

Effect of *Nocardia* on immune response of Heat Killed *Candida albicans* Antigen against systemic candidiasis

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Summary

This study was undertaken to prepare Heat Killed *Candida albicans* Antigen (HK-CA) and to investigate its effect on elicitation of Delayed-type Hypersensitivity (DTH) and protection from virulent *Candida* challenged in mice, and described the major effect of *Nocardia* on the immunogenicity of this Ag. Four groups of 6 males Swiss mice for each group were immunized intraperitoneally (I.p) for 10 days at 5 doses (one day intervals) with 1×10^8 HK-CA plus *Nocardia* cell sap as adjuvant (CFA), the second group immunized with HK-CA plus Freund incomplete adjuvant, the third group immunized with HK-CA only and the fourth group treated with normal saline. Another group of 6 animals was immunized intranasally (I.n) with HK-CA only with the same doses as previously. Two weeks following the last application of Ag, 50% of animals (3 for each group) were challenged intravenously (I.v) with 10^6 viable *C. albicans* to assess the protection rate and the other 50% of mice were used to evaluate the development of DTH response in each immunization method. Greatly percentage survival was observed when mice immunized with HK-CA plus CFA, which challenged with 10^6 live *C. albicans* in comparison with other groups. And also all animals of the 1st group developed highly significant DTH response which was supported by marked infiltration and aggregation of lymphocyte in some internal organs that indicate its intensive involvement in immune response, while in the other groups, mononuclear cells infiltration were present with moderate to less intensive.

Clearly, HK-CA in conjunction with *Nocardia* cell sap as adjuvant afforded significant levels of protection. Furthermore, the novel approach of I.n immunization could be used for the development of an effective vaccine.

Key words : *Nocardia* , *Candida albicans* , Immune response , Candidiasis .

تأثير جرثومة النوكارديا على الاستجابة المناعية لمستضد خميرة المبيضات البيضاء المقتولة بالحرارة ضد الإصابة الجهازية بهذه الخميرة

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الخلاصة

تضمنت هذه الدراسة تحضير مستضد من المبيضات البيضاء المقتولة بالحرارة للتحري عن قابليته في اظهار فرط الحساسية المتأخر كذلك قدرته على حماية الفئران عند اجراء فحص التحدي بالخميرة الحية الضارية. كما انه شملت دراسة تأثير جرثومة النوكارديا على الاستجابة المناعية ضد هذا المستضد اذ تم اخذ 4 مجاميع من ذكور الفئران السويسرية (6 فئران لكل مجموعة) ومنعت داخل الخلب وبخمس جرع لمدة 10 ايام. كانت المجموعة الأولى منعمة بال HK-CA الممزوج بجرثومة النوكارديا المحطمة كعامل مساعد (CFA). اما المجموعة الثانية فقد منعت بال HK-CA الممزوج المساعد غير

الكامل (FIA) والمجموعة الثالثة منعت بال HK-CA فقط. اما المجموعة الرابعة فقد حققت بالمحلول الملحي الفسلجي (مجموعة سيطرة). اخذت 6 حيوانات اخرى ومنعت عن طريق الأغشية المخاطية عبر الأنف بال HK-CA فقط وحسب الجرعة المذكورة اعلاه. وبعد اسبوعين من اخر جرعة, اجري فحص التحدي ل50% من الحيوانات الممنعة (3 فئران لكل مجموعة) وذلك بحقن الخميرة الضارية عن طريق الوريد الذيلي لغرض معرفة نسبة الحماية المتكونة بفعل هذه المستضدات. اما إختبار فرط الحساسية المتأخر فقد اجري لل 50% الأخرى من الحيوانات الممنعة. اظهرت النتائج ان اعلى نسبة حماية كانت في المجموعة الأولى التي تمثلت ببقاء نسبة عالية من الحيوانات حية طيلة مدة التجربة مقارنة مع بقية المجاميع. كذلك فإن حيوانات المجموعة الأولى اظهرت استجابة مناعية عالية لإختبار DTH والذي أدمع بنتائج الفحص النسجي من خلال ملاحظة ارتشاح وتجمع ملحوظ للخلايا المناعية في بعض الأعضاء الداخلية للحيوانات الممنعة والذي يدل على شمول هذه الخلايا في احداث استجابة مناعية قوية, بينما لوحظ ارتشاح لهذه الخلايا في بقية المجاميع لكن بنسب متفاوتة. ومن هذه نستنتج ان HK-CA المرتبط مع جرثومة النوكارديا المحطمة كعامل مساعد اعطى مستوى معنوي عالٍ من الحماية. علاوة على ذلك فإنه يفضل استخدام طريقة التمنيع عبر الأغشية المخاطية في الأنف للحصول على المناعة ضد الأصابة الجهازية بخميرة الكانديدا.

Introduction

Fungal diseases are wide spread and increasing in frequency, especially among immunocompromised patients. Fungal infections caused by *Candida* have been known since the fourth century B.C.E. and are responsible for the majority of fungal infections currently diagnosed. There has been an increase in efforts over the last few years to identify antifungal strategies and compounds (1). The main difficulty is that fungi as eukaryotes, have cellular machinery very close to that of human, making it extremely difficult to identify points that are lethal for the fungus but not the patients. Currently available antifungal agents either toxic or act as a fungistatic manner, and resistance to these agents is already an emerging problem (2). On the other hand, the approach to the development of a vaccine is problematic because the specific immune system mechanisms responsible for protective immunity have not been defined clearly. Candidiasis is a multifaceted disease which may manifest its self at multiple levels, including mucocutaneous tissue and internal organs (3) and the life-threatening forms of candidiasis occur at the systemic level, so the development of vaccine for the disease must be empirical (4).

The best protective effects observed to date have been stimulated by immunization with viable cells from virulent (5) or avirulent (6) strain of *C. albicans*. and even when used non viable *C. albicans*, there have been three reports of significant levels of protective immunity stimulated by *C. albicans* ribosomes, a mannoprotein and antibody to an extracellular candidal proteins (7 and 8).

Aim of present work was to found the effect of *Nocardia* as adjuvant with heat killed *Candida albicans* (HKCA) Ag. on stimulation of immune response against intravenous (I.v) challenge with live, virulent *C. albicans*.

Materials and methods

First experiment:

I- Vaccine preparation: *C. albicans* was obtained from mycological unit of Microbiology department in Veterinary college. This strain was maintained at 4 °C with monthly transfer on Sabouraud dextrose agar (SDA). Cultures of *C. albicans* were incubated for 24 hrs, on SDA at 37°C, inoculated into Brain Heart Broth. The cells were harvested after 18 hrs, and washed three times in normal saline by centrifugation. The final pellet was resuspended in normal saline and the cells were counted in hemocytometer and diluted to 10^8 cell/ml. The cell suspension was heated at 60°C for 6 hrs. The loss of viability of this preparation was confirmed by plating 1 ml of 10^8 cell on SDA and incubating at 37°C overnight. This was Heat Killed *Candida albicans* (HK-CA) according to (9).

II- Preparation of *Nocardia* as an adjuvant: *Nocardia asteroides* was isolated from internal organs of digestive system of chicken and tested to insure the biochemical properties according to (10 and 11). The *Nocardia* was incubated for 5 days at 37°C on SDA. Then the cells were harvested and washed three times by normal saline. The cells were fractionated by ultrasonication for 50 min. and centrifuged by cold centrifuge at 8000 rpm/45 min. to take precipitate as cell wall debris (12).

Freund incomplete adjuvant (FIA) was completed by adding 0.3 mg of *Nocardia* cell wall debris for each 1 ml of FIA. Then 1 ml of this complete adjuvant (CA) was taken and mixed with 1-2 ml of HK-CA then injected into the test animals (13).

III- Immunization procedure: Thirty Swiss mice were taken and divided randomly into five groups. This study consists of two types of immunological methods: The first method was done according to ((14) with some modification) which immunized intraperitoneally (I.p) that contain 4 groups of 6 animals (for each group) at 6-8 weeks of age. The first group was immunized (I.p) for 10 days at 5 doses (one day intervals) with 0.2 ml of 10^8 cells of HK-CA in conjunction with CFA. Second group immunized with 0.2 ml of 10^8 cells of HK-CA in conjunction with FIA. The third group immunized with 0.2 ml of 10^8 cells of HK-CA only, and fourth group inoculated with normal saline. The second method of immunization was intranasally (I.n) in which the mice was lightly anesthetized with ether then immunized with 0.05 ml of 10^8 cells of HK-CA for 10 days at 5 doses as previously (scale 1). Fourteen days after the last immunization of both two methods, 50% of the animals (3 for each group) were challenged intravenously via the lateral tail vein with 10^6 viable *C. albicans* to determine the protection. And the other 50% of animals were used in the 2nd set of experiment to analyze the levels of delayed-type hypersensitivity (DTH).

Second experiment:

The development of DTH in response to each immunization methods was determined by the footpad thickness which was measured 24 and 48 hrs, after 0.02 ml of soluble Ag injected to immunized animals(15)and 0.02 ml of normal saline to control group.The mean of thickness was calculated by subtracting pre-injection measurements from post injection measurement of skin.At the end of this experiment,all the surviving animals were sacrificed for collection of some internal organs(lung,spleen,liver,kidney) to study the histopathological changes in these animals.

Results

Protective immunity following immunization

The result of the first experiments demonstrated that the animals vaccinated intraperitoneally with HK-CA plus CFA were survived for more than 45 days after challenged while all animals in the control group were dead within 48-72 hrs, post challenge and the other groups of animals showed some protection with various levels of immunity,i.e a greater survivor rate was observed for animal immunized with HK-CA plus CFA.however,the HK-CA antigen was stimulated different levels of immune response in these animals.

Cellular immunity in immunized animals

The result of the second experiment indicated that animals immunized with HK-CA plus CFA developed substantial and highly significant of immune response according to the mean of thickness of footpad which measured by caliper at the level $p < 0.05$ which reached to $1.4 \pm 0.2, 1.7 \pm 0.1$ after 24-48 hr, respectively,while the second group (HK-CA plus IFA) the mean of thickness was seemed less than first group.In the third group which represent those animals immunized intraperitoneally with HK-CA alone was developed minimal response,whereas the fourth group that immunized intranasally with HK-CA only gave result relatively similar to the second group,while the mice inoculated with normal saline no DTH was developed .All the results showed that the groups which measured after 24 hr,of inoculation was higher than the groups measured after 48 hr. of inoculation except the first group as shown in table 1

Table 1: DTH in mice immunized with HK-CA mixed with or without adjuvant after 2 weeks of last dose of antigens.

Type of antigen	After 24 hr. of inoculation (mm)	After 48 hr. of inoculation (mm)
HK-CA+CFA	1.4±0.2	1.7±0.1
HK-CA+IFA	1.2±0.2	0.9±0.2
HK-CA only(I.p)	0.03±0.2	0.6±0.2
HK-CA only(I.n)	1.1±0.1	0.87±0.1

LSD= 0.4

Histopathological changes

The organs of first group that immunized with HK-CA+CFA showed: More mononuclear cells infiltration in the interstitial tissue of lung (Fig. 1). The spleen showed more intensive hyperplasia of lymphocytic cells in the periarteriolar sheath with hyperatrophy of muscular layer of central artery,also

the microscopic section showed mononuclear cells aggregation around the central artery (Fig. 2). The kidney showed mononuclear cells aggregation around blood vessel and between renal tubules (Fig. 3).while The liver showed marked mononuclear cells aggregation around the central vein and in the liver parenchyma as well as in the lumen of the central vein (Fig. 4).

The organs of second group that immunized with HK-CA+FIA showed: Large amount of mononuclear cells infiltration and in the capsular layer in the kidney (Fig. 5).and the histological section of spleen and liver was similar to that reported in the previous group but less marked.

The organs of third group that immunized with HK-CA only(I.p) showed: There was no clear pathological lesions seen in the kidney,heart and liver(Fig. 6and7).while The microscopic section of spleen was similar to that reported in the previous group but less intensive (Fig. 8).and Histological examination of the lung showed mononuclear cells aggregation around blood vessels and in the interstitial tissue (Fig. 9).

The organs of fourth group that immunized with HK-CA only(I.n) showed: All Histological examination of the target organs (Kidney,liver,heart) were similar to that of second group.

Discussion

The present study, examined the ability of whole killed *C. albicans* mixed with *Nocardia* cell sap as adjuvant to stimulate high levels of protection against systemic challenge with highly virulent strain of *C. albicans* and this protective immunity was associated with substantial levels of DTH.

The reason of using *Nocardia* in this study was refered to the bacteria possess participated antigenic relationship with *Mycobacteria* especially which was named antigenic 3rd group that consist of 4 Ags shared with *Mycobacteria* (16), so present work could benefit from this antigenic relationship to prepare some types of adjuvants due to easily culturing of this bacteria,shortness of incubation period and available of suitable culture media for it . previously study that carried out to analyse cell wall of *Nocardia*,it was shown that this bacteria has cell wall type IV which contain Meso-diamino-pimilic acid (Meso-DAP),arabinose and galactose also most types of Ags are polysaccharide particularly arabinogalactan and arabinomannose which are similar to cell wall component of *Mycobacteria* and almost of these are responsible for cross-reactions that take place in skin test and serological tests for diagnosis(17).

In this study, observed solid protection against lethal I.v challenged when immunized with HK-CA mixed with this adjuvant due to strong DTH response and this may be refer to presence of peptidoglycan layer which possess immunostimulatory properties like generation of DTH in mice and this agree with(18).

In other hand, because the development of anticandidial cellular immunity in mice is strictly dependant on the activation of CD₄⁺Th₁cells,the HK-CA was an obvious sensible candidate for evaluating the ability of selected *C. albicans* Ags to elicit Th₁-mediated function and protection in the mice and this is

highlighted by(19)which lead to production of lymphokine then infiltration and aggregation of lymphocytes in the site of injection with edema and congestion of blood vessels as pointed by(15),and this ensure results that obtained in this study,when skin thickness occurred in footpad of mice especially in the first group of animals and reached to the peak after 48 hr. of reaction is due to accumulation of these cells in this area which reached to 100 times more than in normal state(20)due to secretion of chemotactic factors that attract macrophages and suppress it's migration via migration inhibition factor which play an important role in cellular response,whereas the occurrence of erythema at site of injection is due to liberation of skin reaction factor then increased blood flow to the injected area in which inflammatory immunosensitization process occur .These results agree with (21and22).The present results could infered that *Nocardia* play an important role when added to oil factors that mixed with vaccines then could be used in vaccination protocols,and this was revealed by (23) which reported that mixing antigenic components of *Brucella abortus* with *Nocardia asteroides* stimulated the immune response better than using the antigen alone.

The second part of the present study which immunized intranasally with HK-CA that stimulated significant level of protection against systemic challenge with a highly virulent strain of *C. albicans*.The result of this group was nearer to that obtained in the group which was immunized intraperitoneally with HK-CA that mixed with IFA when DTH reaction was carried out.This may be explained due to the role of CD_4^+ lymphocyte in resistance to mucosal candidiasis as pointed by(24) because the mucosal exposure stimulates a protective systemic immunity due to T-lymphocytes which are clearly involved in protection mechanisms at both mucosal and systemic levels,so the present result suggested intranasal immunization with HK-CA because smallest dose of inoculum and don't need to any adjuvant.With regarding to histopathological changes,generally it was observed the presence of infiltration and aggregation of lymphocytes with different ratios between the groups.

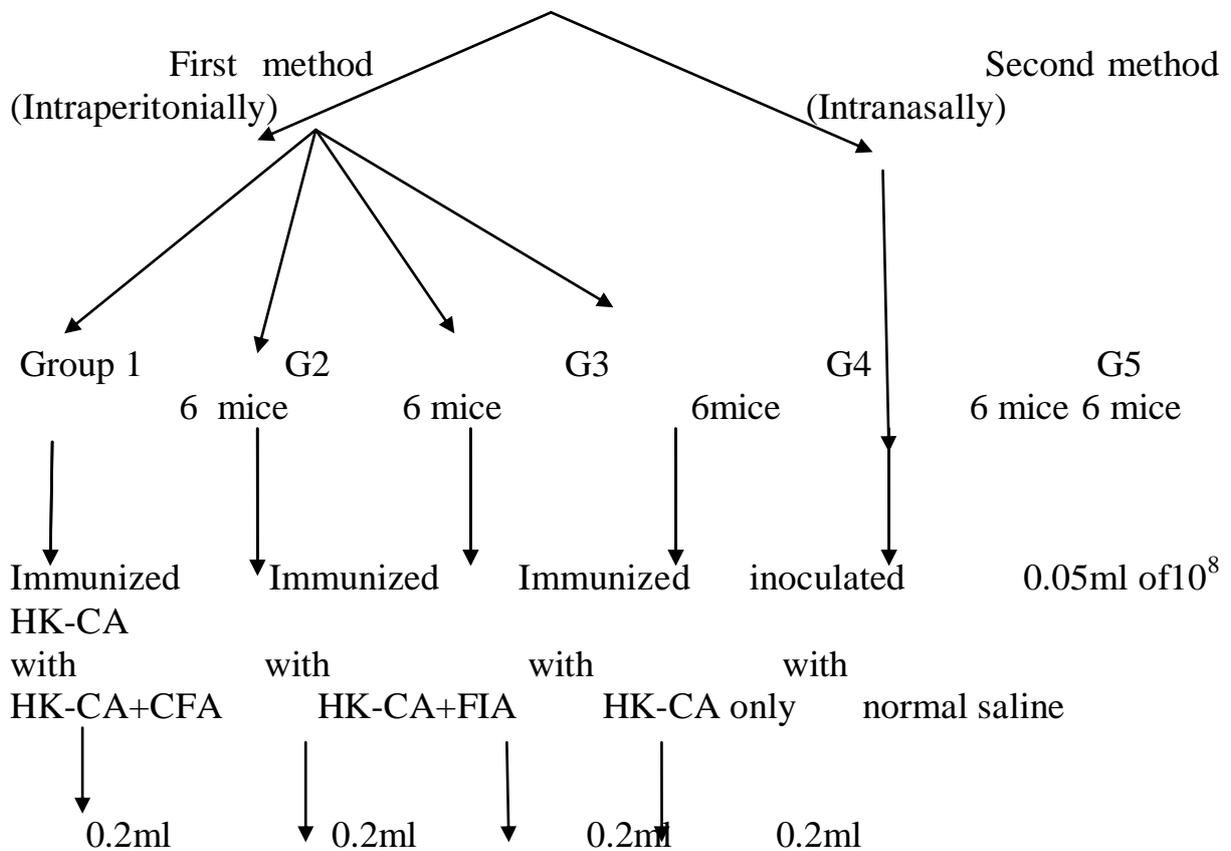
In other studies(25),suggested that muramyl dipeptide(MDP) which is a common structure of peptidoglycan of certain bacterial cell wall stimulated macrophages and increase number of bone marrow macrophage(mononuclear cell) progenitor cells,so the existence of large number macrophage(mononuclear cell) that appeared essentially in the first group indicate it's intensive involvement in the immune response.While other groups,mononuclear cells infiltration are present with moderate to less intensive as mentioned by(26) who observed proliferation of human peripheral blood mononuclear cells was induced by *C. albicans* and it's cell wall fractions.

In conclusion,the *Nocardia* cell sap(as adjuvant) can induce protective immunity when coadministered with whole inactivated *C. albicans* because this adjuvant promote the development of cell mediated immune response against fungi,as well as, there are no vaccines available for human mycosis and there is an urgent need to develop measures of prophylactic immunointervention against

fungal pathogens, the concept of using inactivated *C. albicans* as a component of a potential anticandida vaccine is attractive because of its safety, ease and low cost associated with the preparation of large number of cells particularly when immunized intranasally.

(Scale 1)

Immunization methods



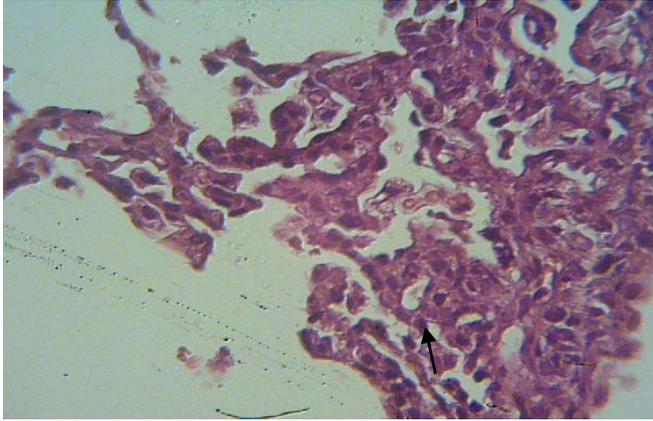


Fig 1: Histological section of the mice lung at 2 weeks post immunization with HK-CA+FCA showed more mononuclear cells infiltration in the interstitial tissue().H&E stain(400X).

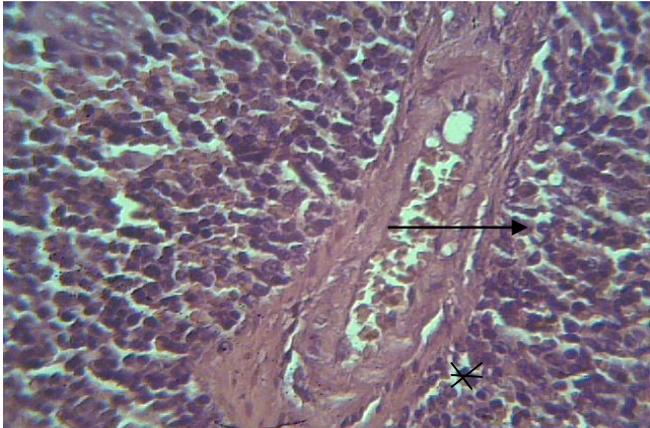


Fig 2: Histological section of the mice spleen at 2 weeks post immunization with HK-CA+FCA showed more intensive hyperplasia of lymphocytic cells in the periarteriolar sheath.(→) with hyper-trophy of muscular layer of central artery(*),also the microscopic section showed mononuclear cells aggregation around the central artery.H&E stain(400X).

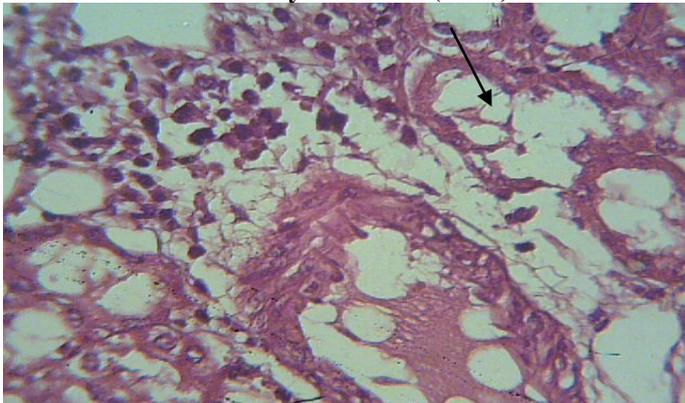


Fig 3: Histological section of the mice kidney at 2 weeks post immunization with HK-CA+FCA showed mononuclear cells aggregation around blood vessel and between renal tubules().H&E.

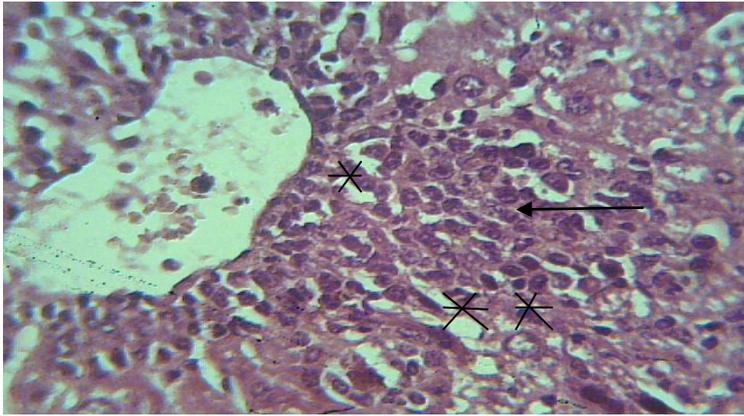


Fig 4 : Histological section of the mice liver at 2 weeks post immunization with HK-CA+FCA showed marked mononuclear cells aggregation around the central vein(→) and in the liver parenchyma(*) as well as in the lumen of the central vein(**).H&E stain.

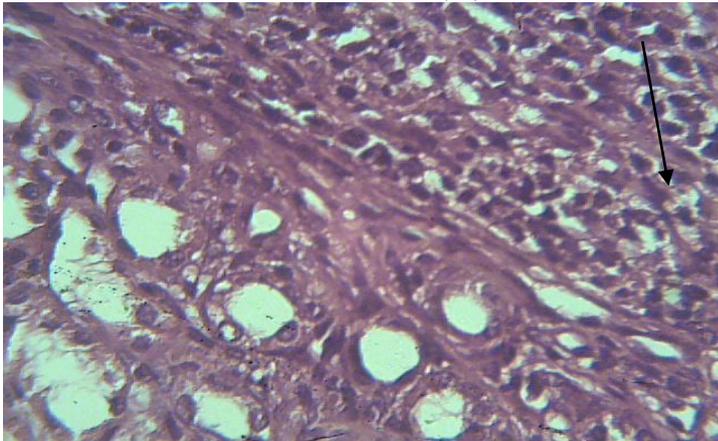


Fig 5: Histological section of the mice kidney at 2 weeks post immunization with HK-CA+FIA showed large amount of mononuclear cells infiltration and in the capsular layer(→)H&E stain.

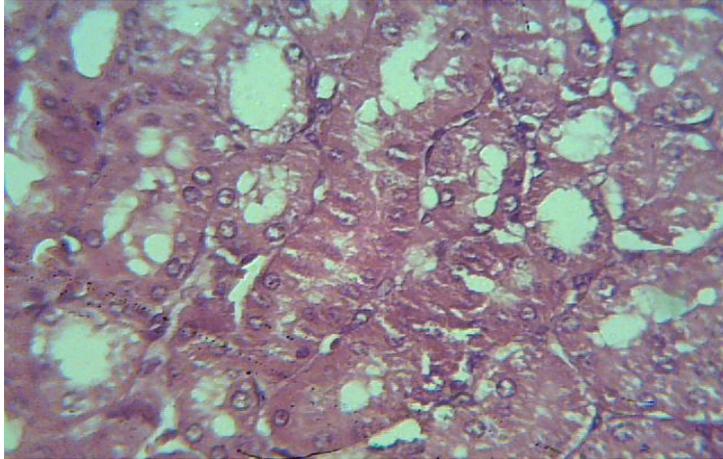


Fig 6 : Histological section of the mice kidney at 2 weeks post immunization with HK-CA showed no clear pathological lesions were reported in the kidney.H&E stain.

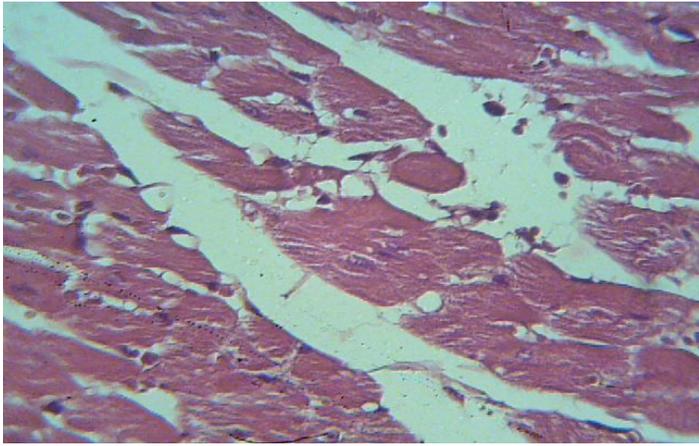


Fig 7 : Histological section of the mice heart at 2 weeks post immunization with HK-CA showed no clear pathological lesions were reported in the heart. H&E stain.

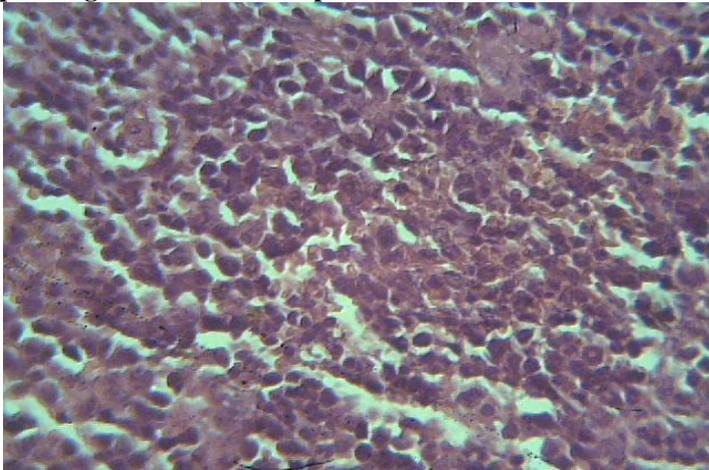


Fig 8: Histological section of the mice spleen at 2 weeks post immunization with HK-CA showed mononuclear cells aggregation around the central artery.H&E stain(400X).

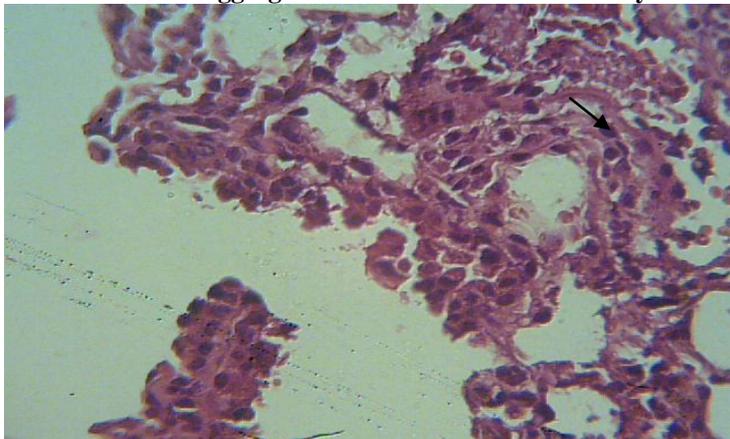


Fig 9: Histological section of the mice lung at 2 weeks post immunization with HK-CA showed mononuclear cells aggregation around blood vessel(→)and interstitial tissue(*).H&E stain.

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