

TOXICITY OF 32.2 kDa MW *Escherichia coli* PILI ADHESIN ISOLATED FROM INFERTILE MALE SEMEN IN REPRODUCTIVE SYSTEM

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ABSTRAK

Escherichia coli (*E. coli*) adalah penyebab utama infeksi saluran kelamin laki-laki tanpa gejala dengan konsekuensi infertilitas. Protein *E. coli* pili hemagglutinin yang diisolasi dari air mani pria infertil dengan 32,2 kDa MW bertindak sebagai adhesin pada spermatozoa. Penelitian ini bertujuan untuk membuktikan apakah *E. coli* pili adhesin 32,2 kDa MW beracun bagi sistem reproduksi laki-laki. Sampel terdiri atas spermatozoa dari 30 marmot yang dibagi tiga kelompok: kontrol, yang diimunisasi protein *E. coli* pili adhesin 32,2 kDa MW, dan yang diinfeksi *E. coli* secara transuretral. Pengamatan motilitas sperma, vitalitas dan morfologi dilakukan di bawah mikroskop. Kadar MDA dan kerusakan DNA sperma diukur dengan spektrofotometer dan metode comet assay dan diamati menggunakan mikroskop fluorescent. Tidak ada perbedaan antara kontrol dan kelompok imunisasi *E. coli* pili adhesin dalam motilitas ($p=0,499$), vitalitas ($p=0,817$) dan morfologi ($p=0,176$); antara kontrol dan kelompok infeksi transuretral dalam motilitas ($p=0,000$), vitalitas ($p=0,000$) dan morfologi ($p=0,000$); dan antara kontrol dan kedua kelompok perlakuan dalam motilitas ($p=0,001$), vitalitas ($p=0,000$) dan morfologi ($p=0,000$). Analisis histologi menunjukkan kelompok imunisasi *E. coli* pili adhesin 32,2 kDa MW tidak mengalami kerusakan jaringan testis, sementara kelompok positif menunjukkan kerusakan sel tubulus seminiferus. Kadar MDA berbeda antara kelompok imunisasi *E. coli* pili, kelompok infeksi transuretral, dan kontrol ($p=0,024$) dan antara kelompok infeksi transuretral dan kontrol ($p=0,007$). Namun, antara kontrol dan kelompok imunisasi protein *E. coli* pili tidak menunjukkan perbedaan ($p=0,251$). Kerusakan DNA berbeda ($p=0,000$) antara kelompok imunisasi *E. coli* pili, kelompok infeksi transuretral dan kontrol; antara kontrol dan kelompok terinfeksi transuretral ($p=0,000$); dan antara kelompok infeksi transuretral dan kelompok imunisasi protein *E. coli* pili ($p=0,000$). Namun, antara kontrol dan kelompok imunisasi *E. coli* pili tidak menunjukkan perbedaan ($p=0,600$). Sebagai simpulan, protein *E. coli* pili adhesin 32,2 kDa MW tidak beracun untuk kualitas sel sperma dan kualitas molekul sperma. (FMI 2018;54:146-154)

Kata kunci: *E. coli* pili adhesin 32,2 kDa MW; sperma marmot; motilitas; morfologi; daya hidup; kerusakan DNA; tingkat MDA

ABSTRACT

Escherichia coli (*E. coli*) is the leading cause of male genital tract infection with no symptoms of infertility. Protein *E. coli* pili hemagglutinin isolated from infertile male sperm with 32.2 kDa MW acts as adhesion in spermatozoa. This study aimed to prove whether *E. coli* pili adhesin 32.2 kDa MW is toxic to male reproductive system. Samples consisted of spermatozoa of 30 guinea pigs divided into three groups: control, immunized with *E. coli* pili adhesin 32.2 kDa MW protein, and transurethral infected *E. coli*. Observations of sperm motility, vitality and morphology were performed under a microscope. MDA levels and sperm DNA damage were measured by a spectrophotometer and comet assay method and observed using a fluorescent microscope. There was no difference between control and immunization group of *E. coli* pili adhesin in motility ($p=0.499$), vitality ($p=0.817$) and morphology ($p=0.176$); between control and transurethral infection groups in motility ($p=0.000$), vitality ($p=0.000$) and morphology ($p=0.000$); and between control and both treatment groups in motility ($p=0.001$), vitality ($p=0.000$) and morphology ($p=0.000$). Histologic analysis showed *E. coli* pili adhesin of 32.2 kDa MW immunization group did not suffer from testicular tissue damage, while the positive group showed a deterioration of seminiferous tubular cells. MDA levels differed between immunization group *E. coli* pili, transurethral infection group, and control ($p=0.024$) and between transurethral and control ($p=0.007$) groups. However, between control and immunized group with *E. coli* pili protein showed no difference ($p=0.251$). DNA damage differed ($p=0.000$) between immunized group with *E. coli* pili, transurethral infection and control group; between control and transurethral infected group ($p=0.000$); and between transurethral infection group and *E. coli* pili protein immunization group ($p=0.000$). However, between control and *E. coli* pili immunization group showed no difference ($p=0.600$). In conclusion, *E. coli* pili adhesin 32.2 kDa MW protein is not toxic for sperm quality and the quality of sperm molecules. (FMI 2018;54:146-154)

Keywords: *E. coli* pili adhesin of 32.2 kDa MW; guinea pigs sperm; motility; morphology; vitality; DNA damage; MDA level

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INTRODUCTION

Male urogenital tract infection, one of the causes of male infertility, is the most important and asymptomatic.

Bacteriospermia plays a major role. Male accessory sex gland infection is a major risk factor in infertility. *E. coli* is a leading cause of prostatitis and

epididymitis (Liu et al 2002). Results of studies found that *E. coli* has negative effect on human sperm motility (Sukarjati et al 2002), human spermatozoa vitality (Sukarjati & Lunardhi 2001), damages human sperm membrane integrity (Sukarjati et al 2006) and increases ROS level (Sukarjati et al 2010).

E. coli negative effect on sperm quality is due to the ability of *E. coli* to attach to sperm using pili. *E. coli* pili proteins from semen of infertile men have been isolated and characterized. *E. coli* pili proteins isolated from the semen of infertile men have a molecular weight (MW) of 32.2 kDa and serves as an adhesin for human spermatozoa (Sukarjati 2008). It has been proven that *E. coli* pili adhesin of 32.2 kDa MW is able to block the attachment of *E. coli* to human spermatozoa in vitro (Sukarjati 2008). It has been demonstrated that *E. coli* pili adhesin with 32.2 kDa MW was immunogenic (Sukarjati et al 2011). This study aimed to test the toxicity of *E. coli* pili adhesin isolated from semen of infertile men with 32.2 kDa MW in the reproductive system of animal models of guinea pigs which included the observation of motility, morphology, vitality, tissue damage of the testes, MDA level and sperm DNA damage.

MATERIALS AND METHODS

Isolation of *E. coli* pili adhesin protein with 32.2 kDa MW

This study used *E. coli* pili adhesin protein of 32.2 kDa MW. *E. coli* was isolated from the semen of infertile males and guinea pigs. *E. coli* pili adhesin protein with 32.2 kDa MW was isolated with following steps:

E. coli multiplication and fimbriae (pili) enrichment

E. coli from stock were rejuvenated first by making cultures in McConkey agar in a temperature of 37°C for 24 hours. Then, the culture from the medium was inoculated into 500 ml Erlenmeyer flask containing BHI medium, and incubated for 24 hours. Then, the bacteria were poured into 50 bottles of 250 ml which already contained 25 ml TGC medium (medium to enrich fimbriae bacteria), each of 10 ml. Incubation was carried out in 37°C for 48 hours. Furthermore, *E. coli* cultures were collected together in a 1000 ml Erlenmeyer flask and were prepared to cut the pili.

Pili cutting

Ready-to-cut *E. coli* was inserted into sterile omnimixer tube and set on omnimixer tool. Then, pili cutting was done using an omnimixer tool at 4°C, 3000 rpm for 30

seconds. Once in omnimixer, the samples were centrifuged at 4°C, 6000 rpm for 15 minutes. The supernatant was collected in a tube and the pellet was suspended with PBS with pH 7.4 in 1:1. Then the cutting was done again with omnimixer at 4°C, 3000 rpm for 30 seconds, then centrifuged at 4°C, 6000 rpm for 15 minutes. The cutting process was repeated up to 5 times. Furthermore, the supernatant was centrifuged 12,000 rpm, in 4°C for 15 minutes to obtain supernatant and pellet. The supernatant, in the form of fraction of pili and pellet, comprised bacterial cells without pili (whole-cell supernatant).

Pili fraction dialysis

Pili fractions obtained were subjected to dialysis process carried out using PBS solution pH 7.4 at 4°C for 2 x 24 hours to remove the remaining TCA. Furthermore, dialysate was precipitated with 35% ammonium sulfate, centrifuged 6000 rpm, 4°C. The supernatant was discarded, pellets were suspended with PBS sufficiently and dialysis was re-done. Results of dialysis were pili proteins and stored at -20°C until used for further examination.

Electrophoresis with SDS PAGE method for multiplication of adhesin protein, electroelution, dialysis and precipitation

Cut I of pili fraction was subjected to electrophoresis using SDS PAGE method. Protein staining SDS-PAGE was done by soaking the gel in a solution of 0.25% Comassie brilliant blue for 30 minutes. SDS-PAGE gel results, which had a 12.5% protein bands with 32.2 kDa MW, were cut horizontally at the top and bottom. Then, the band pieces were collected, dialysis membrane was inserted to carry out electroelution using a horizontal electrophoresis apparatus with an electrophoresis running buffer solution. The electric current was at 125 volts for 2 hours. Results of electroelution were dialysed with PBS solution for 2 x 24 hours. The solution was replaced every 24 hours. The eluate was precipitated with cold absolute ethanol solution overnight, in order to obtain pure 32.2 kDa MW protein that was ready for use.

Toxicity test

Experimental animals used were male guinea pigs. Before being used, they were acclimatized beforehand for 2 weeks. They were grouped into 3 treatment groups as follows: In treated guinea pigs: *E. coli* pili adhesin of 32.2 kDa MW as much as 500 g was suspended with 500 mL PBS, plus 500 µL complete Freund adjuvant, was mixed to form a white emulsion. Then it was injected subcutaneously at 5 points, where the parts to

be injected were first disinfected with 70% alcohol. One week later repeated injection of *E. coli* pili adhesin of 32.2 kDa MW was made mixed with incomplete Freund's adjuvant subcutaneously at 5 points. The injections were continued every week in the same way until the end of week five. Negative control guinea pigs were injected with PBS alone. Positive control guinea pigs were artificially infected transurethrally with *E. coli* isolated from semen of infertile men for 3 times in an interval of 3 days. In the fifth week, guinea pigs were sacrificed, sperm from epididymis was taken and reproductive tract was separated. Furthermore, observation was carried out.

Observation

Motility observation

Guinea pigs epididymal spermatozoa was quickly taken one drop on the object glass, then covered with cover glass and observed under a microscope with a magnification of 400 times. The motility was determined by the following category, according to WHO (1999), namely a. fast forward movement straight ahead, b. slow motion forward, c. not moving forward or moving in place, and d. immotile. The calculation was performed on 100 spermatozoa.

Spermatozoa morphological observation

One drop of epididymal sperm was dripped on object glass, then diluted with a cover glass, then dried in the air. Subsequently, it was stained with safranin, soaked in alcohol 70% for 5 minutes, then dipped quickly with phosphate buffer three times, and finally stained with crystal violet for 10 minutes, washed with water and dried. Furthermore, sperm normality and abnormality was counted on head, tail and the middle at 100 sperm using a microscope of 400 times magnification.

Vitality observation

Epididymal spermatozoa was taken one drop on the object glass, and then dropped with a drop of eosin Y and mixed. Observation was made after 30 seconds under a microscope with a magnification of 400 x. Viable spermatozoa were not stained and died sperms were stained red. It was observed at 100 spermatozoa.

Testicular tissue damage observation

The guinea pigs were sacrificed, then the reproductive tract and accessory sex glands were taken. Furthermore, they were fixed with 7.5% buffered formalin for 15-24 hours, then inserted into ethanol concentration increasing from 70% to absolute. Xylol was inserted for 1 hour

so that the material became transparent. Then, we did the embedding, providing buffer solution so that the material could be cut with a microtome without causing significant distortion of the tissue composition. For that we needed the process of infiltration into the tissue by using paraffin. Sectioning was then performed using a rotary microtome. Results of the cut, which is called ribbon, was taped to the object glass that had been given with polylysine adhesive, and dried. Then deparaffinization was done, in which tissue incision was soaked 2-3 times in xylol each 5-6 minutes, then paraffin in the preparation was lost, soaked in absolute alcohol 2-3 times each for 3 minutes, soaked in 95% alcohol 2-3 times each 2-3 minutes, soaked in 80% alcohol 2-3 times, in 70% alcohol 2-3 times each 2-3 minutes, then soaked with water, soaked in PBS for 5 minutes, soaked with hematoxylin from Meyer (no alcohol) for 5-8 minutes, rinsed with tap water and soaked until became blue colored, dropped with entellan and covered with a glass lid. They were then examined with a light microscope at a magnification of 400 x.

Determining malondialdehyde (MDA) level

Spermatozoa was separated from the protein by the addition of 20% trichloroacetic acid in the same amount and centrifuged for 10 min at 5000 rpm. A certain amount of supernatant was added, namely 0.1 ml of 1% sodium thiobarbituric and 1 N hydrochloric acid to a volume of 10 ml in the flask. The solution was then incubated over a water bath for 135 minutes. The resulting stained solution of 3 ml were observed with a spectrophotometer at a excitation wavelength of 532 nm.

Measuring DNA damage with comet assay method

The surface of the object glass was dropped with 110 mL NMPA (Normal Melting Point Agarose) 0.5% in PBS, covered with a cover glass, kept at a temperature of 4°C for 15 minutes. Cover glass was opened by sliding slowly, then the top of NMPA was dropped with a mixture of 65 mL Low Melting Point Agarose (LMPA) with 10 mL suspension of epididymal sperm (which had been prepared using swim up), closed with a cover glass, kept at a temperature of 4°C until hardened. Furthermore, the glass cover was opened and dropped again with 75 mL NMPA, closed with a cover glass, kept at a temperature of 4°C until hardened. Cover glass was opened, the object glass was placed on the lysis liquid at 4°C for 1 hour. The lysis solution consisted of 100 mM EDTA, 2.5 M sodium chloride, 1% N-lauroylsarcosine, 10 mM Tris base pH 10, Triton X-100, and DNase free proteinase K. Furthermore, the object glass was placed on a horizontal electrophoresis tank that had been filled sufficiently with electrophoresis buffer fluid

until the surface of the object glass submerged for 30 minutes to release the DNA binding. The liquid buffer consisted of 1 mM EDTA and 300 mM sodium hydroxide. Electrophoresis tank was electrified not more than 300 mA at 25 V for 20 minutes. Furthermore, object glass was soaked with buffer for the neutralization process for about 10 minutes. Neutralization solution consisted of 0.4 M Tris pH 7.5. Then, it was put in absolute alcohol for 30 minutes. Furthermore, cyber green staining was done and observed using a fluorescent microscope.

RESULTS

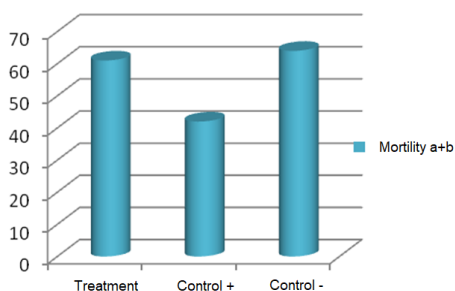


Fig. 1. Sperm motility percentage in treatment group, positive control and negative control.

Data analysis yielded motility differences between treatment, positive control and negative control groups ($p=0.001$). The difference was present between negative control and positive control guinea pigs ($p=0.000$) and between positive control and treatment, the guinea pigs immunized with *E. coli* pili adhesin 32.2 kDa MW ($p=0.001$). Negative control and treated guinea pigs showed no difference ($p=0.499$).

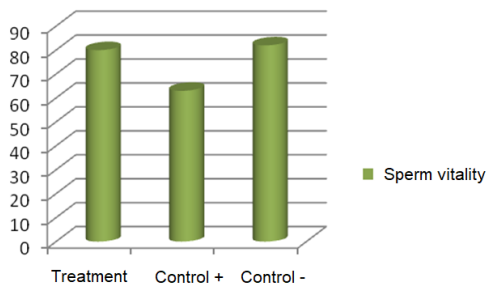


Fig. 2. Percentage of sperm motility in treatment, positive control and negative control groups.

Data analysis showed difference in vitality between treatment, positive control, and negative control groups ($p=0.000$). The difference was found between negative control and positive control guinea pigs ($p=0.000$) and between positive control and treatment groups ($p=0.000$). Whereas, negative control and treated guinea pigs showed no difference ($p=0.817$).

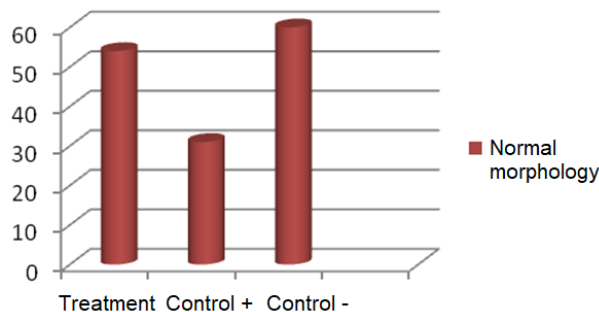


Fig. 3. The percentage of morphologically normal spermatozoa in the treatment group, positive control and negative control.

Data analysis showed normal morphological differences between treatment group, positive control and a negative control groups ($p=0.000$). There was a difference between negative control and positive control guinea pigs ($p=0.000$) and between positive control and treated guinea pigs ($p=0.000$). Whereas, negative control and treated guinea pigs showed no difference ($p=0.176$).

Guinea pig spermatozoa visualization



Fig. 4. Epididymal spermatozoa of treated guinea pigs.

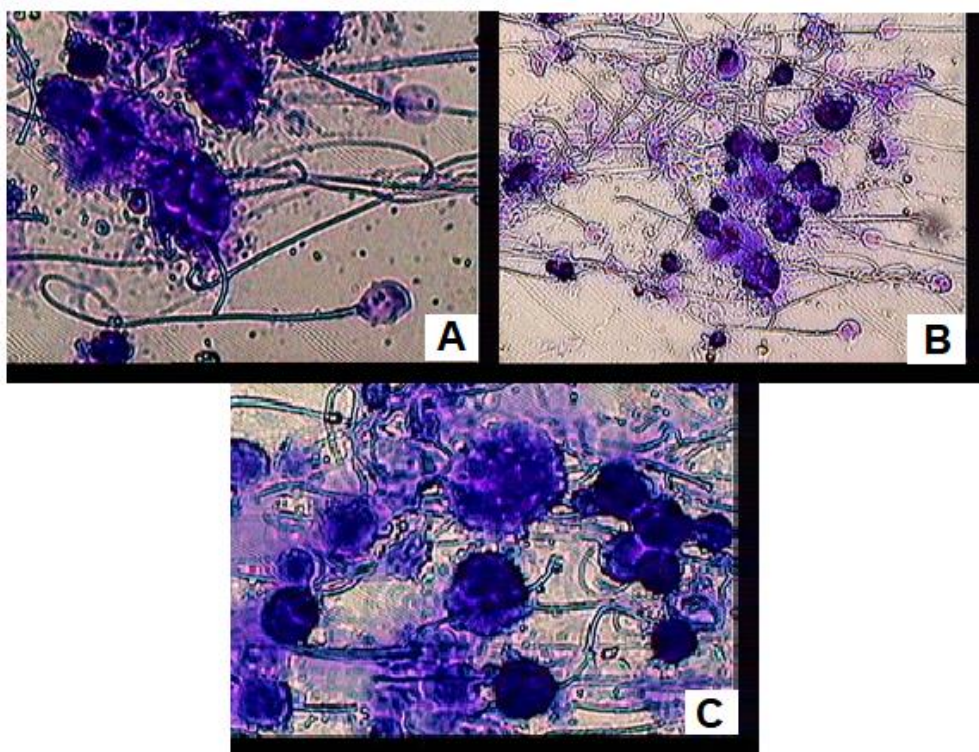


Fig. 5. A and B. Macrophage and *E. coli* in epididymis. C. Guinea pigs epididymal spermatozoa revealing macrophages and *E. coli* attached to the sperm.



Fig. 6. Negative control of guinea pig sperm, showing no macrophages and bacteria.

Guinea pigs spermatozoa MDA level measurement

Data analysis using F test showed differences ($p=0.024$) in MDA levels between groups of treated guinea pigs (immunized with *E. coli* pili protein of 32.2 kDa MW), positive control (guinea pigs infected transurethrally with *E. coli*) and negative control. LSD different test results showed that, compared to control guinea pigs, MDA levels did not differ ($p=0.251$) in guinea pigs

immunized with *E. coli* pili proteins of 32.2 kDa MW. There were differences in MDA levels between guinea pigs infected transurethrally with *E. coli* and those of control ($p=0.007$).

Guinea pigs spermatozoa DNA damage

Data analysis using F test showed differences in DNA damage ($p=0.000$) between guinea pigs in treatment groups (immunized with *E. coli* pili protein of 32.2 kDa MW), positive control (guinea pigs infected transurethrally with *E. coli*) and negative control.

LSD test results showed that DNA damage in control guinea pigs did not differ from DNA damage in guinea pigs immunized with *E. coli* pili proteins of 32.2 kDa MW ($p=0.600$). However, there were differences between DNA damage in control guinea pigs infected transurethrally with *E. coli* ($p=0.000$). Likewise, there were differences between DNA damage in guinea pigs infected transurethrally with *E. coli* and guinea pigs immunized with *E. coli* pili proteins of 32.2 kDa MW ($p=0.000$) and between guinea pigs in treatment groups (immunized with *E. coli* pili proteins of 32.2 kDa MW), positive control (guinea pigs infected transurethrally with *E. coli*) and negative control.

Visualization of guinea pigs spermatozoa DNA damage using Comet Assay method

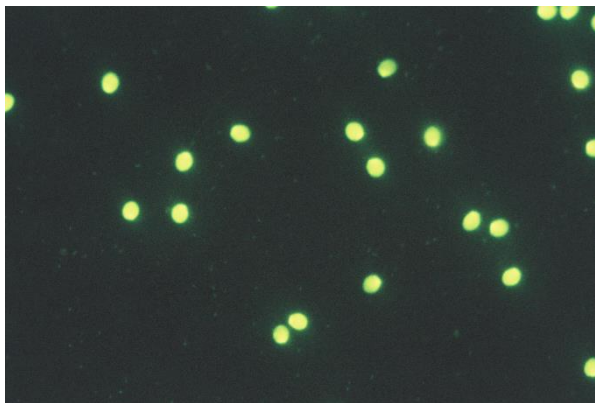


Fig. 7. Sperm DNA damage of control guinea pigs.

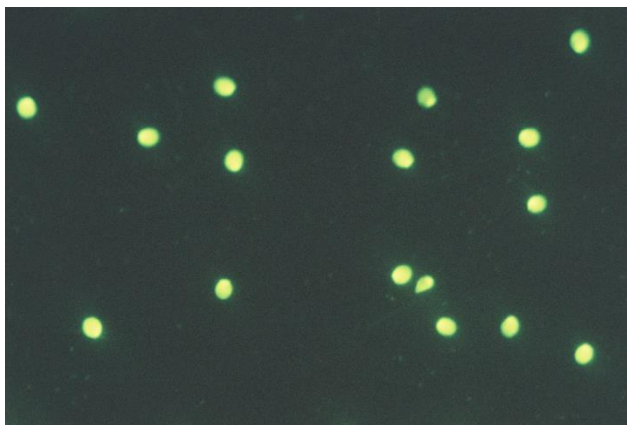


Fig. 8. Sperm DNA damage of guinea pigs immunized with *E. coli* pili protein of 32.2 kDa MW.

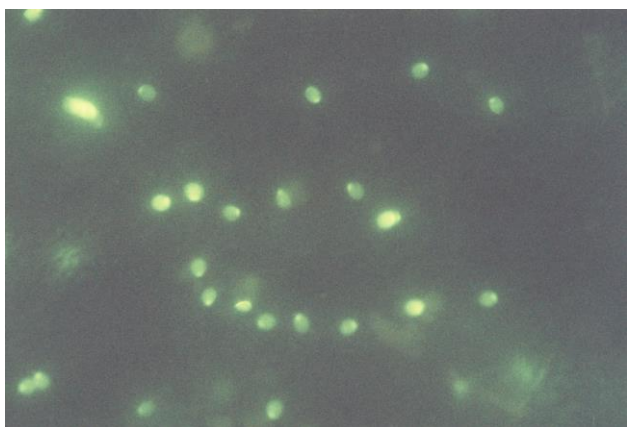


Fig. 9. Sperm DNA damage of guinea pigs infected transurethraally with *E. coli*.

DISCUSSION

Artificial transurethral infection with *E. coli* in guinea pigs (positive control group) can degrade the quality of spermatozoa, which decreases the motility, vitality and morphology of spermatozoa. Artificial transurethral infection in an ascending way can infect the epididymis. This can be confirmed from the results of epididymal sperm visualization of guinea pigs that have the presence of *E. coli*. The *E. coli* are attached to the guinea pig spermatozoa.

The presence of adhesions and toxin produced by *E. coli* causes damage to spermatozoa membrane. It confirms the finding of Monga and Robert (1994) that *E. coli* can reduce sperm quality due to the ability of *E. coli* membrane to attach to spermatozoa. Visually, the attachment is also evident from the results of this study. According to Auroux et al (1991), *E. coli* affects the spermatozoa only when *E. coli* is in contact with spermatozoa. According to Diemer et al (1996), the interaction of *E. coli* and spermatozoa occur in two stages, first the adhesin, then the destruction of spermatozoa membrane. At the end of the pili there is adhesin that mediates the attachment of the bacteria to cell receptors (Schaeffer 1998). By using a electron microscope transmission, Wolff et al (1993) showed that *E. coli* attached to the head and tail of spermatozoa. *E. coli* receptor in the sperm is found on the head and tail, and is part of the sperm membrane protein. *E. coli* adhesion to spermatozoa may cause changes in sperm membrane structure integrity. According to Hafez & Prasad (1976), in general spermatozoa membrane has a function as a medium of transporting all substances required by spermatozoa. According to Zaneveld (1985), spermatozoa membrane, especially the tail, has the function to obtain the substrates needed for energy and function to deliver movement wave. The attachment of *E. coli* on spermatozoa membrane may cause membrane damage.

Membrane damage causes reduced spermatozoa vitality

Spermatozoa membrane damage will also cause disrupted transportation of substances needed as energy source. The energy is needed for sperm movement, so that the disruption of energy sources will cause interference in spermatozoa movement. Toxin and *E. coli* attachment on spermatozoa also have a negative impact on spermatozoa morphology.

Guinea pigs artificially infected with *E. coli* also showed the presence of leukocytes, especially macrophages, in epididymal sperm. This leukocyte phagocytoses sperm, affecting the sperm morphology. In addition,

these leukocytes are the source of Reactive Oxygen Species (ROS). ROS produced by leukocytes can affect sperm quality.

There are several mechanisms that explain the relationship between decreased spermatozoa motility and ROS. Peroxidation of unsaturated fatty acids in membrane lipid is one mechanism that is often implicated (Aitken 1993, Baumber et al 2000). In spermatozoa membrane lipid peroxidation easily occurs because the content of unsaturated fatty acids is high in the plasma membrane. Result of lipid peroxidation is the loss of unsaturated fatty acids associated with the production of lipid hydroperoxide radical, alkoxy radical and peroxy radicals. These radical support the radical chain reaction of lipid peroxidation and causes the production of cytotoxic aldehydes, such as malondialdehyde. The high concentration of unsaturated fatty acids is needed to provide plasma membrane fluidity required for motility. Loss of integrity can lead to increased permeability of the membrane and the loss of the ability to regulate intracellular ion concentration involved in controlling spermatozoa movement.

ROS influence on motility can also occur through the mechanism of changes in mitochondrial function. Mitochondrial membrane potential that is used as a measure of mitochondrial function includes ATP synthesis, mitochondrial protein import, calcium homeostasis and metabolites transport (Baumber et al 2000). Sanocka and Kurpisz (2004) also stated that high levels of ROS is associated with a decrease in mitochondrial membrane potential.

Correlation between high ROS and decreased sperm motility can also be explained in decreasing axonema protein phosphorylation required for sperm motility. ROS inhibits one or more enzymes in oxidative phosphorylation, glycolysis or both, thus limiting the production of ATP by sperm (Baumber et al 2000). Another explanation is that H₂O₂ can diffuse across the membrane to inhibit the activity of enzyme G6PD. This enzyme controls the speed of glucose flow through the hexosa monophosphate shunt, further controls the availability of intracellular NADPH used as a source of electrons by spermatozoa for ROS-producing oxidation by NADPH oxidase enzyme system. G6PD inhibition is also resulting in a decrease in NADPH and, simultaneously, the accumulation of oxidized glutathione into reduced glutathione is taking place. This can reduce antioxidant (Agarwal et al 2003).

Compared to control group, sperm quality in treated guinea pigs group showed no difference. *E. coli* pili adhesin protein with molecular weight of 32.2 kDa immunized to guinea pigs did not degrade the quality of

sperm. Previous studies have proved that *E. coli* pili adhesin 32.2 kDa MW can stimulate immunity. This study has proven that it does not affect the quality of spermatozoa. From these results it can be concluded that the *E. coli* pili adhesin protein of 32.2 kDa MW is not toxic to cellular quality of guinea pig spermatozoa.

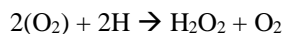
According to Sikka (1996), MDA is the end product of lipid peroxidation induced by Ferro ion. MDA formation can be determined by simple TBA reaction and can be used for diagnosis of lipid peroxidation measurement in vitro and in vivo system. Koksai et al (2003) also stated that MDA is a stable end product of lipid peroxidation.

Lipid peroxidation is widely interpreted as oxidative damage of polyunsaturated fatty acids (PUFA), wherein the fatty acid contains more than two double bonds. PUFAs have unconjugated double bonds which are not separated by methylene. The existence of close bond with methylene makes methylene carbon-hydrogen bond is weak. Furthermore, hydrogen is more prone to breaking. Since abstraction occurs, radicals, which have been stabilized by double bond rearrangement, have been produced and forming a conjugated diene radical that can be easily oxidized. The conjugated diene quickly reacts with O₂ to form lipid peroxy radicals (ROO*), which break the hydrogen atoms from other lipid molecules to form lipid hydroperoxide (ROOH). Lipid hydroperoxide is stable under physiological conditions until they are in contact with transition metals such as Fe or copper. These metals cause lipid hydroperoxide produces alkoxy and peroxy radicals. Furthermore, a chain reaction occurs in the membranes and propagates cell damage (Agarwal et al 2003). This chain reaction can may end in case of the merger of two lipid radicals to form a non-radical fatty acid or radical with a radical repellent compound. The addition of lipid peroxides in spermatozoa plasma membrane may increase structure rigidity and change the ability of sperm to fuse with oocytes (Aitken 1993). High MDA level may lead to decreased sperm motility (Sikka 1996).

In this study transurethral artificial infection with *E. coli* in mice can increase MDA level. This is because infection of the reproductive tract can damage blood testis barrier, which stimulates immune response, both cellular as well as humoral immune responses. In phagocytosis process, granulocytes and macrophages will produce Reactive Oxygen Species (ROS). ROS is an oxidant. One of the targets of ROS is lipid. The main component of sperm cell membranes is the easily oxidized unsaturated fatty acid. MDA is the end product of lipid peroxidation. There is a linear correlation between ROS and MDA. High ROS levels will oxidize

lipids or unsaturated fatty acids, the major component of cell membranes, so that high MDA level is obtained.

Antioxidants are present in seminal plasma and spermatozoa. Antioxidants regulate, challenge and suppress ROS formation. Antioxidant superoxide dismutase (SOD) spontaneously transform superoxide anion radicals ($O_2\cdot^-$) to form O_2 and H_2O_2 . In mammals there are three types of SOD, the cytosolic CuZn SOD superoxide dismutase (SOD 1), intra-mitochondrial manganese superoxide dismutase (SOD 2), and extra-cellular CuZn superoxide dismutase (SOD 3). The three types of SOD catalyze reactions (Sikka 1996):

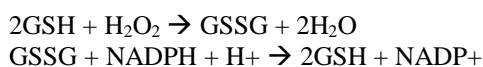


Catalase transforms H_2O_2 into O_2 and H_2O .



SOD protects spermatozoa against spontaneous O_2 and LPO toxicity. SOD and catalase also eliminates O_2 produced by neutrophils oxidase NADPH and plays an important role in lowering LPO and protect spermatozoa during inflammation of the urinary tract (Sikka 1996).

Glutathione peroxidase (GSH) is an antioxidant enzyme that contains selenium and glutathione as an electron donor, and removes peroxy radicals ($ROO\cdot$) from various peroxide, including H_2O_2 . Glutathione reductase then renew the reduced GSH from GSSG (Sikka 1996).



On the state of infection, antioxidants cannot counteract high free radicals. In this study, the levels of spermatozoa MDA in guinea pigs immunized with *E. coli* pili adhesin protein of 32.2 kDa MW was not different from that in control. Low MDA level indicates low peroxidation of guinea pigs' sperm membrane lipid. Thus, *E. coli* pili adhesin protein of 32.2 kDa MW was not toxic.

For several years, conventional semen analysis, which includes concentration, motility, and morphology, is used to diagnose male infertility. However, nowadays test of DNA damage has also to be carried out. In sperm nuclear DNA is contained in the head, which is responsible for carrying genetic information necessary for the fertilization of the egg. Nuclear DNA can be damaged because of the defect at the time of chromatin packaging, apoptosis, oxidative stress, and genetic lesions (Marchesi & Feng 2007). According to Zini and Sigman (2009), the cause of DNA damage is multi-

factorial, ie due to intrinsic factors, such as protamine deficiency, high ROS and apoptosis; or extrinsic factors, ie testicular hyperthermia, and toxic environment. DNA damage is apparently associated with sperm infertility (and abnormal spermatogenesis). However, a small percentage of spermatozoa from fertile men also detect DNA damage. It has recently been reported that DNA fragmentation also appears in sperm with normal motility and morphology (Avendan & Oehninger 2011).

In this study, visualization showed that guinea pigs infected transurethrally with *E. coli* had many granulocytes or macrophages. Granulocytes and macrophages are producing ROS. In this study, DNA damage occurred more severely in the sperm of guinea pigs infected with *E. coli* compared to that of negative control and guinea pigs immunized with *E. coli* pili adhesin protein of 32.2 kDa MW. Linkages between ROS and DNA damage is that high ROS level causes oxidative damage to DNA. 8OHdG is a marker of DNA damage due to oxidation because guanine is a fragile core base, not influenced by diet, and excreted into the urine separately from the other metabolites (Moon et al 2001).

In this study, sperm DNA damage was also detected in control group. Muratori et al (2000) stated that there is DNA fragmentation in the sperm of fertile and infertile men. This refers to the hypothesis put forward by Ollero et al (2001) that there are three hypotheses to explain the occurrence of DNA damage in mature spermatozoa, (a) defect in spermiogenesis, (b) DNA damage occurred after spermiation, (c) a combination of both, the changes in spermiogenesis regulation that cause defects in chromatin packaging and induces ROS, thus damaging mature spermatozoa after spermiation.

CONCLUSION

E. coli pili adhesin protein of 32.2 kDa MW is not toxic, either to sperm cellular quality or to sperm molecular quality.

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