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Preliminary Evidence Regarding the Detection of Cortisol and IL-6 to Assess Animal Welfare in Various Rabbit Housing Systems

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Abstract: The main purpose of this study was to investigate the influence of three different rabbit rearing systems using animal-based measures. Therefore, in 2021, cortisol and IL-6 were assessed during a Biosecurity System pilot program to evaluate rabbit welfare, based on EU Regulation 2016/429 in the Apulia region of Southern Italy. The study was carried out on a total of 45 meat-type rabbits, divided into 3 groups of 15 subjects, aged approximately 60–65 days. Groups came from either: (i) a niche open-air system; (ii) a niche system in a shed with conventional cages, or (iii) an industrial system. Based on our findings, the rearing system had a significant effect ($p < 0.0001$) on the animal-based measures. Cortisol levels were higher in rabbits raised in the niche open-air system compared to those raised in both the niche system located in a shed with conventional cages and the industrial system (11.91 vs. 2.86 and 2.72 ng/mL, respectively). Likewise, IL-6 values were higher in rabbits from the niche open-air system (45.80 ng/mL) and lower in rabbits from the niche system in a shed with conventional cages and those from the industrial system (23.30 and 16.80 ng/mL, respectively). According to the results of the stress indicators measured, cortisol and IL-6, rabbit welfare and meat quality may be affected by rabbit rearing systems.

Keywords: rabbit; rearing system; welfare; cortisol; IL-6

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1. Introduction

The significant decline in global rabbit meat production in the decade of 2010–2019 [1,2] can be explained by considering the changes affecting this form of animal husbandry. The absence of specific legislation regarding meat rabbit welfare on farms negatively impacts the image of this livestock sector, its sustainability, and consumer demand [3]. In addition, the increasing perception of rabbits as being pets also plays a key role [4]. Therefore, there is a need to reassure the consumer regarding animal welfare, to address the current regulatory deficiency, and to inform farmers about better, more innovative rearing and animal management methods that will make the rabbit-rearing sector competitive in the European and global markets [5]. Accordingly, in 2017, the European Parliament approved the resolution "Minimum standards for the protection of farm rabbits" (2016/2077(INI)), in which it called on members of the European Union, as the world's second largest producer of rabbit meat, to submit legislative proposals on the establishment of minimum requirements for the protection of rabbits on farms [5,6]. The most innovative element of the above-referenced parliamentary resolution is the goal set for 2027, which is to eliminate the use of cages by switching to pen/park systems, i.e., alternative housing methods that provide more space for movement and the chance for rabbits to interact in groups. Similarly, the EFSA recently published a scientific opinion entitled "Health and welfare of rabbits farmed in different production systems" (2020) [7]. Based on the mandate received from the European Parliament's Agriculture and Rural Development (AGRI) Committee in drafting the paper, the EFSA Animal Health and Welfare (AHAW) Panel considered six different rearing systems: (i) conventional cages, (ii) structurally enriched cages, (iii) elevated pens, (iv) floor pens, (v) outdoor/partially outdoor systems, and (vi) biological/organic systems. The AHAW Panel also considered three production categories: (a) reproducing does, (b) kits, and (c) growing rabbits [7]. Rabbits' biological, physiological, and ethological traits, as well as prevailing diseases, hygiene, and sanitary measures implemented on farms were considered. These proposals, supported by scientific studies, should be formulated by considering the connection between animal health and welfare [8], ensuring biosecurity on and between farms, and encouraging the prudent and sustainable use of drugs through the ClassyFarm System [3]. In order to assess and compare welfare conditions between animals raised in different rearing systems, direct indicators—also known as animal-based measures (ABMs)—and indirect indicators—i.e., resource- and management-based measures (RBMs and MBMs)—were used [9]. The most frequently used ABMs include cortisol and IL-6. Cortisol release is part of the endocrine self-protection mechanism of the body which deals with stressors [10]. This glucocorticoid is also able to stimulate glycogen mobilization, its conversion into energy, and, consequently, meat acidification [11,12]. Furthermore, blood cortisol content, which is a subjective characteristic of each individual, may affect the amount of body fat, meatiness, and thus, carcass quality, as has been observed in pigs and other animal species [10,13]. The quantification of cortisol or its metabolites—in saliva, plasma, feces, urine, and milk—by ELISA is a physiological indicator for assessing stress [14,15]. IL-6 is a pleiotropic cytokine that activates the JAK/STAT signaling pathway and exerts both pro- and anti-inflammatory action [16].

The contemporary evaluation of plasma cortisol and of IL-6 can be explained by considering the physiological response of the body to stressors, both chemical–physical and cognitive, involving the hypothalamic–pituitary–adrenal (HPA) axis and the immune system [10,17].

Released by the cortical layer of the adrenal glands upon adrenocorticotropic hormone (ACTH) stimulation, cortisol reaches the immune system through the bloodstream. Under physiological stress conditions, the immune system is activated and releases pro-inflammatory cytokines, including IL-6, which reach specific receptors at hypothalamic and pituitary levels via the humoral and neural pathways [16]. The presence within the bloodstream of an adequate amount of these chemical mediators is indicative of an appropriate inflammatory response and leads to deactivation of the HPA. However, under chronic stress conditions, there is an overstimulation of the HPA by the chemical mediators involved and a reduction in levels of specific receptors available in the central nervous system (CNS). Therefore, this self-regulation mechanism loses its physiological balance [17]. Taking into account this information, elevated plasma levels of IL-6 may be found in conditions of acute or chronic stress, but it can also be found in other cases as well. In agreement with some researchers, an increase in this chemical mediator could be associated with (i) long- or short-term inflammatory processes [17], (ii) intense physical exercise [18], or (iii) osteoarticular trauma and multiple organ injury [19].

On the basis of several studies, Pedersen et al. [18,20–22] showed that IL-6, produced and secreted by both skeletal muscle myofibers and adipocytes can be classified as both a myokine and an adipokine. IL-6 is defined as a myokine because it increases over the course of exercise, especially where this is prolonged and without muscle damage. Plasma IL-6 increases would be related to exercise duration, intensity, the mass of muscle recruited, and its endurance capacity [20,21]. The production of this myokine contributes to the protective effect that regular exercise has on chronic diseases (cardiovascular diseases, dementia, depression, and type 2 diabetes). Moreover, IL-6 enables skeletal muscles to act both directly and indirectly on other organs (brain, liver, adipose tissue, and cardiovascular system) with a beneficial effect on health [22].

However, during physical exercise, IL-6 is also produced to a lesser degree by fat tissue, peritendinous tissue, and the brain [18,23]. Its secretion in the brain is performed by astrocytes, which are activated in the presence of brain injury, inflammation, hypoxia, and some diseases [24]. On the other hand, in the hypothalamic nuclei, IL-6 expression is observed, above all, in cases of long-term stress [25,26]. In addition, IL-6 is also involved in the regulation of appetite, body composition, and energy expenditure [27]. Taking into account these considerations, it is possible to theorize that its release is aimed at increasing hepatic glucose production. The brain would, in fact, release IL-6 to avoid hypoglycemia resulting from the increased uptake of glucose into contracting skeletal muscles [28].

Therefore, in 2021, during a Biosecurity System pilot program based on EU Regulation 2016/429 in Apulia (Southern Italy), cortisol and IL-6 were evaluated and compared in three different rabbit rearing systems. The purpose of this study was to investigate the influence of the three distinct rabbit rearing systems on the considered ABMs.

2. Material and Methods

2.1. Sampling

The survey, resulting from a partnership between the Avian Diseases Section of the Department of Veterinary Medicine at the University of Bari "Aldo Moro" (Italy) and three distinct rabbit farms in the Apulia region of Southern Italy, was carried out in November 2021.

The study was carried out on a total of 45 meat-type rabbit hybrids of both sexes, aged approximately 60–65 days when sampling took place. Each group of the considered rabbits, composed of 15 subjects, came from a different rearing system: (i) a niche open-air system (NOAS), (ii) a niche system in a shed with conventional cages (NSS), or (iii) an industrial system (IS) [6]. The three groups of animals received the same commercial feed ad libitum.

The NOAS (Figure 1) was characterized by an enclosure measuring approximately 700 m^2, equipped with an external fence made of 2–3 cm galvanized metal mesh, measuring about 1.8 m high, and buried to a depth of about 20 cm, which was needed to protect the rabbits from wild animals. Inside the enclosure, the rabbits were managed on the ground where there were trees and bushes, which provided shelter and shade for the animals during the warmer periods, as well as a hut in which the drinking troughs were placed and which were managed manually. The enclosure also featured tunnels and galleries dug by the rabbits themselves and used as dens, both by adults and by females with litters. Males and females were reared together, in a ratio of about one male to every three females. Both fertilization and weaning of the young occurred naturally and without any interference from the farmer.

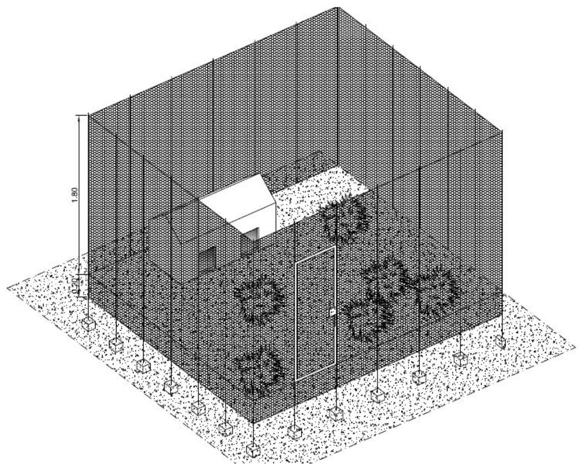

Figure 1. Niche open-air system (NOAS). For the sake of clarity, the enclosure's ceiling has been omitted.

In the NSS (Figure 2), rabbits were housed in conventional cages composed of galvanized mesh and arranged in two rows adjacent to the masonry walls of a shed, equipped with natural lighting and ventilation. The cages measured 60 cm in length by 45 cm in width. As concerns reproduction management, males were reared separately from females in a second prefabricated facility, in single conventional cages of a similar size. In this rearing system, natural insemination was adopted, involving the momentary transfer of a female for 2–3 days to a male's cage to allow natural mating to take place.

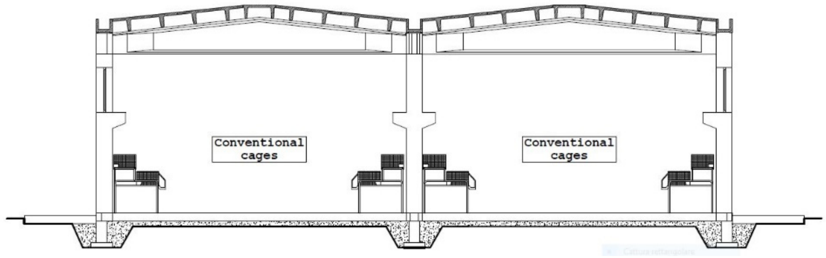

Figure 2. Niche system in a shed with conventional cages (NSS).

In the IS (Figure 3), rabbits were housed in small groups of about 7-8 subjects, corresponding to the brood of rabbits after the reproducing doe had been removed, in enriched cages, also known as WRSA cages, designed in accordance with the World Rabbit Science Association (WRSA) requirements [29].

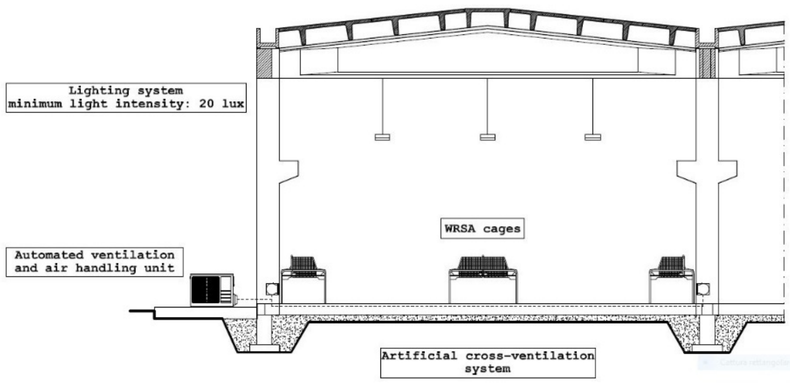

Figure 3. Industrial system (IS).

The cages measured 90 × 30 cm, wider than conventional ones [30], and were equipped with an automatic water and food distribution system and a larger surface area (an extra 74 cm^2), along with a footrest and a raised platform. Furthermore, they were arranged in single-deck rows (a flat-deck system) so as to facilitate handling of the animals.

The shed was equipped with an artificial cross-ventilation system with air intakes positioned at floor-level, thus avoiding direct air flow onto the animals [7]. As regards lighting, a controlled lighting program was adopted, consisting of 16 h of light: 8 h of dark (16L:8D), providing a minimum intensity of 20 lux (according to EFSA guidelines) and with a half-hour crepuscular transition [7].

In this rearing system, the rabbit productive cycle was managed in a cyclical manner, using artificial insemination (AI) of the does upon the weaning of their previous litters, which took place at around 35 days of age.

Blood samples were collected once from each rabbit at the same time (9:00 a.m.) on each farm on 3 consecutive days in order to exclude a circadian variation of cortisol levels.

Although there were few differences between the capture times in the three types of rearing systems considered, the same properly trained personnel were involved, and samples were collected within 2 min. While Massányi et al. [31] showed that sampling techniques do not affect plasma cortisol levels, blood sampling was carried out using the same technique for all of the rabbits. Therefore, as one operator held the rabbit, covering the animal's eyes and head with one hand and holding its pelvis with the other hand, the second operator collected the blood from the saphenous vein [32]. The blood samples were collected in vacutainer test tubes containing ethylenediaminetetraacetic acid (EDTA) and stored on ice at 0 °C for no longer than 60 min, thus avoiding freezing, before being sent to the reference laboratory.

Samples were processed to evaluate cortisol and IL-6 levels, respectively, using a Rabbit Cortisol ELISA Kit and a Rabbit IL-6 ELISA Kit.

2.2. Plasma Cortisol and IL-6 ELISA Test

Plasma cortisol and IL-6 were assessed following the methodology proposed by Ceci et al. [33]. The ELISA cortisol and IL-6 immunoassay were utilized following the manufacturer's guidelines (Rabbit Cortisol ELISA; My-Bio-Source, San Diego, CA, USA, and Rabbit IL-6 ELISA; My-Bio-Source, San Diego, CA, USA, respectively). All reagents were kept at room temperature (25–28 °C) for 30-40 min before being reconstituted. The enzyme conjugate was stored at −20 °C until use in the analyses.

Both ELISA tests were performed using a DYNEX DSX® fully automated four-plate ELISA processing system. DSX® is a proven automated open system able to perform several assays per plate at the same time, boasting optimized efficiency and speed. It uses a perfectly synchronized stem to eliminate plate drift and to ensure premium consistency across four different plate incubators.

2.3. Statistical Analysis

The values for Cortisol and IL-6 were used to compare the three rearing systems, i.e., NOAS, NSS, and IS. Statistical analysis was performed with the GraphPad Prism software, version 9. Data were analyzed with one-way ANOVA to reveal any differences among the three groups.

3. Results

The study assessed two ABMs: plasma cortisol and plasma IL-6. Three different rearing systems for rabbits were evaluated in order to investigate their influence on cortisol and IL-6 as animal welfare indicators [34]. Our findings showed that the rearing methods had a significant impact ($p < 0.0001$) on the stress indicators considered. Indeed, rabbits reared in NOAS showed average plasma cortisol levels of 11.91 ng/mL, whereas rabbits housed in NSS and IS had average plasma cortisol levels of 2.86 ng/mL and 2.72 ng/mL, respectively (Table 1). Regarding IL-6, this cytokine developed in line with cortisol, showing an average value of 45.80 ng/mL recorded in the NOAS-housed rabbits and lower values (respectively 23.30 ng/mL and 16.80 ng/mL) in rabbits housed in NSS and IS (Table 1). In particular, plasma cortisol levels ranged between 2.30 ng/mL and 26.90 ng/mL; 0.50 ng/mL and 10.70 ng/mL; and 0.50 ng/mL and 5.40 ng/mL, in NOAS, NSS, and IS rabbits, respectively (Figure 4). Moreover, plasma IL-6 levels ranged between 39.90 ng/mL and 56.30 ng/mL; 19.10 ng/mL and 27.50 ng/mL; and 12.10 ng/mL and 22.60 ng/mL, in NOAS, NSS, and IS rabbits, respectively (Figure 5).

Table 1. Effect of rearing systems on ABMs.

	NOAS	NSS	IS	*p*-Value
Cortisol	11.91 ^{a} (7.577)	2.860 ^{b} 2.940	2.723 ^{b} (1.714)	<0.0001
IL-6	45.80 ^{a}	23.30 ^{b} (2.942)	16.80 (2.866)	<0.0001

Letters "^{a}" and "^{b}" indicate significant differences among groups. Standard deviations are recorded within parentheses. The cortisol and IL-6 levels are expressed as ng/mL (μ ± standard deviation).

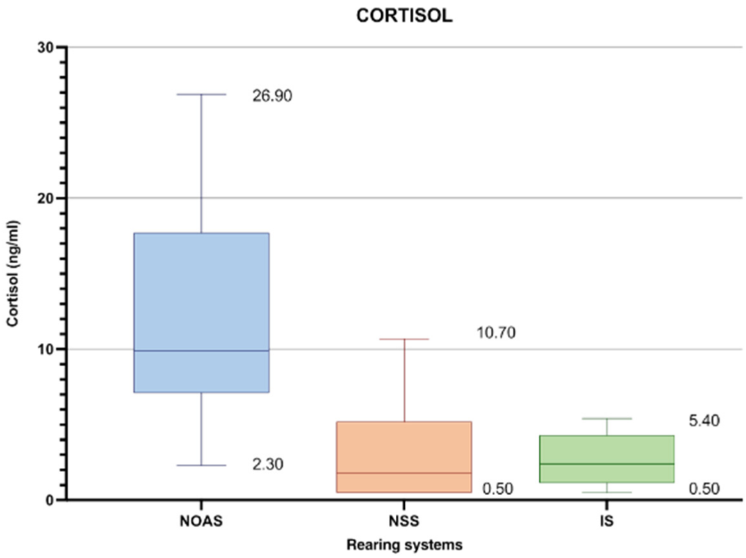

Figure 4. Distribution of cortisol in the three groups of animals.

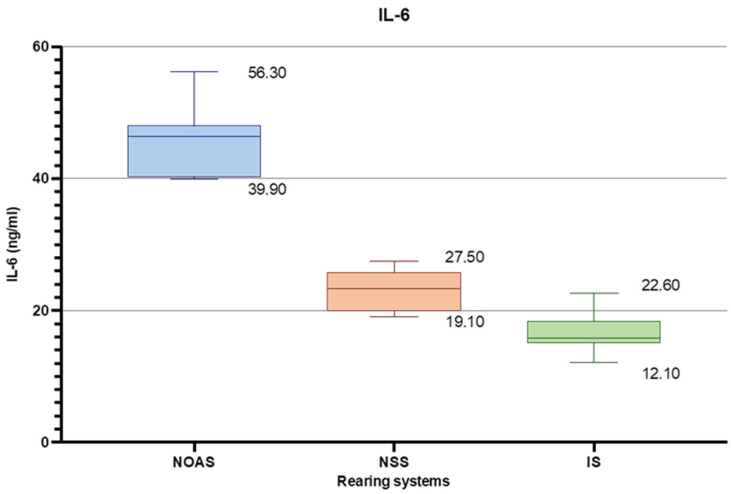

Figure 5. Distribution of IL-6 in the three groups of animals.

4. Discussion

The higher levels of IL-6 observed in the rabbits reared in NOAS could be related to increased exercise, in agreement with Pedersen and Febbraio [18], or to stress due to fights for rank order [35]. Both these situations are part of normal rabbit behavior although they could have negative consequences on the rabbits' welfare. On the other hand, the higher levels of this ABM parameter could be related to stress due to (auditory, olfactory, and visual) perceptions of the presence of predators near the enclosure, despite the presence of the safety fence [35]. At the same time, in this group-rearing system, rabbits have more opportunities to move around, digging holes and tunnels as dictated by their ethological needs [35]. However, as highlighted by other authors [35,36], fights are also frequently observed, with negative effects on (i) reproductive performance, (ii) rabbit lifespan, and (iii) animal welfare. Indeed, aggressiveness is a recognized trait of wild and domestic European rabbits and is one of the main rearing issues in rabbit groups [36]. In addition, living in a group, especially on the ground, involves a greater risk of disease (especially enteric diseases) with potentially higher production costs for therapeutic treatments and/or stock replacement [35].

As concerns rearing systems with conventional cages, such as in the NSS system evaluated in our study, some researchers have shown that, although rabbits have less space to move around and fewer opportunities for social interaction [35,37], there are also fewer injuries and lower mortality rates [38]. Furthermore, in agreement with Perez-Fuentes et al. [38], this system also results in lower ABM levels than does the NOAS system (Table 1).

The IS system, in agreement with Szendrő et al. [35], showed lower concentrations of cortisol and IL-6 than the other two rearing systems considered in our study (Table 1). This could be explained by considering that each rabbit would feel safe in a complex and enriched environment that it can control, one in which it can grow while interacting with the rest of its brood [39].

Consequently, while the IS system with WRSA cages involves quite high investment costs for farmers, it does improve animal welfare levels (providing a wider area of activity and a lower incidence of behavioral stereotypes and fights) [28].

On the basis of our findings, plasma cortisol and IL-6 levels detected in NOAS were more variable (Figures 4 and 5) because this rearing system is less controllable than the other two considered (Table 1). These findings would seem to show that this group-rearing system does not fully meet rabbits' needs and would negatively impact their welfare, even though this type of husbandry system might be expected to ensure the lowest stress levels in rabbits, given that it provides the animals with an opportunity to perform a species-specific behavioral repertoire [35,40]. Indeed, the cortisol levels observed in NOAS-reared rabbits are close to those observed in rabbits during transport and awaiting slaughter [41,42].

As argued by several authors [41–46], meat from transported rabbits frequently has a higher pH (pH > 5.8), a greater ability to bind and retain water, is darker, and is less tender (even after cooking) than that of non-transported rabbits [44]. These characteristics have earned this type of meat the classification of Dark Firm Dry (DFD) [47–49]. The above-mentioned studies showed the existence of a connection between the body's physiological stress response and meat quality [50]. Indeed, cortisol released in response to stress stimulates glycogen mobilization and starts the depletion of glycogen stores in muscles [10,12]. This leads to a decline in postmortem lactic acid production and an increase in the meat's pH [10,51].

Meat produced by stressed animals deteriorates faster than that from resting animals as a direct consequence of the final pH reached at the end of the rigor mortis process. On the other hand, in meat produced by non-stressed animals, after slaughter, the pH value drops because 1% of the glycogen is converted into lactic acid from around 7.4 to approximately 5.6, depending on the species [47]. Moreover, higher pH values make meat more susceptible to microbiological contamination [52]. Variations in pH, resulting from the release of cortisol, not only reduce its tenderness, cut its resistance, and shorten its shelf life, but they also affect its color due to the pH fluctuations in the early post-mortem period [50]. This can be explained by considering that, in high pH conditions, such as stress, muscle proteins can hold a large quantity of water, creating a barrier to oxygen. This allows purplish-red deoxymyoglobin to dominate other myoglobin forms, making the meat darker [53].

Although this is a preliminary study that only analyses different rearing systems based on assessing welfare through ABMs (cortisol and IL-6), it offers plenty of food for thought. Indeed, as illustrated by other authors [54–56], the effects on rabbit growth performance, as well as on carcass and meat quality [56–58], showed that animals housed in cages had: (i) a higher percentage of fat deposits, (ii) better production than rabbits raised in large pens, and (iii) a higher weight at slaughter [59].

5. Conclusions

The results of this survey seem to highlight that rearing systems do have an impact on the ABMs (cortisol and IL-6) assessed. Although NOAS is a rearing system that meets both the European requirement to eliminate cages by 2027 and the ethical concerns of modern consumers [60], the ABMs (cortisol and IL-6) measured in our study showed that rabbits raised in NOAS had greater levels of cortisol and IL-6 than those observed in rabbits raised in cages (whether NSS or IS with WRSA cages). These earliest results need to be validated by further studies in which plasma cortisol levels and IL-6 levels are combined with other animal-based indicators of welfare, such as behavioral, health, and meat-quality data.

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Data Availability Statement: Data is contained within the article.

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Conflicts of Interest: The authors declare no conflict of interest.

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