Voluntary intake of paracetamol-enriched drinking water and its influence on the success of embryo transfer in mice

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DOI: https://doi.org/10.1016/j.rvsc.2016.12.005

Originally published at:
Fleischmann, Thea; Arras, Margarete; Sauer, Mareike; Saleh, Lanja; Rülicke, Thomas; Jirkof, Paulin (2017). Voluntary intake of paracetamol-enriched drinking water and its influence on the success of embryo transfer in mice. Research in Veterinary Science, 111:85-92.
DOI: https://doi.org/10.1016/j.rvsc.2016.12.005

Title:

Voluntary intake of paracetamol-enriched drinking water and its influence on the success of embryo transfer in mice

Short title: Paracetamol for embryo transfer

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Abstract

Embryo transfer (ET) in mice is a key technique in biomedical research, and is carried out mostly via surgery by transferring founder embryos into pseudo-pregnant recipient females. To cover post-operative analgesic requirements in surrogate mothers, oral self-administration of painkillers has several advantages, but its effectiveness has also been criticized as voluntary ingestion of the drug can be uncertain. Additionally, concerns about potential negative side effects of analgesics on embryo viability and development have been raised. In this regard, we investigated the impact of orally administered analgesia by comparing the outcome of ET with and without paracetamol in the drinking water (3.5 mg/ml) of surrogate mothers. Water intake increased significantly when paracetamol, as a sweet-tasting formulation (children's syrup), was added to the drinking water. Measurements of paracetamol concentrations in blood serum confirmed reasonable drug uptake. Success rate of ETs and the body weight of newborn offspring were not different whether paracetamol was administered for two days after surgery or not. In conclusion, paracetamol in drinking water was consumed voluntarily in substantial doses, without detectable side-effects, by freshly operated surrogate mothers, and can therefore be recommended as a feasible method for providing analgesic treatment for surgical ET in mice.

Keywords:

Acetaminophen; Paracetamol; Embryo transfer; Water intake; Mice

1. Introduction

The transfer of isolated embryos into pseudo-pregnant surrogate mothers represents a basic routine procedure for establishing new genetically modified mouse lines, and is used routinely for rederivation of pathogen-contaminated lines or revitalization of archived strains. Embryo transfer (ET) in mice is commonly conducted by a surgical approach (Nagy et al., 2003). Therefore, a laparotomy is performed, i.e. the abdominal cavity is opened under sterile conditions, and the oviduct or uterus horns are exposed for the transfer of embryos, aided by visual control using a microscope. Surgical ET is performed under general anaesthesia, and intra- as well as post-operative pain relief in animals undergoing such invasive surgery is an essential refinement to avoid unnecessary pain and suffering of the affected animals.

Recently, new ET techniques, avoiding the need for surgery and post-operative pain treatment, have been published (Bin Ali et al., 2014; Cui et al., 2014; Steele et al., 2013). In these procedures, embryos (only blastocysts are recommended) are transferred with specialized instruments through the cervix into the uterine horn in conscious surrogate mothers. Although the specifically designed devices required are already available on the market, most laboratories prefer a surgical approach, as it allows for reliable evidence of pseudo-pregnancy by the direct observation of a swollen ampulla or corpora lutea in recipient ovaries. Furthermore, the accurate transfer of the appropriate developmental stages into the oviduct or uterus horns can be confirmed visually. Surgical ET has proven to be a reliable and efficient technique for decades. However, when a surgical approach is chosen, post-interventional analgesia needs to be applied for alleviation of post-operative pain, which can persist for 1–2 days after surgery. The choice of an appropriate pain control should take into account an easy and reliable mode of application, preferably

with the longest possible analgesic affect, but without any negative effects on embryo development or gestation.

Regarding any potential influence on the success rate of ET, different effects have been reported for opioid analgesics. While morphine treatment hampered blastocyst implantation and decreased uterine receptivity (Tang et al., 2015), administering a single dose of buprenorphine during ET surgery did not increase embryonic loss compared to untreated animals (Goulding et al., 2010), and the number of successfully implanted embryos was even greater compared to untreated mice (Krueger and Fujiwara, 2008). Also, application of tramadol after ET in mice did not affect success rate outcomes, and may even have improved pup survival as birth rates and body weight in animals receiving tramadol did not differ from untreated animals, whereas the number of offspring was slightly increased in animals treated with this type of analgesic (Koutroli et al., 2014).

Non-steroidal anti-inflammatory drugs (NSAIDs) are generally not recommended for pain treatment during pregnancy (Nolan, 2000). However, in mice, flunixin treatment was not associated with increased embryonic loss after ET (Goulding et al., 2010). In another study, application of tolfenamic acid or flunixin led to a higher pregnancy rate and higher numbers of offspring than in animals undergoing ET without analgesic treatment (Schlapp et al., 2015). A report on multimodal analgesia (recipient female mice received carprofen together with buprenorphine) also showed no significant adverse effects on the results of ET in mice (Parker et al., 2011).

Besides potential side-effects on gestation and embryo development, duration of analgesic action and route of application are the main criteria when choosing an appropriate pain relief protocol for mice. In small rodents, analgesics are applied mainly by intraperitoneal (i.p.) or subcutaneous (s.c.) injection. With opioids,

however, the duration of action is rather short, and injections have to be repeated several times per day to ensure constant analgesic efficacy (Jirkof et al., 2015). Some NSAIDs are known to induce longer lasting pain relief compared to opioids and may need to be injected only once or twice per day (Flecknell, 1984; Miller and Richardson, 2011). However, mice generally experience stress in response to immobilization and injections (Cinelli et al., 2007; Meijer et al., 2005; Meijer et al., 2006). Therefore, oral self-medication represents a promising alternative to provide stress-free post-operative analgesia. The advantages of oral self-administration via drinking water (or food items) are the considerable reduction in stress and potential pain that might be caused by handling and restraining of mice with fresh wounds. However, food neophobia, where animals abstain from the consumption of unfamiliar substances or food, is a well-known behaviour in small rodents (Bauer et al., 2003). Moreover, food or water intake can be decreased after surgery, thus latency to consume analgesics voluntary could be prolonged, resulting in insufficient post-operative pain relief. Consequently, when adding drugs to food or drinking water, it is advisable to examine whether sufficient amounts of the medicated food or water are in fact consumed voluntarily over time.

In human medicine, paracetamol (acetaminophen) has become a popular and widely used non-opioid drug for treatment of fever, as well as for acute and chronic pain management (Allegaert et al., 2014; Mattia and Coluzzi, 2009; Raffa et al., 2004). While the mechanism of action remains partly unknown, selective inhibition of cyclooxygenase enzymes, as well as interaction with endogenous opioid pathways are unique features of paracetamol. Paracetamol is considered to have analgesic and antipyretic, rather than anti-inflammatory, effects compared to typical NSAIDs (Mattia and Coluzzi, 2009). Its intake in therapeutic dosages is generally regarded as safe in a variety of patients, also in pregnant women, where the use of

other NSAIDs is contraindicated due to potential risk to the unborn child (Aminoshariae and Khan, 2015). However, when overdosed, paracetamol can cause liver injuries, triggered by the hepatotoxic effect of its metabolites (Mattia and Coluzzi, 2009).

Paracetamol is also recommended for pain relief in laboratory animals (Flecknell, 1984; Miller and Richardson, 2011). Acetaminophen was shown to increase the pain threshold in rats (Mickley et al., 2006) and to be effective on bone cancer pain (Saito et al., 2005) or to show a potent, synergistic effect when combined with morphine or NSAIDs in mice (Miranda et al., 2006; Saito et al., 2005). The drug can be administered easily by various routes, e.g. by adding to drinking water (Hayes et al., 2000; Mickley et al., 2006). This makes it an ideal drug for broad application in laboratories when opioids are not considered necessary, or are not available.

In the present study, we investigated the analgesic paracetamol as a means of pain management after surgical ET in mice by adding it to the drinking water. The aim of the present study was to determine whether paracetamol in drinking water would be taken up voluntarily by mice in amounts sufficient to cover post-operative analgesic requirements after laparotomy without any detrimental effect on the ET success rate.

2. Materials and Methods

2.1. Ethics statement

Animal housing and the experimental protocols were approved by the Cantonal Veterinary Office, Zurich, Switzerland, and were in accordance with Swiss Animal Protection Law. Housing and experimental procedures were also conform to *European Directive 2010/63/EU of the European Parliament, and of the Council of*

22 September 2010 on the Protection of Animals used for Scientific Purposes and to the *Guide for the Care and Use of Laboratory Animals* (2010/63/EU, 2010; Balinger et al., 2011).

A preliminary investigation was undertaken to exclude adverse effects of a standardized pain treatment protocol with paracetamol in surrogate mothers during ET. Later, at the request of animal welfare officers and authorities, further investigation was performed to confirm the usefulness and reliability of the administration route, i.e. offering the drug for voluntary uptake. Mice used in the present study were surplus animals from our in-house breeding colony. To reduce animal numbers, no dose response studies or analgesiometric testing were conducted. Since experiments were performed at different time points, surrogate mothers or naïve female mice involved in the study varied with respect to their genetic background, i.e. mice of different outbred stocks were used in the two parts of the study.

2.2. Animals and housing conditions

The animal facility provided standardized housing conditions, with a mean room temperature of 21 ± 1°C, relative humidity of 50 ± 5%, and 15 complete changes of filtered air per hour (HEPA H 14 filter); air pressure was controlled at 50 Pa. The light/dark cycle in the animal rooms was set to a 12h/12h cycle (lights on at 07:00, lights off at 19:00) with artificial light of approximately 40 Lux in the cage. Mice were housed in a barrier-protected specific pathogen-free unit and were kept in Eurostandard Type III open-top plastic cages (425 mm × 266 mm × 155 mm, floor area 820 qcm; Techniplast, Indulab, Gams, Switzerland) with autoclaved dust-free sawdust bedding (80–90 g per cage, LTE E-001 Abedd; Indulab, Gams,

Switzerland). A standard cardboard house (Ketchum Manufacturing, Brockville, Canada) served as a shelter, and tissue papers were provided as nesting material. The animals had unrestricted access to sterilized drinking water, and ad libitum access to a pelleted and extruded mouse diet in the food hopper (Kliba No. 3436; Provimi Kliba, Kaiseraugst, Switzerland). To avoid any possible interference from external factors, all necessary husbandry and management procedures were completed in the room at least 1 day before starting the experiment, and disturbances (e.g., unrelated experimental procedures) were not allowed.

The specific pathogen-free status of the animals was monitored frequently and confirmed according to FELASA guidelines throughout the experiments by a sentinel program. The mice were free of all viral, bacterial, and parasitic pathogens listed in FELASA recommendations (Mahler et al., 2015).

For measurements of water intake and paracetamol concentrations in blood serum, 40 female, naïve Crl:CD-1 mice, 8–16 weeks old, were used. Naïve mice were housed in groups of four to eight prior to the study. During baseline measurements and experiments mice were housed individually.

To determine the impact of paracetamol on the outcome of ET, 15 female Zbz:FM mice were used as embryo recipients. The surrogate mothers were 8–16 weeks old when ET was performed. They were housed in groups of two to six animals until mating with vasectomized Zbz:FM males. Mating took place between 16:00 to 17:00 to induce pseudo-pregnancy. Vaginal plug positive females were isolated on the next morning and subsequently housed individually. Two-cell stage embryos were obtained after standard superovulation of B6D2F1 females, mated with Zbz:FM males according to standard protocols (Rulicke, 2004). Briefly, female mice were treated at about 16:00 by intraperitoneal injection of 5 IU pregnant mare serum gonadotrophin (PMSG, Folligon; Intervet, Boxmeer, the Netherlands), followed 48

hrs later by 5 IU human chorionic gonadotrophin (hCG, Pregnyl; Organon AG, Pfäffikon SZ, Switzerland) and mated. About 40 hrs later, treated females were killed by cervical dislocation and two-cell embryos were flushed from both excised oviducts; embryos were stored in an incubator at 37°C, 5% CO2 in air using M16 medium (Sigma-Aldrich, St. Louis, Missouri, USA) until embryo transfer on the same day.

2.3. Experiment set up and data acquisition

The schedule for the experimental procedure of both parts of the study is shown in Fig. 1.

2.3.1. Naïve mice: Water intake and paracetamol in blood serum

Treatment groups:

Forty naïve female mice were randomly allocated into five groups, each group consisting of eight animals: three groups received paracetamol in the drinking water (PW 1–3). In order to compare serum concentrations between voluntary uptake in drinking water and other ascertained administration routes, two further groups received paracetamol either via oral gavage (group G) or via i.p. injection (group I). These two groups, with paracetamol administered as bolus, served as control groups.

Treatment protocol:

Paracetamol was provided in the drinking water according to the recommended published dosage (Flecknell, 2009; Miller and Richardson, 2011). The amount of paracetamol in drinking water was calculated with the intention to provide the mice

with 200 mg/kg body weight (BW) paracetamol over 24 hrs. Assuming that the water consumption of adult outbred mice is at least 3 ml per day, 28 ml paracetamol syrup, formulated to be applied per orally in children (Dafalgan® Children's Syrup, 30 mg/ml; Bristol-Myers Squibb SA, Steinhausen, Switzerland) was diluted in 212 ml tap water, resulting in a final concentration of 3.5 mg paracetamol per ml drinking water. One hour after onset of the light phase (08:00), mice were provided with a freshly prepared bottle of paracetamol-containing water for 6 hrs (group PW1), 11 hrs (group PW2) or 24 hrs (group PW3).

In control group G, the same dose of paracetamol (200 mg/kg BW) (Dafalgan® Children's Syrup, 30 mg/ml; Bristol-Myers Squibb SA, Steinhausen, Switzerland) was given at 12:00 per gavage as bolus with a tube directly into the stomach of the mice. In control group I, the same dose of paracetamol (200 mg/kg BW) was given i.p. at 12:00 by using a formulation intended for injection delivery (Perfalgan®, 500 mg/ml; Bristol-Myers Squibb SA, Steinhausen, Switzerland). Mice in the control groups were provided with untreated drinking water *ad libitum*.

Water intake:

Water intake was determined by weighing the drinking bottle at 6 hrs (PW1), 11 hrs (PW2) and 24 hrs (PW3) after provision of paracetamol-containing water (day 1). Baseline measurements of water intake without paracetamol in the drinking water were taken in the same mice at the identical time points the day before (day 0) (Fig. 1).

Blood sampling and paracetamol serum concentration measurements:

In groups PW1–3, blood was sampled once per animal, either at 6 hrs (14:00; PW1), 11 hrs (19:00; PW2), or 24 hrs (08:00; PW3) after paracetamol was provided for

voluntary intake with drinking water. In control groups I and G, blood was sampled at 2 hrs (14:00) after administering paracetamol in a single dose via gavage (G) or i.p. injection (I). All mice were bled under sevoflurane anaesthesia and killed after the procedure. Blood was centrifuged and the serum stored at –20°C until further analysis. Paracetamol serum concentrations were determined by DRI® Paracetamol-Serum-Tox-Assay by our in-house laboratory (Institute for Clinical Chemistry, University Hospital Zurich, Switzerland).

2.3.2. Surrogate mothers: Water intake and reproductive parameters

Treatment groups:

Fifteen female mice were randomly allocated to either the untreated (n=8) or the paracetamol treated (n=7) group.

Embryo transfer procedure:

After monogamous mating with vasectomized males at 16:00 to 17:00 on the day prior to ET (day 0), females were checked for successful mating on the following morning (day 1) between 07:00 and 07:30 by vaginal plug control. Plug positive females were assumed to be pseudo-pregnant, and were housed individually in fresh cages. At 08:00 on day 1, they received either a fresh water bottle without medication (n=8) or a preemptive bottle with medicated drinking water (n=7) (Fig. 1).

At 13:00 on day 1, pseudo-pregnant females were transferred from the animal room to the nearby laboratory. Anaesthesia and ET were carried out in a biosafety cabinet, which was equipped with a water-bath-heated operating surface (38°C) and

an inhalation anaesthesia device, as described in detail previously (Rulicke, 2004). Briefly, anaesthesia was induced by restraining the mouse and holding its nose in a cone delivering Sevoflurane (≤8% in oxygen at a flow of 200 ml/min). After 15–20 seconds, loss of protective reflexes was checked (e.g., pedal withdrawal reflex) and the back of the anesthetized animal was shaved and disinfected.

ET was conducted bilaterally under aseptic conditions. The skin was cut in the midline of the back, the abdomen was opened by a small incision in the peritoneum near the ovary, and the reproductive tract was pulled out. Six two-cell stage embryos were transferred in M2 medium (Sigma-Aldrich, St. Louis, Missouri, USA) via the infundibulum in the ampulla on each side, so that each recipient received 12 embryos. After placing the tract back in the abdominal cavity, the peritoneum was sutured with absorbable threads and the skin closed with staples. The anaesthetic gas was then stopped and 100% oxygen was supplied to the animal, which subsequently regained reflexes within 2–3 minutes and started to move away from the face mask. Anaesthesia and ET were completed within 15–20 minutes. The animal was allowed to recover for approximately 10 minutes on the warm surface in the biosafety hood, under a filter cup to prevent it escaping. After recovery, the mouse was returned to its cage and brought back to the animal room. Preparation of embryos, anaesthesia and ET was performed by the same technician, who was blinded to the treatment regimens. Animals of the untreated and treated groups were delivered in a randomized manner by the care taker to the lab technician each day. All ETs were completed within a week.

Data acquisition in surrogate mothers:

Water intake was determined at different time points as shown in Fig. 1.

Water bottles were weighed at 08:00 and 13:00 of day 1, at 08:00 of day 2 and at 08:00 of day 3, i.e. 5, 24 and 48 hrs after starting the experiment. Surrogate mothers were monitored for pregnancy after 9 and 12 days of gestation by checking the appearance of the abdominal girth. From day 20 post coitum onwards, they were checked twice daily for birth, and offspring (including still births) were counted. All newborn offspring were weighed using an analytical balance within 12 hrs after birth, and counted daily until weaning at 21 days of age.

2.4. Statistical analyses:

Statistical analyses were performed using SPSS 22 software (IBM, Armonk, NY, USA). All data were tested for normal distribution and homogeneity of variance, and are presented as mean +/- standard deviation.

Baseline and experimental water intake in ml/h of naïve mice was compared with a paired t-test. Water intake of surrogate mothers (in ml/h), as well as litter size and offspring weight after ET, were compared between treatment groups by independent t-tests. Mean serum concentrations of paracetamol were compared between different administration routes with one-way ANOVA. Post hoc analysis with the Games Howell test was carried out to identify significant differences between groups. Significance for all statistical tests was established at $p \leq 0.05$.

3. Results

3.1. Intake of water with and without paracetamol in naïve mice and surrogate mothers

Fluid intake in ml per hour in naïve mice with and without paracetamol in the drinking water is presented in Fig. 2.

340 During baseline measurements, naïve mice drank approximately 4.5 ± 1.46 ml per
341 day in total. Water intake increased significantly when paracetamol was added to
342 the drinking water. Intake of paracetamol-treated water versus untreated water was
343 2.0 ± 0.74 ml vs. 1.2 ± 0.53 ml (p = 0.017) after 6 hrs, 3.4 ± 0.82 ml vs. 1.8 ± 0.13
344 ml (p = 0.001) after 11 hrs, and 5.8 ± 1.40 ml vs. 3.6 ± 0.71 ml (p = 0.001) after 24
345 hrs of administration.
346 Fluid intake in ml per hour in surrogate mothers with and without paracetamol in the
347 drinking water is shown in Fig. 3.
348 Total intake of untreated water within the 5 hrs prior to ET varied between individual
349 animals, ranging from 0.7 to 2.1 ml. The intake of paracetamol-containing water
350 during this period ranged from 1.3 to 2.9 ml. The difference between both groups
351 was non-significant preemptive to ET (p = 0.243). Total water intake after surgery
352 was notably higher with paracetamol treatment, with a significant increase during
353 the first (6–24 hrs, p = 0.023) and the second (24–48 hrs, p = 0.008) day after ET.
354

355 *3.2. Estimated paracetamol intake calculated from water intake*

356 From the amount of paracetamol-containing water consumed by naïve mice, the
357 following consequential doses can be calculated: group PW1 mice consumed 104–
358 357 mg/kg BW (mean 208 ± 90) paracetamol within 6 hrs. In the first 6 hrs, five mice
359 consumed less than the target dose of 200 mg/kg BW, namely 104–177 mg/kg BW,
360 whereas three mice consumed more than the target dose. In group PW 2, mice
361 consumed 273–506 mg/kg BW (mean 351 ± 85) paracetamol within 11 hrs, i.e. all
362 group PW2 mice consumed more than the target dose. In group PW3, mice
363 consumed 331–636 mg/kg BW (mean 517 ± 106) paracetamol within 24 hrs, and
364 calculated doses exceeded target dose of 200 mg/kg BW in all mice. From the

amount of paracetamol-containing water consumed by surrogate mothers, the following consequential doses can be calculated: before ET started, doses of between 154 and 343 mg/kg BW were consumed within 5 hrs (mean 236 ± 68). Two out of 7 animals consumed doses less than 200 mg/kg BW prior to ET, namely 154 mg/kg BW and 170 mg/kg BW. Following ET, in the remaining 18 hrs of day 1, the amount of paracetamol additionally consumed ranged from 379 to 766 mg/kg BW (mean 590 ± 123). On day 2 after ET, doses from 680 to 1077 mg/kg BW (mean 820 ± 132) were consumed within 24 hrs.

3.3. Serum paracetamol concentrations

After 6 hrs, the mean serum concentration of naïve mice receiving paracetamol with drinking water (PW1) was 11.1 ± 3.0 µmol/L (1681.6 ± 460.1 ng/ml). Serum concentrations of naïve mice receiving paracetamol with drinking water were similarly increased after 11 h and 24 h (PW2: 18.3 ± 5.7 µmol/L, 2777.6 ± 870.0 ng/ml; PW3: 18.5 ± 10.7 µmol/L, 2796.5 ± 1620.0 ng/ml). In control groups, the serum concentration was high 2 hrs after bolus application in the injection group (I), with 29.1 ± 8.14 µmol/L (4402.6 ± 1152.3 ng/ml), as well as in the gavage group (G) with 37.5 ± 14.60 µmol/L (5668.5 ± 2208.3 ng/ml).

Mean serum concentrations differed significantly [$F(4.35) = 9.85$, $p \leq 0.0001$]. Post hoc tests revealed significant differences between the i.p. injection group (I) and PW1 ($p = 0.002$), as well as between the gavage group (G) and PW1 ($p = 0.008$) and PW2 ($p = 0.044$).

Individual serum concentrations of mice of different treatment groups are shown in Fig. 4.

3.4. Outcome from ET: comparison of reproductive parameters

The results are summarized in Table 1.

ET was successful in all surrogate mothers of the untreated group, i.e. without paracetamol in the drinking water. All mice became pregnant and delivered litters of 2–6 pups (chronological order: 6, 5, 3, 2, 3, 6, 6, 4).

In the paracetamol-treated group, one recipient was detected not to be pregnant at day 9 and 12 of gestation. We assume that pseudo-pregnancy in this female, although with a vaginal plug, had not been appropriately induced. However, this negative result was included for calculations and analysis. The remaining 6 recipients of the paracetamol-treated group delivered litters of 3–8 pups (chronological order: 8, 3, 6, 6, 6, 3). The treated surrogate mothers delivered on average slightly more pups per litter; however, differences in the final success of ET were not significant ($p = 0.864$).

The body weight of newborns was not significantly different between the two groups ($p = 0.330$). No dead offspring (or parts of pups) were found in cages around the time of birth, and all pups were reared and developed well, i.e. no losses or aberrations of growth or health were noticed at weaning.

4. Discussion

This study found no evidence of adverse effects on gestation or embryonic development after administration of 3.5 mg paracetamol per ml drinking water for 2 days post-surgery. Interestingly, the water intake of surrogate mothers and naïve mice increased when paracetamol was added to the drinking water in the form of a children's syrup. Measurements of serum concentration of paracetamol in naïve mice confirmed substantial drug uptake after 6 hrs preemptive application (i.e. the

approximate time point of the ET), and drug levels increased further after 11 and 24 hrs (i.e. correlating with the post-operative phase after ET). In summary, mice obviously consumed considerable amounts of paracetamol voluntarily with their drinking water before and after surgery, and the outcome of ET was unaffected by the treatment.

Paracetamol, also known as acetaminophen, is one of the most widely used analgesic and antipyretic drugs in human medicine. It is considered safe in therapeutic dosages to treat fever and pain, and is one of the few pain medications recommended during pregnancy (de Fays et al., 2015; Thiele et al., 2013). For pain treatment in adult human patients, dosages of 325–650 mg paracetamol administered per orally or parenteral every 3–4 hrs (max. 4000 mg within 24h) are generally considered to be effective and safe. In laboratory mice, doses of 110–305 mg/kg BW (Fish et al., 2008; Flecknell, 1984; Hawk et al., 2005) have been used for decades. The most common dose recommended by textbooks for pain treatment in mice is 200 mg paracetamol/kg BW (Flecknell, 2009; Miller and Richardson, 2011). According to these recommendations, for our study, the amount of paracetamol in the drinking water was calculated to be 3.5 mg/ml, with the intention to provide the mice with approximately 200 mg per kg BW. This target dose was reached within the first 5–6 hrs in some of the naïve mice and surrogate mothers after providing paracetamol-enriched drinking water. However, several mice stayed beneath the target dose (104–177 mg/kg BW) of 200 mg/kg BW after 5–6 hrs, i.e. just before the intended ET. Low water intake during the pre-operative phase could have been due to the still unfamiliar taste of the water, and to generally lower water intake at the beginning of the light period. Water consumption during the day time tends to be less and more sporadic than during night time due to circadian rhythmicity (Sauer et al., 2016).

After 11 and 24 hrs, all naïve mice voluntarily consumed more than the target dose. The consumption of medicated water also increased in surrogate mothers during the post-surgery treatment phase of 24 and 48 hrs, resulting in an ingested dose significantly higher than the target dose of 200 mg/kg BW (Figs. 2 and 3). This is likely to be attributed to the fact that paracetamol was added to the drinking water as a children's syrup, which, due to its sweet taste, could have stimulated animals to drink more than usual, even after surgery.

It is well known that paracetamol can cause severe liver damage when overdosed. Damage to the liver is not induced by the drug itself but by the build-up of a toxic metabolite due to oversaturated glucuronidation in the liver (Mattia and Coluzzi, 2009). Due to its hepatotoxic characteristics, paracetamol is used widely in experimental models of acute liver injury in mice. According to safety data sheets for paracetamol, the oral lethal dose (LD) 50 in mice is 338 mg/kg BW (see for example www.caymanchem.com/msdss/10024m.pdf). However, it has been reported that experimentally induced liver injury is also sex- as well as strain-dependent (Mohar et al., 2014; Mossanen and Tacke, 2015). Male mice seem to be more susceptible than female mice (Taguchi et al., 2015), and C57BL/6 mice are more responsive than BALB/c (Mossanen and Tacke, 2015). Mossanen and Tacke recommend a dose of 300 mg/kg BW paracetamol with i.p. injection after a fasting period of 12 hrs to reliably induce acute liver injury in mice. Taguchi et al. administered doses of 300 mg/kg BW or 600 mg/kg BW paracetamol, with i.p. injection after 12 hrs fasting to induce liver injury in 4- to 12-week-old mice (Taguchi et al., 2015). Additionally, a recent study showed that pregnant mice were more sensitive to paracetamol-induced hepatotoxicity (Karimi et al., 2015). In this latter study, a dose of 250 mg/kg BW paracetamol administered as a single bolus injection after 16 hrs of fasting at gestation day 12.5 induced hepatocellular injury and

inflammation, while a dose of 450 mg/kg BW induced lethal effects in pregnant but not in non-pregnant mice. Although paracetamol administration did not affect the fetal loss rate, decreased body weights were found in offspring in the prenatal and neonatal stage (Karimi et al., 2015).

As most of the mice in our study voluntarily consumed, at least during the second part of the experiment, higher doses than the target dose of 200 mg/kg BW, and in some cases even more than the highest recommended dose of 305 mg/kg BW, concern regarding potential liver damage or decreased body weight in offspring due to accidental overdosing arises. However, studies by Hayes et al. and Christy et al. revealed no deaths or apparent signs of liver damage or failure even after mice ingested approx. 320–640 mg/kg BW of paracetamol voluntary via drinking water (Christy et al., 2014; Hayes et al., 2000).

To elucidate further the potential for over-dosage and subsequent toxic effects from paracetamol consumption with drinking water in our study, the concentration of paracetamol in blood serum was determined in naïve mice. In both our control groups, after i.p. injection or gavage of 200 mg/kg BW as a bolus, serum concentrations of paracetamol reached 4402.6 ± 1152.3 ng/ml and 5668.5 ± 2208.3 ng/ml, respectively, at 2 hrs after treatment. In contrast, serum concentrations were significantly lower in all drinking water groups compared to our controls. Here, the maximum level of 2796.5 ± 1620.0 ng/ml was noted after 24 hrs (PW3).

In human patients, if plasma concentrations 4 hrs after drug intake are lower than 120023 ng/ml (794 µmol/L), toxic liver effects are unlikely to result. If plasma concentrations are higher than 120023 ng/ml (794 µmol/L), liver insufficiency could occur, and if plasma concentrations are higher than 300057 ng/ml (1985 µmol/L), liver necrosis is likely (DRI® Paracetamol-Serum-Tox-Assay). As data for toxic plasma concentrations in mice are still lacking, we have to rely on data from human

493 studies: In our study, serum concentrations of paracetamol after bolus application
494 as well as after voluntary intake in drinking water, were always far below critical
495 levels from human tox-assays. Moreover, no cases of death occurred, and no
496 obvious aberrations in appearance and behaviour of animals were noticed at regular
497 routine checking. We therefore assume that toxic effects were unlikely at the doses
498 used.
499 Additionally, in our study, doses of up to about 600–1000 mg/kg BW paracetamol
500 per day in the drinking water of mice on days 1 and 2 of gestation did not lead to
501 any significant impairment of our ET success rate. The number of pups born was
502 related to the number of transferred two-cell stage embryos, and was not
503 significantly different between the paracetamol-treated and untreated surrogate
504 mothers. Although one of the surrogate mothers in the paracetamol treated group
505 failed to get pregnant while all untreated animals gave birth, the litters of treated
506 surrogate mothers were on average larger, thus compensating for the lower rate of
507 pregnancy. In addition, the body weight of newborn pups was comparable after
508 paracetamol treatment of recipients at 2 days of gestation. Altogether, our results
509 provided no evidence for any adverse effects of paracetamol treatment on the
510 overall outcome of ET.
511 The observed lack of detrimental effects on animal health and ET outcome may be
512 the result of constant but low intake of the drug via drinking water. Most mice in the
513 present study consumed high levels of paracetamol; however, the animals ingested
514 the medication distributed over a time span of up to 2 days rather than as a high
515 dose bolus after fasting, as carried out in studies to induce liver damage (Corcoran
516 et al., 1988; Karimi et al., 2015; Mossanen and Tacke, 2015; Taguchi et al., 2015)
517 or for traditional LD50 determination. Paracetamol reaches peak concentrations at
518 30–60 minutes after administration, and its half-life in blood plasma is about 2 hrs

519 (Flower et al., 1985; Mickley et al., 2006), thus reducing concerns regarding toxicity
520 in our study.
521 In the present study, the efficacy of paracetamol in regards to post-operative pain
522 relief was not investigated. The focus was rather on whether mice would voluntarily
523 ingest paracetamol-enriched water in amounts sufficient to achieve commonly
524 recommended doses, and whether the drug had any influence on the success rate
525 of ET and offspring survival. Both strains of mice (Crl:CD and Zbz:FM) consumed
526 similar doses of acetaminophen via the drinking water. However, as food and water
527 intake can differ between strains (Bachmanov et al., 2002), the dosage of
528 acetaminophen may also need to be adjusted due to strain variation (Dickinson et
529 al., 2009). Consequently, no evaluation of pain relief can be drawn from the present
530 study, even though plasma levels of paracetamol were comparable to doses
531 effective in analgesiometric tests (Qiu et al., 2007). Future studies are needed to
532 provide evidence for the degree of pain relief after ET with paracetamol, and to
533 elucidate other possible side-effects of the drug when used for this purpose.
534 For transferring this protocol to other laboratories, specific conditions of each
535 country might be considered. It could be necessary to check for availability of
536 acetaminophen and clarify whether a formulation or commercially available drug is
537 permitted by regulative authorities for the use in experimental animals.
538 With regard to surrogate mothers, specifics of strain and age might be considered,
539 although for ET females in a similar age range and mostly outbred strains are used.
540 Thus, differences regarding dose-response and toxic effects might be negligible.
541 This is underpinned by our observation that both outbred strains (Crl:CD and
542 Zbz:FM) consumed similar amounts of water, i.e. doses of acetaminophen.
543 Furthermore, the uptake of paracetamol with the drinking water might be decreased
544 after anaesthesia and surgery, but data obtained in this study and from other

publications (Cesarovic et al., 2010; Sauer et al., 2016) show, that no relevant alteration of drinking behaviour occurred after inhalation anaesthesia with or without surgery. However, in case of doubts regarding uptake of the drug in the immediate post-anaesthetic phase, one may administer the analgesic then as a single bolus-injection to compensate for a suspected delay in drinking after the intervention.

5. Conclusions

In summary, the animals in our study ingested voluntarily substantial amounts of paracetamol with drinking water that allow the assumption of constant post-operative pain treatment. An extension of the preemptive application phase of the medication in the drinking water or a single i.p. injection of paracetamol might be necessary to assure target plasma concentration immediately before, and during the first hours after ET. High doses of paracetamol were reached already several hours after surgery, supported by the increased consumption of medicated water. The animals received their medication without stress through handling, restraint, or manipulation (e.g. frequent injections), all of which could influence their well-being (Jirkof et al., 2015) and possibly adversely affect pregnancy and the outcome of ET. Although substantial doses of paracetamol were consumed within 2 days after surgery, no side-effects on the overall outcome of ET were detected. Therefore, administering paracetamol in drinking water could be a feasible method for providing pain relief in mice undergoing ET.

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Acknowledgements:

The authors would like to thank Cornelia Albrecht for taking care of our surrogate mothers, Robin Schneider, Hugo Battaglia, and the staff of the Central Biological Laboratory for their valuable support, as well as the Institute of Clinical Chemistry, USZ for their generous help and evaluation of samples.

Legends

Fig. 1: Experimental schedule for surrogate mothers and naïve mice.

Measurements of intake of either untreated (n = 8) or paracetamol-containing water (n = 7) in surrogate mothers took place at 13:00 and 08:00 (i.e. after 5, 24, 48 hrs). ET was performed at 5 hrs after the start of the experiment (13:00 – 14:00).

In naïve mice (n = 8 / group), baseline measurements of untreated water intake took place at 14:00, 19:00 and 08:00 (i.e. after 6, 11, 24 hrs) on the first day. On the following day, measurements of paracetamol-treated water intake as well as blood sampling, took place at 14:00, 19:00 and 08:00 (i.e. after 6, 11, 24 hrs).

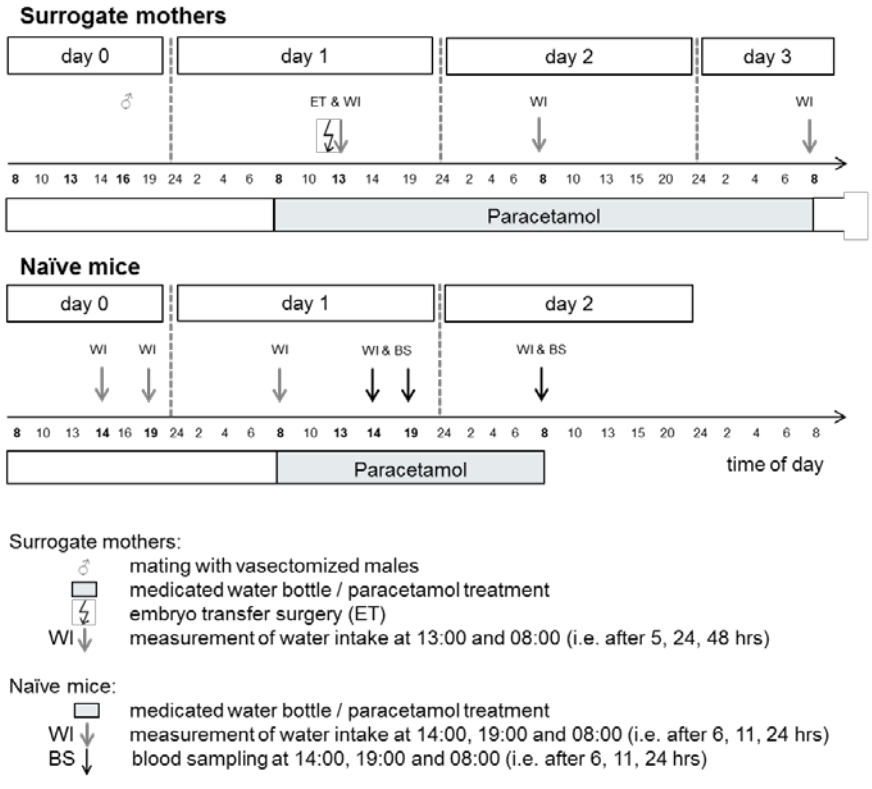

Fig. 2: Comparison of mean water intake per hour with and without paracetamol in naïve mice.

Paracetamol was provided to naïve mice in their drinking water at a concentration of 3.5 mg paracetamol per ml water. Baseline measurements for intake of untreated water were taken the day before. Measurements of water intake was conducted after 6 hrs in PW1, after 11 hrs in PW2, and at 24 h in PW3 (n = 8 / group). Mean values (± SD) of water intake in naïve mice with and without paracetamol in drinking water is traced as ml/h. Bars indicate SD. Significant differences between baseline and experiment were found in all three groups (PW1: p = 0.017; PW2: p = 0.001;PW3: p = 0.001). * p≤0.05 and ** p≤0.01.

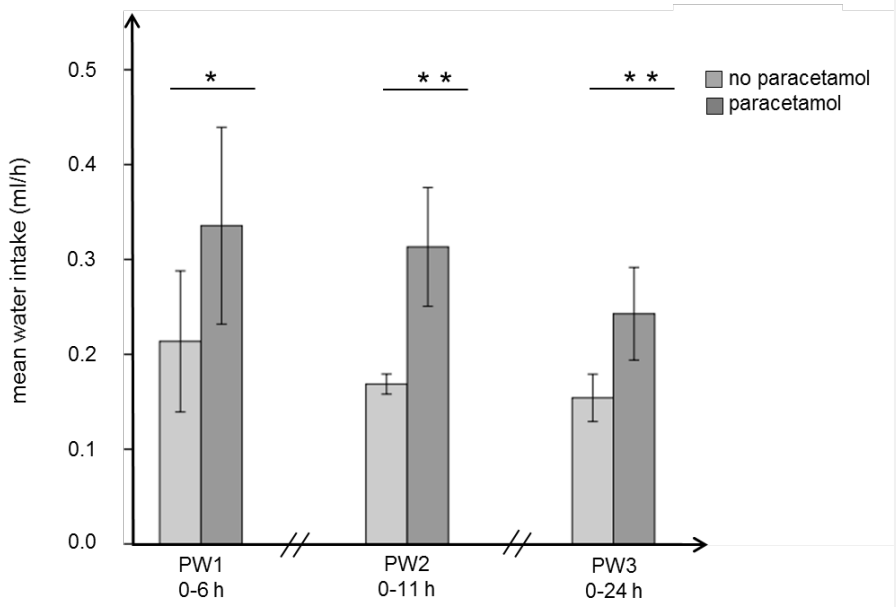

Fig. 3: Comparison of mean water intake per hour with and without paracetamol in surrogate mothers.

Mean water intake in untreated (n = 8) and paracetamol-treated (n = 7) surrogate mothers in the 5 hrs before ET, and during 2 days (≤ 42 hrs) after ET. Water intake was calculated as ml/h. Bars indicate SD. A significant difference was found between treated and untreated groups on the first (18 hrs post ET, p = 0.023) and second (42 hrs post ET, p = 0.008) day after ET. * p≤0.05 and ** p≤0.01.

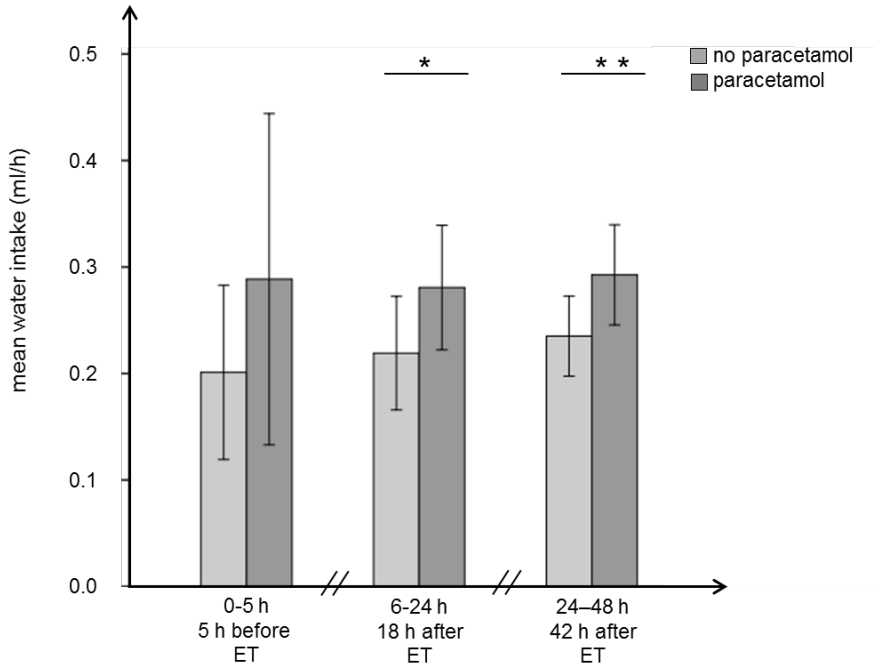

Fig. 4: Individual serum concentrations of paracetamol in naïve mice.

In PW groups, paracetamol was provided to naïve mice in their drinking water at a concentration of 3.5 mg paracetamol per ml water. Blood serum was taken after 6 hrs in PW1, after 11 hrs in PW2, and at 24 hrs in PW3. In control groups, paracetamol was administered as bolus at a dose of 200 mg/kg BW by intraperitoneal injection (I) or gavage (G). Blood was sampled at 2 hrs after bolus application.

Individual serum concentrations for all groups (n = 8 / group) are depicted as one dot for each mouse.

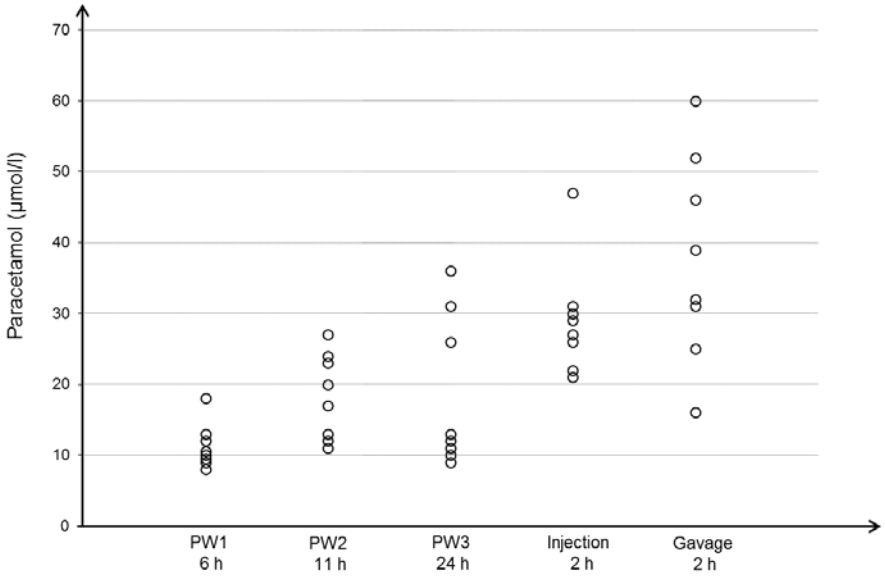

Table 1: Outcome of ET.

One surrogate mother of the paracetamol-treated group was not visibly pregnant and did not give birth, but was included in the calculation of data. Statistical comparison of litter size and offspring weight showed no significant difference whether surrogate mothers received paracetamol or not with their drinking water for 48 hrs (success rate: $p = 0.864$, body weight in newborn offspring: $p = 0.330$).

	without treatment		**with paracetamol**	
number of foster mothers used for ET	8		7	
total number of two cell embryos transferred	96		84	
number of pregnant females at day 9 and 12 of gestation	8		6	
number of litters	8		6	
total number of offsprings	35		32	
mean litter size	4.38 (±1.60)		4.57 (±2.70)	
relation between live offsprings and transferred two cell embryos (success rate)	35/96 (36%)		32/84 (38%)	
mean offspring body weight at birth [g], (±SD)	1.90 (±0.19)	n=35	1.86 (±0.14)	n=32

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