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Developing a puncture-free *in ovo* chicken transfection strategy based on bypassing albumen nucleases

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ABSTRACT

Chicken is a dual-purpose animal important from both agricultural and medical aspects. Even though significant improvements have been made in chicken transgenesis technologies, chicken genome manipulation has not been widely used in developmental biology. This study was aimed to evaluate chicken egg white nuclease properties and thereof plausibility of devising an *in vivo* transfection technology without causing physical damage to the embryo. First, the nuclease activity of egg albumen was assessed. The egg white nucleases were strongly active in degrading DNA and RNA. The egg white DNase activity was comparable to commercially available DNase-I. Nuclease activities were also assessed after heating, proteinase K, or EDTA treatment. Unlike proteinase K, both heating and EDTA were noticeably effective for the nuclease inactivation. Simultaneous application of lipoplex form of DNA (1 μg pDB2: 3 μl Lipofectamine2000) and EDTA showed a synergistic effect in protection against egg white nucleases. Finally, we injected the lipoplexes with or without EDTA close to the embryo at day0, but outside the embryonic epiblast. Implementation of a scrutinized PCR assay indicated that transfection took place only when EDTA was complemented to the lipoplexes. The transfection rate of day4 embryos and the hatched chicks were 54.5 and 30.0%, respectively. EGFP expression was detected in two out of three transgenic chicks. In conclusion, this study provided a detail analysis of chicken egg albumen nuclease properties and suggested the feasibility of developing a puncture-free handmade technology for transfection of the chicken embryo.

1. Introduction

Making transgenic animals with simple and efficient transfection procedure is highly demanded in transgenesis centers. One-step transgenesis by transfection of sperm, oocyte, or embryo can be the best way to solve this issue [1,2]. For chicken transgenesis, oocyte manipulation is not a feasible strategy as it would need conducting an operation in donor hens with a low rate of embryo retrieval [3]. On the other hand, sperm and the developing embryo in the newly laid egg are easily accessible for manipulation. Subjecting sperm cells for transfection has not been a straight-forward strategy for making transgenic animals [4–9]. It has been documented that only membrane-disrupted or dead sperm can stably uptake exogenous DNA while motile spermatozoa are resistant to transfection [5–8]. Therefore, embryo manipulation remains the only way for genetic modification of the chicken embryo.

Retroviral infection of the day0 embryo was the first reported method for chicken transgenesis. However, the drawback of this technique was the very low efficiency of transgene transfer [10]. Afterward, lentiviruses which comprised of HIV elements was efficiently developed as a new class of retroviruses [10,11]. The lentiviral system has two main advantages over the traditional retroviral infection system, including transducing ability of non-dividing cells and reducing the transgene silencing by the host genome [12]. However, limitation of the technology in transferring short-length transgene [12] and more importantly, safety concerns in consumption of lentiviral infected farm animals convinced

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scientists to look for non-viral gene transfer technologies [13]. Transfection of the primordial germ cells derived at day2.5 of embryonic development and their re-introduction at day6 embryo is another reliable technology [14]. However, the method is a multi-staged procedure with the high demand of time, facilities, and expert staff (for review, see Ref. [15]). To circumvent these disadvantages, *in vivo* transfection of circulating primordial germ cells (cPGC) has been developed by microinjecting the lipoplexes into blood vessels [16] [13] of early embryos. However, this newly-modified method, also results in unavoidable damage to chicken embryos during the injection procedure, reducing hatchability, and requiring expert staff for conducting microinjection.

Egg white can be considered as one of the most important factors for chicken embryo development [17] [18]. Its main role starts following the oviposition for formation and expansion of the subgerminal cavity [17]. In addition, egg white is massively absorbed into the ectodermal part of avian embryo throughout the incubation period [18]. Any *in ovo* injected DNA either outside of the embryo or in the subgerminal cavity can be directly confronted with the egg white. Egg white ingredients could interact with exogenous DNA in either a protective [19] or destructive [20] manner via its ample avidin content or its nucleases, respectively. Unsuccessful transfection of the chicken embryo with lipoplexes strongly suggested that there is a stronger transfection barrier which needs to be elucidated in the egg albumen [9]. Therefore, a comprehensive study of chicken egg white nucleases can provide fundamental information about the nuclease properties and potential bypassing methods which can be applicable for direct *in ovo* transfection of the chicken embryo. This study was aimed to evaluate the chicken egg albumen nuclease properties and to assess different treatments for surmounting this hurdle for chicken transgenesis. We devised an efficient handmade transfection technology for one-step chick transfection by injecting the lipoplex-EDTA complex in the egg white surrounding the early blastocyst with no physical damage to the early embryo.

2. Materials and methods

2.1. Ethics statements

The *in ovo* injection approach was carefully carried out in a close vicinity of the day0 embryo to alleviate any physical damage to the embryo. Eggs which did not hatch until day22 of incubation were opened and dead embryos were used for DNA extraction and PCR assay. The day-old chicks were euthanized and used for further analysis. All the experimental procedures were assessed and approved by the Institutional Animal Care and Ethics Committee of Isfahan Branch, Islamic Azad University.

2.2. Constructs

In this study, we used the pDB2 plasmid for evaluation of nuclease activity as well as transgenesis purposes. The 5047 bp pDB2 plasmid contained an enhanced green fluorescent protein (EGFP) cDNA under a human cytomegalovirus immediate-early enhancer/promoter (CMV immearly promoter) along with a CAG enhancer and an SV40 polyA terminator sequences. In addition, we used pTn5 plasmid (15 kb length), E. *coli* as and wild boar genome as prokaryotic and eukaryotic sources, two 60 nt oligonucleotides, as well as total RNA extracted from the honey bee.

2.3. Chicken egg white nuclease activity

To reduce the egg effect on the results, chicken egg whites from 10 eggs were mixed, and a 100 μl of the mixture was randomly chosen for evaluation of nuclease activity. One microgram of DNA, RNA, or oligonucleotides were mixed with the egg white and incubated at 37.5 °C for 1 and 24 h the reaction was stopped by adding 200 μl phenol and two times washing by chloroform to remove the phenol traces. An equal fraction of the aqueous phase was visualized on a 2% agarose gel or quantified by a spectrophotometer machine.

2.4. Egg white nucleases vs. DNase I

To compare the DNase activity of egg albumen with commercial bovine pancreatic DNase I (18068-015; Invitrogen), one micro gram of the plasmid was mixed with 0, 20, 50, 80, and 100% egg albumen in 100 μl final volume. The incubation assay was conducted at 37.5 °C for 30 min. Moreover, one micro gram of the plasmid was incubated with 1 IU of DNAse I at 37 for 30 min. A more subtle range of egg albumen, including 0, 20, 50, 70, 100, 150, 200, 250, and 300 μl, was also incubated with 1 μg plasmid in 300 μl final volume at the before-mentioned condition. In all cases, distilled water was used to adjust the final reaction volume. DNA extraction and evaluation was carried out by the above-mentioned assays.

2.5. Bypassing the nucleases activity

We assayed different inactivation approaches for the chicken albumen nucleases. All of the below experiments were repeated for more than four times. In all experiments, 100 μl of the egg albumen was mixed with 1 μg pDB2 and/or other reagents in 120 μl final volume. Afterward, the mixtures were incubated at 37.5 °C for 1 h and the DNA was extracted and monitored by the above-mentioned phenol-chloroform protocol.

2.5.1. Pretreatment of egg albumen by EDTA, proteinase K, or heating

Egg albumen was mixed with four treatment groups, including of control (distilled water), 25 mM EDTA, Proteinase K (20 mg/ml) (EO0491; Fermentas) at 37.5 °C for 1 h, and heating at 65 °C for 5 min. To prevent any confounding effect between heating and proteinase K activity, the effect of proteinase K on egg white was assessed at 37.5 °C instead of 55 °C which is the optimum temperature for proteinase K activity.

2.5.2. Pre-incubation of egg albumen at 37.5 °C

Egg albumen was extracted from four groups of eggs: newly laid eggs and eggs which were pre-incubated at 37.5 °C for 24, 48, and 72 h.

2.5.3. EDTA concentration

The inhibitory effect of EDTA on albumen nuclease activity was assessed in a series of EDTA concentrations, including 0, 2.5, 5, 10, 15, 20, 25, 30, 35, 40, and 50 mM.

2.5.4. Combined Lipofectamine 2000 and EDTA

In a factorial experiment, the combined effect of Lipofectamine 2000 and 5 mM EDTA was used to evaluate the nuclease inhibitory effect. One microgram of the plasmid was mixed with 1, 2, or 3 μl of Lipofectamine 2000 and incubated at room temperature for 1 h to produce lipoplexes in 1:1, 1:2, or 1:3 ratios, respectively. The lipoplexes were then mixed with 100 μl of the egg albumen supplemented with or without 5 mM EDTA and incubated at 37.5 °C for 24 h. In addition, the lipoplex ratio of 1:3 was further incubated in the presence or absence of 5 mM EDTA for 30 or 50 h; four combined groups of EDTA and time classes were used with lipoplex 1:3 ratio.

2.6. Chicken embryo transfection

Finally, the embryo-mediated transgenesis was evaluated by day0 *in ovo* injection in newly laid eggs. The embryo transfection procedure was carried out as outlined in Ref. [9]. In summary, newly laid fertile eggs from Ross broiler breeders were stored upright at 12 °C for 24 h to translocate the embryo beneath the tapering end of the egg. A small square hole, approximately 1 cm^2, was drilled at the tapering end and *in ovo* injection was conducted in the egg white thoroughly surrounding the embryo by a 100 μl Gastight 1700 Series syringe (Hamilton Robotics, USA). The injection groups comprised of de-ionized water (sham injection), 2 μg pDB2 plasmid (naked DNA), 2 μg pDB2 plasmid combined with 6 μl Lipofectamine2000 (lipoplexes), 2 μg pDB2 plasmid concomitant with EDTA 5 mM (naked DNA + EDTA), and 2 μg pDB2 plasmid, 6 μl Lipofectamine2000 and EDTA 5 mM (lipoplexes + EDTA). The injections were carried out in 50 eggs for each group. The separated shell wall was replaced and pasted gently, and the incubation was carried out at 37.5 °C and 65% humidity for 22 days. At day4 of incubation, a random sample of injected eggs was cracked, and the embryos were transferred into plastic dishes and washed three times with PBS to remove debris and to overcome DNA carryover. The EGFP expression was evaluated by a fluorescent imaging merchandiser (BLS Ltd., Hungary). In addition, the recovered embryos were used for DNA extraction. A PCR approach was carried out with specific primers for EGFP gene to evaluate the transgene transfer into the developed day4 fetuses. At day22 of incubation, all the hatched chicks or dead chicks (developed to > day17) were rinsed to remove debris and evaluated for EGFP expression by *in vivo* fluorescent assay. Moreover, the transgene transfer into their beak, blood, breast muscle, heart, intestine, legs, toes, liver, gonads, and wings was assessed by PCR. It should be noted that animal tissues were kept at −70 °C for further repeats of DNA extraction and PCR. After carefully cleaning steps of the utensils with water and successive exposures to ultraviolet light, PCR reactions were repeated for all the PCR-positive DNA samples in another laboratory in which no plasmid propagation had been conducted. We implemented this highly scrutinized PCR assay, as the only way to evade from pseudo-positive PCR results as the most challenging issue in transgenesis technologies, e.g., during sperm-mediated gene transfer (SMGT) [7,9,21,22]. Therefore, direct visualization of the green fluorescent expression in G0 animals was the only gene expression approach in this study.

3. Results

3.1. Presence of nucleases in chicken egg albumen

After 30 min incubation of chicken egg albumen with various sources of DNA originated from the prokaryotic and eukaryotic genome, plasmids, oligonucleotides, as well as total RNA, a very strong DNase activity was detected in the egg albumen (Fig. 1A). Circular plasmids, pDB2 and pTn5 with 5 and 15 kbp lengths, respectively, and *E. coli* and wild boar genomes were fragmented into small pieces ranging from 50 bp to 1000 bp. The phytase gene amplicons were degraded completely. Moreover, total RNA which was extracted from the honey bee abdomen was also degraded completely following 30 min incubation. Interestingly, the egg albumen was not effective in degrading DNA oligonucleotides. Extension of the incubation time to 24 h led to a complete degradation of pDB2 and the genomes (Fig. 1B). However, the short fragments of the pTn5 plasmid were cleaved to 50 bp fragments which seemed to be resistant to the nucleases. Furthermore, the DNA oligonucleotides remained to be resistant throughout the

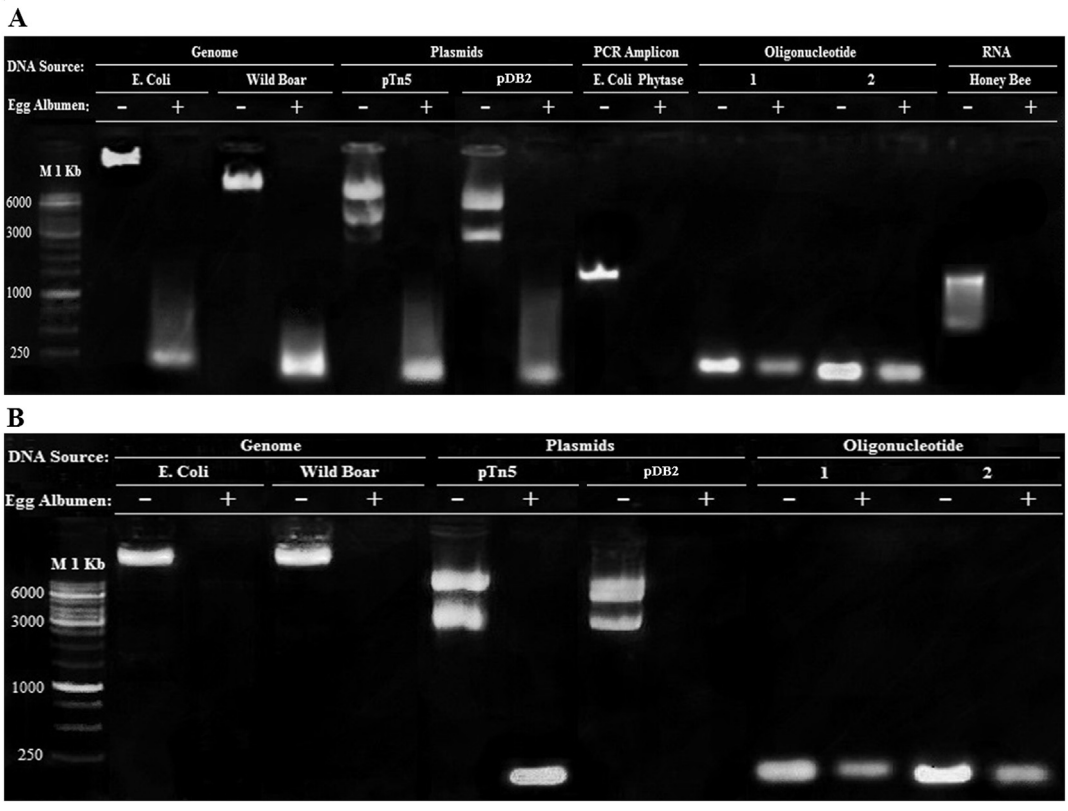

Fig. 1. The presence of nuclease activity in chicken egg white. DNA, total RNA, and oligonucleotides were incubated with 100 μl of egg albumen at 37.5 °C for 30 min (A) and 24 h (B). All samples were treated with phenol and then ran on a 2% agarose gel electrophoresis.

incubation process.

3.2. Evaluation of the chicken egg white nuclease activity

To evaluate the DNase activity of chicken egg albumen, one microgram of pDB2 was treated with a series of egg albumen dilutions in a final volume of 100 μl at 37.5 °C for 30 min. The nuclease activity became obvious when 50 μl of the egg white was used in a 50% dilution (Fig. 2A). Fragments of >500 bp were removed after treatment with 80% dilution of egg albumen whereas the shorter fragments remained even by treatment with 100 μl egg albumen. This experiment was repeated with a more subtle range of dilutions in a larger volume of egg albumen (Fig. 2B). Assessing the nuclease activity of egg white in 300 μl reaction volume indicated that incubation of 50 μl egg albumen, 20% dilution, caused trivial DNA degradation, whereas the original bands were completely divided into 50–3000 bp fragments when 150 μl of egg albumen, 50% dilution, was applied. Moreover, the result from 250 μl egg albumen, 83% dosage, was comparable to the treatment results with one unit of DNase I.

3.3. Bypassing the albumen nucleases activity

Then, we assayed different inactivation approaches for the chicken albumen nucleases using Proteinase K treatment, heat inactivation, and EDTA chelating agent. The nuclease activity was not affected by Proteinase K, whereas both EDTA pre-treatment and the heating approaches could strongly inhibit the nuclease activities (Fig. 3A). In addition, the functionality of egg albumen nucleases decreased by prolonging the egg albumen pre-incubation at 37.5 °C (Fig. 3B). Results of the egg albumen treatment with a series of EDTA concentration showed that final concentration of 5 mM EDTA is sufficient to completely inactivate the nucleases (Fig. 3C). Furthermore, the egg albumen was incubated with a cationic lipid, Lipofectamine2000, in three lipoplex formula comprising 1–3 μl of lipofectamine with 1 μg of pDB2 with or without EDTA for 24 h (Fig. 3D). Used of lipofectamine was very effective in protecting the DNA from degradation for 24 h incubation. Moreover, the EDTA complementation to the lipoplexes strengthened the protection assay. DNA in the lipoplex mixture without EDTA started degradation after 30 h incubation and removed completely after 50 h incubation time, although the EDTA-lipoplex mixture could considerably protect the DNA from degradation even after a 50 h incubation period.

3.4. Chicken embryo transfection

Finally, the embryo-mediated transgenesis was evaluated by *in ovo* injection of lipoplexes containing pDB2: lipofectamine in 1:3 ratio with or without EDTA in the vicinity of the embryo in the newly-laid egg. None of embryos/chicks from either EDTA (n = 22) or lipoplex (n = 25) groups carried the EGFP transgene (Table 1). However, rigorous PCR analysis from the lipoplex-EDTA group showed the presence of EGFP transgene in 6 out of 11 day4 embryos (54.5%) and 3 out of 10 hatched chicks (30%), i.e. the transgenesis efficiency averaged 42.9% in all of the evaluated samples from the lipoplex-EDTA group (Fig. 4A–B). The EGFP expression was confirmed in two of these three chicks by fluorescent imaging (Fig. 4C). Although the transgene integration was detected in the majority of organs in these two chicks, a mosaic pattern was evident for the transgene integration and expression. However, gonads, as the most important organs in the transgenic animals, also carried the transgene.

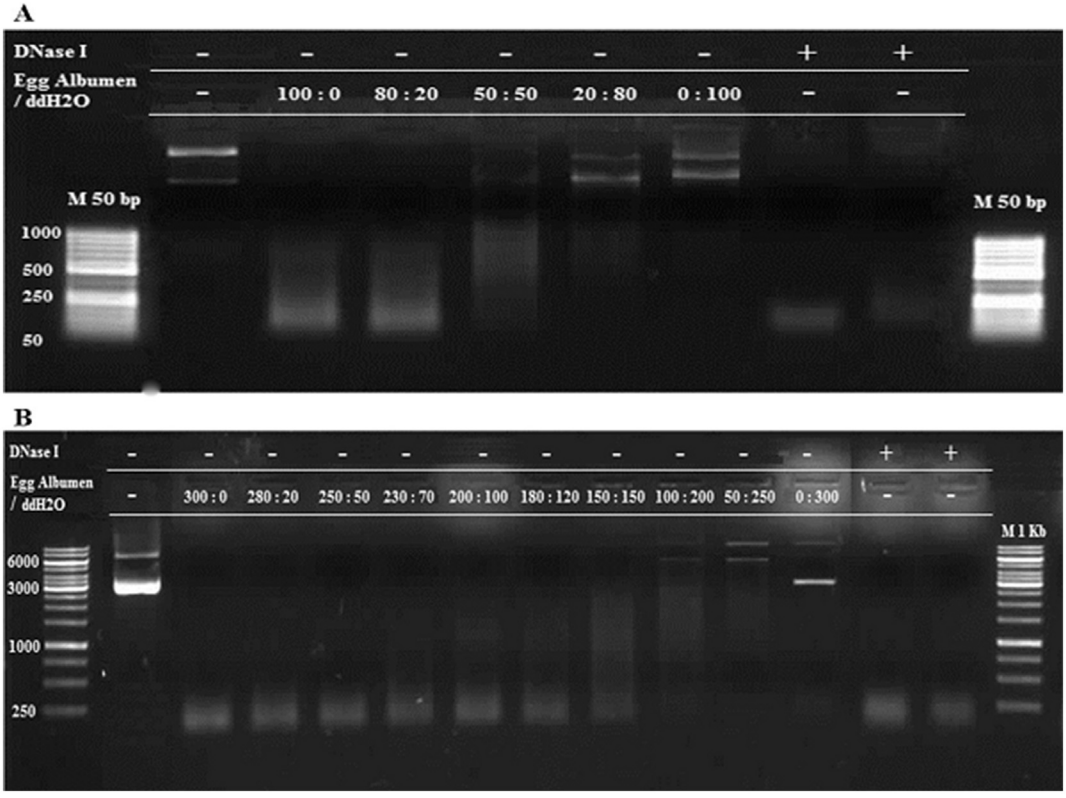

Fig. 2. Evaluation of the chicken egg white nuclease activity compared to a commercial DNase-I. One microgram of pDB2 plasmid was incubated with egg white at 37.5 °C for 30 min in a 100 μl (A) and 300 μl final reaction volume (B). All samples were treated with phenol and then ran on a 2% agarose gel electrophoresis.

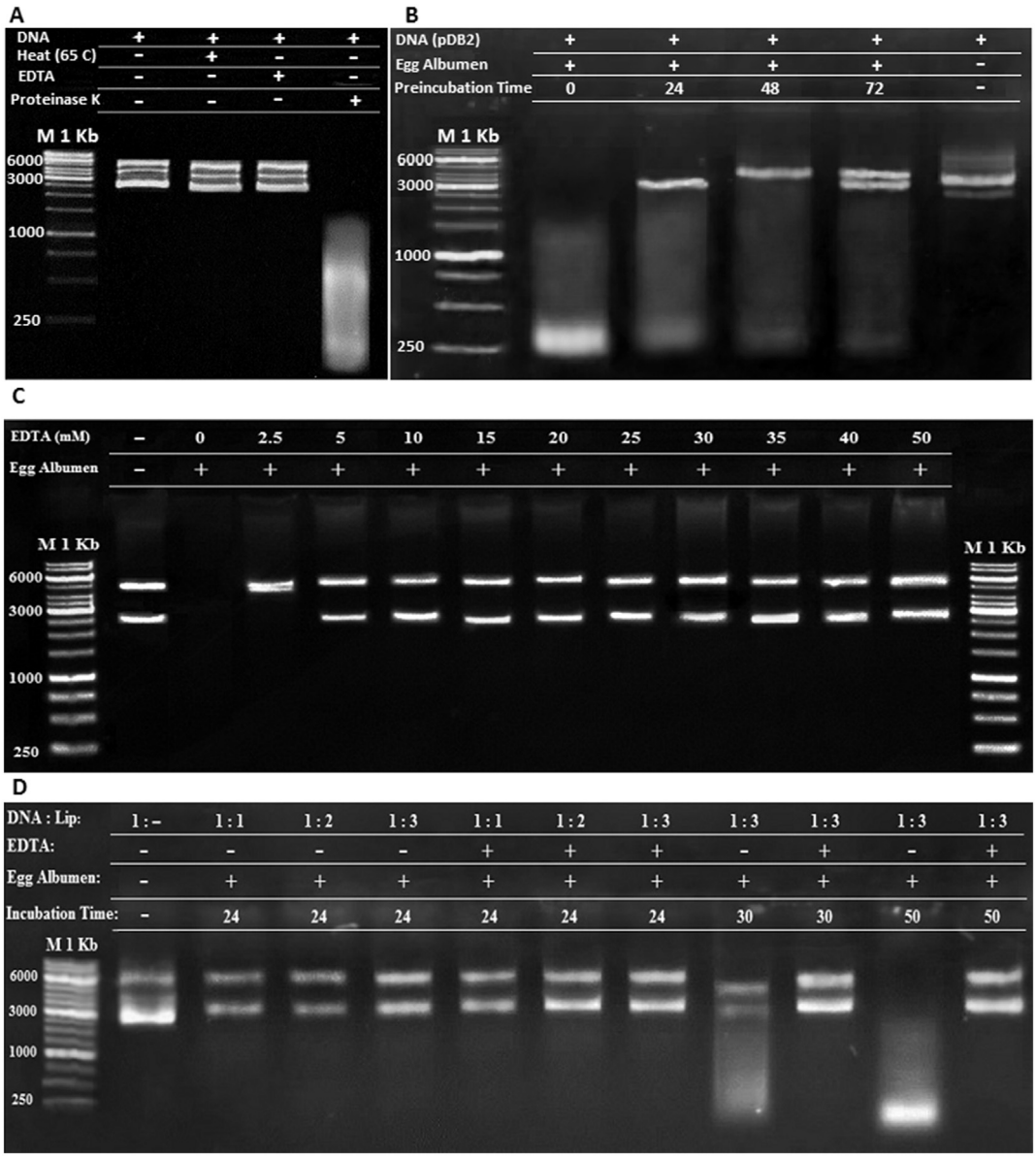

Fig. 3. Assessing different approaches for bypassing the chicken egg albumen nuclease activity. Egg white was treated with 20 mg/ml Proteinase K, heating at 65 °C for 5 min, and 5 mM EDTA (A), the effect of egg white preincubation at 37.5 °C on its nuclease activity (B), the effect of various EDTA concentration on the egg white nuclease property (C), and lipoplex formation (1 μg pDB2: 1–3 μl Lipofectamine2000) with or without complementation to 5 mM EDTA (D). All samples were treated with phenol and then ran on a 2% agarose gel electrophoresis.

Table 1
Chicken embryo development rate and transfection efficiency following a puncture-free injection of lipoplexes with or without 5 mM EDTA in the close vicinity of day 0 embryo.

Factor	Execution time	Treatment				
		Non-injection	Sham-injection	pDB2 + EDTA	pDB2 + Lipofectamine	pDB2 + Lipofectamine + EDTA
No. of injected egg	Day 0	50	50	50	50	50
No. of assessed embryo/chicken	Day 4	15	12	11	12	11
	Day 22	15	18	11	13	10
Transfection efficiency (PCR) (%)	Day 4	$\frac{0}{15}$ (0.0)	$\frac{0}{12}$ (0.0)	$\frac{0}{11}$ (0.0)	$\frac{0}{12}$ (0.0)	$\frac{6}{11}$ (54.5)
	Day 22	$\frac{0}{15}$ (0.0)	$\frac{0}{18}$ (0.0)	$\frac{0}{11}$ (0.0)	$\frac{0}{13}$ (0.0)	$\frac{3}{10}$ (30.0)
EGFP expression efficiency (%)	Day 4	$\frac{0}{15}$ (0.0)	$\frac{0}{12}$ (0.0)	$\frac{0}{11}$ (0.0)	$\frac{0}{12}$ (0.0)	$\frac{0}{11}$ (0.0)
	Day 22	$\frac{0}{15}$ (0.0)	$\frac{0}{18}$ (0.0)	$\frac{0}{11}$ (0.0)	$\frac{0}{13}$ (0.0)	$\frac{2}{10}$ (20.0)

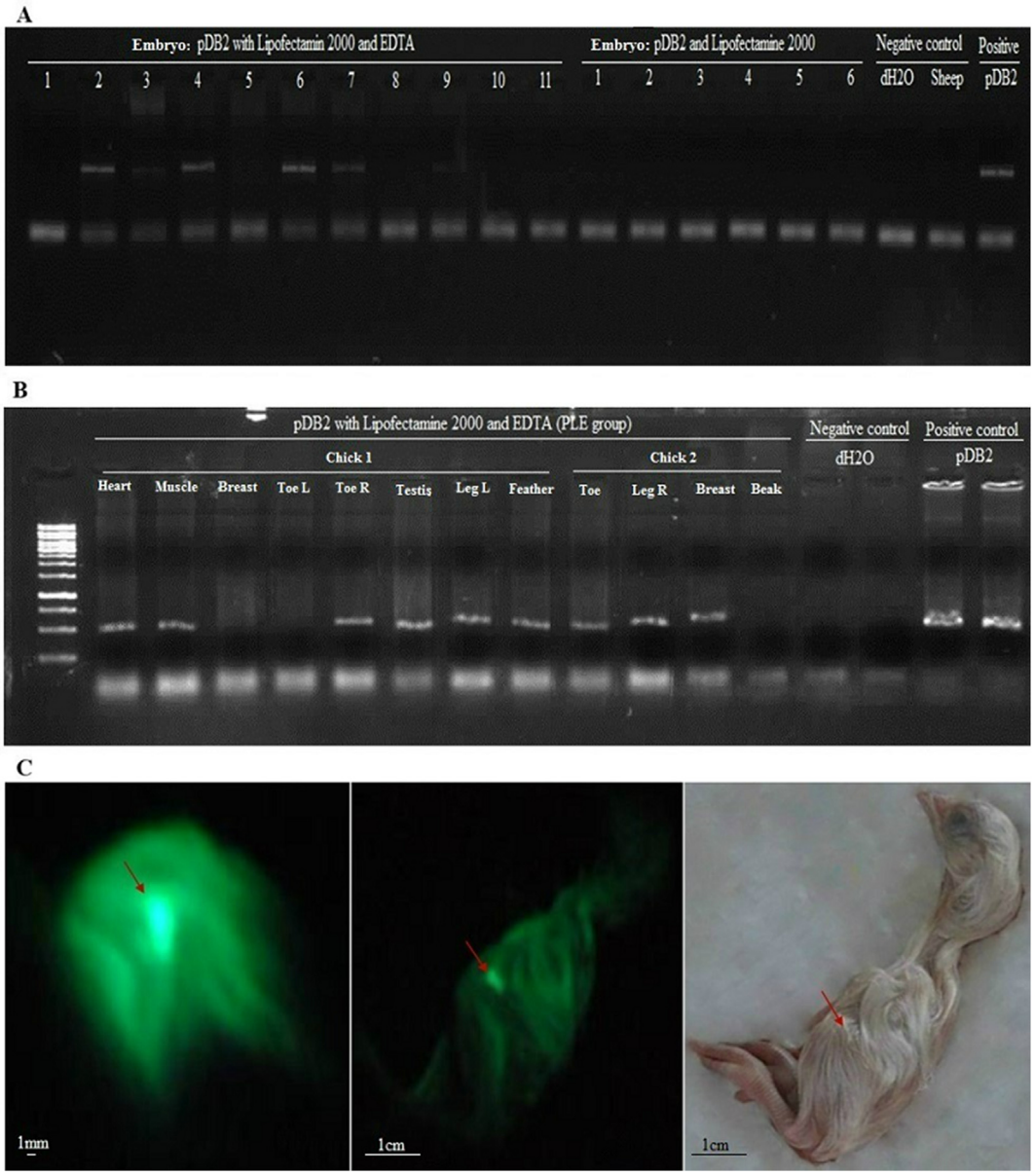

Fig. 4. Puncture-free *in ovo* transfection of chicken embryo using lipoplexes complemented with 5 mM EDTA. PCR results from embryos (A) and different parts of two hatched chicks (B). Water (dH2O) and sheep cells were used as negative controls for DNA extraction and PCR. A day-old dead chick expressing the EGFP transgene (C): C1–C3 is black and white, fluorescent, and the fluorescent image with higher magnification, respectively, from the same chick.

4. Discussion

In this study, we evaluated chicken egg white nuclease activity on various sources of DNA, honey bee total RNA, and two oligonucleotides. Results of this study showed the presence of a strong nuclease activity in the chicken egg white which can degrade DNA and RNA in less than 1 h. Such nucleases at the *in ovo* condition may be essential for preventing bacterial and viral infections and providing a clean environment for the embryo nutrition. Also, our results supported the premise that any injected DNA inside or outside of the chicken embryo can be challenged with the strong nuclease properties of the albumen [9]. Interestingly, the nucleases were not effective on oligonucleotides. This inability of egg white could be utilized *for in ovo* gene silencing strategies using oligonucleotides and morpholinos in chicken. Comparing our results with published literature, we found only one report in which the linearization of a supercoiled plasmid was detected following treatment with chicken egg albumen [20]. Results from the current study are the first report on the presence of strong endo- and exonucleases in the egg albumen. The relatively weak nuclease activity of egg albumen even after 24 h incubation [20] can be partly due to the storing condition of the chicken egg before the albumen separation.

Moreover, comparison of the nuclease activity of chicken egg albumen with the commercial DNase-I indicated that if egg albumen comprises approximately 80% of the reaction volume, the nuclease activity can be comparable to one unit of DNase-I. It should be mentioned that in this study, we also tried to quantify the DNA concentration using spectrophotometer machines. However, we couldn't get reliable absorbance measurements at 260 and 280 nm to report, even though we used three different spectrophotometer machines. This can be due to interference of the egg white with the phenol-chloroform DNA extraction assay. However, to increase the repeatability of gel electrophoresis results, we

repeated each experiment at least four times.

Then, we assessed different strategies to overcome this nuclease activity, including Proteinase K, heat shock, and EDTA treatment of the egg albumen as well as lipoplex formation of the transgene before interacting with the albumen. Proteinase K treatment of egg white did not cause to inactivation of the nucleases, although based on the producer guidelines it was a serine protease which can remove DNases and RNases. This can be due to the ineffectiveness of the proteinase K on the protein structure of nucleases, protection of DNases by egg white calcium [23], or inactivation of the proteinase K by anti-proteases inside of the egg white [24,25]. It has been well-documented that chicken egg white contains a broad range of protease inhibitors [24,25]. Therefore, it is high likely that protease inhibitors blocked the proteinase K before any interaction with the nucleases. In addition, we evaluated heat inactivation of nucleases and showed that the nucleases are sensitive to a heat shock at 65 °C. Moreover, 24 h incubation of egg white at 37.5 °C considerably attenuated the nuclease activity. It might be noteworthy that nuclease activity of egg white from those eggs which were purchased at summer was considerably weaker than those which were evaluated in winter (data not shown). Together, our data indicated that nuclease activity of egg white surrounding a day1 chicken embryo is much weaker compared to that of the day0 embryo. However, because of the quick progress in the embryo development at the early days of incubation, manipulation of the day1 embryo for the aim of transgenesis can increase the propensity of higher mosaic rate compared with that for day0 embryo. Therefore, we need to look for other strategies for inactivation of egg white nucleases. In this study we also evaluated if the nucleases are dependent on the calcium and magnesium ions. EDTA treatment of the egg albumen could completely inactivate the nucleases. It has been shown that high EDTA concentration can prevent integration of the transgene into the host genome [26,27] and postpone the embryo development as well [28]. Therefore, detection of very low dosage of EDTA which inactivates the nucleases while minimizing corresponding mortality rate can be very critical for transgenesis efficiency and embryo development. Then, a series of EDTA concentration was mixed with the egg white, and the nuclease activity was evaluated. The results showed that using 5 mM EDTA can be sufficient to have a strong inactivation of the nucleases.

Beyond the above-mentioned nuclease inactivation assays, lipofectamine effect on DNA protection against the egg white nucleases was also evaluated. The lipoplexes were resistant to the albumen nucleases for 24 h. However, the plasmid started degradation in longer incubation time. Interestingly, 5 mM EDTA supplementation to the lipoplexes extended the protection assay to >50 h. Lipofectamine has a large positive charge which can bind exogenous DNA and makes a heterogeneous multi-layer and condense structure around the DNA, preventing its degradation from inter- and intra-cellular nucleases [29]. Moreover, because of having a positive net charge, the rate of DNA migration through the electrophoresis gel is slower than naked DNA, though it can more efficiently interact with the cell membrane phospholipids layer and facilitate the transfection rate. Overall, based on the results of DNA incubation with the egg white, it can be suggested that egg white nucleases are much stronger than the proposed avidin protection property by Morpago et al. [19].

After evaluation of chicken egg nuclease properties and trying different strategies to overcome the nucleases, we injected the exogenous DNA in a lipoplex form with or without EDTA in the closed vicinity of day0 embryo. Our results showed that irrespective of the injection solution, passing through the egg membrane and injecting materials in the embryo surrounding media deteriorated the embryo development compared to the pierced group without injection. The results of this study also showed that lipoplex injection alone at day0 of embryo incubation is inefficient for making transgenic chicks. This can be due to the nuclease attributes of egg albumen which can degrade lipoplex form of exogenous DNA after 24 h incubation. In this study, concomitant lipoplexes and EDTA injected eggs was the only group in which the DNA transfer was detected by PCR. The overall transfection rate of the embryos was 42.9%. However, as expected, like other chicken transgenesis technologies, the transgenic chicks were mosaic regarding the transgene integration and expression. The transfection rate through this technique was higher than the *in ovo* transfection of primordial germ cells [13] while the transfection rate was comparable with those reported by the lentiviral infection assay [10]. Nonetheless, the results from direct transfection of early embryo with lipoplexes and EDTA were very promising since the majority of the organs including the gonads carried the transgene. However, the technology needs further experiments to improve the embryo development and transfection efficiencies and to evaluate the transgene transfer rate into the next generations.

The results of this study can also be helpful for further clarification of SMGT feasibility in chicken. Injection of sperm-DNA mixture in the vaginal tract of hens has been a controversial strategy for making transgenic chicken [9,30–33]. Moreover, overcoming the semen inhibitors by either sperm washing or lipoplexes was not the only main factor for having a successful transgenesis [9,30]. Indeed, egg white which is secreted in the magnum, the main fraction of female reproductive tract, is another stronger barrier for the transgene transverse from the vagina to the infundibulum. It has been shown that motile sperm cells can superficially interact with exogenous DNA [5–8,34] and only dead sperm stably interact with foreign DNA. Moreover, an embryo can phagocyte dead sperm [35] and dead cells [36] in the uterus after 24 h of fertilization in the media and degrade their genome for high-speed replication demands. Sperm cells have to transverse through the magnum to reach the infundibulum for fertilization. Meanwhile, the superficially unstable bound DNA can be degraded by strong nuclease activity of egg white which is secreted throughout the magnum. In addition, the bypassed exogenous DNA through this section which might attach to the ovulated yolk would re-expose to the egg albumen during the egg formation. Therefore, it is very unlikely that intravaginal insemination of sperm-DNA mixture causes to chicken transgenesis anyway.

5. Conclusion

Results of this study showed the presence of strong nucleases in the chicken albumen which are comparable to commercially available DNase-I, although they were unable to degrade the oligonucleotides. The nucleases were heat sensitive and calcium-magnesium dependent. Moreover, lipoplexes could bypass the nuclease property. Handmade transfection of day0 embryo by concomitant *in ovo* injection of lipoplexes and EDTA in the close vicinity of the embryo could efficiently bypass the chicken egg albumen nucleases and make transgenic chicks. This study demonstrates the feasibility of eliciting a handmade approach for making transgenic chicks without any physical damage to the embryo.

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