In Vitro Study of the Effect of HBV Infection on Early Embryonic Development

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Research Article

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Abstract

Objective: This study aimed to clarify the infection state of early embryos by HBV and the effect of HBV infection on embryo development.

Methods: In vitro culture was carried out on the fresh surplus embryos from infertile couples on the third day (D3) who received routine in vitro fertilization and embryo transfer (IVF-ET) treatment. The embryo cells were infected in vitro by the serum of HBV infected patients with different viral loads. HBsAg quantity in the supernatant secreted by embryo and HBV mRNA in embryo cells were detected. The development of embryo cells after HBV infection was evaluated by embryo morphological parameter score and fragment score in embryo cells.

Results: From May 2013 to July 2015, 238 cases of D3 fresh surplus embryos from 120 infertile couples and 128 samples of embryo culture supernatant were collected. The HBsAg positive rate in the supernatant of embryos in the high viral load infection group was significantly higher than that in the low viral load infection group (40.0% *vs.*15.6%, $p<0.05$). HBV mRNA was detected in 2 of 17 HBsAg positive embryos. The scores of embryos in the high load HBV culture solution group were higher than those in the low load group ($p=0.04$). There was one formed blastocyst in low load HBV culture group, but there was no blastocyst formation in all embryo cells of high load group ($p<0.001$).

Conclusion: The results suggest that embryo cells can be directly infected by HBV in vitro. HBV infection affects the development of embryos.

Introduction

At present, it has been confirmed that germ cell is an important way of vertical transmission of hepatitis B virus (HBV). HBV can infect sperm, integrate into its genome, enter fertilized egg and pass on to offspring. HBsAg, HBcAg and HBV DNA were detected in the ovum of different development stages, suggesting that HBV can infect the ovarian tissue and ovum of patients with chronic hepatitis B and replicate in them[1,2]. HBV can be transmitted to the offspring through the infected ovum. However, it is not clear whether early embryos could be directly infected by HBV in mothers with HBsAg positive.

Early intrauterine infection by HBV, herpes simplex virus (HSV) and human cytomegalovirus (HCMV) could cause abnormal embryo development, early abortion, stillbirth or fetal malformation [3-6]. In our previous study, HBV mRNA was detected in discarded human embryos, and abortion occurred in these patients in the later follow-up [7]. Therefore, it is speculated that integration HBV genes in embryos could interfere with the development of embryos. Furthermore, a study indicated the increased risk of preterm delivery and premature rupture of fetal membranes and the increased possibility of placental abruption, prolonged labor and cesarean section in HBV/HCV infected patients [8]. The fetal perinatal mortality, the incidence of congenital malformation and low birth weight were more significant than those in the control group. Multivariate analysis also showed that fetal perinatal mortality, congenital malformation, and low

birth weight were related with HBV/HCV infection [9]. But some evidences from large population-based cohorts displayed that HBV infection did not affect newborn and pregnancy [10,11]. The relationship between HBV infection and pregnancy outcome is very complex, which is affected by many mixed factors such as maternal conditions and HBV infection state of both parents. Therefore, there is still a dispute about whether there is a difference between the pregnancy outcome of HBV infected people and that of the normal population. The basic study is less involved in this aspect because of ethical reasons.

In order to further explore the clinical significance of HBV infected embryos, micro drop culture was employed in this study to carry out sequential culture in vitro and HBV infection on discarded human early embryos to observe HBV particle infection on early embryos and the influence on embryo development.

Materials And Methods

1.1 Ethics

In accordance with the relevant regulations and ethical principles of the Specification for the Allocation of Human Assisted Reproductive Technology and the Relevant Technical Specifications and Basic Standards for Human Sperm Bank, this study was carried out, and also approved by the Ethics Committee of the First Affiliated Hospital of Xi'an Jiaotong University and the Ethics Committee of Shaanxi Maternal and Child Healthcare Hospital. Volunteers and embryo donors have signed the informed consent.

1.2 Study subjects

From May 2013 to July 2015, D3 fresh surplus embryos were collected from 120 infertile couples receiving routine IVF-ET treatment in the Reproductive Center of Shaanxi Maternal and Child Healthcare Hospital. The age of both husband and wife is less than 35 years and were not infected with HAV, HBV, HCV, HDV and HIV. They voluntarily donated discarded embryos. The quality of D3 embryo was evaluated by PETER cleavage stage embryos scoring system, and 238 embryos of grade I-III were selected from the collected fresh discarded embryos [12].

In vitro infection source of HBV came from the two treatment-naïve chronic hepatitis B patients, who were selected as volunteers in the outpatient department of Infectious Department of the First Affiliated Hospital of Xi'an Jiaotong University. HBV DNA quantitative of these two patients was 3.21×10^5IU/mL, and 2.89×10^8IU/mL respectively. The results of routine biochemical tests showed that the liver function of the patients was normal; HBsAg, HBeAg and HBcAb were positive; HBV genotype was type C; and HIV, HAV, HCV, HDV and EBV infections were excluded.

1.3 Main reagents

Cleavage embryo culture solution G-1TM PLUS, blastocyst culture solution G-2 TM PLUS and mineral oil for culture were purchased from Vitrolife Company, Sweden. SuperScript® III First-Strand Synthesis System was purchased from Thermo Fisher Company, USA. HBV DNA quantitative test kit was purchased from Shenzhen PG Biotechnology Co., Ltd. HBsAg, HBeAg and anti-HBs quantitative test kit was purchased from American Abbott Corporation. RNase inhibitors were purchased from American Invitrogen Company. dNTP（10mmol/L）Platinum Taq DNA polymerase was purchased from American Invitrogen Company. PCR primers were synthesized by Shanghai Sangon Biotech Co.,Ltd.

2 Experimental method

2.1 In vitro culture of early embryos

30μL micro drops were added into a 35mm culture dish, covered with mineral oil, and equalized overnight in a 6% CO_2 incubator at 37 ℃. Fresh embryos were obtained every day and transferred into mineral oil-covered micro drops of embryo culture solution after the equalization overnight according to the experimental groups. On average, one embryo was put into each micro drop to observe the development of embryos and was cultured continuously in the incubator.

2.2 HBV infection in vitro

2.2.1 Source of HBV

15ml venous blood was drawn from the fasting CHB volunteers, and serum was separated. Then the serum was put in the sterile centrifuge tube, and in 56 ℃ water bath case for 30 minutes. After inactivation, they were sub-packaged, and the samples were taken for sterility test and kept in the refrigerator at - 80℃ for standby. The serum of the patient with HBV DNA 3.21×10^5IU/m was used as the source of low viral load infection in vitro, and the serum of the patient with HBV DNA 2.89×10^8IU/mL was used as the source of high viral load infection in vitro.

2.2.2 HBV infection in vitro

The inactivated HBV serum was mixed with embryo culture solution in a ratio of 1:3. Well-equalized embryo was transferred into the culture micro drop containing HBV particles by Pasteur pipette. After infection in 6% CO_2 incubator at 37℃ for 24 hours, the embryo was removed and carefully washed with blastocyst culture solution for 8 times, and HBsAg quantity was detected in the last cleaning solution. If HBV DNA was negative, the embryos were transferred into the micro drops of blastocyst culture solution without HBV serum. After 24 hours, the supernatant was taken and stored at - 80℃ for unified detection.

2.2.3 Experimental groups

According to the principle of randomized grouping, the embryos were divided into four groups: group A1 was negative control group, and the culture solution was embryo culture solution for a total of 38 pieces; group A2 was healthy human serum group, and the culture solution was the mixture of healthy human

serum and embryo culture solution for a total of 32 pieces; group A3 was low viral load serum group, and the culture solution was the mixture of low viral load serum and embryo culture solution for a total of 34 pieces; group A4 was high viral load serum group, and the culture solution was the mixture of high viral load serum and embryo culture solution for a total of 32 pieces. The mixture ratio of inactivated serum and embryo culture solution was 1:3.

2.2.4 Detection of culture supernatant

The supernatant of embryo culture was collected and tested. The supernatant which was less than 20μL in volume or polluted by mineral oil would be discarded. HBsAg quantity was detected by electrochemiluminescence. HBsAg<0.05 IU/mL was negative; HBsAg≥0.05 IU/mL was positive.

2.2.5 Detection of HBV mRNA expression in embryos by single-cell RT-PCR

2.2.5.1 Embryo cleavage

The embryos infected in vitro were washed with PBS buffer for 8 times and then placed in the embryo cleavage solution for decomposition for 2 hours. The last washing solution was reserved for the detection of HBsAg quantity and HBV DNA. The samples with both HBsAg quantity and HBV DNA negative could be used for subsequent experiments.

2.2.5.2 cDNA synthesis

(1) The specifications of Invitrogen's Super-script III First- Strand Synthesis reverse transcription kit was followed to operate. (2) The total volume of the tube was 20μL and heated at 50 ℃ for 50 min. cDNA was synthesized, and then the reaction was stopped by heating at 85 ℃ for 5 min. (3) After centrifugation, RNase H 1μL was added and heating lasted for 20 minutes at 37 ℃ to remove the non-transcribed RNA. The obtained cDNA was stored in -20 ℃ refrigerator, and β- actin was used as housekeeping gene.

2.2.5.3 Nested PCR

(1) According to the results of our previous experiments, we continued to use the primers designed by the mRNA gene sequence of HBV S region [7]. Outer primer：the amplification length was 417 bp, and the nucleotide sequence was P1:5'-CATCTTCTTGTTGGTTCTTCTG-3'; P2:5'-TTAGGGTTTAAATGTATACCC-3'. Inner primer: the amplification length was 206 bp, nucleotide sequence was P3: 5'-TCTATGTTTCCCTCTTGTTGC-3'; P4:5'-TACCACATCATCCATATAACTG- 3'. (2) The nested PCR reaction system and the amplification conditions were the same as those of our published article [7].

2.3 Effect of HBV infection on embryo development

On D4 to D6 in the process of embryo culture in vitro, embryo development status was observed, recorded, and photographed every day. The blastocyst quality based on morphology parameters

including cell count, fragmentation, and embryo expansion, was classified according to Gardner's criteria with the grades (A, B, or C) describes both the trophectoderm and innercell mass of the embryo [13,14].

2.4 Statistical analysis

SPSS 16.0 software was applied for statistical analysis. The measurement data were expressed as $\bar{x}\pm s$. Nonparametric test, Chi-square test or Fisher exact test was used to compare rates. Spearman correlation test was used for the correlation. The difference was statistically significant ($p< 0.05$).

Results

3.1 General conditions of embryo donors

In this study, a total of 120 embryo donors were included. The basic information of embryo donors between different groups was compared. There was no statistical difference in the age of women, BMI index, infertility duration, causes for infertility, ovulation induction time and basic level of follicle stimulating hormone (FSH) ($p>0.05$)(Table 1).

3.2 In vitro culture of embryo cells

A total of 238 D3 fresh discarded human embryos were collected and D4-D6 sequential culture in vitro was carried out. 136 embryos were successfully cultured. After infection in vitro, 128 samples of D5 embryo culture supernatant were collected.

3.3 Detection of HBsAg in embryo supernatant

The embryos were transferred into micro drops of culture solution containing HBV particles. After 24 hours of infection (D4) in vitro, the embryos were washed with blastocyst culture solution for 8 times. After 24 hours of culture (D5), the supernatant was collected and HBsAg quantity was determined by electrochemiluminescence. The quantity of HBsAg in supernatant of all embryos in group A1 and A2 was less than 0.05 IU/mL. HBsAg of 12 (40.0%) cases was detected positive in the embryo supernatant of high load infection group (group A4) and that of 5 (15.6%) cases positive in low load infection group (group A3). The difference between these two groups was statistically significant ($p< 0.05$) (Table 2).

3.4 Detection of HBV mRNA in embryo cells

HBV mRNA was detected and the target fragment was amplified by nested PCR in 17 embryos with HBsAg positive. The length of the amplification product was 206bp (Fig.1). HBV mRNA was detected in 2 of 17 embryos, both of which came from group A4. The result indicated that the positive rate of embryonic HBV mRNA in the high viral load infection group was higher than that of group A3, but the difference was not statistically significant ($p= 0.23$).

3.5 Effect of HBV infection on embryo development

After HBV infection in vitro, the morphology of embryo cells in each group was observed under microscope on D6 of culture in vitro. It was found that blastocyst was observed in both group A1(Fig.2A) and A2(Fig.2B), rating A to C. In group A3, only one embryo developed into blastocyst, and the rest were grade III embryos with different cell morphology (Fig.2C). In group A4, no blastocyst was observed, and most of them were grade IV embryos, and the zona pellucida has been ablated (Fig. 2D). All embryos were scored by embryo cell morphological grading standard. The results indicated that 87.5% of embryos were graded III to IV, and there was statistical difference in embryo cell morphological scoring between the four groups (p=0.022) Table 3. The grades of embryos in group A4 were higher than those in group A3, and the difference was statistically significant (p= 0.04). Furthermore, the number of formed blastocysts of embryo cells between the different groups was compared (Table 4). The highest rate of blastocyst formation was found in the group A1 (13.9%), followed by the group A2, and the difference in blastocyst formation rate between four groups was statistically significant (p<0.001). There was one formed blastocyst in the group A3, but no blastocyst formation in all embryo cells of the group A4, and the difference was statistically significant (p<0.001).

Discussions

The relationship between HBV infection and pregnancy outcome is very complicated. Up to now, the controversy still remains about whether the difference between pregnancy outcome of HBV infected patients and that of normal population exists. Due to the source of samples, little basic research is involved in this aspect.

With the development and popularization of assisted reproductive technology, the number of transplanted embryos by each couple is 2 to 3, and a large number of discarded early embryos appear in the reproductive center. “14 days limit” refers to the blastocyst obtained by in vitro fertilization, somatic cell transplantation, parthenogenetic replication technology or genetic modification, and its culture duration in vitro shall not exceed 14 days from the beginning of fertilization or nuclear transfer [15]. This rule was first put forward by the Ethics Advisory Committee of the U.S. Department of Health, Education and Welfare in 1979. In at least 12 countries, this limit is written in laws supervising assisted reproduction and embryo research [16]. The Ethical Guidelines for Human Embryonic Stem Cell Research issued in December 2003 in China also included this rule, providing legal support for in vitro embryo test.

Many studies have shown that HBV could infect a variety of primary and passage cells in vitro successfully, among which there are many reports about trophoblast cells [17-19]. Our research group has successfully constructed a platform for HBV infecting granular cells in vitro, and the technology is mature [4]. Based on this technology, embryo cell infection by HBV in vitro in this study was carried out. It was found that HBsAg could be detected in the supernatant after early embryos were in the culture solution containing HBV particles for 24 hours, suggesting that embryo cells could be infected directly by HBV in vitro.

It is generally believed that zona Pellucida (ZP) outside embryo cells could protect the embryo from external damage [20]. The surface of ZP presents reticular structure. Under the transmission electron microscope, the reticular structure of the fertilized ZP surface is much sparser, and the average pore diameter of the surface is about 4μm [21]. But the diameter of small HBV spherical particles and tubular particles is about 22nm, and the diameter of Dane particles is about 42nm. Therefore, HBV could pass through ZP and enter the embryo smoothly, and then infect embryo cells.

HBV is a retrovirus, and its replication cannot be separated from the process of reverse transcription [22]. HBV cccDNA is used as a "template" to transcribe all information onto mRNA, and mRNA produces various proteins of HBV by the process of "translation"[23]. Therefore, HBV mRNA is a very important link in HBV replication and transcription. If HBV mRNA is detected in cells, it is indicated that HBV is in the state of active replication, and is performing meaningful expression and transcription. In the early stage of our research, we have detected HBV mRNA positive signals in discarded embryos with father infected by HBV and mother non infected by HBV, which indicates that HBV integrated into embryo cells could have replication activity and could be transcribed and translated under certain conditions [24].

In this study, single cell RT-PCR was used to detect HBV mRNA in embryos with HBsAg positive in supernatant after infection in vitro. The positive signals were detected in two embryos, and HBsAg and HBV mRNA were detected positive at the same time. It indicated that HBV particles could enter embryo cells and exhibit expression and replication activities after embryos were infected in vitro.

Several studies have confirmed that HBV DNA level of pregnant women is an independent risk factor for vertical transmission [25-27]. In this study, we analyzed the relationship between in vitro infection sources （$<10^6$IU/mL *vs.* $>10^6$IU/mL）with different levels of HBV DNA and the number of infected embryo cells. The results showed that with $>10^6$IU/mL serum as in vitro infection source, the number of embryo cells with HBsAg and HBV mRNA positive in D6 supernatant was higher than that of $<10^6$IU/mL/ml group. This result provided more evidences for the relationship between HBV DNA level and germ cell infection. Therefore, the proportion of HBV vertical transmission by way of germ cell could be reduced by controlling HBV load in pregnant women.

Interestingly, we also found that the positive signal of HBV mRNA was detected in only one embryo among the same batch of embryos from the same couple under the same culture conditions, indicating that not all embryo cells in the same body could be directly infected by HBV. This is consistent with the clinical observation that not all the children of the same couple with chronic hepatitis B were infected. We speculate that the experimental results may be related to the high heterogeneity of glycoprotein on the surface of zona pellucida, and this spatial structure of glycoprotein directly affects whether HBV particles could pass through. The specific mechanism of its occurrence needs further study.

We used HBV to infect embryo cells in vitro to evaluate the development of embryo. The evaluation of embryo quality in vitro culture includes two aspects: embryo morphology and developmental potential. During in vitro culture, embryo development will be blocked to different degrees. The proportion of

fragments in cells is an important indicator in evaluating embryo development block. Embryos with more fragments have poor potential in the development to blastocyst and successful implantation, and too many fragments will be accompanied by chromosome abnormalities [28]. The study of Jurisicova et al showed that cell fragments predicted the occurrence of apoptosis [29]. Blastocyst is the final stage of embryo culture in vitro, and is usually formed on D5-D7 after fertilization. In natural state, human embryo is implanted into maternal uterus in the form of blastocyst. Whether blastocyst could be formed is an important sign for early embryo to have development potential. Therefore, we used embryo morphological scoring parameters and blastocyst formation as evaluation indicators. The results showed that the embryo development state and the proportion of blastocyst formation in the groups with HBV infection in vitro were significantly lower than those in the control group. We speculate that blocking and apoptosis occur in HBV infected early embryos during the development process in the pregnant women with HBV DNA positive, which is also the result of natural selection and survival of the fittest. In this study, it is the first time to evaluate whether HBV infection affects embryo development in vitro experiment. The higher the HBV load is, the greater the impact on the development of embryo, which further suggests that pregnant women with high viral load not only have a higher risk of vertical transmission, but also have a greater chance of bad pregnancy outcome.

During the experiment, in order to remove the virus particles on the surface of embryo cells as much as possible to avoid false-positive results, we used PBS to wash thoroughly, and the last washing solution was kept to detect HBV related markers. Only the samples with negative results could be used for subsequent experiments. There still exist some problems in this study: (1) Normal embryo starts to be implanted into the uterus on D6-D7, and it is very difficult to culture the embryo in vitro after D7. So, all the experimental operations need to be completed between D3-D6 of embryo development, which poses strict time limit for the experiment; (2) Because the requirements of environment and technology are very high for the early embryo culture in vitro and the success rate is also low in the actual operation process, our study had the small sample size; (3) The embryos included in the experiment are discarded in the process of assisted reproductive therapy, and their scores and functions are slightly worse than those of the transplanted embryos. The research results of Garrido et al showed that the ovum and embryos with poor quality were more likely to be infected with HBV [30]. In our experiment, the proportion of discarded embryos infected with HBV in vitro could be higher than that of normal embryos. Therefore, we hope to establish a stable embryo stem cell platform in the follow-up study to further study the specific ways of HBV affecting embryo development, the changes of chromosomes after infection and the integrated state of HBV genes.

To sum up, the above studies confirmed that HBV could infect embryo cells in vitro, express and replicate in them, and affect the development potential of early embryos, providing an important research platform for further study of the mechanism of early intrauterine HBV infection and blocking measures.

Declarations

Acknowledgments

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Conflict of interest statement

All authors have no conflict of interest to disclose.

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Tables

Table 1

Comparison of general conditions of embryo donors

	A1 （n=24 ）	A2 （n=30 ）	A3 （n=31 ）	A4 （n= 35）	*Z*	*P*
Age of woman (years old)	32.14±4.32	33.01±4.17	33.24±4.95	32.68±5.19	-0.361	0.643
BMI（kg/m^2）	22.18±2.47	22.04±2.95	21.65±2.81	22.41±2.01	-0.778	0.724
Infertility time (year)	3.24±0.10	3.23±0.09	3.39±0.37	3.32±0.21	-0.195	0.849
Basic FSH （mIU/mL）	8.12±2.69	7.89±2.05	8.17±2.60	8.04±2.11	-0.332	0.972
ovulation induction time（d）	8.05±1.30	8.41±1.25	8.94±1.42	8.27±1.28	-0.117	0.453
Infertilty causes					—	—
Oviduct factor	16	20	19	22		
Uterine factor	2	5	5	7		
Male factor	3	3	2	3		
Compound factor	1	1	3	2		
Unknown factor	2	1	2	1		

Table 2

HBsAg positive rate in embryo cell culture supernatant

Group	Total Samples (piece)	HBsAg（+）samples (piece)	*P*
A3	32	5	0.046
A4	30	12	

Table 3

Morphological grading of HBV infected embryo cells in vitro

Group	Total Samples (piece)	embryo morphological scoring					*P*
		□	□	□	□	*P*	
A1	36	3	2	17	14	0.022[a]	
A2	30	1	5	9	15		
A3	32	0	5	10	17		0.04[b]
A4	30	0	1	6	23		

Note: a is the comparison of embryo cell morphological scoring between four groups, *P* = 0.022; b is the comparison of embryo cell morphological scoring between group A3 and A4, *P* = 0.04

Table 4

Blastocyst formation rate of HBV cultured embryo cells in vitro

Group	Embryo number	blastocyst number	*P*	*P*
A1	36	5	□0.001[a]	
A2	30	3		
A3	32	1		□0.001[b]
A4	30	0		

Note: a is the comparison of blastocyst formation rate between four groups, *P*< 0.001; b is the comparison of blastocyst formation rate between group A3 and A4, *P* < 0.001.

Figures

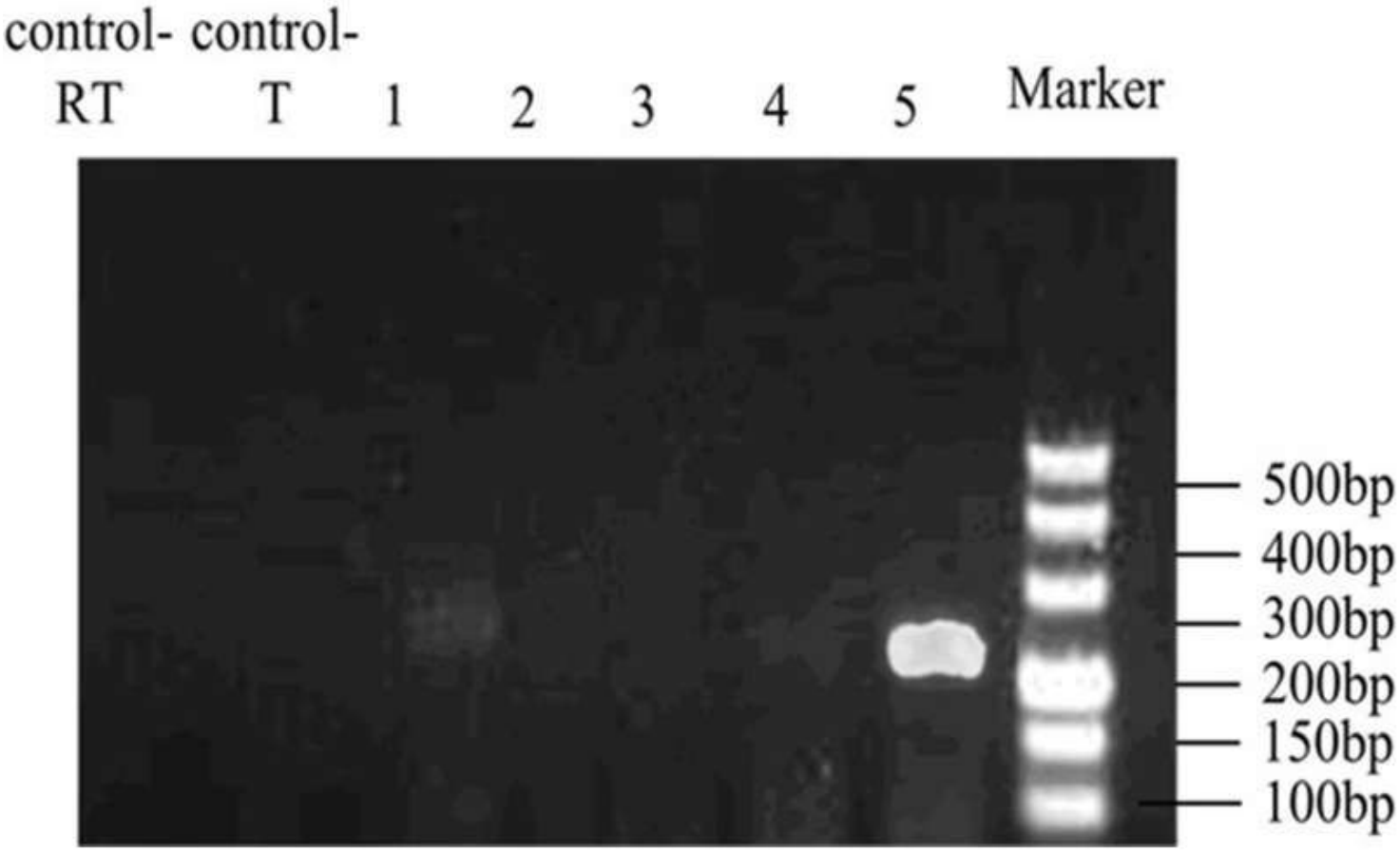

Figure 1

The electropherogram of HBV mRNA amplification products of HBV infected embryo cells in vitro The target fragment was amplified by nested PCR, and the product of second round was 206 bp. Specific HBV mRNA fragments were detected in lane 1 and lane 5.

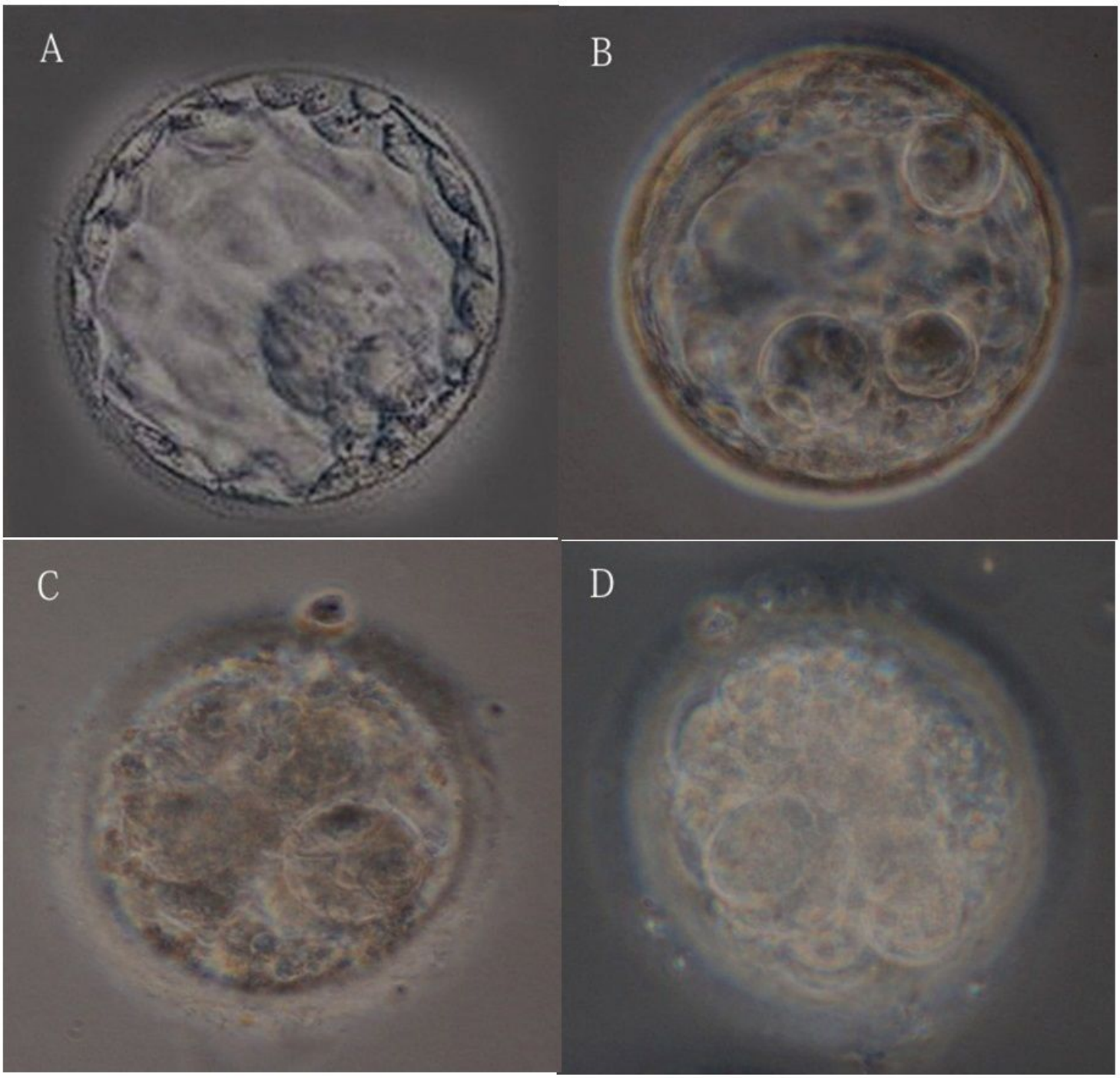

Figure 2

Development state of embryos cultured in vitro (Magnification: 400×) A: grade A blastocysts in group A1; B: grade C blastocysts in group A2; C: grade III embryos in group A3 with different cell morphology; D, grade IV embryos in group A4 with ablated zona pellucida.