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Research Article

Adverse Effects of the Largely Used Diuretic Furosemide Studied on Ants as Models

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Abstract

Working on ants as biological models, we studied the side effects of the largely used diuretic furosemide. This drug is very efficient; it reduces the water recapture occurring in the first part of the Henle's loop. It did not impact the ants' food intake, activity, audacity, sensory perception, social relationships, state of stress, cognition, learning and memory, what is in favor of its use. However, furosemide affected the ants' locomotion, with no adaptation to this side effect; it leaded to water need, and unfortunately to dependence on its consumption. After weaning, furosemide rapidly lost its effect in a in total of 14 hours, but, though still differing from the drug-free situation 12 hours after weaning, its effect already differed from its initial one at about 3 hours after weaning, what is perceived by consumers and leads to dependence. Ototoxicity has also been reported in the literature. We thus concluded that furosemide could be used for treating patients suffering from edema, but at the imperative condition of monitoring the patients as for their movements, their need of water (i.e. their hydration), their amounts of calcium and potassium, their possible deafness occurrence, and above all their possible dependence on this drug consumption. Practitioners should treat their patients with the smallest possible dose of furosemide, and regularly check the state of health of these treated patients.

Keywords: Dependence; Hydration; *Myrmica sabuleti*; Potassium; Sodium; Water Consumption

Abbreviations

ang.deg.: Angular Degrees; ang.deg./cm: Angular Degrees per cm; mm/s: Millimeter Per Second; χ^2= chi-Square; *vs:* Versus; n°: Number; cm: Centimeter; mm: Millimeter; ml: Milliliter; mg: Milligram; s: Second; min: Minute; h: Hour; t: Time; %: Percentage

Introduction

Humans suffering from edema or urinary problems are generally treated thanks to diuretic drugs. There exist three kinds of such drugs: those acting on the kidney loop functioning, those reducing patients' hypertension, and those acting on the aldosterone, a hormone implicated in the ionic balance. We have previously studied the adverse effects of a diuretic which decreases the tension, indapamide, using ants as biological models. We found it has several adverse effects which could affect the daily life of patients [1]. Being treated with that drug, since a few years, we can affirm that these side effects observed in ants occur in humans and impair the quality of life. Indeed, indapamide induces weakness, tiredness, social interactions problems, and there is not adaptation to these side effects. We thus aimed to examine, again on ants as models, the potential side effects of a diuretic acting on the kidney loop functioning, for knowing if such a kind of diuretic could be preferable to those reducing the blood tension. We opted for the study of furosemide, one of the five most frequently prescribed drugs in the USA [2].

Before relating our experimental work, we here below give information available on the pharmacological properties and the unwanted effects of furosemide, briefly explain why we worked on ants, which species we used, what we know on it and which traits potentially impacted by the drug we intended to examine.

Furosemide is a complex molecule (Figure 1) which inhibits the re-capture of sodium in the kidney what soon allows eliminating water. It is thus an efficient diuretic, for most of the treated patients, and is considered as being an essential drug by the WHO. Furosemide is sold under many different labels such as Docfurose, Furodur, Furosemide (the one we here used), Laxis. The pharmacological properties of furosemide have already been largely examined [3-5]. The notice for used joined to the packages of these commercialized drugs report only few and not severe adverse effects, and simply states that furosemide is not recommended for humans having some precise health (hart, liver, kidney) problems. Risks in case of acute kidney injury have been evaluated [6] and cases of deafness have been reported [2]. Let us add that, allowing not seeing the consumption of anabolic products, furosemide is considered as being a doping agent for the people doing competitive sports (notice for use joined to the packages of furosemide). In all this available information, nothing could be found about the impact of the drug on, among others, the individuals' sensory perception, social interactions, cognition, memory, and adaption to side effects of the drug. For us, this gap of information is a one more motivation for studying, on ants as usually, the side effects of furosemide.

The basic biological processes, such as the genetic, muscles contraction, nervous impulses, memorization, and sensory perception mechanisms, are identical for all the animal species, including humans. Several invertebrates and vertebrates are thus used as biological models [7,8]. Invertebrates are advantageously used: they are small, easily maintained in a laboratory, and have a rapid development [9]. Among others, insects are largely used, e.g. the locusts, the mealworms, the fruit flies, the bees [10]. Ants can also be used, essentially because they can be easily maintained at low cost, and because they detain several evolved ethological skills on which the effects of products used by humans can be studied. Among these skills, let us cite the use of specific pheromones for communicating with congeners, the memorization of visual and olfactory cues and the use of these cues for navigating, the establishment of efficient recruitment systems, the brood caring behavior, several specific territorial markings, the building of complex nests and the managing of cemeteries [11].

In this work, we used the species *Myrmica sabuleti* Meinert, 1861 we have already largely studied. We know its recruitment system, navigating strategy, visual perception, visual and olfactory conditioning [12], the ontogenesis of some of their knowhow [13], their self recognizing in a mirror [14]. This species detains several cognitive abilities, e.g. having a number line, acquiring the notion of zero, counting elements, adding numbers, acquiring numerical symbolisms, expecting future events on the basis of previously experienced ones [15,16], associating perceived visual and olfactory cues as well as amounts of elements with their time period of occurrence [unpublished data]. Also, the distance effect, size effect and Weber's law can be applied to their perception [17,18]. Nevertheless, all these cognitive skills remain at a concrete level, the ants never reaching the abstract level.

In the present study, we examined, as we previously did for many other substances (19 and references therein), the potential side effects of furosemide on the ants' food intake, activity, locomotion, orientation ability, audacity, tactile (pain) perception, social relations, stress, cognition, learning and memory. We looked at the ants' adaptation to adverse effects of furosemide, and at their potential dependence on this drug consumption. Finally, we studied the loss of the effect of furosemide after its consumption was stopped. The experimental processes were identical to those used in until now 47 previous works [20-22]. We thus here only briefly related them though however not avoiding some self plagiarism.

Materials and Methods

Collection and maintenance of ants

The experiments were performed on two colonies of the ant *M. sabuleti* collected in May 2021, in the Aise valley (Ardenne, Belgium), in an abandoned quarry. The colonies contained about 600 workers, brood and a queen. In the laboratory, each colony was maintained in one to three glass tubes half filled with water, a cotton plug separating the water from the compartment devoted to the ants. The nest tubes of each two colonies were deposited in a tray (34 cm x 23 cm x 4 cm) which served as a foraging area. In this area, *Tenebrio molitor* larvae (Linnaeus, 1758) cut into two pieces were provided three times per week, and a small tube filled of sugar water (concentration: 15%) and plugged with cotton was set in permanence. The luminosity of the laboratory reached ca 330 lux while working on ants and ca 110 lux during the other time periods. The ambient temperature equaled ca 20°C, the humidity ca 80%, and the electromagnetic field ca 2 μWm^2. These conditions were suitable to the ant *M. sabuleti*. We here often named the ants 'workers' or 'nestmates'.

Figure 1: Realisation of the solution of furosemide provided to the ants. The figure successively shows the chemical structure of the drug, the material used to make the required solution, half a tablet of furosemide duly crushed before being dissolved in 50 ml of sugared water, and a few ants drinking the realized solution.

Solution of furosemide EG® given to the ants

Humans are advised to consume per day generally 1/2 to 1, or if necessary 2 to 3 tablets of Furosemide® containing each one 40 mg of drug (so, 20 to 40 mg or 80 to 120 mg of Furoxemide® per day). A package of Furosemide EG® 40 mg (manufacturer: EG labo- Laboratoires Eurogenerics, Centraln Park, 9-15 Rue Maurice Mallet, 92130 Issy-les-Moulineaux, France) was furnished by the pharmacist Wera (1170 Bruxelles, Belgium). Humans as most of the mammals consume about one liter of water per day. Treated with Furosemide, they consume thus generally 1/2 to 1 tablet of the drug drinking at the same time about one liter of water. The insects, and thus the ants, due to their anatomy (cuticle) and their physiology (excretory system), consume about ten less water than mammals. Consequently, for setting the ants under a diet with furosemide similar to that of humans, we opted to provide them with a solution of one tablet (40 mg of the drug) in 100ml of water or 1/2 such tablet in 50 ml of water. A half tablet of Furosemide® was thus crushed then dissolved into 50 ml of the ants' sugar water, and this solution was provided to the ants in their usual cotton-plugged tubes (Figure 1). The plug of these tubes was refreshed every 2-3 days, and the entire solution was renewed every 7 days. We checked each day if ants drunk the furosemide solution, and they did (Figure 1). The control experiments were firstly performed on the two colonies maintained under normal diet. Then, the tubes containing sugar water were replaced by those contained the sugared solution of furosemide, and the test experiments started after the ants had the drug solution at their disposal during 24 hours.

Meat and sugar water consumption, general activity

While ants were living under normal diet, then while they were consuming furosemide, those of the two colonies which were on the meat food, at the entrance of the sugar water tube, and active everywhere (foraging area, inside the nest etc…) were counted four times (twice during the day, twice during the night) (total = 4 x 2 = 8 different counts each day) during 6 successive days, each day at the same time o'clock. For each diet and each kind of count, the daily mean was established (Table 1, lines 1 to 6). These six daily means obtained for ants under one and the other kinds of diet were compared using the non-parametric test of Wilcoxon [23]. Also, for each kind of diet and of count, the mean of the six daily means was calculated (Table 1, last line).

Linear and angular speeds; orientation to a tied nestmate

These traits were assessed on ants moving in their foraging area, the linear and angular speeds without stimulating the ants, the orientation while stimulating them with a nestmate tied to a piece of paper (Figure 2, A). Such a tied nestmate emits its attractive mandible glands alarm pheromone. For assessing the ants' speeds, then their orientation, 40 trajectories were each time recorded and analyzed thanks to appropriate software [24] created according to the following definitions. The linear speed (in mm/s) was the length of a trajectory divided by the time spent to travel it; the angular speed (in ang.deg./cm) was the sum of the angles made by successive adjacent segment of a trajectory divided by the length of this trajectory; the orientation (in ang. deg.) to a location was the sum of successive angles made by the direction of the trajectory and the direction towards the location, divided by the number of measured angles. An orientation value lower than-90° means that the tested animal tends to orient itself toward the location. An orientation value larger than 90° means that the tested animal tends to avoid the location. For each assessed variables, the median and quartiles of the 40 recorded values were established (Table 2, lines 1, 2, 3) and the distribution of these 40 values obtained for ants consuming furosemide was compared to the corresponding distribution obtained for ants living under normal diet using the non-parametric χ^2 test [23].

Audacity

This ethological trait was assessed through the ants' tendency in coming onto a risky unknown apparatus deposited in their foraging area and made of a cylinder (height = 4 cm; diameter = 1.5 cm) vertically tied to a squared platform (9 cm^2) both in Steinbach® white paper (Figure 2 B). The ants of the two colonies present at any place on this apparatus were counted 10 times over 10 minutes (number of counts: 10 x 2 = 20). The mean and the extremes of the obtained numbers were established (Table 2, line 4). Also, the numbers obtained for the two colonies were correspondingly added, and then chronologically summed by two, what allowed obtaining five successive sums. These five sums obtained for ants under furosemide diet were compared to those obtained for ants under normal diet using the non-parametric Wilcoxon test [23].

Tactile (pain) perception

An ant perceiving the rough character of a substrate walks on it with difficulty, slowly, sinuously, often touching it with its antennae (Figure 2, C 1). An ant poorly perceiving the rough character of a substrate walks on it rather easily, rapidly and not sinuously, and it seldom touches the substrate with its antennae. Consequently, to estimate the ants' tactile (pain) perception, their linear and angular speeds while walking on a rough substrate were assessed according to the method explained in the previous subsection. For each colony, a piece (3 cm x 2 + 7 + 2 = 11 cm) of n° 280 emery paper duly folded was deposited in a tray (15 cm x 7 cm x 4.5 cm) dividing so this tray in a first 3 cm long zone, a second 3 cm long one containing the emery paper, and a last 9 cm long zone. For performing an experiment, 12 ants of each colony were transferred into the first zone of their own apparatus. Their trajectories were recorded when they walked on the emery paper, and their linear and angular speeds were then assessed. The median and quartiles of the obtained 24 values of linear speed on one hand and of angular speed on the other hand were established for each kind of diet (Table 2, lines 5, 6). Also, the distributions of these 24 values obtained for ants consuming furosemide were compared to the corresponding distributions obtained for ants under normal diet using the non-parametric χ^2 test [23].

Brood caring behavior

For each colony, a few larvae were removed from the inside of the nest and deposited in front of the entrance. For each colony, five of these larvae as well as the ants' behavior towards them were duly observed during five minutes (Figure 2, D). The number of these 5 x 2 observed larvae not re-entered after 30 seconds, 1, 2, 3, 4, and 5 minutes were counted (Table 3, line 1). Only five larvae per colony were observed because we had to look at all of them simultaneously. The experiment was made only once because removing larvae out of the nest causes a great social disruption imperiling the brood survival. The six numbers of not re-entered larvae obtained for each colony were correspondingly added, and the six sums obtained for ants under furosemide diet were compared to the six corresponding sums obtained for ants under normal diet using the non-parametric test of Wilcoxon [23].

Social relationships towards nestmates

Ants of a same colony (= nestmates) are not aggressive towards each other. The effect of furosemide on this peaceful social behavior was studied in the course of five dyadic encounters performed for each two colonies maintained firstly under normal then under the drug diet. These encounters (10 in total) were conducted in a cup (diameter = 2cm, height = 1.6cm), the borders of which having been covered with talc to prevent ants from escaping. During each encounter, one ant of the pair was carefully observed during 5 minutes, and the numbers of times it did nothing (level 0 of aggressiveness), touched the other ant with its antennae (level 1), opened its mandibles (level 2), gripped and/or pulled the other ant (level 3), and tried to sting or stung the other ant (level 4) were recorded (Table 3, line 2; Figure 2, E). The numbers obtained for the ten observed ants were correspondingly added, and the distribution of the values obtained for ants under furosemide diet was compared to that obtained for ants maintained under normal diet using the non-parametric χ^2 test [23]. Also, for each two diets, a variable 'a' assessing the ants' social relationships was calculated: 'a' = the number of aggressiveness levels 2 + 3 + 4 divided by the number of aggressive levels 0 + 1 (Table 3, line 2).

State of stress and cognition through escaping from an enclosure

For escaping from an enclosure, an individual must be calm, must not stress, and must cautiously look for an exit. It must also have some cognitive ability for guessing where the exit may be. Consequently, for comparing the state of stress and the cognitive ability of ants maintained under normal diet then under a diet with furosemide, each time, six ants of each colony were enclosed under an enclosure consisted of a reversed cup (made of polyacetate; height = 8cm, bottom diameter = 7 cm, ceiling diameter = 5 cm) deposited in their foraging area. The inside surface of these enclosures were covered with talc to prevent ants climbing on it. A notch (3 mm height, 2 mm broad) was made in the bottom rim of the enclosure for providing to the ants the possibility to escape (Figure 2, F). As soon as the ants were enclosed, those escaped after 2, 4, 6, 8, 10 and 12 minutes were counted for each two colonies, and the numbers obtained for the two colonies were correspondingly added (Table 3, line 3). The six sums obtained for ants consuming furosemide were compared to the six corresponding sums obtained for ants living under normal diet using the non-parametric Wilcoxon test [23].

Cognition

The ants' cognition was estimated by assessing their skill to cross a twists and turns path. Two pieces (two times 4.5 cm x 12 cm) of strong white paper (Steinbach®) duly folded were inserted in a tray (15 cm x 7 cm x 4.5 cm) what created a twists and turns path between a first 2cm long zone in front of this 'difficult' path and a 8 cm long zone beyond it (Figure 3, A). Such an apparatus was built for each two colonies. To make an experiment on a colony, 15 ants were transferred into the first zone of the apparatus lying in front of the twists and turns path, and since that deposit, the ants still in that first zone as well as those having reached the zone located beyond the 'difficult' path were counted after 2, 4, 6, 8, 10 and 12 minutes. The numbers obtained for the two colonies were correspondingly added (Table 3, line 4). For each of the two zones of the apparatus, the six sums obtained for ants consuming furosemide were compared to those obtained for ants living under normal diet using the non-parametric Wilcoxon test [23].

Visual operant conditioning and memory

For each two used colonies provided with furosemide, a green hollow cube, made of strong paper (Canson®), was deposited at a given time above the entrance of the tube filled of sugar water (Figure 3 Ba). Since this deposit, the ants underwent operant visual conditioning. The experiment on ants living under normal diet was previously performed on another similar colony of *M. sabuleti*, collected on the same site because, since a worker of the ant *M. sabuleti* has acquired conditioning to a stimulus, it keeps its conditioning during several days, and even after having lost its learning, the worker more quickly than usually acquires it again. Consequently, it is no longer possible to quantify again this worker's conditioning acquisition. While the experimented ants acquired their conditioning, then after the removal of the green cube, while the ants progressively lost their conditioning, they were tested in a Y-maze. Each colony had its own maze which was built in strong white paper, had its sides slightly covered with talc, was deposited outside the foraging area, in a separated tray (15cm ×7 cm × 5cm), and was provided with a green hollow cube randomly set in its left or right branch. For conducting a test on a colony, 10 workers were one by one deposited inside the maze before its division into two branches, and the ants' choice of one or the other branch of the maze was recorded (Figure 3 Bb). Choosing the branch which contained the green cube was giving the correct response. After a worker had been tested, it was kept in a glass until 10 ones of its colony were tested, and after the 10 workers were tested, all of them were transferred back in their foraging area. Such tests were performed 7, 24, 31, 48, 55 and 72 hours after the start of the ants' conditioning as well as after the green cube removal (Table 4). The responses obtained for the ants of the two colonies during these twelve tests were correspondingly added and the twelve proportions of correct responses were established. The six proportions obtained while ants acquired conditioning, as well as the six proportions obtained while they lost their conditioning, previously recorded for ants under normal diet and later recorded for ants consuming furosemide were compared using the non-parametric Wilcoxon test [23].

Adaptation (tolerance) to furosemide adverse effects

An individual becomes adapted to a drug when over time it less and less suffers from the adverse effects of the drug. Therefore, to quantify an individual's adaptation to a drug, a side impact of the drug must be quantified soon after the individual has consumed the drug, then again later, after it has consumed the drug during some time, and the results of the two assessments must be compared. In the present work, the ants' sinuosity of displacement appeared to be largely impacted by furosemide consumption. So, to study the ants' potential adaption to side effects of furosemide, the ants' angular speed was again quantified after the ants consumed the drug during eight days. The median and quartiles of the obtained values were established (Table 5 upper part), and the distribution of values was compared to that previously obtained after the ants had consumed the drug during one day as well as to that obtained for ants living under normal using the non-parametric χ^2 test [23].

Ants' potential habituation to furosemide

An individual becomes habituated to a drug when, over its consumption of this drug, it becomes less and less cared thanks to the wanted effect of the drug. Examining such a habituation requires assessing a trait favorably influenced by the drug soon after the start of the drug consumption, then after the drug has been consumed during sometime, and the results of the two assessments should be compared. During the present work, no obvious favorable impact of furosemide on ants' physiological or ethological traits could be observed. Consequently, we could not examine if habituation occurs on furosemide use. However, in humans, habituation may occur and should thus be imperatively detected because, if it occurs, patients will be tempted to increase their intake of the drug, and, doing so, to accentuate the adverse effects of the drug.

Ultimate test

This test was required because furosemide was found to largely increase the ants' sugar water intake, and it was unknown if they did so because they wanted the drug, or sugar, or simply water (let us recall that furosemide is a diuretic). The ants' want of drug was examined thanks to the here below described experiment (see the following subsection). Their want of sugar or of water was examined thanks the present explained ultimate test. For each two used colonies, instead of one tube filled of a sugared solution of furosemide, the ants were provided with two smaller tubes (15 cm × 7 cm × 5 cm), one containing again a sugared solution of furosemide, the other containing a pure water solution of the drug (Figure 3, E). The ants coming onto these two tubes were separately counted every two minutes during forty minutes (so 20 times), the numbers obtained for the two colonies were correspondingly added, and for each kind of count, the sum of these added numbers was calculated (Table 5 middle part). This allowed establishing the proportion of ants having chosen the sugar water on one hand and the pure water on the other hand solutions. Also, the two sums (the sum obtained for each two kinds of water) were compared to those expected if ants randomly visited the two provided tubes using the non-parametric χ^2 goodness-of-fit test [23].

Dependence on furosemide consumption

Dependence on a drug occurs when the consumer wants to have always this drug at its disposal, nearly continuously uses it despite its adverse effects, and finally can no longer live without consuming the drug. The ants' dependence on furosemide consumption was here studied after the ants had consumed this drug during 10 days. To do so, 15 ants of each colony were transferred in an own tray (15cm ×7 cm × 5cm) inside of which two small tubes (h = 2.5 cm, diam. = 0.5 cm) plugged with cotton had been deposited, one tube containing sugar water, the other containing the sugared solution of furosemide used during the entire experimental work. The tube containing the drug was set in the left part of the tray for colony A and in the right part for colony B (Figure 3, F). Half a minute after the ants had been transferred into their tray devoted to the present experiment, those being at the entrance of each provided tube were counted 15 times in the course of 15 minutes. The fifteen numbers obtained for each colony were correspondingly added, and the sums of the added numbers relative to the drug-free tube on one hand and to the tube containing the drug on the other hand were calculated. These two sums allowed calculating the proportions of ants counted in front of each provided tube, and thus the proportions of ants having chosen the drug solution and of ants having preferred the drug-free solution (Table 5 lower part). In addition, these two sums were statistically compared to the two numbers which should have been obtained if the ants randomly went to the entrance of each provided tube using the non-parametric χ^2 goodness-of-fit test [23].

Decrease of the effects of furosemide after its consumption was stopped

This decrease was examined after the ants had consumed the drug for 12 days. The trait used for making this study was the ants' angular speed which appeared to be impacted by furosemide. Twelve hours before starting the study, the ants were provided with a fresh solution of the drug, and after these twelve hours, their angular speed was assessed as it had been after 1 and 8 days of furosemide consumption. However, for this preliminary assessment and for all the following ones (see here below), only 20 ants' trajectories were analyzed instead of 40 in order to be able to assess every recorded ants' trajectories all along the decrease of the effect of furosemide and so, to continuously evaluate the current situation. After this preliminary assessment, made at t = 0, the tubes containing the sugared drug solution were removed and replaced by tubes filled of sugar water. This removal and replacement constituted the weaning of furosemide, and consequently the start of the study. Since that time, the ants' angular speed was assessed every two hours until it became similar to the control one, i.e. to that presented by ants living under normal diet. For each of these assessments, the median and quartiles of the 20 recorded values were established (Table 6), and the distributions of the 20 values recorded every two hours were compared to the preliminary distribution obtained at t = 0, as well as to the corresponding distribution obtained for ants living under normal diet (= the control distribution) using the non-parametric χ^2 test for independent samples (Table 6) [23]. Also, the mathematical function best describing the decrease of the effect of furosemide after weaning was researched (see the results and discussion subsection), and the successive median values of the ants' angular speed obtained over this decrease were graphically presented in figure 4, what allowed visualizing the researched mathematical function.

Results and Discussion

Table 1: Effect of furosemide on the ants' food intake and activity. Lines I to VI give the mean numbers of ants counted, over 6 days, on their meat food, on their sugar water, as well as being in activity. Line I-VI gives the means of these six daily means for each of the three considered physiological traits. Furosemide increased the ants' sugar water consumption (may be the water and/or the drug) and very slightly reduced their activity.

Days	Under normal diet			Under a diet with furosemide		
	Meat	Sugar Water	Activity	Meat	Sugar Water	Activity
I	1.50	3.25	16.50	1.50	11.50	12.50
II	2.38	5.13	16.25	2.00	7.50	13.50
III	1.88	4.38	16.50	1.50	9.00	14.50
IV	2.50	2.50	18.75	2.50	7.75	14.00
V	2.13	2.38	19.00	1.25	8.00	17.12
VI	1.50	2.63	20.25	2.00	8.50	19.75
I-VI	1.98	3.88	16.38	1.79	8.71	15.23

Meat and sugar water consumption, general activity

Numerical results are given in table 1. Furosemide appeared to have no impact on the meat consumption (N = 4, T = +3, -7, P = 0.313), to increase the sugar water intake (N = 6, T = +21, P = 0.016), and to very slightly decrease the individuals' activity (meanly 15.23 *vs* 16.38 active individuals at any time; N = 6, T = -21, P = 0.016). Unfortunately, we could not know if the ants under the drug diet drunk more sugar water because they needed water, or wanted sugar, or were dependant on furosemide consumption. The former possibility was examined in a following experiment (see the subsection relative to an ultimate test), and the latter possibility was examined in a last experiment, before studying the loss of the effect of the drug after its consumption was stopped (see the subsection relative to the ants' dependence on furosemide).

Linear and angular speeds

The ants' linear speed was not impacted by furosemide consumption (χ^2 = 0.62, df = 2, 0.70 < P < 0.80) while their angular speed was significantly increased under this drug consumption (χ^2 = 38.65, df = 2, P < 0.001). This was obvious to observers: the ants moved as quickly as usually, but sinuously, erratically, leaving their head or their gaster from time to time. Such a perturbed locomotion resembled that of ants moving near a source of ethanol [25]. It was examined if, after 8 days under furosemide diet, the ants could adapt themselves to this impact of the drug on their locomotion (see the subsection relative to the ants' adaptation to the side effects of furosemide).

Orientation to a tied nestmate

The ants' orientation capability was not affected by furosemide consumption (Table 2, line 3; Figure 2, A). Under that drug diet, the ants duly, correctly and rapidly went towards a tied worker emitting its attractive pheromone. There was no difference between the orientation values obtained for ants living under furosemide diet and for those maintained under normal diet (χ^2 = 0.78, df = 2, 0.50 < P < 0.70). This is in favor of the drug use.

Audacity

This ethological trait was not impacted by furosemide consumption (Table 2, line 4; Figure 2, B). Ants consuming this drug came onto the presented risky apparatus as often and as cautiously as those living under normal diet (N = 5, T = +9.5, -5.5, P = 0.359). This non impact of the drug is in favor of its use.

Table 2: Effect of furosemide on five ants' ethological and physiological traits. The table gives the median (and quartiles) or the mean [and the extremes] of the data recorded for the five examined traits. Furosemide increased the ants' sinuosity of movement, but did not affect any of the other considered traits.

Traits	Under normal diet	Under a diet with furosemide
Linear speed (mm/s)	11.4 (10.0 - 12.7)	10.7 (9.7 - 11.8)
Angular speed (ang.deg./cm)	133 (121 - 146)	172 (161 - 185)
Orientation (ang.deg.)	34.3 (29.0 - 46.3)	34.5 (26.6 - 50.3)
Audacity (n°)	2.70 [1 - 4]	2.85 [1 - 4]
Tactile (pain) perception:linear speed (mm/s)	5.1 (4.5 - 5.7)	5.2 (5.0 - 6.2)
angular speed (ang.deg./cm) on a rough substrate	266 (250 - 286)	278 (249 - 312)

Tactile (pain) perception

Furosemide did not affect the ants' tactile (pain) perception. This was obvious to observers (Figure 2, C) and was confirmed by the numerical results (Table 2, line 5, 6) and the statistical analysis. Indeed, there was no significant difference between the linear and angular speeds of ants walking on a rough substrate and maintained either under a normal diet or a diet with the drug (linear speed: χ^2 = 1.06, df = 1, P ~ 0.30; angular speed: χ^2 = 2.96, df = 2, 0.20 < P < 0.30). However, the ants consuming furosemide had a somewhat larger angular speed than those living under normal diet because such a difference already existed for ants walking on their not rough foraging area (see the subsection relative to the ants' speeds). The fact that furosemide did not affect the ants' tactile (pain) perception is in favor of its use.

Brood caring behavior

Furosemide did not affect the ants' brood caring behavior, and thus their social behavior (Table 3, line 1; Figure 2, D). While consuming the drug, the ants found the larvae removed from the nest, took care of them, held them in their mandibles and transported them towards the nest, just like ants living under normal diet. In each case, all the observed larvae were re-entered after 4 minutes. There was no statistical difference between the social behavior of ants living under one and the other kind of diet (N = 3, T = 6, P = 0.125).

Social relationships towards nestmates

Furosemide did not affect the ants' behavior towards their nestmates (Table 3, line 2; Figure 2, F). We were surprised by the simi-

Table 3: Impact of furosemide on ants' social interactions, stress and cognition. Explanation of these traits assessments is given in the text. The drug did not affect the ants' brood caring and aggressiveness against nestmates, and thus their social relationships, and somewhat increased their ability in escaping from an enclosure and in crossing a twists and turns path, and thus reduced their state of stress and improved their cognition.

Traits	Normal diet	Diet with furosemide
Brood caring: n° of not re-entered larvae over 5 minutes	t: 30s 1 2 3 4 5 min 8 6 4 2 0 0	t: 30s 1 2 3 4 5 min 8 7 6 3 0 0
N° of aggressiveness 0-4 levels towards nestmates, variable 'a'	levels 0 1 2 3 4 'a' 66 31 10 0 0 0.10	levels 0 1 2 3 4 'a' 61 32 14 0 0 0.15
Stress and cognition: n° of ants escaped over 12 minutes	t: 2 4 6 8 10 12 minutes 0 2 4 6 8 10	t: 2 4 6 8 10 12 minutes 0 2 5 7 11 12
Cognition: n° of ants in front and beyond a twists and turns path over 12 minutes	t: 2 4 6 8 10 12 min in front 20 18 16 14 13 9 beyond 0 1 3 8 10 13	t: 2 4 6 8 10 12 min in front 15 12 11 9 8 7 beyond 1 1 5 8 10 11

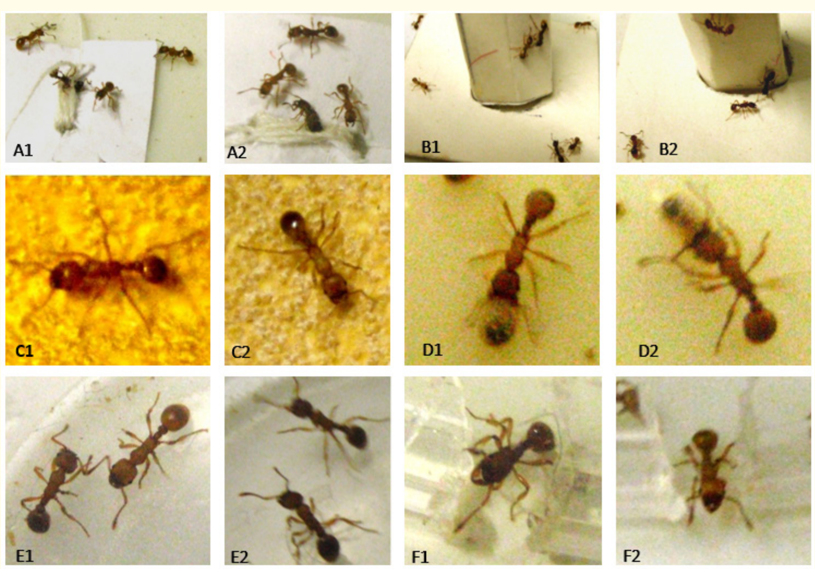

Figure 2: Photos of six experiments made to know the side effects of furosemide. 1: ants under a normal diet, 2: ants under a diet with furosemide. A: ants having duly reached a tied nestmate which emitted its attractive alarm pheromone. B: ants coming onto an unknown risky apparatus. C: ants moving with difficulty on a rough substrate, perceiving thus its uncomfortable character. D: an ant transporting a larva experimentally removed from the inside of the nest. E: two nestmates peacefully staying near each other. F: an ant escaping from an enclosure into which it had been experimentally set. Obviously, furosemide did not affect any of these six examined traits.

larity between the results recorded for ants consuming the drug and those recorded for ants living under normal diet. There was no statistical difference between these two series of recorded data: χ^2 = 0.879, df = 2, 0.50 < P < 0.70. Such a non impact of furosemide on the individuals' social relationships is in favor of its use.

State of stress and cognition through escaping from an enclosure

Unexpectedly, ants consuming furosemide were more able to escape from an enclosure than ants living under normal diet (Table 3, line 3; Figure 2, F), but this difference was at the limit of significance (N = 4, T = 10, P = 0,063). This may be due to the fact that ants consuming furosemide seemed less stressing than ants living under normal diet (personal observation). They duly, calmly walked along the rim of the enclosure, soon found the exit, and went out without hesitating. Also, the cognitive abilities of the ants consuming the drug were probably intact, not impacted by the drug consumption, a presumption checked thanks to the next experiment (see below).

Cognition

Furosemide did not affect the ants' cognition, on the contrary, it seemed to somewhat improve this ability (Table 3, line 4; Figure 3, A). Indeed, the ants consuming this drug and being still in front of the twists and turns path over the 12 experimental minutes were less numerous than those being still there and living under normal diet (N = 6, T = -21, P = 0.016). As for the ants having reached the area lying beyond the difficult path, there was no statistical difference between the ants maintained under one or the other kind of diet (N = 3, T = 6, P = 0.125). Such a result was in agreement with that previously obtained on the ants' escaping ability (see the above subsection), and is in favor of the drug use.

Visual operant conditioning and memory

Furosemide did not impact the ants' conditioning acquisition; it even slightly though not significantly improved this acquisition (N = 3, T = 6, P = 0.125) (Table 4 upper part; Figure 3, B). Also, the drug did not affect the ants' short and middle term memory (Table 4 lower part). The data recorded after the cue removal showed that there was no difference between the responses of ants maintained under a diet with furosemide or under a normal diet (N = 2, NS). Such a non impact of furosemide on the ants' learning and memorization is in favor of its use.

Table 4: Impact of furosemide on the ants' conditioning acquisition and memory. Furosemide did not impact these two important physiological traits, what is in favor of its use.

Time (hours)	Normal diet colony C	Diet with furosemide colonies A; B
7 h	6 *vs* 4 60%	6 *vs* 4; 6 *vs* 4 60%
24 h	6 *vs* 4 60%	7 *vs* 3; 6 *vs* 4 65%
31 h	7 *vs* 3 70%	6 *vs* 4; 8 *vs* 2 70%
48 h	7 *vs* 3 70%	8 *vs* 2; 8 *vs* 2 80%
55 h	8 *vs* 2 80%	8 *vs* 2; 9 *vs* 1 85%
72 h	9 *vs* 1 85%	8 *vs* 2; 9 *vs* 1 85%
cue removal		
7 h	9 *vs* 1 85%	9 *vs* 1; 8 *vs* 2 85%
24 h	8 *vs* 2 80%	9 *vs* 1; 8 *vs* 2 85%
31 h	8 *vs* 2 80%	8 *vs* 2; 8 *vs* 2 80%
48 h	8 *vs* 2 80%	8 *vs* 2; 8 *vs* 2 80%
55 h	8 *vs* 2 80%	8 *vs* 2; 8 *vs* 2 80%
72 h	8 *vs* 2 80%	9 *vs* 1; 8 *vs* 2 85%

Adaptation (tolerance) to furosemide adverse effects

The ants did not adapt themselves to the impact of furosemide on their sinuosity of movement (Figure 3, C). After having consumed the drug during eight days, their angular speed was still larger than that of ants living under normal diet (χ^2 =642.45, df = 1, P < 0.001). Moreover, this sinuosity of movement was even larger than that presented after one day of the drug consumption (χ^2 = 45.83, df = 2, P < 0.001) (Table 5, upper part). The ants' angular speed was thus chosen as the trait affected by the drug for studying its loss of effect after its consumption was stopped (see the subsection relative to this loss of effect). In addition, we observed that the ants' consumption of sugar water remained far larger than the usual one (Figure 3, D), a personal observation not quantified, but examined otherwise, in the here below subsection 'Ultimate test'. The fact that the ants' did not adapt themselves to the side effect of furosemide on their locomotion is not in favor of the drug use.

Ultimate test

The numerical results are given in table 5, middle part, and two photos of the experiment are shown in Figure 3, E. Confronted to

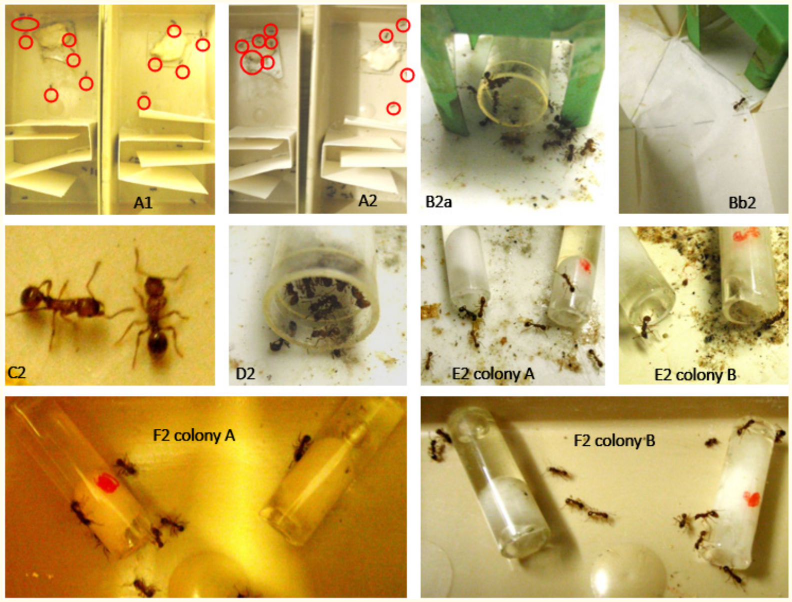

Figure 3: Some photos of six experiments and observations made for examining the side effects of furosemide. 1: ants under a normal diet, 2: ants under a diet with furosemide. A: ants having been able to cross a twists and turns path. B: a: ants' training to a green hollow cube; b: an ant tested in a Y-maze provided with such a cube and giving the correct response. C: ants having consumed the drug during eight days and that on the left having still difficulty walking. D: many more ants as usually drinking the provided sugared solution of furosemmide. E: ants having the choice between a sugared solution of furosemide (red dot) and a pure water solution of this drug, and somewhat preferring the latter one. F: confronted to a drug-free solution and a solution of furosemide (red dot), ants preferring the drug solution, being thus dependent on furosemide consumption.

Adaptation to the side effect of furosemide	Under normal diet	Under a diet with the drug since 1 day	Under a diet with the drug since 8 days
angular speed (ang.deg./cm)	133 (121-146)	172 (161-185)	232 (216-247)
Ultimate test: ants' need of water	pure water solution: colony A: 69 ants, colony B: 23 ants → 56.79% sugared water solution: colony A: 47 ants, colony B: 23 ants → 43.21%		
Dependence on furosemide consumption	drug solution: colony A: 73 ants, colony B: 50 ants → 80.92% drug-free solution: colony A: 15 ants, colony B: 14 ants → 19.08%		

Table 5: Ants' adaptation to the side effect of furosemide on their sinuosity of movement, potential need of water under that drug diet, and dependence on that drug consumption. The ants did not adapt themselves to the side effect of furosemidde; they needed drinking water, but this was not statistically significant; they became dependent on the drug consumption, and this was statistically significant.

a pure water solution and a sugared water solution of furosemide, more ants of colony A preferred the former solution, while ants of colony B similarly went drinking the two solutions. Globally, 56.79% of ants choose the pure water solution and 43.21% choose the sugared water one. There was no statistical difference between such choices and those expected if ants had gone drinking each two presented solutions (χ^2 = 1.24, df = 1, 0.20 < P < 0.30). Nevertheless, the ants rapidly and numerously went onto the two presented solutions, and had some tendency to prefer the pure water solution. They obviously presented some need of water.

Dependence on furosemide consumption

Numerical results are presented in table 5, lower part, and two photos are shown in figure 3, F. The ants developed an obvious dependence on furosemide consumption. During the experiment, 73 ants of colony A and 50 ants of colony B came onto the drug solution, while 15 ants of colony A and 14 ants of colony B went onto the drug-free solution. In total, 80.92% of the tested ants preferred the drug solution, while 19.08% preferred the drug-free solution. These results (123 *vs* 29) statistically differed from those (76 *vs* 76) expected if the ants had equally gone onto the two presented solutions (χ^2 = 30.78, df = 1, P < 0.001). Such dependence is not in favor of furosemide use.

Decrease of the effects of furosemide after its consumption was stopped

Numerical results are presented in table 6 and are illustrated in Figure 4. Note that the value of angular speed obtained at t = 0 was identical to that obtained after the ants consumed the drug during eight days; our assessments were thus reliable. After weaning, the effect of furosemide rapidly decreased, becoming different from its initial one as soon as after three to four hours. It went on rapidly decreasing during 5 to 6 hours, then during two more hours, it more slowly decreased, and finally it very rapidly decreased from 8 to 14 hours after weaning. However, and this is an important observation, furosemide kept an effect statistically different from the control situation until 12 hours after weaning. In other words, even if being soon less active than initially (and this could be perceived by the consumers who may then become dependent on the drug), the drug still kept a valuable effect during 12 hours, and fully lost its effect in a total of 14 hours. The recommended daily dose of 40 mg is thus appropriated, and should not be exceeded for limiting the occurrence of the drug adverse effects. The complex decrease of the effect of furosemide after its consumption was stopped may be due to its chemical structure (Figure 1), and could mathematically be best described by the following quadratic function of the fourth degree:

$E_T = 231.33 - 16.22\ T + 3.15\ T^2 - 0.32\ T^3 + 0.01\ T^4$ with E_T = effect of the drug at the time T, and T = the time in hours.

For example, for T = 2 hours: the effect of furosemide equals:

$E_2 = 231.33 - 16.22 \times 2 + 3.15 \times 2^2 - 0.32 \times 2^3 + 0.01\ 2^4 = 231.33 - 32.44 + 12.6 - 2.56 + 0.16 = 209.09$.

A look at Table 6 and Figure 4 shows that this result is perfectly correct.

Time (hours)	Ants' sinuosity (ang.deg./cm)	Statistics	
		***Versus* t = 0**	***Versus* control**
t = 0	231 (210 - 253)		23.13, 1, <0.001
2 h	210 (199 - 232)	1.02, 2, < 0.70	23.13, 1, < 0.001
4 h	199 (173 - 212)	10.50, 2, < 0.001	16.72, 1, < 0.001
6 h	189 (167 - 197)	14.40, 2, < 0.001	16.72, 1, < 0.001
8 h	182 (152 - 217)	6.27, 2, < 0.05	19.72, 1, < 0.001
10 h	165 (146 - 180)	17.56, 2, < 0.001	14.40, 1, < 0.001
12 h	144 (133 - 179)	17.95, 2, < 0.001	4.84, 1, < 0.05
14 h	130 (117 - 155)	28.60, 2, < 0.001	0.156, 1, < 0.30
Control	133 (121 - 146)		

Table 6: Decrease of the effect of furosemide after its consumption was stopped. The table gives the median (and quartiles) of 20 values of ants' sinuosity recorded at each times, as well as the results of χ^2 tests made to compare these recorded values with those obtained at t = 0 and with the control ones (successively χ^2, df, and P as defined in [23]). The effect of furosemide rapidly decreased in a total of 14 hours; it stayed different from the control situation until 12 hours after weaning, but it already differed from its initial effect as soon as 3 - 4 hours after weaning, what is perceived by consumers and leads to dependence on the drug consumption.

Discussion and Conclusion

The diuretic indapamide having been shown to have perturbing adverse effects [1], we studied the side effects of another kind of largely used diuretic, furosemide. This drug appeared to have far less side effects than indapamide. It did not impact the individuals' activity or their social interactions; it only increased the ants' sinuosity of movement, but it leaded to dependence. Also, it induced need of water and may be of sodium and potassium, and should thus be used for humans under medical supervision. In addition, we here showed that the effect of furosemide rapidly decreased in a total of 14 hours according to a quadratic function of the fourth degree. Below, we provide information on the efficiency of furo-

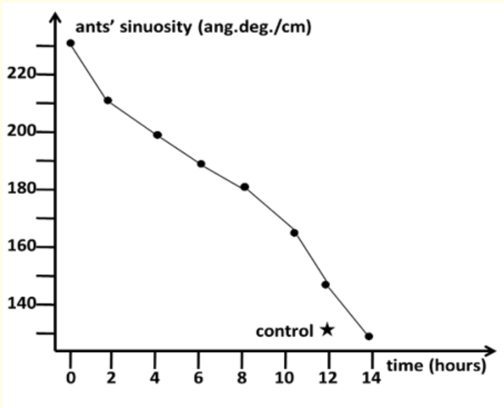

Figure 4: Decrease of the side effects of furosemide after its consumption was stopped. The trait used for making this study was the ants' angular speed. The figure shows that the effect of the drug rapidly decreased after weaning, becoming different from its initial one as soon as after about 3 hours, though staying different from the control situation until 12 hours after weaning. Such a decrease accounts for the development of dependence on furosemide consumption.

semide, its potential adverse effects in humans, and, additionally using our own findings, we conclude.

Furosemide efficiently acts on the ascending part of the Henle's loop, decreasing its recapture of water [26,27]. Patients suffering from edema are thus well cared when using this drug. However, furosemide was found to be sometimes ototoxic, i.e. to lead to deafness, an incapacitating side effect [2,28]. This eventuality must be imperatively taken into account while treating patients with furosemide. A second essential physiological trait which must be closely watched is the amount of sodium and potassium in the blood [27]. These two ions must imperatively be present in adequate amounts and proportions for a proper execution of various functions. In addition, though not reported in the bibliography, but obviously seen during our experimental work, the ants needed to drink water. Consequently, the inside amount of water, the hydrometrics, the hydration of patients cared with furosemide should be carefully and regularly controlled. Also, though once more not reported in the literature, we here revealed that furosemide leaded to dependence; it can thus act as a strong drug, and, according to its few but severe side effects, such dependence may imperil the patients' health. Note that furosemide is considered as being a doping substance for sporty humans (notice for use joined to the drug package and available on internet). We here want to report that we have previously found that dependence on a substance always occurs when the effect of that substance rapidly decreases after weaning [29]. This finding is once more verified in the present work: the effect of furosemide rapidly vanished in 14 hours, becoming different from its initial effect as soon as three to four hours after weaning. However, its effect stayed different from that of a drug-free solution during twelve hours after weaning, what validates its medically advised daily doses.

To finish, let us add that furosemide can be used for experimental medicinal studies, and is thus in fine a useful drug and medicinal substance for the practitioners [30].

To conclude, furosemide is an efficient diuretic and can help lot of patients suffering from edema. However, these patients must be carefully monitored as for five physiological traits: the locomotion, the potential occurrence of deafness, the lack of sodium and/or potassium, some possible dehydration, and the development of dependence on the drug consumption. These five monitoring being carried out, and no more than the smallest effective dose being used, furosemide could surely be an excellent medical diuretic.

Acknowledgements

We are particularly grateful to Mr. Dr Roger Cammaerts who has rapidly and meticulously established the quadratic function of the fourth degree according to which furosemide lost its effect after weaning.

Conflict of Interest

We affirm having no conflict of interest as for the use of the diuretic Furosemide®. We are ethologist, we work on ants and receive no money for making our research.

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