


Evaluation of direct intramural injection to the bladder wall as a method for developing orthotopic tumor models

Masoud Bitaraf¹  | Samad Muhammadnejad² | Ashkan Azimzadeh¹ |
Saman Behboodi Tanourlouee¹ | Erfan Amini³ | Masoumeh Majidi Zolbin¹ |
Abdol-Mohammad Kajbafzadeh¹

¹Pediatric Urology and Regenerative Medicine Research Center, Gene, Cell and Tissue Research Institute, Tehran University of Medical Sciences, Tehran, Iran

²Gene Therapy Research Center, Digestive Diseases Research Institute, Tehran University of Medical Sciences, Tehran, Iran

³Uro-oncology Research Center, Tehran University of Medical Sciences, Tehran, Iran

Correspondence

Abdol-Mohammad Kajbafzadeh, No. 62, Dr. Gharib's Street, Keshavarz Boulevard, Tehran, Iran.
Email: kajbafzd@sina.tums.ac.ir

Funding information

Tehran University of Medical Sciences and Health Services, Grant/Award Number: 98-3-101-45499

Abstract

Background: Bladder cancer poses a great burden on society and its high rate of recurrence and treatment failure necessitates use of appropriate animal models to study its pathogenesis and test novel treatments. Orthotopic models are superior to other types since they provide a normal microenvironment. Four methods are described for developing bladder cancer models inside the animal's bladder. Direct intramural injection is one of these methods and is widely used. However, its efficacy in model development has not yet been studied. We aimed to evaluate the efficacy and success rate of the direct intramural injection method of developing an orthotopic model for the study of bladder cancer.

Method: Tumor cell lines were prepared in four microtubes. Aliquots of 200×10^3 cells were injected through a 27 gauge needle into the ventral wall of the bladders of 4 male and 4 female BALB/c mice following a midline 1 cm laparotomy incision. In addition, 1 million cells from each microtube were injected into the flanks of control mice. To prevent infection and alleviate pain, 5 mg/kg enrofloxacin and 2.5 mg/kg flunixin meglumine, respectively, were injected subcutaneously.

Results: Tumors formed in all mice, resulting in 100% take rate and zero post-operation mortality. Surgery time was ≤ 15 min per mouse. In two mice, tumors were found in the peritoneal space as well.

Conclusion: Direct intramural injection is a rapid, reliable, and reproducible method for developing orthotopic models of bladder cancer. It can be done on both male and female mice and only requires readily available surgical tools. However, needle track can result in cell spillage and peritoneal tumors.

KEYWORDS

animal model, bladder cancer, bladder wall injection, model evaluation, orthotopic model

This is an open access article under the terms of the [Creative Commons Attribution-NonCommercial-NoDerivs](https://creativecommons.org/licenses/by-nc-nd/4.0/) License, which permits use and distribution in any medium, provided the original work is properly cited, the use is non-commercial and no modifications or adaptations are made.

© 2022 The Authors. *Animal Models and Experimental Medicine* published by John Wiley & Sons Australia, Ltd on behalf of The Chinese Association for Laboratory Animal Sciences.

1 | INTRODUCTION

Bladder cancer is the fourth most common cancer globally and the eighth cause of cancer death among men, leading to great morbidity.¹ Its high recurrence rate and high treatment failure rate, as well as the limited treatment options, necessitate better understanding of its molecular-biology basis and pathogenesis, and development of novel therapeutic options. Progress in these areas depends upon development of realistic and relevant models.²⁻⁴

Various in vitro and in vivo models of bladder cancer have been developed. In vitro models are easily established and inexpensive. Although they provide information on mutagenesis and developmental mechanisms, they cannot reproduce the natural micro-environment and 3-dimensional (3D) interactions, and hence do not constitute an ideal model. The only exception to this is tissue engineering models, which are expensive and require specialized equipment and expertise. In vivo models better recapitulate the 3D microenvironment and various cellular interactions, but they are more expensive, time-consuming, and difficult to develop, and raise accompanying ethical issues.^{4,5}

The ideal animal model must be translatable to humans, mimic the histopathology and natural progression of the original tumor, and be easily reproduced.⁶ Orthotopic models, which result from transplantation of cancer cells to the bladder, are closest to this ideal model.⁴ To date, four methods for development of orthotopic bladder cancer models have been reported.^{7,8} After our failed attempt to develop a patient-derived xenograft model of bladder cancer using the direct intramural injection of cancer cells, one of the most widely used methods, we aimed to evaluate the validity of our approach. We therefore utilized a cancer cell line with 100% tumor take rate to evaluate the efficacy and success rate of the direct intramural injection technique, via histopathological assessment, in developing orthotopic bladder cancer models. This paper reports the efficacy and success rate of this technique and discusses and compares the available methods.

2 | METHODS

This study was conducted in accordance with the *Guidelines for the Care and Use of Laboratory Animals in Iran*⁹ and was approved by the

Research Ethics Committees of School of Medicine, Tehran University of Medical Sciences (Approval ID: IR.TUMS.MEDICINE.REC.1398.923).

2.1 | Animals

Ten young BALB/c (5 female and 5 male) mice were used in our study. They were maintained in our research center animal room under standard conditions, with a 12h dark/light cycle and free access to food and water.

2.2 | Cell culture and suspension formation

The anaplastic carcinoma-forming triple-negative murine BALB/c mammary gland cancer cell line 4T1 was selected due to its high tumorigenicity of 100% tumor take rate, hormonal independence, its epithelial origin, and lack of a syngeneic bladder cancer cell line.¹⁰ Cells were cultured in Dulbecco's Modified Eagle's medium (Gibco®; Thermo Fisher Scientific Inc.) plus 10% fetal bovine serum (Gibco®) and 1% penicillin-streptomycin (HyClone; GE Healthcare Life Sciences) and stored in 37°C in a humidified, 5% CO₂. Cells were not allowed to exceed 80% confluence. They also were routinely tested for mycoplasma contamination.

A suspension of cells was prepared by trypsinizing (Gibco®) and centrifugation of cells. The cells were suspended in phosphate buffered saline (PBS) with final concentration of 10 million cells per mL. Suspensions were stored and transferred to surgical room in 0.5 ml volumes in microtubes.

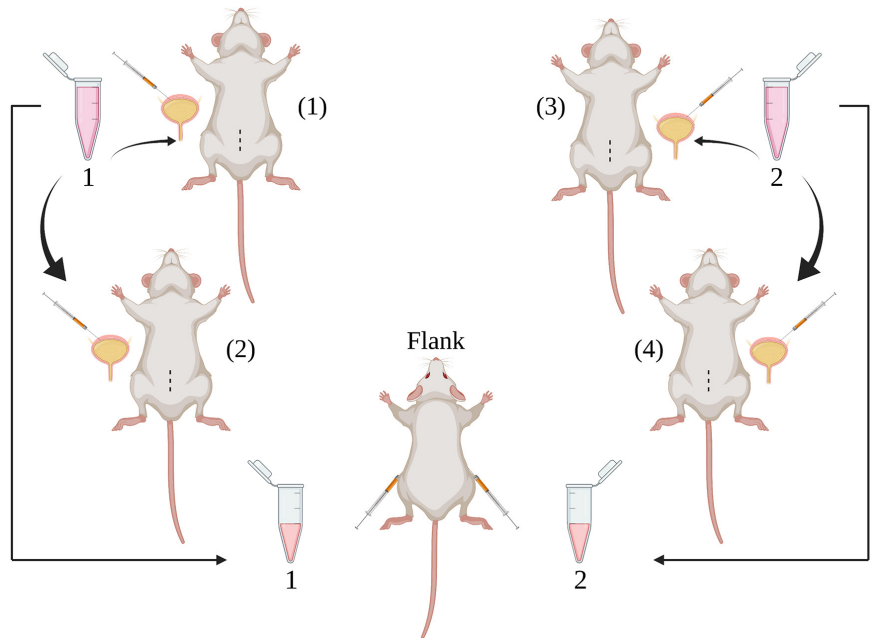
2.3 | Orthotopic tumor implantation

The procedure used in this study was first explained by Dinney et al.¹¹ Each mouse was anesthetized by intraperitoneal injection of 100mg/kg ketamine and 5 mg/kg xylazine. Abdominal skin was shaved and cleaned with povidine-iodine. The mouse was then placed on a surgical board on a heating pad, limbs were fixed using adhesive tape, and eyes were covered with ophthalmic ointment. Prep and drape was done. As shown by [Figure 1](#), a midline 1 cm incision was made at pubic symphysis level. The linea alba was incised



FIGURE 1 The direct intramural injection method. (A), The bladder is exteriorized through a 1 cm midline laparotomy incision. (B), Superficial subserosal injection. (C), Deep intramural/submucosal injection.

FIGURE 2 A microtube containing a tumor cell suspension is prepared for injection into the bladder of two mice. The remaining suspension is injected into one flank of a control mouse. Created with [BioRender.com](https://www.biorender.com)



and the bladder was exteriorized. The bladder dome was grasped with forceps and pulled out so that the ventral wall of the bladder faced upward. There is no difference in technique between an empty and a filled bladder, but it may be easier to empty the bladder to some extent by gently compressing it.

A cell suspension was triturated and a 1 ml tuberculin syringe was filled with the homogenous suspension. Twenty microliters of cell suspension (200×10^3 cells) was injected into the ventral wall, away from ureteral orifices, to avoid causing early obstruction. Based on the Law of Laplace, we used a 27 gauge needle to reduce the chance of cell damage. However, in comparison to a 30 gauge needle, this results in greater chance of canalization and spillage as indicated by Fu et al.¹² Figure 1B,C compares superficial (subserosal) and deep (intramural/submucosal) injections. Though deep injection does not guarantee submucosal placement of cells, it better recapitulates the natural process of tumor formation and invasion of muscle. In superficial subserosal injection, the needle is clearly visible and only covered with a transparent layer of serosa. However, in deep injections, the needle cannot be seen clearly as it is covered with muscle fibers.

The linea alba and skin were subsequently sutured using 5–0 Polyglactin 910 (Ethicon Inc., Johnson and Johnson) suture. To prevent infection and alleviate pain, 5 mg/kg enrofloxacin and 2.5 mg/kg flunixin meglumine, respectively, were injected subcutaneously. In addition, 10 ml/kg of sterile normal saline was injected subcutaneously to prevent post-operative dehydration, since the animal may not be able to stand properly and consume water in the first hours after operation. The mice were placed under a warmer and observed until full recovery.

2.4 | Accuracy check interventions

The accuracy of the injection can easily be tested and validated by injection of 40 μ l of Methylene Blue and concurrent catheterization

and washing of the bladder in a female mouse, using a 24 gauge intravenous catheter lubricated with lidocaine gel. If the injection is accurate, bladder washing using normal saline results in excretion of clear liquid. Also, the injected wall appears blue and its color does not change after washing. However, if the needle has perforated the wall and entered the lumen, washing results in excretion of blue liquid and fading of the wall color.

A new microtube containing a tumor cell suspension was delivered from the culture lab to the surgery room for each two mice. As shown by Figure 2, after injection of the two experimental mice was completed, 100 μ l (1 million cells) of the remaining suspension was injected subcutaneously into one flank of the control mouse to confirm the health and tumorigenicity of the cells.

2.5 | Animal welfare

Sunflower seeds were provided regularly as a compensatory measure for the pain and discomfort caused. The animal cages were enriched by adding paper rolls.

2.6 | Follow-up, regular examinations, and humane end-point criteria

Female and male animals were kept in two separate cages. On even days post-operation, they were weighed; and the surgery site, activity, food and water consumption, reflexes, general appearance and self-hygiene, and urination (for anuria or hematuria) were assessed. The animals were euthanized by ketamine overdose if the following was observed¹: weight loss >20% compared to pre-operation (day 0) value, or² concurrent existence of two or more indicators of illness, or a tumor size of ≥ 20 mm (in flank mice the additive value of

two tumors). Implanted animals that did not meet these criteria underwent daily assessment. All remaining animals were euthanized on day 21 post-operation at the end of the study.

2.7 | Tissue harvest and evaluation

The euthanized animals that underwent bladder wall injection were dissected and the bladder was placed in 10% formalin before paraffin-embedding and sectioning. Sections were stained using hematoxylin and eosin (H&E) for histopathology assessment. If ascites occurred, a smear with Papanicolaou staining was obtained.

3 | RESULTS

Tumors formed in all mice (4 females and 4 males) that underwent intramural cell line injection. The procedure resulted in 100% tumor take rate and zero post-operation mortality. The whole process was performed in less than 15 min per mouse.

Figure 3 demonstrates the gross appearance of the tumor formed in the bladder and histopathological slides of carcinoma cells. Tumors developed between muscle fibers (intramural) or in the submucosal space. Although extension to the serosa was seen, no invasion of adjacent organs was noted.

The relative weight of the mice is charted in Figure 4. On the 12th day, a weight loss of >20% was seen in female mouse No. 2, so it was euthanized and a urinary obstruction was found to be the cause of morbidity. Another female flank-injected mouse lost 15%–20% body weight on day 12 and so underwent daily examination. On day 14, it was euthanized after a deterioration in its general condition. We did not measure the *in vivo* tumor size or weight. The tumor was found in the left flank only, and not in right flank (Figure 5A), and ascites was observed. Fluid was aspirated and a smear was prepared using Papanicolaou stain (Figure 5B). Multiple tumors formed in the peritoneal space after the needle was misplaced in the peritoneum

during subcutaneous injection into the right flank, resulting in peritoneal carcinomatosis.

In the course of the frequent examinations of the mice, male mice Nos 2 and 3 were found to be in moribund state on days 16 and 18, respectively. They were euthanized and urinary obstruction at bladder level, secondary to the tumor, was observed. Male control mouse showed tumors in both flanks (Figure 5C). Multiple tumors were seen in the peritoneal space of 1 male and 1 female mice with the lowest initial body weights.

4 | DISCUSSION

Four principal methods are described in the literature for the development of orthotopic models of bladder cancer. Table 1 summarizes the advantages and disadvantages of these methods.^{7,8}

In this study, we employed direct bladder wall injection of cancer cells and showed that this method results in tumor formation in all the animals that underwent surgery. Normal urothelium hinders cancer cell attachment, presumably through uroplakins and its glycosaminoglycan lining. With direct bladder wall injection, this barrier is bypassed, and hence higher rates of tumor formation are seen.¹³ The urothelium barrier also necessitates physical, electrical, or chemical irritation before instillation of cancer cells using the transurethral catheterization method. This method requires a longer operative time per mouse, partly due to the time needed for irritation. So direct injection also has the advantage of a shorter operative time (≤ 15 min for each mouse).¹⁴ Several factors, other than the normal urothelium barrier, affect the success of tumor formation. These include the aggressive nature of the tumor cells or the take rate of the cell lines used, the number of live tumor cells administered, the method of model development, and the expertise of the operator.

The pathogenesis of bladder cancer begins at in the epithelial layer. Around 70% of cases present with superficial non-muscle-invasive bladder cancer (NMIBC). Only methods with instillation

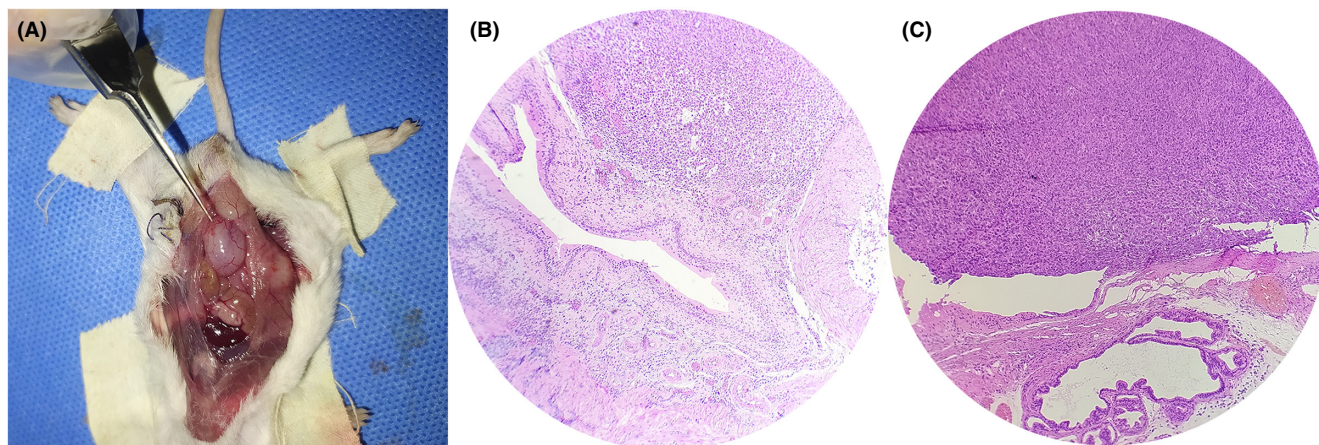


FIGURE 3 Undifferentiated carcinomas formed in the bladder wall. (A), The tip of the forceps points toward the bladder with the tumor. (B), Histopathological slide showing carcinoma in submucosal space. (C), Tumor cells have invaded the serosa but did not invade the prostate.

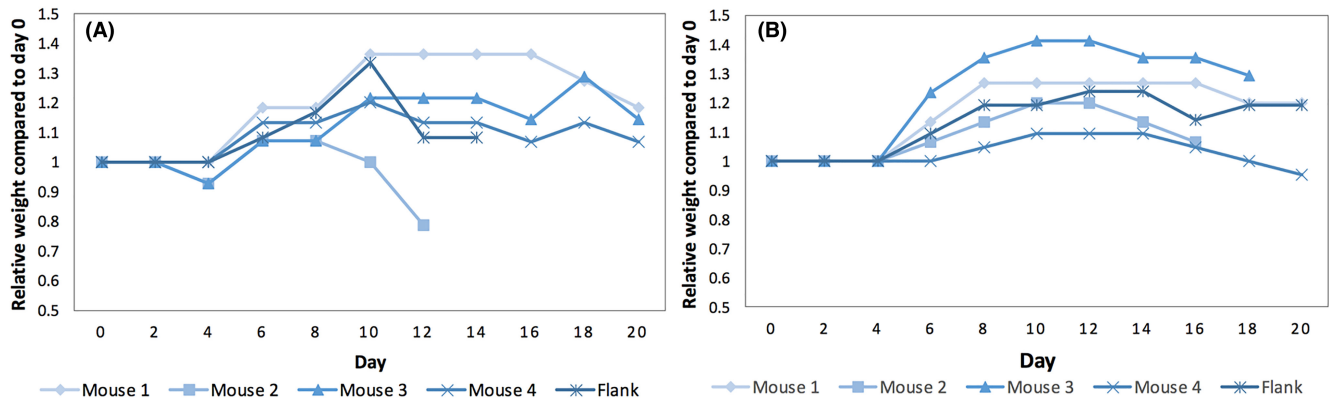


FIGURE 4 The relative weights of mice compared to their pre-operation weights (day 0). (A), Female mice. (B), Male mice.

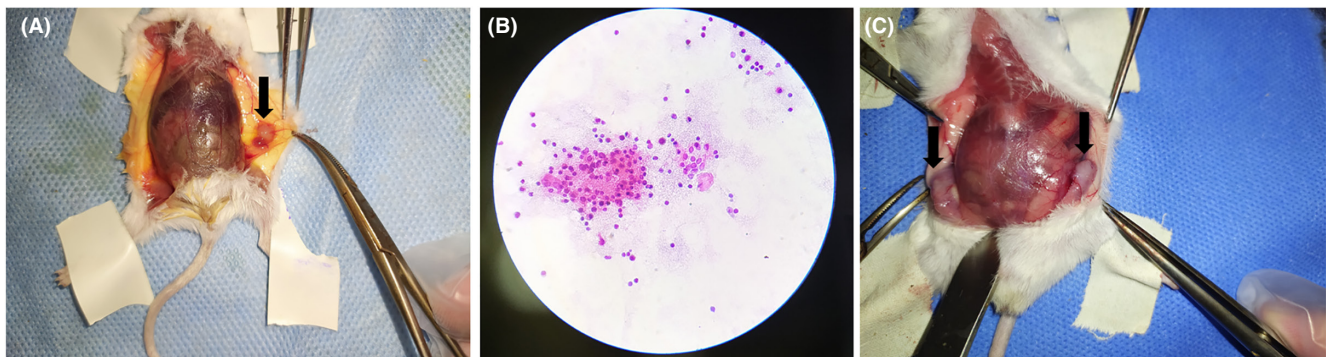


FIGURE 5 Control mice. (A), Female control mouse with a tumor in the left flank and ascites. (B), Papanicolaou stain of ascites fluid smear showing cancer cells. (C), Male control mouse with tumors in both flanks.

of cells into the bladder lumen, either via transurethral catheterization or intraluminal injection, can truly mimic this pathogenesis. However, these methods involve the possibility of ectopic tumor formation, for example in urethra or ureters secondary to evacuation or reflux, respectively. With these methods, tumors can form anywhere, causing early obstruction, whereas, with the intramural injection, cells can be deliberately injected to the anterior wall, away from ureteral orifices to avoid early urinary obstruction and subsequent mortality. This keeps animals alive and non-morbid for a longer period, giving researchers more time to study the effects of their treatments.¹⁵ In our study, three mice deteriorated and were euthanized on the 12th, 16th, and 18th post-operative days. We attribute the early obstructions seen in these cases to the very rapid growth rate of the cell line used, which created a bulging tumor that completely encompassed the bladder lumen. Muscle-invasive bladder cancer (MIBC) accounts for 30% of all bladder cancers. It has a poor prognosis: the 5-year survival rate after radical cystectomy is 40%–60%, and the median overall survival of patients with MIBC who receive no treatment is only 12 month.¹⁶ So it is important to develop models of MIBC to study its pathogenesis and evaluate novel therapies. Although the direct intramural injection method cannot reproduce models of NMIBC, it is valuable in studies of MIBC. Since the cells are injected into the muscular layer or just beneath it into the submucosal space, the

model always represents muscle invasion, and hence is a promising model for MIBC.¹⁷

The absence of mortality following this procedure suggests that this technique is a reliable and reproducible method for developing orthotopic bladder cancer models. However, limitations remain. Cancer cells can spill through the canal created by the injection needle into the peritoneal space and form tumors, as occurred in 2 mice with lowest body weight in our study. This suggests that the smaller the animal, and therefore its bladder, the higher the chance of cell spillage. As bladder cancer occurs more in older age, we suggest the use of bigger and older animals for better disease resemblance, easier handling, and lower spillage and complications. The use of narrower needles, as suggested by Fu and colleagues, also reduces the chance of spillage.^{12,18} Using fibrin glue to block the needle track may be helpful, although we did not try this option.

Another limitation is that this technique requires laparotomy, which is a major form of surgery, so pain control and infection prevention must be carefully addressed. Flunixin meglumine is a non-steroidal anti-inflammatory drug used for pain management. Due to its metabolism in the liver and fecal excretion, it does not interfere with the direct intramural injection method. The fluoroquinolone enrofloxacin is excreted through the liver and kidneys and helps prevent urinary infections and sepsis; the surgical procedures used on

TABLE 1 Advantages and disadvantages of the four main methods of developing orthotopic bladder cancer models

Delivery method of cancer cells	Advantages	Disadvantages
Direct intramural injection	<ul style="list-style-type: none"> • Applicable to both male and female mice • Short procedure time (≤ 15 min per mouse) • Requires simple readily available equipment • High chance of tumor development since mucosa and its uroplakins are bypassed • Exactly location of the tumor 	<ul style="list-style-type: none"> • Animal undergoes laparotomy, which is a major surgery • Cell spillage through needle canal can occur and result in peritoneal tumors • Does not recapitulate the invasion of the tumor from mucosa to upper layers
Intravesical instillation	<ul style="list-style-type: none"> • Better recapitulates the natural progression of tumor from mucosa to upper layers • Requires no major surgery 	<ul style="list-style-type: none"> • Long procedure time (≥ 1.5 h per mouse) • Technically difficult and delicate • Applicable to female mice only • Location of tumor development is not exact and it can develop in ectopic places (e.g. urethra)
Percutaneous intravesical ultra-sound guided submucosal-space injection	<ul style="list-style-type: none"> • Short procedure time (≤ 5 min per mouse after primary setup) • Does not require major surgery • Exactly location of the tumor • Better recapitulates the tumor invasion to upper layers (though does not mimic lamina propria invasion) • High chance of tumor development since mucosa and its uroplakins are bypassed 	<ul style="list-style-type: none"> • Requires advanced and expensive equipment that are not readily available everywhere • Difficult primary setup • Requires expertise in handling the used equipment
Direct intraluminal injection	<ul style="list-style-type: none"> • Applicable to both male and female mice • Short procedure time (≤ 10 min per mouse) • Requires simple readily available equipment • Better recapitulates the natural progression of the tumor from mucosa to upper layers 	<ul style="list-style-type: none"> • Requires major surgery • Cell spillage through needle canal can occur and result in peritoneal tumors • Location of tumor development is not exact and it can develop in ectopic places (e.g. urethra) • Needle canal connects vesical and peritoneal space and can cause peritonitis, hence mortality

the urinary system with this method predispose the animal to such infections.^{19,20}

This study shows that no perfect animal model of human bladder cancer exists, as each has shortcomings. However, the direct intramural injection method is a rapid, feasible, and reproducible method that does not require specialized and advanced equipment. It can be performed on both male and female mice using the same surgical techniques with similar results in both sexes. This is important since bladder cancer is four times more common in men.¹⁸ These advantages and the 100% tumor take rate and zero mortality during the study indicate the efficiency of this technique and its capacity to be used in the field of bladder cancer research.

5 | CONCLUSION

Direct intramural injection is a reliable and reproducible method for developing orthotopic models of bladder cancer. It can be performed on both male and female mice in less than 15 minutes per mouse and only requires basic surgical tools that are readily available.

ACKNOWLEDGMENT

Special thanks to Mr Reza Esmaeili and Mrs Masoumeh Ekhtiari.

CONFLICT OF INTEREST

All authors declare that they do not have any competing interest.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

This study was conducted in accordance with the "Guideline for the Care and Use of Laboratory Animals in Iran" and under approval of Research Ethics Committees of School of Medicine, Tehran University of Medical Sciences (Approval ID: IR.TUMS.MEDICINE.REC.1398.923).

CONSENT FOR PUBLICATION

Not applicable.

DATA AVAILABILITY STATEMENT

All data are presented in the manuscript. Further information is available from the corresponding author on reasonable request.

ORCID

Masoud Bitaraf  <https://orcid.org/0000-0002-3550-2735>

REFERENCES

1. Siegel RL, Miller KD, Jemal A. Cancer Statistics, 2016. *CA Cancer J Clin.* 2016;66(1):7-30.

2. van der Heijden AG, Witjes JA. Recurrence, progression, and follow-up in non-muscle-invasive bladder cancer. *Eur Urol Suppl.* 2009;8(7):556-562.
3. Witjes JA. Management of BCG failures in superficial bladder cancer: a review. *Eur Urol.* 2006;49(5):790-797.
4. Ringuette-Goulet C, Bolduc S, Pouliot F. Modeling human bladder cancer. *World J Urol.* 2018;36(11):1759-1766.
5. Vargo-Gogola T, Rosen JM. Modelling breast cancer: one size does not fit all. *Nat Rev Cancer.* 2007;7(9):659-672.
6. Oliveira P, Colaco A, De la Cruz P LF, Lopes C. Experimental bladder carcinogenesis-rodent models. *Exp Oncol.* 2006;28:2-11.
7. Jäger W, Moskalev I, Raven P, Goriki A, Bidnur S, Black PC. *Orthotopic Mouse Models of Urothelial Cancer.* Springer; 2018:177-197.
8. Lorenzatti Hiles G, Cates AL, El-Sawy L, et al. A surgical orthotopic approach for studying the invasive progression of human bladder cancer. *Nat Protoc.* 2019;14(3):738-755.
9. Ahmadi-Noorbakhsh S, Mirabzadeh Ardakani E, Sadighi J, et al. Guideline for the care and use of laboratory animals in Iran. *Lab Anim.* 2021;50(11):303-305.
10. Pulaski BA, Ostrand-Rosenberg S. Mouse 4T1 breast tumor model. *Curr Protoc Immunol.* 2000;39(1):20.2.1-20.2.16.
11. Dinney CP, Fishbeck R, Singh RK, et al. Isolation and characterization of metastatic variants from human transitional cell carcinoma passaged by orthotopic implantation in athymic nude mice. *J Urol.* 1995;154(4):1532-1538.
12. Fu C-L, Apelo CA, Torres B, Thai KH, Hsieh MH. Mouse bladder wall injection. *JoVE (J Vis Exp).* 2011;53:e2523.
13. Jerman UD, Višnjari T, Bratkovič IH, et al. Attachment of cancer urothelial cells to the bladder epithelium occurs on uroplakin-negative cells and is mediated by desmosomal and not by classical cadherins. *Int J Mol Sci.* 2021;22(11):5565.
14. Chan ES, Patel AR, Smith AK, et al. Optimizing orthotopic bladder tumor implantation in a syngeneic mouse model. *J Urol.* 2009;182(6):2926-2931.
15. Thompson DB, Siref LE, Feloney MP, Hauke RJ, Agrawal DK. Immunological basis in the pathogenesis and treatment of bladder cancer. *Expert Rev Clin Immunol.* 2015;11(2):265-279.
16. Ogawa K, Shimizu Y, Uketa S, Utsunomiya N, Kanamaru S. Prognosis of patients with muscle invasive bladder cancer who are intolerable to receive any anti-cancer treatment. *Cancer Treat Res Commun.* 2020;24:100195.
17. Raven PA, D'Costa NM, Moskalev I, et al. Development of a murine intravesical orthotopic human bladder cancer (mio-hBC) model. *Am J Clin Exp Urol.* 2018;6(6):245.
18. Saginala K, Barsouk A, Aluru JS, Rawla P, Padala SA, Barsouk A. Epidemiology of bladder cancer. *Med Sci.* 2020;8(1):15.
19. Aarnes TK, Muir WW. Chapter 26-pain assessment and management. In: Peterson ME, Kutzler MA, eds. *Small Animal Pediatrics.* W.B. Saunders; 2011:220-232.
20. Papich MG. Chapter 39-antimicrobial drugs. In: Washabau RJ, Day MJ, eds. *Canine and Feline Gastroenterology.* W.B. Saunders; 2013:471-476.

How to cite this article: Bitaraf M, Muhammadnejad S, Azimzadeh A, et al. Evaluation of direct intramural injection to the bladder wall as a method for developing orthotopic tumor models. *Anim Models Exp Med.* 2022;5:575-581. doi:[10.1002/ame2.12293](https://doi.org/10.1002/ame2.12293)