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Breast cancer brain metastasis: evaluating the effectiveness of alginate-based organoids in metastasis modeling to replace matrigel

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Abstract

Background One of the most important and devastating side effects of breast cancer is brain metastasis. Our understanding of cancer heterogeneity is revolutionized by tumoral organoids and seems promising for personalized medicine. This study aimed to generate a hydrogel-based brain metastasis organoid.

Methods Mouse brain metastatic tumor cells (4T1B) were isolated and cultured from the brain metastasis lesions of mice with breast cancer. Different hydrogels, including alginate, carboxymethylcellulose, gelatin, collagen, and matrigel, were prepared. Pre-coated hydrogels in 96-well plates were treated with 4T1B cells. The morphology and viability of metastatic organoids were analyzed after 7 days.

Results According to our results, 4T1B cells formed semi-regular cluster structures in alginate hydrogel. In this group, the cell survival rate and formation of three-dimensional structures were significantly higher than in other groups.

Conclusion For organoid cultures, there's a lot of research on natural and synthetic scaffolds that are chemically or mechanically well-designed. In the present study, we used highly brain metastatic tumor cells and detected that alginate hydrogel is the best choice for organoid formation and breast cancer brain metastasis modeling.

Keywords Organogenesis, Biomaterials, Natural polymers, Breast tumo, Brain neoplasm

Introduction

Among the cancers in women, breast cancer is the most common type of malignancy [1]. Recurrence and metastasis of breast cancer occur in 70% of patients [2]. One of the most common side effects in breast cancer patients is brain metastasis. About one-third of patients who suffer from breast cancer have this complication [3, 4]. Several theories have been proposed to investigate and understand the behavior of metastases. Factors determining the metastatic process will be molecular and genetic characteristics of neoplastic cells, as well as biological conditions [5]. Studies and investigations using samples of affected patients [6] and related animal modeling related to brain metastasis [7, 8] help to better understand the pathophysiology of this disease.

FDA Act Update 2.0, enacted in December 2022, represented a significant departure from the requirement of animal testing for experimental medications. Although the use of animals in scientific studies is not prohibited, the law acknowledges its limits and allows researchers to apply cutting-edge non-animal techniques. In this

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respect, the shift to creating 3D models of human tissues and organs in vitro for the development of precise diagnostics and treatments is emerging as a paradigm shift in biomedicine research [9, 10]. Organoids have over time been widely employed for representing all 3D organotypic cultures deriving from basic human tissue, stem cells, and organ explants [11–13].

The development of tumor organ cultures has gained momentum due to their potential applications for testing and predicting drug responses in the context of precision cancer therapy. For brain metastasis, organoids derived from resected breast cancer brain metastasis were generated for real-time drug testing [14]. Among other things, the huge differences between current models of cancer and the tumor microenvironment (TME) of human patients are one of the obstacles to cancer therapy. By constructing tumor organoids, it is possible to recapture the pathophysiological properties of the TME in vitro [15].

Due to their high biocompatibility, exceptional permeability, and appropriate stiffness, hydrogels with 3D hydrophilic polymer networks are highly suitable as biomaterials for 3D culture. Biomaterials have been deemed effective options for in vitro engineering of tissue and organ models, including organoids, to maintain normal cell function and osmotic pressure. Hydrogels are used in cell culture to obtain soluble salts and growth factors through porosity and water [16, 17]. Hydrogels facilitate the transfer of nutrients, gases, and metabolic wastes between cells and the cellular ECM [18]. Also, hydrogels can imitate the native ECM's microenvironment by regulating their biochemical and physical properties, which can be used to control 3D cell behavior such as adhesion, proliferation, migration, differentiation, and cell-cell/cell-matrix interaction. In tissue/organ model systems and organoid development, functional hydrogels that can react to stress, temperature, pH, or light are a useful way to control the cellular microenvironment [19].

Although Matrigel is commonly used for organoid culture, variations in the chemical properties of different batches have been observed, and this has led to a lack of reproducibility during cell culture experiments. The composition of different commercial matrixes has not been clearly defined. The mechanical properties of matrigel have also been shown to vary according to individual batches. In addition, to achieve specific organ areas for each organ, Matrigel may not be easy to customize [20]. The optimal conditions for organoid culture may not include the necessary components in matrigel [21]. Furthermore, matrigel is susceptible to xenobiotic contaminants due to its animal origin, and the presence of growth factors (GF) and other biological proteins can result in detrimental effects on the cell [22]. Based on these drawbacks, there is a need to develop matrigel-independent organoid culture methods.

Hence, due to Matrigel's aforementioned undesirable properties, the search for fully synthetic and mechanical natural or synthetic scaffolds for use in organoid culture has begun. In the present study, we used highly metastatic brain cancer cells and investigated the potential of different hydrogels in forming organoid structures and their biocompatibility with cancer cells to replace Matrigel in modeling breast cancer brain metastasis.

Materials and methods

Cell culture

Breast cancer cells that metastasized to the brain were isolated and characterized according to our previous laboratory studies [23]. All animal experiments complied with the Ethics Committee of Shahroud University of Medical Sciences (registration number: IR.SHMU.REC.1399.096). Female BALB/c mice weighing 20–25 g were obtained from Royan Institute, Iran. For breast tumor induction, 4T1 cells (10^5 cells in 100 uL PBS) were subcutaneously injected into the flank of the mice. After 35 days, the mice were euthanized with anesthetic overdose of ketamine/xylazine. The brain of cancerous mice was surgically removed and digested mechanically and enzymatically. The digested organs were rinsed with PBS and passed through 70-um cell strainers. The isolated cells were grown in a humidified 5% CO_2 and 37 °C atmosphere in high-glucose Dulbecco's modified Eagle medium (DMEM; Gibco, USA), with 10% FBS and 2% penicillin-streptomycin (all from Gibco, USA).

Hydrogel preparation

The hydrogels used in our work are listed in Table 1. The alginate hydrogel was fabricated by dissolving 1/5% (w/v) sodium alginate in deionized water. Subsequently, 75 mM calcium chloride ($CaCl_2$) was added to the solution to crosslink the alginate, and the prepared mixture was stirred for 4 h. The gelatin hydrogel was fabricated by dissolving 3% (w/v) gelatin in deionized water. Subsequently, 0.05% (w/v) EDC (N-(3-dimethylaminopropyl)-N-ethylcarbodiimide hydrochloride) was added to the solution to crosslink the gelatin, and the prepared mixture was stirred for 2 h. The carboxymethylcellulose hydrogel was fabricated by dissolving 3% (w/v) carboxymethylcellulose in deionized water. Subsequently, 0.05% (w/v) EDC (N-(3-dimethylaminopropyl)-N-ethylcarbodiimide hydrochloride) was added to the solution to crosslink the carboxymethylcellulose, and the prepared mixture was stirred for 2 h. The matrigel and collagen hydrogel were prepared using a stock of matrigel from murine sarcoma (Sigma-Aldrich, St. Louis, MO) and rat tail collagen type I (BD Biosciences, San Jose, USA), respectively.

Table 1 List of hydrogels

Candidate	Ratio	Concentration	Polymer Preparations Information	Cross linker	Crosslinking Process Information
Matrigel	100	-	-	No	-
Collagen	100	-	-	No	-
Carboxymethyl-cellulose (CMC)	100	3% w/v	Water soluble, room temperature, stirring 8 h at 350 rpm	0.05% (w/v) EDC-NHS	Drop wise, room temperature, stirring 2 h at 250 rpm
Carboxymethyl-cellulose (CMC)	100	3% w/v	Water soluble, room temperature, stirring 8 h at 500 rpm	No	-
Gelatin (Gel)	100	3% w/v	Water soluble, temperature 40 °C, stirring 8 h at 450 rpm	0.05% (w/v) EDC-NHS	Drop wise, room temperature (PH = 6), stirring 2 h at 250 rpm
Gelatin (Gel)	100	3% w/v	Water soluble, temperature 40 °C, stirring 8 h at 450 rpm	No	-
Alginate (Alg)	100	1.5% w/v	Water soluble, room temperature, stirring 24 h at 500 rpm	75 mM calcium chloride ($CaCl_2$)	Drop wise, room temperature, stirring 4 h at 350 rpm
Alginate (Alg)	100	1.5% w/v	Water soluble, room temperature, stirring 24 h at 500 rpm	No	-

3D organoid formation

For 3D culture, 4T1B cells were detached from 2D culture flasks using 0.25% Trypsin-EDTA (Gibco), centrifuged at 300 × g for 3 min, and the cell pellet was re-suspended in cell media. Cells were counted using a hemocytometer, seeded at a density of 5 × 104 cells per well in a 96-well plate (100 µL molded gels), and cultured for 1, 7, and 14 days.

Morphological assessment

After the culture of 4T1B cells in different hydrogels, the Olympus CK30 microscope has been used for bright-field imaging of organoids. Different periods were analyzed for the size and morphology of organoid cultures.

Viability by MTT assay

To quantitatively assess the cytotoxicity of treated hydrogels, MTT assays have been used. Briefly, mouse 4T1B cells were cultured at a density of 5×10^3 cells on hydrogel in DMEM/F12 culture medium supplemented with 10% (v/v) FBS, 100 units/ml penicillin, and 100 ml streptomycin in an incubator at 37 °C with 5% CO_2. At each time point (7 days after cell seeding), the culture medium was removed from the 96-well plate, and 200 µl of MTT (0.5 mg/ml) was added to each well. The cells were then incubated at 37 °C for 4 h in the dark. The solution was discarded, and 100 µl of DMSO was added to each well to dissolve the formazan crystals that formed. Using a microplate reader, the absorbance values of the samples were measured at 570 nm after incubation (BioTek Cytation 5). 6 replicates were used for the synthesized and control samples.

Viability by alamar blue assay (ABA)

ABA was used to measure cell growth and proliferation. For Alamar Blue (AB), the stock solution is diluted in a ratio of 1:10 in DMEM/F12 without phenol red, sterilized through a 0.22 µm filter, and preheated to 37 °C. Conditioned media (CM) was removed from each well and replaced with a pre-warmed AB solution. Organoids were incubated in an AB solution for 90 min at 37 °C with 5% CO_2. Using a microplate reader, the absorbance values of the samples were measured at 570 nm after incubation (BioTek Cytation 5). ABA was performed 7 days after cell treatment. 6 replicates were used for the synthesized and control samples.

Statistical analysis

The results obtained in the laboratory are expressed and analyzed as the mean ± standard deviation. GraphPad Prism statistical software 9.0 has been used to analyze the data. (GraphPad Software, La Jolla, CA, USA) using an unpaired sample t-test. $P < .05$ was considered statistically significant.

Results

Brain metastatic tumor cell isolation

A metastatic breast cancer model was used from a Balb/c mouse, which was created after tumor induction within 35 days (Fig. 1A). Metastatic tumors spontaneously develop after injection of 4T1 cells into BALB/c mice so that they can spread to the brain while the primary tumor was proliferating in situ. To stimulate the growth of metastases, it is not necessary to remove the main tumor. On brain metastatic lesions, H&E staining and pathological confirmation were performed (Fig. 1B). In the brains of the cancerous mice, we've properly removed the metastatic tumor cells (Fig. 1B). After first isolation, malignant brain tumor cells form colonies in the culture. The tumor cells from these colonies have been purified after three passages due to their high growth and proliferation rates. These tumor cells, i.e., 4T1B brain metastatic tumor cells, are referred to as such (Fig. 1B).

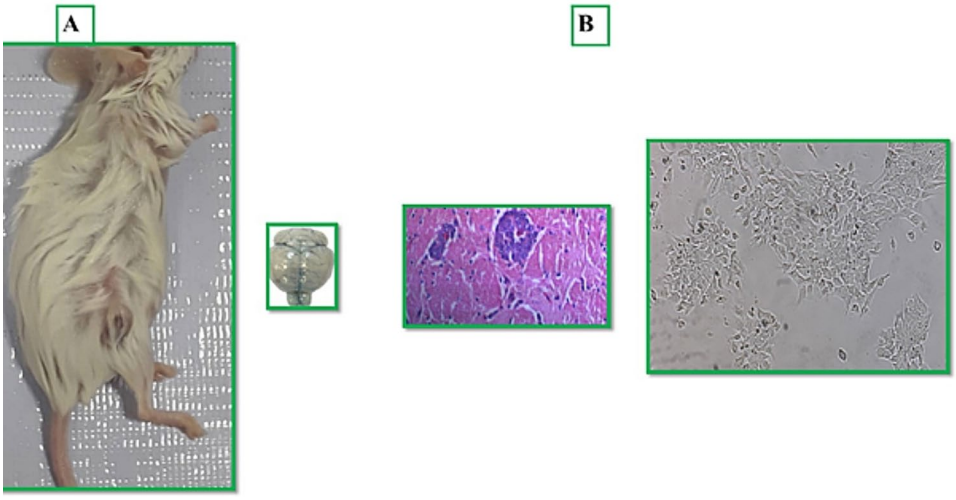

Fig. 1 Brain metastatic tumor cell isolation. (**A**) Metastatic animal model of triple-negative breast cancer was generated after 35 days of tumor induction in Balb/c mice. (**B**) Brain metastatic tumor isolation, H&E staining, and metastatic tumor cell extraction were performed on the brains of cancerous mice

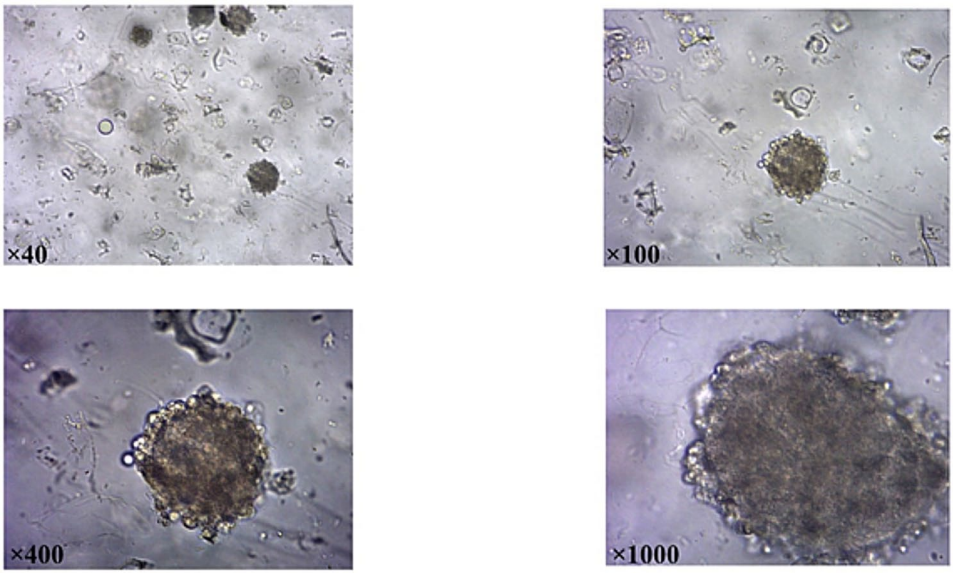

Fig. 2 Establishment of Brain Metastatic Breast Cancer Organoids. Representative images of organoids derived from 4T1B cells in alginate. In alginate hydrogel, 4T1B cells had structurally formed semi-regular cluster structures

Establishment of brain metastatic breast cancer organoids

To explore the properties of hydrogels on organoid formation, we established an organoid model from 4T1B cells. In alginate hydrogels, morphological observations showed that most metastatic organoids had a cystic or solid phenotype, and some cases showed a grape-like morphology. (Fig. 2). In this group, 4T1B cells formed semi-regular cluster structures, and the formation of three-dimensional structures was significantly higher than in all groups. Between different hydrogels, the size and morphology of metastatic tumor cell cultures varied greatly, generating 2D monolayer, solid, cystic, cribriform, and "grape-like" structures.

Evaluation of the cellular viability of 3D organoids

According to our results in alginate hydrogel, the rate of cell survival and formation of three-dimensional structures were significantly higher than in all groups. As shown in Figs. 3 and 4, significantly higher viability of 3D metastatic organoids was detected in the alginate group after culturing for 3 and 7 days.

Discussion

Organoids can be used for a variety of purposes in biology, enabling the modeling of both developmental and disease processes. For all types of ex vivo cultures, Matrigel hydrogel is currently the gold standard. Organoids derived from tissue biopsy would have a strong

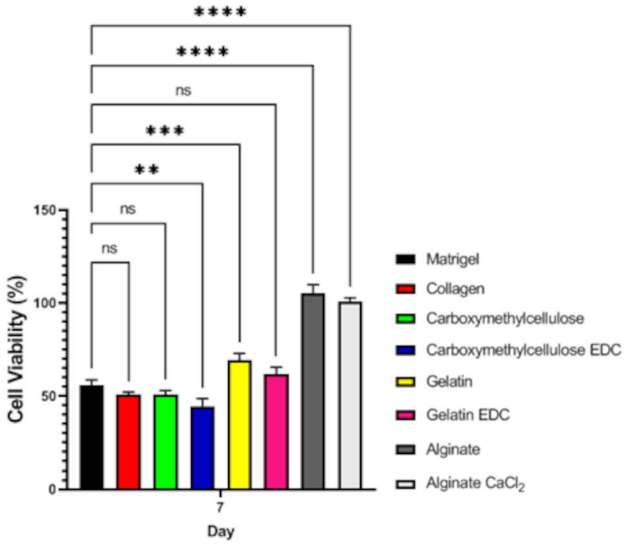

Fig. 3 MTT Viability Assessment of Brain Metastatic 3D Multicellular Organoids on Different Hydrogels. 4T1B cell viability in 3D multicellular organoids after 7 days of culture was assessed with MTT. In the alginate group, the cell survival rate and formation of three-dimensional structures were significantly higher than in all groups. The values indicate the mean ± standard deviation, with a sample size of 6. Significance levels are expressed as: ****$p < .0001$, ***$p < .001$, **$p < .01$, *$p < .05$, and ns = non-significant

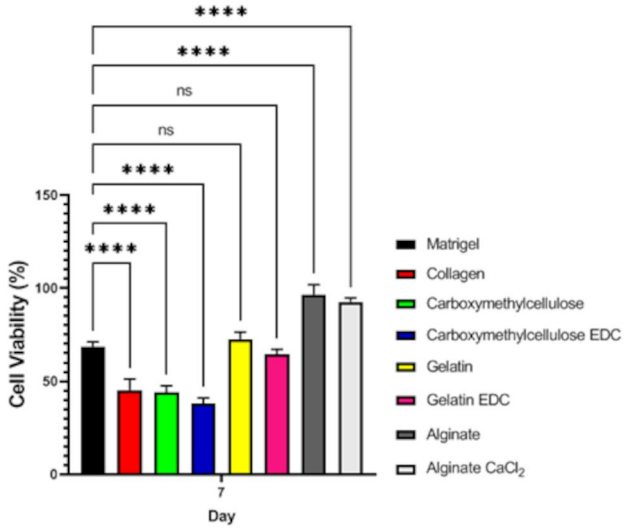

Fig. 4 AlamarBlue (ABA) Viability Assessment of Brain Metastatic 3D Multicellular Organoids on Different Hydrogels. 4T1B cell viability in 3D multicellular organoids after 7 days was assessed with ABA. Compared with other used materials, in the alginate group, the cell survival rate and formation of three-dimensional structures were significantly higher than in all groups. The values indicate the mean ± standard deviation, with a sample size of 6. Significance levels are expressed as: ****$p < .0001$ and ns = non-significant

resemblance to the tumor microenvironment and appear to be better suited for ex vivo modeling of parental tumor tissues. But for each type of tissue organoid, we need to define and customize hydrogel with a combination of naturally occurring and synthesized polymers [20]. In the present study, we used highly metastatic brain tumor cells. After the preparation of different hydrogels, our results showed that alginate hydrogel is the best choice for organoid formation and breast cancer brain metastasis modeling.

Bastien Laperrousaz et al. were found by direct transfection of clonal organoids in matrigel microbeads, which achieved 80% transduction efficiency by electroporation of matrigel-encapsulated organoids using this technique and a morphological study that summarizes the different stages of tumor growth [24]. Matrigel is the most common hydrogel for organoid culture, there have been reports of changes in its biological properties from batch to batch that led to a lack of reproducibility in cell culture studies. The composition of different commercial Matrigels has not been clearly defined [25]. The mechanical properties of matrigel have also been shown to vary from batch to batch, such as elastic modulus. In addition, to obtain the special organ niches of particular organs, Matrigel cannot be easily adapted. In addition, it is not suitable to use organoids based on Matrigel for in vivo studies and cell therapy [26, 27]. Additionally, matrigel is derived from mouse tumors and has not been well characterized, which is not ideal for in vivo tissue regeneration. These undesirable properties of Matrigel triggered the search for chemically and mechanically well-defined natural and synthetic biomaterials for organoid culture. In our study, cultured metastatic tumor cells were unable to form regular 3D organ structures when cultured on Matrigel.

Other types of hydrogels, such as those containing collagen, fibrin, and hyaluronic acid, have several advantages compared to alginate hydrogels for organoid growth. Alginate gel causes little tissue disruption at neutral pH and RT (room temperature) because the gel encapsulates the organic matter [28]. Alginate may also be adapted by altering the stiffness of hydrogels and conjugating hydrogels with adhesion molecules to carry out additional studies on the effects of biophysical and chemical factors on organoleptic maturation [29]. Moreover, it does not react to cellular proteins and thus supports the growth of tissues in a chemically inert manner [30]. Alginate is an ideal alternative to Matrigel, in particular when a well-defined 3D culture condition is desired, according to various studies [20, 31–33].

In a study on high-throughput drug screening and non-adherent alginate breast tumor organoid culture, Guocheng Fang et al. discovered that alginate microbeads exhibit distinct responses to doxorubicin and latrunculin A, with changes in organotic pressure and size affecting the absorption of drugs. This platform offers a versatile and inexpensive strategy for the luminal mechanical analysis, efficient fabrication, and drug screening of breast tumor organoids, but further studies are needed for other

cancer lines and performance evaluation [34]. In another study by Megan M. et al. to investigate breast cancer progression in an immune environment, the researchers developed an organoid model and subsequently discovered that alginate is essentially a minimally supportive hydrogel that lacks cell fusion properties, inhibits the growth of human intestinal organoids (HIO) in vitro, and induces epithelial differentiation of HIOs that is virtually indistinguishable from Matrigel-grown HIOs. Furthermore, alginate-grown HIOs are as mature at transplantation as matrigel-grown HIOs, both of which resemble human fetal gut. As demonstrated in this work, a simple and inexpensive hydrogel can be used to provide mechanical support for HIO survival and development [35].

In our work, when cultured on alginate hydrogel, the metastatic tumor cells had structurally formed semi-regular cluster structures, and the formation of three-dimensional structures was significantly higher than all groups. In addition, the cell viability rate is also higher than in other treatment groups. Alginate is an efficient substitute for Matrigel culture systems, which eliminates the need to produce animal-derived materials and reduces costs, thereby increasing translation potential since it shares similarities with Matrigel. The alginate used in our experiments costs approximately 700 to 900 times less than Matrigel ($0.44) versus $300 to $400 Matrigel per 10 mL, depending on the type of alginate used, presenting a critical cost advantage in basic studies [35].

As a natural matrix, alginate has limitations in batch variability between batches, as with Matrigel [36], However, our experiments have shown that alginate is capable of optimally supporting the growth under a variety of conditions. Therefore, there should be no major change in the matrix efficiency due to slight variations from batch to batch. To support other 3D culture systems simply and cost-effectively for the advancement of regenerative medicine, this system described below will likely be implemented.

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Author contributions
Sepehr Zamani: Performing the Experiments (in vitro Study), writing and reviewing.Fatemeh Sadat Bitaraf: Performing the Experiments (in vitro Study).Mohammad Kamalabadi Farahani: Conceptualization, Methodology, Performing the Experiments (Animal Study, Cell Culture), Supervising the Experimentators, Writing the original draft.

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Data availability
No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate
This study was approved by the Ethics Committee of Shahroud University of Medical Sciences (registration number: IR.SHMU.REC.1399.096). No specific consent was needed for the current investigation. All methods were performed following the relevant guidelines and regulations.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

Conflict of interest
The author declares that they have no competing interests.

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