THE IN VIVO STABILITY OF DIRECTLY INJECTED NAKED DNA AND CALCIUM PHOSPHATE CONJUGATED DNA INTO HENS

Mohammed Baqur S. A. Al-Shuhaib
College of agriculture /Al-Qasim Green University

ABSTRACT:
There is a crucial need for a low cost, non-biohazard horizontal gene transfer method in avian to produce enhanced genetic traits. The delivery of recombinant DNA into hen's tissues has been confirmed for practical use to promote the desired productive features. But still has several limitations such as expense, and the requirement of specialized devices for manufacture. Therefore, a low cost and calcium phosphate tool was devised, and its efficiency was tested by its direct exposure to DNase. Thus, to investigate the stability of the injected DNA through this method, hens were used as biomarkers in this study. Twenty mature hens were randomly chosen. They were directly injected by calcium phosphate – DNA and naked DNA methods. Then, blood samples were withdrawn after one day, three days, and one week of injection. DNA samples were extracted from blood in each case. Afterwards, both methods of direct gene injection were compared using polymerase chain reaction to explore the persistability of injected DNA in both cases. Though the naked DNA has initially shown much more penetration compared with its calcium phosphate counterpart, the later method has shown more stability in vivo. Nevertheless, both in vivo injection methods aren't practical enough for long term in vivo gene expression.

Keywords: comparison, naked DNA, calcium phosphate, direct injection

ثباتية الـ DNA المحقون مباشرة داخل جسم الدجاج بحالته العارية والمقترنة مع فوسفات الكالسيوم

محمد باقر صاحب أحمد حسن الشهيّب

الخلاصة:
هنالك حاجة ماسة لطريقة واطئة الكلفة وغير خطرة بايولوجياً لنقل الجين أفقياً في الطيور لانتاج صفات وراثية محسنة. ولقد تم اثبات ايصال الـ DNA المهندس وراثياً الى أنسجة الدجاج للأغراض التطبيقية وذلك لتحسين الصفات الانتاجية المرغوبة. ولكن مازال هنالك محددات متمثلة في التكلفة والاحتياج الى أجهزة متخصصة للتصنيع. ولهذا، تم تصميم أداة واطئة التكلفة لفوسفات الكالسيوم، والتي قد تم اختبار كفائتها بواسطة التعريض المباشر لانزيم الـ .DNase وهكذا، لنتحرى عن ثباتية وجود الـ DNA المحقون من خلال هذه الآلية، تم استخدام الدجاج كواسمات حيوية في هذه الدراسة. تم اختيار عشرين دجاجة ناضجة بشكل عشوائي. وتم حقنها مباشرة بواسطة طريقة مزيج الـ

ISSN 2072-3875

– DNAفوسفات الكالسيوم وطريقة الـ DNA العاري. ثم تم سحب عينات الدم بعد يوم، وثلاثة أيام، وأسبوع من الحقن. وبعد ذلك، تم استخلاص عينات الـ DNA من الدم في كل حالة. ومن ثم، تم مقارنة كلتا طريقتي حقن الجين المباشر باستعمال تقنية الـ PCR لاستكشاف احتمالية بقاء الـ DNA المحقون في كلتا الحالتين. وعلى الرغم من أن الـ DNA العاري قد بين بداية نفاذية أكثر مقارنة بفوسفات الكالسيوم، فان الطريقة الثانية قد بينت ثباتية أكثر داخل جسم الكائن الحي. مع ذلك، كلتا طريقتي الحقن داخل جسم الكائن الحي هما ليستا عمليتان بشكل كافي للتعبير الجيني لفترات طويلة داخل جسم الكائن الحي.

الكلمات المفتاحية: مقارنة، الدنا العاري، فوسفات الكالسيوم، حقن مباشر

INTRODUCTION:

Direct gene delivery in vivo is a promising alternative route for in vitro transfection techniques, as it would allow sustained expression of the desired gene (Roy et al., 2003). Viral gene delivery systems exposed to several problems, such as the high difficulty of viral gene vehicles production (Wall, 2002), acute inflammatory response that exhibits the limited prospects for repeated administration (Ponder, 2001.), and the significant post-injection biohazard (Cornetta et al., 1991). The method of DNA delivery by calcium phosphate plays a key role in several biomedical applications, and it's very important that the interaction of calcium phosphate with biological systems should be clearly understood for accomplishing a successful gene delivery approach (Houdebine, 2003). Hence, the demand for non-viral gene delivery systems increased. Calcium phosphate was first reported in 1973 for DNA condensation to increase the transfection efficiency with easy preparation (Graham et al., 1973). The calcium phosphate nanoparticles are cheap, safe and significant as compared to viral gene delivery system (Ardekani et al., 2014).

Calcium phosphate has been used for many years as a DNA delivery system (Wigler et al., 1977). Calcium phosphate nanoparticles have been shown to be a lesser irritant than the macroparticles, and they have many advantages over organic ones, such as better keeping quality and also being inexpensive (Tamuly and Saxena, 2012). The area of nanoparticles of inorganic compounds has assumed great significance in entrapping biomolecules in veterinary and medical sciences (Roy et al., 2003).

These nanoparticles have found their way in a number of biomedical applications such as gene therapy, adjuvants and drug-delivery systems (Joyappa et al., 2009; Cui and Mumper, 2003; He et al., 2002). The exact mechanism of action of calcium phosphate nanoparticles as an adjuvant is not clear; however it is believed that the calcium phosphate – exogenous DNA mix settles onto the cells and some of the particles are taken up by endocytosis (Jordan et al., 1996).

In contrast to several gene delivery routes, the calcium phosphate nanoparticles do not stimulate site-specific reaction upon intramuscular injection, and as long as hens have long held promise as a low-cost, high-yield

ISSN 2072-3875

bioreactor for the production of human biopharmaceuticals in egg whites using genetic engineering (Harvey and Ivarie, 2003), the injection methods were focused on these types of animals (Esmaeilzadeh and Farhadi, 2011).

Although calcium phosphate transfection method is highly recommended method for transfection of cells growing in tissue culture (Kingston, 2003), it entails the presence of high cost devices that aid in the process of calcium phosphate nanoparticles construction, such as CO2 incubators and several others cost effective tissue culture equipment. Therefore, a new and simplified mix of calcium phosphate was devised. In which, no sophisticated instrumentation and accessories were recruited to contrast this DNA accompanying nanoparticles. Add to that, this simplified mix was tested in vivo to evaluate its efficiency after injection.

The efficiency of calcium phosphate method of DNA delivery in vivo was focused on in term of its validity in comparison with its corresponding naked DNA delivery route. Nevertheless, the first goal of the present study was to determine to what extent whether a coprecipitates of DNA with a simplified calcium phosphate nanoparticles is an efficient tool to inject DNA in vivo in comparison with naked DNA injection. The second goal of this study is to examine the in vivo linearity of the directly injected intramuscular DNA in both cases.

MATERIALS AND METHODS :

Experimental animals. The mature hens (Gallus domesticus) that used in this study were raised in department of animal resources – college of agriculture – Al-Qasim Green University. All chickens were given a standard cornsoy broiler feed and water ad libitum.

Calcium phosphate DNA transfection mix preparation. Calcium phosphate transfection mixture was prepared according to Sambrook and Rushell (2001) with modifications. Briefly, two transfection tubes were prepared; the transfection tube No.1 (18µl 2M CaCl2 dissolved in 150µl D.W.), and transfection tube No. 2 (150µl 2X Hepes Buffered Saline).

Calcium phosphate DNA transfection mix evaluation. The exogenous DNA entrapment within calcium phosphate nanoparticles was tested by exposing the resulting mix for variable time intervals into DNase (Cat # BS88253, Biobasic – Canada). The exposed amount of the entrapped DNA was equal to the amount of DNase (1 unit of DNase for each one microgram of DNA). The DNase exposed mix was incubated at 37°. Several aliquots were made after each 5 min exposure to DNase treatment. Once degradation to DNase was finished, the resulting mixture was incubated at 65°C for 15min to inactivate DNase activity. Each aliquot was electrophoresed on a prestained (1%) agarose gel unit (Cat. No ON-MS, Mupid – Japan).

Direct DNA injection. The exogenous DNA (gWizGFP vector) was purchased from Aldevron (Cat No. 5006 – USA). Small amounts of the transgene (only

ISSN 2072-3875

10µg in each injection) were used since high concentration of DNA results into lower transfection efficiency (Welzel et al., 2004). Hens were divided into two sections; the first section (No. 1 into No. 10) was injected with 10µg naked transgene treatment, while the second section (No. 11 into No. 20) was injected with calcium phosphate – 10µg transgene conjugation treatment. In the case of naked DNA injection, the gWizGFP vector was diluted into 500µl with D.W. and injected intramuscularly into each animal of section one using he commercially available 1ml insulin syringe. Whereas in the case of calcium phosphate – DNA conjugate, the gWizGFP vector was added into transfection tube No. 1. Then, transfection tube No. 1 solution was very slowly added into transfection tube No. 2 solution until it depleted. Afterward, the mixture was incubated for 30 min at room temperature. Using the same intramuscular naked DNA injection mechanism, calcium phosphate – transgene conjugate was injected through 1ml insulin syringe into the hens section 2 (numbered from 11 into 20).

Blood samples collection. After one day (24hr), three days (72hr), and one week of the transgene injection of the two sections, about 1ml blood samples were collected from the wing vein for each injected animal and placed in anticoagulant (EDTA) tubes. Samples were stored in 4°C overnight until DNA isolation.

Genomic DNA isolation. DNA was isolated from blood by Gnomic DNA mini kit (lot No. JM36410, Geneaid – Taiwan). Then, the concentration and purity of the isolated DNA was assessed by nanodrop (part No. 80-3006-51, Biodrop - UK). The DNA was stored under -20°C until submitting genomic DNA samples to PCR expermins.

Polymerase chain reaction. To check the presence of the injected recombinant DNA PCR experiments were performed. Two specific primers for the transgene green fluorescent protein (GFP) in gWiz-GFP vector were designed according to a free internet software program (http://genamics.com). In which, the length of amplicons was 364 bp, which was extended within the open reading frame of the GFP transgene. After designing was done, forward primer (5´–CCATGCCCGAAGGTTATGTA –3´) and reverse primer (5´–GAAAGGGCAGATTGTGTGGA –3´) were ordered from Bioneer – Korea. PCR reaction was conducted by adding 30 – 50 ng of template DNA and 10pmole of both forward and reverse primers were added into the PCR premix (Cat. No. K-2012, Bioneer – Korea). PCR reaction program was set as the following: step one; initial denaturation 95°C for 5min, step two; (denaturation 95°C for 30 sec, annealing 52°C for 60 sec, polymerization 75°C for 30 sec) x 3, step three; final extension 75°C for 1 min in gradient PCR thermocycler (part No. 6325 000.510, master cycler-nexus, Eppendorf – Germany). The amplicons were electrophoresed on ethidium bromide pre-stained 1.5% agarose gel. Agarose gel were photographed in gel

ISSN 2072-3875

image photodocumentation unit (Cat No. 170-8280, ChemiDoc system, Bio-Rad – USA).

RESULTS AND DISCUSSION :

The longevity of a simplified method of DNA entrapment into calcium phosphate was evaluated – in comparison with naked DNA injection method – through PCR technique using hens as bioreactors. In this method, no special techniques and accessories were used in the mixing of the transfection tubes. However, this easy to make calcium phosphate transfection mix has manifested a significant protection for hens genomic DNA against the aggression of DNase activity for the first 20min (Fig. 1). However, the charge of the DNA interacted with calcium phosphate was neutralized. This obviously related with the counteracting positive charges for some these non-viral compounds. This, in turn, refers to the reactivity of these compounds with regard to DNA entrapments within this sort of nanoparticles. In addition to calcium phosphate kit, there are several manufactured peptides which have potential ability to play crucial role in this approach (Shwatrz et al., 1999). The most popular peptides are cationic peptides; the peptides rich in positively charged amino acids since they counteract the negative charge of DNA molecules. This neutralization of the DNA charge abolishes the repulsion forces in DNA and packs it closely (Khan, 2010). Nevertheless, the protection against DNase activity was never last more than 20min (Fig. 1), but might be enough time for the foreign DNA since its injection into integration into the genome.

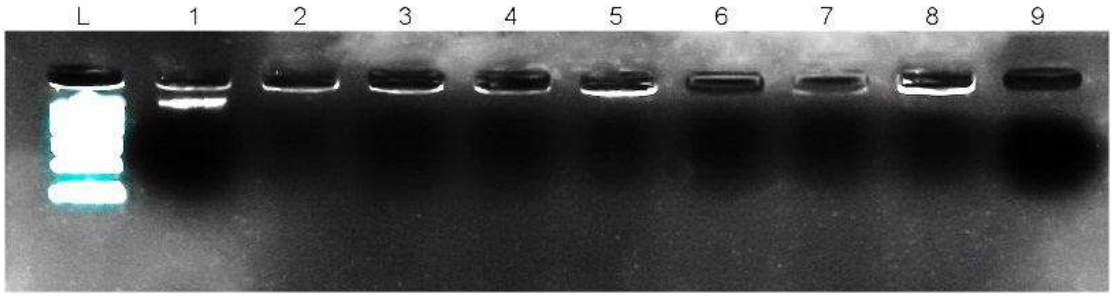

Figure (1): Testing the simplified calcium phosphate – genomic DNA interaction method by direct exposure into DNase activity. Lane "L" refers to 1 kp DNA ladder (Cat. No. D-1040, Bioneer – Korea). Lane 1 refers to unexposed genomic DNA of hens for DNase activity. Lanes 2 – 7 refer to the exposure of calcium phosphate – genomic DNA mix into DNase activity for 5min, 10min, 15min, 20min, 25min, and 30min respectively. Lane 8 refers to DNase unexposed calcium phosphate – genomic DNA mix. Lane 9 refers to the completely degraded naked genomic DNA of hens after its exposure into DNase activity.

ISSN 2072-3875

As long as the in vivo injected transgenes was demonstrated as an obvious and powerful tool in mammals for more than one decade (Butrick et al., 1992; Thierry et al., 1995), the same injection route was tested in this paper in hens by using calcium phosphate as a gene accessory tool. However, PCR experiments were conducted to analyze the fate of recombinant DNA that directly injected into the tissues of hens. The both cases of the injected reporter gene construct gWizGFP were exhibited a significant presence after a 24hr of injection (Fig. 2). Interestingly, the naked DNA state was initially shown excellent in vivo presence within the first 24hr after injection (Fig. 2). Although the complex enables DNA to enter the cell and protects it from nucleases, it was found that only free DNA is available for expression or interaction with the host cell's genome (Twyman, 2005). Add to that, the injection of uncomplexed plasmid DNA in vivo into muscle and skin results in high-efficiency gene transfer and expression (Graham et al., 1973).

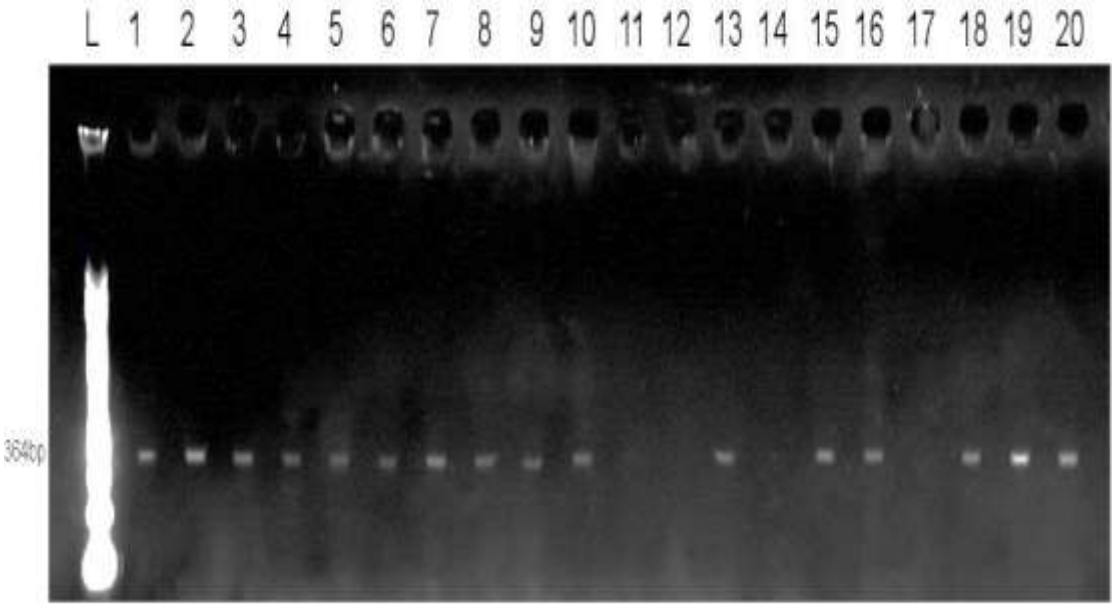

Figure (2): the PCR 364bp amplicons of the transgene (gWizGFP) of two different mechanisms of hens after 24hr of *in vivo* injection. Lane "L" refers to 100 bp DNA ladder (Cat. No. D-1030, Bioneer – Korea). Lanes (1 – 10) refer to the amplicons of the naked DNA *in vivo* injection route, while lanes (11 – 20) refer to the amplicons of the calcium phosphate conjugated DNA *in vivo* injection route.

The conventional PCR technique is not effective tool to determine the exact state of the injected DNA, whether it was localized as an integrated or non-integrated state. Nevertheless, PCR is a non-cost effective tool, through which it was so feasible for any researcher to get a speculation for the genotypic manifestation of the injected gene vehicle.

The results obtained in this paper have shown two main differences. These two

ISSN 2072-3875

differences could be separated by time intervals into two main divisions. The first significant difference is exhibited within the first 24hr after injection. In which, the excellent efficiency of naked DNA to replicate itself in vivo is obviously superseded the calcium phosphate conjugated route. This might be attributed to the fact of the ability of naked DNA to persist efficiently in an episomal state within the first hours that follow the in vivo injection (Ponder, 2001). It was not known however what exactly is the inhibitory role of calcium phosphate in the reducing this initial high efficiency of replication. Moreover, it's not unusual for these results to come in accordance with the notion that indicates the ability of the injected naked DNA to replicate itself within the first 24hr after injection (Sang and Perry, 1989). However, one of the crucial justifications for the low transfection efficiency obtained with non-viral vectors, including the precipitated calcium phosphate, is the partial protection of the vector DNA by the encapsulating material as well as low cell surface accumulation of the plasmid vector (Luo and Saltzmann, 2000). Such partial protection makes the DNA highly susceptible to aggressive DNase attack in the body as well as inside the cell (Roy et al., 2003).

The second significant differences between the two routes of injection were represented after 24hr of injection. This is become an obvious observation when most of the exogenous DNA was lost after 72hr of injection (Fig. 3). However, despite the high efficiency of naked DNA to persist itself in vivo, this persistence is constrained only within the first 24hr after injection. After this, the persistability of the injected naked DNA is lost after this time. Hence, the role of calcium phosphate is emerged. In which, a fierce resistance for the action of nuclease activity might be involved. Nevertheless, No evidence for chromosomal integration of the exogenous DNA was obtained, suggesting that the plasmid DNA persisted episomally (Sang and Perry, 1989).

ISSN 2072-3875

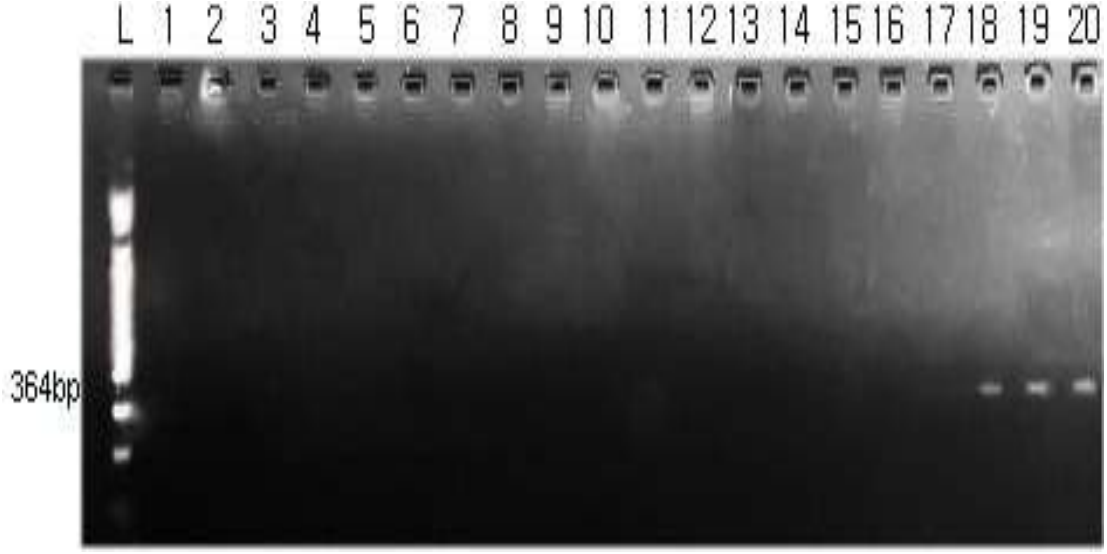

Figure (3): the PCR 364bp amplicons of the transgene (gWizGFP) of two different mechanisms of hens after 72hr of *in vivo* injection. Lane "L" refers to 100 bp DNA ladder (Cat. No. D-1030, Bioneer – Korea). Lanes (1 – 10) refer to the amplicons of the naked DNA *in vivo* injection route, while lanes (11 – 20) refer to the amplicons of the calcium phosphate conjugated DNA *in vivo* injection route.

In conclusion and from the above stated results, it is realized that the direct DNA injection in its naked state is an efficient route in case of the rapid expression of the desired gene in very short period of time. Whereas, the calcium phosphate route might be the favored one in case of longer time of transgene in vivo expression is required. Calcium phosphate nanoparticles can act like gene carriers of the transgene, and therefore this route can be used for more elongated period of time compared with the first one. But when samples were taken after one week of in vivo injection for PCR analysis, the injected transgene was absolutely disappeared from the hens' genome in both injection mechanisms (data not shown). Thus, in both cases, the expression of the desired transgene for one week term was an impractical tool. However, both in vivo methods were not experimentally efficient in the long term gene expression in case of direct horizontal gene transfer. Therefore, much more modifications whether in calcium phosphate of in other nonviral horizontal gene transfer techniques are still required to enhance the stability of the transgene in hens after direct in vivo injection.

REFERENCES :

Ardekani, M. R.; Abden, M. Z.; Nasrullah, N.; Samim, M. (2014). Calcium phosphate nanoparticles; a novel non-viral gene delivery system for genetic transformation of tobacco. Int J Pharm Pharm Sci, 6 (6): 605 – 609.

Buttrick, P.M.; Kass, A.; Richard, N.;Kitsis,R.N.; Kaplan, M.L.; and Leinwand, L.A. (1992). Behavior of Genes Directly Injected Into the

ISSN 2072-3875

Rat Heart In Vivo. Circulation Research 70 (1): 193 – 198.

Cornetta, K.; Morgan, R.A; Anderson, W.F. (1991). Safety issues related to retroviral-mediated gene transfer in humans. Human Gene Therapy, 2(1): 5-14.

Cui, Z.; and Mumper, R. J. (2003). Microparticles and nanoparticles as delivery systems for DNA vaccines. Crit. Rev. Ther. Drug Carrier Syst. 20: 103–137.

Esmaeilzadeh, M.; and Farhadi, A. (2011). An Overview of the Basics of Generate Transgenic Hen 'Bioreactors' . Webmed Central Biology 2(10):WMC002289.

Graham, F.L.; Veldhuis, G.; and Wilkie, N.M. (1973). Infectious Herpesvirus DNA: Nat New Bio 245 (148): 265-266.

Harvey, A.J.; and Ivarie, R. (2003). Validating the Hen as a Bioreactor for the Production of Exogenous Proteins in Egg White. Poultry Science 82:927–930.

He, Q.; Mitchell, A.; Morcol, T.; and Bell, S. J. (2002). Calcium phosphate nanoparticles induce mucosal immunity and protection against Herpes simplex virus type 2. Clin. Diagn. Lab. Immunol., 9, 1021–1024.

Houdebine LM. (2003). Animal transgenesis and cloning. West Sussex, UK: Wiley and Sons.

Jordan, M.; Schallhorn, A.; and Wurm, F.W. (1996). Transfecting mammalian cells: Optimization of critical parameters affecting calcium-phosphate precipitateformation. Nucleic Acids Res 24:596–601.

Joyappa, D. H.; Kumar, C. A.; Banumathi, N.; Reddy, G. R.; and Suryanarayana, V. V. (2009). Calcium phosphate nanoparticle prepared with foot and mouth disease virus P1-3CD gene construct protects mice and guinea pigs against the challenge virus. Vet. Microbiol. 139 (1–2), 58–66.

Khan, K. H. (2010). Gene Transfer Technologies and their Applications: Roles in Human Diseases. Asian Journal of Experimental Biological Science, 1 (1): 208-218.

Kingston, R.E.; Ausubel, F.M.; Brent, R.; Moore, D.D.; Seidman, J.G.; and Smith, J.A.; Struhl, K. Current Protocols in Molecular Biology. Copyright © 2003 John Wiley & Sons, Inc.

Luo, D.; and Saltzmann, W.M. (2000). Synthetic drug delivery systems. Nature Biotechnol. 18: 33-37.

Ponder, K. P. Vectors of Gene Therapy. An Introduction to Molecular Medicine and Gene Therapy. Edited by Thomas F. Kresina. Copyright © 2001 by Wiley-Liss, Inc.

Roy, I.; Mitra, S.; Maitra, A.; and Mozumdar, S. (2003). Calcium phosphate nanoparticles as novel non-viral vectors for targeted gene delivery. International Journal of Pharmaceutics 250: 25-33.

ISSN 2072-3875

Sambrook, J.; and Russell, D. (2001). Molecular Cloning (A Laboratory Manual), Third Edition. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.

Sang, H.; and Perry M.M. (1989). Episomal replication of cloned DNA injected into the fertilised ovum of the hen, Gallus domesticus. Mol Reprod Dev.1(2):98-106.

Schwartz, B.; Ivanov, M.A.; Pitard, B.; Rangara, R.; Byk, G.; Crouzet, J.; and Scherman, D. (1999). Synthetic DNA-compacting peptides derived from human sequence enhance cationic lipid-mediated gene transfer in vitro and in vivo. Gene Therapy 6, 282–292.

Tamuly, S.; and Saxena, M.K. (2012). Preparation of calcium phosphate nanoparticles and evaluation of their effects on muscle cells of rat. Current Science, 102 (4), 610 – 612.

Twyman R.M. (2005). Gene Transfer to Animal Cells.Garland Science/BIOS Scientific Publishers/UK.

Thierry, A.R.; Lunardi-Iskandar, Y.; Bryanwt, J.L., Pabinovich, P.; Gallo, R.C.; and Mahan, L.C. (1995). Systemic gene therapy: Biodistribution and long-term expression of a transgene in mice. Proc. Natl. Acad. Sci. USA 92: 9742-9746.

Wall, R. J. (2002). New Gene Transfer Methods. Theriogenology, 57: 169-201.

Welzel, T.; Meyer-Zaika, W.; and Epple, M. (2004). Transfection of cells with custom-made calcium phosphate nanoparticles coated with DNA. J. Mater. Chem. 14: 2213–2217.

Wigler, M.; Silverstein, S.; Lee, L.; Pellicer, A.; Cheng, Y.; and Axel, R. (1977). Transfer of Purified Herpes Virus Thymidine Kinase Gene to Cultured Mouse Cells. Cell 11:223-232.

ISSN 2072-3875