EFFECT OF USING N-CARbamoyl GLUTAMATE (NCG) AS FEED ADDITIVE ON THE SEMEN QUALITY OF BULL

Adil Jabbar Atiyah¹, Hayder Al-Shanoon¹*, Kreem Iwaid Al-Badry², Faris Faisel Ibrahim³ and Saadam Hussein Mahmoud³

¹Department of Veterinary Public Health, College of Veterinary Medicine, University of Baghdad, Baghdad, Iraq.
²Department of Surgery and Obstetrics, College of Veterinary Medicine, University of Baghdad, Baghdad, Iraq.
³Ministry of Agriculture, Department of Artificial Insemination Center, Baghdad, Iraq.

Abstract

N-carbamoyl glutamate (NCG) has been found to increase the arginine (ARG) level in the blood that is involved in many important biological activities. NCG exhibits lower ruminal degradation compared with ARG. This study aimed to evaluate the potential effects of NCG supplements as a feed additive on the semen quality of bull. The study was divided into two portions. In the first study, the semen was collected over six months (n = 6 Holstein bulls) with the aid of an artificial vagina. The period of experiment was divided into the pre-treatment period (control), treatment period (bull fed NCG 20g/day), and post-treatment period (Stop NCG treatment). In the first study, semen parameters were compared among different periods. The second study examined the semen parameters of the post-treatment group with those of bulls (n = 6) that were not treated with NCG previously (untreated group). From raw semen, the mass activity was determined. For other parameters, the semen was diluted with tris-based extender and evaluated for individual motility (after dilution, after cooling, and post-thawing). The results of the first study indicated that the semen parameters improved significantly (P < 0.05) in the post-treatment group compared with the control (pre-treatment) group. The second study revealed a significant (P < 0.05) improvement in mass activity and individual motility in the post-treatment group compared with the untreated group. These results suggest that NCG supplementation in routine feeding of bulls can improve the quality of semen.

Key words: N-carbamoyl glutamate, semen parameters, feed additive.

Introduction

The essential and non-essential amino acids have a crucial role in an animal’s systemic level. Therefore, adding amino acids in animal feed is necessary for both maintenance and production (Guoyao Wu et al., 2014). Thus, amino acids becomes an essential additive in farm animal feeding due to its positive effect on the animals’ overall performance (Kellens & Church, 2002; Singh, 2015), especially in terms of reproductive efficiency (Mostafa et al., 2014).

Arginine (ARG) is an amino acid that acts as a precursor for the synthesis of nitric oxide (NO) and polyamines. These components regulate the metabolic pathways that involve in the health, reproduction, growth and homeostasis of the animals (Palencia et al., 2018; G. Wu, 2013). ARG is usually considered to be essential amino acid because ARG synthesis is not adequate to meet the animal requirement, specifically during the early stages of growth or for a high level of production (Guoyao Wu et al., 2014). Studies also revealed that ARG used as a feed additive plays a critical role in improving male reproductive efficiency (Ahangar et al., 2017; Chen et al., 2018; Mendez & Hernandez, 1993).

In terms of semen quality, it has been directly proven that administration of ARG helps in the development of sperm, resulting in more ejaculate, higher sperm count, and better motility in men, boars, rabbits, fish, and roosters (Abbaspour et al., 2019; Chen et al., 2018; Kastelic, 2013; Radany & Atherton, 1981; Stanislavov & Rohdewald, 2014). Also, using ARG in vitro can improve semen motility and sperm count in different species (AL-
Ebady et al., 2012; Keller & Polakoski, 1975; Maidin et al., 2014). In bovine, most of the studies have focused on using ARG in vitro, not in vivo, on improving the quality of semen. However, unprotected ARG is not administered orally often to ruminants because of ARG is rapidly degraded in the rumen (Bahram Chacher et al., 2012). Therefore, ARG reaches the small intestine with an insufficient amount where it is absorbed (Taufiq, 2016). Many studies were used rumen-protected ARG widely to increase the level of ARG in the blood of ruminants, such as cows, lambs, and ewes (Hassan et al., 2011; Meyer et al., 2018; H. Zhang et al., 2016). However, using protected ARG seems to be uneconomical due to its high cost (B. Chacher et al., 2013). N-carbamoyl glutamate (NCG), which is a structural analog of N-acetylglutamate, a cofactor of carbamoyl phosphate synthetase, has been used since 2014 to increase the level of ARG in the blood (B. Chacher et al., 2014). NCG exhibited lower degradation in the rumen and had a lower cost than ARG (Bahram Chacher et al., 2012). Moreover, NCG has been used to improve the reproductive efficiency and productivity of ruminant and non-ruminant animals (B. Chacher et al., 2013; Chen et al., 2018; B. Zhang et al., 2014; H. Zhang et al., 2016). However, no study has been conducted that shows the effect of NCG on the semen quality of bulls. This study aims to evaluate how dietary supplementation of NCG would affect semen quality.

Materials and Methods

1. Animal Management

This study was conducted at the artificial insemination center of Abou-Ghareeb, Agriculture Ministry (Baghdad-Iraq) from November 2018 to April 2019 on 12 Holstein bulls. Semen was routinely collected from all bulls weekly with the aid of an artificial vagina. In total, two hundred and ninety-six ejaculates were evaluated during the study period. The average age of the bulls was 4–5 years. All bulls were managed under the same conditions in terms of feeding and watering throughout the study period.

2. Experiment Design

The work was divided into two studies. In the first study, the semen was collected from six bulls during the three periods: the pre-treatment period (from 2018/11/14 to 2019/1/10), which is considered as control; the treatment period (from 2019/1/14 to 2019/3/10), in which each bull was fed 20 g/day of NCG. The dose was determined based on browse study (B. Chacher et al., 2014). The third period is the post-treatment (from 2019/3/11 to 2019/4/8; no NCG treatment). In the second study, the semen parameters of the post-treatment period were compared with those of the other six bulls that were not treated previously.

3. Semen Processing

Immediately after collection, all semen samples were placed in a water bath at 35°C to prevent temperature shock. The volume of the semen was measured using graduated test tubes. The semen concentration was evaluated using a spectrophotometer (Al-Badry et al., 2016). Ejaculates with individual motility of spermatozoa of 50% or more were diluted in Tris–fructose–egg yolk–glycerol extender. Then, the diluted semen was placed in a water bath (37°C). For the freezing step, diluted semen was cooled by transferring it to the cold cabinet (5°C) for four hours. After the equilibration time, the diluted semen was packaged in straws (0.25 mL). Individual motility was assessed before freezing (cold semen). After freezing for 48 h in liquid nitrogen, the straws were thawed in a water bath 37°C for 30 seconds. The semen concentration was calculated using the spectrophotometer (Al-Badry et al., 2016). The semen mass activity and individual sperm activity were evaluated by subjective measurement as follows.

3.1. Mass activity score analysis

The mass activity of spermatozoa was determined directly after semen collection. A drop of raw semen was poured on a worm slide (37°C) to observed under a microscope (magnification of 100×). The score of the mass activity was evaluated based on the following criteria: 0 = No wave, total immobility; 10 = No wave, individual movement; 20–40 = No wave, very slow movement; 45–65 = Wavy appearance, the slow amplitude of wave; 70–85 = Wavy appearance, rapid wave motion, no eddies; and 90–100 = Wavy appearance, rapid wave motion with eddies (Chemineau et al., 1991).

3.2. Individual motility (percentage of motile spermatozoa) score analysis

Sperm motility analysis was conducted after diluting the raw semen with a 2.9% sodium citrate solution, with a ratio of 1:2 (semen/dilution). The mixture was examined under a phase-contrast light microscope (magnification of 400×) for each sample, and three fields were observed for every slide. Sperm motility was considered based on the percentage of sperm with the stander forward progressive movement. Any sperm with circling or oscillating movements were deemed to be immotile. The motility score was calculated based on the stander method described by Bearder et al. (Bearden & Fuquay, 2000).

4. Statistical Analysis

Data analysis was conducted using the SAS software
Effect of Using N-carbamoyl Glutamate (NCG) as Feed Additive on the Semen Quality of Bull

One-way ANOVA was employed to observe the effect of NCG on the semen parameters in bull among the different periods (pre-treatment, treatment, and post-treatment). Pairwise Comparison of mean was performed, employing Tukey’s multiple comparison test. The t-test was applied to analyze the data of semen from the post-treatment group vs. untreated group and pre-treatment group vs. treated group. Data are expressed as means ± SE; differences were regarded as significant at P < 0.05.

Results and Discussion

The sperm parameters of the first study are presented in table 1. Supplementation of NCG to bull significantly improves the sperm parameters except for volume in the post-treatment period compared with the pre-treatment period. The results of the pre-treatment periods varied sperm parameters did not significantly differ from those of the treatment period.

This study found that dietary NCG improves bull semen parameters. NCG’s effect on reproductive efficiency has been studied mainly in females (Palencia et al., 2018). To the best of our knowledge, there have been no previous studies documenting the impact of NCG on male reproductive efficiency, especially the semen parameters in any species. The positive effect of NCG on the semen parameters may be due to an increase in plasma ARG levels. Many studies found that the supplementation of NCG increases the endogenous synthesis of plasma ARG in piglets (Frank et al., 2007), cows (B. Chacher et al., 2014), and ewes (H. Zhang et al., 2016). In many studies, ARG has been found to have a positive effect on sperm quality and activity. In vitro, ARG can stimulate sperm capacitation and acrosome reaction in bovine (O’Flaherty et al., 2004) and sperm motility of human (Keller & Polakoski, 1975; Stanislavov & Rohdewald, 2014), bull (AL-Ebady et al., 2012), and ram (Özer Kaya et al., 2018). In vivo, supplementation of ARG, either orally or by injection, exhibited the same positive effect on sperm parameters. Intramuscular administration of L-arginine for seven weeks every 48 hours increased mass activity, motility, and concentration of ram sperm compared with control. Also, dietary L-arginine improved the semen quality of ram (Kaya et al., 2019), boar (Chen et al., 2018), men (Morgante et al., 2010), fish (Kocabay et al., 2019; Pourkhazaei et al., 2017), and roster (Ahangar et al., 2017).

The results of this study agree with those of the reviews indicated above. The individual motility and the concentration of the sperm significantly increased after the administration of NCG to bulls compared with the pre-treatment group (Fig. 1). The ability of NCG to positively affect semen quality may arise from the nitric oxide (NO) pathway (Buzadzic et al., 2015; Dixit & Parvizi, 2001; Miraglia et al., 2011; Srivastava et al., 2006); as ARG is the substrate for vascular NO formation (Palmer et al., 1988; Rajapakse & Mattson, 2009). Endogenous NO is beneficial to sperm motility (Lewis

<table>
<thead>
<tr>
<th>Sperm parameters</th>
<th>Pre-treatment</th>
<th>Treatment</th>
<th>Post-treatment</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Volume (ml)</td>
<td>5.63 ± 0.23</td>
<td>5.01 ± 0.22</td>
<td>5.46 ± 0.30</td>
<td>0.09</td>
</tr>
<tr>
<td>Sperm concentration (million/ml)</td>
<td>1192 ± 89.55 A</td>
<td>1342 ± 63.22 AB</td>
<td>1453 ± 50.57 B</td>
<td>0.03</td>
</tr>
<tr>
<td>Mass activity (%)</td>
<td>32.53 ± 1.08 A</td>
<td>34.75 ± 1.08 AB</td>
<td>37.63 ± 1.56 B</td>
<td>0.02</td>
</tr>
<tr>
<td>Individual motility (%)</td>
<td>44.51 ± 1.39 A</td>
<td>48.10 ± 1.40 AB</td>
<td>52.50 ± 2.14 B</td>
<td>e^0.01</td>
</tr>
<tr>
<td>Individual motility after cooling (%)</td>
<td>53.50 ± 0.82 A</td>
<td>54.72 ± 0.56 A</td>
<td>56.11 ± 0.82 B</td>
<td>0.001</td>
</tr>
<tr>
<td>Individual motility after freezing (%)</td>
<td>50.53 ± 0.36 A</td>
<td>50.69 ± 0.35 A</td>
<td>53.20 ± 0.86 B</td>
<td>0.03</td>
</tr>
</tbody>
</table>

NCG: N-Carbamoyl glutamate, ^ A, B significantly different among the periods P < 0.05.

Table 2: Comparison of the semen parameters between the untreated group and the post-treatment group.

<table>
<thead>
<tr>
<th>Sperm parameters</th>
<th>Untreated group</th>
<th>Post-treatment group</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Volume (ml)</td>
<td>5.84 ± 0.23</td>
<td>5.46 ± 0.30</td>
<td>0.45</td>
</tr>
<tr>
<td>Sperm concentration (million/ml)</td>
<td>1260 ± 67.60 A</td>
<td>1453 ± 50.57 B</td>
<td>0.01</td>
</tr>
<tr>
<td>Mass activity (%)</td>
<td>55.63 ± 1.83 A</td>
<td>37.63 ± 1.56 B</td>
<td>&gt;0.01</td>
</tr>
<tr>
<td>Individual motility (%)</td>
<td>38.13 ± 1.14 A</td>
<td>52.50 ± 2.14 B</td>
<td>&gt;0.01</td>
</tr>
<tr>
<td>Individual motility after cooling (%)</td>
<td>49.11 ± 2.37 A</td>
<td>56.11 ± 0.82 B</td>
<td>&gt;0.01</td>
</tr>
<tr>
<td>Individual motility after freezing (%)</td>
<td>42.22 ± 2.94 A</td>
<td>53.20 ± 0.86 B</td>
<td>0.005</td>
</tr>
</tbody>
</table>

^ A, B significantly different among the periods P < 0.05.
A study on frozen stallion sperm found a positive correlation between the NO level and sperm motility after thawing (Donnelly et al., 1997; Ortega Ferrusola et al., 2009). NO affects sperm motility via the activation of soluble guanylate cyclase, the subsequent synthesis of Cyclic guanosine monophosphate (cGMP), and the activation of cGMP-dependent protein kinases (Miraglia et al., 2011). Also, NO increases the motility of spermatozoa by increasing energy production originating in the mitochondrial compartment (Otasevic et al., 2013). However, many studies indicated that the effect of NO on sperm motility could be inconsistent, depending on the NO levels (Balercia et al., 2004; Donnelly et al., 1997; Weinberg et al., 1995).

The exciting results of the post-treatment group exhibited a significant increase in all semen parameters,

*Significantly different between groups P < 0.05. NS: Not significant.

**Fig. 1:** Comparison of the semen parameters between the pre-treatment group and treatment group.
except velum, compared with the pre-treatment group, despite the NCG treatment being halted in the post-treatment period. It is expected that long-term dietary supplementation of NCG (around three months) during the treatment period increases the ARG level leads to improve general health status, which may reflect positively on the results of the semen parameters. Other studies found ARG to be involved in many physiological functions and biological activities, such as antioxidant processes (Ma et al., 2010; Wang et al., 2019), maintenance of inter-organ metabolism and ammonia detoxification (Gebhardt et al., 2003; G. Wu et al., 2009), growth promotion (Jobgen et al., 2006; G. Wu et al., 2009; Yao et al., 2011), and hormone secretion enhancement (Chew et al., 1984; Oh et al., 2017). ARG administration also increased the number of Sertoli cells in rabbit testes (Pitaloka, 2016). Sertoli cells have a significant role in regulating spermatogenesis and spermatozoa production (Griswold, 1998). The critical role of Sertoli cells results from providing structural support for sperm cells, regulation of nutrition development, and maintenance of a stable micro-environment for spermatogenesis (Griswold, 2018; Lee & Cheng, 2004). Sertoli cells are the main cellular targets for testosterone signaling, which is required to support male germ cell development and survival (Griswold, 1998). Recently, a study by Abbaspour et al., (2019) reported that dietary supplementation of ARG to old breed rosters increased testosterone concentration, testicular weight, tubular differentiation, spermatogenesis, and repopulation incident.

Based on the above studies, an increase in the number of Sertoli cells and improvement in the health status of the animal may support sperm production and quality after treatment (post-treatment). However, there is still concern that the improvement in sperm quality results from a seasonal effect. In Iraq, studies found that semen quality can change from month to month (Al-Ani & Abdulkareem, 2012; Al-Badry et al., 2016; Mehdi et al., 2012). In this study, individual motility improved significantly during March and April (the post-treatment period) compared with November–January (the pre-treatment period). Therefore, the second study was conducted to answer the following question: Is the improvement in semen quality caused by a seasonal change or by treatment with ARG? As such, the study investigated the quality of bull semen without NCG treatment compared with a post-treatment group (same period of the collection). The results of the second study table 2 indicate that treatment with NCG is the reason for the improvement in semen quality after the post-treatment group compared with a group of bulls that were not treated before. The mass activity, sperm concentration, individual motility, and individual motility after cooling and freezing increased significantly (P < 0.01) in the post-treatment group compared with the untreated group. The second study results reveal that the positive effect of NCG on semen quality can continue for a long time after treatment.

In summary, dietary NCG for bulls improves sperm motility and concentration when used as feed additive over long-term feeding.

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