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REVIEW

Camphor: benefits and risks of a widely used natural product

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ABSTRACT The main aspects of the non-clinical profile of D-camphor, a natural product widely used as a common remedy for several symptoms, are reviewed. The pharmacodynamics and toxicity of this substance are analyzed, with regard to all the literature available, in order to assess a risk profile and better understand the positive and negative results connected with its use. The general conclusion is that the main risks of camphor as a medicinal product are mainly due to a somehow diffused attitude of considering it as "not a real medicine", and to its consequent sometimes not sufficiently careful administration.

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KEY WORDS

D-camphor
pharmacodynamic
pharmacokinetics
risk/benefit assessment
toxicity

Camphor (Figure 1) is a natural product deriving from the wood of the camphor laurel (*Cinnamomum camphora* L.) trees through steam distillation and purification by sublimination; the trees used should be at least 50 years old. It also occurs in some other related trees in the laurel family, notably *Ocotea usambarensis* Eng., and can also be obtained from the plant *Lippia dulcis* Trev., but this is not a major industrial source (Compadre et al. 1986). A major source of camphor in Asia is *Ocimum kilimandscharicum* Baker ex Gurke.

Camphor can also be produced synthetically from vinyl chloride and cyclopentadiene, passing through the intermediate dehydronorbornyl chloride. The naturally occurring form is dextrorotatory and the synthetic form optically inactive (Budavari 1989; Reynolds 1989).

Camphor has a counterirritant, rubefacient and mild analgesic action, and is a major component of liniments for relief of fibrositis, neuralgia and similar conditions. It can be used as a mild expectorant; if ingested, camphor has irritant and carminative properties. Camphorated-oil, a solution in oil given through intramuscular or subcutaneous way, can be used as a circulatory and respiratory stimulant, but this use is considered hazardous. When, in combination with menthol and chenodeoxycholic acid, it has been used to aid dispersal of bile duct stones, although this is no longer recommended (Reynolds 1989).

Aim of the present work is to provide an overview over pharmacological and toxicological aspects of camphor, in order to assess its safety profile and evaluate the level of risk connected with its use.

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Pharmacology

Pharmacodynamics

Camphor, a natural product derived from the wood of the tree *Cinnamomum camphora*, has a long history of use as antiseptic, analgesic, antipruritic, counterirritant and rubefacient (Hercogová 2005; Lynde et al. 2008). Its success and wide medical use, especially in topical preparations, is connected to its mild local anesthesizing effect and to the production of a circumscribed sensation of heat, together with its characteristic and penetrating odour that is by most of people associated to the idea of a strong and effective medicine (Gibson et al. 1989).

Camphor is today mostly used in the form of inhalants and of camphorated oil, a preparation of 19% or 20% camphor in a carrier oil, for the home treatment of colds (Jochen and Theis 1995) and as a major active ingredient of liniments and balms used as topical analgesics (Xu et al. 2005).

The antitussive, nasal decongestant and expectorant action of camphor and of its derivatives was one of the first ones to be systematically investigated (Inoue and Takeuchi 1969).

Its nasal decongesting activity seems to be not purely mechanic, but connected with the stimulation of cold receptors in the nose. The inhalation of camphor vapours (so as the one of eucalyptus and menthol vapours) on a sample of volunteers increased the nasal sensation of airflow through the induction of cold sensation in the nose, despite of actually not affecting nasal resistance to airflow (Burrow et al. 1983).

More recent studies pointed out how camphor efficacy in the treatment of cold is due to its antispasmodic action (Astudillo et al. 2004), and how the effects of camphor on bronchospasm are connected to its anti-histaminergic and anti-cholinergic activities (Görnemann et al. 2008). In fact,

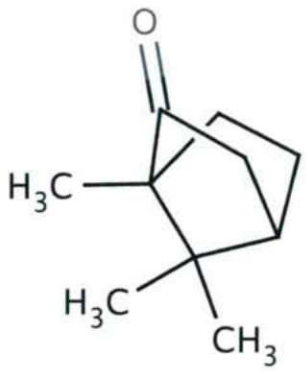

Figure 1. Structural formula of Camphor, a bicyclic monoterpene ketone (1,7,7-trimethylbicyclo [2.2.1] heptan).

camphor appears to be effective to reduce histamine H1 and muscarinic M3 receptor-mediated bronchocostriction (Görnemann et al. 2008), and this action relates also to the inhibition of cough (Kreutner et al. 2000).

Camphor was administered in the form of aromatic vapor, at the concentrations of 50, 133 and 500 μg l^{-1}, to guinea pigs subject to chemically induced cough. No effect were registered at the lowest concentrations, but 500 μg l^{-1} camphor gave a 33% reduction of cough frequency, to which an increase in cough latency coincided (Laude et al. 1994).

The analgesic proprieties of camphor are largely known and applied, but little is known about the molecular mechanisms that are at their basis. (Xu et al. 2005).

Moqrich et al. (2005) demonstrated that camphor activates TRPV3, a member of transient receptor channel superfamily, leading to excitation and desensitization of sensory nerves. The notorious effect of generation of a sensation of heath associated with topic application of camphor (Green 1990) is a consequence of this activation. In fact, TRPV3 is a warm-sensitive Ca^{2+}-permeable cation channel, that once activated originates the warm sensation, actually simulating an effective increase of temperature in the treated area (Xu et al. 2006). This effect, caused by an increase in intracellular Ca^{2+} levels, is typical also of other natural compounds as carvacrol, eugenol and thymol (Xu et al. 2006).

Anyway excessive and repeated application of camphor can lead to sensibilization of TRPV3, in apparent contrast with its analgesic role (Peier et al. 2002; Moqrich et al. 2005).

The antipruritic and counterirritant activity of camphor is instead associated with its capacity of activating TRPV1 - another member of TRP channel superfamily - at the level of dorsal root gangliar [DRG] neurons and inhibiting TRPA1 channels (Moqrich et al. 2005; Nagata et al. 2005), action that is in common with other TRPV1 agonists (Bhave et al. 2002; Xu et al. 2006; Belmonte and Viana 2008). The recently clarified activity of camphor as a TRPA1 inhibitor has been utilized by Lee et al. (2008) for pretreatment of human embryonic kidney cells tested for membrane potential changes elicited by thymol, showing how the response to thymol is blocked by camphor. Bang et al. (2007) showed camphor to suppress acute pain in mouse consequent to intradermal administration of acetaldehyde into mouse footpads.

Capsaicin shares the same action with camphor, but performs it more slowly and less completely; on the other side camphor efficacy is lower, since higher concentrations are required (Xu et al. 2005). Studies on rats demonstrated that the actions of capsaicin and camphor are segregated (Wu et al. 2005), *i.e.* they are mediated by distinct channel regions, and camphor did not activate TRPV1 in capsaicin-insensitive chickens (Xu et al. 2005; Jordt and Julius 2002).

Camphor also inhibits other related TRP channels such as ankyrin-repeat TRP1 (TRPA1), which is a further evidence underlying its analgesic effects (Xu et al. 2005).

Camphor was shown to inhibit mitochondrial respiration. Administration of up to 8 μM of camphor inhibited respiration rate in rat-liver mitochondria, nearly halving the oxygen consumption; this suggests that camphor may be used in oxygenating tumors prior to radiotherapy (Guilland-Cumming and Smith 1979; 1982).

Camphor can also be a potential radiosensitizing agent in radiotherapy. Treatment with camphor (0.5 μmol · body wt^{-1}) 45 minutes before local x-irradiation at the dose levels of 30, 80, 100 or 120 Gy was performed on male C3H/Jax mice bearing transplanted mammary tumours. Sequential measurement of the tumour volumes during 45 days after the irradiation revealed a 4.8 delay of the maximum enhancement ratios in tumour growth (Goel and Roa 1988).

D-camphor (1100 μg ml^{-1}) inhibited oxidative metabolism in *E.coli* (Cardullo and Gilroy 1975). Succinic, lactic and NADH-oxidase activities were inhibited, while NADH and succinic DCPIP oxidoreductase enzymes were unaffected. The restoration of succinic oxidase activity by ubiquinone (Q6) but not by vitamin K1 indicates that D-camphor may operate this inhibition by affecting quinone functions.

Pharmacokinetics

Camphor is readily absorbed from all the sites of administration, after inhalation, ingestion or dermal exposure (Baselt and Cravey 1990). Peak plasma levels were reached by 3 hours post-ingestion when 200 mg camphor was taken alone, and 1 hour post-ingestion when it was ingested with a solvent (Tween 80; Koppel et al. 1988).

In case of dermal application, the volume of the absorption is relatively low in comparison with the speed of the process. After application of different numbers of commercial patches [2, 4 or 8] to the skin of human subjects during 8 hours, the levels of camphor in the plasma were assayed with selective gas-cromatography (Valdez et al. 1999; Martin et

al. 2004). Maximum camphor plasma concentration resulted in a range between 35.2 and 46.8 ng/ml^{-1} in the case of 8 patches, between 19.6 and 34 ng/ml^{-1} for the 4 patches while almost undetectable concentrations were observed when only 2 patches had been applied, showing that dermal absorption is prompt but not massive.

Camphor is distributed throughout the whole body, and can permeate the placenta; for this reason it must be recommended that the use of this product is avoided during pregnancy and lactation (Sweetman 2005).

Its volume of distribution is 2-4 L/kg (Koppel et al. 1988); plasma protein binding has been estimated as 61% (Koppel et al. 1982).

After its absorption and distribution, camphor undergoes hepatic metabolism: it is hydroxylated in the liver into hydroxycamphor metabolites (Sweetman 2005).

Asahina and Ishidate (1933; 1934; 1935) isolated *cis*- and *trans*- -hydroxycamphor and camphor- -carboxylic acid from the urine of dogs that had been fed with camphor; Shimamoto (1934) obtained 3-hydroxycamphor (15%), 5-hydroxycamphor (55%) and trans- -hydroxycamphor (20%) from the urine of dogs, and 5-hydroxycamphor [as major metabolite] and 3-hydroxycamphor from the urine of rabbits.

Robertson and Hussain (1969) observed that (+)-camphor and (-)-camphor increase the content of glucuronide in the urine of rabbits; (+)-camphor was moreover reduced to (+)-borneol as well as being hydroxylated to (+)-5-endo-hydroxycamphor [major product] and (+)-3-endo-hydroxycamphor.

Hydroxylation of camphor, as well as norcamphor, pericyclocamphanone and 5,5-difluorocamphor, is mainly performed by cytochrome P450 (Collins and Loew 1988), a class of heme-containing monooxygenases that are distributed in the whole body (Boxenbaum 1984), by hydrogen abstraction (Wand and Thompson 1986). Cytochrome P450 is responsible for camphor conversion into 5-hydroxycamphor (Gelb et al. 1982), while 3-hydroxycamphor is the primary product of non-enzymatic hydroxylation of camphor (Land and Swallow 1979). Camphor hydroxylation by cytochrome P450 occurs with a different region-specificity for camphor and its related compounds (Collins and Loew 1988).

Hydroxylated metabolites are then conjugated with glucuronic acid and excreted in the urine (Sweetman 2005). The half-life of 200 mg of camphor was 167 minutes when ingested alone, and 93 minutes when ingested with a solvent (Tween 80) (Koppel et al. 1988).

Camphor can modulate the activities of hepatic enzymes involved in phase I and phase II drug metabolism. 50, 150 and 300 mg/Kg^{-1} of camphor dissolved in 0.1 ml of olive oil was administered daily to female Swiss Albino mice during 20 days. At its highest concentration it caused a significant increase in the activities of cytochrome P450, cytochrome b5, aryl-hydrocarbon hydroxylase and glutathione S-transferase, significantly elevating the level of reduced glutathione in the liver (Banerjee et al. 1995).

Interactions

Very few studies of pharmacological interactions between camphor and other compounds are present in literature. In a study combining the administration of D-camphor and an extract from fresh crataegus berries, a synergic action of the two preparations emerged in ameliorating cardiac performances. Both D-camphor and the extract contributed in an increase in total peripheral resistance induced by an increase tone of the arterioles, and while the former appeared to be the main factor in inducing the rapid initial effect, the former added a long-lasting effect (Belz and Loew 2003).

Toxicity

Camphor occurs in nature in its dextrorotatory form (D-camphor), while the laevorotatory form (L-camphor) exists only as a synthetic form. The two enantiomers present different profiles of toxicity.

D-camphor, L-camphor and their racemic mixture were tested for toxicity in mice. At 100 mg · Kg b.w.$^{-1}$ the natural form was non toxic, while the synthetic form induced different kinds of toxic and behavioural effects such as body jerks and hunched posture; the racemic mixture showed similar effects to the L-form (Chatterjie and Alexander 1986).

The oral administration of acute doses of D-camphor to rats and rabbits caused pronounced signs of toxicity. In rats, the consume of food was reduced proportionally to the administered dose, starting from 464 mg · Kg b.w.$^{-1}$ · day^{-1}, and at 1000 mg · Kg b.w.$^{-1}$ · day^{-1} convulsions and pilo-erection were observed, connected with a reduction of motility and weight gain. Reduced body weight gain and food consumption were observed in rabbits treated with 681 mg · Kg b.w.$^{-1}$ · day^{-1} (Leuschner 1997).

Camphor showed porphyrogenic activity in primary cultures of chick embryo - liver cells, with enhanced porphyrin accumulation ranging from 5- to 20-fold (Bonkovsky et al. 1992).

The main problems about camphor toxicity in humans are connected more to the large availability of camphor-containing products and their diffused perception as unhazardous medicines rather than in the intrinsic toxicity of camphor. The daily maximum human therapeutic dose is in fact approximately 1.43 mg · Kg^{-1}, which corresponds to a therapeutic ratio of more than 450 for the endpoint toxicity, reflecting a wide margin of safety (Leuschner 1997). On the other side, as mentioned above, camphor is present in several over-the-counter products, its use as a familiar remedy is commonly accepted, but still some lack of information persists among the consumers.

Cases of camphor intoxication in humans, especially children, are relatively frequent, mostly because of accidental ingestion (Siegel and Wason 1986). More than 100000 cases of ingestion exposures to camphor-containing products were registered between 1990 and 2003 (Manoguerra et al. 2006), causing a range of symptoms that comprises convulsion, lethargy, ataxia, severe nausea, vomiting and coma (Koppel et al. 1988; Manoguerra et al. 2006).

Reproduction toxicity

D-camphor was orally administered to pregnant rats and rabbits during the period of organogenesis to test its embryotoxicity. Doses up to 1000 mg · Kg b.w.$^{-1}$ · day^{-1} to rats and up to 681 mg · Kg b.w.$^{-1}$ · day^{-1} to rabbits showed no teratogenic effects, and in none of the animals were observed higher rates of mutations or malformations (Leuschner 1997).

Mutagenicity and cancerogenicity

In a Salmonella/microsome assay, the upper limit of the dose interval tested for (+/-) camphor resulted to be the highest non-toxic dose, suggesting that the compound is not mutagenic in the Ames test (Gomes-Carneiro et al. 1998).

A single dose of camphor (0.5 μM · g^{-1}) administered 30, 45 or 60 minutes before gamma irradiation significantly reduced the frequency of sister-chromatid exchanges in mouse bone marrow, showing therefore a radiomodifying influence (Goel at al. 1989).

Discussion and Conclusions

Camphor is familiar to many people as a principal ingredient in topical home remedies for a wide range of symptoms, and its use is well consolidated among the population of the whole world, having a long tradition of use as antiseptic, antipruritic, rubefacient, abortifacient, aphrodisiac, contraceptive and lactation suppressant.

In particular, the analgesic and antipruritic action of the compound make it appreciated by a large number of consumers, by whom it is used in the form of essential oil for cutaneous application. Itch is a complex phenomenon, being difficult to localize and quantify (Wahlgren 1995) and involving a variety of skin surface receptors, peripheral and central nerves and specific brain regions. The treatment of itch usually relies on antisthamines, corticoids or various topical remedies (Langner and Maibach 2009) among which camphor has a prominent role. The analgesic action is due to its interacions with members of TRP channel superfamily

Camphor is therefore an important remedy for symptomatic treatment of itching, especially in patients affected by contact dermatitis, because it goes to affect directly the cutaneous nerve ending, as other agents like pramoxine, phenol and menthol do (Burkhart and Burkhart 2003).

Camphor has also an important role in the treatment of cough and colds thanks to its antispasmodic activity, due to anti-histaminergic and anti-cholinergic action that causes depression of bronchospasm coupled with inhibition of cough.

This compound has also a long history of scientific studies on its action and on the way through which it is metabolized in the organisms of both humans and animals, due to the general interest that it has always arisen among common people and scientists. Already in 1879, Schmeideberg and Meyer were analyzing the metabolites isolated from the urine of dogs that had been fed with (+/-) camphor (Schmiedeberg and Meyer 1879), and during the first half of the twentieth century the number of studies focused on its pharmacology and pharmacokinetics has been remarkable.

The bibliographic search that was performed for the compilation of this toxico-pharmacological overview revealed a rich literature existing on camphor, and put in evidence the large amount of works focused on toxic aspects of camphor that were published during the last 30 years; a great number of reports concerning cases of camphor intoxication were also collected. In most cases camphor intoxication occurred following accidental ingestion of camphor-containing product, and sometimes lethal episodes of intoxication of infants due to application of camphor to their nostrils were collected.

As it emerges from all the observed data the toxic risks of camphor-containing products in general, and of camphorated oil in particular, are connected essentially with its improper uses, *e.g.* accidental ingestion, but camphor does not represent a threaten for safety when used on the target patients, following the indicated dosages and the contraindications. Special care must be taken during pregnancy, due to the fact that camphor crosses the placental barrier, and camphor and camphor containing products should be avoided in children who have a history of febrile convulsions or other predisposing factors for convulsions (Galland et al. 1992).

In the past, when camphor was used medicinally, the oral doses ranged from 120-300 mg (Wade 1977), and the parenteral dose range was from 60-200 mg (not recommended anymore).

Camphorated oil can be used with no risks for safety when following the prescriptions. The relatively diffused tendency to the improper use of camphor (high dosages, accidental ingestion, use on infants) is connected with the perception of the product, by many consumers, as a sort of "panacea" with no contraindication. More and more accessible information is therefore necessary to bring to a "responsabilization" of the consume of this product, in order to avoid hazardous situations.

All the above considerations allow the conclusion that camphor in its form of camphorated oil can be safely used at the proposed dosages, on the indicated patients target, for topic application.

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ARTICLE

Protoplast isolation from *Solanum lycopersicum* L. leaf tissues and their response to short-term NaCl treatment

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ABSTRACT Protoplasts were isolated from young leaves of tomato (Solanum lycopersicon L. cvar Rio Fuego). The optimum conditions for protoplast isolation was established by using 2% cellulose R-10 and 0.5% macerozyme R-10 dissolved in 0.4 M sucrose-K3 solution for 12 h cell wall digestion. In order to induce salt stress, the mannitol content of the buffer was partially replaced by NaCl to get an isoosmotic incubation solution containing 100 mM NaCl. It can be concluded that the number of protoplast in unit volume counted by Bürker chamber did not decrease significantly compared to controls due to salt treatment upto 5 hours, but the viability of cells decreased by 55% using fluorescein diacetate staining. Hundred mM NaCl simultaneously enhanced the generation of reactive oxygen species in tomato leaf protoplast. This means that decreases in fluorescein fluorescence is a good and sensitive parameter for the measurement of Na^+-induced decrease in cell viability and cell death in protoplast suspensions.
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KEY WORDS

Solanum lycopersicum
protoplast suspension
viability
salt stress

Salinity is one of the most important environmental stresses. Agricultural productivity is severely affected by soil salinity, therefore it is very important to study the effects of salt stress on cell level. Salt stress disrupts the homeostasis of intracellular ion concentrations. These changes lead to the production of the reactive oxygen species (ROS) and salt stress finally may cause cell death. High salinity also causes changes in water status of cells and it leads to growth arrest (Zhu 2001).

Protoplasts are especially good models to study physiological processes in plant cells. They can be used for in vitro manipulation of tomato to improve salt tolerance (Dorion et al. 1999) or for the analysis of Na^+ sequestration in the cell compartments of protoplasts prepared from salt tolerant and salt sensitive rice cultivars (Kader and Lindberg 2005). There are only few reports on the direct treatment of protoplasts with supraoptimal concentrations of Na^+ (Aditya and Baker 2003), because it has several problems. Number and viability of protoplast may decrease with increasing age of the preparation and with the concentration of Na^+. Thus, a reliable counting of viable cells after treatments is very important.

Fluorescein diacetate (FDA) is a molecule which is widely used to determine the viability of tomato suspension cells (De Jong et al. 2000; Yakimova et al. 2007), tobacco protoplasts (Saunders et al. 1986) and orchid seeds (Pritchard 1985). The principle of staining with FDA is that the non-polar molecule can get through the membrane, so the cells take it up. The non-specific esterases, which are located in the intracellular space, can than hydrolyse the ester bonds, thus non fluorescent FDA becomes fluorescent free fluorescein. Fluorescein is a polar molecule which remains in the cytoplasm, because it can not pass through the intact plasma membrane (Rotman and Papermaster 1966). In contrast with the living cells the dead cells can not hydrolyse the FDA molecule because of the inactivity of the enzymes or even if the cells hydrolyse the dye, the fluorescein simply effuses from the cells. Thus in this staining procedure the viable cells show fluorescence, and the dead cell do not.

In our experiments the counting of cell number in Bürker chamber and the staining with FDA was used to see, how the viability of the tomato leaf protoplasts is affected by 100 mM NaCl as a function of time. We were also interested in the generation of ROS especially of H_2O_2 by NaCl in the protoplast in connection with their viability. Our aim was also to show that decrease in the fluorescence after FDA staining of constant number of tomato protoplast is a suitable parameter of Na^+-induced loss of viability or cell death.

Materials and Methods

The leaves of 3-week-old tomato plants (Solanum lycopersicum Mill. L. cvar. Rio Fuego) were used for protoplast preparation. The seeds were germinated on 26°C, for three days in the dark. The seedlings were grown in a greenhouse in perlite and after seven days they were cultivated in hydroponic culture for two weeks. The nutrient solution contained 2 mM $Ca(NO_3)_2$, 1 mM $MgSO_4$, 0.5 mM KH_2PO_4, 0.5 mM

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Na_2HPO_4, 0.5 mM KCl, micronutrients (10^{-6} M $MnSO_4$, $5 \cdot 10^{-7}$ M $ZnSO_4$, 10^{-7} M $CuSO_4$, 10^{-7} M $(NH_4)_6Mo_7O_{24}$, 10^{-5} M H_3BO_4) and $2 \cdot 10^{-5}$ M Fe-EDTA at pH=5.8. The plants were grown for 12 hours in the light and 12 hours in the dark. The light intensity and relative humidity were 300 µmol $m^{-2}s^{-1}$ and 55-60%, respectively.

Protoplast preparation from tomato leaves

Protoplasts were isolated from the young, terminal leaves of tomato plants. The leaves were cut with scissor and were put upside down into Petri dishes containing 0.4 M sucrose-K3 solution (Nagy and Maliga 1976). Before sucrose was added to the K3 solution (sucrose-K3) it was adjusted to pH 5.5. The leaves were cut into 2-3 mm wide segments and the midrib was cut out. Leaves prepared in this way were put standing straight up into another Petri dish containing 0.4 M sucrose-K3 solution. They were incubated for ten minutes in the solution at room temperature. The solution was then replaced with 15 ml of the enzyme solution, which contained 2% cellulose R-10 (Sigma-Aldrich, St. Louis, MO) and 0.5% macerozyme R-10 (Sigma-Aldrich, St. Louis, MO) dissolved in 0.4 M sucrose-K3. After an overnight incubation at 27°C the enzyme solution was filtered with Pasteur pipette into Erlenmeyer flasks. Then the content of the flasks was placed into Wasserman tubes and a layer of 2-3 ml of W5 washing solution was stratified on the protoplast solution (1L of W5 solution contained: 9 g NaCl, 18.4 g $CaCl_2 \cdot 2H_2O$, 0.8 g KCl, 1 g glucose). Before use it was adjusted to pH=5.8. The Wasserman tubes were centrifuged at low speed for five minutes. The protoplasts were clustered between the two fluid layers creating a ring, and were transferred into new Wasserman tubes. In these new tubes W5 buffers were stratified again and protoplasts were centrifuged at the lowest speed for another five minutes. The protoplasts settled on the base of the tube and the washing solution was replaced with a buffer containing 525.6 mM mannitol, 12.5 mM Na-acetate, 5 mM $CaCl_2$, pH=5.8. The protoplasts were treated with 100 mM NaCl also, in this case we replaced the washing solution with a buffer containing 400 mM mannitol, 12.5 mM Na-acetate, 5 mM $CaCl_2$ and 100 mM NaCl, pH=5,8.

Determination of cell number using Bürker chamber

The protoplasts isolated from young tomato leaves were counted with the Bürker chamber and were analyzed for viability. The viable tomato protoplasts have intact plasma membrane so the cells are spherical, on the contrary, the dead cells have abnormal form, because of the loss of membrane integrity. The cells were counted with a microscope with enlargement of 120. We counted the cells in Bürker chamber, and the percentage of viable cells and the number of cells in 1 ml suspension were determined.

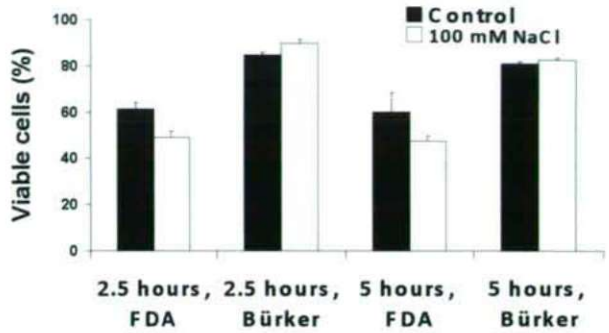

Figure 1. The effect of 100 mM NaCl on the percent of viable protoplasts counted with Bürker chamber or with FDA staining. Means±SD, n=10 for Bürker chamber detection, and 300 cells for FDA staining.

Determination of protoplast viability

Ten µM FDA solution (Sigma-Aldrich, St. Louis, MO) was used for the staining of protoplasts. After 5 minutes of incubation in the FDA dye, the protoplasts were washed for 5 minutes in the W5 buffer and the pictures about the fluorescence of protoplasts was measured with a fluorescence microscope, type Zeiss Axiovert 200M. The excitation wavelenght was λ=495 nm, and the measuring was done at 515 nm (Räthel et al. 2003.). Three randomly placed pictures where taken from the samples with a digital camera (Axiocam HR) and 773 ms exposition time and the pixel intensity was determined on 300 cells. The viability of the protoplasts was analyzed with the Axiovision 4.5 program.

Determination of reactive oxygen species

For the detection of ROS, the protoplasts were dyed with 0.01 µM 2,7-dichlorofluorescein diacetate (H_2DC-FDA) (Sigma-Aldrich, St. Louis, MO) dissolved in MES/KCl buffer for 20 minutes at 37°C, in darkness (Allan and Fluhr 1997). Different derivatives of the dichloro-dihydrofluorescein dye have been used for the detection of ROS *in vivo* (Dickens et al. 1992). These have been reported to react preferentially with $O_2^{\cdot-}$ and peroxynitrite ($ONOO^-$), but they are otherwise

Table 1. The number of viable cells counted in Bürker chamber 2.5 or 5 hours after the isolation of protoplasts in control samples and after 100 mM NaCl treatment .The cell number were calculated for 1 ml protoplast suspension (Means±SE, n=10). There were no significant differences between control and salt-treated samples.

Treatment	2.5 hours (living cells %)	5 hours (living cells %)
Control	625000 ± 180769 (92%)	575000 ± 153292.7 (86.91%)
100 mM NaCl	612500 ± 141888 (88.95%)	575000 ± 117175.5 (91.31%)

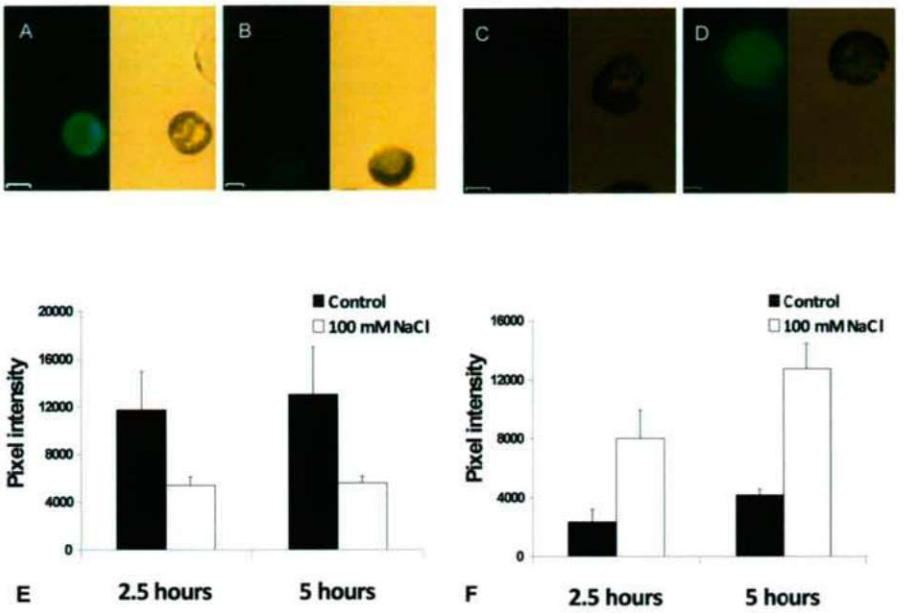

Figure 2. The green fluorescence of tomato protoplasts in control samples (A,C) and in the presence of 100 mM NaCl (B,D) after FDA staining for viability (A,B), and H_2DC-FDA staining for H_2O_2 (C, D) 5 h after protoplast isolation. Mean values±SD of the pixel intensities of protoplasts after FDA (E) and H_2DC-FDA (F) staining in control and 100 mM NaCl-treated samples. The FDA fluorescence indicate the viability of cells and that of H_2DC-FDA shows ROS production. Bars = 5 μm.

relatively unspecific, and their reaction with H_2O_2 cannot be excluded.

Results and Discussion

Protoplasts are considered very important experimental material for genetic transformation, somatic hybridisation, in vitro propagation of horticultural crops, the experimental methods often used for improving salt tolerance of crop plants, but they are rarely used for the investigation of the short-term physiological effects of abiotic stressors, such as high salinity. The protoplast yield depends on various factors such as the plant age, concentration of hydrolytic enzymes and incubation time.

To optimize the production of protoplasts, different factors, such as composition of enzyme solution, incubation period and the age of plants were studied. Good yield of protoplasts were obtained from the youngest leaf of three-week-old plants, with an enzyme solution of 2% cellulose R-10 and 0.5% macerozyme R-10 dissolved in 0.4 M sucrose-K3 solution and 12 h of cell wall digestion.

To find out if tomato protoplasts are suitable for the investigation of salt stress caused by 100 mM NaCl, we replaced the mannitol in the incubation buffer solution to NaCl. After the preparation of protoplasts, the cell number was determined as a function of time with Bürker chamber in order to determine the stability of the protoplast solution in control samples and during stress conditions. Initially the protoplast solution contained about 600000 cells in 1 ml volume. It was found that more than 80% of protoplasts were living five hours after the isolation (Table 1.).

The number of intact, spherical protoplasts were not significantly different between control and NaCl-treated samples 2.5 and 5 hours after protoplast isolation based on counting in a Bürker chamber.

The cell viability after staining with FDA was also measured as a percentage of living cells in the total number of cells. The total cell number and the number of fluorescent cells were determined on the same microscopic area. Comparing 300 cells, 55-60% of the protoplasts were viable five hours after protoplast isolation (Fig. 1). The decreases in the fluorescence of stained cells expressed in pixel intensity showed that the protoplasts treated with the 100 mM NaCl exhibited a significant decrease in cell viability (Fig. 2).

The mean pixel intensity of protoplasts decreased, when they were treated with 100 mM NaCl. This means, that before the disruption of protoplasts, salt stress decreases the viability of the cells. Protoplasts exhibited increased fluorescence under 100 mM NaCl treatment after staining with H_2DC-FDA. Increase in ROS production is a normal reaction of the cells under salt stress. Due to the increased level of H_2O_2, protoplasts considerably lose their viability but maintained the integrity of membranes and spherical shape for 5 hours. We can conclude that the loss of vitality of protoplast under NaCl stress can be demonstrated well with the decrease or loss of

the fluorescence after FDA staining, and tomato protoplasts are good models to demonstrate the effect of salt stress.

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ARTICLE

Contribution to cytotaxonomy of *Silene*: chromosome pairing and unreduced pollen grain formation in sec. *Sclerocalycinae*

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ABSTRACT Meiotic studies of ploidy level, chromosome paring and chiasma frequency were performed on 24 populations of nine *Silene* species belonging to the section *Sclerocalycinae* growing in Iran. The species studied are: 1- *Silene bupleuroides* L., 2- *S. eremitica* Boiss., 3- *S. stapfii* Melzh., 4- *S. shahrudensis* Rech. (two populations), 5- *S. peduncularis* Boiss. (two populations), 6- *S. avromana* Boiss. (three populations), 7- *S. caesarea* Boiss. (seven populations), 8- *S. chlorifolia* SM. , 9- *S. swertiifolia* Boiss. (six populations). The species studied showed 2n = 2x = 24. The chromosome numbers of all species are reported here for the first time. The species and populations studied differed significantly in chiasma frequency and chromosomes pairing indicating partly their genetic differences. When the species were subjected to cluster analysis based on meiotic characters almost the populations of each species were grouped together indicating their distinctness. Meiotic abnormalities including multipolar cell formation formed unreduced pollen grains in some of the species while B-chromosomes occurred in some others.
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KEY WORDS

chromosome paring
ploidy level
cytotaxonomy
Silene

The genus *Silene* L. (Caryophylaceae) with about 700 mostly hermaphrodite species and a few diocious or gynodioceious species shows world-wide distribution but are mainly distributed in the northern hemisphere, Europe, Asia and northern Africa (Bari 1973; Greuter 1995). Chowdhuri (1957), based on morphological characters classified *Silene* species in 22 sections but the results of molecular studies carried out by Oxelman et al. (1997, 2000) and Burleigh and Holtsford (2003) do not support such sectional classifications particularly for the endemic North American taxa.

The *Silene* species are annual, biennial, or perennial herbs most of which are diploid having 2n = 2x = 24, or 2n = 2x = 20 chromosome number (Swank 1932, Bari 1973; Oxelman and Lidén 1995). The species of *S. fortunei* is triploid (2n = 3x = 30; Heaslip 1951), but species showing tetraploid (2n = 4x = 48), hexaploid (2n = 6x = 72), and higher polyploidy levels for e.g. 2n = *c.* 96, 120 and 192 have also been reported (Bari 1973). Moreover 2n = 18 is reported for *S. conica* and *S. lacera* (Sopova and Sekovski 1982), Gvinianidze and Avazneli, 1982), and 2n = 46 is reported for *S. firma* (Zhang 1994), which make x= 9 and x = 23 along with x= 10 and 12, the known basic chromosome numbers for the *Silene*. Extensive cytogenetic studies have been performed on *Silene* species from different parts of the world (Heaslip 1951; Bari 1973; Melzheimer 1978; Markova et al. 2006) but similar studies on *Silene* species of Iran is only confined to karyotype analysis of few species (Gholipour and Sheidai 2008; Sheidai et al. 2008) and no report is available on chiasma frequency and distribution and other meiotic features of these species.

About 110 *Silene* species grow in Iran out of which about 35 species are endemic with very limited geographical distribution (Melzheimer 1980). The section *Sclerocalycinae* Boiss. contains 16 species and 3 subspecies in Iran (Melzheimer 1980), growing mainly in the west and north of the country. Our study reported in this paper is the first cytological analysis of 24 populations of 9 Iranian species of *Silene* species belonging to the section *Sclerocalycinae*.

Material and Methods

Cytological studies were performed on in 24 populations of nine *Silene* species of the section *Sclerocalycinae* Boiss., (Table 1), vouchers specimens are being deposited in the Herbarium of Shahid Beheshti University (HSBU), Iran. The species studied are: 1- *Silene bupleuroides* L., 2- *S. eremitica* Boiss., 3- *S. stapfii* Melzh., 4- *S. shahrudensis* Rech. (two populations), 5- *S. peduncularis* Boiss. (two populations), 6- *S. avromana* Boiss. (three populations), 7- *S. caesarea* Boiss. (seven populations), 8- *S. chlorifolia* SM. , 9- *S. swertiifolia* Boiss. (six populations).

For cytological studies young flower buds of each species were collected from at least 10 randomly selected plants and fixed in acetic acid: ethanol (1:3 v/v) for 24 h after which

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Table 1. Meiotic characteristics of *Silene* species studied.

Species	Locality	2n	TX	IX	TOX	RB	ROD
S. bupleurides ssp.							
bupleuroides	Damavand	24	14.7	15.17	19.88	9.58	2.42
S. eremtica	Khooy	24	17.00	5.23	22.23	9.03	2.93
S. stapfii	Kerman	24	17.18	3.24	20.42	8.06	3.94
*S. shahrudensis*1	Oshtorankooh	24	17.13	4.87	22.00	10.03	1.97
*S. shahrudensis*2	Semnan	24	15.63	6.00	21.63	9.13	2.88
*S. peduncularis*1	Ghooshchi	24	18.29	5.14	23.43	10.57	1.43
*S. peduncularis*2	Tabriz	24	17.74	4.17	21.91	9.09	2.91
*S. avromna*1	Arak	24	17.13	3.23	20.37	8.00	3.93
*S. avromna*2	Uremia	24	15.53	7.06	22.59	10.94	1.06
*S. avromna*3	Gajereh	24	12.41	7.93	20.26	10.11	1.89
*S. caesarea*1	Dena	24	18.84	2.77	21.65	8.61	3.39
*S. caesarea*2	Koohgol	24	17.58	3.87	21.45	8.52	3.48
*S. caesarea*3	Cheshmeh-Mishi	24	16.70	1.55	18.24	5.79	6.18
*S. caesarea*4	Yasooj	24	17.91	2.50	20.41	8.05	3.95
*S. caesarea*5	Khor village	24	16.36	4.50	20.86	8.57	3.43
*S. caesarea*6	Sharestanak	24	16.34	3.97	20.31	8.23	3.77
*S. caesarea*7	Nesa	24	14.69	4.54	18.85	6.23	5.77
S. chlorifolia	Ardakan	24	14.07	6.53	20.60	9.07	2.93
*S. swertiifolia*1	Arak	24	12.64	7.50	20.14	9.18	2.77
*S. swertiifolia*2	Oghlid	24	14.72	6.56	21.28	9.11	2.83
*S. swertiifolia*3	Darbandsar	24	14.54	4.37	18.91	6.94	5.06
*S. swertiifolia*4	Gachsar	24	18.18	2.82	21.00	9.27	2.73
*S. swertiifolia*5	Gajereh	24	14.91	7.91	22.82	8.91	3.00
*S. swertiifolia*6	Touchal	24	12.05	8.64	20.68	9.18	2.82

Abbreviations: TX = Terminal chiasmata, IX = Intercalary chiasmata, TOX = Total chiasmata, RB = Ring bivalent, ROD = Rod bivalent.

they were washed and preserved in ethanol at 4°C until used (Sheidai et al. 2006). For each species, squash slides were prepared and stained with 2% (w/v) aqueous aceto-orcein and the chromosome numbers and chiasma frequency determined from 100 pollen mother cells at diakinesis-metaphase I and 500 anaphase and telophase (Sheidai et al. 2008). Complete pollens which were stained were taken as fertile, while incomplete, shrunken pollens with no stain were considered as infertile (Sheidai and Rashid 2007).

In order to determine any significant difference in chiasma frequency and distribution among the species and populations we performed analysis of variance (ANOVA) by the least significant differences (LSD) method (Sheidai et al. 2008).

Grouping of the species based on meiotic characteristics was performed using various cluster analysis methods including single linkage, unweighted paired group with arithmetic average (UPGMA) and Neighbor Joining (NJ) method as well as ordination based on principal component analysis (PCA) (Podani 2000; Sheidai et al. 2008). Bootstrapping with 100 replications was performed on dendrograms obtained.

In order to detect significant difference between potential unreduced pollen grains and the normal (reduced pollens), t-test was performed. Statistical analyses used SPSS ver. 9 (1998), NTSYS ver. 2.1 (1998) and DARwin ver. 5.0.155 (2006) and PAUP ver. 4.0b10 (2001) software.

Results and Discussion

Chromosome pairing and segregation

The *Silene* species and populations studied by us showed the presence of 2n = 2x = 24 chromosome number (Table 1, Fig. 1. A-G). The chromosome numbers of all species are reported for the first time.

The highest total number chiasmata and ring bivalents

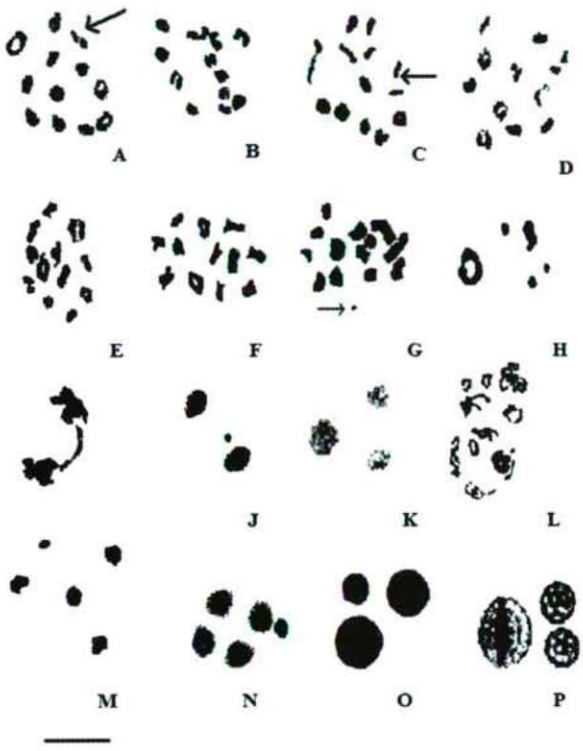

Figure 1. Representative meiotic cells in *Silene* species studied. A-F = Meiocytes showing 2n = 2x = 24 in Kerman population of *S. stapfii*, Tabriz population of *S. peduncularis*, Cheshmeh-Mishi population of *S. caesarea*, *S. bupleuroides* and *S. eremitica* respectively. G = Meiocyte showing one B-chromosome in *S. bupleuroides*. H = Metaphase chromosome stickiness in Khor village population of *S. caesarea*. I = Anaphase bridge in Darbandsar population of *S. swertiifolia*. J = Anaphase-I laggard in Arak population of *S. avromana*. K = Tripolar cell in Cheshmeh-Mishi population of *S. caesarea*. L = Meiocyte with double chromosome number in *S. eremitica*. M = Multipolar cell in *S. shahrudensis*. N = Multipolar cell in *S. eremitica*. O = Unreduced pollen grain (bigger size) in Yasooj population of *S. caesarea*. P = Unreduced pollen grain (bigger size) in Semnan population of *S. shahrudensis*. Scale bar = 10 µm.

occurred in Ghooshchi population of *S. peduncularis* (23.43 & 10.57 respectively), while the lowest values of the same occurred in Cheshmeh-Mishi population of *S. caesarea* (18.24 & 5.79 respectively). Dena population of *S. caesarea* showed the highest number of terminal chiasmata (18.84), while *S. swertiifolia* showed the highest number of intercalary chiasmata (8.64).

ANOVA followed by LSD test showed a significant difference for chiasma frequency and chromosome pairing among *Silene* species and populations studied, indicating that significant change has occurred in the number genes controlling chromosome pairing during species diand populations diversification. Variation in chiasma frequency and localization is genetically controlled (Quicke 1993), and has been reported in several plant species as well as in crop plant varieties (Rees and Dale 1974; Rees and Jones 1977). Such variation between species and populations with the same chromosome number is considered to be a means for generating new forms of recombination which influences the variability within natural populations in an adaptive way (Rees and Dale 1974).

The UPGMA and NJ clustering and ordination based on principal component analysis (PCA) of the species produced similar results (Figs. 2 & 3). In general four major clusters or groups are formed; the populations of the species *S. caesarea* form two of the four major clusters. Their cytogenetic difference with the other species is well documented in the PCA plot (Fig. 3) as these populations are placed in the right side of the plot along PCA1 and PCA2 axes. The populations of *S. swertiifolia a*re distributed in two clusters or groups indicating the presence of intra-specific cytogenetic diversity in this species which also holds true for *S. avromana*. In Flora Iranica (Melzheimer 1980), the species of *S. bupleuroides*, *S. chlorifolia* and *S. swertiifolia* have been placed close to each other, the species of *S. caesarea* and *S. stapfii* being considered related and the same is suggested for the species of *S. peduncularis*, *S. avromana*, *S. shahrudensis* and *S. eremitica*. Grouping of the species based on cytological data reported by us supports the affinity of above said species (Figs. 2 & 3).

Meiotic abnormalities

Metaphase and anaphase chromosome stickiness occurred in some of the species like *S. peduncularis*, *S. shahrudensis* and *S. chlorifolia* (Fig. 1, H & I). The degree of chromosome stickiness ranged from stickiness among two or more chromosomes to the involvement of all metaphase chromosomes forming a complete clump. Chromosome bridges resulting from stickiness were observed in anaphase-I and II as well as telophase-I and II stages (Fig. 1, H). The thickness of bridges observed and the number of chromosomes involved in their formation varied among different meiocytes and in the species studied. Some of the species showed the occurrence of 1 to a few laggard chromosomes in anaphase I and II as well as telophase-I and II. Such laggard chromosomes formed micronucleus in telophase of meiosis II (Fig. 1, j). Genetic, environmental factors and their interaction have been considered as the possible reasons for the occurrence of chromosomes stickiness in different plant species and cultivars (Baptista-Giacomelli et al. 2000).

Multipolar cells and abnormal tetrads were observed in most of the species studied (Fig. 1, K, M & N). Multipolar cells may be formed due to spindle abnormalities. Such meiotic abnormalities may lead to the formation of abnormal tetrads and aneuploid gametes (Villeux 1985; Nirmala and Rao 1996; Sheidai and Attaei 2005; Sheidai and Nouroozi 2005; Sheidai et al. 2005, 2006). The spindle apparatus is normally bipolar and acts as a single unit, playing a crucial role in chromosome alignment during metaphase. Any distortion or breakage in the spindle may result in random sub-grouping of

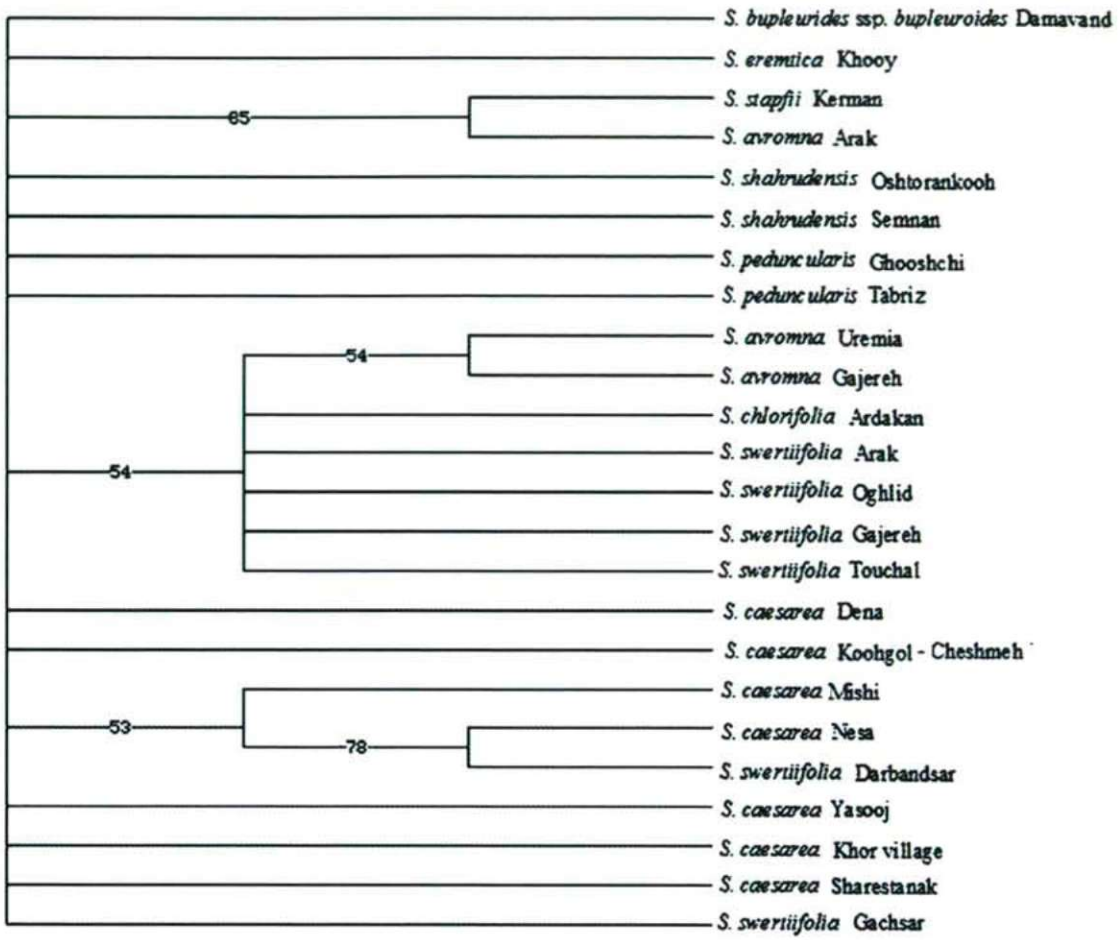

Figure 2. NJ dendrogram of *Silene* species studied. (Numbers above clusters are bootstrap values)

the chromosomes which function independently (Nirmala and Rao 1996). In several instances spindle abnormalities have led to the production of aneuploid gametes for example in polyploidy hybrids and derivatives of *Aegilops* ×*Triticum* hybrids, amphiploid Triticineae, amphiploids of *Solanum* hybrids, etc. Different reasons have been suggested for the occurrence of spindle abnormalities including: duality of nucleus in foreign cytoplasm, environmental influence and disharmonious gene interaction (Nirmala and Rao 1996).

The presence of meiocytes having double the gametic chromosome number as well as bigger size pollen grains (potential unreduced (2*n*) pollen grains), were noticed in most of the *Silene* species studied (Fig. 1, L, O & P). A numerically unreduced diploid or 2n gamete is a meiotic product bearing the sporophytic rather than the gametophytic chromosome number. Such gametes result from abnormalities during either microsporogenesis (2n pollen) or megasporogenesis (2n eggs). Unreduced gametes are known to produce individuals with higher ploidy level through a process known as sexual polyploidization (Villeux 1985). Sexual polyploidization has been considered a major route to the formation of naturally occurring polyploids. Different cytological mechanisms are responsible for the production of 2n gametes, including premiotic doubling of the chromosomes, omission of the first and second meiotic division, post-meiotic division, abnormal spindle geometry, abnormal cytokinesis and desynapsis (Villeux 1985). Detailed cytological investigation of *Silene* species studied revealed that the main cytological mechanisms for production of potential 2*n* gametes are: 1- anaphase-II failure leading to the formation of triads at the end of telophase-II instead of tetrad (one is unreduced, Fig. 1, K); 2- multipolar spindles as discussed earlier and 3- syncyte formation (Fig. 1, L).

The potential unreduced pollen grains, ranged in size from 64-77 µm in diameter and differed from the smaller sized pollen grains (reduced pollen grains) ranging in size from 38-57 µm in diameter. The presence of giant pollen grains has been used as an indication of the production of 2n pollen grains. A variety of methods have been used to detect 2*n* gametes, including morphological screening of 2*n* pollens

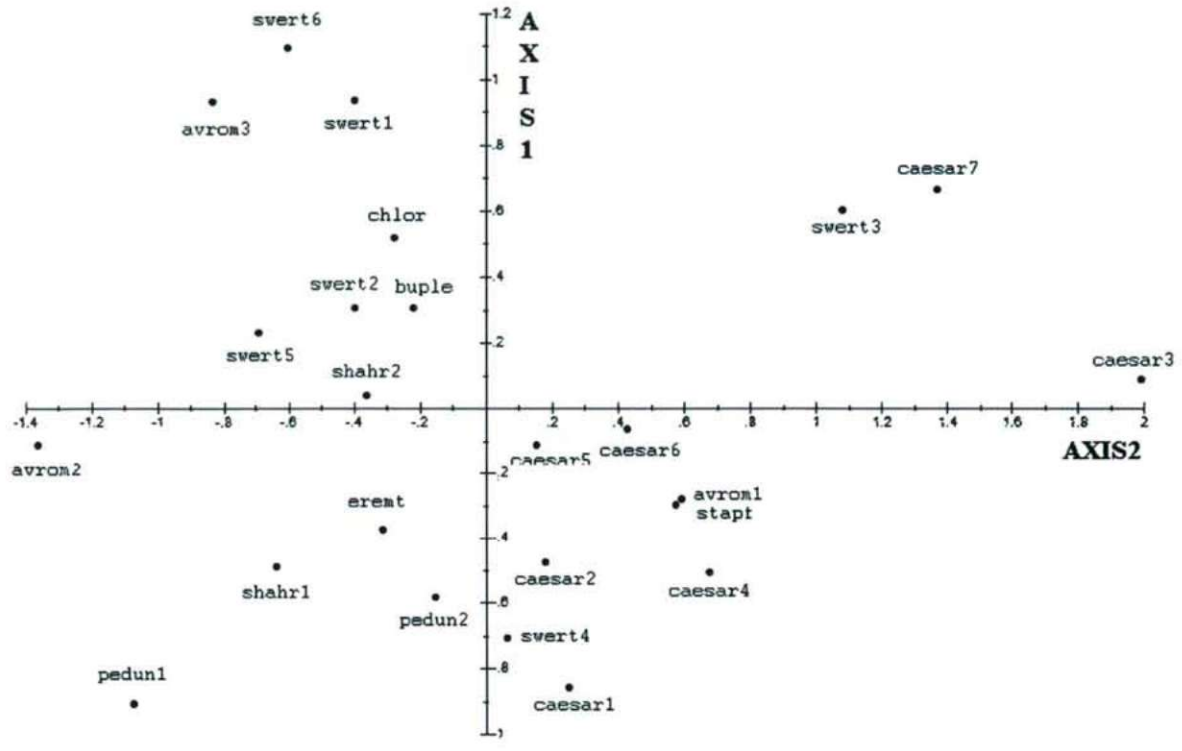

Figure 3. PCA plot of *Silene* species studied. Species abbreviations: buple = *S. bupleuroides*, shar1 & 2 = Ushtorankoon and Semnan populations of *S. shahrudensis*, swert1-6 = Arak, Eghlid, Darbandsar, Gachsar, Gajereh and Touchal populations of *S. swertiifolia*, avrom1-3 = Arak, Uremia and Gajereh populations of *S. avromana*, eremt = *S. eremitica*, pedun1 & 2 = Ghooshchi and Tabriz populations of *S. peduncularis*, stapf = *S. stapfii*, ceasar1-7 = Dena, Koohgol, Cheshmeh-Mishi, Yasooj, Khor village, Sharestanak and Nesa populations of *S. caesarea*, chlor = *S. chlorifolia*.

and flow cytometry analysis of pollen along with cytological investigations (Bretagnolle and Thompson 1995). The measurement of the pollen grains in the species with unreduced meiocytes revealed the presence of a bimodal distribution of pollen grain size. T-test analysis also showed a significant difference between the pollen grains indicating the possible $2n$ constitution of the larger pollen grains. The frequency of potential unreduced pollen grains differed from 2-3% in the species studied. The occurrence of unreduced gametes has been considered important in the evolution of polyploids and also is of economic importance for example in potato for obtaining natural tetraploid by crossing 4x ×2x lines (Bretagnolle and Thompson 1995).

B-chromosomes

The species of *S. chlorifolia*, *S. bupleuroides*, *S. stapfii*, *S. caesarea* and *S. peduncularis showed* the presence of 0-2 B-chromosomes (Bs) (Fig. 1, G). The B-chromosomes were smaller than the A-chromosomes and did not form any meiotic association with them. B-chromosomes are accessory chromosomes reported in many plant and animal species. They show numerical polymorphism and, when present in high numbers, negatively affect the growth and vigor of the plants, while in low numbers they may be beneficial to the plant possessing them (Camacho et al. 2000). B-chromosomes may affect the frequency and distribution of chiasmata as well as chromosome association, however due to low number of meiocytes showing presence of Bchromosomes in the species studied; their effects on chiasma frequency and chromosome associations could not be worked out.

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ARTICLE

Enhanced aglycone production of fermented soybean products by *Bacillus* species

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ABSTRACT This study evaluated the effect of starter culture and fermentation period on the isoflavone content of protein-rich soybeans variety TG145. Initially, soybeans were washed, soaked in water for 16 h and autoclaved at 121°C for 40min. Three different bacterial starter cultures (~10^4 CFU/g) namely *Bacillus subtilis* BEST195, *B. subtilis* Asaichiban and *B. subtilis* TN51 were then added and the fermentation was allowed to proceed at 42°C for 24 h (*natto*-style) and 72 h (*thua nao*-style). The quantities of six major isoflavones (daidzin, genistin, glycitin, daidzein, genistein, and glycitein) were then determined in these fermented soybean products using reverse phase HPLC technique. Generally, our results clearly showed that the content of total isoflavones in the fermented products prepared by *Bacillus* starter cultures greatly increased ranging from 43 t9o-9% compared to that of the unfermented autoclaved soybeans. In addition, a dramatic increase of aglycones was also observed (> 400%) in the soybean products fermented by *Bacillus* sp. strain TN51. This present study suggests a promising use of *Bacillus* starter cultures in improving isoflavone compounds especially the aglycones which would benefit for novel functional food development.
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KEY WORDS

Aglycones
Bacillus
fermented soybean
isoflavones

Soybeans (*Glycine max* (L.) Merr.) are highly regarded as a healthy food in several Asian countries and are widely consumed as soymilk, *tofu* and fermented products. In these soybeans and soy-products, several phytochemicals can beare found and they appear to be the active compounds causing many beneficial health effects (Dixon 2004; Wiseman 2006). Soy isoflavones in particular are a group of natural heterocyclic phenols comprising of aglycones, β-glucosides, acetylglucosides, and malonylglucosides. These isoflavone compounds are of great importance due to their pharmacological and antioxidant properties. For example, several researchers have showed the beneficial use of isoflavones; these include prevention of mammary cancer (Gotoh et al. 1998), reduced risk of cardiovascular diseases (Teede et al. 2001), improvement of bone health and menopause symptoms (Potter et al. 1998; Ishimi et al. 2002), antimutagenic effects (Peterson et al. 1998; Park et al. 2003), and antidiabetic effects (Liu et al. 2006). Of these compounds, it appears that the glucoside forms are predominant in soybeans. However, it should be noted that the content and composition of isoflavones are variable depending on many parameters such as soybean variety (Wang and Murphy 1994; Lee et al. 2003, 2007), geographical plantation (Wang and Murphy 1994; Hoeck et al. 2000;, Lee et al. 2003), storage time (Lee et al. 2003), crop year (Wang and Murphy 1994), and food processing techniques (Jackson et al. 2002; Kao et al. 2004, Lee et al. 2007).

In Asia, there are several traditional fermented soybean products such as Japanese *natto*, Korean *chungkookjang*, Indian *kinema* and Thai *thua nao*. Interestingly, numerous studies have reported that the amount of aglycones is much higher in fermented soybeans compared to that of unfermented soybeans; such products include *natto* (Wei et al. 2008), *miso* (Yamabe et al. 2007), *sufu* (Yin et al. 2004, 2005), *douche* (Wang et al. 2007), and *chungkukjang* (Kwak et al. 2007). At present, the presence of aglycone isoflavones has attracted special interest due to their bioavailability, high rate of absorption in animals and humans (Izumi et al. 2000; Kano et al. 2006) and high antiproliferative activity on human cancer cells (Peterson et al. 1998). Especially for fermented soybeans, several studies have also revealed the role of microbes in soybean fermentation as the active agents to enrich isoflavone aglycones. This conversion of glucoside isoflavones to aglycones during the fermentation is achieved by the activity of microbial β-glucosidase enzymes (Chien et al. 2006;, Kuo et al. 2006;, Chun et al. 2007, 2008). As part of the programme to improve nutritional value of *thua nao*, we previously screened and identified a strong proteolytic bacterium *Bacillus subtilis* sp. TN51 from commercial *thua*

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nao products (Dajanta et al. 2009) and attempted to use such a strain as a pure starter culture in soybean fermentation. This study was therefore undertaken to investigate the content and composition of isoflavone compounds in unfermented soybean and fermented soybean prepared by four *Bacillus subtilis* strains. The content of isoflavones of *thua nao* prepared in traditional manner was also determined.

Materials and Methods

Bacterial strains and culture conditions

Three bacterial strains used in this study were *Bacillus subtilis* TN51 isolated from *thua nao* products (Dajanta et al. 2009), *B. subtilis* BEST195 (Qiu et al. 2004) and *B. subtilis* ASA isolated from Asaichiban *natto* product. These bacteria were routinely cultured on nutrient agar and their stock cultures were maintained at -80°C in 20% glycerol. For inoculum preparation, the bacteria were grown in nutrient broth at 37°C for 24 h. The cells were then harvested, resuspended in sterile distilled water and properly adjusted to obtain a concentration of 10^4 CFU/mL. The suspension was served as the inoculum for soybean fermentation.

Preparation of fermented soybeans

Protein-rich soybean seeds variety TG145 supplied by the Field Crops Research Institute, Department of Agriculture, Chiang Mai, Thailand were used in this study. Initially, the dehulled soybeans were washed and soaked in tap water for 16h at ambient temperature (~25°C). After decanting the water, soaked soybeans were placed in a plastic bag and autoclaved at 121°C for 40 min. To initiate the fermentation, the autoclaved soybeans were inoculated with the *Bacillus* inocula. The fermentation process was allowed to proceed at 42°C for 24 h (*natto*-style) and 72 h (*thua nao*-style). In addition, *thua nao* products fermented in the traditional manner were also prepared; for this, soybeans were cooked by boiling for 2 – 3 h instead of autoclaving.

Extraction of isoflavones

Soybean isoflavones were extracted using the method of Achouri et al. (2005). Two grams of freeze-dried samples were dissolved in 10 ml of 80% methanol in water (v/v) in a 50 mL screw-cap tube, vortexed for 1 min, sonicated in a FB 15046 sonicator (Fisher Scientific, Germany) at 50 – 60 Hz of ultrasonic frequency for 15 min, and centrifuged at 3200 rpm for 30 min. The sample remains were subjected to repeated extractions with 10 mL of 80% methanol. Supernatants from two extractions were then combined, concentrated with an evaporator and dried by nitrogen gas flow at room temperature. Dried isoflavone extracts were subsequently dissolved with 3 mL of 80% methanol and filtered through a 0.2 µm filter membrane prior to HPLC injection.

HPLC analysis

Isoflavones were analysed by reverse-phase HPLC according to the method of Kim and Chung (2007). The Dionex HPLC system used was equipped with a P680 HPLC pump, ASI-100 automated sample injector, thermostatted column compartment TCC-100, Agilent Eclipse XDB-C18 column (4.6 x 150 mm, 5 µm), and PDA-100 photodiode array detector. The mobile phase was composed of 0.1% (v/v) acetic acid in filtered MilliQ water (solvent A) and 0.1% (v/v) acetic acid in acetonitrile (solvent B). The injection volume was 20µl and the components were eluted using the following solvent gradient: from 0 to 50 min 15 – 35% solvent B; then held at 35% solvent B for another 10 min; and from 60 to 65 min re-equilibrated back to 15% solvent B. The flow rate was 1.0 mL/min and UV detector wavelength was set at 254 nm. Stock solutions of six isoflavone standards including daidzin, glycitin, genistin, daidzein, glycitein, and genistein (Plantech-UK) were prepared in 80% (v/v) methanol. Calibration curves were plotted by using peak area and corresponding isoflavone concentration. The identity and purity of isoflavones in the samples were proven by comparing the retention times and UV spectra of the standards.

Statistical analysis

Isoflavone values reported were the means of triplicate determinations with standard deviations (means ± SD). Analysis of variance (ANOVA) was also carried out using SPSS Version 15.0. Duncan's multiple range tests were introduced to determine the significant differences between the treatments ($P<0.05$).

Results and Discussion

A typical HPLC chromatogram of the isoflavone standards, unfermented soybeans, and fermented soybean products are represented in Figure 1. Due to their different chemical structures, these isoflavone compounds were successfully separated by the HPLC system established in this study. The chromatogram of isoflavones extracted from unfermented and fermented soybeans were similar to that of the pure isoflavone standards (Fig. 1). The reliability of the extraction method was also assessed by addition of known concentration of glycitein standard into unfermented soybean before extraction of isoflavones. Recovery percentages for glycitein were calculated as 98% (n = 5). By this means, the correlation coefficients (R^2) of all standard curves of isoflavone standards were over 0.99 (data not shown). Besides, the HPLC soybean chromatograms also showed several peaks of unidentified compounds (X1 – X6). Based on previous reports (Lee et al. 2004; Kim and Chung 2007), these unknown compounds were possibly malonyldaidzin, malonylglycitin, malonylgenistin, acetyldaidzin, acetylglycitin, and acetylgenistin. These compounds are also isoflavone derivatives and their content

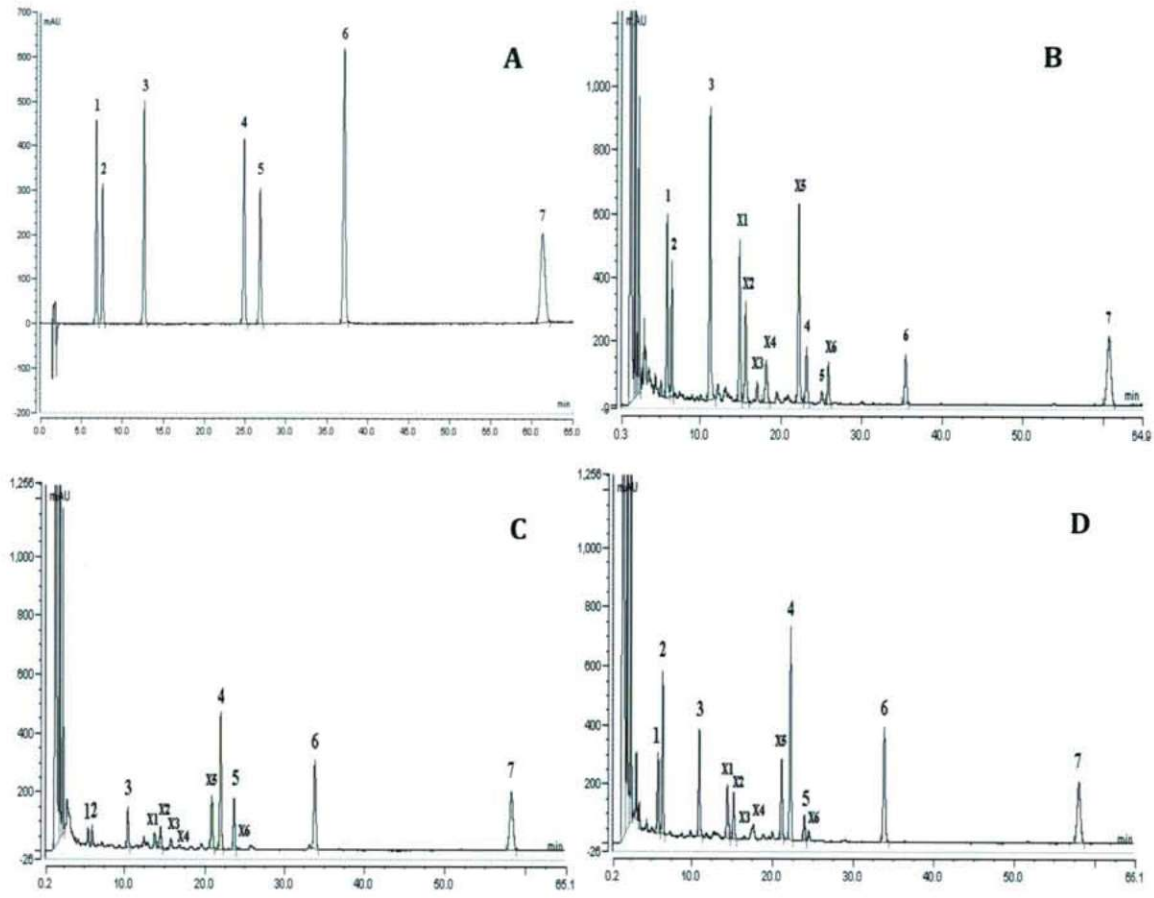

Figure 1. Representatives of HPLC chromatograms showing isoflavone content of (A) standard isoflavones (1 = daidzin, 2 = glycitin, 3 = genistin, 4 = daidzein, 5 = glycitein, 6 = genistein, and 7 = flavone); (B) autoclaved soybeans; (C) traditionally fermented soybeans; and (D) fermented soybeans prepared with B. subtilis TN51. X1, X2, X3, X4, X5, X6 are unidentified compounds that may be malonyldaidzin, malonylglycitin, malonylgenistin, acetyldaidzin, acetylglycitin, and acetylgenistin (see text for details).

appeared to be much higher in unfermented soybeans (Fig. 1B). These glucosides-based compounds have been reported in small amounts and thus not taken into account in our study. It is also evident that these compounds are abundant in soybeans but not in fermented soybeans.

The concentration and composition of isoflavones in unfermented cooked soybeans (boiled and autoclaved) are shown in Table 1. The total isoflavones are expressed as the sum of glucosides (daidzin + glycitin + genistin) and aglycones (daidzein + glycitein + genistein). According to Table 1, the data clearly show that cooking method also affect the isoflavone contents. For unfermented soybeans, autoclaving appears to promote the concentration of total isoflavones especially the glucoside forms. Boiled soybean showed lower level of glucoside isoflavones probably due to heat damage (boiling in water for 4 h). This event is in agreement as previously described by Jackson et al. (2002) and Kao et al. (2004) in that the process of soaking and heating soybeans in water led to a decrease of glucosides. Total glucosides were the largest proportion in both unfermented soybean samples and were accounted for 78 and 84% of the total isoflavones, respectively. This was slightly lower than the 88% proportion as reported by Wei et al. (2008) for unfermented autoclaved soybean. This minor discrepancy was probably due to the difference in the variety of soybean and isoflavones extraction method (Achouri et al. 2005; Yamabe et al. 2007). Genistin was shown to be the most predominant isoflavon in both types of cooked soybeans followed by daidzin and glycitin asin the amount of 116-167, 72-100, and 68-108 μg/g, respectively. For the aglycones group, daidzein (36-37 μg/g) was found at significantly higher level than genistein (23-25 μg/g) and glycitein (10-12 μg/g) ($P<0.05$).

Conversion of glucoside isoflavones into aglycones isoflavones during soaking and cooking has been reported previously. Kao et al. (2004) demonstrated that soaking temperature and time affect the content and conformation of isoflavone compounds in soybean. After soaking soybean at 25, 35, and 45°C for 12 h, the concentration of aglycone iso-

Table 1. Concentrations (μg/g, dry mass basis) of isoflavone components of unfermented soybeans and soybeans fermented with *Bacillus subtilis* strains at 42°C[a].

Isoflavone	Unfermented soybeans[b]		Fermented soybeans[b]			
			thua nao-style		natto-style	
	BSB	ASB	BTN	CTN	BNA	BNB
I. Glucosides						
Daidzin	72 ± 5Bd	100 ± 19Bc	68 ± 8Cd	11 ± 2Ce	169 ± 18Cb	286 ± 21Aa
	(22)	(22)	(11)	(3)	(20)	(32)
Glycitin	68 ± 5Bd	108 ± 15Bc	191 ± 12Ab	24 ± 6Ce	279 ± 13Aa	297 ± 10Aa
	(21)	(24)	(30)	(7)	(32)	(34)
Genistin	116 ± 7Ad	167 ± 12Ac	84 ± 8Be	26 ± 7Cf	214 ± 29Bb	256 ± 8Ba
	(35)	(37)	(13)	(8)	(25)	(29)
Total Glucosides	255 ± 18d	375 ± 42c	343 ± 27c	60 ± 14e	663 ± 47b	839 ± 23a
	(78)	(84)	(54)	(19)	(77)	(94)
II. Aglycones						
Daidzein	37 ± 3Cc	36 ± 2Cc	195 ± 9Aa	135 ± 14Ab	131 ± 4Db	15 ± 2Dd
	(11)	(8)	(31)	(42)	(15)	(2)
Glycitein	10 ± 1Ec	12 ± 1Dc	32 ± 3Db	63 ± 10Ba	13 ± 2Fc	7 ± 2Cc
	(3)	(3)	(5)	(20)	(2)	(1)
Genistein	25 ± 2Dc	23 ± 1CDc	70 ± 4BCa	61 ± 7Bb	57 ± 5Eb	27 ± 3Cc
	(8)	(5)	(11)	(19)	(7)	(3)
Total Aglycones	73 ± 6d	71 ± 4d	297 ± 14a	260 ± 22b	201 ± 11c	49 ± 7e
	(22)	(16)	(46)	(81)	(23)	(6)
Total Isoflavones	328 ± 24d	447 ± 46c	640 ± 31b	320 ± 32d	864 ± 37a	888 ± 21a
	(100)	(100)	(100)	(100)	(100)	(100)

[a]Values are mean ± standard deviation (n = 3) and number in parentheses are percentage of each isoflavone relative to total isoflavones. Data in the same row (small letters) or same column (capital letters) with different superscripts were significantly different ($P<0.05$). [b]BSB = boiled soybean; ASB = autoclaved soybean; BNA = soybeans fermented by *Bacillus subtilis* Asaichiban; BNB = soybeans fermented by *B. subtilis* BEST 195; BTN = *B. subtilis* TN51, which isolated from local Thai fermented soybean (*thua nao*); CTN = control, traditional fermentation.

flavones (daidzein, glycitein, and genistein) clearly increased, conversely there was a decrease of conjugated (malonylglucosides and acetylglucosides) and β-glucoside isoflavones. Such results suggested that malonylglucoside can be converted to acetylglucoside and further converted to glucoside or aglycone isoflavones during the soaking process. Also, Chein et al. (2005) indicated that the conformation of glucosides could be changed during moist heating, with the highest rate of conversion of malonylgenistin to genistin, followed by malonylgenistin to acetylgenistin, and acetylgenistin to genistin. In this study, soybean seeds were soaked in water at 25°C for 16 h and boiled in boiling water for 4 h or autoclaved at 121°C for 40 min; therefore, genistin predominates in all of the cooked soybeans.

Similar results were presented in this study for genistin and daidzein, which are the major glucoside and aglycone forms in unfermented soybean (Kim and Chung 2007) although Lee et al. (2007) showed higher amounts of genistein than daidzein in Ohio soybeans. A number of investigators reported that malonylgenistin is the most predominant in soybeans (Lee et al. 2004; Kim and Chung 2007). However, this study has not calculated the concentration of malonylglucoside and acetylglucoside isoflavones in soybeans.

Apart from physical factors, it is interesting to note that microbial fermentation also plays a key role in isoflavone content variation. In this study, fermentation of bacterial pure starter culture tends to promote the increase of total isoflavones (Table 1). Besides, the fermentation period appears to be another major factor affecting the isoflavone content as observed in the fermented soybeans prepared traditionally (*thua nao*).

All soybeans fermented with pure *Bacillus subtilis* strains showed significantly higher concentrations of total isoflavones than those fermented with mixed natural microorganisms ($P<0.05$). Soybeans fermented with pure culture of *B. subtilis* BEST195 and Asaichiban presented the highest amount of total isoflavone compounds, followed by soybean fermented with *B. subtilis* TN51 and mixed natural microorganisms: 888, 864, 640, and 320 μg/g, respectively. Traditionally fermented soybeans and unfermented cooked soybeans had the similar concentrations of total isoflavones, but differences in the form of isoflavones present. Besides, traditionally fermented soybeans contained higher concentration of aglycone isoflavones, including daidzein (135 μg/g), glycitein (63 μg/g), and genistein (61 μg/g), than unfermented cooked soybeans (36-37, 10-12, and 23-25 μg/g, respectively). Therefore, *B. subtilis* fermented soybeans appear to be a better source of bioavailable soy isoflavones as it has been reported that aglycone isoflavones are absorbed faster and in higher amounts than their glucosides in humans (Izumi et al.

2000; Kano et al. 2006).

Different isoflavone forms were distributed in fermented soybeans; glucoside isoflavone compounds showed the largest proportion in soybean fermented by *B. subtilis* Asaichiban, *B. subtilis* BEST195, and *B. subtilis* TN51, *i.e.* 77, 94 and 54% of total isoflavones, respectively. Of the glucoside isoflavones, glycitin was the most abundant (30-34% of total isoflavones). In contrast, the aglycone contents of traditionally fermented soybeans were very high and accounted for 81% of total isoflavones. After fermentation, total glucosides in fermented soybeans prepared by *B. subtilis* Asaichiban and *B. subtilis* BEST195 increased significantly from 375 μg/g in unfermented autoclaved soybean to 663 and 839 μg/g, respectively. The marked increase of total glucoside isoflavones is probably due to their conversion from malonylglucoside and acetylglucoside isoflavones during fermentation. Indeed, the peak area of malonylglucoside and acetylglucoside isoflavones in both fermented soybeans decreased remarkably when compared with unfermented autoclaved soybean (data not shown). Our results are in agreement with other previous work reporting that the glucoside isoflavone form was a major component in these fermented soybeans (Nakajima et al. 2005; Wei et al. 2008).

Aglycone isoflavones in fermented soybeans presented significantly higher levels than unfermented cooked soybean, excluding *B. subtilis* BEST195 fermented soybeans ($P<0.05$). Content of aglycone isoflavones in fermented soybeans varied with the starter culture; fermented soybeans prepared with *B. subtilis* TN51 showed the greatest abundance of total aglycone isoflavones (297 μg/g), followed by traditionally fermented soybeans (260 μg/g), and *B. subtilis* Asaichiban (201 μg/g), respectively. It has been reported that the increase of aglycone forms during soybean fermentation was observed when using *Bacillus* strains and suggested that β-glucosidase is a key enzyme for the conversion of isoflavone forms in soybean fermented foods *via* deglycosylation. Wei et al. (2008) reported that aglycone isoflavones concentration in *B. subtilis* BCRC14718 fermented soybeans increased significantly after fermentation for 24 h;, conversely, glucoside isoflavone forms were decreased significantly. Likewise, Ibe et al. (2001) reported that isoflavone glucosides in soybean were hydrolysed by β-glucosidase which was produced by *B. sutilis* IF9916, and Kuo et al. (2006) indicated that daidzin and genistin glucoside were converted into aglycone isoflavone, daidzein and genistein by means of deglycosylation by β-glucosidase produced by *B. subtilis* NTU-18 during fermentation of black soybean. Hydrolysis of glucoside isoflavones was started at 8 h after inoculating with *Bacillus* culture. Also, other isoflavone conjugates that are acetylglucoside and malonylglucoside isoflavones contained in soybean might be deglycosylated into aglycone forms; therefore, in this study decreased β-glucosides, malonylglucosides, and acetylglucosides peak areas were observed in fermented soybeans HPLC chromatograms (Fig. 1). It is not only *B. subtilis* that can produce β-glucosidase during soybean fermentation; other microorganisms used for fermented soybeans such as lactic acid bacteria and Bifidobacteria with soymilk (Chun et al. 2007, 2008), *Actinomucor elegans* with *sufu* (Yin et al. 2004, 2005), *Rhizopus* with *tempeh* (Miura et al. 2002), and *Aspergillus* with *miso* (Yamabe et al. 2007) were reported in previous literature. Chien et al. (2006) suggested that β-glucosidase deglycosylation caused a significant decrease of malonylglucoside and acetylglucoside along with a significant increase of aglycone isoflavone during fermentation of soymilk with lactic acid bacteria and bifidobacteria. Malonylglucoside isoflavones are easily converted to glucosides owing to breakdown of weak bonds between sugar and malonyl group caused by heat; moreover, the effect of β-glucosidase, which hydrolyses β-glucosidic linkages of oligosaccharides and other glucosides conjugated compounds to form isoflavone aglycones.

In general, daidzein aglycone isoflavone was found as a larger proportion than genistein and glycitein in fermented soybean. Moreover, *B. subtilis* TN51 fermented soybeans contained significantly higher daidzein and genistein isoflavones than other strains ($P<0.05$), while mixed natural microorganisms fermented soybean showed the highest content of glycitein. Wei et al. (2008) indicated that the concentration of daidzein in soybean fermented with *B. subtilis* BCRC14718 was higher than genistein throughout 48 h of fermentation time. Kuo et al. (2006) demonstrated faster rate of deglycosylation in daidzin (100%) than genistin (75%) by β-glucosidase which was produced from *B. subtilis* NTU-18.

Compared with other fermented soybean products, Thai soybean variety TG145 fermented with *B. subtilis* TN51 showed lower content of total isoflavone compounds than *chungkukjang* which was produced from Korean and Chinese soybean, but exhibited higher amount of aglycone forms. Furthermore, *B. subtilis* TN51 fermented soybean also produced larger amounts of all glucoside and aglycone isoflavone compounds than *doenjang* produced from Korean and Chinese soybeans (Lee et al. 2007). This is probably due to the different soybean variety, fermentation process, strain of microorganisms, and isoflavone extraction method (Miura et al. 2002; Yin et al. 2004, 2005; Yamabe et al. 2007; Wei et al. 2008).

Conclusion

This is the first study reporting content and composition of isoflavone compounds in Thai fermented soybean (*thua nao*). The results indicated that all of fermented soybeans such as *natto*- and *thua nao*-style products contained higher aglycone compounds than unfermented cooked soybeans. Moreover, soybean fermented with *B. subtilis* TN51 showed highest amount of daidzein and genistein. Aglycones are of great interest due to their beneficial properties on human health.

Further work on fermentation improvement using *B. subtilis* TN51 as starter culture is being undertaken in which its use is expected to develop an aglycone-rich fermented soybean.

Acknowledgements

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ARTICLE

Altered stimulus frequency and intensity dependence of the somatosensory evoked potential in rats after acute application of two mitochondrial toxins

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ABSTRACT Mitochondrial toxins are a special group of toxicants with nervous system effects. The resulting nervous system damage could be detected and followed-up by means of functional biomarkers but these still have to be worked out. In this work, adult male Wistar rats were anesthetized with urethane, the left hemisphere was exposed, and a silver recording electrode was placed on the projection area of the whiskers. The whisker pad was stimulated with electric square pulses and the cortical response was recorded. The intensity of the stimulus was varied between 25% and 100% (just supramaximal), and its frequency, between 1 and 10 Hz. Control records were taken, then one of the agents (3-nitrporopionic acid, a mitochondrial toxin of microfungal origin: 20 mg/kg b.w.; or manganese, a heavy metal: 50 mg/kg b.w. in chloride form) was injected ip. and further records were taken. Both agents had an effect on the latency, but on the amplitude, only Mn. Of the relationships between stimulation settings and evoked potential parameters, frequency dependence of latency had the clearest alteration on application of Mn or 3-NP. Such effects may have the potency to be developed to functional biomarkers, applicable in practical toxicology or in animal research.
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KEY WORDS

mitochondrial toxin
electrophysiology
rat
3-nitrporopionic acid

A number of agents - environmental xenobiotics, drugs etc. - act on the human and animal nervous system. Even if the effect is seldom strong enough to cause overt symptoms, long-term exposure leads to subclinical alterations. A modern way to detect such alterations is the use of biomarkers, that is, measurements that indicate exposure to a chemical, the effect of such exposure, or susceptibility to the (usually toxic) effect of such an exposure (Hayes 2001). For neuro-functional alterations, chemical biomarkers are not ideal (Manzo et al. 1996), so the development of markers based on electrophysiological recording may be a promising field of investigation. In this study, two different neurotoxicants, both belonging to the mitochondrial toxins, were used.

Manganese is essential for living organisms in small amounts but toxic when overdosed. Exposure by excess amounts of Mn is typically occupational (metal industries, dry cell manufacturing, use of organo-Mn fungicides) but population-level exposure can also be observed, via drinking water or due to the petrol additive MMT (ATSDR 2000). Long-term exposure causes manganism (an occupational disease resembling Parkinson's disease, characterized by dopaminergic abnormalities) seen mostly in workers exposed to airborne Mn (Bowler et al. 2006), and modelled in animals (Yu et al. 2003). Tyrosine hydroxylation, a crucial step of dopamine synthesis, was blocked by Mn in vitro (Hirata et al. 2001) possibly by a mechanism depending on inhibition of mitochondrial function (Zhang et al. 2003). There are, however, not much reports on Mn effects on spontaneous or evoked cortical activity. Only a few authors found neurological (EEG and/or evoked potential) disturbances following occupational (Sinczuk-Walczak et al. 2001; Sjögren et al. 1996) or accidental (Hernandez et al. 2003) Mn exposure. In our earlier studies, behavioural and electrophysiological changes were found in rats after several weeks oral Mn exposure (Vezér et al. 2005).

The toxin 3-nitropropionic acid (3-NP) is naturally present in leguminous plants, occasionally poisoning grazing livestock (James et al. 1980). Human poisoning may come from consumption of foodstuffs infested with certain moulds (*Arthrinium* , *Aspergillus, Penicillium* spp.) producing 3-NP (Liu et al. 1989; Peraica and Domijan 2001). Most human intoxications were described in China after consumption of mould-infested sugar cane stalks (by children as a delicacy: Liu et al. 1992). The brain damage observed in the victims initiated the application of 3-NP in animal modelling of Huntington's disease (Alexi et al. 2000). Functional deficits described from experimental animals treated with 3-NP involve motility changes (Koutouzis et al. 1995) and low memory performance (Teunissen et al. 2001). Previous works from our

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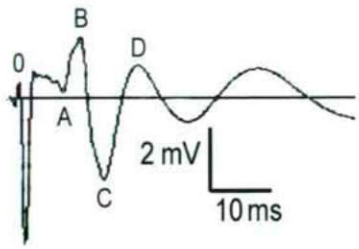

Figure 1. Measurements on the averaged somatosensory evoked potential. Onset latency was determined between 0 and A (stimulus artefact and the start of the main wave); and peak-to-peak amplitude, between B and C.

lab (Szabó et al. 2005) proved that the nervous system effects of 3-NP can be detected by electrophysiological methods.

The common point in the action of the two agents, most probably contributing to their neurotoxicity, is mitochondrial inhibition. 3-NP acts on succinate dehydrogenase (Coles et al. 1979) which is part of mitochondrial complex II. Mn inhibits both complex II (Malecki, 2001) and III (Zhang et al. 2003). The resulting energetic insufficiency may well be reflected in the electrical activity of the nervous system, similarly to what was described in mitochondrial encephalopathy (Scaioli et al. 1998) or experimental hypoxia (Van der Post et al. 2002). Based on this, and on previous experience (Szabó et al. 2003), the aim of the present work was to investigate whether the relationship of the parameters of somatosensory stimulation (intensity and frequency) and somatosensory cortical evoked potential (latency and amplitude) is altered by acute application of 3-NP or Mn.

Materials and Methods

The two toxicants were acutely given to adult (ca. 300 g) male Wistar rats ip. The rats were first anesthetized by ip. injection of urethane (1000 mg/kg b.w.), and the left hemisphere was exposed. Following recovery, a recording electrode was placed on the projection area of the whiskers in the primary somatosensory cortex. A pair of electrodes was inserted in the contralatertal whisker pad, to deliver 0.05 ms wide square electric pulses as stimuli, and cortical evoked potentials (EP) were recorded. The just-supramaximal stimulus strength (around 3 – 5 V) was determined first and taken as 100%. Then, series of 50 stimuli each were given with 25, 50, 75 and 100% strength at 1 Hz stimulation frequency, and 100% stimuli at 2, 5 and 10 Hz. This sequence was repeated 3 times with 30 min interval for control (lines a–c in Figs. 2, 3 and 4). Then, one of the test substances was injected ip. and further 4 records were taken (lines d–f in the figures). Mn was given in form of $MnCl_2$ dissolved in distilled water so that the dose for the pure metal was 50 mg/kg b.w, and the injection volume, 1 ml/kg. 3-NP was given in 20 /kg b.w. dose, also dissolved in distilled water (doses found to have acute effect:

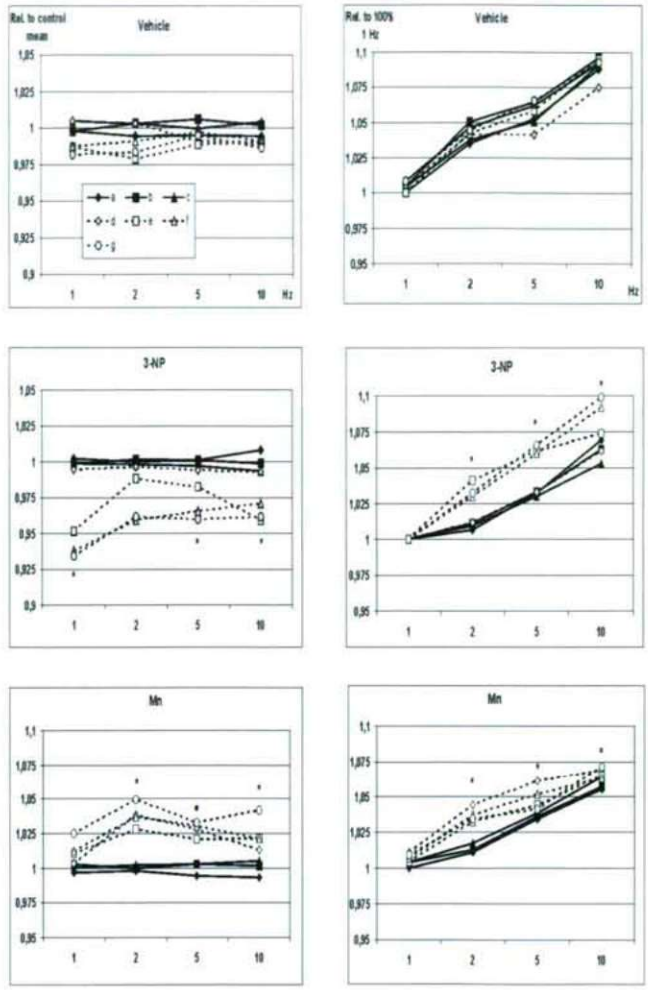

Figure 2. Dependence of the latency of the somatosensory evoked potential on the frequency of stimulation. Abscissa, frequency; ordinate, normalized latency values. Left column: normalized to the mean of the control period (first 3 series), right column: normalized to the value obtained by 1 Hz stimulation (100% intensity). Insert: line symbols for the records: a, 0 min; b, 30 min; c, 60 min (control period); d, 90 min; e, 120 min; 150 min; f, 180 min (administration period). *: $p<0.05$ after vs. before administering the agent.

Pecze et al. 2004; Szabó et al. 2005). Both substances were tested in 8 rats, and another 8 were used as vehicle-treated parallel controls. 3-NP was obtained from Sigma-Aldrich, and $MnCl_2$, from Reanal.

After the last recording, the rats were sacrificed by an overdose of urethane. The principles of the Ethical Committee for the Protection of Animals in Research of the University were strictly followed throughout.

The recorded EPs were averaged, and their onset latency and peak-to-peak amplitude was measured (Fig. 1). Recording, storage and analysis of the EPs was done by the Neurosys 1.11 software (Experimetria, Budapest). To eliminate individual variation, first of all in the EP amplitudes, the

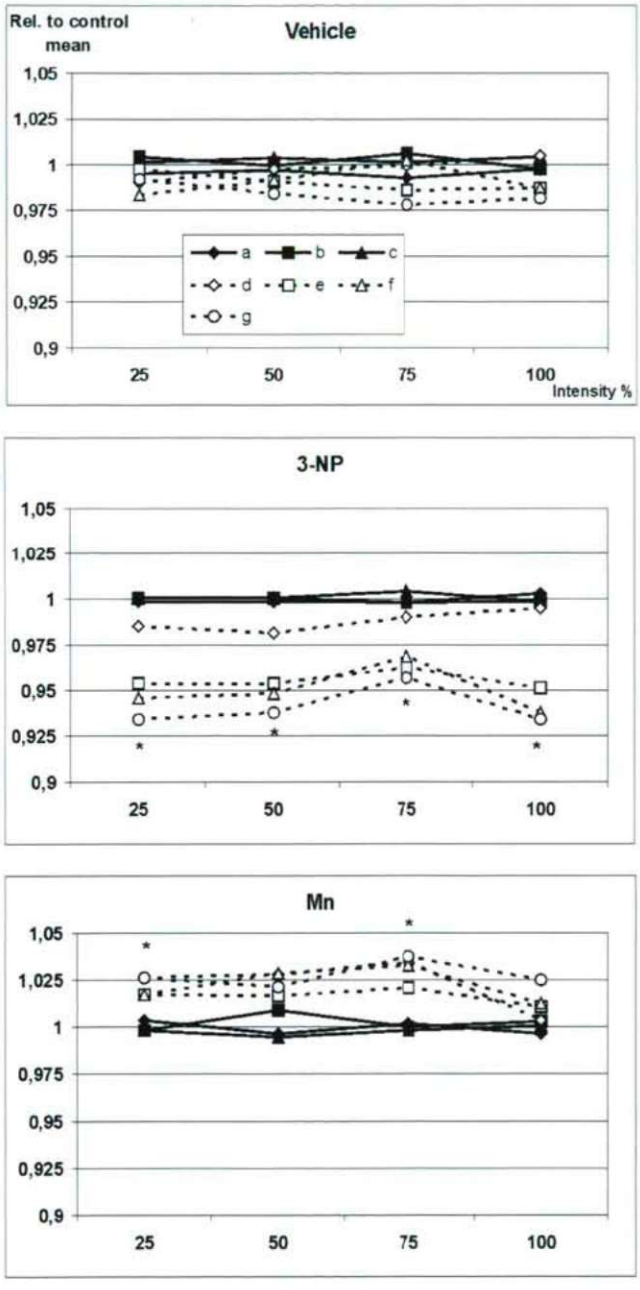

Figure 3. Dependence of the latency of the somatosensory evoked potential on the intensity of stimulation. Abscissa, intensity; ordinate, latency values normalized to the mean of the control period (first 3 series). *: p<0.05 after vs. before administering the agent.

measured values were normalized. The reference base was either the mean of the control period (first 3 series), separately for each stimulus setting. The other way was to normalize to the value obtained by 1 Hz stimulation (100% intensity) in the same series. These normalized data were plotted against the intensity (25 to 100%) or the frequency (1 to 10 Hz) of stimulation and any difference between the resulting plots

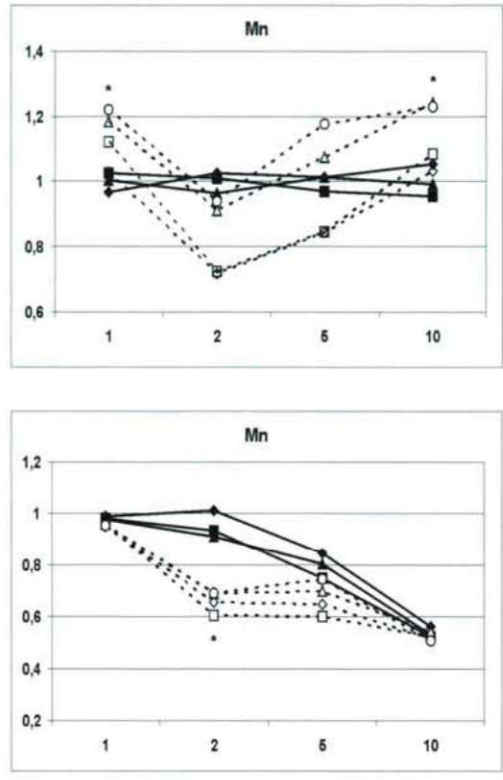

Figure 4. Dependence of the amplitude of the of the somatosensory evoked potential on the frequency of stimulation. Abscissa, frequency; ordinate, amplitude values normalized to the mean of the control period (top), and to the value obtained by 1 Hz stimulation (bottom). *: p<0.05 after vs. before administering the agent.

before vs. after administration of the toxicant was sought for. The significance of before-after differences was tested by two-sample t-test.

Results

In the parallel control (vehicle treated) animals, and in the treated animals in the control period, the latency and amplitude data normalized to control mean and plotted against frequency or intensity of stimulation gave a more or less horizontal line around one (Fig. 2 top left); while after normalizing to 1 Hz stimulation, an oblique relationship was seen (Fig. 2 top right).

EP latency was altered by both agents tested. Compared to control mean, 3-NP caused a shortening of latency in the treated animals (Fig. 2 mid left), which developed with some delay (line d not different from lines a–c) and reached full size at 10 Hz earlier (line e) than at lower stimulus rates. When normalized to 1 Hz stimulation, a steeper frequency-dependent increase of the latency was seen (Fig. 1 mid right). The effect of Mn was lengthening which appeared faster than the effect of 3-NP (Fig. 2 bottom left). The frequency dependence became somewhat steeper also here (Fig. 2 bottom right).

Shortening of EP latency on 3-NP treatment, and lengthening on Mn, was also seen when values recorded at different stimulus strengths were plotted (Fig. 3). On the basis of control records, the changes were similar to those seen as a function of frequency. The intensity dependence itself was, however, not significantly altered (not shown).

On the EP amplitude, only Mn had significant effect (that of 3-NP was observable but less characteristic). Related to control mean, the amplitude gradually increased at 1 and 10 Hz, at 2 Hz and partly 5 Hz, first a clear decrease was seen which changed to an increase later (Fig. 4 top). When normalized to 1 Hz stimulation, the decrease of amplitude after injection of Mn was steeper between 1 and 2 Hz but not at higher frequencies.

Discussion

The changes in the latency and amplitude of the EPs obtained by electrical stimulation of the whisker pad were similar to those observed in earlier works of the Department (Pecze et al. 2004; Szabó et al. 2005) which confirmed also that such acute experiments with short time span are suitable for examining neurotoxic effects. Of especial importance is in this context the stability of the measured and calculated parameters in the parallel control animals and during the control periods, indicating that urethane anaesthesia itself was not responsible for the observed effects.

First of all the shortened EP latency in the 3-NP treated animals suggested that the mechanism responsible is specific, beyond the energetic crisis caused by mitochondrial inhibition. Cortical EPs are due to specific afferent pathways working with glutamatergic excitation. 3-NP is known to inhibit the glial uptake of glutamate (Tavares et al. 2001) resulting in more intense cortical response. 3-NP also affects GABAergic transmission (Erecinska and Nelson 1994) which can contribute to the mentioned effect.

Why the effect of Mn, another mitochondrial toxin, on the latency was dissimilar, was probably due to its other effects. On the one hand, Mn inhibits both the glial uptake (Hazell and Norenberg 1997) and the breakdown of glutamate (Normandin and Hazell 2002); on the other hand, Mn^{2+} ions are inorganic Ca-channel blockers (Büsselberg, 1995), also in cortical neurons (Pumain et al. 1987) – the final effect of which can be slower but higher EPs.

Of all relationships tested, the frequency dependence of the EP latency seemed to be the best choice in terms of a possible biomarker. All the more so, because it is known that natural stimulation of the whiskers at 1 or 10 Hz frequency induces qualitatively different cortical activation (Moore 2004). Practical application in health protection is more likely in case of Mn, where the development of neurological consequences of occupational Mn exposure can possibly be followed-up by non-invasive functional tests. For other metals, the sensitivity of such tests has been already published (lead – Bleecker et al. 2003; mercury – Chang et al. 1995; Lamm and Pratt 1985).

In case of 3-NP, application in the animal model of Huntington's disease is more likely, to check the development of the damage serving as model.

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ARTICLE

The correlation between parameters indicating obesity and certain environmental factors

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ABSTRACT The present study deals with the effects of socio-economic and lifestyle factors on the nutritional status of young adults. 627 university students from the University of Szeged, Hungary participated voluntarily in the survey. In order to illustrate their nutritional status, we determined the body mass index (BMI), the waist circumference (WC) and the waist-to-hip ratio (WHR). According to the BMI, 18.4% of the students are somewhat overweight, 64.8% among them show signs of abdominal fat distribution. Based on the aftereffects of the logistic regression, the most important factors influencing the nutritional status include the parents' level of education, the meals consumed and the frequency of sweets consumption. Among the parameters pointing to obesity, the BMI is the most precise indicator of the external environmental impacts.

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KEY WORDS

body mass index
waist circumference
waist-to-hip ratio
university students
lifestyle factors

Nowadays, obesity is one of the most important health problems. There are about 315 million adults whose body mass index exceeds the limit of the obese category, determined by the WHO (Caterson and Gill 2002). The long-term disorder of the energy balance results in the critical accumulation of adipose tissue, which will eventually lead to overweight and obesity. Furthermore, this will increase the risk of the development of a number of life threatening diseases. In the European Union, most of the death cases are caused by diseases related to obesity, such as cardiovascular diseases, cerebrovascular diseases, diabetes mellitus, cancerous diseases and chronic liver diseases (Elmadfa and Weichselbaum 2004). In addition to healthcare problems, obesity is also a serious financial burden both for the society and on the shoulders of the overweight individual (Gyenis and Joubert 2005).

Today, we have a great number of methods to define accurately the quantity and the distribution of body fat. Underwater weighing (densitometry), multi-frequency bioelectrical impedance analysis and magnetic resonance imaging are to be found among the best-known methods. However, these methods are relatively expensive and their realization is rather difficult when there are so many people involved. In epidemiological and clinical examinations with such large case numbers, the thickness of the skin fold and / or the body mass index (BMI) are primarily determined in order that the scale of obesity can be estimated, because apart from being simple, they also show good correlation with the quantity of the body fat (Dehghan et al. 2005; Chakraborty et al. 2009). Abdominal fatness, as the cause of visceral fat accumulation, is an intensified risk factor of metabolic (such as the diabetes mellitus (type 2), hypertension, dyslipidaemia) and cardiovascular diseases. The distribution of the body fat can easily be estimated through measuring the waist circumference (WC) and calculating the waist-to-hip ratio (WHR), (Tanyolaç et al. 2007).

Although the molecular biological background of obesity is a highly researched field (Rankinen et al. 2006), the basic causes of its epidemiological spread are associated with the exceptional changes in the environmental and lifestyle factors (physical inactivity, excessive calorie intake, bad nutritional habits, urbanization, motorization), (Martínez-González et al. 1999; Bellisle et al. 2004; Dehghan et al. 2005). The purpose of our study is the examination of the correlation between the 3 parameters indicating obesity (BMI, WC, WHR) and certain socio-economic and lifestyle factors in one selected layer of Hungarian young adults.

Materials and Methods

The data to be analyzed were collected among the students of the University of Szeged, from March through April, 2007. We measured and put down four anthropometric characteristic features – the body height, the body weight, the waist circumference and the hip circumference – of a total of 627 students (190 male and 437 female individuals). Weight was measured to the nearest 50 gram on a medical scale, height was measured in millimeters with an anthropometer and the waist circumference (WC) and hip circumference (HC) were taken with an anthropological measuring tape. Measurement of waist circumference was performed midway between the lateral lower ribs and the iliac crests while the subject

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Table 1. Parameters of body measurements and indices of the university students.

Subjects	Boys			Girls		
	n	Mean	SD	n	Mean	SD
Body height**	190	178,9	7,20	437	164,9	6,36
Body weight**	190	75,4	13,60	437	59,8	10,26
BMI**	190	23,5	3,70	437	22,0	3,53
Waist circumference**	190	81,6	9,26	437	70,4	7,65
Hip circumference**	190	88,6	8,87	437	86,2	8,61
Waist-to-hip ratio**	190	0,9	0,05	437	0,8	0,06

** Statistical significance at $p < 0.01$ level.

Table 2. Distribution of university students according to BMI categories.

Subjects	Boys		Girls		Total	
	n	%	n	%	n	%
Underweight	12	6,3	41	9,4	53	8,5
Normal	123	64,7	336	76,9	495	73,2
Overweight**	45	23,7	45	10,3	90	14,4
Obese**	10	5,3	15	3,4	25	4

** Statistical significance at $p < 0.01$ level.

Table 3. Distribution of universty students by the WC and WHR groups.

Subjects	Boys		Girls		Total	
	n	%	n	%	n	%
No health risk	171	90	396	90,6	567	90,4
Health risk	19	10	41	9,4	60	9,6
Abdominal fat distribution*	136	71,6	270	61,8	406	64,8
Peripheral fat distribution	54	28,4	167	38,5	221	35,2

* Statistical significance at $p < 0.05$ levels.

was standing, after a moderate expiration. We measured the circumference of the hip as the grid lined on the anterior iliac spine on the abdomen running all around the body in a horizontal position.

In order to estimate overweight, obesity and other health risks, we calculated the body mass index (BMI) and waist-to-hip ratio (WHR). For the categorization of the BMI and WC values, we took into consideration the recommendations of the World Health Organization (WHO), (James et al. 2001), while in the case of the waist-to-hip ratio, we used the limits of 0.8 for women and 0.9 for men (Rodé 1998) to separate abdominal fat distribution from peripheral fat distribution. During the survey, we used questionnaires to gather the required information about the students' lifestyle and socio-economic background.

We carried out the statistical analysis of the measured data using SPSS for Windows. In order to examine the differences between the averages and frequencies occurring in the particular groups, we referred to ANOVA and Chi-square test. In the first step of the logistic regression all variables were included in the analysis. Afterwards, the "Forward Stepwise" method was used which included in the analysis only variables which had a significant influence on the dependent variables. The two categories of the first dependent variable are the following: BMI 25 (0) and BMI>25 (1). The categories of the second dependent variable are as specified here: based on the waist circumference there is some health risk (1), there is no health risk (0). The third dependent variable shows either peripheral fat distribution (0) or abdominal fat distribution (1). We worked with a 5% significance level throughout the analyses.

Results

Table 1. displays the mean and standard deviation values of body height, body weight, BMI, waist circumference, hip circumference and WHR, in accordance with gender distinction. In the case of male students, the mean values were always significantly higher than those measured for female students ($p<0.0001$). The most significant standard deviation occurred in connection with body weight, which proves very well that extremes could also be found in the sample material ($min_{males}=44.6$; $max_{males}=131.9$). Both the average of the male students and that of the female students lie within boundaries of the normal category, as determined by the WHO. In neither gender group do the averages of waist circumference exceed the limit indicating health risk. On the other hand, the analyzed samples show that, according to the average of the waist-to-hip ratio, the individuals in both gender groups bear higher health risks as they fall into the category of abdominal fat distribution.

In accordance with the BMI categories, the students' proportional distribution figures in Table 2. In the case of male students, the frequency of those being overweight is much higher (29%) than in the case of female students (13.7%), thus the difference is statistically significant ($p<0.0001$). On

Table 4. Results of logistic regression analyses.

Variables	Based on the BMI		Based on the WC		Based on theWHR	
	Coefficient	p	Coefficient	p	Coefficient	p
Size of habitation (>100.000 inhabitants)		0,288		0,208		0,554
<10.000 inhabitants	0,085	0,780	-0,485	0,203	0,290	0,223
10.000 – 50.000 inhabitants	-0,093	0,741	-0,564	0,115	0,245	0,259
50.000 – 100.000 inhabitants	-1,034	0,078	-1,320	0,094	0,327	0,360
Educational level of father (high level)		0,101		0,943		0,035*
Unfinished elementary	2,696	0,031	-19,471	0,999	-0,628	0,497
Elementary	0,574	0,418	0,132	0,890	0,383	0,505
Medium level	-0,127	0,656	-0,194	0,594	0,482	0,006**
Educational level of mother (high level)		0,009**		0,120		0,663
Unfinished elementary	-19,502	1,000	0,852	1,000	-21,662	1,000
Elementary	1,554	0,001**	1,526	0,027	0,617	0,282
Medium level	0,389	0,048*	0,654	0,074	0,194	0,361
Sports activity (every day)		0,332		0,795		0,104
Several times/week	0,908	0,259	-0,475	0,579	0,209	0,683
1-2 occasion/week	0,440	0,579	-0,367	0,657	-0,299	0,541
No sport activity	0,438	0,580	-0,125	0,877	-0,410	0,399
Frequency of daily eating (more than three times)		0,000***		0,001**		0,897
Once a day	-19,163	0,999	-17,795	0,999	-0,182	0,891
Two times	1,591	0,000***	2,191	0,000***	0,149	0,644
Three times	0,574	0,067	1,337	0,007**	0,011	0,960
Randomly	0,346	0,341	0,882	0,116	-0,169	0,520
Time of the main meal of the day (around noon)		0,796		0,972		0,601
In the morning	-0,676	0,410	0,362	0,663	-0,648	0,244
In the afternoon	-0,027	0,940	-0,079	0,866	-0,106	0,701
Randomly	0,146	0,625	0,030	0,940	0,109	0,641
Frequency of sweets consumption (daily)		0,004**		0,079		0,042*
2-3 occasions/week	0,598	0,035*	0,698	0,077	0,033	0,866
Every week	0,915	0,004**	1,199	0,005	0,777	0,004**
Rarely	1,271	0,001**	0,982	0,065	0,110	0,727
Never	-0,454	0,675	0,340	0,768	0,037	0,950
Frequency of fruits consumption (daily)		0,369		0,995		0,643
2-3 occasions/week	-0,341	0,181	0,036	0,912	-0,197	0,322
Every week	-0,701	0,063	-0,163	0,724	-0,197	0,478
Rarely	-0,213	0,773	-19,007	0,998	-0,742	0,177
Never	0,421	0,753	-19,291	0,999	-21,469	0,999

Calculations referred to category where lowest prevalence of overweight and obesity in students is expected, i.e. always to category of the variable in parentheses.
* Statistical significance at $p < 0.05$ levels. ** Statistical significance at $p < 0.01$ level.

he other hand, in the category of underweight students, the proportion of female students is higher, although the difference is not significant (p=0.205).

Table 3. displays the existence of health risk estimated on the basis of the waist circumference, as well as the type of fat distribution. Based on the waist circumference no health risk could be seen in 90.4% of the students we examined, while in 10% of the male students and 9.3% of the female students it could be detected. The difference between the genders is not significant (p=0.320). As far as the waist-to-hip ratio is concerned, in case of both genders, the abdominal type of fat distribution occurred with significantly higher frequency (p=0.018), (males 71.6%, females 61.8%).

The results of the logistic regression are summarized in Table 4. The mothers' education levels have a significant effect of their children's overweight and obesity determined by the BMI. The risk of overweight is higher in the case of the children whose mothers have a lower level of schooling. Ac-

Table 5. Odds ratio and confidence interval of statistically significant risk factors by BMI, WC and WHR.

	BMI		WC		WHR	
	OR	95% CI for OR	OR	95% CI for OR	OR	95% CI for OR
High educational level of mother	4,732	1,889 – 11,857				
Medium educational level of mother	1,476	0,944 – 2,307				
Medium educational level of father					1,620	1,149 – 2,283
Frequency of daily eating: two times	4,909	2,381 – 10,121	8,948	3,128 – 25,601		
Frequency of daily eating: three times			3,808	1,442 – 10,055		
Frequency of sweets consumption: 2-3 occasions/week	1,818	1,042 – 3,172				
Frequency of sweets consumption: Every week	2,497	1,340 – 4,655			2,175	1,286 – 3,679
Frequency of sweets consumption: Rarely	3,566	1,720 – 7,394				

OR-Odds ratio; CI-Confidence interval

cording to our research results, there is a significant correlation between the frequency of daily meals and the prevalence of overweight. Finally, the frequency of sweets consumption also affects the emergence of the risk of overweight (and thereby of the BMI). As for the other independent variables, (the father's level of education, the size of the habitation, the frequency of regular sporting, the time of the main meal, the frequency of fruit and vegetable consumption) the model did not depict any significant effect. We examined the effects of the same independent variables on the existence of health risk estimated on the basis of the waist circumference, as well as on the type of fat distribution. As far as the waist circumference is concerned, only the frequency of the daily meals proved significant, on the other hand, as for the type of fat distribution, the frequency of sweets consumption and the fathers' education levels showed significant effects.

Table 5. presents the values of the odds ratios (OD) and their confidence interval (CI) of those factors witch were included by the Forward Stepwise method in the logistic regression as being significantt. The disposition to overweight according to the BMI is almost five times higher (4.73) when the mothers' level of schooling is only elementary, while it is just one and a half times higher (1.48) when the mothers' education is at a medium level. The consumption of two daily meals almost quintuples (4.9) the risk of overweight. Those who rarely consume sweets have a higher inclination to overweight, which drops progressively with the increase of the frequency of sweets consumption.

Taking in consideration the waist circumference, we can state that the disposition for passing over the limit of health risk is nine times (8.95) higher among those who eat twice a day. As for the waist-to-hip ratio, the children of fathers with a medium level of education are 1.6 times more inclined to abdominal fat distribution than the children whose fathers possess degrees of higher education. Those who do not consume sweets regularly every day have a 2.12 times higher inclination to abdominal fat distribution than the ones whose sweets consumption is on a daily basis.

Discussion

The students' BMI means (male students – 23.5 and female students – 22) fall into the normal category as determined by the WHO. Our results are similar to those reached by Kiss et al. (2008, 2009) during their examinations carried out at Semmelweis University. According to the BMI, the proportion of the overweight students shows a frequency of 18.4%, which includes 29% of the male students and 13.7% of the female students. Antal et al. (2006) involved 264 Budapest university students into their research. They depicted obesity in case of 27% of the male students and 11.3% of the female students; these data correspond with our results. The BMI means in the examined samples and the frequency of the overweight are proportional to the scaling up tendency shown by Hungarian university students that Gyenis (1994) described earlier.

The prevalence of obesity among the adult population of Hungary is rather high: 41.8% of men are overweight and 17.1% of them are obese, while the proportion of overweight women is 31.3%, with 18.2% of obese women (Rodler et al. 2005). Several factors may stand in the background of the fact that in the case of the university students we examined the proportions of overweight and obesity were lower than the national averages. This is partially due to the university students' younger age and higher levels of education and intellectual accomplishment (Halkjær et al. 2003). On the other hand, our personal experiences reveal that more corpulent students simply refused to take part in the survey.

On the basis of the WHR, the proportion of abdominal fat distribution is relatively high in our sample (64.8%), however, according to the WC, the number of students revealing health risk due to abdominal fat accumulation is very low, concerning only 9.6%.

According to our findings, the three parameters indicating overweight and obesity estimate very differently the extent and risks of obesity. The difference between the frequencies defined by WC and BMI is double, whereas WHR reveals that the abdominal fat distribution risk factor is 6.75 times higher in our sample compared to the risk category determined by WC. Although none of the above markers is sufficient enough on its own to determine the amount of total body fat, a great number of studies report that the WC is by far a better indicator than the WHR of the accumulation of body fat and the risks of cardiovascular diseases (Taylor et al. 2000; Dobbelsteyn et al. 2001; Katzmarzyk et al. 2004; Picon et al. 2007).

Among the socio-economic and lifestyle factors we studied, the parents' level of education and some of the nutritional habits (number of the daily meals, frequency of sweets consumption) had statistically proven effects on obesity. In the case of both parents, the lower their level of education is, the higher risks their children have for overweight and the development of abdominal fat distribution. These findings agree with the results of the examinations carried out by Gyenis (1994) and Cho et al. (2009). Unhealthy, irregular eating habits also correspond to the rising prevalence of obesity (Panagiotakos et al. 2008; Berg et al. 2009; Prochnik Estima et al 2009). According to our results, taking a meal twice a day increases considerably the risks of overweight and health problems estimated by WC. People having a day several meals with small portions, as suggested by the principles of the healthy diet, will be less inclined to become overweight or obese. In the background of our findings about sweets consumption there stands the probable fact that students having smaller body weight do not have to worry about overweight, thus the frequency of their sweets consumption does not get influenced.

Our study seems to reveal that among the parameters indicating obesity BMI is the most sensitive to environmental effects, because this showed the most significant correlation with the independent variables. Obesity is also in correlation with several categories of the mothers' level of education, the number of the daily meals and the frequency of sweets consumption.

On the other hand, WC only bore the influence of the number of the daily meals, and WHR was just significantly affected by one category of the father's level of education and one category of the frequency of sweets consumption. Furthermore, even in the case of the regression models, the combination of the independent variables explained the biggest part (18.4%) from the BMI variance, as opposed to the variances of the WC (15.9%) and the WHR (9%). Consequently, the BMI – despite its frequent criticism – is a useful means to explore the exterior environmental factors that may be mentioned in connection with obesity.

In the layer of society university students represent, people do not take part in regular health screening examinations and their daily overload and irregular way of life present a lot of healthcare risks. The number of studies dealing with them is relatively low. Consequently, we should give much higher importance to such research involving university students as well as the extensive information and coverage on the basis of the acquired results.

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ARTICLE

The Paleopathology of specific infectious diseases from Southeastern Hungary: a brief overview

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ABSTRACT The purpose of this study is to review the evidence for the presence of specific infectious diseases in past Hungarian populations. As for treponemal diseases, only few paleopathological cases had been published until relatively recently. New discoveries from the medieval Szeged furnished evidences for the Pre-Columbian occurrence of the disease in this area. Among mycobacterial infections, paleopathological analyses of thousands of skeletons provided a relatively high number of observations of 'classical' skeletal tuberculosis (TB) cases, and some cases of leprosy until the end of the 1990's. The use of DNA assays and the study of early stage traces of mycobacterial skeletal infections highly increased the number of observations during the last ten years. Unfortunately, these results present several biases of the classical osteoarcheological studies, such as the differentiation between the taphonomic and paleodemographic conditions of the series. The evolution of the paleopathological diagnostical methods necessitates the complete re-evaluation of the previously studied materials in order that we can obtain a more realistic paleoepidemiological picture of these diseases
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KEY WORDS

paleopathology
paleoepidemiology
specific infectious diseases
treponematosis
tuberculosis, leprosy

In Hungary, two important and several smaller physical anthropological collections can be used for paleopathological studies. The biggest and best-equipped collection belongs to the Department of Anthropology of the National Museum of Natural History (about 40 thousand skeletons), in Budapest. Another big collection of more then 30 thousand skeletons is disposable for the anthropological and paleopathological research at the University of Szeged.

The Szeged human skeletal material comes from archeological excavations in Southern and Eastern Hungary and represents a large chronological period from the Neolithic Period to Modern Ages. The richest part of this collection is from the Avar Age (6-8th centuries), the Hungarian Conquest Period (10-11th centuries) and the Arpadian Age (11-13th centuries). Actually, our material is stored in 9 premises of 4 distant buildings – we are working in cooperation with the National Museum of Natural History and the Museum of Szeged, to develop a common osteological centre in order to improve storage and research conditions.

Paleopathological research of specific infectious diseases has been one of the main activities of the Szeged team of anthropologists since the 1970's. This activity became more intensive in the 90's when among a large number of ancient TB cases some cases of leprous and treponemal infections were also identified (e.g. Maczel 2004; Marcsik 1972, 1994; Marcsik et al. 1994, 2007; Molnár and Pálfi 1994; Molnár et al. 1998, 2005; Pálfi 1991, 2002; Pálfi et al. 1997, 1999, 2002). In the field of paleomicrobiology, publications represent very successful interdisciplinary cooperation (e.g. Haas et al. 1999, 2000a-b; Donoghue et al. 2005, 2009; Zink et al. 2007).

This interest in specific infectious diseases led us to co-organize 3 parts of the ICEPID series (International Congresses on the Evolution and Paleoepidemiology of Infectious Diseases). We certainly do not want to forget the importance of the ICEPID series, thus we are to organize a second international TB conference in Hungary in 2011 (Szeged and Budapest).

New cases of infectious paleopathology

The recent expansion of the archaeological activity in the Szeged region (new highway excavations, excavations among the ruins of the medieval castle of Szeged, etc.) furnished a lot of new paleopathological cases during the past couple of years. At the same time, the development of the diagnostical methods and the results of the latest cooperation called forth new observations in infectious paleopathology.

The paleopathological evidences of treponemal infections have already been proven from Post-Columbian South-eastern Hungarian archaeological context (e.g. Marcsik 1994; Pálfi et al. 1997; Molnár et al. 1998). Several new treponemal cases have been discovered recently in the anthropological

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Figure 1. Early stage rhinomaxillary changes in leprosy: left maxilla showing periostitis on the nasal surface (Püspökladány, 10-11th centuries AD, Grave No 503, Adult Female).

Figure 2. Facies leprosa: erosion of nasal margins and loss of anterior nasal spine (Szeged-Kiskundorozsma, 7th century AD, Grave No 271, Mature Male).

material from the medieval Szeged Castle excavations (Ősz et al. 2006, 2009a). In these cases, the macro-morphological diagnosis was backed up by paleoradiological and paleohistological analyses; furthermore, the Pre-Columbian origin was suggested both by archaeological and radiocarbon dating (see more details in the present volume of Acta Biologica Szegediensis, Ősz et al. 2009b).

The paleopathological study of mycobacterial diseases (tuberculosis and leprosy) has furnished several important new results from the Szeged Collection. Among others, we have to mention the 2005-2006 results by Donoghue and collaborators: two of the examined Hungarian cases from the Püspökladány series (10th-11th centuries AD) with typical 'early stage' manifestations of *facies leprosa*, included in a larger paleomicrobiological study, were positive both for *Mycobacterium leprae* and *Mycobacterium tuberculosis*. We are presenting here one of these two cases with nasal periostitis (Fig. 1) – as early manifestations of leprous rhinomaxillary changes. Both cases were co-infected by leprosy and tuberculosis (Donoghue et al. 2005, 2009). These cases have been included recently to a phylogeographic analysis of *Mycobacterium leprae* (Monot et al. 2009).

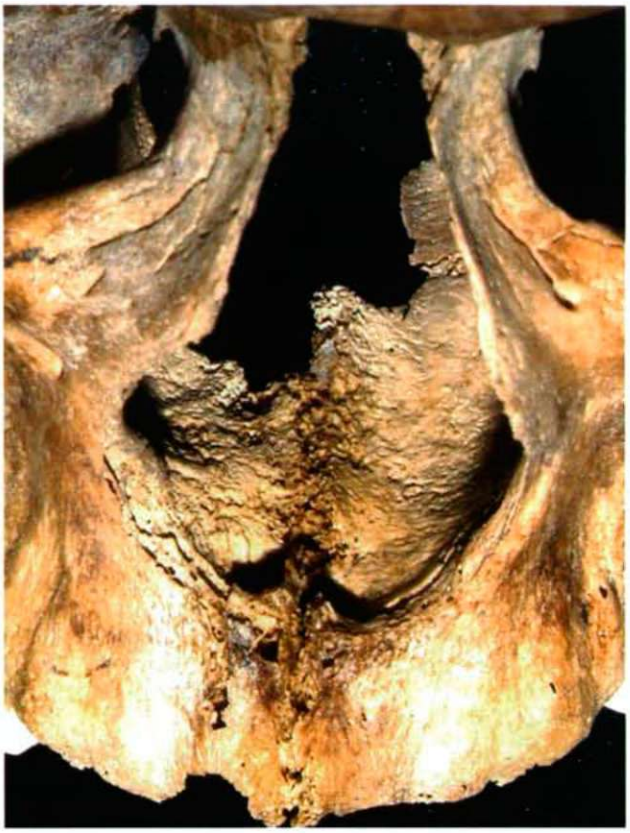

Figure 3. Rhinomaxillary changes in leprosy (Szeged-Kiskundorozsma, 7th century AD, Grave No 517, Adult Male).

The 7th century Avar-Age series of Kiskundorozsma (Szeged) represents a remarkable material for the study of past mycobacterial infections (Molnár et al., 2006). In this small osteoarchaeological sample, 8 of the 94 skeletons revealed traces of the different stages of leprous infection (Fig. 2,3). Paleomicrobiological studies confirmed the *Mycobacterium leprae* infection (Donoghue et al. 2009). These cases permitted to 'move back' in the history of leprosy in the given geographical area: before these finds, the previously discovered earliest Hungarian leprosy cases had been dated to the 10th century (Pálfi et al. 2002; Marcsik et al. 2007, 2009).

Hereinafter, we would like to mention a case of probable leprosy, discovered recently in the Szeged Anthropological Collections. The isolated skull of an adult female specimen

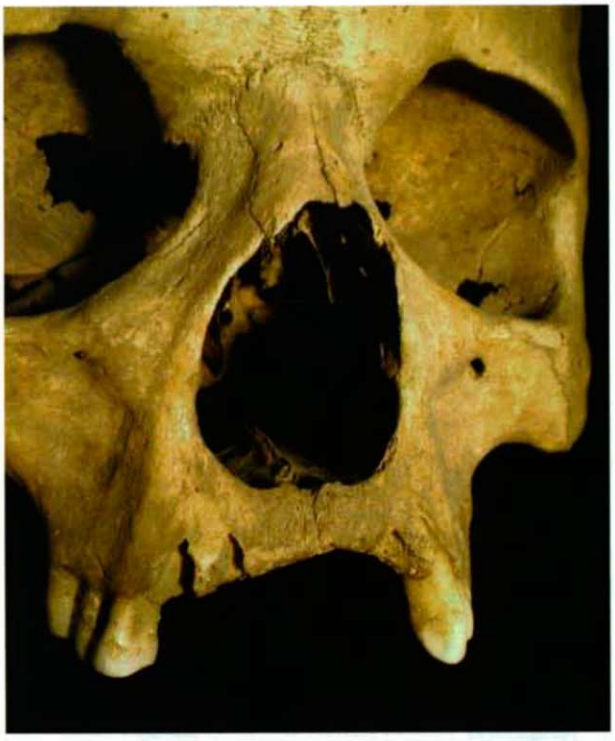

Figure 4. Facies leprosa: rhinomaxillary changes (Szentes-Kistőke, 4-7th century AD, Grave No 11, Adult Female).

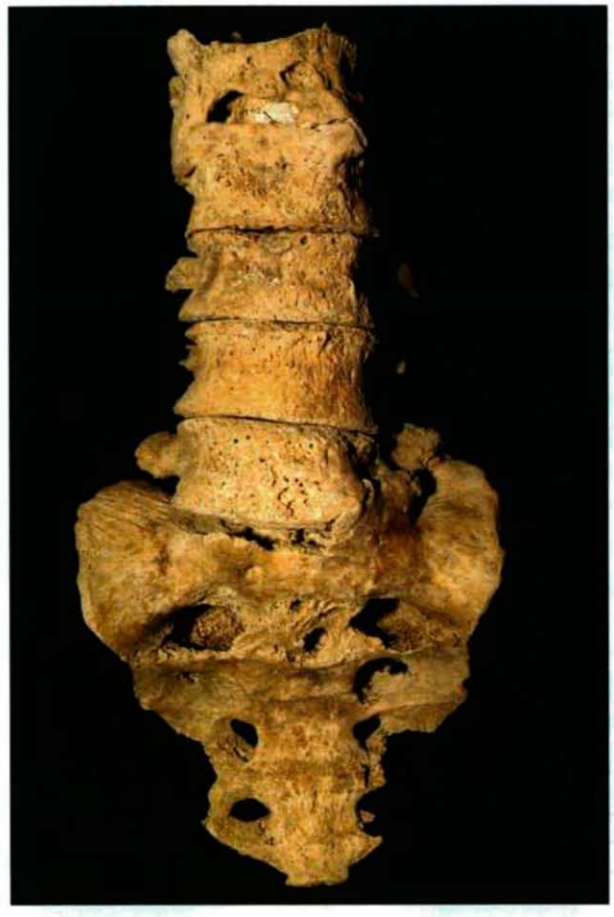

Figure 5. Multifocal spinal TB: lytic lesions, new bone formations, vertebral fusions, traces of cold abscess (Szeged-Kiskundorozsma, 7th century AD, Grave No 176, Senium Male).

(Fig. 4) comes from an archaeological excavation from the first half of the 20th century and has been dated to the relatively large 'Migration Period' (Szentes-Kistőke series, around 4th-7th centuries AD). The morphological aspects of the rhinomaxillary changes are characteristic of a *facies leprosa*. Unfortunately, we do not possess any postcranial bones. Complementary studies and a more precise dating should be necessary. At this moment, we can only conclude that this is a potential early-medieval leprosy case, which may be anterior to the oldest Avar Age cases in Hungary.

Compared to the dozen of new leprosy cases and to the half a dozen of treponemal cases, the number of new TB cases is much higher, especially if we consider both the chronic and the early forms of its manifestations. The above mentioned 7th century Kiskundorozsma series, very rich in leprous cases, furnished a very interesting multifocal spinal TB case too (Fig. 5, 6).

Following the chronology of the archaeological excavations, the paleopathological study of the 16-17th century Bácsalmás-Óalmás anthropological series was carried out in several steps. The first part of the series have already presented a high prevalence of early stage TB cases (e.g. Molnár and Pálfi 1994; Pálfi and Ardagna 2002; Maczel 2003; Fig. 7, 8). Ancient DNA analysis by Haas and co-workers had proved the relationship between the frequent early stage alterations and the TB infection (Haas et al 1999, 2000a). The study of the second part of the series – which was excavated later - is still in progress. The low number of the chronic forms and the high prevalence of early stage cases are to be mentioned. The morphological diagnosis of the Bácsalmás early stage TB cases was partially based on our previous research work, which is still going on, in the Terry Anatomical Collection in Washington DC.

The paleopathological study, still in progress, of the Late Neolithic series of Hódmezővásárhely-Gorzsa (4970 to 4594 BC) furnished several cases of infectious diseases, among them traces of possible TB infections. These cases might be the oldest known TB cases from Hungary – however, complementary biomolecular studies should be necessary (Masson et al 2009).

Some paleoepidemiological conclusions

The intensive study of the paleopathology of specific infectious diseases increased the number of the diagnosed ancient

Figure 6. Vertebral fusion in spinal TB (Szeged-Kiskundorozsma, 7th century AD, Grave No 176, Senium Male).

Figure 7. Early stage TB: periosteal lesions on the visceral surface on the 9th right rib (Bácsalmás-Óalmás, Grave No 61, Juvenile Male).

cases in our collections. As for treponematosis, due to the new discoveries from Szeged, its Pre-Columbian presence in Central Europe is not a question any more. Unfortunately, the low number of cases excludes all attempts of epidemiological reconstruction.

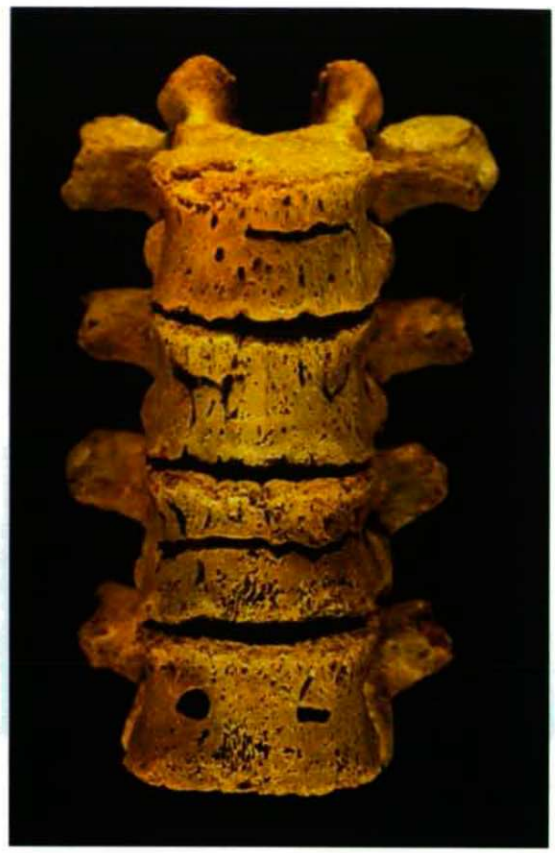

Figure 8. Early stage TB: hypervascularisation and resorptive lesions on the ventral bodies of thoracic vertebrae periosteal (Bácsalmás-Óalmás, Grave No 115, Juvenile Male).

The Hungarian part of the history of leprosy is partially redrawn – or rewritten – based on the new discoveries of the past few years. Leprosy co-existed with tuberculosis in our ancient populations and must have reached the Carpathian Basin before the Ancient Hungarians. This chronic disease was present among the Avars of the 7th century, or may even have been present among other peoples before the Avars during the Migration Period. However, in spite of the higher number of proven cases, this quantity is still insufficient for a serious paleoepidemiological reconstruction.

As for skeletal tuberculosis, the past few years have furnished a great number of data, and a lot of cases have been confirmed by paleomicrobiology. A new tendency is about to emerge: the so-called early stage forms are more frequent in the more recent periods. However, the limits of the osteoarcheological analysis do not allow us to formulate more precise hypotheses. We presented our first attempt at TB paleoepidemiology at the 1994 Copenhagen meeting of the European Anthropological Association (Marcsik et al 1994). In this work, only 11 classical cases were reported from the great number of 3.400 examined skeletons. In 1999, we tried to complete and re-summarize these results (Pálfi and Marc-

sik 1999). More than 5 thousand skeletons were considered from a period of a thousand years between the 7th and 17th centuries. As the evolution of the methodology permitted to recognize more and more cases – a total of 31 TB cases were identified -, the 'virtual prevalence' became higher than in 1994. Finally, Marcsik and co-workers tried to complete this table by another one containing more than 5 thousand skeletons studied between 1999 and 2006 (Marcsik et al 2007). Classical cases and DNA-confirmed early stage cases were considered during that period. Today, we have a lot of data about TB paleopathology in Hungary. However, we need to be extremely critical about our own results. The development of the methodology has continuously been modifying the results about the evolution of the disease.

Some biases

Our results about ancient TB-prevalence have presented certain problems related to the changes of diagnostic criteria, but also to other biases of paleopathological studies. Several of the examined series come from cemeteries of very long periods of occupation. For this reason, if the dating cannot be made more precise, we had better drop some series from the comparative studies. Excavation-related differences in the demographical structures of the series can also be problematic, as in all types of comparative osteoarcheological studies.

As for the biases related to paleoepidemiological studies of specific infectious diseases, solving the problems of 'methodological changes' should be the first task.

We need to establish specific temporary diagnostical packages for each specific infectious disease in order to find out which cases can and which cases cannot be kept in our statistics. Afterwards, we will always have to use the same criteria. When the evolution of the methods reaches an important new step, we must stop and make the necessary changes. And when we start using a new method, unfortunately, we also have to re-evaluate our previous studies.

The last and perhaps also the most problematic element of our comparative studies is the question of taphonomical differences, which is neglected by most of the comparative studies. The establishment of the 'specific diagnostical packages' mentioned above should be completed by the creation of the disease-specific criteria of 'observability'. If we use taphonomy-related correction factors corresponding to the observability of the disease-specific lesions, we can obtain much more realistic prevalence values.

The reconstruction of the infectious diseases of the past is a very complicated task – but one of the most beautiful tasks of human paleopathology.

Acknowledgement

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ARTICLE

Malignant tumors in osteoarchaeological samples from Hungary

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ABSTRACT According to our current knowledge, tumors are the same age as mankind itself. The prevalence of tumorous diseases, however, was seemingly relatively low in the past and apparently increased dramatically in modern times. This theory is based on scattered case studies. However, the majority of these investigations were not carried out using modern diagnostic techniques. The scarcity of data concerning the antiquity of cancer demands new investigations in this field. Future paleopathological discoveries and the application of improved diagnostic techniques may enable „paleo-oncology" to make further contributions to our understanding of cancer. In this study, we present data on the occurrence of malignant bone tumors in 12 anthropological series (3967 individuals) from Hungary dated to the 3rd -16th centuries AD. All skeletons were subjected to a careful macroscopic investigation, complemented by radiological examination and in special case scanning electron-microscopic and histological analyses, too. We identified 13 cases of malignant bone tumors. In most instances, multiple osteolytic lesions with slight osteoblastic reactions, in characteristic skeletal distributions, were strongly suggestive of metastatic carcinoma. However, in some cases multiple myeloma cannot be excluded. A mature male with pronounced osteoblastic reactions, particularly on the hip bones, seemed to be most compatible with the diagnosis metastatic prostate cancer. These observations indicate that carcinomas were present in human populations living on the territory of present-day Hungary over the last two millennia. **Acta Biol Szeged 53(2):117-124 (2009)**

KEY WORDS

paleopathology
osteoarchaeological samples
Hungary
metastatic carcinoma
multiple myeloma

According paleopathological records, tumors have a great antiquity (Capasso 2005). The earliest known unequivocal neoplastic case was noted on the partial skeleton of a North American lower carboniferous (about 300 million years BP) fossil fish, *Phanerosteon mirabile*. The first clear, well-documented case of malignancy dates to the Jurassic, as does the first certain case of metastasis (Rotschild et al 1999; Capasso 2005).

Concerning ancient human populations both benign and malignant forms of tumors are well in evidence certainly from the Neolithic period (Schultz 1989; Brothwell 2008). The prevalence of cancer in ancient populations might have differed from that in modern humans, because of substantial differences in environmental factors (such as tobacco and alcohol use, diet, etc.), life expectancy, and the availability of treatment (Halperin 2004).

According to the generally accepted view, current high rates of malignant tumors in industrialized Western populations have been ascribed to an increase in life expectancy and increasing influence of environmental factors, particularly nutritional intake of potentially carcinogenic substances and air pollution (e.g. Aufderheide and Rodriguez-Martin 1988; Ortner 2003; Capasso 2005; Józsa 2006; Nerlich et al. 2006; Thillaud 2006). It can be assumed that these factors have undergone substantial changes during various periods of time, which seem to have affected historical populations to variable extent.

Paleooncology - as a part of paleopathology - is a new term established by Halperin (2004) and refers to the study of malignant tumors in ancient human populations and their hominid ancestors. These populations provide information of crucial importance concerning the possible influences of morphological and functional evolution, diet, lifestyle, and other environmental factors on tumorous diseases. This new discipline may have deep impact on our knowledge of the natural history of cancer. The application of improved diagnostic techniques (such as paleohistology, micro-ct or proteomic analysis: Schultz 1993; Kuhn et al. 2007; Schmidt-Schultz and Schultz 2004; Schultz et al. 2007; Tóth et al. 2008) may enable paleooncology to make remarkable contributions to our understanding of cancer.

Several cases of bone tumors have been reported from historical anthropological materials, but the majority of pa-

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Table 1. Most important data of the affected individuals.

name of the cemetery (N)	dating of the site	affected individual	pathological alterations	supposed diagnosis	reference
1. Madaras-Halmok (635)	3rd-4th centuries AD	Grave No 216 - fragmentary skeleton of a mature (40-50 yrs) male	several lytic lesions of the cranium	metastatic carcinoma	Marcsik et al. 2002
2. Kiszombor B (55)	5-6th centuries AD	Grave No 45 - cranium of a mature male (40-50 yrs)	circular lytic defects of the cranium	metastatic carcinoma	Marcsik et al. 2002
3. Orosháza Béke TSZ (100)	7-8th centuries AD	Grave No 8 - fragmentary skeleton of an elderly (60+ yrs) male	multiple osteolytic lesions of the calvarium	metastatic carcinoma	unpublished
4. Székkutas-Kápolnadűlő (518)	8th century AD	Grave No 209 - fragmentary skeleton of an elderly (60+) female	osteolytic defects and slight osteoblastic reactions	multiple myeloma or metastatic carcinoma	Pálfi, 1989
5. Székkutas-Kápolnadűlő (518)	8th century AD	Grave No 135 - fragmentary skeleton of a mature male	high number of small osteolytic defects (constant size)	metastatic carcinoma	Pálfi, 1989
6. Pitvaros Víztározó (226)	7-9th centuries AD	Grave No 208 -fragmentary skeleton of a mature female (50-60 yrs)	circular lytic lesions overall on the skeletal remains	multiple myeloma or metastatic carcinoma	Molnár et al. 2006
7. Tiszafüred-Majoroshalom	7-9th centuries AD	Grave No 1092 - cranium and very fragmentary postcranial skeleton of a mature female (50-60 yrs)	lytic defects with a slight superficial, periosteal involvement	metastatic carcinoma	Marcsik et al. 2002
8. Nyíregyháza-Manda (42)	8-9th centuries AD	Grave No 40 - mature male (good state of preservation)	numerous lytic lesions with ragged margins (variable in size)	metastatic carcinoma	Marcsik et al. 2002
9. Hódmezővásárhely-Nagysziget (131)	10-11th centuries AD	Grave No 42 – mature female, (50-55 yrs) good state of preservation	large porous lesions and slight new bone formations on the calvaria	metastatic carcinoma	Bereczki et al. 2003
10. Homokmégy-Székes (195)	10-11th centuries AD	Grave No 94, mature male (50-60 yrs)	number of osteoblastic alterations - especially in the pelvis, vertebrae and ribs	metastatic carcinoma (prostate cancer)	Zink et al. 2004
11. Tiszalök-Köveshalom (270)	11-12th centuries AD	Grave No 42 – no cranium, fragmentary postcranial skeleton of a mature male (45-55 yrs)	several lytic defects without reactive bony margins, concentrated in the hematopoietic areas of each bone	multiple myeloma	Marcsik et al. 2002
12. Csongrád-Ellés (~300)	11-13th centuries AD	Grave No 126 – adult male (30-40 yrs), good state of preservation	large osteolytic lesion (destructive margin; superficial periosteal involvement)	metastatic carcinoma	unpublished
13. Baja-Pető (209)	11-16th centuries AD	Grave No 187-190 - well preserved cranium of a mature male	circular lytic defects of the cranium	metastatic carcinoma	unpublished

leooncological studies only deals with case histories without paleoepidemiological reconstruction of these conditions (e.g. Regöly-Mérei 1962; Pap 1985; Pálfi 1989, Strouhal 1991; Józsa and Pap 1994; Strouhal et al. 1996; Horáčková et al. 1997; Šefčáková 2001; Józsa and Fóthi 2002; Marcsik et al. 2002; Bereczki et al. 2003; Zink et al. 2004; Molnár et al. 2006). The analysis of tumor frequencies in populations from different historical periods may reveal the role of environmental factors on carcinogenesis. To this regard, only few previous reports describe human remains with traces of malignant tumors in historical populations of different time and location (e.g. Schultz 1992; Ricci et al. 1995; Zink et al. 1999; Strouhal 2000; Nerlich et al. 2006; Farkas et al. 2007; Roumelis 2007).

The identification of specific tumor types in bone often depends on careful histological examination of soft tissue components and/or clinical test available to modern pathologists. This information will not usually be available to those engaged in differential diagnosis of tumors in archeological human remains and specific diagnosis of tumor type will not always be plausible. This is particularly the case with metastatic carcinomas affecting bone, which often have very similar skeletal manifestations regardless of the primary site of the tumor (Dorfman and Czerniak 1998; Greenspan and Remagen 1998). However, careful analysis of the variables available to paleopathologists, including the type and distribution of the lesions and other factors such as the age and sex of the skeleton, provide helpful clues. Paleohistology,

Figure 1. Lytic lesions of the cranium (Madaras Halmok cemetery; male, 40-50 yrs).

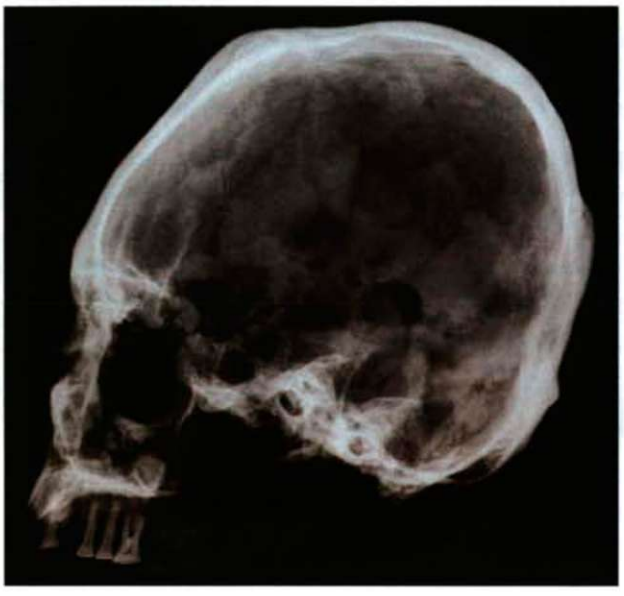

Figure 3. Radiograph - additional lytic loci localized in the diploe (Kiszombor B, male, 40-50 yrs).

Figure 2. Lytic defects of an intentionally deformed skull (Kiszombor B, male, 40-50 yrs)

paleoradiology, proteomic analysis and other new methods can contribute to a more precise diagnosis of the observed tumorous or tumor-like conditions in ancient osteological samples.

Materials and Methods

For the study, the skeletal remains of 12 anthropological series (3967 individuals) from Hungary dated to the 3rd -16th centuries AD were investigated from paleopathological point of view.

All skeletons were subjected to a careful macroscopic investigation, which was complemented by radiological examination and in special case scanning electron-microscopic and histological analyses, too.

Results and Discussion

Skeletal signs of malignant carcinoma or bone metastases were identified in 13 cases in the investigated material. The most important data regarding the affected skeletons are summarised in Table 1.

Our first case is a mature male from the so-called Sarmatian Period of Hungary (Madaras Halmok cemetery). The cranium is affected by several lytic lesions (Fig. 1). The radiograph shows additional lytic loci localized in the diploe. The endocranial table of the frontal bone reveals a large destruction surrounded with reactive bone. The detected pathological alterations seem to fit the diagnosis of metastatic cancer.

The second case is a mature male from the Gepid Phase of Hungary (Kiszombor B cemetery). The intentionally deformed skull reveal lytic lesions on the frontal bone and on the left parietal bone (Fig. 2). The cranium was previously restored using a glue which disturbed the radiographic analysis. In spite of that some additional lytic defects are seen on the radiograph (Fig. 3). The mandible reveals postmortem enlarged circular lytic lesion. The detected alterations could be diagnosed as a possible case of metastatic carcinoma.

The following 7 cases came from the so called Avar Period of Hungary.

Figure 4. Porosity of the bone adjacent to the margin of the lytic lesion (Orosháza Béke TSZ, female, 60+).

Figure 5. Osteolytic defects on the hip bone (Székkutas-Kápolnadűlő, female, 60+).

The cranium of an elderly male from the Orosháza Béke TSZ cemetery is affected by multiple osteolytic lesions. The defects vary greatly in size and some of them have not perforated the entire bone. The close up picture of the lesions shows the porosity of the bone adjacent to the margin (Fig. 4). On the radiograph more lytic loci localized in the diploe could be observed. The detected lesions are probable results of a metastatic carcinoma.

The fragmentary skeletal elements of an elderly female from the Székkutas cemetery reveal osteolytic defects on the frontal bone, on the ribs, on the vertebras and on the hip bones (Fig. 5). The detected alterations suggest the diagnosis of metastatic cancer but the possibility of multiple myeloma cannot be completely excluded.

The fragmentary skeleton of a mature male comes from the same cemetery as the previous case. On the skull a number of small osteolytic lesions are seen which have not perforated the entire bone (Fig. 6). The high number, the small diameter and the constant measure of the lesions seems to fit the diagnosis of multiple myeloma but the diagnosis of metastatic carcinoma cannot be completely excluded.

Figure 6. Numerous small osteolytic lesions on the skull (Székkutas-Kápolnadűlő, male, mature).

Figure 7. Circular osteolytic lesions of the right hip bone (Pitvaros Víztározó, female, 50-60 yrs).

The skeleton of a mature female (Pitvaros-Víztározó cemetery) is affected by widespread osteolytic lesions on the cranium and on the postcranial elements (Fig. 7). The majority of these lesions are circular (from 2 to 36 mm of diameter) and the edges are relatively irregular. Paleoradiological study

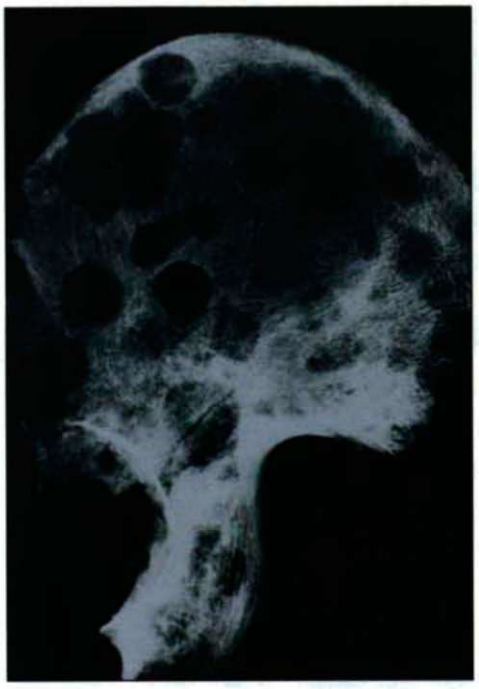

Figure 8. Radiograph - additional lytic defects localized in the trabecular bone (Pitvaros Víztározó, female, 50-60 yrs)

Figure 9. Lytic alterations on the skull (Tiszafüred-Majoroshalom, female, 50-60 yrs)

using plain films and CT investigations, shows the osteolytical aspect of the lesions evidenced by gross examination and the lack of associated osteosclerosis (Fig. 8). The diagnosis is based on the morphological and radiological aspects of the lesions and on the gender and the elderly age of this individual, considering a metastatic process rather than a multiple myeloma.

Figure 10. Large osteolytic defect surrounded by superficial periosteal involvement – hip bone (Nyíregyháza-Manda, mature, male).

Figure 11. Massive osteoblastic alterations on the hip bone (Homokmégy-Székes, 50-60, male)

Our next case is a mature female (Tiszafüred-Majoroshalom cemetey) whose skull reveals circular lytic lesions. Some of the defects affect only the external table of the cranium (Fig. 9). On the inner table of the skull lytic defects with a slight superficial periosteal involvement were observed. The radiograph reveal additional lytic defects in the diploe. The character of the lesions suggests the diagnosis of a metastatic carcinoma.

Our last case from the Avar Period is a mature male from the Nyíregyháza-Manda cemetery. The skeleton is in a very

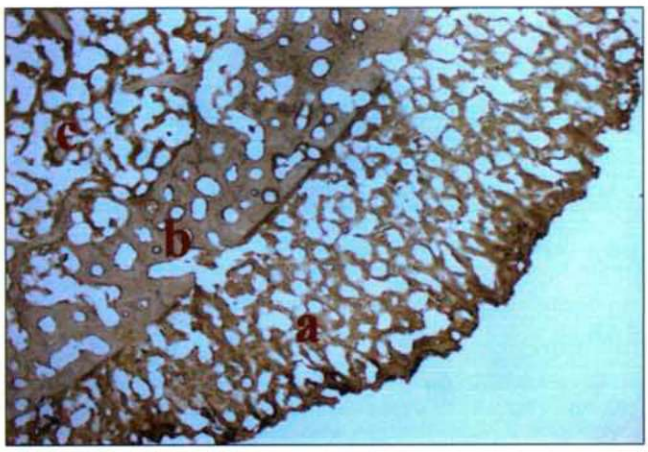

Figure 12. Cross section through a rib. Newly built bone formation (a), remains of original cortical bone (b), filled bone marrow (c), magnification X16 (Homokmégy-Székes, 50-60 yrs, male).

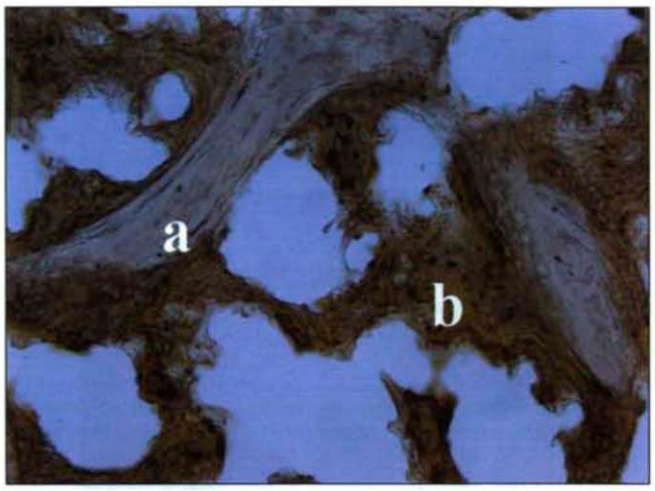

Figure 14. Cross section through a rib. yrs Original spongy bone (a), newly built bone formation (b) magnification X100 (Homokmégy-Székes, 50-60 yrs, male).

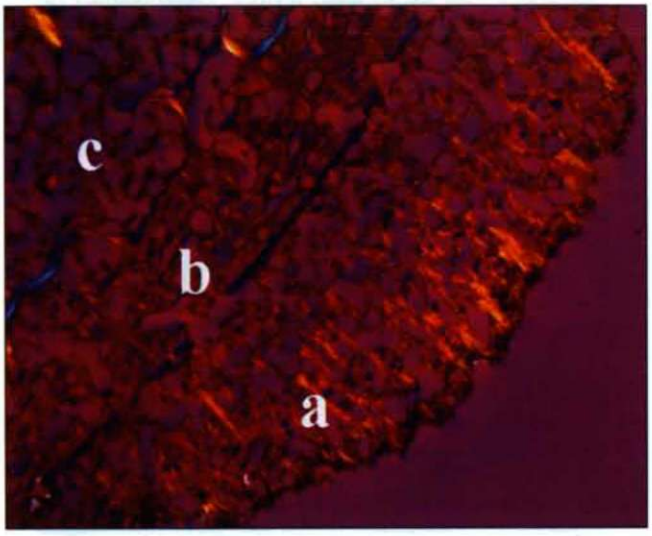

Figure 13. Cross section through a rib. Newly built bone formation (a), remains of original cortical bone (b), filled bone marrow (c),magnification X16, polarized light (Homokmégy-Székes, 50-60 yrs, male).

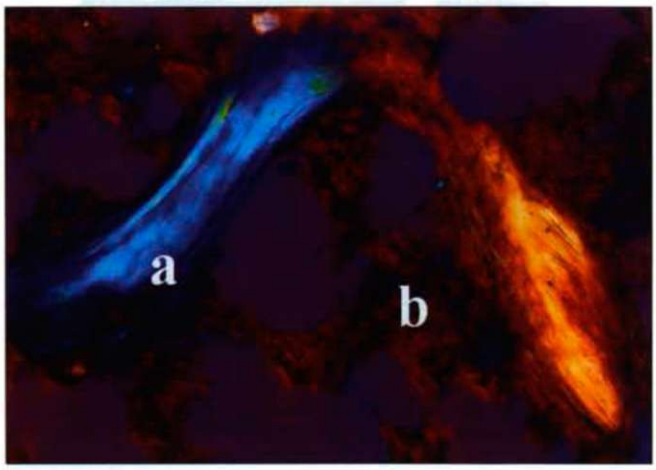

Figure 15. Cross section through a rib. Original spongy bone (a), newly built bone formation (b) magnification X100, polarized light (Homokmégy-Székes, 50-60 yrs, male).

good state of peservation. Numerous circular lytic lesions with ragged margin could be seen on the skull. The mandible shows severe erosive lesion surrounding with periosteal new bone formation. Concerning the detected pathological alterations of the postcranial bones, large osteolytic defects on both innominate bones (Fig. 10) and on the distal quarter of each femur should be noted. These lesions are suggestive of metastatic carcinoma.

The mature female from the 10-11th centuries cemetery of Hódmezővásárhely-Nagysziget reveals pathological alterations on the skull and on the postcranial bones. In spite of some post mortem damage large porous lesion of the calvaria is seen. Another porous lesion surrounding with slight new bone formation could be detected on the temporal surface of the greater wing of the left sphenoidal bone. The radiograph of the frontal bone shows additional lytic lesions in the diploe. The postcranial bones are also affected by slight osteoblastic defects. The number, the character and the distribution of the observed lesions suggest the diagnosis of metastatic carcinoma.

The mature male from the Homokmégy–Székes cemetery reveals severe pathological alterations. A number of osteoblastic alterations are found in many bones of the postcranial skeleton, especially in the pelvis (Fig. 11), ribs and vertebrae. Osteolytic lesions are only present on the base of the skull.

Figure 16. Osteolytic defects of a lumbar vertebra (Tiszalök-Köveshalom, male, 45-55 yrs)

Figure 17. Close up view of a large osteolytic lesion on the skull – destructive margin; superficial periosteal involvement (Csongrád-Ellés, male, 30-40 yrs).

The skeletal lesions were studied using metrical, radiographic and gross morphological observation. Additionally, a detailed histological, immunohistological and CT analysis was performed. These investigations clearly reveal, that the alterations are mainly osteoblastic with the most massive involvement located in the pelvis. Histology shows that the alterations are due to metastasis of a carcinoma (Fig. 12-15). The distribution and the extent of the lesions are most indicative of prostate cancer.

The mature male skeleton from the 11-12th centuries cemetery of Tiszalök-Köveshalom reveal numerous small, purely osteolytic defects on their postcranial bones (Fig. 16). The cranium is missing postmortem. The abscence of

Figure 18. Lytic defect of the right parietal bone (Baja-Pető, male, mature).

sclerotic margin of the defects is seen on the radiograph. The character, the size, the number and the distribution of these lesions seems to fit the diagnosis of multiple myeloma.

The skeletal remains of the adult male from the 11-13th centuries cemetery of Csongrád-Ellés are in a very good state of preservation. The skull reveals severe pathological alterations. Large osteolytic lesion with destructive margin in the left side of the coronal suture is worth mentioning (Fig. 17). Besides this alteration some porous lesions could be seen on the internal and external lamina of the skull. The radiograph shows additional lytic lesions in the diploe. Concerning the postcranial bones, the osteoblastic alterations of the 5th cervical vertebra is worth mentioning. The detected alterations suggest the diagnosis of metastatic cancer.

The last case is a mature male from the 11-16th centuries cemetery of Baja-Pető. The postcranial skeleton was missing postmortem. On the frontal bone circular lytic lesion (~16mm) without superficial periosteal involvement could be observed. Besides the frontal bone the right parietal bone is also affected by a circular lytic defect (Fig. 18). The intra vitam origin of the observed erosion of the mandibular ramus is questionable. The character of the lesions seems to fit the diagnosis of metastatic carcinoma.

Summary

We conducted the paleopathological analysis of the skeletal remains of 3967 individuals deriving from 12 archaeological sites of Hungary with special regards to malignant tumors. During the investigation skeletal evidence of malignant carcinoma or bone metastases were identified in 13 cases. No sign of primary bone tumors was found. Regarding the type

of the observed alterations the predominance of osteolytic lesions has to be mentioned. Concerning sex distribution of the affected individuals it has to be emphasized that males were more often affected (at a ratio of 9 to 4). Apart from one individual the observed cases belonged to older age categories. This observation fits to the generally accepted view that cancer is primarily a disease of old age.

Finally, we can concluded that carcinomas were present in the territory of present-day Hungary over the last two millenia.

Further investigations – applying modern diagnostic methods – in order to define the primary site of the tumors are planned to extend this study.

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ARTICLE

Preliminary report on the paleopathological research of the skeletal material from the Szeged medieval castle excavation

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ABSTRACT This study introduces some diseases occurred among the medieval population of Szeged. Hitherto 641 individuals have undergone general anthropological investigations. The identification of abnormal bone conditions was mainly performed by gross examination, but in several cases further investigations were required.One of the most common pathological disorders was spinal osteoarthrosis. There were some skeletal evidences of trauma identifiable; particularly fractures of the ribs and upper limbs. The incidence of developmental defects in our skeletal population is moderate. We observed some cases of widespread skeletal hyperostosis (DISH) as well as localized cranial hyperostosis (HFI) and also traces of osteoporotic processes. Porotic hyperostosis, a skeletal symptom of some nutritional deficiencies and also specific diseases, is a common phenomenon in our material.We could notice traces of non-specific infections like isolated periostitis or osteomyelitis and also that of slight bone alterations that can be indicative of early stage tuberculosis. However, the typical angular kyphosis found in one case proves that TB was present in medieval Szeged. Three skeletons showed bone lesions caused possibly by acquired syphilis. In two cases the radiocarbon and archaeological dating suggested precolumbian origin. These treponemal cases complete the list of evidences of pre-Columbian treponematosis in the Old World.
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KEY WORDS

paleopathology
Szeged-Castle
Middle Ages
pre-Columbian treponematosis

Paleopathological investigations – of course along with the basic anthropological data – can refer to the people of ancient times; what diseases they suffered from, how patients were treated and what kinds of medicines and therapies were in custom. Learning these data renders it possible for us to come to further consequences about living and health conditions and about the way of life in a given historical population. Thus it can be stated that the synthesis of the results of different paleopathological investigations is beyond individuals but focuses on reconstructing health status at population level (Ortner 2003; Józsa 2006).

Beyond getting to know our ancestors' everyday life, paleopathologic researches can widen our knowledge on medical sciences too, since we can investigate the paleoepidemiology of some special infectious diseases, such as TB, treponematoses or leprosy. These investigations have recently become timely, as TB re-emerges nowadays; not only in the Third World but also in developed countries, often in co-infection with HIV (Pálfi et al. 1999). It is essential to learn as much as possible about pathogens that have great capability of adapting to changes – as it has been proved by the appearance of multidrug-resistant strains of TB bacilli (WHO 2008). Detecting and diagnosing any osseous symptoms related to these infectious diseases might help modern physicians to diagnosing early changes or set new diagnostic criteria.

With this end in view this study concentrates on presenting the preliminary results of paleopathological investigation of the skeletal material from the Szeged-Castle site through demonstrating some special or important cases

The excavation at the former castle of Szeged has been started in 1999. In the middle of the fortress the archaeologists revealed a gothic church built in the first half of the 14th century. The burial place was used first up to 1543, then after when the Turkish occupation in Hungary ended (1686), up to 1713 again (Hajnal et al. 2004; Horváth 2009).

Up to now 970 graves and several other objects such as ossuaries and crypts have been uncovered. The investigation

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Figure 1. Butterfly vertebrae on the thoracic spine (grave 81. female?, 15-17 yrs).

of the human remains began in 2004 at the Department of Anthropology of the University of Szeged and it is going forward as long as any skeletons are unearthed.

In the future, after the excavation has been finished, our results can be completed and in the frameworks of a general study we will be able to understand the pathological processes the medieval population of Szeged suffered from and also to clarify the prevalence of past diseases.

Material and Methods

This presentation focuses on the skeletal material that came to light between 1999 and 2007.

Due to the high burial density in the cemetery, which is a quite common phenomenon in case of medieval graveyards; it is not unusual to find intrusive human bones from other individuals, mixed with the remains of the owner of the grave. It could also happen that remains of two or even more different skeletons could be separated from one grave. Thus the total number of individuals (from burials) might come to higher, though we included in our research only those identifiable as one distinct individual.

Table 1. – Sex- and age groups of the observed Szeged-Castle medieval osteoarchaeological sample.

Age group	Male	Female	Undetermin-able	Total
Neonatus	-	-	17	17
Inf1	-	-	106	106
Inf1/Inf2	-	-	18	18
Inf2	-	-	85	85
Inf?	-	-	2	2
Inf2/Juv	-	-	22	22
Juv	15	15	24	54
Ad	37	46	9	92
Ad-Mat	10	6	1	17
Mat	62	28	11	101
Mat-Sen	21	11	6	38
Sen	9	18	3	30
?(Adult)	12	17	30	59
Total	166	141	334	641

The whole material contains further some hundred individuals coming from large ossuaries and crypts and cannot be identified exactly. We included in our study only the skeletons originating from isolated burial, so the total number of graves examined is 641. All the same, we scrutinized all the osseous material in order to find any interesting pathological alteration.

The general state of bone preservation is quite bad; there are lots of incomplete skeletons and often the available bones are fragmentary at that or their surfaces are also in moderate condition.

To determine sex and age at death we used the accepted anthropological methods (Schour and Massler 1941; Nemeskéri et al. 1960; Éry et al. 1963; Stloukal and Hanáková 1978; Szilvássy 1978; Brothwell 1981; Ubelaker 1984; Lovejoy et al. 1985; Loth and Isçan 1989).

From graves the bones of 337 adults – 151 males, 126 females, 60 undetermined - and that of 304 subadults (younger than 23 years) are available for paleopathological investigations (Table 1.)

The identification of paleopaleopathological conditions was performed particularly by gross observation (e.g. Ortner and Putschar 1985, Ortner 2003; Roberts 2007). In several cases further investigations were required such as radiological or histological analyses. Paleomicrobiological and chemical analyses are still in progress. Radiocarbon dating was carried out by ATOMKI, Debrecen, Hungary and 14C CHRONO Centre Queens University Belfast, Northern Ireland on two samples of considerable importance. As the data collection – and even the excavation - is still in progress and especially because of the commingled, fragmentary or incomplete skeletons, this material is not adequate for precise

Table 2. Localisation and sex-distribution of the observed fracture cases.

Localisation of the fractura	Male	Female	?	Sub-adult	Total
Costa(e)	10	3	-	2	15
Clavicula	4	-	1	-	5
Scapula	1	1	-	-	2
Ulna	8	2	4	-	14
Radius	2	3	-	-	5
Femur	1	-	-	-	1
Fibula	2	-	-	1	3
Os metatarsus	1	-	-	-	1
Vertebra	1	-	-	-	1
Mandibula	-	1	-	-	1
Total	30	10	5	3	48

statistical analyses. It is, however, really valuable, since it furnishes precious data about the occurrences of diseases in medieval Szeged and it also points out general tendencies in their incidence.

Results and Discussion

Skeletal traces of a large series of osteo-articular diseases were identified. As the classification of the paleopathological lesions and diseases is not uniform in the literature (e.g. Aufderheide and Rodríguez-Martín 1998; Ortner 2003; Józsa 2006; Roberts 2007), we prefer to use the following six categories: developmental defects, traumas, joint diseases, metabolic and nutritional disorders, infectious conditions and other diseases. This latter category includes some diseases the incidence of which are relatively low or those which cannot really considered as pathological abnormalities. Therefore we do not detail them in this paper.

Developmental defects

Developmental defects manifest themselves as abnormal structures and they carry the possibility of having pathological function (Ortner 2003; Józsa 2006). As a rule, they can be caused by genetic and non-genetic (environmental) factors. They vary from minor malformations that may go unnoticed by the individual to real life threatening congenital diseases (Roberts 2007).

The frequency of observable developmental anomalies in the Szeged material is low; mainly minor defects on axial skeleton could be detected. The most frequent malformations occurred in our sample affect the sacral region of the spine. Sacralisation when non-sacral elements – mostly the first coccygeal vertebra and less often 5th lumbar vertebra – become similar in shape and fuse or partially fuse with sacrum as well as the incomplete development of the neural arch of one or more vertebrae, which is called partial bifid spine on the sacrum, were common. These alterations did not show any age related occurrence or sexual dimorphism. Our results are similar to those reported in literature, since according to many descriptions malformation of spinal elements are the most common congenital disorders (Marcsik 1998; Molnár 2000; Hegyi 2003; Ortner 2003; Józsa 2006).

Beyond the typical developmental anomalies we detected some rare defects which are important to touch upon since there are only a few reported cases in paleopathological literature.

In case of a juvenile individual (Grave no. 81.) a congenital maldevelopment on thoracic vertebral bodies is seen (Fig. 1). The malformation affects at least two thoracic vertebrae (Th9 and Th11) resulting in congenital division of the vertebral body into two lateral halves with an intervening sagittal cleft. If the cleft occurs in the midsagittal plane it is called "butterfly" vertebra. The background of this anomaly is that chorda dorsalis persists in vertebral body due to bilateral deficiency of sclerotomic substance causing failure in normal ossification in early embryonic period (e.g. Fischer and Vandemark 1945; Müller et al. 1986; Molnár and Marcsik 2003). It is a very rare anomaly, especially when it affects more than one vertebra (Patinharayil et al. 2008), although according to Brasili et al (2002) it may involve two adjacent vertebral bodies. Most instances have been described in lumbar region (Müller et al. 1986). In our case, as it is seen, the defect occurred at the very least in two sites ("hot spots") on the thoracic spine.

The skull remains of an infant about 4-5 years of age (grave 181.) shows abnormal deformity – we observed a remarkable triangular proeminence of the frontal bone This rare condition specified as trigonocephaly is a result of early fusion of the metopic (forehead) suture. It might be related to syndromes but it can occure alone as well (e.g. Flatz et al. 1984; Hegyi 2003). In our case we cannot come to any conclusion regarding its background, since the skeleton is incomplete, only fragments of the skull and the trunk are available. Nevertheless it might be supposed that the typical facial dysmorphism related to "trigonocephaly sequence" were present (Schaap et al. 1992).

Trauma

Trauma most commonly represents extrinsic influences on the skeleton that result from many factors, such as accidental and intentional violence, cultural cosmetic or therapeutic practices that affect bone, and some other pathological conditions (Aufderheide and Rodríguez-Martín 1998; Ortner 2003). Usually fractures, dislocation of joints and many other (rare or less severe) injuries are included in group of traumas.

Fractures are the most frequent traumas observed in our material. In case of 42 affected individuals, the traces of 48 fractures could be detected, particularly breaks of the upper limb and of ribs occurred (Table 2.). Fractures are three times as common in males as in females and adults are most frequently affected. We can say that the most of the injuries

Figure 2. Trauma (ulna fracture) induced pseudoarthrosis (grave 541. male, 30-50 yrs).

healed well, without complications; still we could notice some cases where accompanied infection set in or were followed by severe degenerative alterations.

Sometimes breaks occurred in more than one bone; as it is seen in case of an adult male (grave 185.) where multiple rib fractures as well as break of the clavicle and scapula can be detected. Regarding the fact that all of these injuries occurred on the right side and they are by and large at similar healing stage we might suppose that they can be attributed to the same event.

In case of the individual 441. (undeterminable sex, 50-59 years old) we observed the fracture of the left ulna and at the same time the right one is remodelled, reduced in length, with macroporosity on the distal end of the bone fragment. We assume, although the distal part - as the adjacent radius - is not available, that nonunion (pseudarthrosis) of a fracture was present there.

Another example of complications secondary to trauma is observable on the forearm bones of the skeleton from grave 541.

In case of this middle-aged man we could observe the fracture of the right ulna healed with callus and inflammation, and as concomitant sign a pseudarthrosis developed with the radius (Fig. 2). This defect must have considerably diminished the normal biomechanical function of the forearm. A severe degenerative process occurred on the left elbow joint, probably secondarily to the extreme usage of that arm.

Besides fractures we observed also symptoms of post-traumatic dislocations; two cases of shoulder and one case of hip dislocation.

Except injuries of the postcranial skeleton in six cases there could be detected wounds on cranial bones too; of which one depressed fracture produced by a sharp edged tool might be a perimortem injury (grave 48.).

Joint diseases

Joint diseases are one of the commonest disorders observable in paleopathology (e.g. Jurmain 1977; Aufderheide and Rodríguez-Martín 1998; Roberts 2007). Still, perhaps they are the most difficult to diagnose specifically; since the several hundred diseases known in clinical practice can produce identical bone alterations. So classification of joint diseases in paleopathology differs from that accepted in modern practical rheumatology (Józsa 2006).

The grouping of specific joint diseases is not uniform in paleopathological literature, still bone changes and distribution patterns of different artropathies are clearly described.

On the basis of their characteristics joint lesions observed in Szeged-Castle material could be included in two categories: degenerative joint diseases (DJD - vertebral and extravertebral) and inflammatory joint diseases.

According to many authors DJD – also known as osteoarthritis - can be described as a non-inflammatory, progressive, chronic process that may be bone destroying, bone forming or both (Roberts 2007) and can be usually subdivided as primary or idiopathic, in which no clear etiology is known, and secondary, in which some factors – the most frequent macro- or microtraumatic effects - have already affected the joints as primary causative agent. Differentiation between the two forms is usually complicated and sometimes impossible in paleopathology (Aufderheide and Rodríguez-Martín 1998; Józsa 2006).

Bone changes are principally characterised by loss of articular cartilage and subsequent subchondral lesions due to direct bone-to-bone contact. Eburnation, porosity, osteophyte formation (marginal or central) and joint contour change are the main diagnostic features in DJD (Rogers and Waldron 1987). In vertebral osteoarthrosis degeneration of intervertebral disc (intervertebral osteochondrosis) may lead to osteophyte forming on vertebral rim (spondylosis deformans and with weakening of annulus fibrosus to protruding of nucleus pulposus (Schmorl's nodes). These features can be observed in spinal DJD together with the osteoarthrosis of apophyseal joints (spondylarthrosis deformans; Kerr and Resnick 1984).

DJD – either vertebral or extravertebral or both – was detected on almost one third of the skeletal remains coming from the Szeged-Castle burials. The coincidence of spinal and extraspinal degenerative alterations was high, showing

that they might have been caused by the same factors. Such predisposing factors are principally increased age and maybe constant and severe functional stress, but other background contributing agents must not be ignored, either (Jurmain 1977).

Prevalence of spinal DJD in our material increases with advanced age corresponding the findings of other studies (e.g. Pálfi et al. 1992; Knüsel et al. 1997; Molnár 2000; Rojas-Sepúlvéda et al. 2008). Intervertebral osteochondrosis, spondylosis and spondylarthrosis deformans were seen mostly in of "mature" or middle-aged age group (older than approximately 40 years old) while Schmorls's nodes occurred also in younger – in four cases in subadult - spine elements. That finding is not surprising as intervertebral disc herniation can occur not only as a result of degenerative processes but also can be caused by the presence of congenitally weakened areas in the cartilaginous endplate or also by trauma (Kerr and Resnick 1984).

The incidence of extravertebral DJD is quite high in our material; still most of these alterations are very mild. Some severe secunder arthrosis could be detected subsequently to traumas. Secunder osteoarthrosis in the elbow joint of individual No. 541 has previously described (see "Trauma"), and traumatic dislocations were also followed by degenerative changes in the affected joint in case of two males with shoulder dislocation.

Figure 3. Diffuse idiopathic skeletal hyperostosis (DISH) of the spine (grave 290. male, 50-60 yrs).

Metabolic and nutritional disorders

As a rule, these two groups of diseases are treated as separated categories in the paleopathological literature (e.g. Aufderheide and Rodríguez-Martín 1998; Ortner 2003); nevertheless we think they cannot be categorically divided from each other, since they are often associated, occur together and sometimes they have the same aetiology in the background. We classified here pathologic conditions related to the metabolic and haemopoetic system, such as endocrine diseases, anemias and illnesses caused by insufficient intake of food, vitamins or trace elements.

Increasing bone production just as increasing loss of bone mass are typical symptoms of metabolic disorders. We observed five cases of extensive hyperostosis in which the diagnosis of Diffuse Idiopathic Hyperostosis (DISH) or Forestier disease is very likely. All of them have been determined as middle-aged or older males (above 40 years of age) what is concordant with clinical findings (e.g. Resnick 1976; Rogers and Waldron 2001). Excessive hypertrophic bone formation could be observed at joint margins and the entheses on the appendicular and axial skeleton as characteristic features.

According to Resnick's criteria (Resnick 1976) the most relevant diagnostic feature of DISH is fusion of at least four vertebral bodies. This abnormal ossification takes place under the anterior longitudinal ligament; however, this extensive bony hypertrophy is usually limited to the right side (Fig. 3). Radiological analysis of the block vertebra revealed that vertebral end-plates are intact and the disk spaces are not significantly narrowed (Fig. 4). This phenomenon along with the observation that the apophyseal joints are also unaffected by any pathological changes confirm our diagnosis. The exact aetiology of DISH is unknown but several studies have shown a strong association with obesity and type II. diabetes. Another authors report on the association of DISH with other metabolic disturbances including alterations in lipid metabolism and hyperuricaemia (Rogers and Waldron 2001; Kiss et al. 2002). In archeological studies a high prevalence of DISH has been demonstrated in ancient clergymen and it has been hypothesised that the "monastic way of life" might be a predisposing factor of DISH (Rogers and Waldron 2001; Verlaan et al. 2007).

Another phenomenon manifests in hyperostosis is usually localised at the cranial vault especially on the frontal bone. In our material the skulls of some individuals showed marked thickening on the frontal (or parietal) bone and on the inner table tiny bony outgrows occurred. The female skeleton from grave 133 has already been described (Ősz and Hajnal, 2005) as a case of Hyperostosis Frontalis Interna (HFI) however there are further six suspicious cases found in our skeletal sample related to this disease.

This disorder is part of Morgagni–Stewart–Morel–Moore syndrome which is described as co-occurence of HFI, obe-

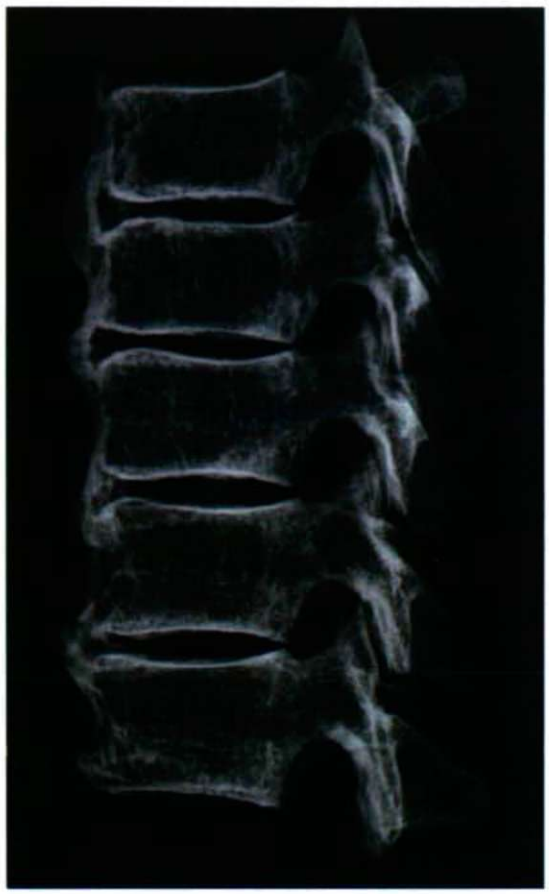

Figure 4. Lateral radiograph of the fused thoracic vertebrae in DISH (grave 290. male, 50-60).

sity, hirsutism, diabetes and other hormonal disturbances in postmenopausal women (e.g. Chaljub et al 1999; Ortner 2003; Belcastro et al. 2006; Glab et al. 2006) and might have a genetic base (Glab et al. 2006). However, according to radiological finding of Chaljub et al. (1999) the presence of HFI itself cannot be considered as a disease; it is a normal and benign phenomenon especially in elder women. There have been only a few cases of HFI published in paleoanthropological literature, though among them there is a higher male prevalence than expected on the evidences of today's clinical findings (Rühli et al. 2004).

Not only increased bone producing processes could be observed among the Szeged-Castle skeletons, but also – and more often – an abnormal reduction of bone mass very probably related to osteoporosis. Mainly a slight loss of bone quantity increasingly with age was detectably associated in some cases with other bone changes. Thus we noticed the symmetrical thinning of parietal bones in five cases and further two individuals had wedged vertebral bodies due to compression fracture.

Porotic hyperostosis (PH) can be regarded as a general

Figure 5. Periosteal new bone formation on the occipital bone (grave 250. infant, ~ 3yrs).

stress factor (Roberts 2007), since it is present when there is an increased demand on producing red blood cells. The main causes of that condition are anaemias resulted by any failure in haemoglobin synthesis, for example in iron-deficiency or in any type of hemoglobinopathies. Anemia can also be the result of abnormal blood loss through bleeding from a variety of causes, including the infection of the gastrointestinal track and menstruation (Ortner 2003). As ascorbic acid has an important role in absorbing iron, vitamin C deficiency may lead to anaemia too. PH, however, can appear as a concomitant symptom of infectious diseases due to decreased iron-level as part of the defence mechanism of the body (Stuart-Macadam 1992).

The bone alterations can occur either on the skull vault (porotic hyperostosis or cribra cranii) or on the orbital roof (cribra orbitalia) and are usually bilateral in distribution. Bone abnormalities are the result of thinning of the outer table of the skull and expansion of the diploë.

The occurrence of PH is reasonably high in the Szeged material; in 90 cases either form of the lesion could be detected from mild (porous) alterations to trabecular outgrowth (though latter occurs only in a few cases). It is conspicuous that the ratio of subadults is outstanding while we don't find any sexual differences among adults.

In case of 5 children cribra orbitalia (which was well-marked in most cases) was accompanied by fine pitting at several region of the skull such as on the jaws, on the frontal and sphenoidal bone and on the basis of the occipital bone. Beyond that endocranial lesions can be noticed mostly on the inner surface of parietal and occipital bones (Fig. 5).

According to our hypothesis the above seen symptoms might be suggestive of some disease resulting by Vitamin C deficiency; as this disorder cause chronic bleeding that

stimulates an inflammatory response resulting in bone lesions, particularly on the skull, scapula, and metaphyseal ends of subadult bones (Ortner et al. 2001). Still we have to consider the possibility of any infectious disease. To confirm our diagnosis chemical analysis of these samples is in progress.

Infectious diseases

In ancient times it was the group of infectious diseases that might have been responsible for most of the deaths. Even among nature people living today infection of the digestive system results in the death of many children. After the development settlements and people lived close to each other often together with animals, any more infectious diseases became endemic in human populations. Civilization, with the development of crowded cities, accompanied epidemics that often killed thousands within a short time (Ortner 2003). Nowadays epidemics have remained a serious problem especially in developing countries. Thus it would be essential to learn about past diseases as much as we can. Paleopathologists still have to face up to the fact that there are only few illnesses which leave indistinguishable traces on bones. Most of the infections are acute processes which can heal or which can lead to death within a few days without affecting the bones at all. However, there are several diseases vestiges of which can be well detected.

Traditionally, infectious diseases include non-specific and specific infections (e.g. Aufderheide and Rodríguez-Martín 1998; Ortner 2003; Roberts 2007, etc.).

Non-specific infections

Non-specific infectious condition can occur on any bone of the skeleton and leaves well identifiable lesions according to its nature. Though, care must be taken when setting a diagnosis, since mechanical stress on periosteum, for example trauma or subperiosteal bleeding as it is seen in scurvy (Ortner et al. 2001; Schultz 2001), can produce very similar alterations.

Infections can affect either the periosteum (periostitis) or the cortex of the bone (osteitis) or it can occur in medullar cavity (osteomyelitis).

Osteomyelitis, the prevalence of which was very low in our skeletal material, is usually caused by pyogenic bacteria in most cases by Staphylococcus aureus. The infection can spread via bloodstream from a distant septic focus (hematogenous osteomyelitis) or from a local infection of adjacent soft tissue or can be a complication of a trauma e.g. compound fracture. Acute hematogenous osteomyelitis occurs predominantly in children, while in adults, osteomyelitis is usually a subacute or chronic infection that develops secondary to a local infection of an open wound (Carek et al. 2001).

One example for the infection of the medullar cavity can be seen on the skeletal remains of an adult male from grave No. 291. resulting in fusion of the right tibia and talus (Fig. 6).

Figure 6. 3D CT-image of the right tibia and talus (grave 291. male, 50-60 yrs). Chronic osteomyelitis of the right tibia with ankylosis of the ankle joint.

The cortex of the tibial diaphysis widened significantly from the proximal towards the distal end with lamellar periosteal new bone formation at some places. In the distal third of the bone an irregular cavity with a partly sclerotic margin can be seen, which opens at the surface through multiple fistulae (Fig. 7). The talus is distorted and it completely fused with the tibia. This picture can be considered as chronic osteomyelitis most likely due to post-traumatic pyogenic infection; however the possibility of haematogenous osteomyelitis (among others tuberculous origin) cannot be disclosed either.

Another instance for chronic inflammation in medullar cavity was detected in case of an old man (grave 288.). On near midshaft of his left femur localised periosteal bone for-

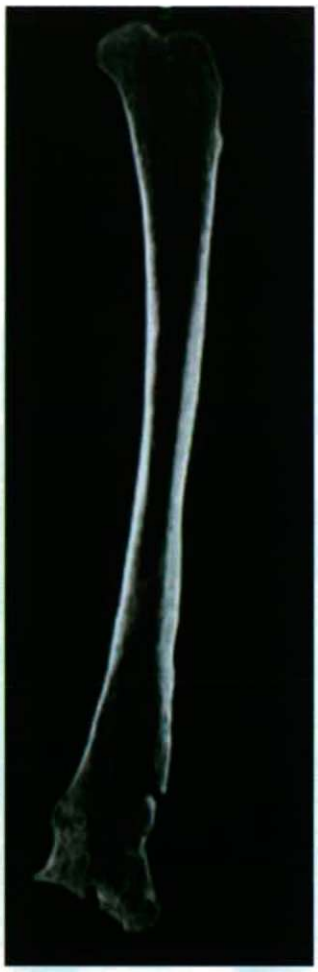

Figure 7. CT-image of tibiotalar ankylosis (grave 291. male, 50-60 yrs). Cloaca with partly sclerotic margin in the distal end of the tibia.

mation with cavitation at the centre has been macroscopically described. The ct-image shows that this cavity penetrates the cortex and sequester can be detected. At the same place the endosteum slightly thickened. The cortex of the diaphysis became a bit more bulky proximal to the lesion. This picture might be indicative of chronic osteomyelitis with sequestration.

Bone lesions due to periosteal reactions were common among our samples and occurred mainly on the bones of the leg. It has to be noted, though, that the very high number of individuals with any osseous changes on the bone surface does not show the real amount of infections, since not all periosteal alterations are of infectious origin (Ortner and Putschar 1985).

Recently it has been suggested that lesions manifesting on certain bones or areas can refer to the aetiology of the alterations. Such predilectional places are for example the maxillary sinuses (evidence for sinusitis), the endocranial

Figure 8. Gibbus resulting from the fusion of seven vertebrae (Th9-L3) and from the collapse of the vertebral bodies Th11-12 and L1-2 (grave 483, female, 30-40 yrs).

surfaces of the skull (referring to meningitis) and the visceral surface of ribs (suggesting the infection of the lung; Schultz 1999; 2001; Roberts 2007).

In the Szeged-Castle material we could observe all of the above mentioned bone changes, although in most cases only slight alterations were seen.

Due to their moderate state of preservation in some cases we could observe periosteal new bone formation on the walls of maxillary sinuses; however it is very likely that the prevalence of maxillary sinustitis was higher in our material.

Specific infections – Tuberculosis (TB) and Treponemal diseases

One of the most widespread specific infectious diseases in the Middle Ages was tuberculosis (e.g. Pálfi et al. 1999; Marcsik et al. 2007) caused by bacteria of the Mycobacterium tuberculosis complex; principally by Mycobacterium tuberculosis and Mycobacterium bovis. The localisation of the primary infection in human hosts is determined by the way of transmission depending on the specific pathogen. TB is transmitted either directly from cattle to human by consuming contaminated cattle products or from human to human usually by droplet infection through the respiratory system. Thus primary TB occurs either in lung or in the intestinal wall. As a rule, bones may be infected secondary via hematogenous dissemination; however the skeleton is affected only in about 3% of TB patients (Ortner 2003). Spinal TB (Pott's disease) is the most common bony lesion in recent clinical patients and also in paleopathological cases; authors report on vertebral involvement of 36-50% of all cases with skeletal involvement (Weber et al. 2004; Roberts 2007). The lesions are localized mostly in the thoracolumbar region and involve the vertebral bodies of one to three maybe four vertebrae at the most (Ortner 2003; Weber et al. 2004). Tuberculous

Figure 9. 3D CT-image of severe angular kyphosis on the thoracolumbar spine (grave 483, female, 30-40 yrs).

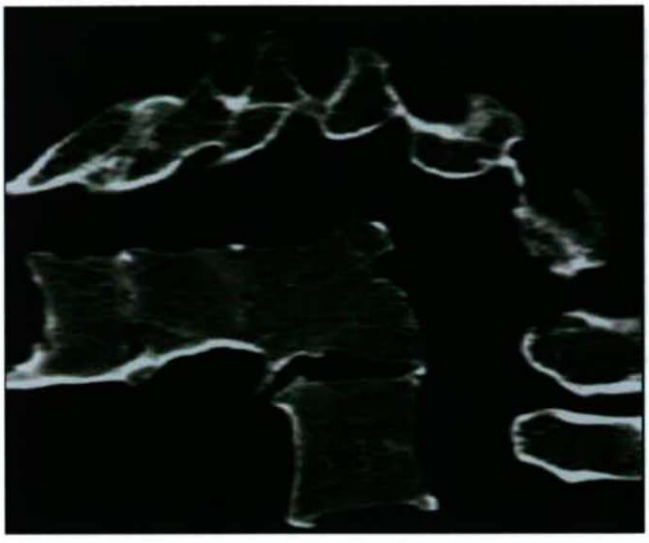

Figure 10. CT-image of the same block vertebra demonstrating the narrowing of the spinal canal and lytic foci in the vertebral bodies (grave 483, female, 30-40 yrs).

changes on posterial element of vertebrae are uncommon, as the bacteria are deposited particularly in areas of cancellous bone, which has a high circulatory rate. The infection may spread over to the paraspinal tissues - involvement of psoas muscle is commonly seen – resulting in forming a paravertebral abscess (Pott's abscess). Bony alterations on the vertebral bodies are mostly purely lytic, reactive new bone formation is rarely seen. Because of the primarily destructive nature of the disease collapsing of vertebral bodies and subsequent angular kyphosis is not uncommon is advanced stages (Burrill et al. 2007).

One example of such an abnormal gibbus formation was found in Szeged-Castle material (Fig. 8), which can undoubtedly be interpreted as a Pott's disease. This individual (grave 483, female, 30-40 years old) had a severe angular kyphosis resulted by the collapse and fusion of seven vertebrae at the thoracolumbar spine. In case of the caudal five vertebrae the bodies are destroyed, collapsed with anterior and central dominance, showing anterior wedging, and forming dorsal kyphotic prominence of the spine as gibbus. The angulation is approximately 90 degrees.

The borders of the vertebral bodies cannot be well differentiated, and the bony structures are abnormal at the maximium of the kyphotic deformity (Fig. 9). At the same level the bony spinal canal is narrowed. The foramina are narrowed too; the facet articulations are ankylotic.

Involving the lower-mid segment there is an asymmetric calcified, paravertebral mass, mainly on the left side in conjunction with the vertebrae (Fig. 10). The amorphous calcification may be due to healed vertebral/paravertebral soft tissue infection.

Another two possible cases of Pott's disease were found among our samples. In case of grave 561. (undeterminable sex, 50-60 years) destruction of at least three lumbar vertebrae was observed accompanied by vestiges of an inflammatory process. Lumbo-sacral TB is the suggested aetiology of the lesion seen on the skeletal remains of individual 487. (male, 40-60 years). The pelvic surface of the sacrum has an abnormal irregular appearance due to periosteal new bone forming and erosive lesions. Traces of an inflammatory process are detected in the retroauricular region of the pelvis. On this skeleton some nonspecific/atypical lesions can also be recognised; diffuse periostitis on the shafts of the long bones and on the bones of the feet as well as slight endocranial changes on the skull bones appeared maybe as concomitant signs of the disease.

Skeletal tuberculosis is not restricted to spine; weight bearing joints (knee and hip) can also be involved due to their good blood supply which provide optimal environment for tubercle bacilli.

In one case (grave 152. adolescent) traces of a serious inflammatory process were seen in the distal end of the right femur. The bone shortened and the distal epiphysis slipped backward and abnormal ossification occurred. Dorsally on the area of the metaphysis remodelled periosteal new bone formation can be noticed; however there is no evidence of sequestering as in osteomyelitis. The femora just as tibiae are postmortally damaged, still remodelling of the cancellous bone is seen. At the same time some other skeletal lesion was present such as marked hypervascularisation of the bodies of Th7-11 vertebrae, slight endocranial lesion and also mild periostitis on the fragments of both tibiae femora and fibulae. On the basis of macromorphological picture the diagnosis of (possibly healed) tuberculous gonitis is presumed. The young age of this individual also supports our assumption, as

Figure 11. Stereo-microscopic picture of parietal bone with serpegious cavitation and perforation with about 1 cm in diameter. (grave 2. female, 45-55 yrs).

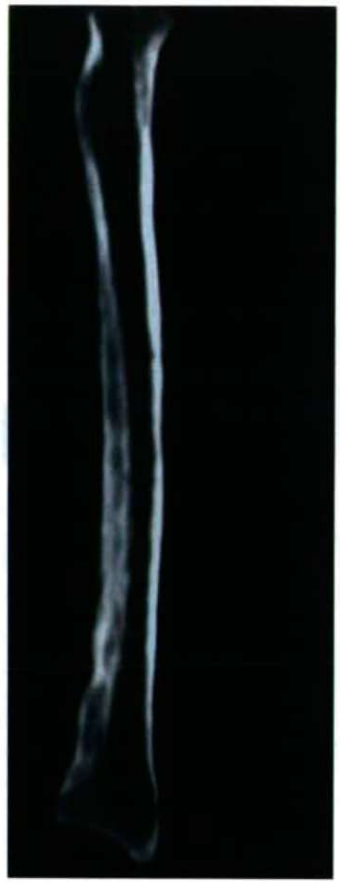

Figure 12. CT-image of the left radius (grave 2. female, 45-55 yrs). Lytic bone defect with sharp edges at the distal end and marked, irregular periosteal thickening on the medial surface almost all along the whole diaphysis.

tuberculosis of the knee affect particularly small children and adolescents (Ortner 2003). Until our previous diagnosis is not confirmed by paleomicrobial and by chemical analyses the possibility of other diseases cannot be precluded, either.

As recently more and more paleopathological reports are published on the diagnostic value of some atypical bone changes, it has become widely accepted among paleopathologists that these minor osseous changes might be considered as early signs of tuberculosis (Maczel 2003).

It is worth to mention that in 59 cases and especially on infant skeletons endocranial changes (abnormal blood vessel impressions and fine new bone formation) on the skull bones – particularly on the inner surface of parietal and occipital bones – could be detected. We have also described irregular bony alterations, possibly traces of old periosteal reactions, on ribs (36 cases). Rib lesions occurred, however, in all age groups, though adults were mostly involved (25 cases).

These bony alterations - endocranial lesions and periosteal remodelling on the visceral surfaces of ribs – along with some other osseous lesions such as diffuse periostitis on one ore more postcranial bones and vertebral hypervascularisation could be associated with tuberculosis (e.g. Roberts et al. 1994; Baker 1999; Schultz, 1999; 2001; Hershkovitz et al. 2002; Pálfi 2002; Maczel 2003; Santos and Roberts 2006).

There are a considerable number of skeletons (at least 30 individuals) in our material in case of which at least two of the above mentioned bony alterations co-occurred. To establish a correct diagnosis biomolecular analyses of these samples have been going on.

Other specific infection detected in Szeged-Castle material was treponematosis. The remains of three individuals showed serious bone lesion related to treponemal disease which was identified as acquired syphilis. The diagnosis was based on morphological observations after the diagnostic criteria of Hackett (1976) and in two cases (grave 2. and 16./4. skull) our findings have already been supplied by further (radiological and histological) investigations. Chemical analysis of the samples (including grave 61.) is in progress.

On the skull of a mature woman (grave 2.) especially on the parietal and frontal bones the typical stages of caries sicca (Hackett 1976) are noticeable (Fig. 11). Clustered pits, confluent clustered pits, focal superficial cavitation, serpiginous cavitaton, depressions and perforations can be recognised (Ősz et al. 2006). Her postcranial bones have been also affected by periosteal alterations; osteomyelitis gummosa as well as periostitis has occured on several long bones. On the AP radiograph of the examined long bones cortical thickening and narrowing of the medullar cavity can be also seen. The ct-image of the left radius shows a lytic bone defect with sharp edges is seen at the distal end (Fig. 12). There is a marked, irregular periosteal thickening on the medial surface almost all along the whole diaphysis. This picture can refer to a chronic process.

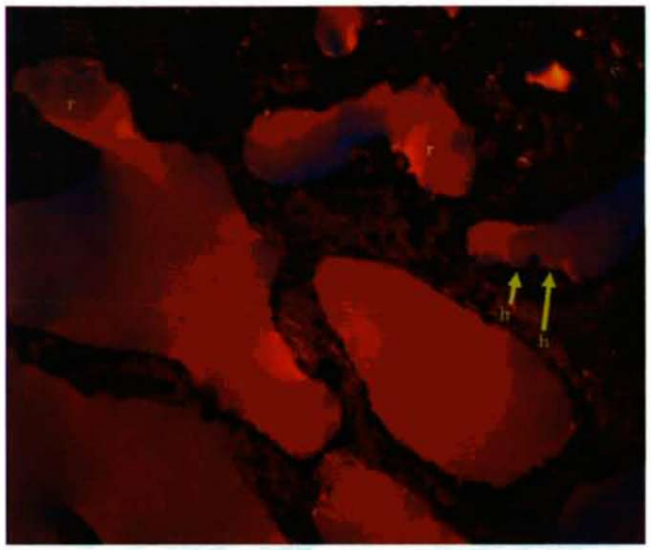

Figure 13. Cross section through the right fibula (grave 2. female, 45-55 yrs). Inflammatory process suggested by the presence of resorption holes (r) with Howship's lacunae (h).– Undecalcified thin ground section, viewed through the microscope in polarized light. Magnification, x 25.

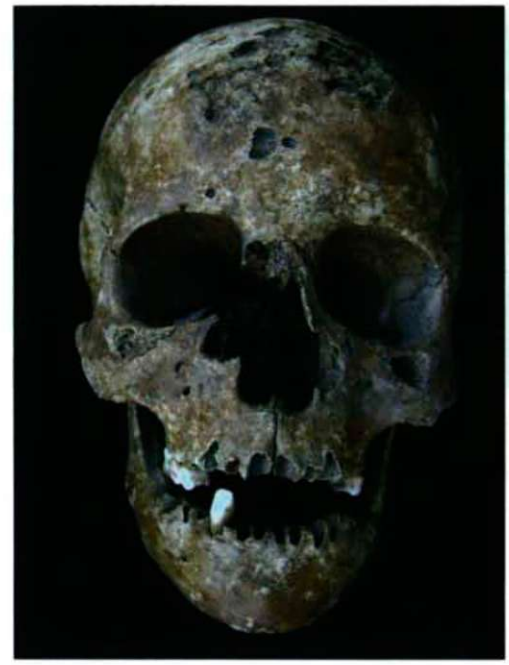

Figure 14. Widespread bony alterations (caries sicca) on the frontal bone and lytic lesions on the facial bones (skull No. 16/4. female young adult).

In case of this individual we had the possibility to carry out histological investigations too (Ősz et al. 2007). Though, unfortunately, the original bone substance was strongly damaged post-mortem due to diagenesis, so the only thing we could establish was that the resorption holes suggested the presence of an inflammatory process, however it is not characteristic of acquired syphilis exclusively (Schultz 2001) (Fig. 13).

The result of the radiocarbon dating suggests that the date of death can be estimated between 1420 and 1490 AD.

Although we include in our study skeletons derived from single graves, we must not disregard any pathological alterations found in skeletal remains coming from ossuaries. During the anthropological and paleopathological examination of the material of ossuary No. 16, remarkable paleopathological lesions were found on a young adult female skull (Fig. 14) The cranial vault exhibits multifocal lytic lesions and wide-spread superficial pitting. On the frontal and parietal bones circumvallate and nodular cavitation appear. The alterations of the external table are typical gummatous lesions characterized by a mixture of new bone formation and destruction creating an irregular lumpy appearance. The mentioned lesions can be identified as different stages of caries sicca. The healed foci of caries sicca leave depressed, sclerotic radial scars (Fig.15 and 16). CT image revealed destructive changes on the basis of the skull and also in the sphenoid body.

On the facial bones multifocal lytic lesions occurred destroying the outer bone surface around the aperture of the nasal cavity.

On the maxilla and on the zygomatic bone lytic lesions as well as naso-palatinal destruction are observable. Traces of periosteal inflammatory reaction (pitting) can be observed around the lytic focus. Both orbital roofs exhibit well-marked cribra orbitalia, which might have been interrelated with the infection (Stuart-Macadam 1992).

The lack of the postcranial skeleton normally decreases the value of a paleopathological diagnosis. However, according to Ortner (2003) the cranial vault and the bones surrounding the nasal cavity are among the greatly predilected areas in acquired syphilis. Together with tibia, these three regions are affected by syphilitic bone lesions in about 70 % of all cases. In spite of the absence of examinable postcranial bones we consider our case as paleopathological example of acquired syphilis, since the examined lesions are highly pathognomic and show all stages of Hackett's classification (eg. radial scars, circumvallate and serpiginous cavitations). The archeological dating places the death of this individual around the second third of the 15th century necessarily anterior to 1490. As according to the archeological evidences the church at the castle of Szeged was restored in the second half of the 15th century during the rule of King Mathias (1458-1490). The graves found in course of the construction had been dug up and put into an ossuary. Crypt No. 16. was filled at that time too, therefore the skeletal material certainly dates back earlier than 1490. The bones might belong to the people deceased in the middle of the 15th century.

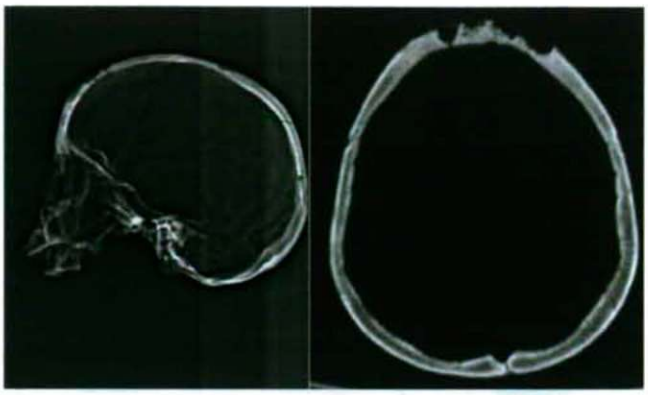

Figure 15. – CT-image of the skull skull No. 16/4. female, young adult). Multiplex lytic lesions with irregular margins on the outer table and in the diploë.

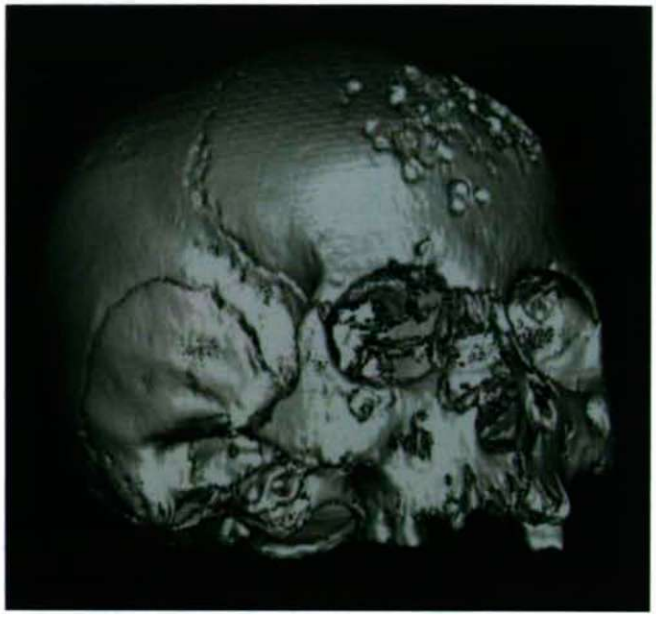

Figure 16. – 3D CT-image of the skull skull No. 16/4. female, young adult). Caries sicca.

The importance of this case required a radiocarbon control of the archeological dating. The 14C investigation of the lower right third molar presumes that the individual must have been entombed between 1423-1512 AD (at 2 sigma range) and within that period the time of the death falls in the range AD 1435-1472 with a high probability (at 1 sigma range). The results of the radiocarbon analysis correspond to the archaeological data.

There has been much debate on the origins and spread of treponematoses caused by the spirochete Treponema pallidum and its subspecies. The paleoepidemiology of acquired syphilis is particularly controversial. The most discussed question has been for years, whether this infectious disease had been present in the Old-World before 1495 when the first well-known and registered epidemic broke out. According to the previously accepted opinion, the disease has been brought from the New World by Columbus's crew. However, there has been more and more paleopathological evidences proving that treponematosis (and among them acquired syphilis) could have existed in our continent even in the pre-Columbian times (e.g. Stirland 1991; Pálfi et al. 1992; Dutour et al. 1994; Mays et al. 2003; Mitchell 2003; Alduc-Le Bagousse et al. 2009).

Conclusions

Paleopathological study of the human osteoarchaeological material from the Szeged-Castle excavation has furnished invaluable information concerning the health status of the medieval inhabitants of Szeged. However, as the investigation is still going on, no definitive conclusions can be drawn yet. Still, fortunately some really interesting and important cases and processes could be detected and also some general tendencies related to their occurrence were recognised in the Szeged-Castle material. These findings, though, allow us to obtain the first insight into the everyday life of the people of the medieval Szeged.

The relatively low incidence of developmental defects and traumas in our skeletal population suggests that the humanity of medieval Szeged was a settled but not endogamous urban population. The cemetery around the castle church was probably used by the citizens and not by soldiers, as only a few evidences of war injuries was observable. Among the detected metabolic disorders, we have to mention the well developed DISH cases, without more precise etiological explanation at the actual level of our studies. Age - and functional stress-related degenerative joint diseases – either in vertebral or extravertebral localisation – was detected on almost one third of the examined skeletal remains.

The observed typical Pott's disease and the other, less typical forms of probable TB infection prove that tuberculosis must have been present among the inhabitants of Szeged, even though we could detect mainly slight or early stage skeletal alterations of the disease. The chemical and molecular analyses of these cases with atypical bony alterations are in progress. Consequently, it is quite possible that the number of confirmed TB-cases from the Medieval Szeged will increase. Two cases of typical form of acquired syphilis could be identified in the examined part of the Szeged-Castle osteoarchaeological material. We have to mention that these early treponemal cases give the first pre-Columbian examples of the disease in Hungary. These observations from the Carpathian Basin complete the list of evidences of pre-Columbian treponematosis in the Old-World that is constantly getting richer and richer. We also have to take into consideration that the osseous symptoms of TB and syphilis develop only

in a small percent of the infected patients. Consequently, the prevalence of these specific infectious diseases could have been higher in the medieval human populations of Szeged.

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