Journal of Genetic and Environmental Resources Conservation, 2021, 9(1):157-167.
www.gercj.com Print ISSN: 2306-8663, Online ISSN: 2306 – 8280

Comparison between Newcastle disease vaccine and liposomal entrapped Newcastle disease vaccine in chicks

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Received: October 17th 2020; Accepted: November 13th 2020

Abstract

The study aimed to evaluate the immune response of chicks against Newcastle disease vaccine between conventional and liposomal Newcastle vaccine, the experiment includes: 120, broiler chicks, one day- old, distrusted equally in to four groups, 30 birds in each; unilamellar, conventional and positive and negative control, the experiment was conducted to determine the ability of unilamellar liposome-encapsulated ND vaccine to protected chicks from field infection comparison with conventional vaccine, the antibody titer against ND vaccine was enhanced vaccination titer of birds at 10 days post the first immunization of conventional ND 639.00±4.82, and unilamellar liposome-encapsulated ND vaccine 729.40±5.64 and the second immunization of conventional ND 720.80±1.49 and unilamellar liposome-encapsulated ND vaccine 1207.90±3.50. The IFN-Y levels in unilamellar liposome-encapsulated ND vaccinated group was higher level antibodies titer than conventional ND and displayed at day 10day post-vaccination 113.00 ±1.83 and at 20 day post-vaccination 151.80 ±1.25 as compared with positive conventional ND vaccine 101.60 ±1.66 and negative control 122.00± 2.05. Challenge test was achieved by virulent ND $10^{7.5}$ ELD_{50} at 40 days old of vaccinated birds and control; post 20 days of second vaccination orally, the protection of vaccines liposome formulate share 90% safeness and become resisted bird to infection higher than conventional control 80% vaccinated bird that endorsed superior formula proper immune cells based on local mucosal immunity and cell mediated immunity were improved protection percent. The study has concluded achieved shorter in onset time protection started and long-lasting immunity against repeated infection in maximized the immunological titer of bird protection as compared with conventional vaccination process.

Keywords: Newcastle disease vaccine, Liposomal entrapped, Chicks.

Introduction

Vaccination is considered one of the chief developments in modern medicine, as the most effective eradication method ever was developed. Vaccination programs were designed to hyperimmunized chickens effectively to protect them from field/virulent strains virus (OIE, 2015 and Han *et al*., 2018).

Several attempts were done for maximizing vaccination efficiency recent advances in Nano-medicine have been studied in the veterinary field and have found a wide variety of applications. Recently has recorded a massive surge of research interest in liposomes delivery therapeutic substances in animal (Sadozai and Saeidi, 2013).

One of the major unconventional approaches to enhance immunogenicity is delivering antigens by liposomal encapsulation as derived vehicles. Liposomes have been considered intensively because of their potential as immune-stimulants and their capacity to deliver antigenic components (Ghaffar *et al*., 2014). Liposomes are synthetic or natural spheres, lamellar of phospholipid. Numerous reports have confirmed that liposomes can act as both vaccine delivery carriers and immune adjuvants and bio-enhancer (Gregoriadis, 1990).

Nano-vaccines are a novel approach to the development of vaccines to combat diseases (Zaman *et al*., 2013) as vaccine development orientates towards less immunogenic "minimalist" composition, a formulation that boosts antigen effectiveness are increasingly needed. The use of nanoparticles in vaccine formulation allows not only improved antigen stability and immunogenicity but also targeted delivery and slow-release (Zhao *et al*., 2014).

On the otherwise the Liposomal-entrapped vaccine formula mostly achieved improved several immune-dynamic and immune-kinetic vaccine behavior firstly; encapsulation protected the vaccine from several passage administration pathways (Bulbake *et al*., 2017), second; increase the potency of immunization activity, third; attained direct to the direct organs active site

detection, fourth; occupying anew pharmacological modifying compartment threshold of steady-state set point and increase circulatory half-life (Schmidt *et al*., 2016 and Chang and Yeh, 2012) with increase time of vaccine release, finally; conclude demined immunization tools for long time and reduce the dosage administered (Schwendener, 2014 and Walve *et al*., 2011). The study was aimed to compare between ordinary (conventional) and liposome carrying Newcastle of attenuated live viral vaccine for arouses immunological reaction, as well as to minimize the viral dose and maximized the immunological titer of bird protection as compared with conventional vaccination process.

Materials and Methods

The experiment was achieved protocol in Collage of Veterinary Medicine and pharmacology Lab. The ethical issued and followed scientific education program (Al-Bayati and Khamas, 2015) of standardizing the laboratory animal in scientific research.

The vaccine standardization and immunity were done in the Veterinary Directorate/Ministry of Agriculture.

Eggs and chicks management: The eggs and chicks were brought from Shuker Hatchery Com.- Baghdad.

Eggs : Fertilized eggs ninety eggs were used for titration of vaccine virus. Eggs were transfer in good condition 37C°and 60%humidity to Department of Biology and Medicine Supervision/ Veterinary Directorate/ Ministry of Agriculture.

Broiler chicks : One day old chicks were transported to the prior cleaned, disinfectant and dry rearing Unit for 3 hr. , Then fumigated with formaalex® along 2 days.The newly hatching chicks were deprived of grain feed for 24 hr. and provided water *to add lib*. rearing unit controlled under temperature condition 31C° and decline of temperature was set down 2 C° degree in every week up to five-weeks of age. Closed fixed temperature room was fixed at 23°C.

Sample collection: Blood samples were collected randomly from jugular vein by disposable syringe 3ml, 28'' and 23''. Blood was immediately kept in clot activator and gel tube in room temperature until serum separated and then frozen at – 20C° until assayed (Allan *et al*., 1978).

Vaccines preparation and standardization: The vaccine was prepared and standardized in two different settling methods showed as follows:

Ordinary vaccines preparation : $ND_{Las.}$ preparation: One vial of lyophilized ND, 2500 dose was dissolved in 2 ml PBS 4C° with vortex for 20 sec (Allan,1978).

$ND_{Las.}$ vaccine EID_{50} standardization: The EID_{50} of vaccine was achieved as following settling maneuvers:

Eggs Inoculation Fertilized eggs used for propagation of live Newcastle disease virus vaccines strains, as described by (Allan *et al.,*1978).

Preparation of red blood cells of the chicken Blood was collected from the wing vein of chicken as described by

by (Alexander,1998).

Rapid Slide Agglutination It was used to detect the growth of the virus in the allantoic fluid (Allan *et al.,* 1978).

Estimation of EID_{50} The EID_{50} was measured by titration of vaccine on chicken embryonated egg; was determined following the method of (Rai,2005) The 10^9 EID_{50}/ 1ml was set in vaccination and Nano-liposome preparations.

Nanoliposomal Vaccine Preparation: The liposomal Nano-sized scale encapsulated vaccine was organized, standardized and achieves bio-enhancer carrier liposome carrying vaccine was done as (Jaafer *et al.*, 2020)a in follows:

Liposome Preparation Technique: The liposome was formulated according to the Bangham thin film technique (Marie and Habeeb, 2012) of settling steps as follows:

Unilamellar liposome: The mixture of both Cholesterol 0.25 g. and 0.25 g. Phosphatidylcholine, 1:1 w/w. The mixture was dissolved in 2 ml methanol prior one drop (50 μl) of Chloroform.

The phosphatidylcholine and cholesterol organic solvent were vortexes continuously for 30 min with evaporation by rotary evaporator; conducted with vaccum pump at 40 °C. The yield was a dry thin film, foam-like appearance deposited on tube wall

The vaccine strain was merged in thin-film liposome and vortexes for 30min. 37°C to prepare liposomal vaccine LipoENDV (Liposome encapsulated Newcastle disease vaccine) entrapment and summarized in figure 1.

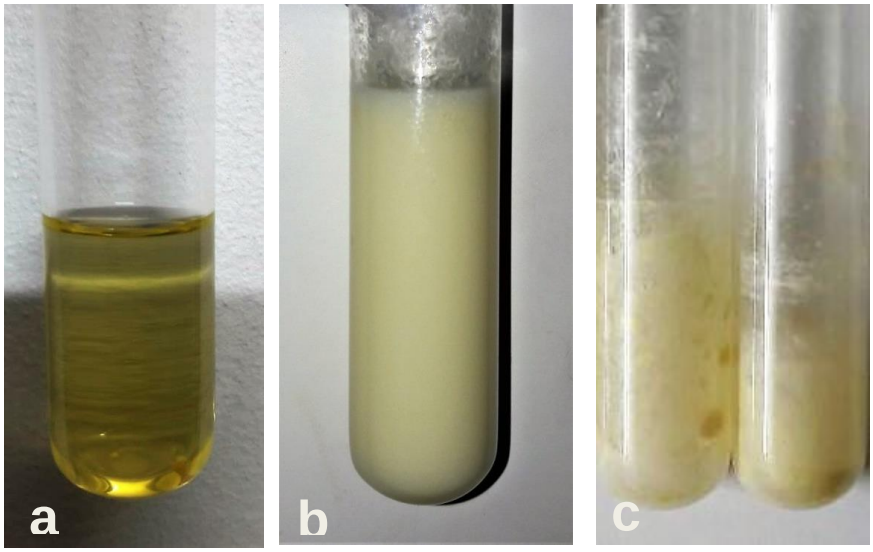


Figure 1: a. phosphatidylcholine and cholesterol dissolve in chloroform, b.The nano ND vaccine liposomal form appearance pale color foamy shape creamy texture, c. Thin-film deposited on the tube wall.

ELISA Technique : The indirect ELISA test was applied for humoral immunity detection according to the method described by Synbiotic Laboratories Incorporation, USA.

Immunization of Ordinary ND vaccine and ULipoEND$_{Las}$V in broiler chicks: This study was conducted to determine the ability of ULipoEND$_{Las}$V to protected chicks from field infection comparison with the ordinary vaccine. After determining the minimum dose vaccine achieved maximized antibody titer in liposomal ND vaccine formula which was 4EID$_{50}$ (Jaafer *et al*, 2020)b.

Experiment design: Broiler chicks 120, one day old, divided into four groups, 30 birds each group . Control negative: Without vaccination, Control positive: Treated with empty Uniliposome, Ordinary ND$_{Las}$: Vaccination by ordinary ND$_{Las,}$ ULipoEND$_{Las}$V: Vaccination by Uniliposome encapsulated Newcastle Disease Lasota Vaccine.

The maternal immunity was checked at 3th day, at 7th day old, the suitable vaccination time detected depending on the titer of maternal immunity. The humoral immunity was checked at day 10th, 20 and 30 post-vaccination by ELISA, IgG, on the way to determine the humoral immunity response, The cellular immunity was detected in 20 and 30-day post-vaccination by ELISA ChINF$_{\gamma}$. Challenge test was conducted in forty days old, clinical signs and postmortem changes were observed.

Titration of virulent viruses on chicken embryonated eggs (Embryo Lethal Dose 50) (ELD$_{50}$) : Titration of the virus in embryos of chicken at age of (9-11) days to calculate the lethal dose of embryos (Embryo lethal dose 50% -ELD$_{50}$) as (Rai, 2005).

Experimental infection : Sixty broiler chicks (Ross 308 Breeders) at 40 days old were divided randomly into 4 groups (15 chicks of each group). Each group was housed under strict hygienic measures (biosafety measures roles) in 4 partitions separated in experimental rooms. Chickens were inoculated intra-ocular, intra-nasal and orally with (0.2ml) ND virus contain 10$^{7.5}$ELD$_{50}$/0.1 for each bird.

Protective Index: Calculated according to (Jackson *et al*.,1997) as :

$$\textbf{Protective Index} = \frac{\text{Mortality percent of the unvaccinated group - Mortality percent of vaccinated}}{\text{Mortality percent in unvaccinated group.}} \times 100$$

Statistics: The statistical analysis was joined with software F ratio variance (ANOVA) variance one and two-way analysis for the study of control and treated groups significant differences through analysis two of variance a portability p≤ 0.05. LSD test used for comparison between groups.

Results and Discussion

Antibodies titers of uniliposome encapsulated vaccine :

Humoral Antibodies titers: Table 1 shows the results of immunization challenge of ordinary ND vaccine and UnilipoENDV were revealed significant increase (P≤0.05) of humoral serum titer of UnilipoENDV group 729.40±5.64 in 17 days old(after 10-day post first vaccination) while ordinary group show 639.00±4.82 comparison with control either positive 272.80±1.44 or negative 274.50±2.28.

In 30 day old (after 10-day post-second vaccination the UnilipoENDV group showed a significant increase (P<0.05) of humoral antibodies titers IgG 1207.90±3.50 while ordinary group show 720.80±1.49 comparison with control.

The highest humoral antibodies titers were shown in 40 days old (after 20-day post-second vaccination) the UnilipoENDV revealed significant increase means 4108.40±5.51 compared with ordinary 3032.60±14.38. The two vaccinated groups were the highest significant rate compared with negative and positive control which reached to 52.30±2.32 and 48.90±2.12 respectively.

Table 1: Humoral antibodies titers of different groups in different periods of age(mean ±SE)

Groups \ Days	17day	30day	40day
Control negative	274.50 A c ± 2.28	153.70 B c ± 2.41	52.30 C c ± 2.32
Control positive	272.80 A c ± 1.44	153.30 B c ± 2.51	48.90 C c ± 2.12
Uniliposome NDV	729.40 C a ± 5.64	1207.90 B a ± 3.50	4108.40 A a ± 5.51
Ordinary NDV	639.00 C b ± 4.82	720.80 B b ± 1.49	3032.60 A b ± 14.38

Means with a different small letter in the same column significantly different (P<0.05)
Means with a different capital letter in the same row significantly different (P<0.05)
NDV=Newcastle disease vaccine

Cellular antibodies titers: The results of evaluating the cellular immunity view by estimate the level of INF_γ of vaccinated groups were illustrated in table 2 showed that the differences among means due to groups and periods were significant (P<0.05). In the first period (30 days) the highest mean of INF_γ uniliposome group 113.00±1.83 and the lowest was shown in control negative group 52.50±1.42. A similar trend was also shown in the second period (40 days). The difference between the mean of the INF_γ in the control negative group was not significant while the control positive group showed a significant (P<0.05) decreasing in the mean of the INF_γ. On the other hand, the uniliposome and ordinary groups showed a significant (P<0.05) increasing in the mean of the INF_γ.

Table 2: Cellular immunity titers measured by ELISA - INF_γ test of different groups in different age

Groups \ Days	30day	40day
Control negative	52.50 A d ± 1.42	55.30 A c ± 2.14
Control positive	59.40 A c ± 1.38	53.00 B c ± 1.97
UniliposomeENDV	113.00 B a ± 1.83	151.80 A a ± 1.25
OrdinaryNDV	101.60 B b ± 1.66	122.00 A b ± 2.05

Means with a different small letter in the same column significantly different (P<0.05)
Means with a different capital letter in the same row significantly different (P<0.05)

Challenge Test:

Mortality percent: Three weeks post-immunization, all groups challenged by vNDV administered via oral and nasal routes $10^{7.5}$ ELD_{50} /0.1 ml, the highest protection was shown in UnilipoENDV that reach to 90% , then ordinary 80%

while non vaccinated group was highly susceptible to vNDV and mortality reach 100% within 7 days post-challenge as shown in table 3.

Table 3 Mortality and Survival percent of challenge test by vNDV $10^{7.5}$ ELD_{50} in 40 days old among groups

Groups	Mortality percent	Survival percent
Control	10/10 (100%)	0/10 (0%)
Ordinary	2/10 (20%)	8/10 (80%)
UnilipoENDV	1/10 (10%)	9/10 (90%)

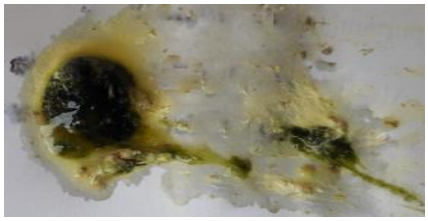
The chi-square value 28.83 P< 0.0001

Post-mortem changes and clinical signs: In the controlled challenged chickens, regular death was first detected 3 days post-challenge and the last death was recorded on the fifth day for all the control chickens. Clinical signs such as the wing or leg paralysis and weakness were observed on the third-day post-challenge and the chickens began to die. After 1 week post-challenge, all the chickens in the controlled groups deceased.

However, chickens immunized with UlipoENDv and ordinary challenged with $10^{7.5}$ ELD_{50} /0.1 ml were more active and showed no signs of disease even after 3 days post-challenge and finally, two chickens survived. Furthermore controlled challenged chickens show depression and loss of appetite, respiratory signs with nasal discharge figure 1 and digestive signs

like greenish diarrhea, Severe manifestations in the digestive system such as hemorrhages of the glandular tip in proventiculus, necrosis of spleen figure 2, heamorrhagic patch and spot of proventiculus glandular tip. figure 4 and cecal tonsils figure 4 was found in dead chickens of the control group, whereas no significant gross lesions were found in the vaccinated group.

Chicken after 3 days post challenge show weakness, the arraw show nasal discharge and conjunctivitis in control group



Chicken after 4 days post challenge show digestive signs illustrated by greenish diarrhea

Chicken after 5 days progress case with nervous signs showed bilateral wing and leg paralysis.

Figure 1: show Clinical signs such as depression and loss of appetite, respiratory signs with nasal discharge bilaterally wing and leg paralysis

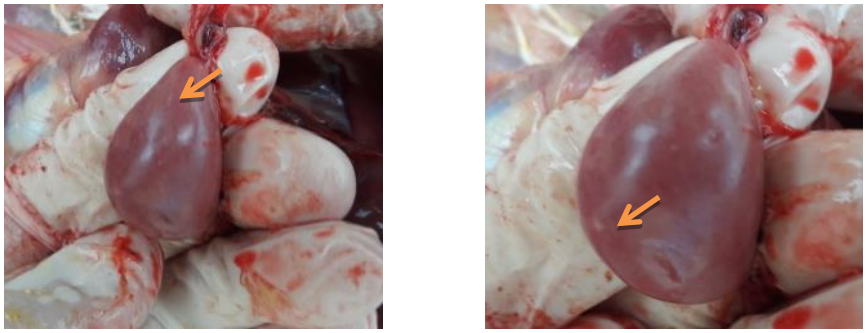


Figure 2: show post-mortum changes 5 days after challenge ND virus infection; the arrow denoted spleen necrosis

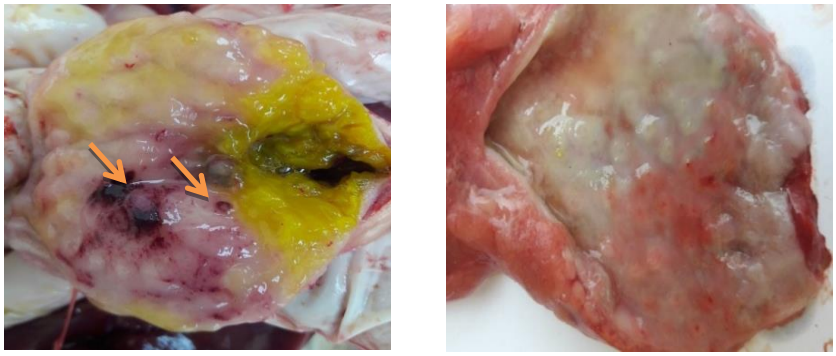


Figure 3: show post-mortum changes 5 days after challenge, the raw pointed one of digestive gross lesion represented by heamorrhagic patch and spot of proventiculus glandular tip.

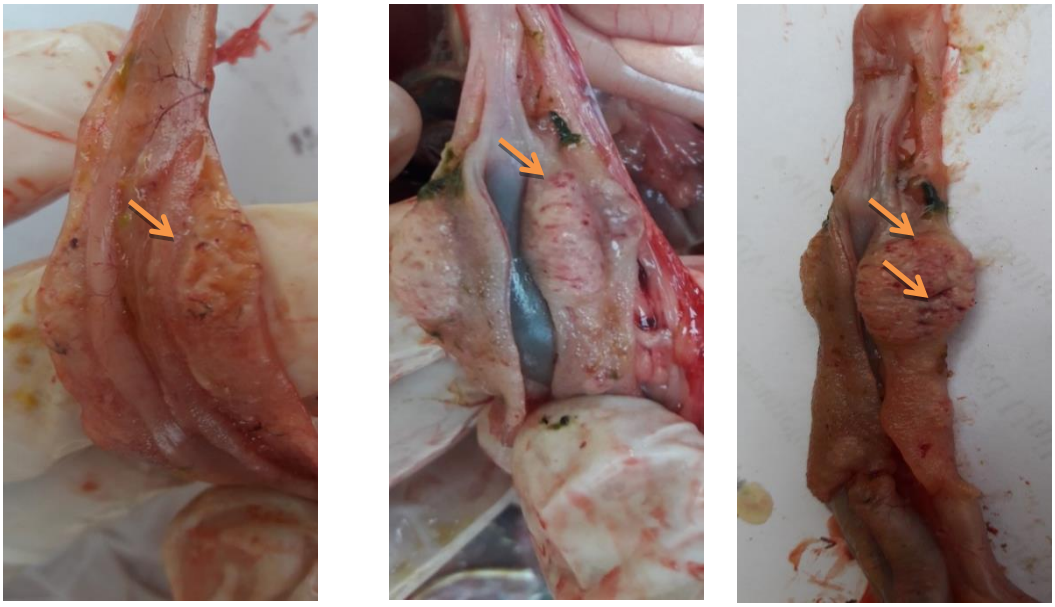


Figure 4: show post-mortum changes 5 days after challenge, the raw pointed one of pathognomic digestive gross lesion represented by heamorrhagic spot and patches of cecal tonsils

The routine program of vaccination in different conventional methodology approved vaccination shared fluctuation of vaccination titer endpoint that supported upstairs the immunity status by many traits of vaccination compromised enhancer of vaccine or implicated new form of delivery of virus as a vaccine base include and reduce the bolus of administration (minimized dose)(Dimitrov *et al.*, 2017).

The nano-material stand new approach to send a fact promotion the vaccine delivery with accepted own nano-behavior carrying vaccine (Onuigbo *et al,* 2012). Liposome promises a one of basic corner of organic Nano; for these reasons, the thesis denoted and reformulated of ND vaccine via encapsulated in liposome and challenged vaccination parameters.

In humoral antibodies titer of Comparison between unilamellar and ordinary: Depending on the previous experiment to detect the prevalence dose and the best formula of LEND either unilamellar or multilamellar to enhancement immunity response for long period, the suitable dose is 10^4 EID_{50} and the formula which gives the long rise of antibodies titers are unilamellar vesicle formula.

The routine vaccine immunization markedly enhances plasma IgG concentration, the antibody titer that corresponding to humoral immunity(Yang *et al.,* 2010). In our study, the antibody titer against NDv were significantly enhanced in vaccinated birds at 10 days after the first immunization of OND and ULEND 639.00±4.82, 729.40±5.64 respectively and after the second immunization of OND and ULEND 720.80±1.49, 1207.90±3.50 respectively, indicating that the humoral immune system enhanced through repeated NDv vaccine either in ordinary or liposomal vaccine compare with control. Pathogen re-exposure often resulted in a more rapid and effective response than the first exposure due to immunologic memory. Also this agrees with Lambrecht *et al*.(2004), reveal to humoral response was detected as early at 7 DPV live attenuated vaccine.

The ULEND group show higher antibodies titer than OND and this related to the liposome, have great potential as vehicles/adjuvants for the targeted delivery of vaccine (Giddam *et al*., 2012). So liposome considered as adjuvant and classified under the vaccine delivery systems(Reed *et al*., 2013 and Altin and Parish, 2006 and Fenske and Culls, 2008),this system function mainly target associated antigen to antigen-presenting cells (APC), which promote the activation state of APCs by up-regulating co-stimulatory signals or MHC expression, inducing cytokine release also enhance antigen processing and presentation to the cells of the immune system (Aguilar & Rodriguez 2007).

Targeting of liposomes can be elicited humoral and cellular response; an enhanced amount of antigen can be delivered to a specific tissue or organ(Immordino *et al*., 2006) uptake by APCs can be enhanced by changing the number of ligand molecules exposed on the liposome surface; and antigen dose can be reduced (Giddam *et al*., 2012 and Foged, 2004).

Some of the reasons why liposomal ND vaccine performed better than the commercial vaccine are that the liposomes formulations are completely biodegradable because their PC (phosphatidylcholines) and cholesterol compositions occur naturally in cell membranes (Gregoridis, 1990) can fuse with cell membranes and that they can evade capture due to their small particle size. Therefore, the liposome-encapsulated ND vaccine was believed to have longer contact and better targeting to the cells of the immune system (Onuigbo *et al*., 2012).

It has been documented that the Bangham ordinary liposome preparation methods could target liposomal vaccine to lymphoid organs sites and provide sustained vaccine release to elevated immunity response more than ordinary that enhancement both of humoral and cellular immunity.

In cellular antibodies titer of Experiment two(Comparison between unilamellar and ordinary: The current study is the first to employ a ChIFN-Y measurement to assess the cellular immune response after vaccination by liposome adjuvant formulation LENDV and ordinary ND to investigated the enhanced cellular immune response. Several studies in poultry have shown that the measurement of chicken interferon(ChIFN- Y) released by T cells after in vitro, stimulation might be a good evaluation of CMI in the chicken after infection and vaccination (Prowse & Pallister, 1989 and Martin et al ., 1994 and Karaca et al ., 1996).

Chicken that stimulated by UENDV seem to progress and have significant elevated IFN-Y level after 10 days P.V. and 20 days P.V. (113.00 ±1.83 and 151.80 ±1.25) compared to ONDV (101.60 ±1.66 and 122.00 ± 2.05) respectively. This agrees with (Lambrecht *et al.,* 2004) that CMI response induced by live Newcastle disease virus (NDV) vaccines has been evaluated sequentially by ex-vivo antigen-specific ChIFN-γ production and cell proliferation of splenocytes from immune chickens. The *ex vivo* data showed that NDV vaccine is capable of stimulating CMI responses to NDV in chickens as measured by the ChIFN-γ ELISA. However, most of the chickens vaccinated with the

live vaccine produced ChIFN-y after antigen recall stimulation, from 2 to 4 weeks after vaccination.

The significant increase of UENDV is indicated to the small liposome vesicles have been shown to induce an enhanced immune response and can provide a depot effect for the sustained release of antigens or can be targeted to deliver the antigen into specific cells also induce potent and long-lasting specific immune response (Giddam *et al*., 2012 and Bhowmick *et al*., 2010 and Alving, 1991). This may be attributed to the association of antigen vaccine with liposomes allows the antigen to gain access to both the MHC class I pathway as well as the MHC class II pathway in antigen-presenting cells (APCs).

The results of our experiment agree with (Khalifa *et al.,* 2014) who reveal to increase production of ChIFN-Y in the encapsulated liposome vaccine superior the non-liposome vaccine compared to control, considering the liposome-encapsulated vaccination might fit in programs that aim specifically to boost the CMI against NDV in addition to primary antibodies response. Other researchers (Fan *et al.,* 2012) show enhance the antibody titer, T- lymphocyte proliferation and the concentrations of interferon-y and interleukin-6 in three preparation of epimedium polysaccharide-propolis flavone immunopotentiator (EPI), as EPI liposome compared with the other two preparations EPI suspension and EPI watery solution, the effect of three preparations on Newcastle disease virus (NDV) infection were compared in chickens vaccinated with ND vaccine then challenged with NDV, These results indicated that liposome could enhance the immune effect of EPI on ND vaccine.

Furthermore some workers (Yu *et al*., 2013) reveal to the adjuvant activity of gypenosides liposome (GPSL) encapsulated with liposome was investigated in vitro and in chickens vaccinated with Newcastle disease (ND) vaccine compared with gypenosides and blank liposome, the results showed that GPSL could significantly enhance lymphocyte proliferation, increase antibody titer, and promote cytokine secretion such as IFN-Y, this indicated that formulations of GPS with liposome can further enhance the immune response against ND vaccine compared with the adjuvant alone. also, GPSL has immunological adjuvant activity in regulating cellular immunity and humoral immunity.

Since mucosa are primary targets for antigens, chickens have extensive mucosa-associated lymphoid tissues (MALT), these include the gut-associated lymphoid tissue (GALT) (Olah and Vervelde, 2008) which is distinct lymphoid aggregates that line the length of the gut, these consist of specialized epithelium containing micro fold cells (M cells) which sample the gut lumen content and deliver them to underling macrophages and dendritic cells (Jeurissen *et al*, 1999; Kitagawa *et al.,* 2000).

The DCS is respond to antigen exposure, When the pathogen-recognition receptors (PRRs) on DCs recognize conserved molecular patterns on virus known as pathogen-associated molecular patterns (PAMPs), then the internalized antigen vaccine will be processed by APCs into smaller pieces including the epitopes (the antigenic determinants) that will be loaded onto MHC (major histocompatibility complex) molecules and then together displayed on cell surface for presentation to T-lymphocytes which takes place within aggregate lymphoid tissue .

The Innate immune system is activated by pathogen-recognition receptors (PRR) that identify pathogen-associated molecular patterns (PAMP) and produce rapid innate responses via danger signals this information is transmitted to T cell via altered cytokine release expression of co-stimulatory molecules, such as CD4 and CD8 (Demento *et al.,* 2011). The extracellular antigens (deliver by vaccine) are taken up phagocytosis into endosomes where proteolysis is followed by presentation via MHC class II molecules to stimulate CD_4 T-helper cells, These cells are coupled with the antigen-MHC complex interact with B cells to activate them by secretion of the cytokines IL-4,IL-5 and IL-10 (Giddam *et al*., 2012 and De Temmerman *et al.,* 2011). B cells then differentiate into antibody-secreting plasma cells and memory cells leading a 'humoral' or Th2-type immune response. Memory cells remember the same antigen for future encounters. CD4 T cells, activated CD8+T cells release IFN-Y and IL-2 cytokine and develop into cytotoxic T cells, also called killer T cells. The cytotoxic T cells initiate a cell-mediated immune response through production of cytokines such as IFN-Y. (Wang *et al*., 2019 and Yuseff and Dumenil, 2015 and Giddam *et al*., 2012).

The results of challenge experiment proved otherwise when both vaccinated and control birds were exposed to velogenic challenge ND virus at 40 day ,{ 20 days (3 wk) second P.V.} through oral rout , 90 % of ULEND and 80% of OND vaccinated birds resisted the challenge, While all control birds succumbed and died, post-challenge signs observed among the unvaccinated birds and the lesions observed post-mortem of deceased chickens were identical those described for ND (Gordon and Jordan, 1988 and Echeonwu *et al*., 2008).

The basis of immunity against NDV is circulating antibodies and cell-mediated immunity. If the stimulating of B-cell was strong it can be enough to elicited T-cell independent induction of IgM antibodies. (Firouzamandi *et al*., 2016).

The results of birds protection was explained by reports of some previous workers (Jayawardane and Spradbrow, 1995ab; Spradbrow, 1992) that following the administration of challenge or vaccine virus by the natural route of infection: oral and/or respiratory, the aim of antibody intervention would be mucosal (secretory) rather than serum. In addition, the local mucosal immune response plays an important role in developing protection for chickens vaccinated against NDV by inhibiting virus replication at the portal of entry for the virus (Ghumman and Bankowski, 1976 and Takada and Kida, 1996).

Apart from serum and secretory antibodies, cell-mediated immunity (CMI) has been reported to contribute to resistance of vaccinated birds to challenge with velogenic viruses, it is believed that basically mucosal immunity and some level of CMI may have been responsible for the protection of the vaccinated birds recorded in this study (Echeonwu *et al*., 2008).

Cell-mediated immune response can be detected as early as 2–3 days post-vaccination with NDV vaccines (Reynolds and Maraqa, 2000 and Ghumman and Bankowski, 1976), So it may be essential for virus clearance (Russell *et al.,* 1997), show that T cells but not B cells may, therefore, be essential for virus clearance, and principally CD8_cytotoxic T cells may be key players in vaccines immunity to NDV. Cytotoxic T cells were detected in the spleen of vaccinated chickens after in vitro re-stimulation (Cannon and Russell, 1986) or in chickens vaccinated twice or vaccinated and challenged with virulent virus (Jeurissen et al., 2000). even though the specific cell-mediated immunity is not sufficient by itself to protect against virulent NDV (Reynolds and Maraqa, 2000). Some workers (Zhao *et al.,* 2012) reveal to in spite of increase level of intestinal Ig A antibodies concentration and reach to 5 weeks of bird after vaccination, but the protection was may be attributed to Ig M, which supports the previous findings that IgM (not IgG or IgA) may be the class of antibody that is most actively involved in the clearance of NDV infection (Russell and Ezeifeka, 1995 and Ewert and Eidson, 1977).

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