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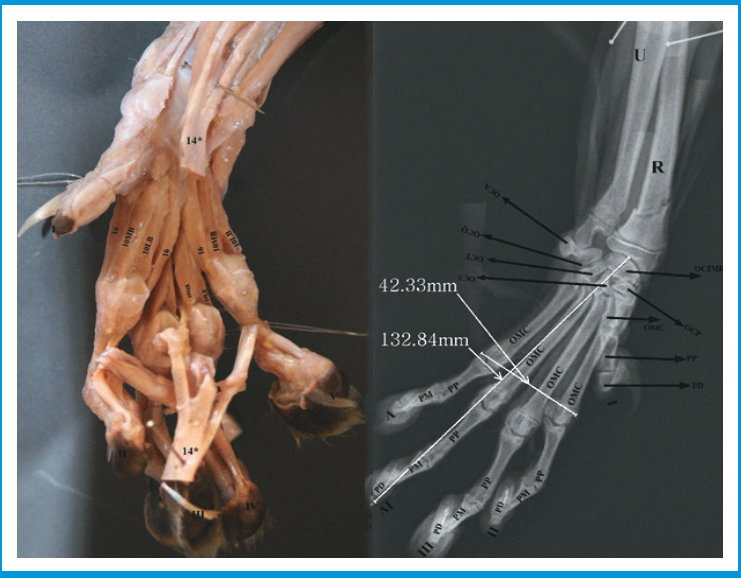

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Original Research Article

THE EFFECT OF TANNIN SUPPLEMENTATION OF MID-LACTATION DAIRY COWS DIETS ON METABOLIC PROFILE PARAMETERS AND PRODUCTION CHARACTERISTICS

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Abstract: The aim of this study was to examine the effect of using tannin supplement in ration for mid-lactation dairy cows (90±17 days in milk) and the number of lactation on metabolic profile parameters values. Additionally, the effect of tannin supplementation on cows' production characteristics in the 2nd lactation was evaluated. Research was conducted on 80 Holstein cows divided into two groups of 40 cows (control and experimental group) that included 16 cows in 2nd and 12 in 3rd and 4th lactation. The tannin supplement Tanimil SCC (40 g/cow/day; 40% of tannins) was added in the total mixed ration (TMR) of experimental group, while the control group of cows was fed ration without the tannin supplement. The trial lasted 60 days. Dairy cows, fed diet supplemented with tannin, had markedly lower values (P<0.05) of ß-hydroxybutyrate concentration (0.79 vs. 0.96 mmol/L) and lower blood urea concentration (5.89 vs. 6.93 mmol/L). Difference in the blood glucose concentrations (3.92 vs. 3.89 mmol/L) was not significant. The lactation number had no significant effect on values of metabolic profile parameters (concentration of glucose, ß-hydroxybutyrate and urea). The effect of interaction of lactation number and tannin supplement on the values of examined parameters of metabolic profile was not significant. Supplementation of tannin in the diet for cows in the second lactation, had a positive effect (P<0.05) on the milk yield (4.42%) and yield of 4% fat corrected milk (FCM, 5.24%). The use of tannin, improved concentration (3.20 vs. 3.12%) and yield (from 1.16 to 1.24 kg/day) of milk protein (P<0.05). Addition of tannin also improved concentration and yield of milk fat, as well as the concentration of lactose. The supplementation of Tanimil SCC to mid-lactation dairy cows had a positive impacts on reduction of blood content of ß-hydroxybutyrate and urea, and improved productive performances of cows in the second lactation.

Key words: tannins; lactating cows; nutrition; metabolic profile; milk yield and composition

Introduction

Maximizing animals' daily gain or milk yield per unit of protein intake is based on improved dietary protein utilization (1). However, one of the main problems in dairy cows nutrition is the excess of ruminal degradable and deficiency of undegradable protein in ration, in absolute and relative figures, relative to the dietary content of carbohydrate fractions and rates of their ruminal degradation.

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Tannins have the potential to protect proteins from ruminal degradation, and to decrease rate of NH_3-N accumulation in the ruminal content, which makes them suitable additives for dairy cows diets with surplus of ruminal degradable protein, and even nonprotein nitrogen (2). High concentrations of tannins (usually higher than 50 g/kg DM) reduce voluntary feed intake and nutrient digestibility to a great extent because they decrease feed palatability, slow down digestion, and development of conditional repulsiveness (3). Intake of low to medium quantity of condensed tannins (CT) (10-40 g/kg DM) may improve feed

digestion and feed conversion mainly due to the reduction of ruminal protein degradation which is related with lower production of NH_3-N and higher availability of essential amino acids and non-ammonia nitrogen for absorption in the small intestine (4). Tannins modify the ruminal fermentation by formation of poorly degradable complexes with proteins that are stable within the pH range of 3.5 to 7.0, and also by reducing quantity of immediately degradable fractions. Such formed complexes, protect proteins from microbial hydrolysis and deamination in the rumen, and increased the quantity of proteins that are degraded in abomasum and small intestine (2). These secondary plant metabolites have also a property to form reversible and irreversible complexes with polysaccharides (cellulose, hemicelluloses, starch and pectin), alkaloids, nucleic acids and minerals (5).

Values of some metabolic profile parameters, β-hydroxybutyrate (BHBA) and urea can serve to estimate cows' health status and to measure the adequacy of dietary protein levels as well as nitrogen utilization efficiency. Subclinical ketosis is characterized by elevated serum β-hydroxybutyrate concentrations (> 1.0 to 1.4 mmol/l) in the absence of clinical signs of ketosis (6). All precursors for the synthesis of milk components, reach mammary gland by blood, so the composition of the milk to the great extent depends of their blood content. Monitoring levels of blood urea nitrogen (BUN) or milk urea nitrogen (MUN) may provide information on how well the ratio of rumen degradable proteins is balanced with energy availability to optimize rumen microbial protein synthesis, as it is known that imbalance in protein and carbohydrate degradabilities may result in suboptimal animal health and lower production (7). However, the increased content of BUN and MUN could be the result of nitrogen inefficiency caused by the surplus of tissues protein supply (8). Szczechowiak et al. (9) reported that using condensed tannins in cattle nutrition significantly increased blood glucose level while concentration of BHBA was unchanged.

Several authors found that using condensed tannins in ruminant nutrition increases milk yield, milk protein, lactose and fat, decreases blood and milk urea nitrogen concentration (1, 10, 11, 12), and protects animal health (13). However, Benchaar and Chouinard (14), Aguerre et al. (15) and Broderick et al. (16) did not find significant effect of low tannins content in cows ration, especially on the feed intake, milk yield, protein content and composition, N concentration, lactose, fat content and fatty acid profile in milk.

The objective of this study was to investigate the effects of supplementing diet for the mid-lactation dairy cows with different lactation number with tannin, and influence of these factors on some blood metabolic profile parameters concentration in cows, and also to explore the effects of tannin supplementation on the production performances of cows in the second lactation.

Materials and methods

Animals and the diets

The trial was conducted on the commercial dairy cattle farm (PKB Corporation —Padinska Skela), Serbia. Research was conducted on 80 Holstein cows (32 in 2nd, 24 in 3rd and 24 in the 4th lactation) that were housed in the individual tie stalls, divided into two groups of 40 cows (control and experimental group) that included 16 cows in 2nd and 12 in 3rd and 4th lactation. Experiment was designed as a 2×3 factorial arrangement of treatments (two rations, with and without added tannin, and three lactation number) for determination the effect on metabolic profile of cows, and as one factorial arrangement with two treatments (ration with and without added tannin) to estimate the effect on production performances of the 2nd lactation cows. The experimental groups were equalized according to the lactation number, stage of lactation (mid-lactation, 90±17 days in milk), the average milk yield (38.6±2.2kg/d) and body condition score (3.2±0.15). A trial period lasted for 60 days. Cows were offered a total mixed ration (TMR) two times a day (in the morning and evening) that were prepared according to the nutritional value and energy content, and balanced to meet nutritional requirements of cows for maintenance and milk production (Table 1). Cows in the control group were fed ration without supplemented tannin, while in TMR for experimental group, tannin (from horse-chestnut bark) based supplement „Tanimil SCC" (Tanin, Sevnica, Slovenia) was added in quantity of 40 g/cow/day (contained 40% tannins). Rations were formulated for the cows of 600 kg BW with milk production of 39 kg/day, containing 3.7% milk fat

Table 1: Composition and nutritional value of the total mixed rations for dairy cows

Ingredients, kg DM/day	Control group	Experimental group
Alfalfa hay	1.78	1.78
Alfalfa haylage	1.77	1.77
Triticale silage	0.75	0.75
Corn silage	6.73	6.73
Beet pulp, ensiled	0.46	0.46
Brewers grain	1.09	1.09
Beet molasses	1.56	1.56
Barley grain	0.89	0.89
Corn grain	2.73	2.73
Wheat grain	1.33	1.33
Canola meal	1.28	1.28
Soybean full-fat, extruded	1.41	1.41
Soybean cake	1.86	1.86
Calcium carbonate	0.09	0.09
Monocalcium phosphate	0.04	0.04
Sodium bicarbonate	0.10	0.10
Salt	0.07	0.07
Vitamin - mineral mix[1]	0.18	0.18
Tanimil SCC	-	0.04
Nutrient composition		
DM, kg	24.1	
CP, g/kg DM	178.0	
RDP, g/kg DM	114.0	
RUP, g/kg DM	64.0	
NDF, g/kg DM	315.0	
Forage NDF, g/kg DM	223.0	
ADF, g/kg DM	192.0	
NFC, g/kg DM	423.0	
EE, g/kg DM	44.0	
Ca, g/kg DM	6.0	
P, g/kg DM	4.0	
Energy value NE_L MJ/kg DM	6.65	

[1]Mixture of vitamins and trace elements, 1 kg contains: Vitamin A – 500000 IU, Vitamin D – 300000 IU, Vitamin E – 1600 IU, Mg – 6.7 g, S – 1g, Fe – 1000 mg, Cu – 1300 mg, Mn – 6600 mg, Zn – 5500 mg, J – 90 mg, Se – 15 mg, and Co – 5 mg.
DM: dry matter; CP: crude protein; RDP: rumen-degradable protein; RUP: rumen-undegradable protein; NDF: neutral detergent fibre; ADF: acid detergent fibre; NFC: non-fibre carbohydrates; EE: ether extract; Ca: calcium; P: phosphorus

and 3.4% protein by using NRC (17) model and programme package Version 1.1.9. The cows had ad libitum access to water.

Analytical procedure

Chemical analysis of feeds and TMRs were performed in the Laboratory for quality management EKO - LAB DOO in Belgrade. For chemical analysis, the TMR samples were collected twice weekly during the trial period. The proximate and detergent fiber analysis parameters were determined according to the AOAC methodology (18).

Blood biochemical parameters

Blood sampling was performed only at the end of trial. Approximately six hours after morning feeding, by venipuncture of *v. coccigea*, 20 mL of blood, from all cows in the experiment, were taken into the test tubes, and immediately transported to the laboratory in a portable freezer. Whole blood samples were centrifuged for 10 minutes at 3000 rpm and serum samples were analyzed by standard biochemical procedures. Determination of values for basic parameters of the cows metabolic profile: concentration of blood glucose, BHBA and urea, was performed using semi-automatic biochemical analyzer RT-1904C (Rayto Life and Analytical Sciences Co. Ltd) in the laboratory for diagnostic analysis of the PKB Corporation - Centre for livestock production, Belgrade.

Milk yield and composition

The milk yield and chemical composition (content of milk fat and milk protein) were determined at the end of the trial. Milk was sampled according to the internationally agreed principles ICAR (19), by using the MK V Milk Meter (Waikato Milking Systems New Zealand Ltd.). The sample (25 g/kg milk) was a proportional part of a total milk quantity. After milking and milk homogenization by the above mentioned sampler, the milk was poured in bottles in an amount of 100 mL and conserved by the addition of 0.2-0.5 % of potassium dichromate ($K_2Cr_2O_7$). Milk chemical composition (content of milk fat and protein) were estimated by infrared spectroscopy using the Milko Scan 104/SN (Foss Electric, Hillerod, Denmark) in the ECO – LAB DOO laboratory in Belgrade.

Statistical analysis

In order to investigate the effects of tannin based supplement and lactation number, and interaction of these factors on metabolic profile basic parameters, the multivariate analysis of variance was used.

The significance of the tannin addition effect on production performances of the 2nd lactation cows was estimated by Student's t-test. Statistical significance was determined at the level of $P<0.05$.

For statistical analysis of the obtained results, a free version of statistical software PASW Statistics 18 SPSS Inc. package was used (20).

Results

Results of the effects of tannin supplementation, lactation number, and interaction of these factors on the values of the examined blood metabolic profile parameters (concentration of glucose, BHBA and urea) of mid-lactation dairy cows are presented in Table 2.

Blood glucose concentration was slightly increased for cows fed ration supplemented with tannin (0.77%), which was not significant. Cows fed TMR supplemented with tannin, had markedly lower average BHBA concentration in blood and content of blood urea ($P<0.05$) in comparison to the control group (by 17.71% and 15.01%, respectively). A significantly lower concentration of BHBA was found ($P<0.05$) in the group fed ration supplemented with Tanimil SCC only for the cows in the second lactation, while the blood urea concentration was significantly lowered ($P<0.01$) in the experimental group only for cows in the third lactation. In the present study it was documented that lactation number did not influence values of glucose, BHBA and urea. Results obtained in this study indicate that there is no interaction of lactation number and tannin supplement addition in TMR of mid-lactation dairy cows, regarding the effect on basic parameters of cows' metabolic profile.

Effects of tannin supplement on the cows production performances in the second lactation are presented in Table 3.

Cows in the second lactation, that were fed tannin supplemented ration had a significantly higher milk yield and higher yield of 4% fat corrected milk (FCM) when compared to cows from the control group (by 4.42 and 5.24%, respectively) ($P<0.05$). Higher

Table 2: Effect of tannin supplementation (T), lactation number (L) and interaction of these factors (T×L) on blood metabolic parameters at mid-lactation dairy cows (mean ± SD)

Parameters[1]	Without tannin			Supplemented tannin			*P values*		
	Lact. 2	Lact. 3	Lact. 4	Lact. 2	Lact. 3	Lact. 4	T	L	T×L
Glucose (mmol/L)	4.10±1.26	3.95±0.36	3.69±0.35	3.77±0.29	3.93±0.59	3.95±0.65	0.856	0.271	0.302
$\overline{X}$		3.89±0.74			3.92±0.53				
BHBA (mmol/L)	1.11±0.34	0.86±0.21	0.94±0.57	0.84±0.19	0.82±0.35	0.70±0.14	0.014*	1.549	0.365
$\overline{X}$		0.96±0.39			0.79±0.26				
Urea (mmol/L)	7.26±3.28	7.15±1.38	6.31±1.24	6.50±1.18	5.73±1.46	5.50±1.76	0.018*	1.732	0.752
$\overline{X}$		6.93±2.09			5.89±1.50				

[1]Mean values ± standard deviation are showed in the same row
BHBA – ß-hydroxybutyrate
* - P<0.05

Table 3: The effect of tannin supplementation on milk yield and chemical composition (mean ± SD)

Parameter[1]	Diet with tannin	Diet without tannin
Milk yield, kg/day	38.95±4.7[a]	37.30±4.1[b]
4% FCM yield, kg/day	37.33±4.2[a]	35.47±4.0[b]
Milk fat concentration, %	3.73±0.38	3.68±0.51
Milk fat yield, kg/day	1.45±0.29	1.37±0.31
Milk protein concentration, %	3.20±0.12[a]	3.12±0.13[b]
Milk protein yield, kg/day	1.24±0.10[a]	1.16±0.11[b]
Milk fat : protein ratio	1.17	1.18
Milk lactose concentration, %	4.63±0.10	4.50±0.12

[1]Mean values ± standard deviation are showed in the columns
FCM: 4% fat corrected milk calculated as (0.4 x kg milk + 15 x kg milk fat)
a, b - Values within the same row not sharing a common letter were significantly different (P<0.05)

milk fat content, fat yield and milk lactose were also found for a group that received tannin in TMR, but determined differences were not significant. The TMR that included tannin provided a higher milk protein concentration by 2.56% (P<0.05) and a higher protein yield by 6.89% (P<0.05) relative to ration without tannin.

Discussion

The aim of this study was to examine the effect of using tannin supplement in ration for mid-lactation dairy cows and the number of lactation on metabolic profile parameters values, as well as, on the production performances of cows in the second lactation. Blood glucose concentration was analyzed since its concentration in the blood of dairy cows is important for energy balance and lactose production in the mammary gland. Determined values were within the physiological range (21, 22). In the present study, similar glucose levels indicated that physiological values exist in all experimental animals. Soltan (11) also reported that replacing of row soybean by processed or tannin supplemented soybean (extruding, addition of plant tannins extract and essential oils, addition of plant tannins and pelleting) in diet for dairy cows did not influence glucose

concentration in blood serum. After addition of leaf meal mixture of different tanniferous plants, the blood glucose level of dairy cows was within the range of physiological values without marked variations (1). It was reported that tannins increase blood glucose concentration, due to their ability to modulate the activity of rumen microorganisms and consequently ruminal fermentation (9). As a result, there is a change in the ratio (%) of volatile fatty acids, with an increase of the ruminal production of propionate, which is a precursor for glucose synthesis. In the present study, values determined for blood BHBA concentration indicate moderate mobilization of body fat reserves, because the physiological concentration of blood BHBA for lactating cows is within the range of 0.6 to 1.0 mmol/L (23). Bjerre-Harpøth et al. (24) stated that increase in the glucose level influence lowering of the blood BHBA, and in the present study a similar tendency was observed.

Concentration of blood urea in both experimental groups of cows was within the physiological range values (2.0-7.5 mmol/L) according to Radostitis et al. (22), with markedly lower average content of blood urea in the group of cows fed TMR supplemented with tannin, which indicated more efficient protein utilization. Obtained results are in line with earlier findings (1, 25) that indicated reduced protein degradation and production of NH_3 in rumen, and therefore, consequently, lower absorption of ammonia through the rumen wall into the blood. Dairy cows fed rations that contained processed or supplemented soybean had reduced blood urea N (4.9, 6.0 and 7.7%, respectively, $P<0.05$) in comparison to the control group fed raw soybean diet (11). The lower blood urea N concentrations reflected improvement in nitrogenous components metabolism from diet and increased absorption of essential amino acids (26). Our results are consistent with those of Sarker et al. (27) reporting that blood urea nitrogen value did not differ significantly between cows in different lactation. However, Roussel et al. (28) found that circulating BUN levels significantly varied in respect to the lactation number. Prpić et al. (29) also found significant effects of the lactation number on metabolic profile parameters of high-yielding cows (higher urea concentration with increased lactation number). Increased urea concentration is an indicator of disproportion between rumen degradable protein intake and fermentable for the rumen microorganisms, as it indicates an increased rate of the ureagenesis in the liver which requires large amounts of energy (30).

The results of our study are in line with those of Dey and De (1) about positive impact of condensed tannins (CT) from tanniferous *Ficus bengalensis* leaves on milk production and milk fat content, and their additive significant positive effect on 4% fat corrected milk yield in crossbred cows during mid-lactation. Same authors also found improved milk production efficiency (kg of feed/kg of FCM yield) ($P<0.05$) for cows fed CT. Wanapat et al. (31) reported that CT rich cassava hay in the diet of dairy cows, may provide additional volatile fatty acids required for the synthesis of milk fat. According to Mabjeesh et al. (26) a higher percent of milk fat, in cows fed ration supplemented with tannin, was the result of additional postruminal digestion. The effect of condensed tannin in improving production characteristics can be explained by increased availability in the intestines and increased supply of tissues with the essential amino acids from protected feed proteins (32). Methionine plays an important role as a methyl donor during the synthesis of milk fat, and also as the precursor for phospholipids components i.e. choline synthesis (33). However, feeding dairy cows with the addition of large quantities of tannin, such as 100 or 200 g/day of commercial tannin product (QT_{100} and QT_{200}, respectively), resulted in a decrease ($P<0.05$) of the daily milk yield from 30.17 to 26.34 and 25.92 kg in the experimental group of cows when compared to the control (13.0 and 14.2%, respectively) (34). Benchaar and Chouinard (14) reported that supplementation of diet with quebracho condensed tannin extract (*Schinopsis balansae*) (150 g/day, 0.45% DM) had no effect on milk fat composition.

Supplemented tannin did not have a profound effect on the milk lactose content. As lactose is synthesized in the mammary gland directly from blood glucose, and in ruminants the gluconeogenesis is predominantly carried out from propionic acid and amino acids, increased availability of amino acids, especially methionine, lysine and amino acids with branched chains, contributes to the extensive synthesis of lactose (35).

In the present study, a somewhat higher values for milk protein concentration were found in cows from the experimental group (3.2%) than the average value of this parameter in Holstein cows, which according to Kirovski et al. (36) is 3.06%. The aim of the modern cattle production

is to achieve protein content of 3.4% in the milk of high-yielding cows, which is in accordance with the tendency for the purchase price of milk to be based on milk protein content (37). The results obtained in our study are consistent with the results of Attia et al. (34) who claimed that using QT_{100} and QT_{200} increased ($P<0.05$) milk protein percentage (3.59% and 3.48%, respectively) when compared to the control group (3.29%). One of the reasons for these effects is an increase in metabolic supply with the undegradable protein due to binding of proteins with CT. The milk fat to protein ratio in our trial did not differ significantly between examined groups of cows. According to Stoop et al. (38), a fat to protein ratio lower than 1.05 reflects milk fat depression, a dietary problem causing an energy imbalance, associated with an increase in the milk fat proportion of total unsaturated fatty acids.

Unlike our results, Aguerre et al. (15) reported that the addition of a quebracho-chestnut tannin extracts (ratio 2:1) did not affect the milk yield, and also determined a trend of linear reduction in branched-chain volatile fatty acid concentration, milk protein concentration and yield, and decrease in fat-and-protein-corrected milk yield, with the increase in tannin levels in the nutrition of cows. Addition of large amounts of tannin caused a decline in milk yield, milk fat and protein concentration, showing a low potential to alter ruminal bio-hydrogenation process and to modify fatty acid profile of milk fat (14). Disagreements in the obtained results may be explained by different sources and levels of added tannins, animal physiological status and diet composition. Effects of mildly excessive CT supplementation can be tolerated for short periods (weeks), but prolonged feeding of CT can slow down digestion rates, increase rumen digesta mass and reduce productivity (39). Moreover, the addition of condensed tannin reduced digestibility of the starch, leading to a decrease in the production of volatile fatty acids, wherein the decrease in the amount of propionate limited glucose synthesis and therefore altered lactose synthesis.

Conclusions

According to the results of this study it may be concluded that mid-lactation dairy cows fed TMR supplemented with tannin had significantly lower concentrations ($P<0.05$) of blood BHBA and urea, with slightly increased concentration of glucose. There was not significant effect of the lactation number, neither interaction of the tannin supplementation in diet and lactation number on values of basic parameters of cows metabolic profile. The addition of tannin in diets for cows in the second lactation had a positive effect on milk production, content and yield of milk proteins ($P<0.05$), while differences in the content and yield of milk fat and lactose were not significant. Results of the present study indicate that tannin supplement „Tanimil SCC“ may be successfully used in nutrition of lactating dairy cows, whereby added to the TMR in quantity of 40 g/cow/day probably has protective effects on rapidly degradable dietary protein and decreases their ruminal degradation. This improves protein utilization, having a positive effect on a significant reduction of blood urea and BHBA concentrations, and improving production performances.

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UČINEK PREHRANSKEGA DODATKA S TANINOM V PREHRANI KRAV SREDI LAKTACIJE NA PARAMETRE PRESNOVNEGA PROFILA IN NJIHOVE PROIZVODNE LASTNOSTI

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Povzetek: Cilj študije je bil proučiti učinek dodajanja tanina v obroku pri kravah molznicah sredi laktacije (90 ± 17 dni v mleku) in števila dojenj telet na nekatere parametre presnovnega profila. Poleg tega je bil ovrednoten tudi učinek dodajanja tanina na proizvodne lastnosti krav v drugi laktaciji. Raziskave so bile izvedene pri 80 kravah pasme Holstein, razdeljenih v dve skupini po 40 krav (kontrolna in poskusna skupina), ki so vključevale 16 krav v 2. in 12 v 3. in 4. laktaciji. Dodatek tanina Tanimil SCC (40 g/kravo/dan; 40 % taninov) je bil dodan v skupni mešani obrok (TMR) poskusne skupine, medtem ko je bila kontrolna skupina krav krmljena brez dodatka tanina. Poskus je trajal 60 dni. Krave molznice, hranjene s krmo z dodanimi tanini, so imele izrazito nižje vrednosti ($P < 0,05$) koncentracije ß-hidroksibutirata (0,79 v primerjavi z 0,96 mmol/L) in nižjo koncentracijo sečnine v krvi (5,89 v primerjavi z 6,93 mmol/L). Razlika v koncentraciji glukoze v krvi (3,92 v nasprotju z 3,89 mmol/L) ni bila statistično značilna. Dojenje ni značilno vplivalo na vrednosti parametrov presnovnega profila (koncentracije glukoze, ß-hidroksibutirata in sečnine). Medsebojni vpliv števila laktacij in dodatka tanina na vrednosti pregledanih parametrov presnovnega profila ni bil statistično značilen. Dopolnilo tanina v krmi krav v drugi laktaciji je imelo pozitiven učinek ($P < 0,05$) na mlečnost (4,42 %) in na količino mleka s popravljeno vrednostjo 4-odstotne maščobe (FCM, 5,24%). Uporaba tanina je izboljšala koncentracijo beljakovin (3,20 v primerjavi z 3,12%) in izkoristek mlečnih beljakovin (od 1,16 do 1,24 kg/dan) ($P < 0,05$). Dodatek tanina je izboljšal tudi koncentracijo in izkupiček mlečne maščobe ter koncentracijo laktoze. Dodatek Tanimila SCC kravam na sredini laktacije je pozitivno vplival na znižanje vsebnosti ß-hidroksibutirata in sečnine v krvi ter izboljšal produktivnost krav v drugi laktaciji.

Ključne besede: tanini; krave v laktaciji; prehrana; presnovni profil; mlečnost in sestava mleka

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Original Research Article

A MACROSCOPIC STUDY ON THE MUSCLES AND TENDONS OF FOREPAWS IN THE ANATOLIAN BOBCAT (*Lynx lynx*)

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Abstract: This study was performed on the tendons and muscles of two Anatolian bobcats (*Lynx lynx*). The research materials were donated by Republic of Turkey Ministry of the Forestry and Water Affairs Sivas Branch Manager. To achieve this objective, dissection and radiography were applied to two dead specimens. Digit I is radiographically rudimentary in the forepaw. The muscles and tendons of the forepaw in the bobcat are located in the cranial and caudal aspect of the antebrachium, the central manus and hypothenar region. The extensor muscles are situated on the cranial aspect of the antebrachium, while the flexor muscles are located on the caudal aspect of the antebrachium. The m. extensor carpi radialis in the extensor group has long and short portions; the m. extensor digitorum lateralis et communis give rise to tendons associated with fingers; the fascia covering the muscles emerges on the lateral and medial border of the m. extensor digitorum lateralis. The m. flexor digitorum superficialis et profundus lie on the caudal aspect of the antebrachium; the superficial digital flexor tendons give rise to the origin of the bellies of the m. flexor digitorum brevis; the m. flexor digitorum profundus form a stout tendon from which the mm. lumbricales originate on the palmar aspect of carpal joints; the tendon of the muscle perforates the tendon of the m. flexor digitorum superficialis. The mm. flexores digitorum profundi breves in the central manus group consist of the lateral and medial portions and insert on the palmar aspect of each proximal phalanx associated with the fingers. Other muscles in the group, adductores digitorum, end to those in the fingers.

Key words: Anatolian bobcat (*Lynx lynx*); forepaw; muscles; tendons; anatomy

Introduction

The bobcat (*Lynx lynx*), which is an endangered species and a medium-sized cat with a short tail, is a predatory carnivorous animal (1, 2). While the carnivore hind limb primarily serves as a locomotor organ, the forelimb may also be involved in both capturing and subduing prey (3, 4, 5). In felids, forelimbs are particularly used to hold onto and position the struggling prey, though small prey specialists make less use of forelimbs in subduing prey. Therefore, the myology of the forelimbs of cats reflects ecomorphological history as well as adaptations to behavior (6).

In terms of insertion, the muscles related to the forepaw in the cranial compartment of the antebrachium are m. extensor carpi radialis longus et brevis, m. extensor carpi ulnaris, m. extensor communis, m. extensor digitorum lateralis, m. extensor pollicis longus, and m. abductor pollicis longus (7, 8). While the functions of m. extensor carpi radialis longus et brevis, and m. extensor carpi ulnaris are involved in the carpal joints, the others affect the digital joints. Forepaw muscles and tendons in the manus can be divided into hypothenar, superficial, and central compartments of the manus (8).

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M. extensor carpi radialis longus originates from the lateral supracondylar ridge of the humerus in the felid (8, 9, 10). After the muscle gives rise to a flat tendon in the distal third of the antebrachium, it passes through the common extensor retinaculum (8) and inserts onto the proximal shaft of the second metacarpal (8, 9, 10).

Like the m. extensor carpi radialis longus, the m. extensor carpi radialis brevis springs from the lateral supracondylar ridge of the humerus in the felis family. The muscle becomes a tendon in the distal quarter of the antebrachium that passes through the common extensor retinaculum before inserting into the third metacarpal bone (8, 9, 10, 11, 12).

The m. extensor carpi ulnaris originates from the epicondyle of the humerus in carnivores (8, 9, 10, 11, 12). The tendon of the muscle passes laterally over the carpus to end on the fifth metacarpal bone (8, 9, 10, 11, 12). In addition, the tendon inserts in both the pisiform bone and its ligament in ocelots (8), while another branch of the tendon radiates into the flexor retinaculum in the cat (11).

It was established that the origin of m. extensor digitorum lateralis was the lateral supracondylar bridge of the humerus in the ocelot (8) and cats (10). The muscle that has three bellies (8, 9, 10, 11, 12) at the level carpal joint becomes a different number (three or four) of tendons in the carnivores. The tendons pass over the carpal joint and then fuse with the m. extensor digitorium communis III-V digits in the carnivores (8, 9, 10, 11, 12).

The m. extensor digitorum communis with a different number of bellies arises from different areas of the humerus, for example, the lateral supracondylar bridge and epicondyle, in the carnivores (8, 9, 10, 11). After the muscle bellies become tendons serving digits II-V at the distal third of the radius (9), the tendons pass through the shared retinaculum on the carpus joint in the carnivores (8, 9, 10, 11).

The m. extensor digiti I (pollicis) longus is only found in carnivores (10) and originates from the distal third of the ulna, covering the tendon of the m. extensor digitorum lateralis, deep into the extensor tendons in the cats (8, 9, 10, 11, 12). In the cat family, its tendon gives rise to two slender branches ending at the first and second digits (8, 9, 10, 11, 12). A third branch that inserts into the third digit may exist in the cat family (8, 9).

The m. abductor digiti I (pollicis) longus that acts as the abductor for the first digit in the carnivores (10) arises from the lateral aspects of the ulna and radius and travels distally and medially to pass superficially into the tendon of the m. extensor carpi radialis in the carnivores. The muscle ends at the base of metacarpal I in the cat family (8, 9, 10, 11, 12).

The muscles of the cranial compartment of the antebrachium that act as forepaw joints comprise the m. flexor carpi radialis et ulnaris, m. flexor digitorum superficialis et profundus, and m. palmaris longus (8, 9, 10, 11, 12).

The m. flexor carpi radialis arises from the medial epicondyle of the humerus in carnivores (8, 9, 10, 11). Its tendon passes over the flexor side of the carpus to end at the palmar aspect of the third and second metacarpal bone in carnivores (8, 9, 10, 11).

The m. flexor carpi ulnaris consists of the ulnar and humeral heads. The caput ulnare arises from the olecranon process of the ulna, while the caput humerale originates from the medial epicondyle of the humerus (8, 9, 10, 11, 12). In the right limb of the ocelot, Julik et al. (8) stated that the caput ulnare consists of the distal and proximal bellies. In carnivores (9, 10, 11), both heads end with a common tendon that is inserted into the accessory carpal bone, while in ocelots (8), both portions pass through a shared flexor retinaculum on the palmar aspect of the carpus to end at the pisiform carpal bone.

The m. flexor digitorum superficialis stems from the medial epicondyle of the humerus in carnivores (9, 10, 11, 12), while the muscle that has three bellies serving digits II–IV emerges from the superficial aspect of the m. flexor digitorum profundus in ocelots (8). In the cat family, the tendon of the muscle that becomes tendinous at the level of the carpus splits into five portions serving digits I–V in the proximal third of the metacarpal bone (9, 10, 11). In the cat family, all of the tendons pass deep into the proximal annular ligament of the digit they serve and divide to allow for the passage called the manica flexoria of the m. flexor digitorum profundus (8, 9, 10, 11, 12). Each of the tendons end at the proximal border of the middle phalanx of the first to fifth digits in the cats (9, 10, 11).

The m. flexor digitorum profundus is composed of three separable heads in carnivores (9, 10, 11, 12) or five heads in the ocelot (Julik et al., 2012). In carnivores (9, 10, 11), the muscle heads are

called caput humerale, radiale, and ulnare, while in the ocelot (8), the heads are named caput humerale laterale, mediale and profundus, caput ulnare, and capita radiale. The caput humerale that originates from the medial epicondyle of the humerus is composed of three distinguishable bellies and the strongest head in the cat (9, 10, 11). The caput ulnare originates from the caudal border of the ulna and olecranon in the cats (9, 10, 11, 12). All the heads of the muscle are joined together to form the strongest tendon just proximal to the carpus in the cat family (8, 9, 10, 12). The conjoined tendon courses through a shared flexor retinaculum and continues distally to serve all five digits. In the proximal metacarpal region, when it detaches a thin tendon to the first digit, the main tendon divides into four powerful limbs for digits II–V in the cat family. They are enveloped by the tube-like cuff of the m. flexor digitorum superficialis, called the manica flexoria, under the metacarpal pad in the cats. After the tendons initially pass through the proximal annular ligament and then the distal annular ligament at the proximal phalanx level of the digits served, they insert into the palmar aspects of the distal phalanx digits (8, 9, 10, 11, 13).

The m. palmaris brevis located in the superficial compartment of the manus originates from the lateral side of the flexor retinaculum. It then run transversely to insert on the metacarpal paw pad in the ocelot (8).

The m. flexor digitorum brevis that is shaped like a cone in cats (11) is composed of two superficial bellies and one deep belly in the ocelot (8). The superficial belly springs from the deep and caudal aspect of the flexor retinaculum, while the deep belly originates from the deep lateral aspect of the tendon of the m. palmaris longus in the distal quarter of the antebrachium. Two superficial bellies fuse and give rise to a shared tendon to digit V, while the deep belly gives rise to a tendon to digit IV (8).

The m. abductor digiti V belonging to the hypothenar compartment of the manus originates from the pisiform in the ocelot (8), while the muscle arises from the accessory carpal bone in cats (11). In both cats and ocelots, the muscle inserts into the base of the phalanx proximalis of digit V (8, 11).

The muscles of the central compartment of the manus are mm. lumbricales, mm. adductors digitorum, and mm. flexores profundi breves. Mm. lumbricales are composed of four distinct bellies serving digits II–V. The bellies originate from the common tendon of the m. flexor digitorum profundus and end at the base of the phalanx proximalis of their respective digits (8, 11).

The mm. adductores digitorum serving digits I, II, and V arises from the transverse carpal ligament, and each muscle inserts onto the base of the phalanx proximalis of its respective digits in the cat family (8, 11).

The mm. flexores profundi breves, except digit I, consist of a medial and lateral belly serving each digit; on the first digit, only a medial belly is present. The bellies of the muscle stem from the transverse carpal ligaments (8).

Because anatomical descriptions concerning the muscles and tendons of the paw in the Anatolian bobcat have not been found, in the present study, we aimed to describe a detailed macroanatomy of the muscles and tendons of the paw in the Anatolian bobcat.

Materials and methods

The study was conducted on two female Anatolian bobcats (cadaver I was 6.9 kg and cadaver II was 7.6 kg in weight, respectively). The animals were obtained from Republic of Turkey Ministry of the Forestry and Water Affairs Sivas Branch Manager. After the animals died of natural causes (14), they were immediately transported to Department of Anatomy of Faculty of Veterinary Medicine at Cumhuriyet University. The animals were fixed with 10% formalin via the carotid common artery to prepare the cadavers. Then, the forelimbs of the cadavers were dissected and photographed with a Canon 50D camera. Radiographic images of manus were taken in dorso-palmar position using PCMAX-100H® (led: input voltage: 3.3 VDC, 2A: Korea). The terminology used in the manuscript is in accordance with the prevailing Nomina Anatomica Veterinaria (15).

Results

The forepaw of first cadaver was 132.84 mm long and 42.33 mm wide, while measurement of other cadavers was 130.20x41.55. Each paw consisted of the first, second, third, fourth, and fifth fingers (Fig. 2F). The first finger, called the dewclaw, was rudimental and composed of the first metacarpal, the proximal and distal phalanx bones (Fig. 2F/I).

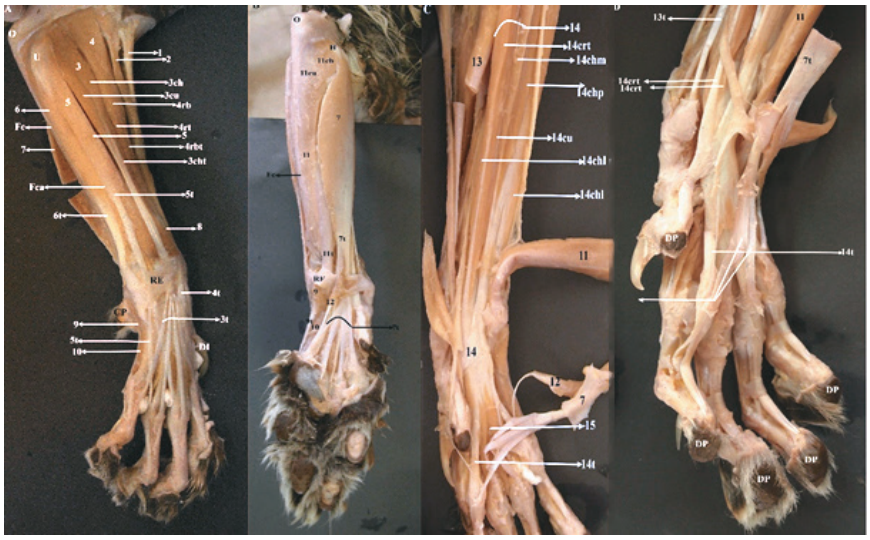

Figure 1: A-The cranial aspect of the antebrachium and manus B- The superficial caudal aspect of the antebrachium and manus C- The deep caudal aspect of the antebrachium and manus D- The deep palmar aspect of the manus

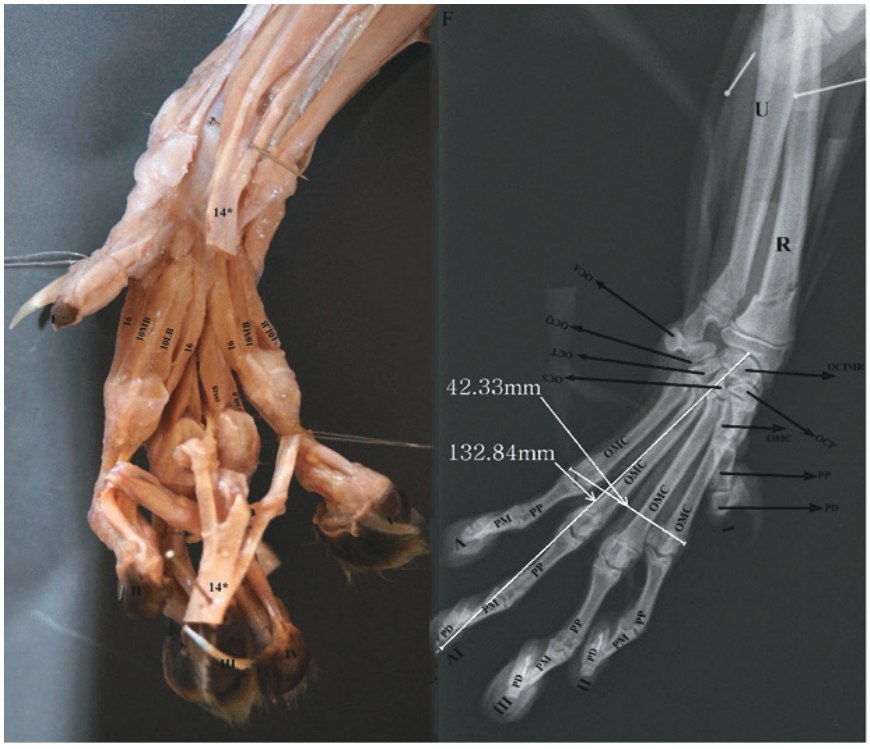

Figure 2: E- The deep palmar aspect of the fingers F- The radiography of the manus

The m. brachioradialis located on the cranial border of the forearm and beneath the skin originated from the caudal aspect of the distal humeral shaft as broad and fan-shaped in the two cadavers (Fig. 1A/2). The muscle that got thinner at the cranial aspect of the elbow joint travelled distally on the cranial aspect of the antebrachium. After it passed over the m. abductor digiti I longus at the level of the distal quarter of the antebrachium, the m. brachioradialis ended on the dorsal aspect of the radial carpal bone.

The m. extensor carpi radial longus that is fused with the m. extensor carpi radial brevis originated from the lateral supracondylar ridge of the distal humerus in both cadavers (Fig. 1A/4rl). In the two cadavers, both muscles distally continued between the m. brachioradialis and the m. extensor digitorum communis on craniolateral aspect of the forearm. While the m. extensor carpi radialis brevis (Fig. 1A/4rb) gave rise to a thin tendon at the mid-antebrachium, the m. extensor carpi radialis longus distally extended,

DI. The first finger
O. The olecranon
U. The ulna
I. The first finger
II. The second finger
III. The third finger
IV. The fourth finger
V. The fifth finger
CP. The carpal pad
RF. The retinaculum extensorum
RF. The retinaculum flexorum
MCP. The metacarpal pad
H. The humerus
R. The radial bone
OCA. The accessory carpal bone
OCIM. The intermedioradial bone
OCP. The first carpal bone
OCS. The second carpal bone
OCT. The third carpal bone
OCQ. The fourth carpal bone
OMC. The metacarpal bone
PP. The proximalis phalanx bone
PM. The second phalanx bone
PD. The distal phalanx bone
DP. The digital pad
dal. The distal annular ligament
pal . The proximal annular ligament
Fc. Fascia

1. N. musculocutaneus
2. The brachioradial muscle
3. The common digital extensor muscle (CDEM)
   3ch. The humeral head of the CDEM
   3cu. The ulnar head of the CDEM
   3cht. The humeral head tendon of the CDEM
   3cut. The ulnar head tendon of the CDEM
4. The radial extensor of the manus (REM)
   4rb. The radial extensor brevis of the manus (REBM)
   4rbt. The tendon of the REBM
   4rl. The radial extensor longus of the manus
5. The lateral digital extensor muscle (LDEM)
   5t. The tendon of the LDEM
6. The ulnar flexor of the manus (UFM)
   6t. The tendon of the UFM
7. The superficial digital flexor muscle (SDFM)
8. The adductor digiti I muscle
9. The abductor digiti V muscle (ADVM)
   9t. The tendon of the ADVM
10. The flexor brevis profundus muscle (FBPM)
    10MB. The medial belly of the FBPM
    10LB. The lateral belly of the FBPM
    10t. The tendon of the FBPM
11. The ulnar flexor of the manus (UFM)
    11ch. The humeral head of the UFM
    11cu. The ulnar head of the UFM
    11t. The tendon of the UFM
12. The flexor digital brevis manus (FDBM)
13. The radial flexor of the manus (RFM)
14. The deep digital flexor muscle (DGFM)
    14chm. The humeral medial head of the DGFM
    14chp. The profound humeral head of the DGFM
    14chpt. The profound humeral head tendon of the DGFM
    14crt. The radial head of the DGFM
    14chl. The lateral humeral head of the DGFM
    14cu. The ulnar head of the DGFM
    14cut. The ulnar head of the DGFM
15. The lumbricalis muscles
16. The adductor digital muscles

then entered deep into the m. abductor digiti I longus at the distal quarter of the antebrachium and divided into two tendons. The tendons passed under a common extensor retinaculum on the dorsal carpus. While the intertion of the thin tendon was at the dorsal aspect of the base of the second metacarpal bone, the stout tendon inserted onto the dorsomedial aspects of the base of the third metacarpal bone in the two cadavers.

The m. extensor carpi ulnaris located at the caudolateral positon in the forearm and enclosed fascia, which emerged from the caudal border of the m. extensor digitorum lateralis, originated from the lateral epicondyle of the humerus in the two cadavers (Fig. 1A/6). The muscle that runs along the caudal border of the lateral digit gave rise to a tendon (Fig. 1A/6t) in the distal quarter of the antebrachium that passed through the retinaculum extensorium in the two cadavers (Fig. 1A/RE). The tendon inserted into the dorsal aspect of the basis of the fourth metacarpal bone.

The m. extensor digitorum lateralis situated in the lateral position in the forearm stemmed from the lateral aspect of the olecranon and from the lateral supracondylar bridge of the distal humerus in the two cadavers (Fig. 1A/5). The muscle travelled distally and then gave rise to a tendon (Fig. 1A/5t) in the mid-antebrachium that passed through the extensor retinaculum on the dorsolateral aspect of the manus. In its course, the fasciae (Fig. 1A/Fc) originate from both the cranial and the caudal border of the muscle. After the course under the retinaculum, the tendon divided into three branches on the dorsal aspect of the fourth and third metacarpal bones in both cadavers (Fig. 1A/5T IV–III). The medial branch run in the distomedial direction on the dorsal aspect of the metacarpal bone and under the common digital extensor tendon to reached the dorsal aspect of the fetlock joint. The branch inserted on the dorsal aspect of the middle phalanx of the third finger in the two cadavers (Fig. 1A/5tiii). The middle branch travelled in the distomedial direction to arrived at the lateral aspect of the fetlock joint in the fourth finger. Then, the branch inserted on the middle phalanx in the fourth finger (Fig. 1A/5iv). The lateral branch, after origin, continued along the caudal border of the fifth metacarpal bone to reached the dorsal aspect of the fetlock joint in the fifth finger. The branch ended at the middle phalanx of the fifth finger in both cadavers.

The m. extensor digitorum communis was composed of three bellies originated from the humerus (caput humerale; Fig. 1A/3ch), the ulna (caput ulnare; Fig. 1A/3u), and the olecranon (capita olecranon) in the two cadavers. The capita olecranon that was the weakest belly emerged from the lateral aspect of the olecranon and then passed under its origin at the lateral digital extensor muscle in both cadavers. The muscle belly fused with the caput humerale in the proximal antebrachium. The humeral head arose from the lateral supracondylar bridge and epicondylus of the humerus to reached the cranial aspect of the elbow joint in the two cadavers (Fig. 1A/3ch). The belly that was covered by the fascia originated from the m. extensor digitorum lateralis in the proximal antebrachium and travelled distally on the cranial aspect in the proximal antebrachium. The caudal portion of the belly became a tendon in the mid-antebrachium, while the cranial portion of the belly became a tendon in the distal radius (Fig. 1A/3cht). The caput ulnare arose from the lateral aspect of the ulna and then run distally between the m. extensor digitorum lateralis and m. extensor carpi radialis longus in the lateral aspect in the antebrachium to turned into a tendon in the proximal antebrachium (Fig. 1A/cut). The tendons of the bellies that were covered by the fascia in the lateral aspect of the forearm, the latter extensor retinaculum in the dorsal aspect of the carpal joint, continue distally to reached the base of the third metacarpal bone in each cadaver (Fig. 1A/3hiii). The common tendon divided into four tendons that served each digit (II–V) at the base of the third metacarpal bone. Each tendon branch run distally along the dorsal aspect of the metacarpal bone to end at the extensor process of the distal phalanx associated with digits II–V (Fig. 1A/3chii-iii-iv-v). During this course, each tendon made the expansion on the dorsal aspect of both the fetlock joint and the proximal interdigital joint associated with the digits.

The m. abductor pollicis originated from the shaft of the radius and ulna in the distal half of the antebrachium in both cadavers. It crossed obliquely in the craniodistal position in the distal quarter of the antebrachium to arrived at the extensor retinaculum (Fig. 1A/8). The muscle that covered the extensor retinaculum in the dorsal aspect of the carpal joint passed between the tendons of the m. extensor digitorum communis and m. extensor carpi radialis longus to become a tendon in the two cadavers (Fig. 1A/8). Its tendon inserted onto the dorsal aspect of the first carpal bone and the base of the first metacarpal bone.

The m. flexor carpi ulnaris composed of two bellies (caput humerale (Fig. 1B/11ch) and ulnare (Fig. 1B/11cu) located on the caudal aspect of the antebrachium. The caput ulnare that was thinner than the caput humerale arose from the caudomedial aspect of the olecranon and travelled along the caudal border of the caput humerale. The muscle got thinner and thinner and became a thin tendon situated caudally to the caput humerale in the mid-antebrachium (Fig1B/11cu). The tendon continued distally to join with the tendon of the caput humerale deep into the carpal pad in the two cadavers (Fig. 1B/11cu). The strongest caput humerale originated from the medial epicondyle of the humerus between the origin of the caput ulnare and the m. flexor digitorum superficialis and became the strongest tendon (Fig. 1B/11cht) in the distal quarter of the antebrachium in the two cadavers. The tendon fused with the tendon of

another head passed through the retinaculum on the lateral side of the palmar carpus and ended at the accessory carpal bone in both cadavers (Fig. 1B/11t).

The m. flexor carpi radialis (Fig. 1B/13) situated on the caudal aspect of the antebrachium, just under the skin and fascia, arose from the medial epicondyle of the humerus. The muscle covered by fascia continued distally in the groove located cranially to the caput humerale of the m. flexor digitorum profundus to gave rise to a tendon on the distal third of the antebrachium in the two cadavers. Then, the tendon passed through a shared retinaculum on the medial side of the palmar manus to insert on the base of the third metacarpal bone in both cadavers (Fig. 1B/13t).

The m. flexor digitorum superficialis covered by the fascia originated from the caudal border of the m. flexor carpi radialis and arose from the medial epicondyle of the humerus (Fig. 1B/7). The muscle located under the skin travelled distally on the caudal aspect of the forearm and became the strongest tendon in the mid-antebrachium in the two cadavers (Fig. 1B/7t). In the distal quarter of the antebrachium, this tendon gave rise to a thin branch serving the carpal pad and the superficial bellies of the m. flexor digitorum brevis, then passed through the common retinaculum in the palmar aspect of the manus to divided into five branches serving each associated digit in the two cadavers (Fig. 1B/7t1-v). A slender branch served the first digit originated from the tendon of the m. flexor digitorum superficialis and inserts on the base of the proximal phalanx in the first digit (Fig. 1B/11t1). Another four branches travelled under the metacarpal pad in the palmar aspect of the forepaw ended at the palmar aspect at the base of the phalanx secunda associated with the digits. Each branch was perforated by the branch of the profound digital flexor tendon at the level of the insertion.

The superficial bellies of the m. flexor digitorum brevis originated from the tendon of the m. flexor digitorum superficialis in the distal quarter of the antebrachium, while the profound bellies arose from the retinaculum flexorum in the palmar aspect of the carpal joint (Fig. 1B/12). The superficial bellies with the tendon of the m. flexor digitorum superficialis passed under the laid retinaculum flexorum and turned into a tendon deep in the metacarpal pad. The tendon laid distally under the metacarpal pad to the palmar aspect of the metacarpal bones IV–V (Fig. 1B/12).

The m. flexor digitorum profundus arose from the distinct area as five heads in the two cadavers (Fig. 1C/14). The heads of the m. flexor digitorum profundus laid distally on the mediocaudal aspect of the antebrachium and inserted deep into the m. flexor digitorum superficialis and became a stout tendon (Fig. 1C/14*) (the common tendon of the heads of m. flexor digitorum profundus) under the metacarpal pad. The belly that originated from the medial border in the proximal third of the radius and called the capita radiale (Fig. 1C/14cr) travelled distally and became a tendon (Fig. 1C/14rdt) in the distal quarter of the antebrachium. The tendon passed through the flexor retinaculum in the palmar aspect of the carpal joint to contributed to the tendon of the m. flexor digitorum profundus under the metacarpal pad. The caput ulnare (Fig. 1C/14cu) arose from the caudal aspect of the proximal ulna, and the muscle laid distally on the caudal aspect of the interosseous membrane. The muscle's superficial portion became a tendon that contributed to the common tendon of the m. flexor digitorum profundus at the level of the mid-antebrachium, while the muscle's profound portion directly inserted on the common tendon of both the right and left paws in the two cadavers. The caput humerale mediale (Fig. 1C/14chm) that originated from the medial epicondyle by a wide tendon (Fig. 1C/14chmt) run distally on the caudal aspect of the antebrachium, deeps into the m. flexor digitorum superficialis, to became a tendon in the distal quarter of the antebrachium. Then, the tendon passed under the flexor retinaculum in the palmar aspect of the carpal joint to contributed to the common tendon of the m. flexor digitorum profundus in both limbs (Fig. 1C/14chmt). The caput humerale laterale (Fig. 1C/14cml) that originated from the medial epicondyle of the humerus in the two heads turned into a tendon (Fig. 1C/14chlt) in the mid-antebrachium, deep into the m. flexor digitorum superficialis in the two cadavers (Fig. 1C/14chlt). The caput humerale profundus (Fig. 1C/14chp) situated in the deepest originates from the medial epicondyle of the humerus and then became a tendon (Fig. 1C/14chpt) in the distal third of the antebrachium. After passed under the flexor retinaculum, the tendon ended to contribute to the common tendon of the m. flexor digitorum profundus in both limbs (Fig. 1C/14*). The common tendon of the m. flexor digitorum profundus laid deep in the metacarpal pad in the carpal tunnel and firstly gave rise to slender a tendon in

the first finger in the mid-metacarpal bone (Fig. 1C/14t1). The common tendon broke off into four branches (II, III, IV, V) (Fig. 1C/14t1-v) to each finger deep in the metacarpal pad in the two cadavers. Each tendon branch laid distally in the palmar aspect of the metacarpal bone associated with the finger to initially passed through the proximal annular ligament in the palmar aspect of the fetlock (Fig. 1C/pal); shortly after, it passed through the superficial digital flexor tendon, and finally, the distal annular ligament (Fig. 1C/dal) in the mid-proximal phalanx, ending at the muscular tubercle of the distal phalanx (Fig. 1C/14t1-v).

The m. abductor digiti V that arose from the palmar aspect of the accessory carpal bone and had a triangle shape turned into a tendon in the mid-first metacarpal bone in the two cadavers (Fig. 1B/9). Then, the tendon laid distally on the lateral aspect of the first finger to inserted onto the palmar aspect of the proximal phalanx of the first finger (Fig. 1B/9t).

The mm. lumbricales situated on the palmar aspect of the profound digital flexor tendon and under the superficial digital flexor tendon arose from the profound digital flexor tendon as four bellies at the metacarpal pad (Fig. 1C/15). Then, the bellies descended distally deep into the metacarpal pad to became a tendon in the palmar aspect of the fetlock joint. Each tendon ended at the palmar aspect of the bases of metacarpal bones II–IV.

The mm. flexores digitorum profundi breves composed of five distinct muscles originated from the distal row of carpal bones and laid distally on the palmar aspect of the metacarpal bone in each finger to ended at the palmar aspect of the base of the proximal phalanx (Fig. 2E/10LB-MB). Each muscle consisted of lateral and medial bellies. The weakest of the mm. flexores digitorum profundi breves was the m. flexor digiti I brevis (Fig. 2E/10LB-MB).

The mm. adductores originated from the distal row of carpal bones and run distally firstly to the palmar aspect and finally to the interdigital aspect of each metacarpal bone associated with the finger to inserted onto the interdigital aspect of the proximal phalanx in each finger (Fig. 2E/16).

Discussion

The muscles and their tendons acting on the digital and carpal joints in the forelimb's paw, as described by Julik et al. (8), are sorted as located in the cranial and caudal compartments of the antebrachium and, the superficial, central and hypothenar compartments of the manus. As has been described in previous literature (8, 9, 10, 11, 12), it was observed that extensor group muscles are located in the cranial aspect of the antebrachium. In this study, the origin of the radial extensor muscles of the manus is similar to that in carnivores, as depicted in the literature (8, 9, 10, 11), but the insertion tendon of the long portion of the muscle ending at the second metacarpal bone is different from what was described in the literature on carnivores (8, 9, 10, 11, 12). It was previously described that the origin of the m. extensor digitorum lateralis was the lateral supracondylar bridge of the humerus and the superficial aspect of the supinator muscle in the ocelot (8). However, in the present study, the origin of the muscle has been observed to be the lateral supracondylar bridge of the humerus and the lateral aspect of the olecranon. The finding that the fascia originate from both the cranial and caudal border of the muscle was firstly obtained in the present study. As was described in the ocelot (8), it has been seen that the muscles turn into a stout tendon in the mid-antebrachium and pass under the extensor retinaculum in the dorsal aspect of the carpal joints. However, it has been revealed that the tendon of the muscle divides into three branches, and each branch inserts onto the dorsal aspect of the second phalanx of the third, fourth, and fifth fingers in the Anatolian bobcat. Although it has been reported that the m. extensor digitorum communis arises from a different area of the humerus in carnivores (8, 9, 10, 11, 12), in the present research, it was found that the muscle originates from the ulna, olecranon, and the lateral supracondylar bridge as three heads. In addition, in this study, it was observed that the ulnar head turns into a tendon in the proximal antebrachium. While becoming a tendon of the cranial portion of the humeral head is the distal radius, those of the caudal portion of the humeral head is in the mid-antebrachium in the Anatolian bobcat. As was described in the ocelot (8), the tendon of the muscle passes under the common extensor retinaculum and divides into four branches for each finger (II–V) in the research. It has been seen that each branch enlarges in the dorsal aspect of fetlock and the proximal interdigital joint and ends at the extensor process of the distal phalanx of the associated finger.

In this study, as described in the literature (8, 9, 10, 11, 12), it has been seen that the flexor muscle group of the forearm situated at the caudal aspect of the antebrachium and their tendons act on the forepaw in the Anatolian bobcat. In this study, it was found that the m. flexor carpi ulnaris arises from both the olecranon and the medial epicondyle of the humerus as two heads, as depicted in the research (8, 9, 10, 11). In the Anatolian bobcat, it has been seen that the region in which the caput ulnare turns into a tendon is in the mid-antebrachium, while for the caput humerale, it is in the distal quarter of the antebrachium. In the study, the findings of the muscle insertion are similar to those described in the literature on carnivores (8, 9, 10, 11). The origin and insertion of the m. flexor carpi radialis in the Anatolian bobcat are similar to what has been reported in the literature. However, in this study, it was observed that the muscle turns into tendon in the distal third of the antebrachium. The findings about the origin and location of the m. flexor digitorum superficialis obtained in this study are similar to the data given in the literature about carnivores (8, 9, 10, 11). However, Julik et al. (8) called the muscle the palmaris longus in the ocelot. As described by many authors (8, 9, 10, 11), it has been found that the tendon of the muscle divides into five branches in the palmar aspect of the carpal joints in the Anatolian bobcat. However, in this study, it was observed that the tendon of the muscle gives rise to the superficial portion of the m. flexor digitorum brevis and a thin tendon serving the carpal pad. In addition, each tendon associated with the fingers is perforated by a deep digital flexor tendon at the level of the palmar aspect of the base of the phalanx media in the Anatolian bobcat. As previously depicted by Julik et al. (8), the m. flexor digitorum profundus in five separate heads arises from the distal humerus, the proximal part of the ulna, and the radius in the Anatolian bobcat. In this study, as in the investigation by Julik et al. (8) in the ocelot, the heads have been named as the radial, ulnar and deep, medial, and lateral humeral heads in the Anatolian bobcat. In this study, it was observed that a stout tendon forms to unite all heads in the palmar aspect of the antebrachium. The tendon gives rise not only to the tendon branch for the muscular tubercle of the phalanx distalis, but also to the mm. lumbricalis in each finger, except the first finger, in the Anatolian bobcat. Furthermore, in this study, it was found that each tendon associated with each finger passes through both the superficial digital flexor tendon and proximal and distal annular ligament related to each finger. The findings about the mm. lumbricalis and its tendons obtained from this study are similar to the data expressed by Julik et al. (8) in ocelots.

The evidence related to the origin and insertion of the central muscles of the manus observed in this study is similar to the outcomes described by Julik et al. (8) for the ocelot. However, in the present study, it was seen that each mm. flexores digitorum profundi breves consists of lateral and medial bellies.

In conclusion, our investigation showed that the fasciae, muscles, and tendons of the forepaw are well-developed in the Anatolian bobcat. In particular, the m. flexor digitorum pprofundus and its stout tendon both work as flexor functions of fingers and give rise the mm. lumbricales. In addition, the flexor muscles in the palmar aspect of the antebrachium, its tendons, and the muscles of the central manus play a role not only in the organs of the locomotor system, but also in subduing struggling prey for the Anatolian bobcat. The muscle and tendon structures of the forepaw may be advantageous for the hunting and survival of the Anatolian bobcat.

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Authors declare that there is no conflict of interests regarding the publication of this article.

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MAKROSKOPSKA ŠTUDIJA KIT IN MIŠIC SPREDNJE ŠAPE PRI RISU (*Lynx lynx*)

H. H. Ari, İ. Yurdakul, G. Aksoy

Povzetek: V raziskavi smo proučili kite in mišice sprednje šape pri dveh risih (*Lynx lynx*). Material za raziskavo je darovalo Ministrstvo za gozdarstvo in vode Turčije. Na dveh kadavrih je bila izvedena radiografija in sekcija. Radiografija je pokazala, da je prvi prst sprednje šape rudimentaren. Mišice in kite sprednje šape se pri risu nahajajo v kranialnem in kavdalnem področju podlahti, centralnega dela dlani in na področju mišic, ki premikajo 5. prst. Mišice iztegovalke se nahajajo na sprednjem delu podlahti, medtem ko so mišice upogibalke nameščene na kavdalnem področju podlahti. Ena izmed mišic iztegovalk *m. extensor* carpi radialis ima dolge in kratke odseke; iz mišice *m. extensor digitorum lateralis et communis* izhajajo kite za prste; fascija, ki pokriva mišice pa se začne na lateralnem in medialnem področju mišice *m. extensor digitorum lateralis*. Mišica *m. flexor digitorum superficialis et profundus* leži na kavdalnem področju podlahti; iz površinskih tetiv digitalnega fleksorja pa izhaja trebuh mišice *m. flexor digitorum brevis*; *m. flexor digitorum profundus* tvori močno tetivo, iz katere *mm. lumbricales* izvirajo na palmarnem področju karpalnih sklepov. Tetiva mišice perforira tetivo mišice *m. flexor digitorum superficialis*. Mišice *mm. flexores digitorum profundi* v osrednji skupini mišic področja dlani so sestavljene iz lateralnih in medialnih delov in se nahajajo na palmarnem področju vsake proksimalne prstnice in so povezane s prsti. Druge mišice v skupini *mm. adductores digitorum*, se končajo na koncih istih prstov.

Ključne besede: ris (*Lynx lynx*); sprednja šapa; mišice; kite; anatomija

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Original Research Article

THE INFLUENCE OF DIFFERENT MOLECULAR WEIGHT SEMINAL PLASMA PROTEIN CONTENT ON SOME FERTILITY PARAMETERS IN BOAR'S EJACULATES

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Abstract: The aim of this study was to investigate the effect of different percentage of seminal plasma proteins with different molecular weight on sperm motility and fertility parameters (farrowing rate (FR), number of live-born pigs (PBA) per litter and percentage of unsuccessful insemination). A total of 50 sperm-rich ejaculate fractions were collected (one per boar) using the gloved hand method. The quality parameters of the semen samples were first evaluated at the farm. Further assessment of sperm quality was performed on a CASA - computer assisted semen analysis by two competent operators. Seminal plasma protein fractions were obtained by AOAC - Association of Official Analytical Chemists as a chemical method. The assessment of reproductive performance was carried out based on collected data of three parameters in selected 9696 sows: FR, PBA per litter and percentage of unsuccessful insemination. Protein fractions were divided in to three groups (10 – 20kDa, 21 – 30kDa and 31 – 40kDa) Proteins with 10 – 20kDa did not have significant effect and correlation with analyzed parameters. Significant differences were recorded in farrowing rate between samples with up to 80 % compared to samples with 10% of proteins with 21 – 30kDa. Significant differences were recorded in unsuccessful insemination between samples with different percentage of proteins with 31 – 40kDa. Results of this study have shown the effect of different percentage of certain fraction of seminal plasma proteins on boar ejaculates fertility potential.

Key words: boars; semen quality; seminal plasma proteins; reproductive results

Introduction

The main reason for using the technique of artificial insemination (AI) is to increase reproductive efficiency and rates of genetic improvement in pig industry. Obtaining good reproductive material for AI includes continuous improvements that follow new technologies in the assessment of good quality semen which have proved that impact on farrowing rate. Farrowing rate in sows and gilts is an important data and commonly used for measure of reproductive performance. The primary determinants of boar fertility that represents the measured progress in pig production are the quantity and quality of semen. Since there is no established gold standard in assessment of good quality semen, prediction of the fertilizing potential is based on objective analysis of semen quality parameters that includes good boar selection and relations with fertility rate data that reflects the reproductive performance. Knowledge of the physiological characteristics of semen, seminal plasma, spermatozoa and the influence of breed, lines and individual boar characteristics are prerequisites for the successful

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improvement of reproductive efficiency and for inheritance of the most desirable semen genotype traits (22).

Boar semen is ejaculated in sequential fractions with a large volume of seminal plasma and the relatively diluted sperm concentration. Seminal plasma is a complex mixture of secretions from testis, epididymis and male accessory glands that may affect the semen quality. An important physiological function of seminal plasma is to carry out spermatozoa through the female reproductive tract, nourishing the sperm, and what is the most important, seminal plasma is directly involved in sperm capacitation and fertilization. This was also supported by the recent observations where the interactions between sperm viability and semen plasma presence or absence were detected during semen storage (2). Therefore, conventional semen evaluation generally includes seminal volume, sperm cell concentration and percentage of motile, viable and morphologically normal spermatozoa, are not sufficient indicators that accurately predict the fertility rate variations among the breeding males (1). Subsequently, the evaluation of semen quality is also directed toward other semen components and seminal plasma as useful predictors of fertility. Given the fact that seminal plasma represents composite secretion, many authors emphasize the importance of seminal plasma protein content since the protein composition could affect the spermatozoa function and this could be considered as valuable biomarker of boar fertility (3, 1, 4, 5) . The SP proteins cover and protect the spermatozoa during ejaculation and the higher protein concentration results in the better protection and preservation of spermatozoa cell membrane stability (21) .

Therefore, recent interest of investigation is pointed at extensive proteomic analysis of boar seminal plasma in order to provide the major characterization of the boar seminal plasma proteins as a first step for further understanding the role of proteins in reproductive outcomes as well as the identification of biomarkers for sperm quality and fertility. Namely, *Perez–Patino et al.,* 2016 (6) found decrypting of the boar seminal plasma proteins challenging since most total protein mass is taken up by a few number of low molecular weight proteins. In order to overcome the limitations, these authors suggested improving the accuracy of later analysis in fractionation of protein according their molecular weight. Using SDS-PAGE electrophoresis, *Karunakaran et al 2016* (7) reported a total of 11 proteins bands in porcine seminal plasma ranging with the molecular weight from 14 – 200kDa, of which, proteins with higher molecular weight were more abundant compared to proteins with lower molecular weight. *Duart and coworkers 2013* (8) reported that the proportion of proteins with molecular weight below 25kDa prevail in boar seminal plasma.

Many studies have shown that proteins with different molecular weight have different individual contribution on sperm quality and fertility potential. In boar seminal plasma protein with molecular weight of 55kDa and 65kDa showed positive correlation with fertilization capacity manifested by a higher percentage of fertilization and number of pigs born alive in the litter. The protein composed of four polypeptides of 43, 55, 65 and 100kDa defined as Platelet-activating factor – acetyl-hydrolase (PAF-AH), that was isolated and purified form boar sperm has been suggested that enhanced sperm motility, capacitation and acrosome reaction (9, 5, 3). The role of protecting sperm against oxidative stress caused by lipid oxidation plays the albumin presented in boar seminal plasma as a series of proteins with the molecular weight in range 76 – 78kDa (5). It has been suggested that protein fraction with greater molecular weight promote binding between sperm and polymorph-nuclear neutrophils (10, 3). Therefore, most of these studies support thesis that proteins with lower molecular weight may be considered as a natural protectors and play crucial role in spermatozoa viability (11). But there is some evidence that proteins with very low molecular weight (5.7kDa) can be deleterious to sperm function. The removal of very low-molecular weight proteins from the seminal plasma enhances sperm motility, plasma membrane integrity, mitochondrial function and sperm exhibit higher ATP content (5).

More than 75% of all proteins in boar's seminal plasma belong to the family of spermadhesins (10). These are multifunctional proteins important in sperm–cell development, sperm capacitation and formation of the oviductal sperm reservoir that preserves the fertilizing capacity of spermatozoa, interaction of gamets and protection of sperm against the female immune system by uterine immunomodulation. Considering the functions of these proteins some authors suggested that

spermadhesins could be considered as potential candidate for markers of boar fertility (5).

Hence, most studies dealing with seminal plasma proteins in boars are aimed at identifying and evaluating its function, but the effect of different abundance of certain seminal plasma proteins on fertility parameters has not been fully understood. Therefore, the aim of this study was to contribute in evaluation of the effects of seminal plasma proteins with different molecular weight in a different proportion in boar's seminal plasma on the spermatozoa viability and fertility.

Materials and methods

Animals and materials

The experiment was conducted at nine large commercial pig farms in Serbia (Autonomous Province of Vojvodina) with the capacity of 1000 sows in the breeding herd. The farms are in radius of approximately 50 km away from the laboratory where the semen quality control was performed. Fifty boars (Large White x Yorkshire n = 22, Swedish Landrace n = 16, Duroc n = 10 and other n = 2) aged between 18 to 36 months when the study period began, were selected. Fifty sperm-rich ejaculate fractions were collected one per boar using the gloved hand method while the boar mounts a dummy sow. Semen samples were filtered through Minitube® filter immediately after collection. The quality parameters of the ejaculate were evaluated, as is the usual practice at the farm.

Semen quality assessment

The fresh native semen samples, with volume range between 60 to 70ml taken from sperm rich fractions prepared for regular use in AI, and placed in sterile plastic flasks with caps and stored in a thermo-box at +17°C. After that, semen are transported to the laboratory at the Scientific Veterinary Institute in Novi Sad, Serbia (within 2-4h after semen collection at the farm), for semen quality assessment on CASA - computer assisted semen analysis. In the laboratory sperm was prepared and diluted for assessment of sperm progressive motility, concentration and morphology evaluation. The average of all measurements per sample was used for data analysis according to *Kommisrud et al. 2002* (13). Only fresh ejaculates with a progressive motility ≥ 65% were used.

Analysis of protein content in seminal plasma

Semen samples were divided in to 20ml samples, placed in 50ml plastic tubes with caps and centrifuged at 1000×g for 10min at 4°C to remove the spermatozoa. The supernatant was re-centrifuged (at 3000×g for 15min at 4°C) to purify the seminal plasma from any residual sperm and other organic particles. The prepared seminal plasma samples were stored in a refrigerator at +4°C. The analysis was performed within 24h after the ejaculates were collected at the farm. Protein analysis was performed in cooperation of the Laboratory or Reproduction at the Faculty of Agriculture -Department of Animal Sciences, and FINS institute from University of Novi Sad. The total protein content in the seminal plasma was determined by the AOAC - Association of Official Analytical Chemists as a chemical method (Official Method 2001.11). The chip-based separations were performed on the Agilent 2100 bio-analyzer (Agilent Technologies, Santa Clara, CA) in combination with the Protein 230 Plus LabChip kit and the dedicated Protein 230 software assay on 2100 expert software. All chips were prepared according to the protocol provided with the Protein 230 LabChip kit. Before loading onto the chip, the samples were diluted with deionized water (1:2 v/v sample: water). After homogenization, samples (4µl) were denatured using 2µL of Agilent denaturing solution (3.5% ß-mercaptoethanol) and heated for 5min at 100°C. After dilution with deionized water, 6µL were applied to the Protein 230+ LabChip. For each investigated sample, analysis was conducted in two independent replications.

All semen samples were divided in to four groups according protein molecular weight including the percentage of each group in total protein content (Table 1).

Assessment of reproductive performance

The assessment of reproductive performance was carried out based on collected data of three parameters in selected 9696 sows: farrowing rate

(FR), number of pigs born-alive (PBA) per litter and percentage of unsuccessful insemination. Insemination of sows was performed with doses which containing in practice on farms in Serbia, 3 bilion Sptz./ 90ml per dose and AI was performed with classic intracervical insemination method. The evaluation was performed on litters immediately after the delivery was completed. The age structure of the sows involved in the experiment was similar.

Statistical analysis

The data was evaluated using the software package "Statistics 12". Mean, standard deviation, minimum and maximum values of the studied traits were determined. Analysis of variance (ANOVA) test with subsequent Kruskal Wallis test, Fisher exact two tailed test was used for comparison of mean values. Pearson's correlation coefficient was examined to evaluate the correlation of the semen parameters and parameters of reproductive performance between the groups. Differences were considered significant at P-values less than 0.05.

Since this research was performed only with biological material, Ethics Committee has not been taken into consideration because the animals used in the research was in the regular intensive production process, from which are used only written results on the end of the reproductive cycle.

Table 1: Average values (Median ± SD) of seminal plasma proteins with different molecular weight (kDa) in ejaculate from different boar breeds

	Molecular weight (kDa) % of Total		
Breed	10-20	21-30	31-40
Large White x Yorkshire	25.12±4.7	51.03±16.3	18.67±16.3
Swedish Landrace Duroc	28.92±9.1	58.12±10.9	9.83±14.1
Other	28.1±7.5	45±19.8	18.63±21.4
	28.4± 7.2	48.45 ± 3.0	21.15 ± 5.3

Table 2: The effect of different percentage of 10-20kD molecular weight proteins on some fertility parameters (means ± SD)

N	Molecular weight (kDa) % of Total			
	10-20% 8 Mean ± SD	21-30% 28 Mean ± SD	31-40% 12 Mean ± SD	p value
Progresive motility	69.37±9.42[a]	72.17±21.14[a]	71.66±15.85[a]	p > 0.05
Unssecsesful inseminations	49.00±19.69[a]	55.32±43.61[a]	64.91±56.96[a]	p > 0.05
FR	68.35±13.16[a]	70.55±21.77[a]	71.66±20.18[a]	p > 0.05
PBA per litter	11.71±1.40[a]	11.69±1.75[a]	11.62±1.35[a]	p > 0.05

Values with a superscript describe statistical significance p value < 0.05

Table 3: Pearson's correlation coefficient between percentage of proteins with 10 – 20kDa molecular weight and progressive motility, unsuccessful inseminations and piglets born per litter

Correlation % Protein molecular weight 10 – 20kDa	r (Pearson's correlation coeficient)	p value
Progresive motility	r = 0.048	p = 0.74
Unssecsesful inseminations	r = 0.073	p = 0.62
PBA per litter	r = - 0.071	p = 0.63

Statistical significance p value < 0.05

Table 4: The effect of different percentage of 21-30kD molecular weight proteins on some fertility parameters (means ± SD): Progressive motility, Unsuccessful inseminations, Farrowing rate (FR), pigs born per litter (PBA)

N	10-20% 4 Mean ± SD	21-40% 5 Mean ± SD	41-50% 8 Mean ± SD	51-60% 17 Mean ± SD	61-70% 12 Mean ± SD	71-80% 4 Mean ± SD	p value
Progressive motility	77.50± 10.408[a]	80.00± 12.747[a]	73.12± 17.100[a]	68.52± 15.588[a]	67.16± 25.992[a]	77.50 ± 6.454[a]	*P*>0.05
Unsuccessful inseminations	43.75± 15.52[a]	39.60± 18.95[a]	29.25± 18.59[a]	36.29± 21.49[a]	32.00± 25.53[a]	18.25± 8.53[b]	*P*<0.05
FR	65.11± 9.28[a]	52.26± 18.81[ab]	72.00± 10.57[a]	57.12± 22.04[ab]	59.03± 22.72[ab]	81.48± 8.20[b]	*P*<0.05
PBA per litter	12.85± 1.26[a]	11.08± 1.33[a]	11.22± 1.40[a]	11.51± 1.75[a]	11.89± 1.71[a]	11.72± 1.75[a]	*P*>0.05

Values with a different superscript, within a row differ (ab p < 0.005)

Table 5: Pearson's correlation coefficient between percentage (%) of proteins with 21 – 30kDa molecular weight and progressive motility, unsuccessful inseminations and piglets born per litter (PBA)

Correlation % Protein molecular weight 21 – 30 kDa	r (Pearson's correlation coeficient)	p value
Progresive motility	r = -0.119	p = 0.42
Unssecsesful inseminations	r = -0.088	p = 0.55
PBA per litter	r = - 0.049	p = 0.74

Statistical significance p value < 0.05

Table 6: The effect of different percentage of 31-40kD molecular weight proteins on some fertility parameters (means ± SD): Progressive motility, Unsuccessful inseminations, Farrowing rate (FR), pigs born per litter (PBA)

N	1-10% 25 Mean ± SD	11-20% 10 Mean ± SD	21-30% 9 Mean ± SD	31-70% 6 Mean ± SD	P value
Progressive motility	68.44± 20.55[a]	75.00± 15.98a	73.33± 16.20a	75.83± 9.70a	*P*>0.05
Unsuccessful inseminations	30.84± 22.68a	47.70± 18.16b	20.00± 10.13bc	42.16± 12.41cd	*P*<0.05
FR	63.07± 20.09a	57.13± 16.83a	63.58± 28.73a	63.78± 7.65a	*P*>0,05
PBA per litter	11.75± 1.45a	10.75± 1.76a	11.90± 1.57a	12.24± 1.37a	*P*>0.05

Statistical significance p value < 0.05

Table 7: Pearson's correlation coefficient between percentage (%) of proteins with 21 – 30kDa molecular weight and progressive motility, unsuccessful inseminations and piglets born per litter (PBA)

Correlation % Protein molecular weight 31 – 40kDa	r (Pearson's correlation coeficient)	p value
Progresive motility	r = 0.194	p = 0.22
Unssecsesful inseminations	r = 0.278	p = 0.078
PBA per litter	r = 0.008	p = 0.96

Statistical significance p value < 0.05

Results

The effect of different percentage of proteins with 10 – 20kDa molecular weight

The effect of proteins with 10 – 20kDa on sperm progressive motility, FR, PBA and %, of unsuccessful insemination shows in Table 2. The effect on progressive sperm motility of proteins with mw of 10 – 20kDa had not been significantly different in samples with different percentage of this fraction of proteins in semen plasma. The similar results were recorded in analysis of parameters for reproductive performance assessment. The analysis on correlation (Table 3) shows that different percentage of the proteins with mw of 10 – 20kDa are not significantly correlated with progressive sperm motility and reproductive performance parameters.

The correlation of different percentage of proteins with 21 – 30kDa molecular weight

The effect of different percentage of proteins with 21 – 30kDa mw on progressive sperm motility was not significant. There was significantly ($p<0.05$) lower percentage of unsuccessful insemination in semen with 71 – 80% of proteins with 21 – 30kDa mw compared to semen with the lowest content of this fraction proteins (10 – 20%). Consequently, the group of boars with the greatest content (17 – 80%) of proteins with 21 – 30kDa mw had the significantly higher FR compared to other groups. No significant differences were recorded in PBA between groups (Table 4). The correlation between the percentages of proteins with mw 21 – 30kDa was not strong and significant though the percentage of protein content was negatively correlated with examined semen parameters (Table 5)

The effect of different percentage of proteins with 31 – 40kDa molecular weight

Significant differences were shown in percentage of unsuccessful inseminations between the groups with different percentage of 31 – 40kDa mw protein content in seminal plasma samples. These differences were not significantly influenced by the different concentration of 31 – 40kDa mw protein according the results of linear correlation between proteins and assessed parameters. No significant differences and no significant correlation were shown in different percentage of this fraction semen plasma proteins to sperm motility and reproductive performance parameters (Table 6; Table 7)

Values with a different superscript, within a row differ (^{abcd} $p < 0.005$)

Discussion

The results of this study have demonstrated that different content of fractionated seminal plasma protein may have effects on sperm that reflects on reproductive performance in boars. These results also confirmed previous findings of the effect of seminal plasma proteins on the ejaculate fertility potential. Namely, earlier studies have clearly shown that seminal plasma proteins exert many functions related to sperm development, maturation, transport and survival in the female reproductive tract, as well as capacitation and acrosome reaction, sperm-egg recognition, and protection against microbial and oxidative damages (12, 8, 14, 23). According to *Apić et al 2016* (3) the ejaculates with higher content of semen plasma protein had significantly higher values of progressive motility, live spermatozoa and spermatozoa with intact acrosomes. Electrophoretic profiles of porcine seminal protein are in range up to 200kDa with different percentage of content in seminal plasma. Some author indicates that seminal plasma proteins in native state, forms high-molecular protein aggregates which may associate or dissociate under some conditions in certain molecular weight peptides (15, 14). In this Investigation, analysis was performed on seminal plasma proteins with the range of molecular weight from 10 to 40kDa since proteins in this bands is with most frequent appearance in boar seminal plasma (15). The protein fractions were categorized in to three groups according the molecular weight in order to analyze the effects of different percentage of each protein fraction. According to results, different percentage of proteins with 10 to 20kDa molecular weight range, do not significantly influence on sperm motility and do not affect significantly on fertility performance. Some authors reported that proteins within this range

may have versatile effect on sperm viability and motility (11) suggested that 10 – 20kDa proteins, as the most abundant protein fraction in boar semen plasma, have mostly protective effect on sperm and maintain the stability of sperm plasma membrane. On the other hand, *Caballero et al.,* 2008 (17) reported that supplementation of boar spermatozoa with low molecular weight protein like heparin-binding (AQN-1, AQN-3, AWN) and non-heparin-binding (PSP-I/PSP-II) spermadhesins, exerted opposite effect on sperm viability, motility and mitochondrial activity. At ejaculation the spermadhesins form a protective coat around the sensitive acrosomal region of the sperm head, thus possibly preventing premature acrosome reaction (24). In our research, higher content of this protein fraction had very weak positive correlation with sperm motility, but insignificant negative correlation was recorded to number of pigs born-alive per litter. This indicate that low fertility rate may be related to higher content of proteins with < 20kDa molecular mass range (15) . Significantly increased farrowing rate and lower percentage of unsuccessful inseminations were recorded in ejaculates with over 70% of 20 – 30kDa proteins in seminal plasma. Thus, the results have shown that 20 - 30kDa protein fractions may influence on sperm fertile potential and are consistent with findings of positive correlation with farrowing rate (16). Similar results are reported in bull ejaculates where 28 – 30kDa seminal plasma proteins are considered as a fertility-associated protein. Some fraction of these range seminal proteins are considered as an antioxidant enzyme (glutathione peroxidase-5) that may protect the sperm membranes from oxidative damage and are also positively related with sperm motility and fertility index (18, 14, 21, 22). *Troedsson et al., 2005* (10) reported that proteins with < 35kDa suppress opsonization to polymorph-nuclear neutrophils and play active roles in protection of viable spermatozoa in mare's female reproductive tract. Therefore, the positive effect of 20 - 30kDa seminal plasma protein on fertility index may be the result of multifunctional role of these proteins in maintaining the viability and motility of spermatozoa in native ejaculate. The percentage of 30 – 40kDa seminal plasma proteins in ejaculates were in accordance to findings of other authors (7). The content of this fraction according to *Strzezek et al.,* 2005 (14) is age related. There are insufficient and inconsistent data about physiological significance of these plasma proteins in boar's ejaculates. Protein with 50kDa described as motility factor inhibitor was purified from boar seminal plasma. Protein recognized as platelet-activation factor acetyl-hydrolase (PAF-AH) composed of four polypeptides (43, 55, 65 and 100 kDa) was isolated and purified from boar sperm, enhances sperm motility, capacitation and acrosome reaction. The results in this study did not show significant effect of 30-40kDa seminal plasma proteins content on sperm motility despite very week positive correlation. But results have shown that higher percentage may have positive effect on farrowing rate and PBA per litter although the correlations were not significant. These results could be supported by the findings of *Flowers et al.,* 2001 (20) who reported that increased concentration of 26 and 55kDa proteins had increased the percentage of farrowing rate and number of pigs born alive.

Conclusion

In summary, our results reinforce the findings that content of different fractions of seminal plasma proteins could influence on fertility potential of boar ejaculates. The results have also shown that proteins with molecular weight less than 40kDa could be considered as fertility marker which is in accordance with other investigations. It could be suggested that percentage of proteins with 20 – 30kDa molecular weight may be the most effective since there was significant differences in analyzed parameters.

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VPLIV VSEBNOSTI BELJAKOVIN SEMENSKE PLAZME RAZLIČNIH MOLEKULARNIH TEŽ NA NEKATERE PARAMETRE PLODNOSTI V EJAKULATIH MERJASCEV

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Povzetek: Cilj opisane raziskave je bil proučiti vpliv prisotnosti beljakovin semenske plazme z različno molekulsko maso na gibljivost semenčic in parametre plodnosti kot so: stopnja prasitev, število živorojenih prašičev na leglo in odstotek neuspešnih osemenitev. Po metodi z orokavičeno roko je bilo zbranih 50 frakcij ejakulata, bogatih s semenčicami (1 na merjasca). Na kmetiji smo najprej ocenili parametre kakovosti vzorcev semena. Nadaljnja ocena kakovosti semenčic je bila izvedena z računalniško podprto analizo semena (CASA), ki sta jo izvedla dva usposobljena operaterja. Semenske frakcije beljakovin v plazmi so bile pridobljene s pomočjo AOAC - metode. Ocena reproduktivne učinkovitosti je bila izvedena na podlagi zbranih podatkov treh parametrov pri izbranih 9696 svinjah. Proteinske frakcije so bile razdeljene v tri skupine (10 – 20 kDa, 21 – 30 kDa in 31 – 40 kDa). Beljakovine velikosti 10 - 20 kDa niso imele značilnega učinka in soodvisnosti z analiziranimi parametri. Ugotovili pa smo statistično značilne razlike v stopnji prasitve med vzorci z do 80 % beljakovin velikosti 21 - 30kDa v primerjavi z vzorci s samo 10 % beljakovin velikosti 21 - 30kDa. Statistično značilne razlike so bile ugotovljene tudi pri uspešnosti osemenitev med vzorci z različnim odstotkom beljakovin velikosti 31 - 40kDa. Rezultati te študije kažejo vpliv različnega odstotka določenega deleža beljakovin iz semenske plazme na potencial plodnosti merjascev.

Ključne besede: merjasci; kakovost semena; semenske beljakovine v plazmi; reprodukcijski rezultati

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Original Research Article

EVALUATION OF INTRA- AND INTER-OBSERVER AGREEMENT OF ULTRASONOGRAPHIC FINDINGS AFTER ADIPOSE TISSUE DERIVED MESENCHYMAL STEM CELLS TREATMENT OF SUPERFICIAL DIGITAL FLEXOR TENDON INJURIES IN HORSES

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Abstract: The present paper assesses the multi-observer evaluation of ultrasonographic response of horses with superficial digital flexor tendon (SDFT) damage to the inoculation of autologous adipose mesenchymal stem cells (AMSCs). The study included 15 horses recruited at an equine hospital following a SDFT damage diagnosis. Horses were implanted with AMSCs obtained from the paracaudal region. Treatment efficacy, in terms of morphological recovery of SDTF considered as degree of echogenicity (TS) and percentage of fibres alignment (FAS), was evaluated using four experts. Inter-observer agreement of experts' judgements on ultrasonographic examinations in different stages of recovery was calculated. The classification of the intra-observer agreement was defined as good for both TS and FAS while the inter-observer agreement was good for TS and moderate for FAS. Ultrasonographic images judged as having a higher TS showed 95 times greater chance of belonging to the final recovery stage than the first and a 50 times greater chance resulted for FAS. This study provided evidence that the inter-observer agreement of ultrasonographic images is a reliable and valid method to evaluate the morphological recovery of injured tendons. In addition, the study demonstrated the morphological recovery of SDFT in horses following AMSCs injection through an independent evaluation system.

Key words: multi-observer agreement; ultrasonographic diagnosis; adipose mesenchymal stem cells; equine tendon desmopathy.

Introoduction

Myo-tendinopathies are common pathological conditions affecting the musculoskeletal system of the horse. The competitive career of sport horses may be shortened because of tendon injuries, especially because full recovery is extremely difficult or impossible. Although several medical and surgical treatments have been proposed over the past years, nowadays, there is a growing interest in the use of stem cells for the recovery of injured tendons as an alternative to the traditional approach. Many papers report the treatment of equine tendinopathies with bone marrow derived mesenchymal stem cells (BMDSC) and their healing is reported by histological, immuno-histochemical findings and clinical observations (1, 2). Follow-up studies confirm the success of this treatment and the functional recovery of racehorses with injuries of the Superficial Digital Flexor Tendon (SDFT) following treatment with BMDSC (3-5). In addition, in the last decades the presence of mesenchymal multilineage stem cells in adipose tissue has been widely documented (6), as well as the multipotency characteristic of the

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Adipose Mesenchymal Stem Cells (AMSCs) (7, 8). The adipose tissue can represent a more suitable substrate in terms of isolation, expansion rate and number of cells available for the implant when compared with BMDSC because of the higher amount of stromal cells present (9). Many authors confirmed the potential benefit of regenerative therapy using adipose-derived nucleated cells in improving equine tendon injuries (10-13).

Ultrasonography diagnosis has been proved useful in the assessment of the equine tendon recovery after stem cell implantation, especially from a qualitative point of view, because of the early detection of tendon fibres regeneration. Nevertheless, clinical trials based on the use of ultrasonographic diagnosis for the evaluation of the efficacy of such approach have not been conducted so far.

The aim of this paper is to assess intra- and inter-observer agreement of the ultrasonographic evaluation of damaged SDFT healing after the inoculation of autologous AMSCs.

Materials and methods

Cases selection

The study was a prospective case series design. During 2008 and 2009, 15 horses were recruited with a total of 17 acute superficial digital flexor tendinopathies, – 7 racehorses, (6 thoroughbred and 1 trotter) and 8 performance horses (6 show jumpers, 1 eventing and 1 quarter horse). Horses with a SDFT lesion were enrolled in the course of routine clinical practice. The 15 horses included in the study had 17 SDFT lesions of different severity. According to the quantitative ultrasonographic assessment proposed by Genovese et al. (14) 3 lesions scored a Severity Rate (SR) of III, 1 scored a SR of IV, 7 scored a SR of V and 6 scored a SR of VI. The research protocol was submitted and approved by the Italian Ministry of Health and its Animal Welfare Unit (RC LT 02/07).

AMSC procedure

Horses were implanted with autologous AMSCs according to the Regulations regarding the use of heterologous cellular and biological products (15, 16). Before starting the study, tests to determine the multipotency characteristics of the horse's AMSC were carried out through the in vivo and in vitro induction for cell differentiation in the presence of lineage-specific induction factors (17).

In previous trials carried out, authors observed that cells survived in fat at 4°C more than 5 days after collection, as well as after cell splitting and harvesting, but with a moderate, diminishing yield. To reduce this variability, delivery of tissue samples from the equine hospital to the laboratory and vice-versa was established to be less than two hours.

During the study, AMSCs were extracted from the subcutaneous adipose tissue that had been surgically collected from the paracaudal region of each horse, under sedation and local anaesthesia. Following collection, a variable amount of adipose tissue sample (5-20 g), depending on the individual fat abundance at the sampling site, was dipped in a vessel containing an antibiotic (100 U/mL penicillin and 100 mg/mL streptomycin), 0,1 M phosphate-buffered saline solution and transported to the laboratory. The isolation and the expansion of cells were performed from the stromal fraction of adipose tissue (6). After shaving and disinfection of the leg with povidone-iodine and alcohol, the expanded cells were injected into SDFT lesion under ultrasonographic control. A variable number of cells, ranging from 0.5 to $2.5*10^6$, were injected, depending on the concentration of the cells obtained during the expansion. Most of the cases were treated with 1 ml. One case was injected with 2 ml because of the extent of the lesion. The injection was performed using a 20 gauge needle within two hours following their re-suspension in 1 ml of autologous plasma, approximately 7 days after their collection, during the subacute phase.

Ultrasonographic diagnosis

All horses were examined using the same ultrasonographic protocol. Ultrasonographic examinations were performed 7-10 days before the injection of stem cells (stage A), that corresponded to the day of surgical adipose tissue collection, 60 days (stage B) and 120 days after the injection (stage C). All horses were examined by the same operator using the same ultrasonographic equipment with a standard procedure in terms of preparation of the leg, probe frequency (10 MHz), gain and focus regulation.

Evaluation of the SDTF morphological recovery

Tendon morphological recovery after the injection of stem cells was evaluated through the measurement of the intra- and inter-observer agreement of judgement on ultrasonographic findings performed by 4 veterinarians, internationally recognized as experts in diagnostic imaging. For this purpose, 3 ultrasonographic exams were collected from each of the 17 damaged tendons at stage A, B and C respectively, counting up a set of 51 images. Experts received a DVD containing the same set repeated 3 times (51x 3=153 images in total) and 2 tables in a purposely developed spreadsheet. In each table, all 153 images were listed and each image was identified by a unique alphanumeric code to assure blind assessment. In addition, the experts were not aware that the set of 153 images was actually made up of 3 repeats of each of the 51 ultrasound scans.

Experts were required to give 2 types of judgement for each ultrasonographic examination: the degree of echogenicity (TS) and the percentage of fibres alignment observed (FAS), according to an ordinal scale of 4 categories: completely anechoic (CA), mostly anechoic (MA), hypoechoic (HP), isoechoic (IS) for TS and 0-25% (AN), 26-50% (LO), 51-75% (MD), 76-100% (HY) of fibres observed for FAS. Before evaluating ultrasonographic images, experts were requested to have an agreement relative to the different mentioned categories corresponding to those reported in figure 1.

Data analysis

The 4 categories of judgement concerning both the degree of echogenicity and the percentage of fibres alignment observed were arbitrarily defined and not outlined by formal specifications. The border between the categories was not clear-cut and the experts might have been affected by a certain degree of subjectivity, thus representing a potential for misclassification. For this reason, TS and FAS were recoded into dichotomous variables:

- recoded TS: “Low” (CA or MA) and “high” (HP or IS) degree of echogenicity;
- recoded FAS: “Moderate” (AN or LO) and “abundant” (MD or HY) percentage of fibres alignment observed.

The Cohen’s kappa coefficient (k) (18, 19), as further implemented (20) and adapted (21), was chosen to evaluate the intra- and inter-observer agreement beyond chance of judgement given on each ultrasonographic image by the single expert and by the entire group of experts, respectively. The same method was then applied to evaluate the intra- and inter-observer agreement of judgements given on ultrasonographic images at the same stage of the lesion (A, B, C). The classification proposed by Landis and Koch (22) was applied for interpreting k: values above 0.61 indicate substantial agreement beyond chance.

The same data were also analysed using a multivariable logistic regression to objectively assess the anatomic recovery following the injection of stem cells.

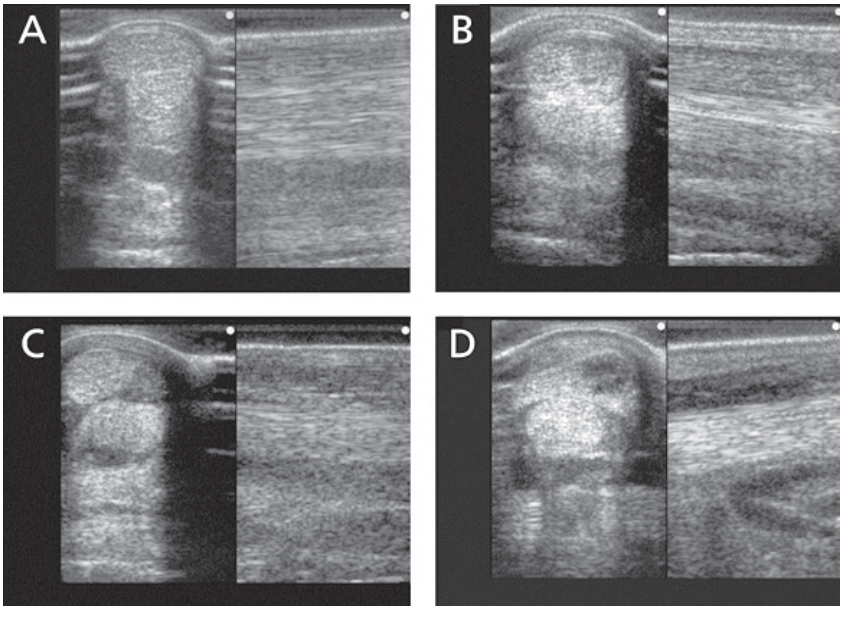

Figure 1: Ultrasonography- Cross Sectional Area (CSA) occupied by the lesion. Examples of degree of echogenicity score (TS) and fiber alignment score (FAS): A = isoechogenic (IS) – fiber 75-100% (HY); B = hypoechogenic (HP) – fiber 50-75% (MD); C = mostly anechogenic (MA)– fiber 25-50% (LO); D = completely anechogenic (CA) – fiber 0-25% (AN)

The probability that a judgement of high TS and FAS was given to ultrasonographic exams at stage B or C compared to images at stage A was also calculated. In detail, the judgements on the recoded TS (high/low) and recoded FAS observed (moderate/abundant) were chosen as outcome variables, while the stage of lesion (A, B, C), the duplication sets (1st, 2nd, 3rd) and the expert (1, 2, 3, 4) as explanatory variables. Odds ratio (OR) and 95% confidence interval (CI 95%) were estimated. The analyses were performed using the software Stata (23).

Clinical evaluation

Following the cell inoculation, the horses were confined in stalls for 2 weeks. Hand walking for 6 weeks was prescribed starting from the third week, during this time the first ultrasonographic control was suggested, before starting a light exercise consisting of walking and trotting increased every 2 weeks up to twentieth week. At that time, if the healing process was satisfactory, the exercise was further increased to racing or competition training, which was started after the thirtieth - thirty-second week depending on the stage of the healing process, assessed through ultrasonographic examination. An ultrasonographic control was recommended every 8 weeks throughout the whole rehabilitation program.

The clinical results, regarding the recovery of their previous athletic activity, were evaluated through the official data for the racehorses and by collecting direct information from the owners in case of the performance horses. For racehorses, to have raced at least four times after treatment was considered as a positive outcome. Any reoccurrence of the SDFT lesion was recorded during the follow-up.

Ethical statement

Clinical procedures described in the present paper did not require approval from an ethics committee. Nevertheless, procedures were performed in accordance with guidelines set by the Committee for Research and Ethical Issues of IASP.

Results

The cell cultures were ready for inoculation about 7 days after adipose tissue collection. In the absence of standardised indications relative to the cell/dose inoculum, even if with a shelf life lasting more than 2 days (personal observations), a variable number of cells ranging from 500,000 to 2,500,000 per horse was injected within 2 hours following their re-suspension in 1 ml of autologous plasma.

In addition, to ensure a low differentiation degree from the first isolation no more than 2 cell passages were made. The number of cells was related to age (faster growth in younger animals) and to the amount of adipose tissue collected, not always abundantly available. After the injection of AMSCs into the SDFT, no local inflammatory reaction reported by the ultrasonographic control.

The total number of judgements given by the 4 experts amounted to 1,224. Each expert gave 306 judgements: 153 on the TS and 153 on the FAS observed.

A preliminary descriptive analysis of data showed a tendency of experts to assign a judgment of higher TS and greater FAS to ultrasound scans performed at stage B and stage C rather than those being performed at stage A (Table 1).

According to the Landis and Koch's table (22), the overall intra-observer agreement of each expert- i.e. the agreement between the judgements given by the same expert on the 3 duplications of the same ultrasonographic image – was classified at least "good" for both judgements, on TS and on FAS, because of k-values always rating higher than 0.61 (Table 2).

As a further evidence of the good level of self-consistency of judgements given by the same expert, the intra-observer agreement of each expert increased when breaking down the judgements by stage of lesion. The overall inter-observer agreement - i.e. the agreement between all experts' judgements given on the same ultrasonographic image - was "good" for judgements on TS (k=0.71), but only "moderate" for those on FAS (k=0.58). Breaking down the experts' judgements by stage of lesion, the inter-observer agreement was

Table 1: Degree of echogenicity (TS) and Percentage of Fibres Observed (FAS). Distribution of the judgments given by each expert by stage of lesion (17 ultrasonographic exams in each stage = 51. 51 * 3 repetition = 153 judgements per expert in total)

		Categories	Recoded categories	Judgements			Total judgements per categories	Total judgements per recoded categories	Expert total judgements
				Stage A	Stage B	Stage C			
TS	Expert 1	CA	Low	14	0	0	14	49	153
		MA		27	8	0	35		
		HP	High	10	20	33	63	104	
		IS		0	23	18	41		
	Expert 2	CA	Low	13	0	0	13	43	153
		MA		21	7	2	30		
		HP	High	17	27	30	74	110	
		IS		0	17	19	36		
	Expert 3	CA	Low	15	3	0	18	55	153
		MA		27	5	5	37		
		HP	High	9	15	17	41	98	
		IS		0	28	29	57		
	Expert 4	CA	Low	17	0	0	17	48	153
		MA		21	10	0	31		
		HP	High	8	11	24	43	105	
		IS		5	30	27	62		
FAS	Expert 1	AN	Moderate	13	0	0	13	39	153
		LO		21	5	0	26		
		MD	Abundant	15	14	25	54	114	
		HY		2	32	26	60		
	Expert 2	AN	Moderate	34	5	2	41	68	153
		LO		8	9	10	27		
		MD	Abundant	9	12	23	44	85	
		HY		0	25	16	41		
	Expert 3	AN	Moderate	19	3	0	22	56	153
		LO		25	5	4	34		
		MD	Abundant	7	19	22	48	97	
		HY		0	24	25	49		
	Expert 4	AN	Moderate	17	0	0	17	51	153
		LO		23	10	1	34		
		MD	Abundant	5	9	21	35	102	
		HY		6	32	29	67		

* AN= 0-25%, LO=26-50%, MD=51-75%, HY=76-100%; CA: Completely Anechoic; MA: Mostly Anechoic; HP: Hypoechoic; IS: Isoechoic

Table 2: Intra-observer agreement by stage of lesion and overall intra-observer agreement (k-value) reached by each expert on judgements given on recoded TS and on recoded FAS by stage of lesion

Recoded TS categories				Recoded FAS categories			
Stage A	**Stage B**	**Stage C**	**TS Overall**	**Stage A**	**Stage B**	**Stage C**	**FAS Overall**
0.92	0.92	1	0.95	0.92	0.92	1	0.95
0.76	0.76	0.92	0.82	0.76	0.76	0.92	0.74
0.69	0.84	0.76	0.76	0.69	0.84	0.76	0.76
0.88	0.84	1	0.87	0.88	0.84	1	0.87

"good" for both the types of judgement only for ultrasonographic images taken at stage B and C (TS k=0.81 and k=0.88, FAS k=0.64 and k=0.84 respectively). Nevertheless, the inter-observer agreement was "moderate" for judgements on ultrasound scans taken at stage A (TS, k=0.44; FAS, k=0.42).

The logistic regression model confirmed that the probability to assign a judgement of "high" echogenicity and "abundant" percentage of fibres alignment observed to ultrasonographic images taken at stage B or C was actually greater than the probability to assign the same judgements to those taken at the stage A. In detail, ultrasonographic images judged as being "high" echogenic have shown 95 times greater chance of being one of those actually taken at stage C (OR=95.8; CI 95%: 41.8-219.5) than being one of those taken at stage A. In addition, ultrasonographic images judged as having an "abundant" percentage of fibres have shown 50 times greater chance of being one of those actually taken at stage C (OR= 49.5, CI 95%: 26.3-93.1) than being one of those taken at stage A.

Table 3. Multivariate logistic regressions: effect of stage of lesion, duplication and expert on the recoded TS and FAS categories

Outcome	Explanatory variables		OR*	p value	95% confidence interval	
TS	***Stage of lesion***	A	1.00	-	-	-
		B	17.42†	0.00	10.53	28.84
		C	95.85†	0.00	41.84	219.54
	Duplication	1st	1.00	-	-	-
		2nd	0.96	0.89	0.54	1.69
		3rd	1.53	0.15	0.86	2.73
	Expert	1	1.00	-	-	-
		2	1.41	0.31	0.73	2.75
		3	0.72	0.32	0.37	1.38
		4	1.06	0.87	0.55	2.05
FAS	***Stage of lesion***	A	1	-	-	-
		B	19.72†	0.00	11.70	33.24
		C	49.46†	0.00	26.28	93.08
	Duplication	1st	1.00	-	-	-
		2nd	1.04	0.89	0.60	1.79
		3rd	1.32	0.33	0.76	2.28
	Expert	1	1.00	-	-	-
		2	0.22†	0.00	0.11	0.43
		3	0.39†	0.01	0.20	0.76
		4	0.51	0.05	0.26	0.99

*OR: Odds Ratio; †: p<0.001

The multivariable analysis excluded - regarding those judgements on TS - any effect related to the experts' subjectivity and the repetition of the ultrasonographic images on the observed outcome, i.e. the ability to systematically select a judgement of high echogenicity to ultrasonographic images taken at later stages (Table 3). Nevertheless, regarding those judgements on FAS, a protective effect of the experts' subjectivity (especially expert 2 and 3) was observed on the outcome. This does not affect the overall tendency to systematically select a judgement of "high" echogenicity to ultrasonographic images taken at later stages (Table 3).

Furthermore, the follow-up of treated horses revealed a good level of recovery. In the racehorses group, 5 out of 7 (71%) horses raced at least 4 times after treatment, while 6 out of 8 (75%) performance horses recovered their previous athletic activity.

Discussion

In the absence of a standardised cell/dose inoculum and taking in consideration the time consuming procedure needed to obtain constant concentrations of cells coming from different samples, the timeliness of inoculation was privileged in order to preserve as much as possible the cells viability. As therapeutic success is influenced by the time elapsing from injuries to cell administration (5), fibrosis and scar tissue formation affect healing, the restriction of this interval period was preferred to the cell inoculum standardisation. In addition, this procedure also reduce the number of cells passages and therefore maintain their original multipotency characteristics. In some cases, to reach concentrations of many millions of cells, at least one more week would have been necessary, compromising the time interval between injury and therapy. Other authors have already shown the difficulty in establishing a significant difference between re-injury rate and amount of cells injected (3).

The collection of fat from paracaudal region has been demonstrated in being an easy and safe procedure in obtaining an appropriate sample for AMSCs culture even if more invasive than collection from the sternum bone marrow, procedure fatal complications.

In the present study the therapeutic success or injury reoccurrence do not seem to be associated to the concentrations of the cells inoculum nor the horse age.

Regarding the re-injury rates observed in this study (29% in racehorses and 25% in other competition horses), these are lower than what reported when using a medical approach (24) and similar to those described in another longer term follow-up (3), carried out after implantation of BMSC, 27% and 25% in race and National Hunting horses, respectively, re-injured.

The no completely satisfactory results of inter-observer agreement may be due to ultrasonographic exams taken at stage A (initial lesions) which could have been more difficult to evaluate for having different degrees of echogenicity and fibres damage, thus, leaving the opportunity of assigning the images of stage A to more classes than those in later stages. In addition, it can not be excluded that judgments on the same ultrasonographic exam might have been affected by a systematic error derived from different factors such as the expert's subjectivity and the technical features of the screens used to display the images. Neverthless, the statistical analysis has shown clear intra- and inter-observer agreement of the judgments.

Even if the study did not enrol a homogeneous cohort of horses regarding age, gender, breed and intended use, the statistical results indicated a significant agreement between the 4 experts. This finding indicates an objective association between ultrasonographic images taken at stage B or C and the judgements of "high" echogenicity and "abundant" percentage of fibres observed, providing evidence of the substantial morphological recovery of the tendon's damage in all the horses subjected to autologous AMSCs inoculation.

The present study provided evidence that the inter-observer agreement of ultrasonographic findings is a reliable and valid method to evaluate the morphological recovery of injured tendons. In addition, the present study also demonstrated the morphological recovery of SDFT in horses following the injection of AMSCs through an independent evaluation system.

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PRESOJA OCEN OPAZOVANJA VEČ STROKOVNJAKOV IN VEČKRATNIH PREGLEDOV ULTRAZVOČNIH UGOTOVITEV PO ZDRAVLJENJU POŠKODBE POVRŠINSKE UPOGIBOVALKE PRSTOV PRI KONJIH Z MEZENHIMSKIMI MATIČNIMI CELICAMI, PRIDOBLJENIMI IZ MAŠČOBNEGA TKIVA

M. G. Sala, F. Canonici, K. Barbaro, E. Aquilini, A. Carvelli, V. Spallucci, M. T. Scicluna

Povzetek: V članku so obravnavane ocene opazovanj več opazovalcev, ki so z ultrazvokom spremljali celjenje poškodovane tetive površinske upogibovalke prstov (SDFT) po zdravljenju z avtolognimi maščobnih matičnimi celicami (AMSC). V raziskavo je bilo vključenih 15 konjev, ki so bili napoteni na Kliniko za zdravstveno varstvo kopitarjev po diagnozi poškodbe SDFT. Konji so bili zdravljeni z vsadki AMSC, pridobljenimi iz parakavdalnega področja. Učinkovitost zdravljenja v smislu morfološke obnove SDTF, ki je bila obravnavana kot stopnja ehogenosti (TS) in odstotka poravnave vlaken (FAS), je bila ovrednotena s pomočjo štirih strokovnjakov. Izračunana je bila klasifikacija ocen strokovnjakov o opazovanju izsledkov ultrazvočnih pregledov v različnih fazah okrevanja živali. Razvrstitev ocen znotraj posameznega opazovalca je bila značilno enotna za TS in FAS, medtem ko je bila podobnost ocen med opazovalci značilno enotna za TS in zmerno enotna za FAS. Ultrazvočne slike, za katere je bilo ocenjeno, da imajo višjo TS, so pokazale 95-krat večjo možnost za doseganje končne faze okrevanja in 50-krat večjo možnost za FAS. Ta študija dokazuje, da je enotnost ocen, ki izhaja iz ocen ultrazvočnih slik več neodvisnih ocenjevalcev zanesljiva in uporabna metoda za oceno morfološke ozdravitve poškodovanih tetiv. Poleg tega je študija s pomočjo opisanega neodvisnega sistema ocenjevanja pokazala morfološko okrevanje SDFT pri konjih po aplikaciji AMSC.

Ključne besede: dogovor ocenjevanja več opazovalcev; ultrazvočna diagnoza; mezenhimske maščobne matične celice; dezmopatija tetive konjev

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Original Research Article

EFFECTS OF MESENCHYMAL STEM CELL TRANSPLANTATION INTO ISCHEMIC BILE DUCT INJURY TISSUES OF RATS ON THE EXPRESSION OF VASCULAR ENDOTHELIAL CELL PHENOTYPE AND THE INCREASE OF VASCULAR DENSITY

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Abstract: We aimed to evaluate the effects of mesenchymal stem cell (MSC) transplantation into the ischemic bile duct injury tissues of rats on the expression of vascular endothelial cell phenotype and the increase of vascular density. The bone marrow of male Wistar rats aged 2-4 weeks was sampled to culture MSCs by the direct adhesion method. Forty-six female rats were randomly divided into a transplantation group (group A) and a non-transplantation group (group B). Group A was transplanted with MSCs (1×10^6 cells/site, a total of 8 sites) into ischemic biliary lesion. After 21 days, the bile ducts with lesions were removed. The transplanted MSCs were tracked by a combination of *in situ* hybridization staining of sex-determining region Y gene with CD34 immunohistochemical staining (double labeling). Microvascular and vascular endothelial growth factors were labeled by immunohistochemical staining to calculate microvascular density (MVD). After 21 days of injection, double labeling revealed that a small portion of Y-chromosome positive cells in group A (brown cell nucleus) had CD34 antigen phenotype (bluish violet). MVD of group A ((63 ± 18)/HP) was higher than that of group B ((53 ± 14)/HP), and the vascular endothelial growth factor (VEGF) expressions of the two groups were significantly different ($P<0.05$). The formation of vascular endothelial cells after MSC transplantation improved the blood supply of bile duct by secreting VEGF.

Key words: mesenchymal stem cell; transplantation; *in situ* hybridization; immunohistochemistry; bile duct injury

Introduction

Bone marrow-derived stem cells have been used clinically to promote myocardial regeneration and angiogenesis (1). However, how to select ideal cell subsets for cell therapy is still controversial owing to different compositions of cell populations (2). Versatile mesenchymal stem cells (MSCs) found in bone marrow can differentiate into neurons, skeletal muscle cells and vascular endothelial cells (3). Therefore, neovascularization can be induced by injecting MSCs into infarcted myocardium (4,5).

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On the other hand, ischemic biliary lesion is a tricky complication in hepatobiliary surgeries, which cannot be satisfactorily treated with repeated intervention or surgical repair hitherto. Zhao et al. reported the preventive effects of transplanting bone marrow mononuclear cells into a mouse model of bile duct ischemia on their ischemic stenosis (6). Considering that false-positive results may be obtained by tracking the cells with 5-bromo-2-deoxyuridine (BrdU), a combination of SRY (sex-determining region Y gene) DNA *in situ* hybridization staining with CD34 immunohistochemical staining (double labeling) was used herein to determine whether transplanted MSCs could differentiate into

vascular endothelial cells. Moreover, vascular endothelial growth factor (VEGF) expression and microvascular density (MVD) were detected by immunohistochemical staining. The experimental results prove that MSC transplantation can prevent stenosis induced by ischemic bile duct injuries.

Materials and methods

Experimental animals

This study has been approved by the animal experiment ethics committee of our hospital, and great efforts have been made to minimize the suffering. Forty-six healthy and clean female Wistar rats aged 8-12 weeks and weighing 200-300 g were provided by the Institute of Experimental Animals, Chinese Academy of Medical Sciences (Animal Certificate No. SCXK (Beijing) 2017-0006). The rats were randomly divided into group A (transplantation group) and group B (non-transplantation group).

Apparatus and reagents

Microscopic artery clamp, ophthalmic scissor, small vessel forcep and microscopic artery forcep were purchased from Shanghai Medical Instruments (Group) Ltd., Corp. Surgical Instruments Factory (China). SRY DNA *in situ* hybridization staining system was obtained from Wuhan Boster Biological Engineering Co., Ltd. (China). Rabbit anti-rat CD34 antigen-antibody IgG and rabbit anti-rat VEGF antigen-antibody IgG were provided by Beijing Boaosen Biological Engineering Co., Ltd. (China). Peroxidase-conjugated streptavidin (SP) staining kit, concentrated dimethyl-benzidine (DAB), nitroblue tetrazolium (400 μg/ml) and 5-bromo-4-chloro-3-indolephosphate (BCIP/NBT) color development kit were purchased from Fuzhou Maxim Biotechnology Co., Ltd. (China).

Separation, culture and identification of MSCs

MSCs were cultured and harvested by using the method of Wexler et al. (7). The femurs and tibias of six-week-old male albino Wistar rats were carefully dissected away from attached soft tissues, the bone ends were cut, and the bone marrow was aseptically flushed with Dulbecco's modified Eagle's medium (DMEM, Gibco, UK). The mononuclear fraction was isolated by density gradient centrifugation at 435 ×g for 30 min at room temperature and seeded at a density of 1×10^6 cells/cm^2 into T75 cell culture flasks (Nunc, Austria). The cells were plated in DMEM-low glucose (Gibco, UK) supplemented with 10% fetal calf serum (Sigma, Germany) and 1% penicillin-streptomycin (Gibco, UK), and cultured at 37°C in a 5% CO_2 atmosphere. After three days, a small number of adherent cells grew into a visible symmetric colony, and non-adherent cells were removed when the medium was refreshed every three days. The remaining purified MSCs were further amplified in a culture flask. The cells were grown to confluence, harvested by incubation with 0.25% trypsin/1 mM EDTA (Gibco, UK), centrifuged at 1200 rpm for 5 min, and subcultured at a 1:3 split ratio in new culture flasks. After confluence was reached for the second time (after eight days), the harvested cells were defined as passage one, and the replated cells were cultured and serially subcultured until passage four. The fourth-passage cells were then transplanted. Cell concentration was maintained at $4\text{-}10\times10^7$/L, and viability was determined as >80% by trypan blue staining.

Animal treatment

Two rat groups were anesthetized by the intraperitoneal injection of pentobarbital (20 mg/kg), and an extrahepatic biliary ischemia model was established by clamping the extrahepatic bile duct (6), leading to an irreversible ischemic injury. The rectal temperature was maintained at (37.0 ± 0.5)°C by a heat lamp. The rats were intramuscularly injected with 500,000 units of penicillin during operation. Afterwards, they were housed in standard conditions and fed with free access to food and water.

Group A: The rats were not fed with food or water 6 h and 3 h before operation. Then they were intraperitoneally injected with pentobarbital (20 mg/kg) for anesthesia and fixed on an operating table, the abdomens of which were disinfected with iodophor. Under sterile conditions, the extrahepatic bile duct was exposed (incision was

made in the middle of the abdomen: 3 cm). After being rinsed by normal saline, the isolated great omentum (about 0.4 cm × 0.4 cm × 0.6 cm) was intermittently stitched by 5-0 silk thread. Then MSC suspension was uniformly injected into the greater omentum at 8 sites by a 1 ml syringe (each site: 25 μl, 1×10^6 cells). The abdominal cavity was sutured with 3-0 silk thread. Group B was treated similarly but injected with 25 μl of PBS instead into the greater omentum. No rats died of anesthesia or improper operations, or received immunosuppression therapy.

Sample collection

Twenty-one days after surgery, the tissues wrapping the greater omentum and those near the bile duct were collected, rinsed with normal saline, fixed in 4% paraformaldehyde and prepared into paraffin-embedded sections.

Immunohistochemical staining

The immunohistochemical SP method was utilized for VEGF and MVD staining. A slice known positive for breast cancer (Beijing Biosynthesis Biotechnology Co., Ltd., China) was used as the positive control, and two rat biliary slices were employed as the negative control, with primary antibody replaced by PBS. The detection was performed according to kit's introductions.

Assessment of immunohistochemical staining results

VEGF staining results were determined by the semiquantitative scoring method (8). Four representative high-power fields were selected, with 100 cells counted for each field. The slices were scored based on the degree of staining and percentage of stained cells: 0 point for unstained, 1 point for light yellow, 2 points for brown, 3 points for dark brown. The percentage of stained cells to counted cells was scored as 0 to 3 points: 0 point for ≤5%, 1 point for 6%-25%, 2 points for 26%-50%, and 3 points for ≥51%. The score of staining degree and that of percentage of stained cells for each slice were added as the final score: 0 point for negative (-), 1 to 2 points for weakly positive (+), 3 to 4 points for positive (++), and 5 to 6 points for strongly positive (+++).

Microvascular measurement

Microvessels were counted according to the Weidner's method (9). If single endothelial cells stained bluish violet existed in clusters and were separated from adjacent blood vessels, parenchymal cells and mesenchymal components, a blood vessel count was determined. There is no need to determine whether they are blood vessels by the appearance of erythrocytes, or by the complete lumen. The complete slice was observed at low magnification to find three regions with high-density blood vessels for counting. The average of microvascular numbers in the three regions was MVD of this sample. Immunohistochemical staining, result determination and MVD calculation were completed by two pathologists blinded to this study.

In situ hybridization and immunohistochemical double-labeled staining

The transformation of transplanted cells into vascular endothelial cells was verified by *in situ* hybridization staining of genes in the sex-determining region of Y chromosome in combination with immunohistochemical CD34 antigen staining. After transplantation, MSCs that did not express CD34 had no phenotypic changes, indicating the transformation into vascular endothelial cells.

Detailed experimental steps: 1) Paraffin sections were rehydrated with gradient concentrations of ethanol solutions; 2) the permeability of tissues and cells was elevated for 10 min at room temperature, allowing the probe to penetrate the cell membrane rapidly; 3) digestion working solution was dropwise added to cover the tissue surface for 10~30 min at 37°C; 4) the sample was incubated in 0.1 mol/L TBS for 15~20 min at 95°C, washed with 0.1 mol/L cold TBS 3 times (5 min each time), placed in 50% formamide on ice bath for 20 min, and washed 3 times with 0.1 mol/L TBS (5 min each time); 5) then the tissue sample was dropwise added pre-hybridization working solution and incubated in a wet box for 1 h at 37°C; 6) it was washed after pre-hybridization; 7) it was dropwise added hybridization working solution and incubated in a wet box for 4 h at 37°C; 8) it was washed again; 9) the tissue sample was dropwise added a mixture of

peroxidase-conjugated avidin working solution (1:800 diluted) and rabbit anti-rat CD34 antibody (1:150 diluted); 10) then it was dropwise added biotin-labeled goat anti-rabbit IgG working solution, incubated for 20 min at 37°C and washed 3 times with PBS; 11) it was thereafter dropwise added alkaline phosphatase-labeled streptavidin working solution, incubated for 20 min at 37°C and washed 3 times with PBS; 12) after DAB color development, it was washed 3 times with PBS and twice with TSM1; 13) color development was conducted with a mixture of NBT (400 μg/ml) and 5-bromine-4-chlorine-3-indole phosphate (200 μg/ml) for 0.5~3 h in dark; 14) the reaction was stopped by adding 20 mmol/L EDTA; 15) nuclear counterstaining, dehydration and mounting with resin were finally carried out.

Statistical analysis

The categorical data were represented as $\overline{X} \pm$ S. The categorical data and numerical data were subjected to the t test and rank-sum test using SPSS19.0 software respectively. $P<0.05$ was considered statistically significant.

Results

Transformation of MSCs into vascular endothelial cells

In the postoperative feeding period, 2 rats in group A and 3 in group B died, respectively. Representative photographs of tissue samples were taken for three different rats from each group. The brown nucleus and unstained cytoplasm by *in situ* hybridization staining revealed that the cell contained Y chromosome and was indeed transplanted cell. The hyacinthine cell membrane and unstained nucleus by immunohistochemical staining showed that the cell membrane expressed CD34, verifying the nature of vascular endothelial cell. The bluish violet membrane of double-labeled cell and brown nucleus suggested that the transplanted cell was transformed into vascular endothelial cell. After 21 days of injection into ischemic biliary lesion, double labeling revealed that a small portion of Y-chromosome positive cells (brown nucleus) expressed CD34 (bluish violet in immunohistochemical staining) phenotype in the vascularized area of group

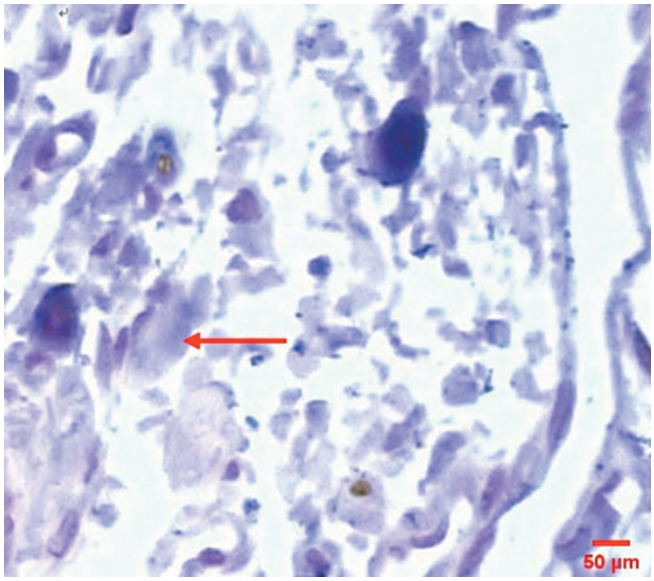

Figure 1: MSC phenotype change and CD34 expression in group A. MSCs were subjected to *in situ* hybridization staining and CD34 immunohistochemical staining (double labeling). Scale bar: 50 μm; magnification: 200×. CD34 was expressed (bluish violet by immunohistochemical staining; red arrow) in a small portion of Y-chromosome positive cells (brown nucleus) in the vascularized area

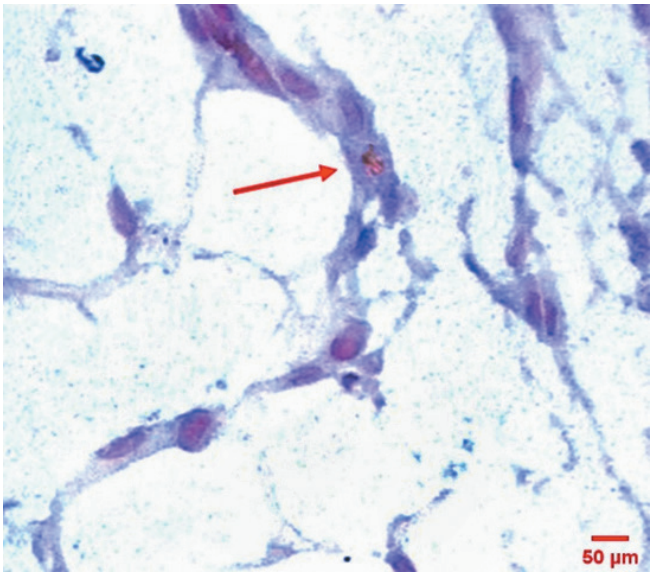

Figure 2: Transformation from MSC into adipose cell in group A. MSCs were subjected to *in situ* hybridization staining and CD34 immunohistochemical staining (double labeling). Scale bar: 50 μm; magnification: 200×. CD34 was expressed (bluish violet by immunohistochemical staining; red arrow) in adipose cells

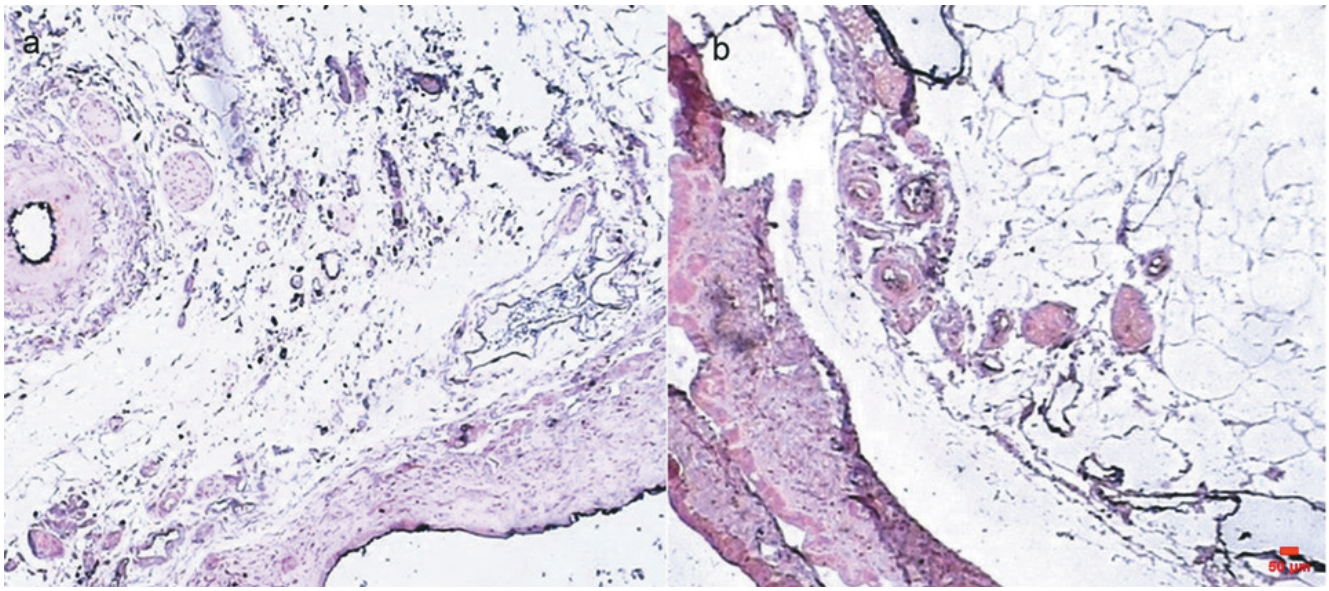

Figure 3: Vascular staining results of (a) group A and (b) group B. MVD of group A was higher than that of group B. Scale bar: 50 μm; magnification: 10×

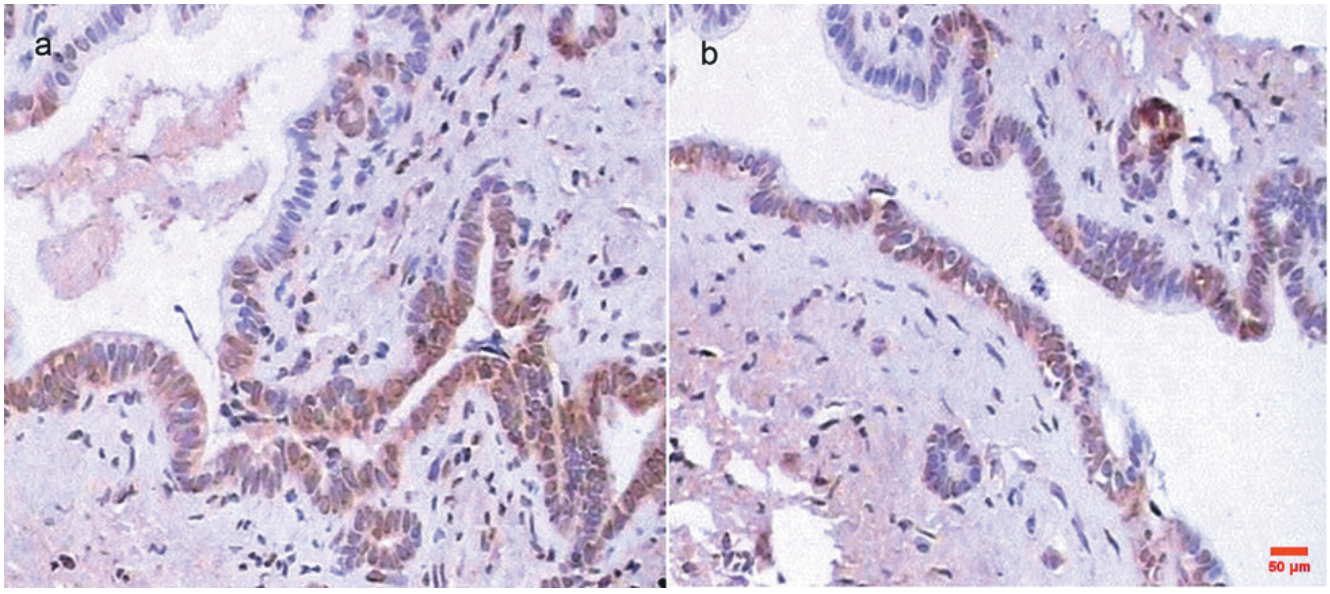

Figure 4: VEGF expressions of (a) group A and (b) group B. VEGF expression in group A was much higher than that of group B. Scale bar: 50 μm; magnification: 100×

Table 1: VEGF expressions of two groups

Group	Case number	VEGF expression scoring			
		-	+	++	+++
A	21	0	2	7	12
B	20	0	3	10	7

Mann-Whitney U Test, $P<0.05$

A (Figure 1; scale bar: 50 µm; magnification: 200×). Although transplanted cell undergoing canalization was absent, Y-chromosome positive cells were found in adipose tissue (Figure 2; scale bar: 50 µm; magnification: 200×), indicating that MSCs turned into adipose cells.

MSCs promoted angiogenesis by increasing VEGF expression

After 21 days of injection into ischemic biliary lesion, vascular staining showed that MVD of group A was higher than that of group B (Figure 3; scale bar: 50 µm; magnification: 10×), which were calculated as (63.3 ± 17.6)/HP and (53.2 ± 13.8)/HP, respectively ($P<0.05$). Moreover, cytoplasmic staining exhibited that the VEGF expression in group A was much higher, mostly in the MSC transplantation area (Figure 4; scale bar: 50 µm; magnification: 100×). Table 1 also shows that the VEGF expression in group A significantly exceeds that in group B ($P<0.05$).

Discussion

MSCs are pluripotent stem cells originating from the early mesoderm and ectoderm, with high heterogeneity and multipotential differentiation capacity. MSCs can express mRNAs of multilineage cells, such as chondrocytes, adipocytes, neurons, myocytes and endothelial cells. Endothelial cells, which exist in the mesoderm, are first differentiated during embryonic development, so MSCs may differentiate into endothelial cells. Kinnaird et al. further proved that bone marrow-derived stem cells secreted vascular cytokines to stimulate the proliferation and migration of endothelial and smooth muscle cells via paracrine signaling, finally promoting arteriogenesis (10). After marrow stromal cell–derived conditioned medium was locally injected, the limb damage and tissue atrophy in a murine model of hindlimb ischemia were relieved.

MSCs may retain some characteristics of stem cells, being capable of differentiating into the structural components of vascular wall *in vitro* and promoting the angiogenesis of ischemic lower limbs *in vivo* (11). Iwase et al. compared the roles of MSCs and myelomonocytes in the angiogenesis of ischemic lower limbs, and found that the angiogenesis of both cells increased, but that of the former was more obvious (12). MSC has stronger viability in the ischemic environment than that of myelomonocyte, which can differentiate into either endothelial cell or vascular smooth muscle cell. The mononuclear cell subset of bone marrow consists of a variety of cells, such as MSCs, hematopoietic cells, endothelial cells and committed cell lines (e.g. NK cells, T lymphocytes and B lymphocytes). Theoretically, the ideal cell type for cell therapy should lack commitment, which can facilitate angiogenesis and trigger vasculogenesis. Collectively, MSCs have both high plasticity and viability (13).

After intramyocardial injection with 1 ml of 5×10^7/ml MSCs, labeled MSCs appear in infarcted areas (14), and the cardiac function is not enhanced with increasing number of transplanted cells. In this study, 4×10^7/ml MSCs were injected. Similarly, Hashemi et al. used 2.5×10^7 cells/ml and found uninjured MSCs in infarcted areas (15).

We herein evaluated the effects of MSCs on the improvement of blood supply for impaired biliary tract after being transplanted to ischemic biliary lesion. Angiogenesis refers to the growth of new blood capillary vessels originating from existing ones and post-capillary venule. Angiogenesis is an extremely complicated process, generally including vascular endothelial matrix degradation, migration and proliferation of endothelial cells, as well as formation of vascular ring and new basilar membrane. Therefore, the enhancing effects of MSCs on the blood supply of biliary tract can be directly assessed by detecting the formation of blood capillary vessels, using markers labeling the vascular endothelium, i.e. F8RA/Vwf, CD31 and CD34. CD34 is a transmembrane glycoprotein with a molecular weight of 110 kD, which is expressed in white blood cells, endothelial cells and stem cells. It has the strongest expression in capillary endothelial cells, followed by the artery, vein, arteriole and venule. $CD34^+$ transplanted cells verify the transformation of MSCs into vascular endothelial cells. Thus, CD34 was selected as the marker in this study. The combination of *in situ* hybridization staining and immunohistochemical double labeling showed that MVD of group A was higher than that of group B, and a small portion of Y-chromosome positive cells (brown nucleus) expressed CD34 (bluish violet in immunohistochemical staining) phenotype in the vascularized area of group A after 21 days of MSC injection. Given that female rats were recipients,

the Y-chromosome positive cells must originate from the transplanted cells of male donor rats. MSCs themselves do not express CD34. Accordingly, the double-labeled positive cells demonstrated that cell phenotype changed after MSC transplantation and turned into vascular endothelial cells. Although no transplanted cells underwent canalization, they expressed CD34 in adipose tissue, confirming their survival and growth around the biliary tract. Group A had significantly higher VEGF expression and MVD than those of group B ($P<0.05$). Thus, MSCs survived after transplantation and promoted angiogenesis by increasing VEGF expression, being consistent with previous literatures (16,17). Previous studies on the transformation of MSCs into vascular endothelial cells have focused on the myocardium and skeletal muscle. Comparatively speaking, the biliary tract has fewer tissues around, and cells after transplantation lack a living environment that they can rely on. Hence, we performed MSC transplantation after using the greater omentum to wrap the biliary tract. After transplantation, MSCs promoted angiogenesis to improve the blood supply for biliary tract by transforming into vascular endothelial cells and secreting angiogenic factor. Besides, another significance of this study is that immunosuppressive therapy is not required for allogenic MSC transplantation. MSCs from human beings have low immunogenicity (18). Moreover, allogenic MSCs can be transplanted into non-myocardial tissues lacking immunodepression on a long-term basis (19). Therefore, allogenic MSCs are potentially superior to autologous hematopoietic stem cells in clinical practice. For example, during liver transplantation surgery in the future, MSCs can be injected around the biliary tract owing to the large possibility of ischemic stenosis.

In conclusion, rat ischemic biliary injury can be safely and effectively treated through local transplantation of MSCs. MSCs improved biliary blood supply by differentiating into vascular endothelial cells, secreting angiogenic factor and increasing blood vessels. MSC transplantation may be an alternative method for treating ischemic diseases.

Acknowledgments

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UČINKI TRANSPLANTACIJE MEZENHIMALNIH MATIČNIH CELIC V ISHEMIJSKO TKIVNO POŠKODBO ŽOLČNIH KANALOV NA IZRAŽANJE ŽILNEGA ENDOTELIJSKEGA FENOTIPA CELIC IN POVEČANJE CELIČNE GOSTOTE

W. Jiang, L. Lu

Povzetek: V študiji smo želeli oceniti učinke presaditve mezenhimskih matičnih celic (MSC) v ishemična tkiva poškodovanih žolčnih kanalov pri podganah na izražanje fenotipa žilnih endotelijskih celic in na žilno gostoto. Celice kostnega mozga podganjih samcev seva Wistar, starih 2 – 4 tedne smo gojili v celični kulturi v ustreznih gojiščih. Šestinštirideset samic podgan je bilo naključno razdeljenih v presaditveno skupino (skupina A) in ne-presaditveno skupino (skupina B). Skupini A smo presadili MSC (1×10^6 celic/mesto, skupaj 8 mest) v ishemično poškodbo žolčnih kanalov. Po 21 dneh so bili poškodovani žolčni kanali odvzeti za histološke preiskave. Presajene MSC so bile spremljane s kombinacijo dveh metod - hibridizacije *in situ*, s katero smo označili gen Y za določanje spola, ter z imunohistokemičnim barvanjem proti beljakvoini CD34 (dvojno označevanje). Mikrovaskularni in vaskularni endotelni rastni faktorji so bili označeni z imunohistokemičnim barvanjem, na podlagi katerega smo izračunali mikrovaskularno gostoto (MVD). Enaindvajset dni po injiciranju je dvojno označevanje pokazalo, da je majhen del celic s kromosomov Y (presajenih celic) v skupini A izražal antigen CD34. MVD skupine A (63 ± 18)/HP) je bil večji od MVD v skupini B ((53 ± 14)/HP) in izražanje žilnih endotelnih rastnih faktorjev (VEGF) se je statistično značilno razlikovalo med skupinama ($P < 0,05$). Tvorba žilnih endotelnih celic po presaditvi MSC je izboljšala preskrbo žolčnih kanalov s krvjo preko izločanja VEGF.

Ključne besede: mezenhimske matične celice; presaditev; hibridizacija *in situ*; imunohistokemija; poškodba žolčnih kanalov

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