The Mongolian Gerbil (*Meriones unguiculatus*) as a Model for Inflammation-promoted Prostate Carcinogenesis[†]

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Running Title: Inflammation-promoted Prostate Carcinogenesis

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Abbreviation list:
MNU: N-methyl-N-nitrosourea
PIN: Prostatic intraepithelial neoplasia
VL: Ventral lobe
DL: Dorsolateral lobe

ABSTRACT

One of the recognized issues in prostate cancer research is the lack of animal models allowing the research of pathological, biochemical, and genetic factors in immunocompetent animals. Our research group has successfully employed the gerbil in several studies for prostate diseases. In the present work, we aimed to analyze the effect of chronic bacterial inflammation on MNU-induced prostate carcinogenesis in gerbils Histopathological assessment of the prostatic complex revealed that treatment combinations with MNU plus testosterone or bacterial infection resulted in a promotion of prostate cancer, with bacterial inflammation being more effective in increasing premalignant and malignant tissular alterations than testosterone in the prostate. Furthermore, chronic bacterial inflammation itself induced premalignant lesions in the ventral lobe and increased their frequency in the dorsolateral lobe as well as malignant lesions in the ventral prostate. These animals showed a rich inflammatory microenvironment, characterized as intraluminal and periductal foci.

These data indicate that chronic inflammation induced by *E. coli* acts as a potent tumor promoter, in the early stages of carcinogenesis in the gerbil, in line with the hypothesis of inflammation supporting several steps of tumor development in the prostate gland.

Keywords: Prostate, Cancer, Inflammation, Gerbil, Androgens

1- INTRODUCTION

During the last decade, there has developed an expanding multidisciplinary body of literature suggesting a strong link between chronic inflammation and prostate cancer (De Marzo et al. , 2007, Sfanos et al. , 2014, Vignozzi and Maggi, 2014). Although population studies have found an increased relative risk of prostate cancer in men with a prior history of certain sexually transmitted infections or prostatitis, only few basic research studies have addressed this hypothesis so far (Elkahwaji et al. , 2007, Quintar et al. , 2010).

One of the well-known deficiencies in prostate cancer research is the lack of working models allowing the investigation of pathological, biochemical and genetic factors of this disease. Spontaneous occurring prostate tumors are rare in most of species, with the possible exception of adenocarcinoma in dogs. Our research group has successfully employed the gerbil (*Meriones unguiculatus*) in several studies involving hormonal treatments (Scarano et al. , 2008), the development of spontaneous neoplasms associated with aging (Campos et al. , 2010) and chemically induced tumors (Goncalves et al. , 2013). One of the main advantages of this model is the short latency period for proliferative lesions in fully immunocompetent animals, with no genetic manipulation.

Multiple different bacterial species are known to infect the human prostate and induce inflammation, with *E. coli* being the most frequently found (Gill and Shoskes, 2016). By using this agent as a pro-inflammatory stimulus, we have tested in the present work the potential tumor promoting action of inflammation in N-methyl-N-nitrosourea (MNU)-induced prostatic lesions in gerbils.

2- MATERIAL AND METHODS

2.1 Animals, Experimental Design, Bacterial Infection, and Carcinogenesis Induction

Three month-old adult male gerbils were housed in plastic cages under conventional conditions (25°C, 40–70% relative humidity, 12 light/12 dark) in pathogen-free conditions, with water and balanced chow supplied *ad libitum*. Animal care and experiments were conducted at São Paulo State University (UNESP) following the NHI Guidelines for the Care of and Use of Laboratory Animals, 1996 and approved by the Ethics Committee of Experimental Animals of Sao Paulo State University (Protocol number: 003/2009).

Gerbils were randomly divided into five groups (Figure 1): Control (comprised of sham injected animals), E (chronic prostatitis by *E. coli*), MNU (a single intraperitoneal injection of MNU 50 mg/kg, CAS 684-93-5 Sigma, St. Louis, MO), E + MNU (MNU injected after 30 days of *E. coli* inoculation), and MNU + T (MNU + weekly doses of testosterone cypionate 2 mg/kg). The chronic model of bacterial prostatitis was performed according to a published protocol (Quintar et al., 2010). Briefly, 20 μl of a solution containing 10^8 CFU per ml of *Uropathogenic E. coli* were inoculated directly beneath the capsule of both ventral lobes using a 30-gauge needle while sham injected controls received PBS. All animals were killed by CO_2 inhalation 3 months after MNU injection. Each animal was subjected to a complete autopsy, the entire prostatic complex was removed, and the dorsolateral (DL) and ventral lobes (VL) were submitted to histopathological assessment.

2.2 Histopathological Classification and Statistics

VL and DL were fixed in 4% paraformaldehyde and embedded in paraffin. Hematoxylin/eosin-stained sections from the entire lobes were subjected to histopathological classification of prostate lesions according to criteria for mouse lesions (Shappell et al., 2004) and adapted for the gerbil prostate (Goncalves et al. ,2013). The number of premalignant lesions, characterized as reactive hyperplasia, prostatic intraepithelial neoplasia (PIN), malignant lesions, as well as the multiplicity of intraluminal and periductal inflammatory cells were determined in a double-blind manner by two researchers, one of them pathologist, as previously described (Goncalves et al. ,2013).

The results were checked for differences among groups and different lobes using the Mann–Whitney and Kruskal–Wallis tests. $P \leq 0.05$ was considered statistically significant. All statistical analyses were performed with Prism 6.0 software (GraphPad).

3- RESULTS AND DISCUSSION

3.1 Bacterial infection incites chronic inflammation in the gerbil prostate

In order to analyze the effect of an inflammatory microenvironment on the development of prostate cancer, the prostate gland was first inoculated with a bacterial stimulus. Compared to PBS, the intraprostatic inoculation of *E. coli* resulted in a chronic infiltration of mononuclear immune cells, as evaluated at 30 days post-infection (n=5). Inflammatory cells, consisting mainly of macrophages and lymphocytes, were scattered diffusely in the stromal compartment of the gland and, less frequently, forming periacinar aggregates. Other morphological signs included focal epithelial hyperplasia, characterized by thickness of the epithelial cell layer and tufting in areas of moderate inflammation, and collagen deposition. Although bacterial inoculation was performed in the ventral lobe, the infection-induced changes were seen in both VL and DL. This inflammatory scenario was the tissue microenvironment where MNU was applied on for the E+MNU group.

3.2 Chronic inflammation accelerates MNU-induced prostate carcinogenesis in gerbils

Based on a previous work (Goncalves et al. 2013), we chose 3 months after MNU inoculation as an adequate time point to analyze the possible promoting effect of bacterial inflammation on prostate carcinogenesis. Even in controls, gerbils exhibited spontaneous premalignant lesions in the DL (Figure 2). Chronic bacterial inflammation plus sham control injection (E group) induced premalignant lesions also in the VL and increased their frequency in the DL. Strikingly, the E group also exhibited some malignant lesions in the VL.As expected, these animals showed a rich inflammatory microenvironment, characterized mainly in the DL as periductal foci. As previously shown, the injection of the

tumor inductor MNU was associated to numerous premalignant lesions, with the DL developing the highest multiplicity, and the induction on malignant lesions in the same lobe (Figure 2).

Treatment combinations with MNU plus testosterone or bacterial infection resulted in a promotion of both premalignant and malignant tissular alterations in the prostate, with E+MNU being more effective in increasing premalignant lesions in both the VL and DL than MNU+T. Interestingly, compared to MNU+T, the number of malignant lesions in the VL was higher in the E+MNU group (Figure 2), suggesting that the initial site of bacterial inoculation could play a role in tumor promotion. Nevertheless, the numerous premalignant lesions in the DL could also progress to malignant lesions eventually in E+MNU, indicating a strong promoting effect for bacterial inflammation on carcinogenesis of the whole gland. In line with this, bacterial induced-inflammation was the only non-combined treatment that generated malignant lesions in the VL, even with the apparent similarity in inflammatory parameters in both lobes.

The promoting effect of testosterone seems to act mostly on the DL while the VL would be more prone to be affected by bacterial inflammation in MNU-induced lesions. However, both prostate tumor promoters, i.e. testosterone and bacterial inflammation, have the same ability in increasing malignant lesions the DL. Likewise, bacterial inflammation might have an intrinsic and direct effect on prostatic carcinogenesis since the weight of the specimens, the relative weights of the prostatic complex and prostatic lobes as well as serum levels of testosterone did not vary significantly after treatments (not shown).

Gerbils appear to be more sensitive to develop prostate proliferative lesions than other rodents. When MNU-induced prostate carcinogenesis was evaluated in Wistar rats, only a 5% of the animals developed proliferative alterations, with the combination of

bacterial inflammation not playing a significant role in promoting premalignant or malignant lesions (n=35, data not shown).

Taken together, these data indicate that the gerbil is an adequate model for prostate cancer initiation and progression. The short latency period and the wide range of lesions from premalignant to malignant could allow to study the whole spectrum of cellular and molecular mechanisms sustaining prostate cancer. On the other hand, chronic inflammation induced by *E. coli* (the most frequent pathogen associated to human bacterial prostatitis) acts as a potent, even more than testosterone, tumor promoter in the early stages of carcinogenesis of this model.

4- ACKNOWLEDGEMENTS

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7- FIGURE LEGENDS

Figure 1: Experimental design. Three-month-old (PND 90) gerbils were divided into five groups. See material and methods for details.

Figure 2: Histopathological evaluation of the Mongolian gerbil's prostatic complex. A: Premalignant lesions in *E. coli*-infected animals and inoculated with vehicle (E) or MNU (E+MNU).**B:** Representative images of normal features and malignant lesions of the prostatic epithelium in the gerbil's dorsolateral lobe after 3 months of treatment. See Material and Methods for group details. **C:** Multiplicity of proliferative lesions and inflammatory disorders in gerbil ventral and dorsolateral lobes.Values are represented as mean±SEM (n=3-5 per group), using Kruskal–Wallis and Mann–Whitney as statistical tests (* indicates P≤0.05).n.s.: non-significant. VL: ventral lobe; DL: dorsolateral lobe.

Figure. 1
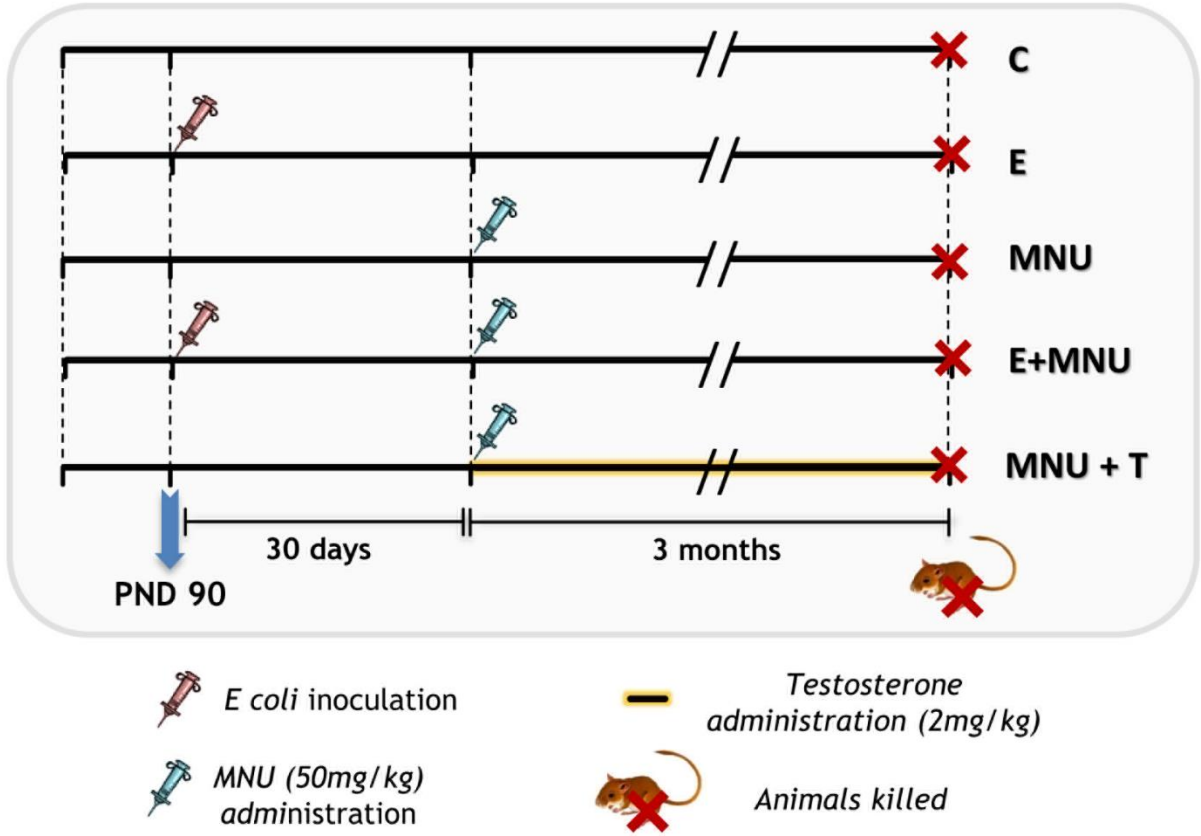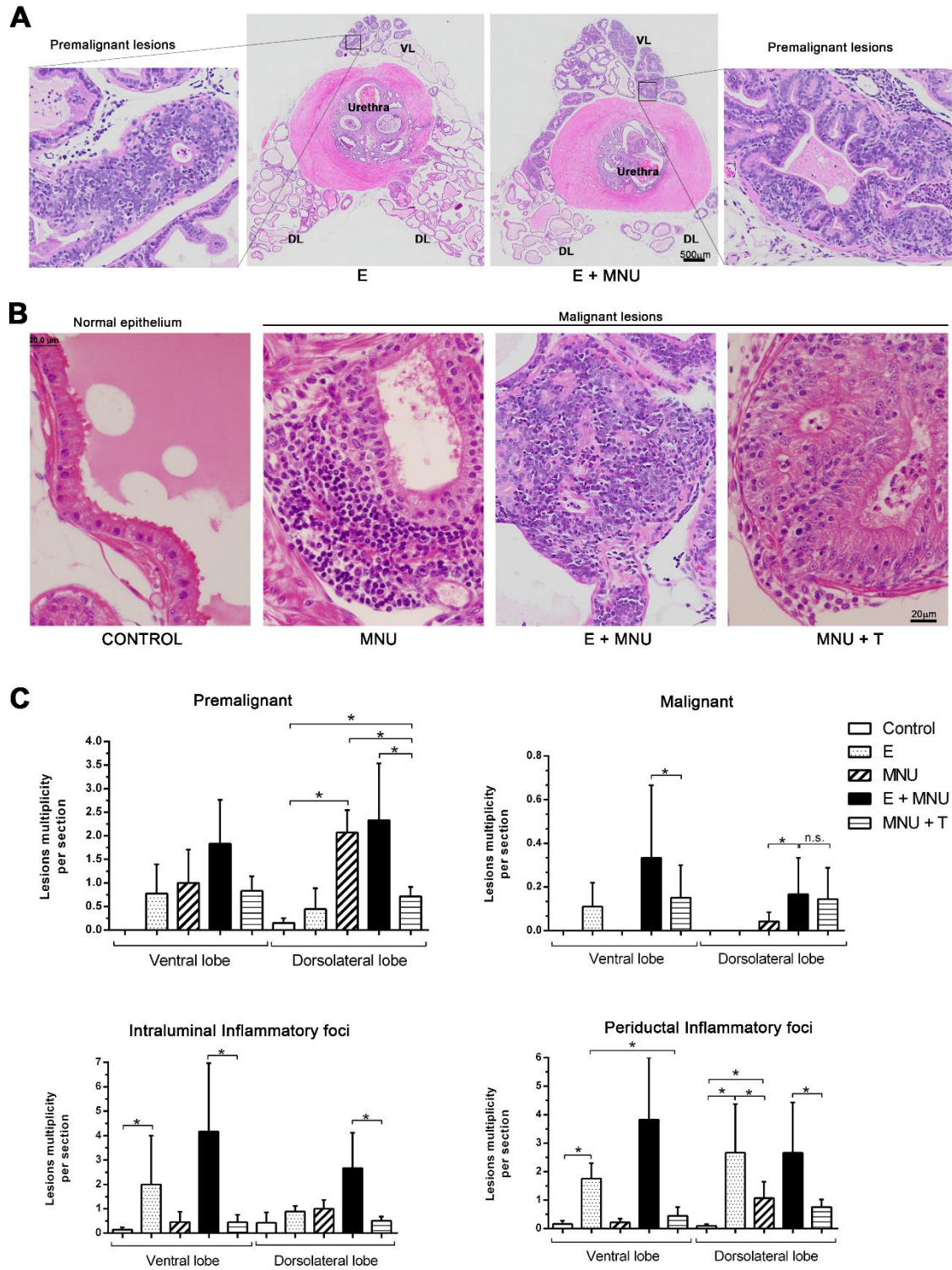
A

Premalignant lesions · VL · Urethra · DL · DL · E · VL · Urethra · DL · DL · 500μm · E + MNU · Premalignant lesions

B

Normal epithelium · Malignant lesions · CONTROL · MNU · E + MNU · 20μm · MNU + T

C

Premalignant · Malignant · Control · E · MNU · E + MNU · MNU + T · Lesions multiplicity per section · Ventral lobe · Dorsolateral lobe · Intraluminal Inflammatory foci · Periductal Inflammatory foci

Figure. 2