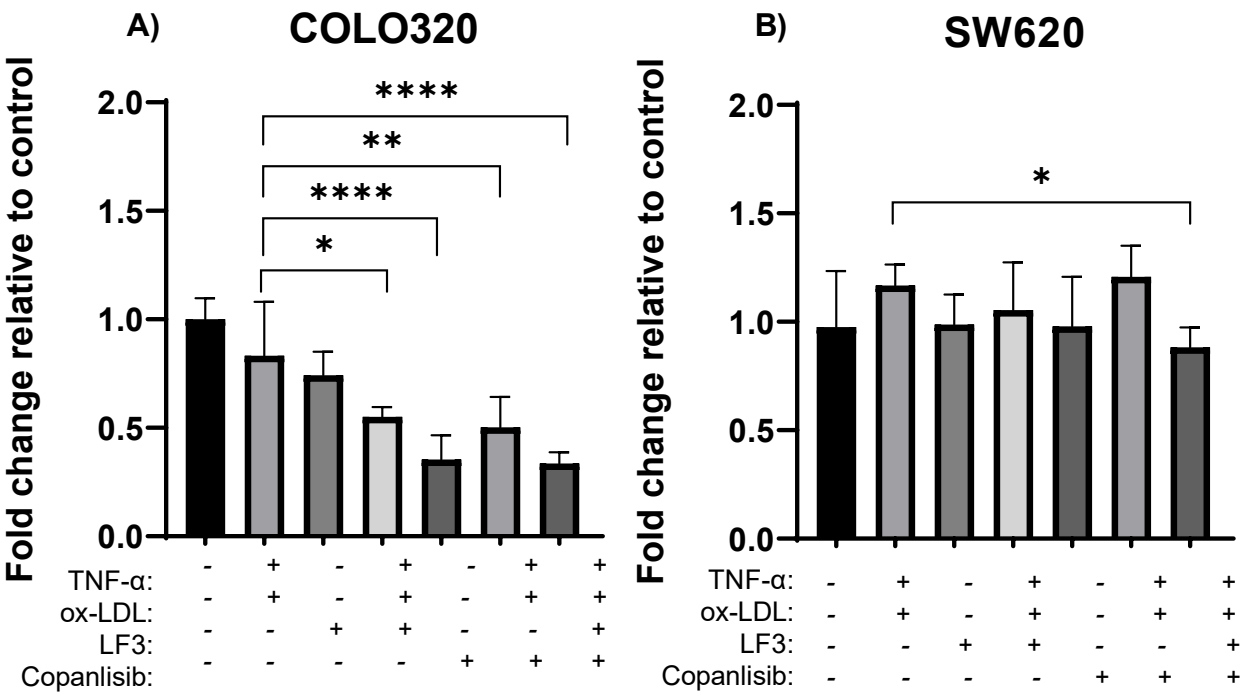

Figure 5. Effect of PI3K/AKT and WNT/β-catenin pathway inhibition on cell viability of (A) COLO320 and (B) SW620 cells. COLO320 cells were treated with 6.25 µg/mL ox-LDL and 15 ng/mL TNF-α, and SW620 cells with 25 µg/mL ox-LDL and 10 ng/mL TNF-α, in the presence or absence of the PI3K/AKT inhibitor Copanlisib (30 nM) and the WNT/β-catenin inhibitor LF3 (10 µM), individually or in combination. Each condition was tested in triplicate. Data are presented as mean ± SD (n = 2). Statistical analysis was performed using one-way ANOVA followed by Tukey's multiple comparisons test; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

DISCUSSION

Colorectal cancer (CRC) is one of the most prevalent invasive malignancies worldwide[20]. In this context, identifying molecular interactions that can act synergistically is essential. These interactions could serve as potential biomarkers for early detection, particularly in patients with risk factors such as inflammation, dyslipidemia, and hypercholesterolemia. They could also guide targeted therapeutic strategies.

Co-treatment with ox-LDL and TNF-α promotes proliferation in CRC cells.

Our results demonstrate that co-treatment with ox-LDL and TNF-α has a synergistic proliferative effect, especially in COLO320 cells. This response is accompanied by increased ROS production and activation of ROS-sensitive signaling pathways. Importantly, the concentrations of ox-LDL used (0–50 µg/mL) did not elicit cytotoxicity in COLO320 or SW620 cells. This finding is consistent with previous studies that have reported low-to-moderate ox-LDL concentrations promote proliferation while higher doses induce cell death or apoptosis. This dose-dependent effect has been observed in various tumor models, including K562/AO2 (leukemia) and EC9706 (esophageal carcinoma) cells, in which proliferation decreased above 20 µg/mL of ox-LDL. In contrast, non-tumor cells, such as human umbilical vein endothelial cells, exhibit enhanced proliferation under the same treatment conditions[21]. In prostate cancer cells (LNCaP, DU-145, C4-2), ox-LDL also stimulates proliferation at concentrations ranging from 10 to 100 µg/mL[22].

Regarding TNF-α, no significant proliferative effects were observed in COLO320 or SW620 cells at 10–15 ng/mL. However, other studies have demonstrated proliferative responses in colorectal (HCT116), breast (MDA-MB-468 and SK-BR3), and vascular smooth muscle cell lines at concentrations up to 20 ng/mL, without evidence of cytotoxicity[23-25]. While co-treatment produced significant increases in proliferation in COLO320 cells and a proliferative trend in SW620 cells, the literature on combined ox-LDL and TNF-α exposure in CRC is limited. One study in HCT116 and COLO320 cells reported that ox-LDL promoted invasion and migration; however, co-incubation with TNF-α showed no additional or synergistic effects[8]. Therefore, our findings highlight a novel proliferative synergy between ox-LDL and TNF-α in selected CRC contexts.

ROS generation is enhanced by co-treatment and differentially modulated by NOX1.

Analysis of ROS dynamics revealed a marked increase following co-treatment, particularly at 30–90 min, in both COLO320 and SW620 cells. These results are consistent with evidence showing that ox-LDL binds to LOX-1, activating NOX1 and downstream pathways such as NF-κB and MAPK[26], while TNF-α stimulates NADPH oxidase activity via NOXO1[27, 28]. Both molecules are pro-inflammatory and pro-oxidative, supporting the idea that their combined effect amplifies ROS production in CRC cells.

Pretreatment with the NOX1 inhibitor ML171 revealed distinct responses between the two lines. In COLO320 cells, ML171 transiently reduced ROS generation, but the cells compensated over time, suggesting contributions from alternative sources such as mitochondria, NOX2, or peroxidases[29]. In contrast, SW620 cells displayed sustained ROS suppression with ML171, indicating a stronger dependence on NOX1 activity. This aligns with reports showing potent NOX1 inhibition by ML171 in CRC cells[30], while organoid studies suggest only partial NOX1 inhibition[8]. The different responses of COLO320 and SW620 highlight the heterogeneity of ROS regulation in CRC. Inhibitor potency, dosage, and compensatory mechanisms may influence outcomes.

Differential impact of PI3K/AKT and WNT/β-catenin pathway inhibition on cell viability.

To investigate whether PI3K/AKT and WNT/β-catenin signaling contribute to co-treatment–induced proliferation, MTT assays were performed using specific inhibitors. In COLO320 cells, both LF3 (a β-catenin/TCF4 inhibitor) and Copanlisib (a PI3K/AKT inhibitor) significantly reduced viability when combined with ox-LDL and TNF-α, with the strongest effect observed under triple inhibition. In contrast, SW620 cells showed reduced viability only under the triple combination, indicating more limited sensitivity.

These differences likely reflect intrinsic molecular profiles. Both cell lines harbor APC mutations, suggesting constitutive WNT/β-catenin activation, yet COLO320 appears to be more functionally dependent on this pathway, making it more sensitive to LF3. Conversely, SW620, which carries KRAS mutations, relies heavily on MAPK signaling, conferring resistance to WNT inhibition[31]. The PI3K/AKT inhibition shows strong sensitivity to Copanlisib in COLO320 cells, consistent with activation of downstream proliferative mechanisms such as SGK1–mTORC1 signaling[32]. In contrast, SW620 cells showed little response to Copanlisib, likely due to constitutive MAPK activity driven by KRAS mutations, which can compensate for PI3K blockade[33]. Nevertheless, combined inhibition partially overcame this resistance, highlighting the therapeutic potential of targeting parallel survival pathways.

In summary, our study demonstrates that exposure to an inflammatory and dyslipidemic environment, modeled by ox-LDL and TNF-α co-treatment, enhances proliferation in CRC cells through ROS generation and the activation of survival pathways. Outcomes depend on the molecular profile of each cell line. Our findings provide evidence of the functional relevance of PI3K/AKT and WNT/β-catenin signaling in mediating

responses to pro-inflammatory and dyslipidemic stimuli. Furthermore, they suggest that therapeutic strategies combining redox modulation with pathway-specific inhibitors could improve treatment efficacy and help prevent or manage CRC in patients with chronic inflammation and dyslipidemia. This translational perspective highlights opportunities for biomarker development, therapeutic personalization, and biotechnological innovation in managing colorectal cancer.

These results provide insight into the mechanisms by which ox-LDL and TNF-α promote proliferation and redox signaling in CRC cells. However, further studies are needed to validate these findings in more physiologically relevant systems. Before transitioning to in vivo models, additional in vitro validation using 3D cultures such as patient-derived CRC organoids would enable a more accurate evaluation of the tumor microenvironment and signaling dependencies. Next, preclinical models should be employed to confirm the contribution of ROS and the PI3K/AKT and WNT/β-catenin pathways to tumor progression under pro-inflammatory and dyslipidemic conditions. Finally, clinical cohort studies could evaluate circulating ox-LDL, TNF-α, and redox-related markers as potential non-invasive biomarkers for early CRC detection and risk stratification. These approaches could bridge the gap between in vitro observations and translational applications, supporting the development of personalized preventive and therapeutic strategies.

CONCLUSIONS

Our findings demonstrate that co-treatment with ox-LDL and TNF-α at low concentrations exerts a synergistic effect that significantly enhances proliferation in the COLO320 cell line. This response is accompanied by a marked increase in ROS generation in both COLO320 and SW620 cells, indicating a redox-dependent mechanism triggered by the combined stimulus. In addition, the differential effects of PI3K/AKT and WNT/β-catenin pathway inhibition across cell lines can be attributed to their distinct molecular profiles, particularly mutations that constitutively regulate these signaling cascades. Together, these results underscore the importance of integrating both molecular alterations and redox status of the tumor microenvironment when designing personalized therapeutic strategies for colorectal cancer.

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