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Review

Application of natural antioxidants in animal reproduction

Aplicação de antioxidantes naturais na reprodução animal

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Abstract
Antioxidants are natural or synthetic substances that delay oxidation through one or more mechanisms, such as scavenging free radicals, inhibiting lipid peroxidation, and complexing with metals, inhibiting tissue destruction via oxidation. Antioxidants are commonly used in animal feed and the food industry to prevent the oxidation of animal-origin products. Moreover, natural oxidants are used increasingly in animal reproduction, especially for semen preservation. In this context, this study aimed to review the applications of natural antioxidants in animal reproduction. We observed that the bulk of the natural antioxidants, approximately 80.4%, were commercially acquired and used mainly for semen cooling/freezing (72%) with promising results (90%) in *Sus scrofa* (boar), *Capra aegagrus hircus* (goat), *Gallus gallus domesticus* (rooster), and *Ovis aries* (ram). However, further studies are needed to help determine the appropriate dosage of natural antioxidants for applications.
Keywords: cryopreservation; free radicals; lipid peroxidation; oxidative stress; vitamin E.

Resumo
Antioxidantes são substâncias naturais ou sintéticas que facilitam o retardo da oxidação por um ou mais mecanismos, como sequestrar radicais livres, inibir a peroxidação lipídica e complexar com metais, inibindo a destruição tecidual via oxidação. Antioxidantes são comumente usados na alimentação animal e na indústria alimentícia para prevenir a oxidação de produtos de origem animal. Além disso, os oxidantes naturais estão sendo cada vez mais aplicados na reprodução animal, principalmente na preservação do sêmen. Nesse contexto, este trabalho teve como objetivo revisar a aplicação de antioxidantes naturais na reprodução animal. Observamos que os antioxidantes naturais foram geralmente adquiridos comercialmente (80,4%) e utilizados principalmente no resfriamento/congelamento de sêmen (72%) com resultados promissores (90%) em *Sus scrofa* (javali), *Capra aegagrus hircus* (cabra), *Gallus gallus domesticus* (galo) e *Ovis aries* (carneiro). No entanto, mais estudos devem ser realizados para ajudar a regular a dosagem de antioxidantes naturais para sua aplicação.
Palavras-chave: criopreservação; radicais livres; peroxidação lipídica; estresse oxidativo; vitamina E.

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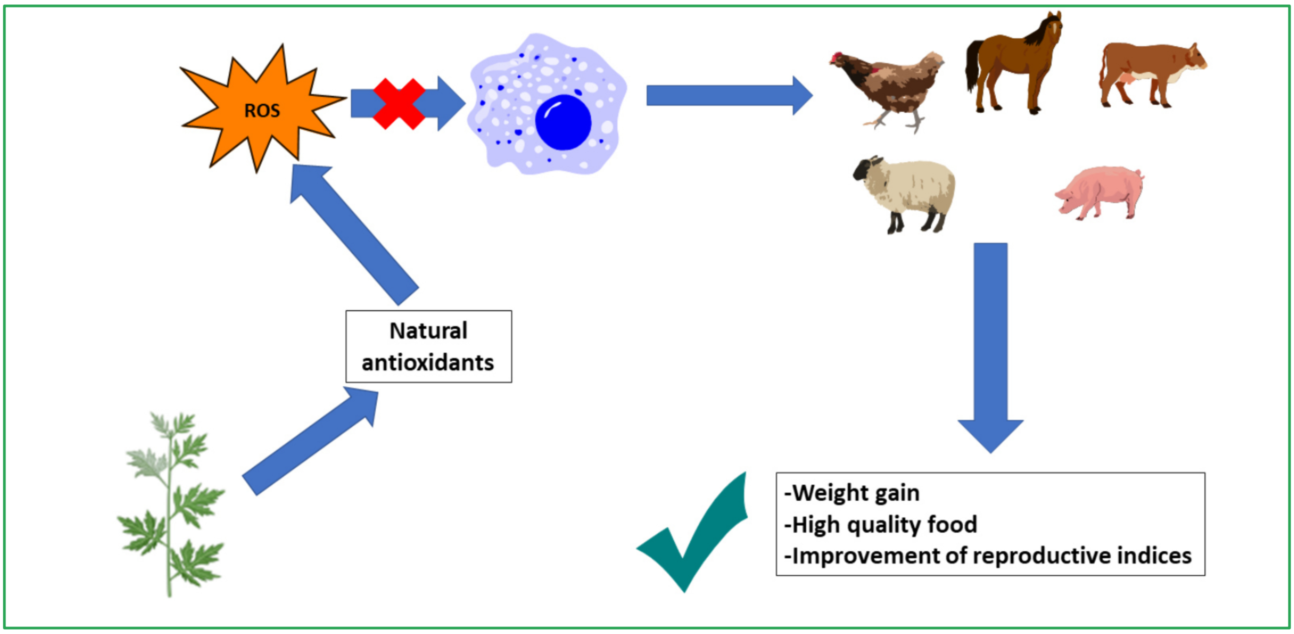

Graphical abstract: Application of natural antioxidants in animal reproduction

Introduction

Free radicals are highly reactive atoms or molecules that contain one or more unpaired electrons [(1)] and are generated during biological processes engendered through endogenous compounds or the metabolism of exogenous compounds. The free radical species are produced primarily in the cytoplasm, mitochondria, and cell membranes, and their cellular targets, i.e., proteins, lipids, carbohydrates, and DNA, are related to the site of their formation [(2)]. During a normal physiological state, a moderate production of reactive oxygen species (ROS) forms an integral part of metabolism. ROS are produced during physiological processes involved in energy production, cell growth, phagocytosis, intracellular signaling, and synthesizing essential substances such as hormones and enzymes [(3,4)].

In response to ROS production and its potential adverse effects, organisms have developed antioxidant systems. However, in situations of an imbalance, compromising the systems of antioxidant defense, the excess ROS generated causes oxidative stress in various cellular structures [(5,6)], and the process is divided into three phases: general adaptation syndrome, resistance, and exhaustion. At first, there is a rapid system alert when an organism attempts to adapt to and recover cellular ROS homeostasis. The absence of such recovery caused by the durability or intensity of stress leads to exhaustion, compromising the health and life of the organism [(7)].

The natural antioxidant defense system of the body partially reduces oxidative stress via the action of enzymes, including superoxide dismutase, catalase, and glutathione peroxidase [(8)], which are involved in the removal of oxygen and hydrogen peroxide [(9)]. In addition, the intake of bioactive compounds containing antioxidant properties stabilizes free radicals, generates competition for the active sites and receptors of various cellular structures, and modulates the expression of genes encoding proteins involved in defense against oxidative and degenerative processes in such cellular systems [(10)]. These bioactive compounds can be nutrients essential for body functions as well as non-nutrients not mandatory for regular body functioning but for improving health through active and protective roles [(11)].

Generally, antioxidants are classified as primers, oxygen scavengers, biological antioxidants, chelating agents, or mixed antioxidants. Primers are phenolic compounds that remove or inactivate free radicals formed during metabolic reactions. Oxygen scavengers capture O_2 from the medium, making it unavailable for autoxidation. Biological antioxidants are enzymes, such as glucose oxidase, which remove oxygen or reactive compounds from ready-to-eat foods. Chelating agents act on the complexation of metal ions, thereby catalyzing lipid oxidation. Finally, mixed antioxidants comprise animal- and plant-derived compounds, such as flavonoids and hydrolyzed proteins [(12,13)].

Phytochemical antioxidants

Most natural antioxidants are plant derived. These phytochemicals are active non-nutrients, which upon entering a cell, eliminate free radicals, generating chemical or electrophilic stress signals, in turn regulating numerous cells signaling pathways [19]. Polyphenols are the primary group of compounds responsible for the antioxidant activities in plants. In addition to playing a role in pigmentation, photoprotection, and defense against microorganisms and insects, polyphenols inhibit free radical production, cell proliferation, and inflammation, and modulate the activity of specific enzymes in plants [20]. Among the phenolic compounds, we can mention phenolic acids, lignans, stilbenes, *e.g.*, resveratrol, and flavonoids, *i.e.*, flavones, flavanones, catechins, and anthocyanins. The distribution and quantity of these compounds vary among plants, influencing the antioxidant capacity of each species[21,22].

The antioxidant activity of natural antioxidants derived from plants is as high as that the activity of synthetic antioxidants, making these products attractive to industries, mainly because of the increased consumer demand for natural ingredients [23]. Extraction of antioxidants using solvents of differing polarities is needed to isolate and identify the antioxidant compounds in natural sources, such as fruits, seeds, and spices. However, before starting the extraction process, a few preliminary steps are required to facilitate the process and preserve the properties of the antioxidant compounds, which are highly sensitive to the action of light, oxygen, and heat [24]. Hence, vegetables are usually dehydrated, freeze-dried or frozen, and sieved or ground before extraction to increase the exposure of the contact surfaces of the substrate to the extraction solvent and to inactivate the enzymes lipoxygenase and polyphenol oxidase, which oxidize phenolic compounds. These enzymes are naturally present in plants and are responsible for enzymatic oxidative rancidity [25].

The antioxidant content in plant-derived samples is determined either in complex mixtures or isolated compounds for bioprospecting or for confirming and quantifying the efficiency of the extractive methods. Antioxidant activity measurements can be performed in several ways [17], the simplest being the total phenol content measurement, which quantifies all phenols in the sample. A standard curve is plotted using a known phenolic compound, i.e., gallic acid, to analyze the total phenol content. Hence, the result is expressed in units of the phenolic compound used, for example, in gallic acid equivalents. However, the analysis is not the most representative as several phenolic compounds have a pro-oxidant rather than an antioxidant function. Methods for determining total anthocyanin and flavonoid content in plant samples also exist. In addition, chromatographic techniques are used for identifying and quantifying specific antioxidant compounds [26].

Several analytical methods have been used to determine the antioxidant activities in plant extracts or isolated compounds based on different redox reactions. Thus, the results of these various methods cannot be compared [17]. In vitro assays determine the ability of plant antioxidants to scavenge free radicals generated in the reaction medium, stable radical species induced by antioxidants, or oxygen radicals generated through enzymatic processes [21].

Among the best-known spectrophotometric methods for determining the antioxidant capacity of plant-derived substances are ferric reducing antioxidant power (FRAP), oxygen radical absorbance capacity (ORAC), cation scavenging diammonium salt 2,2′-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS), and 2, 2-diphenyl-1-picryl-hydrazyl (DPPH) radical scavenging assays. The latter is the most widely used method, suitable for analyzing soluble antioxidants in organic media and applied widely in evaluating the antioxidant activity in fruits [27,28].

The FRAP method is based on direct measurement of the ability of antioxidants to reduce the Fe^{3+}/tripyridyl triazine complex. Pulido et al. [29] described the FRAP method as an alternative for determining the reduction in iron content in the aqueous solutions of pure compounds and biological fluids [21,30].

The ORAC method detects chemical damage to beta- or R-phycoerythrin, proteins that function as light-harvesting components. The decrease in fluorescence caused by reactive species in the presence of free radicals acts as an index of oxidative damage to proteins [21].

The ABTS method measures the ability of antioxidants to quench ABTS cations generated via chemical, electrochemical, or enzymatic reactions, allowing for the measurement of the antioxidant activities of hydrophilic and lipophilic compounds. The scavenging of the cation causes a decrease in absorbance, which is calculated in the radical/antioxidant mixture at different times. A calibration curve is generated using the antioxidant Trolox, and the result is expressed in terms of Trolox equivalent [21,31].

In the DPPH method, based on the scavenging of the DPPH free radical, DPPH acts as an oxidizable substrate reduced by an antioxidant and as an indicator of the antioxidant response. DPPH is an unstable radical with a violet color that receives an electron or a hydrogen radical to become a stable molecule and turns yellow when reduced. Thus, the assay only evaluates the reducing power of the antioxidant, oxidized upon donating an electron, and does not detect pro-oxidant substances [(32,33)].

Several comparative studies have estimated the total antioxidant activity in plant extracts using ABTS, DPPH, FRAP, and ORAC methods. Thaipong et al. [(34)] used *Psidium guajava* (guava) extracts and observed that FRAP was the most reproducible technique for determining antioxidant activity, showing a high correlation with the levels of ascorbic acid and phenolic groups. Ou et al. [(35)] measured the antioxidant properties of freeze-dried vegetable samples, concluding that the ORAC method is chemically more appropriate for determining chain-breaking antioxidant activity, whereas FRAP has certain disadvantages such as interference, reaction kinetics, and quantification techniques. Moreover, Duarte-Almeida et al. [(32)] concluded that fresh acerola fruits possessed the highest capacity to scavenge DPPH free radicals compared with blackberry, açai, and strawberry extracts, in large part, owing to the high content of ascorbic acid present in the fruit, thereby demonstrating the antioxidant capacity of ascorbic acid.

Antioxidants have an essential role in preventing diseases resulting from oxidative stress [(14)]. Thus, the search for substances of natural origin with antioxidant properties and fewer side effects than synthetic antioxidants [(15)] has grown in recent years, primarily because of their economic and clinical importance and the rising appreciation for natural products [(16)]. Over the years, scientific interest in plant extracts has increased owing to the large amounts of biologically active natural substances with antioxidant potential that have been isolated and characterized [(17,18)]. The best-known natural antioxidants are vitamins C and E, carotenoids, and flavonoids such as quercetin, rutin, hesperidin, naringin, naringenin, and sakuranetin [(14)]. Thus, this review highlights the application of natural antioxidants in animal reproduction, considering the evolution of publications in the global scientific literature in the last three decades.

Material and methods

The literature review was conducted between August and September 2022 and focused on retrieving scientific articles on natural antioxidants applied to animal reproduction. The Science Direct database was searched using the following search terms and Boolean operators: "natural antioxidant" AND "animal reproduction" included in the title, abstract, and keywords in articles published between 1991 and 2022. Only research articles were considered in this review. In this sense, from the 93 papers obtained in the initial screening, 51 were analyzed.

Data analysis

Spearman's correlation analysis was used to evaluate if there was an increase in publications over the years (r_s at the 5% significance level). Pettit's test was used to verify the year from which the research articles started to become more frequent and increased in number. A word cloud was prepared using the keywords in articles to demonstrate the focus of the papers. Analyses were performed using R software, version 3.6.1, and RStudio, version 1.2.1335. The analysis results of the distribution of articles by country were depicted on a map created in QGIS, version 3.4. Graphs were generated using GraphPad Prism, version 9.0.0 for Windows (GraphPad Software, San Diego, CA, USA, www.graphpad.com). Figures were created using Adobe Illustrator, version 24.0.1 (www.adobe.com/products/illustrator).

Application of natural antioxidants in animal reproduction

Publication trends

Temporal publication trends on the use of natural antioxidants in animal reproduction have increased significantly since 1991 (Spearman's correlation coefficient, $r_s = 0.74$; $p < 0.0001$), with an annual rate of 2.3% (Fig. 1). The number of citations has also increased over the years (Fig. 1). The changes in publication trends divided the historical series into two periods (Pettit's test, $p < 0.001$). From 1991 to 2009, there were few publications, with an average of 9.8% of the articles published representing the initial stage of natural antioxidant application in animal reproduction. From 2010 to 2022, an average of 90.2% of the papers were published, representing the period of publication growth (Fig. 1). The increasing number of publications may result from an increase in green chemistry movements in this century also reflected in animal science. Green chemistry involves designing and developing chemicals and implementing policies and processes to reduce or eliminate the use or generation of harmful substances [(36, 37)].

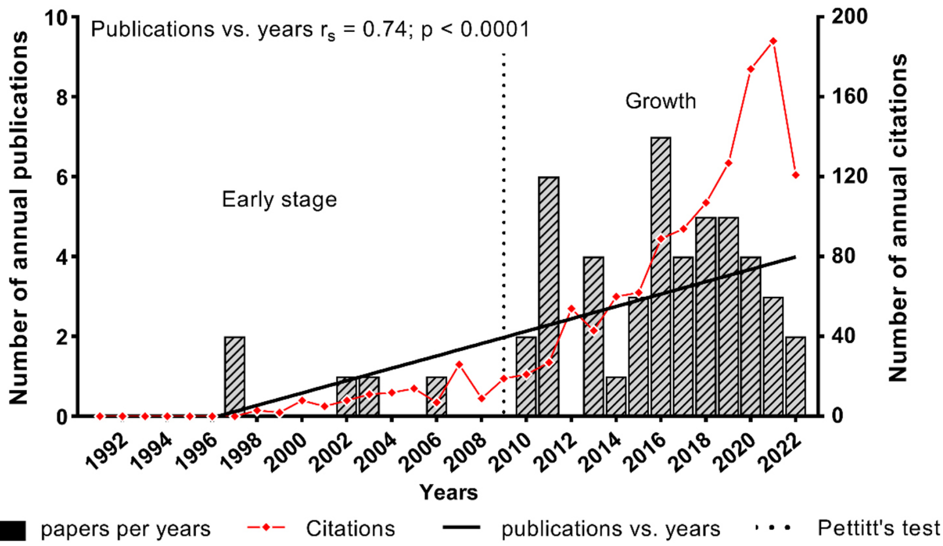

Figure 1. Temporal publication trends on the use of natural antioxidants in animal reproduction.

A keyword analysis is essential for investigating trending topics and the focus of various studies. This study identified 168 different keywords from the 51 publications analyzed. The word cloud highlights the frequency of the use of each word, providing a visible picture for the most prominent terms, *i.e.*, the larger the word, the higher the frequency in the articles. The most cited keywords were antioxidant(s), cryopreservation, vitamin E, and semen (Fig. 2). Thus, our results suggest that natural antioxidants, especially vitamin E, are mainly used for semen preservation during animal reproduction.

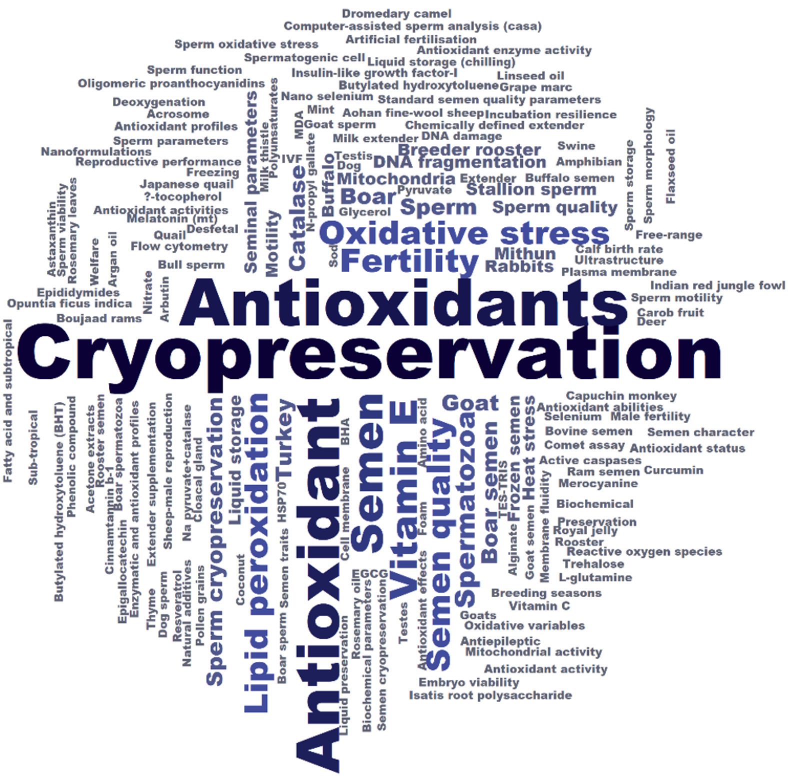

Figure 2. Keyword cloud highlighting the use of natural antioxidants in semen preservation. The keywords retrieved in this search followed a specific trend, i.e., the larger the word, the higher the frequency in the articles.

Regarding the worldwide distribution of publications, 22 countries have studied the use of natural antioxidants for animal reproduction (Fig. 3). China had the highest number of publications (15.7%), followed by Brazil (9.8%) and India (9.8%). China and India have a tradition of herbal medicines, gaining acceptance from the millennials [38, 39], and thus, phytochemicals are accepted widely by the community, and the investigations of other applications of these molecules tend to be greater than those in other countries. Brazil has high biodiversity and an economic focus on agriculture. The challenge is to bring the agricultural sector closer to the biodiversity agenda, thereby making a significant alliance [40]. In search of sustainability, the Brazilian government created the National Bioinputs Program in 2020 to encourage new patterns for production or consumption. Bioinputs are products, processes, or technologies of animal, plant, or microbial origin that can positively affect agricultural production. The adherence of rural producers to sustainable and economic farming practices that use bioinputs is growing in Brazil [41]. Therefore, in the future, the number of studies investigating the utility of natural antioxidants for semen preservation is expected to increase in Brazil.

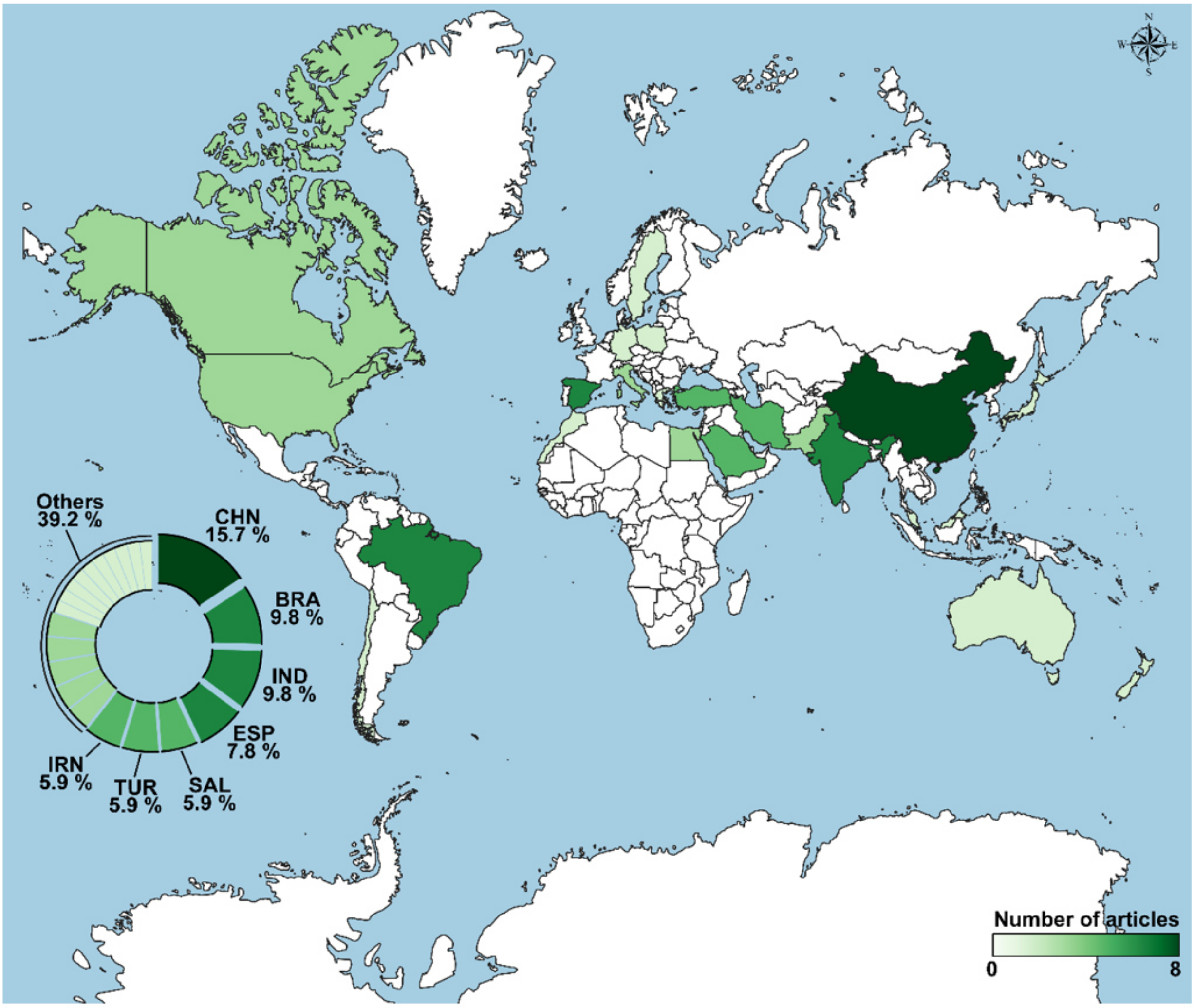

Figure 3. Worldwide distribution of studies performed on natural antioxidants in animal reproduction. The geographic distribution of the published articles is related to the color of each country, proportional to the number of publications.

The promisor application of natural antioxidants in animal reproduction

Oxidative stress has been proposed as one of the primary causes of infertility in animals because it can severely damage sperm and reduce the quality of cryopreserved semen [(42)]. The plasma membrane of sperm is composed of 70% phospholipids, 25% neutral lipids, and 5% glycolipids, which makes sperm susceptible to damage caused by lipid peroxidation, and the vulnerability is increased further upon freezing due to changes in membrane integrity that decrease the motility of the sperm and its capacity to fertilize [(43)].

Cryopreservation has adverse effects on sperm cells, with an important one being irreversible changes in sperm motility and membrane components owing to drastic changes in temperature (cooling/freezing curve) and osmolarity. In addition, the freeze-thaw process induces oxidative stress and increases the production of ROS and reactive nitrogen species [(44)].

The addition of natural oxidants during the cryopreservation process improved the quality of semen and increased the protection of the plasma membrane, reducing freezing-induced damage (Supplementary Table 1). Natural antioxidants were generally commercially acquired (80.4%) and used mainly for semen cooling/ freezing (72%), with promising results (90%) in *Sus scrofa* (boar), *Capra aegagrus hircus* (goat), *Gallus gallus domesticus* (rooster), and *Ovis aries* (ram) (Fig. 4).

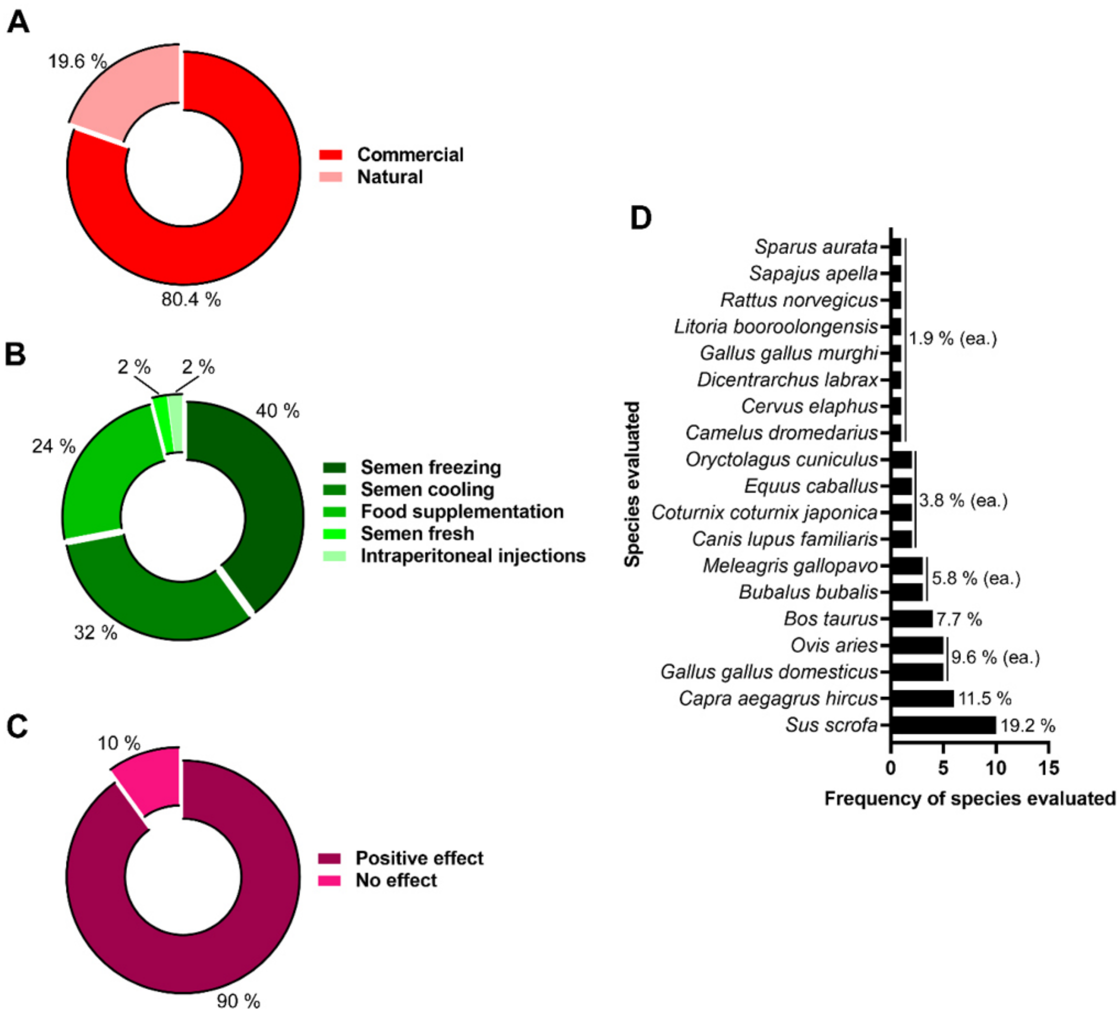

Figure 4. The promisor application of natural antioxidants in animal reproduction. A) The origin of the natural antioxidants (commercial or recently extracted from a natural source). B) The areas of animal reproduction where the natural oxidants were applied. C) The results obtained from the research were generally positive. D) Relative frequencies of the application of antioxidants in the reproduction of various animal species evaluated.

Conclusion

As natural antioxidants do not harm animals and animal by-products owing to their solubility in different media and are not ecotoxic, contributing to the principles of green chemistry, their use is increasing worldwide. Applying these antioxidants in animal reproduction may increase sperm quality and semen viability, facilitating the process of semen cryopreservation. However, further studies need to be conducted to establish legislation regarding the appropriate dosages and regulation of such antioxidant substances for their correct usage and to avoid causing harm to animals.

Conflict of interests

The authors declare no conflict of interest.

Author contributions

Conceptualization: C. S. Castro and L. L. Borges. *Data Curation*: C. S. Castro, C. F. C. Cotrim, I. R. dos Santos. *Formal Analysis*: C. S. Castro, C. F. C. Cotrim and L. M. de Almeida. *Methodolog*y: C. S. Castro, C. F. C. Cotrim, I. R. dos Santos and E. F. L. C. Bailão. *Supervision*: L. L. Borges. *Writing (original draft):* C. S. Castro, C. F. C. Cotrim, I. R. dos Santos, L. M. de Almeida, E. F. L. C. Bailão, S. S. Caramori. *Writing (review and editing):* K. J. G. dos Santos and J. A. M. de Paula

Supplementary material

https://revistas.ufg.br/vet/article/view/73061/38948

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