

Organs of BALB/c mice can be injured in course of tularemia

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Background. *Francisella tularensis* is a biological agent exploitable for bioterrorism and biological warfare purposes due to serious pathogenic progression and easy dissemination. Despite intensive research in the past, some adverse consequences remain unclear. One consequence of this pathogen is oxidative stress.

Aims. The aim of this study was to undertake *ex vivo* assays for monitoring the disease in mice and increase our knowledge of the oxidative stress induced by tularemia.

Methods. The mouse BALB/c model was chosen and the animals were infected by a dose 10⁴ CFU of *F. tularensis*. After five days, the animals were euthanized. Blood immediately processed in plasma, spleen and liver were sampled from the cadavers. Oxidative stress markers, cytokines and histopathological were undertaken.

Results. There was a significant link between oxidative stress and tularemia. Particularly elevated levels of malondialdehyde and decreased levels of low molecular weight antioxidants were found in the liver and spleen of tularemia-infected animals. The histopathological findings correlated well with the oxidative stress markers. The liver and spleen were proven to be significantly at risk from the disease and an association between stress and neutrophils in the affected organs was found. The histopathology excluded risk to other organs such as the kidney and or heart.

Conclusions. Oxidative stress plays a significant role in tularemia infection in mice and this was confirmed by the histology.

Key words: *Francisella tularensis*, intracellular pathogen, liver, spleen, reactive oxygen species, antioxidants

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INTRODUCTION

Francisella tularensis is a potential biological warfare agent causing the infectious disease, tularemia. This has several manifestations based on route of entry of the pathogen.

Tularemia, also known as rabbit-fever or deer-fly fever, is caused by the gram-negative intracellular pathogen *F. tularensis* (ref.^{1,2}). The pathogen is categorized into four subspecies, *F. tularensis* subsp. *tularensis* (called Type A) and *F. tularensis* subsp. *holarctica* (called Type B) which is recognized as the principal causes of human tularemia³⁻⁶. The A type is the most virulent subspecies and it is associated with illness in North America, Canada, and Mexico among others. The B type is less virulent and responsible for almost all tularemia infections in Europe^{7,8}, where it does not usually cause human mortality. The third subspecies is virulent *F. tularensis* subsp. *mediasiatica* and has been isolated in central Asia. The last, *F. tularensis* subsp. *novicida* is generally avirulent in humans, and has been reported in Australia⁹.

F. tularensis is typically found in small mammal species and the most important vectors are rabbits, hares, mice and rodents^{6,10,11}. Humans become infected in several ways, including direct contact with infected dead or live

animals or their products, ingesting contaminated food or water, and inhaling contaminated aerosols^{12,13}. The most common mode of transmission is from blood-sucking arthropods, by ticks, deer flies and mosquitoes. The transmission of *F. tularensis* person-to-person (secondary transmission) is usually considered improbable. The infective dose can be as low as 10 bacterial cells^{3,14,15}.

Tularemia caused by *F. tularensis* has a wide variety of clinical manifestations. This generally depends on the route of entry^{12,16}. The most common clinical forms are ulceroglandular (infection through the skin) and respiratory (infection by inhalation) (ref.^{9,17}). Other forms of the disease are oculoglandular, oropharyngeal, gastrointestinal and typhoidal tularemia^{12,13,18}.

Tularemia is reported primarily in the Northern Hemisphere. Endemic foci of tularemia are found in North America, Europe, Asia, and occasionally in Africa¹⁹. In particular, tularemia cases in Europe are mainly concentrated in Germany, Sweden, British Columbia, Norway and France^{4,20,21}. Other cases are reported in Japan, Alaska, Sudan and Turkey²²⁻²⁴. Currently, outbreaks caused by *F. tularensis* are generally rare and sporadic as the spread and incidence of the disease has been steadily decreasing²⁵.

Though the pathogenesis of tularemia has been extensively investigated²⁶ and its role in oxidative stress was proven in disparate infectious diseases²⁷, the effect of tularemia on oxidative homeostasis and oxidative stress initiation remains unclear²⁸. The present experiment was focused on investigation of oxidative stress and antioxidants levels in laboratory animals infected with tularemia. We hypothesized that tularemia can induce oxidative imbalance in the most endangered organs.

MATERIAL AND METHODS

Bacterium

F. tularensis LVS (ATCC 29684) was cultivated on McLeod agar supplemented with bovine haemoglobin and Iso VitaleX (Becton-Dickinson, San Jose, CA, USA) as described previously²⁹. After two days, cells were harvested and washed in saline solution with centrifugation 2,000×g for 10 min. The concentration of fresh cells was estimated using a cell density meter (WPA, Cambridge, UK) and confirmed by a cultivation test.

Animals and tissues processing

In a total, 20 female three month old BALB/c mice (BioTest, Konarovice, Czech Republic) weighing 20±2 g were divided into two groups. The mice were kept in a stable temperature (22±2 °C) and humidity (50±10%). They had a constant source of light from 7 a.m to 7 p.m and free access to water and chow. The experiment was carried out at the vivarium of the Centre of Biological Defense in Techonin (Czech Republic) and permitted by central ethics commission (Ministry of Defense, Czech Republic). *F. tularensis* was suspended in saline up to a concentration of 10⁵ CFU/mL. 100 µL of *F. tularensis* suspension was applied to the first ten mice. The last ten animals received 100 µL of saline solution (controls animals). The experiment ended five days later when the animals were sacrificed in CO₂ anaesthesia by heart puncture. The spleens and livers were collected immediately. Each 100 mg of organ tissue was placed in 1 mL of phosphate buffered saline and homogenized by mixing at 8,000 rotations per min for 1 min using Ultra-Turrax (Ika Werke, Staufen, Germany). Blood was collected into heparinised tubes by cutting the jugular vein and the plasma was taken after centrifugation at 3,000×g for 15 min.

Ex vivo assays

Thiobarbituric acid reactive substances (TBARS), ferric reducing antioxidant power (FRAP), reduced glutathione (GSH) and glutathione reductase (GR) were assayed in compliance with the previously optimized protocols in the spleen and liver³⁰⁻³². For the TBARS assay, 67 mg of thiobarbituric acid (Sigma-Aldrich) was dissolved in 1 mL dimethylsulfoxide prior to solving in 9 mL of deionized water. Separately, 100 µL of tissue homogenate was treated with 200 µL of 10% trichloroacetic acid for 15 min on ice. After centrifugation at 3000×g for 15 min, 200 µL of supernatant (or equivalent amount of saline solution as

blank) was poured into a thermostable plastic tube, mixed with 200 µL of thiobarbituric acid solution and heated at 100 °C for 10 min. Absorbance was measured at 532 nm shortly after the mixture had cooled. The value of the TBARS (molar scale) was calculated using the extinction coefficient $\epsilon = 156,000 \text{ M}^{-1}\text{cm}^{-1}$.

Low molecular weight antioxidants were assayed as the ferric reducing antioxidant power (FRAP). 2.5 mL of 10 mM 2,4,6-tris(2pyridyl)-s-triazine in 40 mM HCl were poured together with 2.5 mL 20 mM FeCl₃. The mixture was dissolved in 25 mL of 0.1 M acetate buffer pH 3.6 and warmed at 37 °C for 10 min. 200 µL of fresh 2,4,6-tris(2pyridyl)-s-triazine solution were mixed with 30 µL of tissue homogenate (saline solution as blank) and 1 mL of deionized water. After 10 min incubation, the mixture was spun at 10,000×g for 10 min. Supernatant absorbance was measured at 593 nm. The extinction coefficient $\epsilon = 26,000 \text{ M}^{-1}\text{cm}^{-1}$ was used to calculate the FRAP molar value.

The assay of GR: disposable PS cuvette was filled with 100 µL of 10 mM oxidized glutathione (GSSG; Sigma-Aldrich) and the same volume of 1 mM NADPH (Sigma-Aldrich). 650 µL of PBS and 100 µL of 10 mM EDTA diluted the suspension. The kinetics assay was started by addition of 50 µL tissue homogenate. Consumption of NADPH was assayed in a two minute interval and the enzyme activity was calculated from the absorbance change and extinction coefficient $\epsilon = 6,220 \text{ M}^{-1}\text{cm}^{-1}$.

The assay of GSH: 100 µL of homogenate was to remove proteins using 2.5 % (v/v) trichloroacetic acid (600 µL) and spun at 12,000 ×g for 5 min. After centrifugation, 500 µL of the fresh supernatant was neutralized with 125 µL of 0.5 M sodium hydroxide. 400 µL of the precipitated and neutralized sample was mixed with 400 µL of 5,5'-dithiobis-2-nitrobenzoic acid (0.4 mg/mL) and absorbance was measured at 412 nm. Level of GSH was calculated using extinction coefficient $\epsilon = 14,150 \text{ M}^{-1}\text{cm}^{-1}$.

Interferon gamma (IFN- γ) assay: IFN- γ level in plasma was measured using a Mouse IFN- γ Eli-pair kit (Abcam, Cambridge, MA, USA) as recommended by the manufacturer. Pure IFN- γ was used for the calibration.

Histopathology

Samples of liver, kidney, lung, spleen, heart and skin from the site of application were collected into 10% buffered formalin and processed using a standard paraffin histological technique. The samples were then stained by haematoxylin and eosin. The slides were inspected by optical microscopy with a digital output (Olympus BX 35; Olympus; Japan).

Statistical analysis

Origin 8 (OriginLab Corporation, Northampton, MA, USA) was used for data processing. The significance of differences between groups was estimated using one-way analysis of variance with Tukey test. The significance was recalculated for two probabilities levels $P=0.05$ for the group size $n=10$.

RESULTS AND DISCUSSIONS

The FRAP value was assayed in both spleen and liver (Table 1). Against the controls, the FRAP was not significantly shifted in the animals' livers in the course of tularemia. In the spleen the FRAP level was reduced in the animals infected with tularemia. We can infer partial depletion of antioxidants resulting in higher vulnerability to oxidative stress as described further. We found a good example of induced oxidative imbalance in the selected organs. As a second assay for antioxidants, we chose assessment of GSH in the examined organ. The results are summarized in Table 2. The level of GSH was significantly reduced in both spleen and liver. The findings are not surprising as depletion of antioxidants can play a role in the disease progression³³. Moreover, GSH is required by *F. tularensis* for its metabolism. Hence the level can be easily depleted³⁴.

GR was evaluated in tissues samples. The found enzyme activities per kilogram of tissue are shown in Table 3. The liver GR was relatively steady and we found no shifts in its activity indicating that GR probably does not play any extensive role in oxidative stress induced by tularemia. The proven steady level of GR confirms the above mentioned hypothesis that GSH is acquired by *F. tularensis*. The enzyme activity increases with increased level of GSSG (ref.³⁵). The finding can be interpreted that GSH is consumed by metabolism rather than oxidized to GSSG. Direct proof is, however, missing. The found shifts in TBARS level are depicted in Table 4. Both liver and spleen had increased content of malondialdehyde indicating development of oxidative stress. We can use the method for monitoring of stress state with very good results. From a literature search, *F. tularensis* is quite resistant to oxidative stress and it can recovery from stress conditions more easily than the host organism³⁶.

IFN- γ was assessed in plasma samples (Table 5). In accord with expectation, the level was significantly increased in the course of tularemia. IFN- γ is a cytokine necessary for combating the disease. Macrophages are more effective in killing the intracellular pathogen when they are co-stimulated by the IFN- γ (ref.^{37,38}). Here, we used IFN- γ as a marker of the progress of the infection. The controls had minimal levels of IFN- γ . The level was near the limit of detection for the ELISA method. The infected mice had significantly increased IFN- γ level which indicates successful combat of the disease.

The histopathological examination showed significant damage to the tested organs following the tularemia infection. The hallmark of tissue change was pyogranulomatous inflammation. The most pronounced changes were seen in the skin and subcutaneous tissue in the infected animals. Moderate and severe necrosis was also observed in the liver (see Fig. 1) in all animals infected with tularemia. Focal accumulation of neutrophils was found in the spleen together with activation of germinative centers and hyperplasia of white pulp. There were no morphological changes in other tissues (kidney, heart, lungs and brain). The overall impact on tissues is summarized in Table 6.

Table 1. Ferric reducing antioxidant power (FRAP) in examined organs.

	Tularemia	Control
Liver	259±48 (-)	258±24
Spleen	233±21 (*)	266±36

value in $\mu\text{mol/kg}$: mean±standard deviation

* - probability level at 0.05

Table 2. Reduced glutathione (GSH) level.

	Tularemia	Control
Liver	31±9 (*)	51±11
Spleen	39±12 (*)	57±14

value in $\mu\text{mol/kg}$: mean±standard deviation

* - probability level at 0.05

Table 3. Glutathione reductase (GR) activity.

	Tularemia	Control
Liver	6.36±2.42	6.73±1.16
Spleen	9.04±3.14	8.34±1.13

value in $\mu\text{kat/kg}$: mean±standard deviation

Table 4. Thiobarbituric acid reactive substances (TBARS) activity.

	Tularemia	Control
Liver	167±20 (*)	135±26
Spleen	150±19 (*)	115±9

value in $\mu\text{mol/kg}$: mean±standard deviation

* - probability level at 0.05

Table 5. Interferon gamma (IFN- γ) assay in plasma samples.

IFN- γ (pg/mL)	Tularemia	Control
	755±71 (*)	15±13

value in pg/mL: mean±standard deviation

* - probability level at 0.05

The histopathological examination confirmed our hypothesis that the spleen and liver are the most endangered organs during the disease. The presence and activation of neutrophils can cause the generation of reactive oxygen species as the cells are their significant source³⁹.

The proven alterations in oxidative stress markers correspond well with our previous experiment on BALB/c mice⁴⁰. In comparison with the quoted paper, we chose a ten fold lower dose of the pathogen in order to prevent serious nonspecific reactions in the body. The oxidative stress marker findings presented here and in the quoted

Table 6. Histopathology examination.

Group	Tularemia	Controls
Skin and subcutis	pyogranulomatous panniculitis and dermatitis +++	normal morphology
Liver	liver necrosis, multiple focal and confluent	normal morphology
Spleen	focal infiltration with neutrophils, reactive hyperplasia of white pulp	normal morphology
Lungs	normal morphology	normal morphology
Heart	normal morphology	normal morphology
Kidney	normal morphology	normal morphology
Brain	normal morphology	normal morphology

paper point to oxidative injury in the examined organs and provide opportunity for introducing treatment based on oxidative stress reduction. In contrast to the previous work, we chose histopathological of the tested organs and role of neutrophils can be rationalized. Apart from the other animal models, the findings are in compliance with clinical findings on patients infected with tularemia⁴¹.

CONCLUSIONS

This study provides data from *ex vivo* assays for monitoring oxidative stress induced by tularemia in experimental animals. The FRAP method provided fair results for the kidney samples. The GR assay however was not as useful. TBARS is applicable for indicating the development of oxidative stress. We infer that oxidative stress plays a significant role in disease pathogenesis. However, it is not easy to judge whether the stress is a cause or a consequence of tularemia induced pathogenicity.

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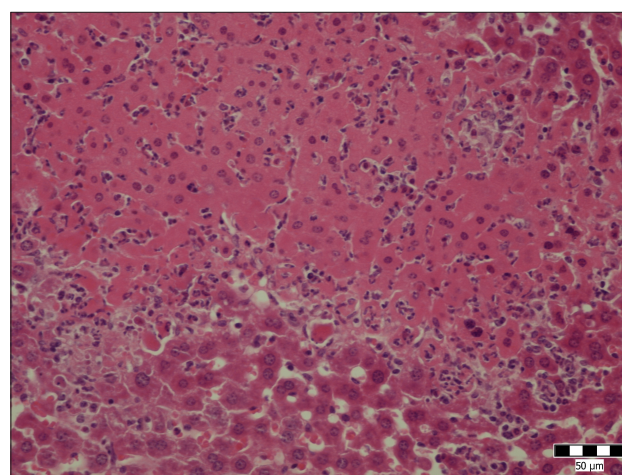


Fig. 1. Histological section of liver in mice infected with tularemia. There is marked necrosis moderately infiltrated with neutrophils. Haematoxylin and eosin staining.

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