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Evaluation of lactoferrin combined hyaluronic acid formulations with the help of hyperthermia on breast cancer cell lines

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ABSTRACT: Lactoferrin is a potential anticancer protein naturally found in mammalian secretions. It shows anti-proliferative effects on various cancer types in vitro and in vivo. Its activity is highly associated with the immune system and its complements. On the other hand, hyperthermia is a type of cancer therapy in which tissues are exposed to heat at a temperature in the range of 40ºC-44ºC which induces apoptosis and necrosis. Hyperthermia normally aims to improve the results of conventional treatment strategies such as chemo or radiotherapy. The study aimed to enhance the lactoferrin effect with hyperthermia. Hyaluronic acid was additionally used in the formulation to augment the anticancer effect of lactoferrin. MTT Assays were performed for cell viability at 24 and 72 hours after the lactoferrin, hyaluronic acid, and hyperthermia treatment on MCF-7, MDA-MB 231, and HDF cell lines in vitro. No anticancer efficacy of lactoferrin was observed. However, it was demonstrated that Hyaluronic acid enhanced the anti-proliferative efficacy of hyperthermia treatment, and significant reductions in the cell viability were observed after 24 hours on MCF-7 cells in a p53-dependent manner whereas no reduction in the cell viability of MDAMB 231 and HDF cells. Mutation on the p53 gene of MDA-MB 231 cells possibly prevented the heat-induced apoptosis. Hyaluronic acid-induced cell death was observed 72 hours after the treatment independently from the heat exposure group. No cytotoxicity was observed on the HDF cell line. The activity could not be obtained from the pure hyaluronic acid solution. Subsequent determination of the chemical responsible for the anticancer efficacy should be performed. This research could lead to the discovery of a new selective chemotherapeutic agent that can be used in breast cancer.

KEYWORDS: Lactoferrin; Cell viability; Hyperthermia; Breast cancer; MTT assay

1. INTRODUCTION

In 2018, The WHO (World Health Organization) reported that 17 million people were newly diagnosed with cancer, 9.6 million patients died because of the cancer and more than 50 million people suffered from cancer worldwide. It indicates that the incidence of cancer is still very high and unfortunately, there is no effective cure yet. The prognosis and treatment get extremely frustrating, especially with patients at advanced stages of the disease. That's why preventing the development of carcinomas is important for reducing the high mortality of cancer [1,2]. Even though the therapeutic approaches were advanced during the last century, breast cancer is still the most common cancer seen in women worldwide. The development of an effective treatment for breast cancer faces a great challenge due to its metastasis incidence and heterogeneity. Thus, the search for new approaches to improve the therapy of breast cancer remains of huge importance.

Lactoferrin as a potential anticancer therapeutic protein Lactoferrin (LF) also called lactotransferrin is an iron-binding cationic multifunctional glycoprotein that belongs to the family of transferrin proteins along with the serum transferrin, melanotransferrin, ovotransferrin and the carbonic anhydrase inhibitor [3]. Its molecular weight is approximately 80-kDa with glycosylation sites and made up of about 700 amino acids. It was discovered in mammary secretions for the first time by Sorensen and Sorensen from the experiments on milk of bovines in 1939. The three-dimensional structure of LF is composed of a single polypeptide chain that symmetrically folds into two separate lobes which are called N (Nitrogen) and C (Carbon) [1]. Physiologically in biological fluids, LF can be found in two forms; either iron-free state called as

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apolactoferrin or iron-bounded state called as hololactoferrin. It can bind up to two ferric ions in its iron-free state. Each ferric ion is bound to the glycoprotein along with two carbonate ions whose protonation causes the weakness of the iron binding efficiency. Nonetheless, the binding site is unavailable only for the ferric ion as LF can bind other metal ions such as Copper, Zinc, and Manganate [3]. LF is found in high concentrations in humans approximately 1.0 to 3.2 mg/mL and in bovines approximately 0.02 to 0.35 mg/mL milk is found in lower concentrations in various secretions derived from epithelial cells, especially in the vaginal fluids, saliva, tears, semen, bronchial and nasal secretions, urine, gastrointestinal fluids, and bile as well as in the second granules of neutrophils. This wide distribution suggests that LF potentially takes a role in numerous crucial biological activities [1].Various relevant physiological functions have been established for LF, including antiviral, antibacterial, antifungal, anti-inflammatory, immune-regulatory, and anticancer activities. Several studies have shown that the iron binding ability of LF and the interaction between its specific receptors are responsible for the diverse biological activities demonstrated thus far. LF is a first-line defense protein against microbial infection following systemic diseases like sepsis or systemic inflammatory response syndrome. Consequent to infection, the monocyte-macrophage system becomes activated and produces inflammatory mediators which further activate the bone marrow to generate immune cells and activate the degranulation of mature neutrophils. Afterward, huge amounts of LF are released from the secondary granules of neutrophils to fight the infection. Moreover, the antimicrobial activity of LF has been reported in recent studies. Two distinct mechanisms are responsible for the activity-dependent prevention of bacterial infections bacteriostatic and lipopolysaccharide-dependent prevention of Gram-negative organisms as bactericidal [4,5]. Interaction of LF with specific cell surface receptors involves danger-signal recognition such as toll-like receptor 4 and CD14. This interaction with those membrane receptors results in the "danger signal" of monocytes and macrophages. For instance, competition between LF and bacterial lipopolysaccharide occurs for the binding 3 sites of CD14 receptor, and binding of LF can attenuate induction of NF-κB dependent gene transcription of diverse inflammatory mediators. Therefore, LF acts as a mechanical feedback mediator of acute inflammation in one way [5]. During the last decade, LF has been reported to take part in immunological activities such as having important roles in the host defense against infective agents and inflammation. Thus, it appears to be a key factor in the mammalian immune system. The cellular and molecular mechanisms that account for the immunomodulatory activities of LF are not completely elucidated, and in vitro and in vivo studies indicate the presence of numerous mechanisms. LF interacts with B cells, well-known antigen-presenting cells, to favor their following interaction with T cells, substantially promoting the increase in the antibody response. The human and bovine LF can interact with surface receptors on the human T cell line which is the Jurkat cell line. Additionally, it has been claimed that LF receptors are expressed in all T cell lines. There are some assumptions that LF plays a role in T cell activation by modulating the function of dendritic cells [3].On a more integrative point, LF can be classified as an acute phase protein acting like an "alarmin," since it is released from neutrophils after the infection taking a crucial part in modulating immune response. With the alarming activity, LF provides conditional interactions between dendritic cells and neutrophils inside a narrow space. This in turn leads to the promotion of a switch from the immature innate to mature adaptive immunity. Furthermore, LF acts as a naturally occurring adjuvant by the increased immunity against every infectious agent because they are present in the body since the beginning of the birth with the lactation by milk. Recent studies have demonstrated that LF has the potential to be used as an anticancer agent. LF treatment reduces carcinogenesis in the colon, metastases, and solid tumor growth in mice. Tumor inhibition by intravenous, intratumoral, and oral routes applied on head and neck squamous cell carcinoma was shown in several studies. In the murine model, LF-induced growth inhibition of floor-of-mouth tumors is equal to that of the most common chemotherapeutic agent used for head and neck squamous cell carcinoma. The effect was associated with increased release of IL-18 in the gut, activation of natural killer cells, and increasing cytotoxic T cells [6]. Although the mechanisms underlying the anticancer effect of LF are not elucidated, they can be mainly classified into three groups which are extracellular effects, intracellular effects, and immunostimulation. The extracellular effects are mostly related to the interaction of LF and cell membrane with membrane receptors the intracellular effects are related mainly to cell cycle arrest and apoptosis. The suggested mechanisms involving the cytotoxicity of LF against cancer cells are various and derived from in vitro or in vivo experiments [1]. Induction of apoptosis by LF is triggered via vital pathways so that cytotoxicity is observed against numerous cancer cell lines. Nonetheless, the triggered apoptosis pathways are either extrinsic such as the Fas-receptor family, or intrinsic such as mitochondria-associated depending on the type of cancer cells.

Akt is an anti-apoptotic signaling molecule that plays a crucial role in the functioning of cancer cells. For instance, If LF treatment was applied to SGC-7901 stomach cancer cells, phosphorylation of Akt and

numerous other key molecules in the signaling pathway was decreased the phosphorylation of glycogen synthase kinase 3β Ser256 and phosphorylation of caspase 9 Ser196 was increased. Therefore, LF seemed to induce apoptosis in SGC-7901 through the Akt pathway. Moreover, induction of stress-related mitogen-activated protein kinase pathway by LF treatment was [5] found in Jurkat T cells where the Jun N terminal kinase (JNK) associated with Bcl-2 was suggested to be the responsible pathway for the induction of apoptosis. LF treatment increased the activated caspase 3 and caspase 9 as well as the level of phosphorylated Bcl-2. It was found that when JNK activation was prevented, Jurkat cells did not experience apoptosis by LF treatment. Oral administration of LF doubled the expression of Fas protein and increased the activation of caspase 3 and caspase [7,8]. Immunohistochemical analysis demonstrated the presence of Fas-positive apoptotic cells at the site of LF-induced tumor inhibition1. Immunostimulation is accepted to be the pivotal element for the in vivo anticancer effects of LF. LF induces the immunoreaction associated with both innate and adaptive immunity. It functions primarily via the activation of powerful Th1 response and the substantial release of cancer killer cells. After the oral administration of LF in mice, the recruitment of lymphocytes, majorly helper T cells and effector T cells, achieved a nearly 20-fold increase. Those tumor-infiltrating lymphocytes can successfully inhibit the progression of cancer cells. The expression of interferon-γ, tumor necrosis factor (TNF) -α, caspase-1, and IL-18, as well as IgM+ and IgA+ B cells, is also considerably increased after LF treatment. Indeed, these mediators show a cascaded effect. Caspase-1 is reported to cleave pro-IL-18 to form mature IL-18. IL-18 is a cytokine responsible for the induction of interferon-γ. Furthermore, IL-18 plays a crucial part in the expression of TNF-γ in T cells and natural killer cells. IL-18 increases Th1 and natural killer cell responses and generates effector T cells. Additionally, LF increases the anticancer activities of natural killer cells and macrophages enhance nitric oxide generation which sensitizes tumors to chemotherapeutic agents. Furthermore, orally administered LF was shown to prevent VEGF165-mediated angiogenesis in rats. The combination of these effects can be to eradicate tumors. LF is believed to prevent oncogenic stimulation via cation-chelating action. As mentioned above, it can bind to metal-related cations like ferric, zinc, and copper. LF is one of the main iron regulators in the human body and maintains systemic iron homeostasis. If iron balance is disrupted, it can lead to free radical formation. Furthermore, they generate oxidative stress, which is assumed to be a strong oncogenic stimulation. Other roles of LF in cancer development and progression have been reported. For instance, downregulation or silencing of lactoferrin genes increases malignant tumors. On the contrary, the proliferation of cancer cells is prevented following the activation of the lactoferrin gene. Besides having nutritional value, lactoferrin or its derivatives may have considerable [1]. Overall, the anticancer activity of LF was reported in various cell lines, animal models, and even in clinical trials. Many in vitro and in vivo studies emphasize that anticancer activity is associated with the induction of apoptosis and modulation of the levels of main apoptotic molecules. On the other hand, proteomic analysis of breast cancer cells revealed that 9% of the proteins upregulated by LF were associated with apoptosis.

In another study with breast cancer, LF modulated main apoptotic molecules primarily Bcl-2 family proteins, inhibitors of apoptosis protein members, like survivin, and their inhibitors. LF is a non-toxic and low-cost dietary protein with powerful anticancer activity, it has a potential application in cancer therapy [2]. Hyaluronic acid (HA) is a linear mucopolysaccharide composed of alternatingly bonded two saccharide units of glucuronic acids and N-acetylglucosamines by β-1,3 and β1,4 glycosidic bonds. This glycosaminoglycan is extensively present in different tissues of the human body. It is the key component of the extracellular matrix in humans' physiological roles [7]. HA binds to its specific receptors and some specific molecules via proteins, named hyaladherins through the extracellular matrix [8]. Over the last decade, HA has become much more widely preferred in the fields of drug research and development, arthritis treatment, and surgery since HA and its derivatives can be used effectively as delivery vehicles for several proteins, peptides, nucleic acids, and various anticancer drugs better than conventional vehicles [9]. Its physical and chemical properties are desirable for drug delivery. HA is also good biocompatibility, biodegradability, high viscoelasticity, plasticity, and non-immunogenicity. Additionally, some functional groups of HA such as carboxyl, hydroxyl, and N-acetyl are convenient for further chemical modifications. As a result of those properties, HA and its derivatives are commonly preferred as drug delivery vehicles contributing to drug thickening, increased stability, sustained release, and transdermal absorption [7]. HA is also a ligand for some particular receptors on the cell surface which are extensively present in various tissues such as the kidney, liver, lymphatic system, and many tumor tissues [7]. Specific binding of HA to its receptors on cancer cells can be beneficial for increasing the targeted drug delivery to cancer tissues [9]. Even though CD44 is the principal receptor of HA, the binding is highly influenced by the type of involved cell. Indeed, the level of interaction between HA and CD44 could be minimal or even additional steps for activating HA could be needed. As aforementioned, the expression of CD44 or CD44-like receptors is

considerably immense in various solid tumors such as breast, pancreatic, and lung cancer. Accordingly, the CD44 and HA polymers could be valuable biomarkers. Nowadays, there are plenty of HA products with FDA approvals. Consequently, HA is investigated in extensive research for cancer treatment owing to its affinity for the widely distributed CD44 receptors [8]. On the other hand, HA and its derivatives are used as vehicles for numerous drug delivery systems. Several researchers evaluate divergent forms of tumor-targeting drug delivery systems based on HA. They include HA-drug conjugates, amphiphilic derivatives of HA polymer, HA surface modifications, and gene-drug delivery with HA [9]. For instance, chemically conjugating HA to lipid-based nanocarriers or polymeric nanoparticles and even developing self-assembly nanocarriers that use a chemical modification of HA as the backbone has already been reported. Until now; non-immunogenicity, biocompatibility, and hydrophilicity of HA have considerably advanced research on developing nanocarrier systems that can target drugs and genes against tumor cells to minimize potential side effects [8]. Hence, many approaches for taking advantage of the targeting ability of HA. HA-based drug delivery systems target and enter cells more effectively through the HA receptors via endocytosis. It is proved that HA provides simple and efficient approaches to achieve active targeting for tumor cells with minimum toxicity profile [8].

Hyperthermia (HT) also called thermal therapy is a type of cancer treatment in which cancerous body tissues are exposed to high temperatures ranging from 40°C to 44°C. In a more general point, it refers to different techniques of heat application or exposure administered as an adjunct to clinically approved approaches particularly radiotherapy and chemotherapy in the treatment of cancer patients. The efficiency of those techniques depends on the degree of temperature, length of the heat exposure, and area of the region included. Previous studies have proved that increasing temperature damages and kills cancer cells, largely with minimum injury to healthy cells. HT can shrink tumor tissues by killing cancer cells and denaturing proteins or related structures within cells [9,10].Whole-body hyperthermia application and hyperthermic perfusion techniques (hyperthermic isolated limb perfusion or hyperthermic peritoneal perfusion) are distinguished from local or regional hyperthermia. However, all HT models are similar in efficacy and it is not enough for clinically approved treatment when applied alone. HT is convenient for the enhancement of the cytotoxicity of chemotherapeutics or radiation. It provides thermal chemosensitization and thermal radiosensitization of tumor tissues. Thus, HT applications aim to improve the outcomes of conventional treatment strategies by implementing a multi-model treatment approach [10]. The combination of HT with chemo/radiotherapy has been studied in numerous clinical trials. They mainly focused on the treatment of various cancer types including sarcoma, carcinoma, melanoma, and diverse locations including breast, brain, lung, head and neck, esophagus, liver, bladder, rectum, appendix, cervix, and peritoneal lining. In most of them, tumor size was significantly reduced with the combination of HT and other treatments [11]. The severity of the damage varies with the type of thermal energy, the application rate, and the sensitivity of the targeted tissue to thermal ablation. The temperatures between 40°C and 44°C lead to irreversible cell damage if the duration of the heat exposure is about 30 minutes to 1 hour. The duration of the heat exposure required for the irreversible cell damage decreases exponentially when the application of temperature is above 60°C. The primary characteristic of injury is the generation of inactivated vital enzymes. Above 60°C, proteins are rapidly denatured, which is extremely cytotoxic and results in following coagulative necrosis. Subsequently, actin filaments and microtubules dysfunctions and in an impaired facilitated diffusion across the cell membrane. Metabolites become accumulated and shifting fluids inside the cell cause cytolysis. As a result, the integrity of the cell membrane changes significantly with HT which is suggested to be the key determinant of HT-induced cell death. In addition, mitochondrial dysfunctions have been reported as heat-induced injury. Rising temperature promotes proton leakage from the inner mitochondrial membrane. On the other hand, extensive structural changes are observed after heat exposure including mitochondrial swelling, formation of dense bodies, and vesiculars in the mitochondrial cristi. Also, DNA replication is inhibited by HT via the denaturation of the essential replication enzymes including DNA polymerase α, which is responsible for semiconservative DNA replication, and DNA polymerase β, which is responsible for DNA repair synthesis. Additionally, as DNA replication stays suppressed after termination of heat another potential mechanism seems to be the denaturation of the polymerase substrate chromatin. Non-histone nuclear matrix proteins are abnormally condensed by heat exposure which physically obstructs DNA replication and repair enzymes. Another suggested intracellular mechanism for heat-induced injury includes the disruption of RNA synthesis, the release of lysosomal enzymes, and the impairment of the Golgi apparatus. Although heat-induced injury may seem to harm the healthy tissue together with tumor tissue by all activities, tumor tissues have been reported to be more sensitive to thermal interventions than normal tissue. It can result from reduced heat dissipating [10] ability of the tumor because of the high levels of tumor metabolic stresses and its acidic interstitial environment [12]. HT has been shown to induce both

apoptotic and necrotic cell death in vitro in a temperature-depending behavior. The susceptibility of cancer cells to apoptosis has been reported particularly in numerous experiments on hematological cell lines. Furthermore, several studies demonstrated that induction of apoptosis on soft tissue and osteosarcoma cell lines has only been observed when heat exposure rises to 43°C. This suggests that some cell types show different susceptibility to apoptosis induced by heat. Subsequently, above a distinct temperature, it is more likely to induce necrosis [10]. Heat shock proteins (HSP) have various complex activities in transformed tumor cells. It is suggested that the activities of Hsps lead to many survival benefits for cancer cells. There is evidence that Hsp70 directly inhibits apoptosis pathways in cancer cells, as shown in human pancreatic, prostate, and gastric cancer cells. Hsps have also been implicated in mediating resistance to potentially cytotoxic hyperthermia. More specifically, the synthesis and accumulation of Hsps in tumor cells exposed to hyperthermia may afford protection from further heat-associated cytotoxic events, as the Hsps may rescue or restore vital cellular proteins. Moreover, there is evidence that Hsps supports the malignant phenotype of cancer cells and affects the cells' survival, but also participates in angiogenesis, invasion, metastasis, and immortalization mechanisms [13]. HSPs protect cells against heat-induced protein damage by their chaperone activity. However, prolonged heating may result in HSP levels regression to normal levels. On the contrary, high temperatures above a threshold may inhibit HSP synthesis, which favors cell death. In more detail, the Hsp90/Hsp70-based chaperone machinery has been demonstrated to control the signaling of protein function, trafficking, and turnover. It has been recently suggested, that Hsp90 and Hsp70 regulate the processing of damaged and abnormal proteins for degradation via the ubiquitin-proteasome pathway. Therefore, HSP90's role in signaling and regulating the fate of damaged proteins is extremely important [14]. The immune response to the HT application alone does not eliminate the tumors. Many studies have investigated strategies for the immune system adjuvants with HT to stimulate a more robust antitumor reaction. Although these investigations are still in preclinical, many have shown promising results [12]. The p53 gene is mutated in 50% of human cancers. p53 is among the most well-established cancer inhibitors DNA repair, and apoptosis. The effect of HT on p53 expression emphasizes an association of p53 in hyperthermic inhibition of tumor growth. Moreover, heat exposure activates p53 and causes the activation of cell cycle checkpoints. It is demonstrated that in human liver carcinoma HepG2 cells which express wild-type p53 gene, HT resulted in DNA damage and induced G2/M arrest through activated p53 and ATM/ATR. It was followed by apoptosis, it was not observed in Huh7 cells expressing mutant p53. These results indicate a critical role of p53 in the cellular effects of HT15.

2. RESULTS

At first, the effect of Lactoferrin treatment was evaluated. Three increasing concentrations of LF treatment were applied to the three different cell lines. A significant increase in cell proliferation after 24 hours in the MDA-MB 231 cell line and 72 hours in the MCF7 cell line was observed at 62.5 µM and 125 µM LF treatment. No significant effect was observed on the HDF cell line. No anti-proliferative or anticancer performance was observed on any cell lines with LF treatment (Figure 1, Figure 2).

To exert the anti-proliferative effect of LF treatment, it was triggered with hyperthermia by heat exposure. Again, the proliferative effect of LF treatment was observed on the MDA-MB 231 cell line after 24 hours with all heat exposures and the MCF-7 cell line after 72 hours with no heat exposure and 44ºC heat exposure of 20 minutes. No significant effect of LF was observed on the HDF cell line except for the proliferation of cells with 44ºC heat exposure of 20 minutes. No anti-proliferative or anticancer performance was observed on any cell lines with LF treatment (Figure 3).

On the other hand, the anti-proliferative effect of HT was observed 24 hours after the heat exposure. A significant reduction in the proliferation was observed in heat exposures in all cell lines. However, the effect of HT was diminished after 24 hours and could not be seen after 72 hours (Figure 4).

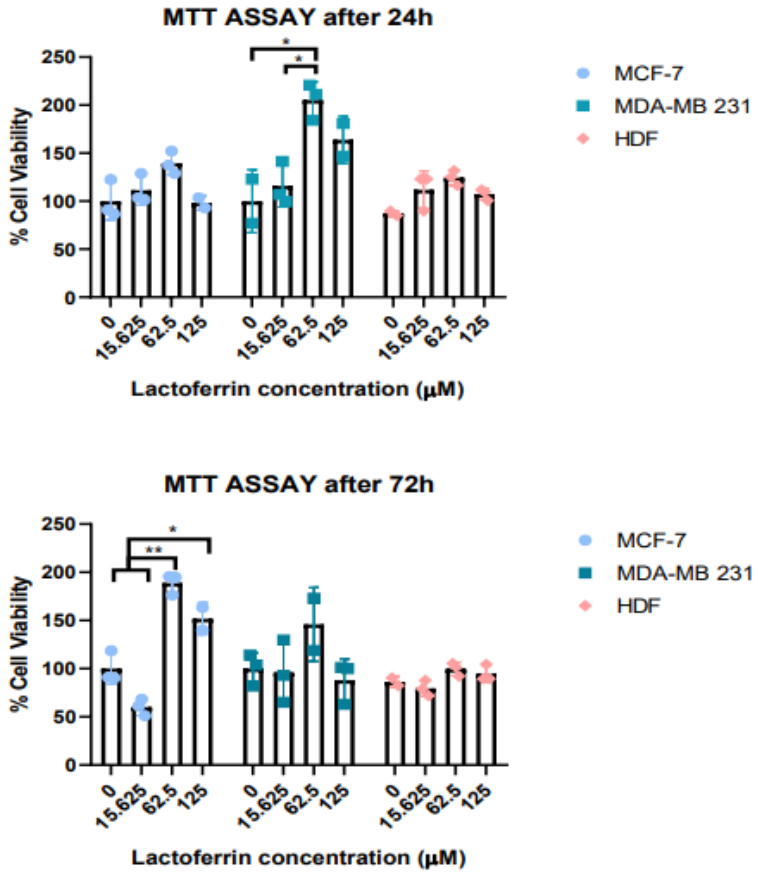

Figure 1. Effect of Lactoferrin treatment (0, 15.625, 62.5, 125 µM) on human breast cancer cell lines (MCF-7 and MDA-MB 231) and human healthy cell line (HDF) a) 24 hours b)72 hours after LF treatment. Results represent an average of 3 technical replicates for each culture. Significant changes compared with control: *P < 0.05; **P < 0.01;(2- tailed unpaired Student's t-test, ANOVA and Tuckey's Multiple Comparison Test)

To exert the anticancer efficacy of LF, it was tried to be stabilized with Hyaluronic acid solution. Unfortunately, proliferative performance but no anticancer effect was seen on all cell lines. However, a major significant decrease in the proliferation was observed on both cancer cell lines after 72 hours with the application of HA formulation solution independent from the LF whereas no toxicity on the healthy cell line (Figure 5, Figure 6). Also, the effect of HT was seen distinctively on the MCF-7 cell line when heat was triggered with the application of HA formulation solution (Figure 6).
Furthermore, the anticancer effect of HT after 24 hours was enhanced with HA formulation solution on the MCF-7 cell line. Also, the HA formulation decreased the cytotoxicity of hyperthermia on the HDF cell line. Additionally, the selective anticancer effect of HA formulation solution application was observed 72 hours after the treatment independent of HT (Figure 7).

To determine the cause of the efficacy of the HA formulation solution, HA püre formulation was applied to MDA-MB 231 and HDF cell lines. No anticancer efficacy was observed with HA pure solution, indeed it increased the proliferation of two cell lines after 24 and 72 hours. A significant reduction in the cell viability of the MDA-MB 231 cell line was achieved only by HA formulation solution 72 hours after the treatment (Figure 8).

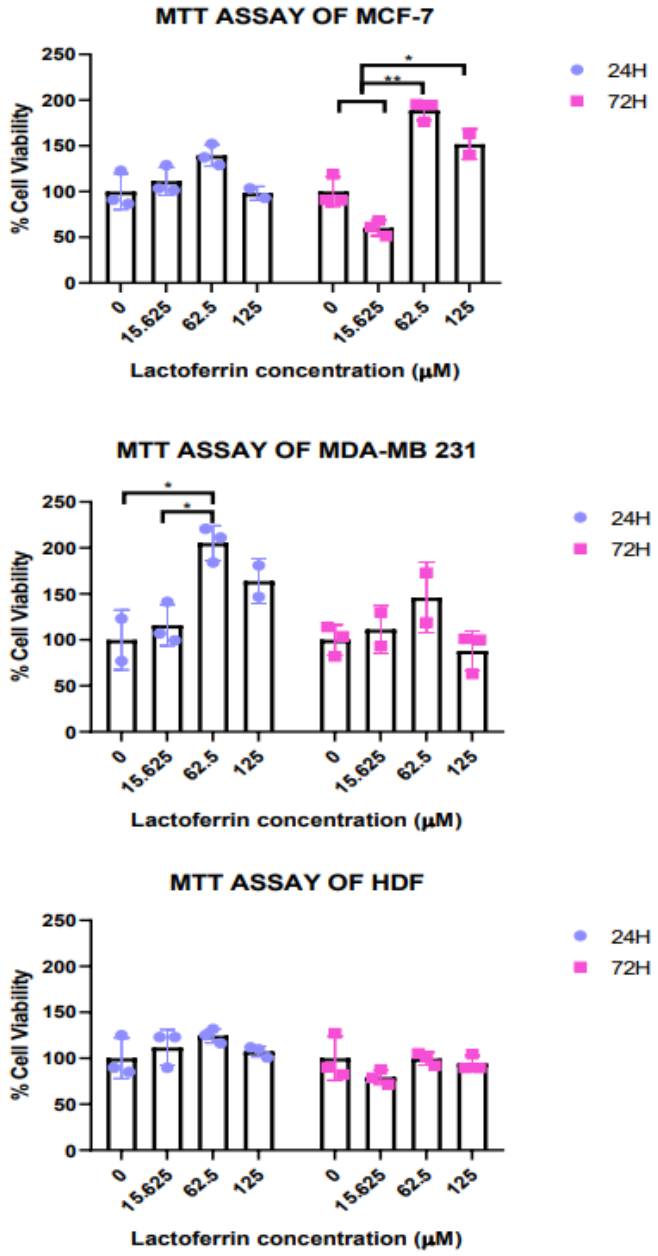

Figure 2. Effect of Lactoferrin treatment (0, 15.625, 62.5, 125 µM) after 2 different times (24h and 72h) on human breast cancer cell lines a) MCF-7 b) MDA-MB 231 and human healthy cell line c) HDF. Results represent an average of 3 technical replicates for each culture. Significant changes compared with control: *P < 0.05; **P < 0.01;(Regular ANOVA and Tuckey's Multiple Comparison Test).

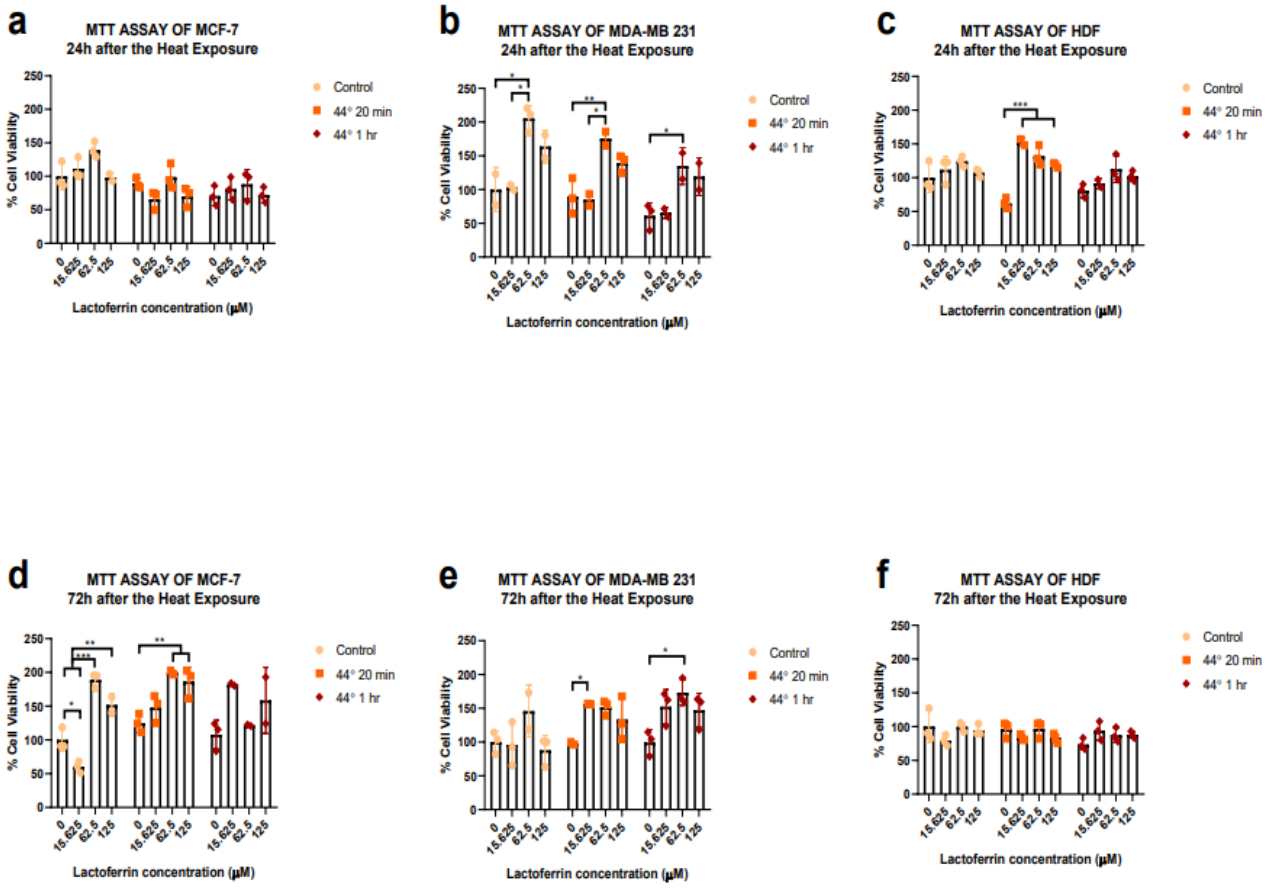

Figure 3. Effect of Lactoferrin treatment (0, 15.625, 62.5, 125 µM) when applied with different heat exposures (Control, 44ºC 20 minutes and 44ºC 1 hour) after 24 hours on a) MCF-7, b) MDA-MB 231, c) HDF cell lines and after 72 hours on d) MCF-7, e) MDA-MB 231, f) HDF cell lines. Results represent an average of 3 technical replicates for each culture. Significant changes compared with control: *P < 0.05; **P < 0.01; ***P < 0.001; (Regular ANOVA and Tuckey's Multiple Comparison Test)

3. DISCUSSION

The effects of LF (15.625, 62.5, 125 µM), HT (44ºC 20 minutes or 1 hour), and HA formulation solution were investigated in vitro on MCF-7, MDA-MB 231, and HDF cell lines. The anticancer or anti-proliferative effect of LF treatment could not be observed. Antiproliferative effects of HT were demonstrated on MCF-7 cell lines having wild type p53 gene after 24 hours which was further enhanced significantly by the application with HA formulation solution. Additionally, the antiproliferative activity of HA formulation solution on MCF-7 and MDA-MB 21 cell lines was reported 72 hours after the treatment. In the literature, many studies reported the anticancer effect of LF in vitro on several cancer cell lines. Therefore, efforts to exert its anti-proliferative efficacy were performed by combining it with HT to obtain a synergistic effect and by formulating it with an HA formulation solution to increase its stability in the aqueous state. However, the anticancer effect could not be achieved by both of the strategies in vitro. First of all, preserving the stability of protein formulations is extremely hard. Proteins are prone to protonation or deprotonation in aqueous solutions. As their protonation state determines the efficacy, creating a stable formulation is one of the key determinants of protein formulations. The formulation of LF with HA was considered in the experiments. HA can hold and capture the LF molecules inside its polymeric structure and prevent further movement more stable formulations of LF are obtained. However, this strategy would be more applicable if LF directly dissolved in HA solution could be used.

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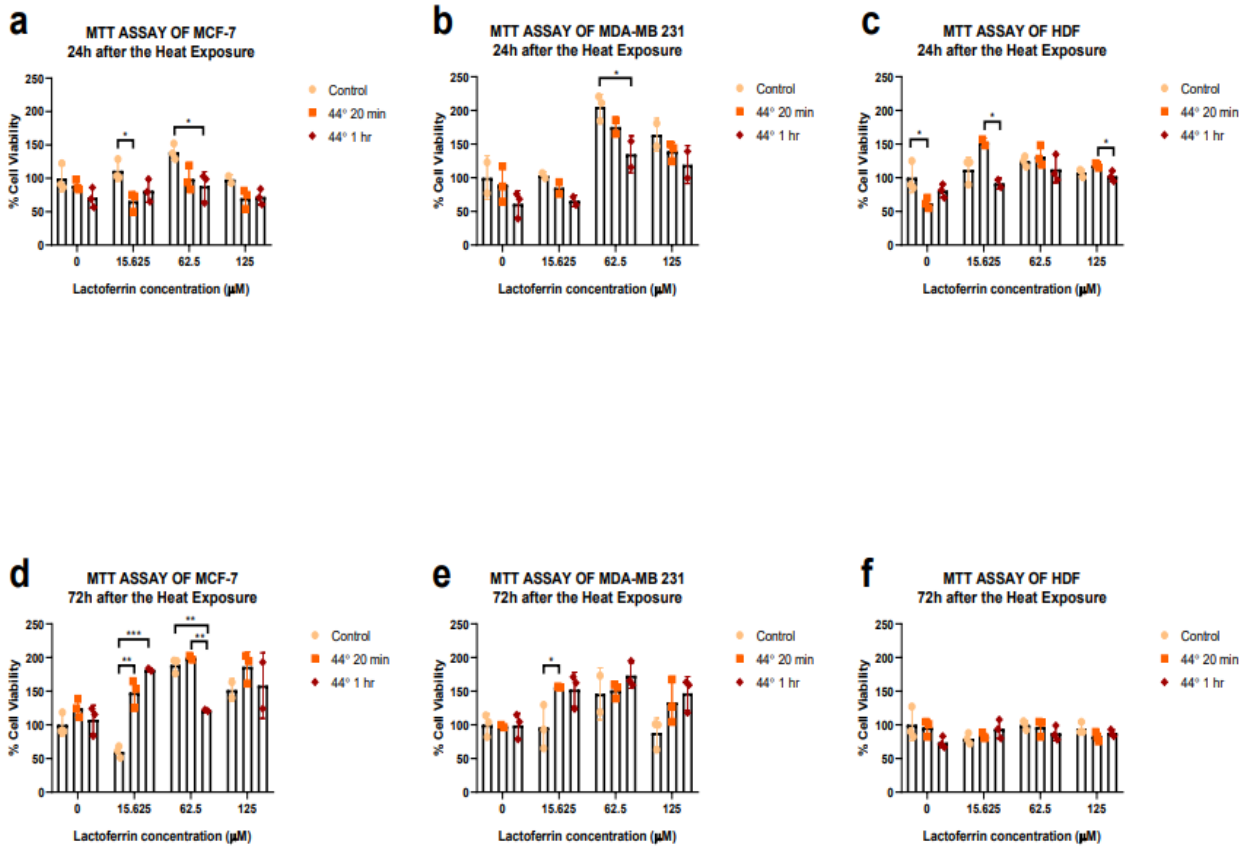

Figure 4. Effect of different durations of heat exposures (Control, 44ºC 20 minutes and 44ºC 1 hour) when applied with different concentrations of Lactoferrin treatment (0, 15.625, 62.5, 125 µM) after 24 hours on a) MCF-7, b) MDA-MB 231, c) HDF cell lines and after 72 hours on d) MCF-7, e) MDA-MB 231, f) HDF cell lines. Results represent an average of 3 technical replicates for each culture. Significant changes compared with control: *P < 0.05; **P < 0.01; ***P < 0.001; (Regular ANOVA and Tuckey's Multiple Comparison Test).

Cell culture requires quantities of chemicals and solutions and automatic pipettes. On the other hand, the gelation process of HA occurs when internal bonds are formed within its structure. Normally this process takes time at room temperature but shortens especially when the quantity of polymer is small. That's why, when LF dissolved directly in %1 HA solution, the solution became gel inside the tip of the automatic pipette and could not be applied to the cell culture. Therefore, HA was mixed with cell media before the addition of LF. As a result, LF could not be trapped inside the HA molecules as HA was diluted. If LF could be dissolved in HA solution and then applied to cell culture, the stability could be preserved more successfully. However, to confirm the hypothesis stability studies and characterization of LF-HA solution must be performed. Secondly, the studies on LF indicated that the main function of LF is associated with the immune system. LF activates the immune system and enhances its response to cancer antigens. It affects T cells and B cells other immune cells in the tumor microenvironment. However, mammalian cell cultures are done with isolated cell lines which are completely homogenous and do not represent heterogeneous tumor tissue. As a result, the immune system is an integral part of an organism's physiological functions. In vitro conditions could not mimic the tumor microenvironment and immune system activities.

Previous research suggested that HT is an effective technique for cancer treatment when used in combination with chemo/radiotherapy. However, both of the therapy modalities involve serious side effects and low patient compliance. Therefore, in this study, the effect of HT on breast cancer cell lines was investigated and a possible strategy to enhance the anticancer efficacy was applied.

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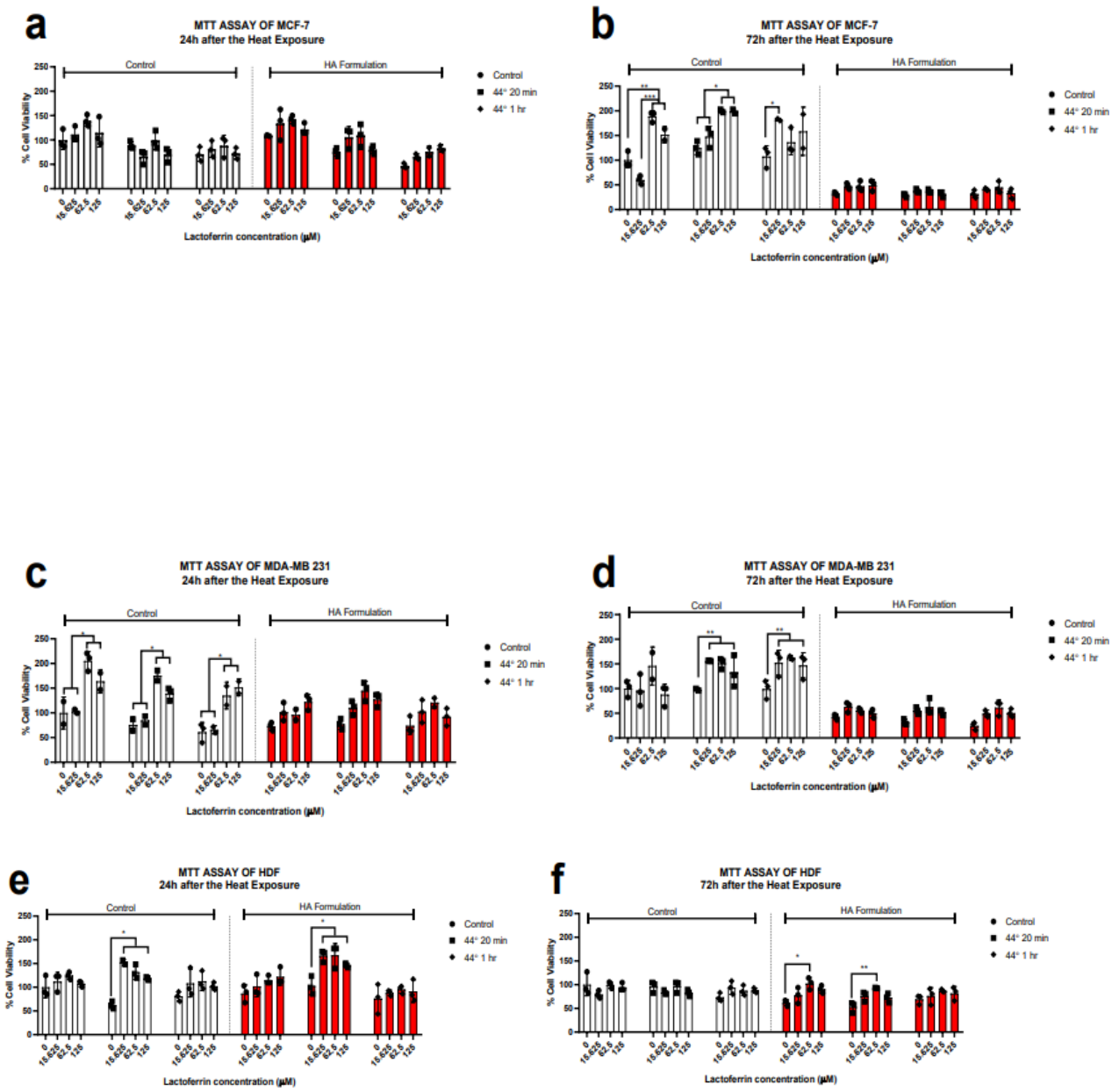

Figure 5. Effect of Lactoferrin treatment (0, 15.625, 62.5, 125 µM) when applied with different heat exposures (Control, 44ºC 20 minutes and 44ºC 1 hour) and Hyaluronic acid Formulation solution after 24 hours on a) MCF-7, c) MDA-MB 231, e) HDF cell lines and after 72 hours on b) MCF-7, d) MDA-MB 231, f) HDF cell lines. Results represent an average of 3 technical replicates for each culture. Significant changes compared with control: *P < 0.05; **P < 0.01; ***P < 0.001; (Regular ANOVA and Tuckey's Multiple Comparison Test)

The heat was applied at 44ºC for either 20 minutes or 1 hour. The maximum applicable temperature was selected to obtain the maximum efficacy out of HT treatment. The gradual decrease in the cell viability was demonstrated on the MCF-7 cell line 24 hours after the heat exposure in a duration-dependent manner but there was no statistically significant change. The results supported the theory that HT is not sufficient alone to hamper tumor progression. Additionally, no significant effect was seen on MDA-MB 231 cell lines a non-significant slight decrease was observed in the cell viability of HDF cell lines. Indeed, it is desired to observe no significant change in the cell viability of HDF cells as they are healthy dermo-fibroblast cells.

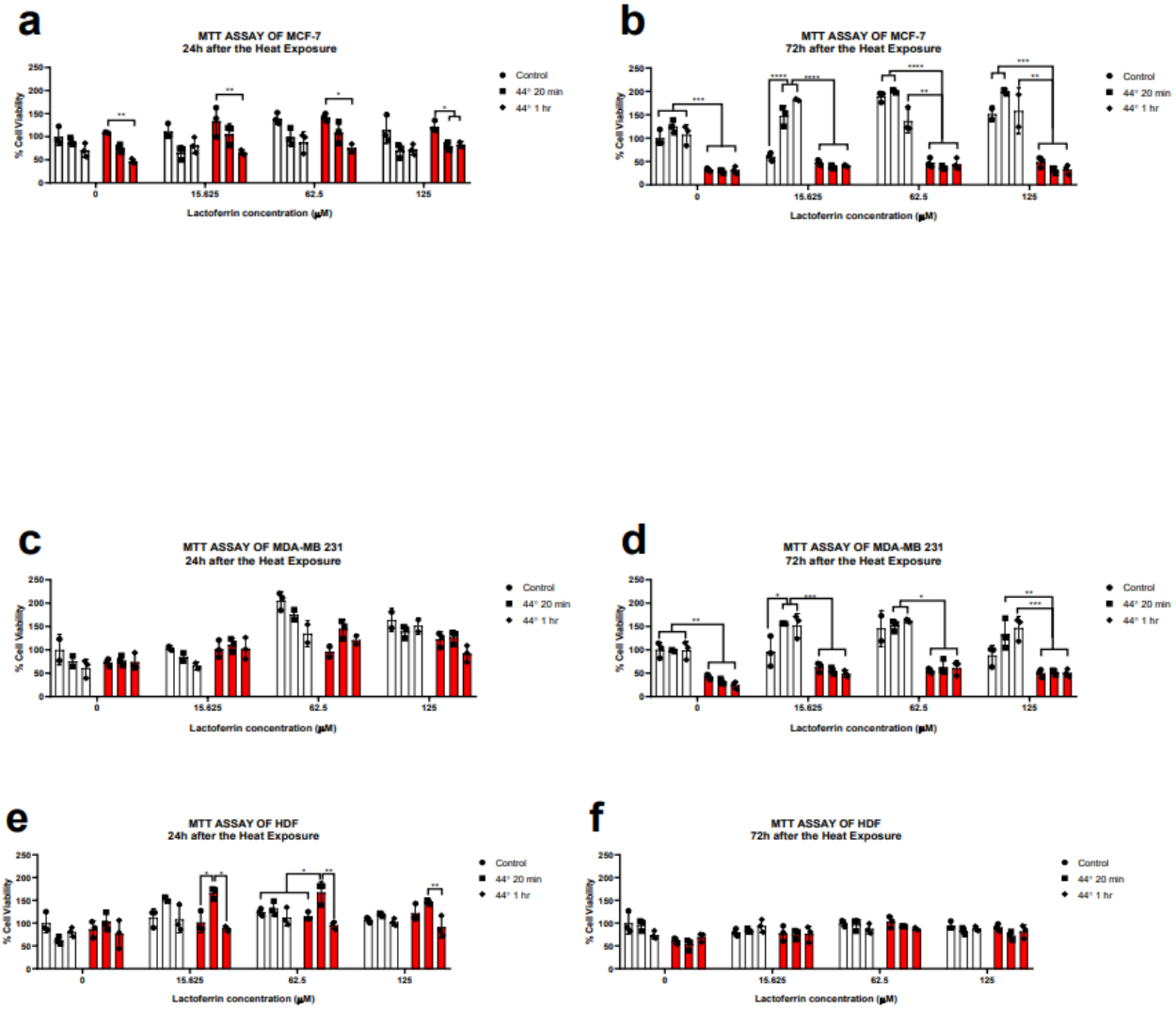

Figure 6. Effect of different durations of heat exposures (Control, 44ºC 20 minutes and 44ºC 1 hour) when applied with different concentrations of Lactoferrin treatment (0, 15.625, 62.5, 125 µM) and Hyaluronic acid Formulation solution after 24 hours on a) MCF-7, c) MDA-MB 231, e) HDF cell lines and after 72 hours on b) MCF-7, d) MDA-MB 231, f) HDF cell lines. Results represent an average of 3 technical replicates for each culture. Significant changes compared with control: *P < 0.05; **P < 0.01; ***P < 0.001; (Regular ANOVA and Tuckey's Multiple Comparison Test)

It showed that the induction of cell death HT was selective for cancer cells except the MDA-MB 231 cell line. MDA-MB 231 cells are triple-negative breast cancer cells that are extremely aggressive. It is initially believed that the lack of efficacy resulted from the aggressiveness of the cell line. One of the main reasons for aggressiveness is known to be associated with the p53 status of the cancer cells. On top of that, an extensive literature search revealed that HT-induced cell death mainly occurs via p53-dependent apoptosis. MCF-7 cell line possesses wild type p53 gene whereas MDA-MB 231 cell line possesses a mutant p53 gene. HT induces the activation of wild-type p53 protein which promotes the cell cycle arrest and further apoptosis in cancer cells. However, when the p53 gene is mutant, the produced p53 protein will be ineffective in promoting cell cycle arrest. Thus, apoptosis could not be promoted. Subsequent experiments on the molecular characteristics of HT-applied MCF-7 and MDA-MB 231 cells could confirm the heat-induced p53-dependent apoptosis. This finding has potential for the future of genetic testing and the prescription of anticancer therapies. p53 status of the patients can be determined by prior genetic testing easily. Therefore, patients with a mutation of the p53 gene will not prescribed any form of HT treatment which would eventually increase the success rate of the HT therapy.

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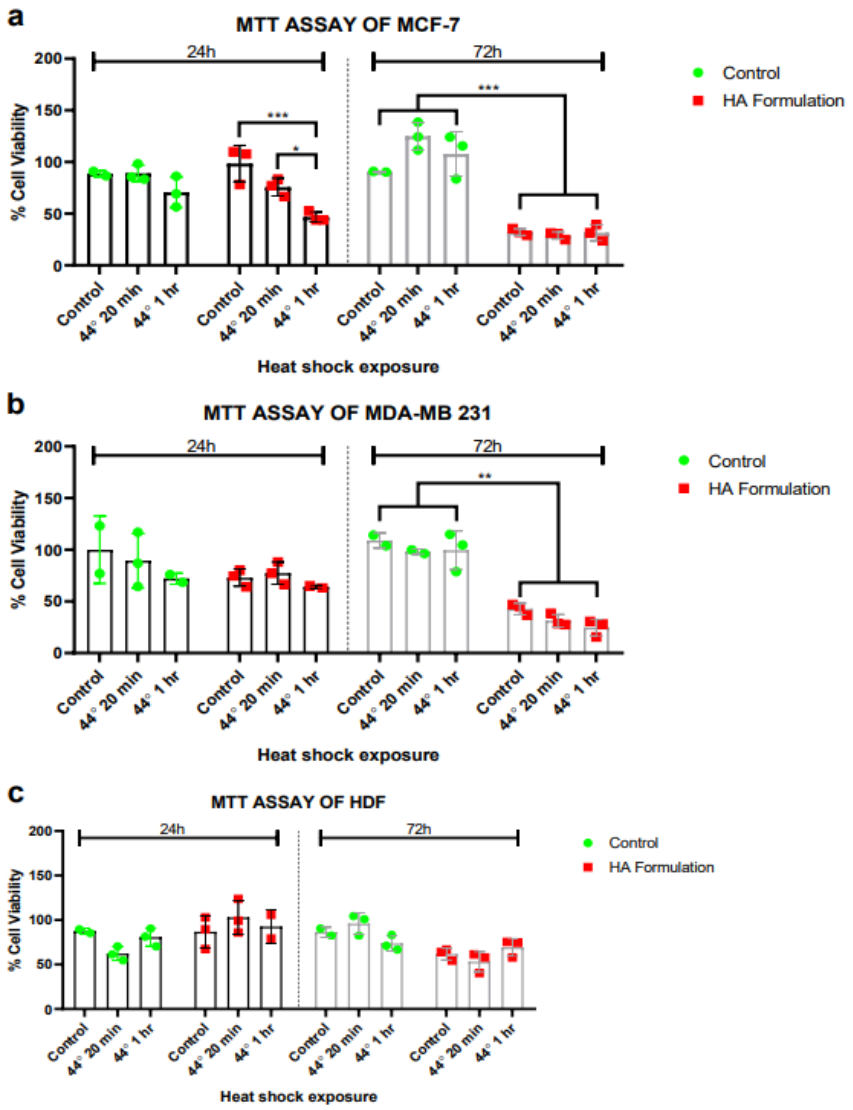

Figure 7. Effect of different durations of heat exposures (Control, 44ºC 20 minutes and 44ºC 1 hour) when applied with Hyaluronic acid Formulation solution after 24 hours and 72 hours on a) MCF-7, b) MDA-MB 231, c) HDF cell lines.Results represent an average of 3 technical replicates for each culture. Significant changes compared with control: *P <0.05; **P < 0.01; ***P < 0.001; (Regular ANOVA and Tuckey's Multiple Comparison Test

Afterward, the HA formulation solution with HT decreased the cell viability of the MCF-7 cell line after 24 hours in a duration-dependent manner. The use of the HA formulation solution increased the anti-proliferative capacity of HT. No significant effect was observed on MDA-MB 231 and HDF cell lines. However, it was clear that the application of HA formulation solution prevented the slight anti-proliferative effect of HT. Also with the heat exposure, the HA solutions show characteristics of hydrogels more than usual. The study was focused on the performance of acute HT applications. According to the results, the anti-proliferative effect of HT was diminished after 24 hours. The heat exposures must be performed periodically called chronic HT. Significant reductions in the cell viability of the cancer cells were observed 72 hours after the HA formulation solution.

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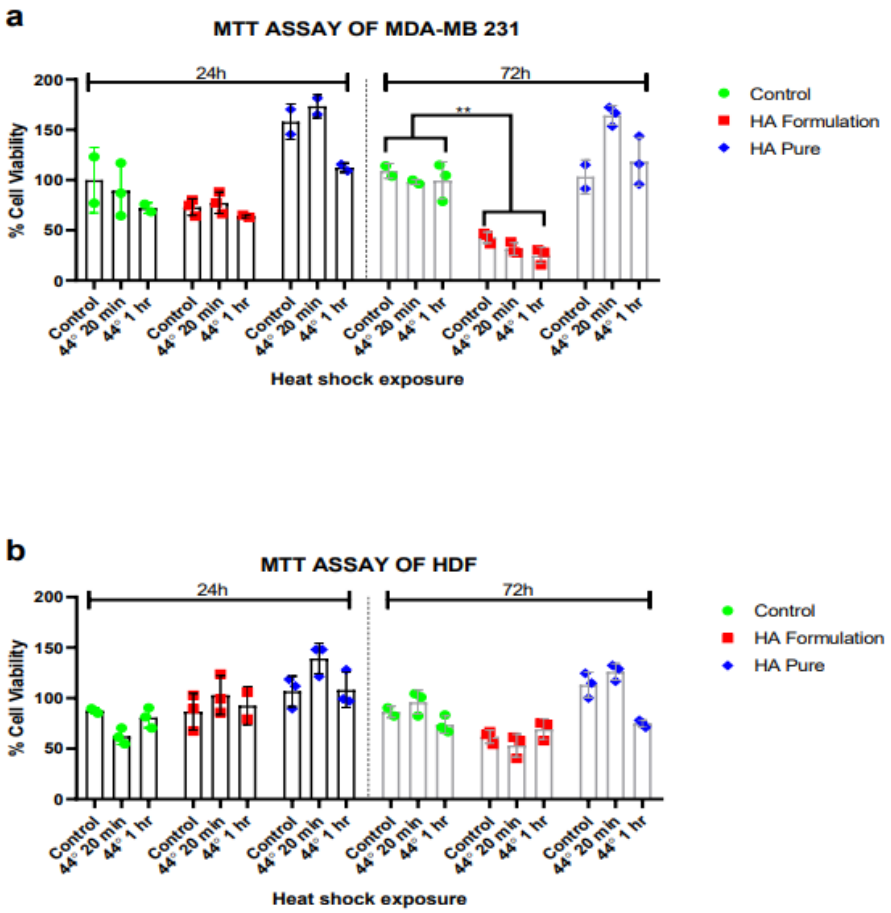

Figure 8. Effect of different durations of heat exposures (Control, 44ºC 20 minutes and 44ºC 1 hour) when applied with either Hyaluronic acid Formulation solution or Hyaluronic acid Pure solution after 24 hours and 72 hours on a) MDAMB 231, b) HDF cell lines. Results represent an average of 3 technical replicates for each culture. Significant changes compared with control: *P < 0.05; **P < 0.01; ***P < 0.001; (Regular ANOVA and Tuckey's Multiple Comparison Test)

No significant change was seen in the cell viability of HDF cells. The effect did not belong to the HT as its efficacy was diminished after 24 hours. The HA pure solution was performed to determine whether the anti-proliferative effect belongs to the HA. The antiproliferative effect was independent of the HA polymer HA itself increased the proliferation of cancer cells. An extensive literature search was performed for other excipients of the HA formulation solution which are Ethyl hexyl stearate, Sebacate, and Sodium isostearate. Neither of the chemicals was reported to cause cytotoxicity at the concentration. However, most studies in the literature on those chemicals were performed for shorter periods than 72 hours or in healthy cell lines. Also, health status is another parameter since it affects the characteristics of cells. Cell membranes of cancer cells are more permeable to chemicals. That's why, any of those chemicals can enter the cancer cells whereas the intact cell membranes of healthy cells prevent the entrance.

4. CONCLUSION

In conclusion, to determine the exact cause of anticancer activity, those previously mentioned chemicals must be further evaluated on MCF-7 and MDA-MB 231 cell lines alone. This research could be a breakthrough for a new selective chemotherapeutic agent with hyperthermia.

5. MATERIALS AND METHODS

Preparation of Lactoferrin and Hyaluronic acid formulations

Lactoferrin powder was purchased from Sigma Aldrich. UV sterilization was performed for the powder. Then, sterilized powder was dissolved in sterile PBS to obtain 12.5 mM as Lactoferrin solution inside a sterile safety cabin. The solution was vortexed for 5 minutes to obtain a completely homogenous solution. The serial dilution obtained other concentrations of lactoferrin solutions (6.25 and 1.525 mM). To preserve the stability of the lactoferrin solutions, they were made the same day before the experiment. The hyaluronic acid powder was purchased from Sigma Aldrich. UV sterilization was performed for the powder. Then, the sterilized powder was dissolved in sterile PBS to obtain %1 Hyaluronic acid solution. The solution was vortexed for 5 minutes to obtain a completely homogenous solution. %1 Hyaluronic acid solution was diluted with each complete cell culture medium (RPMI and DMEM High) to obtain % 0.1 Hyaluronic acid solution. HyaCare® Filler CL Hyaluronic acid solution was purchased from Evonik Corporation. It is a generic formulation that contains %1 Hyaluronic acid and %1 Ethyl hexyl stearate, %0.01 Sebacate, and %0.01 Sodium isostearate. UV sterilization was performed for the solution. This solution was further diluted with each cell culture medium (RPMI and DMEM High) to obtain a %0.1 Hyaluronic acid solution which was called as Hyaluronic acid formulation solution.

Cell Culture

MCF-7, MDA-MB 231, and HDF cell lines were obtained from the Yeditepe University, Molecular Diagnostic Laboratory. All cells were obtained in a frozen state and thawed into T-75 flasks. MCF-7 was cultured in RPMI complete medium with %10 FBS and %2 PSA whereas MDA-MB 231 and HDF were cultured in DMEM High complete medium with %10 FBS and %2 PSA. They were incubated at 37°C with %5 CO_2 and %95 O_2 in an incubator. Every two days the culture media were refreshed. Subcultivation with the ratio of 3:1 was performed by 0.005 M Trypsin solution when the cells reached %80 confluency in T75 flasks.

Lactoferrin, Hyaluronic Acid and Hyperthermia Treatments

MCF-7 and MDA-MB 231 cells were seeded onto a 96-well plate by 20.000 cells per one well while HDF cells were seeded onto a 96-well plate by 7.500 cells per one well on Day 0. They were placed in the incubator for 24 hours. After 24 hours, on Day 1, cell media were refreshed with either cell media (as control), Hyaluronic acid Pure solution, or Hyaluronic acid Formulation solution. Then, 2 µL Lactoferrin solutions with 3 different concentrations were applied to the media. Within 1 hour of the HA and LF application, cells were incubated in a heater at 44ºC for either 20 minutes or 1 hour. After heat exposure, cells were taken back to their incubator. Experimental groups that designed and codded as seen in Table 1.

Table 1. Designed experimental groups and their codes

	Control	44ºC 20 minutes	44ºC 1 hour
Control	Control	HT	HT
15.625 µM	LF	LF + HT	LF + HT
62.5 µM	LF	LF + HT	LF + HT
125 µM	LF	LF + HT	LF + HT
Control + HA	HA	HA + HT	HA + HT
15.625 µM + HA	LF + HA	LF + HA + HT	LF + HA + HT
62.5 µM + HA	LF + HA	LF + HA + HT	LF + HA + HT
125 µM + HA	LF + HA	LF + HA + HT	LF + HA + HT

MTT Assay

At either Day 2 or Day 4; MCF-7, MDA-MB 231, and HDF cell cultures were taken from the incubator and their cell media were removed. Cell media containing %10 MTT reagent was prepared. MTT reagents containing cell media were introduced into the cells and incubated for 2.5 hours. After 2.5 hours, absorbance was measured by the Varioskan LUX Plate Reader device at 590 nm.

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Author contributions: Concept –Resources –Materials –Processing and Interpretation –Literature Search -Writing – Critical Reviews – G.D., G.Z.T.D., F.K.

Conflict of interest statement: The authors declared no conflict of interest in the manuscript.

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