

Yeast Therapy for the Treatment of Breast Cancer: A Nude Mice Model Study

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Abstract. *Background: Studies have demonstrated that phagocytosis of yeast induces apoptosis in human breast cancer (BC) cells in vitro. Here, the in vivo apoptotic activity of the S. cerevisiae against human BC (MCF-7) bearing nude mice was investigated. Materials and Methods: MCF-7 cells were injected into nude mice. Mice were then injected intra-tumorally with yeast on a weekly basis for 45 days. Tumors were excised and analyzed for phagocytosis/apoptosis via histopathological staining, electron microscopy (EM), and flow cytometry. Results: The results demonstrate the ability of MCF-7 cells to phagocytize yeast and the effectiveness of yeast in triggering apoptosis in MCF-7 cells in vivo. Histological sections of yeast-treated tumors show extensive tumor apoptosis/fibrosis. EM studies clearly show apoptotic MCF-7 cells with nuclear margination and fragmentation. Flow cytometry confirmed this result. No noticeable adverse side-effects from the yeast treatment were observed. Conclusion: S. cerevisiae is a promising anti-cancer agent that induces significant levels of apoptosis in malignant cells in vivo. However, yeast therapy for the treatment of breast cancer has yet to pass controlled clinical trials.*

Breast cancer (BC) is the most frequent tumor type among women with an incidence rate of 135.4 per 100,000 women in the United States alone (1). Two characteristics of BC malignancy are their invasive and metastatic potential. Most patients with operable BC now receive postoperative medical treatment in the form of adjuvant chemotherapy, hormone manipulation, or both (2). Despite great

advancements in these treatment methods, BC remains as the second leading cause of cancer-related deaths in women in the United States (1, 2).

Research from the last decade has revealed a promising future for apoptosis-based BC therapies. Several such anti-cancer agents – including tamoxifen, vincristine, doxorubicin and 5-fluorouracil – are widely used in the treatment of BC (3-8). The mechanisms by which these chemotherapeutic agents induce apoptosis in BC are as follows: tamoxifen acts through a rapid mitochondrial cell death program (7), vincristine through (NF-kappaB) activation (8), and both doxorubicin and 5-fluorouracil through caspase activation (9). However, these chemotherapeutic agents are known to cause severe side-effects. For example, medium to high doses of doxorubicin may damage cardiac muscle resulting in congestive heart failure (10). It is therefore of particular interest to explore additional therapeutic approaches of inducing apoptosis of cancer cells that will incur minimal adverse effects.

The ability of of heat-killed *S. cerevisiae* to induce apoptosis in human cancer cell lines *in vitro* has recently been examined. The results of these studies demonstrated that phagocytosis of yeast leads to apoptosis in multiple human cancer cell lines: BC-cells (11), tongue squamous cell carcinomas (12) and colon adenocarcinomas (12). In this study, it was demonstrated that intra-tumoral injection with *S. cerevisiae* induces apoptosis in BC (MCF-7 cells) bearing nude mice. This observation may have future therapeutic implications.

Materials and Methods

Animals. Athymic homozygous nude mice (4-5 weeks old, 20-25 g, female) were purchased from Harlan (Chicago, IL, USA). The mice were maintained in the animal facility at Charles R. Drew University of Medicine and Science, Los Angeles, CA, USA. Mice were housed 2 per micro-isolator cage and were fed sterilized standard cube pellets and water *ad libitum*. All mice were accommodated for 1 wk prior to experimentation. Animal protocols were in compliance with the Guide for the Care and Use of Laboratory Animals in the USA.

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Tumor cell line. Human breast cancer (MCF-7) cells were used in this study. The cells were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA). The cells were cultured *in vitro* within a humidified, 5% CO₂ incubator at 37°C, and were maintained in complete medium at a starting density of 3x10⁵ cells/ml.

Complete medium (CM). This consisted of Roswell Park Memorial Institute-1640 (RPMI-1640) medium supplemented with 10% heat inactivated fetal calf serum (FCS), 100 units penicillin and 100 µg streptomycin.

Preparation of *S. cerevisiae*. Commercially available baker's and brewer's yeast, *S. cerevisiae*, was used in suspensions that were washed once with Hanks balanced salt solution (HBSS). The suspensions were heat-killed *via* incubation for 1 h at 90°C and subsequently washed twice with HBSS. Quantification was carried out using a hemocytometer. Cell suspensions were adjusted to 1x10⁹ cells/ml.

Experimental design and tumor transplantation. MCF-7 cells were grown to 80% confluency and were harvested using a cell scraper. Cell viability was examined using the trypan blue exclusion test and MCF-7 cells were suspended in sterile serum-free RPMI-1640 at concentrations of 10x10⁷ cells/ml. Cell suspensions were then mixed with equal volumes of matrigel (BD Biosciences Discovery Labware, Bedford, MA, USA). Mice were singularly injected *s.c.* into the right axillary (the right back) region with 5x10⁶ tumor cells/mouse in 0.5 ml serum-free RPMI-1640. Mice were examined weekly for tumor growth at the site of injection. Tumor volume (mm³) was measured with a digital caliper and volume estimated as (length x width x height)/2. Mice with tumors reaching 50-100 mm³ were randomly separated into 2 groups of 8 animals. Mice were injected intra-tumorally (*i.t.*) with either yeast (100 µl containing 1x10⁸ cells) or with HBSS once a week for 45 days. After the treatment period, mice were sacrificed and tumor tissues were removed. Tumor tissues were fixed in 10% neutral buffered formalin for histological paraffin sections and pieces about 1 mm³ were fixed in 2.5% glutaraldehyde for electron microscopy studies. Single cell suspensions of tumor tissue were prepared by teasing the tissue with a wire brush and these were used for flow cytometry. Tumors were examined for the presence of phagocytic and apoptotic tumor cells.

Histological studies. The tumor tissues obtained from yeast-treated and control groups were fixed in 10% neutral buffered formalin for at least 24 hours and processed overnight in a Shandon Hypercenter XP Tissue Processor (Pittsburg, PA, USA). The tissues were embedded in paraffin using a routine method. Three-micron histological sections were prepared and stained with Hematoxylin and Eosin (H&E). In addition, other special stains such as Grocott's Methenamine-Silver (GMS) stain and Periodic Acid-Schiff (PAS) stain were used. Sections were examined for histopathological changes and measurements of apoptotic and fibrosis areas under a light microscope.

Measurement of tumor volume. Tumor length (a) and width (b) measurements were obtained on a weekly basis using electronic digital caliper (Control Company, Texas, USA). The collected measurements were entered into the formula (a x b²)/2 to calculate individual tumor volumes.

Measurement of apoptotic areas within the tumor. A series of paraffin sections (3 µm thick) were stained with H & E and examined for tumor cell apoptosis and fibrosis (control n=5, yeast n=5). Section images were captured at x4 magnification using a Leica DMLB microscope (Leica Microsystems Inc., Bannockburn, IL, USA) captured by an Olympus MicroFire-C camera and imported into the computer program MicroFire™ v1.1 (MicroFire-C camera and software from Olympus America Inc., Center Valley, PA, USA). Measurements were carried out for every 10th section using electronic digital calipers. The percentage areas of apoptosis, fibrosis, and viable tumor cells were obtained from 4-20 sections of each tumor. Total percentages were obtained by averaging the values across each tumor.

Electron microscopy (EM). Small fragments (1 mm³ blocks) of tissue were fixed in 2.5% glutaraldehyde, then put into sodium cacodylate buffer the night before processing and postfixed with osmium tetroxide. The tissues were then embedded in resin. Semi-thin sections were cut using a glass knife and stained with toluidine blue. Semi-thin sections are required to confirm the presence of desired cells and to select the best tissue block for thin-sectioning. Finally, thin sections are cut on a diamond knife. Routine staining is performed with uranyl acetate followed by lead citrate.

Flow cytometry. A transactional portion of the tumor was removed that included both stromal as well as epithelial cells. DNA content was measured, following extraction of degraded DNA from apoptotic cancer cells, by propidium iodide (PI) staining as previously described by (13). Briefly, cells (1x10⁶/ml) were fixed in 70% ethanol, washed with PBS and were re-suspended in the DNA extraction buffer (0.2 M Na₂HPO₄ with 0.1 M citric acid pH 7.8). Following extraction, cells were washed and incubated in the DNA staining solution (20 µg/ml PI in PBS containing 50 µg/ml RNase A). Cells were stained for 30 min at room temperature in the dark and were analyzed by FACScan (Becton Dickinson, San Jose, CA, USA). After exclusion of necrotic debris, the sub G0/G1 peak was used to quantify apoptosis.

Statistical analysis. Using the Student's *t*-test, the significance of differences between experimental and control mice groups was tested.

Results

In order to determine the *in vivo* effects of yeast treatments on cell survival, apoptotic MCF-7 cells were identified and evaluated by 3 different methods: histological examination, EM, and flow cytometry. In addition, adverse side-effects from the yeast treatment were also monitored.

Toxicity. Animals were monitored to observe potential toxic side effects of yeast treatment. Daily examinations showed that injections of heat-killed *S. cerevisiae* gave no adverse side-effects as indicated by the fact that: a) normal feeding/drinking and life activity patterns were recorded for the entire treatment period, b) all animals survived the 45-day treatment period, and c) histological sections taken from the liver, kidney, and lung of treated mice had normal appearances comparable to those of healthy, control mice.

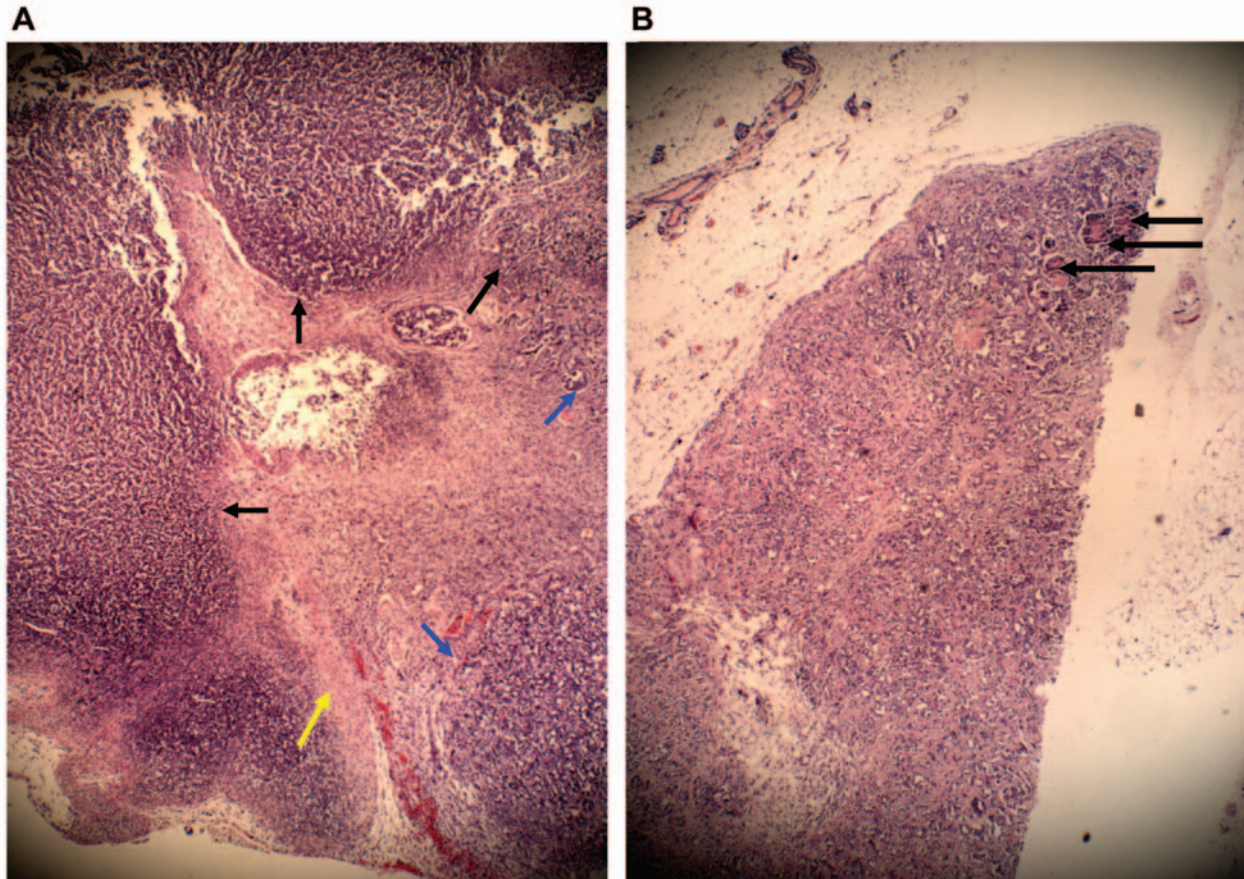


Figure 1. Cross sections of MCF-7 tumor bearing nude mice. A) Section of yeast-treated tumor demonstrates 80% of tumor undergoing extensive apoptosis (black arrows) and fibrous tissue (yellow arrows). Notice that only small foci of residual viable tumor remains (<5%, blue arrows). B) Section of control tumor shows most of tumor are viable except for a few spots of apoptosis (9%, black arrows). (x4 magnification with H&E stain).

Tumor pathology

i) **Tumor volume:** No significant difference in tumor volume was observed between yeast-treated and control mice throughout the examination period (data not shown).

ii) **Histology sections:** Light microscope examination of the paraffin-embedded sections of control and yeast-injected tumors was carried out. Sections demonstrated extensive tumor apoptosis within the lesion (black arrows), with extensive fibrosis (yellow arrow), and small foci of the residual tumor tissues (blue arrows) (Figure 1A). In contrast, sections of the tumors from the control mice show that the tumor predominantly consists of viable tumor cells (Figure 1B, apoptosis noted with black arrows). Under low magnification (x4 with H&E stain), sections of yeast-treated tumors show that approximately 80% of the tumor cells had undergone apoptosis (42%) or fibrosis (38%), and had completely lost their original histological tumor configurations. Control cells showed merely 9% focal apoptosis/fibrosis (Figure 2).

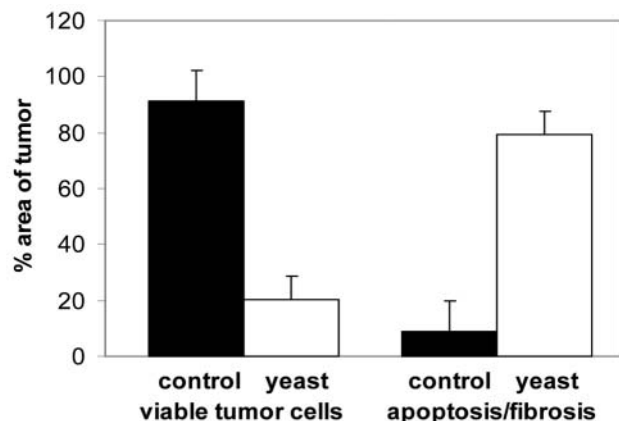


Figure 2. Measurement of apoptotic areas within the tumor. A series of paraffin sections, 30 microns apart, stained with H&E, were used to measure the areas that underwent apoptosis and fibrosis as compared to areas of viable tumor. Section images (x 4) were captured onto a computer and measurements were carried out using electronic digital calipers. Average areas were obtained for each tumor. Bars represent the mean percent area \pm S.D. of viable tumor cells or apoptosis/fibrosis from 5 separate animals in each group.

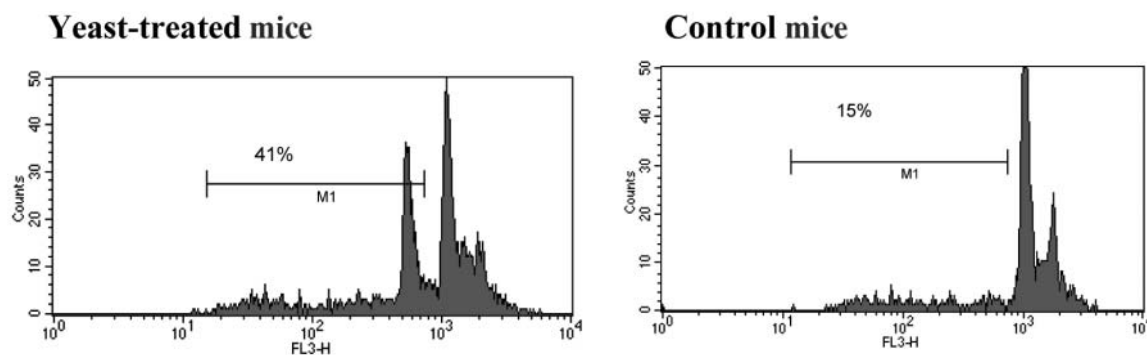


Figure 3. Percentage of apoptotic MCF-7 cells as determined by flow cytometry. Survival of MCF-7 cells was determined by flow cytometry using propidium iodide (PI) technique. The number in the histograms represent the percentage of apoptotic cells. M1 represents % cells with hypodiploid DNA.

iii) *Flow cytometry analysis:* Increase in cancer cell apoptosis as a result of treatment with yeast was further confirmed using flow cytometry analysis. Cancer cell survival was determined using propidium iodide (PI). The data depicted in Figure 3 show that although control animals exhibited a considerable proportion of dead MCF-7 cells (15%), treatment of tumors with yeast resulted in a significant increase in cancer cell apoptosis (41%).

Phagocytosis. The yeast-treated tumors show dramatic numbers of MCF-7 cells phagocytizing yeast. Histological sections stained with PAS and GMS demonstrate numerous malignant MCF-7 cells containing intracytoplasmic pockets of either whole yeasts or fragments of yeasts (Figure 4A). Tumor cells phagocytizing single or multiple yeasts were found throughout the tumor. These observations were further confirmed using EM preparations that clearly demonstrated cancer cells phagocytizing yeast. Cancer cells contained multiple vacuoles of ingested and digested yeast. Lysosomal encirclement and digestion of yeast were noted. The illustration in Figure 4B shows an MCF-7 cell filled with yeasts. Notice marginalized nucleus (N) and presence of vacuoles (V)

Apoptosis

i) *Histological sections:* Tumor cells underwent apoptosis following phagocytosis of yeast. Histological sections of yeast-treated tumors showed a marked increase in the number of apoptotic tumor cells (42% tumor cell death) without neutrophilic infiltration (necrosis). Figure 5A demonstrates an increased number of apoptotic cell clusters in yeast-treated tumors as compared with only a few scattered, individual apoptotic cells in the tumors of control mice (Figure 5B).

ii) *EM studies:* The sequence of apoptotic events is vividly illustrated by EM. Figure 6A shows an EM image of control human BC (MCF-7 cells) where the nucleus (N) is well

defined and occupies most of the cell. Examination of yeast-treated tumors show the malignant BC cell phagocytizing yeasts, triggering the earliest indicators of apoptosis such as nuclear margination and condensation of chromatin (Figure 6B). At the same time, the cytoplasm shows dilation of smooth endoplasmic reticulum as the appearance of numerous intracytoplasmic clear vacuoles (Figure 6B). The presence of residual laminal bodies is also apparent in this photograph. EM studies also showed cells displaying the next stages of apoptosis with nuclear segregation, convulsion and fragmentation, along with the presence of numerous residual bodies (Figure 6B-E). Partially degraded apoptotic bodies with nuclear fragmentation showing decreased density and loss of nuclear membrane integrity were also seen (Figure 6B-E).

Discussion

Many anticancer drugs function by inducing apoptosis (14, 15). However, both chemotherapy and radiation therapy are toxic, immune-suppressive, mutagenic and carcinogenic (16-19), limiting their efficacy as anti-cancer agents. It is therefore of particular interest to find agents that induce apoptosis of cancer cells with minimal side-effects. Results from this study reveal, for the first time, that intratumoral injection with heat-killed non-pathogenic baker's yeast, *S. cerevisiae*, induced highly significant levels of apoptosis in human BC bearing nude mice.

MCF-7 cells proved to be potent phagocytic cells as manifested by their ready engulfment, digestion, and fragmentation of yeast as evident from the histological sections, while EM studies demonstrated that MCF-7 cells phagocytize countless numbers of yeast which are then encircled by lysosomes. This suggests that MCF-7 cells have an effective mechanism for the uptake and ingestion of yeast *in vivo*. Phagocytosis is primarily studied in 'professional' phagocytic cells such as macrophages,

monocytes and polymorphonuclear leukocyte (PMN), which phagocytize particles with ligands such as IgG and/or complement (20) or carry out immune opsonine-independent phagocytosis (21). With respect to BC cells, we used heat-inactivated serum in the phagocytic assay *in vitro*, indicating that serum factor is not required for phagocytosis of yeast by cancer cells *in vitro* (11, 12). Since sera of nude mice lack antibody response, it is likely that phagocytosis of yeast by cancer cells *in vivo* occurs in an opsonine-independent manner.

After phagocytosis of yeast, the tumor cells underwent apoptosis. Histopathological examination of yeast-treated tumors revealed that 80% of the tumor cells had undergone apoptosis/fibrosis resulting in a complete loss of the original histological tumor configurations. Weekly tumor volume data showed no significant difference between the yeast-treated and control groups. However, a separate study examining Swiss albino mice bearing Ehrlich Ascites Carcinoma (EAC) cells *i.t.* injected with yeast for 45 days showed significant suppression of tumor growth by up to 55%, ($p < 0.01$) (unpublished data). The different tumor volume data gathered in these two studies can mainly be attributed to the individual mice models that were employed. Immunocompromised nude mice appear to lack a mechanism for eliminating apoptotic cells that is functional in the normal, Swiss albino mice. Taken together, these data may suggest that an increase in cancer cell apoptosis is associated with a decrease in cell survival.

We employed human BC (MCF-7 cells) because they have proven to be an excellent experimental model for improving the efficacy of various cancer treatment therapies before their use in patients (7, 22). In addition, nude mice were chosen as a model system due to their immune compromised status – as they lack T-cells, resembling the condition of cancer patients. Measurements of splenic NK-cell activity in both control and treated animals revealed no significant difference between the groups (data not shown). This suggests that the significantly high levels of cancer cell apoptosis noted in the current study is unlikely to be due to the activation of T-cells or NK-cells, but rather occurs as a consequence of the phagocytosis of yeast. The mechanism(s) by which yeast induces apoptosis in BC cells *in vivo* is not fully understood. However, the observed active involvement of lysosomes in the phagocytosis and digestion of yeast followed by an increased level of swollen smooth ER and nuclear fragmentation (well-known characteristics of cell apoptosis) may suggest that lysosomes play an important role in BC cell apoptosis.

Phagocytic cells upon performing their function (*i.e.* destruction of phagocytised infectious agent) undergo apoptosis (23-26). We have demonstrated that tumor cells

undergo apoptosis following phagocytosis of yeast (11, 12, 27). The phenomenon of yeast-induced apoptosis in cancer cells is of particular interest for several reasons. First, *S. cerevisiae*-induced apoptosis is selective for cancer cells. While MCF-7 cells in monolayer undergo apoptosis following attachment/ingestion of yeast, no such phagocytic or apoptotic activity was observed in monolayer breast epithelial cells (MCF-10A) (27). Secondly, *S. cerevisiae* exhibits high efficacy for the induction of apoptosis in multiple human cancers: breast cancer cells (11), tongue squamous cell carcinomas (12) and colon adenocarcinomas (12). Additionally, microbial induction of apoptosis in cancer cells is yeast specific as MCF-7 cells do not phagocytize and undergo programmed cell death upon co-culture with fungal mycelia (28). Finally, *S. cerevisiae* showed no toxic characteristics as manifested by the ability of all animals to survive the 45-day treatment period with normal feeding/drinking and life activity patterns.

S. cerevisiae is viewed as a safe, non-toxic, non-threatening agent by the human population. Investigators interested in the effects of daily selenium supplementation against cancer employed selenized yeast as a chemopreventative agent to conduct their clinical trial study (29). Brewer's yeast (*S. cerevisiae*) is sold as a food supplement for human consumption for constipation (30). In addition, another non-pathogenic strain of yeast, *S. boulardii*, has been found to be an effective means of treatment for gastrointestinal infections, such as recurrent *Clostridium difficile* colitis, in human subjects (31-33).

Conclusion

In conclusion, the present study provides the first evidence that heat-killed non-pathogenic yeast induced apoptosis of human BC-cells *in vivo*. The specificity, safety and low cost of heat-killed non-pathogenic yeast may make it an effective and acceptable anti-cancer agent. This data establishes the foundation for *in vivo* optimization studies that could lead to the development of novel therapies.

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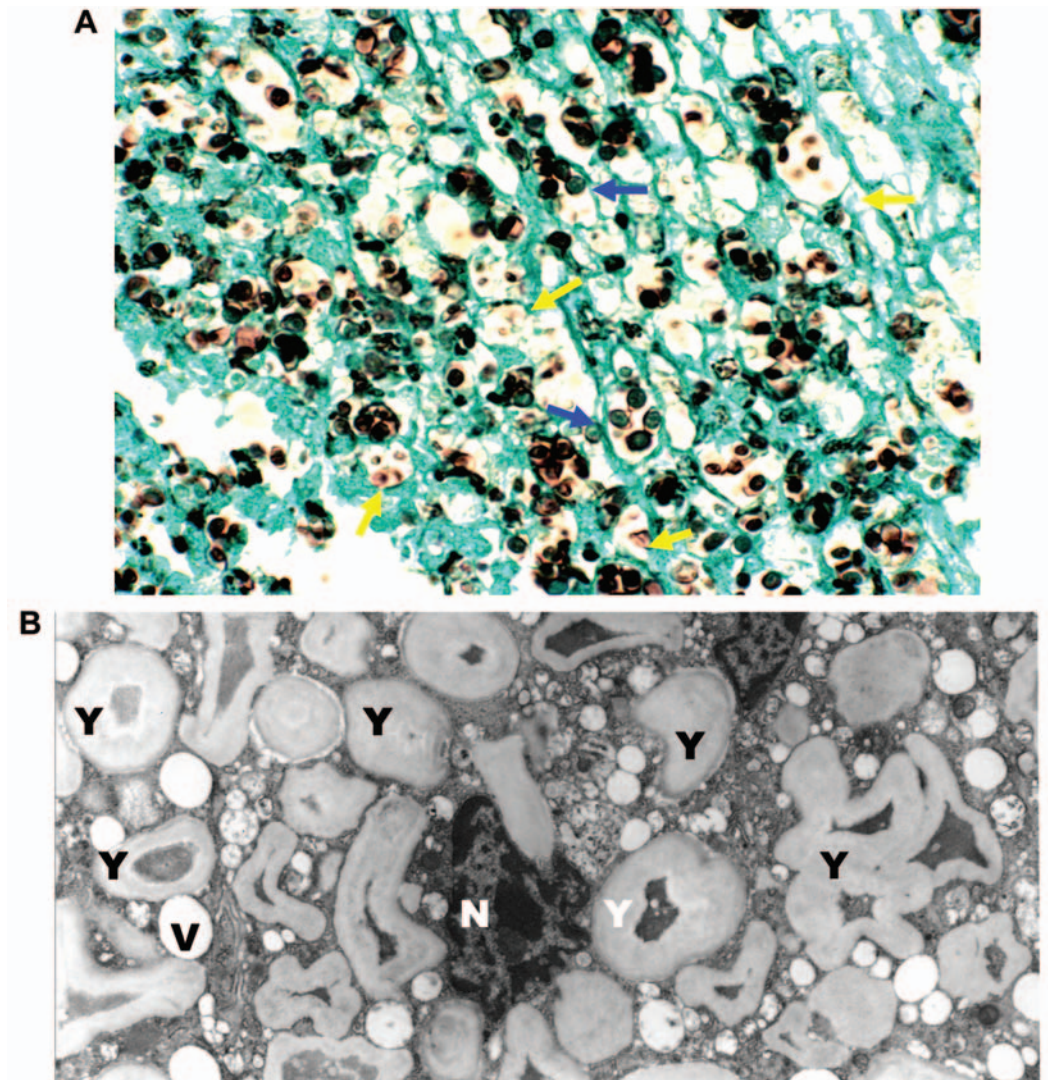


Figure 4. Phagocytosis of yeast by tumor cells. A) Grocott's Methenamine Silver (GMS) stain shows phagocytosis of yeast by tumor cells. Note several pockets of yeast in the cytoplasmic portion of many cancer cells. Blue and yellow arrows point to ingested and digested yeast, respectively (x100 GMS stain). B) EM of the yeast-treated tumor showing a MCF-7 cell phagocytizing countless numbers of yeast (Y). (x3,500 TEM).

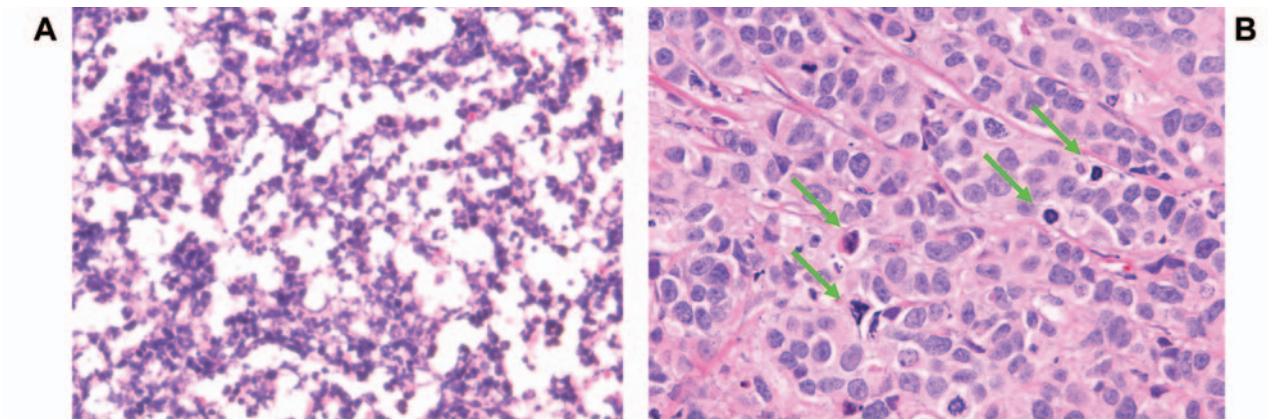


Figure 5. Histological sections show apoptotic cells. A) The yeast-treated tumor demonstrates extensive tumor apoptosis. (x60, H&E stain). B) The control tumor shows only scattered apoptotic cells (green arrows) in the tumor. (x 40, H&E stain).

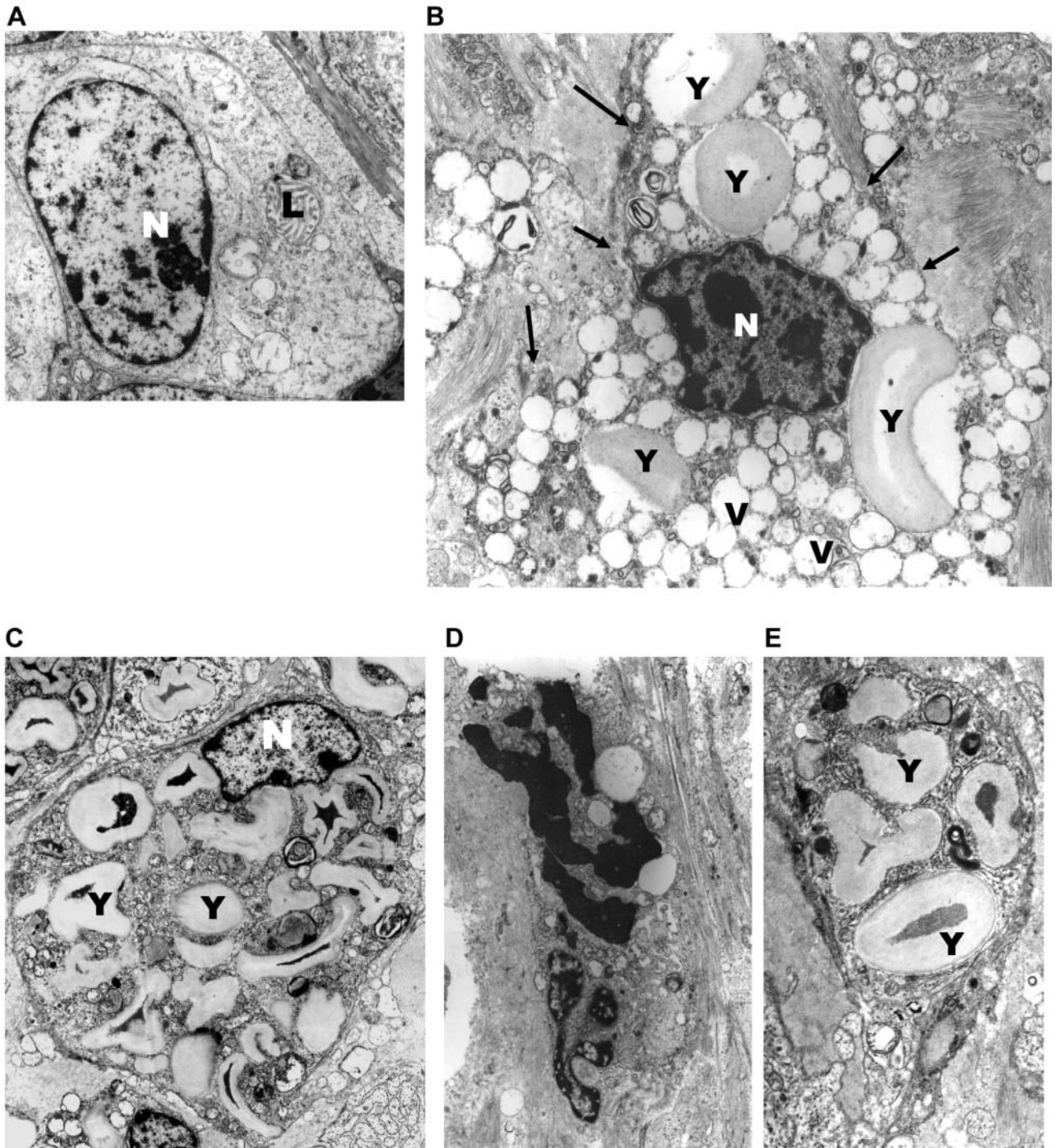


Figure 6. EM examination of apoptotic cells. A) Untreated MCF-7 BC cell. The nucleus (N) of the cell is well defined and occupies most of the cell. The intracytoplasmic lumen (L) is a characteristic feature of breast cancer cells. B) Preparation showing MCF-7 cell phagocytizing four yeasts (Y) that form secondary lysosomes. All the arrows point at the cell membrane. The nucleus (N) demonstrates the earliest changes of apoptosis with segregation and margination of chromatin. The cytoplasm shows dilation of smooth endoplasmic reticulum with features of numerous intracytoplasmic clear vacuoles (V). MCF-7 cells displaying signs of apoptosis with: C) nuclear margination and condensation, D) nuclear fragmentation, and E) the loss of nuclear membrane and dispersal of nuclear fragments. (x3,500 TEM).

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