

Article

Adipocyte–Tumor Crosstalk and CSC-Associated Doxorubicin Response in Breast Cancer Mammospheres: Role of Licorice Root Extract

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Abstract

The response of breast cancer to chemotherapy is strongly influenced by tumor–microenvironment interactions and the presence of cancer stem-like cells (CSCs), which are associated with therapy resistance. The aim of this study was to investigate whether modulation of adipocyte–tumor crosstalk by licorice root extract (LRE) could influence doxorubicin response in CSC-enriched MCF-7 mammospheres. MCF-7 cells were pre-treated with conditioned media derived from preadipocytes, mature adipocytes, or LRE-treated mature adipocytes, or were directly exposed to LRE, and subsequently cultured as mammospheres. CSC enrichment was supported by increased expression of CD44 and CD133. Mature adipocyte-conditioned medium promoted the formation of larger and more compact spheroids, reduced intracellular reactive oxygen species (ROS) and apoptosis, and decreased doxorubicin uptake. In contrast, conditioned medium from LRE-treated adipocytes partially counteracted these effects by increasing oxidative stress, apoptotic response, and intracellular doxorubicin accumulation. Collectively, these findings support the involvement of adipocyte–tumor crosstalk in modulating functional parameters associated with doxorubicin responsiveness and suggest that modulation of the adipose microenvironment by LRE may represent a promising area for further investigation in the context of microenvironment-driven chemoresistance.



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1. Introduction

It is well known that obesity is a risk factor for breast cancer incidence and progression, particularly in postmenopausal women, and is a potent determinant of tumor aggressiveness and therapy resistance [1]. In addition to systemic metabolic alterations, obesity causes substantial changes in the tumor microenvironment via the expansion and functional remodeling of adipose tissue. Adipocytes have been shown to promote tumor progression

by secreting numerous factors such as adipokines, cytokines, growth factors and lipids that can promote cancer cell activity [2].

The crosstalk between adipocytes and cancer cells is an important component of tumor microenvironment-driven progression. Adipocytes may affect redox homeostasis and help create a protective milieu that promotes tumor cell viability under stress conditions. However, to what extent adipocytes enable more aggressive breast cancer cell phenotypes (migration, metabolic adaptation, proliferation, and resistance to therapy) is not well understood. Cancer stem cells (CSCs) are a subpopulation of tumor cells that exhibit self-renewal capacity and tumor-initiating potential [3]. In breast cancer, CSC-like populations can be enriched in vitro using mammosphere formation assays, which provide a three-dimensional model that better recapitulates tumor architecture, cell–cell interactions, and drug response compared to conventional two-dimensional cultures. Because of their heightened resistance to conventional therapies, CSCs are thought to drive tumor recurrence, metastasis, and decreased overall patient survival [4].

There is increasing evidence that adipocyte-derived factors can promote therapy resistance and tumor progression. However, current evidence on adipocyte–tumor crosstalk regarding chemotherapeutic response and CSC-like phenotypes is fragmented and primarily dependent on two-dimensional (2D) and single-endpoint analyses. Overall, how adipocyte-derived signals regulate CSC enrichment and drug response is largely unexplored [5].

Against this background, plant-derived bioactive products are increasingly being explored as multi-target modulators of cancer-related processes and tumor–microenvironment interactions. Because adipocyte–tumor crosstalk is mediated by multiple interconnected signals rather than by a single molecular pathway, chemically complex extracts may provide a useful approach for investigating its modulation. Within this framework, licorice root extract (LRE) was selected because its phytochemical complexity and broad biological profile make it a plausible candidate for influencing multiple components of this interaction [6]. LRE is obtained from the roots of *Glycyrrhiza glabra* L., a medicinal plant with a long history of use in ancient Egyptian, Greek, Roman, and Chinese medicine. Traditionally, licorice root preparations have been used for sore throat, cough, and other respiratory complaints, as well as for gastrointestinal and inflammatory disorders. The root contains a complex mixture of bioactive constituents, including triterpenoid saponins, particularly glycyrrhizin, together with flavonoids, flavonoid glycosides, and other phenolic compounds [6]. The hydroalcoholic LRE used in the present study was previously characterized, with glycyrrhizin and liquiritin apioside identified among its major constituents [7]. However, the qualitative and quantitative phytochemical profile of licorice extracts may vary according to genotype, geographical origin, environmental and cultivation conditions, harvesting stage, and extraction procedure [6], highlighting the importance of interpreting their biological effects in relation to the specific composition of the extract used.

Experimental studies have shown that licorice extracts and their bioactive constituents can exert antiproliferative, pro-apoptotic, and antimigratory effects through the modulation of oxidative stress, autophagy, and signaling pathways involved in tumor progression [6]. Beyond these direct effects, our previous findings demonstrated that the LRE used in the present study can modulate the adipocyte secretome and influence redox homeostasis and tumor-related signaling pathways in breast cancer cells [7]. These observations provided the rationale for considering adipocyte function as an indirect target of LRE. However, whether this modulation extends to CSC-like populations and affects chemotherapeutic response in a three-dimensional context remains unknown.

To address this knowledge gap, a clinically relevant chemotherapeutic agent was incorporated into the experimental model to determine whether adipocyte-derived signals

could influence CSC-associated drug responses. Doxorubicin was chosen because it is an established anthracycline widely used in breast cancer treatment and exerts its cytotoxic effects through DNA intercalation, topoisomerase II inhibition, and reactive oxygen species (ROS) generation [8,9]. Its inclusion was particularly relevant because CSC-enriched populations frequently exhibit reduced responsiveness to doxorubicin through enhanced drug efflux, antioxidant defenses, and resistance to apoptosis [10]. In the present model, doxorubicin therefore served as a clinically relevant experimental challenge for evaluating whether adipocyte-derived signals affect CSC-associated drug responsiveness and whether LRE-mediated modulation of adipocytes can modify these effects.

Accordingly, the present study investigated whether adipocyte-derived conditioned media (CM) and LRE-mediated adipocyte modulation influence CSC-associated phenotypes and doxorubicin response in MCF-7 mammospheres by assessing spheroid morphology, intracellular ROS levels, apoptosis, and intracellular doxorubicin accumulation.

2. Results and Discussion

2.1. Validation of CSC Enrichment in Mammospheres

To verify the enrichment of CSC-like cells under mammosphere-forming conditions, the expression levels of two well-established stemness markers, cluster of differentiation 44 (CD44) and cluster of differentiation 133 (CD133), were measured in parental MCF-7 cells cultured under adherent conditions (2D) and in MCF-7 mammospheres (3D) [11].

Both markers were significantly upregulated in mammospheres compared with parental cells ($p < 0.05$; Figure 1).

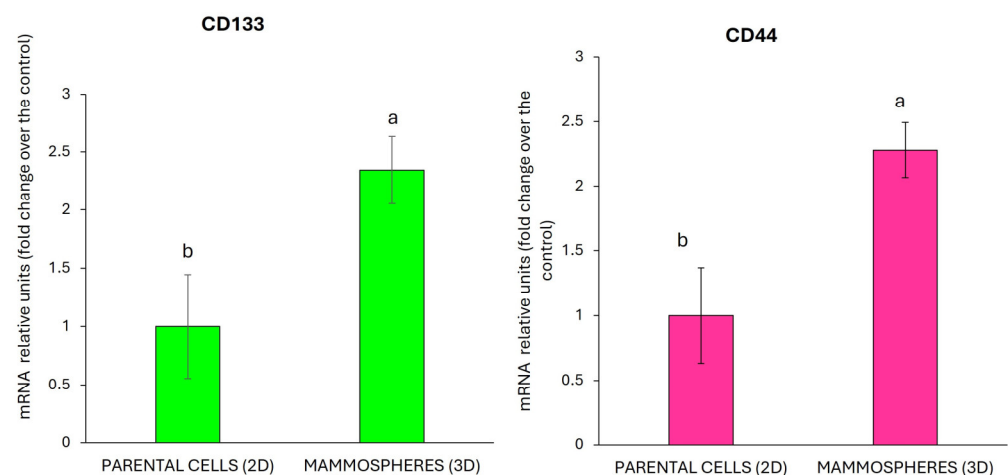


Figure 1. mRNA expression of CD133 and CD44 in MCF-7 breast cancer cells cultured under adherent conditions (2D) and as mammospheres (3D). Gene expression was analyzed by Real-Time PCR and compared between parental cells and mammospheres. Values are expressed as mean $\pm$ SD of three independent biological experiments ($n = 3$). Statistical analysis was performed using one-way ANOVA followed by Fisher's post hoc multiple comparison test. Different letters indicate statistically significant differences between groups ($p < 0.05$).

CD133 expression was increased by approximately 2.2–2.4-fold in mammospheres compared to 2D cultures. Moreover, the mammospheres showed an approximate 2.0–2.3-fold upregulation of CD44 compared to parental cells ($p < 0.05$).

These results show increased expression of CD44 and CD133, which are well-established markers of breast cancer stem-like cells. These observations support the suitability of the mammosphere assay as a model enriched in CSC-like features in MCF-7 cells. CD44 is a transmembrane glycoprotein that is involved in numerous cellular functions important for cell–cell and cell–matrix interactions, migration, adhesion, invasion and

survival signalling pathways. In breast cancer, CD44 expression has been associated with epithelial–mesenchymal transition (EMT), tumour-initiating capacity and therapy resistance [12]. CD133 is a pentaspan transmembrane glycoprotein widely used as a marker of CSC-like populations in several tumor types, including breast cancer. CD133 expression has been correlated with self-renewal, tumorigenic potential, and resistance to chemotherapeutic agents. Additionally, CD133⁺ cells show greater adaptability to stress, which may contribute to tumor resistance and recurrence [13].

The simultaneous upregulation of both CD44 and CD133 in mammospheres suggests the enrichment of a functionally relevant CSC-like population characterized by increased plasticity and potential therapy resistance. This is consistent with the concept that three-dimensional culture systems, such as mammosphere assays, provide a more physiologically relevant microenvironment that may favor the expansion of cells exhibiting stem-like characteristics compared with conventional two-dimensional cultures [14].

Overall, these findings support the suitability of the experimental model for investigating the effects of adipocyte-derived conditioned media and LRE on CSC-associated phenotypes and doxorubicin responsiveness.

CD44 and CD133 are established markers of breast cancer stem-like populations: CD44 is associated with tumor initiation, self-renewal, invasion, and metastatic potential, whereas CD133 identifies aggressive subpopulations characterized by increased tumorigenicity, metabolic adaptability, and resistance to chemotherapy [15,16].

Consistent with our results, transcriptomic and functional studies have shown that three-dimensional MCF-7 mammosphere cultures promote CD44 and CD133 expression and activate pathways involved in self-renewal, survival, and therapeutic resistance [17,18]. Therefore, the simultaneous upregulation observed in the present study supports the enrichment of a functionally relevant CSC-like population and the suitability of this model for investigating microenvironment-mediated drug responses.

2.2. Determination of the Doxorubicin Working Concentration

To determine the appropriate doxorubicin concentration for subsequent experiments, MCF-7 parental cells cultured under adherent conditions were exposed to increasing concentrations (0–4 μ M) of the drug for 24 h, and cell viability was assessed by Methylthiazolyldiphenyl-tetrazolium bromide (MTT) assay. As shown in Figure 2, doxorubicin induced a progressive reduction in cell viability in a concentration-dependent manner. Based on the dose–response curve, the calculated half-maximal inhibitory concentration (IC₅₀) was 1.14 μ M; therefore, 1 μ M was selected as the working concentration for the following experiments.

This concentration was selected because it corresponds to the IC₅₀ value, representing the half-maximal inhibitory effect of doxorubicin on MCF-7 cell viability.

Doxorubicin is the most widely used chemotherapeutic agent in breast cancer and is a known broad-spectrum drug. Its mechanisms of action are multiple and include DNA intercalation, induction of oxidative stress and topoisomerase II inhibition. Doxorubicin has a redox-active structure that promotes reactive oxygen species (ROS) generation, leading to DNA damage and apoptosis [8].

Despite its efficacy, resistance to doxorubicin remains a major limitation, particularly in the context of CSCs [9]. CSCs exhibit increased expression of drug efflux transporters, enhanced DNA repair capacity and greater ability to maintain redox homeostasis—all factors that contribute to a reduced sensitivity to chemotherapeutic agents. The enhanced ability of CSC-enriched populations to survive oxidative stress and evade apoptosis therefore represents an important obstacle to successful therapy [10].

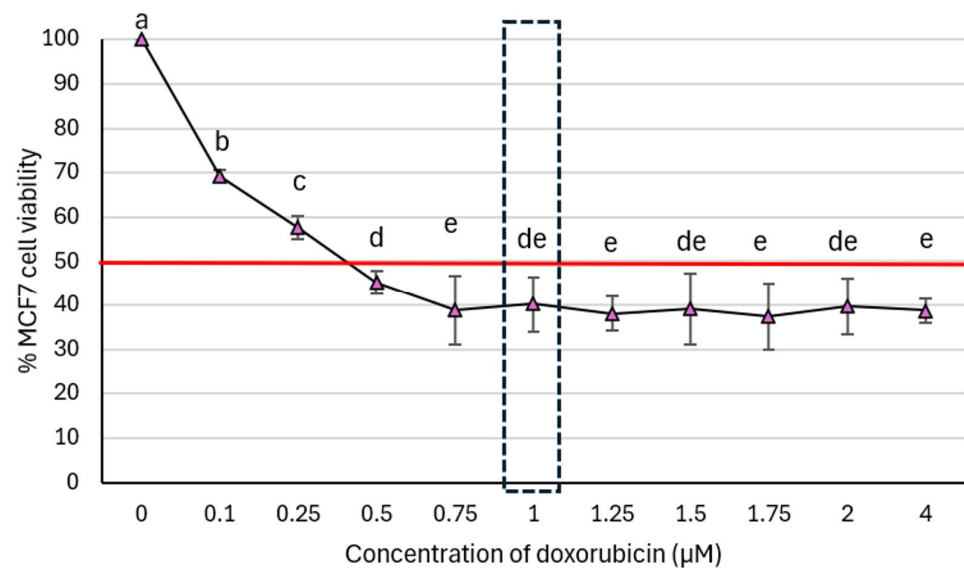


Figure 2. Effect of doxorubicin on MCF-7 breast cancer cell viability. MCF-7 parental cells cultured under adherent conditions were treated with increasing concentrations of doxorubicin for 24 h and analyzed by MTT assay. Values are expressed as mean $\pm$ SD of three independent biological experiments ($n = 3$). Statistical analysis was performed using one-way ANOVA followed by Fisher's post hoc multiple comparison test. Different letters indicate statistically significant differences between groups ($p < 0.05$), whereas groups sharing at least one letter are not significantly different. The red horizontal line indicates the 50% viability threshold, and the highlighted area identifies the doxorubicin concentration selected for subsequent experiments.

The initial dose–response analysis was performed in adherent 2D MCF-7 cells because monolayer cultures provide more homogeneous drug exposure and allow a reproducible assessment of cytotoxicity [19], whereas doxorubicin activity is influenced by drug accessibility and cellular context, which differ substantially between conventional monolayer cultures and three-dimensional tumor models [20–26]. This analysis identified 1 μ M doxorubicin as an appropriate working concentration for evaluating the effects of adipocyte-derived conditioned media and LRE-mediated adipocyte modulation in CSC-enriched mammospheres.

2.3. Intracellular ROS Accumulation

Intracellular ROS levels were evaluated in MCF-7 mammospheres to investigate the effects of adipocyte-derived conditioned media (CM) on redox homeostasis under basal conditions and in response to doxorubicin. As shown in Figure 3, under drug-free conditions, control (CTL) mammospheres displayed intracellular ROS levels of approximately 34.3%. Exposure to LRE alone (27.3%), preAdip-CM (21.7%), and MatAdip-CM (26.3%) were associated with lower ROS levels compared with CTL; however, these groups shared the same or overlapping statistical classes, indicating that these differences were not significant.

In contrast, mammospheres treated with MatAdip + LRE-CM exhibited ROS levels of approximately 39.7%, which were significantly higher than those observed in LRE, preAdip-CM, and MatAdip-CM groups ($p < 0.05$), suggesting that LRE treatment of adipocytes may influence factors present in the conditioned medium that are associated with changes in intracellular ROS levels. These data suggest that, under basal conditions, adipocyte-derived signals tend to maintain a relatively low oxidative state, whereas LRE treatment modifies this equilibrium, promoting a moderate increase in intracellular ROS levels.

This feature is particularly relevant to CSCs, which maintain low intracellular ROS levels through increased antioxidant capacity that may support their self-renewal and

survival under stressful environments. In addition, low ROS levels are frequently associated with increased stemness and decreased responsiveness to anticancer therapies [27].

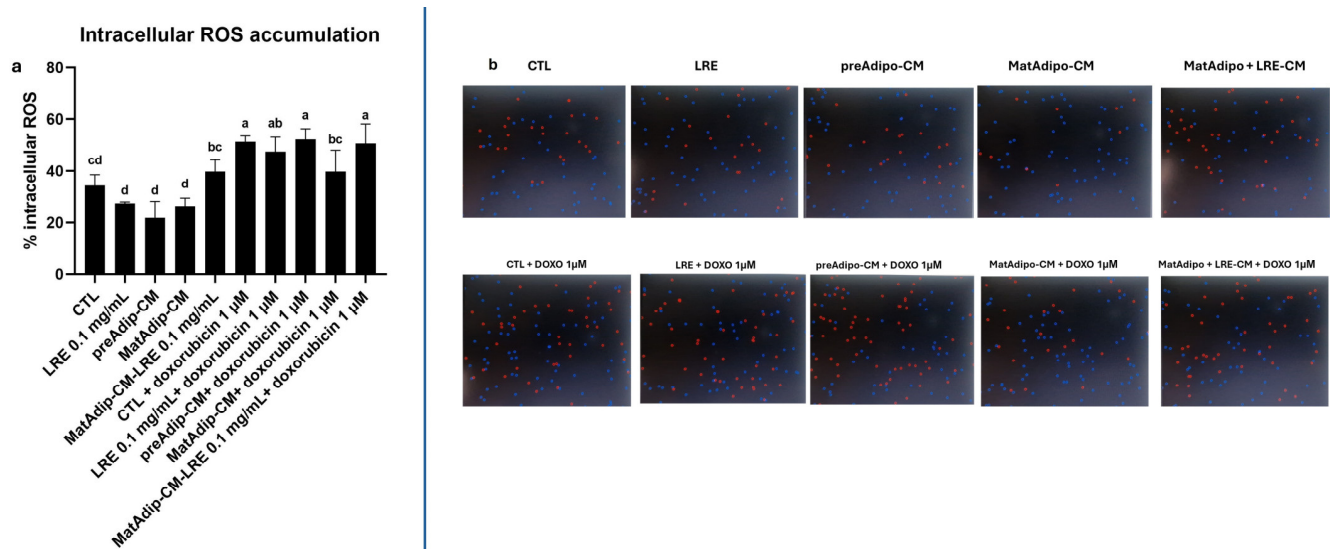


Figure 3. Intracellular reactive oxygen species (ROS) accumulation in MCF-7 mammospheres cultured under different experimental conditions in the absence or presence of doxorubicin. **(a)** Quantitative analysis of intracellular ROS levels in control mammospheres (CTL), mammospheres treated with licorice root extract (LRE, 0.1 mg/mL), preadipocyte-conditioned medium (preAdip-CM), mature adipocyte-conditioned medium (MatAdip-CM), and conditioned medium from LRE-treated mature adipocytes (MatAdip + LRE-CM), with or without doxorubicin (1 μ M). **(b)** Representative fluorescence images acquired with the Tali Image-Based Cytometer showing ROS-positive cells (red fluorescence) and ROS-negative cells (blue fluorescence) under the different experimental conditions. Values are expressed as the mean $\pm$ SD of three independent biological experiments ($n = 3$). Statistical analysis was performed using one-way ANOVA followed by Fisher's post hoc multiple comparison test. Different letters indicate statistically significant differences between groups ($p < 0.05$), whereas groups sharing at least one letter are not significantly different according to Fisher's post hoc multiple comparison test.

The effect of doxorubicin on ROS accumulation was pronounced and consistent with its reported ability to induce oxidative stress. In CTL mammospheres, doxorubicin increased ROS levels from 34.3% to 51.3% ($p < 0.05$). A similar increase was observed in the preAdip-CM + doxorubicin group (52.3%), suggesting that preadipocyte-derived signals do not prevent doxorubicin-induced oxidative stress. Interestingly, the secretome of mature adipocytes was associated with lower ROS levels in conditions of chemotherapeutic stress. Indeed, in the presence of MatAdip-CM and doxorubicin, ROS levels reached only 39.7%, which was significantly lower than under CTL + doxorubicin and preAdip-CM + doxorubicin conditions ($p < 0.05$). The well-established relationship between doxorubicin cytotoxicity and ROS-mediated damage suggests that the reduction in intracellular ROS in the presence of adipocyte-derived factors may be consistent with biological features previously associated with reduced responsiveness to chemotherapy. However, the molecular mechanisms underlying this observation were not directly investigated in the present study.

Importantly, this pattern was not observed when adipocytes were treated with LRE since ROS levels in the MatAdip + LRE-CM + doxorubicin (50.7%) were comparable to those of CTL + doxorubicin and significantly higher than those of MatAdip-CM + doxorubicin ($p < 0.05$). These data suggest that LRE treatment may influence factors within the conditioned medium associated with cellular redox homeostasis, resulting in ROS levels

comparable to those observed following doxorubicin treatment alone in mammospheres. The LRE + doxorubicin group exhibited intermediate ROS levels (47.5%) that were not significantly different from the highest ROS groups.

These results suggest that mature adipocyte-derived conditioned medium is associated with reduced ROS accumulation following doxorubicin exposure in CSC-enriched mammospheres, a feature previously associated with reduced responsiveness to chemotherapy. Conversely, LRE-mediated modulation of adipocytes partially reverses this effect, restoring ROS accumulation to levels associated with effective drug activity. Given the well-established link between low ROS levels and CSC maintenance, the ability of LRE to increase oxidative stress in this context may represent one of the factors potentially involved in the sensitization of tumor cells to chemotherapy.

These findings are consistent with previous evidence showing that CSCs often maintain low intracellular ROS levels through enhanced antioxidant defenses, including superoxide dismutases, glutathione metabolism, and Nrf2-dependent pathways, thereby potentially supporting stemness, survival, and therapy resistance [28–31]. The ability of CSC-enriched populations to buffer oxidative stress more efficiently than differentiated tumor cells further highlights the importance of redox homeostasis in maintaining chemoresistant phenotypes.

The cytotoxic activity of doxorubicin has been widely associated with its ability to induce oxidative stress and disrupt redox homeostasis through ROS generation, redox cycling and mitochondrial dysfunction [32]. The increase in ROS levels observed following doxorubicin treatment in the present study is therefore consistent with its well-established pro-oxidant activity.

In this context, the attenuation of ROS accumulation observed in the presence of mature adipocyte-conditioned medium is consistent with the possibility that tumor microenvironmental factors can promote redox adaptation and limit chemotherapy-induced oxidative damage. Adipocyte-derived factors may enhance the antioxidant capacity in tumor cells, contributing to a lower intracellular oxidative state and increased therapy resistance, particularly in obesity-associated contexts.

Interestingly, bioactive compounds derived from licorice root, such as isoliquiritigenin and glycyrrhethinic acid, have been reported to modulate intracellular redox balance and promote ROS-mediated cytotoxicity in different cancer models [33–35].

Most studies have investigated the direct effects of licorice-derived compounds on tumor cells. The present results suggest that LRE-treated adipocyte-conditioned medium can disrupt the microenvironment-mediated redox shield and restore tumor cell chemosensitivity to chemotherapy-induced oxidative stress.

2.4. Cell Viability, Cell Death and Apoptosis

Cell viability, cell death, and apoptosis were evaluated in MCF-7 mammospheres to determine whether adipocyte-derived conditioned media influence cell survival and apoptotic responses under basal conditions and following doxorubicin treatment.

As shown in Figure 4, under drug-free conditions, CTL mammospheres displayed approximately 32% viable cells, 44.7% dead cells, and 23.3% apoptotic cells. Treatment with LRE alone slightly reduced viability (~15%) and increased cell death (~54%) and apoptosis (~27.7%), although these changes were not consistently associated with clear statistical separation from the control group, suggesting that LRE may exert a modest direct cytotoxic effect on cell viability under basal conditions.

Exposure to preAdip-CM resulted in a marked decrease in cell viability (~10.3%) and a corresponding increase in apoptosis (~38%), suggesting that preadipocyte-derived factors may promote apoptotic processes in mammospheres. In contrast, MatAdip-CM partially

preserved cell viability (~34%) and maintained apoptosis at levels comparable with those of the control group (~24%), consistent with a potential protective influence of mature adipocyte-derived signals.

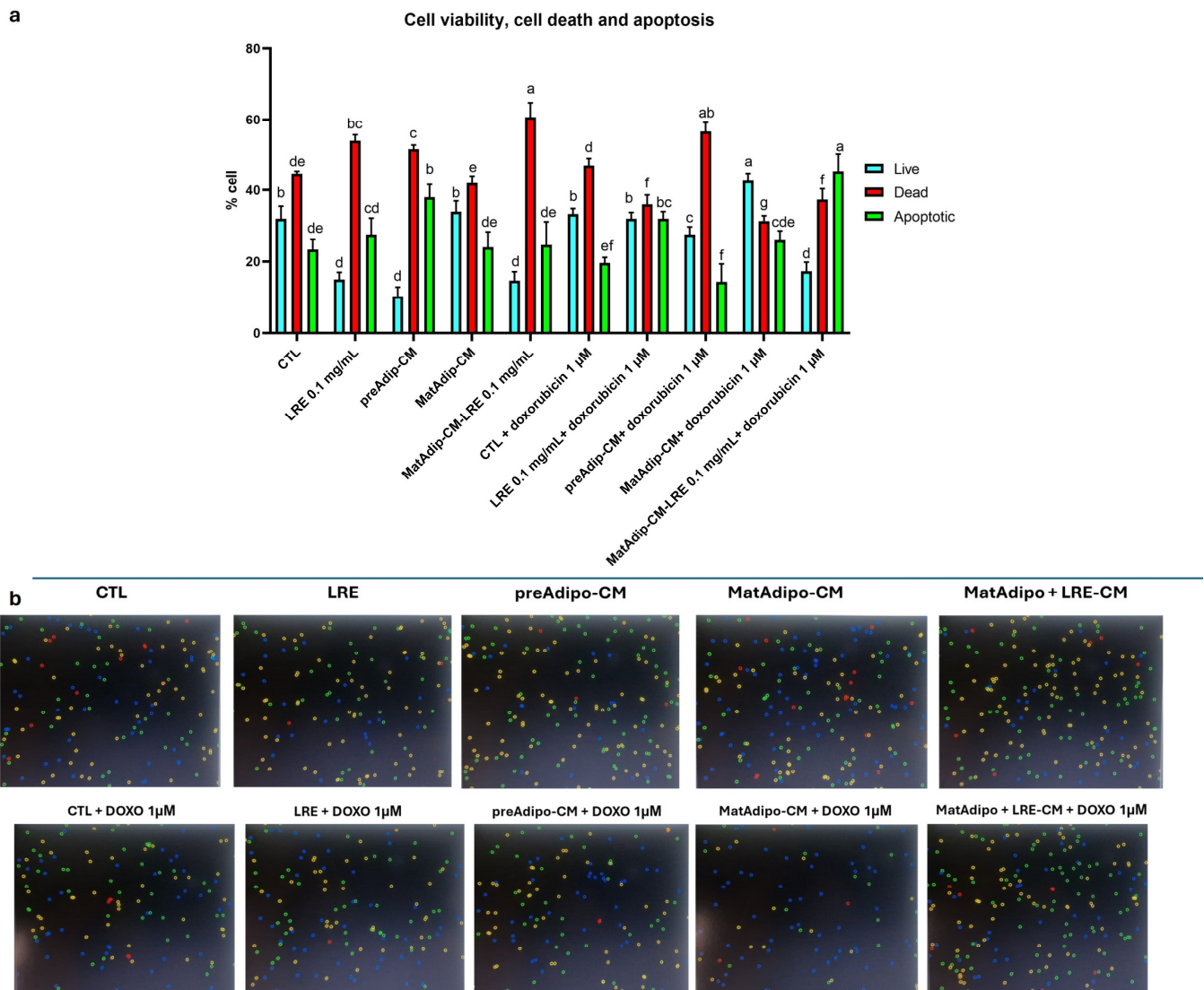


Figure 4. Cell viability, cell death, and apoptosis analysis in MCF-7 mammospheres under different experimental conditions in the absence or presence of doxorubicin (1 μ M). (a) Quantitative analysis of the percentage of live, dead, and apoptotic cells in control (CTL), LRE-treated cells (0.1 mg/mL), preadipocyte-conditioned medium (preAdip-CM), mature adipocyte-conditioned medium (MatAdip-CM), and LRE-treated mature adipocyte-conditioned medium (MatAdip + LRE-CM), with or without doxorubicin. (b) Representative fluorescence images obtained using the Tali Image-Based Cytometer after Annexin V/Propidium Iodide staining. Live cells are shown in blue, apoptotic cells in green, and dead cells in red. Values are expressed as mean $\pm$ SD of three independent biological experiments ($n = 3$). Statistical analysis was performed using one-way ANOVA followed by Fisher's post hoc multiple comparison test. Statistical comparisons were performed independently for live, apoptotic, and dead cell populations. Different letters indicate statistically significant differences between groups ($p < 0.05$), whereas groups sharing at least one letter are not significantly different according to Fisher's post hoc multiple comparison test.

Interestingly, MatAdip + LRE-CM significantly altered this profile, with apoptosis levels (~24.7%) remaining similar to CTL but with a marked increase in dead cells (~60.7%) and reduced viability (~14.7%), suggesting that the secretome of LRE-treated adipocytes

may influence cellular survival, resulting in an increased proportion of dead cells even in the absence of chemotherapy.

The effect of doxorubicin was consistent with its reported cytotoxic mechanism of action. In CTL mammospheres, treatment with 1 μ M doxorubicin increased the proportion of dead cells (~47%) and reduced apoptotic cells (~19.7%), suggesting the activation of cell death pathways. However, the distribution between apoptotic and non-apoptotic cell death suggests a complex response, possibly involving necrotic or late-stage cell death processes.

Importantly, the preAdip-CM + doxorubicin group showed a reduction in apoptosis (~14.3%) and maintained moderate viability (~27.7%), suggesting a partial attenuation of doxorubicin-induced apoptotic signaling. These findings suggest that certain stromal-derived signals may interfere with the activation of programmed cell death pathways.

A particularly relevant observation was made in the MatAdip-CM + doxorubicin group, where cell viability increased (~42.7%) compared with CTL + doxorubicin, while apoptosis remained relatively low (~26%). This finding indicates that mature adipocyte-conditioned medium may exert a protective effect against doxorubicin-induced cytotoxicity, consistent with the reduced ROS accumulation observed in the same condition. Taken together, these findings are consistent with the possibility that mature adipocyte-derived factors may confer a survival advantage and potentially contribute to reduced responsiveness to chemotherapy.

In the MatAdip + LRE-CM + doxorubicin group, this protective effect was partially reversed; in fact, the apoptotic fraction increased to ~45.3%, while viability decreased to ~17.3%. These changes were statistically significant compared with MatAdip-CM + doxorubicin ($p < 0.05$) and suggest that LRE-mediated modulation of adipocytes restores the ability of doxorubicin to induce apoptotic cell death.

CSCs can evade cell death through enhanced anti-apoptotic signaling, efficient DNA repair, and the maintenance of reduced ROS levels. Therefore, the increase in apoptosis observed in the MatAdip + LRE-CM + doxorubicin group suggests that LRE may partially disrupt the protective microenvironment generated by mature adipocytes, thereby potentially increasing the responsiveness of mammospheres to chemotherapeutic stress.

Taken together, these results are consistent with the ROS data, supporting the possibility that mature adipocyte-conditioned medium may promote cell survival and limit both oxidative stress and apoptosis, thereby potentially contributing to reduced responsiveness to chemotherapy. Conversely, LRE-mediated modulation of adipocytes restores oxidative stress and apoptotic susceptibility, ultimately enhancing the effectiveness of doxorubicin in CSC-enriched mammospheres.

These findings are consistent with previous evidence showing that three-dimensional breast cancer models often display reduced sensitivity to chemotherapeutic agents compared with two-dimensional cultures, partly because spheroids develop stronger cell–cell interactions, diffusion gradients, and microenvironmental protection. In breast cancer models, 3D cultures have been shown to alter drug response and apoptotic signaling compared with monolayer cultures, including changes in caspase activation and survival pathways, supporting the concept that spheroid architecture itself contributes to treatment resistance [36,37]. The protective effect observed with MatAdip-CM is also in line with studies showing that adipocyte-derived factors can reduce the efficacy of anticancer treatments in breast cancer cells. Strong et al. demonstrated that cancer-associated adipocytes actively support breast cancer aggressiveness, whereas Lehu     et al. showed that adipocytes promote resistance to several chemotherapeutic agents, including doxorubicin, through a multidrug-resistant phenotype [38,39]. Moreover, the reduced apoptotic susceptibility observed in MatAdip-CM-treated mammospheres is consistent with the known resistance of cancer stem-like cells to cell death. CSCs have been reported to evade apoptosis through

enhanced DNA repair, activation of anti-apoptotic pathways, increased drug efflux, and tight control of intracellular redox balance, all of which contribute to reduced sensitivity to chemotherapy [40,41].

Finally, the increased apoptotic response observed after LRE-mediated adipocyte modulation is consistent with previous studies reporting the pro-apoptotic effects of licorice-derived compounds in cancer models. Licorice phytochemicals, including glycyrrhizin, glycyrrhetic acid, isoliquiritigenin, and licochalcone A, have been reported to regulate oxidative stress, mitochondrial dysfunction, PI3K/Akt/mTOR signaling, and apoptosis in different tumor cell types, including breast cancer models [33,42].

Taken together, these studies support the interpretation that MatAdip-CM may create a microenvironment that favors cell survival and apoptotic escape, whereas LRE-mediated modulation of adipocytes may attenuate this protective signaling and partially restore apoptotic susceptibility under doxorubicin-induced stress.

2.5. Morphological Characterization of Mammospheres

The efficacy of doxorubicin, one of the most widely used chemotherapeutic agents for breast cancer, has been reported to be influenced by the organization of the tumor microenvironment and tumor architecture [43]. In 3D models, the size, compactness and sphericity of a spheroid influence how cells behave and, in particular, how drugs might penetrate and distribute within them [44,45]. This suggests that the tumor architecture may act as a physical and functional barrier to chemotherapy, potentially contributing to reduced treatment efficacy [46]. In this context, a comprehensive morphological characterization of MCF-7 mammospheres was performed to evaluate how adipocyte-derived conditioned media and LRE-mediated adipocyte modulation affect spheroid structure and, potentially, drug responsiveness (Figure 5).

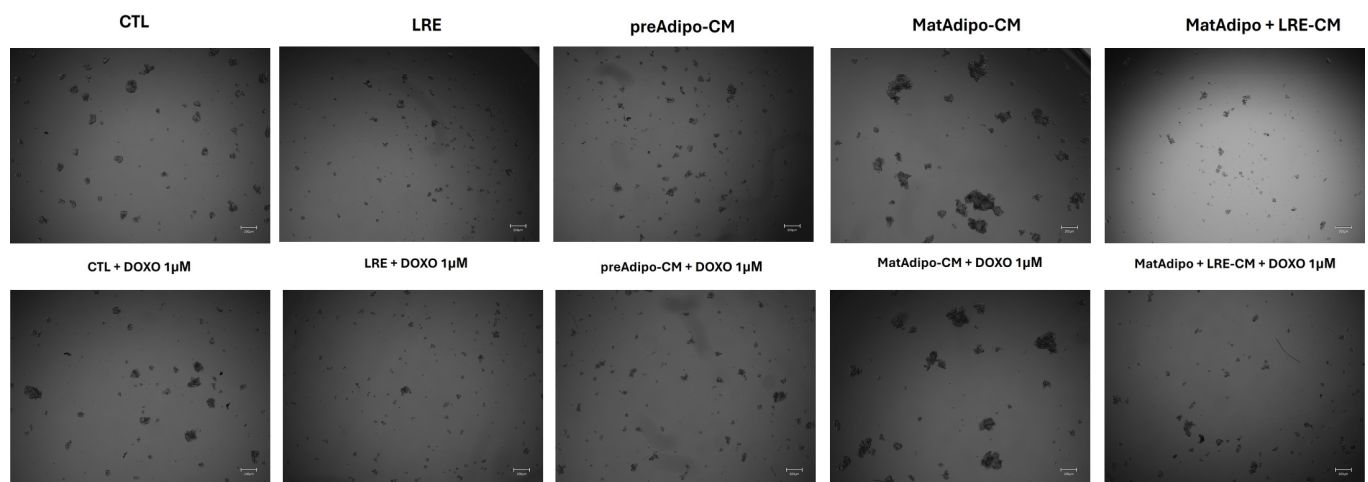


Figure 5. Representative brightfield images of MCF-7 mammospheres cultured under the different experimental conditions. Images were acquired using the EVOS™ M5000 Imaging System at 4× magnification. Representative images illustrate the morphological differences observed among mammospheres under the various experimental conditions, including CTL, LRE, preAdip-CM, MatAdip-CM, and MatAdip + LRE-CM, in the presence or absence of doxorubicin (1 µM). Scale bar = 200 µm.

Morphological parameters (maximum diameter, equivalent spherical volume, circularity, area, and perimeter) were quantified using Celleste™ 6 Image Analysis Software.

Spheroid area is commonly used as an indicator of growth and has been shown to reveal differences between experimental conditions (Figure 6).

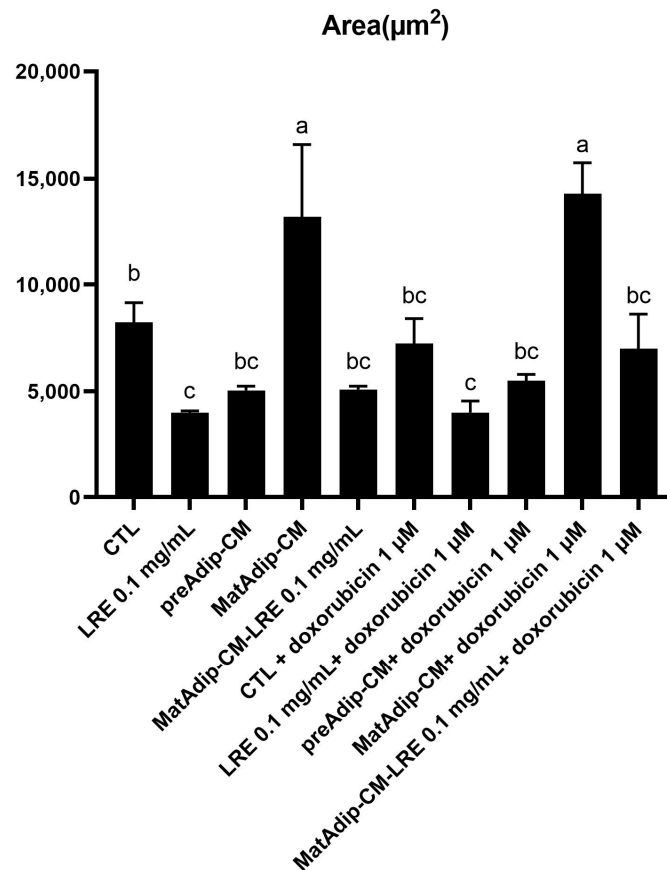


Figure 6. Spheroid area in MCF-7 mammospheres under different conditions ($\pm$ doxorubicin, 1 μ M). Colors identify the different experimental groups, as indicated by the legend included within the figure. Values are expressed as mean $\pm$ SD of three independent biological experiments ($n = 3$). Statistical analysis was performed using one-way ANOVA followed by Fisher's post hoc multiple comparison test. Different letters indicate statistically significant differences between groups ($p < 0.05$), whereas groups sharing at least one letter are not significantly different according to Fisher's post hoc multiple comparison test.

The average spheroid area of CTL mammospheres was $\sim 8230 \mu\text{m}^2$, reflecting a basal proliferative capacity. In contrast, treatment with LRE alone drastically reduced this area to $\sim 3995 \mu\text{m}^2$, suggesting a marked reduction in spheroid growth. Similar values were measured in preAdip-CM and MatAdip + LRE-CM (5039 and $5080 \mu\text{m}^2$, respectively), indicating that these conditions were associated with reduced spheroid growth. The factors secreted by mature adipocytes (MatAdip-CM induced a marked increase in spheroid area to $\sim 13,178 \mu\text{m}^2$, which was maintained in the presence of doxorubicin ($\sim 14,302 \mu\text{m}^2$). Thus, adipocyte-derived factors were associated with the maintenance of larger spheroid structures even in the presence of chemotherapy. In contrast, the spheroid area in the MatAdip + LRE-CM condition remained reduced both in the absence and presence of doxorubicin, reaching approximately $6988 \mu\text{m}^2$ after treatment, a value comparable to that of the control group. This finding suggests that LRE may partially attenuate the protective effect exerted by mature adipocyte-derived factors. Equivalent spherical volume, a more physiologically relevant estimate of spheroid mass and 3D organization, further supported these findings (Figure 7).

While CTL mammospheres displayed a volume of approximately $67,904 \mu\text{m}^3$, LRE treatment dramatically reduced this value to $\sim 20,930 \mu\text{m}^3$, with similarly low values observed in preAdip-CM ($\sim 31,443 \mu\text{m}^3$) and MatAdip + LRE-CM ($\sim 32,057 \mu\text{m}^3$) conditions. In contrast, MatAdip-CM was associated with a substantial increase in volume ($\sim 16,3758 \mu\text{m}^3$),

reflecting larger spheroid size and a more compact structural organization. Importantly, volume remained elevated under doxorubicin ($\sim 18,5987 \mu\text{m}^3$), suggesting that mature adipocyte-derived signals may contribute to the maintenance of spheroid architecture even under cytotoxic conditions. This observation is particularly relevant, as increased spheroid volume has been associated with the formation of internal gradients (oxygen, nutrients, and metabolites), which may contribute to the emergence of resistant cellular subpopulations. The reduction observed in the MatAdip + LRE-CM + doxorubicin group ($\sim 55,928 \mu\text{m}^3$) is consistent with a partial attenuation of these effects following LRE-mediated modulation of adipocytes.

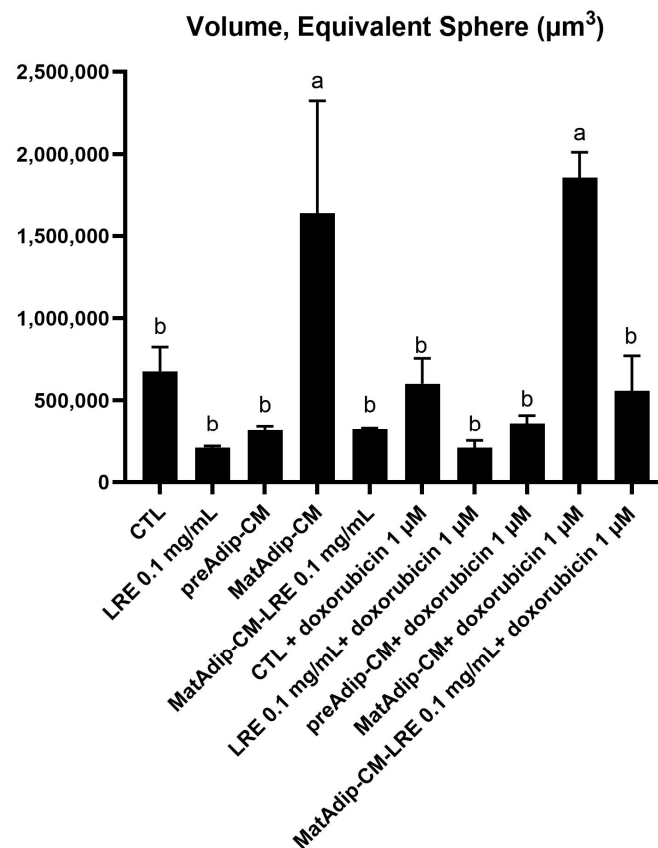


Figure 7. Equivalent spherical volume of MCF-7 mammospheres under different conditions ($\pm$ doxorubicin, $1 \mu\text{M}$). Colors identify the different experimental groups, as indicated by the legend included within the figure. Values are expressed as mean $\pm$ SD of three independent biological experiments ($n = 3$). Statistical analysis was performed using one-way ANOVA followed by Fisher's post hoc multiple comparison test. Different letters indicate statistically significant differences between groups ($p < 0.05$), whereas groups sharing at least one letter are not significantly different according to Fisher's post hoc multiple comparison test.

Maximum and minimum diameter measurements provided a more informative characterization of spheroid size and geometric symmetry (Figure 8). The maximum diameter reflects the overall extent of spheroid growth; the minimum diameter is the shortest axis of the spheroid.

Under control conditions, CTL mammospheres exhibited a maximum diameter of approximately $121 \mu\text{m}$ and a minimum diameter of $\sim 84 \mu\text{m}$, indicating a relatively regular, although not perfectly spherical, structure. Exposure to LRE alone reduced both parameters, with maximum diameter decreasing to $\sim 86 \mu\text{m}$ and minimum diameter to $\sim 59 \mu\text{m}$, consistent with the observed reduction in spheroid size.

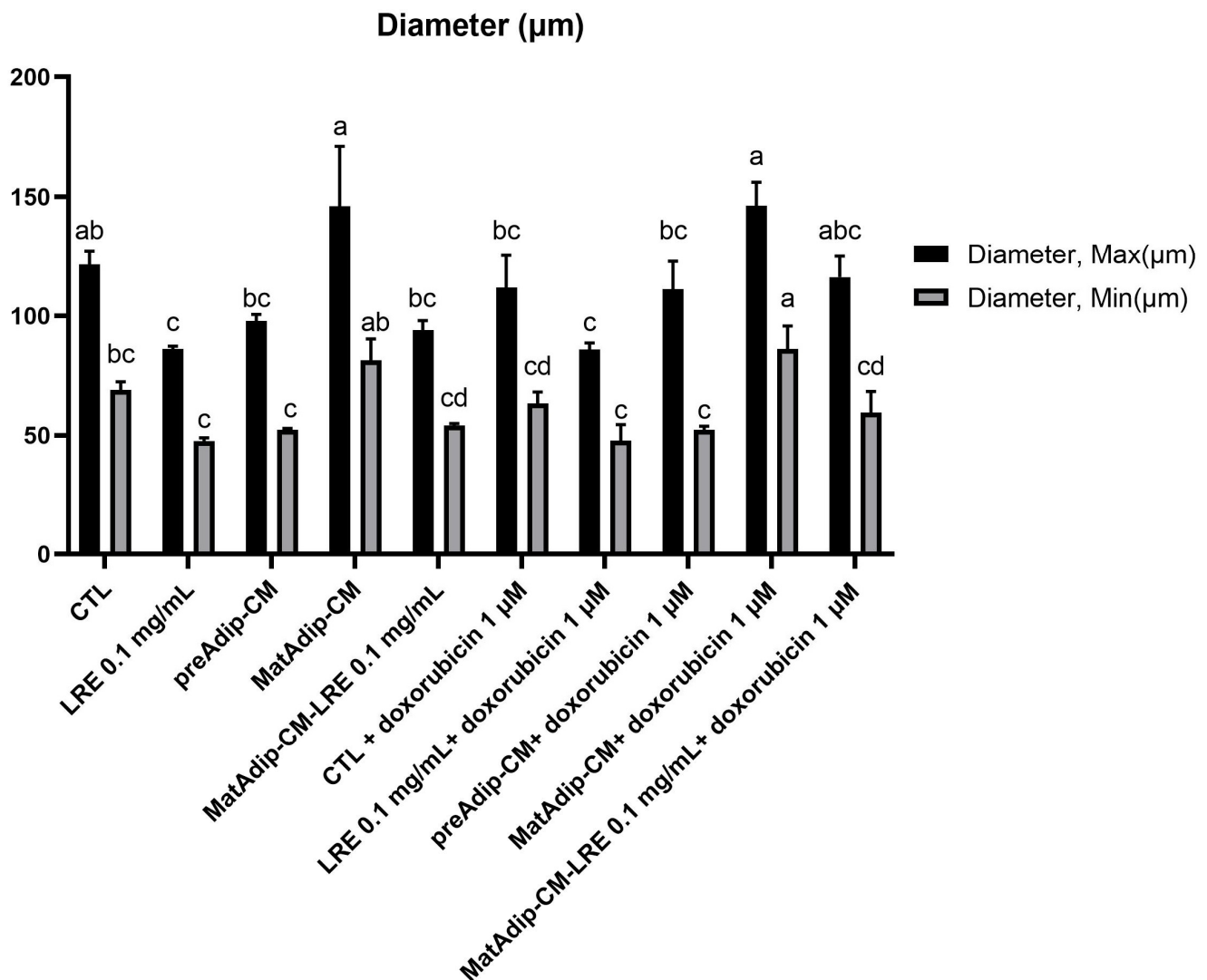


Figure 8. Maximum and minimum diameter of MCF-7 mammospheres under different conditions ($\pm$ doxorubicin, 1 μ M). Values are expressed as mean $\pm$ SD of three independent biological experiments ($n = 3$). Statistical analysis was performed using one-way ANOVA followed by Fisher's post hoc multiple comparison test. Different letters indicate statistically significant differences between groups ($p < 0.05$), whereas groups sharing at least one letter are not significantly different according to Fisher's post hoc multiple comparison test. Statistical comparisons were performed independently within each parameter analyzed.

A similar trend was observed in the preAdip-CM group, where both maximum ($\sim 98 \mu\text{m}$) and minimum ($\sim 65 \mu\text{m}$) diameters remained lower than those of the CTL group, consistent with reduced spheroid size. In contrast, MatAdip-CM was associated with a marked increase in both maximum and minimum diameters ($\sim 146 \mu\text{m}$ and $\sim 99 \mu\text{m}$, respectively), resulting in larger spheroids but also a more isotropic and homogeneous growth pattern. The proportional increase in both axes suggests that spheroids expand in a coordinated manner, maintaining geometric integrity.

Interestingly, in the MatAdip + LRE-CM condition, both diameters were reduced ($\sim 94 \mu\text{m}$ maximum and $\sim 65 \mu\text{m}$ minimum), suggesting that LRE may partially alter this coordinated growth pattern, resulting in smaller spheroids.

In the presence of doxorubicin, CTL spheroids showed a reduction in both maximum and minimum diameters ($\sim 112 \mu\text{m}$ and $\sim 77 \mu\text{m}$, respectively), consistent with the effects of doxorubicin on spheroid morphology. However, MatAdip-CM preserved both parameters

(~146 μm maximum and ~102 μm minimum), suggesting that mature adipocyte-derived signals may contribute to the maintenance of spheroid size and structural symmetry under chemotherapeutic stress.

Conversely, in the MatAdip + LRE-CM + doxorubicin condition, both diameters were reduced (~116 μm maximum and ~72 μm minimum), suggesting that LRE-mediated modulation of adipocytes may partially attenuate the effects of adipocyte-derived factors on spheroid morphology.

From a biological perspective, the combined analysis of maximum and minimum diameters provides important information beyond size alone. MatAdip-CM spheroids were the only group that proportionally increased in both axes, suggesting more homogeneous growth and greater structural cohesion. Both have been associated with increased cell–cell contact and lowered intercellular space, which may limit drug diffusion and potentially contribute to reduced responsiveness to chemotherapy. Reductions, or imbalances, between the two diameters may reflect structural destabilization and increasing irregularity [47].

Beyond size-related parameters, geometric descriptors such as circularity provided critical insights into spheroid organization (Figure 9).

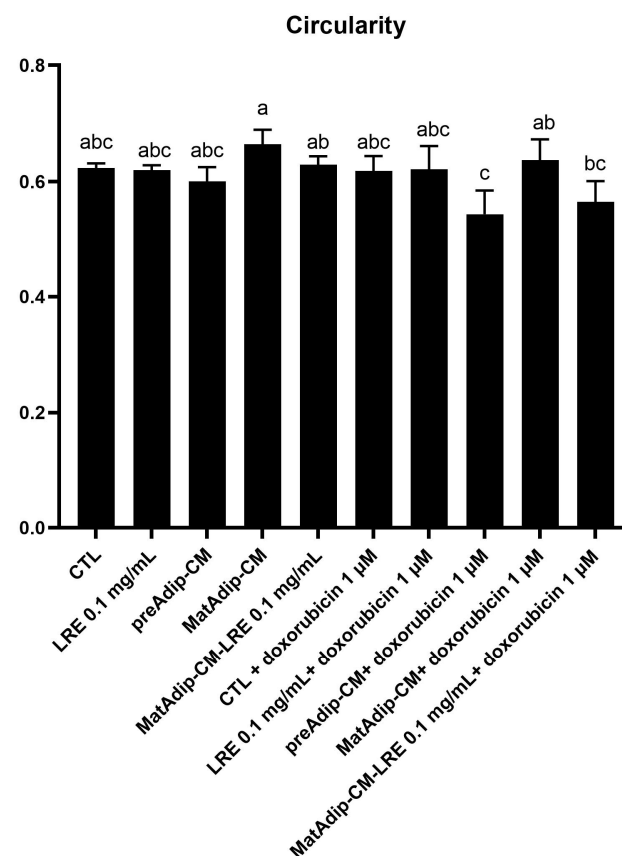


Figure 9. Circularity of MCF-7 mammospheres under different conditions ($\pm$ doxorubicin, 1 μM). Colors identify the different experimental groups, as indicated by the legend included within the figure. Values are expressed as mean $\pm$ SD of three independent biological experiments ($n = 3$). Statistical analysis was performed using one-way ANOVA followed by Fisher’s post hoc multiple comparison test. Different letters indicate statistically significant differences between groups ($p < 0.05$), whereas groups sharing at least one letter are not significantly different according to Fisher’s post hoc multiple comparison test.

Circularity ranges from 0 to 1, with higher values generally indicating a more compact spheroid with greater structural integrity [48]. The circularity of the CTL spheroids was 0.62—suggesting moderately organized structures with a degree of cell–cell

adhesion—which further increased in MatAdip-CM (to ~0.66). These findings suggest that the MatAdip-CM spheroids formed more compact and regular structures, consistent with a higher cellular density. Structural organization itself has been associated with reduced responsiveness to chemotherapy, as densely packed cells may limit drug diffusion [22,44,45]. It is interesting to note that after doxorubicin treatment, spheroids still exhibited relatively high circularity values (approximately 0.61–0.64) in the CTL + doxorubicin and MatAdip-CM + doxorubicin groups. Circularity values were significantly lower in preAdip-CM + doxorubicin and MatAdip-CM-LRE + doxorubicin conditions compared with the MatAdip-CM + doxorubicin condition, suggesting a partial disruption of spheroid integrity and organization (preAdip-CM + doxorubicin = 0.54; MatAdip + LRE_CM- + doxorubicin = 0.56).

Similarly, the perimeter provides additional information on the structural organization of mammospheres (Figure 10).

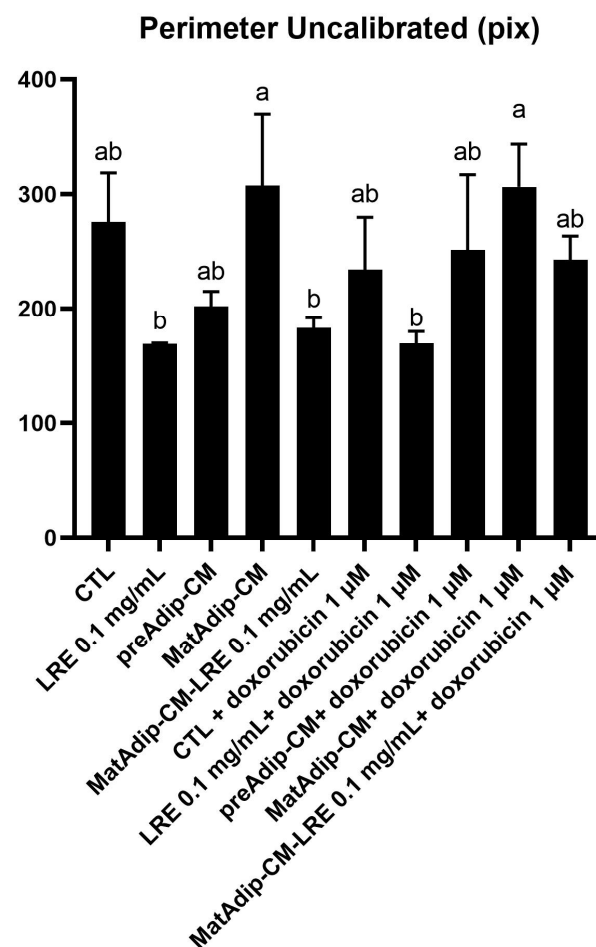


Figure 10. Perimeter of MCF-7 mammospheres under different conditions ($\pm$ doxorubicin, 1 μ M). Colors identify the different experimental groups, as indicated by the legend included within the figure. Values are expressed as mean $\pm$ SD of three independent biological experiments ($n = 3$). Statistical analysis was performed using one-way ANOVA followed by Fisher's post hoc multiple comparison test. Different letters indicate statistically significant differences between groups ($p < 0.05$), whereas groups sharing at least one letter are not significantly different according to Fisher's post hoc multiple comparison test.

The perimeter of CTL spheroids (275 pixels) increased in the MatAdip-CM group (307 pixels), consistent with the formation of larger and more structurally complex spheroids. Following doxorubicin treatment, perimeter values remained elevated in the MatAdip-CM group, at approximately 306 pixels. In contrast, LRE treatment re-

duced perimeter values (~169 pixels), consistent with smaller spheroids displaying a less complex morphology. The intermediate values observed in MatAdip + LRE-CM + doxorubicin (~243 pixels) suggest a partial attenuation of the structural changes induced by MatAdip-CM.

Importantly, larger, more compact, and better-organized mammospheres were observed in the presence of mature adipocyte-derived conditioned medium, and these features were largely maintained even under chemotherapy stress. Functionally, the architecture is relevant in that it poses physical and biochemical barriers to drug penetration, may facilitate the survival of resistant cell populations and may support the formation of protective microenvironments.

LRE-mediated modulation of adipocytes appears to partially alter the 3D spheroid architecture, as indicated by reduced spheroid compactness, structural complexity, and size. This remodeling is not merely structural but may also have functional implications, potentially facilitating drug accessibility and being associated with increased oxidative stress and apoptotic responses.

Collectively, these data suggest that spheroid morphology may represent an important determinant of therapeutic response, and support an association between tumor architecture, cell survival, redox homeostasis, and responsiveness to chemotherapy, with adipocyte modulation potentially playing a modulatory role.

Our results are consistent with previous studies demonstrating that 3D tumor models are more physiologically relevant than traditional 2D cultures [23,46]. Moreover, larger, more compact spheroids have been shown to develop diffusion gradients for oxygen, nutrients and drugs, which can reduce chemotherapy efficacy and drive hypoxic core formation and metabolic heterogeneity [26,49]. Furthermore, morphological parameters beyond size, including circularity and compactness, have been shown to function as indicators of spheroid organization. Highly compact and spherical structures are typically associated with reduced intercellular spaces, stronger cell–cell adhesion, and decreased permeability, whereas less organized spheroids often display enhanced sensitivity to therapeutic agents [37,50]. Importantly, accumulating evidence indicates that both chemotherapeutic agents and bioactive compounds can induce significant morphological alterations in tumor spheroids, including reductions in size, loss of compactness, and disruption of structural integrity, which are frequently associated with decreased cell viability, increased apoptosis, and improved drug sensitivity. In this context, spheroid morphology is increasingly recognized as a functional and integrative readout of treatment response rather than a purely descriptive parameter.

These observations are further supported by studies in other CSC-enriched 3D models. Cianciosi et al. reported that treatment of colorectal cancer colonospheres with Manuka honey resulted in marked morphological alterations, with spheroids appearing smaller and less structurally organized [51].

While most studies on licorice-derived compounds have focused on their direct anti-cancer effects, including modulation of oxidative stress and induction of apoptosis, to the best of our knowledge, no previous studies have specifically investigated the impact of LRE or its major phytochemical constituents on the morphological organization of three-dimensional *in vitro* tumor models, either through direct treatment or via modulation of the tumor microenvironment.

However, the present findings suggest that LRE may also influence tumor behavior indirectly by altering spheroid architecture through modulation of the adipocyte-derived microenvironment. This is consistent with the possibility that bioactive compounds may influence not only intracellular signaling pathways but also higher-order structural features

of tumor organization, which have been associated with drug response and responsiveness to chemotherapy.

2.6. Doxorubicin Uptake in MCF-7 Mammospheres

CSCs have been widely reported to exhibit reduced sensitivity to doxorubicin. This reduced sensitivity has been associated with increased drug efflux, decreased intracellular drug accumulation, and adaptive redox regulation. Accordingly, intracellular doxorubicin uptake was evaluated in CSC-enriched mammospheres to investigate whether the experimental conditions influenced drug accumulation [52].

Doxorubicin accumulation was estimated by measuring intracellular fluorescence intensity in MCF-7 mammospheres exposed to different conditioned media (Figure 11).

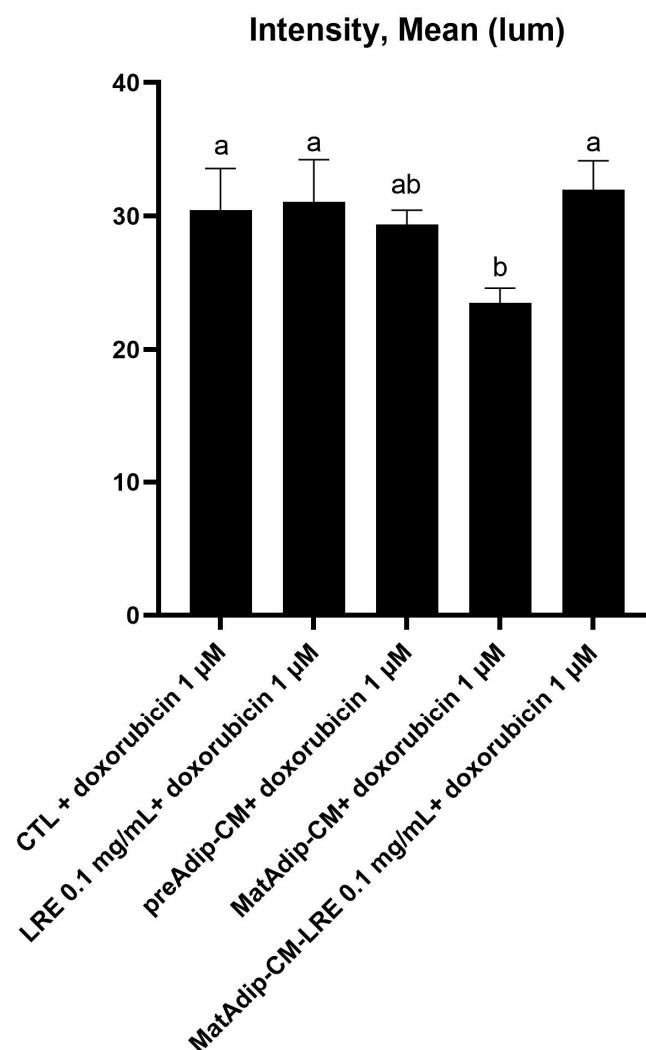


Figure 11. Intracellular doxorubicin uptake in MCF-7 mammospheres under different experimental conditions (1 µM). Uptake was quantified as mean red fluorescence intensity (lum) measured using Celleste™ Image Analysis Software. Values are expressed as mean ± SD of three independent biological experiments ($n = 3$). Statistical analysis was performed using one-way ANOVA followed by Fisher's post hoc multiple comparison test. Different letters indicate statistically significant differences between groups ($p < 0.05$), whereas groups sharing at least one letter are not significantly different according to Fisher's post hoc multiple comparison test.

The differences in fluorescence intensity between the experimental groups suggest that the tumor microenvironment may influence intracellular doxorubicin accumulation.

CTL mammospheres exhibited an average fluorescence intensity of approximately 30.46 lum, representing the baseline level of doxorubicin uptake. A similar value was observed in the LRE-treated group (~31.05 lum), suggesting that LRE alone does not significantly alter intracellular drug accumulation. Likewise, the MatAdip + LRE-CM group (~31.97 lum) displayed fluorescence levels comparable to those of the CTL group, with these groups belonging to the same statistical class, consistent with intracellular doxorubicin accumulation comparable to that observed in the control condition.

In contrast, a marked reduction in intracellular doxorubicin accumulation was observed in mammospheres cultured with MatAdip-CM, where fluorescence intensity decreased to approximately 23.48 lum, representing the lowest value among all experimental conditions ($p < 0.05$). These findings suggest that mature adipocyte-derived conditioned medium may reduce intracellular doxorubicin accumulation. Given that intracellular drug accumulation has been recognized as an important determinant of cytotoxic efficacy, this reduction may reflect the establishment of a microenvironment that limits the intracellular availability of doxorubicin. The preAdip-CM group showed an intermediate response (~29.36 lum), suggesting a modest effect on doxorubicin accumulation, although less pronounced than that observed with mature adipocytes. This observation is consistent with a role of adipocyte differentiation status in shaping tumor–microenvironment interactions.

Importantly, the reduction in intracellular doxorubicin accumulation observed in the MatAdip-CM condition was partially reversed in the MatAdip + LRE-CM group. Under this condition, fluorescence intensity increased to approximately 31.97 lum, reaching values comparable to CTL and significantly higher than those observed in MatAdip-CM ($p < 0.05$). These findings suggest that LRE-mediated modulation of adipocytes may partially restore intracellular doxorubicin accumulation to levels comparable with those observed in the control condition.

Representative fluorescence images of doxorubicin uptake are shown in Figure 12.

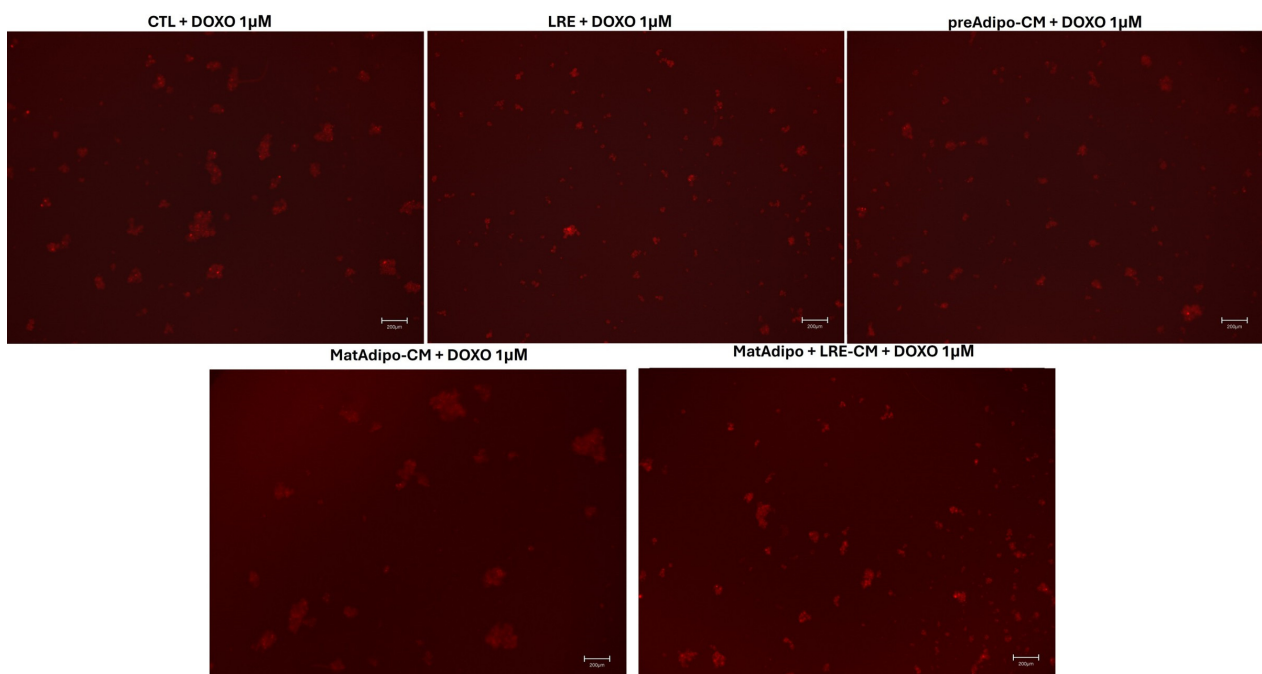


Figure 12. Representative fluorescence images of intracellular doxorubicin accumulation in MCF-7 mammospheres under different experimental conditions (1 μ M). Images were acquired using the EVOS™ M5000 Imaging System at 4 $\times$ magnification under identical fluorescence acquisition settings for all experimental groups. Doxorubicin fluorescence was detected using the RFP channel (excitation: 531/40 nm; emission: 593/40 nm). Scale bar = 200 μ m.

Taken together, these data suggest that adipocyte-derived conditioned media may differentially influence doxorubicin accumulation in MCF-7 mammospheres, with mature adipocytes being associated with a marked reduction in intracellular doxorubicin accumulation that appears to be partially reversed following LRE treatment. These differences in intracellular doxorubicin accumulation may provide a mechanistic basis for the variability in treatment response observed across the different experimental conditions.

These findings are consistent with previous studies demonstrating that reduced intracellular accumulation of chemotherapeutic agents has been recognized as an important mechanism associated with resistance in cancer stem-like cells (CSCs) [40,41]. Although the molecular mechanisms responsible for the altered doxorubicin accumulation observed in the present study were not directly investigated, previous evidence suggests that changes in drug transport, cellular metabolism, and redox regulation may contribute to this phenomenon. Whether these processes are mediated by specific licorice-derived phytochemicals or by the combined activity of multiple constituents present in the extract remains to be elucidated.

The tumor microenvironment, including stromal components such as adipocytes, has been reported to influence drug availability and distribution, potentially contributing to reduced therapeutic efficacy. Adipocyte–tumor interactions have also been reported to promote chemoresistance through multiple mechanisms, including metabolic reprogramming, secretion of protective factors and induction of drug efflux pathways [53].

In recent years, the intrinsic fluorescence of doxorubicin has been extensively used to assess its accumulation and distribution in cancer cells and 3D tumor models [54]. Fluorescence-based techniques in spheroid systems have reliably demonstrated non-uniform drug penetration with reduced accumulation in the densest and most structurally organized regions, supporting the concept that tumor architecture may act as a physical barrier to chemotherapy [55]. The reduction in intracellular doxorubicin accumulation observed in the presence of mature adipocyte-conditioned medium is consistent with previous evidence indicating that adipocyte–tumor crosstalk may contribute to reduced responsiveness to chemotherapy [7].

Several licorice-derived phytochemicals have been reported to modulate drug sensitivity and cellular stress responses in different cancer models. Of particular relevance, glycyrrhizin, a major triterpenoid saponin and one of the main constituents identified in the LRE used in this study [7], and its aglycone glycyrrhetinic acid have demonstrated antitumor activity. Glycyrrhizin has been reported to inhibit cancer cell growth and modulate inflammatory and survival-related signaling, whereas glycyrrhetinic acid can promote oxidative stress, mitochondrial dysfunction, and caspase-3-mediated apoptosis [6,35]. Although evidence directly linking these compounds to doxorubicin sensitivity remains limited, their biological activities suggest that they may contribute to the overall effects of the complex extract. Isoliquiritigenin has been shown to enhance the chemosensitivity of multidrug-resistant uterine sarcoma cells to doxorubicin by promoting apoptosis and autophagy [56]. In addition, licochalcone A has been reported to reverse ATP-binding cassette subfamily G member 2 (ABCG2)-mediated multidrug resistance by inhibiting transporter activity, thereby increasing the intracellular accumulation of chemotherapeutic substrates [57]. However, most studies on licorice-derived compounds have focused on their direct effects on cancer cells. By contrast, the present findings suggest that these phytochemicals may also indirectly influence tumor behavior through modulation of the adipocyte-derived tumor microenvironment, although the underlying mechanisms remain to be elucidated.

Taken together, these results suggest that intracellular doxorubicin accumulation may represent an important factor associated with therapeutic response and support the hy-

pothesis that adipocyte–tumor interactions contribute to its modulation. Furthermore, the present findings raise the possibility that licorice-derived phytochemicals may indirectly influence this process through modulation of the adipocyte-derived tumor microenvironment, although the molecular mechanisms underlying these effects remain to be elucidated.

3. Materials and Methods

3.1. LRE Preparation

LRE was obtained from roots of wild-growing *Glycyrrhiza glabra* L. collected in the Abruzzo region, Italy (41.997485° N, 14.639711° E), and botanically authenticated by Prof. Franco Capocasa (Department of Agricultural, Food and Environmental Sciences, Università Politecnica delle Marche, Ancona, Italy). The extract was prepared according to a previously published protocol [7]. Briefly, dried roots were size-reduced, pulverized using an A 11 basic analytical mill (IKA-Werke GmbH & Co. KG, Staufen, Germany), and extracted with an EtOH/H₂O solution (80:20, *v/v*) under continuous stirring in the dark at room temperature for 72 h. A hydroalcoholic extraction system was selected to allow the recovery of a broad spectrum of licorice bioactive compounds, including phenolic constituents and triterpenoid saponins such as glycyrrhizin, which were previously identified during the phytochemical characterization of the extract [7]. Similar hydroalcoholic extraction systems have been widely employed in phytochemical studies of licorice to recover compounds with different polarity [58].

The extract was then vacuum-filtered and concentrated to dryness under reduced pressure using a rotary evaporator, followed by SpeedVac concentration. The resulting dry extract was stored at −20 °C and resuspended in culture medium at the desired concentration prior to use. The chemical characterization of the extract, including the identification of its main bioactive constituents, has been previously reported in detail [7].

3.2. Adipocyte Differentiation

Murine 3T3-L1 preadipocytes (ATCC CL-173™, American Type Culture Collection, Manassas, VA, USA) were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with fetal bovine serum and antibiotics under standard conditions. Upon reaching confluence, adipogenic differentiation was induced using a hormonal cocktail containing dexamethasone, 3-isobutyl-1-methylxanthine (IBMX), and insulin. After the induction phase, cells were maintained in insulin-supplemented medium until full differentiation, as confirmed by intracellular lipid accumulation. The differentiation protocol was performed as previously described [7].

3.3. Generation of Conditioned Media

Conditioned media (CM) were obtained from three experimental conditions: proliferating preadipocytes (preAdip-CM), fully differentiated adipocytes (MatAdip-CM), and mature adipocytes exposed to LRE (0.1 mg/mL) for 24 h (MatAdip + LRE-CM). The selected LRE concentration was based on our previous concentration–response experiments in 3T3-L1 mature adipocytes, in which LRE cytotoxicity was evaluated by MTT assay over a concentration range of 0–10 mg/mL. Based on these results, 0.1 mg/mL was identified as a non-cytotoxic concentration, maintaining adipocyte viability above 90%, and was therefore selected for conditioned medium generation while preserving biological activity [7]. Prior to CM collection, cells were incubated in serum-free medium. Supernatants were collected, centrifuged to eliminate cellular debris, and stored at −80 °C until further use.

3.4. Cell Culture, Preconditioning, and Mammosphere Formation

MCF-7 human breast cancer cells (ATCC HTB-22™, American Type Culture Collection, Manassas, VA, USA) were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum and antibiotics at 37 °C in a humidified atmosphere with 5% CO₂.

To evaluate the effects of adipocyte-derived conditioned media, MCF-7 cells grown under adherent conditions were pre-treated for 48 h with the respective experimental conditions, including control medium, LRE alone, preAdip-CM, MatAdip-CM, and MatAdip + LRE-CM. Following this preconditioning step, cells were detached using trypsin, washed to remove serum residues, and resuspended in serum-free medium.

For the generation of CSC-enriched cultures, cells were seeded as single-cell suspensions in ultra-low attachment plates (Corning Costar®, Corning, NY, USA) at an appropriate density and cultured in serum-free mammosphere medium. The medium consisted of DMEM/F-12 supplemented with penicillin–streptomycin, epidermal growth factor (EGF, 20 ng/mL), fibroblast growth factor (FGF, 10 ng/mL), insulin (5 µg/mL), hydrocortisone (0.5 µg/mL), and N2 supplement.

Cells were carefully dissociated to obtain a single-cell suspension prior to seeding, to promote clonal spheroid formation rather than cell aggregation. Cultures were maintained under standard conditions, and mammospheres were allowed to form over a period of approximately 10 days, after which they were used for subsequent analyses. This approach is based on the ability of CSCs to survive and proliferate under non-adherent, serum-free conditions, forming spheroid structures, whereas differentiated cells undergo anoikis [59].

3.5. Doxorubicin Treatment and MTT Assay

To determine the appropriate working concentration of doxorubicin, MCF-7 cells under adherent conditions were treated with various concentrations of doxorubicin (0–4 µM) for 24 h. Cell viability was assessed using the MTT assay as previously described [7]. Briefly, 30 µL of MTT solution (10 mg/mL in RPMI medium) was added to each well, and cells were incubated for 2.5 h. Subsequently, 100 µL of DMSO was added to dissolve the formazan crystals, and absorbance was measured at 590 nm using a microplate spectrophotometer. Cell viability was calculated relative to the untreated control as follows:

$$\text{cell viability (\%)} = (\text{absorbance of treated cells} / \text{absorbance of untreated control cells}) \times 100.$$

The IC₅₀ value was calculated from the dose–response curve. Based on these results, a concentration of 1 µM was selected for subsequent experiments.

3.6. Intracellular ROS Measurement

Intracellular ROS levels were evaluated using CellROX® Orange Reagent (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions. CellROX® is a widely used and validated fluorescent probe for the detection of oxidative stress in living cells. Mammospheres were collected, centrifuged to obtain a cell pellet, and washed with phosphate-buffered saline (PBS) prior to staining. The pellet was then incubated with the fluorescent probe for 30 min at 37 °C. After incubation, samples were washed and analyzed using a Tali® Image-Based Cytometer (Thermo Fisher Scientific). Cell classification and fluorescence quantification were automatically performed using the integrated Tali® software (version 2.1) under identical acquisition settings for all experimental groups. The percentage of ROS-positive cells generated by the software was used as an indicator of intracellular oxidative status. Analyses were performed in three independent biological experiments.

3.7. Apoptosis Assay

Apoptosis was assessed using an Annexin V-FITC/Propidium Iodide apoptosis detection kit (Thermo Fisher Scientific) following the manufacturer's protocol. Mammospheres were collected, centrifuged to obtain a cell pellet, and washed with PBS. The pellet was then resuspended and stained with Annexin V-FITC and Propidium Iodide (PI). Samples were analyzed using a Tali[®] Image-Based Cytometer (Thermo Fisher Scientific). Cell classification was automatically performed by the Tali[®] apoptosis analysis software based on Annexin V-FITC and PI fluorescence signals. Cells were categorized as viable (Annexin V−/PI−), apoptotic (Annexin V+/PI−), or dead (Annexin V+/PI+) according to the manufacturer's predefined analysis algorithm. Fluorescence signals were automatically acquired using the predefined channels of the Tali[™] Image-Based Cytometer: the green channel for Annexin V–Alexa Fluor 488 (excitation: 458 nm; emission: 500–550 nm) and the red channel for PI (excitation: 530 nm; emission: 565–620 nm). Early and late apoptotic populations were not analyzed separately.

3.8. RNA Isolation and Quantitative Real-Time PCR

MCF-7 cells were treated with LRE and conditioned media (CM) as described in the experimental setup and subsequently used for mammosphere formation. Total RNA was extracted from mammospheres using the PureLink[®] RNA Mini Kit (Invitrogen, Carlsbad, CA, USA), following the manufacturer's instructions. RNA concentration and purity were assessed using a microplate spectrophotometer (BioTek Synergy HT, Winooski, VT, USA), based on absorbance at 260 and 280 nm. For cDNA synthesis, 100 ng of total RNA was reverse transcribed using the 5X All-In-One RT MasterMix Kit (Applied Biological Materials Inc., Richmond, BC, Canada) in a SimpliAmp Thermal Cycler (Applied Biosystems, Thermo Fisher Scientific). Quantitative real-time PCR (qPCR) was performed using BlasTaq[™] 2X qPCR Master Mix (Applied Biological Materials Inc., Canada) in a QuantStudio[™] 1 Real-Time PCR System (Applied Biosystems, Thermo Fisher Scientific). Gene-specific forward and reverse primers (Sigma-Aldrich, Milan, Italy) were used for amplification. The thermal cycling conditions were set according to the manufacturer's instructions, and a melting curve analysis was performed to verify amplification specificity. Gene expression levels were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and calculated using the comparative cycle threshold (Ct) ($2^{-\Delta\Delta C_t}$) method.

Primer sequences used in this study are reported in Table 1.

Table 1. Primer sequences used for quantitative Real-Time PCR analysis.

Primer	Sequence
GAPDH Forward	5'-GACCCCTTCATTGACCTCAACTACATG-3'
GAPDH Reverse	5'-GTGCACCACCCTGTTGCTGTAGCC-3'
CD44 Forward	5'-AAGGTGGAGCAAACACAACC-3'
CD44 Reverse	5'-ACTGCAATGCAAACCTGCAAG-3'
CD133 Forward	5'-ACCGACTGAGACCCCAACATC-3'
CD133 Reverse	5'-GGTGCTGTTTCATGTTCTCCA-3'

3.9. Morphological Analysis of Mammospheres

Mammospheres were generated and maintained in ultra-low attachment 96-well plates (Corning Costar[®], Corning, NY, USA). Brightfield images were acquired using an EVOS[™] M5000 Imaging System (Thermo Fisher Scientific) under identical acquisition settings for all experimental groups. Morphological parameters, including area, equivalent spherical volume, maximum diameter, minimum diameter, circularity, and perimeter, were quantified using Celleste[™] Image Analysis Software (Thermo Fisher Scientific). For each

mammosphere, a region of interest (ROI) corresponding to the spheroid boundary was automatically identified and analyzed by the software. Since the treatments affected mammosphere formation, number, and morphology, all mammospheres visible within the field of view at 4× magnification were included in the analysis for each experimental condition. Morphological analyses were performed in three independent biological experiments.

3.10. Doxorubicin Uptake Analysis

Mammospheres were generated in ultra-low attachment 96-well plates (Corning Costar®, Corning, NY, USA). Intracellular accumulation of doxorubicin was evaluated by exploiting its intrinsic red fluorescence. Following treatment, mammospheres were imaged using an EVOS™ M5000 Imaging System (Thermo Fisher Scientific) at 4× magnification using the red fluorescence channel (RFP) (excitation: 531/40 nm; emission: 593/40 nm) and identical acquisition settings (exposure, gain, illumination intensity, fluorescence filter configuration) for all experimental groups. The 4× magnification was selected to allow analysis of the entire mammosphere population present within the well. All detected mammospheres were therefore included in the analysis, minimizing field-selection bias.

Fluorescence images were analyzed using Celleste™ Image Analysis Software. No condition-specific thresholding or image enhancement procedures were applied prior to quantitative analysis. Quantification was performed by defining a region of interest (ROI) corresponding to each mammosphere and measuring the mean red fluorescence intensity (lum). The same image analysis parameters were applied to all images to ensure comparability among groups.

Because the experimental treatments directly affected mammosphere formation, number, and morphology, a fixed number of spheroids was not pre-selected. Instead, the entire field of view acquired at 4× magnification was analyzed, and all mammospheres detected within the image were included in the analysis, thereby minimizing selection bias. Mean fluorescence intensity was calculated by the software as the average fluorescence signal of the mammospheres identified within each image.

Quantitative fluorescence analysis was performed in three independent biological experiments, and mean fluorescence intensity was used as an indirect measure of intracellular doxorubicin uptake.

3.11. Statistical Analysis

All experiments were performed in three independent biological replicates ($n = 3$). Data are presented as mean ± standard deviation (SD). Statistical analyses were performed using one-way analysis of variance (ANOVA) followed by Fisher's post hoc multiple comparison test to evaluate differences among experimental groups.

For all analyses, statistical significance was set at $p < 0.05$. Different letters in graphs indicate statistically significant differences between groups.

4. Conclusions

The present study provides evidence that adipocyte differentiation status contributes to shaping biological features associated with the response of CSC-enriched MCF-7 mammospheres to doxorubicin. Compared with preadipocyte-conditioned medium, mature adipocyte-conditioned medium exerted a more pronounced protective effect, being associated with larger and more compact spheroids, reduced intracellular ROS accumulation and apoptosis, and lower intracellular doxorubicin accumulation. Collectively, these findings support the view that signals released by mature adipocytes may generate a microenvironment favoring tumor cell survival and limiting intracellular drug availability. Importantly, conditioned medium from LRE-treated mature adipocytes partially attenuated this phe-

notype by increasing oxidative stress, apoptotic response, and intracellular doxorubicin accumulation while reducing spheroid size and compactness. Thus, LRE-mediated modulation of adipocytes may simultaneously influence several interconnected features associated with doxorubicin responsiveness.

The main contribution of this study is the identification of an indirect mechanism through which LRE may influence breast cancer cell behavior by modulating adipocyte–tumor crosstalk. While previous studies have primarily focused on the direct anticancer effects of licorice-derived phytochemicals, our findings suggest that these compounds may also affect tumor cells through changes in the adipocyte secretome. These observations open new research directions in which the adipose microenvironment, rather than tumor cells alone, is investigated as a potentially modifiable component of chemotherapy response. Given the preliminary nature of this study, the morphological findings should be interpreted together with the functional endpoints examined. Further validation through protein-level analyses, ATP-based measurements of viability and metabolic activity, and assessment of necrotic-core formation will be necessary to characterize the internal biology of the mammospheres more comprehensively. Future studies should identify the specific adipocyte-derived mediators altered by LRE, determine the contribution of individual or combined LRE constituents, and validate these findings across additional breast cancer subtypes and in patient-derived or advanced three-dimensional models.

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