



Gamma Glutamyl Transpeptidase Activity Determination in Epididymis of *Corynorhinus mexicanus* Bat Throughout Its Annual Cycle

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To cite this article:

Arenas-Ríos Edith, Rosado Adolfo, Rodríguez-Tobón Ernesto, Rodríguez-Tobón Ahiezer, León-Galván Miguel Ángel. Gamma Glutamyl Transpeptidase Activity Determination in Epididymis of *Corynorhinus mexicanus* Bat Throughout Its Annual Cycle. *Animal and Veterinary Sciences*. Special Issue: Advances in Bat's Reproduction. Vol. 3, No. 1-1, 2015, pp. 8-12. doi: 10.11648/j.avs.s.2015030101.12

Abstract: GGT initiates the degradation of both oxidized and reduced glutathione at the cell surface by cleaving the unique gamma glutamyl bond. The successive hydrolysis of oxidized or reduced CysGly by aminopeptidase or dipeptidase releases Gly, and cysteine/cysteine is recovered for intracellular synthesis of glutathione. In addition, GGT plays a major physiological role in providing cysteine to cells for GSH synthesis and protein synthesis, thereby playing a major role in antioxidant defense and normal growth. GGT is present in the epididymis, principally in caput. The catalytic activity of GGT is highest in the proximal epididymal regions and decreases toward the distal regions; participating in the epididymal sperm maturation process. In most mammals, epididymal sperm maturation takes place in a period not exceeding ten days, ending in the distal part of the corpus of the epididymis, before reaching the caudal region, which is responsible only for sperm storage; however, in *C. mexicanus* the epididymal sperm maturation ends in caudal epididymal region. For this reason, the main goal of the present study was to determine the relationship between the GGT activity through the caput and caudal epididymal regions, throughout annual cycle of *Corynorhinus mexicanus* bat. We found that the GGT activity appears to be important for epididymal sperm maturation in *C. mexicanus* bat in cephalic region, and important for protection against ROS epididymal caudal region.

Keywords: Bats, Gamma Glutamyl Transpeptidase, Epididymis, Sperm Maturation, Glutathione

1. Introduction

The gamma glutamyl transpeptidase (GGT; EC 2.3.2.2) is an enzyme found in mammals [1, 2]; initiates the degradation of both oxidized and reduced glutathione at the cell surface by cleaving the unique gamma glutamyl bond. The successive hydrolysis of oxidized or reduced CysGly by aminopeptidase or dipeptidase releases Gly, and cysteine/cysteine is recovered for intracellular synthesis of glutathione (GSH) [3].

Has been confirmed that GGT plays a major physiological role in providing cysteine to cells for GSH synthesis and protein synthesis, thereby playing a major role in antioxidant

defense and normal growth [4-12].

GGT is present in the epididymis, principally in caput [13]. The catalytic activity of GGT is highest in the proximal epididymal regions and decreases toward the distal regions [14, 15].

Once the spermatozoa are formed during spermatogenesis and pass to the epididymis, they will carry on morphological and biochemical changes known as epididymal sperm maturation [16], *i.e.* the potential to acquire rectilinear and vigorous movement, to interact with zona pellucida and to fertilize the oocyte [17].

During sperm maturation, in addition to a major role as an antioxidant and in eliminating toxic compounds, GSH has

been implicated in prooxidation processes in various cells, via GGT dependent catabolism [18, 19]. Modulating effects of GSH catabolism have been observed on components of signal transduction pathways, such as those involving cell surface receptors and factors within the sperm cell itself and/or in the epididymis may control sperm protein thiol (PSH) oxidation [18-20].

In most mammals, epididymal sperm maturation takes place in a period not exceeding ten days, ending in the distal part of the corpus of the epididymis, before reaching the caudal region, which is responsible only for sperm storage [21].

However, the Vespertilionidae bat *Corynorhinus mexicanus* (Allen, 1916) provides an interesting experimental model for the epididymal study, because it presents prolonged storage of sperm in the epididymal cauda for several months after the spermatogenesis and regression of testes.

September is the beginning of sperm epididymal storage, therefore since October, there are no new contributions of sperm cells from the testis, and cells remain viable until the copulating season which occurs during November [22, 23]. Earlier research conducted by our group has suggested that in this species the sperm maturation has to be finished in the caudal region of the epididymis and should be dependent on storage time [24].

For this reason the main goal of the present study was to determine the relationship between the GGT activity through the caput and caudal epididymal regions, throughout annual cycle of *Corynorhinus mexicanus* bat.

2. Materials and Methods

2.1. Reagents and Solutions

All chemicals and reagents used were of the highest analytical grade and most of them were purchased from Sigma (St. Louis MO).

2.2. Ethics Statement

Corynorhinus mexicanus is not listed as an endangered species in the Norma Oficial Mexicana NOM-059-ECOL-2010 SEMARNAT-2010 for the protection of native wild species of México [25]. All protocols for the capture and handling of the live animals, as well as the euthanasia procedure used in this study were conducted in strict accordance with the guidelines established by the American Society of Mammalogists for the use of wild mammals in research [26], and were approved by the Ethics Committee of our institution. The capture of specimens studied were approved by the scientific collector's license FAUT-0159 issued to Dr. Miguel Angel León Galván, by the Dirección General de Vida Silvestre de México (an agency of SEMARNAT).

2.3. Capture of Animals

Three adult males of *C. mexicanus* were captured

fortnightly from September to October 2011 using an expand hand-net (Bioquip Tropic net) in a tunnel where they take refuge. This tunnel is located in central México (19°37'14"N, 98°02'02"W; 3320 m altitude). Capture regime includes the period when spermatozoa reach the caput region of the epididymis and the period of sperm storage before the mating season starts [22, 24].

Bats were always captured before or as they left their roost. To ensure adult status of bats, only those animals with complete ossification of the cartilaginous epiphyseal growth plates of the fourth metacarpal-phalangeal joint were selected [22]. In order to ensure the age category and the reproductive condition of the individuals included in this study, according to the report in León-Galván *et al.* [22]; Arenas-Ríos *et al.*, [27]; and Cervantes *et al.* [24], the following variables were determined: body weight registered using an Ohaus® (Ohaus Corporation NJ) portable electronic balance (± 0.01 gr); forearm length measured with a Vernier caliper (± 0.1 mm); and the external aspects of the sexual organs.

2.4. Epididymides Obtention

The animals for each determination were captured on the same day. Immediately after decapitation, the epididymides of the bat were externalized through an incision of the interfemoral skin and tunica vaginalis that covers it, cleared of fat and connective tissue, and dissected. Caput and cauda segments of the epididymides were isolated according to the procedure described by Arenas-Ríos *et al.* [27] and Cervantes *et al.* [24].

Both epididymal segments of the same region (right and left caput and cauda) were pooled and frozen in liquid nitrogen (-170 °C) until utilized. Caput and cauda segments of the epididymides were thawed to 4 °C in ten volumes of cold HEPES buffer (0.1 M, pH 7.4) and homogenized in a Potter-Elvehjem type, glass-glass homogenizer. The obtained homogenates were centrifuged for 10 min at 5000 $\times g$, the precipitates were discarded and all enzymatic activities were measured in the supernatants [27].

2.5. Gamma Glutamyl Transpeptidase Determinations

GGT (EC 2.3.2.21) activity was determined by the method of Yash and Tapani [28], using p-nitroanilide as substrate. Activity is expressed as nmoles of p-nitroaniline produced per min.

The activity of all enzymes tested was calculated either as specific activity, i.e. units of enzyme activity per mg of tissue protein, and as total activity, i.e. units of enzyme activity per total wet weight of the organ.

Some preliminary enzyme determinations were done to ensure appropriate maintenance of enzyme activity after freezing and thawing and to test kinetic conditions. In addition, to ensure zero order kinetics, all enzyme assays were run in duplicate utilizing one and twice the amount of homogenate. All those unusual assays in which duplicates were not satisfactory were repeated using smaller amounts of

homogenate.

Total protein content for the determination of the specific activities of the enzymes was measured using a commercially available bi-cinchoninic acid protein assay kit (Pierce, Rockford, IL, USA).

2.6. Statistical Analysis

Comparisons among groups were made with one-way ANOVA [29] followed by the Bonferroni post hoc test. Homogeneity of variances was tested by Bartlett and Levene tests [29]. Differences were considered statistically significant when $p < 0.05$.

Realization of this work, including the use and handling of animals, was reviewed and approved by the Consejo Divisional de Ciencias Biológicas y de la Salud (Biology and Health).

Sciences Divisional Board) of the Universidad Autónoma

Metropolitana- Iztapalapa. Animals were cared for in accordance to the “Guidelines for the capture, handling, and care of mammals” as approved by the American Society of Mammalogist (Animal Care and Use Committee, 1998).

3. Results

In total 41 adult male bats were used. Mean body mass was 7.5303 (range 7.07-8.0 g) and the mean forearm length was 41.8623 (range 41.37-42.65 mm). Testes were permanently inguinal, and the epididymides, were always bound to the testicles. The epididymis recrudescence occurs in August, with their highest weight in September-October (Fig. 1). Afterwards epididymis weights decreases slowly, reaching their lowest values until March. The separate regions

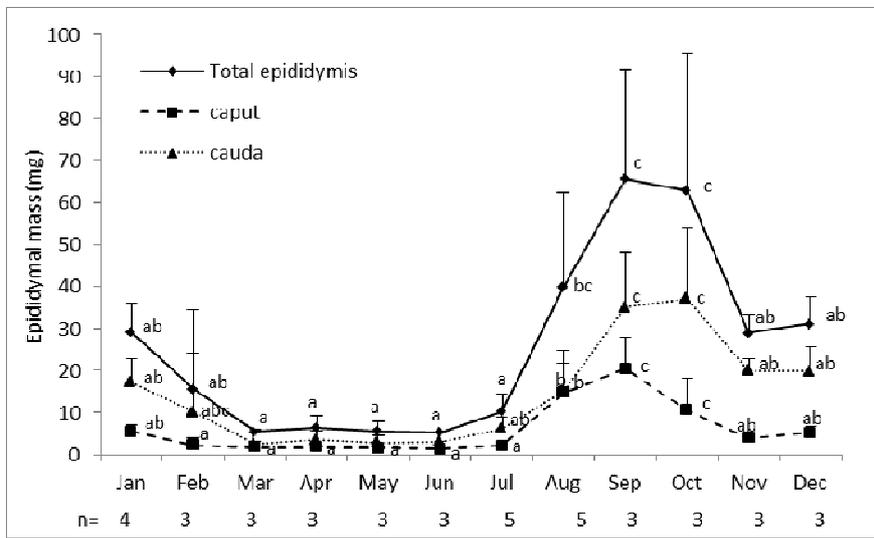


Figure 1. Changes in epididymal mass of *Corynorhinus mexicanus* bat during annual cycle. The epididymal segments were obtained after ligation of epididymides at the deferent-cauda, cauda-corporis, corporis-caput. For simplicity, we report only the mean \pm DS mass of one reproductive organ or epididymal region per bat, using all bats in the sample, and the values in the base indicate the number of bats. Different letters indicate statistically significant differences ($P < 0.05$) between values in the same trace (ANOVA plus Bonferroni post hoc test)

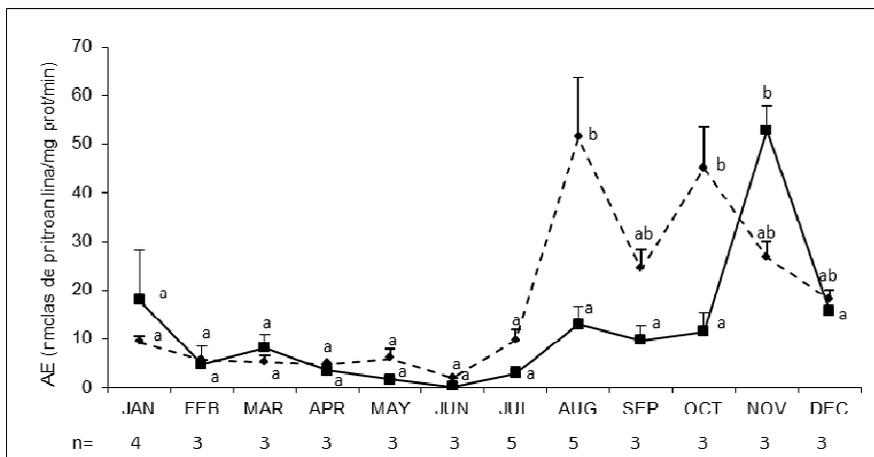


Figure 2. Activity of GGT in the epididymides of *C. mexicanus* bats captured monthly during the annual reproductive cycle; (A) Activity of the enzyme from caput; (B) Activity of the enzyme from cauda. Activity of the enzyme expressed as specific activity, i.e. units of enzyme activity per mg of tissue protein. Data indicate Mean \pm SEM. Numbers indicate the number of bats. Different letters indicate the existence of statistical significant differences between values in the same trace (ANOVA plus Bonferroni post hoc test).

4. Discussion

The Mexican big-eared *Corynorhinus mexicanus* bat, is an endemic species of Mexico, living in temperate zones. It takes a lethargy type "daily torpor" during the cold season, so it has no need to migrate away from their breeding site. Females are seasonal monoestric, observing their mating period principally during November [30, 31]. The Males also have one annual reproductive cycle, with a temporal asynchrony between testis and epididymis; therefore, prolonged sperm storage in epididymis [22, 24, 27].

The testicular development in *C. mexicanus* bat, begins in May reaching its maximum weight in August, and returning to its lowest values in October [32]. The histological data indicate that from November to April testicles have no spermatogenic activity. The seminiferous tubules regressed and Sertoli cells found significantly decreased in number and some show vacuolar degeneration [30].

On the other hand, the maximum epididymis development is in September; a month after the testicle reached its highest values; however, downsizing in the epididymis is observed gradually from December to February, it shows a great development in the caudal region of the epididymis, being evident since September and continuing until January, showing great development in the caudal epididymal region, being evident since September and continuing until January. The record of the presence of epididymal spermatozoa in this specie, was observed from late August until January [31, 33]. In the cephalic region, a clear decrease in sperm concentration was reported in late October [33], which coincides with the weight decrease of this region, while on the same date, a clear tendency to increase sperm concentration in the caudal region was reported [33]. León-Galván et al [22] propose, that increased size of epididymis, the abundant pampiniforme venous plexus system [34] and the absence of hair in this area, could function as an important element and cooling device as part of protection mechanisms of sperm in the sperm long period of storage, along with the participation of antioxidant enzymes: superoxide dismutase, catalase and glutathione peroxidase may be modulating the production of reactive oxygen species (ROS) [27].

However, it has been reported that, unlike other mammalian species in *C. mexicanus*, the epididymal sperm maturation ends in caudal region [24, 35], showing that it is the month of October, the most important, as it was in that month, in which, an important tyrosine phosphorylation of protein in sperm increase, was detected [35], and it is from this date, that sperm were found with the ability of capacitation and to experience acrosome reaction [24, 35].

As mentioned before, GGT is present in the epididymis, mainly in the head region [13], being the high catalytic activity of GGT in proximal epididymal region, and decreases in the distal region [14, 15]. In the present study, it was found that GGT activity is high in the cephalic region, on dates in which sperm are present in the epididymis,

something equivalent to what was reported in rat, where they found that GGT concentration was low in immature rats and increased in sexual maturity of rats. This associated with decreased GSH and L-glutamic acid accumulation levels, which is thought, they could play an important role at the time of the sperm arrival, participating in the epididymal sperm maturation process [28].

GSH Modulators catabolism effects, have been observed in components of signal transduction pathways, such as control of protein oxidation of thiol groups, in epididymal sperm [18-20].

However, if the sperm maturation in *C. mexicanus* bat ends in the caudal region, and this must be accomplished before mating season (November), the results found in this study about the GGT activity, suggest that; the effects associated with GGT activity and related with epididymal sperm maturation, in *C. mexicanus* bat, are only important in the cephalic region [3-12, 18, 19]. In the caudal region, GGT could be involved, only in antioxidant protection of tissue and sperm cells against the attack of ROS [4-12, 27].

It has been reported that, the catalytic activity of GGT is androgen dependent [36]. However, we found an important activity of GGT, even on dates when the testicle reportedly no longer presents activity [22, 23], so this last point deserves to be studied deeply.

Acknowledgments

We would like to acknowledge the entire research group: Morphophysiology and Biochemistry of Sperm (Universidad Autónoma Metropolitana, Iztapalapa) and MarioGabriel Hidalgo. This work was partially financed by CONACYT (Mexico) grants 0105961/M10-A02; UAM-I-CA-136, SEP-PROMEP and scholarship number 233313 to AR.

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