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Investigating the Antioxidant Effect of *Allium cepa* After Exposure to *Escherichia coli* on Biochemical Factors, the Blood Antioxidants, and Testis Tissue in Rats

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Abstract

Objective: Infectious infertility is considered by the World Health Organization (WHO) as a main problem in sexual life and public health. The aim of the present study was to investigate the antioxidant properties and the effect of *Allium cepa* (onion) juice on the tissue of testis and seminiferous tubules affected by *Escherichia coli*.

Materials and Methods: Thirty-Two adult Wistar male rats aging 2.5 to 3 months divided to four groups of 8 rats. Enterotoxigenic *E. coli* (serotype 0114) used to infect the rats. Onions prepared from the district Ilkhichi, Iran which were used for two groups. Following the infection, pathologic samples were prepared from the tissue of the sperms which were investigated through hematoxylin & eosin (H & E) staining. In addition, the motility, vitality, the number of sperms, total antioxidant capacity (TAC), luteinizing hormone (LH), and testosterone were evaluated as well.

Results: Results indicated that in the control group all the seminiferous tubules are sticking together and all the lines of sexual germ cells observed;while, in *E. coli* group were disunited and the line of sexual cells were destroyed. In the groups infected by *E. coli* and treated by *A. cepa* juice, the effects of bacteria reduced considerably. The number of sperms, sperms vitality and motility decreased significantly in *E. coli* infected group, while in the *A. cepa* juice + *E.coli* the effects of infectious was reduced. The results of the study showed that *A. cepa* juice significantly increases TAC and testosterone.

Conclusion: The results indicated *A. cepa* juice has protective effects against *E .coli* bacteria and fertility, testis tissue and antioxidants improvement and the effects of the bacteria decreased significantly.

Keywords: Antioxidant, *Allium cepa*, *E. coli* bacteria, Onion juice, Infertility, Testis tissue, Sperm parameters

Introduction

Fertility is a key element in sexual married life and infertility has been considered by the World Health Organization (WHO) as a public health problem (1). The WHO defines infertility as not getting pregnant after 12 months or more of unprotected sexual intercourse (2). The literature shows that there are 48.5 million infertile couples around the world (3), and one per seven couples suffer from infertility (4). Infertility is a main problem in the lives of 25% of the couples, nearly 60% of the infertility cases are related to men and the remained ones are related to women or both (5,6). A significant trend to postpone the first childbirth has been observed in the developed countries which has been recently seen in developing countries as well (7). The costs of low fertility or infertility have had little decrease in the past 20 years. According to literature male factors contribute in 50% of the infertility cases (8). The data resulted from the analysis of semen indicates that in some patients the reason of the problem is not clear, and in some others experimental treatment has been applied. One reason for the lack of concise therapy for this problem is the lack of fundamental understanding various factors causing infertility. Infertility in men is a problem with various causes associated with different genetic factors and factors including urinary tract infection and genital infection, immunologic diseases and endocrine, damage resulted from reactive oxygen species (ROS), and disorganization caused by endocrine disorders (8).

According to the studies, bacterial infections and the following responses of the immune system are proved to be among the main factors causing infertility in men. Such infections affect different parts of male reproductive system such as testis, epididymis, and the attached glands. Therefore, in different stages of progress, maturity and transfer, these infections affect the quality of sperms negatively, leading to increase of infertility probability (8). Semen infection and reproductive tract infection is one of the main factors causing infertility among men (9). According to a study in 2016 in Britain, infertility in women has been reported as 12.5% in women and 10.1% in men (4). Some pathophysiological mechanisms in infertile men associate with semen infection (bacteriospermia).

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Infection contributes indirectly to unnatural decrease of spermatozoids of semen, decrease of mobility and morphologic changes in sperm, leading to a decrease in the power of fertility. It can also indirectly contribute to infection, testicular damage, inflammation, and consequently stimulating the immune system against its own antigens associated with leukocytospermia which can lead to infertility in men (10).

Studies have shown that *Allium cepa* (onion) as a rich source of phytochemicals, improves antioxidant properties and can either adjust the detoxification system (11, 12). Such performance effects have a significant importance in prevention and treatment in many diseases (13). Biologic activity of onion is due to phenolic compounds and organosulfur. The studies have also shown that *A. cepa* contains endogenous and exogenous antioxidants such as selenium, glutathione, vitamins A, B, and C, and flavonoids like quercetin and isorhamnetin (14). The above antioxidants protect DNA and other important molecules against oxidation and damage, and as a result increase fertility in men (15). In addition, the antibiotic effects of *A. cepa* on *Escherichia coli* bacteria have been proved by a variety of researchers (16-19).

The present study aims was to investigate the effect of antioxidant properties of *A. cepa* juice on biochemical factors, blood antioxidants, and the quality of testicular tissue following an exposure to *E. coli* bacteria.

Materials and Methods

The present study is a laboratory one, performed according to moral principles ruling on work with laboratory animals. A number of 32 Wistar male mature rats weighing 200 to 220 g aging 2.5 to 3 months were used in this study. They were divided into 4 groups, each containing 8 rats which were exposed 12 hours of light and 12 hours of darkness during 28 days. The drink water used for the animals was tap water and they were feed by special food for mice.

To prepare *A. cepa* juice for daily use, a fresh *A. cepa* from the region Ilkhichi was juiced by a manual juice maker, then 3 cc of the juice was given to each rat by gavage method.

Preparing Enterotoxigenic *Escherichia coli*, the Biochemical Properties, Cultivation

Enterotoxigenic *E. coli* (serotype 0114) was prepared from Bou-Ali reference laboratory and was passaged on MacConkey agar and eosin methylene blue (EMB) agar media after being transferred to laboratory. Following incubating in 37°C, the produced colonies were investigated in terms of microscopic, macroscopic, and biochemical conditions. After *E. coli* was cultivated in the medium trypticase soy broth (TSB), it was incubated in 37°C for 12-18 hours to multiply. Then the medium TBS was centrifuged in 2000 rounds for 15-20 minutes, and the produced sediments were washed with PBS for three times. In the final stage the sediments were solved in 0.5 mL PBS. The light absorption was measured by spectrophotometric device with the wave length 630 nm. Due to the absorption of OD-0.5 in the wave length 630 nm, the tube contains $1*10^8$ CFU/mL of bacteria mass, which was filled into 2 mL ampoules and fed to the rats.

Thirty-two male rats were divided into 4 groups;there were 8 rats in each group. The first group was the control group, the second was received *A. cepa* juice, the third group was fed by 0.5 mL *E. coli* suspension every day for 6 consecutive days using sterile syringes, and the fourth group was both infected by the bacteria and fed by *A. cepa* juice. *E. coli* was transferred from testis to the medium MacConkey agar for investigation, separation, and diagnosis. Then it was cultivated on two media violet red bile agar (VRBA) and EMB. After the bacteria grew on the medium EMB, its typical colonies were transferred to the medium triple sugar iron (TSI) and the results were analyzed. Sixty days after the experiment started, blood samples were taken from the eyes of the rats to be investigated in terms of chemical factors and antioxidants.

Investigation of Tissue Samples

E. coli was transferred from the testis to the medium MacConkey agar for investigation, separation, and diagnosis. Then for anesthesia to investigate the testis tissue pentobarbital (40 g/kg) was administrated by intraperitoneal injection, then the intraperitoneal area was opened with an abdominal transverse slot and testis were taken out of the body and weighed in both control and treatment groups. According to animal protection act, the animals were killed at the end of the experiment by CO_2 in 2 hours (9-11 AM). The testis tissue samples were fixed in formalin 10%. After preparing microscopic sections with 5 microns thickness, hematoxylin & eosin (H & E) staining was applied and then Kodak ultra ASA 400 film and optical microscope (model Olympus/3H-Z) made in Japan were used to prepare microscopic images (20).

Investigation of Cytotoxic Effect of *Escherichia coli* on the Tissue

In order to investigate the cytotoxic effect of *E. coli* on testicular tissue, the testes were placed into the medium RPMI without serum;after the capsule around the testis was removed, the seminiferous tubule was opened and chopped in tiny pieces by scissors. The chopped tissues were washed 3-4 times to get completely empty of sperms. Then the tissue pieces were placed into a falcon tube and 10 mL of the medium Hanks' balanced salt solution (HBSS) and 1 mL/mg collagenase were added to it. It was then placed into a 37°C incubator for 30 minutes;during this time the tissues were stirred by agitator several times, so that enzyme and mechanical digestion take place. Then the enzyme digestion stopped by adding 30 mL HBSS. Then, in order for the tissue pieces to settle, the tube was incubated for 10 minutes, the medium on the top was removed, and the pieces were cut in 1 mm dimensions, then trypsin 0.25% was added and incubated for 30 minutes. It

was then centrifuged and added to a medium having serum and once the cells were uniform, about 500 µL of the cellular soup and the medium was removed and placed into plates with 24 spaces. The plates were placed into a CO_2 incubator for 5 days and the cells were cultivated.

To investigate the toxicity of *E. coli* neutral red colorimetric test was used. First, 40 µg/mL neutral red was prepared and placed into a 37°C incubator for one night;the next day it was centrifuged with 1500 rounds for 10 minutes and the crystal sediment was removed from the color medium. A week after the cells were cultivated, the medium on them was removed slowly and a certain volume of neutral red (200 µg) was added to the holes containing cells. The neutral red was incubated in proximity of cells for 3 hours so that the color get absorbed by lysosomes of cytoplasmic membrane of cells. In the phase of cellular fixation 200 µL sodium chloride fixative solution and formaldehyde was added to the holes for washing and fixing cells. Then 200 µL ethanol and acetic acid lubricating solution was added to each hole and incubated for 15 minutes. Then to completely extract the color from the cells, solution was peptized strongly;and then the color intensity was evaluated using ELISA reader in wavelength 540 nm (21).

Results

The results of tissue investigation indicated that all seminiferous tubules of testis are sticking together in the control group and all the ranks of sexual germ cells were observed in this group. On the other hand, in the group infected by *E. coli*, the seminiferous tubules were disunited and sexual cells lines were destroyed. In the group receiving *A. cepa* juice, all seminiferous tubules were sticking together and the sexual germ cells were also observed. Also, inside the seminiferous tubules was full of sperms. In the group infected by *E. coli* and fed by *A. cepa* juice, seminiferous tubules of the testis tissue were less disrupted than the group receiving *E. coli* (Figure 1).

The results of the investigation of the parameters related to sperms including mobility, vitality, and the number of sperms indicated a significant reduction of these parameters in the group infected by *E. coli* compared to the control group ($P<0.05$). In *A. cepa* juice group, all the parameters were significantly higher than the other groups ($P<0.05$). In the group receiving both *E. coli* bacteria and *A. cepa* juice, the parameters reduced insignificantly compared to the control group ($P>0.05$), while they increased significantly compared to the group infected by *E. coli* ($P<0.05$; Table 1).

E. coli decreases the amount of TAC significantly;however consuming *A. cepa* juice in the presence of bacteria can exhibit appropriate protecting effects and increase TAC. Consuming *A. cepa* juice in the absence of the bacteria increases TAC significantly as well. The results also indicated that *E. coli* reduces luteinizing hormone (LH) compared with the control group, while consuming A. cepa juice increased it to the level of the control group. However, LH changes were not significant in the four groups investigated. The results of the study showed that *E. coli* reduces testosterone, while consuming *A. cepa* juice increased it to the level of the control group. The results of the study also indicated that the amount of testosterone was significantly higher in the *A. cepa* juice group compared to the other groups (Table 2).

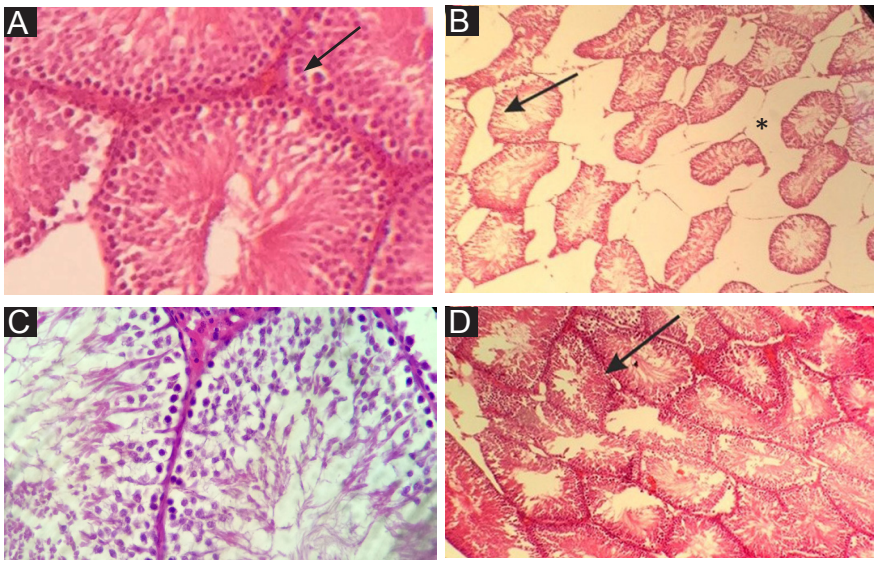

Figure 1. (A) Photomicrograph of testis tissue in control group, seminiferous tubules attached together (arrow), all germinal cells are seen and sperm assemble in lumens of tubules. Haematoxylin and eosin staining, (x640). (B) Photomicrograph of testis tissue of the *E. coli* group, seminiferous tubules the attached (star), germinal cells are not seen and sperm not present in lumens of tubules. Haematoxylin and eosin staining, (x160). (C) Photomicrograph of testis tissue of the group receiving onion juice, seminiferous tubules attached together, all germinal cells are seen and sperm assemble in lumens of tubules. Haematoxylin and eosin staining, (x320). (D) Photomicrograph of the testis tissue of the group receiving *E. coli* and onion juice, seminiferous tubules attached together, spermatogonia is seen and sperm assemble in lumens of tubules, blood vessels in interstitial tissue shows congestion (arrows). Haematoxylin and eosin staining, (x160)

Table 1. Investigating the Effect of *Allium cepa* Juice on the Parameters of Sperm Upon the Infection From *Escherichia coli*

Group	Sperm Motility (%)	Sperm Viability (%)	Sperm Count (10^6)
Control	40 ± 0.05*a	70 ± 0.05*b	62 ± 0.05*b
E. coli	30 ± 0.06 b	60 ± 0.70a	42 ± 0.03a
A. cepa juice	80 ± 0.15c	92 ± 0.05c	70 ± 0.08c
E. coli + *A. cepa* juice	36 ± 0.05a	65 ± 0.05b	52 ± 0.70b
P value	0.002	0.012	0.021

*Different alphabetic indicate significant differences between groups ($P < 0.05$).
Data are presented as mean ± Standard error (SE).

Table 2. Investigating the Effect of *Allium* cepa Juice on the TAC, Testosterone, and LH on the Occurrence of Infection by *Escherichia coli*

Group	TAC (nmol/mL)	LH (nmol/mL)	Testosterone (nmol/mL)
Control	0.56 ± 0.05*b	1.47 ± 0.22	1.63 ± 0.33*b
E. coli	0.48 ± 0.77a	1.41 ± 0.34	1.21 ± 0.33a
A. cepa juice	1 ± 0.07c	1.50 ± 0.33	2.1 ± 0.33c
E. coli + *A. cepa* juice	0.52 ± 0.02b	1.48 ± 0.22	1.51 ± 0.05b
P value	0.011	0.061	0.026

Abbreviations: TAC, total antioxidant capacity; LH, luteinizing hormone.
*Different alphabetic indicate significant differences between groups ($P < 0.05$).
Data are presented as mean ± standard error (SE).

Discussion

E. coli and enterococcus are two common bacterium attacking to intestinal tract. Some researchers have identified both organisms in 13% of their patients (22). They have reported enterococcus in 6.1% of the cases and *E. coli* in 1.7% of the cases in their studies (23). *E. coli* is gram-negative bacilli, mobile, aerobic, anaerobic, optional and non-spore. This bacteria produces a mix of acids in anaerobic conditions including lactate, succinate, ethanol, and carbon dioxide (24). Its optimal growth occurs at 37°C, however can bear to 49°C and continue to grow (25). *E. coli* is the most common factor causing epididymitis – orchitis, transmitted through non-sexual ways and contributes in 65% to 80% of acute or chronic prostatitis cases. Therefore, *E. coli* may contribute to infertility, the fact that can be true for other members of the family enterobacteriaceae belonging to the types of *Klebsiella*, *Salmonella*, and proteus. *Pseudomonas aeruginosa* can cause epididymitis and prostatitis and interfere in this way in fertility of men (26). While, *E. coli* infections in men can lead to infertility by creating factors like immobility of sperms, epididymis and prostate inflammation (22).

Among the men below 35 years with higher sexual activities who have the experience of draining urine tract, the most common pathogen is associated with hydrocele in which the bacterium *Chlamydia trachomatis* and *Neisseria gonorrhoeae* contribute (27). Infection by *E. coli* and other enterobacteriaceae is particularly common in elderly men (28). Genital infections are accompanied with biologic and chemical changes in seminal liquid which can disturb the performance and fertilization of sperms which is related to the testis (29). Hydrocele is a consequence of epididymis in common bacterial infections (30). Male excurrent duct is an entry to microbial infections which can appear as urethritis, prostatitis, or hydrocele. Then it is not surprising that in 13% to 15%, infertility infections with male factor and inflammation of male genital system are considered as the main cause of fertility disorders in men (31). On the other hand testis is an immunological organ whose antigens protect the meiotic germ and haploid cells automatically and have high tolerance (32-35).

The antibiotics have important functions in infectious diseases. Infectious diseases of urinary system are among the factors threatening the life of adult people. Male people in the society suffer at least one of such disease during their lives (36,37). The most important pathogen in this system is *E. coli* with 70%-95%, and *Staphylococcus saprophyticus* with 5% prevalence (38,39). To cure such infections, antibiotics including the antibiotics belonging to the family fluoroquinolones like ciprofloxacin are applied. Such drugs prevent proliferation of bacteria by prohibiting the function of DNA gyrase (topoisomerase) and preventing the DNA strings from opening (40,41).

In a similar study by McGowan et al (10), it was found that the seminal liquid of 16% of fertile men and 25% of infertile men has bacterial infection. Kessler et al reported in their study that if the number of *E. coli* bacterium in semen excesses 10^8, they can destroy the sperm parameters. Yet these experiments were performed in vitro, and it is difficult to generalize them to in vivo conditions (42). In 1995 Merino et al performed microbial experiments on the semen of the men who had referred to their clinic for infertility. Their findings showed that 9% of the aerobic bacterium are *E. coli*. They emphasized in their paper that presence of bacteria in semen has a direct negative impact on the quality of sperms and predicts the potential of azoospermia (43).

The findings by the researchers have shown that cadmium increases lipid peroxidation in testis and glutathione-s-transferase, while it decreases glutathione, superoxide dismutase, catalase, and alkaline phosphatase. Also, due to the toxicity of cadmium the volume of sperm ep-

ididymis and mobility of sperms is reduced and the percentage of unnatural materials in sperm will increase. The results of the above study showed that using union and garlic juice can prohibit the oxidative effects of cadmium, while increased the antioxidant defending mechanism in rats (44). In a study investigating the effect of quercetin and *A. cepa* juice on preventing the consequences of getting poisoned by the smoke of diesel motors, it was found that both quercetin and *A. cepa* juice increase sertoli cells significantly, compared to the poisoned group (45). The findings of this study showed that quercetin and *A. cepa* juice prevent the toxic effect of diesel smoke on reproductive system. The results of many studies have shown that onion is a main source of nutritional phytochemicals and improves antioxidant properties and is able to regulate the detoxification system (11,12). These functional effects are very important in preventing and curing of many diseases (13). S-methyl cysteine sulfoxide is a bioactive organosulfur compound in onions exhibiting a high antioxidant capability. While it can combine with lipid peroxides, it can also prevent lipid peroxidation as an antioxidant or chelating factor or metal transfer (46). Reduction of lipid peroxidation may reduce the ROS, leading to reduced activity of glutathione-s-transferase mediated by ROS. Onion probably acts against some carcinogen factors by regulating phase 2 enzymes like glutathione-s-transferase and p-nitrophenol UDP-glucuronosyltransferase (47). In addition, it seems that improved activity of superoxide dismutase and catalase can decrease the oxidative damage in testis tissue following the consumption of *A. cepa* juice (44). The results of the present study showed that the amount of TAC in onion group was higher significantly than the other groups. It was also higher in infection and onion group compared to the infection group with no onion which indicates the antioxidant properties of onion and is consistent with the results of the previous studies.

The findings of literature have shown that E. coli has a direct impact on spermatozoa through cellular interactions and adhesion phenomenon, changing the mobility of cells and disturbing the integration of cells and molecular structure of spermatozoa (29,48). The findings by researchers showed that *E. coli* bacteria has many significant impacts on human spermatozoa mobility (49).

The findings indicated that the most prevalent factors leading to semen infection in men are *Staphylococcus* and *E. coli*. They are considered as the main factors causing infertility in men. These results are consistent with the findings by Holmes et al. They reported that *E. coli* is one of the main factors causing either marked or unmarked infections in urinary-reproductive systems which can alter sperm parameters like mobility and metabolism;they also showed that *E. coli* leads to death of 15% of sperms outside the body (50).

Ultrastructural damage caused by *E. coli* on the spermatozoa has been recognized by various microscopic methods (51,52). Bonding *E. coli* to the surface structure of spermatozoa by adhesive molecules type 1 leads to the alteration of the structure and damage to plasma layer of human sperms;such damage is exposed on the acrosome part of the sperm as well. Damage to the acrosome part, middle part, and the tail of sperm can decrease the sperm mobility significantly, leading to infertility (51). Bacterial infections make the sperms stick (agglutination), which can lead to immobility of sperms. The degree of immobility depends on accumulation of bacterium in seminal liquid. A bacteria causing agglutination is *E. coli* (48,53). Acute epididymis inflammation resulted from bacterial infection disturbs spermatogenesis process which is resolved by a correct prescription of antibiotics in most cases, while sperm parameters get to their normal status (54,55).

The findings of the present study showed that *E. coli* decreases the sperm mobility, vitality, and the number of the sperms in the infection group. While in the group infected by the bacteria and fed by *A. cepa* juice the degree of mentioned parameters improved, indicating the antioxidant and antibiotic properties of onion which reduces the negative impacts of *E. coli*.

The results have shown that *E. coli* causes epididymis inflammation in rats;and in 20% of the cases infection is extended to testis through lymphatic system. In 50% of cases, the germ cells destroyed on the second day of the study and did not revive even after months. It seems that bacterial toxin impacts and sudden cease of liquid flow into seminiferous tubules lead to these damages in testis (56). The results of the present study also showed that *E. coli* disunites the seminiferous tubules strongly, while in onion + bacteria group this condition was somehow better.

The researchers have reported that LH and testosterone are related to human seminal parameters;they also have explained that the serum level of LH and follicle-stimulating hormone (FSH) is reversely related to sperm concentration (14).

The literature also shows that amount of LH and testosterone influence sperms mobility. LH reduces the concentration, mobility, and morphology of sperms (14). The present study showed that the different between LH among the studied groups is not significant, while testosterone in *A. cepa* juice group increased significantly. The amount of testosterone in infection + *A. cepa* juice group have increased to the level of the control group. The findings of the present study regarding LH and testosterone are consistent with the studies performed previously.

Ethical Issues

The Ethical Committee of Islamic Azad University, Ahar branch approved this study.

Conflict of Interests

The authors declare no conflict of interests.

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