Establishing a new detection system for anti gene doping purposes

Morten Klitgaard Nøhr

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Group Member: Morten Klitgaard nøhr

Written by:

____

Morten Klitgaard Nøhr

Supervisors: Jacek Lichota, Associate professor at the Department of health science and Technology, Aalborg University, and Parisa Gazerani, Associate professor at the Department of health science and Technology, Aalborg University.

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1. 0 PREFACE

This work described in present thesis was undertaken for obtaining the Master of Science (M.Sc.) degree in medicine with industrial specialisation. The experimental work was performed at the Laboratory of Neurobiology, University of Aalborg, Aalborg animal facility, Aalborg sygehus, and Herlev animal facility, Herlev sygehus from 1st September – 31th of May under the supervision of associate professor Jacek Lichota and associate professor Parisa Gazerani.
References are denoted according to the Vancouver Referencing system

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3.0 ABSTRACT

Background Doping becomes more prevalent in the society and the sports world. A relatively new doping method has been added to the prohibited list issued from the World anti-doping agency, which is gene doping. The use of the preproenkephalin gene has the potential of becoming a doping candidate. The gene encodes two enkephalin proteins, that bind to the μ-, δ-, and κ-receptors which exert analgesic effects. It is therefore believed that the insertion of this gene into an athlete can induces higher pain tolerance, which will provide him/her with an unnatural benefit in sports.

AIM To insert the preproenkephalin gene as a potential candidate for gene doping against pain by electroporation method and to establish a detection method in blood and muscle for inserted enkephalins using quantitative polymerase chain reaction.

Method Plasmid encoding the genes for mouse preproenkephalin (PENK) and green fluorescent protein (GFP) was transfected into mouse muscles cells using electroporation. A control group was also electroporated, which received a saline solution instead of plasmid.
The cells were extracted after 24 and 48 hours, which were then analysed using a qPCR assay. An *in vitro* experiment was performed to investigate if PENK plasmid could be efficiently expressed in C2C12 myoblast cells after transfection using a cationic polymer as transfection agent. This was visualised on confocal microscopy.

Results The results showed that it is possible to insert and detect the PENK gene in the muscle. It was not possible to detect the gene in the blood after electroporation. The C2C12 transfection confirmed that the PENK gene could be efficiently expressed. Small clusters were visualised in the C2C12 cells indicating the PENK-GFP proteins are packed in vesicles.

Conclusion This study indicates that it is indeed possible to use PENK as a gene doping candidate. Furthermore, it was impossible to detect this gene using qPCR at other sites then the actual transfected muscle. Thus, blood samples failed to detect the PENK gene, and as such making gene doping by electroporation ideal as a doping method. As such, it is imperative for anti doping purposes to find practically applicable techniques that would be able to detect this type of gene doping

4.0 ABBREVIATIONS

cDNA	Complementary DNA
Ct	Cycle threshold
DAPI	4',6-diamidino-2-phenylindole
DMEM	Dulbecco modified Eagle's medium
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
EPO	Erythropoietin
GFP	green fluorescent protein
LB	Lysogeny broth
mRNA	Messenger RNA
NMDA receptor	*N*-Methyl-D-aspartate receptor
RT-PCR	Reverse transcriptase polymerase chain reaction
PENK	Proenkephalin
qPCR	real-time polymerase chain reaction
RNA	Ribonucleic acid
Rt-	Minus Reverse transcriptase
WADA	World Anti-Doping Agency

5.0 Introduction

Doping has been used by athletes since ancient Greece, and its prevalence has been increasing since, thus showing that some athletes are willing to consider and use performance-enhancing agents, in order to increase their chance of becoming the winner in athletic competitions. According to the Danish anti-doping agency, an investigation showed that 3 % of the average user in fitness centres have either used, or are using anabolic steroids (1). In addition to this, 20 % of the fitness centre users reported that they knew people who were using steroids to enhance physical performance (1). Hence, doping is a great concern both for the sport community and the society.

Different types of performance-enhancing drugs are used in different branches of sports, e.g. erythropoietin is widely used in cycling and testosterone in weightlifting competitions. The world's anti-doping agency (WADA) has therefore formed a list of doping agents, which contains all drugs and methods that can potentially be used as performance-enhancing agents. On this list a relatively new doping method has been added, which is gene doping. This is a technique, in which a gene of interest is inserted into either the cell nucleus transiently or even permanently in the genome of the recipient (2). The inserted gene is capable of producing the desired protein, thus the gene doping user receives the same effect as using conventional doping, but without a risk of efficient detection and disqualification. This is because the doping protein is produced by the users own cellular machinery, and it is therefore impossible to distinguish from the users own specific proteins.

The idea of inserting a gene of interest into the cellular nucleus comes from a potential therapeutic interest. Patients who are unable to produce a specific kind of proteins could have a gene inserted into their cell nucleus, which would then lead to a production of the missing proteins, thus exerting a therapeutic effect. Gene therapy opens up a continuum of new possibilities for modifying the genome and this has the potential to be applied out of therapeutic purposes e.g. for illegal and doping purposes.

In order to insert a gene, different methods have been applied. One of these uses viruses as vectors (3). However, delivery method by viral vectors yields some adverse effects, including a high risk for developing cancer (4). Therefore, studies have used other delivery methods for gene insertion. One of these methods is called electroporation (5), which is a technique that uses electrical pulses and voltage to make the cell membrane permeable to nucleic acid. The method has proven successful, and it is relatively safe to use (6)(7).

In a recent study, the researchers showed that plasmid DNA encoding erythropoietin (EPO) could successfully be transfected into muscle tissues of NMRI mice (8). This was confirmed by measuring the levels of EPO in blood samples. The results showed an increase in EPO, which reached to the desirable therapeutic level (8). This indicates that it is possible to insert a gene of interest and obtain a measurable effect, which could be a therapeutic method for helping patients with protein deficiencies (8). However, this also provides the evidence for a transfection method capable of producing a beneficial effect in sports.

For this reason, WADA is in need of a technique which can detect such new doping methods, in order to prevent athletes from a fraud (9).

In addition to EPO, a number of other genes could potentially be applied for doping purposes. One of the potential candidates would be insertion of a gene that encodes enkephalins. The effect would contribute to a higher pain tolerance while performing heavy sports related tasks. Enkephalins are neuropeptides, which binds to opioid receptors in the nervous system, causing analgesic effects (10). The present study will therefore focus on insertion of a gene encoding preproenkephalin (PENK) into muscle cells of mice, and subsequently will attempt to detect this gene in the plasma and muscles by quantitative polymerase chain reaction (qPCR). The transfection method is electroporation, as this is the most possible method for athletes to use due to its safety and efficiency (6)(7).

To be applied as a routinely detection method in sports, it should be easy and fast to use. The easiest way of obtaining a tissue sample would be to take a blood sample. This can be done rather quickly, with minimal discomfort for the athlete. WADA would therefore in a matter hours be able to analyse and detect gene doping. For this scenario to become a success the leucocytes most be transfected, when the muscle cells are electroporated. Since leucocytes are the only nucleic holding cells in the blood, and would therefore be able to express an inserted gene. Hence, it would be possible to analyse and detect a higher gene expression from a gene doping candidate in the blood.

Since the enkephalins exert analgesic effect, their exact role and contribution in the pain system will be elaborated in further details in the following section.

5.1 The Pain Pathway

The body's ability to recognise pain serves as an alarm system to prevent damage to a tissue. This is an evolutionary trait that has secured the survival of many organisms. (11) Individuals who are born without this sense often suffer infections and cannot survive a full life. This is because that several pathological signs are being unnoticed due to the lack of nociception (12). It is therefore of vital importance that an individual gains a functional nociceptive system.

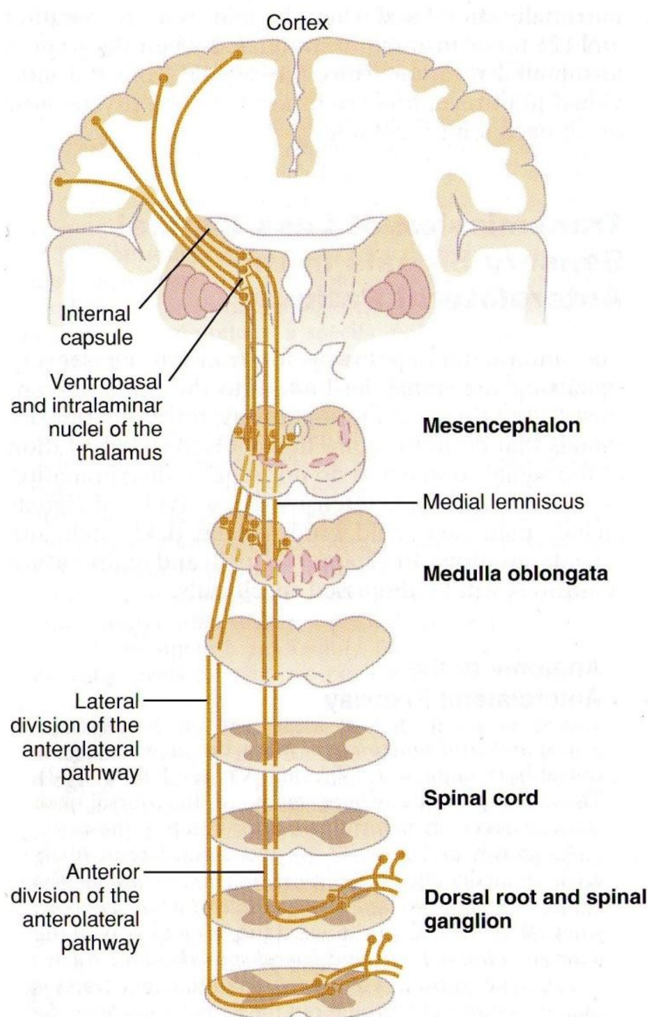

Figure 1, The anterolateral pathway. Part of the pain pathway (transmission) (11).

The pain system of the human nervous system consists of both peripheral and central components with an ability to detect and encode noxious stimuli. The pain is perceived when a noxious stimulus passes through four steps, which are: Transduction, transmission, modulation and finally perception (13)

Pain, for instance muscle pain, arises when nociceptors are stimulated e.g. by a mechanical stimulation (transduction), this mediates a nociceptive signal through III and IV nerve fibers. These nerve fibers (afferents) then synapse with second order neuron in laminae I, II and V in the dorsal horn of the spinal cord (transmission), see figure 1. The next step is modulation, which happens in the dorsal horn of the spinal cord, see figure 2. This step either dampens or amplifies the nociceptive signal. Afterwards, the signal is transmitted through the anterolateral pathway and finally terminates in the somatosensory cortex, where the signal is perceived as pain (perception) (13).

Under normal physiological conditions nerve fibers III and IV remains inactivated by a Mg^{2+} blockade of the N-Methyl-D-Aspartate (NMDA) receptors. This inhibits the Na^{+} and Ca^{2+} influx through the receptor, and thereby maintaining the resting membrane potential. However, when the nociceptors are stimulated, the Mg^{2+} is removed, and an action potential is generated (14).

As mentioned previously, the nociceptive signal can be suppressed by modulation, which is due to the descending pathways from the midbrain that interacts with neurons in the dorsal horn.

When a nociceptive signal is transmitted through the antereolateral pathway, the periaqueductal gray receives the same information, see figure 2. This will activate enkephalin-releasing neurons that project to the raphe nuclei in the brainstem, which activates serotonergic neurons that descends to the dorsal horn in the spinal cord. Here serotonin release activates inhibitory interneurons located in the substantia gelatinosa. These interneurons will release either enkephalins or dynorphins, which bind to either μ-, δ- and κ-opioid receptors (13). The activation of these receptors inhibits the release of substance P from the incoming III and IV nerve fibers. This then terminates the nociceptive signal in the dorsal horn (11).

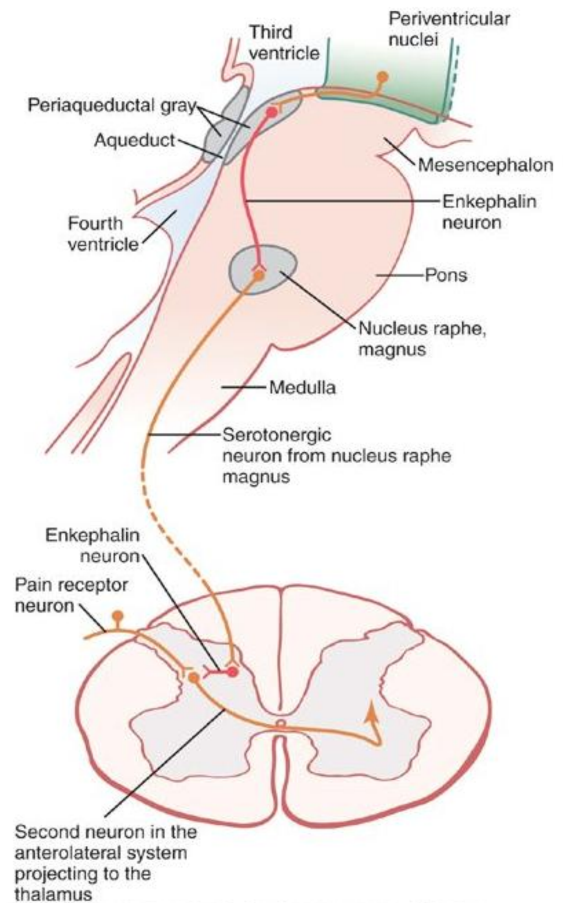

Figure 2, The descending inhibitory pathway exerts analgesic effect via the enkephalin-secreting neurons in both spinal cord and brain (11).

5.2 Enkephalins

The enkephalins consist of met - and leu-enkephalins (13). These two peptides are derived by proteolytic cleavage of the larger precursor peptide preproenkephalin (PENK) (3). The precursor peptide, see figure 3, is processed to produce 6 met-enkephalin (yellow) and 1 leu-enkephalin (light blue) moieties, which are the following peptides; Tyr-Gly-Gly-Phe-Met and Tyr-Gly-Gly-Phe-Leu respectively (13).

```
  1 marfltlctw llllgpglla tvraecsqdc atcsyrlvrp adinflacvm ecegklpslk
 61 iwetckellq lskpelpqdg tstlrenskp eeshllakry ggfmkryggf mkkmdelypm
121 epeeeangse ilakryggfm kkdaeeddsl anssdllkel letgdnrers hhqdgsdnee
181 evskryggfm rglkrspqle deakelqkry ggfmrrvgrp ewwmdyqkry ggflkrfaea
241 lpsdeegesy skevpemekr yggfmrf
```

Figure 3, The protein sequence for Preproenkephalin precursor peptide. Yellow color represents the met-enkephalin peptide and light blue is leu-enkephalin (40).

Enkephalins exert analgesic effects when binds to either μ-, δ- and κ-opioid receptor. However, studies show that met-enkephalin and leu-enkephalin have higher affinity for δ-opioid receptors (13).

Evidence suggests that treating chronic pain patients with the PENK gene may provide an antinociceptive effect (15). To further confirm this hypothesis, the same researchers administered an opioid antagonist, naltrexone which completely blocked the antinociceptive effect of the PENK gene (15).

To the best of our knowledge, no study has been done to elucidate whether the PENK gene expression is reduced in chronic pain patients. However, since the enkephalins have analgesic effects, one could speculate that this might be one of the reasons for development or maintenance of chronic pain.

From a therapeutically aspect, one approach could be administration of the proteins, met- and leu-enkephalins to chronic pain patients. Thereby, well-defined doses of PENK protein, matching the disease stage, can be administered. However, this can be troublesome, because the exogenous

PENK proteins are “foreign” to the body, and an immune response might arise following their administration (11) (13).

Enkephalins can not only be used for therapeutic purposes in chronic paint patients, but can also be used as doping agents. Since enkephalins exert analgesic effects via its modulating ability, an athlete can use it to enhance its levels of present to combat the nociceptive signals in heavy sport of competitions. This would allow the athlete to endure higher levels of pain for a longer period, which would eventually enhance his/her performance. The excessive amount of enkephalins can be either inherited, due to a mutation or following gene doping. Since it is safe, easy and efficient, one can imagine that gene doping would become more popular in the future and this calls for further investigation on detecting methods. To set a reliable method for detection, one should firstly look into the gene insertion methods. The next section focuses on transfection methods in details.

5.3 Transfection

5.3.1 Chemical transfection

Chemical transfection is the most common method of introducing foreign genes into mammalian cell (16). The chemical reagents used in this transfection method are cationic polymers, calcium phosphate, cationic lipid and cationic amino acids. The mechanisms of how these work are similar. The chemical reagents are positively charged, which helps to form a complex with the negatively charged nucleic acid. This chemical/nucleic acid complex remains positively charged and is therefore attracted to the negatively charged cell membrane. The exact mechanism by which the complex passes through the membrane is unknown, but it is believed that it involves either endocytosis or phagocytosis (16).

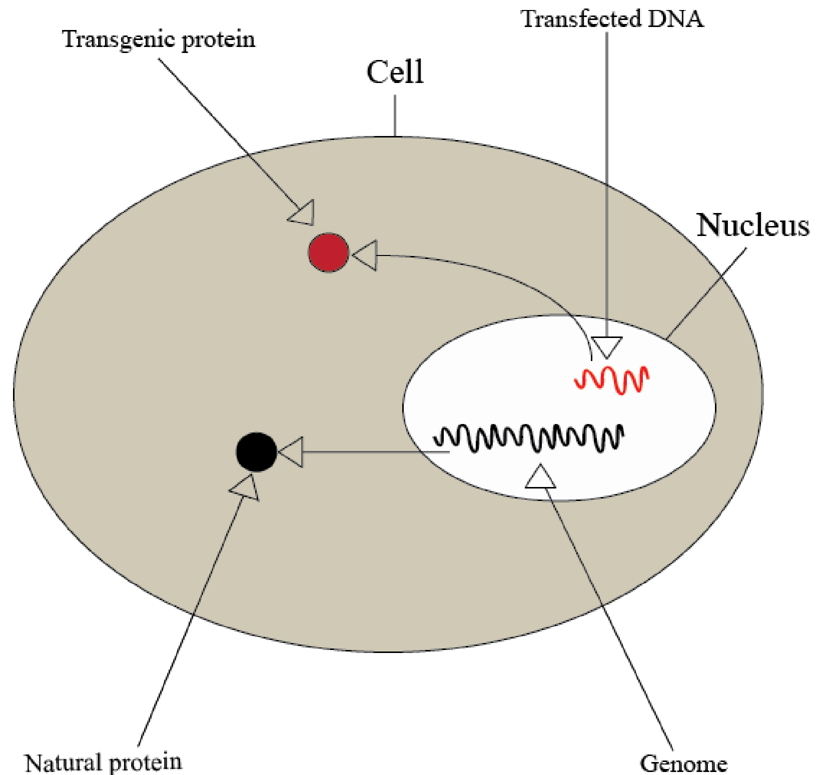

Figure 4, Transient transfection. The transfected DNA is expressed inside the nucleus, but is not integrated into the genome. The transfected DNA is therefore lost after a random number of cell division (16).

The transfection efficiency of the chemical methods is dependent on factors like chemical/nucleic acid ratio and solution pH, which can be regulated *in vitro*. However, this method might have low transfection efficiency *in vivo*, since it is impossible to regulate solution pH (16). Furthermore, this method has low tissue specificity, making it difficult to use as a therapeutic transfection method.
On the other hand, this method has low cytotoxicity, low mutagenesis (16).
The transfected DNA must reach the cell nucleus to be expressed, but the underlying mechanism is unknown (16).

As seen on figure 4, this method is a transient transfection. Thus the transfected DNA is expressed when located inside the cell nucleus, but not integrated into the genome. After a couple of cell divisions the transfected DNA will be degraded (16).

5.3.2 Viral transduction

Viral transduction is another method used for the transferring of genetic material inside cells. The use of viruses as vectors to deliver genetic material shows promising results (15). This is due to the transduction efficiency of the viruses and their ability of integrating a gene of interest into the hosts' genome. This phenomenon, as visualised on figure 5, is also called a stable transfection. In contrary to transient transfection, the inserted gene is inherited (17). A complete virus, known as a virion, consists of nucleic acid surrounded by a protein coat called a capsid. Some viruses have a lipid envelope, which helps entering the host cell (18).

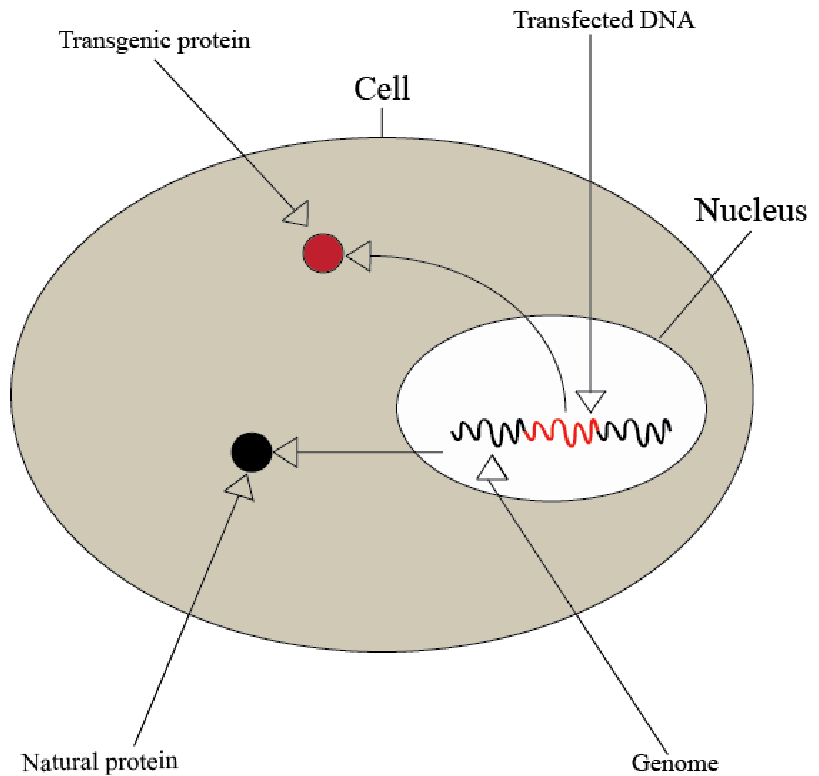

Figure 5, Stable transfection. The transfected DNA are integrated into the hosts' genome. The genetic alteration is therefore carried to the progeny cells (16).

The ideal viral vector should be; safe, of low toxicity, high specificity and easily detectable (19). However, due to the pathogenic nature of viruses, molecular engineering most be employed to circumvent this before using them as vectors. This can be done by depriving the virus of the *cis*-acting elements needed for gene expression and genome replication (19). As an example, retroviruses are used as vectors, because of their ability to become a provirus. This will ensure a stable transfection, see figure 5, and thereby a permanent gene expression. On the other hand, retroviruses are very effective at infecting and replicating, which poses a harmful treat. Therefore,

to design a non-pathogenic retrovirus, a plasmid vector with the gene of interest is transfection into a cell line. Hereafter, a second plasmid with the genes for the regulatory elements and structural proteins is transfected into the same cell line. The cell line then acts as a packaging unit, producing functional non-pathogenic viral vectors (20).

As mentioned earlier, the ideal viral vectors should be safe. However, due to viruses' tendency to mutate, this criterion is not always for filled. (18) Viruses contain single-stranded RNA as their genome, are especially prone to mutation. Since these organisms only contain a single strand of RNA this increases the risk of mutation due to the lack of a backup strand. For that reason, DNA viruses mutate at a lower rate. (18)

As mentioned in the beginning viral vectors can cause cancer. Thus was the case when researchers wanted treat to patients suffering X-linked severe combined immunodeficiency. They used a retrovirus as a vector to deliver a corrective transgene into hematopoietic stem cells. The corrective gene was inserted next to an oncogene, which resulted in 4 of the 20 patients developed T-cell leukemia (3). However, use of lentiviruses shows low genotoxicity in hematopoietic stem cells. (21) This will be a better choice for the above mentioned therapy, and illustrates that further research on viruses as vectors, might prove to be an effective and safe gene delivery method.

5.3.3 Electroporation

Due to the fore mentioned incident with viral vectors, there is a widespread interest in developing a non-viral transfection method.

One of the non-viral transfection methods is electroporation, which is a technique that uses electrical pulses and voltage to increase the permeability of the cell membrane. The higher permeability enhances the entry of transgenic material into the cell.

Studies show that the technique is functional, though the underlying mechanism is not fully understood (22). However, researchers have developed some ideas that might explain the mechanisms underlying this technique. When an electrical field is created around the cell for a short period of time (μs - ms), the transmembrane voltage rises to about 0,250 V – 1,0 V. This is also known as the electroporation phenomena (23) (24). The depolarisation will create some temporary aqueous pathways in the membrane, so-called pores, which enables a free passage of molecules (up to 4kDa) (23).

According to previous studies the electrical field has a critical role in transfection efficacy. The cells inside the field most receive enough voltage and pulses to become permeable. On the other hand, the electrical field strength should not kill the cells. (24)
It is also important that the transfection agent (e.g. plasmid DNA) is located at the electroporation site, before the electrical field is created. Studies have shown that introducing plasmid DNA after the electrical field has been created results in poor transfection (24).
It is also shown in vitro that the pulse amount and duration has an important role in the DNA plasmid-cell membrane interaction. (23).
The cell remains permeable for 5 min. to 4 hours after the electrical field has ended. Some evidence indicates that the uptake of the transgenic agent occurs after the electrical field has ended (24). Electroporation is a efficient method of introducing genetic material into cells, as Gehl. J. et al. showed that the electroporation of a plasmid into muscle cells of mice were expressed for more than 3 months (8).

6.0 AIM

Gene doping is becoming an evadible doping method in sport. Hence, it is required to develop a reliable method to detect gene doping either in plasma or tissues of doped individuals. The aims of the present study were:

1) To insert preproenkephalin gene as potential candidate for gene doping against muscle pain by electroporation method

2) To establish a detection method for inserted enkephalins using quantitative polymerase chain reaction.

7.0 Material and methods

7.1 Animals

All animal experiments were conducted in agreement with the recommendation of the European Convention for the protection of Vertebrate Animals used for Experimentation. The experiments were approved by the animal ethics committee (see appendix 2).

A total of 36, 6 – 8 weeks old female C57BL/6 mice were used. 20 were from Taconic (Ry, Denmark) and were used in Aalborg Hospital. The last 16 were own breed and were used in Herlev Hospital. The animals were maintained in a thermo-stated environment under a 12-hour light/dark cycle and had free access to food and drinking water. The mice were euthanized by cervical dislocation, and blood was collected in EDTA-containing eppendorf tubes. The right tibialis anterior were removed and quickly frozen in liquid nitrogen.

7.2 Cell culture

C2C12 myoblast cells (ATCC, No. CRL-1772) were cultivated in medium consisting of DMEM (Sigma Aldrich, Lot. RNBB9951) with 10 % fetal calf serum (Gibco, Cat. No. 10106-169) and 1 % penicillin/streptomycin (Gibco, Cat. No. 15140-122). Cells were grown in T175 culture flask. When cell confluence reached 60 – 70 %, they were trypsinized and washed twice with sterile phosphate buffered saline after which 0.25 % (w/v) trypsin-0,53 mM EDTA solution was added. The cells where then incubated for 5 minutes at 37 ºC with 5 % CO_2, followed by removal of trypsin-EDTA solution and addition of 8 ml medium. 10 µl of the cell suspension and 10 µl trypan blue were mixed. The cells were then counted on a hemocytometer.

7.3 Plasmid

The plasmid used in this experiment was bought from Origene, USA. As seen on figure 6, the plasmid contained the sequence encoding mouse PENK (NM_001002927.2). A sequence encoding green-fluorescent protein (GFP) is also present in frame with PENK, thereby enabling production of fusion PENK-GFP protein for the visualization of successful transfection and subcellular localization of PENK. The plasmid also contains the sequence for ampicillin, which acts as a selective marker.

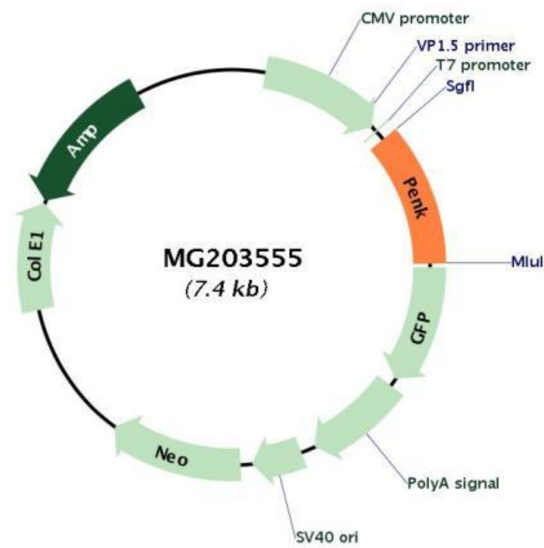

Figure 6, Plasmid map. The plasmid is tagged with the gene encoding green-fluorescent protein. The ampicillin resistance gene is cloned into the plasmid, as selective marker (41).

7.4 Transformation

Both plasmids were transformed by heat-shock into the chemically competent E. coli strain DH5α (Invitrogen, Cat. No. 18265-017), which were plated onto an agar plate containing 100 µg/ml ampicillin. This was recommended by the plasmid manufacturer (Origene, USA). The E. coli were then incubated for 24 hours, resulting in the formation of colonies. After the time period, one colony was picked with a pipette tip, and was submerged into 3 ml lysogeny broth (LB) medium. This mixture was placed on a shaking incubator, which maintain 220 rpm and 37ºc . This allows the bacteria to multiply and after 8 hours the mixture were poured into a pyrex 250 ml flask containing 100 ml LB medium. Hence, the bacteria incubated for 12 hours with 220 rpm and 37 °c in the shaking incubator. The next step was to purify the plasmid by anion ion exchange chromatography

with Nucleobond Xtra midi kit (Macherey-Nagel, Lot. 1110/003). This was done according to manufacturers' protocol.

7.5 Transfection in vitro

7.5.1 Chemical transfection

When there was confluence of 50 %, cells were trypsinized and seeded into a 6 well culture plate at 2500 cells/cm^2. Cells were then allowed to reach a confluence of 70 – 90% (24 hours), before transfection with the turbofect transfection reagent (Thermo scientific, Cat. No. #R0531). This step was performed using 2 µg plasmid DNA diluted in 200 µl of DMEM (Sigma Aldrich, Lot. RNBB9951), the mixture was then vortexed. After 30 sec. mixing, 6 µl of turbofect transfection reagent was added. The final solution was then allowed to incubate at room temperature for 20 minutes, and then pipetted into 2 of the 6 wells in the culture dish. This was done by carefully distributing 100 µl of the solution around each well by rocking the culture dish. The remaining wells did not receive any transfection solution, as they served as controls. These received 2 µg plasmid mixed with 200 µl of DMEM (Sigma Aldrich, Lot. RNBB9951).
The transfection success was calculating using five representative pictures taken from the same transfection. The transfected cells was compared to total number of cells, and calculated as a percentage.

7.5.2 Confocal microscopy

After visual confirmation of transfection using inverted fluorescent microscope (Axiovert 40, Carl Zeiss Microscopy, Germany) , the cells were trypsinized, resuspended and seeded into a chamber slide (C6307, Sigma-Aldrich, USA). The cells were then left for 4 hours, allowing them to attach to the glass slide. They were then fixated with 4 % formaldehyde, and stained with 4',6-diamidino-2-phenylindole(DAPI) . Hereafter, cells were visualised on a confocal microscopy (LSM 700, Carl Zeiss Microscopy, Germany).

7.6 Transfection in vivo

7.6.1 Injection of naked plasmid

Animals were injected with 100 µg, 50 µg, 5 µg, 0.5 µg PENK plasmid into the right anterior tibialis using a 29G insulin syringe. The total volume injected was 20 µl. After 24 and 48 hours mice were euthanized, blood and muscle tissue was extracted. A control group was included which received 20 µl of saline solution.

7.6.2 Electroporation

The animals were anaesthetised 15 min prior to electroporation by injection of Hypnorm/Midazolam combination (2,5 ml hypnorm/2,5 ml Midazolam mixed with 5 ml sterile water). Each animal were injected, subcutaneously, with 100 µl of anaesthesia, after 10 min. they were ready for electroporation. For each mouse 20 µl of plasmid (10 µg) solution was injected intramuscularly, using a 29G insulin syringe, into the tibialis anterior. After the injection a plate electrode with a 4-mm gap in between were fitted around the leg. Hair removal and electrode gel was applied to ensure sufficient contact. A control group had 20 µl of sterile saline injection, instead of plasmid solution.

The electric field was applied using the Cliniporator^{tm} (IGEA, Italy), which applied a combination of a High voltage pulse (800 V/cm(applied voltage = 320 V), 100 µs) and a low voltage pulse (100 V/cm (applied voltage = 40 V), 400µs) with a 1 sec pause between pulses.

This setup was performed for the PENK plasmid, and the control group.

7.6.3 RNA/DNA extraction

Total RNA was extracted from blood samples using QIAamp RNA blood mini kit (Cat. No. 52304, Qiagen, Germany) The RNA concentration was measured using nanophotometer (Implen, Germany).

Total RNA was extracted from muscle samples using AllPrep DNA/RNA Mini kit (Cat. No. 80204, Qiagen, Germany). This was done according to the manufacturer's protocol. The average DNA/RNA yield from muscle tissue was low, therefore the muscle tissue obtained from the 16 mice

from Herlev sygehus was extracted using RNeasy Fibrous Tissue mini kit instead(Cat. No. 74704, Qiagen, Germany). All muscle tissue was pulverised in liquid nitrogen using a morter.

7.6.4 cDNA synthesis

cDNA synthesis was performed using the RevertAid^{TM} Premium First Strand cDNA synthesis kit (#K1651, #1652, Thermo Scientific). This was done using the provided oligo $(dT)_{18}$ and random hexamer primers following protocol by the manufacturer.

7.6.5 PCR purification and DNA sequencing

The qPCR products, a positive sample (1 pg PENK plasmid) and a negative sample (water), from a previously performed qPCR assay was purified using the QiAquick PCR purification kit (Cat. No. 28104, Qiagen, Germany. The Purified PCR product was used in DNA sequencing.
The preparation for DNA sequencing was done by lyophilizing the purified PCR products from a positive sample and a water sample. For the DNA sequencing of the positive sample, 280 ng of purified product was lyophilized and for the water sample 193 ng was lyophlized. These were then sent to Beckman Coulter Genomics (England) together with forward primer (5 µM).

7.6.6 qPCR/RT-PCR

Primers were designed using primer-blast tool from www.ncbi.nlm.nih.gov using the accession number NM_001002927,2 for preproenkephalin. Primers were bought from TAG Copenhagen.

Primers:

Gene	Primer	Sequence
Preproenkephalin mRNA	Forward Reverse	**5'**-GTCCTGCCTCCTGGCTACAGTG-**3'** **5'**-TCCAGTGTGCACGCCAGGAAAT-**3'**
β-Actin mRNA	Forward Reverse	**5'**-CCTCTGAACCCTAAGGCCAACCGTGAA-**3'** **5'**-AGTGGTACGACCAGAGGCATACAGGG-**3'**

The Preproenkephalin amplification product consists of 108 bp, and has a melting temperature of 83,7 °C. The beta-actin amplification product consists of 123 bp, and has a melting temperature of 83 °C.

RT-PCR was performed using 1 µl cDNA, 10 µL Dreamtaq green PCR master mix (2x) (#K1081, Thermo scientific) and 0,8 µl (0,4 µM) of each primer per well. RT-PCR was done using a non-skirted MX3000P$^{(R)}$ 96-well plate (Agilent Technologies, DK.)

After PCR amplification 10 µL of product was mixed with 2 µL 6X DNA Loading Dye (#R0611, Thermo Scientific). This was then loaded on a 1,5 % agarose gel containing 0,5 µg/ml Ethidium bromide, and run in a gel electrophoresis apparatus. After 20 min. the gel was exposed to UV lighting and bands were visualised on Kodak imagestation. Water samples were used as negative control.

qPCR was performed using 1 µl cDNA, 10 µL Brilliant SYBR Green qPCR Master mix (Agilent Technologies, Denmark) and 0,08 µl of each primer (0,4 µM) per well. qPCR was done using a

non-skirted MX3000P(R) 96-well plate (Agilent Technologies, DK). Samples were plated as dublicates.

All reactions were done using qPCR (Mx3000P, Agilent Techonlogies Stratagene, DK). Water samples and samples without reverse transcriptase add (RT-) were used as negative controls.

The thermal profile for qPCR and RT-PCR was 1x: 95º C 10 min; 40x: 95º C 30 sec, 61º C 10 sec, 70º C 30 sec; 1x: 95º C 1 min, 55º C 30 sec, 95º C 30 sec.

Standard curves were made to test primer efficiency for the PENK gene. This was done by adding 10 x 10^8 molecules of plasmid in one well in a non-skirted MX3000P(R) 96-well plate (Agilent Technologies, DK). Then diluting the molecule number 10 times in a second well, etc, this was done with 5 dilutions. The primer/mastermix mixture per well remained the same in all 5 wells (10 µL SYBR green, and 0,4 µM of both forward and reverse primer).

The qPCR results are measured in Ct values, which indicate the number of cycles performed in the qPCR assay before the SYBR Green dye is measurable.

Data normalisation is performed to correct for sample to sample variations. This is done by using a reference gene in the qPCR setup, which acts as baseline.

For normalisation of the results the following equation was used:

$$Normalisation = \frac{\left(1 + E_{target}\right)^{-\Delta Ct_{target}}}{(1 + E_{norm})^{-\Delta Ct_{norm}}}$$

7.7 Data analysis

For the interpretation of the results, SPSS (IBM, USA) was used, as well as Excel (Microsoft, USA) for graph design.
The results are calculated as a ratio between PENK transfection group (PENK 24 and 48) and the average of the total control group, e.g. the average of control 24 hours added to the average of control 48 hours. The data is presented on a graph with standard deviations.
Furthermore, the data were tested for normality and homogeneity. If this showed a P-value> 0,05, a one-way ANOVA was performed. A p-value of < 0,05 was considered statistically significant.

8.0 Results

This study consists of three separate experimental sections: 1) Transfection of C2C12 cells in vitro to confirm efficient expression of PENK; 2) in vivo transfection of mice with PENK and subsequent measurement of transfection efficiency; and 3) plasmid contamination.

8.1 In vitro transfection of C2C12 cells

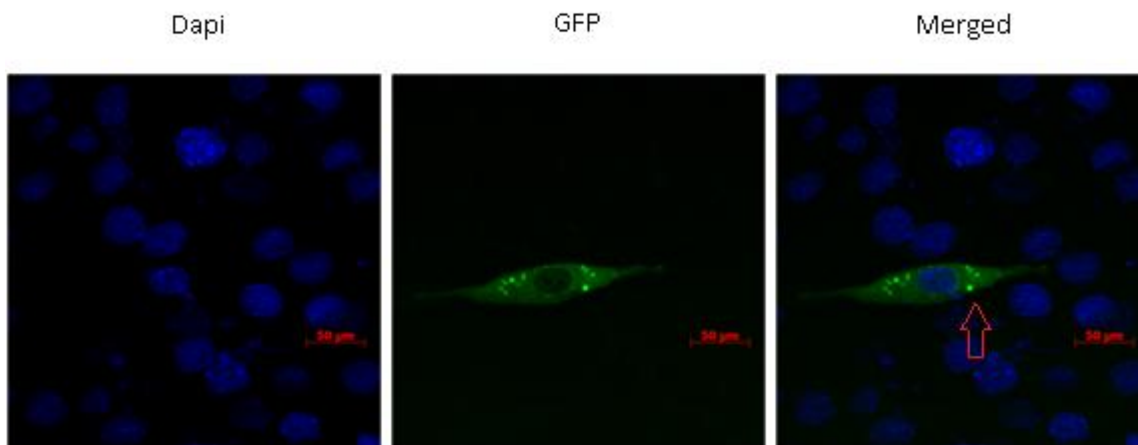

Figure 7, confocal microscopy of transfected C2C12 cells with GFP positive cell.

To evaluate if the PENK plasmid could be efficiently expressed in muscle cells, C2C12 cells were transfected with a plasmid expressing PENK fused with green fluorescent protein (GFP). As indicating in figure 7, GFP and DAPI positive cells were observed, and were found in same focal plane. DAPI allows visualization of the nuclei of cells and was used to estimate the ratio between successfully transfected cells and non-transfected cells.

Small vesicles (see figure 7, red arrow) expressing high degree of GFP were localised in the periphery of the cell, indicating the fused PENK-GFP protein is stored in secretory granules as is the case with the native PENK-protein. The overall expression efficiency was determined to be 7 %, based on the percentage of total cells expressed GFP.

8.2 In vivo

8.2.1 Injection of naked PENK plasmid without electroporation showed no transfection of the muscle or blood.

To determine whether injection of naked PENK plasmid was capable of transfecting cells without electroporation, naked plasmid was injected into the antetior tibialis muscle mice at different concentrations (100 µg, 50 µg, 5 µg and 0,5 µg, and saline solution). Transfection was determined by extracting muscle cells from the right anterior tibialis and blood samples from the right jugular vein, and subsequently analysed using qPCR.

For both muscle and blood, Ct values were above 32 for PENK and water sample, as visualised in figure 8. These shows that there is a PENK expression from the cells, however with the amplification in water it is not possible to distinguish between background and "real" amplification. Thus, PENK transfection without electroporation could not be detected in muscle cells and blood.

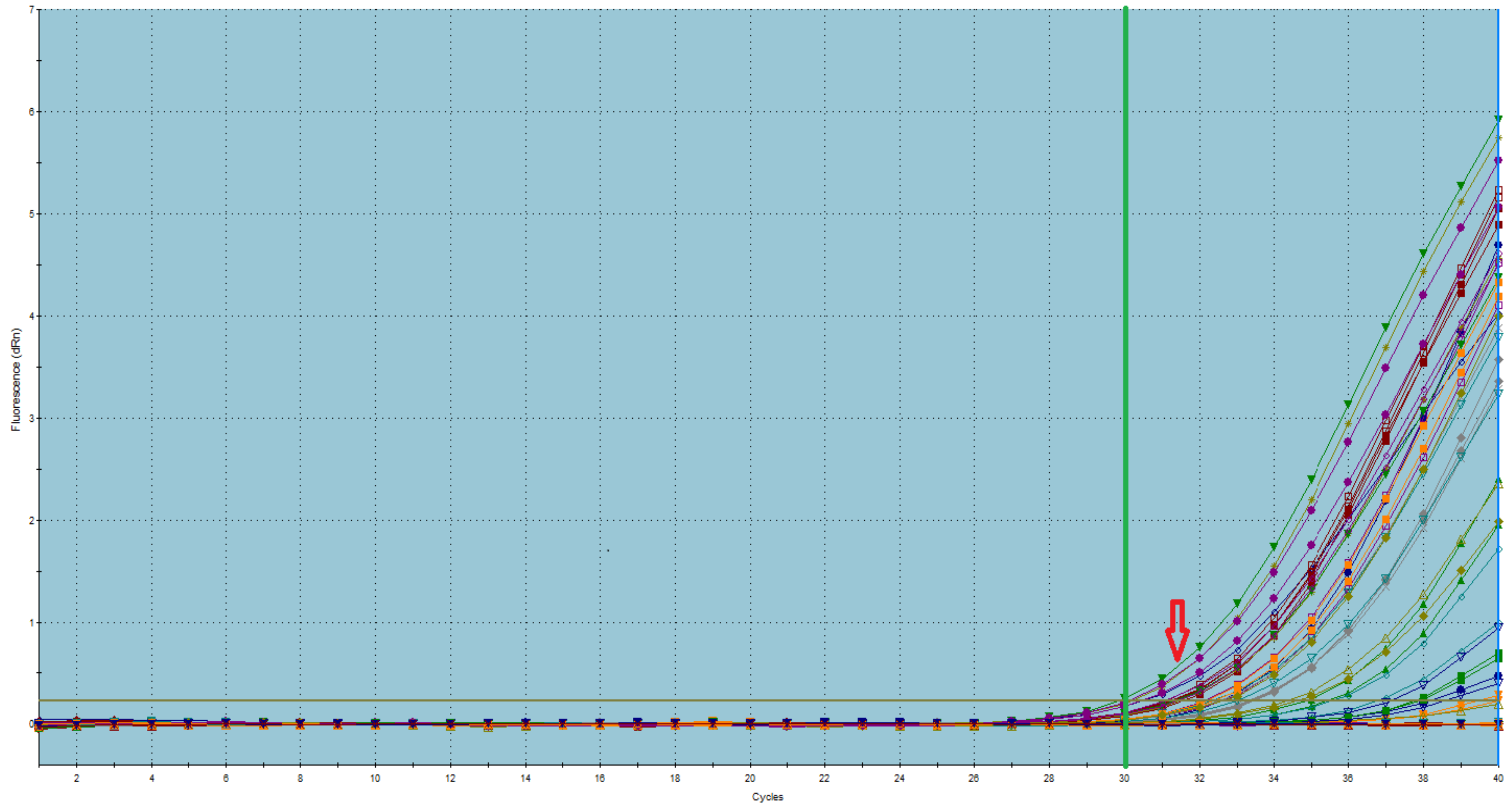

Figure 8, qPCR assay of PENK expression from blood and muscle after naked plasmid injection into the muscle. The curves represent each concentration injected, and also a water sample. Fluorescence is measured on the y-axis and Ct value on the x-axis . The green line represents bagground noise level, and the red arrow points the water sample. 100 µg (N=4), 50 µg(N=4), 5 µg(N=4), 0,5 µg (N=4) and control croup (N=4)

8.2.2 Electroporation increased gene expression after 24 and 48 hours

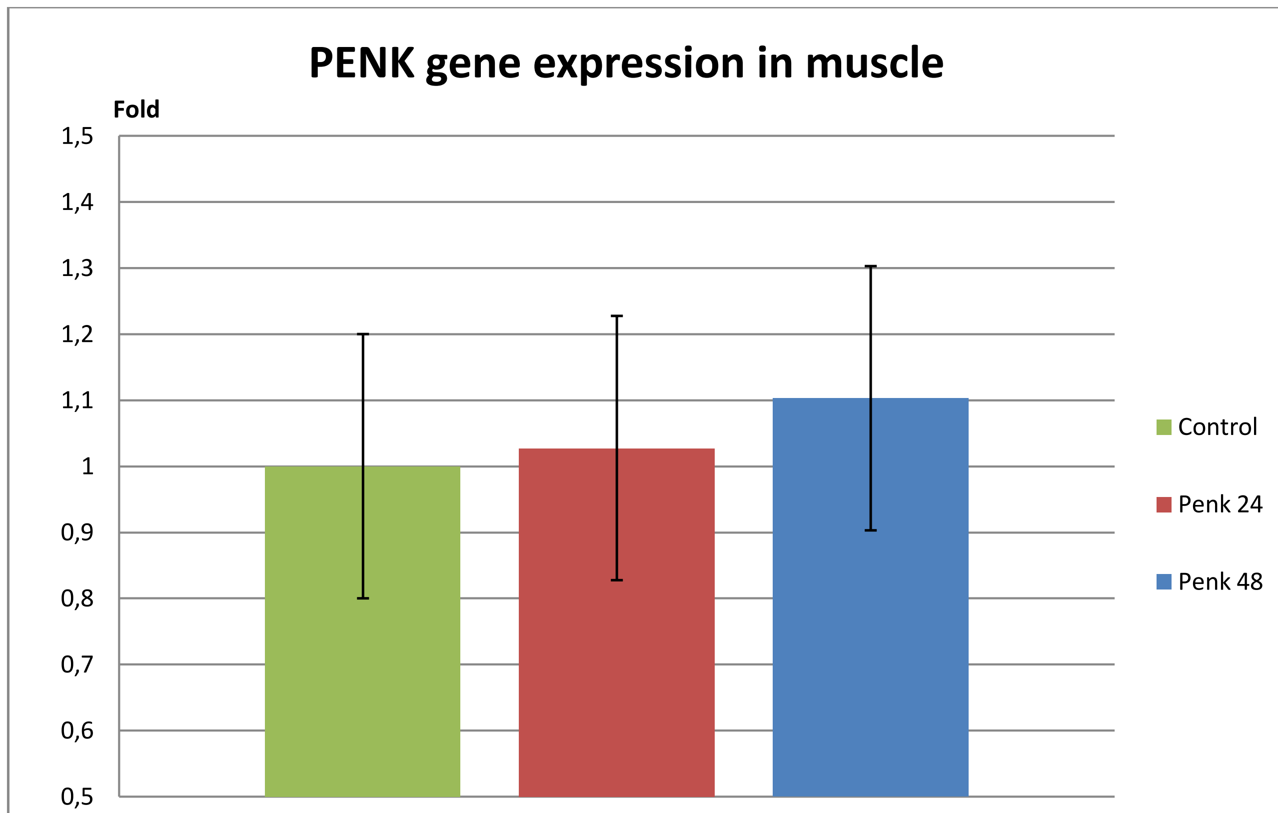

Figure 9, qPCR results from the PENK expression in the muscle tissue 24 h (N=4) and 48 h (N=4) after electroporation compared to the control group (N=8).

To determine if PENK was detectable in blood and muscle after electroporation, mice were euthanized after 24 and 48 hours and tissue was extracted and analysed. The Ct values for PENK 48 hours were 25 – 28, and for PENK 24 the Ct values were 29 – 30, which indicates a higher gene expression in PENK 48. The β-actin all had Ct values of 18 – 20, which indicates that the qPCR reaction was performed successful.

The level of PENK expression was investigated using qPCR and the results were normalized to β-actin and finally compared with a control group that received an injection of saline solution.

The results from muscles are presented in figure 9. 24 hours following electroporation, a 1.03 (±0.2) fold increase in PENK expression was detected compared to control, while at 48 hours a statistically significant 1.10 (±0.2) fold increase was detected compared to control ($P<0,05$). There was no significant increase between PENK expression after 24 and 48 hours ($P=0,560$).

The results from blood are illustrated in figure 10 β-actin had Ct values of 18 – 20 which indicates that the qPCR reaction was performed successful. PENK and water the Ct value was 32-33, thus PENK transfection could not confidently be detected in the blood samples since the water samples had the same Ct value.

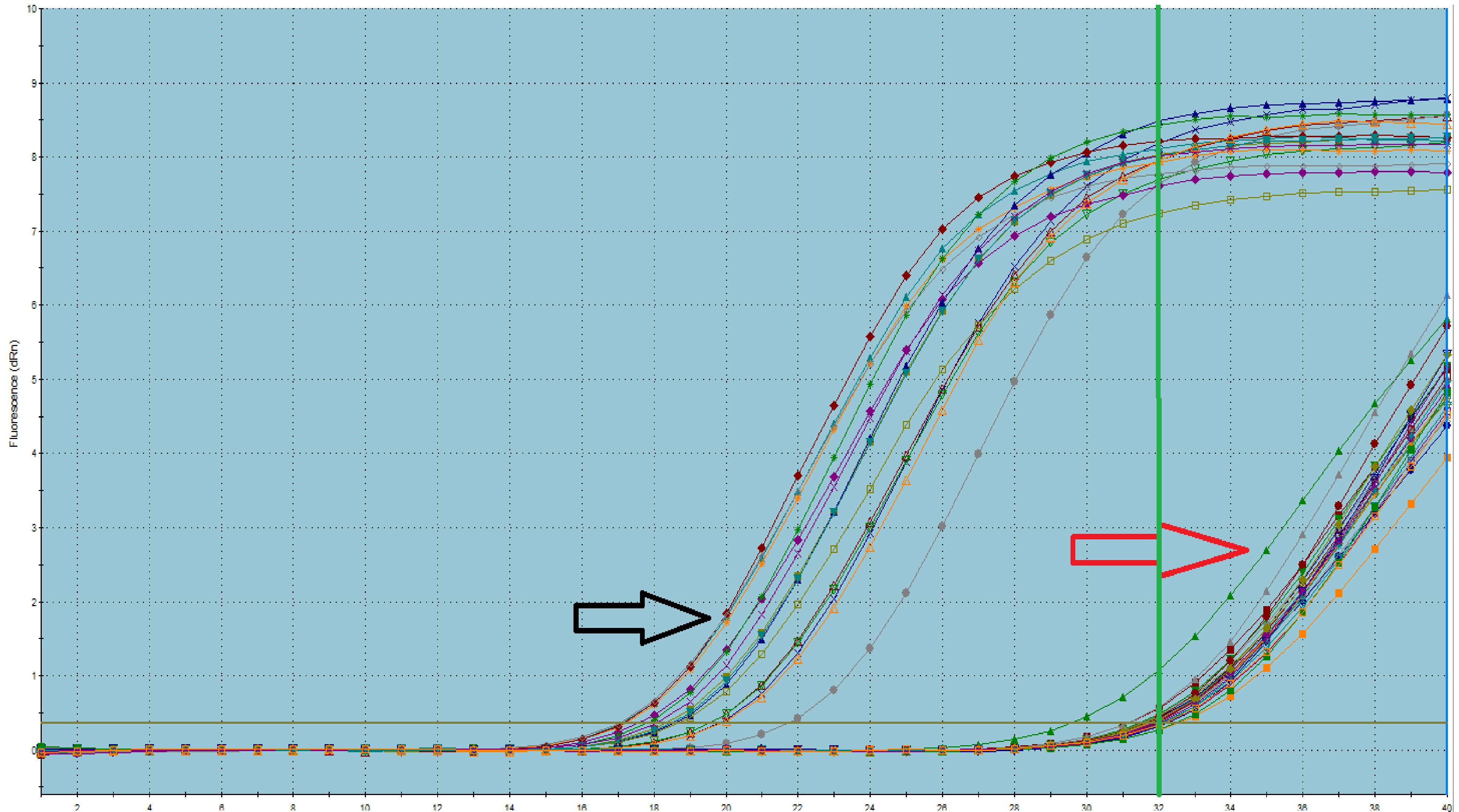

Figure 10, qPCR results showing the Beta-actin gene expression (black arrow) and the PENK gene expression (red arrow) for blood taken 24 and 48 hours post electroporation. Fluorescence is measured on the y-axis and Ct value on the x-axis. The green line represents the bagground noice level. PENK group = 8 mice and control 8 mice.

8.3 Contamination

As can be seen on figure 8 and figure 10 there is amplification in the water samples, and thus contamination was suspected.

8.3.1 Gel electrophorsis

To determine that the amplification in the water samples are not the results of primer-dimers the RT-PCR products was run on an agarose gel, see figure 11.

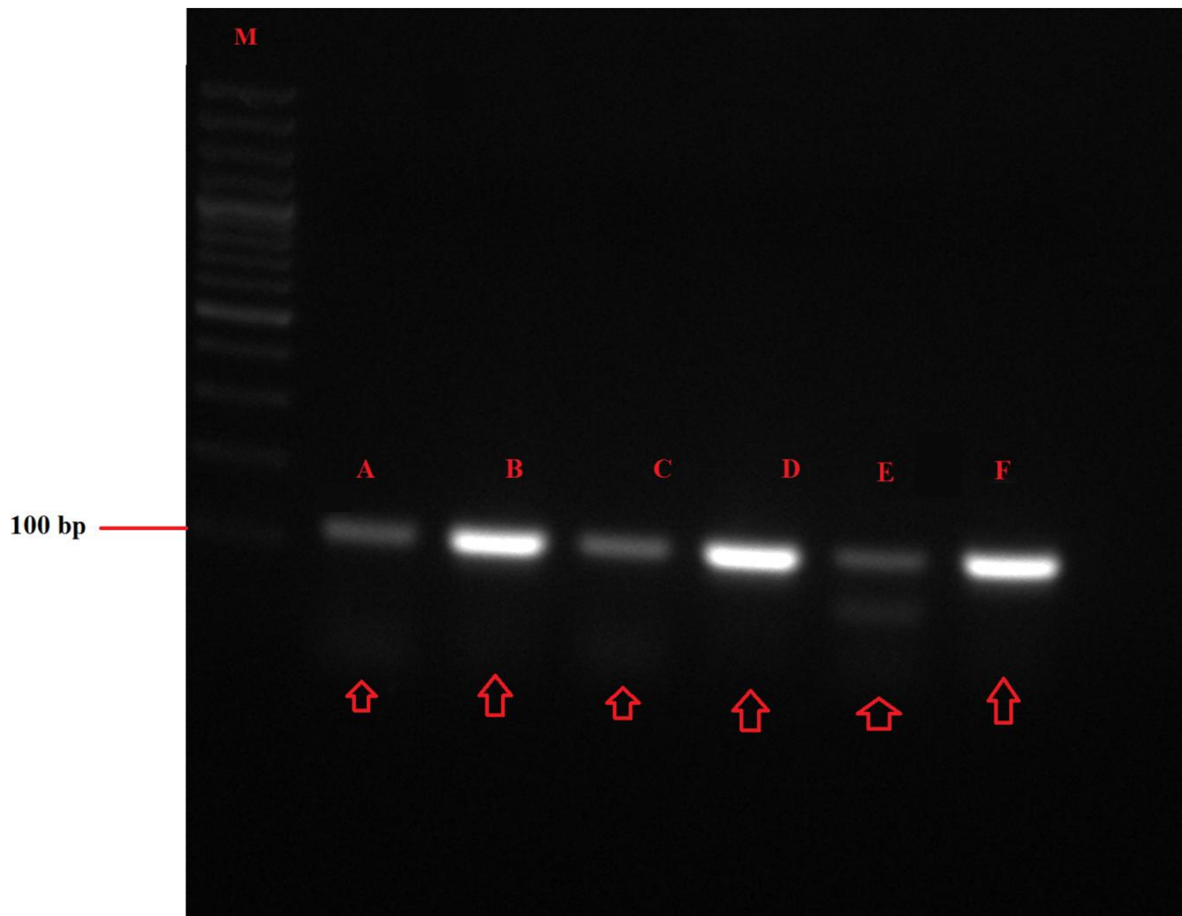

Figure 11, 1,5 % agarose gel with the PCR product from a previous PCR assay. The samples in B, D and F were PCR products from 1 pg PENK plasmid, and A, C, E are from PCR products from the water samples. The red arrows illustrate the formation of primer-dimers. Sample A and B had 60 °C as annealing temperature, C and D had 61 °C and E and F had 62 °C. The size of the PCR product can be seen as the marker (M) represents different DNA lengths.

The agarose gel showed that for both waters samples and samples containing PENK plasmids, regardless of annealing temperature, a band was observed at ~100 bp, This size can be seen as the marker represents different DNA lengths, thus indicating that it is the same PCR products in all the samples. Fainted bands were observed below 100 bp for all samples, which are the formation of primer-dimers.

8.3.2 DNA sequencing

To confirm if the product in water was a result of PENK plasmid or contamination of PCR products and not random contamination, DNA sequencing was performed on the PCR products from a qPCR assay. The DNA sequencing was performed on purified PCR products from a positive sample (1 pg PENK plasmid) and negative sample (water sample) from a qPCR assay.

DNA sequencing and blast tool from www.ncbi.nlm.nih.gov, found that the sequences from the purified PCR products, as indicated in figure 12 and figure 13, were 97 % identical. These sequences were blasted against the genome and transcripts from humans, mouse and rats, using the nucleotide blast tool from www.ncbi.nlm.nih.gov. The blasts results revealed that the DNA sequence of the positive sample was found to be 99 % identical to the mRNA from the mouse preproenkephalin, while for the sample containing water is was found to be 97 % identical to the same mRNA..

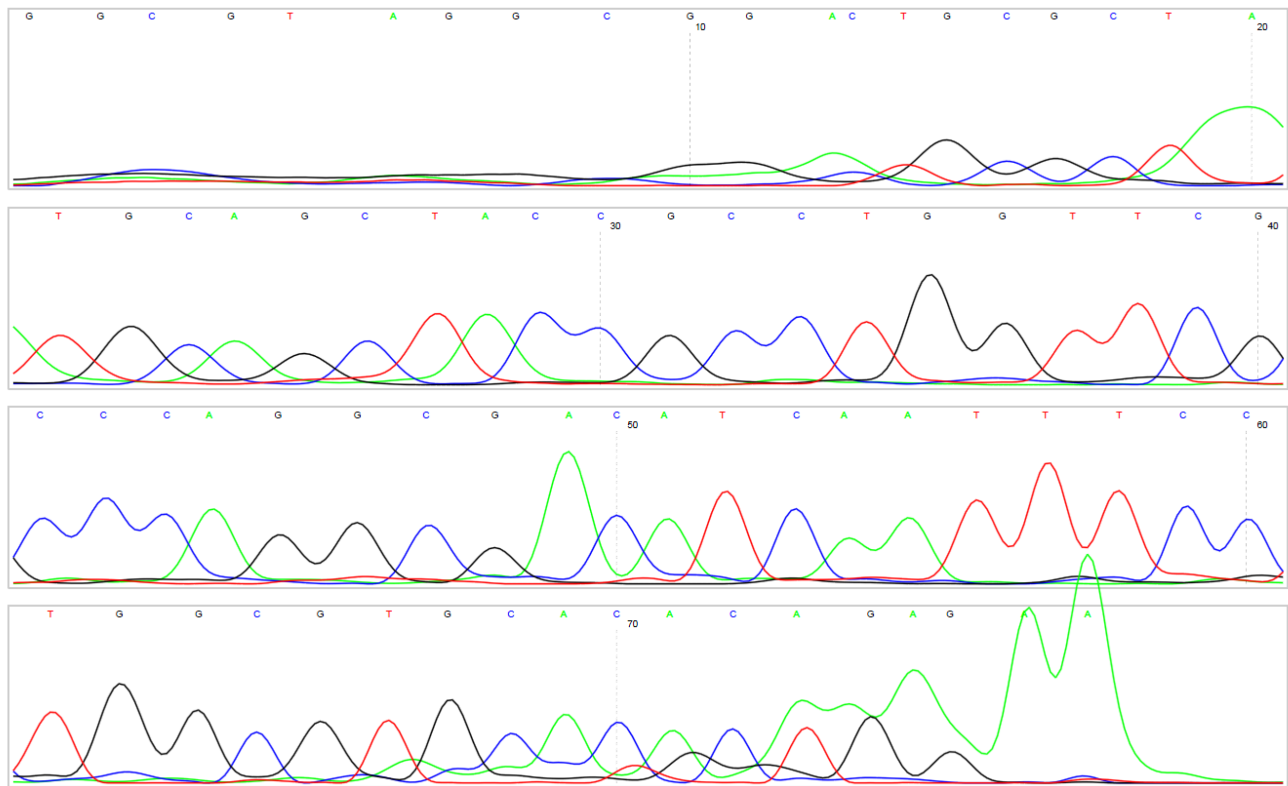

Figure 12, Sequencing of the PCR amplification production from a positive sample. The y-axis indicates fluoresence that corresponds to a specific nucleotide, and the x-axis is the length of the PCR product.

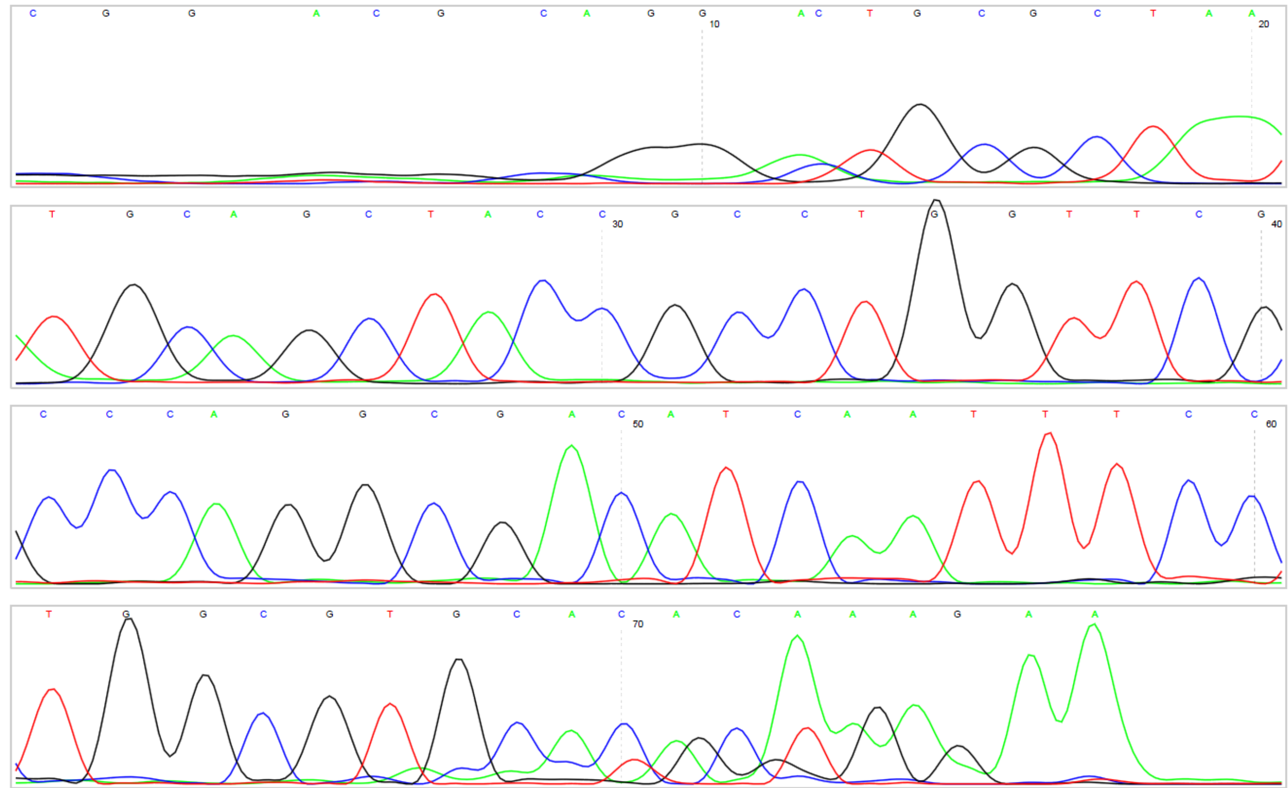

Figure 13, Sequencing of the PCR amplification production from a positive sample. The y-axis indicates fluoresence that corresponds to a specific nucleotide, and the x-axis is the length of the PCR product.

9.0 Discussion

9.1 In vitro

Clear GFP expressing was observed in C2C12 transfected with the PENK-GFP plasmid. Since, the PENK and GFP transcript are fused, successfully transfection and expression of PENK were observed in C2C12 cells. GFP was strongly expressed in small clusters, close to the cell membrane which suggests these are located in vesicles or secretory granules. This corresponds with the fact that PENK normally gets secreted from cells (11). Transfection efficiency was estimated to be 7 %, which was lower than reported by other sources (60 – 80 %) (25)(26). However, the goal of this experiment was to confirm that PENK could be expressed from muscle cells, and since clear GFP expression was seen, the low transfection efficiency in this experiment was irrelevant. These data indicates that it is possible to insert the PENK gene into muscle cells, and make them produce PENK.

9.2 In vivo

Following injection of naked plasmid into mice it was not possible to detect PENK in neither muscle nor blood after 24 and 48 hours. This indicates that the PENK plasmid is not transfected into the cells, when no electroporation were used. Other groups indicate that more than 200 µg of naked plasmid needs to be injected before an increase in gene expression was detectable without electroporation (27).

When injection of PENK plasmid was followed by electroporation, it was possible to detect PENK expression in the muscle after 48 hours post transfection. No significant increase in expression of PENK was observed after 24 hours, indicating that more than 24 hours was needed to reach a significantly higher level than normal. This corresponds to data from other sources (28).
The increased PENK gene expression after 48 hours indicates that the muscle cells have been transfected with the plasmid. Since PENK was undetectable 48 hours following the injection with naked plasmid without electroporation, electroporation seems to be of pivotal importance of in vivo transfection of muscle tissue.

In order to detect PENK in blood it would be required that the leukocytes in the blood were transfected simultaneously as the muscle cells that were the targets of electroporation. However, if PENK is transfected into the leucocytes rapid phagocytosis of the transfected leucocytes (29) might explain why PENK expression from blood was undetectable. The detection of PENK would therefore be impossible if the leucocytes are degraded before 24 hours, making the electroporation method even more efficient as a gene doping method.
When removing the muscle tissue a red tint was detectable in the injection site. This could be a mild form of inflammation or haemorrhage. If this is the case the leucocytes might be present in the muscle tissue, thus when euthanizing the mice these leucocytes would still remain in the muscle. The leucocytes would then be extracted with the muscle tissue, and will increase the gene expression levels from these when analysing the gene expression in the muscle.
The blood samples did not show any expression of PENK after electroporation. This implies that it can be difficult to detect whether a sport competitor have used gene doping, thus indicating that blood is not useful as target for a detection method..
It would be necessary to test if the PENK transfection is sufficient to produce an analgesic effect. In addition to this it would be necessary to establish a dose-response curve on of much PENK would be needed to produce a significant analgesic effect. If higher amounts of PENK gene expression are needed to produce an adequate pain relief it might be possible that the leucocytes would be transfected as well, thus making it possible to detect PENK gene in the blood. However, if higher amounts of plasmid are required to produce a desirable analgesic effect, this could cause toxic problems for the survival of the transfected cells (30). According to a study by Chuang et. al. 40 µg of PENK plasmid was enough to produce an analgesic effect in rats (29), therefore is seems that high amounts are needed to produce pain relief.

The data suggests that the muscles are actively expressing the PENK gene, which could induce an analgesic effect. (31) As mentioned earlier, an increase in pain tolerance would benefit an athlete in a sport competition. When nociceptors in the muscle gets activated the III and IV afferent nerve fibers will mediate the nociceptive signal to the spinal cord. This signal will then continue in the anterolateral system (11). However, if electroporated muscles have produced a sufficient amount of PENK, which translates into the peptides met- and leu-enkaphalins, the athlete would experience increased endurance. Excessive amounts of enkephalins will bind to the µ-, δ and κ-receptors stopping the nociceptive signal. The athlete would thereby feel no pain, and can continue his/her strenuous exercise. This would mean an unequal benefit compared to the competitors. The inserted

PENK gene is therefore a gene doping candidate as this enhances the performance of the athletes as muscle pain is diminished.

Since the doping gene is undetectable in the blood, a big problem arises. If an athlete uses the electroporation method and inserts a gene for doping purposes, the anti doping agencies needs to know exactly what muscle is used as transfection recipient, due to the fact that only the cells in the electrical field would be transfected. Thus, the detection would be very time consuming and nearly impossible to locate the exact transfection site. It would have been a better detection method if it was possible to detect the doping gene in the blood. The anti doping agencies would only need a blood sample to confirm the use of gene doping, regardless of what muscle is used as transfection recipient. This is not the case as proven in this study.

In order to be an effective doping method, expression of the doping gene in the muscles should be long lasting, thus reducing the risk of detection and thereby makes it practical applicable to athletes. Literature indicates that electroporation is a transient transfection method (23), but as Gehl j. et. al. showed, plasmid transfection into muscle tissue via electroporation was detectable for more than 3 months. Thus making electroporation an easy and effective gene doping method, as doping users would have to transfect the gene only a limited amount times to get an effect. The need for an acute and long term detection method is therefore crucial since the long term expression of PENK makes it difficult to detect the electroporation procedure.

Electroporation provides the most plausible gene doping method, compared the other transfection methods. According to other studies (6) (8), electroporation provides a safe and efficient method for the insert of a gene of interest into muscle cells. In according to the findings from this paper, only cells directly within the electrical field would be transfected. The chemical transfection reagents offers high transfection efficiency, but is not localised to a specific tissue, which could lead to unwanted side effects (32). Viral vectors is capable of integrated the gene of interest into the genome (33), however this transfection method can cause adverse effects (3).

To further investigate qPCR as a detection method for the PENK gene it would be interesting to see how long this gene is expressed. The time period should span from weeks to months. The literature indicates that transfected genes can be detected using ELISA months after electroporation (8). For qPCR to be a reliable detection method, this would have to show the same detection properties as ELISA.

This study provides the evidence that it is possible to insert and detect the PENK gene in muscle cells. However, many more genes can be inserted and used as doping candidates (34). Therefore, with highly specific primers, it is possibly to detect other doping genes from electroporated muscles.

From a therapeutic point of view the increase in PENK expression in the muscle illustrates that these can be transfected with a therapeutic gene of interest. Thus using this method as an effective gene delivery method shows promising results (35). In addition to this, the finding that a gene could be efficiently expressed from muscles cells further strengthens the idea that muscles can act as protein factories producing proteins with therapeutic effects. Hence, the insertion of the PENK gene could be a future treatment for patients suffering from chronic pain, which has been postulated by other sources (36).

9.3 Contamination

Some contamination was observed in water samples that were verified by gel electrophoresis and later DNA sequencing. The latter showed a product from the negative sample with a 97 % identity with the transcript encoding mouse PENK. The contamination was quantified to be around 0,001 pg. This little amount of contamination demonstrates how sensitive qPCR/RT-PCR assays are. It also demonstrates how important it is to keep a very clean work area. Many studies indicate that it is necessary to separate the laboratories into a: plasmid preparation area, RNA/DNA, cDNA synthesis area and finally the qPCR/RT-PCR assay area (37). In this study many different methods was used to try to eliminate the contamination. These included change of: lab, labcoat, pipettes, pipet tips and reagents. In addition to this, the qPCR/RT-PCR setup was performed in a flow bench, which eliminates the possibility that the contamination was due to aerosols. The work areas were subsequently cleansed with a DNA contamination reagent (38). The different pipettes that were used were also decontaminated using UV lighting. This method has been described to destroy nucleic acid (39).

10.0 Conclusion

This study indicates that it is indeed possible to use PENK as a gene doping candidate. Furthermore, it was impossible to detect this gene using qPCR at other sites then the actual transfected muscle. Thus, blood samples failed to detect the PENK gene, and as such making gene doping by electroporation ideal as a doping method. Ergo, it is imperative for anti doping purposes to find practically applicable techniques that would be able to detect this type of gene doping.

11.0 Perspective

The use of qPCR for detecting an inserted gene is a method of measuring the gene expression. Since it was not possible to detect PENK in blood following electroporation of muscle, it would be interesting to find another technique that might detect the PENK gene in the blood. It could be worth trying to change the detection method to enzyme-linked immunosorbent assay (ELISA). The idea in gene doping is that a muscle acts as a protein factory producing the doping agent and subsequently secreting this into the bloodstream. Since the results showed higher PENK gene expression in the muscle compared to control, the PENK-GFP proteins would be present in the blood, and thereby detectable using anti-GFP antibodies in an ELISA assay. Therefore, instead of measuring the gene expression in the cells, it would be the amount of PENK-GFP proteins in the blood. Thereby, the ELISA method might able to detect the doping agent in the blood.
Seeing as PENK exerts analgesic effects it could be interesting to investigate the degree of pain relief. This can be done by transfecting the PENK gene into mice via electroporation. Then in a fixed time period the mice pain response could be tested using either the hot plate or tail flick test. This would indicate whether or not the mice have obtained a higher nociceptive threshold. If this shows a higher nociceptive threshold, the results could be adapted to human studies. Hence, indicating a possible treatment capable of exerting analgesic effects in patients suffering from chronic pain.

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Appendix I

Statistical data for Figure 9

Descriptives

Test

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean	
					Lower Bound	Upper Bound
control	8	1,0000	,02733	,00966	,9771	1,0229
penk 24	4	1,0275	,01828	,00914	,9984	1,0566
penk 48	4	1,1029	,07654	,03827	,9811	1,2247
Total	16	1,0326	,05897	,01474	1,0012	1,0640

Descriptives

Test

	Minimum	Maximum
control	,96	1,05
penk 24	1,01	1,05
penk 48	1,04	1,21
Total	,96	1,21

Test of Homogeneity of Variances

Test

Levene Statistic	df1	df2	Sig.

3,342	2	13	,067

ANOVA

Test

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	,028	2	,014	7,743	,006
Within Groups	,024	13	,002		
Total	,052	15			

Robust Tests of Equality of Means

Test

	Statistic[a]	df1	df2	Sig.
Welch	4,261	2	6,126	,069

a. Asymptotically F distributed.

Post Hoc Tests

Multiple Comparisons

Dependent Variable: Test

Bonferroni

(I) gruppe	(J) gruppe	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
control	penk 24	-,02750	,02621	,939	-,0995	,0445
	penk 48	-,10287*	,02621	,005	-,1748	-,0309
penk 24	control	,02750	,02621	,939	-,0445	,0995
	penk 48	-,07537	,03026	,081	-,1585	,0077
penk 48	control	,10287*	,02621	,005	,0309	,1748
	penk 24	,07537	,03026	,081	-,0077	,1585

*. The mean difference is significant at the 0.05 level.

Appendix II

Animal approvals from the animal committee

J.nr. 2013-15-2934-00759 - Blod tapningen

Indhold

Beskrivelse af forsøgsaktiviteten: C 1
Beskrivelse af forsøgsaktiviteten: C 2
Dyreforsøgstilsynets afgørelse

Beskrivelse af forsøgsaktiviteten: C 1

Beskriv forsøget generelt: Denne del af ansøgningen skal kunne forstås af ikke fagfolk og skal omfatte: A) En beskrivelse af forsøgets formål. B) En redegørelse for, at tilsvarende viden kun kan opnås ved anvendelse af levende dyr (B1) og ikke kan opnås ved mindre belastende undersøgelser (B2) eller ved anvendelse af færre dyr (B3). C) En kort redegørelse for antagelsen om, at forsøget er til væsentlig gavn. D) En beskrivelse af samtlige indgreb, der ønskes foretaget på dyrene, herunder en udførlig beskrivelse af den belastning, dyrene herved udsættes for.

A) Formålet med forsøget er at undersøge hvilken effekt et gen, som er indsat i et cirkulært dobbeltstrenget DNA, har på organismen. Det dobbeltstrenget DNA hedder også et DNA plasmid, og det fungerer som en slags bærer, der kan levere et gen ind i musens eget arvemateriale. Dette opnås ved at injicerer et DNA plasmid, som indeholder et gen, ind i en muskel på en mus. Efter 1 og 2 dag(e) vil der blive tappe blod fra musen. Blodet bruges efterfølgende i målemetoden qPCR til at påvise det indførte gens tilstedeværelse og kan således kvantificeres. Tilsvarende tages også det injicerede væv fra musens lårmuskel, der ligeledes bliver analyseret. Denne viden, som opnås ved dette forsøg, skal indgå i et større forsøg (der søges en separat tilladelse til), hvor flere forskellige DNA plasmider skal bruges til at indsætte tre slags gener i musenes DNA. I dette forsøg vil der blive injiceret forskellige koncentrationer af DNA plasmid ind i mus, for derved at undersøge dosis-respons forholdet og sikre den mest optimale dosis af DNA plasmid til det større forsøg. Forsøget vil således bidrage med viden til en behandling af kroniske smertepatienter med genterapi. Dette er muligt, fordi det indførte gen koder for proteinet proenkephalin, som er smertelindrende. Derfor ved at dette gen bliver integreret i musens arvemateriale, kan det blive produceret i en højere mængde, og dertil en lindrende effekt på smerte. Dette forsøg vil også kunne bruges til at teste atleter, om de benytter gendoping, hvilket giver antidopingsagenturer verden over mulighed for at teste for denne form for sportssnyd.

B1) For at måle den effekt genet giver i organismen, er det vigtigt at bruge levende dyr, for at DNA plasmidets optagelse, virkning, omsætning og udskillelse er så tæt på den naturlige proces, som muligt. Dyrenes stofskifte ligner den menneskelige, og resultaterne kan derfor overføres hertil. Derfor bliver den nødvendige viden af DNA plasmidets effekt opnået, da denne har været påvirket af den levende organismes biologi og fysiologi. Herved bliver de ukendte faktorer, som stammer herfra, minimeret og resultaterne er derfor troværdige. Hele det sammenspil af hormoner, immunforsvar, etc., der eksisterer i organismen ville være umulige at efterligne i et in vitro forsøg,

og tilsvarende viden, vil derfor ikke kunne opnås.

B2) Dyrene vil blive udsat for let til ingen belastning af kortere varighed, som vil foregå under bedøvelse. Dernæst vil dyrene leve efter dyrestaldens foranstaltninger. Dyrene aflives i bedøvelse.

B3) Der anvendes så få dyr som muligt, dog så resultaterne vil være troværdige. Der anvendes også informationer fra artikler, hvilket dermed bidrager til at holde dyreantallet nede på et minimum. I en populationsberegning med en power på 80 %, et signifikantniveau på 95 % og en spredning på 8 skal der bruges 6 dyr i hver gruppe. Information heromkring er hentet fra lignende forsøg.

C) Ifølge ugeskrift for læger har 20 % af den danske befolkning mellem 16 - 67 år langvarige/kroniske smerte. Dette tal svarer til 800 000 personer hvori størstedelen er kvinder. Dette forsøg vil være med til at belyse muligheden for at benytte genterapi til smertebehandling og derved give øget livskvalitet hos disse personer. Dvs. en femtedel af den danske befolkning lider af dette, og det må derfor erkendes, at sådan type forsøg er til væsentlig gavn. Forsøget vil være med til at undersøge, om det vil være muligt at indføre et gen pakket i et DNA plasmid ind i kroppen, og derved få den effekt genet koder for. Genet, som vil blive indsat, koder for et smertelindrende protein, og man vil derfor ved at producerer dette i et stort omfang opnå en smertelindrende effekt. Derudover kan man i følge Antidoping Danmarks hjemmeside få oplyst, at 10 % af Danmarks cykel Unions medlemmer har forsøgt og prøvet doping og 20 % i motions- og fitnesscentre. Dette må siges at være et højt antal, og doping bliver derfor et stadigt større problem i sportsverdenen. Metoder hertil bliver hele tiden forbedret, og det er et spørgsmål om tid, inden der tages brug af genterapien (gendoping) for at få øget præstationsevne. Denne viden vil hjælpe sportsagenturer over hele verden med at opdage sportssnyd og derved bevare sportsverdens integritet og ånd. Dette skyldes, de får mulighed for at opdage, når sportsudøvere benytter sig af genetisk doping, og dermed være et skridt foran den ulovlige del af sportsverden.

D) Musene vil blive bedøvet Hypnorm/Dormicum (0,3 ml/100 g) subkutant. De vil herefter blive opdelt i 6x5 grupper, hvor hver gruppe får henholdsvis indsprøjtet 0 µg (6 dyr), 0,5 µg (6 dyr), 5 µg (6 dyr), 50 µg (6 dyr), 100 µg (6 dyr) af DNA plasmid intramuskulært i lårmusklen. De forskellige koncentrationer skal bruges til at registrere et dosis-respons forhold, som skal bruges til at sikre den korrekte dosis til det senere større forsøg. DNA plasmidet vil blive injiceret sammenblandet med sterilt saltvand. Der vil blive anvendt 20G kanyle, og 0,1 ml vil blive injiceret. Der vil være 6 mus i hver gruppe. Musene, som vil få indsprøjtet 0 ug, vil her blive injiceret med 0,1 ml sterilt saltvand. Efter 1 dag og 2 dages ventetid, vil musene blive aflivet (3 dyr for hver koncentration, for hvert tidspunkt) ved cervikal dislokation, hvorefter der vil blive udtaget hjerteblod og væv fra lårmusklen. Blodet (omkring 0,5 - 1 ml) vil blive opsamlet i rør indeholdende EDTA, for at forhindre at blodet koagulerer og bibeholde RNA mængden, og muskelvæv vil blive nedfrosset i rør (ca. 10 mg pr. rør) i flydende kvælstof. Blod mængden vil være nok til at lave 3 RNA oprensninger, som er tilstrækkeligt for at få troværdige resultater. Det samme gælder for muskel væv, idet der vil blive taget 3 gange 10 mg fra den samme lårmuskel. Musene vil i dette forsøg opleve let til ingen smerte, da de bedøves med Hypnorm/Dormicum mens indsprøjtningen foregår, herefter vil de vågne op i

dyrestalden. Musene vil blive aflivet i bedøvet tilstand. Så efter dag 1 vil 3 mus fra hver gruppe blive bedøvet og aflivet. Resultaterne, som kommer herfra vil blive brugt til at måle, hvor effektiv de forskellige koncentrationer af DNA plasmidet har været i at levere genet til muskelcellerne i lårmusklen og blodcellerne, samt hvor stabilt og detekterbare det indførte gen er i blodet.

Skyldes forsøgene krav fra myndighed, som led i godkendelse af stof eller produkt?
Hvis "Ja", beskriv nærmere

Nej

Beskriv forsøgets type, art og forløb, herunder de planlagte indgreb og påvirkning af organfunktioner:
(Der vedlægges evt. forsøgsprotokol. Det angives og begrundes, hvorvidt de givne oplysninger skal hemmeligholdes).

Dette forsøg er et testforsøg, hvor vi vil teste et DNA plasmids evne til at levere et gen ind i en værts arvemateriale. Dette gøres ved, at injicere DNA plasmidet intramuskulært i forskellige koncentrationer (se ovennævnte) ind i mus, og derefter tappe blod og udtage muskelvæv for videre at blive analyseret i en qPCR målemetode. Herved kan man måle genudtrykkelsen af det gen man søger. Injektionen vil være i højre lårmuskel. Musene vil blive bedøvet med Hypnorm/Dormicum (0,3 ml/100 g) s.c. De vil blive nappet i halen og poten for at registrere om anæstesiens virkning er sat ind. De vil derfor ikke opleve smerte, lidelse og lign. Efter 1 dage vil 3 mus fra hver gruppe blive bedøvet med Hypnorm/Dormicum (0,3 ml/100 g) s.c. og blive aflivet. Blod (0,5 -1 ml) fra hjertet og muskelvæv (3 gange 10 mg) fra lårmusklen vil blive opsamlet. Forsøget slutter efter 2 dage med at de sidste mus vil blive aflivet ved cervikal dislokation (i bedøvet tilstand) og herefter vil blodet blive tappet fra hjertet. Yderligere vil 3x10 mg væv fra lårmusklen fra musene blive udtaget og nedfrosset. Blodet vil blive opsamlet i eppendorf rør indeholdende EDTA, således blodkoagulationen mindskes og RNA oprensningen kan lade sig gøre. Musenes organer vil blive påvirket af Hypnorm/Dormicum, og dens celler vil ved indføringssted blive påvirket. Påvirkningen af organfunktioner på hele musen vil reelt kun være af bedøvelsen, og aflivningen, som vil foregå hurtigt. DNA plasmidet, der bliver benyttet, indeholder et gen, som koder for proteinet proenkephalin. Dette er et endogent opioid protein, som virker smertehæmmede i musen og mennesket. Injektionen med DNA plasmid er tidligere blevet lavet jr. artikel " In Vivo Imaging of Far-red Fluorescent Proteins after DNA Electrotransfer to Muscle Tissue" af P. Hojman, J. Eriksen og J. Gehl.

Belastningsgraden:

Ubehag:	Let
	Let/Ingen – Musene vil være bedøvet under forsøget.
Lidelse:	Let
	Let/Ingen – Musene vil være bedøvet under forsøget.
Smerte:	Ingen
	Ingen – Musene vil være bedøvet under forsøget.
Påvirket bevægelsesfrihed:	Nej
Påføres varigt mén:	Nej

Belastningens varighed:

Kortvarigt

Forsøgets varighed:

Døgn

Aflives i bedøvelsen:

Nej

Beskriv den anvendte anæstesi for hver dyreart:

Hypnorm/Dormicum (0,3 ml/100 g) s.c.
Evt. Hypnorm/Dormicum (0,1 ml/100 g) subkutant efter 30 – 40 minutter.

Beskriv den påtænkte smertebehandling og anden lindrende behandling for hver dyreart:

Musene vil være under bedøvelse under hele eksperimentet og vil blive aflivet efter 1 dag og 2 dage

Angiv de velfærdsmæssige kriterier (humane endpoints) for afbrydelse af forsøget for hver dyreart:

Forsøget bliver afbrudt, hvis dyrene begynder at vise tegn på smerte, lidelse eller anden nedsat almen tilstand.

Angiv aflivningsmetoden for hver dyreart:

Cervikal dislokation

Beskriv pasningen af og tilsyn med dyrene, herunder særlige foranstaltninger ved tilsyn:

Musene passes efter dyrestaldens forskrifter med minimum 1 dagligt tilsyn. Dyrene vil have foder og vand ad libitum og vil leve under en 12 timers lys/mørke rytme.

Dyreart og antal pr. år: (Såfremt der påtænkes anvendt genetisk modificerede dyr, udfyldes det særskilte skema for hver anvendelse.)

50, c57black/6 mus. Dette kræves for at kunne lave statistiske udregner. Der tages som beskrevet flere prøver fra samme mus, hvilket giver en større mængde data, som bruges til at give pålideligt data.

Ønsket varighed af tilladelsen til det beskrevne forsøg angivet i antal år:

1 år

Dette forsøg skal bruges for at etablere en dose-response kurve, og projektperioden varer 1 år.

Beskrivelse af forsøgsaktiviteten: C 2

Beskriv forsøget generelt: Denne del af ansøgningen skal kunne forstås af ikke fagfolk og skal omfatte: A) En beskrivelse af forsøgets formål. B) En redegørelse for, at tilsvarende viden kun kan opnås ved anvendelse af levende dyr (B1) og ikke kan opnås ved mindre belastende undersøgelser

(B2) eller ved anvendelse af færre dyr (B3). C) En kort redegørelse for antagelsen om, at forsøget er til væsentlig gavn. D) En beskrivelse af samtlige indgreb, der ønskes foretaget på dyrene, herunder en udførlig beskrivelse af den belastning, dyrene herved udsættes for.

A) Formålet med forsøget er at få en forståelse af transfektionseffektiviten af 1 plasmid i hele organismer. Denne viden skal indgå i et større forsøg, hvor plasmiderne skal indsættes i en vært ved brug af forskellige transfections metoder, og dermed bidrage til forskningen indenfor gendopingsområdet. B1) Når transfektionseffektiviten skal findes, skal der tages højde for mange faktorer f.eks. immunforsvaret. Det er derfor nødvendigt at bruge levende dyr, for at plasmidet bliver påvirket af alle faktorer. Disse faktorer vil være svære at efterligne i et in vitro forsøg. B2) Dyrene vil blive udsat for let belastning, da det er en blodtapning, der kommer til at foregå. Dog er forsøget af nødvendig karakter, og der ønskes tilfredsstillende resultater. B3) Der anvendes mange informationer i form af artikler og tilstrækkelige in vitro forsøg er udført, for derved at holde dyreantallet nede på et minimum. C) Doping bliver et stadigt større problem i sportsverdenen, og da metoder herved bliver forbedret, er det bare et spørgsmål om tid, inden denne tager brug af gendoping (genterapi). Forsøget vil være med til at undersøge, om det vil være muligt at indføre et gen pakket i et plasmid ind i kroppen, og derved få den effekt gen'et koder for. D) Musene bliver bedøvet ved brug af isoflurane og der bliver efterfølgende tappet blod. Derefter bliver C57Black/6 musene injiceret med plasmid DNA i forskellige koncentrationer i lårmusklen, efterfølgende vil musene blive aflivet ved total blod tapning (exsanguination) i bedøvet tilstand efter 15 minutters ventetid (Gothelf, A., Hojman, P., & Gehl, J. (2010). Therapeutic levels of erythropoietin (EPO) achieved after gene electrotransfer to skin in mice. Gene therapy, 17(9), 1077-1084. doi:10.1038/gt.2010.46). Indgrebet her vil være minimalt, da musene er bedøvet. Blodet og muskelvæv vil efterfølgende blive analyseret.

Skyldes forsøgene krav fra myndighed, som led i godkendelse af stof eller produkt?
Hvis "Ja", beskriv nærmere

Nej

Beskriv forsøgets type, art og forløb, herunder de planlagte indgreb og påvirkning af organfunktioner:
(Der vedlægges evt. forsøgsprotokol. Det angives og begrundes, hvorvidt de givne oplysninger skal hemmeligholdes).

Musene bliver bedøvet under førsøget med isoflurane. Herefter får de indsprøjtet 0 µg, 0.5 µg, 5 µg, 50 µg, 500 µg, 5 mg og 50 mg af plamid DNA. Der vil herefter gå 15 minutter og dyrene vil blive aflivet. (Gothelf, A., Hojman, P., & Gehl, J. (2010). Therapeutic levels of erythropoietin (EPO) achieved after gene electrotransfer to skin in mice. Gene therapy, 17(9), 1077-1084.

doi:10.1038/gt.2010.46). Når musene er døde vil væv fra lårmusklen opsamles. Plasmidet, som bruges indeholder gen for preproenkephalin og betragtes som kandidat til gendoping, da genproduktet nemlig har smertenedsættende effekt.

Belastningsgraden:

Ubehag:	Let
	Musene vil være bedøvet under forsøget.
Lidelse:	Let
	Musene vil være bedøvet under forsøget.
Smerte:	Ingen
	Musene vil være bedøvet under forsøget.
Påvirket bevægelsesfrihed:	Nej
Påføres varigt mén:	Nej

Belastningens varighed:

Kortvarigt

Forsøgets varighed:

Timer

Aflives i bedøvelsen:

Ja

Beskriv den anvendte anæstesi for hver dyreart:

5 % Isoflurane

Beskriv den påtænkte smertebehandling og anden lindrende behandling for hver dyreart:

Musene vil være under bedøvelse under hele eksperimentet

Angiv de velfærdsmæssige kriterier (humane endpoints) for afbrydelse af forsøget for hver dyreart:

Forsøget bliver afbrudt, hvis dyrene begynder at vise tegn på smerte eller anden nedsat almen tilstand.

Angiv aflivningsmetoden for hver dyreart:

De bedøvede mus vil blive aflivet ved total blodtapning (exsanguination).

Beskriv pasningen af og tilsyn med dyrene, herunder særlige foranstaltninger ved tilsyn:

Indtil musene skal bruges, passes de efter dyrestaldens forskrifter med minimum 1 dagligt tilsyn. Herefter vil de være bedøvet og termineret efter 30 min.

Dyreart og antal pr. år: (Såfremt der påtænkes anvendt genetisk modificerede dyr, udfyldes det særskilte skema for hver anvendelse.)

50, c57black/6 muse. Dette kræves for at kunne lave statistiske udregner. (Geoff Cumming, Fiona Fidler, and David L. Vaux, Error bars in experimental biology)

Ønsket varighed af tilladelsen til det beskrevne forsøg angivet i antal år:

1 år

Dette forsøg skal bruges for at etablere en baseline af musenes normale genekspression. Derfor er det hensigtsmæssigt at have tilladelsen i en hvis mængde tid. Derved hvis forsøget kræver optimering, er det muligt at refererer til denne tilladelse for at opnå en ny.

.nr. 2013-15-2934-00760 - plasmid med elektro

Indhold

Beskrivelse af forsøgsaktiviteten: C 1
Beskrivelse af forsøgsaktiviteten: C 2
Dyreforsøgstilsynets afgørelse

Beskrivelse af forsøgsaktiviteten: C 1

Beskriv forsøget generelt: Denne del af ansøgningen skal kunne forstås af ikke fagfolk og skal omfatte: A) En beskrivelse af forsøgets formål. B) En redegørelse for, at tilsvarende viden kun kan opnås ved anvendelse af levende dyr (B1) og ikke kan opnås ved mindre belastende undersøgelser (B2) eller ved anvendelse af færre dyr (B3). C) En kort redegørelse for antagelsen om, at forsøget er til væsentlig gavn. D) En beskrivelse af samtlige indgreb, der ønskes foretaget på dyrene, herunder en udførlig beskrivelse af den belastning, dyrene herved udsættes for.

A) Formålet med forsøget er, at undersøge hvorvidt det er muligt ved brug af genindførelsesteknikken, elektroporation at indføre et ønsket gen ind i musenes arvemateriale. Dette gøres ved at injicere DNA plasmid, som indeholder det ønskede gen, ind i mus, hvorefter en elektrisk spænding bliver tilført indførelsesstedet. Herved vil de celler, som bliver påvirket af den elektriske spændingen optage plasmidet og det tilførte gen kan derved blive inkorporeret i arvematerialet. For at teste for transskriptionssucces (at genet er blevet optage i musens DNA), vil blod og muskelvæv blive analyseret ved brug af qPCR. En målemetode hvorved man kan undersøge et bestemt gens udtrykkelse, og således kvantificerer det. Generne (prodynorphin, proopiomelanocortin og preproenkephalin), som vil blive indsat, koder for proteiner, der har smertelindrende effekt. Forsøget vil således bidrage med viden til en behandling af kroniske smertepatienter med genterapi. Dette er muligt, fordi det indførte gen koder for et protein med smertelindrende, og kan ved at blive integreret i musens arvemateriale, blive produceret i en højere mængde. I forlængelse af genterapi kan sådan viden også bruges i ond hensigt, og her tænkes på gendoping. Dette forsøg vil også kunne bruges til at teste atleter, om de benytter gendoping, hvilket giver antidopingsagenturer verden over mulighed for at teste for denne form for sportssnyd.

B1) For at måle den effekt genet giver i organismen, er det vigtigt at bruge levende dyr, for at DNA plasmidets optagelse, virkning, omsætning og udskillelse er så tæt på den naturlige proces, som muligt. Dyrenes stofskifte ligner den menneskelige, og resultaterne kan derfor overføres hertil.

Derfor bliver den nødvendige viden af DNA plasmidets effekt opnået, idet denne har været påvirket af den levende organismes biologi og fysiologi. De ukendte faktorer, som stammer herfra, bliver minimeret og resultaterne er derfor troværdige. Hele det sammenspil af hormoner, immunforsvar, etc., der eksisterer i organismen ville være umulige at efterligne i et in vitro forsøg, og tilsvarende viden vil derfor ikke kunne opnås.

B2) Der vil ikke være belastninger for musene, når genet bliver introduceret i deres arvemateriale. Dette vil foregå under bedøvelse. Musene vil herefter leve under dyrestaldens foranstaltninger i hhv. 1 og 2 dag(e). Elektroporationen kan, ifølge litteraturen, give muskelskader, men at det kun er i form af nedsat muskelstyrke og varigheden er kort. Her henvises til artikel " Efficient and regulated erythropoietin production by naked DNA injection and muscle electroporation" af Rizzuto G, Cappelletti M, Maione D, Savino R, Lazzaro D, Costa P, Mathiesen I, Cortese R, Ciliberto G, Laufer R, La Monica N, Fattori E.

B3) Der anvendes så få dyr som muligt, dog så der stadigt vil kunne dannes troværdige resultater. Der anvendes også informationer i form af artikler, og tilstrækkelige in vitro forsøg er udført, hvilket dermed bidrager til at holde dyreantallet nede på et minimum. Dvs. for at få en power på 80 % og et signifikantniveau på 95 %, og ud fra tidligere artikler, som arbejder med samme type forsøg, have en spredning på 10, skal der bruges 8 mus i hver gruppe.

C) Ifølge ugeskrift for læger har 20 % af den danske befolkning mellem 16 - 67 år langvarige/kroniske smerte. Det tal svarer til 800 000 personer hvori størstedelen er kvinder. Dette forsøg vil være med til at belyse muligheden for at benytte genterapi til smertebehandling og derved give øget livskvalitet hos disse personer. Idet en femtedel af den danske befolkning lider af smerte er et sådan type forsøg til væsentlig gavn. Forsøget vil være med til at undersøge, om det vil være muligt at indføre et gen pakket i et DNA plasmid ind i kroppen, og derved få den effekt genet koder for. Genet, som vil blive indsat, koder for et smertelindrende protein, og man vil derfor ved at producerer dette i et stort omfang opnå en smertelindrende effekt. Derudover kan man i følge Antidoping Danmarks hjemmeside få oplyst, at 10 % af Danmarks cykel Unions medlemmer har forsøgt og prøvet doping og 20 % i motions- og fitnesscentre. Dette må siges at være et højt antal, og doping bliver derfor et stadigt større problem i sportsverdenen. Metoder hertil bliver hele tiden forbedret, og det er et spørgsmål om tid, inden der tager brug af genterapien (gendoping) for at få øget præstationsevne. Dette forsøg vil derfor også hjælpe sportsagenturer over hele verden med at opdage sportssnyd og derved bevare sportsverdens integritet og ånd. Dette skyldes, de får mulighed for at opdage, når sportsudøvere benytter sig af genetisk doping, og dermed være et skridt foran den ulovlige del af sportsverden.

Det er i øvrigt muligt at bruge virus som gen leverings metoden (transfektionsmetode), dog er dette ikke risikofrit (risiko for cancer) og det er svært at forestille sig kronisk smerte patienter/ sportsudøvere vil benytte denne. Derfor vil elektroporationsmetoden åbne en ny mulighed for at indføre gener ind i arvematerialet på en mere sikker måde.

D) C57Black/6 musene vil blive bedøvet med Hypnorm/Dormicum (0,3 ml/100 g). Fra tidligere forsøgs resultater vil den korrekt mængde DNA plasmid blive injiceret med en 20G kanyle intramuskulært i musenes lårmuskel. Der vil blive indsprøjtet 0,1 ml saltvand indeholdende plasmid DNA (den endelige plasmid koncentraionen afhængiger af resultater fra tidligere forsøg). I tiden lige efter injektionen vil der med en elektrodeklemme (en "plate-and-fork" elektrode) blive tilført en spænding på otte gange 50 millisekunder impulser med 12 - 50 V mellem hver elektrode. Musene vil herefter blive opdelt i 2 divisioner, hvori den ene gruppe vil blive aflivet efter 1 dage og den anden efter 2 dage. Efter elektroporationen vil der være en ventetid på 1 og 2 dage hvorefter musene vil blive aflivet ved cervikal dislokation. Blod fra hver mus vil blive taget fra hjertet og vil her blive opsamlet i rør med EDTA, som forhindrer koagulation og nedbrydelsen af RNA (RNA bruges til at kvantificere genudtrykkelsen). Der vil også blive udtaget væv fra lårmusklerne (omkring 10 mg), som vil blivenedfrosset i flyende kvælstof. Dette er for at bevare RNA mængden i muskelvævet. De opsamlede prøver vil derefter blive analyseret og genudtrykkelsen fra disse vil blive belyst ved brug af qPCR. Der skal indsætte i alt tre forskellige gener i forskellige mus. Således, at der i en mus kun vil blive indsat et gen. Der skal således bruges 8 mus for hvert gen og en kontrol gruppe, som ikke vil blive injiceret med DNA plasmid. Disse vil få tilsvarende mængde injiceret, bare i sterilt vand, som de andre grupper. Dvs. der skal bruges i alt 32 mus.

Skyldes forsøgene krav fra myndighed, som led i godkendelse af stof eller produkt?
Hvis "Ja", beskriv nærmere

Nej

Beskriv forsøgets type, art og forløb, herunder de planlagte indgreb og påvirkning af organfunktioner:
(Der vedlægges evt. forsøgsprotokol. Det angives og begrundes, hvorvidt de givne oplysninger skal hemmeligholdes).

Dette forsøg er et transskriptionsforsøg med et translationelt genterapeutiskpotentiale, hvor vi vil teste muligheden for, at indfører nye gener ind i en organismes arvemateriale. Dette gøres ved brug af elektroporation, og med DNA plasmider, der indeholder de ønskede gener. Forsøget starter ved, at C57Black/6 musene vil blive bedøvet med Hypnorm/Dormicum (0,3 ml/100 g) s.c. Der vil blive nappet i halen og poten for at registrere om anæstesiens virkning er sat ind. Musene vil herefter blive injiceret med DNA plasmid, hvor koncentrationen vil blive helt klarlagt efter resultater fra første forsøg er fremkommet (dog vil der ikke blive injicerer højere koncentrationer en 100 µg DNA plasmid). Der skal i alt indsættes tre DNA plasmider i forskellige mus, som vil blive inddelt i grupper. Musene vil desuden blive inddelt i divisioner hvor 1. division er 1 dag og 2. division er 2. dag. Grupperingen foregår således, at for første gen (proopiomelanocortin), vil der være en gruppe

med 8 mus, som modtager dette. For det andet gen (prodynorphin) vil der være 8 andre mus i en anden gruppe, som vil få indsat dette. Det samme gør sig gældende for det tredje gen (preproenkephalin). Der vil desuden være en fjerde gruppe på 8 mus, som vil få injiceret saltvand i stedet for plasmid DNA og vil derfor være kontrolgruppen i forsøget. Injektionen vil foregå intramuskulært i lårmusklen. Lige efter injektionen vil der ved indførelsesstedet blive fastsat en elektrodeklemme (plate-and-fork elektrode), og en spændingen på 12 - 50 V vil blive givet otte gange med 50 millisekunders varighed. Den elektriske spænding gør, at cellerne i spændingsfeltet bliver gennemtrængelige og det indsprøjtede DNA plasmid kan blive optaget. Dette gøres på alle musene inden for samme tidsperiode. Efter elektroporationen vil der være en ventetid på 1 dag, hvor optagelsen af DNA plasmid kan lade sig gøre. Når den 1. dag er gået, vil den første division af musene blive bedøvet med Hypnorm/Dormicum (0,3 ml/100 g) s.c. og aflivet ved cervikal dislokation, og blod fra hjertet vil blive udtaget. Dette vil blive opsamlet i eppendorf rør med EDTA, for derved at bevare RNA og forhindre koagulation. Omkring 10 mg væv fra den lårmuskel injektionen fandt sted, vil også blive udtaget og nedfrosset i kvælstof. Herved bevares RNA og kan således bruges i qPCR. Der vil, fra hver lårmuskel, udtages tre gange 10 mg væv, for at give flere resultater og dermed øge troværdigheden af genekspression herfra. Ved oprensning af RNA er det tilstrækkeligt med små mængder, derfor vil den mængde blod, som bliver tappet fra musenes hjerter, være rigeligt til at få flere resultater. Den anden division af musene vil gå i 2 døgn, før de bliver aflivet ved cervikal dislokation under bedøvelse. Proceduren med elektroporation og indførelsen af gener pakket i DNA plasmid, er udført tidligere. Dette forsøg tager derfor udgangspunkt i artiklen " In Vivo Imaging of Far-red Fluorescent Proteins after DNA Electrotransfer to Muscle Tissue" af P. Hojman, J. Eriksen og J. Gehl. Organer vil blive bevirket af bedøvelsen, men af mindre karakter. Desuden vil disse også påvirkes af aflivningen, men dette vil foregå hurtigt.

Belastningsgraden:	
Ubehag:	Moderat Musene vil være bedøvet, når DNA plasmider introduceres, herved minimeres stress og ubehag for musene. Alle musene vil blive aflivet i bedøvelse (3 fra hver gruppe efter 1 dag og 3 fra hver gruppe efter 2 dage). Som nævnt tidligere kan elektroporation give kortvarig muskelskade i form af nedsat styrke
Lidelse:	Let Let/Moderat Musene vil være bedøvet, når DNA plasmider introduceres. Alle musene vil blive aflivet i bedøvelse (3 fra hver gruppe efter 1 dag og 3 fra hver gruppe efter 2 dage). Som nævnt tidligere kan elektroporation give

	kortvarig muskelskade i form af nedsat styrke
Smerte:	Let
	Musene vil være bedøvet, når DNA plasmider introduceres. Alle musene vil blive aflivet i bedøvelse (3 fra hver gruppe efter 1 dag og 3 fra hver gruppe efter 2 dage).
Påvirket bevægelsesfrihed:	Nej
Påføres varigt mén:	Nej

Belastningens varighed:

Kortvarigt

Forsøgets varighed:

Døgn

Aflives i bedøvelsen:

Nej

Beskriv den anvendte anæstesi for hver dyreart:

Hypnorm/Dormicum (0,3 ml/100 g) s.c.
Evt. Hypnorm/Dormicum (0,1 ml/100 g) subkutant efter 30 – 40 minutter.

Beskriv den påtænkte smertebehandling og anden lindrende behandling for hver dyreart:

Musene vil være bedøvet, når DNA plasmider introduceres. Den første division dyr aflives 1 dag og anden division 2. dag post-transfektion. Dyrene vil blive bedøvet først, og vil derfor ikke opleve nogen smerte. Dyrene smertedækkes det første døgn med buprenofin 3 gange med 8 timers mellemrum. Hvis dyrene viser tegn på smerte, lidelse eller anden nedsat almen tilstand vil de blive aflivet.

Angiv de velfærdsmæssige kriterier (humane endpoints) for afbrydelse af forsøget for hver dyreart:

Forsøget bliver afbrudt, hvis dyrene begynder at vise tegn på smerte, lidelse eller anden nedsat almen tilstand.

Angiv aflivningsmetoden for hver dyreart:

De bedøvede mus vil blive aflivet ved cervikal dislokation

Beskriv pasningen af og tilsyn med dyrene, herunder særlige foranstaltninger ved tilsyn:

Inden musene skal bruges, passes de af uddannede dyreassistenter og lever under dyrestaldens forskrifter med minimum et dagligt tilsyn. Dyrene vil have foder og vand ad libitum og vil leve under en 12 timers lys/mørke rytme.

Dyreart og antal pr. år: (Såfremt der påtænkes anvendt genetisk modificerede dyr, udfyldes det særskilte skema for hver anvendelse.)

50 c57black/6 muse. 8 mus i hver gruppe giver signifikant data til at kunne udfører de statistiske analyser. Jf. beskrevet længere oppe, bliver der for hver blodtapning og muskelvæv lavet et større antal RNA oprensninger. Dette giver nok resultater til at gøre forsøgets udfald troværdigt.

Ønsket varighed af tilladelsen til det beskrevne forsøg angivet i antal år:

1 år

Projektets varighed

Beskrivelse af forsøgsaktiviteten: C 2

Beskriv forsøget generelt: Denne del af ansøgningen skal kunne forstås af ikke fagfolk og skal omfatte: A) En beskrivelse af forsøgets formål. B) En redegørelse for, at tilsvarende viden kun kan

opnås ved anvendelse af levende dyr (B1) og ikke kan opnås ved mindre belastende undersøgelser (B2) eller ved anvendelse af færre dyr (B3). C) En kort redegørelse for antagelsen om, at forsøget er til væsentlig gavn. D) En beskrivelse af samtlige indgreb, der ønskes foretaget på dyrene, herunder en udførlig beskrivelse af den belastning, dyrene herved udsættes for.

A) Formålet med forsøget er, at undersøge hvorvidt det er muligt ved brug af genindførelsesteknikken, elektroporation at indføre et ønsket gen ind i musenes arvemateriale. Dette gøres ved at injicere DNA plasmid, som indeholder det ønskede gen, ind i mus, hvorefter en elektrisk spænding bliver tilført indførelsesstedet. Herved vil de celler, som bliver påvirket af den elektriske spændingen optage plasmidet og det tilførte gen kan derved blive inkorporeret i arvematerialet. For at teste for transskriptionssucces (at genet er blevet optage i musens DNA), vil blod og muskelvæv blive analyseret ved brug af qPCR. En målemetode hvorved man kan undersøge et bestemt gens udtrykkelse, og således kvantificerer det. Generne (prodynorphin, proopiomelanocortin og preproenkephalin), som vil blive indsat, koder for proteiner, der har smertelindrende effekt. Forsøget vil således bidrage med viden til en behandling af kroniske smertepatienter med genterapi. Dette er muligt, fordi det indførte gen koder for et protein med smertelindrende, og kan ved at blive integreret i musens arvemateriale, blive produceret i en højere mængde. I forlængelse af genterapi kan sådan viden også bruges i ond hensigt, og her tænkes på gendoping. Dette forsøg vil også kunne bruges til at teste atleter, om de benytter gendoping, hvilket giver antidopingsagenturer verden over mulighed for at teste for denne form for sportssnyd.

B1) Da det ønskes at påvise gen ekspressionen af et gen, er det derfor nødvendigt at benytte sig af levende dyr. Dette er fordi at gener er påvirket af mange faktorer i organismen, og det er ikke muligt at mimere disse omstændigheder in vitro. B2) Der vil ikke være belastninger for musene, når det smertenedsættende gen bliver introduceret i deres DNA. Dette vil foregå under bedøvelse. B3) Der er forud for forsøget blevet studeret litteratur og lavet undersøgelser in vitro, hvorved at antallet af dyr, som er krævet, holdes på et minimum. Der benyttes det antal dyr, som kræves for at få signifikant data til de statistiske udregninger. C) Doping bliver et stadigt større problem i sportsverdenen, og da metoderne herved bliver forbedret, er det bare et spørgsmål om tid, inden gendoping (genterapi) bliver taget i brug. Forsøget vil være med til at undersøge, om det vil være muligt at indføre et gen, pakket i plasmid DNA, ind i kroppen, og derved få den effekt genet koder for.

D) Musene bliver bedøvet ved brug af isoflurane, herefter bliver C57Black/6 musene transfekteret med DNA plasmid. Dette gøres ved at benytte transfektionsmetoden elektroporation, hvorved det er muligt at få inkorporeret det ønskede gen i musenes arvemateriale. Der vil i alt være 3 plasmider, som skal indsættes. Efterfølgende vil en mus i hver gruppe blive termineret. Musene vil blive euthaniseret ved exsanguination, og deres muskelvæv og blod vil blive opsamlet og analyseret. Gothelf, A., Hojman, P., & Gehl, J. (2010). Therapeutic levels of erythropoietin (EPO) achieved after gene electrotransfer to skin in mice. Gene therapy, 17(9), 1077-1084. doi:10.1038/gt.2010.46)

Skyldes forsøgene krav fra myndighed, som led i godkendelse af stof eller produkt?
Hvis "Ja", beskriv nærmere

Nej

Beskriv forsøgets type, art og forløb, herunder de planlagte indgreb og påvirkning af organfunktioner:
(Der vedlægges evt. forsøgsprotokol. Det angives og begrundes, hvorvidt de givne oplysninger skal hemmeligholdes).

Musene vil i dyrestalden blive bedøvet ved brug af 5 % isoflurane og efterfølgende blive givet 0,5 % pentobarbital 70 mg/kg (Ellrich J and Wesselak M. Electrophysiology of sensory and sensorimotor processing in mice under general anesthesia. Brain Research. Brain Research Protocols 11: 178-188, 2003) Efter musene er blevet fuldt bedøvet vil plasmiderne blive introduceret ind i musen. Dette gøres med elektroporation. Musene vil blive opdelt i 4 grupper, alt efter hvilket DNA plasmid, der er introduceret. Der vil i alt være 3 DNA plasmider, som indeholder gener for hver sit dopingsmiddel, og 1 kontrolgruppe (intet dopingmiddel). Mængden af plasmid, som indføres i musene, vil være bestemt ud fra foregående test. Efter indsættelsen af generne (ca. 15 min.) vil musene bliver aflivet. Musenes blod og væv vil blive opsamlet og analyseret.

Belastningsgraden:

Ubehag:	Let Musene vil være bedøvet, når DNA plasmider introducers, herved minimeres stress og ubehag for musene.
Lidelse:	Let Musene vil være bedøvet, når DNA plasmider introduceres, dog vil de lide en smule, når de udsættes for smerte. Dette vil dog være kortvarigt, så mængden musene vil lide er minimal
Smerte:	Let Musene vil være bedøvet, når DNA plasmider introduceres, så her vil de ikke have smerte.
Påvirket bevægelsesfrihed:	Nej
Påføres varigt mén:	Nej

Belastningens varighed:

Længerevarende

Forsøgets varighed:

Timer

Aflives i bedøvelsen:

Ja

Beskriv den anvendte anæstesi for hver dyreart:

5 % Isoflurane
0,5 % pentobarbital 70 mg/kg (Ellrich J and Wesselak M. Electrophysiology of sensory and sensorimotor processing in mice under general anesthesia. Brain Research. Brain Research Protocols 11: 178-188, 2003)

Beskriv den påtænkte smertebehandling og anden lindrende behandling for hver dyreart:

Musene vil være bedøvet, når DNA plasmider introduceres. Hvis viser tegn på fortsat smerte eller anden nedsat almen tilstand, vil de blive aflivet. Efter transfektionen vil musene blive aflivet.

Angiv de velfærdsmæssige kriterier (humane endpoints) for afbrydelse af forsøget for hver dyreart:

Forsøget bliver afbrudt, hvis dyrene begynder at vise tegn på smerte eller anden nedsat almen tilstand.

Angiv aflivningsmetoden for hver dyreart:

De bedøvede mus vil blive aflivet ved total blodtapning (exsanguination).

Beskriv pasningen af og tilsyn med dyrene, herunder særlige foranstaltninger ved tilsyn:

Musene passes af uddannede dyreassistenter og under dyrestaldens forskrifter med minimum et dagligt tilsyn.

Dyreart og antal pr. år: (Såfremt der påtænkes anvendt genetisk modificerede dyr, udfyldes det særskilte skema for hver anvendelse.)

50 c57black/6 muse. 8 Mus i hver gruppe giver signifikant data til at kunne udfører de statistiske analyser. (Geoff Cumming, Fiona Fidler, and David L. Vaux, Error bars in experimental biology)

Ønsket varighed af tilladelsen til det beskrevne forsøg angivet i antal år:

1 år

Projektets varighed

Appendix III

AMU-Uddannelsesbevis

Morten Klitgaard Nøhr 310887-2127

har med tilfredsstillende resultat
gennemført den kompetencegivende
arbejdsmarkedsuddannelse

FELASA kategori B kursus, i dyreforsøgskundskab

5,0 dage

i perioden

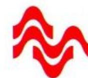
den 19.11.2012 til den 23.11.2012

Uddannelsen er godkendt af Undervisningsministeriet og udviklet af

Mejeri- og Jordbrugets Efteruddannelsesudvalg

HANSENBERG

AMU-uddannelser er erhvervsrettet voksen- og efteruddannelse, som vedligeholder, udbygger og forbedrer kursisternes faglige og almene kvalifikationer, så de passer til arbejdsmarkedets behov både på kort og lidt længere sigt.



Formål/mål:

Indehaveren kan på baggrund af viden om etiske aspekter vedrørende dyreforsøg udføre dyreforsøg i overensstemmelse med gældende lovgivning på området.

Indehaveren kan anvende grundlæggende viden om forsøgsdyr og om dyreværnsmæssige krav ved udførelsen af dyreforsøg

Indehaveren kan på baggrund af en teoretisk og praktisk indførsel i almindeligt anvendte teknikker på forsøgsdyr udfører dyreforsøg.