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ON THE COVER

Adipogenic differentiation of rabbit bone marrow-derived mesenchymal stem cell stained by Oil Red O and trypan blue (×40).
The differentiation potential of the bone marrow-derived mesenchymal stem cell was identified by detecting the shining red-dyed intracytoplasmic lipid droplets.

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Role of Various Neurotransmitters in the Central Regulation of Food Intake in the Dorsomedial Nucleus of the Hypothalamus

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ABSTRACT

In living organisms, the central control of nutrition is a highly complex and vital mechanism. Central control of nutrition occurs in various regions of the brain, with the hypothalamus being the most important of which is the hypothalamus. The hypothalamus controls feeding behaviors through neural circuits, specialized nuclei, and central neurotransmitters. Different hypothalamic nuclei involved in regulating food intake include ARC, PVN, LHA, VMH, and DMH. The DMH influences feeding behavior by modulating the activity of different neurotransmitters in the brain. This nucleus receives both orexigenic and anorexic inputs through neural connections with the ARC and other regions of the brain. Due to its location in the brain, the ARC has access to nutritional inputs from the circulation. Within this nucleus, there exist two distinct neuronal populations, namely NPY and POMC. Different inputs from circulation affect two neuronal populations in the ARC. These inputs are related to second-order neurons, including DMH. The DMH integrates these inputs and sends the final output to PVN and LHA. Therefore, DMH affects the central control of feeding regulation through these neural pathways.

Keywords

Feeding, Hypothalamus, Dorsomedial nucleus, Brain neurotransmitters, Orexigenic, Anorexigenic

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Abbreviations

CNS: Central Nervous System
ARC: Arcuate nucleus
VMH: Ventromedial hypothalamus
PVN: Paraventricular
LHA: Lateral hypothalamus area
DMH: Dorsomedial hypothalamus

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Introduction

The ability to maintain a balance between energy intake and consumption is very important in living things. Researchers have conducted many types of research on this subject [1]. It has been shown that CNS plays a key role in controlling this balance [2]. Research conducted on CNS includes brain neuroanatomical constructions, peripheral and central hormonal and metabolic signals, as well as examining cellular and molecular pathways [3]. Among different brain regions, the hypothalamus plays an important role in controlling the mentioned pathways [4].

Numerous neurotransmitters and neuropeptides affect the central control of feeding via the hypothalamus. This region plays a crucial role in monitoring basic behavior patterns, particularly feeding behavior [5]. Different types of stimulating and inhibitory peptides generated in the CNS affect feeding (Figure 1) [6]. The central regulation of feeding behavior and energy homeostasis in the body is a highly complex process that requires extensive research. Neuroscience researchers have discovered that special hypothalamic nuclei, as well as brain neurotransmitters and neuromodulators, play an important role in the central control of nutritional behaviors [7-15].

Hypothalamus exerts its controlling role through its special nuclei. These nuclei include ARC (first-order neuron), VMH, PVN, LHA, and DMH (second-order neurons) [16-18]. The ARC in the middle eminence is not covered by the BBB, so it has direct access to the signals of energy regulation with blood origin. This nucleus, with its two important neuronal populations (NPY and POMC), plays a very important role in the central control of food intake [19]. By receiving blood signals, the neuronal population in this nucleus sends the necessary message to change the nutritional status and neuronal activity of the second-order neuron [4]. The VMH is called the center of satiety and plays a role in energy homeostasis and body weight. It receives different signals from ARC and PVN through its receptors, modulates these messages, and sends appropriate output to ARC, PVN, and other brain regions involved in the central control of feeding [17, 20]. The PVN is the center of hunger and is the main output center of the hypothalamus. It receives multiple inputs from the ARC and subcortical regions. It then issues the appropriate response to the LHA and other brain regions [16, 21]. The most important task of LHA is to control nutrition according to the changes in the body's energy status. This nucleus receives the necessary messages to control the nutritional status of the body, especially from the ARC, PVN [18], and DMH [22, 23]. Then, by integrating these messages, it issues the appropriate stimulus response to VTA and other brain centers involved in the central control of food intake [24].

The DMH plays an important role in controlling feeding, body weight, and digestive behaviors [22, 23]. It is located in the tubular part of the hypothalamus and the area between the periventricular and lateral regions. In rodents, this nucleus is easily divided into several identifiable sub-regions [25]. The DMH contains two important neural populations, namely NPY and CART neurons, which are the most important ones in the central control of food intake [26]. The DMH receives diverse inputs and integrates them and issues the appropriate response through these two neuronal populations [27]. This nucleus has extensive connections with other hypothalamic nuclei in the central control of food intake. This nucleus receives multiple inputs from all the anterior, middle, and posterior nuclei of the hypothalamus [28]. The DMH also receives signals of blood origin through the cerebellum. The cerebellum transmits various inputs from the vagus nerve and signals of blood origin to the DMH. Therefore, through DMH, the hypothalamus is connected to other neural networks involved in the central control of nutrition, including the cerebellum-vagus nerve [29]. The DMH sends extensive nerve projections to all nuclei of the periventricular zone of the hypothalamus, such as PVN, LHA (except ARC), septum, hippocampus, and amygdala [27].

In the field of neurophysiology, several types of research have been conducted on distinct hypothalamic nuclei, and their roles have been investigated separately. The DMH has received little attention among these nuclei [23]. Therefore, considering the role of DMH in the central control of food input, we review the role of this nucleus in controlling central nutritional behavior.

Abbreviations-Cont'd

BBB: Blood-brain barrier
NPY: Neuropeptide Y
POMC: Pro-opiomelanocortin
VTA: Ventral tegmental area
CART: Cocaine- and amphetamine-regulated transcript
GALRs: Galanin receptors
GPCR: G protein-coupled receptors
PeH: Periventricular hypothalamus
ICV: Intracerebroventricular
GALP: Galanin-like peptide
L-bR: Leptin receptors
DR: Dopaminergic receptors
NPYR: NPY receptor
MCR: Melanocortin receptor
GHSR 1a: Growth hormone secretagogue receptor 1a
MBH: Mediobasal hypothalamic
AgRP: Agouti-related peptide
α-MSH: alpha-melanocyte-stimulating hormone.

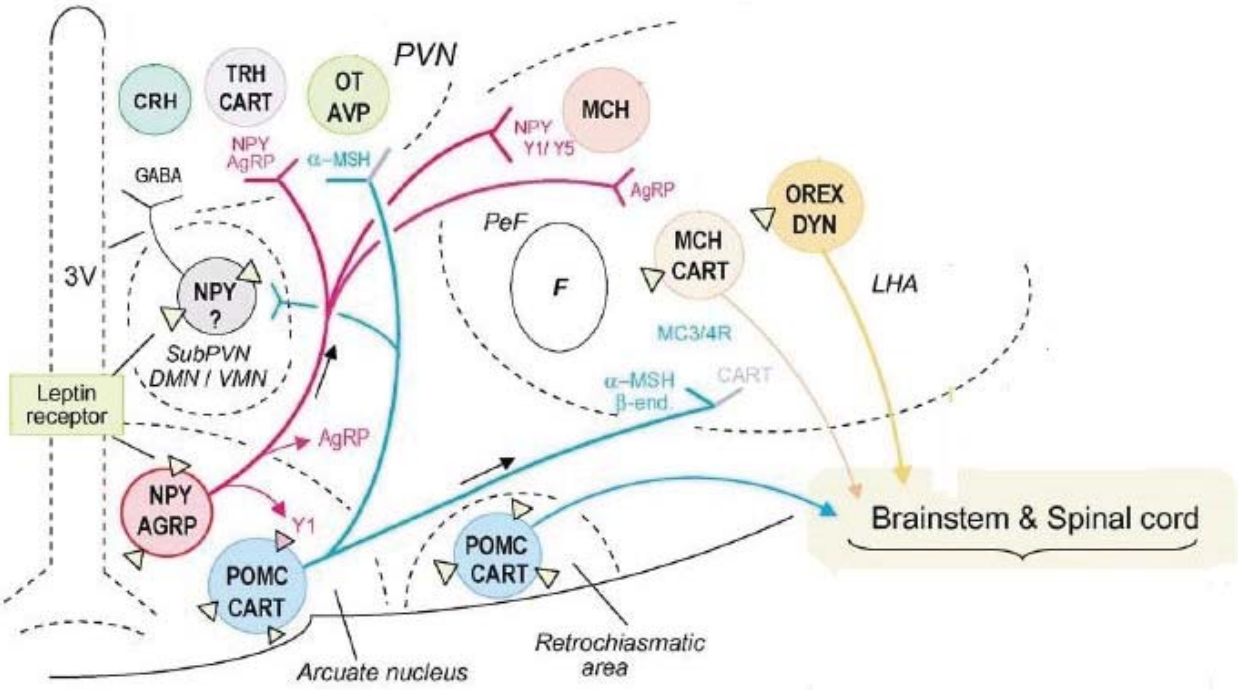

Figure 1.
Nutritional status and energy balance hypothalamic peptidergic circuitry in the rat. Receptors and communities of peptidergic neurons with their projections were defined. The middle group of hypothalamic nuclei, including the arcuate nucleus, retrochiasmatic area, dorsomedial, and ventromedial nuclei have long-form leptin receptors (ObRb, open triangles). Arcuate neuron has the mRNA of neuropeptide Y (NPY) and agouti-related protein (AgRP). These neuron populations project output to the paraventricular nucleus (PVN) and the perifornical/lateral hypothalamus (PeF). These neuron populations project output to autonomic and motor areas of the brainstem and spinal cord, PVN and DMH, and other brain areas (not shown). One neuron group that receives input from these ARC projections in the lateral hypothalamus (LHA) involves MCH, CART, orexin-A (OREX), and dynorphin (DYN). Various populations of these neurochemically special cell groups produce 'ascending' (cortex, amygdala, hippocampus, thalamus) and 'descending' projections to promotor (medullary motor nuclei), locomotor (pedunculopontine locomotor area and spinal cord), and autonomic premotor and motor areas (dorsal motor nucleus of the vagus, A5, RVLM, and ILM) (dorsal motor nucleus of the vagus, A5, RVLM, and ILM) [81].

Study design

Several reliable papers from electronic sources were used in this review article. Creditable articles indexed in the Web of Science, Scopus, PubMed, SID, Google Scholar, and ISI databases using the keywords "feeding central regulation", "hypothalamic dorsomedial nucleus", "brain neurotransmitters", and "brain neuromodulator" were surveyed.

Galanin

Galanin is a neuropeptide found in CNS, especially the hypothalamus, and exerts its effect via GALRs (Table 1). It helps regulate feeding, body weight, reproduction, and growth. The GALRs, as members of the GPCR family, are classified into three types [5, 30]. GALR1 is mostly found in the prefrontal cortex, medial thalamus, and central amygdala. GALR2 is presented in the granule cell layer of the dentate gyrus, cerebellar cortex, and mammillary bodies. GALR3 is found in the hypothalamus [31]. In mice, galanin is distributed in all special nuclei of the hypothalamus, especially VMH. In rats, galanin is most distributed in DMH and PeH [32]. Galanin stimulates eating through various brain regions, especially DMH [33]. Central administration of galanin increased food consumption in rodents. As well as, intracerebroventricular (ICV) administration of galanin stimulates feeding in various hypothalamic nuclei such as DMH in satiated rats [34]. Moreover, the ICV injection of 1 nmol galanin increased feeding in rats with free access to food and water (Table 2). Galanin causes a central increase in food intake via up-regulating c-FOS in GALR1 in DMH [31].

Galanin-like peptide

The GALP, a 60-amino acid neuropeptide, was discovered in the hypothalamus of pigs and is linked to GALRs (Table 1) [30]. GALR1 is mostly distributed in the CNS, while GALR2 and 3 were distributed to a lesser extent in the CNS and peripheral tissues [35]. GALP has a higher affinity for binding to GALR2 than other receptors and functions in the hypothalamus via GALR2 [36]. In mice, the GLAP neurons are

Table 1.
Basic receptors involved in the central control of feeding in DMH.

Receptor	Receptor Category	Receptor Location (s)	Action (s)	Action Mechanism	Ref.
GALR2	GPCR	DMH	Increased food intake	Increased c-Fos expression	[36]
DR2	GPCR	DMH	Decreased food intake	Inhibits the formation of cAMP	[48]
GHSR	GPCR	ARC, and DMH	Increased food intake	Increased c-Fos expression	[60]
Lb-R	Class 1 cytokine receptor	ARC, and DMH	Decreased food intake	Increased c-Fos expression	[79]
NPYR	GPCR	ARC, PVN, VMH, and DMH	Increased food intake	Increase in the GABA release to the POMC neuron	[74]

distributed in the ARC [37]. Leptin receptors (L-bR) are expressed in the GALP neurons. Therefore, GALP has a direct effect on food intake by communicating with leptin [30, 37]. In rats and mice, GALP seems to have different effects. In rats, this leads to a temporary rise in feeding, followed by a reduction in eating and body weight [38]. This transient increase in feeding is linked to the activation of orexin neurons in LHA and NPY neurons in DMH [36, 39]. In mice, it only reduces food intake and body weight [40]. In mice, the repeated intranasal administration of 2 nmol of GALP reduced food intake, water intake, and body weight in 24 hours [30]. Different doses of GALP have diverse effects on feeding. A low dose (1-2 nmol) reduces food intake, whereas a high dose (4 nmol) does not affect feeding. High doses may reduce receptor expression and sensitivity to GALP [41]. In another study, it has been shown that galanin ICV injection increased food intake for the initial 2 hours in rats. GALP upregulates NPY neurons in the DMH. It also raises the level of c-Fos expression in these neurons and augments food intake (Table 2) [36]. As a result, GALP in mice decreased food intake via communication with leptin neurons. In rats, GALP increased food intake via activating orexin neurons in LHA and c-FOS expression in NPY neurons.

Dopamine

Dopamine is a vital neurotransmitter in the CNS, which is produced from tyrosine amino acid [42]. Dopamine neurons are found in the hypothalamus, especially in ARC, DMH, and LHA [43]. Dopaminergic neurons in the hypothalamus communicate with GABAergic, and POMC neurons in ARC and transmit nerve projections to PVN and LHA [44]. Dopamine exerts its effects on feeding control through DRs, which are GPCR. These receptors include DR1-DR5. Dopamine affects feeding via DR1 and DR2 [45]. DR1 was found in suprachiasmatic nuclei, PVN, LHA, VMH, and DMH. DR2 was expressed in LHA, PVN, VMH, and ARC (Table 1) [43]. The impacts of dopamine on the central control of eating depend on the type of nucleus, receptor, and overall energy condition of the body [45]. As well, dopamine seems to have diverse effects on feeding in LHA and VMH [3]. In LHA, dopamine levels are high in response to feeding and during feeding. Dopamine levels in VMH increased during fasting and after feeding. DR2 was found in NPY neurons, ARC, and PVN. When dopamine binds to DR2 in NPY neurons in PVN and ARC, inhibits NPY neurons in ARC and PVN. Consequently, NPY level declines and NPY does not bind NPYRs in DMH. The NPY cannot stimulate DMH. Finally, decreased food intake in rat[43, 46]. As well, dopamine binds to DR1 in POMC neurons, stimulating it. POMC via MCR4 inhibited DMH orexigenic output. As a result, food intake is suppressed in mice via indirect effects (Table 2) [43, 46, 47]. Furthermore, DMH sends these neural projections to LHA and suppresses feeding [48]. Dopamine inhibits feeding by inhibiting NPY neurons and stimulating POMC neurons via DMH.

Ghrelin

Ghrelin is a peptide with 28 amino acids derived from the stomach and released in reaction to a change in nutritional status [49]. It is synthesized and secreted in low volumes in the brain [50]. This hormone is orexigenic and increases in response to a massive decrease in energy [51]. Ghrelin is considered a blood glucose regulator, appetite controller, and anti-depressant [52, 53]. Ghrelin neurons transmit nerve projection to hypothalamic nuclei, including ARC, PVN, VMH, and DMH [54]. Ghrelin exerts its multiple and essential functions through GHSR1a, which is a part of GPCR (Table 1) [55]. This receptor is widely expressed in the hypothalamus, especially in MBH, ARC, PVN, VMH, and DMH [56, 57]. Moreover, ghrelin projection is transmitted to extra hypothalamic regions, namely the amygdala and septum [50]. Among different hy-

Table 2.
The role of main neurotransmitters in the central control of feeding in the DMH.

Substance Type	Animal Type	Administration Type	Co-transmitter	First Order Neuron	Second Order Neuron	Function (s)	Other Central/Peripheral Action (s)	Action Mechanism (s)	Dosage of the drug (s)	Ref.
GALP	Mice	Intranasal	NPY	ARC	DMH, LHA	Decreased feeding	Decreased water intake, and body weight	Interleukin-1 receptor	2 nmol	[30]
GALP	Rat	ICV	NPY	VMH	PVN	Increased feeding	-	Increased in c-FOS expression	0.3 nmol/5µl	[36]
Sulpiride	Rat	Intra hypothalamic	Serotonin	ARC	LAH	Increased feeding	Increased water intake	cAMP level inhibited	8 µg/ 0.5 µl	[48]
Ghrelin	Rat	Intraperitoneal	NPY	ARC	DMH	Increased feeding	Increased c-Fos-like-immunoreactivity in PVN	Increased of c-Fos expression	0.3 nmol	[65]
Leptin	Rat	Intravenous	NPY	DMH	PVN	Decreased feeding	Increased energy consumption	Increased of c-Fos expression	1 mg/Kg	[76]
Angiopoietin-like protein 8	Mice	ICV	NPY	ARC	DMH	Decreased feeding	Decreased body weight	Decreased of c-Fos expression	2 µg/ml	[73]

pothalamic nuclei, DMH is sensitive to the regulation of ghrelin secretion in response to feeding behaviors stimuli [58]. Peripheral and central administration of ghrelin increases feed consumption and body weight [59]. GHSR1a expresses NPY/AgRP neurons in ARC [60] and DMH [61]. It increases the activity of these neurons and upregulates NPY. The NPY neurons transmit orexigenic output to DMH. Therefore, DMH sends orexigenic output to the PVN [62]. The ICV and peripheral injection of ghrelin-induced feeding in rats that had free access to food [63, 64], and also upregulated c-Fos in NPY neurons in ARC. Furthermore, induced c-Fos expression in DMH and PVN. Following the stimulation of NPY neurons in ARC, this nucleus sends excitatory projections to DMH, stimulating it. With DMH activation orexigenic output is sent to PVN [65]. Therefore, via this pathway, DMH exerts its orexigenic effect on feeding in rats (Table 2) [66]. Ghrelin also affects nutrition by reducing signaling from dopamine and serotonin [67]. In the brain, ghrelin neurons interact with dopaminergic neurons, and dopamine modulates an increased effect of ghrelin in nutritional behavior [68]. In addition, ghrelin reduces serotonin release to synaptic cleft [69]. Ghrelin raises NPY activity in ARC and DMH via binding to GHSR1a. In addition to directly increasing the level of NPY in the DMH, the level of this neuropeptide is increased in the ARC and sends excitatory input to the DMH. Next, DMH sends orexigenic messages to PVN.

Neuropeptide Y

The NPY is a vital and strong orexigenic compound in CNS and is synthesized in ARC and DMH. DMH contains NPYR [61]. NPY is a 36-amino acid peptide, which is a member of the pancreatic polypeptide family. NPY is distributed in CNS, especially the hypothalamus [7], and plays an orexigenic role with NPYR. The NPYR belongs to the GPCR family. NPY has multiple receptors, including NPYR1, NPYR2, NPYR4, and NPYR5. The DMH contains NPY1R and NPY5R (Table 1) [70]. NPY exerts orexigenic effects via these receptors. The NPY neurons in DMH are considered gabaergic and non-sensitive neurons to leptin [71]. NPY levels in DMH increase in response to food deprivation and stimulate this nucleus. Now, this nucleus transmits orexigenic output to other nuclei [61]. In mice, NPY neurons in DMH are involved in central feeding regulation [72, 73]. It has been shown that POMC neurons in ARC may have an inhibitory role on NPY neurons in DMH. The POMC-GABAergic neurons in ARC send inhibitory output to DMH. In DMH, MCR4 is expressed. The POMC neuron via MCR4 exerts an inhibitory effect on NPY neurons [1]. During starvation in rats, NPY levels are increased in

the ARC [74] and DMH [75]. Also, the GABAergic inhibitory branch inhibits the POMC neurons in the ARC. As a result, their inhibitory effect is removed from NPY neurons in DMH. Then, NPY exerts its additive effect on food intake by sending excitatory outputs to other brain regions [72]. The main brain neurotransmitter for controlling feeding in DMH is NPY. Central injection of NPY augments feeding and body weight (Table 2) [75]. In response to starvation, the NPY level rises in both ARC and DMH. On the other hand, the inhibitory GABAergic branch of ARC inhibits POMC neurons. As a result, NPY stimulates DMH, and DMH exports the necessary orexigenic message.

Leptin

Leptin is an adipose tissue-derived hormone that inhibits ingestion and facilitates weight maintenance. Lack of leptin or reduced sensitivity to leptin causes obesity. Therefore, leptin is a vital hormone in controlling food intake. The Lb-R, which is found throughout CNS, is a member of the class 1 cytokine receptor family (Table 1) [18]. Leptin is highly expressed in the hypothalamus particularly ARC, VMH, and DMH [71]. Leptin has a receptor on GABAergic neurons in DMH. Therefore, leptin inhibits GABAergic neurons via Lb-R and restrains projection transmitted to PVN. Leptin reduces feeding in rats via this pathway [76, 77]. Furthermore, leptin suppresses feeding and promotes energy consumption by activating other neuron populations [78]. Leptin exerts its effect on nutritional behavior via increased c-Fos expression in ARC, DMH, and PVN [79]. The NPY neurons in ARC transmitted nerve projection to DMH. The main site of leptin action is the hypothalamus. It has been shown that the ICV injection of leptin reduces digestion by affecting the hypothalamus (Table 2). Furthermore, circulating leptin enters CNS through MBH, and then exerts its effect on food intake by transmitting nerve projections from ARC to DMH, and then to PVN. Finally, feeding is reduced. Leptin inhibits the expression of NPY mRNA and increases the level of α-MSH in the hypothalamus. It also reduces the level of this neurotransmitter in ARC, DMH, and PVN. Neurons expressing Lb-R in DMH play a key and essential role in feeding control [80]. Leptin inhibits GABAergic neurons in DMH through Lb-R. It also down-regulates NPY and up-regulates α-MSH in ARC and DMH. As a result, through these pathways, the increasing effects of DMH on food intake are inhibited, and it reduces food intake.

Conclusion

DMH plays an important role in the central control of feeding, but it has received very little attention. NPY in this nucleus plays a critical role in the central stimulation of food intake. Galanin and GALP stimulate central feeding behavior via their receptors in this nucleus. The effect of dopamine on the central control of food intake appears to be highly dependent on the nutritional level, receptor type, and nucleus involved. Dopamine inhibits NPY neurons and stimulates POMC neurons via dopaminergic receptors, resulting in a central decrease in digestion. Ghrelin also increases central food intake by raising NPY levels. Leptin reduces central food intake by decreasing NPY levels (graphical abstract).

Future directions

Considering the effect of DMH on the central control of nutritional behavior, the authors recommend that future research be conducted on the effect of other neurotransmitters on the central control of feed intake via this nucleus.

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Statement of Human and Animal Rights

This study has not been performed on any humans or animals.

Authors' Contributions

Farshid Hamidi, and Shiba yousefvand. Farshid Hamidi presented the main idea and plan. Farshid Hamidi and Shiba yousefvand conducted scientific searches. Farshid Hamidi and Shiba yousefvand performed collecting and analyzing information and articles. Shiba Yousefvand wrote the paper. Farshid Hamidi reviewed the paper. Farshid Hamidi and Shiba yousefvand drew an abstract graphical design.

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Competing Interests

The authors do not have conflict of interest.

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Investigating the Prevalence of *Mycobacterium avium Subspecies Paratuberculosis* (MAP) in Industrial Dairy herds using Ziehl-Neelsen Staining, Culture, and PCR in Mashhad, Iran

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ABSTRACT

Mycobacterium avium subspecies paratuberculosis (MAP) is the causative agent of Johne's disease in wild and domestic ruminants. Clinically, infected cattle show emaciation symptoms, diarrhea, and death. Asymptomatic subclinical cases can intermittently shed MAP through feces and milk and infect other cattle animals, thus increasing the risk of infection. The purpose of the present study was to detect the prevalence of this disease in dairy cattle. For this purpose, 348 samples were randomly collected from 15 cattle farms. All fecal samples were subjected to ZN staining and PCR of nucleotide sequences related to specific MAP gene fragments (IS900, F57) and were cultured in Harold's egg yolk culture medium after being disinfected with solution (0.0% HPC75). PCR testing of 116 fecal samples (33.3%, CI:95% 28.3-38.2), ZN staining of 23 samples (6.6%, CI: 95% 4-9.2), and fecal sample culture of only 15 samples (4.3%, CI:95% 2.3-6.3,) were infected with MAP. Comparison of test results showed poor agreement (kappa statistic: 0.19) between ZN staining and PCR results and poor agreement (kappa statistic: 0.13) between PCR test and fecal culture. This study highlights the advantages of PCR for detecting MAP in subclinically infected cattle compared to ZN staining and fecal culture. Therefore, it can be used for early detection and control of MAP in cattle and at-risk populations.

Keywords

Johne's disease, Mycobacterium avium subspecies paratuberculosis, Ziehl-Neelsen, Iran

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Abbreviations

MAP: Mycobacterium avium subsp. paratuberculosis
ZN: Ziehl-Neelsen staining
JD: Johne's disease
CI: Confidence interval

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Introduction

Johne's disease (JD) is granulomatous enteritis that affects a wide range of domestic and wild animals worldwide [1,2]. In 1895, this disease was first detected in the German state of Oldenburg [3]. Infected animals include cattle, sheep, goats, deer, camels, and wild ruminants [4]. Ingestion of feces, milk, and colostrum contaminated with MAP is the predominant mechanism of MAP transmission in dairy cattle [2]. The pathogenesis of MAP infection has recently been further studied, showing that after MAP enters the small intestine, it invades subepithelial macrophages, interferes with the formation of phagolysosomes, and remains in the phagosome, triggering humoral immune responses. Finally, the disease course changes from the preclinical stages to the period of increased bacterial shedding and the development of obvious clinical symptoms [3]. The infection's livestock and herd performance consequences have been widely studied. Most studies have shown that cows with a positive MAP contamination test have a 2-17% reduction in milk production compared to cattle with a negative test result. Reduced fertility and adverse effects on the reproduction of infected animals can also be another important complication of infected animals [5]. MAP also weakens the livestock's immune system, predisposing animals to develop clinical mastitis or other infectious diseases [6]. One of the problems in controlling MAP infection is that some infected cows may not show clinical symptoms if they are producing enough milk because they frequently shed MAP in the feces before showing symptoms, indirectly contributing to the spread of infection [7]. Due to the long incubation period of JD, clinical symptoms such as resistant watery diarrhea, weight loss, and reduced milk production are usually observed in cows over 2 years of age [8]. Many theories have been proposed regarding the potential of MAP to be involved in human Crohn's disease and infections in non-ruminant animals such as dogs and rabbits [9]. Humans become infected by consuming contaminated milk or dairy products because MAP bacilli survive in pasteurized milk [10]. Usually, no effective measures are taken to control and eradicate this disease, which imposes significant economic losses to the animal husbandry industry and the production of dairy and meat products worldwide [11, 12]. Since there is no cure for JD, herd health control strategies are based on detecting infected hosts and their subsequent elimination to prevent transmission of infection to healthy cattle, which imposes a financial burden on herd management [6, 13]. Due to the slow growth of MAP and the lack of sensitive tests, it is very difficult to detect subclinically infected cattle and diagnose the disease's early stages [10, 14]. It is often impossible to distinguish between infected animals with only one diagnostic method due to their false positive and negative responses [15]. Fecal shedding of MAP organisms can be intermittent. Contamination and low fecal content of microorganisms can reduce culture results by 50% [16]. Therefore, although the culture of samples is the "gold standard," it cannot be used as a single test to accurately detect all infected animals in some herds [17]. Molecular methods based on bacterial DNA and detection of specific sequences in the genome allow hard-to-grow bacteria such as MAP to be detected in samples quickly [15]. MAP-infected animals can be detected directly through fecal PCR and detection of the IS900 or F57 fragment [18]. Comparing the results obtained in similar studies show that the relationship between different diagnostic tests and their combination can improve MAP diagnosis at the herd level [15]. Therefore, it seems that the presence of specific and sensitive diagnostic tools and a better understanding of the prevalence of JD in different parts of the world is necessary to develop control and eradication programs disease [14]. The present study aimed to estimate the amount of MAP contamination in cattle farms of the region using three laboratory detection methods. Comparing the results of these three methods can help to choose the best MAP diagnostic method to obtain accurate results in epidemiological studies and disease control in dairy cattle.

Results

This study tested 348 cow fecal samples for MAP contamination using different techniques. According to PCR, this disease has a prevalence rate of 33.3% (95% CI: 28.2-38.2), a culture prevalence rate of 4.3% (95% CI: 2.3-6.3), and a ZN staining prevalence rate of 6.6% (95% CI: 4-9.2). Three different prevalence rates of MAP infection were detected using three other diagnostic methods. The degree of agreement between the tests results was measured using the Kappa statistic. After analyzing the results of these three tests, it can be concluded that there is a poor agreement (kappa statistic: 0.19) between the results of ZN staining and PCR, as well as a poor agreement (kappa statistic: 0.13) between the results of the PCR test and fecal culture. First, the appearance of the culture medium was investigated by examining colony growth, followed by direct microscopic observation to obtain these results. Figure 1 shows the positive electrophoresis results of PCR products of IS900 and F57 MAP genes in 413-bp and 704-bp samples, respectively. Cows were diagnosed positive in 100% of cases of herds 1, 2, 4, 95% of cases of herd 11, 68% of cases of herd 7, and 10% of the cases of the herd 9. All tests were negative in the other 9 herds.

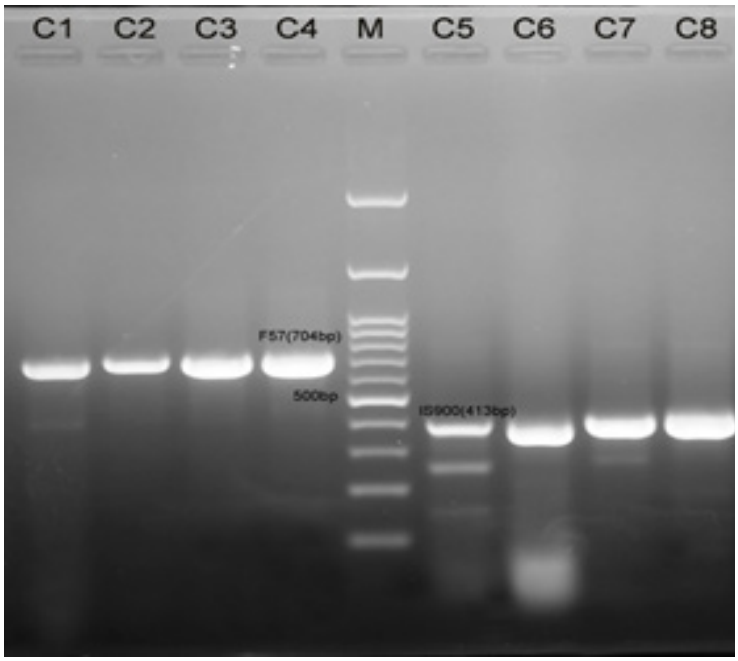

Fig 1.
Confirmation of positive culture results using PCR F57 and IS900 (C1: culture positive F57 PCR; C2 and C3: feces samples positive F57 PCR; C4: positive control (F57); M: 100-bp DNA ladder as the molecular size marker; C5: positive control (IS900); C6 and C7: feces samples positive IS900 PCR; C8: culture positive IS900 PCR).

Table 1 shows the total number of positive animals for each herd in the three tests (based on the number of samples per herd).

Table 1.
The number and proportion of positive PCR results on the feces and feces culture in 15 herds are based on the number of samples taken in each herd (Herd-Level prevalence and total).

Herd (number of samples)	Ziehl-Neelsen stain	feces PCR		feces culture	feces culture PCR
		IS900	F5		
1(41)	15(36.6%)	41(100%)	16(39.02%)	11(26.8%)	11(26.8%)
2(21)	4(19.05)	21(100%)	(0%)	2(9.5%)	2(9.5%)
3(23)	(0%)	(0%)	(0%)	(0%)	(0%)
4(15)	1(6.6%)	15(100%)	2(13.3%)	(0%)	(0%)
5(30)	(0%)	(0%)	(0%)	(0%)	(0%)
6(22)	(0%)	(0%)	(0%)	(0%)	(0%)
7(67)	(0%)	17(25.4%)	(0%)	(0%)	(0%)
8(20)	3(15%)	(0%)	(0%)	2(10%)	2(10%)
9(28)	(0%)	(0%)	(0%)	(0%)	(0%)
10(17)	(0%)	(0%)	3(17.6%)	(0%)	(0%)
11(20)	(0%)	19(95%)	10(50%)	(0%)	(0%)
12(17)	(0%)	(0%)	(0%)	(0%)	(0%)
13(7)	(0%)	(0%)	(0%)	(0%)	(0%)
14(10)	(0%)	(0%)	(0%)	(0%)	(0%)
15(10)	(0%)	(0%)	(0%)	(0%)	(0%)
Total 348	**23(6.6%)**	**113**	**31**	**15(4.3%)**	**15(4.3%)**
		116(33.3%)			

Discussion

This study was carried out using random sampling of cows and the method of direct microscopic observation, culture and molecular detection to understand the contamination status of dairy cattle better. A total of 348 samples were tested, and 136 samples (39.08%) of those tested were positive for at least one of the three tests. Based on the results, only 23 (6.6%) of 116 (33.3%) cows with positive fecal PCR results for one or both genes (IS900, F57) had positive ZN staining. Mahmoudi et al. (2018) sampled 150 fecal samples from apparently healthy dairy cows from industrial cattle farms in Hamadan City and investigated them for the presence of MAP using ZN staining and molecular methods. The positive results for ZN staining and Nested PCR method were 5.33% and 30.23%, respectively [19]. In another study on 200 samples of rectal mucus and serum of cows slaughtered in the Ahvaz slaughterhouse, Zarei et al. (2016) investigated the prevalence of MAP using three staining methods: ZN, molecular, and ELISA. The results showed that the prevalence of MAP in PCR, ZN staining, and ELISA was 13.5%, 4%, and 3%, respectively [20]. The results of the ZN staining test in the two above studies are consistent with the present research results. MAP can be directly observed in samples using (ZN) staining, which is based on the resistance of mycobacteria to acid decolorization by acid alcohol after fuchsin staining. The results are qualitative. However, this method is fast, simple, and inexpensive, with low sensitivity and specificity in milk, colostrum, and fecal samples [21]. The possibility of false negative results increases with the observation of clinical symptoms such as diarrhea in cattle because the MAP number of the samples is low in the lower fecal concentration. Besides, subclinically infected animals also shed large amounts of bacteria in their feces. Due to the possibility of obtaining false positive results from other environmental mycobacteria, this method has low sensitivity and specificity. Herd contamination can be estimated and detected using direct microscopic observation [22]. Fecal culture, the most accurate test for confirming paratuberculosis, is another test in this unique study. The results showed that only 15 (4.3%) out of 348 cultured samples were positive. A study was conducted by Seyedeyin et al. (2010) on the feces of Holstein-Friesian cows with and without clinical symptoms to determine the prevalence of JD in Razavi Khorasan. In this study, 16 and 103 fecal samples were taken from sick and healthy animals, and two different methods analyzed the results. Culture results were positive in 11.7% of asymptomatic samples and 81.3% of clinical samples [21]. In a study by Sultani (2018) in Kerman, the contamination rate was 15.1% in 212 animal fecal samples without clinical symptoms [22]. A low positive culture test rate may be due to several reasons, including:

The need for highly selective culture media, chemical decontamination of the sample (which kills some microbes and reduces cell viability), and long culture durations are just some of the limitations of using culture as a routine diagnostic method [24]. Also, the culture depends on the number of bacteria in the sample. When there are fewer bacteria in the inoculum, culture is more likely to become negative [13]. When investigating the culture results, the optimal contamination rate should be considered. As mentioned, many cells are destroyed during disinfection, or fecal shedding may occur intermittently or rarely. Finally, the only method to detect live MAP is the only culture, although PCR and bacterial DNA can detect it more effectively [15]. Since the sensitivity of MAP bacterial culture is low and the level of MAP shedding in colostrum, milk, and feces in the subclinical phase is below the minimum detection limit, negative results should be interpreted cautiously. Bacterial culture is mainly used to detect live MAP and is considered a diagnostic reference [15, 19, 25]. Based on host infection response, the fecal PCR test had the highest positive results in this study (N=116 cases, 33.3). Of these, 8.91% and 32.47% occurred in F57 and IS900 genes, respectively. IS900 gene was positive in 85 samples, both genes were positive in 28 samples, and the F57 gene was positive in 3 samples. Due to the resistance of the mycobacterial cell wall to lysis and high concentrations of inhibitors in fecal content, PCR-based MAP detection is limited to small numbers of fecal MAP bacilli and has poor DNA recovery [26]. The 14-20 copy of the IS900 sequence in the MAP genome is a suitable target for MAP detection. On the other hand, due to the detection of this sequence in other mycobacteria, its uniqueness for MAP detection has been questioned. Since F57 has fewer copies than alternative genes such as IS900, it can be specifically used as a target gene in PCR to detect MAP in feces, which may be sensitive. The IS900 gene is still a good candidate for PCR, but caution should be exercised when interpreting the results of a diagnostic test based on IS900 PCR alone. Based on IS900 analysis of fecal and milk samples from several regions, the prevalence of this disease is reported as follows:

In a study by Nasiri et al. (2012) in the suburbs of Mashhad, the IS900 gene of MAP using the PCR method was detected in 243 fecal samples (44%) and 56 suspicious raw milk samples (18%) [27]. In another study based on the IS900 Nested PCR method, Seyedeyn et al. (2010) reported the prevalence of MAP in the fecal samples of clinical and subclinical animals as

87.5% and 9.7% in Razavi Khorasan, respectively [21]. In addition, Haghkhah et al. (2008) reported that the prevalence of MAP in the bulk milk of dairy cows in Fars province was 0.11% [28]. The accuracy and sensitivity of this test are excellent due to the similarity and results between the above studies and the present study. However, there are advantages and disadvantages to molecular diagnosis of MAP, including the following:

In situations where bacterial isolation and mass culture are impossible due to long incubation periods and other culture requirements, useful genetic markers F57 and IS900 may be used in vitro [29]. In particular, the F57 sequence seems to be a highly discriminating biological factor that has so far only been detected in MAP. Therefore, detecting this specific MAP gene sequence provides a new technique for finding this organism [30]. As a result, the PCR-based detection of MAP F57 in the sample indicates it is infectious.

Eight of the fifteen herds sampled had no detectable MAP by any of the methods used in the present study. Suppose the infected animals are in the non-infectious phase and the shedding phase has not yet started. In that case, the negative test results in some cattle do not necessarily mean that the animals in the herd are not infected. Although clinical symptoms in any herd are essential as a screening symptom for JD, accurate and sensitive tests are needed both to detect asymptomatic subclinical animals and monitor the health and welfare of the herd. One of the challenges facing this process is the limited sensitivity of the available diagnostic tests to detect the subclinical stages of MAP infection.

Based on the results of the present study, direct fecal PCR testing yielded better results than other tests for rapid MAP diagnosis. Considering the lack of appropriate conclusions in ZN staining tests and fecal culture results in epidemiological studies, the simultaneous use of PCR tests is necessary for achieving accurate and reliable results. Knowledge of the disease prevalence in the target population is the first step for its diagnosis and prevention. The present study was carried out as an epidemiological component of the JD in the suburbs of Mashhad. Eliminating cattle diseases, especially common zoonotic diseases, is one of the ideals and necessities of this industry. The studied area is one of the country's strategic milk and meat production economies.

Further studies on the epidemiology of MAP in herds using advanced laboratory diagnostic techniques are needed to address this major issue. To achieve this important goal, combining the results of this study with other results of previous studies can be a great help. Using current common diagnostic methods and minimal human error, attempts were made to report the exact prevalence rate of the disease in the studied region.

Materials & Methods

Geographic Location

This study was conducted on several industrial cattle farms in the suburbs of Mashhad. The city of Mashhad is located in northeast Iran with a longitude of 59 °and 35' and a latitude of 36 ° and 20'.

Herd selection

Based on geographical distribution and herd management cooperation, 15 herds were selected for this study. The number of samples in each herd was calculated proportionally to the total sample volume and the herd size.
Size formula: n = P * N, where, P = proportion, N = total sample size

Sampling

A total of 348 cows were randomly selected from the study population, and fecal samples were taken from their rectums. Also, the following formula was used to obtain the required sample size. n=Z2×P-exp (1-Pexp)/d2
Z = standard deviation for the given confidence interval for 95% CI, if α = 0.05, Z α=1.96, p = expected proportion = 15% [21, 27, 31] and d = Margin of error (accuracy) = 5
All fecal samples were collected in closed containers and immediately sent to the laboratory. All fecal samples taken by the ZN method were first stained and examined under a direct microscope.

Bacteriological culture

After decontamination, 23 samples that were positive in terms of ZN staining were cultured individually in the culture medium. Other fecal samples (n=325) free of acid bacillus masses were cultured using the strategic pooling method. In the laboratory, fecal samples from 8 to 12 cows per pool were mixed with 2 g of feces from each cow [23, 32]. All fecal samples were disinfected according to the instructions. Three grams of pooled fecal sample was decontaminated with 0.75% (W/V) hexadecyl pyridinium chloride solution (0.75% HPC) for 24 hours, according to standard methods. This suspension was mixed manually by shaking and swirling and stood upright for 5 minutes at room temperature to allow the settling of large particles. Approximately 20 mL of the upper portion of the supernatant was transferred to another sterile 50 mL tube, where the entire suspension was stirred at 200 units/min for 30 min. The tubes were placed vertically in the dark for 24 hours at room temperature. Collected sterile fecal samples were centrifuged at 900 × g for 30 min, the supernatant was discarded and inoculated onto Herrold's egg yolk agar(HEYM) medium containing mycobactin J with 300 µl of the sterile pellet. The slants were incubated at 37°C for up to 20 weeks and investigated at 1-2 week intervals.

Polymerase Chain Reaction (PCR)

Genomic tests were performed using PCR detection methods, and detection of specific MAP IS900 and F57 markers. Only single fecal samples were used for PCR. DNA extraction was performed according to the method proposed by Soolingen et al. [33], and its purity was measured between 1.8-2 using a NanoDrop spectrophotometer. Primers, IS900f (5'-GAAGGGTGTTCGGGGCCGTCGCTTAGG-3'), IS900r (5'GGCGTTGAGGTCGATCGCCCACGTGAC-3') and F57f (5'-ACC GAA TGT TGTTGT CAC CG-3'), F57r (5'-GGA CAC CGA AGC ACA CTC TC-3') derived from IS900 and F57 sequences inserted in the DNA of MAP leads to the production

Table 2.
Sequence and product length of MAP primers used in the study

PCR primers	Nucleotide sequence	Product length(bp)	References
IS900f IS900r	5'-GAAGGGTGTTCGGGGCCGTCGCTTAGG-3' 5'-GGCGTTGAGGTCGATCGCCCACGTGAC-3'	413	[27, 34]
F57f F57r	5'-ACC GAA TGT TGTTGT CAC CG-3' 5'-GGA CAC CGA AGC ACA CTC TC-3'	704	[29, 35]

Table 3.
Reaction conditions used in the study

Target	Thermal reaction condition
IS900	95°C for 5 min; 40 cycles of 94°C for 30 sec, 60°C for 30 sec, 72°C for 1 min; and 72°C for 5 min
F57	95°C for 5 min; 40 cycles of 95°C for 30 sec, 61°C for 30 sec, 72°C for 45 sec; and 72°C for 10 min

of 413bp and 704bp, respectively. The sequence of IS900 and F57 primers, as well as the size of their predicted PCR products, are summarized in Table 2. PCR was performed in a final volume of 20 µl for IS900. It contained a 5 µl sample of DNA mixed with 15 µl of master mix (AMPLIQON or SINACLON). Tubes were placed in a thermocycler (BIO-RAD; MJ Mini Gradient Thermal Cycler, USA), and the amplification was as follows:
One cycle of denaturation at 95°C for 5 min followed by 40 cycles of denaturation at 94°C for 30 s, annealing at 60°C for 30 s, extension at 72°C for 1 min, and final extension at 72 °C for 5 min. Secondary PCR was performed in a final volume of 12 µL for F57. It contained a 3 µl DNA sample and a 9 µl master mix. Tubes were then placed in a thermocycler, and amplification was as follows:
One cycle of denaturation at 95°C for 5 min followed by 40 cycles of denaturation at 95°C for 30 s, annealing at 61°C for 30 s, extension at 72°C for 45 s and final extension at 72 °C for 10 min. The PCR conditions are summarized in Table 3.

Statistical analysis

Data were coded and entered into statistical software for each sampling from each cattle. All statistical analyzes were performed using SPSS ver. 22. Agreement between test combination (ZN staining-PCR tests and PCR tests - culture) were compared by kappa index test [36] (≤0.2, poor; 0.21–0.40, fair; 0.41–0.60,moderate; 0.61–0.80, good agreement; and >0.80, very good agreement).

Authors' Contributions

M.H. and G.R.M. conceived and planned the experiments. T.G.M. carried out the experiments. T.G.M. and M.H. contributed to sample preparation. T.G.M., M.H. and G.R.M. contributed to the interpretation of the results. M.H. took the lead in writing the manuscript. All authors provided critical feedback for analysis and manuscript.

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Competing Interests

The authors declare that they have no conflict of interest according to the work presented in this report.

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Genetic Diversity, Antimicrobial Resistance, and Biofilm-Forming Potential of Equine Fecal Escherichia coli in Northern Iran

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ABSTRACT

This study was carried out to examine the biofilm-forming ability, antimicrobial resistance, frequency of biofilm, and resistance genes, as well as the phylogenic grouping of *Escherichia coli* isolates originating from equine samples. In total, 157 *E. coli* strains were isolated from fresh feces samples of healthy horses in northern Iran. The samples were examined in terms of biofilm formation and antimicrobial susceptibility using a microtiter plate and disc-diffusion test, respectively. PCR amplification was adopted to find the genes that confer biofilm formation and resistance to β-lactam, chloramphenicol, tetracyclines, aminoglycosides, quinolones, sulfamethoxazole, and trimethoprim, and for phylogenetic analysis. More than 50% of isolates showed MDR phenotype. The most significant level of resistance was detected for streptomycin (59.87%), followed by trimethoprim-sulfamethoxazole (29.93%) and oxytetracycline (28.66%). Imipenem and norfloxacin were the most potent antibiotics. Phylogenetic groups B1 (46.50%) and A (21.66%) were the most common groups in isolates, followed by C (6.37%), clade I (5.10%), E (4.46%), D (3.82%), and B2 (2.55%). All isolates in phylogroups B2 and D carried all biofilm-related genes. In addition, antimicrobial resistance genes were common in phylogroups B2, D, A, B1, and E. These findings demonstrate that in northern Iran, healthy horses harbor potential extraintestinal pathogenic and MDR *E. coli* isolates. These animals can be reservoirs for antibiotics-resistant isolates. The obtained data support the current interest regarding antimicrobial resistance, MDR shedding, and managing the use of antimicrobials in veterinary science.

Keywords

Equine, E. coli, Genetic diversity, Antimicrobial resistance, Biofilm

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Abbreviations

PCR: Polymerase Chain Reaction,
MDR: Multi-Drug Resistant
CLSI: Clinical and Laboratory Standard Institute
MBL: Metalo Beta-Lactamase
ESBL: Extended Spectrum Beta-Lactamase
ATCC: American Type Culture Collection, IMP:

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Introduction

The occurrence and expansion of antimicrobial resistance have turned into a major threat to the healthcare of human beings, animals, and the environment worldwide [1, 2]. Having direct or indirect contact with affected animals and contaminated environments can speed up the easy transfer of microorganisms between humans and animals [3]. Therefore, monitoring antimicrobial resistance in animal-originated bacteria, and finding enough data about the paths of the spread of antimicrobial resistance and associated genes and their transfer between different elements of the ecosystem is extremely important [4]. Nevertheless, despite the significant amount of information examining antimicrobial resistance in food-producing animals, a small number of studies have evaluated antimicrobial resistance in bacteria originating from equine [5]. Due to the recurrent use of identical antibiotics for curing human beings and horses, equine-origin antibiotic-resistant microorganisms not only affect their health and limit treatment options, but also endanger the health of people in contact with these animals [4, 6, 7]. *E. coli* is a predominant commensal microorganism that is found in the gastrointestinal microflora of human beings and animals. In comparison with other common bacteria, *E. coli* can easily gain antimicrobial resistance via genetic mutation or horizontal gene transfer through a variety of mobile genetic elements, such as self-transmissible plasmids, transposons, and integrons [8]. These mobile genetic elements may co-carry multi-antimicrobial resistance genes, including ESBL, aminoglycosides, sulfa-derivatives, trimethoprim, and quinolone resistance [9]. During the co-colonization of antibiotic-resistant bacteria along with non-resistant bacteria, resistance genes can quickly spread and exchange between bacterial isolates and may facilitate the appearance of MDR bacteria [8, 10].

One may find a considerable genetic substructure in E. coli species, which has been adopted as an easy and cheap method to assign an *E. coli* isolate to a phylogroup [11]. According to the revised Clermont PCR method for phylotyping *Escherichia coli,* the strains of *E. coli* species could be categorized as eight phylogenetic groups (A, B1, B2, C, D, F, and cryptic clade I) based on the presence or absence of chuA (a gene required for heme transport), yjaA (a gene having unknown performance), arpA, and trpA genes as well as the DNA fragment TspE4. C2 is identified as a putative lipase/esterase gene [12]. It was realized that phylogroup strains are different in terms of phenotypic and genotypic features, ecological niche, traits of life history, and capability to create a disease. For example, virulent extra-intestinal strains are the major members of group B2 and, to a lesser degree, group D, while the majority of commensal strains belong to group A or B1 and strains belonging to phylogroup F are more likely to be implicated as the extra-intestinal pathogens of companion animals, horses, cattle, and humans [11, 12].

There is limited data about antimicrobial resistance and phylogroup distribution in the equine population in northern Iran. *E. coli* is occasionally adopted as a sentinel strain for examining antimicrobial resistance in fecal bacteria [13]. Therefore, the present study was conducted to determine the antimicrobial resistance patterns, biofilm-forming ability, and distribution of resistance and biofilm-associated genes in different phylogroups of fecal *E. coli* isolates in healthy horses in Guilan province, north of Iran.

Results

Bacterial Isolates and Phylogenetic Typing

In this study, a total of 157 *E. coli* strains were isolated. Analysis of PCR results for determining phylogenetic groups among these isolates showed that phylogenetic groups B1 (46.50%) and A (21.66%) were the most common followed by C (6.37%), clade I (5.10%), E (4.46%), D (3.82%), and B2 (2.55%). In 9.55% of isolates, the phylogenetic group was unknown.

Antimicrobial Susceptibility Testing

We observed that 26 isolates were sensitive to all 16 antibiotics used in the assay and 131 isolates (83.4%) were resistant to at least one antibiotic. More than 50% of isolates (83 isolates) showed MDR phenotype (resistant to three classes of antibiotics), three horses (1.9%) were colonized by ESBL-positive isolates, and one isolate (0.63%) indicated an imipenem-resistant phenotype.

The outcomes of testing the antibacterial susceptibility of *E. coli* isolates to common antibiotics have been shown in Table 1 and Figure 1. Overall, 85 diverse patterns of antibiotic resistance have been found in 157 isolates (Supplementary data). The most considerable level of resistance was observed for streptomycin (59.87%) followed by trimethoprim-sulfamethoxazole (29.93%) and oxytetracycline (28.66%). Imipenem and norfloxacin were the most potent antibiotics. All isolates belonging to phylogenetic groups B2, D, and E were MDR. The other MDR isolates were

Abbreviations-Cont'd

Imipenem
CFU: Colony Forming Unit
OD: Optical Density

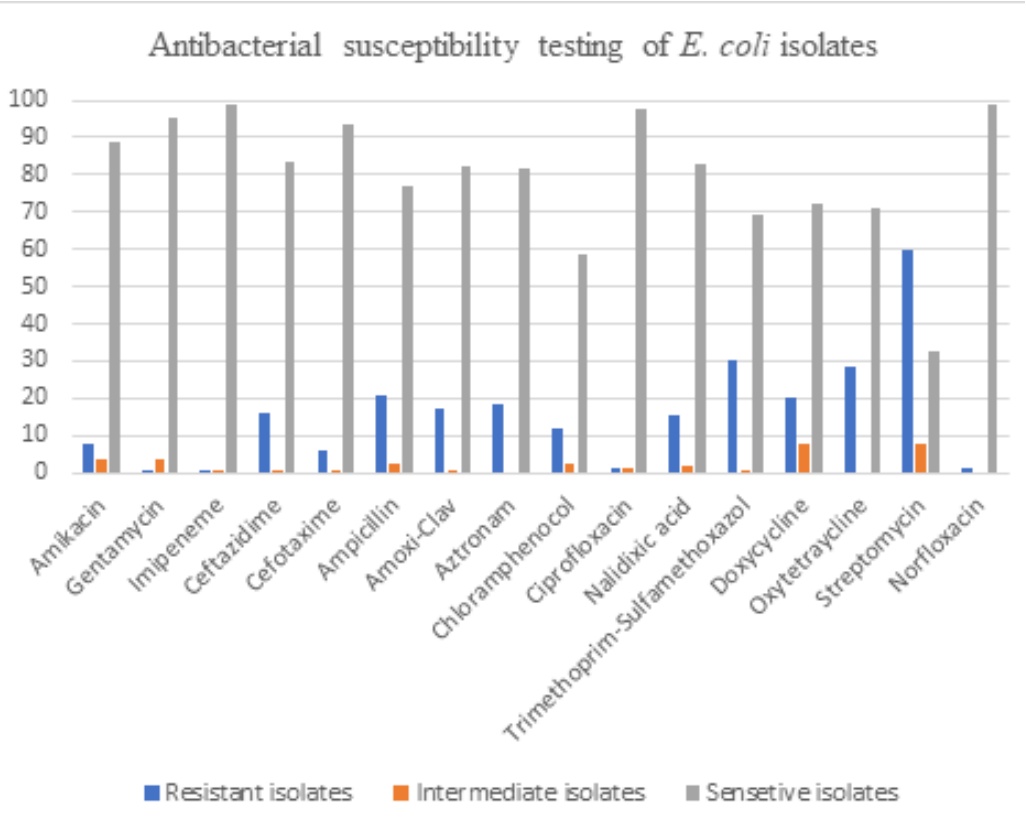

Figure 1.
Antibacterial susceptibility testing of E. coli isolates

Table 1.
Antibacterial susceptibility testing of E. coli isolates

Antibiotic	Resistant isolates	Intermediate isolates	Sensetive isolates
Amikacin	12 (7.64%)	6 (3.82%)	139 (88.53%)
Gentamycin	1 (0.63%)	6 (3.82%)	150 (95.5%)
Imipeneme	1 (0.63%)	1 (0.63%)	155 (98.72%)
Ceftazidime	25 (15.92%)	1 (0.63%)	131 (83.43%)
Cefotaxime	9 (5.73%)	1 (0.63%)	147 (93.63%)
Ampicillin	32 (20.83%)	4 (2.54%)	121 (77.07%)
Amoxi-Clav	27 (17.19%)	1 (0.63%)	129 (82.16%)
Aztronam	29 (18.47%)	0 (0%)	128 (81.52%)
Chloramphenocol	19 (12.10%)	4 (2.52%)	134 (85.35%)
Ciprofloxacin	2 (1.27%)	2 (1.27%)	153 (97.45%)
Nalidixic acid	24 (15.28%)	3 (1.91%)	130 (82.80%)
Trimethoprim-Sulfamethoxazol	47 (29.93%)	1 (0.63%)	109 (69.42%)
Doxycycline	32 (20.38%)	12 (7.64%)	113 (71.97%)
Oxytetraycline	45 (28.66%)	0 (0%)	112 (71.33%)
Streptomycin	94 (59.87%)	12 (7.64%)	51 (32.48%)
Norfloxacin	**2 (1.27%)**	**0 (0%)**	**155 (98.72%)**

in the phylogroups A (20/34), B1 (39/73), C (4/10), and unknown (3/15).

Biofilm-Forming Assay

Of 157 evaluated isolates, 81 (51.6%) were positive in terms of biofilm-forming ability. All MDR isolates were capable of forming a biofilm. The frequency of biofilm-related genes fimH, papC, csgA, and fliC in biofilm former E. coli isolates was 100%, 100%, 77.77%, and 65.43%, respectively. In addition, resistance to all examined antibiotics in biofilm-former strains was higher than biofilm-negative ones (data not shown).

Dissemination of Biofilm-Related Genes in Various Phylogroups

Dissemination of different phylogenetic groups in biofilm-former and biofilm-non-former strains of E. coli is presented in Table 2 and Figure 2. All isolates in phylogroups B2 and D carried all biofilm-related genes. More than half of the isolates in the E and A phylogroups were biofilm-formers and carried variable biofilm-related genes. The allocation of biofilm-related genes in various phylogroups is presented in Supplementary Table 2. There was no significant association between different phylogroups and bio-

film-forming ability among test bacteria ($p < 0.05$).

PCR Screening for Antimicrobial Resistance Genes

Streptomycin resistance-associated genes were more common in test isolates, followed by *tet* and *dfr1* genes as the most frequent ones. The *blaTEM* and/or *blaSHV* genes were detected in three ESBL-positive isolates. Most of the resistance genes were more frequent in biofilm-forming isolates ($p < 0.05$). Antimicrobial resistance genes were common in phylogroups B2, D, A, B1, and E ($p < 0.05$). Each isolate in phylogroups B2 and D carried at least three investigated drug-resistance genes. The distribution of antimicrobial resistance genes in biofilm-former, biofilm-non-former, and different phylogroups of *E. coli* isolates is shown in Table 3.

Table 2.
Distribution of different phylogenetic group in biofilm former and biofilm non former *E. coli* strains

Phylo-group	No. of isolates	
	Biofilm positive	Biofilm negative
A, (34)	18	16
B1, (73)	34	39
B2, (4)	4	-
C, (10)	6	4
D, (6)	6	-
E, (7)	5	2
clade I, (8)	3	5
Unknown, (15)	6	9

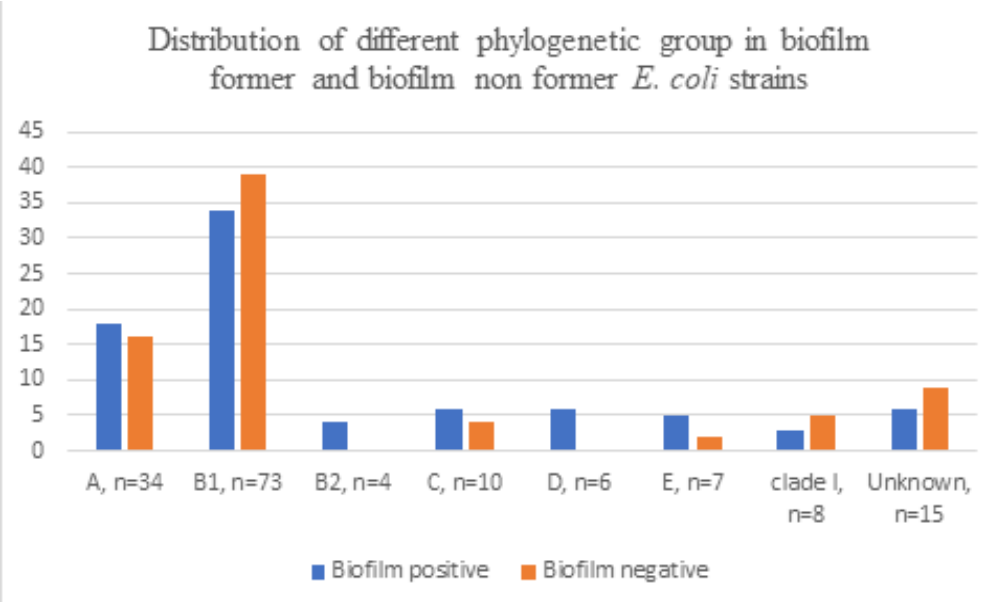

Figure 2.
Distribution of different phylogenetic group in biofilm former and biofilm non former *E. coli* strains

Discussion

Commensal *E. coli* resistance is of special importance to maintaining consumer health as it includes a source of resistance genes. This resistance can be transferred to any other bacteria, such as pathogenic bacteria, through horizontal gene transfer [9, 14]. Veterinary healthcare settings in recent decades have been introduced as the origin of the outbreaks of different multidrug-resistant microorganisms [10, 15, 16]. Injection of antimicrobial agents into horses in both hospital and community contexts is accompanied by a higher risk of the fecal shedding of antimicrobial-resistant *E. coli* [5]. In this study, 85 diverse patterns of antibiotic resistance were detected in 157 isolates, and 131 isolates (83.4%) were resistant to at least one antibiotic. Of 157 *E. coli* isolates examined in terms of antibiotic sensitivity, more than 50% (83 isolates) showed MDR phenotype. Compared to other studies, frequency of MDR in equine fecal *E. coli* was high in the present study. However, our results were comparable with that of Lagarde et al. (2019), which reported 46.3% MDR in *E. coli* isolated from healthy horses [17]. A high level of AMR in domesticated animals is related to the use of antimicrobial agents and is also related to direct exposure to the bacteria responsible for carrying these genes [18].

In the present study, three ESBL-producing isolates harboring *blaTEM* (three isolates) and *blaSHV* (two isolates) genes were identified. Kaspar et al. (2019) detected 9 (4.0%) ESBL-producing *E. coli* positive in

Table3.
Distribution of drug resistance genes among biofilm former and biofilm non former and different phylo-groups of *E. coli* strains

Gene (No.)	Biofilm formation		Phylo-group
	Positive, (81)	Negative, (76)	
TEM, (2)	3 (100%)	-	B2 (2), D (1)
SHV, (1)	2 (100%)	-	B2 (1)
qnr A, (0)	-	-	-
qnr B, (3)	3 (100%)	-	B1 (1), B2 (2)
tetA, (39)	28 (71.79)	11 (28.21)	A (12), B1 (16), B2 (4), D (4), E (3)
tetB, (48)	42 (87.5%)	6 (12.5%)	A (14), B1 (17), B2 (4), D (5), C (2), E (5), clade I (1)
strA-strB, (54)	42 (77.77)	12 (33.33)	A (19), B1 (23), B2 (3), C (2), D (4), E (1), unknown (2)
aadA, (5)	5 (100%)	-	B2 (3), D (2)
aadB, (8)	8 (100%)	-	B1 (2), B2 (3), D (2), E (1)
Cat I, (11)	8 (72.72)	3 (27.28)	A (2), B1 (1), B2 (4), D (2), E (1), unknown (1)
Cat II, (7)	5 (71.42)	2 (27.58)	A (1), B2 (3), D (2), E (1)
Sul 1, (23)	21 (91.3)	2 (8.7)	A (4), B1 (7), B2 (3), D (4), E (2), Clade I (2), unknown (1)
Sul 2, (17)	17 (100%)	-	A (3), B1 (5), B2 (3), D (2), E (1), Clade I (2), unknown (1)
Dfr1, (36)	26 (72.22)	10 (27.28)	A (7), B1 (12), B2 (4), C (2), D (5), E (5), unknown (1)

terms of *blaCTX-M* and/or *blaTEM* in non-hospitalized horses in Northwest Germany [10]. In another research conducted in the United Kingdom, 6.3% of 650 fecal samples from non-hospitalized horses were reported to be positive regarding ESBL-*E. coli* [19]. In Canada, ESBL/AmpC genes were found in *E. coli* obtained from 7.3% of healthy horses [17].

In a higher frequency, Johns et al. (2012) reported 138/228 (60.5%) MDR and 17/228 isolates positive for ESBL production in fecal *E. coli* isolates of horses treated with antimicrobial agents, among which 12/17 isolates had *blaTEM* gene and 4/17 had *blaSHV* [5]. Moreover, examinations of equine patients in a veterinary clinical context revealed even higher percentages (10.7% and 34.2%) of animals that carry ESBL [20, 21]. These findings confirm previous descriptions based on which feces from hospitalized horses and horses cured with antibiotics harbored more antimicrobial-resistant *E. coli* isolates compared to untreated horses and are more likely to harbor MDR [22, 23].

Present results showed the highest frequency was for streptomycin resistance (59.87%) and the most prevalent resistance gene (54/94) was related to this medication. This result is consistent with those obtained by Sato et al. (2020) indicating the highest (30.9%) streptomycin resistance among *E. coli* strains obtained from healthy racehorses in Japan. Furthermore, in accordance with the present results, they reported that almost all *E. coli* isolates have been susceptible to kanamycin and gentamycin (98.8% and 100%, respectively) [24]. In equine medicine, streptomycin is frequently used to cure gram-negative bacterial infections [8]. These can explain the high resistance rate of equine microbiota against this antibiotic. According to the phenotypic assay in this study, *aadA* and *aadB* were detected in five and eight aminoglycoside-resistant isolates, respectively, and *strA-strB* (streptomycin-resistance genes), as the most common resistant genes, were identified in 54 isolates.

In addition, possibly as a result of extensive application of trimethoprim-sulfamethoxazole to cure infections induced by gram-positive and gram-negative bacteria among horses [24], resistance to this antibacterial combination was relatively high in the tested isolates (47/157). However, in South Korea and Portugal, *STX* resistance was found to be 9.8% (5/51) and 2.8% (2/71), respectively [8, 25]. In a study in Japan, 15.8% of *E. coli* isolates were found to be resistant to trimethoprim [24]. Among trimethoprim-sulfamethoxazol-resistant isolates, we detected dfr1, sul1, and sul2 in 76.6%, 48.9%, and 36.2%, respectively. Ahmed et al. (2010) reported that 93% of the trimethoprim-resistant equine fecal *E. coli* isolates were positive regarding at least one dfr gene [22]. These genes are often encod-

ed on mobile genetic elements, leading to this wide dissemination of *dfr* genes.

Among 19 chloramphenicol-resistant isolates identified in this study, *catI* and *catII* were detected in 11 and 7 isolates, respectively. *catI* gene was previously accountable for most of the plasmid-mediated resistance to chloramphenicol [22]. In addition, this article detected 51/157 isolates to be resistant to oxytetracycline and/or doxycycline, among which *tetA* and *tetB* genes were determined in 39 and 48 isolates, respectively. Tetracycline resistance in *E. coli* most often happens among animals, such as horses, and the efflux genes *tetA* and *tetB* are generally disseminated in gram-negative bacteria [22, 26]. This pattern of prevalence was also shown in equine fecal *E. coli* strains from hospitalized horses in Northwest England in which the *tetB* gene showed to be the most common (71%) resistant to tetracycline, followed by tetA (18%), while no other tet gene was detected [17].

In the current study, 17.19% of E. coli isolates were not susceptible to the first generation of quinolones (nalidixic acid) but norfloxacin and ciprofloxacin were potent antibacterial agents (1.27% resistance). Compared to our results, Lagarde et al. reported that 54.1% of isolates from healthy horses were not susceptible to nalidixic acid, and 20.3% of isolates were not susceptible to ciprofloxacin, which is a fluoroquinolone in this collection [17].

Furthermore, the present assay detected biofilm-forming ability in 81/157 (51.6%) of fecal *E. coli* isolates. Resistance to every examined antibiotic in biofilm-former strains was significantly higher than in biofilm-negative antibiotics. The frequency of biofilm-associated genes *fimH, papC, csgA*, and *fliC* in biofilm-former *E. coli* isolates was 100%, 100%, 77.77%, and 65.43%, respectively. However, less data is available on biofilm formation and the distribution of its associated genes in microorganisms isolated from horses. In the USA, the prevalence of fimH and papC genes among 25 horse *E. coli* isolates was 92% and 4%, respectively [27]. Also, P fimbriae structural subunits-encoding genes, including papC, were the most commonly identified (75/164, 45.7%) virulence genes in *E. coli* from companion animals, including horses in the UK [28].

The present article also investigated the phylogenetic group of *E. coli* obtained from horses. In the present assay, commensal strains were predominant, and 68.2% of isolates were related to the phylogroups B1 and A. Among the remaining isolates, 10 (6.4%) were potential extraintestinal pathogens (B2 and D). In accordance with the present assay, in a study conducted in Iran, phylotype B1 was detected as the most common phylotype (76.92 %) among *E. coli* isolated from healthy riding horses in Kerman [29].

In the present study, every isolate in phylogroups B2 and D carried all biofilm-related genes. These findings are in line with the results of Olowe et al. (2019) who reported that phylogroups B2 and D contained 100% of each of the biofilm-related genes [30]. Biofilm-related genes *fimH* and *papC* were found in 100% of biofilm-positive isolates and were prevalent in all phylogroups. The fimH gene was determined to be common in every phylogroup of *E. coli* according to various studies [30-32].

Moreover, several studies have shown that phylogroups B2 and D have more biofilm-related gene characteristics compared to phylogroups B1 and A [30, 33-35]. Our results showed that all isolates belonging to B2, D, and E phylogenetic groups were MDR. However, the isolates in the other phylogroups were also MDR, and several studies have shown that the strains of group B2 are mainly MDR [30, 36, 37].

Conclusion

Our findings demonstrated that in Guilan province, Northern Iran, non-hospitalized, healthy horses harbor potential extra-intestinal pathogenic and MDR *E. coli* isolates. These animals can be reservoirs for ESBL-producing and noteworthy human and veterinary medicine-used antibiotics-resistant isolates. The obtained data emphasized the growing concern regarding antimicrobial resistance, MDR shedding, and management of antimicrobial applications in veterinary.

Materials & Methods

Equine Study Population and Demographic Data

During May-August 2021, fresh feces samples were collected from 23 farms of 157 healthy and non-hospitalized horses that lived on private farms around Rasht, Northern Iran. The studied horses aged 3 months to 20 years, 72 were female, and 85 were male without receiving antibiotic therapy during the past six months.

E. coli Isolation and Antimicrobial Susceptibility Testing

Every single sample was transferred to the laboratory on the ice in 4 h. *E. coli* was isolated on MacConkey (MC) agar and EMB agar media and was evaluated based on standard bacteriology and biochemistry methods and one *E. coli* isolate was selected per sample for further investigation. The disk diffusion assay was adopted to determine the antimicrobial susceptibility pattern for each isolate of *E. coli* based on the Clinical and Laboratory Standards Institute suggestion [38].

The antimicrobials used in these assays were selected based on their use and importance for human and equine medicine as follow: beta-lactams [amoxicillin+clavulanic acid, ampicillin, ceftiofur, cefotaxime, and cefquinome ampicillin (AM; 10 µg), amoxicillin/clavulanic acid (AMC; 20/10 µg), ceftazidime (CAZ; 30 µg),

Table 4.
Nucleotid sequences of primers used in this study

Gene	Primer Sequence (5"-3")	Amplicon size (bp)	Annealing tem. (°C)	Ref.
blaTEM	F-CTAGTATGACGTCTGTCGC	1080	58	[15]
	R-GACAGTTACCAATGCTTAATC			
blaSHV	F-TTATCTCCCTGTTAGCCACC	795	58	[15]
	R-GATTTGCTGATTTCGCTCGG			
qnr A	F-ATTTCTCACGCCAGGATTTG	516	53	[15]
	R-GATCGGCAAAGGTTAGGTCA			
qnr B	F-GATCGTGAAAGCCAGAAAGG	469	54	[15]
	R-ACGATGCCTGGTAGTTGTCC			
tetA	F-CCTCAATTTCCTGACGGGCT	712	55	[15]
	R-GGCAGAGCAGGGAAAGGAAT			
tetB	F-ACCACCTCAGCTTCTCAACG	586	55	[15]
	R-GTAAAGCGATCCCACCACCA			
strA-strB	F- ATG GTG GAC CCT AAA ACT CT	893	60	[16]
	R- CGT CTA GGA TCG AGA CAA AG			
aadA	F- GTG GAT GGC GGC CTG AAG CC	525	60	[16]
	R- AAT GCC CAG TCG GCA GCG			
aadB	F-GAG GAG TTG GAC TAT GGA TT	208	53	[16]
	R- CTT CAT CGG CAT AGT AAA A			
cat I	F-AGTTGCTCAATGTACCTATAACC	585		[17]
	R-TTGTAATTCATTAAGCATTCTGCC			
cat II	F-ACACTTTGCCCTTTATCGTC			[17]
	R-TGAAAGCCATCACATACTGC			
sul 1	F- CGG CGT GGG CTA CCT GAA CG	433	66	[16]
	R-GCC GAT CGC GTG AAG TTC CG			
sul 2	F-CGG CAT CGT CAA CAT AAC CT	721	66	[16]
	R-TGT GCG GAT GAA GTC AGC TC			
dfr1	F-ACGGATCCTGGCTGTTGGTTGGACGC	254	55	[17]
	R-CGGAATTCACCTTCCGGCTCGATGTC			
fimH	F-TGCAGAACGGATAAGCCGTGG	506		[18]
	R- GCAGTCACCTGCCCTCCGGTA			
csgA	F-GCAATCGTATTCTCCGGTAG	418		[18]
	R -GATGAGCGGTCGCGTTGTTA			
papC	F-TGATATCACGCAGTCAGTAGC	501		[18]
	R- CCGGCCATATTCACATAAC			
fliC	F-ATGGCACAAGTCATTAATACCCAAC	1491		[18]
	R- CTAACCCTGCAGCAGAGACA			

cefotetan (CTT; 30 µg), imipenem (IMP; 10 µg)], aminoglycosides [amikacin (AN; 30 µg), streptomycin (S; 10µg) and gentamicin (GM; 10 µg)], tetracyclines (oxytetracycline and doxycycline), sulphonamides/potentiated sulphonamides (sulphamethoxazole and trimethoprim-sulphamethoxazole), fluoroquinolones [nalidixic acid (NA; 30 µg), ciprofloxacin and enrofloxacin], carbapenems (imipenem), and chloramphenicol (C; 30µg), aztreonam (ATM; 30 µg). Plates were incubated at 37°C for 18-20 h. An isolate resistant to at least one antimicrobial agent in at least three antimicrobial classes was considered MDR. *E. coli* ATCC 25922 was included as quality control for the applied antimicrobial agents.

Extended-Spectrum Beta-Lactamase (ESBL) Production

ESBL production was detected by double disk diffusion technique with ceftazidime (30 µg) and cefotaxime (30 µg) disks alone and combined with clavulanic acid (10µg) on Muller Hinton agar. The positive test result was determined as ≥5 mm elevation in the diameter of the zone compared to a disk free of clavulanic acid. Moreover, an imipenem-EDTA double disk synergy assay was adopted for evaluating MBL enzyme production. Improvements in the diameter of the inhibition zone in IMP+EDTA in comparison with IMP-only disks were determined as MBL producers.

Biofilm Formation Assay

This test was carried out on a microtiter plate. Standard overnight cultures (1.5×108 CFU/ml) were diluted 100 times in PBS. A volume of 200 µl of each culture dilution was transferred to separate wells of a 96-well, flat-bottomed polystyrene plate, and was incubat¬ed overnight at 37°C. After the process of incubation, the planktonic bacteria were discarded, the wells were delicately washed three times using sterile physiological saline and were fixed using methanol for 20 min. Then, each well was stained using crystal violet and washed. Biofilm-related crystal violet was destained in 1 ml ethanol-acetone (95:5, vol/vol). Afterwards, the optical density of the mixed solution was found to be 600 nm. Isolates with OD > 0.625 were categorized to be biofilm-positive [39].

Extraction of DNA

A colony of each bacterial strain was purified and bacterial genomic DNA was extracted by a GenElute Bacterial Genomic Kit (Sigma-Aldrich, St Louis, MO).

Phylogenetic Typing of E. coli Isolates

Bacterial phylogenetic groups were determined using the previously described method of quadruplex phylogroup assignment [12]. The isolates were categorized in phylogenetic groups A, B1, B2, C, D, and F via scoring the presence or absence of genes in arpA/chuA/yjaA/TspE4.C2 order according to the following criteria: A: (+---); B1: (+--+); B2: (-++-/-+++/-+-+); F: (-+--); A or C: (+-+-); D or E: (++--/++-+); E or clade I: (+++-). The three last ones were screened using C or E-specific primers.

PCR Screening for Biofilm-Associated Genes

The frequency of four hypothetical biofilm-related genes in biofilm-former *E. coli* isolates has been investigated by Olowe et al. (2019). Table 1 shows the utilized primers. Extracted nucleic acid was adopted as template DNA for PCR. PCR was carried out based on a total volume of 25 mL, including 0.5 ml dNTPs (10 mM), 5 mL enzyme buffer (10X), 3 ml forward/reverse primers (10 pmol), 2 ml template DNA (2 mg), 0.5 mL enzyme (2.5 U), and 14 ml deionized water. Each gene was individually expanded and PCR products were assessed by electrophoresis on 1% agarose gel. Afterwards, PCR products were sequenced to affirm the identity of the amplicon sequence (Bioneer, South Korea).

PCR Screening for Antimicrobial Resistance Genes

Based on the patterns of antimicrobial susceptibility, PCR was performed for evaluating the corresponding antimicrobial resistance genes. Isolates showing resistance to investigated antimicrobial classes were screened regarding the existence of 14 diverse resistance genes by PCR as described before (Table 4). Tetracycline-resistance genes (*tetA* and *tetB*), sulfamethoxazole-resistance genes (sul1, sul2), TMP-resistance genes (dfr1), streptomycin-resistance genes (*strA-strB*), *blaTEM*, and *blaSHV* were screened. Furthermore, the existence of plasmid-mediated quinolone-resistance genes (*qnrA, qnrB*), and genes encoding aminoglycoside-modifying enzymes (*aadA* and *aadB*) were investigated. Table 2 demonstrates the sequences of oligonucleotide primers adopted in this evaluation. The conditions of PCR reactions were as mentioned above.

Statistical analysis

Correlations between the phylogenetic group, biofilm-forming ability, and frequency of resistance-related genes in examined bacteria were assessed by SPSS Statistics and the Chi-square test. For all tests, $p < 0.05$ was considered significant.

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Competing Interests

There is no any conflict of interests.

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Therapeutic Effects of ADU-S100 as STING Agonist and CpG ODN1826 as TLR9 Agonist in CT-26 Model of Colon Carcinoma

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ABSTRACT

Cancer immunotherapy emerged as a novel therapeutic approach to destroy tumor cells, and it has grown toward clinical transition following successful fundamental research and clinical trials. Immunotherapy by efficacious adjuvants is critical for increasing protective immune responses against infectious diseases and cancers. STING and TLR9 agonists are interesting candidates for novel immunotherapies of cancers. In this study, the antitumoral effects of ADU-S100, as a potent STING agonist, and CpG ODN1826, as a TLR9 agonist, in single and combined forms in CT-26 colon adenocarcinoma model were evaluated. This model was induced in female BALB/c mice which were divided into five groups treated with PBS, ADU-S100 (20 and 40 µg), CpG ODN (40 µg), and ADU-S100 (20 µg)+CpG ODN (20 µg). The tumor volumes and weights of mice were measured every other day. On the 30th day, the tumor, spleen, and liver tissues of mice were isolated for histopathological assessment. Hematological analysis was performed on heart blood. Intratumoral injection of agonists induced significant tumor suppression in all treatment groups with profound effect in the combination group that received half concentration of single form. Moreover, the histopathological analysis of tumor tissues showed the presence of apoptotic and inflammatory cells and increased the number of lymphocytes in the blood samples of the treatment groups indicating the effective role of these agonists in clearing the tumor. Therefore, a such synergy of adjuvants may have an effective role in cancer immunotherapy and offer new perspectives on the combination of agonists that trigger innate immune sensors during malignancy.

Keywords

STING agonist, TLR9 agonist, Synergistic effect, Immunotherapy, Colon carcinoma model

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Abbreviations

STING: Stimulator of interferon genes
TLR9: Toll-like receptor 9
CpG ODN: CpG oligodeoxynucleotides
IRF7: Interferon regulatory factor 7
MYD88: Myeloid differentiation primary response 88
DCs: Dendritic cells

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Introduction

There are different treatment methods for cancers, including radiotherapy, chemotherapy, and surgery. However, each procedure has limitations that affect the diagnosis and survival of patients [1]. In recent years, immunotherapy, as a promising treatment approach that triggers the immune system against a broad category of cancers, has had principal efficacy on some patients with metastatic tumors [2]. Recent investigations opened a novel chapter as immunoadjuvants for cancer immunotherapy by the initial activation of innate and subsequent adaptive immune responses [3].

One of the key immunoadjuvants is TLR agonists that bind to the receptors on the endosomal compartments of mainly immune cells and lead to inflammatory reactions and adaptive immune responses. One of the important agonists of TLR9 is CpG ODN as a synthetic TLR9 ligand that can activate the signaling pathway through MyD88 and IRF7 to produce type I interferons and through NF-κB signaling pathway to stimulate immune cells to induce pro-inflammatory cytokines production [4]. CpG ODNs as a potent cancer vaccine adjuvant can increase the proliferation of cytotoxic CD8+ T cells against tumor antigens and trigger Th1-type immune responses that stimulate antigen-specific adaptive immunity [4, 5]. CpG-ODN 1826, as a class B CpG specific for murine TLR9, induces the production of pro-inflammatory cytokine and antibodies from B cells and has demonstrated encouraging outcomes by reducing tumor growth and improving survival in clinical trials [6-8]. Another important immunoadjuvant identified in recent years is STING which is stimulated with cytosolic dsDNA and induces the transcription of numerous innate immune genes [9].

STING is an adaptor protein localized predominantly on the ER membrane. In response to specific agonists, STING induces a signaling pathway that leads to the production of IFN-β as a critical pro-inflammatory cytokine [10]. Consequently, DCs are activated, and cytotoxic T lymphocytes are recruited in TME. There are different agonists for inducing STING, among which MLRRS2 CDA (also called MIW815 or ADU-S100) is known as a powerful vaccine adjuvant. It induces TME activation in a variety of tumor types by priming the antigen-specific CD8+ T cell and lasting immune-mediated tumor rejection [11]. Due to the improved features of ADU-S100 compared to other STING agonists, the therapeutic effects of this agonist in combination with different immunomodulatory agents have been evaluated [12].

In this study, we investigated the synergistic effect of ADU-S100 in combination with CpG ODN1826 in a mouse model of colon carcinoma. Furthermore, we evaluated the ability of single and combination forms of agonists on tumor growth and survival rate in a CT-26 colon carcinoma model. Our results suggest that combined ADU-S100 and CpG ODN1826 have a significant antitumor impact, making them a promising immunotherapeutic agent for the treatment of colon cancer.

Abbreviations-Cont'd

NF-κB: Nuclear factor kappa B
TME: Tumor microenvironment
dsDNA: Double-stranded DNA
ER: Endoplasmic reticulum
IFN: Interferon
SC: subcutaneous
HE: Hematoxylin-eosin
CBC: Complete blood count
cGAMP: Cyclic guanosine monophosphate–adenosine monophosphate
CDN: Cyclic dinucleotides
APC: Antigen-presenting cell

Results

Anti-tumoral activity of CpG ODN 1826 and ADU-S100 in single and combined forms against established CT-26 colon adenocarcinoma

ADU-S100 is a new STING agonist that affects tumor cells by activating the STING signaling pathway in TME and priming APC and CD8+ T lymphocytes. Intratumoral injection of these molecules into various cancer models, including CT26 colon cancer, B16 melanoma, and 4T1 breast cancer models, showed stronger antitumor outcomes [11, 13, 14]. On the other hand, CpG ODN 1826, as a well-known B-type CpG, has been utilized as an adjuvant for vaccines and successfully evaluated in a number of vaccination models [15]. This study investigated whether the combination of CpG-ODN 1826 and ADU-S100 attenuates tumor growth more than the single forms of agonists in the CT-26 cancer model.

In order to induce mice with tumors, CT-26 cell suspensions were subcutaneously implanted into the right flank of animals and treated with 20 μg of CpG ODN 1826 and ADU-S100 together or twice the concentration of single agonists (40 μg). ADU-S100 (20 μg) was used to confirm the synergistic effect of agonists. The volume and weight of tumors in the mice were recorded every other day. As can be seen in Table 1, significant tumor regression was observed in all treatment groups compared to the control group ($p < 0.0001$). The highest suppression of tumor growth was observed with the synergy of two agonists from 1952 to 32 mm3.

In ADU-S100 (40 μg) and combination groups, only one out of seven treated mice did not show a

decrease in tumor volume (Figure 1B and 1D). In CpG ODN (40 µg) group, five mice showed a considerable shrinkage in tumor volume, but in the other two members of this group, tumor volume reached 358 and 624 mm3 on the 30th day (Figure 1C). Although in ADU-S100 (20 µg), the average tumor volume showed a smaller decrease compared to the double concentration of this agonist, the change was significant compared to the control (Figure 1A). These results indicated that the synergistic antitumor effects of ADU-S100 when combined with CpG ODN is approximately equal to those of ADU-S100 when administrated at higher doses (twice the concentration). Images of CT26 tumor-bearing mice and treated mice of all groups on the 30th day are represented in Figure 2.

At the end of the experiment, mean tumor weight was considerably lower in the combination group (0.08 g) and other treatment groups than in the control (2.01g) ($p < 0.05$) (Figure 3A). The curve of spleen weight is shown in Figure 3B and spleen weight was positively correlated with tumor weight. This differ-

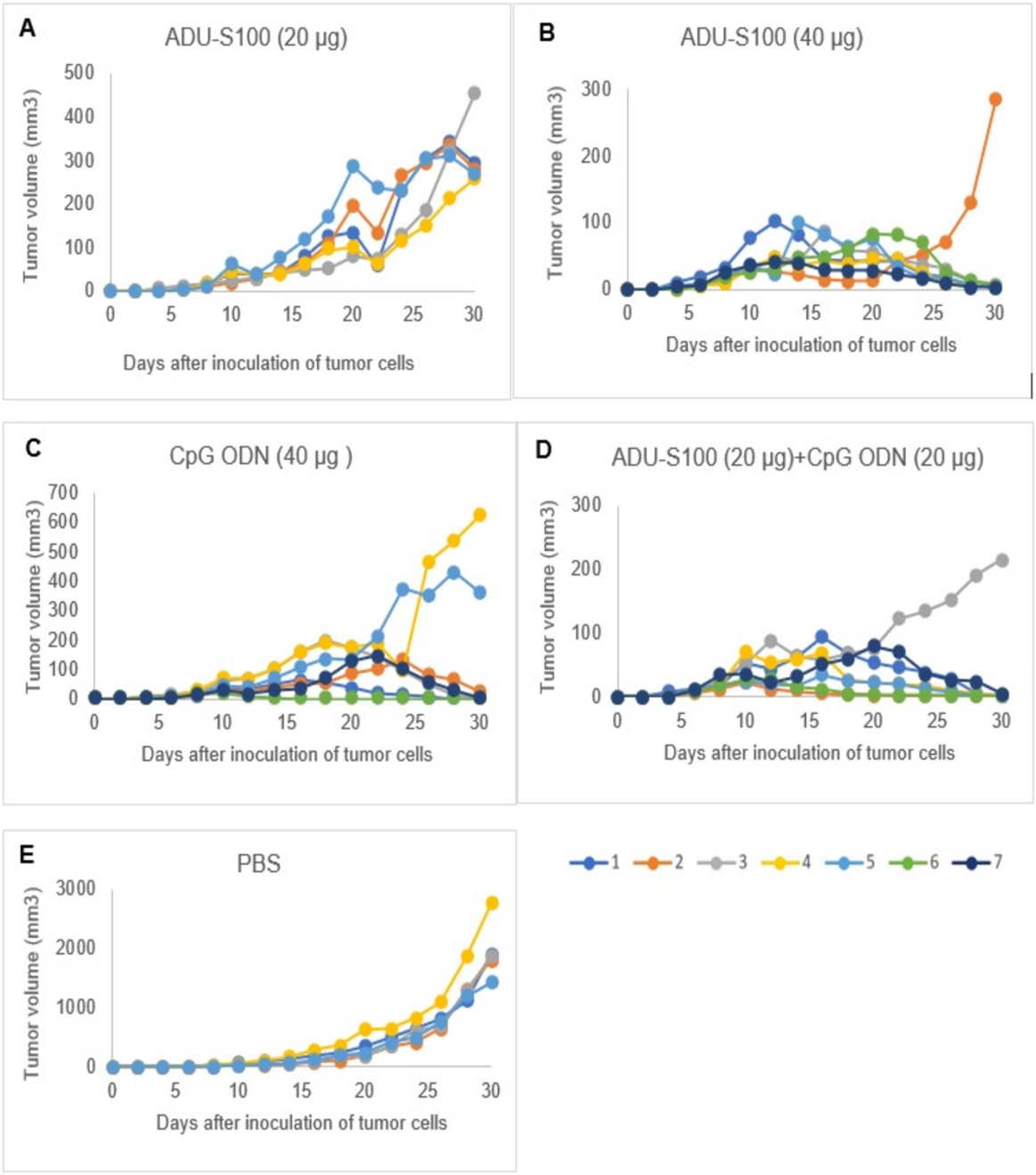

Figure 1.
Intratumoral (IT) administration of STING and TLR9 agonist reduces tumor volume in CT-26 adenocarcinoma model. (A) Mice were injected with 3×105 CT-26 cells (in 100 µl of PBS) SC on day 0. On days 10 and 16, mice were given IT injections of PBS, ADU-S100 (20 and 40 µg), CpG ODN (40 µg), and ADU-S100 (20 µg) + CpG ODN (20 µg) (n=7). tumor volumes were monitored every other day for 30 days.

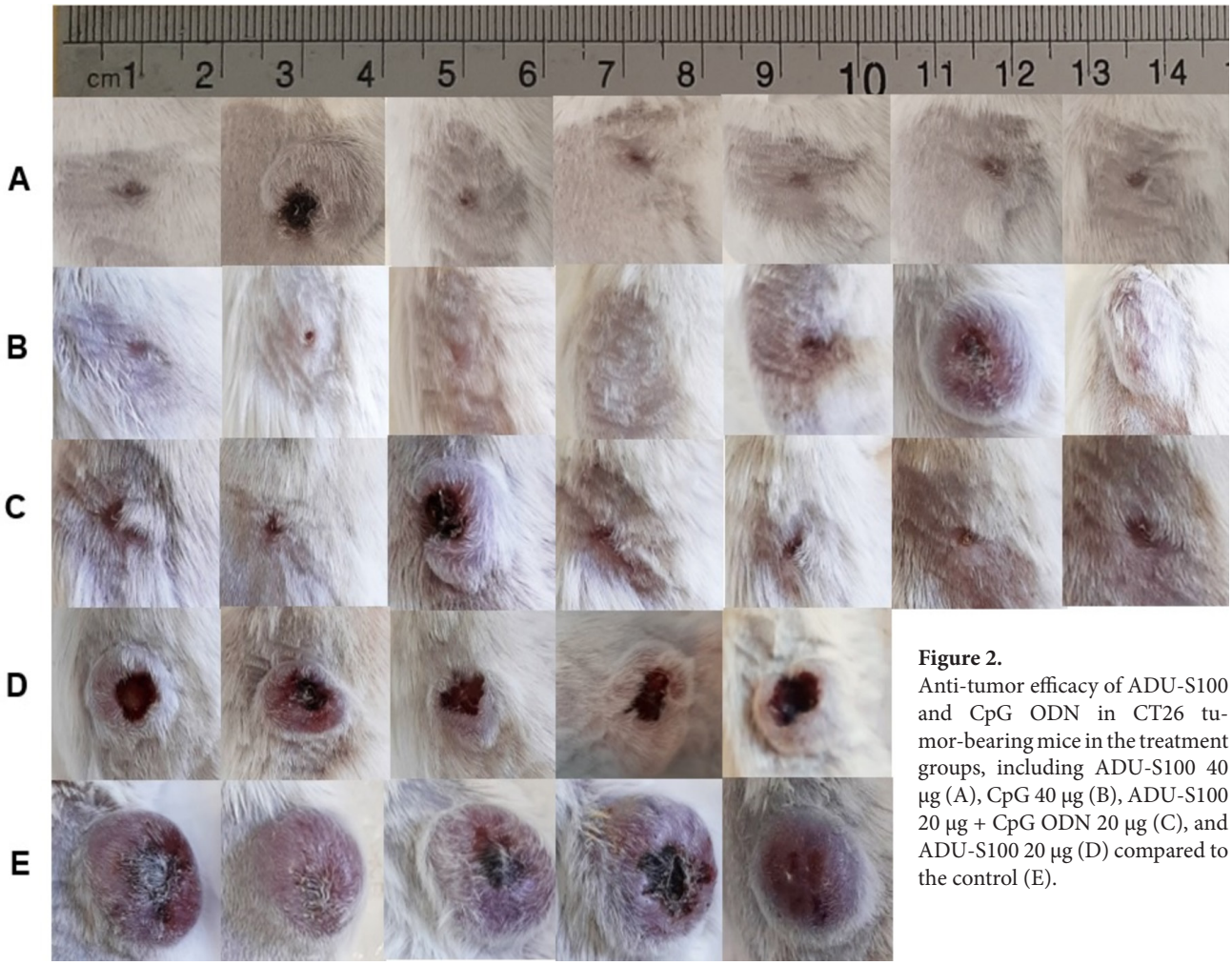

Figure 2.
Anti-tumor efficacy of ADU-S100 and CpG ODN in CT26 tumor-bearing mice in the treatment groups, including ADU-S100 40 µg (A), CpG 40 µg (B), ADU-S100 20 µg + CpG ODN 20 µg (C), and ADU-S100 20 µg (D) compared to the control (E).

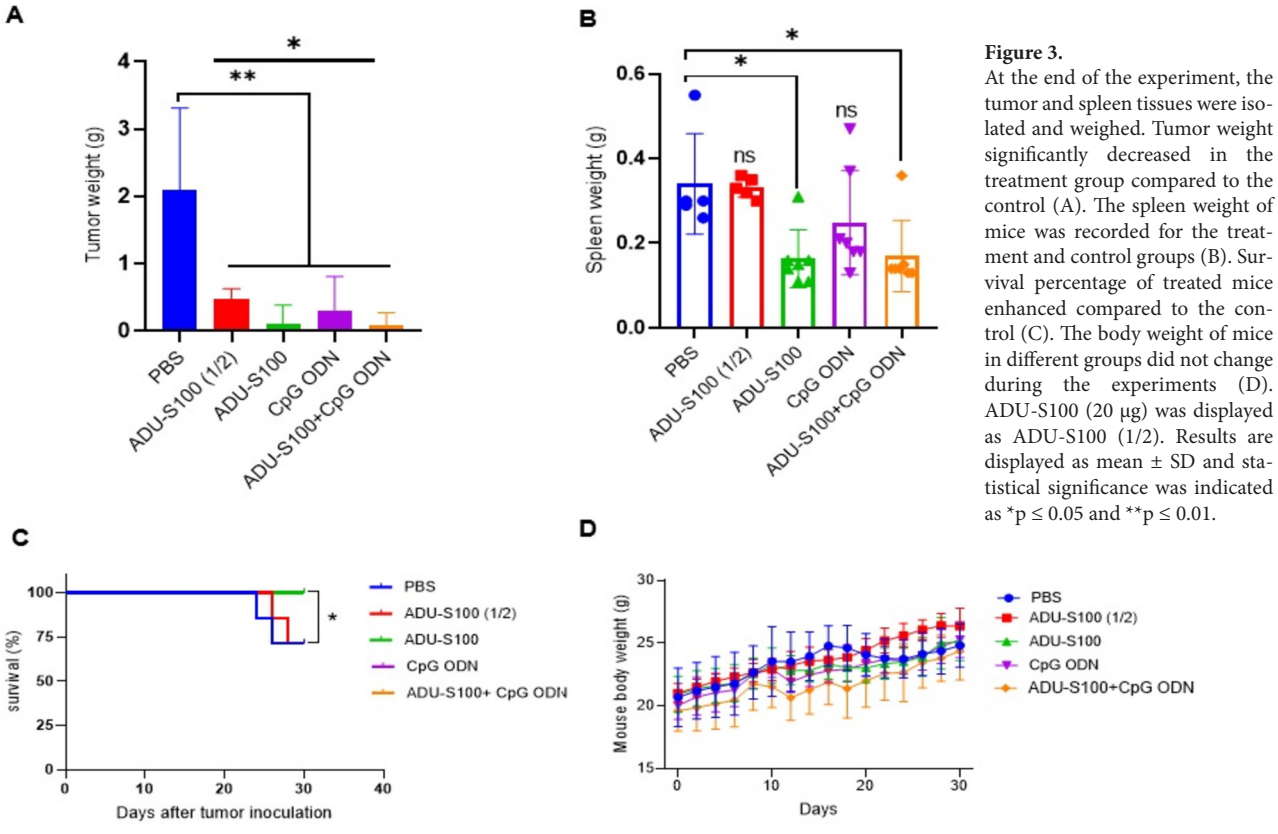

Figure 3.
At the end of the experiment, the tumor and spleen tissues were isolated and weighed. Tumor weight significantly decreased in the treatment group compared to the control (A). The spleen weight of mice was recorded for the treatment and control groups (B). Survival percentage of treated mice enhanced compared to the control (C). The body weight of mice in different groups did not change during the experiments (D). ADU-S100 (20 µg) was displayed as ADU-S100 (1/2). Results are displayed as mean ± SD and statistical significance was indicated as *p ≤ 0.05 and **p ≤ 0.01.

ence was significant in the ADU-S100 (40 µg) and combined groups which had the smallest tumor volume at the end of the experiment. During the study, the mice survival rate increased from 71.4% in the control group and ADU-S100 (20 µg) to 100% in other treatment groups (Figure 3C). Furthermore, the body weight of animals was also monitored during the experiment and no significant weight change was observed compared to the control group (Figure 3D).

Histological examination of the tumor, spleen, and liver tissues

Microscopic examination of the tumor tissues showed round, polygonal, spindle-like, polymorph neoplastic cells of varied sizes, containing prominent one or more nuclei, within a delicate stroma. The control group displayed a large number of tumor cells undergoing mitosis (Figure 4A). In addition, some criteria, such as necrosis and hemorrhage as signs of malignancy, were detected in the control group. Compared to the control group, increased numbers of apoptotic cells were observed in the treatment groups (Figure 4 B-E). Histological analysis of the spleen and liver tissues was performed to assess the abnormal

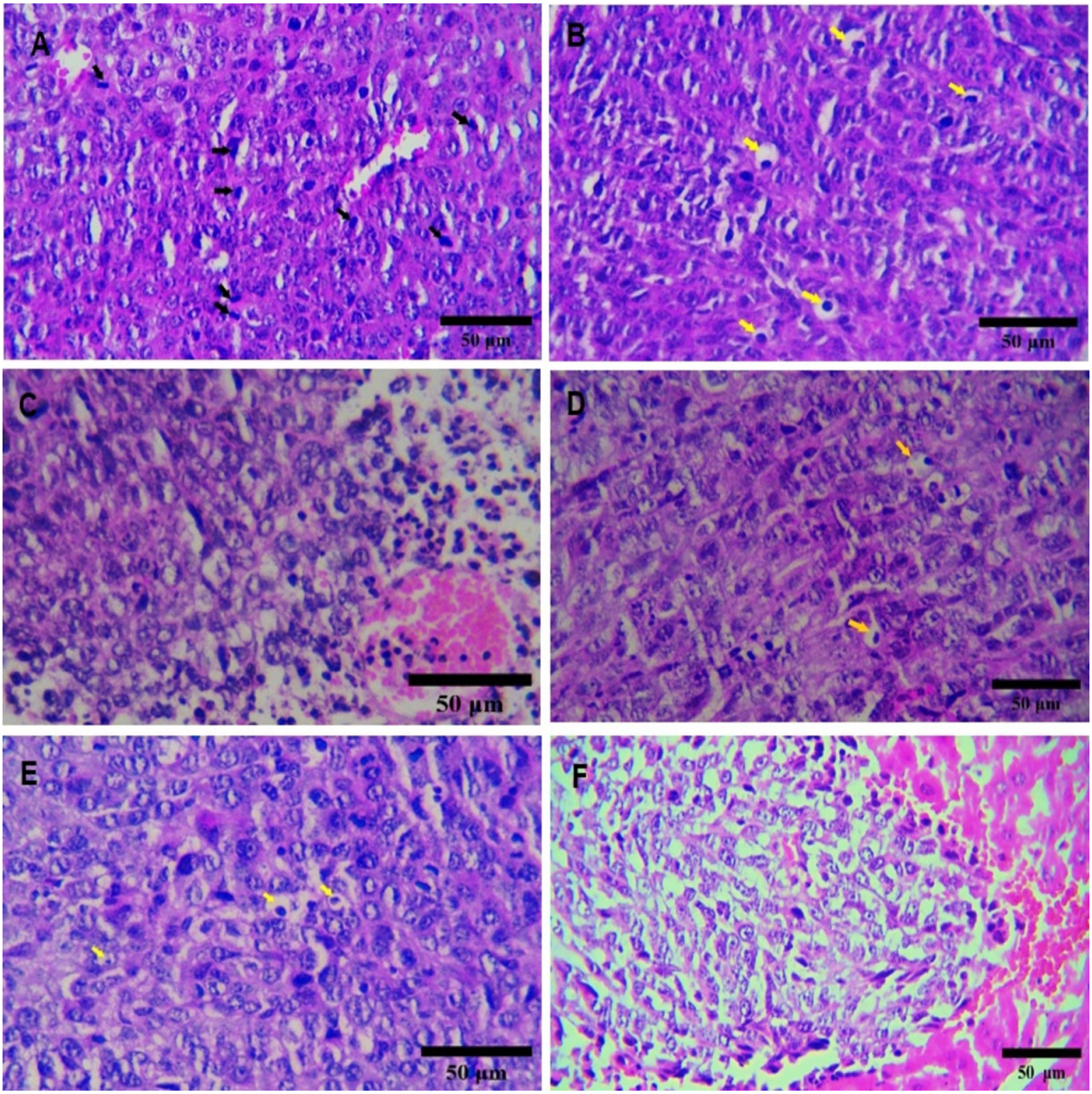

Figure 4.
Hematoxylin-Eosin staining of tumor tissues in the control (A), ADU-S100 20 µg (B), ADU-S100 40 µg (C), CpG 40 µg (D), and ADU-S100 20 µg + CpG ODN 20 µg (E). Liver metastasis in the control group (F). Cells undergoing mitosis in the control group are displayed with black arrows (A). Typical features of apoptosis (B-E, yellow arrows) are shown in the treatment groups.

effects of the agonists on the reticuloendothelial tissues. No obvious histological changes were detected in the spleen tissue in none of the treatment groups. The liver microstructure of the control group revealed tumor metastasis, while the treatment groups were normal (Figure 4F).

Hematological analysis of whole blood

CBC experiments showed that the number of lymphocytes increased significantly in the ADU-S100 (40 µg), CpG ODN (40 µg), and ADU-S100 (20 µg) + CpG ODN (20 µg) groups ($p < 0.05$) indicating the recruitment of immune cells to the TME (Table 2). Other hematological factors measured in this research were not significantly different between the control and treatment groups.

Table 1.
The average tumor volume (mm^3) in control and all treatment groups on 30th day

Group	Average tumor volume (mm^3) ± SEM	*p*- value
Control	1952±194	
ADU-S100 (20 µg)	310±32	<0.0001
ADU-S100 (40 µg)	44.8±36	<0.0001
CpG ODN (40 µg)	144±87	<0.0001
ADU-S100 (20 µg) + CpG ODN (20 µg)	32±28	<0.0001

Mean in each group is significantly different compared to control group ($p < 0.0001$).
SEM: Standard error of means. CpG ODN: CpG oligodeoxynucleotides.

Table 2.
The number of lymphocytes (%) of blood samples in control and all treatment groups on 30th day

Group	Number of lymphocytes (%) ± SEM	*p*- value
Control	13.5 ± 3	
ADU-S100 (20 µg)	21 ± 5	0.17
ADU-S100 (40 µg)	42 ± 7	<0.05
CpG ODN (40 µg)	44 ± 3	<0.05
ADU-S100 (20 µg) + CpG ODN (20 µg)	50 ± 6	<0.05

Mean in three treatment groups is significantly different compared to control group ($p < 0.05$).
SEM: Standard error of means. CpG ODN: CpG oligodeoxynucleotides.

Discussion

In the TME, immune cells and tumor cells can be affected by STING and TLRs activation. In addition, T cells, endothelial cells, fibroblasts, and APCs as the main cells in this pathway, can lead to type I IFN production with STING stimulation [11, 16]. Type I IFN signaling is important for antitumor immune responses through stimulating apoptosis and anti-proliferative responses acting directly on tumor cells leading to tumor suppression [17]. Due to the role of the STING pathway in the generation of spontaneous immune responses against tumors, STING agonists were discovered for direct pharmacologic stimulation of this signaling pathway [18-20]. Among STING agonists, the intratumoral administration of CDNs, such as ADU-S100, into different types of cancer inhibited tumor growth, generated lasting and systemic antigen-specific T cell immunity capable of rejecting distant metastases, improved survival of mice, and induced direct apoptosis of cancer cells [13, 21- 23]. On the other hand, CpG ODNs have been considered widely as a new choice for vaccine adjuvants because of their powerful capacity to enhance vaccine immunogenicity against cancer [3, 24]. In this research, we examined the antitumor effects of single and combined forms of ADU-S100 and CpG ODN1826 in the CT-26 colon cancer model. Our results showed that ADU-S100 at 40 µg dose suppressed the CT-26 tumor growth more effectively than at 20 µg dosage. Moreover, the combination of ADU-S100 and CpG ODN1826 resulted in the smallest tumor volume (Figures 1 and 2). As a result, the intratumoral injection of this combination in half concentration effectively reduced tumor growth in the CT-26 model (Figure 1A). In addition, the survival rate of treated mice (100%) was more than the control group (71.4%). Corrales et al. showed that the intratumoral injection of ADU-S100 in the CT26 colon carcinoma model caused T cell memory and durable tumor regression. When the tumor cell line was injected into mice again, the animals were completely resistant to re-challenge. This study had similar results in the 4T1 breast cancer model [11]. Other STING agonists also showed effective therapeutic results in the colon carcinoma model.

STING deficiency in mouse models bearing colon 26 adenocarcinomas reduced the antitumor effects of cGAMP as a potent STING agonist. The cGAMP displayed remarkable antitumor activity against CT-26 adenocarcinoma (2569 mm3 to 967 post-treatment). This agonist increased the survival rates of mice from 40% up to 90% in 20 days. Furthermore, cGAMP in-

duced the apoptosis of tumor cells and raised the expression levels of critical cytokines, such as IFN-β and IFN-γ, in the mouse serum [20]. The intratumoral injection of cGAMP by inducing CD8+ T cell responses delayed the growth of injected tumors and controlled the growth of distant tumors [21]. Deng et al. explained that STING pathway signaling was effective in type I IFNs induction and promotion of innate and adaptive immune responses upon radiation in MC-38 tumor models. The antitumor effects of radiation were significantly dependent on STING signaling as shown by impairing this antitumor effect in STING-deficient mice. Moreover, cGAMP and radiation synergistically increased the antitumor responses and reduced radiotherapy resistance [25]. The synergistic effects of K3CpG and cGAMP in melanoma, lymphoma, and pancreatic models displayed significant tumor suppression compared to single forms of agonists [26, 27]. Cai et al. investigated the synergism of CpG ODN, CGAMP, and anti-OX40 in TC1 and B16 models. This combination induced tumor regression and cytokines production by activating innate and adaptive immunity [28]. Formulation of recombinant protein HPV with 2'-3'cGAMP CDN and CpG-C ODN induced remarkable tumor suppression in TC-1 harboring mice as a cervical cancer model. This combination caused lymphocyte proliferation and increased the number of IFN-γ secreting cells [29].

In HE staining assays, cell apoptosis was observed in the tumor tissues upon ADU-S100 and CpG ODN treatment concerning the control group which may be displayed that the therapeutic effect of agonists is because of stimulation of tumor cells apoptosis. Circulating Lymphocyte-mediated immune response against tumor cells is critically important in tumor suppression [30]. Hematology experiments displayed that the number of lymphocytes rose in all treatment groups except ADU-S100 (20 μg), especially in the combination group and this lymphocytosis probably played a crucial role in tumor suppression.

In conclusion, our study suggests that the combination of ADU-S100 and CpG ODN with a reduced concentration is a potent adjuvant in tumor regression and antitumor immunity and emphasizes the potency of such combination adjuvants as a potential cancer immunotherapy approach.

Materials & Methods

Animals and cells

Six- to eight-week-old female BALB/c mice were purchased from Royan Institute (Tehran, Iran). All in vivo studies were performed according to the animal experimental guidelines approved by the Institutional Animal Care and Use Committee at Ferdowsi University of Mashhad. The animals were kept in standard cages at a temperature of 20°C-25°C and a 12L:12D lighting cycle. Mouse colon cancer cell line, CT26, was bought from the research institute of biotechnology (Mashhad, Iran), cultivated in RPMI 16-40 medium (Gibco, Grand Island, USA), and supplemented with heat-inactivated fetal bovine serum (10% v/v) and penicillin-streptomycin (1% v/v) (Gibco, Grand Island, USA). The cells were incubated at 37°C with a 5% CO2 atmosphere.

Tumor model

To set up the animal tumor model, CT26 cells (100 μl PBS containing 3105 cells for each mouse) were injected SC into the right flank of the animals (day 0). Tumor-bearing mice were randomly assigned to five groups (n=7): (a) Control group received 20 μl PBS; (b, c) tumor groups treated with ADU-S100 disodium salt (Med Chem Express, New Jersey, NJ, USA) at various dosages of 20 or 40 μg; (d) tumor group treated with 40 μg of CpG ODN (InvivoGen, San Diego, USA), and combination group (e) received 20 μg ADU-S100 and 20 μg CpG ODN simultaneously. The agonists were injected intratumorally on the 10th and 16th days after tumor inoculation.

The tumor volume (V) was estimated every other day with a digital caliper and calculated according to the equation V=(L×W2)/2, where W is the small diameter and L represents the large diameter of the tumor [11, 31, 32]. The animals were sacrificed on day 30 and the body weight of each mouse was measured. The spleen, liver, and tumor tissues were isolated for histological analysis.

Histopathological evaluation

The tumor, spleen, and liver tissues were fixed in 10% buffered neutral formalin for 24 h. The tissues were then dehydrated by enhancing the concentrations of ethanol solution (Merck, Munich, Germany), embedded in paraffin wax, and cut by microtome. The 5-μm sections were mounted on the slides and stained with HE. The sections were surveyed by light microscope (Labomed, Labo America Inc, USA) for the presence of histopathological lesions in all groups.

Hematological parameters

At the end of the study, whole blood was collected from mouse hearts in a tube containing an anticoagulant. Hematological parameters, including white blood cell, red blood cell, hematocrit, hemoglobin, mean cellular volume, mean cellular hemoglobin, mean cellular hemoglobin concentration, platelet count, red blood cell distribution width, plateletcrit, mean platelet volume, platelet distribution width, neutrophil, lymphocyte, monocyte, and total protein were assessed by a cell counter (Nihon Kohden, Nima Pouyesh Teb, Iran)

Statistical analysis

All data were analyzed using GraphPad Prism software version 9.0 (La Jolla, San Diego, California). The Mann–Whitney test was used for the statistical analyses. Results are displayed as mean ± SD or mean ± SEM. The significant differences between groups are shown as **** $p \leq 0.0001$, *** $p \leq 0.001$, ** $p \leq 0.01$, and * $p \leq 0.05$.

Authors' Contributions

S.H. performed the experiments, analyzed data and wrote the main draft of the manuscript., S.A. analyzed the histopathological experiments data., Z.M.F. assisted in in vivo experiments, M.G.S. and H.F assisted in data analysis.,

A.H. conceived the idea, designed experiments, provided reagents, analyzed data, edited and revised the manuscript and supervised the whole study.

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Competing Interests

The authors declare that they have no conflict of interest according to the work presented in this report.

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Intra-periaqueductal Grey Matter Injection of Orexin A Attenuates Nitroglycerin-induced Deficits in Learning and Memory in Male Rats

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ABSTRACT

This study explored the potential contribution of Orx1R within vlPAG to the learning and memory of animals with chronic migraine-like pain. Migraine was induced by repeated i.p. administration of nitroglycerin (5 mg/kg). Passive avoidance adeptness was evaluated in the shuttle box maze. The spatial memory performance was estimated using MWM tests. In the MWM task, NTG-injected rats revealed an imperative increase in escape latency and traveled the distance to catch the stage and a decrease in the time spent to pass into the goal zone in comparison to the control animals. Such NTG-evoked responses were attenuated by the post-treating intra-vlPAG injection of orexin A at 100 but not 25 and 50 pM. Furthermore, in the shuttle box test, NTG-treated rats showed eversion memory retrieval impairment as reflected by decreased phase through latency and longer time spent in the black chambers of the maze. Administration of orexin A at 50 and 100 pM could suppress NTG-related eversion memory deficiency in rats. However, orexin A (100 pM) aptitude to preserve memory performance, in both MWM and shuttle box tasks, was significantly prevented by SB334867 (20 nM) as an Orx1R antagonist. Overall, these data support the role of Orx1R within vlPAG to modulate migraine-related cognition deficits in rats.

Keywords

Migraine, Nitroglycerin, Orexin A, Learning and memory, Rats

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Abbreviations

PAG: periaqueductal grey matter
Orx1R: Orexin 1 receptors
vlPAG: ventrolateral periaqueductal grey matter
MWM: Morris water maze
CGRP: Calcitonin gene-related peptide
NTG: Nitroglycerin

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Introduction

Migraine is a pervasive brain complaint principally defined by unbearable pulsating pain in the head. In addition to headaches, people with migraine typically show some irregular expressions called aura as visual, sensory, or gastrointestinal problems [1, 2]. The management of migraine is multifaceted and migraine therapies annually impose a massive burden on the society and public health systems [3-6].

Migraine brains have structural and functional irregularities in the pain processing centers, including the trigeminocervical complex and PAG [7, 8]. It has been indicated that the functional association between PAG and some of the remaining nociceptive processing is altered in migraine patients [7]. In particular, axon terminals from PAG have been traced in trigeminal sensory nuclei through neurons in the nucleus raphe magnus [9]. In addition, the activation of PAG neurons modulates the spinal trigeminal neurons in a cat model of trigeminovascular pain [10]. During migraine attacks, the irregular activation of trigeminal afferent neurons innervating cerebral blood vessels could induce cerebral vasodilation by releasing vasoactive peptides, such as CGRP and substance P, which may have a role in pain sensitization [11-14].

It has been demonstrated that normal cognitive processing is disrupted in migraine. Although most studies display an elevated risk of cognitive dysfunction in migraine patients, there are some paradoxical data. For example, it has been indicated that migraine induces significant deficits in visual attention and verbal memory [15-17]. However, in the study by Jelicic et al., migraine did not alter cognitive performance in middle-aged or older adults [18]. Moreover, long-lasting migraine headache in middle-aged twins was not accompanied by cognitive dysfunction [19]. A familial hemiplegic migraine mutation inspired significant spatial memory deficiency in contextual fear-conditioning and MWM tests in mice. It could also increase hippocampal excitatory transmission and long-term potentiation [20].

The orexin peptides (A and B) are expressed by different neurons in the hypothalamus. These peptides stimulate distinct G protein-coupling receptors, Orx1R and Orx1R [21, 22]. Distributed cortically and subcortically, orexinergic neurons are involved in nociceptive transmission [23-25]. The manipulation of Orx1R has been shown to alter migraine headache intensity. Intravenous infusion of orexin A might modulate nociceptive neurons to trigeminal nucleus caudalis in rats the model of dural trigeminovascular nociception [26].

It is well documented that Orx1R plays a vital role in learning and memory processing. Activation of Orx1R in the hippocampus improved spatial learning and LTP in rats [27, 28]. In addition, trigeminal pain-associated learning and memory deficits in rats were suppressed by orexin A injection in the trigeminal nucleus caudalis or rostral ventromedial medulla of rats [29, 30]. Moreover, the blockade of orexins receptors in the basolateral amygdala could disturb the passive evasion memory of rats [31].

There are functional connections between PAG circuits mainly vlPAG with cortical and subcortical brain regions in either pain or learning and memory processing, including the thalamus, prefrontal cortex, insular agranular, and amygdala [32-34]. Therefore, the current study aimed to show if manipulating Orx1R in vlPAG could alter the learning and memory values of rats with migraine headaches.

Results

MWM

During acquisition blocks, the groups showed significant alterations in latency scores to find the concealed platform [$F(3, 483)=33.634$, $p = 0.001$]. Post hoc comparisons indicated that NTG administration could significantly increase latency time to catch the platform in the first, third, and fourth blocks of acquisition trials ($p < 0.05$) (Figure 1). Nevertheless, post-training administration of orexin A (100 pM) attenuated latency time in all blocks. In addition, orexin A at 50 pM could decrease escape latency to the platform in the third and fourth blocks compared to the NTG group ($p < 0.05$) (Figure 1). However, orexin A (100 pM) was inhibited by prior treatment infusion of SB334867 (20 nM) (Figure 1).

Moreover, our results indicated a significant differences between groups in the space traveled to find the concealed platform during the acquisition test [$F(3, 483)=28.626$, $p = 0.001$]. As shown in Figure 2, the distance traveled to find the stage significantly increased in NTG or NTG plus normal saline-treated groups in all acquisition trials. Orexin A administration at 100 pM but not 25 and 50 pM could repress NTG-increased moved distance to catch the stage ($p < 0.05$) (Figure 1). Such effects of orexin A (100 pM) were disallowed by SB334867 (20 nM) (Figure 2).

In the probe trial, the groups showed a significant difference in the time spent [$F(6, 41)=8.061$, p

Abbreviations-Cont'd

MAPK: Mitogen-activated protein kinase
ANOVA: One-way analysis of variance
NO: Nitric oxide
ERK: Extracellular signal-regulated kinase
CB1R: Cannabinoid 1 receptors

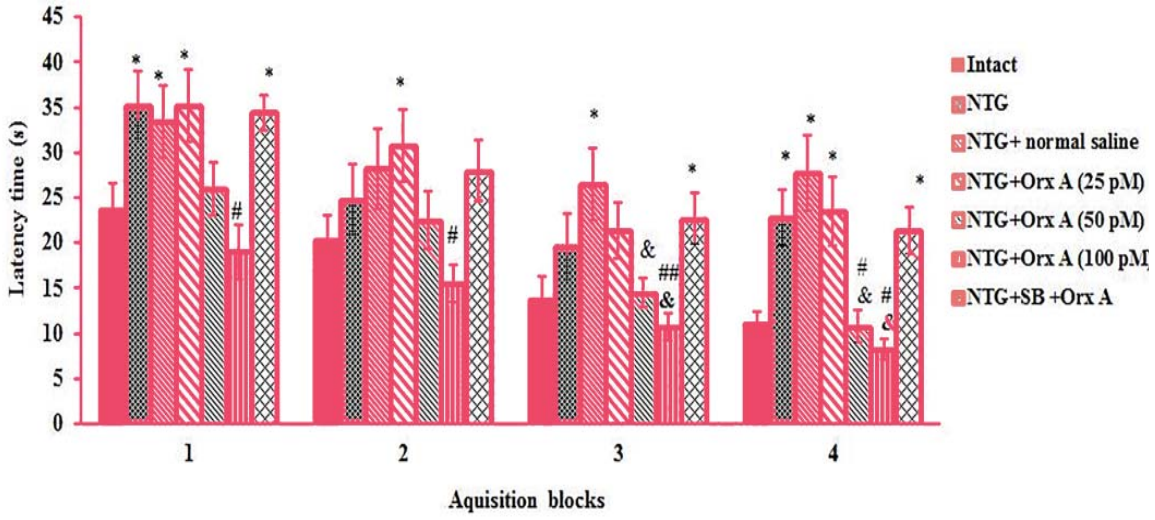

Figure 1.
Comparison rats escape latency during acquisition blocks to reach the hidden platform in MWM test between groups. $^{*}P < 0.05$ vs intact, $^{\#\#}P < 0.01$, $^{\#}P < 0.05$ vs NTG- treated group, $^{\&}P < 0.05$ vs NTG + normal saline group. NTG: nitroglycerin; SB: SB334867; Orx A: orexin A.

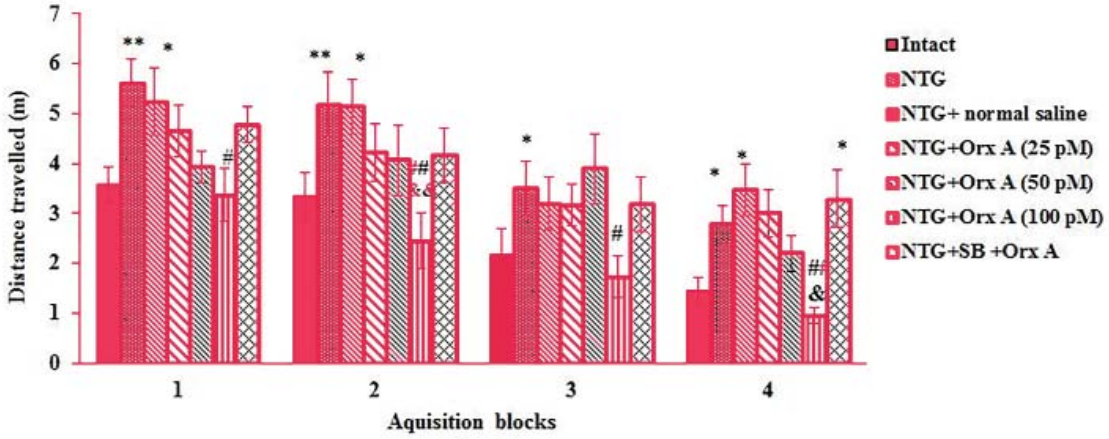

Figure 2.
Comparison distance travelled by rats to reach the hidden platform during acquisition blocks of MWM test between groups. $^{**}P < 0.01$, $^{*}P < 0.05$ vs intact, $^{\#\#}P < 0.01$, $^{\#}P < 0.05$ vs NTG- treated group, $^{\&\&}P < 0.01$, $^{\&}P < 0.05$ vs NTG + normal saline group. NTG: nitroglycerin; SB: SB334867; Orx A: orexin A.

= 0.001] and the number of visits [F (6, 41)=6.408, p = 0.001] across the target area. As shown in Figure 3, the total number of visits and time spent in the goal region significantly attenuated in NTG-treated rats. Orexin A administration at 100 pM but not 25 and 50 pM augmented the number of visits and the time spent in the goal zone compared to the NTG groups ($p < 0.01$ and $p < 0.05$). However, orexin A (100 pM) value to increase the number of visits and the time spent in the goal region was inhibited by pre-treatment administration of SB334867.

Shuttle box

Assessment of passive avoidance memory retrieval in the shuttle box test showed a significant difference between groups in phase through latency and time spent in a dark hall. Post hoc test showed decreased step through latency ($p < 0.05$) and increased time spent in the darkroom ($p < 0.01$) in rats infused by NTG or NTG+ vehicle in comparison to untreated control rats. The NTG-induced reduction in step-through latency ($p < 0.05$) and the rise in the time spent in the dark chamber were attenuated with orexin A (50 and 100 pM). However, orexin A (100 pM) amended effects on memory retrieval prevented by the co-injection of SB334867 (20 nM) (Figure 4).

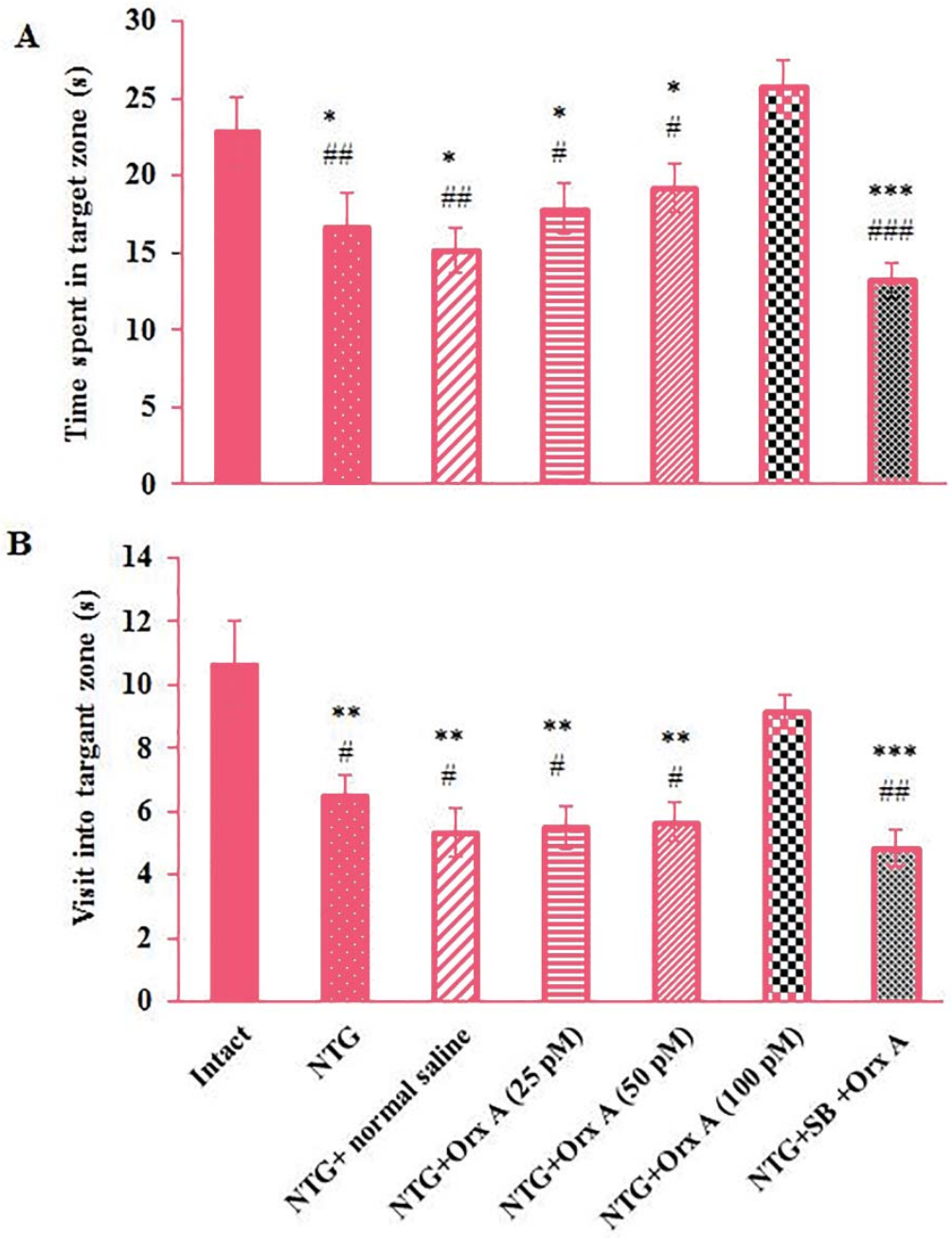

Figure 3.
Comparisons the time spent (A) and the numbers of visit (B) into goal zone among experimental groups. $^{***}P < 0.001$, $^{**}P < 0.01$, $^{*}P < 0.05$ vs intact, $^{\#\#\#}P < 0.001$, $^{\#\#}P < 0.01$, $^{\#}P < 0.05$ vs NTG+Orx A (100 pM) treated group. NTG: nitroglycerin; SB: SB334867; Orx A: orexin A.

Discussion

This study investigated the influence of the post-treatment infusion of orexin A in vlPAG on learning and memory competence in a rat model of NTG-evoked migraine. According to our results, orexin A dose-dependently improved learning and memory indices in rats exposed to migraine. The effects of orexin A were suppressed by the prior injection of Orx1R antagonist SB334867.

Migraine headaches have been shown to interfere with normal cognitive processing in the brain [20, 35]. In this study, the systemic administration of NTG induced severe memory impairments. Consistent with our findings, it has been indicated that NTG-reduced vasodilation disturbs passive avoidance memory performance in mice [36]. The NTG, as a donor of NO, plays a vital role in regulating physiological functions under normal conditions [37, 38]. However, irregularly increased NO levels in the hippocampus disrupted cognitive processing in hypothyroid rats [39]. Furthermore, diminished NO is associated with a reduction in age-associated cognitive deficits in NO

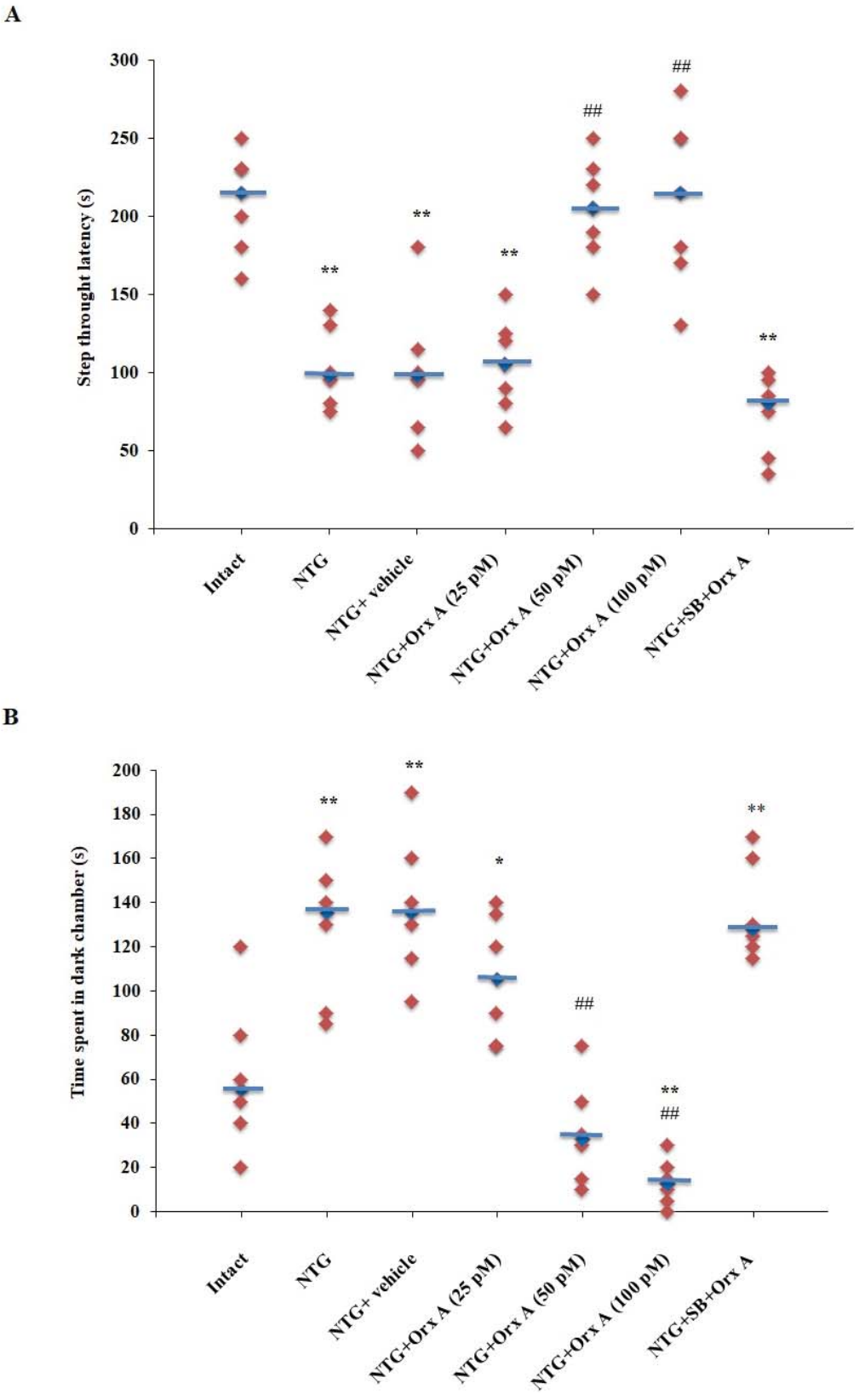

Figure 4.
Comparisons step through latency (A) and time spent in dark chamber (B) in shuttle box test among experimental groups. $^{***}P < 0.001$, $^{**}P < 0.01$, $^{*}P < 0.05$ vs intact, $^{\#\#}P < 0.01$, $^{\#}P < 0.05$ vs NTG+Orx A (100 pM) treated group. NTG: nitroglycerin; SB: SB334867; Orx A: orexin A.

synthase mutant mice [40]. These studies support the idea that NTG administration could induce learning and memory deficits by changing the synthesis or release of NO in the brain.

Here, for the first time, we demonstrated that the pharmacological stimulation of Orx1R in vlPAG decreased learning and memory disruption in migraine model rats. Previous studies have strongly specified that the orexinergic system within vlPAG is involved in the transmission of nociceptive signals, such as trigeminal nociception [41, 42]. Orexin A analgesic activity is accompanied by ameliorating cognitive deficiency in rats. Notably, Orx1R stimulation in the trigeminal nucleus caudalis and restroventral medulla could reduce learning and memory deficiency persuaded by dental inflammation in rats [29, 30]. In addition, it has been indicated that orofacial pain-induced memory disruption is associated with reductions in Orx1R concentration in the hippocampus of rats [43]. Consequently, this study shows that orexin A competence against NTG-induced learning and memory deficiency might be related to the modulation of migraine pain transmission in the vlPAG circuits [41].

Many signaling pathways are triggered by orexin A especially classical ERK/MAPK, and cAMP-PKA cascades in the brain [44]. Noticeably, central infusion of Orx1R agonist suppressed learning and memory deficits in pentylenetetrazol-kindled rats via activating Orx1R and ERK1/2 pathways [45]. In addition, the perifornical hypothalamus and central amygdala orexinergic neurons are involved in the modulation of conditioned fear memory through activating phospholipase C and sodium-calcium exchanger pathways in rats [46]. Moreover, orexin A declined cognitive lack and upregulated BDNF expression in a rat model of Parkinson's disease by inducing phosphatidylinositol 3-kinase and PKC signaling [47]. As a hypothesis, the influence of orexin A efficiency on learning and memory might be partially achieved by intracellular molecules driven by Orx1R activation. Nevertheless, further studies are needed to display the careful mechanism(s) behind such a phenomenon.

The PAG is a nonspecific place for controlling the learning and memory process. However, PAG circuits make direct or indirect influences on the brain areas complicated in-memory processing, including thalamic nuclei, hypothalamus, amygdala, and lateral prefrontal cortex [48, 49]. Functional connectivity has been reported between PAG and prefrontal cortex, CA1 area of the hippocampus as well as amygdala during the formation of contextual fear conditioning memory [50]. In addition, axonal projection from the prefrontal cortex to vlPAG is involved in contextual fear discrimination and generalization [51]. The current study showed that the activation of excitatory Orx1R signaling within vlPAG can manipulate vlPAG afferents to cortical areas involved in the control of learning and memory.

In the vlPAG, orexinergic neurons are found with the neuronal subpopulations that express a variety of neuro-regulatory molecules, such as glutamate, GABA, and cannabinoid [41, 52-55]. It has been indicated that association between Orx1R and CB1R in vlPAG may induce analgesic effects by modulating GABAergic and glutamatergic neurons [41]. In addition, the pharmacological blockage of either Orx1R or CB1R within vlPAG blocked the carbachol-induced antinociception in rats [56]. Furthermore, intranasal orexin A administration raised glutamate flow in the cortex [57]. Consequently, it is hypothesized that Orx1R crosstalk with other receptor systems within PAG could alter subjective behavior as previously reported for nociception in rats.

Conclusion

In conclusion, the results of this study showed that Orx1R in vlPAG is involved in the modulation of learning and memory deficiency induced by the NTG model of migraine headaches in rats. These findings support the potential efficiency of Orx1R medicine in controlling cognitive deficiency comorbid with migraine headaches.

Animals

Adult male Wistar rats were used. The rats were preserved on a 12 h light/dark typical cycle at a specific temperature of 22°C ± 2°C. The rats had ad libitum access to food and water. The experimental processes were certified by Shahid Bahonar University Animal Care and Ethics Committee (IR.UK.VETMED.REC. 1398.003).

Surgical procedure

The rats were anesthetized by ketamine and xylazine solvation (60 and 5 mg/kg/ i.p., respectively) and were sited on a stereotactic device. A hole was punctured in the skull and a stainless steel guide cannula was fixed in vlPAG at stereotaxic coordinates AP: 7.8, LM: 0.6, and DV: 5.9 mm [58]. All the rats were endorsed one week postoperative prior to the start of the tests. At the end of the process, the precise location of the cannula for each rat was certified histologically (Figure 5).

Medications and microinjection

Nitroglycerin was purchased from Caspian Tamin, Rasht, Iran. Orexin A and SB334867 (both Sigma, USA) were liquefied in saline and dimethylsulfoxide, respectively. The medications were infused into vlPAG by an injector needle (30 g) attached to a polyethylene pipe to a 1 µL Hamilton syringe. All medicines were dispersed over 1 min. The needle was left in the site for an additional 60 sec to block backflow and consent medicine distribution.

Experimental design

Chronic migraine pain was prompted by the i.p. administration of NTG (5 mg/kg) (five injections in total). Next, the animals were arbitrarily distributed into different experiment groups as follows:

intact (control), vehicle, orexin A (25, 50, 100 pM), and SB 334868 (20 nM)+orexin A (100 pM). Animals in the vehicle and orexin A groups were infused with the intra-vlPAG administration of normal saline or orexin A (25, 50, 100 pM). The SB 334868 (20 nM)+orexin A (100 pM) group received SB 336747(20 nM) followed by orexin A (100 pM).

Evaluation of learning and memory

MWM test

The MWM contained a dark spherical pool (136 cm in diameter and 60 cm in height). The additional indications were positioned in defined sites on the room test walls which were detectable to the animals. The pool contained water (22°C±1°C) to a depth of 25 cm alienated into four distinct zones by four main instructions. A spherical stage was situated in the middle of one of the zones (2 cm below the water outward). First, each animal was placed in the water from one of the directions. The animal movement was shadowed by a numeral camera fixed above the central area of the pool and was measured using the Ethovision software.

The experiment included learning and probe assessments. The learning test was accomplished on three successive days (four trials per day). The rats were indorsed to swim within the maze to hook the covered stage. When the stage was perceived, the animal had to remain on the stage for 30 sec. The time of escape latency and moved space by each animal were appraised. In the memory test, one day after the learning test, the covered stage was detached from the pool. Afterwards, animals were located in the quadrant as opposed to the goal quadrant and were indorsed to swim for 1 min. The visit to the goal zone and the time spent were noted.

Shuttle box test

The apparatus consisted of two distinctive halls (light and dark). There was a plexiglass door between two halls. The learning experiment comprised acclimatization and achievement trials. In the acclimatization examination, the rats were separately sited in the light hall and permitted to go in the black hall. In the acquisition experiment, each rat was positioned in the bright room, and the behead door was undone. When the rats entered the dim hall, the gate was barred and a persistent electric shock was exerted via the gridiron floor. In case the animal did not arrive in the dark area in 5 min, the success of learning was deliberated effectually. The total number of learning trials was calculated. After one day, in the maintenance trial, the rats were positioned in the light room and indorsed to onset into the dark box. Phase through latency and the time expended in the darkroom were noted for each rat. The maximum cut-off time was set up at 5 min.

Statistical analysis

The data of the MWM test (learning trials) were analyzed by repeated measures ANOVA. Furthermore, the results of the probe trial were assessed using one-way ANOVA. Differences between groups were analyzed by Tukey's post hoc test. In the shuttle box task, the data were analyzed with Kruskal–Wallis H test. In addition, multiple comparisons were performed utilizing the Mann-Whitney-U test. *P*-values less than 0.05 were considered imperative.

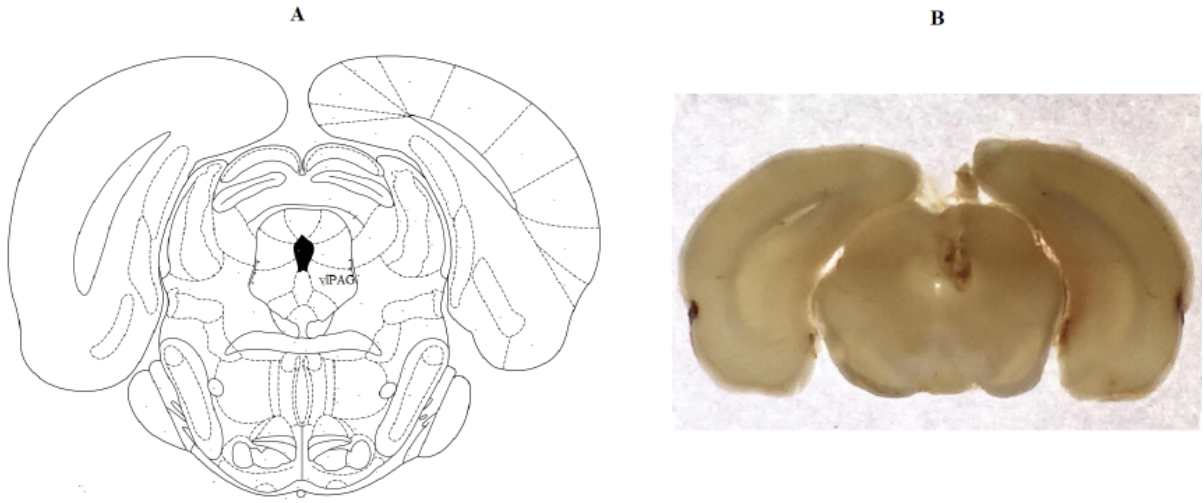

Figure 5.
A representative vlPAG section replicated from rat brain atlas of Paxinos and Watson (A) and a illustrative brain coronal section displaying the injection place (B).

Authors' Contributions

RK intended the experiments, performed the procedures, and analyzed the data. MA supervised the study and drafted the manuscript.

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Competing Interests

The authors declare that they have no conflict of interest according to the work presented in this report.

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Lawsonia inermis possesses a significant analgesic activity compared to *Waltheria indica, Moringa oleifera, Nigella sativa*, and diclofenac in female Wistar rats

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ABSTRACT

Pain is a severe symptom of many diseases, with an increasing percentage of people manifesting various types of pain. Medicinal plants provide analgesic potential with little toxicity. We performed this experiment to compare the analgesic activities of *Lawsonia inermis*, *Waltheria indica*, *Moringa oleifera*, and *Nigella sativa* in Wistar rats using writhing and paw lick responses. We grouped 21 adult female rats into seven groups (n=3), including uninduced and untreated rats (group 1), induced untreated rats (group 2), rats treated by Lawsonia inermis at 200 mg/kg (group 3), rats treated withWaltheria indica at 200 mg/kg (group 4), rats treated with *Nigella sativa* at 200 mg/kg (group 5), rats treated with *Moringa oleifera* at 200 mg/kg (group 6), and rats treated with diclofenac at 10 mg/kg (group 7). We dosed rats for 14 days after inducing the pain. Phytochemical screening showed that methanolic extracts of Lawsonia inermis, Moringa oleifera, and ethanolic extract of *Waltheria indica* contain: Alkaloid, *saponin,* steroid, tannin, flavonoid, phenols, terpene, and glycosides. The rate of weight gain in rats treated with M. oleifera and W. indica was 7%, and with diclofenac was 9% compared to the untreated control. L. inermis and N. sativa possessed a weight gain of 3% and 2%, respectively. All the extracts exhibited analgesic activities by significantly reducing the number of lick and writh in the order of *Lawsonia inermis, Nigella sativa, Moringa oleifera,* and *Waltheria indica.*This study concluded that *Lawsonia inermis* possess significant analgesic activities compared to other plants and the standard drug (diclofenac).

Keywords

Pain, Lawsonia inermis, Nigella sativa, Moringa oleifera, Waltheria indica, Analgesic

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Abbreviations

NSAIDs: Non-steroidal anti-inflammatory drugs
Cox: cyclooxygenase
GCs: Glucocorticoids;
L:Lawsonia
M: Moringa
W: Waltheria
N: Nigella

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Introduction

Scientists describe the pain as an unpleasant sensory experience connected to the potential impairment of tissues [1]. Reports have shown that most pains are usually related to inflammation [2], and inflammation has several mediators like prostaglandins, histamine, serotonin, leukotrienes, and bradykinin that are accountable for inflammatory responses (allergy and hypersensitivity), which results in abnormal production and release of these mediators at an abnormal rate [3]. Analgesic drugs can be produced from natural or synthetic sources. Natural analgesics are mainly derived from *Papaver somniferum* (opium poppy) which is an opioid analgesic (for example, morphine), *Vernonia amygdalina*, and *Zingiber officinale*. These plants can be used for treating acute and chronic pain and are tolerated in geriatrics patients with minimal side effects [4]. However, analgesics like acetaminophen and non-steroidal anti-inflammatories are derived from synthetic sources, predisposing them to toxicities [4, 5]

Reports have shown that medicinal plants have been used as the primary sources of drugs for managing animal and human diseases since ancient times [5]. The World Health Organization (WHO) pronounced that 80% of the world's population depends mainly on medicinal plants for health care delivery and needs, especially those in developing countries [5]. Most plants used for anti-inflammatory purposes also possess analgesic activity. Between 2000 and 2019, about 154 Nigerian medicinal plants have been studied and proven to exhibit analgesic and anti-inflammatory properties [6].

Incessantly untreated pain is one of the most major conditions that can affect the body structure, leading to physical injury and mental disorders [7]. These heart-breaking conditions are the primary cause of body deformities that can lead to death, except treated promptly [8]. Statistical reports have shown that 20% (1 in 5) of adults suffer from pain, and 10% (1 in 10; almost 60 million) of adults are diagnosed with chronic pain yearly [9].

The currently used analgesic drugs include opioids and steroidal and non-steroidal anti-inflammatory agents that can result in severe adverse reactions [10]. Side effects associated with current analgesics (NSAIDs) include gastric ulcers, platelet inhibition, cardiovascular disorder, and organ failure (liver, kidney) [10]. Steroidal anti-inflammatory drugs are related to immunosuppression, muscle weakness, blurry vision, increased weight, and appetite [10]. Side effects of opioids are nausea, vomiting, constipation, drowsiness, dependence, tolerance, and addiction [10]. There is a need for more experimental research to ensure that newer analgesic drugs that possess minimal or no adverse effects are produced from medicinal plants. This study was conducted to evaluate and compare the analgesic effects of four medicinal plants; *Lawsonia inermis* (henna), *Waltheria indica* (sleepy morning), *Moringa oleifera* (Moringa), *Nigella sativa* (black seed) with diclofenac in pain induced Wistar rats.

Results

Phytochemical analysis

The tables below showed the various phytoconstituents present in the methanolic leave extract of Lawsonia inermis Linn, (Table 1), Moringa oleifera (Table 2), and ethanolic root extract of Waltheria indica Table 3.

Table 1.
Phytochemical constituent of *Lawsonia* inermis Linn. leaves

Test	Crude methanol extract
Saponins	++
Tannins	++
Cardiac glycoside[s]	++
Flavonoids	++
Steroids	+
Alkaloids	+
Anthraquinones	+
Terpenoids	+

Interpretations -: Absent, +: Present, ++: Abundantly present

Table 2.
Phytochemical constituent of *Moringa oleifera* Linn. leaves

Test	Crude methanol extract
Tannins	+
Alkaloid	+
Saponin	+
Flavonoid	+
Glycosides	+
Phenol	+
Carbohydrate	+
Terpenoids	+

Interpretations -: Absent, +: Present, ++: Abundantly present

Table 3.
Phytochemical constituent of *W. indica* Linn. roots

Test	Crude ethanol extract
Alkaloids	++
Saponins	+
Tannins	+
Cardiac glycosides	++
Flavonoids	++
Steroids	+
Glycosides	+
Anthraquinones	+
Terpenes	+
oxalates	+
Trypsin	+

Interpretations -: Absent, +: Present, ++: Abundantly present

Weight gain

The changes in the weight of rats treated with Lawsonia inermis, Waltheria indica, Moringa oleifera, Nigella sativa, and diclofenac are listed in Table 4.

After seven days of treatment, the percentage of weight gain in all other treated rats W. indica showed a significant increase (6%.) compared to other treatment groups. The percentage of weight gain in all other treated rats was non-significant except for groups treated with N. sativa that showed considerably low weight gain (0.4%) when compared to all other treatment groups and the two controls. After 14 days of treatment, diclofenac-treated rats showed a significantly increased weight (9%) compared to all other treatment groups and the two control. W. indica and M oleifera treated groups showed moderate weight gain (7%) compared to L. inermis (4%) and N. sativa (1.8%), as shown in Table 4.4.

The results of the analgesic activities of the extracts of Lawsonia inermis, Waltheria indica, Moringa oleifera, and Nigella sativa

The analgesic effect of Lawsonia inermis, Waltheria indica, Moringa oleifera, and Nigella sativa extracts are present in Tables 5 and 6.

Formalin test

Table 5 shows the results of the analgesic effect of the extracts using the formalin-induced paw lick response. The lower the lick, the higher the analgesic potency. L. inermis has the lowest lick response (11.7 ± 5.13) compared to W. indica (37.3 ± 8.62), N. sativa (17.7 ± 6.51), M. oleifera (26.3 ± 3.51), diclofenac (29.0 ± 7.94) and the control (44.7 ± 5.69). All the extract treatment groups had considerable analgesic activities compared to diclofenac.

Percentage inhibition of induced pain

The percentage inhibition is indicated in Figure 1. L. inermis-treated rats had higher analgesic potency than other treatment groups and the control. Rats treated with N. sativa also showed a considerable analgesic effect compared to the control group. The M. oleifera dosed group showed similar analgesic potency with the group treated with the conventional drug (diclofenac) compared to the control.

Acetic acid test

Table 4.6 shows the results of the analgesic properties of the extracts using the acetic acid-induced writhing response. The lower the writh, the higher the analgesic effect. L. inermis showed the highest analgesic activities compared to other extracts and the standard drug

Percentage inhibition

The percentage inhibition is indicated in

Table 4.
Weight gain of rats treated with *Lawsonia inermis*, *Waltheria indica, Moringa oleifera, Nigella sativa* and *diclofenac*

Group	Day 0	Day 7	Day 14
Negative control	166.7 ± 20.11	172.7 ± 21.78(3.6%)	179.7 ± 21.94 (4%)
Positive control	136.3 ± 11.15	141.3 ± 10.12(3.67%)	147.7 ± 18.23 (5%)
L. inermis	138.3 ± 24.13	144.3 ± 25.58(4.34%)	149.7 ± 24.34 (4%)
W. indica	117.7 ± 2.076	124.7 ± 1.528(6%)	133.7 ± 2.518 (7%)
N. sativa	178.3 ± 3.507	179.0 ± 9.167(0.4%)	182.3 ± 12.58 (1.8%)
M. oleifera	197.0 ± 11.27	202.0 ± 11.14(2.54%)	216.0 ± 15.62 (7%)
Diclofenac	118.3 ± 1.528	122.7 ± 7.369(3.72%)	134.3 ± 13.80(9%)

Table 5.
Analgesic effect of the extracts and diclofenac on response to pain using the formalin test

Group	No of licks/10mins	Mean ± SD
Positive control	43	44.7 ± 5.69
	40	
	51	
L. inermis	13	11.7 ± 5.13***
	6	
	16	
W. indica	28	37.3 ± 8.62
	39	
	45	
N. sativa	18	17.7 ± 6.51**
	11	
	24	
M. oleifera	30	26.3 ± 3.51*
	26	
	23	
Diclofenac	26	29.0 ± 7.94*
	38	

Results are shown as Mean ± SD: n=5
*Significant *$p \leq 0.05$ **$p \leq 0.01$ ***$p \leq 0.001$

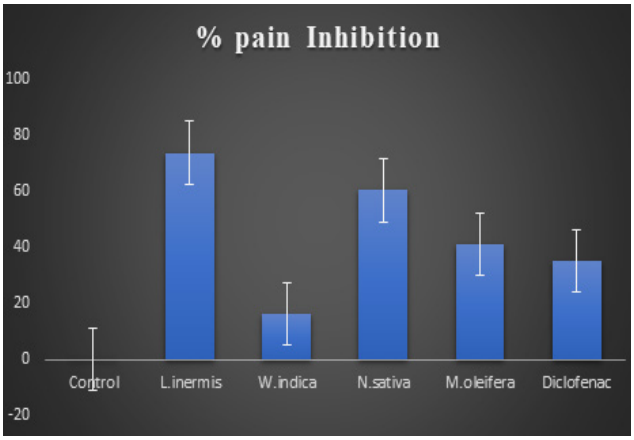

Figure 1.
Percentage inhibition of formalin induce paw lick pain treated with various extracts of *L. inermis, W. indica, N. sativa, M. oleifera,* and *diclofenac*

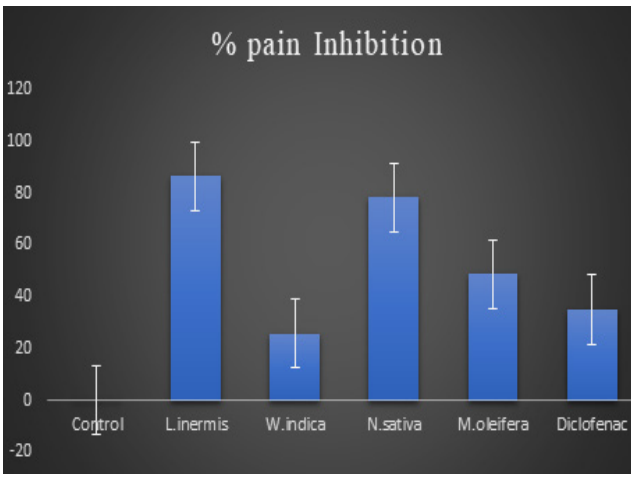

Figure 2.
Percentage inhibition of acetic acid writhing test (pain) treated with various extracts of *L. inermis, W. indica, N. sativa, M. oleifera,* and *diclofenac*

Figure 2. L. inermis group had significantly higher analgesic potency than other treatment groups and the control. Rats treated with N. sativa also showed a considerable analgesic effect compared to the control group. The M. oleifera dosed group showed similar analgesic potency with the group treated with the conventional drug (diclofenac) compared with the control.

Discussion

Bioactive phytoconstituents usually found in medicinal plants include alkaloids, tannins, flavonoids, antioxidants, carotenoids, and phenolic compounds. Phytochemical screening of the extracts used in this study revealed the presence of alkaloids, saponin, steroids, tannin, flavonoids, phenol, terpene, and glycosides. The result obtained from this study conforms with the previous work of Aremu et al. [12] Aremu et al. [16], and Basiru et al., [17] who reported similar phytoconstituents in methanolic extract of Lawsonia inermis Linn leaves, Moringa oleifera Linn. Leaves and ethanolic extract of Waltheria indica Linn. root, respectively.

Most medicinal plants contain alkaloids, saponins, steroids, tannins, flavonoids, phenols, terpenes, and glycosides [17]. These compounds have been reported for their analgesic and anti-inflammatory potential. Alkaloids specifically can be found in the papaver somniferum (poppy plant), a plant that produces opium which is a source of many narcotic analgesics [18], while steroids are essential anti-inflammatory agents [19]. Tannins (tannic acid) are polyphenols that are soluble in water and are found in several plants with marked antioxidant activities [20]. Flavonoids are secondary metabolites that possess multiple functions such as analgesic and antioxidant activities [21]. Phenols are abundantly present in plants and their metabolites possess many physiological functions. Terpenoids are the largest naturally occurring

plant compounds that form the primary component of their essential oil. Glycosides are organic substances made of sugars with alcohol and phenol with major effects on the heart either positively (cardiotonic) or negatively (toxicity) [22].

The result from this study showed that the percentage weight of rats treated with diclofenac had 9% weight gain when compared with W. indica (7%), M. oleifera (7%), N. sativa (1.4%), and the two controls. This result agrees with Allo et al. [23] who reported that moringa improves weight gain. This result also contradicts the outcome of Waterman et al., [24] who reported that isothiocyanate-rich M. oleifera extract reduces weight.

Paw lick and writhing tests are standard methods used to induce experimental pain by injecting irritant substances such as formalin and acetic acid in rats. Analgesic potentials of the test compound are predominantly evaluated through the decreased frequency of paw licking and stomach writhing, respectively [25]. The result of the acetic acid-induced writhing and the formalin paw lick tests obtained from this study disclosed that all the extracts exhibit significant analgesic potentials. This observation is due to the reduction in the number of writhes and paw lick responses as compared to the untreated control rats. L. inermis exhibited the highest analgesic effect, followed by N. sativa oil. This result agrees with Ghannadi et al. [25]) who reported that ethanolic extract of L. inermis Linn. leaf and black seed oil possess a dose-dependent analgesic, anti-inflammatory, and antipyretic effect, respectively. M. oleifera, on the other hand, showed moderate analgesia when compared to both L. inermis and N. sativa. This result conforms with the report of Bhattacharya et al., [26] and Adedapo et al., [15] who ascertained that various extracts of M. oleifera exhibit dose-dependent analgesia when compared to indomethacin. W. indica had the least analgesic effect compared to all other extracts. Nirmala and Sridevi, [27] and Termer et al. [28] attributed the analgesic potency of W. indica Linn. roots to the presence of tiliroside, epicatechin, and quercetin which are flavonoid derivatives as seen from the phytoconstituents reported above.

It was noted that M. oleifera and W. indica showed increased weight and minimal analgesic activities, while L. inermis and N. sativa showed marked analgesic effects with minimal weight changes. Reports have shown that many drugs cause weight gain, which can lead to overweight and obesity [29]. Most conventional analgesic drugs usually enhance body fat redistribution by increasing central adiposity, resulting in resistance to insulin, dyslipidemia, metabolic syndrome, and risk for the non-insulin-dependent type of diabetes (type II) [30]. Thus, from the result above, L. inermis and N. sativa will not cause abnormal weight changes even when used for the long term.

Conclusions

All the evaluated extracts contained phytochemicals with known antioxidant properties. All the extracts exhibited analgesic activities by significantly reducing the number of lick and writh in the order of Lawsonia inermis, Nigella sativa, Moringa oleifera, and Waltheria indica. Lawsonia inermis possesses significant analgesic activities compared to other plants and the standard drug (diclofenac). Lawsonia inermis is effective in the treatment of pain, and its effect on various types of pain could be investigated further by researchers.

Materials & Methods

Drug

Diclofenac sodium was acquired from a reputable pharmaceutical company in Ilorin, Kwara State, Nigeria.

Plant collection, preparation, and authentication

The leaves of Lawsonia inermis, Moringa oleifera, and stem bark of Waltheria indica were harvested from different areas in Kwara State The plants were authenticated at the Herbarium, Department of Botanical Sciences, University of Ilorin, Ilorin with voucher specimen no.134289 which was deposited for reference.

Nigella sativa oil (Hemani 125 mL) was obtained from a reputable store in Ilorin, Kwara State, Nigeria.

Extraction of plants material

The leaf samples of Lawsonia inermis, Moringa oleifera, and stem bark of Waltheria indica were washed, dried, and macerated into powdery form. 500 g of different leaves (L. inermis and M. oleifera) were soaked in 99% methanol (ratio 1:3, w/v), while Waltheria indica was soaked into 98% ethanol at room temperature, for 48 hours respectively. The procedures were repeated twice for the second and third extraction processes. The filtrates were sieved through filter paper (Whatman size no.1) and vaporized using a rotary evaporator. The dry residues were weighed and preserved in a refrigerator at 4oC until used.

Ethical approval

Ethical approval was obtained from the Ethical Committee on Animal Use and Care, Faculty of Veterinary Medicine, University of Ilorin, Nigeria with the approval code number UREC/FVM/15/32TA037.

Phytochemical analysis

Phytochemical screening of the leave samples of Lawsonia inermis, Moringa oleifera, and stem bark of Waltheria indica was conducted to identify the presence of various phytoconstituents following standard analytical procedures [11].

Experimental animals

Adult female rats (120 g) were obtained from the laboratory animal unit, Department of Biochemistry, University of Ilorin. They were housed in hygienic cages in a fly-proof animal house adhering to the internationally accepted standards for laboratory animal use and care laid down by the Canadian Council on Animal Care, (CCAC). The rats were fed with standard pelletized feed (Chikun feeds) and pro-

vided with clean water ad libitum. The rats were allowed to acclimatize for two weeks before the commencement of the experiment. This is in accordance with the guidelines for laboratory animal care and use (Institute for laboratory animal research).

Experimental design

A total of 21 female Wistar rats were divided into seven groups, each consisting of three rats, and the extracts were administered through the oral route using a cannula. Each group was treated as:
Group 1: (Negative control) was untreated and was uninduced.
Group 2: (Positive control) induced and untreated.
Group 3: Treated with Lawsonia inermis extract at 200 mg/kg daily for 14 days orally
Group 4: Waltheria indica extract was administered orally at 200 mg/kg daily for 14 days
Group 5: Nigella sativa oil was administered orally at 100 mg/kg daily for 14 days
Group 6: Moringa oleifera extract was administered at 200 mg/kg daily for 14 days.
Group 7: treated with diclofenac 10 mg/kg daily for 14 days orally.

Weight measurement

The weight was assessed using an automated electronic scale (Sensor Disc Technology, London) from day one and later on a weekly basis. In doing this, a round plastic bowl was positioned on the scale and tared to zero after which a rat was placed inside the bowl and consequently weighed as described by [12].

Assessment of analgesic activities

Two models were used to induce pain in this experiment (the paw-licking test and the acetic-acid-induced writhing test).

Formalin paw lick test

The formalin test was performed as described by (Hunskaar and Hole, 1987). 20 µL of 2.5% formalin were injected into the sub-plantar space of the right hind paw of the mice and immediately kept in a cage where they can be easily monitored. The frequency of lick and licking time of the injected paw was documented for 10 mins [13]. The percentage inhibition of the paw lick response was estimated using the formula;

$$\% \text{ Inhibition} = D0 - Dt / D0 \times 100$$

D0 is the average paw lick response of the control group
Dt is the average paw lick response of the drug-treated groups

Acetic acid-induced writhing test

The writhing test was carried out as first demonstrated by Sigmund et al. The rats were treated with various extracts an hour before the injection of 0.6% acetic acid at 10 mL/kg intraperitoneally. Writhing activity characterized by hindlimb extension, abdominal muscle contraction, and arching of the back was observed in the animals [14]. The number of writhes i.e. abdominal constrictions were recorded for 5mins following the injection of acetic acid [15]. The percentage inhibition of the writhing response was estimated using the formula:

$$\% \text{ Inhibition} = D0 - Dt / D0 \times 100$$

Do is the average writhing response of the control group
Dt is the average writhing response of the drug-treated groups

Data analysis

The values were expressed as mean ± standard deviation (Mean ± SD). The differences within the groups were compared with the Dunnett post hoc method of ANOVA with GraphPad Prism statistical package, San Diego, California, U.S.A (www.Graphpad.Com).

Authors' Contributions

Aremu A and Idris J. F planned the experiments. Akorede G. J, Aremu A and Idris G. J carried out the experimental dosing. Olatunji A. O and Basiru A carried out analgesic test. Aremu A. Idris J. F and Ahmed O. A. contributed to the interpretation of the results. Idris J. F took the lead in writing the manuscript. All authors provided critical feedback and helped shape the research, analysis and manuscript.

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Competing Interests

The authors declare that there is no conflict of interest.

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RESEARCH ARTICLE

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Protective Effect of Celery (*Apium graveolens L.*) Essential Oil on the Experimental Model of Cuprizone-induced Multiple Sclerosis in Male C57BL/6 Mice

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ABSTRACT

Considering the beneficial effects of *Apium graveolens L.* (Celery) on the nervous system, this study elucidates the protective effect of CEO on the experimental model of cuprizone-induced MS in male C57BL/6 mice. Frothy mice were allocated into four experimental groups: control, cuprizone (chew pellet containing 0.2 %CPZ), CEO (800 mg/kg), and CPZ+CEO. Animals received treatments based on their groups for 5 weeks. Finally, reflexive motor behavior and serum antioxidant levels were determined. Based on the findings, ambulation score, hind-limb suspension, front limb suspension, and grip strength significantly decreed in the mice treated with CPZ ($p < 0.05$). Hind limb foot angle, surface rights, and negative geotaxis significantly increased in the animals treated with CPZ ($p < 0.05$). Co-administration of CPZ+CEO significantly reduced the adverse effects of CPZ on ambulation score, surface righting, hind limb suspension, grip strength, and negative geotaxis ($p < 0.05$). Co-administration of CPZ+CEO significantly diminished the adverse effects of CPZ on the number of crosses in the open field test and duration on the rotarod ($p < 0.05$). Serum MDA activity increased while GPx, SOD, and TAS decreased in the mice treated with CPZ ($p < 0.05$). Co-administration of CPZ+CEO significantly reduced the adverse effects of CPZ on serum antioxidant levels ($p < 0.05$). These results suggested the protective effect of CEO against CPZ-induced MS mediated by its antioxidant activity.

Keywords

Celery, Essential oil, Cuprizone, Multiple sclerosis, Mice

Number of Figures: 13
Number of Tables: 0
Number of References:: 34
Number of Pages: 11

Abbreviations

ANOVA: Analysis of variance
CEO: Celery essential oil
CPZ: Cuprizone
GPx: Glutathione peroxidase
MDA: Malondialdehyde
MS: Multiple sclerosis

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Introduction

MS is a complex neurodegenerative disease caused by the demyelination of neurons in the CNS [1]. This condition might emerge due to genetic risk factors and oxidative stress, representing fatigue, muscle weakness, ataxia, cognitive impairment, and depression [2]. As oxidative stress plays a primary role in the development of MS, an imbalance between antioxidant capacity and the production of ROS is responsible for the pathophysiology of MS [3]. Chemical and natural medications are widely used to decrease oxidative stress and cognitive deficits in MS patients. Even though these medications prevent immune cell-driven inflammation and reduce the relapse rate, they are ineffective at controlling the predominant neurodegeneration that happens later in the disease course processes [4].

Apium graveolens L. is a green-branched leaf stalk from the family *Apiaceae*. This plant is rich in phenolic compounds, flavonoids, L-3-n-butylphthalide, limonene, selinene, volatile oil, sedanolide, and linoleic acid [5]. It has several medical properties, mainly inflammatory, antimicrobial, antioxidant, and antiulcerogenic [6]. The beneficial effect of L-3-n-butylphthalide was demonstrated to improve cognitive impairment in Alzheimer's mouse models [7]. Battery test is routinely used to determine neurodevelopmental or neurodegenerative disorders. This method includes limb grasping and placing, cliff avoidance, righting, accelerated righting, gait, auditory startle, and posture [8]. Celery (300 and 600 mg/kg) ameliorates neurobehavioral and neurochemical disorders in perinatal lipopolysaccharides exposure in mice offspring [5].

Recent reports have been growing on the beneficial activity of *A. graveolens* on the nervous system. For instance, it has been reported that celery extract improved cognitive impairment in Alzheimer's [9] and Parkinson-like symptoms in an experimental mouse model [10]. It has been indicated that the oral administration of celery extract (125, 250, and 500 mg/kg) enhanced anxiety-like behavior using a battery of behavioral tests. In addition, celery extract decreased MDA production while increasing GPx levels in the cortex and striatum of the mice [11]. CPZ models were beneficial for the pathophysiology of MS [12]. Due to the lack of a straightforward way to treat this disorder, we investigated the protective effect of CEO on the experimental model of CPZ-induced MS in male C57BL/6 mice.

Results

Based on Figure 1, CPZ significantly decreased ambulation scores compared to the control mice (p = 0.043). Supplementation of CEO significantly amplified ambulation score (p = 0.022). Co-administration of CPZ+CEO significantly reduced the adverse effects of CPZ on ambulation scores compared to the CPZ group (p = 0.034). In this study, the hind limb foot angle significantly enlarged following CPZ treatment compared to the control group (p = 0.021). on the other hand, CEO significantly reduced hind limb foot angle rather than control mice (p = 0.044). Pretreatment with CPZ+CEO significantly minimized the influence of CPZ on the hind limb foot angle compared to the CPZ group (p = 0.036) (Figure 2).

It was observed that hind limb suspension significantly reduced in the CPZ-treated mice (p = 0.043). Hind limb suspension was not influenced by CEO compared to the control animals (p = 0.643). The combination of CPZ+CEO significantly decreased the adverse impact of CPZ (p=0.021) (Figure 3). According to Figure 4, surface righting was significantly raised by CPZ (p = 0.046), while CEO treatment significantly decreased surface righting compared to the control group (p=0.012). Pretreatment with CPZ+CEO significantly opposed the effect of CPZ (p = 0.023).

In the current study, grip strength significantly decreased in mice that received CPZ (p = 0.013). CEO treatment significantly increased grip strength compared to the control mice (p = 0.043). Grip strength significantly improved in the CPZ+CEO group compared to the CPZ group (p = 0.05) (Figure 5).

Based on our findings, CPZ significantly diminished front limb suspension (p = 0.035). The CEO supplementation significantly improved front limb suspension compared to the control animals (p = 0.032). The effect of CPZ on front limb suspension was not suppressed in the group pretreated with CEO (p = 0.67) (Figure 6). As shown in Figure 7, the negative geotaxis significantly rose in the CPZ-treated mice (p = 0.023). Supplementation with CEO significantly decreased negative geotaxis in comparison with the control mice (p = 0.041). Co-administration of CPZ+CEO improved negative geotaxis compared to CPZ only (p = 0.14).

As presented in Figure 8, the number of crosses in the OFT significantly decreased in CPZ group following CPZ administration (p = 0.015). Supplementation with CEO increased the number of crosses in OFT compared to the control animals (p = 0.043). Co-administration of CPZ+CEO decreased the adverse effects of CPZ on OFT compared to the CPZ group (p

Abbreviations-Cont'd

OFT: Open Field Test
ROS: Reactive oxygen species
SOD: Superoxide dismutase
TAS: Total antioxidant status

= 0.024). As mentioned in Figure 9, the duration of stay on the rotarod decreased following CPZ administration (p = 0.021). Supplementation with CEO did not affect rotarod time in comparison with the control group (p = 0.23). Pretreatment with CEO diminished the impact of CPZ on rotarod time (p = 0.042).

According to Figure 10, CPZ administration significantly elevated serum MDA compared to the control mice (p = 0.031). Supplementation with CEO significantly decreased serum MDA (p = 0.027). Co-administration of CPZ+CEO significantly reduced CPZ-induced elevation in the MDA production rather than CPZ (p = 0.034). In this research, SOD activity decreased following CPZ administration in comparison with the control mice (p = 0.023), while SOD activity was enhanced by CEO supplementation (p = 0.014). Co-administration of CPZ+CEO improved serum SOD compared to the CPZ-only group (p = 0.043) (Figure 11).

Regarding the adverse effects of CPZ, the serum GPx activity significantly decreased (p = 0.033), whereas enhanced in CEO-treated mice (p = 0.014). Co-administration of CPZ+CEO reduced the adverse effects of CPZ on serum GPx in comparison with the CPZ-treated mice (p = 0.047) (Figure 12). Finally, serum TAS significantly declined in the CPZ group (p = 0.015) and supplementation with CEO increased its levels (p = 0.023). Co-administration of CPZ+CEO decreased CPZ-induced elevation in TAS compared to the CPZ-only group (p = 0.041) (Figure 13).

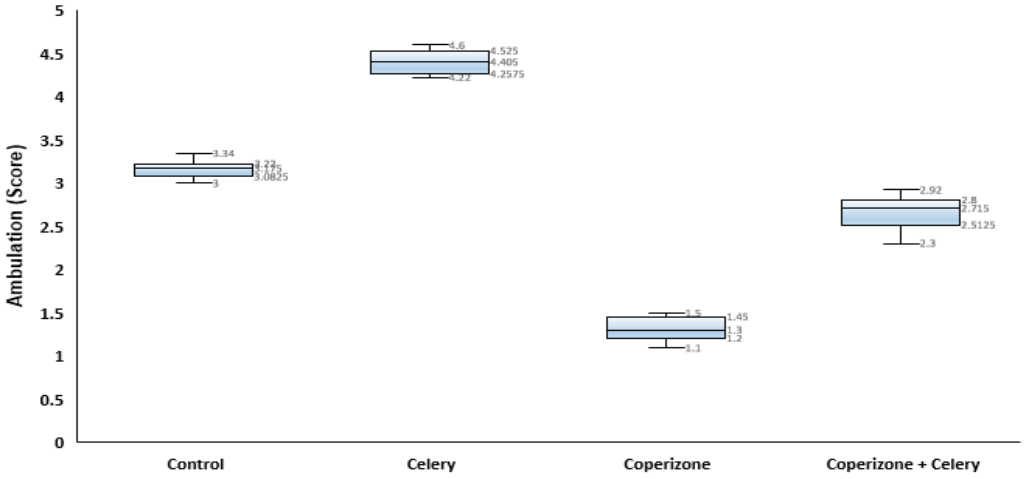

Figure 1.
Effects of cuprizone, celery extract and their combination on ambulation score in Cuprizone-induced model of multiple sclerosis mice (n = 10) (p < 0.05).

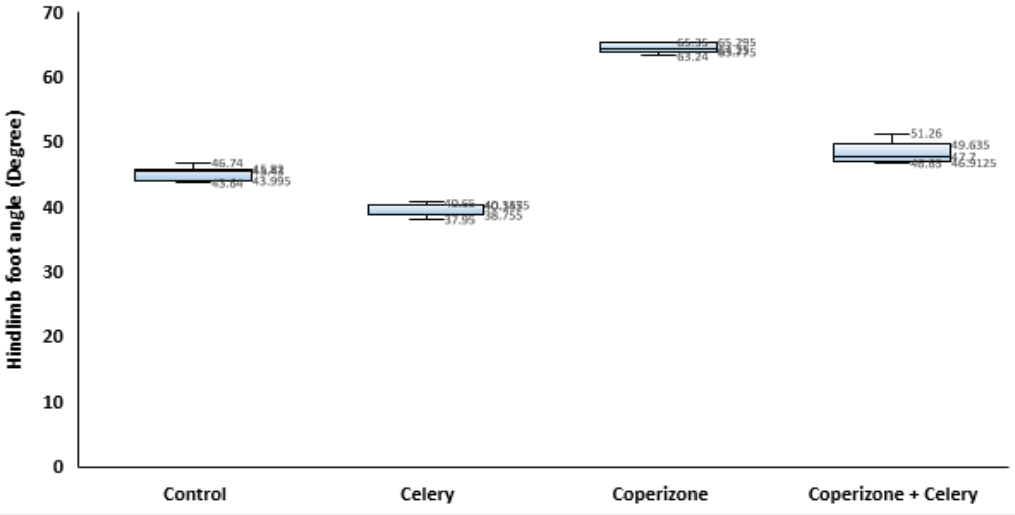

Figure 2.
Effects of cuprizone, celery extract and their combination on hindlimb foot angle in Cuprizone-induced model of multiple sclerosis mice (n = 10) (p < 0.05).

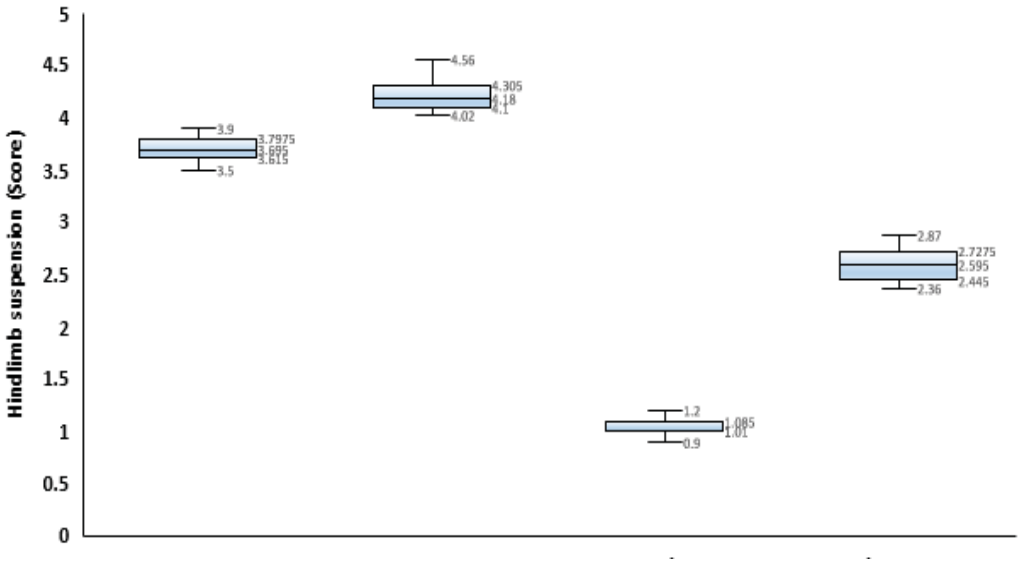

Figure 3.
Effects of cuprizone, celery extract and their combination on hindlimb suspension in Cuprizone-induced model of multiple sclerosis mice (n = 10) ($p < 0.05$).

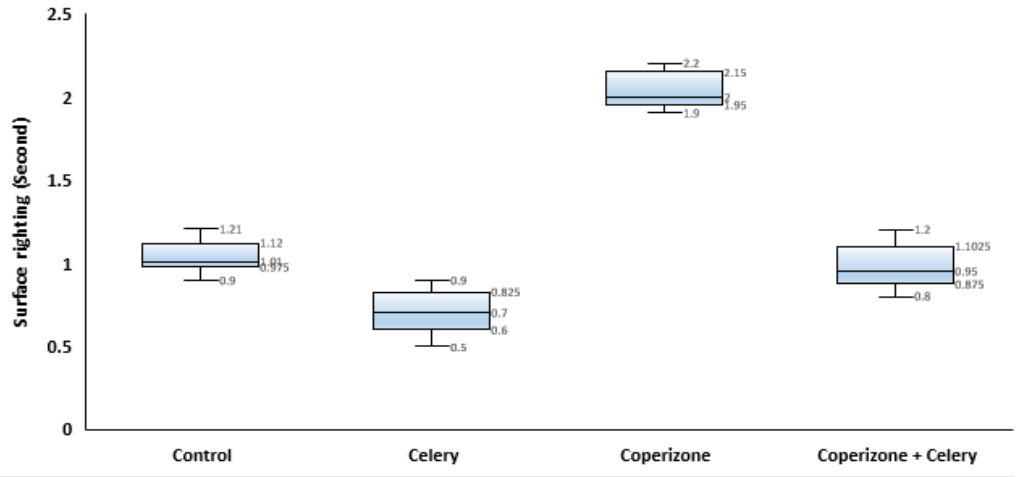

Figure 4.
Effects of cuprizone, celery extract and their combination on hindlimb foot angle in Cuprizone-induced model of multiple sclerosis mice (n = 10) ($p < 0.05$).

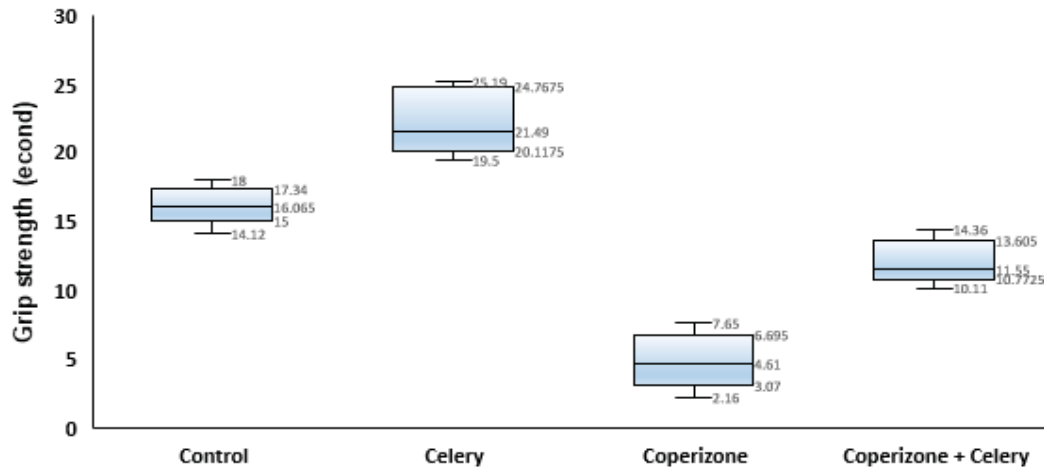

Figure 5.
Effects of cuprizone, celery extract and their combination on grip strength in Cuprizone-induced model of multiple sclerosis mice (n = 10) ($p < 0.05$).

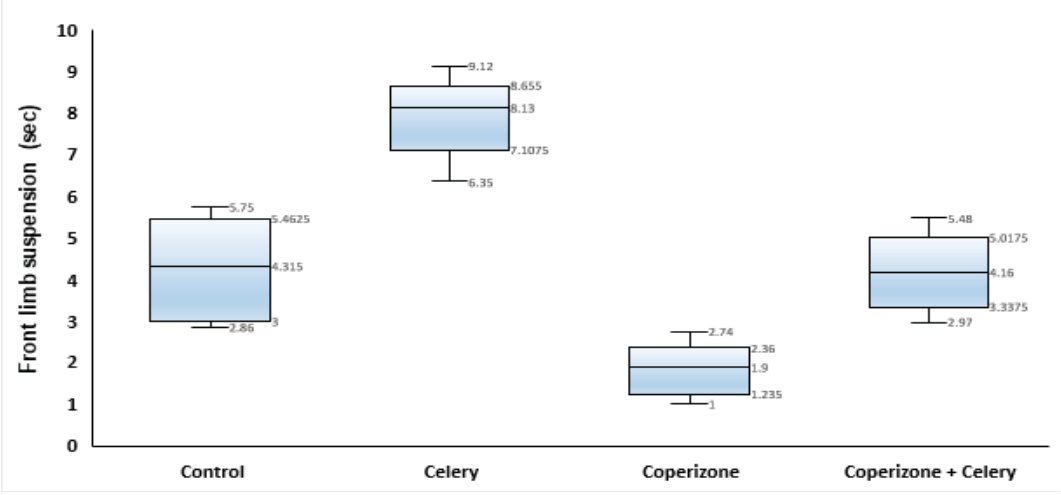

Figure 6.
Effects of cuprizone, celery extract and their combination on front limb suspension in Cuprizone-induced model of multiple sclerosis mice (n = 10) ($p < 0.05$).

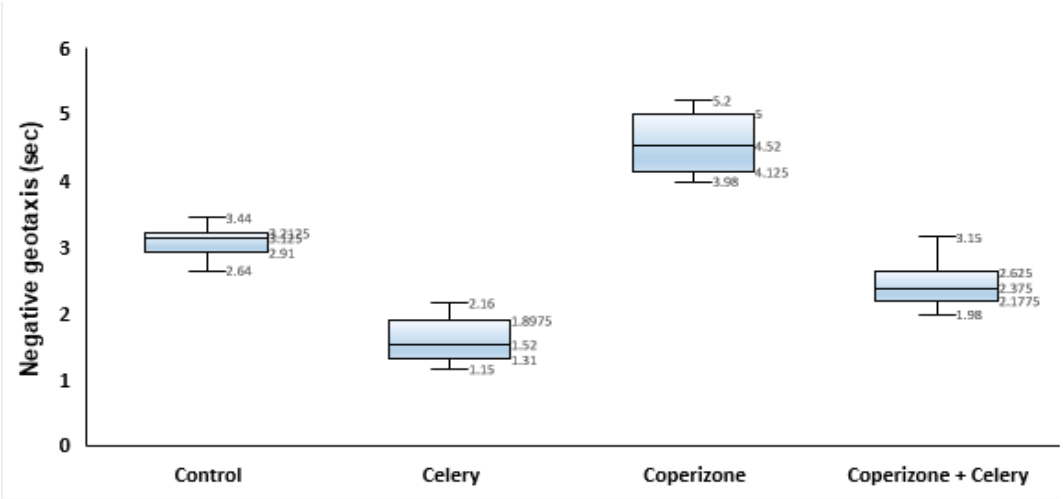

Figure 7.
Effects of cuprizone, celery extract and their combination on negative geotaxis in Cuprizone-induced model of multiple sclerosis mice (n = 10) ($p < 0.05$).

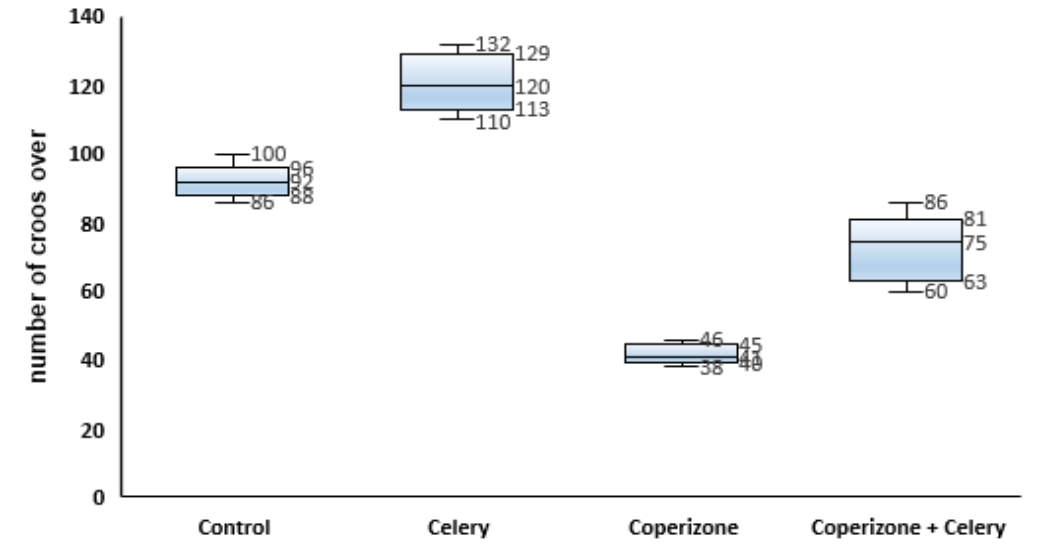

Figure 8.
Effects of cuprizone, celery extract and their combination on number of cross on open field test (OFT) in Cuprizone-induced model of multiple sclerosis mice (n = 10) ($p < 0.05$).

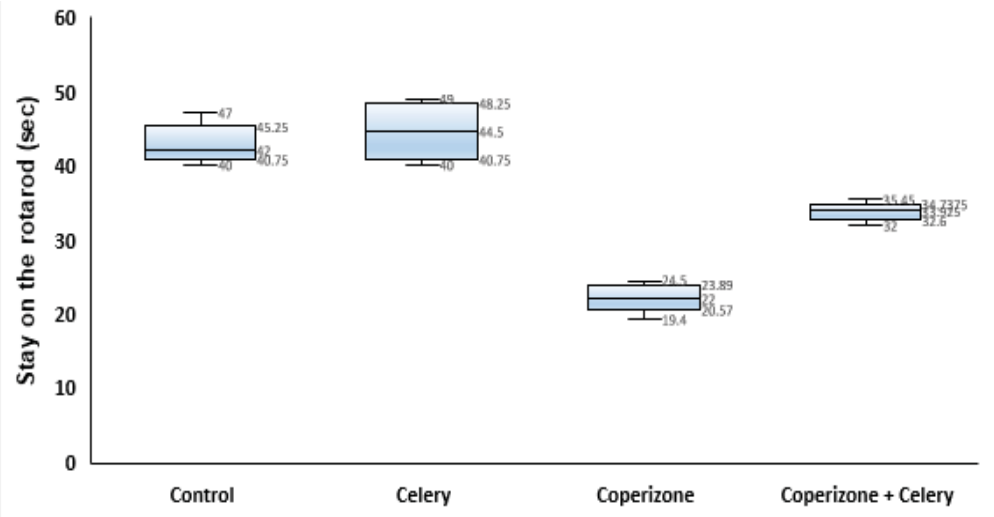

Figure 9.
Effects of cuprizone, celery extract and their combination on stay on the rotarod in Cuprizone-induced model of multiple sclerosis mice (n = 10) ($p < 0.05$)

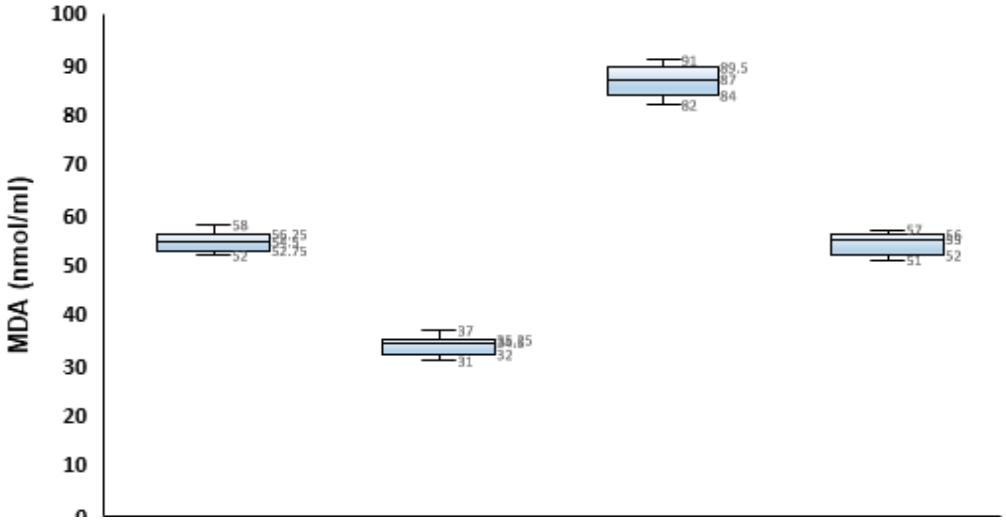

Figure 10.
Effects of cuprizone, celery extract and their combination on serum Malondialdehyde in Cuprizone-induced model of multiple sclerosis mice (n = 10) ($p < 0.05$)

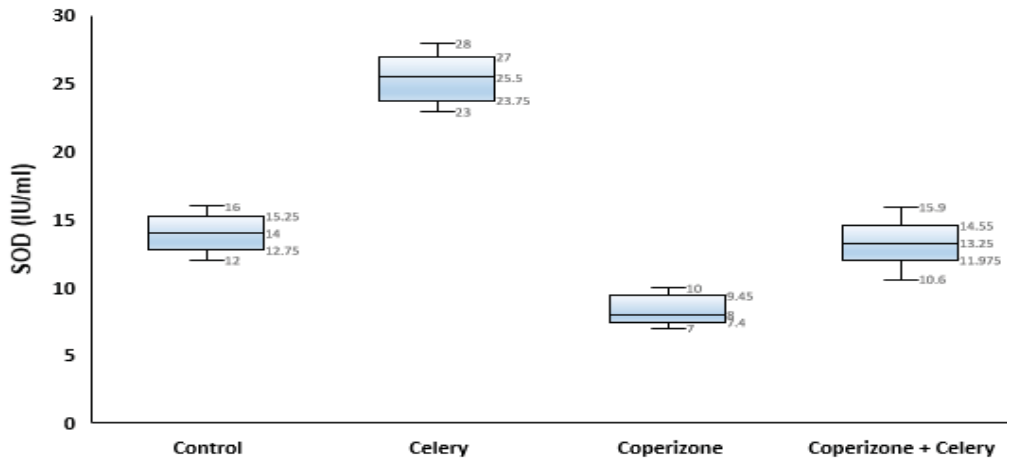

Figure 11.
Effects of cuprizone, celery extract and their combination on serum Superoxide dismutase in Cuprizone-induced model of multiple sclerosis mice (n = 10) ($p < 0.05$)

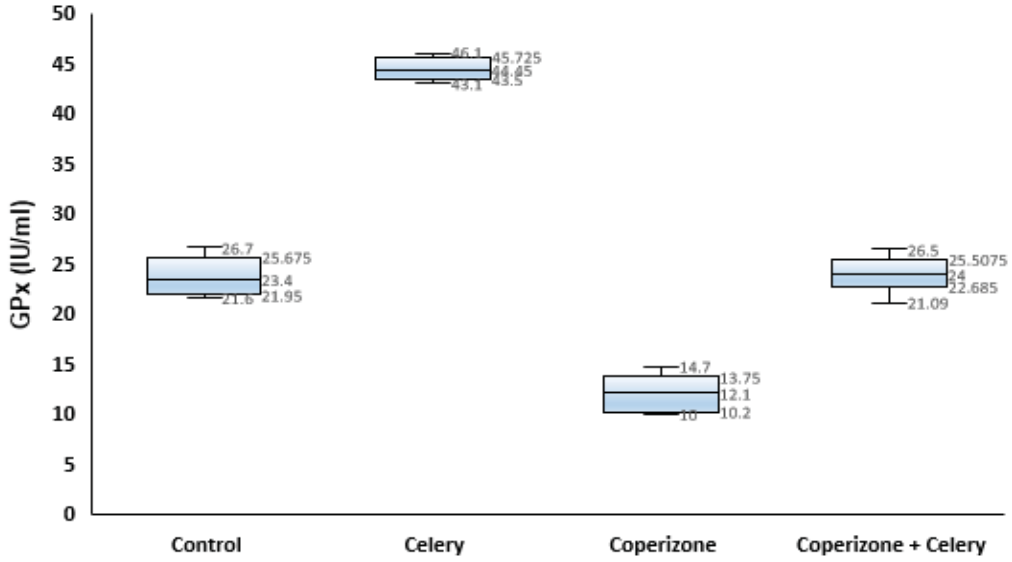

Figure 12.
Effects of cuprizone, celery extract and their combination on serum Glutathione peroxidase in Cuprizone-induced model of multiple sclerosis mice (n = 10) ($p < 0.05$).

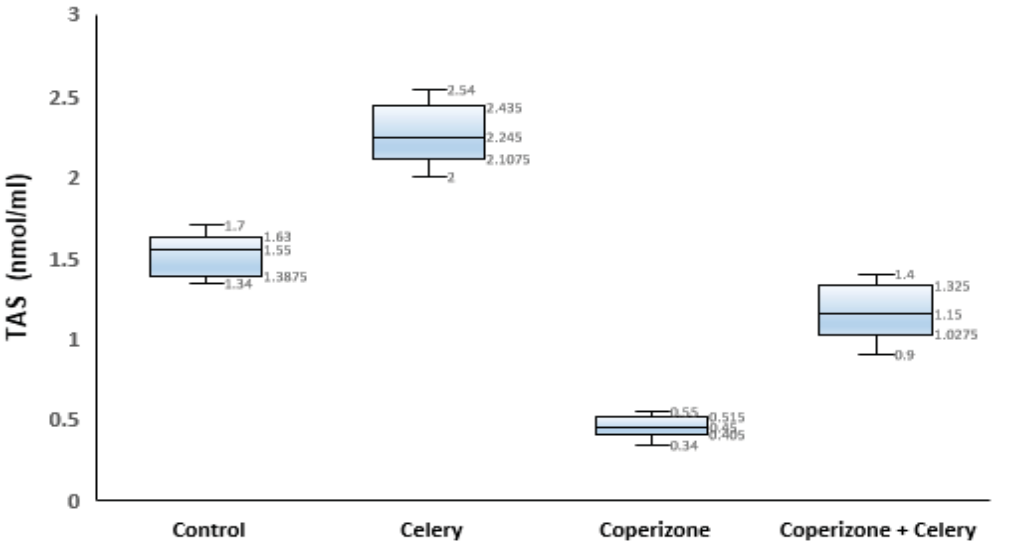

Figure 13.
Effects of cuprizone, celery extract and their combination on total antioxidant status in Cuprizone-induced model of multiple sclerosis mice (n = 10) ($p < 0.05$).

Discussion

Despite several types of research on the effectiveness of celery on brain and nervous system-related disorders, this study was performed for the first time on the effect of celery on CPZ-induced MS in mice. Based on the main findings, CPZ significantly decreased reflexive motor behavior in mice. Several methods were introduced for a model of demyelination in which a CPZ-containing diet for 4-6 weeks is preferred by many researchers [12]. This leads to oligodendrocytes damage, followed by microglia and astroglia activation, which disrupts energy metabolism in the mitochondria. Therefore, C57BL/6 mice are suggested [13], and here, we used a CPZ-containing diet for 5 weeks using C57BL/6 mice. According to our results, CPZ significantly decreased the crosses and rotarod time, and the administration of CPZ with CEO reduced the adverse effects of CPZ. In this regard, Xu et al. [14] reported that the mice exposed to 0.2% CPZ for 4-6 weeks had impaired sensorimotor gating and less social interaction, resulting in staying in the open arms of the maze and more memory impairment. Demyelination mainly occurs in white matter. However, there is evidence of grey matter [15].

As observed, CEO had a governing role in reflexive motor behavior in mice, and even the co-administration of CPZ with CEO significantly reduced the adverse impact of CPZ on reflexive motor behavior. In a similar report, different levels of A. graveolens extract (125-500 mg/kg) amplified activity and decreased anxiety-related behaviors (peak effect at 125 mg/kg) [11]. It has been well documented that antioxidants play a protective role against CPZ-induced demyelination [16]. Natural phenols have antioxidant properties in brain neurodegenerative diseases, such as MS [17]. It has been reported that A. graveolens methanolic extract (125-500 mg/kg) enhanced novel exploration and memories [18]. It seems that l-3-n-butylphthalide is responsible for augmented long-term spatial memory and reduced β-amyloid deposition in transgenic Alzheimer's disease mice [7]. A. graveolens methanolic extract (125 and 250 mg/kg) raises the number of living neurons in the cortex and hippocampus brain areas [18]. However, we could not conduct a histopathological investigation in this study due to limitations. It has been shown that L-3-n-butylphthalide, as the main bioactive component of A. graveolens, increased the transcription of neuroprotective factors, brain-derived neurotrophic factor, and klotho in mice with chronic epilepsy (19). The positive effects of celery extract on MS might be related to its main bioactive components, and we were not able to determine the effect of celery on these trophic factors because of some limitations.

Here, CPZ significantly elevated serum MDA while reducing GPx, SOD, and TAS levels. CEO decreased serum MDA while enhancing serum SOD, GPx, and TAS levels in the CPZ-treated mice. Based on the evidence, there is a close interrelation between the pathophysiology of MS and oxidative stress in humans and animals [4]. Because of high oxygen consumption and ROS production, the brain tissue is highly susceptible to oxidative damage due to polyunsaturated fatty acids constituting the neuronal membranes. Thus, the overproduction of MDA and reduced intracellular antioxidative protection (i.e., catalase, GPx, and SOD) leads to dysfunction and, ultimately, neuronal cell death. Tanasawet et al. [11] reported that A. graveolens (125, 250, and 500 mg/kg) decreased MDA production and enhanced GPx levels in the cortex and striatum of the mouse, and our findings were in agreement with this report. Celery contains several bioactive compounds, such as flavonoids and L-3-n-butylphthalide [5]. The anxiolytic activity of A. graveolens might result from its antioxidant properties [11].

These results suggested the protective effect of CEO against CPZ-induced MS mediated by its antioxidant activity. Further investigation is needed to determine its active constituents and precise mode of action.

Materials & Methods

Animals

Forty male C57BL/6 mice (aged 4-6 weeks and weighting 19 ± 2 g) were kept under laboratory conditions (temperature of 22°C ± 2°C and 12/h light/dark cycle) with adequate food and water in Razi laboratory complex (Islamic Azad University, Science and Research Branch, Tehran, Iran). One week after acclimatization, the mice were randomly allocated into four experimental groups (n=10). The research committee of Islamic Azad University, Science and Research Branch approved all study protocols (IR.IAU.SRB.REC.1401.112).

Preparation of celery crude essential oil

Apium graveolens L. was identified at the Faculty of Agriculture, Science and Research Branch, Islamic Azad University, Tehran, Iran. The Apium graveolens L. leaves (100 g) shade dried at room temperature (25°C ± 2°C). The samples were hydro-distilled by a Clevenger-type apparatus for 3.5 hours until the complete recovery of essential oil. The essential oil on top of the distillate was collected, dried, and stored in a dark glass bottle covered with aluminum foil at 4°C±1°C [20].

Study protocol

The control group received a regular diet. In group 2, acute demyelination was induced by feeding mice with 0.2% (w/w) CPZ (Sigma Aldrich, St. Louis, MO, USA) mixed with ground chow for 5 weeks [15]. In group 3, a regular diet was provided, and mice were administered daily p.o. with CEO (800 mg/kg) for 5 weeks [21]. In group 4, mice received a diet containing CPZ (0.2% w/w) for 5 weeks and were administered p.o. with CEO (800 mg/kg). Finally, reflexive motor behavior and serum antioxidant levels were determined. MS corresponding animal model, experimental autoimmune encephalomyelitis, is widely used to understand disease pathogenesis and test novel therapeutic agents. These defects are quantified using a standard experimental autoimmune encephalomyelitis scoring system on a 0-5 disease severity scale as 0: no disease; 1: loss of tail tone; 2: hind limb weakness; 3: hind limb paralysis; 4: hind limb and forelimb paralysis or weakness; and 5: moribund/death [22].

Ambulation

Ambulation test as crawling behavior is used to determine the ability to walk following MS [23]. In this test, mice are motivated to walk, then a scoring system is used for the quality of walk in which no movement is scored as zero, asymmetric walking is scored as 1, symmetric slow movement is scored as 2, and finally, fast walking mice are given score 3. This test was completed in triplicate at 3 minutes and the average score was recorded [24].

Hind limb foot angle

Following the signs of MS, hind limb positions change wherein walking [24]. Consequently, the movements of mice in an open field box were recorded by a camera. Images obtained from the hind limb positions and pictures in which mice had full stride in a straight line were used. A line was drawn from the end of the heel to the tip of the toe and the angle between them was measured [24].

Front limb suspension

This test was used to assess the ability of animals to hang with front limbs. Briefly, mice were allowed to grasp a wire which was tied to two ends between a wall. After grasping the wire, the time until the

mice released the wire was recorded. This test was repeated in triplicate at 3 minutes and the average time in seconds was recorded [24].

Hind limb suspension

This test described mice's ability to hang over their hind legs [24]. Following the signs of MS, hind limb strength decreases in mice. The hind legs of mice were hung over the wire which was tied at two ends between a wall and a scale system, and hind limb posture was determined. After mice hung over the wire, the hind limb posture score was recorded as not able to grasp the wire (score 0); the hind limb easily released from the grasped wire in a clasped position (score 1); by rising the tail, the hind limb was easily released from the grasped wire and stayed close to each other (score 2); by rising the tail, the hind limb of the mice was easily released from the grasped wire but stayed normal (score 3); by rising the tail, hind limb separation was normal with force [24].

Surface righting

This test assesses the ability of animals to return to their normal position [25]. Briefly, mice were kept in the pine position for 5 seconds. Next, they were released and the time needed for flipping the mice to the normal position onto the feet was recorded [24].

Grip strength

Mice were placed on a 16×18 fiberglass screen and slowly rotated from a horizontal to a vertical location. In this test, mice try to grasp the screen to not release from the surface [26]. The latency to fall was recorded [27].

Negative geotaxis

Mice were placed face down on a 45° surface. Then, they were released and the time needed to face the hill upward was recorded [24].
Open field test
This test describes the locomotor and exploratory activities of animals. Mice were allocated into an OFT apparatus (45×45×30 cm3 with nine divided squares wooden box). Mice were allocated at the center of the box and the number of squares passed was recorded in 6 minutes [28].

Rotarod test

This test evaluates animals' motor coordination and ability to stay running on accelerated rods. Mice were laced on rotarod apparatus and the test was performed with an acceleration of 0-20 rpm in 10 minutes. The time until mice fell off the rod was recorded [29].

Antioxidant activity

After determining the behavioral tests, blood samples were taken from each mouse from cardiac and serum MDA levels, and SOD, GPx, and TAS activities were obtained using Zell Bio GmbH (Germany) assay kits [30-32].

Statistical analysis

Data were analyzed using one-way ANOVA and were presented as mean±SE (standard error) using SPSS version 22.0. For treatments showing significant differences by ANOVA, between-group evaluations were performed using the Tukey posthoc test ($p < 0.05$).

Authors' Contributions

Tahoura Mohammadi-Ghohaki : collect data, draft of paper, Shahin Hassanpour: thesis supervisor, revise paper, study protocol, Morteza Zendehdel : thesis advisor.

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Competing Interests

There is no conflict of interest.

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RESEARCH ARTICLE

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Ameliorative Effects of Melatonin on Exercise-induced Oxidative Stress and Haematological Response of Untrained Arabian Stallions Following a Race Of 2000 m

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ABSTRACT

We performed this study to evaluate the effects of melatonin on oxidative stress and haematological responses following an exercise of 2000 m at maximum speed. Eighteen untrained, clinically healthy stallions of the Arabian breed with a mean body weight of 401 ± 32.11 kg (395–404 kg) and an age range of 5.7–0.54 years (5–6 years) were used in this research. The subjects were divided into three groups of six stallions each. Group I (MTEX) was treated with melatonin at a dose rate of 0.03 mg/kg orally once daily for one month. Group II (NMTX) was not administered melatonin but exercised, while the last group (OMTX) was neither administered melatonin nor exercised. The results obtained show that post-exercise, the biomarkers of oxidative stress evaluated were significantly lower ($p < 0.05$) in the MTEX group than in the NMTX group. The leucocyte count, neutrophil counts, and the ratio of neutrophil to lymphocyte were higher ($p < 0.05$) in the NMTX group than in the MTEX group. Furthermore, it was recorded that packed cell volume and the total erythrocyte count were statistically higher ($p < 0.05$) in the MTEX group than in the NMTX group. Therefore, we concluded that melatonin ameliorated oxidative stress and some haematological parameters will be beneficial to horses subjected to the stress of exercise.

Keywords

Arabian stallion, haematological parameters, melatonin, oxidative stress

Number of Figures: 0
Number of Tables: 3
Number of References:: 31
Number of Pages: 6

Abbreviations

MTEX: Group treated with melatonin before exercise
OMTX: Not treated with melatonin but exercised
NMTX: Not treated with melatonin, not exercised
PUFA: Polyunsaturated fatty acid

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Introduction

The horses are used for a variety of activities, but they need to be extensively trained and exercised for easy restoration and their general well-being [1,2]. Exercise increases the body's need for oxygen, alters metabolic load, and increases the heart rate alongside other physiological responses to withstand the demand for exercise [3,4]. Exercise Physiology of Horses has been widely studied, and research has demonstrated that exercise improves cognitive abilities and health status [5.6]. However, exercise can be a source of stress, and stress is a term used to describe a situation, circumstance, or stimulus that can endanger or affect an organism's homeostasis [7, 8, 9]. When stress is not managed or prevented, it can become maladaptive, impairing the physiological process of the body [10].

Two interconnected branches of the autonomic nervous system that make up the physiological stress response are the sympathetic adrenal medullary network, which reacts quickly to stress and facilitates the release of adrenaline into the bloodstream and ultimately into brain regions, and the slower hypothalamic pituitary adrenal network, which facilitates the release of glucocorticoids [11,12, 13]

Increase in oxidative stress has been defined as an imbalance in the body's oxidant-to-antioxidant ratio. It has been showen that in horses, oxidative stress levels rise, and their antioxidant status changes during endurance and high-intensity competition [7, 14, 15]. All antioxidant substances—vitamins, minerals, enzymes, and proteins—must either be synthesized by the body or obtained through diet [16]. As a result, the oxidative stress and antioxidant state of the horse athlete are both influenced by nutrition and exercise intensity [17, 18, 19]. During endurance and competitive exercise events, horses have demonstrated increased oxidative stress and changes in their antioxidant status [7, 20]. Melatonin and its metabolites, which are potent anti-inflammatory agents and antioxidants, protect mitochondrial integrity by scavenging reactive oxygen species and reactive nitrogen species [21,22]. Melatonin and its derivatives induce several antioxidant enzyme activities. Thus, melatonin is necessary to slow down the process of aging and maintain the cellular redox equilibrium [23].

The objective of this study is to determine the effects of melatonin on biomarkers of oxidative and haematological parameters of stallions of the Arabian breed subjected to an exercise of 2000 m.

Results

The biomarkers of oxidative stress are shown in Table 1. The activity of superoxide dismutase obtained MTEX group (2.49 ± 0.14 u/ml) post-exercise was statistically lower ($p < 0.05$). The activity of the enzyme catalase in the NMTX group (10.05 ± 5.43 u/ml) post-exercise was statistically higher ($p < 0.05$) than the value obtained in the MTEX group (6.04 ± 0.43 u/ml) after exercise. The activity of the enzyme glutathione peroxidase was lower ($p < 0.05$) in the MTEX group than in the NMTX group after exercise (278.13

Table 1.
Biomarkers of Oxidative Stress

Parameters	Time	OMTX	MTEX	NMTX
Superoxide dismutase (u/ml)	Pre-exercise	1.77 ± 0.33	1.62 ± 0.21	2.57 ± 0.37
	Post-exercise	2.41 ± 0.54	2.49 ± 0.14a	8.57 ± 4.78b
Catalase (u/ml)	Pre-exercise	4.57 ± 0.41	4.97 ± 0.23	4.74 ± 1.531
	Post-exercise	4.38 ±0.37	6.04 ± 0.43a	10.05 ± 5.43b
Glutathione peroxidase (U/L)	Pre-exercise	267.13 ± 5.66	259.90 ± 5.32	267.82 ± 7.73
	Post-exercise	264.37 ± 6.31	278.13 ± 8.43a	324.05 ± 19.07b2
Glutathione reductase (U/L)	Pre-exercise	0.38 ± 0.02	0.44 ± 0.16	0.45 ± 0.03
	Post-exercise	0.41 ± 0.03	0.87 ± 0.34	1.58 ± 1.33
Malondialdehyde (µmol/L)	Pre-exercise	5.58 ± 0.27	5.01 ± 0.34	5.72 ± 1.061
	Post-exercise	5.42 ± 0.49	7.26 ± 0.56a	10.24 ± 5.77b

a,b Means for the same row having different superscript letters are significantly ($P < 0.05$) different
Key: OMTX: Not administered with melatonin, not exercised
MTEX: Administered with melatonin, then exercised
NMTX: Not administered with melatonin but exercised

± 8.43 U/L and 324.05 ± 19.07 U/L, respectively). The concentration of malondialdehyde after exercise in the MTEX group (7.26 ± 0.56 µmol/L was lower ($p < 0.05$) than the value obtained in the NMTX group (10.24 ± 5.77 µmol/L).

Table 2 shows that the total count of leucocytes obtained in the MTEX group (4.83 ± 0.14 $\times 10^9$/L) was significantly lower ($p < 0.05$) than the value obtained in the NMTX group (7.31 ± 0.86 $\times 10^9$/L). The neutrophil count recorded in the NMTX group after exercise (5.34 ± 0.85 $\times 10^9$/L) was significantly higher ($p < 0.05$) than the count recorded in the MTEX group after exercise (3.34 ± 0.11 $\times 10^9$/L).

The ratio of neutrophil to lymphocyte calculated in the NMTX group after exercise (3.97 ± 1.64) was statistically higher ($p < 0.05$) than the value calculated in the MTEX group (2.08 ± 0.35) post-exercise.

Table 3 shows the erythrocyte parameters. The erythrocyte count obtained in the MTEX group (12.22 ± 6.74 $\times 10^{12}$/L) post-exercise was statistically higher ($p < 0.05$) than the value recorded in the NMTX group (8.72 ± 0.44 $\times 10^{12}$/L) after exercise. The PCV obtained in the NMTX group was statistically lower ($p < 0.05$) than the value recorded in the MTEX group (26.87 ± 0.76 % and 32.13 ± 6.25 %, respectively).

Table 2.
Biomarkers of Oxidative Stress

Parameters	Time	OMTX	MTEX	NMTX
Leucocytes ($\times 10^9$/L)	Pre- exercise	5.13 ± 0.32	4.21 ± 0.23	5.41 ± 0.37
	Post-exercise	5.07 ± 0.28	4.83 ± 0.14a	7.31 ± 0.86b
Neutrophils ($\times 10^9$/L)	Pre-exercise	2.77 ± 0.08	2.53 ± 0.34	2.83 ± 0.46
	Post-exercise	2.83 ± 0.17a	3.34 ± 0.11a	5.34 ± 0.85b
Lymphocytes ($\times 10^9$/L)	Pre-exercise	1.68 ± 0.23	1.82 ± 0.25	1.72 ± 0.21
	Post-exercise	1.69 ± 0.33	2.68 ± 0.34	1.88 ± 0.42
Monocytes ($\times 10^9$/L)	Pre-exercise	0.13 ± 0.03	0.13 ± 0.05	0.17 ± 0.02
	Post-exercise	0.11 ± 0.02	1.07 ± 0.16	0.18 ± 0.04
Neutrophil/Lymphocyte Ratio	Pre-exercise	1.78 ± 0.14	1.21 ± 0.32	1.86 ± 0.78
	Post-exercise	1.81 ± 0.16	2.08 ± 0.35a	3.97 ± 1.64b.

a,b Means for the same row having different superscript letters are significantly ($P < 0.05$) different
Key: OMTX: Not administered with melatonin, not exercised
MTEX: Administered with melatonin, then exercised
NMTX: Not administered with melatonin but exercised

Table 3.
Erythrocyte indices of stallion

Parameters	Time	OMTX	MTEX	NMTX
Erythrocytes ($\times 10^{12}$/L)	Pre-exercise	5.56 ± 0.62	7.12 ± 0.73	5.98 ± 0.23
	Post-exercise	5.78 ± 0.53	12.22 ± 6.74a	8.72 ± 0.44b
Packed Cell Volume (%)	Pre-exercise	26.11 ± 0.93	32.13 ± 6.25a	26.87 ± 0.76b
	Post-exercise	29.65 ±1.02	63.42± 13.67a	55.42± 4.74b
Haemoglobin (g/dl)	Pre-exercise	8.55 ±1.21	8.71 ± 2.41	7.71 ± 0.36
	Post-exercise	8.62 ± 1.09	11.53 ± 0.74	10.83 ± 0.68

a,b Means for the same row having different superscript letters are significantly ($P < 0.05$) different
Key: OMTX: Not administered with melatonin, not exercised
MTEX: Administered with melatonin, then exercised
NMTX: Not administered with melatonin but exercised

Discussion

Exercise has been reported to be beneficial to horses [24] but extreme exercise without requisite dietary antioxidant supplementation may be deleterious. This is because, during intense exercise, it has been demonstrated that there are increases in oxidative stress response and altered antioxidant status in horses [17]. In this study, we observed that the biomarkers of oxidative stress were higher in the NMTX group than in the MTEX group, indicating a higher level of oxidative stress process in the NMTX group.

Melatonin use has been demonstrated to considerably ameliorate oxidative stress indicators [25]. However, the effect of melatonin on certain oxidative stress markers, such as during exercise in horses, has not been well reported in the literature. In this study, we observed that most of the biomarkers of oxidative stress studied were significantly lower in the MTEX group post-exercise. Melatonin improves the antioxidative potential of the cell by enhancing the synthesis of enzyme-based antioxidants like superoxide dismutase, glutathione peroxidase, and also the enzymes that are precursors in the synthesis of glutathione [26], thereby quenching oxygen free radicals such as superoxide radical, hydroxyl radical, peroxyl radical, and peroxynitrite anion. Melatonin often improves the synthesis of the antioxidative enzymes' messenger RNAs (27) and also chelates transition metal ions and shields cellular membranes from damage. This chain of events helps to explain how melatonin lowered oxidative stress in the MTEX group (28). This is also similar to the findings of Kruk et al. (27).

Due to its interaction with lipids, melatonin can stabilize the erythrocyte membrane, thereby prolonging the life of cells (29). This could explain the higher erythrocyte count recorded in the MTEX group. Intense exercise is known to increase reactive oxygen species, which results in the lysis of the erythrocytes (30), especially in horses not supplemented with antioxidants. This phenomenon may explain the lower erythrocyte count obtained in the NMTX group post-exercise. This is similar to the finding of Krokosz et al. (29). This is because, lipid peroxidation, a phenomenon where oxidants such as free radicals destroy lipids with carbon-carbon double bonds, specifically polyunsaturated fatty acids (PUFAs) occur in the membrane of the erythrocyte making their membranes fragile.

The higher leucocyte and neutrophil count recorded in the NMTX group indicates that there was a higher inflammatory response in the group due to exercise. On the other, the lower counts in the MTEX group suggest a protective role of melatonin as melatonin has been reported to possess a potent anti-inflammatory function [31]. The ratio of neutrophil to lymphocyte (a biomarker of inflammation) was higher in the NMTX group compared to the MTEX group post-exercise, further substantiating the anti-inflammatory effects of melatonin.

Materials & Methods

Animals

For this experiment, a total of 18 clinically healthy Arabian stallions with mean body weights between 401 ± 32.11 kg (395-404 kg) and ages between 5.7 ± 0.54 years (5 - 6 years) were used. The stallions were primarily used for pleasure riding and were acquired from the Royal horse stable. They lived in a stable with concrete floors, corrugated iron roofs, wooden ceilings, and cement brick walls. Before the experiment, they had a four-week pre-conditioning period. They were given hay as their primary food source, with concentrate (groundnut bran) as a supplement, and water was available at all times.

The stallions were divided into three groups of six stallions each. Group I (MTEX) was administered with melatonin once daily for one month before exercise. Group II (NMTX) was not administered melatonin but exercised while the last group (OMTX) was neither administered melatonin nor exercised.

Experimental design

The stallions in MTEX and NMTX groups were conditioned to exercise before the experiment began by putting them through a calibrated exercise protocol every day for two weeks.

Administration of melatonin

Melatonin was purchased from Twinlab in Hauppauge, New York, USA. Melatonin was given to each stallion in the MTEX group at a dose of 0.03 mg/kg body weight orally once daily for one month. The medication was given to the stallions after being dissolved in water and aspirated using the appropriate syringe daily for a month.

Exercise protocol

Each stallion in the MTEX and NMTX group was mounted by a trained rider, with a weight of approximately 70 kg. The stallions were then subjected to a race of 2000 m on a standard race track at maximum speed.

Blood sampling

Each stallion had 10 mL of blood collected from the jugular vein before and after exercise. Exactly 5 ml of the blood samples were dispensed into sample bottles containing potassium ethylenediaminetetraacetic acid, an anticoagulant for haematological analyses, while the remaining 5 ml were collected in plain sample bottles for serum harvesting, which was used for the evaluation of biomarkers of oxidative stress. The blood samples collected were transported to the laboratory in a Coleman box containing ice and analyzed immediately.

Analyses of samples

A standard veterinary automated analyzer (KT-6610 VET, Jiangsu, China) was used to determine the total count of erythrocytes, the total count of leucocytes, haematocrtit value (packed cell volume), and hemoglobin concentration.

The concentration of malondialdehyde, superoxide dismutase, glutathione peroxides, and catalase activities was determined spectrophotometrically using their respective ELISA kits (Cayman Chemicals, Ann Arbor, Michigan, USA). The spectrophotometer manufactured by Spectronic-20, Philip Harris Limited, Shenstone, UK, was used for the analyses.

Data analyses

This study's data were analyzed using one-way analysis of variance (ANOVA), followed by Tukey's posthoc test, and expressed as Mean ± SEM. Values of $P < 0.05$ were regarded as significant. The studies were performed using the program GraphPad Prism (version 5.0).

Conclusions

According to our findings, we concluded that melatonin ameliorates biomarkers of oxidative stress and some haematological parameters following exercise in stallions. Therefore, supplying melatonin as a potent antioxidant is crucial for horses before strenuous exercise.

Authors' Contributions

ASA: Conceptualization, writing the original draft, and carrying out the experiments

DAA: Data analyses, and project administration

CON: Manuscript revision, funding, and carrying out the experiments

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Competing Interests

The authors hereby declare that there was no conflict of interest.

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Abstract in Persian

نقش انتقال دهنده‌های عصبی مختلف در تنظیم مرکزی اخذ در هسته پشتی هیپوتالاموس

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چکیده

در موجودات زنده، کنترل مرکزی تغذیه یک مکانیسم بسیار پیچیده و حیاتی است. کنترل مرکزی تغذیه در نواحی مختلف مغز انجام می‌شود. مهمترین ناحیه مغزی در کنترل مرکزی تغذیه، هیپوتالاموس است. هیپوتالاموس رفتارهای تغذیه‌ای را از طریق مدارهای عصبی، هسته‌های ویژه متعدد و انتقال دهنده‌های عصبی مرکزی کنترل می‌کند. هسته‌های مختلف هیپوتالاموس که در کنترل اخذ غذا نقش دارند عبارتند از ARC, PVN, LHA, VMH, و DMH. از طریق انتقال دهنده‌های عصبی مختلف در مغز بر تغذیه تأثیر می‌گذارد. این هسته ورودی‌های اشتهاآوری و بی‌اشتهایی را از طریق اتصالات عصبی با ARC و سایر مناطق مغز دریافت می‌کند. ARC به دلیل موقعیتش در مغز، به پیام‌های ورودی تغذیه‌ای با منشأ گردش خون دسترسی دارد. در این هسته، دو جمعیت عصبی شامل نوروپپتید (NPY) پرو اپیوملانوکورتین (POMC) وجود دارد. پیام‌های ورودی مختلف از گردش خون، با تأثیر بر این دو جمعیت عصبی در ARC، پیام‌های ورودی مربوطه را به نورون‌های دسته دوم از جمله DMH. ارسال می‌کنند. پس از آن، DMH. این پیام‌ها را ادغام می‌کند. DMH. خروجی نهایی را به PVN و LHA می فرستد. بنابراین DMH. بر کنترل مرکزی تغذیه از طریق این مسیرهای عصبی تأثیر می‌گذارد.

واژگان کلیدی

تغذیه، هیپوتالاموس، هسته پشتی هیپوتالاموس، انتقال دهنده‌های عصبی مغز، اشتهاآور، بی‌اشتهایی

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شیوع *مایکوباکتریوم آویوم* تحت گونه *پاراتوبرکلوزیس* در گاوهای آلوده تحت بالینی در گله های شیری صنعتی مشهد با استفاده از رنگ آمیزی Ziehl-Neelsen، کشت و PCR

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چکیده

مایکوباکتریوم آویوم تحت گونه *پاراتوبرکلوزیس* (MAP)، عامل بیماری یون در نشخوارکنندگان اهلی و وحشی می باشد. از نظر بالینی، گاوهای آلوده علائم لاغری، اسهال و در نهایت مرگ را نشان می دهند، اما مبتلایان تحت بالینی که علائم بالینی ندارند، می توانند به طور متناوب MAP را از طریق مدفوع و شیر دفع کنند و سایر حیوانات گله را آلوده کرده و خطر عفونت را افزایش دهند. هدف اصلی این مطالعه شناسایی شیوع این بیماری در گله های شیری با انجام رنگ‌آمیزی Ziehl-Neelsen، کشت نمونه‌های مدفوع و آزمایش مولکولی بود. برای این منظور، ۳۴۸ نمونه از ۱۵ گاوداری به طور تصادفی جمع‌آوری شد و تمام نمونه‌های مدفوع تحت رنگ‌آمیزی ZN، توالی نوکلئوتیدی PCR مربوط به قطعات ژن اختصاصی MAP (IS900, F57) قرار گرفت و پس از ضدعفونی با محلول (HPCO ۰.۷۵٪) برروی محیط کشت هرولد حاوی زرده تخم مرغ کشت شدند. آزمایش PCR نمونه مدفوع، ۱۱۶ نمونه (شیوع ۳۳.۳٪، ۹۵٪ CI: ۲۸.۳-۳۸.۲)، رنگ آمیزی ZN ۲۳ نمونه (شیوع ۶.۶٪، ۹۵٪ CI: ۴-۹.۲)، و کشت نمونه مدفوع تنها ۱۵ نمونه (شیوع ۴.۳٪، ۹۵٪ CI: ۲.۳ -۶.۳) آلوده به MAP بودند. مقایسه نتایج آزمایش‌ها نشان‌دهنده تطابق ضعیف (آمار کاپا: ۰.۱۹) بین رنگ‌آمیزیZN وPCR و بالاترین ضرایب کاپا (آمار کاپا: ۰.۸۹) بین آزمایش‌های PCR و کشت مدفوع است. این مطالعه مزایای PCR را برای تشخیص MAP در گاوهای آلوده تحت بالینی در مقایسه با رنگ‌آمیزی ZN و کشت مدفوع برجسته می‌کند، بنابراین می‌توان از آن برای تشخیص و کنترل زود هنگام MAP در گله‌ها و جمعیت‌های در معرض خطر استفاده کرد.

واژگان کلیدی

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تنوع ژنتیکی، مقاومت ضد میکروبی و پتانسیل تشکیل بیوفیلم اشرشیاکلی های جدا شده از مدفوع اسب در شمال ایران

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چکیده

مطالعه حاضر با هدف بررسی توانایی تشکیل بیوفیلم‌سازی، مقاومت های آنتی‌بیوتیکی، فراوانی ژن های مرتبط با بیوفیلم و مقاومت های آنتی بیوتیکی و گروه‌بندی فیلوژنیک جدایه‌های اشرشیاکلی با منشاء اسب انجام شد. در مجموع، ۱۵۷ اشرشیا کلی جدا شده از نمونه مدفوع تازه اسب های سالم شمال ایران جمع آوری شد. جدایه ها از نظر تشکیل بیوفیلم و حساسیت به داروهای ضد میکروبی به ترتیب با روش میکروتیتر پلیت و دیسک دیفیوژن مورد آزمایش قرار گرفتند. از واکنش PCR برای آنالیز فیلوژنتیک و تعیین فراوانی ژن‌های موثر در ایجاد بیوفیلم و ژن های مقاومت در برابر بتالاکتام‌ها، کلرامفنیکل، تتراسایکلین‌ها، آمینوگلیکوزیدها، کینولون‌ها، سولفامتوکسازول و تری متوپریم استفاده شد. بیش از ۵۰ درصد جدایه ها فنوتیپ MDR را نشان دادند. بیشترین نسبت مقاومت برای استرپتومایسین (۸۷/۵۹ درصد) و پس از آن تری متوپریم- سولفامتوکسازول (۹۳/۲۹ درصد) و اکسی تترایکلین (۶۶/۲۸ درصد) مشاهده شد. ایمی پنم و نورفلوکساسین قوی ترین آنتی بیوتیک ها بودند. گروه‌های فیلوژنتیک B1(٪۴۶.۵۰) و A(٪۲۱.۶۶) شایع‌ترین گروه‌ها در جدایه‌ها بودند و پس از آن C(٪۶.۳۷)، کلاد ۱ (۰٪۵.۱۰)، E(٪۴.۴۶)، D (٪۳.۸۲) و B2(٪۲.۵۵) قرار گرفتند. تمام ایزوله‌های فیلوگروه‌های B2 و D حامل تمام ژن‌های مرتبط با بیوفیلم بودند و ژن‌های مقاومت ضد میکروبی در فیلوگروه‌های B1, A, D, B2, و E رایج بودند.

این یافته‌ها نشان می‌دهد که در شمال ایران، اشرشیا کلی های جدا شده از اسب‌های سالم دارای پتانسیل ایجاد عفونت های خارج روده ای و فنوتیپ MDR هستند. این حیوانات می توانند مخزن قابل توجه مقاومت به آنتی بیوتیک ها در انسان و دامپزشکی باشند. این داده ها از نگرانی مداوم در مورد مقاومت ضد میکروبی، انتشار سویه های MDR و مدیریت استفاده از ضد میکروبی در دامپزشکی حمایت می کند.

واژگان کلیدی

اسب، اشرشیا کلی، تنوع ژنتیکی، مقاومت ضد میکروبی، بیوفیلم

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Abstract in Persian

اثرات درمانی ADU-S100 به عنوان آگونیست STING و CpG ODN1826 به عنوان آگونیست TLR9 در مدل CT-26 سرطان کولون

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۲ گروه ژنتیک و زیست شناسی ژنوم، بیمارستان کودکان بیمار، تورنتو، کانادا.
۳ موسسه تحقیقات واکسن و سرم سازی رازی، سازمان تحقیقات، آموزش و ترویج کشاورزی (AREEO)، مشهد، ایران.

چکیده

ایمنی درمانی سرطان یک روش درمانی جدید برای از بین بردن سلول های تومور است و پس از آزمایش های بالینی و تحقیقات بنیادی موفق به سمت تغییرات بالینی پیشرفت نموده است. ایمنی درمانی توسط ادجوانت-ها برای افزایش پاسخ های ایمنی محافظتی در برابر بیماری های عفونی و سرطان حیاتی است. آگونیست های STING و TLR9 کاندیدای جالبی برای ایمنی درمانی جدید سرطان هستند. در این مطالعه، اثرات ضد توموری ADU-S100 به عنوان آگونیست STING و CpG ODN1826 به عنوان آگونیست TLR9 به صورت تکی و ترکیبی در مدل CT-26 سرطان کولون مورد بررسی قرار گرفت. این مدل در موش های ماده BALB/c القا شد که به پنج گروه تحت درمان با PBS, ADU-S100 (۲۰و۴۰ میکروگرم) و ۴۰ میکروگرم)، CpG ODN (۴۰ میکروگرم) و ADU-S100 (۲۰ میکروگرم) + CpG ODN (۲۰ میکروگرم) تقسیم شدند. حجم تومور و وزن موش ها روز در میان اندازه گیری شد. در روز ۳۰، بافت های تومور، طحال و کبد موش ها برای بررسی بافت شناسی جدا شد. آنالیز هماتولوژی بر روی خون قلب انجام شد. تزریق داخل توموری آگونیست‌ها باعث سرکوب معنادار تومور در گروه‌های درمان بویژه در گروه ترکیبی گردید که نصف غلظت فرم تک آگونیست ها را دریافت کردند. همچنین آنالیز بافت شناسی نمونه های تومور وجود سلول های آپوپتوز و التهابی و آنالیز هماتولوژی نمونه های خون، افزایش تعداد لنفوسیت ها در گروه های درمانی را نشان دادند که نشان دهنده نقش موثر این آگونیست ها در پاکسازی تومور است. بنابراین، چنین هم افزایی ادجوانت ها ممکن است نقش موثری در ایمنی درمانی سرطان داشته باشد و دیدگاه‌های جدیدی را در مورد ترکیب آگونیست‌هایی که گیرنده های ایمنی ذاتی را در سرطان تحریک می‌کنند، ارائه دهد.

واژگان کلیدی

آگونیست STING ، آگونیست TLR90 ،اثر هم افزایی، ایمنی درمانی، مدل سرطان کولون

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Abstract in Persian

تزریق ارکسین A داخل ناحیه شکمی جانبی دور قنات خاکستری اختلال حافظه و یادگیری القا شده با نیتروگلیسرین را در موش های صحرایی کاهش می دهد

راضیه کوشکی[1*]، مهدی عباس نژاد[2]، بهارالسادات مجدزاده[2]

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۲ گروه زیست شناسی، دانشکده علوم پایه، دانشگاه شهید باهنر کرمان، کرمان، ایران.

چکیده

در این مطالعه نقش گیرنده1 ارکسین(Orx1R) در ناحیه شکمی جانبی دور قنات خاکستری (vlPAG) بر تعدیل اختلال حافظه و یادگیری موش های صحرایی میگرنی بررسی گردید. برای القا میگرن از تزریق داخل صفاقی نیتروگلیسرین (5 mg/kg) (NTG) به صورت یک روز در میان به مدت ۹ روز استفاده شد. حافظه و یادگیری فضایی و اجتنابی غیر فعال به ترتیب، با استفاده از دستگاه ماز آبی موریس (MWM)و جعبه شاتل باکس مورد ارزیابی قرار گرفت. در آزمون MWM، گروه درمان شده با NTG افزایش مدت زمان و مسافت طی شده تا رسیدن به سکو پنهان و کاهش در تعداد ورود و زمان گذرانده شده در ناحیه هدف در مقایسه با گروه کنترل نشان داد. درمان با تزریق داخل vlPAG ارکسین A۵۰(pM) اختلال رفتاری ناشی از NTG را کاهش داد. همچنین در آزمون شاتل باکس، در حیوانات درمان شده با NTG کاهش تاخیر ورود به جعبه تاریک و افزایش مدت زمان گذرانده در جعبه تاریک مشاهده گردید که نشان-دهنده ی اختلال در حافظه اجتنابی غیرفعال می باشد. اختلال حافظه اجتنابی ایجاد شده با NTG با تزریق دوزهای ۵۰ pM و ۱۰۰ pM ارکسین A کاهش یافت. با این وجود، اثرات مثبت ارکسینA (۱۰۰ pM) در بهبود اختلال حافظه و یادگیری موش های میگرنی در آزمون های حافظه و یادگیری MWM و شاتل باکس به دنبال پیش درمان با SB334867 ، آنتاگونیست Orx1R، ممانعت گردید. در مجموع ، نتایج این مطالعه ، نقش موثر Orx1R در ناحیه vlPAG را در تعدیل اختلال حافظه و یادگیری ناشی از میگرن نشان می دهد.

واژگان کلیدی

حافظه و یادگیری، موش های صحرایی ، ارکسینA ،میگرن، نیتروگلیسرین

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IRANIAN JOURNAL OF VETERINARY SCIENCE AND TECHNOLOGY

Guide for authors

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Gene names: The standard gene names, as provided by HGNC (HUGO Gene Nomenclature Committee) should be used. Gene names must be italicized. If the case of mammalian species and if gene names refer to rodent species, they must be upper case; if they refer to non-rodent species they must be written in capitals. If they refer to other species, they must written lower case. Protein names are written in capitals and are not italicized. As an example:

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Quantitative PCR: If the quantitative PCR method has been used, the related section in Materials and Methods must be written following the reference:

Bustin SA, Benes V, Garson JA, Hellemans J, Huggett J, Kubista M, Mueller R, Nolan T, Pfaffl MW, Shipley GL, Vandesompele J, Wittwer CT. The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. Clin Chem. 2009 Apr;55(4):611-22.

The following information must be provided in the section:

Protocol for DNA/RNA extraction, including quantification and determination of purity;

Reverse transcription (if used): amount of RNA, concentration of all reagents: primers concentration (either random primers or oligonucleotides), reverse transcriptase and master mix components;

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An unhealthy diet, obesity and physical inactivity play a role in the onset of type 2 diabetes, but it has been shown that increased physical activity substantially reduces the risk [1], and participation in regular physical activity is one of the major recommendation of the evidence based guidelines for the primary prevention of diseases [2]. According to the 2004-05 National Health Survey, more than half a million Australians (3.5% of the population) have diabetes mellitus which had been medically diagnosed and most of these people have the Type 2 condition [3]. Gestational diabetes is also on the increase, rising steadily between 2000-01 and 2005-06 [4]. Approximately two thirds of those with diabetes have been prescribed medication [3], but it is of concern that a recent review of the literature found that many people do not take their medication as prescribed [5]. Many patients also self monitor the disease by measuring their blood glucose levels with a glucose meter but Song and Lipman [6] have concerns about how well this is managed.

References for the above example:

1. Hull J, Forton J, Thompson A. Paediatric respiratory medicine. Oxford: Oxford University Press; 2015.

2. Eckerman AK, Dowd T, Chong E, Nixon L, Gray R, Johnson S. Binan Goonj: bridging cultures in Aboriginal health. 3rd ed. Chatswood, NSW: Elsevier Australia; 2010.

3. Johnson C, Anderson SR, Dallimore J, Winser S, Warrell D, Imray C, et al. Oxford handbook of expedition and wilderness medicine. Oxford: Oxford University Press; 2015.

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5. Petitti DB, Crooks VC, Buckwalter JG, Chiu V. Blood pressure levels before dementia. Arch Neurol. 2005; 62(1):112-6. Doi: 10.1001/archneur.62.1.112.

6. Liaw S, Hasan I, Wade, V, Canalese R, Kelaher M, Lau P, et al. Improving cultural respect to improve Aboriginal health in general practice: a multi-perspective pragmatic study. Aust Fam Physician. 2015; 44(6):387-92. Doi: 10.1001/archneur.62.1.110.

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The section "Author Contributions" in the manuscript should illustrate and clarify who contributed to the work and how. If a contributor does not meet all four above criteria should be acknowledged in the "Acknowledgements" section of the article.

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Ethical guidelines for Peer reviewers

Iranian Journal of Veterinary Science and Technology (IJVST) follows and adheres to COPE Ethical Guidelines for Peer Reviewers. IJVST peer reviews all submitted manuscripts with contents in the scope of the journal. The process has been explained in the section "Peer Review Process".

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Peer Review Process

PEER REVIEW PROCESS

Iranian Journal of Veterinary Science and Technology peer reviews all submitted manuscripts with contents within the scope of the journal.

Initial assessment

The submitted manuscript will be subjected to a primary review by the editor or a member of the editorial board for suitability and relevance of the findings to the scope of the journal and quality of the science presented in the paper (sufficient originality, having a message that is important to the general field of Veterinary Medicine, quality of data, novelty, English language, and overall manuscript quality) within two weeks. If the paper is evaluated to be relevant to the scope of the journal and having enough scientific rigor and novelty, it will be sent for the next stage. Otherwise, those manuscripts which are evaluated as not-appropriate in the initial review will be rejected at this stage.

Initial screen

The initial screen will be performed by the editorial office for the structure and format of the manuscript.

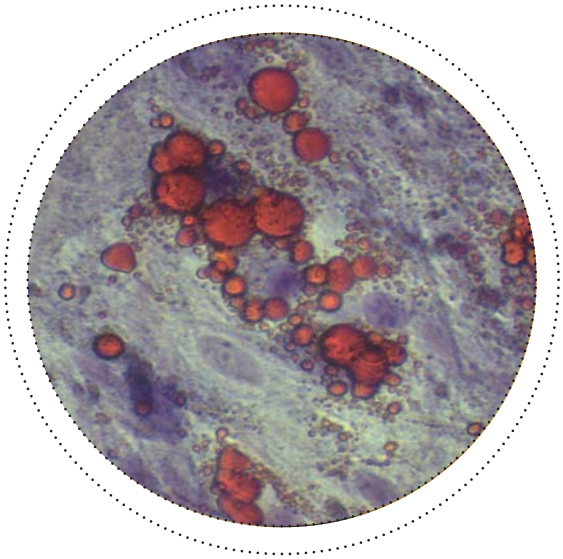
Peer review (double-blind)

The manuscripts which are found to be appropriate after the initial screen will be sent for external review by experts in the related field. We have prepared a checklist for reviewers that summarizes their evaluation of the manuscript. The items in this checklist are:

1. TITLE is clear and adequate
2. ABSTRACT clearly presents objects, methods, and results.
3. INTRODUCTION well-structured and provides a rationale for the experiments described.
4. MATERIALS AND METHODS are sufficiently explained and is detailed enough to be reproduced.
5. RESULTS are clearly presented and supported by figures and tables.
6. DISCUSSION properly interprets the results and places the results into a larger research context, and contains all important references.
7. Conclusions are logically derived from the data presented.
8. English Language/style/grammar is clear, correct, and unambiguous.
9. Figures and tables are of good quality and well-designed and clearly illustrate the results of the study.
10. References are appropriate.
11. Regarding this article are you concerned about any issues relating to author misconduct such as plagiarism and unethical behavior.
12. Comments on the importance of the article.

Final Decision

Based on the reviewers' recommendations a final decision is made by the editor and if needed the help of a member of the editorial board (depending on the field of study). Decisions will include accept, minor revision, major revision with and without re-review, and reject. We aim to reach a final decision on each manuscript as soon as their review results are available.



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