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The Efficiency of Transgenesis by Restriction Enzyme Mediated Integration - Sperm Mediated Gene Transfer (REMI-SMGT)

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Abstract
The present study tends to test the validity of REMI-SMGT and to evaluate the efficiency of REMI-SMGT on generating other mammalian species rather than the sheep that made only by one group and to observe the possibility of doing so by using rabbits as a model for this approach and also to reduce the cost of REMI-SMGT by substituting liposomes and highly cost effective media with a high efficient, non-cost effective substitute.
Direct protective relationship of liposome with DNA and seminal fluid was identified compared with DMSO. While different treatments (linearized DNA – restriction enzyme – liposome complex, DNA – restriction enzyme – DMSO complex, DNA –DMSO complex, DNA –liposome complex, and even naked DNA) were all found to be successful to internalize inside the head of the sperm according to PCR results, only three (one by restriction enzyme – liposome treatment and two by restriction enzyme – DMSO treatment) out of fourteen new born babies were found to be transgenic by PCR.
Despite the absolute ability of exogenous DNA to be internalized inside rabbit's sperm head only few percent of transgenic babies were obtained. This may not reflect the weakness of restriction enzyme mediated transgenesis technique itself but it reflects the inability of recombinant sperm to fertilize superovulated oocyte compared with their normal counterparts. Comparable results were found between liposome and DMSO treatment which may reflect direct relationship of DMSO with the cell membrane instead of with the exogenous DNA itself as what is found with liposome.
Key words : REMI-SMGT **,** PCR **,** DNA – restriction enzyme **,** DMSO

1. Introduction

In the field of animal transgenesis, many attempts have been made recently to simplify these experiments and to reduce the cost and labor required to do such tasks. Although several transgenesis techniques such as DNA microinjection and somatic cell nuclear transfer have been applied successfully to produce transgenic animals (Gordon *et al.*, 1980; Willadsen., 1986), but these traditional techniques are so tedious and have several disadvantages (Wolf *et al.*, 2000; Wilmut, 2002). Retroviral mediated gene transfer has solved some of these usual problems (Khan, 2010), but has, however, inevitable disadvantages represented most prominently by its biological hazard (Cornetta *et al.*, 1991).

Many researchers found that the most simple and non-cost effective way to produce transgenic animals is to focus on the natural ability of the free seminal fluid sperm cells to "carry" the foreign DNA and to "fertilize" the oocyte (Brackett *et al.*, 1971). The most important breakthrough obtained in this aspect is the accumulated information that demonstrated the ability of foreign DNA to be internalized into the sperm head after simple incubation step (Horan *et al.*, 1991; Lavitrano *et al.*, 1992; Kim *et al.*, 1997; Maione *et al.*, 1997). Accordingly, the only manipulation step is restricted into the head of the sperm. Then, nature will be allowed to fulfill its scheduled task of reproduction. This method known as sperm mediated gene transfer or SMGT (Lavitrano *et al.*, 1989). However, simple incubation of naked DNA with sperm head is may not efficient enough to integrate the foreign DNA into the genome of the sperm (Niu & Liang, 2008).
Several enhancements have been made to increase the efficiency of this promising method such as using electroporation (Gagne *et al.*, 1991), linkers (Chang *et al.*, 2002), retroviral vectors (De Miguel & Donovan, 2003), and liposomes (Bachiller *et al.*, 1991). But, according to many data, these approaches don't have the molecular mechanisms that directly working on integrating the exogenous DNA during its incubation with sperm genomic DNA. Several researchers have further simplified SMGT by direct injection of foreign DNA into the testes of animals combined with electroporation or lipofection (Sato *et al.*, 1994). Testis mediated gene transfer of TMGT, however, don't have significant differences compared with the original SMGT because each of which relay's upon sperms as a vehicles to carry the exogenous DNA. Thus, the problem of reduced integration still exists. It has been reported that many enhancement approaches have increased the reproducibility of the original SMGT (Celibi *et al.*, 2003). Nevertheless, it becomes known to many researchers the obvious inefficiency of

SMGT enhancement approaches to "integrate" the foreign DNA into the genome of the sperm (Weeler & Walter, 2001).

Surprising molecular trick that represented by implicating restriction enzymes in this arena has been made in SMGT. This trick has been made on SMGT by Israelite group at 2000 and 2009 (Shemesh *et al.*, 2000; Harel-Markowitz *et al.*, 2009). They have generated transgenic sheep and chickens with a high ratio of transgenesis efficiency. This method is called restriction enzyme mediated integration SMGT or REMI-SMGT. The unusual thing in this aspect is that nobody has tested the validity of this interesting technique after this group. Moreover, no one evaluated the efficiency of this technique on other mammalian species whether on laboratory (such as mice and rats) or on laboratory and domestic (such as rabbits). REMI SMGT is not a weird enhancement of the traditional SMGT but the combination of restriction enzymes made this technique very interesting with respect to the molecular mechanism by which the restriction enzyme enhance" the rate of integration. This mechanism can be simplified by incubation of transgene located within a circular vector with its corresponding restriction enzyme; the enzyme that have only one sensitive site located out of the transgene sequence. After digestion of circular DNA, its linear counterpart is produced. The linearized transgene and the same enzyme then incubated with liposome. The role of liposome here is just to pass the transgene and its corresponding enzyme through the cell membrane of the sperm cells (Sciamanna *et al.*, 2000).

It is believed that once the exogenous DNA encounter the sperm genome its corresponding restriction begins to digest its sensitive sites that located on the hosting genome, meanwhile the exogenous DNA will seize the opportunity in order to integrate itself into the genome of the sperm cell by cellular DNA repair mechanism (Shemesh *et al.*, 2000).

It was found it is so mandatory to use rabbits in this thesis as a model to generate transgenic animals. There are several reasons to use rabbits in REMI-SMGT; 1) rabbits are never tested in REMI-SMGT, 2) rabbits are domestic aside from being considering as laboratory animals. So, to test the efficiency of this technique it is very important to use such model to prove or not to prove its validity, 3) sperm are easier to be collected from rabbits compared with other laboratory animals such as mice and rats. Moreover, sperms that collected from only one male have the ability to fertilize several females. Add to that, collection of rabbit sperms can be done twice a week without effecting on its efficiency, easier super-ovulation of rabbits with continuous reproducibility all over the year, and 5) rabbits have short gestation time which is usually not exceed more than one month after fertilization (Chrenek & Makarevich, 2008).

In REMI-SMGT, as it is made initially, two components should be used to facilitate the task of exogenous DNA. The first one, the most commonly used liposomes, or its cheap substitute, which they are used to facilitate the entry of exogenous DNA through the cell membrane, and the second component, is the restriction enzyme, which is used to facilitate the integration of this DNA into the genome of the sperm. According to this technique, sperm cell repair mechanisms heal the damages introduced by the internalized restriction enzyme and "integrate" the foreign DNA mistakenly into the genome of the sperm. We think it is very necessary to see how much this technique is capable on misleading the molecular repair mechanisms of sperm cell, since this tracking opens the door widely for more exploration of molecular manipulations of the sperm head for the sake of producing a transgenic animal with a minimum efforts and costs. Wall (2002) referred to the absence of any significant disadvantages in REMI SMGT. Nevertheless, despite the evident efficiency of several experiments that increases the rate of exogenous integration for several folds but this is not enough since there is a great necessity to repeat these experiments to make sure from the credibility of these results. However, the numbers of papers concerning REMI-SMGT is very little to judge how much this approach is efficient. Therefore further studies are in the way to elucidate much more details on the validity of this particular approach.

According to our knowledge, this research is the first one which both modifies and evaluate REMI-SMGT success ratio away from the results obtained by the same Israelite group.

2. Materials and Methods

2.1. Materials:

DNA extraction kit; Easy-DNATM Kit (Invitrogen – Cat. # K1800-01). PCR premix; PCR SuperMix (Invitrogen – Cat. # 0572-014). Enzymes; BamH I (Invitrogen – cat # 15201023), DNase I (Fermentas, Cat # EN0521). Hormones; Chorulon (Chorionic Gonadotropin,PMSG (Pregnant Mere Serum Gonadotropin) Intervet – Holland, anaesthetics; Zoletil 50 (Virbac – France). Ladders; TrackIt™ 1 Kb Plus DNA Ladder (Invitrogen – Cat. #10488090), MassRuler™ DNA Ladder Mix (Fermentas – Cat. # SM0403). Oligos; Forward primer (5´– CCATGCCCGAAGGTTATGTA –3´) and reverse primer (5´– GAAAGGGCAGATTGTGTGGA –3´) Invitrogen. Reagent; Liposome ® 2000 (Invitrogen – Cat. # 11668-027). Vectors; gWiz-GFP (green fluorescent protein) vector (Aldevron – Cat. # 5006) and pTZ57R/T vector (Fermentas – Cat. # K1213).

Sperm activation medium; Sperm Tyrode-albumin-lactate-pyruvate (Sp-TALP) medium; It was prepared according to Parrish *et al.*, 1988, with some modifications (Cheng *et al.*, 1996; Bateman, 2011; Boiti, 2005).This

medium was prepared with some modifications represented by preparing sp-TALP medium without being included with bovine serum albumin (BSA). After its preparation, this medium was filtered through 0.22µm filter paper and stored in refrigerator for short period of time. This media contains 100 mM NaCl, 3.1 mM KCl, 25 mM NaHCO3, 0.3 mM NaH2PO4, 21.6 mM Sodium lactate (Sigma – lot # 16H5049), 2 mM CaCl2, 0.4 mM MgCl2, 10 mM HEPES, 1 mM Sodium Pyruvate (Invitrogen – cat # 11360).

Computer software programs; two software programs were used; Genamics Expression; for DNA and protein sequence analysis (http://genamics.com) and PVTech Plasmid for plasmid drawing software for windows (http://www.biovisualtech.com).

Experimental Animals; Eight New Zeeland sexually mature healthy white rabbits and ten sexually mature female were included in this study. New Zeeland white rabbits were raised in the animal house in the school of bioscience and biotechnology / FST / UKM. They were individually housed under controlled conditions of temperature (19 – 21◦C) and standard artificial light (12 hour light and 12 hours dark). A diet of grower rabbits pellets (ad libitum) and fresh water was provided. Animals were cared according to international standards management established for the care and use of laboratory animals in facilities approved by the University Kebangsaan Malaysia Animal Ethics Committee (UKMAEC).

2.2 Methods

2.2.1 gWiz-GFP vector linearization with restriction enzyme: *Bam*HI restriction enzyme was selected according to PVTech plasmid software program. In which, BamHI single and recognition site that located directly downstream of GFP was chosen as a method of choice for gWiz-GFP linearization. The digestion reaction components are assembled in order to digest 50µg (10µl) gWizGFP vector. The linearization components were incubated at 30ºC in a water bath for 30min. An aliquot was removed, and the extent of digestion was analyzed agarose gel electrophoresis.

2.2.2 The ability of liposome and DMSO to change the electrophoretic mobility of circular as well as linear DNA: gWiz-GFP DNA (in its circular and linear form) was electrophoresed on agarose gel in three lanes; alone, mixed with liposome and mixed with DMSO. Picture with taken by photodocumentation unit (Alpha Innotech – USA).

2.2.3 The effect of liposome compared with DMSO on reducing DNase activity:

A. *Preparation of DNA-liposome-DNase mixture:* 10µg gWizGFP vector was mixed with 10µg liposome. Then, 1µl (10x) DNase reaction buffer was added. The mixture was completed to 50ml with deionized water and incubated for 30min at room temperature. Only 1U DNase was added to the DNA-liposome mixture. Two aliquots were made, each one with 25µl; the first aliquot was incubated in water bath for 5 min at 30°C. While the second aliquot was incubated in water bath for 10 min at 30°C. After each incubation, 1µl 50mM EDTA was added to each aliquot, and then each aliquot was incubated at 65°C for 10 min to inactivate the enzyme.

B. *Preparation of DNA-DMSO-DNase mixture*: 10µg gWizGFP vector was mixed with 10µl DMSO. Then, 1µl (10x) DNase reaction buffer was added. The mixture was completed to 50ml with deionized water and incubated for 30 min at room temperature. Only 1U DNase was added to the DNA-DMSO mixture. Two aliquots were made, each one with 25µl; the first aliquot was incubated in water bath for 5 min at 30°C. While the second aliquot was incubated in water bath for 10 min at 30°C. After each incubation, 1µl 50mM EDTA was added to each aliquot, and then each aliquot was incubated at 65°C for 10 min to inactivate the enzyme.

C. *Preparation of DNA-DNase mixture*: 10µg gWizGFP vector was mixed with 10µl deionized water. Then, 1µl (10x) DNase reaction buffer was added. The mixture was completed to 50ml with deionized water and incubated for 30 min at room temperature. Only 1U DNase was added to the DNA solution. Two aliquots were made, each one with 25µl; the first aliquot was incubated in water bath for 5 min at 30°C. While the second aliquot was incubated in water bath for 10 min at 30°C. After each incubation, 1µl 50mM EDTA was added to each aliquot, and then each aliquot was incubated at 65°C for 10 min to inactivate the enzyme.

The three different mixtures were analyzed by agarose gel electrophoresis, pictures were taken by photodocumentation unit.

2.2.4. The effect of liposome on DNase activity profile of rabbit's seminal fluid: Two treatments were performed in this experiment; the first one represented by incubating fixed concentration (6µg) of gWizGFP vector with three different concentrations of rabbits seminal fluid (1µg, 5 µg to 10µg). The second treatment was performed in three concentrations as mentioned in first treatment except the including fixed concentration (6µg) of liposome. In both cases, the incubation was extended for one hour at room temperature. Then, analyzed by agarose gel electrophoresis. The gel was pictured in photodocumentation unit.

2.2.5. Sperm cells incubation with the exogenous DNA and PCR analysis: *Sperm Collection and motility investigation*: Sperm was collected by home-made artificial vagina; 2ml of semen was diluted with 8ml pre-warmed sp-TALP for 10min under 1000xg.Supernatant was discarded and the previous step was repeated by adding 10ml sp-TALP medium. Supernatant was discarded again and the resulting sperm cells were resuspended to100x106 (100 million active cells) sperm / 0.6 ml in the same medium.

A. *Linearized and circular gWiz-GFP vector preparation for incubation with sperm cells*: Different treatments were used to incubate gWiz-GFP vector with sperm cells (each 100µg gWiz-GFP was linearized by 1000U *Bam*H I). First treatment: 200µl of linearized gWiz-GFP vector -BamHI mixture was incubated with 20µl (20µg) liposome. Second treatment: 200µl of linearized gWiz-GFP vector -BamHI mixture was incubated with 20µl DMSO. Third treatment: 10µg circular gWiz-GFP vector was incubated with 10µl (10µg) liposome. Fourth treatment: 10µg circular gWiz-GFP vector was incubated with 10µl DMSO. Fifth treatment: 10µg circular gWiz-GFP vector. All of treatments were incubate for 30min at room temperature.
B. *gWiz-GFP vector incubation with sperm cells:* 300µl sperm cells (in sp-TALP) were added to all the prepared treatments (DNA – BamHI – liposome, DNA – BamHI – DMSO, DNA – liposome, DNA – DMSO, and DNA alone mixtures) respectively, and incubated at room temperature for one hour.
C. *DNase treatment*: Each sperm cells suspension treated with 10µg gWiz-GFP was incubated for 30 min with10 units of DNase mixture at 37ºC in water bath. After 30 min of incubation, 10µl of stop solution was added and the mixture was incubated at 65ºC for 10 min to inactivate the enzyme.
D. *DNA isolation from gWiz-GFP incubated sperm cells*: Genomic DNA was extracted from sperm cells according to Invitrogen instruction manual (Cat # K1800-01). DNA concentration was measured by UV-visible spectrophotometer (Shimadzu – Japan).
E. *PCR design testing:* Two specific primers for the transgene green fluorescent protein (GFP) in gWiz-GFP vector were designed according to *Genamics Expression* software program. In this program, 364 bp PCR fragment chosen for amplification was extended within the open reading frame of the recombinant GFP; from 2156 bps into 2520 bps. PCR amplification was taken place using conventional thermal cycler (Eppendorf Master Cycler - USA). PCR Super Mix was divided into aliquots into individual PCR tubes (each aliquot was 45 µl) and all the reaction components were kept on ice. Upstream and forward primers and DNA template were added to the PCR Super Mix. The PCR tubes were placed on ice and all the components were added to make 50µl final reaction volume. Reactions were placed in the thermal cycler that was preheated to 95°C and previously set up to the following cyclic conditions (table1):
PCR products were analyzed by agarose gel electrophoresis. Photo was taken using photodocumentation unit (fig. 1).
F. *PCR analysis of gWizGFP – sperm cells incubation:* After the testing the success of PCR primers that designed for gWizGFP gene, less than 0.5µg of gWizGFP incubated sperm cells genomic DNA were analyzed by PCR. Resulting PCR reaction mixture of each sample was analyzed by agarose gel electrophoresis. Picture was taken by photodocumentation unit.
2.2.6. Artificial insemination (AI): It was applied with the obtained recombinant sperm in order to produce transgenic rabbits (Vasicek *et al.*, 2003).
A. *Preparation of artificial insemination tools* (Morell, 1995)**:** 1ml capacity (0.5cm X 30cm) Pasteur pipette was slightly bent by heat at the portion that to be inserted into female uterus.
B. *Hormonal pre-treatment:* This step was performed according to Dimitrova *et al.*, 2009. Forty-eight hours before AI 25 females were injected by 40 IU PMSG per doe intramuscularly.
C. *Preparation of treatments:* All 10 females were separated into individual cages. These cages were arranged into two different treatments; gWiz-GFP vector – liposome – BamHI treatment, gWiz-GFP vector – DMSO – BamHI treatment. Each treatment was applied on five superovulated females. The BamHI linearized 100µg gWiz-GFP vector was used in artificial insemination. Two treatments (liposome mediated and DMSO mediated REMI-SMGT respectively) were applied to transfect superovulated females with gWizGFP vector through artificial insemination.
D. *Sperm collection for artificial insemination:* After sperm collection by artificial vagina, sperm investigation under light microscope was taken place in order to eliminate any "non-qualified" semen. 2ml of semen was diluted with 8ml pre-warmed sp-TALP for 10min at 1000xg. Supernatant was discarded and the previous step was repeated by adding 10ml sp-TALP medium. Supernatant was discarded again and the resulting sperm cells were re-suspended to 100x106 (100 million active cells) sperm / ml in the same medium.
E. *Sperm cells – gWizGFP vector incubation:* 2ml diluted suspension of sperm cells (containing 108 cells), was combined with each individual gWizGFP vector treatments, and followed by gentle mixing for 1 hour at room temperature. Each one of these different treatment was sufficiently used to inseminate five females. Certain amounts of these complexes were applied for each superovulated female (table 2).
Each doe submitted to insemination was anesthetized with 150µl Zoletil-50. Two steps was taken place to prevent sperm escape from the uterus; the first one in which doe's body was inverted upside down, and the second in which two ligatures were placed bilaterally (Arion *et al.*, 2001).While the body of superovulated and anaesthetized female in the wanted situation, the slightly bended pipette was surrounded by sufficient amount of glycerol and placed carefully into the vagina (fig. 2. A). Correct insemination position was chosen after careful monitoring to the intended destination of the insemination pipette (fig. 2. B, C and D).

Strict precautions were taken place in order not to damage any nearby tissues during this step. Once the insemination pipette penetrated about 8cm depth into the uterine tract, 500 µl sperm cells – gWiz-GFP complexes (0.5ml of 10 million sperm cells) was injected into the vagina by inserting it into 8cm depth using a slightly bended syringe (figure 2. E) to insure appropriate delivery of sperms to anesthetized female rabbits.
This situation was kept for 15 -30 min or until the anesthetic action was finished. At the time of insemination, ovulation of rabbits was induced by injection of 50 U of chorionic gonadotropin per doe.
Pregnant doe were isolated in separate cages during pregnancy period and nest boxes were made and placed beside them before three days of their kindling.

2.2.7 Transgenesis Detection by PCR: Before undergoing transgenesis detection, the status of suckling of pups was watched carefully for the first two days after birth. Pups were separated from their moms by picking nest boxes up from mom's cages and returning them back and let mothers give milk to suckling pups. Genomic DNA was isolated and purified from 350 µl blood of each new born pup according to Invitrogen instructions manual. PCR was taken place from the DNA isolated from blood. Resulting PCR reaction mixture of each sample was analyzed by agarose gel electrophoresis. Photos were taken using photodocumentation unit.

2.2.8 Detection of Fluorescence by direct exposure to UV: This step was performed according to Epperly, 2007 with modifications. To detect the possible expression ability of gWiz-GFP vector in PCR positive new born babies, liposome mediated REMI-SMGT babies, DMSO mediated REMI-SMGT babies, and negative control babies (two days old) were directly exposed to a source of UV light (UV-translluminator dual intensity) at 365nm (Gentuar – Belgium) under dark conditions.

3. Results and Discussion

Our attempted to test the efficiency of REMI-SMGT technique as well as to lower the total cost of this technique by manipulating some experiments to keep the restriction enzyme active for further digestion steps. The total cost was lowered not only by reducing the amount of restriction enzyme into the half, but by substituting the cost effective liposome and transfection medium with a very low cost alternatives; the transfectants DMSO since REMI-SMGT experiments done by Shemesh and his colleges (2000) were cost – effective because of the large amounts of restriction enzyme and liposome wanted. Moreover, sp-TALP was proved to be efficient enough for such transfection experiments compared with the cost effective commercially available transfection media (Eghbalsaied, *et al.*, 2009). We applied such modified low cost REMI-SMGT on rabbits considering these animals as a model applicable for both domestic and mammals.

3.1. The ability of BamHI to linearize gWizGFP vector in short time with high processivity:

One of the appearing goals of this research was to shorten time and budget required undergoing REMI-SMGT. This was accomplished by reducing the incubation time instructed by the manufacturer company of restriction enzyme into half. This was done by incubating the linearizing enzyme, BamHI, with the circular gWizGFP vector for only 30 min instead of 60 min. complete digestion was shown (figure 3) without drastically effecting on the degree of processivity of BamHI. This protocol was performed to increase the half-life of the enzyme without surrendering to duplicate the added amount of this enzyme and consequently duplicate the cost of REMI-SMGT.
Another step was taken into account to keep the processivity of this restriction enzyme during DNA linearization process was to reduce the temperature at which the enzyme linearize the circular gWizGFP vector. It was known that the recommended higher temperatures of incubation increase the enzyme processivity and decrease the stability (Granner & Weil, 2003). As much as the incubation temperature deceased as much as the enzyme's stability increased and processivity decreased in the same degree since there were a reversal relationship between processivity and stability of the enzyme. Therefore, utilizing a combination between the processive and the stable incubation temperature was represented by applying 30°C for 30 min (fig. 3) instead of 37°C for 60 min.
Eventually, using this procedure, the enzyme was kept active to undergo further manipulation steps that were very necessary later in transgenesis through REMI-SMGT. The reason behind the alteration of the original protocol of DNA linearization by BamHI was taken place in order not to lose the cost effective restriction enzyme in the further REMI-SMGT crucial steps.

3.2. Comparison between the ability of liposome and DMSO on retarding of circular and linear gWizGFP vector electrophoretic mobility: It was clearly noticeable the direct ability of liposome on neutralizing the charge of the DNA with which it bound (figure 4). While, no change in electrophoretic ability of DMSO – DNA were obtained in two cases, the linear and the circular counterparts, no difference was observed between the response of linear and circular form of gWizGFP to the binding with liposome or DMSO. That could be deciphered by the fact that the mode of binding of such chemicals with DNA was possibly taken place irrespective of the existence of the free ends of DNA with which they were intended to be interacted. However, no complete neutralization was accomplished in case of liposome (fig. 4) but this was not clear since the interaction was taken place with only one concentration of the two interacted molecules. Therefore, further

details should be provided. Thus, 30 min incubation of liposome with variable concentrations of gWizGFP DNA wasn't given any complete retardation with all the used concentrations of gWizGFP DNA (results not shown). Therefore, liposome might not possess "absolute" effect to completely retard the electrophoretic mobility of DNA.

3. 3. Comparison between the ability of liposome and DMSO to protect gWizGFP vector from DNase activity: Liposome showed significant protection of DNA from the hydrolytic activity of DNase compared with DMSO (fig. 5). Consequently, no direct binding between DNA and DMSO was observed. This information was not surprising since no direct interaction between DMSO and DNA with respect to DNA neutralization was observed as well (fig. 4).

These results were in accordance with the notion of Chang and his colleagues; they referred to the ability of liposome to partially protect DNA from the hydrolytic action of DNase I (Chang *et al.*, 1999). The more resistible DNA – liposome complex to DNase digestion was agreed with the more stable DNA – liposome complex observed by El-Gendy and his colleges (2006). This observation, in turn, agreed indirectly with the notion in which the liposome stabilized exogenous DNA and keeps it intact, since it was demonstrated that liposome was capable on providing direct stability upon binding with the exogenous DNA (Fellgner *et al.*, 1987; Sato *et al.* 2003).

3. 4. The ability of liposome to protect gWizDNA from DNase activity of rabbit seminal fluid: Since liposome showed direct protection of DNA against DNase activity, DNA – liposome complexes were incubated with seminal fluid and compared with non-liposome bound DNA controls. Liposome was showed interesting power by which it could protect DNA from DNase digestion (Seleva *et al.*, 1981; Kim *et al.*, 2000). The significant results of the protection of liposome interacted DNA observed in (fig. 5) was confirmed in (fig. 6), in which noticeable reduction in hydrolytic degree of liposome – DNA complex that treated with seminal fluid was observed. According to this figure direct liposome – DNA interaction was demonstrated but in the same time, no complete protection was performed. Although complete protection of exogenous DNA from the action of DNase was not happened, liposome provided the best direct transfectants tool through which the exogenous DNA is directly protected from DNase activity. This agreed with Schaefer-Ridder and his college's results; they demonstrated the ability of liposome to protect the foreign DNA from digestion of proteases or DNase present in the cytoplasm of the egg (Schaefer-Ridder *et al.*, 1982).The result obtained in (fig. 6) was possible as well since no complete neutralization was taken place (fig. 3) despite prolonged incubation times were used (results not shown).

3.5. PCR detection of the ability of gWizGFP to internalize the head of rabbit's sperm:

Since certain DNA fragments were easier to deliver than the others (Chan *et al.*, 1995), therefore, different entry mechanisms were used in which liposome and DMSO mediated REMI-SMGT were compared with other treatments in order to get initial clue about to what extent the exogenous DNA was capable on transmitting the sperm cellular membrane. Surprisingly, as shown in PCR results, all the DNA treatments were demonstrated to be quite effective to internalize through cell membrane (fig. 7). In this figure, the PCR products (364bp) were detected in all gWizGFP vector treatments. It was demonstrated by many accumulated data the ability of exogenous DNA to internalize into the head of sperm of several types of mammals such as (Castro *et al.*, 1990; Bachiller *et al.*, 1991; Lavitrano *et al.*, 1992: Francolini *et al.*, 1993).

Despite of the fact that PCR tool don't able to provide any details about the subcellular localization or to give affirmative information about the possibility of transgene integration but the absolutely observed positive results might suggested a possible success of the transgene to integrate into the genome of the sperm. Moreover, the absolutely positive results obtained in this experiment indicate the feasibility of exogenous DNA internalization whatever treatment implied to present this DNA to interact with the surface of the sperm. One more thing these results indicated which was the possibility of the presence of more than one route through it the exogenous DNA could be delivered into the head of the sperm. This piece of information might also be possible since the multiple exogenous DNA mixtures, and consequently the multiple mechanisms, which used to communicate with the surface of the sperm cells, were all proved to be successful in this context. However, this experiment was not the main point of the research since this research was not focused on the internalization process, but rather, it was focused on the ability of the recombinant sperm to transfer its transgene into the next generation through artificial insemination. Although, these results were preliminary but they were promising since all the treatments showed success in the internalization process despite the DNase I digestion was applied on all treatments after the incubation. While the use of liposomes or DMSO easily explained the internalization process, its occurrence with naked DNA opens a question that, does not offer easy proved answers (Gandolfi *et al.*, 1998). The apparent feasibility of this process made some researchers to describe this process as "spontaneous" mechanism in which mature sperm cells have the ability to take up exogenous DNA and could be taken place naturally under the favorable conditions (Francolini *et al.*, 1993).

The absolutely positive results obtained by PCR might add some confusion since false positive results might be expected because of the highly sensitive mode of PCR technique (Chan, 1999). However, PCR as a tool of transgenesis technique remains reliable indicator for transgenesis (Rexroad, 1992). Add to that, this research was not the first one who describe very high rate of transgene internalization into the sperm despite the multitude of treatments used since many papers were mentioned such rate of success using different incubation routes (Lavitrano *et al.* 1989; Khoo *et al.* 1992; Gandolfi 1998; Spadafora 1998; Lavitrano *et al.* 2006; Hoelker *et al.* 2007; Lanes *et al.* 2009, Collares *et al.*, 2010).

3.5. Transgenesis efficiency of recombinant sperm artificially inseminated rabbits: Before undergoing transgenesis through artificial insemination, a considerable time of semen processing was wasted. This time represented by the time of extensive washing taken place in semen to remove seminal fluid from sperm cells and the incubation time of sperm cells with the exogenous DNA. These inevitable pre-insemination steps were wasted significant time from the ejaculation until insemination. These sperm related relatively time consuming manipulation steps might contribute drastically in the reduction of the recombinant sperm activity. Therefore, despite using activation medium, the low success ratio of transgenesis could be attributed to the reduction of quality of semen during the centrifugation, separation, insemination or even in the post-internalization. In the later stage, transgene might be degraded by the silent endogenous nuclease activity that activated once the transgene entered inside the head of the sperm, since such entry renders the metabolically inactive sperm to a highly active metabolic nuclease activity. This, in turn, plays a role in the elimination of the recombinant sperm cells representing a potential danger for the development of the progeny or for its genetic identity (Maione *et al.*, 1997). High capacity to binding with DNA were observed after only 30 min of incubation but the only significant problem was represented with the reduction of viability of recombinant sperm into 50% compared with their normal counterparts. This in turn would drastically compromised fertility (Canovas *et al.*, 2010). Nevertheless, artificial insemination alive new born babies were apparently normal and there were no symptom of any illness.

3.6. PCR detection of transgenesis efficiency of recombinant sperm injected new born babies of rabbits (liposome- *Bam*HI – gWizGFP DNA and DMSO- *Bam*HI – gWizGFP DNA treatments): In this study, it was shown the ability of recombinant rabbit's sperm to generate transgenic animal through artificial insemination. These results were demonstrated by PCR. DMSO mediated and liposome mediated REMI-SMGT were compared with each other to observe the possibility of the non-costly DMSO to replace the costly liposome to generate transgenic animals. In the same time, the efficiency of REMI-SMGT was directly validated in rabbits using GFP as transgene. DMSO and liposome were involved in REMI-SMGT since they were proved to be the best chemical transfectants for GFP vectors (Marta *et al.*, 2009). Although some researchers referred to the increased motility or fertility of recombinant sperm compared with their normal counterparts (Chan, 2000; Nakanishi & Iritani, 1993) while others referred that transfection had no effect on fertilizing capacity on recombinant spermatozoa during insemination (Chrenek *et al.*, 2005), the majority of papers usually noticed that the recombinant spermatozoa was less competent than its normal counterpart with respect to their fertility rate, whether this was attributed only to the incubation time (Feitosa *et al.*, 2009) or to the exogenous DNA itself since several papers were described elaborately the weak fertilizing ability of manipulated sperm (Castro *et al.*, 1990; Rottman *et al.*, 1992; Squires and Drake, 1993; Sasaki *et al.*, 2000; Sciamanna *et al.*, 2000, Lavitrano *et al.*, 2006). While some researchers demonstrated that the loss of sperm activity was a consequence of the removal of seminal fluid and to the extensive washing taken place before being incubated with exogenous DNA (Kang *et al.*, 2008). This fact is not surprising since seminal fluid contained many factors that maintain sperm motility (Dyck *et al.*, 1999; Holody *et al.*, 1999).

The negative role of liposome on the survival rate was deduced clearly since as much as this material increased as much the reduction in sperm fertility was increased as well (El-Gendy *et al.*, 2006), while DMSO has little effect on sperm motility (Shen *et al.*, 2006). However, the only one successful kindling out of five in case of liposome mediated REMI-SMGT possibly related with the reduced developmental rate of liposome - transgene treated embryos compared with their normal counterparts during early embryological stages (Kim *et al.*, 2008).

This study was not the first one which utilized DMSO as an accessory tool to convey transgene since it was successfully used with the transgene to do the same role in rabbits (Li *et al.*, 2006; Shen *et al.*, 2006). Despite the clear observations made in this research about the absence of any direct relationship between DMSO and DNA compared with the direct relationship occurred between liposome and DNA (fig. 4, and fig. 5) the PCR results were not comparable with the previously obtained results. Hong and his colleges (1998) demonstrated the high efficiency of DMSO compared with liposomes. They found the ability of DMSO to transfect 80% of chicken primordial germ cells compared with only 17% regarding to liposomes (Hong *et al.*, 1998). While other researchers discovered evident clue which demonstrated the ability of this material to interact with membranes and lipid vesicles (Hempling & White, 1984; Long *et al.*, 2003). This in turn, might refer to a particular relationship between DMSO from one site and cellular membrane from another site. This relation might be

involved in certain role by which the exogenous DNA would be capable on internalizing into the head of the sperm. This suggestion was supported by the increase of transfection rate of sperm with DMSO in case of using heat shock (Kuznetsov & Kuznetsoza, 1995; Kuznetsov *et al.*, 2000). Moreover, the demonstrated facts of indirect interactions between DMSO and DNA rather than the direct interaction occurred between liposome and DNA might reflected more elegant role played by DMSO, particularly, no data indicated any reduction in activity of DMSO bound sperm compared with liposome bound sperm since the later complex was reported to be much less motile compared with the unbound state (Zoraqi & Spadafora, 1997; El-Gendy et al, 2006).

Though DMSO mediated REMI-SMGT was produced two transgenic babies and liposome mediated REMI-SMGT was produced only one transgenic baby, this transgenesis fact was a result of only three successful kindling. Undoubtedly, this was not enough to deduce an actual percentage of the success of each DMSO or liposome respectively.

Unfortunately, in all SMGT techniques, there was no practical available method by which recombinant sperm could be separated from non-recombinant counterparts. This – off course – made the researcher confused about the final result until performing one of the transgenesis detection methods. However, according to PCR, only three transgenic out of fourteen new born babies were obtained (fig. 8). This ratio didn't exceed 21.4% of alive new born babies obtained from artificial insemination.

However, the ability of the exogenous DNA to be integrated into the head of sperm is somewhat overestimated since the increased efficiency was not necessarily concomitant with an increase of generation of transgenic animals (Naganishi & Iritani, 1993; Gandolfi, 1998).

Although PCR, as a transgenesis detection technique, was not capable of detecting whether transgene was just episome or it was integrated into the genome, it was powerful enough to observe the internalization of transgene into the head of the sperm. Add to that, some researchers relied only on PCR to detect transgenesis (Vasicek *et al.*, 2003). However, information provided by such conventional PCR was just preliminary results, but, they were enough in this context since they demonstrated the internalization of transgene. This, in turn, considered as serious and confident step in the detection of transgenesis.

The age of artificial insemination new born babies at which blood withdraw taken place was only two days since it was demonstrated that PCR amplification bands from gWizGFP DNA was found to become progressively less intense as the animal aged. Thus, an animal that tested gWizGFP DNA – positive at a young age might be tested gWizGFP DNA negative at later screenings, making the rate of PCR – positive animals vary over time (Pittoggi, 2006).

The most critical step toward REMI-SMGT approach was not just to incorporate the exogenous DNA into the head of sperm, but to force it to be integrated into the genome of the sperm. This, possibly, added more regulations on REMI-SMGT as a successful technique since even after the success of the transmission of exogenous DNA, the process of its expression is usually failed. This was attributed to its loss of integration, or even before its integration it might not integrated properly or it is submitted to fragmentation before doing so. This possible failure in the integration was related to several reasons, such as the reduction of viability induced by the transfection process, which in turn, decrease the biological activity of transfected sperm to fertilize oocyte compared with the non-transfected counterparts (Garcia-Vazquez *et al.*, 2010), or the sperm conversion to an immotile form after its transfection (Schellander *et al.*, 1995; Anzar & Buhr, 2006; Feitosa *et al.*, 2009), or to both of them (Canovas *et al.*, 2010). However, even the recombinant sperm were retained their motility after transfection; they were unable – because of this manipulation – to fertilize the oocyte to produce the transgenic offspring (Bachiller *et al.*, 1991).

It was demonstrated that internalized foreign DNA, when presented above a threshold amount, trigger the activation of sperm endonuclease(s) which cleave the exogenous DNA molecules and also degrade the sperm endogenous chromatin. This process eventually led into attenuation of recombinant spermatozoa compared with their normal counterpart (Maione *et al.*, 1997). Moreover, it was shown that sperm interaction with foreign DNA trigger endogenous nuclease(s) that cleave both the exogenous and the genomic DNA, eventually leading to sperm cell death processes which resemble apoptosis (Spadafora, 1998).

3.7. Direct exposure of transgenic new born babies rabbits to high intensity UV light: To generate any transgenic animal, the transgene should be localized inside the cells of the animal that intended to be transgenic (Houdebine, 2003), but the place at which the transgene located was a controversial scene of much more debate. However, in this research, PCR positive results for the three transgenic new born babies might indicate the success of the transgene (gWizGFP vector) to insert itself into the cytosol to behave like episome only. Or it might indicate the ability of transgene to integrate itself into the genome to behave like one of the large genome family. Or eventually it might indicate the ability of such transgene to express on itself to produce its modular protein (GFP). However, the last probable conclusion was the least one since the direct exposure of PCR positive babies and their comparison with PCR negative counterpart did not observe any significant difference between them at all (results not shown). This possible lack of expression might be related to the rearrangement of

transgene after being internalized into the sperm (Khoo, 2000).
Unfortunately, the dark fluorescent room facility that was designed to be optimized at 450 nm – the optimum wavelength at which gWizGFP or any GFP was express normally – was not available. Therefore, the wavelength at which UV light used was not exceeding 330 nm (the fluorescent violate) wavelength that which was designed to visualize nucleic acids which was far away from 450 nm (the fluorescent green) wavelength. Although this fact was a considerable one, but some researchers were exposed their new born PCR positive babies directly to UV light and some of them got negative results, such as the experiments done in rats (Epperly, 2007). It was mentioned that there were several limitations of utilizing GFP biological activity, such as presence of some limitations in GFP stability or to the necessity of the presence of more than 1µM GFP per cell to glow significant fluorescence (Epperly, 2007; March *et al.*, 2003; Tsien, 1998).
The final destination of sperm-bound DNA, after delivery in the oocyte, is still a contradictory issue; particularly, the question of whether foreign molecules of nucleic acids become integrated into the host genome or remain as extra-chromosomal structures was still unsolved. several accumulated data indicated that the fate of the exogenous DNA depend on the procedures through which sperm cells and DNA come together: the generation of non-integrated episomal structures is a highly probable event when foreign DNA molecules were directly incubated with intact spermatozoa that were then used in fertilization assays (Khoo *et al.*, 1992; Khoo, 2000; Kuznetsov *et al.*, 2000; Robinson *et al.*, 2000; Tsai, 2000, and Spadafora, 2008).
Regardless of the low efficiency of transgenesis obtained in this study, but the low cost of this modified technique was an encouraging tool to researchers to undergo transgenesis by improving REMI-SMGT to increase the ratio of transgenesis without increasing the total cost of the technique. Although small number of animals was used in this preliminary study, these results were encouraging since the practical nature of this new modified REMI-SMGT methodology allow of direct production of transgenic animals. However, many results were successfully reported the ability of SMGT in general to generate alive transgenic mammals such as in mice (Lavitrano *et al.*, 1989), rats (Blanchard & Boekelheide, 1997), rabbits (Wang *et al.*, 2003), and cattle (Sperandio *et al.*, 1996), but the production of REMI-SMGT transgenic animals was reported only in bovine (Shemesh *et al.*, 2000). Nevertheless, this is the second report which generated alive REMI-SMGT transgenic mammals and the first report which generated alive REMI-SMGT transgenic rabbits despite the low efficiency of transgenesis reported by this research compared with the research of Shemesh and his colleges that published at 2000.

Conclusion

Although the process of generating high frequency recombinant sperm was not difficult, the process of generating high frequency transgenic animals from such germ cells was a tremendous task. This was because the fertilizing ability of recombinant sperm was much less than it was found in their normal counterparts. Moreover, despite the apparent ability of liposome to relatively protect exogenous DNA from the hydrolytic activity of seminal fluid and DNase activity compared with DMSO, the later one held an indirect ability to internalize exogenous DNA without any direct relationship with it. Eventually, REMI-SMGT was less effective – at least in rabbits – than it was claimed in other mammals.

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Table (1). Thermal cycling conditions for PCR amplification. These guidelines were tested for *Eppendorf* thermal cycler.

Step	Temp (°C)	Time	No. of cycles
Initial denaturation	95	2 min	1
Denaturation	95	0.5 min	30
Annealing	52	1 min	
Extension	75	0.5 min	
Final extension	75	5 min	1

Table (2): concentrations applied during AI for each; gWizGFP DNA, liposome, DMSO, and restriction enzyme

No. of treatments	Type of treatment	Concentration per each treatment			
		Vector	Liposome	DMSO	RE
5	Vector-liposome-RE	10µg	10µg	---	50U
5	Vector-DMSO-RE	10µg	---	10µl	50U
Final volume per treatment		500µl with sp-TALP medium			

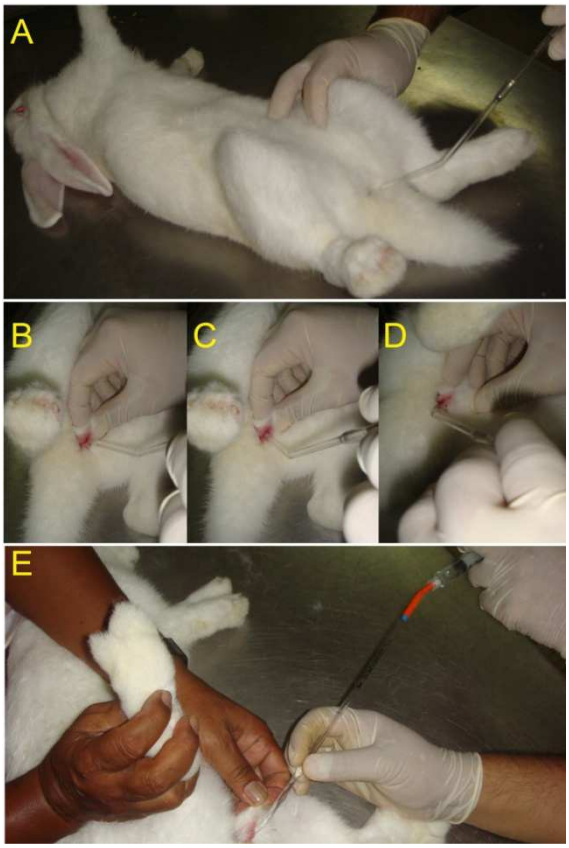


Figure (2): Artificial insemination (AI) in rabbits. When insemination was done by two persons, one retains rabbit by holding her back and the other operated with the glass insemination pipette.

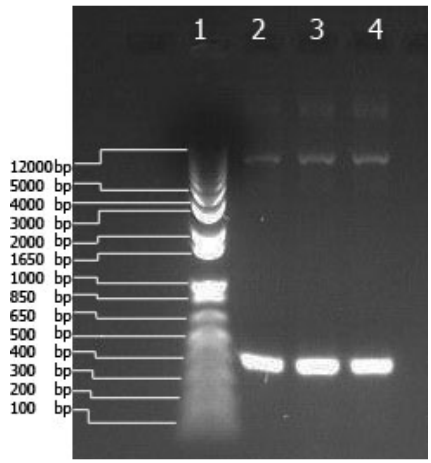
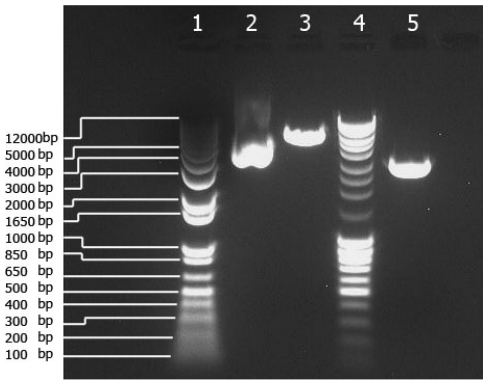


Figure (1): Testing of primer design of gWizGFP 364bp fragment by polymerase chain reaction. Lane 1: 20µl DNA size marker (Invitrogen). Lane 2: 364 bp fragment produced from 0.7µg DNA template (gWizGFP vector) and 0.08µM of each forward and reverse primer. Lane 3: 364 bp fragment produced from 0.8µg DNA template (gWizGFP vector) and 0.10µM of each forward and reverse primer. Lane 4: 364 bp fragment produced from 1 µg DNA template (gWizGFP vector) and 0.12µM of forward and reverse primers. Electrophoresis conditions: agarose concentration 1%, power applied: 5.5 V /cm, time of run: 1 hr. staining dye used: ethidium bromide.



Figure(3). Linearization of gWizGFP vector by *Bam*H I restriction endonuclease during only 30 min incubation at 30°C. Lane 1: 20µl (2.2 µg) DNA Size marker (Invitrogen – USA). Lane 2: 1 µl (5µg) covalently closed circular gWizGFP vector (Aldevron – USA). Lane 3: 4 µl (5µg) linearized gWizGFP vector (Aldevron – USA). Lane 4: 20µl (2µg) DNA Size marker (Fermentas – USA). Lane 5: 20µl (5µg) linear pTZ57R/T vector (Fermentas – USA). Electrophoresis conditions: agarose concentration 1%, power applied: 4.5 V / cm, time of run: 1 hr. staining dye used: ethidium bromide.

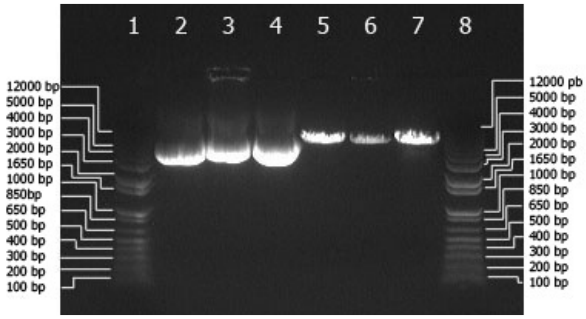


Figure (4). The ability of liposome and DMSO respectively to change the electrophoretic mobility of circular as well as linear DNA. Lane 1: 15µl (1.5 µg) DNA Size marker
(Invitrogen). Lane 2: 2 µl (10 µg) CCC gWizGFP vector alone. Lane 3: 2 µl (10 µg) CCC gWizGFP vector with 10 µl (10 µg) liposome. Lane 4: 2 µl (10 µg) CCC gWizGFP vector with 10 µl DMSO. Lane 5: 10 µl (10 µg)

linear gWizGFP vector alone. Lane 6: 10 µl (10 µg) linear gWizGFP vector with 10 µl (10 µg) liposome. Lane 7: 10 µl (10 µg) linear gWizGFP vector with 10 µl DMSO. Lane 8: 15µl (1.5 µg) DNA Size marker (Invitrogen – USA). Electrophoresis conditions: agarose concentration 0.8%, power applied: 4.5 V / cm, time of run: 50 min. staining dye used: ethidium bromide.

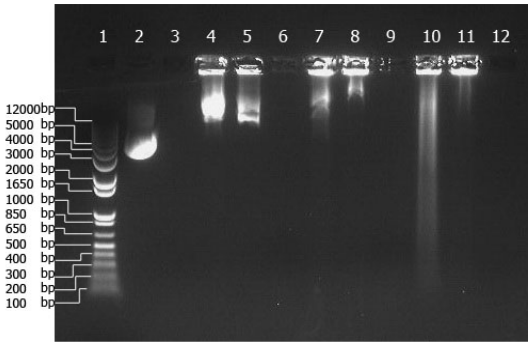


Figure (6). The effect of liposome on DNase activity profile of rabbit's seminal fluid. Lane 1: 20µl size marker (Invitrogen). Lane 2: 2µg gWizGFP DNA (Aldevron). Lane 3: 1µl (1 µg) seminal fluid. Lane 4: 10µl taken from incubation of 6µg gWizGFP DNA, 1µl seminal fluid & 23µl D.W. Lane 5: 10µl taken from incubation of 6µl (6µg) gWizGFP DNA, 6µl (6µg) liposome, 1µl seminal fluid & 17µl D.W. Lane 6: 5µl seminal fluid. Lane 7: 10µl taken from incubation of 6µg gWizGFP DNA, 5µl seminal fluid & 18µl D.W. Lane 8: 10µl taken from incubation of 6µg gWizGFP DNA, 6 µl (6µg) liposome, 5µl seminal fluid & 13µl D. W. Lane 9: 10µl seminal fluid. Lane 10: 10µl taken from incubation of 6µg gWizGFP DNA, 10µl seminal fluid & 14µl D.W. Lane 11: 10µl taken from incubation of 6µg gWizGFP DNA, 6µl (6µg) liposome, 10µl seminal fluid & 8µl D.W. Lane 12: 1µg bovine serum albumin (BSA).

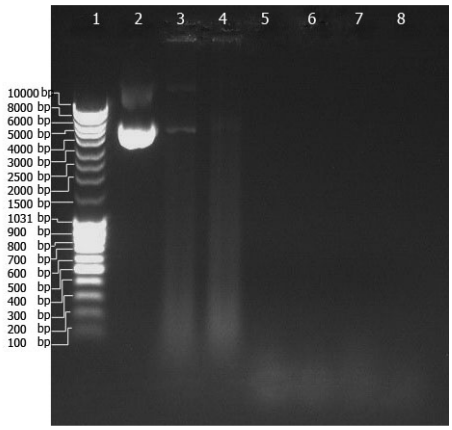


Figure (5). The effect of liposome compared with DMSO in reducing DNase activity. Lane 1: 20µl DNA size marker (Fermentas – USA). Lane 2: 1µg gWizGFP vector. Lane 3: 30min liposome incubated and 5min (1/10) DNase hydrolyzed 3.3µg gWizGFP vector. Lane 4: 30min liposome incubated and 10min (1/10) DNase hydrolyzed 3.3µg gWizGFP vector. Lane 5: 30min DMSO incubated and 5min (1/10) DNase hydrolyzed 3.3µg gWizGFP vector. Lane 6: 30min DMSO incubated and 10min (1/10) DNase hydrolyzed 3.3µg gWizGFP vector.Lane 7: 5min (1/10) DNase hydrolyzed 3.3µg gWizGFP vector. Lane 8: 10min (1/10) DNase hydrolyzed 3.3µg gWizGFP vector. Electrophoresis conditions: agarose concentration 1%

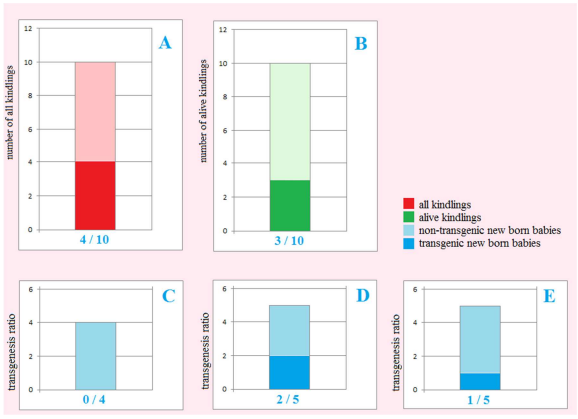


Figure (8). diagram shows the efficiency of transgenesis of in superovulated females after their insemination with sperm cells treated with exogenous DNA. A) The number of kindling obtained after the artificial insemination of recombinant sperm into the superovulated female uteri, in which only four out of ten kindling were obtained. B) The number of alive kindling obtained after the artificial insemination of recombinant sperm into the superovulated female uteri, in which only three out of ten kindling were obtained. C) The number of new born transgenic babies obtained after artificial insemination with gWizGFP linearized vector – DMSO – BamHI of only one of the three alive kindling obtained. D) Number of new born transgenic babies obtained after artificial insemination with gWizGFP linearized vector – DMSO – BamHI of only one of the three alive kindling obtained. E) Number of new born transgenic babies obtained after artificial insemination with gWizGFP linearized vector – liposome – BamHI of only one of the three alive kindling obtained

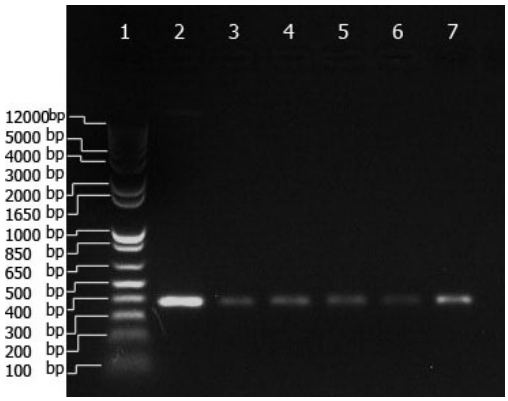


Figure (7). gWizGFP vector integration into the sperm cells after incubation for 1 hour at room temperature. Lane 1: 20µl DNA size marker (Invitrogen – USA). Lane 2: 10µl of 364 bp PCR tested amplified fragment. Lane 3: 364 bp PCR amplified fragment from 1µl sperm genomic DNA isolated after incubation with gWizGFP – liposome- BamH I. Lane 4: 364 bp PCR amplified fragment from 0.51µl sperm genomic DNA isolated after incubation with gWizGFP – DMSO- BamH I. Lane 5: 364 bp PCR amplified fragment from 0.51µl sperm genomic DNA isolated after incubation with gWizGFP – liposome. Lane 6: 364 bp PCR amplified fragment from 0.41µl sperm genomic DNA isolated after incubation with gWizGFP - DMSO. Lane 7: 364 bp PCR amplified fragment from 0.31µl sperm genomic DNA isolated after incubation with gWizGFP only. Electrophoresis conditions: agarose concentration 1.5%, power applied: 5.5 V / cm, time of run: 1.15 hr. staining dye used: ethidium bromide.

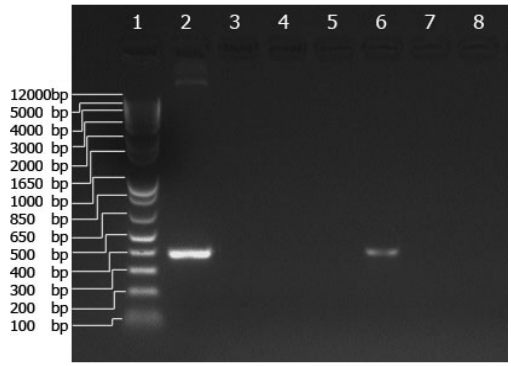


Figure (10). Detection of gWizGFP vector existence into the blood withdrawn from new born artificial insemination babies their mothers were injected with gWizGFP DNA-
liposome-BamHI complex. Lane 1: 20µl DNA size marker (Invitrogen – USA). Lane 2: 10µl of 364 bp PCR tested amplified fragment. Lanes (3 to 7) of 10 µl PCR product of new born babies from No. 10 to No. 14. Lane 8: 10µl of PCR product of negative control new bom baby. Electrophoresis conditions: agarose concentration 1.5%, power applied: 5.5 V / cm, time of run: 45 min. staining
dye used: ethidium bromid

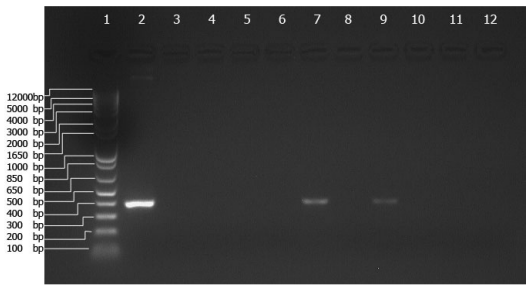


Figure (9). Detection of gWizGFP vector existence into the blood withdrawn from new born artificial insemination babies their mothers were injected with gWizGFP DNA-DMSO-BamHI complex. Lane 1: 20µl DNA size marker (Invitrogen – USA). Lane 2: 10µl of 364 bp PCR tested amplified fragment. Lanes (3 to 11) 10µl PCR product of newborn babies from No. 1 to No. 9 respectively. Lane 12 was 10µl of PCR product of negative control new born baby. Electrophoresis conditions: agarose concentration 1.5%, power applied: 5.5 V / cm, time of run: 45 min. staining dye used: ethidium bromide.