EFFECT OF A GONADOTROPIN-RELEASING HORMONE ANALOGUE ON TESTICULAR AND PERITONEUM HISTAMINE CONCENTRATION IN PUBERTAL MALE RATS

Efeito de um análogo do hormônio liberador de gonadotrofinas na concentração de histamina testicular e peritoneal de ratos machos puberes

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Summary

Mast cells are the main source of histamine. There is a strong correlation between mast cell numbers and histamine content in the tissues. The present study was undertaken to determine the effects of a gonadotropin-releasing hormone agonist (GnRH-A) associated or not with androgen therapy on testicular and peritoneum histamine concentrations of pubertal male rats. Three experimental groups were used in this study: (1) animals submitted to GnRH-A [des Gly10, (DTrp6)-LHRH ethylamide] treatment, (2) animals subjected to GnRH-A plus androgen treatment (testosterone propionate), and (3) animals treated with the vehicle (controls). Animals treated with GnRH-A alone presented significantly higher levels of testicular histamine concentration in comparison with control males. GnRH-A treatment associated with androgen replacement produced a dramatic increase in testicular histamine concentration, which was markedly greater than the increase produced by the treatment with GnRH-A only. Peritoneum histamine concentration was not affected by GnRH-A treatment. These results demonstrate that the effect of GnRH-A in increasing testicular histamine concentration in pubertal male rats, is potentiated by testosterone. Since mast cells and histamine seem to have an important role in the restoration of testis function, the increase of testicular histamine concentration after GnRH-A treatment may be an adaptive response of testis to the impairment of steroidogenesis.

Resumo

Os mastócitos são a principal fonte de histamina. Existe uma forte correlação entre o número de mastócitos e o conteúdo de histamina nos tecidos. O presente estudo foi realizado para determinar os efeitos de um análogo do hormônio liberador de gonadotrofinas (GnRH-A) associado ou não à terapia com andrógeno sobre as concentrações de histamina testicular e peritoneal em ratos machos puberes. Três grupos experimentais foram utilizados neste estudo: (1) animais submetidos a tratamento com GnRH-A [des Gly10, (DTrp6)-LHRH ethylamide], (2) animais submetidos a tratamento com GnRH-A associado a androgenoterapia (propionato de testosterona), e (3) animais

tratados com solução inerte (controle). Animais tratados somente com GnRH-A apresentaram níveis significativamente mais elevados na concentração de histamina testicular em comparação com os animais controle. O tratamento com GnRH-A associado ao andrógeno produziu um forte aumento na concentração de histamina no testículo, sendo nitidamente superior ao aumento produzido pelo tratamento com GnRH-A somente. A concentração de histamina no peritôneo não foi afetada pelo tratamento com GnRH-A. Esses resultados demonstram que o efeito do GnRH-A em aumentar a concentração de histamina testicular em ratos machos puberes, é potencializado pela testosterona. Uma vez que mastócitos e a histamina parecem ter um papel importante no restabelecimento da função testicular, o aumento da concentração testicular de histamina após tratamento com GnRH-A pode ser uma resposta adaptativa dos testículos ao comprometimento da esteroidogênese.

Key terms: GnRH-A, histamina, mastócitos, testosterona.

1-INTRODUCTION

Gonadotropin releasing hormone agonist (GnRH-A) at supraphysiological doses and chronically administrated has been utilized for several clinical applications, including the treatment of prostate cancer (PC) in men and central precocious puberty (CPP) in boys (Wilson *et al*., 2007). In other male mammals species, a chronic administration of GnRH-A has been tested, as in dogs (Vickery *et al*., 1985; Trigg *et al*., 2001), cheetahs (Bertschinger *et al*., 2006), bulls (D'Occhio *et al*., 2000), ferrets (Schoemaker et al., 2008) monkeys (Lunn *et al*., 1992) and wallabies (Herbert *et al*., 2004) as an alternative for surgical castration.

Gonadotropin releasing hormone (GnRH) controls the activity of the gonadotrope cells of the pituitary gland and, as a consequence, controls the release of

gonadotropins, which determine the growth, development, and functional activity of testis (Pawson and McNeilly, 2005). Paradoxical anti-fertility effects may occur when potent agonists of GnRH are delivered continuously to animals, which produces a progressive deactivation of GnRH receptors, with a significant decrease in gonadotropin secretion. This effect leads to a significant decrease in testosterone synthesis by testis; lower testis, prostate and seminal vesicle weights; and an inhibition of spermatogenesis (Adams, 2005; Padula, 2005). The decrease in androgen synthesis by testis is one of the objectives of treatment of PC and CPP, such condition can be achieved by continuous use of GnRH analogues (Sharifi *et al*., 2005; Mericq *et al*., 2009).

Mast cells reside in tissues throughout the body, particularly in association with structures such as blood vessels and nerves, and in proximity to surfaces that interface with the external environment. They have multiple critical biological functions, which include a role in innate immunity, involvement in host defense mechanisms against parasitic infestations, immunomodulation of the immune system, control of local blood flow, angiogenesis and tissue repair (Metcalfe *et al*., 1997; Mekori and Metcalfe, 2000;). These cells are known mainly for both synthesizing and releasing a heterogeneous group of pharmacological mediators. Among the preformed and newly synthesized inflammatory substances released by the degranulation of mast cells, histamine remains the best-characterized and most potent vasoactive mediator implicated in the acute phase of immediate hypersensitivity. Moreover, mast cells seem to be the main source of histamine in the tissues (Metcalfe *et al*., 1997).

It is well-established that mast cells are significantly affected by sex steroids (Payne *et al*., 1982; Shirama *et al*., 1988; Gaytan *et al*., 1990; Padilla *et al*., 1990; Menendez-Pelaez *et al*., 1992; Mayo *et al*., 1997; Di Matteo *et al*., 2000; Lima *et al*., 2000; Oner and Ozan, 2002). Most of these studies were carried out in the Harderian gland of rodents and indicate testosterone as an inhibitory factor of mast cell proliferation, and castration as a method for reposition of the cell population (Payne *et al*., 1982; Shirama *et al*., 1988; Menendez-Pelaez *et al*., 1992). On the other hand, we have reported that bilateral castration produced a marked reduction in peritoneum histamine concentration in pubertal male rats, and androgen replacement prevented the

decrease (Lima *et al*., 2000). The reduction in peritoneum histamine concentration after bilateral castration may be associated with a decrease in mast cell numbers. Histamine concentration can be used as an index of mast cell density, since mast cells are the main source of histamine and there is a strong correlation between mast cell numbers and histamine content in tissues. For instance, peritoneum mast cells of rats contain approximately 10-30 pg histamine/cell, whereas mucosal mast cells have lower amounts (1-3 pg/cell) (Metcalfe *et al*., 1997). By altering steroidogenesis, the treatment with GnRH-A may affect several parameters in testis, including the number and function of testicular mast cells.

The chronic treatment with GnRH-A has been evaluated for diverse application and in several animal species but as far as is our knowledge , there is no report in the literature regarding the effects of GnRH-A at suppraphisiological doses on mast cells and histamine concentration in pubertal male rats. Based on this, the present study was conducted to determine the effects of treatment with GnRH-A on testicular histamine concentration and peritoneum histamine concentration in pubertal male rats, in order to verify whether mast cells are affected.

2-MATERIAL AND METHODS

2.1-**Animals and experimental procedure**

Pubertal male *Rattus novergicus* of the wistar strain were used in this study. All animals were housed in plastic boxes (40 x 32 x 17 cm), eight per cage, in a light-and-temperature-controlled room (12 h of light: 12 h of darkness; 22 ± 2 °C) with food and water available *ad libitum*. All animals were sacrificed by decapitation between 9:00 and 10:00 hours.

In order to verify the effect of GnRH-A treatment on histamine concentration, the following experiment was designed: 22-day old male rats were divided into two groups of eight animals each: (1) animals subjected to GnRH-A treatment without androgen

replacement, and (2) animals subjected to GnRH-A treatment associated with androgen replacement.

GnRH-A treatment consisted in daily subcutaneous injection of LHRH analog [des Gly10, (DTrp6)-LHRH ethylamide] (L-5386) at concentration of 100 μg/100 g body weight from the 22th to 42th. The solvent used for dilution and injection was saline. Androgen replacement consisted of daily subcutaneous injection of testosterone propionate at concentration of 100 μg/100 g body weight from the 30th to the 42th day of age. The solvent used for dilution and injection was oil. The control group consisted of males that were not submitted to GnRH-A treatment. They received the dilution vehicle only. All animals were weighed and sacrificed at 43 days of age.

All reagents were obtained from Sigma Chemical Company (USA).

2.2-**Measurement of plasma testosterone concentration**

After decapitation, blood was collected and plasma was separated by centrifugation and stored at -20 °C for the measurement of testosterone concentration. Testosterone was measured by the radioimmunoassay method described by Bélanger *et al.* (1980). All samples were analyzed in duplicate. The interassay coefficient of variation was 4.8% and the intra-assay coefficient of variation was 5.6%.

2.3-**Measurement of testicular testosterone concentration**

The left testis was removed, weighed and stored at -80 °C for the measurement of testosterone concentration. Testosterone was measured by the double-antibody radioimmunoassay method of Bélanger *et al.* (1980). All samples were analyzed in duplicate. The interassay coefficient of variation was 6.2% and the intra-assay coefficient of variation was 4.3%.

2.4-**Measurement of testicular histamine concentration**

The right testis was removed and immersed in a cold solution of perchloric acid (0.4 N). Residual histamine in the cells was released by disrupting the cells with perchloric acid and centrifugation at 400 g for 10 minutes at 4 °C. After centrifugation, the organic portion was removed and discarded. The supernatant was collected and stored at -20 °C for the measurement of histamine concentration. The histamine content was measured by the *o*-phthalaldehyde spectrofluorometric procedure of Shore *et al.* (1959). The fluorescent intensity was measured at 440 nm (excitation at 360 nm) in spectrofluorometer. The intensity of fluorescence provides a base for the calculation of histamine percentage.

2.5-**Measurement of peritoneum histamine concentration**

After decapitation, 10 ml of saline solution was injected into the peritoneal cavity and the abdomen was massaged for one minute. The peritoneal cavity was carefully opened and the fluid was collected with a Pasteur pipette. The peritoneal fluid was added to 3ml of perchloric acid (0.4 N). Residual histamine in the cells was released by disrupting the cells with perchloric acid and centrifugation at 400 g for 10 minutes at 4 °C. After centrifugation, the organic portion was removed and discarded. The supernatant was collected and stored at -20°C for the measurement of histamine concentration. The histamine content was measured by the o-phthalaldehyde spectrofluorometric procedure of Shore *et al.* (1959). The fluorescent intensity was measured at 440 nm (excitation at 360 nm) in spectrofluorometer. The intensity of fluorescence provides a base for the calculation of histamine percentage.

2.6-**Statistical analysis**

Statistical analyses were carried out by Kruskall-Wallis's multiple-comparison method followed by Dunn's test. P values < 0.05 were considered to be significant. Values are expressed as mean $\pm$ SD (Standard Deviation).

3-RESULTS

3.1-**Plasma and testicular testosterone concentrations**

Table 1 shows plasma testosterone and testicular testosterone concentrations in control and GnRH-A treated male rats. Animals treated with GnRH-A alone presented testicular testosterone concentration significantly lower ($p<0.05$) than control males. Plasma testosterone concentration was also significantly lower ($p<0.05$) in animals submitted to GnRH-A alone in comparison with control males, reflecting a significant decrease in testicular testosterone production. Androgen replacement therapy was sufficient for raising plasma testosterone concentration (15.16 ± 2.3 ng/ml) and testicular testosterone concentration (23.8 ± 3.3 ng/g) to values markedly higher than those presented by the control group.

Table 1. Effects of GnRH-A treatment on plasma and testicular testosterone concentrations in pubertal male rats.

Group (n)	Age (days)	Plasma Testosterone (ng/ml)	Testicular Testosterone (ng/g)
Control (8)	43	2.62 ± 0.5 [a]	11.31 ± 1.3 [a]
GnRH-A only (8)	43	0.32 ± 0.1 [b]	3.72 ± 1.2 [b]
GnRH-A+Testosterone (8)	43	15.16 ± 2.3 [c]	23.8 ± 3.3 [c]

Male rats were treated with GnRH-A without testosterone replacement (GnRH-A only) and GnRH-A associated with testosterone replacement (GnRH-A + Testosterone). The Control group consisted of males that were not submitted to GnRH treatment (intact males). They received the dilution vehicle only. GnRH treatment consisted of daily subcutaneous injection of GnRH-A from 22th to 42th of age. Androgen replacement treatment consisted of daily subcutaneous injection of testosterone propionate at 100 μg/100 g

body weight from 30th to 42th of age. All groups were sacrificed at 43 days old. Data were obtained by radioimmunoassay and are reported as mean ± SD. Different letters indicate statistically significant differences between the means: a ≠ b ≠ c ($P < 0.05$, Kruskall-Wallis test, followed by Dunn's test).

3.2-**Testicular histamine concentration**

Figure 1 shows the testicular histamine and peritoneum histamine concentrations in control and GnRH-A treated male rats. Animals treated with GnRH-A alone presented testicular histamine concentration (287.9 ± 55.6 ng/g) significantly higher in comparison with control males (121.7 ± 35.1 ng/g). The GnRH-A treatment associated with androgen replacement produced a dramatic increase in testicular histamine concentration (792.5 ± 77.6 ng/g), which was markedly greater than the increase produced by GnRH-A alone (287.9 ± 55.6 ng/g).

3.3-**Peritoneum histamine concentration**

As shown in Figure 1, chemical castration did not affect peritoneum histamine concentration. There was no statistically significant variation in histamine concentration among control males (275.6 ± 26.1 ng/ml) and males that received GnRH-A without androgen replacement (260.6 ± 31.4 ng/ml) and males that received GnRH-A associated with androgen replacement (337.5 ± 38.3 ng/ml).

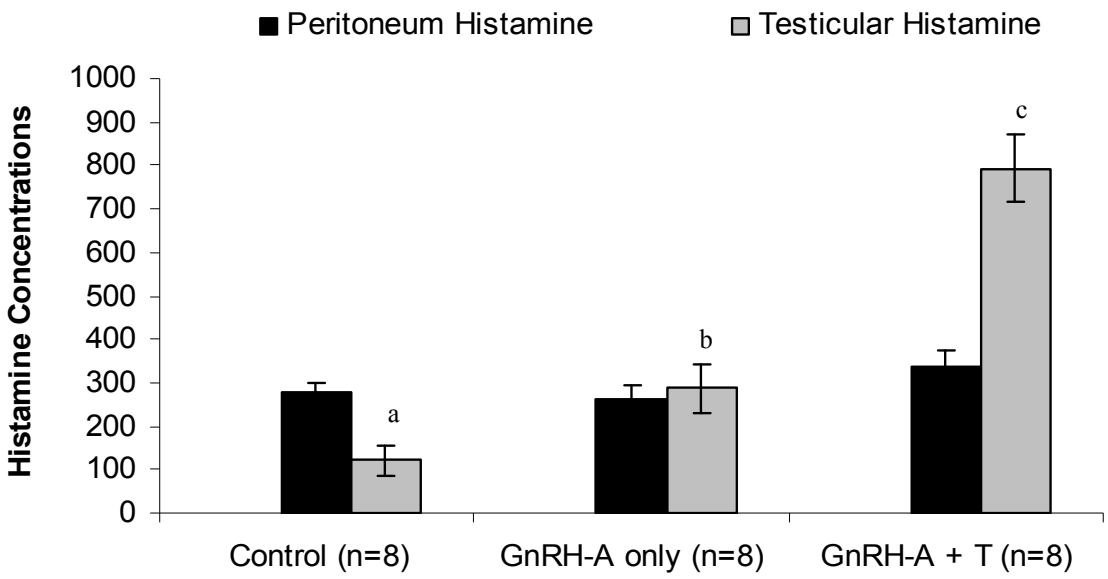

Figure 1. Effects of GnRH-A treatment on peritoneum and testicular histamine concentrations in pubertal male rats. Male rats were treated with GnRH-A without testosterone replacement (GnRH-A only) and GnRH-A associated with testosterone replacement (GnRH-A + T). The Control group consisted of males that were not submitted to GnRH-A treatment (intact males). They received the dilution vehicle only. GnRH-A treatment consisted of daily subcutaneous injection of GnRH-A from 22^{th} to 42^{th} of age. Androgen replacement treatment consisted of daily subcutaneous injection of testosterone propionate at 100μg/100g body weight from 30^{th} to 42^{th} of age. All groups were sacrificed at 43 days old. Data are reported as mean ± SD. Different letters indicate statistically significant differences between the means: $a \neq b \neq c$. Absence of letters indicates there was no significant difference between groups ($P < 0.05$, Kruskall-Wallis test, followed by Dunn's test).

4-DISCUSSION

Peritoneum histamine and testicular histamine concentrations were analyzed in intact pubertal male rats and pubertal males subjected to GnRH-A treatment, in

association with androgen replacement or not. Our results showed that treatment with GnRH-A alone produced a significant increase in testicular histamine concentration in comparison with control males. GnRH-A treatment associated with androgen replacement produced a dramatic increase in testicular histamine concentration, which was markedly greater than the increase produced by GnRH-A alone.

To our knowledge, the present study is the first to report the effects of GnRH-A chronically administrated on peritoneum histamine and testicular histamine concentrations in pubertal male rats. Histamine content in tissues is correlated to the number and maturity of mast cells (Metcalfe *et al*., 1997). Hormonal regulation of mast cells has been extensively studied in different species and in different tissues within the same species. Previous studies on male rodents showed that bilateral castration resulted in a significant increase in mast cell numbers in the Harderian gland of hamsters and mice, while administration of testosterone prevented the increase (Payne *et al*., 1982; Shirama *et al.,* 1988). In addition, administration of testosterone in female Syrian hamsters resulted in a decrease in the number of recognizable mast cells in the Harderian gland (Menendez-Pelaez *et al*., 1992). Mast cells in the female Harderian gland degranulate in response to testosterone and this effect is achieved directly through a receptor in the Harderian gland (Mayo *et al*., 1997). These data suggest that mast cells from rodents are under androgenic control. Previous studies report the effects of sex steroids on mast cell numbers in the testis of the frog Rana esculenta. The authors observed a massive increase in mast cell numbers after 15 or 30 days of treatment with cyproterone acetate, a blocker of androgen receptors, confirming the involvement of androgens in mast cell proliferation and/or differentiation in this species (Di Matteo *et al*., 2000). On the other hand, bilateral castration in male rats significantly decreased the number of mast cells in the thymus, while testosterone replacement prevented the decrease (Barbini *et al*., 1981). After gonadectomy in male rats, testosterone treatment induced an increase in the number of mast cells in the thymus (Oner and Ozan, 2002).

Castration-induced regression and testosterone stimulated regrowth of the vasculature in the rat ventral prostate lobe have also been the subject of study. Previous studies demonstrated that castration decreases and testosterone treatment rapidly

normalizes blood flow of the adult rat ventral prostate. These studies indicate that the vasculature could be regulated, directly or indirectly, by androgens. After castration, testosterone treatment induced a transient but pronounced increase in mast cells in the stroma of the ventral prostate, close to blood vessels, one day after treatment for cell replacement (Franck-Lissbrant *et al*., 1998). This result suggests that mast cells from the stroma of the rat ventral prostate are stimulated by testosterone. This effect may be important in the initial phase of the testosterone-induced vascular response in the ventral prostate, since mast cells are involved in angiogenesis and in the control of local blood flow (Metcalfe *et al*., 1997).

In previous studies we observed that bilateral castration in pubertal male rats produced a marked reduction in peritoneum histamine concentration, and androgen replacement prevented the decrease (Lima *et al*., 2000). Since histamine content in tissues can be used as an index of mast cell density (Metcalfe *et al*., 1997), it is probable that the reduction of peritoneum histamine concentration after bilateral castration may be due to a decrease of mast cell numbers in peritoneum. The analysis of data from literature and our results indicates that testosterone appears to stimulate mast cells from the thymus, peritoneum, testis and prostate of rats, while it seems to play an inhibitory role on mast cells from the Harderian gland of rodents and mast cells from the testis of the frog Rana esculenta. This apparent divergence of results may be due to the fact that mast cells exhibit a diversity of histological, biochemical, and functional properties depending on the species and on their anatomical location within the same species (Chiu and Lagunoff *et al*., 1972; Barret and Metcalfe, 1984; Befus *et al*., 1985). In the present study, we have observed that the treatment with GnRH-A did not affect peritoneum histamine concentration, while it affected testicular histamine concentration.

Therefore, it seems that mast cells from testis are affected by the chronical administration of GnRH-A, while mast cells from peritoneum are not, confirming that mast cells from different tissues may respond differently to the same biological factors. Mast cell subtypes show differences in function, including responsiveness to various secretagogues and stimulation or inhibition by drugs (Metcalfe *et al*., 1997).

As we have observed, the treatment with GnRH-A impairs testicular steroidogenesis and results in a significantly lower production of testosterone. The increase of testicular histamine concentration after treatment with GnRH-A may be an adaptive response of testis to the impairment of steroidogenesis. The number of mast cells increases during puberty in human testis (Nistal *et al*., 1984). Testicular dysfunction correlates with increased testicular mast cells and abnormal spermatogenesis is associated with increased numbers of mast cells in human testis (Apa *et al*., 2002; Hussein *et al*., 2005; Sezer *et al.,* 2005). Moreover, there is expression of the rate-limiting enzyme in histamine synthesis-histidine decarboxylase by human testicular mast cells and there is expression of the histamine receptors by germinal, interstitial, and peritubular cells in the testes of fertile and infertile patients (Albrecht *et al.,* 2005). In rats, the concentration of testicular histamine is considerably higher in the immature gonad than in the adult (Zieher *et al*., 1971). Moreover, mast cells are frequently encountered in rat testes during recovery from impairment of testicular function (Kretser and Keer, 1988) and histamine seems to have the ability to stimulate testicular steroidogenesis in hamsters (Mayerhofer *et al*., 1989). Histamine also can stimulate Leydig cell steroidogenesis in rats, depending on its concentration (Mondillo *et al*., 2005). These data clearly indicate that mast cells and histamine can modulate Leydig cell steroidogenesis and have an important role in the restoration of testis function.

In our results, we have observed that the treatment with GnRH-A only caused low testicular testosterone concentrations and elevated testicular histamine levels. As we discussed previously, the effect caused after GnRH-A treatment, increasing the testicular histamine concentration may be a compensatory response of testis to the impairment of steroidogenesis. In this case, the increase in the testicular histamine concentration would be a secondary response to the reduction of the local testosterone production. Since the inhibitory effect of GnRH-A on testis function (spermatogenesis and steroidogenesis) is reversible with the suspension of therapy both in CP and CPP in humans it is possible to postulate a participation of histamine in testicular function restoration. However, we also observed that the treatment of GnRH-A associated with testosterone propionate led to a dramatic increase in testicular histamine concentration associated with a higher testicular

testosterone concentration. This result raises the possibility that the increase of testicular histamine concentration after GnRH-A treatment may not be simply a secondary response to the impaired testicular steroidogenesis. It is possible that both GnRH-A and testosterone directly stimulate testicular mast cells and histamine production. In this sense, GnRH-A and testosterone may act simultaneously by stimulating testicular mast cells and histamine production, or, GnRH-A may directly stimulate mast cells and testosterone may increase GnRH-A effect.

In conclusion, our results demonstrated that GnRH-A chronically administrated caused a significant increase in testicular histamine concentration in pubertal male rats. The association between GnRH-A treatment and androgen replacement produced a dramatic increase in the testicular histamine concentration, which was markedly greater than the increase produced by GnRH-A alone. Since histamine concentration can be used as an index of mast cells density (Metcalfe *et al*., 1997), the significant increase of testicular histamine concentration after GnRH-A treatment may be associated with an increased number of local mast cells. Further studies are necessary to verify whether the increase observed in testicular histamine concentration in the males treated with GnRH-A is due to an increase in mast cell numbers or whether GnRH-A treatment induced by GnRH-A stimulates mast cell maturation, and consequently, their histamine storage capacity.

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