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Evaluation of Inactivation Efficacy of Sabin Polio Virus Using Different Inactivating Agents and its Immunogenicitypost Nano and Micro Encapsulation

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Abstract: The present study aimed to prepare an inactivated Sabin derived Polio Vaccine (sPV), using different inactivating agents [beta-propiolactone(BPL), ascorbic acid (AA) and ultraviolet light (U.V)] as substitute to formaldehyde for safe and effective immunization against poliomyelitis. Monitoring of inactivation kinetics using Vero cell line indicated that BPL showed the highest response as it was capable of inactivating viruses in 40 minutes at 37°C, while, other inactivants require 55 hr (AA) and 80 hr (U.V) for the same degree of inactivation under the same conditions without critical loss in antigenicity. Swiss mice was used to evaluate nanoparticulate adjuvant composed of calcium phosphate (CAP) compared to the commonly used aluminum phosphate (ALUM) adjuvants for its ability to stimulate immunoregulatory responses and related histopathological changes. Results indicated that CAP was more potent as an adjuvant, in addition it elicited little tissue inflammations which ceased completely by the 4th week, while inflammations accompanied to the use of Alum persisted for 8 weeks. A markedly higher degree of antigenicity was demonstrated with beta-propiolactone-inactivated CAP adsorbed polio virus vaccine than other vaccine candidates.

Key words: Polio Vaccine · Calcium phosphate nanoparticles · Immunity · Pathology

INTRODUCTION

Poliovirus, the causative agent of poliomyelitis, is a human enterovirus and member of family Picornaviridae [1]. The first inactivated virus vaccine was developed in 1952 by Jonas Salk, prepared by formalin inactivation of three Polio virulent strains, Mahoney or Brunenders (PV1),MEF-1/Lansing (PV2),and Saukett/Leon (PV3) grown in Vero cell line Subsequently, Albert Sabin developed another attenuated strains of the three serotypes which introduces mutations in the viral internal ribosome entry site (IRES) and hinders the ability of the virus to infect nervous system tissue [2]. It became obvious that the emergence of large populations of unvaccinated individuals following oral polio vaccine (OPV) cessation could risk re-starting a global polio pandemic caused by either vaccine derived polio virus(VDPV), wild-type polioviruses, or recombinant DNA virus [3], The possibility of using live-attenuated strains for the production of inactivated poliovirus vaccines (IPV) seems like an ideal option in the context of the current effort for the global eradication of wild polio [4]. An adjuvant is an agent that may stimulate the immune system and increase the response to a vaccine, without having any specific antigenic effect itself, some of which are inorganic such as ALUM that carry the potential to augment immunogenicity [5]. A CAP nanoparticle ranging from 1.0 to 1000 nm was documented to produce strong T-helper (Thl T-cell) associated and mucosal immunoglobulin-A(IgA) immunity and lower IgE response, in strong contrast to aluminum adjuvants which generally trigger production of IgE antibody and produce local irritation at the site of injection in animal experiments and human clinical trials [6].

The present study aimed to prepare a vaccine consist of inactivated Sabin polio serotypes (sPV) using different inactivants also evaluation CAP / Alum adjuvated polio vaccine candidates concerning immune potential relatively to the route of administration and related histopathological changes.

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MATERIALS AND METHODS

Vero Cells and Polio Virus Subtypes: Vero cells and live attenuated polio virus serotypes (1, 2 and 3) were kindly supplied from Cell Culture Department and Regional Laboratory for Enteric Viruses' Diagnosis Center, VACSERA-Egypt. Growth Medium (M-199) was decanted from 175cm SA tissue culture flasks (NUNC, Roskilde, Denmark) containing confluent monolayers of Vero Cells (ATCC-CCL-81) of cell concentration (~150×10^3cell/ml), 5ml of M-199 (GIBICO) containing 5% fetal calf serum (FCS)were added, infected with sPV serotypes as 1:100 final dilution for one hour at 37°C with periodic shaking to allow virus attachment, the remaining volume was removed and 60 ml of (MEM with 1% FCS was inoculated and incubated at 37°C in CO_2 incubator (jouan-france) and were daily observed for detection of cytological damage/cytopathic effect (CPE). Bottles showing 90% CPE were subjected to three cycles of Freezing and thawing to gather cell free and cell associated polio viruses; extracted harvest were filtered using 0.22µm membrane filter (Millipore-USA) [7] polio virus serotypes were titrated using 96 well tissue culture well plates, virus infectivity titer were calculated according to Reed and Munech [8].

Inactivation of sPV Using Chemical Inactivants: sPV serotypes were subjected to 0.0035M BPL, 0.5M AA catalyzed with 0.1M Copper sulphate) (sigma-Aldrich) and UV light, where sPV serotypes were individually dispensed in glass dishes supplied with magnetic stirrer, kept on ice bath to avoid external heat effect and subjected to U.V lamp of wave length (7nm and 2.84 W/cm^2). Inactivated sPV serotypes were collected, 10 fold serially diluted 10^{-1}-10^{-8} for determination of residual live virus titer. Growth medium was discarded, dispensed as 0.1 ml of each dilution in 6 wells of 96-well plates (Dynatech,USA), (including negative control), sealed and kept at 37°C in CO_2 incubator (Jouan-france) with daily microscopic recording of the infectivity titer depletion / hr according to Reed and Muench [8].

Vaccine Formulation: Suspension of non adsorbed inactivated sPV serotypes were mixed to contain 10^6 $\log_{(10)}$ P1, 10^5 $\log_{(10)}$ P2 and $10^{5.47}$ $\log_{(10)}$ P3.

Preparation of Adjuvant

Calcium Phosphate Nanoparticle Adjuvant: Solution A was prepared at room temperature by the rapid dissolution of 18.36 g Na_2HPO_4-$12H_2O$; 12.5 g NaOH; 7.5 g $NaHCO_3$ in 325 ml of distilled water. Solution B was prepared at room temperature by rapid dissolution of 10.75 g Ca $(NO_3)_2$ $4H_2O$ in 125 ml of distilled water, both solutions were sterilized. Vaccinal candidates were homogenized in a 0.07 M dibasic sodium phosphate sterile solution. The Vaccine suspension was mixed with solution A prior to mixing to solution B. The precipitate of gel-like amorphous calcium phosphate thus formed was aggitated for approximately 30 seconds. PH determined to be 13.5 [9].

Aluminum Phosphate (Alum) Adjuvant: 0.63 M of $AlCl_3.6H_2O$ and 0.3 M of $Na_3PO_4.12H_2O$ were individually dissolved in 40 ml distilled water, filtered using 0.22 µm millipore, stirred continuously during at 40 to 60 rpm. 0.3 M $Na_3PO_4.12H_2O$ solution was added to a mixing bottle followed by 300 ml sterile distilled water, the vaccine suspension was added followed by addition of 0.63 M $AlCl_3.6H_2O$ solution to the mixing bottle. During which, pH was maintained between 6.5-6.8. Adjust to the final volume one liter with sterile distilled water and Mixed for 2 hr at 37 °C [10].

Scanning of Adjuvant Particle Size and Morphology: JEOL-JEM-1230 Transmission electron microscope (TEM) was used for verification particle size and morphological feature of prepared Alum and CAP adjuvants.

Toxicity of Adjuvants: 20 g male Swiss mice groups were kindly supplied from Helwan Animal House-VACSERA-Egypt. Webster Mice were fed on pelted chow, Mice used in the present study according to the Animal Care and Use Committee (ACUC). Prepared CAP and Alum (1.2 mg CAP nanoparticles and 1.32 mg aluminum phosphates gel) and Normal saline (negative control was included) were administered to three separate groups (10 mice/group) as 0.5 ml intraperitonealy. Following 14-day observation, tissue (liver, kidney and spleen) biopsies were dissected at 24, 72 h and 1, 4 and 8 weeks post immunization, fixed in 10% neutral-buffered formalin, embedded in paraffin, sectioned at 5 μm and stained with hematoxylin and eosin for evaluation of histopathological changes [7].

Immunization of Mice: BPL, AA and UV light inactivated sPV serotypes either CAP/ALUM adsorbed or non adsorbed vaccinal batches were used to immunize experimental animal 19groups (10 each/ cage) adsorbed vaccines were administered both S.C and I.M in the Right and Left thighsas (0.25 ml / mouse). Formalin inactivated standard vaccine (IMOVAX POLIO) was included as a positive control group. Blood samples were collected from retro orbital sinus at 2 week intervals. Sera samples were

separated by cold centrifugation (K 42 Jouan-France) for evaluation of Optical densities (OD) representing to antibody level (Ab) using sandwich ELISA. where sPV serotypes were used to coat ELISA plate in 1 M Carbonate-bicarbonate buffer pH 9.6 individually as 1/100 final dilution. Plates were incubated at room temperature for 18 hrs. Plates were blocked with 4% BSA (Sigma Aldrich) for 1 hr at 37°C, Blocking buffer was decanted and plates washed 3 times (PBS-Tween-20). 1/50 diluted sera samples were1 log $_{(2)}$ diluted. Sera samples were dispensed in the reciprocal wells and plates were incubated for 1hr and anti-mouse conjugate (Sigma-Aldrich-USA) labeled with peroxidase enzyme used as 1/1000 final dilution. Plates were incubated for an hour at 37°C. reacting materials were washed as previous and substrate was dispensed as 0.1 ml / well and plates kept in the dark for 10 minutes.

Reaction was stopped using 2 N HCl (Sigma Aldrich-USA) OD was measured using ELISA reader (Dynatech-MRX) at wave length 450 nm [11,12].

Statistical Analysis: Data were analyzed for statistical significance by a one-way analysis of variance (ANOVA).A P value of <0.05 was considered significant in all parameters.

RESULTS

Toxicity of Chemicals Inactivates: Ascorbic acid (1.5 mg/ ml), 0.0035M Beta-propiolactone was tested for toxicity in mice. It was clear that both ascorbic acid and Beta-propiolactone were completely safe to mice as no mortality detected 7 days post injection.

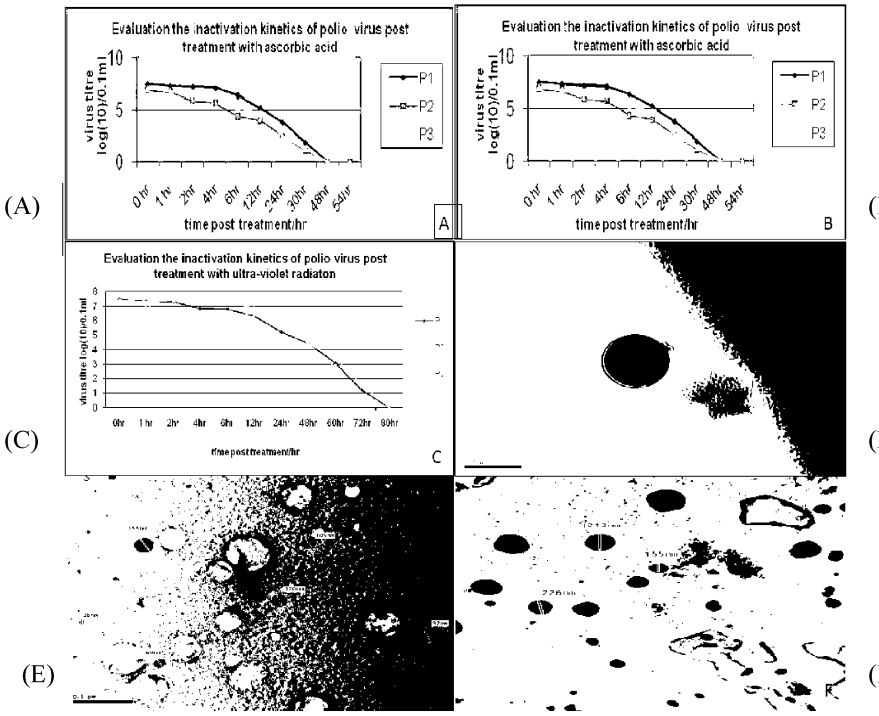

Fig. A: Evaluation the inactivation Kinetics of poliovirus Post treatment with ascorbic acid using.5 mg/ml Final Concentration in cell culture

Fig. B: Evaluation the inactivation kinetics of polio Virus post treatment with 0.0035 M Beta-propiolactone in cell culture

Fig. C: Evaluation the inactivation kinetics of polio virus post treatment with ultra-violet radiation in cell culture.

Fig. D: Showing transmission electrophotomicrograph of nanoparticle Calcium phosphate adjuvant exhibiting spherical double Membrane wall. The bar represents 0.2 µm.

Fig. E: Showing transmission electron photomicrograph of nanoparticle Calcium phosphate adjuvant of particle size˜ 80 nm. The bar represents 0.5 µm

Fig. F: Showing transmission electron photomicrograph of aluminum Phosphate adjuvant of particle size ˜ 208 nm. The bar represents 1µm.

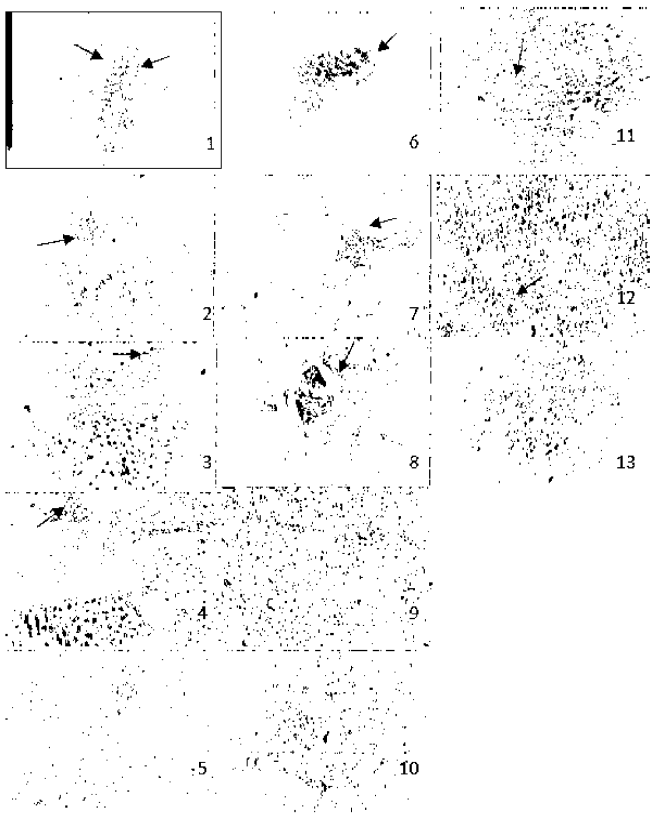

Fig. 1: Liver section of mice treated with aluminum phosphate adjuvant (24 hr) showing portal infiltration with leukocytes (H and E x 200).

Fig. 2: Liver section of mice treated with aluminum phosphate adjuvant (72 hr) showing focal area of hepatic necrosis replaced by leucocytic cells infiltration (H and E x 200).

Fig. 3: Liver of mice treated with aluminum phosphate adjuvant (1 week) showing massive leucocytic cells infiltration in the portal triad (H and E x 200).

Fig. 4: Liver of mice treated with aluminum phosphate adjuvant (4 weeks) showing recent thrombus as well as portal infiltration with leucocytic inflammatory cells (H and E x 200).

Fig. 5: Liver of mice treated with aluminum phosphate adjuvant (8 weeks) showing small focal hepatic necrosis associated with leucocytic cells infiltration (H and E x 200).

Fig. 6: Kidney of mice treated with aluminum phosphate adjuvant (24 hr) showing congestion of renal blood vessels associated with focal leucocytic cells aggregation (H and E x 200).

Fig. 7: Kidney of mice treated with aluminum phosphate adjuvant (72 hr) showing focal area of tubular necrosis completely replaced by leucocytic cells infiltration (H and E x 200).

Fig. 8: Kidney of mice treated with aluminum phosphate adjuvant (1 week) showing thickening of the partial layer of Bowman's capsule (H and E x 200).

Fig. 9: Kidney of mice treated with aluminum phosphate adjuvant (4 weeks) showing vaculation of epithelial lining renal tubules together with pylenosis of their nuclei (H and E x 200).

Fig. 10: Kidney of mice treated with aluminum phosphate adjuvant (8 weeks) showing cellular cast in the lumen of some renal tubules (H and E x 200).

Fig. 11: Spleen of mice treated with aluminum phosphate adjuvant showing lymphocytic depletion associated with appearance of multiple megakaryocytes (H and E x 200).

Fig. 12: Spleen of mice treated with aluminum phosphate adjuvant (4 weeks) showing lymphocytic depletion associated with appearance of multiple megakaryocytes (H and E x 200).

Fig. 13: Spleen of mice treated with aluminum phosphate adjuvant (8 weeks) showing lymphocytic necrosis and depletion (H and E x 200).

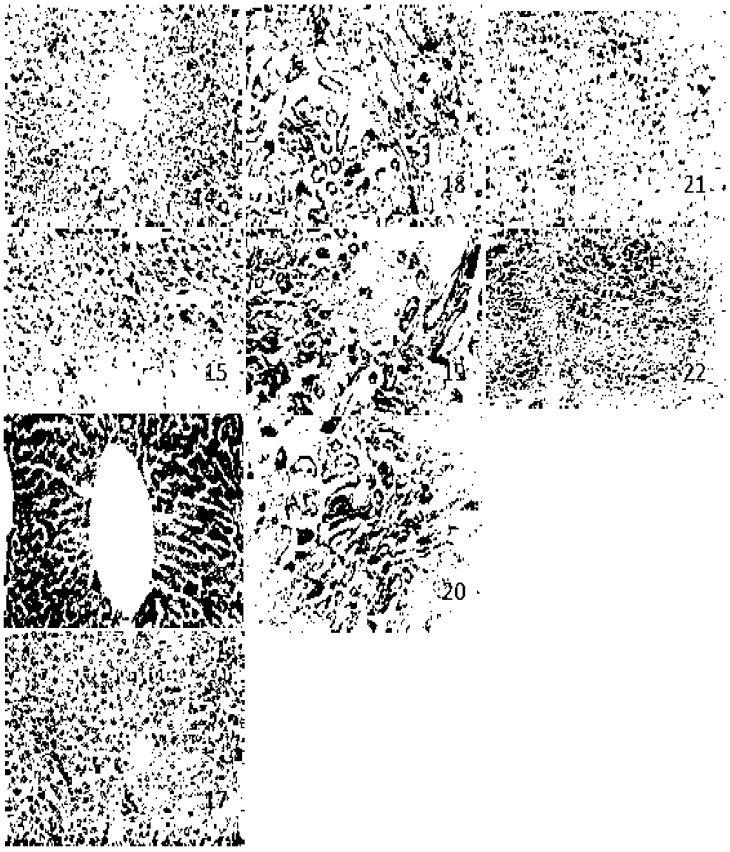

Fig. 14: Liver of mice treated with calcium phosphate nanoparticle adjuvant (24 hr) showing degeneration of hepatocytes (H and E x 200).

Fig. 15: Liver of mice treated with calcium phosphate nanoparticle adjuvant (72 hr) showing multiple small focal areas of hepatic necrosis associated with leucocytic cells infiltration (H and E x 200).

Fig. 16: Liver of mice treated with calcium phosphate nanoparticle adjuvant (1 week) showing small vacuoles in the cytoplasm of some hepatocytes (H and E x 200).

Fig. 17: Liver of mice treated with calcium phosphate nanoparticle adjuvant (4 weeks) showing kupffer cells activation. (H and E x 200).

Fig. 18: Kidney of mice treated with calcium phosphate nanoparticle adjuvant (24 hr) showing congestion of intertubular blood capillaries (H and E x 200).

Fig. 19: Kidney of mice treated with calcium phosphate nanoparticle adjuvant (72 hr) showing focal renal hemorrhage. (H and E x 200).

Fig. 20: Kidney of mice treated with calcium phosphate nanoparticle adjuvant (1week and 4 weeks) showing apparent normal renal parenchyma (H and E x 200).

Fig. 21: Spleen of mice treated with calcium phosphate nanoparticle adjuvant (24 hr and 72 hr) showing no histopathological changes (H and E x 200).

Fig. 22: Spleen of mice treated with calcium phosphate nanoparticle adjuvant (1 week and 4 weeks) showing no histopathological changes (H and E x 200).

Inactivation of Polio Virus Using Chemical Inactivates: Polio virus stock prepared as three separate aliquots each sPV serotype was grown individually on Vero cell line(clone,CCL-81) and inactivated either with 0.0035M BPL, AA (1.5mg / ml) and U.V. Data recorded post treatment revealed that polio virus serotypes were completely inactivated using AA 54 hrs post treatment recording a mean depletion of virus infectivity titer for P-1,P-2 and P-3 in the order of 0.42,0.273 and 0.267 log (10)/hr $TCID_{50,}$ respectively Figure (A), In the meanwhile

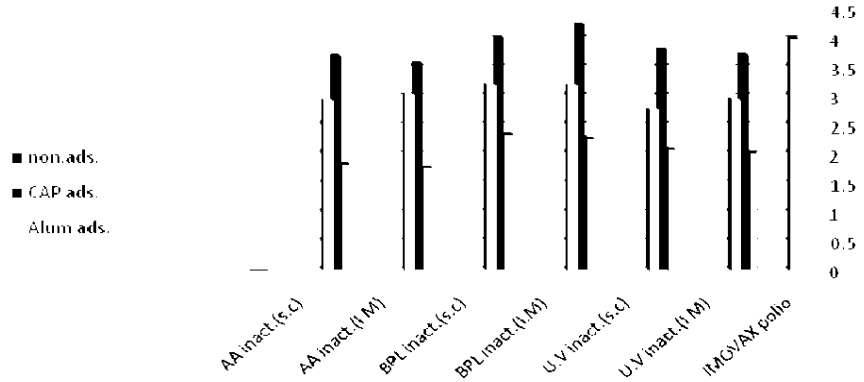

Fig. 23: Evaluation of antibody level post vaccination with CAP/Alum adsorbed or non adsorbed sPV serotypes using ELISA

polio virus serotypes inactivated in shorter time 40 minutes BPL treatment recording a mean depletion of virus infectivity titer for P-1,P-2 and P-3 in the order of 0.547,0.273 and 0.505log (10)/ hr $TCID_{50}$ respectively Figure (B).Finally ultraviolet radiation (U.V) showed the longest time whereas no virus detected 80hrs post treatment recording a mean depletion of virus infectivity titer for P-1, P-2 and P-3 in the order of 0.368,0.522 and 0.49 log (10)/ hr $TCID_{50}$ respectively as shown in Figure (C). Prepared adjuvants were monitored using transmission electron microscope. Figure (D) and Figure (E) Showed photomicrograph of calcium phosphate Nanocapsule adjuvant which exhibits spherical double membrane wall structure having particle size ˜ 80 nm, Vaccine is entrapped in the core and adsorbed onto the exterior. While aluminum phosphate adjuvant revealed spherical particles having particle size ˜ 208 nm as seen in Figure (F).

DISCUSSION

Poliovirus, the causative agent of poliomyelitis, is a human enterovirus and member of the family of Picornaviridae [13]. Researchers developed two polio vaccines inactivated (IPV) and live attenuated (OPV) grown in cultures made from monkey kidneys [14],Because OPV is inexpensive, easy to administer, and produces excellent immunity in the intestine (which helps prevent infection with wild virus in areas where it is endemic), it has been the vaccine of choice for controlling poliomyelitis in many countries[15],Until the first discovery of circulating vaccine-derived polioviruses (cVDPV) occurred in 2000 during investigation of a polio outbreak in the Dominican Republic and Haiti that was caused by a recombinant between a derivative of Sabin 1 strain and an unidentified enterovirus [16]. However, ensuring containment of wild-type polioviruses, from which the current IPV is made, raises major concerns. Thus, development of IPV from non-pathogenic strains becomes a top priority, it would cost less, increase efficacy, and alleviate biosecurity concerns [17], However, the antigenic properties and immunogenicity of sIPV appear different from conventional IPV [18], because the protective antigens of Sabin viruses are significantly less stable upon treatment with formaldehyde [19, 20].There are four possible solutions to the problem of lower immunogenicity of Sabin IPV. First would be to increase antigen content to the level that would ensure adequate seroconversion. However, this would require growing greater quantities of virus, which would work against cost reduction; a second approach might be to stimulate immunogenicity with adjuvants. A third solution could be to explore use of alternative inactivating agents [21] that do not damage antigens as much as formaldehyde, For example BPL used in making rabies vaccine, can rapidly inactivate poliovirus without damaging its protective antigens [22]. Finally, the fourth solution would be to make IPV from strains that have an antigenic structure identical to the currently used by Partial disruption within the 5'-non-coding region of polio RNA which is one of the important determinants of neurovirulence of poliovirus by substitution by weaker base pairs [23].

In the present study vaccine candidates were inactivated using different inactivating agents (AA, BPL and U.V), whereas Ascorbic acid is a water soluble and pre-eminent scavenger of oxygen radicals that undergoing auto oxidation catalyzed by cupric ions to inactivate vaccinial viruses [24]. Ascorbate enhances several functions of the immune system as the production of

interferon, which helps prevent cells from being infected by a virus [25].1.5 mg/ml final concentration AA catalyzed by cupric ions, was monitored and data revealed complete inactivation of polio virus within 54 hrs recording a depletion of virus infectivity titer of P1, P2&P3 in the order of (0.42 log (10)/ hr $TCID_{50}$, 0.273 log (10)/ hr $TCID_{50}$, 0.2675 log (10)/ hr $TCID_{50}$) ,However, Ascorbic acid used at pH 5, could not inactivate sPV due to deproteinization of medium proteins as NaOh was used for pH adjustment. The presented data were considered with many studies carried out by Breindl [26] who stated that the inactivation of Po-1 was greater at pH 5.0 than at pH 7.0 in the presence of 11.2 mM ascorbic acid, At pH 7.0 there was a greater loss of infectivity of Po-1 in 11.2 mM than in 4.5 mM ascorbic acid that mean the inactivation was Ph and Concentration dependent .

Beta-propiolactone is a monoalkylating agent widely used for inactivation of viruses in the preparation of many vaccines which can conveniently be removed by heating, to cause hydrolysis into the non-toxic beta.-hydroxypropionic acid. it causes structural modification by alkylation and depurination of nuclic acid and is capable of inactivating viruses in 10–15 minutes at 37 C whereas formalin and phenol require days for the same degree of inactivation under parallel conditions, a significantly higher degree of antigenicity is demonstrated with BPL -inactivated virus vaccines than with formalin- and phenol-inactivated virus vaccines [27].The inactivation efficacy of 0.0035M BPL revealed that BPL could inactivated sPV serotypes within 40 minutes recording a depletion of virus infectivity titer of P1, P2&P3 in the order of (0.54 log (10)/ hr $TCID_{50}$, 0.273log (10)/ hr $TCID_{50}$, 0.505 log (10)/ hr $TCID_{50}$) .In study using BPL for inactivation of polio serotypes, D-antigen recoveries found to be high (88, 88 and 60%, respectively) but were significantly less when formalin was used (22, 15 and 25%). beta-Propiolactone inactivated virus was purified, combined with Freund's adjuvant and used to hyper immunized rabbits. High titers (50 000-200 000) of specific neutralizing antibody were obtained [22].

U.V inactivates viruses by altering their genome or capsid, In both DNA and RNA viruses [28], At 254 nm, loss of viral infectivity could thus be associated with formation of photoproducts and could affect protein integrity or conformation by breaking disulfur (S-S) bonds or creating cross-links between proteins and the genome [29].Finally, Polio virus serotypes were exposed to U.V of Wavelength 253.7 nm, 13.4 W, 0.355A &2.84W/cm^2, data revealed that U.V inactivated polio virus showing complete inactivation within 80 hr. recording a depletion of virus infectivity titer of P1, P2&P3 in the order of (0.368 log (10)/ hr $TCID_{50}$, 0.522 log (10)/ hr $TCID_{50}$, 0.45log (10)/ hr $TCID_{50}$) .Several studies were conducted on the inactivation effect of ultraviolet on polio virus which states that the U.V dose to inactivate 90% of PFU ($1log_{10}$of inactivation) of PV/milliliter is about 0.96 mW.s/cm^2 (9.6 J/m^2) in clear suspending medium [30]. A study concerning exposure of poliovirus type 1 (PV-1) to UV indicated that viral RNA is a primary target of UV and hypochlorite inactivation and that the sole target of thermal inactivation is the viral capsid [31].

Concerning the adjuvants used in the present study (CAP, ALUM) results revealed that tissue reactions caused by injection of a CAP adjuvant completely ceased by the 4th week, while irritation caused by ALUM persisted for 8 weeks This study agreed with Goto *et al.* [32]. Qing He also reported that CAP adjuvant, in comparison with alum adjuvant, induced a lower level of IgE, decreased the level of local irritation in experiments with animals, and caused fewer variations in human clinical trials [33, 34]. Toxicity and animal studies confirmedthat only minor inflammation occurred at the injection sites in guinea pigs during the first 2 weeks and that CAP did not elicit IgE responses. Moreover they found that the most important consideration is that CAP nanoparticles enhance the immune response to viral antigens. Data strongly suggest that CAP nanoparticles significantly enhance the antibody response to Herpes simplex virus HSV-2 antigen, which is normally poorly immunogenic. The level of protection against an intravaginal HSV-2 infection after immunization with different HSV-2 vaccine formulations was compared and total of 40% of non immunized mice survived a low-dose (10^3-PFU) challenge. In contrast, 100% protection was observed in the group of mice immunized with HSV-2 proteins containing adjuvants. The group of mice treated with HSV-2 plus CAP or HSV-2 plus alum showed comparable neutralizing antibody titers that may have protected the animals from low-dose infection. Particularly high IgG2a titers were observed in the mice immunized with HSV-2 and EBV proteins containing CAP adjuvant but not in mice immunized with HSV-2 and EBV proteins with alum adjuvant or without adjuvants [34].

It was concluded that the total antibody response of I.M followed by S.C administered-BPL inactivated-CAP adsorbed sPV seems equivalent to the reference vaccine IMOVAX POLIO also showing highest immune response recording4.253&4.026 respectively. Regarding inactivation

kinetics of sPV serotypes was faster in case of BPL followed by AA and finally U.V, recording 40 minutes, 54 hrs&80hrs respectively, The presence of adjuvant showed marked increase in antibody level meanwhile the presence of calcium phosphate nanoparticle in vaccine models showed better immune response followed by ALUM where none adsorbed vaccines showed the lowest antibody level.

REFERENCES

1. Ryan, K.J. and C.G. Ray, 2004. "Enteroviruses", Sherris Medical Microbiology, 4th ed., McGraw Hill, pp: 535-7.
2. Dowdle, W.R., D.E. Gourville, O.M. Kew, M.A. Pallansch and D.J. Wood, 2003. Polio eradication: the OPV paradox. Rev. Med. Virol., 13(5): 277-91.
3. Cello, J., A. Paul and E. Wimmer, 2002. Chemical Synthesis of Poliovirus cDNA: Generation of Infectious Virus in the Absence of Natural Template, doi:10.1126/Science,1072266 .
4. Doi, Y., S. Abe, H. Yamamoto, H. Horie, H. Ohyama, K. Satoh, Y. Tano, Y. Ota, M. Miyazawa, K. Wakabayashi and S. Hashizume, 2001. Progress with inactivated poliovirus vaccines derived from the Sabin strains. Dev. Biol., 105: 163-169.
5. Clements, C. and E. Griffiths, 2002. "The global impact of vaccines containing aluminum adjuvants". Vaccine, 20(Suppl) 3: S24-33.
6. Ickovic, M.R., E.H. Relyveld and E. Henocq, 1983. Calcium phosphate adjuvanted allergens. Total and specific IgE levels before and after immunotherapy with house dust and mite extracts. Ann. Immunol., 134D: 385-398.
7. Gujral, J.S., T.J. Bucci, A. Farhood and H. Jaeschke, 2001. Mechanism of cell death during warm hepatic ischemia-reperfusion in rats. Hepatol., 33: 397-405.
8. Reed, L.T. and H.A. Muench, 1938. Simple method of calculating fifty percent end point. Am. J. Hyg., 27: 493-8.
9. Johnson, S., C. WagnerBartak, T. Morcol and S. Bell, 2000. Calcium phosphate nanoparticle adjuvant. Clin. Diagnostic Laboratory Immunol., pp: 899903.
10. Gupta, R.K., 1998. Aluminium compounds as vaccine adjuvants. Adv. Drug Delivery Rev., 32: 155-172.
11. El-Karamany, R., I. Imam and A. Farid, 1981. Production of inactivated RVF vaccine. J. Egypt Publ. Health Assoc., 56: 495-525.
12. Mohamed, A., H. El-Kaschef, M. Reda, M. Tantawey and A. Hassen, 1996. Biological studies for the importance of Rift Valley Fever Vaccine. ph.D thesis, Suez Canal Univ. Zool. Depart.
13. Rümke, H., J. Labadie, N. Elzinga-Gholizadea and T. Mensinga, 1998. IPV-Vero Vaccine Induces a Strong Booster Reaction and is Well Tolerated in Adults, pp: 535-538.
14. Miller, N., 2004. The polio vaccine: a critical assessment of its arcane history, efficacy and long-term health-related consequences, pp: 239-251.
15. American Academy of Pediatrics Committee on Infectious Diseases"."Poliomyelitis prevention: recommendations for use of inactivated poliovirus vaccine and live oral poliovirus vaccine. Pediatrics, 99(2): 300-5.
16. Kew, O., V. Morris-Glasgow, M. Landaverde, C. Burns, J. Shaw, Z. Garib, J. André, E. Blackman, C. Freeman, J. Jorba, R. Sutter, G. Tambini, L. Venczel, C. Pedreira, F. Laender, H. Shimizu, T. Yoneyama, T. Miyamura, H. van der Avoort, M. Steven Oberste, D. Kilpatrick, S. Cochi, M. Pallansch and C. de Quadros, 2002. Outbreak of poliomyelitis in Hispaniola associated with circulating type 1 vaccine-derived poliovirus, Science, 5566(296): 296.356-9.
17. Dragunsky, E.M., A. Ivanov, V. Wells, A. lyshina, G. Rezapkin, S. Abe, S. Potapova, J. enterline, S. Hashizume and K. chumakoy, 2004. Evaluation of immunogenicity and protective properties of inactivated poliovirus vaccines. The J. Infectious Diseases, 190(8): 1404-12.
18. Rezapkin, G., J. Martin and K. Chumakov, 2005. Analysis of antigenic profiles of inactivated poliovirus vaccine and vaccine-derived polioviruses by block-ELISA method. Biologicals, 33(1): 29-39.
19. Tano, Y., H. Shimizu, J. Martin, Y. Nishimura, B. Simizu and T. Miyamura, 2007. Antigenic characterization of a formalin-inactivated poliovirus vaccine derived from live-attenuated Sabin strains. Vaccine, 25(41): 7041-6.
20. Simizu, B., S. Abe, H. Yamamoto, *et al.*, 2006. Development of inactivated poliovirus vaccine derived from Sabin strains. Biologicals, 34(2): 151-4.
21. Chumakov, K. and E. Ehrenfeld, 2008. New Generation of Inactivated Poliovirus Vaccines for Universal Immunization After Eradication of Poliomyelitis. Clin. Infect. BMJ., doi: 10.1136/bmj.a2809 1:1.

22. Jiang, S.D., D. Pye and J.C. Cox, 1986. Inactivation of poliovirus with beta-propiolactone. J. Biol. Standardization, (2): 103-9.
23. Macadam, A.J., G. Ferguson, D.M. Stone, *Et al.,* 2006. Rational design of genetically stable, live-attenuated poliovirus vaccines of all three serotypes: relevance to poliomyelitis eradication. J. Virol., 80(17): 8653-63.
24. Madhusudana, S., R. Shamsundar and S. Seetharaman, 2002. *In vitro* inactivation of the rabies virus by ascorbic acidInt. J. Infect. Dis., 8: 21-25.
25. Karpinska, T., Z. Kawecki and M. Kandefer-Szerszen, 1982. The influence of ultraviolet irradiation, L-ascorbic acid and calcium chloride on the induction of interferon in human embryo fibroblasts, 30: 33-37.
26. Brindl, M., 1988. The Structure of Heated Poliovirus Particles J. gen. Virol. IX, I47-156 McBean, A.M and Etal. Serologic response to oral polio vaccine and enhanced-potency inactivated polio vaccines. Am. J. Epidemiol., 128: 615-628.
27. Schaff, K., 1959. Avian Encephalomyelitis Immunization with Inactivated Virus, 3: 245-256.
28. Durbeej, B. and Eriksson, 2003. On the formation of cyclobutane pyrimidine dimmers in UV-irradiated DNA., 78: 159-167.
29. Simonet, J. and C. Gantzer, 2006. Inactivation of Poliovirus 1 and F-Specific RNA Phages and Degradation of Their Genomes by UV Irradiation at 254 Nanometers. Dec., 72(12): 7671-7.
30. Wetz, K. and K.O. Habermehl, 1982. Specific cross-linking of capsid proteins to virus RNA by ultraviolet irradiation of poliovirus. J. Gen. Virol., 59: 397-401.
31. Nuanualsuwan, S. And D. Cliver, 2002. Infectivity of RNA from Inactivated Poliovirus, 69(3): 1629-1632.
32. Paul, J.R., 1971. A History of Poliomyelitis. (Yale studies in the history of science and medicine). New Haven, Conn: Yale University Press, pp: 16-18 ISBN 0-300-01324-8.
33. Powell, M. and M. Newman, 1995. Adjuvant properties of aluminum and calcium compounds, pp: 229-248.
34. Qing, He., A. Mitchell, S. Johnson, C. Wagner-Bartak, C. Tulin 101. Morcol and J. SteveBell, 2000. Calcium Phosphate Nanoparticle Adjuvant. BioSante Pharmaceuticals, Inc. Smyrna, Georgia 30082.