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DOES SPOTTED PUMPKIN (*LAGENARIA BREVIFLORA* R.) POSITIVELY AFFECT THE HEALTH STATUS OF BROILER CHICKEN?

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Abstract

Coccidiosis poses a significant challenge in poultry production, necessitating the development of effective, natural, and health-promoting alternatives such as *Lagenaria breviflora* to conventional treatments. Two hundred and eight (208) day-old Cobb chicks hatchery-infected with *Eimeria* were divided into four treatment groups and administered: control (0), 50, 100 and 150 grams of *Lagenaria breviflora* R. (Lb) whole fruit per litre of water thrice a week for 6 weeks. Data were obtained on faecal count, haematological indices, serum metabolites and stress biomarkers. There was reduction in *Eimeria* count as Lb increased with the highest reduction (85.20%) in birds that received 150 g. The highest ($P < 0.05$) packed cell volume, haemoglobin and red blood cell count was observed in 150 g Lb while white blood cell count was highest ($P < 0.05$) in 50 and 150 g. Total protein was highest ($P < 0.05$) in 150 g, globulin was highest in 100 and 150 g LB while the serum stress biomarkers (SOD and GSH-Px) was least in 50 g. The administration of Lb up to 150 g/L of water can used in eradicating coccidiosis without compromise on health of birds.

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Introduction

The Nigerian poultry industry is faced by various problems most especially the quality of day-old chicks in terms of health conditions, which is prominent among the issues affecting its positive output apart from quality feed. Prominent among these diseases are salmonellosis, infectious bronchitis and coccidiosis. Salmonellosis and coccidiosis are currently the bane of the Nigerian poultry industry resulting in high mortality and morbidity in the first 5 days and later in life (Balarabe and Obeta 2015). Although, there is no existing evidence or science to suggest the likelihood of a vertical transmission of coccidiosis in birds but recent occurrence of deaths in first few days of chick life may point to the possible infection in the hatchery.

Coccidiosis is caused by coccidian parasites of the genus *Eimeria*, intracellular protozoan parasites of enterocytes resulting in high chicks' mortality. Most anticoccidial drugs currently used in abating the menace exhibit low efficacy and also result in deleterious side effects in birds (Al-Mathal 2010). The consequential side-effect of exposure to conventional drugs and its overuse results in damages to internal organs invariably resulting to system breakdown and death. The consistent use of these conventional drugs has led to the development of resistant coccidian strains, mostly an offshoot of extensive use, under usage (underdose) and adulteration of drugs experienced in most developing countries.

The resistance mostly due to physical or genetic alterations, and the side effects of these drugs led to its consequent vertical shedding to offspring and the rise of transmission coupled with rearing of birds on floor system. Since most meat-type and eggers are reared on floor from first day with continuous access to litter, coccidiosis will continually be a menace to poultry rearers. Coccidiosis provides a good example of the relationship between the number of invading organisms and the severity of the resulting infection, because the morbidity and mortality of the host species are usually proportional to the number of sporulated coccidial oocysts ingested by the bird.

The inability of farmers to access constant veterinary services and effective cocci-vaccines has greatly affected the development of poultry in developing countries leaving the farmers with no choice than alternative options in combating the threat. The continent of Africa is blessed with natural reserves of plant resources of botanical importance for human and animal health management. One of such plant is Spotted pumpkin (SP). SP has been documented and used in rural poultry health management against coccidiosis and Newcastle disease (Sonaiya et al. 1999) likewise in rural human health as herbal remedy for the treatment of some disorders such worm infestation, wound antiseptics and skin infections. The extracts of spotted pumpkin have been documented to contain important chemical constituents such as phenols, flavonoids carotenoids, saponins etc (EKUNSEITAN et al. 2016, ADEYEMI et al. 2017) capable of inducing positive health effect.

Numerous laboratory studies have demonstrated its ethnobotanical properties, such as miracicidal and cercaricidal activities (AJAYI et al. 2002), antioxidant activity (ONASANWO et al. 2011) and antibacterial activity (TOMORI et al. 2007). These findings underscore the importance of integrating these attributes into poultry production systems to evaluate their efficacy and potential for industry-wide application. Recent studies (EKUNSEITAN et al. 2017, 2019) revealed positive responses in orally administered dosages in poultry birds raised on modified housing systems, favouring its usage in animal health management especially with the growing drive for organic products in most developing countries. These positive outcomes include increased immune response to Newcastle disease vaccination in growing pullets (EKUNSEITAN et al. 2019), haematinic and therapeutic activity (ALADE 2000, EKUNSEITAN et al. 2017), antimicrobial activities (EKUNSEITAN et al. 2017), immunomodulatory effect (SABA et al. 2009) and worm control (EKUNSEITAN et al. 2017).

Materials and Methods

The experiment was carried out at the Poultry unit, Teaching and Research Farms, Federal University of Agriculture, Ogun state, Nigeria.

Lagenaria breviflora (spotted pumpkin) whole fruits was washed with clean water, weighed and cut into smaller parts to increase the surface area and then extracted according to the method of EKUNSEITAN et al. (2017) to give concentrations of 50, 100 and 150 grams per litter of water.

Two hundred and eight day-old Cobb broiler chicks, hatchery-infected with *Eimeria spp* were used for the experiment. The chicks exhibited clinical signs of ruffled feather and blood stained to brownish diarrhoea from day one of the experiment, birds were also clinically observed by personnel. Sample birds were selected from the pool, euthanized and accessed for identification and counts of *Eimeria* spp. (REHBEIN et al. 1999). The birds were then randomly assigned to four (4) treatment groups: 0 (control), 50, 100 and 150 grams of Lb per litre of water (weight/ volume) in a Completely Randomized Design (CRD), containing fifty-two (52) birds each. Each treatment was further divided into four replicates of thirteen (13) birds each. *Lagenaria breviflora* was administered determined concentration three consecutive days in a week from day one till the 6th week (day 42) of the experiment via drinking water (Ekunseitan et al. (2017). Birds in the control group were immediately placed on Amprolium + Sulphaquinoxaline (+vit K). Conventional vaccination was given to all treatment groups while medication (antibiotics and coccidiostat) was administered only to the control group. The chicks were housed in a well-ventilated deep litter pen. The chicks were fed with commercial starter feed (Metabolizable Energy 2800 kcal/kg, Crude Protein: 21%) and finisher feed (Metabolizable Energy 2900 kcal/kg, Crude Protein: 19%).

Fresh faecal droppings were collected from each replicate group into a plain bottle weekly for six weeks starting from the first week of the experiment. The oocyte count and identification were carried out using McMaster egg counting technique. The sample was viewed under the compound microscope at 10 x 10 magnification. Oocytes were identified and counted within the engraved area of the counting chambers (Rehbein et al. 1999).

At the 42nd day of the experiment, 10 ml of blood was collected from four pre-selected birds per replicate via the jugular vein with use of hypodermic syringe. Approximately 3.0 ml of blood was collected into EDTA bottles for haematological parameters while the 4 ml was collected into a plain bottle for the serum metabolites determination and the remaining into another labelled plain bottles for quantifying oxidative stress biomarker.

Haematological parameters such as Red blood cell (RBC) and Packed cell volume (PCV) was determined according to Brian et al. (2000), Haemoglobin as reported by Dace and Lewis (1984), White blood cell and differential counts via visual cell count using a haemocytometer.

Serum metabolites (Total protein, Albumin, Globulin, Asparte aminotransferase (AST), Alanine aminotransferase (ALT) and Alkaline phosphate (ALP) was determined using Randox Laboratories Standardized Test Kit (Hitachi Model 917 Multichannel Analyser). Serum superoxide dismutase (SOD) was determined by the method of Oberley et al. (1984), glutathione peroxidase (GSH-Px) by Reddy et al. (2004) and Thiobarbituric Acid Reactive Substances (TBARs) by Keles et al. (2001).

Data on faecal analysis were transposed to base of log 10 and evaluated using repeated measures analysis (SPSS 2010). Haematological and serum data were subjected to one-way Analysis of Variance (ANOVA) in a completely randomized design (CRD). Significant means among other measured variables were separated using Tukey test at 95% probability.

Results and Discussion

Effect of *Lagenaria breviflora* (spotted pumpkin) fruit extract on faecal egg count of broiler chicken

The effect of *Lagenaria breviflora* (spotted pumpkin) fruit extract on faecal egg count of broiler chicken is presented in Figure 1. A continuous reduction of oocysts count was observed as the administration of *Lagenaria breviflora* increased. The highest oocysts count (3.49) in week 2 was observed in birds administered 150 g of *Lagenaria breviflora* while the least oocysts count (3.17) was observed in birds administered 100 g of *Lagenaria breviflora*. The lowest ($P < 0.05$) oocysts count (0.65) in week six was observed in birds administered 150 g of *Lagenaria breviflora* while the highest oocysts count (1.79) was observed in the control group. A 50.54% oocysts count reduction was observed in the control group, 62.51% in birds administered 50 g of *Lagenaria breviflora*. 70.85% in 100 g of *Lagenaria breviflora* group and 85.20% in 150 group. *Eimeria* is the causative agents of coccidiosis and a blight in poultry production causing significant commercial losses in the animal industry (WILLIAMS 2002).

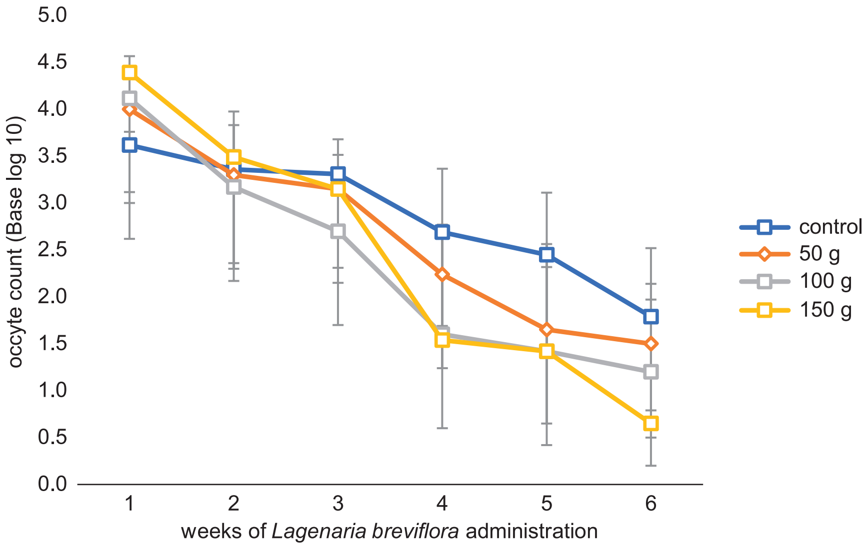

Fig. 1. Effect of *Lagenaria breviflora* (spotted pumpkin) fruit extract on faecal egg count of broiler chicken

The observed decrease in oocyte per gram as the level of the administration of *Lagenaria breviflora* increased may be due to various mechanisms of phytochemicals such as phenols, saponins, alkaloids acting in synergy and capable of inhibiting the life cycle of Coccidia (MOLAN et al. 2009) and decimating *Eimeria* count in the birds. Plants contain immunoregulatory compounds capable of enhancing and inducing immune responses thereby maintaining bird's health (SUGIHARTO et al. 2017).

The phytochemical analysis of *Lagenaria breviflora* has been examined to contain considerable amount of metabolites like flavonoids, saponin, tannin, etc. (EKUNSEITAN et al. 2016, ADEYEMI et al. 2017) while comparable dose-associated responses in faecal count had been reported by researchers using phytobiotics plants as an alternative to conventional drugs (BIU et al. 2010, ELKHTAM et al. 2014, EKUNSEITAN et al. 2017, EKUNSEITAN et al. 2018) to safeguard the welfare of animals and consumers of products. The increase in dosage will invariably result in the amount of metabolites in the extract (ODULANA 2014, AREMU 2014) potentially enhancing its biological activity and therapeutic efficacy. Chemical metabolites present in *Lagenaria breviflora* R. fruit display various mechanisms of action such as inhibiting intestinal motility while some components have been shown to inhibit specific pathogens (AHMAD et al. 2006, EKUNSEITAN et al. 2016, ADEYEMI et al. 2017).

Tannins, has been reported to inhibit the life cycle of Coccidia (MOLAN et al. 2009), reduction in the rate of oocysts hatching and development (HUR et al. (2005) through the penetration of the wall of the oocyst and damage to the cytoplasm as tannins has been posited to weaken endogenous enzymes responsible for sporulation. In addition, tannins have been reported to improve the immune system, and exert bactericidal and coccidiostatic properties in birds (PERIN et al. 2019). *Lagenaria breviflora* whole fruit contains considerable amount of flavonoids (223.00 mg/100 g) and saponins (138.0 mg/100 g) (EKUNSEITAN et al. 2016), flavonoid have been reported to control coccidian infection by induction of oxidative stress through the conversion of hydroxyl groups into pro-oxidant when reacting oxidizing species (ROS) oxidize the lipids and proteins present in inner cell membrane of coccidian thereby resulting in necrosis and apoptosis of damaged cells (BAKKALI et al. 2008). NAIDOO et al. (2008) observed that the use of plant-derived antioxidants when compared to conventional coccidiostat exhibited a similar positive effect observed in the study. In addition, the reduction trend was also in tandem with report of RAKHMANI et al. (2014) which observed proportionally inverse relationship between saponins content and percentage of oocyte per gram count reduction as a consequence of penetration through micropyle cap on polar end of oocyst (WIEDMER et al. 2011).

Effect of extract of *Lagenaria breviflora* (spotted pumpkin) on the haematological indices of broiler chicken

The result of the haematological indices of broiler chicken administered *Lagenaria breviflora* is presented in Table 1. Oral administration of *Lagenaria breviflora* had significant ($P < 0.05$) increasing effect on Packed cell volume (PCV), Haemoglobin (Hb), White blood cells (WBC), Red blood cells (RBC), and Eosinophils. The highest RBC value (3.88) was observed in birds administered 150 g/L of *Lagenaria breviflora* while birds administered 50 g/L of *Lagenaria breviflora* had the least (3.16) value. The highest ($P < 0.05$) PCV value (36.00) and Hb (12.30) value was observed in birds administered 150 g/L of *Lagenaria breviflora* while the lowest value (27.50, 9.20) was observed in birds administered 50 g/L of *Lagenaria breviflora*. The levels of PCV, Hb and RBC count observed in the study might be linked with the *Eimeria* load observed in the treatment groups. Anaemic condition is characterized by decreased PCV, Hb and RBC in infected birds and conceivably due to loss of blood into the gastrointestinal tract with haemorrhages in the ceca and intestine. Lb has been proposed to induce haematinic and therapeutic values in periods of anaemia (Olorunfemi et al. 2014, Ekunseitan et al. 2017), an ability associated with cucurbitaceae family indicating increased production of reticulocytes and suggestive of stimulating erythropoiesis. The significant reduction in the *Eimeria* load correlated with the elevated erythrocytes component counts in Lb dosed groups with greatest counts in the 150 g per litre.

Table 1

Effect of extract of *Lagenaria breviflora* (spotted pumpkin) on the haematological indices of broiler chicken

Parameters	*Lagenaria breviflora* administration				P-Value
	0 g/L Lb	50 g/L Lb	100 g/L Lb	150 g/L Lb	
Packed cell volume [%]	29.00 ±1.41^{c}	27.50 ±0.71^{c}	34.50 ±3.5^{b}	36.00 ±1.41^{a}	0.036
Haemoglobin [g/L]	9.80 ±0.57^{ab}	9.20 ±0.14^{b}	11.55 ±1.20^{ab}	12.30 ±0.71^{a}	0.041
Red blood cells [10^{12}/L]	3.41 ±0.19^{ab}	3.16 ±0.06^{b}	3.78 ±0.23^{ab}	3.88 ±0.07^{a}	0.028
White blood cells [10^{3}/ul]	9.75 ±0.92^{b}	22.20 ±2.97^{a}	12.35 ±1.91^{b}	24.15 ±1.63^{a}	0.005
Heterophils [%]	44.00 ±9.90	47.00 ±16.97	36.00 ±38.18	12.50 ±4.95	0.464
Lymphocytes [%]	51.00 ±11.31	49.00 ±15.56	57.00 ±38.18	85.50 ±6.36	0.409
Basophils [%]	0.00 ±0.00	0.50 ±0.71	0.50 ±0.71	0.00 ±0.00	0.615
Eosinophils [%]	1.50 ±0.71^{ab}	3.00 ±1.41^{ab}	4.00 ±0.00^{a}	0.00 ±0.00^{b}	0.026
Monocytes [%]	3.50 ±0.71	0.50 ±0.71	2.50 ±0.71	2.00 ±1.41	0.125

^{abc}: Means in the same row with different alphabet differ significantly ($P < 0.05$), mean ±SD

White blood cell was statistically highest (P < 0.05) and similar in birds administered 50 g/L (22.20) and 150 g/L (24.15) of *Lagenaria breviflora*. The highest value of Eosinophils (4.00) was observed in birds administered 100 g of *Lagenaria breviflora* while the lowest value of Eosinophils (0.00) was observed in birds administered 150 g/L of *Lagenaria breviflora*. The white blood cell count obtained in the study indicate that oral administration of *Lagenaria breviflora* had a substantial effect on the immune system of the experimental birds. Increase in administration of *Lagenaria breviflora* led to an increase in lymphocytes count, *Lagenaria breviflora* and *Telfairia occidentalis* are part of the Cucurbitaceae family whereas *Telfairia occidentalis* has been reported to have a haematopoietic effect in rats (Alade 2000). Although there was a non-significant increase in lymphocyte counts as the level of LB increased in birds, the increase observed which was dosage-dependent may be ascribed to the induction of immune response by LB in the infected birds (Adeyemi et al. 2017, Ekunseitan et al. 2016). Lymphocytes participate in the first line of defence against many infections especially intra-intestinal epithelial lymphocytes which makeup a relatively large proportion of the total immune system (Rose et al. 1996) thereby participating in resistance to the *Eimeria* infection.

Effect of extract of *Lagenaria breviflora* (spotted pumpkin) on the serum indices in broiler chicken

The effects of extract of Lb on the serum indices of experimental broiler chicken is presented in Table 2. It was observed that Lb extract had significant (P < 0.05) effect on Total protein, globulin and Uric acid. Increasing concentration of Lb extract from 0 to 150 g resulted in an increase in total protein. Globulin was statistically similar and highest in 100 and 150 g dosed group with the least values observed in the control and 50 g group. This affirms the immune-stimulating effect of Lb by influencing synthesis of blood proteins by hepatocytes as observed with the elevated values of serum total protein and globulin. The plant's hepatic influence implies that higher rate of protein synthesis occurs in the liver thereby elevating the synthesis of serum proteins (Jaleel and Nair 2004) resulting in subsequent increase in the concentration of total protein in the blood (Szabó et al. 2005). The serum enzymes were similar (P < 0.05) in all treatment groups indicating no hepatic injuries in birds administered Lb up to 150 g/litre when compared with the control group. Although, Pieme et al. (2006) stated that AST, ALT and ALP enzymes produced by the liver can be influenced by phytochemicals present in plant extracts, only AST and ALT were observed to be numerically higher in birds administered *Lage-*

naria breviflora except ALP where it was numerically highest in the control group. The values obtained for AST, ALT and ALP fell within the range of values reported by (EKUNSEITAN et al. 2018, RUBIO et al. 2019), while the ALT values were higher than that reported CHUSKIT et al. (2024) in broiler chickens at 21 and 28th age of age. Serum electrolytes (Ca^+ and Na^+) are important substances for myriads of homeostatic and physiological functions: cell muscle contraction and bone formation, transfer of impulses in nerve cells and blood clotting. Although, a non-significant reduction ($P < 0.05$) was observed in serum calcium concentration as the level of Lb dosage increased similar to AJANI et al. (2015) thereby negating the possibility of plant toxicity.

Table 2

Effect of extract of *Lagenaria breviflora* (spotted pumpkin) on serum metabolites of broiler chicken

Parameter	*Lagenaria breviflora* administration				P-Value
	0 g/L Lb	50 g/L Lb	100 g/L Lb	150 g/L Lb	
Glucose [mg/dL]	140.45 ±2.33	143.95 ±2.05	138.95 ±9.12	152.90 ±8.49	0.271
Total protein [g/L]	56.90 ±1.41^d	62.00 ±0.42^c	67.70 ±1.70^b	70.85 ±0.21^a	<0.001
Albumin [g/L]	35.75 ±1.48	38.55 ±1.91	34.85 ±2.62	37.45 ±0.64	0.306
Globulin [g/L]	21.15 ±0.05^b	23.45 ±1.65^b	32.85 ±3.05^a	33.40 ±0.30^a	0.015
Aspartate aminotransaminase [U/L]	191.50 ±2.55	222.55 ±7.42	224.90 ±28.71	192.95 ±8.27	0.174
Alanine aminotransferase [U/L]	11.20 ±1.27	13.90 ±3.11	17.10 ±0.57	17.70 ±0.57	0.056
Alkaline Phosphatase [U/L]	295.35 ±7.14	195.00 ±7.78	245.65 ±93.13	209.75 ±12.94	0.289
Calcium [mg/dL]	9.35 ±0.49	9.00 ±0.14	8.80 ±0.14	7.70 ±1.13	0.190
Sodium [mmol/L]	121.20 ±13.01	133.20 ±6.79	136.50 ±8.77	129.65 ±10.82	0.536

^{abc}: Means in the same row with different superscript differ significantly ($P < 0.05$), mean ±SD

Effect of administration of extract of *Lagenaria breviflora* (spotted pumpkin) on oxidative stress marker of broiler chicken

The effect of *Lagenaria breviflora* (spotted pumpkin) on oxidative stress marker of broiler chicken is presented on Table 3. Aqueous *Lagenaria breviflora* extract had significant effect ($P < 0.05$) on the oxidative stress makers except Malondialdehyde (MDA). Superoxide dismutase (SOD) and Glutathione peroxidase (GSH-Px) was lowest ($P < 0.05$) in birds administered 50 g *Lagenaria breviflora* but similar and highest in other treatment groups. Oxidative stress occurs in animals as a consequence of pathogenic occurrence therefore the animal's biological system subsequently promotes the production and expression of antioxidant enzymes as a defence mechanism.

Table 3

Effect of administration of *Lagenaria breviflora* (spotted pumpkin) on oxidative stress marker of broiler chicken

Parameter	Levels of oral administration				P-Value
	0 g/L Lb	50 g/Lb	100 g/Lb	150 g/Lb	
Superoxide dismutase [U/L · 10^{-2}]	1.62 ±0.14^{a}	1.09 ±0.07^{b}	1.23 ±0.00^{b}	1.33 ±0.12^{ab}	0.049
Malondialdehyde [U/L · 10^{-9}]	2.61 ±0.42	2.07 ±0.05	1.90 ±0.04	3.44 ±0.75	0.073
Glutathione peroxidase [U/L]	6.10 ±0.14^{a}	3.90 ±0.92^{b}	4.75 ±0.28^{ab}	5.25 ±0.64^{ab}	0.040

^{abc}: Means in the same row with different alphabet differ significantly (P < 0.05), mean ±SD

The significant (P < 0.05) increase in activities of antioxidant enzymes SOD and GSH-Px in birds administered with 100 and 150 g dosed group statistically *comparable* with the control group is suggestive of the plant capable of directly activating synthesis of antioxidant enzymes to combat the stress induced by Eimeria counts in the bird's system. Similar increment in GSH-Px activity was observed in male wistar rats as a defence response to oxidative stress (FOLORUNSHO et al. 2019), indicating the ability of the fruit of beneficially affecting the activities of some antioxidant enzymes such as Superoxide dismutase and Glutathione peroxidase. The use of natural antioxidants can improve the health status of birds infected with coccidiosis (QUIROZ-CASTANEDA and DANTAN-GONZALEZ 2015) as wide range of herbs and their extracts are capable of inducing potential antioxidant functions (BRENES et al. (2010). *Lagenaria breviflora* contain phenolic compound which increases the activity of antioxidant enzyme thus affecting the concentration of (O_2^- and H_2O_2) free radicals in the serum of the birds (KAURINOVI and VASTAG 2019).

Conclusion

The results of the study revealed that administration of LB at increasing levels demonstrated a dosage-dependent reduction in *Eimeria* counts, with the greatest reduction at 150 g. This dosage also resulted in the improved blood indices (Packed cell volume, haemoglobin, red blood cell count, total protein, and globulin levels), stress biomarkers (SOD and GSH-Px). These findings indicate that LB, administered at levels up to 150 g/L of water, effectively mitigates coccidiosis while supporting the overall health and physiological balance of the birds.

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PRELIMINARY PHYSICOCHEMICAL CONTENTS AND ANTIOXIDANT PROPERTIES OF COCONUT WATER IN TRINIDAD AND TOBAGO

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Key words: antioxidants, coconut water, mineral composition, physicochemical contents.

Abstract

The chemical, mineral, and antioxidant properties of two species of young coconut water (yellow and green) from Trinidad and Tobago were quantitatively analyzed and compared. Parameters such as Total Phenolic Content (TPC), 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity, Total Soluble Solids (TSS), Titratable Acidity (TA), pH, turbidity, and mineral composition were evaluated. The results revealed distinct differences in mineral concentrations, chemical properties, and antioxidant activity between the two coconut species. The green coconut water (Atlantic Tall) exhibited significantly higher antioxidant activity in both DPPH and TPC assays compared to the yellow coconut water (Malayan Dwarf) ($p < 0.05$). Although the crude extract yield was marginally higher in yellow coconut water, this difference was not statistically significant ($p > 0.05$). No significant differences were observed between the two species in terms of TSS and pH ($p>0.05$). However, the titratable acidity was higher in the green coconut water ($p < 0.05$), while the yellow coconut water exhibited greater turbidity ($p < 0.05$). The yellow coconut water also had higher concentrations of Na, K, Ca, and Mg ($p < 0.05$), though there was no significant difference in iron (Fe) content ($p > 0.05$). Overall, the findings suggest that coconut water from Trinidad and Tobago is rich in essential minerals and possesses notable antioxidant properties, with variability between the two species in certain chemical and physical characteristics.

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Introduction

Coconut (Cocos nucifera), a member of the Arecaceae family, is a widely recognized fruit known for its nutritional and therapeutic potential (DEBMANDAL and MANDAL 2011). Coconut trees thrive along tropical, sandy shorelines, where they can withstand salt spray and brackish soils, growing well in a wide range of soil types with a pH between 5 and 8 (BROSCHAT and CRNE 2000). The fruit itself is composed of water, sugars, vitamins, and essential minerals such as potassium, sodium, magnesium, iron, and calcium, though its composition can vary among species (PRADES et al. 2012).

In Malaysia, studies have shown that coconut water can effectively rehydrate the body after exercise, offering a viable alternative to water or carbohydrate-electrolyte beverages (SAAT et al. 2002). Additionally, coconut water has been reported to possess significant antioxidant properties (MANTENA et al. 2003). Oxidative stress, caused by free radicals produced during oxygen metabolism, can lead to cellular damage, contributing to conditions such as liver and heart diseases, as well as certain cancers. Antioxidants neutralize these free radicals, potentially preventing or reducing oxidative damage (WARE 2018). Given its high antioxidant activity, coconut water may serve as a beneficial supplement in combating oxidative stress and associated diseases (AJEIGBE et al. 2023, OJIHA et al. 2022, RETHINAM and KRISHNAKUMAR 2022). However, further research is necessary to validate these claims.

In preclinical studies, green coconut water has been shown to alleviate oxidative stress in hypoglycemic rats (PREETHA et al. 2012). In the Caribbean, Trinidad and Tobago's coconut industry has a long history, with the first coconut estate established in Mayaro in the 1900s, followed by the Cedros and Constance estates. The Woodland estate in Tobago dates to the 1800s. Despite the challenges posed by Red Ring disease, which has impacted coconut tree populations, the industry remains active, producing a variety of coconut-based products, including milk, copra, water, dry nuts, and oil. In Trinidad and Tobago, coconuts are multifunctional, serving culinary, religious, recreational, economic, and medicinal purposes (PEMBERTON et al.1992).

BORDEIX et al. (2020) identified three primary species of coconut palms: tall, dwarf, and hybrid varieties. In Trinidad and Tobago, five known varieties include the Malayan dwarf yellow, Malayan dwarf green, Atlantic tall (Jamaican tall), Malayan dwarf red, and non-descript hybrids. While studies from around the world have explored the bioactive compounds, minerals, and antioxidant properties of coconut water, to the best of our knowledge, such research has not been conducted in Trinidad and Tobago or the broader Caribbean region. Moreover, while studies have compared the antioxidant activity of young and mature coconuts, there has been limited focus on comparing coconut water from different species of young coconuts. For instance, Fonseca et al. (2009) compared the antioxidant activity of green and yellow coconuts in Brazil, but details regarding the specific varieties and ages of the coconuts were not provided.

This study aims to fill this gap by providing preliminary data on the mineral composition and antioxidant content of coconut water in Trinidad and Tobago. It will specifically compare the young coconut water of two distinct species: the Atlantic tall coconut (green coconut) and the Malayan yellow dwarf coconut (yellow coconut).

Materials and Methods

Chemical reagents

The following chemical reagents were purchased from Sigma-Aldrich, Germany: methanol, 2,2-diphenyl-1-picrylhydrazyl (DPPH), Folin-Ciocalteu phenol reagent, gallic acid, sodium hydroxide, malic acid, and distilled water.

Coconut water collection

Ethical approval for the study was obtained from the University of the West Indies, Trinidad and Tobago, with the approval number Ref: CREC-SA.2467/12/2023. Three young green Atlantic Tall coconuts (GCW) were harvested from a coconut tree in Chaguanas, Trinidad, and three young yellow Malayan Dwarf coconuts (YCW) were collected from a tree in Couva, Trinidad. The coconuts were hand-picked and stored at room temperature for one day before being submitted to the laboratory. On the day of sample collection, all six coconuts were opened. The volume of coconut water from each coconut was measured and recorded. The water from the three green coconuts was pooled and labeled as “green” and the water from the three yellow coconuts was pooled and labeled as “yellow.” The samples were stored at 4°C in a refrigerator until further analysis.

Antioxidant activity

Extraction of coconut water. The extraction of coconut water was conducted according to the method described by Mahayothee et al. (2016).

Briefly, 20 g of coconut water was mixed with methanol at a 1: 5 (v/v) ratio and shaken for 3 hours at room temperature using an orbital shaker set at 90 rpm. The mixture was then filtered using Whatman No. 4 filter paper, and the resulting extract was evaporated under reduced pressure at 50°C using a rotary evaporator. The concentrated extract was adjusted to a final volume of 10 mL with methanol in a volumetric flask for further analysis. All experiments were performed in triplicate.

Percentage yield of the coconut water. Percentage yield [%] = Weight of the extracted coconut water sample/Total weight of the sample · 100.

Antioxidant activity (DPPH). DPPH radical scavenging activity was measured using a modified method from MAHAYOTHEE et al. (2016). Briefly, 0.1 mL of the diluted coconut water extract was mixed with 3.9 mL of $6 \cdot 10^{-5}$ M DPPH solution in methanol. The mixture was incubated at room temperature in the dark for 2 hours. Absorbance was measured at 515 nm using a UV-Vis spectrophotometer. The DPPH radical scavenging activity [%] was calculated using the formula:

$$\frac{A_0 - A_S}{A_0} \cdot 100,$$

where A_0 is the absorbance of the DPPH solution without the sample, and A_S is the absorbance of the DPPH solution with the sample.

Total Phenolic Content (TPC) Determination. The total phenolic content (TPC) of the coconut water was measured using the Folin-Ciocalteu method with modifications (TAN et al. 2014). A stock solution of gallic acid (500 mg/L) was prepared in deionized water, and working standard solutions were prepared by serial dilution to final concentrations of 20, 40, 60, 80, and 100 mg/L. For the analysis, 1 mL of coconut water was mixed with 70 mL of distilled water and 5 mL of Folin-Ciocalteu phenol reagent (10-fold dilution). The mixture was incubated for 5 minutes at room temperature, followed by the addition of 15 mL of 7.5% (w/v) sodium carbonate. The volume was then brought to 100 mL with distilled water, and the mixture was incubated for 2 hours at room temperature. Absorbance was measured at 765 nm using a UV-1650 PC UV-Vis spectrophotometer. TPC was expressed as gallic acid equivalents (GAE) in mg/L.

Chemical and Mineral Analysis

Total Soluble Solids (TSS) measurement. The TSS of coconut water was determined using a digital refractometer (at 25°C), and results were expressed in Brix.

Titratable Acidity (TA). Titratable acidity was measured using the Titratable Acidity in Wine or Juice method. Five milliliters of degassed coconut water was transferred to a 250 mL Erlenmeyer flask, and 100 mL of neutralized deionized water was added. The solution was titrated with 0.1 N NaOH until a faint pink color persisted for 30 seconds. TA was calculated using the following equation:

$$NN \cdot MM \cdot VV \cdot 100/Vc,$$

where:
NN – normality of NaOH,
VV – the volume of NaOH used,
*V*c – the volume of coconut water,
MM – the malic acid factor (67.05).

pH measurement. The pH of the coconut water was determined using a pH meter equipped with a pH electrode.

Turbidity measurement. Turbidity was measured using a UV-Vis spectrophotometer at 610 nm. Absorbance was measured relative to distilled water, and the transmittance (T) and turbidity were calculated using the following equations:

$$T = 100 \cdot (10^{\text{Abs}}),$$

where: Abs was the adsorption at a wavelength of 610 nm.

$$\text{Turbidity} = 100 - T,$$

where: T was the transmittance at a wavelength of 610 nm.

Mineral content analysis. The concentrations of sodium (Na), magnesium (Mg), calcium (Ca), potassium (K), and iron (Fe) in coconut water were determined using a flame atomic absorption spectrophotometer, following the method of Tan et al. (2014). All assays were performed in triplicate.

Statistical analysis. Data were analyzed using SPSS statistical software. Descriptive statistics, including means and standard deviations, were calculated. To compare the means of the different coconut varieties, a Student's t-test was used. A p-value of < 0.05 was considered statistically significant. All analyses were conducted in triplicate.

Results and Discussion

Results: yield, DPPH, and TPC of the two coconut species

Our findings indicate that there was no significant difference ($p > 0.05$) in yield (YCW : 2.53% and GCW : 2.24%) between the two species of coconut, as shown in Figure 1. However, the DPPH radical scavenging activity and total phenolic content (TPC) were significantly higher ($p < 0.05$) in the green coconut water (GCW) compared to the yellow coconut water (YCW), as illustrated in Figure 1.

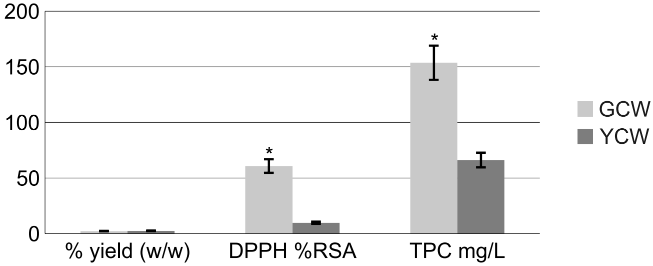

Fig. 1. Total yield of coconut water extract, DPPH radical scavenging activity, and total phenolic content of the two coconut varieties

Each value in the diagram represents a mean ± SD ($n = 3$), *Indicates a significant difference between the two different samples ($p < 0.05$). Explanations: YCW – Yellow coconut water; GCW – green coconut water; DPPH (2,2-diphenyl-1-picrylhydrazyl); TPC – total phenolic content.

Table 1

The volume of coconut water obtained from the coconut water varieties, quantity after extraction and the % yield

Specification	Please remove the asterisk from YCW [ml]	GCW [ml]
1	280	360
2	330	350
3	330	350
Total	940	1060
Quantity obtained after extraction	23.78	23.74
% yield	2.53%	2.24%

YCW – yellow coconut water; GCW – green coconut water

The mineral content of yellow and green coconut water

The Malayan Yellow Dwarf coconut water contained higher levels of all minerals compared to the Atlantic Tall variety ($p < 0.05$). However, no significant difference in iron content was found between the two varieties ($p > 0.05$) – Table 2.

Table 2

The mineral content of yellow and green coconut water

Parameter	YCW [mg/l]	CGW [mg/l]
Sodium (Na)	134.28 ±2.73^{a}	78.20 ±4.10^{b}
Potassium (K)	2143.21 ±11.43^{a}	1546.91 ±45.76^{b}
Calcium (Ca)	288.75 ±1.45^{a}	243.16 ±1.38^{b}
Magnesium (Mg)	82.23 ±0.73^{a}	41.72 ±0.60^{b}
Iron (Fe)	0.108	0.161

$^{a,\ b}$superscripts denote significant differences ($p < 0.05$) within rows

Physico-chemical properties of yellow and green coconut water

No significant difference was observed ($P > 0.05$) in the total soluble solids (TSS) between the two coconut varieties (Table 3). However, a significant difference was found ($P < 0.05$) between the two varieties in terms of acidity and turbidity, as shown in Table 3.

Table 3

Physico-chemical properties of yellow and green coconut water

Parameter	YCW [mg/l]	GCW [mg/l]
Total soluble solids [% Brix]	4.67 ±0.50	4.67 ±0.50
Titratable acidity [%w/v Malic acid]	0.091 ±3.45^{a}	0.076 ±2.99^{b}
pH	4.76 ±0.09	4.66 ±0.02
Turbidity (NTU)	116.67 ±2.08^{a}	138.33 ±0.58^{b}

$^{a,\ b}$ superscripts denote significant differences ($p < 0.05$) within rows

Discussion

Quantifying the antioxidant and mineral content of substances intended for medicinal applications is crucial for assessing their potential health benefits. To the best of our knowledge, this study is the first conducted in Trinidad to identify and quantify indigenous coconut water's antioxidant and mineral content. The findings of this study, including total phenolic content (TPC), DPPH radical scavenging activity, and the yield of crude coconut water extract, are presented in Figure 1.

Our results indicate that the Malayan Yellow Dwarf coconut water produced a slightly higher yield (2.53%) compared to the Atlantic Tall coconut water (2.24%), though this difference was not statistically significant ($p > 0.05$).

While no previous studies have directly compared the antioxidant activity of Atlantic Tall and Malayan Yellow Dwarf coconut waters, a 2012 study in Brazil (SANTOS et al. 2013) demonstrated that Malayan Green

Dwarf coconut water protected against oxidative stress induced by hydrogen peroxide. This suggests that coconut water from Malayan Green Dwarf coconuts may be beneficial in preventing oxidative damage in humans and animals. However, further research is necessary to confirm these potential benefits.

In our study, the Atlantic Tall coconut water exhibited higher antioxidant activity than the Malayan Yellow Dwarf variety. The DPPH analysis showed a 60.7% radical scavenging activity for the Atlantic Tall variety, compared to only 9.8% for the Malayan Yellow Dwarf. Similarly, the TPC was significantly higher in the Atlantic Tall coconut water, with a value of 153.7 mg/L, compared to 66.2 mg/L for the Malayan Yellow Dwarf. Antioxidants play a critical role in scavenging free radicals in the body, thereby reducing oxidative stress and preventing cellular damage (Ware 2018). The DPPH assay is widely used to evaluate antioxidant capacity because it measures the ability of a substance to neutralize free radicals (Baliyan et al. 2022). Phenolic compounds are widely known for their antioxidant properties. Previous research with coconut water in fruit flies shows that the reducing power and scavenging ability of coconut water (CW) could be attributed to the total flavonoid and total phenol content and high concentration of the phenolics which leads to a significant reduction in the generation of free radicals and MDA level in the brain of $Alcl_3$ induced amnesic *D. melanogaster* fed with coconut water supplemented diet (Oluwarotimi et al. 2021).

Our study's findings are consistent with those of Mahayothee et al. (2016) and Santos et al. (2013). Mahayothee et al. (2016) reported TPC values ranging from 6.2 to 10.01 mg/g in coconut water from Thailand, while Santos et al. (2013) found TPC values for Malayan Green Dwarf coconut water to be 99.4 μg/mL, and for Malayan Yellow Dwarf, 23.8 μg/mL. In comparison, the antioxidant content of Trinidadian coconut water appears higher. At a concentration of 0.1 mL/mL, our DPPH radical scavenging activity was also significantly greater, indicating that coconut water from Trinidad has a higher antioxidant capacity than that from Thailand and Brazil.

Coconut water has long been used as an effective oral rehydration drink, thanks to its electrolyte composition, which includes essential minerals such as calcium, potassium, iron, magnesium, and sodium. These minerals are vital for maintaining the body's acid-base balance. In a study conducted in Pakistan, Khan et al. (2003) reported that coconut water can aid in rehydration, especially during mild diarrhea, due to its high glucose content and easy absorption properties. Our study found that coconut water from Trinidad and Tobago contained particularly high concentrations of calcium, potassium, sodium, and magnesium compared to those reported in other studies. Specifically, the Malayan Yellow Dwarf coconut water had higher levels of these minerals than the Atlantic Tall variety ($p < 0.05$). However, no significant difference in iron content was observed between the two varieties ($p > 0.05$). Similar studies conducted in Sri Lanka, Brazil, and Malaysia have also documented high mineral concentrations in coconut water, though the values reported in those studies do not align with our findings.

In addition to antioxidant and mineral content, we also evaluated the chemical properties of the coconut water, including total soluble solids (TSS), titratable acidity (TA), pH, and turbidity. Our results indicated no significant difference in TSS and pH between the Malayan Yellow Dwarf and Atlantic Tall coconut waters ($p > 0.05$). However, significant differences were observed in TA and turbidity ($p < 0.05$), with the Malayan Yellow Dwarf variety having higher TA values and the Atlantic Tall coconut water being more turbid. Titratable acidity is often a more reliable indicator of a substance's perceived acidity and flavor profile than pH alone. Our TA values were 0.091% and 0.076% for the Malayan Yellow Dwarf and Atlantic Tall varieties, respectively, consistent with values reported by MAHAYOTHEE et al. (2016), who found TA values of 0.09% and 0.07%. Our pH values, which were 4.76 and 4.66 for the Malayan Yellow Dwarf and Atlantic Tall coconut waters, respectively, align with those observed in other studies, which reported pH values ranging from 4.84 to 5.84 (MAHAYOTHEE et al. 2016, TAN et al. 2014).The studies by MAHAYOTHEE et al. (2016) and TAN et al. (2014) explored the composition of coconut water at different stages of maturity, with consistent findings regarding pH and total phenolic content (TPC). Specifically, MAHAYOTHEE et al. (2016) highlighted those young coconuts, harvested at 190 days after planting, exhibited moderate acidity, balanced sweetness, and high TPC compared to mature coconuts. This aligns with the findings in this study, which also observed similar characteristics in young coconut water.

Furthermore, TAN et al. (2014) documented that young coconuts have a lower pH and higher TPC values when compared to matured coconut water, reinforcing the trend that the maturity stage of coconuts significantly affects their chemical composition. These results suggest that the age of the coconut at harvest plays a critical role in determining the acidity, sweetness, and phenolic content of coconut water, with younger coconuts being particularly rich in beneficial compounds.

Regarding TSS, our study found values of 4.67 and 4.70 for the Malayan Yellow Dwarf and Atlantic Tall varieties, respectively, which are lower than the TSS values reported in Thailand and Brazil, where young coconuts were found to have TSS values between 5.60 and 7.7. This suggests that coconut water from Trinidad and Tobago may have a lower sugar content compared to coconut water from other regions. The concept of Total Soluble Solids (TSS) in coconut water is important because it directly impacts the concentration of sugars, minerals, and other bioactive compounds present. TSS is typically a measure of the total dissolved solids in a liquid, including sugars, organic acids, proteins, amino acids, and salts. In the case of coconut water, TSS content can be affected by factors like the age of the coconut, its variety, and environmental conditions during cultivation. Low TSS in coconut water means that the sugar content is relatively low compared to other reported. This characteristic makes the Trinidad coconut water an excellent option for rehydration, especially in cases of mild dehydration, as it delivers a significant quantity of electrolytes (e.g., potassium, sodium, magnesium, and calcium) without overloading the body with excessive sugars. Coconut water with low TSS may have a lower glycemic index which helps in controlling blood sugar spikes in individuals with Type 2 diabetes, less disruptive effect on gut microbiota. Coconut water with lower total soluble solids (TSS) has significant medicinal implications, particularly for rehydration, metabolic health, and gastrointestinal function (Prades et al. 2012, Rethinam and Krishnakumar 2022).

Conclusion

The findings of this study clearly demonstrate that coconut water from Trinidad and Tobago possesses notable antioxidant, chemical, and mineral properties. Both the Malayan Yellow Dwarf and Atlantic Tall coconut water (green) exhibit antioxidant activity, with the Atlantic Tall variety showing the most significant antioxidant potential, suggesting it could serve as an effective supplement to combat oxidative stress. On the other hand, the Malayan Yellow Dwarf variety contains higher mineral content compared to the Atlantic Tall, making it a more suitable candidate for fluid therapy and a natural alternative to commercially available rehydration solutions. Both coconut water varieties are slightly acidic with a sweet taste, though the Atlantic Tall is more turbid in appearance. As indicated by the low Total Soluble Solids (TSS) levels, the low sugar content makes coconut water from Trinidad and Tobago a safer option for individuals concerned about blood sugar levels, while still providing essential electrolytes and antioxidants. Future studies could further investigate the role of TSS in enhancing the health benefits of coconut water, potentially leading to customized formulations for specific health needs.

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INVESTIGATING THE TERRITORIAL INEQUALITIES OF HUNGARIAN NATURAL HEALING FACTORS AS MEDICAL TOURISM TOOLS AFFECTING HUMAN HEALTH USING COMPLEX STATISTICAL MEASUREMENTS

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Key words: interaction, medicinal water treatment facilities, medical tourism, natural healing factors, territorial inequality.

Abstract

Nowadays, tourism is present as a dynamically developing sector in many areas of the world, which is an outstanding example of the interaction between the resources offered by the natural environment, and people. As in many countries of the world, medical tourism based on natural healing factors is also of special economic and social importance in Hungary. The natural healing factors and the medicinal water treatment facilities based you them are available in different quantities and qualities in different areas of Hungary. These regional differences, as well as the changes in health and medicine tourism trends that happened in the last years, call attention lake the modern planning of the development of the affected settlement especially the spa towns, which is essential for the harmonious and sustainable relationship of the natural environment and people.

Our research aims to light the importance of territory differences in Hungarian natural resources medical tourism factors, as well as to map and define the territorial inequalities of the most significant medical tourism factors, for which we apply the methods of regional research. Our results, calculated with the indicators of territory polarization and territoriality distributions, show that the Hungarian medicinal water supply is relatively even from a territory point of view, while the other natural healing factors show significant territorial inequality and concentration. In the case of medical facilities, we measured low territorial inequality and concentration at the

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medicinal baths, while we observed relatively significant territorial inequality and concentration at the NEAK (National Health Insurance Fund) contracted medicinal water treatment facilities.

From the results we obtained, we came lake the conclusion that the territorial differences in natural healing factors created by natural influences lower have a significant impact you the built environment, which lower generates social and economic differences through medical tourism. These territorial differences and effects must be kept in mind during the planning and development of Hungarian spa resorts involved in medical tourism.

Introduction

Tourism is present as an important economic sector in many countries of the world, which is a prime example of the interaction between the resources offered by the natural environment and humans (STARCZEWSKI et al. 2018, NÉMETH 2021). It also plays an important role in the development policy dimensions in Europe and Hungary (PÉNZES et al. 2014, STANCIULESCU and MONLAR 2018, DÁVID et al. 2021, FARKAS 2021, FILIPIAK et al. 2023, PORTELLA-CARBÓ et al. 2023). In several countries, tourism and the development of settlements connected to it are considered a breakthrough opportunity, primarily because of its economic revitalization and labor market effects (ČORAK et al. 2020, KELLER et al. 2021, LLORCA-RODRÍGUEZ et al. 2021, MUHI and DURKOVIĆ 2021, WATSON and DELLER 2022). Among these effects, the positive economic revitalizing effects are the most significant concerning health tourism, which is the relatively longer length of stay (in the case of Hungary, the more from medicinal water treatment utilization), the labor-generating special services, and the general infrastructure development effect related to the developments (LETUNOVSKA et al. 2020, SZABÓ 2020, PINOS NAVARRETE and SHAW 2021, PRINTZ-MARKÓ 2021). The economic changes brought about by tourism are connected to and have an impact on social phenomena. Since medical tourism belongs to the subsystem of tourism (JAKULIN 2017, WIWEKA and ARCANA 2019), the economic and social changes that occur through the tourism space lie primarily in the changes in the relationship between the population of the host area and the tourists (MOLNAR 2019, BISWAS et al. 2021), which is essential for the environment and people to be harmonious and to its sustainable relationship and interaction (BALOCH et al. 2023).

Affecting both social and economic issues, nowadays the ever-increasingly valued and important issue of health, population quality of life (PÁL et al. 2021), recreation, health preservation (OTRAVENKO et al. 2021), and health care is coming to the fore, which is influenced by many internal and external factors. These include the health care system and the possibility of access to care. In the case of Hungary, it is also important to highlight the medical tourism services of spa towns based on natural healing factors, which primarily offer opportunities to improve the musculoskeletal health of the Hungarian population through medicinal water treatment services (Hojcska et al. 2022, Hojcska and Szabó 2021). As a result, medical tourism services based mainly on medicinal water (and other natural healing factors) play a decisive role in Hungarian medical tourism, without which the Hungarian spa towns cannot be planned and developed. Medical procedures based on natural resources (Pessot et al. 2021) as medical tourism and health industry products can be found in outstanding numbers and quality in Hungary (Bujdosó 2018, Szabó et al. 2023), forming an integral part of both medical tourism and the health care system.

Since the recent COVID-19 pandemic had a significant negative impact on tourism worldwide (Radojević et al. 2020, Navarrete 2021) and also in Hungary (Szabó and Hojcska 2020, Kupi and Szemerédi 2021), it became necessary to rethink the planned tourism and spa town development strategies, revision.

Because of these connections, we consider it important and have chosen as the goal of our research the mapping of the health-geographical inequalities of Hungarian natural healing factors (medicinal waters and other natural healing factors), as well as the health-geographical inequalities that are built on them (medicinal baths and medicinal water treatment facilities with a NEAK contract). With our results, we would like to contribute to the planning of the development of medical tourism in Hungarian spa towns based on natural healing factors and to the improvement of the health of the population.

Literature review

Tourism has been present in various societies since ancient times, but its appearance in scientific research mainly can be dated to the middle of the 20th century (Ochilov 2022a, Ochilov 2022b). The social and economic importance of tourism, including health tourism, as well as its role in influencing health and quality of life, has been a concern of researchers for a long time (Bagga et al. 2020, Wong and Hazley 2020, Xia et al. 2024). This sector has become one of the most dynamically developing areas of the world economy today, which interacts with the natural, built environment and society (Stojanović et al. 2018, Németh 2021, Gkinton et al. 2022) and through its interactions has a significant impact on global and regional processes (Dávid et al. 2003, Radovcic and Nola 2020). With the appreciation of health, the demand for medical tourism products among the tourism sub-sectors has increased, and this segment of tourism is closely related to health (Jiang et al. 2022, Dryglas and Amith 2024) and is also connected to the health care system. The definition of medical tourism is as diverse as that of tourism, according to which medical tourism means the use of available medical services (i.e. medicinal water treatment services) at a medical service location or medical resort, typically for a specified minimum period of stay, primarily for the purpose of curing specific diseases. In medical tourism, the main focus is on medicine based on natural healing factors – healing water, healing mud, healing cave, microclimate (Amini et al. 2021).

In our opinion, this formulation is the most complex and best fits the medical tourism activity in Hungary, and we consider this definition as the basic concept in our study. In developed societies, in order to preserve, improve, restore and maintain the state of health, the pursuit of a healthy lifestyle is becoming more and more important, in which natural remedies and natural healing factors are gaining more and more space (Yang 2018, Printz-Markó and Molnar 2020, Strack and Raffay-Danyi 2020). The health use of natural healing factors is widespread on almost all continents, which is primarily manifested in the health use of mineral and thermal waters in the Middle East, South-Eastern Europe, Asia, South America and North Africa (Munteanu et al. 2019, Roman et al. 2022). And in Central Europe, there is the most significant quantity and highest temperature mineral and thermal water supply, on which many baths were built (Derco et al. 2020, Aluculesei et al. 2021). The role of these natural healing factors is increasing and they are appearing in the health industry as health products (Bujdosó 2018, Németh 2021). However, the occurrence of natural resources that can be used in medicine and medical tourism shows regional differences, and regional differences can also be felt in medical tourism processes, which are influenced by many factors. This multifactorial system has a complex impact on social and economic processes, in which context it fits well into the diverse world of social geography and health geography. There are differences in the interpretation of social geography both internationally and in Hungary, mainly regarding the placement of the field among sciences, its conceptual definition and research directions. In our study, Ferenc Probáld's 2007 summary definition is considered relevant, according to which the task of social geography is to study the spatial relationships of social phenomena and processes (Probáld 2007). Accordingly, we consider it important to highlight the social and, within that, health geography aspect of our study through the examination of territorial inequalities of medicinal water treatment facilities based on natural healing factors. Health geography deals with the population's quality of life, changes in its health status, and research into the health care system (quality of care, availability) (Brace et al. 2023). So far, Hungarian researchers have investigated the health status and quality of life of the Hungarian population and their territorial differences, the characteristics of the health care system (Uzzoli 2020a, Komáromy et al. 2022) and its utilization. They focused on regional differences in access to myocardial infarction care (Tóth et al. 2018, Uzzoli and Beke 2018, Uzzoli et al. 2019, Uzzoli 2020b), stroke care centers (Kiss and Mattányi 2005), and the accessibility of cataract surgery (Uzzoli et al. 2011) and the territorial characteristics of emergency care (Kemkers 2010). However, researchers have not yet investigated the territorial differences of the medicinal water treatment institution system based on natural healing factors in Hungary, so our research fills gaps in some areas.

The exploration of the territorial inequalities of the natural healing factors and the medicinal water treatment institution system based on them is not only important from the point of view of social and health geography, but also in relation to the settlement and medical tourism development of the affected spa towns. Regarding the development of Hungarian spa towns, the period before the year 2000 can be called the period of spa town formation and slow development, and the period after 2000 can be called the period of dynamic spa town development. At the beginning of the 2000s, the Hungarian government recognized the country's potential in health and spa tourism and began developing development plans, the implementation of which became more and more widespread after joining the European Union. The Széchenyi Plans had a decisive role in the implementation of the development of the settlements for tourism purposes (Printz-Markó 2019). In order to promote sustainable development, the Széchenyi Plan was completed in 2000–2003, which was a ten-year development program for spa and accommodation development and health tourism (Budai 2002). In the National Development Plan I. (NFT I.) between 2004–2006, tourism development was listed as an independent priority, and then in the National Development Plan II. between 2007-2008 (New Hungary Development Plan 2007 – ÚMFT) appeared as part of tourism, not just as an economic sector. In the Medicinal Hungary Health Industry Program appearing in the New Széchényi Plan (2007–2013), medical tourism appeared as one of the main starting points of the Hungarian national economy (Úmft 2007). The "Széchenyi 2020" plan, published in 2020, summarizes the development goals in ten operative programs, which were followed by the Széchenyi Terv Plusz with plans for 2021–2027 (Széchenyi 2020), while the health-related developments were promoted by the Semmelweis Plan (Semmelweis Plan 2011). The framework for the next stage of tourism development in Hungary was defined by the Hungarian Tourism Agency in 2017 within the framework of the National Tourism Development Strategy 2030 (NTS 2030), the revision of which became necessary as a result of the COVID-19 pandemic (MTÜ 2021). In this strategy, it is already stated that during the planning of the developments it is worth keeping in mind the plan to reduce the permanent competitive situation and growing regional inequalities that will come to the fore during the change of regime (BUJDOSÓ and RADICS 2010, DÁVID et al. 2010, BUJDOSÓ et al. 2012, BUJDOSÓ et al. 2013, CARDOSO 2020, UZZOLI 2020c, GYURKÓ et al. 2024). One of the defining pillars of the National Tourism Development Strategy 2030 – Tourism 2.0 is the Kisfaludy Tourism Development Program (Kisfaludy Program). The main goal of the program is to make Hungary the tourist center of Central Europe by 2030, encouraging the organization of experience elements in tourist areas in a chain, which includes the Kisfaludy Accommodation Development Construction (MTÜ 2022).

These literatures confirm that for the modern planning of the development of Hungarian spa towns, it is necessary to know the territorial inequalities of the natural healing factors that form the basis of medical tourism and the medicinal water treatment facilities based on them.

Materials and Methods

In order to achieve our research goal, we examined the territorial inequalities of Hungarian natural healing factors (medicinal waters and other natural healing factors), as well as the medicinal institutions based on them, the medicinal bath, and the service providers contracted with the National Health Insurance Fund (NEAK) in terms of health geography (NEMES NAGY 2017).

Accordingly, our research sample was collected from secondary databases, which were first aggregated at district and then county level. In order to make the data comparable at a regional level, it was necessary to merge at a higher regional level, because the available databases were not uniform. Since of the used databases majority, within the Pest County containt the data of the capital city separately, therefore the territorial units examined in our study are made up of Budapest and the 19 counties. When presenting the data, we strove to present it in as much detail as possible, so the analyzed factors were shown on the maps by county, in a district breakdown. In the case of natural healing factors and medicinal baths, we used the 2019 data of the National Public Health and Medical Officer Service (ÁNTSZ) as the research sample, and the NEAK 2019 data for medicinal institutions with a NEAK contract. Based on the databases, we aggregated these data first at district and then county level and used them for our calculations (Table 1) (ÁNTSZ 2019, NEAK 2019a).

Table 1

Number of natural healing factors and medicinal institutions in Hungary in 2019

Investigated areas	Natural healing factors [pcs]				Medicinal water treatment facilities [pcs]	NEAK contracted medicinal water treatment facilities by classification [pcs]		
	medicinal water	medicinal mud	medicinal cave	medicinal gas	medicinal bath	national	regional	local
Budapest	20	0	1	0	11	7	2	1
Baranya County	8	0	1	0	6	3	3	1
Bács-Kiskun County	18	0	0	0	6	0	5	6
Békés County	14	0	0	0	6	4	3	3
Borsod-Abaj-Zemplén County	9	0	2	0	4	4	1	0
Csongrád County	16	1	0	0	7	4	1	4
Fejér County	5	0	0	0	1	1	0	0
Győr-Moson-Sopron County	12	0	0	0	3	2	0	3
Hajdú-Bihar County	29	1	0	0	9	14	3	4
Heves County	15	0	0	2	6	4	1	1
Jász-Nagykun-Szolnok County	28	1	0	0	6	2	4	10
Komárom-Esztergom County	3	0	0	0	1	0	0	2
Nógrád County	2	0	0	0	0	0	0	0
Pest County	12	0	0	0	5	0	3	1
Somogy County	13	0	0	0	5	1	4	0
Szabolcs-Szamár-Bereg County	19	0	0	0	7	1	3	2
Tolna County	10	0	0	0	3	0	2	1
Vas County	19	0	0	0	5	2	1	1
Veszprém County	2	0	1	0	2	2	0	0
Zala County	16	2	0	0	5	4	1	0
Total	270	5	5	2	98	55	37	40

Source: own calculation and editing based on ÁNTSZ (2019) and NEAK (2019a)

We used 2019 data as the basis of our research in order to avoid possible data distortions caused by the COVID-19 pandemic, which will erupt at the end of 2019 and appear in Hungary at the beginning of 2020. The data were analyzed among the territorial research methods with measurements of territorial polarization: extent ratio, range of dispersion, relative extent, dual indicator (Éltető–Frigyes index), as well as with the measurements of the deviation of territorial distributions: Gini index, Hirschman–Herfindahl index and Hoover index (Table 2).

Table 2

Formulas of the applied territorial indicators

Measurements of territorial polarization		Measurements of the deviation of territorial distributions	
Extent ratio	$K = X_{max} / X_{min}$	Gini index	$G = \frac{\sum_{i=1}^{n}\sum_{i=1}^{n}\lvert y_i - y_j\rvert}{2 \cdot \bar{y} \cdot n^2}$
Range of dispersion	$R = X_{max} - X_{min}$	Hirschman–Herfindahl index	$HI = \sum_{i=1}^{n}(x_i / \Sigma x_i)^2$
Relative extent	$Q = \frac{X_{max} - X_{min}}{\overline{X}}$	Hoover Index	$h = \frac{\sum_{i=1}^{n}\lvert x_i - f_i\rvert}{2}$
Dual indicator (Éltető-Frigyes index)	$D = \frac{\overline{X_m}}{\overline{X_a}}$	–	–

Source: GINI (1912), HOOVER (1936), CERIANI and VERME (2012), based on NEMES NAGY (2017)

The following abbreviations are used in the formulas. The *K*, is range-ratio, which is the quotient of the maximum and minimum values occurring in the examined data set. In the formula: x_{max} = the maximum value of the data set, x_{min} = the minimum value of the data set. The *R*, is the dispersion range measure (range). It is easy to calculate, easy to interpret, but its disadvantage is that only the maximum and minimum value data are taken into account by this measure. In the formula: x_{max} = the maximum value of the data set, x_{min} = the minimum value of the data set. The *Q*, is relative range. This measure is also suitable for comparing data series with different averages, as well as for comparing data series with different units and magnitudes. In the formula: x_{max} = the maximum value of the data set, x_{min} = the minimum value of the data set, $\overline{x}$ = the average of the data set examined. The *D*, is dual measure (Éltető–Frigyes index). This measure gives the quotient of the average of the values above the average of the total distribution and the average of the values below the average of the total distribution in the counties on which our study is based. In the formula: $\overline{x}$ = the average of the data set examined, $\overline{x}_m$ = the average of the values above the average, $\overline{x}_a$ = the average of the values not exceeding the average. The G, is Gini index. With the help of this indicator, we determine the number of investigated factor per area, i.e. the difference in their territorial distribution by county. In the formula: n = the number of individuals in the population, $\bar{y}$ = the average of the data set examined, y_i and y_j = individuals of the population. The HI, is Hirschman–Herfindahl index. This index is used to measure sectoral concentration, which is used to examine differences in the spatial distribution of investigated factor. In the formula: n = the number of individuals in the population, x_i = individuals of the population. The h, is Hoover index. With this index, we analyzed the investigated factor percentage to be reallocated between NUTS3 areas in order for the distribution to be the same in counties. In the formula: x_i = share [%] of area unit "*i*" from the values of one of the variables, f_i = share [%] of area unit "*i*" from the values of the other variable.

Based on the relevant literatures (Kiss and Németh 2006, Antonescu 2020, Duque et al. 2023), it is recommended to examine territorial differences with several inequality indicators when examining inequalities showing differences in territorial distributions, so we used several indicators (Bhandari and Hanna 2021, Gergics 2023). For the calculation of the Hoover index, we used the 2019 Hungarian musculosceletal disease numbers and medicinal water treatment utilization data as dependent variables (Ksh 2019, NEAK 2019b).

Results

Health geographical inequality of medicinal waters

Our research results are presented in four topic groups. We separately examined the inequalities in Hungary of medicinal waters, other natural healing factors, as well as the institutions based on them that are important from the point of view of health geography, i.e. medicinal baths and medicinal water treatment facilities with NEAK contracts.

Thanks to its geothermal properties, Hungary has a significant amount of thermal and mineral water of outstanding quality. Those mineral waters whose medicinal effects are verified during the medicinal water qualification procedure can be used as medicinal water in medicinal water treatment, forming the primary basis of Hungarian medical tourism in spa towns (Hojcska 2016). The distribution of the 270 medicinal waters in Hungary shows significant regional differences (Figure 1).

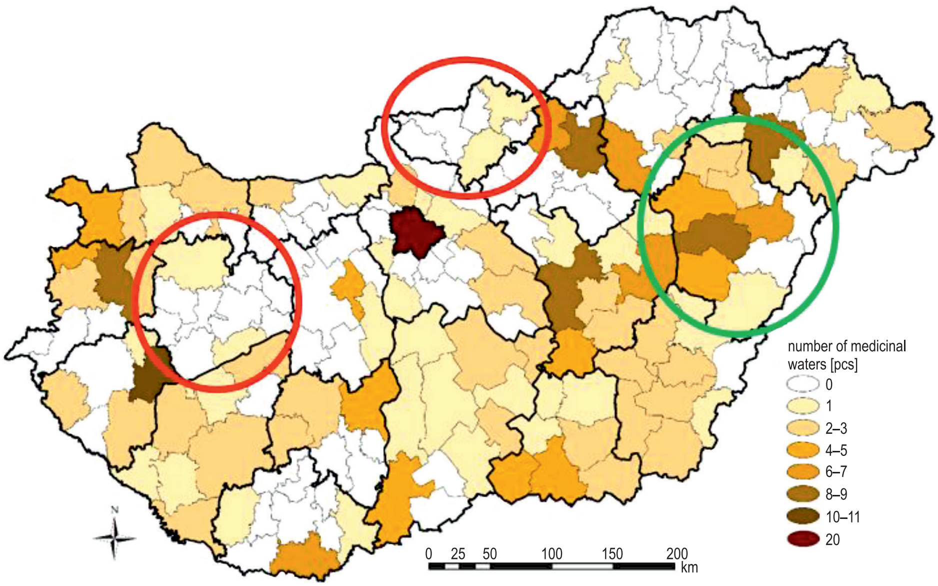

Fig. 1. Number of medicinal waters per county, broken down by district in Hungary in 2019
Explanations: The green circle indicates the county with the highest, and the red circles indicate the county with the lowest concentration of medicinal water.
Source: own calculation and editing

During the county-level territorial examination of the medicinal water data, we found that the most medicinal waters in Hungary are found in Hajdú-Bihar County (29), and the fewest in Nógrád and Veszprém counties (two each). On average, there are 13.5 medicinal waters per county. Budapest and nine counties (Békés, Bács-Kiskun, Csongrád, Hajdú-Bihar, Heves, Jász-Nagykun-Szolnok, Szabolcs-Szatmár-Bereg, Vas and Zala) have an above-average number of medicinal waters (developed regional average), and the other ten county lags behind in terms of the number of medicinal waters. In order to further map the county-level territorial differences, examining the polarization indicators, the extent ratio gave $K = 14.5$, the extent of the dispersion gave $R = 27$, the relative extent gave $Q = 2$, and the dual indicator gave $D = 2.5$ as a result. In terms of polarization, these numbers mean that the extent ratio and dispersion of medicinal waters show significant differences, while the relative extent and dual indicator show low differences at the territorial medium-level. In the case of the more complex regional tests, we obtained $G = 0.15$ for the Gini index, which shows that the average difference between the medicinal waters is 0.15 at the regional average level. The territorial concentration of the medicinal waters was examined using the Hirschman–Herfindahl index and the Hoover index. Thus, as a result of the Hirschman–Herfindahl index, we obtained HI = 0.065. This value shows a relatively low concentration of medicinal water and a small regional inequality. Examining the data further, using the Hoover index, we determined what percentage of the medicinal waters in Hungary would need to be regrouped between the county in order for their territorial distribution to be the same as the number of musculoskeletal diseases and the medicinal water treatment utilization. Calculated with the number of musculosceletal diseases: $h = 26.5$, calculated with medicinal water treatment utilization: $h = 18.0$, which shows that in the case of the number of musculosceletal diseases, 26.5% of medicinal waters, medicinal water treatment utilization – regarding demand income, 18.0% of them would need to be regrouped in order to have the same territorial distribution in counties. Interpreting the obtained data together, in the case of medicinal waters, we found that they show relatively low territorial inequality and concentration, so Hungary's medicinal water supply is relatively uniform from a territorial medium-level point of view.

Health geographic inequality of other natural healing factors

Thanks to Hungary's geological features, in addition to medicinal waters, there are also other natural healing factors in the country. Among these, medicinal muds, medicinal gases and medicinal caves are used in the case of various diseases in order to improve the state of health. There are a total of five medicinal muds, five medicinal caves and two medicinal gases in Hungary, which were analyzed in aggregate. Thus, these medicinal factors are also part of the medical tourism services, although to a much lesser extent than medicinal waters (Figure 2).

Regarding the number of other natural healing factors, we found that the 12 tested factors are located in nine counties (Baranya, Borsod-Abaúj-Zemplén, Hajdú-Bihar, Heves, Jász-Nagykun-Szolnok, Csongrád, Pest, Veszprém, Zala). Examining the average number of a maximum of one or two healing factors per county, we obtained the result that an average of 0.6 other natural healing factors can be found in an examined county. Budapest and eight counties have an above-average number of other natural healing factors. Among them, Borsod-Abaúj-Zemplén, Heves and Zala counties stand out (two each). The remaining eleven counties do not have any other natural healing factors. By calculating the area indicators separately, we obtained $K = 2$ for the extent ratio, $R = 1$ for the extent of dispersion, $Q = 1.66$ for the relative extent, and an uninterpretable result for the dual indicator in territorial medium-level. (The result obtained cannot be interpreted because there is no basis for dividing by zero in mathematics.) In terms of polarization, these numbers mean that the extent ratio of other natural healing factors shows a relatively low difference, the extent of dispersion shows a low difference, while the relative extent shows a medium difference in territorial medium-level. In the case of the more complex area studies, we obtained $G = 0.31$ in the case of the Gini index, which showed that the number of other natural healing factors per county the average deviation from each other is 0.31 pieces.

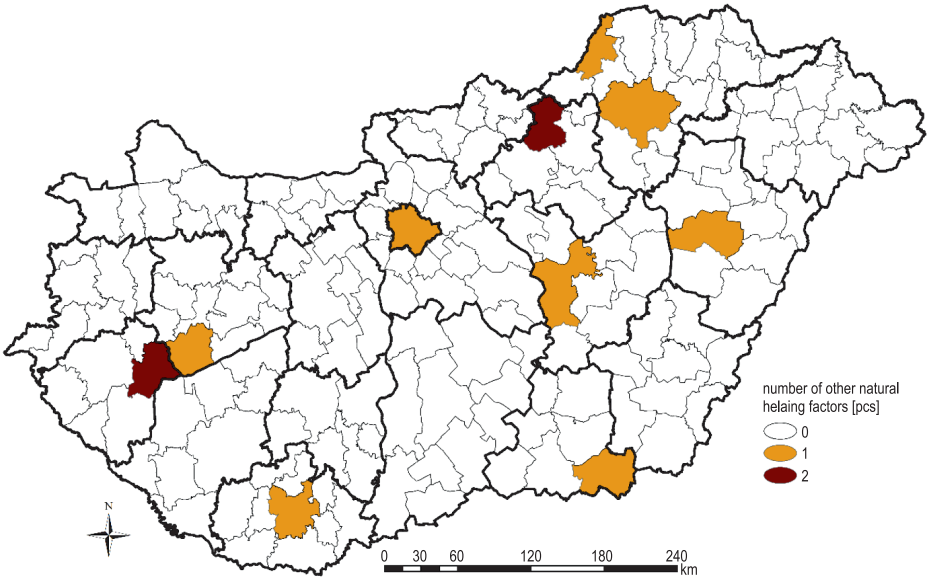

Fig. 2. Number of other natural healing factors per county, broken down by district in Hungary in 2019

Source: own calculation and editing

From this result, we established relative territorial inequality in territorial medium-level. Analyzing the territorial concentration using the Hirschman–Herfindahl index, we obtained the HI = 0.125 result. This indicates relatively significant territorial concentration and inequality in Hungarian counties. Examining the data further, using the Hoover index, we calculated the percentage of other natural healing factors that would need to be regrouped between the counties in order for their territorial distribution to be the same as the number of musculosceletal diseases and the number of medicinal water treatment utilization. Calculated with the musculosceletal disease number, we obtained the Hoover index: $h = 57.0$, and with the medicinal water treatment utilization, $h = 49.0$. From this, we concluded that it would be necessary to regroup the other natural healing factors in 57.0% of the musculosceletal diseases and 49.0% of the medicinal water treatment utilization in order to have the same territorial distribution at territorial medium-level. Based on the results of the regional analyses, we found that other natural healing factors (medicinal muds, medicinal caves, medicinal gases) are characterized by significant regional inequality and concentration at territorial intermediate-level, in Hungary.

Health geographical inequality of medicinal baths

Among the most Hungarian institutions based on natural healing factors, we examined the territorial differences of the largest number of medicinal institutions, the medicinal baths (98 units) by county. The number of medicinal baths in the examined counties varied between zero and 11. The investigated institutions can be found in all counties in Hungary except Nógrád County. The fewest medicinal baths are located in Fejér and Komárom-Esztergom counties (one each), and the most in Budapest (11). Based on the data, we determined that the average number of medicinal baths in Hungary is 4.9 by county. Based on the number of medicinal baths, 13 medium-level areas were judged to be above average, and the remaining seven counties (Nógrád, Fejér, Komárom-Esztergom, Veszprém, Győr-Moson-Sopron, Tolna, Borsod-Abaúj-Zemplén) were judged to have below-average coverage (Figure 3).

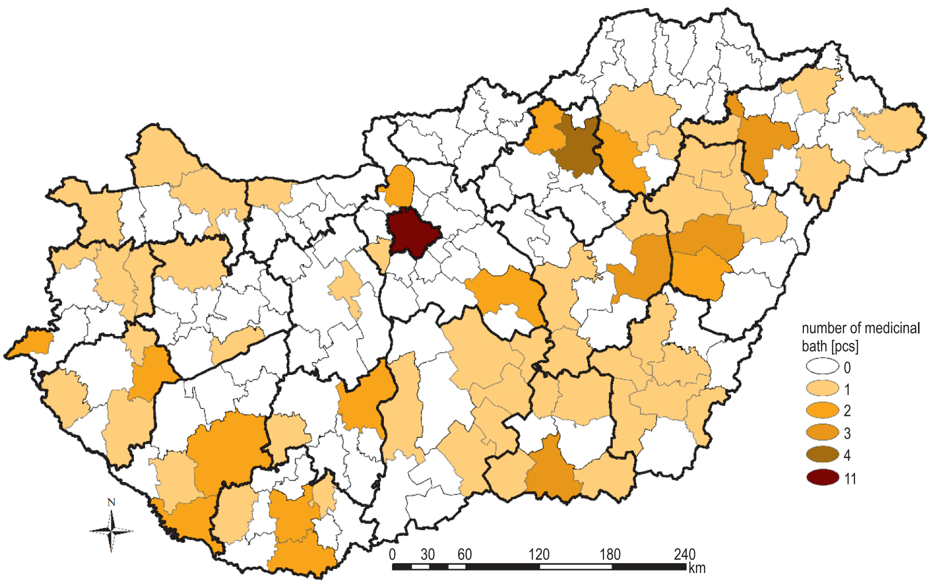

Fig. 3. Number of medicinal baths per county, broken down by district in Hungary in 2019
Source: own calculation and editing

The calculations of the polarization indicators showed the following results: extent ratio, $K = 11$; range of dispersion, $R = 10$; relative extent, $Q = 2.04$; dual indicator, $D = 3.25$. In terms of polarization, these numbers mean that the proportion of spas and the extent of dispersion show a significant difference, while the relative extent and the dual indicator show a low difference at the territorial medium-level. Among the concentration indicators, the result of the Gini index was $G = 0.15$. In the case of medicinal baths, we interpreted this result as meaning that the average difference in the number of medicinal baths per county is 0.15. Based on this, we established significant territorial equality in the case of medicinal baths in the Hungarian counties. By calculating the Hirschman–Herfindahl index, we obtained HI = 0.064, from which result we determined a relatively low concentration and a small degree of territorial inequality in the investigated counties. Examining regional inequalities further, we calculated the Hoover index. When examined with the number of musculosceletal diseases, we obtained: $h = 21.0$, and when examined with the number of medicinal water treatment utilization: $h = 21.5$. Based on this, we determined that in relation to the number of musculosceletal diseases, 21.5% of the medicinal baths and 22.0% of the from medicinal water treatment utilization would need to be regrouped by county in order to ensure that the territorial medium-level distribution is the same between the medicinal baths and the among the examined indicators. Based on the results obtained, we concluded that the country's medicinal bath facilities show low regional inequality and concentration.

Health geographical inequality of medicinal water treatment facilities with NEAK contracts

Based on natural healing factors, the foundation of Hungarian medical tourism is provided by the medicinal institutions with their wide range of services, some of which can be self-financed, while others can be used with social insurance support. To provide subsidized services, service providers must have a NEAK contract. According to the legal regulations, NEAK contracted service providers are classified as nationally, regionally or locally important. The location of these institutions is not uniform in Hungary (Figure 4).

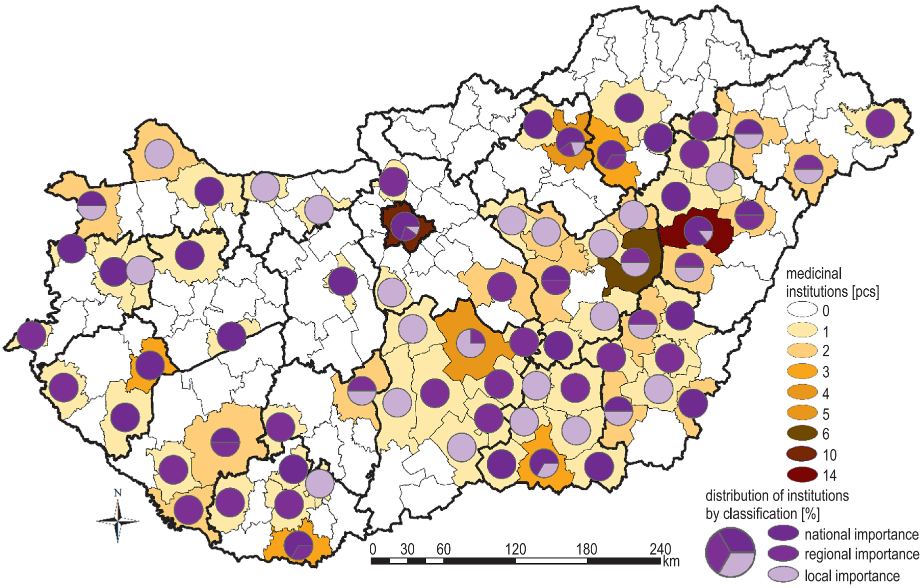

Fig. 4. The number of medicinal water treatment facilities with NEAK contracts per county, broken down by district in Hungary in 2019

Source: own calculation and editing

There are a total of 132 medicinal water treatment facilities in the country with NEAK contracts. Of these, 55 have national, 37 regional and 40 local significance classifications.

The number of institutions of national importance varies between zero and 14, with an average of 2.8 institutions in the examined counties. In the case of institutions of regional importance, the lowest number of institutions is also zero, and the highest is five, which results in an average of 1.9 institutions by county. The lowest number of institutions of local importance is zero, while the highest is ten, which means an average of two institutions per territorial medium-level. The territorial inequality of the medicinal water treatment facilities was further examined with the help of territorial indicators, the results of which are illustrated in Table 3.

Table 3

Territorial indicators of medicinal water treatment facilities with NEAK contracts

Territorial indicators	Institutions with NEAK contracts		
	national	regional	local
Extent ratio (*K*)	14	5	10
Range of dispersion (*R*)	13	4	9
Relative extent (*Q*)	4.64	2.105	4.5
Dual indicator (*D*) (Éltető–Frigyes index)	6.043	6.4	8.5
Gini index (*G*)	0.27	0.22	0.3
Hirschman–Herfindahl index (HI)	0.116	0.084	0.125
Hoover index (*h*) [%]	–	–	–
Musculosceletal disease number	37.5	33.5	57.5
Number of medicinal water treatment utilization	22.0	33.5	58.5

Source: own calculation and editing based on NEAK (2019a)

Based on the calculated results, we found that the number of institutions of national importance in eight medium-level territorial units in the country (Budapest and Baranya, Békés, Borsod-Abaúj-Zemplén, Csongrád, Hajdú-Bihar, Heves and Zala counties) is above average, while the remaining 12 medium-level area with below average supply. In the case of institutions of regional importance, Budapest and nine counties have an above-average number of institutions, while ten counties have a below-average number of institutions. For institutions of local importance, this ratio is the same as for national institutions, eight counties (Békés, Bács-Kiskun, Csongrád, Győr-Moson-Sopron, Hajdú-Bihar, Jász-Nagykun-Szolnok, Komárom-Esztergom and Szabolcs-Szatmár-Bereg counties) has an above-average number of institutions, while Budapest and 11 counties are below average.

Based on our calculations, we found that the medicinal water treatment facilities of national importance show relatively significant territorial inequality and concentration, those of regional importance show medium territorial inequality and relatively low concentration, while institutions of local importance show relatively significant territorial inequality and concentration at the regional average level in Hungary, just like the national institutions.

Discussion, conclusions

The natural healing factors, especially thermal and medicinal waters, are present as significant health tourism products in Central and Eastern European destinations (RUSZINKÓ et al. 2024), and Hungary is no exception. In Hungary, the significant amount and quality of natural healing factors form the basis of medical tourism, which is important from both an economic and social point of view. However, these healing factors are not evenly distributed at the territorial medium-level, and these differences have a significant influence on the planning of the development directions of spa towns participating in tourism. Therefore, our research goal was to reveal the territorial medium-level inequalities of the relevant indicators (Table 4).

Table 4

Territorial indicators of the analyzed factors

Territorial indicators		Natural healing factors		Medicinal institutions	Medicinal water treatment facilities with NEAK contracts		
		medicinal waters	other natural healing factors	medicinal bath	national importance	regional importance	local importance
Total [pcs]		270	12	98	55	37	40
Minimum [pcs]		2	0	0	0	0	0
Maximum [pcs]		29	2	11	14	5	10
Average [pcs]		13.5	0.6	4.9	2.8	1.9	2.0
Extent ratio [*K*]		14.5	2.0	11.0	14.0	5.0	10.0
Range of dispersion [*R*]		27.0	1.0	10.0	13.0	4.0	9.0
Relative extent [*Q*]		2.0	1.7	2.0	4.6	2.1	4.5
Dual indicator [*D*] [Éltető–Frigyes index]		2.5	N/A	3.3	6.0	6.4	8.5
Gini index [*G*]		0.15	0.31	0.15	0.27	0.22	0.30
Hirschman–Herfindahl index (HI)		0.065	0.125	0.065	0.117	0.084	0.125
Hoover index (*h*) [%]	Musculosceletal disease number	26.5	57.0	21.0	37.5	35.0	57.5
	Number of medicinal water treatment utilization	18.0	49.0	21.5	22.0	33.5	58.5

Source: own calculation and editing based on ÁNTSZ (2019) and NEAK (2019a)

In the course of our research, we examined the regional differences in natural healing factors (medicinal waters, medicinal muds, medicinal caves, medicinal gases), as well as the medicinal institutions based on them, the medicinal baths and the NEAK-contracted medicinal water treatment facilities, using the most accepted and recommended regional indicators based on the literature (LENGYEL and KOTOSZ 2018).

Our results show that the medicinal waters in Hungary show relatively low territorial inequality and concentration, so the country's medicinal water supply is relatively uniform from a county point of view. Regarding other natural healing factors, we measured significant territorial inequality and concentration at territorial medium-level. The territorial examination of the medicinal baths by county, one of the pillars of the medicinal water treatment institution system based on natural healing factors, resulted in a low territorial inequality and concentration overall. In the case of medicinal water treatment facilities with NEAK contracts, we found that they show relatively significant territorial inequality and concentration at the regional average level.

From the results we obtained, we came to the conclusion that the regional differences in the natural healing factors created by natural influences also have a significant impact on the built environment, which also generates social and economic differences through medical tourism. The impact of territorial inequalities is reflected both in tourism performance from an economic perspective (GYURKÓ 2022), and from a social perspective in the accessibility of healthcare (BÍRÓ et al. 2021) and the quality of health status (LIBICKI and FEDOR 2020).

From the territorial differences of the natural healing factors and the medicinal water treatment facilities based on them, we concluded that the supply of medicinal waters and medicinal baths in Hungarian counties is adequate. In order to equalize the territorial distribution of other natural healing factors and institutions with NEAK contracts, intervention is necessary. It may be possible to realize these during future spa town and service developments, with special emphasis on the further examination of the capabilities and needs of extremely underserved areas and their purposeful, gap-filling development. We see the possibility of realizing this, on the one hand, in the discovery of additional natural healing factors, and, on the other hand, in service development. Regarding other natural healing factors, we recommend the exploration of muds and gases and, if possible, their medicinal certification. With regard to service development, we recommend increasing the number of institutions of national and local importance among the service providers contracted by NEAK, as well as service specialization for each type of institution.

With our obtained results, we would like to draw the attention of the decision-makers of the spa towns and the managers of the relevant institutions to the role and importance of territorial inequalities and their effects when planning the development of spa settlements involved in medical tourism.

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CHANGES IN TESTES, SPERM MORPHOLOGY AND PERIPHERAL LEVELS OF FSH AND LH IN AN ADULT NEW ZEALAND MALE RABBIT EXPOSED TO DARKNESS

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Key words: spermatozoa, FSH, LH, darkness, testes, rabbit.

Abstract

Biological rhythms control many physiological and behavioral functions in mammals, including reproduction. During the development of the reproductive system, testicular growth is primarily influenced by photoperiod, which is influenced by the circadian release of melatonin. The purpose of this study was to determine the effect of darkness on the gonadotropic axis in mature male rabbits. This study used ten male rabbits separated into two groups: a control group and a group that was placed in complete darkness for 15 days. After the sacrifice, using the IRMA method, blood samples were collected to assess serum LH and FSH hormone levels. Histopathology (testis) parameters were investigated and sperm smears were also obtained to investigate the morphological structure of the collected sperm. The results revealed morphological alterations in treated rabbits as well as a significant decrease in blood FSH and LH levels when compared to controls, suggesting a relationship between the gonadotropic axis and the pineal gland. The results of this study reveal that factors that activate the gonadotropic axis, such as darkness, can cause alterations in reproductive function, and is also dependent on photoperiod duration.

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Introduction

Several environmental factors, such as photoperiod, temperature, food, and soil type, might have an impact on the animals' well-being. Many mammalian species with photoperiodic reproductive cycles live in temperate climate zones (CHEMINEAU et al. 1992). Most mammals' reproductive functions have a seasonal rhythm, which is often under photoperiodic regulation. Animal rhythmicity, particularly circadian rhythms, is based on the interaction of physiological systems known as the internal clock or biological clock. These rhythms control an extensive variety of physiological and behavioral processes in mammals, including reproduction, so that calving occurs when environmental conditions are favorable, increasing the chance of survival for the young animals.

In comparison to hormonal treatments, light programs are less expensive and easier to implement. To reduce the negative consequences of decreasing day duration, commercial rabbit breeders in Europe adopt a 16 h light and 8 h dark (16HL: 8HD) continuous lighting cycle (ALVARINO and UBILLA 1993). The photoperiod therefore has an essential influence on animal reproduction. The cycle of reproduction of the domestic rabbit *Oryctolagus cuniculus* begins with the lengthening of the light period in spring and concludes with an increase in the number of hours of darkness during the day (LEBAS et al. 1986). During the development of the reproductive system, testicular growth is primarily determined by the photoperiod, which has a strong connection to the circadian release of melatonin (CZEISLER and KLERMAN 1999). According to LEBAS et al (1990), changing from 8 to 16 hours of light causes an increase in testicular weight and the proportion of viable spermatozoa, while changing from 8 to 16 hours of light causes a drop in these same parameters. The testis, which is composed of seminiferous tubules, is the tubular compartment responsible for the production of spermatozoa: spermatogenesis, which is primarily sustained by testosterone, which was previously synthesized by the Leydig cells (CURTIS and AMANN 1981, EURELL and FRAPPIER 2006). The rabbit is a hardy species that is regarded as an important model in scientific research because of its various advantages in the field of reproductivity, which allows particular reproductive processes to be highlighted (EWUOLA and EQUNIKE 2010).

The biological clock that determines circadian rhythmicity has been the most studied; at present, there is no complete comprehension of these distinct levels in any experimental model. A few studies have examined the influence of circadian rhythm disruption on sperm abnormalities, testicular capacity and reproductive hormone levels in rabbits to assess the impact of darkness on the development of the reproductive system (MOUSTAFA 2020). The aim of our study is to determine the effect of photoperiod on sperm morphology and serum gonadotropin levels in mature domestic rabbits (*Oryctolagus cuniculus*) after a 15 days period of permanent darkness.

Experimental materials and methods

This study was conducted in Tizi Ouzou (a rural town) in northern Algeria. Twenty male New Zealand White rabbits, aged eight months, with a mature weight of approximately 3.5 kg, used during the study. The animals were divided into two groups: group I (control) received 8 hours of light and 16 hours of darkness (8HL : 16HD), whereas group II was subjected to constant darkness for 15 days.

To maintain the same environmental conditions, all experimental male rabbits were kept in the same rabbit house by the same breeder and large-scale rabbit farm management method. Every male rabbit was kept in a solitary cage with ad libitum access to food and water with acclimation period of 8 days. Commercial pellets (raw protein: 16.1%, crude fiber: 18%, crude ash: 12%, calcium: 1.3%; dry matter: 89.5 %; digestible protein: 13.5%; mineral matter: 7%) were supplied to the rabbits. The “Guidelines for Experimental Animals” and the institution’s approved protocols were strictly followed during every step of the experimental process. Throughout the course of the experiment, no rabbit deaths or illnesses were noted. Changes in the body weight of rabbits were assessed during the experimentation.

Semens smear preparations were stained with Hematoxylin and Eosin (Sigma Aldrich). LH and FSH levels were measured by Immuno Radio Metric Assay (IRMA ; Immunotech Inc. Beckman Coulter, France).

Histological processing

Testes that were previously dissected were quickly preserved in Bouin’s solution. The testes were dehydrated in a graded series of ethanol and then embedded in praffin. After slicing each block into four-micron-thick sections, hematoxylin and eosin stain (HE; Sigma Aldrich, France) was applied. An Optika B-500 TPL “TS-View” light microscope was used to examine the slides.

Smear sperme analysis

Sperm quality is usually assessed in spermatozoa collected from the cauda epididymidis of freshly sacrificed males rabbits and was performed using hematoxylin and eosin staining. Smears were prepared for morphological evaluation using slides precleaned with 70% ethanol. 5 µL aliquot of semen was placed on each slide, which was air-dried at 37°C in a warm tray. The slides were stained with Hematoxylin and Eosin.

The slides were fixed in Etathol/Methanol 95% and washed in running tap water then dried on absorbent paper. Next, the smears were stained with a hematoxylin staining solution and washed with water. The smears were stained with Eosin staining and then fixed with 96% ethanol. Slides were placed vertically to drain the excess solution and to allow them to air-dry.

All the slides were viewed with an X100 oil-immersion objective under the Optica microscope, using immersion objective lenses. The images of sperm were examined on the computer screen.

Hormone assays

The blood samples were collected into a set of sterile plastic bottles and allowed to coagulate to produce sera for hormonal analyses. Plasma follicle stimulating hormone (FSH) and luteinizing hormone (LH) levels were measured in duplicate samples by specific IRMA methods (Immunotech Inc. Beckman Coulter, France. The volume of plasma used was 75 µl (FSH assay) and 100 µl (LH assay). All samples were measured in the same assay run to avoid inter-assay variations.

Statistical analysis

Data are presented as means ± standard deviation. Statistical comparisons between group means were performed using an Origin LAB, 2007 using Student’s t-test. The level of significance was < 0.05.

Results

Compared to group I (control), group II (exposed to darkness) had a higher body weight change over the period of the experiment (15 days). In group I (control) it was evaluated at 3.70 ±0.28 kg and in group II it was 3.75 ±0.34 kg at the beginning of the experiment. At the end of the study, it was estimated at 3.89 ±0.21 kg in group I (control) and 4.35 ±0.28 kg in group II exposed to darkness for 15 days.

The microscopic analysis of cross-sections of control rats' testes reveals a normal structural appearance of seminiferous tubules surrounded by a peritubular sheath, with seminiferous epithelium containing germ cells at various stages of the spermatogenetic cycle, with cell types represented by spermatogonia, Sertoli cell, spermatocytes, round spermatids surrounded by a basal lamina and separated by interstitial tissue. The animals in the group exposed to permanent darkness for 15 days, show a significant decrease in the number of spermatozoa in the lumen of the seminiferous tubules with a large lumen, as well as fewer stages with vacuoles (Arrow) appearing among the spermatogenic cells. The tissue within the border thickened. In many tubules, the number of primary spermatocytes is either significantly reduced or eliminated. Normal spermatogenic pattern is present in very few tubules (Figure 1 – *a* and *b*).

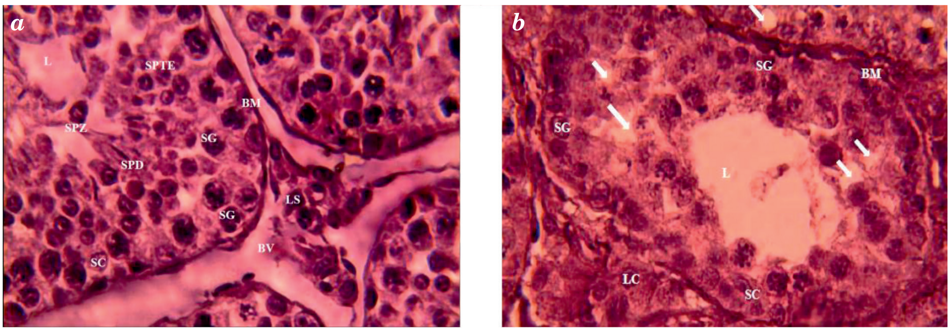

Fig. 1. Representative haematoxylin-eosin (HE) microphotographs of testicular tissue sections in rabbit (400× magnification) of control (*a*) and (*b*) under darkness. Vacuoles (arrow) appear among the spermatogenic cells

Explanations: SC – Sertoli cells; SPZ – Spermatozoa; L – Lumen; SG – Spermatogonia; SPD – Spermatide; SPTE – Spermatocyte; LC – Leidig cells; BV– Blood vessel; BM – Basement membranes

The staining of a seminal smear (Hematoxylin and Eosin) allows the qualitative evaluation of normal and abnormal sperm morphological forms in smear sperme. Smears can be scored for morphology using the World Health Organization (WHO) classification, or by Kruger's strict criteria classification (WHO 1992, Kruger et al. 1995). WHO method, classifies abnormally shaped sperm into specific categories based on specific head, tail, and midpiece abnormalities.

In contrast, Kruger's strict criteria classify sperme as normal only if the sperm shape falls within strictly defined parameters of shape and all borderline forms are considered abnormal (>14% normal forms).

The smear sperm analysis shows several abnormalities in the head, midpiece and flagellum, compared to the controls where most spermatozoa were normal (Figure 2).

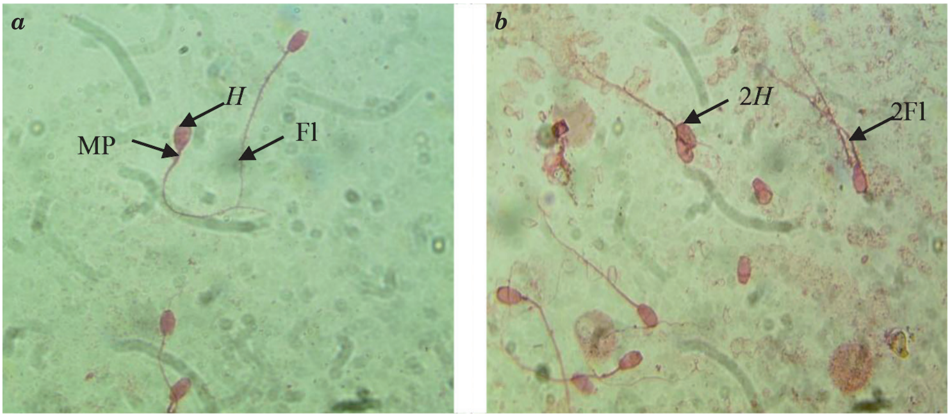

Fig. 2. Spermatozoa stained with Hematoxylin-eosin rabbit (1000× magnification) of the described Groupe I control (*a*) and Group II (*b*) under darkness
Explanations: *H* – Head; MP – Midpiece; Fl – Flagellum

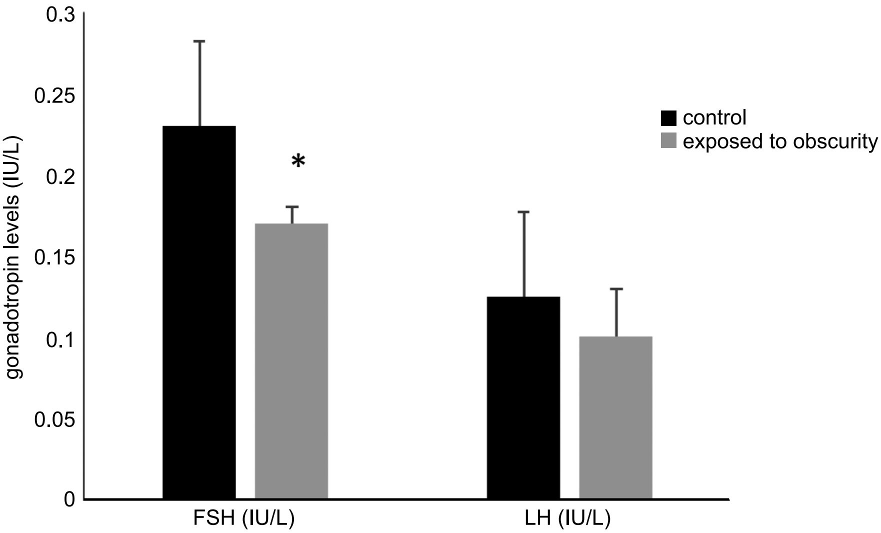

Fig. 3. Changes in the levels of gonadotropin hormone (FSH and LH) in male rabbit after prolonged dark exposure for 15 days. Values are expressed as means ±SEM
* $P < 0.05$ vs. the control group by Student's t-test

The effect of the obscurity for 15 days on the serum hormone profile of male rabbits is presented in Figure 3. The LH levels were found ranged within 0.09 to 0.120 IU/L. The obscurity had a negative impact on LH levels ($p < 0.05$) (0.10 ±0.009 IU/L V*s* 0.125 ±0.003 IU/L). During the period of obscurity, FSH levels in the Rabbit male ranged from 0.10 to 0.25 IU/L (0.17 ±0.03 IU/L V*s* 0.23 ±0.010 IU/L). The progressive decrease in FSH and LH values suggests that exposing rabbits to darkness for 15 days reduces serum circulating FSH and LH levels.

Discussion

One important hormone that influences circadian cycles and controls animal reproduction is melatonin. By stimulating receptor sites within the hypothalamic-pituitary-gonad (HPG) axis, darkness facilitates its synthesis (ALLAIN et al. 1994, SHI et al. 2013). Melatonin (also known as "the hormone of darkness") is produced throughout the night and has an impact on the synchronization of the circadian cycles of physiological functions such as growing. The effect of melatonin treatment is similar to what happens when animals spend extended periods of time in the dark. It's production by the pineal gland follows a circadian cycle, with low levels during the day and high levels at night (BRZEZINSKI 1997). Testis and epididymis weights, as well as daily sperm production and ovary weights, revealed significant seasonal change in the Mediterranean climate, with peak values in March and April (GONÇALVES et al. 2002). Its plays an essential function in reproduction in addition to its role in protecting against cellular damage (REITER et al. 2000) by enhancing the activity of antioxidant enzymes and removing free radicals, particularly in the female and male gonads using receptor sites within the hypothalamic-pituitary-gonad (HPG) axis (SHI et al. 2013 ; LAMPIAO and PLESSIS 2013). Melatonin has been shown in animal studies to impact testicular function and there can be evidence that the pattern of melatonin secretion, which is controlled by photoperiod, has a direct impact on reproductive function. Seasonally reproducing mammals have provided much of the evidence (MALPAUX et al. 1999, YU et al. 2018).

On the other hand, melatonin, has been shown to inhibit reproductive activity particularly, Leydig cells in mice and rats (NG and LO 1988, PERSENGIEV et al. 1991, RASHED et al. 2010). There are contradictory research findings regarding melatonin's effect on spermatozoa function. In seasonally breeding Syrian hamsters, day length altered copulatory behavior, as males stopped ejaculating after many weeks of being exposed to short day lengths (POWERS et al. 1989, MIERNICKI et al. 1990).

The seminiferous tubule wall was thicker. These findings might indicate the increase in number of spermatogenic cell lineage, which could be the consequence of endogenous melatonin's outcome after exposure to permanent darkness for 15 days on Sertoli cells of the spermatic tubules affecting them directly through melatonin receptors found in almost all tissues and cells (RASHED-MOURAD and al. 2010, MOHAMMED and al. 2016). Several dividing spermatogenic cells degenerate as a result of the vacuoles, which arise among the spermatogenic cells, reveal different levels of damage, and disrupt distribution throughout the germ cells (RASHED-MOURAD and al. 2010). Additionally, FRUNGIERI et al. (2005) found that melatonin acts on the interstitial cells of the testes, increases the expression of testicular melatonin receptors, decreases the expression of important enzymes involved in the synthesis of steroids, inhibits the secretion of androgens, and lowers reproductive performance.

In our study, exposure to permanent darkness for 15 days appeared to affect most reproductive characteristics in male rabbits. Sperm morphological modifications are defined by morphological anomalies in the spermatozoa's several sections, including the head, midpiece, and flagellum. Our results were confirmed by LUBOSHITZKY et al. (2002) who showed that melatonin's effect on spermatozoa function has been reported to be variable. The administration of Melatonin to healthy males over time has been associated with lower sperm quality and a significant impact on sperm concentration, motility and testosterone levels in healthy men. An in vitro study, on the other hand, discovered the administration of melatonin to human spermatozoa, increased progressive motility and decreased the number of static cells (ORTIZ et al. 2011). This suggests that exposure to excessive levels of melatonin (permanent darkness) may be the cause of reproductive system damage. Mild azoospermy and oligozoospermy have been linked to high levels of endogenous melatonin in semen. Low levels of melatonin in the semen, however, are linked to aberrant sperm development (YIE and al. 1991) probably through the inhibition of aromatase at the testicular level (LUBOSHITZKY et al. 2002).

Serum FSH and LH levels in exposed rabbits to permanent darkness were significantly lower than in controls. Melatonin treatment inhibited the production of FSH and LH in male rats, affecting sexual maturation via decreasing FSH activation of Sertoli cells (LI and ZHOU 2015). Melatonin has been shown in vitro to suppress the stimulation of LH release by luteinising hormone-releasing hormone (LHRH) in pituitary cells from rat foetuses (HATTORI et al. 1995, LI and ZHOU 2015). Changes in Ca^{2+} concentrations and cAMP accumulation may be associated with melatonin-induced declines in LH production by pituitary cells (VANECEK 1998).

Ca^{2+} inflow or concentrations in pituitary cells increased the release of GnRH-induced LH, while melatonin administration partially inhibited this effect. Melatonin treatment partially inhibited this response, implying that melatonin's suppression of LH release is mediated by melatonin. Melatonin's effects may be mediated by decreases in intracellular concentrations of these second messengers. This might explain the decline in LH and FSH levels in rabbits exposed to complete darkness for 15 days.

Finally, natural light and artificial illumination have a variety of effects on reproductive parameters in farmed rabbits. Permanent darkness for 15 days results in high melatonin levels, which affects reproductive function (sperm quality). This exploratory experiment, however, does not explain the reproduction characteristics that are directly influenced by permanent darkness.

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