Abstract
The main objective of this research is to identify the efficiency of reducing the total cost of the utilizing of the cost-effective liposomes by using non-cost effective substitutes such as DMSO. Direct comparison between two transfectants; the high cost liposome and the low cost DMSO was made on the basis of their direct DNA interaction ability. Direct protective relationship of liposome with DNA and seminal fluid was identified compared with DMSO. Different treatments (linearized DNA, circular DNA, restriction enzyme – liposome – DNA, restriction enzyme – DMSO – DNA, DMSO – DNA and liposome – DNA, and even naked DNA) were all found to be successful to internalize inside the head of the sperm according to PCR results. Despite the clear and significant relationship that were observed between liposome and DNA compared with DMSO, but there was no difference in the efficiency of each of which to escort the exogenous DNA in its way into the head of the sperm.

Comparison between the Efficiency of Liposome and Dimethylsulfoxide to Induce Transfection of the Rabbit’s Sperm

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Introduction
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 [1]. 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 [2-5]. 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 [6]. 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 [7].
Several enhancements have been made to increase the efficiency of this promising method such as using electroporation [8], linkers [9], retroviral vectors [10], and liposomes [11]. 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 [12].

It has been reported that many enhancement approaches have increased the reproducibility of the original SMGT [13]. 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 [14].

An interesting 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 [15, 16]. 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 [17].

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 [15].

We have 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 [18].

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 [19], or its cheap substitute such as DMSO [20], 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 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.

It was referred to the absence of any significant disadvantages in REMI-SMGT [21]. 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.

**Materials and Methods**

**Materials:**
DNA extraction kit; Easy-DNATM Kit (Invitrogen – Cat. # K1800-01). PCR premix; PCR SuperMix (Invitrogen – Cat. # 0572-014). Enzymes; BamHI I (Invitrogen – cat # 15201023), DNase I (Fermentas, Cat # EN0521). Ladders; TrackIT™ 1 Kb Plus DNA Ladder (Invitrogen – Cat. #10488090), MassRuler™ DNA Ladder Mix (Fermentas – Cat. # SM0403). Oligos; Forward primer (5’–CCATGCCCAGGTTATGTA –3’) and reverse primer (5’–GAAAGGCAGATTGTGGGA –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 method with some modifications (22; 23; 24, ).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 Zealand sexually mature healthy white rabbits were included in this study. New Zealand 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).

**Methods**

**gWiz-GFP vector linearization with restriction enzyme:** BamHI 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 50 μg 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.

**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).

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

**Preparation of DNA-liposome-DNase mixture and DNA-DMSO-DNase mixtures:**

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. As for the preparation of DNA-DMSO-DNase mixture, the same procedure mentioned in the previous preparation was taken place but DMSO was included instead of liposome.

**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. 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.

**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.

**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.

**Linearized and circular gWiz-GFP vector preparation for incubation with sperm cells:**

Different treatments were used to incubate gWiz-GFP vector with sperm cells. 100µg gWiz-GFP was linearized by 1000U BamH I Then all DNA restriction enzyme component mixture (DNA-BamHI mixture) was stored at -20°C.

- 200µl of DNA-BamHI mixture was incubated with 20µl (20µg) liposome for 30 minutes at room temperature.
- 200µl of DNA-BamHI mixture was incubated with 20µl DMSO for 30 minutes at room temperature.
- 10µg circular gWiz-GFP vector was incubated with 10µl (10µg) liposome for 30 minutes at room temperature.
- 10µg circular gWiz-GFP vector was incubated with 10µl DMSO for 30 minutes at room temperature.
- 10µg circular gWiz-GFP vector was incubated for 30 minutes at room temperature without any additives.
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.

**DNase treatment:** Each sperm cells suspension treated with 10µg gWiz-GFP was incubated for 30 min with 10 units of DNase mixture at 37°C in water bath. After 30 min of incubation, 10µl of stop solution was added and the mixture was incubated at 65°C for 10 min to inactivate the enzyme.

**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).

**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 polyacrylamide gel electrophoresis. After staining with ethidium bromide, photo was taken using photodocumentation unit (figure 1).

![Table 1](image)

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

**Figure 1** Testing of primer design of gWizGFP 364bp fragment by polymerase chain reaction.

Lane 1: 15µl DNA size marker (Fermentas)

Lane 2: 364 bp fragment produced from 0.7µg DNA template (gWizGFP vector) and 1.5µl of each forward and reverse primer PAGE electrophoresis conditions: polyacrylamide gel electrophoresis. Voltage applied: 200 V (15.38 V/cm), run time: 30 min.

**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.
Results and Discussion

An attempt was made to test the efficiency of lowering the total cost of REMI-SMGT technique. 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 [15] 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 [26]. However, direct comparison between DMSO and liposome was represented the central point of this research. We applied such modified low cost REMI-SMGT on rabbits considering these animals as a model applicable for both domestic and mammals.

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 2). 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 (figure 2) 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.

![Figure 2](image)

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 (figure 3).

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 (figure 2).
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 [27]. 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 [28]. 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 [29].

**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 [30, 31]. The significant results of the protection of liposome interacted DNA observed in figure 3 was confirmed in figure 4, 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; who demonstrated the ability of liposome to protect the foreign DNA from digestion of proteases or DNase present in the cytoplasm of the egg [32].The result obtained in figure 4 was possible as well since no complete neutralization was taken place (figure 3) despite prolonged incubation times were used (results not shown).
**Figure 4**. The effect of liposome on DNase activity profile of rabbit’s seminal fluid

Lane 1: 2µg 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 & 4µl 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).

Electrophoresis conditions: agarose concentration 1%, power applied: 4.5 V/cm, time of run: 1 hr. staining dye used: ethidium bromide.

**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 [33], 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 (figure 5). 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 [34, 11, 35].

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 [36]. 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 [3]. 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 [35].
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 [37]. However, PCR as a tool of transgenesis technique remains reliable indicator for transgenesis [38]. 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 [39; 40; 41; 36; 42; 43, 44].

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References


Figure 5 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
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.
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