Berberine Inhibits Breast Cancer Stem Cells Development and Decreases Inflammation: Involvement of microRNAs and Interleukin-6

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Abstract

Background: Breast cancer (BC) is often related to depressive symptoms, influenced by epigenetics. Elevated interleukin-6 (IL-6) levels in BC correlate with inflammation, dysbiosis, cancer stem cells (CSC) stemness, and depression. In cancer chemoprevention, bioactive food components, like berberine (BBR), were shown to target CSCs via microRNAs (miRNA) in vitro and in vivo. This study investigates BBR's impact on breast CSC stemness and depression modulation through IL-6 and epigenetics.

Methods: Mammosphere assays and miRNA analysis in murine and human BC cell lines challenged with BBR were used. BBR was orally administered to female Balb/c mice, followed by mammary carcinoma injection. Serum IL-6 and depressive-like behaviors were evaluated, and tumor tissues were collected for ex-vivo assays, miRNA expression, and IL-6 detection.

Results: BBR inhibited CSC mammosphere formation and proliferation, upregulated miRNA-Let-7c and miRNA-34a-5p expression in both cell lines. BBR reduced mammosphere formation in breast tumors and increased miRNA-145-5p and miRNA-34a-5p expression, suggesting tumor-suppressive effects. BBR significantly decreased serum IL-6 levels. In the experimental conditions for behavioral studies, BBR did not have any effect on depressive-like behaviors.

Conclusion: This study highlights BBR’s potential as an "Epi-Natural Compound" for BC prevention and inflammation reduction.

Keywords: Breast Cancer, Berberine, Epigenetics, microRNA, Cancer Stem Cells

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Contents

List of Abbreviations

AMPK	**AMP-activated Protein Kinase**
BBR	**Berberine**
BFGF	**Basic Fibroblast Growth Factor**
BL1 & BL2	**Basal-Like 1 & 2**
BC	**Breast Cancer**
CSC	**Cancer Stem Cell**
DIANA-5	**Diet and Androgens-5 Study**
DMSO	**Dimethyl Sulfoxide**
DOHaD	**Developmental Origins of Health and Disease**
EGFR	**Epidermal Growth Factor Receptor**
EGF	**Epidermal Growth Factor**
ER+	**Estrogen Receptor Positive**
FST	**Forced Swim Test**
HNSCC	**Head and Neck Squamous Cell Carcinoma**
HER2+	**Human Epidermal Growth Factor Receptor Positive**
HPA	**Hypothalamic-Pituitary-Adrenal axis**
IBIS	**International Breast Cancer Intervention Study**
IGF-I	**Insulin-like Growth Factor I**
IL-6	**Interleukin-6**
IM	**Immunomodulatory**
LAR	**Luminal Androgen Receptor**
LDH	**Lactate Dehydrogenase**

LPS	**Lipopolysaccharide**
MDD	**Major Depressive Disorder**
MES	**Mesenchymal**
MiRNAs	**MicroRNAs**
MRI	**Magnetic Resonance Imaging**
NO	**Nitric Oxide**
PARP	**Poly (ADP-ribose) Polymerase**
PET	**Positron Emission Tomography**
PR+	**Progesterone Receptor Positive**
SCFAs	**Short-Chain Fatty Acids**
SHBG	**Sex Hormone-Binding Globulin**
Th17	**T helper 17**
TME	**Tumor Microenvironment**
TNBC	**Triple Negative Breast Cancer**
TST	**Tail Suspension Test**
U.S.	**United States**

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Chapter 1: General Introduction

1.1. Breast Cancer (BC)

With an expected 56,500 occurrences of female breast ductal carcinoma in situ and 310,720 cases of invasive disease in 2024, breast cancer is the most frequent noncutaneous cancer among U.S. women. About 42,250 of U.S. women who receive a BC diagnosis will pass away from the illness. In contrast, lung and bronchus cancer, the top cause of cancer death in women in the U.S., will claim the lives of roughly 59,280 women in 2024 (1).

In Canada, it is estimated that in 2024, 30,500 Canadian women will be diagnosed with BC. This accounts for 25% of all new cancer diagnoses in women in 2024. Moreover, 5,500 Canadian women will die from BC. This accounts for 13% of all cancer deaths in women in 2024. On average, 84 Canadian women will be diagnosed with BC each day. Every day, an average of 15 Canadian women will die from BC (2). In other words, BC is one of the most common types of cancer and is responsible for many cancer-related deaths each year (3). BC ranks second in terms of cancer-related deaths among women worldwide, surpassed only by lung cancer. Thus, it remains the most frequently diagnosed cancer in the world female population, with an estimated 2.1 million new cases recorded in 2018 (4).

1.2. BC Risk Factors

Age is the most important risk factor for most malignancies (5). Other risk factors for BC include the following (5): family health history (6), major inheritance susceptibility (7), germline mutation of the *BRCA1* and *BRCA2* genes and other BC susceptibility genes (8), alcohol intake, breast tissue density (mammographic) (9), endogenous estrogen (10), menstrual history (early menarche/late menopause) (11), nulliparity, older age at first birth,

hormone therapy history, personal history of BC (12), and radiation exposure to the breast/chest (13).

Age-specific risk estimates can be used to help plan screening programs for women with and without a family history of BC. The Gail model and the Tyrer-Cuzick model (also called International Breast Cancer Intervention Study (IBIS) model), version 8, are the most regularly used tools, with the latter including family history and breast density to a greater extent than the former (14).

Of all women with BC, 5% to 10% may have a hereditary mutation of the *BRCA1* and *BRCA2* genes (15). Women of Jewish descent are more likely to carry specific *BRCA1* and *BRCA2* mutations (16). Women with *BRCA1* and *BRCA2* mutations have an estimated lifetime risk of developing BC ranging from 40% to 85%. Carriers with a history of BC are at a higher risk of developing contralateral illness, potentially by up to 5% annually (17). Male carriers of the *BRCA1* and *BRCA2* mutations are likewise more likely to develop BC (18).

1.3. BC Subtypes

BC is a highly diverse neoplasm with several subtypes. These subtypes are commonly grouped into four categories based on the immunohistochemical expression of hormone receptors: estrogen receptor positive (ER+), progesterone receptor positive (PR+), human epidermal growth factor receptor positive (HER2+), and triple negative (TNBC), which is defined by the lack of expression of any of the above receptors (19). The estrogen receptor (ER) is an important diagnostic factor, since roughly 70-75% of invasive breast carcinomas have considerably high ER expression (20,21). The ER regulates PR expression (22); hence, physiological PR levels provide information about the working ER pathway. However, both

ER and PR are abundantly produced in breast cancer cells and are recognized as diagnostic and prognostic indicators (23). Higher PR expression is related to improved overall survival, time to recurrence, and time to treatment failure or advancement, whereas lower levels are associated with a more aggressive disease course as well as poorer recurrence and prognosis (24). Human epidermal growth factor receptor 2 (HER2) expression accounts for roughly 15-25% of breast tumors, and its status is primarily relevant in the selection of appropriate treatment (25,26). HER2 overexpression is one of the first events in breast cancer (25). HER2 enhances the detection rate of metastatic or recurring breast tumors by 50% to 80%. Serum HER2 levels are regarded as a promising real-time marker for the presence or recurrence of cancer. HER2 amplification causes enhanced overactivation of proto-oncogenic signaling pathways, resulting in uncontrolled cancer cell proliferation and worse clinical outcomes for HER2+ cases. HER2 overexpression is also associated with a significantly shorter disease-free duration (27). The Ki67 antigen is a cellular proliferation marker that is useful for determining cell proliferation rates. The proliferative activity determined by Ki67 represents the cancer's aggressiveness, response to treatment, and time to recurrence (28). As a result, Ki-67 is critical in determining the most effective treatment strategy and potential follow-ups for recurrence. It could also be viewed as a prognostic element. High Ki67 expression correlates with decreased survival rates (29,30)

The first major subtype of breast cancer is the luminal A subtype. These tumors are distinguished by the presence of ER and/or PR, the absence of HER2, and a low expression of the cell proliferation marker Ki-67 (less than 20%). Clinically, they are low-grade, slow growing, and have the best prognosis, with a lower risk of relapse and a greater survival rate.

These carcinomas respond well to hormone therapy, but chemotherapy provides less benefit (31).

Luminal B tumors are of higher grade and have a worse prognosis than luminal A cancers. They are ER positive, PR negative, and show a high Ki67 expression (more than 20%). They are often of middle to high histologic grade. These cancers may benefit from hormone therapy in addition to chemotherapy. The increased Ki67 causes them to proliferate quicker than luminal A and has a worse prognosis (32). It accounts for 10–20% of luminal cancers. It expresses estrogen receptors at a moderately modest level while increasing proliferation and cell cycle genes. It represents the group of luminal cancers with the poorest prognosis. They benefit from hormone therapy and chemotherapy in a larger percentage than the prior group (33).

The HER2-positive category accounts for 10-15% of breast tumors and is defined by strong HER2 expression in the absence of ER and PR. They proliferate quicker than luminal cells, and the prognosis has improved since the introduction of HER2-targeted therapy. The HER2-positive subtype is more aggressive and rapidly developing. Within this, there are two subgroups: luminal HER2 and HER2-enriched (34). They have a worse prognosis than luminal tumors and require specific drugs directed against the HER2/neu protein and tyrosine kinase inhibitors, in addition to surgery and treatment with precise chemotherapy (35).

Triple-negative breast cancer lacks the ER, PR, and HER2 receptors. They account for around 20% of all breast cancers. It is most common in women under the age of 40, particularly African American women. The TNBC subtype is further divided into many subgroups, including basal-like (BL1 and BL2), claudin-low, mesenchymal (MES), luminal androgen receptor (LAR), and immunomodulatory (IM), with the first two accounting for 50-

70% and 20-30% of cases, respectively (36). TNBC is distinguished by its aggressiveness, early relapse, and predisposition to appear in advanced stages. It has a rapid rate of proliferation, mutations in DNA repair genes, and enhanced genomic instability (37).

1.4. Gut Microbiome, Primary Immune System, and Chronic Inflammation

The gut microbiome acts as a complex ecology of beneficial microbes, influencing both health and illness (38). Dysbiosis, or an imbalance in the gut microbiota, can have a significant impact on gut function and metabolic consequences, as well as distant organs (39). Disruption of gut microbial balance can cause an increase in gut permeability (40). This syndrome causes the intestinal barrier to weaken, allowing lipopolysaccharides (LPS) from Gram-negative pathogenic bacteria to enter the circulation (40). Once in circulation, these LPS take advantage of the damaged intestinal barrier to migrate to other organs, where they might accumulate and cause tissue damage (39,40). The migration and buildup of LPS can activate an immunological response through TLR4 (41), principally involving macrophages (40). The systemic immunological and inflammatory responses are thus activated (42). Chronic inflammation, and dysbiosis-induced leaky gut, might be detrimental to the host (42). It stimulates and raises the expression of numerous inflammatory proteins involved in insulin signaling pathways, raising the risk of insulin resistance (43). Insulin resistance is a major cause of metabolic dysfunction in cancer patients, which is linked to greater cancer recurrence rates and shorter overall survival (44–46). As insulin is thought to be an oncogene, several studies have shown that higher insulin levels and/or higher levels of insulin-like peptide (a biomarker of insulin secretion) associated with insulin resistance increase the risk of recurrence and mortality in women with early stage BC (45,46). An immunological cross-communication occurs between the gastrointestinal tract and extra-intestinal sites, including

exocrine glands such as the mammary glands, as postulated by the common linked mucosal immune system theory (47,48).

1.5. Gut-Breast Axis

The gut is a complicated neuroendocrine organ. It constantly exchanges information with the brain and other organs via a complex network of neurological, metabolic, and endocrine signals (49). Furthermore, the gut microbiota has a significant impact on metabolic health, specifically glucose metabolism, lipid metabolism, and energy balance (50,51). Imbalances in gut microbiota composition have been associated to metabolic diseases including obesity, insulin resistance, and type 2 diabetes (50,51). These metabolic disruptions can affect breast health in a variety of ways, including changes in hormone levels, inflammation, and cellular metabolism (50,51).

In the BC, the microbiome is important in the regulation of estrogen (52). Gut microbial beta glucuronidases convert conjugated estrogen to deconjugated estrogen, which regulates breast dysbiosis and causes chronic inflammation, altering DNA breaks, proliferation, angiogenesis, metastasis, and invasion (52).

1.6. “Estrobolome” in Gut-Breast Axis: Breast Carcinogenesis

The hormone estrogen is thought to cause approximately 40% of all malignancies in women (53). Endogenous estrogen has a significant role in BC, particularly in the postmenopausal phase, where 70% of all breast tumors are estrogen-positive (52). The gut estrobolome, a group of gut bacterial genes that may metabolize estrogens, has been found to have a considerable impact on endogenous estrogen levels, particularly in cases of estrogen-positive BC (54). The increased fecal exudation of conjugated estrogens during ampicillin

medication provides evidence of the gut microbiota's participation in estrogen metabolism (54,55). The enzymes intestinal β-glucuronidase or β-glucosidase activity improves estrogen deconjugation and reabsorption in the enterohepatic blood circulation, leading to higher serum estrogen levels before re-conjugation in the liver (54,55). Microorganisms with estrogen-metabolizing enzymes rely on diet and bacterial context (55–57). As a result, dietary habits can have a direct impact on estrogen metabolism in the gut and are likely to affect systemic estrogen levels (56). Furthermore, certain probiotics, such as lactobacilli, have been proposed to decrease the activity of the gut microbiota's estrogen metabolizing enzymes, lowering estrogen deconjugation within the gut (54).

1.7. Diet and BC

Chronic inflammatory responses are associated with both insulin resistance and elevated free hormone levels, which eventually contribute to BC. Villarini A. et al. (2012) conducted BC studies in the DIANA-5 trials, examining the efficiency of a Mediterranean diet combined with moderate physical activity in reducing BC occurrences in women with invasive BC who are at high risk of recurrence. This investigation examined serum insulin and sex hormone levels, which had previously been linked to breast prognosis (58,59). BC risk is linked to metabolic syndrome, serum insulin, and low sex hormone-binding globulin (SHBG) levels, which result in elevated serum androgens and estrogen (59). SHBG is a protein produced by the liver that binds firmly to steroids such as testosterone and estradiol and transports them in an inactive form in the circulation, reducing free androgens and free estradiol and maintaining the balance of those sex hormones in the blood (60). Sedentary lifestyle, obesity, a high-calorie diet, as well as high saturated fat, high glycemic index foods, and high protein intake are all key risk factors for metabolic syndrome, insulin resistance,

and elevated androgenic activity (59). Insulin stimulates the synthesis of androgens in the ovary, as well as the expression of growth hormone receptors, and inhibits the liver's production of SHBG, increasing the bioavailability of both sex hormones and IGF-I, all of which cause more inflammation and create a favorable environment for CSC, affecting the tumorigenesis and progression of BC and resulting in BC growth (59).

1.8. Cancer Stem Cells

Cancer stem cells (CSCs) are tissue-specific stem cells that specialize and self-renew to generate cancer cells. Mutations that activate oncogenes or deactivate tumor suppressor genes can cause the development of CSCs. CSCs exist in modest numbers, but they are thought to be responsible for tumor growth and maintenance, and they may increase the probability of relapse in some cancers. CSCs are important targets in integrative oncology because they are resistant to conventional cancer therapies (61). Figure 1 shows the difference between conventional therapies and CSC-targeted therapies in tumor formation. CSCs' tumorigenic and aggressive characteristics make them an ideal target for therapy and prevention. New evidence is emerging on the role of breast CSCs in tumor relapse, metastasis, and chemoresistance (62). CSCs increase chemoresistance by secreting specific cytokines. Cytokines can act directly on tumor cells or indirectly via other cell types to stimulate tumor cell proliferation. This contributes to breast CSCs' unique ability to sustain cell proliferation and self-renewal (63). Furthermore, breast CSCs have the unique potential to form mammospheres, which are huge clusters of cells that differentiate into tumor cells (64). As a result, mammosphere development is regarded as a distinguishing feature in BC (65). Breast CSCs are increasingly recognized as the fundamental source of tumor progression, making them one of the most developing areas of scientific research (65).

Numerous medicines are being explored to target CSCs while avoiding resistance (61,66). These include signaling pathway regulation, epigenetic manipulation, and immunotherapy (61).

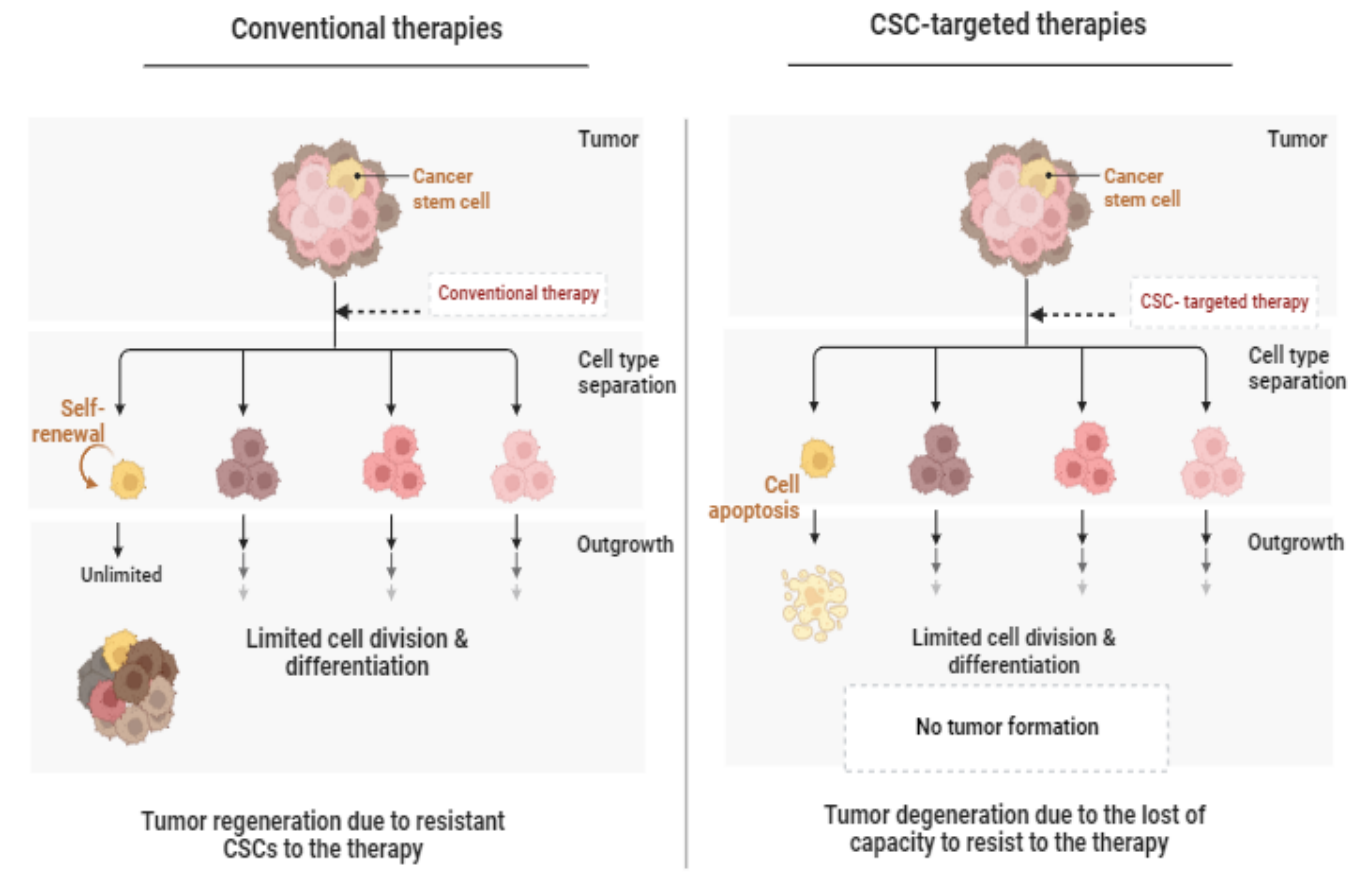

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Figure 1: Conventional therapies vs CSC-targeted therapies in tumor formation (created with BioRender.com)

1.9. The Developmental Origins of Health and Disease

The "Barker hypothesis," also known as the developmental origins of health and disease (DOHaD) hypothesis, emphasizes the role that genetic predispositions and

environmental factors play in determining health outcomes. It focuses on the potential for individualized treatments to support lifelong health and disease prevention (67–69). Studies on epigenetics have shown how behavior and environmental factors affect gene expression. Although epigenetic modifications have the ability to modify gene function, they are reversible (70). Because the epigenome is dynamic, short-term environmental adaptation is possible (71–73). It's interesting to note that instructing stem cells to differentiate into cellular and tissue lineages requires epigenetic changes, including DNA methylation, histone modifications, and non-coding RNAs (74). By removing their capacity to differentiate and retain stem-like characteristics, aberrant epigenetic changes have the ability to transform healthy stem cells into cancer stem cells (66,75).

1.10. microRNAs

A class of small, single-stranded, non-coding RNA molecules with roughly 20–25 nucleotide sequences is known as miRNAs (76). Controlling the expression of genes is the primary role of miRNAs (77). Certain genes can be silenced by miRNAs, and different cancer types frequently change the expression of these genes (77). By attaching themselves to a target miRNA's 3' region and either cleaving it or suppressing its translation into a protein, miRNAs can silence genes (77). This implies that miRNAs are involved in the pathogenesis of cancer (77). miRNAs are major players in the genesis of cancer because they control gene expression, which is critical for cell survival, proliferation, and differentiation (78). Furthermore, rather than their sequence, miRNAs are being recognized as epigenetic modulators due to their function in changing a gene's function (78). While DNA methylation and histone modification are the most prevalent forms of epigenetic alterations, more is being learned about the functions of miRNAs in preserving epigenetic modifications (76).

Additionally, epigenetically changed CSCs are being discovered by researchers, which may explain why they play such important roles in the early stages of cancer (76). Cancer frequently results in the dysregulation of miRNAs, some of which act as tumor suppressors and others as oncogenes (79). Tumor growth is accelerated by oncogenic miRNAs, which are frequently overexpressed in cancer (77). Tumor suppressor miRNAs are frequently downregulated in cancer, which interferes with their ability to support regular biological functions (77). A tumor suppressor miRNA is miRNA-145-5p (80). BC cell proliferation and migration are inhibited because of miR-145's direct or indirect regulation of TGF-β1 expression. In BC, miR-34a suppresses tumors by aiming at genes that support the growth and survival of cells. It stops the expression of oncogenes such as *Bcl2*, *Cdk4*, and *cMyc*, which slows the growth of tumors and arrests the cell cycle (81). Furthermore, miRNA Let-7c is a tumor suppressor miRNA and a potential major depressive disorder diagnostic biomarker (82). Let-7c has been linked to CSCs and could be one of their future therapeutic targets (83).

Gut miRNAs control a wide range of physiological and immunological processes in the intestine and are critical for both innate and adaptive immunity. There are other miRNAs that affect the ratio of Th17 to Treg cells (84). An immunosuppressive environment that fosters tumor development and metastasis is created when these miRNAs are dysregulated in BC, upsetting the delicate balance (85).

1.11. Interleukin-6

Interleukin 6 (IL-6) is a small protein that regulates a variety of homeostatic and pathological processes, including embryonic development, wound healing and aging, inflammation, and immunology (86). IL-6 has been discovered as a cytokine abundantly

expressed in the tumor microenvironment (TME) of different tumor types, including head and neck squamous cell carcinoma (HNSCC) (87,88), pancreatic cancer (89), non-small-cell lung cancer (42), breast cancer (90), ovarian cancer (91), and melanoma (92). In addition to being important during carcinogenesis, IL-6 accelerates the sequence of events required for the creation of a secondary tumor, or metastasis (93). Therapeutic techniques for decreasing IL-6 signaling include targeting intracellular and intercellular signaling pathways. To avoid downstream signaling events, intracellular signaling inhibition may involve inhibiting signal transducer kinase activity (for example, the JAK-STAT pathway). Intercellular signaling techniques aim to disrupt different stages of the IL-6 signaling cascade. This may include limiting cytokine synthesis and release, preventing the shedding of the soluble IL-6 receptor (sIL-6R), neutralizing cytokines or soluble receptors, preventing cytokine binding to receptors, and preventing heterotetrameric complex formation. Furthermore, downstream signaling and gene transcription can be targeted for inhibition to disrupt IL-6-mediated cellular responses. These different treatment approaches present potential opportunities for reducing IL-6-induced inflammation and associated disease processes (93).

IL-6 signaling contributes to the survival and spread of CSC populations in BC (94). IL-6 has also been shown to correlate with treatment resistance in breast cancer patients, highlighting the IL-6/JAK/STAT3 pathway as an essential prognostic marker in breast cancer progression, chemoresistance, and metastasis formation (95). Cytokines, such as IL-6 and TNF-α, regulate estrogen synthesis in peripheral tissues, including normal and malignant breast tissue. IL-6 and TNF-α boost the activity of aromatase, estradiol 17β-hydroxysteroid dehydrogenase, and estrone sulfatase (96).

IL-6 is also essential for epigenetic changes in stem cells (97,98). The IL-6 signaling pathway regulates cancer cell proliferation, CSC renewal, and metastasis. CSCs, in turn, produce IL-6, resulting in a positive feedback loop that keeps CSCs in the tumor microenvironment (99).

Genetic variants in the IL-6 gene have been linked to an increased risk of BC (100). Similarly, IL-6 has been related to the development and progression of BC, with cancer cells activating the IL-6 signaling pathway via proliferation, angiogenesis, invasion, metastasis, and chemoresistance. Drugs designed to suppress IL-6 activity or its receptor have shown promise in the treatment of BC (94). IL-6 has also been studied as a biomarker for BC diagnosis, prognosis, and treatment response (94,101). IL-6 has an important function in modulating inflammation and immunological responses (102), particularly in the gut-brain-breast axis. According to studies, IL-6 may be implicated in the regulation of stress, metabolism, and inflammation, as well as the development of BC (94,102). IL-6 has also been linked to depression in both preclinical and clinical investigations (103). Murine restraint stress is frequently used to study the behavioral and molecular aspects of major depressive disorder (MDD) (103). After one hour of restraint stress, plasma IL-6 concentrations increased before gradually decreasing, demonstrating that restraint stress can raise plasma IL-6 levels (103). A meta-analysis showed that IL-6 is the most elevated cytokine in the serum of the patients with MDD (104). Another meta-analysis found that depressed groups had greater levels of IL-6 than non-depressed groups (105).

1.12. Depression

The most common psychiatric comorbidity among patients with BC is depression, which has a detrimental effect on patients' quality of life, the course of their illness, and their

overall survival (106,107). It is commonly known that depression and mortality are related among patients with cancer, particularly those with BC. Patients with cancer who experience depression worsen their condition and may die from their illness more quickly (108–110). Preclinical research aimed at understanding the neurobiological mechanisms behind depression and testing potential treatment options requires behavioral tests to assess depressive-like behavior in animal models, such as the tail suspension test (TST) and the forced swim test (FST) in female mice (111,112). The Porsolt Forced Swim Test, or FST for short, measures how much time an animal spends swimming and how much time it spends floating in a tall, water-filled cylinder (111,112). The length of time spent floating is indicative of depressive-like behaviors (111,112). The TST is assumed to represent decreased motivation, which is characteristic of human depression (111,112). An animal will stop moving when it feels the fleeting, inevitable discomfort of hanging by its tail (112). The definition of immobility, which is the absence of deliberate movement, includes swaying in a passive manner (111,112).

1.13. Alternative Medicines

As cancer rates rise, a wider range of complementary and alternative medicines are being used as adjunct therapies in primary, secondary, and tertiary prevention settings (113). Many plant-derived compounds have been widely explored as preventative methods or as adjuncts to therapy in BC patients (114,115). This is because of their anticancer effects as antioxidant and anti-inflammatory activity (116).

Precision medicine—a customized treatment strategy based on an individual's specific genetic, genomic, and clinical characteristics—in conjunction with the convergence of prebiotics and probiotics presents a potential route for the advancement of BC therapies.

By understanding how a person's gut microbiome influences breast health, healthcare providers can develop more tailored and effective strategies for preventing, diagnosing, and treating breast-related issues (117). A new frontier in enhancing patient outcomes is the combination of tailored medical methods with nutritional therapies. Still, there are a lot of unanswered questions about the exact mechanics and best combinations for practical use. Thorough efficacy verification is crucial to converting these insights into medical therapies. Beyond typical medical procedures, using the simplicity and accessibility of common food to provide joyful and supportive care is an appealing idea. Integrating dietary methods into BC therapy not only provides a more comprehensive and burden-free approach for patients, but it also highlights the potential synergy between nutritional therapies and medicinal treatments.

1.14. Berberine

Berberine (BBR) is a potent prebiotic for *Akkermansia muciniphila* (118). BBR stimulates *Akkermansia* development by increasing mucin production in the host intestine (118). BBR is a natural isoquinoline alkaloid (119), found in various plants, including European barberry, goldenseal, goldthread, Oregon grape, phellodendron, and tree turmeric. It has a bitter-tasting, yellow molecule and is accessible as a supplement in capsules and other forms. It has been examined for its potential benefits in traditional Chinese medicine for more than three millennia. Nonetheless, it was not until the late twentieth century, particularly in the 1990s, that modern scientific research on BBR began to flourish, drawing attention to its potential therapeutic properties, including but not limited to anti-inflammatory, antioxidant, antimicrobial, and antitumor effects, as well as its potential applications in the treatment of metabolic disorders such as diabetes and obesity (120,121).

Furthermore, BBR functions as an AMP-activated protein kinase (AMPK) activator. Its insulin-independent hypoglycemic impact is due to suppression of mitochondrial function, promotion of glycolysis, and activation of the AMPK pathway. BBR may potentially block the enzyme α-glucosidase (122). It has been shown in newly diagnosed patients with type 2 diabetes to lower blood insulin levels by improving insulin sensitivity (122). It may increase insulin secretion in patients with impaired β-cell activity by rejuvenating tired islets (122). BBR may also have additional benefits for diabetic cardiovascular problems due to its cholesterol-lowering, anti-arrhythmic, and nitric oxide (NO)-producing capabilities (122). The AMPK route may contribute to BBR's ability to limit CSC proliferation, particularly as those cells have been shown to be sensitive to the AMPK pathway (83). The AMPK pathway regulates the cell cycle, proliferation, and survival (83). A prior study demonstrated that BBR might boost the AMPK pathway while inhibiting the mTORC1 pathway, which is involved in cell growth (83). In another investigation, BBR was found to decrease metastasis by regulating the AMPK pathway and inhibiting certain MMPs. BBR regulates the AMPK system and has an anti-inflammatory and antioxidant effect (123).

Numerous studies have been published on BBR's various health benefits, including its potential for the treatment of cancer, cardiovascular disease, and neurological diseases (120,121,124). BBR has the potential to enhance the anticancer properties of EGFR inhibitors in gastric cancer. The researchers discovered that BBR inhibits EGFR signaling and improves the efficiency of epidermal growth factor receptor (EGFR) inhibitors in decreasing gastric cancer cell proliferation (125). Another study on BBR and BC discovered that BBR might cause apoptosis and cell cycle arrest by increasing cytochrome c, caspase-9, and poly ADP ribose polymerase (PARP) cleavage while decreasing levels of *Bcl2* (126).

BBR can affect the gene expression of a variety of proteins involved in apoptosis and the cell cycle, including miRNAs (127). As a result, BBR may have the ability to regulate the expression of specific miRNAs, such as miRNA-145 and miRNA Let-7c (65,83).

1.15. Rationale, Hypotheses, and Objectives

It has already been shown that BBR plays an important role in inflammation and prevention of certain types of cancer, as well as its role in decreasing insulin resistance, but it is yet to be known the effect of BBR in decreasing BC stem cell development and proliferation, the involvement of tumor suppressive miRNAs that prevent BC, the effect of BBR in reducing inflammation in the context of BC, and the effect of this prebiotic on depression. Through my master's, my project was divided into two parts: in vitro and in vivo experiments to reach our specific objectives:

- To study the effect of BBR on CSC proliferation and on modulating miRNAs in vitro and in vivo.
- To analyze the in vivo effect of BBR on preventing mammary carcinoma via the modulation of miRNAs and IL6
- To study the effect of BBR on alleviating depressive-like behaviors in mice with BC.

We hypothesize that BBR will:

- Inhibit CSC proliferation and modulate tumor suppressive miRNAs, thereby preventing BC development.
- Reduce IL-6 levels in both tissue and serum, indicating an anti-inflammatory effect in the context of BC.

- Alleviate depressive-like behaviors in mice with BC, potentially improving overall outcomes and quality of life.

These hypotheses were tested through our comprehensive in vitro and in vivo experimental approaches, providing insights into the multifaceted roles of BBR in cancer biology and mental health.

Chapter 2: Berberine Inhibits Breast Cancer Stem Cells Development and Decreases Inflammation: Involvement of microRNAs and Interleukin-6

Berberine Inhibits Breast Cancer Stem Cells Development and Decreases Inflammation: Involvement of miRNAs and Interleukin-6

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2.1. Abstract

Background: Breast cancer (BC) is a health concern worldwide, often accompanied by depressive symptoms in patients. Epigenetics and cancer stem cells (CSCs) play critical roles in cancer progression. In BC, elevated IL-6 levels contribute to an inflammatory signature, linked to dysbiosis, CSC stemness, and depressive behaviors within the gut. Bioactive food components have preventative effects against BC by targeting CSCs via microRNAs (miRNAs). A prebiotic called berberine (BBR), a natural isoquinoline alkaloid with anti-inflammatory and antitumor characteristics, may inhibit cancer cell proliferation and induce apoptosis in various cancer types. However, the effect of BBR on the stemness of CSC and depressive behaviors by modulating IL-6 through epigenetics deserves further research.

Methods: Mammosphere formation assays were conducted by treating murine 4T1 and human MDA-MB-231 BC cell lines with BBR. Total RNA was isolated from 4T1 CSC exposed to BBR for qPCR analysis of miRNAs Let-7c and miRNA-34a-5p. BBR oral administration to female Balb/c mice was done, followed by injection with mammary carcinoma cells to induce BC. The tail suspension test and forced swim test were conducted to assess depressive-like behaviors. Tumor tissues were collected for ex-vivo mammosphere assays, miRNA expression analysis, and IL-6 detection by ELISA. Serum was also collected for IL-6 analysis.

Results: BBR treatment inhibited mammosphere formation and proliferation of CSC derived from 4T1 and MDA MB-231 cell lines. Additionally, BBR upregulated the expression of miRNAs Let-7c and miRNA-34a-5p in both cell lines, suggesting its involvement in regulating cancer-related gene expression. In vivo, oral administration of BBR led to reduced mammosphere formation in breast tumor tissues, along with elevated expression of miR-145-5p

and miR-34a-5p, indicating its potential tumor-suppressive effects. Furthermore, BBR treatment resulted in a significant decrease in serum IL-6 levels, suggesting anti-inflammatory properties. However, no significant differences were observed in depressive-like behaviors between the control and BBR-treated groups.

Conclusion: The project may shed light on the role of natural compounds such as BBR in preventing BC, highlighting its multifaceted effects on cancer progression and inflammation. Therefore, these substances have the potential to be used as "Epi - Natural Compounds" to prevent cancer.

2.2. Introduction

Breast cancer (BC) is one of the most common types of cancer, accounting for a large number of cancer-related fatalities per year (3). BC is the second-leading cause of cancer-related deaths among women globally, after lung cancer (4). Despite this, BC remains the most often diagnosed malignancy in women, with an expected 2.1 million new cases reported in 2018 (4).

Cancer stem cells (CSCs) are tissue-specific stem cells that differentiate and self-renew, producing cancer cells (61). Mutations that activate oncogenes or inactivate tumor suppressor genes can lead to the formation of CSCs (61). CSCs exist in small populations, but they are assumed to be responsible for the growth and maintenance of a tumor, and they may enhance the likelihood of relapse in some malignancies (61). CSCs are important targets in integrative oncology because they are resistant to standard cancer treatments (61). New research is emerging on the implications of breast CSCs for tumor relapse, metastasis, and chemoresistance (62). CSCs work to promote chemoresistance by secreting certain cytokines (63). Cytokines can function directly on tumor cells, or they can work through other cell types to promote the growth

of tumor cells (63). This adds to breast CSCs' rare ability to maintain cell proliferation and self-renewal abilities (63). Moreover, breast CSCs have the unique ability to create mammospheres, which are large groups of cells that give rise to tumor cells (64). Therefore, mammosphere formation is considered a hallmark of BC (65). Breast CSCs are being increasingly accepted as a primary cause of tumor growth; thus, they are one of the emerging new focuses of scientific research (65). Numerous therapies are being developed to target CSCs and avoid resistance (61,66). These include modulation of signaling pathways, epigenetic manipulations, and immunotherapy (61).

The developmental origins of health and disease (DOHaD) hypothesis, known as the "Barker hypothesis," highlights the involvement of environmental in determining health outcomes, with a focus on the possibility of personalized treatments to promote lifelong health and disease prevention (67–69). Epigenetics studies report how environmental variables and behavior influence gene expression. While epigenetic alterations can alter the function of genes, they are reversible (70). The epigenome is dynamic, allowing for short-term adaptation to the environment (71–73). Interestingly, epigenetic alterations such as DNA methylation, histone modifications, and non-coding RNAs are required for programming stem cells to differentiate into cellular and tissue lineages (74). Aberrant epigenetic alterations have the potential to turn normal stem cells into cancer stem cells by eliminating their ability to differentiate and possess stem-like properties (66,75).

miRNAs are tiny RNA molecules that regulate gene expression, often altered in cancer (76). They silence specific genes by binding to mRNA, affecting protein production. This involvement in gene regulation makes them crucial for cell functions and cancer development (77). Additionally, miRNAs act as epigenetic modulators, altering gene function without

changing the gene sequence (78). Research is also finding CSCs to be epigenetically modified, which may lead to their key roles in the initial progression of cancer (76). miRNAs are often dysregulated in cancer, with some miRNAs functioning as tumor suppressors and others functioning as oncogenes (79). Oncogenic miRNAs are often overexpressed in cancer and act to increase tumor growth (77). Tumor suppressor miRNAs are often downregulated in cancer, and their roles in maintaining normal biological processes are hindered (77). miRNA-145-5p is a tumor suppressor miRNA (80). The expression of TGF-β1 is directly or indirectly regulated by miR-145, resulting in the inhibition of proliferation and migration of BC cells. miR-34a functions as a tumor suppressor in BC by targeting genes that promote cell proliferation and survival. It inhibits the expression of oncogenes such as *cMyc*, *Bcl2*, and *Cdk4*, which slow tumor growth and cause cell cycle arrest (81). Moreover, miRNA Let-7c is a tumor suppressor miRNA, it is also a candidate diagnostic biomarker of major depression (82). Let-7c has been found to be associated with CSCs and may be a potential therapeutic target for CSCs (83).

Interleukin-6 (IL-6) signaling is implicated in the maintenance and expansion of cancer stem cell populations in BC (102). IL-6 has a significant role in regulating inflammation and immune responses (102). IL-6 may be involved in regulating stress, metabolism, and inflammation leading to BC development (100). Similarly, IL-6 has been linked to the development and progression of BC, with the IL-6 signaling pathway activated in cancer cells through cancer cell growth, angiogenesis, invasion, metastasis, and chemoresistance (94). Targeting the IL-6 pathway has shown promise in BC treatment, with drugs developed to inhibit IL-6 activity or its receptor (94). Research has also explored the potential of IL-6 as a biomarker for BC diagnosis, prognosis, and response to therapy (94,101). Also, a meta-analysis showed that depressed groups had more IL-6 levels than non-depressed groups (105).

An increasing variety of complementary and alternative medicines are being used as adjunct therapy and prevention against cancer (113). Specifically, many plant-derived substances have been extensively researched to provide prevention strategies or as an addition to therapy for BC patients (114,115).

Berberine (BBR), naturally found in European barberry and tree turmeric, is an isoquinoline alkaloid, is a yellow-colored, bitter-tasting vitamin (112). It has potential therapeutic benefits—such as anti-inflammatory, antioxidant, antimicrobial, and antitumor effects, and can be applied in the treatment of treatment of cancer, cardiovascular disease, and neurological disorders, and metabolic disorders like diabetes and obesity (120,121,124). BBR is able to induce apoptosis and cell cycle arrest through mechanisms of increasing cytochrome c, caspase-9, and cleavage of PARP while reducing levels of *Bcl2* (126). BBR can alter the gene expression of various proteins involved in apoptosis and the cell cycle, which includes miRNAs (127).

Depressive symptoms are among the reported comorbidities among BC patients and have a negative impact on patients' quality of life, disease progress, and overall survival (106,107). The link between depression and mortality has been well established in cancer patients, including those with BC (108–110). Behavioral tests for evaluating depressive-like behavior in animal models are essential for preclinical research aiming at comprehending the neurobiological mechanisms underlying depression and testing prospective treatment approaches (108,109). Among these tests, two were chosen for behavioral assessments to assess depressive-like behaviors in female mice, the tail suspension test (TST) and the forced swim test (FST) (108,109). The FST, also called the Porsolt Forced Swim Test, compares the amount of time the animal spends swimming with its time floating in a water-filled tall cylinder (108). The TST is

thought to reflect reduced motivation, which is hallmark of depression in humans (109). When an animal experiences the temporary, unavoidable discomfort of hanging by their tail, they will become motionless. Passive swaying is included in the definition of immobility, which is the lack of intentional movement (109).

The goal of this study is to determine the role of BBR in the mechanisms of prevention of BC and mitigating depression. Our specific objectives are to study the effect of BBR on CSC proliferation, modulating miRNAs in vitro and in vivo, analyzing IL-6 level regulation by BBR in tissue and serum of mice fed BBR and challenged with BC cancer cells, and studying the effect of BBR in alleviating depressive-like behaviors in mice with BC.

2.3. Materials and Methods

In Vitro Experiments

BC Cell Culture

Murine 4T1 and human MDA-MB-231 cell lines from the American Type Cell Collection (ATCC; Chicago, IL, USA) were analyzed for this project. Both cell lines were cultured in DMEM medium supplemented with 10% FBS (Sigma-Aldrich, Oakville, ON, Canada) and 1% penicillin/streptomycin. Cells were maintained at 37 ℃ with 5% CO2 in the humidified incubator.

Preparation of BBR Solution for In Vitro Experiments

BBR was purchased from Sigma-Aldrich (BBR chloride hydrate). It was dissolved in dimethyl sulfoxide (DMSO) to create a 5-millimolar stock. The cytotoxicity of BBR was determined by an LDH assay to determine cell viability. Both cell lines were seeded at a density of 5,000 cells per well in a 96-well plate with various concentrations of BBR from 25 μM to 125

uM. The absorbance measurements from the LDH assay were linked with the cytotoxicity levels caused by BBR treatment. Cell cytotoxicity curves were plotted as a function of BBR concentration, and suitable statistical analyses were used to calculate the half-maximal inhibitory concentration (IC50).

Mammosphere Formation in BC Cell Lines

CSCs were extracted from the 4T1 and MDA-MB-231 cell cultures and were put in DMEM F12 medium supplement and supplemented with 500 uL L-glutamine, 500 uL sodium pyruvate, 250 uL hydrocortisone, 50 uL penicillin/streptomycin, 50 uL BFGF, 25 uL insulin, and 25 uL EGF. 5.10^3 concentrations of cells treated with 6 concentrations of 25 μM to 125 uM were added to a low-attachment surface 96-well plate. The cells were monitored, analyzed, and counted at 48 h for mammosphere formation using light microscopy. This experiment was repeated three times independently.

Real-time Quantitative Reverse Transcription PCR for In Vitro Experiments

Total RNA was isolated from the 4T1 and MDA-MB-231 cell lines using TRIzol® reagent after being exposed to 75 uM BBR for 48 h. This was done using the miRNeasy kit (Qiagen, Toronto, ON, Canada). The total RNA was treated with DNase I and reverse transcribed into cDNA. The cDNA was produced by Moloney Murine Leukemia Virus (MMLV) reverse transcriptase (Invitrogen, Burlington, ON, Canada). qPCR was carried out to measure expression of miRNA Let-7c and miRNA-34a-5p using Taqman primers (Applied Biosystems, Burlington, ON) and a FastStart Taq Polymerase (Roche, Mississauga, ON, Canada) in a CFX96 machine (Bio-Rad, Mississauga, ON, Canada). The expression of SNORD-65 was used as an endogenous control.

In Vivo Experiments

BBR Preparation for Oral Administration to the Mice

BBR was dissolved in distilled water. The study involved oral administration of BBR to mice through their drinking water. A daily dosage of 125 mg/kg (130) was calculated based on the average weight of the mice, resulting in each mouse receiving 2.25 mg of BBR daily. Careful handling procedures to maintain freshness and quality were followed. The solution was then stirred with a magnetic stir bar and submerged in a water bath at 37 degrees Celsius to ensure the complete dissolution of BBR. During the feeding regimen, control groups received bottles containing 1% sucrose water, while treatment groups received bottles containing BBR in 1% sucrose water. Water consumption was monitored every day to ensure proper intake of the treatment, and all solutions were refrigerated promptly after use to maintain stability and efficacy.

Study Design for In Vivo Experiment

6–7-week-old female Balb/c mice (Charles River, Montreal, QC, Canada) divided into two groups 12 mice in each were used in the current study (appendix figure 2), following the protocol #HSe-4177. Three mice from the same group were housed together in the same cage with a 12 h light-dark cycle. After acclimation of 1 week, the control group was given a daily dose of 1% sucrose water, and the treatment group was given a daily dose of 125 mg/kg BBR dissolved in 1% sucrose water the whole experimental period till euthanasia. After 2 weeks of dietary intervention, all animals received injections with the 4T1 mammary carcinoma cell line (1400 cells/0.2 mL/mouse) into the mammary fat pad of the Balb-c mice to induce BC for in vivo experiments. Behavioral tests were done on the mice, a TST and a FST separated, to assess depressive-like behaviors. After 3 weeks of 4T1 cell injection, mice were ethically euthanized.

After euthanasia at 12 weeks of age, the tumor was collected and used for several experiments, the tumor was digested, CSC was suspended for ex-vivo mammosphere analysis, proteins were extracted for an ELISA test to assess the IL-6 level, and miRNAs were analyzed by qPCR. Serum was also collected from each mouse for IL-6 analysis by ELISA.

Ex-vivo Mammospheres

During euthanasia, tumor dissection entailed separating tumors into three sections, with the largest part specified for tumor dissociation and subsequent cell culture. 100 mL media was prepared for the cell culture using DMEM F12 knock-out medium (Invitrogen) for stem cells (1000µL sodium pyruvate (sigma) 100 mM, 1000µL of L-glutamine 100 mM, 500µL of Hydrocortisone 100 ug/ml, 100µL of streptococcus penicillin (100 µg /mL – 100ui/mL), 100uL of bFGF (20 µg/ml), 50 µL insulin (human) 10mg/mL, 50µL of EGF 20 ug/mL). Specific enzymes essential for dissociation were used. After the digestion process, the dissociated 4T1 cells from each sample were collected and seeded at a density of 5,000 cells per well in 96-well plates (low attachment). Mammosphere growth and aspect were recorded at several time points (24 h, 48 h, 72 h), including counting and microscope imaging to determine the impact of BBR on mammosphere development.

RNA Extraction and qPCR Analysis from Tumor Tissues

The second part of the tumor was designated for microRNA and PCR analysis. The miRNeasy kit and protocol were also used in this assay, as previously explained. qPCR was carried out to measure the expression of miRNA 145 and miRNA 34a. The expression of SNORD-65 was used as an endogenous control.

IL-6 detection by ELISA Kit in Breast Tumor Tissue and Serum

For protein extraction, a solution of protein inhibitor and lysis RIPA buffer was prepared. Tumor tissue is transferred to the lysis buffer-filled tubes for tissue lysis and protein extraction. Thus, the extracted proteins were isolated and ready for further analysis and experimentation, in IL-6 detection by ELISA kit. Serum and tissue levels of the IL-6 cytokine were measured with the Mouse IL-6 Uncoated ELISA kit from Invitrogen. The experimental procedure begins by coating a Corning™ Costar™ 9018 ELISA plate with capture antibody overnight at 4°C, followed by washing and blocking the wells to minimize non-specific binding. Standard preparation and serial dilutions are carried out to create a standard curve. Subsequently, samples are added to the appropriate wells, followed by incubation. The detection antibody is then introduced, and after another round of washing, avidin-HRP is added. The plate undergoes a final wash before the addition of 1X TMB solution and incubation, followed by a stop solution. The plate is read at 450 nm, with optional wavelength subtraction at 570 nm for data analysis.

Depressive-Like Behaviors

The Forced Swim Test

For six minutes, the mouse is submerged in a plastic cylinder that is 22 centimeters in diameter and 37 centimeters tall. The water temperature was 23 degrees Celsius. A camera records the mouse's movements, which are then examined by the tracking program Ethovision (Noldus). The duration of immobility is the main target to assess, and then the immobility durations of both control and treatment groups were compared.

The Tail Suspension Test

Individual mice were suspended 50 cm above the floor by taping their tails to a horizontal bar for 6 minutes. The mice will actively try to escape, followed by periods of immobility. The total duration of immobility is calculated as the time that the force of the mouse’s movements

was below a threshold criterion; the magnitude of the mouse's movements is recorded in arbitrary units. The amount of time the animal remains immobile or inactive during the test is the outcome measure. In mice, the percentage of time they spend immobile relates to their level of depression-like behavior.

Statistical Analysis

The statistical analysis was conducted using GraphPad Prism 5.0 software (GraphPad Software Inc., San Diego, CA, USA). For the quantification of 4T1 and MDA MB-231 mammospheres at different BBR concentrations, a one-way ANOVA followed by a Tukey multiple comparisons test was employed to determine statistical significance. A Two-way ANOVA was used for the quantification of ex-vivo mammospheres. The analysis of IL-6 serum and tissue concentration, miRNAs, and behavioral tests involved t-tests and one-way ANOVA to compare control and treatment groups. A significance level of $p \leq 0.05$ was chosen, with the data presented as mean ± SEM.

2.4. Results

In Vitro

Cytotoxicity

BBR has negligible influence on cell growth when the concentration is less than 25 µM. However, when the concentration is between 50 and 75 µM, BBR inhibits 4T1 cell growth in a time- and dose-dependent manner, a dose higher than 100 µM was cytotoxic to the cells **(Supplemental 1)**. Therefore, a BBR concentration equal to 75 µM was used in the rest of the in vitro experiments.

CSC Growth and Proliferation

Figure 2 displays the results of 5.10^3 concentrations of CSC extracted from both cell lines treated with 6 concentrations of BBR (0 uM, 25 uM, 50 uM, 75 uM, 100 uM) counted and analyzed after 48 h. Mammospheres are formed significantly in the control group without BBR **(Figure 2A).** The treatment group (with 75 uM BBR) shows significantly smaller groups of cells as well as single cells, but mammospheres are absent **(Figure 2B).** The same results were observed for the second cell line, MDA MB-231 **(Figure 2D, 2E)**. Mammosphere number was significantly decreasing with the increase in BBR concentration in both cell lines **(Figure 2C, 2F).** This marks the highly effective action of BBR in inhibiting mammosphere formation in breast CSCs. Thus, both BC cell lines were able to form mammospheres under non-adhering culture conditions, yet BBR treatment decreased the mammosphere formation and proliferation.

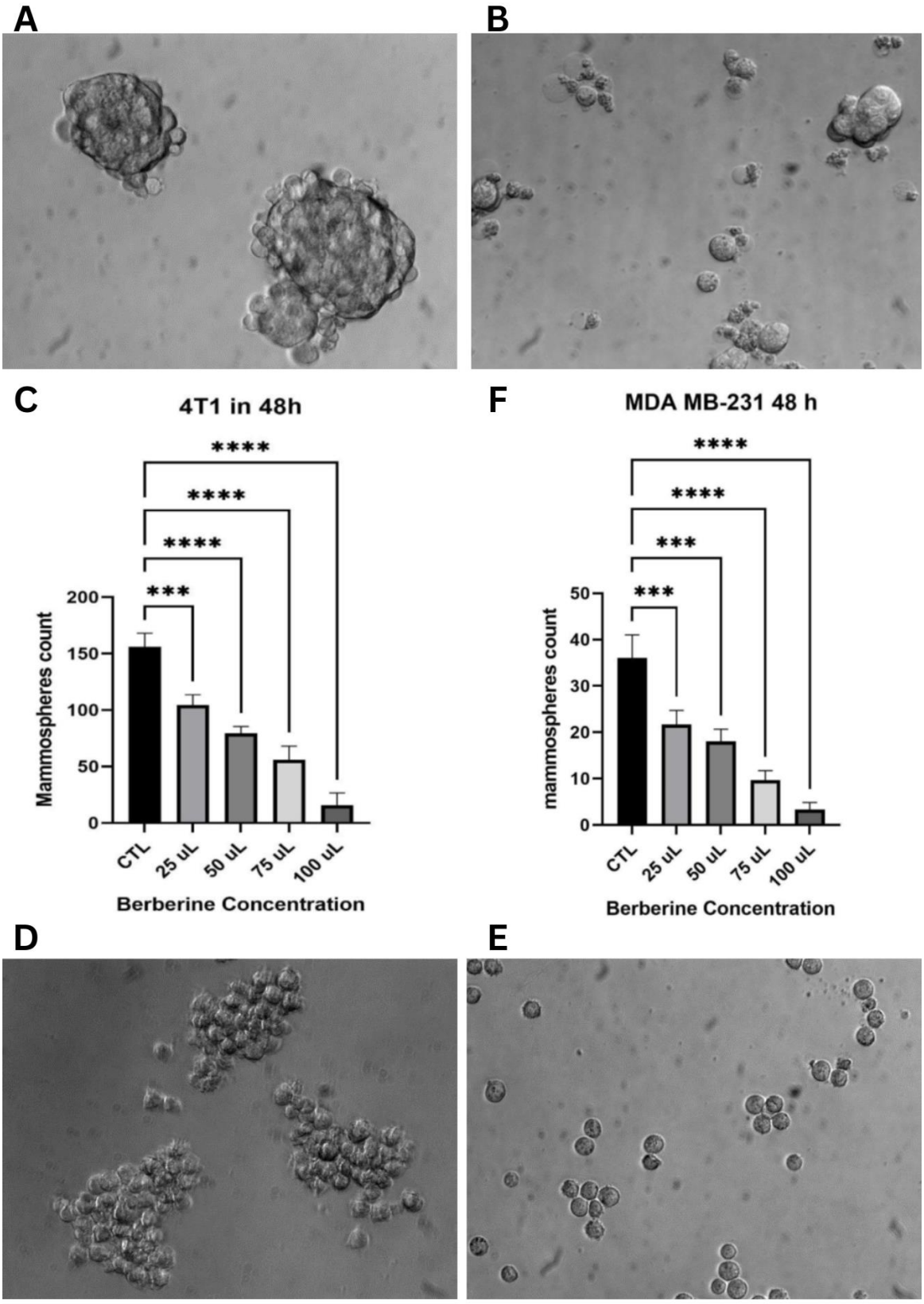
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Figure 2: CSC growth and proliferation from both cell lines. ***(A)*** *Untreated 4T1 CSCs (microscope x10).* ***(B)*** *4T1 CSCs treated with 75 uM BBR (microscope x10).* ***(C)*** *Quantification of 4T1 mammospheres in different BBR concentrations.* ***(D)*** *Untreated MDAMB231 CSC (microscope x10).* ***(E)*** *MDAMB231 CSC treated with 75 uM BBR (microscope x10).* ***(F)*** *Quantification of MDA MB-231 mammospheres in different BBR concentrations. Statistical significance was determined using a one-way ANOVA and Tukey multiple comparisons test. (***=p<0.001) (****=p<0.0001)*

Effect of BBR on miRNA Expression in 4T1 Cell Culture

The miR-34a relative expression significantly increased in the BBR treated 4T1 cells at a concentration of 75 μM compared to the untreated 4T1 cells control group, whereas it showed a non-significant elevation at a concentration of 50 μM compared to the control group **(Figure 3A)**. Similarly, the expression of miR-let-7c significantly increased in the BBR treated 4T1 cells with a concentration of 75 μM, whereas it showed a non-significant elevation at a concentration of 50 μM **(figure 3B).** Thus, miR-34a and miR-let-7c are upregulated by BBR.

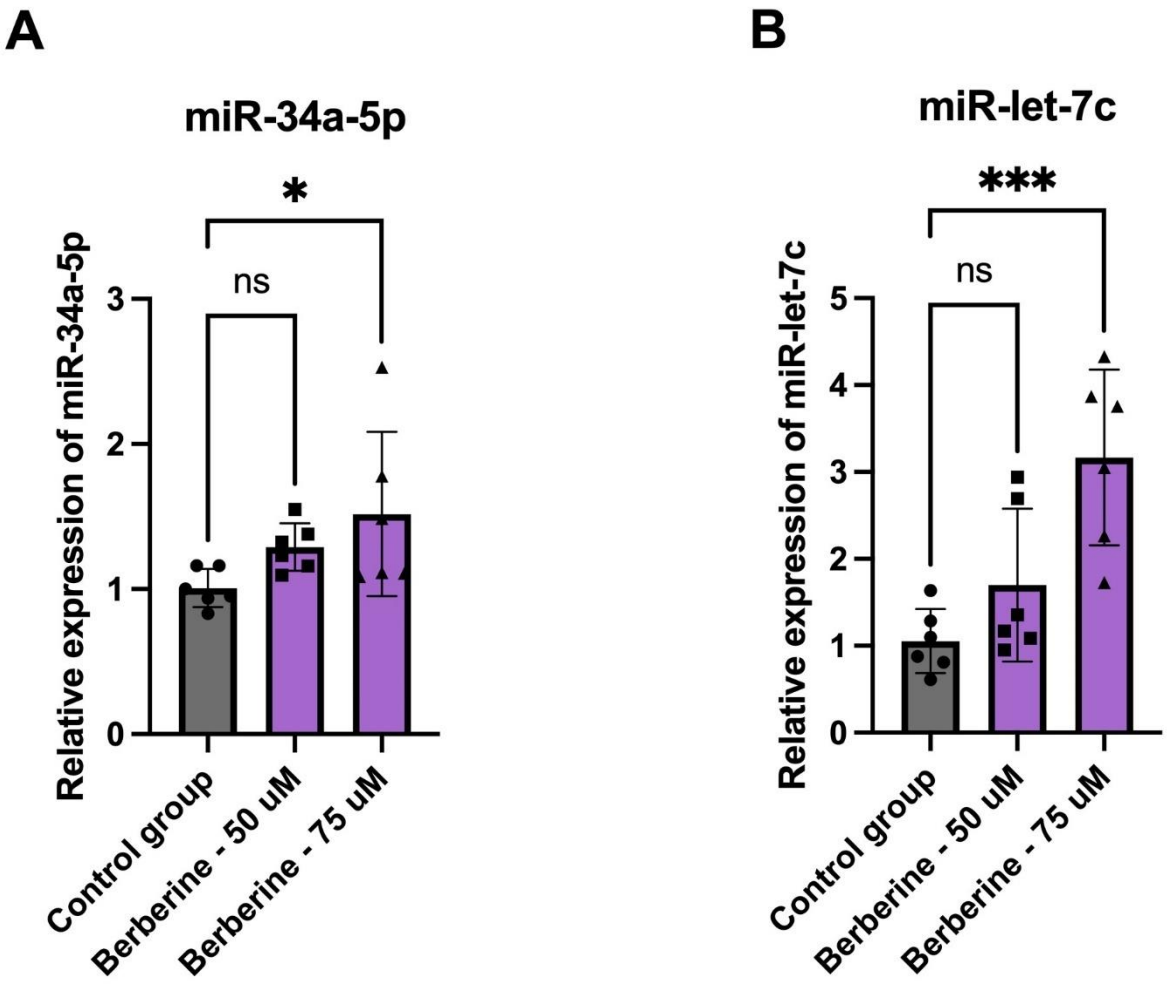

Figure 3: Relative expression of miRs in 4T1 cell culture in vitro. ***(A)*** *The miR-34a-5p relative expression.* ***(B)*** *The miR-let-7c relative expression. Presented as mean ± SEM and statistically evaluated with a simple t-test (*P < 0.05, ***P < 0.001).*

In vivo

At week 12, 10 mice developed mammary tumors in the control group, and 12 mice developed mammary tumors in the treatment group, mice were then sacrificed. Mice's average weights in the control and treatment group were 19.79 g and 20.1 g respectively. The average tumor weight and dimensions for the control groups were 140.9 mg and 0.249 cm^3, respectively, compared to the treatment group with 184,16 mg and 0.312 cm^3 respectively.

Ex-vivo mammospheres: CSC Growth and Proliferation

For this experiment, CSCs were extracted from breast tumors without any further treatment. **Figure 4A** displays the results of 5.10^3 concentrations of cells from the control group counted after 72 h. **Figure 4B** displays the results of 5.10^3 concentrations of cells from the treatment group counted after 72 h. Comparing both mammosphere formations, the control mammospheres are more clustered and larger in number. The treated group mammospheres are smaller clusters and more disintegrated, as well as single cells. There is a significant decrease in ex-vivo mammospheres in the treated group compared with the control group after 24 h and 72 h, while there is no significant decrease after 48h **(Figure 4C)**. The increase in the number of mammospheres in the treatment group is slower than in the control group. The decrease in mammosphere numbers in both groups after 48 h is explained by the attachment of the small clusters to form bigger ones. Thus, BBR inhibits mammosphere formation and proliferation in breast CSCs.

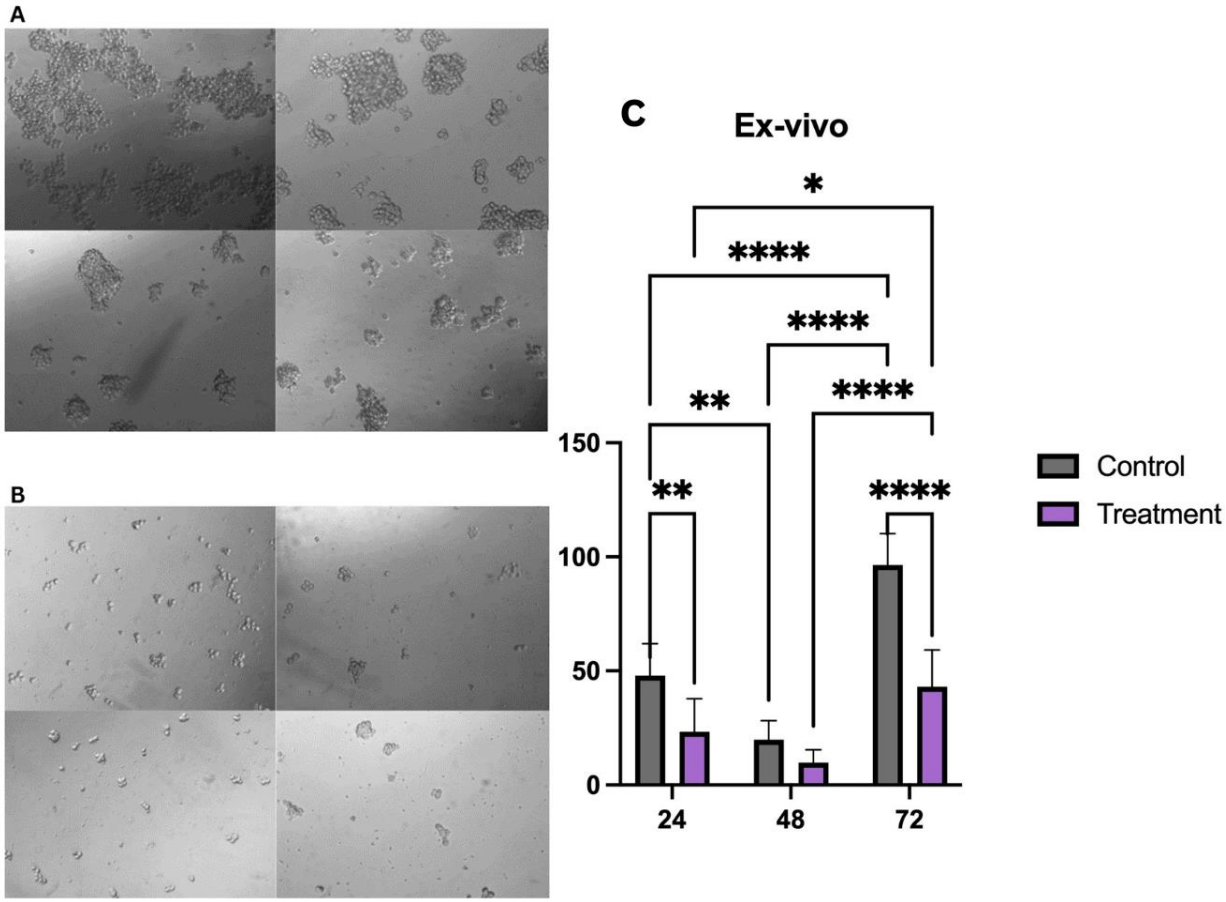

Figure 4: Ex-vivo mammospheres of the breast tumors both groups. ***(A)*** *Mammospheres microscope imaging (X10) of untreated mice drinking 1% sucrose water injected with the 4T1 cell line.* ***(B)*** *Mammospheres of BBR-treated mice drinking BBR solution injected with the 4T1 cell line.* ***(C)*** *Ex-vivo mammospheres count of the breast tumor in the control and treatment groups; presented as mean ± SEM Statistical significance was determined using a two-way ANOVA and Tukey multiple comparisons test. (*P <0.05, **P <0.01, ***P <0.001, ****P < 0.0001).*

Effect of BBR on miRNA Expression in the tumor

Data show a significant elevation in the expression of miR-145-5p in the BBR-treated group compared with the untreated control group **(Figure 5A)**. Similarly, data shows a significant elevation in the expression of miR-34a-5p in the BBR-treated group compared with the untreated control group **(Figure 5B)**. This suggests that BBR significantly upregulates the expression of miR-145-5p and miR-34a-5p in BC tumors.

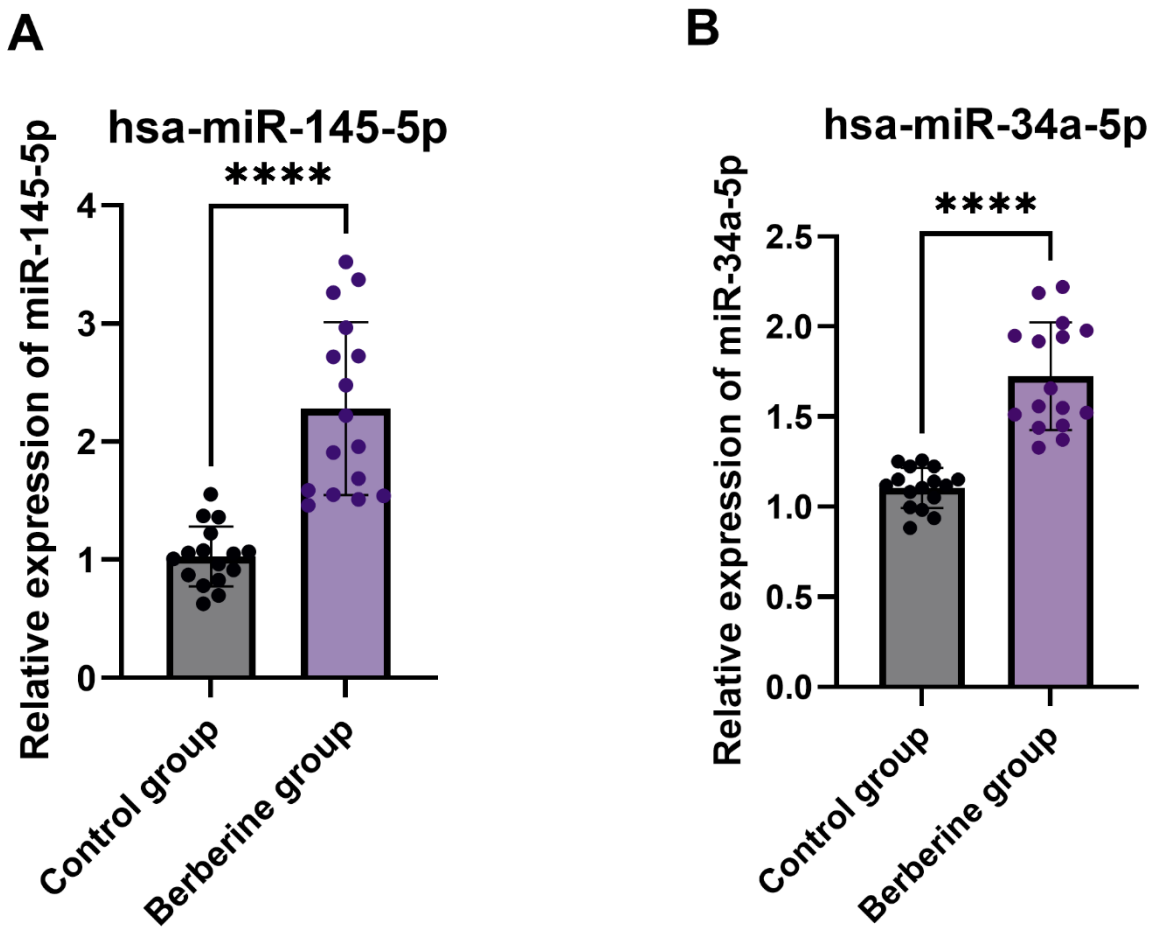

Figure 5: Relative expression of miRNAs in tumor tissues. ***(A)*** *the miR-145-5p relative expression in BC tumor tissues.* ***(B)*** *The miR-34a-5p relative expression in BC tumor tissues. Presented as mean ± SEM and statistically evaluated with a simple t-test (****P < 0.0001).*

IL-6 detection in serum and in breast tumor tissue

Oral administration of BBR for 5 weeks led to a trend but a non-significant reduction in the pro-inflammatory cytokine IL-6 levels in the breast tumor tissue of the BBR-treated group compared with the control group ($p > 0.05$) **(Figure 6A)**. Similarly, there is a significant reduction in the pro-inflammatory cytokine IL-6 levels in the serum of the BBR-treated group compared with the control group ($p < 0.05$) **(Figure 6B)**.

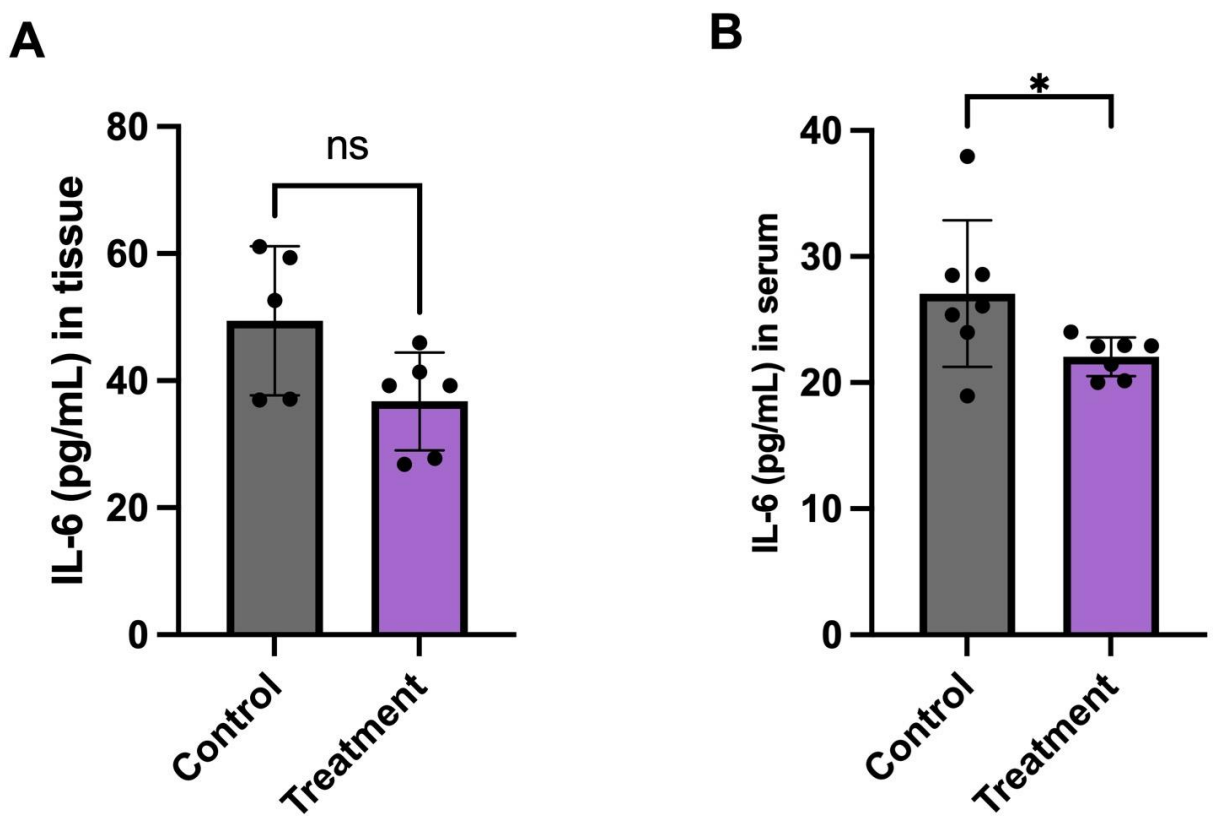

Figure 6: Interleukin (IL) 6 levels measured with ELISA. ***(A)*** *IL-6 levels in Balb-c mouse breast tumor.* ***(B)*** *IL-6 levels in Balb-c mouse plasma pools (pg/ml). The graphs show the mean +/− SEM; statistical significance was tested using t-test analysis (*P<0.05, ns means no significance).*

Depressive-Like behaviors

For the Forced Swim Test (FST), using the Ethovision data, based on the length of immobility, which was measured as the cumulative duration of "activity state inactive", the average periods of immobility for every experimental group were compared. The analysis did not show any statistically significant differences between the control and treatment groups ($p > 0.05$) **(Figure 7A)**. Similarly, for tail suspension, the total amount of time the mouse spent below the activity threshold is the target value to assess depressive-like behaviors. The activity threshold is set by the BEH Core in uOttawa based on it and is considered "inactivity" in this test. The data has no units; it's an arbitrary value calculated off the baseline. The analysis of the average "time

below the lower threshold" revealed no statistically significant difference between the control and the BBR group ($p > 0.05$) **(Figure 7B)**.

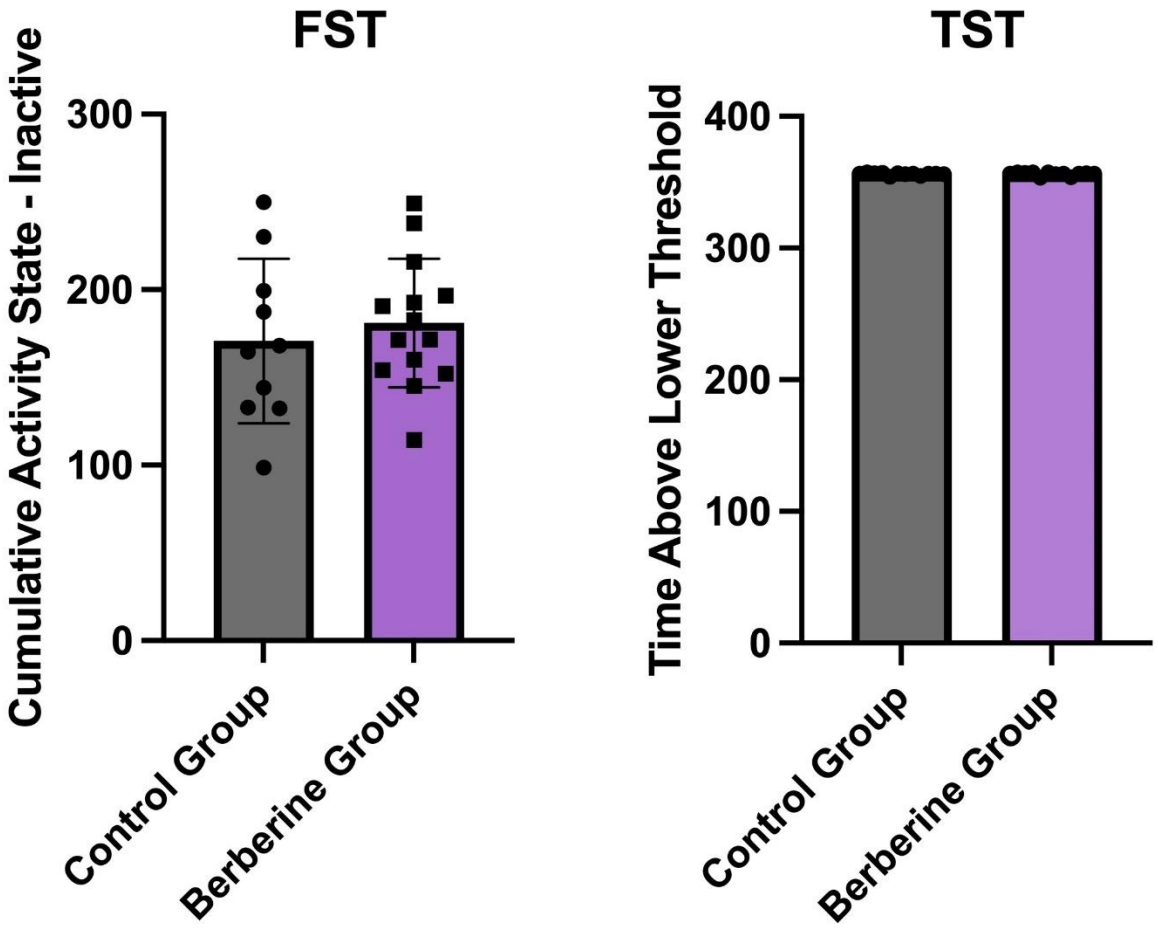

Figure 7: Depressive-like behavior tests in female mice exposed to either 1% sucrose water or berberine in 1% sucrose water treatment at week 11 of age. ***(A)*** *The duration of inactivity in seconds (sec) during the forced swim test.* ***(B)*** *The duration below the threshold in the tail suspension test. Data are represented as mean (±SEM); no significance was shown that differs between the groups; n = 12 for each group.*

2.5. Discussion

Breast carcinogenesis involves dysregulation of tumor suppressor genes and oncogenes. This study investigates the effects of BBR on BC and depressive-like behaviors in mice

including miRNAs and IL-6. The results showed that BBR exhibits promising effects in BC therapy and prevention by targeting multiple pathways involved in tumor progression, apoptosis regulation, and inflammation modulation.

As seen in the study, BBR was targeting CSCs by decreasing mammosphere formation and proliferation. CSC are implicated in tumor initiation, recurrence, metastasis, and resistance to therapy (61,131) . BBR's ability to reduce sphere formation in CSCs may be associated with its ability to induce apoptosis. In a previous study, BBR liposomes were found to induce apoptosis of CSCs through increasing pro-apoptotic proteins such as Bax and decreasing anti-apoptotic proteins such as Bcl-2 (123). It was shown that BBR was able to target the mitochondria of breast CSCs to induce apoptosis (123). Furthermore, the AMPK pathway may contribute to BBR's ability to inhibit the growth of CSCs. Previous research has found CSCs to be sensitive to the AMPK pathway (83). Moreover, BBR has been shown to have the ability to modulate the AMPK pathway (83,123). The AMPK pathway is involved in the regulation of the cell cycle, cell proliferation, and cell survival (83). A previous study found BBR able to stimulate the AMPK pathway and, as a result, inhibit the mTORC1 pathway, which is involved in cell proliferation (83). In addition, BBR was able to inhibit metastasis through regulation of the AMPK pathway and inhibition of certain MMPs in another study (123). In regulating the AMPK pathway, BBR exhibits an anti-inflammatory and antioxidant effect (123).

In this study, the targeted miRNAs were significantly increased in BBR treated groups compared with the control group, both in vitro and in vivo. Since miRNA-145, miRNA-34a, and miRNA-7c are frequently dysregulated in BC, they act as tumor suppressors by targeting critical oncogenes that control cell proliferation, apoptosis, and metastasis (81,132). Dysregulation of these miRNAs is linked to BC progression, aggressiveness, and resistance to treatment.

miRNA-145 targets cell reprogramming genes such as *Oct4* and *Sox2*, which are implicated in CSC maintenance (133). It inhibits human embryonic stem cell regeneration, promotes differentiation, and plays a significant role in carcinogenesis and tumor development (133). Elevated *Oct4* levels are regarded as one of the most important indicators of cancer cell chemotherapy resistance; however, increasing miR-145 levels can reduce expression of *Oct4* and its target gene *Zeb1*, counteracting the increase in *Oct4* levels generated by pemetrexed treatment, a type of chemotherapy (134). Restoration of miR-145 expression can efficiently increase BC cell apoptosis expressing wild-type *TP53* or estrogen receptor, implying that miR-145 therapy may be helpful in patients with BCs expressing wild-type *TP53* or estrogen receptor (135).

Let-7c contributed significantly to cell apoptosis and cell growth suppression in BC, in part by targeting ERCC6 (136). MiRNA let-7c acts as a cancer suppressor in a variety of ways, including preventing early cancer progression by suppressing *Hmga2* expression (137), inhibiting migration and invasion of human non-small cell lung cancer and colorectal cancer (138,139), and inducing cell apoptosis and disrupting the cell cycle in human hepatocellular carcinoma cells. In BC patients, let-7c is downregulated in both tissues and serum, and postmenopausal status influences let-7c expression (140). A higher expression level of let-7c has been linked to a better clinical outcome in individuals with estrogen receptor-positive BC (141).

In this study, miRNA-34a (miR-34a) was significantly increased in BBR treated groups both in vitro and in vivo. This is linked to miR-34a function as a tumor suppressor miRNA and is frequently downregulated in many malignancies (81,142–144). It is recognized to have an important role in causing apoptosis, or programmed cell death. miR-34a promotes apoptosis by targeting genes involved in cell survival, proliferation, and antiapoptotic pathways (145). Several

studies have shown that miR-34a overexpression can induce apoptosis in cancer cells via many pathways (144). For example, miR-34a targets genes such as *Bcl2*, an anti-apoptotic protein (146). miR-34 inhibits *Sirt1* deacetylase implicated in cell survival pathways; it increases acetylated p53 and expresses p21 and PUMA, p53 transcriptional targets that regulate the cell cycle and apoptosis, respectively (147). miR-34a-5p may play a significant role in initiating apoptosis by down-regulating *Snai1* in apigenin-treated lung cancer cells (81). miR-34a causes apoptosis while inhibiting tumor growth by downregulating these genes. Additionally, miR-34a has been linked to the regulation of p53, a master regulator of apoptosis and tumor suppression. miR-34a is a p53 transcriptional target, resulting in a feedback loop that amplifies apoptotic induction in response to cellular stress (144). miR-34a is located on 1p36 and is typically deleted in neuroblastomas. Furthermore, a reduction in miR-34 expression has been associated with resistance to apoptosis caused by p53 activating substances used in chemotherapy (142,144). Its capacity to induce apoptosis emphasizes its utility as a diagnostic and therapeutic agent in cancer treatment.

Interleukin-6 (IL-6) signaling is implicated in the maintenance and expansion of CSC populations in BC (94). IL-6 is also essential for epigenetic alteration in stem cells (97,98). IL-6 activates the NF-κB and STAT3 signaling pathways (148,149). NF-κB and STAT3 have been recognized as major regulators of epigenetic switches in inflammation (150,151). Recently, a positive feedback loop involving microRNA let-7 has been shown to maintain chronic inflammation in malignant cells (151). The feedback loop mediated by IL-6 signaling can activate the NF-κB pathway and its downstream targets, including let-7 and Lin-28. Similarly, IL-6 was shown to be critical in maintaining the inflammatory loop in BC CSCs (150,151). In summary, IL-6 signaling regulates cancer cell proliferation, CSC renewal, and metastasis. CSCs,

in turn, produce IL-6, creating a positive feedback loop that sustains CSC populations within the tumor microenvironment (99). Targeting IL-6 signaling pathways may disrupt CSC function and sensitize breast tumors to conventional therapies, potentially improving patient outcomes. As a result, BBR significantly reduced IL-6 levels in the serum; however, there was only a trend, though not statistically significant, observed in the tissue samples from mice treated with BBR.

The absence of significant results in the Forced Swim Test (FST) and tail suspension tests between the control and treatment groups could be attributed to the study's limited duration or the aggressiveness of the breast tumor that hid BBR's effect on depressive-like behaviors in mice by not giving enough time for behavioral changes. This means the third hypothesis was not confirmed.

Although no results were found regarding depressive-like behaviors, we believe further investigations are needed based on the following rationale. Our study verified the effect of BBR on decreasing IL-6 levels in serum. Hence, it was previously stated that IL-6 plays a role in stress-related psychiatric disorders, proposing that increased gut permeability may lead to the translocation of gut bacteria and their metabolites into the bloodstream, which can activate an immune response and cause inflammation (neuroinflammation) and the implication of the pro-inflammatory cytokine IL-6 that impacts the brain, resulting in mood and behavioral changes (152). It is suggested that controlling IL-6 levels following tumor development could reduce inflammation in the intestine and brain, modulate the gut microbiota, and decrease the risk of developing depression (153). Prebiotics can improve intestinal integrity, immunity, blood glucose and plasma lipid levels, and mineral absorption. Dietary interventions that enhance fiber intake and overall diet quality have been shown to lessen depressive symptoms in people with clinical depression (154). BBR also regulates SCFA metabolism (157). SCFAs contribute to the

integrity of the intestinal barrier by increasing the synthesis of tight junction proteins such as occludin and claudins. Short-chain fatty acids (SCFAs) are known to improve gut function by decreasing histone deacetylase (HDAC) activity and activating the AMP-activated protein kinase (AMPK) pathway and SP1 transcription factor. In the brain, they are known to affect the dynamics of the actin cytoskeleton and its interaction with the tight junction proteins (158). SCFAs help to maintain gut barrier integrity and immunology, hence guarding against peripheral inflammation (neuroinflammation). SCFAs can also improve the blood-brain barrier and regulate neurotransmitter and neurotrophic factor levels (159). SCFAs have been shown to lower the cortisol response to psychosocial stress in humans, indicating a modulating impact on the HPA axis (160).

The limitations of this study include the aggressive nature of the 4T1 cancer model necessitated a three-week period for euthanasia, potentially limiting the duration for assessing depressive-like behaviors. Although IL-6 levels were reduced in the treated group, additional confirmation by IL-6 and other pro-inflammatory cytokines in the brain would provide a more complete knowledge of neuroinflammatory alterations, increasing the depth of the study. The inclusion of a negative control group without BC would have improved the study's comparability, especially in behavioral measures of depressive-like behaviors. Although 1% of sucrose may appear insignificant, the cumulative effect over a five-week period could potentially affect the microbiota composition and other physiological factors in mice. But the sugar addition to both groups aimed to hide the bitter taste of BBR for more palatability and to ensure ingestion.

While our study has some limitations, it also has considerable strengths. First, our comprehensive experimental design includes both in vitro and in vivo tests, allowing for a thorough investigation of BBR’s effects on breast CSC and depressive-like behaviors. This

multimodal approach not only strengthens our findings but also provides a more complete picture of the underlying mechanisms. Furthermore, our findings are significant, providing valuable insights into the potential therapeutic or preventative use of BBR in BC. Moreover, to ensure objectivity and minimize bias, other colleagues were blinded to both groups while analyzing our results for confirmation, further enhancing the credibility of our findings.

The current research suggests various prospects for advancement in the future. Extending the period of behavioral assessments, particularly with a less aggressive cancer cell line, may provide a more complete knowledge of BBR's effect on depressive-like behaviors throughout time. Evaluating SCFA in the feces of BBR-treated mice could help to understand its prebiotic properties and their implications for gut microbiota modification. Furthermore, undertaking microbiome profile research, for example, by fecal analysis, provides a chance to identify specific microbial signatures altered by BBR, clarifying its therapeutic potential. Analyzing apoptosis through cell cycle and apoptosis assays to verify the mechanism that BBR follows to decrease CSCs is also a good approach to consider in future research. Pursuing these prospective initiatives offers potential for expanding our understanding of the effects of BBR on BC, depressive behaviors, neuroinflammation, and gut microbiota, ultimately leading to better preventative measures, therapeutic techniques, and patient outcomes.

2.6. Conclusion

The results of this study suggest the potential role of BBR in preventing BC stem cell formation and proliferation by modulating IL-6, and miRNA expression. Therefore, these substances have the potential to be used as "Epi - Natural Compounds" to prevent BC. It is crucial that further studies are conducted to confirm these results and search further into the role

of BBR as an adjunct to traditional BC therapy and the potential benefits of BBR in alleviating depression. These findings may indicate the antitumor characteristics of BBR as a novel next-generation prebiotic.

Author contributions

N.I. performed experiments, wrote the manuscript, prepared the figures, and performed data analysis; N.A and H.Y.-S. taught all the techniques; M.H., D.K., F.B. collected samples. C.M. designed and supervised the work. All authors have read and agreed to the published version of the manuscript.

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Conflict of interest

The authors declare no conflict of interest.

Chapter 3: General Discussion

3.1. Discussion

Patients with BC frequently look to complementary and alternative medicine approaches to enhance their general health and treatment results. This trend reflects an increasing awareness of the shortcomings of traditional treatments and a need for more all-encompassing methods that consider the intricate interactions between the psychological, emotional, and physical aspects of cancer care. Understanding the relationship between environmental, psychological, and physiological aspects of cancer progression and recovery allows medical professionals to provide more complete and individualized care for patients with breast cancer.

The gastrointestinal system is home to trillions of bacteria called the gut microbiome, which is essential for regulating immune response, inflammation, metabolism, and neurotransmitter synthesis. Dysbiosis, or disruptions in the composition of the gut microbiota, has been linked to several illnesses, including mood disorders and breast cancer (161). Probiotics, prebiotics, and symbiotics can help with gut dysbiosis, so they become more important for treating and managing BC (161). By fostering the growth and activity of beneficial gut bacteria and reestablishing the microbial balance, the “biotics” have the potential to reduce inflammation, boost immunity, and improve treatment results for breast cancer patients by supporting the gut flora (161). Interestingly, berberine functions in the digestive system as a prebiotic. In the intestine, berberine lowers inflammation by controlling the intestinal microbiota and encouraging the formation of butyrate by the strains of bacteria (162).

BC is caused by the abnormal regulation of tumor suppressor genes and oncogenes. My study looks at the impact of BBR on BC and depressive-like behavior in mice. The findings revealed that BBR has promise in BC treatment and prevention by targeting numerous pathways involved in tumor progression, apoptosis regulation, and inflammation modulation.

BBR targeted CSCs by limiting mammosphere development and proliferation in the BBR-treated mice.

As demonstrated in the study, BBR targeted CSCs by reducing mammosphere development and proliferation. CSC are involved in tumor initiation, recurrence, metastasis, and resistance to therapy (61,131). BBR's ability to inhibit sphere formation in CSCs may be linked to its ability to trigger apoptosis. In a recent study, BBR liposomes were discovered to promote CSC apoptosis by boosting pro-apoptotic proteins like *Bax* and lowering anti-apoptotic proteins like *Bcl-2* (123). It was demonstrated that BBR might target the mitochondria of breast CSCs and trigger apoptosis (123). Furthermore, the AMPK pathway may help BBR restrict the formation of CSCs. Previous research has demonstrated that CSCs are responsive to the AMPK pathway (83). Furthermore, BBR has been demonstrated to regulate the AMPK pathway (83,123). The AMPK pathway regulates the cell cycle, proliferation, and survival (83). A prior study demonstrated that BBR could boost the AMPK pathway while inhibiting the mTORC1 pathway, which is implicated in cell growth (83). In another study, BBR was found to decrease metastasis by regulating the AMPK pathway and inhibiting certain MMPs. BBR regulates the AMPK pathway and has an anti-inflammatory and antioxidant impact (123).

BBR upregulated the tumor suppressor miRNAs in tissue samples from BBR-treated mice.

In this study, targeted miRNAs were considerably higher in BBR-treated groups than in the control group, both in vitro and in vivo. miRNA-145, miRNA-34a, and miRNA-7c are often dysregulated in BC; therefore, they operate as tumor suppressors by targeting key oncogenes that drive cell proliferation, apoptosis, and metastasis (81,132). Dysregulation of these miRNAs has been linked to cancer progression, aggressiveness, and resistance to treatment. miRNA-145 targets cell reprogramming genes such as *Oct4* and *Sox2*, which are involved in CSC maintenance (91). It inhibits human embryonic stem cell regeneration, increases differentiation, and contributes significantly to carcinogenesis and tumor development (133). Elevated *Oct4* levels are regarded as one of the most important indicators of cancer cell chemotherapy resistance; however, increasing miR-145 levels can reduce expression of *Oct4* and its target gene *Zeb1*, thereby counteracting the increase in Oct4 levels caused by pemetrexed treatment (134). Restoration of miR-145 expression can effectively promote apoptosis in BC cells expressing wild-type TP53 or estrogen receptor, hinting that miR-145 therapy may be beneficial in patients with breast malignancies expressing wild-type TP53 or estrogen receptor (135). Moreover, Let-7c-5p, in part by targeting ERCC6, greatly increased cell apoptosis and suppressed cell proliferation in BC (136). miRNA let-7c-5p functions as a cancer suppressor in a variety of ways, including preventing early cancer progression by suppressing *Hmga2* expression (137), inhibiting migration and invasion of human non-small cell lung cancer and colorectal cancer (138,139), and inducing cell apoptosis and disrupting the cell cycle in human hepatocellular carcinoma cells. Let-7c-5p

expression is downregulated in both tissues and serum in BC patients, and postmenopausal status modulates it (140). Higher levels of let-7c-5p expression have been linked to a better clinical result in people with estrogen receptor-positive BC (141). In this work, miRNA-34a (miR-34a) levels were considerably elevated in BBR-treated groups both in vitro and in vivo. This is linked to miR-34a's role as a tumor suppressor miRNA, which is typically downregulated in various cancers (81,142–144). It is recognized to have a key role in apoptosis, or programmed cell death. miR-34a induces apoptosis by targeting genes involved in cell survival, proliferation, and antiapoptotic pathways (145). Several studies have demonstrated that miR-34a overexpression can cause apoptosis in cancer cells via a variety of routes (144). miR-34a, for example, targets genes like *Bcl2*, which is anti-apoptotic (146). miR-34 suppresses *Sirt1*, a deacetylase involved in cell survival pathways; it increases acetylated p53 and expresses p21 and PUMA, p53 transcriptional targets that regulate the cell cycle and apoptosis, respectively (147). miR-34a-5p may play an important role in apoptosis by inhibiting Snai1 in apigenin-treated lung cancer cells (81). miR-34a induces apoptosis while suppressing tumor growth by downregulating these genes. Furthermore, miR-34a has been connected to the regulation of p53, a key regulator of apoptosis and tumor suppression. miR-34a is a p53 transcriptional target, which causes a feedback loop that enhances apoptotic induction in response to cellular stress (144). miR-34a is found on 1p36 and is often deleted in neuroblastomas. Furthermore, reduced miR-34 expression has been linked to resistance to apoptosis mediated by p53 activating drugs used in chemotherapy (142,144). Its ability to cause apoptosis highlights its potential as a diagnostic and therapeutic agent in cancer treatment.

BBR decreased the pro-inflammatory cytokine IL-6 in the serum

Interleukin-6 (IL-6) signaling plays a role in the preservation and spread of CSC populations in BC (94). IL-6 is also required for epigenetic modifications in stem cells (97,98). IL-6 promotes the NF-κB and STAT3 signaling pathways (148,149). NF-κB and STAT3 are important regulators of epigenetic switches in inflammation (150,151). Recently, a positive feedback loop involving microRNA let-7 was discovered to maintain chronic inflammation in malignant cells (151). The IL-6 signaling feedback loop activates the NF-κB pathway and its downstream targets, such as let-7 and Lin-28. Similarly, IL-6 was found to be essential for maintaining the inflammatory loop in breast CSCs (150,151). To summarize, IL-6 signaling controls cancer cell proliferation, CSC renewal, and metastasis. CSCs, in turn, create IL-6, establishing a positive feedback loop that maintains CSC populations in the tumor microenvironment (99). Targeting IL-6 signaling pathways may disrupt CSC activity and make BCs more susceptible to conventional therapies, thereby improving patient outcomes. Hence, while BBR showed a significant decrease in IL-6 levels in the serum, there appears to be a trend, albeit not statistically significant, in the tissue samples from mice treated with BBR.

BBR did not alter the depressive-like behaviors

The BBR's influence on depressive-like behaviors in mice may have been obscured by the aggressiveness of the breast tumor or the length of the trial, as evidenced by the lack of significant results in the tail suspension (TST) and forced swim test (FST) between the treatment and control groups. This indicates that the third hypothesis was not supported. The effect of BBR on lowering blood IL-6 levels was confirmed by our investigation. Therefore,

it has been suggested that IL-6 plays a part in stress-related psychiatric disorders. Specifically, it has been suggested that increased gut permeability may result in the translocation of gut bacteria and their metabolites into the bloodstream, which may trigger an immune response, induce inflammation (neuroinflammation), and have an impact on the brain that leads to changes in mood and behavior (152). It has been proposed that regulating IL-6 levels after tumor formation may lessen brain and intestinal inflammation, alter the gut microbiome, and lower the chance of developing depression (153). Prebiotics can enhance the absorption of minerals, blood glucose and plasma lipid levels, immunity, and intestinal integrity. It has been demonstrated that dietary interventions that improve the amount of fiber consumed and the overall quality of the diet can reduce depressive symptoms in individuals with clinical depression (154) and increase depressive symptoms (155,156). BBR controls the metabolism of SCFAs as well (157). Because they promote the production of tight junction proteins like occludin and claudins, SCFAs help maintain the integrity of the intestinal barrier. By reducing histone deacetylase (HDAC) activity, activating the AMP-activated protein kinase (AMPK) pathway, and stimulating the SP1 transcription factor, short-chain fatty acids (SCFAs) are known to enhance gut function. They are known to have an impact on the actin cytoskeleton's dynamics and how it interacts with tight junction proteins in the brain (158). By preserving the integrity of the intestinal barrier and immune function, SCFAs protect against peripheral inflammation, also known as neuroinflammation. Additionally, SCFAs can control neurotrophic factor and neurotransmitter levels, as well as enhance the blood-brain barrier (159). The human cortisol response to psychosocial stress has been demonstrated to be lowered by SCFAs, suggesting a moderating effect on the HPA axis (160).

3.2. Limitations of the Study

This study's limitations include BBR solubility. Overcoming the problem of low BBR solubility required some optimization. We effectively handled the difficulty of BBR solubility by heat-solubilizing the solution. However, further investigations into alternative solubilization methods may enhance drug delivery efficiency.

Furthermore, the aggressive nature of the 4T1 cancer model required a three-week period before euthanasia, which could limit the time available to analyze depressive-like behaviors. This constraint underscores the need for careful planning and optimization of experimental timelines to capture comprehensive data on behavioral outcomes.

Additionally, the absence of a negative control group devoid of breast cancer poses a limitation in evaluating the specificity of observed depressive-like behaviors and other measures. Including such a control group would enhance comparability and strengthen the study's conclusions regarding the impact of breast cancer pathology on behavioral outcomes.

Moreover, the addition of 1% sucrose to both groups may have influenced behavioral testing by enhancing energy and motivation while maybe masking depressive-like behaviors. This factor should be addressed while interpreting the results.

Although 1% sucrose may look insignificant, the cumulative effect over a five-week period could affect microbiota composition and other physiological parameters in mice. However, the sugar addition to all groups was intended to mask the bitter taste of BBR, improve the palatability, and ensure consumption.

3.3. Strengths of the study

Our study has several limitations, but it also has significant strengths. First, our comprehensive experimental design covers both in vitro and in vivo experiments, allowing us to thoroughly investigate BBR's effects on breast CSC and depressive-like behaviors. This multimodal approach not only supports our findings, but it also provides a fuller picture of the underlying mechanisms.

Furthermore, our findings are significant, providing important and reliable information about the possible therapeutic or preventative use of BBR in BC and mental disorders.

Additionally, to uphold the integrity and objectivity of our study, rigorous measures were implemented to minimize bias and ensure the robustness of our findings. One such measure involved blinding other colleagues who reviewed our data for confirmation, thereby mitigating the risk of subjective interpretation and strengthening the credibility of our results. This adherence to scientific rigor enhances the validity and trustworthiness of our study outcomes, bolstering confidence in the reliability of our conclusions.

3.4. Future Directions

The current research suggests a variety of potential possibilities for advancement. Increasing the duration of behavioral assessments, particularly with a less aggressive cancer cell line, may provide a more comprehensive understanding of BBR's effect on depressive-like behaviors over time.

Moreover, leveraging advanced imaging techniques such as functional MRI or PET to measure brain activity holds promise for elucidating neuroinflammatory changes induced by

BBR treatment. By validating these findings at the neurobiological level, researchers can further elucidate the mechanisms underlying BBR's effects on mental health outcomes.

In parallel, evaluating short-chain fatty acids (SCFA) in fecal samples from BBR-treated mice could shed light on their prebiotic characteristics and their impact on gut microbiota composition. This approach offers valuable insights into the reciprocal relationship between BBR, gut microbiota, and host health, potentially uncovering novel therapeutic targets for BC and depressive disorders.

Furthermore, delving into microbiome profile research through fecal examination enables the identification of specific microbial signatures altered by BBR treatment. This not only elucidates BBR's therapeutic potential but also informs the development of personalized treatment strategies targeting gut dysbiosis in BC patients.

Additionally, conducting in-depth analyses of apoptosis using cell cycle and apoptosis assays can provide mechanistic insights into how BBR reduces CSCs. Understanding the precise molecular pathways involved in BBR-mediated inhibition of CSC proliferation is critical for the development of targeted therapies.

To translate these preclinical findings into clinical practice, carefully planned clinical trials are essential to evaluate the effectiveness and safety of BBR in BC patients with concurrent depressive symptoms. By bridging the gap between bench and bedside, these trials can validate the therapeutic potential of BBR and inform evidence-based treatment strategies for BC and mental health disorders.

Overall, pursuing these prospective projects has the potential to significantly advance our understanding of the impact of BBR on BC, depressive behaviors, neuroinflammation,

and the gut microbiota. By elucidating the underlying mechanisms and therapeutic implications of BBR, researchers can develop more effective preventative strategies and therapeutic interventions and ultimately improve patient outcomes in BC and mental health care.

General Conclusion

This study delves into the multifaceted effects of BBR on cancer prevention and its potential implications for BC stem cell development, tumor suppressive miRNAs, inflammation reduction in BC, and depressive-like behaviors associated with BC. Throughout my master's project, I pursued two distinct avenues of investigation, employing in vitro and in vivo experiments to address specific objectives. Firstly, we uncovered how BBR influences CSC proliferation and modulates miRNAs both in laboratory cell cultures and mouse tumor tissues. Secondly, we assessed the in vivo efficacy of BBR in preventing mammary carcinoma by studying its effects on miRNAs and IL6. But our third hypothesis was not proven regarding the potential of BBR in alleviating depressive-like behaviors in mice afflicted with BC.

Appendix

- Supplemental 1: Cytotoxicity Results

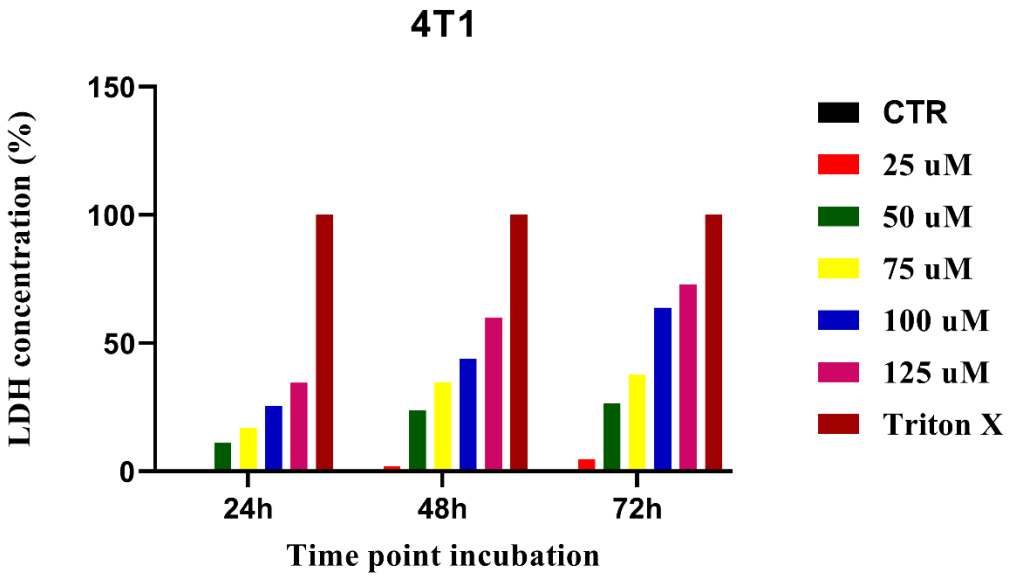

Figure 8: The Percentage of LDH Concentration at Different Time Points for 4T1 Cell Lines Treated with Various Concentrations of berberine

- Supplemental 2: Experimental Timeline

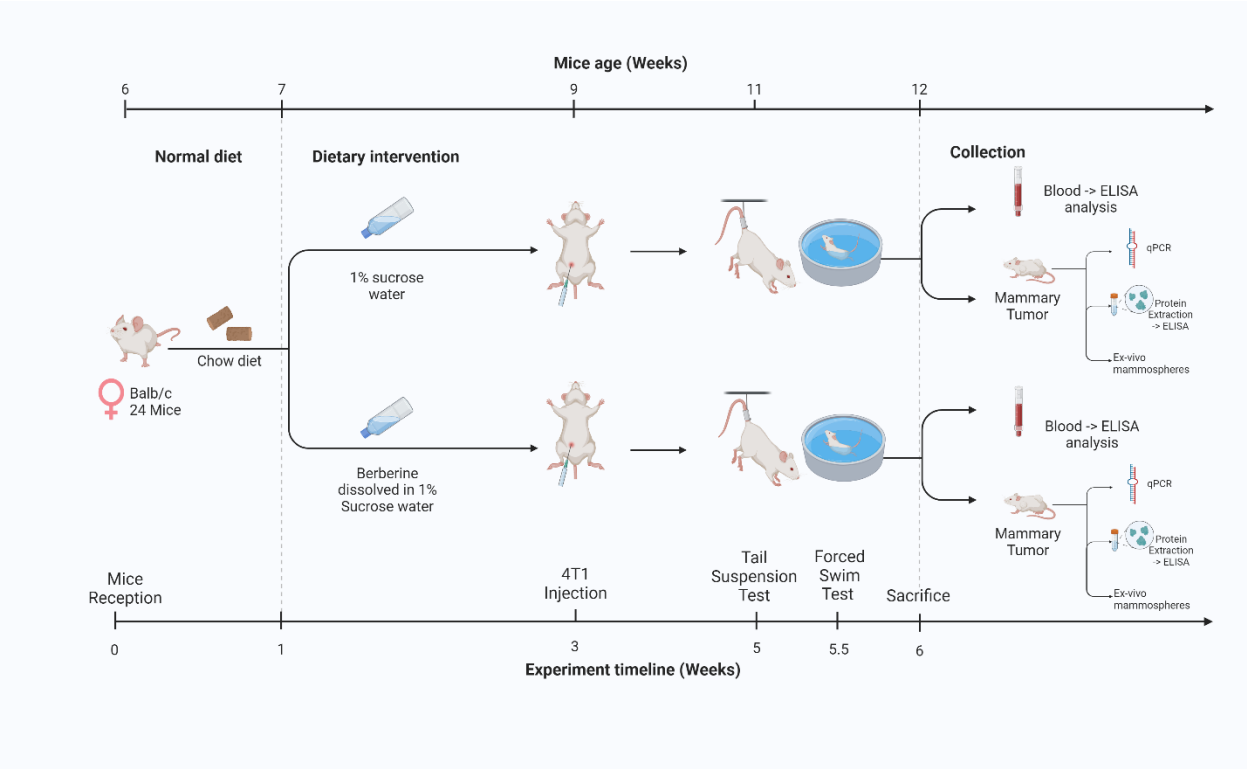

Figure 9: Experimental timeline for in vivo experiments (created with BioRender)

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