

# Effects of CO<sub>2</sub> Euthanasia of C57BL/6 Mice on Sperm Motility, In Vitro Fertilization, and Embryonic Developmental Competence

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Cryopreservation of epididymal sperm collected after euthanasia is a common method to preserve and distribute valuable mouse models worldwide. However, the euthanasia method used prior to sperm collection must not adversely affect sperm quality. The most common method of euthanasia in mice is CO<sub>2</sub> asphyxiation, but its effect on the quality of sperm collected postmortem is largely unknown. The objective of this study was to determine the effects of CO<sub>2</sub> euthanasia of C57BL/6 mice on both freshly recovered sperm and sperm subjected to freezing and thawing. First, sperm concentration, progressive motility, curvilinear velocity (VCL), average path velocity (VAP), and progressive velocity (VSL) were analyzed for mice euthanized by cervical dislocation (CD), high flow CO<sub>2</sub> (100%), or low flow CO<sub>2</sub> (30%) displacement/minute, respectively. Then, *in-vitro* fertilization and embryonic development rates were determined using frozen-thawed sperm from each euthanasia method. Neither fresh nor frozen-thawed sperm showed significant differences in sperm concentration, progressive motility, VAP, or VCL when compared to CD and CO<sub>2</sub> groups. However, frozen-thawed sperm collected from CD mice had higher VCL values than did those collected from the low flow mice ( $P = 0.039$ ). VCL was not different in fresh or frozen-thawed sperm collected after mouse euthanasia by CD as compared with high flow CO<sub>2</sub> or by high flow as compared with low flow CO<sub>2</sub>. Frozen-thawed sperm showed no differences among the 3 euthanasia groups for fertilization ( $P = 0.452$ ) or blastocyst development rates ( $P = 0.298$ ). The results indicate that CO<sub>2</sub> euthanasia can be used as an alternative to CD to obtain optimal quality mouse sperm for cryopreservation while remaining compliant with welfare requirements.

**Abbreviations:** ART, assisted reproductive technologies; CD, cervical dislocation; IVF, in vitro fertilization; VAP, average path velocity; VCL, curvilinear velocity; VSL, progressive velocity

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## Introduction

Mice are by far the animals most commonly used by biomedical researchers<sup>25</sup> and the most commonly used animals for the generation of genetically engineered models of human disease and disorders.<sup>41</sup> To this end, researchers worldwide have created thousands of genetically modified mouse strains to investigate the genetic origins of human diseases and disorders.<sup>23</sup> The numbers of these rodent strains continue to increase and can be costly to create and maintain. Scientists perform a wide spectrum of phenotypic characterization on these mice to characterize the strain for the biomedical community. Germplasm cryopreservation is an effective means of preventing loss of these strains during catastrophic events.<sup>3</sup> Furthermore, scientific collaboration among different institutions, often across the globe, require safe transfer of these strains. Shipping live mice can be challenging and sometimes impossible, both domestically and internationally, due to logistics and international trade laws. Therefore, a more cost effective, efficient, and convenient strategy is to transport cryopreserved germplasm for future rederivation at receiving institutions.

Postmortem collection of germplasm (for example spermatozoa, oocytes, embryos, ovarian, or testicular tissues) from mice requires great care to ensure the quality and functional viability of the recovered cells. High quality germplasm is important to obtaining successful rederivation via assisted reproductive technologies (ART) such as in vitro fertilization (IVF), intracytoplasmic sperm injection (ICSI) and embryo transfers. In addition, one of the main requirements for the use of animals in biomedical research is to comply with the 3Rs of replacement, reduction, and refinement. These principles are employed to improve animal welfare and scientific vigor where the use of animals cannot be avoided.<sup>16,19,29,34</sup> Prioritizing efficient postmortem recovery of germplasm greatly reduces the number of donor mice that are necessary for germplasm bio-banking. Therefore, the methods used to euthanize mice for germplasm harvest must not reduce its quality for future ARTs used to rederive specific strains.

Euthanasia for postmortem germplasm collection for cryopreservation and subsequent recovery of live mice via IVF is commonly performed by either cervical dislocation (CD) (mice) or CO<sub>2</sub> asphyxiation (rats).<sup>43,47</sup> Sperm is abundant, easy to collect, relatively easy to cryopreserve and can be collected from sick, aged or even dead animals. Sperm can be used to preserve transgenic mouse lines that have a single mutation on an inbred background. While the AVMA recognizes CD as an approved method of euthanasia for mice, its guidelines state that CD is technically challenging and requires scientific justification.<sup>5</sup> One

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study found that CD was unsuccessful in 21% of cases, even when performed by highly trained technicians.<sup>10</sup> In addition, euthanasia via CD is aesthetically displeasing and may distress personnel and contribute to compassion fatigue.<sup>44</sup> Thus, CO<sub>2</sub> asphyxiation is the most commonly used euthanasia method for many rodent users due to its convenience, ease and cost effectiveness.<sup>7</sup> Researchers who use CO<sub>2</sub> asphyxiation as a euthanasia method should first confirm it does not have deleterious effects and is suitable for achieving research objectives.

Cryopreservation can have serious detrimental effects on spermatozoa including osmotic, physical, hypothermic, and chemical toxicity of the cryoprotective agent.<sup>51</sup> Epididymal mouse sperm, particularly when derived from the inbred mouse strains (for example C57BL/6, FVB or 129, BALB/C) are more sensitive to these effects than other species, including bull, boar, and ram.<sup>51</sup> Sperm may lose functional integrity if they are subjected to suboptimal conditions either in vivo or in vitro and may then no longer be able to complete fertilization and trigger further embryonic development. Thus, maintaining high quantitative and qualitative yield is important for achieving rederivation using cryopreserved spermatozoa. Germplasm quality is critical for cryo-resuscitation and for minimizing experimental confounds during the development of effective cryopreservation protocols.

Under normal conditions, the body can balance the ions that control pH within the required physiologic levels.<sup>18</sup> In the case of hypoventilation, such as that caused by a prolonged period of CO<sub>2</sub> asphyxiation, respiratory acidosis can occur because the lungs cannot eliminate adequate amounts of CO<sub>2</sub> from the peripheral blood. This excess CO<sub>2</sub> causes the pH of the circulating blood and, potentially, of other bodily fluids to fall, producing an acidic environment. However, mouse oocytes and sperm may remain within the range of 7.3 to 7.4 to obtain optimal fertility both in the female reproductive tract and during IVF.<sup>6,34,39</sup> One study reported that the circulating blood pH of the C57BL/6 female mice euthanized by low-flow CO<sub>2</sub> asphyxiation caused significant reduction in circulating blood pH to 6.5 as compared with 7.3 in mice euthanized by CD and reduced IVF success when using C57BL/6 sperm.<sup>24</sup>

Our group recently reported that CD yields superior quality fertilizable oocytes compared with either low- or high-flow CO<sub>2</sub> asphyxiation.<sup>52</sup> This in-depth subcellular investigation showed that higher rates of premature cortical granule exocytosis (PCGE) are largely responsible for reduced fertilization rates for the oocytes collected from donor mice euthanized by CO<sub>2</sub> asphyxiation before the IVF procedure. These findings collectively suggest that an acidic environment in the oviduct due to CO<sub>2</sub>-induced asphyxiation prior to oocyte recovery impaired successful IVF.<sup>24,53</sup>

To date, few studies have examined the effects of CO<sub>2</sub> euthanasia on rodent epididymal sperm motility characteristics.<sup>9,46</sup> None of those studies found adverse effects of CO<sub>2</sub> asphyxiation euthanasia on rat spermatozoa, but to our knowledge effects of CO<sub>2</sub> euthanasia procedure on mouse spermatozoa have not been investigated. In the current study, C57BL/6 male mice were euthanized by high flow CO<sub>2</sub>, low flow CO<sub>2</sub>, or CD, and then motility characteristics of fresh and thawed sperm were assessed. Furthermore, IVF and subsequent embryo culture experiments were performed using either fresh or thawed sperm to effects on IVF and in vitro embryo developmental competence.

## Materials and Methods

All chemicals used in this study were obtained from Sigma chemical company (St. Louis, MO) or ThermoFisher Scientific (Waltham, MA) unless stated otherwise.

**Animals.** This study was conducted at an AAALAC-accredited facility under an IACUC-approved protocol and in compliance with the 8<sup>th</sup> edition of the Guide for the Care and Use of Laboratory Animals.<sup>28</sup> Twelve- to 14-wk-old male C57BL/6J ( $n = 42$ ) and 7- to 8-wk-old female C57BL/6J ( $n = 56$ ) *Mus musculus* were obtained from an inhouse breeding colony. The housing environment was maintained at  $22 \pm 2$  °C, with a relative humidity of 30% to 70% on a 14:10-h light:dark cycle (lights on, 700 CST). Mice were either single housed in standard polypropylene shoebox cages (7.25 in. L  $\times$  11.75 in. W  $\times$  5 in. H [18.4 cm  $\times$  29.8 cm  $\times$  12.7 cm], Allentown, Allentown, NJ) or group housed in groups of 8 to 10 in large shoebox caging (10.5 in.  $\times$  19 in.  $\times$  6 in. [26.7 cm  $\times$  48.3 cm  $\times$  15.2 cm]) on aspen bedding (Specialty Papers, Watertown, TN) and had unrestricted access to a commercial rodent diet (Formulab Diet 5008, Purina, St. Louis, MO) and water. Colony health was evaluated every 3 mo through sentinel exposure to dirty bedding. All sentinels were seronegative for Mouse hepatitis virus, Minute virus of mice, Mouse parvovirus, Parvovirus NS-1, Theiler murine encephalomyelitis virus, Murine rotavirus, *Mycoplasma pulmonis*, Sendai virus. PCR testing was negative for fur mites and pinworms.

**Euthanasia.** Mice in CO<sub>2</sub> euthanasia groups were placed in a clean 14.6-L polyurethane box connected to a CO<sub>2</sub> tank (38.5 cm L, 19.5 cm W, 19.5 cm H). The flow rate was 30% displacement volume/minute for mice in the low flow CO<sub>2</sub> group and 100% displacement volume/minute for the high flow CO<sub>2</sub> group via Western Medica CO<sub>2</sub> flow meter (Westlake, OH). The percentages were chosen to represent the lowest acceptable flow rate in the 2020 AVMA euthanasia guidelines and the maximal rate at which CO<sub>2</sub> could be displaced.<sup>5</sup> Mice remained in the box until they had stopped breathing for 1 min. Mice were removed from the box and cervically dislocated as a secondary method of euthanasia; the box was then cleaned with 70% ethanol. The time from the start of euthanasia to time of sperm collection was recorded for each mouse. Mice in the CD group were euthanized by trained personnel by simply grasping the skin on the back of the neck by the thumb and forefinger and immediately pulling on the base of the tail in an opposite upward direction from the head in accordance with 2020 AVMA guidelines. The immediate dislocation of the spinal column from the brain ensured sudden death in a few seconds. All female donors for oocyte collection for IVF were euthanized via CD only.

**Computer-Assisted Sperm Motility Analysis (CASA).** Computer-assisted sperm motility analysis (Hamilton Thorne Biosciences, M2030, Beverly, MA) was used to determine sperm motility characteristics in an 80- $\mu$ m deep dual sided chamber (2 $\times$  CELL, Hamilton Thorne Biosciences) at 37 °C. After capacitation, 10- $\mu$ L sperm samples were assessed on an IVOS (Hamilton Thorne sperm analysis system). One fresh and one frozen-thawed sample was assessed from each mouse. Ten representative areas from each sample were used on each slide to measure concentration, progressive motility, curvilinear velocity (VCL), average path velocity (VAP), and progressive velocity (VSL) for each sample.

**Sperm collection and freezing.** After euthanasia, the cauda epididymis was removed, washed in HEPES buffered follicle holding medium (FHM), and external blood and fat removed with clean tissue paper. Each epididymis was secured using fine tweezers, and a small cut was made using sterile microscissors. Densely packed sperm was gently teased from each epididymis using fine tweezers. For freezing, sperm were exposed to 1.2 mL 18% raffinose pentahydrate (w/v) and 3% skim milk (w/v) freezing solution as previously described.<sup>50</sup> After 10 min, freezing solution containing the sperm was loaded into 0.5-mL

French straws and placed in a Styrofoam box containing LN<sub>2</sub>. Straws were placed and cooled in vapor phase of LN<sub>2</sub> on a mesh wire 8 in. (20.3 cm) above LN<sub>2</sub> for 5 min, and then the straws were plunged into LN<sub>2</sub> for long-term storage.

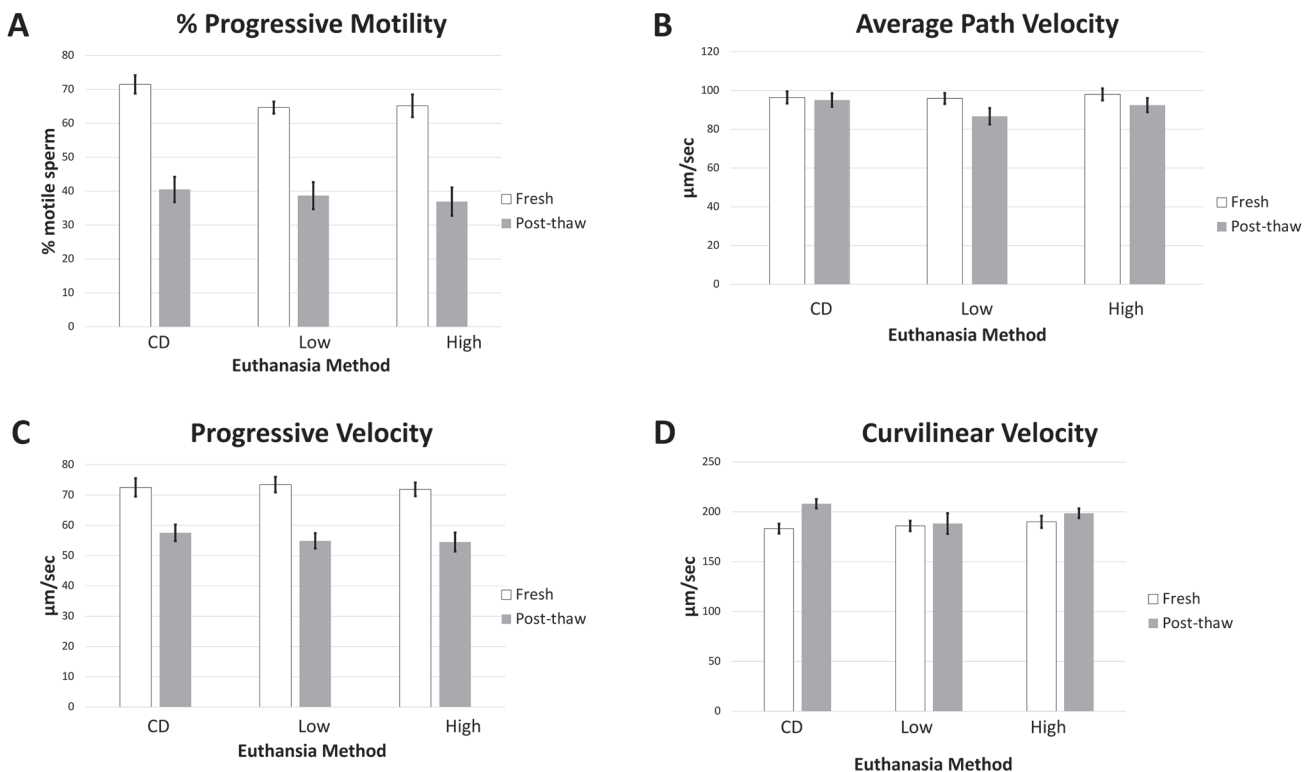
Motility of fresh sperm was analyzed from each donor mouse prior to cryopreservation by diluting a small portion of fresh sperm in HEPES buffered FHM media and using the CASA system. To determine motility characteristics of frozen-thawed sperm, the straws containing frozen sperm were taken directly from LN<sub>2</sub> storage, placed in a warm water bath (37 °C) and allowed to thaw. Freezing solution containing sperm was then expelled from the straw into a 1.5-mL microfuge tube with 1 mL of FHM media for motility analysis. Tubes were spun down in a microcentrifuge at 0.3 relative centrifugal field (RCF) for 5 min. After centrifugation, the supernatant was carefully removed without disturbing the pellet, and 70 µL of FHM was added to the pellet. Tubes were then placed in a hot bead bath at 37 °C and allowed to capacitate for 30 min before motility analysis was performed using the CASA system.

**Superovulation.** Each mouse was injected with 5 IU pregnant mare serum gonadotropin (PMSG) intraperitoneally followed by 5 IU human chorionic gonadotropins (hCG) 48 h later. Clutches of cumulus-oocyte complexes (COCs) were collected from the oviducts 14 to 15 h after hCG injection.

**In vitro fertilization.** The straws containing frozen sperm were removed from LN<sub>2</sub> and thawed in a water bath at 37 °C. The content of the straw was gently expelled on top of 1 mL FHM media containing BSA (4 mg/mL) in microfuge tube and then centrifuged for 5 min at 300 g. The supernatant was removed, and sperm pellet was gently pipetted out and placed into pre-equilibrated 95-µL FERTIUP drop for sperm capacitation for 30 min. IVF was performed as previously described.<sup>48,49</sup> Both FERTIUP and CARD (for in vitro fertilization) media were

pre-equilibrated in a humidified incubator containing 5% CO<sub>2</sub> in air under mineral oil at 37 °C in 35 mm culture dishes. For IVF, the dissected oviducts from the donor females were placed separately into mineral oil next to a CARD drop under a stereomicroscope. The clutches of COCs from the donors were first released into the mineral oil using fine tweezers and a 28 gauge needle. They were then gently pulled into 90-µL CARD drops. For IVF, thawed or fresh sperm was capacitated in FERTIUP and then gently added (approximately 350 × 10<sup>5</sup> motile sperm/mL) to CARD drops that contained the clutches of COCs collected after the 3 euthanasia methods. After about 6 to 8 h of sperm and egg coincubation, the presumptive zygotes were denuded from cumulus cells by pipetting, washed in FHM media containing BSA (4 mg/mL), transferred into KSOM amino acid culture drops, and allowed to develop in an incubator at 37 °C and 5% CO<sub>2</sub>.<sup>26,48</sup> Development rates were assessed at 24 h for 2-cell, 72 h for morula and 96 h for blastocyst development.

**Statistical analysis.** Statistical analysis was performed using SigmaPlot version 14.0 (Systat Software, Palo Alto, CA) and *P* values < 0.05 were considered significant. All values are given as mean ± SEM and represented by error bars in the figures. An unpaired *t* test was used to compare the time from onset of CO<sub>2</sub> to completed euthanasia for the 2 methods of CO<sub>2</sub> euthanasia. A 2-way ANOVA with a Tukey posthoc analysis was used to determine whether euthanasia method affected on sperm quantity and motility parameters, including sperm concentration, progressive motility, VAP, VCL, and VSL. To determine whether exposure to CO<sub>2</sub> affected fresh or frozen sperm quality, an ANOVA was performed on both fresh and thawed samples for each motility parameter (progressive motility, VAP, VSL, and VCL) samples. An ANOVA was used to test for differences between groups. A 2-way ANOVA was used to determine whether euthanasia time affected measures of sperm quality. A



**Figure 1.** The C57BL/6 cauda epididymal fresh sperm and post thaw progressive motility (A) velocity parameters; curvilinear velocity (B), average path velocity (C), and progressive velocity (D) for either fresh or frozen-thawed C57BL/6 sperm. The results are presented as averages across groups (*n* = 12 per treatment group). CD: cervical dislocation. Low: low-flow CO<sub>2</sub>. High: high-flow CO<sub>2</sub>. Error bars represent standard error of mean. \* indicates significance.

one-way ANOVA was used to test whether CO<sub>2</sub> euthanasia of mice affected the embryo developmental competence (2-cell, morula, and blastocyst) of their thawed sperm.

## Results

**Euthanasia time.** Mice ( $n = 12$  per treatment group) in the low-flow rate CO<sub>2</sub> group had a significantly longer euthanasia time (340 s) as compared with the high flow rate group (52 s) ( $P < 0.001$ ). However, euthanasia method did not significantly affect fresh sperm recovery from cauda epididymis ( $P = 0.939$ ). Recoveries for CD, low-flow, and high-flow rate CO<sub>2</sub>, and CD euthanasia methods were, respectively, 16 million/mL, and 17 million/mL, 18 million/mL.

**Fresh and frozen-thawed sperm motility.** To determine whether euthanasia via CO<sub>2</sub> asphyxiation affected sperm motility parameters (progressive motility, VSL, VAP, and VCL), sperm was analyzed by CASA (Figure 1A through D). Progressive motility of freshly collected sperm were 65%, and 65%, and 71% for low-flow, and high-flow rate CO<sub>2</sub>, and CD respectively, with no significant differences between groups ( $P = 0.105$ ). In thawed sperm, progressive motility values were 39%, and 37%, and 40% respectively, for low-flow, and high-flow rate CO<sub>2</sub>, and CD with no significant differences between groups ( $P = 0.817$ ). VAP, VSL, and VCL values were also measured for fresh and thawed sperm. VAP values for the freshly collected sperm were 96, 98, and 96  $\mu\text{m}/\text{sec}$  for low-flow, and high-flow rate CO<sub>2</sub>, and CD respectively, with no significant differences between groups ( $P = 0.877$ ). For the thawed sperm, VAP values were 87, 92, and 95  $\mu\text{m}/\text{sec}$  for low-flow, and high-flow rate CO<sub>2</sub>, and CD respectively, with no significant differences between groups ( $P = 0.295$ ).

VSL values for freshly collected sperm were 72, 73, and 72  $\mu\text{m}/\text{sec}$  for low-flow, and high-flow rate CO<sub>2</sub>, and CD respectively, with no significant differences between groups ( $P = 0.873$ ). For thawed sperm, VSL values were 55, 54, and 58  $\mu\text{m}/\text{sec}$ , respectively, for low-flow and high-flow rate CO<sub>2</sub>, and for CD, with no significant differences between groups ( $P = 0.710$ ). VCL values for freshly collected sperm were 186, 190, and 183  $\mu\text{m}/\text{sec}$  for low-flow, and high-flow rate CO<sub>2</sub>, and CD respectively, with no significant differences between groups ( $P = 0.673$ ). For thawed sperm, VCL values were 188, 198, and 208  $\mu\text{m}/\text{sec}$  for low-flow, and high-flow rate CO<sub>2</sub>, and CD respectively. Thawed sperm collected from the CD group had significantly higher VCL values than did sperm collected from the low-flow group ( $P = 0.039$ ), with no significant differences between sperm collected from the CD and with high-flow groups ( $P = 0.344$ ) or between high-flow and low-flow groups ( $P = 0.548$ ). No significant differences were found for progressive motility, VSL, or VAP between groups for either fresh or thawed samples. No significant differences were found for VCL between groups for freshly collected sperm, but thawed sperm samples from mice euthanized by CD were significantly faster than those obtained from mice euthanized via low-flow CO<sub>2</sub> ( $P = 0.039$ ).

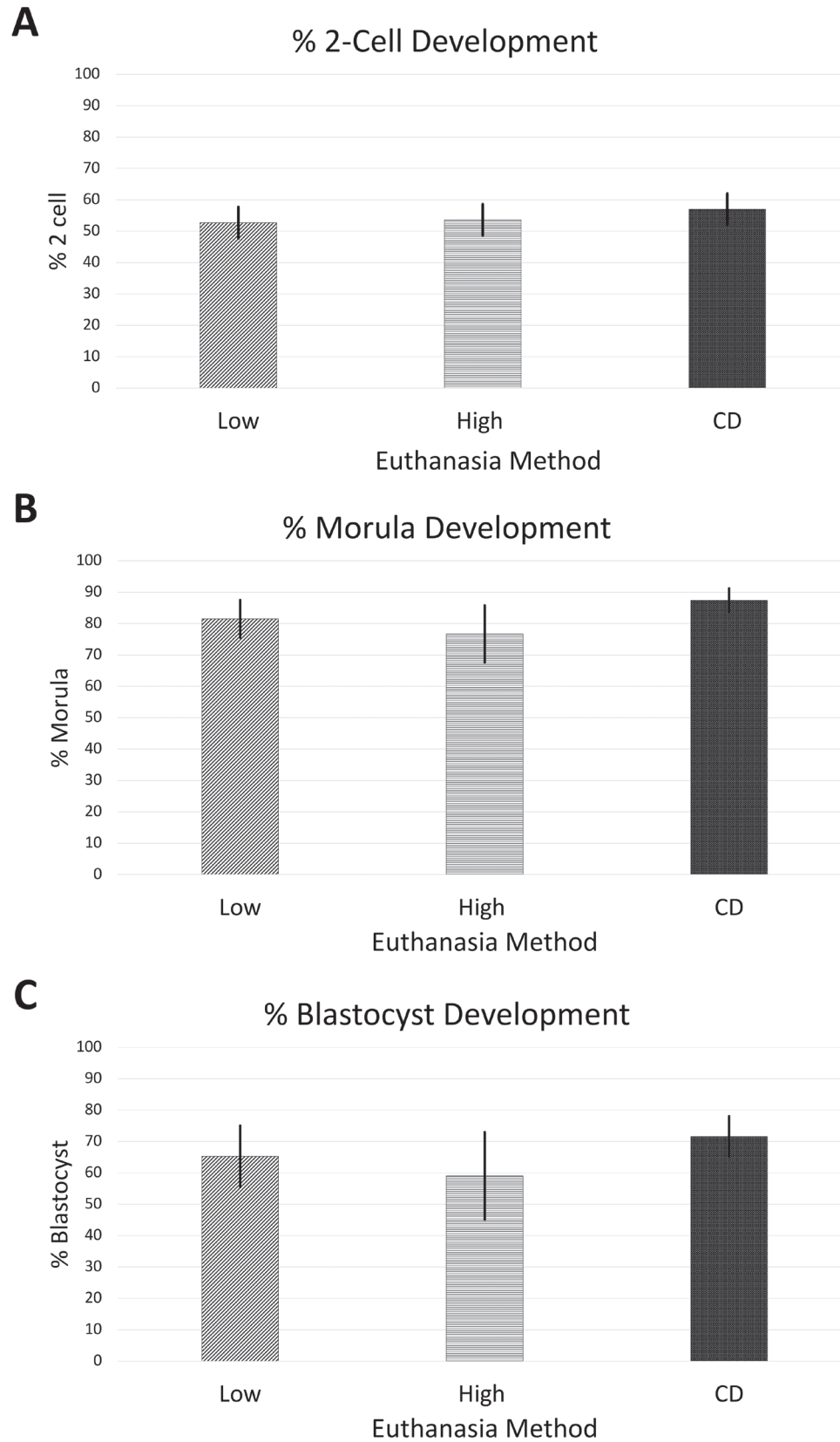
**Embryonic development competency.** To determine whether sperm collected from mice euthanized with CO<sub>2</sub> were altered with regard to IVF and in vitro embryo development, the percentage of embryos that reached developmental milestones were calculated (Figure 2). The percentage of oocytes that developed to 2-cell stage embryos was first calculated for thawed sperm. This value was then used to calculate morula and blastocyst development percentages (Figure 2). The percentages of 2-cell development for high-flow, low-flow CO<sub>2</sub>, or CD were 54%, 53%, and 57%, respectively. Morula development rates for high flow, low flow CO<sub>2</sub>, or CD were, respectively, 77%, 81%, and 87%,

while blastocyst stage development rates were 59%, 65%, and 72%. No significant differences were detected between groups for IVF ( $P = 0.452$ ), morula development rates ( $P = 0.310$ ), or blastocyst development rates ( $P = 0.298$ ) using thawed sperm collected after the 3 euthanasia methods.

## Discussion

Obtaining high quality spermatozoa and oocytes is integral to efficient in vitro fertilization, intracytoplasmic sperm injection and high-quality embryo production for reproductive studies and germplasm cryopreservation. In contrast to other domestic farm and companion animals, the most common method of recovering mouse sperm or oocytes for in vitro reproductive studies or genome cryo-banking requires postmortem collection of spermatozoa or oocytes from the cauda epididymis or oviduct of previously superovulated donor mice, respectively.<sup>2</sup> The euthanasia method is an important determinant of the future developmental competence of the germplasm, and should provide optimal recovery of spermatozoa, oocytes, or preimplantation embryos. Fertilization is a critical period for preimplantation embryonic development in mammals. Thus, the exposure of oocytes or sperm to suboptimal conditions such as hypothermia, acidosis, or various physical stresses, either before or during the course of fertilization, could impair fertilization and ultimately result in lower rates of embryonic development.<sup>21,52,53</sup> One group showed that the blood pH of the female C57BL/6 mice euthanized by using CO<sub>2</sub> at a displacement rate of 20%/L/min was significantly lower (pH 6.5) than that of mice euthanized by CD (pH 7.3).<sup>24</sup> One of our recent studies demonstrated that CD preserved the intact distribution of cortical granules (CGs) and F-actin of metaphase II (MII) mouse oocytes, leading to significantly higher IVF success rates and better embryo development to the blastocyst stage as compared with either high-flow or low-flow rate CO<sub>2</sub>.<sup>53</sup> This suggested that the key factors for harvesting good quality oocytes were maintaining blood and intracellular pH at approximately 7.3 and rapid collection of oocytes after euthanasia by CD. While CD appears necessary to collect quality oocytes, it has been unclear if it was necessary to collect quality sperm.<sup>53</sup>

The current study extended our investigation to compare the effects of low-flow or high-flow CO<sub>2</sub> asphyxiation and CD euthanasia on mouse cauda epididymal sperm motility, in vitro fertilization, and embryonic developmental competence. Several in vitro studies from a number of species suggest that hydrogen ions, bicarbonate ions, and CO<sub>2</sub> can influence sperm motility, viability, and metabolism and thus optimal pH is necessary not only for sperm maturation, but also for proper motility and acrosome reactions upon fertilization.<sup>35,36</sup> The basis of our study was that respiratory acidosis caused by CO<sub>2</sub> retention would detrimentally affect epididymal sperm and impair their fertility after postmortem recovery because these physiologic and subcellular parameters are important in sperm maturation and motility.<sup>20,24,53</sup> Sperm production is protected by the blood–testes barrier, which creates an immune privileged site within the testes and epididymis and prevents the accumulation of harmful substances.<sup>13,15</sup> A previous study showed that sperm maturation in the epididymis is regulated by both absorptive and secretory activities of the epithelial lining.<sup>14</sup> After spermatozoa production in the seminiferous tubules in the testis, sperm transit along the length of the caput, corpus, and cauda epididymis, and are subjected to constant morphologic, ionic, pH and osmotic changes. One group found that 96% of the testicular fluid output and bicarbonate was reabsorbed in the efferent ducts before transit into caput epididymis.<sup>35</sup> In that



**Figure 2.** (A) Percent *in vitro* fertilization rate, (B) morula development rate, and (C) blastocyst development rate (C) using C57BL/6 sperm collected after cervical dislocation, low flow CO<sub>2</sub> and high flow CO<sub>2</sub> euthanized donors following *in-vitro* fertilization using C57BL/6 frozen-thawed sperm. The results are presented as averages across the groups based on replicated 6 replications). Error bars represent standard error of mean. CD: cervical dislocation. Low: low-flow CO<sub>2</sub>. High: high-flow CO<sub>2</sub>.

study, luminal pH and bicarbonate levels in the efferent ducts of the rat are higher than those found in the epididymis, where low pH and bicarbonate contribute to sperm quiescence during storage. The high rate of bicarbonate reabsorption in the efferent ducts is considered a major contributor to the establishment of the low pH and bicarbonate milieu of the epididymis; bicarbonate has a role in balancing pH both in vivo and in vitro.

In vivo studies in rats found significant acidification in caput and cauda epididymis whereas little acidification was detected in the seminiferous tubules in the testis.<sup>31,32</sup> Later, in vivo micro-puncture studies in the rat seminiferous tubules and epididymal duct were devised to study acidification by direct measurement of intraluminal pH by using a highly reliable pH microelectrode.<sup>8</sup> The study found that pH in the proximal caput (pH 6.6), middle caput (pH 6.59), and proximal cauda epididymis (pH 6.8) were significantly more acidic than testicular artery (pH 7.4) or systemic arterial blood (pH 7.4). A considerable amount of information suggests that pH and the bicarbonate concentration in the lumen of the extra testicular ducts have a direct physiologic role in reproductive function. Several previous studies indicated that these factors are involved in the regulation of sperm metabolism and motility.<sup>11,12,30,37</sup> For instance, lower intracellular pH suppresses sperm metabolism and thus motility,<sup>11</sup> while bicarbonate has a role in activating sperm adenylyl cyclase,<sup>38</sup> and thus increases cAMP production. Other reports indicate that pH and bicarbonate levels are regulated in the lumen of the extratesticular ducts and decline along the epididymis.<sup>4,8,42</sup> The low pH and bicarbonate concentration in the cauda epididymis keeps sperm in a quiescent state during tightly packed epididymal storage.<sup>1,4,12,30,38</sup>

Computer-aided sperm analysis (CASA) systems allow analysis of large numbers of spermatozoa and provide values that are representative of the sample, minimizing subjective bias in choosing sperm for motion characterization. During sperm motion analysis, the most important characteristics are the progressive motility and various velocity parameters like curvilinear velocity (VCL, total distance traveled during observation period), straight-line velocity (VSL, net space gain during observation period), and average path velocity (VAP, distance traveled in average direction of movement). The percentage of progressively motile sperm in any given population is a sensitive indicator of adverse effects on sperm motion and ultimately determines how many sperm will reach the oocytes and perform proper fertilization. In addition, VCL, VSL, and VAP are useful and sensitive indicators of adverse effects on sperm motion. For example, decreased VSL values are associated with less in vivo fertilization, and a fall in VSL predicts that sperm will travel a shorter distance.<sup>45</sup>

The results of the current study indicate that mice that have been euthanized with either high flow or low flow CO<sub>2</sub> have few changes in sperm recovery or motility as compared with mice euthanized by CD. Although the VCL was significantly lower in thawed samples from mice euthanized by high flow CO<sub>2</sub> as compared with the CD group, the biologic relevance of this difference is minimal. Our study tested the 30% displacement/minute flow rate recommended by the AVMA and a 100% displacement/minute as this is the highest flow rate possible if had wanted to titrate the flow rate to determine the optimal rate for preserving sperm quality. However, we emphasize to readers that if CO<sub>2</sub> is used to euthanize mice for sperm collection, then a 30% flow rate should be used as it is in accordance with the 2020 AVMA Guidelines for the Euthanasia of Animals, and has been demonstrated to not impact sperm quality.<sup>5</sup> In addition, based on our study we can infer that a flow rate between 30%

to 70% should not affect sperm quality, however, studies using 70% displacement per minute would give further guidance. Our data indicate that CO<sub>2</sub> euthanasia is a viable alternative to CD when collecting sperm for cryopreservation and other downstream applications.

Several early studies investigated the effects of euthanasia using CO<sub>2</sub> and various inhalation agents on Sprague–Dawley rat epididymal sperm motility characteristics. One group tested decapitation, ether or halothane inhalation, and high or low rate CO<sub>2</sub> asphyxiation, but found no significant differences among the treatments with regards to sperm motility.<sup>46</sup> Similarly, another group euthanized male rats using either high-flow rate CO<sub>2</sub>, decapitation, and inhalation anesthetics enflurane, halothane, isoflurane, or sevoflurane, but did not detect any differences in progressive motility among the treatments.<sup>46</sup> Later, another study compared isoflurane and low flow rate CO<sub>2</sub> asphyxiation on rat sperm and did not find significant effects on sperm motility even after 10 min of CO<sub>2</sub> exposure. Thus, our study in mice is consistent with these previous studies on rat sperm collected after CO<sub>2</sub> euthanasia.<sup>9</sup>

One of the most important considerations in any animal experimentation is that the method of euthanasia should not adversely influence the immediate postmortem viability and the function of the collected cells or tissues needed for other in vitro experimentation. This would ultimately ensure quantitative and qualitative assessment of the data collected. Successful assisted reproductive techniques, such as cryopreservation, IVF, or sperm microinjection, require germplasm of high quality and quantity. To date, relatively few studies have investigated the influence of euthanasia methods on postmortem sperm, oocyte and embryos.<sup>24,27,39,43,53</sup> One study examined the influence of delayed dissection of oviducts after CD on MII mouse oocyte viability and embryo developmental competence in vitro.<sup>22</sup> The study determined that retaining the MII oocytes or pre implantation embryos in the oviduct for up to 30 min resulted in lower oocyte viability upon recovery and adversely affected the in vitro developmental potential. Two studies have shown adverse effects of CO<sub>2</sub> euthanasia on IVF ability for C57BL/6 oocytes due to largely premature exocytosis of cortical granules.<sup>24,53</sup> Another group examined the quality of mouse zygotes derived from 3 strains of mice (C57BL/6, B6SJLF1 and FVB/N) after euthanasia by either high-flow rate CO<sub>2</sub> (100% displacement) or CD.<sup>27</sup> They found no difference between high CO<sub>2</sub> and CD with regard to the morphologically normal zygote yield among the 3 mouse strains. In addition, they found no significant difference between high flow CO<sub>2</sub> and CD euthanasia on the in vitro development potential of the zygotes to blastocyst stage. Our study did not find a significant difference in terms of fertilization ability of spermatozoa in development of 2-cell stage embryos between either CO<sub>2</sub> euthanasia method or CD; this further demonstrates their functional viability at the cellular level, as indicated by mitochondrial, plasma membrane and acrosome integrity and is consistent with the progressive motility values, which were also not affected by the euthanasia methods in our study.

The blastocyst stage embryo development rates in our study were also comparable between CO<sub>2</sub> and CD euthanasia. Sperm DNA must be specifically organized in a unique condensed state that differs considerably from that of somatic cells or oocytes. Sperm chromatin undergoes extensive modifications during spermatogenesis, and the addition of protamines lead to very tightly packed chromatin.<sup>33</sup> Thus, the chromatin contained in the nucleus of a fully developed spermatozoa is a very stable structure. Therefore, the chromatin of mature sperm is much more resistant to damages caused by potentially harmful agents

than that of spermatogonia in seminiferous tubules, somatic cells, or oocytes.<sup>17</sup> To date, few other studies have examined the effects of euthanasia method on in vitro embryonic developmental competence.<sup>26,27</sup> A previous, related study investigated the quality of one-cell embryos derived from 3 strains of mice (C57BL/6, FVB/N, and B6SJLFl1) that were euthanized by either high-flow rate CO<sub>2</sub> or CD.<sup>27</sup> That study found no difference between CO<sub>2</sub> and CD in terms of the morphologically normal zygote yield and in vitro development potential of the one-cell embryos to blastocyst stage. These studies clearly suggest that once fertilization takes place, development to later stages such as 2-cell to morula and blastocyst are developmentally more robust and thus are more tolerant of suboptimal conditions.<sup>26,27</sup>

The use of CO<sub>2</sub> rather than CD is beneficial in many ways. One obvious benefit is better animal welfare. As stated earlier, even highly trained professionals can perform incomplete CD euthanasia. In addition, becoming proficient at CD requires practice that typically involves live animals.<sup>10</sup> Replacing CD with CO<sub>2</sub> euthanasia fulfills the 2 of the 3Rs by refining euthanasia methods while conserving sperm quality and by reducing the overall number of animals used in research by choosing a technique that does not require extensive training on live animals.<sup>16</sup> Finally, replacing CD with CO<sub>2</sub> euthanasia can help to reduce compassion fatigue in research staff. Compassion fatigue is a well-known issue for persons those who work with laboratory animals, especially those who perform euthanasia.<sup>40</sup> Replacing a physical method of euthanasia with a more aesthetically pleasing method can perhaps help to reduce compassion fatigue in these individuals.

This study showed that, unlike in oocyte collection from female mice, euthanasia of male mice by CO<sub>2</sub> asphyxiation is suitable for collection of epididymal sperm for cryopreservation or other ARTs. We suggest that various innate biologic mechanisms, including the blood–testes barrier, relatively low pH, and higher osmotic pressure in the cauda epididymis maintain the sperm in a relatively acidic and dehydrated state and likely protect mouse sperm from potential damage at the nuclear, cytoplasmic, and cellular level. Our study used C57BL/6 mice because they are one of the most commonly used strains in research, and a common background strain for transgenic mice. We can infer that our results apply to other mouse strains, but future studies using other strains would provide further insight. Thus, for the purpose of collecting mouse sperm for cryopreservation or other ARTs, euthanasia via CO<sub>2</sub> asphyxiation can be used as an alternative method in circumstances where CD is not preferred due to personnel distress and the potential development of compassion fatigue.

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