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Preclinical study of safety and immunogenicity of combined rubella and human papillomavirus vaccines: Towards enhancing vaccination uptake rates in developing countries

Asmaa Gohar[a], Nourtan F. Abdeltawab[b,*], Nahla Shehata[a], Magdy A. Amin[b]

[a] Biotechnology Control Unit, National Organization of Research and Control of Biologicals, Cairo, Egypt
[b] Department of Microbiology and Immunology, Faculty of Pharmacy, Cairo University, Cairo, Egypt

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ABSTRACT

Rubella vaccine was not part of national immunization programs (NIP) in several countries in the Middle East and North Africa (MENA), South-East Asia (SEA), and South Africa regions until the year 2000. Therefore, immunization coverage of females older than 20 years old in these countries has been the focus of national campaigns for rubella elimination in developing countries. Vaccines against human papillomavirus (HPV) are not part of NIPs in developing countries. To enhance the advantages of rubella-directed immunization campaigns and to increase HPV vaccine uptake in developing countries, this study aimed to test the stability, potency, efficacy and safety of a combined rubella and HPV vaccine. Female BALB/c mice were immunized subcutaneously with proposed combined HPV16/HPV18 VLP and rubella vaccine at weeks (W) 0, 3 then with HPV vaccine at W 7. Immunized mice developed antigen-specific antibodies against rubella and HPV significantly higher than mice immunized with rubella or HPV vaccine alone. The combined vaccine induced significantly higher splenocyte proliferation than control groups. In addition, pro-inflammatory cytokines IL-4, IL-6, IL-2, and IFNγ levels were significantly higher in mice immunized with the combined vaccine than control groups. Overall, the combined vaccine was safe and immunogenic offering antibody protection as well as eliciting a cellular immune response against rubella and HPV viruses in a single vaccine. This combined vaccine can be of great value to females above 20 years old in the SEA, MENA and South Africa regions offering coverage to rubella vaccine and a potential increase in HPV vaccine uptake rates after appropriate clinical testing.

1. Introduction

Women in developing countries experience a shortage in the basic healthcare thus may face life-threatening health issues. Among the main health issues facing women in developing countries is maternal and infant mortality, human immunodeficiency virus (HIV) infection, breast, and cervical cancers [1]. Rubella causes miscarriage, premature delivery, and congenital rubella syndrome (CRS) that can cause fetal retinopathy, cataract, microphthalmia, hearing loss, congenital heart disease and hepatomegaly [2]. Chances of the fetus being affected by rubella are dependent on whether the pregnant mother was infected with rubella before or after conception [3]. Rubella vaccine was FDA approved in 1969 [4] and was introduced as part of combined measles-mumps-rubella (MMR) vaccine in developed countries general immunization programs. However, in most developing countries rubella vaccine was not included in national immunization programs (NIP) until 2000. So, females older than 18 years old in these countries were not immunized with rubella vaccine that may threat their health and their prospective infants. Egypt as a developing country and one of the most populous member countries of the world health organization (WHO) Eastern Mediterranean Region can be considered a model country for studying vaccine implementation. Egypt has achieved significant progress in vaccine preventable disease (VPD) control as it received technical and financial support from Centers for Disease Control and Prevention (CDC) and WHO. It is one of the developing countries with WHO regional office and following surveillance guidelines leading to current statistics on vaccine coverage and uptake rates [5]. Egypt's NIP is one of the most successful programs in the region with national immunization campaigns reaching > 95% coverage of the population. Rubella vaccine was introduced to Egyptian NIP in

* Corresponding author.
E-mail addresses: asma.gohar@yahoo.com (A. Gohar), nourtan.abdeltawab@pharma.cu.edu.eg (N.F. Abdeltawab), lr_egy@yahoo.com (N. Shehata), magdy_biotek3@yahoo.com (M.A. Amin).

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1999 therefore, females older than 20 years old have not received rubella vaccine [5]. In 2002, Egypt established a goal to eliminate measles and rubella and to prevent CRS by 2010, but large-scale rubella and measles outbreaks occurred in 2005–2007 made it difficult for Egypt to achieve the 2010 goals. The Egyptian Ministry of Health (MOH) made a strategy to eliminate rubella and measles by conducting a national catch up immunization campaign in 2008–2009 targeting 36 million children, adolescents and young adults from 2 to 19 years old [6], but females ≥ 20 years old were not included in this campaign and women in child bearing period who were born before 1999 did not get the compulsory vaccine.

Cervical cancer is one of the most common cancers among women worldwide and is highly associated with HPV types 16 and 18 [7,8]. The majority of cervical cancers occur in women in the developing countries, where the accessibility and availabilities of vaccines and preventative screenings such as pap smears are limited [9]. FDA approved HPV vaccines include quadrivalent (HPV types 6, 11, 16, and 18), nonavalent (HPV types 6, 11, 16, 18, 31, 33, 45, 52, and 58), and bivalent (HPV types 16 and 18) vaccines known by trade names Gardasil®, Gardasil9, and Cervarix® respectively [10]. FDA approved HPV immunization in males and females aged 9 through 45 years with continuous research on increasing vaccination rates [11]. Gardasil9 is indicated in males for prevention of anal cancers caused by HPV types 16, 18, 31, 33, 45, 52, and 58 and for prevention of genital warts caused by HPV types 6 and 11. Gardasil9 is also used to prevent anal intraepithelial neoplasia grades 1, 2 and 3. It also showed increased impact compared to the quadrivalent vaccine in preventing HSIL [12]. In addition, bivalent HPV vaccine, Cervarix was shown to have potential to reduce the incidence of grade 3 or greater cervical intraepithelial neoplasia and cervical cancer [13].

In developing countries, HPV vaccine is not part of the routine immunization programs and is regarded as a costly vaccination. Over 25 million women over 15 years old are at risk of developing cervical cancer in Egypt. Moreover, females over 20 years old were not immunized against rubella [6], thus a combination between rubella and HPV vaccines could offer value to increasing vaccination rates of HPV while protecting against anogenital cancers and rubella teratogenicity.

Combination vaccines are considered as one of the solutions to minimize the vaccination cost and increase compliance while offering the same protection as individual vaccines with several successful combination vaccines as DTP and MMR. HPV vaccine is a valuable preventive measure of anogenital cancers, thus, investing in a combined vaccine of HPV with an already successful NIP vaccine, rubella vaccine, can prove valuable in promoting vaccine uptake. Accordingly, protection against cervical cancers with a relatively low public health expenditure compared to cancer treatment expenses. Thus, in this study, we aimed to combine rubella and HPV vaccines into a single vaccine that is safe, effective and potent. We assessed the safety profile and immune responses in a murine mode.

2. Materials and methods

2.1. Vaccines

Bivalent Cervarix ® (HPV types 16 and 18) (GlaxoSmithKline, UK), tetravalent Gardasil® (HPV types 6, 11, 16 and 18) (Merck & Co., USA) HPV vaccines, and rubella reference with a labeled potency of 4 $\log_{10}TCID_{50}$/0.5 mL were obtained through National Organization of Research and control of biologicals (NORCB) (Giza, Egypt). We prepared our proposed combined vaccine by the reconstitution of the lyophilized rubella reference powder with the bivalent HPV vaccine. We used this combined vaccine to assess the potency, stability and abnormal toxicity of the combined vaccine. We diluted the combined vaccine where rubella vaccine was diluted to a dose of 3 $\log_{10}TCID_{50}$/0.5 mL and HPV vaccine was diluted to a dose 2 µg/mL (1/20 the human dose) for animal studies. For cytokines and splenocyte proliferation assay, we used tetravalent HPV vaccine and rubella vaccine.

Table 1
Concentrations of combined vaccine use in MTT splenocyte proliferation assay.

HPV vaccine	Rubella vaccine
2 µg	3 log $TCID_{50}$
2 µg	2 log $TCID_{50}$
2 µg	1 log CCD_{50}
1 µg	3 log $TCID_{50}$
1 µg	2 log $TCID_{50}$
1 µg	1 log $TCID_{50}$
0.5 µg	3 log $TCID_{50}$
0.5 µg	2 log $TCID_{50}$
0.5 µg	1 log $TCID_{50}$

Table 2
Serum biochemical parameters assayed for profiling safety of combined vaccine.

Assay	Combined vaccine (n = 10)	Control (n = 10)	P value
Creatinine (mg/dL)	0.16 ± 0.11	0.13 ± 0.05	0.67
BUN (mg/dL)	12.9 ± 0.35	16.3 ± 0.5	0.02*
Uric acid (mg/dL)	1.7 ± 2	1.6 ± 0.17	0.54
GGT (U/L)	1.33 ± 0.5	1.3 ± 0.57	0.99
GPT (U/L)	37.6 ± 2.08	38.3 ± 1.5	0.67

2.2. Cell culture

Rabbit-kidney (RK-13) cells were purchased from the holding company for biological products and vaccines (VACSERA) (Giza, Egypt). RK-13 cells were cultured in MEM Hanks supplemented with 10% fetal bovine serum (FBS) and% 1% L-glutamine [14].

2.3. Animal experiments

Female BALB/c mice (17–25 g) and guinea pigs (250–350 g) were used for experiments. BALB/c mice (total n = 58) were purchased from Theodor Bilharz Research Institute (Giza, Egypt). Guinea pigs (total n = 4) were purchased from the animal facility of the holding company for biological products and vaccines (VACSERA) (Helwan, Egypt). Animals were housed in accordance with standard laboratory conditions with access to food and water ad libitum, in an environmentally controlled room with 12 h light and dark cycles.

Animal research procedures were conducted in compliance with the principles and recommendations of the National Institutes of Health guide for the care and use of laboratory animals 8th edition (2011). All animal experimental protocols were conducted following regulations and approval of the institutional ethical committee of the Faculty of Pharmacy, Cairo University, Egypt (Protocol number: MI1723).

2.4. In vivo abnormal toxicity test

The test was performed in accordance with European pharmacopoeia monograph 01/2008: 20609, abnormal toxicity, 2005 P. 20609 where two groups of mice weighing 17–22 g, 8–10 weeks old (n = 5 mice/group) and two groups of guinea pigs weighing 250–350 g, (n = 2 guinea pig/group) were inoculated intra-peritoneal with the combined HPV-rubella vaccine at 20µg/0.5 mL and 3 log $TCID_{50}$ of human dose. All the inoculated animals were observed at least twice daily for any signs of ill health for a 7–day observation period.

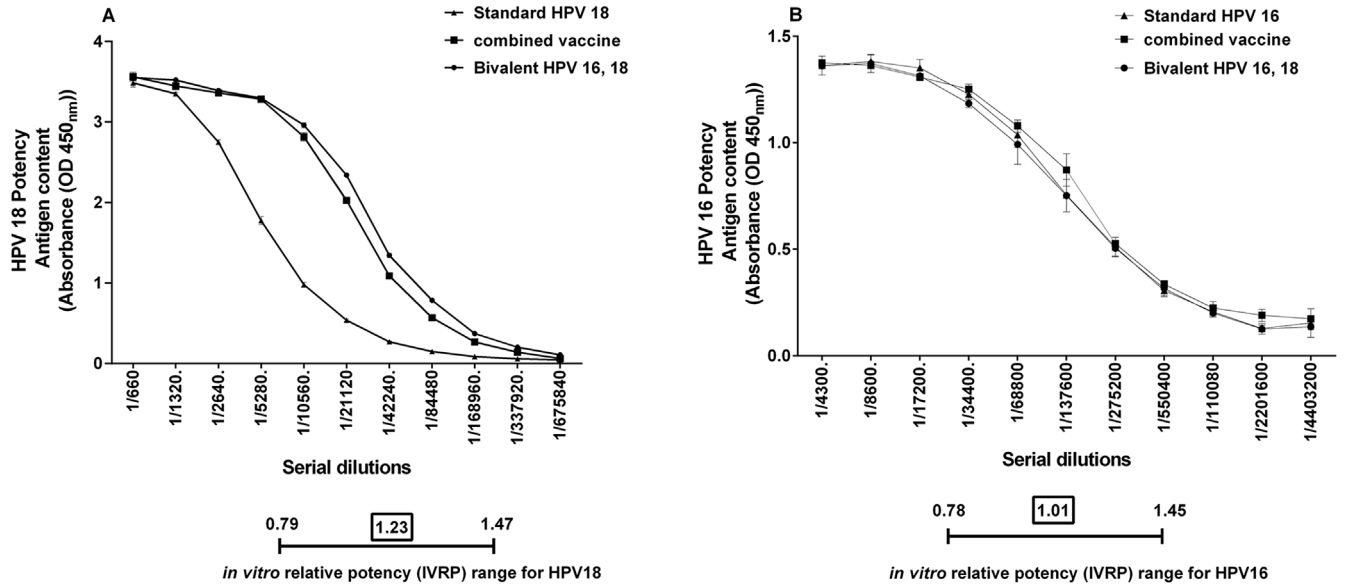


Fig. 1. Combined HPV 16, 18/Rubella vaccine did not affect antigen content as evaluated by *in vitro* relative potency. (A) Serial dilutions of standard type 18 and (B) standard type 16 with bivalent standard and the combined samples were made and tested for antigen content using ELISA immune assay. The *in-vitro* relative potency (IVRP) of combined vaccine was calculated by dividing the estimate concentration of bivalent standard over the estimate concentration of the sample multiplied by factor 1.01 or 0.95 for type 18 and 16 respectively. The results showed that the relative potency of the combined vaccine were within the acceptance criteria (0.78–1.45) for HPV 16 and (0.79–1.47) for HPV 18. The calculations were made using 4 parameters sigmoid model by combistat program. Data represented as means ± SD of at least two independent experiments.

2.5. In vivo evaluation of combined vaccine immunogenicity

Female BALB/c mice, weighing 18–25 g, 8–10 weeks old (n = 10 mice/group) were immunized by subcutaneous injection with different vaccines. First group of mice were immunized with rubella vaccine at a dose of 3 $\log_{10}$ $TCID_{50}$ at weeks 0 and 4. Group two; mice were immunized with bivalent vaccine at a dose 2 μg/mL (1/20 the human dose) at weeks 0, 3 and 7 [15]. For group three, mice were immunized with combined bivalent-rubella vaccine at weeks 0, 3 then at week 7 mice were immunized with HPV alone. For group four, mice were injected with saline that served as a control group. Blood samples were collected from mice two weeks after each immunization, then at week 12 and week 24. At week 24, the mice were challenged by subcutaneous route with 1 μg bivalent standard type 16 and type 18 without adjuvant. The bivalent standard is the bivalent vaccine without the adjuvant to test the vaccine without interference of adjuvant related immune response. Blood samples were collected one week after the challenge at week 25. For the duration of the experiment, the animals were monitored daily at least twice and after 25 weeks, the experiment was terminated and animals were euthanized according to institutional regulations. The antibody response in mice sera was determined as an absorbance value and was compared to the control group.

2.6. Serum biochemical parameters assay

We compared creatinine, BUN, uric acid, GGT and GPT levels in sera from combined vaccine immunized mice versus the control group using Fujifilm DRI–CHEM NX500 (Tokyo, Japan).

2.7. Detection of HPV16, 18 antibodies post mice immunization with the combined vaccine

Blood samples were collected throughout the experiment at specified time points from the mice groups and sera were separated where antibodies titers were measured as detailed before [16] Briefly, ELISA plates (Nunc-Denmark) were coated with 1 μg of standard HPV16 and HPV 18 diluted in PBS. The plates were incubated overnight at 2–8 c refrigerators. After incubation, plates were washed, and then blocked with PBS + 2% BSA. Ten-fold diluted mouse sera (100μL/well) were added and incubated 2 h at 37°c. Plates were then washed and anti-mouse-horseradish peroxidase (1/4000) was added and incubated for 1.5 h at 37°c and at 300 rpm in ELISA shaker. TMB was added after washing and the plate was incubated for 15 min, followed by reaction stop with 1 M H_2SO_4. ELISA plate reader was used to measure absorbance of samples in the wells of the plates at wavelength 450 nm.

2.8. Detection of rubella antibodies post mice immunization with the combined vaccine

We measured levels of rubella antibodies from pooled sera by DRG kit (DRG international, Inc., NJ, USA). Diluted serum (1/40) was added to the purified rubella antigen coated plate and incubated 37°c for 30 min. After washing, the anti-mouse enzyme conjugate was added and the plate was incubated at 37°c for 30 min. TMB was added and the plate was incubated for 15 min. After that, the reaction was stopped by 1NHCL. The O.D was determined and the concentrations of rubella antibodies were detected.

2.9. In vitro potency and stability of combined vaccine

The potency and stability of the vaccine were assessed by indirect sandwich ELISA. The stability of the vaccine was determined by incubating the combined vaccine at 37°c for 7 days. The general procedure of potency and stability was performed according to previously established protocol [17]. We used $TCID_{50}$ based end-point dilution assay to evaluate the *in vitro* potency of rubella virus in the combined vaccine. To do, we diluted rubella vaccine serial dilutions starting from 10^{-1}, $10^{-1.5}$, 10^{-2}, $10^{-2.5}$, 10^{-3}, $10^{-3.5}$, 10^{-4} and $10^{-4.5}$. Each dilution was used to inoculate RK-13 cells at 10 wells (10 replica) with 100μl/well. The plate was covered with sealer and the plate was incubated at 32 ± 1°c for 12 days. Presence of CPE was observed on 4th day until the final reading on the 12th day. The titer in $TCID_{50}$ was calculated using Spearman-Karber method, then expressed as $TCID_{50}$/mL [18,19]. The detected CPE was compared to controls that did not

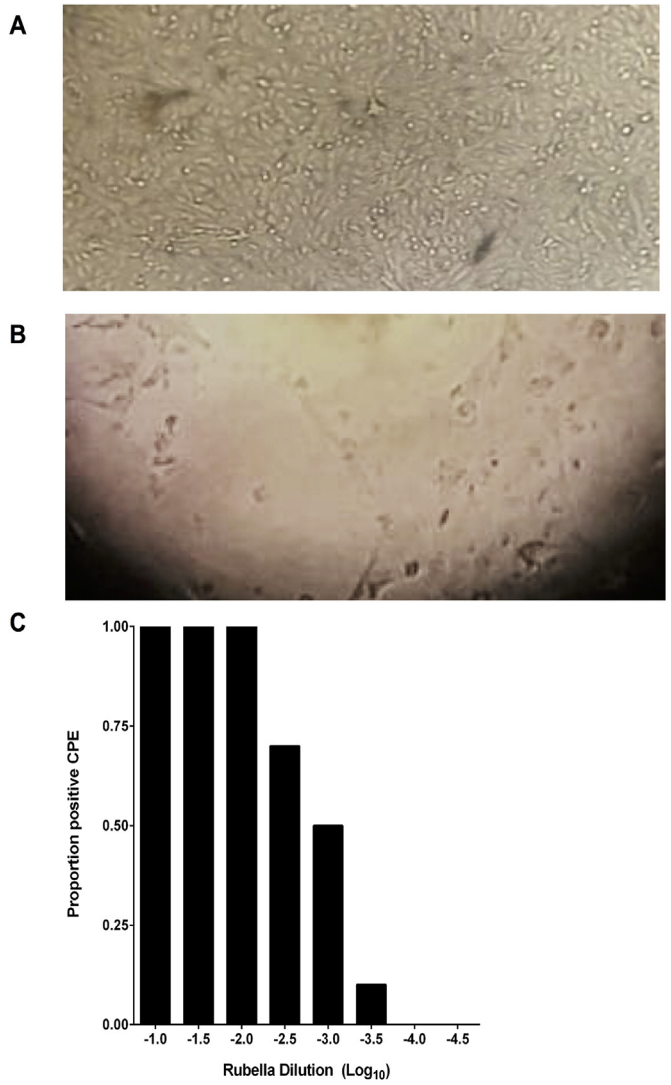


Fig. 2. Potency of rubella was not altered by the combination as measured by $TCID_{50}$ based end-point dilution assay. Combined vaccine was serially diluted and each dilution was used to inoculate RK-13 cell line. The titer in $TCID_{50}$ was calculated using Spearman- Karber method after the end of the incubation period. (A) Normal sheet of RK-13 cells before viral infection. (B) Cytopathogenic effect (CPE) of rubella on RK-13 cells showing cell lysis. (C) Serial dilution of rubella virus and proportional CPE.

show any CPE.

For stability study, the vaccine was incubated for 7 days at 37 °C according to WHO guidelines, and then we completed the procedure as in potency assay by tissue culture technique [18].

2.10. Cytokines assay

We pooled sera of five mice per group prior to assay and assessed levels of IL-6, IFNγ, IL-2 and IL-4 using ELISA method according to the manufacturer's protocol (MyBioSource), (BioLegend), (CUSABIO) and (Cloud-Clone Corp) respectively. Cytokine concentrations were calculated using standard curves (Fig S1).

2.11. Ex vivo cell proliferation assay

We immunized BALB/c mice (n = 4/group) with the combined vaccine at week 0 then HPV alone at week 2 and 4. Mice were euthanized 10 days after the last immunization. We harvested mice spleen cells (400,000/well) and cells were seeded onto 96-well culture plates and treated with 1 μg of HPV vaccine that was combined with 2 log $TCID_{50}$ of/100 μl for 5 days. Cells were incubated with 3-[4, 5-dimethylthiazol-2-yl]-2, 5 diphenyl tetrazolium bromide (MTT) yellow dye (5 mg/mL in PBS) at 37 °C incubator with 5% CO2 for 4 h. The purple formazan product was dissolved in DMSO, and the absorbance was measured at 570 nm using an ELISA reader. PHA was used as positive control. The MTT assay was done in triplicate. The results were expressed as stimulation index (SI) calculated as: OD values of stimulated cells minus relative cell numbers of un-stimulated cells divided by relative OD values of un-stimulated cells.

We used 400,000 cells per well, 1 μg of HPV combined with 2-log $TCID_{50}$/0.5 mL after optimization studies. For optimization studies, we used different concentrations of spleen cells at 100,000, 200,000 and 400,000/well treated with different concentrations of combined HPV and rubella vaccines for 3,5 and 6 days (Table .1).

2.12. Statistical analysis

Data was analyzed using Graph Pad Prism 6.01(Graph-Pad software Inc., California, USA). We compared groups using parametric *t*-test and one-way analysis of variant with Tukey's multiple comparisons test. P-values less than 0.05 were considered significant. Potency test calculations were made by Combistat software using 4 parameters sigmoid curve.

3. Results

3.1. Animals inoculated with the combined vaccine had no mortality or signs of toxicity

We assessed the safety profile of combined vaccine by monitoring two groups of adult mice (17–22 g) (5 mice/group) and two groups of adult healthy guinea pigs (250–350 g) (2 animals/group) for seven days after inoculation with the combined vaccine. All animals survived until the end of the experiment and showed no signs of toxicity or ill health during the experimental period (Fig S2). In addition, there were no significant changes in body weight, activity, or food consumption in the immunized group compared to the control group. This test was made in accordance to the European pharmacopoeia on assessment of toxicity of vaccines and biologicals in animal models.

3.2. Mice immunized with the combined vaccine showed no abnormalities in tested kidney and liver functions

We measured selected parameters of kidney functions, creatinine, BUN, Uric acid, and liver function parameters, GGT and GPT. We found no significant differences between the control and test group in serum biochemical analysis except for BUN (mg/dL), however, all mice results were within the physiological range (Table 2).

3.3. Combined vaccine did not affect Antigen content of HPV as evaluated by in vitro relative potency

To reduce the number of animals used in this study, we employed the *in-vitro* relative potency assay which has been previously developed as an alternative to the mouse potency assay [20]. We measured the potency of HPV-rubella combined vaccine bivalent type 16 and 18 indirect ELISA test. The results indicated that the combination did not affect HPV as it met the criteria for validity. Criteria of validity include that the regression coefficient for the standard curve must be > 0.95, mean of blanks must be < 0.2, the inter-dilution CV must be $\leq 20\%$, non-linearity, non-parallels > 0.5. The test results were calculated using the combistat software and we found that the *in-vitro* relative potency (IVRP) results were within the acceptance criteria (0.78–1.45) for HPV type 16 and (0.79–1.47) for HPV type 18 (Fig. 1). Thus, it can be concluded that the HPV antigen content was not affected by the

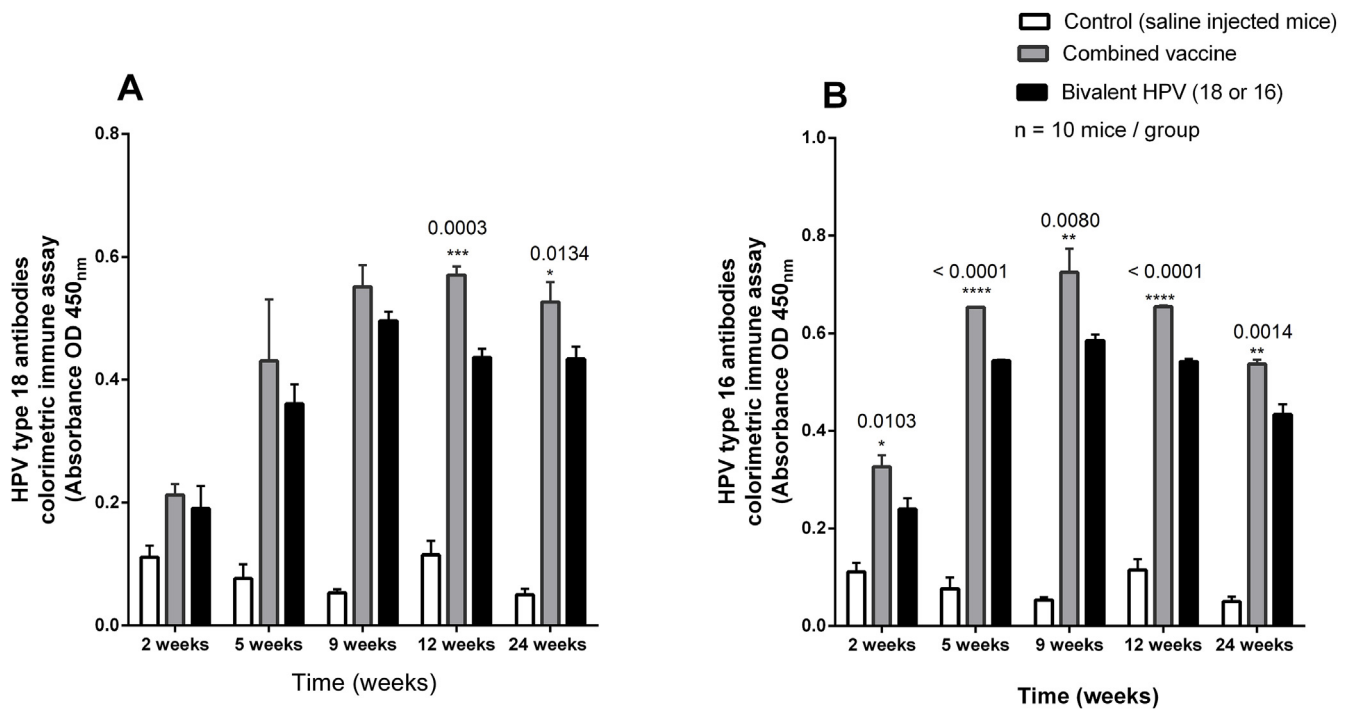


Fig. 3. Combined HPV 16, 18/Rubella vaccine elicited *in vivo* specific IgG antibodies against bivalent HPV 16, 18 measured as antibody absorbance significantly higher than mice immunized with HPV vaccine alone. An antibody absorbance value of the combined vaccine candidate at selected time points were compared to bivalent HPV vaccine at the same selected time points. Specific antibodies against (A) bivalent HPV 18 L1 VLP and (B) bivalent HPV 16 antigen differ significantly between mice immunized with the combined vaccine or those immunized with HPV vaccine alone. Statistical analysis was done using unpaired independent t-test. Data represented as means ± SD of at least two independent experiments.

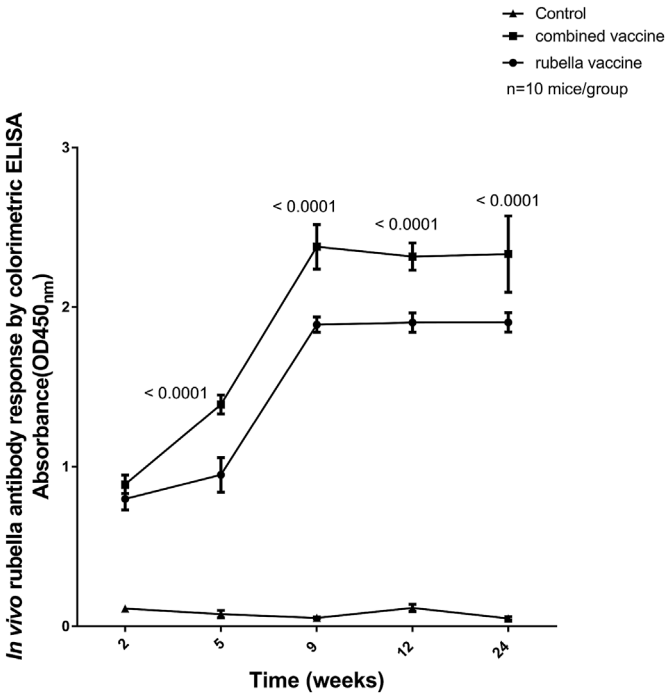


Fig. 4. Combined vaccine elicited *in vivo* specific IgG antibodies against rubella measured as antibody absorbance significantly higher than mice immunized with rubella vaccine alone. Rubella antibody concentrations of the combined vaccine at selected time points were compared to rubella antibody concentration at the same selected time points. Mice immunized with the combined vaccine developed specific antibodies against rubella significantly higher than mice immunized with rubella vaccine alone. Statistical analysis was done using unpaired independent t-test. Data represented as means ± SD of at least two independent experiments.

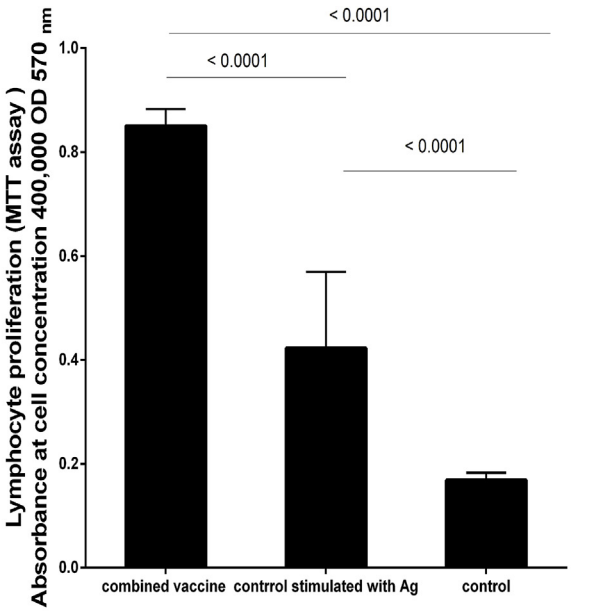
combination.

3.4. Potency of rubella was not altered by the combination

We cultured the combined vaccine on RK-13 cells and compared the results to control cells without any treatment**.** Rubella showed cytopathogenic effect calculated as 3.6 Log_{10} $TCID_{50}$/0.5 mL while the control did not show any CPE (Fig. 2). Rubella titer was within the acceptance criteria (≥3 logs $TCID_{50}$).

3.5. Combined vaccine elicited in vivo specific IgG antibodies against HPV significantly higher than mice immunized with HPV vaccine alone

Sera were collected on weeks 2, 5, 9, 12, and 24 to assess the immune response of the vaccinated groups**.** At 5 weeks after the immunization, the induction of serum IgG antibodies specific for HPV type 16 L1 reached a plateau that was maintained for 24 weeks after the first immunization (Fig. 3). We also found that specific antibodies against HPV 16 or HPV 18 L1 VLP antigen differ significantly between mice immunized with the combined vaccine or those immunized with HPV vaccine alone (Fig. 3). To determine the secondary immune response, mice were challenged with un-adjuvanted antigen at week 24, and then sera were collected one week after the challenge. There was an increase in the immune response (OD_{450nm} = 0.718 ± 0.022) that significantly differed from the pre-challenged response (OD_{450nm} = 0.526 ± 0.032) ($p \leq 0.001$) for HPV 18. For HPV 16, post challenge response (OD = 0.727 ± 0.025) is significantly higher than pre challenge response (OD = 0.537 ± 0.008) ($p \leq 0.001$). These results might suggest that HPV vaccination was able might be able to induce memory B cells.



Mice treated with			
PBS	-	+	+
Combined vaccine	+	-	-
Cells treated with			
1 ug HPV	+	+	-
2 Log Rubella	+	+	-

Fig. 5. Combined vaccine elicited significant splenocytes proliferation compared to control group. Mice (n = 4 mice per group) were immunized on day 0 with combined vaccine, and then at week 2 and 4, mice were immunized with HPV alone. Splenocytes were harvested 10 days after the last immunization. Splenocyte proliferation responses against the combined vaccine using MTT assay were significantly higher than negative control group ($P < 0.05$). Statistical analysis was done using one-way ANOVA. Data represented as means ± SD of at least two independent experiments.

3.6. Combined vaccine elicited in vivo specific IgG antibodies against rubella significantly higher than mice immunized with rubella vaccine alone

Sera from treatment groups and control groups were used to assess for antibody response. At week nine after the first immunization, the induction of serum IgG antibodies specific for rubella reached maximum levels, then a plateau level that was maintained for 24 weeks after the first immunization (Fig. 4). The results showed that combined vaccine serum IgG levels were induced to levels significantly higher to those in the group treated with rubella vaccine (Fig. 4). Standard rubella curve was used to calculate the potency of rubella vaccine (Fig S3).

3.7. Combined vaccine elicited significant splenocytes proliferation compared to control group

Splenocyte proliferation was expressed as stimulation Index (SI) for immunized mice. The results showed a significantly enhanced Ag-specific splenocytes proliferation in the immunized mice group compared with control group using MTT cell proliferation assay ($p < 0.005$) (Fig. 5). Splenocyte proliferation assay was optimized as detailed in supplementary Fig S4.

3.8. Combined vaccine elicited pro-inflammatory cytokines production

We analyzed selected pro-inflammatory cytokines levels in sera of mice immunized with combined vaccine. IL-6, IFN-γ, IL-2 and IL-4 sera levels in mice immunized with the combined vaccine were significantly higher than control mice, the HPV mice group and rubella mice group (Fig. 6).

3.9. Combined vaccine was stable in vitro after exposure to 37 °C for 7 days

We measured the stability of the vaccine by *in vitro* relative potency and we found that the vaccine was stable after incubation at 37 °C for 7 days. The results of the control were compared with the results of the vaccine; HPV relative potency was within acceptable criteria as shown in Fig. 7 and rubella showed CPE in the combined vaccine while the control did not show any CPE. Rubella titer was 3.3 Log $TCID_{50}$/0.5 mL (Fig. 8).

4. Discussion

4.1. HPV and rubella vaccine were mutually compatible in the proposed combined vaccine where stability and potency was not adversely affected

We used IVRP assay instead of mouse potency assay as mouse potency assays require 4–6 weeks for completion, their results are highly variable and not sensitive to minor perturbations of the antigen [20,21]. Our results showed that the potency of the combined vaccine was not affected by the combination (Fig. 1) based on WHO guidelines and previously established protocols [22,23]. Demonstration of vaccine stability is a crucial part of vaccine development, HPV and rubella vaccines were stable under accelerated conditions at 37 °C for 7 days (Fig. 7) complying with WHO guidelines and previous studies [22].

4.2. Combined HPV and rubella vaccine was immunogenic stimulating both humoral and cellular immune responses

Our results demonstrated that administration of a three – dose regimen of combined rubella and bivalent HPV vaccine to Balb/C mice resulted in potent humoral immune responses characterized by serum type-specific anti-HPV and anti-rubella IgG antibodies and immune memory in mice. Our results were in agreement with previous studies where each of HPV and rubella vaccination on their own induces protective and long lasting immunity [24–26]. We also tested several selected cytokines that are known to be involved in inducing humoral immunity and cellular immunity [27]. We found that IL-4, and IL-6 concentrations in mice immunized with the combined vaccine were significantly higher than controls and this is in agreement with previous studies [28–30]. Also, the tested IL-2 and IFN-γ concentrations were significantly higher in mice receiving the combined vaccine and this is in agreement with previous studies [28,31–33]. Moreover, our *ex-vivo* splenocyte proliferation assay revealed the involvement of cellular immune responses to the combined vaccine. This is in agreement with Woo et al. who studied the cell mediated immunity in mice by HPV 16 L1 VLP and found that splenocytes proliferation was significantly increased with a mixed Th1/Th2 response [31].

Although HPV exposure and pregnancy may occur prior to age 20 especially in rural areas, nevertheless, the proposed combined vaccine would likely benefit a significant fraction of the younger women especially with the rise of marital age. This proposed vaccine is especially important as immunization with HPV is not part of the national immunization program in Egypt and many developing countries. So this proposed combined vaccine will benefit significant fractions of women above 20 years old as FDA extended HPV immunization to include females 27–45 years old instead of 9–26 years old. This extension in the immunization program will add significant value to large number of females in Egypt and developing countries. Moreover, women younger

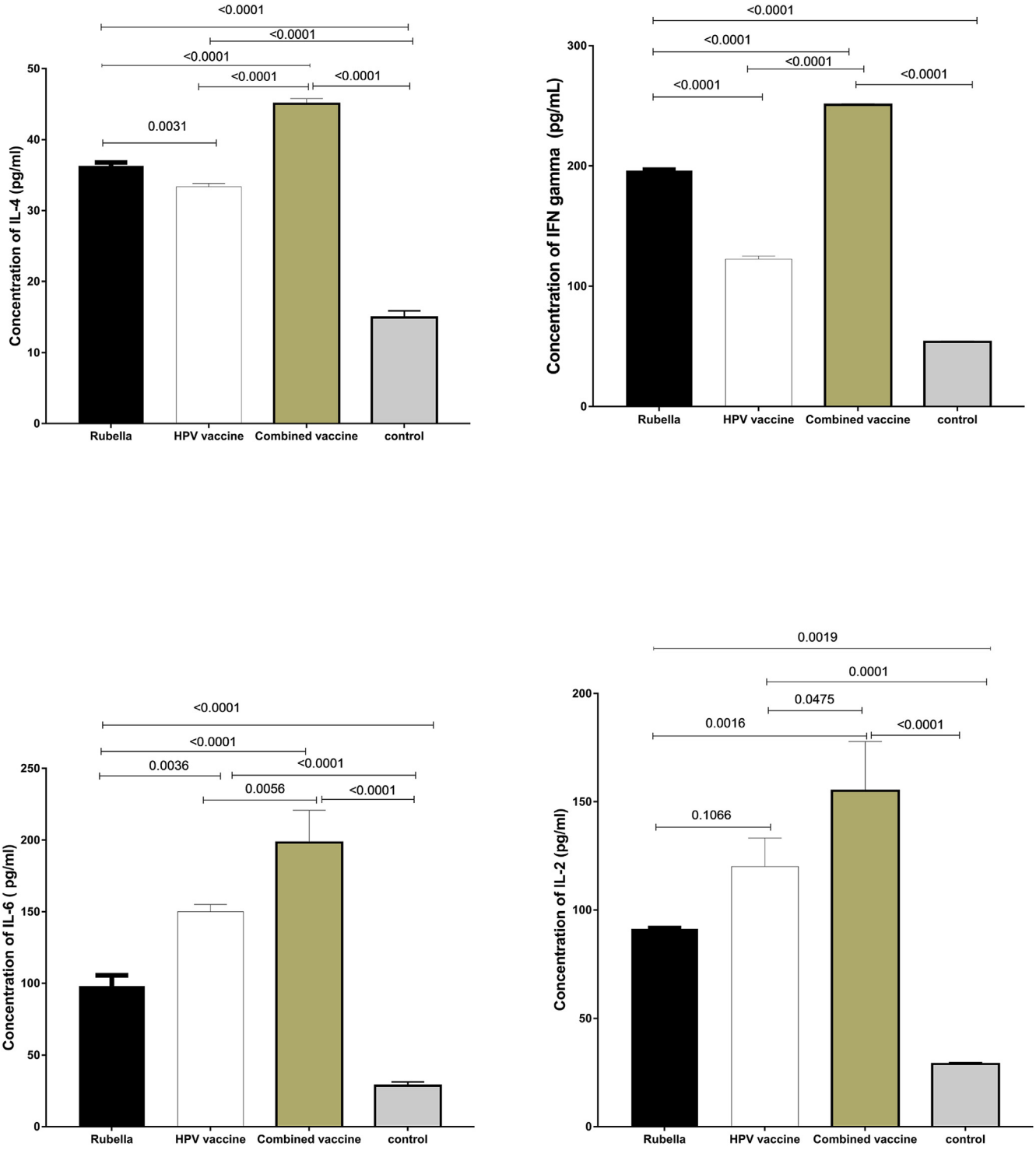


Fig. 6. Combined vaccine elicited pro-inflammatory cytokines production. Balb/C mice (n = 4 mice per group) were immunized with HPV, or rubella vaccines or their combination. Blood sera were collected 72 h after the last immunization. IL-6, IFN- γ, IL-2, IL-4 response were significantly higher than control groups. (A) IL-4, (B) IFN- γ, (C) IL-6, and (D) IL-2. Statistical analysis was done using unpaired independent t-test. Data represented as means ± SD of at least two independent experiments.

than 20 were immunized with MMR vaccine introduced to national immunization programs since 2000 in most developing countries and in Egypt since 1999. The main aim of the present study was to propose a vaccine for rubella and HPV to vaccinate women over 20 years of age as they were not immunized with rubella nor HPV vaccine.

4.3. Adding HPV vaccine to rubella vaccine would have no significant change to public health policy and NIP while offering protection against anogenital cancers

Despite the availability of vaccines to HPV and rubella viruses, vaccine uptake rates are variable depending on each country. One of the major factors known to increase vaccine uptake, reduce cost, time

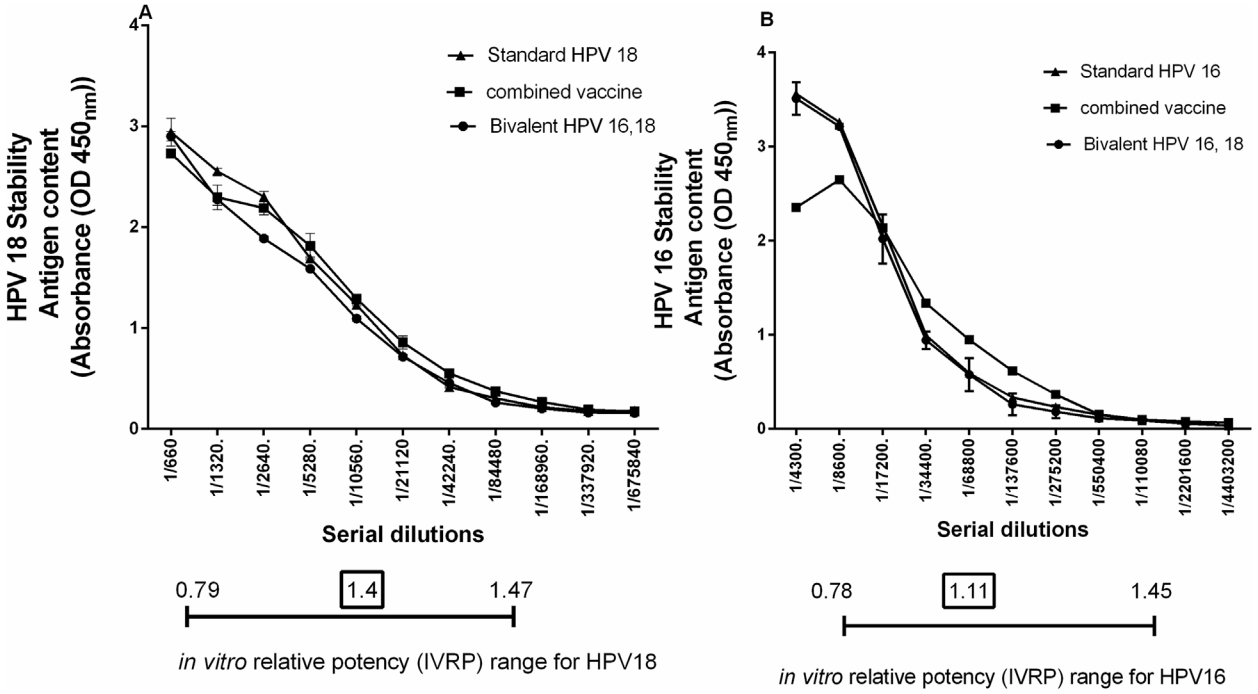


Fig. 7. Combined vaccine was stable *in vitro* after exposure to 37°C for 7 days. We assessed the stability after 7 days of incubation at 37°c. The *in-vitro* relative potency of combined vaccine was calculated by dividing the estimate concentration of bivalent standard over the estimate concentration of the sample multiplied by factor 1.01 or 0.95 for (A) HPV 18L1VLP and (B) HPV 16 respectively. The results showed that the relative potency of the combined vaccine were within the acceptance criteria (0.78–1.45) for HPV 16 and (0.79–1.47) for HPV 18. The calculations were made using 4 parameters sigmoid model by combistat program.

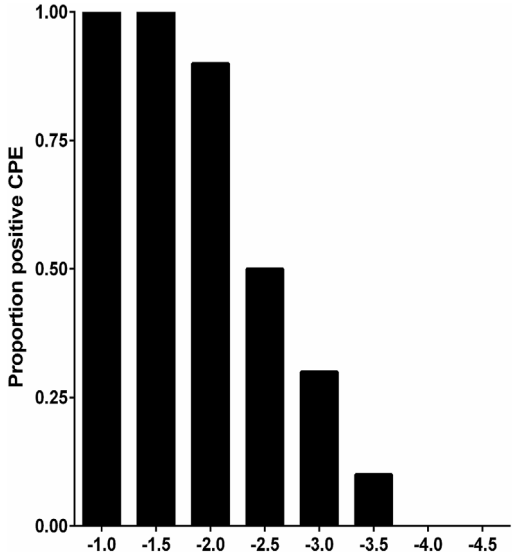


Fig. 8. Stability of rubella was not altered by the combination as measured by $TCID_{50}$ based end-point dilution assay. The vaccine was incubated for 7 days at 37 °C according to WHO guidelines. Serial dilutions of combined vaccine were made. Each dilution was used to inoculate RK-13 cells. The titer in $TCID_{50}$ was calculated using Spearman-Karber method, then expressed as $TCID_{50}$/mL.

and discomfort to the vaccinee is combination vaccine [34]. Moreover, having a combined vaccine improves the process of vaccination as some parents and health care providers raised objections to administering multiple injections during a single visit [35]. Based on this preclinical study, adding this combined vaccine to the immunization schedule would not cause much change in the schedule. Where the combined vaccine would be administered to vaccinee older than 19 years for a first dose, followed by one dose after which a third booster dose of HPV alone if needed. This study also showed that HPV vaccine can be effective with two instead of three doses to induce sufficient humoral immunity, however, the third dose can enhance long term memory response [36]. Appropriate further studies are needed to assess the timing and dosing of the proposed combined vaccine.

The current study focused on bivalent HPV vaccine as a proof-of-principle preclinical study for the feasibility of combining rubella and HPV vaccines for a population in need of such combination. The use of quadrivalent or nonavalent HPV vaccines (Gardasil and Gardasil 9) in the combined vaccine might be of added value and should be studied in details. The associated immune response is expected to be affected by the different adjuvant systems used in the bivalent vaccine (Cervarix) vs. the quadrivalent or nonavalent HPV vaccines (Gardasil and Gardasil 9). Alum or amorphous aluminum hydroxyl phosphate sulfate (AAHS) used in the quadrivalent and nonavalent vaccines as an adjuvant system vs. the combination of aluminum hydroxide and monophosphoryl lipid A (MPL) used in the bivalent vaccine (Cervarix) is expected to stimulate the immune system differently based on previous studies (Ebensen, T. *et al.*, 2019 [37] and Cimica and Galarza, 2017 [38]). It is anticipated that antibody titers produced by Cervarix immunization would be more potent than Gardasil and might elicit higher cellular immunity due to MPL and alum combination adjuvant.

4.4. In conclusion

Our proposed combined vaccine had similar potency and stability of individual HPV and rubella vaccines. Moreover, combination between rubella and HPV was safe in animal model, inducing significant antibody and cellular immunity and immunogenic against both viruses. The presented results support the interest of conducting clinical trials of the combined vaccine towards assessment of dosing and timing of administration of the proposed combined vaccine.

Authorship

All authors attest they meet the ICMJE criteria for authorship.

Conflict of interest statement

The authors declare no conflict of interest or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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