Primordial Germ Cell-Mediated Chimera Technology Produces Viable Pure-Line Houbara Bustard Offspring: Potential for Repopulating an Endangered Species

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Abstract

Background

The Houbara bustard (*Chlamydotis undulata*) is a wild seasonal breeding bird populating arid sandy semi-desert habitats in North Africa. The population has declined drastically during the last two decades and it is classified as vulnerable. Captive breeding programmes have been effective in reviving population numbers and thus radical technological solutions are essential for the long term survival of this species. The purpose of this study is to investigate the use of primordial germ cell-mediated chimera technology to produce viable Houbara bustard offspring.

Methodology/Principal Findings

Embryonic gonadal tissue was dissected from Houbara bustard embryos at eight days post-incubation. Subsequently, Houbara germ cells (gPGCs) was injected into White Leghorn chicken (*Gallus gallus domesticus*) embryos, producing 83/138 surviving male chimeric roosters that reached sexual maturity after 5 months. The incorporation and differentiation of Houbara gPGCs were confirmed using Houbara-specific primers. A total of 31.3% (5/16) gonads collected from the injected chicken embryos showed the presence of donor Houbara germ cells. Semen samples from 34 chimeric roosters were analyzed and eight were confirmed as germline chimeras. Semen samples from these roosters were used to artificially inseminate three female Houbara bustards. Subsequently, 45 Houbara eggs were obtained and incubated, two of which were male live born Houbara; the other was female but died before hatching. Genotyping confirmed that the male chick was a pure-line Houbara rooster.

Conclusion

This study demonstrates for the first time that Houbara gPGCs can migrate, differentiate and eventually give rise to functional sperm in chimeric chicken. This approach may provide a promising tool for propagation and conservation of endangered avian species that cannot breed in captivity.


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Introduction

The Houbara bustard is classified as vulnerable on the IUCN Red List and is listed on Appendix I of CITES [1], belonging to the order Gruiformes and it is the only species of the genus Chlamydotis (Chl.). The Houbara bustard breeding conservationists is to generate a means by which Houbara bustards could be produced with the fecundity of cell-mediated chimera technology is a promising approach with the potential to achieve this.

Avian primordial germ cells (PGCs), precursor of the germ cells, are epiblastic in origin [4] during early development, transferred into another chicken embryonic blood circulating system and can contribute a chimeric germ line, which produces functional gametes when in a chicken background.

Materials and Methods

Animals

Houbara bustards (Chlamydotis undulata undulata) were raised and bred in the Houbara breeding center of the Dubai, United Arab Emirates (UAE). Fertilized Houbara bustard eggs were collected after being artificially inseminated. Houbara bustards for progeny testing were raised under the same conditions. White Leghorn chickens were maintained and were collected after artificial insemination (AI). Chimeric chickens were raised under the same conditions.

Preparation of donor Houbara bustard gonadal cells

Fertilized fresh Houbara bustard eggs were collected and incubated for 8 days at 37.8°C and 60% relative humidity.
bustard embryos to determine the sex before dissecting the gonads, and male embryos were used as gonadal donors. The gonadal tissue was collected individually from Houbara bustard embryos under the stereomicroscope, dissected into small pieces using the tip of 1ml syringes. Dissected tissues were then incubated in Trypsin (0.25%) and EDTA (0.02%) solutions for 30 minutes at 37°C, and dissociated by pipetting with P200 pipetman until there were no obvious tissue clumps observed. The tissue was then incubated in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% Fetal bovine serum (FBS) and antibiotics (L-Glutamine-Penicillin-Streptomycin solution; Sigma, G1146). Cell suspension was collected by centrifuging at 300g for 5 minutes to remove the supernatant and resuspended in 1ml DMEM (10% FBS). A total of 5μl of cell suspension was taken to viability by the Trypan blue exclusion method. The cell concentration was adjusted to 4×10⁶ cells/ml before transfer.

**Transfer of the Houbara bustard gonadal cells into White Leghorn chicken embryos**

Fertilized White Leghorn chicken eggs were incubated with the “sharp end up” for 2.5 days until embryonic stage 15–16. A small window (about 10mm in diameter) was made into the shell to expose the embryo on the sharp end. A total of 8×10⁵ DMEM (10% FBS) was injected into dorsal aorta of each chicken embryo with a fine glass pipette. The injected eggs were fixed firmly by a heated surgical scalpel. All of the recipient eggs were incubated with the blunt end up until hatch. Gonadal tissues were collected from the embryos that died during the week before hatch. Houbara bustard cells were detected using species-specific primers as described below.

**Detection of Houbara bustard PGC-derived sperm from the semen of chimeric roosters**

Chimeric chickens were raised to sexual maturity. Semen samples were collected from 34 chimeras weekly for a period of three months and diluted 20 times in calcium and magnesium-free phosphate buffered saline (PBS) and 50 μl of diluted semen was used to detect the presence of sperm by a PCR species identification test as described below. The sensitivity of the PCR species identification test was determined using a graded ratio from one to 10 million sperm (Table 1, Figure 1b).
Figure 1. PCR gels with species-specific primers showing the detection of Houbara bustard DNA. (a) Detection of bustard DNA in the gonadal tissue of chimeric chicken embryos. Lanes 3, 7, 10, 13, and 16 show the bustard species identification sensitivity test: $6 \times 10^6$ chicken sperm mixed with decreasing quantities of bustard sperm; (c) Detection of bustard DNA in roosters. Lanes 4, 5, 9, 18, 21, 26 and 28 show the bustard DNA.

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Table 1. Detection of donor cell-derived Houbara bustard sperm in the semen of chimeric roosters.

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Progeny test

During the breeding season between January and May, semen samples were collected from 8 male chimeric roosters previously. Fresh samples were re-checked by PCR for presence of Houbara bustard DNA. Doses of semen were inseminated twice a week into three virgin female Houbara bustards. The resulting eggs were collected and incubated as above. The remaining unhatched eggs were opened after 25 days incubation to examine the fertility and development.

Blood was collected from the resulting progeny; a piece of muscle tissue was dissected from the body of dead embryos. Sex genotyping and parentage verification tests were conducted with these samples by molecular analysis as described below.

Table 2. Progeny test of germline chimeric roosters by artificial insemination with female Houbara bustards.

Molecular analysis

DNA Extraction.

Pretreatment was done according to the sample type. a) Whole blood: 50µl of blood, collected in EDTA-vacutair K and 500µl of tissue lysis buffer, incubated at 56°C for 2 hrs. b) Tissue samples: about 25mg of the tissue was tissue lysis buffer, incubated at 56°C for 2 hrs. c) Semen samples: 25–50µl of semen was treated with 25µl of 0.25M Tris-HCl (pH 8). Subsequently, any of the above lysed cells were mixed with 1 Isoamylalcohol (25:24:1). The DNA was precipitated using 1/10 volume of 3M sodium acetate and 2.5 volumes of TE buffer (pH8), quantified by Nanodrop ND-1000 Spectrophotometer V3.5 (Nanodrop, Technologies Inc).

Species Identification.

The specific primers CHN1F and CHN1R, BTD2F and BTD2R (Table 3) were used for identification of chicken and Houbara bustard species. These primers were designed using the sequence on chromosome 11 at the NCBI database. Trace/Gallus_gallus_WGS are designed from the cytochrome b gene found in the mitochondria [19]. The Gene Bank accession number for this sequence is AF07 expected product size and references are given in Table 3. Amplification was performed according to the following conditions: i) 1 min, followed by 40 cycles of 94°C for 45sec, 58°C for 30sec and 72°C for 45 sec. The final extension was carried out at 72°C for 10 min.

Sex Determination.

Sex identification was performed according to a) [20]; primers USP1 and USP3 were used to determine the sex control primers (Table 3). The cycling conditions were as follows: an initial denaturation at 95°C for 5 min was followed by 30 cycles of annealing at 50°C for 30 sec and extension at 72°C for 1 min and a final extension at 72°C for 10 min. b) primer fragment that was restriction digested using the enzyme HaeIII. The thermal cycling conditions included a 5 min 95°C 30sec, 55°C for 15 seconds and 72°C for 15 seconds. This was followed by a 1 minute annealing at 56°C amplified PCR product was digested with HaeIII at 37°C for 1 hour. The digested and the undigested products were then made sure all the samples had been amplified.

Microsatellite analysis.

The genome DNA samples were extracted from blood of the live progeny and related adult birds, and subjected...
verification. The list of primers, their sequences and references are shown in Table 4. PCR was performed in a and Fast Start Taq Polymerase (Roche Diagnostics, USA). M13 F or R tailed primers were used and grouped ir analyzed by running on ABI 3730 XL DNA Analyzer and the genotypes were analyzed using the Genemapper V4.0 (Applied Biosystem

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<th>No.</th>
<th>Primer name</th>
<th>Primer sequence</th>
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<tr>
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<td>Ba2234R</td>
<td>CGA ACG CTA ATT ACG ACC AAC CAA G</td>
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<tr>
<td>2</td>
<td>Ba2234F</td>
<td>CAG GTC TGG TGT TCT AAG GA</td>
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<td>14</td>
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Table 4. Primer list of genotyping analysis using Houbara bustard microsatellites (STR) markers [26]
doi:10.1371/journal.pone.0015824.t004

Results

Harvest of donor Houbara bustard gonadal cells

Chicken embryos developed to embryonic stages 26, 28 and 30 after 5, 6 and 7 days incubation, while Houbara slower development in the same age, approximately 1–2 days delay, and showed significant individual variance period (22–24 days). The total number of gonadal cells in 8dpi Houbara bustard embryos was 102.7±21.2×10^3, embryos, and 114.8±20.5×10^3 cells (n = 23) and 96.9±1.2% in male embryos.

The morphological characteristics of Houbara bustard gPGCs were similar to that of chicken. They can easily be distinguished from somatic cells, which were larger in size (12–15 µm in diameter) as well as richer in granules in the cytoplasm than somatic cells.

Production of chimeric chickens

In total, 138 chicken embryos were injected with Houbara bustard gonadal cells from individual male embryos, with a hatchability of 60.1% (83/138). All of the hatched putative chimeric chicks had a typical White Leghorn phenotype. Under normal conditions, and 35 male and 35 female birds reached to sexual maturity after 5 months. Houbara bustard species-specific primers have been developed and used to detect Houbara bustard sperm prc
identification PCR test was determined with the mixed samples of Houbara bustard and chicken sperm. Certain Houbara bustard sperm was mixed with six million chicken sperm. Results showed that Houbara bustard sperm 10 Houbara bustard sperm in chicken semen containing 6 million sperm (Figure 1b).

A total of 302 semen samples were collected from 34 chimeric roosters. Houbara bustard species-specific DNA 23.5% (8/34) birds. Since the semen does not contain somatic cells, these results indicated that eight chimeric sperm and therefore considered as germline chimeras. Further 95 semen samples were collected from these 8 confirmed as Houbara bustard-DNA positive (Figure 1c; Table 1). These results suggested that Houbara bustard might be able to differentiate into sperms in the testis of chimeric rooster.

Progeny test

Three female Houbara bustards were artificially inseminated 198 times with the semen samples collected from 8 samples inseminated were confirmed containing Houbara bustard sperms by molecular analysis. Subsequently, incubated, of which two eggs were found to be fertile. One successfully hatched after 22 days of incubation, while hatching (Figure 2a–d, Table 2). The chick and the dead embryo showed typical Houbara bustard phenotype, al
Figure 2. Parents, offspring and progeny tests.  
(a) Germline chimeric rooster; (b) Female Houbara bustard (HB020154); (c) Houbara bustard chick generated from donor cells derived sperm produced by chimeric rooster (d) Dead Houbara bustard embryo from chimeric rooster; (e) species ID PCR of the dead Houbara embryo and the live Houbara chick.  
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Species identification and parentage test of the resulting progenies

A Houbara bustard species-specific DNA fragment was amplified from both offspring, however chicken species-providing strong evidence that the resulting offspring were genetically pure Houbara bustard. The hatched chick female (Figure 2f). The genotyping analysis using Houbara bustard and chicken microsatellites (STR) markers provided independent confirmation that the resulting offspring were genetically pure Houbara bustard, not chick produced through parthenogenesis, as their genotypes are not fully derived from their mother. Moreover, it verifies genotypes with the parental (donor) genotype. Unfortunately no sample was kept from the donor animal and thus parents. At the same time one can predict the donor's genotype from its offspring and their mother. By comparing donor's genotype as described in Table 5.

Table 5. Genotyping analysis of the Houbara bustard family using Houbara bustard microsatellites (STR).  
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Discussion

In the present study, a viable Houbara bustard was successfully hatched between a male chimeric rooster and a female (Figure 2f). The study demonstrates that PGCs can be harvested from embryos with a high viability and that germ cells are able...
and differentiate into functional sperm alongside the endogenous chicken sperm. In other words, the male chick spermatogonial development in the testis. The chimeric rooster thus served as a surrogate father of the chick, p including hormonal systems, might be widely conserved in different avian species and orders. This might help u particularly the male spermatogenesis process.

Already pheasant PGCs derived progenies were successfully produced from chimeric roosters [16]. Also donor chicken-quail chimera; however, no progeny was obtained [22]. Highly sensitive molecular sexing and species ii were developed as a result of this research, also providing strong molecular tools for Houbara bustard research sperm, and the tracing of the donor cells in the chimeric embryo or rooster.

The Houbara bustard reaches sexual maturity at about two years (one year at the earliest) [23]; this was confirm sexual maturity in about five months. In the present study, it was confirmed that Houbara bustard sperm could be mature, indicating that donor germ cell differentiation occurs in same time that the recipient spermatozoa forms rooster gonads supports the development of host germ cell as well as of the donor germ cells. The donor Houbara semen sample, but no specific pattern was observed. However, donor DNA was detectable from the host semen negative result on donor sperm in the host semen is due to the PCR sensitivity. Furthermore, Houbara bustard I season. It is also not clear if the donor sperm was generated and released in the host testis by its own spermatogenesis in the host seminiferous tubules needs to be investigated further. Unlike the differentiation pattern, which the Houbara bustard PGCs follow the non-seasonal breed the chance to produce even more Houbara bustards using the domestic male chicken reproduction system all y

The efficiency of the progeny production is still very low, only one live chick and one dead before hatching. It is i pure Houbara bustard, not hybrid, and not from a parthenogenic development. Furthermore we provided strong of the chimeric rooster and the Houbara bustard by the parentage test, which is a strong molecular tool, for the kind of competition between the donor and recipient germ cells in the chimeric body, but still it is not well understood chimeric rooster semen is fluctuating. To increase the number of donor derived sperm few efforts need to be made by purification and culture in vitro [24], [25], 2 reduce the endogenous chicken PGCs using mechanic or chemi

Furthermore, production of female chimeras between chicken and other domestic avian species by PGCs trans PGCs derived offspring has not been achieved from the chimeric hens [27]. If female PGCs could differentiate it bustards could be reproduced through male and female chimeric chicken. This will greatly increase the Houbara from hunting wild birds, and discourage people from trading or smuggling Houbara bustards. In the end, this tec other endangered avian species that cannot be bred in captivity.

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Author Contributions

Conceived and designed the experiments: UW CL I-KC. Performed the experiments: CL VB ZG KAK SS I-KC. Contributed reagents/materials/analysis tools: UW KAK RW CL. Wrote the paper: UW CL KAK JK I-KC DKG.

References


