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Comparative Evaluation of Mx and Alum as Bio and Conventional Adjuvants in Inducing Immune Responses by Influenza DNA Vaccine

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ABSTRACT

Background & Objective: DNA vaccines as a new generation of vaccines require adjuvant to improve vaccine immunogenicity; adjuvants can also increase the DNA vaccine efficacy. In this study, the effects of the host's interferon-inducible Mx protein as bio adjuvant and conventional alum adjuvant were evaluated.

Materials & Methods: The BALB/c mice were immunized by different prime-boost strategies of the alum and Mx adjuvanted-HA2 DNA vaccine; they were challenged with the specific influenza virus. The potency and safety were evaluated. Humoral immune response was assayed by haemagglutination and virus neutralization tests. The induction of cell-mediated immune responses was determined using an MTT assay. The safety of vaccine regarding side effects occurrence was assessed by observation and histopathologic evaluation.

Results: Mx as a host defense peptide was able to increase the immune response against influenza better than alum adjuvant ($p<0.01$). By HA2 and Mx in prime and boost strategy, the highest level of specific antibodies developed; they are capable of inducing cell-mediated immune responses. Results indicated that Mx in the DNA vaccine could induce stronger immune responses without any side effects; but alum had some local and general reactions.

Conclusion: The Mx could effectively enhance immune responses; it has the potential to enhance the vaccine immunogenicity.

Keywords: Influenza vaccine, Humoral and cellular responses, HA2, Mx, bio adjuvant, Alum adjuvant

Introduction

A new generation of influenza vaccine need to be developed for modulating broad-spectrum of immunity against the divergent virus; it can be used in the event of a pandemic. Due to the considerable effects of DNA vac-cine on cellular and humoral response increment, by CD4 and CD8 cell priming, their development has been of interest (1,2); rapid large-scale production is another notable feature of DNA vaccine to meet demand in a pandemic (3).

Among viral proteins, HA comprises major neut-ralizing epitopes; it is considered as a high immunogenic protein (4). The HA DNA vaccine is an attractive alternative approach to induce cytotoxic T lymphocyte (CTL) and antibody response (5). Neutralizing antibodies against the HA2 subunit has proper protection and cross-react with other subtypes of HA virus (3, 6).

The most important influencing factor on vaccination is arguably the type and concentration of the vaccine adjuvants, which enhance and direct the immune response to the vaccine (7). An adjuvant can enhance the immunogenicity with a limited amount of antigen, which is dive-loped to be co-administered with the influenza vaccine; it meets the requirement to prevent regional outbreaks or the next pandemic. Adjuvants have different mechanisms including antigen delivery increment, improvement in the magnitude and breadth of the responses via MHC antigen presentation and also immune-stimulatory signals creation (8).

Several adjuvants have been studied for the flu vaccine. Aluminum hydroxide (generically called alum) is the first adjuvant, which its safety property has been accepted for the use in humans (9). Alum is well-known for forming a depot of antigens; the adjuvanticity of alum that activates immune responses via dendritic cell (DC) interaction has been illustrated recently (10). Another adjuvant for influenza vaccine is an oil-in-water emulsion (11).

Because of post-immunization reactions by almost all of the synthetic adjuvants (12), the efficiency of mole-cular and biological adjuvants, such as: bacterial derivate-ives, cytokines and immune regulators has been studied to increase the vaccine's efficiency as a new strategy (13). These components will induce an effective immune response, without any side effects. Among biological adjuvants, host defense peptides are small and positively

charged peptides, that enhance antibody formation and cell-mediated response in mice (14).

The host cellular protein is called Mx protein, which involves host defense peptides; it plays a well-known role in inducing interferon and immune system regulation (3, 15). We decided to use this protein as a biological adjuvant in the vaccine. Based on previous researches (3, 6, 15), stimulation of immune responses by Mx and alum adjuvants with HA2 vaccine was evaluated in mice in the present study; it was also compared to different DNA prime-boost strategy. Finally, the best strategy and adjuvant for immunization against the influenza virus were introduced.

Materials and Methods

HA2 subunit based vaccine

HA2 subunit based DNA vaccine against influenza has been constructed in our previous study (3). HA2 nucleotide datasets of H9N2 subtypes were designed based on the NCBI database; they were aligned using ClustalW. By Bio edit software, conserved HA2 sequence was determined to be 571bp long. RNA extraction was done from a JX456181.1 virus by Ribospin™ kit (Gene All, South Korea). By the appropriate restriction enzyme sites, cloning was performed into the pcDNA3.1 vector (Invitrogen, USA), between the *Bam*HI and *Nco*I sites. Then plasmid was propagated in *Escherichia coli* and purified using the EndoFree®Plasmid Mega Kit (Qiagen, Germany). The concentration of this construct was adjusted 1 µg/ µl for mouse immunization.

Adjuvants

Bio adjuvant: The Mx bio adjuvant was constructed on the base of our previous study (3). On the base of NCBI database, the Mx sequences were aligned by the ClustalW from Homo sapiens, Mus musculus, and Gallus gallus. According to our previous *in silico* study [20], a conser-ved sequence encodes the motif of 13SGKSSVLEALSG-VALPR30 in interferon; the induced domain reveals better results against HA2 H9N2 viruses by inducing B-cell and T-cell immune responses. Thus the primers were designed, the fragments were amplified and cloned. The ratio of vaccine to Mx was adjusted 7:1 on DNA mass; the dose-finding was done according to our previous research (3).

Alum adjuvant: The alum adjuvant was prepared in the Razi vaccine and serum research institute. The ratio of vaccine to alum was adjusted 7:3 on DNA mass based on experimental dose-finding study (3).

Immunization of BALB/c mice

Eighty female BALB /c mice with the age of 6 – 8 weeks were prepared from the animal laboratory department of Razi vaccine and serum research institute. The mice were housed and tested according to the protocols of the ethics committee of the Razi Institute. The ethical code is as follows: RVSRI.REC.98.005.

They were categorized into eight groups, including four controls and four treatment groups; they were kept in the separate cages. The control groups included A: injected with normal saline as a negative control, group B: Alum receivers, group C: Mx receivers, and group D: HA2 vaccine receivers.

HA2 vaccine and adjuvants were injected to the test groups in the prime-boost manners including group E: Prime by HA2/alum, group F: prime by HA2/Mx, group G: prime and boost by HA2/alum and group H: prime and boost by HA2/Mx. The mice were injected by the intramuscular route in quadriceps. After 14 and 28 days, the boosts were injected (3, 16).

Challenge

Four of each group were challenged by 100 mouse infectious doses (MID50) of influenza A/chicken/Iran SS7/2011H9N2, under anesthesia with diethyl ether intra nasally, two weeks after the last injection; this influenza strain is not deadly. In mice, the virus titers were determined as the 50% cell culture infectious dose (CCID50) in MDCK cells in the lungs, 4 days after challenge.

Immunogenicity evaluation

On the third day before immunization and on the 7th, 14th, 28th, 42th, 56th and 70th days post-injection, sera were collected from all of mice in each group. The humoral immune response was assayed by haemagglutination inhibition assay (HI) (17) and virus neutralization (VN) (18) test. The cell-mediated immune responses were determined by a 3-(4,5-dimethyl thiazol-2-thiazolyl) -2,5-diphenyltetrazolium bromide (thiazolyl-blue; MTT assay) with some modifications (19). The stimulation index (SI) was calculated as the ratio of the average optical density (OD) of antigen-stimulated cells to the average OD value of cells (3, 20).

Safety evaluation

For evaluation the safety of the vaccine and adjuvants, the injected mice were weighed weekly and observed for local reaction at the injection site and also general reactions. In each group spleen and lungs of two mice were sampled aseptically; then the tissue sections were stained with hematoxylin and eosin. They were evaluated histopathologically (21).

Statistical analysis

The results were analyzed by a one-way analysis of variance (ANOVA) by SPSS ver. 11; P-value ≤ 0.01 was considered significant.

Results

HA2 and Mx construction

The constructed pcDNA3.1/HA2 and pcDNA3.1/Mx were transformed into TOP10 *Escherichia coli* and the positive clones were screened using restriction enzyme digestion and sequencing. Digestion confirms the presence of the genes, based on the bands detection of the expected size; it was done based on the previous study (3). (Figure 1) It was also checked with the original sequence of the gene bank database.

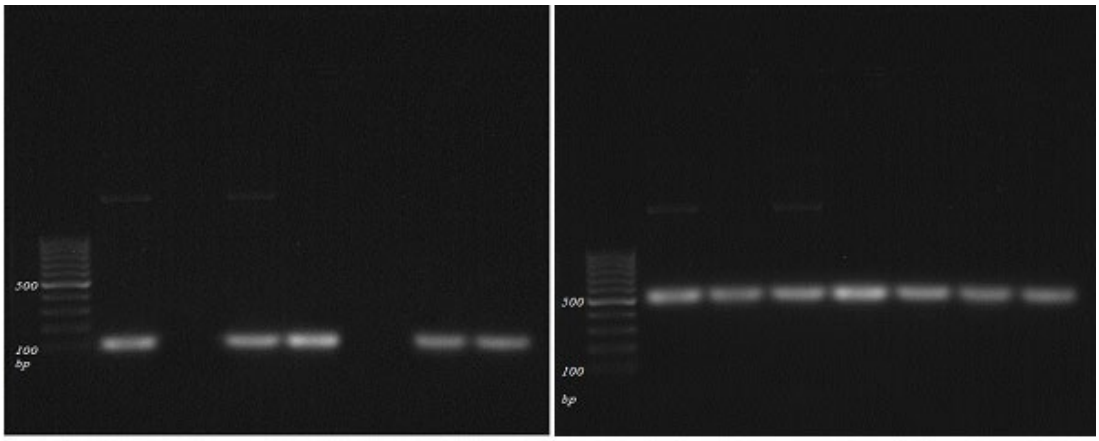

Figure 1. The conformation of Mx (Left) and HA2 (Right) clones by restriction enzyme digestion. Of seven Mx clones, five clones and all of the HA2 clones were confirmed in this assay.

Immunogenicity evaluation

Humoral antibody responses were evaluated in serum samples taken from the control and treated mice, by HI and VN tests. As listed in table 1 and shown in figure 2, immunized group H (injected by HA2 and Mx in the prime and boost manner) developed the highest levels of specific antibodies with HI mean titer about 6.89 Log_2.

In group G (injected by HA2 alum adjuvant in the prime and boost manner) the specific antibodies were lower than group H (5.41) (The titer of the control group was 0.91). The titers significantly differ among the groups, which were boosted by the same regimen or other boosting strategies. Importantly, when Mx was co-administrated with the HA2, the HA antibody responses were significantly higher in the test groups compared to the HA2 vaccine and HA2 with alum adjuvant.

Similar results were found in the VN test. As shown in table 1 and figure 3 the highest neutralizing antibody titer (1.91) was detected in the H group; it was 1.70 in the group G compared to the control group (0.43), at the end of the study. Among the vaccinated groups, the difference could be determined due to the neutralizing antibodies.

Induction of cell-mediated responses was evaluated by SI calculation. The results (Figure 4) showed that the HA2 vaccine by Mx with the same boosting could enhance the immunity with a mean SI of 5.152. The SI for the HA2 alum adjuvant vaccine was 3.508 (The SI in the control group was 0.9 and 1.00. In challenged mice, the virus titer in the lungs was significantly lower in the immunized groups by HA2 bio and conventional adjuvants vaccines compared to the control group ($p< 0.01$).

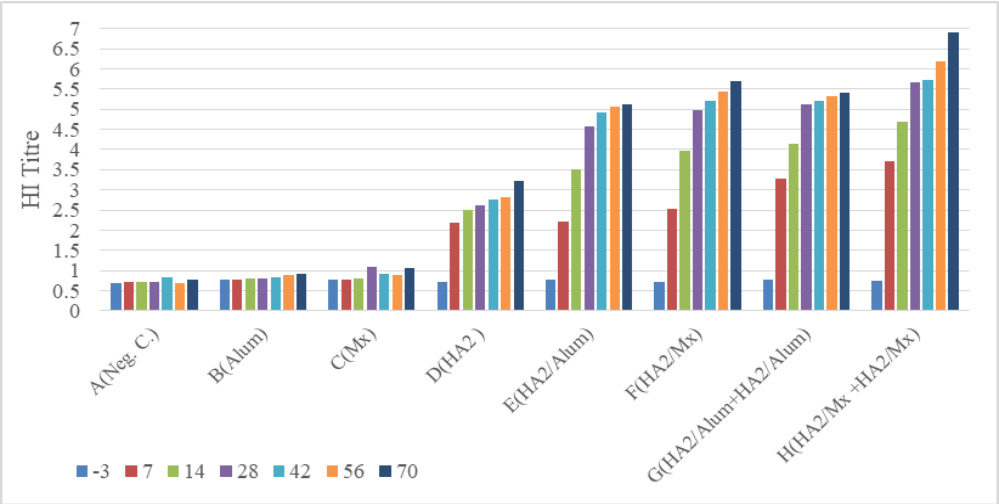

Figure 2. The HI antibody titers of mice groups. There are significant differences between the immunized groups received different regimens.

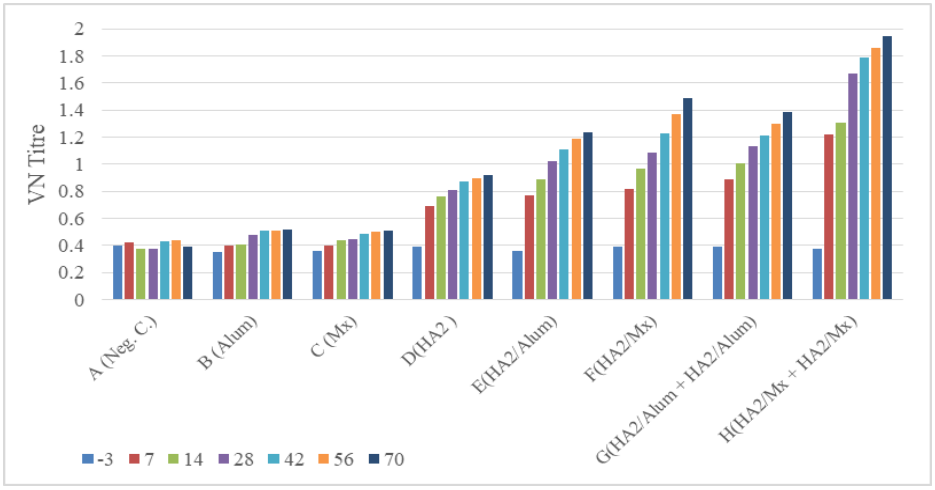

Figure 3. The VN antibody titers of mice groups. There are significant differences between the immunized groups received different regimens.

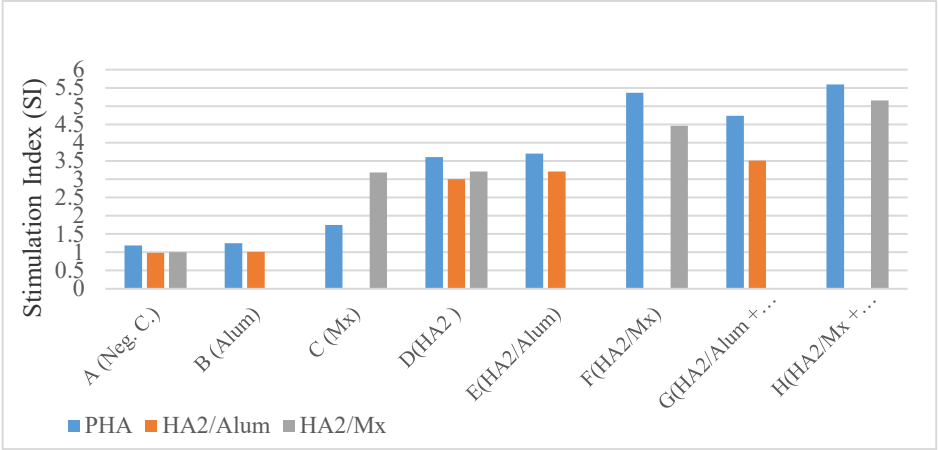

Figure 4. The stimulation index (SI) of lymphocyte proliferation assay in mice groups. The results indicated the adjuvanted vaccine stimulate the lymphocytes in MTT assay similar to PHA.

Safety evaluation

The mean weight of mice in the vaccinated group by Mx bio adjuvant was 31.6 gr, which indicated the safety of both vaccine and the bio adjuvant; the mean weight of mice was 27.7 gr in the groups vaccinated by alum. The results of histopathological analysis in the mice groups indicated, that there were not any alternations including hyperemia, inflammation and deformation following HA2 DNA vaccine and Mx injections (Figure 5). In the vaccinated group by alum, there were some local reactions at the site of vaccine injection.

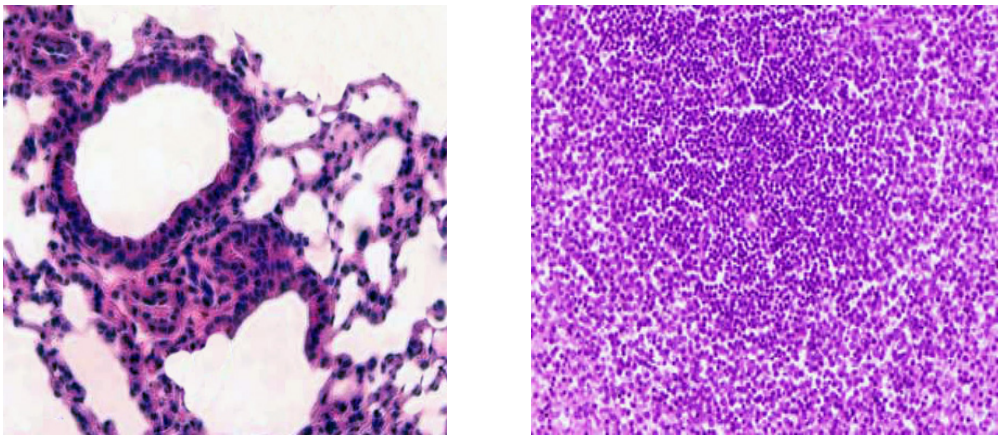

Figure 5. Histopathology of spleen (right) and lung (left) specimens of MX-treated mice. No specific tissue change or inflammatory reaction was seen.

Table 1. The mean antibody titer (±standard deviation) against the influenza virus evaluated in immunized mice. The results of HI and VN tests were compared between the group in three days before vaccination and at defined days post-vaccination.

Groups	Inoculum		Test	Before Injection	Days after Injection					
	Prime	Boost		-3	7	14	28	42	56	70
A	Neg. C.	-	HI	0.70±0.17	0.72±0.20	0.73±0.18	0.73±0.21	0.82±0.19	0.69±0.22	0.78±0.24
			VN	0.40±0.12	0.42±0.10	0.38±0.09	0.38±0.08	0.43±0.08	0.44±0.08	0.39±0.09
B	Alum	-	HI	0.76±0.13	0.77±0.14	0.80±0.20	0.80±0.18	0.83±0.19	0.89±0.21	0.91±0.19
			VN	0.35±0.06	0.40±0.08	0.41±0.07	0.48±0.06	0.51±0.10	0.51±0.9	0.52±0.8
C	Mx	-	HI	0.77±0.18	0.78±0.16	0.81±0.21	1.08±0.22	0.93±0.21	0.89±0.25	1.07±0.24
			VN	0.36±0.10	0.40±0.08	0.44±0.07	0.45±0.06	0.44±0.11	0.50±0.11	0.51±0.07
D	HA2	-	HI	0.73±0.18	*2.18±0.33	**2.49±0.40	**2.63±0.41	***2.75±0.41	***2.81±0.36	***3.23±0.41
			VN	0.39±0.08	*0.69±0.11	*0.76±0.09	**0.81±0.09	*0.87±0.22	**0.90±0.09	**0.92±0.12
E	HA2+Alum	-	HI	0.76±0.21	*2.21±0.27	**3.51±0.39	***4.58±0.43	***4.92±0.39	***5.07±0.39	***5.13±0.38
			VN	0.36±0.07	*0.77±0.12	**0.89±0.11	**1.02±0.12	**1.11±0.14	**1.19±0.13	**1.24±0.14
F	HA2+Mx	-	HI	0.72±0.22	*2.53±0.26	**3.98±0.38	***4.89±0.41	***5.21±0.40	***5.44±0.43	***5.71±0.39
			VN	0.39±0.10	**0.82±0.14	**0.97±0.14	**1.09±0.14	***1.23±0.13	***1.37±0.16	***1.49±0.17
G	HA2+Alum	HA2+Alum	HI	0.77±0.23	*3.27±0.25	***4.13±0.42	***5.12±0.41	***5.21±0.39	***5.32±0.43	***5.41±0.43
			VN	0.39±0.11	**0.89±0.14	**1.01±0.15	***1.13±0.15	***1.21±0.16	***1.30±0.19	***1.39±0.18
H	HA2+Mx	HA2+Mx	HI	0.75±0.22	*3.70±0.24	***4.70±0.41	***5.66±0.40	***5.72±0.38	***6.19±0.41	***6.90±0.40
			VN	0.38±0.11	**1.22±0.14	**1.31±0.15	***1.67±0.15	***1.79±0.16	***1.86±0.19	***1.95±0.18

The data were analyzed by ANOVA (*p<0.05, ** p<0.01, ***p<0.001)

Discussion

The induction robust immune response has opened entirely new horizons in the development of efficacyous DNA vaccines against influenza infection. The major mechanism of the influenza vaccines is based on targeting the viral highly immunogenic HA surface molecule. HA1 recognizing antibodies have simultaneously point mutations, and do not cross-react with other HA subtypes unlike HA2 (22, 23).

The stalk domain (HA2) is a conserved unit of the HA, and vaccination with the subunit elicits immune sera with broader reactivity; it creates protection against influenza disease (24). It has been previously shown that the HA2 subunit antibodies can prepare broad protection against all of the influenza features (25, 26). It has been suggested that antibodies against HA2 can neutralize a broad range of virus strains and subtypes (23). So, inducing an immune response against HA2 could potentially elicit broad inhibitory antibodies (8).

DNA vaccines are mildly immunogenic and need suitable adjuvant along with optimization of the delivery by prime-boost strategies to increase vaccine efficacy (27). Results from our in silico and other studies (28) indicated that host defense peptide (Mx) as bio adjuvant can be exploited to improve immune responses against influenza virus-induced, by HA2 DNA vaccine.

Among non-bio adjuvants, alum is the most effective and common used one. The accurate mechanism of alum is not clear exactly, but the results of some studies have been cited as follows (29). Aluminum adjuvants selectively stimulate Th2 immune response (30); it can stimulate dendritic cells (DC) and other immune cells to secrete interleukin-1β (IL-1β) (an immune signal that promotes antibody production) (31).

Studies have showed that alum is not perfect, since it cannot work with all antigens and it does not stimulate Th1 (32). It is the weakest inducer of Th1 cellular immune responses; Th2-based immune response is not likely to create optimum protection against several important infectious diseases. Besides, recent studies have indicated some concerns about alum safety issues, for example, some descriptions of nodules and erythema (33).

The present in vivo study showed that the administration of Mx enhances the humoral immune response to the HA2 influenza vaccine, especially in the group, which was boosted with the same regimen

more than alum adjuvanted HA2 vaccine ($p<0.01$). Consequently, the potential of Mx as a bio adjuvant and conventional adjuvant was evaluated in the VN assay. The VN results showed that the HA2 vaccine by Mx was relatively high effective; it enhanced the protective effects against influenza infection in comparison to the alum adjuvant ($p<0.01$).

The analysis demonstrated that the humoral immune responses to influenza induced by Mx adjuvanted HA2 vaccine, were higher than the alum adjuvanted HA2 vaccine. The mice challenged the evaluation of the vaccination effects on the virus clearance rate. So, the virus titer was significantly lower in the immunized groups compared to the control group ($p< 0.01$).

Conclusion

In recent years, many components have been proposed to introduce a suitable adjuvant for providing augmentation of vaccine immune responses such as CD40L, MDA5, MF59, IC31 and Ag85A **(8, 13, 25, 34)**. Thus, some researches focus on bio adjuvants such as interferon inducer peptides, cytokines and immune system regulator proteins **(35)**; they have significant effects on promoting chemotaxis of immune cells, regulating metabolism, enhancing vaccine responses and limiting inflammation/sepsis **(14)**. In the present study immunization by HA2 vaccine with Mx adjuvant had sustained the immune responses, with no side effects in comparison to the conventional adjuvant.

Concerning the promising results of this study in inducing immune response, using influenza DNA vaccine with prime-boost strategy, with bio adjuvant leads to better results than conventional adjuvant.

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Conflict of Interest

The authors declare that they have no conflict of interests.

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