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Refinement of Surgical Procedure and Husbandry Practices of Bile Duct Ligation in Mice Model of Cholestatic Disease

R.K. Shakthi Devan[1], Sabarish Babu[1], Vijay Marshal[1], Vijayakumar Kuchibhotla[1], Pravin Hushangabade[2]

[1]Syngene International Limited,
[2]Bristol-Myers Squibb India Pvt. Ltd.
Bangalore, India.

Corresponding author:
R.K. Shakthi Devan,
Biocon-Bristol Myers Squibb R&D Center, Biocon Park,
2 & 3 Jigani Link Road, Bommasandra Phase-IV, Bangalore - 560 099, India.
Phone: +91 80 28084245, Email: sakthi.devan@syngeneintl.com

Abstract

Refinement of procedure plays a pivotal role on improving the well-being of laboratory animals that enables successful outcomes of experiments by obtaining reliable data. In general, identical practices reduce variability among mice and there may be several factors that can confound results which becomes inevitable within the context of long-term experiments. We explored several refinements pertaining to surgical techniques and husbandry practices of ongoing studies with the objectives of reducing early mortality during post-operative period (72h) and ensuring animal welfare as higher priority by providing better alternative husbandry practices until the termination of experiments. The entire refinement process was systematically implemented in mice where surgical intervention of bile duct ligation (BDL) was carried out and allowed to develop liver fibrosis as part of therapeutic requirement then treated with new chemical entities (NCE's) along with standard of care (SOC) upto 14 days. Survival surgery involving large number of mice in different groups was approved by the ethical committee. The notable refinements implemented such as suture patterns along with tissue adhesive to prevent wound dehiscence; replacement of corncob by adopting paper based bedding with nesting pads as enrichments; provision of Individually Ventilated Cages (IVC) with top inlet air supply for uniform flow in order to minimize hypothermia. Other contributing factors which included the use of improved surgical platform with thermo-controlled integral monitoring systems; Provision of infra-red light source immediately after surgery and until recovery; use of food supplement (Diet Gel) along with regular rodent diet; clinical scoring of mice during post-operative care and its management. However, there was no interference on fibrotic disease progression as well as experimental results because these refinements were implemented in a coordinated manner as part of ongoing process. Collectively, refinements of surgical as well as husbandry practices have improved the likelihood of mice and reduced incidences of mortality from 35% to 4% of four subsequent studies conducted at different intervals in mice.

Introduction

The implementation of 3R principle (replacement, reduction and refinement) coined by Russel and Burch has been considered as the essential components of humane treatment of animals in research and gained widespread attention among scientific community (Tannenbaum, 2015). The refinement of experimental procedure provides an opportunity to improve the welfare of laboratory animals (Flecknell, 1994). Although, laboratory animal welfare and ethical practices can minimize experimental variability that eventually improving the validity in biomedical research (Richardson, 2005). It has been emphasized to provide due consideration on humane end points especially in progressive diseases associated with long term experiments where pain and distress becomes inevitable (Franco, 2012). Bile duct ligation (BDL) is a common surgical model employed to induce obstructive cholestatic injury and develop jaundice leading to strong fibrotic reaction in mice (Scholten, 2010), and these ligation of bile duct induces progressive disease and associated with considerable morbidity and mortality (Georgiev, 2008). Moreover, BDL is a painful procedure because abdominal cavity is severed to perform the surgical ligation (Liles, 1993) and uncontrolled pain during post-operative care shown as research interference (Richardson, 2005), in contrast, such kind of pain is not evidenced in human patients. However, it has been recommended to monitor perioperative procedure, particularly until recovery period which is very critical for the successful conduct of study (Flecknell, 2009). Fibrosis is the presence of excess collagen due to new fibre formation that leads to clinical symptoms and eventually causes disturbance of liver cell function (Anthony, 1977). The excessive accumulation of extracellular matrices (ECM's) produced mainly from activated hepatic stellate cells further develops cirrhosis over the period; moreover, the release of proinflammatory transforming growth factor (TGF α) and profibrogenic (TGF β) cytokines in mice plays a key mechanism of fibrogenesis (Weber, 2008; Hara, 2015). The literature survey shown that an increasing number of BDL has been carried out especially with the advent of genetically altered mice strains (Georgiev, 2008; Khan, 2017) and animal model is the gold standard in fibrosis research that prove these findings are highly relevant and can be translated to clinic (Liedtke, 2013). Numerous publications have been reiterated in the context of 3R's by adopting best possible ways of refinements in laboratory animals (Burden, 2018; Graham, 2015). Refinements may include all the aspects of animal use such as enriched housing, habituation, anesthetics, analgesics (Wurbel, 2017) and husbandry practices, improved methodology in invasive techniques, study designs including statistics that can altogether benefit the scientific quality and animal welfare (Llyod, 2007). Considering the above, our objectives were to refine the surgical techniques and husbandry practices to improve the well-being of mice that underwent surgery from the ongoing experimental studies.

Material and Methods

Case Report

The BDC model was under establishment phase especially for liver fibrosis in male C57BL/6 mice (Envigo, The Netherlands) by ligating bile ducts subsequently fibrosis develops over the period and thereafter mice were housed for a period of 14 days. This major survival surgery was performed by laparotomy of mid-ventral approach and post-operative care was provided. In spite of the above, there were mortality during early post-operative period within 72 h (3 days). The observations were discussed among investigators and veterinarians then ruled out the possibilities and decided to prevent such kind of incidence in the forthcoming studies by adopting best possible refinements and/or alternative methods pertaining to surgery as well as husbandry practices.

Husbandry Practices

Syngene Laboratory Animal Research (SLAR) facility at Syngene International Limited (Bangalore, India) has been AAALAC-accredited since 2009 and provides discovery and development supports to various therapeutic areas of pre-clinical research. This barrier facility was built by using clean room panels with epoxy floor and provision of heating ventilation and air conditioning (HVAC) with 15-20 air changes per hour (100% exhaust) controlled by building monitoring system. Photoperiod of light and dark cycles (12:12h) controlled by an automatic timer; provision of autoclaved water, irradiated rodent diet (1320) (Altromin, Germany), bedding (Arbocel-08708 pure cellulose fibre), Germany, Nestlets (Ancare, USA) and supplement (Diet Gel - Clear H_2O, USA) were used. The facility is equipped with dual corridors traffic patterns and two cage washers as well as two autoclaves as redundant backups. The animal use protocol was approved by institutional animal ethics committee (IAEC) for the use of mice including survival surgery, and applicable facility's standard operating procedure, guidelines were followed.

Surgical procedure of BDL

The surgical site was prepared aseptically with povidone iodine and the mice were anesthetized using 2-3 % isoflurane with oxygen as carrier through the calibrated vaporizer system. The mice were placed over the thermo-controlled surgical platform (VetEquip, AF26438) and waste anesthetic gas was scavenged in a scavenging cube (VetEquip). The surgical area was covered with sterile transparent drape and preemptive analgesia (Meloxicam 5 mg/kg b.w.) was given subcutaneously prior to surgery, thereafter every 24h interval for 3 days. A midline ventral incision was made over the skin, thereafter abdominal muscles incision continued upto xiphoid process to identify the bile duct then ligated by using monofilament or braided suture material (Ethicon 5-0). A piece of saline soaked gauze was placed over the liver to maintain moist until the abdominal cavity was closed. The muscle apposition was closed by Vicryl (3-0) and skin was sutured with Silk (3-0) by using simple interrupted pattern followed by Vetbond (3M) tissue adhesive was used for quick opposition of skin to avoid wound dehiscence. The entire surgical procedure was performed in the biosafety cabinet and the animals were transferred to recovery cages where infra-red light (7-10 min) was exposed until its recovery. The mice were group housed with paper based bedding and adequate enrichments provided during post-operative care, thereafter until the experimental period of up to 14 days. Similarly, sham mice also underwent surgery as part of the experiment and dosing was carried out as per the study regimen by investigators. The refinements were followed systematically for BDL surgery of at least 285 mice recruited in four different studies.

Results and Discussion

The BDL in mice were carried out as per the study plan and carefully monitored during intraoperative and until post-operative care. During the entire study process, trained investigators and surgical veterinarians were involved on various activities and ensured that animal well-being was maintained as high priority in line with humane end points. In general, aseptic consideration should be given prior to the surgery, if any improper and/or inadequate procedure can affect the animals by altering their physiological and behavioral responses may lead to infections that eventually interfere with results (Bradfield, 1992; Cooper, 2000). During the process, several refinements have been implemented in order to achieve less mortality without compromising the successful outcomes of experiments (Table 1). The literature survey shown that BDL surgery was conducted in mice for experiments in several institutions (North America - 39%; Europe - 27%; Asia - 24%), and described about anesthetic details and surgical procedure (57%) followed by systematic analgesic agents administration (3.4%) for the use of therapeutic or toxic substance (17.6%) in mice (Secklehner, 2015). At our facility, we used meloxicam (5mg/kg) as preemptive analgesic thereafter until 3 days of post-operative care which was decided by the investigator and veterinarian along with IAEC committee that analgesia was not interfering with this research. However, another investigation confirmed that total bile duct ligation *vs* partial bile duct ligation shown to be comparable in bilirubin, AST and ALP levels during the first week, thereafter, all the aforementioned parameters were increased constantly in tBDL as compared with pBDL shown to be normalized at the end of second week. Furthermore, biliary pressure and serum bile acids have been the predisposing factor of hepatic necrosis showed significantly lower in pBDL than tBDL (Heinrich, 2011).

A report was demonstrated that bile duct-ligated rats were transient, in contrast with fibrotic human patients continue to show progressive disease and become irreversible (Tarcin, 2011). The common bile duct (CBD) ligation in mice showed that early post-operative mortality and the procedure was refined by the investigators by placing a metal clip across lower end of CBD in order to reverse the biliary obstruction. Moreover, the study results revealed that early post-operative survival was 100% (Day 0-2) and 85% (Day 3-5), thereafter survival reversal was recorded as 70% by adopting the refinement (Kirkland, 2010). On the other hand, a comparative methods of three murine model developed for chronic obstructive jaundice such as total ligation of common bile duct (tCL), partial common bile duct ligation (pCL), ligation of left and median hepatic bile duct with gallbladder removal (LMHL) models showed survival rate of 30% (Day 7), 67% and 79%, respectively. The authors described that tCL showed high mortality, whereas pCL may be an appropriate model and LHML model survived high and opined that LHML suitable for long term studies (Aoki, 2016). In addition, a report suggested that soaked diet with warm environment was generally preferred to sick animals and/or unable to access feed (Flecknel, 2009) due to surgical intervention for initial days, the finding was corroborating with our practice that we have offered DietGel to all mice after surgery until 3 days and further duration was decided by surgical veterinarian based on the feed accessibility and health conditions of mice.

We have observed early mortality especially within 3 days of post-surgery and to overcome the situation refined our existing practices in the subsequent studies mainly to avoid hypothermia. The refinement includes, improved surgical platform with thermo-controlled warm water recirculation system while on surgery (Fig 1) and sterile drapes used to cover the surgical areas to avoid hypothermia as BDL surgeries was performed in the biosafety cabinet (Fig 2). Suture patterns (simple interrupted) along with tissue glue (Fig 3) by replacing the wound clips demonstrated reduced wound dehiscence until the termination of experiments. The mice were exposed with infra-red lamp immediately after the surgery to maintain body temperature until its recovery in the designated recovery cages (Fig 4). The provision of individually ventilated cages (IVC) with top inlets avoided direct flow of supply air over the mice and thereby hypothermia incidences were reduced during the post-operative care and helped to maintain the thermoregulation of mice. Apart from the above, corncob was replaced with paper based bedding to avoid prick pain or injury at the surgical sites along with compressed nesting pads to provide warmth and express nesting behaviors were monitored daily twice indicated well-being of mice (Table 1). The routine clinical scoring (appearance, hydration, behavior, and respiration) showed minimal to mild changes of the parameters evaluated (Table 2). A report demonstrated that use of thermoregulatory devices in anesthetized rodents and its effect of maintaining core temperature with consistent on physiologic parameters (Caro, 2013). Although, timely monitoring and attention to problems improves the possibility of a successful surgical outcome (Kuhlman, 2008), and careful consideration may be necessary for fluid replacement on intraoperative therapy depending on surgical intervention and its duration. As a precautionary measure, we administered saline (0.5mL) through subcutaneous route to avoid dehydration prior to the surgery. Additionally, vitamin K1 was given through subcutaneously (40 µg/kg b.w.) (Mennone, 2006) in order to reduce early mortality after BDL surgery in mice. The earlier investigation suggested that administration of Vitamin K1 prolonged the life span of bile duct-ligated rats (Akimoto, 2005), similarly, other investigation revealed that biochemical parameters, fibrotic score and collagen content were significantly reduced by vitamin K1 treatment (Jiao, 2014). It is recommended by guide for the care and use of laboratory animals that veterinarian should be involved in discussions pertaining to the selection of anesthetic agents and doses including perioperative analgesic use, hence, the investigator and veterinarian share responsibility by ensuring that postsurgical care is appropriate (NRC, 2011). Overall, the compiled observations of four ongoing studies and subsequent noteworthy refinements of surgical techniques including husbandry practices, collectively improved the welfare of animals and reduced early mortality up to 72h in mice. Nevertheless, cross functional oversight with a proactive approach by investigators, veterinarians and animal care personnel were enabled to implement timely corrective changes to reduce the incidences of mortality from 35% to 4% (Fig 5) in mice especially during postoperative period of experimental studies.

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References

1. Akimoto T, Hayashi N, Adachi M, Kobayashi N, Zhang XJ, Ohsuga M, Katsuta Y. 2005. Viability and plasma vitamin K levels in the common bile duct-ligated rats. *Exp Anim*. 54(2):155-161.
2. Anthony PP, Ishak KG, Nayak NC, Poulsen HE, Scheuer PJ, Sobin LH: The morphology of cirrhosis: definition, nomenclature, and classification. *Bull World Health Organ*. 1977, 55: 521-540.
3. Aoki H, Aoki M, Yang J, Katsuta E, Mukhopadhyay P, Ramanathan R, Woelfel IA, Wang X, Spiegel S, Zhou H, Takabe K. 2016. Murine model of long-term obstructive jaundice. *J Surg Res*. 206(1):118-125.
4. Bradfield JF, Schachtman TR, McLaughlin RM, Steffen EK. 1992. Behavioral and physiological effects of inapparent wound infection in rats. *Lab Anim Sci*. 42:572-578.
5. Burden N, Chapman K, Sewell F, Robinson V. 2015. Pioneering better science through the 3Rs: An introduction to the National Centre for the Replacement, Refinement, and Reduction of animals in research (NC3Rs). *J Am Assoc Lab Anim Sci*. 54 (2): 198-208.
6. Caro AC, Hankenson FC, Marx JO. 2013. Comparison of thermoregulatory devices used during Anesthesia of C57BL/6 mice and correlations between body temperature and physiologic parameters. *J Am Assoc Lab Anim Sci.* 52 (5): 577-583.
7. Cooper DM, McIver R, Bianco R. 2000. The thin blue line: A review and discussion of aseptic technique and post procedural infections in rodents. *Contemp Top Lab Anim Sci*. 39:27-32.
8. Flecknell PA. 1994. Refinement of animal use-assessment and alleviation of pain and distress. *Lab Anim*. 28; 222-231.
9. Flecknell PA. 2009. Laboratory animal anesthesia 3rd edition. Amsterdam; Elsevier.
10. Franco NH, Correla-Neves M, Olsson IAS. 2012. How "humane" is your endpoint?-Refining the science-driven approach for termination of animal studies of chronic infection. *PloS Pathog*. 8: e1002399.
11. Georgiev P, Jochum W, Heinrich S, Jang JH, Nocito A, Dahm F. 2008. Characterization of time-related changes after experimental bile duct ligation. *Br J Surg*. 95: 646-656.
12. Graham ML, Prescott MJ. 2015. The multifactorial role of the 3Rs in shifting the harm-benefit analysis in animal models of disease. *Eur. J. Pharmacol*. 759: 19-29.
13. Hara M, Inoue I, Yamazaki Y, Kirita A, Matsuura T, Friedman SL, Rifkin DB, Kojima S. 2015. L59 TGF-β LAP degradation products serve as a promising blood biomarker for liver fibrogenesis in mice. *Fibrogenesis & tissue Repair*. 8(17): 2-10.
14. Heinrich S, Georgiev P, Weber A, Vergopoulos A, Graf R, Clavien PA. 2011. Partial bile duct ligation in mice: a novel model of acute cholestasis. *Surgery*. 149(3):445-451.
15. Jiao K, Sun Q, Chen B, Li S, Lu J. 2014. Vitamin K1 attenuates bile duct ligation-induced liver fibrosis in rats. *Scand J Gastroenterol*. 49(6):715-721.
16. Khan Z, Yokota S, Ono Y, Bell AW, Oertel M, Stolz DB, Michalopoulos GK. 2017. Bile Duct Ligation Induces ATZ Globule Clearance in a Mouse Model of α-1 Antitrypsin Deficiency. *Gene Expr*. 17(2):115-127.
17. Kirkland JG1, Godfrey CB, Garrett R, Kakar S, Yeh BM, Corvera CU. 2010. Reversible surgical model of biliary inflammation and obstructive jaundice in mice. *J Surg Res*. 164(2):221-227.
18. Kuhlman SM. 2008. Principles of anesthesia monitoring: Introduction. *J Invest Surg*. 21:161-162.
19. NRC. 2011. Guide for the care and use of laboratory animals. 8th edition, National Academic Press, The Washington DC.
20. Liedtke C, Luedde T, Sauerbruch T, Scholten D, Streetz K, Tacke F, Tolba R, Trautwein C, Trebicka J, Weiskirchen R. 2013. Experimental liver fibrosis research: update on animal models, legal issues and translational aspects. *Fibrogenesis and Tissue Repair*. 6 (19): 1 - 24.
21. Lloyd MH, Foden BW and Wolfensohn SE. 2007. Refinement: promoting the three Rs in practice. *Lab Anim*. 42: 284-293.
22. Liles JH, Flecknell PA. 1993. The influence of buprenorphine or bupivacaine on the post-operative effects of laparotomy and bile-duct ligation in rats. *Lab Anim.* 27: 374-380.
23. Mennone A, Soroka CJ, Cai A, Harry K, Adachi M, Hagey L, Schuetz JD, Boyer JL. 2006. Mrp4 -/- Mice Have an Impaired Cytoprotective Response in Obstructive Cholestasis. 2005 *J Hepatol*. 43 (5): 1013-1021.
24. Richardson CA, Flecknell PA. 2005. Anesthesia and post-operative analgesia following experimental surgery in laboratory rodents: are we making progress? *Alten lab Anim*. 33: 119-127.
25. Secklehner J, Richardson CA. 2015. The reporting of animal welfare details in liver research: A review of studies describing bile duct ligation in mice (2011 - 2013). *J Hepatol*. 62: 238-251.
26. Scholten D, Osterreicher CH, Scholten A, Iwaisako K, Gu G, Brenner DA, Kisseleva T. 2010. Genetic labeling does not detect epithelial-to-mesenchymal transition (EMT) of cholangiocytes in liver fibrosis in mice. *Gastroenterology*. 139: 987-998.
27. Tag CG, Sauer-Lehnen S, Weiskirchen S, Borkham-Kamphorst E, Tolba RH, Tacke F, Weiskirchen R. 2015. Bile Duct Ligation in Mice: Induction of Inflammatory Liver Injury and Fibrosis by Obstructive Cholestasis. *J Vis Exp*. 96: e52438, 1-11.
28. Tannenbaum J, Bennett BT. 2015. Russell and Burch's 3R's Then and Now: The need for clarity in definition and purpose. 54(2):120-132.
29. Tarcin O, Basaranoglu M, Tahan V, Tahan G, Sucullu I, Yilmaz N, Sood G, Snyder N, Hilman G, Celikel C, Tozun N. 2011. Time course of collagen peak in bile duct-ligated rats. *BMC Gastroenterol*. 11(45): 2-7.
30. Weber S, Gressner OA, Hall R, Grunhage F, Lammert F. 2008. Genetic determinants in hepatic fibrosis: from experimental models to fibrogenic gene signatures in humans. *Clin Liver Dis*. 12: 747-757.
31. Wurbel H. 2017. More than 3Rs: the importance of scientific validity for harm-benefit analysis of animal research. *Lab Anim*. 46 (4): 164-166.

Table 1: Optimization of procedures and improvements of surgical outcomes of the studies

Parameters	Assessment of refinements and/or procedures adopted
Surgical Platform and Support systems	Thermo-controlled surgery platform with an in-built warm water recirculation system used for maintenance of body temperature while on surgery.
Skin Suture Patterns	Vetbond (3M) tissue adhesive and simple interrupted suture was used to prevent wound dehiscence by replacing wound clips.
Infrared Lamp	Infra-red lamp heat source used in the home cages immediately after surgery and until recovery in order to reduce hypothermia.
Supplemental Diet	DietGel was provided along with regular chow diet until 3 days of recovery period.
Use of IVC System	Air supply from top of the cages to reduce hypothermia during post-operative period.
Bedding Type	Paper based material (Arbocel) and nesting pads were used by replacing the corncob in order to provide better comfort and warmth after making nesting to maintain thermoregulation.
Clinical Scoring	Nesting pattern, hydration status and well-being of mice were assessed twice daily.

Table 2: Summary of clinical scores during post-operative period in mice

Parameters	Duration	Sham group	Treatment groups
Appearance	24h	0	0
	48h	1	1
	72h	1	2
Hydration status	24h	1	1
	48h	1	1
	72h	2	2
Behaviour	24h	1	1
	48h	2	2
	72h	2	2
Respiration	24h	1	1
	48h	1	1
	72h	1	2
Nesting Pattern (%)	24h	10	12
	48h	25	30
	72h	40	45
Note - n=12 mice per group; clinical scoring monitored twice daily from 4 studies consisting of 280 mice after BDL surgery.			

Scoring criteria:

Appearance - Normal-0, Lack of grooming-1, Porphyrin staining-2, Piloerection-3

Hydration - Normal-0, Slightly dehydrated-1, Moderately dehydrated-2, Severely dehydrated-3

Behaviour - Socially interactive-0, Hyperactive-1, Decreased mobility-2, Increased isolation-3, Vocalisation-4, Ataxia-5

Respiration -Normal pattern-0, Increased-1, Increased with abdominal-2, Labored-3, Agonal-4

Nesting Patterns - Excellent >80%; Good 50 - 80%; Fair 30-50%; lack of well-being < 30%.

Figure 1: Surgical platform with thermo-controlled warm water recirculation set up and active scavenging system

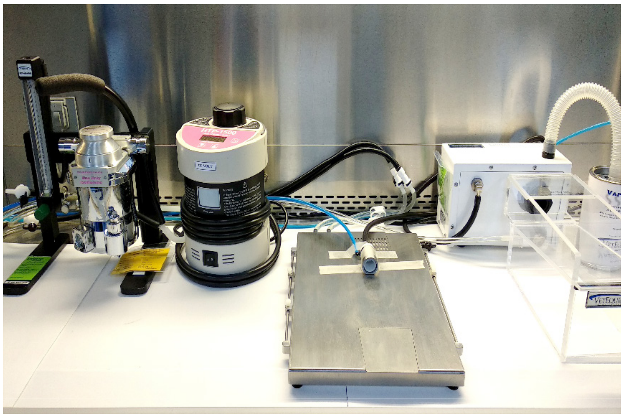

Figure 2: Midventral laparotomy incision and sterile adhesive drape used during surgery to prevent hypothermia

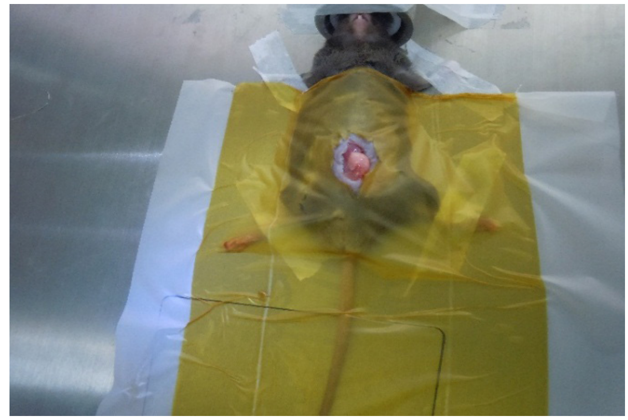

Figure 3: Skin sutured with Silk 3-0 and Vetbond adhesive applied over the surgical incision site to prevent wound dehiscence

Figure 4: Mice exposed under Infra-Red heat source after the surgery to prevent hypothermia until the recovery

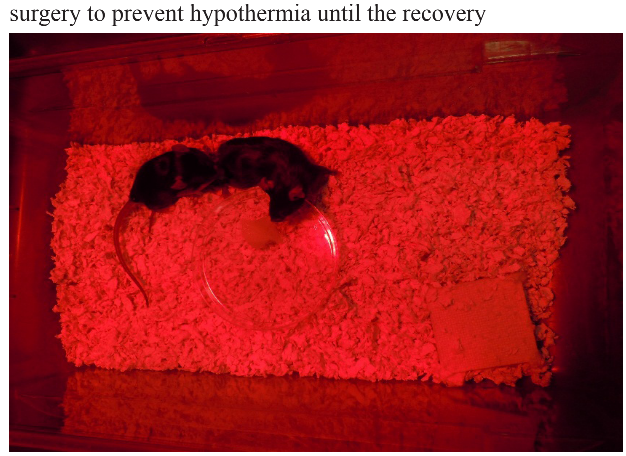

Figure 5: Summary of death incidences during post-operative care (up to 72h) of ongoing studies after BDL surgery in mice