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ORIGINAL ARTICLE

Effects of chronic treatment with the eNOS stimulator Impaza on penis length and sexual behaviors in rats with a high baseline of sexual activity

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Endothelial nitric oxide synthase (eNOS) has an important role in erection, and it also affects aspects of sexual behavior. In this experiment, we determined whether a compound enhancing the activity of eNOS, Impaza, could stimulate any aspect of sexual behavior and increase penis length in rats with a high baseline of sexual activity. For comparison, the PDE5 inhibitor sildenafil was included. Male rats were orally treated with Impaza or sildenafil for 28 days. Impaza (3 ml kg^{-1}) was given daily while sildenafil (3 mg kg^{-1}) was given twice weekly. Tests for sexual incentive motivation and copulatory behavior were performed just before drug treatment and at days 7, 14 and 28 of treatment. In addition, the length of the protruding penis at mount, intromission and ejaculation was measured. Impaza but not sildenafil increased penis length at mount after 14 and 28 days of treatment. The compounds failed to modify sexual incentive motivation or copulatory behavior. It is suggested that Impaza enhanced intracavernous pressure, as such a pressure increase is the most likely explanation for enhanced penis length at mount. This effect, together with an absence of motivational actions, suggests that Impaza may be the most valuable treatment for erectile dysfunction.

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INTRODUCTION

Erectile dysfunction is the most common sexual disorder in older men.[1,2] Despite the availability of a number of proerectile drugs, there are still an important number of men who, for one reason or another, do not benefit from them. Indeed, it has been mentioned that as much as 35% of men suffering from erectile dysfunction do not respond to PDE5 inhibitors,[3] and discontinuation rates have been reported to be high, between 35% and 45%.[4,5] The reasons for non-adherence to the treatment include fear of possible side effects and high drug costs.[6] There is, consequently, a need for the development of alternative pharmacological approaches.

A compound, Impaza, stimulating endothelial nitric oxide synthase (eNOS) has been reported to facilitate erection in men.[7,8] Impaza is also helpful in erectile deficiency secondary to diabetes type II, chronic prostatitis and hypogonadism,[9] and there are data showing that the compound increases the penile vascular response to erotic stimulation.[10] It has been shown to enhance sexual motivation in old male rats after chronic treatment.[11] A similar effect was obtained with the PDE5 inhibitor sildenafil. Furthermore, sildenafil and Impaza were found to enhance sexual incentive motivation in young adult rats with an initially low motivation level while it was ineffective in males with a high baseline level of motivation.[12] This observation seems particularly interesting as erectile dysfunction is frequently associated with low or absent sexual motivation.[13–15] The fact that no effect was observed in animals with a high baseline activity is also of importance, as clinically useful prosexual compounds ideally should restore behavior to a normal level without leading to hypersexuality.

In the present experiment, we evaluated the effects of 28 days of treatment with Impaza or sildenafil on copulatory behavior and sexual incentive motivation in animals with a high baseline level of sexual activity. Moreover, the length of the protruding, erect penis was measured during mount, intromission and ejaculation with the help of a video-recording procedure. Studies in cats have shown that compounds increasing intracavernous pressure also increase penile length.[16,17] This observation combined with data showing that the length of the protruding rat penis *in copula* varies between mount and intromission[12] in the same was as intracavernous pressure does[18] suggests that penis length may be a convenient indicator of the quality of erection in the male rat. It was predicted that penis length at mount, but not at intromission and ejaculation, would be enhanced by proerectile treatment, whereas sexual behavior should be unaffected in these highly active animals. The reason for predicting a specific effect on penis length at mount is that erection during mounting in rats is mainly a vascular response, while intromission and ejaculation are associated with suprasystolic intracavernous pressure because of contraction of the striated penile muscles.[18–20] As there is no reason to believe that this somatic response would be affected neither by altered activity of eNOS nor by a PDE5 inhibitor, no effect was expected during these latter copulatory events.

MATERIALS AND METHODS

Male (300–350 g) and female (250–300 g) Wistar rats from Charles River WIGA, Sulzfeld, Germany were housed in a temperature-controlled room (21 ± 1 °C) with a relative humidity of 55 ± 10% and a reversed

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12 h light/dark cycle (lights on 23:00—11:00). Tap water and commercial rat pellets were freely available. The females were ovariectomized under isoflurane anesthesia at least 2 weeks before use as partners in tests for copulatory behavior and as sexual incentives in the test for sexual motivation. All experimental procedures were approved by the National Animal Research Authority of Norway and animal housing and care obeyed the rules in the European Union council directive 86/609/EEC.

Hormones and drugs

The females were brought into estrus by a subcutaneous injection of estradiol benzoate, 25 μg per rat, followed by progesterone, 1 mg per rat, 48 h later and 4–8 h before tests. Both steroids were from Sigma, St Louis, MO, USA. They were dissolved in peanut oil, and the injection volume was 0.2 ml per rat.

Affinity-purified antibodies to the C-terminal fragment of eNOS at ultra-low doses (Impaza, OOO "NPF "Materia Medica Holding", Moscow, Russia) were provided as a ready-to-use water solution produced according to homeopathic technology. The actual concentration of the antibodies is not known, but the solution used here is identical to the one used in clinical practice. Sildenafil citrate was prepared from commercial Viagra (Pfizer, Pocé-sur-Cisse, France) tablets. A 25 mg tablet was crushed in a mortar and then dissolved in distilled water to a sildenafil concentration of 1 mg ml^{-1}. The solution was always used on the day of preparation. All treatments were given orally by gavage, and the volume was always 3 ml kg^{-1}.

Behavioral tests

Sexual incentive motivation. This test has been described in detail elsewhere.[21,22] Briefly, a rectangular arena (100 × 50 cm, the wall was 45 cm high) with rounded corners was used. There was one opening (25 × 25 cm) on each long side of the arena. Behind each opening, there was an incentive animal cage (25 × 15 × 25 cm high) containing either an intact male or a sexually receptive female. In front of each incentive animal cage, a virtual zone (the incentive zone) of 29 × 21 cm was defined. Indirect white light (about 5 lux in the arena) was used for illumination.

Before each experimental session, the arena and the incentive animal cages were carefully washed with 0.1% glacial acetic acid in water. The incentive animals (a sexually receptive female and an intact male) were then placed in their respective cages. At the beginning of an observation, an experimental subject was introduced into the middle of the arena. Immediately thereafter, the experimenter left the room and did not return until just after the end of the 10-min observation period. The subject was gently removed from the arena, and the following rat was introduced. A videotracking system (Ethovision pro, Noldus, Wageningen, the Netherlands) recorded the experimental subject's position in the arena with a frequency of 5 Hz. Detailed descriptions of this test can be found elsewhere.[21,22]

Copulatory behavior. Black sheet-steel cages (40 × 60 × 40 cm high) with Plexiglas front and glass floor were used. They were positioned over a mirror inclined at 45°. This allowed for a simultaneous side and ventral view of the copulating male. Dim white light (about 5 lux in the observation cages) was produced by lamps placed on both sides of the cages, about 10 cm below cage floor level. This arrangement assured that the ventral part of the rat was clearly illuminated making it possible to view the penis during copulation.

At the beginning of a test, the male was put into the observation cage about 5 min before a sexually receptive female was introduced. Copulatory behavior was then observed until the end of the first postejaculatory interval. Standard behavioral parameters as defined elsewhere[23] were recorded with an in-house software. If no mounting occurred, the test was terminated after 15 min. It was also terminated if the ejaculation latency became >30 min or the postejaculatory interval >15 min. The cage floor was carefully washed with commercial glass cleaning liquid before the introduction of a new rat in order to assure an optimum ventral view. Tests were recorded on DVD (digital video disc) for later analyses.

Penis length measurements

The length of the part of the erect penis protruding from the prepuce during mount and/or following withdrawal after intromission or ejaculation was estimated from the video record using an improved version of a procedure used in an earlier study.[12] Playback was controlled by free video playback software (Media Player Classic 123; downloaded from http://www.topshareware.com/media-player-classic-123-player/downloads/1.htm) and the image was projected on a large screen (68 × 83 cm) with a LCD (liquid-crystal display) projector. From the beginning of a mount with or without intromission/ejaculation until withdrawal, the video was advanced frame by frame. The frame where the erection was maximal was always chosen for measurement of the protruding penis. This was done directly on the projection screen with a digital caliper. Measurement was not possible at every mount or intromission because of an unsatisfactory view. Nevertheless, in most sexually active animals, at least five erections during mount and another five at intromission were measurable. In case the subject displayed >5 measurable mounts and intromissions, only the first five were measured. The erection observed after ejaculatory withdrawal was measured whenever possible. The mean penis length for mount and intromission was then calculated for each animal at each test. This mean was used for statistical analysis. All screen measurements were transformed into actual penis length before analysis. The measurements were made by an assistant blind to the treatments.

Design and procedure

Initially, 60 rats were tested six times for copulatory behavior. Tests were performed twice a week. After the last of these pretests, the mean intromission ratio (number of intromissions/(number of mounts + number of intromissions)) for the last three tests was calculated for each animal. The intromission ratio is often used as an indicator of the efficiency of erection and appropriate contraction of the ischiocavernosus muscles,[23] both of which are required for vaginal penetration (intromission). Provided the number of pre-ejaculatory intromissions is constant, animals with low intromission ratio will perform more mounts than animals with a high intromission ratio, hence copulatory behavior can be considered more intense in the former. In the present study, we selected animals with a low intromission ratio with the purpose of including only subjects with a high level of copulatory activity.

In case an animal had failed to display copulatory behavior during the last three pretests, it was assigned an intromission ratio of 1. The median of all animals was then determined, and the subjects with an intromission ratio below the median were selected for the experiment. Even though this cutoff point is arbitrary, it should assure that the selected animals displayed a higher level of sexual activity than a non-selected sample would. The median ± semi-interquartile range for the entire sample turned out to be 0.60 ± 0.15. The selected animals had a median semi-interquartile range of 0.44 ± 0.09 while the corresponding values for the non-selected animals were 0.73 ± 0.06. The selected and non-selected animals had the same mean number of intromissions at the last three pretests (selected, 13.35 ± 0.62; non-selected, 13.55 ± 0.93; $t(58) = 0.18$, NS) while there was a large difference in mounts (selected, 22.79 ± 2.08; non-selected, 5.01 ± 0.52, $t(58) = 8.31$, $P < 0.001$) at these same tests. Thus, the selected animals displayed a much more intense copulatory behavior than the non-selected animals. This is the reason for maintaining that the subjects used in this experiment had a high baseline sexual activity.

The selected animals were randomly assigned to one of the three groups of 10 animals each. These animals were then familiarized to the sexual incentive motivation test at three sessions of 10 min each, separated by 48 h. A baseline test was then performed. First, the subjects were exposed to the motivation test. Immediately after the 10-min observation period, they were transferred to the copulatory behavior testing room. The test was started 5 min later. The morning after the baseline test, drug treatment was initiated. One group was given 3 ml kg^{-1} of distilled water and another was given 3 ml kg^{-1} of Impaza. The third group received sildenafil, 3 mg kg^{-1}, twice a week and an equivalent volume of distilled water on the other days. Sildenafil was always given on the day of testing. The second weekly sildenafil treatment was performed 3 days later. This frequency of sildenafil treatment was used in order to approximate the typical on-demand clinical use.[24,25]

Treatments were given daily for 28 days, and tests were performed on Days 7, 14 and 28 of treatment. On test days, drug treatment was given 1 h before the test for sexual incentive motivation.

Statistics

Treatment effects were evaluated by determining the difference between the baseline and later tests in each parameter. The value obtained at baseline was simply subtracted from the value obtained at later tests. This procedure allows for a sensitive analysis of treatment-induced changes, corrected for any group differences at baseline. It is commonly used in pharmacological and behavioral studies.

Sexual incentive motivation was expressed as a preference score (time spent in the female incentive zone/(time spent in the female incentive zone ± time spent in the male incentive zone)) and this score was analyzed with two-factor analysis of variance (ANOVA) with repeated measures on one factor. The within-groups factor was test day (Days 7, 14 and 28) and the between-groups factor was treatment (water, Impaza and sildenafil). The time spent in the incentive zones was evaluated by three-factor ANOVA with repeated measures on two factors. Test day and incentive (male, female) were the within-groups factors and treatment was the between-groups factor. Data from the copulatory behavior tests as well as penis length were analyzed by two-factor ANOVA in the same way as the preference score. In case of significance, *post hoc* comparisons were made with Tukey's HSD (honestly significant difference) test. Finally, the difference between the baseline and each of the following tests was evaluated with the one-sample *t*-test in order to determine whether it was significantly different from 0.

RESULTS

Sexual incentive motivation and copulatory behavior

There was no effect of treatment on the preference score and no effect of test and no interaction in test × treatment ($Ps > 0.56$; see Figure 1a). Analysis of the time spent in the incentive zones also failed to detect any significant treatment effect ($Ps > 0.43$). Thus, sexual incentive motivation was not modified by the treatments. Data are illustrated in Figure 1b.

There was no treatment effect on any measure of ambulatory activity ($Ps > 0.13$). However, there was an interaction in test × treatment ($P < 0.05$) with regard to the distance moved during the test. The interaction was due to the fact that the group treated with water moved shorter distances as treatment progressed, whereas the other groups remained at a stable level. Activity data are displayed in Figure 2. Copulatory behavior was unaffected by the treatments (data not shown).

Penis length

ANOVA of penis length at mount revealed that there was no main effect of test and no interaction in test × treatment. On the contrary, there was a treatment effect ($P < 0.01$). *Post hoc* comparison with the Tukey's test showed that the difference from the baseline was larger in the group treated with Impaza than in the groups given sildenafil or water at all tests. There was no difference between the groups given water and sildenafil. Finally, when the observed differences were compared with 0 it turned out to be significant for the group treated with Impaza at the tests performed on days 7 ($P < 0.05$), 14 ($P < 0.01$) and 28 ($P < 0.05$), whereas no significant difference was observed in the other groups. Thus, Impaza enhanced penis length at mount.

When the penis length at intromission was analyzed with ANOVA, no effect reached significance. However, when the difference from the baseline was compared with 0, it turned out to be significant in the Impaza group at Day 28 of treatment ($P < 0.05$). There was no significant effect at any other test and in

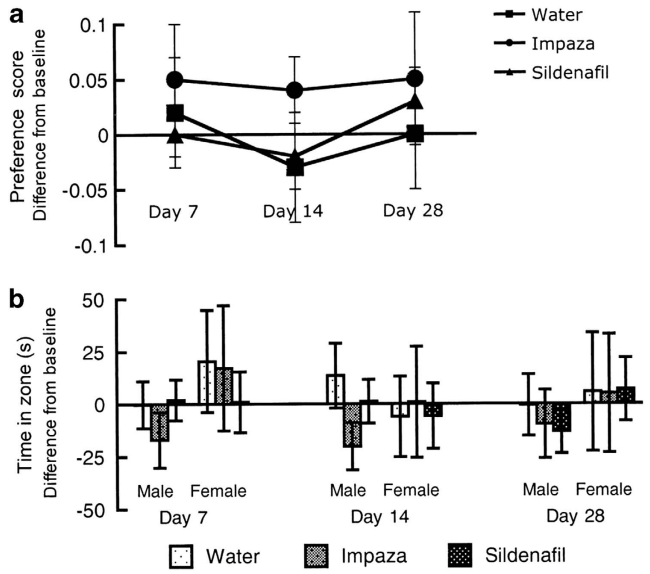

Figure 1. (**a**) Difference in preference score between the baseline and later tests in male rats treated with distilled water (3 ml kg^{-1}), Impaza (3 ml kg^{-1}) or sildenafil (3 mg kg^{-1}) daily for 28 days. Tests were performed just before the beginning of drug treatment (baseline) and at treatment days 7, 14 and 28. (**b**) Difference in time spent in the incentive zones between the baseline and later tests in these rats. Data are mean ± s.e.m.

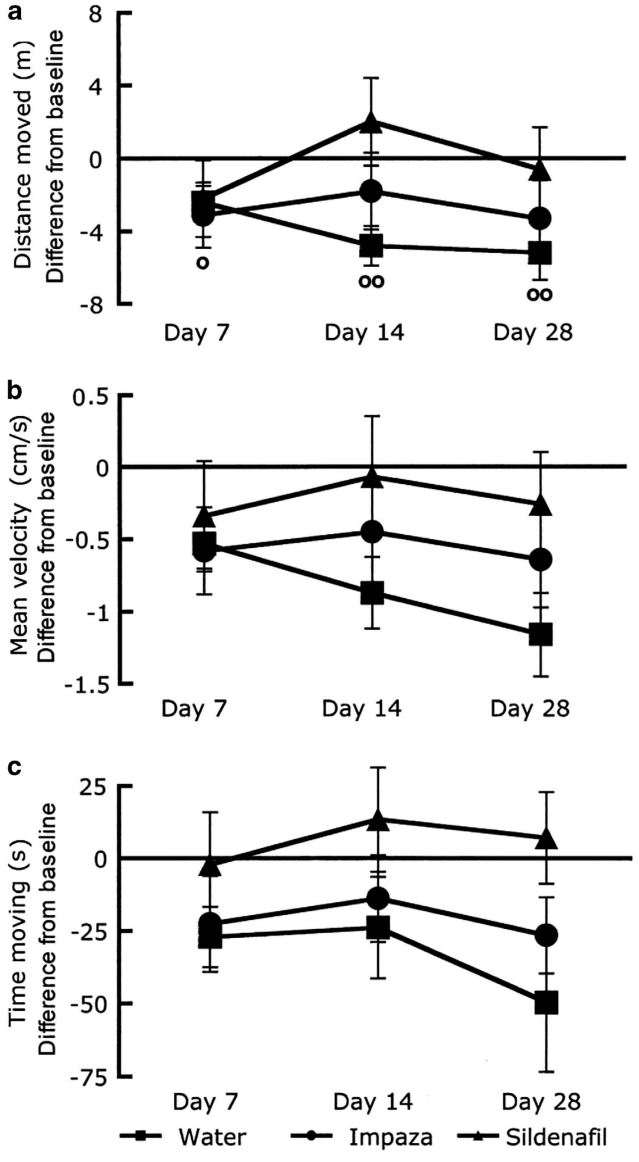

Figure 2. Difference in parameters of ambulatory activity between the baseline and later tests in male rats treated with distilled water (3 ml kg^{-1}), Impaza (3 ml kg^{-1}) or sildenafil (3 mg kg^{-1}) daily for 28 days. Tests were performed just before the beginning of drug treatment (baseline) and at treatment Days 7, 14 and 28. (**a**) Distance moved during the 10 min test; (**b**) mean velocity of movement during the test; and (**c**) time spent moving during the test. Data are mean ± s.e.m. °difference from the baseline > 0, $P < 0.05$; °°$P \leqslant 0.01$.

any other group. None of the treatments affected penis length at ejaculation. The data from the baseline test are illustrated in Figure 3. In Figure 4, data from the three treatment tests are shown as the difference from the baseline.

DISCUSSION

Present results show that none of the treatments modified sexual incentive motivation or copulatory behavior. This is in line with earlier studies where it was found that sildenafil and Impaza only enhance sexual incentive motivation in subjects with a low baseline level of motivation.[11,12] Likewise, copulatory behavior is not modified by these compounds in rats displaying a high level of baseline activity. In agreement with these observations, the experimental treatments used in the present study had no behavioral effects. Although the treatments failed to affect sexual behaviors, Impaza increased penis length at mount on the tests performed on Days 7, 14 and 28. On the contrary, penis length at intromission was not systematically affected by any treatment, although the Impaza group had longer penis on Day 28 than at baseline. It was, however, not different from that recorded in the other groups on Day 28.

As mentioned in the Introduction, the length of the erect penis is dependent on the pressure in the corpora cavernosa and/or corpora spongiosa. In rats, intracavernous pressure during intromission is further enhanced by contraction of the ischiocavernosus muscles.[26] The main function of this contraction is to move the penis forward and upward, thereby bringing it into contact with the vaginal orifice[27] making penetration possible. The contribution of the striated penile muscles to the intracavernous pressure, hence erection, during intromission may mean that drug-induced changes in this pressure are masked. During ejaculation, intense contraction of the bulbospongiosus muscles enhances intracavernous pressure still more, but this occurs during penetration, when the penis is invisible. Thus, although erection at mount is an essentially vascular event, erection at intromission and ejaculation is heavily influenced by activity in striated muscles. Consequently, compounds affecting the vascular contribution to erection should have their most evident effect during mounting.

According to the reasoning exposed in the preceding paragraph, we propose that Impaza causes lengthening of the penis because of enhanced intracavernous pressure. The importance of eNOS in erection is well known,[28] and any compound enhancing eNOS activity could potentially facilitate erection by contributing to increase this pressure.[29] Unfortunately, the molecular mechanisms of the action of Impaza remain unknown, precluding any informed speculation as to the exact nature of the events underlying the effects observed here.

The fact that Impaza enhanced penis length, whereas sildenafil did not requires an explanation. To that end, it is necessary to briefly mention the events underlying the erection response. It is believed that erection is initiated by release of NO from penile autonomic nerves. The ensuing relaxation of cavernous smooth muscle cells and penile arteries leads to a shear stress-induced phosphorylation of eNOS at Ser1177 resulting in endothelial production of additional NO.[30] It appears that eNOS activity is essential for attaining complete erection and for sustaining it.[31,32]

Although sildenafil blocks the phosphodiesterase responsible for the termination of NO action, Impaza stimulates eNOS, leading to enhanced production of NO in endothelial tissue. NO of endothelial origin, like that of neural origin, activates soluble guanylyl cyclase, which enhances the production of 3,5-cyclic

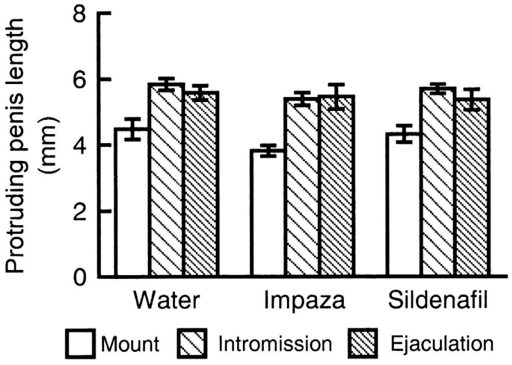

Figure 3. Penis length at mount, intromission and ejaculation at the baseline test. Data are mean ± s.e.m. The length at intromission and ejaculation is superior to that at mount in all the three groups ($P<0.01$).

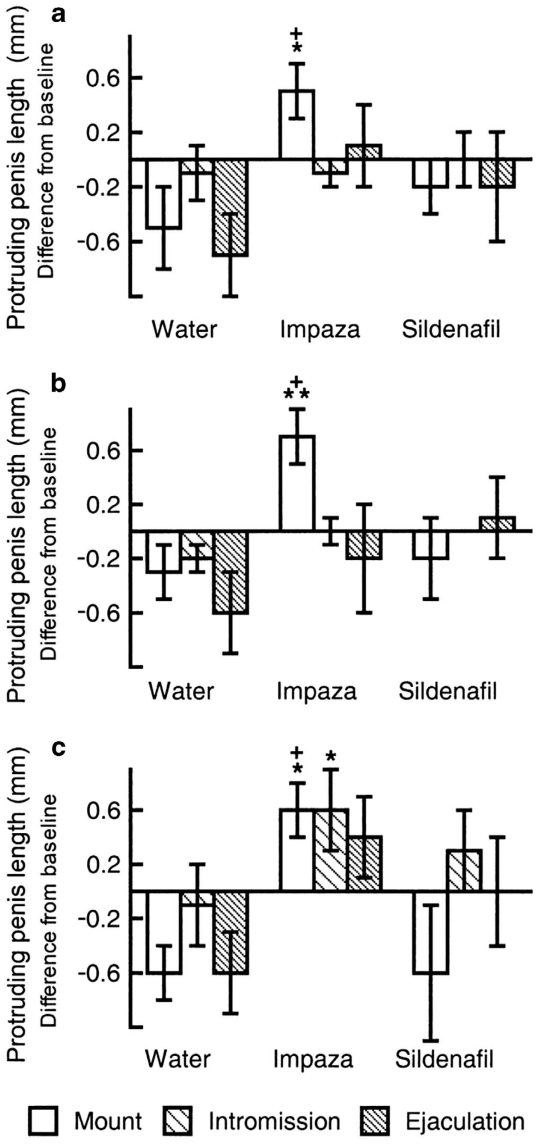

Figure 4. Penis length, expressed as change from the baseline, in male rats treated with distilled water (3 ml kg^{-1}), Impaza (3 ml kg^{-1}) or sildenafil (3 mg kg^{-1}) daily for 28 days. (**a**) The data from the test performed on Day 7 of treatment, (**b**) the data from the test on day 14 and (**c**) the data from the test on day 28. Data are mean ± s.e.m. $^{+}$different from control treatment (water) $P<0.05$; *difference from the baseline > 0, $P<0.05$; **$P<0.01$.

guanosine monophosphate (cGMP). The action of cGMP is terminated by PDE5. An inhibitor, such as sildenafil, should prolong the action of endothelial NO as much as that of neural NO and should, therefore, have an effect quite similar to what is obtained by stimulation of eNOS. Furthermore, there are data showing that chronic administration of PDE5 inhibitors promote phosphorylation of eNOS, enhancing the activity of the enzyme.[33,34] Indeed, it has been proposed that PDE5 inhibitors improve erection through an endothelial-dependent manner.[35] All these observations would suggest that sildenafil should be as efficient as Impaza in increasing penis length.

There are, however, some data that could explain the fact that sildenafil lacked effect, whereas Impaza was effective. In young rats (4 months) chronic sildenafil failed to improve erection whereas an effect was found in old (19 months) rats. The enhanced erection appeared to be mediated by increased expression of phosphorylated eNOS at serine-1177 and phosphorylated Akt at serine-473, effects that were observed in old rats only.[33] The lack of effect on erection in young rats coincides with present data, as the rats employed here were between 4 and 5 months old. Another possible explanation for the lack of effect of sildenafil is that altered endothelial NO availability caused by chronic treatment with Impaza has enhanced the reactivity of the erection promoting RhoA/Rhokinase pathway, whereas sildenafil failed to do so. Indeed, endothelial NO has been suggested to be a regulator of the basal signaling functions of the NO–RhoA/Rhokinase erection pathways.[36] Enhanced reactivity in this pathway could easily lead to a faster increase in intracavernous pressure at mount, which, in turn, could result in a longer penis. This is, for the moment, pure speculation awaiting experimental test.

A simpler explanation for the lack of effect of sildenafil is that the dose used was too low. This dose was the same as in earlier studies,[11,12,37] but it is not impossible that a higher dose would have increased penis length. It should also be noted that sildenafil, at the dose used in the present experiment, failed to enhance penis length in a study with an earlier version of the penis length measurement procedure, showing that the lack of effect is consistent.

Independently of the mechanisms involved in the effects of Impaza and the cause of the lack of effect of sildenafil, present results suggest that Impaza, at the dose used here, had a consistent effect on penis length without modifying sexual incentive motivation or copulatory behavior in the highly active animals used here. This, together with the fact that Impaza stimulates sexual behaviors in animals with low activity,[11,12] indicate that this compound could be efficient for treating erectile dysfunction and associated motivational problems without having aphrodisiac properties.

CONFLICT OF INTEREST

ESZ (Research Associate), JLD (Head of Preclinical Development), IAK (Senior Research Associate), SAS (Chief Scientific Officer) have paid positions in OOO ''NPF ''Materia Medica Holding'', OIE is the General Director and the owner of the company above mentioned. XC and AÅ are employees of the University of Tromsø and have no financial interests in OOO ''NPF ''Materia Medica Holding''.

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