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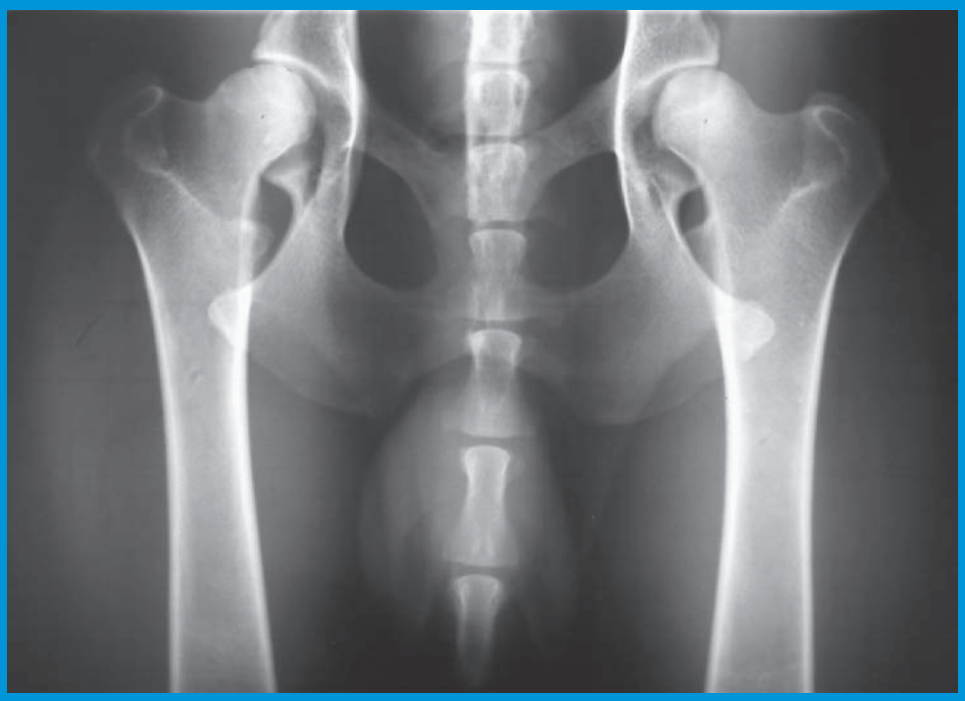

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Address: Veterinary Faculty, Gerbičeva 60, 1000 Ljubljana, Slovenia
Naslov: Veterinarska fakulteta, Gerbičeva 60, 1000 Ljubljana, Slovenija
Tel.: +386 (0)1 47 79 100, 47 79 210, Fax: +386 (0)1 28 32 243
E-mail: slovetres@vf.uni-lj.si

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Review Paper

ACID-BASE AND ION REGULATION DURING EXERCISE WITH EMPHASIS ON HORSES

Modest Vengušt

Address of author: Clinic for Reproduction and Horses, Veterinary Faculty, Gerbičeva 60, 1000 Ljubljana, Slovenia

E-mail: modest.vengust@vf.uni-lj.si

Summary: Exercise induced intracellular and extracellular acidosis requires a number of homeostatic adaptations to return acid base status to its resting level. The purpose of this review is to briefly describe the quantitative approach to these homeostatic adaptations, and to describe intracellular (intramuscular and intraerythrocyte) and extracellular (plasma) ion kinetics during exercise. Special consideration is given to horses.
Ions and carbon dioxide (CO_2) movement between muscle and plasma and output of CO_2 by the respiratory system play a critical role in acid base homeostasis. The skeletal muscle acidosis is largely driven by a fall in intramuscular strong ion difference due to increase in intramuscular lactate concentration (La^-) and reduction in intramuscular potassium concentration (K^+). The increase in intramuscular hydrogen ion concentration is buffered by a reduction in creatine phosphate concentration (CrP^{2-}), and by a small change in the apparent equilibrium constant (K_A) for weak acid buffers. Diffusion of La^- into venous blood and its associated metabolism, re-uptake of K^+, and diffusion of CO_2 from muscle and its pulmonary elimination contribute to the resolution of acidosis. CrP^{2-} is resynthesized, and K_A reverts to its resting value.

Key words: exercise - physiology; muscles - physiology; acid - base equilibrium; acidosis, lactic; horses

Introduction

Rapid increases in ATP turnover during intense, short-term exercise result from substrate and oxidative phosphorylation (1, 2, 3, 4, 5, 6), which causes a series of metabolic and ionic events that contribute to changes in acid base status. Ion and CO_2 movement between muscle and plasma play an essential role in that process. Metabolic processes driven by exercise necessitate rapid increases in gas exchange within the active skeletal muscle and across the lung. They decrease (mildly) intramuscular/intracellular bicarbonate ($_m[HCO_3^-]$) and increase intracellular hydrogen ion concentration ($_m[H^+]$). Changes in $_m[HCO_3^-]$ and $_m[H^+]$ are due to increases in intracellular lactate concentration ($_m[La^-]$) and loss of intracellular potassium concentration ($_m[K^+]$), which reduces the intracellular strong ion difference ($_m[SID]$), thereby generating carbonic acid (1, 7).

Most ionic events during exercise share great similarities between species studied. Horses are specially adapted to exercise and have been extensively studied for acid base physiology. Only differences in acid base regulation that are very particular to horses are emphasized in the text below.

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A quantitative approach to acid-base chemistry

The application of a physicochemical approach to the regulation of acid-base status in intra- and extracellular space clarifies the links between fluid and electrolyte control and the physiological and biochemical events occurring during exercise in muscle, circulation, and the lungs (8, 9, 10). It quantifies the relative contributions of three independent variables: strong ion difference (SID), weak electrolyte concentrations (A_{tot}), and the partial pressure of CO_2 (pCO_2) to changes in dependent variables ($[H^+]$, $[HCO_3^-]$) in aqueous solutions. Changes in $[H^+]$ can be achieved only by changing one or more of these three independent variables. The system is constrained by three fundamental physical laws: conservation of mass (equations 2 & 9), electro-neutrality (equation 12) and the equilibrium constraints on dissociation reactions (equations 5 - 8).

Strong ions are by definition electrolytes that, based on their K_A, completely dissociate in physiological aqueous solutions at physiological $[H^+]$. The net effect of the presence of strong ions can be expressed in terms of the difference between the total concentration of strong base cations and strong acid anions. This is termed strong ion difference (SID):

$$[SID^+] = \Sigma\ [\text{strong cations}] - \Sigma\ [\text{strong anions}] \quad (1)$$

Weak electrolytes are only partially dissociated in H_2O at physiological $[H^+]$. A_{tot} is used to express the total available anionic charge of the weak electrolytes, which consist of associated (HA) and dissociated (A^-) forms:

$$[A_{tot}] = [A^-] + [HA] \quad (2)$$

Carbon dioxide, a major end product of cell metabolism is, under physiological temperature, $[H^+]$, and pressure, moderately soluble in H_2O. It also reacts with H_2O to form several other solute compounds, all of whose concentrations are dependent variables. The amount of dissolved CO_2 (dCO_2) is directly proportional to its partial pressure (pCO_2) in the gas phase and its solubility coefficient (SCO_2):

$$dCO_2 = SCO_2\ (pCO_2) \quad (3)$$

During exercise CO_2 moves down its partial pressure gradient from a working muscle into circulation and is then removed through the respiratory system. Dissolved CO_2 reacts with H_2O to form carbonic acid (H_2CO_3), which further dissociates into H^+ and HCO_3^- (hydration of CO_2); HCO_3^- then further dissociates to form H^+ and CO_3^{2-}. The process is catalyzed by the enzyme carbonic anhydrase (CA):

$$H_2O + CO_2 \xleftarrow{CA} H_2CO_3 \leftrightarrow H^+ + HCO_3^- \leftrightarrow H^+ + CO_3^{2-} \quad (4)$$

The dissociation of the H_2CO_3 can also be formed as the mass action equation:

$$K_a\ [dCO_2] = [H^+] + [HCO_3^-] \quad (5)$$

Where K_a is the dissociation constant, incorporating the dissociation constants for hydration and dehydration of the CO_2. Based on Equation (3), the following can be substituted:

$$K_a\ [SCO_2\ (pCO_2)] = [H^+] + [HCO_3^-] \quad (6)$$

The dissociation constant incorporating hydration and dehydration of CO_2 (K_a) can be combined with SCO_2 to form the constant K_C:

$$K_c\ [pCO_2] = [H^+] + [HCO_3^-] \quad (7)$$

As mentioned above, HCO_3^- further dissociates to form H^+ and CO_3^{2-}:

$$K_3\ [HCO_3^-] = [H^+] + [CO_3^{2-}] \quad (8)$$

Where K_3 is the equilibrium dissociation constant for HCO_3^-.

H^+ homeostasis in physiological aqueous solutions is most readily described in H_2O:

$$H_2O \leftrightarrow OH^- + H^+ \quad (9)$$

The law of mass action further on transforms Equation 9:

$$Kw\ [H_2O] = [H^+]\ [OH^-] \quad (10)$$

The concentration of H_2O is 10^9 greater than $[H^+]$ and the dissociation constant of water (K_w) is small. Therefore, $[H_2O]$ itself can be considered a constant (K'_w):

$$K'w = [H^+]\ [OH^-] \quad (11)$$

K'_w changes with temperature; therefore, temperature changes will manipulate the $[H^+]$. The increase in temperature will increase $[H^+]$, and vice versa.

Based on the information above water interacts with the weak electrolyte system (Equation 2), as well as the CO_2 system (Equations 7 and 8). It will also interact with strong electrolytes:

$$[SID] + [H^+] - [HCO_3^-] - [A^-] - [CO_3^{2-}] - [OH^-] = 0 \quad (12)$$

The above equations can be rearranged and combined into a single equation for $[H^+]$ in terms of independent variables and the equilibrium constants of each system that interacts in the solution:

$$[H^+]^4 + (K_A + [SID])\ [H^+]^3 + (K_A \times ([SID] - [A_{tot}]) - (K_C \times pCO_2 + K'w))\ [H^+]^2 - (K_A \times (K_C \times pCO_2 + K'w) + K_3 \times K_C \times pCO_2))\ [H^+] - (K_A \times K_3 \times K_C \times pCO_2) = 0 \quad (13)$$

In conclusion, in the above series of equations independent variables (SID, A_{tot}, pCO_2) interact with the concentration of four dependent variables (H^+, HCO_3^-, CO_3^{2-}, OH^-) employing three

fundamental physical laws: conservation of mass, electro-neutrality, and equilibrium dissociation of weak acids and water (8, 9, 10, 11).

Ion regulation in exercise - intracellular events

The intramuscular acidosis develops mainly due to a large fall in $_{m}[SID]$, secondary to increases in $_{m}[La^{-}]$ and reductions in $_{m}[K^{+}]$. Further, the $_{m}[H^{+}]$ is increased by a reduction in $_{m}[CrP^{2-}]$, and partly due to a large increase in $_{m}PCO_{2}$. Intramuscular A_{tot} may (12) or may not (7) contribute to the intracellular acid-base changes. The rise in $_{m}[H^{+}]$ is also modulated by a change in the K_{A} for weak acid buffers (7).

A rapid increase in $_{m}[H^{+}]$, decrease in $_{m}[K^{+}]$, and the accumulation of $_{m}[La^{-}]$ contribute to changes in sarcolemmal and transverse tubular membrane potential, which further alters the $_{m}Ca^{2+}$ homeostasis and contractile function of skeletal muscle (12, 13, 14). K^{+} release from skeletal muscle depends on the intensity of contraction and is proportional to number of action potentials per unit of time (15, 16, 17, 18). In skeletal muscle, K^{+} kinetics are regulated by Na^{+}-K^{+} ATPase and K^{+} channels (19, 20). During muscle contraction the high rate of K^{+} efflux cannot be compensated by slower inward K^{+} transport by the Na^{+}- K^{+} ATPase; therefore, voltage gated K^{+} channels that open during the repolarization phase of the action potential must primarily modulate the high rate of K^{+} efflux (19). ATP sensitive K^{+} (K_{ATP}) channels located in the sarcolemma may contribute to the net loss of K^{+} from muscle (19, 21, 22, 23). It was originally suggested that the K_{ATP} channels are only activated in metabolically exhausted muscle fibers (24) and that the activity of the channels contributes to the decrease in force during fatigue (22). However, the fact that glibenclamide, the selective inhibitor of K_{ATP} channels, has no effect on interstitial $[K^{+}]$ during exercise, suggests that K_{ATP} channels are not important for K^{+} release during muscle contractions in human muscle (25). Another group of K^{+} channels, the large Ca^{2+} activated channels, are reported to be present in rat skeletal muscle T-tubules (26). The function of these channels in muscle is unknown (25).

La^{-} is removed from skeletal muscle by a bi-directional monocarboxylate carrier that is responsible for 70-80% of the La^{-} flux. La^{-} diffusion as undissociated lactic acid according to the prevailing transmembrane $[La^{-}]$ and $[H^{+}]$ gradients across the plasma membrane and nonspecific anion exchange, plays a minor roles in La^{-} removal from skeletal muscle (27, 28, 29, 30, 31).

While the intramuscular proton-buffering mechanisms contribute over the short term to the regulation of $_{m}[H^{+}]$, its restoration after exercise depends on the recovery of $_{m}[K^{+}]$ and on the removal of $_{m}La^{-}$. Hence diffusion of La^{-} into venous blood and its associated metabolism, re-uptake of K^{+}, and diffusion of CO_{2} from muscle and its pulmonary elimination accomplish the resolution of acidosis. CrP^{2-} is re-synthesized, and K_{A} reverts to its resting value (1, 6, 32). However, based on a quantitative approach to acid-base chemistry the recovery from intracellular acidosis develops as an interaction between SID, A_{tot}, and pCO_{2} between the intracellular (ICF) and extracellular fluid (ECF), resulting in restoration of normal $_{m}[H^{+}]$. The restoration of $_{m}[H^{+}]$ occurs at the expense of increasing the $[H^{+}]$ in interstitial fluid, plasma, and erythrocytes. Plasma and erythrocytes contribute to recovery by redistributing CO_{2} to the lungs and SID to other tissues. It may appear that erythrocytes provide a first line defense within the blood to attenuate the large and abrupt increase in plasma $[La^{-}]$ and $[H^{+}]$ that occur with high-intensity exercise (33, 34, 35, 36). However, with regards to $[K^{+}]$, erythrocyte $[K^{+}]$ ($_{e}[K^{+}]$) in studies by McKelvie et al. (33) and Lindinger et al. (34) was calculated from whole blood and plasma K^{+} content as well as hematocrit. When $_{e}[K^{+}]$ is established from direct measurements of K^{+} content in a fixed volume of sedimented (packed) cells, the changes in $_{e}[K^{+}]$ of arterial and venous blood during exercise appeared to be due to water shifts and not due to fluxes of K^{+} between erythrocytes and plasma (37, 38). It has been proposed that contracting muscle and, partially, the inactive tissue, can take up K^{+} probably by a combination of K^{+} and hormone activation of the Na^{+}- K^{+} pump (38). However, it appears that Juel *et al.* (38) neglected the importance of erythrocyte volume (EV), as changes in EV will influence the hematocrit and therefore [ion] (33).

Ion regulation in exercise - intravascular/extracellular events

Changes in plasma [SID] occur as a consequence of fluid shifts between different compartments, and exchange of strong ions across the capillary and erythrocyte membrane. Changes in plasma volume between the vascular and extravascular compartments are forced by changes in hydrostatic and osmotic forces acting between these compartments. The increase in the

plasma [K^+] may be contributed to a decrease in plasma volume and an efflux of [K^+] from the active skeletal muscle (33, 34, 37, 38). Plasma [La^-] changes are balanced between release of La^- into circulation from the active muscle, and its uptake and metabolism (34, 39). Na^+ and Cl^-, despite their relative increase in plasma during exercise, seem to move out of the vascular space, as the relative increases in plasma is less than that predicted from the change in plasma volume only (34, 39).

In recovery plasma [K^+] ($_p[K^+]$) decreases promptly and $_p[Na^+]$ increases as a consequence of a high rate of Na^+- K^+ ATPase activity in skeletal muscle and other tissues. Plasma [La^-] decreases gradually as La^- continues to move from skeletal muscle down its concentration gradient. The magnitude of La^- movement is governed by the intensity of muscle work (1, 40, 41, 42, 43, 44, 45, 46, 47, 48). In horses, erythrocytes have been suggested to function as a lactate sink (35, 36); up to 50% of horse blood lactate is in erythrocytes (35, 49, 50), whereas the corresponding value in man is 17% (51).

Changes in A_{tot}, an ion equivalent of the total available amino acids from proteins, also influence the plasma acid base status (10, 52). In horses the anionic equivalent for plasma proteins is 0.21 mmol/L of plasma protein (53). The increase in $_p[A_{tot}]$ during exercise is accounted for by the decrease in plasma volume (7, 52).

Conclusion

Several ionic events regulate the acid base equilibrium in living organism. Both, the intracellular and extracellular compartments must be regulated in an integrated manner to enable strong ion exchange between the muscle, plasma, and erythrocytes, which contributes to the resolution of acidosis. Quantitative approach to acid base clarifies the acid base chemistry by recognizing the acid base independent variables (SID, PCO_2, A_{tot}) and their influence on acid base dependent variables ([H^+], [HCO_3^-]). It quantifies the contribution of particular ions or CO_2 to acid base homeostasis at rest and during exercise.

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REGULACIJA ACIDOBAZNEGA RAVNOTEŽJA IN RAVNI IONOV PRI TELESNEM NAPORU S POSEBNIM OZIROM NA KONJE

M. Vengušt

Povzetek: Pri telesnih obremenitvah (športu) nastala acidoza znotraj in zunaj celičnega prostora zahteva številne fiziološke prilagoditve za uravnoteženje acidobaznega sistema na fiziološki nivo, ki je značilen za mirovanje. Namen tega prispevka je opisati kvantitativen pristop k razumevanju acidobazne fiziologije pri telesnih obremenitvah in razložiti dinamiko ionov znotraj (eritrociti, mišična celica) in zunaj celice (plazma), s posebnim poudarkom na konjih.

Prehajanje ionov in ogljikovega dioksida (CO_2) med mišicami in plazmo ter odstranjevanje CO_2 z dihanjem igrata pomembno vlogo pri ohranjanju acidobaznega ravnotežja. Acidozo oz. povečano koncentracijo vodika ($[H^+]$) v skeletni mišici povzroči znižanje razlike v koncentraciji močnih ionov (SID). SID se zniža zaradi povečane koncentracije laktata (La^-) in znižane koncentracije kalija (K^+) v mišici. Preveliko povečanje $[H^+]$ v mišici pri obremenitvah pa se ublaži z zmanjšanjem koncentracije kreatin fosfata (CrP^{2-}) in z manjšo spremembo navidezne ravnotežnostne konstante (KA) šibkih kislin. Prehod La^- in CO_2 iz mišice v vensko kri ter porast K^+ v mišicah po prenehanju obremenitve prispevajo k odpravi acidoze. Koncentracija CrP^{2-} se obnovi, KA pa se vrne na svojo izhodiščno vrednost.

Ključne besede: napor - fiziologija; mišice - fiziologija; acido - bazno ravnotežje; acidoza, laktatna; konji

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Original Research Paper

THE EFFECT OF HIGH ANTICOAGULANT K_3-EDTA CONCENTRATION ON COMPLETE BLOOD COUNT AND WHITE BLOOD CELL DIFFERENTIAL COUNTS IN HEALTHY BEAGLE DOGS

Alenka Nemec [1]*, Marinka Drobnič–Košorok [2], Janoš Butinar [1]

Addresses of authors: [1] Clinic for Small Animal Medicine and Surgery, [2] Institute of Physiology, Pharmacology and Toxicology, Veterinary Faculty, Gerbičeva 60, 1000 Ljubljana, Slovenia

* Corresponding author, Email: alenka.nemec@vf.uni-lj.si

Summary: The sodium or potassium salts of ethylenediaminetetraacetic acid (EDTA), anticoagulants recommended for routine haematological analyses, are known to have deleterious effects on platelets, erythrocytes and leukocyte counts even at recommended concentrations (1,8 mg/mL of blood). Under-filling of tubes containing K_3EDTA due to difficulties in sampling that produces higher K_3EDTA concentrations than recommended may occur in severely hypotensive, restless, very small or obese dogs and cats. The aim of the present study was to investigate the effect of high anticoagulant K_3EDTA concentration on complete blood count (CBC) and white blood cell differential count (WCDC) in healthy beagle dogs. The recommended volume of 3 and 1 mL of blood were put in K_3EDTA containing tubes to obtain recommended (1.8 mg/mL) and high (5.4 mg/mL) K_3EDTA concentrations (under-filled tubes), respectively. CBC and WCDC were determined with a laser haematology analyser with species-specific software. Presence of platelet clumps was determined by examination of blood smears. The results showed a significant (P<0.05) decrease in hematocrit (HCT) from 0.55 ± 0.043 to 0.48 ± 0.042 (13.4 ± 4.8% decrease) and mean corpuscular volume (MCV) from 74.4 ± 1.1 to 65.4 ± 3.0 fL (11.9 ± 4.2% decrease), and a significant increase in mean corpuscular haemoglobin concentration (MCHC) from 306.5 ± 5.7 to 349.7 ±17.8 g/L (14.0 ± 5.9% increase) in samples with high K_3EDTA concentration in comparison with the recommended concentration. No platelet clumping was detected in all examined blood smears. According to the results of our study, we recommend respecting the required blood to anticoagulant ratio, as under-filling of tubes leads to a high final concentration of K_3EDTA, which affects values of HCT, MCV and MCHC and thus interpretation of haematological results in dogs.

Key words: anticoagulants; edetic acid - pharmacology; hematologic tests; blood cell count; leukocyte count; dogs

Introduction

Several studies have revealed that various anticoagulants used for collection of blood specimens produce different effects on results of haematological analyses (1, 2, 3, 4, 5). Ethylenediaminotetraacetic acid (EDTA) is the anticoagulant recommended for complete blood cell counts (CBC) and white blood cell differential count (WCDC) by the National Committee for Clinical Laboratory Standards (6), principally for its cell preservation properties. Among sodium (Na) and potassium (K) salts of EDTA, the International Council for Standardization in Hematology (ICSH) currently recommends the dipotassium salts of EDTA (K_2-EDTA) as the anticoagulant for CBC (7). Under optimal conditions (appropriate anticoagulant concentration and analysis within 1-4 h of sample collection), the choice of K_2 or K_3-EDTA has little effect on the results of CBC and WCDC (8).

However, EDTA, even when using recommended EDTA concentrations, is known to have deleterious effects on platelets (9, 10, 11, 12, 13), erythrocytes (1, 13, 14, 15, 16) and leukocyte counts (5, 17, 18, 19) and occasionally dogs and cats have strong aggregations of platelets or white blood cells (WBCs) in EDTA blood tubes (20, 21, 22). The collection of blood in other anticoagulants (e.g., heparin, citrate) sometimes prevents or

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slows the aggregation allowing more accurate WBC counting (23).

The phenomenon of *in vitro* EDTA-induced platelet (PLT) clumping or agglutination is a well-recognized artefact, commonly reported in human medicine (10, 12, 15, 17, 18, 24, 25). The mechanism of agglutination or adherence (clumping) appears to involve EDTA-dependent platelet agglutinins (specific antibodies) present in the plasma (10, 26, 27, 28, 29, 30). There are few reports on EDTA-induced platelet clumping in veterinary medicine (31, 32, 33). EDTA-induced platelet clumping causes spuriously low PLT counts (pseudothrombocytopenia - PTP), which may lead to erroneous diagnosis, unnecessary and costly additional laboratory examinations, and inappropriate medical and surgical therapy or the unjustified withdrawal of essential medication. PTP may be accompanied by either spuriously high white blood cell (WBC) counts (pseudoleukocytosis) or occasionally spuriously low WBC counts (PLT-WBC adherence - pseudoleukopenia). Pseudoleukocytosis is due to the formation of PLT clumps large enough to mimic white cells on the WBC side of an impedance counters (Coulter S Plus IV/V, Technicon H*6000, Ortho ELT 8), whereas pseudoleukopenia may be due to the "gating out" of large PLT-WBC masses (5, 17, 18).

Blood sampling following a recommended blood to EDTA anticoagulant volume ratio is necessary to prevent coagulation. Under-filling is probably the most common phenomenon in clinical laboratories due to problems during blood sampling, particularly when blood is collected from very small, obese, severely hypotensive or restless patients. Under-filling of the tubes containing EDTA results in an excess of EDTA, which can reduce PCV and affect other haematological parameters (1, 2, 15, 23, 34).

The aim of the present study was to investigate the influence of high anticoagulant K_3EDTA concentration, produced by under-filling of tubes, on CBC and WCDC (white blood cell differential count) in healthy beagle dogs.

Material and methods

Animals

Thirty beagle dogs, eighteen females and twelve males, ranging from 8 months to 3 years, were selected and deemed healthy on the basis of history, results of physical examination and serum biochemical profiles (urea, creatinine, sodium, potassium, total bilirubin, albumin and alanine-aminotransferase - results not shown).

Sample collection

Venous blood samples were collected from fasted dogs using 5 mL syringes; 3 mL and 1 mL blood samples were put in two separate vacuum tubes containing K_3EDTA (5.40 mg) as anticoagulant, prepared for the collection of 3 ml blood. The final concentrations of K_3EDTA were: 1.8 mg/mL (recommended concentration) and 5.4 mg/mL (high concentration).

Both K_3EDTA blood samples for CBC and WCDC determination were stored at room temperature until analysis. Venous blood samples were also collected into plain tubes, which stood for 30 min at 4°C to clot, prior to centrifugation (1200 g for 10 min) and separation of serum.

Determination of haematological parameters – CBC and WCDC

CBC and WCDC were determined by an automated laser haematology analyser Bayer - Technicon H*1 with species-specific software (H*1 Multi-Species V30 Software). The measurements were performed within 1-4 h after venipuncture (5). CBC included white blood cells (WBC), red blood cells (RBC), haemoglobin concentration (HGB), haematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC) and platelets (PLT). WCDC represented a six-part differential (neutrophils (NEUT), lymphocytes (LYMP), monocytes (MONO), eosinophils (EOS), basophils (BASO) and large unstained cells (LUC). The LUC category consists of a heterogeneous population of all large cells that fail to exhibit any peroxidase activity (atypical lymphocytes, immature granulocytes and blasts).

Blood smears were made from single drops of blood anticoagulated with EDTA. Staining with May Grunwald-Giemsa solution was followed by microscopic examination of the smears for the presence of platelet clumps.

Statistical evaluation

Statistical analyses were done by use of the statistical programme SPSS 10.0 for Windows (SPSS – statistical package for social sciences). Means and standard deviations were calculated for all haematological and biochemical parameters. A paired *t* test was used to determine differ-

ences in CBC and WCDC parameters between blood samples with recommended and high K_3EDTA concentration. A value of $P<0.05$ was considered significant.

Results

The results of haematological analyses showed the influence of high K_3EDTA concentration on the following parameters of CBC (HCT, MCV and MCHC), while the parameters of WCDC were not affected (Tables 1 and 2). There was a significant ($P<0.05$) decrease in HCT of 13.4 ± 4.8% (from 0.55 ± 0.043 to 0.48 ± 0.042) and MCV of 11.9 ± 4.2% (from 74.4 ± 1.1 to 65.4 ± 3.0 fL), and a significant increase in MCHC (from 306.5 ± 5.7 to 349.7 ±17.8 g/L) of 14.0 ± 5.9% in samples with high K_3EDTA concentration compared to samples with the recommended final concentration of K_3EDTA.

Platelet clumping was not detected in any of the blood smears. No significant differences in PLT count (Table 1) were found between samples with recommended and high K_3EDTA concentrations. Despite the significant differences in HCT, MCV and MCHC between samples with recommended and high K_3EDTA concentrations, the CBC and WCDC parameters (mean values) in both types of samples remained within the normal reference ranges given by the producer of the analyser and data from the literature (35, 36, 37).

Discussion

Despite appropriate sample collection techniques, under-filling of tubes with blood and platelet clumping remains a common problem in veterinary medicine (31, 38, 39). Under-filling of tubes containing anticoagulant EDTA results in higher concentration of EDTA, which is known to cause spurious interferences, even at the recommended concentration, on platelets (5, 9, 10, 11, 12), erythrocytes (1, 13, 14, 15, 16) and leukocyte counts (5, 17, 18, 19). Occasionally, dogs and cats have strong aggregations of platelet or white blood cells (WBCs) in EDTA blood tubes (20, 21, 22, 23).

The results of the present study in healthy beagle dogs showed no significant changes in MPV and PLT count between samples with recommended and high K_3EDTA concentration. Unlike the present study, significantly lower PLT count has been measured in bulls in samples with high K_3EDTA concentrations due to EDTA-induced platelet clumping observed in blood smears (33).

In the present study, a significant decrease in HCT and MCV and a significant increase in MCHC was found in under-filled samples with high K_3EDTA concentration compared to the recommended K_3EDTA concentration. The significant changes of HCT, MCV and MCHC are ascribed to shrinking of erythrocytes in a hypertonic medium. Our results are consistent with the study of Chen et al. (1) who investigated the influence of under-filling of EDTA tubes using different human blood collection volumes. All salts of EDTA are hyperosmolar, which causes water to leave the cells, resulting in cell shrinkage. The higher the concentration of EDTA, the greater the osmotic withdrawal of water from the cells, leading to a reduction in PCV. This discrepancy will lead to a reduction in MCV and increase in MCHC (34). When insufficient blood is collected, such as frequently happens in small, obese, hypotensive and restless dogs, and also in cats, accuracy of HCT, MCV and MCHC is affected if K_3EDTA is used as the anticoagulant.

Following the results of our study, we recommend respecting the required blood to anticoagulant ratio, as under-filling of tubes leads to a high final concentration of K_3EDTA, which affects values of HCT, MCV and MCHC and thus interpretation of haematological results in dogs.

Table 1: Influence of K_3-EDTA on CBC in dogs

	WBC ($x10^9$/L)	RBC ($x10^{12}$/L)	HGB (g/L)	HCT* (L/L)	MCV* (fl)	MCH (pg)	MCHC* (g/L)	PLT ($x10^9$/L)
1.8 mg/mL K_3EDTA	9.04± 1.55	7.38± 0.60	168.1± 13.1	0.55 ± 0.043	74.4 ± 1.1	22.8± 0.5	306.5 ± 5.7	299.4± 65.5
5.4 mg/mL K_3EDTA	9.48 ±1.71	7.27± 0.55	166.0± 11.9	0.48 ± 0.042	65.4 ± 3.0	22.8± 0.4	349.7± 17.8	271.8± 70.6

*P< 0.05, paired t-test

The comparison of CBC values (mean ± SD) in blood samples of 30 healthy dogs with recommended concentration (1.8 mg/mL) and high concentration (5.4 mg/mL) of anticoagulant K_3EDTA

Table 2: Influence of K_3-EDTA on CBC in dogs

	NEUT %	LYMP %	MONO %	EOS %	BASO %	LUC %
1.8 mg/mL K_3EDTA	59.09±7.03	32.50 ± 7.09	2.86 ± 1.19	4.78 ± 3.21	0.18 ± 0.06	0.57 ± 0.24
5.4 mg/mL K_3EDTA	58.21±6.83	33.33 ± 6.69	3.13 ± 1.54	4.52 ± 3.23	0.28 ± 0.07	0.52 ± 0.19

The comparison of WCDC values (mean ± SD) in blood samples of 30 healthy dogs with recommended concentration (1.8 mg/mL) and high concentration (5.4 mg/mL) of anticoagulant K_3EDTA. Statistical comparison using paired t-test did not indicate any significant difference

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VPLIV VISOKE KONCENTRACIJE ANTIKOAGULANTA K_3-EDTA NA KRVNO SLIKO IN DIFERENCIALNO BELO KRVNO SLIKO PRI ZDRAVIH BIGLIH

A. Nemec, M. Drobnič-Košorok, J. Butinar

Povzetek: Kalijeve in natrijeve soli EDTA (etilendiaminotetraocetna kislina) se kot antikoagulanti največ uporabljajo v rutinski hematologiji, kljub temu da negativno vplivajo na trombocite, eritrocite in levkocite že pri priporočeni koncentraciji (1,8 mg/mL krvi). Pogosto se zgodi, da zaradi težav pri odvzemu krvi pri zelo majhnih, debelih, hipotenzivnih ali nemirnih psih in mačkah, odvzamemo premajhno količino vzorca krvi v epruvete z antikoagulantom K_3-EDTA, kar povzroči višjo koncetracijo EDTA, kot je priporočena. Namen naše raziskave je bil, ugotoviti vpliv visoke koncentracije K_3-EDTA na krvno sliko in diferencialno belo krvno sliko pri psih pasme bigel. V epruvete s K_3-EDTA smo odvzeli 3 mL (priporočena količina) in 1 mL (premajna količina) krvi in s tem dobili priporočeno (1.8 mg/mL) in visoko (5.4 mg/mL) koncentracijo K_3-EDTA. Krvno sliko in diferencialno belo krvno sliko smo določili z laserskim hematološkim analizatorjem, ki je zasnovan na računalniškem programu, s pomočjo katerega lahko določamo krvno sliko in diferencialno belo krvno sliko ljudem in živalim različnih vrst. Z analizo krvnih razmazov smo ugotavljali morebitno prisotnost skupkov trombocitov. V vzorcih z visoko koncentracijo K_3-EDTA smo, v primerjavi z vzorci s priporočeno koncentracijo K_3-EDTA, ugotovili statistično značilno (P 0,05) znižanje vrednosti hematokrita z 0.55 ± 0.043 na 0.48 ± 0.042 (13.4 ± 4.8% znižanje) in MCV s 74.4 ± 1.1 na 65.4 ± 3.0 fL (11.9 ± 4.2% znižanje) ter statistično značilno zvišanje MCHC s 306.5 ± 5.7 na 349.7 ± 17.8 g/L (14.0 ± 5.9% zvišanje). Analiza krvnih razmazov ni pokazala prisotnosti skupkov trombocitov v vseh analiziranih vzorcih. Na osnovi rezultatov raziskave priporočamo, da se pri odvzemu krvi upošteva zahtevano razmerje med količino odvzete krvi in količino antikoagulanta, saj visoka končna koncentracija K_3-EDTA, ki je posledica premajhne količine odvzetega vzorca krvi, vpliva na vrednost hematokrita, MCV in MCHC ter s tem na interpretacijo hematoloških rezultatov pri psih.

Ključne besede: antikoagulanti; edetska kislina - farmakologija; hematološki testi; krvnička, štetje; levkociti, štetje; psi

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Original Research Paper

PROGRESSION OF HIP DYSPLASIA IN 40 POLICE WORKING DOGS: A RETROSPECTIVE STUDY

Bojan Zorko [1]*, Teodora Ivanuša [1], Rok Pelc [2]

Addresses of authors: [1] Small Animal Clinic, Veterinary Faculty, Gerbičeva 60, 1000 Ljubljana; [2] Department for Police Working Dogs of Ministry for Internal Affairs, Zlatek 6, 1000 Ljubljana, Slovenia

* Corresponding author, Email: bojan.zorko@vf.uni-lj.si

Summary: The aim of the study was to evaluate the effects of progression of CHD and degenerative joint disease on a working capability of population of police working dogs. In the first part of the study, we reviewed the whole health documentation of all dogs and gathered all necessary radiographs of hip joints that were taken at first initial procurement. In the second part, clinical examination and radiographs of all dogs were taken 60 months after first examination and the progression of hip dysplasia was evaluated by the FCI scheme. All dogs were male, 35 German shepherds and 5 Rottweilers. The Norberg-Olsson angle in the first set of radiographs was 105.54 ± 3.22° in 37 dogs without hip dysplasia and 100.17 ± 2.99° in 3 dogs with initial mild dysplasia. In the second set of radiographs taken after 60 months of service the Norberg-Olsson angle was 105.60 ± 3.67° in 23 dogs with no signs of hip dysplasia and 101.62 ± 4.49° in 17 dogs with hip dysplasia. On the first set of radiographs, secondary degenerative changes were found in 3 dogs with initial mild dysplasia (7.5 %) and in 14 dogs (35 %) on second radiographs. The position of the centre of the femoral head on first radiographs was outside of dorsal acetabular rim in 25 (31.25 %) of estimated hip joints (n = 80); at the level of the rim in 30 (37.5 %) and inside in 25 (31.25 %). On second radiographs it was outside in 41 (51.25 %); at the level of the rim in 26 (32.5 %); and inside in 13 (31.25 %) of estimated hip joints. The mean femoral angle of inclination for all 40 dogs was 132.50 ± 4.39?°. If it has occured, the increase in degree of hip dysplasia was generally more than one degree. In 57.5 % of cases hip dysplasia was not determined on second radiographs. All dogs with some degree of hip dysplasia were German shepherds, but only one of them had clinical symptoms connected to CHD. The progression of hip dysplasia did not correlate with work period of the dogs, and has no influence on working ability of these dogs, despite their active duties. No dog in this study was lost due to CHD.

Key words: veterinary medicine; bone diseases, developmental; hip dysplasia, canine - radiography; dogs

Introduction

Hip dysplasia is a complex disease that affects humans and most domestic animals. The word dysplasia literally means abnormal development. Canine hip dysplasia (CHD) has been studied extensively since it was first reported in 1935 (1). The majority of research has been done on early diagnosis of CHD and its treatment.

CHD is not a congenital disease and cannot be diagnosed by conventional methods at birth. The hips are normal at birth but uneven growth between the skeletal and muscular system results in a femoral head that is forced out of the acetabular cup. Subluxation of the femoral head occurs at an early age when the components of the joint are still cartilaginous and thus soft and malleable. This results in deformation of the acetabulum and remodelling of the femoral head leading to the development of a painful osteoarthrosis. The course of the disease in a particular dog is influenced by growth rate, body weight, pattern of exercise and by other environmental factors (2).

The actual incidence of CHD is unknown, but the disease is known to affect large and giant breeds more commonly. Large-breed dogs are used by the police and armed Forces. Loss of military working dogs due to CHD has been reported to be 7 % in one study (3) and 12.9 % in another (4).

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A long-term retrospective study of the effects of CHD on a random population of police working dogs was not found in the literature. A retrospective study of CHD in military dogs in San Antonio Texas was done in 1996 (5) and 1971 (6). Numerous authors have suggested that a significant percentage of performance dogs are lost as a result of CHD. In one study 35 % of working dogs were lost due to CHD (7).

The present study was done to evaluate the possible effects of CHD on a population of working dogs and to answer the following questions: Did dogs initially graded as normal develop hip dysplasia or degenerative joint disease (DJD); was there any influence of CHD and degenerative joint disease on working capability; how many dogs were lost due to CHD; is present system of initial procurement radiographs and examinations of Slovenian police dogs appropriate.

Material and methods

Complete medical records and radiographic files of 40 police working dogs were selected from files maintained at the Department for police dogs of Ministry for Internal Affairs in Slovenia. The two breeds selected were German shepherd dog (35 dogs) and Rottweiler (5 dogs). For all 40 dogs medical records were required to be complete, and dogs had to be procured in 1992 or later. Al dogs were male.

On initial evaluation dogs were approximately 20 months old (19.75), on final examination they were 7 years old (6.78). Period between first and final radiographs was approximately 60 months.

All dogs performed similar duties and were kept in similar environment. All dogs had been active as police working dogs despite the presence or absence of CHD.

For each dog pelvic radiographs made at initial procurement and on final pelvic examination were graded. Each dog was clinically examined for any signs of lameness, pain or neurologic deficit. Before taking radiographs all dogs were sedated with medetomidin (0.05 mg/kg Domitor Finland).

A data sheat was prepared and each dog was assigned a number from one to 40. A complete review was performed of each dog's medical record. Data recorded included name and tattoo number, breed, sex, type of dog (patrol, detection...), age at procurement, date of procurement, origin of dog, total years of service, all clinical history and data relating to hind limb lameness or inability to perform to mission standards.

After all data was obtained two radiologists reviewed the pelvic radiographs for FCI grading and for the presence of DJD.

Pelvic radiographs of each dog were checked for proper labelling, dates and position and were verified in the medical record. They were graded according to the FCI current grading system from A to E. The Norberg-Olsson angle and angle of inclination were measured at first. Then the position of the centre of the femoral head relative to the dorsal acetabular rim was estimated. Each measurement was repeated three times and the mean value was recorded. The dogs with the presence of DJD were divided in six groups according to the location of osteophyte formation: Sclerosation of the femoral neck, changes on the femoral head, changes on the femoral neck, changes of the acetabulum, changes on the dorsal acetabular rim and Morgan line.

Dogs were considered to have CHD if they did not rate as A or B grade.

Norberg-Olsson angles, angles of inclination and position of the centre of the femoral head measurements were plotted against age and the time of examination. The relationship between angles, measurements and advancing age were investigated using correlation analysis.

The mean month of work for normal and dysplastic dogs were evaluated using Student t-test. The correlation between Norberg-Olsson angle and progression of DJD was estimated with Pearson's correlation coefficient.

Results

Out of the 40 evaluated, 3 dogs (7.5 %) were considered dysplastic at initial examination. All of them were German shepherd dogs and were graded as C1. They already had mild degenerative changes on femoral neck.

Grading of the second set of radiographs resulted in a total of 23 (57.5 %) normal dogs and 17 (42.5 %) dysplastic dogs. All dysplastic dogs were German shepherd dogs, 8 of them were graded as C1, 6 as C2, two as D1 and one as D2 (Fig. 1). 14 of them had mild to moderate secondary degenerative changes, mainly on the femoral neck and head. The increase in degree of hip dysplasia in all dogs was mainly just over one degree, if it has occurred (Fig. 2 and 3).

The total number of months worked by the normal dogs was compared with the total months worked by the dysplastic dogs. No significant difference was found between the two groups (p greater than 0.05). The mean working period in dysplastic dogs was 61.29 ± 22.84 months. The

mean working period in normal dogs was 59.87 ± 22.40 months.

On final clinical and neurological examination, 20 dogs (50 %) showed signs of hind-limb lameness or hind-limb ataxia and difficulty with obstacles. But only in one dog (2.5 %) this was due to DJD of the hip joint, in all other cases this signs were due to lumbosacral disease (typical back pain, hyperesthesia, anal hyporeflexia, pelvic limb paresis, self mutilation, tail paresis). No dog in this study was lost due to CHD.

At first radiographs mean Norberg-Olsson angle was 105.54 ± 3.22° in 37 dogs with no hip dysplasia and 100.17 ± 2.99° in 3 dogs with initial mild dysplasia. At second radiographs after sixty months of service mean Norberg-Olsson angle was 105.60 ± 3.67° in 23 dogs with no signs of hip dysplasia and 101.62 ± 4.49° in 17 dogs with CHD. In 14 dogs (35 %) initially considered normal and with signs of CHD on final radiograph, there was significant difference between Norberg-Olsson angle measurements (P = 0.041). Mean Norberg-Olsson angle in these dogs on initial radiographs was 104.54 ± 2.66° and 102.68 ± 3.90° on final examination.

On first radiographs secondary degenerative changes were found in 3 dogs (7.5 %) and in 14 dogs (35 %) on second radiographs. Only 3 of them had symmetrical changes. Mean Norberg-Olsson angle in dogs with CHD and DJD was 100.84 ± 4.54°. Mean Norberg-Olsson angle in dogs with signs of CHD but without signs of DJD was 102.60 ± 4.39°. Norberg-Olsson angle does not correlate with progression of DJD.

The mean femoral angle of inclination for all 40 dogs was 132.50 ± 4.39°. There was no significant difference between normal and dysplastic dogs, nor between first and final radiographic examination.

The position of the centre of femoral head on first radiographs was outside of dorsal acetabular rim in 25 (31.25 %) of estimated hip joints (n = 80); at the level of the rim in 30 (37.5 %); and inside in 25 (31.25 %) hip joints. On second radiographs it was outside in 41 (51.25 %); at the level of the rim in 26 (32.5 %); and inside in 13 (31.25 %) of estimated hip joints. The difference is significant.

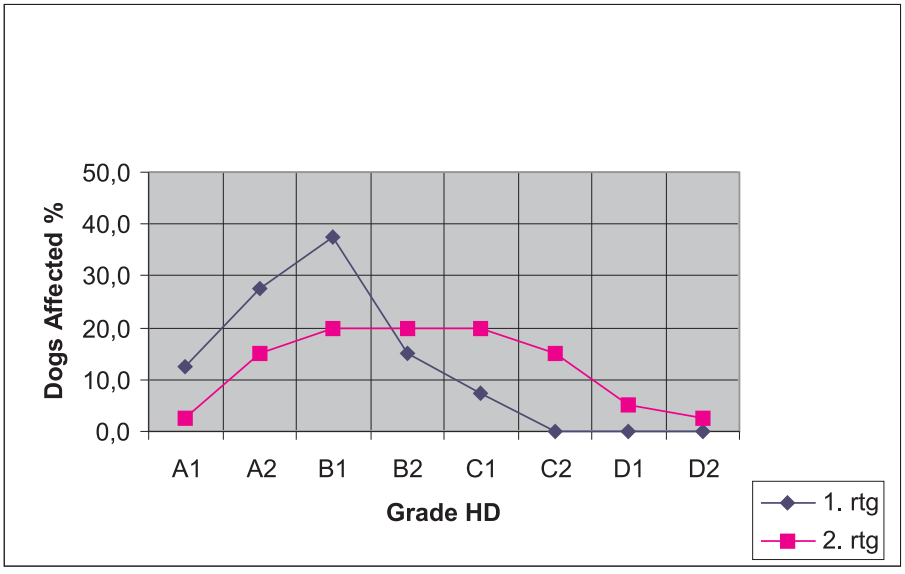

Figure 1: Hip dysplasia grades on initial (1. rtg) and final (2. rtg) radiographic examination

Discussion

All dogs in this study performed similar duties including attack work, tracking, foot patrols, which demands a great deal of jumping and physical exertion. All dogs are maintained in excellent physical condition by routine obstacle course work and specialty training. Most dogs work 8 to 12 hours daily several times a week. They were fed a standard diet. Each dog's weight is regulated and kept within standard limits established by the Police Veterinary Department.

Numerous authors have suggested that a significant percentage (between 7 % and 35 %) of performance dogs are lost as a result of CHD (3,4,6,7,8). This study demonstrates a significantly lower percentage. Only one dog (2.5 %) was lame because of CHD and DJD, but none was euthanized because of CHD.

Of the 40 dogs evaluated, 3 dogs (7.5 %) were

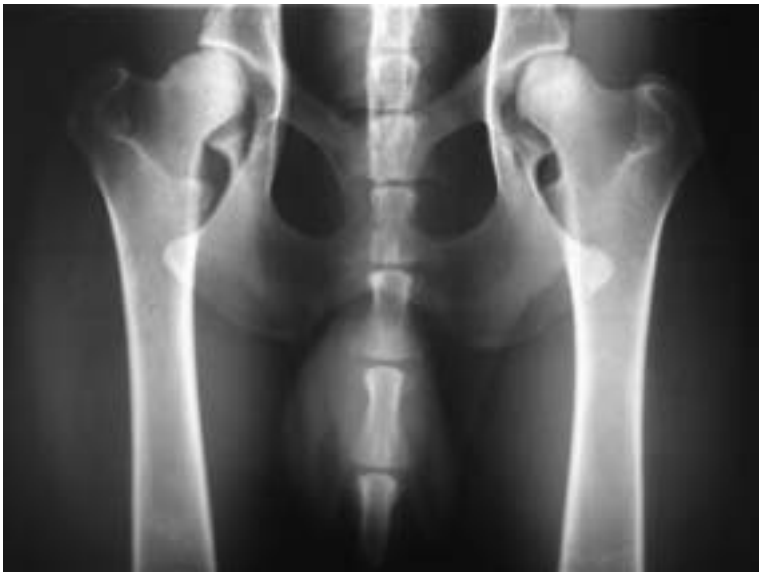

Figure 2: Initial radiograph of 26 month old german shepherd dog graded as L = C1 R = B2

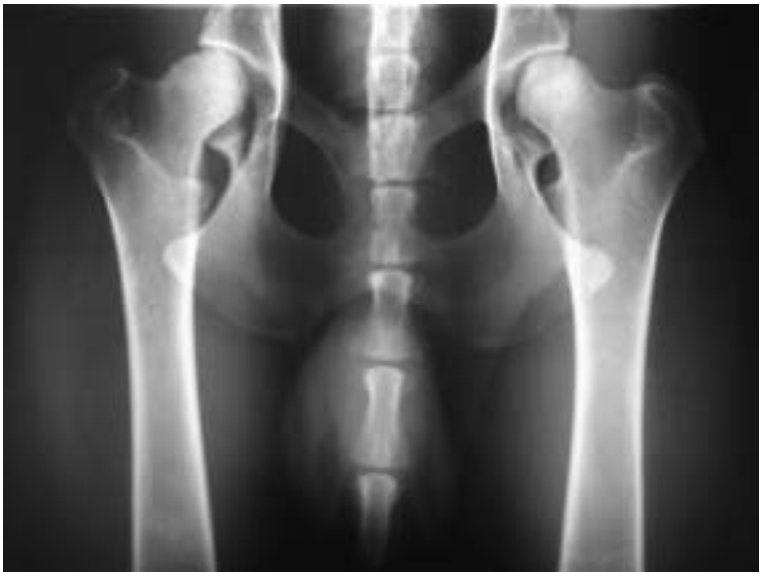

Figure 3: Final radiograph of the same dog (8 years old) graded as L = D1 R = C2

considered dysplastic on initial examination. All of them were German shepherd dogs and were graded as C1. Dogs that are severely dysplastic are rejected on initial procurement based on the results of pelvic radiography and physical examinations. Final grading resulted in a total of 23 (57.5 %) normal dogs and 17 (42.5 %) dysplastic dogs, 14 of dysplastic dogs (35 %) already developed mild to moderate degenerative changes. All dysplastic dogs were German shepherd dogs. It is not known if the percentage of dysplastic dogs of each breed seen in this study is similar to the actual incidence of dysplasia in each breed, because more rottweilers with normal pelvic radiographs were available for procurement than German shepherd dogs. However, it may be presumed that the incidence of CHD in German shepherd dogs was over 50 % on final examination and all rottweilers were normal. Despite high percentage of dogs with CHD and DJD all of them but one were in good physical condition. Dogs selected with temperaments compatible for this type of training may have high pain tolerance (2).

Several authors have suggested an upper age limit for radiographic evaluation of CHD. Six years of age has been suggested, because it is thought that normal dogs would show degenerative changes due to aging and that these changes

would be associated incorrectly with CHD (2). In our study dogs on final examination were approximately 7 years old (6.78), and 11 (27.5 %) of 37 dogs that had normal hip joints initially developed mild to moderate DJD, whereas 100 % of dogs initially diagnosed with hip dysplasia developed moderate DJD, which put their relative risk for development of DJD much higher that of dogs with normal hips.

The mean Norberg-Olsson angle for normal dogs in our study was 105.54 ± 3.22° and 101.62 ± 4.49° for dysplastic dogs which is similar to 104.99 ± 4.12° for normal and 100.84 ± 5.47° for dysplastic dogs in another study (5).

A significant difference existed between Norberg-Olsson angle measurements in 14 dogs (35 %) which were initially considered normal and as dysplastic on final examination ($P = 0.041$), which is similar with a previous studies (3,5,6). The Norberg-Olsson angle in these dogs showed tendency to decrease with progression of dysplasia, whereas in normal dogs it showed tendency to increase with age, which may put the prognostic value of Norberg-Olsson angle in question.

Norberg-Olsson angle did not correlate with progression of degenerative changes. The appearance of subluxation does not appear to lead inevitably to secondary degenerative changes which agrees with a previous study (5). Further studies appear to be indicated to follow dysplastic and normal dogs over their lifetimes to better determine a cause and effect relationship between subluxation (joint laxity) and the development of secondary degenerative changes.

The mean angle of inclination in our study was 132.50 ± 4.39° which is similar to 132.49 ± 5.00° in another (5). Angles of inclination did not correlate significantly with CHD ($P > 0.05$) in this study. This agrees with previous studies which concluded that femoral angles of inclination did not influence the development of CHD (5,9).

There was a significant difference in the position of the centre of the femoral head in relation to the dorsal acetabular rim between first and final evaluation ($P < 0.05$), which gives this grade very important place.

There was no significant difference in the total number of months worked between normal and dysplastic dogs ($P > 0.05$). This also agrees with a previous study (4).

Canine hip dysplasia appears to be a problem that will not limit most police working dogs severely until old age. Of course this takes into account the fact that almost all severely dysplastic dogs on initial procurement radiographs and examinations are excluded from the police ser-vice. A significant percentage of police working dogs are nowadays lost due to lumbosacral disease.

This study has confirmed that the present system of initial procurement radiographs and examinations of Slovenian police working dogs is appropriate.

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RETROSPEKTIVNA ŠTUDIJA NAPREDOVANJA KOLČNE DISPLAZIJE PRI 40 POLICIJSKIH DELOVNIH PSIH

B. Zorko, T Ivanuša, R. Pelc

Povzetek: Proučevali smo napredovanje kolčne displazije pri 40 policijskih delovnih psih. Namen našega dela je bil, ugotoviti učinke kolčne displazije na populacijo delovnih psov, še posebej, ali ima kolčna displazija in posledična degenerativna bolezen sklepa vpliv na delovno sposobnost teh psov. Najprej smo pregledali celotno zdravstveno dokumentacijo vseh psov in zbrali vse rentgenograme kolčnih sklepov, ki so bili narejeni ob sprejemu psov v policijo. V drugem delu raziskave smo vse pse ponovno klinično pregledali po 60 mesecih službe in naredili kontrolne rentgenske slike kolkov. Ugotavljali smo napredovanje kolčne displazije z ocenjevanjem po shemi FCI. Vsi psi so bili moškega spola, 35 je bilo nemških ovčarjev, pa 5 rotvajlerjev. Na prvih rentgenogramih je bil pri 37 psih brez znakov displazije Norbergov kot 105,54 ± 3,22°, pri treh psih z začetno blažjo stopnjo displazije pa 100,17 ± 2,99°. Na kontrolnih rentgenogramih po 60 mesecih dela je bil pri 23 psih brez displazije Norbergov kot 105,60 ± 3,67°, pri 17 psih, z displazijo pa 101,62 ± 4,49°. Na začetnih rentgenogramih smo našli degenerativne spremembe pri 3 psih (7,5 %), na kontrolnih rentgenogramih pa že pri 14 psih (35 %). Položaj centra stegnenične glavice je bil glede na dorzalni acetabularni rob na prvih rentgenogramih zunaj roba pri 25 (31,25 %) sklepih (n = 80), na robu pri 30 sklepih (37,5 %) in znotraj roba pri 25 sklepih (31,25 %). Na kontrolnih rentgenogramih je bil zunaj roba pri 41 sklepih (51,25 %), na robu pri 26 sklepih (32,5 %) in znotraj roba pri 13 sklepih (31,25 %). Če se je stopnja displazije, povečala, se je večinoma za več kot eno stopnjo. Pri 57,5 % psov na kontrolnem slikanju displazije nismo našli. Vsi psi z znaki displazije so bili nemški ovčarji, vendar je imel samo eden (2,5 %) klinične znake bolezni. Kljub aktivni uporabi napredovanje kolčne displazije ni bilo v korelaciji s starostjo ali časom uporabe psov, prav tako ni vplivalo na delovno sposobnost teh psov. Noben pes v tej raziskavi ni bil izločen zaradi kolčne displazije.

Ključne besede: veterinarska medicina; kost, bolezni razvojne; kolk, displazija psa - radiografija; psi

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Original Research Paper

METHOD AGREEMENT OF QUANTITATIVE MEASUREMENTS – STABILITY OF BUTANOL EXTRACTS OF RESAZURIN AS A MODEL

Petra Zrimšek *, Janez Kunc, Marjan Kosec, Janko Mrkun

Address of authors: Clinic for Reproduction and Horses, Veterinary Faculty, University of Ljubljana, Gerbičeva 60, 1000 Ljubljana, Slovenia

* Corresponding author, E-mail: petra.zrimsek@vf.uni-lj.si

Summary: A resazurin reduction assay depends on the ability of metabolically active spermatozoa to reduce resazurin-redox dye to resorufin, which may then provide valuable information for predicting sperm fertilizing capacity. We investigated whether it was possible to accurately measure the resazurin reduction in butanol extracts a day and as late as a week after an assay was performed on boar semen.

According to the scatter diagrams with fitted regression line, it was evident that the paired measurements, i.e. A_{610} at day 0 and day 1 as well as A_{610} at day 0 and day 7, were close to the line of equality. It was also established that the percentage biases were in the range of the within-run coefficient of variation. From the absolute bias plots, it was evident that there was no proportional bias. In order to assess how well the measurements agreed at the individual level, we also determined the limits of agreement. More than 95% of the absolute differences were within the limits of agreement, confirming that the level of agreement between the methods was satisfactory for both of the investigated comparisons.

The stability of the butanol extracts confirmed that the resazurin reduction could be spectrophotometrically measured up to one week after an assay was performed. The usefulness of the assay is, therefore, greatly enhanced as it can be used in on-farm AI laboratories that do not have immediate access to spectrophotometers.

Key words: semen - analysis; comparative study; statistics - methods; fertility agents - analysis

Introduction

Traditionally, boars have been chosen as AI (artificial insemination) studs based upon their genetic excellence. More often than not, these genetic merits are related to improved meat quality and days to market rather than to reproductive performance. A routine examination of a boar's semen quality is very important as an insurance against reproductive disorders. The costs arising from using poor quality semen are high, as it has a negative impact on a herd's farrowing rate, its litter size and non-productive days as well as on the culling of sows and gilts (1).

Although various analytical techniques have been developed to evaluate sperm quality, including sperm concentration, motility, viability and morphology, there is no single method that provides a complete picture about semen quality (2, 3). Another important issue is that for some on-farm AI laboratories, these same routine semen evaluations tend to be impractical because of limitations of equipment, skilled laboratory staff or time. Therefore, a reliable, simple, cost effective and rapid method of assessing the quality of boar spermatozoa would be of benefit to both livestock producers and veterinary practitioners (4).

Resazurin (7-hydroxy-3H-phenoxazin-3-one 10-oxide) is a redox dye used as an indicator of dehydrogenase activity (4, 5). Vital spermatozoa produce reducing factors such as NADH + H^+ during the metabolic process of glycolysis and the citric-acid cycle, and these factors participate in the redox reaction. The diaphorase enzyme transfers electrons to the resazurin dye, which becomes reduced to resorufin and then to dihydroresorufin and manifests as a visual colour change from blue to pink and then to white (6, 7). We developed a spectrophotometric application of resazurin reduction assay for

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boar semen to quantitatively measure the change from blue to pink in butanol extracts (8). After developing the assay, we wondered if it was possible to measure the absorbance at a later date, i.e. within a day or even a week of the assay. In this study, we evaluated the simila-rity between the measurements of absorbance of butanol extracts measured immediately following an assay (day 0), 24 hours after the assay (day 1) and a week after the assay (day 7). Moreover, a comparison between two clinical laboratory met-hods was required because we adapted an existing method to make it more convenient to use. A sa-tisfactory level of agreement would indicate that the modification was successful, which in turn would greatly enhance the usefulness of the assay as it could then be performed even if a spectrophotometer was not immediately available.

Material and methods

Resazurin reduction assay

The resazurin reduction assay was performed as previously described (8). Briefly, 30-µl of 1.8 mM resazurin (Sigma, Germany) diluted in physiological saline was added to 3 ml semen sample (that had been maintained at 37 °C) diluted 1:2 with BTS semen extender (Beltsville Thawing Solution, Netherlands) and incubated at 37 °C in a water bath for 10 minutes. After incubation, two sub-samples of 1 ml were added to 1.5 ml of butanol (Merck, Germany).

Butanol extracts

After the resazurin reduction assays, the developed colour was extracted with butanol. After rapid vortexing, the samples were centrifuged at 3000 g for 10 minutes. The extract's absorbance at 610 nm was then measured in the clear, upper layer of butanol. There were 112 butanol extracts included in this study. We measured the A_{610} immediately (day 0), one day (day 1) and one week (day 7) after the assays were performed. In the meantime, the butanol extracts were kept at 4 °C.

Bias determination

While comparing the measurements between days 0 and 1 as well as between days 0 and 7, we developed scatter graphs to which we fitted regression lines in order to establish the type/s of bias that might be present. Absolute bias is present when data points lie a similar distance to one side of a 45° line in a conventional plot. Proportional bias is present when the distance of the data points from the 45° line increases with the measured value but would be negligible at zero, whereas combined bias is present when the data points diverge increasingly from the 45° line but indicate a measurable value on one axis when the other is zero (9). The least squares linear regression method is appropriate for statistically assessing absolute and/or proportional bias because the x values can be regarded as having no error. Linear regression is applied to paired measurements with the object of fitting the best straight line that can pass through the plotted points to predict the value of the dependent variable, to be expected at any value of the other independent variable (9).

Method agreement

The differences between the pairs of measurements – day 0/day 1 and day 0/day 7 – were calculated for absorbance at 610 nm for each butanol extract. In absolute bias plots, the biases were plotted against their average value for each sample. In order to assess how well the paired measurements agreed with each other, we determined the limits of agreement. The upper limit of agreement was calculated as being $\overline{d} + 2s_{diff}$ and the lower limit of agreement as $\overline{d} - 2s_{diff}$, where $\overline{d}$ was the mean of differences for all the samples (average bias) and s_{diff} was the standard deviation of the differences; $2s_{diff}$ is also referred to as British Standard Institution repeatability (or, reproducibility, as relevant) coefficient and indicates the maximum difference likely to occur between two measurements. This coefficient is the value below which the bias between paired results may be expected to lie with 95% certainty (10).

Results

Using linear regression, we described the relationship between absorbance on day 0 and absorbance on days 1 and 7, respectively, by determining the straight line that most closely approximates the data points on a scatter diagram. The scatter diagram of absorbance at 610 nm on day 0 and absorbance at 610 nm on day 7 is similar to that shown in Figure 1 (data not shown); the data, with a regression line fitted, corresponded to the regression equation: A_{610} (day

7) = 0.00164 + 0.99689 x A_{610} (day 0); R=0.99877, P<0.0001. The estimated linear regression equations indicate that the points are close to the line of equality, i.e. the 45° line.

The mean percentage bias was 0.996 ± 2.820% and 1.380 ± 8.056% for measurements of A_{610} on day 1 and day 7. In the relative bias plot (Fig. 3), a greater number of percentage biases were detected at low absorbance values. However, these biases are in the range of the within-run coefficient of variation of the test, calculated as 7.79 ± 4.06% (8). From the absolute bias plot (Fig. 2), it is also evident that the scatter of the points is random indicating that the size of the discrepancy between the two absorbance values is not related to the size of the absorbance. Therefore no proportional bias is detected.

Average absolute biases were close to zero and were calculated as 0.0045 ± 0.0066 and 0.0007 ± 0.0076 for the measurements of A_{610} on days 1 and 7, respectively. Measurements obtained on day 0 and those obtained on day 1 and day 7 agree; 99.1% and 95.54% of the differences lie within the limits of agreement, respectively.

Therefore, we can measure A_{610} of butanol extracts at any time up to one week after an assay is conducted, confirming the highly practical value of this method.

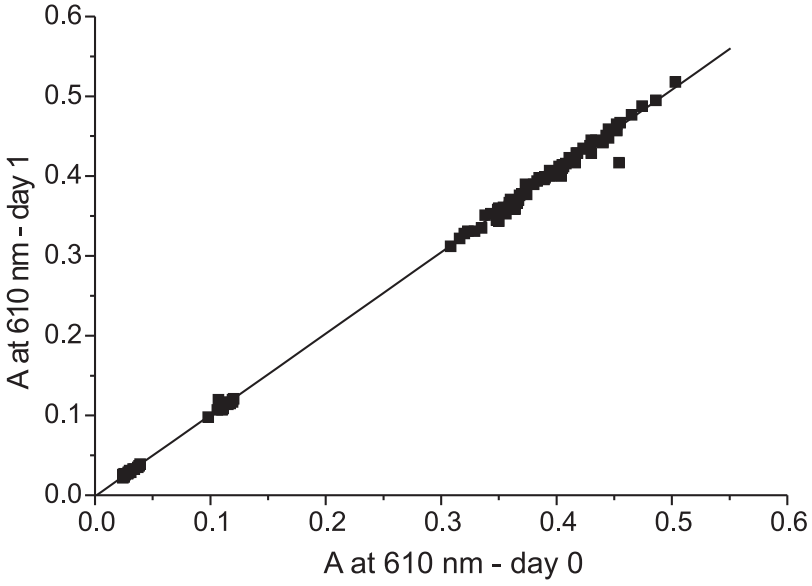

Figure 1: Scatter diagram of A_{610} on day 0 versus A_{610} on day 1 with regression line fitted
Regression equation: A_{610} (day 1) = -0.00125 + 1.01896 x A_{610} (day 0), R=0.99926, P<0.0001

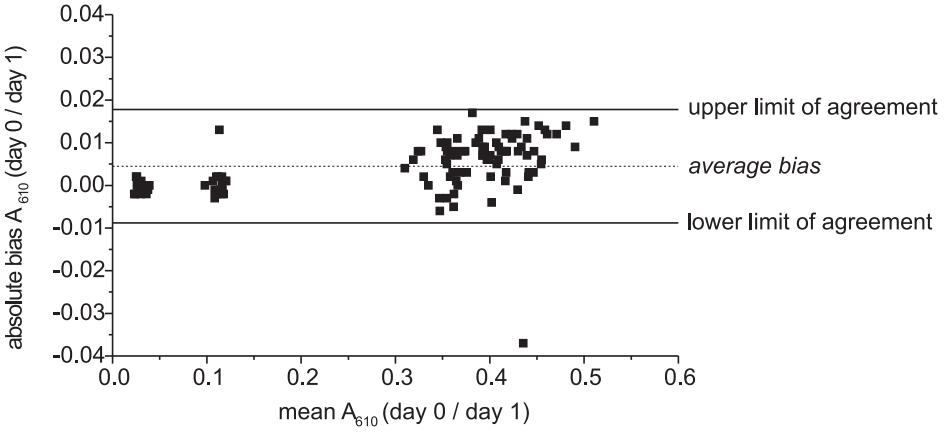

Figure 2: Absolute bias plot of A_{610} on day 0 versus A_{610} on day 1 showing average bias and limits of agreement

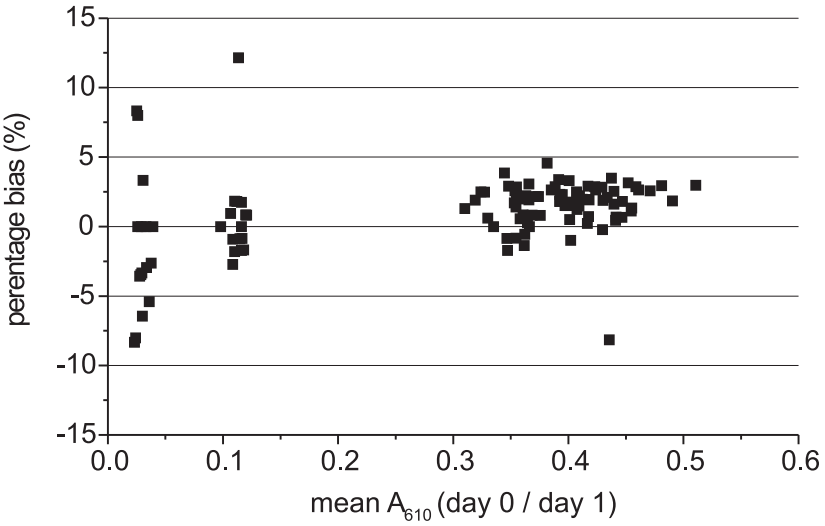

Figure 3: Relative bias plot of A_{610} on day 0 versus A_{610} on day 1

Discussion

It has been reported that the colour change in a resazurin reduction assay correlates significantly with the concentration of motile spermatozoa (5, 8, 11). A high degree of discrimination between good quality and poor quality boar semen samples was also achieved utilizing the sperm index (SI = sperm concentration multiplied by the square root of the percentage of motility multiplied by the percentage of normal morphology) (8). The colour change can be matched by a colour chart but varies between evaluators; therefore a spectrophotometric application of the resazurin reduction test is preferable, enabling the metabolic activity of the sperm to be measured quantitatively (12). It has been mostly used for the evaluation of human semen (6, 7, 12, 13, 14), however, to the best of our knowledge, it has only been used in veterinary medicine for evaluating the quality of ram (12) and boar (8) semen. Following Zalata et al. (7), we used butanol to extract the developed colour after the boar semen assay and measured the level of absorbance in the clear, upper layer of butanol, thereby eliminating the problem of sample turbidity. The resazurin reduction assay is reliable, easy to perform and does not require sophisticated laboratory equipment. On the other hand, a spectrophotometer is not standard laboratory equipment in the majority of on-farm AI laboratories. Therefore, we explored the possibility of measuring the absorbance up to a week after the assay had been performed.

It is essential to establish that a method is repeatable before comparing two measurements for reproducibility (10). The within-run coefficient of variation, calculated as 7.79 ± 4.06%, confirmed that the method had a satisfactory level of repeatability (8), therefore, the pairs of measurements of A_{610} were valid for comparison.

Scatter plots and absolute and relative bias plots give the best overview of comparative data (15, 16). Using scatter diagrams with regression lines fitted, we established that the paired measurements, i.e. A_{610} at day 0 and day 1 (Fig. 1) as well as A_{610} at day 0 and day 7, were close to the line of equality.

According to the available literature, a very common way of investigating method agreement is by performing a paired t-test or by calculating a correlation coefficient to provide a measure of the agreement, however, in this instance, neither method is appropriate for the reasons listed below (10). The paired t-test tests the null hypothesis that means the difference is zero. If the differences between the pairs are large – indicating that the methods do not agree – but are evenly scattered around zero, then we will obtain a non-significant result. We can only conclude that there is no bias, not that the methods agree. Correlation is a statistical method used for quantifying any association between two continuous variables (17). The correlation coefficient provides a measure of the linear association between the measurements obtained by the two methods. It gives us an indication of how close the observations in the scatter diagram are to a straight line. However, to assess agreement, we need to know how close the points are to the line of equality, i.e. the 45° line (10).

We were interested in assessing the similarity between A_{610} on day 0 and day 1 as well as

between day 0 and day 7, so we compared pairs of measurements. Therefore, we calculated the differences between A_{610} at day 0 and day 1 and between day 0 and day 7 for each butanol extract. The mean of these differences ($\overline{d}$) is an estimate of the average bias of one method relative to the other. If this bias is zero, then the two measurements agree on average. However, this does not imply that they agree for each individual measurement. In order to assess how well the measurements agree on an individual basis, we determined the limits of agreement (10). More than 95% of the absolute differences were less than the reproducibility coefficient, confirming that the level of agreement between the methods was satisfactory for both of the investigated comparisons. Therefore, we can measure the level of absorbance up to one week from the time that a test is performed. The usefulness of the assay is, therefore, greatly enhanced as it can be used in on-farm AI laboratories that do not have immediate access to spectrophotometers.

The resazurin reduction assay has been shown to be reliable, simple and easy to perform and would, therefore, be of benefit to both livestock producers and veterinary practitioners in evaluating the quality of boar semen.

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UJEMANJE METOD PRI KVANTITATIVNIH MERITVAH - STABILNOST BUTANOLNIH EKSTRAKTOV RESAZURINA KOT MODEL

P. Zrimšek, J. Kunc, M. Kosec, J. Mrkun

Povzetek: Redukcijski test z resazurinom temelji na sposobnosti metabolično aktivnih semenčic, da reducirajo redoksno barvilo resazurin v resorufin. Test lahko prispeva k ugotavljanju oploditvene sposobnosti semenčic. Ugotavljali smo možnost določanja redukcije resazurina v butanolnih ekstraktih dan ali celo teden po izvedbi testa.
Na podlagi razsevnih grafov s premico linearne regresije lahko ugotovimo, da so parne meritve, to je A_{610}, izmerjene na dan 0 in dan 1, kot tudi parne meritve, opravljene na dan 0 in dan 7, blizu premici enakosti. Relativne razlike med parnimi meritvami so znotraj koeficienta variacije za ponovljivost v testu. Grafi absolutnih razlik dokazujejo, da med primerjanimi metodami ni proporcionalnih napak. Ujemanje posameznih parnih meritev je zadovoljivo, kar potrjuje dejstvo, da je več kot 95 % absolutnih razlik znotraj meja ujemanja.
Merjenje absorbance pri 610 nm v različnih časih po opravljenem testu potrjuje stabilnost butanolnih ekstraktov resazurina. To pomeni, da lahko redukcijo resazurina izmerimo v času enega tedna po izvedbi testa, kar zvišuje njegovo uporabno vrednost. Test bi namreč lahko uporabljali tudi v terenskih pogojih, kjer spektrofotometer med izvajanjem testa ni na voljo.

Ključne besede: sperma - analize; primerjalna študija; statistika - metode; plodnostni faktorji - analize

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Original Research Paper

BACTERIAL CONTAMINATION OF SHELLFISH IN SLOVENIA

Majda Biasizzo *, Andrej Kirbiš, Janez Marinšek

Address of authors: Institute for Food hygiene and Bromatology, Veterinary Faculty, University of Ljubljana, Gerbičeva 60, 1000 Ljubljana, Slovenia

*Corresponding author, Email: majda.biasizzo@vf.uni-lj.si

Summary: In 2003 and 2004 we examined 182 shellfish samples according to the present applicable rules. The samples were tested for the presence of *Salmonella* and enumeration of *Escherichia coli* with the methods recommended by European Community. More then 90 % of samples fulfilled the veterinary conditions about placing live shellfish on the market, concerning the level of 230 *E. coli* in 100 g of flesh. We detected the presence of *Salmonella* at 0.5 % of samples. That represents a rare but possible occasional appearance of this bacteria in shellfish. Concerning other contaminants that may cause poisoning by shellfish consumption, like pathogenic strains of *Vibrio parahaemolyticus*, *Norovirus* or hepatitis A virus, a lot of studies have been done, in a view to prepare new microbiological criteria for foodstuffs in Europe. It is now well recognised that bacterial indicators of faecal pollution (*E. coli* and faecal coliforms) do not adequately indicate the presence of enteric viruses. Another problem is that depuration process is more effective in removing *E. coli* and coliforms than viruses from shellfish.

Key words: food contamination; shellfish - microbiology; food analysis - methods; legislation, food

Introduction

Harvesting and shellfish consumption is an actual problem in Europe concerning seafood safety. Shellfish can be treated as risky food with high probability of poisoning. Bio – toxins have occurred occasionally in shellfish tissue as result of accumulation when growing and feeding in water containing high levels of toxin – producing plankton or pathogenic micro – organisms.

The Slovenian Rules on the veterinary conditions for the production and placing on the market of live shellfish (16) are based on the Directive 91/492/EEC of the European Community and entered into force in January 2004. The Rules set the veterinary conditions for the production and placing on the market of live shellfish intended for direct human consumption or for further processing (purification procedure) before use. Shellfish must fulfil microbiological criteria and criteria about containing toxins. Shellfish must also meet the requirements concerning the content of radionuclides and the presence of toxic and other disputable components appearing naturally or added to the environment, the intake of which could exceed the admissible daily intake, or which could deteriorate the taste of the molluscs (8).

The criteria shellfish must fulfil before marketing are the level of fecal coliform microorganism below 300/100 g of flesh and the level of *E. coli* below 230/100 g of flesh, and should not contain salmonellas in 25 g of flash. Shellfish with higher level of these bacteria can be put on the market after depuration in order to reach acceptable limits (16).

In the Rules on the veterinary-sanitary control of food production establishments, veterinary-sanitary checks and the conditions for health suitability of foodstuffs and raw materials of animal origin, in force from February 2000 (15) until the adoption of the above mentioned Rules, both criteria applied to live shellfish.

The prescribed method for the determination of *E. coli* and Coliform bacteria is determination of their levels by means of the most probable number (MPN) method, with five test tubes and triple dilution. The European Community recommends the modified method according to Donovan (5), based on the determination of the emergence of acids from the lactose.

The Directive (8) recommends the ISO 6579 method for the determination of the presence of salmonellas.

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Material and methods

In 2003, we examined 66 shellfish samples according to the Rules applicable at that time (15). In 2004 we examined 116 samples altogether, 6 samples before and 110 samples after the Rules on the veterinary conditions for the production and placing of live shellfish on the market came into force (16).

All samples were tested for *Salmonella spp.* and *E. coli* level, 6 samples were tested for the coliform count. The origin (harvested in Slovenia or imported) and the time of sampling (before or after depuration) of the shellfish varied.

Detection of Salmonella spp.

The presence of salmonellas in shellfish was determined in accordance with the recommended EU method, i.e. in line with the ISO 6579 standard (12).

The presence of salmonellas was determined in 25 g of whole flesh. The prepared samples were pre-enriched by means of 9-times the amount of peptone water (Buffered peptone water; Biolife, Milano, Italy) and incubated for 16 - 20 h at 37 °C. Afterwards, the samples were subcultured to two liquid enrichment media: Rappaport - Vassiliadis soya broth or RVS (Biokar, Allonne, France) and Mueller Kaufman tetrationat with novobiocin (MKTTn) (Biokar). The first medium was incubated at 37 °C, and the second at 41.5 °C for 24 h. From the enrichment media samples were subcultured to the selective media xylose lysine desoxycholate agar XLD (Merck, Dornstadt, Germany) and Rambach agar (Merck). The plates were incubated for 24 h at 37 °C. After incubation, the plates were examined for the presence of any characteristic colonies. If they were found, the biochemical and serologic confirmations followed.

Enumeration of Escherichia coli

From two options set by the Rules (16), we decided to determine the level of *E. coli* as recommended by the EU expert committee for this area.

The number of *E. coli* bacteria was determined by the method recommended by the EU Central Reference Laboratory (CRL) responsible for the area of microbiological contamination of the shellfish (CEFAS, Weymuth Laboratory, UK).

The samples were prepared according to the standard procedure for the preparation of stock dilutions (ISO 6887-2) and the examination was conducted according to the ISO/TC 34/SC 9 N 587 method (modified Donovan's method) (5).

In order to determine the number of *E. coli* bacteria, the primary enrichment medium of minerals – modified - glutamate broth - MMGB (Oxoid, Basingstoke, England) was used. From three consecutive dilutions (10^{-1}, 10^{-2}, 10^{-3}), one millilitre of the sample was inoculated into five test tubes containing the medium. By using these dilutions, the method can be used for the determination of the *E. coli* count higher than 200 cfu/100 g. To register lower level contamination (over than 20 cfu/100 g), 10 millilitres of the sample from the 10^{-1} dilution was inoculated into an additional series of five test tubes with MMGB. This way, 1 g of the sample was applied to each of them. The test tubes were incubated for 24 h at 37 °C and then the visible as a change in the colour of the medium was observed decomposition of lactose into acids.

The samples from all test tubes with a change of colour of the medium (positive reaction) were subcultured individually to a chromogenic medium: 5-bromo-4-chloro-3-indolyl-β-D-glucuronide tryptone bile agar - BCIG (Oxoid). The plates were incubated for 20-24 h at 44 °C. The blue colonies indicate the action of the β-glucuronidase enzyme, which is the characteristic for most of the *coli* bacteria. The result was interpreted by MPN tables with the number and dilutions of the test tubes in which acid was produced and which were also confirmed as positive by using the BCIG medium, taken in account. The result was given as the number of the *E. coli* bacteria in 100 g of whole flesh.

Results

Aifty-four out of 66 samples examined in 2003 suited the provisions of the Rules (15) concerning marketing. Among the samples that failed to meet the desired criteria, the acceptable number of *E.coli* bacteria was exceeded in all 11 and the coliform bacteria count exceeded in four cases. In one case, the examination only showed a greater number of *E. coli* bacteria. In all 12 samples not suiting the conditions for marketing, the level of 6000 per 100 g was not exceeded; in two of these samples, the value of 6000 was exceeded for the coliform micro-organism count.

Salmonella spp. was present in one sample out of the 116 examined in year 2004. The number of *E. coli* exceeded the maximum limit set in the Rules in 12 samples out of 116 examined in 200;

Table 1: Presence of salmonellas in shellfish samples examined in 2003 and 2004

Year	*Salmonella spp.*/25 g		
	No. of examinations	**not detected**	**detected**
2003	66	66 (100%)	0 (0%)
2004	116	115 (99.1%)	1 (0.9%)
∑	182	181 (99.5%)	1 (0.5%)

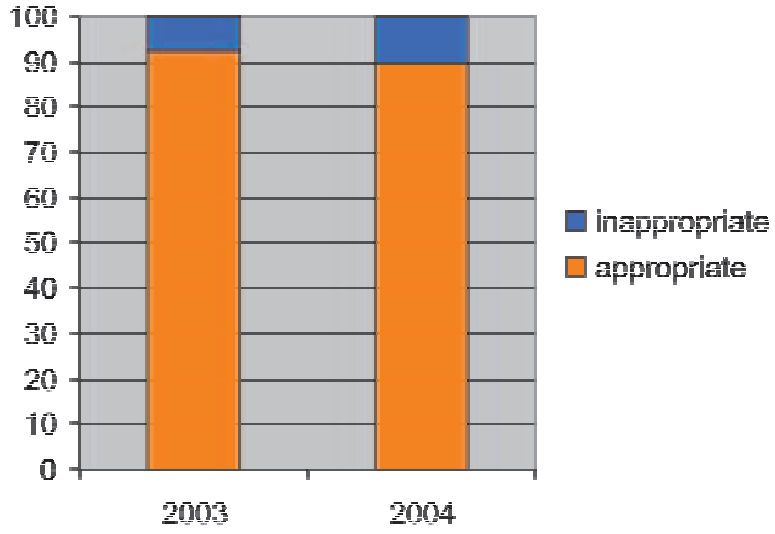

Diagram 1: Percentage of appropriate and inappropriate shellfish samples according to the Rules for direct human consumption of shellfish, regarding demands for *E. coli* number in years 2003 and 2004.

in 11 of these cases the noumber of bacteria present in the sample was between 230 and 4600 and in one sample the number of bacteria was between 4600 and 60000 *E. coli* per 100 g of whole flesh. The other 104 samples examined suited the requirements for the marketing of shellfish.

Discussion

IShellfish feed themselves by filtering sea water and capturing phytoplankton and other nutrients. Through filtration they also consume various contaminants that influence their quality as foodstuffs. Shellfish can contain pathogenic microbes, like salmonellas, *Vibrio parahaemolyticus*, viruses (Hepatitis A virus, *Norovirus*) or parasites (*Giardia*) (4, 9). The main source of shellfish contamination is the faecal pollution of the sea. Faecal contamination of shellfish is related to the organic pollution arriving from inland surface waters, distance of harvest area from the coast, sea temperature and saltines as well as weather – washing of organic substances and micro-organisms deriving from soil into the sea is stronger during heavy rain (14).

The countries along the Mediterranean Sea have reported occasional appearance of salmonellas and the above mentioned viruses in shellfish and shellfish consumption has led to food poisoning in humans (3, 14). In most cases poisoning resulted in mild to moderate gastrointestinal symptoms lasting hours to days, but severe infections like salmonelpsis or hepatitis A occasionally occurred (17).

E. coli and faecal coliforms are not very pathogenic themselves and the purpose of determining their numbers in shellfish is to establish the level of sea pollution. As shown by the parameters set in the Directive, the higher the level of pollution, the greater is the probability of presence of other pathogenic micro-organisms. The procedure is relatively simple, and the result can be obtained as soon as within 48 hours. In this way, one avoids more complicated, time-consuming and expensive determinations of individual pathogenic microbes, like the pathogenic strains of *Vibrio parahaemolyticus*, *Norovirus*, or hepatitis A virus. Shellfish growers want a prompt final result of

examinations, so that they may decide to purify the shellfish in the case of their excessive contamination, and to quickly place the shellfish on the market after purification and re-examination.

The harvesting areas or the sea are classified regarding the content of coliform bacteria or *E. coli* bacteria. The classification is performed by the competent authority after investigating the results of the microbiological examinations of shellfish from a certain area (8). Only shellfish from category A waters, with maximum levels of 230 *E. coli* or 300 coliforms per 100 g flesh, may be marketed for direct human consumption. Shellfish from category B areas must not exceed the limit of 4600 *E. coli* or 6000 coliforms per 100g flesh in 90 % of samples. Such shellfish can only be placed on the market after treatment in a purification station or after relaying so as to meet the category A standards. Live bivalve molluscs from class C areas must not exceed the limits of 60000 fecal coliforms per 100 g of whole flash and should be collected but placed on the market after relaying over a long period (at least two months) in order to meet the category A standards. Where the results of sampling show that the health standards for shellfish are exceeded (more then 60000 faecal coliforms), or that there may be otherwise a risk to human health, the competent authority must close the production area.

The proposal for the new European Directive leaves out the requirement concerning coliform bacteria, but includes the same provision with regard to the number of *E. coli* bacteria (7).

Shellfish we examined were harvested in the Adriatic Sea. In more then 90 % of samples the level of *E. coli* was below limits determined by the Rules. We can't estimate a quality of shellfish on the market, because the shellfish were harvested in different areas and sampled for different purpose (after or before harvesting). We isolated *Salmonella* at 0.5% of examined samples in two years. In Italy, *Salmonella spp*. was detected in 0.7 % of samples analysed between 1996 and 2000 for purposes of microbiological monitoring. Both results reflect a rare but possible presence of these pathogenic bacteria in shellfish from Adriatic Sea (14).

It has been established that bacterial methods do not always reveal the presence of viruses or the presence of members of genus *Vibrio* (13, 10). Depuration is currently commercially practiced and was shown to be adequate for reducing *E. coli*, but ineffective for the elimination of viruses. The shellfish that meet the *E. coli* standards for human consumption may contain human enteric viruses that cause gastroenteritis and hepatitis (11, 6). Hence, there is a need for indicators of viral faecal pollution in order to improve the microbiological control of shellfish.

The EU study (6) discovered that after purification, the level of *E. coli* decrease for 75 %, the level of viruses (by determining the reduction of FRNA – bacteriophage) fell by 43% (28 - 60%) on the average. The reports from Italy show that as much as 50% of the shellfish contaminated by the hepatitis A virus were also positive after purification. Hepatitis A virus was present on average in 10 % of the shellfish tested prior to depuration in Spain and Italy and reduced to 7 % after depuration. It has been also reported that the number of the *Vibrio parahaemolythicus* bacteria may actually increase at temperatures above 20 °C.

All these findings show that current legislative standards for *E. coli* do not effectively protect the consumers from the risk of exposure to pathogenic viruses and other pathogens.

Therefore, in our view, direct determination of *Vibrio* and viruses should be introduced.

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BAKTERIJSKO ONESNAŽENJE ŠKOLJK V SLOVENIJI

M. Biasizzo, A. Kirbiš, J. Marinšek

Povzetek: V letih 2003 in 2004 smo pregledali 182 vzorcev školjk, pregledi pa so bili opravljeni v skladu s trenutno veljavnimi predpisi. V vzorcih smo z uporabo metod, ki jih priporoča Evropska unija, ugotavljali pristotnost bakterij vrste Salmonella spp. in število bakterij E. coli. Glede na dovoljeno število E. coli (230 bakterij v 100 g mesa školjk) je veterinarska merila za primernost izdelka za prodajo izpolnjevalo preko 90 % vzorcev. Ta odstotek kaže na majhno verjetnost pojavljanja omenjenih bakterij v školjkah oziroma možnost občasno povečanega števila teh bakterij v školjkah. Pri pripravi novih mikrobioloških meril za živila v Evropski uniji je bilo narejenih veliko raziskav tudi o drugih patogenih dejavnikih, ki lahko povzročajo zastrupitev po zaužitju školjk; mednje sodijo Vibrio parahaemolyticus, Norovirus in virusa hepatitisa A. Znano je, da E. coli in fekalne koliformne bakterije kot bakterijski kazalci onesnaženosti s fekalijami za nadzor okužbe z enteričnimi virusi niso dovolj zanesljivi. Ob tem je pomembno tudi to, da postopki čiščenja oziroma depuracije odstranijo E. Coli in fekalne koliformne bakterije bolj učinkovito kot patogene viruse.

Ključne besede: hrana, onesnaževanje; lupinarji - mikrobiologija; hrana, analize - metode; zakonodaja, hrana

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Original Research Paper

CHANGES IN HISTOCHEMICAL PROPERTIES OF MUSCLE FIBRES IN DEVELOPING CANINE SKELETAL MUSCLES

Malan Štrbenc

Address of author: Institute for Anatomy, Histology and Embryology, Veterinary Faculty, Gerbičeva 60, 1000 Ljubljana, Slovenia

E-mail: malan.strbenc@vf.uni-lj.si

Summary: In the study changes in muscle fibres of canine skeletal muscles were observed during development from perinatal period to 6 months of age. Emphasis was put on the histochemical fibre type classification and general morphological properties. In neonates muscle fascicles contained one centrally located primary fibre which in some cases still retained a central space as seen in developing myotubes. These fibres started to stain differently from surrounding secondary fibres on foetal day 55. The classification of muscle fibres according to the myosin ATPase (mATPase) method was possible after third week post partum; prior to this the majority of fibres seemed to be undifferentiated. Between the third and the sixth week 7 different fibre phenotypes were found and in two-month-old dog the usual adult composition of muscles with 4 fibre types was first noted. The glycolytic and oxidative capacities were weak in neonates but increased gradually with age. The metabolic differentiation between fibres was first noted at the third week. The diameter of fibres was increasing constantly. The number of muscle fibres assessed by ratio between primary and secondary fibres increased in perinatal period.
When compared to data in the literature, we ascertained that dog skeletal muscles are relatively immature at birth. There were parts of muscles which developed even more slowly and still had a myotubal morphology in neonates. Some muscle-dependent differences were noted: the diaphragm developed faster and an early distinction between slow (m. rhomboideus) and fast muscles (m. extensor carpi radialis and m. tibialis cranialis) was observed. Mature morphology with a random distribution of fibre types inside muscle fascicles and a defined metabolic profile was observed in all muscles in two-month-old dogs. The standard mATPase method became applicable to determine fibre types by this time.

Key words: anatomy, veterinary; muscle, skeletal; muscle fibres - growth and development; myosin ATPase; dogs

Introduction

Canine skeletal muscles have been studied mainly with histochemical methods. On the basis of muscle fibre type numbers and distribution one can presuppose the muscle predominant function and the state of activity. Fibre types according to the mATPase reaction found in dogs were slow type I, hybrid IIC and fast IIA. Instead of the conventional type IIB rather an unique fast subtype of fibres was described (1), labelled as type IIDog by Lattore et al. (2). These fibres strongly express the myosin heavy chain (MHC) isoform IIx, a protein expressed only in some muscles (3, 4). Type IIDog fibres could be therefore also named IIX fibres. Another dog peculiarity is a high degree of oxidative activity in the muscle fibres suggesting that dog muscles are adapted to endurance activity (1, 4, 5).

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During development fibre types are more ambiguous. It is well known that different MHC isoforms are present in the fibres during development, hence the fibre types do not comply with the traditional (adult) classification (6). New fibre types were proposed, such as IB and IC. However, in developing canine muscles a high proportion of IIC fibres was reported by several authors (7, 8). These fibres retain a high mATPase activity in alkaline and acid preincubations. IIC fibres in adults are hybrid fibres since they contain fast and slow MHC isoforms. It is obvious that developing muscle fibres are hybrid as well, but co-expressing developmental MHC isoforms, i.e. embryonic and neonatal (MHC-emb, MHC-neo). These developmental isoforms are replaced by adult MHC isoforms, before and/or after birth, depending on the length of gravidity and subsequently adult fibre types are established. More appropriate designation for darkly stained fibres according to the mATPase method in developing muscles is therefore "undifferentiated fibres" (7, 9, 10).

The degree of muscle fibre differentiation or muscle maturity at the time of birth is correlated with the length of gravidity and general maturity of neonates, which reflects animal's physiological needs right after birth (9, 11). While most of the studies were performed on laboratory animals (including cats), some information is available for domestic animals as well. In cattle the total number of muscle fibres is fixed at foetal day 230. Primary generation consists of slow fibres as in other animals and humans, except of purely fast muscle m. cutaneus trunci, in which primary myotubes expressed fast isoform from the beginning. In mid-gestation various types of secondary fibres were observed, differentiating to slow or fast fibres. Only the third generation of fibres was still undifferentiated just before birth. The precocity of differentiation was muscle-type dependent (12, 13). Also in m. tibialis cranialis of neonate sheep all primary fibres were slow. Secondary fibres started to express adult fast isoforms in mid-gestation - some of them only transiently since they transformed into slow fibres by day 20 post partum (14). In newborn horses a lot of fibres seem to be type IIX and transformed into IIA in the next 48 weeks. By week 10 after birth all fibres differentiated (15, 16). Pig muscles are unique in the way that the central location of slow fibre remains visible throughout the adult life and is accompanied by a rosette of secondary slow fibres that are established in the third postnatal week. A third generation of small diameter fibres was noted only after birth. Dramatic changes were described in the first postnatal week in piglets: the disappearance of undifferentiated fibres (decrease in developmental MHC), formation of proper type I and II fibres (increase in MHC- I and MHC-IIa) and remodelling of energy metabolism (17, 18).

Although immunohistochemistry and immunoblotting provide additional and less ambiguous information on the fibre composition, the enzyme-histochemistry, namely the mATPase fibre type classification, oxidative and glycolitic capacity still present quick, cost-efficient and species-universal methods in quick diagnostics of muscle pathology, regeneration or training efficiency. Postnatal changes in canine skeletal muscles were histochemically assessed in the past, but one study concentrated on a single muscle (10) while in the other the glycolitic capacity assessment of the fibres was lacking and an interval between postnatal weeks 5 and 12 was not studied (7). The aims of our study were therefore to compare several different skeletal muscles in perinatal and postnatal period, determine the time of muscle maturation with regard to morphological characteristics of muscle fibres, and compare the fibre type classification in young dogs to other animals. The muscle characteristics of prenatal and pubertal dogs were also assessed for the first time.

Material and methods

Muscle samples were obtained from 6 fetal dogs after histerectomy or cesarean section on foetal days 50, 55 and 60 (F50, F55, F60; gestational period is on average 63 days) and from 16 puppies with an age range between 1 day and 6 months (1, 3, 5, 11, 15, 22, 28, 42, 60 and 180 days) which died of natural causes or were euthanised due to severe trauma. The puppies had no apparent neuromuscular deficiencies. Five adult dogs were included in the study for comparative purposes. All dogs were of medium size (pure-breeds or mongrels with known parents). The samples were frozen in liquid nitrogen and stored at -80°C. The middle portions of the following muscles were extracted: m. rhomboideus (p. capitis), m. longissimus dorsi (at the level of the last rib), the diaphragm, m. triceps (c. longum), m. extensor carpi radialis, m. sartorius (p. cranialis), m. semitendinosus, m. rectus femoris, m. tibialis cranialis and m. masseter. Transverse serial cryosections (10 μm) were cut on Leica CM 1800 cryostat at -17°C, mounted on APES-covered slides and air-dried.

To determine fibre types in dog skeletal muscles the sections were processed for the mATPase reaction following some of the procedures described by Latorre et al. (2). The sections were incubated either in 0.1M Na-acetate at pH 4.3 and 4.35 or in 0.2M Na-acetate at pH 4.4, 4.5 and 4.6 for 5 minutes at room temperature. For the alkaline preincubation the solutions of 0.1M $CaCl_2$, 0.07M Na-acetate and 0.075M Na-barbital adjusted to pH 9.8 and 10.2 were used (15 min, RT). Sections were then incubated in medium containing 0.1M $CaCl_2$, 0.07M Na-acetate and 0.075M Na-barbital, pH 9.65 and ATP 1.5 mg/ml for 60 min folloving the acid preincubation or 30 minutes folloving alkaline preincubation, both at 37°C. After washing in 0.2M $CaCl_2$ visualization was performed by incubation in a 2% (w/v) cobalt chloride solution (5 min), followed by fresh 1% (w/v) ammonium sulphide solution for 30 seconds.

The diameter of muscle fibres was measured by Lucia M imaging software (Optoteam Wienna). Minimum diameter was selected as a measure of fibre diameter to avoid errors due to possible section obliquity.

To estimate fibres' basic metabolic profile the presence of active oxidative enzyme succinate dehydrogenase (SDH) and glycolytic mitochondrial menadion-linked α-glycerophosphate dehydrogenase (α-GPDH) was demonstrated as previously described by Nachlas et al. (1957) and Dubowitz and Brooke (1973), respectively.

The age-dependant differences in enzyme activities was followed by biochemistry. Frozen muscle samples were cut on microtome and homogenised (Ultra-turrax, IKA-Werke) in 20 volumes of 100 mM KPO_4, 5 mM EDTA and 5 mM EGTA (pH 7.4) while kept on ice. The homogenate was sonicated on ice to further disrupt mitochondrial membranes and frozen. The procedure was repeated and after second thawing the samples were further diluted with ice-cold 100 mM KPO_4, 5 mM EDTA and 5 mM EGTA (pH 7.4) to achieve final dilution 1:20 (wet tissue mass : buffer volume). Citrate synthase (CS) activity was determined by the standard method of Srere (19) and lactate dehydrogenase (LDH) by Bergmeyer and Bernt (20) spectrophotometrically at 25°C using UV/VIS Spectrofotometer Perkin Elmer Lambda 12.

The appearance of CoA-SH was measured at wavelength 412 nm to asses CS activity in the following reaction: acetyl-CoA + oxaloacetate + H_2O ↔ citrate + CoA-SH + H^+ (side reaction CoA-SH + DTNB → mercapptide ion). Aliquots of the diluted homogenate were used for the assay in duplicates. They were kept on ice just prior to the analysis and then submerged in water bath at 25°C to increase reaction kinetics. 975 µl of 100 mM Tris (pH 8,1) used as a buffer was dispersed into 1.5 ml quartz cuvette and the following reagents added: 75 µl 3 mM acetyl-CoA, 150 µl 1 mM DTNB, 150 µl oxaloacetate and 150 µl of sample homogenate.

The LDH activity was determined by the rate of oxidation of NADH (pyruvate + NADH + H^+ ↔ lactate + NAD^+) as decrease in extinction at 340 nm. To 1.5 ml of buffer-pyruvate solution (50 mM phosphate, pH 7.5 and 0.63 mM pyruvate), 25 µl of reduced NADH (ca. 11.3 mM β-NADH obtained by dissolving 14 mg NADH-Na_2 and 15 mg $NaHCO_3$ in 1.5 ml distilled water) and 50 µl of sample homogenate ere added. Readings were taken at 20-s intervals and plotted against time. Enzyme activities were calculated from the rate of change of assay absorbance at the maximal linear slope and expressed as micromoles per minute per gram (wet mass) of tissue.

Results

In foetuses and newborns all muscles were composed of fascicles which had one centrally located primary myotube/myofibre, surrounded by secondary fibres with smaller diameter. In the diaphragm myotubes were transforming into myofibres already on F50, but m. rectus femoris and m. triceps brachii were still composed solely of myotubes on F50. Between fascicles and individual fibres of foetuses (F50, F55) there were wide intercellular spaces with loose connective tissue that diminished just before birth (F60). Fibres were more or less rounded compared to typical polygonal morphology of mature fibres. In other muscles myotubes (seen as rings or crescents) remained visible in some parts of the muscle in neonates while other parts of the same muscle were maturing faster. The tubal morphology of primary fibres was therefore noted up to postnatal day 5 and that of the secondary fibres up to postnatal day 1 in majority of the muscles. Typical changes in myofibre morphology are shown in Fig. 1. In some parts of m. rhomboideus, m. rectus femoris, m. triceps brachii and m. semitendinosus tubal morphology was noted up to postnatal day 11 (Fig. 2).

No distinction between primary and secondary fibres could be made on the basis of the mATPase staining method on fetal days F50 or F55 (se panels A and B in Fig. 1). On F60 and in newborns two different fibre types were observed according to mATPase staining method: primary fibres had a low mATPase activity and secondary fibres retained a high mATPase activity after both, alkaline and acid preincubation (for example of acid preincubation see Fig. 1C). On the third day post partum a few intermediately stained fibres occurred in some muscles. The rhomboideus muscle had the highest number of intermediately-stained fibres on postnatal days 11 and 22 after acid preincubation at 4.4 (Fig. 2 and Table 1). By the third week a mosaic appearance in mATPase staining with acid preincubation was noted in most muscles (Fig. 1E).

A spectrum of staining intensities was observed between weeks 3 and 6 and seven different fibre phenotypes could be established in the majority of the muscles. They did not completely comply with the standard classification of fibre types in adults, but the destination of differentiation could be proposed (Table 2). Fibres with the smallest diameter retained a high mATPase activity after all preincubation media used and they were referred to as undifferentiated fibres. M. rectus femoris and m. triceps brachii had more undifferentiated fibres than the other muscles. Proper type I fibres were

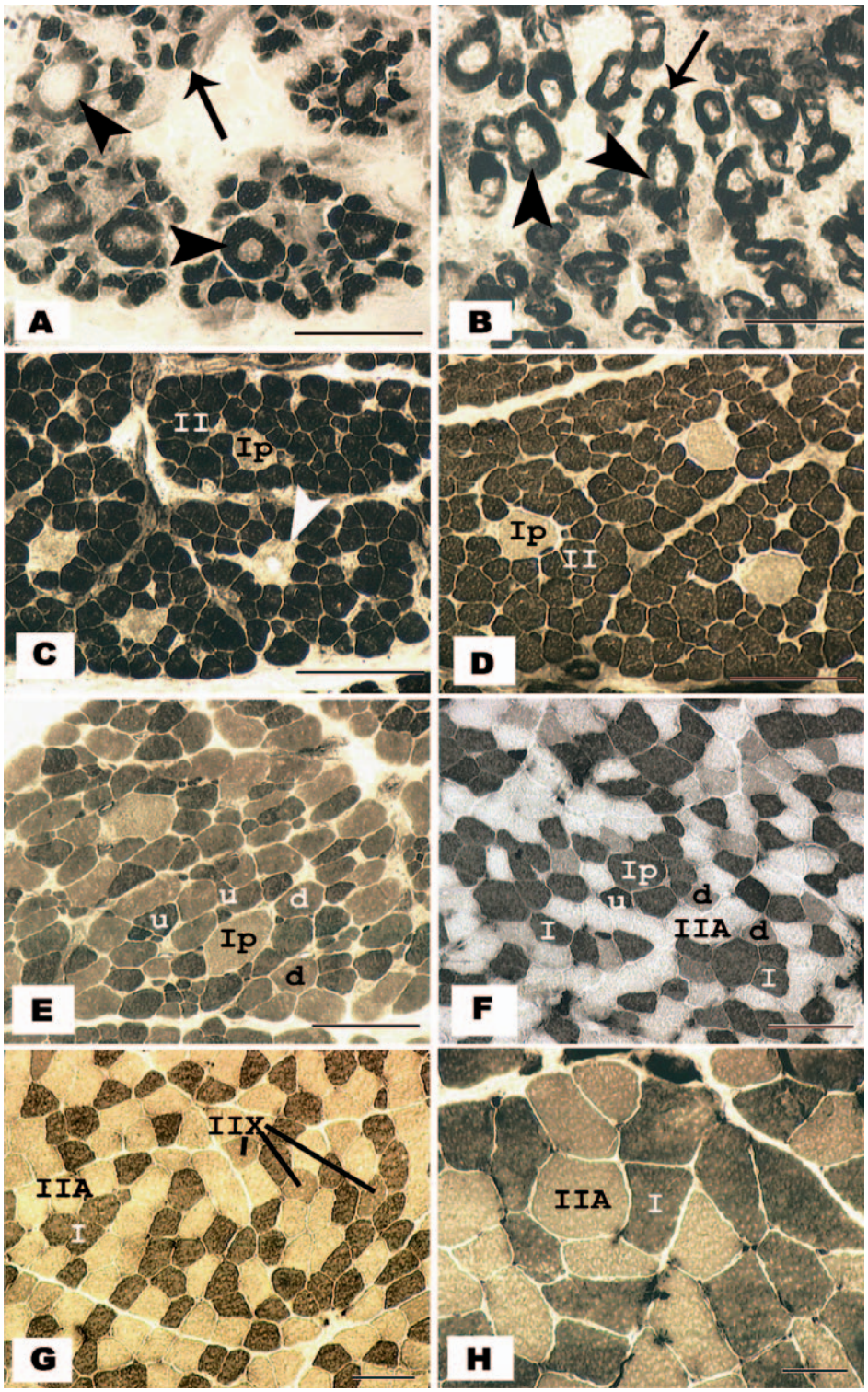

Figure 1: Canine skeletal muscle fascicles in m. sartorius (A) and in m. triceps brachii (B) on foetal day 50 (F50); m. sartorius on F60 (C), on postnatal day 11 (D), the third week (E), the sixth week (F), the second month (G) and in adult dog (H) according to the mATPase reaction at pH 4.4. Few days before birth big primary myotubes (arrowheads) and smaller secondary myotubes (arrows) can be seen clearly. After birth, there is a centrally located primary myofibre (Ip), surrounded by smaller secondary ones (II). The number of secondary fibres increased after birth. After the third week undifferentiated (u) and differentiating (d) fibres can be observed and adult types start to appear (I, IIA). IIX fibres were first noted at two-month-old dog. By postnatal week 6 the inversion of the staining properties of primary slow fibres (Ip) occurred (compare panels C, D and E with F). Scale bars = 50 μm.

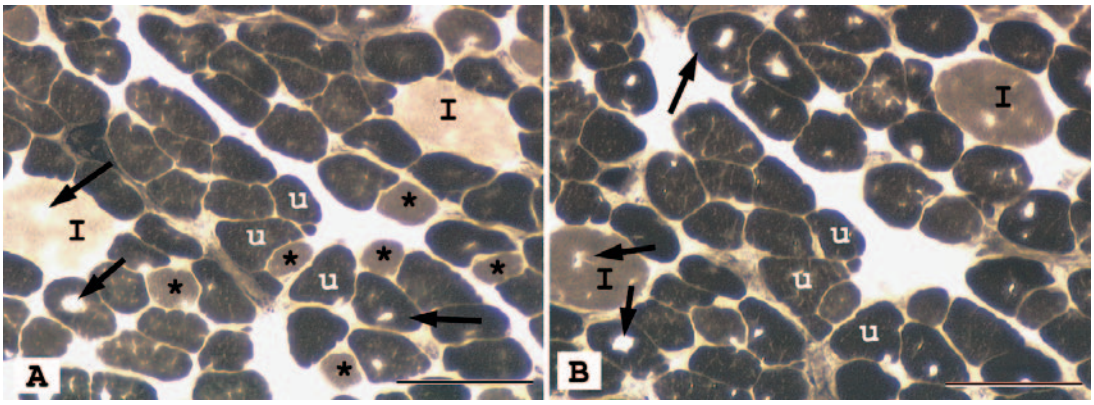

Figure 2: The mATPase demonstration of fibre types in serial sections of m. rhomboideus on postnatal day 11; acid preincubation at pH 4.4 (A) and 4.6 (B). After acid preincubation at pH 4.4 (and 4.3, not shown) about 11% of the fibres stained with intermediate intensity (*). They possibly represent the future slow fibres but stain dark at 4.5 and higher, the same as the rest of secondary fibres, classified as undifferentiated (u). At this age big type I fibres (I) are weakly stained after 4.2 - 4.4 pH values of preincubation media (also in alkaline, not shown) and intermediately in pH 4.5 (and 4.6). Some fibres still have a tubal morphology (arrows). Scale bar = 50 μm.

Table 1: Three different dog fibre phenotypes established by the mATPase method after foetal day 55 and up to the third postnatal week. Gray to black circles represent the staining intensity of muscle fibres. Big primary fibres had different staining properties than any adult fibre type. Intermediately stained fibres (differentiating) occurred in some parts of m. extensor carpi radialis, m. tibialis cranialis and m. rhomboideus at postnatal day 3, but in most of the other muscles at postnatal day 11, except in m. triceps brachii, m. semitendinosus and m. rectus femoris, where only big fibres and undifferentiated fibres were seen.

developing muscles between postnatal days 1 and week 3			
	preincubation		
fibre type	alkaline **10.2**	acid **4.5**	acid **4.3**
big primary fibres	○	◉	○
differentiating	◉	◉	●
undifferentiated	●	●	●

Table 2: General staining scheme of seven different fibre phenotypes established by the mATPase method on weeks 3, 4 and 6 in investigated canine muscles excluding the masseter. The undifferentiated fibres had the same staining properties as adult IIC fibres but were the smallest in the diameter. With the three fibre types which did not fall into normal adult category a proposed differentiating direction is given. Primary fibres failed to comply with proper type I until the second month of age as well as proper IIDog were not found during this period.

developing muscles between postnatal weeks 3 and 6			
	preincubation		
fibre type	alkaline **10.2**	acid **4.5**	acid **4.3**
primary slow (big type I)	○	●	●
secondary slow (small type I)	○	●	●
undifferentiated (IIC)	●	●	●
differentiating IIC → I	◉	●	●
differentiating IIC → IIA	●	●	◉
differentiating IIC → IIDog (IIX)	●	◉	●
IIA	●	◉	○

observed between weeks 3 and 6 (Fig. 1 E and F). They retained a high mATPase activity after the acid preincubation (dark stain) and had lost it after alkaline preincubation (no stain), however, the big primary fibres stained slightly after alkaline preincubation until week 6. In m. rhomboideus there was already 43% of type I fibres in six-week-old dog, while other muscles had between 12 and 19% of type I fibres. In adults m. rhomboideus was the slowest muscle, with the ratio between fast and slow fibres about 1:1.

At two months of age the muscle fibre type composition resembled those found in adult animals (Fig. 1 G). Fibre types I, IIA, IIC and IIX (IIDog), irregularly distributed inside muscle fascicles, were found (except in m. extensor carpi radialis, see below), and their proportions were similar to previously established patterns in adult animals (3). Type IIM fibres in m. masseter, as seen in adults, strongly resemble the undifferentiated fibres: they stained dark after acid and alkaline preincubations.

The number of fibres per muscle fascicle was increasing. The ratio between primary and secondary fibres increased in average from 1:9 on F55 to 1:25 on F60 and to 1:44 at day 5 post partum in most of the muscles. In the masseter these numbers were lower, in average 1:5 fibres on F50, 1:15 on F60 and 1:19 on day 5. M. rectus femoris and m. triceps brachii had slightly higher number of fibres in one muscle fascicle after postnatal day 5; in average ratio between primary and secondary fibres was 1:47.

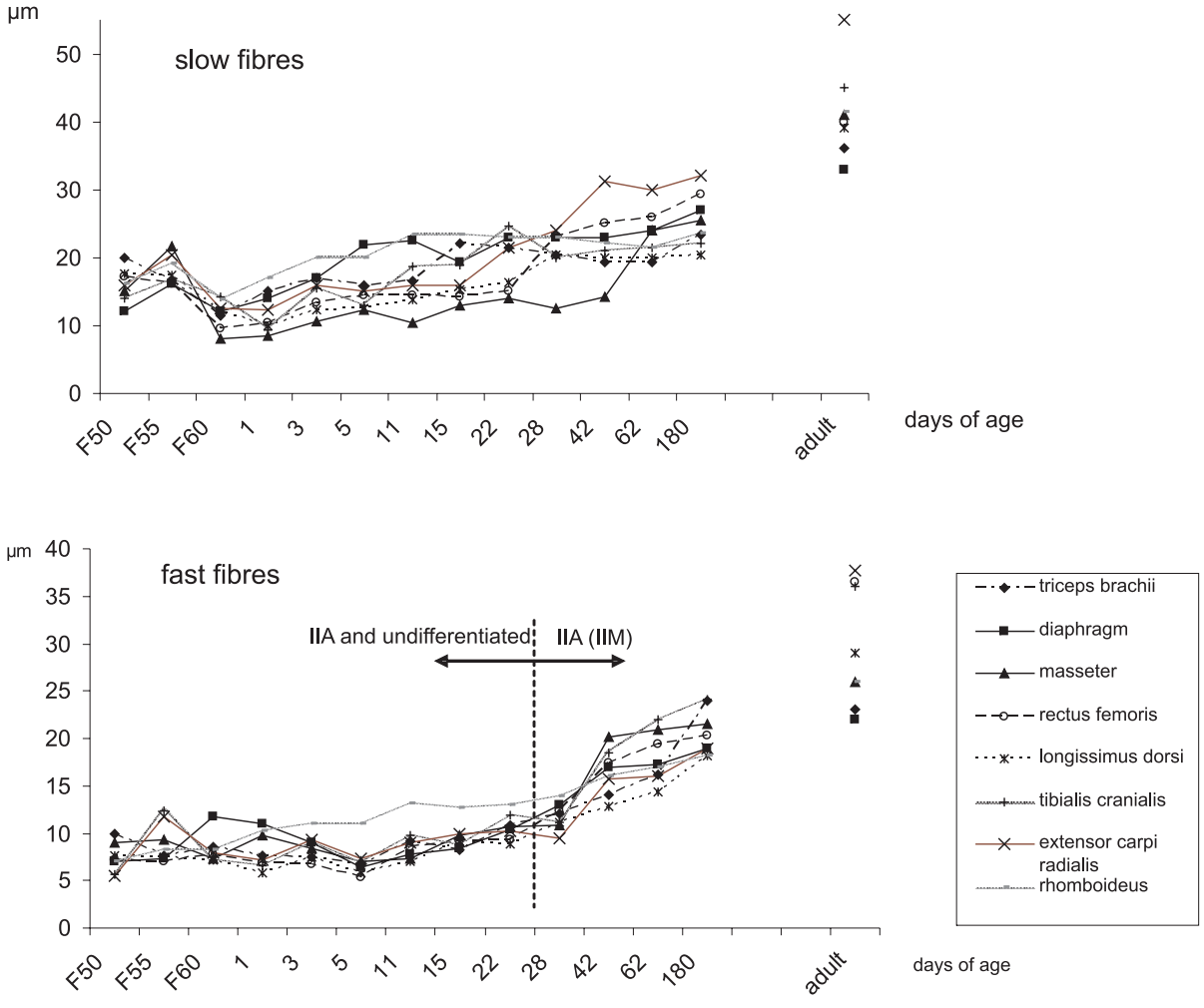

Figure 3: Average diameter of slow and fast muscle fibres. Up to postnatal day 28 all of the secondary fast fibres were measured for the fast fibres. In older dogs, only the dia-meter of type IIA fibres (in m. masseter the IIM fibres) is shown as these fibres are the most numerous ascendant of secondary fibres. IIX (IIDog) fibres, where present, had in average 30% bigger diameter than IIA fibres.

Fibre's diameter gradually increased in the postnatal period, as shown in Fig. 3. Primary fibres were on average twice as big as secondary fibres in the same muscle until six weeks of age. While the central space of primary myotubes closed in neonates, the average diameter transiently decreased. A slight decrease of the average diameter was also noted in secondary fibres between F55 (F60 in the diaphragm) and postnatal day 5. For comparison, type I fibres in adults were 40 - 100% bigger than type I fibres in a pubertal dog (6 months of age) with the exception of diaphragm in which an increase of only 22% was noted. As for type IIA fibres an increase of average fibre diameter between 20 and 80 % (as much as 100 % in m. extensor carpi radialis) was determined in adults compared to six month-old dog. The type IIM fibres in m. masseter had only 15% bigger diameter in adults compared to pubertal dog.

Extensor carpi radialis was the "fastest" muscle in our study. In adults it was composed in average of 11 % of slow fibres and 85 % of fast fibres. The ratio between fast type IIA and IIX (IIDog) fibres varied greatly between the individuals but it was always in favour of IIA fibres. In general the muscle fascicles retained their foetal fibre type distribution - the central position of slow fibre surrounded by fast fibres remained visible in adult muscle. In some superficial fascicles no type I fibres were detected in pubertal and adult dogs (Fig 4), although all fascicles had a slow primary fibre in neonates and young dogs. Similar composition, but only with higher number of slow fibres had m. tibialis cranialis.

The enzyme-histochemical reactions demonstrating the activity of metabolic enzymes SDH and α-GPDH were relatively weak in prenatal and neonatal muscles with the reaction for α-GPDH being just slightly more intense (Fig. 5A and B). The staining in both methods intensified thereafter and the first differences in α-GPDH staining intensity among muscle fibres were noted in the diaphragm at the third week and in m. extensor carpi radialis and m. tibialis cranialis at the fourth week (Fig 5C and D). In other muscles slight differentiation in SDH reaction and stronger in α-GPDH was noted by sixth week (Fig. 5 E and F). At two months of age a prominent differentiation between glycolytic and oxidative fibres was observed, the same as at six-month-old (Fig. 1G) and adult dogs (Fig.1H). All fibres had relatively strong activity of oxidative enzyme without a distinct differentiation between type I and II fibres except in the m. tibialis cranialis and m. extensor carpi radialis. Glycolytic activity was much more prominent in type IIA and IIDog fibres.

Increase of enzyme activities was confirmed by biochemistry. As shown in Fig.6, the activities of LDH (glycolytic metabolism) and CS (oxidative metabolism) measures in m. semitendinosus significantly increased in a similar fashion. The LDH and CS activities were maximal by fourth week and sixth week, respectively, and these values were similar to adult dogs. However, in a samples of two-month-old and six-month-old dog the values were significantly lower.

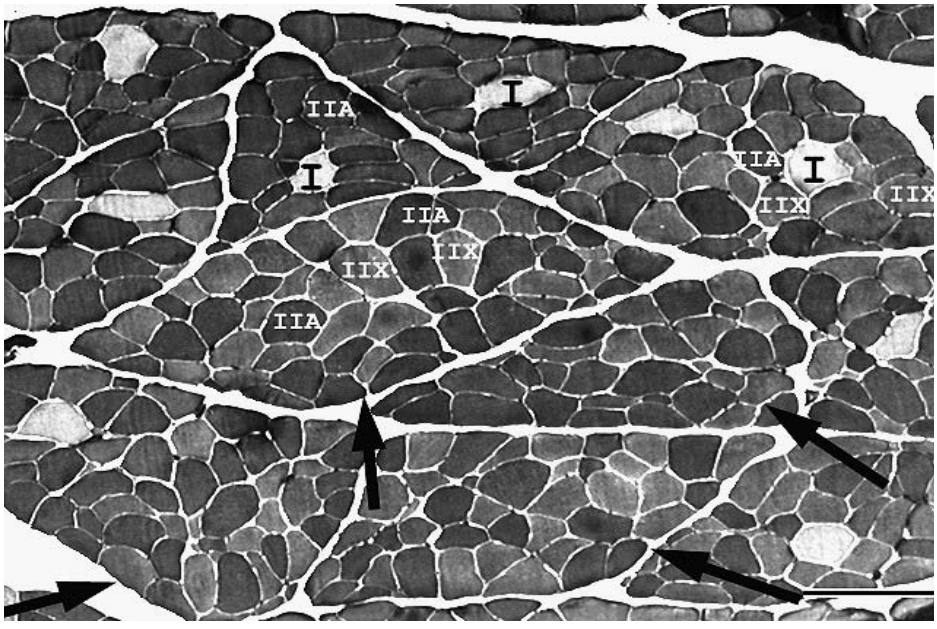

Figure 4: M. extensor carpi radialis in pubertal (6-month-old) dog; mATPase method at pH 4.6. Most of the muscle fascicles retained their developmental distribution of fibre types - i.e. one centrally located slow fibre (I), surrounded by fast fibres (IIA and IIX). However, some fascicles contained no slow fibres at all (arrows), such fascicles were located superficially in the muscle belly. Scale bar = 100 μm.

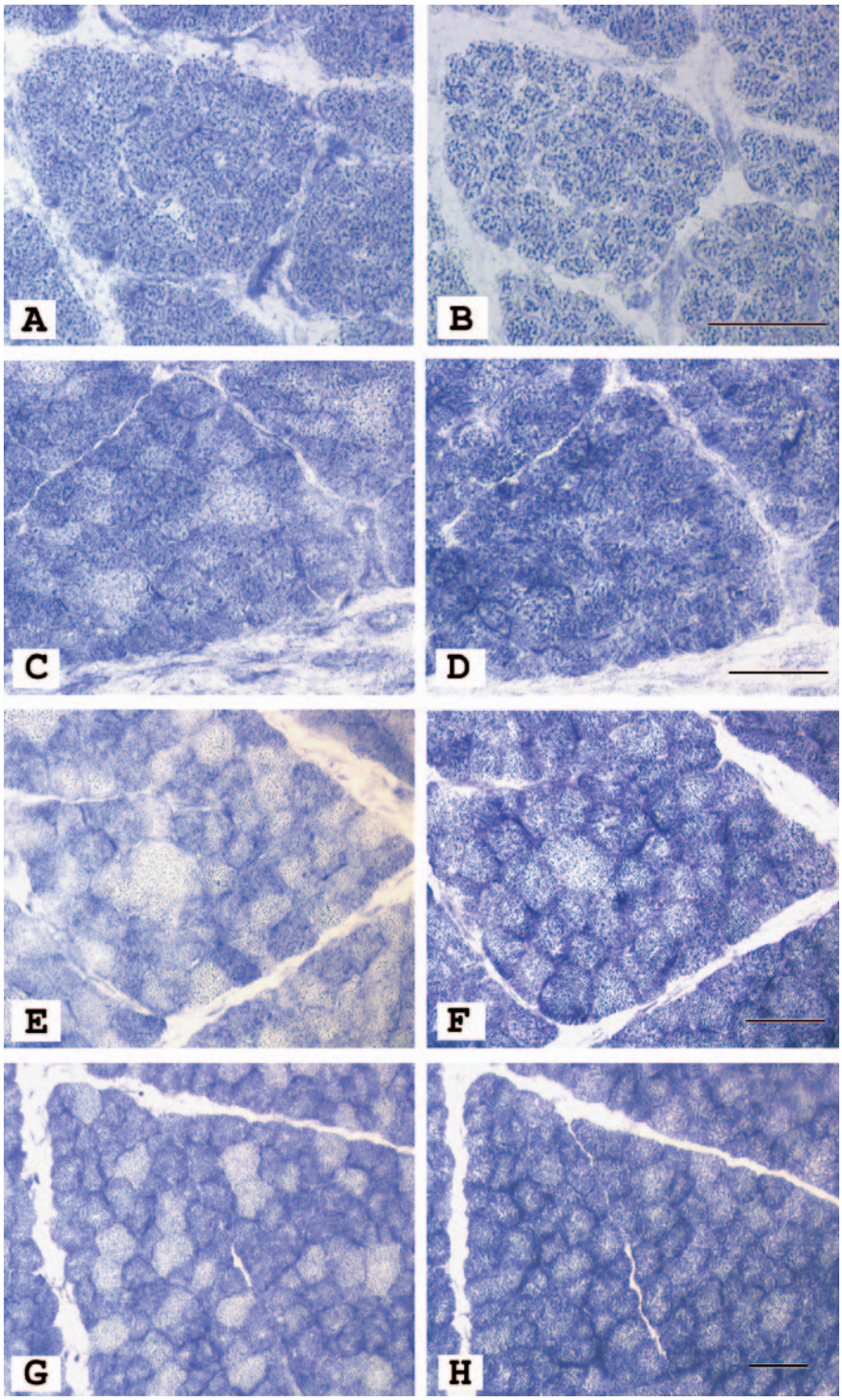

Figure 5: Demonstration of glycolytic enzyme α-GPDH (left column) and for oxidative SDH (right column) in serial sections of m. semitendinosus on fetal day 60 (A, B), 6-week-old dog (E, F) and 6-month-old dog (G, H), and m. tibialis cranialis in a 4-week-old dog (C, D). In prenatal, neonatal and early postnatal period the staining was uniform among fibres and the staining intensity was increasing with age. First glycolytic differentiation was noted in diaphragm at the third week and in m. extensor carpi radialis and m. tibialis cranialis at the fourth week (second row), while in other muscles this was notable by the sixth week (third row). At two months and later the differentiation was the same as in the adult animals - i.e. slow fibres had lower glycolytic activity but all fibres had a high oxidative activity. Scale bars = 50 μm.

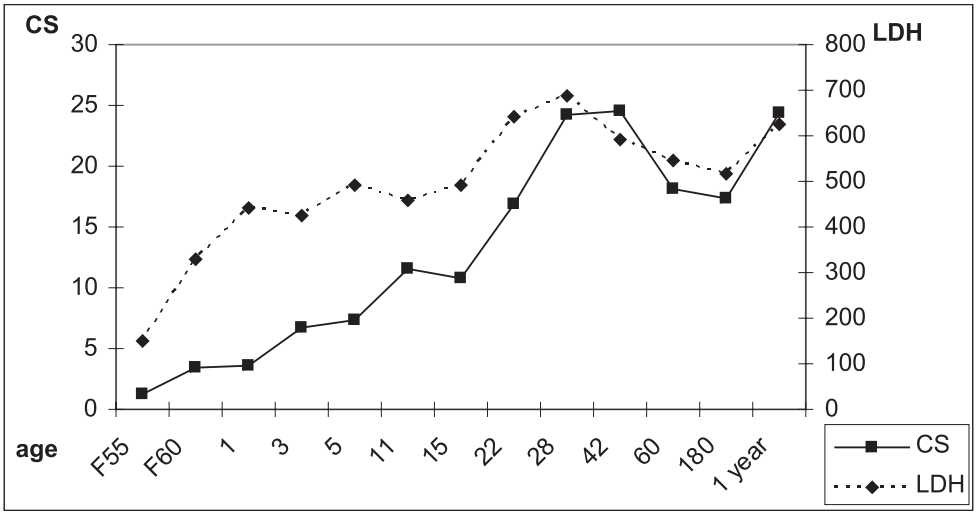

Figure 6: Increase of maximum enzyme activities in m. semitendinosus with age. Cytrate synthase (CS) and lactate dehydrogenase (LDH) activities in μmol per minute per g wet mass of muscle tissue.

Discussion

The described central organisation of skeletal muscle fascicles and the presence of fibres with internal nuclei or central perinuclear space (tubal morphology) in neonatal dogs speaks of relative immaturity at the time of birth. However, a quick maturing was noted in perinatal period: most of the muscle fascicles were loose and composed of myotubes few days before birth but the majority of them transformed into organised and more tightly packed units by postnatal day 5. Just in some parts of the muscles myotubes were still seen after birth. It seems that the functional parts of the muscle mature faster in neonates. The general maturation was also slightly delayed in m. rhomboideus, m. triceps brachii, m. rectus femoris and m. semitendinosus.

Fibre types in neonate canine muscle are different from adult fibre types. The majority (91-97%) of fibres was undifferentiated; their staining properties were comparable to the adult type IIC. Only one fibre per muscle fascicle, located in the centre of the fascicle was the slow type fibre. They were classified as primary type I in contrast to secondary (normal) type I. Primary fibres didn't obtain the typical mATPase staining properties untill sixth week post partum. Another, intermediate type of fibres started to appear few days after birth but it was not up to postnatal weeks 3 and 4 that 7 universal fibre phenotypes were established. The early appearing differentiating or "intermediate" fibres were ambiguous since they appeared in functionally different muscles i.e. they were the most numerous in m. rhomboideus which had a high number of type I fibres 3 weeks later, but they appeared early also in m. tibialis cranialis and m. extensor carpi radialis which became typical fast muscles with IIA fibres predominance in next four weeks. Although these differentiating fibres resembled the slow type staining, it is unlikely that there was an early differentiation into slow type in fast muscles since the established transition of MHC isoforms goes in direction from developmental → IIa → I (6, 22). The staining properties of early differentiating fibres obviously only reflected the loss of developmental components but their intended adult profile has not been acquired yet.

Some muscle-dependant differences were noted in establishing the metabolic profile as well. Optical density of SDH and α-GPDH staining technique increased with age and was at first uniformly distributed among fibres. Increasing enzyme activity was confirmed by biochemistry – the CS and LDH activities were increasing up to fourth and sixth week, respectively, but were again lower in a two-month-old and six-month-old dogs. This might be explained by prominent differentiation between oxidative and glycolytic fibres on tissue sections which occurred by sixth week and coincided with appearance of different mATPase fibre types. As a consequence the joint enzyme activity in the whole muscle could decrease. It might, however, also represent the individual or breed-dependant peculiarity and

more samples of different breeds from this period (2 – 6 months of age) would be needed before conclusions could be made. Precocity of metabolic maturation was again observed in the diaphragm, m. tibialis cranialis and m. extensor carpi radialis. The differentiation was more prominent with the α-GPDH. This reflects the adult profile where all fibres have relatively high oxidative capacity (1, 2, 3).

If we compare our results of time-dependant transformations with data known for cats (11, 21) we can conclude that the two species develop similarly. In cats the most prominent transformations occurred between days 30 and 40 which is comparable to weeks 4 and 6 in our research. After this period the maturation of dog muscles seems to be slower, i.e. the appearance of IIX fibres occurred later, i.e. in two-month-old dog, most likely because on average the puberty in dogs develops later in life than in cats.

Compared to bigger domestic animals, in perinatal period the dog muscles are morphologically and functionally immature. The morphology of neonate puppy muscles resemble the situation found at mid-gravidity in cattle. A neonate calf is able of standing and walking and its muscle fibres types are randomly dispersed with only traces of developmental isoforms expressed (12, 13). A high content of IIX fibres was reported in neonate foals (15, 16), while in our research they did not appear until the second month. Piglet muscles seem to be much less developed at birth if compared to calf, foal or lamb, but still more mature than that of the dog. The classification of fibre types according to mATPase method in pig was applicable already few days post partum (17). On the other hand, small mammals like rodents and rabbits have almost foetal morphology of skeletal muscles in perinatal period but quickly undergo dramatic changes. In rabbits mature morphology of muscle fibres was established relatively early, at postnatal day 40 (23, 24, 25).

The results of our study indicate that the number of dog muscle fibres is not definite at the time of birth. Such late formation of muscle fibres was described in mice and rats, where myogenesis is completed in the first week post partum (26). In bigger animals and humans the myogenesis is supposed to conclude in foetuses but there are reports of a late-forming third generation of fibres, at least in bigger muscles (12, 27). In sheep and pig the third generation of fibres with small diameter and expression of developmental isoforms was noted only after birth (17, 28, 29). We observed undifferentiated fibres with very small diameter in dog m. triceps brachii, m. longissimus dorsi and m. rectus femoris muscles as late as in 2 and 6 month-old dogs. The fibres with small diameter in dogs were described before (30), nevertheless, the formation of the third generation of myofibres in dogs remains to be established.

The apparent decrease in an average fibre diameter as seen in Fig. 3 can be explained. First, in the case of primary fibres, this happens in neonates when myotubes close completely and transform into myofibres. Second, in the case of secondary fibres, this happens by postnatal day 5 which coincided with the noted increase of secondary to primary fibres ratio. The average diameter obviously decreased due to the appearance of new fibres with a very small diameter.

While reading previous studies on developing canine muscle we got an impression that enzyme-histochemical methods are suitable for assessing the development of fibre types in the early postnatal period (7, 10). However, on closer inspection it was obvious that it was almost impossible to classify the fibres before the tenth week and becomes reliable after the twelfth week. In our study the classification became quite reliable in two-month-old dog (week 9) through there was still a certain number of the undifferentiated fibres. The mATPase method is also inappropriate to follow the development of m. masseter since the IIM fibres strongly resemble the undifferentiated fibres. Using this method it was impossible to detect transformation from undifferentiated to the adult state of the masseter muscle. It is not likely that muscle fibres were undifferentiated for so long since studies of masticatory muscles of other animals showed an active transformation on the basis of the MHC isoform content (21, 24) and similar was reported on canine pharyngeal muscles, which share the embryonic origin with the masticatory muscles (8).

We conclude that in developing canine muscles there is an active transformation from undifferentiated foetal into directed developmental stage in the first three weeks post partum. Between the third and the sixth week an active differentiation of fibre types takes place and by the second month mATPase fibre classification technique becomes applicable. Some early differences among muscles were seen i.e. faster maturation of fully active muscle – the diaphragm and fast muscles (m. extensor carpi radialis and m. tibialis cranialis). Fast and slow muscles show fibre-type differences relatively early, by the third week post partum. M. triceps brachii, m. longisimus dorsi

and m. rectus femoris retained a certain proportion of undifferentiated fibres longer than other muscles studied. It is to be expected that all muscles with big diameter are likely to retain differentiation and growth capacity up to the pubertal age.

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SPREMEMBE HISTOKEMIČNIH ZNAČILNOSTI MIŠIČNIH VLAKEN V PASJIH SKELETNIH MIŠICAH V POROJSTVENEM RAZVOJU

M. Štrbenc

V raziskavi smo proučili spremembe v pasjih skeletnih mišičnih vlaknih med razvojem od prerojstvenega obdobja do šestih mescev starosti. Poudarek je bil na histokemičnih metodah določanja tipov mišičnih vlaken in osnovnih morfoloških značilnostih. Pri novorojenih živalih smo v mišičnih snopih opisali eno centralno ležeče počasno vlakno, ki v nekaterih primerih vsebuje centralni prostor, kar je značilno za razvijajoče se miotube. Primarna vlakna so se na podlagi dokazovanja aktivnosti miozinske ATPaze pričela ločevati od sekundarnih 55. dan brejosti. Do tretjega tedna po rojstvu so bila vsa vlakna neizdiferencirana, po tem času pa je bila možna omejena klasifikacija mišičnih vlaken. Med tretjim in šestim tednom starosti smo določili 7 fenotipov mišičnih vlaken na podlagi metode z miozinsko ATPazo, pri dvomesečnem psu pa so imele mišice večinoma zrel profil s štirimi običajnimi tipi vlaken. Glikolitična in oksidativna kapaciteta je bila v vlaknih novorojencev šibka, je pa zlagoma naraščala in prve razlike med vlakni glede glikolitične kapacitete smo opazili tretji teden po rojstvu. Premer mišičnih vlaken je naraščal ves čas porojstvenega razvoja in še po šestem mesecu. Tudi skupno število vlaken, ocenjeno kot razmerje med primarnimi in sekundarnimi vlakni, se je v obporodnem obdobju povečalo.

Če primerjamo razvoj mišičnih vlaken pri psu s podatki o razvoju pri drugih domačih živalih, lahko ugotovimo, da so pasje mišice ob rojstvu relativno nezrele. Nekateri deli mišic so se razvijali še celo počasneje od ostalih, saj so imela vlakna cevkasto strukturo še pri novorojencih. Opazili smo tudi določene razlike med mišicami. Trebušna prepona (diafragma) se je kot polno dejavna mišica razvijala hitreje od ostalih, relativno zgodaj pa smo ugotovili tudi razlike med počasno (m. rhomboideus) in hitrima mišicama (m. extensor carpi radialis in m. tibialis cranialis). Zrelo morfologijo mišic z naključno razporejenimi različnimi mišičnimi vlakni znotraj snopov in izoblikovanim presnovnim profilom smo ugotovili v vseh mišicah pri dveh mescih starosti, v tem obdobju pa je tudi že možno uporabljati klasično metodo ugotavljanja aktivnosti mATPaze in presnovnih encimov za določanje mišičnih tipov.

Ključne besede: anatomija, veterinarska; mišica, skeletna; mišična vlakna - rast in razvoj; miozinska ATPaza; psi

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Examples of references

Book: Hawkins JD. Gene structure and expression. Cambridge: University Press, 1991: 16.

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Article in a journal or newspaper: Fuji J, Otsu K, Zorzato F, et al. Identification of mutation in porcine ryanodine receptor asociated with malignant hyperthermia. Science 1991; 253: 448-51.

Article in proceedings of a meeting or symposium: Schnoebelen CS, Louveau I, Bonneau M. Developmental pattern of GH receptor in pig skeletal muscle. In: the 6th Zavrnik memorial meeting. Lipica: Veterinary Faculty 1995: 83-6.

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Načini citiranja

Knjiga: Hawkins JD. Gene structure and expression. Cambridge: University Press, 1991: 16.

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