BMC Veterinary Research

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Hepatocyte production across embryonic stages in chicken: an in vitro approach

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Abstract

Background Chicken hepatocytes are a valuable resource for cell-based assays and investigating the underlying mechanisms of diseases.

Objective In this study, we examined in vitro generation of chicken hepatocytes from three embryonic developmental stages.

Methods Hepatocytes were isolated from 5-, 7-, and 10-day-old chicken embryos and cultured in DMEM/F12 + 10% FBS. After 3 days, we measured proliferation rate, the expression of hepatocyte-specific genes (AFP, ALP, FOXA2, CYP3A4, CXCR4, OCT4, NANOG, SOX17), enzyme activity (ALT, AST), albumin production, and urea secretion.

Findings Morphological examination of individual hepatocytes exhibited a characteristic hexagonal structure with prominent nuclei and nucleoli. E10 embryos exhibiting a markedly higher proliferation rate in comparison to those from E5 and E7 stages (until 20 days). Whereas, approximately 50% of E5 and E7 hepatocytes showed reduced proliferation after three days. In suspension culture, hepatocytes formed spheroids or hepatospheres. The expression of hepatocyte-specific genes (AFP, ALP, FOXA2, and CYP3A4) was higher in E10 compared to E5 hepatocytes. The expression of stemness/early developmental markers (CXCR4, OCT4, NANOG, and SOX17) was significantly lower in E10 than E5 hepatocytes. E10 hepatocytes revealed significantly increased ALT and AST expression and urea secretion. While, Albumin production was significantly lower in E10 hepatocytes.

Conclusion Our results invested that E10 is the optimal developmental stage for the derivation and proliferation of chicken hepatocytes in vitro.

Keywords Hepatocytes, Chicken embryo, Proliferation

Introduction

Birds have long been important in basic and applied scientific research due to their unique biological features and wide-ranging applications [1, 2]. Fertilized eggs, along with cells from embryonic and adult tissues, are invaluable for investigating fundamental concepts in developmental biology, embryology, pharmaceutical biotechnology, and vaccine production [3–6]. Among avian organs, the liver stands out as one of the largest and most complex, playing multifaceted roles essential for the organism's survival and well-being. From facilitating hematopoiesis during the fetal period to orchestrating

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the production of essential proteins, regulating fat metabolism, bile production, nutrient homeostasis, and detoxification processes, the liver is indispensable for maintaining overall physiological balance [7]. At the cellular level, hepatocytes are the primary constituents of liver tissue, working alongside other specialized cells like fat storage cells and satellite cells [8].

Chicken hepatocytes serve as valuable tools in avian research for elucidating drug metabolism and predicting the pharmacokinetics and toxicity of pharmaceutical agents [9, 10]. By providing critical insights into drug clearance mechanisms, they inform drug development and enhance safety assessments in avian pharmacology [11, 12].

In avian development, embryonic day 5 (E5; Hamburger-Hamilton (HH) stages 26–28) is the stage of hepatic specification and budding and is composed predominantly of hepatoblasts– bipotent progenitor cells. E7 (HH stages 30–32) is a period of lineage commitment and morphogenesis that shows a peak period of hepatoblast proliferation. Functional maturation then occurs by E10 (HH stages 36–38), where albumin production becomes more pronounced [13–15].

Despite significant advancements in avian embryonic research, including the establishment of highly efficient methods for deriving and maintaining pluripotent stem cells and germ cells [16–18], achieving reproducible in vitro culture of embryo-derived hepatocytes has been difficult. This study aims to bridge this gap by investigating the derivation of hepatocytes from these three distinct developmental stages: progenitor (E5), transitional (E7), and committed (E10).

Materials and methods

Ethics statement

Fertilized eggs from the White Leghorn (Gallus gallus domesticus) were sourced from a commercial farm in Ahvaz, Iran, known for adhering to industry standards to ensure optimal bird performance. The study protocol strictly adhered to the guidelines for the ethical care and use of experimental animals as established by the Institute for Laboratory Animal Research (ILAR) at Ahvaz Jundishapur University of Medical Sciences (Ethical no. IR.AJUMS.ABHC.REC.1402.065).

Cell culture

Liver tissue was isolated from chicken embryos at embryonic days 5, 7, and 10 (E5, E7, E10) following the incubation of fifteen embryonated eggs (5 eggs for each stage) at stage X under controlled conditions (37.5 °C, 60–65% humidity). Sterile dissection techniques were employed to separate the embryos from the yolk and extraembryonic tissues. The isolated liver tissue was dissected into small pieces and digested with 0.25% trypsin-EDTA (Sigma, USA) for 15 min at 37 °C with gentle agitation. The enzymatic activity was halted by adding a double volume of complete culture medium containing 10% fetal bovine serum (FBS), and the resulting cell suspension was centrifuged before being plated onto gelatin-coated plates.

Cell culture media

The conventional medium comprised DMEM/F12 basal medium (DMEM, Invitrogen) supplemented with 10% FBS (HyClone), 2 mM glutamine (Invitrogen), 1 X non-essential amino acids (Invitrogen), 0.16 mM β-mercaptoethanol, and 100 U/ml penicillin and 100 mg/ml streptomycin (Invitrogen). Following media preparation, the plates were incubated at 37 °C with 5% CO2. The medium was replaced every day and the growth and proliferation of hepatocytes were meticulously monitored over a five-day period using an inverted microscope. after 6–7 days the chicken hepatocytes were dissociated with trypsin and the cells were plated on new gelatin-coated dishes.

Cell proliferation assay

To monitor the number of hepatocytes, conventional method, such as cell counting using a hemocytometer counting chamber, was used to calculate the cell density every 24 h.

Cell viability assessment

We measured cell viability using the Trypan blue exclusion method right after isolating the cells and found it was consistently over > 90% prior to plating them.

Cell spheroid formation

A suspension of 3×10^5 cells/mL was prepared in DMEM/F12 + 10% FBS medium. Low attachment bacterial plates (60 mm) were used for cell seeding. Each plate was filled with 4 mL of the prepared cell suspension. Besides, 1% agarose solution was prepared by dissolving agarose powder in sterile phosphate-buffered saline (PBS; Invitrogen) or cell culture medium. The solution was heated until complete dissolution of agarose, then cooled to approximately 37 °C. A layer of the prepared agarose solution was added to the bottom of a sterile culture dish. The agarose layer was evenly distributed and allowed to solidify. After solidification of the agarose layer, 3×10^5 cells were transferred onto the surface of the agarose in the culture dish. The culture dish containing the agarose-seeded cells was placed in a humidified incubator set at 37 °C and 5% CO2.

Gene expression analysis

Total RNA extraction and cDNA synthesis

Total RNA extraction from tissues and standard 2D monolayer cultures was performed using TRIZol reagent (Invitrogen) according to the manufacturer's protocol. RNA quality assessment was conducted using both UV spectrophotometry (Eppendorf) and agarose gel electrophoresis. The purity and integrity of RNA samples were evaluated based on the absorbance ratios of 260/280 and 260/230. A 260/280 ratio between 1.8 and 2.2 and a 260/230 ratio between 2.0 and 2.2 are indicative of high-quality RNA, with absorbance values above 280 indicating the presence of protein and above 230 potentially indicating residual phenol contamination.

For gel electrophoresis analysis, the presence of distinct and sharp bands corresponding to 28 S and 18 S ribosomal RNA was observed, confirming RNA integrity and quality. The absence of degraded RNA bands further validated the high quality of the extracted RNA samples. The PrimeScript RT reagent Kit (TaKaRa) was utilized for first-strand cDNA synthesis using 1 μg of total RNA, and the resulting cDNAs were stored at −20 °C for subsequent analysis.

Quantitative real-time PCR (qPCR)

qPCR was performed to quantify the expression levels of liver genes using a QuantStudioTM Real-time PCR system (Applied Biosystems). The qPCR reaction consisted of an initial denaturation step at 95 °C for 10 min, followed by 40 amplification cycles of denaturation at 95 °C for 15 s and annealing/extension at 60 °C for 1 min. High-resolution melting analysis was then conducted on the PCR amplicons to ensure the specificity of the amplification. Dissociation curve analysis was performed using Dissociation Curve 1.0 software (Applied Biosystems) to detect and eliminate potential primer dimer artifacts. Melting curve analysis and direct sequencing of the amplicons confirmed the presence of a single PCR product for all primer pairs, ensuring the specificity of the qPCR assay. Data analysis was performed using the $2-\Delta\Delta CT$ method, and the expression levels were normalized to the housekeeping gene β-actin.

Quantification of liver enzyme activity

The medium was changed daily. After 4 days, FBS-free medium was added for 48 hours. The supernatant was harvested on the sixth day of culture and preserved at −20°C. A sandwich enzyme-linked immunosorbent assay (ELISA) protocol was employed to measure the enzyme activity of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) in hepatocytes. Initially, the Capture Antibody was diluted in Coating Buffer, and 100 μL of this solution was added to each well of a 96-well microplate. The microplates were then incubated overnight at 4 °C to allow for antibody coating. Subsequently, the plates were washed four times with phosphate-buffered saline-Tween (PBS-T) 0.05% to remove unbound antibodies. To prevent non-specific binding, the plates were blocked with 5% bovine serum albumin (BSA) diluted in PBST (200 μL/well) and incubated for 2 hours at 37°C. After another washing step, 100 μL of the sample solution was added to each well, and the microplates were incubated for 1 hour at 37 °C to allow for antigen binding. Following antigen binding, horseradish peroxidase-conjugated anti-ALT and anti-AST antibodies (100 μL) were added to each well and incubated for 1 hour at 37°C. After three additional washing steps to remove unbound antibodies, 100 μL of 3,3',5,5'-tetramethylbenzidine (TMB) substrate solution was added to each well and incubated for 15 min at room temperature in the dark to initiate the enzymatic color reaction. The color development was then halted by adding 2 M sulfuric acid (50 μL/well). Finally, the absorbance of the samples was measured at a wavelength of 450 nm using a microplate reader, and all samples were processed in duplicate to ensure accuracy and reproducibility.

Albumin (ALB) and urea production assays

The medium was changed daily. After 4 days, FBS-free medium was added for 48 h. The supernatant was harvested on the sixth day of culture and preserved at −20 °C. The quantification of secreted ALB in the culture media was conducted using Chicken Albumin ELISA Kit from Assay Genie following the provided protocols, and measurements were taken using purified chicken albumin (C7786, BioReagent). The amount of urea generated was assessed using a colorimetric assay kit (Biorexfars, BXC0126A, 941210). To standardize the results, normalization to total protein content was performed using a Total Protein Kit (Biorex, BXC0173A, 921239) in accordance with the manufacturer's guidelines. Each experiment was performed in triplicate to ensure reliability and reproducibility.

Experimental design and statistical analysis

All experiments were performed with a minimum of three biological replicates (cells isolated from six independent embryos for each developmental stage). Within each biological replicate, technical replicates were used. Results were presented as the mean ± standard deviation (SD) of biological replicates. For normally distributed quantitative data, comparisons between two groups were performed using the t-test, while the ANOVA test was employed for multiple group comparisons. Non-parametric equivalents were utilized for data that did not follow a normal distribution. Significance was set at *P*-values less than 0.001 (***) and less than 0.05 (**)

between groups. SPSS software (version. 21) was used for data analysis.

Results

Culture of hepatocytes from three developmental stages

Liver tissue was isolated from chicken embryos at E5, E7, and E10 using a well-established protocol as described in previous study [19], to identify the optimal embryonic stage for hepatocyte proliferation. Liver cells underwent enzymatic digestion and plated on 0.1% gelatin-coated plates in DMEM/F12 + 10% FBS. Figure 1 illustrates isolation and cultivation process from three distinct developmental stages.

Morphological analyses of hepatocytes isolated from three developmental stages

After a 2-day incubation, phase-contrast microscopy revealed that the cells had formed small mono-layer colonies. The individual hepatocytes within these colonies exhibited a characteristic hexagonal structure, with prominent nuclei and nucleoli and a marginal cytoplasm (Fig. 2A). After 3 days of incubation, Cell Counting Kit-8 (CCK-8) was used to determine the number of viable cells in each group. E10 hepatocytes showed a markedly higher proliferation rate for up to 20 days in comparison to those from E5 and E7 stages ($P < 0.001$) (Fig. 2B).

Suspension cultivation of hepatocytes derived from E10 embryos

In the suspension culture, E10-derived hepatocytes were carefully plated onto both agar-coated and bacterial dishes, facilitating distinct environments for cell growth and interaction. Over time, the formation of spheroids or hepatospheres plates became evident. Phase contrast microscopy revealed no significant differences in spheroid morphology or number between the agar-coated and bacterial dishes cultures ($P > 0.05$) (Fig. 3).

Gene expression in chicken-derived hepatocytes

We compared gene expression profile between E5 and E10-derived hepatocytes on the 5th day of culture using qRT-PCR. The results revealed a significant differences in the expression levels of eight genes. Hepatocyte-specific markers (AFP, ALP, FOXA2, and CYP3A4) were

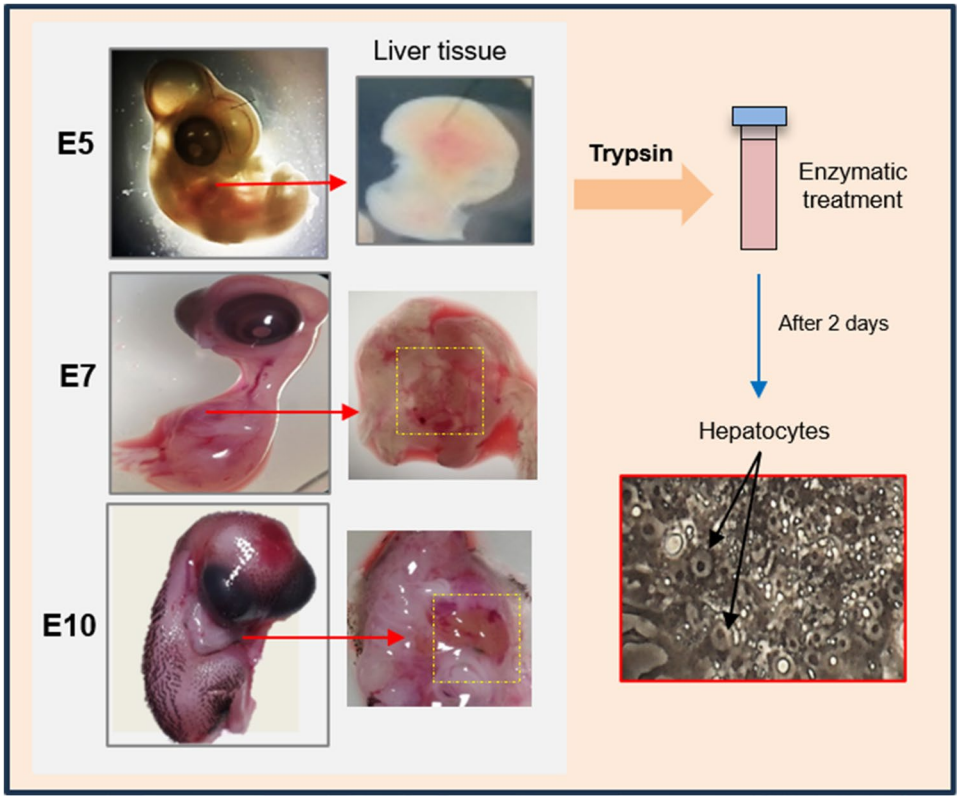

Fig. 1 The methodology involved in isolating and cultivating chicken embryo-derived hepatocytes from chicken embryos at embryonic days 5 (E5), 7 (E7), and 10 (E10) encompassed several key steps. Initially, liver tissue was extracted from the embryos at the specified embryonic days using established protocols. Subsequently, the isolated liver tissue underwent enzymatic digestion to obtain single-cell suspensions. These hepatocytes were then plated onto culture dishes precoated with 0.1% gelatin and cultured in a medium comprising DMEM/F12 supplemented with 10% FBS. Following a suitable incubation period, typically 2 days, the cultured cells were examined under phase-contrast microscopy to assess their morphology and growth characteristics. This process facilitated the evaluation and comparison of hepatocyte behavior and development across different embryonic stages, namely E5, E7, and E10

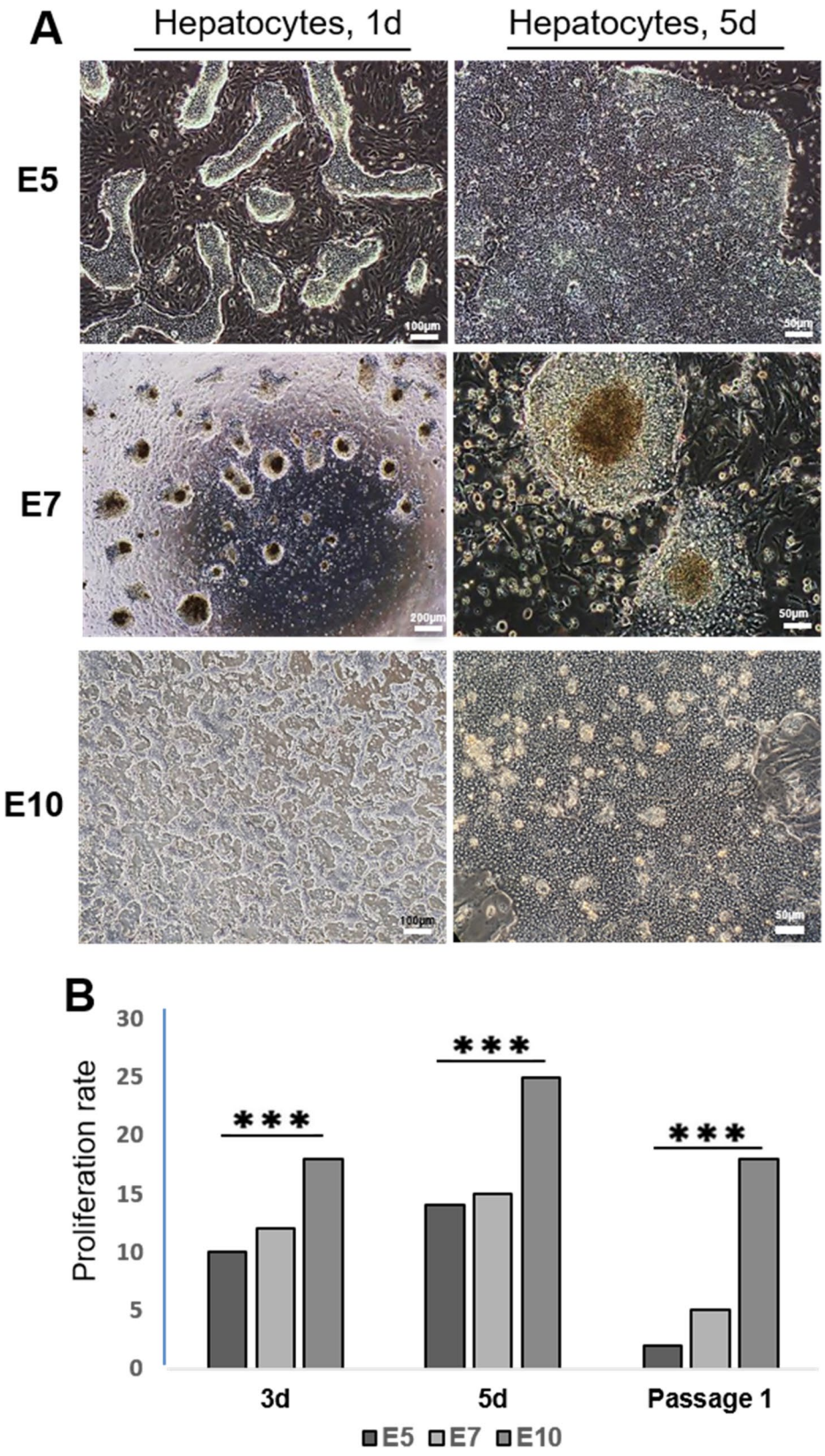

Fig. 2 (**A**) The morphological characteristics of hepatocytes after 24 h and subsequent to 5 days of incubation, across three distinct developmental stages. Upon morphological examination, individual hepatocytes within the colonies displayed a distinctive hexagonal structure characterized by well-defined nuclei and nucleoli, encased within a marginal cytoplasm. (**B**) The proliferation rates of hepatocytes were compared across three developmental stages during primary culture and passage one (*** $P < 0.001$)

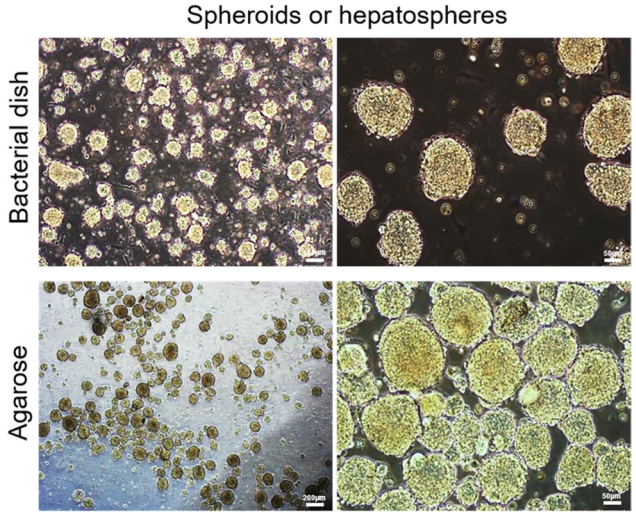

Fig. 3 Formation of spheroids or hepatospheres in bacterial dishes and on agarose-coated plates in DMEM/F12 supplemented with 10% FBS medium ($P > 0.05$)

markedly higher in E10-derived hepatocytes compared to E5 ($P < 0.001$). Conversely, the expression of stemness and early development genes (CXCR4, OCT4, NANOG, and SOX17) were significantly downregulated in E10-derived hepatocytes ($P < 0.001$) (Fig. 4).

The function of chicken-derived hepatocytes

ELISA assay was used to quantify the activities of ALT and AST enzymes in the hepatocytes. The expression of ALT and AST in E10-derived hepatocytes were significantly higher compared to E5-derived cells ($P < 0.001$) (Fig. 5A). Furthermore, functional analysis demonstrated that Albumin production was significantly lower in E10-derived hepatocytes compared to E5-derived hepatocytes ($P < 0.001$). Conversely, urea secretion was higher in E10-derived hepatocytes compared to E5-derived cells ($P < 0.01$) (Fig. 5B).

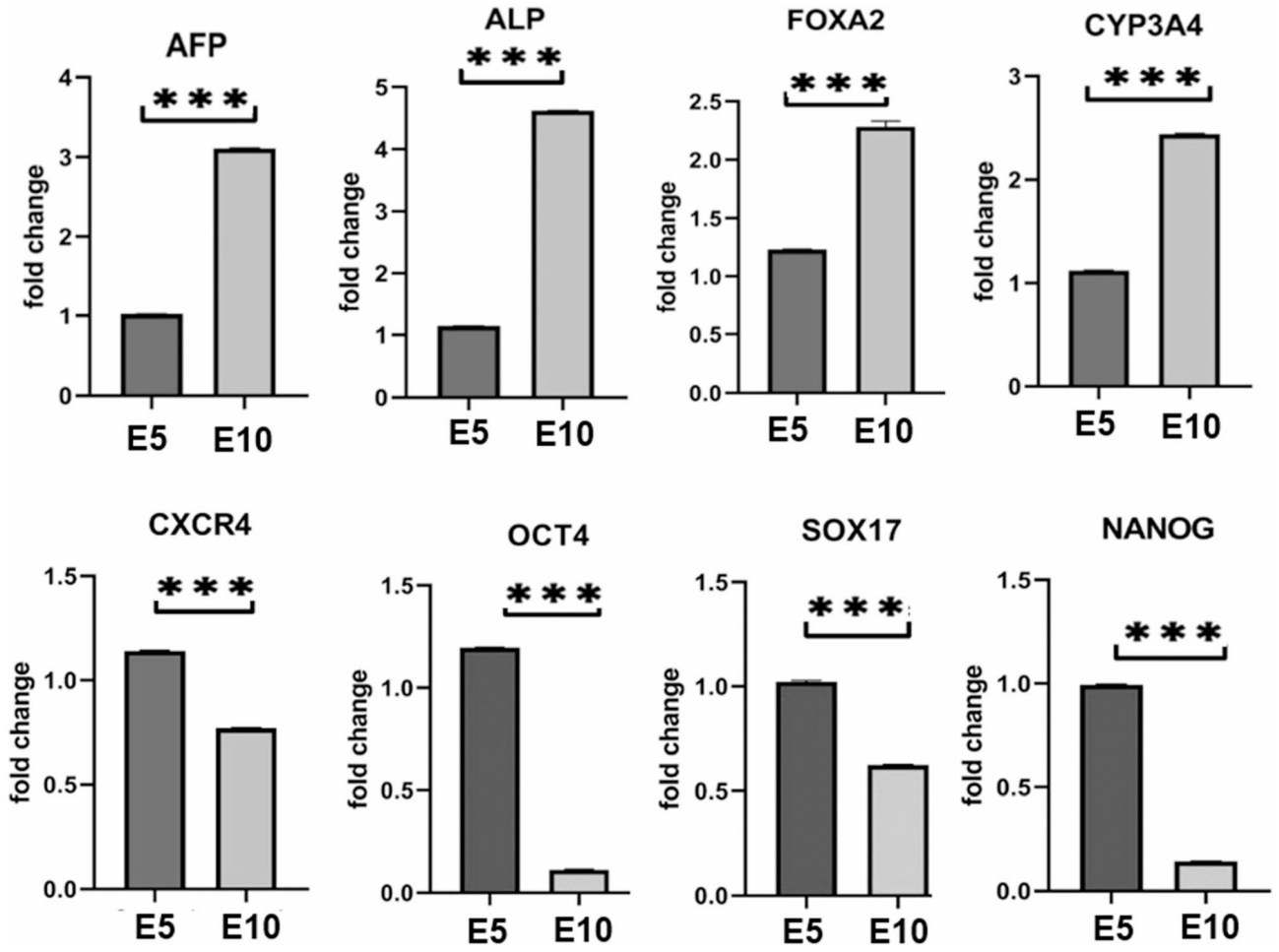

Fig. 4 The expression of AFP, ALP, FOXA2, CYP3A4, CXCR4, OCT4, NANOG, and SOX17 from standard 2D monolayer cultures in E10-derived hepatocytes compared to E5-derived hepatocytes (*** $P < 0.001$)

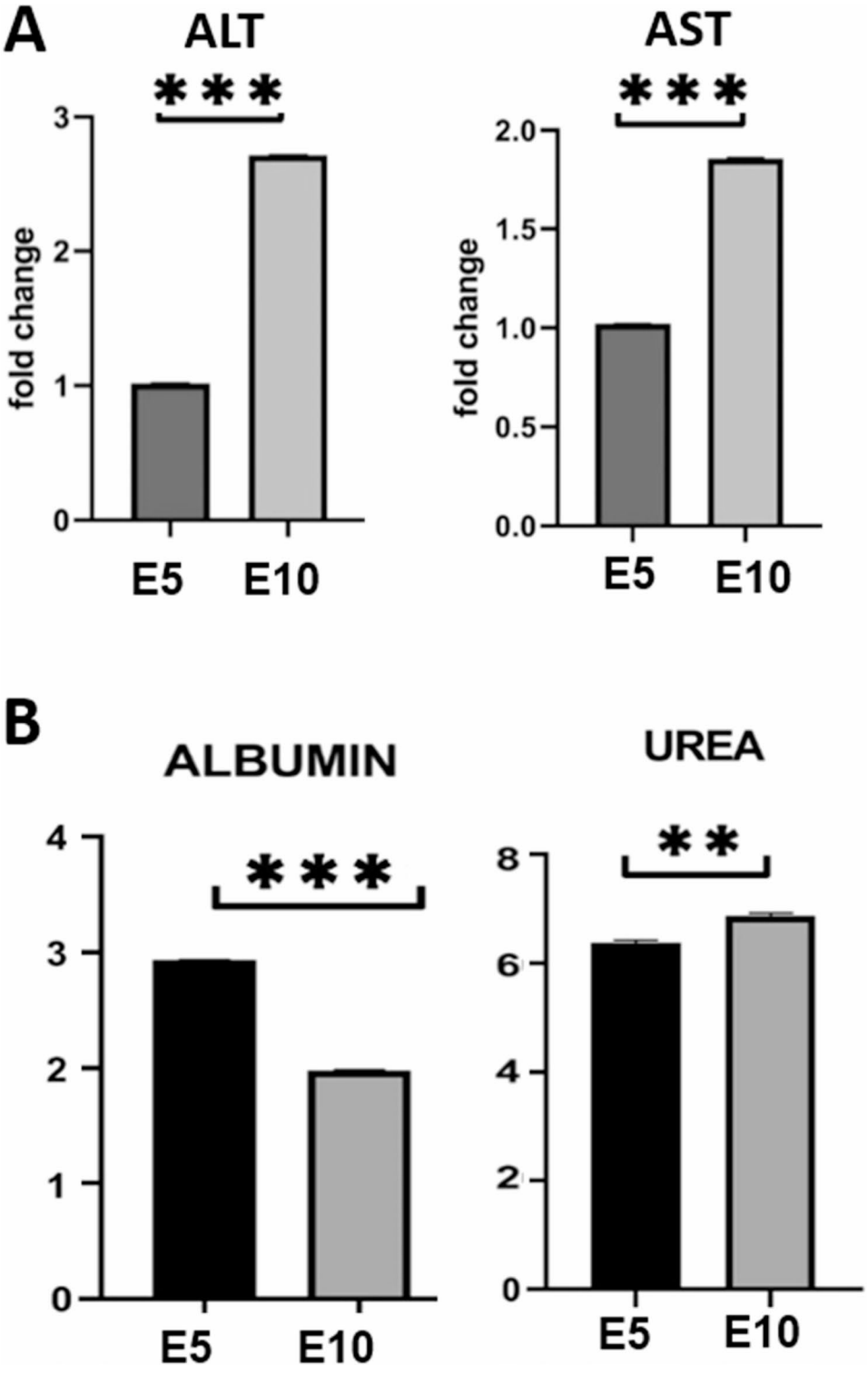

Fig. 5 Analysis the function of chicken-derived hepatocytes. (**A**) The activities of ALT and AST enzymes in E5- and E10-derived hepatocytes. (**B**) The production of albumin and urea by hepatocytes (***$P < 0.001$, **$P < 0.01$)

Discussion

Our findings indicate that E10 emerges as the most suitable stage for hepatocyte derivation. This suggests that the embryo's developmental stage determines hepatocytes characteristics and proliferative capacity. Morphologically, the hepatocytes illustrated a characteristic hexagonal structure with prominent nuclei and nucleoli, indicative of a well-developed and functional phenotype [20].

Hepatocytes from different developmental stages exhibited distinct proliferation rates. In primary culture, E10-derived hepatocytes demonstrated a significantly higher proliferation rate compared to those from E5 and E7. Upon passage to the first generation, the proliferation rates of hepatocytes continued to vary across the developmental stages. E10-derived hepatocytes maintained a significantly higher proliferation rate compared to those from E5 and E7. This indicates that the developmental stage exerts a sustained influence on the proliferative capacity of hepatocytes, even after subculturing.

In culture, the cells aggregated into spherical clusters, forming a three-dimensional architecture known as spheroids or hepatospheres, demonstrate the cells ability to form complex multicellular arrangements [21]. A similar process occurred on both agarose-coated plates and bacterial dishes, where the cells proliferated and created some aggregates as spheroids or hepatospheres.

Furthermore, the expression profile of hepatocyte-specific genes (AFP, ALP, FOXA2, and CYP3A4) was significantly higher in E10-derived hepatocytes. The decreased expression of pluripotent-related genes (OCT4 and NANOG) aligns with lineage commitment and to allow for functional specialization. The downregulation of OCT4 and NANOG is consistent with previous studies highlighting their role in maintaining pluripotency and self-renewal in embryonic stem cells, with their expression typically diminishing upon cellular differentiation [22]. This transition from a pluripotent state to a more committed cell fate is crucial for the development and maturation of hepatocytes, allowing them to acquire functional properties essential for liver physiology [23].

We observed higher expression of both ALT and AST in E10-derived hepatocytes compared to those derived from E5, indicating a heightened metabolic activity in the E10 group. This elevation suggests enhanced liver function and increased cellular metabolic [24]. Albumin production was significantly lower in E10-derived hepatocytes than in their E5-derived counterparts, which may reflect differences in cellular maturity or differentiation status between the two developmental stages. Conversely, E10-derived hepatocytes exhibited higher level of urea secretion. This increase may indicate enhanced urea cycle activity or nitrogen metabolism in E10-derived cells, potentially reflecting their greater metabolic activity.

We selected E5, E7, and E10 stages to capture key maturation checkpoints. The E10 cells are dialing down genes linked to a primitive, multipotent state while ramping up classic hepatocyte markers such as *AFP*, *ALP*, *FOXA2*, and *CYP3A4*. This genetic transition shows a firm commitment to the hepatocyte lineage. To really be confident that these cells are mature, more tests, including western blots to check levels of important CYP450 enzymes, a lidocaine clearance assay (for CYP1A2 activity), and functional assays for ammonia detoxification will require.

Several studies have explored the isolation and culture of avian liver cells for various research applications. For instance, Lee et al., isolated fetal liver cells from 15-day-old chick embryos by digesting fetal liver tissues with VT solution (a 1:1 ratio of 0.25 trypsin and PBS) for 30 min. The cells were cultured in Dulbecco's medium (0.45% glucose) supplemented with 10% FBS at 39 °C in a 5% CO2, ultimately reaching a population of 6×10^5 cells [25]. Feng et al. highlighted the utility of chicken liver cells for cultivating four types of adenoviruses [26]. Other research has suggested the utility of liver cells, particularly hepatocytes, as a suitable source with high viral receptor expression for propagating ALV-J (avian leukosis virus) [27]. Mackei et al. demonstrated the suitability of chicken hepatocytes as a model for studying inflammatory response and liver stress in cell culture systems [28]. In a related avian model, Bao et al. investigated liver development and hepatocyte culture in duck embryos. They tracked morphological changes in the liver from the embryonic period through the first week post-hatching. They successfully isolated liver cells from 21-day-old duck embryos and revealed that the cultured liver cells exhibited robust growth. These findings enhance our understanding of in vivo liver growth, in vitro hepatocyte culture, and the study of hepatic lipid metabolism [29].

Conclusion

Overall, our findings provide valuable insights into the stage-specific characteristics of chicken hepatocytes and highlight their potential for cell-based assays and disease modeling. Further studies are warranted to elucidate the molecular mechanisms behind these developmental differences and to explore the clinical implications of our results for disease research and regenerative medicine.

Acknowledgements
Not applicable.

Authors' contributions
Investigation and formal analyses, writing draft, and editing were performed by Maryam Farzaneh and Shirin Azizidoost.

Funding
This manuscript was financially supported by the Cellular and Molecular Research Center, Medical Basic Sciences Research Institute, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran (Grant No: CMRC-0233).

Data availability
Not applicable.

Declarations

Ethics approval and consent to participate
This experimental study was approved by the Ethics Committee of Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran (Ethical no. IR.AJUMS.ABHC.REC.1402.065).

Competing interests
The authors declare no competing interests.

Received: 22 March 2025 / Accepted: 13 October 2025
Published online: 17 November 2025

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