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Three-dimensional Culture of Buffalo (*Bubalus bubalis*) Mammary Epithelial Cell Line

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ABSTRACT

Background: The mammary gland is a crucial organ in dairy animals, responsible for milk production, regulated by intricate hormonal and cellular interactions. Traditional two-dimensional (2D) cell culture systems used in mammary gland research fail to replicate the complex *in vivo* environment, limiting the understanding of mammary gland biology. The study explores the advantages of three-dimensional (3D) culture systems for Buffalo Mammary Epithelial Cells (BuMECs), aiming to enhance morphological and functional differentiation compared to 2D cultures.

Methods: BuMECs were cultured in Dulbecco's Modified Eagle's Medium (DMEM/F12) supplemented with 10% FBS and specific growth factors. For 2D culture, BuMECs were grown on a plastic substratum. For 3D culture, cells were cultured on top of and embedded in growth factor-reduced (GFR) Matrigel. Morphological differentiation was observed under phase-contrast microscopy. Total RNA was extracted and cDNA was synthesized to measure β-casein gene expression using quantitative real-time PCR (qPCR). Protein expression was analyzed by Western blotting, targeting total casein.

Result: BuMECs grown in 3D cultures exhibited enhanced morphological differentiation, forming alveolar and ductal structures, unlike the monolayer formation observed in 2D cultures. Rapid formation of duct-like structures was noted within 24 hours in 3D cultures. Functional differentiation was significantly improved, evidenced by a five-fold increase in β-casein mRNA expression and higher protein levels of α, β and κ-casein in 3D cultured cells compared to 2D cultures. Western blot analysis confirmed the elevated casein protein expression in 3D cultures.

Key words: Buffalo, Mammary Epithelial Cell Line, Mammary Gland, Three-dimension.

INTRODUCTION

The mammary gland, a key organ in dairy animals, is responsible for milk production, a process intricately regulated by hormonal control and cellular architecture. Understanding mammary gland biology is essential for improving lactation efficiency and milk quality in livestock, notably in buffaloes, which are significant milk producers in many parts of the world, including South Asia and the Mediterranean (Nanda and Nakao, 2003). Traditional studies on mammary gland biology have predominantly utilized two-dimensional (2D) cell culture systems. However, these systems fall short of replicating the complex *in vivo* environment, limiting our understanding of cellular interactions and functions within the mammary tissue (Barcellos-Hoff *et al.,* 2013). The emergence of three-dimensional (3D) cell culture technologies marks a significant advancement in mammary gland research. 3D cultures provide a more physiologically relevant environment, allowing cells to maintain their native polarity and to interact with their surroundings in a manner that closely mimics *in vivo* conditions (Weigelt *et al.,* 2010). This approach has been validated by various studies to enhance the functional differentiation of mammary epithelial cells by providing a more physiologically relevant environment (Nurye and Mummed, 2023). Additionally, studies have shown that DNA methylation plays a crucial role in the inflammatory response and gene expression in mammary epithelial cells, impacting milk production and quality (Dong *et al*., 2021). Furthermore, the role of hormones in mammary gland activity and its correlation with milk components underscore the importance of understanding the biochemical environment for optimizing lactation (Li *et al*., 2020). This is particularly important for mammary epithelial cells (MECs), which exhibit distinct functional behaviours in response to the 3D architecture, including differentiation and milk production capabilities (Roskelley *et al*., 1994).

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Buffalo mammary epithelial cells (BuMECs) present a unique model for studying lactation biology due to the species' high milk fat content and disease resistance. However, research on BuMECs has been constrained by the limitations of 2D culture systems, which do not adequately represent the mammary gland's complex structure and microenvironment. This gap underscores the need for 3D culture systems that can more accurately model the buffalo mammary gland, facilitating studies on tissue development, function and disease. Three-dimensional culture systems, including spheroids, organoids and matrices, have been employed to recreate the mammary gland architecture. These systems have provided insights into the cellular and molecular mechanisms underpinning mammary gland development, differentiation and pathogenesis (Schedin and Keely, 2011). For instance, organoid cultures derived from primary mammary cells have elucidated pathways involved in mammary gland morphogenesis and the role of the extracellular matrix in guiding cell differentiation (Simian *et al.,* 2001). 3D cell culture systems have been adapted for bovine mammary epithelial cells, using primary cells isolated from enzymatically digested mammary tissue. Various studies have demonstrated the effectiveness of different ECM components, such as collagen and laminin, in promoting cell differentiation and milk protein synthesis. For instance, primary bovine mammary epithelial cells cultured in collagen and floating collagen gels have shown polarized conformation and differentiated status, producing key milk proteins like casein (Talhouk *et al.,* 1993). Research has extended the use of 3D culture systems to include bovine mammary epithelial cell lines, such as BME-UV1 and MAC-T, alongside primary mammary epithelial cells. However, a 3D culture system developed from an established buffalo mammary epithelial cell line has not been reported. Adapting 3D culture techniques for BuMECs could significantly enhance our understanding of buffalo mammary gland biology, with implications for dairy production and mammary gland health. By offering a more representative model of the mammary tissue, 3D cultures can facilitate in-depth studies on the effects of hormones, growth factors and mechanical cues on BuMEC function. Furthermore, these cultures can serve as valuable platforms for investigating mammary gland diseases, such as mastitis, which poses a significant challenge in buffalo dairy farming (Sharma *et al.,* 2011). Moreover, cellular agriculture technology has highlighted the potential for cell-cultured milk production, emphasizing the structural, functional and productive aspects of the 3D culture of mammary epithelial cells (Kwon *et al.,* 2024). The present study attempts to grow BuMECs on top and embedded in ECM for their morphological and functional differentiation.

MATERIALS AND METHODS

Preparation and culture of buffalo mammary epithelial cell line (BuMECs) in plastic surface and in extracellular matrix

The buffalo mammary epithelial cell line (BuMEC) established by Anand *et al.* (2012) at Animal Biotechnology Centre, National Dairy Research Institute, Karnal was used as a model system. The current investigation was conducted between early 2022 and mid-2023. The BuMECs were cultured in Dulbecco's Modified Eagle's Medium (DMEM/F12) supplemented with 10% FBS, 5 µg/ml bovine insulin (Sigma, USA), 1 µg/ml hydrocortisone (Sigma, USA), 10 ng/ml EGF (Sigma, USA) and 50 ng/ml amphotericin. After reaching confluency, the cells were rendered competent for hormonal induction by cultivation in serum starvation medium lacking fetal bovine serum and additional growth factors to promote differentiation for 24 hours. Subsequently, the cells were induced by incubation at 37°C under 5% CO_2 for 3 days in a medium containing 1 µM dexamethasone (Sigma), 5 µg/ml insulin and 5 µg/ml prolactin (Sigma). For on-top culture over ECM, tissue culture plates were coated with growth factor-reduced (GFR) Matrigel (BD Biosciences, USA). Matrigel was thawed at 4°C and coated on prechilled culture plates at a concentration of 50 µl/cm² using prechilled tips. The coated plates were incubated at 37°C for 30 minutes. BuMECs were seeded at a density of 50,000 cells/well in complete growth medium with hormonal supplements. The medium was replaced every 48 hours until the end of the experiment. For growing BuMECs embedded in ECM, BuMECs were suspended in 50 µl of GFR Matrigel at a concentration of 2,000 cells/well and seeded in a 96-well plate. The Matrigel was allowed to solidify and growth media was added on top of the gel. Morphological changes were observed under a phase-contrast microscope.

RNA isolation and cDNA preparation

Total RNA was extracted from proliferating and functionally differentiated buffalo mammary epithelial cells (BuMECs) using Trizol Reagent (Invitrogen, USA) following the manufacturer's protocol. The extracted RNA was reverse transcribed into cDNA using SuperScript™ Reverse Transcriptase (Invitrogen, USA), according to the manufacturer's

Table 1: The primer sequences used in real-time PCR analysis.

Genes	Primer sequences	Accession number
β- Casein	5′AATATCCAGTTGAGCCCTTTAC3′ (F)	DQ317447
	5′CTTAGACAATAATAGGGAAGGG3′ (R)	
β- Actin	5′AAGATGACCCAGATGATGTT 3′ (F)	NM_001290932
	5′GGATCAGCAAGCAAGCAGGAGTAC3′ (R)	

instructions. The beta-casein gene fragment was amplified from the synthesized cDNA using specific primer pairs, while the beta-actin gene, used as an internal control, was also synthesized using specific primer pairs (Table 1). PCR amplification was performed for 25-30 cycles under the following conditions: initial denaturation at 95°C for 5 minutes, denaturation at 95°C for 30 seconds, annealing at 58°C for 30 seconds and extension at 72°C for 1 minute. Relative quantification of the PCR products was conducted by comparing the threshold cycle (Ct) values of different samples using SYBR Green master mix (Roche, USA) and a Light Cycler (Roche, USA). The comparative Ct method (ΔΔCt method) (Livak and Schmittgen, 2001) was employed to analyze the relative expression levels of the beta-casein gene, normalized to the beta-actin gene. This method allowed for the quantification of gene expression changes between BuMECs grown on plastic and those grown on ECM.

Western blot

The 2D and 3D cultured BuMECs were washed with Dulbecco's Phosphate Buffered Saline (DPBS) and protein lysates were prepared from the monolayered cells as well as the cells cultured on top of the matrix and embedded in the matrix using RIPA lysis buffer (Sigma, USA). For 3D growth, the cells were recovered using Dispase enzyme and lysates were prepared using RIPA buffer. Protein lysates were subjected to SDS-PAGE and the resolved proteins were blotted onto PVDF membranes and blocked overnight with NAP-Blocker (G Biosciences, USA) at 4°C. The membrane was then probed with rabbit total casein primary antibody at a dilution of 1:2,000 for 1 hour at room temperature. Membranes were washed three times, each for 15 minutes, with TBST, then incubated with HRP-conjugated goat anti-rabbit secondary antibody diluted 1:5,000 in blocking buffer for 1 hour at room temperature. After washing three times (15 minutes each) with TBST, chemiluminescent detection was performed using a substrate (Pierce, USA) and the results were scanned with a Typhoon Trio+ laser imager (GE, USA).

Statistical analysis

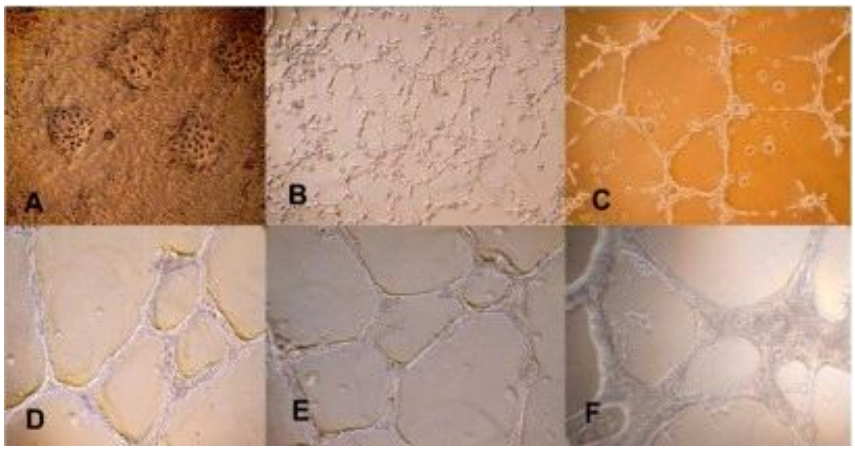
The results were analyzed by using Graph Pad Prism 5 (Graph Pad Software, San Diego, CA, USA). Real-time PCR experiments were performed in triplicate and data were expressed as mean±Standard deviation (SD). Statistical significance was assessed using the student's t-test, with p-values< 0.05 considered significant.

RESULTS AND DISCUSSION

Morphological differentiation of BuMECs in 2D and 3D cultures

The buffalo mammary epithelial cells (BuMECs) were cultured on both plastic substratum and in an extracellular matrix (ECM) to assess their morphological differentiation capabilities. In the 2D culture system, BuMECs grown on plastic formed a confluent monolayer, displaying typical epithelial cell morphology with cobblestone appearance (Fig 1A). In contrast, when cultured on top of ECM, the BuMECs exhibited rapid morphological changes, forming duct-like structures within a short time frame. Phase contrast microscopy revealed the progression of duct-like formations at different time points: After 30 minutes (Fig 1B), 2 hours (Fig 1C), 8 hours (Fig 1D and 1E) and 24 hours (Fig 1F), indicating dynamic cellular reorganization and early stages of tissue structure formation.

In the 3D culture system, BuMECs embedded in ECM demonstrated significant morphological differentiation, forming complex alveoli-like and duct-like structures, which are characteristic of mammary gland architecture. Within 24 hours, initial alveolar clusters began to form (Fig 2A)



A Monolayer on plastic substratum. B-F Rapid Duct-like formation on the top of extracellular matrix. Phase contrast micrographs were taken after 30 min (B), 2 h (C), 8 h (D, E) and 24 h (F) in culture. Magnifications A-F (×100).

Fig 1: Two Dimensional growth of BuMECs on plastic and on top of ECM.

and by 48 hours, these structures became more pronounced (Fig 2B). Over the course of six days, the cells further organized into distinct alveolar (Fig 2C, 2D and 2F) and ductal structures (Fig 2E), highlighting their ability to recreate *in vivo*-like tissue architecture. The magnifications of the images (100x, 200x and 400x) provided detailed visual evidence of the three-dimensional organization of BuMECs in ECM.

Formation of duct-like structures in ECM

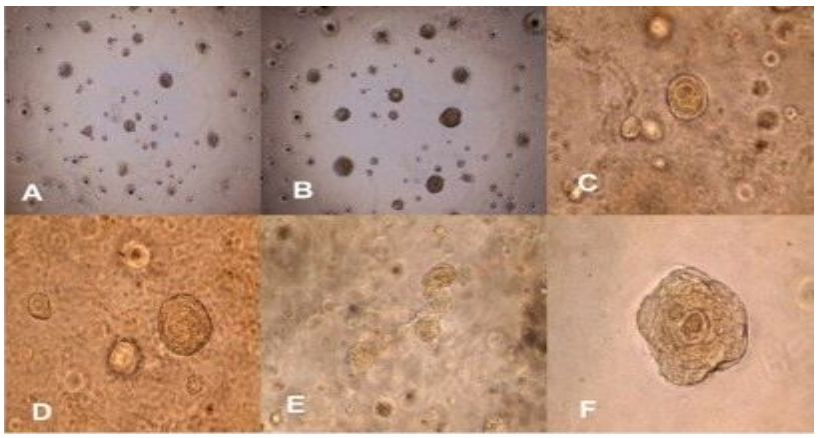
Further analysis of the morphological differentiation revealed that BuMECs cultured in 3D ECM developed duct-like structures after seven days of culture (Fig 3). These structures were visible under phase contrast microscopy, indicating successful tissue organization and the formation of functional units reminiscent of mammary gland ductal systems. The development of these duct-like structures further confirms the capability of BuMECs to undergo significant morphological differentiation in a 3D microenvironment.

Gene expression analysis of differentiated BuMECs

To evaluate the functional differentiation of BuMECs, quantitative real-time PCR (qPCR) was performed to measure the expression levels of β-casein, a key marker of mammary epithelial cell differentiation. The results showed a significant upregulation of β-casein mRNA in BuMECs cultured in 3D ECM compared to those grown on plastic substratum. Specifically, the expression of β-casein was five-fold higher in the 3D cultured cells, as depicted in the qPCR analysis (Fig 4). This substantial increase in β-casein expression underscores the enhanced differentiation capacity of BuMECs in a 3D microenvironment.

Protein expression analysis *via* western blotting

Western blot analysis was conducted to further confirm the functional differentiation of BuMECs by detecting casein protein levels. Protein lysates from both 2D and 3D cultured BuMECs were prepared and Western blotting was performed to analyze the expression of total casein. The results demonstrated distinct bands corresponding to α-casein,



Development of alveoli-like (A, B, C, D and F) and duct-like structures (E). Phase contrast micrographs were taken after 24 h (A), 48 h (B), 6 days (C, D, E and F) in culture. Magnifications A, B, E (×100), C, D (×200) and F (×400).

Fig 2: Three-Dimensional growth of BuMECs grown embedded in ECM.

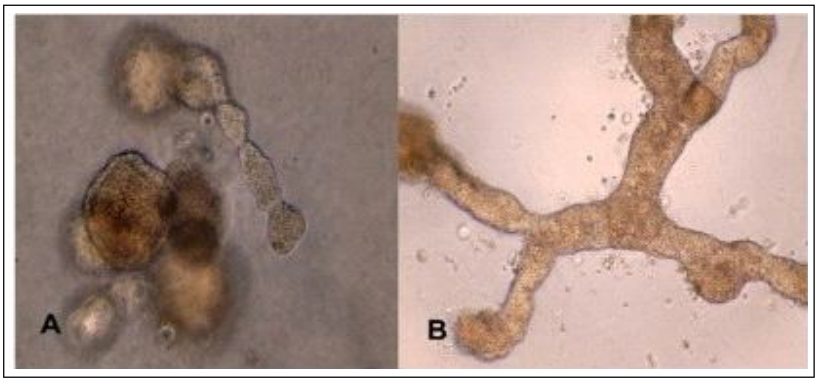


Fig 3: Development of Duct–Like structures after 7 days in culture. A, B (×100).

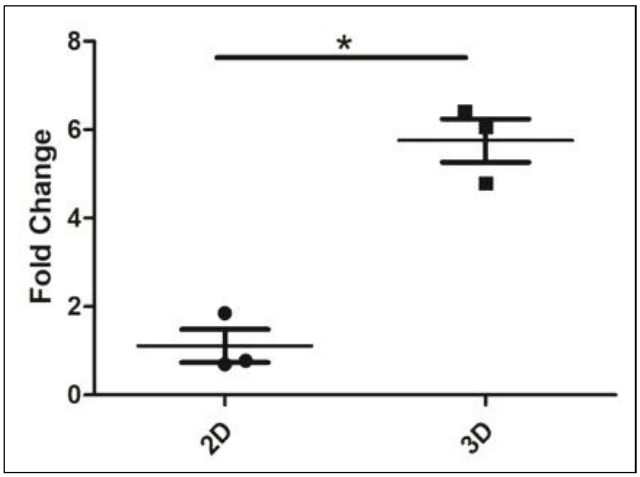
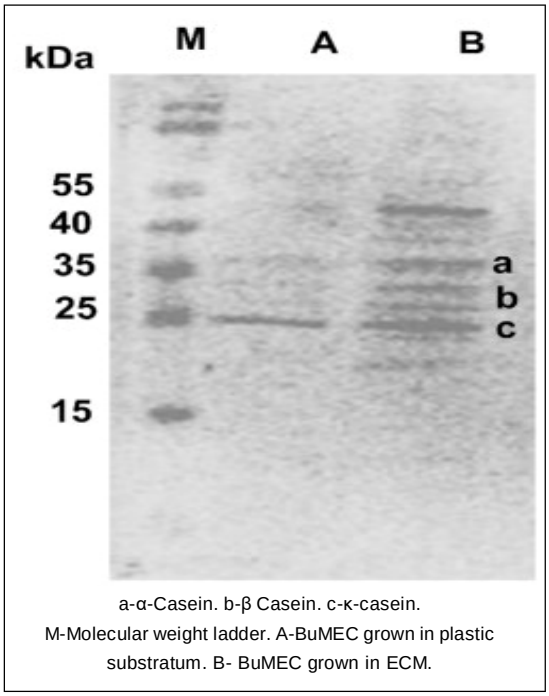


Fig 4: qPCR analysis revealed a 5-fold increase in Beta-casein expression in BuMECs grown embedded in ECM compared to those grown on plastic substratum.



a-α-Casein. b-β Casein. c-κ-casein.
M-Molecular weight ladder. A-BuMEC grown in plastic substratum. B- BuMEC grown in ECM.

Fig 5: Western blot analysis for casein in BuMEC lysates.

β-casein and κ-casein, with significantly higher expression levels observed in the 3D cultured cells compared to the 2D monolayers (Fig 5). This differential expression pattern highlights the increased secretory activity and functional maturation of BuMECs in the 3D ECM environment.

This study underscores the significant advantages of employing three-dimensional (3D) culture systems for the buffalo mammary epithelial cell line (BuMECs), revealing enhanced morphological and functional differentiation compared to traditional two-dimensional (2D) cultures. In 2D cultures, BuMECs formed a typical monolayer with a cobblestone appearance, reflecting the limitations of 2D systems in replicating the complex architecture of mammary glands. Conversely, 3D cultures facilitated the formation of alveolar and ductal structures, more closely mirroring *in vivo* conditions. This observation aligns with Weigelt *et al.* (2010), who reported that mammary epithelial cells in 3D cultures maintain native polarity and form glandular structures similar to those observed *in vivo*. The rapid formation of duct-like structures within 24 hours in our study supports the dynamic reorganization capabilities of 3D cultures, as noted by Barcellos-Hoff *et al.* (2013). Functional differentiation was significantly enhanced in 3D cultures, evidenced by the upregulation of β-casein mRNA and increased protein expression of casein variants (α, β, κ-casein). This finding is consistent with Roskelley *et al.* (1994), who demonstrated that mammary epithelial cells in 3D cultures exhibit differentiated phenotypes and milk production capabilities. Our observation of a five-fold increase in β-casein expression in 3D cultured cells underscores the superior differentiation potential of 3D environments. This study found higher mRNA induction of milk casein genes in 3D cultures compared to 2D cultures, which concurs with Shandilya *et al.* (2016), who reported that 3D cultures of primary buffalo mammary epithelial cells (BMECs) showed significantly higher expression of milk protein and fatty acid metabolism genes, indicating enhanced functional differentiation and the formation of lumen and dome-like structures by day 5 and polarized acinus-like structures within 15 days further supports the efficacy of 3D culture systems in mimicking *in vivo* conditions. However, the current study differs from earlier reports in using an established cell line subjected to more than 25 passages and cryopreserved for more than 10 years (Anand *et al*., 2012). Kozlowski *et al.* (2009) demonstrated that BME-UV1 cells form polarized acinar structures, termed mammospheres, within 16 days using Matrigel®. These mammospheres recreated cell-to-cell junctions with tight junction proteins like ZO-1 and E-cadherin. Finot *et al.* (2021) compared Matrigel® and ultra-low attachment supports with 2D cultures, finding that cell line profiles significantly influenced their performance. These studies provide valuable insights into cell line suitability for 3D culture, enhancing the understanding of bovine mammary gland biology. Our findings align with those of Schedin and Keely (2011), who reported that 3D culture systems provide critical insights into mammary gland development, differentiation and disease. The formation of acini-like structures and subsequent differentiation in our BuMECs model parallels the mammary gland morphogenesis described by Simian *et al.* (2001). Similarly, Krause *et al.* (2008) high lighted the advantages of 3D cultures in mimicking the microenvironment of mammary tissues, which is crucial for studying cellular interactions and differentiation. Moreover, our results resonate with Sharma *et al.* (2011), who explored the role of ECM in

mammary gland pathogenesis, including mastitis. The successful formation of duct-like structures in our 3D cultures suggests potential applications in studying mammary gland diseases and improving dairy production in buffaloes. This 3D model provides a platform to explore the genetic regulation of milk production traits, as highlighted by the role of miRNAs in balancing nutrition and milk yield in buffalo (Pang *et al.,* 2022). Also, our findings are consistent with Zielniok *et al.* (2014), who emphasized the importance of stromal-epithelial interactions in mammary gland biology and disease progression. Implementing 3D culture systems for BuMECs enhances our understanding of buffalo mammary gland biology and opens new avenues for investigating the effects of hormones, growth factors and mechanical cues on mammary epithelial cell function.

CONCLUSION

Our study demonstrates that 3D culture systems offer a more physiologically relevant environment for BuMECs, promoting enhanced morphological and functional differentiation. These findings underscore the potential of 3D cultures to advance mammary gland research, with significant implications for dairy science and animal health. Future research should leverage these 3D models to explore the molecular mechanisms underlying mammary gland development and disease, particularly in buffaloes, which are economically significant milk producers. Furthermore, further studies could explore the application of 3D culture systems in high-throughput drug screening and developing therapeutic interventions for mammary gland diseases. Integrating advanced imaging and omics technologies with 3D cultures could provide deeper insights into the cellular and molecular dynamics of mammary epithelial cells, ultimately contributing to improved livestock management and dairy production.

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Conflict of interest

The authors declare no conflict of interest.

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