Punicalagin is beneficial for spermatogenesis in male New Zealand White rabbits

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ABSTRACT
Taking into account that punicalagin (PUN) is a very powerful antioxidant; the current study evaluated the potential positive effects of oral PUN on spermatologic parameters of male New Zealand White rabbits. A total of 24 male bucks was housed individually and trained for semen collection. After the training for 2 wk, rabbits were assigned into 4 groups and received daily gavages of 0, 1, 2, and 10 mg/kg PUN in tap water for 9 wk. Semen was collected weekly from each rabbit and samples at d 1 and 63 of the experiment were analyzed separately. Initial values (ejaculate volume, ejaculate weight, ejaculate pH, sperm concentration, percent progressive motility, and seminal plasma protein levels) tested at d 1 were similar among the groups. There were also no differences in ejaculate volume, ejaculate weight, ejaculate pH and seminal plasma protein concentrations at the end of the experiment. Libido and reproductive organ weights were not affected by the treatments. However, sperm concentrations (P<0.01) and percent progressive motility (P<0.04) were significantly improved for bucks in 2 and 10 mg/kg PUN groups. Thus, the current study suggested that as low as 2 mg/kg/day PUN can be beneficial for spermatogenesis and motility in male rabbits.

Key words: Male reproduction, Nutrition, Spermatogenesis.

INTRODUCTION
Punicalagin (PUN) is an ellagitannin, especially found in pomegranate (Punica granatum L.), and leaves of the tropical almond tree (Terminalia catappa L.). Pomegranate juice has been reported to have about 2 g/L PUN (Gil et al., 2000). PUN is thought to be responsible for the most important part of the antioxidant properties found in pomegranate juice. Due to the high antioxidant properties of pressed pomegranate juice and tropical almond tree leaf, number of in vivo and in vitro studies with these products can be found in the literature. However, the studies made with PUN, which is thought to be the direct active substance, are limited.

It is possible to find in vivo studies on the protective effects of PUN. PUN has hepatoprotective (Lin et al., 1999), anti-inflammatory (Lin et al., 1999; Jean Gilles et al., 2013), antidiabetic (Nagappa et al., 2001), antimicrobial (Machado et al., 2002; Silva et al., 2015) and neuroprotective (Yaidikar et al., 2014) effects in different laboratory animals. Furthermore, in another study, no toxic effects of punikalajin were observed when administered orally for 37 days to rats (Cerda et al., 2003). Although studies have been carried out on the spermatologic effects of pomegranate juice and pomegranate juice concentrate in rats (Fedder et al., 2014; Leiva et al., 2011; Turk et al., 2008), we did not find a study that directly examines the effect of punikalajin on spermatologic properties of male mammals.

Since it is possible to acquire full ejaculates in male rabbits, it is actually the most suitable model for the investigation of spermatologic effects of chemicals in humans (Foote, 2002). Nevertheless, the rabbit model has not been used before in investigating the antioxidant properties of PUN on spermatologic parameters. Therefore, the aim of the current project was to determine the effects of PUN on some reproductive parameters of male New Zealand White rabbits.

MATERIALS AND METHODS
Experiment: The current study was approved by the ethics committee of Mehmet Akif Ersoy University (2015.25.11/159) and supported by TUBITAK (project no: 116O027). The experiment was carried out at the Experimental Animal Units of Mehmet Akif Ersoy University, Faculty of Veterinary Medicine. At the beginning of the study, 24 male New Zealand White rabbits were randomly placed into individual galvanized cages and kept under standard laboratory conditions (humidity of %50-55, room temperature of 22 ±2 °C and 14:10 hours of light:dark cycle). The age and weight of the rabbits were 7-10 months and 2.4 to 3.7 kg, respectively.

Feed and water were supplied ad libitum. The rabbits were fed standard commercial rabbit pellets (Korkuteli Yem Gıda San. Korkuteli, Antalya, Turkey; 89% dry matter, 6.93% ash, 17% crude protein, 12.68% crude fiber, 3.67 crude fat, 0.49% calcium, 0.46% phosphor) during the
entire experimental period. Prior to the experiment, bucks were adapted to the laboratory conditions and trained to use an artificial vagina for 10 days. Subsequently, the bucks were divided randomly into 4 groups of 6 rabbits each. The control group (CON) received tap water daily for 9 weeks. During the same time periods, rabbits in PUN1, PUN2 and PUN3 groups received 1, 2 and 10 mg/kg/day of PUN in tap water, respectively. Oral gavages were performed between 08:00 and 09:30 hours before the morning feeding each day. During the experiment, weekly body weights for individual animals were recorded at 07:00 hours and dose adjustments were made accordingly. Ejaculates were collected once a week by means of an artificial vagina and samples on day 1 (initial) and 63 of the experiment were immediately transferred to the laboratory and used for the analysis. At the end of the experimental period, rabbits were euthanized and wet weights of testes and epididymides were recorded. **Semen collection:** Semen evaluations were performed by a single researcher as single-blinded fashion. Prior to the semen collection, rabbits were allowed 2 false mounts and at the subsequent mounting, the artificial vagina was adequately positioned for penis intromission. The ejaculate was collected into graded warm tubes. After removal of gel mass, volume and the weights of the ejaculates were recorded and kept in a water bath. **Semen evaluation:** Immediately after removal of gel mass, initial pH of semen samples was determined by a pH cooperative paper (pH-IndikatorpapierNeutralit pH 5.5–9.0; Merck, Darmstadt, Germany). Spermatozoa concentration, sperm motility and sperm morphology were calculated as previously described by Ata et al. (2007). Sperm counts were made in the sperm suspension in 0.1 ml formalin saline solution (4% formalin in 0.9% saline), with the aid of a Thomahemocytometer (Marienfeld GmbH & Co. KG, LaudaKönigshofen, Germany) at x400 magnification. The percentage of motile sperm was estimated by visual examination under x400 magnification using a phase-contrast microscope with heated stage (37.8°C). To measure seminal plasma protein concentrations, 200 µl of semen from each collection was centrifuged for 20 min at 800 g to separate spermatozoa and clear seminal plasma. Then, these seminal plasma samples were used to evaluate the total seminal plasma protein levels by using a refractometer (Atago, SPR-N, Japan). **Statistical analyses:** The data were analyzed by Proc T-Test procedure of the SAS statistical package. The individual treatment group means were compared by Dunnett post hoc analysis. The minimum level of significance was set at p<0.05. **RESULTS AND DISCUSSION**

Pomegranate (Punica granatum L.) is a fruit that has been consumed since ancient times and has become very popular today because of its antioxidant properties. Antioxidants and reproductive health are one of the most popular topics of today’s world and will continue to receive a great deal of interest. Currently, the number of literatures evaluating the association between antioxidants and male reproduction is rising. Therefore, in our study, we evaluated the possible positive effects of PUN on some reproductive parameters in male New Zealand White rabbits. Overall, no negative health problems or significant body weight changes were observed among the groups due to PUN treatment (data not shown here). Initial values for spermatologic parameters are represented in Table 1. The parameters were within the normal range for male rabbits (Campos et al., 2014) and did not differ among the treatment groups. Since these were the initial values, we were not expecting to see any difference and this indicated that the distribution of rabbits across the groups was uniform. The data on the wet reproductive organ weights of rabbits are in Table 2. In the current study, oral PUN treatments had no significant effect on the weights of the testes and epididymides. In other studies, different doses of pomegranate juice did not significantly alter the reproductive organ weights in rats (Al-Olayan et al., 2014; Turk et al., 2008). Moreover, another antioxidant, vitamin C, did not cause a change in testicular weights in rabbits (Ata et al., 2007).

The PUN treatments did not cause any difference in ejaculate volume, ejaculate weight or ejaculate pH values (p>0.1). However, progressive motility was significantly increased in all three PUN doses (Table 2; P<0.05), whereas the spermatozoa concentration was higher in the two highest PUN doses compared to the control rabbits (P< 0.05). Proper nutrient supply is crucial to ensure optimal spermatogenesis and increase in sperm count and percent motile spermatozoa.

**Table 1:** Initial spermatologic parameters (mean ± SD) of male New Zealand White rabbits prior to the experiment.

<table>
<thead>
<tr>
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<th>CONT</th>
<th>PUN1</th>
<th>PUN2</th>
<th>PUN3</th>
<th>P&lt;</th>
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<tbody>
<tr>
<td>Ejaculate volume (ml)</td>
<td>0.55 ± 0.07</td>
<td>0.57 ±0.15</td>
<td>0.56 ±0.08</td>
<td>0.58 ± 0.09</td>
<td>NS</td>
</tr>
<tr>
<td>Ejaculate Weight (mg)</td>
<td>0.56 ± 0.10</td>
<td>0.59 ±0.15</td>
<td>0.57 ±0.08</td>
<td>0.59 ± 0.10</td>
<td>NS</td>
</tr>
<tr>
<td>Progressive Motility (%)</td>
<td>57.5 ± 8.21</td>
<td>55.8 ±7.35</td>
<td>55.0 ±7.07</td>
<td>53.3 ± 5.16</td>
<td>NS</td>
</tr>
<tr>
<td>Ejaculate Ph</td>
<td>7.11 ± 0.07</td>
<td>7.11 ±0.11</td>
<td>7.16 ±0.10</td>
<td>7.10 ± 0.11</td>
<td>NS</td>
</tr>
<tr>
<td>Sperm concentration (x10⁶/ml)</td>
<td>233.3 ± 21.3</td>
<td>238.2 ±28.6</td>
<td>232.5 ±29.9</td>
<td>244.2 ± 24.4</td>
<td>NS</td>
</tr>
<tr>
<td>Seminal Plasma Proteins (g/dl)</td>
<td>3.30 ± 0.22</td>
<td>3.26 ±0.16</td>
<td>3.26 ±0.08</td>
<td>3.20 ± 0.21</td>
<td>NS</td>
</tr>
</tbody>
</table>

CONT=control; PUN1=1 mg/kg/day punicalagin; PUN2=2 mg/kg/day punicalagin; PUN3=10 mg/kg/day punicalagin. NS=not significant.
are important for overall sperm quality (Ramachandran and Singh, 2017). It is imperative to have a certain number of motile sperm in the ejaculate for successful fertilization. In our study, daily oral PUN caused an increase in sperm number and motility, especially in PUN2 and PUN3 groups. The positive effects of PUN on spermatologic parameters could possibly be due to PUN’s antioxidant properties. Number of in vivo studies have shown that antioxidants such as vitamin C and E, increase the number of spermatozoa in mice (Acharya et al., 2003; Mishra and Acharya, 2004; Rao and Sharma, 2001; Sato and Ishikawa, 2004) and rabbits (Ata et al., 2007). Moreover, in an in vitro study, addition of pomegranate juice to the liquid storage of Bos frontalis semen decreased the percentages of abnormal spermatozoa at different hours of storage periods at 5°C (Perumal and Rajkhowa, 2015). Ellagitannins from plants and fruits were potent O₂⁻ scavengers (Coballase et al., 2011) and PUN depicts the ability to scavenge nitric oxide, H₂O₂ and ferrous chelating activity (Al-Olayan, 2014; Aloqbi et al., 2016). Oxidative stress can lead to the loss of specific protein function in the tissues. Significant increase in superoxide radicals known to stimulate lipid peroxidation (Al-Olayan, 2014). Thus, oxidative stress can cause alteration in the spermatogenic cycle and degeneration in the seminiferous tubules (Chandra et al., 2012). In the current study, it is possible that PUN reduced the oxidative damage in the reproductive tract of the rabbits by preventing the excessive generation of free radicals and protected the testis or sperm cells against oxidative damages.

Although the literature lacks studies on the direct effect of PUN on spermatologic parameters, there are studies on the spermatological effects of pomegranate juice and pomegranate juice concentrate in rats (Turk et al., 2008; Leiva et al., 2011; Fedder et al., 2014). Oral pomegranate juice given up to 1 ml per day has been reported to improve spermatologic parameters in rats (Turk et al., 2008). Administration of the pomegranate ethanol extract at 500 mg/kg also resulted in an increase in sperm counts in rats (Leiva et al., 2011). In another study, it was implicated that pomegranate extracts increased sperm quality in men (Fedder et al., 2014). Similar to the previous reports, sperm concentration and motility of PUN treated rabbits were higher than control rabbits in our study.

**CONCLUSION**

Including the highest oral dose of 10 mg/kg/day, PUN had no negative effect on the parameters we looked at in male New Zealand rabbits. On the other hand, the effects of PUN, especially at doses of 2 and 10 mg/kg/day, on sperm concentration and motility were encouraging. Thus, it can be said that long-term intake of PUN can positively alter sperm quality and should be further evaluated for the possible effects on men with low sperm counts.

**REFERENCES**


**Table 2:** Effects of punicalagin treatments on testis weights, epididymides weights and some spermatologic parameters (mean ± SD) of male New Zealand White rabbits.

<table>
<thead>
<tr>
<th></th>
<th>CONT</th>
<th>PUN1</th>
<th>PUN2</th>
<th>PUN3</th>
<th>P&lt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Right Testis Weight (g)</td>
<td>2.95 ± 0.45</td>
<td>2.87 ± 0.40</td>
<td>2.84 ± 0.47</td>
<td>2.76 ± 0.42</td>
<td>NS</td>
</tr>
<tr>
<td>Right Epididymides (g)</td>
<td>1.06 ± 0.27</td>
<td>1.00 ± 0.11</td>
<td>1.13 ± 0.22</td>
<td>1.14 ± 0.08</td>
<td>NS</td>
</tr>
<tr>
<td>Ejaculate volume (ml)</td>
<td>0.85 ± 0.19</td>
<td>0.86 ± 0.18</td>
<td>0.86 ± 0.13</td>
<td>0.87 ± 0.10</td>
<td>NS</td>
</tr>
<tr>
<td>Ejaculate Weight (mg)</td>
<td>0.89 ± 0.17</td>
<td>0.91 ± 0.18</td>
<td>0.94 ± 0.15</td>
<td>0.94 ± 0.11</td>
<td>NS</td>
</tr>
<tr>
<td>Progressive Motility (%)</td>
<td>73.3± 6.05</td>
<td>79.1± 3.76</td>
<td>80.0± 4.47</td>
<td>81.6± 4.08</td>
<td>0.04</td>
</tr>
<tr>
<td>Ejaculate Ph</td>
<td>7.11 ± 0.07</td>
<td>7.08 ± 0.14</td>
<td>7.08 ± 0.07</td>
<td>7.10 ± 0.10</td>
<td>NS</td>
</tr>
<tr>
<td>Sperm concentration (x10⁹/ml)</td>
<td>296.5± 38.2</td>
<td>322.3± 40.1</td>
<td>363.6±31.1</td>
<td>371.3± 31.4</td>
<td>0.01</td>
</tr>
<tr>
<td>Seminal Plasma Proteins (g/dl)</td>
<td>3.38 ± 0.37</td>
<td>3.45 ± 0.19</td>
<td>3.43 ± 0.34</td>
<td>3.45 ± 0.37</td>
<td>NS</td>
</tr>
</tbody>
</table>

CONT=control; PUN1=1 mg/kg/day punicalagin; PUN2=2 mg/kg/day punicalagin; PUN3=10 mg/kg/day punicalagin. NS=not significant.


