Tamoxifen exacerbates morbidity and mortality in mice receiving medetomidine anaesthesia

Victoria S. Rashbrook[1], Laura Denti[1] and Christiana Ruhrberg[1,2]

[1] UCL Institute of Ophthalmology, University College London, 11-43 Bath Street, London EC1V 9EL, UK

[2] author for correspondence: c.ruhrberg@ucl.ac.uk.

Abstract

Tamoxifen-induced CreER-LoxP recombination is often used to induce spatiotemporally controlled gene deletion in genetically modified mice. Prior work has shown that tamoxifen and tamoxifen-induced CreER activation can have off-target effects that should be controlled. However, it has not yet been reported whether tamoxifen administration, independently of CreER expression, interacts adversely with commonly used anaesthetic drugs such as medetomidine or its enantiomer dexmedetomidine in mice. Here we report a high incidence of urinary plug formation linked to morbidity after ketamine-medetomidine anaesthesia in male mice previously injected with tamoxifen. As dexmedetomidine causes morbidity and mortality in male mice at higher rates than medetomidine, our findings suggest that both medetomidine and dexmedetomidine are both contra-indicated for male mice after tamoxifen treatment.

Introduction

The Cre-LoxP system is often used to induce gene deletion in mice, whereby the P1 bacteriophage Cre recombinase (hereafter referred to as Cre) is inserted together with a promoter into the mouse genome to excise the LoxP- flanked genetic material in cell types that express Cre [1]. CreER is a modified version of Cre, in which Cre is fused to the oestrogen receptor (estrogen receptor, ER) to retain it in the cytoplasm, but CreER translocates to the nucleus after binding the tamoxifen metabolite 4-hydroxy tamoxifen (4-OHT) [1]. Thus, treating mice with tamoxifen or 4-OHT enables temporally controlled gene deletion [2, 3].

Although CreER-mediated gene modification is now a widely used model to study gene function in the mouse, both tamoxifen and CreER induction have been reported to cause toxicity, with the extent of toxicity dependent on the tamoxifen dose, because tamoxifen itself can be toxic at high concentrations, but also because a higher tamoxifen dose induces more CreER nuclear translocation and thus more off-target effects [4-7]. For example, the intraperitoneal injection of

pregnant dams with tamoxifen at embryonic day (E) 9.75 caused a high incidence of limb abnormalities in E17 mouse embryos [8]. Tamoxifen also causes intrauterine haemorrhage and increases the mortality of pregnant mice injected at E5.5 [9], but tamoxifen treatment of non-pregnant mice does not increase mortality [9].

CreER-modified transgenic mice may also be used in procedures that require anaesthesia after tamoxifen administration to induce gene deletion. Thus, the α2-adrenoreceptor antagonist medetomidine or its active enantiomer dexmedetomidine are commonly included in injectable anaesthetic regimes that are used in both veterinary medicine and research studies using live mice [10]. However, it has not been reported whether tamoxifen exacerbates adverse effects of dexmedetomidine or medetomidine. Here we describe urinary plug formation after ketamine-medetomidine anaesthesia in tamoxifen-treated male mice. As dexmedetomidine can cause morbidity and mortality in male mice at higher rates than medetomidine [12, 13], our findings suggest that medetomidine, even though preferable over dexmedetomidine in male mice, is also contra-indicated for male mice after tamoxifen treatment.

Methods

Adult male and female mice (defined as over 2 months of age) on a mixed C57Bl/6J and 129/Sv background were housed in individually ventilated cages and fed with a regular chow diet. All animal work was carried out with a licence provided by the UK Home Office after local ethical and veterinary review. All mice described in this report were originally intended to provide controls for gene deletion experiments, or used to study the effects of gene deletion with Cre or CreER [11]. 7 and 3 days, or 7 and 0 days, or 28 and 14 days prior to anaesthesia, mice were injected intraperitoneally on two occasions with 0.5 mg tamoxifen (Sigma-Aldrich) dissolved in 85 µl or 250 µl vegetable oil (Sigma-Aldrich). This tamoxifen dose was chosen as a reported effective dose to induce genetic deletion in adult mice with *Cagg*-CreER™ [11]. As per local recommended anaesthetic practice to induce deep anaesthesia prior to surgery, mice were then given 75 mg/kg of ketamine (Narketan; Vetoquinol) and 0.5 mg/kg of medetomidine (Dormitor; Vetoquinol), both diluted in sterile water. Intraperitoneal injection of 8.3 mg/kg atipamezole (Antisedan; Zoetis) was used to resolve medetomidine action. Mice were kept on a warming pad until fully recovered from sedation. Mice were returned to their home cages and monitored daily for 7 days after anaesthesia. Mice were monitored daily for clinical signs such as hunching and grimacing, mobility and signs of a hard or distended bladder. Mice were euthanised by an appropriate schedule 1 method at the end of the experiment or upon reaching their humane endpoints. Humane endpoints were defined as an

67 inability to urinate, severely reduced mobility or signs of distress or pain as evidenced by the grimace
68 scale. Statistical analysis was performed using GraphPad Prism v9.2. Survival curves were analysed
69 for difference using a Log-rank (Mantel-Cox) test. P-values of ≤0.05% were considered significant.
70

71 **Results**

72 Adult male and female mice were injected intraperitoneally with 0.5 mg tamoxifen on two occasions
73 prior to anaesthesia with 75 mg/kg ketamine and 0.5 mg/kg medetomidine and the resolving
74 anaesthesia using atipamezole (**Fig. 1A**). We unexpectedly observed that male mice injected with
75 the second tamoxifen dose 0-3 days prior to induction of anaesthesia had a 50% incidence of
76 adverse effects compared to 9.4% in mice that had received their second tamoxifen dose 14 days
77 prior to anaesthesia, and male mice not given tamoxifen had no adverse effects (**Fig. 1B, C**). Severe
78 adverse effects were seen in male mice expressing CreER and in CreER-negative control male mice.
79 Moreover, female mice showed no obvious adverse effects, regardless of their tamoxifen dosing
80 schedule (**Fig. 1B, C**). Mice found at their humane endpoints presented with an inability to urinate
81 when scruffed. Of the male mice euthanised at their humane endpoint, 3 mice were analysed post-
82 mortem for gross abnormalities, and all were found to have distended bladders (**Fig. 1D**). Some
83 mice were found dead, thereby exceeding the pre-specified humane endpoint of the study,
84 requiring the filing of a report sent to the named animal welfare office, local veterinarian and
85 legislator. Specifically, we observed a significantly decreased survival rate up to 72 hours post-
86 anaesthesia in male mice injected with tamoxifen compared to male mice not injected with
87 tamoxifen (**Fig. 1B**). By contrast, female mice did not have decreased survival after tamoxifen dosing
88 followed by anaesthesia (**Fig. 1B**).
89

90 ***Note: This report was produced to document and alert other researchers to the adverse effect of***
91 ***tamoxifen administration prior to medetomidine anaesthesia in male mice. We will not repeat the***
92 ***above experiments to increase statistical power for ethical reasons.***
93

94 **Discussion**

95 Our study has established that the combination of tamoxifen injection with the anaesthetic agent
96 medetomidine increased mortality in male mice 24-72 hours post-anaesthesia. Medetomidine is
97 comprised of a combination of 2 enantiomers, dexmedetomidine and levomedetomidine, whereby
98 dexmedetomidine is thought to be the active form and typically used at half the dose of
99 medetomidine [10]. Both medetomidine and dexmedetomidine have been suggested to be

100 comparable in anaesthetic effect, and each needs to be combined with ketamine for deep
101 anaesthesia [10]. Even though medetomidine and dexmedetomidine are commonly used in both
102 veterinary medicine and research with live mice [10], a literature search after observing adverse
103 effects identified two published studies reporting that medetomidine and dexmedetomidine cause
104 mortality in C57Bl/6J male mice at an incidence of 3% and 67%, respectively [12, 13]. In agreement with
105 the lower incidence of 3% mortality with medetomidine compared to dexmedetomidine, we did not
106 observe any deaths in the small cohort of male mice that had received ketamine-medetomidine but
107 no other intervention. By contrast, we observed increased mortality in 50% of male mice treated
108 with tamoxifen within 3 days and in 9.4% of male mice treated with tamoxifen within 14 days of
109 receiving medetomidine. These findings suggest that tamoxifen exacerbates medetomidine toxicity
110 and that a shorter interval between administering both drugs is the most detrimental.

111 Medetomidine has been used in a range of domestic animal species for veterinary semen
112 collection with a catheter under anaesthesia and has been associated with increased sperm counts
113 [14, 15]. In agreement, the prior study reporting increased mortality in male mice linked this to the
114 formation of seminal coagulum that blocked the urethra [13], presumably because ejaculation was
115 stimulated but the ejaculate was not released or manually removed, as is the case in veterinary
116 practice for semen collection. As previously described for medetomidine-anaesthetised male mice
117 in a study not using tamoxifen [13], the medetomidine-anaesthetised male mice treated with
118 tamoxifen in our study also had distended bladders on post-mortem. Nevertheless, it is unclear why
119 tamoxifen treatment increased the incidence of this medetomidine-induced adverse effect. As
120 tamoxifen treatment in male mice decreases the proportion of mature elongated sperm cells at the
121 expense of rounded, immature spermatogonia in the Sertoli cells [9], it is conceivable that immature
122 sperm cells released into the seminal fluid may increase the incidence of urethral plug formation.

123 It cannot formally be excluded that elements of the anaesthetic regimen other than
124 medetomidine increased mortality in tamoxifen-treated mice, or that mechanisms other than
125 seminal coagulum are responsible; however, both possibilities seem unlikely, because female mice
126 did not show mortality in our study. Moreover, a prior report showed that medetomidine toxicity in
127 male mice was mitigated by replacing medetomidine with xylazine, whilst retaining ketamine and
128 atipamezole in the anaesthetic protocol [13]. Further work would be required to determine whether
129 tamoxifen can also exacerbate the side effects of other anaesthetic regimens.

130 Notably, the mortality and morbidity associated with tamoxifen use prior to medetomidine-
131 based anaesthesia decreased with a longer duration between the last tamoxifen dose and the
132 induction of anaesthesia. Thus, mortality in male mice was reduced from 50% to 9.4% when the last

133 dose of tamoxifen was administered 14 days before anaesthesia, as opposed to 0 or 3 days before
134 anaesthesia. Nevertheless, we consider a 10% mortality incidence unacceptable. An even longer
135 duration between tamoxifen administration and induction of anaesthesia may further reduce the
136 mortality rate but may be unsuitable for some experimental models due to recovery from the
137 intended purpose of tamoxifen administration, which is to induce gene deletion. Thus, a population
138 of recombination-resistant cells may have an advantage over recombined cells and out-compete
139 them (e.g. Fantin et al. Blood, 2013[16]). Therefore, we suggest that alternative injectable anaesthetic
140 formulations not containing medetomidine should be used when using male mice, particularly in
141 combination with tamoxifen administration to induce gene deletion, and when inhalation
142 anaesthesia is unsuitable.
143
144 **Declaration of conflicting interests:** The authors declare that there is no conflict of interest.
145
146 **Funding:** V.S.R., L.D. and C.R. were supported by research grants from the British Heart Foundation
147 [PG/19/37/3439, FS/18/65/34186] and Wellcome [205099/Z/16/Z].
148
149 **Acknowledgements:** Figures were created with a license from BioRender.com.
150
151 **References:**
152 1. Nagy A. Cre recombinase: the universal reagent for genome tailoring. *Genesis* 2000; 26: 99-109.
153 2. Feil R, Brocard J, Mascrez B, et al. Ligand-activated site-specific recombination in mice. *Proc Natl*
154 *Acad Sci U S A* 1996; 93: 10887-10890. DOI: 10.1073/pnas.93.20.10887.
155 3. Metzger D, Clifford J, Chiba H, et al. Conditional site-specific recombination in mammalian cells
156 using a ligand-dependent chimeric Cre recombinase. *Proc Natl Acad Sci U S A* 1995; 92: 6991-
157 6995. DOI: 10.1073/pnas.92.15.6991.
158 4. Loonstra A, Vooijs M, Beverloo HB, et al. Growth inhibition and DNA damage induced by Cre
159 recombinase in mammalian cells. *Proc Natl Acad Sci U S A* 2001; 98: 9209-9214. 2001/08/02.
160 DOI: 10.1073/pnas.161269798.
161 5. Bersell K, Choudhury S, Mollova M, et al. Moderate and high amounts of tamoxifen in
162 alphaMHC-MerCreMer mice induce a DNA damage response, leading to heart failure and death.
163 *Dis Model Mech* 2013; 6: 1459-1469. 2013/08/10. DOI: 10.1242/dmm.010447.
164 6. Brash JT, Bolton RL, Rashbrook VS, et al. Tamoxifen-Activated CreERT Impairs Retinal
165 Angiogenesis Independently of Gene Deletion. *Circ Res* 2020; 127: 849-850. 2020/07/09. DOI:

166 10.1161/CIRCRESAHA.120.317025.
167 7. Rashbrook VS, Brash JT and Ruhrberg C. Cre toxicity in mouse models of cardiovascular
168 physiology and disease. *Nat Cardiovasc Res* 2022; 1: 806-816. 9/9/2022. DOI: 10.1038/s44161-
169 022-00125-6.
170 8. Sun MR, Steward AC, Sweet EA, et al. Developmental malformations resulting from high-dose
171 maternal tamoxifen exposure in the mouse. *PLoS One* 2021; 16: e0256299. 2021/08/18. DOI:
172 10.1371/journal.pone.0256299.
173 9. Ved N, Curran A, Ashcroft FM, et al. Tamoxifen administration in pregnant mice can be
174 deleterious to both mother and embryo. *Laboratory animals* 2019; 53: 630-633. DOI:
175 10.1177/0023677219856918.
176 10. Burnside WM, Flecknell PA, Cameron AI, et al. A comparison of medetomidine and its active
177 enantiomer dexmedetomidine when administered with ketamine in mice. *BMC Vet Res* 2013;
178 9: 48. 2013/03/19. DOI: 10.1186/1746-6148-9-48.
179 11. Brash JT, Denti L, Ruhrberg C, et al. VEGF188 promotes corneal reinnervation after injury. *JCI*
180 *insight* 2019; 4 2019/11/02. DOI: 10.1172/jci.insight.130979.
181 12. Cagle LA, Franzi LM, Epstein SE, et al. Injectable Anesthesia for Mice: Combined Effects of
182 Dexmedetomidine, Tiletamine-Zolazepam, and Butorphanol. *Anesthesiol Res Pract* 2017; 2017:
183 9161040. 2017/02/18. DOI: 10.1155/2017/9161040.
184 13. Wells S, Trower C, Hough TA, et al. Urethral obstruction by seminal coagulum is associated with
185 medetomidine-ketamine anesthesia in male mice on C57BL/6J and mixed genetic backgrounds.
186 *J Am Assoc Lab Anim Sci* 2009; 48: 296-299. 2009/05/30.
187 14. Madrigal-Valverde M, Bittencourt RF, Ribeiro Filho AD, et al. Quality of domestic cat semen
188 collected by urethral catheterization after the use of different alpha 2-adrenergic agonists. *J*
189 *Feline Med Surg* 2021; 23: 745-750. 2020/11/19. DOI: 10.1177/1098612X20973183.
190 15. Zambelli D, Cunto M, Prati F, et al. Effects of ketamine or medetomidine administration on
191 quality of electroejaculated sperm and on sperm flow in the domestic cat. *Theriogenology* 2007;
192 68: 796-803. 2007/07/31. DOI: 10.1016/j.theriogenology.2007.06.008.
193 16. Fantin A, Vieira JM, Plein A, et al. NRP1 acts cell autonomously in endothelium to promote tip
194 cell function during sprouting angiogenesis. *Blood* 2013; 121: 2352-2362. DOI: 10.1182/blood-
195 2012-05-424713.
196
197
198

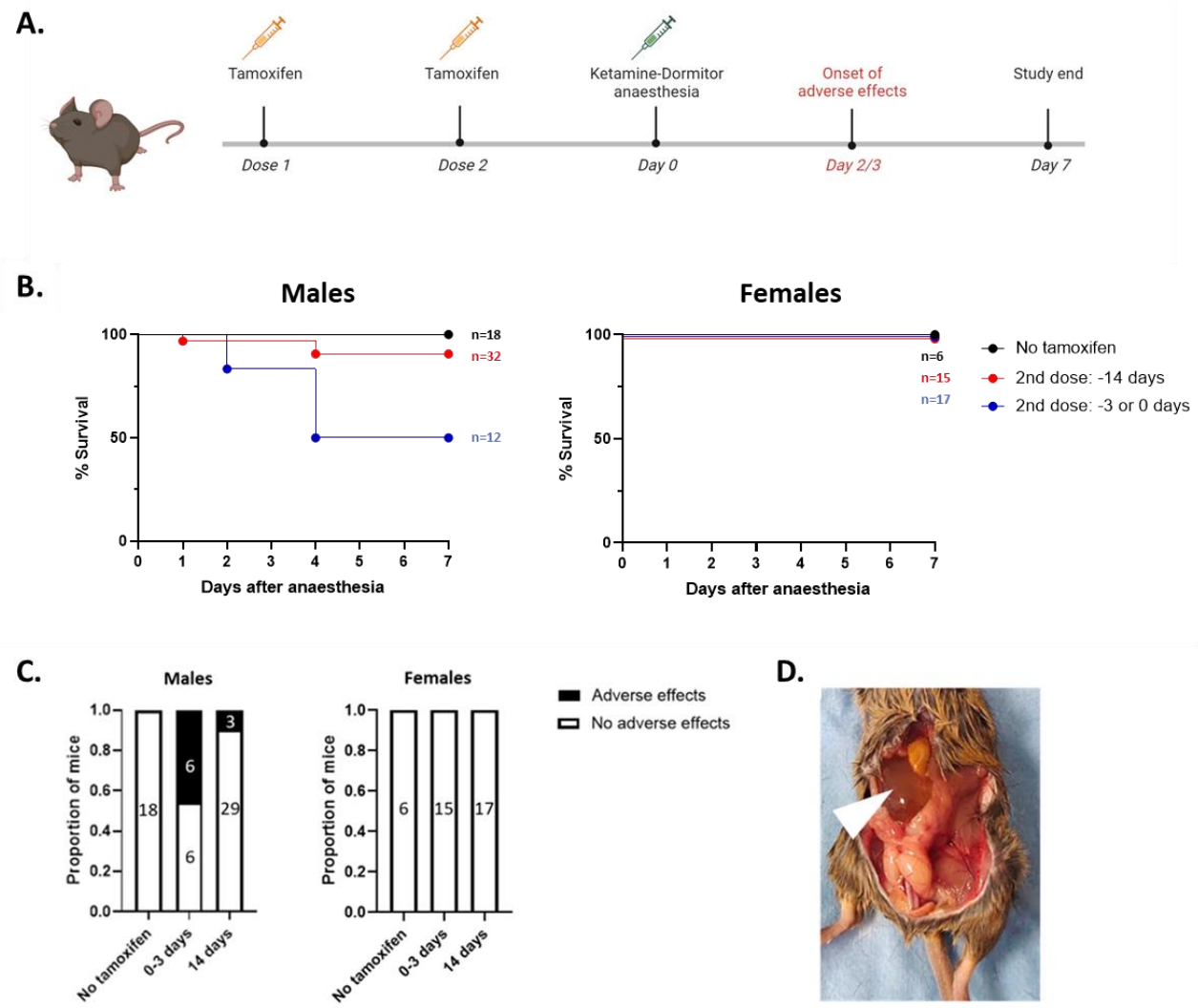

Figure 1: Tamoxifen injection followed by dexmedetomidine-ketamine anaesthesia causes morbidity and mortality in adult male but not female mice.

Mice were either not injected (n = 18 males, n = 6 females) or injected intraperitoneally with tamoxifen (n = 12 males, n = 15 females) prior to anaesthesia with ketamine-dexmedetomidine (n = 32 males, n = 17 females). Mice were monitored daily for signs of adverse effects.

(**A**) Treatment and analysis timeline: In some studies, mice were injected with tamoxifen on day 0 or 3, and on day 7 prior to anaesthesia. In other studies, mice were injected intraperitoneally with tamoxifen on days 14 and 28 prior to anaesthesia with ketamine-dexmedetomidine. Where mice were injected with tamoxifen on day 0, tamoxifen was given prior to anaesthesia. Male mice injected with tamoxifen on days 0/3 and 7 prior to anaesthesia had significantly reduced survival compared to uninjected male mice (6 out 12 mice lost, P=0.0009). Male mice injected with tamoxifen on days 14 and 28 prior to anaesthesia did not have significantly different survival compared to uninjected male mice (3 out of 32 mice lost, P=0.1861).

(**B**) Survival curves of male and female mice up to 7 days after anaesthesia.

(**C**) Proportion of adverse effects in uninjected and tamoxifen-injected male and female mice within 7 days of anaesthesia.

(**D**) Example of a male mouse culled due to severe adverse effects, sprayed with ethanol before dissection to prevent fur shedding; the arrowhead indicates a distended bladder.