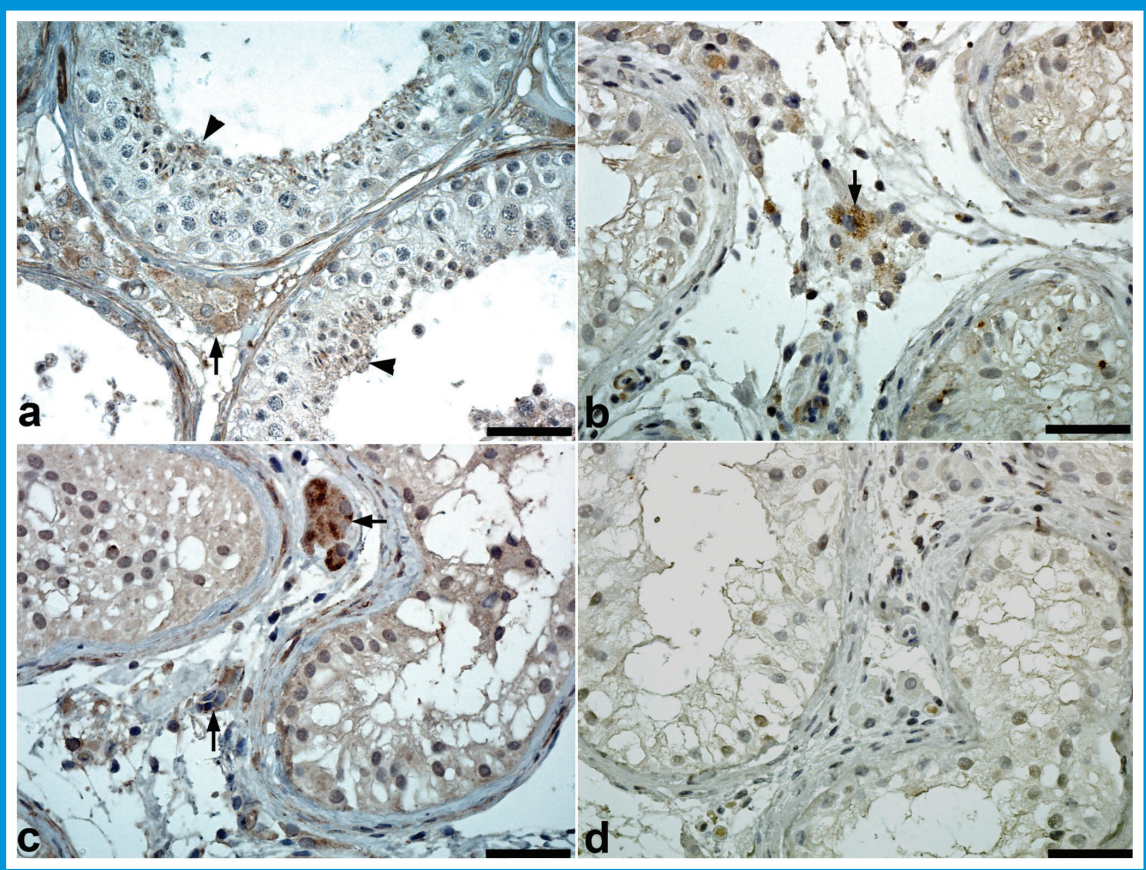
ISSN 1580-4003

THE SCIENTIFIC JOURNAL OF THE VETERINARY FACULTY UNIVERSITY OF LJUBLJANA

SLOVENIAN VETERINARY RESEARCH

SLOVENSKI VETERINARSKI ZBORNIK



Slov Vet Res 2008; 45 (3): 79-116

Volume 45 3

Slov Vet Res • Ljubljana • 2008 • Volume 45 • Number 3 • 79-116

THE SCIENTIFIC JOURNAL OF THE VETERINARY FACULTY UNIVERSITY OF LJUBLJANA

SLOVENIAN VETERINARY RESEARCH

SLOVENSKI VETERINARSKI ZBORNIK

Volume 45 3

Slov Vet Res • Ljubljana • 2008 • Volume 45 • Number 3 • 79-116

The Scientific Journal of the Veterinary Faculty University of Ljubljana

SLOVENIAN VETERINARY RESEARCH
SLOVENSKI VETERINARSKI ZBORNIK

Previously: RESEARCH REPORTS OF THE VETERINARY FACULTY UNIVERSITY OF LJUBLJANA
Prej: ZBORNIK VETERINARSKE FAKULTETE UNIVERZA V LJUBLJANI

4 issues per year / izhaja štirikrat letno

Address: Veterinary Faculty, Gerbičeva 60, 1000 Ljubljana, Slovenia
Naslov: Veterinarska fakulteta, Gerbičeva 60, 1000 Ljubljana, Slovenija
Tel.: +386 (0)1 47 79 100, 47 79 129, Fax: +386 (0)1 28 32 243
E-mail: slovetres@vf.uni-lj.si

Sponsored by the Slovenian Research Agency
Sofinancira: Agencija za raziskovalno dejavnost Republike Slovenije

ISSN 1580-4003
Printed by / tisk: Birografika Bori d.o.o., Ljubljana
Indexed in / indeksirano v: Agris, Biomedicina Slovenica, CAB Abstracts, IVSI
Urlich's International Periodicals Directory, Thomson Reuters Scientific
http://www.vf.uni-lj.si/vf/index.php/Slovenski-veterinarski-zbornik.html

SLOVENIAN VETERINARY RESEARCH

SLOVENSKI VETERINARSKI ZBORNIK

Slov Vet Res 2008; 45 (3)

Slov Vet Res 2008; 45 (3): 83-8
UDC 636.7.09:616-083.98:616-005.1

Review Article

FLUID THERAPY IN HAEMORRHAGIC SHOCK

Alenka Seliškar

Clinic for Small Animal Medicine and Surgery, Veterinary Faculty, Gerbičeva 60, 1000 Ljubljana, Slovenia

E-mail: alenka.seliskar@vf.uni-lj.si

Summary: Haemorrhagic shock is a clinical syndrome manifested by inadequate tissue oxygenation due to poor perfusion because of decreased effective circulating blood volume. Isotonic and hypertonic salt solutions and various colloids have proven efficacy in resuscitation. Isotonic crystalloids freely cross capillary membrane and large quantities must be infused to restore plasma volume. The poor intravascular retention of isotonic crystalloids supports intravascular volume transiently but later can cause tissue oedema formation with impaired oxygen perfusion. The addition of colloids considerably reduces total fluid requirements, prolongs volume expansion, and obtains haemodilution with increased microvascular perfusion. Hypertonic salt solutions with or without colloids have been shown to expand plasma volume three to four times the volume infused through an endogenous fluid redistribution. They also have positive inotropic effects and the increasing cardiac output is apparently mediated by a reflex arc involving the vagus nerve with receptors in the lung. Microvascular blood flow is not only enhanced by haemodilution but also by hyperosmolar shrinkage of blood and endothelial cells. Regardless of the fluid therapy used, the treatment of haemorrhagic shock reqiures rapid restoration of circulating volume to limit potential complications of long-standing hypoxia. It is important to recall that the endpoint of fluid therapy for an animal in haemorrhagic shock is the normalization of the vital signs rather than administration of a specific volume of fluids.

Key words: haemorrhagic shock; fluid therapy; crystalloids; colloids

Introduction

Haemorrhagic shock is a clinical syndrome manifested by reduced perfusion of vital organs leading to inadequate delivery of oxygen and nutrients necessary for normal tissue and cellular function (1). Haemorrhagic shock occurs when circulating blood volume decreases due to haemorrhage, which leads to decreased venous return to the heart. This in turn causes decreased cardiac output and activation of a variety of homeostatic mechanisms including hormonal modulation and cardiovascular neurologic reflexes. The compensatory mechanisms help to improve blood volume, sustain the blood pressure, and maintain perfusion to the vital organs such as heart and brain. The perfusion can be maintained for a short period of time following mild to moderate volume loss, however without the intervention the compensatory mechanisms eventually fail and the complications of shock ensue. Oxygen delivery decreases and the tissues switch to anaerobic metabolism and accumulate the oxygen debt. The goal of treatment is the improvement of tissue perfusion and oxygenation, and the mainstay of the treatment for the haemorrhagic shock is the expansion of the intravascular volume by intravenous fluid therapy (2).

The average estimated adult dog blood volume represents 8% to 9% of body weight (77 to 78 ml/kg) and in cat 6% to 7% of body weight (62 to 66 ml/kg). Estimating blood loss is complicated and to help guide volume replacement, haemorrhage can be divided into four classes. Class I is a non-shock state, such as occurs when donating a blood (up to 15% of estimated blood volume). Class II haemorrhage occurs at estimated blood volume loss of 15 to 30% and class III at 30 to 40%, respectively. Class IV is a pre-terminal event requiring immediate therapy (3). Massive haemorrhage may be determined as loss of total estimated blood volume within a 24-hour period, or loss of half of the estimated blood volume in a 3-hour period (4).

Received: 28 May 2008
Accepted for publication: 2 September 2008

Controlled and uncontrolled haemorrhagic shock

Haemorrhagic shock occurs when a patient loses 30% or more its estimated blood volume (5). The source of the haemorrhage should be determined and controlled as soon as it is possible during the initial stabilization. Although the basic approach to fluid therapy of dogs and cats in haemorrhagic shock remains the same for the most situations, special consideration is given to the circumstances in which the fluid therapy itself may cause the problems (e.g. head trauma) (2).

Studies from 1960s and 1970s using Wiggers animal model of graded controlled haemorrhagic shock demonstrated that a large extracellular fluid deficit occurred in prolonged severe haemorrhagic shock (6). Only the infusion of both shed blood and lactated Ringer's solution was effective in terms of replacing the red cell mass, plasma volume, and extracellular fluid. The advocates of early fluid resuscitation with large volumes of fluids, i.e., isotonic crystalloids in volumes 2 to 3 times the estimated blood loss, argued that the need for increasing cardiac output and oxygen delivery to maintain microvascular perfusion and oxygenation exceeds any risk of accentuating haemorrhage. Aggressive fluid resuscitation during the Vietnam War with crystalloid solutions and blood products allowed patients who previously would have succumbed to haemorrhagic shock to survive. Likewise, renal failure became less frequent clinical problem, but acute respiratory distress syndrome appeared as an early cause of death after severe haemorrhage (1).

Large volume fluid resuscitation has been challenged in clinical trials (7, 8) and experimental models (9, 10, 11, 12) of uncontrolled haemorrhagic shock. Any attempts to increase blood pressure to normal by aggressive fluid resuscitation resulted in increased bleeding from injured vessels, haemodynamic decompensation, and increased mortality, when compared to no fluid resuscitation (8,13, 14) or hypotensive resuscitation (15, 16). Aggressive fluid infusion to achieve normal haemodynamic parameters is therefore contraindicated in uncontrolled haemorrhagic shock because it may renew internal bleeding; it is withheld until the time of surgical intervention.

The route of fluid administration

The main goals of resuscitation are to stop the source of haemorrhage and to restore circulating blood volume. Vascular access must be obtained to begin adequate volume resuscitation, because fluids administered subcutaneously or into peritoneal cavity are not considered adequate for shock therapy. The neurohormonal response to low cardiac output results in peripheral vasoconstriction and poor absorption of fluids administered subcutaneously or intraperitoneally. Central veins (v. jugularis) allow larger volumes of crystalloids to be administered faster, but usually the catheterization of a peripheral vein is completed before placing a catheter in the jugular vein. The intraosseous route (trochanteric fossa, tibial crest, iliac wing or proximal humerus) of administration may come into consideration in animals weighing less than 2 kg, for instance puppies and kittens (17).

Isotonic crystalloids

Isotonic crystalloids have been the most common type of fluid recommended initially for the shock patient. Examples of isotonic crystalloids include lactated Ringer's solution and physiologic saline or 0.9% NaCl. Lactated Ringer's solution is probably the most widely and frequently used balanced salt solution for fluid resuscitation in haemorrhagic shock. It provides a source of bicarbonate as a result of metabolism of lactate to CO_2 and H_2O, which is particularly beneficial in shock patients with clinically relevant metabolic acidosis. Hyperchloraemic metabolic acidosis has been reported after administration of large doses of physiologic saline, therefore it is less suitable for the acute volume resuscitation (17).

Isotonic crystalloids freely cross capillary membranes and large quantities must be infused to restore plasma volume. The poor intravascular retention of isotonic crystalloids supports intravascular volume transiently but later can cause tissue oedema with impaired oxygen perfusion. Approximately 75% to 80% of isotonic crystalloids move to the interstitial space within the first hour after intravenous administration, therefore four times the amount of crystalloids (compared with blood loss) should be administered to support the circulation (17, 18). Large volumes of isotonic crystalloids decrease intravascular oncotic pressure due to dilution of impermeant protein anions. Decreased oncotic pressure impairs maintenance of intravascular volume and promotes extravasation of fluids into the interstitial space (17).

Several studies have raised questions regarding the effects of resuscitation regimens on aspects of

the immune response to haemorrhagic shock. It was observed, that lactated Ringer's solution exacerbated neutrophil superoxide burst activity and increased neutrophil adherence (19). Also, it has been shown that aggressive crystalloid resuscitation was followed by increased cytokine activation including IL-1, IL-6 and TNF (20).

Colloids

Colloid solutions are divided into biologic (e.g., whole blood, albumin, plasma) and synthetic (hydoxyethyl starch derivatives, dextrans, gelatins, haemoglobin-based oxygen carriers). They have been advocated for the treatment of haemorrhagic shock as they tend to remain in the intravascular compartment. The addition of colloids to crystalloids considerably reduces total fluid requirements, prolongs volume expansion, and obtains haemodilution with increased microvascular perfusion. Ideally, a shock bolus of crystalloids and synthetic colloids is an excellent starting point to increase intravascular volume, venous return, cardiac output, and tissue perfusion. As these fluids are given, fresh whole blood or blood constituents can be obtained to be used for transfusion (2).

Whole-blood transfusion may be warranted as a first choice biologic colloid during haemorrhagic shock. Packed red cells together with fresh frozen plasma may be used as well. Adequate haemoglobin levels are required to maintain oxygen-carrying capacity and to deliver oxygen to the tissues. In addition, plasma is an excellent source of coagulation factors and proteins such as albumin, which are required for drug binding and buffering. The colloid oncotic pressure of albumin is nearly identical to that of plasma (20 to 25 mmHg). However, albumin distributes throughout the extracellular space, and the amount of time it spends in the intravascular space is longer than that of crystalloid but shorter than that of hetastarch. The plasma half-life of albumin is 16 hours, and more than 90% of infused albumin remains in the intravascular space (21).

In animals with acute blood loss of more than 30% estimated blood volume and haematocrit values below 25%, packed red blood cells are administered at doses of 15 to 20 ml/kg, fresh frozen plasma at doses of 10 to 15 ml/kg, and whole blood at doses of 20 to 25 ml/kg (2). Under most of circumstances, the recommended rate of whole-blood transfusion has been reported not to exceed 22 ml/kg/h, but a clinical situation such as haemorrhagic shock may require quicker administration (22).

Synthetic colloids are high molecular weight substances that remain in the vascular space. They fall into following major groups: the hydoxyethyl starch derivatives, the dextrans, the gelatins, and haemoglobin-based oxygen carriers (to be discussed later). The hydroxyethyl starches are synthesized by partial hydrolysis of amylopectin (the branched form of plant starch), the dextrans from a macromolecular polysaccharide produced from bacterial fermentation of sucrose, and the gelatins from hydrolysis of bovine collagen followed either by succinylation or linkage to urea (23).

Among the synthetic colloids, hydoxyethyl starches (HES) have the fewest anaphylactoid reactions and are the most commonly used colloids in Europe. The extent of hydoxyethylation (degree of substitution), and its pattern, determine the degradation of HES by serum α-amylase, and therefore account for the pharmacological differences between various HES specifications (24). The average molecular weight plays only a minor role in determining the pharmacological profile of HES solution (25, 26). HES may interfere with coagulation and accumulate in plasma and tissues (23).

The most pronounced side effects were found with large and highly substituted HES molecules, like hetastarch HES 450/0.7 which has an average molecular weight of 450,000 dalton and high degree of molar substitution (i.e., ratio of hydroxyethyl groups to glucose residues) of 0.7 (27). HES 450/0.7 prolongs partial thromboplastin time, but clinical episodes of bleeding have not been reported in human or veterinary patients when daily administration does not exceed currently recommended guidelines (10 to 20 ml/kg in the dog and 10 to 15 ml/kg in the cat). The dosage may be administered as a rapid bolus in dogs and over 10 to 15 minutes in cats because rapid administration has been reported to cause nausea in cats. HES 450/0.7 at a dosage of 20 ml/kg results in 70% to 200% (average 141%) increase in plasma volume, and the duration of plasma volume expansion is 12 to 48 hours, with longer retention time with higher doses (17).

In Europe, several hydroxyethyl starch products are available with smaller average molecular weights and smaller degree of molar substitution, including pentastarch or HES 200/0.5 with average molecular weight of 200,000 dalton and degree of molar substitution of 0.5 (HAES-steril 6%, Fresenius Kabi, Bad Homburg, Germany) and a novel HES type 130/0.4 with an average molecular weight of

130,000 dalton and a degree of substitution of 0.4 (Voluven, Fresenius Kabi, Bad Homburg, Germany). The molecular weight of the latter is the narrowest of all available HES types, i.e., the proportion of very large and very small molecules is reduced. Voluven® has a volume effect of approximately 100% (of the infused volume) and a 4- to 6-hour duration, which is comparable with the current European standard HES, i.e., HAES-steril 6%®. These products have been developed to maximize volume expansion effects while minimizing the risk of adverse effects on the haemostatic system by reducing the number of large molecules (22, 26).

Hypertonic saline

Hypertonic salt solution, i.e., 7.5% NaCl in a dose of 4 ml/kg has been shown to expand plasma volume three to four times the volume infused through an endogenous fluid redistribution (28, 29). It also has positive inotropic effects and the increase in cardiac output is apparently mediated by a reflex arc involving the vagus nerve with receptor in the lung. Microvascular blood flow is not only enhanced by haemodilution but also by hyperosmolar shrinkage of blood and endothelial cells (30). Small-volume hypertonic resuscitation is effective in achieving haemodynamic stability, restoring splanchnic organ perfusion (31, 32), attenuating neutrophil margination (33) and reducing the increases in postresuctitation intracranial pressure (34, 35). The use of hypertonic saline requires preexisting normal hydration and serum sodium concentration as it causes considerable increases in serum sodium concentration leading to salt poisoning (17).

The duration of effects is similar to that of isotonic crystalloids, and additional intravascular support with colloids is required to maintain effective volume expansion. Complications of hypertonic saline administration may occur when solutions are infused too rapidly (not faster than 1 ml/kg/minute) and include bradycardia, hypotension, bronchoconstriction, and rapid, shallow breathing. Cellular dehydration is another potential complication of administering hypertonic solutions, an effect that is more likely when multiple doses are used or when hypertonic saline is used in dehydrated patients (17).

Oxygen-carrying blood substitutes

Oxygen-carrying blood substitutes hold a promise as effective resuscitation fluids that may improve oxygen carrying capacity without problems of storage, compatibility, and disease transmission that are associated with standard blood transfusion.

The haemoglobin-based oxygen carrier Oxyglobin (Biopure Corporation, Cambridge, Massachusetts, USA), which is a polymerized haemoglobin of bovine origin, has been released to the veterinary market. Oxyglobin® exerts a colloid effect in blood, potentially making it the ideal fluid for resuscitation. Supplemental oxygen is not required for the positive effects on oxygen-carrying capacity, and it has prolonged shelf life (approximately 2 years at room temperature) (17). Disadvantages include short plasma-half life, potential renal toxicity, hypertensive effects, and the potential of immunogenic effects (1).

Conclusion

Shock is defined as inadequate tissue oxygenation due to poor perfusion. Haemorrhagic shock ensues when circulating blood volume decreases secondary to haemorrhage and a patient loses 30% of its blood volume. The main goal of therapy in haemorrhagic shock is obtaining adequate tissue oxygenation. Intravenous fluids, crystalloids, and/or colloids should augment cardiac output, thus increasing tissue perfusion and potential oxygen delivery to the tissues. Blood products may be necessary to improve haemoglobin content and oxygen-carrying capacity.

References

1. Krausz MM. Initial resuscitation of hemorrhagic shock. World J Emerg Surg 2006; 1: 14.

2. Mandell DC, King LG. Fluid therapy in shock. Vet Clin North Am Small Anim Pract 1998; 28: 623-44.

3. Committee on trauma: advanced trauma life support manual. Chicago: American College of Surgeons 1997: 103-12.

4. Gutierrez G, Reines HD, Wulf-Gutierrez ME. Clinical review: hemorrhagic shock. Crit Care 2004; 8: 373-81.

5. Guyton AC. Textbook of medical physiology. 7th ed. Philadelphia: WB Saunders, 1986: 326.

6. Bacter CR, Canizaro PC, Shires GT. Fluid resuscitation of hemorrhagic shock. Postgrad Med 1970; 48: 95-9.

7. Kaweski SM, Sise MJ, Virgilio RW. The effect of prehospital fluids on survival in trauma patients. J Trauma 1990; 30: 1215-8.

8. Bickell WH, Wall MJ Jr, Pepe PE, et al. Immediate versus delayed fluid resuscitation for hypotensive patients with penetrating torso injuries. N Engl J Med 1994; 331: 1105-9.

9. Gross D, Landau EH, Assalia A, Krausz MM. Is hypertonic saline safe in »uncontrolled« hemorrhagic shock? J Trauma 1988; 28: 751-6.

10. Bickell WH, Briuttig SP, Wade CE. Hemodynamic response to abdominal aortotomy in anesthetized swine. Circ Shock 1989; 28: 321-32.

11. Solomonov E, Hirsh M, Yahiya A, Krausz MM. The effect of vigorous fluid resuscitation in uncontrolled hemorrhagic shock following massive splenic injury. Crit Care Med 2000; 28: 749-54.

12. Gross D, Landau EH, Klin B, Krausz MM. Treatment of uncontrolled hemorrhagic shock with hypertonic saline solution. Surg Gynecol Obstet 1990; 170: 106-12.

13. Bickell WH, Bruttig SP, Millnamow GA, O'Benar J, Wade CE. The detrimental effects of intravenous crystalloids after aortotomy in swine. Surgery 1991; 110: 529-36.

14. Krausz MM, Bar Ziv M, Rabinovici R, Gross D. »Scoop and run« or stabilize hemorrhagic shock with normal saline or small-volume hypertonic saline? J Trauma 1992; 33: 6-10.

15. Kowalenko T, Stern SA, Dronen SC, Wang X. Improved outcome with hypotensive resuscitation of uncontrolled hemorrhagic shock in a swine model. J Trauma 1992; 33: 349-53.

16. Dronen SC, Stern SA, Wang X, Stanley M. A comparison of the response of near-fatal acute hemorrhage with and without a vascular injury to rapid volume expansion. Am J Emerg Med 1993; 11: 331-5.

17. Day TK, Bateman S. Shock syndromes. In: DiBartola SP, editor. Fluid, electrolyte, and acid-base disorders in small animal practice. 3rd ed. St Louis: Saunders Elsevier, 2006: 540-64.

18. Griffel MI, Kaufman BS. Pharmacology of colloids and crystalloids. Crit Care Clin 1992; 8: 235-53.

19. Rhee PD, Burris C, Kaufman M, et al. Lactated Ringer's solution causes neutrophil activation after hamorrhagic shock. J Trauma 1998; 44: 313-9.

20. Hierholzer CB, Harbrecht JM, Menezes J. Essential role of nitric oxide in the initiation of the inflammatory response after hemorrhagic shock. J Exp Med 1998; 187: 917-24.

21. Kirby R, Rudloff E. The critical need for colloids: maintaining fluid balance. Compend Contin Educ Vet 1997: 19: 705.

22. Crystal MA, Cotter SM. Acute hemorrhage: a hematologic emergency in dogs. Compend Contin Educ Vet 1992, 14: 1.

23. Hughes D, Boag AK. Fluid therapy with macromolecular plasma volume expanders. In: DiBartola SP, editor. Fluid, electrolyte, and acid-base disorders in small animal practice. 3rd ed. St Louis: Saunders Elsevier, 2006: 621-34.

24. Treib J, Baron JF, Grauer MT, Strauss RG. An international view of hydoxyethyl starches. Intensive Care Med 1999; 25: 258-68.

25. Ferber HP, Nitsch E, Förster H. Studies on hydroxyethyl starch. Arzneimittelforschung 1985; 35: 615-22.

26. Förster H. Physical and chemical properties of hydroxyethyl starches. In: Baron JF, editor. Plasma volume expansion. Paris: Arnette, 1992: 105-20.

27. Langeron O, Doelberg M, Ang ET, Bonnet F, Capdevila X, Coriat P. Voluven®, a lower substituted novel hydroxyethyl starch (HES 130/0.4), causes fewer effects on coagulation in major orthopedic surgery than HES 200/0.5. Anesth Analg 2001; 92: 855-62.

28. Gala GJ, Lilly MP, Thomas SE, Gann DS. Interaction of sodium and volume in fluid resuscitation after hemorrhage. J Trauma 1991; 31: 545-56.

29. Dubick MA, Wade CE. A review of the efficacy and safety of 7.5% NaCl/6% dextran 70 in experimental animals and humans. J Trauma 1994; 36: 323-30.

30. Corso CO, Okamoto S, Laiderer R, Messmer K. Resuscitation with hypertonic saline dextran reduces endothelial cell swelling and improves hepatic microvascular perfusion and function after hemorrhagic shock. J Surg Res 1998; 80: 210-20.

31. Maningas PA. Resuscitation with 7.5% NaCl in 6% dextran 70 during hemorrhagic shock in swine: effects on organ blood flow. Crit Care Med 1987; 15: 1121-6.

32. Scalia SV, Taheri PA, Force S, et al. Mesenteric microcirculatory changes in nonlethal hemorrhagic shock: the role of resuscitation with balanced electrolyte or hypertonic saline/dextran. J Trauma 1992; 33: 321-5.

33. Angle N, Hoyt DB, Cabello-Passini R, Herdon-Remelius C, Loomis W, Junger WG. Hypertonic saline resuscitation reduces neutrophil margination by suppressing neutrophil L selectin expression. J Trauma 1998; 45: 7-13.

34. Anderson JT, Wisner DH, Sullivan PE, et al. Initial small volume hypertonic resuscitation of shock and brain injury: short and long term effects. J Trauma 1997; 42: 592-601.

35. Quereshi AI, Suarez JI. Use of hypertonic saline solutions in treatment of cerebral edema and intracranial hypertension. Crit Care Med 2000; 28: 3301-13.

TEKOČINSKA TERAPIJA HEMORAGIČNEGA ŠOKA

A. Seliškar

Povzetek: Hemoragični šok je klinični sindrom, ki se kaže z nezadostno oskrbo tkiv s kisikom zaradi slabe prekrvitve kot posledice zmanjšanega efektivnega volumna krvi. Pri zdravljenju hemoragičnega šoka so se kot učinkovite izkazale izotonične in hipertonične raztopine soli in različni koloidi. Izotonični kristaloidi neovirano prehajajo skozi kapilarno membrano, zato jih moramo injicirati v veliki količini, da nadomestimo izgubljeno količino plazme. V znotrajžilnem prostoru ostanejo le kratek čas, kasneje pa zaradi prehajanja v zunajžilni prostor lahko povzročijo edem tkiv in njihovo okrnjeno oskrbo s kisikom. Dodajanje koloidov znatno zmanjša potrebe po skupni količini tekočin, podaljša volumensko ekspanzijo, zaradi razredčitve krvi pa se zveča mikrovaskularna prekrvitev. Hipertonične raztopine soli z dodanimi koloidi ali brez njih z endogeno preraz-poreditvijo tekočin zvečajo količino plazme tri do štirikratno glede na injicirano količino raztopine soli. Učinkujejo pozitivno inotropno, k zvečanju minutnega volumna srca pa naj bi prispevala tudi aktivacija refleksnega loka, ki vključuje n. vagus in receptorje v pljučih. Mikrovaskularni pretok se po dajanju hipertoničnih kristaloidov ne zveča samo zaradi razredčitve krvi, temveč tudi zaradi hiperosmotskega skrčenja krvnih in endotelijskih celic. Ne glede na vrsto tekočine, ki jo uporabimo pri zdravljenju hemoragičnega šoka, je nujno čimprej vzpostaviti učinkovito cirkulacijo, s čimer omejimo morebitne zaplete zaradi dalj časa trajajoče hipoksije. Poudariti je tudi treba, da je cilj tekočinske terapije normaliziranje življenjskih funkcij živali in ne zgolj dajanje vnaprej določenega odmerka tekočin.

Ključne besede: hemoragični šok; tekočinska terapija; kristaloidi; koloidi

Slov Vet Res 2008; 45 (3): 89-102
UDC 576.314:57.088:577.12:66.095.261.42

Review Article

SEVEN TRANSMEMBRANE RECEPTORS (7TM) IN THE VIEW OF DIMERIZATION AND EXPERIMENTAL METHODS TO STUDY THEIR DIMERIZATION AND CROSS-TALK

Valentina Kubale[1, 2]*, Azra Pogačnik[1], Thue W. Schwartz[2, 3], Milka Vrecl[1]

[1]Institute of Anatomy, Histology and Embryology, Veterinary Faculty of University of Ljubljana, Gerbičeva 60, 1115 Ljubljana, Slovenia; [2]Laboratory for Molecular Pharmacology, Department of Neuroscience and Pharmacology, The Panum Institute, University of Copenhagen, Blegdamsvej 3, DK-2200, Copenhagen, [3]7TM Pharma, Fremtidsvej 3, DK-2970, Hørsholm, Denmark

*Corresponding author, E-mail: valentina.kubale@vf.uni-lj.si

Summary: Seven transmembrane (7TM) receptors constitute the largest and the most diverse superfamily of proteins encoded in the human genome comprising at least 850 putative members. These receptors are widely expressed in the body and play a fundamental role in physiology and pathophysiology. Not only do they regulate many physiological processes, but drugs that target these receptors and either activate or inactivate them, account for the most prosperous drugs sold worldwide. Of approximately 500 currently marketed drugs, more then 30 % are modulators of 7TM receptor function. In the last two decades, impressive progress in the understanding of 7TM receptor function has been achieved, though dimerization or oligomerization of 7TM receptors is still a novel and controversial concept. Although a large quantity of data, obtained by different biophysical, biochemical, structural and functional approaches e.g. coimunoprecipitation, Western blot, bioluminescence and fluorescence resonance energy transfer (BRET and FRET, respectively) argue for dimerization or oligomerization of these receptors, several publications criticized the applied methods and challenged the concept. In this paper the main and the most important techniques are presented and complemented with our ideas.

Key words: 7TM receptors, structure, dimerization, experimental methods

Introduction

With its 865 members, according to recent bioinformatics analysis of human genome (1) the group of seven transmembrane (7TM) receptors, also known as G protein-coupled receptors constitute the largest superfamily of proteins in humans, as well as in animals. As the first name implies, these integral membrane molecules share a common tertiary structure characterized by seven membrane-spanning α- helices connected by alternating intra- and extracellular loops. As the second name implies, the receptors are able to signal by activating heterotrimeric intracellular guanine-nucleotide binding proteins (G-proteins) (2, 3).

Received: 2 September 2008
Accepted for publication: 20 September 2008

The 7TM receptors are targets for an extraordinary repertoire of extracellular endogenous ligands, which show a vast variation in structure, size and chemical nature. This includes large proteins, peptides, amino acids, lipids, biogenic amines, nucleotides and even single ions. This diverse group of ligands illustrates the involvement of these receptors in a great variety of physiological processes. What these diverse ligands have in common is their ability to functionally activate 7TM receptors and thereby propagate signals to the intracellular environment and in such way to serve as molecular switches for cells, which is crucial for cellular survival and cellular adaptation. As a result, receptors exert a wide range of physiological functions, for instance they mediate neurotransmission, hormone response, inflammation, regulate heart rate and blood pressure, modulate skin pigmentation, direct the site-specific

trafficking of leukocytes, control neuronal activity in specific areas of the brain, contribute to the progression of infectious diseases, or are involved in recognizing light-sensing molecules in the eye, several hundred of distinct odorants in the olfactory system and a large number of taste molecules in taste buds (2, 4, 5, 6, 7).

Defects in receptor signalling systems and their regulation are responsible for many diseases. These include defects in heart rhythm and motor skills, metabolic diseases like obesity and type II diabetes, mental disorders, as well as some viral infections, like those observed with influenza, herpesvirus infections and some forms of cancer. Given this functional diversity, it is not surprising that approximately a third of the commercially available drugs modulate 7TM receptor activity and 7TM receptors remain highly interesting, both from a biological perspective and as future drug targets (7). 7TM receptor agonists and antagonists have therapeutic benefit across a broad spectrum of diseases including pain (opioid receptor agonists), asthma (β_2-adrenergic receptors agonists), peptic ulcers (histamine receptors antagonists) and hypertension (angiotensin receptor antagonists) (6, 8). They have also been proven as relatively easy drug targets to modulate cellular responses, since these drugs can act without entering the cell. However, around 100 7TM receptors, according to the list maintained by the International Union of Basic and Clinical Pharmacology (IUPHAR), have not yet been paired with their endogenous ligands and are known as "orphan" receptors (9).

7TM receptors have no overall sequence homology (10); though, they share a basic common architecture of seven hydrophobic TM domains consisting of 20-25 amino acids, while the total length of 7TM receptors is generally 400-480 residues. The TMs form α helices connected by alternating three intracellular loops (IC-loops) and three extracellular loops (EC-loops) and are flanked by an extracellular amino-terminal tail and intracellular carboxyl-terminal tail. Apart from the overall common architecture, most 7TM receptors have structural similarities also designated as "fingerprint residues", which are distributed as clusters of conserved residues throughout the receptor. Based on conserved key residues and structural motifs, the 7TM receptor superfamily has been subdivided into three major families denoted A, B and C. In addition, three minor families exist namely the frizzled/smoothened, taste TR2 and pheromone VR1 receptor families (6). The A family receptors are by far the most studied. Rhodopsin (light receptor of the eye) and β_2-adrenergic receptors (receptors that target cateholamines) are prototypical members and their structures have been determined at the molecular level. Rhodopsin was the first 7TM receptor crystallized (11) and several years later two groups have successfully crystallized β_2-adrenergic (12) and β_1-adrenergic receptors (13). Other important members of the A family are chemokine receptors and receptors regulated by viruses. The B family of receptors are characterised by a large (~ 100 amino acids) N-terminal domain to which peptide hormones bind and activate this group. Metabotropic glutamate receptors (mGlu) are the prototypical receptors that make up the C family. Family C members have a very large N-terminal domain (> 350 residues), which is responsible for binding their endogenous ligands.

The 7TM receptors primarily signal through the family of G-proteins. These heterotrimeric compounds consist of the three subunits, designated α, β and γ, of approximately 40, 35 and 7 kDa, respectively. Agonist activation of a 7TM receptor induces conformational change within the receptor resulting in increased affinity for the G-protein and exchange of GDP and GTP on the Gα subunit. Subsequently, both the Gα-GTP and Gβγ are activated and able to modulate signalling molecules. The intrinsic activity of the Gα subunits hydrolyzes the GTP back to GDP, which promotes reassociation with the Gβγ and both signalling molecules are returned to their inactive states. Diversity of G-protein signalling leads to a wide range of possible biological responses including cell proliferation, differentiation, development, survival, angiogenesis, hypertrophy or oncogenesis. This is accomplished by a plethora of Gα and Gβγ subunits, which stimulate a variety of intracellular signalling pathways and other downstream targets (14, 15). Traditionally, G-proteins have been classified into four families based on sequence homologies among the α-subunits, namely $G\alpha_s$, $G\alpha_i$, $G\alpha_q$ and $G\alpha_{12}$ (16). A total of 16 genes encode Gα proteins and further diversity is generated through transcriptional splice variation (17). The Gβ and Gγ subunits are tightly linked and can be considered as a single entity. There are five Gβ genes and twelve Gγ genes and also many splice variants have been described. Multiple pairings between Gβ and Gγ subunits are possible.

Members of the $G\alpha_s$ family primarily induce the activity of adenylyl cyclase (AC), leading to cAMP formation. On the contrary, most members of the $G\alpha_i$ family inhibit the activity of AC. The $G\alpha_q$ subunits

primarily stimulate different subtypes of phospholipase C (PLC) β, which results in inositol 1,4,5 triphosphate (IP3) and diacylglycerol (DAG) accumulation, while the $G\alpha_{12}$ subunits regulate the ras homology guanine exchange factor activity. As opposed to the Gα families, the action of the βγ-subunits does not primarily act upon one type of effector. The βγ-subunits are capable of regulating a multitude of effector molecules including all the abovementioned as well as phophoinositide 3-kinases, G-protein coupled receptor kinases (GRKs), tyrosin kinases and ion channels among others (11). In addition to signalling through G-proteins, 7TM receptors can also transduce signals through G-protein independent mechanisms. This was first reported in 1988, when acetylcholine was found to induce a potassium current through muscarinic receptors in the absence of G-protein (18). However, this remained highly controversial until recently as the tools available to rule out a G-protein mediated response have made G-protein-independent signalling unequivocal. The existence of G protein-independent, GRK/β-arrestin-mediated signal transduction has been established for several 7TM receptors (19) and may represents a potentially important mechanism by which 7TM receptors modulate physiologic processes as recently suggested in the cardiovascular system (20).

Idea about 7TM receptors dimerization and their cross-talk

7TM receptors are traditionally thought to act as monomers that engage their cognate G-proteins with a stochiometry of 1:1 (6). The traditional view of 7TM receptors signalling (described above), a cell-surface receptor is activated by the binding of a single agonist ligand, which further activates one G-protein (21, 22). Rhodopsin, the first 7TM receptor which was purified, was characterized as a single polypeptide that functions as an isolated monomer 30 years ago. Initially it was assumed that it binds like other 7TM receptor members to one heterotrimeric G-protein, thus a single receptor can activate sequentially a large number of G-proteins. However, this idea has been challenged during the 1970s and 1980s, with the usage of radiation inactivation as well as accumulating pharmacological and biochemical data regarding the association of many 7TM receptors types into higher-ordered structures (23, 24). On the other hand, crystalographic analyses of two new crystal forms of rhodopsin (25) revealed an interaction surface which may be involved in the formation of functional dimers or oligomers and implicates TM-I, TM-II, and helix 8 as the main contact surface stabilizing the dimers; this supports a stochiometry of 2:1 (2 receptors, 1 G-protein). Most evidence suggests that 7TM receptors dimerize, although formations of higher 7TM receptor complexes are also probable, as receptor dimers may represent the "building blocks" of higher order oligomers. This idea that receptors might dimerize was first proposed in 1982 (26) although the idea only gained some acceptance after more than a decade. 7TM receptors can be either connected to identical partner(s), which results in formation of homodimers (or homo-oligomers), or to structurally different receptor(s), which results in formation of heterodimers (hetero-oligomers) (27).

7TM receptor dimerization was proposed to play a potential role in i) receptor maturation and correct transport to the plasma membrane, ii) ligand-promoted regulation, iii) pharmacological diversity, iv) signal transduction, and v) receptor internalization and desensitization (28, 29).

Dimerization interface and domain swapping

Many different interfaces of dimerization are reported in literature, involving the transmembrane domains and ectodomains, such as C-termini. A considerable amount of evidence supports a TM-V and TM-VI dimerization interface (30), however on the other hand, functional site was identified also in helices TM-II and TM-III. Nevertheless, the interface may depend on additional stabilizing factors such as the coiled-coil interactions reported in the $GABA_B$ receptor and the disulfide bridge interactions in the muscarinic and the other class C receptors. $GABA_B$ receptors are an example of an interaction involving their C-termini (31). The evidence from several laboratories (reviewed in 30) support the TM-V and TM-VI dimerization interface, known to play a role in activation. Helices TM-V and TM-VI can form two dimer types: TM-V, TM-VI-contact dimers, and TM-V, TM-VI-domain-swapped, dimers. Simple TM-V, TM-VI contact dimer would use exactly the same V-VI interface and would differ only in the relative orientation of IC loop3. Domain swapping was proposed by Gouldson et al. (32) as an explanation for the functional rescue observed on co-expression of adrenergic-muscarinic chimeric receptors (33). The ability of helices TM-I and TM-V and TM-VI and TM-VII to function as separate A and B domains has been shown for the rhodopsin, adrenergic, muscarinic,

vasopressin, and GnRH receptors. Such evidence for a dynamic structure reinforces the idea that these receptors could domain-swap (30). The functional interaction site identified in helices TM-II and TM-III is not as well-defined as the site on helices TM-V and TM-VI and there are not many experiments that clearly implicate the external face of helices TM-II and TM-III in dimerization activity (34).

Methods applied for studying 7TM receptor dimerization and cross-talk

Different methods have been employed in order to resolve how 7TM receptors work, showing that they might not act as single monomeric units. Despite many different techniques applied to resolve this issue, this new concept is still debated (35). One concern has been that 7TM receptors homo-/heterodimerization might be an artefact of receptor over-expression and/or a result of the techniques used to study receptor associations. Some of these methods are discussed below.

Sucrose density gradient fractionation and immunoblot analyses

Initially, analysis of the receptor species for neurotransmitters, such as dopamine, opioids, and other peptides present on plasma membranes obtained by sucrose density gradient fractionation and visualized by immunoblot, revealed receptor monomers, dimers, and higher molecular weight forms. Tetramers of 7TM receptors have also been reported in immunoblot analyses of membranes from cells expressing the receptors and in native brain tissue (36). This method never gained general acceptance, partly due to the fact that there were observations of some oligomers with odd an number of receptors (e.g. trimers, pentamers) (23).

Co-immunoprecipitation and Western blot

One of the most frequently used approaches was co-immunoprecipitation of differentially-tagged receptors, which has also been used to track other protein partners. Immunoprecipitation is a useful method for isolating proteins of interest from cellular extracts using specific antibodies. Following immunoprecipitation of a protein of interest, it can be determined via Western blot whether any other proteins have co-immunoprecipitated. 7TM receptors can be tagged with different epitopes (e.g. HA-, c-myc-) and the same 7TM receptor can be co-expressed in two different foms in a heterologous expression system. Initially this technique was used to co-express c-myc- and HA-tagged forms of β_2-AR in insect Sf9 cells and demonstrated interaction between the two isoforms of the receptor as both were present in imunoprecipitates generated using either anti-HA or anti-c-myc antibodies (37). These studies also noted the resistance to monomerization during SDS/PAGE resolution of simple membrane preparations, while higher-ordered structures were observed, under denaturating conditions (in the presence of sodium dodecyl sulfate; SDS), which were interpreted as potential artefacts. Because of hydrophobic nature of the receptor TM domains there were opinions that co-immunoprecipitation might reflect nonspecific aggregations due to detergent extraction of membranes or cells. However, Salim et al. (38) showed interactions between several 7TM receptors. The first widely accepted demonstration of 7TM receptor heterodimerization came from the $GABA_B$ (GB_BR) receptors, which only responded to GABA when co-expressed (39).

Atomic force microscopy (AFM)

Rhodopsin is the only 7TM receptor for which the presumed higher-order oligomeric state has been demonstrated in the native disk membrane. Recent atomic force microscopy (AFM) studies revealed an oligomeric arrangement of rhodopsin and opsin in the form of large paracrystalline arrays, which shows receptors organized into rows of dimers (40, 41).

AFM is a very high-resolution scanning probe microscope, with demonstrated resolution of fractions of nm, more than 1000 times better than the optical diffraction limit. The AFM can be used to image and manipulate atoms and structures on a variety of surfaces. The atom at the apex of the tip "senses" individual atoms on the underlying surface when it forms initial chemical bonds with each atom. Because these chemical interactions gently alter the tip's vibration frequency, they can be detected and mapped. AFM has also been used to measure the forces required to unfold single rodopsin molecules, thereby revealing which residues are responsible for its stability. Recent functional analyses of fractions from solubilized disk membranes revealed that higher-order rhodopsin oligomers are the most active species. Based on these data and the X-ray structure, an atomic mod-

el of rhodopsin dimers has been proposed, a model that is currently analyzed and utilized in various ways (42).

Fluorescence resonance energy transfer (FRET) technique

Fluorescence resonance energy transfer (FRET) is a biophysical method that enables proximity assessment of appropriately tagged receptor proteins in live cells. Initially it was used to demonstrate conformational changes in 7TM receptor monomers upon agonist binding and was later used to detect 7TM receptor dimers. Förster resonance energy transfer (RET) is based on non-radiative transfer of energy between suitable donor and acceptor that are less than 100 Å apart and properly orientated (43, 44). The basic principle (described in 45) of this method of detection uses two fluorescently labeled proteins, the green fluorescent protein (GFP) and a mutation of this protein with altered spectral characteristics, e.g. cyan fluorescence protein (CFP), yellow fluorescence protein (YFP) or others variants. The transfer of energy is a consequence of the spectral overlap between the donor emission and acceptor excitation profiles. Although, FRET enables the visualization of protein interactions in living cells, the problems associated with FRET (e.g. high fluorescent background, autofluorescence, photobleaching) make BRET, which uses bioluminescence donor instead of a fluorescent one, the technology of choice for several applications. Also a distinct time-resolved FRET-based approach to monitor interactions between 7TM receptors at the cell surface is based on using anti-epitope tag antibodies labeled with suitable donors and acceptors, which are modifications of earth elements lanthanides. N-terminally epitope-tagged forms of 7TM receptors should only be accessible to such antibodies in intact cells if they have been delivered successfully and inserted in plasma membrane (46). Advanced version is a combination of time-resolved FRET and SNAP-tag technologies, which enables quantitative analysis of protein-protein interactions. SNAP-tag technology enables direct covalent labeling of the proteins of interest, eliminating the need for antibodies and reducing the assay complexity. It consists of a protein tag that reacts covalently with a labeled substrate, forming a stably labeled fusion protein. Substrates are derivatives of O_6-benzylguanine (BG) that can carry a wide range of different labels (46, 47).

Bioluminescence resonance energy transfer (BRET)

BRET is principal method by which scientists currently investigate 7TM receptors oligomerization (28). BRET was first described in a study on dimerization of the bacterial Kai B clock protein (43). Subsequently, BRET was introduced in the 7TM receptor and tyrosine kinase receptor field demonstrating β_2-AR dimerization (48) and ligand-induced conformational changes in the insulin receptor (49). Further on it has been suggested as a suitable tool for characterization and detection of 7TM receptors homo/heterodimerization (50). Since then, existence of several homo- and heterodimers has been reported in living cells. Renilla luciferase (RLuc) and green fluorescent protein (GFP) used as energy donor an acceptor, respectively are fused to the proteins of interest and co-expressed in the same cells (described in 45). In the presence of the substrate (e.g. coelenterazine, DeepBlueC), RLuc emits light at a specific wavelength and after interaction of labeled proteins RET occurs, when energy is transferred from RLuc to GFP and light is emitted at different wavelength. Following BRET, an advantageous version, termed BRET^2 was developed with improvements in the substrate (instead of coelenterazine h, a coelenterazine analog DeepBlueC was employed) and Stokes shift (GFP was replaced by GFP mutant GFP^2), which gives better separation of RLuc and GFP light and hence higher sensitivity (51). However, quantification of the fraction of 7TM receptors existing as momoner vs. dimer or higher-order oligomer remains a challenging task. With a modified form of the Veatch and Stryer model (52) and using BRET, we proposed a theoretical model to distinguish between these possibilities (50).

Controlled dimerization system

This system was developed by Patricia Hinkle group (53) in order to study the functional importance and consequences of homodimerization of thyrotropin-releasing hormone receptor (TRH). In this system, which allows artificially induced homodimerization of TRH receptor, the C-terminal tail of receptor was linked to HA-tagged human FK506 binding protein (FKBPv). Furthermore, a dimeric ligand was created by linking together to monomeric ligands, which each bind to a single FKBPv and used to induce dimerization of FKBPv-TRH receptor fusion protein.

Chimeric approach - employment of quality control system of GB_BR

The most direct and convincing evidence for receptor dimerization came from studies on the GABA (GB) receptors, the first 7TM receptors shown to be functionally combined of two distinct subunits, $GABA_{B1}$ (GB_{1a}) and $GABA_{B2}$ (GB_2), as an obligatory constitutive heterodimer (39, 54, 55, 56), thus representing a good model for studying the functional relevance of 7TM receptor dimerization. The GB_{1a} found to be largely nonfunctional in the terms of ligand binding when expressed alone and GB_2 nonfunctional in signalling properties. Co-expression of the two subunits is required for proper insertion of the heterodimer to plasma membrane. The GB_{1a} subunit contains an endoplasmic reticulum (ER) retention RSRR signal in its C-terminal tail, preventing it from reaching the cell surface as a monomer. Only when associated with GB_2, GB_{1a} retention signal is masked and the GB_{1a} subunit can reach the cell surface as a functional receptor (56). Although no covalent linkage between the subunits has been observed, these dimers are likely to be very stable due to α coiled-coil interaction. To determine whether the GB_{1a} subunit is required for all GB_BR pharmacology, transgenic mice were generated with a GB_{1a} receptor deletion. In these mice, pre- and postsynaptic GB_BR function was absent, which suggests that GB_{1a} is essential for all GB_BR pharmacology. In addition, the epileptic phenotype of heterozygote animals indicated that both GB_BR agonist and antagonists might have therapeutic benefit in treatment of neurological and psychiatric disorders (57). In a study on mGlu5 receptor (58), a member of C family of 7TM receptors, chimeric mGlu5 receptor was engineered by exchange of the mGlu5 receptor tail with GB_{1a} and GB_2 tails, developing mGlu5-GB_{1a} and mGlu5-GB_2 chimeras (receptors that have combined attributes from different sources). The study demonstrated that the quality control system of GB_BR could be transferred to other family C members. In our study this principle was transferred to a member of the A family of 7TM receptor (59).

Concatameric approach

For studying dimerization, a new approach of forced obligatory dimers, also termed concatamers can be used. The term concatamer or concatameric receptors is used when two or more linear molecular units are covalently linked in tandem. Previously this system was used to study the stoichometry of acetylcholine ion gated channel - a heteromer consisting of five subunits (60) and was later transferred to the 7TM receptor field by Terpager (61). In the latter study two 7TM receptors were forced together by linkage with an artificial transmembrane region, since 7TM receptors have an uneven number of transmembrane segments, which places the N-terminal and C-terminal parts on the opposite side of membrane, we can not fuse them together in a classical way. Concatamers were engineered by linking the C-terminal end of "receptor A" to the N-terminal end of "receptor B" through a spacer construct comprising of an artificial transmembrane helix. As model systems, two unrelated 7TM receptors e.g. NK_1-R and β_2-AR were chosen, which, at present, are not expected to form heterodimers by themselves, but homodimers of the β_2-AR were (37, 48) and were also connected together in a fusion protein. The same system was used to study homodimerization of the ghrelin receptor (unpublished).

Dimerization observed in different families of 7TM receptors

Heterodimerization in the C family of receptors has been most extensively studied and for some experts in the field of 7TM receptors the only one demonstrated to form real dimers. In this family of 7TM receptors heterodimerization is important for either receptor function, proper expression on the cell surface or enhancing receptor activity. In the A family of receptors, dimerization was extensively studied, however it remains extensively controversial. On the other hand, this view my change due to recent compelling evidence for the dimerization in the family A 7TM receptors that was elegantly demonstrated for the first time *in vivo* by Huhtaniemi's group who was able to rescue the LH receptor knockout phenotype by complementation i.e. co-expressing two nonfunctional receptor mutants in the knockout mice (62). Review of examples is presented in Table 1.

Clinical relevance of 7TM receptor homo- and heterodimerization

Importance of 7TM receptor dimerization is already obvious in cross-talk between different classes of drugs. Beta blockers can, in some cases, block signalling by both β_2-AR and angiotensin AT_1R. Furthermore, the interaction between σ-OPR and μ-OPR

Table 1: Overview of dimerizing 7TM receptors

7TM receptors FAMILY C	Type/ Mechanism	Effect	Method	Reference
GABA receptors $GB_{1a}R$, GB_2R	heterodimerization via C-terminal tail coil-coiled interaction	$GB_{1a}R$ binds the ligand, GB_2R is required for cell-surface expression, together are functional	yeast two-hybrid screening; co-immuno- precipitation	(54, 63, 64)
glutamate receptor mGluR1	*homodimerization* via a disulfide bridge in the N-terminal domain	each bind a ligand, might be required for proper expression on the cell surface	different crystal structures of the extracellular ligand-binding region of mGluR1	(65)
Taste receptors (T_1R_2), (T_1R_3)	heterodimerization	sweet response	transgenic rescue experiments	(66)
Taste receptors T_1R_1 and T_1R_3	heterodimerization	umami taste responses effect on pharmacology of receptors	transgenic rescue experiments	(66)
FAMILY A				
adrenergic receptors (AR) β_2-AR	*homodimerization*	targeting of receptor to the cell surface	coimmunoprecipitation; rescue of a constitutively desensitized form of the receptors; BRET; mutation of a putative dimerization motif	(37, 48, 50, 67)
- β_2-AR - β_1-AR or β_3-AR	heterodimerization	reduction in the rate of agonist-induced internalization of β_2-AR; reduced ability of the receptor to stimulate ERK phosphorylation; **proper regulation of cardiac contractility**	co-immunoprecipitation; BRET; knock out mice; co-immunoprecipitation	(68, 69, 70)
β_1-AR/ β_2-AR α_2-AR	heterodimerization	cross-internalization of the receptors following agonist stimulation	BRET	(71)
β_2-AR opioid receptors	heterodimerization	significant cross-internalization of the receptor complex by adrenergic and opioid ligands	BRET	(72)
dopamine receptors D_1R D_2R	heterodimerization	heterodimer exhibits different function than the individual momomer receptors	transgenic mice experiments	(73)
opioid peptide receptor (OPR) family	*homodimerization*	interconversion between the dimeric and monomeric forms plays a role in opioid receptor internalization	BRET; agonist-induced internalization with differentially (Flag and c-Myc) epitope-tagged receptors; biochemical and pharmacological methods	(72, 74, 75)
opioid peptide receptor (OPR) family δ-OPR κ-OPR	heterodimerization	change in pharmacological properties of the receptors	biochemical and pharmacological methods	(75)

δ -OPR μ-OPR	heterodimerization	influences receptor pharmacology and results in synergistic signalling, delta opioid receptor ligands enhance morphine's potency	co-immunoprecipitation; *in vivo* experiment on mice	(76)
δ-OPR/κ-OPR β_2-AR	heterodimerization	influences receptor internalization	BRET;	(72)
μ-OR somatostatin receptor $SSTR_2A$	heterodimerization	significant co-internalization and cross-desensitization	co-immunoprecipitation;	(77)
μ-OR NK_1-R	heterodimerization	significant co-internalization and cros-desensitization	co-immunoprecipitation; BRET	(78)
somatostatin receptor family $SSTR_5$, $SSTR_1$ D_2R	heterodimerization	significant co-internalization and cross-desensitization	biochemical and pharmacological methods	(79)
purinergic receptor subtypes A_1R, $P2Y_1R$	heterodimerization	exhibits a distinct pharmacological profile, which may contribute to the diversity of purinergic receptor binding sites	co-immunoprecipitation; confocal laser microscopy	(80)
A_1R D_1 dopamine receptors	heterodimerization	exhibits a distinct pharmacological profile	co-immunoprecipitation; confocal laser microscopy	(81)
A_1R mGluR1	heterodimerization	new molecular and functional interaction between two functionally unrelated types of G protein-coupled receptors and receptors cross-talk	co-immunoprecipitation	(82)
$A_{2A}R$ mGluR5	heterodimerization	synergistic signaling between adenosine receptor and glutamate receptor agonists	co-immunoprecipitation; confocal laser microscopy; *in vivo* experiment on mice	(83)
olfactory receptors (ORs) M71 β_2-AR	heterodimerization	a striking enhancement in the surface trafficking of M71 in heterologous cells	co-immunoprecipitation; co-internalization	(84)
vasopressin oxytocin receptor (OTR) subtypes $V_{1a}R/V_2R$, $V_{1a}R$/OTR, V_2R/OTR $V_{1a}R$//OTR	heterodimerization	two receptors can be endocytosed as stable heterodimers	co-internalization	(85)
vasopressin bradykinin B_2 receptor	heterodimerization	effect on the cell surface expression	co-immunoprecipitation; BRET	(86)
muscarinic acetylcholine M_2 M_3	heterodimerization	functial interaction between receptors, cross-interaction between receptor subtypes	chimeric receptors	(87)
M_3 α_2C	heterodimerization	functial interaction between receptors, cross-interaction between receptor subtypes	chimeric receptors	(87)

thyrotropin-releasing hormone receptors TRHR1 TRHR2	heterodimerization	interactions of TRHRs with beta-arrestin may be altered by hetero-oligomer formation	BRET; confocal microscopy	(88)
Angiotensin AT_1 bradykinin B_2	heterodimerization	alteration in the hetero-dimerization of 7TM receptors was **linked to preeclampsia, first known disorder caused by heterodimerization of 7TM receptors**	pharmacological methods; *in vivo* experiment on mice	(89)
angiotensin AT_1 and AT_2	heterodimerization	AT_1 antagonises AT_2 receptor	pharmacological methods; *in vivo* experiment on mice	(90)
cholecystokinin type A and B	heterodimerization	heterodimeriaztion enhances signalling and promotes cell growth	pharmacological methods; co-internalization	(91)
endothelin type A and B receptors	heterodimerization	influence on ligand induced internalization	co-immunoprecipitation; co-internalization	(92)
thyrotropin (TSHr) and lutropin (LH/CGr) receptors	*homodimerization* and heterodimerization	link between homodimerization and negative cooperativity	BRET; TR-FRET: functional and biochemical approaches	(93)
relaxin family peptide receptor 1 and 2	*homodimerization* and heterodimerization	link between homodimerization and negative cooperativity	BRET	(94, 95)
FAMILY B				
gastric inhibitory polypeptide receptor (GIP-R)	*homodimerization*	new insight into the ability of family B 7TM receptors to dimerize	BRET	(50)
(GIP-R with β_2-AR	heterodimerization	heterodimerization between members of different families of 7TM receptors	BRET	(50)
vasoactive intestinal polypeptide receptors (VPAC1 VAPC2)	*homodimerization* and heterodimerization	new insight into the ability of family B 7TM receptors to dimerize	BRET	(96)
(VPAC1 and VAPC2) with secretin receptor	heterodimerization	new insight into the ability of family B 7TM receptors to dimerize	BRET	(96)
Calcitonin-like receptor protein (CLRL9, receptor-activating-modifying protein (RAMP) family	dimerize with non-7TM receptor proteins	expressed alone, it seems to be non-functional, but when co-expressed with a member of the receptor-activating-modifying protein (RAMP) family, becomes functional receptor	co-immunoprecipitation	(97)

(**bold** – known relevance in organism, *italic* - homodimerization)

has been postulated to account for the well-known effects of σ-OPR on μ-OPR mediated-analgesia. Synergistic and antagonistic interactions between drugs are extremely important to consider in a clinical setting and heterodimerization between receptors represents a specific mechanism that may potentially underlie certain drug-drug interactions at the molecular level. In the field of chemokine receptors, two subtypes of chemokine receptor, CCR5 and CXCR4 are known to act as co-receptors for HIV entry into cells. Evidence exists that dimerization or cross-talk between 7TM receptors in host cell and viral 7TM receptor can enable the activity of viruses.

Further evidence for the clinical significance of 7TM receptor heterodimerization has also come from recent studies on Wnt receptors. Wnts are

secreted glycoproteins that play diverse roles in regulating cell fate and proliferation via activation of 7TM receptors belonging to the frizzled (Fz) family. Mutations to the Fz4 subtype have been found to underlie an autosomal-dominant form of a disease known as familial exudative vitreoretinopathy (FEVR), which is characterized by impaired growth of retinal capillaries leading to eventual retinal degeneration. The mutant Fz4 receptor in FEVR can form homodimers with wild-type Fz4 leading to ER retention of these receptors (98). Preeclampsia was the first disorder associated with altered 7TM receptor heterodimerization of AT_1/B_2 receptors (89). AT_1 is a receptor for the vasopressor angiotensin II and the B_2-receptor for the vasodepressor bradykinin 2. Upon AT_1–B_2-receptor heterodimerization, angiotensin- II–stimulated signaling is strongly enhanced. AT_1–B_2-receptor heterodimerization in preeclampsia is mediated by a 4–5-fold increase in B_2-receptor protein levels. A significant induction of B_2 receptors can be observed with the major clinical signs of preeclampsia.

7TM receptor homo- and heterodimerization could therefore represent a novel aspect of 7TM receptor biology that has exciting potential in drug discovery.

Acknowledgements

The authors would like to kindly thank Kate Whitfield for English proofreading of the manuscript. V.K., A.P. and M.V. thank the Slovenian Research Agency for financial support (programme P4-0053; Slovenian-Danish collaboration grant BI-DK/07-09-002).

References

1. Fredriksson R, Schioth HB. The repertoire of G-protein-coupled receptors in fully sequenced genomes. Mol Pharmacol 2005; 67:1414-25.

2. Schwartz TW. Molecular structure and function of 7TM G-protein coupled receptors. In: Foreman JC, Johansen T, eds. Textbook of receptor pharmacology. Boca Raton: CRC Press; 2003: 81-111.

3. Hamm HE. The many faces of G protein signaling. J Biol Chem 1998; 273: 669-72.

4. Brink CB, Harvey BH, Bodenstein J, Venter DP, Oliver DW. Recent advances in drug action and therapeutics: relevance of novel concepts in G-protein-coupled receptor and signal transduction pharmacology. Br J Clin Pharmacol 2004; 57: 373-87.

5 Drews J. Drug discovery: a historical perspective. Science 2000; 287: 1960-4.

6. Gether U. Uncovering molecular mechanisms involved in activation of G protein-coupled receptors. Endocr Rev 2000; 21: 90-113.

7. Pierce KL, Premont RT, Lefkowitz RJ. Seven-transmembrane receptors. Nat Rev Mol Cell Biol 2002; 3: 639-50.

8. Wise A, Gearing K, Rees S. Target validation of G-protein coupled receptors. Drug Discov Today 2002; 7: 235-46.

9. Levoye A, Jockers R. Alternative drug discovery approaches for orphan GPCRs. Drug Discov Today 2008; 13: 52-8.

10. Bockaert J, Pin JP. Molecular tinkering of G protein-coupled receptors: an evolutionary success. EMBO J 1999; 18: 1723-9.

11. Palczewski K, Kumasaka T, Hori T, et al. Crystal structure of rhodopsin: a G protein-coupled receptor. Science 2000; 289: 739-45.

12. Rasmussen SG, Choi HJ, Rosenbaum DM, et al. Crystal structure of the human β_2 adrenergic G-protein-coupled receptor. Nature 2007; 450: 383-7.

13. Warne T, Serrano-Vega MJ, Baker JG, et al. Structure of a β_1-adrenergic G-protein-coupled receptor. Nature 2008; 454: 486-91.

14. Neves SR, Ram PT, Iyengar R. G protein pathways. Science 2002; 296:1636-9.

15. McCudden CR, Hains MD, Kimple RJ, Siderovski DP, Willard FS. G-protein signalling: back to the future. Cell Mol Life Sci 2005; 62: 551-77.

16. Offermans S. G-proteins as transducers in transmembrane signalling. Prog Biophys Mol Biol 2003; 83: 101-30.

17. Milligan G, Kostenis E. Heterotrimeric G-proteins: a short history. Br J Pharmacol. 2006; 147: S46-55.

18. Olesen SP, Davies PF, Clapham DE. Muscarinic-activated K+ current in bovine aortic endothelial cells. Circ Res. 1988; 62: 1059-64.

19. Urban JD, Clarke WP, von Zastrow M, et al. Functional selectivity and classical concepts of quantitative pharmacology. J Pharmacol Exp Ther 2007; 320:1-13.

20. Lutrell LM, Lefkowitz RJ. The role of β-arrestins in the termination and transduction of G-protein-coupled receptor signals. J Cell Sci 2002; 115: 455-65.

21. Cerione RA, Codina J, Benovic JL, Lefkowitz RJ, Birnbaumer L, Caron MG. The mammalian β_2-adrenergic receptor: reconstitution of functional interactions between pure receptor and pure stimulatory nucleotide binding protein of the adenylate cyclase system. Biochemistry 1984; 23: 4519-25.

22. Cerione RA, Sibley DR, Codina J, et. al. Reconstitution of a hormone-sensitive adenylate cyclase system. The pure β-adrenergic receptor and guanine nucleotide regulatory protein confer hormone responsiveness on the resolved catalytic unit. J Biol Chem 1984; 259: 9979-82.

23. Salahpour A, Angers S, Bouvier M. Functional significance of oligomerization of G-protein-coupled receptors. Trends Endocrinol Metab 2000; 11: 163-8.

24. Devi LA. Heterodimerization of G-protein-coupled receptors: pharmacology, signaling and trafficking. Trends Pharmacol Sci 2001; 22: 532-7.

25. Lodowski DT, Salom D, Le Trong I, et al. Crystal packing analysis of Rhodopsin crystals. J Struct Biol 2007; 158: 455-62.

26. Agnati LF, Fuxe K, Zoli M, Rondanini C, Ogren SO. New vistas on synaptic plasticity: the receptor mosaic hypothesis of the engram. Med Biol 1982; 60: 183-90.

27. Angers S, Salahpour A, Bouvier M. Dimerization: an emerging concept for G protein-coupled receptor ontogeny and function. Annu Rev Pharmacol Toxicol 2002; 42: 409-35.

28. Terrillon S, Bouvier M. Roles of G-protein-coupled receptor dimerization. EMBO Rep 2004; 5: 30-4.

29. Gurevich VV, Gurevich EV. Rich tapestry of G protein-coupled receptor signaling and regulatory mechanisms. Mol Pharmacol 2008; 74:312-6.

30. Dean MK, Higgs C, Smith RE, et al. Dimerization of G-protein-coupled receptors. J Med Chem 2001; 44: 4595-614.

31. Marshall FH, Jones KA, Kaupmann K, Bettler B. $GABA_B$ receptors - the first 7TM heterodimers. Trends Pharmacol Sci 1999; 20: 396-9.

32. Gouldson PR, Higgs C, Smith RE, Dean MK, Gkoutos GV, Reynolds CA. Dimerization and domain swapping in G-protein-coupled receptors: a computational study. Neuropsychopharmacology 2000; 23 (Suppl 4): S60-77.

33. Maggio R, Vogel Z, Wess J. Coexpression studies with mutant muscarinic/adrenergic receptors provide evidence for intermolecular "cross-talk" between G-protein-linked receptors. Proc Natl Acad Sci U S A 1993; 90: 3103-7.

34. Lavoie C, Mercier JF, Salahpour A, et al. β_1/β_2-adrenergic receptor heterodimerization regulates β_2-adrenergic receptor internalization and ERK signaling efficacy. J Biol Chem 2002; 277: 35402-10.

35. Chabre M, le Maire M. Monomeric G-protein-coupled receptor as a functional unit. Biochemistry 2005; 44: 9395-403.

36. Peterson GL, Rosenbaum LC, Broderick DJ, Schimerlik MI. Physical properties of the purified cardiac muscarinic acetylcholine receptor. Biochemistry 1986; 25: 3189-202.

37. Hebert TE, Moffett S, Morello JP, et al. A peptide derived from a β_2-adrenergic receptor transmembrane domain inhibits both receptor dimerization and activation. J Biol Chem 1996; 271: 16384-92.

38. Salim K, Fenton T, Bacha J, et al. Oligomerization of G-protein-coupled receptors shown by selective co-immunoprecipitation. J Biol Chem 2002; 277: 15482-5.

39. Kuner R, Kohr G, Grunewald S, Eisenhardt G, Bach A, Kornau HC. Role of heteromer formation in $GABA_B$ receptor function. Science 1999; 283: 74-7.

40. Fotiadis D, Liang Y, Filipek S, Saperstein DA, Engel A, Palczewski K. The G protein-coupled receptor rhodopsin in the native membrane. FEBS Lett 2004; 564: 281-8.

41. Liang Y, Fotiadis D, Filipek S, Saperstein DA, Palczewski K, Engel A. Organization of the G protein-coupled receptors rhodopsin and opsin in native membranes. J Biol Chem 2003; 278: 21655-62.

42. Lee SP, O'Dowd BF, George SR. Homo- and hetero-oligomerization of G protein-coupled receptors. Life Sci 2003; 74: 173-80.

43. Xu Y, Piston DW, Johnson CH. A bioluminescence resonance energy transfer (BRET) system: application to interacting circadian clock proteins. Proc Natl Acad Sci U S A 1999; 96: 151-6.

44. Eidne KA, Kroeger KM, Hanyaloglu AC. Applications of novel resonance energy transfer techniques to study dynamic hormone receptor interactions in living cells. Trends Endocrinol Metab 2002; 13: 415-21.

45. Kubale V, Vrecl M. Novi postopki za ugotavljanje proteinsko-proteinskih interakcij v živih celicah = Novel tools to identify protein-protein interactions in living cells. Vet Nov 2004; 30: 271-8; erata 30: 360-1.

46. Maurel D, Comps-Agrar L, Brock C, et al. Cell-surface protein-protein interaction analysis with time-resolved FRET and snap-tag technologies: application to GPCR oligomerization. Nat Methods 2008; 5: 561-7.

47. Regoes A, Hehl AB. SNAP-tag mediated live cell labeling as an alternative to GFP in anaerobic organisms. Biotechniques. 2005; 39: 809-10, 812.

48. Angers S, Salahpour A, Joly E, et al. Detection of β_2-adrenergic receptor dimerization in living cells using bioluminescence resonance energy transfer (BRET). Proc Natl Acad Sci U S A 2000; 97: 3684-9.

49. Boute N, Pernet K, Issad T. Monitoring the activation state of the insulin receptor using bioluminescence resonance energy transfer. Mol Pharmacol 2001; 60: 640-5.

50. Vrecl M, Drinovec L, Elling C, Heding A. Opsin oligomerization in a heterologous cell system. J Recept Signal Transduct Res 2006; 26: 505-26.

51. Heding A. Use of the BRET 7TM receptor/β-arrestin assay in drug discovery and screening. Expert Rev Mol Diagn 2004; 4: 403-11.

52. Veatch W, Stryer L. The dimeric nature of the gramicidin A transmembrane channel: conductance and fluorescence energy transfer studies of hybrid channels. J Mol Biol 1977; 113: 89-102.

53. Song GJ, Hinkle PM. Regulated dimerization of the thyrotropin-releasing hormone receptor affects receptor trafficking but not signaling. Mol Endocrinol 2005; 19: 2859-70.

54. Ng GY, Clark J, Coulombe N, et al. Identification of a $GABA_B$ receptor subunit, GB_2, required for functional $GABA_B$ receptor activity. J Biol Chem 1999; 274: 7607-10.

55. Pagano A, Rovelli G, Mosbacher J, et al. C-terminal interaction is essential for surface trafficking but not for heteromeric assembly of GABA(b) receptors. J Neurosci 2001; 21: 1189-202.

56. Margeta-Mitrovic M, Jan YN, Jan LY. A trafficking checkpoint controls $GABA_B$ receptor heterodimerization. Neuron 2000; 27: 97-106.

57. Prosser HM, Gill CH, Hirst WD, et al. Epileptogenesis and enhanced prepulse inhibition in $GABA_{(B1)}$-deficient mice. Mol Cell Neurosci 2001; 17: 1059-70.

58. Kniazeff J, Bessis AS, Maurel D, Ansanay H, Prezeau L, Pin JP. Closed state of both binding domains of homodimeric mGlu receptors is required for full activity. Nat Struct Mol Biol 2004; 11: 706-13.

59. Kubale, V. The role of endocytosis and dimerization on the intracellular trafficking and signaling of G protein-coupled receptors. Ljubljana: University of Ljubljana, Veterinary Faculty, 2007:63. PhD thesis

60. Zhou Y, Nelson ME, Kuryatov A, Choi C, Cooper J, Lindstrom J. Human $\alpha_4\beta_2$ acetylcholine receptors formed from linked subunits. J Neurosci 2003; 23: 9004-15.

61. Terpager, M. Covalent linkage of 7TM-G-protein coupled receptors - a tool to study dimerization. Copenhagen: Department of Pharmacology, Faculty of Health sciences University of Copenhagen, 2005: 36. PhD thesis

62. Rivero-Müller, A, Ji, Lajic, S, Rahman, N, Ji, TH, Huhtaniemi, I. Saving LHR knockout males. In: 15th European Testis Workshop, Naantali, Finland, 2008: 5S.II. OP.

63. White JH, Wise A, Main MJ, et al.Heterodimerization is required for the formation of a functional $GABA_B$ receptor. Nature 1998; 396: 679-82.

64. Galvez T, Duthey B, Kniazeff J, et al. Allosteric interactions between GB_1 and GB_2 subunits are required for optimal GABAB receptor function. EMBO J 2001; 20: 2152-9.

65. Kunishima N, Shimada Y, Tsuji Y, et al. Structural basis of glutamate recognition by a dimeric metabotropic glutamate receptor. Nature 2000; 407: 971-7.

66. Nelson G, Hoon MA, Chandrashekar J, Zhang Y, Ryba NJ, Zuker CS. Mammalian sweet taste receptors. Cell 2001; 106: 381-90.

67. Hebert TE, Bouvier M. Structural and functional aspects of G protein-coupled receptor oligomerization. Biochem Cell Biol 1998; 76: 1-11.

68. Lavoie C, Mercier JF, Salahpour A, et al. b1/b2-adrenergic receptor heterodimerization regulates β_2-adrenergic receptor internalization and ERK signaling efficacy. J Biol Chem 2002; 277: 35402-10.

69. Breit A, Lagace M, Bouvier M. Hetero-oligomerization between β_2- and β_3-adrenergic receptors generates a b-adrenergic signaling unit with distinct functional properties. J Biol Chem 2004; 279: 28756-65.

70. Zhu WZ, Chakir K, Zhang S, et al. Heterodimerization of β^1- and β_2-adrenergic receptor subtypes optimizes b-adrenergic modulation of cardiac contractility. Circ Res 2005; 97: 244-51.

71. Xu J, He J, Castleberry AM, Balasubramanian S, Lau AG, and Hall RA. Heterodimerization of α_2A- and β_1-adrenergic receptors. J Biol Chem 2003; 278: 10770–7.

72. Ramsay D, Kellett E, McVey M, Rees S, Milligan G. Homo- and hetero-oligomeric interactions between G-protein-coupled receptors in living cells monitored by two variants of bioluminescence resonance energy transfer (BRET): hetero-oligomers between receptor subtypes form more efficiently than between less closely related sequences. Biochem J 2002; 365: 429-40.

73. Kobayashi M, Iaccarino C, Saiardi A, et al. Simultaneous absence of dopamine D1 and D2 receptor-mediated signaling is lethal in mice. Proc Natl Acad Sci U S A 2004; 101: 11465-70.

74. Cvejic S, Devi LA. Dimerization of the delta opioid receptor: implication for a role in receptor internalization. J Biol Chem 1997; 272: 26959-64.

75. Jordan BA, Devi LA. G-protein-coupled receptor heterodimerization modulates receptor function. Nature 1999; 399: 697-700.

76. Gomes I, Gupta A, Filipovska J, Szeto HH, Pintar JE, Devi LA. A role for heterodimerization of μ and δ opiate receptors in enhancing morphine analgesia. Proc Natl Acad Sci U S A 2004; 101: 5135-9.

77. Pfeiffer M, Koch T, Schroder H, Laugsch M, Hollt V, Schulz S. Heterodimerization of somatostatin and opioid receptors cross-modulates phosphorylation, internalization, and desensitization. J Biol Chem 2002; 277: 19762-72.

78. Pfeiffer M, Kirscht S, Stumm R, et al. Heterodimerization of substance P and μ-opioid receptors regulates receptor trafficking and resensitization. J Biol Chem 2003; 278: 51630-7.

79. Rocheville M, Lange DC, Kumar U, Patel SC, Patel RC, Patel YC. Receptors for dopamine and somatostatin: formation of hetero-oligomers with enhanced functional activity. Science 2000; 288: 154-7.

80. Yoshioka K, Hosoda R, Kuroda Y, Nakata H. Hetero-oligomerization of adenosine A1 receptors with P2Y1 receptors in rat brains. FEBS Lett 2002; 531: 299-303.

81. Gines S, Hillion J, Torvinen M, et al. Dopamine D1 and adenosine A1 receptors form functionally interacting heteromeric complexes. Proc Natl Acad Sci U S A 2000; 97: 8606-11.

82. Ciruela F, Escriche M, Burgueno J, et al. Metabotropic glutamate 1a and adenosine A_1 receptors assemble into functionally interacting complexes. J Biol Chem 2001; 276: 18345-51.

83. Ferre S, Karcz-Kubicha M, Hope BT, et al. Synergistic interaction between adenosine A_{2A} and glutamate mGlu5 receptors: implications for striatal neuronal function. Proc Natl Acad Sci U S A 2002; 99: 11940-5.

84. Hague C, Uberti MA, Chen Z, et al. Olfactory receptor surface expression is driven by association with the β_2-adrenergic receptor. Proc Natl Acad Sci U S A 2004; 101: 13672-6.

85. Terrillon S, Durroux T, Mouillac B, et al. Oxytocin and vasopressin V_{1a} and V_2 receptors form constitutive homo- and heterodimers during biosynthesis. Mol Endocrinol 2003; 17: 677–91.

86. Devost D, Zingg HH. Homo- and hetero-dimeric complex formations of the human oxytocin receptor. J Neuroendocrinol 2004; 16: 372-7.

87. Maggio R, Barbier P, Colelli A, Salvadori F, Demontis G, Corsini GU. G protein-linked receptors: pharmacological evidence for the formation of heterodimers. J Pharmacol Exp Ther 1999; 291: 251-7.

88. Hanyaloglu AC, Seeber RM, Kohout TA, Lefkowitz RJ, Eidne KA. Homo- and hetero-oligomerization of thyrotropin-releasing hormone (TRH) receptor subtypes. Differential regulation of β-arrestins 1 and 2. J Biol Chem 2002; 277: 50422-30.

89. AbdAlla S, Lother H, el Massiery A, Quitterer U. Increased AT(1) receptor heterodimers in preeclampsia mediate enhanced angiotensin II responsiveness. Nat Med 2001; 7: 1003-9.

90. AbdAlla S, Lother H, Abdel-tawab, AM, Quitterer U. The angiotensin II AT_2 receptor is an AT_1 receptor antagonist. J Biol Chem 2001; 276: 39721-6.

91. Cheng ZJ, Harikumar KG, Holicky EL, Miller LJ. Heterodimerization of type A and B cholecystokinin receptors enhance signaling and promote cell growth. J Biol Chem 2003; 278: 52972-9.

92. Gregan B, Jurgensen J, Papsdorf G, et al. A. Ligand-dependent differences in the internalization of endothelin A and endothelin B receptor heterodimers. J Biol Chem 2004; 279: 27679-87.

93. Urizar E, Montanelli L, Loy T, et al. Glycoprotein hormone receptors: link between receptor homodimerization and negative cooperativity. EMBO J 2005; 24: 1954-64.

94. Svendsen AM, Zalesko A, Kønig J, et al. Negative cooperativity in H2 relaxin binding to a dimeric relaxin family peptide receptor 1. Mol Cell Endocrinol 2008 Aug 3. Doi:10.1016/j.mce.2008.07.014.

95. Svendsen AM, Vrecl M, Ellis TM, et al. Cooperative binding of insulin-like Peptide 3 to a dimeric relaxin family peptide receptor 2. Endocrinology 2008; 149: 1113-20.

96. Harikumar KG, Morfis MM, Lisenbee CS, Sexton PM, Miller LJ. Constitutive formation of oligomeric complexes between family B G protein-coupled vasoactive intestinal polypeptide and secretin receptors. Mol Pharmacol 2006; 69: 363-73.

97. McLatchie LM, Fraser NJ, Main MJ, et al. RAMPs regulate the transport and ligand specificity of the calcitonin-receptor-like receptor. Nature 1998; 393: 333-9.

98. Prinster SC, Hague C, Hall RA. Heterodimerization of g protein-coupled receptors: specificity and functional significance. Pharmacol Rev 2005; 57: 289-98.

RECEPTORJI S SEDMIMI TRANSMEMBRANSKIMI OBMOČJI (7TM) V LUČI DIMERIZACIJE TER EKSPERIMENTALNI PRISTOPI ZA PROUČEVANJE NJIHOVE DIMERIZACIJE IN MEDSEBOJNIH VPLIVOV

V. Kubale, A. Pogačnik, T. W. Schwartz, M. Vrecl

Povzetek: Receptorji s sedmimi transmembranskimi območji (receptorji 7TM) oblikujejo največjo in najbolj raznoliko superdružino proteinov, kodiranih v človeškem genomu, ki šteje vsaj 850 domnevnih predstavnikov. Receptorji 7TM so v organizmu splošno izraženi in igrajo temeljno vlogo v fiziologiji ter patofiziologiji. Ker uravnavajo mnogo fizioloških procesov, so pomembna tarča za zdravila, ki jih bodisi aktivirajo ali inaktivirajo in so posledično najbolj uspešno prodajana zdravila. Od približno 500 trenutno prodajanih zdravil jih več kot 30 odstotkov vpliva na delovanje receptorjev 7TM. V zadnjih dveh desetletjih je znanje na področju delovanja receptorjev 7TM močno napredovalo, kljub temu pa je koncept njihovega združevanja v bodisi dimere ali oligomere še vedno sporna ter neobičajna tematika. Kljub temu da obstaja mnogo podatkov o njihovi dimerizaciji, pridobljenih z različnimi biofizikalnimi, biokemijskimi, strukturnimi in funkcionalnimi pristopi, kot so na primer tehnike koobarjanja, prenos po metodi western ter metodi bioluminiscenčnega in fluoroscenčnega prenosa resonančne energije (BRET, FRET), so številne objave kritizirale obstoječe metode in izzvale omenjeno zamisel. V članku predstavljamo osrednje in najpomembnejše metode za proučevanje dimerizacije, ki so dopolnjene še z našimi pristopi.

Ključne besede: 7TM receptorji; struktura; dimerizacija; eksperimentalni pristopi

Slov Vet Res 2006; 43 (2): 103-8
UDC 619:616-002.525:636.2/.3

Original Research Paper

IMPAIRED EXPRESSION OF FOXA3 IS NOT A COMMON CAUSE OF SERTOLI CELL-ONLY SYNDROME IN HUMAN INFERTILE PATIENTS

Katerina Čeh[1], Branko Zorn[2], Jasna Šinkovec[2], Gregor Majdič[1]*

*[1]Center for Animal Genomics, Veterinary faculty, Gerbičeva 60; [2]Andrology Centre, Department of Obstetrics and Gynecology, University Medical Centre, 1000 Ljubljana, Slovenia

*Corresponding author, E-mail: gregor.majdic1@guest.arnes.si

Summary: FoxA3 knockout mice develop Sertoli cell-only syndrome in adult life. In human infertile patients, the underlying cause of Sertoli cell-only syndrome is often unknown. In the present study, immunoexpression of FoxA3 in human testes biopsy samples was examined to determine whether expression of FoxA3 is impaired in human infertile patients. Twenty six human testes samples (20 with Sertoli cell-only syndrome, 3 with maturation arrest and 3 with normal spermatogenesis) were examined using immunocytochemistry with specific antibodies against FoxA3 protein. All human samples were obtained during diagnostic biopsy procedure because of azoospermia at outpatient infertility clinic. In all 26 human testis samples examined, immunoexpression of FoxA3 was detected in Leydig cells regardless of the histopathological findings. Suggesting that impaired expression of FoxA3 is not a common cause of Sertoli cell-only syndrome in human infertile patients.

Key words: testes; infertility; FoxA3; immunohistochemistry

Introduction

FoxA proteins are members of the winged helix/forkhead transcription factor gene family. The FoxA transcription factors (a1, a2 and a3, formerly known as hepatocyte nuclear factor HNF 3α, -β, -γ) are important for endodermal development during embryogenesis. During formation of the definite endoderm, *FoxA2* is activated first, followed by *FoxA1*, and finally *FoxA3* (1). In addition to their presumed role in endoderm development, *FoxA3* is the dominant regulator of *GLUT2* gene expression in the hepatocytes. The targeted null mutation of the *FoxA2* gene results in a missing or abnormal neural node and endoderm, which leads to early embryonic lethality (2). Embryos deficient in *FoxA1* develop to term but have abnormal glucagon secretion and die due to hypoglycemia around postnatal day 10 (3). In contrast, *FoxA3*$^{-/-}$ mice develop normally and are fertile at young age, although through analyses revealed some differences in liver gene expression between WT and FoxA3 knockout mice suggesting that FoxA3 is an important activator of hepatocyte specific genes (4).

Behr et al. (5) recently reported impaired male fertility and atrophy of seminiferous tubules in adult *FoxA3* knockout mice. Testes of FoxA3$^{-/-}$ mice show loss of germ cells secondary to an increase in germ cell apoptosis, ultimately leading to Sertoli cell-only syndrome. Interestingly, both FoxA3$^{-/-}$ and FoxA3$^{+/-}$ mice exhibit loss of germ cells, reduced fertility and testes weights. Microarray analysis of testis transcriptome from WT and FoxA3 knockout mice revealed several interesting changes in the gene expression. Among others, adult Sertoli cells from FoxA3$^{-/-}$ mice expressed anti-Müllerian hormone (5), what is similar to some previous reports about human infertile patients (6).

Infertility affects 13-18% of couples and growing evidence from clinical and epidemiological studies suggests an increasing incidence of male reproduc-

Received: 8 July 2008
Accepted for publication: 9 September 2008

tive problems. Half of these couples have a component of male factor infertility and almost 30% of infertilities will be caused solely by male factors (7, 8). In 40 % - 50 % of cases, the male partner has quantitative or qualitative abnormalities of sperm production and about 25 % of male patients with non-obstructive azoospermia are diagnosed with Sertoli cell only syndrome, where some or all tubules are completely devoid of germ cells. The etiopathogenesis of testicular failure remains unknown in about half of the cases, although it is presumed that in many cases the real causes are yet undiscovered genetic defects (9, 10).

Histological findings in the murine testes reported in the study by Behr et al. (5) were similar to some cases of Sertoli cell-only syndrome in men. Therefore, in the present study, immunoexpression of FoxA3 protein was examined with the aim to determine whether the lack of FoxA3 expression could be a cause of Sertoli cell-only syndrome in men.

Materials and methods

Mice

Adult mouse testes were obtained by perfusion fixation of adult (60 days old) Balb/c WT mice. Mice were anesthetized, perfused with Bouins' solution and testes postfixed in Bouins' solution for 24 hours before processing into paraffin wax using standard procedures. All animal experiments were approved by the Veterinary commission of Slovenia and were done according to the ethical principles and in accordance with the EU directive (86/609/EEC).

Infertile men

Human testis samples were obtained during diagnostic biopsy of infertile patients with azoospermia attending the Andrology Centre at the outpatient infertility clinic, Department of Obstetrics and Gynecology, University Medical Centre Ljubljana, before intracytoplasmic sperm injection was attempted. Samples were fixed in 4% paraformaldehyde and processed into paraffin wax using standard procedures.

Twenty-six human testes biopsy samples with different pathology, 20 with Sertoli cell-only syndrome, 3 with maturation arrest and 3 with normal spermatogenesis were examined. Average age of patients with Sertoli cell-only syndrome was 35.7 years (range 30 to 47 years). None of the patients was diagnosed with hypogonadotrophic hypogonadism. The use of human samples was approved by the Slovenian national medical ethics' committee.

Immunohistochemistry

Sections (7 microns) were mounted on slides coated with 3-aminopropyl triethoxy-silane (TESPA; Sigma, Taufkirchen, Germany) and dried overnight at 50°C. Before incubation with primary antibodies, sections were dewaxed, rehydrated in graded ethanols, washed in water and phosphate buffer saline (PBS) followed by blocking endogenous peroxidase by incubating the section for 30 min in 1% H_2O_2 in PBS. Antigen retrieval was performed by microwaving the sections in 0.01M citrate buffer (pH 6.0) on full power (750W) for 20 min, and thereafter left standing for 20 min without disturbance. Sections were then washed for 5 min in PBS and blocked using normal goat serum diluted 1:5 in PBS. Polyclonal rabbit antibodies raised against synthetic peptide corresponding to amino acids 287 – 299 of *FoxA3* protein (Abcam, Cambridge, UK) were used at a dilution of 1:500. All primary antibodies were diluted in PBS containing 20% normal goat serum. Sections were incubated with primary antibodies overnight at 4°C in a humid chamber. The following day coverslips were removed, sections washed twice in PBS (5 min each wash), incubated for 30 min with goat anti-rabbit immunoglobulins (Jackson immunochemicals, West Grove, PA, USA) diluted 1:100 in PBS and then washed again in PBS (2 times 5 min). For detection of bound antibodies, sections were first incubated with rabbit peroxidase-antiperoxidase complex (Jackson immunochemicals) for 30 min and washed 2 times in PBS (5 min each). Color reaction product was developed by incubating sections in a mixture of 0.05% (w/v) 3,3'-diaminobenzidine tetra-hydrochloride (DAB, Sigma) in 0.05M Tris-HCl, pH 7.4 and 0.01% hydrogen peroxide. After 15-30 min, sections were washed in distilled water, counterstained with haematoxylin, dehydrated in graded ethanols, cleared in xylene and coverslipped using Pertex mounting medium (CellPath plc, Hemel Hempstead, UK). Specificity of the antibody was controlled by using non-immune rabbit serum instead of primary antibodies.

Photomicrographs were captured onto computer using a Nikon Eclipse 80i microscope and Nikon DS-Fi1 digital camera.

Results

Immunoexpression of FoxA3 in murine testis

In adult murine testis, FoxA3 protein was expressed in Leydig cells and in postmeiotic elongated spermatids. Expression in elongated spermatids was first present in stage I-II and persisted until stage VII, when spermatids are shed into the tubule lumen (Fig. 1a and 1b).

Immunoexpression of FoxA3 in human testis

In the human testes samples, FoxA3 immunoexpression was detected in Leydig cells in all 26 samples examined, irrespective of their pathology (normal spermatogenesis, spermatogenic arrest, Sertoli cell-only syndrome; Fig. 1a, b and c). Similarly to the expression pattern in the mouse testes, immunoexpression of FoxA3 was also detected in the postmeiotic spermatids in human samples with complete spermatogenesis (Fig. 1a).

Discussion

In the present study, immunoexpression of FoxA3 protein was studied in the human testes to determine whether FoxA3 protein expression could be involved in the development of Sertoli cell-only syndrome in men.

Male infertility is a widespread, although still poorly understood health problem. In recent years, many genes that contribute to male infertility/subfertility have been identified. However, in many cases the underlying genetic cause of infertility is unknown (7-10). Mice with targeted mutation of FoxA3 gene became subfertile with age. Although young animals are healthy and fertile, throughout adult age germ cells in their testes degenerate and this ultimately leads to the Sertoli cell-only syndrome in many, but not all, tubules within the testis (5). This histopathological finding is very similar to some cases of human infertility/subfertility where mixed seminiferous tubule phenotype is observed. In biopsy samples from such patients, tubules with complete spermatogenesis as well as tubules with Sertoli cell-only syndrome can be found (11).

Antigen used to raise the antibodies was synthetic peptide corresponding to aminoacids 287 – 299 of human FoxA3 protein. To determine the specificity of the antibodies used in our study, we first performed immunocytochemical staining on mouse testes samples. Results were similar to the results from study by Behr et al. (5), in which they utilized CRE – β-galactosidase system to detect sites of expression of FoxA3 protein. In the mouse testis, FoxA3 was detected in both Leydig cells and germ cells with stage specific expression in the elongated spermatids, suggesting that antibodies indeed recognized FoxA3 protein.

In all 20 human testes samples with Sertoli cell only syndrome examined, we also detected the expression of FoxA3 protein at similar levels to those found in patients with normal spermatogenesis, suggesting that lack of FoxA3 expression was not a cause for the development of Sertoli cell-only syndrome in the patients examined. However, since immunocytochemistry is not a quantitative method, differences in the expression levels, perhaps due to haploinsufficiency, between patients with normal spermatogenesis and patients with Sertoli cell-only syndrome cannot be ruled out. Therefore, further genetic studies will be needed to confirm whether there is any role for a FoxA3 gene in human infertility. Furthermore, in the present study we examined 20 patients with Sertoli cell-only syndrome so it is still possible that there are patients with mutations in FoxA3 gene that were not found in our study due to relatively small sample size.

Kariotyping and genetic analyses of microdeletions on Y chromosome were not performed in this group of patients. This could potentially implicate other underlying causes for development of Sertoli cell only syndrome. However, in patients with Klinefelter's syndrome, seminiferous tubules usually undergo degeneration during puberty and in adult patients they are usually severely affected with strong hyalinization (12). In patients we examined in this study, tubules were well preserved but lacked germ cells, suggesting that Klinefelter's syndrome was unlikely cause for Sertoli cell only syndrome in these patients. Microdeletions on Y chromosome could also result in Sertoli cell only syndrome and this could not be excluded as a possible cause for the infertility in some of the patients examined in this study. However, microdeletions on Y chromosome are usually cause of infertility in about 5 - 10 % of azoospermic patients and within these, pathology is quite varied from Sertoli cell only to maturation arrest (13). Therefore, even though we cannot rule out microdeletions as underlying cause for histopathological findings in our patients, it is likely that such patients will represent only a minority of cases and therefore suggesting that majority of pa-

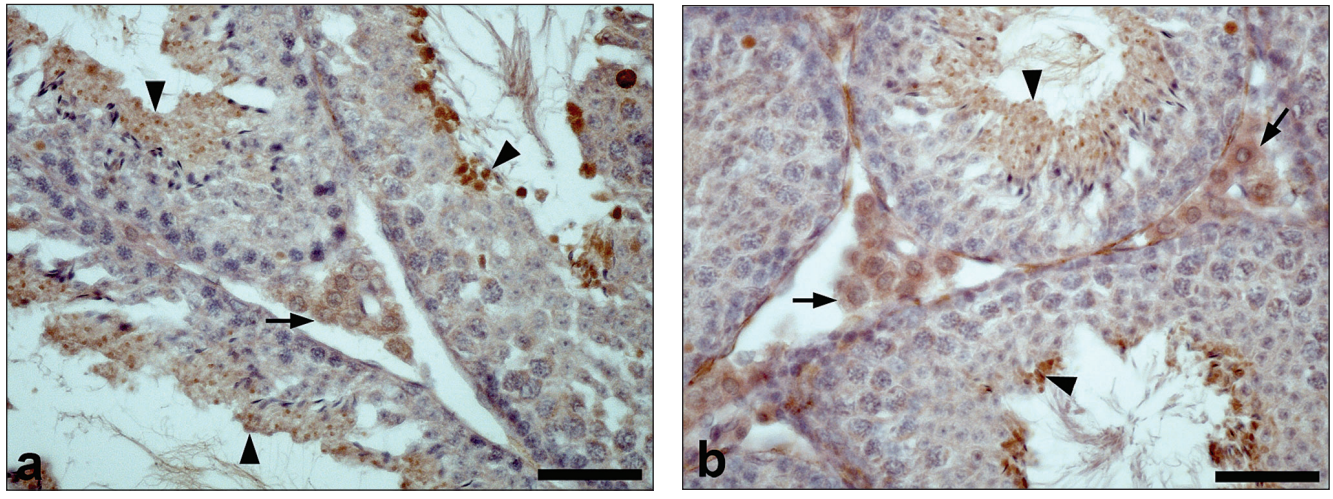


Figure 1: FoxA3 immunoexpression in the normal adult mouse testis (a and b). FoxA3 protein was detected in Leydig cells (arrows) and in elongated spermatids (arrowheads) within the tubules (bar = 50 μm)

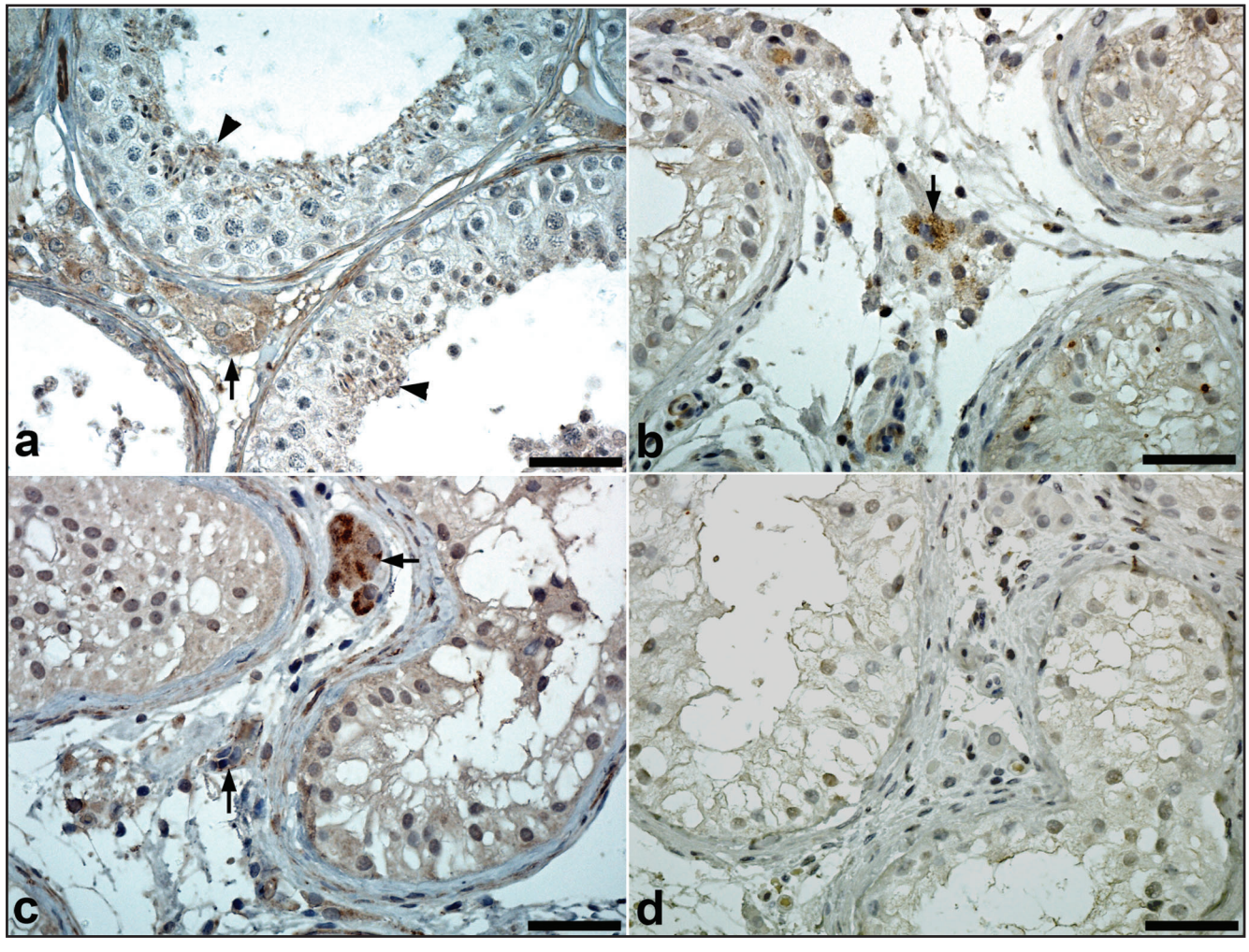


Figure 2: FoxA3 immunoexpression in human testes samples obtained during the diagnostic biopsy for azoospermia. FoxA3 protein was detected in Leydig cells (arrows) in patients with normal spermatogenesis (a) and in patients with Sertoli cell-only syndrome (b, c). In a sample with normal spermatogenesis, FoxA3 immunoexpression was also detected in the elongated spermatids (a, arrowhead), similarly to the mouse testis. No immunostaining was detected in section incubated with normal rabbit serum (d; bar = 50 μm)

tients had unknown cause of Sertoli cell only, which could be also lack of FoxA3 protein.

In conclusion, FoxA3 immunoexpression was detected in all 20 patients with Sertoli cell-only syndrome examined, therefore, our study suggests that inactivating mutations of FoxA3 gene are not a common cause for this condition in the human infertile patients.

Acknowledgement

This study was supported by ARRS (Slovenian Research Agency) grant P4-0053 and Katerina Ceh is supported by ARRS graduate fellowship.

References

1. Shen W, Scearce LM, Brestelli JE, Sund NJ, Kaestner KH. FoxA3 (hepatocyte nuclear factor 3gamma) is required for the regulation of hepatic GLUT2 expression and the maintenance of glucose homeostasis during a prolonged fast. J Biol Chem 2001; 276: 42812-7.

2. Weinstein DC, Ruiz i Altaba A, Chen WS, et al. The winged-helix transcription factor HNF-3 beta is required for notochord development in the mouse embryo. Cell 1994; 78: 575-88.

3. Kaestner KH, Katz J, Liu Y, Drucker DJ, Schutz G. Inactivation of the winged helix transcription factor HNF3alpha affects glucose homeostasis and islet glucagon gene expression in vivo. Genes Dev 1999; 13: 495-504.

4. Kaestner KH, Hiemisch H, Schutz G. Targeted disruption of the gene encoding hepatocyte nuclear factor 3gamma results in reduced transcription of hepatocyte-specific genes. Mol Cell Biol 1998; 18: 4245-51.

5. Behr R, Sackett SD, Bochkis IM, Le PP, Kaestner KH. Impaired male fertility and atrophy of seminiferous tubules caused by haploinsufficiency for FoxA3. Dev Biol 2007; 306: 636-45.

6. Steger K, Rey R, Louis F, et al. Reversion of the differentiated phenotype and maturation block in Sertoli cells in pathological human testis. Hum Reprod 1999; 14: 136-43.

7. Iammarrone E, Balet R, Lower AM, Gillott C, Grudzinskas JG. Male infertility. Best Pract Res Clin Obstet Gynaecol 2003; 17: 211-29.

8. Isidori A, Latini M, Romanelli F. Treatment of male infertility. Contraception 2005; 72: 314-8.

9. Bhasin S. Approach to the infertile man. J Clin Endocrinol Metab 2007; 92: 1995-2004.

10. Krausz C, Giachini C. Genetic risk factors in male infertility. Arch Androl 2007; 53: 125-33.

11. McLachlan RI, Rajpert-De Meyts E, Hoei-Hansen CE, de Kretser DM, Skakkebaek NE. Histological evaluation of the human testis–approaches to optimizing the clinical value of the assessment: mini review. Hum Reprod 2007; 22: 2-16.

12. Aksglaede L, Wikstrom AM, Rajpert-De Meyts E, Dunkel L, Skakkebaek NE, Juul A. Natural history of seminiferous tubule degeneration in Klinefelter syndrome. Hum Reprod Update 2006; 12: 39-48.

13. Krausz C, Forti G, McElreavey K. The Y chromosome and male fertility and infertility. Int J Androl 2003; 26: 70-5.

MOTNJE V IZRAŽENOSTI BELJAKOVINE FOXA3 NISO POGOST VZROK ZA RAZVOJ SINDROMA SCO PRI NEPLODNIH MOŠKIH

K. Čeh, B. Zorn, J. Šinkovec, G. Majdič

Povzetek: Pri miših brez gena FoxA3 se v odraslem življenju v modih razvije sindrom SCO (prisotnost samo sertolijevih celic). Pri neplodnih moških pacientih z enako histološko sliko mod je vzrok za sindrom SCO običajno neznan. V opisani raziskavi smo ugotavljali prisotnost beljakovine FoxA3 v modih zdravih miši ter v biopsijskih vzorcih človeških mod pacientov s popolno spermatogenezo in pacientov s sindromom SCO, da bi ugotovili, če je motena izraženost gena FoxA3 lahko vzrok za sindrom SCO pri ljudeh. Šestindvajset biopsijskih vzorcev (20 vzorcev s sindromom SCO, 3 vzorci s hipospermatogenezo in 3 vzorci z normalno spermatogenezo) smo pregledali z metodo imunohistokemije s specifičnimi protitelesi proti beljakovini FoxA3. Vsi vzorci človeških mod so bili zbrani z rutinsko biopsijo ob pregledu pacientov na kliniki za neplodnost. V vseh 26 preiskanih vzorcih smo ugotovili izraženost beljakovine FoxA3 v lejdigovih celicah, ne glede na histološko sliko. Rezultati naše raziskave kažejo, da napake v izraženosti beljakovine FoxA3 niso pogost vzrok za nastanek sindroma SCO pri neplodnih pacientih.

Ključne besede: modo; neplodnost; FoxA3; imunohistokemija

Slov Vet Res 2008; 45 (3): 109-6
UDC 579.62:986.5-097:612.46

Original Research Paper

DEVELOPMENT AND EVALUATION OF MONOCLONAL ANTIBODY-BASED ENZYME LINKED IMMUNOSORBENT ASSAY FOR BORRELIA GARINII ANTIGEN DETECTION IN URINE

Igor Gruntar, Tadej Malovrh

Institute for Microbiology and Parasitology, Veterinary Faculty, Gerbičeva 60, 1000 Ljubljana, Slovenia

*Corresponding author, E-mail: igor.gruntar@vf.uni-lj.si

Summary: We developed antigen capture double sandwich ELISA (ACDS ELISA), based on borrelia flagellin-specific monoclonal antibody. Nested PCR analysis for borrelial DNA in the urine samples was used for comparison. Results obtained by ELISA and nested PCR analyses of urines from mice, experimentally infected with *B. garinii* by different routes of inoculation, confirmed the presence of borrelial antigens (antigenuria), while whole spirochetes or their DNA could not be detected or were not released into the urine (absence of spirocheturia). Such ELISA might become a good supplementary method in the diagnostics of Lyme disease, especially in seronegative subjects suspected to be infected with *B. burgdorferi.*

Key words: Borrelia antigens; urine; immunoenzime assay

Introduction

Borrelia burgdorferi sensu lato spirochete (B.b.) is the causative agent of Lyme borreliosis, a multisystemic illness and the most prevalent tick-borne disease. The diagnosis of this disease is achieved by evaluating clinical, epidemiological and laboratory findings (1, 2). Unfortunately, according to the protein electrophoresis patterns and specific monoclonal antibodies (mAbs') reactivity, B.b. is antigenically extremely heterogeneous microorganism which induces highly variable humoral immune response and is consequently difficult to confirm by serological methods (3-10). Serological diagnosis of Lyme disease is therefore unreliable and a direct method for detection of B.b. in presumably infected patients is needed to improve and to simplify borreliosis diagnostics. PCR is a method of choice (11-14) but unfortunately still too expensive, too elaborate and inaccessible for some laboratories. Since *B. burgdorferi* antigens have been detected in urine, blood and many tissues of infected hosts (15, 16) we tried to develop a simple but sufficiently sensitive and specific monoclonal antibody based ELISA for B.b. antigen detection in urine specimens. The method was evaluated by probing urine samples from experimentally infected BALB/c mice, analysing also the dynamics of.*B. burgdorferi* antigenuria, humoral immune response and the importance of inoculation route. The results were compared to those obtained with nested flagellin gene targeted polymerase chain reaction performed on the same samples.

Received: 12 August 2008
Accepted for publication: 10 September 2008

Materials and methods

B. burgdorferi antigen detection ELISA

The method we developed is an antigen capture double sandwich (ACDS) ELISA based on a monoclonal antibody (mAb) 1G6/G6/D12, directed against genus specific 41 kDa protein flagelin. MAb used in the test is of our own production (17) and can be substituted with adequatly purified mAbs specific for other borrelial proteins. In the first step 100µl per well of mAb (5 µg/ml, carbonate-bicarbonate buffer, pH 9'6) is adsorbed onto solid phase (96 microwell ELISA plate for antibody adsorbtion; Nunc Maxisorp, Copenhagen, Denmark) at 4oC overnight. After washing the wells with 3 x 350 µl of Phosphate

Buffered Saline (PBS) with 0,05% Tween 20 (washing solution, PBS -T; Tween 20: Sigma, St. Louis, USA), nonspecific antigen adsorption is limited by blocking unoccupied binding sites in the wells with 200 µl of 2 % Bovine Serum Albumine (Sigma, St. Louis, USA) in PBS, pH 7,2 (PBS-BSA) for one hour at 37oC. The wells are then washed as described above and borrelial antigens in the urine samples (if present), diluted 1:4 in PBS-BSA, are allowed to react with the mAb (100 µl per well, one hour at room temperature, shaking). The eventually bound flagellin is, after washing, recognized by previously standardized (crossboard titration in indirect ELISA) hyperimmune polyclonal anti B.b. rabbit serum at 1:800 dilution in PBS-BSA (100 µl, one hour at room temperature, shaking). Rabbit antibodies, specific for the *B. burgdorferi* flagellin (if present in the sample), react in the next step (after the washing of the wells) with pig anti-rabbit immunoglobulins conjugated with horse-radish peroxidase (HRP-conjugate; Dako, Copenhagen, Denmark; 1:1000 dilution in PBS-BSA, 100 µl per well, one hour at room temperature, shaking) and are revealed consequently, after washing the wells, by the Tetra Methyl Benzidine HRP substrate (TMB, Sigma, St. Louis, USA; 100µl, 10 minutes at room temperature) evaluating the results spectrophotometrically by ELISA test plate reader (Behring EF-200 ELISA reader, Germany; double OD at 450 and 630 nm) after the peroxidase activity in the wells was stopped by adding 50 µl per well of 0,5 M H_2SO_4. Positive (previously tested negative urine spiked with 30 ng/ml of sonicated *B. garinii*) and negative controls (negative urine) are included on every test plate, 4 wells each. Samples yielding optical densities higher than 3 SD of the negatives' average are considered positive.

The method was tested by probing different types of samples (PBS, BSK medium, urine) spiked or not with borrelial antigens (sonicated or whole borrelia cells) in known concentrations (two-fold dilutions starting at 4µg/ml to 2 ng/ml and $7{,}2x10^6$ to 3,5x103 *B. burgdorferi* spirochetes/ml, respectively). Samples spiked with sonicated *Leptospira spp., E. coli* and *Salmonella* spp. at same concentrations were used to detect eventual cross reactions due to other bacteria flagellin.

Experimental infection of mice with B. garinii and sampling procedures

Fifteen 6-week old female BALB/c mice divided in 3 separately caged groups of.5 animals were infected by different routes of inoculation with the 3rd passage *B. garinii* M3/S (cultured from a tick infested mouse heart on our institute and determined as *B. garinii* by RFLP analysis after MluI digestion by dr. Eva Ruzic-Sabljic from Institute for microbiology, Medical Faculty, University of Ljubljana). The first group of mice (group A) was infected intraperitoneally, the second (group B) subcutaneously and the third (group C) intravenously with.0,1 ml BSK-H medium containing $1x10^7$ M3/S spirochetes. Additional 5 mice (group D) were inoculated with 0,1 ml sterile BSK-H medium and served as negative control. Blood and urine samples were taken at the beginning of the experiment (day 0), and on days 4, 8, 15 and 30 after initial inoculation of spirochetes. Serum and urine samples were stored at - 20oC immediately after sampling.

ELISA for the detection of B. garinii-specific antibodies in sera from experimentaly infected mice

B. garinii-specific antibodies in mouse sera were detected by indirect ELISA as described previously (17). Shortly, sonicated *B. garinii* was adsorbed onto the wells of ELISA plate overnight. Unoccupied binding sites in the wells were blocked by 2% BSA. Serum samples at 1/400 dilution reacted then with an adsorbed antigen and bound *B. garinii* specific antibodies were detected by anti mouse HRP conjugated rabbit immunoglobulins and TMB/stop substrate, measuring optical density at 450 nm in the test wells. Sera from uninfected group D mice served as negative controls. Sera yielding optical densities higher than calculated average + 3 SD of sera derived from uninfected controls were considered positive.

Detection of borrelial DNA in urine samples

A nested PCR, developed by Schmidt et al. (13), claimed to be capable of detecting less than 5 *B.burgdorferi* organisms was applied to detect *B. garinii* DNA in urine of experimentally infected mice. The target for this PCR was a specific region of the flagelin gene, common to all three pathogenic species of genus *Borrelia: B. burgdorferi* sensu stricto, *B. afzelli* and *B. garinii.* The primers and most of the PCR procedure were identical to the one described by Schmidt. Mouse urines collected during one month experimental infection were thawed and brought to room temperature. 50 µl of each sample was centrifuged at 14,000 g for 30 min. The pellet was resuspended in 600 µl PBS, pH 8. Half (300 µl)

of this suspension was used for PCR analysis. 200 *B. garinii* M3/S spirochetes in 50 µl of PBS, previously counted in Hauser - Petroff chamber by darkfield microscopy, were added to the other half to evaluate eventual inhibitory activity of each urine sample. Both suspensions were then centrifuged at 14,000 g for 20 min. The resulting pellets were dissolved in 100 µl of a 5% Chelex-100 suspension (Perkin-Elmer, USA) in PBS, heated at 100oC for 5 min and subsequently chilled on ice. After centrifugation (3000 g for 1 min), 10 µl of each supernatant was used as a template DNA in the first PCR.

Primers used in the first PCR were BBSCH31 (CAC ACC AGC ATC ACT TTC AGG GTC T) and BBSCH42 (CAA CCT CAT CTG TCA TTG TAG CAT CTT TTA TTT), targeting the chromosomal flagellin gene of *B. burgdorferi* (GenBank accession No X15661-2) and representing positions 477 to 501 and 913 to 881, respectively. The reaction mixture contained 50 mM KCl, 1,5mM MgCl2, 10mM Tris-HCl (pH 8,3), 0,05% glycerol, 0,1% TritonX-100, 0,1 mM EDTA, 00,1% BSA, 200µm (each) desoxynucleotide triphosphate (dAMP, dCTP, dTTP, dGTP), 50pM (each) outer primers, 2 U of *Taq* polymerase (Promega, USA) and 10µl of the DNA preparation (total volume 60 µl). The mixture was then overlaid with one drop of mineral oil. The amplification reactions were performed in 0,5 ml tubes with attached cap in a PTC-200 thermocycler (MJ Research, Watertown, USA). The first PCR with the outer primers was run for 25 cycles with denaturation at 94°C (30 s),.annealing at 55oC (30 s) and elongation at 72°C (30 s). Thermocycling was preceeded by 1 min at 95°C and followed by final 10 min extension at 72°C. After amplification, 3 µl of.the first PCR mixture was transferred to a second PCR mixture, consisting of the same master mixture as described abov; instead of outer primers, 25 pm (each) of inner primers FL-59 (TTT CAG GGT CTC AGG CGT CTT) and FL-7 (GCA TTT TCA ATT TTA GCA AGT GAT G), representing positions 491 to 511 and 767 to743, respectively, were used, yielding a 277-bp amplicon. The second PCR was done with 35 cycles of the same duration and same temperatures used for the first PCR for the denaturation and elongation, but the annealing temperature was increased to 58°C.

Blank controls, containing 10 µl of water instead of urine samples, as well as positive controls (10 fg of purified borrelial DNA) were run in parallel with each amplification assay.

DNA extraction, PCR mixture preparation, amplification, and analysis of amplicons were each done in separated areas. Filter barrier pipette tips and a dedicated set of pipetors were used for sample and PCR mixture preparation.

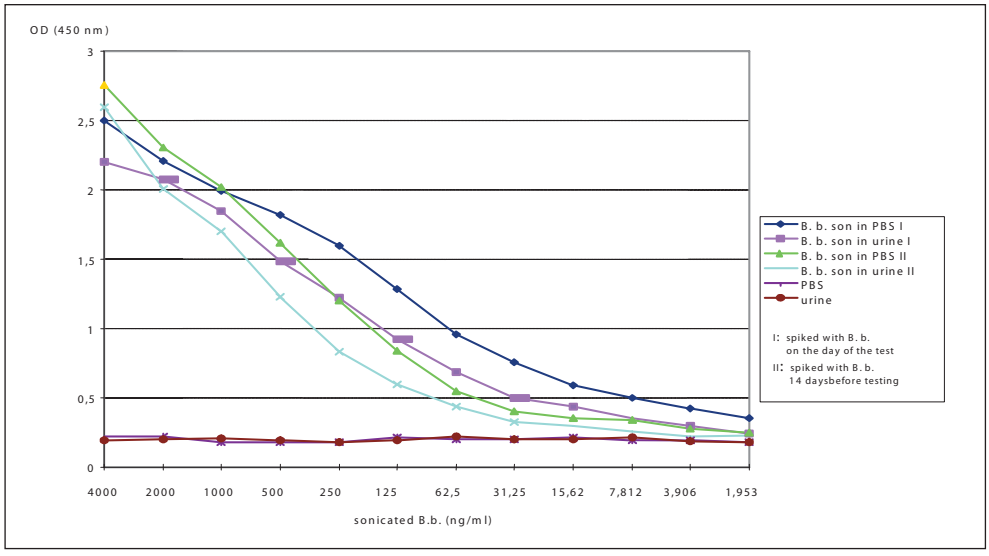
Amplified products were resolved by 3% agarose gel (NuSieve/SeaKem, 3:1; FMC Corporation, Rockland, Maine, USA) at 150 V for 25 min. The gel was then stained with ethidium bromide and the bands observed under UV illumination at 254 nm.

Results

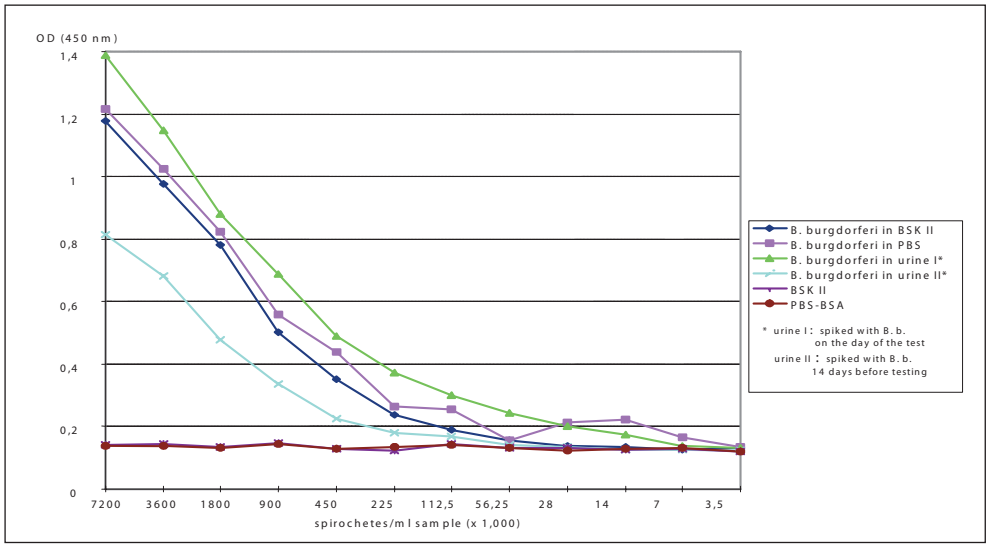
Results of our ACDS ELISA sensibility testing with serial dilutions of sonicated borrelial antigens and whole *B. garinii* cells in different types of samples are shown in Graphs No. 1 and 2, respectively. The detection limit was less than 0,5 ng/100 µl sample for sonicated borrelial antigens and cca 2,000 spirochetes/100 µl sample for whole borrelial cells. The type of the sample did not interfere with the results. Urine samples spiked with borrelial antigens reacted positively even after 14 days of storage at 4oC indicating that urine had no deterioration effect on B.b. flagelin. Cross reactions to the other bacterias' flagelin were not observed (Graph No. 3).

The detection limit of.the flagellin targeted nested PCR was in our hands slightly higher than described by the authors (13). The method was capable of detecting from 10 to 20 *B. garinii* borrelias per sample, as determined by assaying mouse urine samples spiked with known numbers of spirochetes, and thus prooved to be sufficiently sensible and specific (according to the authors) tecnique for *B. burgdorferi* DNA detection in urine. Yet, when urines obtained from mice from our experiment were examined, borrelial DNA could not be detected by this method. In view of the fact that the same samples, when spiked with 200 *B. garinii*, always.yielded the expected 277 bp amplicon, mouse urine tested were not found to be inhibitory.

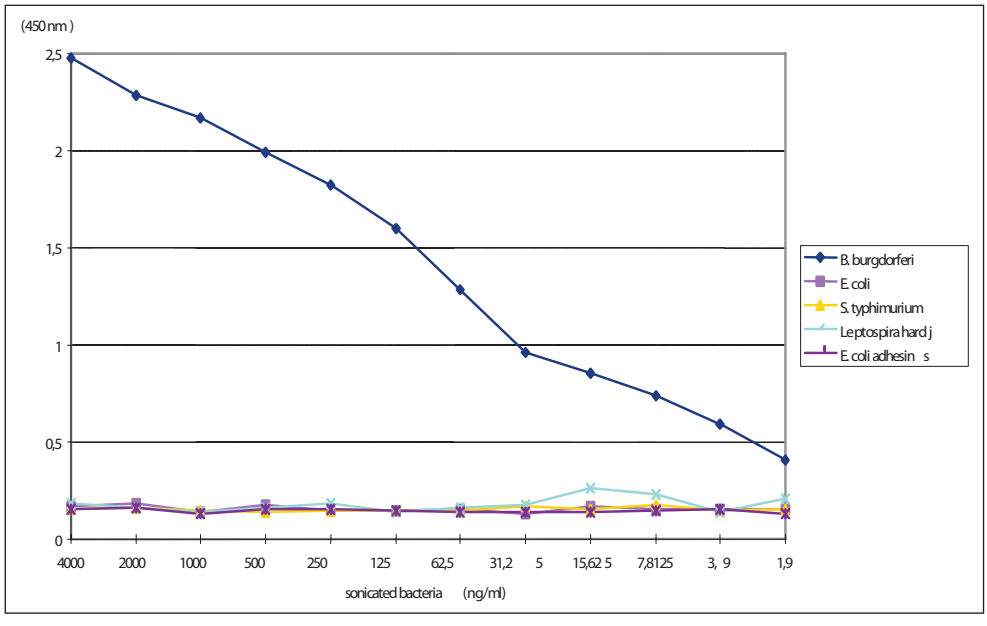
Contrariously, when the same samples were tested in ACDS ELISA, borrelial antigens could be detected in urine of virtually every *B. garinii* infected mouse at least in one of four samples taken (OD-450 nm > 0,192; cut off = average of negative controls + 3 SD = 0,145 + 3 x 0,015). The dynamics and the extent of *B. garinii* flagellin shedding in urine, together with humoral response of each animal in intraperitoneally, subcutaneously and intravenously infected groups of mice during the first month of.experimental infection, are shown in graphs 4, 5 and 6, respectively.



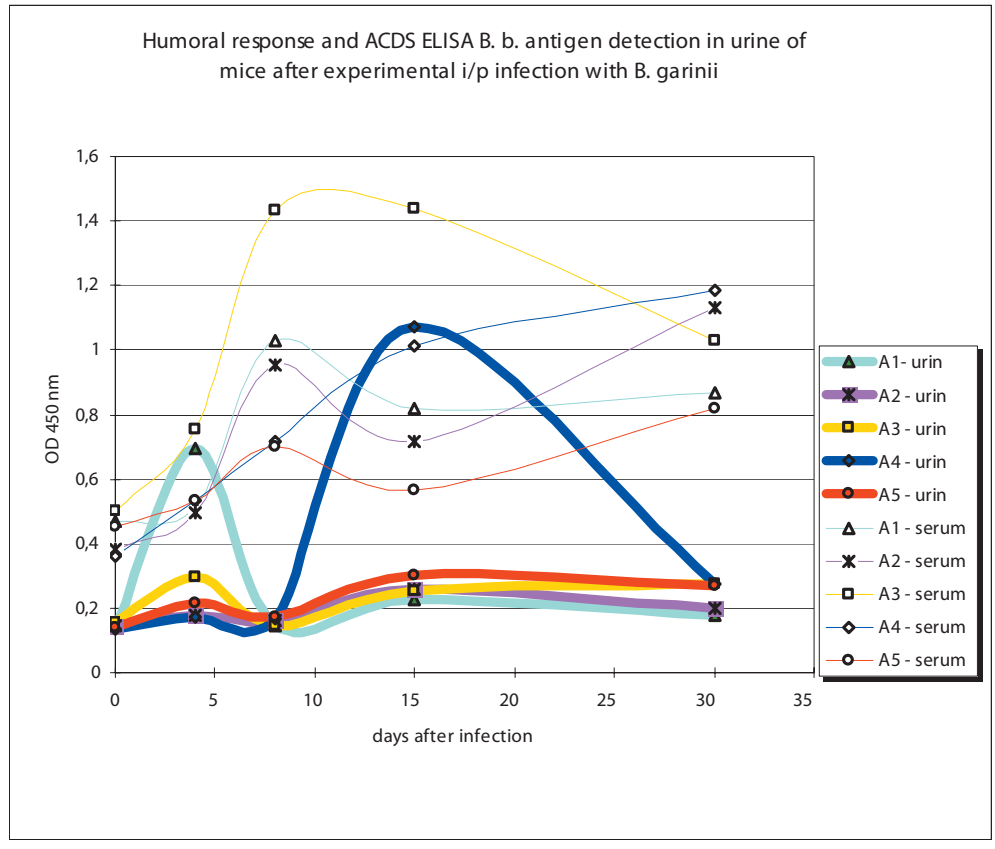
Graph 1: ACDS-ELISA *B. burgdorferi* flagelin detection in BSK II medium, PBS-BSA and urine spiked with sonicated *B. burgdorferi*



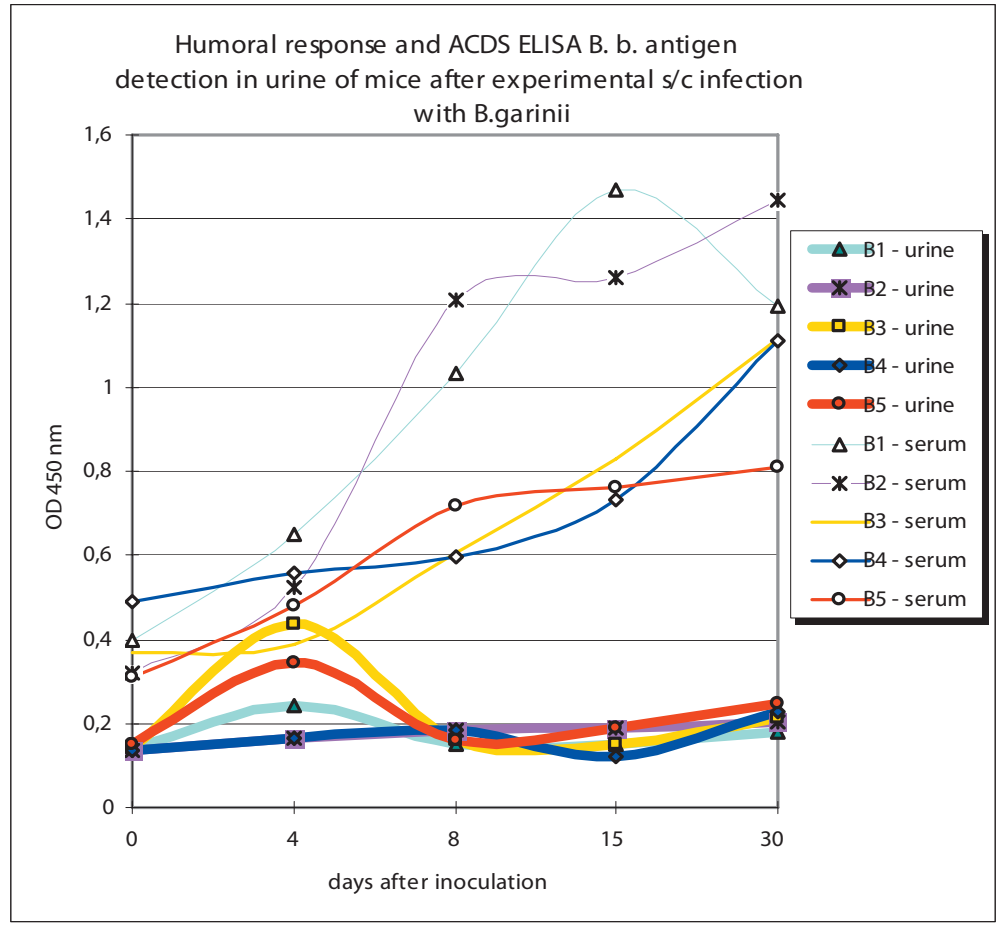
Graph 2: ACDS-ELISA *B. burgdorferi* flagelin detection in BSK II medium, PBS-BSA and urine spiked with whole *B. burgdorferi* cells



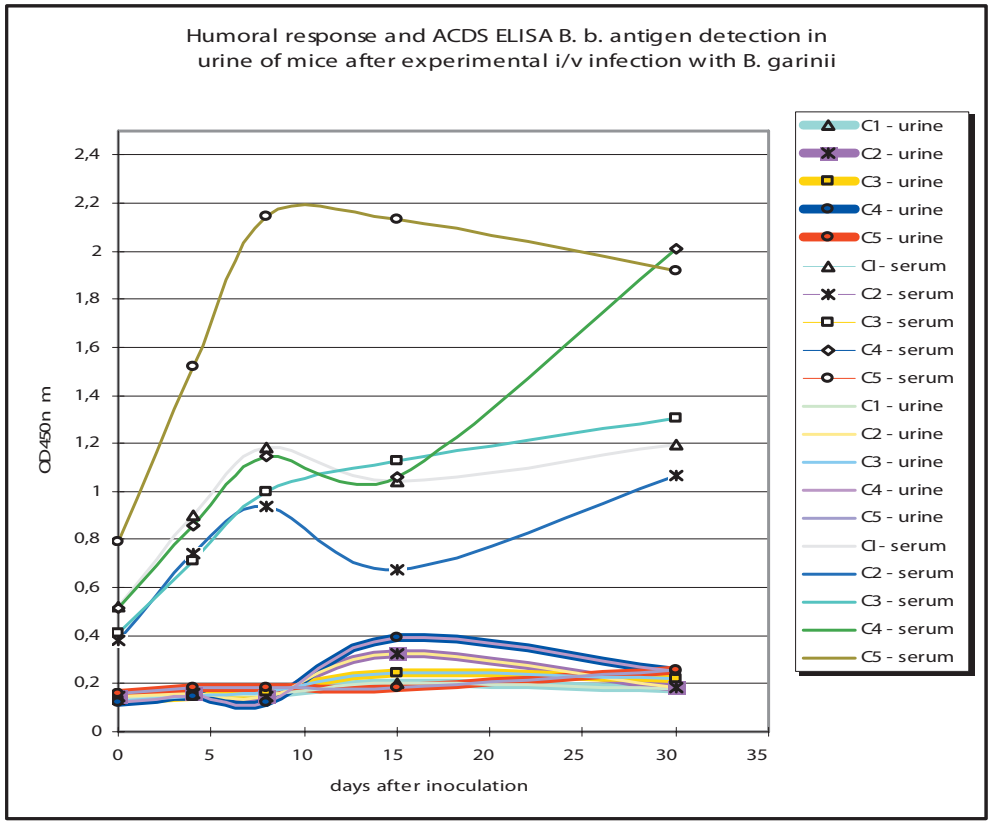
Graph 3: Reactivity of sonicated heterologous bacteria in ACDS-ELISA for *Borrelia burgdorferi* detection in urine samples



Graph 4: Humoral response (indirect ELISA) and borrelial antigen detection in urine samples (ACDS ELISA) of mice after experimental intraperitoneal infection with *B. garinii*



Graph 5: Humoral response (indirect ELISA) and borrelial antigen detection in urine samples (ACDS ELISA) of mice after experimental subcutaneous infection with *B. garinii*



Graph 6: Humoral response (indirect ELISA) and borrelial antigen detection in urine samples (ACDS ELISA) of mice after experimental intravenous infection with *B. garinii*

Discussion

Our developed antigen capture ELISA, grounded on the flagellin-specific monoclonal antibody might be, at least in our view, a tool for improving the complex Lyme disease diagnostics. Being capable of detecting less than 0,5 ng of sonicated borrelial antigens, the method offers substantially better sensibility than equivalent inhibition ELISA (18). The detection limit was higher when whole spirochetes were used as an antigen in sensitivity testing; this fact can be explained by periplasmic and not surface localization of *B. burgdorferi* flagella in intact spirochetes. The catching mAb can consequently not react with the flagellin molecules at the same extent as when sonicated borreliae were used. Although not tested with comparable *B. burgdorferi* s. s. and *B. afzelli* antigens, the assay should be, due to flagellin protein invariability.among B. burgdorferi sensu lato genomospecies, able to detect all the causative agents of Lyme borreliosis, too. This is not the case with the OspA antigen capture ELISAs, which can recognize only a limited number of *B. burgdorferi* Osp A serotypes (19), even if the detection limit of the method is superior in comparison to our method. In addition to this, OspA detection in urine and other biological fluids might be questionable since this protein is generally down-regulated in infected hosts (20). Cross reactions to the other bacterias' flagellin were not observed (Graph No. 3), but further investigations in this field are necessary. Reports on cross-reactivity of antibodies raised against other bacteria to this protein exist (21); other authors however claim that these antibodies do not react with 41 kDa flagellin but with another borrelial protein of 40 kDa (22). The type of the sample (urine or PBS) did not interfere essentially with the results. Urine samples spiked with borrelial antigens reacted positively even after 14 days of storage at 4°C indicating that urine had no serious deterioration effect on *B.burgdorferi*.flagellin.

Results obtained by ACDS ELISA and nested PCR analysis of urines from mice, expeimentally infected with *B. garinii* by different routes of inoculation confirm the presence of.borrelial antigens (including flagellin) in urine from infected mice (antigenuria), while whole spirochetes or their DNA could not be detected or were not released into the urine (absence of spirocheturia). Our data correlate well with available articles on borrelial antigenuria in mice (15,16, 18), inasmuch as our negative PCR results combine well with reports on failed attempts

of *B. burgdorferi* cultivation from mouse urine during experimental infection, despite the fact that the spirochetes were easily recovered from the walls of obviously infected urinary bladders (23, 24, 25)..As far as we know, with an exception of one work where *B. burgdorferi* has been succesfully isolated from mouse urine, but only from mice coinfected with *Babesia microti* (26), this spirochete has not been cultivated from, nor has its DNA been detected in mouse urine yet.

B. burgdorferi flagellin has been found in at least one of four urine samples taken from each of experimentally infected mice. The dynamics and the extent of flagellin shedding in urine appeared to be dependent on inoculation mode, which apprently influenced the humoral response development. Flagellin concentration in urine seemed to be inversely proportional to the.amount of.*B. burgdorferi* specific antibodies present in serum: flagellin could be detected in higher levels when the humoral response was still weak (in the first 4 days of infection) and a slight rise-ups in flagellin concentration were observed when specific antibody levels fell, usually around day 15. This antibody level - flagellin urine shedding relationship was most transparent in animals inoculated intravenously, which.responded immediately and with higher levels of specific immunoglobulins than those infected intraperitoneously or subcutaneously, and shed evidently less flagellin in urine than mice belonging to the other two groups (graphs 4-6). One month after initial inoculation antibody levels were elevated in all of the infected mice and flagellin could not be detected in urine any of the animals in the experiment. Urine and sera derived from the negative control group of mice reacted negatively throughout the study in the ACDS and antibody ELISA, respectively. These results suggest that the infection with *B. garinii* is being controlled by the specific humoral response level. Tiny rise-ups in flagellin in urine present in parallel with temporary regress in antibody titers might be a sign of multiplication of *borreliae*, possibly escape variants with new antigens expressed, and are then followed by a new rise in antibody titer..

The assay might become a good supplementary method in the Lyme disease diagnostics, especially in seronegative subjects suspected to be infected with *B. burgdorferi.* Further cross reactivity testing with broader range of bacteria, particularly other spirochetes, is necessary in order to calculate the exact specificity of the test, and the test needs to be evaluated also by probing urine samples from humans and other animal species. Although not so perfectly sensitive and specific as diverse PCR technique, the method demonstrates by these preliminary results an acceptable sensitivity for the *B. burgdorferi* antigen detection in the urine specimens with the possibility of improving it by concentrating the urine samples. In contrast to PCR, it does not suffer from unknown inhibition substances present in the samples and can detect borrelial antigens even if (or when) the spirochetes are not released into urine. Besides this, it is cheap, simple and fast and thus easily adaptable to every laboratory.

References

1. Steere AC. Lyme disease. N Engl J Med 1989; 321: 586-96.

2. Steere AC, Grodzicki AN, Kornblatt JE et al. The spirochetal etiology of Lyme disease. N Engl J Med 1983; 308: 733-40.

3. Barbour AG, Heiland RA, Howe TR. Heterogeneity of major proteins in Lyme disease borelliae: a molecular analysis of North American and European isolates. J Infect Dis 1985; 152: 478-84.

4. Barbour AG. Antigenic variation of a relapsing fever Borrelia species. Ann Rev Microbiol 1990; 44:155-71.

5. Bundoc VG, Barbour AG. Clonal polymorphisms of outer membrane protein Osp B of B. burgdorferi. Infect Immun 1989; 57: 2733-41.

6. Lam TT, Nguyen TPK, Montgomery RR et al. Outer surface proteins E and F of Borrelia burgdorferi, the agent of Lyme disease. Infect Immun 1994; 62(1): 290-8.

7. Padula SJ, Dias F, Sampieri A et al. Use of recombinant OspC from B. burgdorferi for serodiagnosis of early Lyme disease. J Clin Microbiol 1994; 32: 1733-8.

8. Shoberg RJ, Jonsson M, Šadziene A et al. Identification of a highly cross-reactive outer surface protein B epitope among diverse geographic isolates of Borrelia spp causing Lyme disease. J Clin Microbiol 1994; 32: 489-500.

9. Fingerle V, Hauser U, Liegl G et al. Expression of outer surface proteins A and C of B. burgdorferi in Ixodes ricinus. J Clin Microbiol 1995; 33: 1867-9.

10. Probert WS, Allsup KM, Le Febvre RB. Identification and characterization of a surface-exposed 66-kilodalton protein from B. burgdorferi. Infect Immun 1995; 63: 1933-9.

11. Goodman JL, Jurkovich JM, Kramber JM, Johnson RC. Molecular detection of persistent Bor-

relia burgdorferi in the urine of patients with active Lyme disease. Infect Immun 1991; 59: 269-78.

12. Pachner AR, Ricalton N, Delaney E. Comparison of polymerase chain reaction with culture and serology for diagnosis of murine experimental Lyme borreliosis. J Clin Microbiol 1993; 31: 208-14.

13. Schmidt B, Muellegger RR, Stockenhuber C et al. Detection of Borrelia burgdorferi - specific DNA in urine specimens from patients with erythema migrans before and after antibiotic therapy. J Clin Microbiol 1996; 34: 1359-63.

14. Lebech AM, Hansen K. Detection of Borrelia burgdorferi DNA in urine and cerebrospinal fluid. samples from patients with early and late lyme neuroborreliosis by polymerase chain reaction. J Clin Microbiol 1992; 30: 1646-53.

15. Dorward DW, Schwann TG, Garon CF. Immune capture and detection of Borrelia burgdorferi antigens in urine, blood or tissues from infected ticks, mice, dogs and humans. J Clin Microbiol 1991; 29: 1162-70.

16. Hyde FW, Johnson RC, White TJ, Shelburne CE. Detection of antigens in urine of mice and humans infected with Borrelia burgdorferi, etiologic agent of Lyme disease. J Clin Microbiol 1989; 27: 58-61.

17. Gruntar I. Preparation and characterization of monoclonal antibodies against Borrelia burgdorferi. Zb Vet Fak 1999; 36: 15-30.

18. Magnarelli LA, Anderson JF, Stafford III KC. Detection of Borrelia burgdorferi in urine of Peromyscus leucopus by inhibition enzyme-linked immunosorbent assay. J Clin Microbiol 1994; 32: 777-82.

19. Burkot TR, Wirtz RA, Luft B, Piesman J. An OspA antigen-capture enzyme-linked immunosorbent assay for detecting North American isolates of Borrelia burgdorferi in larval and nymphal Ixodes dammini. J Clin Microbiol 1993; 31(2): 272-8.

20. Suk K, Das S, Sun W et al. Borrelia burgdorferi genes expressed selectively in infected hosts. Proc Natl Acad Sci USA 1995, 92: 4269-73.

21. Shin SJ, Chang YF, Jacobson RH et al. Cross-reactivity between B. burgdorferi and other spirochetes affects the specificity of serotests for detection of antibodies to the Lyme disease agent in dogs. Vet Microbiol 1993; 36:161-73.

22. Bruckbauer HR, Preac-Mursic V, Wilske B. Cross-reactive proteins of Borrelia burgdorferi. Eur J Microbiol Infect Dis 1992; 11: 224-32.

23. Schwann TG, Burgdorfer W, Schrumpf ME, Karstens RH. The urinary bladder, a consistent source of Borrelia burgdorferi in experimentally infected white-footed mice (Peromysces leucopus). J Clin Microbiol 1988; 26: 893-5.

24. Czub S, Duray PH, Thomas RE, Schwann TG. Cystitis induced by infection with the Lyme disease spirochete, Borrelia burgdorferi in mice. Am J Pathol 1992; 141: 1173-9.

25. Petney TN, Hassler D, Bruckner M, Maiwald M. Comparison of urinary bladder and ear biopsy samples for determining prevalence of Borrelia burgdorferi in rodents in central Europe. J Clin Microbiol 1996; 34: 1310-2.

26. Bosler EM, Schulze TL. The prevalence and significance of Borrelia burgdorferi in the urine of feral reservoir hosts. Zentralb Bakteriol Mikrobiol Hyg A 1986; 263: 40-4.

RAZVOJ IN PREIZKUŠANJE METODE ELISA Z MONOKLONSKIMI PROTITELESI ZA UGOTAVLJANJE ANTIGENOV SPIROHETE BORRELIA GARINII V URINU

I. Gruntar, T. Malovrh

Povzetek: V prispevku je opisan razvoj antigenske lovilne metode ELISA, ki temelji na načelu dvojnega sendviča z monoklonskimi protitelesi, specifičnimi za borelijski flagelin. Za primerjavo metod smo uporabljali vgnezdeni PCR specifičen za borelijsko DNK v vzorcih urina. Rezultati pridobljeni z ELISA in z vgnezdenim PCR v vzorcih urina eksperimentalno okuženih miši z različnimi načini inokulacije *B. garinii*, potrjujejo prisotnost borelijskih antigenov (antigenurija), medtem ko celotnih spirohet ali njihove DNK ni bilo možno dokazati ali pa celih spirohet ni bilo prisotnih v urinu (ni spiroheturije). Takšno metodo ELISA bi torej lahko uporabljali kot dopolnilno metodo v diagnostiki lajmske borelioze, še posebej pri seronegativnih primerih, pri katerih se domneva, dag re za okužbo z *B. burgdorferi.*

Ključne besede: borelijski antigeni; urin; imunoencimske analize

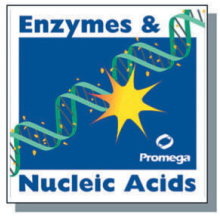
KEMOMED

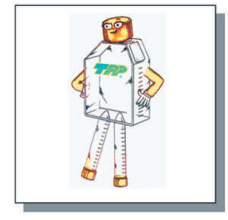
PE: Stritarjeva 5, 4000 Kranj, Slovenija
tel.: (0)4/ 2015 050, fax: (0)4/ 2015 055
e-mail: info@kemomed.si
www.kemomed.si

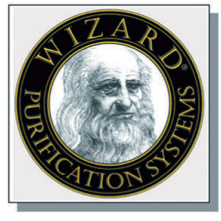
Promega

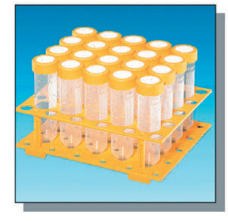
SYNGENE

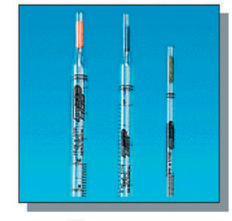
TRP®











IZDELKI ZA MOLEKULARNO BIOLOGIJO

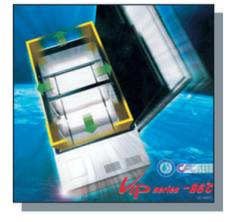
DOKUMENTACIJA IN ANALIZA GELOV

PLASTIKA ZA CELIČNE KULTURE

ELGA LABWATER

SANYO

Invitrogen life technologies



ČISTA VODA ZA LABORATORIJ

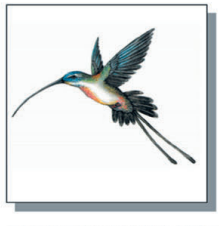
SKRINJE IN HLADILNIKI

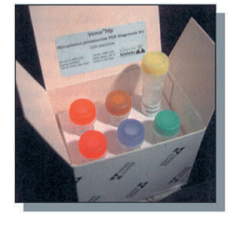
CELIČNE KULTURE, GELI IN MOLEKULARNA BIOLOGIJA

BIOHIT

minerva biolabs®

phenomenex®
...breaking with tradition™





ELEKTRONSKE IN MEHANSKE AVTOMATSKE PIPETE

DIAGNOSTIKA MIKOPLAZEM IN LEGIONEL

HPLC in GC POTROŠNI MATERIAL

INSTRUCTIONS FOR AUTHORS

Slovenian Veterinary Research contains original articles which have not been published or considered for publication elsewhere. All statements in the articles are the responsibility of the authors. The editorial policy is to publish original research papers, review articles, case reports and abstracts of theses, as well as other items such as critical reviews of articles published in Slov Vet Res, shorter scientific contributions, letters to the editor, etc. Authors should send their contributions to the editorial board's address. All articles are subjected to both editorial review and review by an independent referees selected by the editorial board. The editorial board reserves the right to translate titles, summaries and keywords that have not been translated into Slovene by the authors.

Contributions should be written in English and should not exceed 12 pages (27 lines per page, approx. 75 characters per line). They should be submitted electronically (preferably to E-mail address, slovetres@vf.uni-lj.si), written in any word processor for Windows. Authors are requested to provide names of three potential reviewers. The text should be double spaced and the lines should be numbered on the left-hand side. The margin on the left-hand side of the page should be 4 cm.

The front page of a manuscript should start with the title, followed by the name and surname of the author(s). If there is more than one author, their names should be separated by commas. The next line ('Addresses of authors:') should contain the authors' full names and addresses (institution, street and number, postcode and place) after the colon. All the given data should be separated by commas. The name, address and E-mail and/or phone number of the corresponding author should be written in the next line.

The Summary of 200-300 words should follow on the next page.

Under 'Keywords:' (after the colon), keywords should be given. Individual words or word combinations should be separated by semicolons. Scientific papers and papers which present the author's research and findings should also include the following obligatory headings assigned by the author to appropriate parts of the text: Introduction, Materials and methods, Results, Discussion, and References. Review articles should consist of an introduction, sections logically titled according to the content, and references. Information on fund-providers and other matters important for the paper (e.g. technical assistance) should be supplied under 'Acknowledgements', which should be placed before the references. Figure legends should follow the references.

Tables, graphs and diagrams should be logically incorporated in the text file. Original photographs or drawings should be sent as separate files in bmp, jpg or tif format. They should be referred to by type and using Arabic numerals (e.g. Table 1:, Figure 1:, etc.). The colon should be followed by the text or title. All references cited in the text should appear in the References. They should be numbered in the text in the order in which they appear, marked with Arabic numerals placed in parenthesis. The first reference in the text should determine the number and order of the respective source in the References. If the author refers again to a source which has already been used in the text, he should cite the number the source had when it was referred to for the first time. Only works which have been published or are available to the public in any other way may be referred to. Unpublished data, unpublished lectures, personal communications and similar should be mentioned in the references or footnotes at the end of the page on which they appear. Sources in the References should be listed in the order in which they appear in the text. If the source referred to was written by six authors or less, all of them should be cited; in the case of seven or more authors, only the first three should be cited, followed by 'et al.'.

Any errata should be submitted to the editor-in-chief in good time after publication so that they may be published in the next issue.

Examples of references

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

Chapterorar ticle in a book: Baldessarini RJ. Dopamine receptors and clinical medicine. In: Neve KA, Neve RL, eds. The dopamine receptors. Totowa: Human Press, 1996: 475-98.

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.

NAVODILA AVTORJEM

Slovenski veterinarski zbornik (Slovenian Veterinary Research) objavlja izvirne prispevke, ki še niso bili objavljeni oz. poslani v objavo drugam. Za vse navedbe v prispevkih so odgovorni avtorji. Uredniška politika obsega publiciranje znanstvenih člankov, preglednih znanstvenih člankov, strokovnih člankov, povzetkov disertacij in drugih prispevkov, kot so kritične presoje o vsebini razprav, objavljenih v zborniku, kratke znanstvene prispevke, pisma uredniku in drugo. Avtorji pošljejo prispevke na naslov uredništva. Glavni urednik pregleda vse prispevke. Za vse članke je obvezna strokovna recenzija, za katero poskrbi uredništvo.

Prispevki naj bodo napisani v angleškem jeziku, z naslovom, povzetkom in ključnimi besedami tudi v slovenščini. Obsegajo naj največ 12 strani, kar pomeni 27 vrstic na stran s približno 75 znaki v vrstici. Prispevki naj bodo poslani v elektronski obliki v katerem koli urejevalniku besedil za okensko okolje. Zaželjena je uporaba elektronske pošte (slovetres@vf.uni-lj.si) in avtorji naj predlagajo tri možne recenzente. Besedilo naj ima dvojni razmik med vrsticami, pri čemer naj bodo vrstice na levi strani oštevilčene. Besedilo naj bo na levi strani od roba oddaljeno 4 cm.

Naslovna stran prispevkov se začne z naslovom, sledi ime in priimek avtorja. Kadar je avtorjev več, jih ločimo z vejicami. V naslednjih vrsticah je v rubriki Addresses of authors: za dvopičjem treba navesti polno ime in priimek ter naslov(e) avtorja(ev), tj. ustanovo, ulico s hišno številko, pošto in kraj. Vse navedene podatke ločujejo vejice. Sledi vrstica, kjer je treba navesti ime ter elektronski (E-mail:) in poštni naslov ter telefonsko številko (Phone:) odgovornega avtorja.

Sledi besedilo povzetka Summary v obsegu 200 do 300 besed. V naslednji rubriki Key words: se za dvopičjem navedejo ključne besede. Posamezne besede ali sklopi besed morajo biti ločeni s podpičjem.

Znanstveni članki in tisti, ki so prikaz lastnih raziskav in dognanj, morajo vsebovati še naslednje obvezne rubrike, s katerimi avtor sam naslovi ustrezne dele besedila v prispevku: Introduction, Material and methods, Results, Discussion in References. Pregledni članki naj vsebujejo uvod, poglavja, ki so glede na vsebino smiselno naslovljena, in literaturo. Podatke o financerjih ali drugih zadevah, pomembnih za prispevek, npr. o tehnični pomoči, avtorji navedejo v rubriki Acknowledgements, ki se uvrsti pred rubriko References. Za rubriko References sledijo spremna besedila k slikam.

Priloge, kot so tabele, grafikoni in diagrami naj bodo smiselno vključene v besedilo. Slikovni material naj bo poslan posebej v obliki bmp, jpg, ali tif.

Priloge in slike morajo biti poimenovane z besedami, ki jih opredeljujejo, in arabskimi številkami (npr. Table 1:, Figure 1: itn.). Za dvopičjem sledi besedilo oziroma naslov. Vsi navedki (reference), citirani v besedilu, se morajo nanašati na seznam literature. V besedilu jih je treba oštevilčiti po vrstnem redu, po katerem se pojavljajo, z arabskimi številkami v oklepaju. Prvi navedek v besedilu opredeli številko oziroma vrstni red ustreznega vira v seznamu literature. Če se avtor v besedilu ponovno sklicuje na že uporabljeni vir, navede tisto številko, ki jo je vir dobil pri prvem navedku. Citirana so lahko le dela, ki so tiskana ali kako drugače razmnožena in dostopna javnosti. Neobjavljeni podatki, neobjavljena predavanja, osebna sporočila in podobno naj bodo omenjeni v navedkih ali opombah na koncu tiste strani, kjer so navedeni. V seznamu literature so viri urejeni po vrstnem redu. Če je citirani vir napisalo šest ali manj avtorjev, je treba navesti vse; pri sedmih ali več avtorjih se navedejo prvi trije in doda et al.

Da bi se morebitni popravki lahko objavili v naslednji številki, jih morajo avtorji pravočasno sporočiti glavnemu uredniku.

Načini citiranja

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

Poglavje ali prispevek v knjigi: Baldessarini RJ. Dopamine receptors and clinical medicine. In: Neve KA, Neve RL, eds. The dopamine receptors. Totowa: Human Press, 1996: 475-98.

Članek iz revije ali časopisa: Fuji J, Otsu K, Zorzato F, et al. Identification of mutation in porcine ryanodine receptor asociated with malignant hyperthermia. Science 1991; 253: 448-51.

Članek iz zbornika referatov: 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.

Slov Vet Res 2008; 45 (3)

Review Articles

Original Research Papers

Slov Vet Res 2008; 45 (3): 79-116