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Original Article

Babesiosis Causes Reproductive Dysfunction in Splenectomized Mice: A Proof of Concept *in Vitro* Study

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ABSTRACT

Babesias as the second common blood parasite in mammals after trypanosomes have aroused wide concern particularly due to having zoonotic potential. This study was implemented to scrutinize epididymal sperms characteristics and *in vitro* fertilizing (IVF) capacity as well as subsequent pre-implantation embryos developmental potential following experimental babesiosis (EB) induction by *Babesia bigemina* in mice. In this experimental study, twenty-four adult male mice were randomly categorized into four equal groups including untreated control, sham, splenectomy, and EB. Experimental babesiosis was induced in splenectomized mice through 2 ml intraperitoneal injection of an infected heparinized blood sample belonging to a cow with confirmed *B. bigemina* infection. All animals were euthanized after 5 days and epididymal sperms characteristics and IVF abilities along with early embryo development were analyzed following infection confirmation. Experimental babesiosis resulted in epididymal sperms quantity, quality, and IVF potential reduction as well as pre-implantation embryos developmental retardation compared to control, sham, and splenectomy groups. These findings revealed that *B. bigemina* infection can result in male subfertility and/or infertility in mice leading to pre-implantation embryos developmental arrest.

Introduction

Babesiosis as an emerging zoonotic disease is caused by infection with hemoparasites of the protozoan genus Babesia being the second most common blood-borne parasites of mammals following trypanosomes.[1] Hemolytic anemia, a major clinical manifestation of babesiosis progression, can lead to blood supply disturbance, tissue hypoxia and ultimately cellular damage.[2] Accordingly, compelling evidence has confirmed that blood flow reduction is linked to testicular germ cell degeneration as well as spermatogenesis arrest,[3] and testicular ischemia related oxidative stress can trigger inflammatory cells activation leading to increased vascular permeability, edema and cellular death.[4, 5]

Reportedly, it has been suggested that experimental babesiosis (EB) induced hemolytic anemia can cause

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marked histo-architectural disorganizations along with hepatic and renal dysfunctions in rats,[6] as well as severe hypo-spermatogenesis and spermatogenic maturation arrest in mice.[7] Correspondingly, epidemiological reports have highlighted the non-negligible association of protozoan parasitic diseases and fertility disorders.[8,9]

In line with that, this study was designed to examine epididymal sperms characteristics and *in vitro* fertilizing (IVF) capacity as well as subsequent pre-implantation embryos developmental potential following EB induction by *Babesia bigemina* in mice.

Materials and Methods

Experimental Design

For this experimental study, twenty-four adult male mice were obtained from Animal Resources Center of Faculty of Veterinary Medicine, Urmia University, Urmia, Iran, and housed under standard housing conditions of 25±2° C, relative humidity 50 ± 10% and photo period of 12 hr dark/12 hr light. Food and water were available *ad libitum* (No. 1600, 2018.07.21).

Following one-week acclimatization, adult male mice were randomly assigned into four equal groups including untreated control, sham, splenectomy and EB. Sham mice received normal saline [0.200 ml; intra-peritoneally (IP)]. Experimental babesiosis was induced in splenectomized mice through injection of infected heparinized blood sample belonging to a cow with confirmed *B. bigemina* infection (2.00 ml; IP). All animals were sacrificed following anesthesia with 5% ketamine and 2% xylazine (40 mg/kg and 5 mg/kg; IP, respectively) after 5 days and epididymal sperms characteristics and IVF capacities along with pre-implantation embryos developmental potential were recorded.[7,10]

Splenectomy

Surgical procedures were executed under IP injection of cocktail consisting of 0.1 ml xylazine (Trittau, Germany), 1 ml ketamine (Alfasan International, Woerden, Holland) and 8.9 ml distilled water at a dose of 0.1 ml/10 g body weight. [11] A ventral midline incision was made, the peritoneum was opened, the spleen was recognized and splenic blood vessels and ligaments were ligated. The spleen was then removed by transecting the vessels just distal to the ligature. Finally, The *linea alba* and skin incisions were closed with absorbable and non-absorbable monofilament suture materials, respectively (Figure 1).[12]

Epididymal Sperm Sampling

Following animals' euthanasia, a caudal ventral midline incision was made and epididymides were dissected out in a sterile condition under a 20-time magnification provided by a stereo zoom microscope (Model TL2, Olympus Co., Tokyo, Japan). Then, epididymal tails were cut into small pieces, transferred to 1 ml of human tubal fluid (HTF; Sigma-Aldrich, USA) medium and incubated for 10 min at 37° C in an atmosphere of 5% CO_2 incubator.[13]

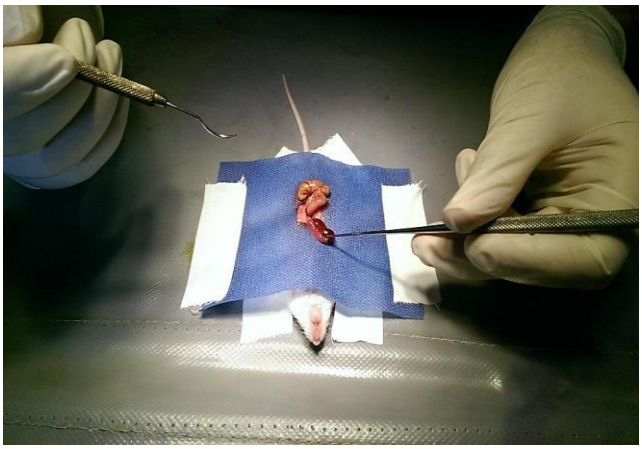

Figure 1. Splenectomy in a mouse.

Epididymal Sperm Characteristics Evaluation

The epididymal sperm count was determined using standard haemocytometer method as described formerly.[13] To analyze abnormality percentages, sperm smears were prepared on clean and grease free slides, allowed to be air-dried overnight, stained with 1% eosin-Y/5% nigrosin and examined at 400×.[13]

Epididymal Sperm IVF and Pre-Implantation Embryos Developmental Potentials Determination

Female mice were super-ovulated with pregnant mare's serum gonadotropin (Folligon, Netherlands; 7.50 IU; IP) followed by injection of human chorionic gonadotropin (hCG; Folligon, Netherlands; 7.50 IU; IP) after 48 hr interval.[13]

Females were sacrificed and ovulated oocytes were collected after 13 hr of hCG administration. Fifteen µl of capacitated sperm suspension was added to the fertilization medium and fertilization rate was monitored through male and female pronuclei observation using inverted microscope (Olympus, Japan) six hr later. Blastulation rate was also

determined via recording the number of embryos reaching the blastocyst stage.[14]

Statistical Analysis

The data were analyzed using Statistical Package for Social Sciences (SPSS Inc., Chicago, IL, USA), statistical significance was set at $p < 0.05$ and findings were expressed as mean ± standard error of mean. One-way Analysis of Variance (ANOVA) followed by Tukey post-hoc test was also used to compare the differences among experimental groups.

Results

Spermatology

Significant reduction in sperm count was observed following EB induction compared to control, sham and splenectomy groups (Figure 2); meanwhile, significant lower percentages of normal sperm morphology were also seen in experimentally infected animals compared to control, sham and splenectomy groups (Figure 3).

Epididymal Sperm IVF and Pre-Implantation Embryos Developmental Potentials

Epididymal sperms IVF capacities in all experimental groups are shown in Figure 4. Experimental babesiosis caused a significant reduction in epididymal sperms IVF potential compared to control, sham and splenectomy groups.

Moreover, EB resulted in blastulation rate decline compared to control, sham and splenectomy groups (Figures 5 and 6).

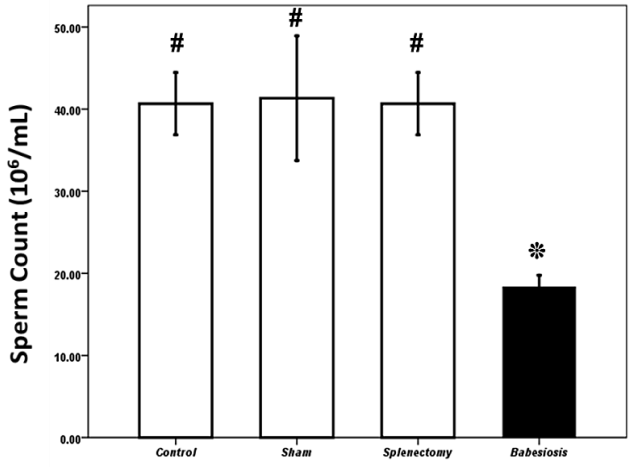

Figure 2. Epididymal sperms concentration in all experimental groups.
Different signs indicate statistical difference at a $p < 0.05$.

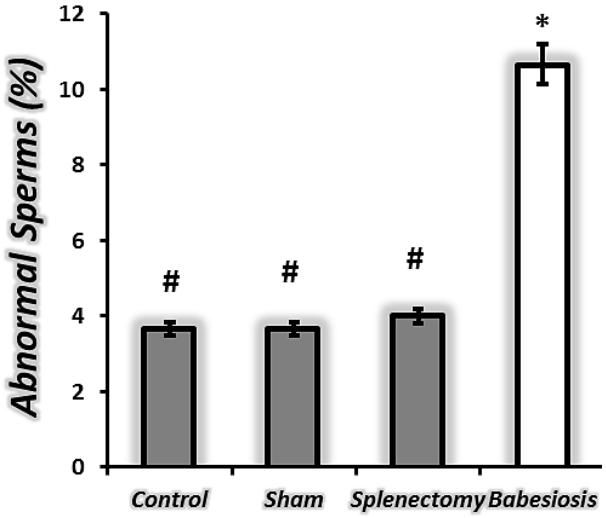

Figure 3. Epididymal sperms morphology in all experimental groups.
Different signs indicate statistical difference at a $p < 0.05$.

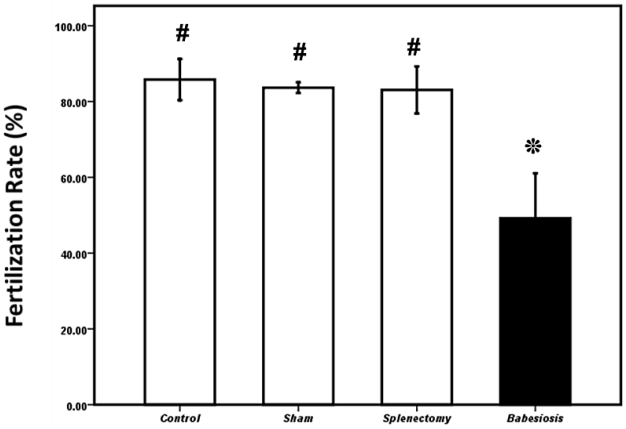

Figure 4. Epididymal sperms fertilizing potential in all experimental groups.
Different signs indicate statistical difference at a $p < 0.05$.

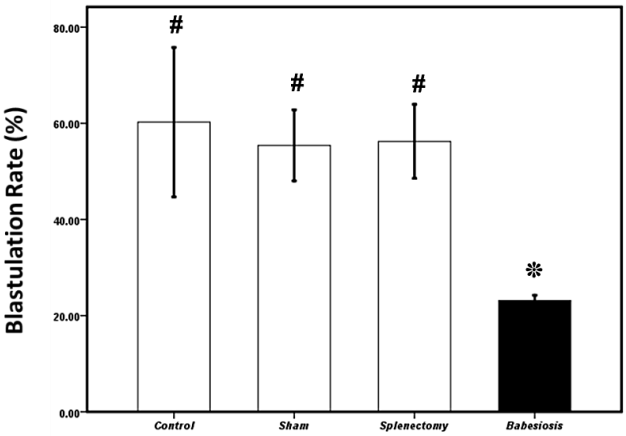

Figure 5. Blastulation rate in all experimental groups.
Different signs indicate statistical difference at a $p < 0.05$.

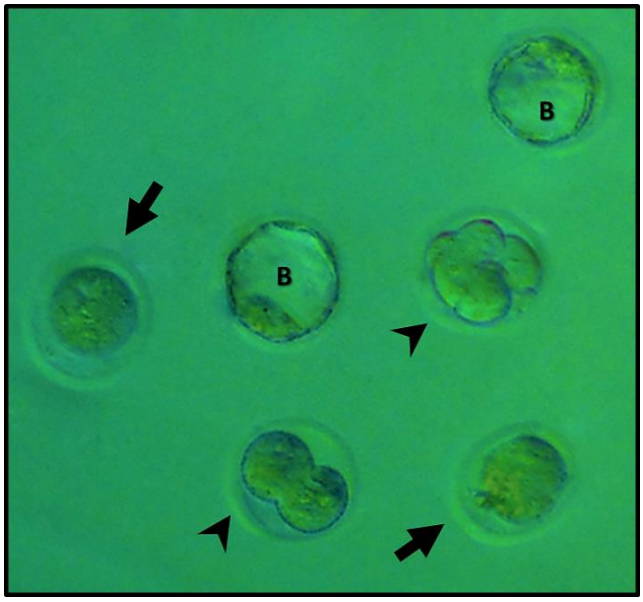

Figure 6. Photomicrographs of pre-implantation embryo development in an experimental babesiosis group. Blastocysts (B) can be observed along with unfertilized oocytes (arrows) and arrested embryos (arrowheads) (200).

Discussion

Reportedly, it has been highlighted those protozoan parasitic infections may be an important causative factor of infertility in human populations.[15] Considering that, the current study was designed to reveal the spermatological and early embryological aspects of EB in mice. As a result, EB led to epididymal sperms quantity, quality and IVF potential decreases along with pre-implantation embryos developmental arrest in mice compared to controls. It has been shown previously that testicular blood supply disruption results in oxidative stress leading to germ cell damages and spermatogenesis impairments.[3,16] Further, it was found that testicular ischemia induced oxidative stress can cause leukocyte activation and chemotaxis as well as leukocyte-endothelial adherence leading to increased micro-vascular permeability, edema and eventually spermatogenic cells death.[4,5,17]

A growing body of evidence indicates that mammalian sperm cells are vulnerable to oxidative damages due to their plasma membrane structural nature and free radicals over-generation can result in sperm abnormalities and necrospermia confirming our findings.[18]

It is noteworthy to mention that abnormal sperms are the major sources of excessive free radical production in semen and free radicals producing sperms can cause fertilization and embryo development failures.[19,20] Based on this concept, fertilization and blastulation rates reduction following EB induction in the present study can be ascribed to the abnormal sperm cells effects during *in vitro* insemination of oocytes. Accordingly, former studies have shed light on the role of parasitic infections evoked oxidative stress in sperm abnormalities leading to reproductive failures.[21]

On the whole, it seems that Babesia infection can lead to spermatogenesis impairments and male subfertility and/or infertility in mice probably through testicular micro-circulatory disruption and blood flow reduction.[22] Consistent with these findings, it has been reported that experimental *Borrelia crocidurae* inoculation can lead to pre- and post-capillary blood vessels blockage in rats testicular tissue causing testiculopathy via normal testicular blood flow disruption.[23] In line with that, it was also found that experimental visceral leishmaniasis in BALB/c mice can cause marked testicular cyto-architectural disarrangements and damages.[24]

Acknowledgments

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Conflict of Interest

The authors declare that there are no conflicts to disclose.

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