Chlorambucil and fludarabine as a new pre-transplant conditioning for patients with chronic lymphocytic leukemia: results of *in vivo* experiments

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ABSTRACT: Chronic lymphatic leukemia (CLL), incurable by standard treatments, may be potentially cured by allogeneic hematopoietic stem cell transplantation. Since CLL affects predominantly older people, there is a need for some low-toxicity conditioning with, on the other hand, strong antileukemic activity. Since there are very encouraging results with busulfan + fludarabine conditionings in myeloid malignancies and since the clinical study with the combination treatment with chlorambucil and fludarabine was stopped prematurely for myelotoxicity, we hypothesized that this chlorambucil + fludarabine combination would have the potential as a good conditioning for high-risk lymphoid malignancies. The aim of this study was to test the chlorambucil + fludarabine combination *in vivo* (in rats) for toxicity. Male Wistar rats were used in all experiments. First, the maximum tolerated dose (MTD) of each drug was tested. For fludarabine, doses of 0.75–60 mg/kg/day, and for chlorambucil, doses of 0.15–50 mg/kg/day were used, all administered for five days. Then, the combination treatment was tested: (1) fludarabine and chlorambucil simultaneously (F+CH), (2) fludarabine followed by chlorambucil (F-CH), (3) chlorambucil followed by fludarabine (CH-F); all drugs were administered for five days. For fludarabine alone, the MTD was not reached. Clinically, the rats tolerated well even the highest doses. Moreover, no myelotoxicity was seen. However, pneumotoxicity, hepatotoxicity, nephrotoxicity, and gastrointestinal toxicity were found by a histological examination. For chlorambucil alone, the MTD is about 40–50 mg/kg/day. Pneumotoxicity, nephrotoxicity, gastrointestinal toxicity, and myelotoxicity were observed. The combination treatment tested a fixed dose of fludarabine (3 mg/kg per day) and three doses of chlorambucil (1, 2, and 4 mg/kg/day). Clinically, the best tolerated combination was fludarabine followed by chlorambucil (F-CH). Haematological toxicity was observed, usually affecting predominantly lymphocytes, and interestingly, it was most pronounced in the clinically best tolerated regimen. Rats can tolerate extremely high doses of fludarabine and chlorambucil. Based on these experiments, for further development, hopefully into the clinical usage, we could recommend the administration of fludarabine, followed by chlorambucil. This combination will further be tested together with monoclonal antibodies and total lymphoid irradiation as a conditioning regimen for allogeneic bone marrow transplantation.

Keywords: rat; haematological toxicity; non-haematological toxicity; antileukemic drugs

B-cell chronic lymphocytic leukemia is the most commonly diagnosed haematological malignity in the Western world. Disorders of apoptosis and differentiation of B lymphocytes are involved in pathogenesis as a consequence of this progressive accumulation of functionally incompetent leukemic

cells in blood, bone marrow and lymphoid organs. The majority of therapeutic regimens are only palliative, directed at reducing the symptoms (Abbott, 2006; Palma et al., 2006). Allogeneic hematopoietic cell transplantation is a potentially curative method. CLL is a predominant disease of older people, therefore the regimen with low toxicity and strong antileukemic potential is required. Good results in the therapy of myeloid and lymphoid malignancies have been achieved with busulfan-fludarabine combination (Slavin et al., 1998; Krejci et al., 2006). Fludarabine-chlorambucil combination seems to be an applicable regimen for achieving the disease remission of lymphoid malignancies. Clinical studies of this combination were stopped in the 1990s because of severe myelotoxicity and subsequent infections (Rai et al., 2000; Morrison et al., 2001). In regard of the improved supportive and anti-infective therapy of immunocompromised patients we expect that this combination can be used as a nonmyeloablative pretransplant regimen in the therapy of lymphoid leukemia.

Chlorambucil – 4-{4-[bis(2-chlorethyl)amino] phenyl}butyric acid (Czech Pharmacopeia, 1997) and its derivates are alkylating agents – substances able to form covalent bonds with proteins and DNA. Structural and functional damage to DNA leads to cytotoxic, mutagenic and carcinogenic effects (Calabresi and Schein, 1993). Cells affected by tumorous transformation are more susceptible to the effects of alkylating substances than the healthy ones. This fact is being employed in the therapy of oncologic diseases. Chlorambucil was developed in the 1950s as a nitrogenous derivative of yperite. At present, it is used for the therapy of chronic lymphatic leukemia, in particular, low-grade non-Hodgkin's lymphoma and Hodgkin's disease (Pangalis et al., 2002; Nicolle et al., 2004; Palma et al. 2006). Toxicity is relatively low, the most severe side effects are haematological toxicity and neurotoxicity, described rarely in older patients and children with nephrotic syndrome or at an accidental intoxication or overdosing (Salloum et al., 1997; Nicolle et al., 2004).

Fludarabine (9-β-arabinofuranosyl-2-fluoro-adenine-5'-mono-phosphate) belongs to the group of purine analogues, drugs able to inhibit DNA and RNA synthesis (Gandhi and Plunkett, 2002). Its tolerability is relatively good. The main side effects are haematological toxicity, immunosuppression increasing the risk of severe infections and neurotoxicity if high doses are administered (Chun et

al., 1991; Solal-Celigny et al., 1996; Morrison et al., 2001). Fludarabine is used in the first and second line of therapy of lymphoid malignancies and may be combined with other drugs.

The aim of this study was to determine the maximum tolerated dose (MTD) of chlorambucil and fludarabine and the haematological and non-haematological toxicity of each of these drugs and of their combination in an animal model. Results of this study are intended for use in the testing of this combination as a nonmyeloablative preparation regimen for bone marrow transplantation.

MATERIAL AND METHODS

Animals

All experiments were performed using SPF outbred Wistar rats (males) ranging in body weight from 250 to 335 g, purchased from Anlab (Prague, Czech Republic). Acclimatization lasted for two weeks. Animals had free access to drinking water and complete food (Biostan Mypo, Biosta, Blucina, Czech Republic) supplied *ad libitum* during the whole experiment. Experiments were carried out under an institutionally approved protocol in accordance with ethical principles and Act No. 246/1992 on the Protection of Animals against Cruelty and follow-up rules.

Drugs

Chlorambucil (LKT Laboratories, Minnesota, USA) was dissolved in 96% ethanol, divided into aliquots in micro test tubes and kept at –20°C until use. Shortly before use, the solution was diluted with water for injection down to the ethanol concentration ranging between 50 and 60% and administered by a gastric tube to rats anaesthetized for a short period by ether.

Fludarabine: 50 milligrams of fludarabine were dissolved in 2 ml of water for injection in aseptic conditions. This solution was diluted with saline to the required concentration and administered intraperitoneally. First, the maximum tolerated dose (MTD) of each of the drugs was tested. Animals were randomly divided into experimental groups. Each group included four animals and was used for testing one of the drug concentrations; control group included four animals as well. Chlorambucil

was administered in the following doses – 0.15; 0.25; 0.5; 0.75; 2; 4; 8; 12; 20; 30; 40 and 50 mg/kg per day, respectively – once daily for five consecutive days. Fludarabine was administered in the following doses 0.75; 1.5; 2.25; 3; 5; 7; 9; 11; 20; 40 and 60 mg/kg/day, respectively – once daily for five consecutive days. Then, the combination treatment was tested. Doses were established according to the results of testing of MTD. We used a fixed dose of fludarabine (3 mg/kg/day) and three doses of chlorambucil (1, 2, and 4 mg/kg/day). Three application regimens were tested: (1) fludarabine and chlorambucil simultaneously for five days (F+CH), (2) chlorambucil (for five days) followed by fludarabine (for five days) (CH-F), and (3) fludarabine (for five days) followed by chlorambucil (for five days) (F-CH). 70% ethanol (0.7 ml per individual/day, perorally) and saline (0.9 ml per individual/day, intraperitoneally) were administered to control animals (C) for five days.

Haematological and biochemical parameters

Animals were examined clinically on daily basis during the experiment. Haematological examination was performed before the administration of drugs started (initial values) and three times after the therapy stopped (days 2–3, 9–11 and 19–20). Haematological parameters such as red blood cell count, white blood cell count, platelet count, differential leukocyte count, packed cell volume and haemoglobin concentration were determined using the Celltac alpha MEK 6318 analyzer, Nihon Kohden, Japan. To evaluate differential leukocyte count blood smears were stained according to Pappenheim. Biochemical parameters (urea, creatinine, ALP, ALT, AST, Na, K, and Cl) were determined before the therapy started and twice after the therapy stopped using a Konelab 20i automatic analyzer (Thermo Scientific, Finland).

Histopathological examination

Samples for histopathological examination (heart, lungs, kidneys, adrenals, liver, spleen, oesophagus, stomach, duodenum, ileum, caecum, colon descendens, urinary bladder and brain) were collected from individuals that died during the experiment or that were euthanized by ether. Histological specimens were stained by haematoxylin-eosin and Azan and examined by light microscopy.

Table 1. Total leukocyte count $\times 10^9$ (mean ± SD)

Regimen	Initial values	Days after therapy stop		
		2–3	9–11	19–20
CH+F	8.85 ± 1.69	2.00 ± 0.85**	5.95 ± 1.91	7.35 ± 0.92
F-CH	8.95 ± 1.41	2.63 ± 1.71**	5.10 ± 0.22	7.83 ± 1.12
CH-F	9.75 ± 0.81	5.05 ± 1.06*	7.50 ± 0.57	8.30 ± 0.00
CH 40	10.30 ± 0.58	0.33 ± 0.06**	8.10 ± 0.00	9.20 ± 0.00
CH 50	9.82 ± 2.61	1.30 ± 0.00[b]	#	#
F 60	9.00 ± 1.51	10.27 ± 2.25	9.67 ± 0.61	8.75 ± 0.26
C	8.75 ± 2.72	17.65 ± 7.13	13.13 ± 2.35	13.00 ± 2.14

CH+F = chlorambucil (4 mg/kg/day) and fludarabine (3 mg/kg/day) simultaneously for 5 days

F-CH = fludarabine (3 mg/kg/day) for 5 days followed by chlorambucil (4 mg/kg/day) for 5 days

CH-F = chlorambucil (4 mg/kg/day) for 5 days followed by fludarabine (3 mg/kg/day) for 5 days

CH 40 = chlorambucil (40mg/kg/day) for 5 days

CH 50 – chlorambucil (50 mg/kg/day) for 5 days

F 60 = fludarabine (60 mg/kg/day) for 5 days

C = control

= the value was not assessed

[b]without statistical evaluation due to a low number of animals

*statistically significant decrease ($P < 0.05$)

**statistically significant decrease ($P < 0.01$)

Statistical analysis

We estimated arithmetic mean and standard deviation. Student's *t*-test was used for the evaluation of significance of differences between pre-treatment and post-treatment values (significant $P < 0.05$, highly significant $P < 0.01$). The evaluation was performed using Microsoft Excel software.

RESULTS

Fludarabine

For fludarabine alone, the MTD was not reached. Clinically, the rats tolerated well even the highest doses (60 mg/kg/day). Moreover, no myelotoxicity was seen. However, the histological examination showed pneumotoxicity, hepatotoxicity, nephrotoxicity, and gastrointestinal toxicity.

Chlorambucil

For chlorambucil alone, the MTD is about 40 to 50 mg/kg/day. Seventy-five percents of individuals (three out of four) died in the group dosed with 50 mg per kg/day and 50% of individuals (two out of four) in the group dosed with 40 mg/kg/day. Pneumotoxicity, nephrotoxicity, gastrointestinal toxicity (histological changes without a marked impact on the clinical state or biochemical parameters) and significant leukopenia ($P < 0.01$) with dominant lymphocytopenia ($P < 0.001$) were observed (Table 1 and 2). RBC and platelets were not affected.

F-CH regimen

Clinically, the best tolerated combination was fludarabine followed by chlorambucil. No individuals died in this group. Haematological toxicity was mild, manifested by transient leukopenia ($P < 0.01$) with dominant lymphocytopenia ($P < 0.001$) and insignificant drop of neutrophils and monocytes depending on the dose (Table 1 and 2). RBC and platelets were not affected. Changes in biochemical parameters were not significant. The histological examination revealed nephrotoxicity with predominant affection of proximal renal tubules, pneumotoxicity and gastrointestinal toxicity with affection of the jejunum and ileum.

CH-F regimen

There died 50% of rats (two out of four) in the group of CH-F regimen with the highest dose (4 mg/kg/day of F and 3 mg/kg/day of CH for five days) and 50% of rats (two out of four) in the group dosed 4 mg/kg/day of F and 1 mg/kg/day of CH for five days. Haematological toxicity was characterized by significant leukopenia ($P < 0.05$) and lymphocytopenia ($P < 0.05$), unlike the F-CH regimen accompanied by neutrophilia ($P < 0.01$) (Tables 1 and 2). Urea and creatinine showed an insignificant increase depending on the dose and persistent for three weeks after the therapy finished. The activity of AST and ALT insignificantly increased (in all doses). Histopathology revealed pneumotoxicity, hepatotoxicity and nephrotoxicity.

CH+F regimen

The simultaneous administration of chlorambucil and fludarabine was tolerated worst. There died 50% of individuals (two out of four) in the group with the dose 3 mg/kg/day of F and 4 mg/kg/day of CH for five days and 50% (two out of four) of individuals in the group dosed with 3 mg/kg/day of F and 2 mg/kg/day of CH. Clinically apparent changes (diarrhoea, apathy and inappetence) and haematological toxicity occurred in all groups. Haematological toxicity was manifested by leukopenia ($P < 0.01$) with predominant lymphocytopenia ($P < 0.01$) (Table 1 and 2). Mild, non-significant
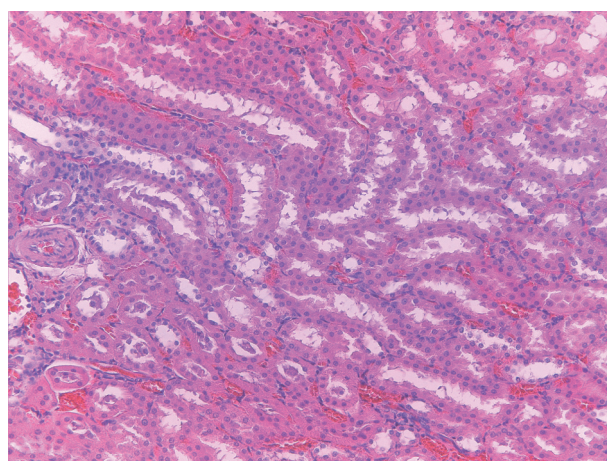

Figure 1. Kidney (haematoxylin-eosin staining). Damaged epithelium in distal tubules. There is detritus in the lumen of some tubules. CH-F regimen (4 mg/kg chlorambucil, 3 mg/kg fludarabine); magnification 200×

Table 2. Differential leukocyte count × 10^9 (mean ± SD)

Regimen	Initial values			Days after therapy stop								
				2–3			9–11			19–20		
	lymphocytes	neutrophils	monocytes	lymphocytes	neutrophils	monocytes	lymphocytes	neutrophils	monocytes	lymphocytes	neutrophils	monocytes
CH+F	8.13 ± 1.84	0.66 ± 0.24	0.02 ± 0.02	1.25 ± 0.57**	0.65 ± 0.18	0.08 ± 0.06	3.83 ± 1.61	1.93 ± 0.42	0.17 ± 0.15	5.33 ± 0.05	1.66 ± 0.83	0.22 ± 0.03
F-CH	7.91 ± 1.28	0.91 ± 0.31	0.07 ± 0.07	1.79 ± 1.12**	0.78 ± 0.60	0.02 ± 0.02	3.70 ± 0.23	1.21 ± 0.42	0.07 ± 0.05	5.93 ± 0.89	1.71 ± 0.30	0.07 ± 0.03
CH-F	8.88 ± 0.62	0.74 ± 0.25	0.07 ± 0.11	2.32 ± 1.03*	2.54 ± 0.15[a]	0.13 ± 0.01	5.33 ± 0.40	1.80 ± 0.14	0.12 ± 0.01	6.50 ± 0.00	1.78 ± 0.00	0.00 ± 0.00
CH 40	8.56 ± 0.67	0.74 ± 0.29	0.99 ± 0.54	0.07 ± 0.05**	0.23 ± 0.14	0.05 ± 0.04	5.27 ± 0.00	1.94 ± 0.00	0.73 ± 0.00	#	#	#
CH 50	8.15 ± 2.47	0.65 ± 0.38	0.86 ± 0.52	0.34 ± 0.00[b]	0.88 ± 0.00	0.08 ± 0.00	#	#	#	#	#	#
F 60	7.55 ± 1.35	1.08 ± 0.13	0.28 ± 0.17	7.15 ± 1.26	2.84 ± 0.31[a]	0.34 ± 0.11	6.67 ± 0.33	2.37 ± 0.55	0.24 ± 0.06	#	#	#
C	7.85 ± 2.25	0.78 ± 0.45	0.04 ± 0.03	11.04 ± 3.14	6.17 ± 7.63	0.34 ± 0.35	9.11 ± 1.81	3.59 ± 0.44	0.30 ± 0.18	10.05 ± 1.99	2.48 ± 0.20	0.22 ± 0.10

CH+F = chlorambucil (4 mg/kg/day) and fludarabine (3 mg/kg/day) simultaneously for 5 days

F-CH = fludarabine (3 mg/kg/day) for 5 days followed by chlorambucil (4 mg/kg/day) for 5 days

CH-F = chlorambucil (4 mg/kg/day) for 5 days followed by fludarabine (3 mg/kg/day) for 5 days

CH 40 = chlorambucil (40mg/kg/day) for 5 days

CH 50 = chlorambucil (50 mg/kg/day) for 5 days

F 60 = fludarabine (60 mg/kg/day) for 5 days

C = control

= the value was not assessed

[a]statistically significant increase ($P < 0.05$)

[b]without statistical evaluation due to a low number of animals

*statistically significant decrease ($P < 0.05$)

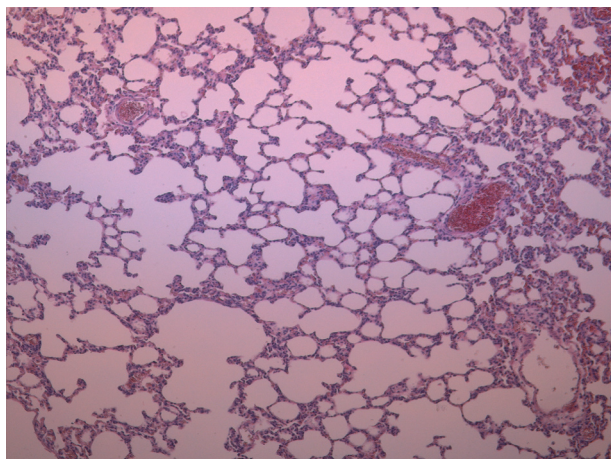**statistically significant decrease ($P < 0.01$)

Figure 2. Lung (haematoxylin-eosin staining) Damaged septa on the periphery, lymphoreticular tissue in surroundings of vessels. The epithelium of terminal bronchi is damaged, there are cells in the lumen. F-CH regimen (4 mg/kg chlorambucil, 3 mg/kg fludarabine); magnification 200×
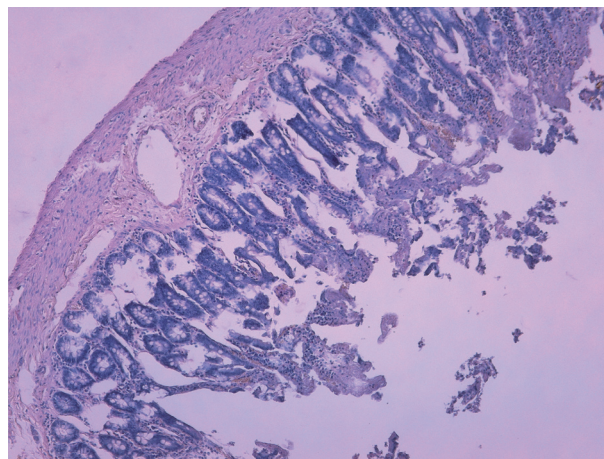

Figure 3. Destruction of villi – ileum (haematoxylin-eosin staining). CH-F regimen (4 mg/kg chlorambucil, 3 mg/kg fludarabine); magnification 200×

monocytosis was observed. Histological changes included damage to renal tubules, intestinal epithelium, alveolar and bronchial epithelium, vacuolization of liver parenchyma and degenerative changes in the cerebrum and cerebellum in two cases.

Controls

Non-significant leukocytosis and neutrophilia occurred in the control group (Tables 1 and 2). Changes in biochemical parameters were insignificant.

DISCUSSION

The most severe histological toxicity of fludarabine, chlorambucil and their combination we revealed was pneumotoxicity and nephrotoxicity. These types of toxicity were apparent in all histologically examined specimens.

The manifestations of nephrotoxicity were damage to the epithelium of proximal tubules, in the most severe cases of distal tubules, and haemostasis and bleeding in the medullar and cortical area (Figure 1). Nephrotoxicity is not a frequent side effect of either chlorambucil or fludarabine therapy in human medicine although a case of acute renal failure (acute tubular necrosis) due to chlorambucil overdosing was reported (Blank et al., 1983).

Toxic effects were found in the lung parenchyma – haemorrhage within bronchi, damage

to the structure of the bronchial and bronchiolar epithelium, damage to the alveolar epithelium and focal atelectasis of the pulmonary parenchyma (Figure 2). Pneumotoxicity as a side effect of chlorambucil therapy has scarcely been described in humans; most commonly it results in interstitial pneumonia, fibrosis, and damage to the alveolar epithelium, or pneumonia due to the obliteration of bronchi (Weiss and Muggia, 1980; Khong, 1998; Kalambokis et al., 2004). Interstitial pneumonia occurring during the fludarabine therapy, usually as a consequence of infections, can result in a respiratory failure (Chun et al., 1991; Solal-Celigny et al., 1996; Foran et al., 1999).

It can be stated in general that the histological changes were milder after the combination of drugs than after monotherapy with large doses. This damage, with only minor exceptions, was not accompanied by clinical manifestations.

Neurotoxicity of fludarabine has been described in literature, but it was not observed clinically in our experiment. However, the histological examination revealed changes in the cerebellum (degenerative changes and vacuolization of cells in *stratum granulosum*) and in the cerebrum (focal atrophy of cortex, vacuolar changes of cells in lamina pyramidalis) in groups with the highest doses of combined therapy. Neurotoxicity described in humans and also in laboratory animals manifests itself regularly as changes in an electroencephalograph only (Pradhan, and Marsan, 1963). Clinically apparent neurotoxicity of chlorambucil is usually a

consequence of an overdosing or accidental intoxication. Typical symptoms are apathy, or increased excitability on the contrary, dizziness and seizures (Nicolle et al., 2004). There were reported epileptiform seizures and clonic convulsions in several cases of intoxication or overdosing of chlorambucil in older people or in children with nephrotic syndrome (Byrne et al., 1981; Salloum et al., 1997). Neurotoxicity is a serious side effect of the fludarabine therapy. The most frequent signs are lethargy, depression, asthenia and peripheral neuropathy in patients treated with lower doses of fludarabine, severe neurological toxicity (coma, cortical blindness) were observed after the administration of high doses (Chun et al., 1991; Elias et al., 1993; Solal-Celigny et al., 1996).

Gastrointestinal toxicity, especially damage to epithelial cells of villi in the jejunum and ileum (Figure 3), was clinically manifested as a diarrhoea.

Clinically, haematological toxicity was the most apparent toxicity. Leukopenia with dominant lymphocytopenia appeared, however regeneration was fast (two weeks). Red blood cells and platelets were not significantly affected.

Haematological toxicity is one of the most frequent and serious side effects of therapy with chlorambucil, fludarabine or their combination. Myelosuppression in humans due to fludarabine therapy is transient, manifesting itself as neutropenia, thrombocytopenia or pancytopenia resulting in severe infections (Chun et al., 1991; Elias et al., 1993; Solal-Celigny et al, 1996; Foran et al., 1999; Rai et al., 2000). Pancytopenia or anaemia are a frequent consequence of chlorambucil monotherapy (Summerfield et al., 2002; Nicolle et al., 2004). Combined therapy in human medicine was accompanied by severe suppression of bone marrow, resulting in pancytopenia and consequential serious infections (Gram-positive bacteria, *Candida* spp., Herpesvirus) (Elias et al., 1993; Morrison et al., 2001).

Combined therapy with chlorambucil and fludarabine is usable in CLL treatment and in regard of myelosuppression it appears as a possible pretransplantation regimen in patients with this type of leukemia.

Based on these experiments, for further perspectives, hopefully into the clinical usage, we could recommend the administration of fludarabine, followed by chlorambucil. This combination will further be tested together with monoclonal antibodies and/or total lymphoid irradiation as a pretreatment regimen for the allogeneic transplantation of haemopoeitic cells.

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THE VETERINARY BIOTECHNOLOGY, EPIDEMIOLOGY AND FOOD SAFETY NETWORK (CENTAUR)

The CENTAUR network aims at upgrading the standards of economically significant priority animal diseases control in the region with particular emphasis on transboundary animal diseases, animal health and consumer protection.

The CENTAUR is willing to achieve it through dissemination of scientific information, training, links with the international centres of excellence and cooperation. The important task is also to present the problems, personalities, institutions, and scientific achievement of the region. Efficient utilization of Internet, e-mail and improvement in English language proficiency is followed, too.

Under the CENTAUR network the CENTAUR NEWSLETTER FLASH INFORMATION (CNFI), an international electronic bulletin (ISSN 1213-368X), is published, providing subscribers with instant information in the form of e-mail messages relating to fields of interest which subscribers define themselves during the process of registration. CNFI covers global animal disease-related events and is distributed to the registered readers from all over the world. The number of subscribers has been growing rapidly and new registrations are always welcome. More than 1200 registered members of the CENTAUR network from 70 countries receive the e-mail information at present. The web page http://centaur.vri.cz is requently visited by colleagues from countries of all continents.

The forms of CNFI are as follows:

E-MAIL MESSAGES are distributed to field specific registered members. Sometimes identical information is distributed to more fields of interest. Therefore second mail with identical subject and time of dispatching should not be opened but immediately deleted.

CNFI BULLETIN: approximately 10 issues per year with general information for the CENTAUR network members are distributed to all registered addresses as an attachment to e-mail. This bulletin is also available for downloading from the CENTAUR web page http://centaur.vri.cz

CENTAUR network members are welcome as authors of original papers or reviews submitted for publication in an international peer reviewed jornal for veterinary medicine and biomedical sciences Veterinarni medicina, indexed is the Web of Science, Current Contents and other databases. Papers published in this journal are free in full text at http://vetmed.vri.cz

CENTAUR network members can request the Editor for search from the published papers if their intentions are oriented towards to contributions for CNFI or submission the manuscript for publication in the journal Veterinarni medicina.

CNFI subscription is free. Register your "fields of interest" according to the instructions available at http://centaur.vri.cz/default.asp?page=cent_reg.asp and you will receive instant confirmation of your choice by e-mail. To unsubscribe or change the selected fields of interest, send an e-mail to the CNFI editor <hruska@vri.cz>. Contributions, comments and requests of the subscribers are welcome.

CNFI and the CENTAUR network are your tools!