Antioxidative protection of squalene adjuvant and rabies vaccine with adjuvant

Anna Ondrejková\textsuperscript{a}, Judit Süli\textsuperscript{b}, Jarmila Harvanová\textsuperscript{b}, Róbert Ondrejka\textsuperscript{a}, Marián Prokeš\textsuperscript{a,*}

\textsuperscript{a}Department of Epizootiology and Parasitology, University of Veterinary Medicine and Pharmacy in Košice, Komenského 73, 041 81 Košice, Slovak Republic
\textsuperscript{b}Department of Chemistry, Biochemistry and Biophysics, University of Veterinary Medicine and Pharmacy in Košice, Komenského 73, 041 81 Košice, Slovak Republic
* Correspondence e-mail: marian.prokes@uvlf.sk
Summary

The authors verified the possibility of antioxidative protection of squalene adjuvant emulsions by the antioxidants α-tocopherol and β-carotene. They determined the influence of β-carotene on the stability and antigenic effectiveness of adjuvant emulsion in combination with rabies vaccine.

The composition of the adjuvant emulsions or vaccines was: 2.5% squalene; 6% detergents; 0.5% antioxidant; 91% water phase. The oxidative injury after UV-irradiation was followed by the detection of the peroxide value of the emulsions. The stability of the emulsions was evaluated by the determination of the emulsion’s particle size. The level of rabies antibodies (RAB) in mice sera until day 90 after vaccination, was determined by the rapid fluorescent focus inhibition test.

In the in vitro system of squalene adjuvant, α-tocopherol acted as a prooxidant, while β-carotene effectively reduced the oxidative injury. The homogenization of the squalene adjuvant during a prolonged period from 8 to 10 minutes did not change the particle size. The oxidation processes were efficiently reduced by β-carotene during the preparation process and also during the 70-day storage.

The vaccine with β-carotene induced a gradual increase in the RAB levels with the highest value on day 28. While the inactivated rabies vaccine with adjuvant without β-carotene developed a rapid formation of RAB, the application of the vaccine with β-carotene induced a slower but more uniform production of RAB. The level of RAB was significantly higher after the application of the vaccine with β-carotene and reached the protective value of 0.5 IU/mL, in contrast to the vaccine without β-carotene.

Keywords: adjuvant; squalene; β-carotene; antioxidative effectiveness; rabies vaccine
INTRODUCTION

One of the possibilities to achieve an increase in an inactivated vaccine’s effectiveness is the use of adjuvants. Adjuvant substances have been used for nearly 90 years in order to increase the effectiveness of various vaccines.

A number of combined adjuvants have been prepared up to now. Even though adjuvants based on mineral oils caused various post-vaccinal reactions of a local or even general character in vaccinated individuals,¹ Freund’s incomplete adjuvant played an important role in commercial veterinary vaccines. Some of the local reactions may be eliminated by using metabolizable natural oils (soya, sesame, olive, etc.) in adjuvant formulations.² At the present time, aluminium hydroxide adjuvant is still often used in veterinary medicine,³ although its potentiating effect fails to reach (in general) the level of oil adjuvants and its security is also questionable.⁴ In the development of new adjuvant formulations for human and veterinary use, squalene based oil-in-water (O/W) emulsions are promising.⁵ Squalene (SQ) is a polyprenyl compound naturally occurring in the animal and plant world⁶ and it is the major component of human sebum.⁷ Squalene emulsions are generally relatively stable, but the squalene due to its high degree of unsaturation, is sensitive to oxidation.⁸ The oxidation process may disturb the stability of the adjuvant emulsion and also affect its efficacy. The greatest risk of squalene oxidation arises during the preparation of the emulsions, particularly for homogenization⁹ and during the prolonged storage of emulsions. Therefore, an antioxidant, usually α-tocopherol, is added to some SQ adjuvant formulations (e.g. Stable emulsion – SE, AS03 – GlaxoSmithKline Biologicals).¹⁰ α-tocopherol (vitamin E) is the most common antioxidant for lipids. Since SQ itself is an effective antioxidant, the positive effect of the addition of another antioxidant to the emulsion formulation in in vitro system is questionable.¹¹ The protection mechanisms in vivo and in vitro differ from each other and it is not possible to apply automatically the experience and knowledge from one to another. Mueller and Boehm¹² compared the antioxidant activity of β-carotene and other carotenoid compounds with the antioxidant effectiveness of α-tocopherol in in vitro lipid systems and found that β-carotene was characterized in in vitro conditions by a significantly better antioxidant activity than that of α-tocopherol.

The aim of this study was the verification of the antioxidative protection of SQ by α-tocopherol and β-carotene. Additionally, we examined the preparation of adjuvant
formulation with the more effective antioxidant in our conditions and determined the influence of antioxidant on the stability and antigenic effectiveness of adjuvant emulsion in combination with rabies vaccine.

MATERIALS AND METHODS

**Antioxidative protection of squalene emulsion by antioxidants**

**Composition of adjuvant emulsions:**

Oil phase: *squalene* (2, 6, 10, 14, 18, 22-tetracosahexene; Merck-Schuchardt, Germany), content 2.5% (w/w), which is the ideal final content for SQ in adjuvant formulations.\(^{13}\) Emulsifiers, final content according to Süli *et al.*\(^{14}\):

- **Poloxamer 105**, non-toxic, well-resorbable emulsifier (polyethylene-polypropylene polyol; ICI, Great Britain), HLB = 18.5; final content 4% (w/w);
- **Abil-Care 85** (Bis-PEG/PPG-16/16 PEG/PPG-16/16 Dimethicone (and) Caprylic/Capric Triglyceride; Evonik Industries AG, Germany) HLB \(\approx\) 10; final content 2% (w/w), available for emulsion preparing at decreased temperature (up to 37 °C).

Antioxidants:

- **α-tocopherol** (Sigma, USA); final content 0.5% (w/w), or
- **β-carotene** (Sigma, USA); final content 0.5% (w/w).

Commercial products contain α-tocopherol in a wide concentration range: SE – 0.01%, AS03 – 2.5%. The antioxidant concentration in our experiments was chosen following our previous results about the antioxidative effectiveness of α-tocopherol, β-carotene and rutin in the protection of squalene against oxidative injury.\(^{15}\)

Water phase: *Dulbecco’s Modified Eagle’s Medium* (D-MEM) with L-glutamine, 1 g/L glucose, 25 mmol/L HEPES (PANTM Biotech UK Ltd., Great Britain); final content of water phase in the adjuvant formulation without antioxidants 91.5% (w/w), with antioxidant 91% (w/w).

**Preparation of adjuvant formulations:**

The adjuvant emulsions were prepared by homogenizing with an ultrasonic disintegrator Soniprep 150 MSE (MSE, Great Britain) with a probe ULT-210-515D at a frequency of 23 kHz. The time of homogenization was 8 min., temperature of preparation 37 °C, according to our previous experiments.\(^{14}\)
Oxidation of SQ in emulsions:

Photo-oxidative injury of SQ emulsions and their potential prevention by antioxidants (α-tocopherol or β-carotene) were investigated in the in vitro adjuvant emulsion systems, and were exposed to various doses of UV-radiation (16, 48, 96, 144 and 192 kG). The oxidation was induced by 30 W germicidal lamp without UV-A and UV-B specification.

Determination of the peroxide value (PV) of the adjuvant emulsions:

Oxidation processes of lipids originated primarily in lipid hydroperoxides, which were reacted in acidic pH with potassium iodide to form iodine. Iodine was determined by titration with sodium thiosulphate:

\[
\begin{align*}
R-O-OH + 2 \text{KI} + 2 \text{CH}_3\text{COOH} &\rightarrow R-OH + I_2 + 2 \text{CH}_3\text{COOK} + \text{H}_2\text{O} \\
I_2 + 2 \text{Na}_2\text{S}_2\text{O}_3 &\rightarrow 2 \text{NaI} + \text{Na}_2\text{S}_4\text{O}_6
\end{align*}
\]

The peroxide values of SQ emulsion and SQ emulsions with antioxidants were observed immediately after the preparation. The mean PV was always calculated from 5 titrations.

Effect of β-carotene on the emulsion stability

Following the results from the antioxidative effectiveness of α-tocopherol or β-carotene, the next investigation was only aimed at β-carotene.

Determination of particle size in adjuvant emulsions:

In order to ensure the correct emulsion stability, the suitable choice of emulsion homogenization time was very important. According to our further investigative results,\textsuperscript{14} to achieve a stable SQ emulsion, 8 min homogenization time was adequate. We wondered whether the addition of β-carotene might affect the particle size in the emulsion, which would be an important indicator of emulsion stability. So prolonged, 10 min homogenization was also tested. The particle size analysis was performed on the instrument Mastersizer 3000 (Malvern Instrument Ltd., Great Britain).

Following of the oxidation changes in emulsions after preparation and during storage:

The oxidation changes in emulsions were followed by peroxide value determinations. The emulsions without β-carotene and with β-carotene were tested immediately after
preparation and during a 10-week storage period at weekly intervals. The emulsions were stored in polyethylene containers at 4 °C, which were preferable to glass.16)

Effect of β-carotene on the antigenic activity of inactivated rabies vaccine with squalene adjuvant

Preparation of inactivated rabies vaccines with SQ adjuvant:

The preparation of the rabies vaccines was similar to the preparation of the adjuvant emulsions mentioned above. However, the difference was in the aqueous phase. It was prepared using the inactivated rabies virus suspension, instead of the cell medium D-MEM. The rabies virus suspension from strain Vnukovo-32 at the level of 107th serial passage cultivated in a stable cell line BHK-21/13 in the culture medium D-MEM was used. The titre of rabies virus was 10^-6.2 MICLD50/0.03 mL. The inactivation of the rabies virus was performed with β-propiolactone in a final content of 0.025%.17) Rabies vaccines with adjuvant without β-carotene and with 0.5% β-carotene were prepared.

Vaccination scheme and sampling for rabies antibody level determinations:

In this study, we used female mice of ICR lines, 3 weeks of age and weight 10 g. All procedures and protocols of husbandry and manipulation were approved by State Veterinary and Food Administration of the Slovak Republic according to the Act of the Slovak National Council No. 39/2007 Coll. on veterinary care, pursuant to Government Regulation No. 23/2009 Coll. and legal Directives EU, legislation for the protection of animals used for scientific purposes. Seventy-two (72) random-bred white mice were used in the experiments: 36 animals were vaccinated with the vaccine without β-carotene, and 36 animals with the vaccine with β-carotene included. The vaccines were administered intraperitoneally in a dose of 0.1 mL. After 7, 14, 21, 28, 60 and 90 days, 6 mice from both groups were killed using cervical dislocation and thereafter were bled. The level of rabies antibodies was determined in the blood sera.

Determination of rabies antibodies level

The rabies antibody level in the blood sera was detected in accordance with the recommendation of WHO18) by the rapid fluorescent focus inhibition test (RFFIT).19) In the assay a standard laboratory reference strain of rabies virus CVS11/Paris (Institute Pasteur, Paris, France), adapted to the cultivation on the BHK21/C13 cell culture was used. The
titre of the rabies virus was $10^{6.0}$ MICLD$_{50}$/0.03 mL. In the test, the standard reference positive and negative control sera were used.

**Statistical evaluation of results**

The statistical evaluation of the results were performed by Student’s t-test independent by groups.\textsuperscript{20}

**RESULTS**

**Antioxidative protection of squalene emulsions by antioxidants**

The squalene emulsions were very sensitive to the provoked oxidation. After UV-irradiation, a high increase in peroxide value was observed, depending on the radiation dose. α-tocopherol in this *in vitro* system acted as a prooxidant, while β-carotene reduced the oxidative injury at all of the radiation doses used (Table 1). In view of the results obtained, further investigations were aimed at the testing of the antioxidative properties of β-carotene only.

The peroxide value of squalene adjuvant with β-carotene was significantly lower ($p<0.01$) in comparison with adjuvant without β-carotene. The prolongation of homogenization time caused a slight increase in PV of both adjuvant emulsions (Table 2).

**Effect of β-carotene on the emulsion stability**

**Determination of particle size in adjuvant emulsions:**

The particle size analysis of SQ and SQ-β-carotene adjuvant emulsions showed good stability of the prepared emulsions, and β-carotene did not affect the particle size. The prolonged homogenization did not result in a decreased particle size (Table 3), so in terms of particle size, the 8-min homogenization period was satisfactory.

**Following of the oxidation changes in emulsions after preparation and during storage:**

In each following period, the peroxide value of the adjuvant emulsion with β-carotene was significantly lower ($p<0.01$) than the value of the emulsion without β-carotene (Table 4). Despite the antioxidative protection, the peroxide value of the emulsions containing β-carotene until day 14 of storage increased approximately twice in
comparison with the value immediately after preparation and this level remained almost unchanged until the end of the following period. During 10 weeks of storage, the peroxide value of the non-protected emulsions gradually increased, and on day 70 (10 weeks) it was nearly 5-times higher in comparison with the protected one.

**Effect of β-carotene on the antigenic activity of inactivated rabies vaccine with squalene adjuvant**

The course of the development of the rabies antibody (RAB) formation was different in both vaccines. Seven days after vaccination with both vaccines, the levels of RAB were similar. After using a vaccine without β-carotene, the RAB started to form rapidly and on day 14 the levels of RAB were significantly higher (0.42 IU/mL; p<0.01) than after the application of vaccine with β-carotene (0.19 IU/mL). This level was rather increased until day 28, but did not reached the protection level (0.5 IU/mL) and then had a decreasing trend. On day 90, the value of RAB was moderately decreased (0.38 IU/mL).

With the application of the vaccine with β-carotene, the RAB levels gradually increased until day 28. On day 21, it was already 1.2-times higher than the vaccine without β-carotene and reached the protective value. The highest value was achieved on day 28 (0.86 IU/mL), it was 1.8-times higher in comparison with the vaccine without β-carotene. At the next intervals, the RAB level was slightly decreased, but it was still 1.8-times higher compared to the RAB level after vaccination without β-carotene (p<0.001) and was over the protective value (Fig. 1).

**DISCUSSION**

The effectiveness and the stability of the prepared product is very important for the manufacturer of drugs and vaccines. Squalene is a good choice for adjuvant emulsions of oil-in-water type, because of the diffusion of squalene molecules through the aqueous medium and consequently the origination of Ostwald ripening is unlikely. On the other hand, the squalene molecule contains six unsaturated bonds and is a strong antioxidant due to its large electron exchange capacity without being exposed to molecular disruption. Squalene is very sensitive to the oxidation process, which primary results in the formation of various hydroperoxide position isomers. However, some authors suggested a lower sensitivity of squalene to peroxidation. Squalene in emulsion form is much more sensitive than the oil alone. As the preparation process occurs, the increase in
temperature and aeration of emulsions during homogenisation supports the formation of peroxides.

The oxidation process may influence the properties of the squalene adjuvant emulsion. In the first place, the emulsion stability characteristics may be changed, and in the second place, the oxidation products may be harmful for humans and animals. Just for easy oxidizability, the squalene itself is considered an antioxidant. According to our results, the addition of \( \alpha \)-tocopherol is not a good choice for antioxidant protection of squalene adjuvant emulsions. Surprisingly, \( \alpha \)-tocopherol had a prooxidant effect for squalene emulsion after UV-irradiation in all doses, except the highest dose – 192 kGy. This is an alarming fact, because due to its antioxidant properties, \( \alpha \)-tocopherol is often added to the products containing lipids (adjuvants, dermatological and cosmetic creams). We have more data about the positive effects of \( \alpha \)-tocopherol on squalene in vivo for the protection of skin against UV radiation, but little is known about the behaviour of systems containing squalene and other antioxidant in in vitro conditions when exposed to UV radiation.

The increased peroxide value could be even caused by oxidation products of \( \alpha \)-tocopherol. Therefore, it should be noted that although \( \alpha \)-tocopherol is known as the most active interrupter of radical chain reaction in human tissues, in in vitro systems, where reparative mechanisms are absent, it is necessary to ensure its regeneration. In a comparative study, it was found that in in vitro systems, all isomers of carotenes were twice as active in the interruption of radical chain reactions compared to \( \alpha \)-tocopherol. The activity of radical catching was 10—25 times higher and in the monitoring of the iron reduction, \( \beta \)-carotene was approximately twice as potent as \( \alpha \)-tocopherol.

Kohno et al. and Psomiadou and Tsimidou, on the basis of their photo-oxidation studies even claimed that squalene regenerates \( \alpha \)-tocopherol and not conversely. In our former experiments, there was observed a low antioxidant activity of \( \alpha \)-tocopherol only if it was added to the system after irradiation. In this case, we determined the lowest peroxide value 30 min. after the addition of \( \alpha \)-tocopherol to irradiated squalene oil. Therefore, before the automatic addition of other antioxidants, it would be appropriate to perform studies of their suitability for this particular system.

The particle size analysis of adjuvants with \( \beta \)-carotene and without \( \beta \)-carotene indicated a good stability of the emulsions prepared. Prolongation of the homogenization period did not have an impact on the reduction of the particle size, so in terms of particle size, it is irrelevant to homogenize for longer than 8 min. The results of squalene oxidation
(peroxide values) also suggested that prolonged homogenization may not be needed. Prolonged homogenization leads to aeration of the samples, which may cause potentiation of the oxidative processes. Oxidative processes occur furthest during storage, which is reflected in the increasing peroxide value of emulsions. β-carotene effectively protects squalene emulsions also during the storage period.

Various inactivated adjuvant vaccines with α-tocopherol induced rapid and higher production of antibodies in test animals compared to control vaccines without adjuvants. However, we have no data about vaccines containing β-carotene. The immune enhancing effects of dietary administered carotenes against infectious disease was first revealed during the early 1930s. The antioxidant protection of squalene by β-carotene in our experiments were satisfactory, but it was also important to know whether the addition of the antioxidant altered the effectiveness of the vaccines. Our results suggested that the adjuvant effectiveness of β-carotene was also adequate. The addition of β-carotene to the vaccine caused a slower, but more uniform increase in antibodies during the observed period. In the vaccine without β-carotene, the highest levels of antibodies were detected on day 14, and then had a decreasing trend. While in the vaccine with β-carotene, the antibody levels on day 21 were already higher than the vaccine without antioxidant and gradually increased until day 28. The protective level of rabies antibodies recommended by WHO i.e. 0.5 IU/mL (as determined by virus neutralization tests on mice or cell culture), was reached only by vaccination with the vaccine with β-carotene on day 21 and the protective level was maintained during the observed period. From our results, it was clear that β-carotene, in addition to the antioxidant protection of rabies vaccine with adjuvant, affects also its antigenic effectiveness. In a recent study, the relationship of the plasma concentrations of six major carotenoids (β-carotene, α-carotene, β-cryptoxanthin, lycopene, lutein, and zeaxanthin) with the incidence and severity of acute respiratory infections was determined. The results indicated that β-carotene may exert the most significant immune enhancing effects. Originally, it was thought, that the immune enhancing properties of carotenes were due to their conversion to vitamin A. Now it is known that carotenes exert many immune system enhancing effects independent of any vitamin A activity. β-carotene enhance many aspects of immune functions, including proliferation, induction of specific effector cells, as well as the secretion of cytokines. The mechanisms for immuno-enhancement may include the quenching of reactive O₂ intermediates which are immunosuppressive and can be immunotoxic. When O₂ intermediates reacted with lipids, the lipid peroxides generated were also
immunosuppressive. The peroxidation of cell membrane lipids can decrease membrane fluidity and, thus, depress proliferation. Membrane receptors, required for antigen recognition, can also be damaged by peroxidation of membrane lipids. Reactive O₂ intermediates can generate and are products of the arachidonic acid cascade. Certain prostaglandins and leukotrienes depress immune responses. Carotenoids may affect immune function because of their antioxidant and singlet O₂-quenching capacities.³⁶—³⁸)

Considering that vitamins (or provitamins) have immunomodulatory and adjuvant properties in vaccines,³⁹) β-carotene as antioxidant in squalene adjuvants seems to be a good choice. Our laboratory developed a technological procedure of preparation of an inactivated rabies vaccine with adjuvant. We anticipate that the experimental adjuvant may also be used to potentiate the effectiveness of other antigens which will enable the preparation of additional liquid adjuvant veterinary vaccines.

CONCLUSIONS

In the study of the in vitro system of the squalene adjuvants, α-tocopherol acted as a prooxidant, while β-carotene was able to effectively reduce the oxidative injury during all of the UV-irradiation doses used. The authors found that the homogenization of squalene adjuvants during prolonged periods of time between 8—10 min, did not change the particle size, which is an important indicator of the stability of the vaccine emulsions. However, prolonged homogenization may lead to an increased oxidation of squalene. The oxidation processes were efficiently reduced by β-carotene not only in time of the preparation process, but also during the 70-day storage.

The influence of β-carotene on the antigenic activity of inactivated rabies vaccine with adjuvant was followed. The vaccine with β-carotene induced a gradual increase in the levels of rabies antibodies, with the highest antibody levels on day 28. While the inactivated rabies vaccine with adjuvant without β-carotene developed a rapid formation of rabies antibodies (on day 14), the application of vaccine with β-carotene induced a slower but more uniform production of rabies antibodies. The level of rabies antibodies was significantly higher after application of the vaccine with β-carotene and, by contrast to the vaccine without β-carotene, the levels of antibodies reached the protective value of 0.5 IU/mL.
Acknowledgment
This work was supported by scientific project APVV-0605-12 (Slovak Republic).

Conflict of interest
The authors declare no conflict of interest.
REFERENCES


10) Fox CB. Squalene emulsions for parenteral vaccine and drug delivery. Molecules, 14, 3286–3312 (2009).


Table 1
Peroxide value of SQ and SQ-antioxidant adjuvant emulsions after UV irradiation. Number of titrations of each sample: n = 5

<table>
<thead>
<tr>
<th>Dose of UV radiation</th>
<th>0 kGy</th>
<th>16 kGy</th>
<th>48 kGy</th>
<th>96 kGy</th>
<th>192 kGy</th>
</tr>
</thead>
<tbody>
<tr>
<td>emulsion (SQ)</td>
<td>0.37 ± 0.04</td>
<td>20 ± 0.28</td>
<td>25 ± 0.75</td>
<td>46 ± 1.06</td>
<td>97 ± 2.38</td>
</tr>
<tr>
<td>emulsion (SQ + α-T)</td>
<td>0.29 ± 0.02</td>
<td>22 ± 0.37</td>
<td>37 ± 0.89</td>
<td>53 ± 1.37</td>
<td>65 ± 1.41</td>
</tr>
<tr>
<td>emulsion (SQ + β-C)</td>
<td>0.15 ± 0.01</td>
<td>10 ± 0.25</td>
<td>12 ± 0.76</td>
<td>15 ± 0.89</td>
<td>18 ± 0.72</td>
</tr>
</tbody>
</table>

α-T: α-tocopherol
β-C: β-carotene

Table 2
Peroxide value of SQ and SQ-β-carotene adjuvant emulsions after preparation by 8-min and 10-min homogenization. Number of titrations of each sample: n = 5

<table>
<thead>
<tr>
<th>Time of homogenization</th>
<th>Vaccine without β-carotene</th>
<th>Vaccine with β-carotene</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>8-min</td>
<td>0.370 ± 0.104</td>
</tr>
<tr>
<td></td>
<td>10-min</td>
<td>0.380 ± 0.076</td>
</tr>
</tbody>
</table>

** p<0.01

Table 3
Particle size in SQ and SQ-β-carotene adjuvant emulsions after preparation by 8-min and 10-min homogenization

<table>
<thead>
<tr>
<th>Diameter value</th>
<th>SQ-emulsion 8 min</th>
<th>10 min</th>
<th>SQ-emulsion + β-carotene 8 min</th>
<th>10 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dv (10)</td>
<td>316 µm</td>
<td>315 µm</td>
<td>314 µm</td>
<td>314 µm</td>
</tr>
<tr>
<td>Dv (50)</td>
<td>423 µm</td>
<td>420 µm</td>
<td>420 µm</td>
<td>418 µm</td>
</tr>
<tr>
<td>Dv (90)</td>
<td>561 µm</td>
<td>561 µm</td>
<td>556 µm</td>
<td>553 µm</td>
</tr>
</tbody>
</table>

Dv (10) – over 10% of the particles had a diameter of below …
Dv (50) – over 50% of the particles had a diameter of below …
Table 4
Peroxide value of adjuvant emulsions immediately after preparation (d 0) and during 10 weeks of storage. Number of titrations of each sample: n = 5

<table>
<thead>
<tr>
<th>Time after preparation</th>
<th>SQ-adjuvant without β-carotene</th>
<th>with β-carotene</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>± 0.104</td>
<td>0.130 ± 0.057 **</td>
</tr>
<tr>
<td>d 0</td>
<td>0.370 ± 0.104</td>
<td>0.160 ± 0.042 **</td>
</tr>
<tr>
<td>d 7</td>
<td>0.380 ± 0.076</td>
<td>0.330 ± 0.027 **</td>
</tr>
<tr>
<td>d 14</td>
<td>0.550 ± 0.018</td>
<td>0.335 ± 0.034 **</td>
</tr>
<tr>
<td>d 21</td>
<td>0.530 ± 0.027</td>
<td>0.620 ± 0.027 **</td>
</tr>
<tr>
<td>d 28</td>
<td>1.270 ± 0.148</td>
<td>0.310 ± 0.042 **</td>
</tr>
<tr>
<td>d 35</td>
<td>1.310 ± 0.103</td>
<td>0.320 ± 0.036 **</td>
</tr>
<tr>
<td>d 42</td>
<td>1.560 ± 0.154</td>
<td>0.320 ± 0.042 **</td>
</tr>
<tr>
<td>d 49</td>
<td>1.940 ± 0.126</td>
<td>0.390 ± 0.040 **</td>
</tr>
<tr>
<td>d 56</td>
<td>1.850 ± 0.090</td>
<td>0.395 ± 0.038 **</td>
</tr>
<tr>
<td>d 63</td>
<td>1.970 ± 0.112</td>
<td>0.395 ± 0.038 **</td>
</tr>
</tbody>
</table>

** p<0.01
Fig. 1. Level of rabies antibodies in mice vaccinated with inactivated rabies vaccine with adjuvant, without and with β-carotene

--------- protective level of rabies antibodies (0.5 IU/mL)

* statistical significance $p<0.05$

** statistical significance $p<0.01$

*** statistical significance $p<0.001$