Spring 5-10-2024

CANNABINOID RECEPTORS 1 AND 2 IN EQUINE TESTICULAR TISSUE

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by

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B.S., Southern Illinois University Carbondale, 2019

A Research Paper
Submitted in Partial Fulfillment of the Requirements for the
Master of Science Degree

School of Agricultural, Life, and Physical Sciences
in the Graduate School
Southern Illinois University Carbondale
May 2024

RESEARCH PAPER APPROVAL

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for the Degree of

Master of Science

in the field of Animal Science

Approved by:
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Southern Illinois University Carbondale
March 28, 2024

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LIST OF ABBREVIATIONS

Abbreviation	Definition
CBD	Cannabinoid
THC	Tetrahydrocannabinol
2-AG	Arachidonoylglycerol
CAGR	Compound Annual Growth Rate
ECS	Endocannabinoid System
CBRs	Cannabinoid Receptors
CB1	Cannabinoid Receptor 1
CB2	Cannabinoid Receptor 2
GPCR	G-protein coupled receptors
FAAH	Fatty acid amide hydrolase
GABA	Gamma-aminobutyric acid
IHC	Immunohistochemistry
HPG	Hypothalamic–pituitary-gonadal axis
FSH	Follicle-stimulating hormone
LH	Luteinizing hormone
DTT	Dithiothreitol
TCE	Trichloroethylene
TBST	Tris-buffered saline with 0.1% Tween® 20 detergent
DM	Dry Matter

CHAPTER 1

CANNABINOIDS IN EQUINE SUPPLEMENTS

Cannabinoid (cbd) oil is extracted from plants in the cannabis strain containing elevated levels of cannabidiol [center for disease control]. Cannabis, such as hemp, is grown for a variety of purposes. The substance abuse and mental health services administration advisory in february of 2023 stated that cbd has no psychoactive properties. Most people ingest cbd for a variety of therapeutic reasons, such as to relieve anxiety, to manage pain, or to treat inflammatory conditions.

Cannabis and its Genus

Hemp plants contain 20% CBD on average by weight [1]. Ingestion of CBD may alleviate anxiety, treat cognitive and movement disorders, pain, and epilepsy.
Plants in the cannabis genus are annual flowering plants. In general, cannabis is classified into three species: *Cannabis sativa*, *Cannabis indica*, and *Cannabis ruderalis*. However, there are some discrepancies in this classification, and some believe they are all subspecies of the same original species. Depending on the variety, *Cannabis sativa,* also commonly referred to as hemp, tends to be moderately branched and can range from 5 to 18 feet tall. *Cannabis indica* tends to be more compactly branched and can grow between 2 and 4 feet tall. *Cannabis ruderalis* typically contains very low levels of THC and is not usually grown as a crop. Generally, *Cannabis ruderalis* is used to enable auto-flowering in hybrid plants [2].

More than 60 of the 426 chemical compounds found in hemp/cannabis are cannabinoids. Of those 60 compounds, the four most researched major compounds are d-9-THC, CBD, d-8-THC, and cannabinol. Alongside the varying numbers of

compounds, preparation or extraction methods of cannabinoids can impact the potency of cannabinoid-containing products [3].

With regards to CBD's antagonistic roles, in addition to having a low affinity for CB1 orthostatic sites, CBD is also a CB1 antagonist [1]. Although Laprairie et al. reported that CBD may largely function as an antagonist of 2-Arachidonoylglycerol (2-AG), the primary endogenous ligand for the CBD receptor, and Tetrahydrocannabinol (THC), the principal psychoactive constituent of cannabis [3]. More information detailing CB1 and CB2 receptors can be found in Chapter 2.

Cannabinoid industry and research

Cannabis products are legal for medical use in 37 states, three territories, and the District of Columbia as of February 3, 2022, according to the National Conference of State Legislatures. Since the expansion of legalization of CBD products, animal owners are asking if the same health benefits can be observed if cannabinoids are given to their pets. Even livestock owners are inquiring about the uses of cannabis plants and products in production scenarios. The anti-inflammatory and mood mitigative properties of human cannabinoid supplementation are typically the two main targets for animal CBD supplements.

Along with the growth of the CBD industry, the number of research studies being published about CBD has increased. As well as the number of studies with animals and CBD. Below is a table that illustrates the increase in both areas of research. Number of articles sourced from PubMed as of 02/22/2024.

Table 1 CBD and CBD Supplement Research 2018-2022 as of 02/22/2024

Year Published	CBD Studies	CBD Animal Studies
2018	634	145
2019	813	181
2020	1,120	226
2021	1,335	269
2022	1,398	236

More specifically, PubMed reported just 44 published studies which included both CBD and Equines (As of 02/22/24). This low volume of research, along with the increasing administration of CBD to pets by owners, gave rise to the topic of this study.

Equine CBD Supplements

With many CBD products claiming to have inflammation-mitigating properties, equine owners have recently looked to CBD supplements for recovery and training purposes.

Consumers might assume from many CBD products' advertisements and testimonials that the motivation for supplementing performance horses with Hemp-based CBD products may have certain beneficial results, including inflammation reduction, anxiety reduction, and pain reduction. Typical advertised uses for equine Hemp CBD supplements include athletic recovery, injury recovery, and anxiety reduction in cases such as trailering, and arthritis.

Typically pelleted, hemp is consumed by equines as a source of CBD supplementation Hemp is a class of *Cannabis sativa* botanicals that are grown specifically for medicinal use and typically does not contain THC. Other routes of administration are feed top dress liquid CBD oil, oral paste, and transdermal methods

such as a poultice. Products such as these are provided by companies such as LameAway or VetCS.

The route of administration for equine CBD supplements has not been thoroughly researched. Studies centered around different CBD forms for humans have been a topic of research, but not specifically horses. Given the different digestion system of equines, studies should be conducted detailing inclusion rates as well as the digestibility of CBD in horses compared to humans. This information could provide better statistical data needed for formulating CBD products for horses in the future.

Hemp seeds and oil are the two most commonly researched food additives with regard to cannabis in foodstuffs. Hemp oil contains high levels of antioxidants, studies have shown these antioxidants can help in reducing the incidence of some cancers as well as neurologic disorders [4]. Hemp seeds also contain elevated levels of proteins, lipids, insoluble fiber, and carbohydrates, omega 3, and omega 6 [5]. With regards to the hemp plant itself, the plant can have a crude protein content from 5.3% to 24.5% DM (dry matter), 1.0% to 5.7% calcium DM, non-digestible fiber 28% to 80% DM, acid detergent fiber 18% to 65% DM, and total digestible nutrients 19.8 to 61.5% [6] .

The European Food Safety Authority wrote in their EFSA Journal in 2011, *Scientific Opinion on the safety of hemp (Cannabis genus) for use as animal feed*, detailing the possibility of issues arising from improper CBD/THC feed product feeding and or concentration on livestock health. Concentrations of CBD can vary among products and manufacturers. Pelleted CBD supplements tend to be 1% dry matter CBD. Although many companies do not provide a CBD value, instead they provide a value of the CBD-containing ingredient. For example, GoGreen Hemp Pellet, a pelleted hemp

supplement with claims to reduce inflammation, pain, and age-related issues, states that it provides 37.5mg of hemp extract per one tablespoon serving according to the label. This statement does not provide any analysis for CBD volume or percentage in the product or what extraction, or fractioning methods are used.

Similarly, the company Lame Away provides products including topical poultices, washes, lotions, oils, and salves all containing "full-spectrum" CBD oil. In full-spectrum CBD, every extract found in the cannabis plant is included, including at times very small amounts of THC, the psychoactive ingredient that gives marijuana its high. [7]. This could raise an important question about many CBD supplements that utilize full-spectrum CBD extracts for CBD products. With these products containing full spectrum CBD active compounds, how consistent and dependable of a product is it? Does it contain THC? These are questions that need to be answered.

CHAPTER 2

ENDOCANNABINOID SYSTEM AND CANNABINOID RECEPTOR PHYSIOLOGY

Although there has been research published on the distribution and function of endocannabinoids in rats, mice, and humans, little research has been done on horses. The rapid expansion of Endocannabinoid System (ECS) research throughout the body has gained momentum, thanks to advances in biotechnology. An overview of the components and tissue distributions of this complex endocannabinoid system is presented in this chapter.

Cannabinoid receptors regulate physiologic processes such as appetite, pain perception, mood, and memory as part of the endocannabinoid system. Cannabinoid receptors are members of the G protein-coupled receptor superfamily. For this paper, two Cannabinoid Receptors (CBRs) will be discussed, Cannabinoid Receptor 1 (CB1) and Cannabinoid Receptor 2 (CB2).

Several mammalian species exhibit similarly distributed and well-characterized CBRs, but equine tissue distribution and expression patterns remain to be examined in many areas [8].

There is ample evidence that mammalian species contain an endocannabinoid system (ECS). Invertebrate species, as primitive as the Hydra, have been described as possessing this trait. Several organ systems, including those of the nervous system, immune system, and many other organ systems, are provided with homeostasis by this otherwise ubiquitous system. Three components make up the ECS: endogenous ligands, GPCRs, and enzymes that degrade and recycle endogenous ligands. Two

endogenous molecules act as ligands in the ECS. The endogenous ligands are anandamide (arachidonoyl ethanol amide) and 2-AG. There are other putative GPCRs being considered as part of this system, in addition to GPCRs described as part of this system [9].

Endogenous Ligands

N-arachidonoyl ethanolamine (anandamide, AEA) and sn-2 arachidonoyl glycerol are two of the most recognized endogenous cannabinoid ligands [10]. At least ten enzymes are involved in the biosynthesis or catabolization of AEA and 2-AG. Upon release from the postsynaptic sites, their ligands bind to the presynaptic cannabinoid receptors, modulating the release of neurotransmitters.

The two compounds are synthesized on demand because of the activation of GPCRs in response to an increase in intracellular calcium levels. [11,12]. N-acyltransferases and phospholipases D are involved in the synthesis of anandamide, while diacylglycerol lipases and phospholipases C are involved in the synthesis of 2-AG. Neither endocannabinoid has a long duration of action because of its rapid degradation. During degradation, phospholipids play an important role. Fatty acid amide hydrolase (FAAH) degrades anandamide primarily into ethanolamine and arachidonic acid, resulting in anandamide being converted into ethanolamine. It has been suggested that endogenous cannabinoid ligands play several physiological roles, including modulating sleep, appetite, reward, and immune function [13].

The peripheral CB2 endocannabinoid system plays both an inflammatory and an anti-inflammatory role, as indicated by the downregulation of inflammatory mediators and cells resulting from activation of the CB2 receptor [14]. A central and peripheral pain

circuit is affected by antinociceptive properties through CB1 receptors. It has been shown that endocannabinoids influence appetite and reward in complex ways through the regulation of GABAergic and glutamatergic input to dopaminergic brain regions through the CB1 receptor, which leads to a decrease in avoidance behavior and an increase in appetitive responses to rewarding stimuli like food or substance abuse [15].

Cannabinoid Receptor 1 (CB1)

Structurally, CB1 receptors contain seven transmembrane helices, a glycosylated N-terminus, and a cellular matrix-enclosed C-terminus. CB1 receptors have been found in high numbers in neural tissues such as the hippocampus and the basal ganglia [16,17,18]. In the central nervous system, the CB1 receptors are most abundant in the amygdala, hippocampus, cortex, basal ganglia outflow tracts, and cerebellum [19]. GABA (gamma-aminobutyric acid) and glutamate are both released by glutamatergic and GABAergic cells upon their activation [20]. Researchers have discovered that CB1 receptor activity activates calcium and potassium ion channels, thus modulating neurotransmitter release in a pertussis toxin-dependent manner. The receptor can exist as homodimers or as heterodimers or hetero-oligomers attached to other GPCRs. The CB1 receptor features an allosteric modulatory binding pocket in addition to its main binding site. CB1 receptors have also been found in extra neural sites including the testes, eyes, vascular endothelium, and spleen.

Cannabinoid Receptor 2 (CB2)

Like CB1 receptors, CB2 receptors also contain seven transmembrane helices, a glycosylated N-terminus, and a cellular matrix-enclosed C-terminus. Cannabinoids' immunomodulatory properties are partially explained by the CB2 receptors discovered

in 1993 [21]. CB2 receptors are found in a wide range of cell types, including immune system cells, astrocytes, microglia, and neurons in the central nervous system [21,22]. In addition to maintaining bone mass and reducing inflammation, CB2 activation plays a key role in neuro-defense functions. The use of CB2 agonists has been investigated as a treatment for neurological disorders such as Huntington's chorea and Alzheimer's disease [23,24]. GI-Goa subunits of CB2 receptors stimulate the adenylyl cyclase enzyme by inhibiting its activity [19] by coupling with gai/o subunits, which results in elevated cAMP levels inside cells, and by affecting the Ras-Ref-MEK-ERK pathway [25,26].

CB2 receptors have a more defined presence in the brain than CB1 receptors. CB2 receptors are typically associated with inflammation and are primarily located on the resident macrophages of the central nervous system [27,28]. CB2 and CB1 receptors in neural tissues work independently of each other [29]. A low level of CB1 receptors has been detected by immunohistochemistry in human post-meiotic germ cells, spermatocytes, and spermatids as well as Leydig cells. [30]. CB1 receptors have been detected via Western Blotting and IHC (immunohistochemistry) in rodent spermatogonia and Leydig cells [31].

CB2 receptors were the target of a veterinary study that focused on treating chronic pain linked to osteoarticular equine disease. The focus of this study was to examine the effects of CB2 agonists on chronic pain in horses compared to the standard route of treatment which typically includes steroid and non-steroidal treatment. Using CB2 agonists as a treatment for chronic pain was intended to minimize the negative side effects associated with long-term steroid and non-steroid use [32].

CHAPTER 3

MAMMALIAN SPERMATOGENESIS

Cannabinoid receptors have been localized in Leydig cells, spermatocytes, and spermatids [33], therefore it is important to understand how these cells impact the spermatogenesis cycle as well as male fertility, in general.

Spermatogenesis is the process by which the male gametes, called spermatozoa, are formed in mammalian testes. Much of this process takes place in the parenchymal tissue of the seminiferous tubules within the testes. There are two types of cells in the seminiferous tubules: somatic cells and germ cells. Somatic Nurse Cells include Sertoli cells and Myoid cells. Germ cells include the stages of spermatozoa development: spermatogonia, spermatocytes, and spermatids.

Spermatogenesis in Stallions, Humans, and Bucks (male mice)

Spermatogenesis begins at 13-18 months of age for stallions, 7-8 weeks of age in mice, and 9-15 years of age for humans [34,35]. We included data from human and mouse studies due to them being the predominant model used in research studies. Production quantity and efficiency, while constant throughout the animal's lifespan, slowly declines as the animal progresses in age. Spermatogenesis in mammals never fully ceases, unless interrupted by trauma or disease.

Semen Evaluation

Semen evaluation is a combination of volume, sperm concentration, sperm morphology, and motility. Each of these parameters is assessed to predict the animal's breeding efficiency. Neither of these parameters by themselves 100% reflect the animal's fertility, but their combination provides a viable representation of the animal's

fertility. Improper sperm morphology or motility would possibly result in the sperm being unable to swim to the oocyte. Also, low semen volume not only would provide an inadequate concentration of sperm but could also reduce sperm viability in the female reproductive tract due to a lack of supporting nutrients from the semen. Low sperm concentration would lead to dwindling numbers of viable sperm, leading to either low fertility rates or embryos with defects due to being fertilized by malformed sperm. Low percentages of progressively motile sperm would have similar effects as low sperm concentrations, low sperm numbers are just as detrimental as sperm being unable to reach the oocyte entirely [35].

CBD Cellular Pathways in Male Reproduction

Barchi, Marco, et al, in 2019, showed that CB receptors play a very large role in male reproduction at central and gonadal levels. CB receptors have been shown to positively modulate the hypothalamic–pituitary–gonadal axis (HPG) axis, Sertoli and Leydig cell function, germ cell differentiation, and sperm function. It was also shown that CB receptors play a regulatory role in spermatogenesis by promoting the meiotic entry of spermatogenesis and spermiation. Specifically, for CB1 receptors, studies have shown that CB1 receptors are actively correlated with Leydig cell differentiation, steroidogenesis, spermiogenesis, and sperm maturation. For CB2 receptors, the receptors have been located predominantly in the testes concerning the male reproductive system. However, the normal physiological function of CB2 receptors in the male reproduction system is still being researched. Studies suggest that CB2 receptors could work in conjunction with CB1 receptors to regulate sperm motility [36].

CHAPTER 4

MALE HORMONES AND CBD

Researchers are still studying the effects of cannabis on male sexual and reproductive functions. Direct inhibition of testicular spermatogenesis and/or steroidogenesis may cause some effects, while altered hormone levels may cause others.

Male Hormones and CBD Research Overview

A male's entire reproductive system depends on hormones to function properly. In other words, hormones are chemicals that stimulate or surpass the activity of cells or organs. Male reproductive hormones such as luteinizing hormone (LH), follicle-stimulating hormone (FSH), and testosterone participate in the process of reproduction in males.

The pituitary gland is responsible for the production of FSH and LH and is located at the base of the brain, the pituitary gland is responsible for many functions. As a result of LH being released, testosterone is produced, which is essential to carry out the process of spermatogenesis in the body. In addition to influencing muscle mass and strength, testosterone has also been shown to play an important role in developing male characteristics, such as fat distribution, bone mass, and sex drive [37]. Long-term cannabinoid exposure in male mice resulted in altered sperm morphology and disrupted spermatogenesis [38] and may modulate sperm function during fertilization [39]. Chronic exposure to or use of cannabinoids in humans leads to alterations in spermatozoa production by the testis [40], inhibits spermatogenesis [41], and can reduce the mass or size of testes and accessory reproductive glands [42].

There are two distinct morphological compartments in the testis: interstitial tissue and seminiferous tubules. A sizable portion of interstitial tissue is comprised of vascular, lymphatic, and connective tissue elements, macrophages, fibroblasts, and androgen-secreting cells called Leydig cells. Differentiating germ cells reside in the seminiferous epithelium, which is supported and protected by Sertoli cells. In rat-isolated Sertoli cells, THC has been reported to reduce FSH-induced cAMP accumulation at concentrations that do not affect ATP levels or cytotoxicity. Cannabinoid receptor activation causes adenylyl cyclase inhibition, which, in turn, triggers this effect.

There are three phases of spermatogenesis: mitotic renewal and proliferation of spermatogonia, meiosis, and spermiogenesis. Humans exposed to cannabinoids can sustain a decrease in the number of spermatozoa produced in the testes, a reduction of the amount of testosterone produced and secreted by Leydig cells, a reduction in the size of the testes and reproductive accessory organs [43].

The male reproductive system has consistently been shown to be affected by cannabinoids and THC in animal studies. Both acute and chronic doses of CBD cause significant reductions in testosterone production by rat testis microsomes [44] and decreased testicular weight [45].

Several studies have suggested that CBD reduces testosterone synthesis in rodents, which increases abnormally formed sperm [46]. Animal studies have shown that the functioning of the male gonadotropin is altered as a result. A dose of CBD resulted in a significant reduction in serum testosterone concentrations in rhesus monkeys [47]. CBD has been seen to cause significant reductions in testosterone production by rat testis microsomes at acute and chronic doses [44], as well as loss of

testicular weight [47]. Reduced testosterone synthesis may be responsible for the decreased testicular weight.

The effects of cannabis smoking on serum LH are similar to those of non-smoking controls or baseline levels prior to smoking cannabis. There is an association between chronic marijuana use and decreased plasma testosterone levels. However, other studies have not supported these findings. As a result of chronic exposure to or use of cannabinoids, Leydig cells produce less testosterone.

CHAPTER 5

LABORATORY RESEARCH

Hypothesized Side Effects of Cannabinoid Ingestion by Horses

CBD has been shown to negatively impact male reproduction in humans and rodents. Giving CBD-based supplements to intact male horses (stallions) could lead to negative impacts on the reproduction of the stallion. Effects could be reduced sperm viability [36,46] and reduced levels of male sex hormones including FSH, LH, and testosterone [44,47]. All mentioned side effects could lead to lowered fertility of the stallion. Beyond physical side effects to the stallion, these side effects could financially impact equine breeding facilities.

Limitations on the number of available stallions prevented direct administration of CBD products. Therefore, this research uses laboratory techniques utilizing harvested equine testicular tissues to determine the absence or presence of CB receptors.

Laboratory Research

Partially successful benchtop laboratory research was conducted on equine testicular tissues to grossly identify cannabinoid receptors in the equine testis. These research methods included Western Blotting and Reverse Transcription Polymerase Chain Reaction. Reverse Transcription Polymerase Chain Reaction and its Sanger Sequencing results for the Equine CB1 receptor showed the presence of the RNA necessary to produce the Equine CB1 receptor.

Animals And Testicular Tissue Processing

Four equine Testes were collected during routine castrations via a licensed veterinarian. Equine Breeds included were quarter horse and Dutch pony. Ages ranged from 9 months to 36 months. Testes were immediately placed on ice in sample bags for transport to the lab. The transport time to the lab was 1 hour. The testes were manually sliced, into approximately 1/8” transverse sections and frozen at -20°C.

RNA Extraction for rtPCR

The GeneJET RNA Purification Kit (Thermo Scientific (REF# K0731 LOT# 01067803) using the provided protocol (Chomczynski, P. and N. Sacchi. 1987) was used to isolate RNA. Thirty micrograms of testicular tissue were disrupted using three hundred microliters of lysis buffer supplemented with dithiothreitol (DTT) and forceps in a 1.7ml microcentrifuge tube. Once homogenized, 600µl of Proteinase K was added to digest sample proteins. The sample was then centrifuged, using an Eppendorf 5415c centrifuge, for 10 minutes at >12,000 x g, the supernatant (lysate) was then transferred to a new RNase-free microfuge tube.

Seven hundred microliters of the lysate were then transferred to the included GeneJET Purification Column and centrifuged for 1 min at >12,000 x g, the flow through was discarded and the remaining lysate was transferred to the column and centrifuged. Three individual wash steps using the included Wash Buffer 1 and Wash Buffer 2 were conducted to purify the extraction sample. One hundred microliters of the included nuclease-free water were added to the center of the GeneJET Purification Column and centrifuged for 1 minute at >12,000 x g. Following extraction, RNA concentration was determined with an Implen NanoPhotometer N60 MicroVolume. The RNA-containing

flow through was stored at -20°C.

Reverse Transcription

Reverse transcription was conducted utilizing the NoScript Reverse Transcription Mix, Random Primers from Promega (REF# ADA2800 LOT# 00004504713), and the included protocol. Incubations were performed using the MiniPCR Mini8 thermal cycler (SKU: QP-1000-01).

For each cDNA reaction, 10µl of GoScript Reverse Transcription mix (A5001 kit) was prepared as follows: 4µl of Nuclease free H_2O, 4µl of GoScript Reaction Buffer with the Random Primer mix, and 2µl of GoScript Enzyme Mix. The 10µ of reaction mix was added to 5µl of sample RNA (at 1.1305µg/µl concentration) and 5µl of Nuclease free H_20 for a final volume of 20µl.

The Reverse transcription mix was aliquoted into 4 x 5µl PCR tubes and incubated in the MiniPCR Mini8 thermal cycler with the time and temperature protocol in *Table 2*. Post-reverse transcription samples were consolidated into a single 1.7ml microcentrifuge tube stored at -20°C.

Table 2 Reverse transcription temperatures and cycle lengths

Step	Temperature	Time	Cycles
Anneal Primer	25°C	5 minutes	1 cycle
Extension	42°C	60 minutes	1 cycle
Inactivation	70°C	15 minutes	1 cycle

Primer Design

Primer sequences were designed by searching GenBank for known CB1 and CB2 sequences from bovine, rat, and mouse sequences. We then utilized these known sequences to identify regions of homology. The regions were used as templates to

create forward and reverse primers using the Pick Primers software feature. Ten forward and reverse pairs of primers were assessed (See Table 2).

Table 3 Primers and Sequences

Batch #	Oligo Name	Oligo #	Sequence (5'-3')
WD 09647264	1F EqCanR1 784	3027345662000090	GCTTGTTTGTTTTCCCTCCCA
WD 09647265	1R EqCanR1 784	3027345662000100	CTTAGGCCTGGTGA CGATCC
WD 09647258	2F EqCanRec2 331	3027345662000030	ACCCACCTACG TACAAAGCC
WD 09647259	2R EqCanR2 331	3077345662000040	TTGGCCAACCTCAC ATCCAG
WD 09647256	1F EqCanR2 114	3027345662000010	TTCCCCCTGATCCCCAATGA
WD 09647257	1R EqCanR2 114	3027345662000020	TACATGCTGATGGGCCTTCC
WD 09647260	F EqGADPH187Smits	3027345662000050	CAGAACATCATCCCTGGCTTC
WD 09647261	R EqGADPH187Smits	3027345662000060	ATGCCTGCTTCACCACCTTC
WD 09647262	F EqUBC 206Smits	3027345662000070	GCAAGACCATCACCCTGGA
WD 09647263	R EqUBC 206Smits	3027345662000080	CTAACAGCCACCCCTGAGAC
WD 09647266	4F EqCanR1 649	3027345662000110	GCTTGTTTGTTTTCCCTCCCA
WD 09647266	4R EqCanR1 649	3027345662000120	ATTGGGGCTATCTTTGCGGT
WD 09504108	3F EqCnbndRec1 317	3026933030900010	CATTAACACGTGATGGGACCG
WD 09504109	3R EqCnbndRec1 317	3026933030900020	TCCGTCGGTCGGTCAATAAG
WD 09504110	2F EqCnbndRec1 618	3026933030900030	ATGGGACCGCGCTTCATAAAT
WD 09504111	2R EqCnbndRec1 618	3026933030900040	GAGGAAGAGACCCGCAACAG
WD 09504112	4F EqCnbndRec1 322	3026933030900050	GGCTATTAACACGTGATGGG
WD 09504113	4R EqCnbndRec1 322	3026933030900060	CTCCGTCGGTCGGTCAATAA
WD 09504114	5F EqCnbndRec1 608	3026933030900070	ACCGCGCTTCATAAATGGGAC
WD 09504115	5R EqCnbndRec1 608	3026933030900080	GAGACCCGCAACAGGGAATC

Reverse Transcription PCR

Reverse Transcription PCR was conducted on the equine teste DNA sample utilizing the MiniPCR 5X EZ PCR Master Mix Load Ready mix (SKU: RG-1000-01) and included protocol. Once the concentration of the template DNA was determined utilizing an Implen NanoPhotometer N60 MicroVolume Spectrophotometer, a master mix was made using the template DNA, the selected forward and reverse primers, and the MiniPCR 5X EZ PCR Master Mix Load Ready-mix. Volumes are shown in *table 3*.

Table 4 Volumes and Reagents of PCR Master Mix

Reagent	Volume
MiniPCR 5X EZ PCR Master Mix Load Ready	32 µl
Forward Primer (10µM)	3.2 µl
Reverse Primer (10µM)	3.2 µl
DNA Sample (12.5ng/µl)	2 µl
Distilled H_2O	119.6 µl

The PCR master mix with template DNA was aliquoted into eight 20µl PCR reaction tubes and incubated in the MiniPCR Mini8 thermal cycler using the cycle length and temperature protocol in *Table 4*. PCR products were then directly loaded on a 2% agarose gel for gel electrophoresis.

Table 5 PCR cycle lengths and temperatures

Step	Temperature	Time	Cycles
Initial Denaturation	95°C	100sec	1
Denaturation	95°C	30sec	30
Annealing	62°C	30sec	
Elongation	72°C	180sec	
Final Elongation	72°C	300sec	1

Gel Electrophoresis

Gel electrophoresis was conducted utilizing the MiniPCR blueGel Electrophoresis System and included protocol. A 100bp DNA ladder (LOT# LAD1910) was used as a DNA band size reference. A 2% agarose gel was prepared from 0.4g of MiniPCR agarose powder (LOT# B20191111) and 20 mL of 1x TBE solution. The 9-well comb was utilized and 20µl of PCR product was loaded into each well. The gel

electrophoresis ran until the ladder extended the length of the gel. Gels were visualized via the MiniPCR blueBox Illuminator (Model: QP-1700-01).

Gel Extraction

Band extraction following gel electrophoresis was conducted utilizing the PureLink® Quick Gel Extraction Kit from Invitrogen DNA (Catalog numbers K2100-12 and K2100-25, Publication Part number 7015017 and MAN0003719).

Before extraction, a water bath was equilibrated to 50°C. A minimal area of gel was excised containing the DNA fragment of interest. The excised gel slice was weighed and the included gel solubilization buffer L3 was added in a 1.7mL polypropylene tube with a 3:1 ratio of L3 buffer to gel slice (1.2ml Buffer L3: 400 mg gel). The tube containing the gel slice and L3 buffer was then placed into the 50°C water bath. Incubation time at 50°C was 10 minutes, and the tubes were inverted every three minutes to ensure the gel dissolved. The tube containing the gel and L3 buffer was then incubated at 50°C for 5 minutes.

The dissolved gel was then loaded into the included Quick Gel Extraction Column inside of a wash tube, one Quick Gel Extraction Column was used per 400mg of dissolved gel. The extraction tube was then centrifuged, using an Eppendorf 5415c centrifuge, at >12,000g for 1 min, and the flowthrough was discarded. The remaining dissolved gel was processed through the column, as stated above. Following gel extraction, 500µl of the included Wash Buffer W1, was added to the column, the column was then centrifuged at >12,000g for 1 min, and the flowthrough was discarded. The column was then centrifuged at maximum speed for 2 min to remove the ethanol, the flowthrough was then discarded. The extraction column was then placed into a recovery

tube and 50µl of the included Elution Buffer E5 was added to the center of the column and incubated for 1 min at room temperature. The purified DNA was then collected from the extraction column via centrifuge at >12,000g for 1 min, and stored at 4°C for immediate use or -20°C for long-term storage.

Sequencing

DNA sequencing was conducted at the Core DNA Sequencing Facility at the University of Illinois, Urbana-Champaign. The sequencing center required 5µl of template DNA sample for sequencing. Samples were sent for both CB1 and CB2 receptors labeled as EqCanR1 and EqCanRec2. The samples were labeled with the same name as the primer used during PCR, as well as the sample tissue species and age. CB1 concentration was 4.98 ng/µl and CB2 was 5.25ng/µL. These concentrations were lower than the sequencing center suggested for the kilobase pair level of the samples, sample concentration was not applicable. The concentration of the submitted samples was achieved using gel extractions from a complete 9-well gel.

Sequencing result sequences were run through the NCBI Basic Local Alignment Search Tool (BLAST) program.

Protein Homogenization for Western Blotting

Testicular tissues intended for western blotting were stored at -80°C before homogenization following slicing. Samples were thawed in an ice water bath for approximately 6 hrs. Once thawed enough to manipulate, the testes were decapsulated and approximately 1g samples were excised. The samples were homogenized using an Ultra Turrax tissue blender and SDS Lysis buffer. The homogenate was then centrifuged at 12,000 G for 5 min and the supernatant containing the proteins was collected and

allocated before being stored at -20°C.

Western Blot Protocol

Western blots were conducted utilizing the protocol provided by Dr. Dale Hales of Southern Illinois University Carbondale and a Bio-Rad Mini Trans-Blot® Cell Tetra Electrophoresis system. 30µg of homogenized protein with Laemmli buffer was denatured at 85°C for 10 min before being loaded into a 2% acrylamide gel. Gels were electrophoresed in the Bio-Rad chamber filled with 1X running buffer at 100 volts until the loading dye reached the top of the resolving gel. The gels were subsequently electrophoresed at 125 volts until the loading dye reached the bottom of the resolving gel. The gels were removed from the casting plates and the stacking gel containing the loading wells was cut off and discarded. 2,2,2-trichloroethane (TCE) was then activated using the Bio-Rad Gel Doc EZ Gel Documentation System stain-free gel protocol.

Transfer from gel to blot was conducted using the transfer cassette and sponges included in the Bio-Rad Mini Trans-Blot® Cell Tetra Electrophoresis System. The transfer was conducted in a chilled 1X transfer buffer solution at 100 volts for 1 hr. Trichloroethylene (TCE) was then measured using the Bio-Rad Gel Doc EZ Gel Documentation System stain-free blot protocol. Primary antibodies were sourced through Abcam and Secondary antibody was sourced through ThermoFisher. (Table 5)

Table 6 Antibody Information

Name	Source	Product Number	Host Species
Anti-Cannabinoid Receptor I antibody	Abcam	AB23703	Rabbit
Anti-Cannabinoid Receptor II antibody	Abcam	AB3561	Rabbit
Goat anti-Rabbit IgG (H+L) Cross-Adsorbed Secondary Antibody, FITC	ThermoFisher	F2765	Goat

Following TCE measurement, the blot was blocked with soy milk for 5 minutes and rinsed with Tris-buffered saline with 0.1% Tween® 20 detergent (TBST). Each primary antibody was incubated on the blot at 1:250 concentration at 4°C for 24 hours. Following incubation, the blot was rinsed 3 times for 5 minutes each with TBST. The secondary antibody was then incubated over the blot at 1:500 concentration for 1 hour at room temperature. The blot was rinsed 3 times for 5 minutes each following incubation with the secondary antibody. Blot imaging was conducted using the LiCor Odyssey CIX imaging system.

rtPCR Results

Current results are centered around rtPCR. We sent off a CB1 and CB2 receptor DNA template from gel extractions following gel electrophoresis, to the University of Illinois Sequencing Lab. Results after sequencing were run through the NCBI BLAST program, which reported a sequence match for the CB1 receptor DNA. Indicating the presence of the DNA sequence synonyms for cannabinoid receptor 1. Sequencing for CB2 was not successful.

The CB1 and CB2 gels showed crisp bands for both receptors. The CB1 gel (figure 4) presents a solid band across all 8 replication wells roughly near the 700-900 base pair ladder bands. The CB2 gel (figure 5) also presented crisp bands with less

random binding visible than the CB1 evident by less "streaking" trailing the bands. The CB2 gel showed solid bands across all 8 replication wells between the 300 and 400 base pair ladder bands. The CB2 gel indicated a closer base pair band to the intended 331bp than the CB1 gel did.

Table 7 Sequencing BLAST Results from 12/21/21

Sample Name	***Intended Result***	***Blast Results***
EqCanR1	Equine Cannabinoid Receptor 1	Select seq NM_001257151.1 Equus caballus cannabinoid receptor 1 (CNR1), mRNA
EqCanRec2	Equine Cannabinoid Receptor 2	Procambarus clarkii isolate 11 cytochrome oxidase subunit I (COI) gene, partial cds; mitochondrial

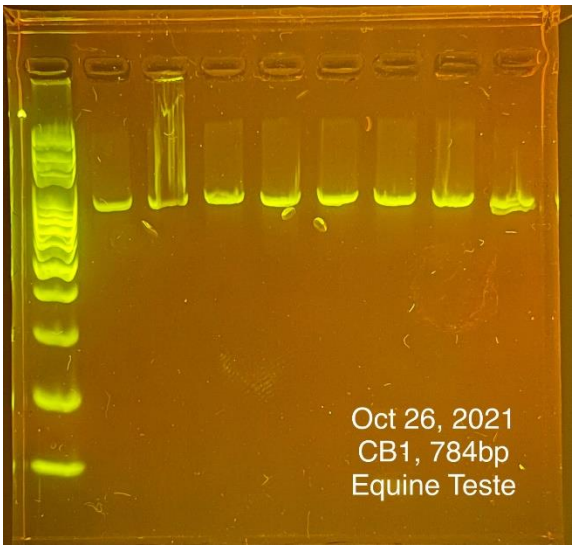

Figure 1 PCR Gel 10/26/21 CB1, 784BP Equine Teste

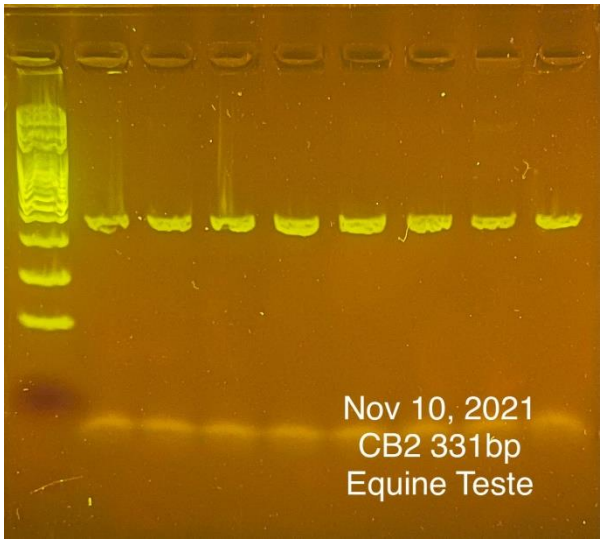

Figure 2 PCR Gel 11/10/21 CB2, 331BP Equine Teste

Western Blotting Results

Western blotting did not render clear results. Protein transfer seemed to be successful following the gel-to-blot transfer step, although no bands would be visible following the incubation period with antibodies. A beta-tubulin positive control was conducted and showed a positive band for beta-tubulin (see images below).

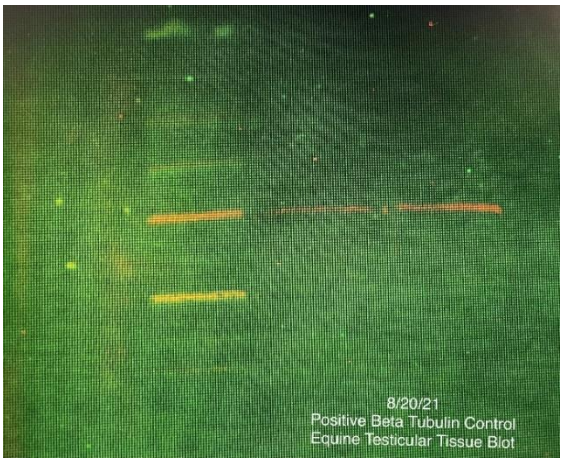

Figure 3 Positive equine tissue beta-tubulin western blot

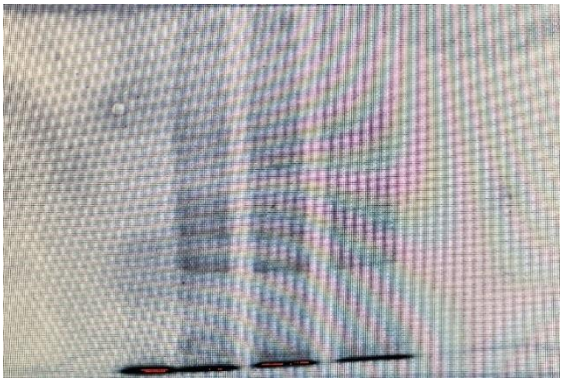

Figure 4 Positive western blot transfer blot with equine and rat teste proteins

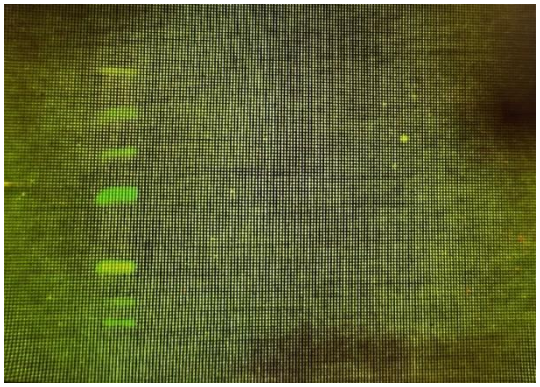

Figure 5 Western blot with the absence of fluorescence for cannabinoid receptor proteins

Along with the beta-tubulin control, a rat teste homogenate control was conducted with western blotting. This blot was conducted following the same western blotting protocol as stated previously while utilizing the same volume of homogenate as equine teste homogenate. Results were identical as before; proteins would be visible present on the blot following the gel-to-blot transfer (figure 8), though no bands would be present following the antibody incubation period (figure 7).

CHAPTER 6

CONCLUSION AND FUTURE RESEARCH

Conclusion

In this article, we were able to shed light on a possible side effect of CBD supplementation on the fertility of intact male equines. Given the research that has shown impacts on male fertility parameters such as sperm count, spermiation, and male hormone fluctuation, future research studies could be used to show an impact on these same parameters in male equines.

Through rtPCR, our laboratory research was able to show that mRNA for CB1 receptors was present in equine testicular tissues. This result indicated that the building blocks for the CB1 receptor are present in the equine teste tissue. Further research should be conducted to better determine their presence.

Potential future laboratory procedures

Due to the failed antibody validation by Raybiotech, and the lack of in-house western blotting results, the first major next step would need to be conducting a more specific antibody selection targeting equine tissues specifically. This could be conducted via Raybiotech, or a similar biotechnology company, purchasing third-party antibodies seeing the absence of an equine-specific CB1 or CB2 antibody from many online purveyors.

Chiocchetti et al. cited research about cannabinoid receptors in the equine dorsal root ganglia, these receptors were found in neural tissues with highly concentrated cannabinoid receptors in the human body [26]. This brought into question the efficacies

of the tissue homogenate concentration used in the attempted western blot procedure about the concentration of the intended CB receptors.

Upon consultation with a neural professional (Dr. Joseph Cheatwood (SIUC), personal communication), who hypothesized that the procedure used by Galiazzo et al. in 2021 was viable except when comparing the concentration of tissue homogenates of equine dorsal root ganglia and equine testes. Given the teste is not a neural organ, it could be hypothesized that the receptors are present in the tissue homogenate, simply at a low concentration undetected by western blot analysis. An approach to resolve this issue could be a procedure to concentrate the intended structures in the testes containing the receptors. This procedure could be as simple as the reduction of moisture in the homogenate sample or as specific as isolating Leydig or Sertoli cells from the testes.

Recommended research projects

Future studies could be to direct feeding trials combined with semen collections and evaluations to provide a direct proof of concept, although the number of stallions needed to produce an appropriate level of statistical confidence may be unattainable without substantial funding support.

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Research Paper Title:
Cannabinoid receptors 1 and 2 in Equine Testicular Tissue

Major Professor: Karen L. Jones