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PROTECTIVE IMMUNITY AGANIST BRUCELLA INFECTION FOLLOWING IMMUNIZATION WITH PERIPLASMIC PROTEINS OF BRUCELLA MELITENSIS

(With 4 Tables and 23 Figures)

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المناعة الواقية ضد العدوى بالبروسيلا بعد التحصين بالبروتين البريبلازمى للبروسيلا ميلتنسس

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في هذه الدراسة تم تقييم المناعة السائلة والخلوية والتأثير الواقي في فئران التجارب المحصنة بالبروتين البريبلازمى ٥٠ المرسب بالامنيوم سلفات المشبعة ٥٠% أو بالبروتين البريبلازمى ٧٠ المرسب بالامنيوم سلفات المشبعة٧٠%. النتائج السيرولوجية لاختبار الاليزا أوضحت زيادة بالأجسام المضادة (ج) نتيجة لتأثير هذه الانتيجينات. وكذلك الليمفوسيت والمناعة المتأخرة للحساسية الزائدة زادت أيضا نتيجة للتحصين بالبروتين البريبلازمى ٥٠ والبروتين البريبلازمى ٧٠(المجموعة الثانية والثالثة). وكذلك التحصين بالبروتين البريبلازمى ٧٠ والبروتين البريبلازمى ٥٠ تسبب فى تثبيط عدوى الطحال مما أدى إلى قلة عدد البكتريا في الطحال مقارنة بالفئران الغير محصنة (مجموعة التحكم الايجابي). وقد أوضحت النتائج أن البروتين البريبلازمى ٥٠ أكثر فاعلية من البروتين البريبلازمى ٧٠ فى أحداث مناعة واقية ضد العدوى بالبروسيلا ميلتنسس النوع الثالث. الفحص الهستوباثولوجى للفئران فى المجموعة الأولى اظهر نقص في الحويصلات الليمفاوية وزيادة في خلايا الجهاز الشبكى البطانى بالطحال، وزيادة فى عدد الخلايا البلعمية مع وجود استحالة بخلايا الكبد مصحوب بتراكمات حبيبية بنسيج الكبد. كذلك وجد التهاب رئوي بيني وزيادة فى سمك الطبقة الطلائية المبطنة للشعب الهوئية بالرئة، استحالة وتنكرز بالخلايا المبطنة للأنابيب البولية مع وجود زيادة في حجم الكوبيبات الكلوية. ظهرت التغيرات الهستوباثولوجية سالفة الذكر بصورة محدودة في كل من المجموعة الثانية والثالثة المحصنة بالبروتين البريبلازمى ٧٠ ، ٥٠ مما يعزز دورة المناعى ضد الاصابة بميكروب البروسيلا ميلتنسس والمتبلور بصورة أوضح فى المعاملة الاخيرة.

SUMMARY

In this study the humoral and cell mediated immune responses and protective effect induced in BALB/C mice immunized with *Brucella melitensis* periplasmic proteins precipitated with ammonium sulphate at 50% saturation ($BCSP_{50}$) or precipitated with ammonium sulphate 70% saturation ($BCSP_{70}$) were evaluated. Serological results of an enzyme linked immunosorbent assay (ELISA) indicated that the immunoglobulin G increased as a response to inoculation of these antigens. Lymphocyte proliferation (LP) and delayed type hypersensitivity (DTH) also increased due to immunization with $BCSP_{70}$ and $BCSP_{50}$. However, immunization of mice with $BCSP_{70}$ and $BCSP_{50}$ suppressed splenic infection resulting in reduction in the numbers of CFU per spleen compared to non immunized controls. Overall results suggested that $BCSP_{50}$ was more effective in inducing protective immunity to *Brucella melitensis* biovar3 infection than $BCSP_{70}$. Histopathological examination of mice of group (1) revealed deplesion of white pulp and proliferation of reticuloendothelial cells in spleen, activation of kupffer cells with degenerative changes in hepatocytes and granulomatous reaction in liver, interstitial pneumonia and hyperplastic proliferation of bronchial epithelium in lung, degenerative changes of renal tubules and hypercellularity of glomeruli. While group II and III which were immunized either by $BCSP_{70}$ or $BCSP_{50}$ revealed lesions in all specimens were of much moderate nature indicating the protective effect of them against *Brucella melitensis* with attention that the later has more protective effect.

Key words: *Brucellosis, B. melitensis, vaccination, immunogenicity*

INTRODUCTION

Brucella organisms are facultative gram negative intracellular pathogens cause zoontic diseases of importance throughout the world (Thoen and Enright, 1986).

Brucella organisms enter the body via breaks in the skin or mucous membranes or via inhalation. *Brucella melitensis* may be opsonized by normal human serum promoting their phagocytosis by polymorphnuclear leukocytes. Polymorphnuclear leukocytes can kill *Brucella abortus* but not *Brucella melitensis*, factors that probably account for the greater virulence of the latter. Organisms not killed by polymorphnuclear leukocytes travel to the regional lymph nodes, then enter the circulation and localize in organs of monocyte and macrophage system where they are ingested by macrophages some organisms survive and multiply inside cells, but when macrophages are activated intracellular organisms are killed releasing endotoxin from their cell walls (Wood *et al.,* 1993).

Jones *et al.* (1997) reported that the organisms localized in a varity of tissues with a particular affinity for male and female reproductive organs, placenta, fetus, mammary glands and other tissues such as liver, Joints and bone resulting in diverse clinical signs.

The severity of the disease produced and the subsequent immune responses induced are dependent on the biochemical nature of the bacterial factors involved and their interactions (Beauclair and Khansari, 1990).

Many of these factors are proteins, while others are simple or complex polysaccharides, lipopolysaccharides (LPS) or lipoproteins (Jacques *et al.,* 1991).

The acquired immune resistance to infection by the facultative intracellular *Brucella abortus* involve both humoral and cellular immunity (Pavalv *et al.,* 1982).

Antibodies are partially protective against Brucella infection but the importance of cellular immunity in Brucella infection has been clearly demonstrated by Kable *et al.* (2006).

Vaccination of cattle and sheep with *B. abortus* strain 19 and Rev1 vaccines respectively are recommended practice for controlling bovine and ovine brucellosis (Blasco *et al.,* 1987)

Several factors including undesirable postvaccinal titres which cause diagnostic problems (Huber and Nicoletti, 1989) and pathogenicity for man, have led to renewed interest in the development of an effective non viable vaccines for brucellosis (Blasco *et al.,* 1987). Although there are reports of a potentially useful vaccine involving the use of a rough mutant (RB_{51}) of *B. abortus*, there are serious problems associating with existing Brucellosis vaccine (Stevens *et al.,* 1995).

Previously in attempts to develop better non living vaccines against *B. abortus* infection, killed attenuated cultures crud fractions of whole cells and partially purified cellular fractions of the members of Brucella genus have been tested as vaccines (Pugh *et al.* 1991).

However, studies were directed at the use of outer membrane proteins, cell surface proteins, polysaccharides and lipopolysaccharides (Jacques *et al.,* 1991 and Tabatabai *et al.,* 1992).

Brucella melitensis biovar 3 is the most prevalent serobiovar in Egypt as reported by El-Diasty (2004). Therefore, we have focused in this study on preparation of periplasmic proteins from this serobiovar and to study their immunogenic properties and protective effect which confirmed by the histopathological examination in attempts to develop better non living subcellular vaccine.

MATERIALS and METHODS

Bacterial strain:

Lyphoilized stock cultures of *Brucella melitensis* biovar 3 (isolated in Brucella department in Animal Health Research Institute) were used as inocula. Cells used for extraction procedures were from cultures transferred once on tryptose agar and then grown in liquid media as described previously (Alton *et al.,* 1975). Cultures used for challenges were maintained and grown on potato infusion agar and consisted of 40 -48 h growth diluted and adjusted turbidometirically to contain the described colony forming units (CFU).

Extraction of periplasmic proteins according to Zhan *et al.* 1993 and Pugh and Tabatabai, 1996:

Bacteria were harvested and washed once with saline. Hot saline extracts were obtained by suspending organisms in saline and autoclaved at 132 °C for 12 minutes. The autoclaved suspension was

centrifuged at 12.000xg for 15 minutes and the supernatant was collected by precipitation with ammonium sulphate (50% saturation). After centrifugation at 8000 xg for 15 minutes, the precipitate was dissolved in 0.01M PBs (7.2 pH) and dialyzed against 0.01 M PBs for 48h. This preparation was designated $BCSP_{50}$. The supernatant from 50% ammonim sulphate was further precipitated with ammonium sulphate 70% saturation. The resultant pellets were dissolved in PBS and dialyzed as described above. This preparation was termed $BCSP_{70}$.

Both preparations were centrifuged at 12.000 xg to remove insoluble material and sterilized by filtration. The protein concentration was determined by the procedure of lowry *et al.* (1951).

These preparations were subjected to sodium dodoecyle sulphate electrophoresis (SDS-PAGE) (Laemmli 1970). and stained with coomassie blue stain according to(Lugtenberg *et al.*, 1975).

Immunization:

BALB/C mice from vacsera (Helwan) were used as a model for protection conferred by periplasmic proteins of *B. melitensis* to challenge inoculation with *B. melitensis* biovar 3. Four mice groups (30 mice each) weighing 21 ±5 grams were used.

1. Group I: was inoculated with 0.2 ml 0.15 NaCl (kept as control positive group).
2. Group II: was immunized I/P with 10 µg $BCSP_{70}$ dissolved in 0.2 ml 0.15 NaCl (Pugh and Tabatabai, 1996).
3. Group III: was immunized I/P with 10 µg $BCSP_{50}$ dissolved in 0.2 ml 0.15 NaCl.

4. Group IV: kept as control negative.

Challenge of mice:

Four weeks after immunization, the immunity of mice was challenged with virulent culture of *B. melitensis* biovar 3. Each mouse (in immunized and control positive groups) was given approximately 10^4 CFU intraperitonial (I/P) in 0.2 ml of saline solution (Tabatabai *et al.,* 1992).

ELISA:

Specific serum IgG antibody concentration against the antigen was determined on 1, 2, 3 and 4 weeks after immunization. The $BCSP_{70}$ and $BCSP_{50}$ assays were performed with 1:100 dilution of serum with 0.1 µg of BCSP $_{70}$ or $BCSP_{50}$ as the test antigen (Pugh and Tabatabai, 1996). Results of the IgG antibody concentration were expressed as absorbance unit A410 and obtained with an ELISA reader.

Lymphocyte blastogensis:

Lymphocyte proliferation (LP) of immunized and control animal groups was assessed from heparinized blood samples using 3-(4, 5 dimethylthiazol-1, 2)-2, 5 diphenyl tetrazolium bromide (MTT). MTT is reduced to a blue formazon compound by succinate dehydrogenase mitochondria enzyme produced by liver cells. MTT blastogenesis microassay was conducted as described by Maslak and Reynlods (1995).

Delayed-type hypersensitivity (DTH): This reaction was determined by the method described by Arya *et al.* (1989)

Ten mice from each group were injected 28 days after immunization with either $BCSP_{50}$ or $BCSP_{70}$ (20 µg in 20 µl of PBS) in the right foot bad and 20 µl of PBS in the left foot pad. Foot pad thickness was measure 48 h later with Hauptmer dial caliper. A difference in foot pad thickness of ≥ 2 U (1u = 0.1 mm) was regarded as positive reaction.

Quantitation of bacterial number:

Two weeks post challenge mice were killed by CO2 asphexiation. Spleens were homogenized, diluted serially and plated (Pugh *et al.,* 1989). *Brucella melitensis* colonies were counted after inocubation for 3 days at 37 ° C.

Application of Fluorescent Antibody Technique (FAT):

FAT was used for detection of *B. melitensis* in spleen of examined mice. FAT was carried out as described by Jones *et al.* (1978).

Histopathological studies:

Specimens from lungs, liver, spleen and kidneys were collected from immunized control groups 2 weeks post challenge. These organs were fixed in 10% neutral buffered formalin. Processed by routine paraffin embded in and sectioned at 4 µ. Sections were stained with hematoxylin and eosin and examined by light microscopy according to Bancroft (1996).

RESULTS

A representative pattern of $BCSP_{70}$ and $BCSP_{50}$ on SDS-PAGE is shown in Fig. (1). $BSCP_{70}$ showed protein bands at 36, 31, 26, 21, 19, 14 and 12 KDa. Whereas $BCSP_{50}$ showed protein bands at 45, 38, 31, 26, 21, 19, 14 and 12 KDa.

Mice in group II and III immunized with $BCSP_{70}$ and $BCSP_{50}$ respectively develop increased IgG antibody titre begin 1st week till 4th week post immunization before challenge of immunity with *B. melitensis* biovar 3.

It is clear that $BCSP_{50}$ stimulate more antibody production than $BCSP_{70}$ (Table 1). Both $BCSP_{70}$ and $BCSP_{50}$ were tested for their ability to stimulate lymphocyte blastogensis in immunized mice. Lymphocyte proliferated in response to both antigens in immunized mice greater than non immunized controls (Table 2).

On day 28 post immunization, the DTH responses for $BCSP_{70}$ and $BCSP_{50}$ in immunized mice were higher than control responses.

Overall, mice receiving 10 µg $BCSP_{50}$ had the highest DTH response (Table 3).

When CFU of control mice (Table 4) were compared with those of immunized mice, mice given 10 µg of $BCSP_{70}$ and $BCSP_{50}$ had decreased number of CFU. However mice immunized with $BCSP_{70}$ had higher number of CFU than mice immunized with $BCSP_{50}$.

FAT applied on spleen of examined mice revealed reduction in *Brucella melitensis* antigen in mice immunized with $BCSP_{50}$ followed by mice immunized with $BCSP_{70}$ compared with control group (Fig. 2-4).

However spleen weight in all groups of immunized mice following challenge with *B. melitensis* biovar3 lower than spleen weight in *B. melitensis* biovar 3 challenged non immunized.

The most intensive lesions among organs of infected non-immunized mice was spleen. The gross findings revealed that the spleen showed slight enlargement as well as a multiple grayish necrotic foci were noticed on its surface. No gross lesions could be detected in liver. The lungs appeared congested and some of them had edematous heamorrhagic appearance. Most of kidneys showed no gross abnormalities except in few cases grey white pin head sized necrotic foci were observed on the surface of the kidneys.

The histopathological examination of the spleen of mice in group (I) revealed severe depletion of the white pulp (Fig. 5) and proliferation of reticuloendothelial cells which appeared as clusters together with degenerated lymphocytes (Fig. 6). Splenic blood vessels appeared dilated with thickening and festooning of its wall (Fig. 7). Thickening of splenic capsules and trabeculae were observed in few cases (Fig. 8). The microscopic examination of spleen of group (II) revealed slight depletion in white pulp with mature lymphocytes (Fig. 9). But group (III), the spleen showed apparent normal structure (Fig. 10).

Liver of infected mice showed activation of kupffer cells. The activated cells appeared small rounded with deeply stained nuclei (Fig. 11) the portal areas showed dilation and hyperplastic proliferation of bile ducts which infiltrated by macrophages and lymphocytes with fibrous connective tissue proliferation (Fig. 12). Some liver showed vaculization of hepatocytes and different degenerative changes, dilated central vein and perivascular granulomas were observed which formed of macrophages, epitheloid cells, lymphocytes and giant cells with no fibrosis could be detected (Fig. 13). In liver of group (II), hepatic Parenchyma appeared slight normal with focal areas of infiltration by mononuclear inflammatory cells (Fig. 14). While liver of group (III), there were no significant changes in hepatic structure than normal except slight congested central vein and sinusoides (Fig. 15).

Lungs of group (I) revealed congestion of the blood vessels and perivascular infiltration by mononuclear inflammatory cells with interstitial pneumonia, proliferation of pneumocyte type 1 and II (Fig. 16). Some cases showed haemorrhagic pneumonia where the alveoli appeared filled with eosinophilic material interlacing with different types of inflammatory cells accompanied with compensatory emphysema (Fig. 17). In most cases, the epithelial lining bronchi showed hyperplastic proliferation and peribronchial infiltration by mononuclear inflammatory cells (Fig. 18). In mice of group (II), the lung appeared with mild changes than the infected cases where the alveoli filled with slight amount of edema (Fig. 19). While In mice of group (III), the lung appeared with apparent normal alveoli and bronchi (Fig. 20).

The kidneys of mice in group (I) showed degenerative changes of the proximal and distal convoluted tubules together with congestion of the blood vessels and infiltration by mononuclear inflammatory cells (Fig.

21). Focal areas of coagulative necrosis in cortex characterized by pale eosinophilic material were also observed, the glomeruli were enlarged showed hypercellularity (Fig. 22). In kidneys of mice of group (II & III), the lesions appeared the same with mild granular degeneration of the renal tubules (Fig. 23).

Table 1: Antibody response in mice immunized with $BCSP_{70}$ and $PCSP_{50}$.

Animal groups	Weeks after immunization				
	0	1	2	3	4
I	0	0	0	0	0
II	0	0.205±0.016	0.429±0.023	0.523±0.036	0.540±0.029
III	0	0.261±0.014	0.609±0.031	0.728±0.044	0.698±0.046
IV	0	0	0	0	0

Group I: non immunized mice (control positive). Group II: immunized mice with $BCSP_{70}$.

Group III: immunized mice with $BCSP_{50}$. Group IV: control negative mice

Table 2: Lymphocyte transformation in immunized and control animals.

Animal groups	Weeks after immunization				
	0	1	2	3	4
I	1.39 ±0.09	1.23 ±0.09	1.09 ±0.08	1.25 ±0.11	1.31 ±0.13
II	1.41±0.11	2.11 ±0.18	2.21 ±0.12	2.31 ±0.13	2.44 ±0.19
III	1.45 ±0.12	2.36 ±0.12	2.59 ±0.10	2.66 ±0.12	2.53 ±0.22
IV	1.35 ±0.17	1.30 ±0.11	1.21 ±0.11	1.19 ±0.09	1.40 ±0.10

See footnote Table1

Table 3: DTH responses to $BCSP_{70}$ and $BCSP_{50}$ antigens.

Animal groups	Elicitation (hours after antigen inoculation)	
	24 h.	48h.
I	2.14 ±0.10	2.09 ±0.2
II	6.21 ±0.3	6.91 ±0.3
III	8.16 ±0.7	11.34 ±0.8
IV	2.06 ±0.19	2.31 ±0.21

The mean represented the footpad thickness (0.1 mm unit after antigen inoculation).

See footnote Table1

Table 4: Comparison of postchallenge exposure CFU numbers and splenic weights of mice immunized with $BCSP_{70}$ and $BCSP_{50}$.

Animal groups	Immunogen	Log10 CFU per spleen	Spleen weight (mg)
I	Non	7.33 ±0.21	229 ±40
II	BCSP70	4.59 ±0.51	149 ±16
III	BCSP50	3.85 ±0.63	121 ±11
IV	Non	- ve	105 ±9

See footnote Table1

LEST OF FIGURES

Fig. 6: Spleen of mice infected with *B. melitensis* showing proliferation of reticuloendothelial cells together with degenerative lymphocytes in white pulp (H & E, x 400).

Fig. 7: Spleen of mice infected with *B. melitensis* showing dilated splenic blood vessels with thickening and festooning of its walls. (H & E, x 400).

Fig. 8: Spleen of mice infected with *B. melitensis* showing thickening of splenic capsule and trabeculae (H 7 E, x 400).

Fig. 9: Spleen of mice immunized with $BCSP_{70}$ showing white pulp with mature lymphocytes and slight depletion of its number (H & E, x 400).

Fig. 10: Spleen of mice immunized with $BCSP_{50}$ showing apparent normal white pulp and red pulp (H & E, x 200).

Fig. 11: Liver of mice infected with *B. melitensis* showing activation of van kupffer cells (H & E, x 400).

Fig. 12: Liver of mice infected with *B. melitensis* showing portal areas with dilation and hyperplastic proliferation of bile ducts, infiltration by macrophages and lymphocytes with proliferation of fibrous connective tissues (H & E, x 400).

Fig. 13: Liver of mice infected with *B. melitensis* showing dilated central vein with perivascular granulomatous reaction and vacuolar degeneration of hepatocytes (H & E, x 400).

Fig. 14: Liver of immunized mice with $BCSP_{70}$ showing apparent normal hepatocytes and small focal areas infiltrated by mononuclear inflammatory cells. (H & E, x 200).

Fig 15: Liver of immunized mice with $BCSP_{50}$ showing apparent normal hepatic cords with slight congested central vein and sinusoids (H & E, x 400).

Fig. 16: Lung of mice infected with *B. melitensis* showing interstitial pneumonia with perivascular infiltration by mononuclear inflammatory cells and thickening of their walls (H & E, x 200).

Fig. 17: Lung of mice infected with *B. melitensis* showing hemorrhagic pneumonia where alveoli appeared filled with eosinophelic material interlacing with erythrocytes, macrophages and lymphocytes accompanied with emphysema. (H & E, x 400).

Fig. 18: Lung of mice infected with *B. melitensis* showing hyperplastic proliferation of the epithelial cells lining the bronchi which surrounded by mononuclear inflammatory cells (H & E, x 400).

Fig. 19: Lung of immunized mice with $BCSP_{70}$ showing alveoli and bronchi with slight edema (H & E, x 400).

Fig. 20: Lung of immunized mice with $BCSP_{50}$ showing apparent normal alveoli and bronchi (H & E, x 400).

Fig. 21: Kidney of mice infected with *B. melitensis* showing degenerative changes in cells lining the renal tubules, congestion of the blood vessels as well as infiltration of mononuclear inflammatory cells. (H & E, x 400).

Fig. 22: Kidney of mice infected with *B. melitensis* showing variable degree of degenerative changes in the cells lining the renal tubules, focal areas of coagulative necrosis with hypercellularity of the glomeruli (H & E, x 400).

Fig. 23: Kidney of immunized mice with $BCSP_{70}$ and $BCSP_{50}$ showing slight granular degenerative of the cells lining the renal tubules. (H & E, x 200).

DISCUSSION

Brucellosis is a contagious, zoonotic bacterial disease that affects several species of domestic animals causes abortion, manifests itself in humans as a systemic, febrile illness (Kreeger *et al.,* 2004).

The development of a subunit vaccine for animal brucellosis for replacement live attenuated vaccines (S_{19}, Rev_1) is an important research objective in several laboratories world wide. The success of a subunit brucella vaccine will depend on the ability of the vaccine components to stimuate an effective host immune response (Cassatero, *et al.,* 2007).

Several investigators have examined the immunogenicity of surface proteins of *B. abortus* (Confer *et al.,* 1987; Winter *et al.,* 1988 and Winter and Rowe, 1998).

A defined subunit vaccine would allow differential diagnosis of infected cattle from vaccinates, since vaccinates would have high antibody levels only to the particular subunits from *B. abortus* included in the vaccine (Confer *et al.,* 1987). This would leave other bacterial antigens as targets for new diagnostic probes which will be responded to only in the event of actual field infection. However the molecular composition of such a subunit vaccine has yet been determined (Winter *et al.,* 1988).

In the present study we evaluated the serum antibody and cell mediated immune responses (CMI) to periplasmic proteins extracted from *Brucella melitensis.*

The prechallenge exposure serologic results with two antigens ($BCSP_{70}$ and $BCSP_{50}$) indicated that increase IgG titre begin from 1^{st} week post immunization till 4^{th} week.

Serum antibody responses don't correlate with immunity since heifer vaccinated with S_{19} may be immune to challenge despite low antibody titers within few months after vaccination, in fact high antibody titres correlate with chronic infection rather than resistance (Kreutzer *et al.,* 1979).

Although humoral immune responses may contribute to protection in the murine model of brucellosis (Winter *et al.,* 1988) adoptive transfer of resistance in mice is mediated by immune T cell (Pavalv *et al.,* 1982).

Immunity in brucellosis is due to mainly cellular immune mechanisms and the induction of increased antibody doesn't correlate with cellular immunity and could act antagonistically. Thus, acquired resistance to brucellosis requires antigen specific T cells and activated macrophages (Pugh *et al.,* 1991).

Both $BCSP_{70}$ and $BCSP_{50}$ were tested for their ability to stimulate lymphocyte proliferation (LP) and delayed type hypersensitivity (DTH). Arise in LP and DTH responses to periplasmic proteins were observed in immunized groups suggesting an anamnestic CMI response to the previous antigenic stimulation. These results agree with Zhan *et al.* (1993) who proved that brucella immune spleen cell proliferated in response to these antigens and produced IL2 and INF-Y.

The CFU and splenic weight results indicated that $BCSP_{70}$ and $BCSP_{50}$ lead to protection in mice when inoculated at dose of 10 µg. The unequal reduction in CFU and spleen weight induced by 10 µg of $BCSP_{50}$ and the same dose of $BCSP_{70}$ indicated that $BSCP_{50}$ was more effective than BSCP70 in inducing protection at the same dose. $BCSP_{50}$ stimulate more protection than did $BSCP_{70}$, this may be related to different pattern of protein bands seen in SDS-PAGE (Fig.1) although specific proteins corresponding to the observed activities were yet to be identified (Zhan *et al.* 1993).

The results from these experiments indicated that $BCSP_{50}$ and $BCSP_{70}$ are immunogenic suppressed splenic infection and resulting in reduced number of viable organisms per spleen when used to immunized BALB/C mice against *Brucella melitensis.*

These results also confirmed by the histopathological examination which revealed that the spleen of group I, was enlarged and its surface was granular. This may be attributed to engorgement of splenic sinuses and pulp spaces with red cells and macrophages. This results was similar to that described by Mense *et al.* (2001) who recorded splenomegaly in mice intranasal inoculated by *B. melitensis.*

Microscopically spleen showed severe depletion of white pulp and proliferation of reticuloendothelial cells. This may be attributed to the main immunological function of the spleen is to guard the body's vascular compartment by generating T cells and dependant IgM antibody responses to bacterial polysaccharides and by exerting an enormous phagocytic power that is especially important in the case of intravascular pathogenic microorganisms before antibody formation and subsequent opsonization occurs (Male *et al.,* 1996). The lymphocytes compartment and monocytes mainly lodged in lymphoid organs as the blood circulation contain only 1% of total lymphocytes from there cells can move to sites of infection or inflammation. This concept explains the depletion happened in the white pulp of the spleen (Wanda and colin, 1998). These results come in accordance with Palmer *et al.* (1996).

The liver of all mice of group (I) showed slight enlargement due to congestion. The histopathogical examination showed proliferation of kupffer cells in the form of increase in size and number as it considered the reticuloendothelial cells of the liver that play a major role in opsonization and phagocytosis of brucella organism (Moslen, 1996). These results agreed with Mense *et al.* (2004). Other alterations observed in liver may be attributed to the toxic effects of Brucella microorganisms. These results were coincided with Izadjoo *et al.* (2000). The granulomatous reaction observed in infected mice, also previously recorded with sterba (1984). This granulomatous reaction appeared to be due to immunity mediated by T and/or B cells is required for clearance of bacteria from spleen and lung and control of bacteria replication in the liver (Izadjoo *et al.,* 2000).

Lungs of group (I) revealed interstitial pneumonia, some alveoli filled with inflammatory cells and edemas as well as hyperplastic proliferation of bronchi. These lesions may be due to toxic effect of the brucella on lung tissue which leads to injury of the alveolar epithelium followed by proliferation of type II

epithelial cells whose normal function is surfactant secretion, newly divided type II cells can differentiate into type I which are incapable of division or mature type II cells (Jones *et al.,* 1996). Our results come in accordance with Rhyan *et al.* (2001).

Kidneys of group (I) showed no specific histopathological changes detected during the experiment other than nephrosis observed in the convoluted tubules together with increased celluerity glomerular tuft. However glomerular changes are regarded as a manifestation of cellular defense of renal tissues against bacteramic stage of brucella infection (Montaser, 1995). Slightly the same lesions were reported by Mense *et al.* (2004).

Microscopically lesions in spleen, liver, lung and kidneys of mice in group (II & III) were of much moderate nature indicating the protective effects of $BCSP_{70}$and $BCSP_{50}$ against *Brucella melitensis* with the attention that the latter was more protective.

REFERENCES

Alton, G.G.; Jones, L.M. and Pietz, D.E. (1975): Laboratory techniques in brucellosis. World Health Oganization, Monograph series No. 55, Geneva, Switzerland.

Arya, L.N.; Elzer, P.H.; Rowes, G.E.; Enright, F.M. and Winter, A.J. (1989): Temporal development of protective cell mediated and humoral immunity in BALB/mice infected with *B. abortus*. J. Immunol., 143: 3330-3337.

Beauclair, K.A. and Khansari, D.N. (1990): Protection of mice against *Brucella abortus* by immunization with polyclonal anti-idiotype antibodies. Immunobiology, 180: 208-220.

Blasco, J.M.; Marin, C.M.; Barberan, M.; Morigon, I. and Diaz, R. (1987): Immunization with *Brucella melitensis* Rev_1 against *B. ovis* infection in rams.Vet. Microbiol., 14:181-198.

*Bancroft, D.*J.; Cook, C.H.; Stirling, R.W. and Turner, D.R. (1996): Manual of histopathological techniques and their diagnostic application. Churchill Livingstone, Edinburgh. .

Cassatero, J.; Vetikovsky, C.A.; Brunol, E.; Stein, S.M.; De la Barrera, S.; Bowden, R.; Fossati, C.A. and Giamartolomei, G.H. (2007): Improved immunogenicity of vaccination on regimen combing a DNA vaccine encoding *B. melitensis* omp_{31} and Recombinant omp_{31}. Boostering Clin. Vaccin. Immunol., 14(7): 869-874.

Confer, A.W.; Tabatabai, L.B.; Deyoe, B.L.; Oltjen, S.L.; Hall, S.M.; Oltjen, J.W.; Morton, R.H.; Funlenchele, D.L.; Smith, R.E. and Smith, R.A. (1987): Vaccination of cattle with chemically modified and unmodified salt-extractable proteins from *Brucella abortus*. Vet. Microbiol., 15:325-339.

El-Diasty, M.M. (2004): Epizootological and immunological studies on bovine brucellosis. M. V. Sc. Thesis, Infectious Disease, Fac. Vet. Med., Suez Canal University.

Huber, J.D. and Nicolettii, P. (1989): Comparison of the results of card, Rivanol, complement firation and milk ring tests with the isolation rate of *Brucella abortus* from cattle. Am. J. Vet. Res., 47: 1529-1531.

Jacques, I.; Oliver, B.V. and Durbray, G. (1991): Induction of antibody and protective response in mice by Brucella polysaccharide-BSA conjugate. Vaccine, 9: 896-900.

Jones, G.L.; Herbert, G.A. and Cherry, W.B. (1978): Flourescent antibody technique and bacterial application use of departmet of health education welfare.Puplic Health Service Center for Disease Contorl.

Jones, T.C.; Dungworth, D.L. and Mohr, u., eds. (1996): Respiratory system 2nd Ed.ILSI Press,Washington D.C.

Jones, T.C.; Hunt, R.D. and King, N.W. (1997): Veterinary Pathology.6th Ed, Phladelphia Vol. 3, Baltimore, New York p: 444-448.

Kable, M.M.; McDonald, T.J. and Ficht, T.A. (2006): Evaluation of protection afforded by *B. abortus* and *B. melitensis* unmarked deletion mutants exhibiting different rates of clearance in BALB/C mice. Infec. Immun., 74(7): 4048-4057.

Kreutzer, D.L.; Dreyfus, L.A. and Robertson, D.C. (1979): Interaction of polymorphnuclear leukocytes with smooth and rough strains of *Brucella abortus*. Infect. Immun., 23: 737-742.

Kreeger, T.J.; Cook, W.E.; Edwards, W.H. and Cornish, T. (2004): Brucellosis in captive Rocky Mountain Bighorn SHEEP (Ovis Canadensis) caused by *Brucella abortus* biovar 4. J. Wildlife Dis, 40(2): 311-315.

Izadjoo, M.J.; Pololsky, Y.; Mense, M.G.; Bhattacharier, A.K.; Paranvitana, C.M.; Hadfield, T.L. and Hoover, D.L. (2000): Impaired Control of Brucella meltensis Infection in Rag 1-Deficient Mice. Infec Immun., 68(9): 5314-5320

Laemmli, U.K. (1970): Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) 227; 680-685**.**

Lowry, O.H.; Rosebrough, N.T.; Farr, A.L. and Randall, R.J. (1951): Protein measurement with the folin phenol reagent. J. Biol. Chem., 193: 265.

Lugtenberg, B.; Meijers, J.; Peters, R. Hoek, P.V.O and Alplen, V. (1975): Electrophoretic resolution of the major outer membrane proteins of Escherchia coli k/2 into four bands FEBS Lett., 85: 254 -258

Male, D.; Champion, B. and Cooke, A. (1996): Advanced Immunology. 3rd ed., Mosby, Baltimore.

Maslak, D.M. and Reynlods, D.L. (1995): Mitogenic responses of the associate lymphoid tissues of chicken. Avian Dis., 39:1-8.

Mense, M.G.; VanDeverg, L.L.; Bhattacharjee, A.K.; Lindler, L.E.; Hodfield, T.L. and Hoover, D.L. (2001): Bacteriologic and histologic features in mice after intranasal inoculation of Brucella melitensis. Am. J. Vet. Res., Mari, 62(3): 398-405.

Mense, M.G.; Broschel, R.H.; Wilhelmsen, C.L.; Pitt, M.L. and Hoover, D. L. (2004): Pathologic changes associated with brucellosis experimentally induced aerosol exposure in rhesus macaques (Macaca mulatta). Am. J. Vet. Res., Ma, 65(5): 644-52.

Montaser, A.M. (1995): Morphopathological and Immunopathological studies on animals naturally and experimentally infected by Brucella. Thesis Ph. D. Pathology, Fac. Vet. Med., Cairo University

Moslen, M.T. (1996): Toxic responses of liver. In "Casarett and Doull's Toxicology. C. D. Klaassen Ed., 5th ed., 403-416, McGrow Hill, New York.

Palmer, M.V.; Cheville, N.F.; Tatum, F.M. (1996): Morphometeric and histopathologic analysis of lymphoid deplesion in murine spleens following infection with Brucella abortus strains, 2308 or RB51 or anhtr A deletion mutant. Vet. Path., May, 33(3): 282-9.

Pavalv, H.; Hogarth, M.; Mckenzie, I.F. and Cheers, C. (1982): In vivo and vitro effects of monoclonal antibody to Ly antigens on immunity to infection. Cell Immunol., 71: 127-138.

Pugh, G.W. Jr. and Tabatabai, L.B. (1996): Variation in *Brucella abortus* 2308 infection in BALB/C mice induced by prior vaccination with salt –extractable periplamsic proteins from *Brucella abortus* 19. Infect. Immun., 64(2): 548-556.

Pugh, G.W.Jr.; Tabatabai, L.B.; Bricker, B.H.; Mayfield, J.E.; Phillips, M.; McDonald, T.J. and Xehr, E.S. (1989): Identification of virulence factor for *Brucella abortus* infection in BALB/C mice. Am. J. Vet. Res., 50: 887-892.

Pugh, G.W.; Phillips, M.; Tabatabai, L.B. and McDonald, T.J. (1991): Unresponsiveness of vaccinated BALB/C mice to a second inoculation of lipopolysaccharide from *Brucella abortus* strain 2308. Vet. Microbiol., 62: 5327-5334.

Rhyan, J.C.; Gidlewski, T.; Roffe, T.J.; Aune, K.; Philo, L.M. and Ewalt, D.R. (2001): Pathology of brucellosis in Bison from yellow stone National Park. J. Wildlife Dis., 37(1), 101-109.

Sterba, F. (1984): Morphological changes in brucellosis in guinea pigs due to experimental infection with Brucella suis. Vet. Med. (Proha) Jun; 29(6): 353-71.

Stevens, M.G.; Olsen, S.C. and Chevelle, N.F. (1995): Comparative analysis of immune responses in cattle vaccinated with *Brucella abortus* strain 19 or RB51. Vet. Immunol. Immunopathol, 44: 223-235.

Tabatabai, L.B.; Pugh, G.W.; Stevens, Jr. M.G. and Philips, M. (1992): Monophosphoryl lipid A-induced immune enhancement of *Brucella abortus* salt-extractable protein and lipopolysaccharide vaccines in BALB/C mice. Am. J. Vet. Res., 53: 1900-1907.

Thoen, C.O. and Enright, F. (1986): Brucella, p:160-171. In C. L. Glyes and C. O. THoen (eds). Pathogenesis of bacterial infection in animals. Iowa State University Press, Ames, Iowa.

Wanda, M.H. and Colin, G.R. (1998): Fundementals of Toxicologic Pathology. Academic Press, San Diago, London, New York p: 233-237.

Winter, A.J. and Rowe, G.E. (1998): Comparative immune responses to native cell envelope antigens and the hot sodium dodecyl insoluble fraction (PG) of *Brucella abortus* in cattle and mice. Vet. Immnunol. Immunopathol., 18: 149-163.

Winter, A.J.; Rowe, G.E.; Duncan, R.J.; Eis, M.J.; Widom, J.; Ganen, B. and Marein (1988): Effectiveness of natural and synthetic complexes of protein and O polysaccharides as vaccines against *Brucella abortus* in mice. Infect. Immun., 56: 2808-2817.

Woods, G.L.; Gutierrez, Y.; Walker, D.H.; Puritilo, D.T. and Shanley, J.D. (1993): Diagnostic pathology of infectious disease. Lea and Febiger, Philadelphia, London p: 295-296.

Zhan, Y.; Yang, J. and Cheers, C. (1993): Cytokine response of T- cell subsets from *Brucella abortus* -infected mice to soluble Brucella proteins. . Infect. Immun., 61(7): 2808-2817.