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In Vitro Model for Evaluation of Cancer Cell Proliferative Activity under Simulated Acidosis and Using Chitosan Microparticles

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Abstract: This investigation provides experimental data related to the development of a useful in vitro model allowing for a deeper analysis of invasive (metastatic) cancer cells using the effect of forced acidosis on the behavior of an epithelial colon cancer cell line. The results demonstrate that incubation in the medium with different pH values, adjusted by hydrochloric or lactic acids, increased more than 20 fold the proliferative activity of cancer cells at 48 h of incubation without affecting their cell viability. The newly synthesized chitosan micro-formulations were tested as a potential system for the screening of cancer progression, based on the attachment of cancer cells to the chitosan surface, without radically disturbing their viability. The latter could allow us to develop a versatile and useful in vitro model for the detailed investigation of the biological and molecular biochemical processes of invasive (metastatic) cancer cells.

Keywords: chitosan micro particles; cancer cells; acidosis; cancer progression



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1. Introduction

Colon cancer is one of the most frequent human malignancies in the world [1–3]. Despite the progress in surgical techniques and systemic treatments, the majority of diagnosed patients die from the metastatic spread of tumor cells, generally into the liver and the peritoneum [2,4–6]. The biology of metastasis formation is described as an invasion–metastasis cascade in which tumor cells leave their primary site and form new colonies in distant tissues [7,8]. This process consists of five steps: local invasion of tumor cells into the surrounding matrix (Step 1), intravasation of tumor cells into the circulatory system (Step 2), systemic transportation of tumor cells (Step 3), extravasation of tumor cells into parenchyma of distant tissue sites (Step 4), and colonization of distant organs and the establishment of macroscopic tumors (Step 5). The pivotal roles in the promotion of the invasion–metastasis cascade are the mutual and interdependent interactions between cancer cells and their microenvironment [9]. Although it is known that the tumor environment is a complex network of inflammatory and immune cells, carcinoma-associated fibroblasts, hypoxia, signal molecules, and extracellular matrix components [9], the initial mechanisms responsible for the initiation of tumor cell invasion are still poorly understood.

Evidence suggests that environmental acidification in cancers occurs during the initial avascular phase of a carcinoma [10–12]. During the phase of rapid growth, in which tumors become vascularized, the supply of oxygen is limited because of the blood's sluggish perfusion through the heterogeneously distributed vascular network [13]. These cells encounter an oxygen-poor environment and, through the Pasteur effect, switch from

oxidative phosphorylated metabolism toward anaerobic glycolysis [14,15]. As a result, the proton (H^+) concentration increases within the lumen due to the diffusion limitation and increased production of lactate from hypoxic–glycolytic cells [16]. Consequently, a highly acidic lumen medium is achieved.

An appropriate intracellular pH has many diverse effects on cells, including an effect on proliferation rate [16,17], differentiation [18–20], metabolism [21,22], protein synthesis, and glycosylation [23]. For example, studies have demonstrated a relation between changes in pH and cell proliferation activity using human peripheral blood mononuclear cells (T cells) and murine lymphocytes. The results indicate that the increased proliferation rate induced a reduction in the pH values [18,24]. At the same time, intracellular acidification has been shown to be cytotoxic because of the induction of apoptosis [25,26]. To overcome the low intracellular pH, as a result of accelerated metabolism, adequate for uncontrolled cell proliferation activity as well as cancer progression, cancer cells use a broad range of mechanisms for removing acids in order to maintain an appropriate intracellular pH and avoid cancer cell death. In this respect, lactic acid and H^+ efflux through monocarboxylate transporters and Na-driven proton extrusion have been described, respectively [27,28]. As a consequence, a reverse pH gradient is formed and the pH of the extracellular space of cancer cells becomes more acidic compared with the lumen [13,28–30]. An earlier study by Hooly et al. included a comparative analysis of the extracellular pH values of 77 different human tumors and normal subcutaneous tissue. According to the study, the average pH values, corresponding to the tumor environment, were within the pH range of 7.25 ± 0.99 , whereas the pH value in normal tissue was $pH = 7.54 \pm 0.09$. They also reported that extracellular pH values are not related to the tumor histology, degree of differentiation, tumor size, patient age, or treatment history [31]. Because extracellular pH can affect cultured cancer cells in so many ways, it is important to clarify whether it plays a role in metastasis formation.

Recently, scientific efforts have been focused on continuing to develop screening methodologies and technologies for the prediction of cancer disease progression. The chemical structure of chitosan, including the abundance of amino and hydroxyl groups on its polypeptide chain, gives it plasticity, functional groups that are easily modifiable during chemical processing, and an increased positive charge under changes in pH environments [32]. The latter physicochemical property of chitosan prompted us to test it as a possible in vitro screening model for the rapid identification of invasive cancer cells that could lead to metastasis formation. Due to the wide range of biological properties of chitosan, including its biocompatibility [33,34], biodegradability [33,35,36], and antitumor activity [37–39], it can be successfully used as a microfluidic system. The results from an experimental study indicate that, due to its capacity to fix and support the adhesion, motility, and expression levels of gene markers of cancer stem cells [40], it could be used as a platform for researching the biology and biochemistry of invasive and potentially metastatic cancer cells.

The aim of this preliminary investigation was to develop a versatile and useful in vitro model allowing for a deeper analysis of the molecular processes in invasive (metastatic) cancer cells. The experiment was focused on two different aspects: exploring the effect of simulated acidosis on the proliferative activity of a colon cancer cell line (Colon 26) and investigating these cells' viability, and assessing the possible applicability of the newly synthesized chitosan micro-formulations as a potential screening method for the identification of invasive (metastasis) cancer cells.

2. Materials and Methods

Chemicals: Chitosan (medium molecular weight, CAS No.: 9012-76-4), lactic acid ($CH_3CH(OH)COOH$) (80%, Chintex, Dimitrovgrad, Bulgaria), Tween[®] 80 (CAS No.:9005-65-6, Sigma-Aldrich, Darmstadt, Germany), sodium tripolyphosphate (TPP) (CAS No.:7758-29-4, Acros Organics, Geel, Belgium), phosphate-buffered saline (PBS, P-3813, Sigma-

Aldrich), and hydrochloric acid (CAS No.: 7647-01-0, Sigma-Aldrich, Darmstadt, Germany) were used in the experiments.

Chitosan particle synthesis: The chitosan particles were synthesized by the ion gelation method [41] with modifications. A 0.5 % chitosan solution (40 mL) was prepared in 1% lactic acid under magnetic stirring. Then, 1% Tween 80 (20 mL) was added to the transparent acidic chitosan solution and 2% sodium tripolyphosphate (TPP) (32 mL) was gradually added drop by drop to the mixture, which was agitated for 40 min under magnetic stirring at 500 rpm. The obtained suspension was sonicated for 4 min in an ultrasonic homogenizer (SONOPULS HD 2070.2, BANDELIN electronic GmbH & Co. KG, Wertheim Germany) and subsequently centrifuged for 15 min at $5300 \times g$ in a Heraeus Labofuge 200 (Thermo Electron Corporation, Waltham, Massachusetts, USA). The obtained chitosan particles were washed with milli-Q water three times, freeze-dried in a laboratory freeze dryer (Biobase, BK-FD10P, Jinan City, Shandong Province, China), and stored in a desiccator for further use. The synthesized biopolymer particles were eco-friendly because they were made from natural products that are ecological and have no toxic side effects. The chitosan microparticles were stable after storage for 6 months, which was proven by periodic measurements of their size and an exploration of their morphology under a digital microscope.

Cells and treatment protocol: The experiments were performed on a colon cancer epithelial cell line (Colon 26) kindly provided by Prof. Bilyana Nikolova of the Institute of Biophysics and Biomedical Engineering, Bulgarian Academy of Sciences. The cells were cultured in Dulbecco's Modified Eagle's medium (DMEM, Sigma-Aldrich, Germany) with a high concentration of glucose (4500 mg/L) and supplemented with 10 % heat-inactivated fetal bovine serum (FSB, Sigma-Aldrich, Germany) and antibiotics (100 U/mol penicillin and 100 µg/mol streptomycin) in a humidified atmosphere at 37 °C with 5% CO₂. Twenty-four hours before the experiments were conducted, adhesive cells were trypsinized, centrifuged ($800 \times g/5$ min), counted (using the trypan blue method), and re-suspended in fresh medium without antibiotics but with specific pH values obtained previously. The re-suspension of the cancer cells in the medium without antibiotics was included in the treatment protocol in order to eliminate their potential to have an influence on the biochemistry of the cultured cells and an impact on the experimental endpoint [42]. Colon 26 epithelial cells were seeded in 12-well plates at a density of 5×10^4 cells per 1 mL of cultured medium and incubated in a humidified atmosphere at 37 °C with 5% CO₂. Untreated cells (cells incubated only in fresh medium without antibiotics and with a pH = 8.0) were used as controls for all experiments.

The cells were divided into three groups. To simulate different acidosis conditions, cells were exposed to a medium with a corrected pH. Two types of acids were used in the experiments: 1% lactic acid (group 1) and 0.1 mol/L hydrochloric acid (group 2). Different volumes from both types of acids were added drop by drop into a cell medium (pH = 8.0) under permanent mixing to obtain six different specific pH values (pH = 6.0, pH = 6.6, pH = 7.0, pH = 7.4, pH = 7.8, and pH = 7.9). Group 3 contained cells exposed to normal and pH-corrected mediums and treated with the newly synthesized chitosan microparticles applied at concentrations of 1000 mg/mL, 500 mg/mL, 200 mg/mL, 100 mg/mL, 50 mg/mL, 25 mg/mL, and 10 mg/mL. To determine the effect of the adjusted pH on cell viability and proliferative activity and to study the effect of the chitosan formulations on colon cancer progression, three incubation periods (24, 48, and 72 h) were studied in the colon cancer cell line.

Cell proliferation and metabolite activity: For the identification of viable, proliferate, and invasive cancer cells, the 3-(4,5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide (MTT) assay was used. MTT (5mg/mL) was dissolved in PBS (Sigma-Aldrich, Germany) and a volume of 50 µL was added to each well. After 3 h of incubation at 37 °C, 150 µL of MTT solvent was added to each well and again incubated for 15 min at room temperature in the dark in a shaker for the complete dissolution of MTT formazan crystals. The absorbance was measured at 570 nm using a Zenyth 200rt microplate reader (Biochrom).

The cell viability (percentage) of two or more dependent and independent experiments was calculated using the following equation:

$$\text{Cell viability (\%)} = (\text{OD of treated cells}) / (\text{OD of control cells}) \times 100,$$

where OD is the optical density of the culture.

Cell viability: Cell viability was analyzed by trypan blue staining method with automated counting, using an Automated Cell Counter (Invitrogen, OR, Waltham, Massachusetts USA). Summarily, 10 μL from cells suspension were mixed with 10 μL trypan blue in concentration 0.4% and the mixture was incubated for 30 seconds on room temperature. After that 10 μL from mixture was placed in a Countess® (Invitrogen) glass chamber and the cell viability was counted automatically.

Statistical analysis. All results are expressed as the mean $\pm$ standard deviation (SD) from three independent experiments with two parallel samples for each experiment ($n = 6$ for experiments with acids and $n = 10$ for experiments with chitosan particles). The comparisons between samples/groups were performed using Student's *t*-test and XL Stat for the Excel-One-test. Values of less than 0.05 were considered statistically significant.

3. Results

3.1. Effect of Simulated Acidosis on Cell Proliferation Activity

Figure 1 A, B demonstrate the “behavior” of cancer cells cultured on a medium with different pH values (from 6.0 to 8.0) achieved by lactic acid (1%) and hydrochloric acid (0.1 mol/L), respectively. The results shown in Figure 1A indicate an abrupt increase in the viability and proliferative activity of colon cancer cells after 48 h of incubation in the pH-corrected cell medium (from pH = 6.6 to pH = 7.9) adjusted with lactic acid as compared with the control group cells (pH = 8.0). An exception to the general tendency was noticed at pH = 6.0, where the proliferative activity was lower than in the control cells incubated in the normal medium. A decrease in cancer activity was observed after 72 h of incubation, as compared with incubation for 48 h, but these results are related to over-crowding of the cell mass due to the rapid cell proliferation. A deviation from this behavior was observed at pH = 6.0, where the proliferative activity of the colon cancer cells after 48 h of incubation was lower than that at 72 h. There was no change in the proliferative activity and viability after 24 h of incubation of cancer cells exposed to the medium containing lactic acid (from pH = 6.0 to pH = 7.9) compared to the control group (pH = 8.0), where a slight elevation in the proliferative activity was observed.

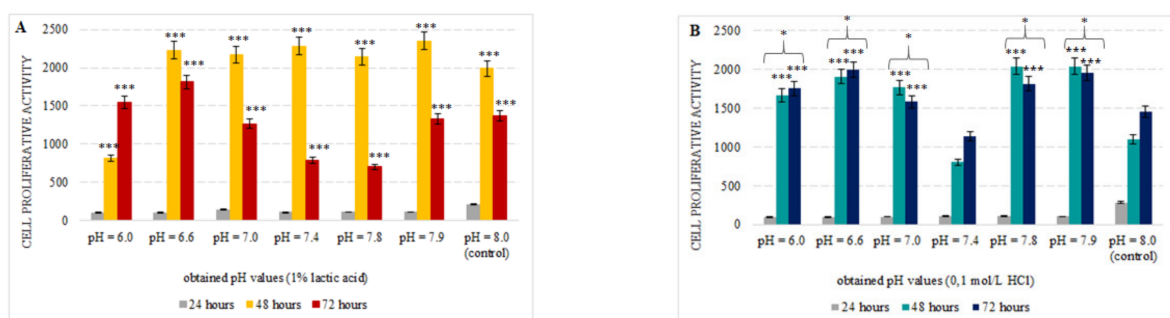


Figure 1. Cancer cell proliferation activity (Colon 26) after correction of pH values of the culture medium by 1% lactic acid (A) and by 0.1 mol/L hydrochloric acid (B) after 24, 48, and 72 h of cultivation. The levels of statistical significance ($*** p < 0.001$) in the individual group experimental series ($n = 3$) with lactic acid (group 1) or HCl (group 2) for 48 and for 72 h were determined. The level of statistical significance ($* p < 0.05$) between two periods of incubation (48 h and 72 h) with HCl was also determined.

A tendency for increased cell division and viability of colon cancer cells was also demonstrated after incubation with the medium containing 0.1 mol/L hydrochloric acid (Figure 1B). Generally, the obtained results in the medium with HCl (Figure 1B) are lower than those noticed in the medium containing lactic acid (Figure 1A). According to Figure 1B, the acceleration in cell proliferative activity occurred after 48 h of incubation in all tested pH-corrected cell medium ranges (from pH = 6.0 to pH = 7.9) compared with the absence of such activity under the same conditions but after 24 h of treatment. Compared to the results shown in Figure 1A, the cancer cells exposed to HCl did not show dramatic variations in cell proliferative activity and viability after 48 and 72 h of incubation, but a slight suppression of proliferative activity was noticed at pH = 7.0, pH = 7.8, and pH = 7.9. A special case was observed at pH=7.4, where a suppression of cell proliferative activity was established after 48 and 72 h compared with other tested conditions.

The data in Figure 2 A, B show the viability of colon cancer cells after incubation in the pH-corrected cell medium adjusted with lactic acid and hydrochloric acid, respectively. The obtained results are similar to those observed in the previous analysis.

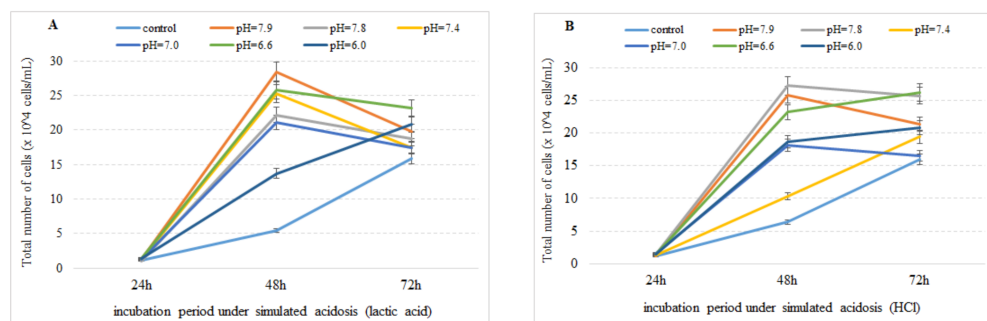


Figure 2. Colon cancer cell viability after seeding in a pH-corrected cell medium adjusted by lactic acid (A) and hydrochloric acid (B).

The characteristic sign of the viability of epithelial colon cancer cells is the formation of colonies by adhesion-molecule-mediated cell forces. A surprising tendency for elevated survival, but in a cell suspension, was observed for epithelial colon cancer cells exposed to the pH-corrected cell medium (under acidosis conditions) after 48 and 72 h of incubation. This finding was registered during the microscopic study (Figure 3), in which the cell suspension was formed from live cells, and a dramatic change in the shape of cells was observed (round cells). Such a morphological change, provoked by extracellular signals, is indicative of an increased tendency for metastasis formation and could be a possible reason for the release of cancer cells into the lymphatic system or blood stream.

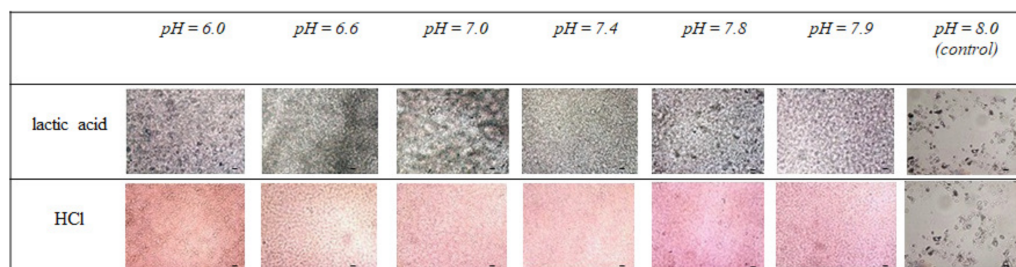


Figure 3. The formed cell suspension and induced morphological changes in the epithelial colon cancer cell line incubated for 48 h in a cell environment whose pH values were corrected with lactic and hydrochloric acids (magnification, 40× scale bar, 50 μm).

3.2. Chitosan Microparticles as a Potential Model for the Examination of Cancer Progression

Our research represents a pilot study whose results could provide a background for future in vitro investigations. The method for the synthesis of the novel chitosan

microparticles applied in this study was described in detail earlier (see Section 2). An examination established that the chitosan microparticles were stable after 6 months of storage. Their size was also determined using an Automated Cell Counter (Invitrogen, OR, Waltham, Massachusetts USA) with the option for bead counting. The results are displayed in Figure 4.

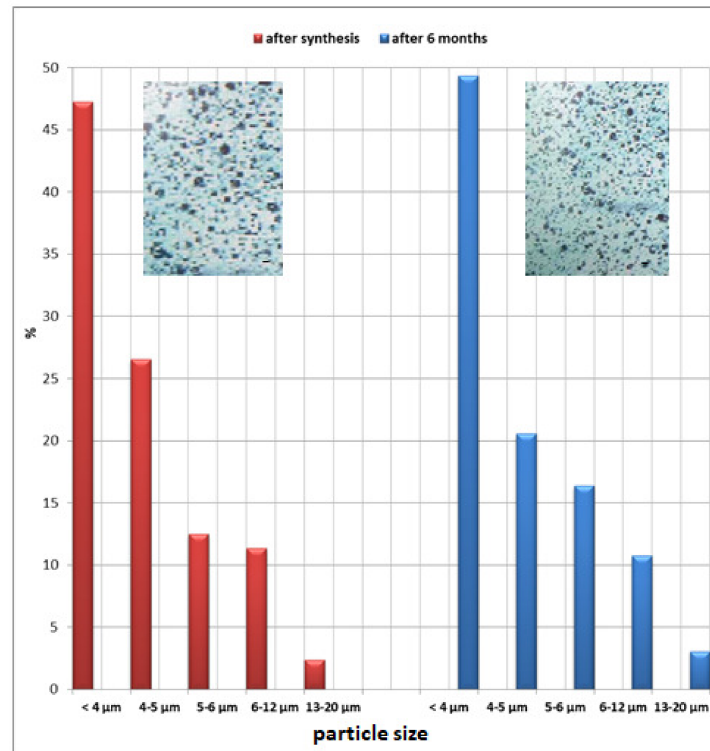


Figure 4. Size distribution of chitosan microparticles (scale bar: 50 μm).

The results shown in Figure 5 indicate that the synthesized chitosan particles, applied at different concentrations in the medium with corrected pH values on the colon cancer cell line, did not affect the viability of cancer cells after 24 h of incubation, similarly to the control cells (the red line in Figure 5). A lack of cancer cell–chitosan particle interactions was observed. However, after 48 h of incubation, during which the stimulation of proliferative activity due to acidosis was registered (Figure 1A,B), the chitosan particles showed a suppression of cell cancer division but did not affect cell viability. An exception was observed at a chitosan particle concentration of 1000 mg/mL and a pH = 6.0, where an increase in proliferative activity, similar to that induced by HCl, was registered. The tendency of particles to suppress cell proliferation was maintained after 72 h of incubation, but the induction of the signal pathway for cell death was not observed because the level of cell viability was not reduced compared to the level at the beginning of the experiment (the red line).

The microscopic study performed on the epithelial colon cancer cells exposed to the medium with corrected pH values (simulated acidosis) showed re-attachment and colonization of cancer cells on the chitosan surface in all tested concentrations after 48 and 72 h of incubation, without an effect on their viability (Figure 6). According to this observation, we suggest that cancer cell–chitosan particle interactions occurred at all tested concentrations of chitosan. Re-attachment of cancer cells to the bottom of the flask was not registered.

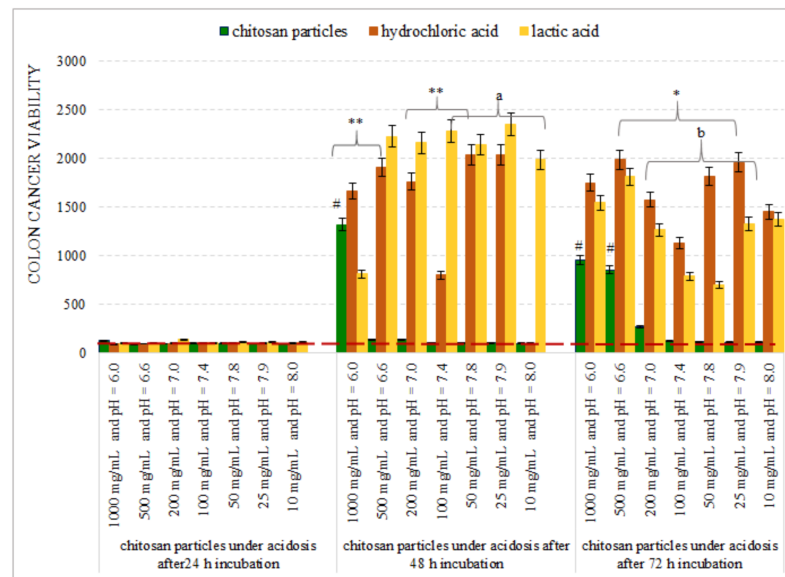


Figure 5. The effect of chitosan microparticles on the proliferative activity and viability of a colon cancer cell line incubated in a medium with pH-corrected values for 24, 48, and 72 h. The red line indicates the viability of colon cancer cells at the beginning of the experiment. Two levels of statistical significance between pH values adjusted by hydrochloric acid (* $p < 0.05$ and ** $p < 0.01$) or lactic acid (a $p < 0.05$ and b $p < 0.01$) were determined between the different applied concentrations of chitosan particles. The levels of statistical significance (# $p < 0.01$) in the experiments with chitosan particles were determined.

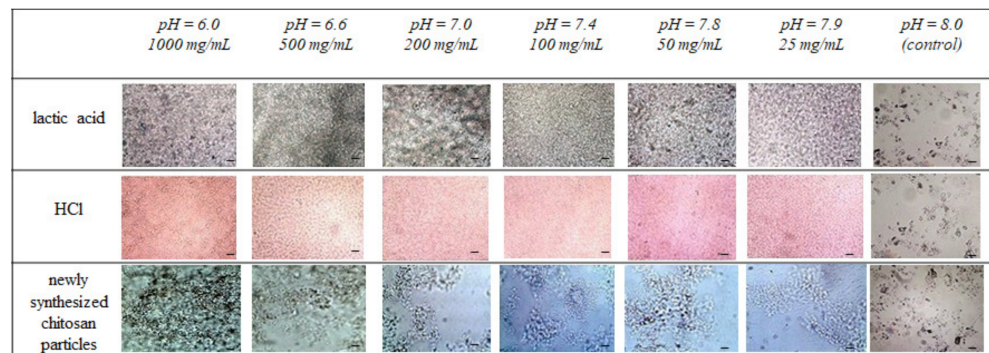


Figure 6. A microscopic photo of the effect of chitosan particles applied in different concentration ranges to colon cancer cells exposed to a forced acidosis cell environment for 48 h (magnification, 40 $\times$; scale bar, 50 μ m).

4. Discussion

Despite the progress in diagnostic and surgical techniques and systemic chemotherapy, the majority of patients diagnosed with primary colon cancer die due to metastasis formation, typically in the liver and peritoneum [2,4–6]. Hematogenous metastasis is a universal pathway by which tumor cells detach from the extracellular matrix in the primary tumor and spread via the bloodstream or lymphatic system in a suspension state. The tumor cells that circulate into the bloodstream are called circulating (invasive) tumor cells [43]. The evidence from studies on metastasis has demonstrated that, once the cells detach from the primary tumor, the circulating tumor cells seem to undergo dramatic morphological changes. Some of the reported scientific data are related to the rapid rounding of cells because of cell-adhesion-mediated properties, which are probably due to a decrease in the cytoskeletal tension and the degeneration of stress filaments as well as the loss of almost all biochemical and biophysical signaling from the extracellular matrix of

the primary tumor [44,45]. There are data that have demonstrated that the suspension state of hematogenous metastasis provides conditions conducive to the migration and re-attachment of cancer cells in vitro and metastasis formation in vivo [45,46].

Obviously, our results from the exposure of a colon cancer cell line to a simulated acidosis environment indicate that the minimal changes in pH values could be initiators of a radical increase in cell division and could provoke cancer progression. The latter observation was confirmed with a microscopic photo showing the existence of a typical adherent colon cancer cell line in a suspension state that is not typical for those cells as well as the formation of round live cells after 48 and 72 h of incubation. The suppression of cancer cell division after 24 h of incubation in the medium with corrected pH values was shown, and at 48 and 72 h an intense increase in proliferative activity occurred. These results could be interpreted using the circadian cell cycle, which for most cells finishes after 24 h. This period could be considered an adaptation period for cancer cells to new conditions. The high concentration of viable cancer cells in the suspension state up to 72 h could be associated with an increased probability for migration and probable metastasis formation. This study provides experimental results that indicate that the induction of acidosis in solid tumors is a major and critical factor in the formation of metastasis. In addition, acidosis may also be a necessary condition to avoid the native immune response. The observed results support the “acid-mediated tumor invasion” hypothesis, in which the progress of tumor cells is associated with the acquirement of resistance to acid-induced toxicity during carcinogenesis because of the changed bio-chemical metabolism compared with normal cells [47]. Additionally, the research of Estrella et al. has convincingly demonstrated that colon tumors secrete acid into their surrounding stroma and it is necessary for their local invasion [48].

The invention of an ideal, versatile, and useful model for the investigation of the biology and biochemistry of invasive (metastatic) cancer cells must satisfy some of the most general requirements. For example, it must be convenient, as it has to allow for mass production and cell cultivation for prolonged time periods. In this respect, the natural polysaccharide chitosan seems to provide favorable conditions for cell cultivation for long periods of time, as it has already been implicated in methods for 3D cultivation [49–51]. However, the involved interaction mechanisms between cells and biopolymers are still under investigation. Moreover, there are research data that have demonstrated the induction of a relationship between circulating (invasive) tumor cells and the chitosan biopolymer. For example, Rao et al. established a similarity between the chemical properties of chitosan and hyaluronan, a tissue polysaccharide, because of their capacity to bind cancer stem cells via the CD44 receptor, which is a major target gene of Wnt signaling [52]. Additionally, Chang et al. reported that chitosan has the ability to promote cancer stem cell-related characteristics and the progression of not only CD44-positive colon cancer cells but also CD44-negative hepatocellular carcinoma cells [40]. However, we do not currently have clear results depicting the influence of unloaded chitosan particles on the activity of invasive (metastatic) cancer cells.

Our study provides information on the effect of unloaded chitosan microparticles on the potential for the generation of an invasive metastatic epithelium colon cancer cell line, obtained by incubation in a pH-corrected cell medium adjusted by lactic or hydrochloric acid. We reported that the applied chitosan microparticles induced a radical reduction in the proliferative activity of colon cancer cells as compared with those induced by the individual application of both acid types, but the biopolymer formulation did not affect the cell viability. The microscopic study showed re-attachment of cells in the suspension and the formation of colonies from possibly invasive (metastatic) colon cancer cells.

The unique metabolic behavior of cancer cells that enables them to maintain accelerated anaerobic glycolysis is responsible for the generation of strongly negative charges on their surface [53]. Our results suggest that the deepening of glycolysis processes and the induction of acidification in the cell environment are tightly connected to invasive cancer progression and metastasis induction. The specific chemical structure of chitosan

as well as its physicochemical properties in acidic, neutral, and alkaline conditions are responsible for chitosan's complexation ability [54–56]. In more acidic conditions, specific to invasive cancer cells, the primary amine groups ($-\text{NH}_2$) on chitosan-based microparticles are protonated and thus acquire a positive charge ($-\text{NH}_3^+$) [57,58]. Thus, the specific electrostatic interactions between strongly negatively charged invasive (metastasis) cancer cells and positively charged chitosan microparticles could be initiated (Figure 7). Such a tendency provides a basic framework for conducting a deeper scientific analysis for the possible application of chitosan particles as a microfluidic system for the screening of cancer progression.

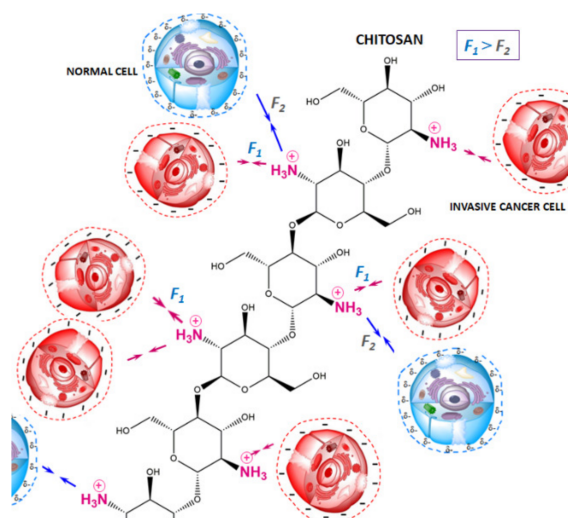


Figure 7. Possible electrostatic mechanism for the screening of invasive cancer cells and their differentiation from normal cells using chitosan-based microparticles. F_1 represents the strength of cancer cell–chitosan particle interactions. F_2 represents the strength of interactions between normal cells and chitosan particles.

5. Conclusions

To the best of our knowledge, this is the first time a study has provided evidence of the effect of forced acidosis on the behavior and proliferative activity of an epithelial colon cancer cell line. We demonstrated that incubation for 48 h in a medium whose pH values have been adjusted by hydrochloric or lactic acid increased more than 20 fold the proliferative activity of cancer cells without affecting their cell viability. Moreover, survival behavior that is not typical of colon cancer cells in a cell suspension and dramatic changes in shape were observed, which could be indicative of metastasis formation. The newly synthesized chitosan micro-formulations provide an opportunity for the re-attachment of colon cancer cells in a suspension to the chitosan surface without a radical disturbance of their viability. This provides a basis for deeper research into the possible application of chitosan-based particles as a screening agent and a component in microfluidic systems. The latter could allow us to develop a versatile and useful *in vitro* model for the detailed investigation of the biological and molecular biochemical processes of invasive (metastatic) cancer cells.

Author Contributions: Conceptualization, D.I. and Z.Y.; methodology, D.I., T.T. and Z.Y.; validation, D.I., Z.Y., T.T., S.S. and R.P.; formal analysis, R.P.; investigation, D.I. and Z.Y.; writing—original draft preparation, D.I.; writing—review and editing, S.S. and T.T. All authors have read and agreed to the published version of the manuscript.

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Informed Consent Statement: Not applicable.

Data Availability Statement: The data presented in this study are available on request from the corresponding author.

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Conflicts of Interest: The authors declare no conflict of interest.

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