Polyclonal antibody production against bovine serum albumin conjugated artemisinin in rabbit

Kaboutari, J.[1], Arab, H.A.[1]*, Foroumadi, A.[2], Nikbakht, G.R.[3], Taheri, M.[4]

[1]*Department of Basic Sciences, Faculty of Veterinary Medicine, University of Share Kord, Share- Kord-Iran.*

[2]*Department of Pharmacology, Faculty of Veterinary Medicine, University of Tehran, Tehran-Iran.*

[3]*Pharmaceutical Sciences Research Center, Faculty of Pharmacy, Tehran University of Medical Science, Tehran-Iran.*

[4]*Department of Pathobiology, Faculty of Veterinary Medicine, University of Tehran, Tehran-Iran.*

[5]*Central Research Laboratory, Faculty of Veterinary Medicine, University of Tehran, Tehran-Iran.*

Abstract: **The aim of the present study was to produce a polyclonal antibody against bovine serum albumin (BSA) conjugated with artemisinin. To gain an immunogenic character of artemisinin, a carboxyl group was added to it using mixed anhydride method. Then, the reactive compound of artemisinin was conjugated with BSA. The BSA+artemisinin were injected to white female New Zealand rabbits for two times. In the first injection, the Fraud's complete adjuvant was used, and two weeks later, a booster injection was carried out with a Fraud's incomplete adjuvant. Two weeks later, blood samples were collected; their serum were separated and frozen until assessment. The production of antibody against BSA+artemisinin was assayed by Immunoblot technique. Antibody was separated and concentrated by saturated ammonium sulfate. The assay confirmed the successful production of an antibody against BSA+artemisinin as a fundamental step in investigation of pharmacodynamics and pharmacokinetics of artemisinin.**

Key words: *Artemisia sieberi*, artemisinin, polyclonal antibody, BSA-conjugation.

Introduction

Artemisinin and its several derivatives have impressive parasiticidal activity against malaria agents including strains of *Plasmodium* resistant to conventional anti malarial compounds and cerebral malaria, with high efficiency and negligible toxicity (Li *et al*., 1994). Artemisinin was extracted, for the first time, from a Chinese medical plant Qinghao (*Artemisia annua*). The plant has been used as antipyretic and anti malarial agent in traditional Chinese medicine for more than 2000 years (Toufigh Gordi, 2001). It is a sesquiterpene trioxane lacton containing a peroxide bridge that is essential for its therapeutic activities (China Cooperative Research

on Qinghaosu, 1982; Klayman *et al*., 1985; Woerdenbag *et al*., 1994).

Coccidiosis is an intestinal infection caused by the intracellular protozoan parasite of the genus *Eimeria*. It is a major parasitic disease of poultry with substantial economic burden, estimated to cost around 2 billion $ in annual losses world wide (Zhang and Zeng, 2005).

For many years, prophylactic use of different chemical drugs has been the primary mean of controlling this disease (Patricia and Fetter, 2002), but the increasing problem of *Eimeria* resistance against many drugs has prompted a majority of research efforts to seek an alternative methods of coccidiosis control (Williams, 2002). As apart of such efforts *Artemisia* plant has been a candidate

* **Corresponding author's email: harab@ut.ac.ir, Tel: 21-61117086, Fax: 21- 66933222**
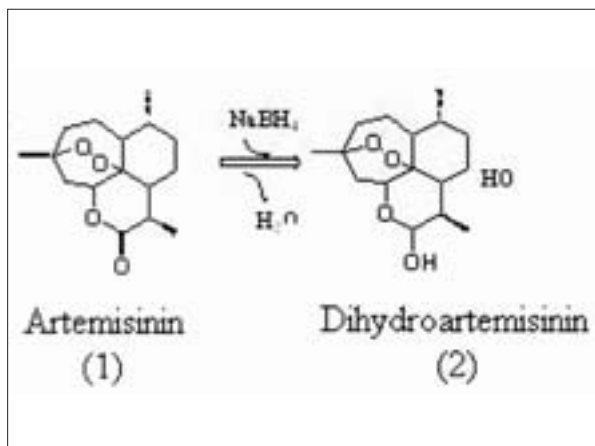
Fig. 1- Reduction of artemisinin to yield dihydroartemisinin in the first stage of artemisinin derivatization.
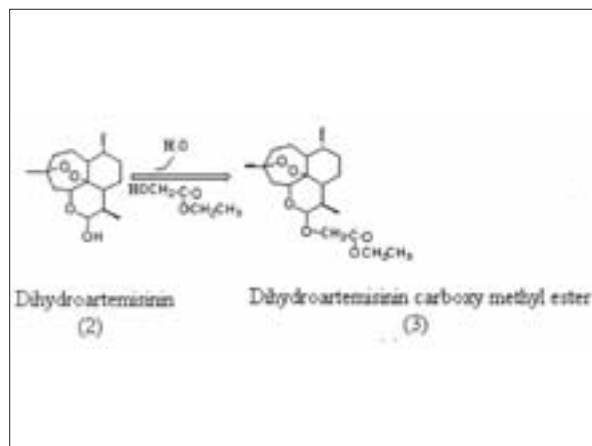

Fig. 2- Addition of an ester moiety to dihydroartemisinin to achieve dihydroartemisinin carboxy methyl ether in the second stage of derivatization.

being a potential source of anti coccidian activities in chickens.

Patricia and co-workers (1997) for the first time showed the protective effect of the dried leaves of *Artemisia annua* and pure artemisinin against coccidiosis experimentally induced by different species of *Eimeria*. It was shown that the dried leaves of *A. annua* were able to protect the birds against *Eimeria tenella* (Patricia *et al*., 1997). Arab *et al* determined and quantitated the amount of artemisinin in another species of *Artemisia- Artemisia sieberi-* and showed that the plant extract was able to reduce the coccidian infection in broilers (Arab *el al*., 2006).

Despite the numerous studies on the pharmacodynamics of artemisinin in malaria, there is no evidence to show the mechanism (s) of the action of this compound in avian coccidiosis. It seems that the first step to understand the mechanism of action of artemisinin is to determine the site of action of drug and tracing it in the body tissues. A simple and reliable method to fulfill this assignment is to trace the administrated drug by Immunohistochemistry method using polyclonal antibodies.

Since, artemisinin is too small to be immunogenic (mol. wt 282), it must be conjugated to a large carrier protein to take action as the primary immunogenic agent. Furthermore, it lacks a reactive group to bond to a carrier protein. To overcome this, Ferreira and Janick (1996) derivatized artemisinin to

dihydroartemisinin carboxymethylether in three steps without disturbing the peroxide bridge, and then linked this reactive compound to either Thyroglobulin (TGB) or BSA. The conjugates were mixed and emulsified with complete or incomplete Freund's adjuvant, and then they were injected to female New Zealand rabbits for six to seven times. The ELISA showed that the polyclonal antibodies induced against artemisinin-TGB conjugate in rabbits but not against artemisinin-BSA (Ferreira and Janick, 1996). The aim of this study was to produce a polyclonal antibody against BSA conjugated artemisinin using Ferreira and Janick modified method. This antibody can be used as a research probe to trace the artemisinin in the intestinal tissue by Immunohistochemical technique.

Materials and Methods

Artemisinin derivatization: To insert a reactive compound on the artemisinin structure, it was derivated to a reactive compound in three phases (Ferreira and Janick, 1996).

Phase 1. Reduction of artemisinin: Five hundred mg (1.77 mmol) artemisinin (Alexi's biochemicals, France) was dissolved in 40 ml of methanol (Merck, Germany) at room temperature. The solution was cooled to 0-5°C and then 300 mg (17.9 mmol) of $NaBH_4$ was added over a period of 30 minutes. A TLC system (2% methanol in $CHCl_3$) was used to monitor the reaction until artemisinin was completely
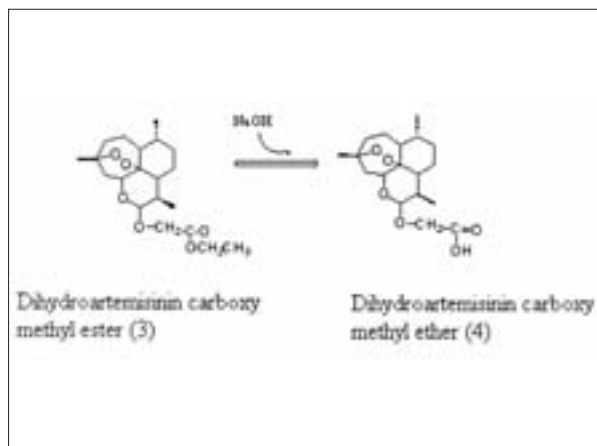
Fig. 3- Hydrolysis of the ester moiety of dihydroartemisinin carboxy methyl ester to yield dihydroartemisinin carboxy methyl ether in the third stage of artemisinin derivatization.

Fig. 4- Immunoblot of preimmune serum that shows there is no spot on place of BSA, BSA-Artemisinin conjugate and Artemisinin indicating that the antibody has not been induced against these compounds in serum.

reduced during 2-3 hours. The reaction was stopped by decreasing the pH to 5-6 using 2.6 ml of 30% acetic acid in methanol. Methanol was evaporated by a vacuum system and then the reduced artemisinin (dihydroartemisinin) was washed out three times by 50 ml of ethyl acetate (Sigma -Aldrich, USA). Each wash was separately filtered through a Whatman filter paper no.1 (Schiecher-Schuell, Germany), mixed with others and refiltered under vacuum. The clear ethyl acetate solution was evaporated to yield 84% of dihydroartemisinin. (Fig. 1)

Phase 2. Addition of an ester moiety to dihydroartemisinin: The reaction was initiated by dissolving 420 mg (148 mmol) of dihydroartemisinin in 25 ml of anhydrous diethyl ether (Merck, Germany), dried with Na-benzophenone, and then 3 gr of dry molecular sieve (3A$^{\circ}$, Sigma, USA) and 7.4 mmol of ethyl glycolate (700µl, Aldrich,USA) were added to the mixture. The temperature of the solution was reduced to 0°C, and then 3 drops of 50% solution of boron trifluoride etherate (Merck, Germany) in anhydrous diethyl ether were added to it. The mixture was stirred for 5 hours under nitrogen and monitored by TLC (20% ethyl acetate in petroleum ether). An additional 15 ml of anhydrous diethyl ether, 700 µl of ethyl glycolate and 1 drop of boron trifluoride etherate were added to the mixture. The mixture was stirred for additional 3 hours until no signs of dihydroartemisinin could be detected on TLC (20% ethyl acetate in petroleum ether). The molecular

sieves were filtered and washed out with anhydrous diethyl ether, and then the solvent was evaporated to yield a yellowish oily compound. This oily compound was extracted 3 times with petroleum ether (Merck, Germany) and then the petroleum ether was evaporated to obtain a colorless oily compound. (Fig.2)

Phase 3. Hydrolysis of the ester to yield the carboxy group: One equivalent NaOH (2.86 mmol or 114 mg) was dissolved in 50 ml of 95% ethanol, and then it was added to dihydroartemisinin carboxymethylester compound obtained at phase 2. The mixture was stirred at room temperature over night to produce the sodium salt of the ester. Ethanol was evaporated under vacuum and the remaining solid was washed out with ethyl acetate. To remove the remaining starting materials, the sodium salt of the ester was dissolved in water and the clear solution was acidified using 1N HCl. The resulting product which was a white solid, was extracted with ethyl acetate, and then washed with water and dried with Na_2SO_4. The solvent was evaporated to obtain 230mg of a crude epimeric mixture (α , β) of the desired carboxylic acid (dihydroartemisinin carboxymethylether) (Fig.3). This compound was purified by column chromatography using Silica gel (Merck, Germany 230-400 mesh) with a mobile phase of 40% ethyl acetate, 59% hexane (Merck, Germany), and 1% acetic acid. Phosphomolibdic
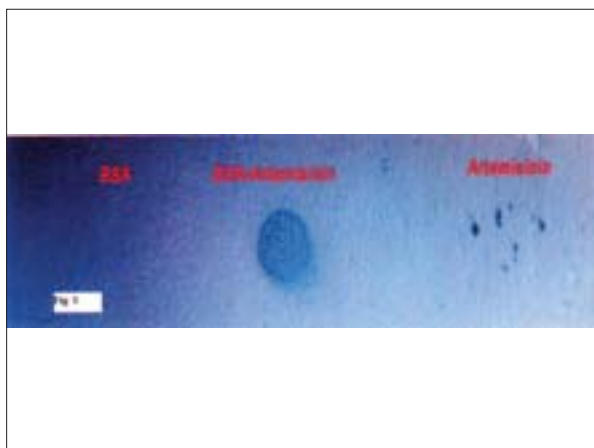
Fig. 5- Immunoblot of immune serum illustrating two clear blue
spots where artemisinin alone and artemisinin-BSA conjugate were
fixed and reacted with immune serum.

Fig. 6- Immunohistochemical staining of the cecal tissue in which
entrocyte of the epithelium in brown color indicating the presence of
artemisinin and inflammatory cells are evident.

acid (PMA, Fluka, USA) solution was used to stain
the spots in TLC.

Conjugation of dihydroartemisinin carboxy
methyl ether to BSA: When TLC confirmed the
derivatization process, the conjugation of
dihydroartemisinin carboxymethylether to BSA was
started using mixed anhydride method (Ferreira and
Janick, 1996). 40 mg (0.117 mmol) of
dihydroartemisinin carboxymethylether was
dissolved in 2 ml of anhydrous dioxane and kept on
ice. 100 mg (0.0015 mmol) BSA fraction V (Sigma-
Aldrich, USA) was dissolved in 5 ml of doubled
distilled water while pH was adjusted to 9.5, cooled
to 10°C, and then 2 ml of anhydrous dioxane was
added to the solution. 50 μl of tri-n-butyl amine
(Merck, Germany) was added to dihydroartemisinin
carboxymethylether solution and stirred under
nitrogen for 30 minutes at 10°C. Thirty μl isobutyl
chloroformate (Merck, Germany) was added to this
solution and then it was continually stirred under
nitrogen for 20 minutes at 10°C. After adding of
another 2ml anhydrous dioxane to this mixture, it was
added drop wise to the BSA solution in H_2O/dioxane
(5:2) whilst it was stirred in an ice-cold temperature.
When two solutions were mixed, the pH of the
mixture decreased to 6.9, and then it was adjusted to
7.4 by addition of 1ml $NaHCO_3$. The solution was
stirred overnight in a cold room and then it was
centrifuged at 13000g for 5 minutes. The supernatant
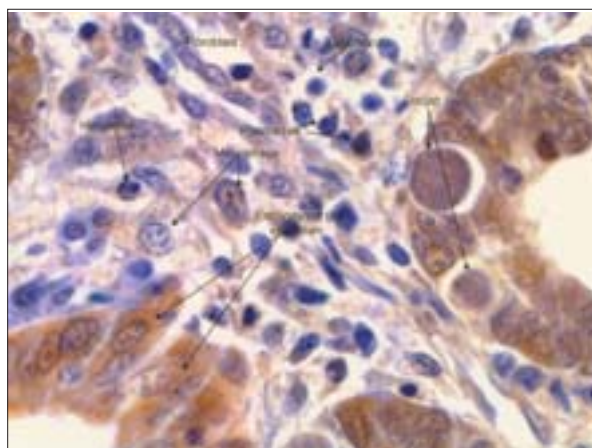was dialyzed in water in a cold room for 3 days (2

changes of 1 liter water per day).

Animal immunization: Three white adult female
New Zealand rabbits were prepared from Pasteur
institute (Iran Pasteuv Institute, Tehran,Iran) and
kept on a standard diet and environmental conditions.
A blood sample was taken from each animal as
negative control and then two intramuscular
injections carried out to induce immunization. The
first injection was consisted of 500 μg
BSA+artemisinin conjugate (1:1 ratio) in PBS, and
then it was mixed and emulsified with complete
Freund's adjuvant to a final volume of 1ml for each
rabbit. Two weeks later, a booster injection was done
with 500 μg BSA+ artemisinin conjugate mixed with
incomplete Freund's adjuvant (1:1) to a final volume
of 1ml. After 14 days, 20-30 ml blood sample was
collected from each rabbit via jugular vein, and then
they were centrifuged to separate the sera.

Antibody production assessment: A standard
Immunoblots method (Ausubel *et al*., 2002) was used
to assay production of antibody against BSA-
artemisinin conjugate. Aliquots of 5 μl artemisinin, 5
μl BSA-artemisinin conjugate and 5 μl of BSA were
spotted on PVDF membranes (0.45-mm pore size,
Roche, Laboratories, Germany) and positively
charged Nylon membrane (Roche, laboratory,
Germany). Membranes were blocked with 5% skim
milk in physiologic buffer solution (PBS, 0.02 M Tris
base-0.385 M NaCl-0.1%) and washed with Tris-
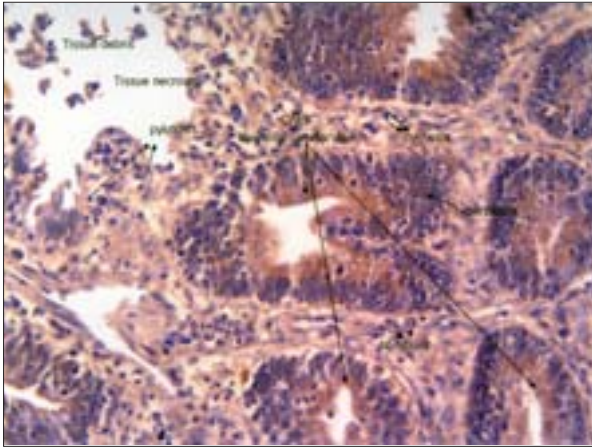buffered saline containing 0.05% Tween 20 (TPBS).

Fig. 7- Immunohistochemical staining of the cecal tissue in which entrocyte of the crypt of Liberkoen in brown color indicating the presence of artemisinin and trace of inflammation are seen.
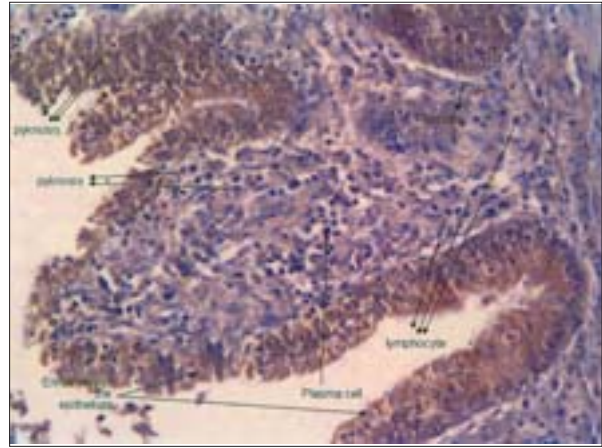

Fig. 8- Immunohistochemical staining of the duodenal tiuuse in which epithelium of the vilus is stained brown color indicating the presence of artemisinin.

The membranes were incubated separately with specific polyclonal antibody provided against artemisinin, and then preimmune and immune serum samples diluted in TPBS-BSA (PBS 0.02%, Tween 20 and 3% BSA) at 37°C for 2h and it was washed again. The labeled protein G recombinant-proxidase (1:500) (Sigma, Germany) was used in place of secondary antibody for primary sera. The membranes were incubated with protein G for 1h at 37°C, and washed out three times with TPBS. The reaction was developed with 0.3mg α-chloronaphtol (Sigma, Germany) in 100ml of PBS containing 30μl of 30% H$_2$O$_2$, and then the sheet paper membranes were placed in running tap water for 5min to stop the reaction.

When Immunoblot confirmed the production of antibody, saturated ammonium sulfate was added for antibody separation and concentration (Hudson and hay, 1989). Finally the concentrated antibody was labeled using Fluorscein Isothiocyanate (FITC) (Gosling, 2000). IHC examination by using Avidin-Biotine Complex technique and produced polyclonal anti artemisinin antibody was done on intestinal samples of chickens infected with experimental coccidiosis and received artemisinin orally. (Data not shown).

Results

Artemisinin derivatization and conjugation

At the first phase of derivazation, dihydroartemisinin

was obtained by evaporation of clear ethyl acetate solution. The second phase of derivazation resulted in the attachment of an ester moiety to dihydroartemisinin. After the extraction of the oily compound with petroleum ether, a colorless oily compound containing a mixture of $\alpha.\beta$ epimers of dihydroartemisinin carboxymethylester was obtained. In the third phase of artemisinin derivatization, a carboxy group was added on the dihydroxyartemisinin through hydrolysis of the ester. The dilydoryartemisinin carboxy methyl ether was purified by column chromatography and its structure was confirmed by TLC spot. The TLC analysis was used for monitoring of the progress of the reactions during all phases.

Antibody production and IHC: The inoculation of conjugated dihydroartemisinin carboxymethylether +BSA to rabbit successfully induces antibody production against artemisinin. During Immunoblots, it was found that the Nylon membrane was better than PVDF membrane, since PVDF could not absorb artemisinin. Therefore, here we have shown only the result obtained from the experiments using Nylon membrane. The results of the Immunoblots assessments have been shown in Figure 4 (preimmune spots) and 5 (immune serum). The antibody production assay showed while the blue spots were not appeared on the membrane one, there were two spots on the membrane two; one in the place of artemisinin alone and the second was on the place of BSA-artemisinin conjugate. As Figure 4

and 5 shows there were no spot on place of BSA, indicating that there was no antibody against this compound in preimmune and immune serum. However, Figure 5 illustrates that there are two clear blue spots where artemisinin alone and artemisinin+BSA conjugate were fixed and reacted with immune serum indicating the presence of anti artemisinin and anti BSA+artemisinin conjugate in the immune serum.

Figures 6 and 7 show the IHC of the cecal tissue of chicken received artemisinin and Figure 8 shows the ICH of Duodenal tissue in which brown colored area of the epithelial tissue is positively stained with artemisinin.

Discussion

In the present study, we successfully produced a polyclonal antibody against BSA+artemisinin conjugate that is useful for Immunohistochemistry work. Because of the hapten nature of the artemisinin, at first a reactive carboxy group was added on it, and then it was conjugated with a suitable carrier, BSA, using mixed anhydride method. TLC studies confirmed the artemisinin derivatization while an endoperoxide group remained intact. The compound obtained at the end of the derivatization had a reactive carboxy group appropriate for conjugation to BSA and induction of immunization in rabbit. After injection of conjugated compound, the Immunoblot assay showed the success of immunization, leading to production of antibody against artemisinin. The production of antibody against artemisinin showed that the BSA successfully linked to dihydroartemisinin carboxymethylether compound by its carboxyl group using the mixed anhydride method.

Development of drug resistance against conventional anti coccidian drugs has paid the increasing attention toward the development of new groups of drugs including artemisinin. The effects of artemisinin on avian coccidiosis especially against *E. tenella* have been shown by few studies (Patricia *et al*., 1997; Arab *et al*., 2006). However, there is no evidence to show the mechanism(s) by which artemisinin exerts its anti coccidian effect. One of the

simplest ways to understand the mechanism of anti coccidian effects of artemisinin is to produce antibody against it and then trace the site of action of the compound by Immunohistochemistry technique.

Ferreira and Janick (1996) in an attempt to produce polyclonal antibodies against artemisinin extracted from *Artemisia annua* linked the dihydroartemisinin carboxymethylether to either TGB or BSA. The artemisinin+TGB and -BSA conjugates were injected to rabbits, but only artemisinin+TGB was able to induce the antibody production which was attributed to failure in suitable conjugation of artemisnin to BSA. Ferreira *et al* used an ELISA method to confirm the production and specificity of the antibodies. (Ferreira and Janick, 1996). While they failed to make an active conjugation of BSA+artemisinin to induce antibodies, we could successfully conjugate artemisinin with BSA using a modified immunization. In our work the raise of artemisinin polyclonal antibody was confirmed by Immunoblot method.

Artemisinin is a hapten and by itself can not be used to induce antibody. Hapten is a non antigenic compound, usually with low molecular weight that can not stimulate the immune system. But if it be conjugated with an antigenic compound (carrier), an antigenic character can be created in the conjugation. Hapten-carrier complex or new antigen is able to induce antibody production so that the specificity of antibody is preserved for hapten (Lansteiner, 1962).

If an animal is immunized against hapten+carrier complex, in addition to the anti hapten antibody and anti carrier antibody, there are specific antibodies which are directed against epitopes resulted from hatpen-carrier conjugation. Thus, when a complex of hapten-carrier is injected to animals, different antibodies will be produced against hapten, carrier and carrier-hapten complex (Lansteiner, 1962). In the present study, the immunoblots test showed that antibodies were raised against both artemisinin (hapten) and artemisinin+BSA (hapten carrier complex). BSA can not stimulate rabbit immune system and thus will not produce antibody against itself. Because, it is a heterologus antigen and there

are many epitopic similarities between BSA and rabbit serum albumin (Lansteiner, 1962). There is evidence that BSA cleared of its larger phagocytable particles, is less immunogenic than the regular one. In addition, BSA is sometimes able to induce tolerance at dose 1mg in the rabbit, whereas at 0.1 mg induced slightly less tolerance but the difference was not significant (Frei *et al*., 1968). The concentration of BSA used in our study was in the range to induce tolerance and thus no antibody was induced against it.

Artemisinin and its derivatives are found to be changed into an active metabolite, dihydroartemisinin, during their metabolisms in the body (Navartnam *et al*., 2000). Therefore, antibody produced against artemisinin+BSA conjugate would be useful if it can react with this metabolite. Ferreira and Janick (1996) have shown cross reactivity of anti artemisinin antibody against artemisitene, dihydroartemisinin, arteannuin B, and artmisinic acid using polyclonal antibodies induced by injection of artemisinin+TGB conjugation in the rabbit (Ferreira and Janick, 1996). In addition suitable results obtained from IHC examination using produced anti artemisinin antibody practically confirmed the usefulness of the antibody and its cross reactivity with artemisinin metabolite(s). Thus, it seems that the polyclonal antibody induced against artemisinin can be used to detect artemisinin and different derivatives including dihydroartemisinin in the body tissues.

In conclusion, we could successfully raise a poly clonal antibody against BSA conjugated artemisinin in rabbits. This production can be a fundamental tool to investigate pharmacodynamics and pharmacokinetics of artemisinin and its derivatives in different animals by immunohistochemical technique.

Acknowledgement

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تولید آنتی‌بادی پلی کلونال علیه آرته میزینین الحاق شده به آلبومین سرم گاو در خرگوش

جهانگیر کبوتری ۱   حسینعلی عرب ۲*   علیرضا فرومدی ۳   غلامرضا نیکبخت ۴   مهدی طاهری ۵

۱)گروه علوم پایه دانشکده دامپزشکی دانشگاه شهر کرد، شهر کرد ـ ایران.

۲)بخش فارماکولوژی دانشکده دامپزشکی دانشگاه تهران، تهران ـ ایران.

۳)مرکز تحقیقات علوم دارویی دانشکده داروسازی، دانشگاه علوم پزشکی تهران، تهران ـ ایران.

۴)گروه پاتوبیولوژی دانشکده دامپزشکی دانشگاه تهران، تهران ـ ایران.

۵)آزمایشگاه مرکزی دانشکده دامپزشکی دانشگاه تهران، تهران ـ ایران.

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چکیده

هدف از مطالعه حاضر تولید یک آنتی‌بادی پلی کلونال علیه آرته میزینین الحاق شده به آلبومین سرم گاو بوده است. برای ایجاد خاصیت ایمونوژنیک در آرته میزینین با استفاده از روش آنهیدرات مخلوط، یک گروه کربوکسیل به ساختمان شیمیایی آرته میزینین اضافه شد. سپس، این ترکیب واکنشگر آرته میزینین با سرم آلبومین گاو متصل گردید. ترکیب آرته میزینین با آلبومین دوبار به خرگوش سفید نیوزلندی تزریق شد. در تزریق اول از ادجووانت فروند کامل استفاده شد و دو هفته بعد یک تزریق یادآور با ادجووانت ناکامل فروند انجام گردید. دو هفته بعد از تزریق دوم، نمونه‌های خونی جمع‌آوری و سرم‌ها جدا گردیدند و تا زمان آنالیز در فریزر نگهداری شدند. آنتی‌بادی تولید شده پس از ارزیابی با تکنیک ایمونوبلات، از سرم‌ها جدا و سپس با استفاده از روش سولفات آمونیوم اشباع شده تغلیظ گردید. سنجش انجام شده بر روی آنتی‌بادی جدا شده از سرم خرگوش، حاکی از آن بود که این آنتی‌بادی علیه آرته میزینین الحاق شده با آلبومین تولید شده است. این تولید می‌تواند یک قدم اساسی در جهت مطالعات فارماکودینامی و فارماکوکینتیک آرته میینین در حیوانات مختلف باشد.

واژه‌های کلیدی: درمنه سیبری، آرته میزینین، آنتی‌بادی پلی کلونال، آلبومین الحاق شده.

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*) نویسنده مسؤول: تلفن: ۶۱۱۱۷۰۸۶ ـ ۰۲۱   نمابر: ۶۶۹۳۳۲۲۲ ـ ۰۲۱   Email: harab@ut.ac.ir