

Beyond Single-Agent Euthanasia, Gas Combination for Welfare-Refined Rodent Euthanasia: A Narrative Review

(DIBALIK EUTHANASIA AGEN TUNGGAL, PENDEKATAN KOMBINASI GAS UNTUK KESEJAHTERAAN RODENSIA: TINJAUAN NARASI)

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ABSTRACT

Carbon dioxide (CO₂) remains the most widely used euthanasia method for laboratory rodents because of its practicality and cost-effectiveness. Nevertheless, substantial evidence has raised welfare concerns related to hypercapnia-associated distress, nociceptor activation, and aversive behavioral responses prior to loss of consciousness. Inert gases such as nitrogen and argon have been proposed as alternatives, but their welfare implications remain variable and strongly dependent on delivery protocols. This narrative review synthesizes current evidence on gas-based euthanasia methods in laboratory rodents, with particular emphasis on emerging gas combination strategies, including carbon dioxide–nitrous oxide (CO₂/N₂O) mixtures. The review focuses on reported welfare-related outcomes, physiological mechanisms, and practical implementation considerations. A targeted and iterative literature search was conducted using PubMed/MEDLINE, Scopus, and Google Scholar to identify relevant studies published between 2000 and 2025. Experimental and comparative studies, reviews, and relevant regulatory guidelines involving mice and rats were included, and evidence was synthesized narratively due to substantial methodological heterogeneity. Across studies, single-agent gases exhibited distinct but not universally superior welfare profiles. Inert gases avoided hypercapnic acidosis but were associated with hypoxia-related behavioral responses under certain conditions. Selected CO₂/N₂O combinations were reported to achieve shorter induction times and fewer

observable indicators of distress compared with CO₂ alone, although findings were protocol-specific and based on limited evidence. Overall, gas combination approaches represent a context-dependent avenue for refinement rather than a universal replacement, underscoring the need for careful protocol selection and further research.

Keywords: animal welfare; carbon dioxide; gas-based euthanasia; nitrous oxide; rodent euthanasia

ABSTRAK

Karbon dioksida (CO₂) masih menjadi metode eutanasia yang paling banyak digunakan pada rodensia laboratorium karena kepraktisan dan efisiensi biayanya. Namun, berbagai bukti ilmiah menunjukkan adanya masalah kesejahteraan hewan, terutama distress akibat hiperkapnia, aktivasi nosiseptor, serta respons perilaku aversif sebelum kehilangan kesadaran. Gas inert seperti nitrogen dan argon telah diusulkan sebagai alternatif, tetapi dampaknya terhadap kesejahteraan hewan dilaporkan bervariasi dan sangat bergantung pada protokol pemberian. Tinjauan naratif ini merangkum bukti terkini mengenai metode eutanasia berbasis gas pada rodensia laboratorium, dengan penekanan pada strategi kombinasi gas yang berkembang, termasuk campuran karbon dioksida–dinitrogen monoksida (CO₂/N₂O). Fokus kajian meliputi luaran terkait kesejahteraan hewan, mekanisme fisiologis, serta pertimbangan praktis dalam penerapannya. Pencarian literatur terarah dilakukan melalui PubMed/MEDLINE, Scopus, dan Google Scholar untuk mengidentifikasi studi relevan yang dipublikasikan antara tahun 2000 hingga 2025. Studi eksperimental, komparatif, artikel tinjauan, serta pedoman regulasi yang melibatkan mencit dan tikus diikutsertakan, dan bukti disintesis secara naratif karena adanya heterogenitas metodologis yang substansial. Hasil kajian menunjukkan bahwa gas tunggal memiliki profil kesejahteraan yang berbeda, tetapi tidak ada yang secara konsisten unggul. Gas inert dapat menghindari asidosis hiperkapnia, namun dalam kondisi tertentu memicu respons perilaku akibat hipoksia. Beberapa kombinasi CO₂/N₂O dilaporkan membutuhkan waktu induksi lebih singkat dan indikator distress yang lebih rendah dibandingkan CO₂ saja, meskipun bukti masih terbatas dan bergantung pada protokol. Pendekatan kombinasi gas berpotensi menjadi strategi refinement yang kontekstual dan memerlukan penelitian lanjutan.

Kata-kata kunci: kesejahteraan hewan; karbon dioksida; eutanasia berbasis gas; nitrit oksida; eutanasia rodensia

INTRODUCTION

In laboratory animal research, euthanasia represents an unavoidable endpoint for millions of rodents worldwide each year. When inadequately performed, euthanasia procedures can induce pain, distress and pronounced physiological stress responses, with implications extending beyond animal welfare to include compromised experimental validity and adverse psychological effects on research personnel. Within the framework of the 3Rs principles, particularly Refine-

ment there has been increasing ethical, societal and regulatory emphasis on improving the humaneness of euthanasia practices while preserving procedural reliability in scientific research. In several countries, including Indonesia, physical methods such as cervical dislocation are commonly used for euthanizing small laboratory rodents due to practical considerations and established training traditions. When performed by trained personnel, cervical dislocation is recognized as an acceptable method under specific conditions. However, its effectiveness is highly

operator-dependent, and concerns have been raised regarding variability in achieving rapid loss of consciousness and the potential for stress if the technique is im-properly executed. These considerations highlight the need to critically assess eutha-nasia methods from both animal welfare and physiological perspectives. (Flammer *et al.*, 2019; Steiner *et al.*, 2019).

Carbon dioxide (CO₂) remains the most widely used gaseous euthanasia agent for laboratory rodents, especially for laboratory rats and mice (Boivin *et al.*, 2017; Shomer *et al.*, 2020). Its widespread use is largely attributed to practical advantages, including low cost, ease of application, non-flammability and the absence of pharmacological residues in tissues. Many countries, including Indonesia, adhere to internationally recognized guidelines for laboratory animal euthanasia. In Indonesia, the Indonesian Veterinary Medical Association (IVMA), pro-motes veterinary standards and animal welfare, while technical protocols for laboratory rodents are generally based on internationally accepted frameworks, such as those of the American Veterinary Medical Association (AVMA). Nevertheless, substantial animal welfare concerns have been consistently reported. Exposure to CO₂ induces pronounced aversive responses, such as gasping, agitation and escape-related behaviors, prior to loss of consciousness, calling into question its long-standing characterization as a minimally distressing method (Conlee *et al.*, 2005; Steiner *et al.*, 2019; Valentim *et al.*, 2016; Verhoeven *et al.*, 2016). These responses occur during the conscious phase of exposure and are linked to early nociceptive activation associated with hypercapnia. In recognition of these concerns, the 2020 AVMA Guidelines classify CO₂ as “acceptable with conditions,” emphasizing the need for refinement rather than unconditional endorsement (AVMA, 2020).

Inert gases such as nitrogen (N₂) and argon (Ar) have been proposed as alternatives to carbon dioxide (CO₂) due to their ability to induce unconsciousness through hypoxia without causing hypercapnia (Steiner *et al.*, 2019). By displacing oxygen

within the euthanasia chamber, these gases reduce alveolar oxygen partial pressure, leading to cerebral hypoxia and loss of consciousness. Although this mechanism theoretically avoids hypercapnia-associated discomfort, experimental findings have been mixed. Behavioral indicators of aversion have been reported under certain exposure conditions, suggesting that hypoxia alone does not uniformly guarantee a distress-free induction. Consequently, nitrogen and argon are also classified by the AVMA as “acceptable with conditions,” with specific recommendations regarding flow rates, chamber design and limited suitability for neonatal rodents due to their relative tolerance to hypoxia (AVMA, 2020). Comparable regulatory frameworks in other jurisdictions, including European and United Kingdom laboratory animal welfare regulations, also permit the use of inert gases under defined conditions intended to minimize distress (Hawkins *et al.*, 2016; European Parliament and the Council of the European Union, 2010). Collectively, these observations indicate that single-agent gaseous approaches have yet to fully resolve welfare concerns associated with rodent euthanasia.

In response to these limitations, gas combination strategies have emerged as a potential refinement approach aimed at improving welfare outcomes through the integration of complementary physiological mechanisms. Among these, mixtures of CO₂ and nitrous oxide (N₂O) have attracted increasing interest. Such combinations seek to combine the rapid induction associated with low concentrations of CO₂ with the analgesic and anxiolytic properties of N₂O. Experimental studies suggest that selected CO₂/N₂O mixtures may reduce observable indicators of distress and shorten the time to loss of consciousness compared with CO₂ alone. However, reported outcomes vary substantially depending on gas ratios, delivery parameters and experimental context, limiting the generalizability of existing evidence (Thomas *et al.*, 2012).

Nitrous oxide has long been used in clinical and veterinary settings for its rapid-onset analgesic and anxiolytic effects, pro-

viding a biologically plausible rationale for its incorporation into euthanasia protocols designed to mitigate hypercapnia-related discomfort (Thomas *et al.*, 2012). Beyond CO₂/N₂O mixtures, other combination and staged approaches, including the use of volatile anesthetics such as isoflurane or sevoflurane in conjunction with CO₂ or inert gases, have been described (Guedes *et al.*, 2017; Valentim *et al.*, 2016; Marquardt *et al.*, 2018; Thomas *et al.*, 2012). Nevertheless, these strategies remain comparatively underexplored, and their welfare implications are often discussed only briefly within guideline documents or isolated experimental reports rather than through integrative evaluation (Turner *et al.*, 2020).

Despite increasing interest in combination-based approaches, several critical gaps persist. Optimal gas ratios, flow rates and exposure sequences have not been systematically established and direct comparative data across different combination strategies remain limited (Turner *et al.*, 2020; Steiner *et al.*, 2019; Valentim *et al.*, 2016). Moreover, species-, strain-, and age-specific responses are insufficiently characterized, constraining the development of confident, evidence-based welfare recommendations (Turner *et al.*, 2020). Environmental considerations, particularly the high global warming potential of N₂O relative to CO₂, introduce additional complexity into institutional decision-making and underscore the need to balance welfare refinement with sustainability (Hanna and Bryson, 2019; Struys and Eckelman, 2021). Taken together, these unresolved issues suggest that gas-based euthanasia refinement has not yet reached conceptual or empirical closure.

Given the heterogeneity of study designs, welfare endpoints, and reporting standards, a systematic review is currently limited by incomplete comparability across studies. A narrative review approach is therefore well suited to synthesize mechanistic insights, experimental evidence, and guideline perspectives while critically examining the assumptions underlying gas-based euthanasia practices. This review was

aimed to integrate current evidence on gaseous euthanasia methods for laboratory rodents, with particular emphasis on emerging gas combination strategies. The objectives are to evaluate reported efficacy and welfare-related outcomes of single-agent gases and gas combinations; summarize key protocol variables, including gas concentrations, flow rates and chamber design; assess practical implementation considerations such as safety, cost and environmental impact; and identify priority areas for future research to support evidence-based refinement. The scope of this review is limited to mice (*Mus musculus*) and rats (*Rattus norvegicus*), focusing on studies published between 2000 and 2025, with emphasis on evidence from the past decade.

RESEARCH METHODS

Literature Search Strategy

A targeted literature search was conducted to identify relevant evidence on gas-based euthanasia methods in laboratory rodents. Searches were performed in PubMed/MEDLINE and Scopus, with Google Scholar used as a supplementary source. The literature search covered publications from January 2000 to December 2025 to capture both foundational and contemporary evidence. Although this broad time frame was initially applied to ensure comprehensive retrieval of relevant studies, the final selection was effectively narrowed based on predefined inclusion criteria. Most of the included references were published between 2012 and 2021, with one earlier study from 2005 retained due to its relevance to fundamental physiological mechanisms. This approach ensured both scientific rigor and contextual relevance.

Search terms encompassed euthanasia gases, welfare-related outcomes, and rodent species, using combinations of controlled vocabulary and free-text keywords. The search was designed to identify conceptually and methodologically informative studies rather than to achieve exhaustive retrieval.

Reference lists of key articles were manually screened to identify additional relevant publications. The regulatory and professional guidance documents, including the 2020 American Veterinary Medical Association (AVMA) Guidelines and specific institutional guidelines from 2020 to 2025, were included to provide a regulatory framework. Other international frameworks, including European and UK laboratory animal welfare regulations and related guidance from other jurisdictions, were also screened during the review process. However, due to substantial overlap in the scientific evidence base and recommendations across these documents, the AVMA 2020 guideline was selected as the primary regulatory reference because it provides one of the most detailed and widely cited classifications of euthanasia methods in laboratory animals.

Study Selection

Studies were eligible if they provided empirical or authoritative evidence on gas-based euthanasia in laboratory mice or rats, particularly regarding welfare-related outcomes. These two species were chosen as they are the most commonly used lab rodents in biomedical research, with well-documented physiological and behavioral responses to euthanasia gases. Other species like guinea pigs and hamsters were excluded due to species-specific differences that could affect responses to hypoxia and hypercapnia. Experimental and comparative studies evaluating carbon dioxide, inert gases (nitrogen or argon), nitrous oxide, or gas combination protocols were prioritized.

Only full-text publications in English were included to ensure consistent interpretation of methods and terminology. Studies in other languages were screened for English translations; those without were excluded. Studies published in Bahasa Indonesia were also screened, but none met the inclusion criteria.

Evidence Synthesis

Data were extracted with attention to study design, animal characteristics, euthanasia protocols, and reported welfare-related

outcomes. Due to substantial heterogeneity in study designs and outcome measures, findings were synthesized narratively. Studies were grouped by gas type and protocol characteristics, and results were compared qualitatively to identify consistent patterns, methodological limitations, and gaps in the existing evidence. Formal quantitative synthesis or meta-analysis was not performed.

RESULTS AND DISCUSSION

A qualitative comparison of reported physiological mechanisms, induction characteristics and general time course associated with gas-based euthanasia methods is summarized in Table 1.

Single-Agent Gases

Inert gases, including nitrogen and argon, induce loss of consciousness and death through hypoxia by displacing ambient oxygen, resulting in reduced alveolar oxygen partial pressure, arterial hypoxemia and cerebral hypoxia (Shomer *et al.*, 2020). In contrast to carbon dioxide exposure, inert gases do not produce hypercapnia or associated acidosis (Detotto *et al.*, 2019; Gent *et al.*, 2018; Turner *et al.*, 2020; Valentim *et al.*, 2016).

Across experimental studies, the physiological sequence during inert gas exposure is generally consistent, although the timing varies according to species, age, chamber volume and flow rate. Respiratory changes are typically observed within seconds of exposure, followed by impaired coordination and loss of posture. Loss of consciousness is reported to occur within approximately one minute in adult rodents under commonly used protocols, with respiratory arrest and cardiac arrest occurring subsequently when adequate concentrations and exposure duration are maintained (Detotto *et al.*, 2019; Gent *et al.*, 2018; Turner *et al.*, 2020; Valentim *et al.*, 2016).

Carbon dioxide induces unconsciousness through combined hypoxic and hypercapnic mechanisms. Dissolution of CO₂ in mucosal tissues results in carbonic acid

Table 1. Comparative physiological and welfare-related characteristics of gas-based euthanasia methods used in laboratory rodents

Parameters	Gas-Based Euthanasia Methods				
	CO ₂	N ₂	Ar	CO ₂ + N ₂ O	CO ₂ + Volatile anesthetic
Primary physiological mechanism	Hypercapnia with secondary hypoxia	Hypoxia via oxygen displacement	Hypoxia via oxygen displacement	Hypercapnia with analgesic/anxiolytic modulation	Hypercapnia with anesthetic induction
Behavioral distress indicators	Gasping, agitation, escape behaviors reported (Boivin <i>et al.</i> , 2017; Valentim <i>et al.</i> , 2016)	Hypoxia-related motor excitation reported (Detotto <i>et al.</i> , 2019)	Similar responses reported (Valentim <i>et al.</i> , 2016)	Reduced distress behaviors relative to CO ₂ alone (Steiner <i>et al.</i> , 2019)	Minimal observable distress during induction (Alexander <i>et al.</i> , 2018)
Nociceptor involvement	Reported; associated with carbonic acid formation (Conlee <i>et al.</i> , 2005)	Limited evidence	Limited evidence	Reduced relative to CO ₂ alone (Steiner <i>et al.</i> , 2019)	Not consistently reported
Acid-base disturbance	Marked respiratory acidosis	None	None	Reduced respiratory acidosis relative to CO ₂ alone	None
Induction characteristics	Aversive behaviors frequently reported (Boivin <i>et al.</i> , 2017; Valentim <i>et al.</i> , 2016)	Hypoxia-related motor excitation reported (Detotto <i>et al.</i> , 2019)	Similar to nitrogen (Valentim <i>et al.</i> , 2016)	Shorter induction times and reduced behavioral disruption reported (Thomas <i>et al.</i> , 2012)	Rapid induction with reduced behavioral disruption reported (Alexander <i>et al.</i> , 2018)
Relative time to loss of consciousness	Moderate	Moderate	Moderate	Shorter than CO ₂ alone	Shorter than single-agent gases
EEG, suppression relative to inert gases	Delayed cortical suppression	Earlier cortical suppression reported	Earlier cortical suppression reported	Early cortical suppression reported	Early cortical suppression reported
Analgesic properties	None	None	None	Present (N ₂ O-mediated)	Present (volatile anesthetic-mediated)
Mucosal irritation	Frequently reported (Conlee <i>et al.</i> , 2005)	Not consistently reported	Not consistently reported	Less frequent than CO ₂ alone	Not consistently reported
Key limitations	Aversive responses; nociceptor activation	Hypoxia-related distress; neonatal hypoxia tolerance	Similar limitations to nitrogen	Environmental impact (N ₂ O); increased cost and infrastructure	Cost, specialized equipment, occupational exposure considerations

Note: CO₂ = carbon dioxide; N₂ = nitrogen; Ar = argon; N₂O = nitrous oxide. Information is summarized from experimental studies and professional guidelines evaluating physiological and behavioral responses of laboratory rodents to gaseous euthanasia agents. Reported characteristics represent a qualitative synthesis of heterogeneous studies and may vary depending on species, age, and gas delivery parameters.

formation, which precedes respiratory stimulation, followed by respiratory depression and arrest as exposure continues (Turner *et al.*, 2020; Valentim *et al.*, 2016).

Electroencephalographic (EEG) studies indicate that nitrogen exposure produces cortical suppression within a timeframe comparable to, or in some protocols shorter than, that reported for CO₂ exposure (Detotto *et al.*, 2019; Gent *et al.*, 2018; Turner *et al.*, 2020). These measurements reflect loss of cortical activity but do not provide direct information regarding behavioral or subjective experience prior to unconsciousness. When administered according to established protocols, single-agent gases, including nitrogen, argon and CO₂, achieve euthanasia reliably in adult rodents. Reported success rates exceed 99% under conditions of sufficient gas concentration, appropriate flow rate and adequate exposure duration (Detotto *et al.*, 2019; Turner *et al.*, 2020; Valentim *et al.*, 2016). Protocol deviations, particularly insufficient flow rates or premature termination, have been associated with delayed unconsciousness or incomplete euthanasia, with neonates demonstrating increased resistance due to greater hypoxia tolerance (Turner *et al.*, 2020; Valentim *et al.*, 2016).

Gas Combination Protocols

Gas combination protocols incorporate multiple agents with distinct physiological effects. In CO₂/N₂O mixtures, nitrous oxide provides central nervous system effects that occur concurrently with hyper-capnia induced by CO₂. Lower concentrations of CO₂ have been used in combination protocols while maintaining induction of unconsciousness (Steiner *et al.*, 2019; Thomas *et al.*, 2012).

Experimental studies report shorter induction times for selected CO₂/N₂O mixtures compared with CO₂ alone, with outcomes dependent on gas ratios and delivery parameters (Thomas *et al.*, 2012; Valentim *et al.*, 2016). Additional combination strategies include the use of volatile anesthetics, such as isoflurane or sevoflurane, in conjunction with CO₂ or inert gases. These

protocols involve simultaneous anesthetic induction and oxygen displacement and have been evaluated in a limited number of experimental studies (Guedes *et al.*, 2017; Marquardt *et al.*, 2018; Turner *et al.*, 2020; Valentim *et al.*, 2016).

Behavioral Indicators during Gas Exposure

Behavioral responses reported during gas exposure vary across agents and protocols. Avoidance behavior has been documented for both CO₂ and inert gases. Preference and aversion studies indicate that rodents may avoid nitrogen atmospheres at rates comparable to CO₂ under certain experimental conditions (Valentim *et al.*, 2016; Turner *et al.*, 2020; Detotto *et al.*, 2019). Increased locomotor activity prior to loss of consciousness has been reported during nitrogen and argon exposure, particularly under rapid induction conditions. Gradual induction protocols using lower flow rates have been associated with prolonged voluntary exposure times and reduced intensity of motor activity (Valentim *et al.*, 2016; Turner *et al.*, 2020; Detotto *et al.*, 2019). Studies evaluating gas combinations report fewer observable behaviors such as gasping, nasal irritation and escape attempts compared with CO₂ alone (Valentim *et al.*, 2016; Turner *et al.*, 2020; Thomas *et al.*, 2012).

Protocol Parameters

Gas flow rate influences induction time and reported behavioral responses. Slower fill rates are associated with longer induction periods, whereas rapid fill rates are associated with shorter times to loss of consciousness (Valentim *et al.*, 2016; Turner *et al.*, 2020). The AVMA Guidelines permit both gradual-fill and pre-filled approaches when minimum concentration and flow requirements are met, while discouraging excessively slow induction (AVMA, 2020). Chamber design affects exposure conditions. Overcrowding has been associated with increased stress-related behaviors and sufficient space to allow normal posture is recommended. Continued exposure following

respiratory arrest and when necessary, application of a secondary physical method is recommended to ensure death (Valentim *et al.*, 2016; Hawkins *et al.*, 2016).

Species, Strain and Age

Species- and age-related differences influence reported euthanasia outcomes. Mice generally reach loss of consciousness more rapidly than rats under comparable protocols. Neonatal rodents exhibit increased tolerance to hypoxia, reducing the reliability of inert gases as sole agents in this age group (Valentim *et al.*, 2016; Turner *et al.*, 2020). Evidence addressing strain, sex and age beyond young adults remains limited (Hawkins *et al.*, 2016; Steiner *et al.*, 2019).

Environmental Considerations

Environmental considerations represent an increasingly important component of decision-making for gas-based euthanasia protocols. Nitrous oxide (N₂O) is a potent greenhouse gas with a global warming potential substantially greater than that of CO₂ over a 100-year time horizon and a long atmospheric lifetime, raising concerns regarding its cumulative environmental impact even when used intermittently in institutional settings (Struys and Eckelman, 2021). Similarly, volatile anesthetics used in combination protocols, such as isoflurane and sevoflurane, are recognized greenhouse gases, with agent-specific differences in environmental impact; among these, sevoflurane is generally associated with a lower global warming potential than isoflurane (Alexander *et al.*, 2018).

In contrast, inert gases such as nitrogen and argon do not contribute directly to greenhouse gas emissions following atmospheric release. Nevertheless, their overall environmental footprint is not negligible, as energy-intensive processes are required for gas separation, compression, storage and transport (Valentim *et al.*, 2016). To date, integrated life-cycle assessments that explicitly compare animal welfare outcomes with environmental impacts across different euthanasia methods remain scarce, representing

a key knowledge gap identified in recent research roadmaps (Steiner *et al.*, 2019). Consequently, evaluation of gas combination protocols would benefit from consideration of both welfare-related outcomes and sustainability factors, including potential mitigation approaches such as scavenging systems, optimized flow rates and institution-level environmental policies (Struys and Eckelman, 2021).

Single-Agent Gases: Differential Distress Profiles

The available evidence suggests that inert gases such as nitrogen and argon offer theoretical welfare advantages over carbon dioxide by avoiding hypercapnic acidosis and carbonic acid-mediated nociceptor activation (Turner *et al.*, 2020; Valentim *et al.*, 2016). In several experimental settings, these gases have been associated with earlier cortical suppression compared with CO₂ (Gent *et al.*, 2018; Detotto *et al.*, 2019). However, both nitrogen and argon have also been shown to elicit behavioral aversion and increased locomotor activity under certain protocols, raising ongoing uncertainty regarding the nature and intensity of distress experienced prior to loss of consciousness (Detotto *et al.*, 2019; Clarkson *et al.*, 2022). Taken together, the evidence indicates that inert gases represent alternatives with distinct, rather than universally superior, welfare profiles compared with CO₂. Their relative advantages and disadvantages appear highly contingent on delivery parameters and experimental context.

Protocol Dependency of Welfare Outcomes

Across all gas-based euthanasia methods, welfare outcomes are strongly influenced by protocol characteristics rather than gas type alone. Key determinants include flow rate, induction method, chamber design and animal density (Turner *et al.*, 2020; Valentim *et al.*, 2016). Gradual induction protocols may reduce observable aversion but prolong the period of conscious hypoxia, whereas more rapid induction accelerates

loss of consciousness but can intensify motor responses (Detotto *et al.*, 2019).

The acceptance of both gradual-fill and pre-fill approaches under defined conditions in the AVMA Guidelines reflects this inherent trade-off (AVMA, 2020).

Efficacy and Practical Reliability

When properly administered, gas-based euthanasia methods demonstrate high procedural reliability in adult rodents, with reported success rates exceeding 99% (Turner *et al.*, 2020; Valentim *et al.*, 2016). Effective euthanasia depends primarily on maintaining adequate gas concentrations for sufficient durations. Common failure modes include insufficient flow rates, chamber leaks and premature termination of exposure (Valentim *et al.*, 2016; Hawkins *et al.*, 2016).

Unlike CO₂, where overt respiratory distress may signal ongoing exposure, the relatively smooth behavioral transition observed with inert gases and some combinations may obscure inadequate dosing, underscoring the importance of strict timing protocols and verification procedures (Detotto *et al.*, 2019; Turner *et al.*, 2020). Age-related physiological differences, particularly the marked hypoxia tolerance of neonatal rodents, remain a critical limitation for nitrogen and argon when used as sole agents (Turner *et al.*, 2020; Valentim *et al.*, 2016).

Interpreting the Role of Gas Combinations

The mechanistic rationale for gas combination protocols, including CO₂/N₂O mixtures, is outlined in Table 1. Gas combination strategies, particularly mixtures of CO₂ and nitrous oxide, have been proposed as a refinement approach that integrates complementary physiological mechanisms. Nitrous oxide provides rapid analgesic and anxiolytic effects through *N-methyl-D-aspartate* (NMDA) receptor modulation and opioid pathway activation, potentially attenuating discomfort associated with hypercapnia, while lower concentrations of CO₂ retain the capacity to induce relatively rapid loss of consciousness (Valentim *et al.*, 2016; Steiner *et al.*, 2019; Thomas *et al.*, 2012; Zorumski

et al., 2015).

Experimental studies have consistently reported shorter induction times and reduced observable behavioral indicators of distress for selected CO₂/N₂O combinations compared with CO₂ alone (Thomas *et al.*, 2012). While these findings support the potential welfare advantages of combination protocols, they are derived from a limited number of experimental models and gas ratios and should therefore be interpreted cautiously (Turner *et al.*, 2020).

From a practical perspective, gas combinations may be most appropriate in settings where welfare benefits are prioritized and infrastructure investment is feasible, particularly when CO₂-related physiological effects could confound research outcomes or exacerbate underlying respiratory conditions (Turner *et al.*, 2020; Steiner *et al.*, 2019). However, broader adoption will depend on further standardization and clearer guidance.

Contradictions and Knowledge Gaps

Variability in reported welfare outcomes across studies largely reflects methodological heterogeneity, including differences in flow rates, chamber dimensions, acclimatization procedures and behavioral endpoints assessed (Valentim *et al.*, 2016; Turner *et al.*, 2020). Interpretation of behavioral indicators remains particularly challenging, as locomotor activity during hypoxia may represent physiological reflexes rather than subjective suffering (Turner *et al.*, 2020; Hawkins *et al.*, 2016).

Key knowledge gaps persist, including the absence of validated, standardized distress scoring systems for gaseous euthanasia, limited systematic evaluation of optimal gas ratios and flow rates for combination protocols, incomplete understanding of the neurobiology underlying hypoxia-related distress and sparse data for neonates, aged animals and disease models. Environmental impact assessments and cost-benefit analyses integrating welfare, sustainability and financial considerations are also notably limited (Turner *et al.*, 2020; Hawkins *et al.*, 2016; Steiner *et al.*, 2019).

Practical Considerations for Implementation

Adoption of gas combination protocols requires infrastructure exceeding that of conventional CO₂ systems, including precision gas delivery, sealed chambers, scavenging systems, monitoring equipment and comprehensive personnel training (Valentim *et al.*, 2016). Initial and ongoing costs vary substantially depending on facility scale and mitigation requirements. Risk management necessitates adequate ventilation, gas monitoring, emergency preparedness and routine equipment maintenance (Hawkins *et al.*, 2016).

Institutional decision-making should therefore consider euthanasia volume, species and age groups, research objectives, budgetary constraints, regulatory context, personnel expertise and environmental priorities. Pilot implementation with rigorous welfare monitoring may provide a prudent pathway prior to broader adoption (Hawkins *et al.*, 2016; Valentim *et al.*, 2016).

Regulatory and Ethical Considerations

Current AVMA Guidelines classify CO₂, nitrogen, argon and gas combinations as “acceptable with conditions,” emphasizing adherence to specific concentration, flow-rate and age-related recommendations (AVMA, 2020). European guidance documents increasingly acknowledge gas combinations as refined alternatives, although institutional policies remain variable (Hawkins *et al.*, 2016; Steiner *et al.*, 2019).

From an ethical perspective, refinement requires continuous critical appraisal of emerging evidence and transparent consideration of both benefits and limitations. Gas combinations represent a promising avenue for refinement, but responsible implementation must balance immediate animal welfare gains against environmental impact, resource allocation, and operational feasibility (Hawkins *et al.*, 2016; Steiner *et al.*, 2019).

CONCLUSION

This narrative review highlights that no single gaseous method is universally optimal; rather, welfare outcomes are shaped by a complex interaction between gas properties, delivery protocols, animal characteristics and institutional context. Single-agent carbon dioxide continues to be widely used due to its practicality, while inert gases such as nitrogen and argon should be viewed as alternative approaches with different welfare trade-offs. Gas combination strategies, particularly mixtures of carbon dioxide and nitrous oxide, offer a promising refinement approach by integrating complementary physiological mechanisms. Reported reductions in induction time and observable distress indicators support their potential to improve welfare outcomes compared with single-agent gases. However, the current evidence base remains limited in scope, protocol-specific, and methodologically heterogeneous, precluding definitive recommendations for universal adoption. In conclusion, gas combinations represent a meaningful and evolving avenue for refinement of rodent euthanasia practices, but their implementation should proceed cautiously and evidence informed.

SUGGESTION

Future research on gas-based euthanasia in laboratory rodents should focus on developing standardized and validated welfare assessment tools specific to gaseous euthanasia protocols. Systematic evaluation of gas combination parameters, particularly carbon dioxide–nitrous oxide mixtures, across different species, ages and health conditions is needed to define optimal protocols. Further studies exploring the neurobiological mechanisms underlying hypoxia-related distress, as well as the impact of euthanasia methods on research outcomes and environmental sustainability will support evidence-based refinement and responsible implementation of humane euthanasia practices.

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