Baker’s yeast induces apoptotic effects and histopathological changes on skin tumors in mice

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Abstract: The current study investigates the apoptotic effect of Baker's yeast (Saccharomyces cerevisiae) on chemically-induced skin cancer in mice. Intratumoral treatment with yeast caused: increases in Ca2+ in skin homogenate, as well as modulation of the intrinsic/extrinsic pathways by downregulating Bcl-2 and FasL, upregulating Bax, and increasing the expression of cytochrome-c and caspases 9, 8, and 3. Histopathological changes were detected, including mild dysplasia, atypia, tumor regression, and absence of basaloid cell proliferation. No toxic effects were detected, as examined by histopathological, biochemical, and body weight analysis. These results show that yeast exerts anti-skin cancer activity, suggesting its possible use for treatment of human skin cancer.

Keywords: skin cancer; baker’s yeast; apoptosis

1. Introduction
Skin cancer is the most common of all cancer types. In the United States, more than 87,000 cases of melanoma (the most serious form of skin cancer) are expected to be diagnosed in 2017 (Retrieved March 12, 2017 from https://www.cancer.org/research/cancer-facts-statistics/all-cancer-facts-figures/cancer-facts-figures-2017.html). In Australia, skin cancer is especially prevalent: by the age of 70, two in three Australians will have been diagnosed with skin cancer, and more than 434,000

ABOUT THE AUTHOR
Mamdooh Ghoneum is an internationally recognized immunologist, who is an expert in the area of cancer immune therapy. He has published over 100 articles and 130 abstracts, as well as 11 books in Japanese and in English related to cancer. He holds 2 patents for 2 biological response modifiers (BRMs) for the treatment of cancer. Mamdooh Ghoneum's contribution to immunotherapy has been to introduce several new non-toxic BRMs that activate different arms of the immune system and induce the death of cancer cells. Another approach he discovered is the use of heat-killed Baker’s yeast as a novel therapy for the treatment of cancer. Most recently, Mamdooh Ghoneum showed that Baker’s yeast is an effective chemosensitizer and can enhance the killing capacity of the chemotherapeutic agent paclitaxel against breast cancer cells. Baker’s yeast may represent a novel, non-toxic adjuvant for chemotherapy treatment.

PUBLIC INTEREST STATEMENT
Baker’s yeast is an essential component for the production of fermented foods like bread and beer. Our earlier studies showed that heat-killed baker’s yeast exerts anticancer activity against different types of cancer. This is based on the observation that cancer cells can engulf yeast, which ultimately shuts down and kills the cancer cell from within. This study aims to assess baker’s yeast as a novel natural product that can cause the death of skin cancer cells in animals. Several parameters were examined and show skin tumors stopped growing and regressed in mice treated with yeast compared to those untreated. Though several treatments for skin cancer exist, these are known to have severe side effects. We show the anticancer effects of yeast as a safe, non-toxic agent, which may suggest its possible use as treatment against skin cancer in humans.
Australians are treated for one or more non-melanoma skin cancers each year (Retrieved April 30, 2016 from http://www.cancer.org.au/about-cancer/types-of-cancer/skin-cancer.html). Incidences of skin cancer are expected to increase worldwide, mainly due to excessive sunlight exposure, which contains harmful ultraviolet (UV) radiation, as well as changes in dietary habits and lifestyle. Treatment of skin cancer can involve surgery, chemotherapy, and radiation therapy, and combination therapy is always preferred over a single treatment modality.

Though several treatments have been discovered for skin cancer, including chemotherapy (Gartel, 2012; Sosman & Puzanov, 2006) and interferon alpha (IFN-α) and its pegylated forms, these are known to have severe side effects (Mocellin, Lens, Pasquali, Pilati, & Chiarion Sileni, 2013). While new cancer immunotherapy (immune checkpoint inhibitors) represents a possible breakthrough for treatment (Bourke, O’Sullivan, & Khattak, 2016; D Aniello et al., 2017; La-Beck, Jean, Huynh, Alzghari, & Lowe, 2015), most cancer patients, including melanoma patients, developed immune-related adverse events (Bertrand, Kostine, Barnetche, Truchetet, & Schaeeverbeke, 2015; Horvat et al., 2015). Unfortunately, current treatments are not enough to effectively and safely combat the disease. Therefore, the need for new agents that exert anti-skin cancer activity with minimal side effects has become an emerging area of research.

Another method that has been used to treat skin cancer is the injection of microbes, including Corynebacterium parvum and Bacillus calmette Guérin (BCG) (Fahey, Brosman, Ossorio, O’Toole, & Zighelboim, 1976; Lipton et al., 1983, 1991). However, the clinical use of these BRMs has been severely limited because of their in vivo cytotoxicity (Fritze et al., 1984; Hilal et al., 1981; Hirshaut, Pinsky, Wanebo, & Braun, 1984; Vázquez-Lavista, Flores-Balcázar, & Llorente, 2007). In the current study, we examined the effect of a safe microbe that is a known food supplement, baker’s yeast, Saccharomyces cerevisiae (S. cerevisiae), for possible use as an anticancer agent against skin cancer in mice. S. cerevisiae is an essential component for the production of fermented foods like bread and beer. Our earlier studies showed that heat-killed baker’s yeast exerts anticancer activity against human breast cancer cells. This is based on the observation that cancer cells phagocytize yeast and that yeast then subsequently triggers apoptosis in cancer cells in vitro and in vivo (Ghoneum & Gollapudi, 2004; Ghoneum, Wang, Agrawal, & Gollapudi, 2007). This study aims to assess baker’s yeast as a novel natural product that can induce an apoptotic effect, associated with histopathological changes, on skin cancer in animal bearing tumor. Results show anti-cancer effects of yeast treatment as a safe, non-toxic agent and may suggest its possible use as treatment against skin cancer in humans.

2. Materials and methods

2.1. Animals
One hundred male Swiss albino mice aged 6–7 weeks (20 ± 2 g body weight) were used in this study. The animals were obtained from the animal house at National Research Centre, Giza, Egypt, and acclimatized for one week prior to the commencement of the study. Mice were housed in plastic cages and allowed to acclimate in standard conditions (under 12 hr light/dark cycle). The animals were given free access to distilled water and commercialized food throughout the experiment. The environmental conditions were standardized with respect to temperature, humidity, and light. This study was approved by the Animal Ethical Committee at National Research Centre, Giza, Egypt.

2.2. Preparation of S. cerevisiae
Baker’s and brewer’s yeast, S. cerevisiae, was purchased from ACH Food Companies, Inc., Memphis, TN, USA. The yeast was used in suspensions that were washed once with Hanks balanced salt solution (HBSS). The suspensions were heat-killed via incubation for 1 hr at 90°C and subsequently washed twice with HBSS. Quantification was carried out using a hemocytometer. Yeast cell suspensions were adjusted to $1 \times 10^7$, $10^8$, and $10^9$ cells/ml and mice received IT injection of 100 μl yeast.
2.3. Tumor induction
Mice were shaved on the dorsal skin using an electrical clipper and painted once with 7,12-dimethylbenz[a]anthracene (DMBA) on one side (120.0 nmol DMBA in 0.2 ml acetone). One week later, mice were painted with 12-O-tetradecanoylphorbol 13-acetate (TPA) twice weekly for 14 weeks (4.0 nmol TPA in 0.2 ml acetone) (Abel, Angel, Kiguchi, & DiGiovanni, 2009). DMBA and TPA were purchased from Abcam. Inc., Cambridge, MA, USA.

When the tumor volume reached ~100 mm³, the mice bearing tumor alone without yeast treatment were sacrificed, along with the normal control mice without tumor. The rest of the mice bearing tumor were injected intra-tumorally (IT) for 16 weeks with 100 μl yeast (2 times/week) at three concentrations: 10⁷, 10⁸, and 10⁹ cells/ml of yeast respectively, and then sacrificed. Each skin tumor was injected with 100 μl of yeast. Thus, an animal bearing 2 skin tumors received 200 μl of yeast treatment, 100 μl into each tumor site.

2.4. Experimental design
This study comprised 100 mice, divided into 5 groups (20 mice/group) as follows: Group 1 served as normal control mice without tumors, Group 2 were mice bearing tumors, and Groups 3–5 were mice bearing tumors receiving yeast through IT injection 100 μl (2 times/week) at concentrations of 10⁷, 10⁸, and 10⁹ cells/ml, respectively. The mice in Group 2 were sacrificed when the tumor volume reached ~100 mm³; roughly two weeks following tumor induction. From our previous studies, this size of tumor has been shown to be an optimal tumor volume suitable for treatment with yeast. Delaying yeast treatment results in devastating rapid growth of the tumor and subsequently the animal prompting mice to be sacrificed; The rest of the mice bearing tumors in Groups 3–5 were sacrificed at 16 weeks post treatment with yeast. The tumor volume was measured horizontally and vertically by using digital caliper. Volume of the tumor \( V \) was determined by Carlsson's formula \( V = \frac{ab²}{2} \) where \( a \) and \( b \) are the longest and shortest diameters of the tumor, respectively. For these 5 groups, the following investigations of biochemical analysis for the apoptotic pathway were carried out: calcium ions, FasL, Bcl2, Bax, cytochrome-c, and quantification of caspases 8, 9, and 3. In addition, toxicity studies involving histopathological, biochemical analysis, and changes in body weight were completed.

2.5. Preparation of tumor/skin homogenates
Tumor homogenates were prepared as follows: mice bearing tumor, with and without yeast treatment, were sacrificed, samples of tumor tissue were cut, and weight of the tissues were recorded. Tumor tissues were then rinsed with ice-cold PBS (0.01 M, pH = 7.4), weighed, minced, and then homogenized in PBS with a glass homogenizer on ice (9 ml PBS per gram tissue). The homogenates were centrifuged at 3,000 rpm for 10 min and supernatant was removed and frozen at −70°C. Similarly, normal control mice without tumor were shaved and samples of skin (~100 mm³) were treated as above.

2.6. Biochemical analysis for the apoptotic pathway
The homogenates of tumor tissues under different treatment conditions and skin homogenates of normal control mice were analyzed for the levels of different parameters of apoptotic pathway: calcium ions, cytochrome-c, FasL, Bcl2, Bax, and caspases 8, 9, and 3, using enzyme-linked immunosorbent assay (ELISA) (Sun Red Biotechnology Company, Shanghai).

2.6.1. Calcium ion concentration
Calcium ion levels were analyzed with a Calcium Ionized Detection ELISA Kit (Kono Biotech Co., Ltd.) according to the manufacturer's instructions. Monoclonal antibodies were incubated with skin homogenate, followed by the addition of Calcium binding protein antibodies labeled with biotin and combined with Streptavidin-HRP to form immune complex. Incubation was then carried out and antibodies were washed again to remove the uncombined enzyme before Chromogen Solution A and B were added. The chroma of color and the concen of Humanon of the different homogenate of Calcium Binding Protein B of sample were positively correlated. Calcium concentration in the supernatant materials was estimated according to the ELISA Kit datasheet.
2.6.2. Cytochrome-c, FasL, Bcl2, Bax, and caspases
Homogenates of tumor and healthy skin tissues from mice were analyzed using ELISA. The levels of cytochrome-c, FasL, Bcl2, Bax, and caspases 3, 8, and 9 activities were measured according to the ELISA kit instructions (Sun Red Biotechnology Company, Shanghai, People's Republic of China).

2.7. Histopathological analysis of skin cancer
At the end of the experiment, animals were sacrificed. The skin samples from negative control and animal bearing tumors, with and without yeast treatment, were excised and fixed in 10% buffered formalin, processed through ascending grades of alcohol, cleared in xylene and prepared into paraffin blocks. Serial sections 4 microns thick were prepared and stained with hematoxylin and eosin (H&E) for routine histopathological study. The sections were examined for histopathological changes using an Olympus CX41 microscope. Slide tissue microphotography was done using CCD digital camera Olympus DP-12 attached to the Olympus CX41 research microscope. Digital photomicrographic sections were taken at various magnifications.

2.8. Toxicity studies

2.8.1. Histopathological analysis
Normal control mice and mice bearing tumor were treated IT with yeast (100 μl) at different concentrations (10^7, 10^8, 10^9 cells/ml) for 16 weeks. Mice were then killed and different organs (liver, kidney, and testis) were excised, and sections were prepared and stained with H&E for histopathological analysis.

2.8.2. Biochemical analysis
Biochemical analysis in healthy control mice with and without yeast treatment was carried out. Biochemical analysis of liver function was analyzed by serum aspartate aminotransferase (AST), serum alanine aminotransferase (ALT), and alkaline phosphatase (ALP), while kidney function was measured by creatinine levels. Other parameters, including blood urea and lipid peroxidase were examined. All of the above mentioned assays were conducted using colorimetric determination by the Biodiagnostic Kit, Egypt. We used previously described methods to determine AST and ALT levels (Wang, Auyeung, & Zhang, 2014), Lipid Peroxide quantification (Satoh, 1978), creatinine levels in serum (Bartles, Bohmer, & Heirli, 1972), and Alkaline Phosphatase levels (Belfield & Goldberg, 1971). The blood urea was determined according to the Urease-Berthelot method (Fawcett & Scott, 1960).

2.8.3. Statistical analysis
All statistics were performed using SPSS 14 for Windows package (SPSS Inc., Chicago, IL, USA). The differences between means were performed using student “t” test and the comparison of the study groups was done by using analysis of variance (ANOVA), followed by Dunnett post hoc comparisons. This was done to compare changes between groups of the study, and the data obtained in the present work were represented in tables as average (Mean) ± Standard Error. In all tests p value was considered significant when less than or equal to 0.05 and highly significant when less than or equal to 0.01.

3. Results

3.1. Biochemical analysis for the apoptotic pathway

3.1.1. Calcium ions
Data in Figure 1 shows a significant decrease in the concentration of ionized calcium (Ca^{2+}) in the skin homogenate of tumor-bearing mice as compared to normal control mice (p ≤ 0.05). However, treatment with yeast resulted in an increase in the concentration of ionized calcium in the skin homogenate of mice that received 100 μl yeast at concentrations 10^7 (p ≤ 0.01), 10^8 (p ≤ 0.001), and 10^9 (p ≤ 0.001) in comparison to tumor-bearing mice.
3.1.2. Analysis of apoptotic regulators

The expression of pro-apoptotic proteins FasL and Bax and anti-apoptotic protein Bcl-2 were examined in mice under different treatment conditions: normal control skin, tumor skin alone, and yeast-treated (100 μl) tumor skin at concentrations 10^7, 10^8, and 10^9 cells/ml.

3.1.2.1. FasL: Figure 2 shows a significant increase in the level of FasL in tumor homogenate in mice bearing tumor alone as compared with normal control skin homogenate (p ≤ 0.001). In contrast, the expression of FasL significantly decreased post treatment with yeast in a dose-dependent manner: 10^7 (p ≤ 0.05), 10^8 (p ≤ 0.01), and 10^9 (p ≤ 0.01) in comparison to tumor-bearing mice alone.

3.1.2.2. Bcl2: The expression of Bcl2 was significantly increased in tumor homogenate in comparison with normal control tissue (p ≤ 0.001). However, treatment with yeast caused a decrease in Bcl2 expression in a dose-dependent fashion when compared with animal bearing skin cancer alone. A decrease in Bcl2 expression was detected post treatment with 100 μl yeast concentration of 10^7 (p ≤ 0.01), and the effect became highly significant at concentrations of 10^8 and 10^9 cells/ml in comparison to tumor skin homogenate (p ≤ 0.001) (Figure 2).

3.1.2.3. Bax: Data in Figure 2 shows that the Bax level was significantly decreased in tumor homogenate in animal bearing tumor in comparison with normal control tissue (p ≤ 0.001), while treatment with yeast caused an increase in Bax level in a dose-dependent fashion when compared with animal bearing skin cancer.

3.1.3. Cytochrome-c

Results depicted in Figure 3 show a slight difference in the levels of cytochrome-c (pg/ml) between homogenates of normal control skin and tumor (p ≤ 0.05). Treatment with 100 μl yeast at low concentration (10^7 cells/ml) resulted in a slight increase in the level of cytochrome-c. However, higher concentrations of 10^8 and 10^9 cells/ml resulted in a dramatic increase in cytochrome-c by over 15-fold (p ≤ 0.001).

3.1.4. Quantification of caspases 9, 8, and 3

The level of caspases 9, 8, and 3 were examined in mice bearing tumor and after treatment with yeast. Mice bearing tumor demonstrated a decrease in the level of caspases 9, 8, and 3 as compared with normal control group. On the other hand, treatment with yeast resulted in an increase in the expression of caspases 9, 8, and 3 in a dose-dependent fashion (Figure 4).
3.2. Histopathological analysis

Histopathological changes were examined in tumor-bearing mice with and without yeast treatment.

3.2.1. Skin of normal healthy mice and animal bearing tumor
Figure 5(A) shows skin from normal healthy mice while Figure 5(B)–(F) shows skin from tumor-bearing mice. In Figure 5(A), healthy mouse skin shows ordinary benign stratified squamous epithelium with the underlying dermal connective tissues showing adnexal structures. The skin of tumor-bearing mice in Figure 5(B) shows marked epidermal-epithelial proliferation and hyperplasia. Moderate dysplasia with dense chronic inflammatory infiltrate can be seen underneath the covering of proliferating epidermis at the epidermal–dermal junction. Figure 5(C) shows epidermal papilla hyper-keratosis, elongated rete ridges, bulbous ends, and prominent dysplasia. In addition, the focus of a crater-like ulcer...
was seen filled with keratin with an adjacent overhanging epidermal edge (Figure 5(D)). This appearance was consistent with early malignant changes in the form of low grade, well-differentiated squamous cell carcinoma. Surface capillary projection of tumor tissue can be seen in Figures 5(E) and (F) with mild acanthosis, hyperkeratosis, and focal mild dysplasia within lining epithelium.

3.2.2. Tumor skin of mice treated with yeast

Figure 6 illustrates histopathology of the tumor skin treated with 100 μl of yeast at concentrations of $10^7$, $10^8$, and $10^9$ yeast/ml. Treatment with a concentration of $10^7$ yeast/ml resulted in dense inflammatory cell aggregates infiltrating lobules of mildly proliferating basaloid cells with atypia (A and B). After treatment with $10^8$ yeast/ml, there was a decrease in the thickness of the epidermal cell lining being formed of differentiated keratinocytes, and mild dysplasia was noticed. Mitosis, polymorphism, and atypia were diminished. Tongues of proliferating basaloid cells invading the dermis were minimal, and dermal inflammation was also diminished (C and D). Treatment with yeast at higher concentration of $10^9$ yeast/ml caused tumor regression (E). There was also a decrease in the thickness of the epidermal cell layers. Lining consisted of well-differentiated keratinocytes, and foci of glassy keratinocytes were observed. Diminished keratin was also seen. We noticed the absence of basaloid cell proliferation, tongues, lobules, and cords of basaloid cells invading the dermis in this group. Atypia was markedly regressed with absence of dysplasia, mitosis, and polymorphism.

3.3. Toxicity

Toxicity studies were carried out in order to determine if yeast treatment is safe to use. The following parameters were examined: (A) animal behavior, (B) animal survival, (C) histopathological analysis of internal organs, and (D) biochemical analysis.

3.3.1. Monitoring animal behavior

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 life activity patterns, including feeding/drinking of yeast-treated mice, were recorded for the entire treatment period and showed to be normal as compared with normal control.

3.3.2. Monitoring animal survival

Results of this study showed that all animals receiving yeast treatment survived for the duration of the treatment period of 16 weeks. On the other hand, mice bearing skin tumor without yeast survived for only 2–3 weeks. Figure 7(A)–(D) shows mice under different treatment conditions. (A) shows healthy mice, (B) shows mice bearing tumor post treatment with DMBA and TPA for 14 weeks, (C)
Figure 8(A)–(D) shows a dose-dependent relationship between yeast treatment and tumor growth. Mice treated with a low dose of yeast (100 μl at a concentration of 10^7 yeast) showed multiple nodules (A & B). Mice treated with a higher dose of yeast (100 μl at a concentration of 10^9 yeast) resulted in a significant reduction in tumor nodules (C & D).

3.3.3. Histopathological analysis
At the end of the experiment, normal control mice and tumor-bearing mice treated with yeast (100 μl at a concentration of 10^9 yeast) were sacrificed. Kidney, liver, and testis were dissected and
extracted from sacrificed animals. The H&E sections were examined under light microscope. Figure 9 shows that histology sections from yeast-treated mice had normal appearances comparable to those of healthy control mice without treatment.

3.3.4. Biochemical analysis
Results depicted in Table 1 revealed that mice bearing tumor showed significantly higher levels of AST, ALT, ALP, urea, and creatinine as compared with normal mice. However, treatment with yeast reversed the levels of these parameters to the values of normal mice. Furthermore, data in Table 1 showed significant differences among mouse groups receiving different yeast doses, for example: (1) the levels of AST U/l: Yeast 10^9 vs. Yeast 10^7, p ≤ 0.05, (2) the levels of ALT U/l: Yeast 10^9 vs. Yeast 10^8, p ≤ 0.01, and (3) the levels of ALT U/l: Yeast 10^9 vs. Yeast 10^7, p ≤ 0.01.

Results in Table 2 show that animals bearing tumor had a 15-fold increase in lipid peroxidase levels, however treatment with yeast decreased the levels of lipid peroxidase in a dose-dependent manner and reached the limits of the normal control values at the high dose of yeast (100 μl yeast at a concentration of 10^9 cells/ml).
3.3.5. Body weight
Changes in BW in mice under different treatment conditions were examined. As shown in Figure 10, animals bearing skin tumor demonstrated a significant decrease in body weight as compared to normal control mice ($p \leq 0.001$). On the other hand, IT injection of yeast (100 μl) resulted in a significant gain in BW. The effect of yeast treatment was dose dependent. The effect was less remarkable with lower concentrations (10$^7$ cells/ml) and maximized at a concentration of 10$^9$ cells/ml, bringing the body weight close to the normal control mice.

4. Discussion
The ability of Baker's yeast ($S. cerevisiae$) to trigger cancer cell apoptosis is one of the most remarkable features of antitumor activity shown in our lab. We have previously shown the apoptotic effect of heat-killed Baker's yeast against several human cancer cell lines of the breast, tongue, and colon in vitro (Ghoneum & Gollapudi, 2004, 2005a, 2005b; Ghoneum, Hamilton, Brown, & Gollapudi, 2005), in nude mice bearing human breast cancer, and Swiss albino mice bearing Ehrlich Ascites Carcinoma (EAC) cells (Ghoneum, Badr El-Din, Noaman, & Tolentino, 2008; Ghoneum, Brown, & Gollapudi, 2007; Ghoneum et al., 2007), and without inducing a significant effect in normal cells (Ghoneum & Gollapudi, 2005b). Results of this study show the ability of yeast to induce apoptosis in skin cancer in mice bearing tumors. Yeast-injection IT (100 μl) for 16 weeks at concentrations of 10$^7$, 10$^8$, and 10$^9$ cells/ml caused

Figure 6. (A–E) Histopathological examination of H&E-stained tumor tissue from yeast-treated mice. (A & B) Skin of mice showed dense inflammatory aggregates infiltrating lobules of mildly proliferating basaloid cells with diminished atypia ($\times 200$); (C & D) showing a decrease of epidermal cell lining and mild dysplasia was noticed. Mitosis, polymorphism and atypia were diminished ($\times 200$); (E) Showing marked diminution of the thickness of epidermal layers without atypia ($\times 200$).
apoptosis of cancer cells in a dose-dependent manner via modulation of the level of the Bcl-2 family, downregulating FasL levels, and activation of caspases.

Microbes, including *C. parvum* and Bacillus calmette Guérin (BCG), have been used to treat skin cancer (Fahey et al., 1976; Lipton et al., 1983, 1991). *C. parvum* and BCG act as immune modulators as manifested by activation of murine splenic macrophages via type I interferon production (Neumann, Macher, & Sorg, 1980). It has been suggested that T cells play a key role in the activation of macrophages (Mori, Mihara, Uesugi, Nagai, & Koda, 1994). These microbes also trigger apoptosis in cancer cells via TLRs (Simons, O’Donnell, & Griffith, 2008).

Calcium ions (Ca$^{2+}$) play a vital role in the induction of apoptosis (Green & Reed, 1998; Smaili, Hsu, Youle, & Russell, 2000). The endoplasmic reticulum (ER) is a major storage site for calcium. During phagocytosis, intracellular calcium rises after being released from the ER (Bernardi & Rasola, 2007; Di Virgilio, Meyer, Greenberg, & Silverstein, 1988; Greenberg, El Khoury, Di Virgilio, Kaplan, & Silverstein, 1991; Myers & Swanson, 2002). In the current study we examined the concentration of Ca$^{2+}$ in tumor homogenates with and without yeast treatment. Results demonstrated a significant decrease in the
concentration of Ca$^{2+}$ in animal bearing skin cancer as compared to skin from normal control mice. However, treatment with yeast IT resulted in a significant increase in the concentration of Ca$^{2+}$, relative to animals with tumor alone, in a dose-dependent manner. This data is in accordance with our earlier in vitro studies, which showed that yeast-induced apoptosis against human metastatic breast cancer cells, MDA-MB-231, was Ca$^{2+}$ dependent (Ghoneum, Matsuura, Braga, & Gollapudi, 2008). This was indicated by the elevation of Ca$^{2+}$ post culture of cancer cells with yeast, and by a significant suppression of apoptosis of cancer cells upon addition of 2-aminoethoxydiphenyl borate (2APB), a pharmacological inhibitor of Ca$^{2+}$ release from the ER (Ghoneum et al., 2008). The increase of Ca$^{2+}$ due to IT yeast treatment of mice bearing skin cancer is mainly due to Ca$^{2+}$ released from the ER and Golgi of cancer cells, and not to the presence of intracellular Ca$^{2+}$ from the yeast, since the amount of Ca$^{2+}$ in the cytosol of yeast is 50–200 nM and therefore considered negligible (Aiello, Fu, Miseta, & Bedwell, 2002; Dunn, Gable, & Beeler, 1994; Miseta, Kellermayer, Aiello, Fu, & Bedwell, 1999). In addition, we measured the Ca$^{2+}$ concentration in the yeast solution at concentration of 10$^7$ cells/ml and found it to be negligible compared to the Ca$^{2+}$ concentration in the tumor tissue.

Apoptosis (programmed cell death) is a physiologic form of cell death that plays an important role in normal development, tissue homeostasis, and pathological situations (Arends & Wyllie, 1991; Kerr, Wyllie, & Currie, 1972). There are two major pathways of apoptosis: (1) the mitochondrial (intrinsic) pathway, and (2) the death receptor (extrinsic) pathway (Green & Reed, 1998; Gupta, 2001). A key feature of the intrinsic pathway of apoptosis is the disruption of mitochondrial membrane
potential (MMP) and release of cytochrome-c, which activates the caspase cascade leading to apoptosis. Ca^{2+} triggers apoptotic signals directly via the mitochondrial pathway of apoptosis (Bernardi

### Table 1. Effect of yeast treatment on serum aspartate aminotransferase (AST), serum alanine aminotransferase (ALT), alkaline phosphatase (ALP), blood urea, and creatinine

| Parameter | Normal control mice | Tumor bearing mice |
|-----------|---------------------|--------------------|
|           | Untreated           | Yeast 10^7 | Yeast 10^8 | Yeast 10^9 |
| AST U/l   | 117.4 ± 13.48       | 176.3 ± 4.94*   | 99.6 ± 3.33** | 109 ± 9.11 | 114 ± 9.27** |
| ALT U/l   | 38.6 ± 5.87         | 95.5 ± 5.80*    | 32.8 ± 5.50*   | 54 ± 8.15* | 54 ± 8.09*   |
| ALP U/l   | 66.2 ± 0.22         | 93.7 ± 2.08†    | 45.2 ± 6.64†   | 54.4 ± 6.77** | 57.4 ± 9.54** |
| Urea mg/dl| 17.6 ± 4.82         | 42.5 ± 3.81†    | 14.6 ± 3.09†   | 18.2 ± 3.63* | 20.6 ± 4.26** |
| Creatinine mg/dl | 0.46 ± 0.13 | 1.026 ± 0.03†  | 0.5 ± 0.14**  | 0.52 ± 0.13** | 0.58 ± 0.13** |

Notes: Normal healthy mice were treated with yeast (100 μl) at different concentrations (10⁷, 10⁸, 10⁹ cells/l). One-way ANOVA with Dunnett post hoc tests were used to control for alpha-error inflation for multiple comparisons.

* p ≤ 0.0001—significant to normal control mice.
† p ≤ 0.001—significant to normal control mice.
‡ p ≤ 0.001—significant to untreated tumor bearing mice.
* p ≤ 0.05—significant to normal control mice.
† p ≤ 0.05—significant to untreated tumor bearing mice.
** p ≤ 0.001—significant to untreated tumor bearing mice.
* p ≤ 0.01—significant to yeast-treated tumor bearing mice at doses 10⁷ and 10⁸ cells/l.
† p ≤ 0.05—significant to yeast-treated tumor bearing mice at dose 10⁹ cells/l.

### Table 2. Effect of yeast treatment on lipid peroxide (nmol/ml)

| Groups                  | Mean ± S.E | Compared to normal control mice | Compared to tumor bearing mice |
|-------------------------|------------|----------------------------------|--------------------------------|
| Normal control mice     | 43.59 ± 1.95 |                                  | p ≤ 0.001                     |
| Untreated tumor bearing mice | 656.38 ± 62.99 | p ≤ 0.001                      | p ≤ 0.001                     |
| Yeast 10⁷               | 99.88 ± 3.06 | p ≤ 0.001                       | p ≤ 0.001                     |
| Yeast 10⁸               | 63.12 ± 3.97 | p ≤ 0.01                        | p ≤ 0.001                     |
| Yeast 10⁹               | 47.4 ± 3.09  | p ≤ 0.05                        | p ≤ 0.001                     |

Notes: One-way ANOVA with Dunnett post hoc tests were used to analyse data. Significance was defined by a p-value ≤ 0.05.
and Rasola, 2007; Green and Reed, 1998; Smaili et al., 2000). Ca\(^{2+}\) appears to facilitate disruption of MMP via activation of permeability transition pores (PTP) which ultimately results in cell apoptosis (Bernardi, 1999; Kroemer & Reed, 2000). Our earlier study showed that phagocytosis of yeast by human breast MCF-7 cancer cells led to disruption of MMP (Ghoneum and Gollapudi, 2004). In this study, the increase of Ca\(^{2+}\) post treatment with yeast was associated with an increase in the levels of both cytochrome-c and the expression of caspases 9, 8, and 3 in a dose-dependent manner, relative to mice bearing tumor alone.

Anti-apoptotic and pro-apoptotic effectors can modulate the mitochondrial pathway of apoptosis, and it is widely accepted that the predominance of the pro-apoptotic protein (Bax) over the anti-apoptotic protein (Bcl-2) promotes apoptosis (Salomons et al., 1997; Wolter et al., 1997). Results of this study showed significant decrease in the expression of Bcl-2 and an increase in the Bax level. The effect was dose dependent relative to animals with tumor alone. These results coincide with the previous results that showed yeast-induced apoptosis in breast cancer, MDA-MB-231 cells, was associated with an increase in Bax and a substantial decrease in expression of Bcl-2 resulting in alteration in the Bax:Bcl-2 ratio (Ghoneum et al., 2008), suggesting that yeast induces apoptosis of MDA-MB-231 cells in vitro by a mechanism involving intracellular Ca\(^{2+}\) and Bax:Bcl-2. Several studies showed that Bcl-2 and Bax can modulate Ca\(^{2+}\) mobilization from the ER to the cytosol and mitochondria during apoptosis through different mechanisms, including interaction with InsP (Sosman & Puzanov, 2006) receptors (Lao & Chang, 2007; Lewis, Hayashi, Su, & Betenbaugh, 2014).

Fas-ligand (FasL), a member of the tumor necrosis superfamily, plays a central role in the induction of the apoptotic effect of CD8 + T cells and NK cells against cancer cells (Kojima et al., 2002; Mehmut et al., 2005). FasL is strongly expressed in many types of cancer cells, and is considered a mechanism by which cancer cells can escape the attack of the immune system (Erb, Ji, Kump, Mielgo, & Werlini, 2008; Massari, Kastelan, & Gruber, 2007). The engagement of Fas/FasL interaction in the phenomenon of escape strategy has been reported in many types of malignancies, including hepatocellular carcinoma (Fabregat, 2009), endometrial cancer (Chaudhry & Asselin, 2009), leukemic cells (Komada & Sakurai, 1997; Wang, Zhao, Wang, & Zou, 2010), and lung cancer (Huang, Nishimoto, Yang, & Kleinerman, 2014). Similar observations were also described in melanoma and non-melanoma skin cancer (Erb et al., 2008; Massari et al., 2007). Data of this study shows a significant increase in the level of FasL in tumor as compared with normal control skin (P ≤ 0.001). However, mice bearing tumor that were treated with yeast showed significant decrease in the expression of FasL. This was associated with a significant increase in the expression of caspases 8 and 3 in a dose-dependent fashion as compared to mice bearing tumor alone. Caspase 8 is the initiator of the cascade reaction of cell apoptosis. It can transmit apoptotic signals and activate downstream effector caspase to induce cell apoptosis. This suggests that yeast can enhance apoptosis in skin cancer cells by downregulating FasL levels.

Taken together, yeast treatment resulted in a decrease in the expression of both FasL and Bcl2, and an increase in the Bax level relative to animals with tumor alone. In addition, yeast-treated mice showed an increase in the level of cytochrome-c as well as an increase in the expression of caspases 9, 8, and 3. This suggests that yeast treatment induces apoptosis in skin cancer cells by both the mitochondrial pathway and the death receptor pathway.

The yeast-induced apoptotic effect on tumor cells was associated with histopathological changes of the tumors. Yeast-treated mice, in a dose-dependent manner, showed significant histopathological tumor regression changes as compared with mice bearing tumor alone. Yeast-treated mice receiving high doses (10\(^6\) cells/ml) showed a decrease in the thickness of the epidermal cell layers. The lining consisted of well-differentiated keratinocytes and foci of glassy keratinocytes. Diminished keratin and marked regression of atypia with absence of dysplasia, mitosis, and polymorphism was also seen. These histopathological changes reflect the ability of yeast to induce an apoptotic effect on skin tumors.
It is of interest to note that *S. cerevisiae* can act as a chemosensitizer. Our recent study showed that Baker's yeast in the presence of chemotherapy (paclitaxel) *in vitro* increased the sensitivity of metastatic murine 4T1 cells to chemotherapy (Badr El-Din, Mahmoud, Hassan, & Ghoneum, 2017). This exemplifies the additional capability of yeast to act as an anti-cancer agent.

The toxic effect triggered by administration of any foreign substance to animals or humans is a very important consideration. In our earlier studies, we examined the *in vitro* effects of Baker's yeast against non-tumorgenic human fibrocystic mammary tissue cells, MCF-10A, and found that these cells did not exert phagocytic ability, and yeast did not induce an apoptotic effect on that particular cell line (Ghoneum & Gollapudi, 2005a). This suggests that the apoptotic effect of yeast is a selective phenomenon that is directed mainly towards phagocytic cells including cancer cells. Other studies on animals have revealed that animals can tolerate relatively high doses of yeast without any pathology detected up to at least 21 days post treatment, including rats (Fahrig, 1975), mice (Frezza, Zeiger, & Gupta, 1979) and monkeys (Maejima, Shimoda, Morita, Fujiwara, & Kitamura, 1980). The effect of yeast on humans has also been studied. For example, i.v. injections of yeast Glucans have been given to humans to boost the immune system of patients undergoing major surgery (Babineau et al., 1994, 1994) and patients with *Paracoccidioidomycosis* (PCM), an endemic disease in most Latin American countries; and for elimination of *P. brasiliensis* (Wanke & Londero, 1994). Results of these studies revealed no adverse side effects associated with yeast infusion (Babineau et al., 1994; Meira et al., 1996). In the current study, we have examined the potential toxic effect of yeast on mice bearing skin cancer, and the following observations were recorded: (1) animals treated with yeast IT survived the 16-week treatment period with normal feeding/drinking and life activity patterns: while mice untreated with yeast only survived 2-3 weeks, (2) histopathology of different organs derived from yeast-treated mice was similar to normal control mice, (3) biochemical analysis of yeast-treated mice, which included liver function, kidney function, and other parameters, were within the limits of normal healthy mice, and (4) changes in body weight showed that treatment with yeast resulted in countering the significant decrease in body weight due to cancer. Weight loss is common among people with cancer, and it has been also noticed in animal studies that adipose tissue wasting can occur as soon as the tumor is palpable (Costa & Holland, 1962).

In conclusion, Baker's yeast (*S. cerevisiae*) is a safe, non-toxic agent that exerts anti-skin cancer activity in mice via intrinsic and extrinsic apoptotic pathways. This may suggest its possible use for the treatment of skin cancer in humans after investigations by clinical trials.
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