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Behavior analysis of clones and its offspring as detecting dog

복제견과 자손의 탐지견 자질에 관한 행동 분석 연구

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Behavior analysis of clones and its offspring as detecting dog

by Ji Hyun Lee

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ABSTRACT

Behavior analysis of clones and its offspring as detector dog

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Dogs are used as a companion animal, hunter, shepherd, rescuer, courier, postman, border patrol, criminal investigator, customs officer, natural resource etc. Korea customs service has been using detector dog in airport and seaport to screen illegal narcotics and explosives since 1988. In order to produce a better detector dog, it is most important to procure a better candidate dog for training. Korea customs service has been conducting breeding program between dogs that have excellent detection ability, but

there have been obstacles including availability range, population, age of possible candidates, and hereditary. Above all, possession of male and female's excellent traits did not guarantee inheritance of their outstanding qualities to the offspring.to solve these problems, seven cloned dogs (named "Toppies") were cloned by somatic cell nuclear transfer (SCNT) using fibroblasts derived from a male Labrador retriever possessing superior drug detection ability. Although genetic identity of the clones with the donor dog was confirmed, similarities in behavior and drug-detecting ability of the clones were not examined. Therefore, I hypothesized that cloning of excellent dogs can produce dogs that have proper behavior temperaments for the detection training. Furthermore, this study investigated the behavior related to the training of offspring of cloned male.

In first experiment, behavior traits of seven cloned dog puppies and four control puppies at 9-12 week old age were analyzed by Campbell test which has commonly been used for puppy selection and evaluated tendency of dominance. Campbell test consists of social attraction, following, social domination, elevation and dominance, then, according to the results, the dogs are categorized into one of six types; Type 1 is excessive dominant, type 2 is dominant, type 3 is balanced submissive, type 4 is submissive, type 5 is excessive submissive and type 6 is independent Among these types, type 1, type 2 and type 3 are regarded as suitable for working dog. In second

experiment, behavior traits of cloned dog's offspring at seven weeks old age was assessed by Volhard puppy aptitude test (modified Campbell test), Toman litter test, and mirror test. 10 offspring were produced by breeding between one cloned male (Toppy-Tuesday) and a female in Korea Customs Service detector dog training center (KCS DDTC). Volhard puppy aptitude test consisted of nine subtests; social attraction, following, restraint, social dominance, elevation dominance, retrieving, touch sensitivity, sound sensitivity and sight sensitivity. According to the results, puppies are categorized to six groups, the same as in the experiment 1.Toman litter test consists of moving a puppy into a strange building, arrival of test leader (TL), bringing food, throwing an object into puppies, a strange noise, TL stays with the dogs continuously and bring food and is used to determine the level of socialization and domination in a litter. In Toman litter test, puppies were divided into four types (L type, F type, R type, and A type) and L type is suitable for working dog. Mirror test was conducted to identify the socialization level of puppies, and results were used to evaluate normality of reaction against a mirror. In both experiments, all puppies were trained according to the Korea Customs Detector Dog Training Center's training manual after the tests, then final selection test was performed. For statistical analysis, individual scores of Campbell test were compared by general lineal mixed model using SAS 9.3. Correlation between final training score and

mature weight of cloned dog's offspring was analyzed by Fisher's exact tests using GraphPad Prism 4.02. Significance level was 0.05.

In the first experiment, Campbell test results showed that although To-Wedn is significantly different from To-Mon (P=0.0031) and To-Thur (P=0.0098), scores of all subtests between cloned and control groups were significantly different (P<0.0001). The success ratio for detector dog training in the cloned puppies was higher (86%) than in control dogs (30%). In the second experiment, cloned male had normal reproductive abilities; his offspring were healthy and had no abnormalities in morphology and development. Volhard puppy aptitude test results showed that only six dogs received type 2 or 3 among 10 offspring (60%). In Toman litter test, only three dogs exhibited type 2 which was same as the cloned male. In mirror test, 7 puppies received normal grade and 3 puppies reacted abnormally. Success rate for training of the 10 offspring was 60%, which was significantly higher than mean success rate (42.5%) of Korea Customs Service detector dog training center.

In conclusion, cloned puppies derived from drug detection dog that possessed superior ability had behavioral consistency and higher average of success rates compared to natural-bred puppies. The cloned dog showed normal reproductive performance, but its offspring showed inconsistent behavior. Therefore, drug detection dogs with high performance can be

produced more efficiently using SCNT than conventional breeding.

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Keywords: Puppy aptitude test, Cloned dog, Drug detection dog, Selection rate, Reproductive normality, Behavioral traits

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CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

DDTC	Detector Dog Training Center
KCS	Korea Customs Service
SCNT	Somatic Cell Nuclear Transfer
TL	Test Leader

PUBLICATION LIST

PBLICATION PAPERS

1. **Jihyun Lee**, Jin Choi, Hyun Ju Oh, Min Jung Kim, Geon A Kim, EunJung Park, Young Kwang Jo, Sang Im Lee, Do Kyo Hong, Byeong Chun Lee. Behavioral analysis of cloned puppies derived from an elite drug detection dog. Behavior Genetic (2014) 44:68-76.
2. **Jihyun Lee**, Geon A Kim, RakSeung Kim, Jong Su Lee, Hyun Ju Lee, Min Jung Kim, Byeong Chun Lee. Puppy aptitudes test for prediction suitability as drug detection dogs in Labrador Retrievers(*submitted*).
3. **Jihyun Lee**, Geon A Kim, Byung Chun Lee. Reproductive ability of cloned male and behavior traits of offspring(*submitted)*

PART 1

GENERAL INTRODUCTION

1. Literature review

1.1. Early Selection test for working dog

Dogs have been assisting in many areas of human life [1]. Dogsare used as a companion, hunter, shepherd, rescuer, courier, postman, border patrol, criminal investigator, customs officer, natural resources detector, children guardian, disabled people's assistance, healer(the dog can detect human emotions and successfully recognizes psychotic states) and odourlogist(in service of the police) [2]. Especially,among at least 30 different detection tasks that trained dogs could perform, detecting narcotics and explosives is their most common use [3, 4]. In particular, due to their superior scent detection, dogs are generally trained for drug detection in airports or similar specific tasks.

Temperaments of dogs are very important factor for successful trainingas a working dog, because it influences an individual's behavior and response to the environments [3, 5].According to the theory of behavioral development stage, socialization starts within 3-12 weeks, and most of puppy test has been conducted before 12 weeks. During this sensitive period, a puppy

easily forms attachment and can establish aspects of future behavior of that dog [6]. It also has been reported that the period between six and eight weeks of development may facilitate certain testing since puppies are motivated to approach unknown people in contrast to usual wariness [7]. These findings are the basis for the development of early selection tests, and failure rate of dogs entered in the training process but unable to complete it, decreased to approximately 50% with the use of selection test [8].

Domination of a dog considered as the most important temperament and can be used as early criteria to predict future success. Campbell assume that dominance is a stable trait of dog behavior, which is fixed from young age [9]. He designed a test to evaluate the domination level between puppies and human, that is social domination [10].Principals of Campbell test [11] have been become *v*ery popular for predicting personalities of working dogs as well as pets.

Based on the Campbell test, Pfaffenberger developed specific puppy aptitude test for guide dog [12] and it consisted of reactions to various startling stimuli, new situations, tactile stimuli and willingness to fetch [13] and it made raise a success ration as a guide dog.

Another common puppy selection test is Volhard puppy aptitude test developed by Jack and Wendy Volhard in 1996 [14], and it is partially similar compared to that of Campbell test. While Campbell's test is to evaluate basic traits related to social domination between dogs and human being, Volhard test added obedience factors to the Campbell test. Volhard puppy aptitude test included retrieving and 3 kind of sensitivity test (touch, sound, and sight).

Government agencies or organization related working dogs such as guide dog, police dog, detector dog, sled dogs have developed their own specific puppy aptitude test based on the principals. Most of these puppy tests were added one or two special situations according to their special task or the breed used for their work to the Campbell test. For example, for guide dog test, response to the training and being walked on leash and aggression or sensitivity test for German shepherd in Police dogs etc.

Goddard studied guide dog group and conducted a series of behavior test between four weeks and six months old dogs to predict the performance as a guide dog in the future. This early selection test evaluated, the response to a human handler, the response to unusal objects, the response to simple training, the behavior while being walked on a leash. The result showed that

fearfulness(the response to a strange person, a strange dog, a strange place and certain unusual objects) is strongly associated with the performance [15].

The study of South Africa police dogs, puppy test designed for selection was used it consisted of obstacle test (8 weeks), retrieval test (8 and 12 weeks), startle test for temperament (12 weeks and 16 weeks), gunshot test (12 weeks) and aggression test (six months and nine months). They showed that the some subtest(retrieving at eight weeks and 12 weeks old age and the startle test at 16weeksassociated with the performance in future [16].

965 German shepherd puppies bred in Czech Republic Police Breeding Facility, Prackovice, Czech Republic were evaluated at seven weeks. In this study, test was consisted of independent movement and interactions with the testers, negotiating obstacles, response to distracting stimuli by a shovel, entering a room, behavior toward a person, behavior in new environment, response to a distracting noise while left alone in a room, response to loud distracting stimuli, retrieval, tug of war. According to the result, the puppies showed heavy willing to chase, catch and fetch a tennis ball, and puppies followed a rag drawn away from them while puppies those responded weakly against distracting noise in various situations and showed low activity while negotiating obstacles and moving and interacting with the

tester were successful performance in adulthood [13].

In the study of Asher, Puppy Profiling Assessment was designed for evaluating behavior of potential guide dog puppies prior to placement with puppy walking, volunteers, at 6-8 weeks of age, by presenting puppies with a series of controlled stimuli and scoring their reaction on a scale [9].

A study of Champness in Melbourn university, puppy test devised aptitude test for six week old conducted but there was no correlation between performance at this age and maturity. However, older and older, the accuracy and validity of test was increased [17].

For investigating extent of socialization, mirror test and Queinnec test was applied in three breeds(German dog, doberman and riesenschnuatzer) [18]. The result shows that the puppies of German dog breed possessed the highest socializing capabilities and which means that the representatives of German dog breed would require the least amount of training time in order to form specific behavior [18].

Mentioned tests above were carried out against individual puppy, but the domination and socialization among puppies are also important. In study

ofUzunova, puppies were categorized intofour group and the tendencies of socialization were evaluated [19]. Temperament type is an important the extent of socialization and the formation of specific behavior [20, 21].

1.2. Heritable behavioral traits in working dogs

Generally, behavior is influenced by environment and genetic factors; Genetic factors are transmitted by inheritance, but the traits can be modified by interacting genetic and environment factors [22].The importance of genetics in dog behavior and personality was recognized by Pavlov [23]and genetics can influence the innate patterns of reactivity [24], tolerance of frustration [25], physiologic effects from emotion-provoking stimuli [26-28] response to punishment [29] and reaction to isolation [30, 31].

Likewise, in dogs, it has been suggested that the traits related detector dog training such as boldness, concentration etc. are thought to be heritable, that is, associated with genetic factors. For example, heritability of traits essential for training was assessed using 1310 German shepherds and 797 Labrador retrievers in Swedish police dog training center, and the results showed that affability in German shepherd and ability to co-operate in Labrador retriever was highly heritable [32]. Goddard and Beilharz analyzed

the trainer's score of 249 Royal Guide dog association-bred Labrador retrievers between 1970 and 1976 reported heritability of fearfulness [33]. Unites States Army's Division of Bio-Sensor Research reported heritability of temperament (ability to chase and attack a decoy and the tendency to use its olfactory abilities) for military working dog by analyzing575 German shepherd [34]. Study of Melbourn university showed low to moderate (h^2=0.00-0.15) heritability of important traits, chase and retrieving, metal possession, physical possession, activities for detector dog, except for independent possession (h^2=0.23) [17].

Therefore, it can be assumed that environmental factors are controlled, majority of specific traits may be influenced by genetic factors.

In genetically identical mice and control mice group bred naturally, a series of test, Morris water task and Krushnsky test etc., were conducted in order to investigate activity level, learning memory and motor abilities [35]. The result showed that activity level, learning memory and motor abilities were normal.

To investigate whether the somatic cloning procedure has an influence on locomotion, exploratory, vocal and social behavior, five cloned heifer and

five naturally bred control heifers were used [36]. In this study, it was failed to find the influence of SCNT in behavior of cloned heifers. However, in cattle, four cloned heifers exhibited a higher level of curiosity, more grooming activities and were more aggressive and dominant than control [36, 37]. In this study, these clones derived from the same donor preferred each other as companions to unrelated conspecifics, which may suggest a process of kin recognition.

And, there's no report regarding behavioral similarities in dogs having same genetic information.

1.3. Somatic cell nuclear transfer to conserve valuable genetic resources

SCNT is a reproductive process using somatic cell instead of sperm and egg in SCNT procedure, somatic cells derived from donor are injected into enucleated oocyte, and the cell-oocyte couplet is fused electrically. After activation of fused embryos, they were transferred to a surrogate mother. Successful reprogramming of cloned embryos leads to healthy offspring, which is genetically identical with the cell donor. Cloned dogs having same genetic information showed similar growth characteristics including body weight, height, development and hematologic profiles within normal

reference range [38].In livestock, cloned cattle had a normal muscle contractile and metabolic characteristics comparing with the normal bred control cattle [39] and normal telomere length found in cloned cattle [40].

Several studies investigated reproductive normality in cloned animals. Nigerian Dwarf Goat (*Capra hicus*) clones developed sexually within the normal timeframe for their breed and were fertile [41].Reproductive characteristics of cloned heifers were not different in estrous cycle, length, ovulatory follicle diameter, number of follicular waves, or profiles of hormonal changes with non-cloned heifers [42]. Semen characteristics including motility of fresh and frozen-thawed and Percoll-treated spermatozoa, as well as *in vitro* fertilization ability and embryo quality of cloned bulls was not different with their cell donor [43]. Cloned male cats also had normal reproductive fertility including normal range of gonadal hormone production, and their kitten produced by natural breeding had a normal growth pattern [44]. In line with these results, cloned dogs displayed not only normal patterns of hormone levels and morphologic changes of the vaginal epithelium, but also fertile reproduction resulting healthy puppies [45].

Based on the normality and reproductive ability of cloned animals derived

from SCNT, this technique has been applying conserve valuable genetic resources. Because most of genetic information, except mitochondrial DNA, is transmitted from a cell donor to a clone, characteristics related to nuclear genetic information can be passed onto the clones. Therefore, cloning can be the powerful tool to produce dogs with an outstanding talent identical to that of their donors than other artificial reproductive technologies, if the talent is based on genetic factors. Assuming temperaments related to detector dog training was genetic and seven cloned narcotic detector dog were produced, however, behavioral similarities in cloned animals has not been investigated yet.

2. General objective

The aim of this study was 1) to examine similarities in behavior of cloned detector dogs derived from an elite drug–detector dog and 2) to analyze behavior related to detection training of offspring of cloned male dog.

In Part 1, as a general introduction, it was written the literature review related to early selection tests and heritable behavioral traits in working dog, and somatic cell nuclear transfer to conserve valuable genetic resources. In

part 2, I described general methodology of this thesis. InPart 3 and 4, the study of behavior analysis regarding cloned puppies derived from an elite drug-detection dog and behavior analysis of offspring of cloned male was written respectively. In these parts, I explained detailed methodology, result and discussion. In Part 5, I described final conclusion,

PART 2.

General Methodology

1. Study sample

Cloned and age matched Labrador retrievers at 9-13 weeks old were used to compare behavior of cloned dogs. Seven cloned dogs, named To-Sun, To-Mon, To-Tue, To-Wedn, To-Thur, To-Fri, To-Sat, and To-Sun were produced by canine SCNT [46]. Donor cells were established from ear skin of an elite drug detecting dog (seven years old) and a total of 544 In vivo matured dog occytes were collected from 51 bitches in natural estrus cycle. Donor cells were injected into the perivitelline space of enucleated *in vivo* matured dog oocytes, then fused with electric stimulation using an Electro-Cell Fusion apparatus (NEPA GENE Co., Chiba, Japan) and activated chemically. A total of 400 fused couplets were transferred into 18 naturally synchronous recipient bitches, three of which delivered a total of seven cloned puppies. Four of 18 bitches were pregnant, however one delivered one live pup and one dead pup and the live one died within five days. One of rest three surrogate bitches delivered naturally, one was delivered naturally and gave birth by cesarean section. Others delivered by cesarean section only. Microsatellite analysis that the cloned puppies and the donor dog are genetically identical [46]. Four control puppies were naturally bred by another elite drug detection dog (male) and a breeding dog (female). The

somatic cell donor dog and the parents of the controls have no genetic relationship. The parents of control puppies and the donor dog were not tested because we systematized this evaluation course after they were grown up. After the tests they experienced more evaluations and training courses at similar age according to the manual. Their ages of starting various activities were similar also. All of the puppies were cared for by the same persons and their living quarters were identical. Puppy care system was followed by guidelines of Detector dog training center (DDTC).

Among seven puppies, To-Tue was bred with a female dog having a good personality for studying behavioral traits of offspring of a cloned dog. For predicting fertile period and delivery time, serum progesterone was measured from observation of estrus bleeding. Natural breeding for this study was performed at the 3rd observed estrus. Progesterone concentrations were measured with a DSL-3900 ACTIVE® Progesterone Coated-Tube Radioimmunoassay Kit (Diagnostic Systems Laboratories, Inc., Webster, TX). The place of mate was DDTC. After mating, the bitch was lived in her kennel with other dogs until 30 days and then, she was moved the delivery room located in DDTC. She was fed normal dry food until 45-50 days, after that time, she was fed puppy dry food or liquid type commercial food till the delivery. The diagnosis of pregnancy was detected by palpation of detector

dog veterinarian at 45-50 days of pregnancy. Specialized veterinarian and technician monitored the pregnant bitch from mating to delivery and also monitored progesterone concentration and body temperature.

2. Training and evaluation

2.1. Campbell test and selection test

2.1.1. Test area and age of puppies for Campbell test

Puppies were evaluated individually at the DDTC using the same test area (10m X 4.4m) for each animal. The test was conducted by only one test leader (TL)in the absence any other object, animal or person that could attract the puppies' attention. The duration of the test for each puppy was the same: each subtest lasted 30 sec so the whole test period was about 3 min. The TL was a stranger to the puppies and 4 people (TL and 3 handlers) evaluated the all puppies at the same time. Only the TL directly watched the puppies during each test; the other three evaluators observed them from outside the test area through a window so as nothing was seen by the puppies, and they also checked the results after the test with recorded video

and discussed the exact responses of the tested individuals. The evaluators classified the results according to the responses defined by Campbell [11].This test was supposed to be conducted at age 8 weeks, however a total 11 puppies were 9-13 weeks old because of movement procedure of puppies.

2.1.2.Cmpbell test and response type

Each puppy stayed alone for about 3 min in the empty test areabefore the test started, to allow time for it to be comfortable. The test consists of five subtests and the order of the subtests conducted as follows: (1) Social Attraction, (2) Following, (3) Restraint, (4) Social Dominance, (5) Elevation Dominance. Bartlett (1979) described the test in more detail so we applied her description of the response interpretation [47].

In social attraction test, TL placed puppy in test building and observed them from a few feet away. TL coaxed the puppy to him by clapping hands gently and kneeling down. TL had to coax in a direction away from the point where the puppy entered the testing building. In following test, TL walked away from the puppy in a normal manner, and made sure the puppy saw him walk away. In restraint**,** TL crouched down and gently rolled the puppy on his back and heldit one hand for a full 30 sec. And then, in Social dominance, at

first, let puppy stood up and TL gently stroked puppy from the head to back while TL crouched beside him. TL continued stroking until a recognizable behavior is established, in elevation domination, TL bended over and cradled the puppy under its belly, fingers interlaced, and palms up and elevated the puppy just off the ground. TL held it there for 30 sec.

According to Bartlett's interpretation [47], which was described Campbell test in more detail, the puppy's responses indicate the degree of dominance: If the puppy receives mostly scores of 1 (type 1 dog), this dog is extremely dominant and has to be trained by an experienced handler because of extreme dominance and aggressive tendencies. This dog can be a good detection dog with him or her. In case of mostly scores of 2, the puppy is dominant and can bite. Firm, consistent, and fair handling is needed. It may be too active for elderly people and too dominant for small children. Type 2 is generally regarded as the most appropriate aptitude for working dogs at the DDTC because this kind of dog has an active and outgoing temperament. If the dog receives mostly scores of 3, it is best for the average owner and also good with the elderly and children. Type 3 is good for a working dog as well. This dog adapts well to changes. A dog with mostly scores of 4 is submissive and slightly less outgoing than a dog that scores mostly 3. This dog gets on famously with children and trains well. However, types 4 and 5

dogs are not supposed to be good prospects for working dogs since they are submissive and less active. Scores of mostly 5 mean that the puppy is extremely submissive and needs special encouragement in handling. It experiences difficulties with changes and frightens easily, so it is not good for a beginner. The puppy that receives mostly scores of 6 is independent and not affectionate. It is difficult to train as a working dog or make it into a pet.

2.1.3.Selection test and evaluation

After classification by the Campbell test, the puppies were trained for about a year according to the detector dog training and test manual. Selection tests were conducted when the training course ended. The test areas were the building used for training and Incheon airport. There were five evaluation items: boldness, concentration, detecting process, response of detection and possessiveness. Each item was scored as 4 (poor), 8 (fair), 12 (average), 16 (good), and excellent (20) and the full score was 100. When the puppies were tested, more than 3 kinds of narcotics were used; hemp, ecstasy, methamphetamine and cocaine. These were hidden in glass or vinyl bottles and concealed in cloths, boxes, hard cases and a person’s forearm. The aim of this final test is the selection of drug detection dogs that will work

successfully in the field. This study was on results of the Campbell test and the final selection of candidate dogs for drug detection work.

2.2. Volhard puppy aptitude test, Toman litter test and Mirror test

2.2.1.Training and evaluation course in DDTC

Offspring of the cloned dog at 7-8 weeks was assessed by Volhard puppy aptitude test, Toman litter test and Mirror test, instead of Campbell test, in order to evaluate their traits for detector dog training. These tests were only for study and we did not use for selection for further training. Except those tests, every training and evaluation course was the same with cloned dogs according to training manual of DDTC.

2.2.2. Puppy aptitude test and response types

To predict the possibility as a detector dog, Volhard puppy aptitude test, which is modified Campbell test, and evaluation were used [48]. TL who conducted the test was an experienced instructor of DDTC, a perfect stranger against puppies. The place of test was one of the training building in DDTC and it was the first time of visiting for puppies. The duration of

time in each subset was 30 sec. Volhard puppy aptitude test consisted of 9 subtests; (1) Social attraction. (2) Following, (3) Restraint. (4) Social Dominance, (5) Elevation dominance, (6) Retrieving, (7) Touch Sensitivity,(8) Sound sensitivity and (9) Sight Sensitivity. Method for Social attraction, Following, Restraint, Social dominance, Elevation dominance are same with Campbell test.

To evaluate the retrieving**,** TL crouched beside puppy and attracted his attention with crumpled up ball. When the puppy showed interest and was watching, tossed the ball 4-6 feet in front of puppy. TL attracted puppies' attention to retrieve the ball or training aids. In touch sensitivity test, TL took the webbing of one front foot and pressed it between finger and thumb lightly then more firmly until you got a response, while you counted slowly to 10, in sound sensitivity test, TL placed puppy in the center of test building, assistant made a noise outside of the test building. A large metal spoon struck sharply on a metal pan twice works. For the last test of sight sensitivity, TL placed puppy in the center of room, and suddenly unfolded an umbrella in front of puppy. TL observes the reaction of puppies.

According the interpreting method used in Volhard puppy aptitude test, puppies are categorized to 6 groups.Type 1 puppies that consistently receive

score 1 in temperament section of the test are extremely dominant, aggressive puppy who can easily to be provoked to bite. A puppy receives mostly 2 is dominant and self-assured. He can be provoked to bite; however he readily accepts human leadership that is firm, consistent and knowledgeable. A puppy in Type 3 obtains mostly 3 and is outgoing and friendly. He will adjust well in situations in which he receives regular training and exercise. He has a flexible temperament that adapts well to different types of environment, provided he is handled correctly. In Type 4, a puppy receives mostly 4, is an easily controlled, adaptable puppy whose submissive nature will make him continually look to his master for leadership. A puppy receives mostly 5 is Type 5 and this type of pup who is extremely submissive and lacking in self-confidence. He bonds very closely with his owner and requires regular companionship and encouragement to bring him out himself. A puppy that scores 6 consistently is Type 6 and he is independent and uninterested in people. He will mature into a dog who is not demonstrably affectionate and who has a low need for human companionship. In DDTC, puppies that belong to Type 1, 2, 3 were suitable for detector dog training.

2.2.3.Toman litter test and response type

In DDTC, one of method to find out the level of socialization and domination of puppies among the litter, Toman litter test which has been applied since 1984 [49, 50] was used. All the puppies that belong to the same litter were tested as a group at one of the training building, DDTC, which is a strange place for every puppy. TL was not familiar with puppies and was different person from the one conducting Volhard puppy aptitude test. According to the method of Toman litter test mentioned as below, puppies were divided into 4 categories. Category L is for Leader, which represents the dominant members of the litter, Category F is for faithful, which are subordinate dogs, namely docile and obedient, Category R is for Remote, dogs that are somewhat indifferent, and Category A is for antisocial. The category in which groups together the bitter and rebellious dogs. The group test is divided into 7 subtests; (1) Moving a puppy into an strange test building, (2) Arrival of TL, (3) Bringing food, (4) Throwing an unknown object, (5) A strange noise, (6) TL stays with the dogs continuously, and (7) Bring food.

At first, assistant moved puppies in to a strange building and TL observed puppies behind the screen. The bitch of this group was far enough away from her litter for the puppies to be unable to hear or see her. The technician of who took care will also distance himself. Type L did not bark and

explored an new area, Type F barked and wanted to join a game, Type R just sat down and looked around and seemed be confused, then followed other puppies, and Type A was crying in a circle or crawled around the ground. Then, TL approached test group and observes the behavior of puppies: Type L just sat and stared at the TL or looked for another interesting thing or stuff. Type F came cross the TL slowly. Type R and Type A ran toward the TL quickly. TL brought one small container and inducing competition among puppies. Although they were not hungry, Type L and Type F started eating, and they attacked Type R and Type A. Next, TL threw a unknown object (etc. plastic bottle) into the middle of the litter without touching puppies. Type L jumped and grabbed it. Type F pulled the object carefully and played with it together with Type L. Type R was frightened at the object or sometimes runs away. Type A was in panic and ran away and did not access it. After finishing throwing , assistant made a sharp noise behind the wall.Type L lifted its head and stared in the direction of noise, pricking up its ears. Type F, Type R and Type A ran away or in confusion. And then, TL stayed with the dogs continuously. TL was immediately recognized by the puppies and stays for enough time to evaluate this group. Type L was not interested in the TL. Type F stayed around TL. Type R and Type A moved toward the TLIn this final situation, TL must prepare enough food bowl and each puppy had to access to the food. Type L ate the food calmly (did not attack) and

was not disturbed by any of them. Type F ate it while moving around but remained comparatively calm. Type R and Type F age but struggled with others.

2.2.4.Mirror tests and evaluation

After Toman Litter Test was done, TL placed puppies in a room with a large mirror in which it can see its own image in full. They stayed enough time to identify their characteristics in the room.The puppies' reactions will be observed in order to evaluate its level of social breeding. Puppies that are sociable, well balanced, confident and dominant will display some tension as a hierarchical ladder is put into place. In normal reaction, the puppy will look at itself in the mirror and it will spend periods of time motionless. It will go up to the mirror and move back considerably. Abnormal reaction includes crying, crawling, staying in front of mirror and other reaction.

3. Statistical Analysis

Individual scores were compared between and within groups: cloned or

control puppies. To analyze differences among the puppies, the general linear mixed model was used with SAS 9.3 (SAS Institute Inc., Cary, NC, USA). Differences between cloned and control puppies were analyzed. A score of between 1 and 6 was used as a response to each Campbell subtest: (1) excessive dominance; five kinds of subtest scores were used as dependent variables. Independent variables were individuals and groups. Evaluators were calculated as a random factor to reduce their effect on the data since they can be different from each other. By pairwise comparison each individual dog was analyzed to find if anyonewas significantly different from another. The same procedure was carried out for all 5 Campbell subtests. The sum of scores for each type was divided by the total number of individuals to determine the frequency distribution of scores of each type. To determine the average number of scores, a method for obtaining average values [51] was used with modification. Pairwise comparison was used to analyze the differences in Least Square Post Hoc test. P values were corrected by Tukey-Kramer adjustment.

Correlation between final training score and mature weight of cloned dog's offspring was analyzed by Fisher's exact tests using GraphPad Prism 4.02. Significance level was 0.05.

PART 3.

Behavioral analysis of cloned puppies derived from an elite drug-detection dog

1. Introduction

Dogs are generally superior to other animals and as technological devices in scent detection. The dogs that are especially proficient in this regard are often used to detect targets by scent and, are called detector or search dogs. Detector dogs are used for detecting dangerous materials such as explosives or drugs (narcotics) [52, 53]. For example, drug detecting dogs are employed in airports and prisons and are trained to scan large numbers of people for the presence of narcotics [54]. The use of such dogs has increased in recent years, especially because of modern phenomena such as drug trafficking and terrorist threats. Although selection procedures for producing drug detection dogs are established within individual organizations, only a small minority of animals can be successfully trained for their specific roles. Using this approach, there is a very low probability of finding the best or "elite" detector dogs. Although there have been several attempts to establish breeding programs for specialist detection dogs, thereis an insufficient supply of elite dogs [54]. Alternative ways of providing such animals are needed, and in the present study, SCNT technique was examined as a method to produce elite drug-detecting dogs.

SCNT is a unique reproductive engineering technology that can yield a newborn that is virtually identical to the somatic cell donor. Since the birth of a cloned sheep, SCNT has become available as a method used for cloning of several species [55-59]. In previous study, even cloned puppies derived from a somatic cell of an elite drug-detecting dog were produced *via* SCNT[46]. The seven cloned puppies have the same genotype as the donor dog, but that study did not examine their behavior patterns or drug-detecting potential. Therefore, the present study performed the Campbell test for behavioral analysis on the dogs and compared the outcomes with their selection results as drug detection dogs.

The Campbell test performed by the DDTC was established primarily to evaluate underlying aptitudes of puppies for drug detection (Detector Dog Test Manual, 2009, Customs Detector Dog Training Center, Customs Border Control Training Center, Korea Customs Service). By better predicting which dogs can be successfully trained, burdens on trainers and costs can be reduced. Following the Campbell test, the puppies are trained for about 14 months according to the Korea Customs Detector Dog Training Center's training manual. The training course consists of subjecting the dogs to various environments, motivation by reward, improving concentration, distinguishing between drugs and other chemicals, etc. In this study, during

standard training procedures, judgments as to the relative ability of cloned dogs, and whether they should continue training or be rejected, were made by experienced trainers.

Therefore, the aim of this study was to determine whether cloning of a genotype by SCNT can affect the behavioral patterns of cloned dogs, with a special emphasis on cloned drug-detecting dogs.

2. **Results**

2.1. **Comparison between Cloned and Control puppies**

Eleven puppies from cloned and control groups were compared, and four observers were statistically processed as a random effect. This procedure is to reduce the bias according to observers because four different observers evaluated the puppies. Five subtest scores were used as dependent variables. Independent variables were individuals and groups. Fig. 1 presents differences of the average test scores ($F_{1,\ 30}$=66.11, P<0.0001), Table 1 shows the factors and least squares means. The scores were significantly different between the groups (cloned or control). The average score of

control puppies was 3.0937 (Standard error=0.1400) while that of cloned puppies was 1.6929(Standard error=0.1073). The scores of all 5 subtests were significantly different between the two groups (Fig. 2-(A), (B), (C), (D), (E)).

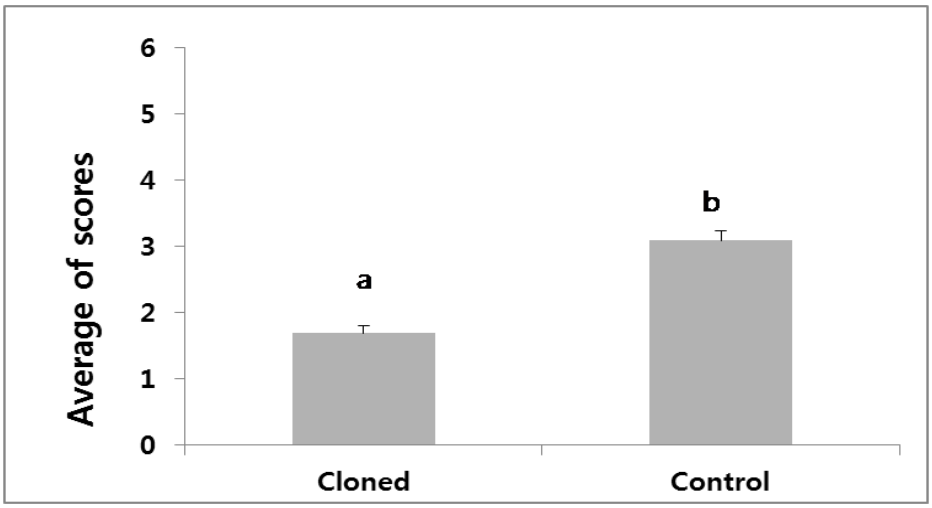

Figure 1. Average scores of Campbell tests in cloned and control puppies. Eleven puppies from cloned and control groups were compared, and four observers were statistically processed as a random effect. Five subtest scores were used as dependent variables. Independent variables were individuals and groups. The average score of the cloned puppies is 1.6929 (Standard error=0.1073) and that of controls is 3.0937 (Standard error=0.1400). Average scores between cloned and control puppies were significantly different ($F_{1, 30}$=66.11, P<0.0001).

Table 1. Factors and least squares meansof average scores of Campbell tests in cloned and control puppies.

Type 3 Tests of Fixed Effects				
Effect	**Num DF**	**Den DF**	**F Value**	**P > F**
Group	1	30	66.11	<.0001

Least Squares Means						
Effect	**Group**	**Estimate**	**Standard Error**	**DF**	**t Value**	**P> |t|**
Group	Cloned	1.6929	0.1073	30	15.78	<.0001
Group	Control	3.0937	0.1400	30	22.10	<.0001

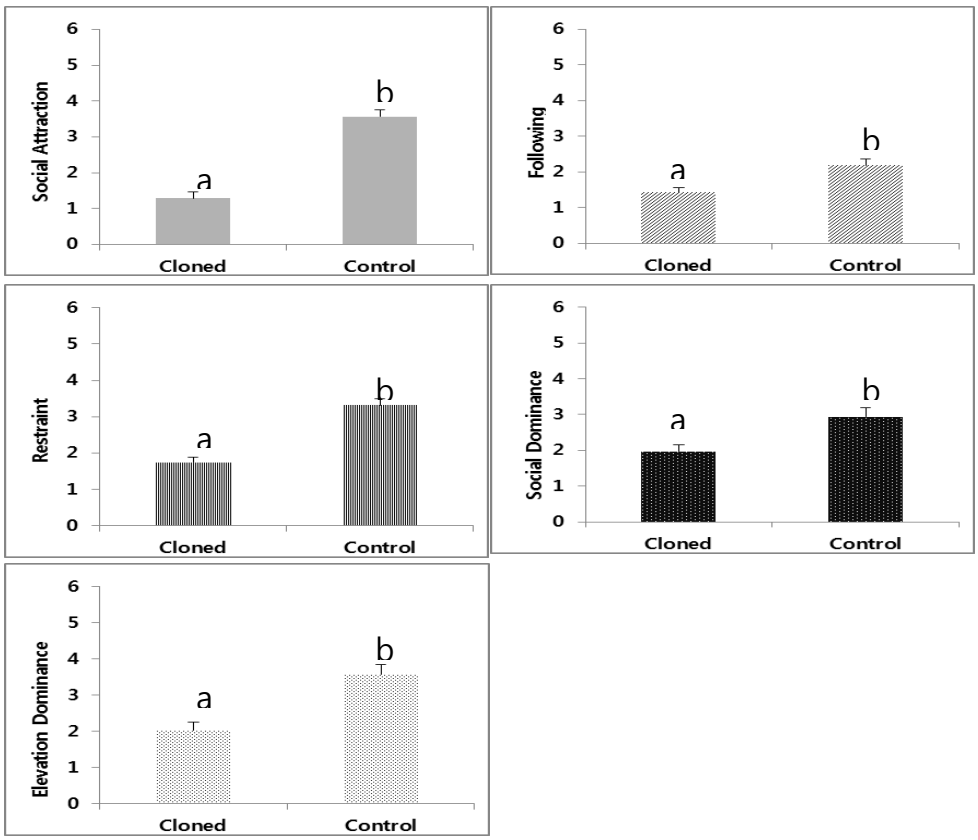

Figure2. Scores of five subtest in Campbell test**(A)**Scores of the Social Attraction subtest in cloned and control puppies. Five subtest scores were used as dependent variables. Independent variables were individuals and groups. Four observers were statistically processed as a random effect. The scores were significantly different ($F_{1, 30}$=121.74, P<0.0001). **(B)**Scores of the Following subtest in cloned and control puppies. The scores were significantly different ($F_{1, 30}$=14.89, P=0.0006).**(C)**Scores of the Restraint subtest in cloned and control puppies. The scores were significantly different ($F_{1, 29}$=53.7, P<0.0001). **(D)**Scores of the Social Dominance subtest in cloned and control puppies. The scores were significantly different ($F_{1, 29}$=10.23, P=0.0033). **(E)**Scores of the Elevation Dominance subtest in cloned and control puppies. The scores were significantly different ($F_{1, 29}$=22.3, P<0.0001).

The factors and least squares means are presented in Table 2-(A), (B), (C), (D), (E).For the subtest Social Attraction, the score for controls was 3.5626 and for cloned it was 1.2857 ($F_{1, 30}$=121.74, P<0.0001); for the subtest Following, the control score was 2.1875 *vs*. 1.4286 for cloned ($F_{1, 30}$=14.89, P=0.0006); for Restraint, the control score was 3.3229 *vs*. 1.75 for cloned ($F_{1, 29}$=53.7, P<0.0001); for Social Dominance, the controls scored 2.9369 *vs*. 1.9643 for cloned ($F_{1, 29}$=10.23, P=0.0033); and for Elevation Dominance, the scores were 3.5795 for control and 2.0357 for cloned ($F_{1, 29}$=22.3, P<0.0001).

These results show that cloned puppies achieved more dominant scores than control puppies in the five subtests. In addition, I confirmed that there were also differences in frequency distribution of scores between cloned and control puppies. Table 3 shows that the cloned group achieved (2) type most frequently, while the control group achieved (3) type most frequently in the Campbell test. Variation of assessed types was higher in the control than in the cloned group, since the former obtained 4 kinds of type 6 – (2), (3), (4), (5), while the latter scored 3 kinds of the type – (1), (2), (3), though the sample size of controls was smaller than that of cloned. In comparing individuals within the cloned group, only To-Wedn was significantly different from To-Mon and To-Thur.

Table 2. Factors and least squares means ofScores of the Elevation Dominance subtest in cloned and control puppies.

Type 3 Tests of Fixed Effects

Effect	Num DF	Den DF	F Value	P > F
Group	1	29	22.30	<.0001

Least Squares Means

Effect	Group	Estimate	Standard Error	DF	t Value	P> |t|
Group	Cloned	2.0357	0.2123	29	9.59	<.0001
Group	Control	3.5795	0.2742	29	13.06	<.0001

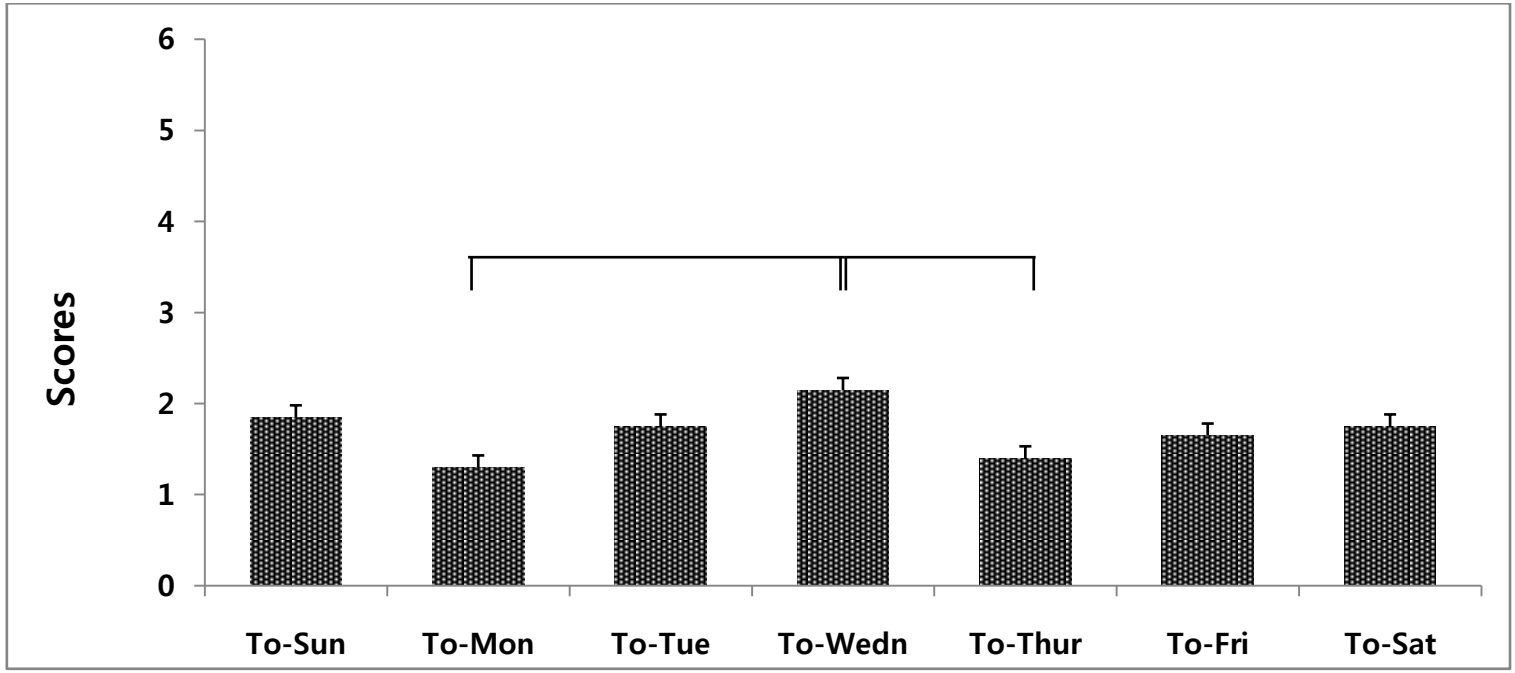

Figure3. Scores of five subtests in seven cloned puppies ($F_{6, 18}$=4.81, P=0.0043). Seven puppies of cloned group were compared by general linear mixed model, as four observers were considered as a random effect. Five kinds of subtest scores were used as dependent variables. Independent variables were cloned individuals. To-Wedn is significantly different from To-Mon (P=0.0031) and To-Thur (P=0.0098). P values were corrected by Tukey-Kramer adjustment.

2.2. Evaluation of Score Frequency of Each Type of the Campbell test

I compared the number of the subtest type score obtained between cloned and control puppies. Scores were graded by the same four evaluators for all of the puppies. Table 3 shows the average value of scores frequency of each type and the averages of cloned and control animals. Range of these values is from 0 to 1 because this table presents the rate of the whole number of times. The type of the highest average of cloned dogs was type (2) (0.5500) and that of control dogs was type (3) (0.4581);the highest average in total was type (2) (0.4334). In case of type (4) submission and type (5) excessive submission, control puppies scored 0.2875 (4) and 0.0250 (5), however cloned puppies did not obtain (4) or (5). Variation of assessed types was higher in the control than in the cloned group, since the former obtained 4 kinds oftype6 – (2), (3), (4), (5), while the latter scored 3 kinds of the type – (1), (2), (3), although the sample size of control is smaller than that of cloned.

2.3.Comparison within Cloned Group

Seven puppies of cloned group were compared by general linear mixed model, as four observers were considered as a random effect. Five kinds of

subtest scores were used as dependent variables. Independent variables were cloned individuals. There were differences within the cloned group. Differences between To-Wedn and To-Mon (P=0.0031), To-Wedn and To-Thur (P=0.0098) were significant. Figure 3 shows the differences ($F_{6, 18}$=4.81, P=0.0043). P values were corrected by Tukey-Kramer adjustment. The factors and least squares means are presented in Table 4.

Table 3. Frequency distribution of scores of each type of the Campbell test in cloned and control dogs. (1 : excessive dominance, 2 : dominance, 3 : balanced dominance, 4 : submission, 5: excessive submission, 6 : independent)

Type	**1**	**2**	**3**	**4**	**5**	**6**
Cloned	0.3786	0.5500	0.0714	0.0000	0.0000	0.0000
Control	0.0000	0.2294	0.4581	0.2875	0.0250	0.0000
Individual	**1**	**2**	**3**	**4**	**5**	**6**
To-Sun	0.1500	0.8500	0.0000	0.0000	0.0000	0.0000
To-Mon	0.7000	0.3000	0.0000	0.0000	0.0000	0.0000
To-Tue	0.3500	0.5500	0.1000	0.0000	0.0000	0.0000
To-Wedn	0.0500	0.7500	0.2000	0.0000	0.0000	0.0000
To-Thur	0.6000	0.4000	0.0000	0.0000	0.0000	0.0000
To-Fri	0.4000	0.5500	0.0500	0.0000	0.0000	0.0000
To-Sat	0.4000	0.4500	0.1500	0.0000	0.0000	0.0000
C-1	0.0000	0.1177	0.8824	0.0000	0.0000	0.0000

C-2	0.0000	0.2000	0.4000	0.4000	0.0000	0.0000
C-3	0.0000	0.2000	0.2000	0.6000	0.0000	0.0000
C-4	0.0000	0.4000	0.3500	0.1500	0.1000	0.0000
Average	**0.2409**	**0.4334**	**0.2120**	**0.1046**	**0.0091**	**0.0000**

Cloned is the average value of cloned puppies (To-Sun, To-Mon, To-Tue, To-Wedn, To-Thur, To-Fri, To-Sat), Control is the average value of control puppies (C-1, C-2, C-3, C-4). Control puppies achieved (3) type most frequently (average 0.4581), however, cloned puppies achieved (2) type most frequently (0.5500).

Table 4. Factors and least squares meansof scores of five subtests in seven cloned puppies.

Type 3 Tests of Fixed Effects				
Effect	**Num DF**	**Den DF**	**F Value**	**P > F**
ID	6	18	4.81	0.0043

Least Squares Means						
Effect	**ID**	**Estimate**	**Standard Error**	**DF**	**t Value**	**P> |t|**
ID	To-Fri	1.65	0.1323	18	12.47	<.0001
ID	To-Mon	1.30	0.1323	18	9.830	<.0001
ID	To-Sat	1.75	0.1323	18	13.23	<.0001
ID	To-Sun	1.85	0.1323	18	13.98	<.0001
ID	To-Thur	1.40	0.1323	18	10.58	<.0001
ID	To-Tue	1.75	0.1323	18	13.23	<.0001
ID	To-Wedn	2.15	0.1323	18	16.25	<.0001

Differences of Least Squares Means

Effect	ID	_ID	Estimate	Standard Error	DF	t Value	Pr>|t|	Adjustment	Adj P
ID	To-Fri	To-Mon	0.3500	0.1828	18	1.91	0.0716	Tukey-Kramer	0.4962
ID	To-Fri	To-Sat	-0.1000	0.1828	18	-0.55	0.5910	Tukey-Kramer	0.9976
ID	To-Fri	To-Sun	-0.2000	0.1828	18	-1.09	0.2883	Tukey-Kramer	0.9219
ID	To-Fri	To-Thur	0.2500	0.1828	18	1.37	0.1882	Tukey-Kramer	0.8112
ID	To-Fri	To-Tue	-0.1000	0.1828	18	-0.55	0.5910	Tukey-Kramer	0.9976
ID	To-Fri	To-Wedn	-0.5000	0.1828	18	-2.74	0.0136	Tukey-Kramer	0.1453
ID	To-Mon	To-Sat	-0.4500	0.1828	18	-2.46	0.0241	Tukey-Kramer	0.2302
ID	To-Mon	To-Sun	-0.5500	0.1828	18	-3.01	0.0075	Tukey-Kramer	0.0883
ID	To-Mon	To-Thur	-0.1000	0.1828	18	-0.55	0.5910	Tukey-Kramer	0.9976
ID	To-Mon	To-Tue	-0.4500	0.1828	18	-2.46	0.0241	Tukey-Kramer	0.2302
ID	To-Mon	To-Wedn	-0.8500	0.1828	18	-4.65	0.0002	Tukey-Kramer	0.0031
ID	To-Sat	To-Sun	-0.1000	0.1828	18	-0.55	0.5910	Tukey-Kramer	0.9976
ID	To-Sat	To-Thur	0.3500	0.1828	18	1.91	0.0716	Tukey-Kramer	0.4962

ID	To-Sat	To-Tue	0	0.1828	18	0.00	1.0000	Tukey-Kramer	1.0000
ID	To-Sat	To-Wedn	-0.4000	0.1828	18	-2.19	0.0421	Tukey-Kramer	0.3481
ID	To-Sun	To-Thur	0.4500	0.1828	18	2.46	0.0241	Tukey-Kramer	0.2302
ID	To-Sun	To-Tue	0.1000	0.1828	18	0.55	0.5910	Tukey-Kramer	0.9976
ID	To-Sun	To-Wedn	-0.3000	0.1828	18	-1.64	0.1181	Tukey-Kramer	0.6597
ID	To-Thur	To-Tue	-0.3500	0.1828	18	-1.91	0.0716	Tukey-Kramer	0.4962
ID	To-Thur	To-Wedn	-0.7500	0.1828	18	-4.10	0.0007	Tukey-Kramer	0.0098
ID	To-Tue	To-Wedn	-0.4000	0.1828	18	-2.19	0.0421	Tukey-Kramer	0.3481

2.4.Final selection test of Cloned and Control puppies

Six cloned dogs that finished the training course were evaluated by a final drug-detection dog selection test and all of them passed. The pass level was a score of 60. To-Tue was graded as Excellent (score 90) and the remaining five dogs were evaluated as Good. In age matched-controls, seven puppies finished the training course and one of them passed the test. One of the eight puppies died before the training course was over. The pass rate of cloned dogs was 86% since six puppies passed among seven cloned ones. That of controls was 13% in the aggregate since one passed among eight control ones. This value was lower than generally found as 30% [5] or 50% [8].

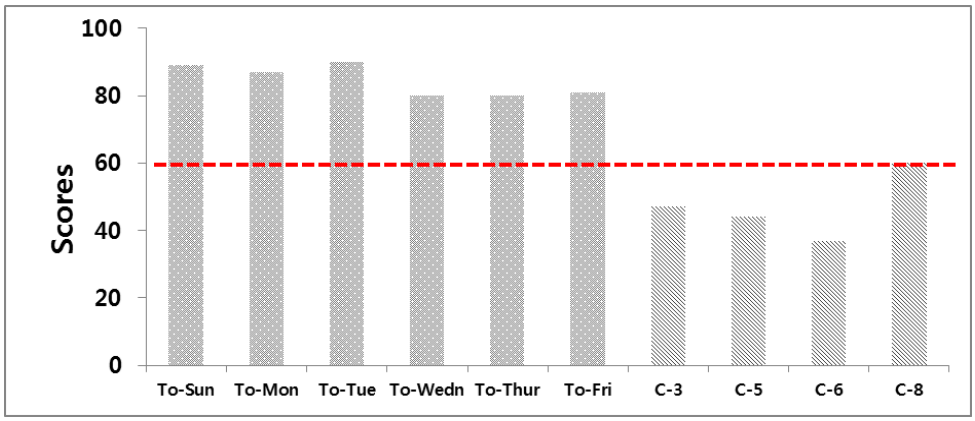

Figure4. Result of the detector dog selection test in six cloned dogs and four control dogs. All of the six trained cloned dogs passed the test. One puppy of seven controls (C-8) also passed the test. I have the data of success or failure of all of eight control dogs, however precise figures of four of them (C-1, C-2, C-4, C-7) could not be obtained. Therefore we indicated only the accurate marks in this Fig.4. The red line is the pass mark.

3. Discussion

The present study investigated for the first time the behavior of cloned dogs derived from a somatic cell of an elite drug detection dog. Although seven dogs is a small number, the present study has a relatively large significance because the dogs are genetically identical. All the clones and age-matched-controls were born within a short time period, making behavioral comparisons possible.

First, I attempted to determine whetherany differences in behavioral trends existed among cloned dogs with the same genotype. Secondly, the Campbell test was performed to evaluate the importance of prior puppy behavior in adult working dogs. Puppy behavior testing has become a valuable tool to select individuals for specific tasks[16]. The puppies are classified into six kinds of aptitude types by the Campbell test scores.

Among 6 kinds of aptitude type, the cloned puppies belonged to type 2 (dominance), while the control puppies belonged to type 3 (balanced submission). It is believed that dominant behavior of all clones might be a heritable characteristic derived from the genetics of an elite drug

detectingdog by SCNT. In English Cocker Spaniels, the Campbell test showed an associationbetween dominant behavior patterns and genetic factors [60]. A study in mice reported that genotype might significantly affect the aggressive behavior [61]. Consistent with theaptitudetypes results, all of the five subtest type scores were significantly different between the two groups. Possibly, the cloned dogs showed slight variations in their scores on the Campbell test because of their genetic identity. This observation indicates the possibility of a correlation between genotype and dominant behavior. In agreement with our results, cloned cattle exhibited behavioral trends that indicated a genetic influence. Similar behavioral trends were observed in grooming, curiosity, win–loss interaction, and dominance and aggressiveness, as well as inter-suckling and front mounting [62].

Based on the above results, we hypothesized that the cloned dogs would be selected as drug detection dogs through training and the selection test. This possibility was verified in this study using the selection test of the Korea Customs Detector Dog Training Center. Here, we demonstrated the ability of cloned dogs as drug detectors. Six trained cloned puppies successfully completed the drug detection dog selection test (Fig 4). Fig 4 shows marks of six cloned and four control dogs.I have the data of success or failure of all

of eight control dogs, however precise figures of 4 of them could not be obtained. Therefore we indicated only the accurate marks in this Fig. In the Korea Customs Detector Dog Training Center, the general pass mark of the selection test is a score of 60, and six of seven cloned dogs exceeded the pass mark. The one failure was To-Sat who had the appropriate attributes for a detection dog; nevertheless he could not complete the training course because of a leg fracture from an accident. In contrast, only one of eight control dogs passed the selection test, an efficiency of only 13%, a very low rate compared to that of the cloned dogs. In addition to the present study, other studies [11, 47] have reported a low selection rate of 30% up to 50% with dogs produced from natural breeding [5, 8, 63]. In this study the results of puppy testing appear related to adult selection testing since all the cloned puppies passed the selection test after they achieved type 1 or 2 in the Campbell test. However, in the control puppies there was no correlation between the Campbell test and the selection test. Therefore, we propose that cloning of a detection dog with high performance can be a better way to produce outstanding working dogs.

In conclusion, the present study demonstrated that genetically identical clones are more consistent in their behavior than naturally bred animals, and that cloned dogs can be classified into the same behavioral groups by the

Campbell test. Another important outcome of the present study is the successful evaluation as drug detection dogs of cloned dogs derived from a donor detection dog with excellent ability. Related administrative agencies presume that cloning an elite service dog can be an economic way to produce excellent dogs. In addition, test-failed dogs can be a matter because it is not easy to find foster families even though they are good dogs as a pet. At this moment, drug detecting cloned dogs are five years old now and doing outstanding works at the airport and the harbor in South Korea. They do not show the quick aging now and we are studying further about that.

PART 4.

Reproductive ability of a cloned male

Andbehavior traits for its offspring

1. Introduction

It is possible to propagate of specific genetics or elite genetics through SCNT. In particular, a number of breeds dogs and elite service dogs have been generated by SCNT [46, 59, 64-66]. It has been demonstrated that cloned animals, both male and female have normal reproductive characteristics, can reproduce normally [67-69]. In dogs, one report demonstrated that cloned females have normal reproductive hormone levels and ovarian follicle development [70]. Furthermore, another study. reported that healthy offspring were produced when cloned females were artificially inseminated using semen of cloned males, both derived from adult somatic cells [45]. However, up until now, there has been no reported study on the the behavioral characteristics of cloned dog offspring.

Despite rapid progress in analytical technology for detection of drugs and explosives, dogs are considered the gold standard in detection areas [71]. Multiple professional organizations have recommended screening for detection of drug and explosives. However, this is difficult without the superior detection capability of dogs. Generally, dogs are trained successfully as odor detectors and the breeds such as the blood hounds,

pointers and retrievers [17].

Canine behavioral traits are driven by a complex interaction of endocrine and neuroendocrine factors. It was provided for evidence that heredity is a major contributor to physiognomy and physique [72] and the environment is influential in the final expression of these traits [17]. Behavioral traits and aptitude for various roles in dogs are also likely to be influenced by genetic factors [5]. It was demonstrated in five dog breeds that genetic factors contribute significantly to several behavioral traits, for example, fearfulness, aggressiveness, reactivity and general activity [73, 74]. Especially, it was reported that behavioral traits such as courage, nerve stability, hardness and affability has positive genetic correlation in Labrador retrievers [75]. Moreover, dogs exhibiting superior drug or explosive detection ability are recommended for breeding to increase the production of such animals, thereby reducing the burdens (cost and time) associated with training and selection of detection dogs. Because of its high efficiency, SCNT, using donor cells from dogs with superior talent, represents an excellent approach for producing drug detection [76].

In this study, I studied the reproductive ability of cloned male dog by natural breeding. Accordingly, we bred the cloned dog with a bitch in the DDTC and produced puppies. The second aim of this study was investigation of

these puppies and their performance in the selection test, to evaluate their normality.

2. Results

2.1. Breeding and birth of puppies by breeding a cloned male dog and female dog

Natural breeding was performed for evaluation of normal fertility of cloned male dog, To-Tuesday. The fertile period of female dog was confirmed by checking progesterone concentration as shown in Fig.5A and breeding was performed within fertile periods. After 61 days of first mating, female dog showed hormonal changes in progesterone typical of pregnancy and offspring were born by natural delivery (Fig.5B). 10 offspring were produced which consisted of five males and five females (Table 5). They were healthy, had yellow coat color, no abnormalities in morphology and development (Table 5).

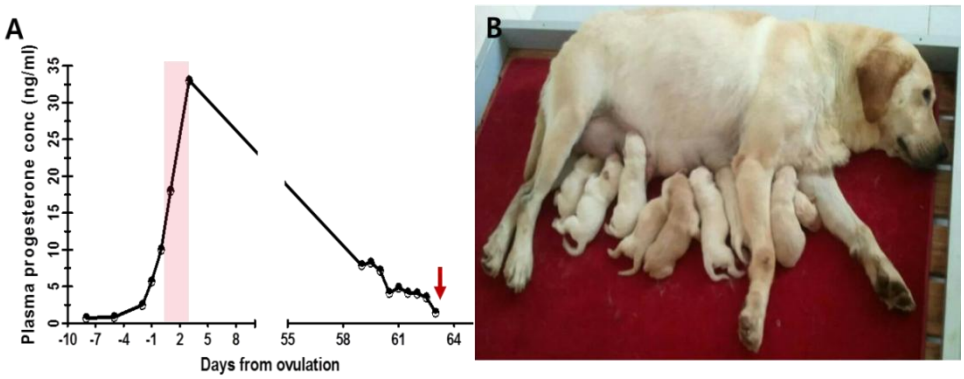

Figure 5. Birth of puppies by natural mating between cloned male and normal female(A) Progesterone concentration of a female dog from estrus to delivery for determine the day of ovulation and parturition. Red square means the fertile perid of a female dog and stayed with a male cloned dog in a room. Red arrow means the delivery time(progesterone is 1.46 ng/ml). (B) Apperance of puppies after one day of delivery.

Table 5. Characteristics including sex, coat color and mature weight in parent dogs (a cloned male, a wild female) and F1 puppies (P1-P10) produced bynatural mating

ID of dog (Name)	Sex	Coat Color	Mature Weight (kg)
Cloned male (F0,Toppy-Tuesday)	Male	Yellow	28
Wildfemale (Taemi)	Female	Yellow	29
P1 (San)	Male	Yellow	26.5
P2 (Sam)	Male	Yellow	27
P3 (Solgae)	Male	Yellow	29
P4 (Suri)	Male	Yellow	27
P5 (Siwon)	Male	Yellow	28
P6 (Sarang)	Female	Yellow	25.8
P7 (Saerom)	Female	Yellow	20
P8 (Satbyul)	Female	Yellow	19
P9 (Sowon)	Female	Yellow	20
P10 (Swlgi)	Female	Yellow	28

Figure 6. Mature appearance of F1 offspring produced by breeding a male cloned dog and female dog. F1 offspring developed without health problem until the end of training course. The images were takenat 2 years of age.

2.2. Final score of offspring forselectionof detector dogs

Among ten dogs, six dogs that passed with scores above 60 in detector dog training course. All the offspring received lower final score in the training program than the parent cloned male dog. Final selection success rate for offspring evaluated for drug detection was 60% (Table 6).

2.3. Correlation analysis between final training score and mature weight

Within ten cloned offspring of the cloned dog, I examined whether final training score and mature weight are significantly correlated (Table 6). Mature weight of puppies at 2 years of age had no significant correlation to final selected outcome score (Fig.7).

2.4. Behavioral traits of Volhard tests in puppies

Among ten puppies, six obtained a total score of Type 2 and Type 3 as indicated Table 3. Four puppies achieved total scores of Type 4, Type 5 and Type 6.

Table 6. Mature weight and final score as the training outcomeofF1 puppies at 2 years of age

ID of dog (Name)	Mature Weight (kg)	Final score
Cloned male (F0, Toppy-Tuesday)	28	90 (Success)[a]
P1 (San)	26.5	81 (Success)
P2 (Sam)	27	74 (Success)
P3 (Solgae)	29	37 (Fail)
P4 (Suri)	27	86 (Success)
P5 (Siwon)	28	74 (Success)
P6 (Sarang)	25.8	65 (Success)
P7 (Saerom)	20	68 (Success)
P8 (Satbyul)	19	34 (Fail)
P9 (Sowon)	20	48 (Fail)
P10 (Swlgi)	28	53 (Fail)

[a]Final training outcome of cloned male dog, Toppy-Tuesday data is suggested in previous study (Choi et al., 2014)

Figure 7. Correlation analysis between final score and mature weight of F1 offspring at 2 years of age. They have no correlation with significance.

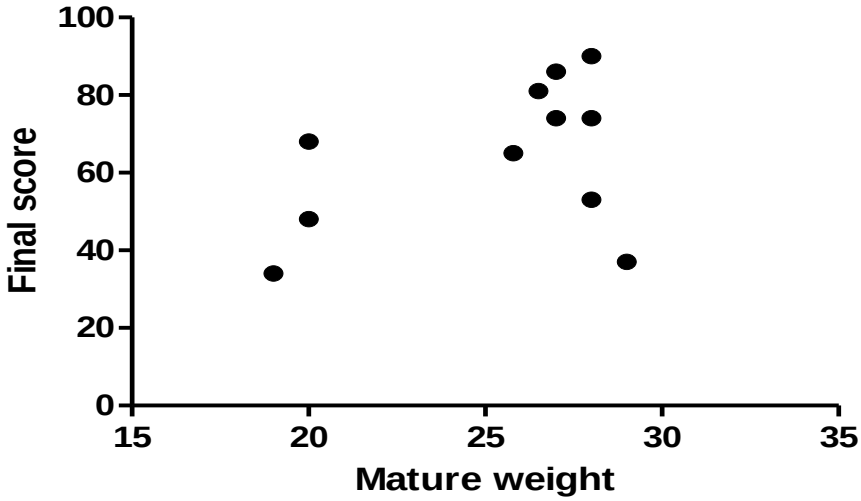

Table 7. Puppy Volhard tests of F0, a cloned male dog and F1 puppies at 8 weeks of age

ID of dog (Name)	Social Attraction	following	restraint	Social Dominance	Elevated Dominance	Retrieving	Touch Sensitivity	Auditory Sensitivity	Sight Sensitivity	Total type of Volhard tests
Cloned male (F0, Toppy-Tuesday)	1	1	2	2	3	-	-	-	-	2
P1 (San)	3	2	5	3	3	6	1	6	1	3
P2 (Sam)	1	2	2	2	3	3	4	4	4	2
P3 (Solgae)	3	2	5	4	3	6	1	6	3	4
P4 (Suri)	2	2	3	2	3	3	5	4	3	3
P5 (Siwon)	1	2	3	3	3	4	4	4	3	3
P6 (Sarang)	5	6	6	6	3	6	1	4	5	6
P7 (Saerom)	4	4	4	4	3	5	5	4	3	4
P8 (Satbyul)	1	2	4	1	3	3	5	4	3	3
P9 (Sowon)	1	2	4	1	3	4	5	4	3	3
P10 (Swlgi)	6	5	5	4	3	5	6	5	4	5

[a]Finaltraining outcome of cloned male dog, Toppy-Tuesday data is suggested in previous study (Choi et al)

2.5. Behaviroal traits of Mirror and Toman tests in puppies

Table 8 shows that threeF1 puppies achieved Type L scores ; six obtained Type F and one scored Type R in the Toman litter test. In Mirror test, seven puppies showed normal reaction and three puppies exhibited abnormal reactions.

Table 8. Behavior andTomann litter of F0, a cloned male dog and F1 puppies (P1-P10) at 8 weeks of age

	Mirror	**Tomann litter Test**
Cloned male	NR	L
(F0, Toppy-Tuesday)		
P1 (San)	NR	F
P2 (Sam)	NR	L
P3 (Solgae)	NR	F
P4 (Suri)	NR	F
P5 (Siwon)	A	F
P6 (Sarang)	A	F
P7 (Saerom)	NR	F
P8 (Satbyul)	NR	L
P9 (Sowon)	A	L
P10 (Swlgi)	NR	R

NR means normal response(the puppy looks at inself in the mirror and spends periods of time motionless, may go up to the mirror and move back considerably). A means abnormal response(Except for normal reaction)

3. Discussion

In the study, I examined the reproductive ability of male cloned dog, mature weight and behavior traits of his offspring. Several previous studies reported that cloned animal had a normal reproductive ability [41, 45, 76]. As a result of natural mating between a cloned male dog (Toppy-Tuesday) and a female dog in DDTC, 10 healthy puppies were produced. It was previously reported that although first cloned male dog exhibited normal libido, natural mating could not be performed due to his inexperience in the clasping method [45]. However, I found that the cloned male dog of Labrador retriever dog had a normal libido along with other mating behavior and reproductive ability.

His puppies showed normal development and matured well without congenital defect or infectious disease until the final selection. Positive genetic correlations between mature weight and mature height were found for Labrador retrievers [77]. Inthis report, among ten puppies, seven dogs (70% offspring) showed mature weight above 25kg which are similar with those of their parent dogs. All of these offspring had similar mature weight within normal reference range [77, 78]. Among failed four puppies in final selection test, two dogs (50%) showed lower weight than other dogs.

Although I could not find any correlation between final score and mature weight or height in puppies, I suggest that this was because I investigated only ten puppies, small number to provide conclusive analysis.

The behavioral traits of the puppies were monitored to evaluate trainability and compare the similarity between cloned parent dog and his puppies because it is assumed that behavioral traits of puppies are inherited from the cloned male dog. To determine the behavioral traits of puppies, Volhard test, Toman litter test and mirror test were performed. As in our previous report, a cloned male dog gets subtype 2 in Campbell test [11]. However, among all the puppies only one, Solgae, scored subtype 2 which was identical to his father cloned dog in the Volhard test. Interestingly, six puppies showed 2 or 3 subtype score in the Volhard test, indicating suitability for training.. Notably, all puppies receiving 2 or 3 subtype score in Volhard test were not among the final selected test final. In the Toman liter test and the Mirror test, a cloned dog got both L type and Normal response, respectively. As in the Volhard test, only one puppy, P2 Saem, showed the same response in the Mirror test and L type of the Toman Litter test. Because Toman litter test examined dominance within one litter groups during socialization [19, 79], I suppose that cloned male dog has no relationship with the result from puppies in Toman litter test. Results of the present study are consistent with

the conclusion that it is difficult to predict adult behavior before puppies one year old [3]. This is also supported by studies that compared behavior between one and four years old [80], as well as between eight week and three year old dogs [81].

Furthermore, in the present study, the cloned dog's offspring achieved 60% success rate in final selection tests. Comparing 42.5%, the mean success rate of KCS normal bred dogs (Lee et al., 2014, Applied Animal Behavioral Science submitted) and approximately 30% success rate of Japan detector dog training[5], 0-40% success rate of US customs service [17] were reported, our 60% training outcome success rate was remarkable. However, Offspring of the donor of cloned male "Chase" scored mean 60% (success ratio) in breeding with normal female. Hence, it was thought that the breeding score of offspring of cloned male was higher than mean success ratio but it was similar as breeding score of their donor dog, that is, elite dogs.

Cloned detector dog received an excellent grade in training, so it was concluded that cloning elite dogs suing the SNCT technique of was a better approach for producing qualified detector dogs [76]. However, it is very hard to find a good candidate dogs for drug detection training in all

countries.I showed that we could produce superior cloned dogs using skin cells from superior dogs and breed them, which led to us hypothesize that this method will allow us to generate better candidate dogs with higher possibility to pass training course.

Furthermore, while the behavioral traits of puppies correlate with the genetic factors of a cloned male dog, and although only one litter was investigated for this study, it is generally accepted that genetic factors are also important in training dogs.

In conclusion, cloned Labrador retriever male dog have normal reproductive characteristics and can produce puppies, which later succeeded in the training course for drug detection above a 60% success selection rate.

PART 5.

General Conclusion

This study was carried out to investigate the behavior related to detector dog training in cloned dogs derived from an elite dog and cloned dog's offspring. In order to perform it, Campbell test, most popular puppy test, was used to evaluate the similarity among cloned puppies. Volhard puppy aptitude test, modified Campbell test, Toman litter test and mirror test was also applied to analyze behavior of cloned male's offspring. According to results of Campbell test, cloned dog group showed a similar behavior patterns compared with control group. Furthermore cloned dogs had higher score and success ratio than control group. Offspring of cloned male was healthy and had no abnormalities in development and morphology. And the success ratio of offspring was higher than mean of natural bred dog in DDTC but lower than cloned dog group. And the result of behavioral analysis shows that cloned male and its offspring had a relationship in above mentioned tests.

Therefore, in this study, cloned detector dogs those were genetically identical with their elite donor showed a consistent behavior patterns and suitable for detector dog training. So, using SCNT technique, detector dogs those have even performance level can be produced and it will be helpful to raise a success ratio of detector dog and it leads us to promote the quality.

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국문 초록

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개는 반려 동물, 사냥, 목양견, 구조견, 전달견, 국경관리, 범죄수사, 세관, 자연물 등에 사용되어 왔다. 1988년부터 관세청은 공항만에서 불법 마약류와 폭발물을 단속하기 위해 사용해 왔다.

보다 우수한 탐지견을 생산하기 위해서는 훈련에 적합한 보다 나은 후보견을 확보하는 것이 가장 중요하다. 관세청은 우수견을 이용해 번식 프로그램을 실시하여 왔으나 우수한 견의 확보, 가능 연령 유전적 문제 등 여러 가지 장애가 있었다. 무엇보다도 우수한 능력을 지닌 부견과 모견의 형질이 그 자손에게서 발현되지 않을 수 도 있다는 것이었다. 이러한 문제를 해결하기 위하여 우수 탐지견 유래 섬유아세포를 이용한 체세포 핵 이식 방법(SCNT)으로 복제견 7두를 생산하였다.
원본견과의 유전적 동질성은 확립되었지만 복제견의 탐지견 능력과 행동에 대한 동질성은 아직 검증되지 않았다. 따라서 우수견

복제로 탐지견 훈련에 적합한 행동 특징을 가진 견을 생산할 수 있다는 가설을 세웠다. 더 나아가 이 연구에서 복제견 수컷의 자손의 훈련과 관련된 행동을 조사하였다.

첫 번째 실험에서는 강아지 선별에 대중적으로 사용되어 온 Campbell test를 이용하여 9-12 주령에 7두의 복제견 강아지와 4두의 일반 번식 강아지의 우위성을 평가하였다. 캠벨 테스트는 사회적 친화도(social attraction), 따르기(following), 사회적 우위성(social domination), 거상 우위성(elevation dominance)로 구성되어 있으며 결과에 따라 6가지 중 타입으로 결정된다 ; Type 1은 극단적 우위성, Type 2는 우위, Type 3는 조화된 우위성, Type 4는 복종적, Type 5는 극단적으로 복종적이며, Type 6는 독립적이다. 이 세가지 타입 중 Type 1 Type 2, Type 3가 사역견에 적합하다고 간주된다.

두 번째 실험에서 복제견 자손의 행동 성향을 판별하기 위하여 7 주령에 볼하드 강아지 태도 테스트 (변형 캠벨 테스트), 토만 리터 테스트, 거울 테스트를 사용하였다. 복제견 (투피-튜즈데이)와 관세청 탐지견센터의 모견 사이에서 10두의 자손이 탄생하였다. 볼하드 테스트는 변형 캠벨 테스트로 사회적 친화도(social attraction), 따르기(following), 견인(restraint), 사회적 우위성(social dominance), 거상 우위성(elevation dominance),

회수(retrieving), 촉각 민감성touch sensitivity), 청각 민감성(sound sensitivity), sight sensitivity(시각 민감성)으로 구성되어 있다. 결과에 따라 강아지는 캠벨 테스트와 같이 6개의 집단으로 구분된다. 토만 리터 테스트는 강아지를 낯선 공간을 데리고 가서, 실험자가 도착하여 관찰한 후 사료를 주고, 강아지에게 물체를 던져 보고 낯선 소음을 들려 주고 지속적으로 실험자가 강아지와 같이 시간을 보낸 후 다시 한번 사료를 준다. 토만 리터 테스트는 한 배에서 강아지들 사이에서 사회성 수준과 우위성을 판단하기 위해서 사용된다. 토만 리터 테스트에서 강아지는 4가지 타입으로 나뉘며(L type, F type, R type, F type) 그 중 L type 이 사역견에 적합하다.

거울 테스트는 강아지의 사회성 수준을 판별하기 위해 시행 되었으며 결과는 거울에 대한 반응의 정상성을 판단하는 것이다. 이 두 가지 실험에 참여한 모든 강아지들은 관세청 훈련 매뉴얼에 따라 훈련 후 최종 선별 테스트가 실시 되었다. 통계 분석을 위하여 각각의 캠벨 테스트 점수는 SAS 9.3을 이용한 일반 선형 혼합 모델을 이용하여 분석하였다. 최종 훈련 점수와 복제견 자손의 성숙 체중 사이의 관련성은 GraphPad Prsim 4.02을 사용하여

Fisher의 추출 테스트로 분석하였으며 유의성 수준은 0.05이다.

첫 번째 실험에서 캠벨 테스트 결과는 To-Wednes의 결과가 To-Mon(P0.0031)과 To-Thur(P=0.0098) 과 매우 달랐지만 복제견 군과 대조군의 모든 테스트 결과는 유의성 있게 달랐다 (P<0.0001). 복제견 강아지에서 탐지견 훈련 성공율 (86%)은 대조군(30%) 보다 매우 높았다.

두번째 실험에서 복제견 수컷은 정상적인 생식 능력을 가졌으며 ; 그 자손은 건강하고 발생과 발달에 있어서 비정상적인 면이 없었다. 볼하드 강아지 태도 테스트 결과는 10두 중 6두 만이 Type 2 혹은 Type 3를 받았다(60%). 토만 리터 테스트에서 3두 만이 복제견 수컷과 동일한 Type 2를 보여 주었다. 거울 테스트에서 7두의 강아지가 정상 반응을 3두는 비정상 반응을 보여 주었다. 10두의 복제견 자손의 탐지견 훈련 성공율은 60%였으며 이 결과는 평균 관세청 탐지견 성공율(42.5%) 보다 매우 높았다.

결론적으로 우수한 능력을 지닌 마약 탐지견 유래 복제 강아지는 자연 번식견에 비해 행동에 있어 일관성을 지니고 있었으며 높은

성공율을 보여 주었다. 복제견은 정상적인 생식능력을 가지고 있으나 그의 자손과의 행동에서 연관성을 찾을 수는 없었다. 그러므로 일반적인 번식 보다는 SCNT를 사용한 체세포 복제를 이용하면 보다 효과적으로 우수견을 생산할 수 있다.

주요어 : 강아지 태도 테스트, 복제견, 탐지견, 선별 테스트, 선별률, 생식 정상성, 행동 특징

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